1 Infections by *Pasteuria* do not protect its natural host *Daphnia magna* from subsequent infections

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Abstract:

The existence of immunological memory in invertebrates remains a contentious topic. Exposure of *Daphnia magna* crustaceans to a noninfectious dose of the bacterium *Pasteuria ramosa* has been reported to reduce the chance of future infection upon exposure to higher doses. Using clonal hosts and parasites, we tested whether initial exposure of the host to the parasite (priming), followed by clearing of the parasite with antibiotic, protects the host from a second exposure (challenge). Our experiments included three treatments: priming and challenge with the same or with a different parasite clone, or no priming. Two independent experiments showed that both the likelihood of infection and the degree of parasite proliferation did not differ between treatments, supporting the conclusion that there is no immunological memory in this system. We discuss the possibility that previous discordant reports could result from immune or stress responses that did not fade following initial priming.

Introduction

Priming a host, i.e. exposing the host's immune system to a pathogen for the first time, may result in host protection upon subsequent exposures to the same pathogen (Masri and Cremer, 2014; Schmid-Hempel, 2011). The existence of this specific acquired protection has been demonstrated only in a few animal phyla and its mechanisms are rarely known. There exist many possible scenarios leading to a potential specific acquired protection (Masri and Cremer, 2014; Schulenburg et al., 2007): 1) a long lasting response, i.e. a response initiated during the first exposure that persists and is still actively ongoing during the second exposure; 2) a leftover effect of a unique response, where the long-lived effector molecules produced after the first exposure protect the host against a second infection; or 3) a true memory, similar to that of the vertebrate acquired immune system, where the response to the first exposure disappears, and the host reacts with the production of immune factors and/or proliferation of specific cell populations, which protect against a second infection (Schmid-Hempel, 2011). Those scenarios are to some degree distinct in their evolutionary significance. A true memory is a selected mechanism to protect against reinfection with the same parasite strain even after a long time delay. A long lasting response or a leftover effect are selected for when the risk of reinfection is immediate, i.e. within a relatively short time interval after the first challenge. These two scenarios do not exclude each other and may act at the same time. Disentangling these possibilities would greatly advance our understanding of the analogies and homologies between the vertebrate immune system and that of invertebrate taxa.

Originally, the specificity and memory of the immune response of several invertebrate phyla (echinoderms, nemertean, arthropods, sponges, and cnidarians) were measured by studying the recognition of tissue grafted from the same (Cooper and Roar, 1986; George et al., 1987; Karp and Hildemann, 1976), or different species (Cooper, 1968; Langlet and Bierne, 1982). The results were often conflicting mostly because the strains of animals used in the experiments were not or poorly genetically defined. The absence of consistent evidence from these studies lead to reduced efforts as no case could be made for specificity or memory in these animals. Later studies of invertebrate immunity focused on host-parasite interaction rather than opportunistic or other antigenic materials, and suggested that

priming the invertebrate immune system can lead to memory (Kurtz and Franz, 2003; Little et al., 2003; McTaggart et al., 2012; Moret and Siva-Jothy, 2003; Pham et al., 2007; Pope et al., 2011; Rodrigues et al., 2010; Roth et al., 2009; Sadd and Schmid-Hempel, 2006; Tidbury et al., 2011; Witteveldt et al., 2004). However, experiments have been criticized because they were condition dependent, and assessed fitness traits, such as the survival and fecundity of the challenged hosts, rather than immunological criteria, such as the expression of immune factors and the reduction of parasite success (Hauton and Smith, 2007; Little et al., 2008; Rowley and Powell, 2007). Another example is given by Rodrigues et al (2010 and 2015), who showed that the immune priming of mosquitoes to *Plasmodium falciparum* lasted 14 days due to an adapted mechanism of hemocyte differentiation and revealed the molecular mechanism underlying it. Due to the diversity of approaches and systems used, it is currently difficult to make generalities, however, the existence of immune priming in some invertebrate taxa is likely.

