

Immunocompetence increases with larval body size in a phytophagous moth

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Abstract. Despite the obvious benefit of an immune system, its efficacy against pathogens and parasites may show great variation among individuals, populations and species. Understanding the causes of this variation is becoming a central theme in ecology. Many biotic and abiotic factors are known to influence immunocompetence (temperature, age, etc.). However, for a given age, size among individuals varies, probably as a result of accumulated resources. Thus, these variable resources could be allocated to immune defence and, consequently, body size may explain part of the variation in immune responsiveness. However, the influence of body size on immune defence is often overlooked. The present study investigates variations in haemocyte count and phenoloxidase activity in larvae of the phytophagous vine moth *Eupoecilia ambiguella* Hübner of the same age, although differing in body size. The measurements of immune function are made both when the insects are immunologically naïve and 24 h after a bacterial immune challenge. The base levels of these immune parameters do not covary with body size in naïve larvae. After the bacterial immune challenge, more haemocytes and phenoloxidase enzyme are mobilized, and the mobilization of these immune effectors is correlated positively with individual body size. Thus, larger larvae exhibit higher immunocompetence than smaller ones, suggesting that smaller larvae might be more vulnerable to infection. These results suggest that body size is probably an underestimated variable, which nevertheless modulates the insect immune system and should thus be considered as a covariate in insect immune system measurement. It is recommended therefore, that body size should be taken into account in ecological immunity studies with insects.

Key words. *Eupoecilia ambiguella*, haemocyte count, immune-challenged larvae, larval body size, larval immune defence, phenoloxidase enzyme cascade.

Introduction

Fungal pathogens, bacteria, nematodes and parasitoids are the greatest biotic causes of mortality in insects (Hawkins *et al.*, 1997). However, the success of insects with respect to avoiding and surviving infections is in part attributable to their

immune system (Zuk & Stoehr, 2002). To combat infection, insects rely on immune effector systems, including haemocytes and enzymes of the phenoloxidase (PO) cascade that are the frontline of physiological defence against a large range of pathogenic insults. The immune responses provide rapid clearance of more than 99% of the bacterial pathogens that enter the haemocoel (Haine *et al.*, 2008) and may even deal with large pathogens such as nematodes and parasitoid eggs by forming melanotic capsules around them (Blumberg & Luck, 1990; Strand & Pech, 1995; Blumberg, 1997; Kraaijeveld *et al.*, 2001; Smilanich *et al.*, 2009). Melanotic capsules result

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from haemocytes smothering the invader and the activity of the PO in melanizing the resultant cell mass (Cerenius & Söderhäll, 2004; Siva-Jothy *et al.*, 2005). The enzyme is stored in the haemolymph and haemocytes as an inactive proenzyme, prophenoloxidase (PPO), which is rapidly activated upon infection. Although the amount of naturally inactive and active enzymes in the haemolymph (PO plus PPO) is a measure of the maintenance of the PO cascade, the amount of 'naturally activated' PO gives an estimate of its use. The activation of the cascade is often accompanied by the release of reactive oxygen species that have a cytotoxic oxidative effect on pathogens (Carton *et al.*, 2008). All of these processes generally lead to the death of the pathogen.

Despite the obvious benefit of having an efficient immune system, large individual differences exist with respect to immune ability (Sadd & Schmid-Hempel, 2009). Such variation is often linked negatively to other life-history traits (Gwynn *et al.*, 2005), hence influencing individual fitness, and potentially affecting population dynamics (Blumberg, 1997; Schmid-Hempel, 2005; González-Santoyo & Córdoba-Aguilar, 2012). Therefore, a deeper understanding of the immune response and the causes of variation in immunocompetence is a central theme in ecology (Rolff & Siva-Jothy, 2003; Schmid-Hempel, 2005; Schulenburg *et al.*, 2009). Many biotic and abiotic variables that affect insect immune responses are reported. For example, temperature (Lynn & Vinson, 1977; Blumberg, 1997; Linder *et al.*, 2008), host plant quality, in the case of phytophagous insects (Klemola *et al.*, 2007; Smilanich *et al.*, 2009; Vogelweith *et al.*, 2011), sex (Zuk & Stoehr, 2002), and physiological conditions (González-Santoyo & Córdoba-Aguilar, 2012) affect immune responses in many groups of insects. Immune response may vary during the life of an individual at different developmental stages, as well as with age (Blumberg, 1997; Ryder & Siva-Jothy, 2001; Grove & Hoover, 2007; Eleftherianos *et al.*, 2008; McNeil *et al.*, 2010). Furthermore, accumulated resources may also be of importance in determining the potential strength of an immune response developed by an individual upon challenge. Indeed, maintenance and use of the immune system imposes resource-based trade-offs (Rolff & Siva-Jothy, 2003), high resources (e.g. lipid reserves) and this may provide more energy to mount a better immune response against pathogens (Cheon *et al.*, 2006). Insect larval body size reflects accurately body condition in terms of protein, lipid and carbohydrate content (Timmermann & Briegel, 1999; Glazier, 2005). Although insect larvae vary in body size between different developmental stages, they also vary in size within the same developmental stage or age, probably because of variable ability to compete for resources.

