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The expression of virulence during double infections by different parasites with conflicting host exploitation and transmission strategies

F. BEN-AMI*, T. RIGAUD† & D. EBERT‡

*Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel
†Equipe Ecologie Evolutive, Laboratoire Biogéosciences, Université de Bourgogne, UMR CNRS 5561, Dijon, France
‡Zoologisches Institut, Evolutionsbiologie, Universität Basel, Basel, Switzerland

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Abstract

In many natural populations, hosts are found to be infected by more than one parasite species. When these parasites have different host exploitation strategies and transmission modes, a conflict among them may arise. Such a conflict may reduce the success of both parasites, but could work to the benefit of the host. For example, the less-virulent parasite may protect the host against the more-virulent competitor. We examine this conflict using the waterflea *Daphnia magna* and two of its sympatric parasites: the blood-infecting bacterium *Pasteuria ramosa* that transmits horizontally and the intracellular microsporidium *Octosporea bayeri* that can concurrently transmit horizontally and vertically after infecting ovaries and fat tissues of the host. We quantified host and parasite fitness after exposing *Daphnia* to one or both parasites, both simultaneously and sequentially. Under conditions of strict horizontal transmission, *Pasteuria* competitively excluded *Octosporea* in both simultaneous and sequential double infections, regardless of the order of exposure. Host lifespan, host reproduction and parasite spore production in double infections resembled those of single infection by *Pasteuria*. When hosts became first vertically (transovarilly) infected with *O. bayeri*, *Octosporea* was able to withstand competition with *P. ramosa* to some degree, but both parasites produced less transmission stages than they did in single infections. At the same time, the host suffered from reduced fecundity and longevity. Our study demonstrates that even when competing parasite species utilize different host tissues to proliferate, double infections lead to the expression of higher virulence and ultimately may select for higher virulence. Furthermore, we found no evidence that the less-virulent and vertically transmitting *O. bayeri* protects its host against the highly virulent *P. ramosa*.

Introduction

Host–parasite interactions rarely involve a one-to-one association in nature. Instead, in many populations, hosts are commonly found to be multiply infected by two or more parasite species (Lello *et al.*, 2004; Decaestecker *et al.*, 2005; Rutrecht & Brown, 2008; reviewed by Petney & Andrews, 1998; Cox, 2001; Read & Taylor, 2001; Rigaud *et al.*, 2010) or by different strains of the same parasite species (Werren *et al.*, 1995; Lagrue *et al.*, 2007; López-Villavicencio *et al.*, 2007). Numerous theoretical and empirical studies have highlighted that interactions between genetically diverse parasites may influence both within- and between-host selection and consequently shape the evolution of parasite traits. In particular, under conditions of frequent multiple infections, lineages of virulent parasites are predicted to be more competitive than those exploiting their host more prudently, leading to an overall increase in virulence (Antia *et al.*, 1994; Bonhoeffer & Nowak, 1994; van Baalen & Sabelis, 1995;

Correspondence: Frida Ben-Ami, Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel.
Tel.: +972 3 6406080; fax: +972 3 6405347; e-mail: frida@post.tau.ac.il

Frank, 1996; Mosquera & Adler, 1998). These theoretical predictions received empirical support, notably from studies of multiple infections involving several strains of a single parasite species (Davies *et al.*, 2002; Wille *et al.*, 2002; Hood, 2003; Hodgson *et al.*, 2004; Hughes *et al.*, 2004; Vizoso & Ebert, 2005a; Jäger & Schjørring, 2006; Wargo *et al.*, 2007; Ben-Ami *et al.*, 2008) and to a lesser extent from studies of multiple infections by different parasite species (Thomas *et al.*, 2003; Lohr *et al.*, 2010). The latter are at the centre of this study and hereafter referred to as double infections.

Virulent effects of parasites can take diverse forms, with very different consequences for potential conflicts with other parasites or the host. Among the most fitness-devastating parasites for the host are castrating parasites, here defined as parasites whose primary effect on the host is to curtail host reproduction, while having relatively little effect on host mortality (Baudoin, 1975). Castrating parasites convert the resources the host invests into its offspring into their own growth and reproduction (Baudoin, 1975; Obrebski, 1975; Jokela *et al.*, 1993; Jaenike, 1996; O'Keefe & Antonovics, 2002). By doing so they can gain a substantial biomass (Baudoin, 1975). The high needs of castrators for resources put them in conflict with other parasites. This may select castrators to eliminate competitors, because sacrificing resources to other parasites may have strongly detrimental effects. Castrators rely solely on horizontal transmission, and rapid castration was suggested as a means to maximize their fitness (Obrebski, 1975; O'Keefe & Antonovics, 2002; Ebert *et al.*, 2004). This life history strategy places castrators in direct conflict with vertically transmitted (VT) parasites, which rely on host reproduction.

