Viability, infectivity and fatty acid synthetic activity of *Perkinsus marinus* meront cells incubated in estuarine and artificial seawater

Fu-Lin E. Chu*, Eric D. Lund

Virginia Institute of Marine Science, College of William and Mary, 1208 Greate Road, Gloucester Point, Virginia 23062, USA

ABSTRACT: We investigated the viability and fatty acid synthetic activity of *in vitro* cultured *Perkinsus marinus* (Dermo) in lipid-free medium and estuarine water, and the infectivity of *P. marinus* maintained in artificial seawater (ASW). Viability and fatty acid synthetic activity in 7 d old *P. marinus* meronts maintained in lipid-free medium and estuarine water were tested. The infectivity of meronts incubated in ASW was examined by first incubating *P. marinus* meronts in ASW for 2, 3 or 7 d, and then inoculating viable ASW-incubated meronts into the shell cavity of individual oysters *Crassostrea virginica. P. marinus* infection prevalence and intensity in oysters were determined 9 wk post-inoculation. Heavy mortality occurred in meronts maintained in estuarine water, a drop from an initial value of 100 % viable to 7.8 and 6.1 % after 3 and 14 d incubation, respectively. Viability was 85 and 67 % in meronts maintained in lipid-free medium for 3 and 24 d, respectively. Meronts kept in lipid-free medium for 14 d retained their ability to synthesize fatty acids. Viable meronts incubated in ASW remained infective for up to 7 d. The infection prevalences were 85, 48 and 100 %, in the treatments inoculated with viable meronts that were incubated in ASW for 2, 3 and 7 d, respectively. Infection prevalence in the group inoculated with viable meronts immediately after they were transferred to ASW ranged from 61 to 85 %. Our results suggest that in nature meronts can survive for at least 14 d outside the host. Viable meronts are not only infective, but are also able to replicate and retain their fatty acid synthetic ability for 7 d.

KEY WORDS: *Perkinsus marinus* · Viability · Infectivity · Fatty acid synthesis

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INTRODUCTION

Perkinsus marinus (Dermo) is the parasite of a sessile host, the eastern oyster *Crassostrea virginica*. This parasite has continuously caused severe loss of eastern oyster populations on the US East and Gulf Coasts since the 1950s, and 4 life stages — meront (trophozoite), prezoosporangia (hypnospores), zoosporangia, and biflagellated zoospores — have been identified and described (Perkins 1966, 1988). The meront stage comprises the majority of the parasite load in tissues of heavily infected oysters. When tissue-associated meronts are placed in fluid thioglycollate medium (FTM) for 4 to 5 d, they develop into prezoosporangia. Zoosporulation (production of biflagellated zoospores) usually occurs after incubating FTM-cultured pre-

zoosporangia in estuarine or seawater (a salinity of 20 to 22) for 5 to 6 d. Prezoosporangia are sometimes found in tissues of infected oysters. However, it is unclear whether prezoosporangia released from moribund and deceased oysters are able to zoosporulate in nature. The meront stage is believed to be the primary agent for disease transmission (Perkins 1988, Chu 1996). Although exactly how *P. marinus* infective cells (e.g. meronts) enter a new host is not well understood, the disease caused by *P. marinus* is transmitted via dispersal of infective cells into the water column from live and moribund/dead infected oysters (e.g., Chu 1996, Bushek et al. 2002, Chu et al. 2002, Ragone Calvo et al. 2003).

In previous studies, we revealed that, unlike other parasitic protozoans, *Perkinsus marinus* has a unique

fatty acid synthetic capability (Soudant & Chu 2001, Chu et al. 2002, 2004). The meront stage of this parasite differs from all other parasitic protozoans that rely on their host for essential lipids. *P. marinus* meronts can synthesize a range of saturated and unsaturated fatty acids, including the essential fatty acid, arachidonic acid (AA) (Soudant & Chu 2001, Chu et al. 2002). The ability of *P. marinus* to synthesize AA is novel. No other parasitic protozoan has been reported to have such a capability. This capability may be related to the disease transmission processes, since lipids are essential membrane components and comprise energy reserves for growth and development and at times of no or limited nutrient supply.

It has long been hypothesized that *Perkinsus marinus* infective cells can survive in nature for a considerable length of time and remain infective when they enter a new host. However, no study has been conducted to examine the viability and infectivity of water-borne *P. marinus* infective cells, how long the primary transmission stage meront remains viable in the water column, and whether or not the 'viable' meronts are infective. No intermediary host is known to act as a vector for disease transmission by this parasite. Although Dermo disease could be transmitted via scavengers (Andrew & Hewatt 1957, Hoese 1963), and the snail *Boonea impressa* could be a potential vector for Dermo transmission (White et al. 1987), there is accumulated evidence supporting transmission through water-borne infective particles as the primary mechanism (e.g. Chu & Volety 1997, Bushek et al. 2002, Ragone Calvo et al. 2003). As such, the length of time that *P. marinus* cells disassociated from the host can remain viable and infective in the water column is critical for disease transmission and has a direct effect on their ability to infect distant host populations. The objectives of the present study were (1) to investigate the length of time that the parasite remains infective and viable in estuarine water and in a lipid-free environment, and (2) to determine, by incubating meront cells in estuarine water, if the meront stage of *P. marinus* retains its fatty acid synthetic ability and can proliferate outside the host in the wild.

