

Needle in a haystack: involvement of the copepod *Paracartia grani* in the life-cycle of the oyster pathogen *Marteilia refringens*

C. AUDEMARD¹, F. LE ROUX¹, A. BARNAUD¹, C. COLLINS¹, B. SAUTOUR²,
P.-G. SAURIAU³, X. DE MONTAUDOUIN², C. COUSTAU⁴, C. COMBES⁴
and F. BERTHE^{1*}

¹Laboratoire Génétique et Pathologie, IFREMER, BP 133, 17390 La Tremblade, France

²Laboratoire d'Océanographie Biologique, UMR 5805 – Université Bordeaux I – CNRS, 2, rue du Professeur Jolyet, 33120 Arcachon, France

³Centre de Recherche en Ecologie Marine et Aquaculture, L'Houmeau, B.P.5, 17137 L'Houmeau, France

⁴Laboratoire de Biologie Animale, CNRS UMR 5555, CBETM, Université de Perpignan, 66860 Perpignan, France

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SUMMARY

Marteilia refringens is a major pathogen of the European flat oyster, *Ostrea edulis* Linnaeus. Since its description, the life-cycle of this protozoan parasite has eluded discovery. Attempts to infect oysters experimentally have been unsuccessful and led to the hypothesis of a complex life-cycle involving several hosts. Knowledge of this life-cycle is of central importance in order to manage oyster disease. However, the exploration of *M. refringens* life-cycle has been previously limited by the detection tools available and the tremendous number of species to be screened in enzootic areas. In this study, these two restrictions were circumvented by the use of both molecular detection tools and a mesocosm with low biodiversity. Screening of the entire fauna of the pond for *M. refringens* DNA was systematically undertaken using PCR. Here, we show that the copepod *Paracartia* (*Acartia*) *grani* is a host of *M. refringens*. Not only was DNA of *M. refringens* consistently detected in *P. grani* but also the presence of the parasite in the ovarian tissues was demonstrated using *in situ* hybridization. Finally, successful experimental transmissions provided evidence that *P. grani* can be infected from infected flat oysters.

Key words: *Marteilia refringens*, *Ostrea edulis*, *Paracartia grani*, PCR, *in situ* hybridization, experimental transmission.

INTRODUCTION

Marteilia refringens (Grizel *et al.* 1974), a major pathogen of the European flat oyster, *Ostrea edulis* Linnaeus, is responsible for important mortalities and economic losses in the oyster industry (Alderman, 1979; Figueras & Montes, 1988). During the past 3 decades of biological investigation, emergence of control strategies has been impeded by a major obstacle, which is elucidating the life-cycle of this protozoan parasite. Early field observations led authors to suspect the involvement of other host species in the transmission of *M. refringens* (Balouet, 1979; Balouet, Cahour & Chastel, 1979a; Balouet *et al.* 1979b; Grizel, 1985; Lester, 1986). This hypothesis was further supported when authors repeatedly failed to infect healthy oysters with oyster-derived parasites after cohabitation, injection or feeding (Balouet, 1979; Balouet *et al.* 1979a; Berthe *et al.* 1998). Although extensive efforts were

made to identify potential hosts, these studies were strongly impeded by the technical limits of histology, transmission electron microscopy and immunohistochemistry as screening tools, and complicated by the hundreds or thousands of species present in enzootic areas (Balouet *et al.* 1979b; Grizel, 1985).

In the present study, we circumvented the problem of species diversity by choosing particular natural oyster ponds, locally named 'claires', in the Marennes-Oléron Bay. Environmental characteristics of claires strongly limit the number of inhabiting species as compared with intertidal areas and oyster beds. Claires host less than 100 recognizable species (Reymond, 1991; Audemard *et al.* 2001), versus more than 1000 on open shores as Marennes-Oléron Bay (de Montaudouin & Sauriau, 2000). In addition, we recently developed a PCR-based tool allowing the detection of *Marteilia* DNA. Based on the sequence of the small subunit ribosomal RNA gene of *M. refringens*, specific polymerase chain reaction primers and *in situ* hybridization probes were designed (Le Roux *et al.* 1999; Berthe *et al.* 2000). PCR and *in situ* hybridization represent powerful tools for the detection of the parasite in potential

* Corresponding author: Laboratoire Génétique et Pathologie, IFREMER, BP 133, F- 17390 La Tremblade. Tel: +33 5 4636 9843. Fax: +33 5 4636 3751. E-mail address: fberthe@ifremer.fr

hosts, because they are sensitive, rapid and independent of both developmental stages of the parasite and location in host species (Mialhe *et al.* 1995). Here, we first used PCR to screen every species sampled in the claire ponds for the presence of *M. refringens*. When consistent detection of *M. refringens* was observed in a given species, presence of the parasite was assessed using *in situ* hybridization. Finally, experimental transmission of the parasite was performed to confirm involvement of the candidate species in the *M. refringens* life-cycle.

