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Variation in phenoloxidase activity and its relation to parasite resistance within and between populations of *Daphnia magna*

Patrick T. Mucklow[1], Dita B. Vizoso[1,2], Knut Helge Jensen[2], Dominik Refardt[2] and Dieter Ebert[1,2*]

[1]*Institut für Zoologie, Universität Basel, Rheinsprung 9, 4051 Basel, Switzerland*
[2]*Departément de Biologie, Ecologie et Évolution, Université de Fribourg, Chemin du Musée 10, 1700 Fribourg, Switzerland*

Estimates of phenoloxidase (PO) activity have been suggested as a useful indicator of immunocompetence in arthropods, with the idea that high PO activity would indicate high immunocompetence against parasites and pathogens. Here, we test for variation in PO activity among clones of the planktonic crustacean *Daphnia magna* and its covariation with susceptibility to infections from four different microparasite species (one bacterium and three microsporidia). Strong clonal variation in PO activity was found within and among populations of *D. magna*, with 45.6% of the total variation being explained by the clone effect. Quantitative measures of parasite success in infection correlated negatively with PO activity when tested across four host populations. However, these correlations disappeared when the data were corrected for population effects. We conclude that PO activity is not a useful measure of resistance to parasites or of immunocompetence within populations of *D. magna*. We further tested whether *D. magna* females that are wounded to induce PO activity are more resistant to infections with the bacterium *Pasteuria ramosa* than non-wounded controls. We found neither a difference in susceptibility nor a difference in disease progression between the induced group and the control group. These results do not question the function of the PO system in arthropod immune response, but rather suggest that immunocompetence cannot be assessed by considering PO activity alone. Immune response is likely to be a multifactorial trait with various host and parasite characteristics playing important roles in its expression.

Keywords: phenoloxidase; *Daphnia*; immunity; resistance; microsporidia; immunocompetence

1. INTRODUCTION

Invertebrates show a variety of immune functions against parasites and pathogens. Among them, the prophenoloxidase (ProPO) activating system plays several roles in invertebrate immunity and is considered to be one of the most important defence mechanisms. The oxireductase phenoloxidase (PO) is part of a complex system of proteinases, pattern-recognition proteins and proteinase inhibitors that constitute the ProPO activating system (Millar & Ratcliffe 1994; Söderhall & Cerenius 1998). It is thought to be part of the invertebrate's immune response against parasites because the conversion of ProPO to active enzyme can be initiated by molecules such as lipopolysaccharide, peptidoglycan and beta-1,3-glucans from invading micro-organisms. PO is the final enzyme in this cascade and the bottleneck in the melanization reaction (Söderhall & Cerenius 1998), which is a common response to parasite entry in many invertebrates. During a successful immune reaction, melanin encapsulates the parasites (including pathogens and parasitoids) and kills them.

Evidence for the role of PO in arthropod immunity comes from three lines of research. First, molecular studies have helped to clarify the mechanisms of the ProPO activating system (Millar & Ratcliffe 1994; Söderhall & Cerenius 1998), with one study demonstrating reduced resistance in a mosquito transduced with antisense RNA of the PO gene (Shiao *et al.* 2001). Second, everything else being equal, hosts have been shown to increase PO activity when facing conditions associated with a high risk of parasitization. For example, increased PO activity was observed under conditions of high density (Reeson *et al.* 1998), after wounding of the hosts (Nayar & Knight 1995; Mucklow & Ebert 2003), after injection of lipopolysaccharides (Moret & Schmid-Hempel 2000) and after infection with parasites that are unable to avoid recognition (Hagen *et al.* 1994; Gomes *et al.* 1999). In counteradapting, some parasites and parasitoids inhibit host PO activity, presumably to avoid being encapsulated (Beckage 1998; Dowd 1999; Shelby *et al.* 2000). Third, correlational evidence suggests that resistance is associated with high levels of PO activity and melanin production (Nigam *et al.* 1997; Siva-Jothy 2000). Taken together, these studies clearly demonstrate that the PO system plays an important role in arthropod immunity.

Based on this evidence, it has been suggested that PO activity can be a general indicator of the host's competence to resist parasites, often referred to as immunocompetence (Hauton *et al.* 1995, 1997; Adamo *et al.* 2001; Kurtz & Sauer 2001; Moret & Schmid-Hempel 2001). Immunocompetence is understood as the reaction of an organism to minimize the fitness costs of an infection (Owens & Wilson 1999). With respect to the PO system, this idea assumes, first, that variation in resistance is largely explained by variation in PO activity across hosts

* Author for correspondence (dieter.ebert@unifr.ch).

(neglecting other components of the immune system) or that variation in PO activity covaries positively with other defence components, thus serving as a general indicator of the host's ability to fight parasites. Second, it assumes that variation among parasite genotypes or species plays only a minor role, i.e. that there are negligible interactions between host and parasite genotypes. Both of these assumptions seem to, at least partly, contradict current understanding of arthropod immunity (see Schmid-Hempel & Ebert (2003) for a review). For example, several studies have demonstrated that the outcome of a host–parasite encounter depends on both the host genotype and the parasite genotype (Files & Cram 1949; Lively 1989; Ebert 1994; Imhoof & Schmid-Hempel 1998; Carius *et al.* 2001). However, this finding alone does not invalidate the idea that PO activity indicates immunocompetence. The validity of the two above-mentioned assumptions must be judged on a quantitative basis, because small deviations from these assumptions (e.g. some variation introduced by interactions between host and parasite genotypes) may not invalidate the use of PO activity as a measure of immunocompetence.

