Specificity, genetics of resistance and eco-immunology in *Daphnia*-microparasite interacions

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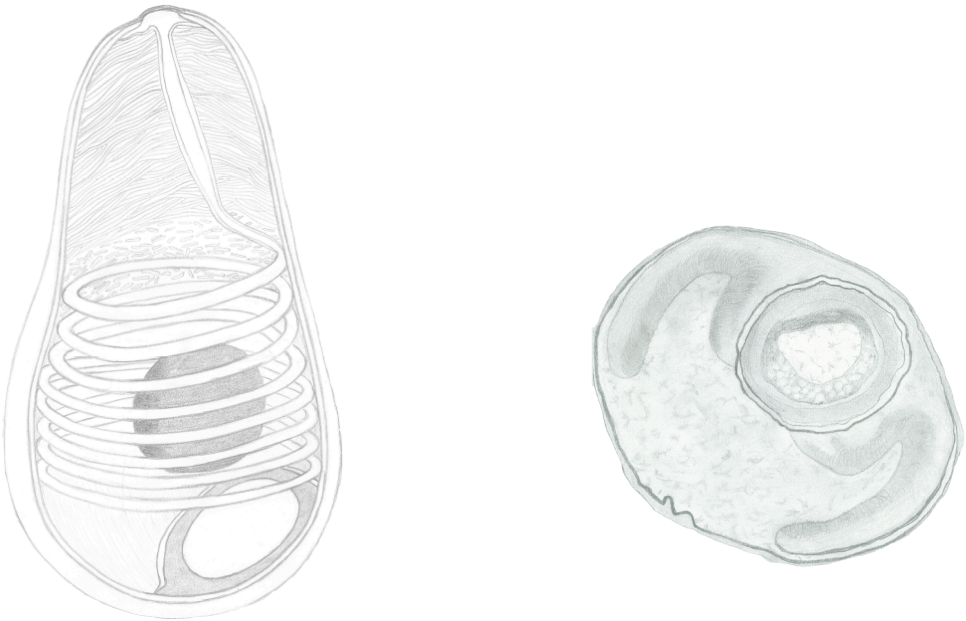

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Cover page, left handside :
Drawing of *Gurleya vavrai*. Drawn after microphotographs from Friedrich, Winder, Schaffler & Reinthaler (1996) European Journal of Protistology and with the help from the book by Wittner & Weiss (1999) « The Microsporidia and Microsporidiosis ».

Cover page, right handside :
Drawing of *Pasteuria ramosa*. Drawn after microphotographs by the Zentrum für Mikroskopie (ZMB) der Universität Basel from samples prepared by Sebastian Gygli (Zoologisches Institut, Universität Basel) and microphotographs from Duneau, Luijckx, Ben-Ami, Laforsch & Ebert (2011) BMC Biology

powaqqatsi: (from the Hopi language) sorcerer life, an entity, a way of life that consumes the life forces of other beings in order to further its own life.

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I.

Opening words

"Introduction & Outline"

Arguably the most common life strategy on earth is that of the parasite. The diversity of parasites is far greater than that of hosts (Dobson et al. 2008). Humans alone may be infected by more than 1'400 parasites (Cleaveland et al. 2001), among which about 300 species of helminths, 70 species of protozoa, 300 species of fungi, 500 species of bacteria and rickettsia, and 200 viruses and prions (Taylor et al. 2001). Infectious diseases accounted for 25% (14 million) of all human deaths worldwide in 2000 and remain among the leading causes of human morbidity and death globally (World Health Organization 2000, 2008). The burden of parasitism as a driving force of selection on host populations, with special reference to human evolution, was first proposed by J.B.S. Haldane (Haldane 1949), one of the founders of population genetics (Dronamraju 2004). In response to the strong selection imposed by parasites on their hosts, hosts have evolved resistance in the form of their various immune systems. Parasites have responded in like by evolving further ways of infecting hosts. This principle of reciprocal evolution, where species impose and respond to selection on each other, is known as co-evolution (Janzen 1980). Co-evolution of hosts and parasites has been suggested, and in certain cases found, to provide the explantory mechanism for a wide range of phenomena such as local adaptation, but also local maladaptation (Gandon 2002), speciation (Kawecki 1998), the maintenance of polymorphism (Frank 1993), community structure (Hudson et al. 2006), the evolution of sexual reproduction (Jaenike 1978; Hamilton et al. 1990), and species invasions (Prenter et al. 2004). The relationship between parasites and their hosts is thought to be largely governed by the specificity of their interaction (Lambrechts 2010). Parasites with low specificity may have a broad host range in which they may infect phylogenetically unrelated hosts (Antonovics et al. 2013). *Encephalitozoon cuniculi*, one of the few microsporidian species to have been observed infecting mammals, has a very broad host range infecting unrelated species found within the orders lagomorpha, rodentia, insectivora, carnivora, primates, and perissodactyles (Canning and Hollister 1987). Such parasites may be of little consquence for the evolution of specific host resistance because they are unlikely to be tightly co-evolving with any one particular host species. Such intimate

co-evolutionary relationship would lead to specialisation of the parasite on the host, thereby reducing the parasite's host range. *Nosema lymantriae*, and *Vairimorpha disparis* are two examples of microsporidia with a very narrow host range that are thought to infect only one host species, the moth Lymantria dispar (Solter et al., 2010). Very specific parasites, are usually thought to be more likely to co-evolve with their hosts. In **Chapter II** of this thesis, we investigated the specificity of a poorly studied microsporidian parasite of *Daphnia*: *Gurleya vavrai*. This parasite was first described infecting *Daphnia longispina* in two locations in England. Although it has not been much studied yet, most of the few studies using this parasite have observed it to infect *D. pulex* (Friedrich et al. 1996; Stirnadel and Ebert 1997). We investigated the host-range of *G. vavrai* by exposing a range of putative hosts to it. We thereby address the questions of the breadth of *G. vavrai*'s host-range, asking whether it is a specialist similarly to *N. lymantriae* or *V. disparis* or whether it has a broader host-range within the *Daphnia* genus. We discuss our results in light of the theory of host-ranges and explain how both local adaptation and phylogeny may play a role in shaping the specificity of *G. vavrai* to its hosts. Parasite host-ranges are thought to be driven by 'non-host resistance' and 'co-evolved resistance' or a mix of both (Antonovics et al. 2013). Non-host resistance describes the observed tendency of parasites to be very successfull at infecting a target host, and with decreasing host relatedness from that target host be less able to infect hosts. For this principle, phylogeny is the explanatory component. In co-evolved resistance, the parasite and the host must meet frequently enough so that each of them responds to the selective pressure imposed by the other by evolving traits to cope with each other. By encountering its parasite frequently enough, the host will evolve resistance traits. In turn the parasite, encountering this particular host frequently enough, and more often than other hosts, will evolve infectivity for it, and thereby counteract the evolved resistance of the host.

In a co-evolutionary host-parasite interaction, particularly, the host-parasite interaction is determined by the genetic architecture of traits pertaining to infection and resistance of hosts and parasites (Lambrechts et al. 2006). Therefore characterizing the genetic architecture of host resistance may shed light on the

mechanisms of infection and resistance between both the parasite and the host, but most importantly it may help in understanding the type of co-evolutionary dynamics involved (Lambrechts 2010).

Co-evolving hosts and parasites are generally thought to adopt two main co-evolutionary dynamics : directional co-evolution or cyclic co-evolution. These two types of antagonistic co-evolution differ in many respects, such as the time scale at which change can be seen, their mechanism and the underlying genetics. Directional co-evolutionary dynamics are characterized by the successive appearance of new beneficial resistance traits in the host population or beneficial infection traits in the parasite popultation (Woolhouse et al. 2002). In other words, the host evolves new defense or avoidance mechanisms and the parasite responds in kind by evolving new infection mechanisms. In such directional co-evolutionary dynamics, selective sweeps may be observed, where successive resistance alleles or infectivity alleles appear in the host or parasite populations respectively and rapidly go to fixation (Woolhouse et al. 2002). A constant increase in resistance of the host and in infectivity of the parasite is observed (Buckling and Rainey 2002) although, as this process relies on the arisal of new beneficial mutations, it is a slow process and change may only be observed over long periods of time (Ebert 2008).

Cyclic co-evolutionary dynamics arise when traits of the host-parasite interaction are under negative frequency-dependent selection. In a negative-frequency dependent selection system, rare host genotypes have a selective advantage, as they are less frequently parasitized than common host genotypes. Under negative-dependent selection, host alleles for resistance and parasite alleles for infectivity are selected for when they are rare, but selected against when they are common. Negative frequency-dependent selection leads to co-evolutionary cycling of host and parasite alleles for resistance and infectivity. Such co-evolutionary dynamics are also called Red Queen dynamics, in reference to Lewis Carroll's Alice in Wonderland, in which the Red Queen said to Alice « It takes all you can run to stay in the same place ».

The determining factor leading either to directional co-evolution or cyclical co-evolution is the underlying genetic interaction. Two classes of models of genetic

interaction between hosts and parasites are commonly used to describe co-evolutionary dynamics : matching-allele models (MAM; Frank 1993), and gene-for-gene models (GFG; Thompson & Burdon, 1992). In a GFG, the parasite may recognize the host and successfully infect it if one of its ligands is able to recognize at least one of the multiple receptors of the host. Similarly the resistant host needs only to recognize one of the parasite ligands to mount a successfull immune response. Therefore, a given parasite genotype is able to infect a broad range of host genotypes, although not all of them with the same efficiency. Similarly host genotypes may be susceptible to a large range of parasite genotypes (Frank 1992). Evidence from natural host-parasite systems for GFG models of genetic interaction are many and come principally from plant-parasite systems (Flor 1971; Thompson and Burdon 1992).

In MAMs, infection genetics are based on the idea of a unique matching between effectors and receptors, where successful infection requires a specific match between the parasite and the host genotypes (Frank 1993). Only a small subset of host genotypes may be successfully infected by a particular parasite genotype (Frank 1993). In contrast to GFG models, MAMs preclude the existence of universal parasite infectivity or universal host resistance and they lead to co-evolutionary cycling of alleles for host resistance and parasite infectivity without the need to invoke further mechanisms to explain the observed cycles (Sasaki 2000; Agrawal and Lively 2002). Empirical support for this class of models is still scarce. In **chapter III** of this thesis, we build upon previous work by Luijckx et al. (2011, 2012, 2013) to pursue the description of the genetic architecture of resistance in the *Daphnia*-*Pasteuria* host-parasite system. We produced three genetic crosses of the host, in which we crossed a parent resistant to two clones of the parasitic bacteria *Pasteuria ramosa* to three other parents, that had different phenotypes for resistance to these two parasite clones. One parent was susceptible to both parasite clones, another parent was susceptible to one but resistant to the other parasite clone, and the last parent had the opposite resistance phenotype. Previously the presence of 'double resistant' hosts in nature was not explained and seemed to contradict the proposed model of genetics of resistance for this system (see Luijckx

et al, 2013 and **chapter III** for details). Our results confirm the suggestion of Luijckx et al. (2013), that a MAM is the likely underlying genetic model in this system. We could further reject one of the previously proposed genetic architectures for resistance, a one loci model (Luijckx et al. 2013). The second proposed genetic architecture for resistance, a two loci model with two alleles for each loci, could not explain the existence of double resistant hosts (Luijckx et al. 2013). Modifying this model to incorporate a third loci with two alleles allowed us to explain double resistance. Furthermore, and most notably, our study brings the first empirical evidence for negative epistasis between linked loci, a key requirement of MAMs. *Daphnia-Pasteuria* is possibly the first host-parasite system in which all the conditions for MAM and Red Queen dynamics have been demonstrated (strong to extreme genotype-by-genotype interactions Carius et al. 2001; Luijckx et al. 2011; absence of costs of resistance Little et al. 2002, **chapter IV** of this thesis; simple genetics Luijckx et al. 2012; closely linked loci Luijckx et al. 2013, Routtu & Ebert in prep, **chapter III**; negative epistasis **chapter III**).

While MAMs readily lead to cycling of host and parasite alleles for resistance and infectivity respectively, without the need of other mechanisms maintaining such cycles (Frank 1993), GFG models lead to directional selection (Thompson and Burdon 1992). However, when resistance is a costly trait, GFG models may also lead to co-evolutionary cycles and thereby maintain polymorphism for host resistance (Sasaki 2000; Agrawal and Lively 2002). Therefore misinterpretation of the absence of selective sweeps or co-evolutionary cycles as indications of an underlying MAM may arise without prior knowledge of the presence or absence of costs for resistance traits. Evidence for cycling and costs of resistance simultaneously suggest an underlying GFG. Respectively, the absence of costs for resistance strengthens the likelihood of a MAM. Therefore costs of resistance are one of the key differences between the MAMs and the GFGs in cycling co-evolutionary host-parasite dynamics. In **chapter IV**, we competed pairs of hosts of known genetic architecture for resistance and of different resistance phenotype (i.e. susceptible or resistant) in absence of the parasite and looked whether the susceptible hosts had a fitness advantage and resistant hosts endured costs of being resistant. As we could not find

any effect of the resistance phenotype on the outcome of the competition assays, this chapter does not bring evidence that may question the conclusions of **chapter III** and concurs with several other studies that could not find indications for costs of resistance in this system (Little and Ebert 2000; Little et al. 2002; see Labbé et al. 2010 for a discussion). While the absence of an observation is not a proof of the absence of the phenomenon of interest, only through the accumulation of studies finding no evidence can we increase our confidence in the absence of that phenomenon. In addition to our study and that of Little et al. (2002), which both specifically looked for evidence of costs of resistance in the *Daphnia magna*-*Pasteuria ramosa* host-parasite system, further studies that were not specifically looking for costs of resistance, but due to the design of their experiments could have observed indications consistent with costs of resistance but did not, increase our confidence that costs of resistance to *Pasteuria ramosa* are absent in *Daphnia magna* or so low that they are undetectable by measuring fitness related traits. Therefore our study along with others, do not invalidate the proposed MAM underlying the genetics of resistance in the *Daphnia magna* – *Pasteuria ramosa* host-parasite system.

Finally, **chapter V** deviates somewhat from the notion of host-parasite specificity, whether broad or fine, but uses the 'mechanistic specificity' of the host-parasite interaction between *D. magna* and *P. ramosa* to explore structural resistance of the parasite spore. In this chapter, we exposed dormant spores to different temperatures and later looked whether these treatments had affected the parasite's ability to infect the host by examining the several steps of the infection process. Namely we looked whether the parasite retained its ability to detect the host and activate (shedding its exosporium and thereby deploying its parasporal fibers that will enable it to attach in the next step), whether temperature inhibited the parasite's ability to attach to the host, and finally whether it remained infectious by looking if hosts became diseased and new spores were produced. The most remarkable result was that the parasite appears to be tailored to resist temperatures that the host could endure and still remain perfectly infectious (no effect at all on any of the steps of the infection process at these temperatures) but

that at higher temperatures, the parasite started to sustain damages from the heat treatments. Furthermore, we explore the relationship between structure, as inferred from model organisms such as *Bacillus subtilis*, and resistance to heat in order to understand better the major steps of the infection process of *P. ramosa* and the biology of this highly virulent and potentially very prevalent parasite of *Daphnia magna* (Stirnadel and Ebert 1997; Carius et al. 2001; Duncan et al. 2006).

Hosts

Throughout the chapters of this thesis several species of hosts have been used. Principally *Daphnia magna* was used in **chapters II, III, IV** and **V**. In **chapter II**. a range of hosts were used in testing the host-range of a poorly studied microsporidian parasite. The main hosts of interest in this chapter were *D. pulex*, *D. pulex arenata* and *D. longispina*, but it also included *D. magna, D. barbata, D. lumholzi* and *D. galeata*.

Daphnia are freshwater planktonic crustaceans (Phyllopoda: Cladocera) that inhabit small temporary water bodies, such as rock pools or ponds, to large permanent lakes. Their size varies between species with the largest, *D. magna*, reaching 5 mm bodylength. Many *Daphnia* reproduce by cyclic parthenogeny induced by environmental cues (5 of the species we used do, one, *D. pulex*, is subdivided into two lineages, one obligate parthenogen and one cyclic parthenogen). During beneficial periods *Daphnia* reproduce asexually producing clones and when environmental cues, such as the photoperiod, indicate disadvantageous conditions for *Daphnia*, they will produce males and reproduce sexually. The outcome of the sexual reproduction are highly resistant resting eggs, called ephippia, that can lay in the sediment for decades until environmental conditions are restored and then hatch. Resting eggs release one to two female offspring, which after a few days (variable from species to species and also dependent on temperature and food availability) will already be able to produce their first asexual clutch. Clutches are variable in size, depending on the species but also on the quality and availability of food and on the quality of the environment.

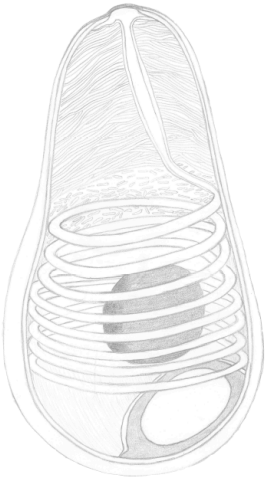
Parasites

Gurleya vavrai (**Chapter II**)

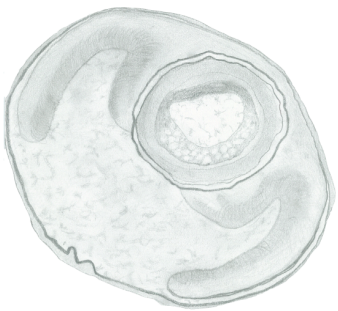
Gurleya vavrai Green, 1974 is a microsporidium endoparasite of Daphniids. It was described by Green in his seminal paper on parasites and epibionts of Cladocera (Green 1974). Originally sampled in *Daphnia longispina* in Hertfordshire and in Norfolk, England, in 1971 and 1972, it has since been described infecting *D. pulex* by various authors (Stirnadel & Ebert 1997, Friedrich et al. 1996, and see **chapter II** of this thesis). *G. vavrai* forms oval and slightly pear-shaped spores. Spores lay in the sediment of freshwater bodies, where filter-feeding and deposit feeding *Daphnia* pick them up and ingest them. Upon ingestion, the spores recognize cues from the host and they germinate. Germination leads to the hydration of the spores, the inflation of their vacuole and finally ends with the eversion of the polar tube that pierces by mechanical action through the epithelium of the host into a host cell. As all described microsporidia, *G. vavrai* is an obligate intracellular parasite. Instantly following the extrusion of the polar tube is the expulsion of the spore contents through the everted tube into the host cell. There the microsporidan cell will go through two developmental phases (the proliferative phase and the sporogonic phase) leading to the production of new spores that will be released into the environment after host death by the decaying host body. *G. vavrai* infects the hypodermis of the host (Green 1974; Stirnadel and Ebert 1997). Symptoms are visible with the naked eye as small white masses, cloud-like, that appear at the foci of infection throughout the body. As the infection spreads, the whole body becomes cloudy white and at last, shortly before host death, propagules spread to the head of the host and it too becomes cloudy white. When the host's body surface is 60-100% infected, estimated by the coverage of white spore masses, host death occurs. *G. vavrai* reduces strongly host fecundity and shortens host lifespan, therefore it has the potential to inflict strong selective pressure on hosts (Stirnadel and Ebert 1997; Little and Ebert 1999). (See microphotographs in the Appendices, page 132)

Pasteuria ramosa (**Chapters III** through **V**)

Pasteuria ramosa is a Gram-positive bacteria from the *Bacillus-Clostridium* clade (Firmicutes:Pasteuriaceae). It is an endospore-forming parasite of the body cavity of *Daphnia*. Highly resistant transmission stages, spores, of the bacteria lay in the sediment (sometimes for decades) of the water bodies inhabited by the host. Upon filter-feeding or deposit-feeding the host picks up the bacteria and ingests them. During this process *P. ramosa* recognizes cues from the host and activates by shedding its exosporium and deploying its parasporal fibers. During passage through the feeding host's oesophagus *P. ramosa* attaches to the oesophagus cuticle, provided the host is susceptible. After attachment, the spore is thought to germinate and grow an infection peg that pierces through the host epithelium into the host body cavity. The bacteria injects itself into the host body cavity, where it will go through outgrowh and vegetative growth producing millions of daughter cells. After some time, triggers likely induce sporulation, and new transmission stages are produced. Finally, the host dies and millions of spores are released into the environment by the decaying host body (Ebert et al. 1996; Duneau et al. 2011). *P. ramosa* may exert a strong selective pressure on the host as it castrates and shortens host lifespan (Ebert et al. 2004). (See microphotographs in **chapter V**, p. 118)



Gurleya vavrai



Pasteuria ramosa

(Drawings based on microphotographs and textual descriptions, see page 4 for details)

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II.

Specificity of host-parasite interactions on a broad scale:

"Host-range of the microsporidium *Gurleya vavrai* from a Daphnia meta-population."

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Abstract

Background: *Gurleya vavrai* is a microsporidian parasite of Daphnia (Crustacea: Phyllopoda). Due to its apparent high infection rate and virulence, it is expected to play an important role in the population dynamics of its host community. Experimental data on its host range are not available. We present here a 5-year field survey of a 3 species Daphnia metacommunity, combined with infection trials under controlled conditions.

Methods/Principal Findings: In total 854 field samples were tested for the presence of *G. vavrai*. Only in 11 populations of *D. pulex* the parasite was detected, never in *D. magna* or *D. longispina*, although the later is a recorded host of this parasite at other places. We exposed clones from various species of Daphnia to the parasite. We find that *Daphnia pulex* and *D. longispina* are susceptible to *G. vavrai*. The fully sequenced clone of the closely related North-American *D. pulex arenata* (TCO) was also susceptible. In contrast, phylogenetically more distant Daphnia species were not susceptible to the parasite.

Conclusions: Our findings reveal that two of the three most frequently found hosts in the Daphnia metapopulation of the rock pools of the Baltic Sea from which the parasite originated are susceptible and broaden its host-range to include *D. pulex arenata* and the asexual form of *D. pulex* from the Baltic Sea metapopulation, which likely has origins in North America. We discuss a possible link of host phylogeny, local adaptation and host-parasite co-evolution to host susceptibility to *G. vavrai*.

Introduction

Parasites are important players in ecological and evolutionary processes and may play a major role in shaping host communities (Minchella & Scott, 1991; Hatcher et al., 2006; Poulin, 2007). Repeated interaction between host and parasite are believed to lead to the specialization of the parasite on its host (Kawecki, 1998; Poulin, 2007), which, in turn, may cause selection for resistance in the host. Subsequently the parasite may evolve ways to circumvent the novel resistance mechanisms of the host, thus resulting in a co-evolutionary arms race (Kawecki, 1998; Antonovics et al., 2013). If a parasite evolves in a multi-host community it is expected to evolve in proportion of the frequency of encounters, leading to different degrees of adaptation, to each host species. A generalist parasite is expected to be less adapted to any particular host since it spends less time adapting to each host type and adaptations that are beneficial in one host might not be in another host. In contrast, a specialist parasite evolving with only one host is expected to be better adapted (Kawecki, 1998). On the level of host communities, the exploitation of multiple host species might be advantageous because this presents unspecific parasites with a larger array of hosts to persist in. This is important during periods of asynchrony of host generations or in cases of temporary or permanent local extinctions of individual host species due to biotic or abiotic factors, as is the case in metapopulations and metacommunities.

Metapopulations are dynamic systems where local extinctions, founding events and migration between patches happen regularly (Hanski, 1998). Multi-species metapopulations are metacommunities, which are of particular interest if the different species can serve as host for the same parasite species. Since the host and parasite species composition of each patch may vary under the influence of specific colonization-extinction dynamics over time, knowledge of the specificity of each parasite is essential to understand the long-term epidemiology of such a system. Biotic and abiotic factors can affect host abundance in various ways, leading to occurrence period asynchrony or local extinctions. In such cases parasites might

evolve to retain some infectivity towards one or more alternative hosts (i.e. evolve a broader host-range). In case of environmentally transmitted parasites that survive poorly in the environment, having a larger host-range would facilitate long-term persistence. Furthermore, in meta-communities, where related host species occur in sympatry and parasites have abundant opportunities for cross-species transmissions, parasites might be expected to evolve a wider host range (Woolhouse et al., 2001; Antonovics et al., 2013). Taken together, parasites in metacommunities with frequent local extinction/colonization dynamics are expected to have a wide host range.

Microsporidia are obligatory intracellular parasites that infect almost every major clade of eukaryotes including humans (Mathis, 2000). Their host-ranges can span from very wide, infecting many species from different genera or classes, to very narrow, infecting only one species of host (Didier et al., 2000, and references therein; Mathis, 2000; Solter et al., 2010). The microsporidian parasite *Gurleya vavrai* Green, 1974, was initially described from two populations of *Daphnia longispina* (Green, 1974) in England and has since been reported to infect not only *D. longispina* but also *D. pulex* in England and Central Europe (Stirnadel & Ebert, 1997; Little & Ebert, 1999, see also .Friedrich et al 1996: in this study called *G. daphniae,* see Refardt et al., 2002). Stirnadel & Ebert (1997) never found the parasite on sympatric *D. magna*. *G. vavari* was also observed to occur in a *Daphnia* rockpool metacommunity (D. Ebert, unpublished observations), providing the opportunity to test the prediction, that parasites in a metacommunity should evolve to be generalists.

The *Daphnia* metacommunity of the rock pools of the Baltic Sea comprises *D. longispina* Müller, 1785, *D. magna* Straus, 1820, and two clades of *D. pulex* Leydig, 1860, one facultative parthenogenetic and one exclusively parthenogenetic (Hanski & Ranta, 1983; Bengtsson, 1989; Pajunen & Pajunen, 2003; Lehto & Haag, 2010). The parasites of this metacommunity have been studied intensively (e.g. Green, 1957; Altermatt et al., 2007; Ebert et al., 2001; Zbinden et al., 2008). An earlier study focusing on *D. magna* established that the microsporidia *Hamiltosporidium*

tvaerminnensis (formerly called *Octosporea bayeri*) and *Ordospora colligata* are specific to *D. magna*, while an undescribed *Larssonia* species infected all *Daphnia* host species (Ebert et al., 2001). So far, *G. vavrai* was only observed in a *D. pulex* population (D. Ebert, unpublished observation). *D. pulex* occurs more rarely in this metapopulation than *D. magna*, thus being able to infect further host species might be beneficial for *G. vavrai*'s long-term persistence. Using an observational (field survey) and experimental approach (infection trials) we examine the specificity of *G. vavrai* to the 4 sympatric host taxa of the rock pool metacommunity. To obtain a more comprehensive picture of the specificity of *G. vavrai* we expand to include host clones from other regions in Europe and also further potential host species within the genus *Daphnia*. Since parasites are often dependent on host genotypes for their ability to mount a successful infection (Schulenburg & Ewbank, 2004; Lambrechts et al., 2005; Schmid-Hempel, 2011; Luijckx et al., 2011), we also address the question of host specificity at the level of host genotypes by testing multiple genotypes for each of the *Daphnia* species found in the Baltic meta-population.

