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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.

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;

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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