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Differential expression of candidate immunity genes in *Daphnia magna*, in response to the castrating parasite *Pasteuria ramosa*

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Summary

The main objective of the present study was to investigate the expression of 4 candidate immunity genes in the crustacean *Daphnia magna,* when exposed to the castrating bacterial parasite *Pasteuria ramosa*.

Specifically, we looked at the following genes:

i) Prophenoloxidase (pPO), involved in the production of melanin
ii) Nitric oxide synthase (NOS1 and NOS30) and Arginase, involved in the production of nitric oxide

The interaction between *D.magna* and *P.ramosa* is characterized by a very high level of specificity (Carius *et al* 2001). We used two different *D.magna* host clones, "GG4" and "GG7", which differ in their response to the parasite isolate applied: "GG4" is highly susceptible, whereas "GG7" is resistant. Thus, an additional objective of the present study was to gain further insight into the molecular background of the specificity found in this host-pathogen system.

Melanisation and the production of nitric oxide represent two of the best characterized general effector mechanisms of the invertebrate immune response. The activity of the genes investigated in the present study should therefore, once confirmed as immunity genes, reflect whether an immune response has been triggered or not, and potentially the strength and timing of such a response.

We found significant increases in the expression of pPO in response to *P.ramosa* at two different time-points: An early response, 1-2 hours after exposure, and a late response, 16 days after exposure. However, contrary to our expectations, the response was only seen in the susceptible genotype "GG4". We therefore hypothesise that there are two levels of resistance in the *D.magna-P.ramosa* interaction: first, the parasite must successfully enter the host, and second, the immune response of the host must be combated. Thus, the response specificity could be interpreted as a failure of the parasite to invade the resistant clone. We are currently investigating the response of pPO in other clone-parasite combinations, to test this hypothesis.

We did not find any significant effects of exposure to *P.ramosa* in either NOS1, NOS30 or Arginase. We found a significantly higher basic level of expression of NOS1 in the resistant clone compared to the susceptible clone, but since no effect of exposure was found, more studies are needed to assess the relevance of this feature. As a consequence of the specificity of the response seen for pPO, other clone-parasite combinations will be investigated, also to evaluate the importance of NOS1, NOS30 and arginase, in the immune response of *D.magna.*

Contents

1. Introduction

1.1 Parasites and Evolution

And the red queen said to Alice: "Now, here, you see, it takes all the running you can do to stay in the same place" (Lewis Carroll, 1872).

In 1973, Van Valen was the first to use the popular quote from Lewis Carrolls book "Through the looking glass, and what Alice found there" (1872) in an evolutionary context. In his paper "A new evolutionary law", Van Valen proposed that all organisms are locked in a perpetual arms race with their competitors and therefore need to evolve constantly to avoid extinction. Later, the "Red Queen Hypothesis" was more specifically established by Bell (1982) to describe the influence of parasites in driving selection, and in particular the maintenance of sexual reproduction.

Parasites are ubiquitous on all living organisms. Because many of them also have severe consequences for their hosts, parasites have been predicted to influence several aspects in evolution, such as driving genetic change, the maintenance of gene flow, speciation and the maintenance of sexual reproduction (e.g. Altermatt *et al.* 2007, Bell, 1982, Ebert and Hamilton 1996). For parasite-driven selection to occur, genetic variation in susceptibility (the probability of becoming infected) must be present, and parasites must have a negative impact on host fitness (Ebert and Hamilton, 1996). These requirements have been abundantly demonstrated (reviewed by Little, 2002).

However, although the potential for parasite-driven selection appears to be present, it has proved harder to find direct empirical evidence (but see e.g. Decaestecker *et al.* 2007, Duncan and Little 2007). For example, responses to selection in the wild can be hard to predict, because the cost of infection often depends on environmental variables (e.g. Mitchell *et al.* 2005). Furthermore, hosts may respond to parasite challenge by behavioural changes (avoidance), or reduce the cost of infection through changes in life history traits, e.g. the timing of reproduction, which can be difficult to monitor (Duncan and Little 2007). Another problem, common for most evolutionary studies, is the time scale, i.e. following a sufficient number of generations to detect selection.

Invertebrates are convenient for research in the evolution of host-pathogen interactions, because they tend to have very short generation times and often are amenable to laboratory experiments. Parasites may interact with their hosts through the immune response, and therefore a direct effect of parasite-driven selection would be expected on at least some genes involved in the immune response. However, the genes underlying the immune response of the invertebrates are generally not well characterized, and particularly genes determining host-pathogen specificity are entirely lacking (Duncan and Little 2007). This project takes another step to help that elucidation, by looking for immune response genes in the crustacean *Daphnia magna.*

1.2 The immune system of invertebrates

Research on the immune response of invertebrates has increased considerably during the past decade (Rivero 2006). Invertebrates do not have an adaptive immune response, such as the one which characterize the vertebrates, but instead they deal with most of their parasites using a non-specific defence mechanism termed the innate immune response. "Innate" refers to the fact that this type of immune response is genetically inherited, in contrast to the adaptive immune response, which continuously evolves during the life-time of an organism, and is capable of remembering previously encountered pathogens. The innate immune response is also found in vertebrates, but is usually interlinked with the adaptive response, which complicates the separate characterization of each response. The innate immune response is believed to be the more ancient of the two responses (Cerenius and Söderhall 2004), and an understanding of the innate immune response is therefore essential for understanding the evolution of the immune response as a whole.

The invertebrate immune response can be partitioned into three steps: Recognition, Transduction (through signalling pathways) and Attack (through effectors) (see fig.1.1) (Schmid-Hempel 2005). Recognition of bacteria and fungi can be mediated by pattern recognition receptors (PRRs), a group of proteins capable of binding pathogen-associated molecular patterns (PAMPs), such as conserved compounds of the bacterial or fungal cell wall. After the initial binding of PAMPs, a number of different pathways may be induced, ultimately leading to the production of effectors, such as antimicrobial peptides, melanin or reactive oxygen species.

Although a large number of genes have been confirmed to function in the immune response of invertebrates, most studies have been carried out in model species, and therefore it is still an open question how conserved the genes are, and whether they have the same functions in all invertebrates. This problem is further exacerbated by the fact that some of the effectors of the invertebrate immune response also have additional non-immune-related functions.

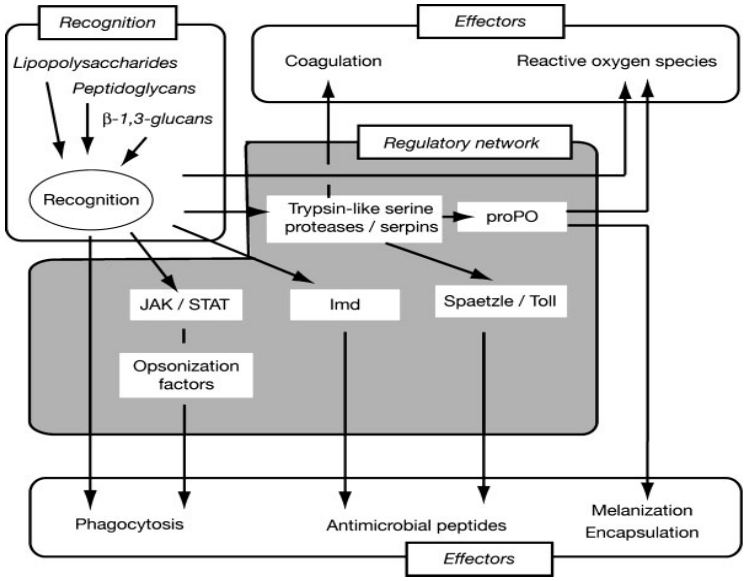

Fig. 1.1: Schematic representation of the immune response of Drosophila melanogaster (Schmid-Hempel 2005).

1.3 Effectors of the invertebrate immune response

1.3.1 Prophenoloxidase (pPO)

Melanisation, mediated by pPO, is probably the most widely investigated mechanism of the invertebrate immune response, and is thought to be unique to the invertebrates (Cerenius and Söderhall 2004). “Melanisation” refers to the deposition of melanin around foreign substances. The melanin will physically shield an intruder, and equally important, melanin formation is accompanied by the production of toxic intermediates. Melanin is also involved in other aspects of invertebrate biology, including wound healing, pigmentation and sclerotization (Cerenius and Söderhäll 2004). The production of melanin is initiated by the prophenoloxidase-activating system, as the end product of an enzymatic cascade in which pPO is the bottleneck enzyme (see fig.1.2 for a schematic representation of the enzymatic cascade).

pPO is a zymogene (inactive enzyme precursor), which must be activated by cleavage to yield the functional PO. This activation is accomplished by the pPO-activating enzyme (ppA), which is also produced as a zymogene and must be activated by yet other enzymes further upstream in the cascade. In some cases additional cofactors are needed together with active ppA to activate the pPO (Cerenius and Söderhall 2004). Once active, PO converts phenols into quinones, which subsequently polymerize into melanin. Melanin is eventually deposited onto a parasite, and, when further haemocytes are recruited, can lead to encapsulation.