Materials and Methods

Parasite

Gurleya vavrai infects the hypodermis of its host (Green, 1974; Stirnadel & Ebert, 1997) where it produces symptoms visible with the naked eye. In early stages small white masses appear at the foci of infection, where environmental transmission stages (spores) aggregate. With time the spore masses grow and propagules spread through the hypodermis until the host is almost entirely infected rendering its hypodermis whitish. At last, propagules spread to the hosts' head. Host death occurs usually when its body surface is 60 to 100% infected, estimated by the coverage of white spore masses. *G. vavrai* has the potential to inflict strong fitness consequences as it reduces host fecundity (Stirnadel & Ebert, 1997; Little & Ebert, 1999).

Field survey

From 2009 to 2013, we visited each summer 560 rock pools on 17 islands of the Tvärminne archipelago in Southwestern Finland and assessed the presence of *Daphnia magna, D. longispina* and *D. pulex*. These rock pools are part of a long-term metapopulation project (Pajunen & Pajunen, 2003). When *Daphnia* were found in a rock pool, a sample was taken to the laboratory, kept cool (about 10-12°C) and searched within 4 days for the presence of *G. vavrai*. This was done by examining ~20 animals per host species per pool using a dissection microscope. Advanced infections with *G. vavrai* are easy to discover, as the carapace is dark and non-transparent against the light and white in reflected light. We produced squash preparations of symptomatic animals and checked at 400x magnification (phase contrast microscopy) for parasite spores. If at least one animal was infected, the entire population was scored as infected.

Parasite isolate

G. vavrai was isolated from infected *D. pulex* sampled from a rock pool population (FI-RO1, island Rovholmen, 59°50'17''N 23°14'57''E) near Tvärminne Zoological Station, Finland, in summer 2010. Parasite spores were produced by exposing 8 uninfected *D. pulex* (clone FI-RO1-6-d, originating from the same population as the parasite) to a concentrated suspension of spores (obtained by crushing and suspending in ADaM medium an infected FI-RO1-6-d host from the field) in 20 mL of the culture medium ADaM (modified from Klüttgen et al., 1994, as detailed in Ebert et al., 1998) during one week. After a week, hosts were transferred to 80 mL of fresh medium twice per week and fed daily 5 Mio cells of the algae *Scenedesmus* sp. until infection was easily detectable by eye. Infected hosts were crushed and suspended in ADaM and the spore concentration was estimated with a Thoma haemocytometer. Prior to their use spore suspensions were stored up to 30 days in the dark at 4°C.

Hosts

Daphnia (Phyllopoda: Cladocera) are small planktonic crustaceans, between 0.2 and 5 mm bodylength, found in small temporary ponds to large permanent lakes. In this study we used 6 species of Daphnia, five of which are cyclic parthenogens and one, *Daphnia pulex*, is subdivided into three clades, a european obligate parthenogenetic clade (*D. pulex* asexual clade), a European cyclic parthenogenetic clade (*D. pulex* sexual clade) and a north-american cyclic parthenogenetic clade (*D. pulex arenata*). Altogether species from three subgenera of Daphnia were used in this study. From the Daphnia *sensu stricto* subgenus we tested the three clades of *D. pulex*. *D. pulex* Leydig, 1860, the host from which the parasite was isolated, is widely distributed throughout Eurasia, North and South America and South Africa (Flössner, 2000). The asexual clade of *D. pulex* is only found in North America (Hebert et al. 1988) and Fennoscandia (Ward et al., 1994), it is hypothesized to have colonized Fennoscandia from North America (C.R. Haag, unpubl.). Due to its importance for further research, we chose to include in this study the *D. pulex arenata* genotype used for the *D. pulex* genome project (*D. pulex arenata* US-TCO; Colbourne et al., 2011). *D. pulex arenata* is phylogenetically closely related to *D. pulex*, but its distribution is restricted to North America (Pfrender et al., 2000). From the subgenus Hyalodaphnia we used *D. longispina* and *D. galeata*, which are two common species in Eurasia, the former being commonly found together with *D. pulex* and *D. magna* in the Daphnia rock pool metacommunity of the Baltic Sea (Pajunen 1986). From the Ctenodaphnia subgenus we tested *D. lumholzi*, *D. barbata* and *D. magna*. For the same reasons as before, we included the *D. magna* genome project genotype (*D. magna* FI-X-inb-3). All host species and clones used in this study were collected between 2000 and 2011 and kept in our lab stocks since. Experiments were run in 2012.

Experimental design

Hosts were kept for at least 5 parthenogenetic generations in the laboratory before use. The experiment was set up by preparing, for each host tested, 16 replicated jars containing each 20mL of medium (ADaM modified from Klüttgen et al., 1994, as detailed in Ebert et al., 1998) and 4 genetically identical individuals (3 to 8 days

old). To 8 of these replicates 100'000 spores of the parasite were added, and the other 8 replicates were left untreated as controls. Replicates were randomized and kept in an incubator with a 16:8 light:dark cycle and at a constant temperature of 20±0.5°C. One week after exposure jars were topped up to 80 mL medium. Afterwards hosts were transferred to new jars with fresh medium and their offspring discarded once per week until the end of the experiment. Animals were fed daily with 5 Mio cells of the unicellular algae *Scenedesmus* sp. Replicates were randomly rearranged within the incubator once per week to minimize positional effects. Dead hosts were stored at -20°C in 0.5 mL medium. The experiment was terminated after 40 days with the collection and freezing (-20°C in 0.5 mL medium) of all remaining hosts. Samples were later thawed, homogenized and checked by phase contrast microscopy (magnification 400x) for parasite spores (Thoma haemocytometer, depth 0.02 mm, square width: 0.05 mm). We defined successful infections by the presence of environmental stages (spores) of the parasite.

Results

Field survey

In total 854 population samples were collected and checked for *G. vavrai*. In 17 *D. pulex* samples (127 pools; 13.4% infection rate) the parasite was found, while it was never seen in any sample of *D. longispina* (n=316 pools) and *D. magna* (n=411)(Fisher exact test, $p<0.0001$). Pools with populations scored infected occurred only on 3 of the 17 studied islands (Fyrgrunded, Melanskär and Skallotholmen) in total in 11 different pools (Table 1). Pools with infected *D. pulex* populations were found in 3 cases to have at least one other Daphnia species present at the same time: in all three cases with *D. magna* and in one case also with *D. longispina* (Table 1). But none of these other *Daphnia* species showed signs of *Gurleya* infections. Four pools with infected *D. pulex* on the island Skallotholmen had the asexual form of *D. pulex*.

Experimental infections

Infection trials revealed that every genotype of *D. pulex* from the Finnish metapopulation was susceptible while only one *D. longispina* genotype was and none of the *D. magna* genotypes of the Finnish rock pools were (Table 2).

From the non-Finnish *D. pulex* tested, 1 of 3 genotypes from France was susceptible, 1 of 6 genotypes from England was susceptible, the only genotype sampled in Germany was not susceptible and all three genotypes sampled in Iran were susceptible (Table 2). The swiss *D. longispina* genotype was susceptible, as was that from Germany, but the genotype from England was not (Table 2). None of the *D. magna* genotypes were susceptible, neither were the genotypes of *D. barbata*, *D. galeata* or *D. lumholzi* (Table 2). The North-American *D. pulex arenata* (US-TCO) was susceptible. Altogether susceptibility was only found in the three *D. pulex* clades, *D. pulex arenata* and *D. longispina*, but not in other Daphnia species (Figure 1)

Discussion

Knowing the specificity with which parasites infect different hosts is essential for understanding meta-community dynamics. In this study we experimentally confirm previous field observations (Green, 1974; Stirnadel & Ebert, 1997; Little & Ebert, 1999) that *Gurleya vavrai* is able to infect *Daphnia pulex* and *D. longispina*, two freshwater crustacean hosts that are often found sympatrically, as is the case in the Daphnia metacommunity of the Baltic Sea (Pajunen, 1986). Surprisingly, a 5 year field survey of this Daphnia metacommunity did not uncover a single infected *D. longispina* population, not even in one case where infected *D. pulex* coexisted with *D. longispina* in a rock pool. The third commonly found sympatric host, *D. magna*, did not get infected, a result consistent with earlier field surveys (Stirnadel & Ebert, 1997; Little & Ebert, 1999). The same was true for three other Daphnia species, of which however, only single clones were tested.

Is D. pulex the main host of G. vavrai?

Previously *G. vavrai* was reported from *D. pulex* and/or *D. longispina* from England, Austria and France (Green 1997; Little & Ebert, 1999; Stirnadel & Ebert 1997; Friedrich et al 1996). Here we extend this range to Finland, the most northern location yet studied. Generally the parasite seems rather rare. Our study found the parasite in 13.4% of *D. pulex* populations sampled. An earlier survey examined 34 Daphnia populations (16 of which were *D. pulex* and 10 were *D. longispina*) throughout central Europe and found *G. vavrai* only in one *D. pulex* population at a prevalence of 4.2% (Little & Ebert, 1999). Medium to large scale surveys of Daphnia parasites in other countries and even different continents did not reveal any *Gurleya* or *Gurleya*-like parasite (Bengtsson & Ebert, 1998; Killick et al., 2008) but not all surveys included samples of *D. pulex* or *D. longispina* (Duffy et al., 2010; Goren & Ben-Ami, 2013).

Only one study reported *G. vavrai* from *D. pulex* as well as *D. longispina*. Stirnadel & Ebert (1997) found *G. vavrai* in three ponds in Southern England, with average prevalences of 11, 29 and 9% in *D. pulex* and 0.5, 3 and <0.5% in the sympatric *D. longispina* respectively. This higher susceptibility of *D. pulex* to the parasite seems also to be reflected in our experiments, where 16 out of 24 (66.7%) *D. pulex* clones (all origins) were susceptible, while 3 out of 7 (43%) *D. longispina* were susceptible. This might indicate that *D. pulex* is more likely the target host and *D. longispina* either an accidental host by spillover or an alternative host to which the parasite is less adapted.

Some parasites while being mainly adapted to a major host often show some degree of infectiousness towards minor hosts (Poulin, 2007). Cross-species transmission is more likely to happen between closely related host species (Charleston & Robertson, 2002; Perlman & Jaenicke, 2003; Streicker et al., 2010; Longdon et al., 2011) and thus in metacommunities composed of closely related host species, parasite spillover from a source host to an accidental host may happen at higher rates than otherwise expected due to the combination of high host species densities (leading to more frequent opportunities of cross-species transmission) and to high physiological similarity (due to smaller genetic divergence) between

hosts. This model could explain some level of detection of infections in *D. longispina*. The only records which seem inconsistent with this idea are the findings of Green (1974), who stated in his 4-line species description of *G. vavrai*, that the parasite was found in two populations of *D. longispina*. He did not mention other Daphnia species in these ponds. Thus, an alternative explanation might be that the parasite is able to adapt locally to the available hosts within a small set of related hosts. Yet, another hypothesis is that Green's (1974) original description is a different parasite species. Given his very short description and the fact that there are many very similar microsporidian parasites in *Daphnia*, this is not unlikely. If so, then Green's parasite would remain, *G. vavrai* infecting *D. longispina* and the *D. pulex* infecting parasite would be *G. daphniae*, which was formally described by Friedrich et al (1996). *G. daphniae* is currently seen as a synonym of *G. vavrai* (Refardt et al 2002). Sequence data (16S rDNA) indicate that the parasite of Friedrich et al., other isolates from South England and the parasite in the finish metacommunity are the same (all sampled from *D. pulex*)(Refardt et al. 2002; D. Ebert unpublished data). Sequence data are not available for a *D. longispina* derived form.

Phylogenetic pattern

The phylogenetic position of the susceptible host clades shows a clear clustering (Figure 1) with resistance increasing with genetic distance from our source host, a sexual *D. pulex* population from Finland. In other systems, hosts most related to the source host have also been found more likely to be susceptible (Perlman & Jaenicke, 2003; Streicker et al., 2010; Longdon et al., 2011; reviewed in Antonovics et al 2013). Here, possible proximal causes include that a common feature important for parasite infection was present before the separation of the Pulex and the Longispina groups. The lineage leading to *D. galeata* might have lost this feature but with one *D. galeata* clone we have little power to support this. An alternative explanation could be that species from the Pulex and the Longispina sub-clades independently evolved features allowing the parasite to infect them, which seems however less parsimonious. The pattern seen within the *D. pulex* clade is also consistent with the ancient trait hypothesis. The sexual *D. pulex* from Finland, which represents the

European *D. pulex* lineage, the asexual *D. pulex* lineage from Finland, which is believed to have arrived from North-American ancestors after the last ice-age (Ward et al. 1994) and the closely related, but geographically far distant *D. pulex arenata* from North America (Pfrender et al., 2000; Colbourne et al., 2011). If the parasite has its center of origin in central Europe, it is presumably adapted to the European *D. pulex* lineage. Its ability to infect other *D. pulex* lineages would then be supportive of the hypothesis that the parasite takes advantage of an ancient trait of its host. This trait might be as old as the split between the Longispina and the Pulex clade.

Local adaptation

Within the *D. pulex* clade, the susceptibility found was strongly concentrated within the clones from the Finnish meta-population, which were all susceptible (Table 2). These genotypes originated from different ponds located on different islands within the metapopulation. Outside of the finnish meta-population susceptibility was lower (Table 2) and rather patchy. This pattern might indicate local adaptation of *G. vavrai* to its host at the metapopulation level, a well known phenomenon in diverse host-parasite systems considered an indication of co-evolution (Morgan & Koskella, 2011). This phenomenon was also found in other microsporidian parasites in Daphnia (Ebert 1994; Altermatt et al., 2007). It has been suggested that specialist parasites with severe fitness consequences for their hosts, such as *G. vavrai*, are more likely to be locally adapted (Lively, 1999; Gandon, 2002).

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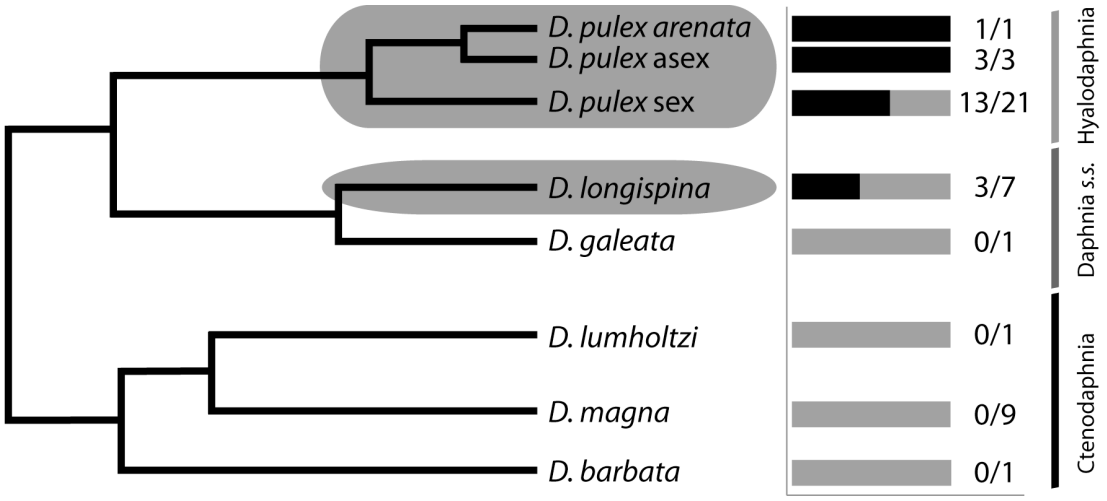
Table 1. Daphnia samples from 11 pools (out of 560) in which at least once during the period of 2009 to 2013 an infection with *G. vavrai* was observed. For each year the *Daphnia* species present in a sample are given (m: *D. magna*, p: *D. pulex* and l: *D. longispina*). Capital P (and grey background) mark the samples in which the *D. pulex* population was infected. Populations Skallotholmen 39 to 45 harboured the asexual form of *D. pulex*. A dash (-) indicates that either no *Daphnia* were present at the time of sampling, or it was not possible to take a sample, because the pool was dry.

Island-Pool, reproduction	**2009**	**2010**	**2011**	**2012**	**2013**
Fyrgrundet-4, sex	-	-	p	P	p
Fyrgrundet -5, sex	p	p	P	p,l	p
Melanskär-61, sex	p	-	P	p	P
Skallotholmen-1, sex	m	-	m, l	p, m, l	P, m, l
Skallotholmen -10, sex	l	l	l	l	l, P
Skallotholmen -36, sex	P	-	-	-	P
Skallotholmen -37, sex	-	-	P	-	P
Skallotholmen -39, asex	p	p	P	P	P
Skallotholmen -42, asex	p	p	p	-	P
Skallotholmen -44, asex	p	-	P, m	m	P
Skallotholmen -45, asex	p, m	p	P, m	m	p, m

Table 2. List of host genotypes (clones) tested with number of replicates scored positive for infection. Clones with * were produced by sexual reproduction in the laboratory. Clones marked with **§** are the clones used for genome sequencing by the Daphnia Genome Consortium.

Species	Genotype	Origin	Replicates: infected / total
D. pulex arenata	US-TCO **§**	USA	**7/8**
D. pulex asex	FI-FU1-57-a	Finland	**3/8**
	FI-SK-44-b	Finland	**8/8**
	FI-SYN-4-a	Finland	**3/8**
D. pulex sex	DE-S2-2	Germany	0/8
	FI-ALB-7-c	Finland	**4/8**
	FI-ALO-1-d	Finland	**8/8**
	FI-KV1-1-d	Finland	**7/7**
	FI-LAG-3-c	Finland	**8/8**
	FI-LH-3-c	Finland	**6/8**
	FI-M-60-a	Finland	**8/8**
	FI-N-69-b	Finland	**4/8**
	FI-RO1-6-d	Finland	**16/16**
	FR-CON-1	France	**2/8**
	FR-CON-2	France	0/6
	FR-CON-3	France	0/8
	GB-A23-2	England	**3/8**
	GB-A23-3	England	0/8
	GB-A23-7	England	0/8
	GB-S8-4	England	0/8
	GB-S8-7	England	0/8
	GB-S8-9	England	0/8
	IR-GG1-2	Iran	**5/8**
	IR-GG1-3	Iran	**3/8**
	IR-GG1-4	Iran	**3/8**
D. galeata	DE-WG2	Germany	0/8
D. longispina	CH-BEL-11	Switzerland	**3/8**
	DE-EG-10-4	Germany	**8/8**
	FI-F-6-a	Finland	0/16
	FI-FS-26-a	Finland	**1/15**
	FI-LA-13-c	Finland	0/8
	FI-N-20-d	Finland	0/8
	GB-S8-1	England	0/8
D. barbata	ZW-BAR	Zimbabwe	0/7
D. lumholzi	ZW-LUM	Zimbabwe	0/6
D. magna	DE-G1-06	Germany	0/8
	DE-Iinb1 *	Germany	0/8
	HU-HO-2	Hungary	0/8
	FI-AL1-4-4	Finland	0/8
	FI-SK-58-2-18-4	Finland	0/8
	FI-SP1-2-3	Finland	0/6
	FI-Xinb3 * **§**	Finland	0/8
	FI-FA-XFA-46 *	Finland	0/8
	RU-RM1-21	Russia	0/7

Figure 1. Schematic Daphnia phylogeny of tested taxa. Susceptible taxa are highlighted in dark grey. Adjoined to the phylogeny is a mosaic graph showing the proportion of infected to uninfected genotypes, black = infected, light grey = uninfected, numbers of infected over uninfected genotypes are indicated to the right. (for a complete phylogeny of Daphnia: see Adamowicz et al., 2009)



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Author contributions

César M.J.A. Metzger[a] – designed and performed the experiment, collected laboratory data, analyzed the data and wrote the manuscript.
Pepijn Luijckx[a,b] – designed the experiment, supervised and helped in the laboratory during the first third of the project, helped write the manuscript.
Frida Ben-Ami[c,d] – surveyed field populations and collected field data.
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III.

The genetics of resistance in *Daphnia magna* – *Pasteuria ramosa*:

"Strong negative epistasis and physical linkage control resistance against a bacterial parasite."

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Abstract

Key evolutionary processes related to infectious diseases are often assumed to be driven by specific genetic architectures of host and parasite interactions. There has been much debate about the underlying infection genetic models, such as gene-for-gene (GFG) or matching-allele models (MAM), best explaining evolutionary dynamics of hosts and parasites. Multi-locus MAM represent a specific genetic architecture of host and parasite interactions and have been widely used in the theoretical development of important evolutionary questions related to antagonistic coevolution, maintenance of sex, sexual selection and speciation. However, empirical evidence for these models is still elusive. Using genetic crosses we describe the genetic architecture of resistance in the *Daphnia-Pasteuria* host-parasite system and show that resistance is based on few closely linked loci with strong interactions. We provide the first experimental evidence for negative epistasis between resistance loci, a key assumption of MAM. Our findings corroborates previous phenotypic evidence that *Daphnia* and *Pasteuria* co-evolve as predicted by the Red Queen Theory, thus bringing evolutionary theory closer to real biological systems.

Introduction

Parasites are ubiquitous and their interaction with their hosts plays a significant role in ecology, evolution and agriculture. Host-parasite interactions affect biodiversity (e.g. chestnut blight, Anagnostakis and Hillman 1992), community structure (Hatcher et al. 2006), nutrient cycling (Suttle 2007), spread of invasive species (Torchin et al. 2003), and infectious diseases remain among the two leading causes of human morbidity and deaths worldwide (World Health Organization 2008). The relationship between parasites and their hosts is believed to be largely governed by the specificity of their interaction (Lambrechts 2010), which in turn is determined by the genetic architecture of disease related traits of hosts and parasites (Lambrechts et al. 2006).

Theory aiming to explain the evolution of key processes related to host-parasite interactions often rely on specific genetic architectures of host and parasite traits. The most prominently used genetic models are Gene-For-Gene Models (GFG) and Matching-Alleles Models (MAM). In some aspects, these models represent extremes of the genetic architectures used to model host-parasite co-evolution (Agrawal and Lively 2002).

In a GFG, resistance is conferred by the recognition of a virulence product of the parasite by a host receptor. Under this model each parasite locus coding for a virulence product has a corresponding locus in the host coding for a receptor. Absence of a recognizable product (due to null alleles on parasite loci or a new unrecognized parasite product for which no host receptor exists) results in the presence of parasites that are universally virulent (able to infect all hosts), a key characteristic of all models using a GFG genetic architecture. Empirical evidence for such models is abundant in plant-parasite systems (Thompson and Burdon 1992).

In a MAM, a genetic match between the host's genotype and the parasite's genotype leads to infection whereas mismatches lead to resistance (vice versa in case of an inverse MAM) (Frank 1993). Thus while in a GFG the corresponding host and parasite loci act independently (each parasite locus interacts with one host

locus), in a MAM host loci and their alleles act together to determine the susceptibility status against a parasite strain (multiple host loci interact with multiple parasite loci). Thus the characteristic feature of a MAM, not seen in GFG, is that the substitution of one allele on a locus changes the genotypic matching between hosts and parasites which can lead to a change in resistance (Luijckx et al. 2013). Consequently under the MAM no parasite can be universally infective and no host can be universally resistant. These features are shared by all MAMs regardless of their different implementations, e.g. matching vs. inverse matching (Dybdahl and Storfer 2003), variable number of matching loci (Hamilton et al. 1990; Frank 1993) and different levels of ploidy (Otto and Nuismer 2004; Agrawal 2009). In multi-locus MAMs, which are most prominently used, a match on basis of (the shared action of) multiple loci requires the presence of epistasis between linked loci. In absence of either epistasis or linkage the loci could act independently from each other, similar to a GFG, and thus violate a key assumption of a MAM; the requirement for genotypic matching between host and parasite.

Although empirical evidence for negative epistasis between linked resistance loci is lacking, MAMs have been extensively used to address longstanding questions in evolutionary biology such as speciation, local adaptation and sexual selection (Hamilton and Zuk 1982; Gandon et al. 1996; Kawecki 1998; Dybdahl and Storfer 2003). The Red Queen theory states that time-lagged negative frequency-dependent selection by parasites gives common host genotypes a disadvantage, thus favoring rare host genotypes (Jaenike 1978; Hamilton 1980; Salathé et al. 2008). Such antagonistic co-evolutionary dynamics leads to cycling of resistance alleles in the host and virulence alleles in the parasite. The Red Queen Theory heavily relies on MAMs due to the presence of epistasis in these models. As coevolution builds up linkage disequilibrium in hosts and parasites, in turn recombination breaks such linkage disequilibrium by shuffling of genes, thus maintaining genetic polymorphism in both host and parasite populations (Frank 1994) and favoring sexual over asexual reproduction (Lively 2010), two phenomena which have long puzzled evolutionary biologists.

Recently, the first empirical evidence congruent with a MAM was found in the *Daphnia magna* – *Pasteuria ramosa* model system. Using genetic crosses Luijckx et al. (2013) showed that the genetics underlying host resistance was consistent with a MAM. The authors proposed two putative genetic models that could explain their findings, a one-locus hierarchical dominance model with 3 alleles (with allele x>y>z) or a multilocus model. A theoretical model using a single locus MAM confirmed that the observed genetics could lead to Red Queen dynamics and showed an advantage for sex by segregation. Most of evolutionary theory, however, has focused on multilocus MAMs where an advantage for sex is provided by an advantage for recombination (Hamilton et al. 1990; Otto and Nuismer 2004; but see Agrawal 2009). In addition, the proposed genetic models cannot account for double resistant animals observed in natural populations (Andras and Ebert 2013). Their presence seem to invalidate a key property of MAMs: the absence of universal resistance.

In the present study we explicitly test for the inheritance of double resistance (resistance to two parasite genotypes, C1 and C19) and further develop the proposed genetic models. We show that 1) a multilocus model can explain the data while ruling out a one locus model, 2) a third locus is responsible for double resistance, 3) however, this third locus does not confer universal resistance, and 4) we provide the first empirical evidence for negative epistasis between linked resistance loci. Together these findings give strong support for the key assumptions of multi-locus MAMs and indicate that antagonistic coevolution can lead to Red Queen dynamics.

