



Viral exposure effects on life-history, flight-related traits, and wing melanisation in the Glanville fritillary butterfly

Luisa Woestmann^a, Melanie Gibbs^b, Helen Hesketh^b, Marjo Saastamoinen^{a,*}

^a Organismal and Evolutionary Biology Research Programme, Faculty of Biological and Environmental Sciences, PO Box 65, Viikinkaari 1, 00014 University of Helsinki, Finland

^b NERC Centre for Ecology & Hydrology, Maclean Building, Benson Lane, Crowmarsh Gifford, Wallingford, Oxfordshire OX10 8BB, United Kingdom



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ABSTRACT

Infections represent a constant threat for organisms and can lead to substantial fitness losses. Understanding how individuals, especially from natural populations, respond towards infections is thus of great importance. Little is known about immunity in the Glanville fritillary butterfly (*Melitaea cinxia*). As the larvae live gregariously in family groups, vertical and horizontal transmission of infections could have tremendous effects on individuals and consequently impact population dynamics in nature. We used the *Alphabaculovirus* type strain *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and demonstrated that positive concentration-dependent baculovirus exposure leads to prolonged developmental time and decreased survival during larval and pupal development, with no sex specific differences. Viral exposure did not influence relative thorax mass or wing morphometric traits often related to flight ability, yet melanisation of the wings increased with viral exposure, potentially influencing disease resistance or flight capacity via thermal regulation. Further research is needed to explore effects under sub-optimal conditions, determine effects on fitness-related traits, and investigate a potential adaptive response of increased melanisation in the wings due to baculovirus exposure.

1. Introduction

The immune system is a crucial component of all organisms, as it defends a host against infections. Such effects are widespread and can cause substantial fitness losses and damage to hosts. Immune defence has evolved in an ecological context, being under strong selection pressure due to ongoing host-parasite co-evolution in an evolutionary “arms race” (Decaestecker et al., 2007; Mone et al., 2010). With increasing global changes due to climate warming and spread of invasive species, alterations in not only prevalence but also severity of some infectious diseases have been suggested to increase significantly (Jones et al., 2008; Shikano and Cory, 2015; Roy et al., 2017), and thus the understanding of how infections impact performance of individuals, especially in wild populations, is of crucial importance. Herbivorous insects are a relevant group for ecological immunological studies, as they are often very sensitive to their environment, and thus might be greatly impacted by any changes in disease dynamics. In the last two decades, eco-immunological studies have expanded also outside model systems, and with increased availability of genomic tools and techniques, our understanding of the impact of biotic and abiotic factors on variation in immunity has greatly improved (Schulenburg et al., 2009;

Adamo & Lovett, 2011).

Infection by pathogens often impacts a wide range of host phenotypic traits, such as physiology, morphology, and behaviour (e.g. Poulin & Thomas, 1999; Hoover et al., 2011). Life-history theory suggests that investment in pathogen defence and resistance, however, comes at a cost of other physiological processes, important for individual quality, such as growth and reproduction (Stearns, 1992; Roff, 2002). Fluctuating asymmetry (FA; the stochastic differences between right and left halves of bilaterally symmetrical organisms; Palmer and Strobeck, 1986) has been suggested to represent one measure of developmental instability, and good quality individuals are predicted to show less FA as their genome is able to buffer against environmental influences (Møller & Swaddle, 1997). In many species females choose males based on sexual secondary characters, like courtship, symmetry or wing patterns, that generally reflect individual's disease resistance (Rantala et al., 2000; Rantala & Kortet, 2003). Pathogens, such as deformed wing virus and nematodes, can provoke drastic effects on morphology in bees and midges (*Chironomus*), respectively (Wülker, 1985; de Miranda & Genersch, 2010). Studies on Monarch butterflies have also shown that pathogenic infection can directly reduce flight ability, however this was not shown to be related to changes in wing morphological traits per se

* Corresponding author.

E-mail addresses: luisa.woestmann@helsinki.fi (L. Woestmann), mela1@ceh.ac.uk (M. Gibbs), hhesketh@ceh.ac.uk (H. Hesketh), marjo.saastamoinen@helsinki.fi (M. Saastamoinen).

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(Bradley & Altizer, 2005). Similarly, in several insects, including Lepidoptera, stressful conditions during development have been shown to impact flight-related traits including wing-morphology (Gibbs et al., 2010; Saastamoinen & Rantala, 2013). In general, the observed responses are often pathological side (or stress) effects but in some cases they have also been shown to represent adaptations of the host or the pathogen (Dingemans et al., 2009), which are often not investigated and hard to demonstrate (Hughes et al., 2012; van Houte et al., 2013). The responses further depend on the pathogen in question, and are likely coupled with consistent behaviours tightly intertwined with life-history (Kortet et al., 2010). Assessing individual fitness costs in regards to changes in life-history or morphology due to an upregulated immune defence is important in larger context, as it allows us to understand the role of pathogens in regulating natural populations.

In insects, much of the immunity research has focused on model systems, such as *Drosophila* (Lemaître & Hoffmann, 2007). In Lepidoptera, moths, on the other hand, have been the main focal system of immune studies, whereas immunity in butterflies is less well understood (e.g. Reeson et al., 1998; Wilson & Graham, 2015, but see e.g. Lindsey & Altizer, 2008). Baculoviruses are DNA viruses that are highly pathogenic and obligate killers of insects and other arthropods. They represent a good model system for exploring viral infections in insects as they predominantly infect lepidopteran species (Cory & Myers, 2003). Baculoviruses also have the potential to influence host population dynamics (Dwyer & Elkinton, 1993) and they are widely used as control agents for insect pests through inundative applications (Cory and Myers, 2003; Lacey et al., 2015). Viral horizontal transmission is achieved via environmentally stable occlusion bodies, which are produced at the end of the infection cycle in host tissues and released into the environment where they can be ingested by new hosts. Horizontal transmission is known to have an important role in pathogen disease progression (e.g. McCallum et al., 2001). With increasing host density, the influence of pathogens on population dynamics is often more dramatic (although simple mass action models are influenced by many factors including spatial structure, pathogen clumping and behaviour (e.g. d'Amico et al., 2005).