The proPO cascade is known to be inducible by microbial carbohydrates, such as ß-1,3-glucans, peptidoglucans and lipopolysaccharides, which are recognized by pattern-recognition receptors (PRRs), but the steps from the recognition by these proteins to the triggering of the proPO cascade are still not well understood (Cerenius and Söderhall 2004), and other inducers might also exist. The activation of pPO appears to be tightly regulated, and various protein inhibitors (mainly serpins) along the activating cascade have been identified (see fig.1.2)

The pPO gene has been cloned from a large number of invertebrates, and the importance of the melanisation process in the immune response has been demonstrated in several species (Cerenius *et al.* 2008). Most studies have investigated the activity of the PO enzyme directly, and the importance of transcriptional regulation of the pPO gene is still not well understood. However, a number of studies have shown that the transcription of the gene can be increased when hosts are challenged with parasites or immunostimulants (e.g. Cerenius *et. al* 2003, Ko *et al.* 2007, Rajagopal *et al.* 2005).

How parasites deal with the active PO also awaits further investigation. It has been shown that venoms from endoparasitoids can interfere with the pPO activation (Asgari *et al.* 2003), and it is thus plausible that other parasites have developed similar strategies. Another specific example of adaptation is seen for the parasite *Aphanomyces astaci* (crayfish plague), which remains viable in its host *Pastifastacus leniusculus* (crayfish) while encapsulated by a sheath of melanin, and can resume growth if the host gets weakened by other factors (Cerenius *et al.* 2003).

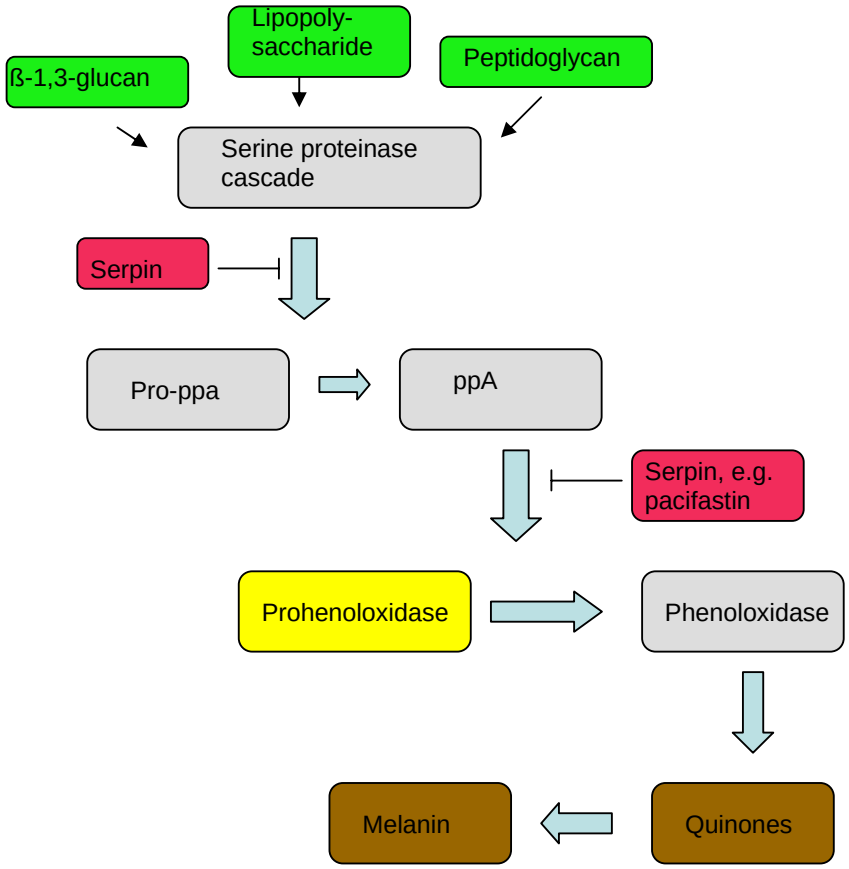

Fig.1.2 The prophenoloxidase activating cascade (Adapted from Cerenius and Söderhäll 2004, fig.1). Enzymes which are part of the cascade are marked in grey or yellow. Inducers are marked in green, inhibitors in pink and end products in brown. The gene for which the expression was investigated in the present project is highlighted in yellow.

1.3.2 Nitric oxide synthase and Arginase

Nitric oxide (NO) is a toxic compound, produced by nitric oxide synthase (NOS) (Rivero 2006). This enzyme is well known in vertebrates, where it exists in three different copies: eNOS (endothelial), nNOS (neuronal) and iNOS (inducible) (Tripathi *et al.* 2007). eNOS and nNOS are produced constitutively, at low levels. In contrast, iNOS is inducible, and can produce NO at very high levels, the expression being determined by the *de novo* synthesis and stability of iNOS mRNA and protein (Tripathi *et al.* 2007). iNOS has been implicated in the innate immune response of vertebrates, particularly in connection with inflammation (a general response to infection in vertebrates) (Tripathi *et al.* 2007). The induction of iNOS is presumably under strict control, since large quantities of NO is toxic to both hosts and pathogens (Rivero 2006).

NO has been firmly established as a signalling molecule in invertebrates, where it plays a role in a large number of physiological, developmental and behavioural processes (Yeh *et al.* 2006). More recently, evidence of the involvement of NOS in the immune response of invertebrates has been obtained in a number of organisms such as mosquitoes (Herrera-Ortiz *et al.* 2004, Luckhart *et al.*

1998), fruit fly (Foley and O´Farrell 2003), silk worm (Imamura *et al.* 2002), triatomid bug (Whitten *et al.* 2007), blow fly (Faraldo *et al.* 2005) and molluscs (Wright *et al.* 2006). The exact function of the NO produced by NOS is still unclear. NO is toxic to most pathogens on its own, but has also been shown to induce the production of antimicrobial peptides, thus functioning as a signalling molecule rather than killing the parasite directly (Foley and O´Farrell 2003, Imamura *et al.* 2002). The details of the regulation of NOS and its possible induction of other immune mechanisms remain to be elucidated.

Homologues of NOS have only been found in one copy in most of the invertebrates investigated so far (Rivero 2006). However, two copies of the NOS gene have recently been identified in *Daphnia pulex* and *Daphnia magna* (unpublished results). These copies are highly diverged, suggesting that they are not the product of a recent duplication event (personal communication, Pierrick Labbe). It therefore seems plausible that only one of the genes should be involved in the immune system of the *Daphnia.*

In the evolutionary arms race between host and pathogen, pathogens have evolved various means to defend themselves against NO produced by their hosts. Some pathogens appear to have moved to tissues rich in NO-scavenging proteins, such as haemoglobin and myoglobin (Rivero 2006). Other parasites induce the enzyme arginase, which uses the same substrate as NOS, namely arginine, in this way both inhibiting the production of NO and getting compounds important for proliferation (Vincendeau 2003).

1.4 The host-pathogen system: *Daphnia magna* and *Pasteuria ramosa*

In the present study we investigated the expression of the genes encoding pPO, NOS and arginase in a well studied host-pathogen interaction, the *Daphnia magna- Pasteuria ramosa* interaction.

1.4.1 *Daphnia magna*

Daphnia are planktonic crustaceans, belonging to the order cladocera. The genus *Daphnia* contains more than 100 known species of freshwater plankton organisms around the world. *Daphnia* are found in many types of freshwater habitats, ranging from large lakes to temporary pools, such as rock pools (Ebert 2005). Most *Daphnia* reproduce both sexually and asexually (i.e. with cyclic parthenogenesis), their life-cycle is shown in fig.1.3. During the warm season, females produce a clutch of parthenogenic eggs after each adult molt. Sexual reproduction is triggered by factors such as low temperature, high *Daphnia* density or reduced food availability (Ebert 2005). The sexually produced eggs, also called “resting eggs” or “ephippia”, are encapsulated in a strongly melanised protective structure, and can survive long periods of cold or drought (Ebert 2005).

