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Experimental transmission of *Marteilia refringens* with special consideration of its life cycle

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ABSTRACT: Ever since its first description the paramyxean parasite *Marteilia refringens* has been recognised as one of the most significant pathogens of bivalve molluscs. The existence of a complex life cycle has been postulated by many authors. However, the transmission route of *M. refringens* and its life cycle remain unresolved. Experimental observations are presented here that may help lead to a satisfactory hypothesis of the *M. refringens* life cycle. Trials were performed to test the infectivity of purified early and late stages of *M. refringens* by different routes of inoculation. Horizontal transmission was also tested by cohabitation with carriers in experimental tanks. Field cohabitation and exposure to natural sources of the parasite helped examine the role of the environment in the transmission of *M. refringens*. Lastly, naturally infected oysters were monitored in experimental tanks, in order to study infection progression in the oysters through serial samples. These experiments provide no evidence to support direct horizontal transmission of *M. refringens*, but rather suggest that intermediate or alternative hosts, or free-living stages, are essential in the life cycle of the parasite.

KEY WORDS: *Marteila refringens* · *Ostrea edulis* · Transmission trial

INTRODUCTION

Marteilia refringens is the aetiological agent of 'Aber Disease' (Grizel et al. 1974). Since 1968, this parasite has caused serious, recurring mortalities in the European flat oyster, *Ostrea edulis* industry (Alderman 1979, Grizel 1985) and among cultured molluscs in several locations (Robert et al. 1991, Tiscar et al. 1992, Lama et al. 1993, Martin et al. 1993, Robledo et al. 1994, Fuentes et al. 1995).

Marteilia refringens is the type species of the genus *Marteilia* (Grizel et al. 1974). *Marteilia sydneyi* (Perkins & Wolf 1976) is also responsible for mortalities of *Saccostrea commercialis* in Queensland, Australia. In Europe, *M. refringens* has been observed in *Ostrea edulis*, *O. angasi*, *O. chilensis* (Grizel et al. 1974, Grizel et al. 1982, Bougrier et al. 1986), and in *Mytilus edulis* and *M. galloprovincialis* (Tigé & Rabouin 1976, Claver-Derqui 1990, Villalba et al. 1993). *M. refringens* cells were also found in the Pacific cup oyster *Crassostrea gigas* (Cahour 1979). Another species of the genus *Marteilia*, *M. maurini*, has been described in both mussels *Mytilus galloprovincialis* and *M. edulis* from France (Comps et al. 1982, Auffret & Poder 1985). *Marteilia* sp. has been observed in cockles *Cardium edule*, clams *Tapes rhomboides* and *T. pullastra* (Comps et al. 1975, Poder et al. 1983, Figueras et al. 1996), and mussels *Modiolus modiolus*, *Mytilus edulis* and *M. galloprovincialis* (Comps et al. 1975, Poder et al. 1983, Auffret & Poder 1985, Figueras et al. 1991, Ceschia et al. 1992). Lastly, the *Marteilia christenseni* was described in *Scrobicularia* [*piperata*] *plana* (Comps 1983) on the Atlantic coast of France.

In spite of numerous papers published on *Marteilia* spp., 2 main questions remain unresolved: taxonomic relationships within the genus *Marteilia*, and the life cycle of the parasite. However, these questions are important in developing effective programmes to control this pathogen and to prevent the transfer of infected stocks between European countries.

The diagnoses of the 2 main species of *Marteilia* found in Europe (*M. refringens* and *M. maurini*) were

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based on ultrastructural characteristics and host specificity (Grizel et al. 1974, Comps et al. 1982, Figueras & Montes 1988). However, since host specificity was discarded when *M. refringens* was found in *Mytilus galloprovincialis*, *M. maurini*, which was described as the only *Marteilia* species parasitizing mussels, may not be a true different species from *M. refringens*.

The existence of an intermediate host was postulated early on as a result of field observations by many authors (Balouet 1979, Balouet et al. 1979a, b, Grizel 1985, Lester 1986) but has never been demonstrated.

The aim of this work was to assess whether support could be given to the hypothesis of an intermediate host or free-living stages in the life cycle of *Marteilia refringens* based on experimental observations. This study is descriptive because, in some of the trials, true negative or positive controls were difficult to design. We present herein experimental observations that may help lead to a satisfactory hypothesis of the *Marteilia* life cycle.

