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Exploring sub-lethal effects of exposure to a nucleopolyhedrovirus in the Speckled Wood (*Pararge aegeria*) butterfly

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Abstract

This study investigated the sub-lethal effects of larval exposure to baculovirus on host life history and wing morphological traits using a model system, the speckled wood butterfly *Pararge aegeria* (L.) and the virus *Autographa californica* nucleopolyhedrovirus. Males and females showed similar responses to the viral infection. Infection significantly reduced larval growth rate, whilst an increase in development time allowed the critical mass for pupation to be attained. There was no direct effect of viral infection on the wing morphological traits examined. There was, however, an indirect effect of resisting infection; larvae that took longer to develop had reduced resource investment in adult flight muscle mass.

Keywords: *Pararge aegeria;* baculovirus; sub-lethal; wing morphology; development time

1. Introduction

The cost to an insect host of surviving a sub-lethal pathogen infection may be measured through changes in different fitness traits (Zuk and Stoehr, 2002). This has implications for designing effective biological control strategies and for understanding the role of pathogens in regulating natural populations of insects (Hesketh et al., 2010; Roy et al., 2009). Therefore, increasing attention is being focused on the contribution of immune defence to adult fitness in insect systems (Schmid-Hempel, 2005). We have investigated the consequences of overcoming a viral infection on life history and wing morphological traits in the speckled wood butterfly *Pararge aegeria* (L.). This species has been used extensively as a model system for studies of insect ecology and life history evolution (e.g. Van Dyck and Wiklund, 2002). Although, sub-lethal effects of baculovirus infection on life history traits in Lepidoptera have been well recorded (e.g. Goulson and Cory, 1995; Sood et al., 2010; Sporleder et al., 2007), effects on wing development and morphology are less well considered; changes related to baculovirus infection have been crudely quantified through measurements of wing deformities (e.g. Milks, 1997; Vail and Hall, 1969). Any potential change to wing formation that could affect flight and/or dispersal ability will be particularly important to recognize in species such as *P. aegeria* that have experienced shifts in distribution and dispersal in response to climate change and habitat fragmentation over the last few decades (e.g. Hill et al., 1999; Gibbs & Van Dyck, 2010).

We specifically selected flight morphology traits commonly used in butterfly studies and known to be correlated to flight performance in *P. aegeria* (Berwaerts et al., 1998; Hill et al., 1999). We hypothesized that a concentration-dependent response against infection in the larval stage with *Autographa californica* multiple capsid nucleopolyhedrovirus (*Ac*MNPV) would reduce investment in morphological traits associated with flight. To test this hypothesis, we examined changes in *P. aegeria* larval, pupal and adult development traits as well as sex differences in response to sub-lethal infection with baculovirus.

2. Materials and Methods

2.1. Bioassay

A stock of *Ac*MNPV was obtained as described in Gibbs et al. (2010a) and the concentration of occlusion bodies was estimated by counting 3 times in an improved Neubauer haemocytomter at magnification x400 (<10% error in counts). Larvae starved overnight were inoculated individually in Petri dishes (5cm diameter) containing a piece of damp filter paper and 5 x 1cm pieces of *Poa trivialis* (L.) leaf with 1 µl of viral inoculum (log concentration of virus between $1x10^3$ and $1x10^9$ occlusion bodies ml^{-1}) or sterile distilled water added. Thirty (bioassay 1) or 25 (bioassay 2) larvae were inoculated overnight. The following day, larvae were transferred individually to bagged potted plants of *P. trivialis* where they were maintained in controlled conditions (18°C; photoperiod 16:8 light:dark hours). Mortality was monitored daily and suspected viral deaths collected and frozen at -20°C. Larvae that died of baculovirus infection were opaque and flaccid but remained intact. The presence of OB's was confirmed by staining with Giemsa solution. Pupae were weighed and placed in individual plastic tubs on a piece of filter paper until eclosion. Larval development times to pupation and time to adult eclosion were recorded. Adults were sexed, and fresh total body weight was recorded after wing expansion and meconium (pupal waste products) had been released. Adults were subsequently frozen at -20°C.