Here, we test experimentally whether PO activity can be used as an indicator of resistance against parasites in an arthropod–microparasite system. In a first experiment, our goal was to test the relative importance of clonal variation and parasite-species variation for resistance on the outcome of host–parasite interactions. To do so, we measured the PO activity levels and the resistances of 33 host genotypes from four host populations against four endoparasites: one bacterium and three microsporidians. These four parasites are very common parasites of *Daphnia magna* and represent a substantial fraction of the parasites that *D. magna* females encounter in central and northern Europe (Green 1974; Little & Ebert 1999; Ebert *et al.* 2001; Decaestecker *et al.* 2002). The host is the water flea *D. magna*, which can reproduce clonally, allowing us to focus on clonal variation and covariation in PO activity and resistance within and between host populations and across parasite species. Clonality of the host is particularly important here, as it allows us to test for resistance to several parasites and to estimate PO activity on the same host genotypes, thus excluding variation resulting from segregation of polymorphic genes within populations.

In a second experiment we tested whether induced PO activity results in higher resistance or slower disease progression. This experiment is based on the observation that, 24 h after wounding, wounded *Daphnia* have higher PO activity levels than control animals (Mucklow & Ebert 2003).

2. MATERIAL AND METHODS

Daphnia magna Straus is a planktonic freshwater crustacean usually found in eutrophic shallow ponds. It is attacked by various bacterial, microsporidial and fungal parasites (Green 1974; Stirnadel & Ebert 1997; Ebert *et al.* 2000). *Daphnia magna* reproduces by cyclical parthenogenesis and can be kept in the laboratory in a state of clonal reproduction. At 20 °C maturity is reached *ca.* 10 days after birth.

We used 33 host clones originating from four different populations of *D. magna* that had been kept in the laboratory for at least six months (more than 10 generations) prior to the experiments. Nine clones were hatched from ephippia (resting eggs) collected from a mud sample from a carp-breeding pond near Munich, in southern Germany. Nine clones were isolated from a pond near Gaarzerfeld in northern Germany. Four clones were collected from a small pond near Kniphagen in northern Germany. Finally, 11 clones were isolated from rock-pool populations on the Skerry islands of the Tvärminne Archipelago in southern Finland (Ebert *et al.* 2001). All clones differed genetically from each other, as determined by cellulose–acetate electrophoresis (Hebert & Beaton 1993) at the loci aminoaspartate transferase (EC 2.6.1.1), mannose-6-phosphate isomerase (EC 5.3.1.8), malate dehydrogenase (EC 1.1.1.37) and glucose-6-phosphate isomerase (EC 5.3.1.9).

We used four parasite species (table 1): *Pasteuria ramosa* Metchnikoff 1888 (Ebert *et al.* 1996), a bacterium infecting the host's body cavity; *Octosporea bayeri* (Jirovec 1936), a microsporidium that invades the fat body and the ovaries; and *Ordospora colligata* (Larsson *et al.* 1997) and *Glugoides intestinalis* (Larsson *et al.* 1996), two intracellular microsporidian parasites of the gut epithelium. We used two isolates of *O. bayeri* collected from two rock-pool populations, both located in southern Finland, *ca.* 100 km apart. These populations were different from the populations that provided the Finnish host clones but belong to the same metapopulation system. As both *O. bayeri* isolates behaved similarly, we report only their mean infection success. *Pasteuria ramosa* and *Or. colligata* were isolated from the same Gaarzerfeld population as some of the host clones. *Glugoides intestinalis* was isolated from the same population near Munich that provided some of the host clones.

Isolates of all parasites were obtained as explained earlier (Ebert & Mangin 1997; Ebert *et al.* 2000; Decaestecker *et al.* 2002; Mucklow & Ebert 2003). The isolates of each of these parasites may not consist of single genotypes, as multiple infections of individual hosts may occur (Ebert & Mangin 1997). However, as they experienced a genetic bottleneck during isolation and during subsequent culturing in monoclonal host populations, their genetic diversity is expected to be much lower than that of the natural populations from which they were taken. The *D. magna* clones used to culture the parasite isolates were different from the 33 clones used in the experiments and originated from the same populations as the parasites. For each parasite, spore suspensions were produced by grinding infected animals. Spores were counted by using a bacterial-counting chamber with a cell depth of 0.02 mm at magnification ×600. Suspensions were prepared so that each aliquot contained the same amount of ground *Daphnia* tissue. The placebo contained only ground tissue from uninfected *Daphnia*.

Throughout the experiments, the *Daphnia* were kept under standardized conditions: artificial culture medium (Klüttgen *et al.* 1994) modified according to Ebert *et al.* (1998), the unicellular green algae *Scenedesmus* sp. as the only food, constant photoperiod (16 L : 8 D) and a water temperature of 20 ± 1 °C. Before starting the infection experiment, we created 14 replicate lines (maternal lines) per clone (462 lines in total) and kept them isolated for three generations under the same environmental conditions. This allowed us to randomize maternal effects across clones (Ebert *et al.* 1993). We used the third-generation females when they were 21 days old to estimate PO activity. We had previously collected the offspring of six out of the 14 replicate lines per clone for use in the infection experiment.

