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## Dose effects and density-dependent regulation of two microparasites of *Daphnia magna*

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**Abstract** Individual hosts constitute a limited resource for parasites, suggesting that density-dependent effects may play a role in within-host growth and parasite regulation. This hypothesis has been tested for several helminth parasites, but not for microparasites. We therefore examined dose-response patterns for the microparasitic bacterium *Pasteuria ramosa* and the fungus *Metschnikowiella biscuspidata* infecting the planktonic crustacean *Daphnia magna*. With increasing numbers of transmission stages administered to the host we found that host fecundity and survival and parasite transmission-stage production declined. Using a *k*-value analysis, a method that quantifies the strength of density dependence, we found for both parasites that density dependence acted at all doses, indicating the absence of a minimum density below which parasite fitness is density-independent. At low doses density was exactly compensated, but it was overcompensated at high doses. Overcompensation at high doses was weak for *P. ramosa*, but high for *M. biscuspidata*. At the two highest doses *M. biscuspidata* killed its hosts before any transmission stages were produced. Our data indicate that density dependence is expressed through retarded spore development in *P. ramosa*, but through both host mortality and reduced parasite fecundity in *M. biscuspidata*. A further experiment (*P. ramosa* only) revealed that in well-fed hosts more parasite transmission stages are produced than in poorly fed hosts, suggesting that competition for host resources retards *P. ramosa* development. Our data for *P. ramosa*, but not for *M. biscuspidata*, are largely consistent with assumptions made in models on microparasite epidemiology. We draw attention to the relevance of dose effects and within-host competition for the evolution of virulence.

**Key words** Within-host regulation · Microparasites · Cladocera · Multiple infections · Evolution of virulence

### Introduction

Parasite transmission stages are often more clustered in space than expected by random. This is particularly true for parasites which release millions of transmission stages at once. Such clusters of transmission stages may form local foci of infection and can result in very intense infections, where many parasites enter a single host. As a consequence, hosts may differ strongly in the intensity of infection, with a distribution of parasites per host which is best described as overdispersed, i.e. a few hosts carry the majority of the parasites (Anderson and May 1991; Cox 1993). It is usually observed that the more parasites infect a host individual, the stronger are the parasite-induced effects on host fecundity and survival (Anderson and May 1978; Keymer 1982). Likewise, parasite *per capita* fitness, measured as fecundity, survival or body growth, is usually negatively correlated with the number of parasites entering a host (Anderson and May 1978; Keymer 1982; Michael and Bundy 1989; Nie and Kennedy 1993; Ashworth et al. 1996). On a population level, it has been suggested that these patterns regulate parasite abundance by preventing an unchecked increase in parasite number within individual hosts and host populations (Anderson and May 1978, 1991; Jaenike 1996). This is because parasites in the most intensely infected hosts suffer the highest costs, either due to increased host mortality or due to reduced parasite fecundity and survival. Such density-dependent within-host regulation may be a strong factor regulating parasite populations.

A number of processes may generate density dependence within parasite life cycles, the most important of these being the effect of the host immune system, intra-specific within-host competition for resources, and, possibly, parasite-induced host mortality (Keymer 1982; Quinnell et al. 1990), all of which have been intensively studied in helminth parasites. In contrast to helminths

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and other macroparasites, within-host regulation of microparasites, such as viruses, bacteria and most unicellular parasites has rarely been addressed. The operational definition of microparasitic infections (Anderson and May 1991) includes the idea that “contest” competition (*sensu* Nicholson 1954) dominates within-host population growth, i.e. regardless of how many parasite transmission stages entered the host, reproductive success of the parasite (i.e. the total number of transmission stages produced per infected host) will be approximately the same. This view is based on the observation that microparasite populations usually grow until they reach a certain density (carrying capacity) or are limited by the immune system (e.g. Castaneda et al. 1987; Mock 1987). As a consequence the number of secondary infections resulting from a primary infection (a measure for parasite fitness) is independent of the infective dose of the primary infection. These assumptions can be tested experimentally by plotting a measure of density dependence as a function of spore dose, for example the  $k$ -value (see examples in Varley et al. 1973; Begon et al. 1990). Positive  $k$ -values are found when the number of mature spores found in a host of dose level  $i$  is lower than the number expected by multiplying the spores found in the lowest infective dose level by the dose  $i$ ;  $k$  equals 0 when a doubling in spore dose results in a doubling of the resulting spores. If contest competition dominates within-host population-growth, the slope of  $k$  over the spore dose equals 1 (Varley et al. 1973).

Indirect evidence for density-dependent within-host regulation of microparasites comes from a number of systems. Castaneda et al. (1987) reported that the within-host carrying capacity of the fungus *Paracoccidioides* in mice was dose-independent, but that the time taken to reach the carrying capacity was shorter for higher doses. Higher virulence as a consequence of higher dose has been observed for trypanosomes in mice (Diffley et al. 1987) and a baculovirus of insect larvae (Hochberg 1991). The spread of measles within small communities (e.g. families) is characterised by an increase in dose from the index case to secondary and tertiary infections, with the consequence that case mortality increases on average 3-fold (Garenne and Aaby 1990; Aaby 1991). To our knowledge there are no studies which have quantified directly density-dependent regulation of microparasites.