We tested whether priming with its natural parasite, *Pasteuria ramosa*, leads to long term protection in the host crustacean *Daphnia magna*. It is established that *D. magna* and *P. ramosa* coevolve in nature (Decaestecker et al., 2007) and that their interaction is host genotype - parasite genotype specific (Duneau et al., 2011; Luickjx et al., 2011). It has been reported that offspring of infected *D. magna* mothers have higher fitness when challenged with the same isolate of *P. ramosa* that caused the maternal infection, compared to a challenge with a different isolate (Little et al., 2003). This result implies that *D. magna* is able to develop some form of specific memory. Furthermore, two other studies suggest that individuals exposed to a non-infective dose of *P. ramosa* (i.e. a dose that does not result in infection) are less likely to get infected by a second exposure within 48 hours to the parasite (Garbutt et al. 2014; McTaggart et al., 2012). The infection process in the *Daphnia-Pasteuria* system follows several steps (such as encounter, attachment, penetration, within-host growth), each of which could manifest a form of resistance (Duneau et al., 2011; reviewed in Ebert et al., 2016). It is not clear from the experimental design of the previous studies when resistance occurs during infection (entering the host or within-host proliferation step). If priming seems likely, there is no support for any immunological *Daphnia* features involved in the regulation of *P. ramosa* during the step of the parasite proliferation within the host (Decaestecker et al., 2011; Labbé and Little, 2009; Labbé et al., 2009) and

therefore it is not clear how priming may work. In our current experiment, we used host genotype - parasite genotype combinations where each bacterium was known to be equally able to enter the host (i.e. we overcame variation at the steps before within-host growth). We controlled for the capability of the specificity of the innate immunity of the host (i.e. **genetically encoded resistance**), by exposing the host to one of his natural parasites. In this system, we conducted experiments that would test for the reactivation of a response and of its impact on parasite fitness.

Here, we test the following hypotheses: 1) exposed *D. magna* **individuals** can be primed and subsequently **are** protected from *P. ramosa*, and 2) priming is specific to the parasite genotype causing the initial infection.

Results and discussion

Each experiment consisted of three experimental treatments and four control treatments (Figure 1). We infected *D. magna* with *P. ramosa* following three experimental treatments: 1) hosts where infected, then cured with tetracycline and then exposed to the same parasite strain (homologous challenge), 2) hosts where infected, then cured with tetracycline and then exposed to a different parasite strain (heterologous challenge), 3) no early challenge, but a tetracycline treatment followed by an exposure to a parasite (naïve exposure).

A number of control treatments were included to verify that each of the steps in the experimental procedure (“Early infection”, “Cured”, and “Late infection”) was effective and that the antibiotic did not produce unwanted side effects. We quantified the effect of priming by measuring the host’s susceptibility to infection (proportion of hosts infected) and by counting parasite transmission stages produced during the late infection. We compared the host’s susceptibility to the parasite across the three experimental treatments. Increased resistance in non-naïve (previously exposed) hosts relative to naïve (previously unexposed) hosts would suggest immune priming. Furthermore, increased resistance in the homologous challenge treatment relative to the heterologous challenges would suggest specificity in immune priming with respect to parasite genotype. Each experimental treatment included 36 replicates,

i.e. individually-kept and treated female *D. magna*, and each control treatment included 15 individuals. This experiment was conducted twice with two different *D. magna* genotypes. In both cases we found that the first exposure led to 100% host infection in the absence of antibiotics and that the antibiotics cured 100% of the *Daphnia* hosts (Table 1).

Clearance of Pasteuria ramosa

D. magna's ability to naturally clear *P. ramosa* infection typically lasts a few days after exposure (i.e. there is never clearance once symptoms are visible (Ebert et al., 2016)). To ensure that the host was exposed but also that the parasite was cleared, we exposed *D. magna* to a dose that resulted in 100% (see control) of infection before treating the infection with tetracycline (Figure 1, "Cure" control treatment). Tetracycline is a bacteriostatic antibiotic and therefore stop bacterial activity (by stopping protein synthesis) without killing or even – at the given dose – harming hosts (Chopra and Roberts, 2001). In contrast to the untreated controls (Figure 1, "Early infection"), antibiotic-treated hosts were free of the parasite 25 days after antibiotic treatment (Table 1). Under our experimental conditions, *Daphnia* seem to be able to eliminate *P. ramosa* only when exposed to antibiotic. Therefore the clearance of the bacteria "inactivated" by the antibiotic would be consistent with the hypothesis that normally, *P. ramosa* is able to circumvent the host immune system, either by suppression or active manipulation. This would be consistent with the absence of *D. magna* humoral immune response upon *P. ramosa* infection (Decaestecker et al., 2011; Labbé and Little, 2009; Labbé et al., 2009). Because bacteriostatic antibiotics do not kill bacteria, host clearance of the "inactivated" bacteria implies that, although the modalities are unknown, the host immune system encounters the bacteria, thereby increasing the chance of an immune response and possibly priming during the within-host proliferation step.

