Dynamics of the parasite *Marteilia refringens* (Paramyxea) in *Mytilus galloprovincialis* and zooplankton populations in Alfacs Bay (Catalonia, Spain)

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SUMMARY

Since the first description of *Marteilia refringens* (Paramyxea) in flat oysters *Ostrea edulis* in 1968 in the Aber Wrach, Brittany (France), the life-cycle of this parasite has remained unknown. However, recent studies, conducted in the 'claire' system, have proposed the planktonic copepod *Acartia grani* as a potential intermediate host for the parasite. Nevertheless, experimental transmission of the parasite through the copepod has failed. Recent studies in this field have reported the presence of the parasite in zooplankton from the bays of the Delta de l'Ebre, a more complex and natural estuarine environment than that of the claire. As a result, 2 new *Marteilia* host species were proposed: the copepods *Oithona* sp. (Cyclopoida) and an indeterminate Harpaticoida. Consequently, the objective of the present work was to study the dynamics of *Marteilia* in the zooplankton community from one of the bays, Alfacs Bay, as well as the dynamics of the parasite in cultivated mussels during 1 complete year. Six different zooplankton taxa appeared to be parasitized by *M. refringens*, including copepods (3 Calanoida, *Acartia discaudata*, *A. clausi* and *A. italica*; 1 Cyclopoida, *Oithona* sp.; and 1 Harpacticoida, *Euterpina acutifrons*), and larval stages of decapod crustaceans (zoea larvae of Brachyura, probably *Portumnus* sp.). These taxa are thus proposed as new subjects for study, since they could be intermediate hosts in the infection process of mussels by *Marteilia*.

Key words: *Marteilia*, *Mytilus galloprovincialis*, intermediate host, zooplankton copepods, Brachyura larvae.

INTRODUCTION

Marteilia is a protozoan parasite belonging to the phylum Paramyxea which affects some marine invertebrate species. In Europe, this parasite has been responsible for important mortalities of flat oysters *Ostrea edulis* in culture areas (Goulletquer and Héral, 1997). As far as cultivated mussels *Mytilus galloprovincialis* are concerned, several authors have reported less mortality but a loss of condition and negative effects on reproduction in parasitized individuals (Villalba *et al*. 1993; Camacho *et al*. 1997). Due to the detrimental impact of this parasite on flat oyster culture since the 1970s, *M. refringens* infection has been listed as a notifiable disease to the OIE (the World Organization for Animal Health). Consequently, the parasite has become a subject for scientific research since its first description in 1968 in the Aber Wrach, Brittany (Comps, 1970).

In Europe, 2 species or types of *Marteilia*, *M. refringens* (type O) and *M. maurini* (type M), are responsible for marteiliosis of oysters and mussels, respectively (Le Roux *et al*. 2001). Nevertheless, recent data, based on molecular studies, suggest that both parasites, *M. refringens* and *M. maurini*, are conspecific and correspond to 2 types of the same genomic species called *M. refringens* (López-Flores *et al*. 2004), most probably through an alloxenic speciation process, and this nomenclature is used in the current study. Furthermore, a molecular typing study has recently emphasized the possible cross-detection of the parasite types and the potential lack of specificity for their hosts (Novoa *et al*. 2005). However, these molecular results need confirming by robust epidemiological and ecological datasets, as well as by biological aspects, such as a study of the complete life-cycle of the parasite.

After some unsuccessful attempts to infect mussels and oysters horizontally, the hypothesis of

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a heterogenic life-cycle for *M. refringens* was proposed (Berthe *et al*. 1998). Moreover, development of molecular techniques for *M. refringens* detection has facilitated life-cycle studies (Le Roux *et al*. 1999, 2001). The copepod *Acartia grani* (*Paracartia grani*) has been suggested as an intermediate host in the life-cycle of *M. refringens* infecting flat oysters (Audemard *et al*. 2002). This copepod appeared infected by *M. refringens* when the dynamics of the parasite were studied in the 'claire' system in Marennes-Oléron. 'Claires' are shallow ponds traditionally used for fattening and greening oysters, and the life-cycle of *M. refringens* was demonstrated to be completed in this environment (Audemard *et al*. 2001). However, results obtained in the 'claire' model need to be corroborated in a more complex and natural ecosystem (Berthe *et al*. 2004).

On the other hand, to date, no intermediate hosts have been proposed for *M. refringens* infecting mussels. The flux of both *M. refringens* type O (usually infecting flat oysters) and type M (usually infecting mussel) through the copepod *A. grani* therefore represents an interesting subject of study in order to improve the understanding of the relationship of the two parasite types with potential intermediate hosts. The experimental infection of *A. grani* from infected flat oysters and mussels has been successful but the infection patterns of *M. refringens* in *A. grani* were different for copepods infected *via* mussels or *via* flat oysters. While the development of the parasite was obvious in copepods infected from flat oysters, only early stages of infection were found in *A. grani* infected from mussels. Neither oysters nor mussels were infected after cohabitation with infected copepods (Carrasco *et al*. 2005), as reported in previous studies (Audemard *et al*. 2002; Berthe *et al*. 2004). Therefore, research on other zooplanktonic species as candidates for *Marteilia* intermediate hosts was proposed for further work.