Results & Discussion

Daphnia can reproduce both sexually and asexually allowing for genetic crosses to be performed and the resulting offspring to be phenotyped with replication. We performed multiple crosses with three *D. magna* genotypes previously investigated by Luijckx et al. (2013) and a fourth uninvestigated genotype, all originating from the same metapopulation (Tvärminne, Finland). These four *D. magna* genotypes had different resistance profiles to *P. ramosa* clones C1 and C19 (SS, SR, RS, RR, where S stands for susceptible and R for resistant to C1 and C19 respectively). Selfing of these genotypes confirmed that all, but the SR genotype were homozygotes for their resistance phenotypes (Luijckx et al. 2013). The SS, SR and RS clones, were each crossed with the RR clone and for every cross multiple F1 were selfed to create multiple F2 panels (see Figure 1). We did not detect differences in hatching success or segregation of infertility alleles (as described by Routtu et al. 2010) in any of the crosses. Four clonal replicates of each parent, F1 and recombinants of the F2 panels were then phenotyped for resistance to *Pasteuria* genotypes C1 and C19 using the method developed by Duneau et al. (2011), which visualizes the attachment of spores to the oesophagus of the host, the step in the infection process responsible for host susceptibility. In all tests, all 4 clonal replicates were consistent either showing attachment or absence of attachment of spores to the oesophagus. Nevertheless to verify rare recombinant phenotypes we increased the within-genotype replication number to 12.

The F1 of all crosses were resistant to both C1 and C19 (i.e. double resistant, RR) and every F2 panel showed segregation of resistance (Figure 1). In the cross between SR*RR (F2-n_{tot}=719, Table 1), the SR parent was known to be heterozygous and two different segregation patterns were expected and indeed observed. Both patterns (70.9% RR & 29.1% SS and 76.3% RR & 23.7% SR) were consistent with double resistance being inherited as a Mendelian trait with dominant resistance (P-value = 0.077 & P-value = 0.71). The SS*RR cross (F2-n_{tot}=207, Table 1) was identical to one of the two panels in the SR*RR cross and indeed gave similar results

(77.7% RR & 23.3% SS, P-value = 0.41). From the second panel in the SR*RR cross we hypothesized that resistance against C19 was either recessive to double resistance or coded for by a separate locus with 2 alleles and dominant resistance. A single locus hypothesis was rejected (p-value < 0.0005, Supplementary Table 2) due to the observation of recombinant genotypes: 2% SR and 0.1% SS in the RR*RS cross (F2-n_{tot}=866). The finding of both SR and SS recombinants suggests that at least 3 loci might be involved in the inheritance of resistance. Linkage between loci is corroborated by results from previous crosses (Routtu & Ebert in prep and Luijckx et al. 2013; see Table 3). Here we propose a multilocus model based on our data that can explain both our observations and those of previous studies (Luijckx 2012, Luijckx 2013, Little 2006). Under this model resistance is coded for by 3 linked loci with each 2 alleles, dominant resistance and epistasis between dominant resistance alleles. The first locus codes for resistance against *P. ramosa* strain C1 (RS) which was clearly demonstrated to exhibit Mendelian segregation with 2 alleles and dominant resistance by Luijckx et al. (2012) and corroborated by our results. Furthermore, as evident from our observations, a second and third locus code respectively for resistance to C19 (SR) and double resistance (RR). In addition to the masking of the C1 and C19 resistance loci by the dominant allele on the RR locus, our results also indicate negative epistasis between the C1 and C19 resistance loci (Figure 2). Individuals carrying a dominant allele (A) on the first locus are always susceptible to *Pasteuria* C19 regardless of the resistance allele on the second locus. In addition to the support from our RR*RS cross, the presence of negative epistasis is also congruent with data of Luijckx et al. (2013) who also found that resistance to C19 reversed in presence of resistance to C1. Further support comes from observations of Little et al. (2006) who found apparent recessive resistance against one *Pasteuria* isolate and dominant resistance against another, a pattern that could also be explained by the presence of negative epistasis between dominant resistance loci.

To determine the recombination rate and the arrangement of the three loci we computed the expected values best explaining the observed results (Supplementary Figure 1). Model results on basis of the observed recombinants in the RR*RS cross

indicated that the three linked loci are organized in the genome in the order A-B-C with more frequent recombination between loci B and C (r_2 = 0.231 ± 0.040, genetic distance = 23.1 ± 4.0 cM) than between A and B (r_1 = 0.085 ± 0.052, genetic distance = 8.5 ± 5.2 cM) (Figure 2). With these recombination estimates we expected to find some recombinants in crosses 1 & 2, but no recombinants were observed. This could be explained if recombination frequencies between crosses differ (Smukowski and Noor 2011). Indeed differences in recombination rates within species have been reported for other systems (e.g human, Kong et al. 2010; house mouse Dumont et al. 2011; fruit fly, Brooks and Marks 1986). Alternatively as our estimates are based on only few observations with large variance we may have overestimated the true recombination rate. Indeed, other studies did not find any recombinants between loci A and C (Routtu & Ebert in prep) and loci A and B (Luijckx et al. 2013). Our genetic model with three diallelic loci and dominant resistance is consistent with other studies on invertebrates where dominant resistance is common (Carton et al., 2005). The finding that three loci underlie resistance is in line with studies on other animal hosts where on average 2.47 (± 1.18) resistance loci are found (Wilfert and Schmid-Hempel 2008). Linkage between resistance loci also seems to be a frequent occurrence (see review: Wilfert and Schmid-Hempel 2008). Evidence for interactions between linked resistance loci as revealed by our crosses is, however, absent in animals and exceedingly rare for plants with only one additive by dominance interaction reported for resistance to cucumber mosaic virus (Ben Chaim et al. 2001). However, epistasis between resistance loci has been suggested in other systems, such as in a snail and its trematode parasite (Dybdahl et al. 2008) and flour beetles and their microsporidium parasite (Wegner et al. 2008). The here found genetic architecture of resistance has striking similarities with other immune related genes that are found in physical linkage. Examples include the massively duplicated and diversified 'Chicken Ig-like Receptors' (CHIR) with its 103 or more clustered loci found on a single microchromosome (Laun et al. 2006), the 'Natural Killer Cell' gene clusters in mammals (Kelley et al. 2005; Carrington and Martin 2006) and the gene clusters of R-genes in plants (Young 2000).

The finding of epistasis between linked resistance loci provides the first empirical evidence for a key assumption of the genetic architecture underlying multiloci MAMs. The presence of the dominant resistance allele on the C locus apparently contradicts a MAM because a MAM does not include universal resistant genotypes. Therefore, we tested the resistance of the RR-parent to five uncharacterized *Pasteuria* isolates. These field-isolates may contain multiple genotypes and thus cannot be used to infer genetic patterns of inheritance. We found that four out of the five natural isolates tested were able to infect the RR-parent, thus showing that the double resistant genotype, and associated C allele, are not universally resistant (Table 2). This is further supported by the absence of evidence for selective sweeps from a study using resting stages of both *D. magna* and *P. ramosa* from lake sediment cores (Decaestecker et al. 2007) and the persistence of SS, SR and RS phenotypes in natural populations (Andras et al. in prep, Luijckx unpublished) Furthermore specificity in this host-parasite system has been extensively studied and universal resistance has never been found. On the contrary, all previous studies have described either strong (Carius et al., 2001; Decaestecker et al., 2003) or extremely (Luijckx et al., 2011) specific interactions between *Daphnia* and *Pasteuria.* Although the dominant C allele is clearly not universally resistant, it also deviates from a strict MAM by its ability to act independently from the A and B loci as it masks their expression (thus violating MAM). However, if alleles on the A or B locus provide resistance against an untested strain of *P. ramosa* to which the C locus is susceptible, masking of A and B by C would again result in strong negative epistasis. Although our results may slightly deviate from a pure MAM, our main finding, the presence of negative epistasis between linked loci, demonstrates that the key mechanism underlying a MAM genetic architecture is realistic. Furthermore, small deviations are unlikely to affect the overall evolutionary dynamics as across most of the continuum between MAM and GFG the highly dynamical features of a MAM dominate (Agrawal and Lively 2002).

Observations of strong genotype-genotype interactions between numerous *D. magna* and *P. ramosa* isolates and absence of universal virulence (Carius et al.

2001; Decaestecker et al. 2007; Duneau et al. 2011; Luijckx et al. 2011, 2012, 2013, 2014; Andras and Ebert 2013), may suggest that similar genetics as described here may apply to untested *Daphnia-Pasteuria* combinations. Further support for the general applicability of our model, is that all *Pasteuria* spores, even divergent strains adapted to other species of *Daphnia* (Luijckx et al. 2014), depend on the attachment to their host's oesophagus for successful infection (Duneau et al. 2011; Luijckx et al. 2011, 2014). Since attachment is controlled by the here described loci, the outcome of untested *Daphnia-Pasteuria* combinations are likely under similar genetic control. A putative mechanism of how the here described loci could act together to resist *P. ramosa* may involve the formation of protein complexes. The loci may code for different subunits of a protein complex that is involved in preventing attachment of *P. ramosa* to the host esophagus either by actively disrupting parasite spores or by blocking receptors targeted by *P. ramosa*. MAM have played a critical role in the development of evolutionary theory and have been used to address questions related to antagonistic coevolution, the maintenance of sex (Jaenike 1978; Otto and Nuismer 2004), the maintenance of genetic variation (Frank 1993), speciation (Kawecki 1998) and sexual selection (Hamilton and Zuk 1982), topics that have been longstanding interests of biologists. With the finding of negative epistasis between closely linked resistance loci, the genetic feature responsible for the highly specific nature of MAMs, the *Daphnia-Pasteuria* system can now be used to test specific predictions of these theories. Furthermore, theories building up on MAMs often make the assumption of binary resistance patterns. This assumption is fulfilled by the *Pasteuria – Daphnia* system, while many other empirical studies have often observed resistance patterns that are more quantitative (reviewed in Wilfert and Schmid-Hempel 2008). This may in part be due to our ability to separate the different steps in the infection process (Duneau et al. 2011) and potentially other systems may observe a more binary pattern if methods similar to the ones used in our study would be applied. Indeed, empirical evidence for theories that have a MAM at their basis has already been found in the *Daphnia-Pasteuria* system, as is the case for antagonistic coevolution (Decaestecker et al. 2007). Furthermore, with high virulence through castration (Ebert et al. 1996; Little and Ebert 2000), high

prevalence in natural populations (Duncan et al. 2006), strong genotype-by-genotype interactions (Carius et al. 2001; Luijckx et al. 2011) and evidence for negative frequency dependent selection (Decaestecker et al. 2007) the *Daphnia-Pasteuria* system meets all the assumptions of the Red Queen Theory suggesting that antagonistic coevolution in this system can maintain genetic variation and overcome the two fold cost of sex.

Conclusion

The majority of theory surrounding host-parasite coevolution is based on MAMs but empirical evidence for such genetic architecture is still lacking. In contrast, there has been considerable empirical work done supporting GFG in plant-parasite interactions. As both these genetic models lead to very different disease and evolutionary dynamics, adopting the right genetic model is critical for our understanding of key ecological and evolutionary concepts (e.g. local adaptation, Gandon et al. 1996; antagonistic coevolution, Jaenike 1978; and maintenance of genetic variation, Frank 1993). The results presented here provide the first empirical evidence for negative epistasis between linked resistance loci, the key feature responsible for a MAM genetic architecture, providing substantial credibility for MAM based theories. Unlike plants, the genetic architecture of resistance of animals, and especially invertebrates, has received less attention and remains poorly understood making it hard to predict which of the genetic models (GFG vs. MAM) should be applied to animal-parasite interactions. However as host-parasite genotype-genotype interactions and absence of universal virulence and resistance, both potential indicators of a MAM, have been reported for numerous animal-parasite systems (Lively and Dybdahl 2000; Carius et al. 2001; Schulenburg and Ewbank 2004; Lambrechts et al. 2005) genetic architectures congruent with MAMs may be widespread.

Material and methods

Host & parasite material

Four iso-female/clonal lines with different resistance phenotypes were used as parents: FI-SK-58-2-18-4, the RR-parent (C1 and C19 resistant); FI-Fa-XFa6-inb-46, the SS-parent (C1 and C19 susceptible); FI-Fainb3, the SR-parent (C1 susceptible, C19 resistant); and FI-Xinb3, the RS-parent (C1 resistant, C19 susceptible). All parents are descendants from the same *Daphnia magna* metapopulation near Tvärminne (Finland). Two of the parents (SR-parent and RS-parent) were sampled and inbred three times (selfing) in a previous study. The SR-parent is known to be heterozygous for one of the resistance loci while the RS-parent is homozygous (for details see Luijckx et al. 2013; therein named parent 1 and parent 2).

The SS-parent resulted from a complex crossing scheme using FI-Fainb3 and FI-Xinb3 (see Luijckx et al. 2013). The RR-parent was chosen from a set of 31 selfed candidate *D. magna* clones collected from the field. Both the SS-parent and RR-parent were confirmed to be homozygous; for the SS-parent no segregation of resistance was detected after selfing it once (number of phenotyped offspring, n=39) while the chosen RR-parent was selfed twice to confirm homozygosity (number of phenotyped offspring, n=19). The two *Pasteuria* clones used, C1 and C19, were obtained from natural spore isolates by single-spore infections as detailed in Luijckx et al. (2010).

Crosses & phenotyping

Details of the crossing procedure were described previously (Luijckx et al. 2012, 2013). In short, males of one parent were placed together with females from the other and all asexually produced offspring were removed every three days to prevent selfing. Sexual eggs were collected, dried for 1 week, rehydrated, bleached (5% aqueous solution) for 2-4 min to stimulate hatching and subsequently hatched under daylight lamps in artificial *Daphnia* medium (ADaM: Klüttgen et al. 1994 modified after Ebert et al. 1998). Resulting F1 lines were asexually propagated and

were then allowed to produce resting eggs by selfing. These eggs were collected and hatched as described above and the resulting F2 recombinants were kept as asexual lines until tested for resistance. In total we produced thirteen F1 clones, each of which was used to produce an F2 panel: 2 for the RR-parent x SS-parent cross, 8 for the RR-parent x SR-parent cross and 3 for the RR-parent x RS-parent cross (Figure 1). The resistance phenotype of each clonal line was determined by individually exposing 4 animals of each clone to 5000 fluorescently labeled spores of the parasite (C1 or C19) in 2.5 mL of medium for 30 min. This was done in the dark to avoid bleaching of the dye. After incubation hosts were checked for spore attachment to their oesophagus with a fluorescence microscope. Attachment indicates successful infection, while lack thereof indicates resistance (for details see Duneau et al. 2011).

Infection trials

Each of the four parents (RR, RS, SR and SS) and three *D. magna* clones collected from Germany, Hungary and Finland (DE-G1-06, HU-HO-2, FI-Kela-18-10) were exposed individually to five different *P. ramosa* isolates (sampled from Russia (isolates 1, 2, 3 & 4) and Switzerland (isolate 5)). Eighteen 4-6 days old females per treatment (parent * *P. ramosa* isolate combinations and control * *P. ramosa* isolate) combinations were individually placed into 100-mL jars containing 20 mL of ADaM. One hundred thousand spores of the isolates were added to each jar and after one hour the exposed hosts were fed 2 Mio algae cells each. The animals were fed every day 2 Mio algae cells for one week, jars were then topped up to 80 mL ADaM and feeding was increased to 3 Mio algae cells per day. From then on, all hosts were transferred to fresh ADaM in weekly intervals and feeding was increased to 5 Mio algae cells per day. After 42 days, the experiment was terminated and the infection status of each host was checked under a phase contrast microscope (magnification 400x). Hosts that died during the experiment were also checked for their infection status.

Statistics

Statistical analysis was performed with the software R (R Development Core Team 2010). P-values were obtained by computing chi-square tests with a random re-sampling procedure (Monte Carlo simulations, number of iterations = 2000) for observed resistance phenotypes in F2.

To estimate the recombination rates of cross 1 – r_1 between loci A and B and r_2 between loci B and C – we constructed a model in R with which we searched which combination of recombination rates gives the best fit to the observed data. Starting from a population of all possible parental genotypes (e.g. aBC, Abc), we constructed a matrix of possible offspring genotypes. We constructed a second matrix of identical size and filled it with the phenotypes corresponding to the offspring genotypes from each cell of the first matrix (Supplementary Table 3). We then created a vector containing our observed phenotype count data (n_{tot} = 866 offspring). Assuming that recombination rates are r_1 and r_2 and that we start from heterozygote parents, the frequencies of both male and female gametes should be: $(1-r_1)*(1-r_2)$ for genotypes aBC and Abc (no recombination), $(1-r_1)*r_2$ for aBc and AbC (recombination between B and C only), $r_1*(1-r_2)$ for ABC and abc (recombination between A and B) and finally r_1*r_2 for ABc and abC (recombination between both A and B and B and C). Using these gamete frequencies, we computed first the expected frequencies of all F2-genotypes. We then converted them to expected counts of phenotypes RR, RS, SR and SS in our offspring population using the genotype – phenotype map previously described. The expected counts were compared to observed counts using the chi-square distance. The numeric values r_1 and r_2 that minimize this distance are r_1=0.085 ± 0.052 and r_2=0.231 ± 0.040. Confidence intervals were computed by bootstrap procedures (n=1000). For those values of r_1 and r_2, the p-value of a chi-square test is larger than 0.53 and indicates that the observed counts (RR: 631, RS: 217, SR: 17, SS: 1) are compatible with the model and expected counts (RR: 649, RS: 200, SR: 16, SS: 1).

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Table 1. Percentages of the four phenotypes (observed and expected) of the 13 F2 panels used in this study. Chi-square tests for the difference between observed and expected counts are given. Expected values in crosses 1 and 2 are mendelian proportions, in cross 3 they were calculated using a statistic model computed in R.

P	F1 replicates	F2 (N_{total})	F2 ($\%_{observed}$)				F2 ($\%_{expected}$)				Chi^2	p-value
			RR	RS	SR	SS	RR	RS	SR	SS		
cross 1 pattern A												
SR-parent * RR-parent	cross 3 selfed 1	98	76.5	0	23.5	0	75.0	0	25.0	0	0.12	0.81
	cross 3 selfed 2	85	76.5	0	23.5	0	75.0	0	25.0	0	0.10	0.81
	cross 3 selfed 3	103	74.8	0	25.2	0	75.0	0	25.0	0	0.00	1.00
	cross 3 selfed 4	40	77.5	0	22.5	0	75.0	0	25.0	0	0.13	0.86
	cross 3-A pooled	326	76.3	0	23.7	0	75.0	0	25.0	0	0.20	0.71
cross 1 pattern B												
SR-parent * RR-parent	cross 3 selfed 5	98	70.4	0	0	29.6	75.0	0	0	25.0	1.10	0.35
	cross 3 selfed 6	105	72.4	0	0	27.6	75.0	0	0	25.0	0.38	0.58
	cross 3 selfed 7	94	69.1	0	0	30.9	75.0	0	0	25.0	1.72	0.23
	cross 3 selfed 8	96	71.9	0	0	28.1	75.0	0	0	25.0	0.50	0.55
	cross 3-B pooled	393	71.0	0	0	29.0	75.0	0	0	25.0	3.37	0.08
cross 2												
SS-parent * RR-parent	cross 2 selfed 1	102	77.5	0	0	22.5	75.0	0	0	25.0	0.33	0.67
	cross 2 selfed 2	104	77.9	0	0	22.1	75.0	0	0	25.0	0.46	0.57
	cross 2 pooled	206	77.7	0	0	22.3	75.0	0	0	25.0	0.78	0.41
cross 3												
RS-parent * RR-parent	cross 1 selfed 1	341	72.7	26.1	0.9	0.3	75.0	23.1	1.8	0.1	4.12	0.22
	cross 1 selfed 2	146	71.9	24.0	4.1	0	75.0	23.1	1.8	0.1	4.62	0.21
	cross 1 selfed 3	379	73.4	24.5	2.1	0	75.0	23.1	1.8	0.1	1.13	0.75
	cross 1 pooled	866	72.6	24.9	2.4	0.1	75.0	23.1	1.8	0.1	2.14	0.51

Table 2. Outcome of infection trials with 5 isolates of *Pasteuria ramosa* and the 4 parent clones used in this study. In contrast to the parasite clones used to test the genetic crosses, **i**solates may contain more than one parasite genotype, which can result in a much more continuous infection range. Insufficient spore material was available for isolate 2 to expose all host genotypes (therefore the RS-parent and DE-G-106 were omitted). Cell entries are the number of infected animals over the number of all tested animals.

	Parasite				
Host genotype	isolate 1	isolate 2	isolate 3	isolate 4	isolate 5
RR-parent	**2**/15	**9**/15	**11**/16	**6**/18	0/15
RS-parent	3/11	-	9/14	3/14	0/10
SR-parent	5/14	9/15	5/15	6/18	0/16
SS-parent	4/17	10/13	5/14	5/14	0/8
DE-G1-06	3/11	-	4/13	9/16	1/16
HU-HO-2	2/13	10/15	3/11	10/11	0/11
FI-Kela-18-10	6/12	9/15	2/11	8/12	0/11

Table 3. Direct tests for recombination: comparison between three studies. This work and that of Luijckx et al. (2013) and Routtu & Ebert (in prep) all investigates genetic crosses with respect to resistance to *P. ramosa*. Comparing the outcome of the selfing of double heterozygotes of these studies is a direct test for detecting recombination. Genotypes of the double heterzygotes (F1), the loci between which recombination may be detected by these crosses, the number of recombinants found and the total number of offspring produced are indicated. Only 'cross 3' from the present study found recombinants.

Dataset	**F1 genotype**	**expected recombinating loci**	**F2 $N_{recombinants}$**	**F2 N_{total}**
Cross 1B and 2 *This study*	aaBbCc	B-C	0	600
Cross 3 *This study*	AaBbCc	A-B & B-C	18	866
Complex crossing scheme *Luijckx et al. (2013)*	AaBbcc	A-B	0	118
QTL Panel *Routtu & Ebert (in prep)*	AabbCc	A-C	0	800

Supplementary Table 1. Comparison of two previous (1-locus and 2-loci) models and the new (3-loci) model for *Daphnia-Pasteuria* interactions. A "-" stands in for an allele that has no influence on the final phenotype. * indicates models as hypothesized by Luijckx et al. (2013).

Host genotypes			Exposure outcome with parasite genotypes		Infection Phenotype
One-Locus Model*	Two-Loci Model*	Three-Loci Model	C1	C19	Abbreviation
w-	-	----C-	resistant	resistant	RR
xx	AA--	AA--cc	resistant	susceptible	RS
xy/xz	Aa--	Aa--cc	resistant	susceptible	RS
yy/yz	aaB-	aaB-cc	susceptible	resistant	SR
zz	aabb	aabbcc	susceptible	susceptible	SS

Supplementary Table 2. Statistical tests of cross 1 under three different expectations. Chi-squares were performed with 2000 Monte Carlo simulations in the software R. F2 expected values for 'no linkage' and 'full linkage' are mendelian proportions, for 'optimal' they were calculated using a statistic model computed in R.

P	$F2\ (N_{total})$	$F2\ (\%_{observed})$				$F2\ (\%_{expected})$				Chi^2	p-value
		RR	RS	SR	SS	RR	RS	SR	SS		
cross 1: no linkage RS-parent RR-parent	866	72.67	24.87	2.37	0.10	75	18.75	4.69	1.56	7.8341	0.0511
cross 1: full linkage RS-parent RR-parent	866	72.67	24.87	2.37	0.10	75	25	0	0	3.35E+13	0.0005
cross 1: optimal RS-parent RR-parent	866	72.67	24.87	2.37	0.10	75	23.05	1.82	0.12	2.14	0.5112

Supplementary Table 3. Visual representation of the two matrices built in the R model. Upper matrix: matrix of possible F2 genotypes. Lower matrix: matrix of phenotypes corresponding to the upper matrix. Green denotes double resistance (RR), yellow resistance to C1 and susceptibility to C19 (RS), blue susceptibility to C1 and resistance to C19 (SR) and red double susceptibility (SS).

Matrix of possible offspring genotypes

	CBa	cbA	cBa	CbA	CBA	cba	cBA	Cba
CBa	CCBBaa	CcBbaA	CcBBaa	CCBbaA	CCBBaA	CcBbaa	CcBBaA	CCBbaa
cbA	cCbBAa	ccbbAA	ccbBAa	cCbbAA	cCbBAA	ccbbAa	ccbBAA	cCbbAa
cBa	cCBBaa	ccBbaA	ccBBaa	cCBbaA	cCBBaA	ccBbaa	ccBBaA	cCBbaa
CbA	CCbBAa	CcbbAA	CcbBAa	CCbbAA	CCbBAA	CcbbAa	CcbBAA	CCbbAa
CBA	CCBBAa	CcBbAA	CcBBAa	CCBbAA	CCBBAA	CcBbAa	CcBBAA	CCBbAa
cba	cCbBaa	ccbbaA	ccbBaa	cCbbaA	cCbBaA	ccbbaa	ccbBaA	cCbbaa
cBA	cCBBAa	ccBbAA	ccBBAa	cCBbAA	cCBBAA	ccBbAa	ccBBAA	cCBbAa
Cba	CCbBaa	CcbbaA	CcbBaa	CCbbaA	CCbBaA	Ccbbaa	CcbBaA	CCbbaa

Matrix of phenotypes corresponding to offspring genotypes

	CBa	cbA	cBa	CbA	CBA	cba	cBA	Cba
CBa	RR	RR	RR	RR	RR	RR	RR	RR
cbA	RR	RS	RS	RR	RR	RS	RS	RR
cBa	RR	RS	SR	RR	RR	SR	RS	RR
CbA	RR	RR	RR	RR	RR	RR	RR	RR
CBA	RR	RR	RR	RR	RR	RR	RR	RR
cba	RR	RS	SR	RR	RR	SS	RS	RR
cBA	RR	RS	RS	RR	RR	RS	RS	RR
Cba	RR	RR	RR	RR	RR	RR	RR	RR

3

Figure 1. – Crossing scheme annotated with resistance profiles of *Daphnia magna* genotypes against two *Pasteuria ramosa* genotypes. Each pedigree represents a summary of multiple replicate crosses (Individual replicate crosses can be found in table 1). Phenotypes are indicated as two halves of a circle with the first half representing resistance to *P. ramosa* C1 and the second resistance to *P. ramosa* C19. White means resistance, black susceptibility, such that a fully black circle represents susceptibility against both C1 and C19 (SS). Hypothesized genotypes under our genetic model (found in Supplementary Table 1) are located next to the half circles, a letter indicates determining alleles and neutral positions are indicated by a dash (-).