We report here on experiments designed to test and quantify density dependence during within-host growth of two microparasites in the planktonic crustacean *Daphnia magna*. We address the following questions:

1. Is within-host growth of microparasites density-dependent? Specifically, we use  $k$ -value analysis to quantify the strength of density dependence.
2. Density dependence in many organisms acts only above a certain minimum density (threshold density). We ask whether such a density threshold also applies to a microparasite. We postulate that, in contrast to macroparasites (e.g. Shostak and Scott 1993), there

should not be a density threshold for microparasites, since even a single transmission stage may found large within-host parasite populations. In contrast, macroparasites can only grow in size, but not in number, within their host.

3. Does parasite-induced host mortality affect parasite population growth? Specifically we test whether more intense infections lead to higher host mortality and how host death reduces parasite reproductive success.
4. Does the host nutritional status limit parasite reproductive success?

The *Daphnia*-microparasite system employed provides several advantages for this project. The two microparasites used, the yeast *Metschnikowiella biscuspidata* and the bacterium *Pasteuria ramosa*, form spores in the hemolymph, and are only transmitted after the host is killed. Thus, the number of spores (transmission stages) produced up to host death is a good estimate of the lifetime reproductive success of these semelparous parasites. Further, it is possible to distinguish immature and mature spores in both species, which enables an assessment of the impact of treatment on spore development. Finally, the host can be kept clonally and therefore the confounding effects of genetic variation among hosts can be excluded.

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## Materials and methods

### Host and parasites

Clonally reproducing lines of the cladoceran *Daphnia magna* Straus were used throughout this study. The clone used in the two dose experiments was isolated from a pond south of Oxford (UK). The other clones were bred from resting eggs isolated from a mud sample from a pond in Moscow Zoo, Moscow, Russia. All clones were kept in the laboratory under standardised conditions prior to the experiments. All experiments were done with asexually replicated females. Thus, all hosts were genetically identical, except when clone effects were of interest (see nine-clone experiment below). Culture conditions were: 16/8 h light/dark cycle, 20°C and artificial culture medium (Klüttgen et al. 1994).

We used two endo-microparasites, the bacterium *Pasteuria ramosa* Metchnikoff (Ebert et al. 1996) and the fungus *Metschnikowiella biscuspidata* (Green 1974). Both parasites were obtained from the sample from which the host clone was isolated. Infection occurs when the filter-feeding host ingests the waterborne spores of the parasites. The parasites reproduce in the body cavity of the host and spores are only released after the death of the host. We accumulated spores by keeping infected hosts in 1 l medium (about 30–60 *D. magna* per beaker) and harvested dying hosts from the bottom of the culture over a period of about 4 weeks. The cadavers were kept at 4°C until the start of the experiments. To produce a spore suspension, we carefully homogenised cadavers and filtered (50 µm mesh width) the solution. To obtain a placebo solution for the controls we carried out the same treatment with uninfected hosts. Using a haemocytometer (Thoma chamber) we determined the parasite spore concentration and diluted the spore suspension to the desired concentrations. In both parasites it is easy to distinguish mature and immature spores (Green 1974; Ebert et al. 1996), and we counted only mature spores for the preparation of the spore suspensions.

For the nine-clone and the two-food-level experiment only *P. ramosa* was used. Host clones and the *P. ramosa* strain used in

these experiments were isolates from the population in Moscow Zoo, Russia.

### Dose experiments

For both dose experiments a cohort of 150 juvenile females (12–60 h old), collected from one large mass culture, were split over 30 beakers each with five females in 400 ml medium. Water was replaced every 2nd day and food supply was  $10^5$  cells of the green alga *Chlamydomonas reinhardtii*  $\text{ml}^{-1} \text{day}^{-1}$ . Newborn *Daphnia* were removed daily. After 20 days all newborn *Daphnia* that were born within one night (12 h) were pooled in one beaker (randomisation of maternal effects) and then placed singly in 40-ml beakers, filled with 20 ml medium. To each beaker we added either a defined spore suspension or a control (parasite-free) solution. Three types of controls were used: medium (control 1), a solution produced from uninfected hosts (control 2) or a solution produced from an infective spore suspension which was heat-treated for 60 min at  $90^\circ\text{C}$  (control 3). Controls 1 and 2 were used in both dose experiments, control 3 was only used in the *P. ramosa* dose experiment. Spores and control solutions were added on 2 successive days.

The *M. bicuspidata* dose experiment included five spore doses. The highest spore dose was 37,500 spores  $\text{ml}^{-1}$  medium (in 20 ml medium). This spore concentration was successively diluted 4 times by factor 5 to produce the next lower concentrations, resulting in five dose levels from 60 to 37,500 ( $=60 \times 5^4$ ) spores  $\text{ml}^{-1}$  medium. There were 20 replicates per dose level. For the *P. ramosa* experiment we used 13 dose levels. We started with a concentration of 100,000 spores  $\text{ml}^{-1}$  medium and diluted this solution successively 12 times by factor 2 to achieve the lower concentrations, resulting in 13 dose levels ranging from 24.4 to 100,000 ( $=24.4 \times 2^{12}$ ) spores  $\text{ml}^{-1}$  medium. Nine replicates were used for each dose. Both experiments were terminated 5 days after the last infected host died. At this time, none of the control animals had died.

During the experiments we counted all offspring. We recorded time to host death and counted the number of mature and immature spores in the cadavers by homogenising the cadaver in 0.3 ml water and counting spores with a haemocytometer. In the *P. ramosa* experiment we also sacrificed two infected hosts from each treatment at day 20 and counted the mature and immature spores.

