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Coexistence of three microsporidia parasites in populations of the freshwater amphipod *Gammarus roeseli*: evidence for vertical transmission and positive effect on reproduction ☆

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Abstract

We investigated the prevalence, transmission mode and fitness effects of infections by obligatory intracellular, microsporidian parasites in the freshwater amphipod *Gammarus roeseli*. We found three different microsporidia species in this host, all using transovarial (vertical) transmission. All three coexist at different prevalences in two host populations, but bi-infected individuals were rarely found, suggesting no (or very little) horizontal transmission. It is predicted that vertically-transmitted parasites may exhibit sex-specific virulence in their hosts, or they may have either positive or neutral effects on host fitness. All three species differed in their transmission efficiency and infection intensity and our data suggest that these microsporidia exert sex-specific virulence by feminising male hosts. The patterns of infection we found exhibit convergent evolution with those of another amphipod host, *Gammarus duebeni*. Interestingly, we found that infected females breed earlier in the reproductive season than uninfected females. This is the first study, to our knowledge, to report a positive effect of microsporidian infection on female host reproduction.

Keywords: Microsporidia; Vertical transmission; Sex ratio distortion; Host reproduction; Crustacea

1. Introduction

Transmission mode is an important component of parasite life history strategies that determines their spread in host populations (Ebert and Herre, 1996; Galvani, 2003). Vertical transmission occurs in many microparasite groups (Werren and O'Neill, 1997; Bandi et al., 2001; Terry et al., 2004). Such parasites transmit transovarially through females, and male hosts represent an evolutionary dead end (Bandi et al., 2001). A number of predictions can be made about the consequences of infection by vertically-transmitted parasites on host fitness. First, a vertically-transmitted parasite is predicted to employ sex-specific virulence by altering host sex ratio to increase the proportion of the transmitting sex (Hurst, 1993; Bandi et al., 2001). Indeed, in insects, some microorganism infections result in male-killing (Kellen et al., 1965; Hurst et al., 1994). Another example of sex-specific virulence is seen in crustacean hosts, where infection results in feminisation of genetic males (Bulnheim, 1978; Dunn et al., 1993; Bouchon et al., 1998). Second, since vertically-transmitted parasites rely on successful host reproduction for their transmission, it is predicted that, everything else being equal, vertically-transmitted parasites will have either a positive or neutral effect on host fitness and, in particular, on host reproductive success, e.g. fecundity and sexual behaviour (Ebert and Herre, 1996; Hurst et al., 1994; Dunn and Smith, 2001). Both positive (e.g. *Buchnera* bacteria, Douglas, 1996; or *Wolbachia* bacteria, Dedeine et al., 2001), and neutral fitness effects (e.g. microsporidia, Dunn et al., 1993) on

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hosts have been documented for several different vertically-transmitted microorganisms.

Microsporidia are obligate intracellular parasites thought to be closely related to fungi (Baldauf et al., 2000; Mathis, 2000). They infect a diverse array of animal phyla and exhibit a range of transmission strategies (Dunn and Smith, 2001). As with other parasites, the degree of virulence they exhibit is correlated with transmission mode (Ebert and Herre, 1996). Most microsporidia are pathogenic, responsible for numerous infectious diseases in both vertebrate and invertebrate hosts, and are transmitted horizontally (Canning, 1993; Wittner and Weiss, 1999). In invertebrates, however, some microsporidia exhibit mixed transmission strategies, and associated variations in their degrees of virulence (e.g. Agnew and Koella, 1999; Vizoso and Ebert, 2004). For example, in a mosquito species females transmit the microsporidian infection vertically, but it kills, and subsequently transmits horizontally through, male hosts (Becnel and Andreadis, 1999). An intriguing case of vertical transmission was recently reported in a gammarid crustacean (Galbreath et al., 2004), where the microsporidia *Fibrillanosema crangonycis* exhibits a strong pathogenic effect in its host, yet transmits vertically and localises in eggs. It is probable in this case that the pathogenic effect causes horizontal transmission of the parasite, increasing its transmission power.

Microsporidian infections of the amphipod crustacean *Gammarus duebeni* are a very-well documented example of purely vertically-transmitted parasites that induce host feminisation (Dunn et al., 1993, 1995; Terry et al., 1998; Rodgers-Gray et al., 2004). There are at least three different vertically-transmitted parasites in different host populations (Terry et al., 2004), and interestingly, in some locations two different microsporidia infect a single population (Ironside et al., 2003b). However, no double infections in the same individual have been reported (Hogg et al., 2002), and this lends support to evidence obtained from mitochondrial markers that microsporidian transmission in *G. duebeni* is purely vertical (Ironside et al., 2003a). Direct tests of the impact of microsporidian infections on the reproduction of their *G. duebeni* host found negative effects of infection on host fitness (Terry et al., 1998; Kelly et al., 2003), and no evidence for parasite manipulation of host mating success (Kelly et al., 2001). However, sex-ratio distortion is associated with two of these microsporidian infections (Ironside et al., 2003b), a phenomenon due to parasite-induced feminisation of putative males (Dunn and Rigaud, 1998). In employing this strategy, the parasites increase their probability of spreading in the infected host populations, by increasing the proportion of their transmitting host (females) (Hatcher and Dunn, 1995; Rigaud, 1997). In this context, the transmission advantage conferred to the parasite feminising its host compensates for the deleterious effect of infection (Kelly et al., 2003).