MATERIALS AND METHODS

In this study, numerous trials were performed on transmission of *Marteilia refringens*. Inoculation of purified parasites (Trials 1 to 4) tested the infectivity of early and late stages of *M. refringens* by different routes of inoculation. Then, possible horizontal transmission was tested by cohabitation with carriers in experimental tanks (Trials 5 to 8). Lastly, field cohabitation and exposure to natural sources of the parasite tested the role of the environment in transmission (Trials 9 to 11). Also, naturally infected oysters were monitored in experimental tanks (Trials 12 and 13), with a view to studying the infection development in oysters through serial samples.

Origin of the molluscs included in the study. Molluscs were selected according to results of histological observations of sampled individuals. A minimum of 30 molluscs from each batch were sampled at the beginning of every trial and checked for *Marteilia refringens* by means of both digestive gland imprints and histological sections. Digestive gland imprints were useful because of the quick results obtained; however, results were then confirmed by histology. Imprints were stained using a Giemsa modified staining method (Hemacolor kit, Merck). Tissue samples, fixed with Davidson's fixative, were processed following established protocols and stained with hematoxylin-eosin. Infected individuals were obtained from endemic zones of marteiliosis. Highly infected flat oysters *Ostrea edulis* were collected from the Golfe du Morbihan and Aber Wrac'h (Brittany, France), and Noirmoutiers island (Vendée, France). Infected blue mussels *Mytilus edulis* were obtained from the La Trinité river (Brittany, France), and *M. galloprovincialis* from the Ria de Vigo and the Ria de Pontevedra (Galicia, Spain).

'Non-infected' oysters were collected from areas considered non-enzootic for *Marteilia refringens* and checked for the absence of the parasite: Sète, Port Leucat (Var, France), La Rochelle (Charente maritime, France). 'Non-infected' *Mytilus edulis* were obtained from Royan (Charente maritime, France), and Weymouth (Dorset, United Kingdom), and *M. galloprovincialis* from Thau (Var, France). Hereafter the term '*Marteilia*-free' is used to mean those molluscs from samples or locations in which *M. refringens* was not found in preliminary sampling.

Artificial production of infection by *Marteilia refringens* cell inoculation. ***Purification of the parasite:*** *M. refringens* cells were obtained from naturally infected flat oysters. Prior to purification of cells from oysters, prevalence was individually checked by means of digestive gland imprints. About 850 digestive glands were processed to assess the presence of *M. refringens* in oysters. The infection level ranged from 2 to 80% among the flat oyster populations sampled from *M. refringens* endemic areas, along the Atlantic coast in France. Purification of the parasite was carried out following Mialhe et al. (1985) and Robledo et al. (1995) with minor modifications. Briefly, digestive glands were homogenized in sterile sea water with 1% Tween 80 (SSWT) using an ultraturax. Homogenates were filtered through 250 µm followed by 75 µm filters. The resulting filtrate was centrifuged at 2000 × *g*, for 30 min at 8°C. The pellet was then resuspended in 5 ml SSWT and placed on a 5/35% sucrose gradient to be centrifuged (2000 × *g*, 30 min, 8°C). The 5/35% fraction containing sporangia primordia (early stage) and the pellet fraction containing the sporangia (late stage) were collected. Each fraction was then washed with SSWT to remove sucrose. Cells were counted on a Malassez hemacytometer and the cell number was adjusted for inoculation experiments by dilutions in sterile sea water.

Inoculation experiments: Oysters were maintained and fed in experimental tanks containing full-strength sea water. The water temperature of the latter was approximately 20°C throughout the trials, which were performed in summer. During the experiments, oysters were fed on a micro-algae mixture of the genera *Chaetoceros*, *Tetraselmis*, *Isochrysis*, and *Pavlova*. Mortality was checked daily; *Marteilia refringens* infection was assessed in moribund or dead oysters by means of both digestive gland imprints and histological sections. For each trial, 30 oysters of the same origin were held as a negative control. The experiments performed to artificially reproduce infection by *M. refringens* cell inoculation are listed in Table 1.

Table 1. *Ostrea edulis*. Experiments performed to artificially reproduce infection by *Marteilia refringens* cell inoculation

Trial	No. of inoculated oysters	Route of inoculation	Inoculated with	No. of inoculated cells per individual	Experiment duration
1	14	Forced ingestion	Sporangia	5×10^6	1 mo
1	11	Forced ingestion	Sporangia primordia	8×10^6	1 mo
2	4	Pallial cavity	Sporangia	5×10^6	1 mo
2	3	Pallial cavity	Sporangia primordia	5×10^6	1 mo
3	12	Pallial cavity	All stages	Undetermined	1 mo
4	9	Pallial cavity	Sporangia	1.5×10^6	Up to 3 wk
4	9	Pallial cavity	Sporangia primordia	7×10^6	Up to 3 wk

Trial 1. After oysters had been anaesthetized with $MgCl_2$ (Culloty & Mulcahy 1990) *Marteilia refringens* cells were inoculated by forced ingestion through the mouth. Two experiments were performed. First, 14 flat oysters *Ostrea edulis* were each inoculated with approximately 5×10^6 sporangia cells and later, 11 oysters with 8×10^6 sporangia primordia. The number of inoculated oysters and the number of parasite cells inoculated depended on the number of sporangia and sporangia primordia obtained by purification.