Due to their physical and environmental characteristics, the bays of the Delta de l'Ebre in Catalonia (Spain), which are endemic areas for the *Marteilia* parasite, were proposed as a model study site for *Marteilia* dynamics and life-cycle studies (Carrasco *et al*. 2007). The Ebre bays are larger and more representative of other infected areas than the 'claire model', especially for *Marteilia* infecting mussels because mussel culture is very important in this area. Furthermore, recent studies have reported zooplankton infected by *M. refringens* in these bays. Preliminary results in such a complex estuarine environment suggested the implication of some zooplankton species in the parasite life-cycle. The Cyclopoida *Oithona* sp. and an indeterminate Harpaticoida appeared to be infected by the parasite (Carrasco *et al*. 2007). A recently developed molecular diagnostic technique based on the intergenic spacer of rDNA (IGS) (López-Flores *et al*. 2004) showed high sensitivity and was therefore considered to be an interesting tool for the detection of *M. refringens* in small organisms such as zooplanktonic species (Carrasco *et al*. 2007). In order to improve the understanding of the life-cycle and ecology of the parasite, screening of *M. refringens* infection in the zooplankton community, using IGS nested PCR, was carried out in Alfacs Bay (Delta de l'Ebre) for a whole year (October 2004-October 2005). The dynamics of the parasite in mussel cultures were also studied and compared with the results obtained from zooplankton.

MATERIALS AND METHODS

Study site

The Delta de l'Ebre is located in the North Western Mediterranean (Fig. 1). Sediments from the River Ebre form and shape this delta, which is mainly comprised of 2 semi-enclosed bays: Fangar Bay in the northern half, and Alfacs Bay in the southern half. Both are exploited for mollusc aquaculture and fisheries. The dynamics of *M. refringens* in mussels and the zooplankton community were studied in Alfacs Bay for 1 complete year from October 2004 to October 2005. For the purpose of this study, 2 critical environmental parameters, temperature and salinity, were recorded weekly at a depth of 2 metres using a manual conductivity meter (WTW) during the experimental period.

Mytilus galloprovincialis *samples*

For this study, mussels *Mytilus galloprovincialis* were collected from experimental ropes, hung from a commercial raft stocked with Alfacs spat collected naturally during the spring of 2004. Sixty mussels were randomly collected each month during the study period between October 2004 and October 2005. Half of each individual was fixed in 10% formaldehyde in filtered sea water (v/v) for 24–48 h, and afterwards conserved in 70% ethanol (v/v) for histological studies in order to detect the presence of *M. refringens*. Samples were dehydrated and embedded in paraffin wax. Paraffin blocks were cut at 3 μm and stained with haematoxylin-eosin. Slides were studied under light microscopy at a magnification of 40X, and the prevalence and infection intensity were recorded.

The prevalence was calculated as the number of mussels found infected divided by the number of mussels histologically tested. Infection intensity was calculated according to Villalba *et al*. (1993) in a range from 0 to 5, which represented null infection to high infection intensity. Parasite stages in each positive case were also recorded by following Robledo and Figueras (1995): young stages in the epithelium of the stomach or primary digestive

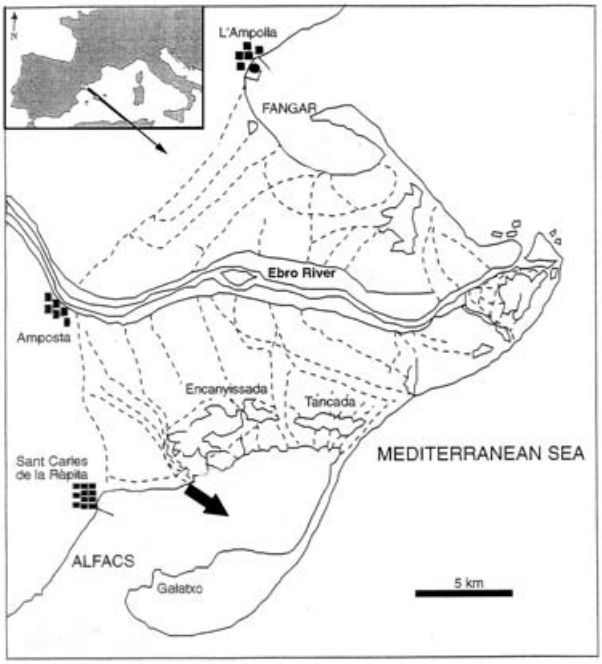

Fig. 1. Study site: Delta de l'Ebre, Catalonia, North East Spain (arrow shows the sampling site).

tubules (plasmodia containing 1 or 2 cells), first stages of maturation in primary and secondary tubules (plasmodia containing 2 or more cells), middle stages of maturation (sporangiosori with sporania primordia), mature stages (sporangiosori with mature sporangia) and mature sporangia released in the lumen of hepatopancreatic tubules.