In other amphipod crustaceans, microsporidian infections have been inferred from PCR-based screening, and sex ratio distortion has been suggested from the relative frequencies of infected males and females (Galbreath et al., 2004; Terry et al., 2004). However, only in *G. duebeni* has the transmission pattern been studied in detail.

In this study, we investigated microsporidian infections in two populations of the amphipod crustacean, *Gammarus roeseli*. *Gammarus roeseli* and *Gammarus duebeni* have geographically distinct ranges, the former is distributed across South-Central Europe, while the latter is distributed in Northern Europe (Barnard and Barnard, 1983). Consequently, any similarities in microsporidian infection patterns between these two host species are unlikely to be consequences of ecological proximity. Based on a previous PCR screening and phylogenetic analysis, one population of *G. roeseli* has been shown to be infected by two microsporidia species closely related to the parasites in *G. duebeni* (Terry et al., 2004). We therefore aimed to determine whether the pattern of microsporidia infection in natural populations of *G. roeseli* shows different or convergent evolution compared to *G. duebeni*, by asking the specific questions: (i) How many microsporidia strains are there in *G. roeseli*, and do they coexist in the same populations? (ii) Do microsporidia have the same pattern of transmission in *G. roeseli* as *G. duebeni*? (iii) Do they induce host sex ratio distortion? (iv) Does microsporidia infection exert any fitness effect on host reproduction?

2. Material and methods

2.1. Collection and maintenance of Gammarus roeseli

Gammarus roeseli were sampled in two rivers: The River Ouche (site: Colombière, Dijon, Côte d'Or, France) and the River Tille (site: Maillys, Côte d'Or, France), using the kick-sampling technique (Hynes, 1954). Sampling took place in January and March 2002, and in February, March and April 2003. All animals collected were kept for further analysis without discrimination of size, sex or reproductive status. When present, reproductive pairs were separated from free individuals (*G. roeseli*, like many gammarids, form precopula pairs several days before mating, during which the male holds on to the female; Pockl, 1993). Free individuals were transferred into large containers filled with river water, while each pair was transferred to an individual container. The containers were kept at low temperature on ice packs during transfer to the laboratory. For each sample, free individuals were counted, sexed, and maintained in the laboratory in an aquarium ($35 \times 23 \times 20.5$ cm). Pairs were counted and maintained in individual containers filled with 150 ml river water. Gammarids were maintained in a room at $14\,^{\circ}\mathrm{C} \pm 1$, under a constant photoperiod regime of light:dark 12:12. Rotted elm leaves were provided for food.

2.2. Prevalence and molecular identification of microsporidia parasites

In order to investigate the prevalence of microsporidia in *G. roeseli* at each site, a PCR-restriction fragment length polymorphism (PCR-RFLP) test using microsporidian 16S rDNA primers was made, following Hogg et al. (2002) and Ironside et al. (2003b).

Each collected host individual was measured under a stereoscopic microscope using a video-analysis system (linear dimension of the fourth coxal plate, measured from the base to dorsal limit, see Bollache et al., 2000). Individuals were then dissected to remove the gonads, which were then stored in 100% ethanol awaiting DNA extraction. Care was taken to sterilise dissecting forceps between individuals to avoid contamination (serial baths of hypochlorite solution and 70% ethanol). Tissues were crushed, and digested in 300 µl CTAB buffer (100 mM Tris–HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% CTAB; 0.2% 2-Mercaptoethanol) with 10 µl proteinase K (20 mg/ml) for 4 h at 60 °C. After cooling, 2 µl of RNaseA were added, the solution was incubated at 37 °C, then mixed with 300 µl chloroform:isoamyl (24:1) and centrifuged. One hundred microlitres of protein precipitation solution (Promega) was added to the supernatant, gently vortexed and centrifuged. The supernatant was transferred into tubes containing 300 µl isopropanol, and frozen overnight. After centrifugation, pellets were washed in 70% ethanol, dried and resuspended in 50 µl pure water.

A fragment of the microsporidian 16S ribosomal gene was amplified with the primers V1f and 530r (Baker et al., 1994; Weiss et al., 1994). PCR reactions were performed in 25 µl reaction mixtures adjusted to a final concentration of 1.5 mM $MgCl_2$, 0.2 µM of each primer, 200 µM dNTPs, 0.02 µg/µl BSA and 1 U/µl of Promega Taq polymerase, with buffer according to manufacturer's instructions. After initial denaturing at 95 °C for 5 min, 35 cycles of 95 °C (1 min), 57 °C (1 min) and 72 °C (1.5 min) were performed. The final extension was at 72 °C for 10 min. Microsporidian 16S amplification revealed two distinct sizes of PCR products (around 480 and 420 bp). To distinguish between the parasite types, the amplified products of seven large and seven small fragments were sent for sequencing to MWG Biotech after purification following the company's instructions. Sequencing was performed with the primers used for PCR. Sequences were aligned using the ClustalW algorithm in BioEdit v5.0.9 (Hall, 1999), and were compared to microsporidian sequences isolated from *G. duebeni*: *Nosema granulosis* (GenBank AJ011833) and *Microsporidium* sp. A (GenBank AJ438956) (Hogg et al., 2002) (this microsporidia has been subsequently named *Dictyocoela muelleri*, Terry et al., 2004). A restriction map was produced for each sequence, using BioEdit, to reveal specific enzyme cutting sites. These enzymes were then used to digest PCR products, following the manufacturer's instructions (MBI Fermentas), allowing us to determine the prevalence of each parasite strain in our samples.