MATERIALS AND METHODS

Study site

Claire ponds in the Marennes-Oléron Bay (S-W France) are ponds with natural bottoms traditionally used for growing oysters (Korringa, 1976). Ponds which were used in this study, were located on the East Coast of Ile d'Oléron in the central zone of the Bay (Audemard *et al.* 2001). Water temperatures ranged from 8 °C in February to 25 °C in summer. From May to late September, mean values were higher than the 17 °C threshold necessary for infection of flat oysters by *M. refringens* (Grizel, 1985). Salinity ranged from 28 in spring (April) to 37 in summer (August). Species from the pond were sampled on a monthly or weekly basis for macrofauna, meiofauna, or zooplankton respectively (Audemard *et al.* 2001).

Screening by PCR

For each species sampled in the pond analysed individually or by batches in the case of small species, tissues were suspended in 10 volumes of extraction buffer (NaCl 100 mM, EDTA 25 mM, pH 8, SDS 0.5%) containing proteinase K (100 µg/ml). Following an overnight incubation at 50 °C, DNA was extracted using a standard protocol involving phenol/chloroform, and precipitation with ethanol. PCR was performed as described by Le Roux *et al.* (1999), with 2 different primer pairs: one pair CS1/CAS1, was common to 50 eukaryotic species examined including *Marteilia* (universal primers) providing an internal control of amplification experiments. The other, SS2/SAS1, was specific for *M. refringens* 18S rDNA (Berthe *et al.* 2000; Le Roux *et al.* 1999). PCR was performed in 50 µl with 10 ng of purified DNA mixed with 5 µl of PCR buffer 10×, 5 µl of 25 mM MgCl₂, 5 µl of 2 mM of each dNTP, 0.5 µl of each primer at 100 µM and 0.25 µl (1 unit) of *Taq* DNA polymerase (Promega). Samples were overlaid with mineral oil, denatured for 5 min at 94 °C and amplified by 30 cycles: 1 min at 94 °C for denaturation, 1 min at 55 °C for primers annealing, and 1 min at 72 °C for elongation in a thermal cycler apparatus (Appligene). Polymerization at 72 °C was then extended for 10 min to ensure completion of the amplified products.

In situ hybridization

Animals were placed in Davidson's fixative AFA (10% glycerine, 20% formalin, 30% 95° ethanol, 30% H₂O, 10% glacial acetic acid) for at least 24 h and then embedded in paraffin. The sections were subsequently treated by conventional histological procedures. *In situ* hybridization was performed as described by Le Roux *et al.* (1999). Sections were treated with proteinase K (100 µg/ml) in TE buffer (50 mM Tris, 10 mM EDTA), at 37 °C for 20 min. Slides were dehydrated by immersion in an ethanol series and air dried. Sections were placed in 50 µl of hybridization buffer (4× SSC, 50% formamide, 1× Denhardt's solution, 250 µg/ml yeast tRNA, dextran sulphate 10%) containing 10 ng of the labelled probe. Two probes were used: (1) the digoxigenin-labelled probe, called Smart 2 specific for *M. refringens* and (2) a host probe, labelled with digoxigenin by PCR with universal primers and host DNA as template to assess the quality and accessibility of targetted nucleic acid (Le Roux *et al.* 1999). Sections were covered with *in situ* plastic cover-slips and placed on a heating block at 95 °C for 5 min. Slides were then cooled on ice for 1 min before overnight hybridization at 42 °C in a humid chamber. Sections were washed twice for 5 min in 2× SSC at room temperature, and once for 10 min in 0.4× SSC at 42 °C. The detection steps were performed according to the manufacturer's instructions (Dig nucleic acid detection kit, Boehringer Mannheim).