MATERIALS AND METHODS

Meront cell culture. *Perkinsus marinus* meront cells were cultivated as previously described (Chu et al. 2002) in the medium defined by La Peyre et al. (1993). Medium was prepared with artificial seawater (ASW) and adjusted to an osmolarity of 590 (equivalent to a salinity of 20, Lund et al. 2004). The medium was then sterilized by 0.22 µm filtration and stored at 4°C until use. Meronts were inoculated at a concentration

of 1×10^6 ml^{-1} and cultured in 10 ml aliquots of medium in T-10 tissue culture flasks at 28°C. Meronts at exponential growth phase (7 d old) were harvested and used for all experiments.

Oysters. To avoid potential confounding effects due to the presence of the parasite, adult oysters *Crassostrea virginica* were obtained from Pemaquid Oyster Company, Waldoboro, Maine, where oysters are rarely infected by *Perkinsus marinus* (Ford 1996). Oysters were maintained in a closed seawater system at ambient temperature (20°C) and salinity (15 to 20) in 2 µm-filtered York River water. Upon arrival, 15 oysters were tested for *P. marinus* infection using total body burden assessment (Choi et al. 1989, Bushek et al. 1994b). No infection was detected in any of the sampled oysters. Oysters were fed an algal paste (Instant Algae Shellfish diet 1800, Reed Mariculture) at a ration of 0.2 g oyster^{-1} day^{-1} and allowed to acclimate for 12 d prior to experimental use.

Lipid and fatty acid analyses. Total lipids were extracted from meronts and oyster tissues according to the procedure described by Bligh & Dyer (1959). Fatty acid composition and contents of meronts and oyster tissues were analyzed using gas chromatograph/flame ionization detector (GC/FID). Briefly, total lipids were transesterified according to Metcalfe & Schmitz (1961) and fatty acid methyl esters (FAMEs) extracted with carbon disulfide (Marty et al. 1992). The organic phase was evaporated, redissolved in hexane, and analyzed by a GC (Varian 3300; Varian Analytical Instruments) equipped with a flame ionization detector, using a DB-WAX capillary column (25 m × 0.32 mm; 0.2 µm film thickness; J&W Scientific). Identification of FAMEs was based on the comparison of their retention times with those of authentic standards and confirmed by gas chromatograph-mass spectrometry (GC/MS). The quantity of each component was calculated based on the internal standard.

Neutral red assay. Neutral red (NR) assay (La Peyre & Chu 1994) was used to assess the viability of *Perkinsus marinus* meront cells. The NR assay examines uptake of liquid droplets or fluid-phase endocytosis by living cells; thus it is an assessment of cell viability. Briefly, meront cells were incubated with NR solution (Sigma N2889) in 1:9 ratio for 30 min and the number of viable cells (stained) and dead (non-stained) cells were counted using a Bright-Line hemocytometer (Reichert).

Diagnosis of *Perkinsus marinus* infection. Prevalence and intensity of *Perkinsus marinus* infection in experimental oysters were determined using total body burden assessment (Choi et al. 1989, Bushek et al. 1994b). Tissues from individual oysters were removed. The total wet weight of oyster tissue and dry shell weight were recorded, and whole oyster tissues

were homogenized in 0.22 µm-filtered York River water (the maintenance water). Tissue homogenates were added to FTM containing antibiotics (penicillin/streptomycin), incubated in the dark at room temperature for 5 to 7 d. This medium stimulates *P. marinus* meront cells to develop into prezoosporangia, which are larger and easier to identify than meronts. Subsequent treatment with sodium hydroxide digests the oyster tissue, but not the prozoosporangia, which can be stained with Lugol's solution and counted. Triplicate tissue suspensions of each oyster were assayed for body burden. Results were expressed as cell number g^{-1} wet tissue weight (mean ± SD).

Expts 1A and 1B. Viability, proliferation and fatty acid synthetic activity of meronts maintained in estuarine water and lipid-free medium. We conducted 2 experiments to determine the length of time that *Perkinsus marinus* meronts remain viable in estuarine water and lipid-free medium, and if meronts proliferate in lipid-free medium and estuarine water.

Expt 1A: To test how long meronts remain viable and whether they replicate in estuarine water and lipid-free environment, 7 d old meronts were inoculated in regular medium, lipid-free medium (medium without added lipid concentrate), or 0.22 µm-filtered estuarine water (York River water, YRW) adjusted to a salinity similar to that of the regular medium (salinity = 20; n = 3). Their viability and proliferation were monitored for 24 d at 28°C. Samples were taken at 3, 7, 14 and 24 d post-inoculation to count cell number and test viability, using a NR assay.

