Multiple infections of a host by different strains of the same microparasite are commonly observed in natural populations (Lipsitch and Moxon 1997; Read and Taylor 2001). In contrast to single infections in which the lifetime transmission success of an infection is believed to be the primary determinant of parasite fitness (see Anderson and May 1991, and the references therein), it was suggested that evolution under conditions of frequent intrahost competition proceeds differently. Under conditions of frequent multiple infections, parasite success depends on several interconnected factors, such as relatedness among parasite genotypes (Frank 1992; Brown 1999; Gardner et al. 2004; Lively 2005), host resistance to infection (Gandon et al. 2002), sublethal effects on the host that feedback to reduce parasite fitness (Schjørring and Koella 2003), the mechanism of parasite-induced pathogenesis...
sive. For example, in the rodent malaria are fairly limited, and more importantly the results are inconclu-
sion model assumes that the most virulent strain outcompetes
reduced virulence is preferred when all parasite strains gain the
resource (i.e., any individual in a group can profit from it) that
can be exploited or manipulated by the competing parasite strains
(Dionisio and Gordo 2006). Whereas increased virulence is fa-
vored when individual parasite strains are rivals and the most
competitive individuals gain a disproportionate share of the host
(tragedy of the unmanaged commons; Hardin 1994; Frank 1998),
reduced virulence is preferred when all parasite strains gain the
same benefits from host manipulation, even though some do not
bear the costs (cf. public goods dilemma; Olson 1965; Brown
1999). An alternative dependence of the predicted course of evo-
olution on the specific form of the assumed competition was also
described by Nowak and May (1994, 1995). Their superinfection
model assumes that the most virulent strain outcompetes
less virulent ones in a multiply infected host (Nowak and May
1994). Assuming a positive and saturating relationship between
infectivity and virulence, Nowak and May (1994) showed that su-
perinfection favors higher virulence of coexisting strains, beyond
the level maximizing parasite reproduction ratio. Conversely, the
coinfection model assumes that all parasite strains within a host
are transmitted, irrespective of their virulence (May and Nowak
1995). Given an analogous relationship between infectivity and
virulence, coinfection also results in a higher virulence but with a
larger number of coexisting parasite strains than superinfection,
that is, a polymorphism for virulence arises (May and Nowak
1995). It should be noted that superinfection and coinfection are
two extremes of a continuum of possible models, each with its
respective assumptions regarding within-host competition (Adler
and Mosquera Losada 2002; Nowak and Sigmund 2002).

In light of the diversity in the experimental results, the present
study examines simultaneous and sequential mixed-strain infec-
tions in the Daphnia–Pasteuria host–microparasite system. Se-
quential infections of two different strains in particular may un-
cover mechanisms of within-host dynamics, because they allow to
manipulate the relative advantage of one strain over the other. This
system is suitable for studying multiple infections because clonal
reproduction of the host, Daphnia magna, allows us to control
effects of host heterogeneity with regards to susceptibility.
Additionally, virulence and the expected lifetime transmission
success of its directly transmitted semelparous endoparasite, Past-
teuria ramosa, can be accurately measured based on the host’s
time of death and the subsequent release of spores, respectively.
Genetic markers allow us to quantify the relative success of coin-
fected parasite isolates.

**Materials and Methods**

**HOST–PARASITE SYSTEM**

*Daphnia magna* Straus is a cyclical parthenogenetic, freshwater
cladoceran that often inhabits eutrophic ponds. Sexual reproduc-
tion is triggered environmentally (Kleiven et al. 1992). In nature
*D. magna* is frequently found to be infected by a wide variety of
bacterial, microsporidial, and fungal parasites (Green 1974; Ebert
2005), with severe impact on host fitness (Stirnadel and...
Ebert 1997; Ebert et al. 2000b). Age at first reproduction is about 10 days (at 20°C) but the life span of uninfected Daphnia is up to 100 days, whereas parasitized individuals live much shorter (Ebert et al. 2000a). We used a single clone (HO2) originally from a pond in Hungary, 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 Daphnia in 400 mL glass beakers, each containing eight individuals with artificial medium (Klüttgen et al. 1994; modified as per Ebert et al. 1998), where they were fed daily with about $1.5 \times 10^5$ cells mL$^{-1}$ of the chemostat-cultured unicellular algae Scenedesmus gracilis.