Experimental infections

Uninfected oysters. Uninfected oysters, *Ostrea edulis* L., originating from the laboratory hatchery, were kept under laboratory conditions. Oysters were 2 years old at the time of the experiment. In total 30 oysters were checked at the time of the experiment for absence of *M. refringens* by means of digestive gland imprints.

Infected oysters. A batch of uninfected oysters originating from the laboratory hatchery, were placed in claire ponds during summer 1999 which led to their infection by *M. refringens*. They were then placed in laboratory tanks at 12 °C until the beginning of the experiment in summer 2000. At this temperature, the parasite does not develop further in its flat oyster host and is not released into the environment (Berthe *et al.* 1998). Oysters were 2 years old at the time of the experiment. *M. refringens* prevalence in these oysters was 87%. Before the experiment, oyster shells were cleaned with a brush to remove epibionts.

Uninfected copepods. Uninfected copepods, *Paracartia (Acartia) grani*, were reared in the laboratory

following previously described protocols (Guerrero & Rodriguez, 1998). A batch of 50 of these copepods was checked for the absence of *M. refringens* by means of PCR, as described above.

Infected copepods. Infected copepods, *P. grani* from the claire pond, were sampled every 4 days from August to September 2000. *In situ* hybridization was performed to test the presence of the parasite in *P. grani* on different sampling dates. Copepods were rinsed in filtered sea water and were directly added in tanks containing uninfected oysters.

Physical parameters of the experiments

The experimental and control tanks contained 20 l of filtered sea water. During the experiments, they were kept in the laboratory under natural photoperiod with temperature ranging from 22 to 28 °C. Algal food (*Isochrysis galbana*, *Chaetoceros pumilum* and *Tetraselmis suecica*) was produced in the laboratory and added every day to each tank. Water from the tanks was partially renewed every 4 days with filtered seawater.

Trial 1: Oyster to copepod transmission. The experiment started on the 24 July 2000 and lasted 25 days. This duration approximately corresponds to the longevity of an adult calanoid copepod stage.

Five infected flat oysters were placed on a 500 µm mesh located in the upper part of the tank. The mesh minimized the effect of oyster filtration currents and allowed contact of *M. refringens* sporonts shed with oyster faeces with the copepods. Uninfected copepods were added to the tanks at the beginning of the experiment. Three replicates of the experiment were performed. During the experiment, copepod samples were preserved in Davidson fixative for further *in situ* hybridization. The control experiment was designed as described above, but included uninfected flat oysters also placed on a mesh. Three replicates were performed.

Trial 2: Copepod to oyster transmission. Thirty uninfected flat oysters were placed on mesh in tanks. Copepods from the claire pond were added every 4 days, from August to September 2000. On 9 sampling dates of *P. grani* from the claire pond used in this trial, *M. refringens* was detected 3 times (on the 28 August, 4 and 11 of September) with prevalences below 12%. The experiment ended 1 month after the last copepods were added. At the end of the experiment, flat oysters were analysed by *in situ* hybridization. Three replicates were performed. The control consisted of tanks containing 30 uninfected flat oysters as described above, but no

copepods were added. At the end of the experiment, oysters were analysed by *in situ* hybridization. Three replicates of the experiment were performed.

RESULTS

Screening by PCR

The results are given in Table 1. For each species or taxon sampled in the claire pond, the number of individuals analysed is indicated in column 2. PCR was performed on individuals or groups of individuals with primers (column 3). The number of positive PCRs (assessing possible DNA amplification) is indicated in column 4. For each sample in which amplification had been obtained with CS1/CAS1 primers, PCR was performed with SS2/SAS1 primers specific for *M. refringens* and the number of positive detections is indicated in column 5.

Based on the PCR amplifications, 4 groups of species can be distinguished: the species showing 1/ no detection of *M. refringens*, 2/ very rare detection, 3/ consistent detection, or 4/ undetermined status (column 5).

Among the 62 taxa or species analysed by PCR, 36 taxa or species (Table 1) belonged to the first group (no detection) and 11 species belong to the second group (very rare detection). In the third group there were 2 species (the cnidarian *Cereus pedunculatus* and the copepod *Paracartia grani*), in which there was repeated detection. In the case of *C. pedunculatus*, *M. refringens* DNA was detected 48 times out of 273. Regarding *P. grani*, *M. refringens* DNA was detected in 5 batches out of 6. The last group consisted of 13 species where no DNA amplification had been obtained by PCR, when performed with universal primers.