Experimental test for host immune priming

Unlike most previous studies on the invertebrate immune system, which involved non-natural parasites and routes of infection (Pham et al., 2007; Sadd and Schmid-Hempel, 2006), we infected *Daphnia* using a natural parasite and the natural route of infection (i.e. exposing hosts to waterborne transmission stages

of the parasite, which are ingested by the filter feeding host). All experiments were done with cloned parasite lines, avoiding the problem of parasite genotype cocktails - as has been reported from natural isolates (Luickjx et al., 2011; Mouton and Ebert, 2008). These cloned *Pasteuria* are known to be compatible with the host and thus made sure that the parasite was entering the host body cavity. There was no difference in the likelihood of the late infection among the three experimental treatments in the two experiments (Table 2), and no difference in the number of parasite spores produced by infected hosts (Figure 2, linear model, “spore number” controlled for variance due to “clone used in the challenge”: for experiment 1: $df=2$, $F=0.29$, $p=0.75$; for experiment 2: $df=2$, $F=0.05$, $p=0.95$). These results do not support the hypothesis of a protection by immune factors in *D. magna* over the two weeks following the exposure. The similarity in the infection outcomes of naïve and non-naïve individuals suggests that the host does not become more resistant after clearing an infection 12 days before a second challenge. Our data also do not suggest an inhibition of the immune system by the antibiotic as an explanation for our results. The naïve exposure treatment and the “late infection” control, which are identical except that the former includes an antibiotic treatment, were equally susceptible for the clone Kela-20-13 (Pearson’s Chi-squared test, $\chi^2=0.23$, $df=3$, $p\text{-value}=0.97$). For the clone HO2, the treatment “Naïve exposure”, which has received antibiotic, were even marginally more resistant (Pearson’s Chi-squared test, $\chi^2=9.22$, $df=3$, $p\text{-value}=0.03$) (see Tables 1 and 2).

A previous study in the same system reported that a second exposure immediately or 48h after the first one decreased the likelihood of being infected (McTaggart et al., 2012) but did not show specificity. Such a short-term effect maybe elicited by wounding at the site of infection when the spores penetrate into the host body cavity (Duneau et al., 2011) during the first exposure, and not due to immune priming. Increased PhenolOxydase (PO) response after wounding has been described in *D. magna* by cutting off the spina (Mucklow and Ebert, 2003), and up-regulation of the ProPhenolOxidase (proPO) gene and hemocyte recruitment have been also reported shortly after infection (Auld et al., 2012; Labbé and Little, 2009). These observations correspond well with the observation that it takes several hours for *P. ramosa* to cross the foregut epithelium of the host (Duneau and Ebert, 2012). The ongoing activity of hemocytes and PO around the wounded epithelium may explain why, shortly after exposure, parasites

from a second challenge cross the epithelium at a lower rate. It could also be that attached spores from the first exposure may obstruct attachment by spores in a second challenge and may result in a specific form of priming. The presence of an ongoing cellular immune response would also explain why McTaggart et al. (2012) did not detect specificity in the host response. A later study report a lasting of seven days under the same conditions but details of the results reveal that only one *Daphnia* clone over four showed this effect which call for further investigation (Garbutt et al., 2014.).

