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Infection cycle of *Marteilia pararefringens* in blue mussels *Mytilus edulis* in a heliothermic marine oyster lagoon in Norway

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ABSTRACT: Agapollen is a traditional heliothermic marine oyster lagoon in western Norway, representing the northernmost site of any *Marteilia* sp. protists detected in Europe. The semi-closed lagoon is a unique site to study the life cycle and development of *M. pararefringens* in naïve mussels. Two baskets with uninfected mussels were deployed in the lagoon outlet in May and October 2018, respectively, and sampled every 6 wk. The parasite was first detected in the mussels by PCR in early July and by histology in late August. By then, *M. pararefringens* had developed into mature stages, indicating a rapid development during mid-summer. Sporulation occurred during autumn. Mussels deployed in October never became infected, indicating that transmission was restricted to the warmest period of the year. Pronounced pathology was observed in infected mussels, including degenerated digestive tubules and infiltration of haemocytes. Mussel mortality was observed in the baskets, but whether this was due to infections of *M. pararefringens* or other environmental factors could not be determined. Plankton samples from the lagoon were also collected for PCR analysis. These samples, dominated by copepods, were positive for *M. pararefringens* in summer. In sorted samples, *M. pararefringens* was detected in the *Acartia* spp. and *Paracartia grani* fractions between July and October. These plankton copepods are therefore potentially involved in the life cycle of *M. pararefringens*.

KEY WORDS: *Marteilia pararefringens* · *M. refringens* M-type · *Marteilia maurini* · *Mytilus edulis* · Pathogen · Life cycle · Parasite · Development · Histopathology · Norway

1. INTRODUCTION

Marteilia spp. are protists belonging to the Ascetosporea, a class of unicellular eukaryotes that exclusively parasitize invertebrates (Bass et al. 2019). *Marteilia* spp. infect several species of bivalve molluscs (reviewed by Carrasco et al. 2015, Ward et al. 2016), and pathology associated with heavy infections (marteiliosis) may lead to high mortality in reared molluscs world-wide (Berthe et al. 2004). The first described species was *Marteilia refringens* in flat oysters *Ostrea edulis* in France (Grizel et al.

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1974), causing significant mortalities. Due to the severity of the disease, *M. refringens* was included in the World Organisation for Animal Health (OIE) list of notifiable diseases (https://www.oie.int/en/what-we-do/standards/codes-and-manuals/aquatic-code-online-access/?id=169&L=1&htmfile=chapitre_marteilia_refringens.htm) as well as the EC directive 2006/88 (EC 2006). In the 1970s, *Marteilia* sp. was also detected in European blue mussels *Mytilus edulis* (Comps et al. 1975) and later in *Mytilus galloprovincialis* (Comps et al. 1982). The *Marteilia* species infecting mussels was described as a separate species, *Marteilia maurini*, based on ultrastructural characteristics and host specificity (Comps et al. 1982). However, these criteria were challenged by Longshaw et al. (2001), who found no significant difference in ultrastructural characteristics between the 2 species, and López-Flores et al. (2004) and Novoa et al. (2005), who found both parasite species in both hosts. Based on the lack of evidence to keep them separated, *M. refringens* and *M. maurini* were officially synonymized under *M. refringens* in 2007, referred to as 'O-type' in oysters and 'M-type' in mussels, respectively (Le Roux et al. 2001, Balseiro et al. 2007).

A phylogenetic study of *Marteilia* spp. infecting *Mytilus* spp. and *O. edulis* in Norway, Sweden, and the UK found strong evidence for biologically distinct genotypes and proposed that *M. refringens* O-type and *M. refringens* M-type (Le Roux et al. 2001) were 2 separate species. The latter was renamed *Marteilia pararefringens* (Kerr et al. 2018), the former is referred to as *M. refringens* sensu stricto. Earlier records that could refer to either, are referred to as *M. refringens* sensu lato. There is insufficient evidence and extant material to test whether *M. maurini*, as described by Comps et al. (1982), is the same lineage as *M. pararefringens* (Kerr et al. 2018), and M-type (=*M. pararefringens*) is not exclusively a parasite of mussels, on which the original description of *M. maurini* relied to distinguish it from *M. refringens*. Therefore, the decision was made by Kerr et al. (2018) to erect a new species, *M. pararefringens*, to avoid these ambiguities, and the confusing (and possibly incorrect) scenario of 'resurrecting' the name *M. maurini* for the 'M-type'.

M. refringens sensu stricto has not yet been found in any northern European host or environmental sample, while *M. pararefringens* has been frequently detected infecting mussels (only) and in environmental samples in the UK, Norway, and Sweden. Hence, the distribution ranges of *M. refringens* and *M. pararefringens* also appear to differ: while co-occurring in more southerly European regions, only *M. pararefringens* is present in northern regions, or at least *M. refringens* is so far undetectably rare in the north.

Studies on the life cycle of *M. refringens* and *M. pararefringens* indicate the presence of an intermediate host. The complete life cycle is still unknown, although several copepod species have been identified as potential host species (Audemard et al. 2002, Carrasco et al. 2007a, Carrasco et al. 2008, Boyer et al. 2013, Arzul et al. 2014).

In Scandinavia, *Marteilia* sp. was detected for the first time in 2009, when the disease surveillance programme in Sweden included blue mussels aiming to obtain disease-free status for bonamiosis and marteiliosis (EURL 2010). During the first year, 30 blue mussels were collected from each of 5 sites along the Swedish west coast. In Stigfjorden, in the archipelago south of the island Orust, 5 out of 30 mussels collected in September were infected with *Marteilia* sp., verified as *M. refringens* M-type by the European Union Reference Laboratory (EURL). In Norway, Aarab et al. (2011) reported *Marteilia* sp. in histological samples of *M. edulis* used for environmental monitoring, collected from Tysværvågen, Rogaland, but this finding was not notified and went unnoticed until *Marteilia* sp. was detected in *M. edulis* collected in Agapollen, a traditional 'oyster poll' (a heliothermic marine lagoon) at Bømlo, western Norway (Fig. 1). This discovery (in October 2016) occurred during a surveillance programme for *Marteilia* spp. and *Bonamia* sp. in Norwegian blue mussels and flat oysters. The prevalence of *Marteilia* infection in mussels was 50%, with most affected animals exhibiting heavy infections with sporulating *Marteilia* cells in the digestive epithelia (Mortensen et al. 2017). PCR and sequencing identified the parasite as *M. refringens* M-type (Le Roux et al. 2001). The M-type diagnosis was verified by EURL, and OIE and EU were notified.