Expt 1B: This experiment repeated Expt 1A, with similar experimental conditions, with the exception that the meronts were incubated for a different time (14 d). Also, in addition to taking samples for measurements of viability of meronts at 3, 7 and 14 d post-inoculation, meronts were also sampled at the beginning and 3, 7 and 14 d post-inoculation and stored at −80°C for later lipid and fatty acid analyses. In this experiment, only viable meronts were counted, but all meronts were collected for lipid and fatty acid analysis.

Expts 2A and 2B. Infectivity of *Perkinsus marinus* meronts maintained in host-free environment. We conducted 2 separate experiments to test the infectivity of 3 different *Perkinsus marinus* cultures incubated in artificial seawater (ASW).

Expt 2A: After incubation in ASW for 0, 2 or 7 d, *Perkinsus marinus* cell viability was assessed using an NR assay prior to use for artificial infection. A concentration of 1.5×10^6 viable 0, 2 and 7 d ASW-incubated *P. marinus* cells was injected into the shell cavity of oysters (n = 21 oysters for each incubation period). Negative controls were inoculated with filtered ASW only. Artificially infected oysters were maintained individually in 2 l jars for 9 wk at 20 to 25°C. Oysters were

fed an algal paste (Instant Algae Shellfish Diet 1800, Reed Mariculture) at a daily ration of 0.2 g oyster^{-1}, and water was changed 3 times per week. At the end of the experiment (9 wk after *P. marinus* meront cell inoculation into the shell cavity), *P. marinus* infectivity was determined using a body burden assay (Choi et al. 1989, Bushek et al. 1994b).

Expt 2B: The experimental conditions were similar to those described for Expt 2A, except that different concentrations of viable *Perkinsus marinus* cells were used for infection and they were incubated in ASW for different lengths of time. In this experiment, *P. marinus* meront cells were incubated in ASW for 0, 3 or 7 d prior to use for infection. A concentration of 4.0×10^6 of viable 0, 3 and 7 d ASW-incubated cells was injected into the shell cavity of oysters (n = 21 oysters per incubation period). As for Expt 2A, negative controls were inoculated with filtered ASW only. We diagnosed the oysters for prevalence and intensity of *P. marinus* infection 9 wk after artificial infection.

Statistical analysis. Weight percent and mg ml^{-1} DW (dry weight) fatty acid compositions from Expt 1B were analyzed by 2-way analysis of variance (ANOVA) (independent factors of time and medium type). Data on prevalence of infection from Expt 2 were analyzed by chi square. When chi square results were significant (p < 0.05), treatments were compared by orthogonal contrasts. All percentage data were arcsine transformed prior to analysis.

RESULTS

Expts 1A and 1B examined the proliferation and the length of time that *Perkinsus marinus* meront cells remain viable in lipid-free medium and estuarine water, Expts 2A and 2B their infectivity in a host-free environment.

Expt 1A. Proliferation and viability of meronts

Meronts proliferated in both regular and lipid-free media during the course of the experiment (Fig. 1). Cell number in the regular medium increased from a Day 0 density of 6.5×10^6 cells ml^{-1} to a peak of 30.5×10^6 cells ml^{-1} at Day 14, with a density of 28.3×10^6 cells ml^{-1} on Day 24. Cell number in the lipid-free medium increased from a Day 0 density of 7.5×10^6 cells ml^{-1} to a peak of 13.3×10^6 cells ml^{-1} at Day 14, with a density of 9.2×10^6 cells ml^{-1} on Day 24 (Fig. 1). Mortality in meronts maintained in lipid-free medium was much lower than for those maintained in estuarine water, but higher than for those maintained in regular medium. Viability at 24 d was 80 and 67 % in meronts

maintained in regular medium and lipid-free medium, respectively (Fig. 2). The meront cells maintained in estuarine water (York River water, YRW) declined from an initial density of 7.4×10^6 to 6.63×10^5 cells ml^{-1} by Day 3 and continued to decrease to 5.4×10^5 cells ml^{-1} by Day 14 (Fig. 1). The cell viability for this treatment dropped from an initial value of nearly 100 to 7.8% at Day 3 and 6.1% at Day 14 (Fig. 2).

Expt 1B. Proliferation and fatty acid content composition of meronts

Proliferation

Although only viable cells were counted in Expt 1B, results were consistent with those of Expt 1A (Fig. 3). Meronts cultivated in regular medium continued to proliferate over the course of the experiment, increasing from an initial concentration of 1.3×10^6 to 43.5×10^6 cells ml^{-1} at Day 14 (Fig. 3). Meront cell number in the lipid-free medium treatment increased from an initial concentration of 1.5×10^6 to

24.1×10^6 cells ml^{-1} at Day 3, then declined to 14×10^6 cells ml^{-1} by Day 14 (Fig. 3). Meront number declined in estuarine YRW from an initial concentration of 1.5×10^6 to 0.64×10^6 cells ml^{-1} by Day 3, but declined only slightly thereafter. At Day 14, there were still 0.5×10^6 viable cells ml^{-1} in the YRW treatment (Fig. 3).