Zooplankton samples

Zooplankton samples were collected every 15 days throughout the study period by means of horizontal hauls made with a Juday-Bogoroy zooplankton net fitted with 100 μm mesh. The net was hauled at 3 knots for a period of 5 min around the mussel ropes. Each zooplankton sample was split into 2 aliquots: one was immediately fixed for 24–48 h in 10% formaldehyde in filtered sea water for taxonomic studies; the second was fixed and conserved in 100% ethanol for molecular analyses.

The predominant zooplankton groups of each sample were taxonomically classified under a stereomicroscope. A minimum of 10 individuals from the most abundant taxa were sorted and transferred into Eppendorf vials containing 96% ethanol for further molecular analysis.

DNA extraction and amplification by PCR

DNA extraction from zooplankton samples was carried out as follows: ethanol-fixed animals were ground and suspended in 10 volumes of extraction buffer (NaCl 100 mM, 10 mM Tris, pH 8, 2·5 mM EDTA, pH 8, SDS 0·5%) containing proteinase K (100 μg/ml). Following an overnight incubation at 55 °C, DNA was extracted using a standard protocol involving phenol/chloroform, and precipitation with ethanol. Integrity and quantity of DNA were measured by spectrometry.

Nested-PCR targeting the IGS was performed in 2 consecutive PCR reactions using the 2 primer pairs previously designed by López-Flores *et al*. (2004).

The primers MT-1/MT-2 (first round PCR) and MT-1B/MT-2B (second round PCR), were used to amplify a fragment of the intergenic spacer of the ribosomal genes (IGS) of the parasite. First round PCR assay was carried out according to the standard conditions for Silver-star *Taq* polymerase (Eurogenetec, Seraing, Belgium) using 10 ng of DNA. After DNA denaturation at 94 °C for 5 min, 30 cycles were run with a PTC-100tm thermocycler (MJ. Research, Inc.). The protocol was comprised of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 1 min. A final elongation for 10 min at 72 °C was also performed. In the second round PCR assay, DNA denaturation at 94 °C was carried out for 5 min, and 24 cycles were run with a PTC-100tm thermocycler (MJ. Research, Inc.) as follows: denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and elongation at 72 °C for 30 sec. A final elongation for 10 min at 72 °C was also performed.

DNA extracted from an infected oyster was used as a positive control for both PCRs. Amplified products were analysed electrophoretically on 1% agarose gels and purified using the QiaClean sequencing kit (QIAGEN). The same protocol was performed for each separate zooplankton taxon in order to evaluate the presence of the parasite.

Nucleotide sequences of purified PCR fragments were determined by Sanger's method (Sanger *et al*. 1977) using the ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems). The identity of the sequences was obtained by comparison with sequences stored in the GenBank data base using BLAST (Altschul *et al*. 1997).

RESULTS

Environmental characteristics

Temperatures decreased from October 2004, when values reached 18 °C, to January 2005, which was one of the coldest months (around 8 °C) (Fig. 2). During March 2005, water temperatures increased from 8 °C to 16 °C, and reached 17 °C in April. Maximum temperature values were observed during June (29·4 °C) and July (28·8 °C). Temperature values began to decrease again from 23 °C in September to 19 °C at the end of October 2005.

Salinity was usually between 34 and 36 p.s.u., although specific decreases in November 2004 and the end of August 2005 brought salinity values down to around 23 p.s.u.