Like horizontally transmitted (HT) parasites, VT parasites have been shown to exhibit diverse effects on their hosts. For example, VT microbes may feminize their host, alter the sex ratio of host offspring (Werren *et al.*, 1995; Bouchon *et al.*, 1998; Weeks *et al.*, 2003; Terry *et al.*, 2004) and manipulate or 'sabotage' host behaviour (Thomas *et al.*, 2002; Haine *et al.*, 2005). In recent years, an increasing number of examples have been presented, which show that VT microbes protect their hosts against other parasites (Gil-Turnes *et al.*, 1989; Tsuchida *et al.*, 2002; Oliver *et al.*, 2003; Ferrari *et al.*, 2004; Zchori-Fein & Perlman, 2004), and there is growing appreciation that VT defensive symbionts play an important role in the ecology of host–parasite interactions (Scarborough *et al.*, 2005; Hedges *et al.*, 2008; Jaenike *et al.*, 2010). This protection can render the parasitic effect of a VT microbe into a mutualistic effect, potentially explaining the persistence of exclusive VT parasites (Haine, 2008; Brownlie & Johnson, 2009). The advantage for the VT microbe in protecting its host from other parasites is the increased lifetime reproductive success of the host and the VT parasite.

The conflict between coinfecting parasites is most extreme when the two parasites employ strategies that exclude each other. This is the case when a VT parasite encounters a castrating parasite: whereas the former transmits via host offspring, the latter suppresses host reproduction. As outlined previously, there is considerable evidence demonstrating that VT parasites protect their host against other parasites, whereas at the same time virulent parasites, and in particular castrators, were suggested to be superior competitors. These contrasting predictions of the outcome of the conflict between VT parasites and castrators are at the centre of this study.

The study system

The present study examines double infections in *Daphnia magna*, a cyclical parthenogenetic crustacean parasitized by a wide variety of bacterial, microsporidial and fungal parasites (Green, 1974; Ebert, 2005), with severe impact on host fitness (Stirnadel & Ebert, 1997; Ebert *et al.*, 2000). In field populations, many parasites may coexist in the same pond and multiple infections of host individuals are often observed (Stirnadel & Ebert, 1997; Decaestecker *et al.*, 2005). A common and widely spread parasite of *D. magna* is the bacterium *Pasteuria ramosa* Metchnikoff 1888, an obligate endoparasite with strict horizontal transmission, in which infective stages (i.e. spores) are released from the decaying cadaver of the host (Ebert *et al.*, 1996; Ebert, 2005). It has a strong castrating effect on the host, which rarely produces any offspring after infection (Jensen *et al.*, 2006). It is also capable of infecting other *Daphnia* species (e.g. *D. dolichocephala*; Duneau *et al.*, 2011). The microsporidium *Octosporea bayeri* Jirovec 1936 is host specific to *D. magna* and is very common in rock pool habitats of the Baltic Sea (Ebert *et al.*, 2001). It has a mixed transmission strategy, because infection can be acquired horizontally via waterborne spores released from a host cadaver or vertically from a mother to her offspring (Vizoso *et al.*, 2005). Vertical transmission is 100% efficient to asexual host offspring, but lower to sexually produced host eggs (Ebert *et al.*, 2007). Vertical transmission to sexual eggs is important for the parasite to survive host diapause (Ebert *et al.*, 2007). Following infection, *O. bayeri* develops in the host's fat cells and ovaries. Infected hosts are able to reproduce nearly normal, but suffer about 20% loss in competitive ability (Vizoso & Ebert, 2004; Bieger & Ebert, 2009).

The present study investigates a clearly identifiable conflict: *P. ramosa* requires host resources to grow and therefore castrates its host, whereas *O. bayeri* needs host offspring for vertical transmission. We examine differences between the parasite species and their associated transmission strategies. Both parasites coexist in the same *D. magna* metapopulation in southwestern Finland, although *O. bayeri* is much more abundant in these *D. magna* rock pool populations (Ebert *et al.*, 2001). As the virulence and the expected lifetime transmission

success of the two parasite species can be accurately quantified in the laboratory, we are able to test for conflicts between the parasites over when should the *Daphnia* host be castrated and when should it be killed. We also attempt to identify possible benefits incurred on *D. magna* by *O. bayeri* during its horizontal and vertical transmission phases. Taken together, these tests will determine whether (i) the concurrently transmitted *Octosporea* can protect the *Daphnia* host against *Pasteuria* and whether (ii) *Pasteuria*, being a castrator, is a stronger competitor than *Octosporea*.

Materials and methods

Host and parasite collections

We used a single *D. magna* clone (SP1-2-3) originally collected from a rock pool in the Tvärminne archipelago of southwestern Finland, by isolating parthenogenetic eggs from the brood chamber of an uninfected adult female and raising the clonal offspring in isolation under standardized laboratory conditions. In preparation to the experiment, we stock-cultured *D. magna* in 400-mL glass beakers, each containing eight individuals with artificial medium (Klüttgen *et al.*, 1994; Ebert *et al.*, 1998), where they were fed daily with 1.5×10^5 cells mL^{-1} medium of the chemostat-cultured unicellular algae *Scenedesmus gracilis*.