Total fatty acid content in cultures

Meronts in both lipid-free medium and regular medium increased in total fatty acid content g^{-1} DW of meronts over the duration of the experiment, while the meronts maintained in 0.22 µm-filtered estuarine YRW declined from initial values (Fig. 4). The fatty acid content of the positive control increased at every sampling point from an initial concentration of 11.9 to 28.8 ± 2.4 mg g^{-1} DW (mean $\pm$ SD) at Day 14. The lipid-free medium treatment increased from an initial value of 11.9 to 22.0 ± 4.7 mg g^{-1} DW by Day 7, then remained relatively static (20.5 ± 2.1 mg g^{-1} DW) until Day 14. The total fatty acid content in the YRW
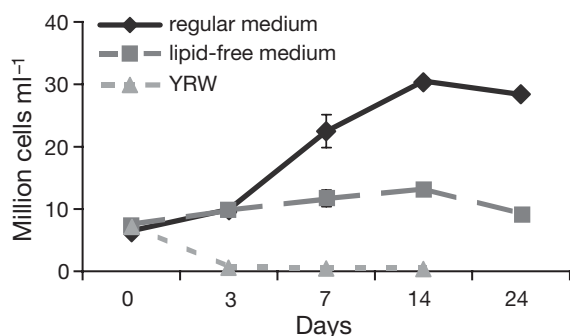

Fig. 1. *Perkinsus marinus*. Growth (cell numbers, mean $\pm$ SD, n = 3; Expt 1A) of meronts maintained in regular medium, lipid-free medium and 0.22 µm-filtered estuarine water (York River water, YRW)

Fig. 3. *Perkinsus marinus*. Meront cell number (mean $\pm$ SD, n = 3; Expt 1B) cultivated in regular medium, lipid-free medium and YRW
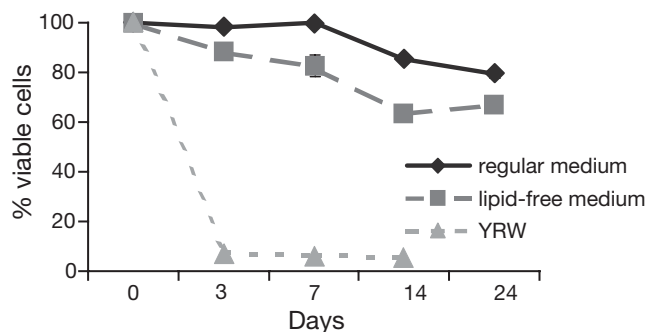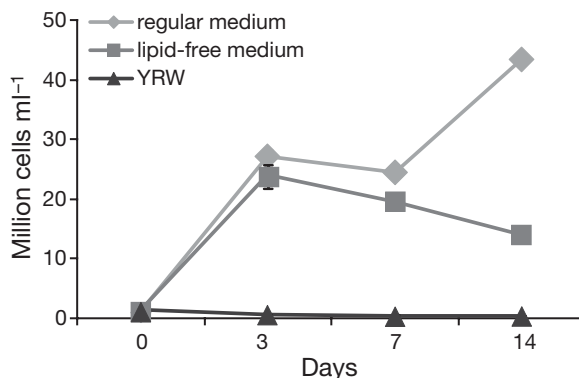

Fig. 2. *Perkinsus marinus*. (mean $\pm$ SD, n = 3; Expt 1A). Viability of meronts maintained in regular medium, lipid-free medium and 0.22 µm-filtered YRW, measured by neutral red viability assay
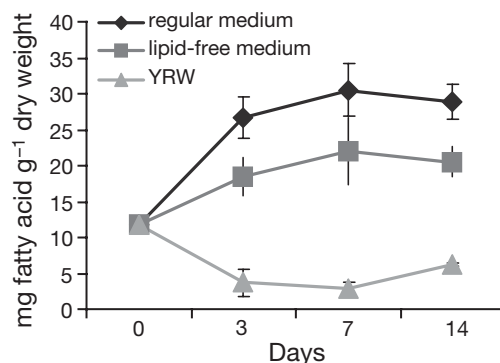

Fig. 4. *Perkinsus marinus*. Total fatty acid content (mean $\pm$ SD, n = 3; Expt 1B) of meronts in regular medium, lipid-free medium and YRW

Table 1. *Perkinsus marinus*. Weight percent (mean ± SD, n = 3) of major fatty acids in meronts maintained in regular medium, lipid-free medium and filtered York River water for 14 d (Expt 1B). Only major fatty acid components are shown (i.e. >2%). FAME: fatty acid methyl ester; RM: regular medium; LF: lipid-free medium; YRW: 0.2 µm-filtered York River water. [a]: Significantly different for 'treatment' (p < 0.05); [b]: significantly different for 'day' (p < 0.05); nd: non detectable