Daphnia have been subject of ecological research for several decades, because of their key position in freshwater ecological food webs; as grazers they are an important food source for many fish species (Ebert 2005). Research has traditionally been focused on topics such as phenotypic plasticity, behaviour, toxicology and the evolution of sexual and asexual reproduction (Ebert 2005). *Daphnia* are also convenient as laboratory organisms. They have a short generation time, and they can be maintained in a state of clonal reproduction for years. Furthermore, *Daphnia* are transparent throughout their lifetime, making it possible to follow tissue-specific changes. A large number of parasites have been described for the *Daphnia* (Ebert 2005). In the present project we focused on the interaction between *D.magna* and the parasite *Pasteuria ramosa.*

1.4.2 *Pasteuria ramosa*

P. ramosa is a gram-positive bacterium, belonging to a distinct clade within the family of *Alicyclobacillaceae* (Ebert *et al.* 1996). There are currently 8 known species of *Pasteuria,* all of which are endoparasites of nematodes, except *P. ramosa,* which is an obligate parasite of *Daphnia* (Bishop *et al.* 2007). More well-known close relatives include other endospore-forming bacteria, such as *Bacillus* and *Clostridium* (Ebert 2005). *P. ramosa* mainly infects *D. magna,* but occasionally also *D. pulex* and *D. longispina* (Ebert 2005). So far, *P. ramosa* has been recorded in Europe and North America (Ebert 2005).

While other *Pasteuria* species enter their hosts by penetrating the host epidermis, it is likely that *P. ramosa* is ingested by *D. magna* while filter-feeding (Ebert 2005). Inside the host, *P.ramosa* remains extracellularly, replicating in the haemolymph of the host. At the time of death, a single *Daphnia* contains 10-20 million spores, and an infection can be reliably assessed by the naked eye from about 16 days post-infection (see fig.1.3). *P. ramosa* does not kill the host immediately, but instead sterilizes the host, and utilizes host resources for its own development. Infected hosts stop reproducing about 5-15 days post-infection. In the lab, death often occurs as late as 40-50 days post-infection. Accordingly, the parasite takes many days to develop and go through several spore stages. Transmission has been found to be strictly horizontal (waterborne) (Ebert *et al.* 1996).

P. ramosa can survive as endospores in the sediment, which therefore effectively functions as a spore bank. Exactly how long the spores can survive is unknown, but infectious spores have been recovered in mud samples, several decades old (e.g. Decaestecker *et al.* 2007).

1.4.3 Previous studies on the *D. magna*-*P. ramosa* interaction

The interaction between *D. magna* and *P.ramosa* has already been much investigated at the ecological level. It has been shown that *P. ramosa* is capable of driving selection of *D.magna* in the wild (Duncan and Little, 2006, Decaestecker *et al.* 2007), that the virulence of *P. ramosa* on *D.magna* can be influenced by environmental factors such as temperature (Mitchell *et al.* 2005), and that switching between clonal and sexual reproduction may be influenced by *P. ramosa* (Duncan *et al.* 2006). Furthermore, a number of clones and spore isolates have been characterized, showing not only large variation in susceptibility among *D.magna* clones to the spore isolates, but also that this susceptibility is dependent on parasite isolate, i.e. genotype by genotype interactions (Carius *et al.* 2001). It appears that *P. ramosa* susceptibility is a stably inherited trait, which can evolve rapidly, and which is most likely controlled by a small number of genes in *D.magna* (Little *et al.* 2006)

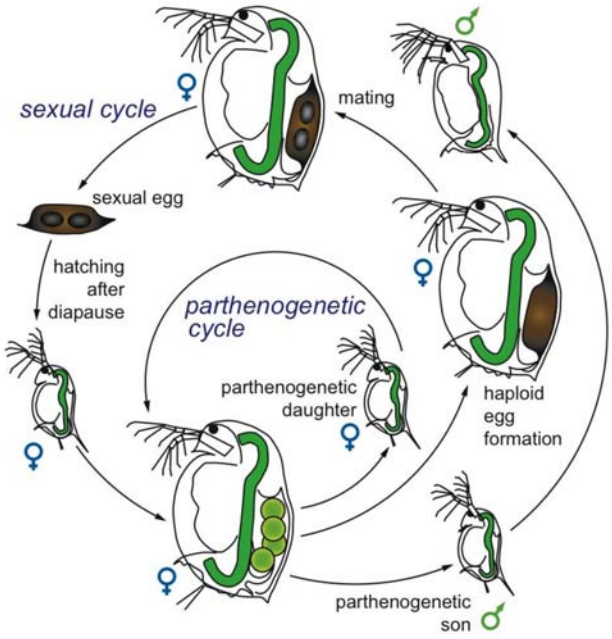

Fig.1.3 Life cycle of a cyclic parthenogenetic Daphnia (Ebert 2005, fig. 2.9)

Fig.1.4 D. magna infected with P. ramosa (Ebert 2005, fig. 3.1). The individual to the left is healthy, and has a new clutch on the way. The individual to the right is infected: the brood chamber is empty, and a dark brownish mass fills the rest of the body. Furthermore, the infected one is bigger, which is thought to be another parasite-induced manipulation.

1.4.4 Molecular research in *Daphnia*

Recently, the draft genome of *D. pulex* was published, opening up for new possibilities of molecular studies in the *Daphnia.* The genome of *D.magna* is not yet available. Several candidate immunity genes in *D. pulex* have been identified, based on homology to immunity genes identified in other invertebrates (Unpublished results, see table 1.1). While this is not likely to represent the full repertoire of the *Daphnia*, it does provide the tools for beginning the elucidation of the molecular basis of the immune response in the *Daphnia.* It should be stressed though, that the list in table 1.1 consists of candidate genes, which await experimental confirmation.

Table 1.1 Immunity genes in D. pulex (Unpublished data), with data for mosquito (Anopheles) and fruit fly (Drosophila) for comparison. The gene copy number for each species is specified. The "attack" genes, Prophenol oxidase and Nitric oxide synthase were investigated in the present study

Function	Gene	Anopheles	Drosophila	Daphnia
Recognition	PGRP	7	13	1
	TEP/a-2-macroglobulin	15	6	8
	GNBP	6	3	11
	Scavenger A	5	5	6
	C-type-lectin	22	11	9
	Galectin	8	5	3
Transduction	Two Toll/Toll related	10	9	7
	Relish	2	3	1
	MyD88	1	1	1
	Tube	1	1	1
	Pelle	1	1	1
	Cactus	1	1	1
	Imd	1	1	1
	STAT	1	?	1
Attack	Gambicin	1	0	0
	Chitinase	1	18	17
	Prophenol Oxidase	9	3	1
	Caspases	12	9	9
	Nitric oxide Synthase	1	1	2
Others	Argonaut	2	2	2
	Dicer	2	2	2
	DSCAM	1	1	1
	Gemini	?	1	1
	Dorsal	?	1	1

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1.5 Project aims

The main objective of the present project was to identify the genes implied in the immune response of *D. magna,* when faced with *P. ramosa* attack. To do that, we have chosen a candidate-gene approach, where we have selected a subset of genes, which have been much investigated in the immune response of other invertebrates, namely pPO, NOS and arginase. We followed the expression level of these genes by comparing the quantity of RNA produced by individuals exposed to *P. ramosa* with control individuals, using qPCR.

Furthermore, we compared the behaviour of two *D.magna* clones known to be highly susceptible and highly resistant respectively, to the *P. ramosa* isolate applied, in the hope of getting some insight into the molecular basis for the specificity found in this host-pathogen interaction. We will present the results of these analyses for each gene, and discuss their possible role in the *D. magna* immune response. We will also discuss the insights gained in this study into the mechanisms of the invertebrate immune response.

2. Materials and methods

2.1 Host clones and parasite isolates

Two *Daphnia magna* host clones were used, named "GG4" and "GG7", originating from *Pasteuria ramosa* – infected *Daphnia magna,* from a pond in Gaarzerfeld, northern Germany, in 1997 (Carius *et al.* 2001). The clones have been maintained in a state of asexual reproduction, un-exposed to the parasite, since the collection.

We applied one spore isolate of *Pasteuria ramosa,* named Sp1 (Carius *et al.* 2001). This spore isolate is highly infective on the "GG4" clone, but not infective on the "GG7" clone (Carius *et al.* 2001). Sp1 was also collected from the pond in Gaarzerfeld in 1997 (Carius *et al.* 2001). Propagation of Sp1 has been done *in vivo* several times since the collection in 1997, mainly on highly susceptible genotypes.

2.2 Experimental design and protocol

The basic set-up for the experiments is shown in table 2.1. For each of the two host clones, "GG4" and "GG7", we had two treatment groups: i) exposure to a solution consisting of *P. ramosa* spores (from crushed *Daphnia*), or ii) a sham-solution consisting of crushed *Daphnia* only. Each treatment was repeated 18 times, to provide 6 time points, each with 3 replicates. Additionally, 3 replicates were set-up for each genotype as "time-zero" controls. Each replicate consisted of 4 or 6 *Daphnia,* thus a total of 312 or 468 *Daphnia* were used for the short-term and the long-term experiment, respectively.

