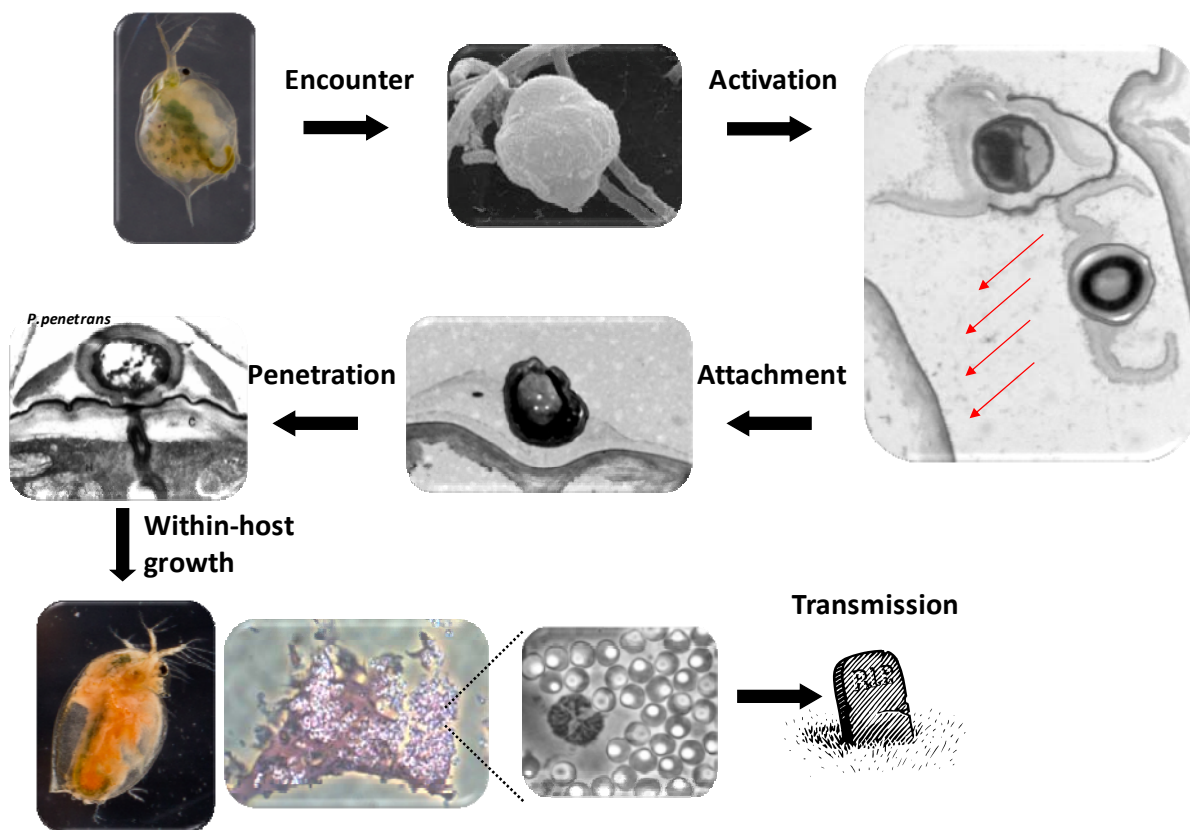


# Evolutionary and proximate mechanisms shaping host-parasite interactions: The case of *Daphnia magna* and its natural bacterial parasite *Pasteuria ramosa*.

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PhD thesis – Universität Basel, Schweiz



Dedication

To my deceased grandfather Marcel Duneau

Dédié à

Mon défunt grand père Marcel Duneau

Cover : Sequence of the steps shaping the interaction of *Daphnia magna* with its parasite *Pasteuria ramosa*.

**Evolutionary and proximate mechanisms shaping host-parasite interactions:  
The case of *Daphnia magna* and its natural bacterial parasite *Pasteuria ramosa*.**

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## Summary

Host-parasite interactions are composed of a sequence of steps, all necessary for successful infection: parasites need to encounter their hosts, to enter into their bodies, and to proliferate within them. Selection will act on the mechanisms used in each of the steps; the parasite being selected to increase their efficiency, and the host selected to reduce it. I have proposed, and shown, that explicitly analyzing the factors that influence each of the steps and their impact on host and parasite fitness is of crucial importance for a complete understanding of host-parasite interactions. In my Ph.D. research work, I identified markers of different steps of the interaction between the host crustacean *Daphnia magna* and its natural bacterial parasite *Pasteuria ramosa*, and investigated factors influencing different steps, as well as the contribution of each of them to shaping the interaction between the two species.

I established that the infection of *Daphnia magna* by *Pasteuria ramosa* could be decomposed in at least five sequential steps (Chapter 1): 1) the encounter between the host and the parasite, 2) the activation of the parasite transmissible, resting stage, which happens once it contacts the host, 3) the attachment of the parasite to the host cuticula, 4) the penetration of the parasite into the host body cavity, and 5) the proliferation of the parasite within the host. The factors affecting the likelihood of encounter between host and parasite had been investigated before, in a study that revealed that there is a host genetic component, and polymorphism for the ability of the host to avoid encountering the parasite. Resolving the interaction into its different steps and focusing on steps affect the encounter allowed me to see that: i) different steps are under the influence of different factors (Chapter 1), ii) the traits underlying some steps, but not all, do not seem to be polymorphic (Chapter 1), iii) the parasite genotype specificity of the success of the attachment step can explain the genotype specificity of the host susceptibility (Chapter 1), iv) the speed with which the parasite penetrates the host body after attachment is crucial for the parasite success (Chapter 2), v) the molting, usually seen as a cost against parasite, can be beneficial to reduce the likelihood of infection, vi) once in the host body, the parasite will adapt to the environment that is characteristic of the most common host sex, here female characteristic (Chapters 3 and 4), vii) the success of proliferation of *P. ramosa* inside *D. magna* hosts is not influenced by previous host exposure to that same parasite (Chapter 5). All in all, I show that considering each of the steps explicitly provides new light into the mechanisms and selective pressures on hosts and their parasites. Each of the two interacting parties will, indeed, be under more or less strong selection to maximize their success at each of the steps. Below I will elaborate on this idea in relation to my specific findings and the research perspectives they open.





## Introduction

### Background

The costly exploitation of one species by another (*i.e.* parasitism) is one of the most abundant lifestyles and the antagonistic interactions between host (the exploited species) and parasite (the exploiting species) are a key structuring force in natural populations of all organisms. The coevolution of hosts and their parasites is the result of multiple adaptations (*e.g.* for the parasite to infect the host) and counter-adaptations (*e.g.* for the host to avoid infection) evolving in concert at several stages of the interaction [1].

From phages to ectoparasites, the success of the infection, or its failure, depends on the success of each of the sequential steps which compose the whole interaction. First, the parasite must encounter its host. During this step, the parasite will be selected for traits that increase the likelihood of this encounter. For example, it is known that humans carrying the transmissible stage of the parasite responsible for Malaria, *Plasmodium falciparum*, attract more the mosquito vector, *Anopheles gambiae*, than those individuals uninfected or carrying non transmissible stages [2]. On the other hand, the need to avoid parasites acts as a major selection pressure on animal behavior [3] and elements of their migratory [4], social [5], and foraging strategies [6] are important for parasite avoidance.

Once encounter has taken place, parasites need to enter the host tissues, either partially (*e.g.* bloodsucking ectoparasites) or entirely (in endoparasites). For many parasites, this step is preceded by the attachment to the host protective layer (*i.e.* the cuticula/skin). Many hosts have evolved mechanisms to prevent this attachment. For example, some species produce extra layers upon their cuticula/skin – the usual first barrier against infection – that obstruct parasite penetration [mucus that functions in coral protection, 7, *e.g.* salivary mucins that preserve oral cavity health, 8]. Host can also have other means to remove the recently encountered parasite (*e.g.* grooming behavior, local immune

inflammations after a bite of ectoparasite). Such defense mechanisms impose strong selection on the parasite to develop adaptations to cross the host epithelium quickly, minimizing the chances of being noticed and removed by the host. For example, blood-sucking arthropods, like ticks and mosquitoes, have a modified rostrum to penetrate through the skin of their vertebrate hosts and saliva that disrupts the recognition by the host's dermal immune system [9]. As a more extreme example of a parasite adaptation to penetrate the host quickly, microsporidian parasites evolved a host invasion apparatus that rapidly pierces the host cell membrane, and serves as channel for sporoplasm passage into the new host cell, thereby skipping any attachment to the host external cuticula [10].

After the penetration into the host, the next step of the infection process is the parasite proliferation inside the host's body. During this step, the parasite will adapt to maximize the exploitation of the host's resources, under the conditions that the most common host type provides as environment [11]. To counter this proliferation, the host can adapt to be able to modify the parasite environment and make it less suitable [*e.g.* iron-withholding strategy in innate immunity of vertebrates and invertebrates, 12] or actively defend itself with an immune response. The immune system is a "mobile organ" resulting from hosts having adapted to avoid parasite establishment after penetration, or to reduce the parasite proliferation and its cost. Thus, the immune system, both innate and acquired, confers a fitness advantage to the individual using it and is, therefore, always adaptive. In counterpart, under the specific selection pressures of different components of host immunity, parasites evolved strategies to disrupt or hide from the host immune recognition [13]. For example, the Gram-positive bacterium, *Bacillus anthracis*, produces anthrax toxins that disarm the host's immune response repertoire [14].

Each step of the infection process involves different traits (*e.g.* particular aspects of morphology or physiology) of both the host and the parasite. For example, the mosquito sense organs are used to find their host, their rostrum is used to penetrate through the host skin, and their saliva to avoid being detected by the host while they feed. Each of these traits is selected for the successful exploitation of the host resources. Because the different steps of the infection involve distinct sets of parasite and host traits, those different steps are probably under the control of different genes, and may be influenced by the environment to different degrees. Still, and even though, each step may contribute to the success of the infection in a different manner, studies of host-parasite interactions typically investigate the success of the whole process of infection and do not take explicit account of each of the steps of that process. For example, hosts are characterized as resistant regardless of which step, or steps, of the interaction might be failing. I will argue that the intermingling of the effects of all steps has limited the interpretation of results of previous studies. Understanding the origin of variation in parasite success, central for controlling disease, will require understanding variation in success of each step. Therefore, the polymorphism of the traits involving in each step, their role and the strength of selection acting on them are important to be determined to fully understand the host-parasite interactions.

### Aims of the thesis

As explained above, the successful infection of one host by a parasite depends on the success of each of a sequence of steps. Because these steps are at least partially independent from each other, they can make distinct contributions to the coevolution of hosts and parasites. Yet, surprisingly, very few studies of host-parasite interactions take explicit account of this. The aim of my Ph.D. research work was to show that disentangling the process of infection can help to better understand host-parasite interactions, their specificity, their dynamics, and their evolution. The first objective of the thesis is to characterize

the sequence of steps of the interaction, develop a method to disentangle them easily, and test the effect of genetic and environmental factors on the success of the initial steps (Chapter 1). The second objective is to investigate which type of adaptations can occur at different steps to avoid or favor the infection. I investigate whether the host can be adapted to avoid the penetration once in contact with the parasite (Chapter 2), and whether the parasite can specifically adapt to proliferate in the common host physiology, more specifically, related to differences between male and female hosts (Chapters 3 and 4). The third specific objective was to find out whether the host can reduce the likelihood of infection after recurrent exposure to the same parasite (Chapter 5).

### Experimental model

I used the host *Daphnia magna* and its natural bacterial parasite *Pasteuria ramosa*. This system has been investigated in both field and laboratory studies. It has been shown that *P. ramosa* evolves tightly with *Daphnia*, and it imposes strong selection on *Daphnia* [15,16]. This is one of the few systems with empirical evidence for frequency-dependent selection in nature (Decaestecker *et al.* 2007). Recently, the possibility of working with clonal strains of the parasites in laboratory revealed that the interaction is very *D. magna* genotype - *P. ramosa* genotype specific [17]. The knowledge about the conditions of infection associated with the control of both parasite and host genotypes have been crucial in this thesis.

**The host** *Daphnia magna* is a planctonic crustacean. *Daphnia* have been intensively studied for 250 years for eco-toxicology, phenotypic plasticity, and behavior, and, more recently, for the interactions with their natural parasites, with emphasis on issues of antagonistic coevolution [reviewed in 18]. *Daphnia* provides both extensive genetic and genomic resources (including the fully sequenced genome of *D. pulex* [19], and genetic maps for *D. magna* [20]; see *Daphnia* Genomics Consortium at <https://wiki.cgb.indiana.edu/display/DGC/Home>) and solid knowledge on its ecology. All there

make it a powerful model system (also in the official list of NIH model systems), including for modern evolutionary ecology.

*Daphnia* have a wide, nearly cosmopolitan, distribution and colonize most of still freshwater bodies [21]. The environmental conditions of their habitats can range from very stable (*e.g.* large temperate lakes whose water depth and temperature changes relatively little throughout the seasons and years) to extremely unstable (*e.g.* rockpools, which can dry or be covered with snow within the same year, with sometimes more than 15°C difference within the same day). They are small transparent crustaceans (Figure 1) whose body is covered with a carapace, mainly made of chitin, which is shed at regular intervals [22]. The transparency of the body facilitates checking infected and reproductive status, and was of great relevance for the work described in chapter 1 of this thesis. The shedding of the whole carapace (molting) was important for the work described in chapter 2. *Daphnia* are planctonic filter feeders, eating mainly planctonic algae and, in our laboratory, they are kept in a freshwater medium on a diet of unicellular green algae (*Scenedesmus obliquus* during my experiments).

The majority of *Daphnia* species reproduce by cyclical parthenogenesis. They reproduce asexually for most of the season and sexually when conditions deteriorate (*e.g.* high densities) or predict future deterioration (*e.g.* change in photoperiod before winter). The asexual eggs are kept for several days in the female brood pouch (several dozen per clutch) and are released into the environment when the offspring are able to swim (Figure 1A). These eggs will produce mainly female offspring, and occasionally males. Males and females are, thus, genetically identical (and also identical to their mothers) and sex is environmentally determined [24]. Adult males and females differ in size, morphology (Figure 2), physiology, behavior and, of course, in their roles in reproduction. The predominantly asexual reproduction has as consequence that the sex ratios in *Daphnia* populations are typically very strongly female biased for most of the year. The differences between males and females in phenotype and in abundance were of great



Figure 1: **Asexual and sexual reproductive female.** A- Female carrying asexual embryos in the brood pouch. Adult females can carry several dozens of offspring genetically identical of herself. B- Female carrying an ephippium, *i.e.* is a melanized structure derived from the carapace that protects sexual eggs from harsh conditions. Ephippia are shed during molting. [Picture from 23].

relevance for the work described in chapters 3 and 4.

Induced by changes in the environment, females can also produce haploid eggs that need fertilization by males. Similarly to asexual eggs, sexual eggs are laid in the brood pouch (maximum two per clutch). Whether fertilization occurs before or after this event is unknown. Unlike the asexual eggs which develop without interruption, the embryos resulting from the sexual eggs stop developing around the gastrula stage (Elham Sheikh-Jabbari, personal communication). During development, the brood pouch becomes dark because of the formation of two chitinous layers

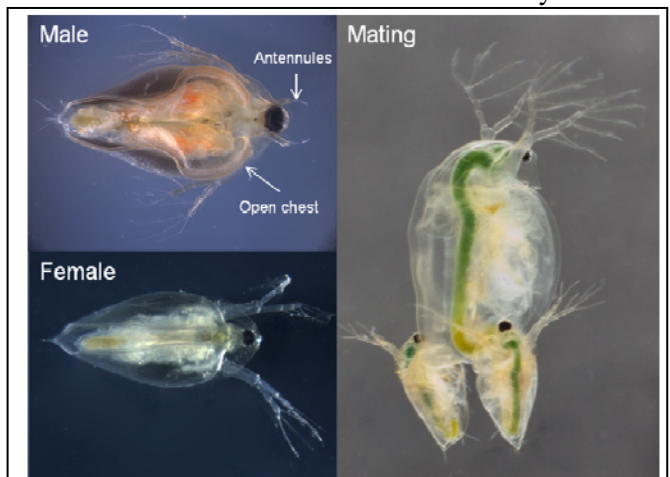


Figure 2: **Morphological differences between male and female *D. magna*.** Males are distinguished from females (here both in ventral view and during mating) by their smaller size, larger antennules (typically too small to be seen easily in females), modified post-abdomen (open chest) and first legs, which are armed with a hook used in clasping during mating.

surrounding the eggs (Figure 1B). This structure, which is part of the carapace, is called an ephippium and it will be released with the eggs inside once the mother sheds her carapace. Because of the protective role of the ephippium, protecting the embryos from desiccation and allowing *Daphnia* revival when the pool is refilled with water, the sexual eggs are also called resting eggs.

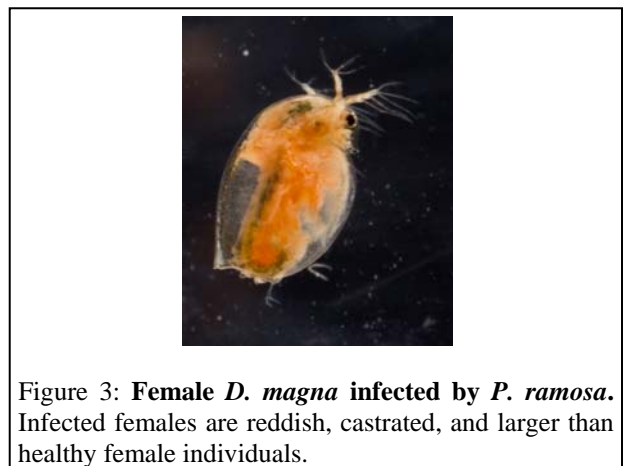
Asexual reproduction is the means of reproduction in normal conditions, and sexual reproduction is inducible with environmental conditions. The cyclical parthenogenesis of *Daphnia* can be controlled in the laboratory, adding to their value as an experimental system in evolutionary ecology. On the one hand, the asexual mode of reproduction allows for keeping hosts as clonal lineages, and to record phenotypic traits like fecundity, growth and survival on multiple replicates of the same genotype. This has been crucial for all the experiments in the thesis. On the other hand, the sexual mode of reproductions allows for performing crosses to, for example, study the genetic basis of variation in different traits. The durability of the sexual eggs allows for recovering natural genotypes conserved in mud which can be hatched and studied decades later.

**The parasite** In nature, *Daphnia magna* is frequently found to suffer from bacterial, fungal and microsporidial infections [23,25], among them by the bacterium *Pasteuria ramosa* [25]. This parasite is a common parasite of several *Daphnia* species [23,25,26], and infections have been reported from both Europe and North America (Ebert 2005).

*Pasteuria ramosa* is a Gram-positive, endospore-forming bacteria closely related to *Bacillus* and *Clostridium*, both of which include species responsible for human diseases (e.g. Anthrax acute disease). *P. ramosa* is an extracellular endoparasite, proliferating within the hemocoel and the musculature of the *Daphnia* host [27]. It infects susceptible hosts when the waterborne endospores are ingested while the hosts filter the water for food procurement. This

endospore is a resting stage of the parasite that can remain dormant in the ground for decades [28] thanks to the protection of an external layer called exosporium. The activation of the dormant endospores was addressed in chapter 1. After infection, it is only after 12 to 14 days that the first parasites can be detected under microscope. This explains why in laboratory the *Daphnia* infection status cannot be determined reliably before 14 days after host and parasite are put into contact.

Shortly after the start of the proliferation inside the host body, the parasite castrates the host [29]. This induces host gigantism, which increases the host carrying capacity for proliferating parasite spores. This point was crucial for the work in chapter 4. The *Daphnia* infection status starts to be reliably noticeable by visual inspection thanks to the obvious symptoms that includes host castration, reddish body color and gigantism (see Figure 3). Once infection is noticeable, *Daphnia magna* can generally not recover from a *P. ramosa* infection, unless treated with antibiotics [30]. This was crucial for the experimental design of the work described in chapter 5. Parasite proliferation leads to the production of several millions of endospores which will be transmitted horizontally only after host death. This parasite is not transmitted vertically.



### Thesis outline

The consecutive chapters of this thesis more or less follow the sequence of steps in the *Daphnia magna*-*Pasteuria ramosa* infection process, to explore different aspects of the interaction and coevolution of the system.

**Chapter 1** The success of each of the sequential steps that compose the infection process is necessary for parasite transmission. Each step can have a different impact on the interaction between hosts and parasites. In this chapter, I present a case study where I characterize a series of consecutive steps of an infection process and distinguish the effects of different factors (environment, genetics and phylogeny) on each of the steps. I developed a new method using the transparent *D. magna* hosts and fluorescently-labeled spores of its parasite *P. ramosa* to identify easily markers of the steps. My key finding is that different consecutive steps of the infection process, notably, the activation and attachment of the parasite spores after encountering the hosts, are influenced by different factors, and thus, can make different contributions to shaping host-parasite interactions and coevolution. More precisely, I found that the activation and attachment steps are not affected by environmental factors like temperature, food level and population density, and occur in both female and male hosts. On the other hand, the two steps differ in the way they are affected by genetic factors. Activation does not depend on host genotype - parasite genotype combinations, and the cues triggering it are phylogenetically conserved. On the other hand, the attachment step is highly host genotype - parasite genotype specific. With my data, I showed that infection success, a process which is generally considered to show a quantitative outcome (*i.e.* the likelihood of infection can be any number between 0% and 100%), can in fact be reduced to a Yes/No outcome (*i.e.* the likelihood of success at any of the steps can be either 0% or 100%) when the right step in the infection process is looked at. These binary outcomes, presumably based on binary underlying mechanisms, are often key assumptions of theoretical models of host-parasite co-evolution (*e.g.* the Red Queen model and the Selective Sweep model). My results show that an approach that disentangles the contribution of the individual steps to the success of the whole infection process can help reconcile empirical data with predictions based on such evolutionary models and reveals why previous attempts had difficulties in doing so.

**Chapter 2** The attachment of the parasite to its host body is a crucial step in most host-parasite interactions, where it precedes penetration of the parasite into the host. In invertebrate hosts, this attachment often occurs onto the protective layer that surrounds the body cavity, called the cuticula. The complete shedding of this layer, a process called molting, is a crucial feature in the life-cycle of many invertebrate phyla (and of some vertebrates). In this chapter, I investigated whether host molting can contribute to resistance to parasites, and whether it can be manipulated by infected hosts for that purpose. I used *D. magna* hosts which molt at regular intervals and its parasite *P. ramosa* which attaches to the host cuticula before penetrating into the host body cavity where it can proliferate. I show that molting does rid the host of attached parasites, and by doing so, reduces the likelihood of infection. My data shows that for this to be effective, host molting has to occur within the first 12hr after infection, before parasite penetration into the host. Because molting can reduce parasitism, I asked whether infected *Daphnia* hosts could actively manipulate timing of molting. Parasite-induced delay of molting has been shown in other invertebrates. However, my results show that exposure to the parasite does not affect molting interval in *Daphnia magna* hosts. I discuss the implications that molting as a passive mechanism of resistance may have on parasite evolution.

**Chapter 3** Once the parasite is inside the host, it will face whatever challenges are imposed by the host's internal environment. Common, clear, and consistent differences between host individuals can be seen in cases of sexual dimorphism, which is common in bisexual species. Males and females typically differ for all sorts of traits, including morphology, physiology and behavior. In this chapter, I combined conceptual thinking with a review of the literature on host sex-specific parasitism to make the case that host sex differences are likely to represent different challenges and different opportunities for parasites. I propose that host-sex driven selection on the parasite can lead to three different

scenarios in terms of parasite evolution: 1) sex-specific adaptation, 2) single sex-specialization, and 3) sex-specific phenotypically plastic expression of parasite traits. Which of these scenarios will dominate depends mainly on two variables: the degree of host sexual dimorphism and the likelihood that the parasite encounters hosts of each sex. Taking parasite evolution into account this chapter might contribute towards explaining the widespread phenomenon of host sex-biased parasitism and disease expression. With this chapter, I hope to have contributed novel insight and to have opened new perspectives to studies of host-parasite interactions.

**Chapter 4** In this chapter, I explore experimentally the ideas developed in Chapter 3, *i.e.*, that parasites might adapt to the most common host sex. I hypothesized that divergent selection on parasites, imposed by differences between male and female hosts, could result in parasite adaptations specific to the most common host sex, and possibly neutral or disadvantageous in the rare sex. I used a parasitic clone of *Pasteuria ramosa* isolated from a female host individual of a strongly female-biased population of *D. magna*, and tested whether it was better adapted to female than male hosts. My main results suggest that parasite-induced host castration leading to gigantism, which increases carrying capacity for parasite proliferation, is a parasitic trait that seems to have been selected for in the female host environment. My data shows that while parasite-induced castration, so far described only for female hosts, also occurs in males, it does not result in male gigantism, the described adaptive value of female host castration. Thus, it seems that the parasite's ability to induce castration is an adaptation in female hosts which does not have an adaptive value in male hosts. To my knowledge, this is the first report of specific adaptation to the most common host sex of a horizontally transmitted parasite. I predict that many more will be found as researchers start looking for them.

**Chapter 5** After the parasite penetrates into the host body cavity, the host's immune system is expected to reduce the chance of, or limit, parasite proliferation. The higher efficiency of the immune response upon a second exposure to a parasite is the principle of vaccination, and has been intensively studied in both vertebrate and invertebrate organisms. But while that type of memory property of the immune system is well established for vertebrates, controversy remains about its occurrence in invertebrates. In this chapter, I took into account common criticisms on previous studies investigating the presence/absence of specific memory in invertebrate immunity, and investigated the possibility of vaccination of the relatively short-lived *Daphnia magna* against its natural bacterial parasite *Pasteuria ramosa*. Using clones of the host and clones of the parasite, I tested whether a first exposure ("priming") of a host to a parasite, followed by clearing of the parasite with antibiotic, gives an advantage to the host upon a later challenge with the same parasite clone. My results showed that there is neither memory nor better protection following priming. I discuss the predictability of such results in relation to host lifespan, and natural parasites able to adapt to the host immune system.

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## CHAPTER 1

### RESOLVING THE INFECTION PROCESS REVEALS STRIKING DIFFERENCES IN THE CONTRIBUTION OF ENVIRONMENT, GENETICS AND PHYLOGENY TO HOST-PARASITE INTERACTIONS

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#### Abstract

##### *Background*

Infection processes consist of a sequence of steps, each critical for the interaction between host and parasite. Studies of host-parasite interactions rarely take into account that different steps might be influenced by different factors and might, therefore, make different contributions to shaping coevolution. We designed a new method using the *Daphnia magna* – *Pasteuria ramosa* system, one of the rare examples where coevolution has been documented, to resolve the steps of the infection and analyze the factors that influence each of them.