Agapollen represents the northernmost site of any *Marteilia* sp. detected in Europe to date. The detection of *M. pararefringens* with high prevalence in the semi-closed, heliothermic oyster lagoon represents a unique opportunity to study the life cycle and parasite progression of *M. pararefringens* in blue mussels, the results of which are reported here. The objectives of this paper were to (1) determine periods within which *M. pararefringens* is transmitted to mussels, (2) describe the progression of *M. pararefringens* infection in mussels, and (3) examine the occurrence of *M. pararefringens* in plankton samples from Agapollen.

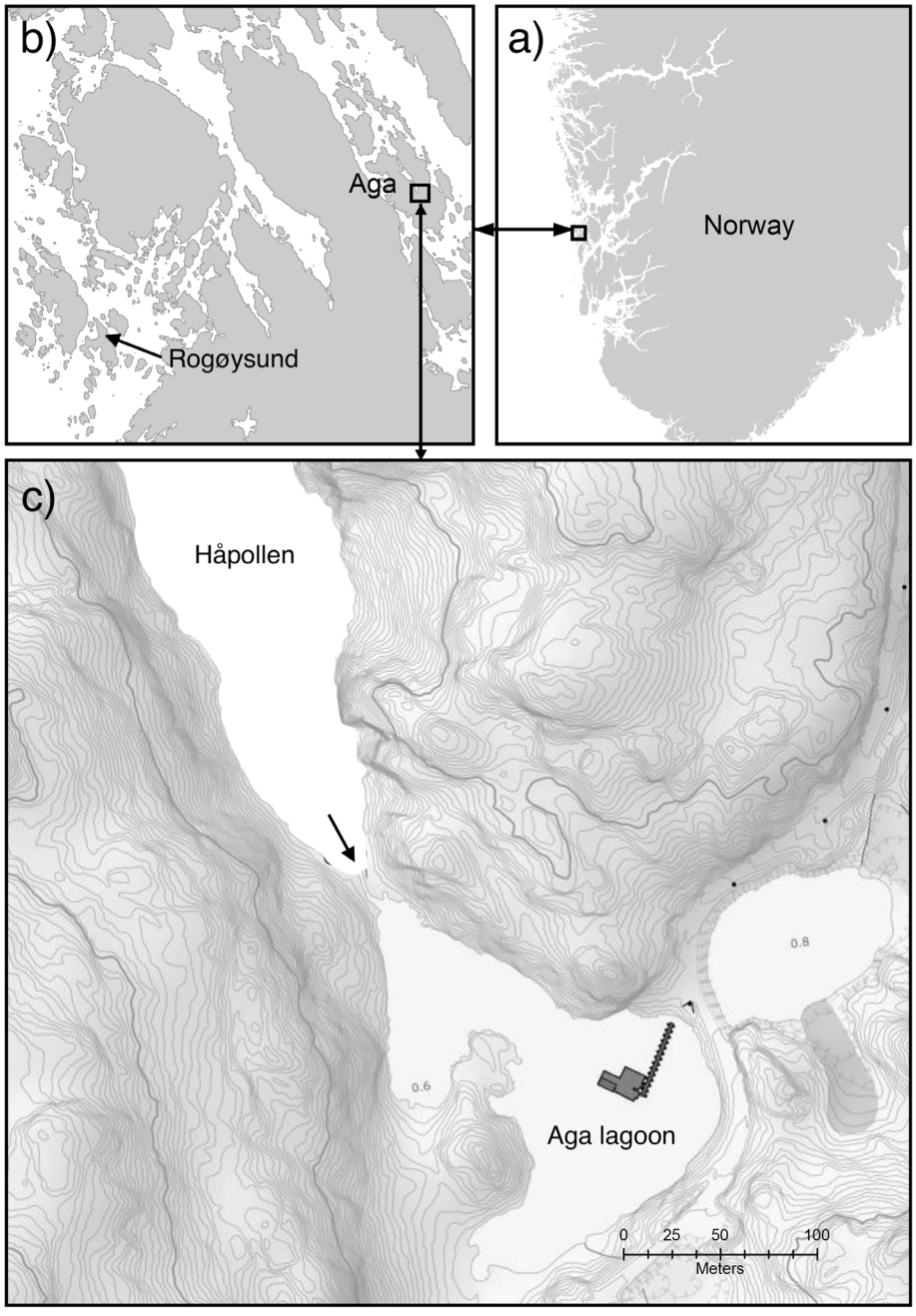

Fig. 1. (a) Western Norway. (b) Part of the island of Bømlo with Rogøysund (origin of the healthy mussels) and Aga (where mussels are infected with *Marteilia pararefringens*). (c) Heliothermic oyster lagoon (poll) at Aga. Healthy mussels in oyster baskets were deployed in the channel (arrow) connecting the Aga lagoon to the outside Håpollen

2. MATERIALS AND METHODS

2.1. Site

Agapollen is in Bømlo municipality, Hordaland, western Norway (59° 50.4′ N, 5° 14.8′ E) (Fig. 1). The site is a heliothermic marine lagoon (oyster poll), previously used to produce flat oyster spat. It contains 2 conduits for water transmission: a connection to a freshwater pond and a channel where saltwater circulates with the tide. The channel is connected to Håpollen, which leads to open waters in Bømlafjorden. The lagoon is around 150 × 200 m wide, with a maximum depth of 6 m, and has soft sediments. When the lagoon was used to produce oyster spat, the water flow through the channel was controlled

with a stem-gate. Oyster farming ceased around 2010, and the channel is now open, allowing a tidal current.

To determine the onset and progression of the infection, 2 deployments of non-infected blue mussels were performed in Agapollen in 2018/2019. The mussels (2–3 yr old; mean length ± SD = 58 ± 12 mm) were collected at a nearby bivalve farm at Rogøysund: 280 in May 2018 for the first deployment and 280 in October 2018 for the second deployment. Before placement, 30 mussels were PCR screened for each group to confirm that they were negative for *Marteilia* spp. Mussels were put in SEAPA Ltd 15 l/20 mm mesh oyster baskets and placed at the sampling site in the subtidal zone. The mussels were equally divided (roughly 125 mussels) in 2 oyster baskets per deployment. The secondary, parallel study in October was used to determine whether transmission of the parasite could occur during sporulation in autumn. October was chosen because data from 2017 showed sporulation stages in this period (Mortensen et al. 2018).