Table 2.1 Experimental set-up

	GG4 (Highly Susceptible)	GG7 (Highly Resistant)
Exposed (*P. ramosa* solution)	6 time points x 3 replicates	6 time points x 3 replicates
Non-exposed (sham solution)	6 time points x 3 replicates	6 time points x 3 replicates

Newly hatched *D. magna* were collected from the same cohort, and differed no more than 24 hours in age. The *Daphnia* were randomly distributed in 200ml jars, with 4-6 *Daphnia* per jar, and maintained in artificial medium (Aachener Daphnien Medium), as described by Klüttgen *et al.* (1994). They were fed 0.5 absorbance (at 665nm) of *Chlorella* sp. per *Daphnia,* per day.

At day four, the *Daphnia* were transferred to new jars with medium only, as a short starvation prior to the exposure treatment, to promote filter-feeding during parasite exposure. The exposure was initiated on day five. Two independent experiments were performed, differing in the time-series collected and the exposure protocol.

a. *Long-term experiment*
D. magna were distributed in 200ml jars at the start of the exposure treatment. Approximately 60.000 spores of *P. ramosa* per *D.magna* were added to the "exposed" samples, and an equal volume of sham-solution was added to the "non-exposed" samples. Sand was added to all samples and stirred after 24 hours, to promote contact between host and parasite.

After an exposure period of 48 hours, the *Daphnia* were transferred to new jars without spores and sand, and kept under normal conditions. *D.magna* were fed as prior to exposure, through-out the exposure period and after.

The following time-points were collected randomly among the samples for each treatment: 0.5, 1, 2, 4, 8, and 16 days counting from *the start* of the exposure treatment. Three replicates of each *Daphnia* clone were also taken out before the exposure treatment, as a "time-zero" control. The infection status of the *Daphnia* was checked in the day 16 sample before collection (infection status with *P. ramosa* can be reliably assessed by the naked eye at this time after exposure). At each time point the *Daphnia* were transferred to eppendorf tubes with "RNAlater" solution (Ambion), and stored at -20ºC for later extraction.

b. *Short-term experiment*
In order to look at gene expression patterns in more detail during the first 48 hours after exposure, and to be able to estimate the time of contact between host and parasite more precisely, a new exposure protocol was developed.

At day five, the *Daphnia* were transferred to 1.5ml Eppendorf tubes, 3-4 in each, with 1ml medium. Approximately 100.000 spores of *P. ramosa* per *Daphnia* were added to the "exposed" samples, and an equal volume of sham-solution was added to the "non-exposed" samples. A small volume of algae was also added to promote filter feeding. The tubes were turned up-side down every 10min through-out the 2-hour exposure period, to promote contact between host and parasite. After this period, the *Daphnia* were transferred, first to a set of medium-filled jars, and then to a second set, to remove all spores

The following time-points were collected randomly among the samples for each treatment: 1, 2, 6, 12, 24, 48 hours counting from *the end* of the exposure treatment. Three replicates of each *Daphnia* clone were taken out before the exposure treatment as a "time-zero" control. At each time-point, the *Daphnia* were transferred to eppendorf tubes with "RNAlater" solution (Ambion), and stored at -20ºC for later extraction. Additionally, three replicates of each treatment were kept to day 16, to estimate the infection success with the new exposure protocol.

2.3 Molecular analysis

2.3.1 The qPCR method

We applied qPCR (quantitative real-time PCR) to determine gene expression. The central idea of this method is to get an estimate of the amount of mRNA (for the genes of interest) which is present in an organism under specific conditions, by following a PCR amplification of the reverse-transcribed mRNA in "real time" with a fluorescent marker.

2.3.2 RNA extraction and reverse transcription

Samples with whole *D. magna* in frozen RNAlater solution **(**Ambion) were thawed and RNAlater solution was removed by pipetting. RNA was extracted with Rneasy mini kit (Qiagen), according to manufacturers instructions (purification of total RNA from animal tissue).The RNA was further purified with a DNAse treatment (Promega), to remove possible traces of co-extracted DNA.

2 µl RNA was reverse-transcribed into cDNA, using "Reverse transcription system" kit (Promega) according to manufacturers instructions. cDNA was diluted 5 times by adding 80µl of H_2O to each tube. The concentration and purity of the cDNA was checked with PCR, using specific primers on a region containing an intron, to distinguish between DNA and cDNA products.

2.3.3 qPCR

qPCR was done on a LightCycler 480 (Roche), using SYBR Green I Master mix (Roche). We used 1µl cDNA for the amplification of actin, prophenoloxidase and arginase, and 2µl cDNA for the amplification of the nitric oxide synthase genes. Specific primers available in the laboratory were used for each gene. Their sequences are listed in table 2.2. All forward primers are 5'-3' oriented, and reverse primers are 3'-5' oriented.

Table 2.2 Primers used for qPCR

Gene	Primer sequence
Actin*	Act-F: CCACACTGTCCCCATTTATGAA Act-R: CGCGACCAGCCAAATCC
Prophenol-oxidase (pPO)	pPO_Q_F2: TAACCCAGGAATGCCCTTCAC pPO_Q_R2: GCGGGATATTGGACGAAAAGT
Nitric oxide synthase (NOS1)	NOS1QF2: AGTCCGATTTTCGTGTCTGG NOS1QR2: ACCTCGGTGAATTGGACATT
Nitric oxide synthase (NOS30)	NOS30QF1: GAGCTCTTCAACCACGCTTT NOS30QR1: AGACGTCACGATCATCACCA
Arginase	ArgQF1: TGGTCTCCGGGATGTAGAAC ArgQR1: GACGGCTTCTTTGATGCCTA

* Designed by Heckmann *et al.* (2006)

Cycling conditions were as follows: 95 °C, 5 min. followed by 45 cycles of 95 °C for 10s, 58°C for 10s and 72°C for 10s, for the actin, pPO and arginase genes, and 50 cycles for the NOS1 and NOS30 genes. After the last amplification cycle a melting curve was generated.

Quantification was done with the Roche software provided, using the maximum secondary derivative method.

2.4 Statistical analysis

We analyzed our data using a generalized linear model (GLM). The complete model was:

Log(Activity) = Genotype + Exposure + Time + Genotype*Exposure + Genotype*Time + Exposure*Time + Genotype*Exposure*Time

Genotype and Exposure were treated as categorical variables (with two levels: exposed/non-exposed and “GG4”/”GG7” respectively), and Time was treated as a continuous variable. The data was log-transformed before the analysis to ensure the normal distribution of residuals. The initial model was pared down to the minimal adequate model for each gene, according to Crawley (2007). We started by removing the three-way interaction (when not significant), then two-way interactions, and then the explanatory variables themselves. The *p*-values were estimated with an *F*-test. Analysis was performed using the R package (http://www.r-project.org/), and the assumptions of the model were checked using residual plots.

3. Results

3.1 Prophenoloxidase (pPO)

3.1.1 Short-term experiment

The graphical representation of the expression of pPO for the short-term experiment is given in fig.3.1. An approximately 2-fold increase in pPO expression occurred one hour after the exposure time (the earliest collected time-point), which was maintained at the two-hour time-point, and had disappeared at the six-hour time-point (see fig.3.1). A significant three-way interaction between Genotype, Exposure and Time was found (table 3.1), and therefore no terms could be removed from the model. We continued by analyzing the data for each genotype separately.

For the susceptible genotype “GG4, the minimal model was: Log(Activity) = Exposure + Time + Exposure*Time (table 3.1). For the resistant genotype “GG7”, the minimal model was: Log(Activity) = 1 (no explanatory variables were found significant; table 3.1).

3.1.2 Long-term experiment

The graphical representation of the expression of pPO for the long-term experiment is given in fig.3.2. An increase in the expression of pPO was found in the exposed individuals at the “day 16” time-point, relative to the non-exposed individuals. This effect was less pronounced in the resistant genotype (hence the significant “Genotype” effect). Furthermore, there was a gradual increase in the expression of pPO over time, for both genotypes (fig.3.2). The minimal model was: Log(Activity) = Genotype + Exposure + Time + Exposure*Time (table 3.2).

Table 3.1 P-values from the GLM analysis for the short-term experiment (the first 48 hours after exposure to P.ramosa). A p-value below 0.05 indicates that the corresponding explanatory variable explained a significant part of the variation in the data.