*Pasteuria ramosa* Metchnikoff 1888 is a bacterial obligate endoparasite of Daphnia (Ebert et al. 1996; Ebert 2005). Transmission occurs through contact between the host and the waterborne spores, and most likely requires the ingestion of the endospores by a filter-feeding host. Following infection the bacterium grows in the body cavity of its host, causing sterilization, and ultimately millions of endospores are released from the decaying cadaver of a formerly infected host (Ebert et al. 1996). Castration can occur before the host produces any clutches, and mature spores (about 5–6 μm spherical endospores) are found within the host from about 20 days following infection. The *P. ramosa* isolates for this experiment were obtained from infected *D. magna* individuals from Germany (isolate P1), Finland (P3), and Belgium (P4). These hosts were well-fed until they died, upon which their parasite spores were used to repeatedly propagate infection via the same host clone HO2 of our experiment, until there were enough spore-carrying cadavers to produce sufficient amounts of spore suspensions for the experiment. Spore suspensions for each parasite isolate were prepared by grinding five previously infected HO2 cadavers. All cadavers were carefully homogenized and spore concentrations were determined using a haemocytometer (Thoma ruling).

**EXPERIMENTAL SETUP**

We followed a cohort of 616 *D. magna* individuals and examined the outcome of single infections as well as multiple infections, both simultaneously and sequentially. We used offspring of the third generation of the HO2 isofemale line and employed a split-brood design to minimize maternal effects. In total there were 22 treatments, each with 28 replicates, as depicted in Table 1: nine single infections, three simultaneous multiple infections, three sequential single infections, six sequential multiple infections, as well as an uninfected control group. “Multiple” hereafter refers to the multiplicity of isolates, not to the multiplicity of exposure. Throughout this experiment and on a daily basis, we monitored *Daphnia* survival, release of offspring, and the amount of *Pasteuria* spores following the host’s death. We defined virulence as host mortality, that is, time-to-host-death (since birth). Host fitness was defined as the lifetime number of offspring produced and parasite fitness as the number of spores at the time of host death, which is equal to the lifetime spore production of an infection.

In detail, to start the experiment we separated newborns from the HO2 mother generation (0–24 h old, third clutch offspring) into four 400 mL beakers and fed them daily with $1.5 \times 10^5$ algae cells mL$^{-1}$ medium. On day four we singly placed all *Daphnia* in 100 mL jars, filled with 20 mL of artificial medium, and initially fed them $2 \times 10^6$ algae cells per animal per day. The first infection of the relevant treatments was performed on day five. 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 the appropriate *Pasteuria* 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 $3 \times 10^6$, $5 \times 10^6$, $6 \times 10^6$, $7 \times 10^6$, $8 \times 10^6$, $9 \times 10^6$, and $10 \times 10^6$ algae cells per day, respectively.

