

Microphallids in *Gammarus insensibilis* Stock, 1966 from a Black Sea lagoon: manipulation hypothesis going East?

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SUMMARY

Patterns of parasite site selection, variation in infection parameters and interspecific associations are examined in the light of new field data on larval microphallids in *Gammarus insensibilis* from a Black Sea lagoon. These patterns are discussed in relation to the predictions for the manipulative effect of *Microphallus papillorobustus* and its relationships with the other microphallid species based on studies on the French Mediterranean coast. Four species were recovered: *Maritrema subdolum*, *Microphallus hoffmanni*, *M. papillorobustus* and *Levinseniella propinqua*. The latter two were located in both corporal and cephalic segments, but the selection of brain appeared stronger for *L. propinqua*. *M. subdolum* was the first colonizer of amphipod population recruits, and unequivocally the dominant species in the lagoon. There was a significant positive relationship between the parasite load of all 4 species. Concurrent infections were exceedingly frequent, and no departures from random association were detected. We found no evidence that the cerebral metacercariae of *M. papillorobustus* consistently predict the parasite load of any of the other species in the system and identify sources for heterogeneity that may account for the differences between the Black Sea and the Mediterranean system: habitat heterogeneity, bird diversity and host-parasite systems used to infer relationships between microphallids.

Key words: Microphallid metacercariae, amphipods, *Gammarus insensibilis*, interspecific associations, Black Sea.

INTRODUCTION

The intermediate host-manipulation hypothesis predicts a significant alteration of the host phenotype caused by the parasite which enhances its transmission to the final host *via* predation. Whether adaptive or not (Poulin, 2002) this feature has the potential to affect the transmission of parasites co-occurring with the manipulator in multispecies communities in intermediate hosts. Changes in behaviour of 2 species of amphipods, *Gammarus insensibilis* and *G. aequicauda*, caused by the metacercariae of the microphallid, *Microphallus papillorobustus* (Rankin, 1940), and its relationships with 3 other microphallid species utilizing its transmission route to the final hosts is a frequently cited digenean example for manipulation of intermediate hosts. It is based on field data from 3 brackish lagoons along the Mediterranean in Southern France and a laboratory infection and predation experiment (Helluy, 1983, 1984, see Combes, 2001, for a better presentation of these data). The effect of the manipulator species on the demographic structure and reproduction of 2 intermediate gammarid hosts, *G. insensibilis* and

G. aequicauda, and its relationships with the co-occurring microphallid species were further examined by Thomas and co-authors in Thau Lagoon, France (e.g. Thomas *et al.* 1995*b, c*; 1996*c*; Thomas, Renaud and Cézilly, 1996*b*; Thomas *et al.* 1997; Thomas, Poulin and Renaud, 1998*a*, Thomas *et al.* 1998*b*, see also reference list).

The hypothesis of the manipulative effect of *M. papillorobustus* and its relationships with the other 3 microphallid species was developed on the basis of 3 main findings. (i) Cercariae of *M. papillorobustus* systematically migrate to the brain of *G. insensibilis*, encyst in the cerebroid ganglia and alter host behaviour by inducing a positive phototaxis, a negative geotaxis and aberrant 'suicidal' evasive behaviour (Helluy, 1983). (ii) Infected gammarids are more vulnerable to predation and, therefore, parasite transmission to aquatic birds is favoured (Helluy, 1984). As a result, field samples of *G. insensibilis* show decreasing mean parasite abundance and dispersion (measured by variance-to-mean ratio) in older individuals (Thomas *et al.* 1995*b*), providing a good example of prevalence-dependent mortality (Rousset *et al.* 1996). (iii) Cercariae of 3 other sympatric microphallid species, *M. hoffmanni* Rebecq, 1964, *Maritrema subdolum* Jägerskiöld, 1909 and *Levinseniella brachysoma* (Creplin, 1837) (syn. *L. tridigitata* Deblock *et al.* 1958), do not migrate to the brain, always encyst in the amphipod abdomen, never alter host behaviour and have no significant

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effect on its survival (Thomas *et al.* 1995*a*; Thomas, De Meeûs and Renaud, 1996*a*; Thomas *et al.* 1997, 1998*a, b, c*). However, *M. subdolum* enhances its transmission to the final host by parasitizing amphipods previously infected with *M. papillorobustus*, utilising the so-called ‘hitch-hiking’ strategy (Thomas *et al.* 1997, 1998*b*) whereas the other 2 species infect hosts at random (and are ‘lucky passengers’ according to the terminology in host-as-vehicle analogy, see Lafferty, 1999) (Thomas *et al.* 1998*a*).

