Physiology of Immunity in the Water Flea *Daphnia magna*: Environmental and Genetic Aspects of Phenoloxidase Activity

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ABSTRACT

In an attempt to understand the ecological correlates of immunocompetence in *Daphnia magna* (Crustacea, Cladocera), we tested for variation in immune function in relation to feeding conditions, host conditions, and host genotype. We investigated both phenotypic (environmental dependent and condition dependent) as well as genotypic aspects of the prophenoloxidase activating system (Pro-POAS), which has been described as a key factor in invertebrate immunity. *Daphnia magna* is an ideal study system to disentangle phenotypic and genetic variation because females can reproduce clonally. Well-fed *Daphnia* showed higher phenoloxidase (PO) activity than *Daphnia* kept at a low food level. Wounding provoked a higher level of PO activity, indicating that the Pro-POAS was condition dependent. Further, we found clonal variation in PO activity among four clones of *D. magna* isolated from four different populations. The same four clones were tested for their resistance to the bacterial pathogen *Pasteuria ramosa.* High resistance corresponded to high PO activity. Our results suggest adaptive variation in PO activity and suggest that its expression is costly. These costs may influence the evolution of the PO activity level and the maintenance of its genotypic variation.

Introduction

There are increasing numbers of reports on parasite epidemics in natural zooplankton populations (Green 1974; Brambilla 1983; Vidtmann 1993; Stirnadel and Ebert 1997). Given such epidemics, one may expect that zooplankton have evolved a well-developed immune system to cope with their natural enemies. We know of a variety of immune mechanisms in other invertebrates: cellular responses such as phagocytosis, encapsulation, nodulation formation, and cytotoxicity as well as acellular mechanisms such as antibacterial proteins, agglutinins, and other defenses (Millar and Ratcliffe 1994). An important immune function in invertebrates is the prophenoloxidase activating system (Pro-POAS), which acts in cellular and acellular immunity. The Pro-POAS has been demonstrated in ascidians, molluscs, and many arthropods (Millar and Ratcliffe 1994; Hata et al. 1998; Söderhäll and Cerenius 1998; Deaton et al. 1999). The ability to produce phenoloxidase (PO) is considered a key aspect of invertebrate immunity (Aspan et al. 1995; Hauton et al. 1997; Reeson et al. 1998; Barnes and Siva-Jothy 2000) and helps hosts defend against invading parasites in a variety of ways, such as providing opsonins for recognition of nonself, initiating capsule and nodule formation, enabling microbial killing, and mediating in cell-cell communication. The Pro-POAS is an enzyme cascade in which β-1,3-glucans, lipopolysaccharides, and peptoglycans turn the inactive enzyme prophenoloxidase (Pro-PO) into the active form PO via several steps. PO oxidizes phenols, which leads to the formation of melanin, which is believed to be important in killing bacteria (Söderhäll and Cerenius 1998). The formation of melanin can be observed in a spectrophotometrical reaction in vitro and therefore can be used to quantify the activity of PO. PO activity thus can be used to measure the function of the Pro-POAS, since PO is the key enzyme of this system (Söderhäll and Cerenius 1998). Here we present the first investigation of the PO system in *Daphnia.*

It has been argued that a well-functioning immune response must be costly; otherwise all organisms would be resistant to all kinds of pathogens (Lochmiller and Deerenberg 2000). Nevertheless, the nature of the constraining factors for a perfectly functioning immune system is still not clear. Many studies report on environmental-dependent and condition-dependent factors that affect immune function (Li et al. 1992; König and Schmid-Hempel 1995; Hauton et al. 1997; Reeson et al. 1998; Siva-Jothy et al. 1998; Moret and Schmid-Hempel 2000; Rigby and Jokela 2000) whereas others focus on genetic determination as a constraining factor in host defense (Fryer and Bayne 1989; Carton et al. 1992; Boots and Begon 1993; Kraaijeveld and Godfray 1997; Langland et al. 1998).