(a) *Estimation of phenoloxidase activity*

To measure PO activity, we collected haemolymph from *Daphnia* of all 14 replicate lines per clone. The method is

Table 1. Summary information of the four *Daphnia magna* parasites used in this study.

parasite species, taxon	mode of transmission	infected tissue	effect on host
Pasteuria ramosa, bacteria (Ebert *et al.* 1996)	horizontal (water-borne spores are released from dead hosts)	haemolymph, body cavity	total host castration, induces gigantism
Octosporea bayeri, microsporidia (Jirovec 1936; Green 1957)	horizontal (water-borne spores are released from dead hosts) and vertical (transovarial)	fat cells, ovaries, intracellular	reduces fecundity and survival by 30–70%
Ordospora colligata, microsporidia (Larsson *et al.* 1997)	horizontal (water-borne spores are released from the living host with the faeces)	gut epithelium, intracellular	reduces fecundity and survival by 5–20%
Glugoides intestinalis, microsporidia (Larsson *et al.* 1996)	horizontal (water-borne spores are released from the living host with the faeces)	gut epithelium, intracellular	reduces fecundity and survival by 5–20%

described in full elsewhere (Mucklow & Ebert 2003). First, we measured the body length of each female (from the tip of the head to the base of the spina) using a dissecting microscope at magnification ×25. Then we rinsed the *Daphnia* briefly in deionized water to remove algae and dried it on filter paper until no medium was left. We then pricked the *Daphnia* in the heart with a needle. One microlitre of haemolymph was collected immediately as it oozed out of the body cavity. Haemolymph samples of the different clones were collected in alternating sequence.

The PO activity was recorded in the form of dopachrome production, a product of the oxidation of L-dopa by the enzyme PO. Absorbance at 475 nm was measured with a spectrophotometer (Nayar & Knight 1995; Reeson *et al.* 1998). We used a modified version of Reeson *et al.* (1998): 1 µl of haemolymph was deposited in an Eppendorf tube with 150 µl of phosphate-buffered saline (PBS) (0.15 M of NaCl, 10 mM of $Na_2HPO_4{\cdot}2H_2O$ at pH 7.4 with *o*-phosphoric acid). Then 450 µl of 20 mM L-dopa was added, and the whole mixture was transferred into a microcuvette to measure absorbance in the spectrophotometer. Absorbance was measured immediately after adding the L-dopa and again after 4.5 h. Previous tests have shown that the relatively strong dilution of the haemolymph samples does not significantly influence the precision of PO activity measurement.

In addition, 10 'non-haemolymph' controls were measured, using PBS and L-dopa, but without haemolymph. The mean of the activity units of the 'non-haemolymph' controls was subtracted from all data (subtracting background absorbance). As the 'non-haemolymph' controls are independent of the treatments, they have no influence on the differences among treatment groups reported here. PO activity estimates were then calculated from the change in absorbance over the 4.5 h time period (absorbance after 4.5 h − absorbance at time 0). During the first 5 h the increase in absorbance over time is linear (P. Mucklow, unpublished data). For convenience, we multiplied this difference by a factor of 1000. A further control consisting of PBS and haemolymph only was not measured during this assay, as earlier experiments showed no difference in oxidation compared with the 'non-haemolymph' controls (Mucklow & Ebert 2003). Activation of PO with $CaCl_2$ and trypsin did not change the PO activity estimates. Therefore all experiments were conducted without trypsin and $CaCl_2$.

(b) *Infection experiment*

Out of the 14 maternal lines used to produce the females for the PO assay, we randomly chose six lines per clone for the infection experiment. Seven female offspring of each replicate line were collected from the third or fourth clutch. For infection, each female (a total of $1386 = 33$ clones × six lines × seven offspring) was placed individually into a well of a 24-well plate with 2.5 ml of algal solution (medium with 10^5 algal cells ml^{-1}). These females were 3 to 5 days old. All treatments were represented on each well plate, with the arrangement of the treatments being randomized between plates. We used a split-brood design for this experiment, with each of the seven daughters from the same mother assigned to a different treatment (including an uninfected control). All animals received 0.2 ml of the respective spore suspension or the placebo. The six parasitized sisters were infected with *P. ramosa* (two spore doses), *O. bayeri* (two isolates), *G. intestinalis* or *Or. colligata*. The seventh sister (control) received the placebo suspension. Spore doses were 2.5×10^5 spores per animal for the *P. ramosa* high spore dose, both strains of *O. bayeri*, *G. intestinalis* and *O. colligata*, and 2.5×10^3 spores per animal for the low-dose *P. ramosa* treatment. On the third and fourth (last) days in the 24-well plates, each *Daphnia* was fed 2.5×10^5 cells of algae. After 4 days, the females were individually transferred into 100 ml jars and fed 2×10^6 cells of algae per day. The medium was changed after every adult instar. The position of the trays containing the jars in the climate chamber was changed daily to minimize gradient effects. *Daphnia* from all microsporidian treatments were frozen 26 days after infection. Infection status in the *P. ramosa* treatments was verified in living animals at day 30 after infection. At this stage less than 5% of animals had died, and there was no difference in mortality among the treatment groups. The four parasites used here typically kill their host only later during an infection (Ebert *et al.* 2000).

(c) *Quantification of susceptibility*

As the four parasite species differ in their biology, we used different methods to test for infection or to quantify the strength of the infection.

(i) Octosporea bayeri

From previous experiments (D. Ebert, unpublished data), we knew that the number of spores produced by *O. bayeri* varies strongly among clones and that spore production is a good indicator of how much the host suffers from the infection. Therefore, we determined the success of infection as the number of parasite spores per host 26 days after infection. By this time infections are well developed, and spores are easy to quantify. To quantify the number of *O. bayeri* spores we ground up whole individual hosts, homogenizing them in 0.5 ml of medium. We then counted a subsample in a counting chamber using phase-contrast microscopy at a magnification of ×300.