2.2. Sampling protocol

2.2.1. Mussels

Samples were taken from the oyster baskets at Aga every 6 wk from 28 May 2018 to 8 May 2019 (Table 1). A sample size of 30 mussels (per deployment) of no specific size, 15 from each basket, was chosen based on the sampling protocol used by the Institute of Marine Research (IMR) in the surveillance programme of bivalve diseases. Due to the conservative number of mussels used, exactly 30 mussels were gathered during each sampling. However, as some mussels happened to be empty when opened for dissection in the laboratory, final sampling numbers varied (29 in August and October 2018). Only 23 mussels were sampled in November 2018 because the remaining mussels were deceased. A total of 321 mussels were sampled for the field study: 141 mussels from the first deployment that lasted from 28 May to 19 November 2018 and 180 mussels from the second deployment that lasted from 9 October 2018 to 8 May 2019.

Table 1. Overview of sampling dates and sample size for the mussels in deployments 1 and 2, as well as all plankton sampling, from 28 May 2018 until 7 August 2019. Due to mortality, final sampling from the first deployment was in November 2018. Final sampling from the second deployment was in May 2019. Notes specify which samples were used in sequencing and restriction fragment length polymorphism (RFLP) analysis. No mussels: no mussels available for sampling; no plankton: no plankton in water column. Dates are given as d.mo.yr; NA: not applicable

Date	Mussel deployment 1				Mussel deployment 2				Plankton	
	Sample size	Prevalence (%) PCR	Prevalence (%) Histology	Note	Sample size	Prevalence (%) PCR	Prevalence (%) Histology	Note	PCR	Note
28.05.18	30	0	0	Null sample	NA			Mussels not deployed	NA	No plankton
04.07.18	30	27	0	Mussel 8 and 15 sequenced	NA			Mussels not deployed	Positive	
29.08.18	29	69	90	Mussel 6 and 24 sequenced; Mussel 7 used for RFLP analysis	NA			Mussels not deployed	Positive	
08.10.18	29	83	86	Mussel 5 and 25 sequenced; Mussel 15 used for RFLP analysis	30	0	NA	Null sample; no histology sample taken	Positive	
19.11.18	23	87	87	Mussel 19 and 22 sequenced; Mussel 12 used for RFLP analysis	30	0	0		NA	No plankton
03.01.19	NA			No mussels	30	0	0		NA	No plankton
11.02.19	NA			No mussels	30	0	0		NA	No plankton
25.03.19	NA			No mussels	30	0	0		NA	No plankton
08.05.19	NA			No mussels	30	3	0		Negative	
28.05.19	NA			No mussels	NA			No mussels	Positive	Sequenced
19.06.19	NA			No mussels	NA			No mussels	Positive	Sequenced; separated into *Paracartia grani* and *Acartia* spp. — both fractions positive for *M. pararefringens*
10.07.19	NA			No mussels	NA			No mussels	Positive	
07.08.19	NA			No mussels	NA			No mussels	Positive	

The mussels were transported to Bergen and processed at the IMR laboratory, according to standard methodology and quality assurance procedures. All mussels were processed for PCR and histological analysis, except the mussels in the null sample taken before the mussels were set in the baskets for the second deployment, which were only PCR analysed.

2.2.2. Plankton

Sampling of plankton occurred every 6 wk from 28 May 2018 to 7 August 2019 (Table 1). Zooplankton were collected from the water column in the poll using a WP2 plankton net equipped with a 180 µm mesh cup. The collected plankton was sieved through a 1 mm plankton mesh to remove debris and jellyfish, collected on another 180 µm mesh and transferred to 100 % ethanol in tubes. The zooplankton samples collected from June, July, and August 2019 were sorted, separating the copepods *Paracartia grani* and *Acartia* spp. as the fractions of interest. All plankton samples were analysed by PCR as described in Section 2.3.

2.3. Sample processing

2.3.1. PCR

DNA was extracted from fixed digestive tissue of mussels and plankton samples using a QIAamp DNA mini kit (Qiagen), after Proteinase K digestion at 56°C overnight. All DNA was quantified and checked for purity using Nanodrop (Thermo Scientific). PCR was performed using Pr4/Pr5, the internal transcribed spacer-1 (ITS-1) primers of Le Roux et al. (2001). PCR reactions were performed in 50 µl volumes consisting of 1× PCR buffer (Qiagen), 1× Q solution, 25 pmol primer solution, 0.2 mM of each dNTP, 1.25 U HotStarTaq polymerase, and 100 ng DNA template. Amplifications were performed on an Applied Biosystems GeneAmp 9700 thermal cycler using the following program: 95°C for 15 min; followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 minute; followed by 72°C for 10 minutes and held at 4°C.

To identify the *Marteilia* species (*M. refringens* sensu stricto or *M. pararefringens*) present in the positive samples from PCR analysis, restriction fragment length polymorphism (RFLP) analyses were performed on ITS-1 amplicons (Pr4/Pr5 primers) (see Le Roux et al. 2001) from 1 heavily infected mussel per sample period (Table 1). The DNA was extracted from the same sample used for PCR analysis.

In addition, regions of the parasite DNA were sequenced from 2 heavily infected individuals from each sampling period where prevalence was >0 (July, August, October, and November 2018; Table 1) to confirm the *Marteilia* species. Sequencing was not performed on the 1 PCR-positive mussel in May 2019. PCR was performed with the primers Pr1 to Pr5 from Le Roux et al. (2001) that target the ITS-1 region. PCR was also performed with the primers MT-1 and MT-2 from López-Flores et al. (2004) that target the intergenic spacer (IGS) region. PCR was performed as described above, but the annealing temperature was 55°C for all PCRs except Pr4/Pr5. The PCR products were cleaned using Illustra ExoProStar 1-step kit and sequenced using Sanger's method (BigDye v3.1 kit) at the Sequencing laboratory at the University of Bergen (https://seqlab.uib.no).