##### *Results*

Using the transparent *Daphnia* hosts and fluorescently-labeled spores of the bacterium *P. ramosa*, we identified a sequence of infection steps: encounter between parasite and host, activation of parasite dormant spores, attachment of spores to the host, and parasite proliferation inside the host. The chances of encounter had been shown to depend on host genotype and environment. We tested the role of genetic and environmental factors in the newly described activation and attachment steps. Hosts of different genotypes, gender, and species were all able to activate endospores of all parasite clones tested in different environments; suggesting that the activation cue is phylogenetically conserved. We next established that parasite attachment occurs onto the host esophagus independently of host species, gender and environmental conditions. In contrast to spore activation, attachment depended strongly on the combination of host and parasite genotypes.

##### *Conclusions*

Our results show that different steps are influenced by different factors. Host-type-independent spore activation suggests that this step can be ruled out as a major factor in *Daphnia-Pasteuria* coevolution. On the other hand, we show that the attachment step is crucial for the pronounced genetic specificities of this system. We suggest that this one step can explain host population structure and be a key force behind coevolutionary cycles. We discuss how different steps can explain different aspects of the coevolutionary dynamics of the system: the properties of the attachment step explaining the rapid evolution of infectivity, and the properties of later parasite proliferation explaining the evolution of virulence. Our study underscores the importance of resolving the infection process to better understand host-parasite interactions.

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## Background

Host-parasite coevolution is the result of multiple adaptations and counter-adaptations evolving in concert within the constraints of a particular system. Hosts use diverse defense mechanisms that coevolve with the offensive mechanisms of the parasite. From phages to ectoparasites, the success of infection depends on a series of steps and for each of them, the hosts may have specific defense mechanisms [1,2]. The following steps may be distinguished, with more or fewer steps potentially existing depending on the system, and the level of resolution: The host encounter with the parasite is the first step. During this step, the host may exhibit particular behaviors to avoid the parasite [3], and there may be polymorphism for such behaviors within species [4]. Once encounter has taken place, parasites with a dormant stage may need to be activated to terminate diapause and initiate the infection process, for example, by endospore germination [e.g. 5]. After the activation step, endoparasites need to enter the host tissues. For many parasites, including the one studied here, this occurs through the attachment of the parasites to the host tissues. Hosts may evolve to prevent this attachment. For example, plants often have very specific mechanisms to prevent fungal pathogens from entering leaf tissue [6], and some species produce layers upon their epithelium - the first barrier against infection - to obstruct parasite penetration [e.g. mucus in coral protection, 7, e.g. salivary mucins to preserve the oral cavity health, 8]. After attachment and entering its host, the next step of infection is proliferation. To counteract parasite growth, the host adapts physiologically [e.g. iron-withholding, 9] or actively defends itself with an immune response. In a final step of infection, the

parasite releases transmission stages, to infect other hosts.

It has been argued that the fact that infection trials often intermingle the effects of different infection steps strongly influences our interpretation of host-parasite interactions [1,10,11]. For example, if only one of the steps is specific, the entire infection process will be specific. The same is true for environmental effects and host genotype-parasite genotype interactions. Furthermore, even if each of the steps is under simple genetic control (*i.e.* one or few loci) the combination of all of them might behave as a quantitative genetic trait and become more difficult to investigate. Resolving the infection process into its component steps simplifies the complexity of the infection process and helps to better understand host-parasite interactions. Evolutionary models of host-parasite interactions are usually based on relatively simple assumptions about the underlying genetics and the impact of the environment. They commonly consider binary (Yes/No) infection outcomes (e.g. matching-allele matrix [12,13,14]), even though available experimental data suggests more quantitative outcomes when looking at host and parasite interactions [15,16,17]. Explicit analysis of individual steps of infection can help bring in line theoretical models and data concerning the entire infection.

Because little is known about the degree of specificity of individual steps, the specificity attributed to host-parasite interactions is usually the combined effect of all steps. Although it is reasonable to assume that different steps are under the control of different genes and are influenced by the environment to different degrees, it is possible that a single component of the infection pathway may explain most of the observed variation in host-parasite interactions. This is

particularly important because understanding variation in host susceptibility is central for controlling disease and understanding evolution. Here, we use the *Daphnia-Pasteuria* host-parasite system to investigate which step(s) best explains the high degree of host genotype by parasite genotype interactions reported for this system [18,19,20]. We analyze the contribution of host and parasite genetics, host gender, host phylogeny and of the environment for the dynamics of host-parasite co-evolution.

Reproduction in planctonic crustacean *Daphnia* is primarily clonal, which is very suitable for dissociations of genetic and environmental effects of its interactions with parasites. *Daphnia* are frequently found to suffer from bacterial, fungal and microsporidial infections [21,22], among them the Gram-positive bacterium *Pasteuria ramosa* [21,22,23]. *P. ramosa* produces endospores for transmission [Fig. 1A and B; 21] that can remain dormant for decades [24]. Transmission is waterborne and endospores do not have flagella. The infection process is unknown, but penetration of the host cuticula has been observed for the congeneric species *P. penetrans*, a parasite of root-knot nematodes [25]. Inside the host, *P. ramosa* proliferates in the hemocoel and musculature, castrates females and is transmitted horizontally after the release of endospores from the dead host [26,27]. The interaction of *D. magna* clones and *P. ramosa* clones has been shown to be specific [20]. *Pasteuria* was shown to impose strong selection on its host [28] and there is evidence for coevolution [29]. Furthermore, strong effects of the environment and genotype-environment interactions were reported for the overall infection process [30,31]. The goal of this study is to disentangle the different steps of the infection process and to analyze how they

are shaped by host and parasite genetics, and the environment. We aim at finding the step which explains the most variance for the strong host-parasite interactions reported for the overall infection process.

We consider the following steps of the infection process and will investigate in details the second and the third, previously undescribed: (i) Encounter. (ii) Activation (*i.e.* once in contact with *Daphnia*, parasite endospores need a signal to germinate). (iii) Attachment (*i.e.* the parasite must attach to the host and cross the host epithelium). (iv) Proliferation (*i.e.* Parasite proliferation and spore production). (v) Termination (*i.e.* killing the host to release spores). For the encounter and the proliferation steps environmental and host clone effects have been shown [4,30,32,33,34,35]. However, neither of them can explain the strong host genotype by parasite genotype interactions described for the overall infection process in this system. Here, we localize where the activation and attachment steps take place and test for genetic and environmental factors influencing those steps.

## Results

### *Spore activation*

We developed a new method that traces fluorescently-labeled spores of *Pasteuria ramosa* in the transparent *Daphnia magna* hosts to investigate the activation of parasite spores and the attachment of the parasite to the host. Within minutes of exposing *Daphnia* host to *P. ramosa* spores, we observed a characteristic change in spore morphology. Spores acquire a “sombbrero”-like structure (Figure 1C and D) which corresponds to the shedding of the exosporium and the extension of the peripheral fibers. This morphology was never observed in spores not exposed to hosts.

We call this morphological change in spore shape “activation.” Activation was found to happen regardless of the host clone or *Pasteuria* clone used and was observed in both resistant and susceptible *D. magna* clones (Table 1).

### Spore attachment

We used different combinations of hosts and parasite clones previously characterized to be resistant or susceptible to given *Pasteuria* clones [20]. We observed the fate of fluorescent spores of three parasite clones exposed to 14 *D. magna* host clones with the aim to identify differences which correlate with the compatibility of a given host-parasite combination (Table 1). The parasites attach to the host esophagus for all susceptible (compatible) host-parasite combinations,

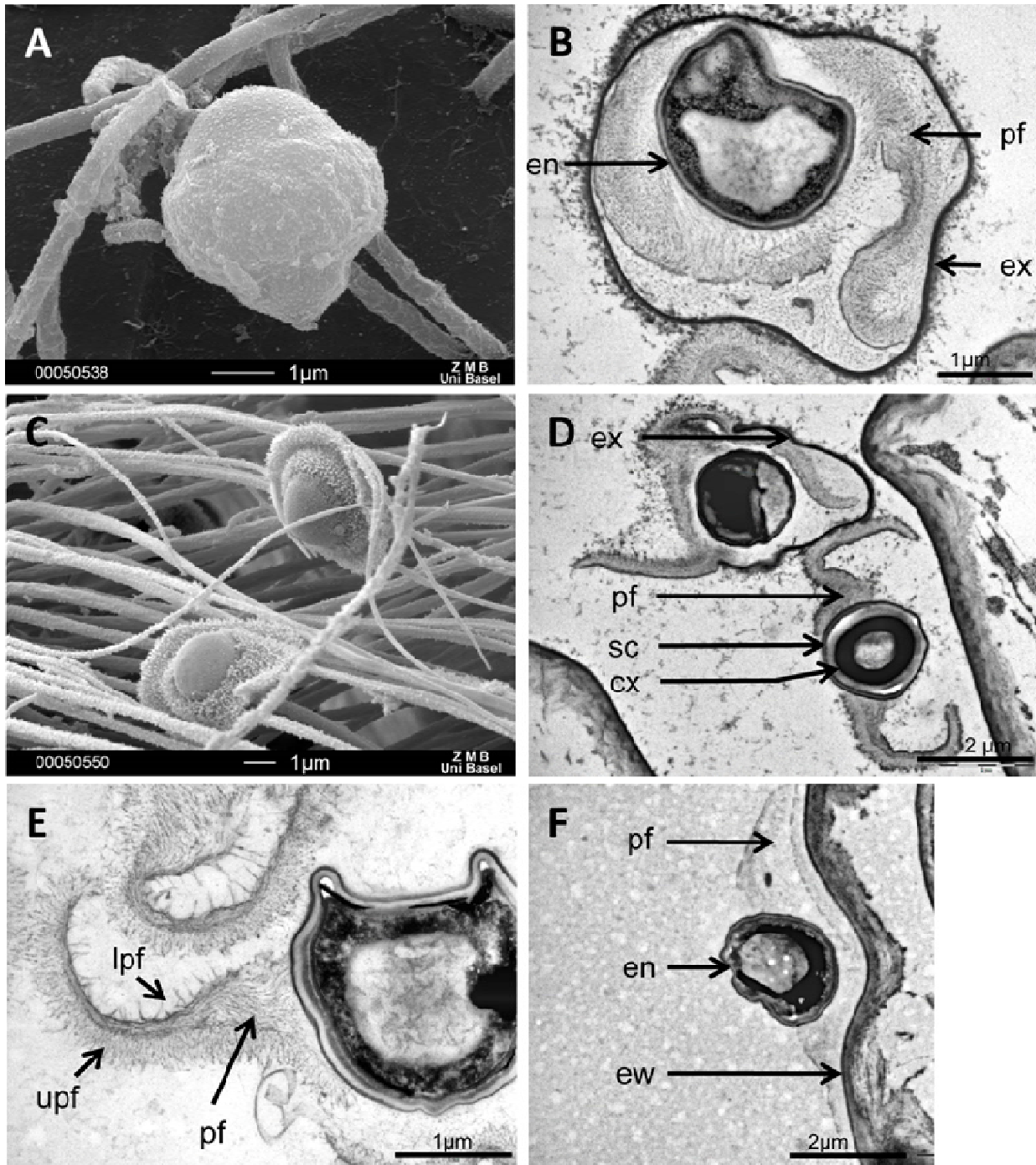
while they never do so for the resistant combinations (Table 1, Figure 1F, 2A). Thus, the result of this attachment-test was 100% consistent with the results of infection trials (Table 1). For susceptible combinations the host esophagus was densely covered with spores forming a dense layer in the esophagus, while there were no spores attached in resistant combinations. We never observed ambiguous cases, e.g. only few spores attached. While spores in the mid and end gut moved with the flow of the food, those attached to the esophagus were not to the esophagus and all spores passed with the flow of the food through the gut (Figure 2B).

Thus, spore attachment in the esophagus was very specific to the *D. magna* and *P. ramosa* genotype and consistent with resistant/susceptibility status for each combination.

**Table 1: Results of infection trials, spore activation tests, and attachment-tests.**

Clones of <i>D. magna</i>	Origin	<i>Pasteuria</i>	Infectivity trail			Spore activation			Attachment-test (attached out of five)		
			C19	C1	C14	C19	C1	C14	C19	C1	C14
HO1	Hungary	R	R	R	Yes	Yes	Yes	0	0	0	
HO2	Hungary	<b>S</b>	<b>S</b>	<b>S</b>	Yes	Yes	Yes	<b>5</b>	<b>5</b>	<b>5</b>	
HO3	Hungary	R	R	R	Yes	Yes	Yes	0	0	0	
M5	Belgium	R	R	R	Yes	Yes	Yes	0	0	0	
M10	Belgium	<b>S</b>	<b>S</b>	<b>S</b>	Yes	Yes	Yes	<b>5</b>	<b>5</b>	<b>5</b>	
linb1*	Germany*	R	R	R	Yes	Yes	Yes	0	0	0	
Mu12	Germany	R	R	R	Yes	Yes	Yes	0	0	0	
DG-1-106	Germany	<b>S</b>	R	R	Yes	Yes	Yes	<b>5</b>	0	0	
AL144	Finland	R	<b>S</b>	<b>S</b>	Yes	Yes	Yes	0	<b>5</b>	<b>5</b>	
Xinb3*	Finland*	<b>S</b>	R	R	Yes	Yes	Yes	<b>5</b>	0	0	
XI*	Finland*	R	R	R	Yes	Yes	Yes	0	0	0	
Xfa6*	Finland*	<b>S</b>	R	R	Yes	Yes	Yes	<b>5</b>	0	0	
Kela-39-09	Finland	R	<b>S</b>	<b>S</b>	Yes	Yes	Yes	0	<b>5</b>	<b>5</b>	
Kela-18-10	Finland	<b>S</b>	R	R	Yes	Yes	Yes	<b>5</b>	0	0	

We tested all combinations of three *P. ramosa* clones (C19, C14, C1) with 14 *D. magna* clones. Infectivity trials results are defined by exposing *Daphnia* to the parasites and determining the infection status after 20 days. Resistant means that none of the replicates were infected. Activation was determined by observing spores in the gut of the host with a sombrero-like shape. R means that the host clone is totally resistant to the concerned parasite clone. S means that the host clone is susceptible to the concerned parasite clone. Yes means that the spores were activated. \* *Labcross*: “linb1” is “Mu11” (Belgium) selfed once; “Xinb3” is “X” (Finland) selfed 3 times; “Xfa6” is “AL144” selfed 3 times and crossed with “Xinb3”; “XI” is a cross between “linb1” and “Xinb3”.



**Figure 1: Scanning (SM) and transmission (TM) electron microscopic images of the activation and the attachment step of the infection process of *Pasteuria ramosa* in *Daphnia magna*.** A.) SM image of a resting stage of *Pasteuria ramosa*. B.) TM image of resting stage before activation. The exosporium (ex) encloses the two peripheral fibers (pf) and the endospore (en). C.) SM image of activated spores trapped by *Daphnia* phyllopods. D.) TM image of activated spores in *Daphnia* esophagus. Top left, spore is in the process of activating and shedding the exosporium. Bottom right, activated spore with its sombrero-like structure in cross-section. Spore coat (sc) surrounding the cortex (cx). E.) TM image of peripheral fibers (pf) and its microfibers on the upper side (upf) and on the lower side (lpf). The upper side is more furnished in microfibers and is likely to play a role in the attachment. F.) TM image of *Pasteuria* attached to the *Daphnia* esophagus wall (ew). The nomenclature were defined according to the nomenclature of *Pasteuria penetrans* in [36].

**Table 2: Influence of the environment and host gender on spore attachment, as determined by the attachment-test.**

		Treatments											
		LF, 20°C, single		HF, 20°C, single		HF, 10°C, single		HF, 15°C, single		HF, 25°C, single		HF, 20°C, crowded	
Pasteuria clone	Daphnia clone	Kela 39-09	Kela 18-10	Kela 39-09	Kela 18-10	Kela 39-09	Kela 18-10	Kela 39-09	Kela 18-10	Kela 39-09	Kela 18-10	Kela 39-09	Kela 18-10
C1	Female	<b>6/6</b>	0/5	<b>9/9</b>	0/9	<b>10/10</b>	0/10	<b>10/10</b>	0/10	<b>10/10</b>	0/10	<b>10/10</b>	0/10
	Male	<b>10/10</b>	0/10	<b>9/9</b>	0/9	<b>9/9</b>	0/10	<b>10/10</b>	0/10	<b>9/9</b>	0/10	<b>10/10</b>	0/10
C19	Female	0/10	<b>7/7</b>	0/8	<b>8/8</b>	0/9	<b>10/10</b>	0/10	<b>10/10</b>	0/9	<b>9/9</b>	0/10	<b>10/10</b>
	Male	0/5	<b>10/10</b>	0/10	<b>9/9</b>	0/10	<b>10/10</b>	0/10	<b>9/9</b>	0/9	<b>9/9</b>	0/10	<b>10/10</b>

Infection trials (see Table 1) showed that *D. magna* clone Kela-39-09 is susceptible to *P. ramosa* clone C1, but resistant to C19. Kela-18-10 is resistant to C1, but susceptible to C19. LF= low food condition, HF = high food condition, single = *Daphnia* raised single in a 100 ml jar, crowded = *Daphnia* randomly picked from crowded cultures (high density). The bold characters highlights results where *P. ramosa* were attached to the *D. magna* esophagus.

### *Influence of gender and culture conditions*

Activation of spores was observed in all treatments and in all host clone-*Pasteuria* clone combinations (Table 2). In contrast, the specificity revealed by the attachment-test was found to be independent of host gender, temperature and culture conditions (*i.e.*, single vs. crowded; high vs. low food, Table 2).

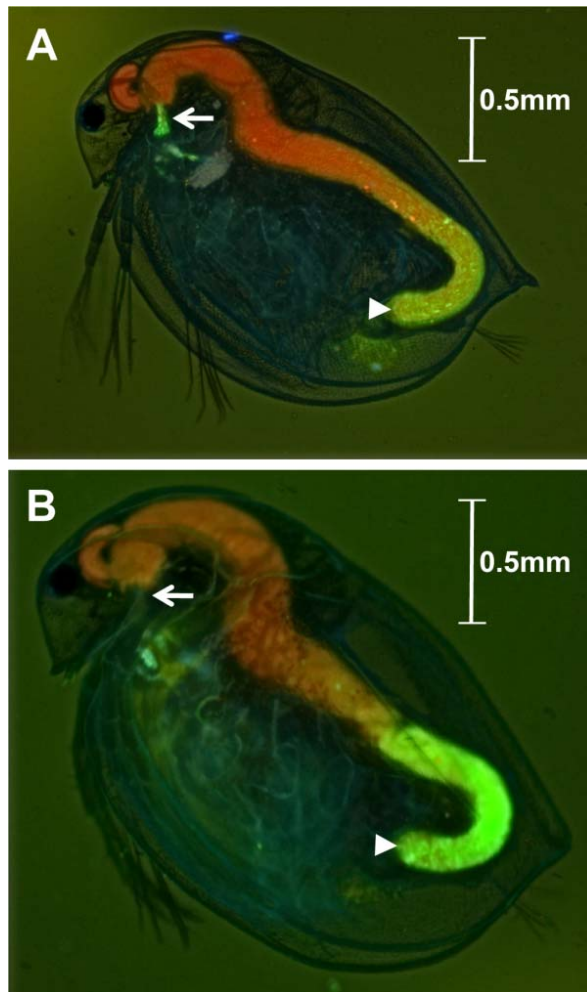
### *Spore activation and resistance of other Daphnia species*

Spores were found to be activated after exposure to all *Daphnia* species tested (Table 3). We found that spores of the *P. ramosa* clone C19 were able to attach to the esophagus and infect *D. dolichocephala* (Table 3) but did not stick to the esophagus or infect *D. arenata*, *D. galeata*, *D. barbata*, *D. similis* or *D. lumholtzi*. We also tested other species for spore activation of *P. ramosa*. Upon exposure to *Simocephalus vetulus* (Daphniidae) spores were readily activated, but did not attach to the esophagus nor did they infect any of the individuals tested. Upon exposure to mosquito larvae (*Culex* spp.), which are also filter-feeding but are not

crustaceans, *P. ramosa* spores were neither activated nor attached to the host.

### Discussion

The aim of the current study was to analyze two steps in the life cycle of a bacterial parasite, characterize the specificity of the interaction with regard to genetic and environmental factors, and relate these findings to what is known about host-parasite coevolution in this system. We focused on the activation of the parasite's resting stages, and on the attachment of the activated spores to the host tissue where it enters the host. Our study revealed that *P. ramosa* spores captured by the filter feeding *Daphnia* are indiscriminately activated by every *Daphnia* clone and *Daphnia* species tested (Table 1 and 3). Furthermore, activation was not only found to be independent of the host genotype or species and host gender, but also of the environmental conditions (namely, density, temperature and food conditions). The following step of the infection process, however, the attachment of the activated spore to the esophagus wall of the host, depended strongly on the combination of the *D. magna* and parasite genotype, but not on



**Figure 2: Fluorescently labeled parasite spores attach to the oesophagus of susceptible, but not resistant, *Daphnia* clones.** A.) Picture of a susceptible *Daphnia magna* exposed to fluorescently labeled spores. The entire animal is shown. Parasites are attached on the epithelium of the esophagus (arrow). Other labeled spores can be seen with the rest of the food in the end gut (arrowhead). B.) Picture of a resistant *Daphnia magna* exposed to fluorescently labeled spores. The entire animal is shown. The esophagus is free of parasite (arrow). Labeled spores can be seen with the rest of the food in the end gut (arrowhead). Note the autofluorescence of the mandibule. Extended focus images obtained by the camera Leica DFC 300FX and the program Leica Application Suite (Version 3.4.0, package “Montage”). Intensity, contrast and sharpness were increased with the same strength.

**Table 3: Relationship between one *D. magna*-derived clone of *Pasteuria ramosa* (clone C19) and several *Daphnia* species belonging to three different subgenera (*Daphnia magna* belongs to the subgenus *Ctenodaphnia*).**

Clones of <i>Daphnia</i> species	Sub-genus	Origin	Infectivity trail	Spore activation	Attachment-test (attached out of five)
<i>D. arenata</i>	Daphnia	USA	R	Yes	0
<i>D. galeata</i>	Hyalodaphnia	Germany	R	Yes	0
<i>D. barbata</i>	Ctenodaphnia	Zimbabwe	R	Yes	0
<i>D. similis</i>	Ctenodaphnia	Israel	R	Yes	0
<i>D. lumholtzi</i>	Ctenodaphnia	Zimbabwe	R	Yes	0
<i>D. dolichocephala</i>	Ctenodaphnia	South Africa	S	Yes	4

Legend as in Table 1.

the host's gender, nor the environmental conditions which they were kept (Table 1, 2 and 3).

Previous studies with the *Daphnia-Pasteuria* system were not able to disentangle activation, attachment and proliferation step. Thus, variation in infection success as reported in earlier studies [19,32,35,37,38,39,40,41] may be explained by the combined effects of these steps. However, the binary polymorphism found in infection trials with high doses of single parasite clone [20] correlates perfectly with the results of our attachment-test (Table 1). This suggests that only *Pasteuria* clones able to attach to the esophagus are able to infect the host. Ben-Ami et al. [39] proposed that *D. magna* might be either completely resistant or susceptible to *P. ramosa* depending on the genotype-genotype interaction. They called this the “binary infection hypothesis.” Our data are consistent with this hypothesis and further pinpoint which specific step of the infection process is responsible for the high degree of specificity. For a given combination of host and parasite genotypes, the activated spores are either able to attach and then infect, or they do not attach and do not infect. We did not see any evidence for a graded (quantitative) form of interaction.

#### *Spore attachment is a key step in Daphnia-Pasteuria coevolution*

The *Daphnia-Pasteuria* system has become one of the prime examples of antagonistic coevolution. Host and parasites show strong genetic effects for resistance, virulence and infectivity; genotype x genotype interactions have been reported within and across populations, and selection acts rapidly in natural populations [18,19,41,42]. Our study suggests that the parasite-dependent [28] host population structure and the coevolution [29]

described for this system are mainly driven by the properties of a unique step, the attachment step. First, this step revealed very strong host genotype by parasite genotype interactions (Table 1). Second, the attachment step is independent of the environmental conditions. Third, a recent study of *D. magna - P. ramosa* coevolution using resurrected host and parasite isolates from lake sediments showed a signal of fluctuating selection only for infectivity, but not for parasite virulence [29]. Virulence (the parasite's effect on infected hosts) was observed to evolve as well, but at a slower rate [29]. The authors proposed that the difference between the evolution of virulence and infectivity resulted from different genes contributing to these traits. Here we give a mechanistic explanation for this finding. Infectivity depends on the attachment and most likely on the ligands present on the host and on the parasite. On the other hand, expression of virulence may depend on the host's immune response during the within-host proliferation step. It is likely that these processes are determined by different sets of genes.

The identification of the attachment step as the key step in the coevolutionary dynamics in this system will allow us to improve our understanding of the patterns of antagonistic coevolution. For example, evolutionary models studying the coevolution of the infectivity and the virulence steps [43] can fit our system in relation to the coevolution of the attachment and the proliferation steps. Those models typically characterize infection outcomes as binary (Yes/No), while empirical data suggest they are more quantitative [15,16,17]. Here we show that we can observe a binary outcome when individual steps of the infection process are considered. Furthermore, our method provides a fast and reliable way to test



individuals and populations for their susceptibility to *Pasteuria*. Ongoing research in our group showed that up to 400 *Daphnia* individuals can be tested in a day (P. Luijckx in preparation). The assay we developed makes it possible to test for susceptibility without the potentially confounding effect of the within-host proliferation step in the infection trials.

#### *From the environment to the host body cavity*

The resting endospores of *P. ramosa* can remain dormant for decades under harsh environmental conditions [24,29]. Before attachment to the host, the spores need to be activated (Fig. 1D). The filter-feeding *Daphnia* capture particles, including parasites, from the water and transport them on a mucus-layered pathway from the phyllopod to the mouth. During this process, the parasite's exosporium opens by an unknown trigger, releasing the activated spore form within less than 10 minutes (Fig. 1). Despite spore activation is a necessary step for the infection; this step is entirely unspecific with regard to *Daphnia* species and clone, host gender and the environmental conditions (Tables 1, 2 and 3). The signal that triggers spore activation may be related to chemical substances in the mucus of the filtering apparatus, but other factors (*e.g.* mechanical) cannot be excluded.

Once the activated spore enters the esophagus, it will attach to the esophagus wall, if host and parasite genotype are compatible. There it presumably penetrates the gut wall and enter the host's body cavity. A similar attachment process on the cuticula is also known from *P. penetrans*, but in this case the parasite seems to be able to attach to any area of the nematode's body surface [25]. It has been proposed that the lower part of *P.*

*nishizawae* attaches to the host, because this part is densely covered by microfibrils [44]. In contrast, *P. ramosa*, it is the upper part of the peripheral fibers (Fig. 1E) that are most densely covered with a layer of microfibrils. These fibers may be involved in the attachment (Fig. 1F).