### Nine-clone experiment

In the *P. ramosa* dose experiment described above, infectivities at the lowest dose levels were above 50%. To extend the range of dose effects to even lower levels of infectivity, we reanalysed spore count data from a previously published experiment (Ebert et al. 1998). Here we perform a  $k$ -value analysis of these spore count data, to test whether  $k$ -values at very low infectivities increase with dose. The details of this experiment are described by Ebert et al. (1998). Briefly, nine *D. magna* host clones were challenged with four doses of *P. ramosa* (1000, 12,500, 25,000 and 50,000 spores  $\text{ml}^{-1}$ ). Ten replicates were used for each dose and clone combination. We used  $10^5$  cells per ml medium of the green algae *Scenedesmus gracilis* as food. The experimental set-up was otherwise the same as that described here for the dose experiment.

### Two-food-level experiment

In this experiment we used a high food level ( $2 \times 10^5$  cells of *S. gracilis* per 1 ml medium) and a low food level ( $5 \times 10^4$  cells per 1 ml medium) to test the effect of host condition on reproduction of the parasite. For this experiment one *D. magna* clone was randomly chosen from the nine Russian clones. The same *P. ramosa* isolate as in the nine-clone experiment was used. The experimental set-up was otherwise the same.

All females received the same *P. ramosa* spore dose of 1000 spores  $\text{ml}^{-1}$ . For each food level 16 females were exposed to spores and 16 females were used as controls. Controls received water instead of parasite spores. We measured host body length (including the spina base, but excluding the spina) at day 44 and subsequently killed the animals and counted the number of mature parasite spores. Day 44 was chosen because it is relatively close to the expected death of the infected hosts. Of the 64 replicates 8 were lost, leaving us with 12–16 replicates per treatment group.

### Data analysis

Analysis was done with the SAS statistical package (SAS Institute 1990). For the *P. ramosa* dose experiment we fitted linear and polynomial regressions for the spore counts in relation to the  $\log_{10}$ -transformed doses (PROC REG, SAS Institute 1990). We present only the linear regression in those cases where the quadratic term was not significant.

Evidence for density dependence can be found by analysing  $k$ -values (Varley et al. 1973) in relation to density. An increase of  $k$  with dose indicates density-dependent growth of the parasite.  $k$  can be calculated in various ways. We use a method employed in plant science, where  $k$  for seed production at any density is calculated relative to the seed production at the lowest density (see for example Palmblad 1968).  $k$  was calculated as

$$k_i = \log_{10} (M_i D_i / M_0 D_0) \quad (1)$$

where  $k_i$  is the  $k$ -value at dose  $i$ ,  $\log_{10}$  is the decadian logarithm,  $M_i$  is the number of mature spores produced by a host challenged with dose  $i$ ,  $D_i$  is the dose level (=number of spores  $\text{ml}^{-1}$ ),  $M_0$  is the number of mature spores produced by a host challenged with the lowest dose and  $D_0$  is the number of spores of the lowest dose level. The lowest dose with respect to  $k$  is defined as the dose which resulted in at least one infected host. Equation 1 implies that positive values for  $k$  are found when the number of mature spores found in a host of dose level  $i$  is lower than the number expected from multiplying the spores found in lowest infective dose level by the dose  $i$ .  $k$  equals 0 when a doubling in spore dose results in a doubling of the resulting spores, or in other words, when the *per capita* reproductive success of the parasite is independent of the dose. For the lowest dose level,  $D_0$ ,  $k$  is defined as 0. If the parasite is not able to reproduce at all (i.e.  $M_i=0$ ),  $k$  is infinite. Only infected hosts were used to calculate the  $k$ -values.

In the nine-clone experiment infectivity among clones varied widely (Ebert et al. 1998). Therefore we calculated  $k$ -values for each clone and used the lowest dose which resulted in at least one infected host as the reference point (determining clone-specific  $D_0$  and  $M_0$ ). For the analysis of the average slope of  $k$  over the  $\log_{10}$ (spore dose  $\text{ml}^{-1}$ ) we pooled all data and plotted them against  $\log_{10}$ (spore dose  $\text{ml}^{-1}$ ), such that the reference points for each clone had the coordinates 0,0. A regression, forced through the origin (i.e. regression without intercept), was used to calculate the slope of  $k$  over the  $\log_{10}$ (spore dose) (SAS, PROC REG, option NOINT; SAS Institute 1990). Since in this experiment infectivity for many clones was only around 10% at the lowest dose level, this  $k$ -value analysis is informative even at low levels of infectivity.

To calculate the expected number of spores resulting from an infection (parasite lifetime reproductive success) it is necessary to include the likelihood of host survival until parasite-induced host death. We calculated for each infected host the likelihood of survival until parasite-induced death (we assume that death in our experiments was always caused by the parasite, which is supported by the near zero mortality among the controls), using three mortality rates:  $m=0.001$ , 0.01 and 0.1  $\text{day}^{-1}$  (calculations with  $m=0$  result in estimates nearly the same as those for  $m=0.001$  and are therefore omitted here). For each host, parasite lifetime reproductive success was estimated as (probability of infection for a given dose)  $\times$  (probability of host survival until the parasite killed it)  $\times$  (number of mature spores produced), given that the host survived until mature spores were produced. This fitness calculation

assumes that parasite-independent host death rate is constant over time and that hosts which die before the parasite kills them do not contribute to parasite fitness (i.e. no spores produced). The resulting numbers are estimates of expected spore production per infection. To obtain *per capita* spore production rates, these numbers were divided by the dose. To obtain more realistic fitness estimates for the parasite, the estimates should be multiplied by the likelihood that a single spore will cause a secondary infection. However, if the quality of the spores produced during an infection is independent of the dose that caused this infection, this would only result in a different scaling of the fitness estimates, and was therefore omitted here.