Thus, for a given age, larger larvae are expected to have accumulated more resources (e.g. by eating more) and should therefore be able to allocate more resources to immune defences than smaller larvae. Unfortunately, in many studies investigating the immune ability of insect larvae, larval body size is often not measured or reported (for exceptions, see Lee *et al.*, 2008; Bukovinszky *et al.*, 2009; Shikano *et al.*, 2010; Vogelweith *et al.*, 2011). Examination of the effects of larval body size on immunocompetence could improve the general understanding of the key factors that drive variation

of immunocompetence in natural populations. Furthermore, it may also be useful to determine the extent to which body size should be taken into account for the design of experiments, as well as for statistical analysis in ecological immunity studies.

To test the effect of larval body size on immunocompetence, larvae of the European Grape Berry Moth larvae *Eupoecilia ambiguella* Hübner (Lepidoptera, Tortricidae) are studied at the same stage and age. Similar to many other highly immobile fruit-inhabiting larvae, immune defence for *E. ambiguella* probably represents an important selective advantage against parasitoid attacks, as opposed to escape behaviour. In the present study, the effect of larval body size on the haemocyte count and on the maintenance and use of the PO enzyme cascade in the haemolymph of immunologically naïve larvae is investigated. Moreover, the strength of the immune response to bacterial infection is measured, and the potential relationship between immune response and larval body size is examined.

Materials and methods

Insects

Insects used were obtained from an inbred stock of *E. ambiguella* reared at the Institut National de la Recherche Agronomique, Bordeaux, Aquitaine, France, for several years. This culture is based on a great number of caged adults (several thousand per week) to which wild adults are periodically added. This laboratory strain has conserved genetic variability because considerable variation is found in immune parameters between larvae (Vogelweith *et al.*, 2011). In addition, in preliminary experiments, a very similar pattern in terms of basal immunity level and parasitoid escape behaviour has been found between inbred stock and wild lines sampled in French vineyards (F. Vogelweith, unpublished observations). Therefore, the results obtained with this laboratory strain could be extrapolated to field populations. Larvae were maintained in boxes (18 × 11.5 × 7 cm) on a semi-artificial diet, as described by Thiéry & Moreau (2005), with the exception of an LD 16 : 8 h photoperiod at 22 ± 1 °C and 70 ± 10% relative humidity, with a density of 100 individuals per 300 mL of diet.

Experimental protocol

Larvae of the same larval stadium (fifth stadium = L5 instar larvae) and age (27 days post-hatching) were collected from the stock culture. After being chilled for 10 min on ice, a 1-µL sample of haemolymph was collected and flushed into a microcentrifuge tube containing 20 µL of sodium cacodylate, CaCl₂ buffer (0.01 M sodium cacodylate; 0.005 M CaCl₂; pH 6.5) to measure levels of immune defence in naïve larvae. Larvae were then immediately wounded in the posterior part of the ventral side of the abdomen with a sterile needle that had been dipped either into sterile Ringer's solution or into a concentrated suspension of heat-killed *Arthrobacter globiformis* (approximately 10⁹ cells per mL⁻¹). Larvae were kept individually in microcentrifuge tubes containing diet, under standard