An endospore adhesin epitope, situated on the exosporium of *P. ramosa*, has been identified and suggested as a ligand that might be responsible for the recognition and the binding onto the host [45]. However, according to our results, it is unlikely that this epitope is involved in the attachment because the exosporium of *P. ramosa* is removed during the activation step. A later study, analyzing surface proteins of *P. ramosa* spores by two-dimensional gel electrophoresis, proposed collagen-like protein as responsible for the binding onto the host but might suffer the same problem of the previous study [46]. We propose that latter studies on candidate proteins responsible for the specific attachment to the host in this system investigate the spores once activated.

The development of *Pasteuria*, from the moment they attach to the esophagus until the vegetative stage can be detected in the hemolymph (about 8 days at 20°C [47]), is unknown. Also, the penetration mechanism is poorly described. Sayre and Wergin [25] show a transmission electron micrograph of *P. penetrans* with a structure they call a germ tube crossing the host epithelium. Our hypothesis is that the endospore makes a hole across the host epithelium and injects its cortex into the host. As one response of *Daphnia* to wounding is an increase of Phenoloxidase (PO) activity [48], one might expect the penetration process to trigger an immune response, but this remains an open question. However, resolving the infection process will allow studying the immune

response during the proliferation step without the confounding effect of genetic variation in the attachment step.

#### *Environment effects and the proliferation step*

We found that environmental effects do not influence the activation and attachment step (Table 2). Excluding these steps, we suggest that the proliferation step is the one responsible for the reported sensitivity of the overall infection process for environment effects [32,34]. The activation and the attachment step seem independent of the host's immune system (defined as a system that is potentially able to kill parasites), while the proliferation step is likely to be governed by the host's immune system. The immune system may lead to variation between and within those *Daphnia* clones that allow *Pasteuria* attachment (and thus the parasite to enter the host), thereby contributing to local and temporal adaptation, maternal effects and induced resistance [29,34,49]. We suggest that future studies on host immunity should use only *Pasteuria* clones that can attach to a given clone of *Daphnia* so that all variation observed is likely to originate from variation during the proliferation step. These factors highlight the importance of controlling the host and parasite genotypes and breaking down the infection process to understand the respective role of each step in host-parasite interactions.

#### *Resolving the infection process leads to better understand host-parasite interactions*

Resolving the infection process in its sequential steps has been proposed in a number of theoretical models [10,11] but experimental data are scarce. Our approach is transferable to other host-parasite systems and our results suggest that this can provide

important new insights about host-parasite interactions and their evolution. Increasing the degree of resolution of the infection processes highlights the universe of possibilities of the different levels at which host and parasites interact. The different steps might differ in how they are influenced by the environment. They might also differ in which sets of genes regulate them. As is probably the case for our study system, different steps of the infection process might follow distinct evolutionary dynamics and be explained by different model (*e.g.* balancing selection, directional selection) [10,11]. However, because of the sequentiality of the steps, it is possible that the selection on one might depend on the selection on other steps. We propose that analyzing infection as a succession of well characterized steps will help to reconcile empirical data with predictions based on alternative coevolutionary models (*e.g.* Red Queen and Selective Sweep models).

Spores of all *P. ramosa* clones tested, and which were isolated from natural *D. magna* populations, were activated by all *D. magna* clones as well as by six other *Daphnia* species (Table 3) and even a more distantly related Cladoceran, *Simocephalus vetulus*. Also, aside from the natural host *D. magna*, *D. dolichocephala*, too, became infected following attachment of the activated spores to the host esophagus. This suggests that the triggers for spore activation and, to a lesser extent, for attachment are phylogenetically conserved. This may facilitate host range evolution of the parasite. Indeed, despite its high specificity on the level of the host clone, *P. ramosa* infections have been reported in several species within the family Daphniidae [50]. It will be necessary to test more clones of different *Daphnia* species to determine their pattern of susceptibility and resistance to

the parasite. Importantly, phylogenetically conserved steps of the infection process can be ruled out as major factors in coevolution, but are perhaps the most appropriate targets for vaccine and drug development. In fact, the genes involved in some infections steps have been worked out for some systems [51,52] and can be of use in biomedicine for diseases control [53,54].

## Conclusion

Our study highlights the explanatory power of resolving the steps of the infection process to better understand host-parasite interactions and coevolution. Attachment appears to be the crucial step for the previously observed high specificity in the *Daphnia-Pasteuria* system and we speculate that it is the crucial step for coevolution as observed in this system [29]. Our results reveal that each step can involve different interactions between host, parasite and environment and that certain steps can be phylogenetically conserved. With this knowledge at hand, it will be easier to apply simple models of host-parasite interactions to this system and identify the mechanistic basis of trade-offs, maternal effects, genotype x environment interactions and coevolution. The logic of this procedure can equally be applied to other host-parasite systems but also to study other types of biotic interactions.

## Methods

### *Host and parasite*

We used 14 isofemale lines (here after referred as clones) of *Daphnia magna* and one clone each of six other *Daphnia* species (Tables 1, 3). Unless otherwise stated, *Daphnia* clones were kept in standard medium (ADaM) [55, modified by using only 5% of the recommended Selenium dioxide

concentration] at 20°C and fed with the chemostat cultured unicellular algae, *Scenedesmus obliquus*.

The parasites used were single genotypes of *Pasteuria ramosa*, C1, C14 and C19, characterised as clones in Luijckx et al. [20] and originated from *D. magna* populations in Moscow (Russia), Tvärminne (Finland), and Gaarzerfeld (Germany), respectively. Spore suspensions of *Pasteuria* were obtained by homogenizing infected *D. magna* in ADaM and quantifying spore density. The status of resistant or susceptible *D. magna* were defined previously [20]. The infection status of two further Finnish *D. magna* clones (“Kela-39-09” and “Kela-18-10”) exposed to *Pasteuria* clones were tested with the same protocol. All infections in these experiments were done with naïve individuals born to naïve mothers, kept under high food conditions. These conditions were applied because they are known to minimize triggering of immune effect [34,35,56].

### *Fluorescence labeling of spores*

Fluorescently labeled spores of *P. ramosa* were produced by homogenizing infected *Daphnia* in ADaM, followed by centrifugation at 10 000 g for 5 min at room temperature. The spore pellet was suspended in 0.5 ml of 0.1 M sodium bicarbonate (pH 9.1) containing 2.0 mg/ml of fluorescein-5(6)-isothiocyanate (F3651-100MG, Sigma-Aldrich), a green fluorescent dye that stains proteins unspecifically [57]. Spores were incubated in the dark for 2 hours at room temperature with occasional vortexing. The suspension was centrifuged at 10 000 g for 5 min, and the supernatant removed. The spore pellet was suspended in distilled water and again subjected to centrifugation. This process was repeated until the supernatant

was clear. Labeled spore suspensions can be stored at 4°C in the dark for several months.

The shape and location of the green labeled spores were examined in the transparent *Daphnia* using a microscope with fluorescent light (Leica DM 2500, at magnification 200 x and 400 x) and filter cubes Leica B/G/R (bandpass filter excitation 420/30nm; 495/15nm; 570/20nm – band pass filter suppression 465/20nm; 530/30nm; 640/40nm). We increased the color contrast by adding a red fluorescent dye to the medium in which the *Daphnia* were observed. This was done by preparing a solution of concentrated red dye (0.05 ml of DMSO with 0.0015 g of Tetramethylrhodamin-5-isothiocyanate; T0820-5MG by Sigma-Aldrich), which was homogenized in PBS to make the diluted dye (1 µl of concentrated solution with 10 ml of PBS). We added 1 µl of this solution to the *Daphnia* medium 10 minutes before observing the *Daphnia*. We obtained extended focus images using a camera Leica DFC 300FX and the program Leica application Suite (Version 3.4.0, package “Montage”).

#### *The separation of the different steps and their specificity*

Adult *Daphnia* were put individually in 1 ml of medium in 24-well-plates and exposed for at least 1 hour to around 17,000 labeled *P. ramosa* spores. Susceptible hosts exposed to labeled spores become infected, suggesting that the dye does little or no harm to the spores (data not shown).

#### *Spore activation*

Pilot trials revealed that the labeled spores remain in their typical spherical shape as long as they are not in contact with a host. Upon

contact with the host phyllopod (swimming and respiratory appendages of branchiopod crustaceans), spores with a “sombbrero”-like shape are observed (Figure 1C and D). We called this process spore activation. We tested all combinations of 14 *D. magna* clones and three *P. ramosa* clones for spore activation (Table1). The same was done for one clone each of six further *Daphnia* species, but only in combination with one *P. ramosa* clone (Table 3). We used five replicates for each host-parasite combination (in total  $(14 \times 3 \times 5) + (6 \times 1 \times 5) = 190$ ; details in Table 1).

#### *Spore attachment*

After exposure, *Daphnia* were placed on a microscopic slide, and we examined the complete *Daphnia* body under a fluorescent microscope. The transparent body of *Daphnia* allowed us to determine in which body region activated fluorescent spores attach in the living animals. Once we determined the specific area, we tested resistant and susceptible *Daphnia magna* clones (five replicates of 14 clones, details in Table 1) for differences in attachment. The same was done with clones of other *Daphnia* species (five replicates of one clone per species; details in Table 3). To validate the assignment of individuals with apparently no spores attached to their esophagus, we viewed the esophagus of slightly squashed animals at 400 x magnification. For each experiment, the examiner was not informed whether the animals belonged to a susceptible or to a resistant clone. To confirm that the *Daphnia* ingested spores, the gut content was inspected for the presence of spores. All exposed animals had spores in the feces. We call this procedure to test for spore attachment the “attachment-test.”

### *Influence of gender and culture conditions*

To analyze if the specificity pattern observed in the attachment-test was dependent on host sex or culture conditions, ten host individuals of each sex were tested in each of six treatments. This was done with *D. magna* clones “Kela-39-09” and “Kela-18-10” because these two *Daphnia* clones have the reverse pattern of infectivity to the two *P. ramosa* clones used, and they are easily induced to produce male and female offspring in the laboratory. *Daphnia* were raised either at one of four temperatures (10°C, 15°C, 20°C, 25°C, with high food), two food levels (at 20°C, fed daily with 2.5 or 5 million algae) or two density levels (at 20°C, high food level, single *Daphnia* or *Daphnia* from crowded stock cultures) (see Table 2). These conditions were chosen to represent various environments that are common in natural *Daphnia* populations. We did not employ a full factorial design, as our interest was not in establishing reaction norms but in testing for the influence of non-genetic conditions in general. *Daphnia* of both clones raised under these conditions were exposed to fluorescently labeled spores of *P. ramosa* C1 and C19. Given the very clear effects observed with the 4 combinations of hosts and parasites used and the range of conditions tested, we do not believe that other combinations would change our results drastically. Still, we cannot exclude with certainty that some combinations might lead to a different result.

### *Resistance or susceptibility of other Daphnia species*

One clone of each of six other *Daphnia* species (*D. arenata*, *D. dolichocephala*, *D. galeata*, *D. barbata*, *D. similis* and *D. lumholtzi*) were assayed for their propensity

of esophageal spore attachment using *P. ramosa* clone C19. For this assay, groups of five conspecific individuals were exposed to 200,000 *P. ramosa* spores of clone C19 in 20 ml medium. Four replicates per species were used. After 5 days we filled the jars to 100 ml, and changed medium on a weekly basis. Animals were fed daily with 5 to 10 million algal cells per jar depending on the size of the *Daphnia* species. The infection status was investigated under a microscope with phase contrast (magnification 400 x), at host death or 29 days after exposure.

### *Electron microscopy*

To prepare *Daphnia* for transmission electron microscopy (TEM), infected individuals were fixed on ice in 4% glutaraldehyde buffer in Sorensen's phosphate buffer (0.1 M KH<sub>2</sub>PO<sub>4</sub> and 0.1 M Na<sub>2</sub>HPO<sub>4</sub>) and kept in the dark for several hours. The animals were then rinsed five times on ice using the same buffer for a total of 5 min. Post-fixation was carried out with 1% OsO<sub>4</sub> in Sorensen's phosphate buffer on ice. After post-fixation, the *Daphnia* were again washed in Sorensen's phosphate buffer on ice, dehydrated in a graded acetone series, and finally embedded in the epoxydic resin EPON.

Transversal and sagittal sections were made through the esophagus. Semi-thin sections (diamond knife, 0.7-1 µm) were cut to approach the right spot on the resin block using a RMC MT 6000-XL (RMC Inc.) ultramicrotome. To identify regions of interest for transmission electron microscopy, the tissue was stained using Richardson's dye [58] and examined under a light microscope. To see parasite structures using transmission electron microscopy, 5-8 ultrathin sections (diamond knife, 60 nm) were cut after every 10 semithin sections. The ultrathin sections

were mounted on Formvar-coated copper grids and stained with uranyl acetate and lead citrate to enhance the contrast. Ultrathin sections were analyzed using a FEI Morgagni<sup>TM</sup> transmission electron microscope at 80 kV equipped with a digital camera.

For scanning electron microscopy (SEM), *D. magna* were fixed in 3% glutaraldehyde buffer in 0.1 M phosphate buffer for 2 hours at 20°C. Samples were washed two times in distilled water for 5 to 10 seconds, dehydrated in graded ethanol series, and critical point dried overnight (16 hours). The specimens were coated with gold (20 nm) and viewed using a Philips XL 30 ESEM under high voltage conditions from 5 to 15 kv.

#### Author's contributions

DD conceived and designed the study, performed the experiment, performed data analysis, and drafted the manuscript. PL participated in the design of the study. FB performed the scanning electron microscopy. CL performed the transmission electron microscopy. DE conceived of the study, participated in its design and participated in drafting the manuscript. All authors read and approved the final manuscript.

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## CHAPTER 2

### THE ROLE OF MOLTING IN THE DEFENSE AGAINST AN ENDOPARASITE

David Duneau, Dieter Ebert

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#### Abstract

Parasitic infections consist of a succession of steps during which hosts and parasites interact in specific manners. At each step, hosts can use diverse defense mechanisms to counteract the parasite attempts to invade and exploit them. The penetration of parasites into the host body cavity is a key step for a successful infection, which leads to the proposition that the epithelium is a line of defense against parasites. The shedding of this protective layer that surrounds the body cavity (molting), a crucial feature in the life-cycle of several invertebrates and vertebrates, is generally considered as a cost for hosts exposed to parasites. Here, we used the crustacean *Daphnia magna* to test whether molting can be beneficial for the host by decreasing the likelihood of infection by the bacterial pathogen *Pasteuria ramosa*. This parasite is known to attach to the host cuticula before penetrating into its body. We found that the likelihood of successful parasite infection is strongly lowered if the host molts within 12 hours after parasite attachment. We further show that exposure to the parasite does not induce hosts to molt earlier. We discuss that such a passive mechanism of resistance may have implications for host and parasite evolution and epidemiology.

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## Introduction

All multicellular organisms have an external layer, called cuticula or skin. This layer serves a protective role by forming a physical barrier against external biotic and abiotic attacks, as well as an immune shield [e.g. mammals, 1]. This barrier has been shown effective against parasites, and mechanical damage to it correlates with an increase in probability of infection [2]. Parasites evolve elaborate adaptations to cross this barrier, such as the specialized ovipositors of parasitoids that lay eggs inside insect hosts, the unique adaptations of fungal pathogens to cross the cell wall of their plant hosts [3], and the modified rostrum of blood sucking arthropods that exploit vertebrate hosts. Parasites also have diverse strategies to penetrate into the host body quickly [e.g. invasion apparatus of microsporidia allowing the penetration into the host cell without ever attaching to the host integument, 4] and minimizing notice by host defense mechanisms [e.g. the saliva of the bloodsucking arthropods disrupts the recognition by the dermal immune system, 5]. Thus, parasites commonly evolve adaptations to efficiently cross the host skin/cuticula on one side, and host evolves ways of reducing the likelihood of parasite invasion through the barrier on the other side.

The ecdysozoans (e.g. arthropods, nematodes) and the squamata (*i.e.* lizards and snakes) need to shed their cuticula/skin for growing, a process called molting or ecdysis. There are costs and benefits to this process. One of its consequences is that shortly after having shed the barrier, the new barrier is temporarily soft and thin with individuals sometimes unable to walk or fly. The individuals are, therefore, vulnerable to predators, competitors, and parasite penetrations until the barrier is fully re-established [2,6]. On the other hand, molting at regular intervals benefits the host by regularly removing the accumulation of epibionts [7,8] and wounds [9]. Given the costs and benefits, the timing of molting is crucial. The crustacean *Gammarus pulex* adjusts the time of its molt cycle in response to parasitic infection risk, elongating it by several days when the individuals are exposed to “micro-organism-enriched” water [10]. This

result raised the exciting possibility that, despite likely developmental constraints on the molting process, hosts might be able to alter the moment of molting as an adaptation to avoid infection. Such mechanism might highlight the role of parasites on ecdysozoan development.

Many ecto- and endoparasites need to attach to their hosts before penetrating the cuticula. If the molting occurs when the parasite is already attached to the host epithelium but before it penetrates the barrier, it could interfere with the penetration and prevent infection. Here we test this hypothesis. Using the Gram positive bacterium *Pasteuria ramosa* and its host *Daphnia magna*, we investigate the possibility that molting interferes with the success of infection. Parasitic bacterium of the genus *Pasteuria* attach and penetrate the host cuticula before proliferating within the body in nematodes and crustaceans [11,12]. The host susceptibility is explained by the specific attachment of the parasite to the host esophagus which precedes it entering the host body cavity [12]. In arthropods, the esophagus is part of the ectoderm and is, therefore, shed during molting [13]. Thus, we predicted that if molting occurred shortly after the attachment of the parasite to the host, the parasite might not have enough time to penetrate into the host’s body. If this is the case, molting could be an effective mechanism for freeing hosts of attached parasites and might be an important selective pressure on endoparasite penetration speed. Moreover, if molting interferes with parasite penetration, it is conceivable that hosts might respond to parasite attachment by shortening the time to the molting. We test these hypotheses in this study.

## Material and methods

### *Biological material*

We used different genotypes (clones) of the transparent crustacean *Daphnia magna* (Kela 39-09, Kela 18-10 and Xinb3 from Finland, HO2 from Hungary and M10 from Belgium). Host clones were kept in standardized medium [ADaM, 14, modified by using only 5% of the recommended Selenium] at 20°C, and fed daily with chemostat cultured unicellular algae,

*Scenedesmus obliquus*. The parasites used were *Pasteuria ramosa* clones C1 and C19, originally sampled from infected *D. magna* in natural populations in Moscow (Russia) and Gaarzerfeld (Germany), respectively [15]. Parasite suspensions for experimental exposure were produced from homogenized infected *Daphnia*.

#### *Parasite removal with host molting*

To test whether parasite spores attached to the host cuticula can be found in the esophagus of the shedded carapace after molting (=exuviae), we exposed 23 *Daphnia magna* females from the laboratory stock of clone Kela 39-09 and Kela 18-10 to 20000 fluorescently labeled spores (cf. Duneau et al. 2011) of each of the *Pasteuria ramosa* clones C1 and C19. The two *Daphnia* clones were chosen because they have an opposite infection pattern for the two parasite clones. The clone Kela 39-09 is susceptible to *P. ramosa* C1 but not to C19, and the clone Kela 18-10 is susceptible to C19 but not to C1 [12]. *Daphnia* were raised in mass culture and then placed individually in 24-well plates, where exposure to the parasite took place. Thirty six hours after exposure to parasites, we checked all host individuals for molting by visual inspection. For the 30% of individuals that had molted within the 36 hours (susceptible combinations: Kela 39-09 / C1 n=5, Kela 18-10 / C19 n=13; resistant combinations: Kela 39-09 / C19 n=6, Kela 18-10 / C1 n=6), we checked for presence or absence of parasite spores attached on the esophagus of the exuviae under a fluorescence microscope (Leica DM 2500) with RGB filter cubes (Leica, bandpass filter excitation 420/30 nm; 495/15 nm; 570/20 nm - band pass filter suppression 465/20 nm; 530/30 nm; 640/40 nm).

#### *Effect of molting on parasite infection*

To test whether molting interferes with the process of infection, we conducted two independent experiments in which we exposed 196 (experiment 1) and 160 (experiment 2) *D. magna* individuals from clone HO2 to *P. ramosa* clone C19. HO2 is known to be susceptible to C19 [12]. We used 28 additional *Daphnia* as control (non exposed). Individual *D. magna* juveniles, not older than 3 days, were placed individually in 20

mL ADaM with 20000 spores (juveniles molt approximately every 36 hours at 20°C). Because the experiment 1 revealed a short time window for results to be observed, we conducted the experiment 2 with a reduced duration for parasite exposure from 12 (exp. 1) to 4 hours (exp. 2). After the exposure, host individuals were transferred to 80 mL of parasite-free medium. In both experiments, each individual was checked for molting every 4 hours, between 0 and 36 hours after exposure to *P. ramosa* spores. After 36 hours, all individuals that had molted were kept individually in 80 mL for 25 days during which the medium was renewed weekly. After this period the individuals were checked for infection status. The design of experiment 2 was modified based on the experience with of experiment 1. First, in experiment 1, juveniles originated from a mass culture, thus, their mothers were unknown. In experiment 2, we took four juveniles per individually-kept mother. Second, individuals which molted during the exposure phase were excluded. And finally, to reduce the possibility that spores passing the gut are present in the medium, we transferred all host animals a second time, after one hour, into parasite-free medium. We ended up with total sample sizes of 157 (exp. 1) and 98 (exp. 2) individuals. The number of molting *Daphnia* for each 4 hour interval varies between intervals, but was generally larger than ten (see Figure 2).

To study the influence of the time between exposure and molting on the probability that the host became infected, we used a generalized linear model [GLM; 16] with a binomial error distribution, and logit link constructed as: *Infection status* ~ *Experiment* \* *Time between exposure and molting*. “Infection status” is either 0 (uninfected) or 1 (infected), and “\*” indicates that the effects were tested of both main factors as well as their interaction. The assumption on the error distribution was checked by estimating dispersion parameters in GLM. No significant overdispersion was detected. For the experiment 2, we tested separately the effect of the mother by taking “mother” as a random factor in a general mixed model. As the factor “mother” did not affect the outcome concerning the time period between exposure and molting and its relationship

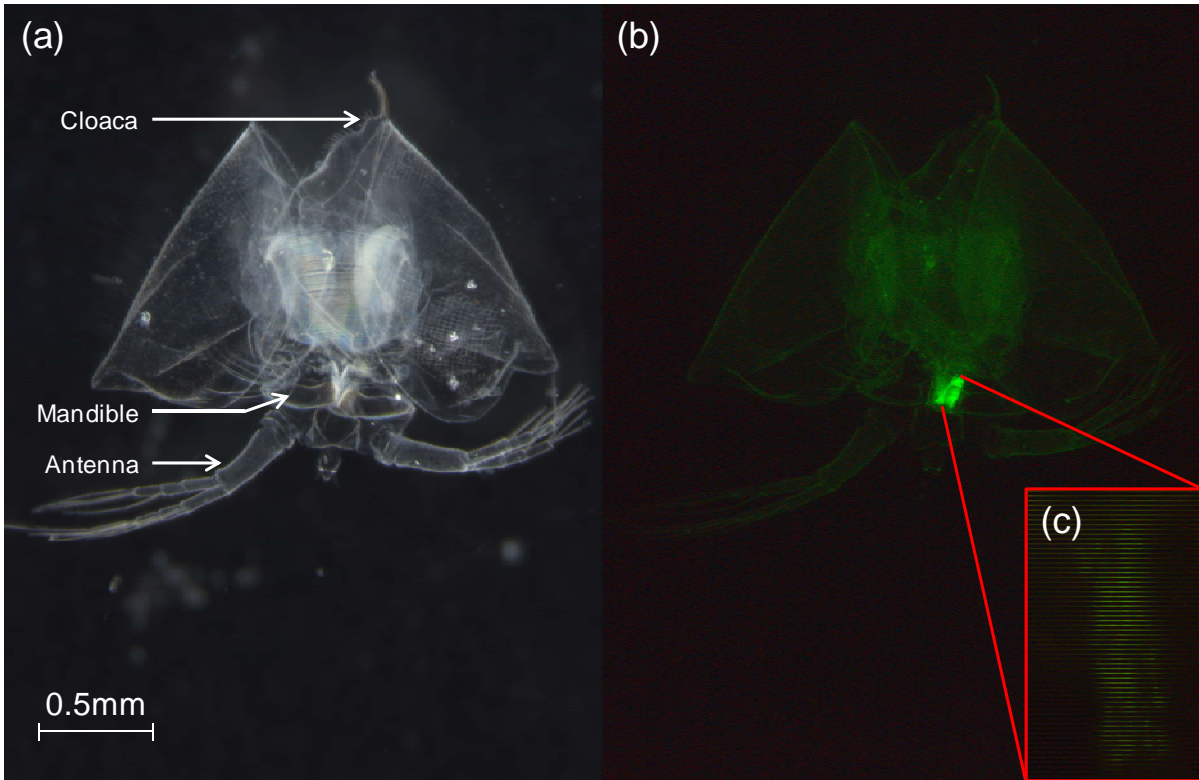


Figure 1: Exuviae (= shed cuticula) of *D. magna* exposed to *P. ramosa*. Pictures represent the same exuviae under light (a) and fluorescent (b) stereomicroscopy. Picture (c) represents the magnification (x 200) of the esophageal region. Spores were found attached to the esophagus part of the exuviae in 100% for susceptible hosts, but not in resistant hosts.

to the probability of infection, we present here the simplest generalized linear model combining the two experiments and excluding “mother” as factor.