## Results

### *M. bicuspidata* dose experiment

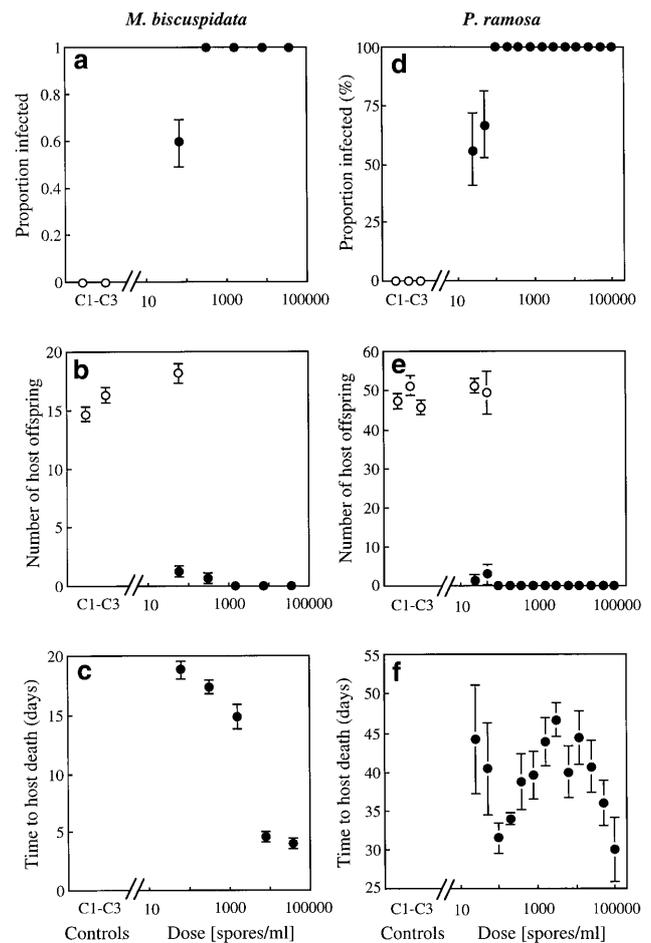
The proportion of infected hosts was 60% at the lowest dose, but was 100% in all higher dose treatments (Fig. 1). All controls were uninfected. Host fecundity and survival were reduced in infected hosts and this effect was stronger the higher the dose (Fig. 1). In the two highest dose treatments all hosts died without producing any offspring. The non-infected hosts from the lowest dose treatment had fecundities similar to that of the control females (Fig. 1).

The number of spores recovered from infected hosts at death declined rapidly with increasing dose, and no mature spores were recovered from hosts in the highest dose levels (Fig. 2). However, at least some immature parasite spores were found at all dose levels. The proportion of mature spores was around 60% for the three lower dose levels, but fell to zero for the two highest dose levels (Fig. 2).

### *P. ramosa* dose experiment

The proportion of infected hosts was 100% at all but the two lowest dose levels, where infectivities were 55.5% and 66.7% (Fig. 1). Host fecundity was zero at all but the lowest two dose levels where infected hosts succeeded in producing a few eggs (Fig. 1). Uninfected hosts from the two lowest dose levels had fecundities similar to the controls (Fig. 1). Survival of infected hosts showed an unexpected non-linear relationship with dose level (Fig. 1). With increasing dose level, time to death first declined, then increased and finally declined again. However, since variances within dose levels for time to death were high for the two lowest doses, the initial decline should be interpreted with caution.

The number of immature spores at day 20 first increased with increasing dose, but then declined steadily (Fig. 2, Table 1). In contrast, at host death, the number of immature spores increased steadily with dose. Comparing their numbers at day 20 and at host death shows that over time the number of immature spores declined at low doses (an expected consequence of spore maturation), but increased at high doses (Fig. 2). The number of mature spores at day 20 was low at all doses, but lowest



