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Phenoloxidase but not lytic activity reflects resistance against *Pasteuria ramosa* in *Daphnia magna*

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The field of ecological immunology strongly relies on indicators of immunocompetence. Two major indicators in invertebrates, the activity of phenoloxidase (PO) and lytic activity have recently been questioned in studies showing that, across a natural range of baseline levels, these indicators did not predict resistance against a manipulated challenge with natural parasites. We confirmed this finding by showing that baseline levels of PO and lytic activity in the host *Daphnia magna* were not related to spore load of the parasite *Pasteuria ramosa*. Yet, PO levels in infected hosts did predict spore load, indicating PO activity can be useful as an indicator of immunocompetence in this model parasite–host system.

Keywords: ecological immunology; immunocompetence; lytic activity; parasites; phenoloxidase

1. INTRODUCTION

There is increasing attention for the contribution of immunity for the evolution of life-history strategies in invertebrates [1]. The corresponding research field of ecological immunology has made considerable progress in exploring trade-offs between immunological variables and life-history components by relying upon specific indicators of immunocompetence, the ability of a host to prevent and control infection by pathogens. One frequently used indicator of immunocompetence in invertebrates is the activity of phenoloxidase (PO). This enzyme oxidizes phenols, resulting in the formation of melanin, which plays a role in killing bacteria [2]. Strong support for a role of PO in parasite resistance comes from 'binary' studies, often *in vitro*, in which parasite resistance is found to be lower in animals in which PO has been inhibited (e.g. [3]). Besides this cellular defence, another major component of the immune response is the lytic activity of inducible antibacterial peptides [4].

Although both parameters are widely acknowledged as major components of the arthropod innate immune system, some recent experimental studies questioned their usefulness in reflecting immunocompetence. They showed that across a natural range of baseline levels, these immune parameters did not predict resistance against a manipulated challenge with a natural parasite *in vivo* ([5], PO and lytic activity in crickets; [6], PO activity in the water flea *Daphnia magna*). Furthermore, a trade-off between both parameters has been documented [7,8], potentially weakening their link with an organism's immunocompetence.

The complexity of the immune response and the lack of strong support for the link between measurements of immune parameters and immunocompetence are particularly disturbing, as in evolutionary ecological studies one typically deals with a relatively small natural gradient in immune response. This raises the general question as to what extent the natural variation in immune levels is biologically significant. Studies that found no relation between potential indicators and parasite resistance were using baseline immune levels, yet some immune parameters only reflect immunocompetence when upregulated [5]. Therefore, we set out to evaluate the role of PO and lytic activity in the immunocompetence in the water flea *D. magna*, by also quantifying immune levels in infected hosts. *Daphnia magna* is a model system in evolutionary ecology and the study of parasite–host interactions [9,10]. Support for parameters of immunocompetence in this host is therefore particularly relevant for studies on life-history evolution. We here assess immunocompetence by measuring PO and lytic activity together with the spore load after infection with *Pasteuria ramosa*, a common parasite of *D. magna*.

2. MATERIAL AND METHODS

(a) *Experimental design*

To test for an upregulation of immune defence in infected animals and to relate the resulting immune defence levels to spore load, we exposed four different *Daphnia* clones to one of two parasite treatments: a placebo control and a suspension of parasite spores. We will refer to levels of immune defence in the control treatment as baseline levels. For each clonal line, four sets of 10 juveniles, all born within a 24 h interval, were transferred into 20 ml jars (containing parasite spores or placebo solution) in standardized laboratory conditions (for details see electronic supplementary material, appendix S1). Juveniles were exposed during the first 5 days. Thereafter, they were transferred to 50 ml dechlorinated tap water, which was changed daily together with the removal of offspring. The experiment was stopped after 21 days, and all measurements of immune variables and spore loads were carried out at this point.

(b) *Response variables*

Phenoloxidase was activated with chymotrypsin and quantified spectrometrically following Mucklow *et al.* [6], and lytic activity was determined using a lytic zone assay [7]. *Pasteuria ramosa* spores were counted to quantify individual spore load, and we calculated individual performance based on number of offspring per day (see electronic supplementary material, appendix S1).