2.3.2. Histology and categorisation of the stages of infection

Dorso-ventral cross sections of mussel soft tissue were fixed in Davidson's fixative, embedded in paraffin, sectioned at 3 µm, and stained with haematoxylin-eosin-saffron. The infection intensity in each mussel was scored using a modified version of the scoring system described by Villalba et al. (1993) for *M. refringens* sensu lato in *Mytilus galloprovincialis*. Here, we categorised the cells into early, intermediate, and advanced stages. Only entire digestive tubules (in the field of view) and with a visible lumen were counted. This was done by random selection of 10 sites at 400× magnification and counting the number of non-infected and infected digestive tubules for each mussel. To calculate the mean number of parasites and the relative percentage of each stage for each infected mussel, every *Marteilia* cell was counted and categorised into either early, intermediate, or advanced stage for each randomly selected site. Furthermore, each mussel was scored based on the degree of host haemocytic reaction, based on an arbitrary scale ranging from 0 (no infiltration of haemocytes in the digestive gland) to 3 (heavy infiltration of haemocytes in the digestive gland); 1 and 2 were intermediate values (light and moderate, respectively).

2.4. Temperature profile

The oyster lagoons often have an elevated temperature profile compared with the surroundings, due to

a 'greenhouse effect' caused by a top layer of brackish water. To record a profile, temperature in the oyster pool was logged from May to October 2019. Loggers (Onset HOBO Pendant temp/light) were placed at low tide level in the channel (at the mussel deployment site) and at 30 and 200 cm depth in the middle of the lagoon. Temperature was recorded every 12 h.

2.5. Statistical analyses

Results were analysed using Statistica (13.3) software (StatSoft). Temporal changes in infection intensity (not intensity score, i.e. '0' values removed) were examined using Kruskal-Wallis non-parametric ANOVA. Correlations were examined using Spearman rank correlation coefficients (R_S). Double-zeros (i.e. non-infected individuals) were excluded in order to avoid spurious correlations (Ludwig & Reynolds 1988). Fisher's exact test was used when comparing prevalence.

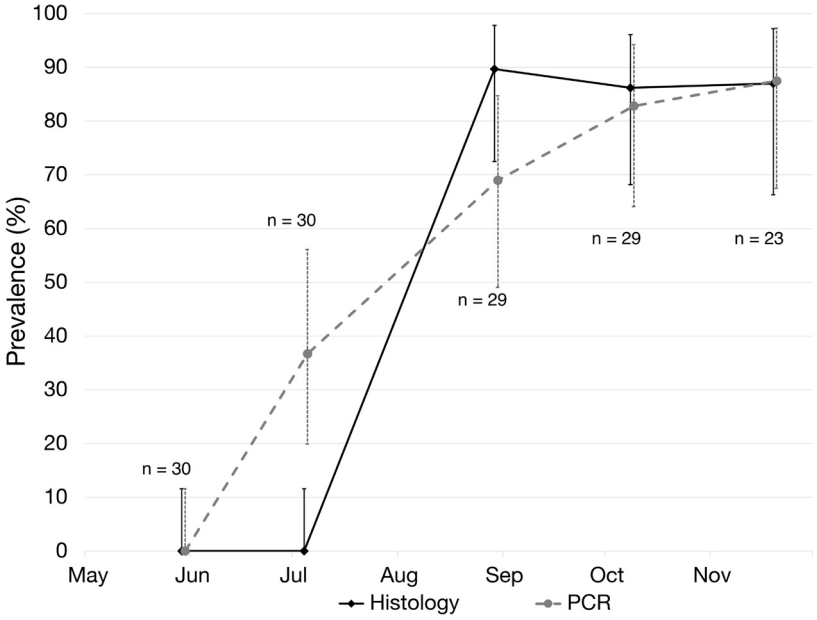

Fig. 2. Prevalence of *Marteilia pararefringens* in the blue mussels deployed in the heliothermic lagoon at Aga. Prevalence was based on molecular analyses (dotted line) and histology (solid line) from the first group of mussels, deployed in May 2018 (molecular prevalence shifted +1 d to avoid error bar overlap). Error bars represent 95% binomial confidence intervals. Data from the second deployment from October 2018 to May 2019 are not included

3. RESULTS

3.1. Infection trial of deployed mussels

3.1.1. Transmission period and prevalence

Marteilia-specific ITS-1 rDNA PCR first detected *Marteilia* sp. in 11/30 (27%) animals tested on 4 July 2018, i.e. 6 wk after deployment of the first group of mussels. The PCR prevalence rose to 20/23 (87%) positive animals by 19 November 2018. Histological evidence of infection did not appear until 29 August 2018, where 26/29 (90%) were positive for the parasite. The histological prevalence remained high until final sampling of the first group on 19 November 2018. PCR-RFLP analyses performed on 1 positive mussel per sampling group confirmed that mussels were infected with *Marteilia pararefringens*. Prevalence using both PCR and histology is shown in Fig. 2 for the first group. In the second group, deployed on 9 October 2018, no sampled animals were positive for the parasite until the final sampling on 8 May 2019 where 1 mussel was PCR positive.

3.1.2. Progression of infection in the host

All infected mussels had varying degrees of parasite development during each sampling period. However, the first sign of infection was the presence of parasites in the stomach epithelium (Fig. 3a). The *Marteilia* cells found in this organ were all early stages and never matured at this site. Early stages were also present in the ducts and digestive tubules. Mussels that were infected in the digestive tubules (infection score 2–5) were not always infected in the stomach or duct epithelia. The prevalence of the parasite in the stomach epithelium or ducts did not significantly change with increasing infection intensity (Fisher's exact test, $n = 59$, $p > 0.05$). The mean number of parasites per infected digestive tubule significantly increased with higher infection intensities ($R_s = 0.85$, $p < 0.001$).

Intermediate stages were found mainly in the digestive tubules (Fig. 3b), but ducts were also infected with this stage in some individuals. This stage had darker secondary cells, with a light boundary surrounding each. The secondary cells seemed to cluster in groups.