However, spatial variation in both the behaviour of uninfected hosts and host-parasite interactions apparently exists (e.g. Mouritsen, 1997; Mouritsen and Poulin, 2002; Latham and Poulin, 2002), thus raising the question as to whether patterns and processes coming from studies in one ecosystem (e.g. Thomas and Renaud, 2001) are readily extendible to others. It is possible that a higher diversity of patterns will be revealed as data accumulate on a larger geographical scale. Unfortunately, very little can be extracted from published data on marine amphipod-microphallid systems in other geographical localities (e.g. Belopol’skaya, 1957; Mordvinova, 1978, 1985, Zander *et al.* 2002) that can be used in testing this prediction, since they provide only summed data across individual hosts.

Our study aims partially to fill the gap in knowledge of larval microphallid communities in amphipods by providing quantitative field data on 4 species parasitizing *Gammarus insensibilis* in a Black Sea lagoon. Here we examine a large ‘Spring’ sample of gammarids, collected in the course of a wider sampling programme, in an attempt to reduce at least the seasonal bias while testing the generality of predictions of Thomas and co-authors inferred from studies on *Gammarus* spp., originating from both a geographically distant and an ecologically distinct environment. We provide novel data on an amphipod-microphallid system that is characterized by a substantially higher transmission rate than previously observed, focusing on (i) parasite site selection, (ii) variation in infection parameters with size, and (iii) interspecific associations.

MATERIALS AND METHODS

Study site

Pomorie lagoon is a small (850 ha), shallow (mean depth 1 m; max. depth 1.6 m) lagoon (range of salinity 26–44 vs 17–18‰ of the Black Sea surface waters, Vasilev and Mitrofanova, 1998), located on the Bulgarian Black Sea Coast (42°35’N, 27°38’E). The lagoon is an attractive resting place for a wide variety of aquatic birds, especially important during their spring and autumn migrations along the East European migration route (Via Pontica), as well as an overwintering area. It was designated as an

Important Bird Areas Program site in 1997, protected site in 2001, and a new Ramsar site in 2002 (Michev and Profirov, 2003).

Data from regular bird counts carried out at the lagoon (Michev and Profirov, 2003) were used as an approximation for the diversity and abundance of birds, temporarily utilizing the lagoon.

Parasite samples

A large sample of *G. insensibilis* ($n=427$) was collected with a fine mesh net along a transect on the southern shore of the Pomorie Lagoon on 26 May 2000. Amphipods were fixed in 4% borax-buffered formaldehyde in sea water. In the laboratory the amphipods were sexed, measured (length from rostrum to telson) and dissected under a stereoscopic microscope. The sample was stratified by size into 8 size classes with a step of 1 mm. Due to the high abundance of the metacercariae it was difficult to correctly assign their location by segments (especially in the thorax); therefore a distinction was only made between metacercariae located in the head, thorax and abdomen; present analyses are based on summed data for the latter sites. All microphallid metacercariae were identified and counted. A small separate sample of amphipods brought alive in the laboratory served as the source of metacercariae (*ca* 100) for initial species identification. The morphology of the metacercariae was studied in both live specimens and in Canada balsam whole-mounts after staining with iron-acetocarmine (Georgiev, Biserkov and Genov, 1986) and was compared with the descriptions of Kostadinova and Gibson (1994).

Statistical analyses

Ecological terms are used according to Bush *et al.* (1997). Spearman rank correlation (r_s), and standard parametric tests (ANOVA, multiple regression) were applied for statistical comparisons. Using a Bonferroni correction in *post hoc* tests, following main analysis, more conservative values (data in text) were taken to indicate significance of pairwise comparisons. Where parametric tests were used, the data were $\ln(x+1)$ transformed (Sokal and Rohlf, 1981). Prevalences and frequencies were compared with Fisher’s exact test.

The degree of association between the 4 parasite species was assessed by a 2-fold approach. First, we tested for departures from random association by comparing the observed and expected frequencies of double infections. Expected number of double infections was calculated by multiplying the number of hosts and the prevalence of the species forming the pair (Kuris, 1990; the so-called host-centred measure of association of Outreman *et al.* 2002).

Table 1. Summarized infection parameters of the 4 microphallid species recovered in the spring sample of *Gammarus insensibilis* from Pomorie lagoon

	Total metacercariae	<i>Microphallus papillorobustus</i>	<i>Microphallus hoffmanni</i>	<i>Maritrema subdolum</i>	<i>Levinseniella propinqua</i>
Prevalence (%)	99.3	89.9	94.6	99.3	47.1
Mean abundance \pm s.d.	39.45 \pm 31.4	7.18 \pm 7.9	8.49 \pm 8.4	22.98 \pm 18.9	0.81 \pm 1.2
Median abundance	31	5	6	17	0
Intensity range	1–209	1–59	1–84	1–152	1–8
Mean intensity \pm s.d.	39.73 \pm 31.4	7.98 \pm 8.0	8.97 \pm 8.3	23.14 \pm 18.8	1.73 \pm 1.2
Median intensity	31	5	7	18	1

Secondly, in order to control for the effect of amphipod size on interspecific association (size-abundance relationship was highly significant for all four species; r_s range: 0.162–0.533, $n=427$, $P<0.001$), the residuals of the regressions of parasite abundance of each species *vs* host size were used as measures of parasite load independent of size (e.g. see Poulin, Steeper and Miller, 2000). Association between species was then inferred from Spearman's rank correlation coefficients between these variables.

