Developmental and Comparative Immunology 57 (2016) 120–125

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

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

David Duneau*, Dieter Ebert, Louis Du Pasquier

University of Basel, Zoological Institute, Vesalgasse 1, Basel, Switzerland

ARTICLE INFO

Article history:
Received 23 September 2015
Received in revised form
7 December 2015
Accepted 8 December 2015
Available online 19 December 2015

Keywords:
Immune priming
Host–parasite interaction
Invertebrate
Daphnia
Pasteuria

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.

1. 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;

* Corresponding author. Current address: Laboratory Ecologie et Diversité Biologique (EDB), Université Toulouse III Paul Sabatier, Bâtiment 4R1, 118, route de Narbonne, 31062 Toulouse cedex 9, France.
E-mail addresses: david.duneau@univ-tlse3.fr, david.duneau@gmail.com (D. Duneau).

http://dx.doi.org/10.1016/j.dci.2015.12.004
0145-305X/

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 Ramirez et al. (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.

2. Results and discussion

Each experiment consisted of three experimental treatments and four control treatments (Fig. 1). We infected *D. magna* with *P. ramosa* following three experimental treatments: 1) hosts were infected, then cured with tetracycline and then exposed to the same parasite strain (homologous challenge), 2) hosts were 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).

2.1. 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 (Fig. 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 (Fig. 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 antibodies 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.

2.2. 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 (Fig. 2, linear model, "spore number" controlled for variance

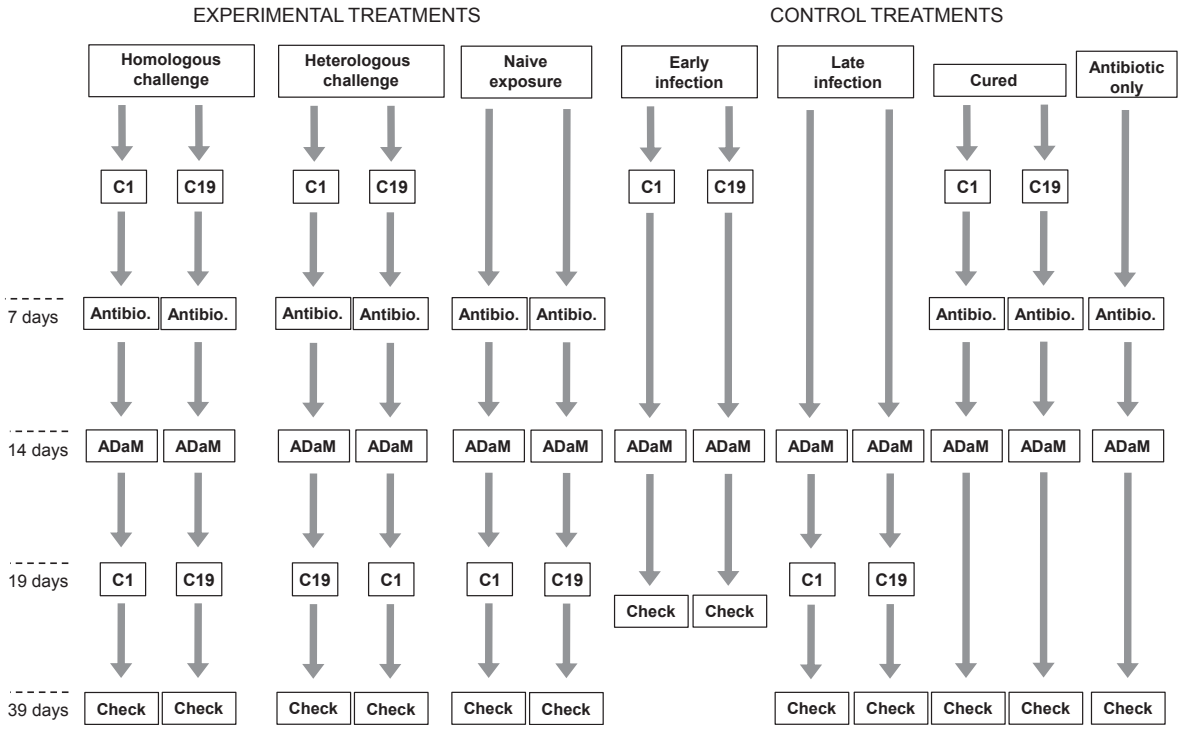

Fig. 1. Experimental design to test for the effect of priming. C1 and C19 are two *P. ramosa* clones. "Antibio." is the treatment with tetracycline. "ADaM" is the artificial *Daphnia* culture medium free of parasite spores and antibiotic. "Check" is the moment where we killed the hosts to quantify the 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.