(c) *Statistics*

The effects of clone and parasite treatment on spore load, PO levels and lytic activity were tested using two-factor ANOVAs. We tested whether clones with higher immune levels (baseline or after 21 days of infection) had a lower spore load using product moment correlations on clonal means. Additionally, we tested this hypothesis with—as unit of replication—the clonal lines (four per clone), using an ANCOVA with clone as random categorical factor, PO and lytic activity (baseline or after 21 days) as covariates, and spore load (log-transformed) as dependent variable. The interaction between clone and covariate was never significant, and was removed from the final models.

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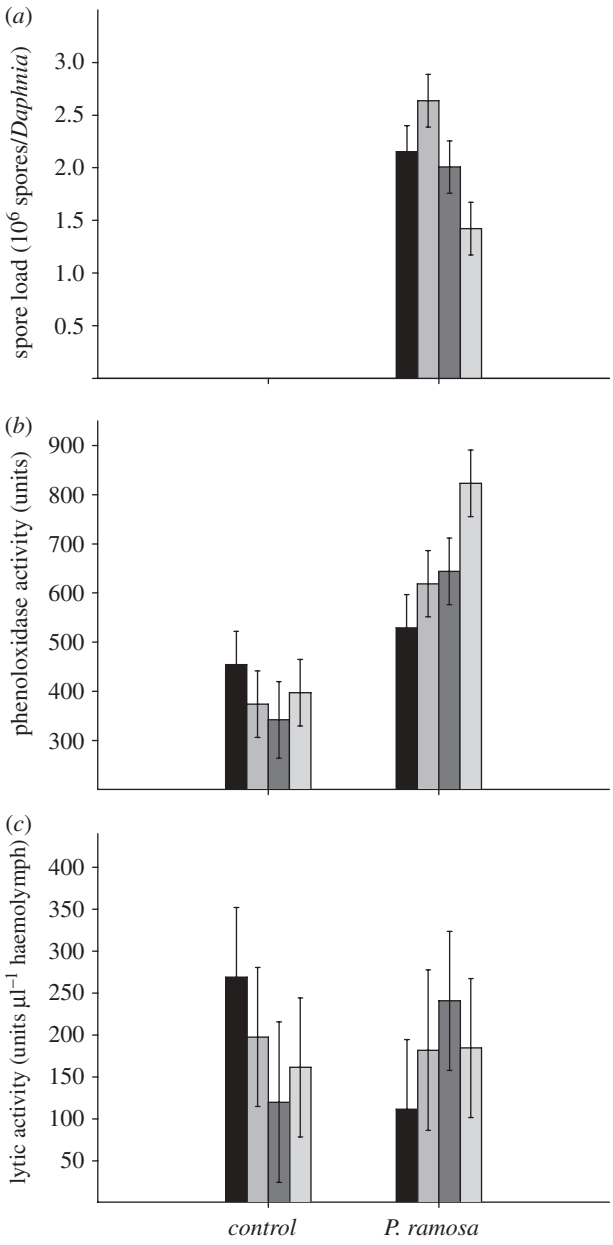


Figure 1. (*a*) Spore load (*b*) phenoloxidase activity and (*c*) lytic activity for four *D. magna* clones after 21 days of infection with the parasite *P. ramosa*. Given are least square means ± 1 s.e.

3. RESULTS

All *Daphnia* exposed to *P. ramosa* spores as juvenile were heavily infected and castrated after 21 days. This resulted in a strong accumulation of parasite spores in the infected animals ($F_{1,22} = 250.1$, $p < 0.0005$; figure 1*a*). Infected animals showed higher PO levels ($F_{1,22} = 33.1$, $p < 0.0005$; figure 1*b*). No upregulation was found for lytic activity ($F_{1,22} = 0.01$, $p = 0.91$; figure 1*c*). Clones did not differ in these variables (all $p > 0.10$), nor were there any clone × parasite treatment interactions (all $p > 0.08$).

Clones with a higher baseline PO activity did not have a lower spore load ($r = -0.07$, $p = 0.93$; figure 2*a*). Yet, clones with a higher PO activity measured 21 days after infection did show a significant lower spore load ($r = -0.95$, $p = 0.046$; figure 2*b*). A stronger test at the clonal line level (see electronic supplementary material, appendix S2) confirmed that clonal lines with higher PO levels after infection showed a lower spore load (ANCOVA: slope −0.0006, s.e. = 0.00020, $t_{11} = -2.78$, $p = 0.018$; figure 2*b*), while this was not the case for baseline PO levels (ANCOVA: slope 0.00031, s.e. = 0.0003, $t_{10} = 1.01$, $p = 0.34$; figure 2*a*).