Our study mainly differs from previous studies on immune priming of *Daphnia* by the use of an antibiotic to ensure that the priming dose was no longer present when the host received the challenge dose. The use of antibiotic has the disadvantage that it also clears other, eventually beneficial, bacteria from the host. As shown previously in the *Anopheles-Plasmodium* system, it is possible that *Daphnia* gut microbiota plays a role in immune priming (Rodriguez et al., 2010). However, our antibiotic treatment occurred only after the host could have been primed (i.e. the microbiota was intact during the time priming could occur). Additionally, we wanted to reduce the effect of the absence of microbiota during the response to the challenge dose. Therefore, we allowed for recolonization of the host gut by environmental bacteria over a period of five days, before doing the challenge dose. Recent work has shown that re-association of bacteria with juvenile *Daphnia* for six days restores the cost of not having a microbiota (Sison-Magnus et al., 2015). One day under non-sterile laboratory conditions is enough for a recolonization of the gut (Dieter Ebert personal communication). Finally, reciprocal transplant of microbiota among clones of *Daphnia magna* did not influence their interaction with *Pasteuria* (Sison-Magnus & Ebert, in preparation). Thus, while we could not exclude a possible role of a change in microbiota community due to our antibiotic treatment, we do not have any evidence that this might have been the case in the current experiment.

Another study reported that the offspring of infected *D. magna* mothers were better able to cope with reinfections with the same *P. ramosa* isolate (Little et al., 2003). The capability of parents, usually mothers, but not exclusively (Zanchi et al., 2011), to influence the resistance of their offspring against the parasites they have previously been exposed to, has been described several times, even in invertebrates (Moret, 2006; Sadd et al., 2005; Sadd and Schmid-Hempel, 2007). This phenomenon is

especially important for organisms with asexual reproduction, such as *D. magna*, because it might provide specific protection to the offspring that have the same genotype as their mothers, and are therefore equally susceptible to parasites. Little et al. (2003) found that offspring had higher fitness when exposed to the same (homologous) parasite isolates as their mother, when compared to exposure to other (heterologous) isolates. The authors interpreted this as evidence for a maternal transfer of strain-specific immunity in *D. magna*. Offspring fitness is a very indirect way to determine immune competence, as it depends on the assumption that fitness is related to the ability to fight parasites. Trade-offs, which are known to play a strong role in the immune function of arthropods, may undermine this assumption (Dowling and Simmons, 2012; Kraaijeveld et al., 2001; Moret, 2000; Sheldon and Verhulst, 1996; Short et al., 2012). To avoid this problem, we measured infection rates and parasite spore production rather than host fitness, as it gives a better measure of immune function. Furthermore, the alleged immune priming seems to be in contrast to the results of the present study, which indicate an absence of specific immune priming within a generation. The reported specific maternal transfer also seem to contradict the reported absence of immune response specificity reported in another study from the same lab (McTaggart et al., 2012). The findings of Little et al. (2003) could nevertheless be explained by other causes than the maternal transfer of specific somatic immune factors (e.g. epigenetic effects) but it would remain in contrast with the absence of a maternal effect when the mother is infected with a fungal parasite (Prior et al., 2011).

Further study in this system will require the use of other methodologies, and the consideration of other facets of infection, including: the elucidation of the early phase of parasite penetration, the possibility of intracellular immunity mechanisms, more elaborate kinetic studies of cellular and humoral parameters and their involvement in parasite clearance, more refined gene expression studies, and the identification of the genes responsible for resistance in resistant *D. magna* clones. A better understanding of immune mechanisms in invertebrates would represent an advance in epidemiology (Tidbury et al., 2012), evolutionary biology (Best et al., 2013), and would benefit to the aquaculture industry.

Experimental procedures

The two (mostly identical) experiments in this study included three experimental treatments for each of the two parasite clones and seven control treatments. The three experimental treatments are “Homologous challenge”, “Heterologous challenge” and “Naïve exposure” (Figure 1).