**Table 1.** Experimental design. For single infections only one isolate is shown; for multiple infections only treatments per one pair of isolates are shown. All possible combinations for three isolates of *P. ramosa* were produced, resulting in a total of 21 treatments plus a control treatment (without parasite exposure). X and Y stand for 1, 3, and 4, with X ≠ Y.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Type of infection</th>
<th>First infection (day 5)</th>
<th>Second infection (day 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PX+0</td>
<td>Single</td>
<td>50,000 spores of PX</td>
<td>None</td>
</tr>
<tr>
<td>PX+PX</td>
<td>Single, double dose</td>
<td>100,000 spores of PX</td>
<td>None</td>
</tr>
<tr>
<td>0+PXd</td>
<td>Single, delayed</td>
<td>None</td>
<td>50,000 spores of PX</td>
</tr>
<tr>
<td>PX+PXd</td>
<td>Single, sequential</td>
<td>50,000 spores of PX</td>
<td>50,000 spores of PX</td>
</tr>
<tr>
<td>PX+PY</td>
<td>Multiple, simultaneous</td>
<td>50,000 spores of PX and of PY</td>
<td>None</td>
</tr>
<tr>
<td>PX+PYd</td>
<td>Multiple, sequential</td>
<td>50,000 spores of PX</td>
<td>50,000 spores of PY</td>
</tr>
<tr>
<td>PY+PXd</td>
<td>Multiple, sequential</td>
<td>50,000 spores of PY</td>
<td>50,000 spores of PX</td>
</tr>
</tbody>
</table>
Table 2. Repetitive DNA PCR primer sequences.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr1*</td>
<td>ACCTAAAGAACAGGAATATCTGGA</td>
<td>GCATGGAATGATTTTTGCTG</td>
</tr>
<tr>
<td>Pr2*</td>
<td>CTGCTGGATGGATGGACTACGTGA</td>
<td>ACGGTTCCCGTAGGTATAGG</td>
</tr>
<tr>
<td>Pr3*</td>
<td>GGAACCAATCGAACCAGGTAT</td>
<td>AACGGTTTCTTCGCTTGTTG</td>
</tr>
<tr>
<td>Pr7</td>
<td>AACGTACTGACAAACCAAACA</td>
<td>AATTTTTCTTAGATTGCTAGGTTGA</td>
</tr>
<tr>
<td>Pr9</td>
<td>ATACGACGAACGGAACAAGA</td>
<td>AACAAAGAATTAACGCCATT</td>
</tr>
</tbody>
</table>

*Indicates the primer sets used for the relative quantification of the isolates in the competitive experiment.

The temperature was 20 ± 0.5°C and the light:dark cycle was 16h:8h. 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 were dissected and checked for disease under a phase contrast microscope (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 and for DNA analysis.

GENETIC MOLECULAR ANALYSIS

To trace the relative success of Pasteuria isolates during multiple infections we used isolate-specific genetic markers (VNTRs: variable number of tandem repeats). Bacterial DNA extraction from spores was done with the EZNA Tissue DNA kit (Peqlab, Erlanger, Germany). Bead mill homogenization was performed before DNA extraction using a high-speed (frequency: 1/30 s) bead beater MM300 (Retsch Technology, Haam, Germany), after suspending spore solution in 200 μl of TL buffer and 25 μl of proteinase K in 1.5 mL tubes containing glass beads (0.5, 0.1 and 1 mm diameter). Tubes were subjected to bead beating at 50×100 rpm for 1×10 s, 1×20 s and 3×30 s successively, and then centrifuged at 45×100 rpm for 15 min at 10°C to pellet the bead mix. The supernatant was transferred to a new tube and DNA extraction was performed following the kit procedure. Incubation lasted at 55°C for 2–3 h. Final elution volume was 100 μl. PCR amplification and automatic genotyping are as described elsewhere (for details, see Mouton et al. 2007). The relative quantification of the different P. ramosa isolates within a single host was determined by calculating the ratio of respective peak heights of the isolate-specific alleles on the electropherogram. Three primer sets were used (primers marked with a star in Table 2). We tested the reliability of this method by mixing different proportions of P1 spores with P3 spores: 0.02, 0.05, 0.1, 0.25, 0.5, 0.75, 0.90, 0.95, and 0.98. The correlation coefficients of linear regression were close to 1 for all primers tested, indicating that the empirical values obtained were good estimates of the relative quantification of alleles (Fig. 1).