For lytic activity, no relations with spore load were found. Neither clones with a higher baseline lytic activity ($r = -0.36$, $p = 0.64$; figure 2*c*), nor with a higher lytic activity after infection showed a lower spore load with *P. ramosa* ($r = -0.33$, $p = 0.67$; figure 2*d*). We found no correlation between PO levels and lytic activity, neither between baseline levels (ANCOVA: slope −0.01, s.e. = 0.19, $t_{11} = -0.06$, $p = 0.95$; figure 2*e*) or after infection (ANCOVA: slope 0.06, s.e. = 0.38, $t_{11} = 0.15$, $p = 0.86$; figure 2*f*; full ANCOVA statistics are included in the electronic supplementary material, appendix S2).

4. DISCUSSION

The finding that baseline PO levels and lytic activity in *D. magna* are not related to resistance against the parasite *P. ramosa* confirms the PO results of Mucklow *et al.* [6]. Similarly, Adamo [5] reported that baseline PO levels and lytic activity of the cricket *Gryllus texensis* are not related to resistance against three pathogenic bacteria. Based on these observations and the fact that some studies showed that total PO cannot be upregulated after infection (e.g. [11]), these authors questioned whether PO levels reflect immunocompetence. Infection with *P. ramosa*, however, resulted in an upregulation of PO levels that remained 21 days after infection. This cannot be an artefact of age-specific expression of PO levels [12] because in the control animals, not exposed to the parasite and therefore not infected, we also measured PO levels 21 days after the start of the experiment. Although not general (see above), upregulation of total PO indicating de novo synthesis has been demonstrated, also in *D. magna* [13]. In our study, higher PO levels in infected hosts (while still very low compared with other taxa) were associated with lower spore loads. One may argue that this negative covariation of PO level and spore load reflects the fact that animals with a higher spore load have lower PO levels because they suffer more from the infection. However, as we found no relationship between performance and PO or spore load, this is unlikely (see electronic supplementary material, appendix S3). Admittedly, we lack information about the immune response during infection, but is seems reasonable to assume that individuals with higher PO levels after 21 days also had higher levels earlier. Therefore, our results indicate that *D. magna* which upregulate PO to a higher level when infected with *P. ramosa* have a higher resistance to that parasite.

For lytic activity, our data suggest no upregulation after 21 days of infection, despite the accumulation of spores of the Gram-positive bacteria *P. ramosa*. This is in contrast with other studies showing a long-lasting (up to 44 days in dragonflies) upregulation of lytic activity after an immune challenge [14,15]. The latter study suggested antimicrobial peptides as a second line of defence, after the constitutive immune