Multiple regression was run attempting (i) to test the predictive value of the presence and abundance of cerebral metacercariae of *M. papillorobustus* for the abundance of the other microphallid species and (ii) to assess the functional relationship among the abundance of the 4 species in the studied system. Partial regression coefficients resulting from 3 independent regression bouts were used to infer relationships between species based on abundance independent of size. All analyses were carried out using SPSS[®] 11.0 (SPSS Inc., Norušis, 2002) and the programme Quantitative Parasitology (QP1.0, Rózsa, Reiczigel and Majoros, 2000).

RESULTS

Microphallid community

A total of 4 microphallid species, *Microphallus papillorobustus*, *M. hoffmanni*, *Maritrema subdolum* and *Levinseniella propinqua* Jägerskiöld, 1907, was recovered. Overall prevalence of microphallids in *G. insensibilis* at Pomorie lagoon was 99.3% with 91.1% of the hosts harbouring 3 and 4 species. Overall, 16 846 metacercariae were recovered. The number of microphallid metacercariae per infected individual ranged from 1 to 209, with equal mean and median abundance and intensity (Table 1). *Microphallus* spp. and *M. subdolum* occurred at very high prevalence among the gammarids sampled whereas *L. propinqua* exhibited moderate frequency of occurrence. Infection parameters differed highly significantly ($P<0.001$) among the 4 species (Fisher's test and bootstrap test for mean intensity, pairwise $P<0.0002$; ANOVA, $F_{(3, 1704)}=744.52$, $P<0.0001$) with the exception of the prevalence and intensity of the 2 *Microphallus* species which were

close. *M. subdolum* and *L. propinqua* had the highest and the lowest prevalence, abundance and intensity, respectively, while *Microphallus* spp. occupied an intermediate position (Table 1).

Location of metacercariae in gammarids

All metacercariae of *M. subdolum* and *M. hoffmanni* were located in the thoracic and abdominal segments (including gnathopods and pereopods, respectively, in some cases). In addition to thorax and abdomen, the 2 other species were also frequently located in the head of hosts. *M. papillorobustus* was recovered in the head with a higher prevalence (54.1 *vs* 31.6%, $P<0.0001$) and mean intensity (1.57 *vs* 1.13, $t=8.063$, $P<0.0001$) than *L. propinqua*. However, the overall prevalence of cephalic infection with *M. papillorobustus* was substantially lower than corporal (54.1 *vs* 87.8%, $P<0.0001$) while differences were not significant in the case of *L. propinqua* (31.6 *vs* 26.7%, $P=0.14$). Finally, the metacercariae were present solely in the head in 2.3% and 43.8% of the hosts infected with *M. papillorobustus* and *L. propinqua*, respectively.

A total of 363 metacercariae (11.8%) of *M. papillorobustus* and 152 (43.8%) of *L. propinqua* were found in the head. The first size class showed the highest proportion of cephalic metacercariae of both species; the values for *M. papillorobustus* generally decreased with size, reaching a minimum in size class 8, whilst those for *L. propinqua* did not follow a specific pattern. The abundance distributions of the 2 species in the head did not show a significant correlation ($r_s=0.0441$, $P>0.05$).

Size-dependent variations in parasite populations

The infection parameters of the 4 microphallid species in the 8 size classes of *G. insensibilis* are given in Table 2. Mean amphipod length was significantly correlated with both prevalence and mean abundance of the metacercariae (range of $r_s=0.755$ –0.976, $P<0.05$) across the size-class range, except for the prevalence of *M. subdolum* ($r_s=0.655$, $P>0.05$). As in the overall sample, the 4 species showed differing patterns of variation of infection parameters in the

Table 2. Prevalence and abundance of the 4 microphallid species in the sample of *Gammarus insensibilis* stratified by size