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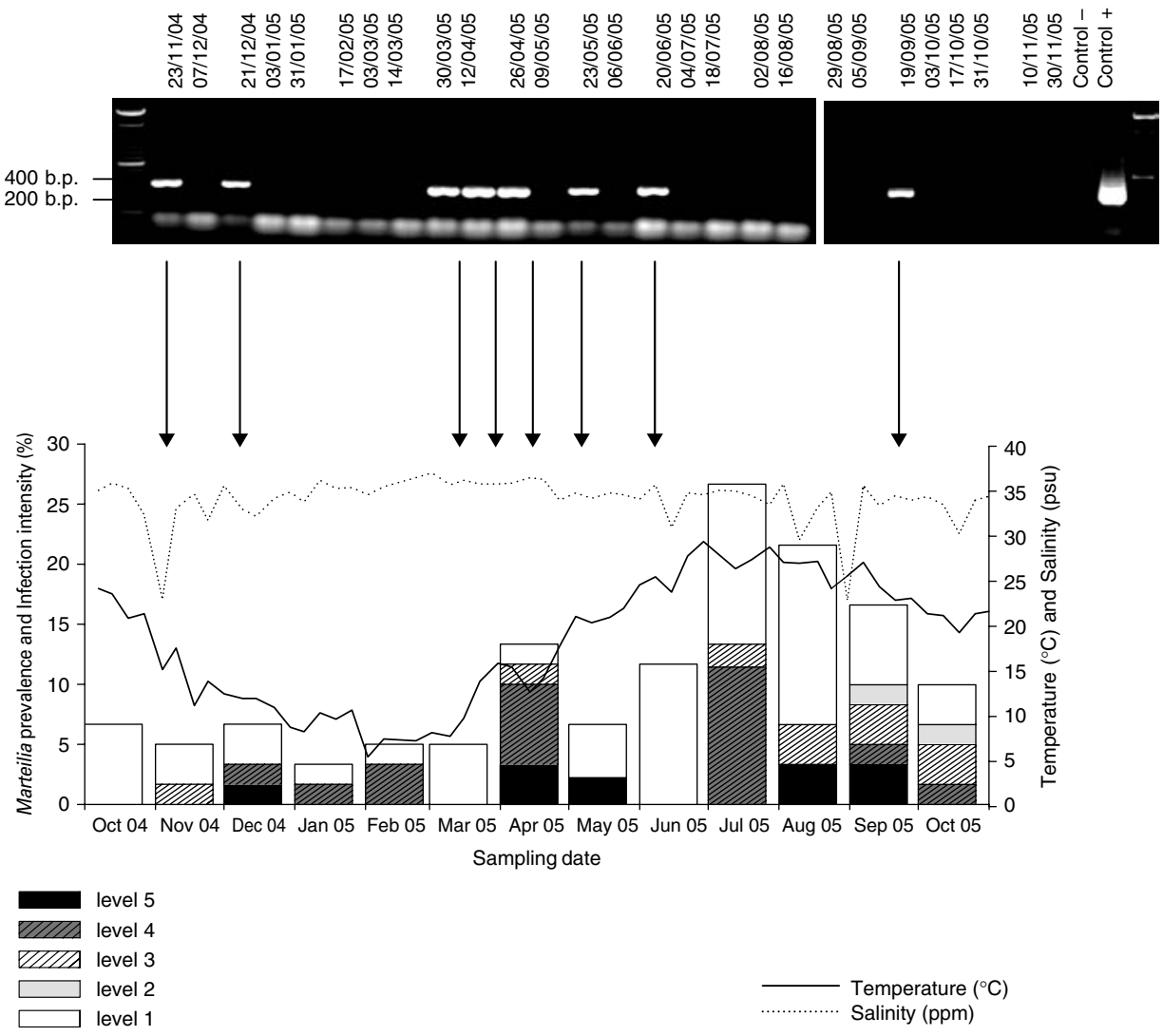

Fig. 2. The graph shows the prevalence of *Marteilia refringens* and the infection intensity level from October 2004 to October 2005 in Alfacs Bay. Temperature and salinity are also represented. The electrophoresis gel shows the zooplankton samples detected as positive by nested PCR during the same period. The positive control corresponds to DNA extracted from a *Marteilia*-infected flat oyster.

Marteilia refringens *dynamics in* Mytilus galloprovincialis

The prevalence of *M. refringens* in *M. galloprovincialis* showed a clear seasonal cycle throughout the year (Fig. 2). During autumn and winter (October 2004–March 2005) prevalences (the % of infected mussels) were low, ranging from 6·67% in October and December 2004 to 3·34% in January 2005. In spring, prevalences increased from 5% in March to 13·34% at the end of April. Infection prevalences in May decreased to 6·67% but they increased again during the summer period, reaching 11·67% in June and 26·67% in July, which was the maximum value observed during the year. During September and the autumn season, prevalences of infection began to decrease again (16·67%) and in October 2005 only 10% of the individuals were found infected.

Other parasites, such as some Turbelaria individuals and the Trematoda *Protoeces* sp. were also recorded in most of the monthly samples, whereas ciliates were only observed in a few cases. Two individuals (one from the June sample and the other one from the October 2005 sample) were affected by neoplasia.

Infection intensity and parasite stages in mussels

Infection intensity was high, level 5 (more than 90% of infected tubules), during April and May, August, September, and also in December (Fig. 2). In these samples, an important part of the infected individuals had most of the digestive tubules infected by *M. refringens*. Furthermore, all the known developmental stages of *Marteilia* were present in these samples, from young stages in