The two parasite species used in this experiment were each obtained from a singly infected *D. magna* individual (*P. ramosa* P1 isolate from Germany and *O. bayeri* FUNR-8-5 isolate from Finland), different from the host clone used for the actual experiment. In the case of *P. ramosa*, the infected individual was well fed until it died, upon which its parasite spores were used to repeatedly propagate infection via the highly susceptible *D. magna* clone HO2 from Hungary. In the case of *O. bayeri*, the parthenogenetic offspring of the 'initial' *D. magna* clone were used to repeatedly propagate infection, until there were enough spore-carrying cadavers to produce sufficient amounts of spore suspensions for the experiment. All cadavers were carefully homogenized, and spore concentrations were determined using a haemocytometer (Thoma ruling).

Experimental design and setup

The experiment had two phases: in the first phase, we examined double infections that resulted from horizontal infections by both parasite species, whereas in the second phase, we exposed vertically infected *D. magna* (by *O. bayeri*) to horizontally infecting *P. ramosa*. In the first phase, we followed a cohort of 448 *D. magna* individuals and examined the outcome of single infections as well as of double infections, both simultaneously and sequentially. In total, there were eight treatments, each with 56 replicates as listed in Table 1A. In the second phase, we followed a cohort of 224 *D. magna* individuals, half of which were already vertically infected by *O. bayeri* and the other half was naïve. These vertically infected offspring were obtained from 24 mothers exposed as juveniles to horizontally infecting *O. bayeri*. Our aim was to assess the effects of horizontal infection (by *P. ramosa*) on hosts previously infected vertically by *O. bayeri*. In this phase, we had four treatments, each with 56 replicates as described in Table 1B.

Throughout both phases of the experiment and on a daily basis, we monitored *D. magna* survival, release of offspring and the amount of *P. ramosa* and *O. bayeri* spores following the host's death. We defined virulence as time-to-host-death-since-first-exposure (i.e. host longevity). This can be time-to-host-death-since-age-12 days for

Table 1 Overview of the treatments in the (A) first and (B) second phase of the experiment. The use of different dose levels (50 000 in single infections vs. 100 000 spores in double infections) has been previously found to be insufficient to produce significant effects on any of the variables in this study, i.e. host longevity, host reproduction and parasite spore production (Ben-Ami *et al.*, 2008).

Treatment A	Type of infection	First infection (day 5)	Second infection (day 12)
P	Single	50 000 spores of *Pasteuria ramosa*	None
O	Single	50 000 spores of *Octosporea bayeri*	None
N+Pd	Single, delayed	None	50 000 spores of *P. ramosa*
N+Od	Single, delayed	None	50 000 spores of *O. bayeri*
P+O	Double, simultaneous	50 000 spores of each parasite species	None
P+Od	Double, sequential	50 000 spores of *P. ramosa*	50 000 spores of *O. bayeri*
O+Pd	Double, sequential	50 000 spores of *O. bayeri*	50 000 spores of *P. ramosa*
Control	None	None	None
Treatment B	**Type of infection**	**First infection**	**Second infection (day 5)**
P	Single	None	Horizontal infection using 50 000 spores of *P. ramosa*
Ov	Single	Vertical infection by *O. bayeri* from mother	None
Ov+P	Double, sequential	Vertical infection by *O. bayeri* from mother	Horizontal infection using 50 000 spores of *P. ramosa*
Control	None	None	None

delayed single infections; time-to-host-death-since-age-5 days for single, simultaneous and sequential infections; and time-to-host-death-since-birth for control and vertically infected hosts. Host fitness was defined as the lifetime number of offspring produced. *Pasteuria* fitness was estimated from the number of spores at the time of host death, which is equal to the lifetime spore production of an infection. *Octosporea* fitness was estimated from host reproductive success (vertical transmission) and from spores counted in killed hosts (horizontal transmission success).

In both phases of the experiment, we used third clutch offspring of the *D. magna* clone line. To start the experiment, we separated newborns from the *D. magna* clone line (0–24 h old) into four 400-mL beakers and fed them daily with 1.5×10^5 algae cells mL^{-1} medium. On day four, we singly placed female-only *Daphnia* in 100-mL jars, filled with 20 mL of artificial medium, and initially fed them 2×10^6 algae cells per animal per day. The first infection treatment was performed on day 5. A week later, on day 12, we replaced the medium of all animals with 20 mL of fresh medium and exposed the appropriate treatment groups to *P. ramosa* or *O. bayeri* spores (those with a delayed challenge). A week after the second infection, we replaced the medium of all animals with 100 mL of fresh medium and thereafter medium was replaced on a weekly basis. To accommodate the growing food demands of the growing animals, on days 9, 15, 18, 22, 27, 30 and 37, we increased the daily food level for all individuals to 3×10^6, 5×10^6, 6×10^6, 7×10^6, 8×10^6, 9×10^6 and 10×10^6 algae cells per day, respectively.