For single-isolate treatments we randomly tested five infected individuals, to confirm that hosts were indeed carrying spores of one isolate only. For mixed-isolate treatments, we tested 11–20 infected Daphnia and determined the relative presence of each isolate. Hence, we could ascertain the outcome of simultaneous and sequential mixed infections, based upon the isolate’s relative presence in the host and use it as a measure of its relative proportion of P1 spores with P3 spores: 0.02, 0.05, 0.1, 0.25, 0.5, 0.75, 0.90, 0.95, and 0.98. The correlation coefficients of linear regression were close to 1 for all primers tested, indicating that the empirical values obtained were good estimates of the relative quantification of alleles (Fig. 1).
competitive success. The VNTRs had been developed only after the here-described experiment had been conducted. Although isolates P3 and P4 show clear phenotypic differences in their infection profile in response to various host clones (and also in this study against HO2), we did not find any markers to distinguish between them in the treatments.

**STATISTICAL ANALYSES**

All statistical tests were done using SPSS for Windows release 13.0.1 (SPSS Inc. 2005). The effects on virulence, host and parasite fitness were investigated using general linear models (GLM). Because time-to-host-death and spore production were normally distributed and the experiment ended only after all hosts had died, there was no need for censoring data and using specific survival analysis procedures (e.g., Kaplan-Meier or Cox regression). Offspring production failed to meet the normality and equality-of-variances assumptions and was thus analyzed using nonparametric tests. In GLM procedures all factors were considered fixed, except parasite isolate which was treated as a random factor. Models were minimized by removing nonsignificant ($P > 0.10$) terms from the model, starting with interactions (Zar 1999). Contrasts were used to test specific hypotheses in subsets of the total dataset.

Infectivity data were analyzed using binary logistic regression, with infection treatment (nondelayed single infections as the reference category, delayed single infections, simultaneous, sequential) and parasite isolate coded as indicator variables (Hosmer and Lemeshow 2000). We initially constructed a full model that included all variables and their interactions. We then one-by-one removed variables with nonsignificant coefficients, starting with interactions, thereby deriving a reduced model.

### Results

**GENERAL EFFECTS**

Throughout the 22 treatments (including control), some *Daphnia* individuals died for unknown reasons during the first two weeks of the experiment (in total 171 of 616 died or 27.8%). This mortality was unrelated to treatments as there were no differences among treatments ($F_{21,171} = 1.31, P = 0.18$). In some infection treatments a few hosts remained uninfected (up to 24% in the late-exposed treatments). These uninfected *Daphnia* were not included in the analysis of parasite traits and traits related to infected hosts. None of the control *Daphnia* became infected. Host longevity in the control group was on average almost twofold higher than that of other treatments (Table 3[A], Fig. 2A). Host control animals produced 323.4 ± 12.1 offspring per individual (±SE), whereas the pooling of all infection treatments resulted in only 10.2 ± 2.3 offspring per individual ($\chi^2 = 49.49, P < 0.001$; Fig. 2C).

Thirteen of 21 infection treatments (61.9%) resulted in all individuals becoming infected. A logistic regression with