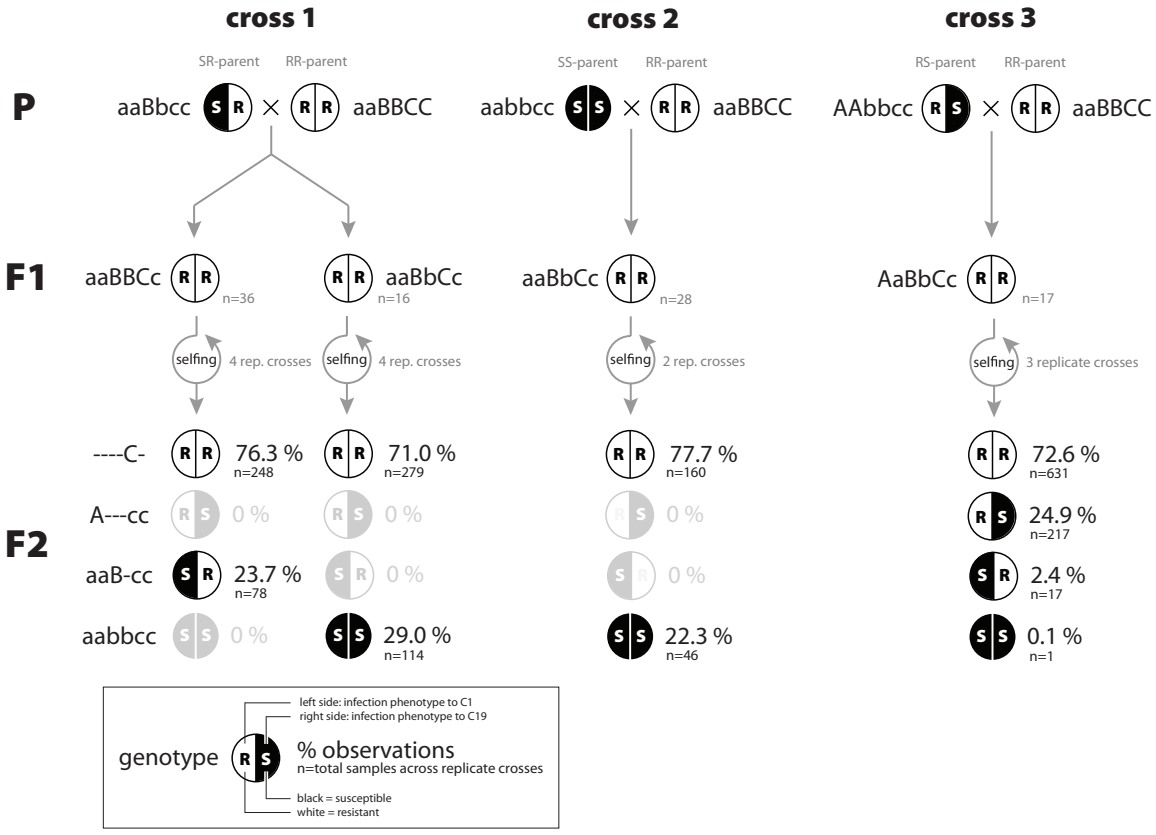
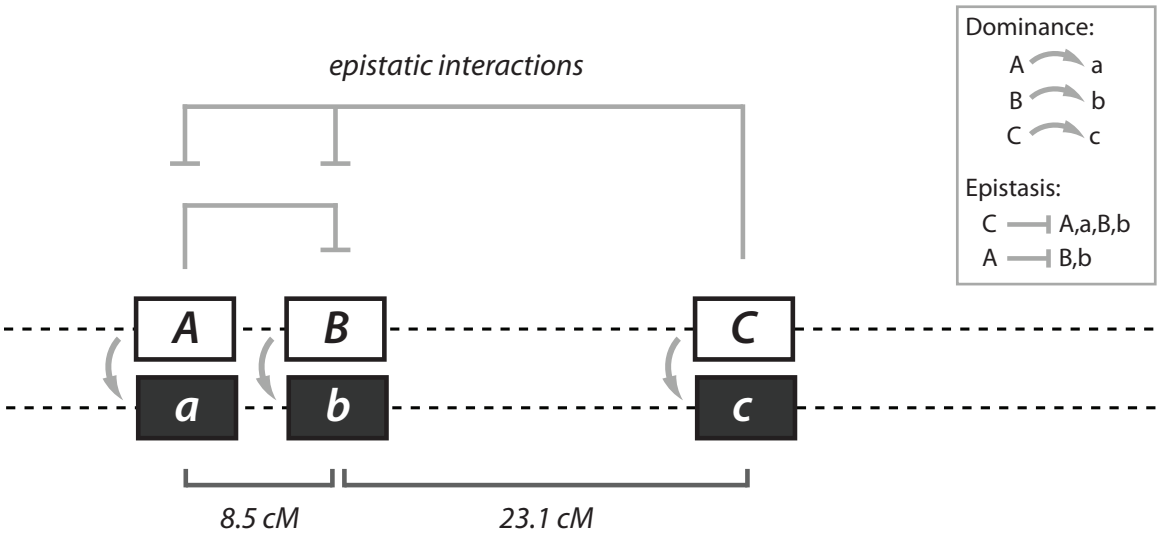
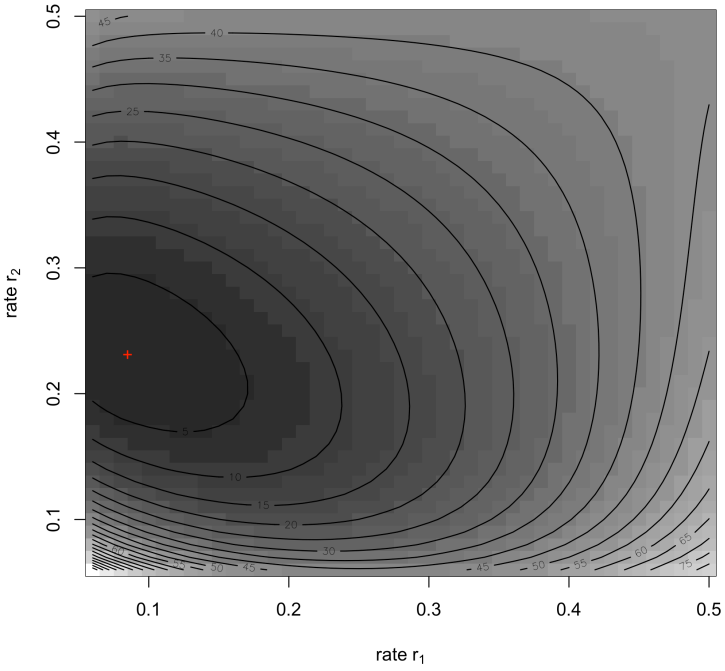


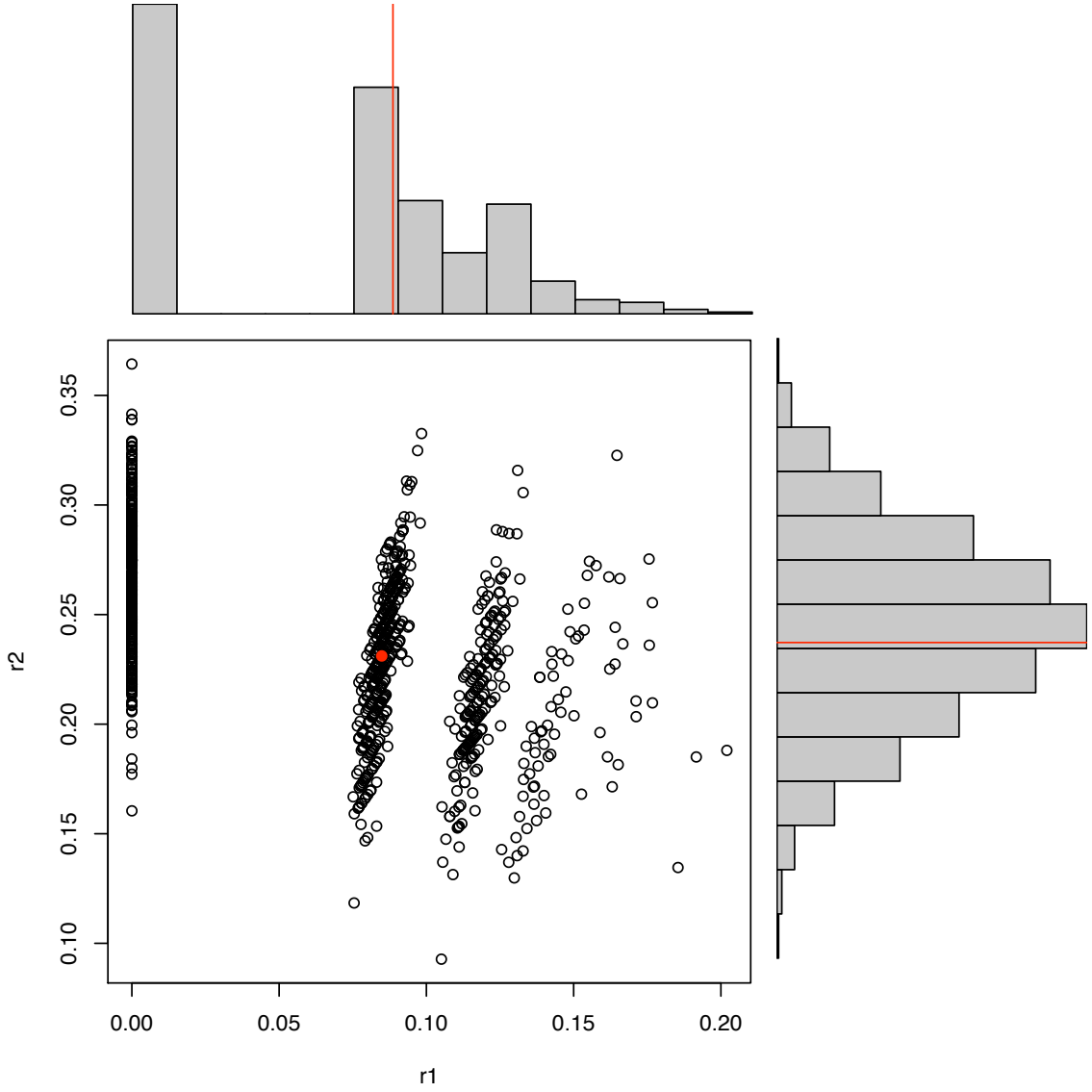
Figure 2. Genetic architecture of host resistance. Host genetics underlying *Pasteuria ramosa* resistance are based on 3 linked loci represented by the 3 pairs of boxes. Each locus has a dominant resistance allele (upper case letter in a white box) and a susceptible allele (lower case letter in a black box). Epistatic interactions among loci are symbolized by lines between loci, where the dominant resistance alleles from loci C and A affect the expression of loci A&B and B respectively.



Supplementary Figure 1. Chi-square estimates for all possible combinations of recombination rates between the A and B and the B and C loci. A red cross indicates the optimal estimate for values of recombination rate 1 and recombination rate 2 to explain the proportions of phenotypes obtained in cross 3.



Supplementary Figure 2. Bootstrap estimates of recombination rates r_1 and r_2. The filled red circle indicates the values of r_1 and r_2 for which the chi-square distance is minimized. The estimates are arranged in clusters, because for the given sample size, one recombinant more or less makes a big differences in the estimated recombination rate r_1.



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Author contributions

César M.J.A. Metzger[a,*] – performed the experiment, collected the data, analyzed the data, and wrote the manuscript.

Pepijn Luijckx[a,b,*] – designed the experiment, crossed the parental isofemale lines, wrote the manuscript.

Gilberto Bento[a] – contributed to the analyses and helped write the manuscript.

Mahendra Mariadassou[a,c] – produced the statistical model and helped write part of the manuscript.

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IV.

Eco-immunology of *Daphnia* resistance:

"The cost of evolved resistance in the absence of the disease: a competitive fitness assay in a host-parasite system of known genetic architecture does not support the cost of resistance hypothesis."

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Abstract

Background: Parasites are ubiquitous and represent a strong selective force leading to the evolution of host resistance. However, host-parasite populations often display polymorphism for resistance. The cost of resistance hypothesis posits that immune traits are costly to evolve, maintain or deploy, and thus resistant hosts will have a disadvantage in the absence of the parasite. Using a host-parasite system of known resistance genetic architecture, we investigated the presence of a cost of resistance.

Methods/Principal Findings: We collected *Daphnia magna* hosts from a natural population in Switzerland known to contain only two resistance phenotypes – one that is more resistant to local strains of its bacterial parasite *Pasteuria ramosa*, and one that is more susceptible to the parasite. We paired hosts of opposing resistance phenotype and competed them in meso- or microcosms under semi-natural or controlled conditions in the absence of the parasite. After two months the proportion of resistant hosts was quantified. Our experiments did not show any indication of a cost of resistance in this system.

Conclusions: Our findings do not indicate that resistance is detectably disadvantageous in the absence of the parasite. Thus we do not think that costs of resistance play a major role in the maintenance of polymorphism for resistance and co-evolution in this *Daphnia* population. In light of our results, we discuss putative structures of resistance genes and implications for the genetics underlying host-parasite co-evolution in this system.

Introduction

Understanding the mechanisms of co-existence of hosts and parasites and their consequences for the evolution of resistance and infectivity is one of the central questions of the evolutionary study of infectious diseases. This question is far reaching with implications not only in the fields of immunology and epidemiology, but also agriculture, ecology and conservation biology (Galvani 2003; Hudson et al. 2006; Morgan and Wall 2009). With higher reproduction rates, shorter generation times, usually larger population sizes and haploid genomes, parasites have the potential advantage of rapidly increasing the frequency of favorable genotypes in their population (Hamilton et al. 1990; Gandon and Michalakis 2002). Polymorphism for resistance in the host population and the high reproduction rate of parasites leads to the rapid spread of particular infectious parasite genotypes able to infect susceptible hosts with the consequence of their elimination from the population, ultimately leading to the elimination of the parasite due to the absence of susceptible hosts. Given these dynamics, the maintenance of susceptible genotypes in parasitized host populations (Carius et al. 2001; Poulin 2007; Wolinska and Spaak 2009), appears paradoxical.

The stable coexistence of hosts and parasites necessitates that neither universally resistant host genotypes nor universally infectious parasite genotypes go to fixation in the population. Two competing hypotheses can explain how polymorphism for resistance and infectivity can be maintained in a host-parasite system. The cost of resistance hypothesis is based on trade-offs (Stearns 1992) and posits that resistance traits are costly to evolve, maintain or deploy (May and Anderson 1983). The costs incurred by resistant hosts are compensated by the advantage that resistance provides in the presence of the parasite. In absence of the parasite, these costs burden resistant hosts competing with their susceptible counterparts for resources. If parasite occurrence varies in space and time, or in strength, resistance polymorphism can be maintained in the host population

without the need for genetic polymorphism in the parasite population (Agrawal and Lively 2002) or host-parasite co-evolutionary dynamics (Sasaki and Godfray 1999).

The second commonly proposed mechanism to maintain polymorphism for resistance and infectivity in host-parasite populations is co-evolution by negative frequency-dependent selection. This mechanism, often referred to as Red Queen dynamics, posits that there is an advantage for rare host and parasite genotypes that results in a constant turnover in host and parasite genotypes and prevents common genotypes from going to fixation (Jaenike 1978; Hamilton 1980; Frank 1994). These dynamics require that host populations, which often have longer generation times and slower mutation rates than parasites, generate genotypic variation through some mechanism other than mutation frequently enough to keep pace with their rapidly evolving parasites. Thus the maintenance of polymorphism for resistance in the host population may be coupled with the maintenance of sexual reproduction as a mechanism of creation of genetic diversity via recombination (Hamilton 1980; Lively 2010).

As resources are limited, their investment in costly traits such as resistance may come at the expense of other traits (Sheldon and Verhulst 1996). Therefore costs of resistance should be detectable as negative genetic covariances between different traits. Resources allocated in longevity or fecundity have previously been found to be redirected towards immunity in resistant animals (Webster and Woolhouse 1999; Ahmed et al. 2002; McKean et al. 2008). Measuring reproductive rate, lifetime reproduction, longevity, or any combination of these between resistant and susceptible hosts may thus be revealing of the existence of costs of resistance.

The *Daphnia magna* – *Pasteuria ramosa* host-parasite system is ideally suited for investigating potential costs of resistance as 1) the host, *D. magna*, can reproduce clonally allowing for replication at the genotypic level, 2) resistance to *P. ramosa* is a binary trait (yes/no) that can be easily determined with a phenotype assay, and 3) the genetic architecture of resistance was recently described (Luijckx et al. 2012, 2013 and Metzger et al. in prep). Altogether this allows replicated competition experiments to be conducted between *D. magna* clones of differing

resistance phenotypes to determine if the more resistant phenotype bears a significant competitive disadvantage.

In the present study we attempt to detect fitness costs by measuring the outcome of competition experiments where artificial populations started with 10 resistant host females and 10 susceptible host females were kept in semi-natural conditions in mesocosms or in controlled conditions in microcosms for 2 months. At the end of the experiment, all individuals in each population (including parents and parthenogenetic offspring) were assayed to determine which phenotype of each pair had a higher fitness. We did not find any indication for the existence of a cost of resistance.

Materials and Methods

Study system

Daphnia magna, the host, is a facultative parthenogenetic freshwater crustacean (Phyllopoda: Cladocera). Sexual reproduction is environmentally induced, and females reproduce asexually when maintained in optimal environmental conditions (e.g. day length, temperature, water volume). Asexual reproduction can yield up to ~50 offspring every 4 days (at 20°C), and time to maturation is usually 6 to 12 days or longer when diet is restricted. *D. magna* feeds by filtering particles out of the water such as bacteria and algae.

Pasteuria ramosa, the parasite, is a gram-positive endospore forming bacterium that infects *D. magna*. Infection occurs during filter and deposit feeding when spores of the bacterium are picked up from the sediment and ingested. During this process the spores activate and attach to the cuticle of the susceptible hosts' oesophagus. In resistant hosts, attachment does not occur (Duneau et al. 2011). As an obligate endoparasite, *P. ramosa* will reproduce clonally within the host body cavity and ultimately produce new spores, a costly process for the host that induces castration and gigantism and ends with host death. After death, the decaying corpse

of the host releases the spores of the bacterium back to the sediment. The spores of the bacterium can remain infectious in the sediment for decades (Decaestecker et al. 2007; Andras et al. in prep).

Screening for resistance

We collected hundreds of live hosts from Lake Aegelsee (47°33'29"N, 8°51'43"E) near Frauenfeld, Switzerland, raised them individually in the lab, and let them reproduce parthenogenetically to obtain isofemale lines. All lines were initially treated with the broad-spectrum antibiotic tetracycline to clear potential infections and then kept clonally under controlled conditions for several generations in order to minimize maternal effects. In addition, clonal cultures were examined regularly to ensure that they were free of infection prior to their use in experiments. Parasite material was obtained from *in vivo* clonal cultures in our lab. Spores of the parasite can be labeled with fluorescent dye and, after exposing hosts to these labeled spores, fluorescence microscopy can be used to detect attachment of the spores to the oesophagus of susceptible hosts (Duneau et al. 2011). By exposing hosts to different *P. ramosa* strains labeled with different colored dyes, it is possible to simultaneously measure host resistance with respect to multiple parasite genotypes. Using this simple assay, we characterized the resistance phenotype of each *D. magna* clone with respect to two clonal genotypes of *P. ramosa*, clones C1 and C19 (Luijckx et al. 2011). These two clones were collected in Russia and Germany respectively, and are not known to occur in Lake Aeglesee. However, they are useful for broad characterization and distinction of host resistance phenotype (hereafter called *resistotype*). Based on this assay there are four possible host resistotypes, yet after phenotyping thousands of isofemale lines over the course of five years, only two of these resistotypes have ever been observed in the Aeglesee population (~70% of genotypes were C1 & C19 resistant; ~30% were C1 & C19 susceptible; Andras et al. in prep). Infection experiments challenging *D. magna* from the Aeglesee with strains of *P. ramosa* from the same population, have found that one of these two resistotypes (C1 & C19 resistant) is significantly more resistant to

local *P. ramosa* than the other resistotype (C1 & C19 susceptible)(Anselm 2013). This population, containing only two resistotypes, one of which is more resistant to local parasites, is ideally suited to testing costs of resistance in a system that has been coevolving naturally for decades or more. Hereafter, *D. magna* clones of the more resistant phenotype will be referred to as "resistant", and clones of the more susceptible phenotype will be referred to as "susceptible".

Experimental setup

In three separate experiments we competed resistant against susceptible hosts.

1) In August 2012, we prepared 47 mesocosms on the roof of the Zoological Institute in Basel, Switzerland. Each mesocosm contained 75L of medium (ADaM, as in Ebert, Zschokke-Rohringer, & Carius, 1998 modified from Klüttgen, Dülmer, Engels, & Ratte, 1994) and was introduced with 10 individuals of a susceptible *D. magna* genotype from Lake Aeglesee and 10 individuals of a resistant host genotype. All pairs of genotypes were selected randomly. Of the 47 mesocosms, 27 contained unique genotypes and 20 contained one of 4 genotype pairs that were replicated in 5 tanks each. The position on the roof of each genotype pair was randomly assigned. At the beginning of the experiment, $4x10^8$ algal cells (*Scenedesmus* sp.) were added to each mesocosm - a concentration that had been previously shown to create a self-sustaining low level of food. Mesocosms were left undisturbed for 9 weeks until the end of the experiment (October 2012), whereupon up to 50 hosts were randomly sampled from each mesocosm for phenotyping.

2) The second experiment was similar in principle but was performed in 400 mL jars in a controlled climate chamber (20°C ± 1°C, light:dark 16:8) A total of 47 jars were introduced with competing pairs resistant and susceptible *D. magna* clones. Most of the host clone pairs were repeated from the first experiment, with the exception of 5 pairs where one of the genotypes had died in the meantime. In these cases we replaced the missing genotype with another genotype of the same phenotype. As before, four host genotype combinations were replicated in five jars

each. All other jars contained unique genotype combinations. We added $4x10^8$ algal cells (*Scenedesmus* sp.) to each jar once at the beginning of the experiment and left them to compete for 2 months, whereupon all individuals in each jar were phenotyped.

3) In the third experiment we set up 31 jars of 400 mL ADaM in a climate chamber (20°C ± 1°C, light:dark 16:8) with the exact same combinations of genotypes as Experiment 2, only this time we did not replicate any combination and thus had only unique genotype combinations. To reduce mortality during the experiment, feeding rates were increased in to $8x10^7$ algal cells per week. Cultures were maintained for 2 months whereupon all individuals in each jar were phenotyped.

Stasitical analyses

Prior to statistical analysis, the proportions of phenotypes in the final populations were arcsine-square-root transformed for the purpose of normalization (Sokal and Rohlf 1995). To test for an effect of host phenotype on the outcome of competition we compared the proportions of resistant and susceptible hosts with *paired t-tests* (Sokal and Rohlf 1995). For the comparison of the outcomes of competition between Experiments 1 and 3, to detect whether the same genotypes consistently won in both experiments, a *Chi² test* was performed (Sokal and Rohlf 1995). Statistical analyses were performed using Microsoft Excel (2008) and the statistical software R (R Development Core Team 2010). Post-hoc power analyses were performed using the program G*Power v 3.1.2 (Faul et al. 2007) with the following parameters: α = 0.05, power (1-β) = 0.8, two-tailed t-tests.

Results

Experiment 1

Out of 47 mesocosm populations of resistant and susceptible host pairs, five were discarded from the analysis due to low population size (8 or fewer individuals sampled). 42 populations yielded enough hosts for analysis (Mean = 20.21, sd = 4.08). We found 19 populations dominated by resistant clones, 27 populations dominated by susceptible clones and one population with the same quantity of each phenotype (p = 0.085, Figure 1). To ensure proper detection at the 0.05 level with our sample size, we performed a post-hoc power analysis that revealed the minimal sample size for effect detection to be 7 populations (actual power = 0.846). With an actual sample size of 42 it is unlikely that an effect was not detected. In this setup, some of the populations were replicated 5 times. If these pairs are removed from the analyses, the total sample size is reduced to 23 populations 16 of which were dominated by resistant clones, 10 of which were dominated by susceptible clones, and 1 of which had the same number of each phenotype *(*p = 0.42). A post-hoc power analysis revealed a minimal required sample size of 6 populations (actual power = 0.813). Thus the overrepresentation of the replicated pairs of genotypes did not change the outcome of the analysis.

We tested for a consistent effect of genotype pairs on the outcome of the competition assays by looking whether the populations within the four groups of replicated populations were more likely to yield the same competition outcome. We found a significant effect indicating consistency among population with the same phenotypes prevailing in competition more often then not between replicated populations (p = 0.005). A post-hoc power analysis indicated a minimal required sample size of 6, our actual sample size was 19 (actual power = 0.828).

Experiment 2

Due to the severely restricted feeding regime, only nine of the 47 populations in Experiment 2 survived until the end of the experiment. Of these nine populations, three were started with an identical composition and four were unique combinations. Two of the replicated populations had to be discarded from analyses since they contained less than 5 hosts at the time of sampling. All other populations had enough hosts and were used in the analyses (Mean = 19.4, sd = 7.7). In this setup, no effect of host phenotype could be detected either (p = 0.381, Figure 2). A post-hoc power analysis revealed a minimal required sample size of 3 (actual power = 0.964), indicating that the severely reduced sample size of this experiment would likely not have hindered effect detection at the 0.05 level.

Experiment 3

Twenty-seven of the 31 experimental populations established at the start of the experiment survived and yielded enough hosts for analysis (Mean = 36.7, sd = 11.9). As in the two previous experiments, no effect of host phenotype was detected (p = 0.697, Figure 3). Post-hoc power analysis revealed a minimal required sample size of 5 populations in order to reach statistical significance at 0.05, thus the non-detection of an effect is unlikely attributable to limited sample size (actual power = 0.826).

Comparison across experiments

In addition to the separate comparisons performed for each experiment, we also looked for possible genotype effects across experiments. By comparing the outcome of competition between different trials with the same host combinations we can detect a consistent effect of the host genotypes. This analysis was performed between Experiment 1 and Experiment 3. Experiment 2 was not included because it had too few shared host pairs. We did not find a difference in the outcomes of competition between the two experiments (Chi^2 = 1.23, p = 0.77; Figure 4), indicating that winning genotypes in experiment 1 also won competition in experiment 3.