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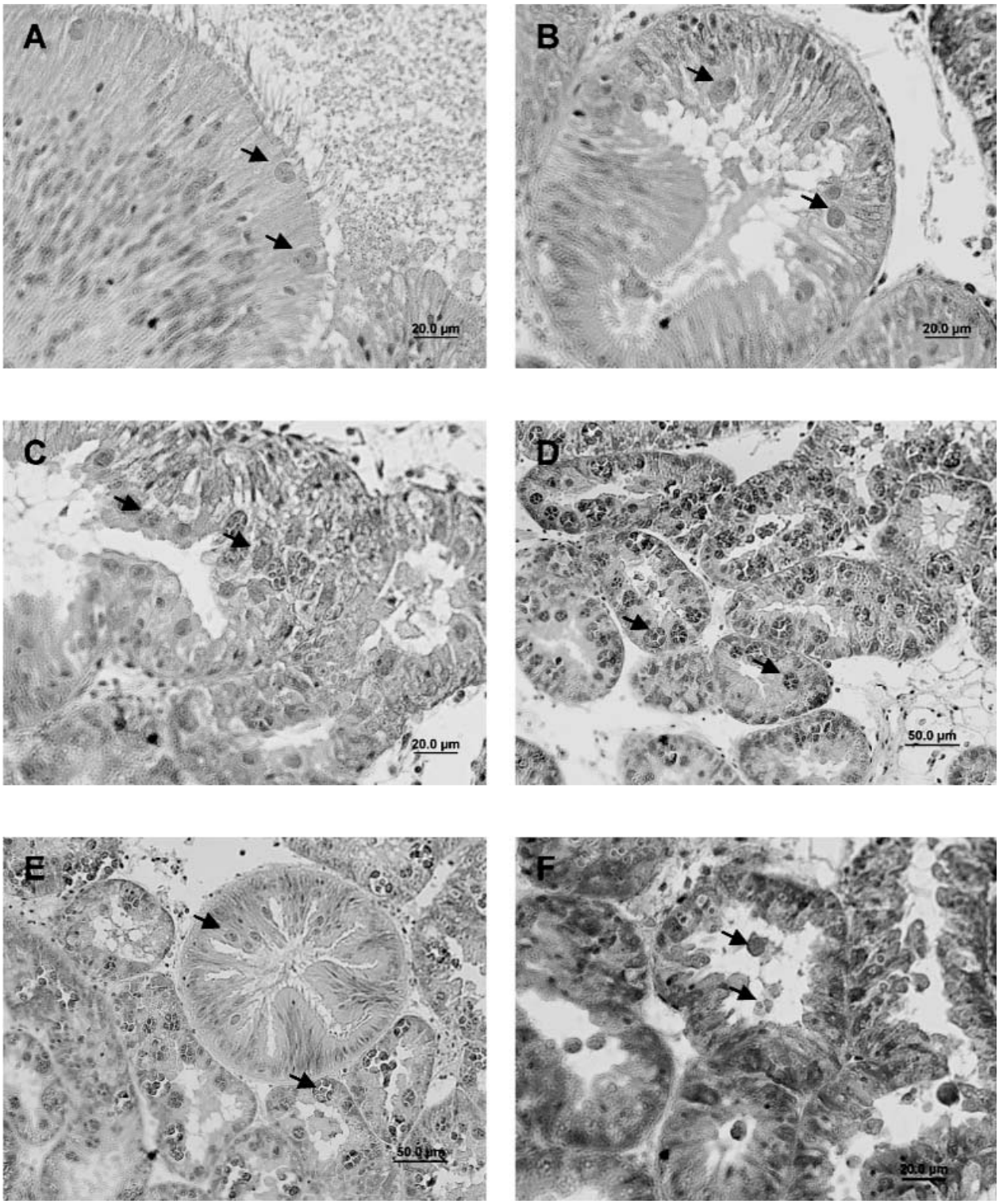

Fig. 3. Different stages of *Marteilia refringens* development in its host *Mytilus galloprovincialis* observed with light microscopy (H&E stained). (A) Young stages, plasmodia containing 1 or 2 cells in the epithelium of the stomach. (B) First stages of maturation in primary and secondary tubules, plasmodia containing 2 or more cells. (C) Middle stages of maturation, sporangiosori with sporangia primordial. (D) Mature stages, sporangiosori with mature sporangia. (E) Sporangiosori with mature sporangia in secondary tubules and young stages in a primary tubule. (F) Mature sporangia released in the lumen of hepatopancreatic tubules.

the epithelium of the stomach (plasmodia containing 1 or 2 cells) to mature stages (sporangiosori) containing sporangia (Fig. 3). Sporangiosori containing mature sporangia and free sporangia were released, in some of the cases, into the lumen of the digestive tubules.

In July, 50% of the individuals infected by *Marteilia* spp. reached level 4 infection intensity

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Table 1. Taxonomic classification and relative abundance of taxa constituting zooplankton samples found to be infected by *Marteilia refringens* using nested PCR

(+, Weak abundance; ++, medium abundance; +++, high abundance.)

Date	Taxon	Abundance	*Marteilia* PCR
23/11/04	*Oithona* sp.	+++	Negative
	Acartia clausi	+++	Negative
	Lamellibranchia larvae	+++	Negative
	Oithona sp. Nauplius	+	Negative
	Euterpina acutifrons	+++	Negative
21/12/04	*Oithona* sp.	+	Negative
	Ctenophore	+	Negative
30/03/05	*Euterpina acutifrons*	++	**Positive**
	Apendicularia	+	Negative
	Oithona sp.	+++	**Positive**
	Acartia clausi	+++	Negative
	Acartia discaudata	+	Negative
12/04/05	*Centrophages* sp.	++	Negative
	Acartia clausi	++	**Positive**
	Acartia discaudata	+++	**Positive**
	Acartia italica	+	**Positive**
26/04/05	*Acartia clausi*	+	Negative
	Acartia discaudata	+	**Positive**
23/05/05	*Acartia latisetosa*	++	Negative
	Acartia discaudata	+	**Positive**
	Acartia italica	+	**Positive**
	Acartia discaudata	+	Negative
	Brachyura zoea	+	Negative
20/06/05	*Paracalanus* sp.	+	Negative
19/09/05	*Paracalanus* sp.	+	Negative
	Oithona sp.	++	**Positive**

(between 50% and 90% of infected tubules). Most of these mussels showed 85% infected tubules and in some of these individuals only first stages of maturation (plasmodia in the epithelium of the stomach and in secondary digestive tubules) could be observed. About 40% of the mussels collected in July were infected by young stages located in the stomach epithelium (level 1) and 10% of the samples reached level 3 infection (between 10% and 50% of infected tubules). In January and February, 50% and 75% of the infected individuals, respectively, also reached level 4 infection intensity and the presence of mature stages (sporangiosori with mature sporangia) was common.