<table>
<thead>
<tr>
<th>Table 3. Analysis of variance (ANOVA) contrasts for time-to-host-death and parasite spore production. $F$ statistic and significance in contrasts in which the degrees of freedom are greater than one are for the joint contrast. Bold typeface indicates significant effects.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Corrected model</td>
</tr>
<tr>
<td>A. Control vs. infection treatments</td>
</tr>
<tr>
<td>Single-strain infection contrasts</td>
</tr>
<tr>
<td>B. Parasite isolate effect (PX+0 vs. PY+0)</td>
</tr>
<tr>
<td>C. Dose effect (PX+0 vs. PX+PX)</td>
</tr>
<tr>
<td>D. Age effect (PX+0 vs. 0+PXd)</td>
</tr>
<tr>
<td>E. Delayed dose effect (PX+PX vs. PX+PXd)</td>
</tr>
<tr>
<td>Simultaneous infection contrasts</td>
</tr>
<tr>
<td>F. Between-simultaneous treatments (PX+PY)</td>
</tr>
<tr>
<td>Sequential infection contrasts</td>
</tr>
<tr>
<td>G. Same-isolate treatments (PX+PXd vs. PY+PYd)</td>
</tr>
<tr>
<td>H. Different-isolate treatments (with P1 vs. without P1)</td>
</tr>
<tr>
<td>I. P1+P4d vs. P1+P1d and P4+P4d</td>
</tr>
<tr>
<td>J. P4+P1d vs. P1+P1d and P4+P4d</td>
</tr>
<tr>
<td>K. P1+P3d vs. P1+P1d and P3+P3d</td>
</tr>
<tr>
<td>L. P3+P1d vs. P1+P1d and P3+P3d</td>
</tr>
<tr>
<td>M. P3+P4d vs. P3+P3d and P4+P4d</td>
</tr>
<tr>
<td>N. P4+P3d vs. P3+P3d and P4+P4d</td>
</tr>
<tr>
<td>Error</td>
</tr>
</tbody>
</table>
Figure 2. Mean ± SE of (A) Time-to-host-death, (B) Parasite spore production, and (C) Host offspring in single infections, and in sequential and simultaneous multiple infection treatments.
Table 4. Logistic regression analysis of the effects of infection treatment, parasite isolate, and their interactions on *P. ramosa* infectivity. Bold typeface indicates significant effects. All interactions were insignificant (*P* > 0.9).

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>df</th>
<th>Change in -2LL</th>
<th><em>P</em></th>
<th>Odds ratio (exp[B])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delayed single infections</td>
<td>1</td>
<td>16.19</td>
<td>&lt;0.001</td>
<td>0.095</td>
</tr>
<tr>
<td>Simultaneous infections</td>
<td>1</td>
<td>&lt;0.01</td>
<td>&gt;0.99</td>
<td></td>
</tr>
<tr>
<td>Sequential infections</td>
<td>1</td>
<td>1.03</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Presence of P1</td>
<td>1</td>
<td>13.57</td>
<td>&lt;0.001</td>
<td>0.088</td>
</tr>
<tr>
<td>Presence of P3</td>
<td>1</td>
<td>0.33</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Presence of P4</td>
<td>1</td>
<td>3.09</td>
<td>0.08</td>
<td></td>
</tr>
</tbody>
</table>

infectivity as the dependent variable, and infection treatment, parasite isolate and their interactions as independent indicator variables, suggests that only delayed single infections and the presence of the parasite isolate P1 (both which reduced infectivity – Table 4, Fig. 2B) had significant predictive power.

**SINGLE INFECTIONS**

We found a significant difference in virulence among the three parasite isolates, with P1 being the most virulent and P4 the least, irrespective of the infective dose used (Table 3[B/C], Fig. 3). Similarly, there was a significant difference in the production of parasite spores, with P4 producing more spores followed by P3 and P1, regardless of dose (Table 3[B/C], Fig. 3). Delayed single infections did not affect the ranking of the parasites in terms of virulence or spore production, and even though time-to-host-death increased significantly, time-to-host-death-since-exposure (*F*~1,373~ = 1.79, *P* = 0.18) and spore production did not change significantly (Table 3[D], Fig. 3). Host fecundity traits did not differ between dose levels or among parasite isolates (Fig. 2C), except for delayed infections that resulted expectedly in an increase in the number of host offspring (*χ*² = 63.14, *P* < 0.001). Dose level did not affect any of the variables in this study, that is, virulence and spore production in single infections using 100,000 spores were similar to those found when *Daphnia* were exposed to two sequential dose levels of 50,000 spores (Table 3[E], Fig. 3). Apparently, in contrast to much larger dose differences (Ebert et al. 2000b), a difference in dose by a factor of two is not enough to produce a significant effect. Therefore, in the following we only compared the higher dose, single infection treatments (100,000 spores) with multiple infections consisting of 50,000 + 50,000 spores.