Advanced stages, including the sporulation stage, were only found in the digestive tubules (Fig. 3b,c). In heavily infected individuals, however, spores and

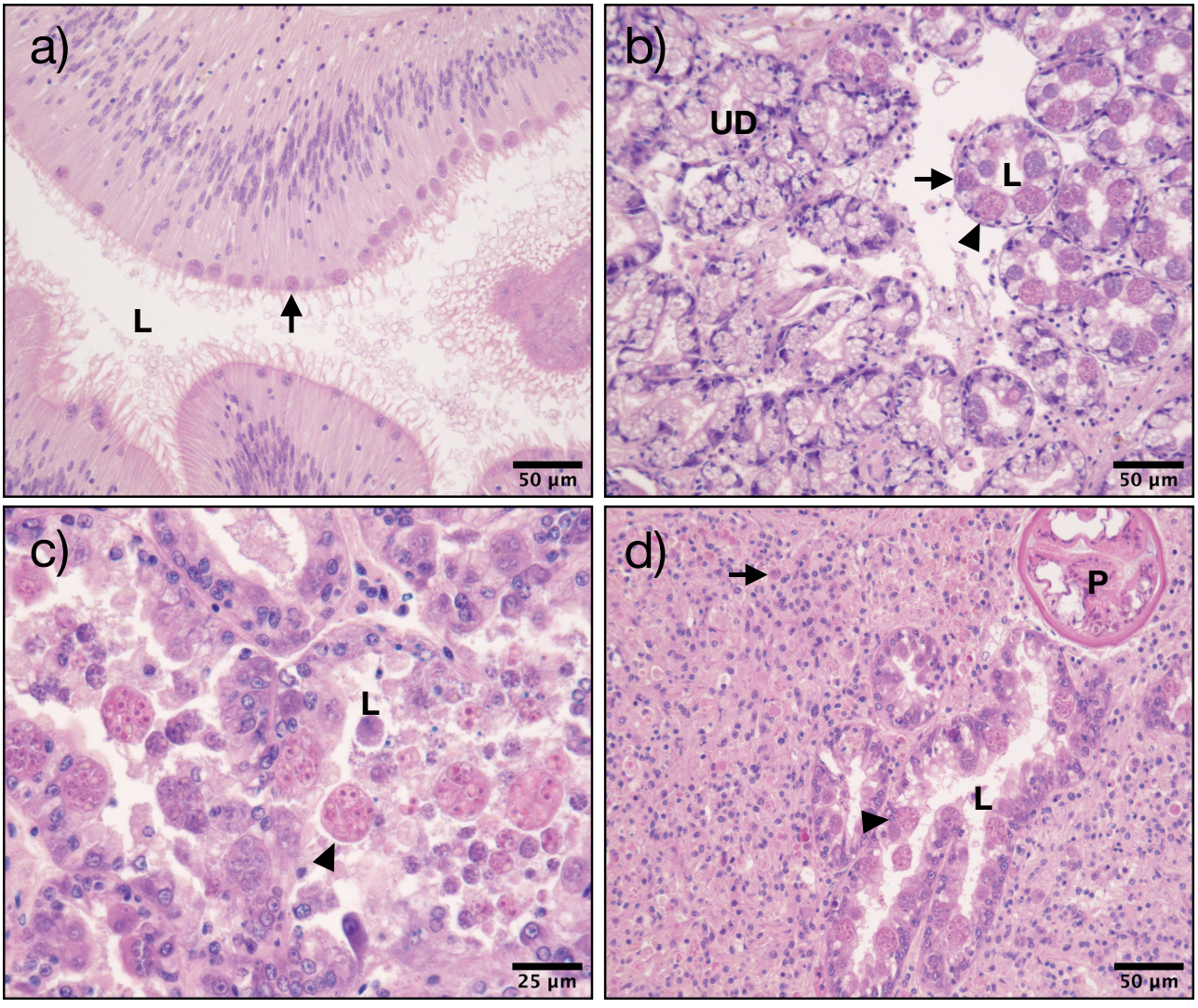

Fig. 3. Histology of blue mussels infected with *Marteilia pararefringens* (haematoxylin-eosin-saffron stained). Lumen indicated by L in all photos. (a) Part of mussel stomach, with ciliated epithelium invaded by early stages (arrow). (b) Area of digestive gland with intermediate stages (arrow) and advanced stages (arrowhead) on the right and uninfected digestive tubules (UD) on the left. (c) Infected digestive tubule where mature sporangia (arrowhead) are liberated into the lumen. (d) Massive haemocytic infiltration of infected tissues in the digestive gland. Some of the digestive tubules had been destroyed, and the space is filled with haemocytes, cell debris, and *Marteilia* spores (arrow). The intact digestive tubules are heavily infected with mature stages (arrowhead). Mussels were often infected with other parasites, such as trematode metacercariae (P)

sporonts enclosing mature spores were present in the lumen of the stomach. In such cases, the stomach epithelium was intact and not disrupted as observed in the digestive tubules. The intestinal epithelium was never infected with the parasite in any sample, nor were there spores or sporonts present in the intestinal lumen.

Early, intermediate, and advanced stages were present for the duration of the positive sampling period between August and November 2018. Early stages were most abundant, remaining above 70% of the total number of *Marteilia* cells observed throughout the sampling periods. The intermediate stages remained at around 20% of the total number of parasites, only decreasing in November 2018. The relative percentage of advanced stages remained low, peaking in October 2018 at 11%.

The mussels showed varying infection intensities, ranging from infections only occurring in the stomach epithelium to heavy infections where more than 90% of the digestive tubule profiles were infected. However, the intensity of infection did not significantly change between sample periods (Kruskal-Wallis, $H_2 = 5.62$, n = 69, p = 0.06).

Histopathological changes increased with infection intensity. Focal necroses in the digestive tubules were seen in light infections, and multi-focal necroses and complete degeneration of digestive tubules in heavy infections (Fig. 3d). The host haemocytic reaction consisted of infiltration in the connective tissues between digestive tubules, ducts, and stomach, and correlated with intensity in all months ($0.60 < R_s < 0.66$, $p < 0.002$).

Out of the 280 mussels in the first deployment, a total of 141 mussels were sampled. The remaining 139 mussels (50%) died of unknown causes between May and November 2018.

3.2. Detection of *M. pararefringens* in plankton

Plankton was sampled every 6 wk from 28 May 2018 to 7 August 2019 (Table 1). There was no plankton available for sampling after 8 October 2018 to 8 May 2019. There was very little plankton in early May 2019, so a second sampling 3 wk later, on 28 May 2019, was done so as not to miss the preliminary algal bloom in the poll. Sampling continued every 6 wk until 7 August 2019.

The dominant zooplankton in the plankton samples were the copepods *Paracartia grani* and *Acartia* spp., based on morphological observations. The detection signal of *M. pararefringens* in plankton samples was higher in July 2018, August 2018, and June 2019 than in October 2018. Plankton samples from May 2018 were not positive for the parasite. Plankton samples from June to August 2019 were sorted into *P. grani* and *Acartia* spp., with both genera showing positive PCR results for the parasite in all months.