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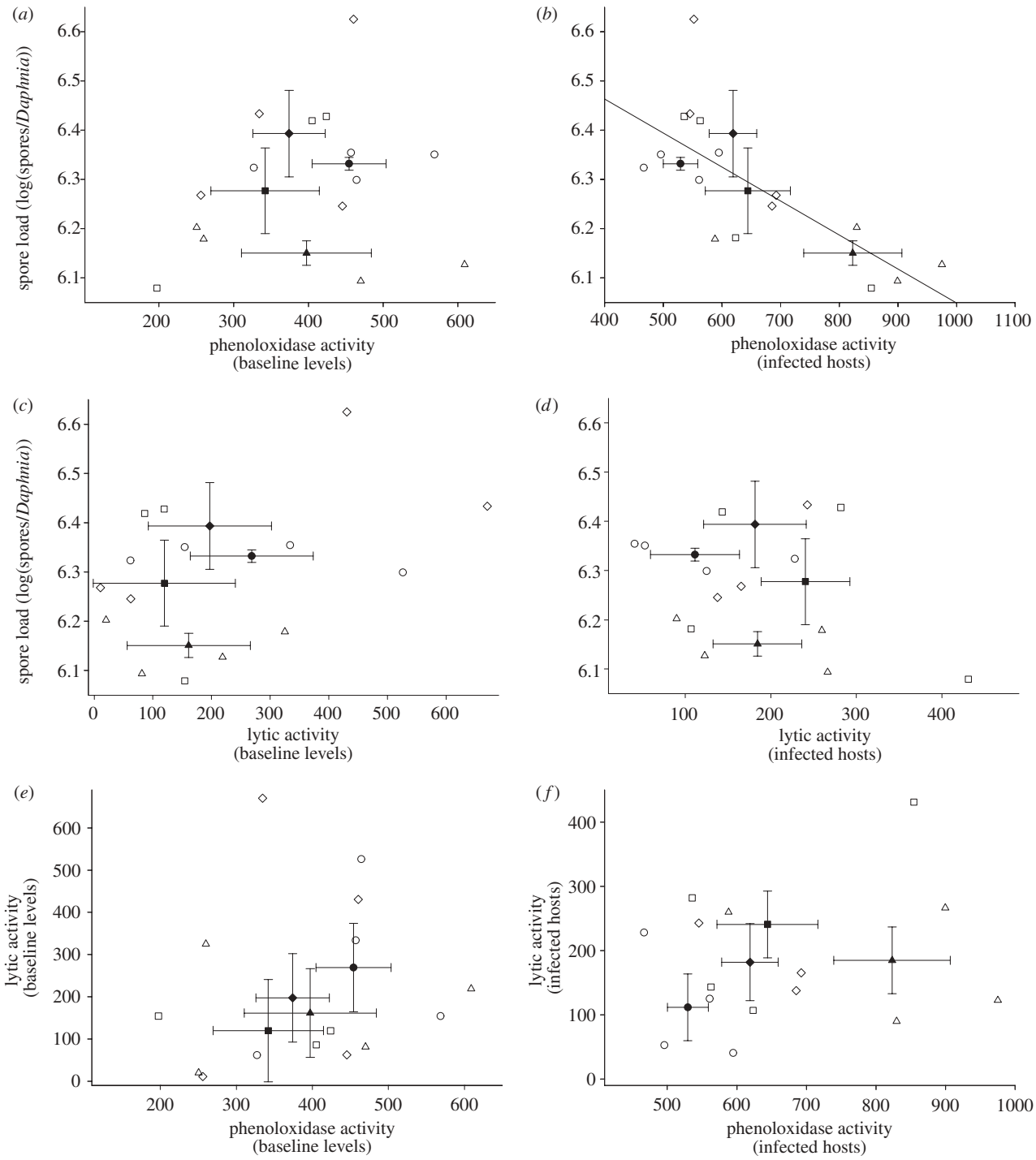


Figure 2. Relationships between (log-transformed) spore loads of the parasite *P. ramosa* in the host *D. magna* and (*a*) baseline PO levels, (*b*) PO levels in infected hosts, (*c*) baseline lytic activity, and (*d*) lytic activity in infected hosts. Relationships between PO levels and lytic activity in (*e*) not-infected hosts and (*f*) infected hosts. Symbols represent the different clones, and clonal means are plotted $\pm$ 1 s.e.(full symbols).

response or rapidly activated enzyme cascades (such as PO) have cleared most of the bacteria. It therefore might be that in the case of a chronic-proliferating infection as in *P. ramosa*, lytic activity (at least against *Micrococcus luteus*) is not a primary mechanism to clear infections. But we acknowledge that while we cannot exclude the possibility that still other components of the invertebrate immune system helped to generate the pattern of decreasing spore load, our data suggest that PO but not lytic activity plays a significant role in resistance against *P. ramosa* in *D. magna*.

That PO levels in infected hosts and not baseline PO levels can be useful as an indicator of immunocompetence in this parasite–host system may not be that surprising. For example, Adamo [5] showed for lytic activity that the upregulated levels but not the baseline levels reflected resistance to parasites in a cricket species. Similarly, Liu *et al.* [16] concluded that the capacity for production of PO activity is an important component for increased resistance against bacterial infection in crayfish. Okado *et al.* [17] more generally reported that among nine *Drosophila* strains,

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differences in resistance were related to differences in upregulation of the innate immune response. Our study shows that higher PO levels are associated with lower number of parasites in the host, using a natural gradient of PO levels after challenge with a natural parasite *in vivo*. Our study adds to previous support for a role of PO in parasite resistance coming from studies, often *in vitro*, that reported a lower parasite resistance in hosts with manipulated low PO levels. We also directly scored host resistance in terms of spore load, which is important as other end points like survival may not necessarily covary directly with parasite load [18] and may depend on other factors like the cost involved in upregulating the immune system [12]. Importantly, our approach involved infecting animals using the natural pathway of parasite infection rather than injecting them with the parasite, which causes wounding and may thus interfere with the PO system [19].

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