We used a species from the *Alphabaculovirus* genus of baculoviridae, specifically the virus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), to investigate the effects of viral exposure on survival, developmental life-history, flight-related traits, wing melanisation and wing symmetry in the Glanville fritillary butterfly (*Melitaea cinxia*). Even though viral infections are likely to be important in natural populations of this species, little is known about the full range of pathogens the Glanville fritillary encounters in nature. This study is hence the first to experimentally test the response of this butterfly to an exemplar viral infection. Moreover, as effects of a baculovirus infection in the larval stage on adult traits are not widely investigated so far, our study also provides new information more generally. Host-pathogen interactions are likely to be relevant in this species due to its life-history: females lay eggs in clusters and the larvae live gregariously throughout most of their development. This means that horizontal transmission of pathogens may be particularly effective, as parasite transmission is usually positively density dependent (Anderson & May, 1981; McCallum et al., 2001, but see Wilson et al., 2003). Furthermore, as in Finland the Glanville fritillary persists as a classical metapopulation in the Åland islands, dispersal represents a key life-history trait. Any potential effects of viral infection on flight-related traits that influence adult flight ability could therefore have important fitness consequences for this species. The specific aims of this study were to 1) determine the viral concentration-mortality relationship in sixth instar larvae of the Glanville fritillary butterfly exposed to AcMNPV in the laboratory, 2) assess virus concentration-dependent effects on larval developmental traits and 3) investigate whether adults show changes in flight-related wing morphology or body allocation related to viral exposures during development that could potentially influence their flight ability. We hypothesized that physiological constraints caused by

resources being allocated to immune defence during growth would result in resource allocation trade-offs across life stages with fewer resources available to invest in life-history traits, such as developmental time or daily mass acquisition within the larval stage, and/or fewer resources available to invest in wing morphological traits in the adult stage. Little is known about the effects of baculovirus infection on wing development and wing morphological traits, but previous studies on another butterfly species found that exposure to viral infection during development affects resource allocation to thorax mass (Hesketh et al., 2012; Gibbs & Weir, 2017). We therefore predicted that effects of baculovirus infection on wing development (i.e. wing symmetry) and flight-related wing morphology (i.e. forewing length, forewing area, loading, forewing aspect ratio, thorax ratio) may also be apparent in the Glanville fritillary. In addition, we hypothesized that due to constraints, increased need of melanin for immune defence would result in reduced melanisation of adult wings. Finally, as immune response is thought to vary generally based on different life-histories for males and females (Rolff, 2002), we expected to observe sex-dependent differences in response to viral exposure in the Glanville fritillary.

2. Material and methods

2.1. Study species

In Finland, the Glanville fritillary butterfly, *Melitaea cinxia* (Melitaeini: Nymphalidae), is present only in the Åland islands in the south-western edge of the mainland. The habitat of the butterfly is highly fragmented in the Åland islands, and the butterfly persists there in a classic metapopulation. The metapopulation consists of a network of around 4000 habitat patches of dry meadows within an area of 50 × 70 km (Hanski, 1999; Nieminen et al., 2004). Larvae utilize two plants as their food source, *Plantago lanceolata* and *Veronica spicata*. Having a univoltine life-cycle, larvae feed during the first five instars on the host plant and overwinter in a silken web during diapause. They continue feeding in spring, moult into two to three more instars and pupate in May followed by adults in June to mid-July, which feed on nectar. Females emerge about 2–3 days later than males and carry the full number of oocytes in their ovarioles, which they lay in several clutches on the larval host plants (Boggs & Nieminen, 2004). Females show higher dispersal rates than males (Kuussaari et al., 1996) and the longest colonization distances recorded are about 4–5 km (Van Nouhuys & Hanski, 2002). Males, which typically perform short flight bouts and rapid take-offs establishing or defending their mating territory, are, however, also able to fly more continuously in search for females (Boggs & Nieminen, 2004).

2.2. Baculovirus production

A stock suspension of the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) was produced as described previously in Gibbs et al. (2010) and Gibbs and Weir (2017). To enumerate the concentration of the stock, occlusion bodies (OBs) were counted in an improved Neubauer haemocytometer at magnification 400× (< 10% error in counts) and this was replicated three times to give an estimated average concentration of OBs ml⁻¹. The stock suspension was stored at -20 °C until required. Serial dilutions were prepared in sterile distilled water from the stock suspension for experiments to give seven different final concentrations ranging between 1 × 10³ and 1 × 10⁹ OBs ml⁻¹ of 10-fold differences at each concentration.

2.3. Experimental design

2.3.1. Experiment 1: effects of increasing concentration exposure to AcMNPV

This experiment was designed to examine the impact of exposure of sixth instar larvae to increasing concentrations of AcMNPV on larval

developmental time (as the number of days from viral inoculation to pupation), mass acquisition (calculated as (pupal mass – mass at inoculation)/larval development time; after Hesketh et al., 2012), pupal mass and larval survival until pupation and eclosion as an adult. We also measured the mass of the adult dry thorax in relation to body mass (thorax ratio) as an indicator of resource allocation to flight muscles (Marden, 1987). The body of each individual was dried for 24 h at 60 °C and weighed (Precisa 2625MA-FR analytical balance accuracy: ± 0.1 mg). The thorax was then carefully removed and weighed and used as a measure of investment in flight muscle mass (after Hughes et al., 2012). The results of experiment 1 were also used to obtain the dose level of AcMNPV, to be used in Experiment 2 (see below).