Size class	1 (n=23)	2 (n=69)	3 (n=84)	4 (n=51)	5 (n=65)	6 (n=52)	7 (n=45)	8 (n=38)
Length range (mm)	7.7-10.0	10.1-11.0	11.1-12.0	12.1-13.0	13.1-14.0	14.1-15.0	15.1-16.0	16.1-18.4
Mean length ± s.d. (mm)	9.2 ± 0.8	10.6 ± 0.3	11.5 ± 0.3	12.5 ± 0.3	13.5 ± 0.3	14.5 ± 0.3	15.5 ± 0.3	16.8 ± 0.5
Prevalence (%)								
<i>M. papillorobustus</i>	56.5	89.9	85.7	80.4	96.9	96.2	100	100
<i>M. hoffmanni</i>	60.9	95.7	92.9	96.1	98.5	100	97.8	97.4
<i>M. subdolum</i>	91.3	100	98.8	100	100	100	100	100
<i>L. propinqua</i>	30.4	37.7	41.7	51.0	50.8	42.3	62.2	63.2
Mean abundance ± s.d.								
<i>M. papillorobustus</i>	2.65 ± 3.7	6.20 ± 6.1	6.02 ± 6.7	4.41 ± 4.9	5.42 ± 4.6	7.00 ± 7.1	12.11 ± 12.2	15.34 ± 10.2
<i>M. hoffmanni</i>	1.87 ± 2.1	6.36 ± 4.8	6.51 ± 5.9	5.61 ± 4.5	7.15 ± 6.1	8.71 ± 5.4	13.73 ± 8.9	20.34 ± 14.7
<i>M. subdolum</i>	8.39 ± 6.1	15.20 ± 9.7	16.92 ± 11.3	16.55 ± 10.1	21.68 ± 13.0	26.98 ± 18.8	37.89 ± 22.0	47.03 ± 29.2
<i>L. propinqua</i>	0.39 ± 0.7	0.65 ± 1.1	0.80 ± 1.4	0.80 ± 1.0	0.97 ± 1.4	0.75 ± 1.3	1.11 ± 1.3	0.87 ± 0.9
Median abundance								
<i>M. papillorobustus</i>	1	5	3.5	2	4	5	8	14
<i>M. hoffmanni</i>	1	5	5	4	6	8	12	18
<i>M. subdolum</i>	8	13	14	15	18	22	38	43.5
<i>L. propinqua</i>	0	0	0	1	1	0	1	1

samples stratified by size. *L. propinqua* exhibited comparatively moderate prevalence; no significant differences were detected in its abundance among the 8 size groups ($F_{(7, 419)} = 1.667, P = 0.115$). The 3 remaining species exhibited a trend of increase in infection with size (see Table 2) with highly significant differences in the distributions of worms among the 8 size classes ($F_{(7, 419)} = 27.71, P < 0.001$; $F_{(7, 419)} = 15.84, P < 0.001$; and $F_{(7, 419)} = 22.69, P < 0.001$, for *M. subdolum*, *M. papillorobustus* and *M. hoffmanni*, respectively). These were generally due to the substantially higher levels of infection in the largest size classes (7 and 8) and the lowest levels recorded in size class 1. Significant differences (pairwise $P < 0.002$) in prevalence were only detected between size class 1 and 5-8 for *M. papillorobustus* and between class 1 and 2-8 for *M. hoffmanni*. Although some decrease in both prevalence and abundance of *M. papillorobustus* in size class 4 was noted (see Table 2), no significant differences were detected between this and the adjacent size-groups for all 3 species.

We examined the size-related variations in the parameters of infection with cephalic and corporal metacercariae of *M. papillorobustus* separately in order to test for departures from the overall pattern described above. The prevalence of cephalic versus corporal infections was similar in the first size class only ($P > 0.05$), the remaining size groups generally showed substantially higher prevalence of corporal metacercariae (size classes 2-7, $P < 0.001$; size class 8, $P = 0.029$). Both prevalence and abundance of the 2 types of metacercariae of *M. papillorobustus* showed a linked pattern of variation with host size (Fig. 1). Infection with both types of metacercariae generally increased reaching maximum levels in the largest size classes (mean abundance 10.87 and 13.95 vs 1.24 and 1.39 for larvae in the body and head in size classes 7 and 8, respectively; prevalence 100% vs 68.9-78.9%, respectively).

The partitioned picture of the prevalence and mean abundance of *M. papillorobustus* presented in Fig. 1 shows that the slight decrease of infection in size class 4, as depicted in Table 2, concerned both cerebral and corporal metacercariae. However, the prevalence of both types of metacercariae was not significantly different from that of the adjacent size groups (size class 3, 5 and 6; setwise $P > 0.05$, pairwise $P > 0.002$). Similarly, differences in abundance distributions of both types were not significant among the host groups of intermediate length, with the exception of the slightly higher mean abundance of the corporal metacercariae in size class 6 vs 4 ($P < 0.05$).