The temperature was 20 ± 0.5 °C and the light/dark cycle was 16 h : 8 h. All treatments were randomly distributed across the shelves of the incubator, and their position was rearranged frequently to avoid position effects. Offspring counts and dead animals were recorded daily. Animals that had died after day 16 (since birth) were dissected and checked for disease using phase-contrast microscopy (300–600×). Animals that had died earlier could not be reliably checked for infection and were thus excluded from the analyses. The experiment was terminated after all animals had died. The dead *Daphnia* were then frozen in 0.1 mL of medium at −20 °C for subsequent parasite spore counting with a haemocytometer.

Statistical analyses

All statistical tests were performed using SPSS for Windows release 15.0.1.1 (SPSS Inc., 2005). Host longevity was analysed using Cox regression with the relevant treatments dummy-coded as categorical covariables. No censoring was required because all *Daphnia* had died during the experiment. Offspring data failed to meet the normality and equality-of-variances assumptions and were thus analysed using nonparametric Kruskal–Wallis ANOVA. For multiple comparisons, the Mann–Whitney *U*-test was used after adjusting the *P*-value with the Bonferroni method. Although this method is over conservative, it did not cause a statistically significant result to become nonsignificant. Spore production was analysed using parametric ANOVA and *t*-tests, except when the data set was too small, in which case we used nonparametric tests. Infection rates were compared using Fisher's exact test.

Results

Phase 1 – horizontal infection of previously uninfected hosts

During the first 2 weeks of the experiment, 49 of 448 *Daphnia* individuals died for unknown reasons (10.9%). This mortality was unrelated to treatments ($\chi^2_7 = 11.41$, $P = 0.12$). None of the control *Daphnia* became infected. Controls were excluded from the analyses of infection rates and parasite spore production. Uninfected *Daphnia* in the infection treatments were excluded from all analyses except infection rates.

Host reproduction

Host control animals produced as many offspring as hosts singly infected with *Octosporea* (control, O, N+Od; Kruskal–Wallis $\chi^2_2 = 0.17$, $P = 0.92$; abbreviations as in Table 1, Fig. 1a), but considerably more than hosts singly infected with *Pasteuria* (control, P, N+Pd; Kruskal–Wallis $\chi^2_2 = 41.1$, $P < 0.001$). Hosts exposed to *Pasteuria* on day 12 produced significantly more offspring than those exposed on day 5 (P, N+Pd; Mann–Whitney $U = 387.5$, $P = 0.001$). This day-of-exposure effect was not observed in *Octosporea* (O, N+Od; Mann–Whitney $U = 68.5$, $P = 0.64$). In simultaneous and sequential double infections when *Pasteuria* was first to infect, offspring production followed largely *Pasteuria* single infection (P, P+O, P+Od; Kruskal–Wallis $\chi^2_2 = 4.45$, $P = 0.11$). However, in sequential double infections when *Octosporea* infected first, the number of offspring was similar to delayed single infection by *Pasteuria* (N+Pd, O+Pd; Mann–Whitney $U = 781.5$, $P = 0.86$).

Host longevity

Host-longevity-since-exposure in treatments involving *Pasteuria* with or without *Octosporea* did not differ significantly (P, N+Pd, P+O, P+Od, O+Pd; Cox regression $\chi^2_4 = 6.69$, $P = 0.15$; Fig. 2a). Host-longevity-since-birth in the control group and in treatments involving only *Octosporea* was also similar (control, O, N+Od; Cox regression $\chi^2_2 = 0.53$, $P = 0.77$). However, as can be clearly seen in Fig. 2a, we found considerable differences between the *Pasteuria* treatments (with or without *Octosporea*) and the remaining *Octosporea*-only treatments pooled within each group (Cox regression $\chi^2_1 = 63.56$, $P < 0.001$).