**SIMULTANEOUS MULTIPLE INFECTIONS**

Time-to-host-death and total spore production in simultaneous multiple infections followed the more virulent strain, that is, individuals in the treatments P1 + P3 and P1 + P4 died much earlier than those in the treatment P3 + P4 (Table 3[F], Fig. 4). However, total spore production among the three simultaneous multiple infection treatments was similar (Table 3[F], Fig. 4). Although hosts exposed simultaneously to P1 and P3, or to P1 and P4, died earlier than those exposed to P1 only (60.3 and 61.6 vs. 66.6 days, respectively, see Fig. 4), these differences were not statistically significant (P1 + P3 vs. P1 + P1: *F*~1,373~ = 0.03, *P* = 0.87; P1 + P4 vs. P1 + P1: *F*~1,373~ = 0.01, *P* = 0.92). In addition, there was a significant difference in host fecundity among simultaneous infection treatments (*χ*² = 8.05, *P* = 0.018).

**SEQUENTIAL INFECTIONS**

Sequential infections using the same isolate resembled the results of simultaneous same-isolate infections (i.e., PX + PXd vs. PY + PYd; Table 3[G], Fig. 3). Correspondingly, spore counts were higher in *Daphnia* infected by P4 than by P3, followed by P1.

---

**Figure 3.** Time-to-host-death (virulence) versus parasite spore production (transmission) of single-isolate treatments with the parasite isolates P1, P3, and P4.

**Figure 4.** Virulence versus transmission of single and simultaneous multiple infections by the parasite isolates P1, P3, and P4.
Host life-history traits did not differ among sequential same-isolate treatments (results not shown).

Time-to-host-death in sequential infection treatments with different isolates (PX + PYd or PY + PXd) was dependent on whether the virulent parasite isolate P1 was present (Table 3[H]), although total spore production did not vary significantly among the treatments. In spite of the fact that time-to-host-death was notably longer in the treatments P3 + P4d and P4 + P3d, offspring counts were appreciably lower ($\chi^2 = 13.61, P = 0.018$).

We also contrasted sequential same-isolate infections (PX + PXd) against sequential infections with different isolates (PX + PYd or PY + PXd). Similar results were obtained when comparing single, double-dose infections (PX + PX) against sequential infections with different isolates (PX + PYd or PY + PXd), which is consistent with the lack of delayed dose effect in Table 3 (E).

In the case of P1 and P4 (the most and least virulent isolates), when P1 had prior residency (P1 + P4d), virulence and total spore production mirrored the virulent isolate P1 (Table 3[I], Fig. 5A). However, when P4 had prior residency (P4 + P1d), virulence and total spore production resembled an average of the two parasite isolates (Table 3[J], Fig. 5A).

In single infections P3 had intermediate levels of virulence and spore production, as compared to P1 and P4. Consistent with this observation, when P3 had prior residency to P4 (P3 + P4d), total spore production was even lower than that of P3 alone (Table 3[M], Fig. 5B). On the other hand when P4 was first to infect (P4 + P3d), time-to-host-death mirrored the virulence of P4, whereas total spore production resembled the more virulent isolate P3 (Table 3[N], Fig. 5B). Similar though nonsignificant patterns for total spore production and virulence were obtained using P1 and P3 (Table 3[K/L], Fig. 5C).

**COMPETITIVE OUTCOME**

We used genetic markers to test for the relative success of the competing parasite isolates within individual hosts. This was done only for multiple infections with P1 and P4, and P1 and P3, as our markers did not allow us to distinguish between P3 and P4. Simultaneous infections with the virulent isolate P1 resulted in an almost complete exclusion of either P4 (Figure 6A) or P3 (Fig. 6B). Consistent with these results, sequential infections in which P1 had prior residency caused the displacement of the less virulent isolates P4 (Fig. 6A) and P3 (Fig. 6B). On the other hand, when the less virulent isolate was first to infect, both isolates succeeded in producing spores, but both isolates suffered by producing fewer transmission stages than expected from single strain infections (Fig. 5A, B).