In situ hybridization

Given the consistent detection of *M. refringens* DNA in *C. pedunculatus* and *P. grani*, *in situ* hybridization was performed with Smart2 probe in order to localize the parasite in potential host tissues.

Cereus pedunculatus

No hybridization was observed with Smart2 in the tissues of 70 *C. pedunculatus* analysed (result not shown). Quality and accessibility of targeted nucleic acid was assessed by controls.

Paracartia grani

Observations were made on copepodid (juveniles) and adult stages. Hybridization with Smart2 was observed within the tissues of *P. grani*. The parasite

Table 1. Screening by PCR of the total fauna sampled in the claire pond model

(Results of the screening by PCR of species sampled in the claire pond. PCR was first performed with 'universal' primers (PCR CS1/CAS1) and, in the case of amplification, with SS2/SAS1 primers. 'A', Ascidia; 'An', Annelida; 'C', Crustacea; 'Ch', Chaetognatha; 'Cn', Cnidaria; 'E', Echinodermata; 'F', Pisces; 'I', Insecta; 'M', Mollusca; 'N', Nematoda; 'Ne', Nemertea; 'Pl', Platyhelminthes.)

Genus species	Number of individuals analysed	Number of PCR using CS1/CAS1 primers	Number of positives with CS1/CAS1 primers	Number of Group PCR using SS2/SAS1 primers	Group
<i>Microdeutopus grillotalpa</i> (C)	731	61	49	0/49	1
<i>Palae monetes varians</i> (C)	287	53	44	0/44	1
<i>Pomatochistus microps</i> (F)	250	44	42	0/42	1
<i>Orchestia gammarella</i> (C)	140	53	37	0/37	1
<i>Hediste diversicolor</i> (An)	496	117	28	0/28	1
<i>Gammarus insensibilis</i> (C)	146	29	27	0/27	1
<i>Paleomon serratus</i> (C)	190	38	26	0/26	1
Harpacticoid spp. (C)	862	17	15	0/15	1
<i>Corophium achuresicum</i> (C)	334	16	15	0/15	1
<i>Notomastus latericeus</i> (An)	133	15	15	0/15	1
<i>Melita palmata</i> (C)	201	27	14	0/1	1
<i>Hydrobia ventrosa</i> (M)	462	16	12	0/12	1
Decapod larvae (zoe I) (C)	204	15	12	0/12	1
<i>Holothuria</i> sp. (E)	242	11	11	0/11	1
<i>Corophium</i> sp. (C)	102	20	8	0/8	1
Nematoda spp. (N)	514	10	8	0/8	1
<i>Balanus improvisus</i> (C)	30	7	7	0/7	1
<i>Tubificoides benedeni</i> (An)	47	7	7	0/7	1
<i>Leucophytia bidentata</i> (M)	56	14	6	0/6	1
<i>Hydrobia ulvae</i> (M)	129	21	6	0/6	1
<i>Spionidae</i> sp. (An)	39	6	4	0/4	1
<i>Anguilla anguilla</i> (F)	4	4	4	0/4	1
Gastropod larvae (M)	116	4	4	0/4	1
Platyhelminth spp. (Pl)	14	4	4	0/4	1
<i>Acartia discaudata</i> (C)	42	2	2	0/2	1
<i>Amphipholis squamata</i> (E)	7	2	2	0/2	1
<i>Chlamys varia</i> (M)	2	2	2	0/2	1
<i>Haliplanella luciae</i> (Cn)	2	2	2	0/2	1
<i>Nephtys humbergii</i> (An)	4	2	2	0/2	1
Ascidian larva (A)	14	2	1	0/1	1
Polychaeta annelid larva (An)	49	2	1	0/1	1
<i>Acartia bifilosa</i> (C)	35	1	1	0/1	1
Chaetognath sp. (Ch)	9	1	1	0/1	1
<i>Pseudopolydora antennata</i> (An)	8	1	1	0/1	1
<i>Alkmaria romijni</i> (An)	349	16	1	0/1	1
<i>Abra tenuis</i> (M)	32	5	1	0/1	1
<i>Molgula manhanntensis</i> (A)	167	116	112	1/112	2
<i>Lineus gisserensis</i> (Ne)	118	35	24	4/24	2
Decapod larvae (zoe II) (C)	570	28	23	1/23	2
<i>Canuella perplexa</i> (C)	966	19	19	1/19	2
<i>Haminoea hydatis</i> (M)	161	24	15	1/15	2
Juveniles of <i>Pomatochistus microps</i> (F)	282	17	15	3/15	2
Cyclopoid spp. (C)	362	15	14	1/14	2
<i>Euterpina acutifrons</i> (C)	362	15	14	2/14	2
Ostracod spp. (C)	287	10	10	1/10	2
<i>Streblospio shrubsolei</i> (An)	44	8	6	1/6	2
<i>Abra segmentum</i> (M)	166	29	2	1/2	2
<i>Cereus pedunculatus</i> (Cn)	732	345	273	48/273	3
<i>Paracartia grani</i> (C)	285	8	6	5/6	3
<i>Corophium volutator</i> (C)	32	6	0	ND	4
<i>Chironomus salinarius</i> (I)	30	3	0	ND	4
<i>Praunus flexuosus</i> (C)	23	1	0	ND	4
<i>Idothea chelipes</i> (C)	22	5	0	ND	4
<i>Carcinus maenas</i> (C)	13	13	0	ND	4
<i>Crangon crangon</i> (C)	11	6	0	ND	4
Isopod sp. (C)	8	2	0	ND	4
<i>Doris verrucosa</i> (M)	5	5	0	ND	4
<i>Eulalia veridis</i> (An)	3	1	0	ND	4
<i>Cerastoderma edule</i> (M)	2	2	0	ND	4
<i>Ephydriidae</i> sp. (I)	1	1	0	ND	4
<i>Facellina coronata</i> (M)	1	1	0	ND	4
<i>Polydora ciliata</i> (An)	1	1	0	ND	4