(ii) Pasteuria ramosa

In a previous study, variation in resistance to *P. ramosa* was mainly found as a binary response—hosts are either infected or not—rather than as variation in the number of transmission stages produced in an infected host (Carius *et al.* 2001). Therefore, we scored each female simply as being infected or not. This is easily determined by sight, as infected females cease reproduction, suffer from gigantism and acquire a characteristic coloration. In cases of doubt, we tested for the presence of spores under the microscope. For each of the two dose levels of *P. ramosa*, we calculated the proportion of the six replicates with infections and used the average proportion of infected hosts across the two dose levels as a quantitative estimate of parasite success.

(iii) Ordospora colligata *and* Glugoides intestinalis

These two microsporidian parasites produce small spores (*ca.* 3 µm in length), making it laborious to quantify infection success with a light microscope. Instead, we used quantitative PCR to quantify the presence of parasite DNA in each host. DNA was extracted from homogenized thawed samples using the DNeasy Tissue Kit (Qiagen, Basel, Switzerland). Real-time quantitative PCR was performed on a LightCycler (Roche Molecular Biochemicals, Rotkreuz, Switzerland) using FastStart DNA Master SYBR Green I chemistry (Roche Molecular Biochemicals, Rotkreuz, Switzerland). Primers GTGATGTTGAGTATTTGA GCG and CACTGACTCTTTCATCGTCC were used to amplify a 236 bp fragment of the small-subunit rDNA gene of *Or. colligata*. Primers CAGAAGCGGGGAACATCAGTC and CCAATATCGTCGTGGGGAAGG were used to amplify a 202 bp fragment 431 bp downstream of the small-subunit rDNA gene of *G. intestinalis*. The reaction volume was 20 µl with 2 µl of template DNA, 500 nM of each primer and 1 mM of $MgCl_2$. Amplification was performed with an initial incubation step of 5 min at 95 °C and 50 cycles consisting of 15 s at 95 °C, 5 s at 60 °C, 15 s (10 s for *G. intestinalis*) at 72 °C and a fluorescence acquisition at 84 °C (81 °C for *G. intestinalis*). A melt with constant fluorescence acquisition was carried out from 65 °C to 95 °C. In every run, we included two standards (respective fragments cloned into a plasmid) with a 1000-fold concentration difference to obtain a calibration equation. The concentrations of the samples were then calculated relative to these standards. Only specific amplifications (as determined with the melting curve) were analysed. All calculations were done with LightCycler software v. 3.

(d) *Wounding experiment*

Daphnia magna females from one clone (Gaarzerfeld clone, DG-1-106, northern Germany) were raised in mass cultures at low density under standardized conditions as described above. From these cultures we collected a cohort of 224 offspring born within 24 h and placed them individually in 20 ml of medium. After 4 days we randomly assigned them to two treatment groups (112 females in each). One group was wounded by placing females under a dissecting microscope at a magnification of ×25 and puncturing the carapace at the centre of the side of the body with a 0.1 mm diameter needle. Females in the other group were treated the same but were not wounded. Twenty-four hours after wounding, half of the animals in each group were exposed to 8.23×10^3 spores of the bacterium *P. ramosa*. This spore concentration was chosen because we aimed for a *ca.* 50% infection rate (Regoes *et al.* 2003). The non-exposed animals received a placebo solution. Spore suspensions and placebo solutions were prepared as described for the other experiment (§ 2b). The experiment now had four different treatment groups: 'control', 'wounded', 'parasite exposed' and 'wounded plus parasite exposed'. Four days later, each female was placed in 80 ml of medium and fed daily, and the medium was changed every 5 days. We checked each female daily for signs of infection and offspring produced. *Pasteuria ramosa* is a castrating parasite, and infections are clearly visible because host reproduction ceases completely *ca.* 8 to 20 days after infection (Carius *et al.* 2001). The time it takes until hosts are castrated can be used as a measure to quantify disease progression. The experiment lasted 25 days. Eleven (out of 224) animals died before the end of the experiment, but in only five of them were we not able to determine whether they were infected or not.

(e) *Statistical analysis*

The effect of host origin on the *Daphnia* PO activity was tested with a nested analysis of variance, with clones nested in populations. Residuals were normally distributed. To account for differences in host body length we also tested PO activity after dividing it by host length. Using the binary infection data, we tested for population and clone effects on parasite resistance for each parasite separately (we used only the high-dose data for *P. ramosa*, because less than 20% of the females in the low-dose treatment were infected). We used a generalized linear mixed model for binomially distributed data using the logit-link function (Glimmix macro for SAS 6.12 software; see Clutton-Brock *et al.* (2001) and Krackow & Tkadlec (2001) for a similar approach). Clone was treated as a random effect and was nested in population.

To answer our main question, whether a genetic correlation exists between PO activity and the parasite's reproductive success in the hosts, we calculated Pearson correlations between clonal means of PO activity and parasite success. Calculating average parasite success across the four species was not meaningful, as the means and variances of parasite success varied greatly across parasite species. Therefore, we standardized the parasite data for each species to a mean of 0 and a variance of 1 to calculate the average parasite success. In the next step of the analysis, we corrected for host population effects. To do this, we performed one-way analyses of variance with the clonal means as the dependent variable and host population as a factor for each parasite species, for the average parasite success and for PO activity (ANOVAs not reported here). We then used the residuals for the correlation analysis between PO activity and parasite success.