In separate experiments, we exposed two *D. magna* clones (isofemale lines HO2 from Hungary, and Kela-20-13 from Finland) to two *P. ramosa* clones (C1 from Russia and C19 from Germany). The parasite clones correspond to different *P. ramosa* genotypes that are capable of infecting both of the host clones (Luickjx et al., 2011). Host-parasite genotype combinations were used which are known to be fully compatible at the attachment step, i.e. show no variation in the first line of defense (see Duneau et al., 2011). The offspring of 36 female *D. magna*, reared in individual jars in identical conditions, were divided among the 13 treatments (i.e. split-brood design, Figure 1). Therefore, each individual within a treatment had a different mother, and maternal effects should be uniform across the 13 treatments. Each experimental treatment consisted of 36 individuals, while each of the seven control treatments consisted of 15 individuals. Due to mortality associated with the handling of very young *Daphnia*, the sample size were slightly smaller one day after the start of the experiment. There was no difference in mortality between exposure sequences (For Controls: GLM binomial distribution: Line: deviance= 0.10, df= 1, p-value=0.75, Exposure sequence: deviance= 5.64, df= 5, p-value=0.34; For Treatment: GLM binomial distribution: Line: deviance= 0.01, df= 1, p-value=0.89, Exposure sequence: deviance= 4.76, df= 5, p-value=0.34). During the experiment, *D. magna* were kept in a standardized medium (ADaM) (modified after Ebert et al., 1998; Klüttgen et al., 1994) at 20°C, and fed daily with chemostat-cultured unicellular algae, *Scenedesmus obliquus*. We provided 2.5 million algae cells per individual daily for the first three days, 3 million daily for the next four days, and 5 million daily on all subsequent days. The presence of infection was detected visually, as the symptoms of infection are apparent (i.e. reddish color and gigantism). The number of spores per infected *D. magna* was assessed using a Thoma counting chamber under a phase contrast microscope (Leica DM 2500, at magnification 400x). In the Experiment 2, some individuals of the Kela 20-13 clone, had still many bacteria in the cauliflower stage, making bacterial quantification difficult. For this reason we remove those individuals from the count dataset. The presence of cauliflower was not different between treatments (Chi-square test: $\chi^2 = 1.16$, $p = 0.55$).

In the experimental treatments with **priming**, we exposed 36 one-to-three-days-old *D. magna* to 50,000 spores of the same clone of *P. ramosa*. Each *D. magna* was exposed to the pathogen separately (one *D. magna* in 20 mL of ADaM). We used 15 additional individuals per parasite clone to control for the early successful infection of the experimental treatments (Figure 1, “Early infection”). These controls were inspected for infection 22 days after exposure. Another 36 individuals were individually kept not exposed at that age (Figure 1, “Naïve exposure”). Seven days after the first exposure, we treated *D. magna* in a solution of 10 mg/L tetracycline antibiotic (from Sigma-Aldrich) in 80 mL ADaM to stop bacterial growth from the first exposure (concentration based on manufacturer’s recommendation). *D. magna* were treated with the antibiotic for seven days. Because this antibiotic is sensitive to the light, the solution was changed every other day. Jars were randomly arranged in the incubator. Fifteen host individuals per parasite clone were used to control that the tetracycline treatment had cured the hosts from the first infection (Figure 1, “Cured”). Animals of this control treatment were inspected at the end of the experiment to be sure that the parasite did not proliferate after removing the antibiotic. We tested for the impact of the antibiotic on *Daphnia* survival with 15 additional *Daphnia* (Figure 1, “Antibiotic only”). In order to reduce the probability that the antibiotic was active during the second exposure, and to let the host’s microbiota recolonize, we kept *D. magna* in antibiotic-free medium for five days. A second parasite exposure was done with 5,000 spores of the same clone of *P. ramosa* that was used for the initial infection (Figure 1, “Homologous challenge”). However, to study the specificity of a potential immune memory effect, 36 *D. magna* individuals were challenged with different *P. ramosa* clones in the first and second exposures (Figure 1, “Heterologous challenge”). We reduced the spore dose in order to avoid a 100% infection rate, which would have hidden variation in infection success. These spores came from the same *P. ramosa* stock used for the first exposure. Because the *D. magna* were larger than during the first exposure, the second exposure took place in 40 mL medium. Twenty days after the challenge, we checked for infection and counted the number of spores per infected individual. We tested for the successful infection of the second exposure with 15 additional individuals (Figure 1, “Late infection”). This experimental design was carried out reciprocally with two *P. ramosa* clones.

Statistical analysis

We conducted a logistic regression for each experiment, to test whether the probability of *D. magna* infection after the second parasite exposure differed between the three experimental treatments. We used a generalized linear model (GLM) with a quasibinomial error distribution and logit link. Assumptions about the error distribution were checked by estimating dispersion parameters in GLM; the slight overdispersion recorded with a binomial error distribution was corrected by using a quasibinomial distribution. In the model, we included the parasite clone used in the challenge (Parasite clone: C1 and C19) and the treatment (Treatment: Homologous challenge, Heterologous challenge, Naïve exposure) as factors. *Infectious status ~ parasite_clone + Treatment*.