2.3. Effect of microsporidia on host sex ratio

Following Galbreath et al. (2004) and Terry et al. (2004), we compared the prevalence of infection between males and females, and compared the number of male and female hosts collected at random, to determine whether microsporidia alter host sex ratio. In addition, Rodgers-Gray et al. (2004) identified morphological features of *G. duebeni* intersexes, i.e. males imperfectly feminised by microsporidia, that included genital papillae at the external termination of the vas deferans. Fifty-eight females were randomly collected from Colombière and examined under a steroscopic microscope for the presence of genital papillae.

2.4. Evidence for microsporidia vertical transmission

To detect parasites within freshly-laid embryos, the fluorescent staining technique developed by Terry et al. (1997) was used. Pairs of animals, collected from the wild or formed in the laboratory, were isolated in individual containers, and checked daily until eggs were laid in the female's brood pouch. Eggs were flushed out from the pouch with a gentle water flow, permeated with 5 M HCl, rinsed in distilled water, fixed in acetone at −20 °C for 3 min, and transferred onto a microscope slide. After air-drying at room temperature, they were stained with 10 µl of DAPI (4,6 diamidino-2-phenylindole) at 0.04 mg/ml, and diluted in crustacean Ringer solution with 10% glycerol. Eggs were squashed by exerting gentle pressure on a cover slip, and incubated for 10 min at room temperature. After rinsing with a drop of pure water, the cover slip was sealed with nail varnish. The eggs were screened using a Nikon E600 fluorescent microscope. Infected embryos were characterised by the presence of diplokaryotic nuclei of the microsporidia around the nuclei of host cells (Terry et al., 1997). The number of cells per embryo and the proportion of infected embryos were counted. In addition, parasite number (intensity) was counted in five embryos selected at random from within each infected clutch. After checking the embryos, DNA from female gonads was extracted, and PCR-RFLP screened to identify the parasites (see above).

2.5. Effect of microsporidia on female host fecundity

During the screening of parasite vertical transmission, and during dissections for analysis of parasite prevalence, the number of embryos laid in female brood pouches was counted. Only females carrying eggs at stages 1 and 2 (i.e. at the beginning of development, before partial clutch mortality in the incubating pouch, Pockl, 1993) were taken into account. These data, combined with the measure of female size and PCR-RFLP screening allowed us to

analyse the effect of size and microsporidian infection on female fecundity.

2.6. Effect of microsporidia on female host reproduction

During 2002 at Colombière and 2003 at Maillys, precopular pairs were first observed in March, suggesting that, in agreement with other studies on *G. roeseli* (Pockl, 1993), the reproductive season starts in early spring. The prevalence of microsporidia was compared between paired and unpaired females. The observed differences between the two categories (see results) led to further tests to determine whether infection by microsporidia favours female reproduction.

At Colombière, no pairs were observed in the field sample collected in February 2003. Males and females were maintained together in the same tank in the lab after collection, and after 8 days pairs were observed (pair formation was accelerated by the higher temperature in the lab compared to the field; Pockl, 1993). The pairs were removed and isolated in individual containers.

Females in pairs at the beginning of the reproductive season were either more attractive to males, or, alternatively, matured earlier in the season. To discriminate between these two possibilities, the diameter of the oocytes in the unpaired female ovaries was measured using the video-analysis system (as above), during dissection of each sample for DNA extraction. In gammarids, oocyte diameter is a measure of ovary maturity, i.e. the reproductive status of the female (only maturing oocytes, accumulating vitellus, will be laid during the next moult, and this indicates female receptivity to pairing; Le Roux, 1933). We measured the average oocyte diameter of paired (i.e. mature) females, close to their moult, in order to obtain a reference value of 455.5 μm ± 25.6 ($n = 8$ females, 10 oocytes measured per female). This measure allowed us to determine which unpaired females were potentially receptive to mating, but that had not yet been chosen by males. The two predictions we tested were: (i) if males chose microsporidian-infected females over uninfected females, the proportion of infected females (I) would be greater than that of uninfected females (U) among females in pairs (i.e. $I > U$). Conversely, among females not in pairs, there would be more mature uninfected females than mature infected females (i.e. $I < U$); (ii) if infected females mature earlier in the season, overall there should be more mature infected females than mature uninfected females (mature females are those in pairs plus those not in pairs with mature oocytes).

2.7. Statistical analysis

Frequency data were analysed either with Pearson χ^2 test or with Fisher exact test where possible. Continuous data were analysed using ANOVA or ANCOVA when data were normally distributed, or using non-parametric statistics otherwise (Wilcoxon and Kruskall–Wallis tests or Spearman correlations).

A logistic regression analysis was also made to test the factors influencing the probability of pairing (see Section 3.4), taking into account the date and microsporidian infection status as factors, size of females as covariable, and their interactions. A stepwise procedure (backward elimination) allowed the removal of non-significant ($P < 0.05$) factors and interactions.