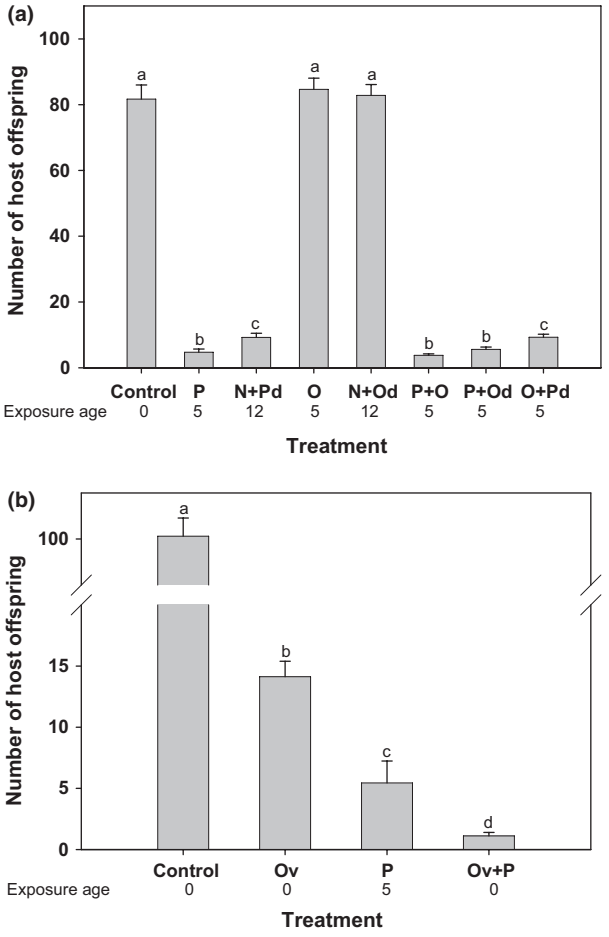

Fig. 1 Mean ± SE of host lifetime offspring production in the treatments of the (a) first and (b) second phase of the experiment. Means with the same letter are not significantly different.

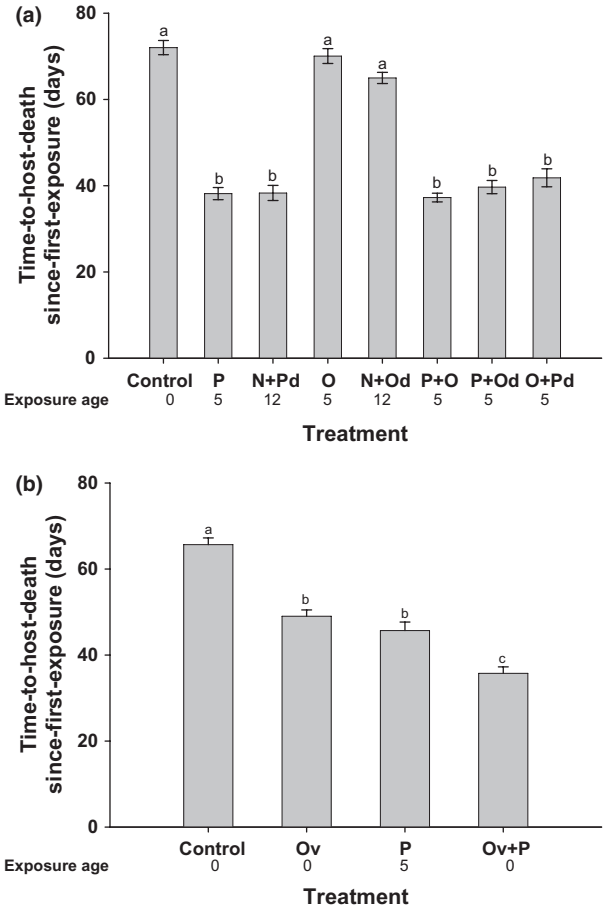

Fig. 2 Mean ± SE of time-to-host-death-since-first-exposure (or since-birth for controls) in the treatments of the (a) first and (b) second phase of the experiment (see Materials and methods for details). Means with the same letter are not significantly different.

Infection rates

The infection rate in treatments involving *Pasteuria* was similar (varying from 80.0% to 92.7%; Fisher's exact test, $P = 0.39$) and independent of the presence of *Octosporea*. In contrast, infection rates significantly differed among *Octosporea* treatments (Fisher's exact test, $P < 0.001$) and they were strongly influenced by the presence of *Pasteuria*. No *Daphnia* were infected with *Octosporea* when *Pasteuria* was first to infect (P+Od), and only two individuals were infected by *Octosporea* (but also by *Pasteuria*) in simultaneous double infections (P+O). Because of these low infection rates, these treatments were not further analysed with regard to *Octosporea* fitness. The infection rate in the remaining *Octosporea* treatments (O, N+Od, O+Pd) varied between 14.0% and 33.3%.

Parasite spore production

Parasite spore production varied considerably between parasite species and among treatments. Spore production by *Pasteuria* was relatively similar in single, delayed single, simultaneous and sequential double infections (P, N+Pd, P+O, P+Od, O+Pd; $F_{4,183} = 2.19$, $P = 0.072$; Fig. 3a). Spore production by *Octosporea* was much higher in single and delayed single infection than in sequential double infections when *Octosporea* was first to infect (O, N+Od, O+Pd; Kruskal–Wallis $\chi^2_2 = 14.85$, $P = 0.001$). In summary, *Pasteuria* fitness is largely unaffected by the presence of *Octosporea*, whereas *Octosporea* strongly loses fitness when *Pasteuria* is present.