During June 2005, October 2004 and March 2005, only young stages located in the stomach epithelium were detected.

Marteilia *in mixed zooplankton samples*

Eight out of 27 zooplankton samples were found positive using the *Marteilia* IGS nested PCR (Fig. 2). Five of them were collected during spring 2005 (from the end of March to the beginning of June). However, isolated positive samples could also be detected in autumn. The analysed sequences, one sequence from each positive sample, showed a similarity of between 94% and 100% with the *Marteilia* IGS fragment. Sequences were submitted to GenBank and have the following Accession numbers: AM504129, AM504130, AM504131, AM504132, AM504133, AM504134, AM504135 and AM504136.

Table 2. Prevalence (%) of taxa in all zooplankton samples detected to be infected by *Marteilia refringens* using nested PCR

(For example, *Acartia discaudata* was present in 50% of the *Marteilia*-positive zooplankton samples.)

Taxonomic group	Taxon	Prevalence (%) in the total sample
Calanoida copepoda	*Acartia discaudata*	**50%**
	Acartia clausi	**50%**
	Acartia italica	25%
	Acartia latisetosa	12·5%
	Paracalanus sp.	25%
	Centrophages sp.	12·5%
Cyclopoida copepoda	*Oithona* sp.	**50%**
Harpaticoida copepoda	*Euterpina acutifrons*	25%
Other groups	Lamellibranchia larvae	12·5%
	Ctenophore	12·5%
	Apendicularia	12·5%
	Brachyura zoea	12·5%

The zooplankton of Alfacs Bay and Marteilia *in zooplankton species*

The zooplankton community consisted of groups of congeneric species (genera *Acartia* and *Oithona*), harpacticoids (*Euterpina, Clitemnestra*), with the occasional presence of *Centropages, Clausocalanus* and *Paracalanus* species. Radiolarians (*Aulacantha*), Cnidarians, ctenophores and appendicularians completed the list of Holoplankton groups. The meroplankton was dominated by larval stages of decapod crustaceans, polychaetes, echinoderm and mollusc larvae, as well as others which were less abundant. Highest zooplankton abundances were recorded in autumn, winter and spring.

The taxa identified in each positive zooplankton sample, as well as their relative abundance, are shown in Table 1. In some samples, up to 4 different taxa were abundant enough to be sorted into Eppendorf vials for further analysis. The Cyclopoida *Oithona* sp., and different Calanoida species, including *Acartia clausi* and *Acartia discaudata*, were present in 50% of the samples found infected by *M. refringens* (Table 2). Furthermore, other Calanoida such as

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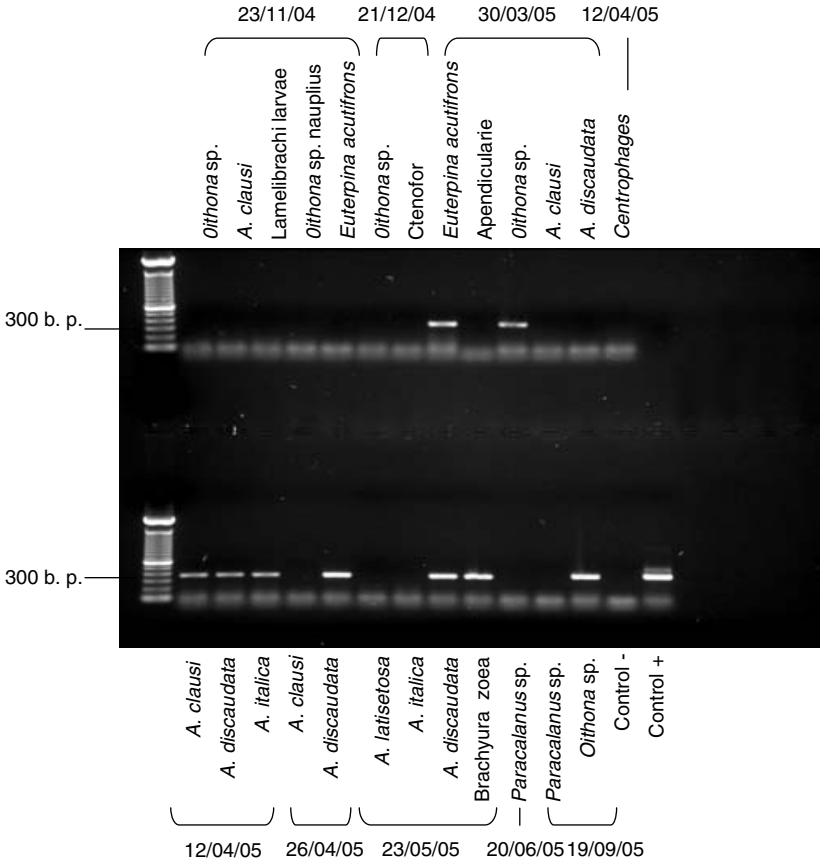