In spring 2016, 361 fifth instar larvae from 14 different families (4–40 individuals/family, on average 25.8 ± 2.9) were encouraged to break diapause by increasing the surrounding temperature. These larvae were offspring of individuals that were reared under laboratory conditions during post diapause development but that had been wild-collected originally as larvae pre-diapause. The larvae used in the present experiment, were reared *ad libitum* on *P. lanceolata* in family boxes for ten days until they all started to feed and grow. The presence of conspecifics is thought to act as feeding simulant in these gregarious larvae and they generally benefit from the presence of group members (Rosa et al., 2017). At this stage (i.e. prior to the inoculation) larval body mass was recorded (Precisa 2625MA-FR analytical balance accuracy: ± 0.1 mg) and the larvae were randomly divided into eight different treatment groups (virus concentration exposure) across the families. Weighing larvae prior to inoculation allowed us to examine (during analyses) potential differences in response due to variation in larval mass at inoculation (Gibbs et al., 2010; Gibbs and Weir, 2017). Following a 24 h starvation period, larvae were exposed to AcMNPV (during the sixth instar). Larvae were inoculated individually in single cups (200 mL) containing a piece of damp filter paper and a small circular piece of *P. lanceolata* leaf cut with a metal cork borer (diameter: 5 mm) with 1 μ l of viral inoculum (concentration of virus between 1×10^3 and 1×10^9 OBs ml^{-1} ; 43–48 larvae per treatment group) or a 1 μ l droplet of sterile distilled water as a control applied on the leaf disc using a pipette. The majority of the larvae consumed the leaf disc within 24 h and in case the individual had not they did not receive fresh food until the day after (independent of treatment). After the second day, we no longer followed whether the larvae consumed the food or not. The larval rearing continued with *ad libitum* feeding in single containers and controlled conditions (28:15 °C, 12:12 h, D:N). Survival was checked daily. The day after pupation, pupal mass was recorded (Precisa 2625MA-FR analytical balance accuracy: ± 0.1 mg), eclosing butterflies were sexed, weighed, frozen at -20 °C and dried at 60 °C for the assessment of dry thorax mass.

2.3.2. Experiment 2: response to viral exposure

This experiment was designed to examine the effects of AcMNPV on larval developmental time, mass acquisition, pupal mass, pupal developmental time, sex ratio, and larval survival to pupation and eclosion as an adult. We further assessed the effects of viral exposure on wing morphology, wing symmetry and wing melanisation (see 2.4). Based on experiment 1, we found generally very low survival rates until adult eclosion, with $\sim 30\%$ survival rates for the lowest concentration tested (1×10^3 OBs/mL of AcMNPV). As our aim here, was to use a concentration that would only moderately influence survival, we chose the lowest concentration of AcMNPV for experiment 2.

For this experiment, 621 larvae from a laboratory produced generation from 24 different families (2–94 individuals/family, on average 25.9 ± 4.5) were encouraged to break diapause by increasing the surrounding temperature. Larvae were reared *ad libitum* on *P. lanceolata* (28:15 °C, 12:12 h, D:N) in family groups for 11 days, after which they were split between the exposure treatment with 1 μ l AcMNPV (1×10^3 OBs/mL, $n = 309$) or a control treatment (sterile distilled water, $n = 316$). Individual larvae were inoculated with AcMNPV as

described under experiment 1. Individuals were kept in single containers (200 mL) after exposure until pupation. Survival was checked daily and pupal mass was recorded on the day following pupation. Eclosing butterflies were sexed and frozen at -20 °C the following day to ensure full wing expansion and release of pupal waste products (meconium) for assessment of wing morphological traits.

2.4. Flight-related morphological traits, wing symmetry, and wing melanisation

Fore- and hind-wings of adults that eclosed from experiment 2 ($n_{\text{adults}} = 84$) were carefully removed. Digital images were taken of dorsal and ventral wing surfaces from all four wings under controlled light conditions (for details on the methodology see Breuker et al., 2010). For all measurements ImageJ software (National Institutes for Health, Bethesda, MD, USA) was used and the forewing surface area (cm^2) and forewing length (cm) were measured, with any damaged wings excluded from analyses. From these values, forewing loading (mg/cm^2) was calculated as adult wet mass at day 1 after eclosion (mg)/total forewing area (cm^2). A higher wing loading thus indicates an individual has smaller wings in relation to its body mass compared to individuals with low wing loading. Forewing aspect ratio was calculated as mean forewing length²/mean forewing area. A high aspect ratio indicates that a wing is more long and slender in shape, while a low value indicates that wings have a more shorter and stubby appearance. We further assessed the degree of basal melanisation of the left dorsal forewing which was quantified as the average grey value (0 = black; 255 = white) of the area of the distal wing cell (described in Talloen et al., 2004). Symmetry was assessed as the absolute value of the difference between right and left wing area (Palmer & Strobeck, 1986). Wing length as well as melanisation was measured twice to test for repeatability (forewing length $R^2 = 0.993$; melanisation $R^2 = 0.997$).