3.3. Molecular characteristics of the parasite

The samples (n = 8) used to sequence regions of the parasite DNA were chosen based on infection intensity score and PCR signal quality (thick bands) (Table 1). The obtained ITS-1 sequences (Pr4/Pr5 region; GenBank accession nos. MW250245–6) were identical but showed a single ambiguous site in 2 sequences. These sequences showed 98.7–100% identity to *M. pararefringens* ITS-1 sequences, and 96.8–97.4% identity to *M. refringens* sensu stricto (377–378 positions compared). The partial ITS-1 sequences from zooplankton copepod samples were identical to those from the mussels (390 positions compared). The 8 IGS sequences obtained (MW250247–8) were also identical, with a single ambiguous position in one of them. These sequences showed 99.5–99.8% identity with *M. pararefringens* from *Mytilus edulis* (MH304851–2). The identity with *M. refringens* sensu stricto was 97.3% or less (486 positions compared).

3.4. Temperature profile

Under the brackish top layer, the temperature in the lagoon remained relatively stable throughout the summer season (until August–September) and until prolonged periods of cold weather eliminated the 'greenhouse effect'. The surface layer, as well as the water running through the narrow channel, fluctuated with climatic conditions (Fig. 4).

4. DISCUSSION

The detection of *Marteilia pararefringens* infecting *Mytilus edulis* in the oyster pond at Aga, Bømlo, Norway, has provided a unique opportunity to study this parasite in a semi-closed system, with an experimental

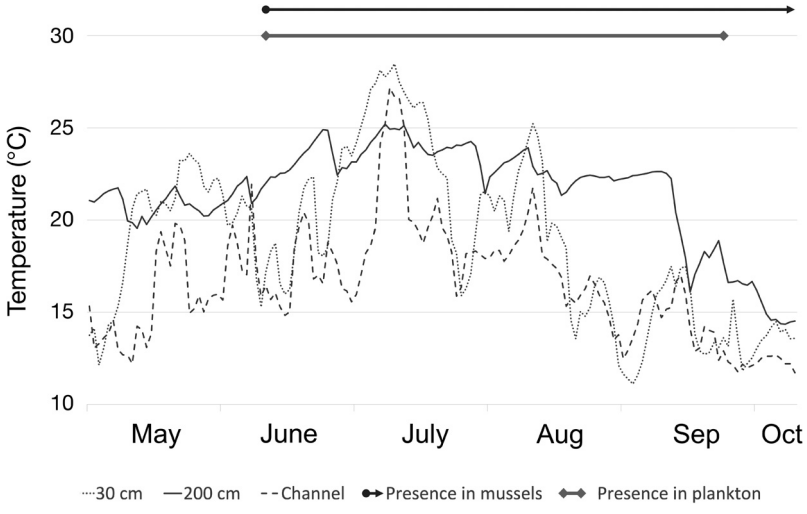

Fig. 4. Temperature profile in the lagoon from May until October; at 30 cm below the water surface (brackish top layer), 200 cm below the water surface (saltwater), and in the channel where the mussels were deployed. The first detection of the parasite, based on PCR data, was in early July 2018 in both mussels and plankton. Plankton could be sampled until October 2018 (not present after this time), and results showed that the samples were positive for the parasite up to this point. The final sample of mussels was collected in November 2018 (not shown), and the parasite was still present then

approach comparable to the claire ponds in France (Audemard et al. 2001). Through the trial with healthy mussels at Aga, the transmission window of *M. pararefringens* was found to be between May and September 2018. Based on the presence of advanced stages in the digestive tubules and sporonts in the lumen of the digestive tract, sporulation of the parasite was determined to occur between July and November 2018. We did not find evidence that transmission of the parasite occurred from October 2018 to March 2019. Since only the digestive gland was PCR analysed, it is possible that infection began earlier in other organs, such as the gills or palps. This was demonstrated recently by Charles et al. (2020). However, no *Marteilia* cells were observed by histology in either of these organs.

Plankton samples were positive from July to October 2018 and again from late May to August 2019 (final sampling). There was no plankton in the water column between November 2018 and early May 2019. No histology has thus far been performed to verify whether the presence in the plankton samples represented actual infections.

4.1. *M. pararefringens* infection in blue mussels

The development and sporulation of *M. pararefringens* is consistent with the description of the phylum Paramyxae (Desportes & Perkins 1990), now classified as the ascetosporean order Paramyxida (Ward et al. 2016, Bass et al. 2019).

The transmission of infective spores from the unknown source to the mussels occurred between May and October 2018, as evidenced by positive PCR results in July and histological confirmation in August. The first infections demonstrated a range of intensities, with some mussels only showing an infection in the stomach epithelium while others were heavily infected in the stomach epithelium, ducts, and digestive tubules. Contrary to what Villalba et al. (1993) described for *M. refringens* sensu lato, we did not detect *M. pararefringens* infection in the stomach epithelium of all infected individuals. It is not clear whether this was due to the absence of *Marteilia* cells or if it was an issue with sectioning.

The most severe infections in the stomach epithelium were most often found in the November 2018 samples. If one assumes that the development of *M. pararefringens* is similar to *M. refringens* sensu stricto, where the infection in the digestive system first appears in the stomach and later progresses to the digestive tubules, the high prevalence of the parasite in the stomach epithelium could be the result of new infections occurring just before the temperature in the oyster lagoon drops below what is necessary for parasite transmission. Based on the data gathered from the lagoon, the temperature drops around September–October. No mussels from the second group, deployed on 9 October 2018, became infected. This means that the *M. pararefringens* cells found in the stomach epithelium in the mussels gathered in November 2018 must have infected the hosts before October 2018, coinciding with the temperature drop. It has been postulated that the transmission and development of *M. refringens* is dependent on a thermal threshold at 17°C (Grizel 1985, Audemard et al. 2001), and it is likely that *M. pararefringens* is also subject to a thermal threshold.

The absence of true infections in July 2018 compared to the moderate to heavy infections in August 2018 indicates that rapid development and maturation occur within a relatively short time (6 wk). The development coincides with the highest temperatures in the oyster lagoon.