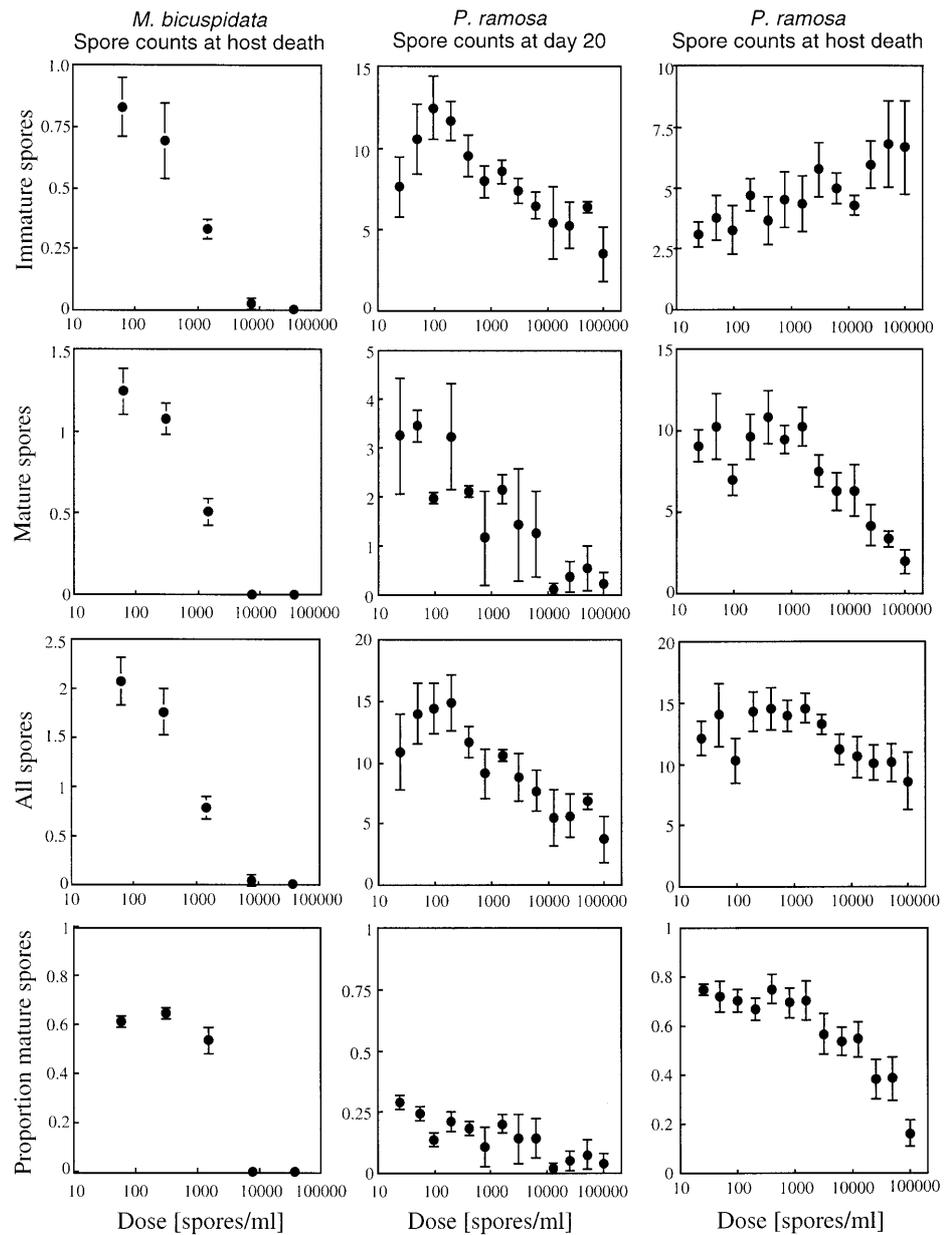
**Fig. 1a–f** Proportion of infected hosts, total host fecundity and time to host death in relation to parasite spore dose. **a–c** Data for *Metschnikowiella bicuspidata*, **d–f** data for *Pasteuria ramosa*. C1, C2 and C3 refer to the different control treatments without parasites. In **a, b, d, e** filled symbols indicate infected hosts, open symbols indicate uninfected hosts. Means and SEs are given

(close to zero) at the highest doses (Fig. 2, Table 1). There was a trend for levels of mature spores at host death to be highest at intermediate spore doses (significant quadratic regression term, Table 1). At high doses very few spores were found (Fig. 2, Table 1). The proportion of fully developed spores at day 20 declined steadily with increasing dose, but was generally low for all dose levels (Fig. 2, Table 1). The proportion of fully mature spores at host death did not change across the seven lower dose levels (about 70%), but declined steadily thereafter (Fig. 2, Table 1).

### *k*-value analysis

The results of both dose experiments indicate density-dependent spore production, as the number of spores produced did not increase proportionally to the dose levels. A *k*-value analysis confirmed this. The *k*-value for mature spores in the *M. bicuspidata* experiment in-

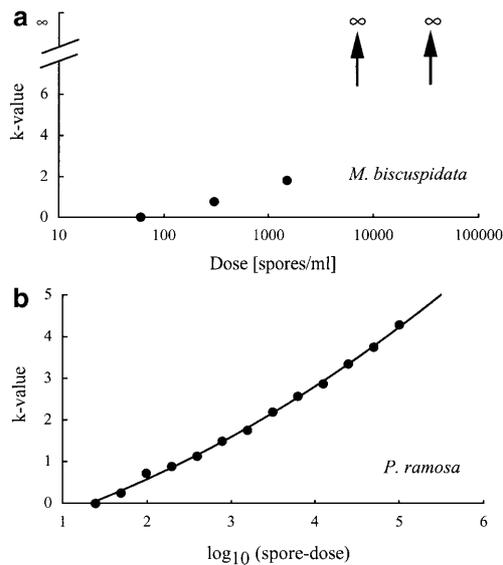
**Fig. 2** Number of parasite spores (in millions) found in hosts at death or at day 20 post-infection (*P. ramosa* only) in relation to parasite dose. Means and SEs are given



**Table 1** Regression equations for spore production of *Pasteuria ramosa* in relation to spore dose. The quadratic term was only included when it was significant in a stepwise regression. The regression equation is:  $y=a+bx+cx^2$ , where  $x=\log_{10}$  (spores  $\text{ml}^{-1}$ ). For traits for which the c parameter was not significant, we list only the parameters for a linear regression (compare with graphs for *P. ramosa* in Fig. 2)

Trait	Regression parameters ( $y=a+bx+cx^2$ )			
	a	b	c	$r^2$
<b>Counts at day 20</b>				
Immature spores	8,872,761	1,650,872	-544,335	0.55***
Mature spores	4,562,221	-914,919		0.60***
All spores	13,966,772	355,612	-484,789	0.62***
Proportion mature spores	0.340	-0.062		0.52***
<b>Counts at host death</b>				
Immature spores	3,135,264	13,483	139,006	0.12**
Mature spores	3,599,855	5,250,933	-1,132,838	0.43***
All spores	6,735,119	5,264,416	-993,832	0.15**
Proportion mature spores	0.461	0.258	-0.061	0.51***