Trial 2. Two experiments were conducted. First 4 and then 3 unanaesthetized non-infected flat oysters were inoculated via the pallial cavity with purified sporangia (5×10^6) and sporangia primordia (5×10^6) respectively. After the inoculation, oysters were held for a period of 3 h out of the water to force them to retain the parasite. The oysters were then returned to the experimental tank.

Trial 3. Twelve flat oysters were inoculated, without $MgCl_2$ anaesthesia, via the pallial cavity with a digestive gland homogenate obtained from 2 highly infected oysters. After inoculation oysters were held for 3 h out of the water. The oysters were then placed back into the experimental tank.

Trial 4. Two experiments were conducted. In the first, 9 flat oysters were inoculated via the pallial cavity with 1.5×10^6 sporangia cells per individual without using $MgCl_2$ anaesthesia. In the second, 9 oysters were inoculated, under the same conditions, with 7×10^6 sporangia primordia.

In Trials 1, 2, and 3 the *Marteilia refringens* infection was assessed, after 1 mo, by means of both smears and histological sections. In Trial 4, *M. refringens* presence was assessed after 2 h (3 oysters from each batch), 24 h (3 oysters from each batch), and 3 wk (3 oysters from each batch).

Infection by cohabitation. By and large, experimental conditions were the same as described above for inoculation experiments. For each trial, animals of the target species were held as negative control. The trials performed to reproduce the infection by cohabitation in experimental conditions are listed in Table 2.

Trial 5. Fifty *Marteilia*-free mussels *Mytilus edulis* were placed in the same tank as 30 infected mussels *Mytilus galloprovincialis* (10/30 were found to be infected when digestive gland smears were examined in sampled mussels from the same batch). After 6 wk, 20 *M. edulis* were sacrificed and the presence of *Marteilia* spp. was assessed by the examination of digestive gland smears and histology. At the end of the experiment (6 mo) all animals were processed for histology.

Trial 6. Forty-eight *Marteilia*-free oysters *Ostrea edulis* were held together with 44 infected mussels *Mytilus galloprovincialis* (10/30 were found to be infected when digestive gland smears were examined in sampled mussels from the same batch). After 6 wk and 6 mo the presence of the parasite in *O. edulis* was assessed as described above.

Trial 7. Thirty *Marteilia*-free oysters *Ostrea edulis* were placed in a tank with 30 *O. edulis* infected with

Table 2. Experiments performed to transmit *Marteilia* spp. by cohabitation of infected and non-infected molluscs

Trial	Source species (infected)	n	Target species (non-infected)	n	Duration
5	*Mytilus galloprovincialis*	30	*Mytilus edulis*	50	6 mo
6	*Mytilus galloprovincialis*	44	*Ostrea edulis*	48	6 mo
7	*Ostrea edulis*	30	*Ostrea edulis*	30	1 mo
8 (tank 1)	*Ostrea edulis*	30	*Mytilus galloprovincialis*	30	4 mo
8 (tank 1)	*Ostrea edulis*	30	*Mytilus edulis*	30	4 mo
8 (tank 1)	*Ostrea edulis*	30	*Ostrea edulis*	30	4 mo
8 (tank 2)	*Mytilus edulis*	30	*Mytilus galloprovincialis*	30	4 mo
8 (tank 2)	*Mytilus edulis*	30	*Mytilus edulis*	30	4 mo
8 (tank 2)	*Mytilus edulis*	30	*Ostrea edulis*	30	4 mo

M. refringens (16/30 were found to be infected when digestive gland smears were examined in sampled oysters from the same batch) for 1 mo. After 1 mo the presence of the parasite was assessed in both groups as described above.

Trial 8. Two experimental tanks were used in this experiment. In tank 1, 30 infected flat oysters (15/30 were found to be infected when digestive gland smears were examined in sampled oysters from the same batch) were held with 30 *Marteilia*-free oysters, 30 *Marteilia*-free mussels *Mytilus edulis*, and 30 *Marteilia*-free mussels *Mytilus galloprovincialis*. Tank 2 contained 30 infected *M. edulis* (8/30 were infected when digestive gland smears were examined in sampled mussels from the same batch) together with 30 *Marteilia*-free *Ostrea edulis*, 30 *Marteilia*-free *M. edulis*, and 30 *Marteilia*-free *M. galloprovincialis*. After 4 mo the presence of the parasite was assessed in all molluscs as described above.