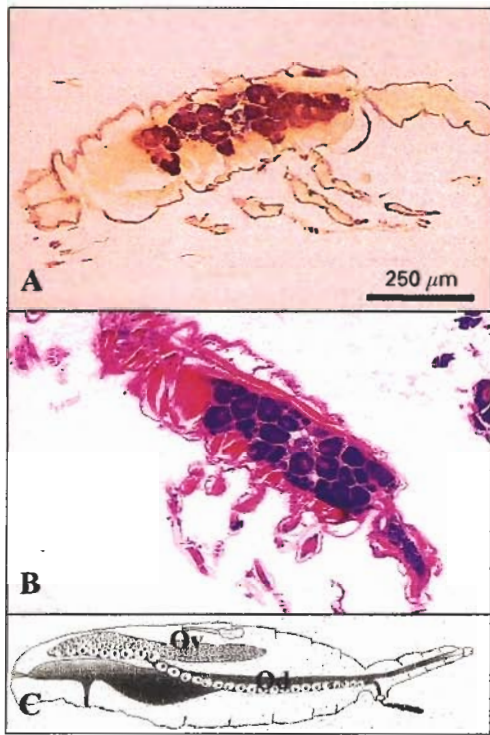


Fig. 1. Slides of a female *Paracartia grani* treated by *in situ* hybridization with Smart 2 probe (A), and treated for conventional histology (Hemalum Eosin) (B). Schematic representation of a female copepod reproductive system (C) with the germinal site of ovary (ov) and oviduct (od). On each figure, the anterior part of the copepod is on the left.

was detected (brown staining) in copepodid and adult females and located in the ovary (Fig. 1A, B, C). *M. refringens* was never detected in male copepods. The maximum prevalences of the parasite were 26%, ($n = 85$) in copepodid females and 23% ($n = 66$) in adult females in August.

Experimental transmission of *Marteilia refringens*

Trial 1. After 7 days of cohabitation with infected flat oysters as described above, females of *P. grani* were infected by *M. refringens* with a prevalence of 3% ($n = 65$). During the experiment, the prevalence ranged from 1.5% ($n = 65$) to 3% ($n = 33$, Table 2) depending on the day of experiment (Table 2). Oysters and both male and female copepods from the controls remained uninfected during the experiment.

Trial 2. After 72 days of cohabitation with copepods sampled in the claire pond, the 90 flat oysters from the trial remained uninfected when analysed by *in situ* hybridization.