3. RESULTS

The effects of host population and clone on the *Daphnia* PO activity were highly significant at both levels (population: mean squares = 7.983, $F = 24.12$, $p < 0.0001$; clone (nested within population): mean squares = 0.3309, $F = 3.74$, $p < 0.0001$; figure 1). The population effect explained 41.3% of the total variance and the clone effect explained 9.8%. A minimal estimate for the repeatability of our PO activity assay can be obtained from the pooled proportion of the genetic-variance components. Ignoring the population effect, clones explained 45.6% of the total variance ($p < 0.0001$), indicating a high repeatability. Infectivity data for each of the parasites showed significant variation on the host population or clone level (table 2).

Table 2. Result of a generalized linear mixed model (GLIMMIX macro in SAS) for binomially distributed infection data from four parasite species (including two strains of *Octosporea bayeri*) testing for host-population and host-clone effects.
(The abbreviations ndf and ddf are the nominator and denominator degrees of freedom, respectively. For the population effect, type III pseudo *F*-values and significance levels are given. For the clone effects (random effect nested within population) a pseudo *Z*-value is calculated.)

parasite	ndf, ddf	type III *F*-values	*p*-value (population)	*Z*-value (clone)	*p*-value (clone)
Octosporea bayeri 2	3, 26.24	10.31	0.0001	1.80	0.07
Octosporea bayeri 3	3, 33.14	9.19	0.0001	0.68	0.49
Pasteuria ramosa	3, 27.77	0.97	0.42	2.87	0.004
Ordospora colligata	3, 25.62	3.69	0.024	2.10	0.035
Glugoides intestinalis	3, 22.38	1.81	0.17	2.44	0.014

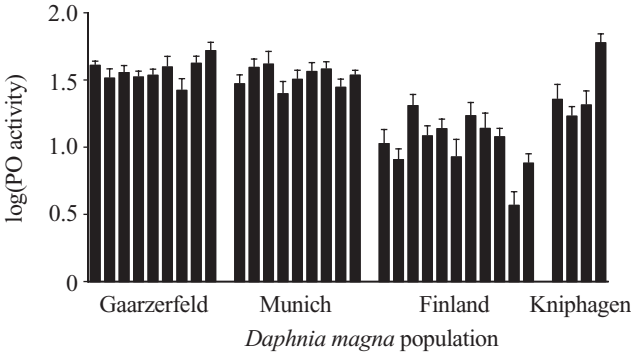

Figure 1. Mean PO activities (and standard errors) for 33 clones of *Daphnia magna* from four populations. PO activity was log-transformed prior to analysis to normalize distributions.

Figure 2 shows the clonal means of parasite success plotted against the clonal means of PO activity for each parasite species. Negative correlations were found for all parasites and were significant for all but *G. intestinalis*. This general negative trend is even more apparent when the average parasite success (average of standardized parasite-success measures) is plotted against PO activity (figure 3). However, further analysis shows that these negative correlations are a result of strong population-level effects. When we repeated the correlation analysis with the residuals of the clonal means corrected for population effects against the residuals of PO activity, all correlation coefficients were close to zero and non-significant (figures 4 and 5). To test the power of these non-significant correlations, we performed two additional analyses. First, we asked whether the decline in the correlation coefficient after correcting for the population effect was significant. The decline was significant for the average standardized parasite success (compare figures 3 and 5; $r_1 = 0.578$, $r_2 = 0.095$, $N = 33$, $z = 2.189$, $p = 0.028$), indicating that, after correcting for population effects, a substantial amount of covariance between resistance and PO activity was lost. Second, we asked how many clones would have been needed to achieve significance ($p < 0.05$) given the observed correlation coefficients. For *P. ramosa*, *O. bayeri*, *Or. colligata*, *G. intestinalis* and the combined dataset these numbers were 385, 763, 35, 3753 and 429, respectively (for alpha *z*-power of 50% for double-sided tests). For most of our parasite species and for the combined dataset, these numbers greatly exceed the experimental possibilities.

Figure 6 explains the discrepancy between figures 3 and 5. The *D. magna* clones from Finland (upper-left mean in figure 6) not only are much more susceptible to parasite infections, but also have much lower PO activity levels. This yields negative correlations between PO activity and the susceptibility measures across populations, but not within populations.

Our PO activity estimates are based on the same amount of haemolymph from each individual female. However, larger individuals may have larger total amounts of enzyme or have higher or lower enzyme activities. There was no significant correlation between *Daphnia* body length and PO activity ($r = -0.046$, $p > 0.3$). Further, accounting for *Daphnia* body length did not change the correlation results presented above (analysis not shown).

The wounding experiment did not reveal an effect of wounding on resistance. We found 31 infected females (out of 52 surviving females, 59.6%) in the wounded treatment and 34 infected females (out of 55 surviving females, 61.8%) in the non-wounded treatment. This difference was not significant (Fisher's exact test: $p > 0.5$). All non-exposed females remained uninfected. To support the hypothesis that wounding boosts resistance (given our experimental design), we would have had to find fewer infected females in the wounded group. Everything else being equal, we would have achieved significance with 22 or fewer infected wounded females, clearly much less than the observed 31 females. Wounding did not influence the general well-being of the *Daphnia*. Among the non-exposed females, wounded animals produced on average 37.2 offspring, while the non-wounded controls produced 36.0 offspring (*t*-test: $t = -1.4443$, d.f. = 107, $p = 0.15$).