2.5. Statistical analysis

A linear mixed-model approach (R 3.1.2 for Windows; The R Project for Statistical Computing; lmer from package lme4; Bates et al., 2015) was used to analyse the effects of viral exposure on developmental life-history, flight-related morphological traits, wing symmetry and wing melanisation. In all models, backward model selection was used, starting with a full model including all meaningful second-order interactions and stepwise removing non-significant terms. Family was included as a random term in all analyses and R^2 values have been extracted from the final model. The difference between R^2 marginal and R^2 conditional was used to determine the percentage of variation explained by the random term family (Nakagawa & Schielzeth, 2013).

For experiment 1, the explanatory variable for viral concentration was \log_{10} transformed and included as a fixed factor in all analyses. We excluded data for larvae inoculated with a concentration of 1×10^9 OBs/mL, as viral mortality in this group led to a biased subsample of the original dataset, with only two females surviving the treatment. In experiment 2, viral treatment was used as a fixed factor, as only one viral concentration was used. In the analyses for larval survival until pupation and eclosion viral concentration was used as a fixed factor and larval mass at inoculation as a covariate. Larvae that survived and developed to the pupal stage could be sexed, and thus sex was also added as a fixed effect to the models for larval development time, larval daily mass acquisition, pupal mass and developmental time from pupation to eclosion as an adult. Larval survival to pupation and from pupation to eclosion as an adult and effects on sex ratio were analysed with a glmer model and binomial distribution. A posthoc test (Tukey's Honest Significant Difference test) was used to compare the effects within the viral concentrations used (experiment 1).

All flight-related morphological traits, wing symmetry, and wing melanisation rates were analysed using viral treatment and sex as fixed

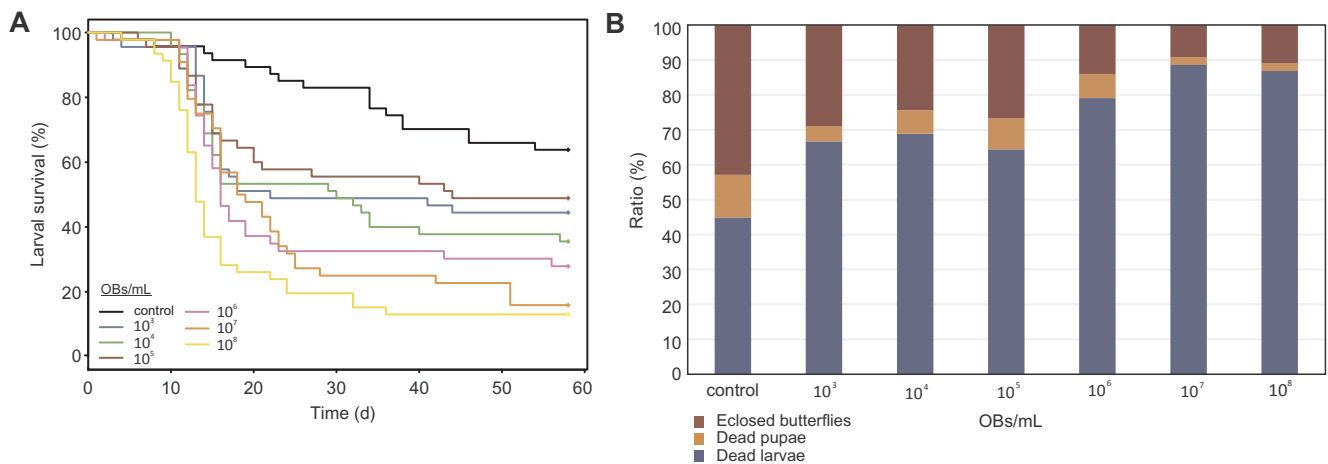


Fig. 1. Survival of larvae exposed to the different concentrations of AcMNPV from experiment 1. A) Larval survival until pupation over time (in days). B) Shown are the percentages for dead larvae (blue), dead pupae (orange) and survived individuals (i.e. eclosing butterflies; red) (sample size: control $n = 48$, 10^3 OBS/mL $n = 45$, 10^4 OBS/mL $n = 45$, 10^5 OBS/mL $n = 45$, 10^6 OBS/mL $n = 43$, 10^7 OBS/mL $n = 44$, 10^8 OBS/mL $n = 46$).

factors and initial larval mass at inoculation and total wet mass as covariates. Investment into flight muscles was assessed via thorax ratio (thorax mass/whole body mass).

3. Results

3.1. Experiment 1: effects of increasing concentration exposure to AcMNPV

Exposure to virus reduced larval survival to pupation ($F_{1,314} = 4.09$, $P < 0.0003$) and from pupation to eclosion as an adult ($F_{1,314} = 3.18$, $P < 0.004$, Fig. 1). Based on a post-hoc comparison survival was significantly reduced only in the three highest concentrations tested (survival percentage until pupation relative to control group: 10^6 OBS/mL 37.9%, 10^7 OBS/mL 20.7% and 10^8 OBS/mL 23.8%) and survival rates were highest in dose 10^5 OBS/mL. Larvae that were lighter at the time of inoculation were more susceptible to viral exposure as they were less likely to survive ($\chi^2_{1,202} = 8.99$, $P = 0.00271$). Larval developmental time was prolonged by exposure to the virus ($F_{1,89} = 8.01$, $P = 0.0004$, Fig. 2A) and females had longer development times than males ($F_{1,89} = 23.38$, $P > 0.00001$). A post-hoc comparison showed that larvae from doses 10^5 , 10^7 and 10^8 OBS/mL had significantly longer development times than larvae exposed to a dose of 10^3 OBS/mL

and only individuals from the group 10^7 OBS/mL had a longer developmental time compared to control individuals (see Fig. 2). Larval daily mass acquisition or pupal mass did not differ across viral doses ($\chi^2_{1,70} = 9.8$, $P = 0.134$ and $\chi^2_{1,70} = 3.24$, $P = 0.778$, respectively).