Interspecific associations

Overall, there was a significant positive relationship (range of $r_s = 0.180-0.571, P < 0.002$) between the parasite load corrected for amphipod size of all 4

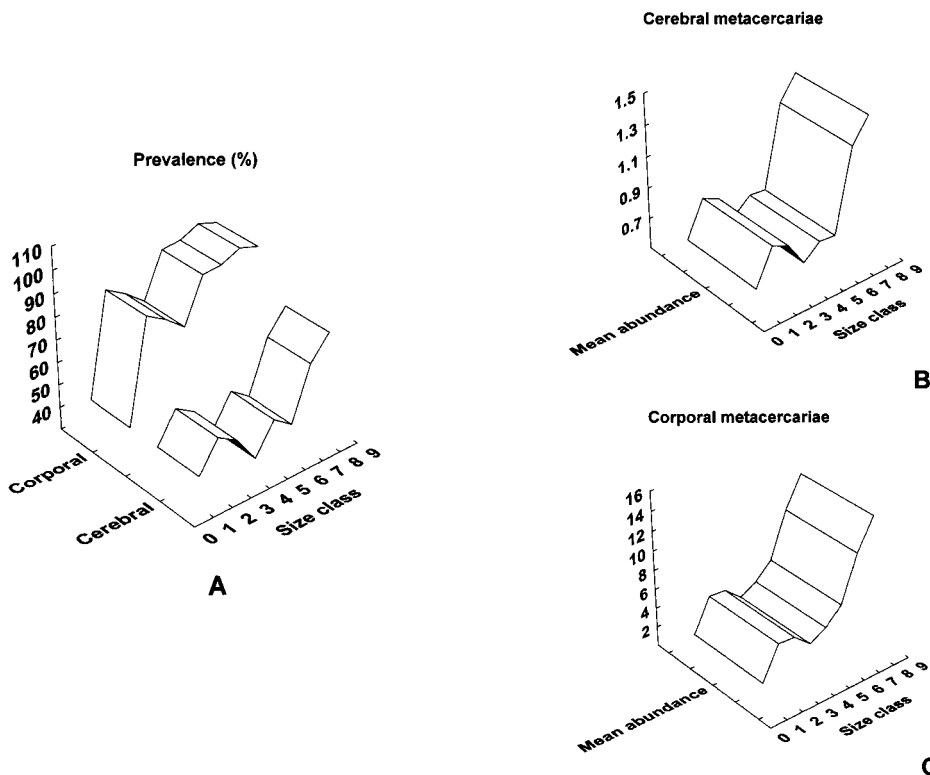


Fig. 1. Variation with host size in prevalence (A) and abundance of the cerebral (B) and corporal (C) type of metacercariae of *Microphallus papillorobustus*.

species with the highest levels of association between *M. subdolum* and *Microphallus* spp. (Table 3). However, double infections were exceedingly frequent among all 4 microphallid species in *G. insensibilis* and no departures from random association were detected by comparing the expected with the observed frequency of co-occurrence (see Table 3).

The abundance of the corporal metacercariae of *M. papillorobustus* exhibited a significant positive correlation with both, the cerebral metacercariae of this species ($r_s=0.465$, pairwise $P<0.0002$ and *M. subdolum* ($r_s=0.504$, pairwise $P<0.0002$) when the residual parasite numbers of the 2 types of metacercariae (cerebral and corporal) were examined separately. Residuals of both cerebral and corporal numbers of metacercariae of *M. papillorobustus* had the same sign in 286 amphipods, a substantially higher frequency than expected by chance ($\chi^2=49.24$, D.F. = 1, $P<0.001$).

A multiple stepwise regression was run in order to test the predictive value of the presence/absence and/or parasite load of *M. papillorobustus* (corporal and cerebral metacercariae considered separately) for the variations in parasite load (corrected for amphipod size) of the other species in our system. The results shown in Table 4 revealed that (i) both the presence and the number of cerebral metacercariae of *M. papillorobustus* do not predict the variations in abundance of *M. subdolum*; (ii) a strong positive association exists between *M. subdolum* and *M. hoffmanni*; (iii) *M. papillorobustus* (both corporal and

cerebral metacercariae) explain *ca* 4.6% of the variation of *M. hoffmanni* in addition to *ca* 35% explained by *M. subdolum*; (iv) the variation in abundance of *L. propinqua* is poorly explained and is related solely to that of *M. subdolum*.

DISCUSSION

Summed data on microphallid infracommunities in *G. insensibilis* indicate that Pomorie lagoon is an important site of infection for Charadriiformes during their spring migration. The overall prevalence (*ca* 100%) is substantially higher than the data of Mordvinova (1978, 1985) who reported 51 and 44%, respectively, as an overall maximum prevalence of the microphallid metacercariae in *G. insensibilis* sampled during summer in the bays of Sevastopol. The overall prevalence of *M. papillorobustus* at Pomorie lagoon is similar to the maximum values (87.0%) recorded by Naidenova and Mordvinova (1985) in *Gammarus subtypicus* and *G. aequicauda* along the Ukrainian Black Sea coast, whereas those of *M. subdolum*, *M. hoffmanni* and *L. propinqua* (as compared to *L. bucephalae*) are substantially higher than the maxima observed by these authors in the Ukraine (99.3 *vs* 50–71%, 94.6 *vs* 57%, and 47.1 *vs* 6%, respectively). Although a comparison with samples from Thau lagoon (France) is not straightforward, the overall infection parameters of the 4 species in our sample suggest that parasite transmission rates in Pomorie lagoon are higher than