If immune function were costly, one would expect the expression of the immune activity to depend on the environment. This dependency might manifest itself in various forms. First,

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if immune function were resource dependent, we would expect it to be reduced when resources were limited (Sheldon and Verhulst 1996). Here, we test experimentally whether a reduced food level decreases immune function. Second, immune function might increase if there is a high likelihood of contracting disease (Li et al. 1992; Nordling et al. 1998; Siva-Jothy et al. 1998). For a wounded organism, it is vital to allocate more energy into immunity to prevent infection (Nayar and Knight 1995; Møller et al. 1998). We tested whether wounded hosts show a stronger immune response than healthy controls. Third, as the environment changes geographically, one might expect that hosts taken from different areas differ in their immune function. Genetic diversity in resistance against parasites has been shown for several systems (Boots and Begon 1993; Henter and Via 1995; Kraaijeveld and Godfray 1997), including *Daphnia* (Ebert et al. 1998; Little and Ebert 1999; Carius et al. 2001), and genetic variance for resistance is one of the main assumptions for host-parasite coevolution (Ebert and Hamilton 1996; Sasaki and Godfray 1999). We tested whether host genotypes from different populations differ in their immune function in the absence of real parasites and whether this correlates with these clones' resistance to the bacterial pathogen *Pasteuria ramosa*.

The water flea *Daphnia magna* Straus is a cyclic parthenogenetic cladoceran whose clones can be maintained in the laboratory. Because females reproduce clonally, it is a suitable organism in which to study the immune system. We used the water flea *D. magna* in this study to investigate the effects of environmental, condition-dependent, and genetic factors on the Pro-POAS.

Material and Methods

Origin of Clones

For the two experiments in which phenotypic plastic responses of immune function were tested (food levels and wounding), one *Daphnia magna* clone was randomly chosen from a sample collected from a pond near Gaarzerfeld (north Germany). In the four-clone experiment, four arbitrarily chosen clones originating from populations in different geographical locations (a pond near Gaarzerfeld in north Germany, a pond in Regents Park in London, Ismaninger Teiche near Munich, and a rockpool near Tvärminne in southern Finland) were used. At the time of the experiments, all clones had been kept in the laboratory for at least 10 generations.

General Experimental Procedure

In all the experiments, *Daphnia* were kept at 20°C and a 16L : 8D interval in artificial culture medium (Ebert et al. 1998). For each of the two nongenetic experiments (food levels and wounding), five mothers from the same *D. magna* clone were raised together in the same 400-mL jar and fed 10^7 cells of the unicellular algae *Scendesmus gracilis* per individual per day. The medium was changed every 2 d. Clonal offspring, 0–24 h old, from the third or fourth clutch of these mothers were then collected within 24 h. Sixty female offspring were randomly chosen for the experimental generation and raised individually in 100-mL jars. In each experiment, 30 individuals were randomly assigned to each of the two treatments.

To randomize maternal and culture effects of the different clones in the four-clone experiment, 20 maternal lines per clone ($4 \times 20 = 80$ lines) were maintained for two generations individually in 100-mL jars and fed 5×10^6 cells of *S. gracilis* per day per individual. At birth, 5 d after birth, and then after every adult instar (ca. every 3 d), the females were placed into clean 100-mL jars filled with fresh medium. The second-generation females were all born within a 2-d period and matured within a 4-d period. These females were offspring from the third, fourth, or fifth clutch of the previous generation. Newborn females (one from each maternal line) were then randomly collected for the experimental generation from the fifth clutch of the second-generation mothers. All 80 females in the experimental generation were raised individually in a 100-mL jar.

Experiments

Two-Food-Level Experiment. In this experiment, we tested whether well-fed *Daphnia* show a higher PO activity than poorly fed *Daphnia*. In each treatment group, 30 individuals of *D. magna* were raised individually in 100-mL jars. During the first 6 d, *Daphnia* were fed a high level of food (5×10^6 cells of algae per *Daphnia* per day) or a low level of food level (1.67×10^6 cells of algae per *Daphnia* per day). On day 7, food amounts were doubled to 10^7 cells of algae per *Daphnia* per day (high food) and 3.3×10^6 cells per *Daphnia* per day (low food) to accommodate the higher needs of the *Daphnia*. Both these feeding rates are relatively high compared with natural conditions (Frost 1985) because we required *Daphnia* to be of a minimum size to handle during hemolymph collection. The high food level is double the standard feeding rate in other experiments, whereas the low food level is well below this standard (Ebert et al. 1998). The medium was changed every 2 d. In this and the following two experiments, all females were bled 21 d after birth.