#### *Molting as a passive defense against parasites*

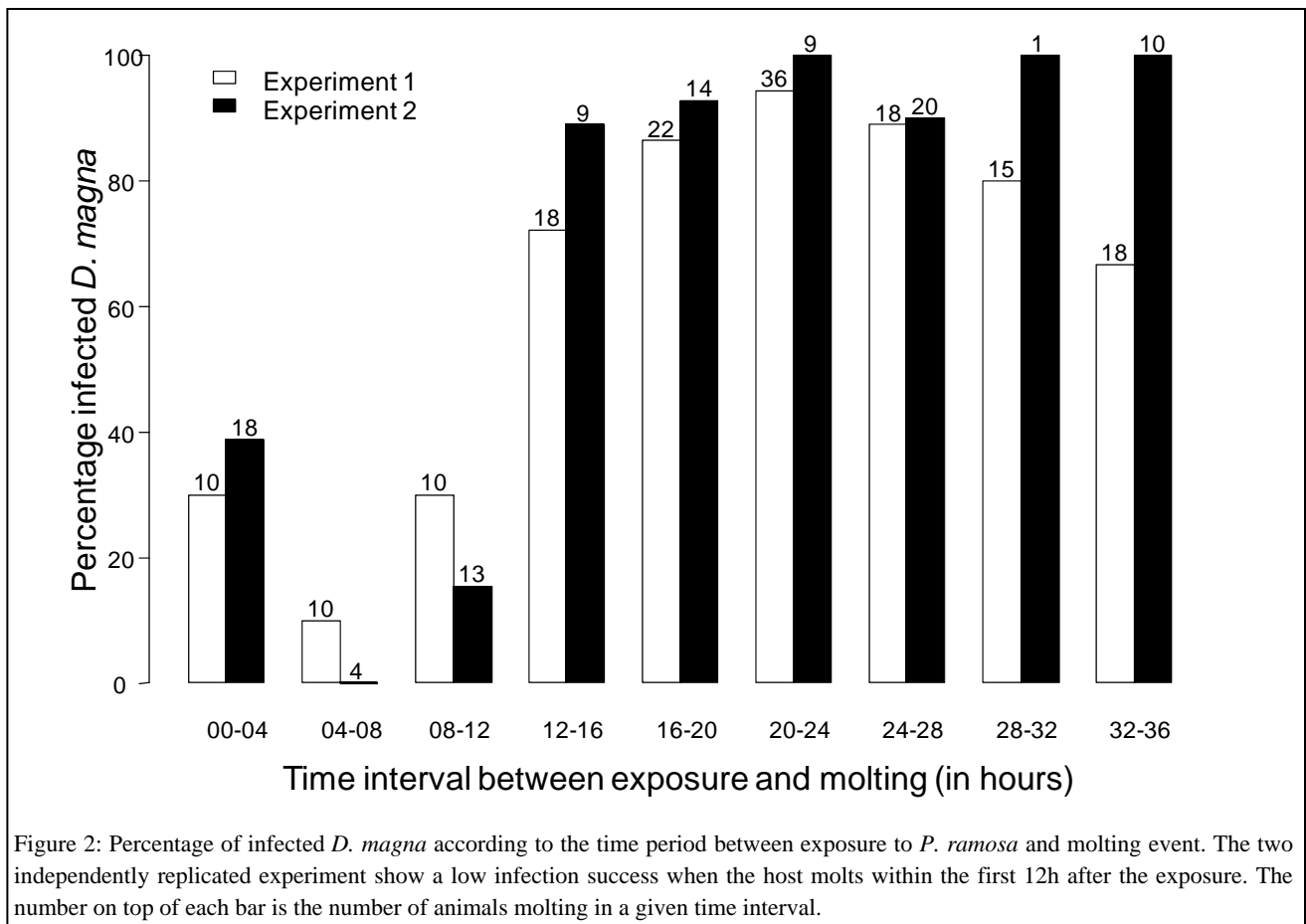
We investigated whether hosts exposed to *P. ramosa* shed their cuticula earlier than those not exposed. We used three *Daphnia* clones from very distinct geographical regions (HO2, M10, and Xinb3) and the *P. ramosa* clone C19. These combinations are known to be compatible [12]. For each host clone, we used 50 pairs of offspring, each taken from one clutch from a different mother, and exposed one offspring to the parasite and the other not (exposed to healthy *Daphnia* homogenized in ADaM to control for the exposure to *Daphnia* tissue). Individuals were kept in 24-well plates and were checked for molting every 2 hours during the 30 hours after exposure. The total amount of replicates having molted within the 30 hours for the clones HO2, M10, and Xinb3 *Daphnia* were 43, 33 and 39 pairs respectively.

#### Results

We used different *Daphnia* genotypes and protocols to test whether molting can help reducing infection and whether it can be manipulated by exposed hosts for that purpose. We conclude that molting does get rid of attached parasites (Figure 1) and reduces the likelihood of infection (Figure 2), but it is not accelerated by the exposure to parasites (Figure 3).

#### *Parasite removal with molting*

Because the cuticula of *Daphnia* esophagus is shedded during molting, we hypothesized that spores attached to this part might be in the exuviae. Microscopic examination of the exuviae of *D. magna* that had been exposed to parasites revealed that the parasite was attached to the cuticula of the esophagus in 100% of the susceptible host individuals (n= 18, Figure 1) and in 0% of the resistant ones (n=12).



### Effect of molting on parasite infection

Because attached *P. ramosa* might need time before penetrating into the body, we hypothesized that molting shortly after parasite exposure would interfere with infection. Our data showed that if the host molts within 12 hours after exposure, the probability of infection is strongly reduced (Figure 2). The time between exposure and molting was a factor contributing significantly to the likelihood of infection (GLM,  $n=255$ ,  $df=1$ , deviance= 56.21,  $p<0.0001$ ), while its interaction with the factor *Experiment* (GLM,  $n=255$ ,  $df=1$ , deviance=3.59,  $p=0.06$ ) was not significant. The two experiments did show consistent results (GLM,  $n=255$ ,  $df=1$ , deviance= 0.2,  $p=0.66$ , Figure 2).

### Molting as a passive defense against parasites

The time interval between parasite exposure and host molting was not significantly different between the three host clones (ANOVA,  $n=115$ ,  $df=2$ ,  $F=2.15$ ,  $p=0.12$ ). Thus, we tested whether the exposed group molted before the non exposed

group without taking host clone into account. We found no significant difference in molt interval between *Daphnia* exposed versus not exposed to the parasite (Paired t-test,  $df=114$ ,  $t=-0.41$ ,  $p=0.68$ , Figure 3).

### Discussion

Parasitic infections consist of a succession of steps (e.g. encounter between host and parasite, attachment of the parasite to the host, penetration of the host body cavity, and proliferation within the host) during which hosts and parasites can interact in specific manners. The penetration into the host body cavity is a key step for a successful infection of endoparasites. Selection during this step should lead parasites to evolve mechanisms to rapidly cross the host skin/cuticula, and hosts to evolve ways of reducing the likelihood of parasite invasion. The shedding of the complete protective layer that surrounds the body cavity is a crucial feature in the life-cycle of several invertebrate phyla (the Ecdysozoa) and some vertebrates of the order of Squamata (snakes and lizards). It is

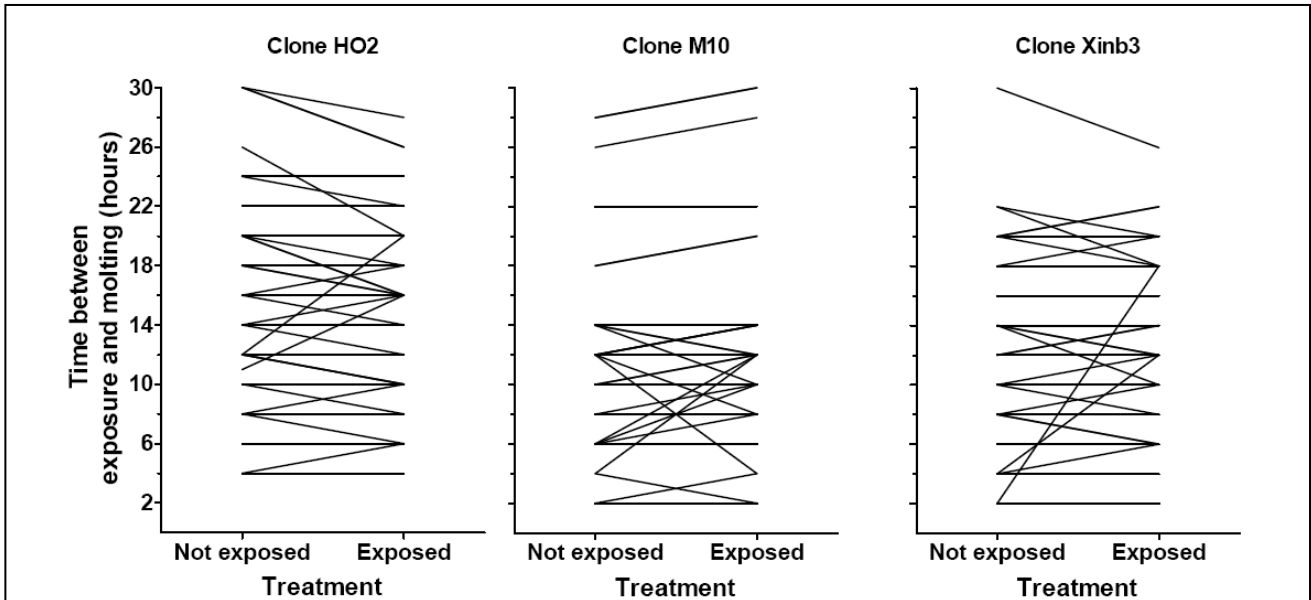


Figure 3: Time between exposure and molting for *D. magna* either exposed to *P. ramosa* or not exposed. The lines connect offspring from the same clutch: HO2, M10 and Xinb3, respectively 43, 33 and 39 pairs. In case of induction of molting by parasite encounter, the offspring exposed to the parasite should have a time between the beginning of the experiment (exposure) and the molting event shorter than the offspring not exposed. The exposure to parasite did not affect the timing of molting.

generally assumed that molting during exposure to parasites is costly because it increases the probability of successful infection to endoparasites [2,10,17]. Here, we investigated whether this shedding of the cuticula can be beneficial for the host and play a role in parasite resistance.

We show that host molting soon after parasite exposure does rid hosts from parasites attached to their cuticula (Figure 1) and reduces the likelihood of successful infection (Figure 2). To our knowledge, this is the first time that host molting is reported to interfere directly with the success of infection by a parasite. The attachment of the bacterial parasite *P. ramosa* to the esophagus of its *D. magna* host was described before [12], but the mechanism the parasite uses to cross the cuticula after the attachment is still unknown. The strong increase in likelihood of infection when hosts did not molted within the 12 hours following the parasite exposure (Figure 2) suggests that it takes about 12 hours for the parasite to penetrate into the host's body cavity and penetration has to occur before host molting. At 20°C, the interval between *Daphnia magna* molts is about 36 hours in juveniles and 3-4 days

in adults [18]. Molting-related disposal of parasites is therefore not trivial for parasites, considering a constant exposure to the parasite, about one third of all spores would be lost before penetration in host juveniles, and 10 to 20% in adults. Thus, it is likely that molting impose selection on parasite to penetrate shortly after being attached.

The probability of failure of parasite infection due to host molting has important implications for experiments with the *Daphnia-Pasteuria* system, which has advanced to a major system for studies of host-parasite evolutionary ecology [19,20,21,22]. Putative variation in host molting among experimental groups can lead to increased noise, or even spurious results, in infection rates. For example, poor resource intake lengthens the intermolt period in *Daphnia* [18] and would thus increase the likelihood of infection. Furthermore, molting in cohorts of exposed animals may be synchronized (e.g. in groups of animals born in the same time) and thus can cause systematic biases, rather than just random noise. Our results suggest that, to minimize these effects, experimental designs should expose *Daphnia* to *P. ramosa* for longer than 12 hours, the approximate time for the



parasite to penetrate into the host. Alternatively, to reduce the chance of losing the spores with the molt, it seems appropriate to expose *Daphnia* several times to a smaller dose of parasite spores.

The protective role of molting is likely to be relevant also in other host-parasite interactions. One of these interactions might involve vector borne disease agents who take advantage of the adaptations of their bloodsucking vectors to cross the skin of their host and to be transmitted. For example, in the case of the etiological agents of Lyme disease, *Borrelia burgdorferi* s.s., the endoparasite needs that its tick vector is attached for around 78 hours before being transmitted to its mammalian hosts [23]. Many vector borne zoonoses (e.g. mites transmitting haemogregarin blood parasites, ticks transmitting *Borrelia* sp.) parasitize snakes and lizards [24]. The here discussed mechanism suggest that regular molting of these vertebrates might have consequences for their likelihood to become infected, especially when the time before transmission takes several days. If the host can shed its skin with the vector before the transfer of the endoparasite, it might explain in part the observation that lizards are less good hosts for certain parasites than other vertebrates [24].

Exposure to “micro-organism-enriched” water has been shown to increase molting intervals in other systems [10]. Therefore, timing of molting can be plastic and, in our system where molting shortly after exposure reduces the likelihood of infection, it is likely that *D. magna* exposed to *P. ramosa* accelerate molting cycles. The results represented in Figure 3 suggest that parasite exposure does not induce the shedding of the cuticula. The induction of molting may be physiologically constraint, either altogether or within the limit of 12 hours during which molting could help minimize infection. However, somatic growth of crustaceans, thus molting cycles, is known to depend on environmental conditions [e.g. food, 25, and temperature, 26] and the reaction norms are different between genotypes [27]. In *Daphnia-Pasteuria* system as in many others, environmental factors are known to affect infection outcomes differently according to the host genotype, the parasite genotype or their

combination [28]. Our results suggest that host molting, correlating strongly with somatic growth in crustacean, may lead to an interaction between parasite success, host clone and environment.

In summary, we confirmed the hypothesis that when an Ecdysozoa host molts shortly after parasite exposure, the parasite infection process is compromised. Therefore, molting can be advantageous to prevent parasite infections and might select for higher parasite penetration speed. It also shifts the cost-benefit calculation for molting further in the direction of the benefits. We showed that in our system this process is not accelerated by the contact with the parasite. However, it might be an issue for other organisms.

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**CHAPTER 3**  
**HOST SEXUAL DIMORPHISM AND PARASITE ADAPTATION**  
David Duneau, Dieter Ebert

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**Abstract**

In species with separate sexes, parasite prevalence and disease expression is often different in the two host sexes. This effect has mainly been attributed to differences in immune response and resource allocation between the two host sexes, but other host sex differences, including morphological, physiological, and behavioral, may also contribute to these observed patterns. Here we make the case for how properties of parasites themselves can also matter. Specifically, we suggest that differences between host sexes can impose selection on parasites and might, therefore, contribute to explaining host sex-biased disease prevalence and expression. We propose that host-sex driven selection on parasites can lead to three different scenarios in terms of parasite evolution: 1) sex-specific adaptation leading to dimorphism in the parasite population, 2) single sex-specialization of parasites, and 3) phenotypically plastic sex-specific expression of parasite traits leading to sex-specific disease. Considering these possibilities will be a significant step forward in the study of host-parasite interactions, with potentially great impact on epidemiological and biomedical studies.

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## Introduction

In populations of sexual species, it is often observed that parasite prevalence, disease symptoms and virulence are different in males and females [see review in 1, recent examples in 2,3,4,5]. This effect of host sex, recorded even in humans, has mainly been attributed to differences in immune response, hormones and resource allocation between the two host sexes [1,6,7,8,9,10,11], but other host sex differences including morphological, physiological, behavioral, dietary, and life history traits, may also contribute to these observations. Here we suggest that differences between host sexes can impose selection on the parasite itself, which in turn might contribute to variation in disease prevalence and expression among males and females.

We propose that host-sex driven selection on the parasite can lead to three different scenarios of parasite evolution: 1) sex-specific adaptation leading to dimorphism in the parasite population, 2) single sex-specialization of parasites, and 3) phenotypically plastic sex-specific expression of parasite traits leading to sex-specific disease. We think that considering these possibilities will contribute to understand the commonly observed differences in the distribution of infectious diseases among host sexes. We begin by explaining three possible evolutionary scenarios. Then, we discuss how host demographic properties, notably host sex-ratio and social structure, can influence the extent to which the parasite evolves. Specifically, differences between host sexes can affect the

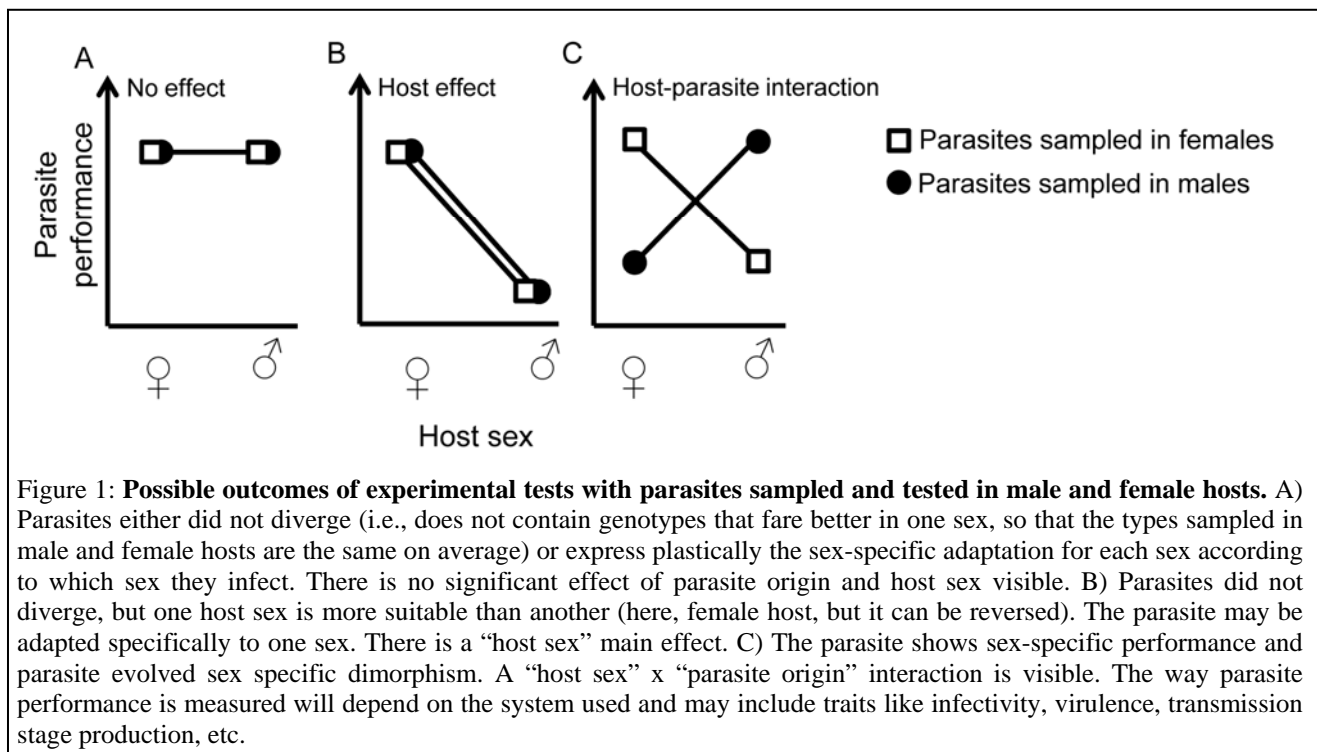
likelihood and extent of transmission within and among host sexes and determine how host sexes represent different selective environments for the parasites. We conclude by considering the implications of host sex-specific adaptation for studies for ecology and evolutionary biology but also for applied subject such as biomedicine, veterinary medicine, agriculture.

## Host sexes and parasite evolution

Males and females are under divergent selection resulting in sexual dimorphism in many traits including morphology, physiology, life history, and behavior. In fact, the most extreme differences described within species are often those between sexes and, typically, sex differences explain most of the phenotypic variation between adults in a sexual population. Parasite populations are expected to be adapted to the characteristics of their most common host type [12]. Therefore, when a parasite population is evolving mainly in one host sex, some of the host sex-specific characteristics may be of relevance for parasite adaptation (Table 1). Without considering the host sex in which the parasite primarily evolved, it is difficult to disentangle whether sex-biased parasitism is due to host and/or parasite characteristics. The hypothesis of host sex specific parasite adaptation may be tested in systems where hosts and parasites can be used in experimental infections and where parasite isolates can be obtained from both host sexes. Such experiments have to our knowledge, never been done, apparently because it is generally assumed that sex-biased disease prevalence and severity are only due to host properties.

**Table 1: Examples of sexually dimorphic traits, which might influence parasite evolution.**

<b>Sexually dimorphic traits</b>	<b>Implications for parasites</b>	<b>Examples</b>
Sex specific tissue	- Parasite adaptation to the tissue only present in one host sex. [e.g. ovarian parasites of fish, 49, and testicular parasites of fish, 50]	Primary sexual traits.
Sex specific properties of tissue	- Parasite adaptation to the specific host properties of a tissue existing in both host sexes. This may results in specific parasite communities adapted to the sex specific properties [e.g. different microbial community on hands of different sexes, 92]	- Different skin properties [e.g. men sweating more than women, 93]. - Differences in diet with implication on digestive apparatus [e.g. American bison males eat relatively more C4 plants and females more C3 plants, 40]
Sex specific need/metabolism	- Parasite adaptation to resources available in each sex.	- Males with wings and females wingless [e.g. Velvet ants, 94] might have different physiology and different needs. - Differences in diet for different needs [e.g. Male capucin monkeys eating more animals than females, 41]



Experiments could be conducted by sampling parasites from female and male hosts (“parasite origin” in figure 1) and exposing them to uninfected females and males (“host sex” in figure 1) in a factorial 2 x 2 experiment and score parasite performance. Some outcomes of such an experiment are shown in figure 1, which reveals that breaking down parasite origin in combination with host sex is the powerful way to reveal host sex-specialization. An absence of a difference in parasite performance (Figure 1A) may suggest an absence of divergence between parasite populations or that parasites evolved phenotypes expressed plastically depending on the host sex they infect. Figure 1B shows a difference among host sexes and may indicate the specialization of the parasite population to one host sex. An interaction between “parasite origin” and “hosts sex” (Figure 1C) would reveal parasite specific adaptation to the two host sexes. Population genetic methods using genetic marker analysis of parasites collected from male and female hosts is an alternative method to detect a dimorphism in the parasite population, but does not allow unambiguous conclusions. In the best case it reveals that the parasite populations are to some degree subdivided into sex-specific sub-populations [13]. Below we elaborate three possible scenarios of parasite adaptation to host-

sex which we call “host sex-specific dimorphism”, “single sex specialization” and “plastic sex-specific disease expression”.

**Host sex-specific dimorphism.** Male and female hosts may represent two sex-specific environmental conditions to which lines of the parasite may adapt specifically. An analogous situation can occur in a geographical setting: When resident genotypes in each environment have on average a higher fitness in their local environment than genotypes originating from other environments, the population is said to be locally adapted [14]. Local adaptation implies antagonistic pleiotropy, whereby the selected alleles have opposite effects on fitness in different environments (trade-off in performance between the environments) [15]. As a consequence, if the two host sexes are viewed as two different environments, this trade-off is expected to result in parasite origin x host sex interactions for parasite fitness (Figure 1C). In that context, the evolution of parasite divergence in a sexual host depends mainly on two parameters, the degree of host sexual dimorphism (difference between environments) and the likelihood to encounter the opposite sex at each transmission event (Figure 2). The later is conceptually the same as gene flow between environments. In the scenario where

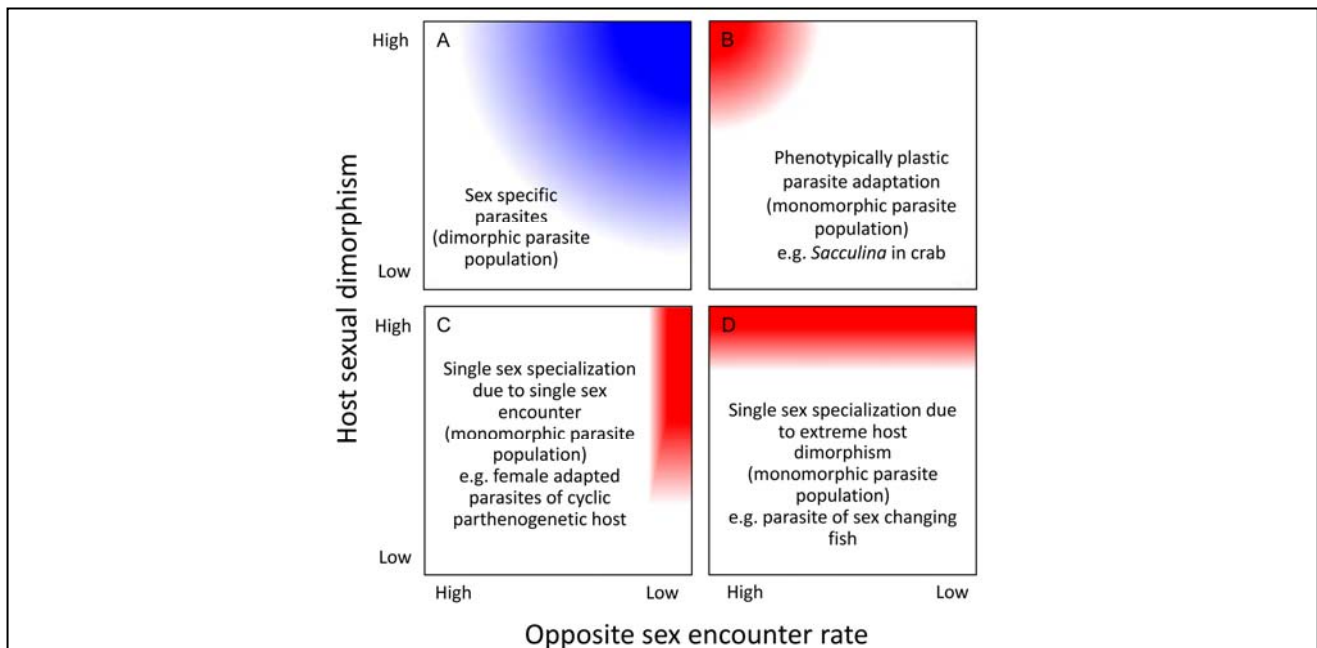


Figure 2: **Parasite evolution in relation to host sexual dimorphism and likelihood of encountering the other host sex.** In red and blue are parameter combinations, which lead to monomorphic or dimorphic parasite populations, respectively. The higher the degree of host sexual dimorphism and the lower the probability to encounter the same host sex, the higher is the likelihood for a parasite to adapt specifically to its common host sex (panel A). When males and females are very different from the parasite's point of view and the parasite encounters both sexes equally often (panel B), the parasite might evolve phenotypic plasticity (e.g. *Sacculina* in crab). When one host is different from the other and so rare, that a parasite cannot persist in it (e.g. males in a facultative sexual species like many rotifers, cladocerans, and aphids) then the parasite species may specialize entirely on the common sex (panel C). When one host is very different from the other in a trait important for the parasite (e.g. ovaries in sex changing fish) then, disregarding the rate at which the opposite sex is encountered, the parasite may specialize entirely on the more suitable host (panel D).

parasite populations are structured by host sex, the parasite populations may adapt to the conditions specific to the host sex they encounter predominantly. Thus, the parasite would evolve a host sex-specific dimorphism (top left panel in figure 2).

**Single sex specialization.** Two scenarios may lead to the specialization on one host sex. In extreme cases, one host sex may be so rare (e.g. males in cyclically parthenogenetic species) that the parasite rarely encounters them (bottom left panel in figure 2). In such cases, the parasite may specialize only on the host sex they encounter. In this case, parasites sampled in the rare host would be adapted to the common (opposite) host sex and an experiment would not yield an effect of “parasite origin” but of “host sex”. Parasites from both origins will have a higher fitness in the common host sex (Figure 1B). In another scenario, the parasite adapts to a host trait that is only found in one host sex. The parasite populations may adapt only to this sex, disregarding the likelihood of encountering the

other (bottom right panel in figure 2). In this case, parasites sampled in the host sex to which they are not adapted would perform better in the opposite host sex (Figure 1B).