Only early stages of *M. pararefringens* were found in the stomach epithelium, regardless of sampling period. Such results have also been described in *Ostrea edulis* and *Mytilus galloprovincialis* infected with *M. refringens* sensu lato (Grizel 1979, Villalba et al. 1993), *Saccostrea glomerata* infected with *Marteilia sydneyi* (Kleeman et al. 2002), and *Crassostrea gigas* infected with *M. refringens* (Montes et al. 1998). It is unclear whether the stomach epithelium is an 'accidental' infection site where cells in the early stages are unable to propagate, or an initial site from which the parasite can reach the digestive tubules and initiate further propagation.

Extrasporogenic development was first confirmed in a study on *M. sydneyi* infecting *S. glomerata,* where it was found in the gills and palp epithelium (Kleeman et al. 2002). *M. refringens* sensu lato has been detected in the gills of *M. galloprovincialis* (Robledo & Figueras, 1995), as well as the gills and labial palps in *M. edulis* (Garcia et al. 2009), but such findings are uncommon and without any evidence of extrasporogenic development. However, an occurrence of extrasporogenic development could explain the relationship seen in the mean number of parasites in relation to the percentage of infected digestive tubules, indicating that the number of parasites per infected tubule increases as the infection progresses. This progression implies a rapid increase in the number of parasites as more digestive tubules are infected with more parasites. This development could be explained by (1) exposure to new spores that con-

stantly infect the mussels over the duration of the transmission period, (2) fewer spores infect first and proliferate through vegetative multiplication, increasing the intensity of infection, or (3) proliferation of cells in extrasporogenic host tissues that migrate to the digestive tubules.

If (1) is true, a constant infection of early stages in the stomach epithelium could be expected as long as there are infective spores in the water column (i.e. in the transmission period). However, this assumes that infective stages always infect the stomach epithelium first, followed by migration to ducts and digestive tubules. Since infected individuals were not always infected in the stomach epithelium, we have not found evidence to support this claim. In heavily infected individuals where all digestive tubules were infected, several hundred parasites were consistently observed. If hypothesis (1) was valid, it would mean that each parasite would have to originate from a single infective spore, each undergoing sporulation. It seems more likely that the high number of parasites originate from fewer infective spores that initiate an infection and then replicate in the host. This is consistent with findings reported by Villalba et al. (1993), but no replication other than sporogony in the target tissues has been conclusively demonstrated for *M. refringens* sensu lato (Carrasco et al. 2015). However, it is possible that *M. refringens* sensu lato can proliferate in non-target tissues as suggested by hypothesis (3), and this has indeed been demonstrated for *M. sydneyi* in the palps and gills of *S. glomerata* (Kleeman et al. 2002).

Sporulation of *M. pararefringens* in the mussels occurred from August to November 2018, based on the presence of mature stages in the digestive tubule epithelium and spores in the lumina of the digestive tract. One sporulation cycle in a year contrasts with past studies by Carrasco et al. (2007b), Robledo & Figueras (1995), and Villalba et al. (1993), who all found sporulation occurring twice a year in *M. refringens* sensu lato (likely *M. pararefringens*) in lower latitudes (Spain). However, temperature seems to play an important role in the development of *Marteilia* spp. Low winter temperatures at higher latitudes might allow only 1 sporulation cycle of *M. pararefringens*, when temperature peaks during summer. Sporulation twice a year could likely be due to mature, advanced stages persisting in a dormant state in the host during the winter and sporulating when the temperature increases again the following spring. Since no infected mussels used in the study survived until winter, we cannot exclude the possibility of this also occurring in *M. pararefringens* in Norway.

4.1.1. Pathology and host haemocyte reaction

Our data show a correlation between the intensity of infection by *M. pararefringens* and the host haemocytic reaction. This host response fails to prevent parasite proliferation and increases as the infection intensity increases. However, there seems to be variation between mussels. Several mussels were heavily infected with only light haemocytic infiltration, while others had a moderate infection with heavy haemocytic infiltration. This could be explained either by a rapid infection and progression of the parasite that evades the host's immune response, or that the host defence (in some specimens) is overwhelmed. Massive haemocyte infiltration has also been reported in *M. galloprovincialis* infected with *M. refringens* sensu lato (Figueras et al. 1991, Villalba et al. 1993), and variable haemocytic reactions were observed by Alderman (1979) in *O. edulis* infected by *M. refringens* sensu lato. The host haemocytic reaction was concentrated around the digestive tract, including stomach, ducts, and digestive tubules. The observed pathology presumably caused mortality of affected mussels.

4.1.2. Healthy mussels in the affected population

The finding of *Marteilia*-negative 2–3 yr old mussels from the local population indicates that some mussels may be resistant to infection by *M. pararefringens*. Healthy mussels were negative both by histopathological examination and PCR. The presence of heavily infected and healthy mussels in the same clusters in the narrow channel clearly indicates a genetic component that affects the susceptibility of *Marteilia* infection. The sampled mussels have filtered the same water flowing through the channel every tidal cycle for 2–3 yr and must have experienced the same infection pressure as the other deployed mussels. Although *M. edulis* is considered the dominant mussel species or genotype along the Norwegian coast (see e.g. Hummel et al. 2001, Mathiesen et al. 2017), the Norwegian mussel populations show a particularly patchy pattern (Brooks & Farmen 2013). This presumably reflects the complexity of the *Mytilus* species and their hybrids in the subarctic region (Mathiesen et al. 2017), and the establishment of populations where the genotypes with the highest fitness will dominate. It is apparent that selection of mussels resistant to the parasite occurs in endemic areas, reaching a host–parasite equilibrium that facilitates successful propagation of *Marteilia* without eradicating the host population (Carrasco et al. 2015). Obviously, adaptation to local

environmental conditions plays an important role in host susceptibility, as shown by Fuentes et al. (2002) who studied the effect genetic origin has on the resistance of *M. galloprovincialis*, *M. edulis*, and hybrids to *M. refringens* infection. Hybrids have been shown to be more susceptible to *Marteilia* infection (Fuentes et al. 2002). However, the resilience of mussel populations with long historical association with *Marteilia* spp. does not explain the variation in infection intensity and prevalence of *M. pararefringens* in the mussels in this study. The observed differences may be due to individual variation where random genetic differences in naïve mussel beds affect resistance to marteiliosis. It is also possible that the difference is due to drifting larvae that have inherited traits from mussel beds subjected to *Marteilia*-resistance selection pressure, for example from Aga (or an unknown location). In any case, genetic analyses should be conducted on all infected and non-infected mussels from affected sites to further investigate this observation.