Thorax ratio was not affected by the exposure to any of the virus concentrations ($\chi^2_{1,78} = 8.29$, $P = 0.218$). However, while females had increasing thorax ratio with increasing mass at inoculation, this was opposite for males (sex * mass at inoculation: $\chi^2_{1,78} = 5.73$, $P = 0.017$). Family as a random term explained between 7 and 21% of the variation in the developmental and morphological traits (see Table S1). Detailed information for all measured traits including non-significant results can be found in the Supplementary material (see Table S1).

3.2. Experiment 2: response to virus exposure

3.2.1. Life-history and survival

Larval developmental time was not influenced by the viral treatment with a dose of 10^3 OBS/mL ($F_{1,228} = 0.87$, $P = 0.506$). Initially heavier individuals at the time of inoculation ($F_{1,228} = 32.04$, $P < 0.00001$) and males developed faster compared to females ($F_{1,228} = 42.6$, $P < 0.00001$). Viral treatment alone had no effect on daily mass acquisition rates ($\chi^2_{1,197} = 0.37$, $P = 0.543$). However,

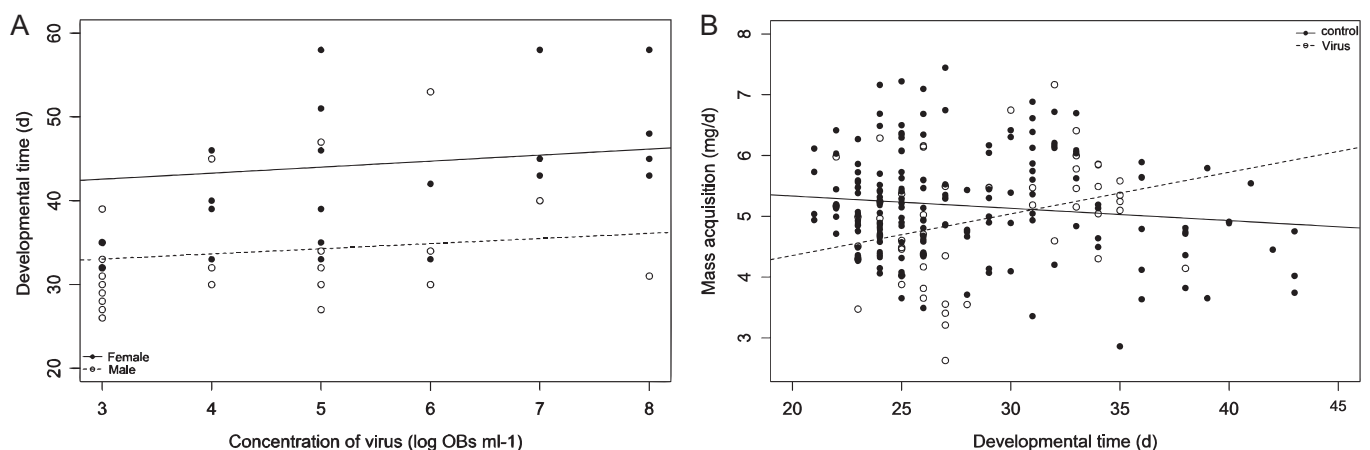


Fig. 2. A) Changes in larval developmental time until pupation (post-inoculation number of days) in experiment 1. Females are represented by a solid line and filled circles and males by a dashed line and open circles. Developmental time increased with increasing concentrations of the virus ($F_{1,89} = 8.01$, $P = 0.0004$) and females had longer developmental times than males ($F_{1,89} = 23.38$, $P > 0.00001$). B) Changes in mass acquisition in experiment 2. The control group is represented by a solid line and filled circles and the viral group by a dashed line and open circles. Virally exposed individuals had increased mass acquisition with increasing developmental time (interaction between treatment and developmental time on mass acquisition: $\chi^2_{1,197} = 5.54$, $P = 0.0018$).

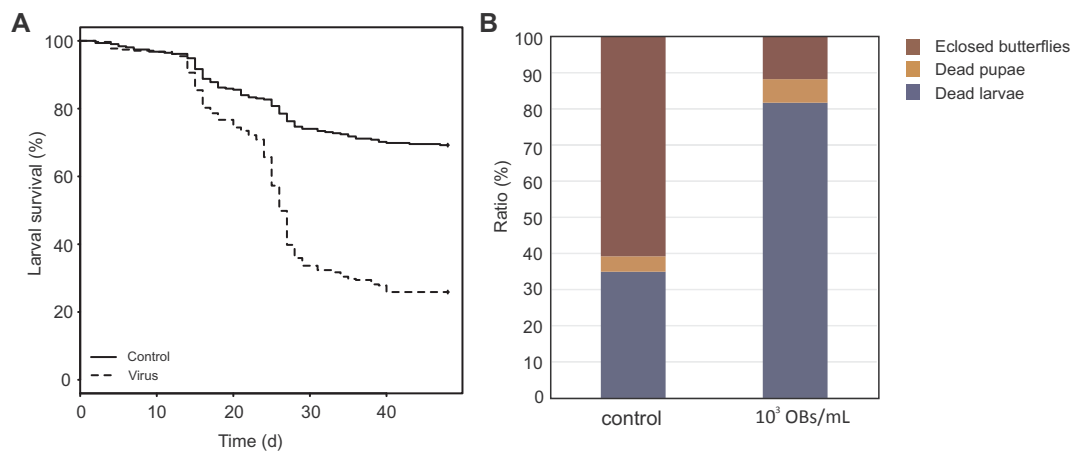


Fig. 3. Survival of larvae exposed to 10^3 OBs/mL of AcMNPV compared to control individuals from experiment 2. A) Larval survival until pupation over time (in days). B) Shown are the percentages for dead larvae (blue), dead pupae (orange) and survived individuals (i.e. eclosing butterflies; red) (sample size: control $n = 316$, 10^3 OBs/mL $n = 309$).