Contrary to our expectation, the progression of the disease did not differ between the exposed groups either. Figure 7 shows the cumulative proportions of females with clear signs of infection (total castration) for both treatment groups. The slopes of the two treatment groups do not differ significantly (analysis of covariance with time as a covariate, a significant interaction between time and treatment would reveal a difference in slope of treatments: *p*-interaction > 0.3).

4. DISCUSSION

Our studies revealed that, across host populations and parasite species, innate PO activity was negatively correlated with the success rate of the parasites (figure 3). This correlation was not found when we corrected for the host population effect (figure 5).

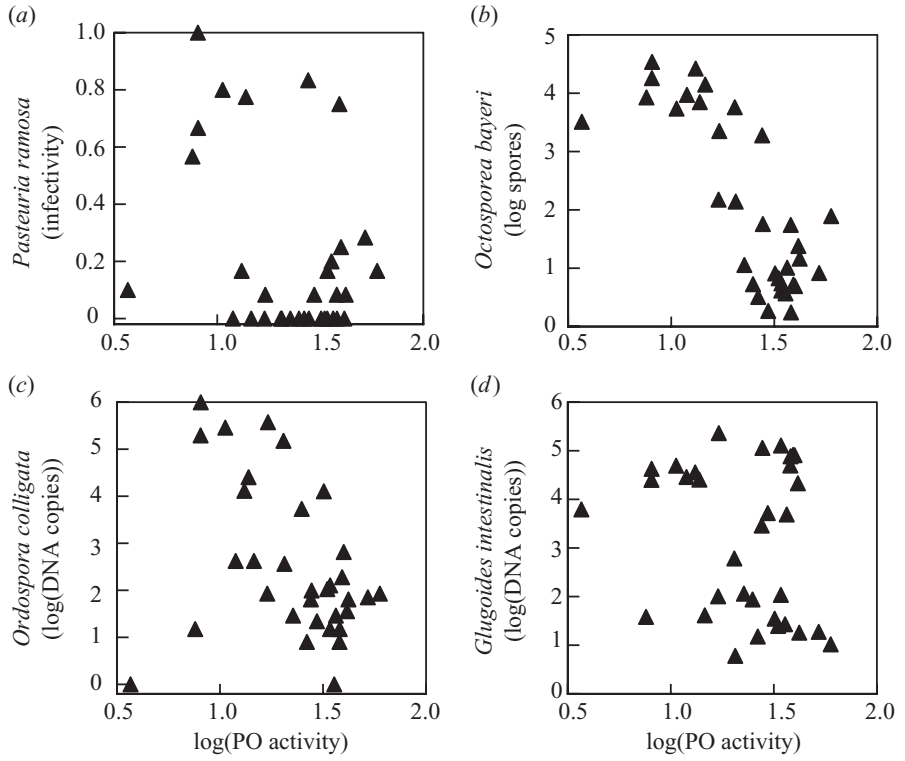

Figure 2. Quantitative measures of parasite success plotted against log(PO activity). Each symbol represents a clonal mean. (*a*) *Pasteuria ramosa* ($r^2 = 0.12$, $p < 0.05$); (*b*) *Octosporea bayeri* (average of two strains; $r^2 = 0.63$, $p < 0.001$); (*c*) *Ordospora colligata* ($r^2 = 0.12$, $p < 0.05$); and (*d*) *Glugoides intestinalis* ($r^2 = 0.04$, n.s.).

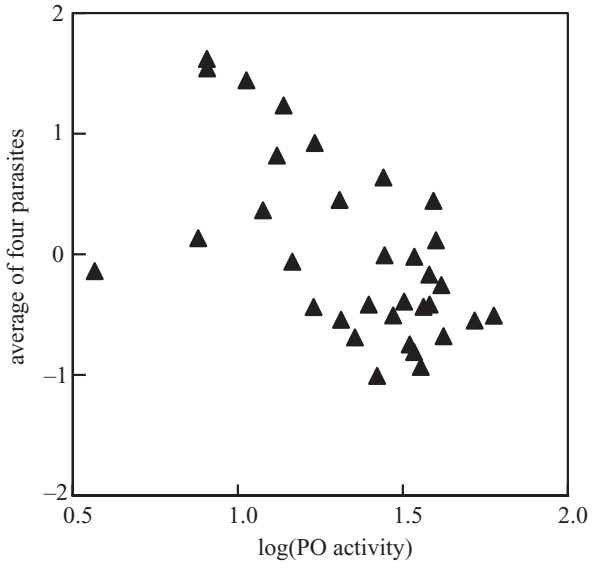

Figure 3. Average parasite success plotted against log(PO activity). Average parasite success is the mean success across four parasite species after the data for each parasite species were standardized to a mean of 0 and a variance of 1; $r^2 = 0.335$, $p < 0.001$.

The main aim of this study was to test whether innate PO activity levels could be used to predict a *Daphnia* genotype's resistance to infection, i.e. as a measure of immunocompetence. Our data clearly show that innate PO activity levels do not allow this conclusion within populations of *D. magna*. As the concept of immunocompetence refers to the average resistance of a host genotype to an 'average parasite', it has a statistical element. Individual parasite isolates may deviate from the 'average parasite' and may thus lead to a rejection of the hypothesis. We found largely consistent results whether we analysed the data for each of the four parasite species separately (figures 2 and 4) or together as the mean parasite success across species (figures 3 and 5).