conditions (LD 16 : 8 h, $22 \pm 1^\circ\text{C}$, $70 \pm 10\%$ relative humidity) for 24 h. A second sample of haemolymph was collected as described above to measure levels of immune defence after wounding (control and bacterially challenged). The samples of haemolymph for the haemocyte count were measured immediately. The haemolymph samples used to measure the levels of maintenance and use of the PO enzyme cascade were stored immediately at -27°C to await assay. Larval body size was estimated by measuring the distance between the most distant lateral sides of the head capsule margins (Delbac *et al.*, 2010), using a SMZ-10A stereoscopic microscope (Nikon, Japan) and a VTO 232 video analysis system (Linkam Scientific Instruments, U.K.). This indicator of larval body size is commonly used in Lepidoptera and allows differentiation between larval instars in *Lobesia botrana* (Godin *et al.*, 2002; Panzavolta, 2007; Delbac *et al.*, 2010). In the model in the present study, body length and weight measurements were not used because sizing and weighing living larvae is extremely difficult, especially in the case of old larvae, which move vigorously and are thus difficult to handle. Indeed, measurement of head capsule width of dead larvae is the most reliable measurement of size in *L. botrana*.

As a result of the small size of the larvae and the fact that two haemolymph samples were collected within 24 h, it was not feasible to collect a sufficient volume of haemolymph to measure both haemocyte count and PO enzyme cascade activity in the same individual. Consequently, two distinct experiments were undertaken to assess these two key immune parameters. In total, 30 larvae were used to measure the haemocyte count (16 larvae injected with a suspension of *A. globiformis* and 14 controls) and 21 larvae were used to measure PO enzyme activity (10 larvae injected with a suspension of *A. globiformis* and 11 controls).

Immune parameters

The haemocyte count was measured using a Neubauer improved haemocytometer under a microscope (magnification $\times 400$). The activity of naturally activated PO enzyme alone (hereafter PO activity) and the activity of the proenzyme (PPO) combined with that of the PO (hereafter total-PO activity) were both measured using a spectrophotometer in accordance with the method described by Cornet *et al.* (2009). PO activity was quantified without further activation, whereas total-PO activity required activation of the PPO with chymotrypsin to produce active PO. Accordingly, frozen haemolymph samples were thawed on ice and centrifuged (4000g for 15 min at 4°C). Five microlitres of supernatant was added to a microplate well, containing $20\ \mu\text{L}$ of phosphate-buffered saline (8.74 g of NaCl, 1.78 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1 L of distilled water, pH 6.5) and either $140\ \mu\text{L}$ of distilled water to measure PO activity alone, or $140\ \mu\text{L}$ of chymotrypsin solution (C-7762; Sigma, St Louis, Missouri; $0.07\ \text{mg mL}^{-1}$ of distilled water) to measure total-PO activity. Then $20\ \mu\text{L}$ of L-3,4-dihydroxyphenylalanine solution (D-9628; Sigma; $4\ \text{mg mL}^{-1}$ of distilled water) was added to each well. The reaction was allowed to proceed at 30°C in a microplate reader (Versamax, Molecular Devices

LLC, Sunnyvale, California) for 40 min. Readings were taken at 490 nm every 15 s and analyzed using SOFT-MAX PRO, version 4.0 (Molecular Devices LLC). Enzyme activity was measured as the slope (V_{max} : change in absorbance unit min^{-1}) of the reaction curve during the linear phase of the reaction and recalculated as the activity of $1\ \mu\text{L}$ of pure haemolymph.

Statistical analysis

Relationships between immune parameters and the body size of naïve larvae were examined using Pearson's correlation tests with a 95% confidence interval (CI) on natural-log transformations. When the 95% CI included 0, the correlation was not significant. Changes in the haemocyte count and in PO and total-PO activity after a bacterial immune challenge were analyzed using repeated measures analysis of variance (ANOVA) with the immune challenge as factor and larval body size as covariate. The assumption for parametric tests was ensured by the natural-log transformations of immune parameters. The results of the repeated ANOVA were confirmed using Pearson's correlation tests between change of immune effectors and larval body size with a 95% CI. All data were analyzed using R, version 2.11.1 (R Development Core Team, 2010).