All tests were performed using JMP© Software version 5 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Microsporidia identification, prevalence in the wild and host sex ratio

Seven small PCR fragments of microsporidian 16S were sequenced: three from Colombière and four from Maillys. All seven were identical over a 360 bp region and were very closely related to *N. granulosis* isolated from *G. duebeni* (1.9% nucleotide divergence, seven out of 361 bases). The similarity of these sequences led us to consider that the parasite of *G. roeseli* was a variant of *N. granulosis*. A restriction map revealed a unique restriction enzyme cutting site by *Bgl*II (compared to the two other microsporidia in these populations, see below). Seven larger PCR fragments were sequenced, five from Colombière and two from Maillys. Four out of five sequences from Colombière were identical to *D. muelleri*, previously found in *G. roeseli* and also found in *G. duebeni* (Hogg et al., 2002; Terry et al., 2004, Genbank AJ438956). The remaining sequence diverged from *D. muelleri* by 5.2% (21 out of 403 bases). Taking into account both the variation within the *Dictyocoela* genus and the nomenclature for this genus, which proposes that the name of the parasite is assigned according to its specific host (Terry et al., 2004), we refer to this provisional new parasite species as *Dictyocoela* sp. (roeselum). Restriction maps identified unique cutting sites in the *D. muelleri* sequence by *Vsp*I, and the *Dictyocoela* sp. (roeselum) sequence by *Bst*1107I. The two sequences from Maillys were identical to the *Dictyocoela* sp. (roeselum) sequence found at Colombière.

PCR-RFLP screening revealed the prevalence of the three parasite species in the two sampling sites. At Colombière, all three parasite species were present at all sampling dates, with different prevalences according to parasite type (Table 1). *Nosema granulosis* and *D. muelleri* were found at relatively high prevalence, while *Dictyocoela* sp. (roeselum) was rare. The prevalence of *N. granulosis* and *D. muelleri* were significantly higher in female hosts than in male hosts (prevalence of *Dictyocoela* sp. (roeselum) was too low to allow accurate analysis). There was no significant variation in the relative proportions of microsporidia species in females according to sampling date

Table 1
Table of microsporidia prevalence according to collection location, date, parasite type (*Dictyocoela muelleri, D. m.; Dictyocoela* sp. (roeselum), *D.* sp.; *Nosema granulosis, N. g.*), and host sex

Population	Date	Infection status	Prevalence in females (%)	Prevalence in males (%)	Comparison of parasite prevalence between sex (Fisher exact test)	Comparison of prevalence in females between dates ($\chi 2$)
Colombière	January 2002	*D. m.*	19.0	0.0	*	
		D. sp.	11.9	3.8	NS	
		N. g.	26.2	3.8	*	
		Uninfected	42.9	92.3		
		Total	100.0 ($n=42$)	100.0 ($n=26$)		
	March 2002	*D. m.*	32.8	nd		
		D. sp.	6.8	nd		
		N. g.	27.6	nd		
		Uninfected	32.8	nd		
		Total	100.0 ($n=58$)			*D. m.*: NS
	February 2003	*D. m.*	36.3	2.0	***	*D.* sp.: NS
		D. sp.	5.9	0.0	NS	*N. g.*: NS
		N. g.	26.5	10.2	*	
		Uninfected	31.3	87.8		
		Total	100.0 ($n=102$)	100.0 ($n=49$)		
	April 2003	*D. m.*	35.7	0.0	***	
		D. sp.	6.0	0.0	NS	
		N. g.	22.6	3.8	*	
		Uninfected	35.7	96.2		
		Total	100.0 ($n=84$)	100.0 ($n=26$)		
Maillys	January 2002	*D. m.*	1.7	0.0	NS	
		D. sp.	56.9	4.0	***	
		N. g.	1.7	0.0	NS	
		Uninfected	39.7	96.0		
		Total	100.0 ($n=58$)	100.0 ($n=25$)		*D. m.*: NS
	March 2003	*D. m.*	6.5	0.0	NS	*D.* sp.: NS
		D. sp.	58.4	9.1	***	*N. g.*: *
		N. g.	9.1	13.6	NS	
		Uninfected	26.0	77.3		
		Total	100.0 ($n=77$)	100.0 ($n=22$)		

NS: $P>0.05$, *$P<0.05$, ***$P<0.001$, nd: not done.

(Table 1). One female was found bi-infected at Colombière, harbouring both *N. granulosis* and *D. muelleri*. All other individuals harboured only one parasite species. Under the hypothesis of random horizontal infection by the parasites, and considering that 25.87% females were infected by *N. granulosis* and 32.87% were infected by *D. muelleri*, the proportion of individuals bi-infected by *N. granulosis* and *D. muelleri* should have been 8.5%. The observed proportion (0.35%) is significantly different from the expected result under random infection (Fisher exact test, two-tail, $P<0.0001$).