DISCUSSION

Marteilia refringens belongs to the phylum Paramyxia, which contains protistan parasites, infectious for marine invertebrates (Desportes & Perkins, 1990;

Berthe *et al.* 2000). Paramyxian parasites are characterized by the formation of spores consisting of several cells enclosed inside one another, which result from internal cleavage within a stem cell. In the phylum Paramyxia, the species *M. refringens* in Europe and *M. sydneyi*, a parasite of *Crassostrea glomerata* (*Saccostrea commercialis*) in Australia (Perkins & Wolf, 1976; Adlard & Ernst, 1995) have been extensively studied but their life-cycle has remained uncharacterized.

As an approach to identifying potential host species participating in the transmission of *M. refringens*, we performed an exhaustive screening of the fauna of claire pond. Using PCR amplification of *M. refringens* DNA, we distinguished 4 groups of species: species where the parasite DNA was never, occasionally, or consistently amplified, as well as species where DNA amplification had not been obtained (even using universal primers). The distinction between these 4 groups allowed species presenting consistent detection of the parasite to be selected as the most probable candidates as *M. refringens* hosts. To draw conclusions on the status of a species as a host of *M. refringens*, several parameters have to be taken into account, such as the number of individuals sampled, the number of analyses performed, possible DNA amplification, and the presence of the parasite. For example, a limited number of individuals showing poor DNA amplification and no detection of the parasite does not allow a decision on the status (potential host or not) of the species.

The first limiting factor can be seen in the number of individuals sampled. This number mainly depends on the abundance of the species in the claire biotope and on the sampling strategy. As a consequence, it was difficult to decide on the status of scarce species such as *Anguilla anguilla*, platyhelminth species, *Amphipholis squamata*, *Chlamys varia*, *Haliplanelle luciae*, *Nephtys humbergii*, chaetognath species, *Pseudopolydora antennata*, *Doris verrucosa*, *Eulalia veridis*, *Cerastoderma edule*, Ephyridiidae, *Facellina coronata*, *Polydora ciliata*. For these species, additional efforts should be made on the sampling frequency in order to increase the number of animals to be analysed. Nevertheless, if these species were infected by *M. refringens*, one can assume that the naturally low abundance of animals should be balanced by a higher parasite prevalence and infection level, therefore allowing the detection of *M. refringens* in the course of this study.

A second limiting factor is the number of analyses performed. For small size species (especially species from the meiofauna and zooplanktonic species), DNA extraction was realised on a pool of individuals. This could result in a dilution of the targeted DNA (i.e. *M. refringens* DNA) and, possibly induce an underestimate of the parasite presence. As an example, in the case of Harpacticoid copepods, pooling reduced 862 sampled individuals into 17

Table 2. Results of the experimental transmission of *Marteilia refringens* from infected oysters to *Paracartia grani*

(Results of the experimental transmission of the parasite *M. refringens* between infected flat oysters and uninfected copepods. Prevalence of the parasite obtained by *in situ* hybridization on female copepods from the trial and the control (with the number of infected animals/the total number of females analysed). Disappearance of copepod was noted in the control on the 17th and 25th days of experiment.)

Time of exposure of copepods to flat oysters (day)	Prevalence of <i>M. refringens</i> in copepods from the control	Prevalence of <i>M. refringens</i> in copepods from the trial
0 (beginning of the experiment)	0% (0/60)	0% (0/60)
3	0% (0/44)	0% (0/30)
7	0% (0/41)	3% (2/65)
10	0% (0/17)	1.5% (1/65)
17	No copepod	3% (1/33)
25	No copepod	0% (0/10)

performed analyses. On the other hand, reducing 285 individuals of *P. grani* to 8 analyses yielded 5 positive PCR results.

The third limitation encountered during this study, came from the absence or the low rate of DNA amplification during PCR when performed with universal primers. The absence of DNA amplification could be explained by the presence of inhibitory factors contained in the shell of crustaceans or molluscs (Wilson, 1997; Le Roux *et al.* 1999). For example, shells of the shrimp *Palaemon serratus* were removed during this study, and led to a substantial improvement of the PCR reaction. However, shells of small crustaceans (*Corophium volutator*, *Idothea chelipes*), insects (Ephydriidae) or molluscs (*Abra segmentum*, *Hydrobia ulvae*) could not be removed because of their small size, and they probably inhibited PCR amplification. For some species such as *Hediste diversicolor*, and *Carcinus maenas* the low rate of DNA amplification probably resulted from DNA degradation. In future studies, the time between sampling and sample preservation (which could last for 3 h due to the sorting of species) would have to be shortened to avoid DNA degradation. Similarly, alternative DNA extraction methods would have to be tested (Ferraris & Palumbi, 1996).