Table 3. Double test for associations among the microphallid species parasitizing *Gammarus insensibilis* from Pomorie lagoon

Species pair	Obs/Exp* ($P > 0.05$)	Spearman's rho ($P < 0.002$)
<i>M. papillorobustus</i> – <i>M. subdolum</i>	384/381	0.519
<i>M. papillorobustus</i> – <i>M. hoffmanni</i>	376/363	0.469
<i>M. papillorobustus</i> – <i>L. propinqua</i>	187/181	0.180
<i>M. subdolum</i> – <i>M. hoffmanni</i>	404/401	0.571
<i>M. subdolum</i> – <i>L. propinqua</i>	223/200	0.233
<i>M. hoffmanni</i> – <i>L. propinqua</i>	196/190	0.180
Cerebral <i>M. papillorobustus</i> – <i>M. subdolum</i>	231/231	0.391
Cerebral <i>M. papillorobustus</i> – <i>M. hoffmanni</i>	228/220	0.400
Cerebral <i>M. papillorobustus</i> – <i>L. propinqua</i>	119/110	0.159

* Number of observed (Obs) and expected (Exp) frequencies of double infections.

Table 4. Summary of the results of the regression analyses

(The variable presence/absence of cephalic *Microphallus papillorobustus* was not included in either model.)

Dependent variable	<i>M. subdolum</i>			<i>M. hoffmanni</i>			<i>L. propinqua</i>		
	$R^2 = 0.429$ $F_{3, 423} = 105.74, P < 0.001$			$R^2 = 0.395$ $F_{3, 423} = 92.06, P < 0.001$			$R^2 = 0.071$ $F_{3, 423} = 32.68, P < 0.001$		
Predictor variables	B	β	P	B	β	P	B	β	P
<i>M. papillorobustus</i> (cerebral)	excluded	excluded	0.068	0.197	0.130	0.004	excluded	excluded	0.319
<i>M. papillorobustus</i> (corporal)	0.203	0.272	0.0001	0.139	0.157	0.001	excluded	excluded	0.194
<i>M. subdolum</i>	—	—	—	0.548	0.464	0.0001	0.221	0.267	0.0001
<i>M. hoffmanni</i>	0.375	0.442	0.0001	—	—	—	excluded	excluded	0.148
<i>L. propinqua</i>	0.149	0.123	0.001	excluded	excluded	0.264	—	—	—

previously observed in gammarid-microphallid systems.

Overall, our results show a picture largely departing from the observations of Thomas and colleagues carried out in a Mediterranean lagoon. Although not unexpected, the novel data on the site selection of *L. propinqua* are intriguing in that they show that selective encystment in the amphipod's brain is not a prerogative of a single microphallid species but has also evolved in other taxonomically distant lineages. Although not detected in our sample, *M. subdolum* may also opt for the amphipod's head (Ginetsinskaya, 1988).

Surprisingly, it appears that the selection of the brain as a location for encystment is stronger in *L. propinqua*, in spite of the higher prevalence and mean intensity of the cephalic metacercariae of *M. papillorobustus*, which evidently reflect the overall differences in the transmission rates of the 2 species in the lagoon. The size-related patterns of the 2 species also differ, exhibiting a single common feature, namely the highest proportion of cephalic metacercariae in the smallest individuals. Evidence from an experimental infection of *G. aequicauda* with *M. papillorobustus* (Helluy, 1983) indicates that at the initial stages of infection most of the metacercariae

(84.2%, data from Combes, 2001) localize in the brain whereas 3 months after continuous exposure to infected snails, metacercariae found in the brain represent only 25.4% of the total (see p. 256, Combes, 2001). Although it is very difficult to infer the exact timing of infection in the field, our data tend to support the hypothesis of preferential brain location by the first colonizers of both species.

The gradual increase in infection parameters with size, reaching substantially higher values in the largest individuals, indicates accumulation with age of the metacercariae of *M. subdolum* and *Microphallus* spp. *M. subdolum* appears unequivocally a dominant species in the lagoon and most likely, judging from the prevalence/abundance data of the smallest size classes, the first colonizer of the amphipod population recruits. The 2 types of metacercariae of *M. papillorobustus* (i.e. cerebral and corporal) exhibited concerted variation with host size reaching maximum values in the largest size class that suggests linked accumulation with age.