Phase 2 – horizontal infection of vertically infected hosts (by Octosporea)

In all four treatments (including control), 11 of 224 *Daphnia* individuals died for unknown reasons during the first 2 weeks of the experiment (4.9%). This mortality was unrelated to treatments as there were no differences among treatments ($\chi^2_3 = 4.88$, $P = 0.18$). All controls remained uninfected, and uninfected *Daphnia* (belonging to infection treatments) were not included in the

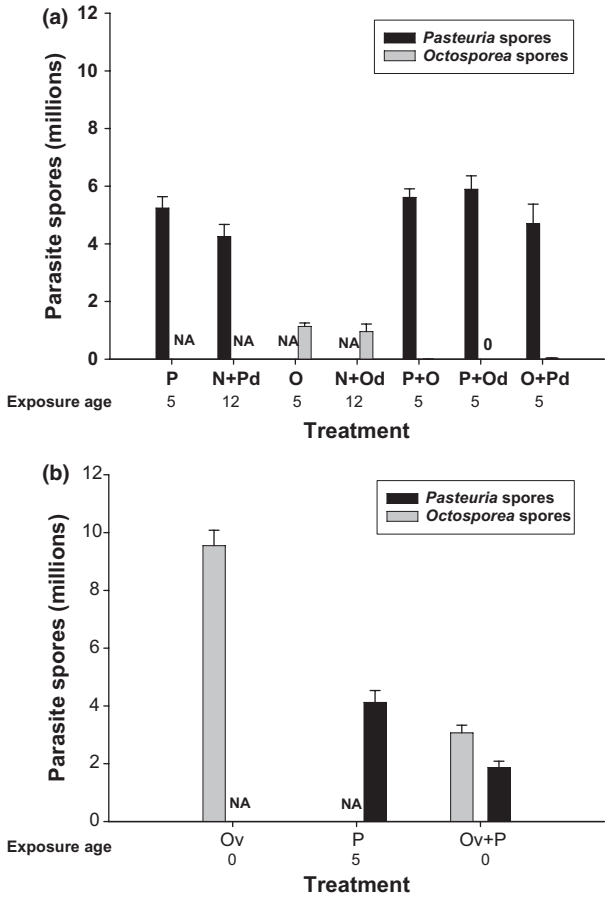

Fig. 3 Mean ± SE of parasite spore production in treatments of the (a) first and (b) second phase of the experiment.

analyses of host reproduction, host longevity and parasite spore production.

Host reproduction

Host control animals produced the highest number of offspring per individual, followed by single infection by *Octosporea*, then by single infection by *Pasteuria*, and finally by double infections by both parasite species (Kruskal–Wallis $\chi^2_3 = 130.58$, $P < 0.001$; Fig. 1b). All Mann–Whitney pairwise comparisons were significant at $P < 0.001$.

Host longevity

Both parasites substantially reduced host longevity relative to the control group (Table 2, Fig. 2b), although there were no differences in virulence between the two single infection treatments. Double infections were even more virulent than single infection by either parasite.

Infection rates

The infection rate of *Pasteuria* was 97.6% in previously uninfected hosts and 90.9% in hosts vertically infected by *Octosporea* (Fisher's exact test, $P = 0.19$). At birth, all newborn hosts (except controls) were vertically infected by *Octosporea*. This was confirmed by examining clonal siblings of offspring used in all infection treatments – these extra control siblings were always found to be infected by *Octosporea*. Moreover, upon death, all individuals in the Ov treatment were still infected, and the infection rate of *Octosporea* after exposure to *Pasteuria* decreased to 86.4% (Fisher's exact test, $P = 0.01$), indicating that *P. ramosa* clears *O. bayeri* in some cases.

Table 2 Results of survival analysis (Cox regression) showing treatment effects on the survival of *Daphnia* in the second phase of the experiment. We used repeated contrasts whereby each category of the predictor variable except the first category is compared to the category that precedes it. The negative sign of all estimated regression coefficients indicates that the hazard of mortality increases in comparison with the preceding category, e.g. the hazard of mortality in the Ov treatment is higher than that of the control treatment. exp(*B*) is the relative risk, e.g. the risk of death in the control treatment is less than a fifth of the risk of death in the Ov treatment. Bold typeface indicates significant effects. See Fig. 2b for comparison.

Contrast	d.f.	*B*	Wald	*P*	exp (*B*)
Ov vs. Control	1	−1.66	43.70	**< 0.001**	**0.19**
P vs. Ov	1	−0.03	0.02	0.88	
Ov+P vs. P	1	−0.83	13.68	**< 0.001**	**0.48**

Parasite spore production

Parasite spore production in double infections was considerably lower than in single infection by either parasite species. In the case of *Octosporea*, single infection produced more than three-fold *Octosporea* spores than double infections (Ov, Ov+P; unequal variances $t_{65.6} = 10.82$, $P < 0.001$; Fig. 3b), whereas single infection by *Pasteuria* produced twice as much *Pasteuria* spores as double infections (P, Ov+P; unequal variances $t_{60.9} = -4.84$, $P < 0.001$).