FAME	Day 0 (initial)	Day 3 RM	LF	YRW	Day 7 RM	LF	YRW	Day 14 RM	LF	YRW
14:0[ab]	6.5	5.7 ± 0.1	4.4 ± 0.1	7.8 ± 0.5	4.4 ± 02	2.9 ± 0.1	8.2 ± 0.4	3.7 ± 0.1	2.0 ± 0.1	5.1 ± 1.3
16:0[ab]	11.0	13.3 ± 0.1	11.9 ± 0.2	14.1 ± 0.3	11.3 ± 0.2	9.1 ± 0.1	13.7 ± 0.3	11.5 ± 0.1	9.1 ± 0.4	12.9 ± 1.2
16:1(n-7)[ab]	3.3	2.1 ± 0.0	1.0 ± 0.0	3.6 ± 0.1	1.3 ± 0.0	0.8 ± 0.0	3.8 ± 0.1	1.2 ± 0.0	0.7 ± 0.0	3.3 ± 0.0
18:0[ab]	6.5	6.8 ± 0.3	6.9 ± 0.1	6.2 ± 0.1	7.7 ± 0.1	5.0 ± 0.1	5.5 ± 0.1	6.2 ± 0.1	3.6 ± 0.0	5.3 ± 1.0
18:1(n-9)[ab]	33.5	22.8 ± 0.2	24.3 ± 0.3	34.1 ± 0.8	28.2 ± 0.5	31.9 ± 0.1	35.2 ± 0.2	31.6 ± 0.3	34.5 ± 0.8	34.8 ± 2.5
18:1(n-7)[ab]	4.7	2.8 ± 0.0	1.6 ± 0.0	4.4 ± 0.0	2.1 ± 0.0	1.2 ± 0.0	4.4 ± 0.1	1.9 ± 0.0	1.1 ± 0.0	4.5 ± 0.2
18:2(n-6)[ab]	2.7	4.1 ± 0.1	4.9 ± 0.1	3.3 ± 0.1	2.9 ± 0.0	3.9 ± 0.0	3.5 ± 0.0	3.0 ± 0.1	4.2 ± 0.1	3.4 ± 0.2
20:1(n-9)[ab]	9.5	5.6 ± 0.0	6.0 ± 0.1	7.4 ± 0.2	7.3 ± 0.2	7.5 ± 0.1	6.9 ± 0.1	6.1 ± 0.2	5.5 ± 0.3	7.1 ± 0.6
20:4(n-6)[ab]	6.5	16.8 ± 0.6	21.5 ± 0.7	6.1 ± 0.3	14.1 ± 0.1	19.7 ± 0.0	6.2 ± 0.2	15.0 ± 0.2	22.1 ± 0.1	7.0 ± 0.3
20:5(n-3)[a]	3.2	2.8 ± 0.1	0.9 ± 0.1	2.8 ± 0.1	2.4 ± 0.0	0.8 ± 0.0	3.1 ± 0.1	2.6 ± 0.0	0.9 ± 0.0	3.4 ± 0.2
22:1(n-9)[a]	2.0	0.7 ± 0.1	nd	nd	0.8 ± 0.1	nd	nd	0.5 ± 0.0	nd	1.2 ± 0.2
24:0[a]	2.5	5.1 ± 0.2	5.9 ± 0.3	2.5 ± 0.5	5.0 ± 0.3	61. ± 0.1	3.1 ± 0.1	5.0 ± 0.1	6.2 ± 0.5	3.1 ± 0.1

treatment had declined over 3-fold by Day 7, but interestingly, the total fatty acid content had increased to 6.0 mg g^{-1} DW by Day 14.

Weight percent fatty acid composition in cultures

The weight percentage of all major fatty acids differed significantly (p < 0.05) with treatment, but 20:5 (n-3), 22:1(n-9) and 24:0 did not vary significantly with sampling day. Although there was little change in fatty acid compositions within each treatment over time, the fatty acid profiles differed in several aspects among the 3 treatments (Table 1). Most notably, the percentage of 20:4(n-6) was highest in meronts from the lipid-free medium treatment (19 to 22%) (Table 1), followed by meronts in regular medium (14 to 17%), and was lowest in the YRW treatment (6 to 7%). The percentage of n-3 polyunsaturated fatty acids (PUFAs) was much lower in the lipid-free medium treatment (0.8 to 09% or <0.2%) than in the regular medium and YRW treatments (both 3.2 to 4.5%). The surviving meronts in YRW maintained a relatively similar weight % fatty acid profile as those at Day 0 (initial). The fatty acid 18:1(n-9), precursor of 20:4(n-6), was abundant in all treatments. However, only meronts in YRW maintained consistently levels of this fatty acid similar to the initial (34 to 35%) over time.