Finally, the absence of detection of *M. refringens* can be seen as a failure of the overall strategy (sampling and screening). When taking into account the different factors cited above, most of the species included in this study cannot be firmly excluded from the hypothetical life-cycle. In contrast, the consistent detection of the parasite obtained in *P. grani* and *C. pedunculatus* strongly supported the hypothesis of their involvement in *M. refringens* life-cycle.

In order to discriminate between true parasitism and presence of *M. refringens* in the digestive tract or on the body surface, *in situ* hybridization was

performed to localize the parasite in tissue sections. *In situ* hybridization experiments demonstrated presence of *M. refringens* in the ovary of *P. grani*. The ovarian tissue was frequently observed to be overwhelmed by *M. refringens* in the form of large numbers of small cells within oocytes. Observed stages of *M. refringens* in *P. grani*, appeared to be morphologically different from the known stages observed in oyster digestive gland, although further transmission electron microscopy studies are obviously needed to characterize these developmental stages. The ovarian location of *M. refringens* may be surprising. However, other paramyxean parasites are known to target gonads of marine invertebrates: *Marteilioides chungmuensis* in *Crassostrea gigas* (Comps, Park & Desportes, 1986) and *Paramarteilia orchestiae* in *Orchestia gammarellus* (Ginsburger-Vogel & Desportes, 1979). The ovary appears during the moult of the first copepodid stage and keeps developing until the sexually mature adult stage (Schram, 1986). Infestation of the ovaries of some copepods has already been described in the case of microsporidian parasites (Andreadis, 1988; Micieli, Garcia & Becnel, 2000) and infection of ovarian tissues does not seem to impede seriously normal metabolic processes (Andreadis, 1988).

Involvement of *P. grani* in the life-cycle of *M. refringens* seems consistent with both the ecology of this copepod and the epidemiology of the disease. *Paracartia grani* is mainly observed in bays and estuaries during spring and summer season (Alcaraz, 1983; Rodriguez & Jimenez, 1990; Rodriguez, Guerrero & Bautista, 1995; Guerrero & Rodriguez, 1998) as it is the case in the claire ponds where it may represent 100% of the total copepod abundance. The seasonal cycle of *M. refringens* appears to match the life-cycle of *P. grani*, as it was shown that transmission occurs during summer (Grizel & Tigé, 1977; Grizel, 1985). During winter, sporangia

primordia of *M. refringens* observed in flat oysters do not develop. In spring, increase of water temperature fosters *M. refringens* development (Balouet, 1979; Berthe *et al.* 1998). Similarly, *P. grani* is absent from the water during winter (Rodriguez & Jimenez, 1990; Rodriguez *et al.* 1995; Guerrero & Rodriguez, 1998) to reappear in spring from resting benthic eggs. These eggs could play a role in overwintering of the parasite as reservoir for the parasite which will be investigated in future studies.

The geographical range of *P. grani* also matches *M. refringens* distribution. In Europe, marteiliosis has been reported to be restricted to north-western Brittany, the Bay of Biscay and the Mediterranean Sea, including the Atlantic coasts of Spain and the Adriatic coasts of Italy and Croatia. *Paracartia grani* is a warm temperate species originating from the tropical and temperate Atlantic coast. It is a calanoid copepod typical of coastal, semi-confined ecosystems, conditioned by instability of both physical parameters (temperature and salinity) and biological conditions (quality and quantity of available food). This species is often observed in the vicinity of oyster beds (Gallo, 1981) and has been observed within the geographical range of the disease; in estuaries or bays where the parasite has been reported (Lubet, 1953; Paulmier, 1965, 1969; Alcaraz, 1983; Elbéé (D') & Castel, 1995; Siokou-Frangou *et al.* 1997; Razouls & Bovée, 1999).