Wounding Experiment. In this experiment, the PO activity of wounded *Daphnia* was compared with that of nonwounded *Daphnia*. Two groups of 30 *Daphnia* each were used: wounded and nonwounded, the latter acting as the control group. All individuals were fed 5×10^6 cells/d of *S. gracilis* per *Daphnia*, and the medium was changed every 3 d. The spina of all *Daphnia* from the wounded group were cut off with a sterile scalpel under a dissecting microscope 24 h before hemolymph was collected. All individuals in the nonwounded group were handled under the dissecting microscope for the same amount of

time as the wounded *Daphnia*. At bleeding (24 h later), a small dark rim (melanization) at the base of the cut off spina was observed, indicating that a melanization reaction had taken place to close the wound. The decision to bleed the *Daphnia* 24 h after wounding was based on a pilot experiment in which we found that PO activity peaked 24 h after wounding (PO activity was measured every 6 h for 48 h).

Four-Clone Experiment. Four *D. magna* clones were examined for genetic variation in PO activity; 20 maternal lines of each of the four clones were maintained for this experiment. All *Daphnia* from the experimental generation were kept under constant, identical conditions to detect clonal variation. Each female was fed 5×10^6 cells/d of *S. gracilis,* and the medium was changed at birth, after 5 d, and from then on after every adult instar (ca. every 3 d). At 28 d after birth, we measured the length of all animals (from the base of the spina to the tip of the head) and collected their hemolymph to assess PO activity.

Collecting Hemolymph

Individual female *Daphnia* were first rinsed briefly in deionized H_2O and placed on filter paper for drying until all the excess medium trapped between the carapace was removed. The hemolymph of the *Daphnia* was then collected by pricking the carapace close to the heart with a needle. A small droplet of hemolymph immediately oozed out, and 1 μL was collected with a microcapillar tube (BLAUBRAND, intraMark cat. no. 708707). If 1 μL could not be collected immediately, bleeding was enhanced by pressing softly against the base of the second antenna with a pair of tweezers. This procedure minimized damage to the *Daphnia* and thus avoided contamination by digestive fluids or other sources. Collecting 1 μL hemolymph required some training but was possible with precision microcapillar tubes (producer-given precision <0.6%). We rejected samples in which we obtained less or more hemolymph (i.e., more than 2 mm below or above the 1-μL line). We estimate our error in volume to be less than 5%. To reduce sampling bias across treatments within experiments, hemolymph samples of the animals belonging to different treatments were collected in alternating sequence.

Phenoloxidase Assay

The activity of the enzyme PO was used to measure the immune function of *D. magna*. The PO activity was recorded in the form of dopachrome production, which is brought about by the oxidation of L-dopa by the enzyme PO. As established in other studies, absorbance at 475 nm can be measured with a spectrophotometer (Nayar and Bradley 1994; Reeson et al. 1998). We used a modified version of Reeson et al. (1998): 1 μL of hemolymph was deposited in an Eppendorf tube with 150 μL of phosphate-buffered saline (PBS; 0.15 M NaCl, 10 mM $Na_2HPO_4 \cdot 2H_2O$, 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 at 475 nm immediately after adding the L-dopa and again after 4.5 h. Additionally, 10 controls were performed in each experiment, using PBS and L-dopa, but without hemolymph. The PO activity of the controls and treatments was calculated as the difference of the absorbance values measured (absorbance after 4.5 h − absorbance at time 0). For convenience, we multiplied this difference by a factor of 1,000 to yield activity units. The activity units of the "nonhemolymph" controls were subtracted from all data in each experiment. This allowed us to verify whether the observed activity was due to the enzyme present in the hemolymph or to a nonenzymatic reaction of the chemicals themselves. Because the nonhemolymph controls are independent of the treatments, they had no influence on the differences among treatment groups reported here. A further control consisting of PBS and hemolymph only was not performed during this assay because preliminary experiments showed no difference in oxidation compared with the nonhemolymph controls (P. T. Mucklow, unpublished data).