At Maillys, the most abundant parasite was *Dictyocoela* sp. (roeselum) (Table 1). Both *D. muelleri* and *N. granulosis* were present at low prevalence at both sampling dates. The prevalence of *D. muelleri* was significantly higher in females than in males. Prevalences of the two other parasites were too low to allow accurate analysis, except for *N. granulosis* in March 2003, where no significant difference was found between sexes. In females, only the difference in *N. granulosis* prevalence according to sampling date was significant (Table 1). Two females were found bi-infected at Maillys, one by *N. granulosis* and *D. muelleri*, and one by *N. granulosis* and *Dictyocoela* sp. (roeselum). All others harboured only one parasite species. Under the hypothesis of random horizontal infection by the parasites, and considering that 6.67% were infected by *N. granulosis*, 4.44% by *D. muelleri*, and 58.52% by *Dictyocoela* sp. (roeselum), the proportion of individuals bi-infected by *N. granulosis* and *D. muelleri* should be 0.30% and the proportion of individuals bi-infected by *N. granulosis* and *Dictyocoela* sp. (roeselum) should be 3.90%. The observed proportion for each bi-infection (0.74%) was not significantly different from the expected result under random horizontal infection for bi-infections *N. granulosis–D. muelleri* (Fisher exact test, two tail, $P=0.31$), but was significantly different for bi-infections *N. granulosis–Dictyocoela* sp. (roeselum) (Fisher exact test, two tail, $P=0.004$).

Between Colombière and Maillys, the differences among infected females in the proportion of the different microsporidian species was significant ($\chi^2=151.32$, $P<0.0001$ after grouping all dates for each site).

In all host samples where analysis was possible, *G. roeseli* females were in excess, their proportion varying between 65 and 80% (significant deviations from 1:1 sex ratio were observed using a χ^2 test, calculations not given) (Table 1). We examined 58 females at random from Colombière for the presence of genital papillae that may indicate which phenotypic females are actually intersex (i.e. imperfectly feminised males) (Rodgers-Gray et al., 2004). No genital papillae were observed in these females.

3.2. Evidence for microsporidian vertical transmission

We analysed vertical transmission using samples infected by *N. granulosis* and *D. muelleri* collected at Colombière, and samples infected by *Dictyocoela* sp. (roeselum) from Maillys. Most clutches laid by infected females were also infected by microsporidia. However, two females lacked parasites in their embryos despite a positive PCR test. Thus, parasites were not transmitted to the eggs in these cases. Conversely, and more surprisingly, infected embryos were observed in clutches of two PCR-negative females. The quality of DNA extraction was controlled by successfully amplifying host mitochondrial ribosomal DNA following Bouchon et al. (1998). In broods of these females, the intensity of infection was quite low, with only one embryo infected out of 20 and 17, respectively (5 and 6% transmission), and the concentration of parasite DNA could have been too low for PCR detection. These females were removed from the analysis. The differences in average transmission rate (i.e. the average proportion of infected embryos) between the three parasites were highly significant (Kruskall–Wallis test: $\chi^2_2 = 19.83$; $P < 0.0001$): *N. granulosis*: median = 1, interquartile 0.91–1 ($n = 18$); *D. muelleri*: median = 0.88, interquartile 0.46-–1 ($n = 26$); *Dictyocoela* sp. (roeselum): median = 0.43, interquartile 0.35–0.92 ($n = 24$). *Dictyocoela* sp. (roeselum) parasites were transmitted at a significantly lower rate than the two other parasites (Wilcoxon test $Z = -2.93$, $P = 0.003$ for comparison with *D. muelleri* and $Z = 4.24$, $P < 0.0001$ for comparison with *N. granulosis*), but the transmission rates of *D. muelleri* and *N. granulosis* did not differ significantly ($Z = 1.70$, $P = 0.09$).

The mean number of parasites per embryo (intensity) differed according to parasite species. Embryos in clutches infected by *N. granulosis* harboured 122.8 parasites ±8.2 (mean ± s.e.m., $n = 80$), while those infected by *D. muelleri* harboured 173.8 parasites ±12.3 ($n = 116$) and those infected by *Dictyocoela* sp. (roeselum) harboured only 8.2 parasites ±1.5 ($n = 106$) (Kruskall–Wallis test: $\chi^2_2 = 165.07$, $P < 0.0001$). The difference in intensity between each parasite was significant (Wilcoxon tests: $Z = 2.77$, $P = 0.005$ between *N. granulosis* and *D. muelleri*; $Z = 10.44$, $P < 0.0001$ between *N. granulosis* and *Dictyocoela* sp. (roeselum); $Z = -11.36$, $P < 0.0001$ between *Dictyocoela* sp. (roeselum) and *D. muelleri*).