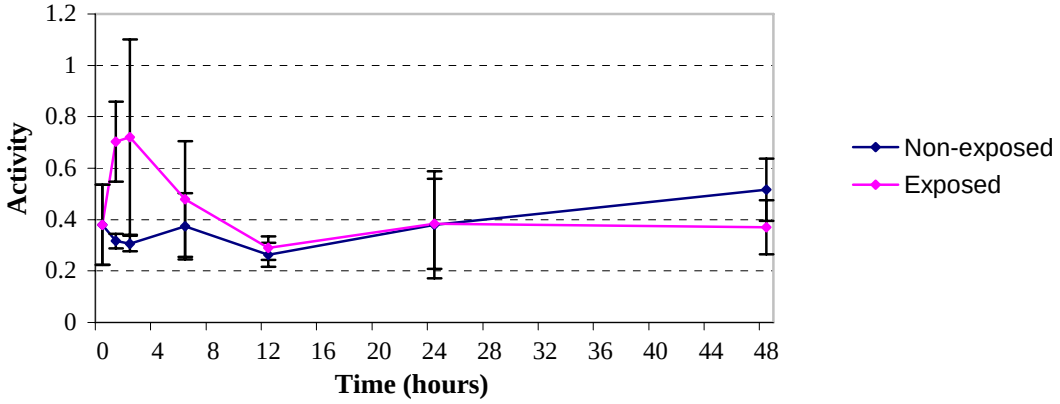
Explanatory variables	pPO[a]		NOS1	NOS30	Arginase[a]	
Genotype	-	-	**<0.001*** **	0.438	-	-
Exposure	**0.002****	0.696	0.204	0.618	0.746	0.723
Time	0.109	0.788	0.754	0.074	0.054	0.092
Genotype*Exposure	-	-	0.657	0.143	-	-
Genotype*Time	-	-	0.642	0.394	-	-
Exposure*Time	**0.012***	0.703	0.761	0.520	0.053	0.245
Genotype*Exposure* Time	**0.027***		0.985	0.107	**0.033***	

a. The data of each genotype were analyzed separately, because the analysis with the complete model yielded a significant three-way interaction, as indicated

Table 3.2 P-values from the GLM analysis for the long-term experiment (from 0.5-16 days after exposure to P.ramosa). A p-value below 0.05 indicates that the corresponding explanatory variable explained a significant part of the variation in the data.

	pPO	NOS1	NOS30	Arginase
Genotype	**0.019***	**<0.001*** **	0.126	0.831
Exposure	0.943	0.460	0.720	0.252
Time	**<0.001*** **	**0.012***	**<0.001*** **	**<0.001*** **
Genotype*Exposure	0.334	0.630	0.396	0.503
Genotype*Time	0.298	0.419	0.620	0.905
Exposure*Time	**0.030***	0.573	0.396	0.339
Genotype*Exposure* Time	0.223	0.215	0.664	0.737

A



B

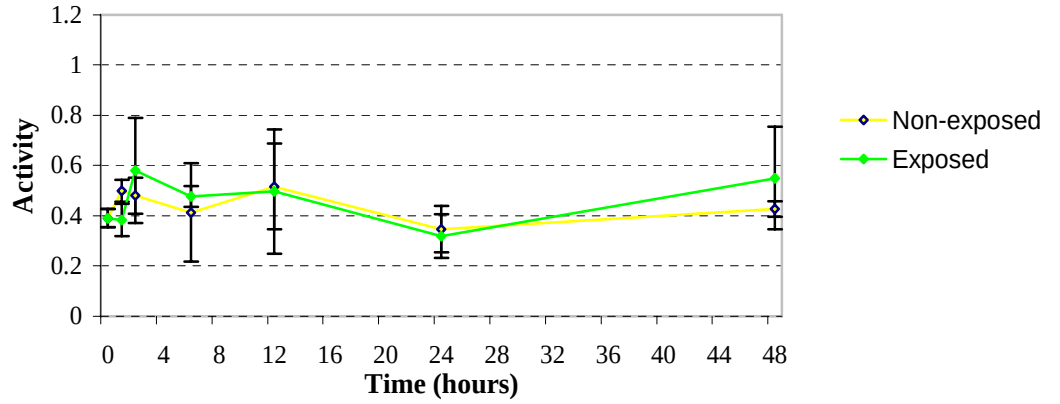


Fig.3.1 Short-term expression of pPO for A) the susceptible genotype "GG4" and B) the resistant genotype "GG7". Gene expression is given as relative activity (as compared to β-actin). Each point corresponds to the mean of 3 independent replicates, the error bars shown are +/- one standard deviation.

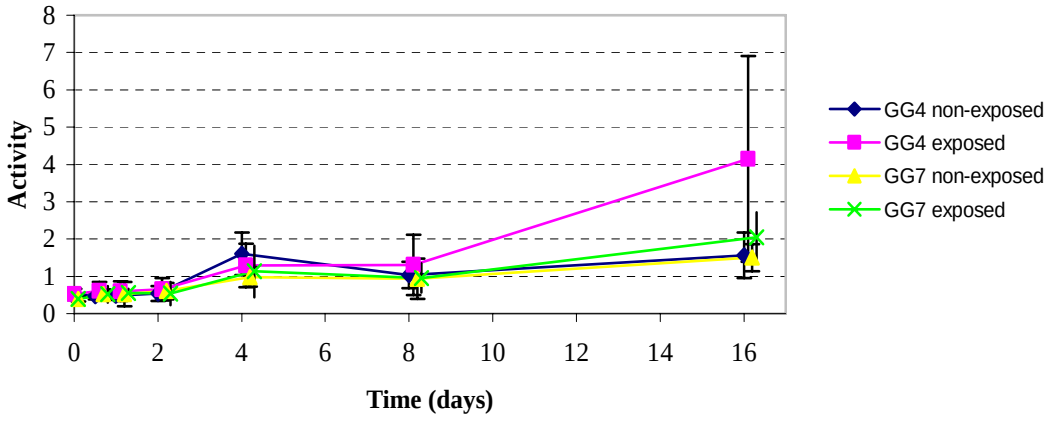


Fig.3.2 Long-term expression of pPO. Gene expression is given as relative activity (as compared to β-actin). Each point corresponds to the mean of 6 independent replicates, the error bars shown are +/- one standard deviation

3.2 Nitric oxide synthase genes (NOS)

3.2.1 Short-term experiment, NOS1

The graphical representation of the expression of NOS1 for the short-term experiment is given in fig.3.3. As seen in fig.3.3, the expression of NOS1 was highly variable. However, the resistant genotype, “GG7”, showed a higher level of expression compared to the susceptible genotype “GG4”. No effect of exposure was found. The minimal model was: Log(Activity) = Genotype (Table 3.1).

3.2.2 Long-term experiment, NOS1

The graphical representation of the expression of NOS1 for the long-term experiment is given in fig.3.4. As for the short-term experiment, the expression of NOS1 was highly variable, but with an overall higher level of expression in the resistant genotype “GG7”, compared to the susceptible genotype “GG4”. Furthermore, there was a gradual increase in expression over time for both genotypes (fig.3.4). The minimal model was: Log(Activity) = Genotype + Time (Table 3.2).

No effect of exposure was found. There was a trend towards a higher expression of NOS1 at the day 2 time-point (fig.3.4), but this trend was not reproducible in the short-term experiment (fig.3.3).

3.2.3 Short-term experiment, NOS30

The graphical representation of the expression of NOS30 for the short-term experiment is given in fig.3.5. No explanatory variables were found significant (Table 3.1), therefore the minimal model was: Log(Activity) = 1.

Although the Exposure variable was not significant, there was a trend towards a higher level of expression of NOS30 at the 1-2 hour time-points in the exposed individuals of the susceptible genotype “GG4”, compared to the non-exposed individuals. This pattern coincided with the time and specificity of the induction of the pPO gene (fig.3.1 versus fig.3.5).

3.2.4 Long-term experiment, NOS30

The graphical representation of the expression of NOS30 for the long-term experiment is given in fig.3.6. As seen in fig.3.6, there was a gradual increase in the expression of NOS30 over time for both genotypes. No effect of exposure was found. Thus, the minimal model was: Log(Activity) = Time (Table 3.2).