larvae had higher mass acquisition with increased developmental time when exposed to the virus (treatment*developmental time: $\chi^2_{1,197} = 5.54$, $P = 0.0018$; Fig. 2B). Females acquired more mass than males, especially when they were heavier at the time of inoculation (sex * initial mass before starvation: $\chi^2_{1,197} = 4.77$, $P = 0.029$). Females were heavier at pupation than males (mean males = 139.87 ± 2.08 , females = 180.88 ± 4.03 ; $\chi^2_{1,197} = 63.39$, $P < 0.00001$). Viral treatment alone had no effect on the pupal mass ($\chi^2_{1,197} = 0.1$, $P = 0.757$). However, larvae reached higher pupal mass with increased developmental time when exposed to the virus (treatment * developmental time: $\chi^2_{1,197} = 7.47$, $P = 0.0063$). Initial larval mass had no influence on the pupal mass ($\chi^2_{1,197} = 0.95$, $P = 0.331$). Viral exposure had no influence on the length of the pupal period ($\chi^2_{1,197} = 0.03$, $P = 0.861$) but initially heavier larvae had longer pupal stages ($\chi^2_{1,197} = 4.73$, $P = 0.0296$). There was no difference between females and males in the length of the pupal period ($\chi^2_{1,197} = 0.2$, $P = 0.654$) and no effect of viral treatment on the sex ratio of eclosing adults ($F_{1,197} = 0.2$, $P = 0.086$). Viral exposure did, however, reduce larval survival to pupation by 47% ($F_{1,617} = 90.77$, $P < 0.00001$; see Fig. 3) and survival from pupation to eclosion as an adult by 49% ($F_{1,617} = 106.12$, $P < 0.00001$) compared to control individuals. It is noteworthy, however, that the survival of the control individuals was also low in this experiment. In general, initially heavier larvae had significantly higher survival rates to pupation ($F_{1,617} = 30.19$, $P < 0.00001$) and to eclosion ($F_{1,617} = 24.73$, $P < 0.00001$). This was especially prominent in the control group, whereas the effect of initial body mass was less evident in the viral exposed group (treatment * initial larval mass: until pupation $F_{1,617} = 5.42$, $P = 0.0301$; until eclosion $F_{1,617} = 5.27$, $P = 0.0174$). Family as a random term explained between 0 and 9% of the variation in the traits (see Supplementary Table S2 for specific details).

3.2.2. Wing morphological traits, wing symmetry and wing melanisation

There was no direct effect of AcMNPV exposure on any of the measured wing morphological traits in this study; viral exposure had no influence on mean forewing length ($\chi^2_{1,74} = 0.63$, $P = 0.428$), total wing area ($\chi^2_{1,61} = 0.45$, $P = 0.503$), forewing aspect ratio ($\chi^2_{1,67} = 1.66$, $P = 0.198$), nor forewing loading (wet wing loading: $\chi^2_{1,61} = 0.68$, $P = 0.411$). Females had significantly longer forewings ($\chi^2_{1,74} = 15.97$, $P < 0.0001$), larger total wing area ($\chi^2_{1,61} = 13.73$, $P = 0.00021$) and higher forewing loading ($\chi^2_{1,61} = 17.71$, $P < 0.00001$) than males. There was no difference in forewing aspect ratio ($\chi^2_{1,67} = 0.02$, $P = 0.879$) or forewing symmetry ($\chi^2_{1,61} = 0.23$, $P = 0.63$) between the sexes. Heavier adults, in general, had larger wing area ($\chi^2_{1,61} = 27.14$, $P < 0.0001$), longer forewings

($\chi^2_{1,74} = 43.96$, $P < 0.00001$) and higher forewing aspect ratio ($\chi^2_{1,67} = 4.26$, $P = 0.039$). Heavier individuals also had less symmetrical wings ($\chi^2_{1,61} = 4.81$, $P = 0.0283$), but viral treatment had no influence on symmetry ($\chi^2_{1,61} = 0.57$, $P = 0.451$). Wing melanisation, on the other hand, was significantly higher in individuals exposed to the virus ($\chi^2_{1,78} = 7.89$, $P = 0.005$). Males had darker wings than females ($\chi^2_{1,78} = 4.71$, $P = 0.0299$). Initial larval mass had no effect on any of the wing measurements (see Supplementary Table S2). Family as a random term explained between 0 and 6% of the variation in these traits (see Table S2).