Our dismissal of the PO–imunocompetence concept for *D. magna* may be challenged along various lines. One might argue that the parasites we chose are not controlled by PO, while other parasite species might support the PO–immunocompetence concept. However, the four species used here are very common parasites of *D. magna* and represent a substantial fraction of the parasites that *D. magna* females encounter in central and northern Europe (Green 1974; Little & Ebert 1999; Ebert *et al.* 2001; Decaestecker *et al.* 2002). If the concept does not work with them, it is questionable whether it has any value for *D. magna*. A similar argument could be made with respect to the tissue specificity of PO. We measured PO activity in the haemolymph, but only the bacterium *P. ramosa* grows in the haemolymph; the other parasites infect the gut and the ovaries (table 1). However, resistance against the four parasite species seems similar across host populations, and we do not expect a different result when using different parasites.

Further, it could be argued that PO activity may work as a measure of immunocompetence if, instead of using the innate PO activity, one measured the induced PO response. This is difficult to test because specific PO induction by parasites is difficult to quantify. Parasites are known to avoid immune induction in various ways, or even to inhibit PO activity (Beckage 1998; Dowd 1999; Shelby *et al.* 2000). Therefore, we tested the effect of induction by inducing PO activity through unspecific

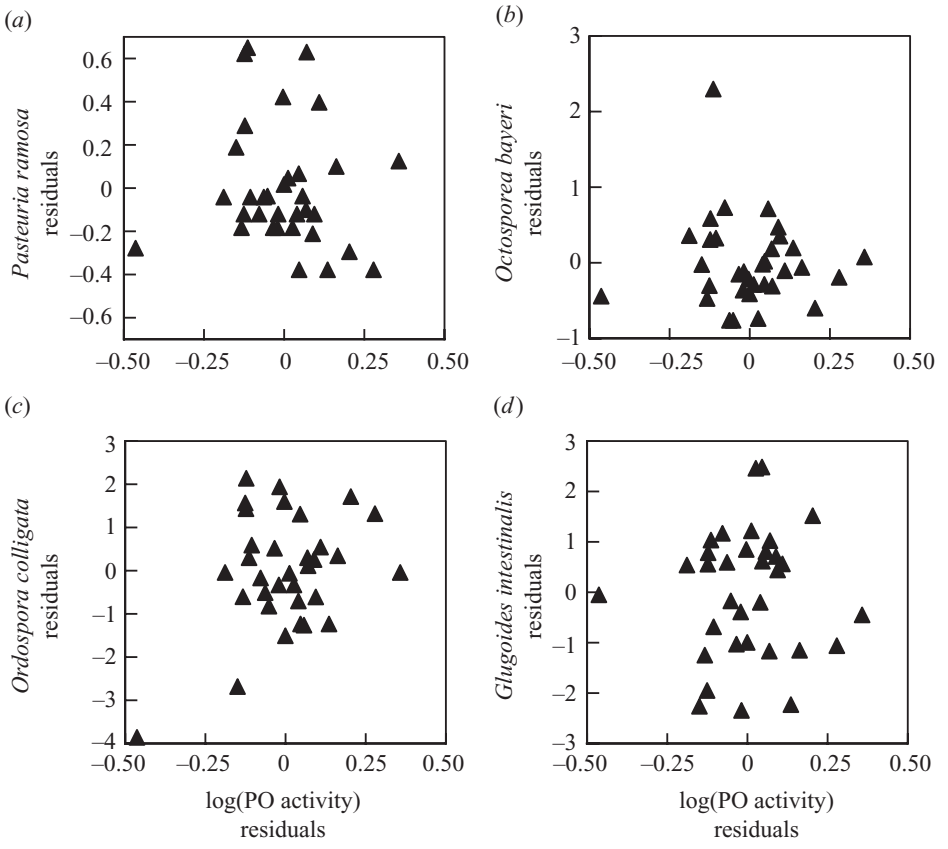

Figure 4. As figure 2, but residuals of parasite success and log(PO activity) were used after the data were corrected for population effects. (*a*) *Pasteuria ramosa* ($r^2 = 0.01$, n.s.); (*b*) *Octosporea bayeri* (average of two strains; $r^2 = 0.005$, n.s.); (*c*) *Ordospora colligata* ($r^2 = 0.11$, n.s.); and (*d*) *Glugoides intestinalis* ($r^2 = 0.001$, n.s.).

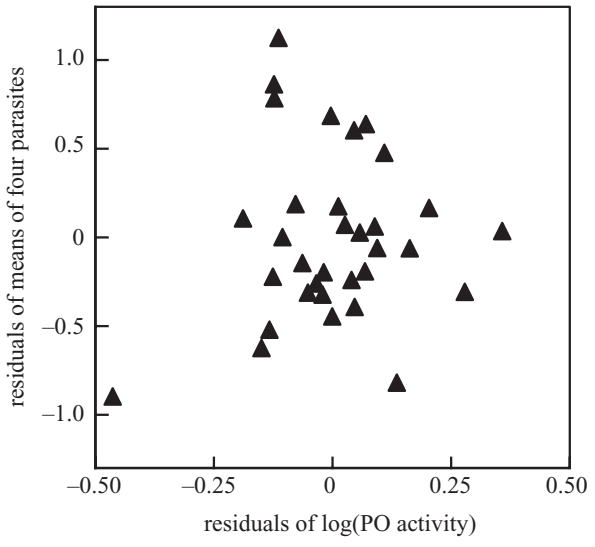

Figure 5. As figure 3, but residuals of average parasite success and log(PO activity) were used after the data were corrected for population effects; $r^2 = 0.009$, n.s.