2.2. Morphological measurements

Adult fore- and hindwings were removed and digital images were taken of the dorsal wing surface under controlled light conditions (detailed methodology; Breuker et al., 2010). Using these digital images forewing surface area (cm^2) and forewing length (cm) were measured using the image analysing software ImageJ (Abramoff et al., 2004; (http://rsb.info.nih.gov/ij/)). Forewing loading (mg/cm^2) was calculated as; adult wet mass at eclosion (mg)/total forewing area (cm^2) and forewing aspect ratio was calculated as; mean forewing $length^2$/mean forewing area. Damaged wings were excluded from analyses. The degree of basal melanisation of each dorsal forewing was measured using ImageJ and quantified as the average grey-value (scaled from 0, i.e. black, to 255, i.e. white) of the area of the distal wing cell (after Talloen et al.,

2004). After wing removal, adults were dried to constant mass and weighed (total adult body mass and thorax mass, after Hughes et al., 2003).

2.3. Statistical analysis

The explanatory variable for virus exposure in all analyses was viral concentration $\log_{10}$ transformed. Data for larvae inoculated at concentrations of $1x10^8$ and $1x10^9$ OB's ml^{-1} were excluded as viral mortality in these treatments meant that the resulting dataset of survivors was a biased subsample of the original dataset. Where necessary to meet model assumptions, data were transformed prior to analysis; inverse square root dry forewing loading, $\log_{10}$ wet forewing loading and $\log_{10}$ basal wing melanin. In all analyses, data were blocked by bioassay occasion and analysed using Generalised Linear Modeling. To take account of allometry effects, total dry mass was included as a covariate when analysing investment in flight (wing area and thorax mass). In each analysis, a full model with all interaction terms was fitted and then simplified by sequentially removing terms with high, non-significant, p-values.

3. Results and discussion

Pararge aegeria was susceptible to infection with *Ac*MNPV at the two highest viral concentrations (see Bishop et al., (1995) for comparative susceptibility of other Lepidoptera to *Ac*MNPV). Mean viral mortality was greater at $1x10^9$ OB's ml^{-1} (46.3%) compared to $1x10^8$ OB's ml^{-1} (21.8%) and larvae died significantly more quickly at the higher concentration (days to death post-inoculation; $F_{1,34}=4.53$, $p=0.041$). There was no viral mortality in control insects.

Generally, adult females had significantly longer larval development times than males ($F_{1,219}=60.33$, $p<0.001$; Fig. 1a; non-significant interaction between sex and log concentration $F_{4,215}=1.07$, $p=0.373$). In line with other studies of sub-lethal baculovirus effects on Lepidoptera, an increase in *P. aegeria* larval development time was positively related to baculovirus concentration ($F_{4,219}=3.21$, $p=0.014$; Fig. 1a; e.g. Monobrullah & Shankar, 2008; Goulson & Cory, 1995; Lee et al., 2006). There was also a significant reduction in larval

mass acquisition per day in those larvae that were exposed to higher concentrations of virus ($F_{4,217}$=3.14, p=0.016; Fig. 1b; non-significant effect of sex $F_{1,217}$=2.94, p=0.088). Taken together, these results suggest that rather than increase their daily resource intake, *P. aegeria* larvae compensate for reallocation of resources from growth to resisting viral infection by feeding over longer time periods. Longer larval development in *P. aegeria* is often associated with sub-optimal growth conditions and periods of larval stress (e.g. Talloen et al., 2004; Gibbs et al., 2004, 2010b) so it is possible that compensatory growth is a typical response in *P. aegeria* to resource stress, although further work would be required to substantiate this specifically in relation to viral infection.