Fig. 4. Electrophoresis gel for the IGS nested *Marteilia refringens* PCR (358 base pairs) analyses of the different species of zooplankton from the *M. refringens*-positive zooplankton samples.

Acartia italica, *Paracalanus* spp. and the Harpaticoida *Euterpina acutrifons* were also present in 25% of the infected samples. The Calanoida *Acartia latisetosa* and *Centrophages* sp. were only present in 12·5% of positive samples. Appendicularians, lamellibranch larvae, ctenophores and larvae of crustacean Decapoda (zoea of Brachyura, probably *Portumnus* sp.) were also present in some samples, but in low abundances.

Six taxa, *Acartia discaudata*, *A. clausi*, *A. italica*, *Oithona* sp., *Euterpina acutifrons* and Brachyura larvae (zoea of *Portumnus* sp.) appeared infected by *M. refringens* when tested by IGS nested PCR analysis (Fig. 4). Therefore, ecologically diverse marine crustacean groups, such as calanoida, cyclopoida and harpaticoida copepods, and decapod crustacean larvae were parasitized by *M. refringens*, although *A. discaudata* and *Oithona* sp. were the most frequently detected positive species. The analysed sequences, 1 sequence from each parasitized species sample, showed a similarity of between 94% and 100% with the *Marteilia* IGS fragment. Sequences were submitted to GenBank and have the following Accession numbers: AM504137, AM504138, AM504139, AM504140, AM504141, AM504142, AM504143, AM504145 and AM504144.

DISCUSSION

Recent studies have shown the relevant role that zooplankton species can play in the life-cycle of certain marine protozoan parasites (Audemard *et al.* 2002; Skovgaard and Saiz, 2006). Implication of zooplankton species in the life-cycle of other protozoan bivalve parasites, such as *Bonamia ostreae* (Haplosporidia), are also currently being studied (Lynch *et al.* 2006*a*, *b*). The results of this current study suggest the existence of closely-linked dynamics for the parasite *M. refringens* in mussel populations and the zooplankton community in the Alfacs Bay ecosystem.

Prevalence values of *M. refringens* in *M. galloprovincialis* showed a seasonal cycle. The results suggest that this seasonal cycle is related to the annual water temperature cycle, as already proposed by other authors (Grizel, 1977; Balouet, 1977; Berthe *et al.* 1998, 2004; Audemard *et al.* 2001). The lowest *M. refringens* prevalence was observed in January, one of the coldest months, whereas in spring, when water temperatures reached values higher than 17 °C in April, *M. refringens* prevalences also increased to double the March values by the end of April. The maximum annual prevalence value was reached

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in July, which corresponded to the hottest month of the studied period, and prevalences of infection began to decrease again during August. Nevertheless, an unexpected and sudden decrease of prevalence occurred from 13·34% in April to 6·67% in May, while temperatures were increasing to 25 °C. This phenomenon could be explained by a release of mature sporangia to the environment. This hypothesis is supported by observation of histological slides from mussels collected during this period that also coincided with the month for which reported intensities were the highest (in April). It could be that, after releasing all mature sporangia, some animals could recover or, alternatively, animals could keep some latent stages of the parasite which are not easy to detect by histopathology.

Compared to the dynamics of *M. refringens* in flat oysters, in which mature stages are usually not observed during the winter time (Balouet, 1977), mature sporangia could be detected in mussels throughout the year in the present study. This observation is in line with previous observations (Robledo and Figueras, 1995). This difference could support the hypothesis of a differentiated ecological behaviour for both types of *M. refringens* (Carrasco *et al*. 2005) or a differentiated susceptibility of the hosts to the infection.

Previous studies undertaken in the Delta de l'Ebre bays have suggested the involvement of zooplankton in the *M. refringens* life-cycle in this particular ecosystem (Carrasco *et al*. 2007). In the present study, using nested PCR, zooplankton appeared to be infected by *M. refringens* mostly during the spring months, when prevalences began to increase just before the peak of parasitism. This period may correspond to the infection period that some authors suggest for *M. refringens* in flat oysters, when temperatures reach more than 17 °C from June to August (Balouet, 1977; Audemard *et al*. 2001). In Alfacs Bay, water temperatures reached 16 °C at the end of March, when the first zooplankton sample appeared infected in spring. Previous observations on *Marteilia* spp. dynamics in mussel populations from the Delta de l'Ebre bays (Carrasco *et al*. 2007) also supported the fact that new infections could occur between the end of May and August. Similar observations in cultivated mussels from Galicia were recorded by Robledo and Figueras (1995). During the months preceding the increase of prevalence, the parasite may infect zooplankton populations, thereby leading to further transmission.