Individual fatty acid content in cultures

The masses (µg) of all major fatty acids differed significantly (p < 0.05) between treatments, but 16:0, 16:1 (n-7), 18:0, 18:1(n-7), 18:2(n-6) and 22:1(n-9) did not vary significantly with sampling day (Table 2). Individual fatty acid contents in meronts maintained in regular and lipid-free media increased as the meront cell

number increased compared to initial values, while those maintained in YRW decreased over time due to declining cell numbers (Table 2). The increase in 16:0, 18:0, 18:1(n-9) and 20:4(n-6) was particularly obvious in meronts cultured in regular medium. Similarly, total saturated, mono-unsaturated, n-3 and n-6 fatty acids and total fatty acids increased with increasing meront number in regular and lipid-free media, whereas all in YRW decreased. However, the increase in the culture in lipid-free medium began to level off after Day 7.

Expt 2A. Infectivity of meronts in host-free environment

All treatment groups except the negative controls contained oysters with *Perkinsus marinus* infection (Table 3, Fig. 5). Prevalence of infection ranged from 80 to 100% and no infection was detected in negative controls. The oysters inoculated with meronts previously maintained in ASW for 7 d had the highest infection prevalence (100%). The oysters inoculated with viable meront cells that had just been transferred to ASW had significantly lower prevalence (p < 0.05) than those inoculated with viable 7 d ASW-incubated meronts. All infections in oysters were light. Average infection intensities were 59 ± 83, 299 ± 512 and 5767 ± 1190 cells g^{-1} wet tissue in oysters inoculated with meronts kept in ASW for 0, 2 and 7 d, respectively.

Expt 2B. Infectivity of meronts in host-free environment

Results were similar to those of Expt 2A, with 100% infection prevalence in oysters inoculated with meronts that had been kept in ASW for 7 d, while significantly (p

Table 2. *Perkinsus marinus*. Fatty acid content (µg, mean ± SD, n = 3) of meront cells in cultures (10 ml) maintained in regular medium, lipid-free medium and filtered York River water for 14 d (Expt 1B). Only fatty acids comprising >2% of total fatty acids are shown. Sat: total saturated fatty acids; Mono: total mono-unsaturated fatty acids; n-3: total n-3 unsaturated fatty acids; n-6: total n-6 unsaturated fatty acids; Total: total fatty acids; other abbreviations as in Table 1. [a]: Significantly different for 'treatment' ($p < 0.05$); [b]: significantly different for 'day' ($p < 0.05$)

FAME	Day 0 (initial)	Day 3 RM	Day 3 LF	Day 3 YRW	Day 7 RM	Day 7 LF	Day 7 YRW	Day 14 RM	Day 14 LF	Day 14 YRW
14:0[ab]	3.4	12.4 ± 0.9	6.8 ± 0.8	1.7 ± 0.4	12.4 ± 0.6	6.0 ± 0.5	1.5 ± 0.1	11.2 ± 0.4	3.8 ± 0.5	0.9 ± 0.3
16:0[a]	5.7	28.7 ± 2.0	18.6 ± 1.8	3.0 ± 0.7	32.1 ± 0.9	18.9 ± 1.1	2.5 ± 0.1	35.3 ± 1.5	17.1 ± 2.3	2.4 ± 0.3
16:1(n-7)[a]	1.7	4.5 ± 0.3	1.6 ± 0.1	0.8 ± 0.2	3.8 ± 0.1	1.6 ± 0.1	0.7 ± 0.0	3.6 ± 0.1	1.3 ± 0.2	0.6 ± 0.0
18:0[a]	3.4	14.8 ± 1.5	10.8 ± 1.0	1.3 ± 0.3	21.9 ± 0.3	10.4 ± 0.5	1.0 ± 0.0	18.9 ± 1.2	6.8 ± 0.6	1.0 ± 0.2
18:1(n-9)[ab]	17.5	49.3 ± 3.5	38.1 ± 3.3	7.3 ± 1.7	80.0 ± 2.3	66.0 ± 3.4	6.4 ± 0.1	96.9 ± 4.0	64.9 ± 7.8	6.4 ± 0.3
18:1(n-7)[a]	2.4	6.0 ± 0.4	2.5 ± 0.2	0.9 ± 0.2	5.9 ± 0.1	2.5 ± 0.1	0.8 ± 0.0	5.9 ± 0.3	2.1 ± 0.3	0.8 ± 0.0
18:2(n-6)[a]	1.4	8.9 ± 0.5	7.7 ± 0.5	0.7 ± 0.2	8.1 ± 0.2	8.2 ± 0.4	0.6 ± 0.0	9.3 ± 0.3	7.8 ± 0.9	0.6 ± 0.0
20:1(n-9)[ab]	5.0	12.0 ± 0.8	9.4 ± 0.8	1.6 ± 0.4	20.7 ± 0.3	15.5 ± 0.5	1.2 ± 0.0	18.8 ± 1.5	10.3 ± 0.7	1.3 ± 0.1
20:4(n-6)[ab]	3.4	36.3 ± 1.0	33.8 ± 4.0	1.3 ± 0.3	40.0 ± 0.4	40.7 ± 2.1	1.1 ± 0.0	46.1 ± 2.8	41.5 ± 4.2	1.3 ± 0.1
20:5(n-3)[ab]	1.7	6.0 ± 0.1	1.5 ± 0.2	0.6 ± 0.1	6.7 ± 0.2	1.7 ± 0.1	0.6 ± 0.0	7.9 ± 0.2	1.7 ± 0.2	0.6 ± 0.1
22:1(n-9)[a]	1.1	1.6 ± 0.2	Trace	Trace	2.4 ± 0.3	Trace	Trace	1.6 ± 0.1	Trace	0.2 ± 0.0
24:0[ab]	1.3	11.0 ± 0.2	9.2 ± 0.4	0.5 ± 0.1	14.0 ± 0.6	12.6 ± 0.4	0.6 ± 0.0	15.3 ± 0.9	11.7 ± 0.6	0.6 ± 0.0
Sat[ab]	15.24	73.0 ± 5.0	50.7 ± 4.3	6.8 ± 1.4	93.9 ± 1.2	55.8 ± 2.8	5.8 ± 0.2	94.0 ± 4.7	46.4 ± 4.5	5.5 ± 0.8
Mono[ab]	29.05	80.1 ± 6.1	56.1 ± 4.9	11.2 ± 2.6	120.2 ± 2.5	91.7 ± 4.4	9.6 ± 0.3	135.2 ± 6.4	84.1 ± 9.1	9.7 ± 0.5
n-3[ab]	2.51	9.9 ± 0.2	1.5 ± 0.2	0.9 ± 0.1	10.8 ± 0.1	1.7 ± 0.1	0.8 ± 0.0	11.4 ± 0.4	1.7 ± 0.2	0.9 ± 0.1
n-6[ab]	4.80	51.0 ± 1.7	46.2 ± 4.9	2.0 ± 0.4	54.5 ± 0.7	54.2 ± 2.8	1.8 ± 0.0	62.1 ± 3.6	53.4 ± 5.2	1.9 ± 0.1
Total[ab]	52.2	216.0 ± 13.2	156.5 ± 13.6	21.4 ± 4.6	283.3 ± 3.8	206.9 ± 10.2	18.2 ± 0.4	306.5 ± 15.3	188.2 ± 19.4	18.4 ± 1.0