4.2. Sympatric carriers of *M. pararefringens*

The proposed life cycle of *M. refringens* sensu lato includes one or several planktonic, marine copepods (Audemard et al. 2002, Carrasco et al. 2007a,b, Boyer et al. 2013, Arzul et al. 2014). The widespread absence of *M. pararefringens* in Norwegian mussels (Mortensen 1993, Mortensen et al. 2021), and confinement to a lagoon with elevated temperatures could reflect that the life cycle of the parasite is dependent on the presence of an exotic copepod species, or a thermophilic native species. Here we found that 2 copepod fractions harboured *M. pararefringens* DNA: *Paracartia grani* and *Acartia* spp. Of these, *P. grani* has previously been found to harbour *Marteilia* sp. in the female gonad (Audemard et al. 2002, Carrasco et al. 2008). In the case of Carrasco et al. (2008), however, it appeared that only *M. refringens* and not *M. pararefringens* (*M. maurini* in that study) readily infected and propagated in the gonadal tissue of *P. grani*. Nevertheless, we cannot exclude *P. grani* as a potential intermediate host of *M. pararefringens* considering the apparent co-dominance of the copepod in the oyster poll and the consistent PCR-positive findings. Further research must be conducted to clarify whether the copepods harbour true infections or not.

Sporulation in mussels occurred between July and November 2018, which coincided with the presence of plankton in the oyster lagoon (from May to October 2018). This indicates that direct passing of spores from mussels to plankton could occur in this period. Arzul et al. (2014) described a decline in prevalence of the parasite (predominantly *M. refringens* M-type in that study) in mussels concurrently with the first detection in the zooplankton, which they argued could be due to the transmission of the parasite from the former to the latter. Our results show that the prevalence in the mussels during sporulation remained high in August, October, and November 2018, with no change in parasite intensity, regardless of plankton presence. This does not seem to support the findings of Arzul et al. (2014).

Since all the mussels in the first deployment were either sampled or perished by November 2018, no conclusion could be reached as to what occurred when the plankton was absent between October 2018 and early May 2019. However, the absence of *Marteilia* cells in the second deployment from October 2018 to March 2019 demonstrates that no transmission to mussels from an intermediate host occurs at this stage, coinciding with the disappearance of the plankton in the water.

It is possible that the high prevalence and presence of sporulation stages in the mussels between July and November 2018 ensure the transmission of infective spores to the zooplankton that then lie dormant in the diapause eggs in the sediment until the bloom in spring the following year. Copepod eggs undergo a diapause in the sediment, and it has been proposed that this allows an overwintering of the parasite, which continues its life cycle when the eggs hatch during spring and early summer (Berthe et al. 2004). The presence of *M. refringens* sensu lato in the gonadal tissue of *Paracartia letisetosa* (Arzul et al. 2014) and *P. grani* (Audemard et al. 2002), and the verification of PCR-positive copepod eggs released from PCR-positive *P. grani* females (Boyer et al. 2013), further demonstrates the possible role overwintering diapause eggs have on the life cycle of the parasite. This area must be investigated further by histological analysis of the plankton samples and studying the sediment at Aga.

Though not directly part of this study, it is important to note that flat oysters populating the same area were not infected with *M. pararefringens*, indicating that the flat oyster is not susceptible to *M. pararefringens* in this geographical region, a similar situation to that observed in the UK (Kerr et al. 2018). This is contrary to what has been reported for *M. refringens* sensu lato, where cohabitations of both types have occurred in both mussels and oysters (e.g. Le Roux et al. 2001, López-Flores et al. 2004, Balseiro et al. 2007).

Flat oysters from Agapollen have been sampled as part of the national surveillance programme for

bivalve diseases from 1995 to 2019 (Hellberg & Mortensen 2000, Mortensen & Skår 2020). After the initial sampling of 300 oysters yearly for 2 yr, routine sampling occurred semi-annually in which 30 oysters were collected from trays, mats, ropes, and other substrates from the poll that had been previously used in production of spat. All flat oysters were sampled for histological analysis, while PCR has only been routinely performed since 2016 (Mortensen et al. 2017). It was in October 2016 that some oysters collected from Agapollen were shown to be PCR-positive but still histologically negative for the parasite (Mortensen et al. 2017). Sequencing showed that the positive signal represented *M. pararefringens* (Kerr et al. 2018). However, oysters were PCR-negative in April, July, and October 2017 and February 2018 (Mortensen et al. 2018, 2019). The positive PCR signals in October 2016 corresponded to the time of *M. pararefringens* sporulation in the mussels and may therefore be due to filtration of spores released from the infected mussels, i.e. contamination and not infection (Mortensen et al. 2017). Oysters and mussels collected at Rogøysund, a farm which yearly received oyster spat from Aga, were negative (Mortensen & Skår 2020). This indicates that *M. pararefringens* has not regularly been moved to the environments or mussels in other open-water oyster farms with oyster spat from the poll.

The historical data mentioned above and what we describe in this study are in accordance with results from studies of wild mussel and flat oyster populations in Sweden (EURL 2010), and with reports from Croatia (Zrnčić et al. 2001) and the west coast of Spain (Figueras & Robledo 1993), where infected mussels cultured side-by-side with flat oysters did not transmit the disease to the oysters. This host distribution could be explained by (1) 2 species of *Marteilia* that have different host tropisms, (2) a different intermediate host necessary in the transmission of the parasite between the 2 bivalve hosts might not be present, or (3) the flat oysters could be resistant to infection. However, Norwegian flat oysters originating from Aga became infected with *M. refringens* sensu lato after being seeded in the Thau lagoon in southern France (Berthe et al. 2004). Based on historical information and the present results, the flat oyster is not likely a host species for *M. pararefringens*.

5. CONCLUSIONS

Through the trial at Aga, we were able to describe the development of *Marteilia pararefringens* in *Mytilus edulis*. There was evidence for transmission of *M. pararefringens* to the mussels from May through September. Development and sporulation occurred from August to November, resulting in the presence of mature sporangia released in the digestive tubules. Mussels showed varying degrees of susceptibility, with some individuals never being infected while others showed heavy parasite infection and host haemocytic reaction in the same sample period.

No evidence of transmission of the parasite to healthy mussels was found from October 2018 to March 2019, indicating one annual infection cycle. Analysis of samples from plankton identified copepods belonging to the genera *Acarti*a and *Paracartia* to contain *M. pararefringens* DNA, and these should be further examined with respect to the life cycle of the parasite.

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