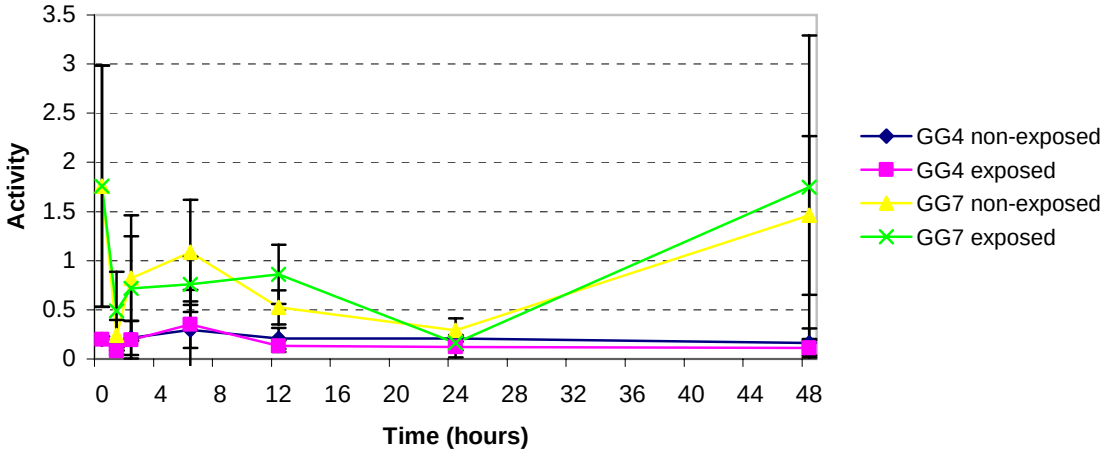


Fig.3.3 Short-term expression of NOS1. Gene expression is given as relative activity (as compared to β-actin). Each point corresponds to the mean of 3 independent replicates, the error bars shown are +/- one standard deviation

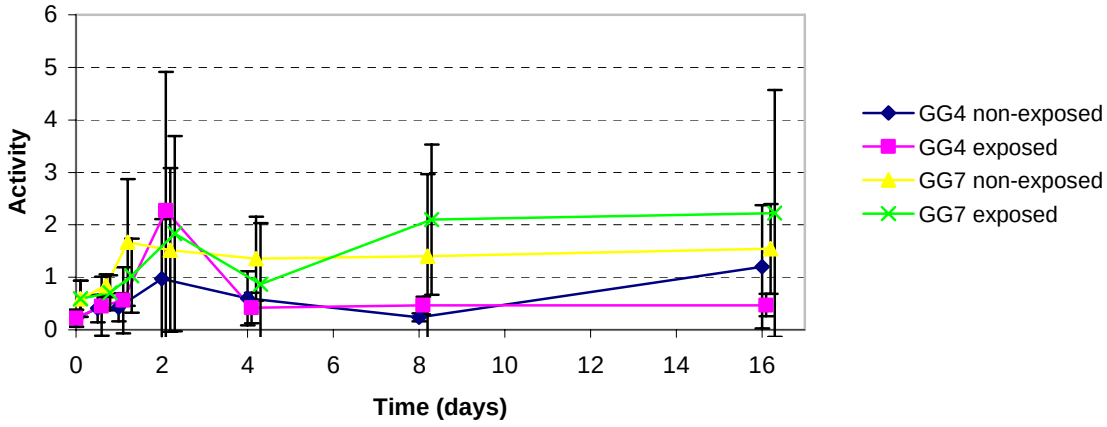


Fig.3.4 Long-term expression of NOS1. Gene expression is given as relative activity (as compared to β-actin). Each point corresponds to the mean of 6 independent replicates, the error bars shown are +/- one standard deviation

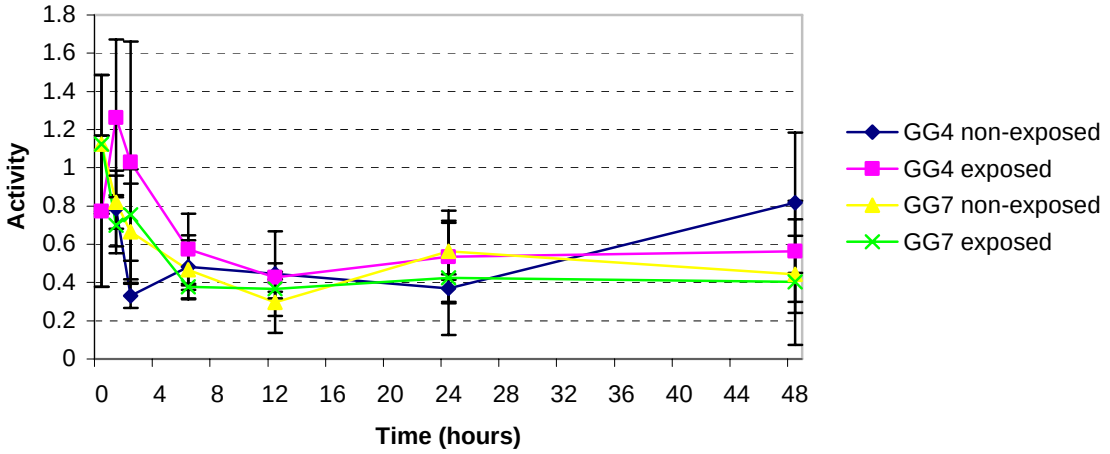


Fig.3.5 Short-term expression of NOS30. Gene expression is given as relative activity (as compared to β-actin). Each point corresponds to the mean of 3 independent replicates, the error bars shown are +/- one standard deviation

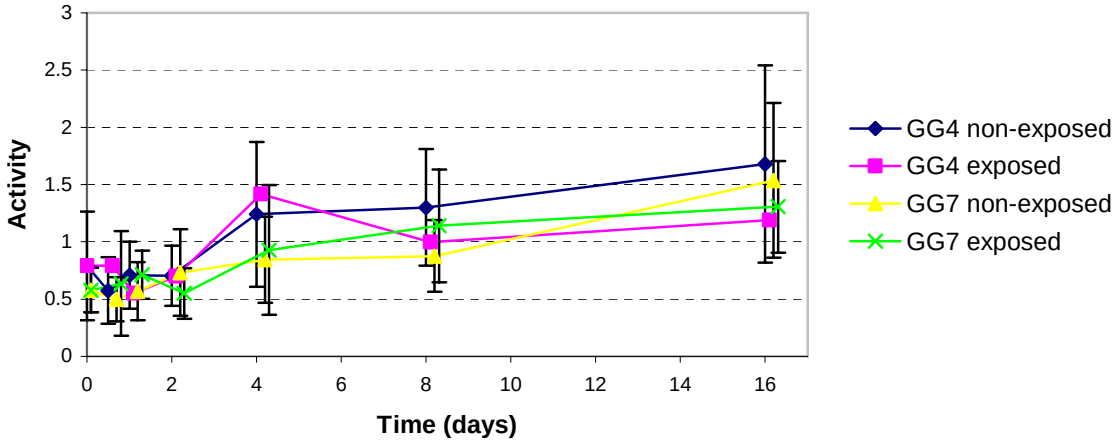


Fig.3.6 Long-term expression of NOS30. Gene expression is given as relative activity (as compared to β-actin). Each point corresponds to the mean of 6 independent replicates, the error bars shown are +/- one standard deviation

3.3 Arginase

3.3.1 Short-term experiment

The graphical representation of the expression of arginase for the short-term experiment is given in fig.3.7. A significant three-way interaction between Genotype, Exposure and Time was found (table 3.1), and therefore no terms could be removed from the model. We continued by analyzing the data for each genotype separately, which resulted in a loss of all significant terms. For both genotypes, the minimal model was: Log(Activity) = 1 (table 3.1).

The significant three-way interaction was probably due to the 48 hour time-point: In “GG4”, the expression was increased in the non-exposed individuals compared to the exposed ones, whereas in “GG7” the opposite trend was seen (see fig.3.7).

3.3.2 Long-term experiment

The graphical representation of the expression of arginase for the long-term experiment is given in fig.3.8. As seen from fig. 3.8, there was a gradual increase in the expression of Arginase over time, for both genotypes. No effect of exposure was found. The minimal model was: Log(Activity) = Time (table 3.2).

3.4 Estimate of the infection success in the experiments

Three replicates of a time-point corresponding to “day 16” were checked for infection, for all experiments. In the long-term experiment, the replicates were used for RNA extraction afterwards, in the short-term experiment the replicates were used as an estimate of infection success only.

The percentage of infected individuals of the susceptible genotype “GG4” was 72.2% in the short-term experiment, and 100% in the long-term experiment. No infection was observed in resistant genotype or in the control treatments.