Contrary to other studies (e.g. Goulson & Cory, 1997), costs incurred in the larval stage did not affect pupal development and morphology. Viral concentration had no effect on final pupal mass ($F_{4,225}$=0.26, p=0.904; females heavier than males $F_{1,225}$=80.31, p<0.001) or duration of the pupal stage ($F_{4,219}$=1.04, p=0.389; no significant relationship with sex $F_{1,219}$=0.27; p=0.602). Costs could possibly be due to increased deployment of physiological processes such as haemocyte encapsulation of viral infected tracheal tissues (Trudeau et al., 2001) and apoptosis of infected mid gut epithelial cells (McNeil et al., 2010). However, in this study we did not identify the mechanisms involved and equally, midgut sloughing or damage may have caused changes in development as opposed to a change in allocation to immune defence.

There was no direct effect of baculovirus infection on any of the flight morphological traits that we examined. There was no relationship between viral concentration and adult butterfly mean forewing length ($F_{4,208}$=0.68, p=0.606), mean forewing surface area ($F_{4,208}$=0.41, p=0.804), forewing aspect ratio ($F_{4,209}$=1.27, p=0.282) or forewing loading (dry wing loading $F_{4,202}$=0.26, p=0.905; wet wing loading $F_{4,203}$=0.13, p=0.973). Females had significantly longer forewings ($F_{1,208}$=46.98, p<0.001), larger forewing surface area ($F_{1,205}$=5.43, p=0.021), heavier body masses ($F_{1,227}$=115.07, p<0.001) and higher forewing loading (dry forewing loading $F_{1,202}$=416.84, p<0.001; wet

forewing loading $F_{1,203}$=89.88, p<0.001) than males. *Pararge aegeria* are sexually dimorphic in their mass and wing size and this accounts for the significant differences we observed between the sexes (Van Dyck & Wiklund, 2002). Females also had paler forewings than males ($F_{1,208}$=52.37, p<0.001) but there was no effect of viral exposure on forewing melanin in either sex ($F_{4,208}$=0.16, p=0.958). It is possible that baculovirus infection in *P. aegeria* has a more subtle effect on wing development, which we were unable to detect in the current study (e.g. Breuker et al., 2007). Interestingly, other butterfly/pathogen studies that have directly demonstrated reduced flight ability in pathogen-infected adults were unable to correlate this reduction to changes in wing morphological traits (Bradley & Altizer, 2005).

The thorax is comprised predominantly of flight muscle (Marden, 1987) and therefore dry thorax masses can be used as a measure of investment in flight muscle mass (Srygley & Chai, 1990). Females had significantly larger thorax masses than males ($F_{1,201}$=86.49, p<0.001) but there was no relationship between thorax mass and log concentration of virus ($F_{4,201}$=1.42, p=0.229). However, there was a correlation for both sexes between larval development time and adult thorax mass; larvae that had long developmental periods became adults with reduced thorax mass ($F_{1,200}$=10.17, p=0.002; Fig. 2). It has been suggested that flight ability in butterflies is improved in individuals with a higher relative thorax mass (Berwaerts et al., 2002; Thomas et al., 1998). Our observations therefore indicate that there is potential for baculovirus infection to indirectly reduce *P. aegeria* flight ability via its effects on thorax mass, but further experiments would be needed to substantiate this. Larvae infected with virus grew for longer to obtain the same overall body mass, but had reduced investment in thorax mass. This indicates that infected individuals allocated relatively more resources to their abdomen which could potentially increase reproductive output. Although it is unknown whether baculovirus infection directly influences reproduction in *P. aegeria*, studies in other Lepidoptera have demonstrated that viral infection reduces reproductive output (Sait et al., 1994).

In conclusion, there was no direct effect of sub-lethal baculovirus infection on *P. aegeria* wing morphology, but larval development was prolonged. There was, however, an indirect effect of resisting infection; larvae that took longer to develop had reduced resource investment in adult flight muscle mass. Further work is required to ascertain whether these changes in flight muscle mass will result in functional changes in *P. aegeria* flight ability.

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