The activity of PO was also determined in samples activated by detergent and by freezing in liquid nitrogen, which destroyed all cells in the hemolymph. No further activation of PO was observed. Subsequent samples were therefore analyzed as described previously without additional activation procedures.

Resistance to Bacterial Pathogen

To test for a correlation between the PO activity and pathogen resistance, we tested the same four clones used in the four-clone experiment for their resistance to the bacterium *Pasteuria ramosa* Metchnikoff 1888 (Ebert et al. 1996). This experiment closely followed the experiment of Carius et al. (2001), with the same system. The pathogen isolate was from the same population as the one from which the Gaarzerfeld clone was isolated. We keep 30 lines per clone under the same conditions as described previously for three generations. From the third generation we collected two female offspring from each line and kept them singly in 20 mL of medium (total 4 clones × 2 treatments [control and spore exposed] × 30 replicates = 240). When they were 3 d old, we added a suspension without spores (as a placebo for the control group) to one female and a suspension containing 10,000 pathogen spores to the medium of the other female offspring. The suspensions had been produced by grinding up heavily infected hosts and diluting them with a suspension of ground-up uninfected hosts, so that the spore suspension and the placebo (control) contained the same amount of host tissues, although they differed in the presence of parasite spores. Animals were fed daily, and the medium was filled up to 100 mL at day 6. In the following

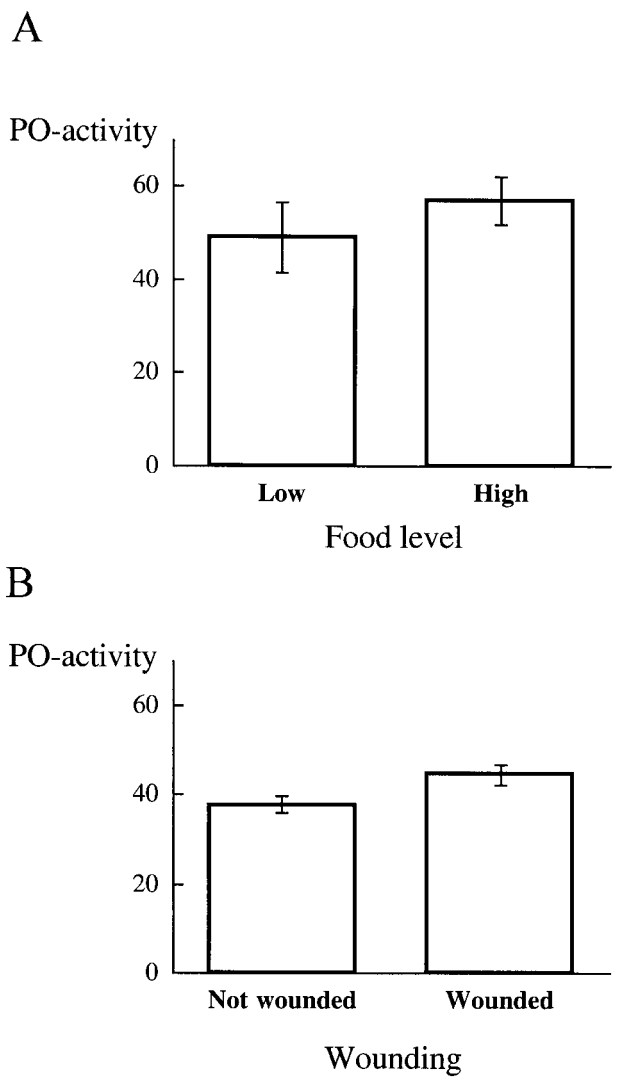

Figure 1. Levels of phenoloxidase (PO) activity in the food-level experiment (*A*) and the wounding experiment (*B*). Means of PO activity (± SEM, $N = 30$) are given.

days we changed the water every 5 d until day 30. Then all females were tested for *Pasteuria* infections. *Pasteuria ramosa* infections can be seen with the naked eye because the infected hosts turn brownish red in color and stop reproducing. None of the controls were infected. We lost 31 replicates due to a handling mistake, leaving us with 89 replicates in the infection treatment.