4. Discussion

4.1. Viral exposure and individual performance during development

Little is known about immune response in the Glanville fritillary butterfly, and the few studies that do exist have focused on responses to bacterial infection (Laurentz et al., 2012; Woestmann et al., 2017). Here, for the first time, we exposed Glanville fritillary larvae to a baculovirus under laboratory conditions. We show that they are susceptible to exposure to the baculovirus AcMNPV, which reduces survival during both larval and pupal stages. Based on visual observations, no viral mortality was observed in the control (unexposed) group, in contrast to the viral treatment groups where baculovirus mortality in larvae was observed with typical symptoms of infection noted such as liquefaction of larval cadavers. The experiment was not designed to test for behavioural changes due to viral infection, thus we do not know if the fact that many viral exposed larvae climbed up the cups prior death is demonstrating an adaptive response of the host or the parasite. There was also a baculovirus concentration-dependent effect on mortality, with lower larval survival at higher concentrations (experiment 1). The mortality levels in the control group in this experiment were, however, higher than general. This suggests that there may have been another source of mortality during our experiment, in addition to the observed viral mortality. We used traditional morphological diagnosis of baculovirus induced mortality but did not perform additional tests to confirm the presence of baculovirus OBs, or check for the presence of another virus or pathogen. Thus, we cannot completely rule out that other infections could have caused additional mortality in our experiment. Nevertheless, while control mortality became problematic later during the assay, mortality in viral infected groups was evident in the first 15 days post-inoculation (~5% for control in comparison to ~63% for treated larvae). The bioassay in general is rather long due to the long developmental period of this butterfly species, and future studies might benefit from making adjustments to account for this. Future studies

could also test for potential covert viruses that may be triggered in treated larvae (Williams et al., 2017). Notably, the mortality rates also differed between the two experiments. Due to the constraint in number of larvae that we are able to collect from the wild, we did not have the possibility of performing replicates to investigate the potential mechanisms underlying such differences. From an ecological perspective, such mortality differences are interesting, and may reflect differences in individual condition, for example due to length of diapause or subtle changes in unmeasured aspects of environmental conditions (e.g. here the experiments were conducted five weeks apart). This also highlights how using non-model organisms from wild populations add complexity, yet may still be more realistic test organisms for such bioassays. In general, family explained 1–12% of the variation in survival rate in both experiments, suggesting that susceptibility to baculovirus infection may have a moderate genetic basis.

Resistance to pathogens often depends on resource availability (Lee et al., 2006), as resource availability improves body condition as well as immune system deployment of organisms (Lee, 2002). Previous studies have shown that negative effects of infection are often weaker in older or larger animals (e.g. Grove and Hoover, 2007; Gupta et al., 2007; Gibbs et al., 2010; Tseng and Myers, 2014; Gibbs and Weir, 2017). Here we show that even though all larvae were inoculated the same number of days after the diapause was broken, those larvae that had heavier body masses at the time of inoculation with AcMNPV developed faster during the larval stage, had longer pupal stages and higher survival to pupation. These data suggest that larvae with heavier masses may have increased resistance and are able to buffer some of the costs associated with responding to AcMNPV exposure. Nevertheless, it is generally interesting that larvae could be successfully infected during a rather late life stage, as individuals at this stage often have a well-developed peritrophic membrane that usually prevents virus infection (McNeil et al., 2010). Initial variation in body size stems from genetic differences, conditions experienced during the pre-diapause stage and/or maternal effects (e.g. Saastamoinen et al., 2013a). It is known that families differ in body mass and that body mass is a heritable trait in this species (Kvist et al., 2013). Previous research in the Glanville fritillary butterfly has additionally shown that females change their resource allocation patterns during reproduction in response to various stressors such as food-deprivation or bacterial infection: under stress, females increase investment to offspring quality (i.e. size) rather than quantity (Woestmann & Saastamoinen, unpublished data). Our finding that heavier larvae have increased resistance to baculovirus infection, indicates that maternal resource allocation towards heavier offspring would be beneficial under conditions when offspring themselves are challenged with pathogens. Further studies are needed, however, to distinguish whether the positive effect of body mass on susceptibility to a baculovirus results from more general developmental resistance of larger and hence better-quality offspring or from more specific increased resource allocation to immunity.

In accordance with the findings of a previous study in a Speckled Wood butterfly (Hesketh et al., 2012) we observed an increase in larval development time with increasing viral concentration. In general, shorter developmental times have been suggested to offer less time for repair of damage caused by infection and can result in higher fitness costs for an individual (Johnson et al., 2012). Increased developmental time is associated with poor growth conditions in the Glanville fritillary, for example when larvae experience food-restriction during development (Saastamoinen et al., 2013b). Notably, however, low concentration of viral exposure had no detectable effects on larval developmental time or pupal mass in the second experiment, even though it still induced increased mortality. In general, studies of the effects of baculovirus infections on larval developmental time show inconsistencies with either no, little or significant effects when infecting early instars (Milks et al., 1998; Matthews et al., 2002; Monobryllah & Shankar, 2008; Gibbs & Weir, 2017). In this study, larvae also reached heavier pupal mass when developing for a longer time, especially after

being exposed to AcMNPV. In general, higher mass acquisition rates were apparent with increased developmental time. This may indicate that virus exposed individuals, via increased developmental time, are compensating and consequently reach comparable pupal mass to control (unexposed) individuals. However, as initially smaller larvae were more likely to die due to the viral exposure, only the most robust individuals survive. This suggests that the larger body size and reduced variation in it, is in fact due to phenotype-dependent survival against viral exposure. In general, males and females are expected to vary in their investment to immunity due to difference in life-histories (Rolff, 2002), and as such we expected sex-specific differences in response to exposure to AcMNPV in the Glanville fritillary. However, we found no differences in response to the viral exposure between the sexes (and for a similar result for *P. aegeria* see Gibbs & Weir, 2017, but see Hesketh et al., 2012). This is somewhat surprising and suggests that although males and females have different growth patterns and also differ, for example, in adult lifespan (Boggs & Nieminen, 2004), this does not necessarily result in sex-dependent responses to viral infection (but see Páez et al., 2015). In the Glanville fritillary butterfly, no strong sex-dependent responses in terms of immunity are known so far: some immune genes respond differently for the two sexes upon bacterial exposure, however, there is so far only an indication that females perform better when being infected (Woestmann & Saastamoinen, unpublished results).