Discussion

Using both semi-natural and controlled laboratory conditions, we tested for a cost of resistance in the host-parasite system *Daphnia magna-Pasteuria ramosa*. This was done using resistance to two strains of the parasite *P. ramosa* as phenotypic marker for broader resistance to sympatric *P. ramosa* and competing resistant hosts against their susceptible counterparts. In none of our experiments did we find evidence for an advantage of being susceptible in the absence of parasitism (Table 1, Figures 1-4). The strength of selection by way of resource availability imposed on competing genotypes has been discussed as a revealing parameter in the study of life-history trade-offs (Stearns 1992). Studies on the cost of resistance in bumblebees and in fruit flies found that trade-offs were revealed when measured under stressful conditions such as starvation or strong intraspecific competition for food (Kraaijeveld and Godfray 1997; Moret and Schmid-Hempel 2000). We used restricted feeding regimes in each of our experiments to create conditions of scarce resource availability. Competing hosts under these stressful conditions for two months (representing ~15 host clutches) was expected to reveal costs of resistance by lowering compensatory resource intake (Moret and Schmid-Hempel 2000). Yet there was no observable difference in fitness between resistant and susceptible hosts under any of the three feeding regimes. These results do not support the existence of a cost of resistance to *P. ramosa* in this population of *D. magna.* Our results are in agreement with that of previous research that could also not find indications of a cost of resistance in other *Daphnia* populations against the bacteria *P. ramosa* (Little et al. 2002). *P. ramosa* is expected to exert strong selective pressure on host populations as it can attain high prevalence and has strong impacts on host fitness (Little and Ebert 2000; Ebert et al. 2001; Duncan et al. 2006; Jensen et al. 2006). Consequently, alleles for resistance to these parasites should be strongly selected for, even if the cost of resistance is high.

Host-parasite systems are commonly divided into two alternative categories based on the underlying genetic mechanism of coevolution – those driven by

matching-alleles models (MAM; Frank 1993), and those that are governed by gene-for-gene models (GFG; Thompson and Burdon 1992). MAM describe the genetics of infection as an exact effectors-receptors match, where infection requires a specific match between a given parasite genotype and a host genotype. Only a subset of host genotypes may be successfully infected by a particular parasite genotype (Frank 1993). GFG does not require such specificity, there a given parasite genotype is able to infect a wider range of host genotypes, albeit not all as efficiently, as it only needs to recognize one among many host receptors. Similarly hosts may be more or less susceptible to a wide array of parasites, and mounting an immune response requires only the recognition of one among many parasite effectors (Frank 1992). Evidence from natural systems for GFG dynamics are numerous and come mostly from plant-parasite systems (Flor 1971; Thompson and Burdon 1992), whereas empirical evidence for MAM mechanisms is still sparse. MAMs underlie the maintenance of polymorphism and sexual reproduction as predicted by the Red Queen theory (Frank 1993). These models readily lead to co-evolutionary cycling of alleles for host resistance and parasite infectivity and preclude the possibility of universal parasite infectivity or universal host resistance. Alternatively, GFG models tend to lead to directional selection where alleles for resistance in the host, or for infectivity in the parasite, can sweep through the population to fixation (Thompson and Burdon 1992). However, if resistance comes at a cost, the GFG model may also maintain polymorphism for host resistance and lead to co-evolutionary cycling (Sasaki 2000; Agrawal and Lively 2002). Therefore indications of cycling, such as the absence of selective sweeps (Woolhouse et al. 2002), may be misinterpreted as evidence for an underlying MAM. Taken together, evidence for both cycling and costs of resistance would suggest an underlying GFG model, while the absence of costs would reinforce the likelihood of MAM. Because costs of resistance are one of the key distinguishing features between GFG and MAM, this is an especially important characteristic for understanding which genetic mechanisms underlie host-parasite systems and the type of co-evolutionary dynamics involved (directional selection, co-evolutionary cycling).

Based on evidence of coevolutionary cycling in natural populations of *D. magna* and *P. ramosa* (Decaestecker et al. 2007, Andras et al. in prep) and the simple and highly specific genetics of host resistance (Carius et al. 2001; Luijckx et al. 2011, 2013, Routtu & Ebert in prep, Metzger et al. in prep), this host-parasite system has been proposed as one of the few documented examples of MAM dynamics (Luijckx et al. 2013). In the present study we observed no cost of resistance in a simple natural population of *D. magna* that was polymorphic for resistance. These findings reinforce the hypothesis that *D. magna* and *P. ramosa* coevolve based on a matching-allele model leading to Red Queen dynamics.

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Table 1. Summary table of summed results of all three experiments.

Experiment	SS	RR	Total
1)	27	19	46
2)	5	3	8
3)	13	14	27
Total	45	36	81
Percentage	55.56	44.44	100

Figure 1. Experiment 1 - Outcome of competition assays in mesocosms in semi-natural conditions.
Estimated proportion of susceptible (red) and resistant (green) hosts.

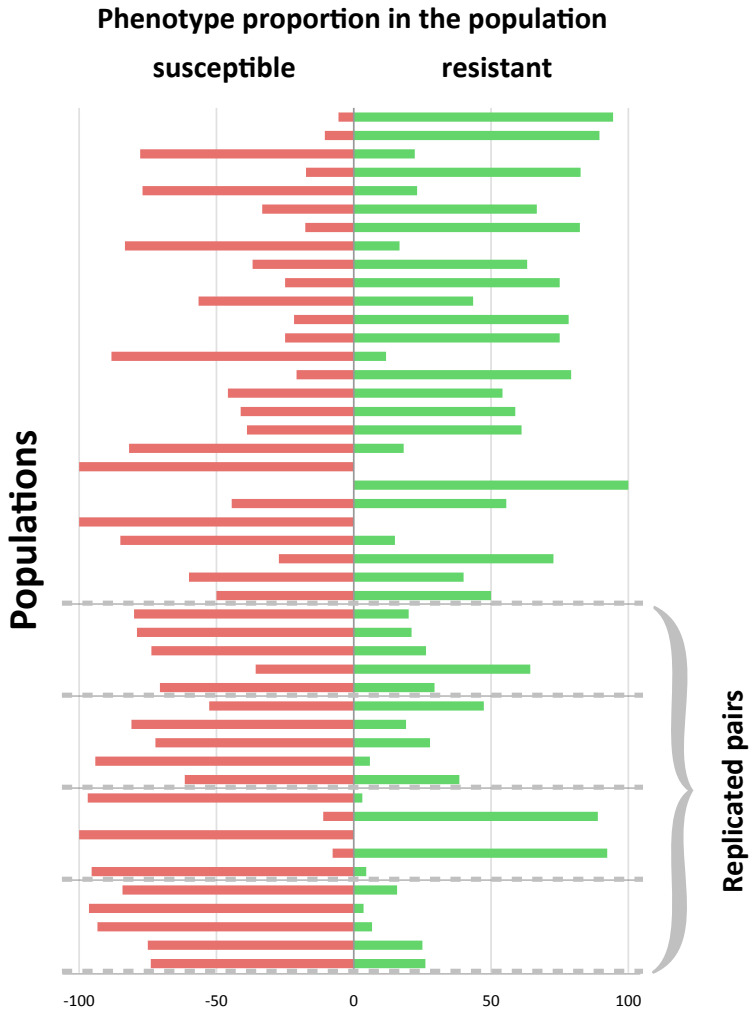


Figure 2. Experiment 2 - Outcome of competition assays in 400mL jars in controlled conditions under scarce feeding.
Estimated proportion of susceptible (red) and resistant (green) hosts

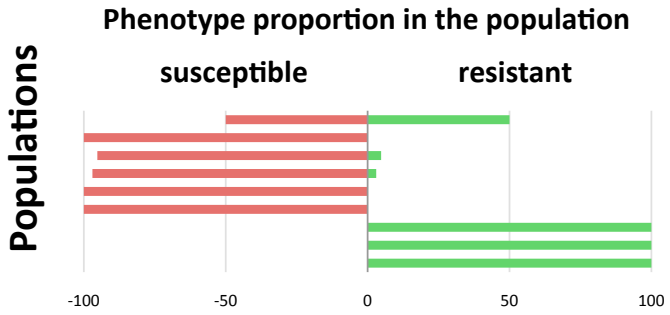
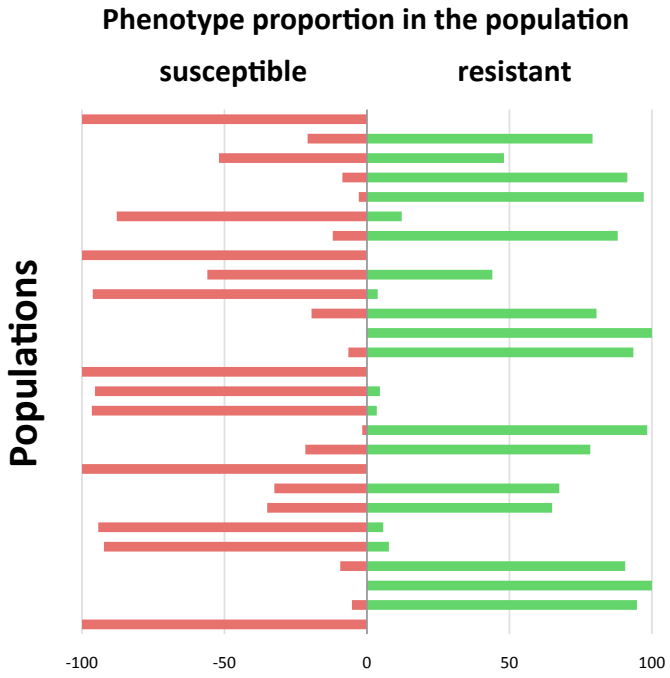


Figure 3. Experiment 3 - Outcome of competition assays in 400mL jars in controlled conditions under regular low-food feeding.
Estimated proportion of susceptible (red) and resistant (green) hosts.



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Author contributions

César M.J.A. Metzger[a] – designed & performed all experiments, collected the data, analyzed the data, and wrote the manuscript.

Jason P. Andras[a,b] – designed & helped perform the experiments, helped analyze the data, helped write the manuscript.

Dieter Ebert[a] – helped write the manuscript, and provided supervision.

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V.

Biophysical aspects of the *Pasteuria ramosa-Daphnia magna* interaction:

"Temperature differentially affects the three-step infection process of a spore forming bacteria"

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Abstract

Spore-forming parasites can resist harsh environmental stressors for long periods of time. By resisting certain disinfection procedures and pasteurization, they are an aggravating threat to health care and the food industry resisting. In natural populations, they can survive desiccation and freezing and lead to epidemics when favorable climatic conditions are restored. We investigated thermoresistance of spores of the bacterial parasite *Pasteuria ramosa*, which causes infection in the ecologically important freshwater crustacean *Daphnia magna*. We found that three steps of the infection process were differently affected by temperature. Activation of the spore resisted high temperatures (up to 99°C) while attachment and infection were inhibited at lower temperatures (68 and 66°C respectively).

Introduction

Evolution provided spore-forming microorganisms with a solution to resist prolonged periods of time in a state of dormancy against acute environmental stressors (Kennedy et al. 1994). A number of pathogenic microorganisms, such as diverse Firmicutes bacteria and Microsporidia, are spore formers, which helps them survive outside their target hosts and thus allows them to await successful transmission to new hosts for many years (Poulin 2007). Pathogenic Gram-positive bacteria are good examples of such organisms, with endospores of *e.g. Bacillus anthracis* or *Clostridium botulinum* lasting many years in the environment before being picked up by a host and resuming metabolism and growth, leading to infection (Manchee et al. 1994). Due to their hardness, spores are of high concern for the food industry, resisting pasteurization procedures at potential risks for health (Andersson et al. 1995). Their ubiquity in the environment contributes also to the role in food spoilage and food-borne disease they play (Setlow 2006). Economic and agronomic relevant species of spore-formers, such as the biocontrol agent *Pasteuria penetrans* used in the control of nematode crop pests, present the advantage of easy and safe storage before field application as well as offer more persistent and targeted treatment in the field as they normally resume metabolism only upon encounter with their control target (Chen and Dickson 1998).

Pasteuria ramosa is a highly virulent and potentially very prevalent parasite of the ecologically important freshwater crustacean *Daphnia magna* (Ebert et al. 1996; Duncan et al. 2006). *P. ramosa* is closely related to *P. penetrans* and is an ancestral member of the *Bacillus-Clostridium* clade (Eubacteria:Firmicutes, Charles et al. 2005; Schmidt et al. 2008), which comprises iconic human pathogenic and long-lasting endospore-forming *B. anthracis*, *C. botulinum*, and *C. tetani*. *P. ramosa* also forms endospores that lay dormant alongside resting eggs of its host for decades in pond sediment, thus archiving the genetic diversity of both host and parasite, a valuable resource for evolutionary biology (Ebert et al. 1996; Decaestecker et al. 2004, 2007; Andras et al. in prep). Upon ingestion by the filter-

feeding host the bacterial spore activates and, provided the parasite and host genotypes match (Luijckx et al. 2011), it attaches to the susceptible hosts' oesophagus where it is thought to germinate and penetrate the host cuticle leading to infection (Duneau et al. 2011). This series of sequential events leading to the infection of the susceptible host were described as the steps of the infection process (Duneau et al. 2011) and comprise at least the activation, the attachment and the proliferation steps (also sometimes referred to as the infection step). The proliferation step may be further divided into the developmental stages of germination, penetration, outgrowth, vegetative growth and sporulation; although the distinction between some of these stages may be difficult to observe due to some overlap in time of onset and the order of the first two of these stages is still unknown. Gene conservation within the *Bacillus-Clostridium* clade was found for several spore proteins, an indication of a potential ancient common origin for much of the spore structure and function in this clade (Setlow 2006). Structural properties of spores, such as the composition of the different spore layers, have been shown to play a role in resistance to stressors such as chemicals and radiation (Driks 1999). In *Bacillus subtilis*, as well as other species of the *Bacillus-Clostridium* clade, protection against heat is most likely independent of structural aspects of the external layers of the spore, but rather dependent on the concentration of certain molecules found within the spore core, such as water, dipicolinic acid (DPA), small acid-soluble proteins (α/β-type SASP) and mineral ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, K^{+} and Na^{+}) (Setlow 2006). No information is available on the role of these components in *Pasteuria*. Resistance of *P. penetrans* spores to heat was found to stop after 1h treatment at 130°C as spores were not able to attach to their nematode host anymore, although infection was already impaired after 30 min at 80°C (Dutky and Sayre 1978). Several optimal growth temperature studies were conducted and showed that *P. penetrans* developed quicker within their nematode host at 30°C and 35°C than at 25°C or lower (Hatz and Dickson 1992), but these studies did not investigate extreme temperature ranges. *B. subtilis* spores treated at pasteurization temperature of 85°C were found to have delayed germination time and delayed first

vegetative doubling, however, after that vegetative growth resumed at a normal rate (Pandey et al. 2013). Investigating the role of temperature does not only serve our proximate understanding of spore structure and resistance but also informs our ultimate understanding of host-parasite dynamics and epidemiology.

In 2003, during one of the hottest recorded summers in Europe (Schär et al. 2004), heat was observed to influence population dynamics of *P. ramosa* with high temperatures throughout the epidemic season leading to extreme prevalence (100%) of the parasite and a severe epidemic. This observation was possibly due to *P. ramosa*'s greater infectivity and virulence at higher temperatures or to a reduction of water level in the pond leading to higher encounter rates between planktonic hosts and the sporebank in the sediment (Duncan et al. 2006). Understanding optimal growth temperature and growth-limiting temperatures of parasites has profound implications for the study of epidemiology in natural systems, and *P. ramosa* was found to be temperature-dependent for infection success as well as the fitness consequences it imposes upon its host (Mitchell et al. 2005; Duncan et al. 2006; Vale and Little 2009). Predictive meteorological scenarios point towards higher mean temperatures as well as more variability, leading to more frequent occurrence of abnormally warm summers (Schär and Jendritzky 2004). In this respect, predictive epidemiology and host-parasite ecology require a solid understanding of the temperature limitations and growth optima of parasitic bacteria. In light of these observations, and due to high conservation of structural genes of the spore within the *Bacillus-Clostridium* clade, the findings of heat resistance in *P. penetrans*, and the finding of continued infectiousness of spores of *P. ramosa* after decades of dormancy in the environment, we suspect that *P. ramosa* may exhibit similar structural resistance to heat as other related spore-formers such as *B. anthracis* and *B. subtilis*.

In the present study, we investigate spore heat resistance of the unculturable parasite *Pasteuria ramosa* and its consequences on the infection process. In seven experiments (Table 1), we expose spores of the parasite to temperatures covering the range of temperate (20°C) to hot environmental conditions and beyond (up to 99°C), and examine separetely the effect of these treatments on three major steps of

the infection process in the host *Daphnia magna*: the activation of the parasite spore as it is filtered from the water column by its freshwater zooplankter host, the attachment of the spore to the oesophagus of the host, and the success of infection of the host by the parasite. Strikingly, we find that activation is highly resistant to extreme temperatures but attachment and infection success are more sensitive. We discuss implications for the mechanistic understanding of these steps and the spore structure, and propose that the parasite's resistance to heat is well adapted to always be infectious in conditions in which hosts may be active.

Materials and Methods

Study system

Pasteuria ramosa Metchnikoff, 1888, is an endospore-forming Gram-positive bacteria from the *Bacillus-Clostridium* clade (Firmicutes:Pasteuriaceae) and a parasite of the planktonic freshwater crustacean *Daphnia* (Phyllopoda:Cladocera). Waterborne spores of the bacteria are picked up from the environment by the host during filter-feeding or deposit feeding. Upon ingestion the spores activate and attach to the epithelium of the host's oesophagus (Figure 1). It is thought that at this stage the bacteria germinate and penetrate the host cuticle into its body cavity, similarly as observed in the closely related *P. penetrans* that penetrates the cuticle of its nematode host after attachment (Chen and Dickson 1998). This then leads to the bacteria's vegetative growth phase within the host body cavity. This phase of bacterial proliferation, which may last from 20 to 70 days approximately, is followed by the sporulation process and finally ends with the entire host body filled with millions of spores (Jensen et al. 2006). At this stage the host dies and spores are released into the environment by the host's decaying body (Ebert 1996; Duneau et al., 2011; Sayre et al., 2009). The vegetative growth of *P. ramosa* induces castration and gigantism of the host, which leads to strong fitness consequences for the host and benefits parasite reproduction (Ebert et al. 2004).

The host, *Daphnia magna* Straus, 1820, has a holarctic distribution (ranging from the subtropical zone to near arctic regions) where it inhabits small temporary pools to medium permanent lakes. *D. magna* reproduces through environmentally induced cyclical parthenogenesis and its sexual eggs (resting eggs called ephippia) may stay dormant for decades in the sediment (Decaestecker et al. 2004, 2007; Andras et al. in prep). It is considered a keystone species in freshwater ecosystems (Ives et al. 1999) and is a well-established model species in ecotoxicology, ecology and evolutionary parasitology (Ebert 2011).

In this study we test heat-resistance of spores of two different genotypes of the parasite, called C1 and C19, that were isolated in a previous study from infected animals sampled in Russia and Germany respectively (see Luijckx et al. 2011). As the parasite is unculturable, all observations were performed by exposing the bacteria to live hosts and examination of the state of the bacteria within the host. Two different genotypes of the host were used, HU-HO2 and BE-M10, originally sampled from Hungary and Belgium respectively. These two host genotypes were chosen because they are well studied and have often been used in combination with C1 and C19 to study host-parasite interactions (Duneau et al. 2011; Luijckx et al. 2011; Duneau and Ebert 2012; Hall and Ebert 2012; Clerc et al. in prep).

Temperature calibration

Spores were heat-treated with an Eppendorf Thermomixer "comfort" (Vaudaux-Eppendorf AG). Prior to experiments we calibrated the Thermomixer using a TES 1300 (Type K) thermometer. This was done by measuring the temperature after 5 min, with the thermometer probe inside an eppendorf tube placed into the Thermomixer. The temperature precision of the Thermomixer was evaluated for three temperatures and was found to have the following deviations from the expected temperature: 20°C + 0.1°C, 60°C -0.5°C, and 99°C – 1.7°C.

Preparation of spore solutions

For all experiments described hereafter, spore solutions were obtained from laboratory cultures in which the parasites are grown in live hosts. This standardized

procedure repeats the transmission process as it happens in nature, in that uninfected hosts ingest spores released from a dead infected host and become infected. This is done by collecting spores through crushing of an infected host's body and suspending the spores in the rearing medium of uninfected hosts. From these parasite cultures, spore solutions of two parasite genotypes (C1 and C19) were obtained. Using a Thoma haemocytometer, concentrations were estimated and solutions were adjusted by dilution with ADaM (ADaM: artificial freshwater medium for zooplankton modified from Klüttgen et al. 1994 by using only 5% of the recommended SeO_2 concentration).

Spore activation (Experiment 1)

Aliquots of the spore solutions (C1 and C19) were heat-treated at 5 different temperatures (20, 40, 60, 80 and 99°C) for 1 hour and were then left to cool down at room temperature for 10 min (20°C ± 2°C). For each temperature treatment x parasite combination two genotypes of hosts (HU-HO2 and BE-M10) were each exposed to 50'000 treated spores in 500 µL ADaM in Eppendorf low-bind tubes for 1 hour at room temperature. Each treatment was replicated 10 times. Control treatments, in which spores were prepared similarly as before but without host exposure, were replicated only twice. As parasites are filtered from the water and ingested by the feeding host, the bacteria detect the host and activate. During ingestion, many bacteria will attach to the host oesophagus but most will directly pass in the host gut, therefore we examined the bacteria in the host's gut in squash preparations to assess the spore status (activated or non-activated) with phase-contrast microscopy (400x magnification). Prior to their examination, hosts were individually washed in fresh ADaM to avoid carrying on non-ingested spores from the medium onto the squash preparation. Activated spores were recognizable by their distinctive deployed peripheral parasporal fibers (that give the activated spore a sombrero-like shape)(Figure 1). When activation takes place, all the spores observed in the host gut are activated, only rare isolated cases may be found non-

activated, therefore we did not quantify the number of activated to non-activated spores.

Spore attachment (Experiments 2, 3 & 4)

C1 and C19 parasite spore solutions were fluorescently labeled following the protocol detailed in Duneau et al. (2011). After this procedure, concentrations were re-estimated using a Thoma haemocytometer and solutions were adjusted by dilution with ADaM. For each parasite spore solutions, aliquots were incubated for 5 min at the chosen treatment temperatures. The range of temperatures used differed between experiments, with 21 heat-treatments (20, 40, 50 to 80 in increments of 2, 90, 95 and 99°C) in Experiment 2, 5 heat-treatments (68, 69, 70, 71, 72°C) in Experiment 3 and 8 heat-treatments (20, 60, 66, 68, 70, 72, 74 and 80°C) in Experiment 4 (Table 1). After incubation tubes were left to cool down 10 min in the dark, to avoid photobleaching of the fluorescently dyed spores, at room temperature (20°C ± 2°C). Host exposure was performed in 96-wells plates with one host per well in ADaM (also in the dark) and 5'000 heat-treated labeled spores. The number of hosts replicates to each parasite and temperature combinations varied between experiments with respectively 4, 8 and 8 hosts per host genotype x parasite genotype x temperature combination in experiments 2, 3 and 4 (Table 1). After 45 min of incubation, the medium was removed from the wells and each host was checked for presence of attached parasite spores in the oesophagus with a fluorescence microscope (magnification 400x). Details of the procedure and scoring of the attachment phenotype are described in Duneau et al. (2011).

Effect of temperature on infection (Experiments 5, 6 & 7)

Similarly to our attachment experiments, different ranges of temperature were used in three experiments assessing infection success after heat-treatment of spores. We treated spores at 21 temperatures (20, 40, 50 to 80 in increments of 2, 90, 95 and 99°C) in Experiment 5, 3 temperatures (54, 62, 70°C) in Experiment 6 and 8 temperatures (20, 60, 66, 68, 70, 72, 74 and 80°C) in Experiment 7. For each

parasite genotype (C1 and C19) x temperature treatment combinations, parasite spore solutions were aliquoted into low-bind Eppendorf tubes for each temperature treatment, and were heat-treated for 5 min and then left for 10 min to cool down at room temperature (20°C ± 2°C). In 12-wells plates, 6-8 days old hosts (of two genotypes, HU-HO2 and BE-M10) were individually exposed to 10'000 (increased to 50'000 in Experiment 6, see Table 1) treated spores in 2mL ADaM. The number of host replicates exposed to each parasite and temperature combinations varied between experiments with respectively 5, 20 and 7 hosts per host genotype x parasite genotype x temperature combination in experiments 5, 6 and 7 (Table 1). Well-plates were kept in an incubator at 23°C ± 1°C and light:dark cycle of 16:8. Hosts were fed every two days 5 Mio algae cells (*Scenedesmus* sp.) and transferred to new well-plates and fresh medium every week. After one month, all hosts were crushed and checked with phase contrast microscopy (magnification 400x) to assess infection status.

Statistics

Statistical analyses were performed in the program R v.2.12.0 (R Development Core Team 2010). Generalized linear mixed models were performed to analyze the effect of temperature, host and parasite on the response variables: activation, attachment or infection. When interactions were not significant we excluded them from the model to increase power.

Results

Effect of temperature on the activation step (Experiment 1)

Treated spores fed to *Daphnia* were found activated in 92.0±2.6% of all examined host guts (Figure 2). Host and parasite genotypes did not influence activation (Table 2). A significant effect of temperature (p = 0.019, Table 2) was found, with one replicate (C19 – BE-M10) without activated spores at 20°C (97.5±1.5%) and 1 or 2 replicates per temperature treatments without activated spores at higher temperatures (100% at 40°C, 95.0±1.8% at 60°C, 87.5±3.0% at 80°C and 90.0±2.5% at 99°C). Spore activation was not observed in the absence of the *Daphnia* host (data not shown).

Effect of temperature on the attachment step (Experiments 2, 3 & 4)

In Experiment 2, attachment was completely inhibited when spores had been treated at 72° C and above (Figure 3). Host and parasite genotypes did not have an effect on attachment (Table3). A significant effect of temperature (p <0.0001) was found (Table3). In Experiment 3, spores treated at 71°C and above did not attach. Spores of C1 treated at 68°C attached in 57% of replicates in HU-HO2 but not when treated at 69°C or above. Spores of C1 attached in BE-M10 at 87.5, 25 and 25% when treated at 68, 69 and 70°C respectively. In the host HU-HO2, spores of C19 treated at 69°C attached in 12.5% of replicates but none treated at 68°C or above 69°C attached. Spores of C19 did not attach in the host BE-M10 independent of the heat-treatment (Figure 3). A significant effect of temperature (p < 0.0001) and parasite (p < 0.0001) was found but host did not have an effect (Table 4). In Experiment 4, attachment was inhibited in spores treated at 70°C or higher. Spores of C1 treated at 68°C did not attach in the host HU-HO2 but attached in BE-M10 in 12.5% of replicates. Spores of C19 treated at 68°C did not attach in either host. Spores of C1 treated at 66°C attached in 87.5% of replicates in HU-HO2. For all other heat treatments, spore, and host combinations, attachment was observed in all

replicates (Figure 3). A logistic model found a significant effect of temperature ($p < 0.0001$) but neither host nor parasite had an effect (Table 5).