We observed positive interspecific associations based on parasite load corrected for size which could be interpreted as strong evidence for hitch-hiking (e.g. Poulin *et al.* 2000 and references therein). However, our data show that double infections result

from independent random co-occurrence of any of the microphallid species pair. Furthermore, in contrast to our expectations based on the hypothesis of the manipulative effect of *M. papillorobustus*, we found no evidence that the cerebral metacercariae of the latter predict consistently the parasite load of any of the species in the system when all species were entered in a multiple regression. On the other hand, the abundance of *M. subdolum* was associated with that of the other microphallid metacercariae, including corporal metacercariae of *M. papillorobustus*. Our results, therefore, tend to suggest that the 4 species utilize host resources in a concerted manner and the interspecific associations observed reflect their high transmission rates in the Pomorie lagoon.

The only species showing significant association with the cerebral metacercariae of *M. papillorobustus* in the studies of Thomas and colleagues was *M. subdolum*. Helluy (1983) was the first to note the presence of *M. subdolum* in some of her field samples and concluded that both *G. aequicauda* and *G. insensibilis* showing modified behaviour contain more metacercariae of this species than 'normal' hosts. Thomas *et al.* (1997) also found a close association (based on a presence-absence index) between *M. papillorobustus* and *M. subdolum* in a sample of *G. insensibilis* from Thau lagoon. These authors observed the behaviour of the cercariae of the 2 species and suggested that *M. subdolum* were swimming high in the water column where they were more likely to encounter amphipods already infected and manipulated by *M. papillorobustus*, thus increasing their probability of transmission by hitch-hiking. However, the prevailing proportion of the cercariae of both species (74% and 86% for *M. subdolum* and *M. papillorobustus*, respectively) were found 'crawling' in the bottom (0–1 cm) fraction in this experiment (see Thomas *et al.* 1997).

Mouritsen (2001) using a more sophisticated experimental design provided evidence for the opposite, i.e. that the cercariae of *M. subdolum* have a high affinity for the sediment-water interface, possibly due to positive geotactism, and are therefore well-adapted to reach bottom-dwelling intermediate hosts. Based on previous data recording negative photo- and positive geotactism in *M. subdolum* (Ginetsinskaya, 1988) and his own results, Mouritsen's conclusion is more convincing. It is also possible, that Thomas *et al.* (1997) misidentified another microphallid cercaria as *M. subdolum* (see Mouritsen, 2001; Hust *et al.* 2004). Thus, the hypothesis of Thomas *et al.* (1997, 1998a) of the hitch-hiking transmission strategy of this particular species may be based on an incorrect premise. The generally low prevalence situation in Thau lagoon might have also contributed to the confusion.

One important point that should be considered in studies on parasite associations is an evaluation of the kinds of species entering a possible interspecific

interaction. The wealth of data on *M. subdolum*, a cosmopolitan species with a very wide range of final hosts, may therefore, help clarify the situation. Although rather old, the notion of Ginetsinskaya (1988) that *M. subdolum* is the only microphallid (out of the 26 species with a second intermediate hosts in the life-cycle whose life-histories were known by the time of the original publication) which does not show a narrow specificity to the second intermediate host (5 host species, data from Deblock, Capron and Rosé, 1961), appears to be valid, since recent studies have added to this list 10 species (see Mordvinova, 1985; Meissner and Bick, 1997; Mouritsen and Jensen, 1997; Zander *et al.* 2002). Curiously, a recent record shows that in the brackish environments of Schlei Fjord, cercariae of *M. subdolum* encyst within the first intermediate host (Kesting, Gollasch and Zander, 1996).

Gammarids do not seem preferred hosts of *M. subdolum*, in fact its main hosts are probably *Idothea* spp. Thus Mordvinova (1985) recorded a prevalence of 100% in *Idothea baltica* (vs 50–70% in *Gammarus* spp.) in the Ukrainian Black Sea coast. Recently Zander *et al.* (2002) suggested that the Orther Bight (Baltic Sea) is an epizootiotope for *M. subdolum*, estimating the density of this parasite in summer as 17 681/m² (based on data on *Idothea chelipes* only) and concluded that even if many isopods were preyed on by fish, 'a very rich source of infectious metacercariae was still presented to its final hosts, birds'. *M. subdolum* is also a strong mortality agent of corophiid amphipods (Mouritsen and Jensen, 1997; Meissner and Bick, 1997; 1999), and therefore, poorly adapted for hitch-hiking (see Mouritsen, 2001). All the above considerations suggest that *M. subdolum* is 'flooding' the marine coastal habitats of birds, utilising an extreme-opportunist transmission strategy, and this offers the most parsimonious explanation of any association with this species.