Presence of parasite infective stages in the environment. The experiments performed to search for infective stages of the parasite in the environment are summarized in Table 3. These experiments were performed in claires, which are coastal ponds with a natural bottom which fill with sea water during high tide periods. They are traditionally used as shellfish stocking or culture ponds.

Trial 9. One hundred and ten *Marteilia*-free flat oysters were placed in a claire pond, located on Oléron island (Charente maritime, France, an enzootic area for *Marteilia*), with naturally infected flat oysters (2/50 were infected when digestive gland smears were examined in sampled oysters from the same batch). *Marteilia* infection was assessed after 4 mo exposure by examination of tissue smears and histological sections.

Trial 10. Three hundred *Marteilia*-free flat oysters were placed in a claire pond (different to the one used in Trial 9), located on Oléron island. *M. refringens* infection was assessed after 4 mo exposure by examination of tissue smears and histological sections of 30 specimens.

Trial 11. Ninety *Marteilia*-free flat oysters were placed in experimental tanks. Thirty were used as negative controls and 60 (2 batches of 30) were placed in a tank which contained unsieved mud from the bottom of the claire from Trial 9. *M. refringens* infection was assessed after 1 and 1.5 mo exposure by examination of tissue smears and histological sections.

***Marteilia refringens* population dynamics in naturally infected oysters under experimental conditions.**

Trial 12. Seven hundred naturally infected flat oysters (19/30 were infected when digestive gland smears were examined in sampled oysters from the same batch) were placed in an experimental tank for 4 mo during spring. The water temperature naturally increased from 10 to 24°C during the experiment. Oysters were sampled every 15 d (n = 30) and histological sections were prepared as described above.

Table 3. Experiments performed to infer presence of infective stages of *Marteilia refringens* in the environment

Trial	Source of the parasite	Target species	n	Duration
9	Infected *Ostrea edulis* in a claire[a]	*Ostrea edulis*	110	4 mo
10	Claire pond	*Ostrea edulis*	300	4 mo
11	Claire pond sediment	*Ostrea edulis*	60	1.5 mo

[a]Coastal ponds (see 'Materials and methods')

Trial 13. One hundred and seventy flat oysters, naturally infected with *Marteilia refringens*, were placed in an experimental tank. The initial level of *M. refringens* was 2/30 positive when digestive gland smears were examined in sampled oysters from the same batch. Several authors (Grizel & Tigé 1977, Balouet et al. 1979a, Grizel 1985) stated that the temperature regime fosters the development of infection in nature. To mimic natural temperature changes between summer and winter, water temperature was maintained at 20°C for about 1 mo, then decreased to 15°C for 1 mo and increased again to 20°C. Following this, oysters were sampled for *M. refringens* detection by means of digestive gland smears, every 2 wk for 10 wk.

Statistical methods of analysis. The confidence intervals of the frequencies of infection were calculated when Npq was <20 (where N is the sample size, p the estimated frequency and $q = 1 - p$) using the binomial distribution table (at the level $\alpha = 5\%$) (Lazar & Schwartz 1987). When Npq was >20, the Normal approximation was used at the $\alpha = 5\%$ level.

To test the evenness of the distribution, a non-parametric test was used with the calculation of Spearman's rank correlation coefficient, because the sample size was too small to test the hypothesis for a classical regression (Schwartz 1984, Haccou & Meelis 1994). Two frequencies that appeared different on each graph were chosen and tested for significant difference with a normal approximation, or with comparison of the confidence intervals at the $\alpha = 1\%$ or $\alpha = 5\%$ level. These tests could not be repeated for the same trial because of the contingency of tests between them (Laplanche et al. 1993).

RESULTS

Inoculation experiments

Purification of the parasite. The modified purification protocol yielded large quantities of *Marteilia re-*

fringens cells from naturally infected oysters. Parasite cell numbers obtained by purification varied from 8 to 16×10^6 sporangia, and from 10 to 60×10^6 sporangia primordia per gram of digestive gland.

Inoculation experiments. *Marteilia refringens* transmission was not detected in any of the inoculation experiments (Trials 1 to 4), regardless of size of inoculum, developmental stage of parasite cells inoculated, or mode of inoculation (Table 4).