Effect of temperature on the proliferation step (Experiments 5, 6 &7)

In Experiment 5, infection was inhibited when spores had been treated at 66°C and above. Below this temperature the number of replicates with successful infection observed augmented with lower treatment temperatures (Figure 4). A logistic model found a significant effect of temperature ($p = 0.004$) and parasite ($p = 0.001$) and an interaction between temperature and parasite ($p = 0.006$) but host had no effect (Table 6).

In Experiment 6, infection success of C1 spores treated at 70°C was observed in 46.7% and 42.1% of replicates in HU-HO2 and BE-M10 respectively, whereas spores of C19 did not lead to infection when treated at this temperature. Spores of C1 heat-treated at 62°C infected 71.4% and 60% of HU-HO2 and BE-M10 respectively, whereas spores of C19 infected 90.9% and 94.7% respectively. Spores of C1 treated at 54°C infected 71.4% and 85% of HU-HO2 and BE-M10 respectively, and spores of C19 85.7% and 88.9% respectively (Figure 4). A logistic model did not find a significant effect of temperature nor host, but parasite ($p = 0.02$) had an effect and temperature and parasite showed an interaction ($p = 0.02$)(Table 7).

In Experiment 7, infection was inhibited in spores treated at 66°C and above. Spores of C1 treated at 60°C did not attach in HU-HO2 and attached in only 33.3% of replicates in BE-M10. Spores of C19 treated at 60°C attached in only 16.7% of replicates in HU-HO2 and did not in BE-M10. Spores of C1 and C19 treated at 20°C attached in 40% of replicates in HU-HO2 and in 71.4% of replicates in BE-M10 (Figure 4). A significant effect of temperature ($p = 0.0005$) and host ($p = 0.009$) were found but parasite did not have an effect (Table 8). Temperature and host showed an interaction ($p = 0.02$).

Discussion

Arguably the defining attribute of spore-forming bacteria is their ability to resist to environmental stressors. The spore provides structural resistance over long periods of dormancy until germinants are detected leading to the activation and germination of the spore (Foster and Johnstone 1989; Setlow 2003). The bacterial cell, then, resumes metabolic activity and vegetative growth. Reverting to the vegetative cell requires several physiological and biochemical events to take place (Foster and Johnstone 1989; Setlow 2003; Moir 2006), each of which may be affected by stressors endured before activation, during dormancy, and as early as during sporulation (Palop et al. 1999). We find that three steps of the infection process of *Pasteuria ramosa*, as described in Duneau et al. 2011, are differentially affected by a history of high temperature during spore dormancy. We first discuss activation (Experiment 1), then discuss attachment Experiments 2, 3 & 4 pooled together as their results were similar, followed by infection Experiments 5 & 7 pooled together and finally discuss infection Experiment 6 separately as its results were somewhat different.

Effect of heat-treated spores on the activation step

The first step of the infection process of *P. ramosa* in *Daphnia*, the activation of the spore, remained unaffected by temperatures as high as 99°C in more than 87% of replicates. In a few isolated cases (1 or 2 replicates per host-parasite-treatment combinations, Figure 2) we observed that spores were present in host guts but had not activated. Although a statistical test found an effect of temperature, we cannot exclude that other factors are responsible for this result as we also found solely non-activated spores in 1 replicate of spores of C19 treated at 20°C and exposed to the host BE-M10 (Figure 2). The apparent imperviousness of this mechanism to temperature suggests thermostability of structural elements of the *P. ramosa* exosporium. The exosporium of *P. ramosa* has not yet been characterized, but in related bacteria of the genus *Bacillus* it was found to be a chemically complex

structure constituted mainly of proteins (more than 50%), but also of amino and neutral polysaccharides, lipids and ash (Matz et al. 1970). Some of these proteins are thought to be important for the interaction of the bacteria with the environment or with their host (Sylvestre et al. 2002) and to play a role in spore activation and germination (Redmond et al. 2004). Therefore denaturation of certain proteins through heat may be expected to lead to impaired environment-spore or host-spore interactions, in which the spore may not recognize its proximity with the host and stays dormant. Alternatively protein denaturation may lead to inhibited activation, where, even though activation signals are recognized, the process does not occur. Understanding the thermostability of structural elements of the exosporium and of the activation mechanism will most likely require the characterization of the exosporium of *P. ramosa*.

Effect of heat-treated spores on the attachment step (Experiments 2, 3 & 4)

The second step of the infection process is the attachment of the activated spores to the oesophagus of the host. In all three experiments, the ability of activated spores to attach to their host was not affected by temperature below 67°C. Inhibition of attachment was first observed when the spores had previously been treated at 68°C and complete inhibition was found above 70°C (Figure 3). Unlike Experiments 2 and 4 where a clear cut-off temperature could be observed, the results in Experiment 3 were more variable, therefore the cut-off temperature is not clear. In Experiment 3 an effect of parasite was also found, indicating a difference in thermoresistance between parasite genotypes. This difference may be due to a difference in the compounds involved in attachment between parasite genotypes, a distinct possibility since both genotypes have been shown to infect different ranges of hosts (Luijckx et al. 2011), or to a small differences between the conditions in which the spore crops of both parasite genotypes were cultured as conditions during sporulation have been shown to influence later spore properties (Palop et al. 1999; Mah et al. 2008). Small changes in conditions *e.g.* temperature or minerals, during sporulation of the spore crops may also account for the ~4°C shift in temperature at which the attachment stopped among experiments (Figure 3).

Temperature or concentration of minerals during sporulation may strongly influence spore resistance to stressors, affect germination or even later steps in bacterial development (Palop et al. 1999; Mah et al. 2008). The concentration of certain ions, such as $CaCl_2$, in solution during sporulation has been found to affect both sporulation time and spore resistance to heat in *Clostridium* (Mah et al. 2008). Heat resistance in spores of *B. subtilis* has been shown to be correlated to core dehydration, a process thought to be favored by higher concentrations of minerals in the spore core (Setlow 2006). Notably calcium plays an important part in the process as it chelates dipicolinic acid (DPA), a contributing compound to dehydration representing ~10% of the spore mass (de Vries 2004). Therefore even slight differences in calcium and salts in the host rearing medium (ADaM), and thus possibly the host body cavity, during sporulation may have affected heat resistance of the *P. ramosa* spores used. Furthermore, studies of various species of *Bacillus* found that temperature during sporulation influenced heat-resistance of the obtained spores, with higher sporulation temperatures leading to higher temperature tolerance of the spore, although this relationship was not always linear (discussed in Palop et al. 1999). This may contribute to explain differences in attachment found between the results of Experiments 2-4. Further investigation of heat-resistance of *P. ramosa* using different concentrations of minerals and other compounds in the host-rearing medium during sporulation may shed light on the variability of the process. Our findings indicate the need for more stringent culturing protocols for the production of spores depending on the expected utility of the spores.

Little is known about the molecular mechanisms that allow *P. ramosa* to attach to its hosts' oesophagus. Using electron microscopy Duneau et al. (2011) showed the presence of microfibers on the surface of the activated spore's disc, the parasporal peripheral fibers (ppf, Figure 1), that are thought to allow spores to attach to the cuticle of the host oesophagus. The exact composition of these fibers is not known. Our results indicate that some components of the attachment mechanism are impaired by temperature above 68°C, this may be due to the

denaturation of certain compounds, such as the microfibers, and warrants further investigation.

Effect of heat-treated spores on the proliferation step (Experiments 5 & 7)

The last step of the infection process is commonly referred to as the proliferation step or infection step but may be subdivided in five further steps corresponding to developmental stages of the bacterium. These stages are germination, penetration (of the host), outgrowth, vegetative growth and sporulation. As we only scored the presence or absence of newly grown spores in hosts one month after exposure, we cannot discriminate between these stages and are effectively only measuring infection success (i.e. infected or not infected). In Experiment 5 and 7, we found that spores heat-treated at temperatures of at least 66° and above, did not lead to infection in hosts (Figure 4). At lower heat-treatments we found variation in infection success (Figure 4); therefore damages by heat may already have been inflicted at lower temperatures. The magnitude of the effect on the proliferation step may depend on the localization of the damages among spore compounds (proteins or DNA), whether spores are able to repair the damages early during germination (Setlow 1995) and on differences of sensitivity or repair capacity between parasite genotypes. Furthermore, an effect of parasite genotype but not host genotype was found in experiment 5 and conversely an effect of host genotype but not parasite genotype was found in experiment 7. A study using the same combination of parasite (C1, C19) and host (HU-HO2, BE-M10) genotypes demonstrated that both host and parasite genotypes have an influence on the proliferation step, although the respective effects varied temporally during the proliferation step (Hall and Ebert 2012). Unlike the activation and attachment steps, the proliferation step is expected to be very complex. It comprises physiological and biochemical processes such as germination, resuming bacterial metabolism and development, growing the infection peg, piercing the host cuticle, outgrowth, vegetative growth and finally sporulation. It requires transcription and translation to operate as well as enzymatic activity to resume during early germination.

Therefore, in comparison to activation and attachment, much more variation in the effects of heat damages may be expected (as observed in Experiment 5).

Heat-treating the spores may have killed the bacteria or inhibited one of the five stages of the proliferation step. In *Bacillus* sp., it is thought that proteins embed during sporulation in the spore coat may initiate the cracking of the coat thereby starting the germination process (Driks 1999), the first stage of the proliferation step. Following a similar mechanism coat proteins of *Pasteuria* may locally crack the coat and initiate germination, followed by the extrusion of *Pasteuria*'s infection peg that penetrates its hosts cuticle, as was observed in *P. penetrans* (Bird et al. 2003). Heat-treating spores may have denatured certain coat proteins, thereby inhibiting the initiation of germination and preventing the penetration of the host. While spore heat resistance in *Bacillus* bacteria is thought to be mainly dependent on water content of the spore core (Gerhardt and Marquis 1989), spore DNA was found to be protected by specific proteins, small acid-soluble spore proteins (α/β-type SASP), that also play a role in resistance to heat (Setlow 1988). Spores of bacterial mutants lacking these proteins were found to be more sensitive to heat than wild-type spores (Mason and Setlow 1986; Hackett and Setlow 1988). α/β-type SASP are highly abundant, constituting 3-6% of the total spore proteins (Setlow 2006), and, together with γ-type SASP (which make up another ~8% of spore core proteins), their degradation provides the spore with the necessary amino acids to resume protein synthesis during germination and outgrowth (Hackett and Setlow 1988). Heat-induced denaturation of these proteins during spore dormancy may therefore have led to heat-induced damages to the DNA, causing a later dysfunction of bacterial development. Another possible involvement of α/β-type SASP in developmental inhibition may be if their degradation did not succeed because the enzymes required for the process were damaged by heat; their continued saturation of the DNA may have prevented transcription leading to arrested development (Sanchez-Salas et al. 1992; Hayes and Setlow 2001).

Effect of heat-treated spores on the proliferation step (Experiment 6)

While Experiments 5 and 7 had similar results, Experiment 6 did not yield the same results (Figure 4). In Experiment 6, infection by C19 was inhibited at 70°C, as was expected after the observations of Experiment 5 and later confirmed with those of Experiment 7, but infection by C1 was not inhibited. After performing Experiment 5, as we did not expect infection to be possible after treating spores at 66°C or more, for Experiment 6 three treatment temperatures (54, 62 and 70°C) were chosen to re-test the effect of heat-treating spores on the proliferation step. Due to the noisy results of Experiment 5 (Figure 4) and the observation that infection success is dose-dependent (Ebert et al. 2000), we decided to perform Experiment 6 with a higher dose of treated spores and a higher replication (using 20 instead of 5 hosts per host genotype x parasite genotype x temperature combinations). Using 50'000 spores instead of 10'000 (Experiments 5 and 7) we obtained successful infections with C1 even after treating spores at 70°C. These results indicate that not all bacteria had their ability to infect inhibited by the temperature treatment. Using a higher dose of spores may have lead to a higher amount of less heat-damaged spores exposed to the host in the same volume of medium (ADaM). In turn this may have lead to a higher encounter rate between these less-damaged spores and the host and thus to more infections. As C19 did not yield infections after treatment at 70°C, we suspect that it was less resistant to heat-induced damages.

Is there a temperature at which spores attach but do not infect?

Comparing attachment (Experiments 2-4) and infection (Experiment 5 and 7, excluding Experiment 6 due to the higher spore-dose used), it appears that a range of temperature treatments (66-68°C) may inhibit the ability of *P. ramosa* spores to infect while preserving its ability to attach to the host. This suggests the possible use of heat-treated infection-inhibited *P. ramosa* spores as phenotypic markers to detect resistance phenotype of hosts without risking infecting them. In the *D. magna – P. ramosa* host-parasite system, attachment is correlated with infection (Luijckx et al. 2011). Because of the variability observed in the infection experiments and the shift

of ~4°C between attachment experiments for the temperature of inhibition of attachment, refinements of the experimental parameters (treatment temperature, spore dose, volume of medium for host exposure and duration of exposure) is required before the method may be safely used. Once refined, this method may be useful for *Daphnia* research as *Daphnia* is a small transparent animal with few phenotypic features usable to distinguish individual genotypes. Using color-labeled non-infective spores that are easily detected by fluorescence microscopy will allow the rapid discrimination of *Daphnia* individuals based on their resistance phenotype to genotypes of *P. ramosa*.

Comparison with upper temperature limitations of the host

Daphnia magna cannot survive more than a few hours at a temperature of 37°C and, throughout its geographic range, the highest average yearly temperature measured is 30°C (Yampolsky et al. 2014). Therefore the results reported in the present study indicate that *P. ramosa* has the potential to always be capable of activation (activation rate of 100% at 40°C), capable of attachment (attachment rate of 100% up to 67°C) and be infectious throughout *D. magna*'s geographic range, as *P. ramosa* spores are heat resistant at the highest temperatures of that range.

Conclusion

Environmental stressors may strongly reduce the survival of organisms. Spore-forming bacteria have championed resisting environmental stressors for long periods of time by growing several thick protective layers (Driks 1999; Setlow 2003), stopping their metabolism, protecting their DNA by saturating it with proteins (Setlow 2007), and dehydrating their core to resist high temperatures (Setlow 2006). Spores are the reason why, even with modern pasteurization techniques, food spoilage and foodborne disease remain of high concern, and human pathogens like *Bacillus anthracis* can resist decontamination procedures. *Pasteuria ramosa*, a relative of other typical spore-formers in the *Bacillus-Clostridium* clade, can survive decades in pond sediment and still be infectious (Decaestecker et al. 2004, 2007; Andras et al. in prep). Strikingly, *P. ramosa* spores may readily resist

temperatures as high as 60-70°C while remaining infectious. In the present study, we examined three major steps of the infection process of *P. ramosa* and found them differentially sensitive to a history of temperature, reflecting their underlying mechanisms.

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Table 1. Summary of experiments performed.
The various parameters of the experiments are indicated such as: spore dose, number of replicates, temperature range, and number of heat-treatments. Corresponding figures and tables are also indicated.

Step of the infection process	Experiment	Quantity of spores	Number of replicates	Temperature range	Number of heat-treatments	Figure	Table
Activation	1	50'000	10	20-99	5	2	2
Attachment	2	5'000	4	20-99	21	3	3
Attachment	3	5'000	8	68-72	5	3	4
Attachment	4	5'000	8	20, 60-80	8	3	5
Proliferation	5	10'000	5	20-99	21	4	6
Proliferation	6	50'000	20	54, 62, 70	3	4	7
Proliferation	7	10'000	7	20, 60, 66-74, 80	8	4	8

Table 2. Activaion step: summary of statistics for experiment 1.
Results of a generalized linear model for the effect of temperature treatment, host and parasite on spore activation. Significant *p*-values are highlighted by asterisks on the right. (* $p < 0.05$, *** $p < 0.001$). Interactions were not significant and were therefore taken out of the model.

	Estimate	Std Error	t value	*P-value*	
Intercept	1.0132759	0.0454612	22.289	<0.0001	***
Temperature	-0.0013926	0.0005904	-2.359	0.0193	*
Host	0.0600000	0.0330674	1.814	0.0711	
Parasite	-0.0400000	0.0330674	-1.210	0.2279	

Table 3. Attachment step: summary of statistics for experiment 2.
Results of a generalized linear model for the effect of temperature treatment, host and parasite on spore attachment. Significant *p*-values are highlighted by asterisks on the right. (*** $p < 0.001$). Interactions were not significant and were therefore taken out of the model.

	Estimate	Std Error	t value	*P-value*	
Intercept	1.9631887	0.0717527	27.360	<0.0001	***
Temperature	-0.0205307	0.0009859	-20.824	<0.0001	***
Host	0.0059524	0.0351510	0.169	0.866	
Parasite	0.0059524	0.0351510	0.169	0.866	

Table 4. Attachment step: summary of statistics for experiment 3.

Results of a generalized linear model for the effect of temperature treatment, host and parasite on spore attachment. Significant *p*-values are highlighted by asterisks on the right. (*** $p < 0.001$). Interactions were not significant and were therefore taken out of the model.

	Estimate	Std Error	t value	*P-value*	
Intercept	5.92766	1.07289	5.525	<0.0001	***
Temperature	-0.08246	0.01532	-5.383	<0.0001	***
Host	0.07202	0.04276	1.684	0.0942	
Parasite	-0.17434	0.04276	-4.077	<0.0001	***

Table 5. Attachment step: summary of statistics for experiment 4.

Results of a generalized linear model for the effect of temperature treatment, host and parasite on spore attachment. Significant *p*-values are highlighted by asterisks on the right. (*** $p < 0.001$). Interactions were not significant and were therefore taken out of the model.

	Estimate	Std Error	t value	*P-value*	
Intercept	1.506867	0.097014	15.532	<0.0001	***
Temperature	-0.017399	0.001375	-12.655	<0.0001	***
Host	0.001493	0.047959	0.031	0.975	
Parasite	-0.029757	0.047959	-0.620	0.536	

Table 6. Proliferation step: summary of statistics for experiment 5.

Results of a generalized linear model for the effect of temperature treatment, host and parasite on the infection success. Significant *p*-values are highlighted by asterisks on the right. (** $p < 0.01$, *** $p < 0.001$)

	Estimate	Std Error	t value	*P-value*	
Intercept	0.5324008	0.1346317	3.954	<0.0001	***
Temperature	-0.0057242	0.0019819	-2.888	0.00411	**
Host	-0.0270843	0.1893874	-0.143	0.88636	
Parasite	0.6270741	0.1949491	3.217	0.00142	**
Temp:Host	-0.0005356	0.0027888	-0.192	0.84781	
Temp:Parasite	-0.0078374	0.0028614	-2.739	0.00648	**
Host:Parasite	-0.3890852	0.2684939	-1.449	0.14819	
Temp:Host:Parasite	0.0053238	0.0039387	1.352	0.17735	

Table 7. Proliferation step: summary of statistics for experiment 6.

Results of a generalized linear model for the effect of temperature treatment, host and parasite on the infection success. Significant *p*-values are highlighted by asterisks on the right. (* $p < 0.05$, ** $p < 0.01$)

	Estimate	Std Error	t value	*P-value*	
Intercept	1.601124	0.603701	2.652	0.0087	**
Temperature	-0.015650	0.009654	-1.621	0.1067	
Host	0.686920	0.795323	0.864	0.3889	
Parasite	2.038903	0.904601	2.254	0.0254	*
Temp:Host	-0.011198	0.012747	-0.878	0.3808	
Temp:Parasite	-0.033557	0.014646	-2.291	0.0231	*
Host:Parasite	-0.231582	1.171107	-0.198	0.8435	
Temp:Host:Parasite	0.004273	0.018890	0.226	0.8213	

Table 8. Proliferation step: summary of statistics for experiment 7.

Results of a generalized linear model for the effect of temperature treatment, host and parasite on the infection success. Significant *p*-values are highlighted by asterisks on the right. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

	Estimate	Std Error	t value	*P-value*	
Intercept	0.5132417	0.1355336	3.787	0.000207	***
Temperature	-0.0072665	0.0020514	-3.542	0.000505	***
Host	0.4672593	0.1773457	2.635	0.009150	**
Parasite	-0.0259852	0.1905528	-0.136	0.891682	
Temp:Host	-0.0060747	0.0026821	-2.265	0.024706	*
Temp:Parasite	0.0010499	0.0028757	0.365	0.715478	
Host:Parasite	-0.0239657	0.2504906	-0.096	0.923884	
Temp:Host:Parasite	-0.0008981	0.0037917	-0.237	0.813041	

Figure 1. (Legend on next page)

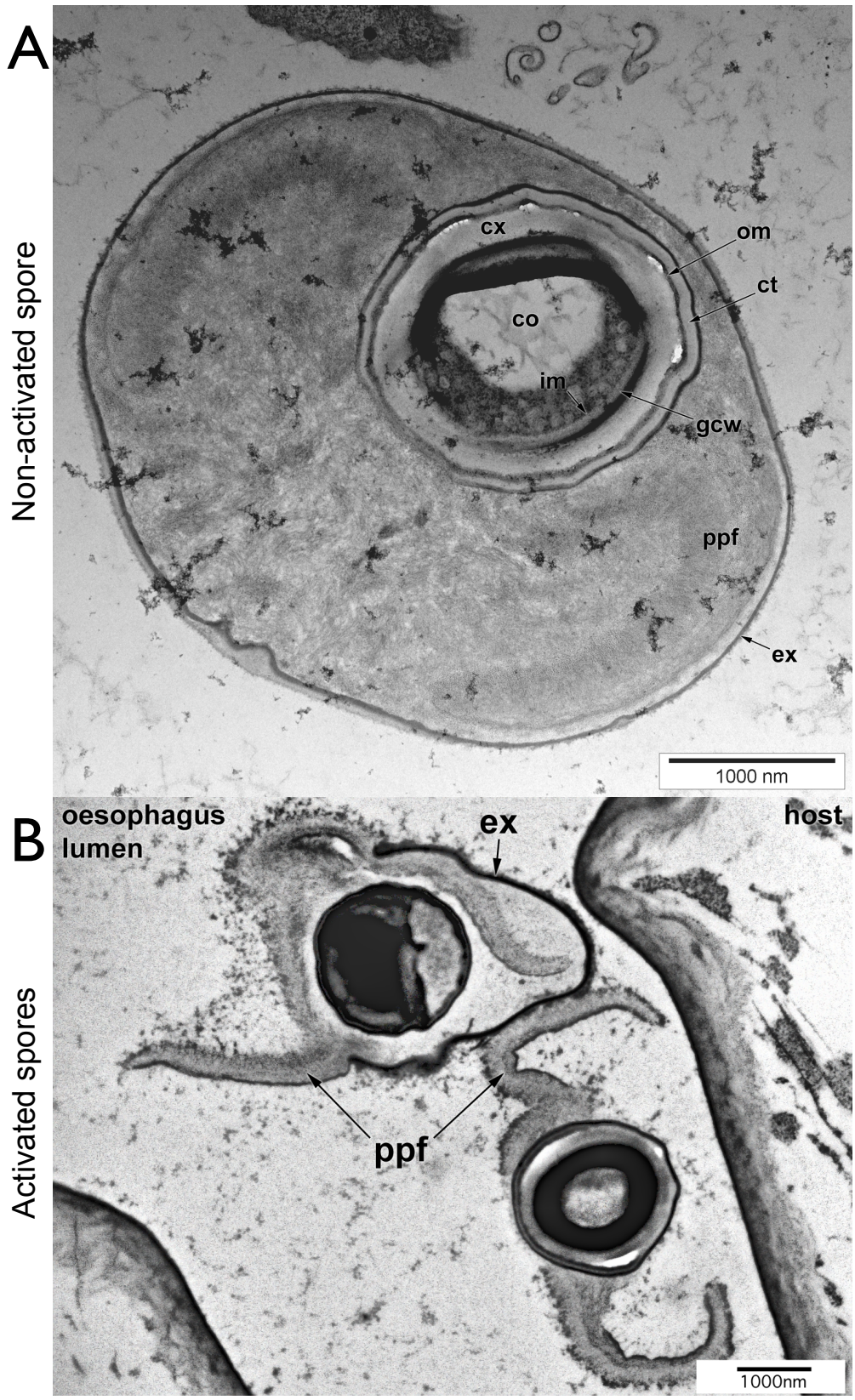


Figure 1. (Figure on previous page) Microphotograph of *Pasteuria ramosa* spores before (A) and after (B) activation (B: originally from Duneau et al. 2011).
(A) The spore before activation is enclosed in the exosporium (ex). It is constituted of two main parts: the peripheral parasporal fibers (ppf), and the endospore in the center. The endospore is constituted by: a coat (ct), an outer membrane (om), a cortex (cx), an inner membrane (im) and finally the core of the spore (co). (B) The spore after activation with shed exosporium (ex) and deployed peripheral parasporal fibers (ppf) which are used to attach to the host oesophagus.

Figure 2. Effect of heat-treated spores on activation (Experiment 1).
Temperature treatments (X-axis, in degrees Celsius) and proportion of replicated hosts in which activated spores were observed in the gut (Y-axis, in percent) are indicated. Full line with round symbols: host genotype HU-HO2, dashed line with square symbols: host genotype BE-M10, empty symbols: parasite genotype C1, filled symbols: parasite genotype C19. Temperatures that were not tested are denoted in grey.

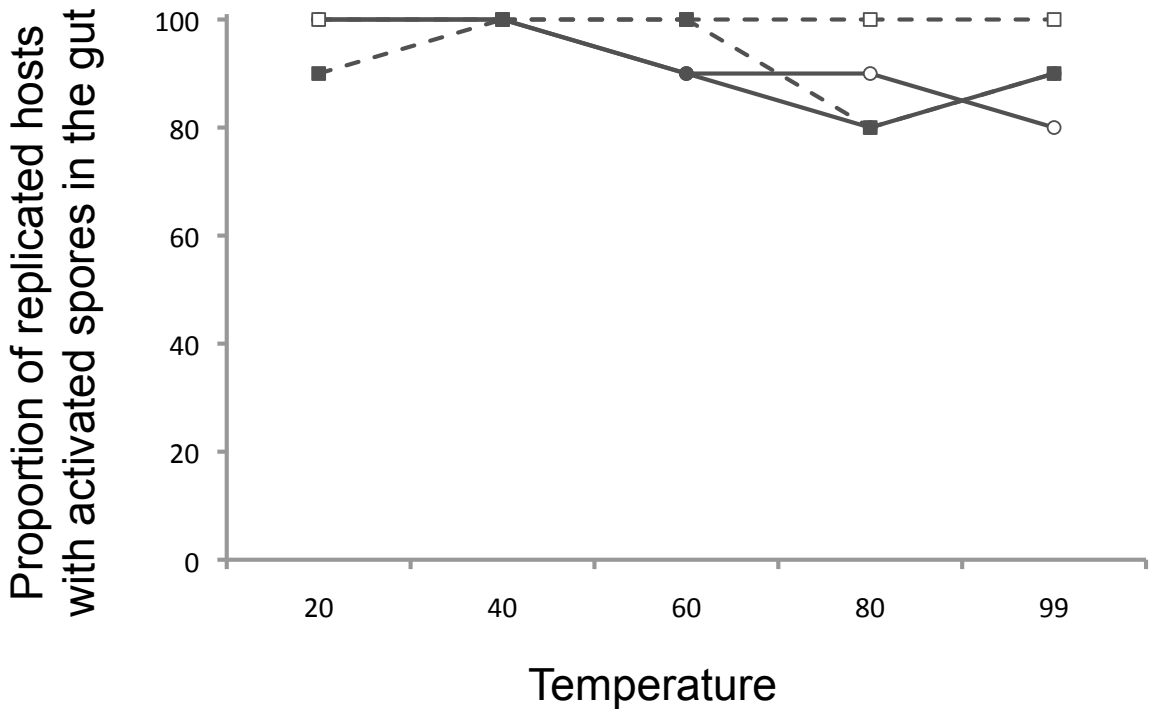


Figure 3. Effect of heat-treated spores on attachment (Experiments 2-4).
Temperature treatments (X-axis, in degrees Celsius) and proportion of replicated hosts in which spore attachment was observed (Y-axis, in percent) are indicated. Full line with round symbols: host genotype HU-HO2, dashed line with square symbols: host genotype BE-M10, empty symbols: parasite genotype C1, filled symbols: parasite genotype C19. Temperatures that were not tested are denoted in grey.