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 Fig. 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:	1st Exposure	2nd Exposure	Infected/Total	Proportion infected
Experiment 1:	Early infection	C1	Ø	14/14	100%
Host clone		C19	Ø	15/15	100%
HO2	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:	Early infection	C1	Ø	14/14	100%
Host clone		C19	Ø	15/15	100%
Kela20-13	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%

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-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-value = 0.03) (see Tables 1 and 2).

A previous study in the same system reported that a second exposure immediately or 48 h 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

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

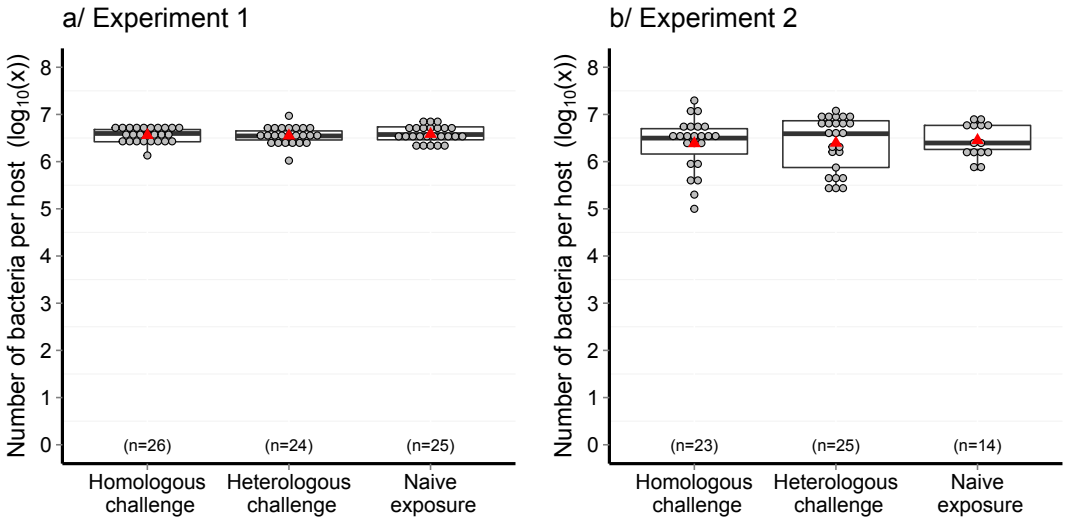

Fig. 2. Number of *P. ramosa* spores resulting from infections after homologous challenge, heterologous challenge and naïve exposure. Means are represented by triangles in the boxplots. Number of replicates per group is indicated in brackets.

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., 2013).

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 (Rodrigues 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 *D. 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.

3. 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” (Fig. 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, Fig. 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 removed 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 (Fig. 1, “Early infection”). These controls were inspected for infection 22 days after exposure. Another 36 individuals were individually kept not exposed at that age (Fig. 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 (Fig. 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* (Fig. 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 5000 spores of the same clone of *P. ramosa* that was used for the initial infection (Fig. 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 (Fig. 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 (Fig. 1, “Late infection”). This experimental design was carried out reciprocally with two *P. ramosa* clones.

3.1. 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).

Acknowledgments

This study was supported by the Swiss National Science Foundation. We thank Jürgen Hottinger for lab support and Patrícia Beldade, Nicolas Buchon, Suzanne Claflin, Adam Dobson and Brian Lazzaro's lab for comments on the manuscript.