#### **Plastic sex-specific disease expression.**

Phenotypic plasticity, a property whereby the same genotype translates into distinct phenotypes depending on the environment, is a common way for organisms to deal with fluctuating environments [16]. Parasites having to face distinct male and female host environments, might have evolved plasticity in relation to those environments and be able to express host sex-specific traits accordingly. Following Scheiner [17], the plastic expression of a trait is favored when 1) environmental variability among environments is high, 2) environments are equally abundant, 3) the strength of selection is equal in both environment, 4) the environmental cue determining the phenotype is highly correlated with the environment of selection, and 5) the cost of plasticity, which is the cost of maintaining the



genetic and cellular machinery necessary to be plastic, is compensated by its advantage. If these conditions are met phenotypic plasticity is expected to evolve (Figure 2B) otherwise a single generalist phenotype will be favored. In case of the evolution of a plastic response, parasites from different origins will not have different fitness when tested in the same sex environment (Figure 1A & B).

The conditions under which these three scenarios may evolve differ strongly. Figure 2 is an attempt to pinpoint conditions, which are likely to play a crucial role for the evolution of sex-specific parasite adaptation and lead either to monomorphic parasite populations (red in figure 2) or to dimorphic parasite populations (blue in figure 2). The two decisive variables are the likelihood that a parasite is transmitted within, rather than between sexes (x-axis in figure 2) and the degree of sexual dimorphism of the host trait(s) the parasite is exposed to (y-axis in figure 2). These two variables are discussed next.

#### Host population structure and parasite transmission

The evolution of sex specific parasite adaptation is affected by the likelihood of the parasites to be transmitted within or among host sexes (Figure 2). This likelihood depends strongly on the host species and the ecological circumstances (Table 2). Here, we focus mainly on cases where the likelihood of encountering a host of the opposite sex is low. A low likelihood of intersex transmission can result from very different situations. Males and females are not always equally abundant and, therefore, parasites might be predominantly transmitting among the common sex. Biased sex-ratios are often observed in natural populations [18,19,20,21], and are even an intrinsic characteristic of certain species, for example the abundance of females in cyclically parthenogenetic species (e.g. aphids, cladocera, rotifers), and in many haplodiploid species such as ants, bees, wasps and mites. Parasites infecting social bees, wasps and ants will face mostly female workers and will only rarely encounter males. For bumble bees, it has been shown that foraging female workers are more infected by

tracheal mites than foraging males [22]. Female-biased sex ratios can also result from sex-ratio distorters such as *Wolbachia* bacteria, infecting at least 20% of all insect species [23].

In species where sex ratios are unbiased, social structures can lead to spatial segregation of males and females and, consequently, of parasites being associated with them. In many species, males and females live in mixed social groups only for limited periods of their life cycle. This is the case for species with matriarchal social organization, such as African elephants, *Loxodonta africana*, where mature males leave the group to be solitary or gather with other males [24]. Sexual segregation is also common in groups such as ungulates [25; Table 2], for example the American bison, where bulls and cows are not in contact for 11 months of the year [26]. This segregation has been proposed to be related to a strategy whereby females can avoid contact with parasitized males [27], supporting our suggestion that parasite populations may remain isolated within a host sex.

#### Host sexual dimorphism and parasite transmission

Sex-specific host traits may affect the rate at which hosts of different sexes encounter parasites and as a consequence the likelihood for parasites to encounter both host sexes (Table 2). For example, body size, which is often dimorphic, has been suggested as part of the reason why parasites in mammals more often infect the generally larger males than females [28]. In many taxa, males are larger than females [e.g. many birds, 29] but the reverse is not rare in some groups [e.g. insects, 30] and can be extreme as is the case with dwarf males [31,32], potentially reversing or exaggerating the pattern of infection bias observed in mammals. Certain types of sex-biased behaviors are also linked to an increased risk of exposure to parasites. For example, in mice and other mammals, male-specific sniffing of urine and feces used to assess social hierarchy can increase contact with pathogens [33,34]. In domestic cats, the feline immunodeficiency virus (FIV), a virus mainly transmitted via bites is twice

more prevalent in males because of the sex differences in social behaviors. Males have a

**Table 2: Examples of host sex differences, which might influence parasite evolution.**

Host sex difference	Examples and their implications for parasites
Exposure	Differences in visited areas. <ul style="list-style-type: none"> <li>- Male spadefoot toads spend many nights in water while females go only once for few hours which results in males being the common hosts for aquatic parasites such <i>Pseudodiplorchis americanus</i> [95].</li> <li>- Cormorant males and females forage in different places which results in parasites of one sex more likely to infect more this particular sex [36].</li> </ul>
	Differences in behavior increasing parasite encounter risk. <ul style="list-style-type: none"> <li>- Male of mammals sniff urine and feces for establishment of social hierarchy which results in increasing the contact with pathogens [33,34] and male to male transmission.</li> <li>- House finch males prefer contact with less aggressive males while females have no preference which results in increasing the likelihood of infection between males when the less aggressive males are more heavily infected [96].</li> </ul>
	Differences in host availability <ul style="list-style-type: none"> <li>- Biased sex-ratio in cyclically parthenogenetic species and in many haplodiploid species such as ants, bees, wasps and mites may results in parasites more likely to infect only one host sex.</li> </ul>
	Difference in social structures <ul style="list-style-type: none"> <li>- Spatial segregation of male and female hosts such as most ungulates which results in the segregation of the parasite populations they carry.</li> </ul>
Susceptibility	Differences in host body size <ul style="list-style-type: none"> <li>- Males are larger than females (e.g. mandrills, elephants, sea lions). Male <i>Bonellia viridis</i> (annelids) are drastically smaller than females (Agius et al. 1983) [see dwarf males, 31]. Strong sex size dimorphism increases the likelihood to encounter the larger host sex [e.g. mammals, 28].</li> </ul>
	Differences in immunocompetence <ul style="list-style-type: none"> <li>- Interaction between endocrine and immune system [44,45,46,47,48] which results in males and females differing in ability to fight off parasites [43] and parasites having a greater opportunity to spread within male hosts [e.g. twice for the striped plateau lizards, 48].</li> </ul>
	“Haploid-susceptibility hypothesis” <ul style="list-style-type: none"> <li>- In haplodiploid species, females are diploid, males are haploid. The “Haploid-susceptibility hypothesis” predicts that, the haploid males are more susceptible [97] and might be the host type the most commonly successfully infected.</li> </ul>
Development	Differences in lifespan <ul style="list-style-type: none"> <li>- Females living longer than males [e.g. male hymenoptera live for days, certain females for years, 52, male marsupials of the species <i>Antechinus</i> sp. die shortly after the breeding season, while females live for years, 53] which may result in more parasite generations within same female host and a higher probability for female host to get infected during their life time.</li> </ul>
	Differences in development <ul style="list-style-type: none"> <li>- In bees, <i>Apis cerana</i>, larvae development is longer in drones (males) compare to workers. <i>Varroa destructor</i> mites have a developmental time matching those of drones. Mites on worker host larvae cannot reproduce [63,64]. <i>Varroa</i> mites can actively choose the drone brood cells [70].</li> </ul>

higher propensity to bite each other [35]. Parasites transmitted in this way will more often be transmitted between male individuals. Conversely, parasites associated with nests (e.g. fleas and ticks) will encounter mature females or juveniles (which, typically, have no pronounced sex differences) more often than they will encounter male hosts. Some sexually dimorphic behaviors have been proposed to explain differences in exposure to parasites (Table 2) including where males and females forage [e.g. Cormorants, 36] and what they eat [e.g. Fore people’s cannibalistic practices, 37]. There are many examples of sex differences in foraging [e.g. squirrel monkeys, 38, and blue-footed and brown boobies, 39], diet [e.g. the American bison, 40, and capucin monkeys, 41] and behavior [e.g. sex biased dispersal, 42]. However, the effects of these differences on the evolution of parasites and to the

likelihood of parasite adaptation to specific host sex remains to be explored.

The likelihood of successful infection upon exposure defines the host’s susceptibility to parasites and can be different for males and females. This susceptibility depends, among others, on the suitability of the host for the parasite to grow and the likelihood to overcome the host immune system. Differential susceptibility due to host immunity has been proposed many times in vertebrates and is attributed to the interaction between endocrine and immune systems [43]. Sex hormones also regulate innate and acquired immunity [44,45], and the interaction between testosterone and the immune system presumably explains the higher parasite susceptibility of male rodents [46,47] and lizards [48]. The likelihood that a parasite can infect a host depends also on host physiology and

on the resources that the parasite can exploit. In extreme cases where the parasite infects a primary or secondary sexual trait [and fish ovary parasite, 49, e.g. fish testis parasite, 50], only one sex is a suitable host. Upon infection, the host physiology that the parasite will be confronted with can be significantly different in males and females. Males and females differ in the type and concentrations of hormones and metabolites (Table 1 and 2) such as body fat, which can be an important resource for parasites. In insects, for example, the females are larger [30] and often have a higher proportion of body fat. Host body size and nutrient composition may be important for the growth of the parasite within the host because space and nutrition are key components of the host's carrying capacity for the parasite population. This will have an impact on the number of generations a parasite can grow within the same host individual in the same way that host longevity will. Longer host lifespan can increase the number of generations that the parasite will have within it, which increases the likelihood of parasite adaptation to its host's characteristics [51]. Sex differences in lifespan are quite common and can be extreme [in some Hymenoptera, 52; see Table 2, e.g. marsupial from the genus *Antechinus*, 53]. By affecting exposure and susceptibility, differences between male and female hosts in morphology and life history traits can influence the likelihood that a parasite encounters one or the other host sex and, therefore, the probability that it evolves host sex-specific adaptations (Figure 2).

### Evidence for parasite sex-specific adaptation

The examples above suggest that male and female hosts can represent different selective environments, with distinct challenges but also different opportunities for parasite growth. In addition, parasites might not be equally likely to encounter both sexes and may even be genetically isolated within host sexes. As a consequence, the scenario of the parasite forming two sub-populations becoming adapted to the sexes they infect the most appears reasonable. There are few documented examples of parasite adaptation to host sex, and to our knowledge no example of a host sex-specific dimorphism has been described.

This scarcity may be a consequence of the absence of studies where this has been explicitly investigated.

Recent examples of parasites actively choosing to infect the sex they most commonly encounter and, where they have the highest fitness, make a strong case for single-sex specialization of parasites. In nature, the ectoparasitic mite *Spinturnix andegavinus* is exposed mainly to female individuals of its host bat *Myotis daubentoni*. Experimental studies have shown that these mites are specialized on female hosts. These mites are only capable of growing on females (example as in figure 1B) and actively choose females when given the choice [54]. Host sex discrimination might be more widespread than is commonly believed. In fact, active choice for host species [i.e. crustaceans, 55, ticks, 56, fleas, 57, nematodes, 58, trematodes, 59,60], or host individuals [61] has been documented for many endo- and ectoparasites. In a context of sex-specific parasite adaptation, the sense organs of these organisms may evolve to discriminate between host sexes.

The mite *Varroa destructor*, an ectoparasite of bees and a great problem in apiculture, provides an exciting example of the evolution of a parasite specifically adapted to one host sex and of mechanisms to select host individuals of the suitable sex. This parasite has a life-cycle that includes a phase on adult bees, when the parasite spreads, and a phase on the developing host larvae inside the brood cells, where it reproduces [62]. In its original host, the Eastern honey bee *Apis cerana*, the mite reproduces exclusively in the presumptive drone (male bee) cells [63,64,65]. Mites carried into the brood cells by the adult nursing workers will leave the adult to stay with the presumptive drone larvae, but not those with workers or queens (they are repelled by a substance in the queen larva's diet of royal jelly [66]). Worker cells are typically much less frequently visited by nursing workers [67], and this might have been the original trigger of the sex-bias in parasite infection. In the more recent host *Apis mellifera*, where the parasite can reproduce in both drone and worker larvae, the difference in nurse care can partly explain that

drone cells are around 10 fold more infected than worker cells [67,68]. We speculate that this host species illustrates what was possibly the ancestral situation in *A. cerana*. Then, the bee worker's sex-biased nursing behavior resulted in males being the most common host for mite reproduction. In the following mites may have become adapted to the male environment. In *A. cerana*, the parasite is specifically adapted to drone larvae life history and physiology and no longer infects worker larvae. The differences between worker and drone larvae that are relevant here include differences in hemolymph composition (reproducing mites appear to need drone hemolymph for fertility [65]), and development time (mites match the developmental time of drones, but not of the faster developing workers [69]). Once adapted for reproduction on male larvae, *Varroa* evolved to recognize drone chemical volatile signals and actively choose to colonize drone brood cells for reproduction [70].

Infection of the wrong host type can carry high fitness costs for the parasite, for example, if one specific aspect of the male or female bauplan/anatomy is necessary for parasite growth or transmission. For parasites commonly exposed to both host sexes such cost might be compensated by a plastic response. This is the case for parasitic barnacles of the genus *Sacculina* which infect and sterilize crabs [71]. The parasite grows in the place where the eggs are generally incubated (underside of the rear thorax), and spreads when female hosts perform egg-laying behavior. When these parasites infect male crabs, they manipulate host traits, feminizing both their morphology and behavior and as a consequence can be transmitted. The details on the mechanism of this feminization are not well understood, however, if this barnacles secrete an endocrinal hormone specifically in males as suggested in [72], it would represent a phenotypically plastic response to host sex (Figure 1A, 2B).

Bacteria from the large group of the Rickettsia, e.g. *Wolbachia* [73] and sex-ratio distorting Microsporidia [74] are well known and widespread examples of vertically transmitted parasites that are sex-specifically adapted. These endosymbionts transmitted transovarially can

infect male and female progeny and have specific behaviors depending on the host sex they infect. This is an example of phenotypically plastic response to host sex (Figure 2B). *Wolbachia* may induce feminization of genetically-male hosts, specifically kills infected males to favor infected female of a same brood, or induces a form of cytoplasmic incompatibility [73]. In the last case, it appears that *Wolbachia* in a male modifies the sperm in such a way that paternal chromosomes are destroyed in the zygote unless a *Wolbachia*, genetically identical, in the egg cytoplasm "cures" the modification. *Wolbachia* is extremely widespread in insects and is a compelling illustration of the importance of sex-specific parasite adaptations. It is likely that many other cytoplasmic parasites show sex-specific adaptations to increase their transmission.

#### Implications of parasite sex-specific adaptation

Host sex-specific parasite divergence has implications for both host and parasite populations and for the dynamics of the interactions between them. Between-sex differences can represent a challenge for parasites, making it difficult to fully adapt to both sexes in well mixed populations.

Host sex-adapted parasites can sometimes encounter a high proportion of the host sex that they are not adapted to. For example, in organisms with cyclical parthenogenesis (e.g. *Daphnia*, aphids, rotifers), males are rare most of the time but common during a particular period of the year and/or under certain environmental conditions. Likewise, in many ungulates, males and females live apart most of the time but come together during the breeding season. During such periods, sex-adapted parasites are faced with the less suitable host sex. This can have evolutionary consequences for the parasites: 1) a reduction or elimination of sex-specific adaptations, when sex-generalist parasites are favored over sex-adapted parasites, 2) it may lead to selection of parasite traits that allow discrimination between sexes to avoid the wrong host type (e.g. active host choice), or sex-manipulations (e.g. feminization of male host). It may also lead to the expression of disease symptoms apparently maladaptive for the

parasite, as they have been observed in some local adaptation studies of host-parasite systems[75]. In extreme situations, parasite populations adapted to one or the other host sex might become isolated from each other (dimorphic parasite population, Figure 2A) and, eventually, lead to the establishment of different parasite species, each specialized on one host sex (monomorphic parasite population, Figure 2C,D).

Parasite sex-specific adaptations and the possibility for host sex-change may be exploited by the host itself. For example, in the sequentially hermaphroditic fish *Thalassoma bifasciatum*, when the hosts are females they can be infected with the parasite *Kudoa ovivora*, which is specialized on exploiting only the host ovaries (Figure 2D). Interestingly, when infected, the hosts are able to change sex, removing the only resource the parasite can exploit and bringing it to a dead end [76]. Sex-specific adaptations of parasites may also influence the evolution of other host traits. For example, if more naturally masculinized females produce fewer offspring but are better able to resist parasites, a female-adapted parasite might select for more masculinized females. On the other hand, it could be argued that very distinct sex characteristics represent more of a challenge to parasites, and exaggerated female versus male traits might be favored in the host population. This type of argument might be thought of as a selective advantage for the evolution of sex dimorphism in hosts. Parasites may spread less rapidly in species with two distinct sex host-types than in species with only one sex (asexual populations). This effect is similar to the monoculture effect, whereby there is more rapid parasite spread in monoclonal than the genetically diverse host populations [77,78].

Parasite adaptation to host sex can have important implications for host-parasite coevolution. We have argued how host sex can drive parasite sex-specific adaptation when parasite subpopulations evolve mainly in one host sex. For the host, however, selection on one sex only can be impaired by intra-locus sexual conflict [79,80] when alleles that confer parasite resistance or tolerance in the affected sex, decrease fitness of the other sex. The expression

of traits associated with parasite resistance may thus become sex limited.

Host sex-specific adaptation of one parasite might also lead to sex-specific adaptation of other, associated parasites. This is the case, for example, for endoparasites transmitted by host sex-biased ectoparasitic vectors. The *Varroa destructor* ectoparasite introduced above is a vector for a number of viruses that infect its bee hosts [78,79]. Because the vector reproduces exclusively in male bee larvae, the viruses will also more often infect male larvae and be selected in that environment.

Host sex is a key factor in studies in biomedicine and disease control. In humans, there are well documented host-sex differences in parasite prevalence and infection symptoms, as well as prevention and treatment of infection. For example, the immune system of men and women was shown to react differently to vaccines [81]. This difference can be vaccine-strain specific [e.g. men exhibited a higher antibody response than women for yellow fever vaccines from two of three different virus strains, 82]. While this is undoubtedly related to intrinsic male versus female differences, if parasites behave differently in male versus female hosts, either because of genetic divergence related to sex-adaptation or because of phenotypic plasticity, then parasites in females and males might not be targeted by the same antibodies/drugs. Whatever the cause, failure to immunize/cure one fraction of the host population might create a reservoir for the parasites, and immunizing/curing one or the other sex can also have distinct effects on disease prevalence. Studies on the yellow-necked mouse showed that while treatment of male hosts reduced parasite prevalence in both sexes, treatment of females reduced parasite prevalence only in females [83]. A disproportionate contribution of male yellow-necked mice to parasite transmission has been confirmed even in the absence of sex-biased infection [84]. Finally, parasite sex-specific adaptation is a strong argument that both sexes need to be included equally in clinical trial, currently an important concern in biomedicine [85,86,87,88,89].

### Concluding remarks

Different types of host heterogeneity have been demonstrated to affect the evolution of infectious diseases [77,90,91]. Here we argue that host sex is likely to be another important factor in parasite evolution. Documented host sex differences in parasite prevalence or effect [see 1] support the idea that the probability that parasites spread (within and between hosts) is not always equivalent with regard to host sex. These differences are generally attributed to intrinsic characteristics of the host individuals [1,6,7,8,9]. Here, we argue that the observed sex biased disease prevalence and/or severity might be due to the host's intrinsic heterogeneity but can also be due to the parasite having adapted to infect and grow in specific host sexes. Unequal host susceptibility and sex-specific adaptation by the parasite are not mutually exclusive explanations for sex-biased prevalence, and, in fact, must work together. The likelihood and extent of host-sex adaptation depends on many factors. These include characteristics of the host populations or host individuals that determine how different the male and female environments are, and how often the parasite experiences them. We discussed examples of each of these to illustrate how they can impact parasite evolution and lead to the divergence and specialization of parasite populations in different host sexes. Parasite characteristics, particularly the mode of transmission, will also have an impact on the likelihood of divergence between parasite populations in male and female hosts. Therefore, transmission mechanisms will affect sex-specific adaptation. For example, sexually-transmitted parasites will typically have to deal with both host sexes and are less likely to adapt to any sex (represented by the left hand side of the x-axis in the figure 2). Maternally transmitted parasites will be more likely to be adapted to females. To conclude, the sex bias of disease prevalence and severity is of a major current concern in parasitological studies, notably in medical trials [85,86,87,88,89]. We propose that by taking the possibility of host-sex-specific parasite adaptation into account, we will gain a better understanding of host-parasite dynamics and thus the possibility of parasite control and more generally of sex related disease expression.

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## Chapitre 4

### HOST SEX-SPECIFIC ADAPTATION OF A HORIZONTALLY TRANSMITTED PARASITE

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#### Abstract

Parasites are believed to adapt to the most common host type. We hypothesize that in host populations with strongly biased sex-ratios, selection induced by differences between male and female hosts could result in parasite adaptations specific to the common host sex. Here, we investigate this hypothesis on a horizontally transmitted parasite of the cyclic parthenogenetic host, *Daphnia magna*, with strongly female-biased sex-ratio. The parasite is known to castrate female hosts which results in host gigantism, presumably by diverting resources from the germline into host somatic growth. This is beneficial for the parasite because larger hosts have higher carrying-capacity for parasite proliferation. Whether this also happens in male hosts is unknown. In five complementary experiments, we exposed male and female *Daphnia* of different genotypes to *Pasteuria ramosa* parasitic spores. First, we showed that the parasite infected female hosts more successfully than male hosts, but not until host sexual dimorphism developed. Second, we showed that the parasite had higher fitness (spore production) in female hosts. Third, we established that some level of castration also occurs in infected males, indicating similarities in the way male and female *Daphnia* are castrated by *P. ramosa*, but showed that male castration does not result in gigantism. We showed that *P. ramosa* is able to castrate both female and male *D. magna* hosts, but that this is associated with increased parasite fitness only in females. Thus, we argue that castration is a parasite adaptation to exploit female hosts. Our findings support the hypothesis that parasites might evolve sex-specific adaptations when one host sex is rare. The occurrence of such adaptations is a novel finding with important implications for parasitology. Parasite sex-specific adaptations can help understand observed host sex differences in the distribution of infectious diseases and disease symptoms.

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## Introduction

Males and females of the same species typically differ in many traits, including morphology, physiology, behavior and life-history. The key difference is their distinct roles in the courtship and reproduction. When cooperating in joint tasks, male and female interests are rarely identical [1]. This goes hand in hand with differences in gametes, primary and secondary sexual characters, quality and quantity of hormones secreted, but also with behavior, somatic structures (e.g. *D. melanogaster* brain [2]), immune response [3,4], and gene expression [5]. Usually, the most striking differences among members of the same species are those between the sexes.

As parasites are commonly expected to be better adapted to the most common host type [6], it is reasonable to assume that parasites adapt to the characteristics of one host sex if it encounters this sex more often. In populations with strongly biased host sex-ratios, the parasite encounters one host sex more frequently than the other. We hypothesize that host sex differences might be important in parasite evolution, leading to parasite populations specifically adapting to the characteristics of the common. Such sex-specific adaptations of parasites may have implication for epidemiology, evolution and biomedical research. Parasites adapted to one sex might show features of maladaptation, e.g. sub-optimal virulence or non adaptive symptoms in the other sex.

This study had the aim to test whether a parasite evolving mostly in one host sex shows sex-specific adaptations. In populations of a cyclical parthenogenetic host, the sex-ratio is typically strongly female biased, either with long periods of asexual reproduction without males being present, followed by short periods of both sexes, or by strongly female biased sex ratios throughout the season. Cyclic parthenogenetic animals are found in several taxa including insects, crustacean and rotifers [7]. We hypothesize that due to the rarity of

males, parasites specialize to exploit females. Using the cyclical parthenogenetic *Daphnia magna* and its bacterial pathogen *Pasteuria ramosa*, we tested the hypothesis that the parasite is specifically adapted to female hosts. Female and male *D. magna* hosts are genetically identical (sex is environmentally determined [8]) but adult males and females differ in size, morphology, physiology and, of course, in their roles in reproduction. Natural populations of the cyclic parthenogenetic host are strongly female biased, potentially allowing parasites to adapt specifically to female hosts. Infection success of the Gram positive bacterium *P. ramosa* depends on both host and parasite genotype [8] and susceptibility is explained by the specific attachment of the parasite to the host esophagus which is known not to be affected by the sex of the host [9]. It is however likely that after successful penetration into the host, the difference in male and female environment has resulted in sex-specific adaptations during the proliferation in haemocoel and musculature [10].

The first aim of this study was to investigate if, despite being genetically identical, the two host sexes represented different environments for the parasite. We recorded the differences between host sexes in probability of infection, and in parasite virulence, proliferation and fitness. The second aim was to test if the typical symptoms described for *P. ramosa* infecting *D. magna* reflect specific adaptations to the female host. *P. ramosa* is adapted to castrate female hosts, which induces the reallocation of the resources usually spent in egg production into the production of somatic tissues. The adaptive value of host castration inducing gigantism is the increase of the carrying capacity of the parasite environment [12,13,14]. We studied whether *P. ramosa* is able to castrate *Daphnia magna* males, and if a reallocation of resources spent in spermatozoa production would trigger male host gigantism. The absence of male castration, or the occurrence of male castration without induction

of gigantism, would suggest that female castration and gigantism is a female-specific adaptation.

## Materials and methods

### *Biological materials*

We used different genotypes (clones) of *Daphnia magna* (details below) isolated from a metapopulation in South-Western Finland. The occurrence of *Pasteuria ramosa* is usually low in this area [15] and the female hosts are not expected to be particularly adapted to this parasite. Host clones were kept in the laboratory in standardized medium (ADaM) [16] at 20°C, and fed daily with chemostat cultured unicellular green algae *Scenedesmus obliquus*. The parasite used was the *Pasteuria ramosa* clone C19 originally sampled from an infected female of *D. magna* in a population in Gaarzerfeld, Germany [9].

### *General methods*

Infections were performed by placing individual hosts either in 100-mL jars filled with 20 mL of medium or in 24-well plates containing 1 mL of medium and exposing them to the appropriate dose of parasites. Spore suspensions used for exposure of *Daphnia* to the parasite were obtained by homogenizing infected *D. magna* and quantifying the amount of spores by counting with a haemocytometer (Neubauer improved) by phase contrast microscopy (Leica DM 2500, magnification 400x). Controls received placebo infections by exposure to a suspension of homogenized uninfected *Daphnia*. For infections performed in 100-mL jars we filled up the jars to 80 mL medium 3 to 4 days after exposure and for those in well plates we transferred all *Daphnia* individually to jars containing 80 mL medium 48 hours after exposure. Hereafter, medium and jars were changed on a weekly basis unless otherwise mentioned. Infection status at the end of experiments was assessed by phase-contrast microscopy unless otherwise mentioned and where appropriate the number of spermatozoa and parasite spores was

estimated by homogenizing the *Daphnia* individual in 0.05 (for spermatozoa counting) and 0.5 mL (for spore counting) of medium and counting a subsample of this suspension using a haemocytometer (Neubauer improved). Individuals that died within 14 days after exposure could not be reliably checked for infection and were excluded from analysis. Body length of *Daphnia* individuals was measured from the top of the head to the basis of the apical spine. An overview of the experiments reported in this article is given in Table 1.