Determination of the role of *P. grani* in the life-cycle of *M. refringens* requires further experimental validation. Transmission of *M. refringens* from flat oysters to copepods was proved to be effective and rapid, as it was detected after 7 days of exposure to infected oysters. However, the prevalence of *M. refringens* in *P. grani* obtained by the experimental transmission was low. This could be due to a weak inoculum of parasites since the source of *M. refringens* consisted of oysters previously maintained at 12 °C and placed in experimental aquaria. They experienced a sharp increase of water temperature, which induced a synchronous release of sporangia in the faeces (Berthe *et al.* 1998). Consequently, the experimental design may have led to a unique flash of parasites, while a continuous release of parasite sporangia may occur under natural conditions. We therefore cannot assume that experimental conditions were optimal for transmission of the parasite from oysters to copepods.

No transmission of *M. refringens* was observed from copepods to oysters in our experiments. This also could result from non-optimal experimental conditions. The source of parasite may have been too weak to enable transmission. Presence of *M. refringens* in females of *P. grani* sampled in the summer 2000 was rare and the prevalence was low (less than 12%). In 2000, both copepods and oysters from the claire pond exhibited low prevalence of *M. refringens* as compared with the previous year where prevalence

in adult copepods and oysters was respectively, 26% and 87%.

The absence of parasite transmission in trial 2 could also reflect the involvement of a third species in the life-cycle of *M. refringens*. When considering this hypothesis, a candidate was *C. pedunculatus* in which the parasite was recurrently detected. *In situ* hybridization performed on 70 individuals never demonstrated *M. refringens* in tissues of *C. pedunculatus*. Consequently, detection of the parasite by PCR could be attributed to phoresy or ingestion of sporangia, infected copepods, or free-living stages of *M. refringens* because cnidarians are non-selective feeders (Van Praët, 1985). Among the 273 PCRs performed, 45 out of 190 (23%) were positive when *C. pedunculatus* was sampled on oysters while 3 out of 83 (4%) were positive when sampled on the sediment of the claire pond (data not shown). Thus, detection of the parasite DNA in *C. pedunculatus* seems to be correlated with the geographical distance from *M. refringens*-infected oysters. However, considering recurrent detection of the parasite, we can not exclude that *C. pedunculatus* may play a role in the *M. refringens* life-cycle, for example, in allowing capacitation or maturation of the parasite within the digestive cavity of this species. Future transmission experiments would have to investigate the role of this species in the *M. refringens* life-cycle.

From a more general point of view, the case of the cnidarian *C. pedunculatus* illustrates the confinement situation of claire ponds. In this ecosystem, species leading to occasional detection of *M. refringens* are numerous and it is difficult to discriminate accidental carriers from inconspicuous hosts. In the claire, *M. refringens* is present in oysters and copepods, as well as in sporangia released within oyster faeces. Other stages of *M. refringens* may exist, as free-living stages or within copepod eggs. *M. refringens* may be part of the food web of the claire pond, directly or indirectly. Considering the size of *M. refringens* sporangia ranging from 9 to 19 µm (Grizel *et al.* 1974; Perkins & Wolf, 1976), they could potentially be ingested by various pond microphageous species, including decapod larvae, copepods (cyclopoids, *Canuella perplexa* and *Euterpina acutifrons*), and ostracods (Dusart & Defaye, 1995; Vannier, Abe & Ikuta, 1998). The parasite could also be present within the digestive tract of annelid *Streblospio* spp., as they feed at the water interface upon suspended, resuspended and deposited material where the parasite can be present (Dauer, Maybury & Ewing, 1981). Nematode (*Lineus* spp.) or fish, *Pomatostichus* sp, may ingest not only free-living stages but also infected copepods. Phoresy of the parasite on the body surface may also explain occasional detection of the parasite. Basically, as *M. refringens* may be present on the sediment (at least as sporangia within oyster faeces) and in the water column, transport is conceivable by any benthic or pelagic species. If

experimental transmissions from *P. grani* to *O. edulis* recurrently fail, these species will have to be studied further as potential host of *M. refringens*.

In conclusion, we demonstrated in the course of this study, that at least 2 species are involved in the life-cycle of *M. refringens*: *O. edulis* and *P. grani*. Transmission experiments between *O. edulis* and *P. grani* were successful, demonstrating that the 2 species are contiguous in the life-cycle of the parasite. First attempts of reverse transmission failed. Design of this experiment should be improved by enhancing the inoculum of *M. refringens*. This could be achieved by improving transmission rate from oysters to copepods reared in the laboratory or relying on better environmental conditions to increase naturally prevalence in copepods populations.

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