Results

In immunologically naïve larvae (i.e. before wounding), the haemocyte count was negatively correlated with larval body size (Pearson's correlation coefficients: $r = -0.43$, $P = 0.018$, 95% CI = -0.68 to -0.08). Larger larvae had fewer haemocytes than smaller ones. No correlation was found for larval body size with either PO or total-PO activity (Pearson's correlation coefficients: for PO activity, $r = -0.02$, $P = 0.94$, 95% CI = -0.42 to 0.44 ; for total-PO activity, $r = -0.30$, $P = 0.19$; 95% CI = -0.64 to 0.17).

Wounding larvae with either a clean glass needle or a needle bearing heat-killed bacteria caused an increase both in haemocyte count and in total-PO activity, 24 h after the first collection of haemolymph. However, PO activity showed no change (Pearson's correlation coefficients: control larvae, $r = 0.33$; $P = 0.25$; 95% CI = -0.21 to 0.75 ; challenged larvae, $r = 0.09$; $P = 0.85$; 95% CI = -0.67 to 0.82) (Table 1). Enhanced haemocyte count and total-PO activity after wounding were dependent on larval body size (Table 1). These immune parameters increased more in larger larvae than in smaller ones (Figs 1 and 2). However, this size-dependent immune response to wounding was also dependent on whether the needle was clean or contaminated with bacteria (Table 1). The increased haemocyte count in the haemolymph was positively related to body size only among the larvae that had been immune-challenged with heat-killed bacteria (Fig. 1) (as indicated by the significant interaction Time \times Larval body size \times Treatment) (Table 1).

Table 1. Results of the analysis for repeated measurements of haemocyte count (haemocyte), phenoloxidase (PO) activity and total-PO activity in *Eupoecilia ambiguella*.

Source	Haemocyte		PO activity		Total-PO activity	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Between subjects						
Larval body size	$F_{1,26} = 0.02$	0.89	$F_{1,17} = 0.87$	0.36	$F_{1,17} = 1.04$	0.32
Treatment	$F_{1,26} = 5.37$	0.03	$F_{1,17} = 1.75$	0.20	$F_{1,17} = 0.91$	0.35
Larval body size × Treatment	$F_{1,26} = 0.11$	0.75	$F_{1,17} = 0.05$	0.82	$F_{1,17} = 0.09$	0.77
Within subjects						
Time	$F_{1,26} = 6.98$	0.01	$F_{1,17} = 0.38$	0.54	$F_{1,17} = 6.12$	0.02
Time × Larval body size	$F_{1,26} = 8.58$	0.007	$F_{1,17} = 0.53$	0.48	$F_{1,17} = 6.82$	0.02
Time × Treatment	$F_{1,26} = 1.28$	0.27	$F_{1,17} = 0.56$	0.47	$F_{1,17} = 0.10$	0.75
Time × Larval body size × Treatment	$F_{1,26} = 7.54$	0.01	$F_{1,17} = 0.06$	0.80	$F_{1,17} = 0.04$	0.84

Statistical significance ($P \leq 0.05$) is indicated in bold.

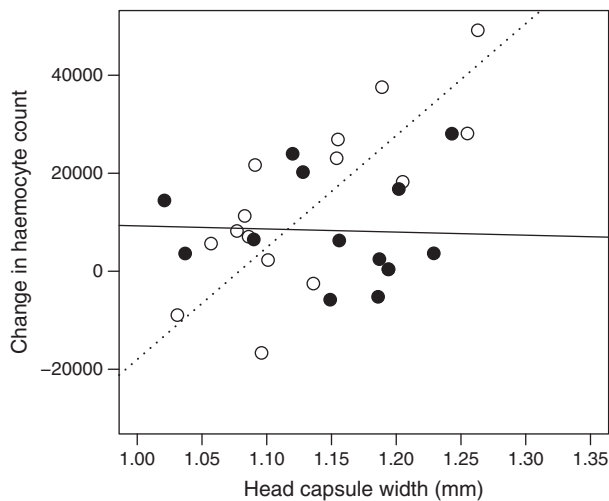


Fig. 1. Change in haemocyte count after an immune challenge (difference between measurement before and after challenge) in relation to larval body size in the European grape berry moth *Eupoecilia ambiguella*. Although haemocyte count covaries positively with the body size of bacterially treated larvae (open circles, dashed line; Pearson's correlation coefficients: $r = 0.68$; $P = 0.003$; 95% CI = 0.28 to 0.88), no relationship with body size was found in control larvae (full circle, solid line; Pearson's correlation coefficients, $r = 0.04$; $P = 0.88$; 95% CI = -0.50 to 0.56).