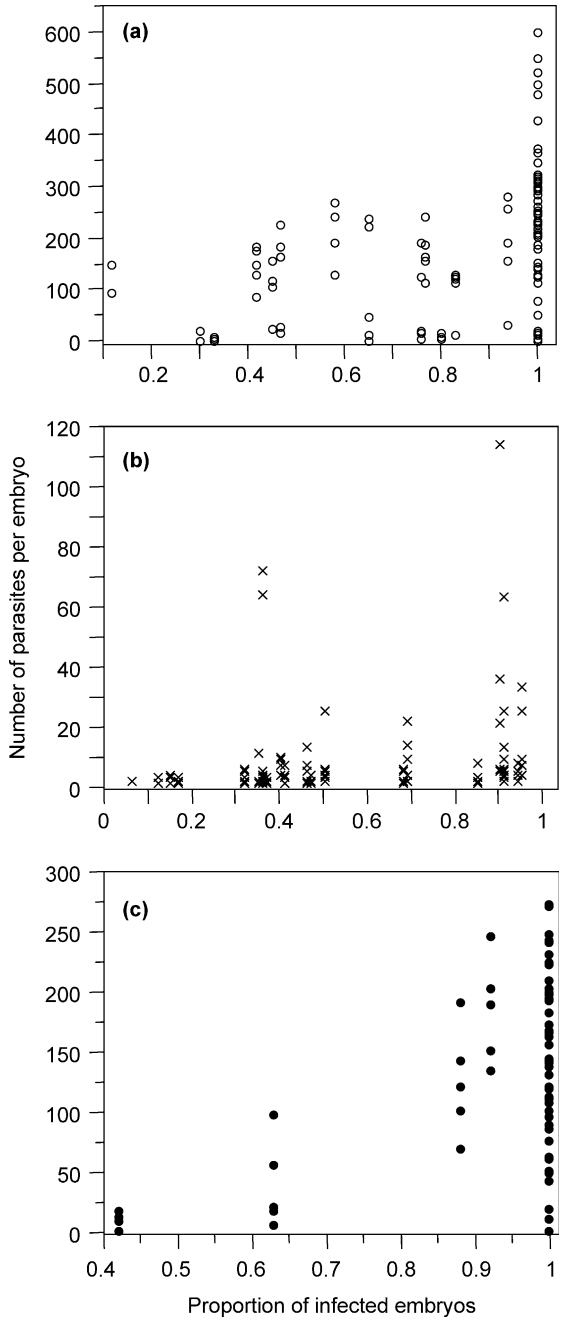

Fig. 1. Relationship between the number of parasites per embryo (intensity) and the proportion of infected embryos (vertical transmission rate) in clutches infected by (a) *Dictyocoela muelleri*, (b) *Dictyocoela* sp. (roeselum) and (c) *Nosema granulosis*. The number of parasites per embryo was counted for five embryos from each clutch, each point represents one embryo.

For each type of parasite, there was no correlation between the developmental stage of the embryos (estimated by counting the number of cells) and the number of parasites per embryo ($r_s = 0.15$, $P = 0.18$ for embryos infected by *N. granulosis*, $n = 80$ embryos with cell numbers between one and 100; $r_s = -0.17$, $P = 0.06$ for embryos infected by *D. muelleri*, $n = 116$ embryos with cell numbers between one and 20; $r_s = 0.10$, $P = 0.29$ for embryos infected by

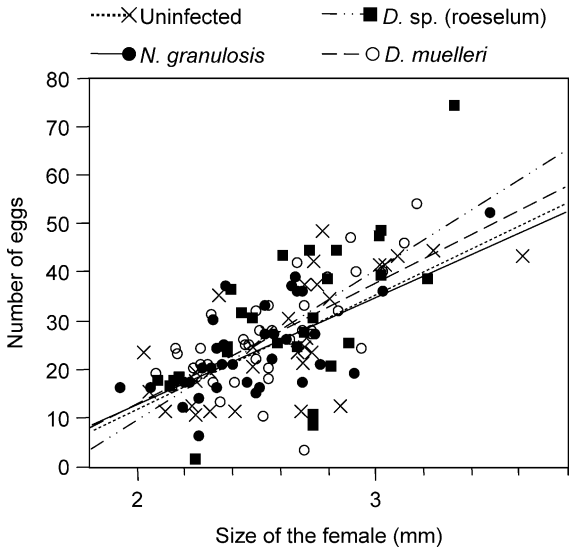

Fig. 2. Relationship between female size and fecundity (number of eggs), according to microsporidian infection status. There was no significant effect of infection either alone or in interaction with female size on host fecundity.

Dictyocoela sp. (roeselum), $n=106$ embryos with cell numbers between two and 100). However, there was a significant positive correlation between the number of parasites per embryo (intensity) and the proportion of infected embryos (vertical transmission rate), in the clutches infected by *D. muelleri* ($r_s=0.50$, $P<0.0001$) (Fig. 1a), *Dictyocoela* sp. (roeselum) ($r_s=0.46$, $P<0.0001$) (Fig. 1b) and *N. granulosis* ($r_s=0.31$, $P=0.005$) (Fig. 1c).

3.3. Effect of microsporidia infection on female host size and fecundity

There was no significant difference in size between all females carrying eggs according to their infection status (ANOVA: $F_{3,133}=1.27$; $P=0.29$). As in many crustaceans, fecundity is related to female size; however, no effect of infection, either alone or in interaction with female size was detected (Fig. 2; ANCOVA: effect of size: $F_{1,133}=105.35$, $P<0.0001$; effect of the infection: $F_{3,133}=0.49$, $P=0.69$; interaction: $F_{3,133}=0.55$, $P=0.65$). Thus, microsporidian infection did not appear to affect female size and fecundity.

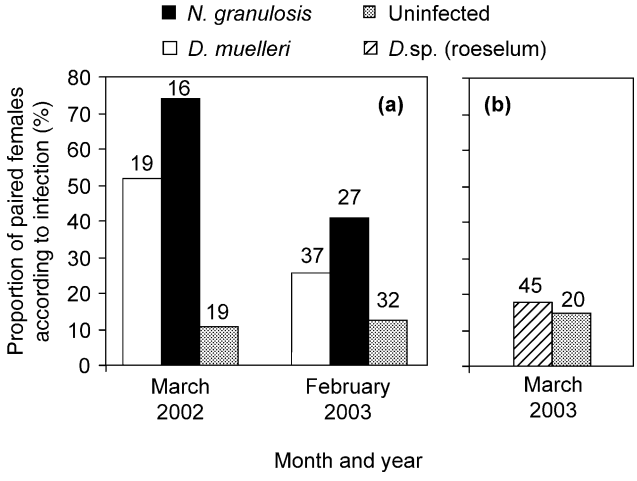

Fig. 3. Proportion of paired females out of all females sampled, at the start of the reproductive season, according to their microsporidian infection status, at (a) Colombière, and (b) Maillys. Numbers above bars indicates total number of females sampled in that infection category.