Significance of regression: \*\* $P<0.01$ , \*\*\* $P<0.001$



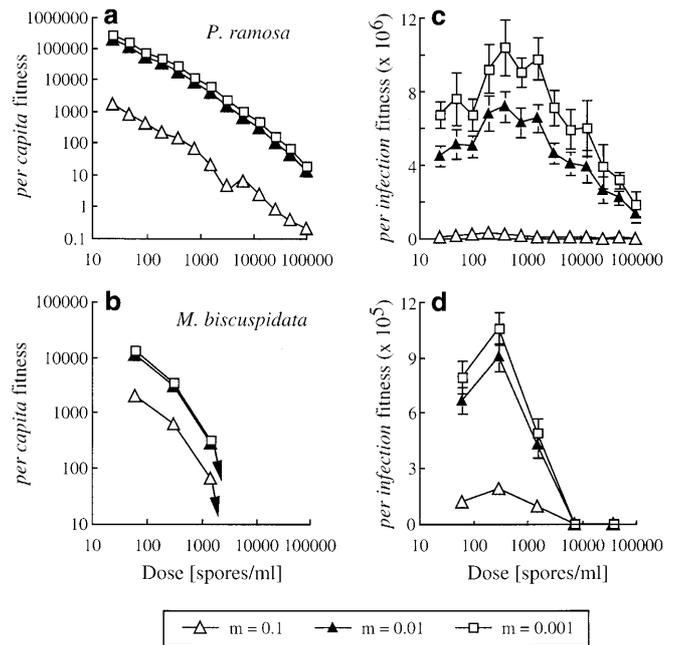
**Fig. 3**  $k$ -values for the reduction in spore production of **a** *M. bisкупidata* and **b** *P. ramosa* in relation to spore dose

creased rapidly (Fig. 3), reaching infinity for the two highest dose levels, indicating very strong overcompensating density dependence. Even for the lowest dose levels,  $k$  increased with dose.

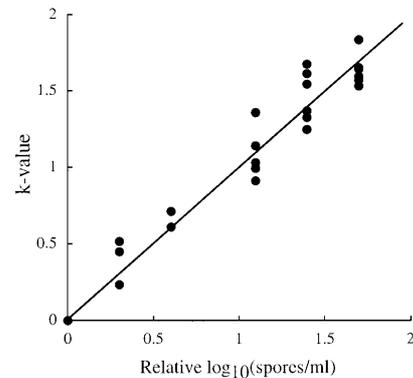
The  $k$ -values for mature spores in the *P. ramosa* experiment also increased with dose (Fig. 3), as indicated by a polynomial regression:  $k=a+bx+cx^2$ , where  $x=\log_{10}(\text{spore dose ml}^{-1})$ ;  $a=-1.045\pm 0.125$ ,  $P<0.0001$ ;  $b=0.792\pm 0.0603$ ,  $P<0.0001$ ;  $c=0.100\pm 0.016$ ,  $P<0.0001$ ;  $r^2=0.99$ ,  $n=13$ . The significant quadratic term indicates that density dependence for *P. ramosa* became stronger as the dose increased. To allow comparison of the slope of  $k$  with the regression of the nine-clone experiment (see below) we also performed two linear regressions, one for the lower six dose levels and one for the remaining higher levels. The slope of  $k$  over  $\log_{10}(\text{spore dose})$  was  $0.970 (\pm 0.0572)$ ,  $P<0.001$ ,  $n=6$ ,  $r^2=0.98$  for the lower dose levels and  $1.360 (\pm 0.0407)$ ,  $P<0.001$ ,  $n=7$ ,  $r^2=0.99$  for the higher dose levels. While the slope for the lower dose levels was not significantly different from 1 ( $P>0.3$ ), the slope of  $k$  for the higher levels was significantly greater than one ( $P<0.001$ ). This indicates nearly exact density compensation for the lower dose levels, but density overcompensation for higher dose levels.

#### Parasite expected fitness

Parasite spore production *per capita* was highest at the lowest doses followed by a rapid decline with increasing dose (Fig. 4), for both parasites. In contrast, parasite fitness estimates *per infection* indicate that intermediate dose levels resulted in the highest expected spore numbers (Fig. 4). The initial increase of the curves is not only an effect of the reduced infectivity at low doses, but



**Fig. 4** Estimates of parasite fitness **a, b** *per capita* and **c, d** *per infection* for **a, c** *P. ramosa* and **b, d** *M. bisкупidata*. Each graph shows fitness estimates calculated for three different host mortality rates,  $m$  (mortality day<sup>-1</sup>)



**Fig. 5**  $k$ -values plotted against  $\log_{10}(\text{spore ml}^{-1})$  for data from the nine-clone experiment. The  $x$ -axis is scaled so that for each clone the lowest dose which resulted in at least one infection is set to 0. The diagonal line has a slope of 1

also an effect of the age at host death. The later the host dies, the higher its chance of dying for reasons independent of parasitism.

#### $k$ -value analysis of nine-clone experiment

The  $k$ -values calculated for each of the nine clones and plotted to a common reference point (zero in Fig. 5) showed a linear increase over dose with a slope of  $1.0004 (\pm 0.0205)$ ,  $P<0.001$ . This slope is not significantly different from 1, indicating nearly exact density compensation for this range of dose levels. The range of

infective doses for this experiment represents approximately the range of the lower dose levels of the *P. ramosa* dose experiment, for which a linear regression revealed a slope for  $k$  of 0.970 ( $\pm 0.0572$ ) (Fig. 3). The slopes of the two experiments did not differ significantly ( $P > 0.2$ ).