### *Likelihood of infection after exposure*

We tested for a difference in infection rate between female and male *Daphnia* hosts in two experiments (Experiment No. 1 and 2 in Table 1). For a long exposure (4 days in 20 mL + 7 days in 100 mL) we used 30 females and 30 males of *Daphnia* clone “SP1-2-3”, three-days-old, that were placed individually in jars (Experiment No. 1). We used five different treatments: control, 5000, 12500, 31300, 78100 parasite spores per jar. Eleven days after exposure, *Daphnia* were transferred to fresh medium and thereafter medium was changed every week. After 21 days, we inspected all individuals for the presence of infection (n=264). We also performed short-exposure experiments with very young animals to exclude that male and female differences were already present during exposure. This avoids a putative effect of difference (e.g. in body size) on the likelihood of infection. For this experiment we used seven *D. magna* clones: “Kela 08-10”, “Kela 10-01”, “Kela 12-06”, “Kela 18-11”, “Kela 20-13”, “Kela 28-08” and “Kela 39-01” (Experiment No. 2 in Table1). For each clone, 40 newborn (approximately one-day-old) of each sex were exposed for 2 days to *Pasteuria* in 24-well plates each (20 individuals to 5000 or to 20000 spores). As controls, we used 14 animals per clone and sex. Dead individuals were recorded daily and stored for later analysis. We stopped the experiment 120 days after exposure (when all infected and most controls had died) and checked infection status of every individual (n=582).

Table 1: **Summary of the study**

No. of the experiments	Experiments	Age at exposure (days)	Exposure duration (days)	Nr. of host clones	Nr. of parasite doses	Total sample sizes	Parameters measured	Results figure
1	Likelihood of infection with sex dimorphism during exposure	3	11	1	5	264	- Infection rate	- Fig. 1
2	Host gigantism, parasite fitness and virulence	1	2	7	2	582	- Infection rate - Survival - Spore amount at death (2 clones) - Body length 21 days post exposure (2 clones)	- Fig. 2 - Fig. 4 - Fig. 5
3	Within host parasite proliferation	1	2	1	1	142	- Spore numbers 20 and 27 days post exposure - Body length 20 and 27 days post exposure (for spore density)	- Fig. 3a - Fig. 3b
4	Host gigantism	3	11	3	1	184	- Body length 21 days post exposure	- Fig. 5
5	Male castration	1	2	1	1	230	- Spermatozoa counting 13 to 26 days post exposure	- Fig. 6

### *Gigantism, parasite fitness and virulence*

To test for parasite sex-specific adaptation we determined parasite fitness (= spore production), the ability of our *P. ramosa* clone to induce gigantism and the reduction of host lifespan in female and male hosts. For a survival analysis we used data collected on seven *Daphnia* clones during the short-exposure experiment whereby we excluded six female controls still alive after 120 days. We measured body length at day 21 post exposure and counted the number of spores at death in two of the seven host clones (Experiment No. 2 in Table1, “Kela 08-10” and “Kela 20-13”).

We tested for parasite induced gigantism in an additional experiment. Three-day-old males and females from three *D. magna* clones (Experiment No. 4 in Table1, “Xinb3”, “SP1-2-3”, “XFa6”) were exposed to 30000 spores of *P. ramosa* for 11 days. We used 25 replicates and 13 controls for each treatment combination. Twenty-one days post exposure, we measured the body length of all individuals still alive (n=184) and recorded their infection status.

### *Parasite proliferation*

To test for differences in the rate of within-host proliferation between the host sexes, we counted spores in two groups stopped at two different times. Newborn (1 day old) *Daphnia* (Experiment No. 3 in Table1, clone “SP1-2-3”) were exposed to 20000 spores in a 24-well plate for 2 days. We quantified the amount of parasite spores in host 20 days (female, n= 37; male, n= 29) and 27 days (female, n= 40; male, n= 36) post exposure. Infecting newborn hosts reduces to a large extend physiological and morphological (notably body size) differences between sexes at the time of exposure. We calculated the difference in spore number counted at day 20 and 27 post exposure, and the spore density for each individual by dividing the amount of spores by the host body volume at the respective sampling day (body volume =  $0.2418 \times \text{body length}^{2.593}$  [17]).

### *Spermatozoa counting*

We estimated the number of spermatozoa of males of age 13 days (approximate age at sexual maturity) to 26 days. We exposed one-day-old males *D.*



*magna* (Experiment No. 5 in Table1, “SP1-2-3”), individually in 20 mL, either to 100000 spores (expected to result in 100% infection rates) of *P. ramosa* or to a placebo suspension (control). To obtain spermatozoa, we exposed males to 50  $\mu$ L of 2.5% nicotine for 15 min in the dark. Nicotine stimulates muscle contractions and therefore is an efficient way to make *Daphnia* release mature spermatozoa. Spermatozoa counts were estimate in infected (n=120) and uninfected (control, n=110) individuals at 13, 16, 19, 22, 24, 26 days after exposure. Each animal was only used for one estimate.

### Statistical analysis

To compare the proportion of *P. ramosa* infection between sexes, we used a generalized linear model (=GLM) [18] with a binomial error distribution, and logit link (long-exposure, n= 264, 1 host clones, 1 parasite clone “C19” (Experiment No. 1 in Table1), short-exposure, n=582, 7 host clones, 1 parasite clone “C19” (Experiment No. 2 in Table1)).

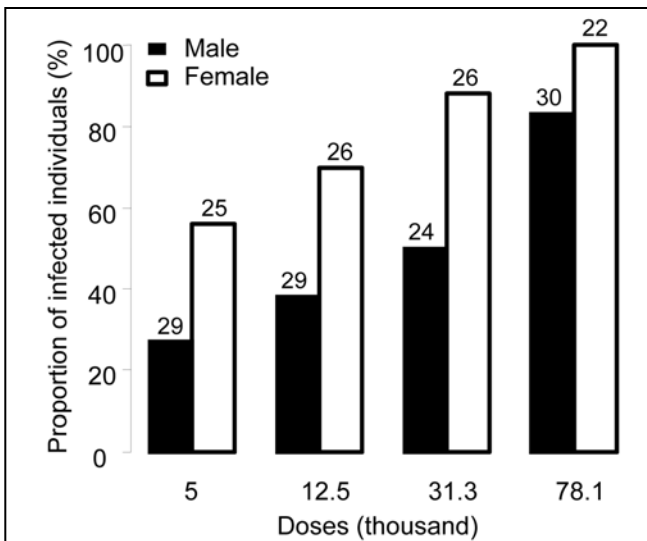


Figure 1: **Proportion of infected male and female hosts when exposed to parasite when sexes are dimorphic.** (long-exposure time; 11 days). The infection rate increased with the dose in both sexes and is always higher in females *D. magna* than in males. Numbers of replicates are given on top of each bar.

Assumptions on the error distribution were checked by estimating dispersion parameters in GLM; no significant overdispersion was detected. To study the impact of *Pasteuria* on female and male *Daphnia* survival (Experiment No. 2 in Table1), we used the log-rank test (package “Survival” in the software R [18]). The estimation of the impact of the parasite on the host lifespan for each sex was estimated by the difference in median survival between the infected and uninfected *Daphnia* within each sex. To test for the difference of parasite spore production in male and female hosts, we used non-parametric tests (Experiment No. 2 and 4 in Table1). For the other tests, we considered parametric assumptions, checked normality and homoscedasticity of residuals, and transformed data when appropriated.

## Results & Discussion

### Higher rates of infection in female

We investigated whether male and female hosts are equally infected by the parasite when exposed to the same dose. Our results show that *P. ramosa* has higher infection rates in females when three-days-old individuals of both sexes, were exposed to the parasite for 11 days (Linear mixed model, Experiment No. 1 in Table1, factor *Sex*, df= 1, deviance= 27.4,  $p < 0.00001$ , Figure 1). The infection rate increased with the dose (factor *Dose*, df= 3, deviance= 34.9,  $p < 0.00001$ , Figure 1) and the sex difference was not different across doses (interaction *Sex x Doses*, df= 1, deviance= 1.35,  $p = 0.45$ , Figure 1). However, when we exposed one-day-old hosts for a short period (48 hours) we did not observe a difference in the proportion of infected females and males (Linear mixed model, Experiment No. 2 in Table1, factor *Sex*, df= 1, deviance= 1.6,  $p = 0.21$ ). Differences in morphology and physiology between male and female *Daphnia* seem small in very young *Daphnia*, with sexual dimorphism developing from the third instar onwards. For example, within the first three days of life there is no size difference (data not shown).

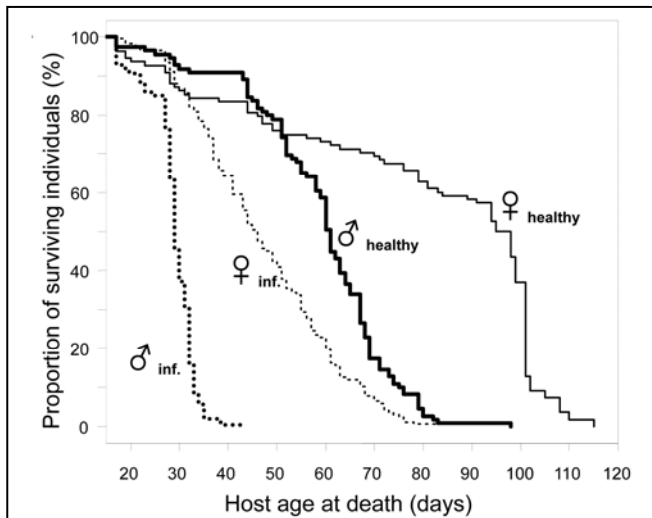


Figure 2: **Survival of male and female hosts either uninfected (solid lines) or infected (dotted lines).** Uninfected *D. magna* live about twice longer than infected ones. *P. ramosa* reduces the lifespan (difference in medians between infected and uninfected) of the females as much as of the male *Daphnia*.

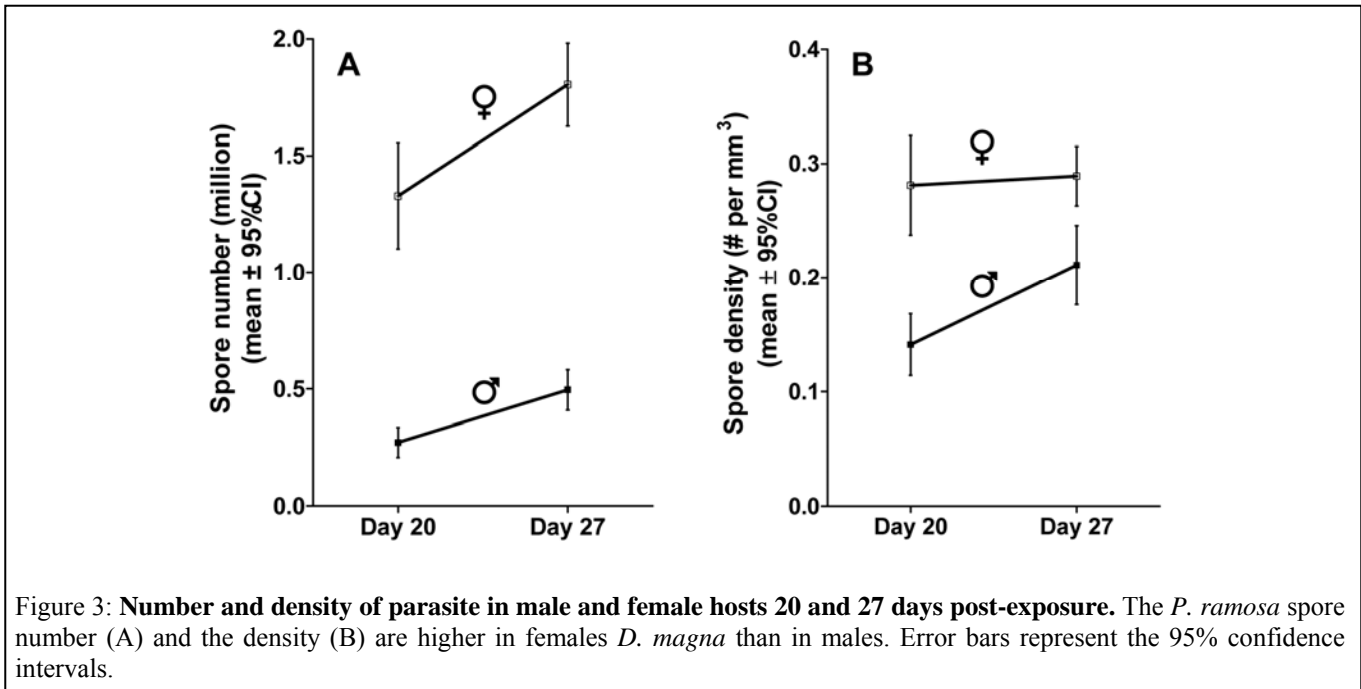
*Daphnia* passively capture *P. ramosa* spores from the water by filter feeding and larger animals have higher filtration rates. Thus, as females grow faster and to larger size they may take up more spores with the food than males. Although the effect of body size on the probability of *P. ramosa* infection is only correlational, the bias in parasites exposure due to sex size dimorphism has been already proposed to explain a part of the sex bias in infection rates in other animals [19]. Such difference in sex-specific encounter may lead to sex-specific parasite adaptations, as parasites might find itself and evolve more in the larger female than in male hosts even when the sex ratio is not biased. In *Daphnia* body size differences were also used to explain the general trend, that larger host species have more parasites [20,21].

#### Higher fitness in female hosts

We monitored lifespan of infected and control male and female *Daphnia* hosts (Experiment No. 2). There was no significant difference in premature mortality (before 14 days post exposure) between male and female hosts and between host clones

exposed to parasite (Two-way ANOVA [log(number of dead individuals before 14 days post exposure)]: *Host clone*,  $df=10$ ,  $F=2.5$ ,  $p=0.08$ , *Sex*,  $df=1$ ,  $F=1.41$ ,  $p=0.26$ ). Uninfected *Daphnia* of both sexes lived longer than their infected counterparts (for females, Log-rank test:  $n=274$ ,  $df=1$ ,  $\chi^2=125$ ,  $p<0.00001$ ; for males, Log-rank test:  $n=300$ ,  $\chi^2=230$ ,  $df=1$ ,  $p<0.00001$ , Figure 2). The costs of being infected appear to be the same in male and female hosts (Figure 2), with the median lifespan being reduced by about 50 % in both sexes. However, infected female *D. magna* lived about 1.5 times longer (median) than infected males (Figure 2). This difference alone is expected to influence the number of parasite cell divisions within the same host individual, and consequently, can increase the chance of the parasite adapting to females [22]. Sex differences in lifespan are quite common and can be extreme [in some Hymenoptera, 23, e.g. marsupial "mice" from the genus *Antechinus*, 24] and is expected to increase the likelihood of parasite sex-specific adaptation (Duneau and Ebert, submitted).

The parasite density was higher in females than in males, which suggests that female hosts tolerate the parasite better than males (Fig. 3B). Spore counts at 20 and 27 days post exposure were also higher in females (Figure 3A). This suggests that the rate of spore production in females was higher than in male in the first 20 days post exposure. As all animals were exposed to the parasite before sex differentiation took place, this difference is unlikely to be caused by differences in the number of spores being ingested by male and female hosts. Thus, we interpret these results as evidence for faster parasite proliferation in female than in male hosts during the parasite's exponential proliferation phase. During the later phase however, the differences in proliferation disappears (between the 20<sup>th</sup> and the 27<sup>th</sup> day post exposure) (Two-way ANOVA [log( spore number)]:  $n=142$ ; factor *Sex*  $df=1$ ,  $F=289.37$ ,  $p<0.00001$ ; factor *Day*  $df=1$ ,  $F=31.96$ ,  $p<0.00001$ ; interaction *Sex* x *Day*  $df=1$ ,  $F=1.62$ ,  $p=0.2$ , Figure 3A). At host death, females

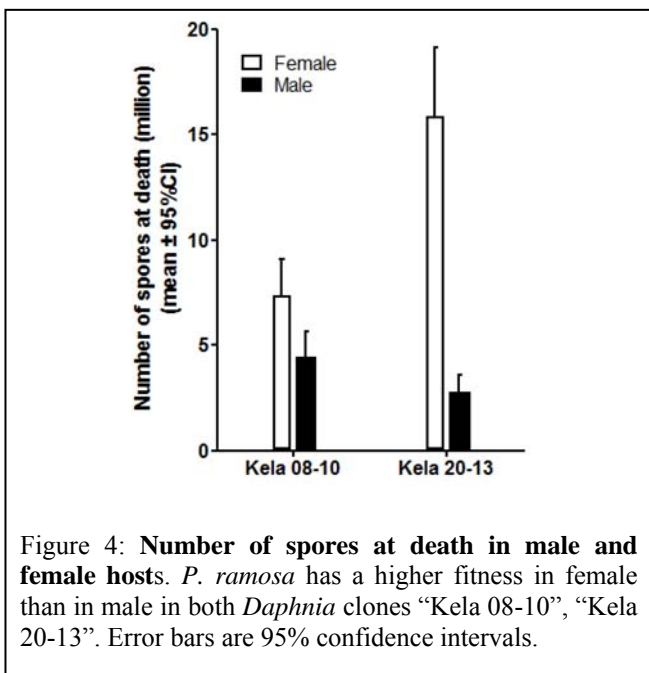


released more spores than males reflecting a higher parasite fitness in the female environment (Experiment No. 2, Clone “Kela-08-10”; Kruskal-Wallis rank test,  $n=49$ ,  $df=1$ ,  $\chi^2=6.2$ ,  $p=0.01$ ; Clone “Kela-20-13”, Kruskal-Wallis rank test,  $n=46$ ,  $df=1$ ,  $\chi^2=32.1$ ,  $p<0.00001$ , Figure 4). Thus, taken together, higher parasite fitness in the female environment may be explained by a combination of

the longer female lifespan (Figure 2), the higher spore production rate in the first 20 days of infection, and the larger female body size, which was shown to increase the carrying capacity up to which the parasite is able to produce spores [13].

#### Host gigantism

Female *Daphnia* are not only larger in general, they were also reported to exhibit gigantism, i.e. enhanced body growth, upon infection with *P. ramosa*. Parasite-induced host gigantism has been observed in diverse taxa, including mollusc, crustacean, vertebrate, and plant hosts and bacterial, fungal, and helminth parasites [25,26,27,28,29]. In the *Daphnia-Pasteuria* system, gigantism has been proposed as a parasite adaptation to increase the parasite’s lifetime reproductive success and, *per se*, the number of spores produced until host death [13]. Following this argument *Pasteuria* may be adapted to induce female gigantism to increase its carrying capacity. This may reduce the cost of rapid exploitation in the host. Here we tested the hypothesis that *P. ramosa* is adapted to induce gigantism in females but not in males. In two independent experiments, infected females were



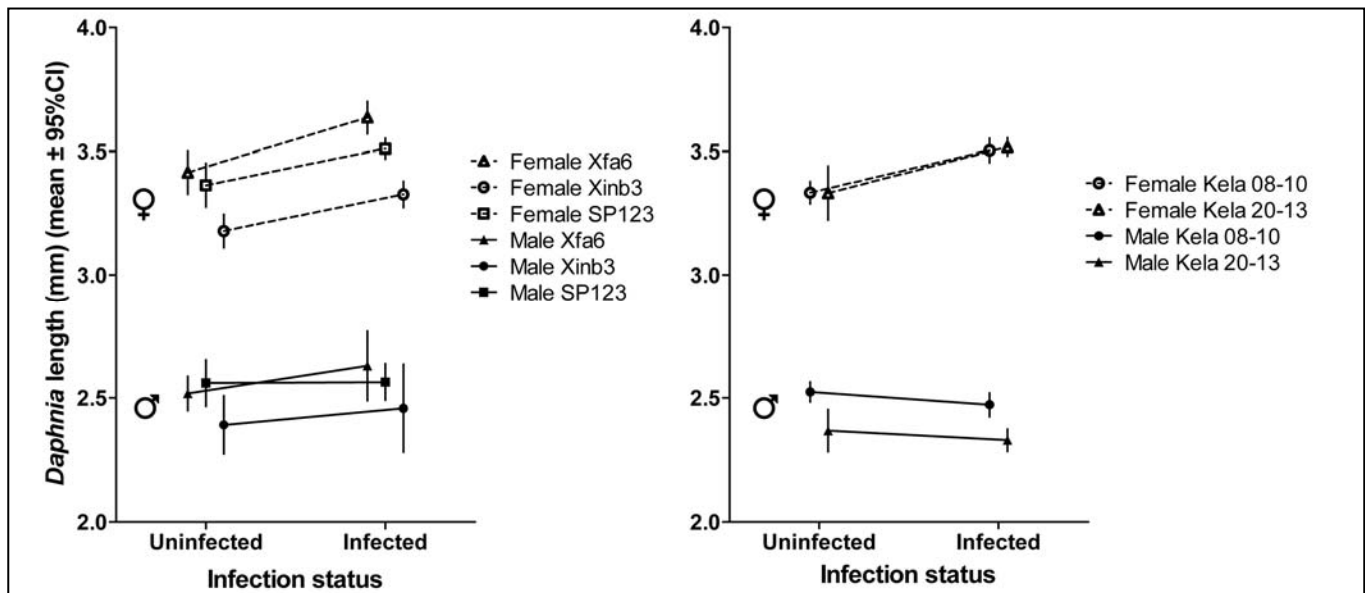


Figure 5: **Body length of infected and uninfected male and female host, 21 days post-exposure.** *P. ramosa* induced gigantism in female *D. magna* but not in males (see Table 2 for statistical results). Error bars show 95% confidence intervals.

larger than the uninfected females, confirming that gigantism is induced by *Pasteuria* in female hosts (Table 2, Figure 5). In contrast, the body size of the infected and of the uninfected male *Daphnia* were not significantly different (Table 2, Figure 5). This shows that *Pasteuria*, or at least the clone we worked with, is only able to increase the body size of female hosts. As a consequence of the absence of gigantism in males, the parasite density increased in

males during parasite proliferation (Welch's t-test:  $df= 61.67$ ,  $t=-3.23$ ,  $p=0.002$ ; Figure 3B), but did hardly so in females (Welch's t-test:  $df= 59, 03$ ,  $t=-0.29$ ,  $p=0.77$ ; Figure 3B). The rate at which parasite density increases is expected to correlate negatively with host lifespan, as it correlates negatively with the amount of host resources per unit of volume depleted by the parasite.

Table 2: **Summary of differences in body length between infected and uninfected female and male hosts.** Based on t-test, the parasite induced gigantism only in female hosts. The results for females remain significant ( $p < 0.01$ ) when corrected for multiple testing. The means of body length are represented in the figure 5.

	<i>Daphnia</i> clone	Sex	N	t-ratio	P-value
Exp. No. 2	Kela 08-10	Female	42	5.24	< 0.0001
		Male	38	-1.62	0.95
	Kela 20-13	Female	45	3.71	< 0.0001
		Male	34	1.34*	0.21
Exp. No. 4	Xfa6	Female	27	4.53	< 0.0001
		Male	27	0.95	0.35
	Xinb3	Female	27	4.73	< 0.0001
		Male	27	0.7	0.49
	SP-1-2-3	Female	27	3.74	< 0.0001
		Male	37	0.08	0.53

\* Result obtained with a "Welch's t-test" to control for unequal variances.

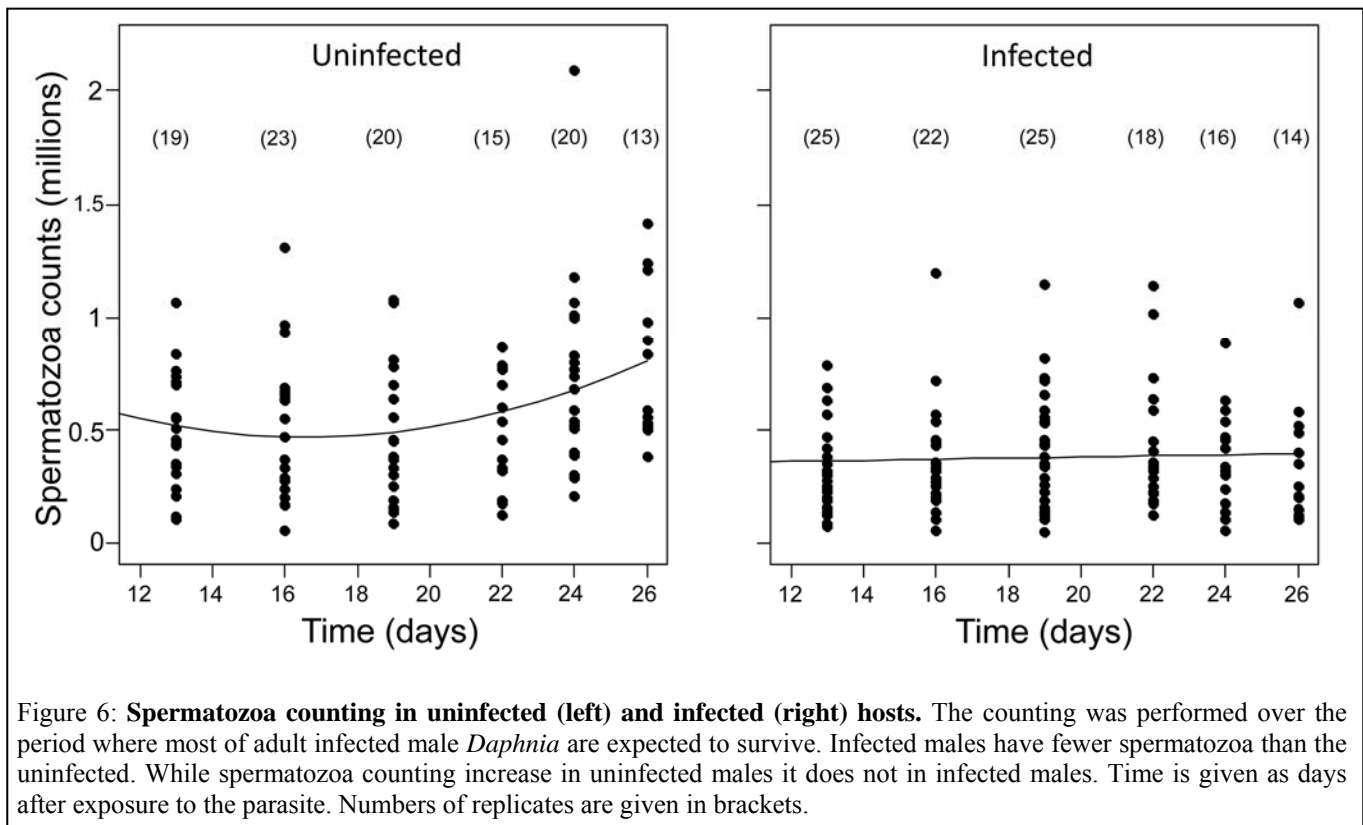


Figure 6: **Spermatozoa counting in uninfected (left) and infected (right) hosts.** The counting was performed over the period where most of adult infected male *Daphnia* are expected to survive. Infected males have fewer spermatozoa than the uninfected. While spermatozoa counting increase in uninfected males it does not in infected males. Time is given as days after exposure to the parasite. Numbers of replicates are given in brackets.