3.4. Effect of microsporidia on female host reproduction

At Colombière, pairs were first found in the wild in March 2002, and out of all females, the proportion of females in pairs was significantly different according to their infection status' (Pearson $\chi^2_2=15.41$, $P=0.0004$), with a strong deficit of uninfected paired females (Fig. 3a) (the few females infected by *Dictyocoela* sp. (roeselum) were removed from the analysis). In February 2003, after 8 days of storage with males in the laboratory, 24 out of the 95 females sampled in the wild were found in pairs. The global logistic regression testing the factors influencing the probability of pairing revealed an effect of date, in that the proportion of paired females was greater in March 2002 (natural conditions) than in February 2003 (experimental conditions) (Table 2, Fig. 3a). Females infected by *N. granulosis* were more frequently paired than others, and among the remaining females, females infected by *D. muelleri* were more frequently paired than uninfected females (Table 2, Fig. 3a).

During 2003, oocyte diameter was measured in unpaired females, in order to compare their receptivity to mating according to their microsporidian infection status. Among these unpaired females, there was no significant difference in the proportions of mature females between the different infection status' (uninfected females: 11.1%, $n=27$; *N. granulosis*: 12.5%, $n=16$; *D. muelleri*: 7.4%, $n=27$; Pearson $\chi^2_2=0.35$, $P=0.84$). There was therefore no excess of uninfected females among unpaired females. However, on the global female sample (i.e. paired and unpaired), differences in female maturity according to their infection status were not significant (Uninfected: 22.6%, $n=31$; *N. granulosis*: 48.2%, $n=27$; *D. muelleri*: 32.4%, $n=37$; $\chi^2_2=4.27$, $P=0.12$).

Table 2
Logistic regression comparing the effect of date, size and infection status on the reproductive status of *Gammarus roeseli* females at the beginning of the reproductive period, at Colombière. A stepwise procedure allowed us to remove non-significant factors and interactions (see text). Only the resulting model is given in the table

Source of variation	d.f.	Likelihood ratio χ^2	P
Date	1	5.40	0.02
Infection: *N. granulosis* vs. others	1	13.99	0.0002
Infection: *D. muelleri* vs. uninfected	1	8.50	0.003

Global model: L–R $\chi^2=24.91$, 3 d.f., $P<0.0001$.

At Maillys, the March 2003 sample revealed no significant difference in *Dictyocoela* sp. (roeselum) prevalence according to reproductive status of females (Fig. 3b; $\chi^2_2 = 0.18$, $P = 0.67$) (the rare females infected by *N. granulosis* and *D. muelleri* were removed from analysis).

4. Discussion

We surveyed two populations of *G. roeseli* and found that the overall pattern of microsporidian infection in this species exhibits convergent evolution with the pattern found in *G. duebeni*. We nevertheless found a fundamental difference: infection by two of the microsporidia infecting *G. roeseli* favours female host reproduction, while no such positive fitness effect has been observed in *G. duebeni*.

We found three different parasite species infecting the same populations, of which one is identical to *D. muelleri* isolated from *G. duebeni*, one very similar to *N. granulosis* also found in *G. duebeni*, and a new species (*Dictyocoela* sp. (roeselum)) that is closely related to microsporidia infecting other gammarids (Terry et al., 2004). All three parasite species are transmitted vertically, two at rates close to those observed in *G. duebeni* (Ironside et al., 2003b), however, the transmission rate of *Dictyocoela* sp. (roeselum) was lower. The latter parasite also exhibits a lower intensity of infection. Since we found a positive relationship between infection intensity and vertical transmission rate for the three parasites, we suggest that transmission is dosage-dependent, i.e. the higher the parasite burden in the female the greater its transmission to offspring.

In both populations there was a deficiency of infected males relative to infected females, and there was a large excess of females in host samples, suggesting that the three microsporidian species are sex ratio distorters (Galbreath et al., 2004). Parasitic sex ratio distortion may be due to male-killing or feminisation (Werren and O'Neill, 1997). The phylogenetic relatedness of microsporidia found in *G. roeseli* and *G. duebeni*, leads us to favour the feminisation hypothesis, but this remains to be tested experimentally (see Dunn and Rigaud, 1998). Given that a few infected males were found, the feminising effect is not complete, and this is consistent with the feminising pattern of *N. granulosis* in *G. duebeni* (Kelly et al., 2002). However, we failed to observe intersex morphology in *G. roeseli*, as observed in *G. duebeni* (Rodgers-Gray et al., 2004). Assuming that microsporidia do feminise *G. roeseli*, these observations suggest that specific host–parasite interactions determine the phenotypic outcome of parasite-induced host feminisation. This was observed in woodlice, where the feminising trait of *Wolbachia* bacteria may have intermediate penetrance in some host species, generating various degrees of intersexuality (Rigaud and Juchault, 1998). Alternatively, there is an all-or-nothing effect in other host species, resulting in the presence of males infected with the bacteria but no intersexes (Rigaud et al., 1999). In addition, at Maillys, a similar number of males and females were infected by *N. granulosis*, suggesting that this parasite either exerts a very weak feminising effect (if any) or that it may exercise a mixed strategy and sometimes transmit horizontally (e.g. Vizoso and Ebert, 2004). Thus, there may be variation in the strategy with which *N. granulosis* exploits its host in different populations.