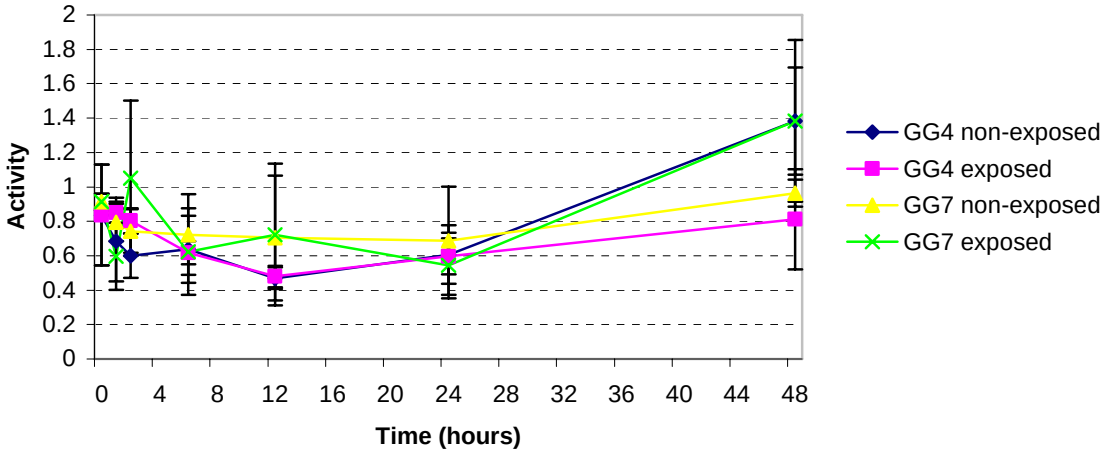


Fig.3.7 Short-term expression of Arginase. Gene expression is given as relative activity (as compared to β-actin). Each point corresponds to the mean of 3 independent replicates, the error bars shown are +/- one standard deviation

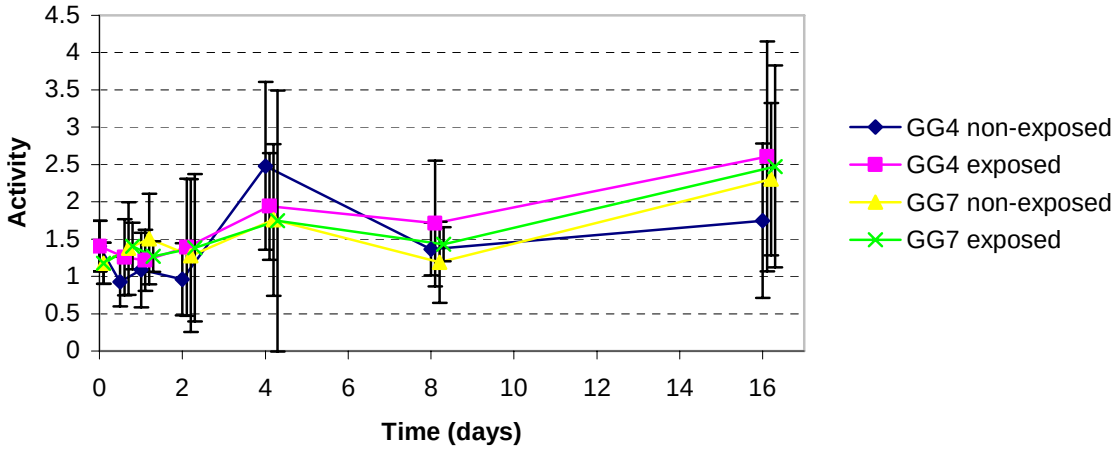


Fig.3.8 Long-term expression of Arginase. Gene expression is given as relative activity (as compared to β-actin). Each point corresponds to the mean of 3 independent replicates, the error bars shown are +/- one standard deviation

4. Discussion

In the present study, we investigated the expression of four candidate immunity genes, pPO, NOS1, NOS30 and arginase, potentially implied in the *D.magna* immune response against the pathogen *P.ramosa.* We used two *D.magna* host clones together with a parasite isolate, to which the clones were known to be susceptible and resistant respectively, to gain insight into the molecular basis of the specificity found for this host-pathogen interaction. We followed the expression of the various genes in *D.magna* after exposure to *P.ramosa* or a sham-solution, using qPCR, and compared the expression pattern in exposed individuals to control individuals. The genes we have chosen for the present project represent general effector mechanisms of the invertebrate immune response. Their activity, if they are part of the immune response of *D.magna*, should therefore reflect whether an immune response has been triggered or not, and potentially the strength and timing of such a response.

4.1 Prophenoloxidase (pPO)

4.1.1 Differential expression of pPO in *D.magna* clones

We found a significant increase in the expression of pPO when *D. magna* were exposed to *P. ramosa* at two different time-points:

i) An approximately 2-fold induction occurred 1-2 hours after the exposure treatment, but only in the susceptible *D. magna* clone, "GG4" (fig.3.1).
ii) Later, 16 days after exposure, another increase in expression was found, which was higher in the susceptible genotype than in the resistant genotype (fig.3.2).

Finally, the expression level of pPO increased with the age of the *Daphnia.*

If resistance is determined by an immune response, our expectation was to see a response in the resistant genotype, indicating a successful and efficient immune response, and either no response or a weaker response in the susceptible genotype. Incompatible with this prediction, we found a significant effect of *P.ramosa* exposure on pPO only for the susceptible genotype. We therefore hypothesise that there are two levels of resistance in this host-pathogen interaction. First, the parasite must successfully enter the host, and second, the host immune response must be combated. Thus, the pattern we see here could be interpreted as a failure of the parasite to invade the resistant clone, whereas in the case of the susceptible clone, the parasite is able to both enter and out-compete the rapidly launched immune response.

Currently, there is still some uncertainty as to whether the parasite is ingested by the filter-feeding host, or enters by penetrating the host epidermis (Ebert 2005). *P.ramosa* develops in the haemolymph (Ebert *et al.* 1996), and if the parasite is ingested the first barrier which must be crossed is the gut. A possibility could then be that the resistant clone in some way prevents the parasite from entering the haemolymph, either passively (e.g. the absence of a receptor needed by *P.ramosa* to enter), or actively (e.g. a defence mechanism in the gut which efficiently kill the parasite). Further studies on the entry mechanism of *P.ramosa* will be needed to answer that question.

Several *D.magna* clones and *P.ramosa* isolates with different levels of susceptibility and infectivity are available in our lab. Therefore, to test the hypothesis of two levels of resistance in the *D.magna-P.ramosa* interaction, we are currently testing the expression of the genes in other *D.magna* clones and *Pasteuria* strains that also differ dramatically in the level of infection achieved following exposure.

pPO is part of an enzymatic cascade involving several genes, and a number of them have been characterized, also in crustaceans. If pPO can be confirmed as an immunity gene in *D.magna,* it could be very useful to follow or even manipulate the expression of some of the important regulators of pPO, such as the pPO-activating enzyme ppA, or pacifastin (a proteinase inhibitor specific against the PO-activating enzyme of crayfish, see fig.1.2, introduction). It would also be interesting to investigate the response of pPO in *D.magna* when challenged with other parasites than *P.ramosa,* to evaluate if the gene is employed in a similar manner in other host-pathogen interactions. Several pathogens of *D.magna* have been described, which are amenable to infections in the lab (Ebert 2005), making such studies feasible. Similarly, it could be tested whether *D.magna* responds to heat-killed *P.ramosa* or other immunostimulants, to avoid any confounding effects of host-pathogen interactions.

4.1.2 pPO and the invertebrate immune response

Melanisation has been confirmed to be important for the outcome of several specific host-pathogen interactions, but the decisive role of pPO in combating pathogens is still being debated for a number of species (Cerenius *et al.* 2008). Some insects contain several copies of pPO, e.g. the fruit fly contains 3 copies, and 9 copies are found in the mosquito *A.gambiae* (Cerenius and Söderhäll 2004), indicating that the pPO genes may have evolved to have different functions in different species.

Compelling evidence for the importance of pPO in the immune system of the crustacean *Pacifastacus leniusculus* was recently found by Liu *et al.* (2007). Similar to the present study, they found an increase in pPO mRNA when *P.leniusculus* was challenged with the pathogenic bacterium *Aeromonas hydrophila,* to which it is susceptible. The response was not quantified, but was demonstrated 3 hours post-challenge, coincident with the timing of the response found in the present study. They continued by silencing the pPO gene through RNA interference, which resulted in an increase in bacterial growth, and host mortality. In contrast, silencing of pacifastin (an inhibitor in the pPO-activating cascade, see fig.1.2, Introduction) resulted in an increase in survival time, and enhanced bacterial clearance (Liu *et al.* 2007). Thus, the authors were able to establish a direct link between host susceptibility and pPO expression, demonstrating that pPO expression can be of importance for the outcome of host-pathogen encounters.

4.2 The NOS genes and arginase

4.2.1 Differential expression of the NOS genes and arginase in *D.magna* clones

Whereas all invertebrates investigated so far contain one copy of the NOS gene (Rivero 2006), *D. pulex* and *D. magna* contain two copies, which are highly diverged, and therefore likely to have different functions. In the present study, the two genes showed different responses:

i) There was a difference in the constitutive level of NOS1 between the two genotypes investigated, both in the short-term and long-term experiments (fig.3.3 and 3.4); A non-significant trend towards an increase in NOS1 was seen at day 2 in the long-term experiment, but this trend was not reproducible in the short-term experiment (fig. 3.3 and 3.4),

ii) NOS30 showed a trend towards an early induction in the short-term experiment (though not significant), in the susceptible genotype only (fig.3.5).