Discussion

Haemocytes and the PO enzyme cascade (PPO and PO activity) are the frontline of immune defence against parasites entering the insect haemocoel (Lavine & Strand, 2002; Cerenius & Söderhäll, 2004; Haine *et al.*, 2008; Strand, 2008). In this context, three parameters are measured in relation to larval body size for *E. ambiguella* of the same age to determine the effect of larval body size on immunocompetence. The results show that basal levels of PO and total-PO activity in larvae are independent of larval body size, whereas basal levels of haemocyte load decrease with larval body size. Although there is no change in PO activity when the larval immune system is challenged (i.e. with a single injection of bacteria),

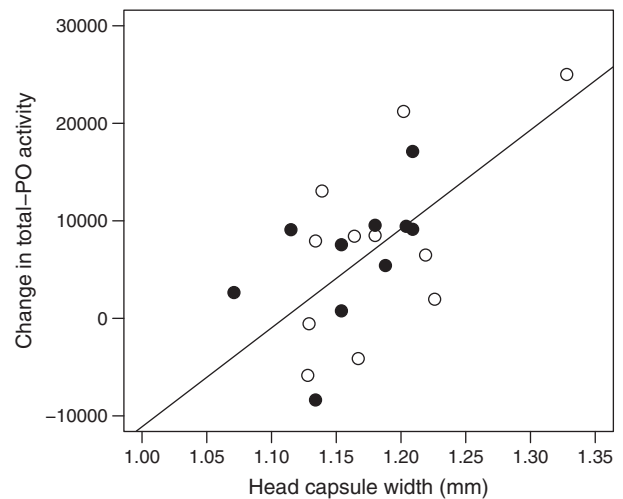


Fig. 2. Change in total-phenoloxidase (PO) activity after an immune challenge (i.e. difference between measurement before and after challenge) in relation to larval body size in *Eupoecilia ambiguella*, both for control groups (full circles) and bacterially-treated groups (open circles) (Pearson's correlation coefficients on pooled data, $r = 0.61$; $P = 0.005$; 95% CI = 0.25 to 0.83).

the amplitude of the immune response involving haemocytes and total-PO activity is significantly stronger in large immune-challenged larvae than in small larvae of the same age. These results suggest that a higher immunocompetence in larger larvae relies on mobilizing haemocyte production and the PPO system enzymes in response to an immune challenge, rather than maintaining high basal levels of immune defences constantly. This may indicate that maintaining high levels of immune effectors when naïve (immunologically non-challenged) could be more costly than inducing the production of these immune effectors rapidly when exposed to an infection.

Haemocytes are certainly the most important functional elements involved in the recognition and encapsulation of pathogens (Lavine & Strand, 2002). In *E. ambiguella*, after an immune challenge, there is a clear increase in the numbers of haemocytes in the haemolymph, probably released from the hematopoietic organ and/or by continued division of circulating

haemocytes (Ratcliffe *et al.*, 1985; Lavine & Strand, 2002). Such haemocyte recruitment is very rapid (within 24 h) and is greater in larger larvae, despite the fact that larger larvae harbour fewer haemocytes when naïve. No change is found in PO activity 24 h after the immune challenge. This result is not unexpected because previous studies show that PO activity appears to be highly variable in comparison to PPO (Cornet *et al.*, 2009). Indeed, the level of PO activity depends on the concentration of the enzyme that is naturally or spontaneously activated in the haemolymph, whereas the level of PPO is the total concentration of enzyme (activated and non-activated) that is present in the haemolymph. Therefore, levels of PPO probably reflect better the total potential strength of the immune response, rather than PO, which represents the current use of the PO cascade (Cornet *et al.*, 2009). A positive relationship is found between larval body size and total-PO activity in response to an immune challenge (injection of bacteria), although the same relationship is also found for the control (injected with Ringer's solution). This lack of difference in response could be explained by the effect of the mechanical puncture performed during the first haemolymph sampling for the two groups. In both groups, these puncture wounds in the cuticle are rapidly healed by a melanotic plug and coagulation of the haemolymph. Several studies report that mechanical injury induces a deposition of melanin around the damaged tissue (Cerenius & Söderhäll, 2004; Nappi & Christensen, 2005). Previous studies and recent findings confirm that melanin is synthesized from the PO enzyme cascade (Lai *et al.*, 2002) and is associated with factors stimulating the coagulation response in insects (Eleftherianos & Revenis, 2011). The mobilization of the PO enzyme cascade for wound healing and coagulation is probably at such a level that the additional stimulation by the bacterial challenge produces no further effect.