Geographical variations in host-parasite interactions may appear more frequent than previously believed, as indicated by our observations on virtually the same microphallid community which largely depart from the patterns of infection and association observed in the same gammarid species in Thau lagoon. Three main factors may account for the differences of microphallid infection between the two studies: habitats, birds and host-parasite systems used to infer relationships between microphallids. Spatial heterogeneity can affect transmission and thus abundance of trematodes (see Bartoli and Holmes, 1997 for a marine example). The small and shallow Pomorie Lagoon is characterized by both horizontal and vertical structural homogeneity (Vasilev and Mitrofanova, 1998) whereas Thau Lagoon offers a number of sources for such heterogeneity. With its surface of 7000 ha, mean depth of 3.8–4.5 m (maximum 10 m), Thau is one of the largest Mediterranean lagoons and a leading site of

shellfish aquaculture. The presence of large amounts of shellfish induces significant changes in the lagoonal cycle and has caused disastrous episodic events killing benthic populations (Deslous-Paoli *et al.* 1998). Further, activities associated with shellfish farming may also induce a periodic disturbance (in addition to any effects of bivalve aquaculture on the ecosystem) and this may influence the use of habitats by water birds. Undoubtedly, the high infection rates at Pomorie lagoon reflect the spatial homogeneity of the habitat ensuring close proximity of the intermediate hosts and therefore, homogeneity of exposure of the gammarid population to microphallid cercariae.

Bird diversity and abundance are very important in determining infection levels in microphallid intermediate hosts. We found no published data on the bird community at Thau but a long-term bird monitoring programme in the Camargue (www.tourduvalat.org) indicates that gulls, *L. cachinnans* in particular, are dominating in coastal lagoons of southern France. On the other hand, data from bird counts carried out in the course of our study at Pomorie lagoon revealed a substantial diversity and abundance of both anatid and charadriiform final hosts for microphallids, to which the input of gulls was rather small. Eight and 16 bird species (out of 22 and 24 possible host species registered during Winter and Spring preceding our sampling, bird counts 7469 and 11 092, respectively) have been reported as final hosts of the microphallid species recovered in the present study. Although the main input of microphallid infections occurs during the two migration waves, we have detected seasonal variations in both the prevalence and abundance of the 4 species, suggesting that they may have differential effect on the gammarid population in the lagoon (Kostadinova and Mavrodieva, unpublished observations).

It appears that *G. insensibilis* from Thau Lagoon was infected with *M. papillorobustus* and *M. subdolum* only. Thus, whereas the hypothesis of host mortality induced by *M. papillorobustus* (Thomas *et al.* 1995*b*, reiterated by Rousset *et al.* 1996) and its association with *M. subdolum* (Thomas *et al.* 1997, 1998*b*) was derived from a study of population samples of *G. insensibilis*, the hypotheses for the associations between *M. papillorobustus* and the other two digeneans (but not *M. subdolum* possibly absent in this sample, see Thomas *et al.* 1998*a*) and the differential host immune response (Thomas, Guldner and Renaud, 2000), were inferred from the *G. aequicauda* – microphallid system. However, although Thomas *et al.* (1998*a*) stated that “manipulated individuals were probably more likely to be collected” the choice of *G. aequicauda* was rather inappropriate for inferring associations of the microphallid species with the cerebral *M. papillorobustus*, since the metacercariae of the latter “were mainly found in the body” in this sample of *G. aequicauda* (see Thomas *et al.* 1995*b*).

In addition to identity, the size of hosts sampled (3–13 mm) could also provide an explanation for the low levels of interspecific association observed by these authors.

Amalgamation of patterns inferred from different host-parasite systems adds an artificial extra-level of variation and this may have also contributed to the dissimilarity between our observations and those of Thomas and co-workers. A comparative study on the biology and distribution of *G. insensibilis* and *G. aequicauda* in a nearby lagoonal system (Bages-Sigean, ca 50 km south to Thau) has revealed differences in their distribution and abundance in relation to depth, temperature salinity and substrate preferences. Thus, *G. insensibilis* was most frequent in the upper (0–1 dm, 25.1%) and lower levels of the water column (below 11 dm, 42.1%), whereas *G. aequicauda* was predominantly found at intermediate depths (2–11 dm, 72.9%) (Janssen *et al.* 1979). It is possible that the differences in habitat utilization of the 2 sympatric amphipod species reflect in their differential role in microphallid transmission. Therefore, combining the patterns observed in the 2 host-parasite systems under the reference ‘same intermediate hosts’ (e.g. Lafferty, Thomas and Poulin, 2000) appears somewhat preliminary.

All the above considerations, although approximate, seem to provide a plausible explanation for the sources of variation resulting in observed differences between patterns of infection of *G. insensibilis* in the 2 lagoons. With regard to the manipulative parasite, *M. papillorobustus*, we found little evidence to support the hypothesis of its preferential location in the host’s brain which may cause prevalence-dependent mortality thus affecting interspecific interactions in the microphallid community. Evidently, integration of further comparative data from natural populations of both intermediate (mollusc and crustacean) hosts would help the development of ideas on interspecific interactions and assembly rules in microphallid communities.

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