Data Analysis

PO data were analyzed as PO activity units using JMP IN (Sall et al. 1996). Wilcoxon tests were performed for the food level and the wounding experiments. The PO activity of the four-clone experiment was analyzed with a one-way ANOVA, using log-transformed data. The residuals were normally distributed. Infection data were analyzed with a logistic regression (binary data for infection [I/0] with a logit link function), with clone as main effect. This analysis included only the infection treatment.

Results

After subtracting the nonhemolymph controls from the experimental data, all measures of PO activity were positive. This indicates that there was PO activity in all samples containing hemolymph. Well-fed *Daphnia magna* displayed a significantly higher PO activity than poorly fed individuals (Wilcoxon χ^2 approximation = 4.54, df = 1, $P = 0.03$; Fig. 1*A*), although the difference of mean values was not large: 56.87 (± 5.29 SEM) activity units for well-fed *Daphnia* and 48.87 (± 7.63 SEM) activity units for poorly fed animals. At time of bleeding, well-fed animals were larger than poorly fed animals ($P < 0.0001$, df = 1). This size difference is unlikely to confound our results because we used the same amount of hemolymph from all animals and we could not detect a correlation between body size and PO activity (Pearson correlation between PO activity and body length in high food: $r = 0.03$, $P > 0.5$; in low food: $r = 0.11$, $P > 0.5$).

A significant difference in the PO activity was found between wounded and nonwounded *Daphnia*, with wounding resulting in higher values of PO activity units than nonwounding (Wilcoxon χ^2 approximation = 6.04, $P = 0.01$; Fig. 1*B*).

In the four-clone experiment, different clones of *D. magna* varied significantly in PO activity ($F = 15.31$, df = 3, $P < 0.0001$; Fig. 2). All clones in the four-clone experiment showed much lower mean values of PO activity than any treatment of the other experiments, possibly because of the higher age of the infested animals (28 d vs. 21 d at bleeding; P. T. Mucklow, personal observation).

The four clones also differed strongly in their resistance to the bacterial pathogen *Pasteuria ramosa*, ranging from 7% to 94% (Fig. 2; $\chi^2 = 48.5$, df = 3, $P < 0.0001$). There was a perfect negative correspondence between resistance and PO activity such that the Finnish clone with the lowest PO activity suffered the highest infection rate, whereas the south German clone was most resistant and had the highest PO activity. Due to the small number of clones ($n = 4$), this correlation was only marginally significant (Pearson correlation: $r = 0.92$, $P < 0.1$).

Discussion

Our study addresses three important aspects of PO activity levels, which are thought to relate to immune function: first, these activity levels depend on the resource level; second, they are upregulated following wounding; and third, they differ among genotypes. The difference measured in the food-level experiment suggests that the maintenance of a high PO activity level may be energy consuming. Without food limitation, *Daph-*

nia expressed PO activity on a high level, but when confronted with low food availability, immune function appeared to be somewhat compromised. The difference in food levels in our experiment was only threefold, whereas in a natural environment, the resource levels of planktonic crustaceans may vary by several orders of magnitude (Frost 1985), suggesting that the effect of food on PO activity may be even stronger. We used a reasonably high resource level in the low-food treatment so that we could maintain large enough *Daphnia* to gain at least 1 μL of hemolymph when bleeding. As low resource levels retard growth in *Daphnia*, acquiring this much hemolymph would not have been possible under lower food levels. The dependency of immune function on resources has been reported in a number of other organisms (for review, see Lochmiller 1996; Lochmiller and Deerenberg 2000), and a link between resource level and immune function is consistent with the idea of a cost of resistance. For example, Kraaijeveld and Godfray (1997) found that after increasing intraspecific competition in *Drosophila*, which also involves food stress, the survival of flies selected for high resistance decreased more than the survival of unselected controls. A direct link between fitness and PO activity was, however, not addressed in our study.