A closer examination of the relative success of the competing isolates when the less virulent isolate had prior residency (i.e., P3 + P1d and P4 + P1d) revealed, however, a high variance in the relative success. In the case of P1 and P3 ($n = 20$), 55% of the hosts carried primarily P1 spores (> 75%), 30% contained mostly P3 spores (> 75%), and only 15% of the hosts had a fairly equal share of both (40–60%). A similar pattern was observed for P1 and P4 ($n = 11$), where 45% of the hosts carried mainly P1 spores (> 75%) and the remaining 55% contained predominantly P4 spores (> 75%).

**Figure 5.** Virulence versus transmission of single, sequential and simultaneous multiple infections by the parasite isolates (A) P1 and P4, (B) P1 and P3, and (C) P3 and P4.
Figure 6. Relative parasite spore production in single, sequential, and simultaneous multiple infections by the parasite isolates (A) P1 and P4 and (B) P1 and P3.

HOST FECUNDITY

Hosts produced more offspring in mixed-isolate treatments (both simultaneous and sequential) than in single infections ($\chi^2 = 6.46$, $P = 0.011$).

Discussion

We found that the three arbitrarily chosen isolates of *P. ramosa* differed in virulence and spore production, so that later host killing correlated with higher transmission stage production. In simultaneous multiple infections, as well as in sequential infections in which the more virulent strain had prior residency, virulence and spore production resembled those of the more virulent strain. Consistent with this finding, the more virulent strain almost completely prevented the less virulent one from producing any spores, thus emphasizing the relationship between virulence and intrahost competitive success. Only when the more virulent strain infected the host seven days after the less virulent strain, the less virulent strain was able to produce on average a considerable proportion of the transmission stages. In comparison with single infections, multiple infections per se neither resulted in overexploitation by the parasites, nor entailed additional costs upon the host. On the contrary, mixed-isolate infections were less harmful to the host, in terms of lower fecundity costs.

Models of multiple infections often assume that competing parasites lead to an overall increased host exploitation and thus higher virulence (Frank 1996). However, empirical evidence concerning the expression of virulence as a result of mixed-strain infections is varied, ranging from no effect on virulence (Hodgson et al. 2004; Hughes et al. 2004; Vizoso and Ebert 2005) to increased virulence (Taylor et al. 1998; Davies et al. 2002). Our results that mixed-strain infections kill the host as early as single infections, but were not more virulent, fit within the spectrum of attainable virulence levels of other studies, and in particular with other studies using semelparous pathogens with strictly horizontal transmission (Hodgson et al. 2004; Hughes et al. 2004).

Our data provide support for some of the assumptions made by theoretical studies on the evolution of virulence under multiple infections. The less virulent isolates P3 and P4 were competitively suppressed in simultaneous infections as well as in sequential infections in which the virulent isolate P1 was first to infect. Hence, these results are consistent with the assumption made in the superinfection model (Nowak and May 1994), and to some extent with preemptive competition, at least in sequential infections with prior residency of the virulent isolate (Bremermann and Thieme 1989). However, when the less virulent isolates were first to infect, we observed some level of coexistence of different isolates, as assumed by van Baalen and Sabelis (1995). Mixed infections were found to produce less transmission stages per isolate than single infections, which does not support the coinfection assumption (May and Nowak 1995).