In arginase, an effect of exposure to *P.ramosa* was indicated by a significant 3-way interaction between genotype, exposure and time in the short-term experiment (fig. 3.7); However, the significance was lost when the data for each genotype was analyzed separately, indicating that the effect of exposure in this case was rather weak (table 3.1). Finally, as for pPO, the expression of all the genes increased with the age of the *Daphnia.*

Since a higher level of expression of NOS1 was found in the resistant genotype, it could be speculated that the constitutive level of expression of NOS1 might be an important determinant of resistance. We have previously investigated the expression of NOS1 in the absence of *P.ramosa* exposure in four different *D.magna* clones, confirming that there appears to be differences in the basic level of expression for this gene (results not shown). However, in the absence of an effect of exposure, the interpretation of these differences is complicated. NOS is known to have non-immune-related functions in crustaceans, and, since we used total RNA extracted from whole *D. magna,* we cannot separate the NOS expression in different organs, such as brain and haemocytes. Therefore, further experiments are needed to evaluate the importance of NOS1 in connection with the immune response of *D.magna.*

One possibility could be to investigate the basic level of expression of NOS1 in a broad collection of *D.magna* clones, to see if there is a general correlation between susceptibility and basic NOS1 expression. Several clones are available in our lab for such an analysis, for which the susceptibility to various isolates of *P.ramosa* is known, or could be assessed. However, the interaction between *D.magna* and *P.ramosa* is characterized by a high level of genotype-by-genotype interactions, (Carius *et al.* 2001), making it hard to accurately determine a general level of resistance. The most efficient way to evaluate the importance of NOS1 would be to manipulate the expression of NOS1 directly, and then assess the effect on susceptibility. This approach is complicated by the fact that *D.magna* contains two NOS genes, and it is therefore necessary to manipulate the genes independently. But it may be possible in the future to use specific RNA interference to that end.

The trend of induction in the NOS30 gene is interesting, because it coincided with the timing and specificity for the early response seen for pPO; Thus, we already have a strong indication that the susceptible *D.magna* are responding to *P.ramosa* at that time-point. Other clone-parasite combinations should therefore be investigated to evaluate the function of NOS30.

The analysis of the data for arginase indicated that there may be an effect of exposure to *P.ramosa,* albeit not a strong one. According to the hypothesis that arginase is induced by the parasite, as a way to avoid exposure to NO, we expected to see an induction mainly in the susceptible genotype; This trend was not observed. However, since we did not find a significant increase in NOS expression in any of the clones tested, the present clone-parasite combination may not be adequate for testing the role of arginase in regulating NOS activity. It will be interesting to see if arginase comes into play in other clone-parasite combinations, currently being investigated in our lab.

4.2.2 Nitric oxide synthase, arginase and the invertebrate immune response

So far, all studies of NOS in connection with the invertebrate immune response have found NOS to be inducible. The timing and level of NOS induction has been shown to be highly variable in different invertebrate species. In the silkworm, an increase in NOS mRNA started 1 hour after challenge with LPS and peaked 6 hours post-challenge (Imamura *et al.* 2002). Similarly, an elevated level of NOS protein was found in the fruit fly as early as 5 hours after infection (Foley and O´Farrell 2003), consistent with the timing of the trend seen for NOS30 in the present study. In contrast, the increase in NOS mRNA in mosquitoes was found primarily between 24-48 hours post-challenge (Luckhart *et al.* 1998, Herrera-Ortiz *et al.* 2004), consistent with the trend presently seen for NOS1 in the long-term experiment. In those cases where the increase in NOS transcript was quantified, the strength of the response varied from about a 2-4 fold increase in the mosquitoes (Luckhart *et al.* 1998, Herrera-Ortiz *et al.* 2004) to an impressive 34-fold induction in the crop of the insect *Rhodnius prolixus*! (Whitten *et al.* 2007).

In crustaceans, the importance of NOS for the immune response has not yet been established. Yeh *et al.* (2006) found inducible NOS activity in haemocytes (cells involved in the immune response of arthropods) of the crustacean *Procambarus clarkii*, indicating an immune function, but studies using hosts and parasites in a natural context are lacking. In *D.magna,* it was previously found that addition of arginine, the substrate of NOS, increased the resistance to *P.ramosa*, whereas addition of L-NAME (a specific inhibitor of NOS) caused a higher susceptibility to *P.ramosa* (Unpublished results), indicating that at least one of the NOS genes in *D.magna* is of importance in this interaction.

Activation of arginase has been proposed to be a common mechanism employed by parasites, to avoid exposure to the toxic nitric oxide (Vincendeau *et al.* 2003). Evidence for the importance of arginase-activation has been found e.g. in the mouse-*Leishmania major* interaction, where the level and timing of arginase expression was correlated with susceptibility to the parasite (Iniesta *et al.* 2005). However, no studies have yet investigated whether arginase-activation can be an important strategy for parasites of invertebrates.

4.3 Experimental considerations, and limitations

4.3.1 qPCR

A crucial assumption for relative quantification of gene expression using qPCR is that the reference gene applied has a non-variable expression pattern (Heckmann *et al.* 2006). We applied ß-actin, which is a commonly used reference gene. We found an increased expression in all the genes over time (most evident in the long-term experiment). It is possible that *D.magna* increases the expression of immunity genes with age, but it could also be that the expression of ß-actin changes with age in *D.magna.* Other reference genes are available (Heckmann *et al.* 2006), which could be tested to resolve that question.

4.3.2 Different levels of regulation

In the present study, we have focused on the transcriptional level of regulation, but several other levels of regulation can be envisaged. In the case of pPO, the enzyme must be cleaved by another enzyme to become active, and a plausible level of regulation would therefore be post-

translationally, controlling the activation of the enzyme rather than the transcription of the gene. Regulation at the post-translational level has the advantage of being a much faster response, compared to an induction starting at the transcriptional level. Indeed, various inhibitors have been identified, acting either at the level of pPO activation or directly on PO activity (Cerenius *et al.* 2008). Thus, another possible explanation for not seeing a response in the resistant genotype could be that a sufficient amount of active enzyme was generated to eliminate the pathogen, without the need for transcriptional induction. Alternatively, it could be speculated that the genotypes differ at the sequence level for the pPO gene, with the resistant genotype producing a more efficient version of the enzyme. Genotyping of *D.magna* clones with respect to the genes of the present study is currently underway in our lab.

Induction of pPO expression in response to parasites or immunostimulants has been found in several studies, indicating a functional importance of transcriptional regulation (Cerenius *et al.*2003, Gai *et al.* 2008, Ko *et al.* 2007, Liu *et al.* 2007, Lu *et al.* 2006, Rajagopal *et al.* 2005). However, that does not preclude an importance of post-translational regulation, rather the two may complement each other (Gai *et al.* 2008, Lu *et al.* 2006).

In contrast to pPO, the NOS enzyme does not require activation, and the enzymatic activity of the inducible NOS does not appear to be subject to any major regulation (Kleinert *et al.* 2004). A permanent high level of NOS protein would seem a rather hazardous immune defence strategy, given that NO is a non-specific, and highly toxic compound. Nevertheless, the cost of a high level of constitutive NO may be compensated, if the probability of encountering the parasite is high. Another consideration is that the translation of NOS mRNA or protein stability may be subject to regulation (Kleinert *et al.* 2004). It would therefore be interesting to investigate whether the difference found for NOS expression at the RNA level is also reflected at the protein level.

4.3.3 The candidate gene approach

In the present study we have focused on a limited number of candidate genes, representing functions which have been abundantly demonstrated to be of importance in the invertebrate immune response. However, the molecular characterization of the immune response of crustaceans is still in its infancy, and the first crustacean genome has only just been sequenced (*D. pulex*). It is therefore possible that other mechanisms than the ones represented in the present study will prove to be more important for this group.

Several genes must be involved in the immune response of the *Daphnia,* and a number of candidate genes have already been identified (table 1.1, Introduction). The list given in table 1.1 is preliminary, and the experimental testing of each of the genes is a long process. We are currently working towards a transcriptome analysis of *D.magna*, both exposed and non-exposed to *P.ramosa,* to get a more comprehensive picture of the various genes implied in the process of resistance.

5. Acknowledgements

First, I would like to thank Pierrick Labbé, who has been immensely helpful during this project. Pierrick has taught me all I know about qPCR, with much patience and enthusiasm, and has furthermore taken time to answer all my questions and give me constructive critique, during the whole project.

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Last, but certainly not least, I would like to thank all the great, smart and entertaining people who have contributed to make my stay in Edinburgh a memorable experience: Stuart, Desiree, Sarah, Andrew, Pedro, Martin, Laura, Anna, Darren, .. Well, all of you, you know who you are!

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