*Castration, a sex-specific adaptation of the parasite to induce host gigantism*

The mechanism by which *Pasteuria* induces gigantism is still unknown. Yet, previous studies proposed that gigantism of female hosts is a consequence of castration [12], including in our system [13]. The idea is that the resources that the host does not invest in germinal tissues are invested in somatic growth. We tested if the absence of gigantism in male host individuals was correlated with an absence of castration. All adult males were found to have spermatozoa, but infected males had significantly lower counts (linear regression controlling for variance due to the *Sampling day*; *Infection status*,  $df = 1$ ,  $F = 25.2$ ,  $p < 0.001$ , Figure 6). The presence of spermatozoa in infected male is possibly related to the fact that an important part of spermatozoa production happens early in development [30], and it might have taken place before the parasite gained control over the host. Spermatozoa counts increased with age for uninfected individuals (linear regression with

quadratic term,  $(Sampling\ day)^2$ ,  $df = 1$ ,  $F = 10.35$ ,  $p = 0.001$  and *Sampling day*,  $df = 1$ ,  $F = 3.39$ ,  $p = 0.07$ , Figure 6A), but not for infected individuals (linear regression, *Sampling day*,  $df = 1$ ,  $F = 0.05$ ,  $p = 0.82$ , Figure 6B). The absence of male gigantism despite the reduced investment in sperm cells is possibly related to the considerably lower investment in spermatozoa production in males compared to egg production in females. While female fitness depends largely on the quality and the quantity of eggs, male fitness depends on a trade-off between expenditure on ejaculate and expenditure on obtaining matings [31, p. 7 in 32]. While the castration of female hosts allows re-allocation of significant amounts of resources to somatic tissues (and may, thus, lead to gigantism), that of males has apparently no consequence for body size. Thus, gigantism seems to be a parasite adaptation selected for its effect on females but not males. In order to divert resources to male somatic growth, the parasite might gain more from reducing male activity (e.g. searching mates).

## Conclusion

We provide evidence consistent with our hypothesis that *P. ramosa* is specifically adapted to female hosts. The biased host sex-ratio associated with a strong sexual dimorphism may drive parasite specialization and lead to female-specific adaptations in the *Pasteuria* – *Daphnia* system. The adaptations may be neutral or even costly in the rare males. In our system the parasite has higher fitness in female host and host castration induces gigantism only in females. Sex-specific parasite adaptations have been observed in a few other systems (e.g. *Wolbachia* and *Sacculina*) but these adaptations are not explained with positive frequency dependent selection. To our knowledge, *Pasteuria* may be the first example of a parasite evolving specifically to the more common host sex. We also show genetic differences between male and female hosts do not explain parasite adaptation, but rather physiological differences among the sexes.

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## CHAPTER 5

### PRIMING OF A SHORT LIVED CRUSTACEAN WITH ITS NATURAL PARASITE DOES NOT VACCINATE IT

David Duneau, Dieter Ebert, Louis Du Pasquier

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#### Abstract

The potential higher resistance upon a second exposure to a parasite is the principle of vaccinations and has been intensively studied in both, vertebrate and invertebrate organisms. The likelihood for short lived organisms to encounter several times the same parasites being generally low, the existence of such an immune memory is currently debated. Previous studies were criticized because they were based on phenomena such as advantages in term of survival and reproductive capacity of challenged hosts, rather than on immunological criteria. Moreover most of experiments used non natural parasites. However, a specific memory in invertebrates that do not generate the repertoire of pathogen receptor somatically is expected to be selected in the germline across generations to resist against natural parasites. It could be less efficient against non natural parasites. Here, we investigated the possibility of vaccination of a short lived crustacean (*Daphnia magna*) by its natural bacterial parasite (*Pasteuria ramosa*) by recording the capacity of the host to reduce the parasite success. Using clonal hosts and parasites we tested whether a first exposure of the host to its parasite (“priming”), followed by clearing of the parasite with antibiotic, gives an advantage to the host after a challenge with the same parasite. Our approach included three experimental treatments: homologous challenge (first exposure and challenge with the same parasite clone), heterologous challenge (first exposure and challenge with distinct parasite clones), and naïve hosts (exposure of a host not previously exposed to any parasite). The experiment was replicated twice independently with two different host clones. The probability of host infection was not significantly different between treatments and parasite proliferation was similar between infected and control hosts. We conclude that there is neither memory nor better protection following a challenge in our system. We discuss the predictability of our results based on the lifespan of our organism and on the use of a natural parasite in such study.

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## Introduction and rationale of the experiments

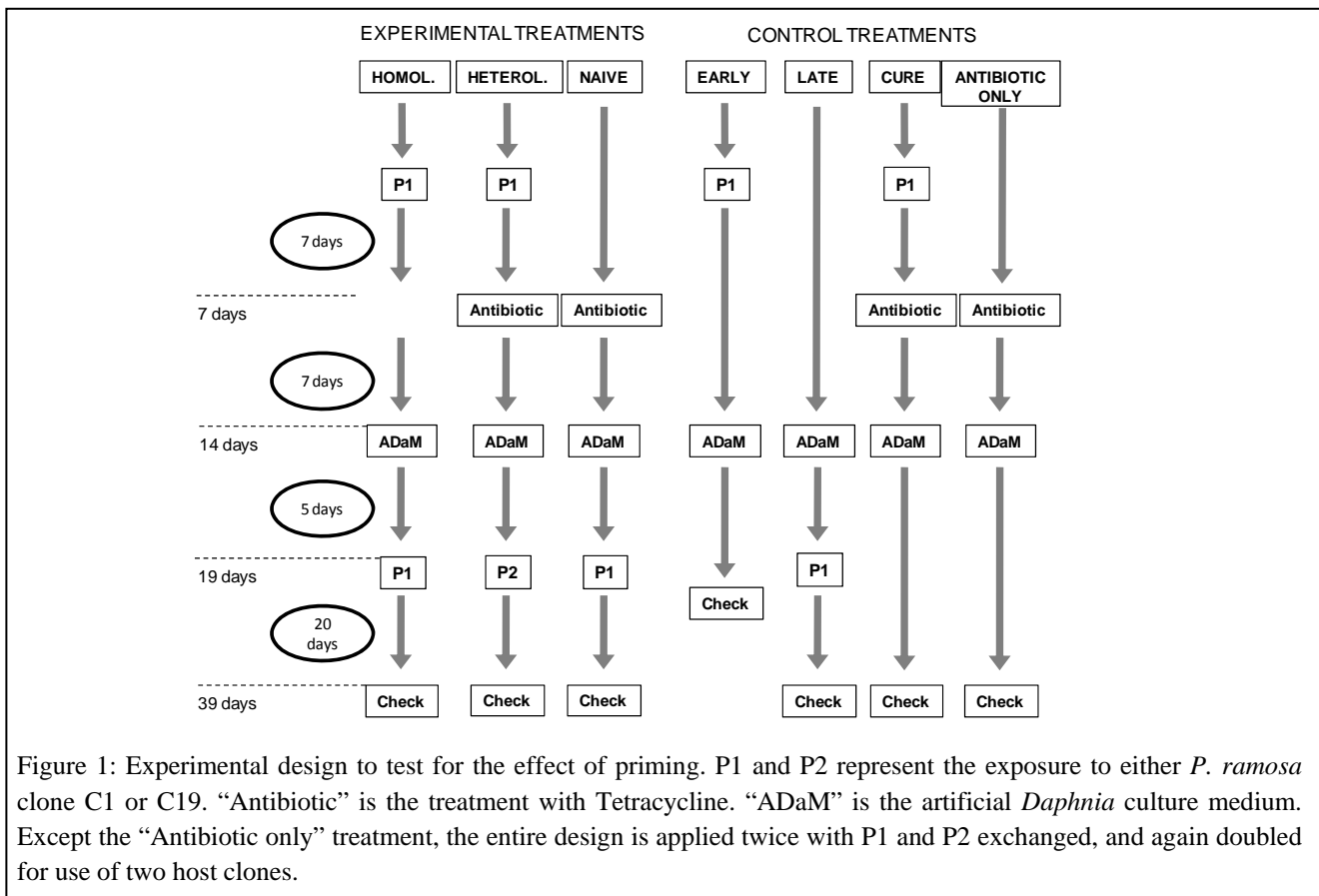
Higher resistance upon a second exposure to a parasite (challenge) is the land mark of many immune responses. In vertebrates, this is due to anamnestic typical specific “memory” based on lymphocyte proliferation. In other phyla, the phenomenon has been observed in some cases [1,2,3,4,5], but its specificity and its nature remain mysterious, not to say controversial [6,7,8]. The mechanisms behind this apparent vertebrate / non vertebrate analogy could imply different types of phenomenon with different implications: 1) a “long lasting response” (*i.e.* an initiated response which persists and is still ongoing at the moment of a second exposure), 2) a “leftover effectors” of a unique response (*i.e.* the activity of long lived effector molecules produced during a first response) and/or 3) a true memory (*i.e.* a second initiation of a response with transcription of immune factors and/or of a proliferation of specific cell populations). The presence of specificity and memory in the immune system of arthropods, sponges, echinoderms, and cnidarians, has been intensively studied. Prior studies investigated specificity and memory of the immune system by studying the recognition of graft either from the same individual (*i.e.* autograft, referenced as control in each experiment involving transplantations), from different individuals of the same species [*i.e.* allograft, 9,10,11], or from different species [*i.e.* xenograft, 12]. More recently, studies investigated the effect of the priming by parasites. However, the read out of these previous studies demonstrating memory in invertebrate organisms has been often criticized [7,8]. The conclusions on the existence of specific memory and the implicit analogy with the acquired response of vertebrates was blamed because they were based on phenomena such as advantages in term of survival and reproductive capacity of challenged hosts rather than immunological criteria.

The use of natural parasites is a crucial issue although neglected in previous studies. In

invertebrate organisms, the specific memory is expected to be based on a repertoire of molecules of recognition belonging to the innate immune system and, therefore, selected across generations to resist against natural parasites, unlike the acquired immune system of vertebrates whose somatically generated repertoire can in principle react with any pathogenic determinant. Thus, non natural parasites may not be (or poorly) recognized by the host repertoire of molecules of recognition. In addition, because natural parasites evolve with their host, they had the opportunity to develop adaptations to avoid to be recognized by the immune system.

Lifespan and social structures of organisms have impact on their likelihood to be exposed several times to the same parasite over time. These aspects are important to predict host adaptations and their specificity to recognize parasites. Among the most convincing studies using immunological parameters, the models are either long-lived [2] or eusocial insects [3], but investigations in short-lived organisms remain to be done with appropriate parameters and conditions.

Understanding better the mechanisms of immunity in non-vertebrate organisms would represent an advance in evolutionary biology but it could also have interesting economical consequences. Considering the economic importance of aquaculture, especially in crustaceans, and the constraints of high intensity cultivation, the understanding and controlling of the memory of the immune response is indeed crucial for developing vaccines. Therefore, it is capital to confirm (or infirm) and extend the earlier findings with more precision, *i.e.* with a better read out (immunity and/or parasite parameters), with natural parasites and under genetically defined conditions. Here, we propose to use the host crustacean *Daphnia magna* that live maximum 3 months under laboratory conditions and its natural parasite *Pasteuria ramosa* to investigate whether a parasite priming vaccinate the host.



In the crustacean *Daphnia magna*, it has been reported that offspring have a higher fitness when challenged with the same genotype cocktail of the parasites *Pasteuria ramosa* that their mothers than when they are challenged with a different cocktail [13]. This result suggested that adult *Daphnia magna* are able to develop specific memory and transfer it to their offspring. However, the participation of *bona fide* immunity in the process has never been tested. It is known that *D. magna* and *P. ramosa* coevolve in nature [14] and that their interaction is host genotype - parasite genotype specific due to the attachment of the bacteria to the host esophagus [15,16]. However, nothing is known about the specificity of the putative immunity. In fact this system is very suitable to test the hypothesis of specific memory in short lived crustaceans with a natural parasite.

## Results and discussion

We tested twice independently whether a first exposure of *Daphnia* to a parasite clone

(“priming”), followed by clearing with antibiotic, gives an advantage to the host after a challenge with the same parasite clone. Our approach included three experimental treatments: homologous challenge (first exposure and challenge with the same parasite clone), challenge (first exposure and challenge with distinct parasite clones), and naïve hosts (exposure of a host not previously exposed to any parasite).

Table 1: Infection records among the seven control treatments in the two experiments.

	Exposure sequence*	Infected/Total	Control group
Host clone: HO2	C1.Ø	14/14	early infection
	C19.Ø	15/15	
	C1.ab	0/14	cured
	C19.ab	0/15	
	Ø.C1	11/13	late infection
	Ø.C19	9/14	
	Ø.ab	0/15	antibiotic only
Host clone: Kela20-13	C1.Ø	14/14	early infection
	C19.Ø	15/15	
	C1.ab	0/13	cured
	C19.ab	0/13	
	Ø.C1	7/14	late infection
	Ø.C19	4/15	
	Ø.ab	0/14	antibiotic only

\* 1<sup>st</sup> exposure. 2<sup>nd</sup> exposure. “Ø” means no parasite exposure. “C1” and “C19” are the parasite clones. “ab” means antibiotic.

Table 2: Infection records among the experimental treatment groups in the two experiments.

Exposure sequence*	Host clone: HO2						Host clone: Kela 20-13					
	C1.C1	C19.C19	C1.C19	C19.C1	∅.C1	∅.C19	C1.C1	C19.C19	C1.C19	C19.C1	∅.C1	∅.C19
Infected/Total	17/29	10/31	14/31	11/31	13/31	12/31	20/34	14/32	18/33	13/32	12/25	9/29
Treatment	Homologous		Heterologous		Naïve		Homologous		Heterologous		Naïve	
Percent infected	45		40		40		51		48		39	
Logistic regression	df=2 deviance=0.4 p=0.82						df=2 deviance=1.22 p=0.55					

\* 1<sup>st</sup> exposure. 2<sup>nd</sup> exposure. “∅” means no parasite exposure. “C1” and “C19” are the parasite clones.

Contrary to previous studies on the immune system in invertebrates which involved injections of non-natural parasites [3,4] or cocktail of parasites [13], we exposed hosts, by the natural way (passive ingestion during filter feeding), using two distinct natural parasite clones. Because the immune system is an adaptation to stop infection and, upon infection, to limit parasite proliferation, we investigated the possible advantage of priming by evaluating the resistance to infection (proportion of infected individuals) and the reduction of parasite proliferation (number of spores). A difference between naïve and non-naïve exposures would reveal the increase in resistance due to the challenge. A difference between homologous and heterologous challenges would reveal specificity in relation to parasite genotype. A number of control treatments were included to make sure that each of the steps in the experimental procedure (early exposure, antibiotic cure, and late exposure) was effective. Figure 1 illustrates all the experimental and control

treatments. We used 36 individually-kept *Daphnia* for each of the experimental treatments and 15 for each of the control treatments.

Assessment of the infection rates in the control treatments shows that all the steps of the experimental procedure were successful: early exposure to parasites resulted in 100% infected *Daphnia*, and exposure to antibiotic resulted in 100% cured *Daphnia* (Table 1). For both host clones, there was no difference in the likelihood of infection between the three experimental treatments (Table 2), and no difference in the number of bacterial spores within infected hosts (Figure 2, ANCOVA, for host HO2, df= 2, F= 0.29, p= 0.75; for host Kela-20-13: df= 2, F= 0.05, p= 0.95, Figure 3). These results show that there is no memory in the immune response of *Daphnia magna* exposed previously to *Pasteuria ramosa*.

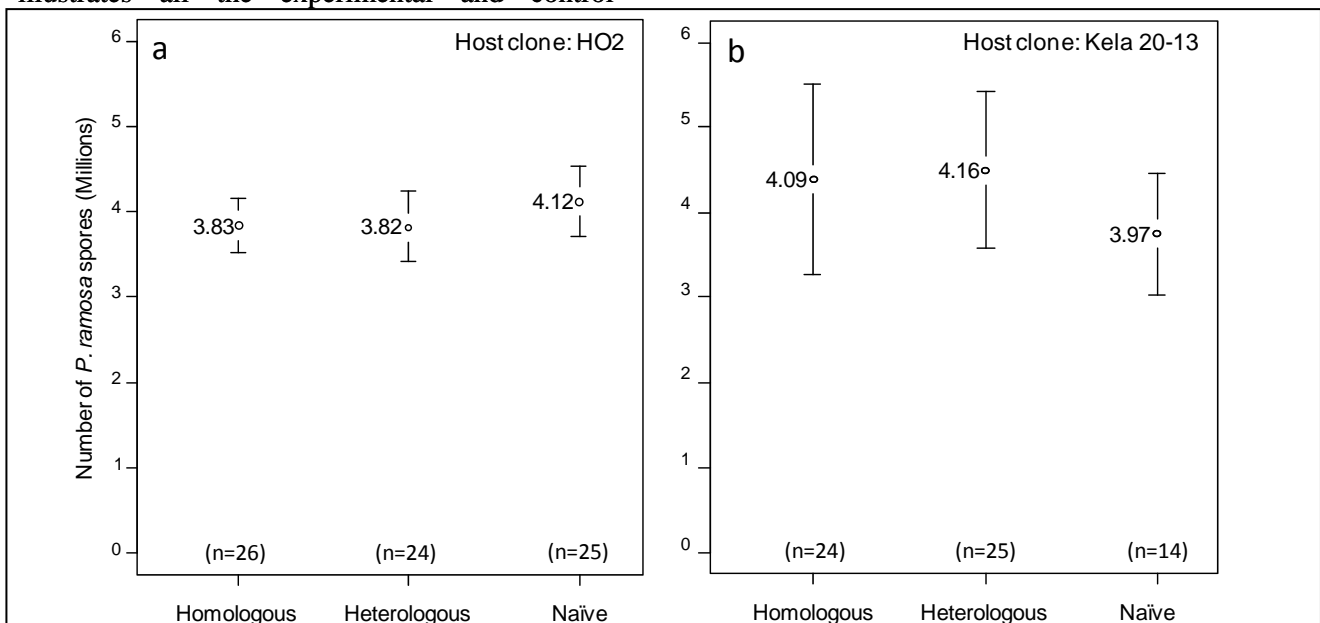


Figure 2: Number of *P. ramosa* spores after Homologous, Heterologous or Naïve treatment. Error bars represent 95% confidence intervals. Number of replicates per group is indicated between brackets.

The absence of difference in infection outcomes between naïve and non-naïve exposures reveals that the host is not more resistant when he already experienced the antigens. It is obvious that if the host is not able to “memorize” an exposure, no specificity can be expected as revealed by the absence of difference between the homologous and heterologous challenges. It is unlikely that our design failed twice to detect the differences. All control treatments revealed that every step of the experiment went as expected (Table 1). In addition, the complete overlaps of the rather small 95% confidence intervals show the robustness of the results (Figure 2). An inhibition of the immune system by the antibiotic treatment seems also unlikely as the groups “Naïve” and “Late infection” did not significantly differ in infection rate (Logistic regression, Host df= 1, Deviance = 3.52, p= 0.06; Antibiotic or not df= 1 Deviance= 3.38, p= 0.07, Table 1 and 2).

An immune response, in whichever organism, requires time. The kinetics of the response (initiation, unfolding, down regulation) may extend over weeks or even months in many species. The word “memory” is used only when the second response is initiated after the down regulation of the primary response [17]. Organisms living several years would probably profit from a specific memory functionally analogous to that of vertebrates because the likelihood to encounter the same parasite after a whole cycle of immune response is higher. Very promising models of long lifespan insects and crustaceans may reveal mechanisms of specific advantages of priming or of maternal challenges (e.g. cockroach and mealworm beetle, shrimps). However, it is possible that in many species, whose lifespan (say a few days or weeks) is shorter than the lifespan (say a month or two or more) of an average immune response, there will be no opportunity for such a “true memory response”. Therefore, the absence of specific memory is not surprising in short lived organisms such as *Daphnia*. A better protection (specific or not) following a second exposure might still be possible 1) because the first response is ongoing at the metabolic/cellular level (*i.e.* “long lasting response”) or 2) because the effector molecules produced during the active phase of the response

are long-lived (*i.e.* “leftover effectors”). In our experiment the time spent in the antibiotic and in “sterile” medium (12 days) was long enough to allow a comeback at the pre-challenge level of the immune system.

Parasites can often avoid host immune responses using a variety of strategies. Among such strategies one finds the mimicking the host “self” or the suppression the immune response. This can be direct suppression via the production of effectors targeting the immune effectors or indirect suppression via the disturbance of the host (neuro)hormones [reviewed in 18] regulatory networks. It is likely that *P. ramosa* uses such strategy to avoid *Daphnia* immune system. Chemical manipulation by *P. ramosa* has already been suggested to explain host castration [19]. Such secretion might interfere with the gamete production by disturbing the host hormones [20] and such a disturbance may have pleiotropic effects on the immune system [21]. Our treatment with Tetracycline stops bacterial growth by interfering with protein translation without killing bacteria [22]. Since no spores were found after the antibiotic control treatment (Table 2), *Daphnia* is able to eliminate the bacteria by means that remain to be elucidated. The arrest of protein synthesis by the bacteria correlated with the clearance of the bacteria, suggesting that the host is able to clear *P. ramosa* when inactive. This support the hypothesis that active *P. ramosa* is able to manipulate or avoid the host immune system. Such strategy would not be completely surprising as the avoidance of the host immune system is well known in a closely related bacteria species, *Bacillus anthracis*. This bacterium has the ability to produce antrax toxins, a non poisonous parasite-produced molecules, that are finely tuned to disarm the host’s immune response repertoire [23]. The identification of proteins responsible for both the castration and the eventual immune system alteration in *P. ramosa* will have to be the focus of future studies.

In summary, testing within one generation, our results show that there is neither memory nor better protection following a challenge in the short lived crustacean *Daphnia magna* against the natural bacterial pathogen *P. ramosa*. The

previous finding of an advantage of maternal priming, measured at the level of offspring, could be due to other factors than the transfer of specific immune factors.

We believe that the question whether the advantage of priming is an analogy of the memory as in vertebrates or a long lasting response needs to be investigated in non vertebrate organisms with a long lifespan looking at the presence of one or two immune activations after two challenges by a natural parasite.

### Experimental procedures

Our experiments were designed to answer the question of whether prior parasite exposure change resistance against a challenge. Additionally we tested whether resistance can be specific, in other words, whether a prior homologous exposure (i.e. challenged with the same parasite clone) is different as compared to a prior heterologous exposure (i.e. challenged with a different parasite clone). Three experimental treatments were designed in relation to which parasites the two *Daphnia* clones were exposed to: “Homologous” (Figure 1, HOMOL.), “Heterologous” (Figure 1, HETEROL.), and “Naïve” (Figure 1, NAIVE). We exposed each of two *Daphnia magna* clones (isofemale lines HO2 from Hungary, and Kela-20-13 from Finland) to each of two *Pasteuria ramosa* clones (C1 from Russia and C19 from Germany) identified as having different genotypes [24]. Offspring of 36 female *D. magna* raised under the same conditions, but singly in a jar, were divided over the 13 groups of the experiment (one offspring per group). Therefore, individuals within a group have a different mother and groups are identical among each other. Each experimental group consists in 36 individuals each, while each of the seven control groups consists in 15 replicates. During the experiment, *D. magna* was kept in standardized medium (ADaM) (Klüttgen et al. 1994; modified after Ebert et al. 1998) at 20°C, and fed daily with chemostat cultured unicellular algae, *Scenedesmus obliquus*. We provided 2.5 million algae cells daily per individual for the first three days, 3 million daily for the next four days,

and 5 million daily afterwards. The presence of infection was detected visually. The amount of spores per infected *Daphnia* was assessed using a Thoma counting chamber under a phase contrast microscope (Leica DM 2500, at magnification 400 x).

We exposed each of the 36 one-to-three-days-old *individuals*, to 50 000 spores of the same clone of *P. ramosa*. Each *Daphnia* was exposed separately to the pathogen (one *Daphnia* in 20 mL of water). Furthermore, 36 individuals were kept not exposed at that age (Figure 1, Experimental treatments). We used 15 additional individuals per parasite clone as controls for the infection treatment (Figure 1, “Early”). These controls were inspected for infection 22 days after exposure. Seven days after the first exposure, we treated *Daphnia* with a solution of Tetracycline (antibiotic) in 80 mL ADaM at 10 mg/l to kill the parasite from the first infection. *Daphnia* were treated during seven days and, because this antibiotic is sensitive to the light, the solution was changed every other day. Fifteen other individuals per parasite clones were used to test whether the Tetracycline cured the hosts properly from the first infection (Figure 1, “Cured”). Animals of this treatment were inspected at the end of the experiment to be sure that the parasite did not proliferate after removing 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 will not be active during the second exposure, we kept *Daphnia* during five days in antibiotic free medium. A second parasite exposure was done with 5 000 spores of the same clone of *P. ramosa* which was used for the first infection. These spores came from the same *P. ramosa* stock used for the first exposure. Because the *Daphnia* 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 amount of spores per infected individuals. We tested for the success of the second exposure with 15 additional individuals which were kept in ADaM as all other individuals (Figure 1, “Late”).

In order to study the specificity of a potential memory effect, 36 *Daphnia* individuals were challenged with a different *P. ramosa* clone in the first and in the second exposure (“Heterologous” treatments, Figure 1). This experimental design was carried out reciprocally with two *P. ramosa* clones. The entire experiment was repeated with a second host clone.

### *Statistical analysis*

All statistics were performed in R [25]. In each replicated experiment, to test whether the three experimental treatment groups differ in probability of being infected after the second exposure to the parasite, we conducted a logistic regression. We used a generalized linear model (GLM) with a quasibinomial error distribution and logit link. Assumption on the error distribution was checked by estimating dispersion parameters in GLM; and the slight overdispersion recorded with a binomial error distribution were corrected by using a quasibinomial distribution. In the model, we included as factors the clone of the parasite used in the second exposure (Parasite clone: C1 and C19) and the treatment (Treatment: Homologous, Heterologous, Naïve).