In each experiment, we used a two-way ANOVA to test whether the three treatments differed in parasite proliferation. The number of spores was log-transformed, and we included as factors first, the clone of the parasite clone used in the challenge (Parasite clone: C1 and C19) and then, the treatment (Treatment: Homologous challenge, Heterologous challenge, Naïve exposure). *Spore number ~ parasite_clone + Treatment*. Normality and homoscedasticity of the residuals were verified. All control treatments produced the expected results. All statistics were performed with R (R Core Team 2012).

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398

Table 1: Summary of the infection records among the four control treatments in the two experiments. We aimed for 15 replicates per control treatment combination, but in some combinations we lost 1 or 2 host individuals due to unexplained early mortality. “Late infection” corresponds to an exposure 19 days after the start of the experiment (see figure 1). “Ø” means no parasite exposure. “C1” and “C19” are the parasite clones used for the exposures. “Antibio.” means the treatment with tetracycline.

Control for:		1 st exposure*	2 nd exposure	Infected/Total	Proportion infected
Experiment 1: Host clone HO2	Early infection	C1	Ø	14/14	100 %
		C19	Ø	15/15	100 %
	Antibiotic cure	C1	Antibio.	0/14	0 %
		C19	Antibio.	0/15	0 %
	Late infection	Ø	C1	11/13	85 %
		Ø	C19	9/14	64 %
	Only antibiotic	Ø	Antibio.	0/15	0%
Experiment 2 : Host clone Kela20-13	Early infection	C1	Ø	14/14	100 %
		C19	Ø	15/15	100 %
	Antibiotic cure	C1	Antibio.	0/13	0 %
		C19	Antibio.	0/13	0 %
	Late infection	Ø	C1	7/14	50 %
		Ø	C19	4/15	27 %
	Only antibiotic	Ø	Antibio.	0/14	0%

Table 2: Summary of infection records among the experimental treatment groups in the two experiments. Antibiotic treatment occurred in all six treatments between 1st and 2nd exposure. “Ø” means no parasite exposure. “C1” and “C19” are the parasite clones used for the exposures.

	Treatment	1 st exposure	2 nd exposure	Infected/Total	Proportion infected	Proportion infected per treatment	Logistic regression
Exp 1: Host clone HO2	Homologous	C1	C1	17/29	59 %	45 %	df=2 deviance=0.4 p=0.82
		C19	C19	10/31	32 %		
	Heterologous	C1	C19	14/31	45 %	40 %	
		C19	C1	11/31	35 %		
	Naïve	∅	C1	13/31	42 %	40 %	
		∅	C19	12/31	39 %		
Exp 2: Host clone Kela 20-13	Homologous	C1	C1	20/34	59 %	51 %	df=2 deviance=1.22 p=0.55
		C19	C19	14/32	44 %		
	Heterologous	C1	C19	18/33	55 %	48 %	
		C19	C1	13/32	41 %		
	Naïve	∅	C1	12/25	48 %	39 %	
		∅	C19	9/29	31 %		

Figure legends:

Figure 1: Experimental design to test for the effect of priming. C1 and C19 are two *P. ramosa* clones. “Antibio.” is the treatment with tetracycline. “ADaM” is the artificial *Daphnia* culture medium free of parasite spores and antibiotic. “Check” is the moment where we killed the hosts to quantify the number of parasite spores in the host and/or for their infection status. Group “Early infection” controls for the success of the infection after the first exposure in the experimental treatments. Group “Late infection” controls for the success of the infection after the second exposure in the experimental treatments. Group “Cured” controls for the success of the antibiotic to cure the infected *Daphnia*. Group “Antibiotic only” controls for the effect of the antibiotic on the host survival. The entire experiment was independently replicated with two host clones.

Figure 2: Number of *P. ramosa* spores resulting from infections after homologous challenge, heterologous challenge and naïve exposure. Means are represented by red triangles in the boxplots. Number of replicates per group is indicated in brackets.

EXPERIMENTAL TREATMENTS

CONTROL TREATMENTS

a/ Experiment 1

b/ Experiment 2