Infection by cohabitation

The results of the different cohabitation experiments conducted in experimental conditions (Trials 5, 6, and 7) were negative. *Marteilia* spp. cells were not detected by histological section examinations of the target species *Mytilus edulis* (Trial 5), *Ostrea edulis* (Trials 6 and 7), after 1 and 6 mo of cohabitation. During Trial 8, the target species had detectable infections at the end of the exposure, but the same was true of controls (Table 4).

Presence of parasite infective stages in the environment

During Trial 9 (cohabitation of infected and non-infected oysters in a claire), 50% of the oysters had died by the end of the experiment. At the end of the experiment, there was a 93% *Marteilia refringens* infection level among surviving oysters which were originally *Marteilia*-free.

In Trial 10 (non-infected oysters placed in a claire), 47% of sampled oysters were positive for *Marteilia refringens* infection by histological examination after 4 mo.

In Trial 11 (use of claire bottom mud as a source of infection in experimental tank), no *Marteilia refringens* infection was detected (see Table 4) after 1 and 1.5 mo exposure.

Marteilia refringens population dynamics in naturally infected oysters under experimental conditions

In Trial 12 (Table 5), the prevalence of *Marteilia refringens* decreased from 66% ($N = 30$) at the beginning of the experiment to 10% ($N = 30$) at the end (Fig. 1). Spearman's rank correlation coefficient (between time and prevalence) was $r_S = 0.973$. The hypothesis of stochastic independence was thus rejected at the $\alpha = 1\%$ level and the distribution was significantly decreasing. Sporangial stages of the parasite were detected in 53% of the oysters at the beginning of Trial 12, and decreased to 3% at the end of the experiment. The 2 wk mortality rate during the experiment increased from 7% to 23.8%, from April to the end of June, and decreased to 2% in August (Fig. 2). The Spearman's rank correlation coefficient was $r_S \cong 0$; the hypothesis of stochastic independence was not rejected at $\alpha = 5\%$. However the distribution does not seem to be even. Between 10 June (6.7% mortality) and 25 June (23.8% mortality) the death frequencies are significantly different ($\alpha = 1\%$).

During Trial 13, the prevalence of *Marteilia refringens* increased from 3% ($N = 30$) to 78% ($N = 20$) of infected molluscs (Table 6), which was a significant difference ($\alpha = 1\%$). Calculation of the confidence intervals using the binomial distribution exhibited that the intervals differed significantly at $\alpha = 5\%$.

Table 4. Results of trials of experimental transmission of *Marteilia* spp.

Trial	Source/route of contamination	Target species	Trial duration	Result +/–	n
1	Forced ingestion	*Ostrea edulis*	1 mo	–	0/25
2	Pallial inoculation	*Ostrea edulis*	1 mo	–	0/7
3	Pallial inoculation	*Ostrea edulis*	1 mo	–	0/12
4	Pallial inoculation	*Ostrea edulis*	Up to 1 mo	–	0/18
5	Cohabitation	*Mytilus edulis*	6 mo	–	0/50
6	Cohabitation	*Ostrea edulis*	6 mo	–	0/48
7	Cohabitation	*Ostrea edulis*	1 mo	–	0/30
8	Cohabitation	*Mytilus galloprovincialis*	4 mo	+[a]	1/57
8	Cohabitation	*Mytilus edulis*	4 mo	–	0/57
8	Cohabitation	*Ostrea edulis*	4 mo	+[a]	10/60
9	Field cohabitation	*Ostrea edulis*	4 mo	+	28/30
10	Field exposure	*Ostrea edulis*	4 mo	+	14/30
11	Sediment exposure	*Ostrea edulis*	1.5 mo	–	0/60

[a] In these trials, negative controls were also found positive at the end of the experiment

Table 5. Results of the study of *Marteilia refringens* population dynamics in naturally infected flat oysters *Ostrea edulis* under experimental conditions (Trial 12)

	Apr 29[a]	May 13	May 28	Jun 10	Jun 25	Jul 8	Jul 21	Aug 5
Infection level (n = 30)	66	56	60	53	36	20	20	10
Sporangia primordia (n = 30)	13	6	6	6	0	0	3	6
Sporangia (n = 30)	53	50	53	46	36	20	16	3
Water temperature (°C)[b]	10	11	11	17	22	21	22	23
Mortality rate (%)[c]	nd	7	6.3	6.7	23.8	9	7.3	2

[a]Time zero; [b]Average water temperature per sampling interval; [c]Mortality per sampling interval; nd: not done