< 0.05) fewer oysters were infected in the group injected with meronts that had not been kept in ASW and in the treatment injected with meronts that had been in ASW for 3 d, (62 and 48%, respectively) (Fig. 6). However, unlike Expt 2A, 1 oyster was detected with 3 *Perkinsus marinus* cells g^{-1} wet tissue in the negative control (Fig. 6). Average infection intensities were 23 ± 35, 15 ± 18 and 316 ± 560 cells g^{-1} wet tissue weight in the treatments inoculated with meronts kept in ASW for 0, 3 and 7 d, respectively (Table 3).

Low mortality was noted in both Expts 2A and B: 1 oyster was found dead in the treatment group in-

oculated with meronts incubated in ASW for 2 d in Expt 2A, and 1 death occurred in the treatment injected with meronts incubated in ASW for 7 d in Expt 2B.

DISCUSSION

Perkinsus marinus is transmitted between oysters via water-borne infective cells (Ray 1954, Mackin 1962, Andrews 1988, Volety & Chu 1994, Ragone Calvo et al. 2003). While cells released from degraded tissues of infected dead oysters are the primary source of infective cells for *P. marinus* transmission (Mackin 1962, Andrews 1988, Ragone Calvo et al. 2003), dispersion of infective cells via feces and pseudo-feces may also be sources of infective particles (Bushek et al. 2002). Infective cells released from a host during summer (the period during which disease prevalence and intensity peak) are believed to be able to survive in the water column through the winter and are infective when they enter a new host in the following spring (Mackin 1962). Our study is the first to investigate how long the primary transmission stage, meront, remains viable in the water column and whether or not the 'viable' meronts are infective. Our results show that viable meronts remained infective in ASW at least for 7 d. Meronts kept in lipid-free medium for 14 d

Table 3. *Crassostrea virginica* infected by *Perkinsus marinus*. Parasite density (mean ± SD; n = 21 oysters in each incubation period) in oysters experimentally infected with viable *P. marinus* meront cells incubated in artificial seawater (ASW) for 0, 2 or 7 d (Expt 2A) and for 0, 3 or 7 d (Expt 2B). Average intensities calculated for infected oysters only. No *P. marinus* infection was detected in any oyster in the control group in Expt 2A; 1 oyster in control treatment was infected with 3 cells g^{-1} wet tissue wt in Expt 2B