#### Two-food-level experiment

Infection rates were 50% (6 of 12) in the high food level and 60% (9 of 15) at the low food level ( $\chi^2 = 0.27$ ,  $P = 0.6$ ). Under high-food conditions hosts harboured 3.84 times as many spores as did low-food hosts [high food (mean  $\pm$  SE): 22.75 million  $\pm$  4.05, low food: 5.92  $\pm$  3.63,  $t$ -test on  $\log_{10}$ -transformed data:  $t = 3.49$ ,  $df = 13$ ,  $P = 0.004$ ]. There were strong food and parasite effects on host fecundity ( $\log_{10}$ -transformed total fecundity, food:  $F = 207.33$ ,  $df = 1$ ,  $P < 0.0001$ ; infection:  $F = 190.63$ ,  $df = 1$ ,  $P < 0.0001$ ; MS error = 0.0265;  $r^2 = 0.91$ ). Although the parasite produced more transmission stages in well-fed hosts, the well-fed infected hosts produced about 4 times more offspring than the poorly fed infected hosts (high food: 46.66 offspring  $\pm$  4.46, low food: 11.44  $\pm$  2.35,  $P < 0.001$ ,  $df = 13$ ). Thus, improved host conditions increased reproductive success of both the host and the parasite.

## Discussion

#### Density dependence of within-host parasite growth

For two microparasites of *D. magna* the number of transmission stages resulting from an infection was less than proportional to the increase in the infective dose. This indicates the presence of within-host density-dependent regulation of parasites. In case of the yeast, *M. biscuspidata* the  $k$ -value analyses indicates that density dependence is "scramble"-type, i.e. parasite reproductive success falls to zero beyond a certain dose (Figs. 2,3). In contrast, for the bacterium *P. ramosa*, density dependence is more "contest"-type (Figs. 3,5), i.e. the number of transmission stages produced does not change very strongly with increasing dose. For higher dose levels of *P. ramosa*, density is only weakly overcompensated (Fig. 3), i.e. the higher the dose the lower the spore production per infection. Thus, in contrast to *M. biscuspidata*, *P. ramosa* appears to better fit the operational definition of a microparasite, which includes that competition within hosts is contest-type (Anderson and May 1991).

The two parasites appear to differ in the mechanism which leads to density dependence. With increasing dose, an increasing proportion of immature spores were found at death of the hosts infected with *P. ramosa*, suggesting that density dependence in *P. ramosa* acted mainly through retarded spore development. This effect is already visible 20 days after infection. By contrast, density dependence in *M. biscuspidata* is mainly deter-

mined by parasite-induced host death. At the two highest dose levels of *M. biscuspidata*, hardly any immature and no mature spores were found. It seems that hosts died so quickly that the parasites had not enough time to produce and mature spores. The ascospores of *M. biscuspidata* are rather large (up to 45  $\mu$ m) and pointed and enter the host by penetrating the gut wall into the hemolymph. The impact of this mechanical damage could be fatal at high doses, producing the observed effect. At the lower dose of *M. biscuspidata* within-host competition appeared to reduce the number of spores which grew and matured. However, as the proportion of mature spores did not decline across the three lower dose levels (as was the case for *P. ramosa*), retarded spore development seems not to have played much of a role in regulating spore number.

The differences between the two parasites might also be explained by the size differences of the parasites. As *M. biscuspidata* spores are much larger than *P. ramosa* spores, it is plausible that resource usage per parasite spore is much higher for the fungus than for the bacterium. Therefore, it is possible that, given the same spore dose, intraspecific competition for the fungus would be more pronounced than for the bacterium. This is consistent with our finding of a more rapid increase of  $k$  over dose for the fungal parasite than for the bacterium.

#### Density dependence acts without a threshold

Density-dependent reproductive success has been shown for many plants and free-living animals as well as macroparasites and parasitoids (see examples in Varley et al. 1973; Begon et al. 1990; Shostak and Scott 1993; Godfray 1994). Typical of many examples is the existence of a density threshold, below which density dependence does not act. Below the density threshold resources are presumably superabundant and the *per capita* fitness is therefore independent of density. However, microparasites reproduce inside their hosts and therefore a single parasite can give rise to a huge parasite population. Thus, density dependence of the *per capita* fitness can be expected for any number of invading parasites, suggesting that a threshold density, below which density dependence does not act, is unlikely. Our data conform to this expectation. Even at doses so low that only a few percent of the challenged hosts become infected, the slope of  $k$  was 1 (Fig. 5).

#### Does parasite-induced host mortality affect parasite population growth?

Evidence for parasite population regulation comes largely from long-term studies of helminth parasites (reviewed in Quinnell et al. 1990). One mechanism of regulation which has been discussed is parasite-induced host mortality. Our data indicate that this mechanism might be important for *M. biscuspidata*, as hosts infected with

high doses died without producing any parasite transmission stages. Thus, parasite population growth is not only regulated by within-host density-dependent processes, but also by the increased mortality of intensely infected hosts.

In contrast, all hosts infected with *P. ramosa* produced spores and there was no simple relationship between host death and spore dose. If we ignore the estimates for the two lowest spore doses (very high standard errors!), a humped shaped relationship between time to death and dose level results (Fig. 1). We speculate that the absence of a simple relationship between host mortality and dose is due to the complex interactions between this parasite and its host. During infection resource allocation of hosts is changed drastically, as the parasite induces castration (Fig. 1) and gigantism (infected hosts are larger than uninfected hosts: Ebert et al. 1996; Zschokke 1998). We have currently no satisfactory model to bring these aspects of *P. ramosa*-*D. magna* interactions into one framework.