Prior (or late) residency (Hood 2003; de Roode et al. 2005a; Jäger and Schjørring 2006), as well as the relative inoculum of the parasite clones (Taylor et al. 1997), was suggested to be an important facilitator of intrahep competition, especially for the less virulent strains. In our study, on average, when the less virulent isolate was first to infect, the competitive outcome appears to resemble scramble competition in terms of relative spore production. However, on a per host basis, host resources were not equally shared by the different isolates across all *Daphnia* individuals. In fact, the relative distribution of each pair of isolates (i.e., P1 and P3, or P1 and P4) was bimodal, with either isolate dominating (> 75%), but not always excluding, the other. This cannot be explained with stochastic effects of very few spores causing the actual infections, as in this case one would find more often total dominance of one strain. Why then was the
competitive outcome of sequential infections so variable, especially when the less virulent isolate had prior residency? Even though dose level was controlled during the experiment, a differential number of successful infections (i.e., number of spores entering the host) could affect the competitive outcome, and in particular the resulting sporeloads per parasite isolate in mixed infections (Ebert 1998; Hochberg 1998). However, it is unlikely that the distribution of isolates would be bimodal. Moreover, this cannot be a result of genetic variability of hosts as all hosts belong to the same clone. A more likely answer is phenotypic heterogeneity among hosts, which may result in differential efficacy of the immune system across Daphnia individuals. A possible mechanism creating variability even within clones could be alternative splicing, which may produce within-clone variation in immune response (Watson et al. 2005) and has recently been discovered in D. magna as well (D. Brites, unpubl. ms.). At present, however, our limited knowledge of the intrahost dynamics of the Daphnia–Pasteuria system prevents us from disentangling the effects of apparent versus interference competition (e.g., Hughes and Boomsma 2004).

Total spore production in multiple versus single infections varied considerably in comparative studies of semelparous pathogens (Hodgson et al. 2004; Hughes et al. 2004; Vizoso and Ebert 2005). In our experiment we found a positive correlation between time-to-host-death and spores produced. In the same Daphnia host horizontally infected by the microsporidium Octosporea bayeri, the parasite isolates were equally virulent, but production of spores varied significantly (Vizoso and Ebert 2005). In the leaf-cutting ant system the parasite strains differed only by virulence (Hughes et al. 2004). Lastly, in the pine beauty moth system the plant species, upon which the hosts were fed, significantly affected the virulence and yield of single infections (Hodgson et al. 2004). Future work should thus extend the range of isolates used, and allow for diverse combinations of virulence and transmission stages.

We observed increased host fecundity in mixed- versus single-strain treatments, alongside with an equivalent production of transmission stages by the more virulent parasite. It may be that upon infection the parasite isolates hinder each other and by doing so delay castration and allow the host to channel yet-unused host resources toward increased fecundity. Later in the infection process, however, parasites may gain control and eventually total spore production reaches a level comparable to single infections. This delay in host exploitation during multiple infections may be important for the host D. magna, as it is often sterilized without laying a single clutch throughout its life span.

Multiple infections of Daphnia clones by genetically diverse P. ramosa strains have been reported from natural populations (L. Mouton, unpubl. ms.). Our experiment demonstrates that virulent strains are better competitors, so that mixed infections will give the more virulent strain an advantage and, if frequent enough, select for higher levels of virulence (Nowak and May 1994; van Baalen and Sabelis 1995; Frank 1996). On the other hand, we found that less virulent strains were superior in terms of spore production when infecting alone, and thus would have an advantage under conditions of low incidence of multiple infections. Infection prevalence in natural populations of D. magna varies widely and may reach in certain ponds or years 100% (Little and Ebert 1999; Mitchell et al. 2004; Duncan et al. 2006; Duncan and Little 2007). Thus in natural populations, it seems likely that the degree with which multiple infections occur varies among ponds, which may lead to spatial differentiation in virulence. If however, the extent of multiple infections varies across years within the same site, a clear spatial differentiation may be blunted by fluctuating selection.

The prediction that frequent multiple infections select for higher virulence hinged on the assumption that more virulent strains are better competitors and that other trade-offs are not important, that is, every thing else is supposed to be equal. This may, however, not always be the case. Less virulent strains may counterbalance their competitive inferiority by having a higher infectivity. This was the case for our strains, where the most virulent strain P1 had the lowest infectivity. Whether this reduced infectivity would offset the benefits of higher competitive ability can only be assessed quantitatively, as it will depend on the exact shape of this multidimensional trade-off. Multidimensionality of trade-offs influencing virulence has been suggested to limit the scope of current models on the evolution of virulence (Ebert and Bull 2003).

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BACTERIAL MULTIPLE INFECTIONS


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