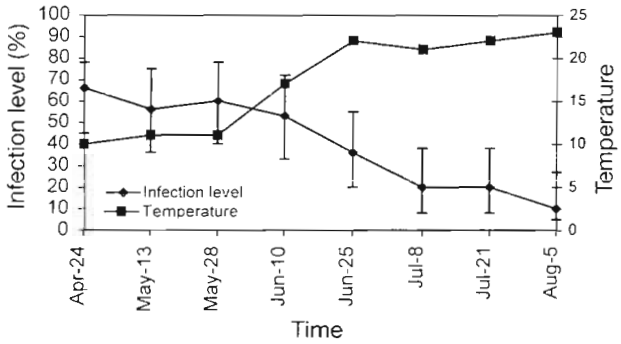

Fig. 1. *Ostrea edulis*. Percentage of *Marteilia refringens* infected oysters during Trial 12, with reference to water temperature. Calculation of the confidence intervals of the frequencies was made at $\alpha = 5\%$

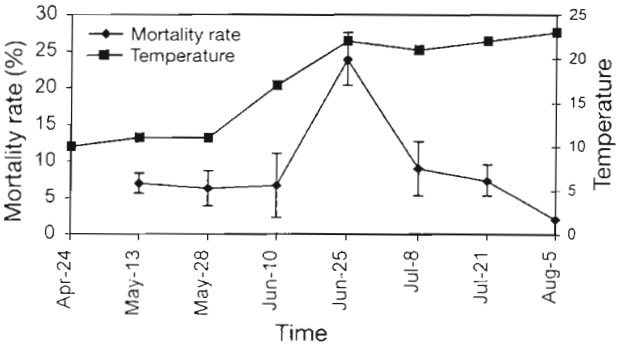

Fig. 2. *Ostrea edulis*. Mortality rate among *Marteilia refringens* infected oysters during Trial 12, with reference to water temperature. Calculation of the confidence intervals of the frequencies was made at $\alpha = 5\%$

DISCUSSION

Ever since its first description, the paramyxean parasite *Marteilia refringens* (Grizel et al. 1974), aetiological agent of Aber disease, has been recognised as one of the most important pathogens of the European flat oyster *Ostrea edulis*. Numerous studies on the subject led to the detection of *Marteilia* spp. in various hosts and locations. The transmission route of *Marteilia* spp., and consequently their life cycle, remain unknown. However, these questions are of central importance in developing effective control of these pathogens. Although oysters resistant to *Bonamia ostreae* exist (Martin et al. 1993), they are not resistant to *M. refringens* infection. Because traditional flat oyster areas are infected by *M. refringens*, this parasite will be a major obstacle to the re-development of flat oyster culture, even if based on *Bonamia ostreae*-resistant oysters. *Marteilia* spp. also reduce mussel yield in Galicia, one of the most productive mussel areas of the world. More recently, an extended range of *Marteilia sydneyi*, causative agent of marteiliosis in *Saccostrea commercialis*, was reported in Australia (Adlard & Ernst 1995).

The existence of an indirect life cycle was postulated by many authors, because transmission of *Marteilia* spp. using various methods has been unsuccessful. These methods were cohabitation of oysters, injection and feeding of spore suspensions (Balouet et al. 1979a, Grizel 1985), transplantation of *M. sydneyi*-infected digestive gland to *Saccostrea commercialis* (Lester 1986), and feeding of *M. refringens* spores to potential intermediate hosts (Van Banning 1979). A different approach was developed by Balouet et al. (1979b), who

Table 6. Results of the study of *Marteilia refringens* population dynamics in naturally infected flat oysters *Ostrea edulis* under experimental conditions (Trial 13)

	Aug 3[a]	Aug 16	Dec 18	Jan 16	Jan 30	Feb 14	Feb 27
Infection level	3 (n = 30)	3 (n = 30)	13.3 (n = 30)	27.6 (n = 20)	40 (n = 20)	20 (n = 20)	78 (n = 20)
Water temperature (°C)[b]	20	15	20	20	20	20	20

[a]Time zero; [b]Average water temperature per sampling interval

looked for possible infective stages in oysters collected in *M. refringens* enzootic areas. Indeed, experimental trials conducted by these authors discarded the horizontal transmission hypothesis in favor of the existence of an intermediate host. Grizel (1985) also suggested that spores may need a period of maturation in sediments prior to becoming infective. However, most of these papers are poorly detailed concerning the description of methods used to attempt transmission of *Marteilia* spp. under experimental conditions. For example, some of the injected parasites were cultured in thioglycolate medium prior to injection (Balouet et al. 1979a), perhaps resulting in loss of virulence. Also, some of these trials were performed during winter, which could inhibit *Marteilia* transmission. Furthermore, Comps & Joly (1980) claimed transmission of *M. refringens* by cohabitation. This result casts some doubt upon the adequacy of the life cycle hypotheses under consideration here.