4.2. Flight-related morphological traits, wing symmetry and wing melanisation

Compared to males, females had longer forewings and higher total wing area yet lower wing area relative to their body mass (i.e. higher wing loading). These sex-specific differences are in accordance with a previous study on the Glanville fritillary (Breuker et al., 2007), and are expected due to differences in their life-history and morphology, with females being larger than males (Kuussaari et al., 1996; Hanski et al., 2002). We observed no direct effect of viral exposure on any of the flight morphological traits examined in this study, consistently with a previous study on the Speckled Wood butterfly (Hesketh et al., 2012). Wing morphological traits or allocation to thorax mass may not, however, be the best proxies of flight capability and dispersal (Stevens et al., 2012), and traits, such as flight metabolic rate, known to be a good proxy of dispersal in the field and that is significantly reduced by food limitation during developmental stages in the Glanville fritillary butterfly (Saastamoinen & Rantala, 2013) should be tested in the future. In any case, the fact that we found no differences in wing size fluctuating asymmetry in response to viral exposure, potentially suggests the high developmental stability, even when experiencing infections during the larval developmental stage. Interestingly, we did find that heavier and thus larger individuals had less symmetric wings, suggesting that faster development may incur some costs during the development of wing discs. We cannot rule out the possibility that more subtle measures of fluctuating asymmetry (e.g. geometric morphometrics) or examination of traits like wing shape could have detected more subtle differences in wing asymmetry across treatment groups (e.g. Breuker et al., 2007). Generally insects are, however, very capable of compensating for directional asymmetry and often even possess asymmetric wing shape or length which may potentially be even adaptive (Goulson et al., 1999; Windig & Nylin, 1999).

By contrast to lack of changes in the wing morphology due to the viral infection, the melanisation of the basal portion of wings, an area known to be important for thermoregulation in dorsally basking butterfly species (Wasserthal, 1975), was significantly increased in response to viral exposure in general. Such a result is generally assumed to be due to a trade-off between melanin-based immune response and dark melanised wing or cuticular patterning (Talloe et al., 2004; Kangassalo et al., 2016). Consistently, with our results, previous studies in the large white butterfly (*Pieris brassicae*) and the yellow mealworm

beetle (*Tenebrio molitor*) have similarly shown that increased melanisation in the wings can occur in response to an immune challenge (Barnes & Siva-Jothy, 2000; Freitak et al., 2005). Melanin production in animals has been thought to be beneficial for warning coloration patterns, camouflage, sexual display, disease resistance and thermal regulation (reviewed in True, 2003). Thermal melanism is especially relevant in ectotherms, such as butterflies, that depend on the environmental temperature and climatic conditions, particularly for those temperate regions. An increase in melanisation of the wings due to viral exposure therefore could result from an adaptive response by which infected individual's improve thermal regulation. A potential faster absorbance of heat due to increased melanisation patterns could positively improve the capacity to escape unfavourable habitats. Additionally, an increase in temperature has been shown to positively affect immune response and survival in response to infection in e.g. the cricket (*Gryllus texensis*) and the greater wax moth (*Galleria mellonella*) (Wojda et al., 2009; Adamo & Lovett, 2011). Alternatively, an increase in wing melanisation could simply be a carry-over effect to the adult stage from viral exposure during larval development. Hence, it would represent a side-effect from the viral exposure and not an adaptive response for the individual. Thus its significance and potential advantageous or disadvantageous effects on individual fitness should be tested under field conditions.

4.3. Conclusions

This study is the first to show effects of Baculovirus exposure in the Glanville fritillary and provides interesting new data on effects on adult traits, which have also not been widely investigated in other insect systems. Our results show that there was a cost of resisting baculovirus infection within the larval stage resulting in decreased survival to both pupation and eclosion as an adult, and prolonged development time at higher viral concentrations. Exposure to lower concentrations of AcMNPV did not result in observable negative effects on the developmental life-history or the flight-related morphological traits measured in this study. We found that viral exposure affected wing development; increased melanisation of the basal part forewings with viral exposure, which may have a positive effect on thermoregulation by allowing individuals to absorb heat faster and thus influence flight ability. Such response might be beneficial and allow individuals to escape the pathogen reservoirs within a habitat. However, further studies are needed to fully demonstrate whether the responses are adaptive in nature. In general, infections can be pervasive with no obvious effects on a host. We assessed effects under optimal conditions, in terms of temperature and food availability. In general, optimal conditions can suppress some costs that would be evident under more constrained conditions and previous studies have shown that dietary composition can alter immune function in insects (Simpson et al., 2015). The Glanville fritillary larvae live gregariously in family groups, and are likely to be affected by vertical and horizontal transmission of viruses in nature (but see Wilson et al., 2003). While horizontal transmission occurs between infected and naïve con-specific larvae, in vertical transmission adult butterflies potentially move viruses to their offspring via transmitting it *transovarially* or via faeces to uninfected habitats. Vertical transmission is still less-well studied (but see e.g. Myers et al., 2000) but could potentially have a huge impact on population dynamics. Further studies are needed to investigate whether viral infections effect fecundity as well as the potential vertical transmission of viruses in the Glanville fritillary butterfly.

Ethics statement

The Glanville fritillary butterfly is not classified as an endangered or protected species in the Åland islands and no permits are required for the collection of individuals in the Åland islands.

Data accessibility statement

The datasets supporting this article and required to replicate the analyses will be deposited on Dryad.

Competing interest statement

The authors declare that they have no competing interests.

Author's contribution statement

LW, MG, HH & MS designed the study, LW & MG performed the experiment, LW analysed the data, LW, MG, HH & MS wrote the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jinsphys.2018.03.009>.

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