This is the first study to find three different species infecting the same populations in gammarids. Multiple species of vertically-transmitted microsporidia infecting the same populations have been identified before (Ironside et al., 2003b), but their existence confounds theoretical models, because a less efficient parasite will be deterministically excluded from a population by a more efficient one (efficient in terms of vertical transmission and sex ratio distortion, Taylor, 1990). Long-term persistence of multiple species could be due to a small amount of horizontal transmission of one of the parasites (Ironside et al., 2003b), a possibility not ruled out in *G. roeseli*. In addition, Ironside et al. (2003b) predicted that many generations may be required for parasite exclusion, and spread of a feminising microsporidian in a population may be limited by variability among hosts (Dunn et al., 1993), seasonal processes (Kelly et al., 2002) and host resistance (Taylor, 1990). In our survey we found some evidence to suggest that the three infections are at transient equilibrium within each population. First, *D. muelleri* is more prevalent at Colombière, while *Dictyocoela* sp. (roeselum) is more prevalent at Maillys. Second, at Maillys the prevalence of *Nosema* increased between 2002 and 2003. Finally, *Dictyocoela* sp. (roeselum) undergoes vertical transmission less efficiently and may be outcompeted by the two other species. We could envisage a scenario where the prevalence of the three parasites at Colombière and Maillys may represent different stages of the evolutionary dynamics of parasite frequency changes.

While there were a few bi-infected individuals, their proportion was lower than expected under random horizontal transmission of the parasites. This is consistent with other studies showing that gammarid microsporidia are transmitted vertically (Hogg et al., 2002; Ironside et al., 2003a). The presence of bi-infected individuals in *G. roeseli* nevertheless contrasts with observations from *G. duebeni* (Hogg et al., 2002; Ironside et al., 2003b). Bi-infection could be due either to a primary occurrence of bi-infected individuals in the population followed by segregation of the two parasites during recurrent bottlenecks during vertical transmission (Poinsot et al., 2000), or to some occasional horizontal transmission, as observed in other microsporidia (Agnew and Koella, 1999; Vizoso and Ebert, 2004). These hypotheses should be tested experimentally in future studies.

We did not find any negative effect of infection on female host size or fecundity. This differs from observations in *G. duebeni* (Terry et al., 1998; Kelly et al., 2003), where females infected by *N. granulosis* were smaller than

uninfected ones, and therefore produced fewer eggs. In contrast, we found evidence that the infection by two of the parasites in *G. roeseli* are beneficial for female host reproduction. At the beginning of the reproductive season, both in the field and the laboratory, there were more females infected by *N. granulosis* and *D. muelleri* in precopula pairs than uninfected females. Our data do not allow us to clearly distinguish between male choice and early female maturity as the reason for this parasite-mediated reproductive advantage. However, there was no excess of mature uninfected females among unpaired females, which suggests that male choice alone does not explain the pairing success of infected females. Infected females could also mature earlier than uninfected females, but the differences found were not significant. Whatever the mechanism, parasites induce earlier reproduction in their female hosts, and this is, to our knowledge, the first study to find a positive effect of a microsporidian infection on a component of host fitness. There are two possible transmission advantages to the microsporidian parasite in employing this strategy. The first is a direct advantage, in that earlier host reproduction increases the number of host broods (Pockl, 1993) and therefore increases parasite transmission. The second advantage could be that where there is a high probability of infection by pathogenic parasites, earlier reproduction of the host ensures microsporidian transmission before pathogenic infection occurs. That is, vertically-transmitted microsporidia share the host's interest in investing in reproduction before damage by a pathogen (see Agnew et al., 2000). The prevalence of harmful parasites in *G. roeseli* populations remains to be determined in order to verify this hypothesis. Infection by *Dictyocoela* sp. (roeselum) did not favour host reproduction and this suggests that there are differences in host exploitation strategies between parasite species.

The patterns of microsporidian infections in *G. roeseli* and *G. duebeni* exhibit some degrees of convergent evolution. They are both species of Eurasian origin, but have non-overlapping geographic ranges (Barnard and Barnard, 1983). Consequently, similarities in microsporidian infection patterns are likely to be a result of phylogenetic relatedness rather than an ecological link, and these similarities could be the trace of an ancient infection of the Eurasian gammarid group by these microsporidian lineages. However, the differences in parasite exploitation both within *G. roeseli*, and between *G. roeseli* and *G. duebeni*, highlight some contrasts in the coevolutionary trajectories between host–parasite couples. In addition, the microsporidian infection patterns in *G. duebeni* and *G. roeseli* are very different from the one of the North American gammarid *Crangonyx pseudogracilis*, which hosts a phylogenetically distant, pathogenic, but nevertheless vertically-transmitted microsporidia (Galbreath et al., 2004). This suggests that North American and Eurasian amphipod fauna experienced different infection histories by microsporidian parasites.

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