Our study also revealed that 24 h after injury, wounded *Daphnia magna* have a higher PO activity than nonwounded individuals. This supports the idea that immune function may be costly and mounted only when necessary (Lenski 2002). Being wounded and therefore at high risk of infection, *Daphnia* allocate increased resources to host defense and/or wound healing. The PO of wounded *Daphnia* might have a double function (Millar and Ratcliffe 1994). First, PO may eliminate microorganisms that pass through an open wound into the body. Second, it is also thought that certain components of the Pro-POAS may be involved in attracting hemocytes to an open wound, thus enhancing wound repair. The immune function can then be expected to return to a lower level after the wound is healed or pathogens have been successfully eliminated. Facultative investment into defense and healing allows for the allocation of resources into other traits, such as reproduction (Nordling et al. 1998; Siva-Jothy et al. 1998; Rigby and Jokela 2000), when not needed for immunity. Our results correspond to other studies that suggest the condition dependency of immune function in insects (Li et al. 1992; König and Schmid-Hempel 1995; Nayar and Knight 1995; Siva-Jothy et al. 1998).

Along with showing that Pro-POAS was influenced by environmental and conditional influences, our study also showed genetic variation for PO activity within natural isolates of a species. Since genetic variability exists in PO activity, natural selection can act on immune function. The correlation between resistance to *Pasteuria ramosa* and PO activity (Fig. 2) suggests an adaptive function of high PO levels, although we cannot exclude that other differences among the four clones confound this result. Other studies on the relationship between PO level and resistance are in line with our finding (Gomes et al. 1999; Shiao et al. 2001), strengthening our proposition that high PO activity is adaptive for *Daphnia* to resist infections. However, it should be noted that we used clones from four different populations and thus are unable to make a statement about evolution of PO activity within populations.

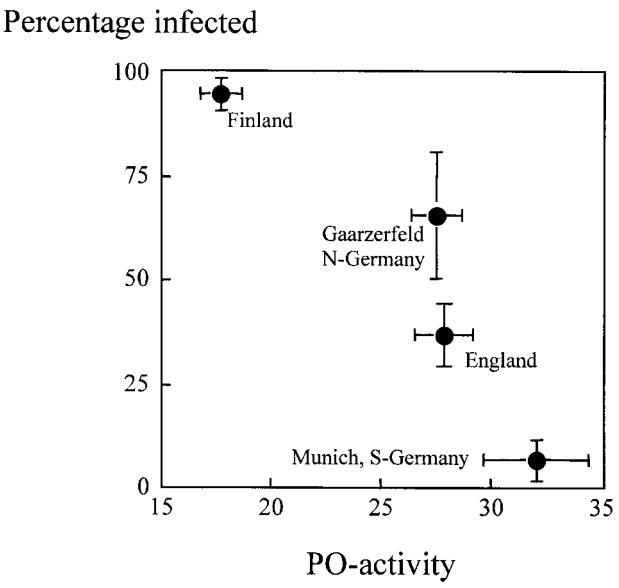

Figure 2. Percentage of infected females from each of four clones ($\pm$binomial error) plotted against the mean phenoloxidase (PO) activity for female *Daphnia magna* from the same four clones. The labels in the figure refer to the origin of the four clones.

If PO has a protective function, one would expect natural selection to increase PO activity to ever higher levels unless there are costs for its expression. In this light, the observed variation among the four clones from four populations may reflect local differences (e.g., in parasite abundance) that would alter the cost-benefit ratios for PO activity. Various levels of parasite prevalence in natural populations of *Daphnia* have been shown (Stirnadel and Ebert 1997), and Kraaijeveld and Godfray (1999) discuss similar geographical variation for the ability of encapsulation in *Drosophila*. Since encapsulation involves the production of melanin (Millar and Ratcliffe 1994), Kraaijeveld and Godfray's findings might be due to the genetic variation of the ability to produce PO.

Because we used only four clones, each coming from different populations with different geographical locations (north and south Germany, England, and Finland), it was not possible to investigate the extent to which PO activity varied genetically within a population. The aim of further studies is to gain more information about the within-population and between-population genetic variation of PO activity. Clonal variation in PO activity might indeed explain the *D. magna*'s differences in resistance against various parasites (Ebert 1994; Ebert et al. 1998). In future studies, we will test for such correlations.

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