Discussion

We found that under conditions of strict horizontal transmission, *P. ramosa* competitively excluded *O. bayeri* in both simultaneous and sequential double infections, regardless of the order of inoculation. Consistent with this finding, host longevity, host reproduction and parasite spore production in double infections resembled those of single infection by *P. ramosa*. Only when *O. bayeri* is VT, it is able to resist to some degree competition by *P. ramosa* and produce a considerable proportion of transmission stages that can be used for horizontal transmission. In this case, both parasites and the host suffer from the competition in comparison with single infections: the host *D. magna* suffers from lower fecundity and longevity, and the two parasites suffer from reduced spore production. Despite the slightly lower

susceptibility of *D. magna* to *P. ramosa* under conditions of double infections, and the substantially lower *Pasteuria* and *Octosporea* spore production in vertically infected hosts, it did not translate into a relative increase in host fitness. Therefore, *O. bayeri* does not appear to confer protection to *D. magna* against *P. ramosa*.

These results suggest that on a population level, *Pasteuria* may outcompete *Octosporea* in the long run. The current geographical distribution of the two species overlaps only in the rock pool metapopulation of *D. magna* on the Baltic Sea. There, *Octosporea* is found in about 50% of the *D. magna* rock pool populations, whereas *Pasteuria* in about 1% (Green, 1957; Ebert *et al.*, 2001). In regions where *Pasteuria* is common (most of Central, Eastern and Western Europe), *Octosporea* is absent. Whether these distributions are shaped by the relative competitiveness of the two parasites or by other factors such as habitat quality or history is unclear. As both *Pasteuria* and *Octosporea* can persist outside the host in sediments under dry and wet conditions, vertical transmission (via parthenogenetic and sexual offspring) certainly provides *Octosporea* an advantage in the highly dynamic rock pools along the Baltic Sea (Vizoso *et al.*, 2005), possibly explaining the dominance there (Ebert *et al.*, 2001).

The interactions between a strictly HT parasite and a concurrently transmitted parasite (i.e. HT+VT) have not been modelled, especially when the transmission of the HT parasite is density dependent (a function of the absolute density of infected hosts in the population). Under conditions of frequency-dependent transmission (transmission success is a function of the frequency of infected hosts in the population, as in vector and sexually transmitted parasites), Altizer & Augustine (1997) showed that adding vertical transmission capabilities to an HT parasite significantly broadens the conditions for parasite invasion. When both HT and VT parasite strains are allowed to coexist within a host population, Lipsitch *et al.* (1996) showed that a VT strain can persist if it provides protection against a more-virulent HT strain. Similar predictions have been made by Lively *et al.* (2005) and Faeth *et al.* (2007). These models, however, assume that an HT parasite cannot superinfect a host that is already infected by a VT parasite – an assumption that does not hold for many host–parasite systems (reviewed in Haine, 2008) including the here used *Daphnia*–*Pasteuria*–*Octosporea* system. If one allows for superinfection by the HT parasite, and assuming that the VT parasite reduces the transmission ability of the HT parasite (as *O. bayeri* did via a two-fold reduction of *P. ramosa* spore production in double infections), Jones *et al.* (2007) showed that VT parasites are more likely to persist with HT parasites that prevent host reproduction (such as *P. ramosa*) than with those that allow it. Jones *et al.* (2010) also suggested that competition between HT and VT parasites critically depends on life history trade-offs the two parasites face, which may be highly specific to their particular biology (e.g. feminization vs. virulence, transmission efficiency vs. virulence). To explore these conjectures further, one could, for instance, compare the present results with studies of double infections by *O. bayeri* and a noncastrating HT parasite of *Daphnia*.

In several studies of double infections, the parasite species employed were not in conflict over transmission. For example, using the desert locust and two HT fungal entomopathogen species, Thomas *et al.* (2003) found that the avirulent parasite can alter the virulence and reproduction of the virulent parasite, depending on the order of infection and environmental conditions. Consistent with our findings, Lohr *et al.* (2010) showed that double infections of *D. galeata* by an intestinal protozoan and a haemolymph fungus (both HT) were more virulent than single infections and that prior residency does not always provide a competitive advantage. Given that sympatric coexistence of different parasite species is common in nature, these diverse results and in particular the role of abiotic factors emphasize the importance of studying double infections with the same 'vigour' as studies of multiple infections involving several strains of a single parasite species.

Another aspect of double infections that has often been overlooked concerns the mechanisms employed by each parasite to penetrate and infect the host. In the case of *P. ramosa*, Duneau *et al.* (2011) showed that spore penetration and activation in single infections are independent of genetic and environmental factors, whereas parasite attachment to the oesophagus is strongly influenced by host and parasite genotypes. Possible dependencies of *O. bayeri* infection steps on genetic or environmental factors have not been examined. Vizoso *et al.* (2005) found that in single infections, *O. bayeri* spores accumulate not only in fat cells but also in the ovary, eventually spreading throughout the entire body cavity. It remains to be determined how *P. ramosa* and *O. bayeri* interact with each other and/or with the host immune system. For instance, it is unknown whether double infections increase *D. magna* phenoloxidase (PO) activity beyond already-higher PO levels found in single infections (Pauwels *et al.*, 2011).