In this respect, the results of Trial 13 are of particular interest. The percentage of oysters infected with *Marteilia refringens* increased from 3% to 78% during the course of this experiment (Table 6). Results of Trial 13 suggest the following hypotheses: (1) During this period, *M. refringens* may have been transmitted by cohabitation of infected and non-infected oysters, or (2) *M. refringens* cells previously present but undetected by means of digestive gland imprints may have become detectable. This last hypothesis is in accordance with observations made by Comps (1979), in a population of infected *Ostrea edulis* transferred from Brittany to the Mediterranean Sea. Similar observations led Balouet et al. (1979a) to conclude that young plasmodia of *M. refringens* are a permanent infective form of the parasite within the oysters. Balouet (1979) also suggested that sporangia primordia corresponded to a chronic infection all year long, while sporangia corresponded to seasonal stages, probably responsible for the spread of the disease. *M. refringens* infection usually begins in May, peaks from June to August, and decreases in December. In winter and early spring, *M. refringens* is usually absent or found in small numbers in the host. Apparently, the seasonal *Marteilia* spp. life cycle is partially ruled by temperature (Grizel & Tigé 1977, Balouet et al. 1979a, Grizel 1985). The water temperature increase during spring could foster the development of overwintering sporangia primordia. This could explain the results of Trial 13 (Table 6). However, we hypothesized that horizontal transmission of the parasite by cohabitation could have occurred during this experiment. This would be in accordance with the observations made by Comps & Joly (1980), during an experimental cohabitation of infected *O. edulis* and presumed *Marteilia*-free *Mytilus galloprovincialis*.

To test this possibility, cohabitation experiments were performed (Trials 5, 6, 7, and 8). From the examination of histological sections, we concluded that, in fact, none of the target species used in these experiments, *Mytilus edulis*, *Ostrea edulis*, or *M. galloprovincialis*, became infected with *Marteilia* spp. after 1 and 6 mo of cohabitation. These results are similar to those previously reported (Balouet et al. 1979a, Grizel 1985).

Grizel & Tigé (1977) discussed the results of a series of experiments in which oysters were transferred from *Marteilia*-free areas to infected ones, and vice versa. An extremely clear account of this work, conducted from 1977 to 1979, is given by Grizel (1985). These experiments led to the conclusion that infection by *M. refringens* occurs within 30 d maximum, during summer. Grizel (1985) reported the rapid establishment of *M. refringens* infection in field conditions. In these experiments, the detection of *M. refringens* in the target species was possible within 1 mo. Although the duration of Trial 7 may have been too short to allow the detection of *M. refringens* in the target species, Trials 5, 6 and 8, which lasted for 4 and 6 mo, also resulted in negative results.

In Trial 8, *Marteilia refringens* was detected in flat oysters (10/60). In our opinion, as the parasite also appeared in the negative controls (12/30), this result should be regarded as undetected, previous natural infection, rather than an actual transmission. Flat oysters originating from the same area, hitherto presumed a *Marteilia*-free area, were later found to be infected by *M. refringens* (Y. Pichot pers. comm.). Similarly, much of the explanation for the infection in the mussels *Mytilus galloprovincialis* (1/57) rests with previous infection. This points out the need for sensitive diagnostic methods that will allow the detection of very low *Marteilia* spp. infection levels. In the present case, the method used to diagnose *Marteilia* spp. in experimental transmission trials may not have been sensitive enough to detect low levels or early stages of the parasite infection. However, it should be mentioned that sample size, averaging around 30 in this study, could be too small to detect very low *Marteilia* spp. prevalences. Because of the lack of negative controls and the small sample size in their experiment, the positive result obtained by Comps & Joly (1980) could also be explained as a previous infection in the light of our results.

The results of the experimental transmission trials performed by inoculation (Trials 1, 2, 3, and 4) are similar to those cited by Balouet et al. (1979a) and Grizel (1985). In view of the fact that conditions were conducive for *Marteilia refringens* transmission (temperature, duration, period of the year), we should consider that, in these trials, the injected stages of *M. refringens* were not the infective stage of the parasite. This is