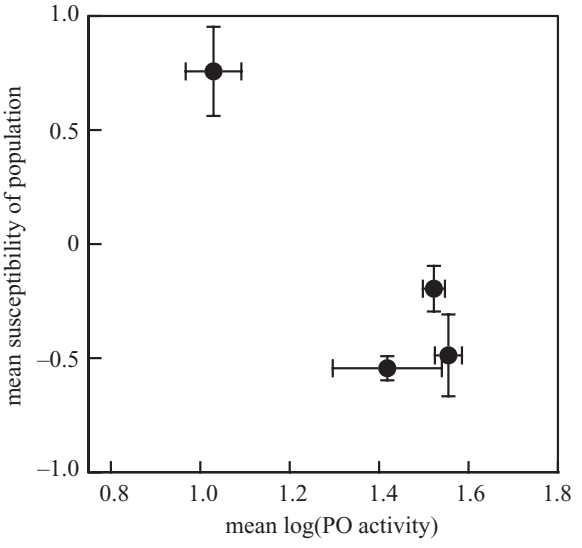

Figure 6. Average parasite success plotted against population mean of log(PO activity) for each of the four host populations. Populations from left to right: Finland, Kniphagen, Munich and Gaarzerfeld.

wounding of the carapace. Our earlier work showed that wounding of the carapace induces a significant (+19%) increase in PO activity within 24 h (Mucklow & Ebert 2003). However, wounded *D. magna* showed the same levels of resistance and disease progression (figure 7) as the non-wounded controls. A problem with wounding is that it may not only affect the PO system, but also induce various other immune components, such as antibacterial compounds. At the same time, wounding may decrease the general well-being of the host, which could further influence susceptibility. As we did not find evidence for a wounding effect on host life history, we believe that the absence of a wounding effect on resistance and disease progression indicates that wounding does not alter host resistance to a reasonable degree. Thus, no evidence so

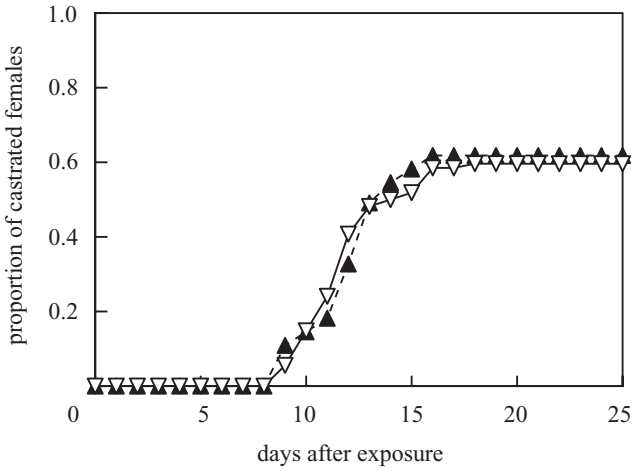

Figure 7. Progression of disease in two cohorts of *Daphnia magna* females exposed to *Pasteuria ramosa*. The *y*-axis shows the cumulative proportion of totally castrated females from the two treatment groups: wounded (open symbols) and non-wounded (filled symbols). Beyond 18 days after exposure, no further infection was recorded.

far suggests that inducing PO activity would change our findings. Nevertheless, further experimentation may be needed before we can definitively state that induced PO does not alter immunocompetence.

Our results indicate that a negative correlation between susceptibility and PO activity may exist across host populations. This finding agrees to some extent with the results of an earlier study in which we used single clones (different from the clones used here) from four populations (three out of the four were the same as the populations in this study) of *D. magna* and one parasite species (Mucklow & Ebert 2003). This earlier study did not, however, allow us to distinguish between population and clone effects or to consider different parasite species (Mucklow & Ebert (2003) used only one clone per population and only *P. ramosa*).

Although four populations might not be enough to support the idea that a cross-population correlation exists, our data are consistent with the idea that a negative covariance may be found by increasing the overall variance for both PO activity and susceptibility, by including host clones from different geographical origins. This may work in favour of the PO–immunocompetence concept, as it is often possible to detect a significant correlation only if the variances are large enough. A problem with the biological interpretation of a cross-population correlation is that other immune traits (e.g. as revealed by haemocyte counts and antibacterial and agglutination tests) may covary with PO activity across host populations, because they may not evolve independently of each other. It is clear from our data that the *D. magna* clones from the Finnish population (upper-left mean in figure 6) show the highest levels of susceptibility to all four parasite species. However, determining whether this is a result of the low PO activity of these clones or of the combined effect of various immune components expressed in these clones is beyond the scope of this study. Low resistance or lower investment in resistance mechanisms may be found in an environment with low rates of parasitism. The Finnish *D. magna* population seems to have comparable levels of parasitism to other populations (Green 1957, 1974; Stirnadel & Ebert 1997; Ebert *et al.* 2001). Thus, we hesitate to propose a correlation between exposure to parasites in the field and PO activity. It seems likely that any attempt to produce such a correlation must consider the temporal patterns of parasite epidemics and, in the case of the Finnish population, possibly the complex population structure of this metapopulation. Further, the coevolutionary history of the host–parasite system may play a role as well (Sasaki & Godfray 1999). Currently this is beyond our knowledge of the infection dynamics of these populations.

Our results do not question the role that the PO system plays in arthropod immunity, for which there is solid evidence (see § 1). However, they do question whether PO activity assays are a useful tool for judging the immunocompetence of hosts. There are certainly other relevant immune components that cause resistance to vary within and across host populations, just as there are various factors across parasite species. The combined effects of different immune components and other genetic factors that influence host fitness may make it impossible to use PO activity as a single measure of immunocompetence in *Daphnia*.

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