The widespread persistence of strictly VT parasites is often attributed to their ability to kill males or induce feminization and thus alter host population sex ratio (Bandi *et al.*, 2001), as well as to protect their host against more-virulent HT parasites (Haine, 2008; Brownlie & Johnson, 2009). This is because vertical transmission alone does not allow a virulent parasite to persist (Lipsitch *et al.*, 1995). Despite the somewhat lower susceptibility to *Pasteuria* of hosts vertically infected by *Octosporea*, when compared to single infection by *Pasteuria* (P vs. Ov+P: 97.6% vs. 90.9%), VT *O. bayeri* kill their host around the same time as *P. ramosa*. Additionally, double infections (i.e. P+O, P+Od, O+Pd, Ov+P; Fig. 1a, b) considerably reduced host reproduction regardless of the mode of transmission. Animals vertically infected

with *Octosporea* indeed carried a significantly lower sporeload of *Pasteuria*, probably due to the prior residency of *Octosporea*, but it remains to be determined whether such a reduction is sufficient to offset the harm caused by *Octosporea*. Hence, although VT *O. bayeri* does not appear to protect *D. magna* against *P. ramosa*, VT *O. bayeri* at least produces spores that can propagate via HT, which emphasizes the importance of horizontal transmission for *Octosporea*. It could perhaps be argued that concurrent transmission evolved as an alternative mechanism for some strictly VT parasites to offset their inability to confer resistance to their host against HT parasites. To attest this hypothesis, one would need (i) to repeat the present experiments with different combinations of *Daphnia/Pasteuria/Octosporea* clones, (ii) to demonstrate that *O. bayeri* does not benefit *D. magna* in other ways (e.g. reduced susceptibility to predation) and (iii) to investigate potential long-term effects of *O. bayeri* on *P. ramosa* in doubly infected *Daphnia*.

Consistent with previous studies of the *Daphnia–Octosporea* system (Vizoso and Ebert, 2005a, b), sporeloads were higher in vertically vs. horizontally infected hosts. However, we found that a VT *O. bayeri* kills its host earlier and reduces fecundity more strongly than an HT *O. bayeri*, whereas previously it was found that horizontally infected hosts died earlier than vertically infected ones and that the infection route did not influence host fecundity (Vizoso and Ebert, 2005a,b). There are several possible explanations for these differences. First, in our study, the host clone was naïve to the *O. bayeri* isolate, whereas in previous studies, the parasite had been adapted to the host clone for several host generations. Previous work on *O. bayeri* indicated rapid adaptation of *O. bayeri* to its host clones (Altermatt *et al.*, 2007). The increased virulence during the second phase of our experiment may thus be a result of the parasite adapting to the new host clone. *O. bayeri* adaptation may also 'purify' the parasite isolate by eliminating less-competitive or less-infective strains from the cocktail. Passaging and purifying prior to an experiment could result in more infective and virulent strains (spores) for horizontal infections (Ebert, 1998; Luijckx *et al.*, 2011). Second, *O. bayeri* strains may differ in their within-host replication rates, insofar that some strains may benefit from increased virulence after being transmitted vertically, because their within-host replication rate is very high. In accordance with this prediction, *O. bayeri* sporeloads in vertically infected *Daphnia* (single infection) were more than two-fold higher in the present study than in previous *Octosporea* experiments. Third, horizontal transmission success is sensitive to the environmental conditions of the experiment. In particular, spore dose and culture conditions, which differed strongly between Vizoso & Ebert (2005a,b) and the current study, may influence the results and thus may explain part of the differences. Fourth, the *Daphnia* clone used in this experiment was different from that used in previous experiments. Strong host genotype × parasite genotype interactions, which have been observed in the *Daphnia–Pasteuria* system (Carius *et al.*, 2001), could also account for the differences.

Conclusions

Our study demonstrated that within-host competition between different parasite species can be severe, even when the two parasites utilize different host tissues to proliferate (*Pasteuria* is an extracellular blood parasite and *Octosporea* is an intracellular parasite of fat cells and ovaries). Frequent competition of this form may select for higher virulence of any of the two parasites. We have also shown a strong asymmetry in competitive success; though, it remains to be determined whether this asymmetry is general or isolate specific. Our data do not support the idea that VT parasites may prevent later-infecting HT parasites from establishing successful infections. Neither do our results suggest that prior residency predicts the competitive outcome. Future work should focus on reconfirming our results using a wider range of *Daphnia* genotypes and parasite clones, as well as the long-term implications of double infections on the persistence of the two parasites and on the evolution of their virulences.

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