The greater immune response to a challenge may indicate that larger larvae can invest more in their immune system because the energy required to mount an immune response is more readily available in larger larvae. Many studies indicate that PO is a costly trait, implying that set-up and maintenance of the PPO activating system is condition-dependent (Barnes & Siva-Jothy, 2000). Several studies indicate that individuals in better physiological condition produce higher levels of PPO and/or PO (González-Santoyo & Córdoba-Aguilar, 2012) and that the strength of the immune response is down-regulated by nutritional stress or starvation (Siva-Jothy & Thompson, 2002; Rantala *et al.*, 2003a, 2003b; Yang *et al.*, 2007). In a sibling species, larval body size reflects body condition because it predicts adult fitness (Thiéry & Moreau, 2005; Moreau *et al.*, 2006a, 2006b). Therefore, a positive relationship is expected between size and the ability to mount a costly immune response. The results of the present study are consistent with this prediction, indicating that the strength of immune responses is condition-dependent. Furthermore, these results may provide an explanation for a previous finding, in which higher body weights show a correlation with higher encapsulation levels in larvae of *Pieris rapae* (Bukovinszky *et al.*, 2009).

The ability of insects to mobilize haemocytes and enzymes of the PO cascade in response to an immune challenge is related to their ability to encapsulate parasitoid eggs; for example, in *Drosophila melanogaster* (Eslin *et al.*, 1996; Eslin & Prevost, 1998) and the tobacco hornworm *Manduca sexta* (Jiang *et al.*, 2010). Therefore, it may be hypothesized that larger individuals present an increased ability to withstand attacks by pathogens during their larval lifetime. The present findings may have important implications for the evolution of plant–herbivore–parasitoid interactions (i.e. tritrophic relationships). Several studies report the influence of host plants on rates of parasitism in the field (Fuentes-Contreras *et al.*, 1996; Helms *et al.*, 2004; Thompson *et al.*, 2005; Ode, 2006). Among the different factors that could explain this variable susceptibility to parasitoid attack, the ability of phytophagous insects to mount an effective immune response against their natural enemies is proposed (Ojala *et al.*, 2005; Kapari *et al.*, 2006; Klemola *et al.*, 2007; Karimzadeh & Wright, 2008; Vogelweith *et al.*, 2011). The results of the present study suggest that larval body size, possibly mediated by food consumption, could be a potential factor for explaining why some larvae are more vulnerable to parasitoids on a given host plant. In line with this, in the sibling species (*L. botrana*), it is found that two species of parasitoids emerge from smaller pupae (Moreau *et al.*, 2010). The findings of the present study may therefore provide explanation for these observations *in natura*, and it is anticipated that size-dependent immunocompetence could have serious consequences in natural populations. For example, it could affect the expected efficacy of released parasitoids in a biological control context and also explain failures of parasitoid establishment in relation to the size of target larvae.

Finally, these findings emphasize that larval body size is an important (but underestimated) factor that could influence the immunocompetence of an individual, suggesting that smaller larvae might be more vulnerable to infection. Unfortunately, in many studies assessing the immune ability of larvae, larval body size is not controlled, producing some baseline variability in the results, especially when comparing different species or different experimental groups. Therefore, in light of the results of the present study, it is recommended that larval body size should be taken into account in ecological immunity studies, or at least considered as a covariate when measuring insect immune defence systems. Clearly, some additional experiments in other biological systems should address this issue, with the aim of defining the importance of body size in immune ability.

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