In each replicated experiment, we tested whether the three treatments differ in the proliferation of the parasite, we did an ANCOVA for each experiment. The number of spores was “log-transformed”, we included as factors the clone of the parasite used in the second exposure (Parasite clone: C1 and C19) and the treatment (Treatment: Homologous, Heterologous, Naïve). Normality and homoscedasticity of the residuals were checked. All control treatments produced the expected results. They were not included in the analysis.

### Acknowledgments

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## Chapter 6

### Summary, conclusions and perspectives

Host-parasite interactions are composed of a sequence of steps, all necessary for successful infection: parasites need to encounter their hosts, to enter into their bodies, and to proliferate within them. Selection will act on the mechanisms used in each of the steps; the parasite being selected to increase their efficiency, and the host selected to reduce it. I have proposed, and shown, that explicitly analyzing the factors that influence each of the steps and their impact on host and parasite fitness is of crucial importance for a complete understanding of host-parasite interactions. In my Ph.D. research work, I identified markers of different steps of the interaction between the host crustacean *Daphnia magna* and its natural bacterial parasite *Pasteuria ramosa*, and investigated factors influencing different steps, as well as the contribution of each of them to shaping the interaction between the two species.

I established that the infection of *Daphnia magna* by *Pasteuria ramosa* could be decomposed in at least five sequential steps (Chapter 1): 1) the encounter between the host and the parasite, 2) the activation of the parasite transmissible, resting stage, which happens once it contacts the host, 3) the attachment of the parasite to the host cuticula, 4) the penetration of the parasite into the host body cavity, and 5) the proliferation of the parasite within the host. The factors affecting the likelihood of encounter between host and parasite had been investigated before, in a study that revealed that there is a host genetic component, and polymorphism for the ability of the host to avoid encountering the parasite [1]. Resolving the interaction into its different steps and focusing on steps affect the encounter allowed me to see that: i) different steps are under the influence of different factors (Chapter 1), ii) the traits underlying some steps, but not all, do not seem to be polymorphic (Chapter 1), iii) the parasite genotype specificity of the success of the attachment step can explain the genotype specificity of the host susceptibility (Chapter 1), iv) the speed with which the parasite penetrates

the host body after attachment is crucial for the parasite success (Chapter 2), v) the molting, usually seen as a cost against parasite, can be beneficial to reduce the likelihood of infection, vi) once in the host body, the parasite will adapt to the environment that is characteristic of the most common host sex, here female characteristic (Chapters 3 and 4), vii) the success of proliferation of *P. ramosa* inside *D. magna* hosts is not influenced by previous host exposure to that same parasite (Chapter 5). All in all, I show that considering each of the steps explicitly provides new light into the mechanisms and selective pressures on hosts and their parasites. Each of the two interacting parties will, indeed, be under more or less strong selection to maximize their success at each of the steps. Below I will elaborate on this idea in relation to my specific findings and the research perspectives they open.

Thanks to a new method I developed, I was able to follow fluorescently labeled *P. ramosa* parasites from the moment they encounter the host to the moment they penetrate into its body cavity [2]. I showed that activation of the parasite endospore, the resting stage which is also the transmissible stage, correlates with a clear alteration of parasite morphology (Figure 1 in Chapter 1), and occurs regardless of host genotype, sex and species (Table 1, 2 and 3 in Chapter 1). These results suggest that the activation cue, *i.e.* whatever signal that the parasite might be detecting in the host after encounter, is phylogenetically conserved. Parasite activation occurs in the *Daphnia* phyllopod, the apparatus used to swim and collect food, which are covered by a mucus, presumably used to catch and degrade particles in suspension. I speculate that the activation is triggered by a very conserved molecule of the digestion of crustaceans. Whatever the molecular trigger, the apparent absence of polymorphism for this step (*i.e.* lack of differences between host individuals in whether they do, or do not, activate parasites upon encounter) implies that activation can be ruled out

as a major factor in the coevolution of hosts and parasites. Such non polymorphic steps in disease establishment can, however, be valuable targets for vaccine and drug development.

Once the activated parasite crosses the *Daphnia* mouth, it attaches specifically to the host esophagus. I showed that the success of this attachment correlates perfectly with the possibility of infection of a host genotype by a certain parasite genotype. The simple “attachment-test” I developed can rapidly and effectively detect whether parasites of different genotypes attach to whatever host (Duneau *et al.* 2011, Chapter 1) and has become the standard method in the Ebert Group to rapidly screen many *D. magna* genotypes for susceptibility to different *P. ramosa* genotypes in both, field and laboratory experiments (many unpublished studies). Parasite clones attaching to the esophagus do not attach anywhere else on the host cuticula. The reason for this specificity is still unknown, and further investigations on the specific properties of the esophagus are needed. Indeed, nothing is known about the actual mechanism of attachment of *P. ramosa* to the esophagus wall of *D. magna*. Because the passage of the water through the *D. magna* esophagus is very quick, and the bolus of food is constantly touching the attached parasites, it seems that the attachment should happen quickly and be strong. The strength with which the parasite attaches to the host is a trait for which there will likely be variation in the parasite population. I speculate that each microfiber of the parasite (Figure 1E in Chapter 1) carries specific receptors (one or several) able to recognize and bind to specific receptors on the host esophagus, and that the total number of specific receptors on the bacteria is responsible for the efficiency of the attachment to a susceptible host. Thus, the investment in the number of microfibers might be crucial for the success of the attachment but might also limit investment in other traits (*e.g.* for traits required for penetration). In addition, the range of host genotypes a bacterium can attach to may depend on the diversity of receptors at its surface. Therefore, there might also be a trade-off between being able to attach strongly to a few host genotypes (*i.e.* specialist strategy) and being able to attach poorly to many host genotypes (*i.e.*

generalist strategy). The selected strategy might be driven by host genotypic diversity in natural populations. In a monomorphic host population, the parasite with few kinds of microfibers/receptors but with great specificity to the common host genotype should be selected. On the other hand, in polymorphic host populations, parasites with microfibers of different kinds, even if less efficient to attach to any one specific host genotype, might be selected. Such a trade-off should be the focus of future studies.

The penetration of the parasite into the host body is a step which has never really been characterized in the *D. magna*-*P. ramosa* system. One available picture of the penetration of the congeneric *P. penetrans*, a parasite of root-knot nematodes, shows a tube connecting the bacterial spore and the host insides [3]. This observation, however, has not been repeated and it remains uncertain to what extent that tube is not some sort of an histological artifact from sample preparation. Hopefully, ongoing research on this species within the platform “*Pasteuria Bioscience*” (Alachua, USA) will clarify this (Liesbeth M. Schmidt, personal communication). Based on the morphology of the activated spores (Figure 1 in Chapter 1), I propose one hypothesis about the mechanism whereby *P. ramosa* penetrates into the body cavity of the host *D. magna*. Activated parasite endospores have a sombrero-like shape, with a central part which protects the cortex (*i.e.* the part containing the genetic material). The external parasporal fibers are the structure that actually attach to the host cuticula. Unlike the central part of the parasite spore, its parasporal fibers are covered with a dense layer of microfibers which are involved in the attachment to the host (Figure 1 in Chapter 1). When a bacterium attaches itself onto the host epithelium, the next step is to digest the host cuticula and transfer the cortex into the host body. For this, the central microfiber-free area might secrete enzymes, like chitinase, that digest the host cuticula and allow the penetration of the parasite cortex into the host, without digesting the cuticula where the microfibers are attached to. I have shown data suggesting that penetration takes about 12 hours to occur (see Chapter 2). The mechanisms that determine how long it takes for a

parasite to cross the host epithelium, whatever they are, are presumably under strong selection. Parasites able to do it faster will have an advantage if that reduces the chances of them being removed from the place of attachment before penetrating the host body where they can proliferate.

The periodic shedding of the cuticula, a process called molting, is characteristic of arthropods. I showed that such molting rids *D. magna* hosts of the attached *P. ramosa* parasites, and reduces the probability of infection, if it occurs before the parasite penetrates the cuticula into the host body cavity (Chapter 2). The ability to molt soon after parasite attachment could, thus, be advantageous for *D. magna*. Because it has been shown that parasites are associated to changes in timing of host molting in other systems, I tested whether *D. magna* individuals could speed their own molting when exposed to the virulent natural parasite *P. ramosa*. This seems not to be the case, at least for the host genotypes tested and in the condition the experiment was done. There are many reasons why this ability might not have been selected for in *D. magna*, including ideas about developmental constraints and about possible, counter-acting selective pressures. In *Daphnia*, molting is tightly connected to development and growth (in juveniles), and to reproduction (in adults when egg laying depends on molting). So, it is possible that there are plentiful constraints on changing the timing of such a crucial process. On the other hand, it is also possible that molting in the presence of parasites might be selected against. This would be the case if during molting individuals are somehow more vulnerable to infection by the parasites still in the medium. Even if parasite-induced reduction of the interval between molting events was not observed for *D. magna* exposed to *P. ramosa*, I believe this remains an exciting possibility, which might be effective in other host-parasite systems.

Once *P. ramosa* penetrates into the body cavity of its host, it proliferates in the host's hemolymph and musculature [4 and histological data not shown]. At the proliferation step, parasites presumably adapt to maximize the

effective use the resources of their most common host type. My work on infection across *Daphnia* species revealed that *P. ramosa* sampled from *D. magna* is able to proliferate inside *Daphnia dolichocephala* (Chapter 1). However, proliferation of *P. ramosa* from *D. magna* has been recorded only in this other *Daphnia* that is a non natural host species, and is phylogenetically closely related to *D. magna*. Further investigations in collaboration with Pepijn Luickjx showed that even though *P. ramosa* is able to attach to different *Daphnia* species, it actually does not proliferate in most of them. We showed that parasites isolated either from *D. longispina* or *D. magna* were able to proliferate only in their original host species, and not in any of the others species tested (Luickjx *et al.* in preparation). Thus, it seems that *P. ramosa* parasites infecting different species will probably never co-occur in the same host individual, where chances for exchange of genetic material are potentially higher. Because of this, our results suggest that *P. ramosa* might be a complex of species, each on one host species, rather than a unique species. Resolving the steps of the infection process allowed us to reveal that the ancient polymorphism for the trait involved in the step of attachment may allow a host shift. It also revealed that adaptation to proliferate in a particular host type (*i.e.* species) might have isolated parasite populations in different host species and resulted in parasite speciation.

As I have argued in chapter 3, adaptation for proliferation in particular host conditions can also take place at the level of intra-specific differences in the host population. The largest differences between two host individuals of the same population are generally those distinguishing males and females. I have proposed and elaborated on an argument of why parasites, even if horizontally transmitted, can be specifically adapted to one host sex (Chapter 3), and tested this idea empirically for the *D. magna*-*P. ramosa* species pair (Chapter 4). In nature, female *D. magna* are generally much more abundant than males. Consequently, *P. ramosa* sampled in *D. magna* should have evolved mostly in female hosts. This makes this interaction a very suitable model to test the existence of specific

parasite adaptations to the most common host sex. One of the major adaptations of *P. ramosa* in *D. magna* is its ability to increase the host individual's carrying capacity for parasite proliferation (via a significant increase in host body size, called gigantism). This gigantism, well documented to happen in infected females, happens via their castration. Presumably, the resources that the castrated host individuals do not invest in reproduction are used for growth [5]. I investigated whether *P. ramosa* was able to induce gigantism, via castration, also in male hosts. I found that while castration does occur in both infected male and female *D. magna*, it induces gigantism in females but not in males (Chapter 4). This suggests that the ability to induce host gigantism is a parasite adaptation tuned for proliferation in female, but not male, hosts. To my knowledge, this is the first explicit demonstration that, as I hypothesized in Chapter 3, horizontally transmitted parasites can be specifically adapted to hosts of one sex.

I also found that, exposed under certain conditions, female *Daphnia* tend to be more often infected by *P. ramosa* than males (Chapter 4). These data were collected from experiments where relatively old *D. magna* males and females were being exposed to the parasite. At these ages, we expect males and females to differ in many traits, body size being one of the most obvious (Figure 2 in Introduction of the thesis). The larger bodies of females are probably associated to higher water filtration rates and, consequently, to higher exposure to the parasite spores in suspension. *D. magna* males are also generally more reddish than females, a color that may be attributed to a difference either in hemoglobin or in carotenoid concentration. In 2008, I started a collaboration with Dr. Stephane Cornet (Dijon, France) to investigate whether male and female *D. magna* differ in carotenoid concentration. The role of carotenoids as antioxidants and immunostimulants is well established in vertebrates [e.g. 6] and suggested in invertebrates [7,8]. Thus, differences between male and female in carotenoid concentration could correlate with the differences in body color and in susceptibility to parasites. Although preliminary results did not show any differences (unpublished data), this

question would deserved further investigation with methods more sensitive to small carotenoid concentrations.

The fact that not all hosts to which parasites attach become infected suggests that parasite penetration or proliferation are failing. Host immunity is one of the key factors that help limiting or preventing parasite proliferation. It is well established that both vertebrate and invertebrate systems have some form of innate immunity mechanisms, with particular cell types dedicated to fighting off invading pathogens. However, it remains controversial whether immune specificity and memory against the parasites which are in fact encountered are properties exclusive of vertebrates [9,10]. There are a number of reports of immune specificity and memory (i.e. better immune response against a specific parasite type encountered previously) for a few invertebrate systems [11,12,13,14]. This includes the *D. magna* – *P. ramosa* system [15], which is often cited as an example of specific memory in invertebrate immunity [14,16,17].

In this thesis, I decided to revisit this topic by 1) incorporating new knowledge and tools which have become available, after the previous reports of immune specific memory, and 2) addressing the experimental shortcomings of previous studies (Chapter 5). The cloning of *P. ramosa* genotypes [18] allowed me to control for the genotype of the parasite, which is of relevance to address the specificity of the host response. I found no evidence for specific memory, or even for a long lasting protection in *D. magna*, upon repeated challenge with *P. ramosa* clones. This result suggests that there is either no specific memory in *D. magna* immunity, or that *P. ramosa* is adapted to avoid or disrupt it. Such a disruption could be, for example, an effect of the parasite-induced hormonal disruptions that are likely to underlie host castration (see above). Parasite adaptations to avoid or disrupt the host's immune system are likely to occur only when parasite and host co-evolve naturally. Thus, tests of immune specific memory that do not use natural host-parasite species pairs can be of limited value for investigating the potential of vaccination against natural parasites. In order to better understand the

importance of the different steps in shaping the *Daphnia magna*-*Pasteuria ramosa* interaction, I considered each step independently in different experiments. Even if their mechanisms are to some extent independent, because the different steps are part of the whole interaction, they are not expected to evolve fully independently. Obviously, the success of any step depends on the success of the step that precedes it, and only a sequence of successful steps will result in a successful infection. On the other hand, the optimization of any particular step might come at the cost of optimizing another, if there are trade-offs between them. In the interaction between *D. magna* and *P. ramosa*, both species have to deal with such potential trade-offs. I can speculate about different scenarios where this might occur. For example, the parasite needs to invest in a protective layer (exosporium) to survive in harsh conditions outside the host, but this layer also needs to open as soon as the endospore encounters the host. The attached parasite needs to secrete what are probably costly molecules to digest the host cuticula before penetrating into the host body, and once inside the host it needs to produce other costly molecules to, for example, castrate the host. Thus, the complete characterization of the interaction between a parasite and its host should involve the characterization of the investment that each of the two interacting parties allocates to each step. Because there will be many different strategies of investment that result in successful infection, I expect there to be genetic polymorphism for the host and parasite traits that underlie their interaction at each step. Such heritable phenotypic variation in each step is the raw material that natural selection can use to shape host-parasite co-evolution.

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## Curriculum vitae

### EDUCATION

- ☞ **June 2007 to present:** PhD Student in Evolutionary Biology, Zoological Institute in Basel, supervised by Dieter Ebert
- ☞ **2005-2006:** MSc in Evolutionary Biology and Ecology, University of Montpellier II
  - 2<sup>nd</sup> year: Biology Ecology Evolution (BEE)
  - 1<sup>st</sup> year: Biology Geosciences Agrosources Environment (BGAE), speciality Ecology Biodiversity Evolution (EBE)
- ☞ **2004:** Licence (year 3), Biology of Organisms, University of Montpellier II (France), speciality: Animal Biology
- ☞ **2003:** DEUG Life Sciences (year 1 & 2), University of Montpellier II (France) (options: Geology, Botany, Oceanography)
- ☞ **2001:** High School Diploma, speciality: Biology

### RESEARCH EXPERIENCE

- ☞ **October to December 2006:** Research assistant, CNRS. « Vector specialisation and the diversification of functional genes: the case of the seabird tick *Ixodes uriae* and the Lyme disease bacterium *Borrelia burgdorferi sensu lato*. » Employed by Karen McCOY, UMR CNRS/IRD 2724, Montpellier (France). (**Activity:** Characterisation of Osp genes in the bacterium *B. burgdorferi s.l.*).
- ☞ **June & July 2006:** « Divergence of vectors, micropathogen evolution and epidemiological consequences, the case of the bacteria *Borrelia burgdorferi s.l.* in the tick-seabird system », fieldwork in northern Norway (English-speaking environment) directed by Karen McCOY, CNRS/IRD, Montpellier (France). (**Activity:** reciprocal transplantation experiment, banding, blood sampling, resighting of color-marked seabirds).
- ☞ **January to June 2006:** Research for the Master 2. « Cascading host effects in vector-borne systems: an example of the bacterium *Borrelia burgdorferi s.l.* and the seabird tick *Ixodes uriae*. » directed by Karen McCOY, CNRS/IRD, Montpellier (France). (**Activity:** extraction and amplification of a housekeeping gene (flagellin)).
- ☞ **July 2004, July & August 2005:** « Study of the parasitic manipulation in the orthoptera (*Nemobius sylvestris*) - nematomorpha (*Paragordius tricuspidatus*) system », directed by Frédéric THOMAS, CNRS/IRD Montpellier (France). (**Activity:** field, experimental studies, breeding)
- ☞ **Spring 2005:** « Study of the impact of maternal parasitism on fitness in the gammarid (*Gammarus insensibilis*) - trematode (*Microphallus papillorobustus*) system », directed by Frédéric THOMAS, CNRS/IRD, Montpellier (France) (**Activity:** field, experimental studies, morphometry)
- ☞ **June & December 2004:** « Phylogeny of the arvicoline group with vWF gene », directed by Thomas GALEWSKI, Ph.D student of Emmanuel DOUZERY, ISEM, University of Montpellier II (France) (**Activity :** extraction, cloning, amplification and sequencing of gene for biogeography)
- ☞ **October 2003 to May 2004 (1 day / week):** « Study of parasitic manipulation in the Gammarid (*Gammarus insensibilis*) - trematode (*Microphallus papillorobustus*) system », directed by Frédéric THOMAS, CNRS/IRD, Montpellier (France) (**Activity:** field, experimental studies, spectrophotometry)

## PEER-REVIEWED PUBLICATIONS

- Published

- ☞ Luijckx P., Fienberg H., **Duneau D.**, Ebert D. (2011) *Resistance to a bacterial parasite in the Crustacean *Daphnia magna* shows mendelian segregation with dominance* Heredity Recently accepted
- ☞ **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 Biology 9:11
- ☞ Ponton F, Otalora-Luna F, Lefevre T, Guerin PM, Lebarbenchon C, **Duneau D.**, Biron DG, Thomas F (2011) *Water-seeking behavior in worm-infected crickets and reversibility of parasitic manipulation* Behavioral Ecology doi: 10.1093/beheco/arq215
- ☞ Gómez-Díaz E, Doherty P Jr, **Duneau D.**, McCoy KD (2010) *Cryptic vector divergence masks vector-specific patterns of infection: an example from the marine cycle of Lyme borreliosis.* Evolutionary Applications 3: 391-401. (cover photo)
- ☞ **Duneau\* D.**, Ponton\* F, Sanchez M, Courtiol A, Terekhin A, Budilova, EV, Renaud F, Thomas F (2009) *Effect of parasite-induced behavioral alterations on juvenile development.* Behavioral Ecology 20: 1020-1025 (\* authors contributed equally to the work)
- ☞ **Duneau D.**, Boulinier T, Gomez-Diaz E, Petersen A, Tveraa T, Barrett RT, McCoy KD (2008) *Prevalence and diversity of Lyme borreliosis bacteria in marine birds* Infection, Genetics and Evolution 8: 352-359
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- ☞ Ponton F, Lebarbenchon C, Lefèvre T, Biron DG, **Duneau D.**, Hughes DP, Thomas F (2006) *How parasitic Gordian worms cut the Gordian knot: a novel solution to predation upon the host.* Nature 440: 756
- ☞ Ponton F, Biron DG, Joly C, **Duneau D.**, Thomas F (2005) *Ecology of populations parasitically modified: a case study from a gammarid (*Gammarus insensibilis*)-trematode (*Microphallus papillorobustus*) system.* Marine Ecology-Progress Series 299: 205-215

## COMMUNICATIONS

- Talks (only when speaker)

- ☞ **Duneau D**, Luijckx P, Ben Ami F, Laforsch C, Ebert E *Zooming into interspecific interactions to better understand coevolution dynamics* (2011), ESEB 2011 Tübingen, Germany
- ☞ **Duneau D**, Luijckx P, Ben Ami F, Laforsch C, Ebert E *Partitioning the Infection Process of Parasites Reveals Striking Differences in the Contribution of Phylogeny, Genetics and Environment* (2010), Swiss-Russian Seminar, Fribourg, Switzerland
- ☞ **Duneau D**, Luijckx P, Ben Ami F, Laforsch C, Ebert E *Partitioning the Infection Process of Parasites Reveals Striking Differences in the Contribution of Phylogeny, Genetics and Environment* (2010) 16th EMPSEB, Wierzba, Poland
- ☞ **Duneau D**, Luijckx P, Ben Ami F, Laforsch C, Ebert E *Partitioning the Infection Process of Parasites Reveals Striking Differences in the Contribution of Phylogeny, Genetics and Environment* (2010) invited speaker Institut de Recherche du Developpement (IRD), Montpellier, France
- ☞ **Duneau D**, Luijckx P, Ebert E *Sex-specific adaptation of the parasite *Pasteuria ramosa* to its host *Daphnia magna**. (2009) 15th EMPSEB, Shoorl, Netherlands
- ☞ **Duneau D**, Luijckx P, Ebert E *Sex-specific adaptation of the parasite *Pasteuria ramosa* and female weakness of its host *Daphnia magna**. (2009) Annual meeting between host parasite group from Zurich (ETH) and Basel (university). University of Basel, Switzerland
- ☞ **Duneau D**, Luijckx P, Ebert E *Females and males, two different protagonists in host-parasite interaction, the example of *Daphnia magna* and *Pasteuria ramosa**. (2008) Research seminar, university of Basel, Switzerland
- ☞ Talk in Denmark invited for a PhD proposal by Dr. Boomsma's team. (2006) Center of Social Evolution at Copenhagen, Denmark

- Poster

- ☞ **Duneau D**, Luijckx P, Ebert E *Sex-specific adaptation of the parasite *Pasteuria ramosa* to its host *Daphnia magna**. (2010) Biology10, Neuchatel, Switzerland
- ☞ **Duneau D**, Luijckx P, Ebert E *Sex-specific adaptation of the parasite *Pasteuria ramosa* to its host *Daphnia magna**. (2009) 12th ESEB, Turin, Italy
- ☞ Ponton F, Joly C, **Duneau D**, Thomas F (2004) *The strange behaviour of 'crazy' gammarids: host manipulation or host collaboration*. Conférences Jacques-Monod, Roscoff, France

- Others

- ☞ **Scientific documentary** (52min) : « *Toto le nemato.* », Price Buffon 2008 « Festival Paris science »

## TEACHING EXPERIENCE and STUDENTS SUPERVISION

### ○ Teaching experience (in English)

- ☞ Teaching assistant, exercises in *Ecological and Evolutionary Genetics* (Fall 2009 and 2010). Level: Master, University of Basel. Time: 6h per year (1h every other week) creation of exercises [volonter]
- ☞ Teaching (tutoring), *Introduction to biology* (Fall 2009). Level: 1<sup>st</sup> year Bachelor, University of Basel. Time: 16h (2h every other week) + correction of students home work [volonter]
- ☞ Organisation of the interaction seminar for the zoological institute and Master student. (From fall 2009 to now) [volonter]
- ☞ Teaching assistant, *practical of Animal Biology (Embryology)* with Prof. Louis Du Pasquier (Spring 2009). Level: 3<sup>rd</sup> year Bachelor, University of Basel. Time: 12h [volonter]
- ☞ Teaching assistant, *practical of Animal Biology (Entomology)* with Prof. Dieter Ebert (Spring 2008). Level: 3<sup>rd</sup> year Bachelor, University of Basel. Time: 8h [volonter]

### ○ Training and supervision of undergraduate students (in English)

- ☞ **Ruder L** *Sexual selection in Daphnia magna*. 2<sup>nd</sup> year Bachelor (from January 2011 to now)
- ☞ **Ruder L** *Effect of a commonly parasite of female hosts on male hosts*. Level: 1<sup>st</sup> year Bachelor (from July 2010 to December 2010)
- ☞ **Supervisor of scientific projects** during a 1-month course focused on undergraduate research skills (Spring 2008, 2009 and 2010). Level: 3<sup>rd</sup> year Bachelor, University of Basel. Time: 60h every year
- ☞ **Hofer L.** *Effect of the temperature on the different steps of the infection process of Pasteuria ramosa, parasite of Daphnia magna*. Level: 3<sup>rd</sup> year Bachelor, University of Basel (1 month in 2010)
- ☞ **Eichin D.** *Specific immune system in Daphnia magna*. Level: 3<sup>rd</sup> year Bachelor, University of Basel (1 month in 2009)
- ☞ **Gygli S.** *Temperature tolerance of Octosporea bayeri, parasite of Daphnia magna*. Level: 2<sup>nd</sup> year Bachelor, University of Basel (1 month in 2009)
- ☞ **Hofer L.** *Temperature resistance of Pasteuria ramosa, parasite of Daphnia magna*. Level: 2<sup>nd</sup> year Bachelor, University of Basel (1 month in 2009)

## POPULAR SCIENCE (in French)

- ☞ De Dinechin D, Deguine JP, **Duneau D** (2006) *L'Homme de Florès. La découverte d'une nouvelle espèce humaine*. Annales de la Société d'Horticulture et d'Histoire Naturelle de l'Hérault 146 (2), 38-45
- ☞ **Duneau D**, Deguine JP, De Dinechin M, Blondel J (2006) *L'homme de Flores. Nanisme et gigantisme insulaire*. Annales de la Société d'Horticulture et d'Histoire Naturelle de l'Hérault 146 (3), 57-66
- ☞ Deguine JP, De Dinechin M, **Duneau D** (2006) *L'Homme de Florès. L'évolution de l'Homme et Homo floresiensis*. Annales de la Société d'Horticulture et d'Histoire Naturelle de l'Hérault 146 (4), 87-94

## REVIEWING WORK

- ☞ Animal behaviour
- ☞ BMC evolutionary biology