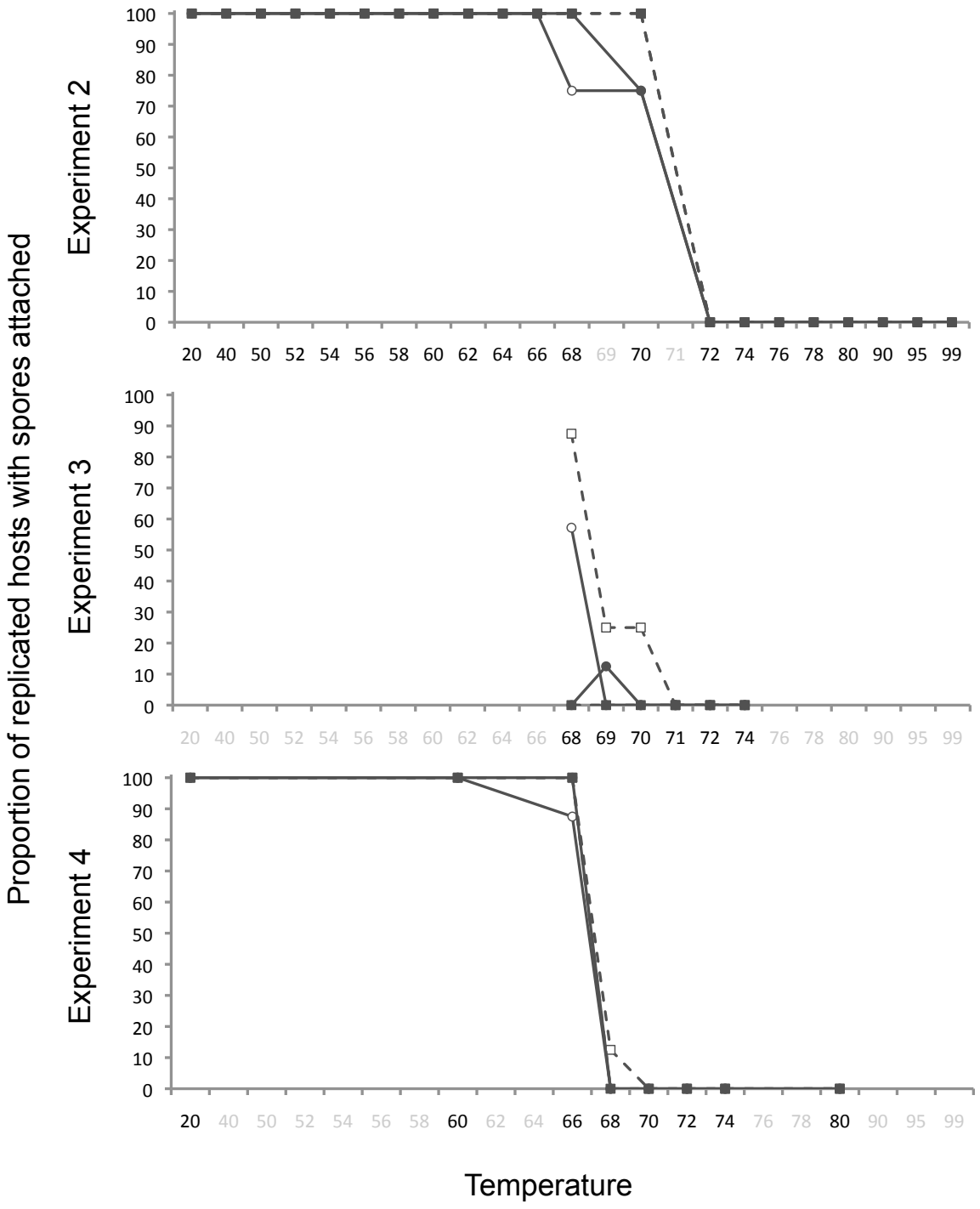
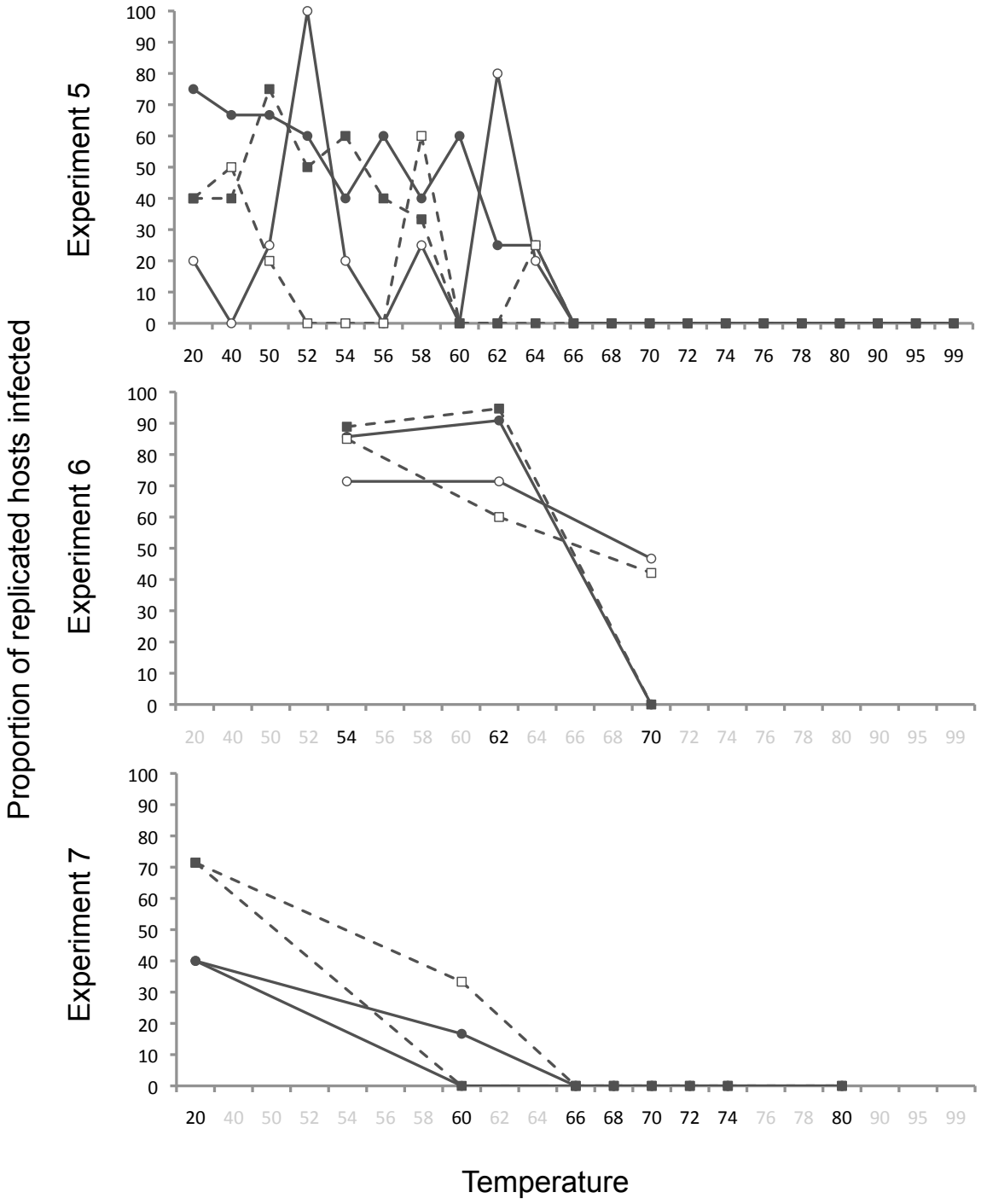


Figure 4. Effect of heat-treated spores on infection success (Experiments 5-7). Temperature treatments (X-axis, in degrees Celsius) and proportion of replicated hosts in which infection was observed (Y-axis, in percent) are indicated. Full line with round symbols: host genotype HU-HO2, dashed line with square symbols: host genotype BE-M10, empty symbols: parasite genotype C1, filled symbols: parasite genotype C19. Temperatures that were not tested are written in grey.



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Author contributions

César M.J.A. Metzger[a] – designed & performed all experiments, collected the data, analyzed the data, and wrote the manuscript.

Sebastian Gygli[a,b] – helped design the experiments & helped perform the activation experiment, provided a microphotograph of a non-activated *Pasteuria ramosa* spore.

Lorenz Hofer[a] – performed a pilot experiment that led to this study under the supervision of David Duneau.

David Duneau[a,c] – performed a pilot experiment that led to this study, provided a microphotograph of an activated *Pasteuria ramosa* spores.

Dieter Ebert[a] – provided supervision.

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VI.

Concluding words

"Summary and Perspectives"

Host-parasite interactions are thought to underlie many important evolutionary phenomena such as the maintenance of polymorphism, the evolution and maintenance of sexual reproduction, local adaptation or maladaptation, population dynamics, community structure and speciation (Hamilton 1980; Hamilton and Zuk 1982; Frank 1993; Kawecki 1998; Lively 1999; Hudson et al. 2006; Gandon et al. 2008). The mechanisms behind these evolutionary phenomena rely on the specificity of the interaction between hosts and parasites (Lambrechts 2010).

In this thesis I have, with the help of my collaborators and that of my supervisor, examined several levels of interaction between hosts and parasites and the specificity of these interactions. I explored the host-range of a poorly studied microsporidian parasite, *Gurleya vavrai*, and was able to expand its known range to include the geographically distant but phylogenetically related species *Daphnia pulex arenata* as well as the obligate sexual lineage of *D pulex*. The results of this study concurred with host-range theory in that we were able to show a role of phylogeny but also a role of local adaptation in shaping the parasite's host-range (Antonovics et al. 2013). As we exposed several genotypes of different species of hosts to the parasite, we were able to see that infection was not an isolated event of one susceptible genotype of the host but that many or all tested host genotypes were susceptible. By testing a range of genotypes of each susceptible host species, from different geographic origins and with sufficient replication, further information to confirm our proposed target host for this parasite may be uncovered, host genotype variation for resistance to the parasite may be found, and the role of encounter rate between host and parasite may be furhter explored. According to host-range theory, the parasite may be better at infecting hosts that are found in sympatry than those outside of the parasite's distribution. As this parasite may exert strong selective pressure on its host, due to the reduction in reproductive function and shortening of the host lifespan, it is surprising that field observations have not found this parasite more often, nor observed dramatic epidemics. Therefore further investigation of the interaction between the parasite and its host may explain the lack of prevalence observed in the field.

In the third chapter of this thesis, we examined the unexplained existence of 'double resistant' *Daphnia magna* hosts found in field surveys (Luijckx et al. 2014, Andras et al. in prep). Double resistance, in this case, refers to the ability of a host genotype to be resistant to two genotypes of the parasite *Pasteuria ramosa*. By crossing a host genotype of the double resistant phenotype with three other host genotypes displaying the three other possible resistance phenotypes (namely double susceptible, susceptible to one parasite genotype but not the other, and vice versa) and testing all F1 offspring and all F2 offspring against both parasite genotypes, we were able to explain how double resistance is inherited. Furthermore we could reject a previously proposed model of genetic architecture of resistance for this system and modify another proposed model (Luijckx et al. 2013) to incorporate double resistance. The genetics of resistance in the *Daphnia-Pasteuria* host-parasite system are now explained by a three linked loci with two alleles per loci model. This constitutes the first animal-parasite example with evidence of interactions between linked loci for resistance. Evidence for interactions between linked resistance loci are also extremely rare in plant-parasite systems with only one additive by dominance interaction found in plant resistance to the cucumber mosaic virus (Ben Chaim et al. 2001). Furthermore, and most strikingly, we were able to give the first empirical proof of the existence of negative epistasis in this model of resistance genetics. Negative epistasis is a key feature of MAMs and had never been empirically demonstrated until now, only indirect indications had been found in other systems such as in a flour beetle-microsporidium host-parasite system (Wegner et al. 2008) or in a snail-trematode host-parasite system (Dybdahl et al. 2008). Our work has confirmed the great potential of *Daphnia magna* to study evolutionary phenomena that rely on MAMs such as the maintenance of genetic polymorphism (Frank 1993) and the evolution of sexual reproduction (Hamilton 1980). Building upon our results by testing crossing schemes with additional parasite genotypes will be required in the future, as it appears that more parasite genotypes with different host-genotype-ranges exist (David Duneau & Dieter Ebert, unpublished data). Explaining inheritance of resistance for additional genotypes of the parasite may require a further amendment of the current genetic architecture

model, by addition of further loci for instance. With the current model, furthering the investigation of Red Queen dynamics should be possible, for example by examining negative frequency-dependent cycling of alleles in natural populations or in experimental populations seeded with hosts of known resistance genotype.

In the fourth chapter of this thesis, informed by the results of the previous chapter, we raised the question of the existence of costs of resistance in hosts from a natural population of the *Daphnia-Pasteuria* host-parasite system. In the absence of the parasite, we competed hosts of opposing resistance phenotypes (either susceptible to two genotypes of the parasite or resistant to both) in experimental populations started with 50% of one host genotype and 50% of the other. We could not find any indication of costs of carrying alleles for resistance, as resistant hosts did not do worse than susceptible hosts in these competitive setups. The experiment was repeated three times with differing environmental conditions (semi-natural mesocosms vs. laboratory microcosms, and three different feeding regimes) and results were consistent through all experiments. Our findings concur with those of a previous study that also competed hosts in the absence of the parasite and could not find any indication of costs (Little et al. 2002). Further studies that could indirectly ask the question of the existence of costs for resistance also did not find indications for such costs (see Labbé et al. 2010 for a discussion), therefore it is unlikely that costs exist for resistance against the parasite *P. ramosa* or if they exist they are negligible (as they consistently could not be detected on host fitness) and thus should not play a role in the co-evolution of *Daphnia* and *Pasteuria*. As the absence of a phenomenon is harder to prove (and may never be perfectly proven) than the existence of the phenomenon, repeated experiments in different conditions, using similar or difference experimental approaches is required to satisfactorily exclude the existence of the phenomenon. Therefore future endeavors to examine costs of resistance in this system may be warranted to provide more confidence and confirm our results and those of other studies.

The previous three chapters examined various aspects of host-parasite interactions pertaining to host-parasite specificity and their co-evolution. Chapter V takes a more mechanistic, structural, approach at the *Daphnia-Pasteuria* host-

parasite system, by examining the effects of a history of temperature on the highly resistant transmission stages of the parasite, the spores. Spores of *P. ramosa* have been found to remain infectious for decades in pond sediment (Decaestecker et al. 2004, 2007; Andras et al. in prep). Spores of related bacterial parasites have also been found to resist extreme environmental and experimental conditions. Already over 100 years ago Koch demonstrated that spores of *Bacillus anthracis* remained infectious after boiling them (Driks 1999). In several experiments we treated spores at different temperatures (between 20°C and 99°C) and subsequently exposed hosts to these treated spores. We examined three main steps (activation, attachment, proliferation) of the infection process of *Pasteuria* in *Daphnia* to detect an effect of the temperature treatments. Our results indicated that activation was not sensitive to the tested temperature range as spores retained their ability to shed their exosporium (activate). In contrast, the attachment step was inhibited at a temperature of 68°C and the proliferation step was inhibited at a temperature of 66°C, leaving a small range of temperature at which infection of the host is inhibited but recognition and attachment to susceptible hosts is not. As *P. ramosa* spores may readily resist temperatures as high as 66°C but *Daphnia* hosts cannot survive more than a few hours at a temperature of 37°C (Yampolsky et al. 2014), it appears that *P. ramosa* is always capable of recognition, activation, attachment, and successfully infecting susceptible hosts within the natural range of temperatures at which the hosts live. In the light of our results and with the help of the extensive knowledge from the literature of decades of thermoresistance and structural research with the model spore-forming bacteria *B. subtilis* (Driks 1999; Moir 2006; Setlow 2006) we discuss possible structural explanations for resistance and sensitivity to temperature of the different steps of the infection process of *P. ramosa*. As *P. ramosa* is an unculturable bacterial parasite, structural analysis of its bacterial development may prove difficult, however with the recent advances in extracting and sequencing DNA from spores of *P. ramosa* (Sebastian Gygli, Jean-Claude Walser & Dieter Ebert, unpublished data), a first step towards a better understanding of its germination and development may be through a comparative bioinformatic approach, looking at annotated genes known to be involved in these processes in related and model

spore-formers such as *B. subtilis* or *Clostridium* sp. Further work involve refining the experimental conditions in which to grow and heat-treat spores of the parasite to be able to use the 'window of opportunity' in which host infection is inhibited but attachment is not. Attached spores are shed with the oesophagus cuticle when the host molts (Duneau and Ebert 2012) returning hosts to a completely unparasitized state. This would allow the use of spores as phenotypic markers to recognize the resistance phenotype (and by extension gather partial information on resistance loci) of hosts without risking loosing the hosts to the infection, a method that may be essential when working with rare field samples.

The *Daphnia*-microparasite system has yielded many important findings in past decades, and remains one of the best systems to study most aspects of host-parasite interactions. More specifically the *Daphnia magna* – *Pasteuria ramosa* host-parasite system has become the model of choice to study co-evolution and species interactions, on the phenotypic as well as the genetic level. Combining classical evolutionary and genetic approaches, as were used throughout this thesis, with modern molecular and genomic approaches will allow the thorough description of the genetics underlying resistance of the host and possibly infectivity of the parasite. Taking a dual host and parasite approach is essential to understand host-parasite interactions, and the *Daphnia-Pasteuria* system offers the rare possibility that many other systems do not have to do so.

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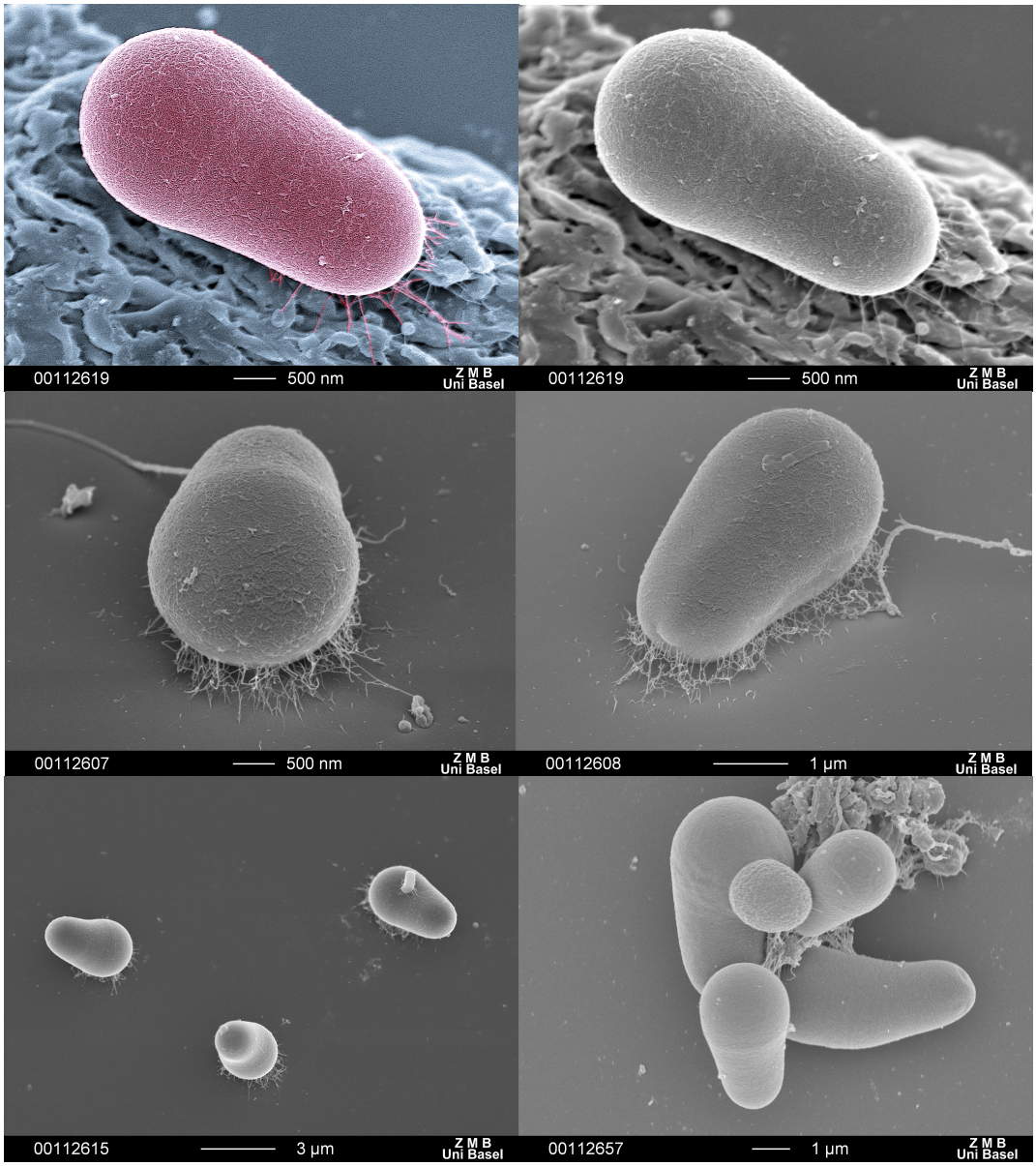
Appendices

Microphotographs of *Gurleya vavrai*,
Acknowledgements,
Curriculum vitae

Microphotographs of *Gurleya vavrai*

César M.J.A. Metzger & Eva. Bieler

Few microphotographs of *Gurleya vavrai* spores are available, and the ones that are were produced almost 20 years ago. Since then, the quality of microscopic photography has improved and so did the microscopes. I tried to obtain scanning electron microscopy (SEM) for spore surface morphology and transmission electron microscopy (TEM) for internal structure microphotographs, unfortunately only SEM yielded good photographs. SEM microphotographs were taken at the University of Basel's Center for Microscopy (ZMB) with the help of SEM-microscopist E. Bieler. The colorized microphotograph was colored by C. Metzger, using the software Adobe Photoshop.



Acknowledgements

The years of research, of late night work, of weekends spent in the laboratory, of few to no results and the frustration, but also of occasional successes and jubilation, could not be possible without the many people that at some point or another, or for some all throughout the thesis, have influenced or crossed the doctoral candidate's path. These people may be friends, other doctoral students, or post-docs, sometimes Master students, often laboratory technicians and other permanent staff members. Hereafter I want to say a few words about these people, who played a role for me. It may at times sound a bit too emotional, but deep down I'm a big sensitive guy, so there you go. This is my way of saying thanks, not only for the period of the doctorate, but also beyond.

First I would like to acknowledge Prof. Dr. Dieter Ebert, for giving me a position as doctoral candidate and research assistant in his group. I had come to him, after having read a review he had written years before. I sent him my résumé as well as a letter of motivation, even though there were no positions advertised or open in his group at the time. Prof. Ebert invited me to come present my Master Thesis research at one of the Zoological Institute's seminars and to get interviewed on the same day. At the end of the process he offered me a position. Due to my impending military service, I would only start one year later, in February 2010.
As I arrived at the Zoological Institute, I was given a seat next to Niklaus 'Niki' Mehring, who was a master student of another group leader, Jean-Claude Walser. Niki, a native from the region, soon became my guide, not only in the Institute, but also in living in Basel. He opened his group of friends to me and showed me around. Thanks to him I could achieve very quick integration among the Bebbis (Citizens of Basel) and starting from some very elementary knowledge of it managed to learn swiss-german in record time. Soon after Niki, I got to know Dita B. Vizoso. Dita was also my desk neighbor in the office, lab manager of another research group, she had done her doctoral studies with Dieter Ebert too. She quickly became a very helpful councilor and source of information about the lab and science, as well as life. Dita has a wonderfully odd creative mind that combined with my own oddness led us through amazing imaginary adventures and quite unique observations about the world around us. We've shared many discussions on many topics, probable or improbable, real or unreal, serious or comical. I have enjoyed every interaction and treasure the fact that I was seated between these two funny persons that Dita and Niki are. I hope they keep the oddness and the craziness, never letting the seriousness dampen these qualities. Sharing the same office was Elham 'Eli' Sheikh-Jabbari, a contemporary doctoral student, on whom I could always count to share calm words of reassurance. Eli and I, being contemporary in our studies, could experience similar phases of success and disappointment at approximately the same time. While not solving anything, knowing that you are not alone has helped both of us I suppose, I know it has helped me much and for this I am really grateful. About six months after I had settled in the office, came the tornado, what a ride with her as my desk neighbor! Marinela Dukiç is everything you'd expect from a Dalmatian and more. She is filled with such life energy and passion, always wanting to share her home country and culture. Sometimes she can be a bit invasive, yes, but it is little nuisance in comparison to her unconditional love for her friends and colleagues. I am sure she will find the swiss banker of her dreams (☺) and marry him, and with her PhD in one pocket, her swiss banker in the other, will be off to further adventures. Last but certainly not the least from the office, a very special person to me, Tobias Schär has also been a great support and friend throughout my whole PhD studies.