probably true for sporangia primordia [although Balouet (1979) considered sporangia as responsible for the spread of the parasite]. Studying *Marteilia sydneyi*, Roubal et al. (1989) showed that sporangia passed into the environment via the alimentary tract. Similar observations were made by Grizel et al. (1974) and Grizel (1975, 1985) on *M. refringens*. Sporangia are found in the lumen of digestive gland tubules and posterior intestine, and in sediment. Wolf (1979) and Franc (1980) suggested that the sporangia of *M. sydneyi* and *M. refringens* ruptured and spores were released into the lumen and surrounding tissue. These considerations could explain the failure of Trials 1 and 2 (inoculation of purified *M. refringens* cells), as maturation of *Marteilia* forms could occur during their release through the digestive tract. However in Trial 3 (inoculation of a digestive gland homogenate obtained from 2 highly infected oysters), the very last developmental stages of *M. refringens* in the oyster were inoculated, leading to the same negative results. In Trial 4, neither sporangia nor sporangia primordia of *M. refringens* established detectable infections. Roubal et al. (1989) hypothesized that *M. sydneyi* sporangia, which pass into the environment, infect filter- or bottom-feeding animals. These latter are probably not oysters themselves. The authors failed to transmit *M. sydneyi* using fish as a potential vector.

In Trial 9 (cohabitation experiment in a claire) and Trial 10 (storage in a claire), it was possible to infect *Marteilia*-free flat oysters. Similar results were obtained by Tigé & Rabouin (1976), Grizel & Tigé (1977), Balouet et al. (1979a).

In Trial 11, the sediment of a claire was used as a source of contamination for oysters. After 6 wk of exposure, the results remained negative. A possible criticism is that the duration of this experiment may have been too short to allow infection or spore maturation. Although Grizel (1985) reported the establishment of *Marteilia refringens* infection within 1 mo, this author also suggested that spores may need to mature in sediments prior to becoming infective.

There are major discrepancies among field and experimental observations of *Marteilia* spp. infections. Possible physiological differences in oysters held in the 2 types of environment should be considered. Indeed, transmission under artificial laboratory conditions could fail because either the environment or the oysters are simply not providing the right stimuli. The local environment may play a role in the virulence of the pathogen (Figueras & Montes 1988). *Marteilia* spp. may be very fastidious parasites requiring very specific conditions to infect, which may not have been met in the course of our experiments.

In Trial 12, the prevalence of *Marteilia refringens* in the studied population decreased from 66% at the beginning of the experiment to 10% at the end of the experiment (Table 5). This was mainly due to the disappearance of sporangial stages of *M. refringens* during the experiment: the sporangial stages of the parasite were detected in 53% of the oysters at the beginning of the trial, and decreased to 3% at the end of the experiment (Fig. 1). Similar results were obtained by His et al. (1976), probably due to both death of infected oysters and loss of *M. refringens* sporangia through release by infected oysters. The 2 wk mortality rate during the experiment increased from 7% to 23.8%, from April to the end of June, and decreased to 2% in August. In parallel, water temperature increased from 10°C to 23°C (Fig. 2). Massive release of *M. sydneyi* sporangia associated with oyster mortality was described by Roubal et al. (1989). In our experiment, we infer that *M. refringens* was released in high numbers by the oysters and that this release may have been associated with an increase in temperature. Based on field observations, Grizel & Tigé (1977) and Balouet et al. (1979a) described a decrease in *M. refringens* prevalence as occurring in December. This was not the case in this experiment as prevalence decreased during July (Fig. 1). If *M. refringens* sporangia released by oysters were the infective stages, the prevalence of *M. refringens* infection would have been expected to increase or at least remain constant until the termination of the trial. Comparing results from laboratory and field studies, we can conclude that under laboratory conditions the disease is not maintained as long as in the field. This is probably due to the lack of new infections which, in the field, are established from May to August. The lack of infective stages of *M. refringens* for oysters maintained under laboratory conditions should then be considered.

The evidence from these inoculation and cohabitation experiments, does not support a direct life cycle in *Marteilia refringens*. Because the natural environment appears to be necessary to achieve contamination of oysters by *M. refringens*, results support the hypothesis of an intermediate or alternative host, or free-living stages of *M. refringens*. Roubal et al. (1989) considered that there was no evidence that the spores may survive for prolonged periods within the sediment. Consequently, the existence of infective areas, as shown in Trial 10, would strongly suggest that an intermediate host is essential in the life cycle of *M. refringens*.

However, *sapiens nihil affirmat quod non probet* (a wise man never insists on what has not been proved). Clearly, more work needs to be done. This will be conducted using field experiments on oysters in a claire. Subsequently, recent advances in the use of molecular probes and related assays could provide powerful tools in the search for potential intermediate hosts although this remains a difficult task. An interesting result of our

work consists in the ability to make naturally infected oysters release *M. refringens* cells. This result could be used as a laboratory model to study the survival rate and development of released *M. refringens* sporangia. Current research is directed towards developing such approaches.

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