The hypothetically important role played by the zooplankton community in the transmission of the parasite would also be supported by the relative proportion of the different developmental stages of *M. refringens* in mussel samples in spring. In April, the month when most of the zooplankton samples were parasitized, mussels were infected mainly by mature stages of the parasite and, as discussed previously, mature stages were released into the environment with mussel faeces, that in turn could infect zooplankton (Audemard, 2001). In June, parasite stages observed in mussels were only represented by primordial stages, probably because of recent new infection. Similarly for July, only young, and not mature, *M. refringens* stages were detected in half of the parasitized individuals.

Other infected zooplankton samples were detected in November and December 2004 and September 2005. In September 2005, temperatures were still over 20 °C and the infection period was possibly still running. However, the prevalences of *M. refringens* were beginning to decrease in mussels in August. This decrease shows that further transmission of the parasite was not occurring during this period.

Two other infected zooplankton samples were detected in November and December 2004. These months are supposed to be outside the infection period with temperatures between 10 and 15 °C and the occurrence of low *M. refringens* prevalences in mussels (around 6%). When Audemard and coworkers (2004) studied the dynamics of *M. refringens* in *Acartia grani* populations in the 'claires' system between May and October, one positive sample could still be detected in September. This event was attributed to two possible phenomena: a false positive due to the presence of the parasite in the digestive tract following ingestion or its presence on some external part of the body; or the lack of development of the infective stage for the bivalves and production of resistant eggs (Audemard *et al*. 2004). In any case, the hypothesis of the role of zooplankton populations as alternative hosts cannot be discarded until the transmission of the parasite is experimentally demonstrated.

Nested PCR performed on zooplankton taxa after sorting, revealed the presence of *M. refringens* in the species which were the most abundant in the Ebre delta. These species also occur in most of the semi-landlocked marine areas in temperate systems, since harbours, estuaries and enclosed bays are usually occupied by groups of congeneric copepods, most of them belonging to the genera *Acartia* (Alcaraz, 1979) and *Oithona* (Turner, 2004), as well as by Appendicularians and larval stages of benthonic organisms, decapod crustaceans, cirripeds, molluscs, amongst others (Riley *et al*. 1949). These zooplankton species are characterized by their ability to feed on a wide size-range of suspended food particles (from $<1\,\mu$m, Appendicularians, to $>50\,\mu$m, *Oithona*), such as phytoplankton, components of the microbial loop, detritus, faecal pellets, etc. Some are non-selective filter-feeders, like the mollusc larvae, whereas others create feeding currents that entrap food particles in their capture area, or they are ambush predators preying on relatively large, mobile prey (Alcaraz, 1980). The size range of *Marteilia* (8–40 μm) fits into the optimum feeding size-range of the

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zooplankton community that occupies the Ebre Delta bays. Previous studies have demonstrated the experimental infection of *A. grani* by *M. refringens via* feeding, and differences in the susceptibility of the copepod to the *M. refringens* type would explain the advance or suppression of the infective process (Carrasco *et al.* 2005). Consequently, further studies, including the use of *in situ* hybridization, will help to discriminate real infections from accidental, *cul de sac* infections caused by ingestion. Complementary experimental transmission experiments will also be required to demonstrate the nature of zooplankton as potential intermediary hosts as opposed to their ability to act just as an alternative host.

Six different zooplankton taxa appeared infected by *M. refringens* using nested PCR, including copepods (3 Calanoida, *A. discaudata*, *A. clausi* and *A. italica*; 1 Cyclopoida, *Oithona* sp.; and 1 Harpacticoida, *Euterpina acutifrons*), and zoea larval stages of Brachyuran decapods (probably *Portumnus* sp.). Copepod species more frequently found infected in the present study were *A. discaudata* and *Oithona* sp. This is the first detection of *M. refringens* in *A. discaudata*, a congeneric species of *A. grani*, which both coexist in most coastal, landlocked marine systems with similar and superimposed ecological niches (Alcaraz, 1983). *Oithona* sp., a fundamental component of zooplankton in similar environments, was already reported as parasitized in a previous study carried out in this same area (Carrasco *et al.* 2007). Moreover, this genus belongs to the Cyclopoida which appeared as infected in the study undertaken in the 'claire' model (Audemard *et al.* 2001).

This study shows that the dynamics of *M. refringens* in mussels and zooplankton appeared to be linked in Alfacs Bay. A number of zooplankton species were found to be infected by the parasite using nested PCR. Nevertheless, complementary transmission experiments are required to test the involvement of these species as potential intermediate hosts in the parasite life-cycle. However, if these studies do not give conclusive results, other non-planktonic species may need to be taken into consideration as potential hosts in the life-cycle of *M. refringens*, such as *Cereus pedunculatus* (Cnidaria) and *Lineus gisserensis* (Nematoda) that were previously found infected in the 'claire' model (Audemard *et al.* 2002).

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