References

Auld, S., Edel, K., Little, T.J., 2012. The cellular response of under host-parasite genetic variation and variation in initial dose. Evolution 66, 3287–3293.
Best, A., Tidbury, H., White, A., Boots, M., 2013. The evolutionary dynamics of within-generation immune priming in invertebrate hosts. J. R. Soc. Interface e10, 20120887.
Chopra, I., Roberts, M., 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. R. 65, 232–260.
Cooper, E.L., 1968. Transplantation immunity in annelids I. Rejection of xenografts exchanged between *Lumbricus terrestris* and *Eisenia foetida*. Am. Zool. 6, 322–327.
Cooper, E.L., Roar, P., 1986. Second-set allograft responses in the earthworm *Lumbricus terrestris*. Transplantation 41, 514–520.
Decaestecker, E., Labbé, P., Ellegaard, K., Allen, J., 2011. Candidate innate immune system gene expression in the ecological model *Daphnia*. Dev. Comp. Immunol. 35, 1066–1075.
Decaestecker, E., Gaba, S., Raeymaekers, J.A., Stoks, R., van Kerckhoven, L., Ebert, D., de Meester, L., 2007. Host–parasite "Red Queen" dynamics archived in pond sediment. Nature 450, 870–873.
Dowling, D.K., Simmons, L.W., 2012. Ejaculate Economics: testing the effects of male sexual history on the trade-off between sperm and immune function in australian crickets. PLoS ONE 7, e30172.
Duneau, D., Luijckx, P., Ben-Ami, F., Laforsch, C., Ebert, D., 2011. Resolving the infection process reveals striking differences in the contribution of environment, genetics and phylogeny to host-parasite interactions. BMC Biol. 9, 11.
Duneau, D., Ebert, D., 2012. The role of moulting in parasite defence. P. Roy. Soc. B-Biol Sci. 279, 3049–3054.
Ebert, D., Zschokke-Rohringer, C., Carius, H., 1998. Within-and between-population variation for resistance of *Daphnia magna* to the bacterial endoparasite *Pasteuria ramosa*. P. Roy. Soc. B-Biol Sci. 265, 2127–2134.
Ebert, D., Duneau, D., Hall, M.D., Luijckx, P., Andras, J.P., Du Pasquier, L., Ben-Ami, F., 2016. A population biology perspective on the stepwise infection process of the bacterial pathogen *Pasteuria ramosa* in *Daphnia*. Adv. Parasit. http://dx.doi.org/10.1016/bs.apar.2015.10.001 (in press).
Garbutt, J., Scholefield, J., Vale, P., Little, T.J., 2013. Elevated maternal temperature enhances offspring disease resistance in *Daphnia magna*. Funct. Ecol. 28, 424–431.
Garbutt, J.S., O'Donoghue, A.J.P., McTaggart, S.J., Wilson, P.J., Little, T.J., 2014. The development of pathogen resistance in *Daphnia magna*: implication for disease spread in age-structured population. J. Exp. Biol. 217, 3929–3934.
George, J., Howcroft, T., Karp, R., 1987. Primary integumentary allograft reactivity in the American cockroach, *Periplaneta americana*. Transplantation 53, 514–519.
Hauton, C., Smith, V., 2007. Adaptive immunity in invertebrates: a straw house without a mechanistic foundation. BioEssays 29, 1138–1146.
Karp, R., Hildemann, W., 1976. Specific allograft reactivity in the sea star *Dermasterias imbricata*. Transplantation 22, 434–439.
Klüttgen, B., Dülmer, U., Engels, M., Ratte, H.T., 1994. ADaM, an artificial freshwater for the culture of zooplankton. Water Res. 28, 743–746.
Kraaijeveld, A., Limentani, E., Godfray, H.L., 2001. Basis of the trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. P. Roy. Soc. B-Biol Sci. 268, 259–261.
Kurtz, J., Franz, K., 2003. Innate defence: evidence for memory in invertebrate immunity. Nature 425, 37–38.
Labbé, P., Little, T.J., 2009. ProPhenolOxidase in *Daphnia magna*: cDNA sequencing and expression in relation to resistance to pathogens. Dev. Comp. Immunol. 33, 674–680.
Labbé, P., McTaggart, S.J., Little, T.J., 2009. An ancient immunity gene duplication in *Daphnia magna*: RNA expression and sequence analysis of two nitric oxide synthase genes. Dev. Comp. Immunol. 33, 1000–1010.
Langlet, C., Bierne, J., 1982. Immune characteristics of graft rejection in nemerteans of the genus *Lineus*. Eur. J. Immunol. 12, 705–708.
Little, T.J., O'Connor, B., Colegrave, N., Watt, K., Read, A.F., 2003. Maternal transfer of strain-specific immunity in an invertebrate. Curr. Biol. 13, 489–492.
Little, T.J., Colegrave, N., Sadd, B., Schmid-Hempel, P., 2008. Studying immunity at the whole organism level. BioEssays 30, 404–405.
Luickjx, P., Ben-Ami, F., Mouton, L., Du Pasquier, L., Ebert, D., 2011. Cloning of the unculturable parasite *Pasteuria ramosa* and its *Daphnia* host reveals extreme genotype–genotype interactions. Ecol. Lett. 14, 125–131.