#### Host nutritional status limits parasite success

Often parasites do better in hosts fed a poor diet, presumably as a consequence of an impaired host immune system (Dobson and Bawden 1974; Slater and Keymer 1986; Wakelin 1986). However, *P. ramosa* in well-fed infected *D. magna* did markedly better than those in poorly fed hosts. Well-fed hosts produced not only more parasite spores, but also more offspring than poorly fed hosts (the parasite is not vertically transmitted), indicating that within-host resources are limiting factors for the host and the parasite. Our results indicate that good nutrition seems not to improve the hosts' immune response, either in terms of reducing the incidence of infection, or in limiting within-host parasite propagation.

#### Density dependence and aggregation

The infection of individual hosts with many parasites can influence individual parasites and their epidemiology in two different ways: through its effects on host and thus parasite fitness and through within-host density-dependent reduction in parasite reproductive success (Anderson and May 1978, 1991). Jaenike (1996) pointed out that the shape of the relationship between parasite fitness and infection intensity is of crucial importance for parasite epidemiology. For the two parasites studied here, host and parasite *per capita* fitnesses decline about exponentially with increasing spore dose. Jaenike (1996) showed that for exponential fitness functions, but not for sigmoidal fitness functions, the net deleterious effect of the parasites on host populations is indeed reduced by parasite aggregation and that mean parasite fitness is reduced as well. Thus, it is conceivable that, if aggregation is the rule for *P. ramosa* and *M. bicuspidata*, their populations might be regulated as a consequence of within-

host density dependence (Anderson and May 1978, 1991; Jaenike 1996). Given the strong effect of high doses of *M. bicuspidata* on survival of *D. magna* (Fig. 1), population regulation would be even more effective for this species than for *P. ramosa*.

Aggregation of microparasites has hardly been investigated and we can only speculate whether it is found in *Daphnia* microparasites. The spatial distribution of certain types of microparasite transmission stages is rather patchy in nature, and such patchiness might lead to aggregation of parasites in a few, heavily infected hosts. Patchiness is a consequence of the mode of spore release of *P. ramosa* and *M. bicuspidata*, and is likely to be found in other semelparous parasites with a "first grow-later kill" strategy as well (for examples see Ebert and Weisser 1997). Spores from such parasites are only released after the parasite mediated host death. A cadaver of a *P. ramosa* infected *D. magna* might harbour up to 100 million parasite spores. Locally dense patches of spores can be seen at places where host cadavers decay in laboratory cultures (D. Ebert, personal observations), forming highly localised foci of infection. The normally plankton-feeding *D. magna* encounter these spores when they browse over the sediments to stir up food particles, which are then ingested by filter feeding (Horton et al. 1979; Freyer 1991; Ebert 1995). Although this might not be the only mode of infection, it would produce infections of high dose in those hosts which encounter patches of spores. Other examples of parasites with similar life-history are granulosis viruses in *Plodia interpunctella* (Indian meal moth), nuclear polyhedrosis virus and the fungus *Entomophaga maimaiga* in *Lymantria dispar* (gypsy moth) and the bacterium *Pasteuria penetrans* in *Meloidogyne* sp. nematodes.

The range of spore doses used in this study is rather high, encompassing nearly 4 orders of magnitude. However, as for a number of vertebrate macroparasites, very high numbers of parasites per host have been observed (Anderson and Michel 1977; Rau 1979), indicating that the ranges used here are not unrealistic under natural conditions.

#### Density dependence and the evolution of virulence

Hochberg (1998) drew attention to the problem of dose and density dependence for understanding the evolution of microparasite virulence. Our work contributes to this discussion in several ways. First, virulence of both parasites studied was dose-dependent, with lower host survival and fecundity at higher doses. As the evolution of virulence will occur within the range of parasite doses normally encountered by hosts, the interpretation of data from the laboratory and from natural populations must account for dose effects. Second, dose effects have important evolutionary implications when different parasite strains infect the same host, because within-host competition is a major factor in the evolution of virulence (Bonhoeffer and Nowak 1994; Nowak and May 1994;

Frank 1996; Ebert and Mangin 1997; Ebert 1998a). Our data indicate that multiple infections of microparasites have high *per capita* costs and therefore could lead to intense within-host competition (Fig. 4). In contrast, if the parasites that enter the host are related to each other, lower levels of virulence might be favoured because of cooperation among relatives (Frank 1996). Third, the “cost of virulence” model is based on the idea that virulence is a costly, but necessary consequence of parasite reproduction and/or survival (Ewald 1980; Anderson and May 1982; May and Anderson 1982). Our data indicate that *M. bicuspidata* can kill its host before any transmission stages are produced. Thus, selection might act against excessive virulence. Fourth, the number of parasites entering a host is not only a function of the number of spores the host encounters, but also of the hosts’ susceptibility. Given the same dose, a susceptible host might receive many more spores than a less susceptible host (Ebert 1998b; Hochberg 1998). Thus, the more infectious a parasite strain is, the stronger is the impact of density dependence. For parasites with overcompensating density dependence (slope of  $k$  over  $\log_{10}$ (spore dose) greater than 1), this could result in lower reproductive success in the more susceptible host, or to lower reproductive success of more infectious parasite strains. Our data show overcompensation for both parasite species, suggesting that higher infectivity might have costs in form of reduced reproductive success.

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