Further out of the office I would like to mention Matt D. Hall and Jason P. Andras. Both were postdocs during almost the entire time of my PhD and, to me, were like the bigger brothers in the lab. Both provided the experienced council needed to keep on track, the scientific advice to save the day, and the personal tap on the shoulder to reassure. Great buddies, to borrow a word from Matt, and hopefully friends for a long time to come. I miss the deep investigative early morning US vs. EU political discussions that Jason and I used to have every other morning. Not only did these sharpen my mind, but also gave me the opportunity to compare notes and understand the differences

between two cultures often mistakenly believed to be quite similar. I've been collecting facts and notes for the time Jason and I will meet again, because I already know a long deep political discussion will ensue. David Duneau was finishing up his PhD thesis when I was beginning mine. We quickly found common ground in the few shared similitudes between the Romand and the French cultures and quickly developed a connection. Although I will never be able to understand the whole eating hearts and other organs thing I will always remember fondly our discussions and our shared hours of pipeting. Early in my PhD, we worked together on an experiment, even though it did not yield the results we had hoped for, the experience of it, and David's guidance through it, taught me much about the experimental methods of the lab and working with Daphnia.
Another of Jean-Claude Walser's students deserves my recognition, Sebastian Gygli, has been a great friend to talk to about many topics, including science, politics and the military service. He has a witty and sharp inquisitive mind, and I had the pleasure of working with him on a few projects, one of which became a chapter of this thesis. Sebastian is a very good fellow, he enjoys the proper things in life such as whiskey and camaraderie. His Irish descent is probably not totally innocent in this character trait of his.

During my third year I got a new office mate, Marilou Sison-Mangus. Marilou, with her Americano-Philippino touch, has been a very good motivational coach and a great friend. We shared our taste in ethereal celtic music and our passion for gossiping. I am really thankful I can count Marilou among my friends; I know I'll be able to visit sunny California and always find a friend there. Quickly after Marilou came Sasha Mushegian, a great baking chef, and a good friend. A sharp thinker and very knowledgeable, she is always of good counsel and ready to joke around when appropriate. Andrea Kauffmann, I got to know her as I was her Blockkurs teaching assistant, got to know her better as she was doing her Masters degree in our group and even better yet as she was beginning her own doctoral work in our group and joined our office. Andrea is a very helpful and friendly person, she has been a good friend, and I think we are quite like-minded on many if not most topics. She's been a great desk neighbor during the last part of my thesis and I already miss her presence in my new office. I've also enjoyed Friday Beer o'clock with her and Sebastian Gygli, but also Urs Stiefel, Viktor Mislin and Daniel Lüscher. And the latest addition to our office team, was Karen Sullam, whom I have not had the chance of knowing for long, but in the short, yet intense, months we shared in the office (most of the time alone together in the office, both working on our writing, her to publish her PhD articles, and me finishing my thesis manuscript) we've developed quite the friendship, and shared much captivating politico-cultural discussions as well as our sense for gossip! As a fellow 'Berner' I know we'll get together from time to time, and I am already looking forward to it! At about the same time that Matt and Jason departed onto new adventures, became Gilberto Bento a good source of guidance and counseling to me. I am very thankful for his experienced, calm and reflective support as well as his friendship.

Janine Wong, Lucas Marie-Orléach, Joël Meunier, Nikolas Vellnow. These are the names of the people that constituted the hunter-gatherer nucleus. With them I went on countless trips to gather trophic resources (lunch). They were good and patient ears, listening to my daily rants and provided much needed external perspective as they were all doctoral students or postdoc from other research groups of the institute. With time passing, friendship grew. Sharing our respective doctoral adventures brought us closer. Janine and Lucas have started and ended their doctoral work in about the same timeframe as I have; therefore in a sense we are like friends of the same "school year". That, I am sure, we will not forget quickly, and it will always remain a special connection.

I have two night owls, one night mum and one nameless night guard to thank for the company during the countless nights spent working until the last bus, 00:32. Even though work at night is much an individual endeavor, isolated in a corner of a dark laboratory or in our respective offices, but occasionally the night owls we are cross paths near the coffee machine and share a few words, a comprehensive smile, a comforting hug. These night owls are Marinela and Roberto Arbore. The night mum is Dragoslava, the cleaning lady who, after hours, swoops through the offices and cleans everyone's personal mug and brings it back to the exact same spot, never mixing up mugs, so that come morning mugs always appeared to have magically become clean overnight. And the last

sleepwalker is the nameless guard. Well, he probably has a name, but I never knew it. Even though I saw him, and we greeted each other, exchanging a few polite words, almost every night I spent at the lab for the better of four years.

Two other persons have become very good friends, Anne Roulin and Jean-Claude Walser. Not only do we understand ourselves well, but we share a mostly common view of the world, science, and politics. We also share a taste for the good things in life (cheese, wine, traveling). I have a lot of respect for their opinion and treasure our discussions and our friendship.

A great many other great personalities have come along throughout the years I was at the "Vesalianum". Many have become good friends and often shared good advice, and I would like to thank them hereafter: Samuel Pichon, Yann Bourgeois, Karen Haag, Jarkko Routtu. A special mention is in order to thank those permanent members of the staff of the institute who have helped me the most and with whom I've shared coffee-break complicity: Yasmin Picton, Viktor Mislin, Brigitte Aeschbach, and Urs Stiefel.

Now Urs Stiefel more than earned his very own mention. Urs is a great friend, and a reliable source of knowledge and help. He likes to sell himself a little bit short, but in reality he knows a lot more than he says. He has a great sense of humor and is also one of those odd minds that I appreciate so much. I have a lot of respect for him and complicity with him. I truly hope our friendship will carry on, and really I don't see why it wouldn't.

Jürgen Hottinger I would like to acknowledge and thank for his assistance in logistical considerations and his help with technical questions, Daphnia raising questions, and the behind the scenes work that he and his team (Urs Stiefel, Elodie Burcklen, Dragoslava, Kristina Müller, Tobias Schär, Nicole Kalberer, Andrea Kauffmann) have done to ensure that the show could go on. Exactly one floor under my office is a place that is precious to have in an institute like ours, the "Werkstatt". Of course the reason it is so, is not only because this workshop contains all sorts of tools and machines allowing high quality woodworking, metal and plastic working as well as electronics but much more because it is host to the magical hands of the very knowledgeable mechanics or "Werkstatt-Menschen" as I have unofficially called them over the years. These men are Viktor Mislin, and his successor Daniel Lüscher. Thanks to their contraption building capabilities the work of the scientists of our institute is made possible.

There is one special friend of mine that I haven't mentioned yet. Pepijn Luickx. Pepijn has been a friend, a colleague, a supervisor, a mentor, a co-author and a careful pair of ears. Without him I think much wouldn't have been possible, as he coached me through my whole first year of doctoral studies and some more after that. We performed multiple experiments together, some with more success than others, but always with great fun being at the bench in the laboratory or on the roof of the institute where we could let our scientific megalomania run free. Many evenings of playing board games, watching movies or simply enjoying a culinary treat at a local restaurant made for much needed and much appreciated changes of pace. Pepijn is a kind person, with whom we will remain friends for years to come. Pep, thank you very much, you were precisely (note the wording) the colleague and friend I needed for this PhD.

Until now I have only mentioned people I befriended during my thesis. But there are other biologists, from before the thesis, that I met while studying in Lausanne, who merit a strong and clear mention. All three of them are very close friends of mine, we've done all sorts of shenanigans together and also share much personal views of life and the world. Gabriel Cisarovsky, Frédéric Laurent and Pierrick Buri. We were all students together, and we were all doctoral candidates in separate institutes, or even universities, for the (almost) same time. I cherish our friendships, and am glad to know that these continued even after we left the common grounds of the University of Lausanne.

I would like to mention two friends that do not fit into the other categories, both of them I have known for a long time now. I cannot categorize them because I have known them from different

situations and groups, and I cannot attribute them more to one or the other origin. Luc Jotterand and Sébastien Moret are two persons whose opinions and points of view I respect very much, I have always been able to refer to them to help with life decisions with their rational, fact-checking, logic. Great friends in the military as well as in the civilian life.

The friends from back home, these are the guys I've known for the longest, and they have stood by my side for all this time, through school, studies and the doctorate. I am really lucky to be able to count on them anytime and all the time, for anything and everything. I was never a fan of the concept of the "best friend" instead I think they are all my "best friend", each and every one of them in their own right: Frédéric Andreae, Stéphanie Devanthéry (although I must say Fred and Steph are more like the brother and sister I never had), Kévin Diserens, Cécile Diserens (whom I incidentally knew as Cécile Roux before she and Kévin entrusted me to perform their marriage!), my good 'mellon' Pascal Martin and stone-licking gossip-sharing long-standing friend Céline Longchamp.

I would like to thank Sebastian Imhof, not only for helping me once during a stormy evening to work on the roof of the institute on one of my larger scale experiments, but for standing by my side these past two years, and being an amazing 'partner in crime'. For giving me all the support he has been giving me, especially during the writing phase of the thesis, when time was scarce and the daunting task of meeting a deadline that was set earlier than first expected due to my finding of a new working position had to be dealt with. He is an amazing person with a great personality and sense of humor, I cannot even begin to estimate the hours we've spent goofing and laughing together. Two peas in a pod, really. He is an irreplaceable part of the whole. Sebi, I am so glad that you are part of the adventure now.

And finally, my parents.

My mother, she who sacrificed the little business she had built on her own to come back home and be a stay-at-home mum because her son, growing, was requiring more attention and because he wanted to have a cat. She who has spent hours helping me learn languages, recite my vocabularies, keep on track with my homework. She who fed me and my father, with food of a quality that most people can ever only dream of, every single day. She who spent hours comforting me, motivating me, supporting me, in person or over the phone throughout the years, during good and bad times. She who gave me all her attention, and devotion, during my childhood and youth and continued to do so during my early adulthood. She has been my very best friend and the strongest of shoulders to lean on when needed. I cannot begin to express how much I appreciate what she's done and sacrificed for me over the years, especially now that I am big enough to really grasp the magnitude of it.

And my father. The man who worked seven days a week, well over twelve hours per day, at least 340 days per year to ensure that my mother and I had a roof and clothes to put on, that we could enjoy a certain level of life, and that I could get the education he had hoped for me to get. He made it all possible, not only through the pragmatic financial support, but also because very early already, as a small child, he took me to social events, to his work, to see his clients, to see his business partners and always requested from people to talk to me, as he did, as if was an adult. This allowed me to develop very early on the language of an adult, instead of that of a small child and it went a long way to shape my character. He gave me a proper quality education, a rare thing that most people do not seem to be lucky enough to get. My father always promoted an atmosphere of learning and never once let a doubt come into my mind about working towards the goal of achieving a university degree. I suspect he would have been happy with a Master degree, but thanks to his unconditional support I made it to the doctoral degree. I know he has always regretted not being able to go to the university himself, even though he became one of the foremost experts in his field in Switzerland, but his dreams were to become a writer. I am sure he will be able to become one the day he will retire. For one, he could start by writing down all the historical knowledge he has gathered in his field, I am sure there would be a broad readership interested. I am so grateful to have had such an understanding and yet firmly directive father, he taught me much, and keeps doing so. He remains my reference in case of doubt or misunderstanding and we often discuss politics and life, sometimes quite animated

discussions, but always very enjoyable ones. I hope my father knows how much respect I have for him and all he did, not only for me but for everyone he has helped throughout all of his life, and how much I am grateful.

Papa, Maman, merci.

Curriculum Vitae

Cesar Metzger

César M. J. A. Metzger

Education

2010 – 2014	PhD Student Group for Evolutionary Biology, Zoological Institute, University of Basel Thesis Main Topic: Host-parasite interactions in the model system *Pasteuria ramosa – Daphnia magna*. Supervisor: Prof. Dr. Dieter Ebert
2009	*Mandatory active duty in the Swiss Army*
2007 – 2009	Master of Science in Evolution and Conservation Biology (M.Sc.) Department of Ecology and Evolution, University of Lausanne Master Thesis: "Testing the Competitive Exclusion Principle using various niche parameters in a native (*Natrix maura*) and an introduced (*N. tessellata*) colubrid." Supervisor: Dr. Philippe Christe Introductive research project: "Local adaptation and color polymorphism distribution in Tawny Owls." Supervisor: Prof. Dr. Alexandre Roulin
2003 – 2007	Bachelor of Science in Biology (B.Sc.) University of Lausanne, Lausanne, Switzerland.
2003 January to April	Studying stay in "Environmental, Population and Organismic Biology" University of Colorado, Boulder, CO, USA.
1998 – 2002	Swiss Federal Maturity Diploma (High School) majoring in Sciences Ecole Nouvelle de la Suisse Romande, Lausanne, VD, Switzerland.

Complementary Education

2008 November	Swiss Mammals Identification Course Organizer: Musée Cantonal d'Histoire Naturelle (Sion, Valais, Switzerland), CSCF (Centre Suisse de Cartographie de la Faune) and SSBF (Société Suisse de Biologie de la Faune)
2008 October to December	Evolutionary Game Theory: An Introduction Reading & Discussion Group Group Leaders: C. Clavien (Sociobiology) & C. Sachse (Philosophy/Epistemology), University of Lausanne
2008 April	Introduction to Field Animal Experimentation Organizer: Inter-University Doctoral Program in Ecology and Evolution of the Universities of Bern, Fribourg, Geneva, Lausanne and Neuchâtel

Professional Experience *(science only)*

2009-2010 December to January	Scientific collaborator Section of Conservation Biology, Dept. of Environmental Sciences, University of Basel Project: Establishment of microsatellite markers for *Vipera ursinii* Supervisor: Dr. Sylvain Ursenbacher
2009 April to August	Field Research Assistant Group Fumagalli, Dept. Ecology and Evolution, UNIL Laboratoire de Biologie de la Conservation, UNIL Bureau d'études AMAIBACH Project: "Study of the hybrid zone of *Triturus c. cristatus* and *T. c. carnifex* in Switzerland"
2007 & 2008 April to September	Field Research Assistant KARCH – Koordinationsstelle für Amphibien- und Reptilienschutz in der Schweiz Project: "Population dynamics control via experimental alien species removal" & Monitoring of the *Natrix maura* and *N. tessellata* populations of the Lavaux."
2007	Undergraduate Research Assistant to Prof. Dr. Claus Wedekind Testing computer-operated Game Theory experimental setups with humans Department of Ecology and Evolution, University of Lausanne
2003 February to April	Undergraduate Laboratory Assistant to Prof. Dr. David A. Chiszar Herpetology Lab, Department of Psychology, University of Colorado, USA

Teaching Experience *(academic)*

2013	Zoology "Blockkurs" – Locust Dissection *(~7h)* Position: Teaching-Assistant 3rd year Bachelor of Sciences in Biology Students, Course Leader: Prof. Dr. D. Ebert, University of Basel
2012	Zoology "Blockkurs" – Locust Dissection *(~7h)* Position: Teaching-Assistant 3rd year Bachelor of Sciences in Biology Students, Course Leader: Prof. Dr. D. Ebert, University of Basel
2011	Zoology "Blockkurs" – Undergraduate Research Projects *(~60h)* Position: Supervisor of a research project (supervising 2 students) 3rd year Bachelor of Sciences in Biology Students, Course Leader: Prof. Dr. D. Ebert, University of Basel
2011	Zoology "Blockkurs" – Locust Dissection *(~7h)* Position: Teaching-Assistant 3rd year Bachelor of Sciences in Biology Students, Course Leader: Prof. Dr. D. Ebert, University of Basel
2010	Evolutionary Bioinformatics – Hybrid Course Plenum + Exercises *(~6h)* Position: Teaching Assistant Master of Sciences in Animal Biology Students, Course Leader: Dr. J.-C. Walser, University of Basel
2010	Introduction into Biology – Tutorial Course *(6x 1.5h)* Position: Teaching-Assistant/Tutor 1st year Bachelor of Sciences in Biology Students, Course Coordinator: Prof. Dr. M. Hall, University of Basel
2010	Zoology "Blockkurs" – Undergraduate Research Projects *(~60h)* Position: Co-supervisor of a research project (supervising 2 students) 3rd year Bachelor of Sciences in Biology Students, Course Leader: Prof. Dr. D. Ebert, University of Basel
2010	Zoology "Blockkurs" – Locust Dissection *(~7h)* Position: Teaching-Assistant 3rd year Bachelor of Sciences in Biology Students, Course Leader: Prof. Dr. D. Ebert, University of Basel
2008	Zoology Practicals *(~30h)* Position: Teaching-Assistant 1st year Bachelor of Sciences in Biology Students, Course Leader: Prof. Dr. T. Kawecki, University of Lausanne
2008	Animal Keeping in a University Animal Keeping Facility *(2x 45 minutes)* Position: Invited Teaching-Assistant (Keeping of Reptiles) 1st year Animal Keeper Trainees, Course Leader: J. Notari, EPSIC & UNIL
2007 & 2008	Swiss Amphibian Fauna Fieldtrips *(8 to 10 fieldtrips/year)* Position: Organizer and First Assistant 1st and 2nd year Bachelor of Sciences in Biology Students, Course Leaders: Dr. A. Maeder (2007) & Prof. Dr. T. Kawecki (2008), UNIL

2007 & 2008 — Swiss Amphibian Fauna Lecture *(45 minutes/year)*
Position: Invited Lecturer
1st and 2nd year Bachelor of Sciences in Biology Students,
Course Leaders: Dr. A. Maeder (2007) & Prof. Dr. T. Kawecki (2008), UNIL

Professional Affiliations & Services

2012 – 2013 — Biology13 Organization Steering Comitee
Planification and coordination of the National Symposium in Organismal Biology
Host: University of Basel
Duration of the Event: 3 Days
Target Public: Students (MSc., PhD.), PostDocs and Group Leaders in Evolution, Ecology and Conservation Biology in Switzerland.

2012 — Host-Associated Microbiota 2012
Member of the organizing committee, co-writer of the grant applications, translator
Host: University of Basel
Duration of the Event: 3 Days
Target Public: Students (MSc., PhD.), PostDocs and Group Leaders in Host-Associated Microbiota Evolution and Ecology research.

2011 — Tenure Evaluation Commission – Member of the commission
Evaluation of a tenure-track Assistant Professor for promotion to Extraordinarius
(Evaluated Professor: Prof. Dr. Walter Salzburger)

2011 – present — Swiss Zoological Society – Member of the board
Deputy for Student Affairs (Undergraduate, graduate and PhD students)

2011 — Professorship Nomination Appellate Commission – Student body delegate (*invited position)*
Nomination of a Full Professor in Evolutionary Botany at the Department of Ecology and Evolution, University of Lausanne
(Nominated Professor: Prof. Dr. John Pannell)

2011 – 2014 — Doctoral Program in Population Genomics – Member of the Scientific Advisory Comitee
Deputy for the University of Basel

2011
April — International Macrostomum Meeting - Helper
Helper during the symposium

2010 – 2012 — Co-organisator (together with Lucas Marie-Orléach) of the Interaction Seminars of the Zoological Institute, University of Basel, Switzerland.
1 to 2 hours seminar series taking place every other week during the semester.

2010 – 2011 — SymBioSE 2011 – Member of the Committee for Board and Lodging
– Member of the Committee for Corporate Design
Cooperatively run 10-day european annual biology students (Levels: BSc, MSc and PhD) meeting, hosted in Switzerland this year by the University of Basel's students.

2008 – 2009 — Dean commission in charge of motivating candidatures and nominating a candidate for the position of Director of the School of Biology – Student body delegate (*invited position*)

	Defining conditions for applicants, inviting applicants, reviewing of applications, proposition of a candidate to the Faculty of Biology and Medicine of the University of Lausanne. (Nominated Director: Prof. Dr. Winship Herr)
2008	Professorship Nomination Commitee – Student body delegate (*invited position)* Nomination of an Assistant or Associate Professor Tenure-track in Evolutionary Botany at the Department of Ecology and Evolution, University of Lausanne (Nominated Professor: Prof. Dr. John Pannell)
2007 – 2008	Biology Students Association – President LAB (L'Association des étudiants en Biologie), University of Lausanne
2007	Professorship Nomination Commitee – Student body delegate Nomination of an Assistant Professor Tenure-track at the Department of Fundamental Microbiology, University of Lausanne (Nominated Professor: Prof. Dr. Justine Collier)
2007 – 2008	Students Consultative Commission – Master students delegate Ecole de Biologie, Faculté de Biologie et Médecine, University of Lausanne
2006	Professorship Nomination Commitee – Student body delegate Nomination of an Associate Professor Tenure-track at the Department of Molecular Vegetal Biology, University of Lausanne (Nominated Prof.: Prof. Dr. Niko Geldner)
2006	Professorship Nomination Commitee – Student body delegate Nomination of an Associate Professor in Zoology (Prof. Dr. Peter Vogel succession) and an Associate Professor in Behavioral Ecology at the Department of Ecology and Evolution, University of Lausanne (Nominated Professors: Prof. Dr. Tadeusz Kawecki and Prof. Dr. Alexandre Roulin)
2006 – 2007	Students Consultative Commission – Third year bachelor students delegate Commission consultative des étudiants (CCE) Ecole de Biologie, Faculté de Biologie et Médecine, University of Lausanne
2005 – 2007	Biology Students Association – Co-President LAB (L'Association des étudiants en Biologie), University of Lausanne
2005 – 2006	Federation of the Student Associations – Student delegate for Biology FAE (Fédération des Associations d'Etudiants), University of Lausanne
2004 – 2008	Committee Leading the Bachelor of Sciences in Biology Reform – Co-initiator & Student delegate Ecole de Biologie, Faculté de Biologie et Médecine, University of Lausanne
2003 – 2005	Biology Students Association – Member of the committee (Chief of Events) LAB (L'Association des étudiants en Biologie), University of Lausanne
2003 – 2008	Council of the School of Biology – Student delegate Ecole de Biologie, Faculté de Biologie et Médecine, University of Lausanne

Symposia attendance

2013	Biology13
7-8 February	Symposium of the Swiss Doctoral Students
Basel, CH	Host: University of Basel
	Organizer: Swiss Zoological Society, Swiss Botanical Society and Swiss Systematics Society
2012	Host-Associated Microbiota 2012
12-14 September	Workshop and mini-symposium
Basel, CH	Host: University of Basel
	Organizers: Dr. Marilou Sison-Mangus, Dr. Samuel Pichon, Alexandra Mushegian, César Metzger and Prof. Dr. Dieter Ebert.
2012	Biology12
8-10 February	Symposium of the Swiss Doctoral Students
Fribourg, CH	Host: University of Fribourg
	Organizer: Swiss Zoological Society, Swiss Botanical Society and Swiss Systematics Society
2011	Biology11
3-4 February	Symposium of the Swiss Doctoral Students
Zürich, CH	Host: University of Zürich
	Organizer: Swiss Zoological Society, Swiss Botanical Society and Swiss Systematics Society
2010	Swiss-Russian Cladoceran Meeting
Autumn	Swiss-Russian Symposia on Evolution, Ecotox, Ecology and Systematics of Cladoceran
Fribourg, CH	Host: University of Fribourg
	Organizer: Dr. Christoph Haag & colleagues
2010	DGC meeting 2010
26-30 March	Symposium of the Daphnia Genomics Consortium
Leuven, B	Host: University of Leuven
	Organizer: DGC meeting 2010 organizing committee.
2010	Biology10
11-12 February	Symposium of the Swiss Doctoral Students
Neuchâtel, CH	Host: University of Neuchâtel
	Organizer: Swiss Zoological Society, Swiss Botanical Society and Swiss Systematics Society
2009	Biology09
12-13 February	Symposium of the Swiss Doctoral Students
Bern, CH	Host: University of Bern
	Organizer: Swiss Zoological Society, Swiss Botanical Society and Swiss Systematics Society

Publications

Metzger C., Christe P., Ursenbacher S. (2011) Diet variability of two convergent natricine colubrids in an invasive-native interaction. *Mertensiella*. (*invited contribution*)

Metzger C., Ferchaud A.-L., Geiser C., Ursenbacher S. (2011) New Polymorphic Microsatellite Markers of the Endangered Meadow Viper (*Vipera ursinii*) Identified by 454 High-throughput Sequencing: When Innovation meets Conservation. *Conservation Genetics Resources*.

Metzger C., Ursenbacher S. and Christe P. (2009) Testing the competitive exclusion principle using various niche parameters in a native (*Natrix maura*) and an introduced (*N. tessellata*) colubrid. *Amphibia-Reptilia*

Popular Press

Metzger C. (2010) La Suisse protège ses animaux. *24heures*. 228:20. (Newspaper: Guest of the day)

Posters

Metzger C., Ursenbacher, S., and Christe, P. (2010) Evaluating the potential for trophic competition between two colubrids, one native (*Natrix maura*) and one invasive (*N. tessellata*) – Koordinationsstelle der Amphibien- und Reptilienschutz der Schweiz (KARCH) – Annual Herpetological Symposium, Goldau (Kanton Schwyz), Switzerland

Metzger C., Ursenbacher, S., and Christe, P. (2009) Trophic regime analysis in two congeneric water-snakes, one native and one introduced. – D.Day – University of Lausanne. (*Poster – Awarded with the Prize of the Fondation Hainard*)

Talks, Seminars & Conferences

Metzger C. (2013) Genetic architecture of resistance in Daphnia – Research Seminar, Zoological Institute, University of Basel, Switzerland. (*In-house research seminar*)

Metzger C. (2013) Host-range of the microsporidium *Gurleya vavrai* – Research Seminar, Zoological Institute, University of Basel, Switzerland. (*In-house research seminar*)

Metzger C. (2012) Going for the throat: survival of the stickiest – Interaction Seminar, Zoological Institute, University of Basel, Switzerland. (*In-house interaction seminar*)

Metzger C. (2012) Overview of Research into the Evolution and Biology of Homosexuality – Talk/Lecture, Association Frei Denken Uni Basel, University of Basel, Switzerland.

Metzger C., Luijckx P. (2010) Experimental co-evolution by negative frequency-dependent selection under semi-natural conditions – Interaction Seminar, Zoological Institute, University of Basel, Switzerland. (*In-house interaction seminar*)

Metzger C. (2009) Comparaison des régimes alimentaires de *Natrix maura* et *N. tessellata* en sympatrie sur les bords du Lac Léman (résultats un an après) – Koordinationsstelle der Amphibien- und Reptilienschutz der Schweiz (KARCH) – Annual Herpetological Symposium, Bern, Switzerland. (*Invited Talk*)

Metzger C. (2009) Testing the Competitive Exclusion Principle using various niche parameters in a native (*Natrix maura*) and an introduced (*N. tessellata*) colubrid – Zoology Institute, University of Basel. (*PhD candidate hiring talk*)

Metzger C. (2008) Comparaison des régimes alimentaires de *Natrix maura* et *N. tessellata* en sympatrie sur les bords du Lac Léman – Koordinationsstelle der Amphibien- und Reptilienschutz der Schweiz (KARCH) – Annual Herpetological Symposium, Bern, Switzerland. (*Invited Talk*)