Masri, L., Cremer, S., 2014. Individual and social immunisation in insects. Trends Immunol. 35, 471–482.
McTaggart, S.J., Wilson, P.J., Little, T.J., 2012. *Daphnia magna* shows reduced infection upon secondary exposure to a pathogen. Biol. Lett. 8, 972–975.
Moret, Y., 2000. Survival for immunity: the price of immune system activation for bumblebee workers. Science 290, 1166–1168.
Moret, Y., 2006. "Trans-generational immune priming": specific enhancement of the antimicrobial immune response in the mealworm beetle, *Tenebrio molitor*. P. Roy. Soc. B-Biol Sci. 273, 1399–1405.
Moret, Y., Siva-Jothy, M.T., 2003. Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle, *Tenebrio molitor*. P. Roy. Soc. B-Biol Sci. 270, 2475–2480.
Mouton, L., Ebert, D., 2008. Variable-number-of-tandem-repeats analysis of genetic diversity in *Pasteuria ramosa*. Curr. Microbiol. 56, 447–452.
Mucklow, P.T., Ebert, D., 2003. Physiology of immunity in the water flea *Daphnia magna*: environmental and genetic aspects of phenoloxidase activity. Physiol. Biochem. Zool. 76, 836–842.
Pham, L.N., Dionne, M.S., Shirasu-Hiza, M., Schneider, D.S., 2007. A specific primed immune response in *Drosophila* is dependent on phagocytes. PLoS Pathog. 3, e26.
Pope, E.C., Powell, A., Roberts, E.C., Shields, R.J., Wardle, R., Rowley, A.F., 2011. Enhanced cellular immunity in shrimp (*Litopenaeus vannamei*) after vaccination. PLoS ONE 6, e20960.
Prior, N., Washington, C., Housley, J., Hall, S., Duffy, M., Cáceres, C., 2011. Maternal effect and epidemiological traits in a planktonic host-parasite system. Evol. Ecol. Res. 13, 401–413.
Rodrigues, J., Brayner, F.A., Alves, L.C., Dixit, R., Barillas-Mury, C., 2010. Hemocyte differentiation mediates innate immune memory in *Anopheles gambiae* mosquitoes. Science 329, 1353–1355.
Ramirez, J.L., de Almeida Oliveira, G., Calvo, E., Dalli, J., Colas, R.A., Serhan, C.N., Ribeiro, J.M., Barillas-Mury, C., 2015. A mosquito lipoxin/lipocalin complex mediates innate immune priming in *Anopheles gambiae*. Nat. Commun. 6, 7403.
R Core Team, 2012. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL: http://www.R-project.org/.
Roth, O., Sadd, B.M., Schmid-Hempel, P., Kurtz, J., 2009. Strain-specific priming of resistance in the red flour beetle, *Tribolium castaneum*. P. Roy. Soc. B-Biol Sci. 276, 145–151.
Rowley, A.F., Powell, A., 2007. Invertebrate immune systems–specific, quasi-specific, or nonspecific? J. Immunol. 179, 7209–7214.
Sadd, B.M., Kleinlogel, Y., Schmid-Hempel, R., Schmid-Hempel, P., 2005. Trans-generational immune priming in a social insect. Biol. Lett. 1, 386–388.
Sadd, M., Schmid-Hempel, P., 2006. Insect immunity shows specificity in protection upon secondary pathogen exposure. Curr. Biol. 16, 1206–1210.
Sadd, M., Schmid-Hempel, P., 2007. Facultative but persistent trans-generational immunity via the mother's eggs in bumblebees. Curr. Biol. 17, R1046–R1047.
Schmid-Hempel, P., 2011. Evolutionary Parasitology: the Integrated Study of Infections, Immunology, Ecology, and Genetics, first ed. Oxford University Press, New York.
Schulenburg, H., Boehnisch, C., Michiels, N.K., 2007. How do invertebrates generate a highly specific innate immune response? Mol. Immunol. 44, 3338–3344.
Sheldon, C., Verhulst, S., 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol. Evol. 11, 317–321.
Short, S.M., Wolfner, M.F., Lazzaro, B.P., 2012. Female *Drosophila melanogaster* suffer reduced defense against infection due to seminal fluid components. J. Insect Physiol. 58, 1192–1201.
Sison-Mangus, M.P., Mushegian, A.A., Ebert, D., 2015. Water fleas require microbiota for survival, growth and reproduction. ISME J. 9, 59–67.
Tidbury, H.J., Pedersen, A.B., Boots, M., 2011. Within and transgenerational immune priming in an insect to a DNA virus. P. Roy. Soc. B-Biol Sci. 278, 871–876.
Tidbury, H.J., Best, A., Boots, M., 2012. The epidemiological consequences of immune priming. P. Roy. Soc. B-Biol Sci. 279, 4505–4512.
Witteveldt, J., Cifuentes, C.C., Vlak, J.M., van Hulten, M.C., 2004. Protection of *Penaeus monodon* against White Spot Syndrome virus by oral vaccination. J. Virol. 78, 2057–2061.
Zanchi, C., Troussard, J., Martinaud, G., Moreau, J., Moret, Y., 2011. Differential expression and costs between maternally and paternally derived immune priming for offspring in an insect. J. Anim. Ecol. 80, 1174–1183.