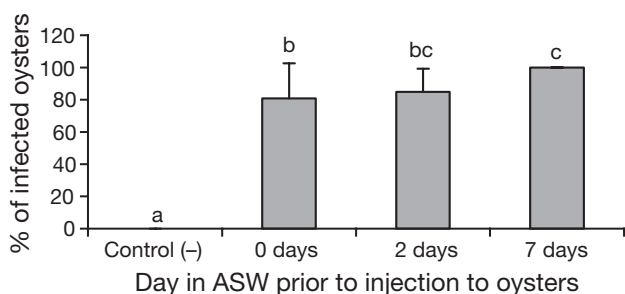|	Day 0 (initial)	Days incubated in ASW 2 d	3 d	7 d
Mean density (cells g^{-1} wet tissue wt)				
Expt 2A	59 ± 83	299 ± 512		767 ± 1190
Expt 2B	23 ± 35		15 ± 18	316 ± 560
Range				
Expt 2A	2–278	3–1770		8–3795
Expt 2B	3–110		3–63	4–1864

Fig. 5. *Crassostrea virginica* infected by *Perkinsus marinus*. *P. marinus* prevalence (% infected oysters, Mean ± SD, Expt 2A) in oysters inoculated with 100 µl of artificial seawater (ASW) containing 1.5×10^6 viable *P. marinus* cells that had been incubated in ASW for 0, 2 or 7 d (n = 21 oysters per incubation period). Control (–): negative control, oysters inoculated with 100 µl ASW. Different letters denote significant differences between treatments (p < 0.05)

Fig. 6. *Crassostrea virginica* infected by *Perkinsus marinus*. *P. marinus* prevalence (% infected oysters, Mean ± SD, Expt 2B) in oysters inoculated with 100 µl ASW containing 4.0×10^6 viable *P. marinus* cells that had been incubated in ASW for 0, 3 or 7 d (n = 21 oysters per incubation period). Further details as in Fig. 5

retained their ability to synthesize fatty acids, including arachidonic acid [20:4(n-6)]. The fatty acid synthetic ability of meronts in lipid-free medium may be related to disease transmission processes.

In addition to temperature and salinity, the *in situ* concentration of *Perkinsus marinus* meronts is critical for disease transmission and development (Volety & Chu 1994, Chu 1996, Chu & Volety 1997, Ragone Calvo et al. 2003). Dose-dependent transmission of various *P. marinus* life stages has been demonstrated in laboratory experiments (Volety & Chu 1994). In a study investigating *P. marinus* transmission in oysters in relation to water temperature, oyster mortality and water column abundance of anti-*P. marinus* antibody-labeled cells enumerated by flow cytometry, it was noted that peak *P. marinus* infection acquisition coincided with elevated abundance of water-borne *P. marinus* cells and was highest at the time of maximum cell abundance (Ragone Calvo et al. 2003). However, a significant relationship between cell abundance and infection-acqui-

sition rate was not observed. Ragone Calvo et al. (2003) speculated that the insignificant correlation between cell abundance and infection incidence was because (1) the enumerated cells were not equally infective and/or (2) through cross-reaction of anti-*P. marinus* antibody with other microbes. It is also possible that not all the enumerated cells were viable, since the viability of the immuno-stained cells was not determined. Our results revealed heavy mortality in meronts as soon as they were transferred to YRW. Viability was reduced from an initial value of nearly 100 to 7.8 % at Day 3 and 6.1 % at Day 14. However, those meronts that did survive the initial high mortality were still viable after 14 d. Also, although some mortality occurred in meronts maintained in lipid-free medium, the surviving viable meronts were capable of replicating (Figs. 1 & 3), and survived for a relatively long time (24 d, Fig. 3).

In the present study, oysters were experimentally infected with similar doses of surviving/viable meronts. Apparently viable *Perkinsus marinus* meronts are infective for at least 7 d in ASW outside the host. Higher *P. marinus* infection prevalence (100 %) in the treatment inoculated with meronts that had been kept in ASW for 7 d is particularly interesting, although difficult to interpret. One possible explanation is that it takes time for the parasites from one environment (culture medium) to adjust to a new environment (ASW). The initial high mortality in meronts kept in YRW and in meronts maintained in lipid-free medium supports this. If it is assumed that the culture medium environment is a simulation of the host condition, then one would expect that heavy mortality would occur when meronts become host-disassociated, i.e. released to the water column via decaying of dead infected oyster tissues and/or feces and pseudo-feces. Possibly, those surviving can remain viable in the water column (i.e. ASW in the present study) for a long time and have 'physiologically adapted' to the new environment.

In the present study, infectivity was tested in meronts held in ASW up to only 7 d. We believe that infectivity of meronts in nature may well persist beyond 7 d. In the present study, after initial mortality, meronts in lipid-free medium and YRW survived for up to 24 and 14 d, respectively (Fig. 2). As indicated in the 'Introduction', although potential transmission vectors and reservoirs for *Perkinsus marinus* have been proposed (Andrew & Hewatt 1957, Hoese 1963, White et al. 1987), no secondary host has been reported and its host is a benthic sedentary bivalve *Crassostrea virginica*. Thus, *P. marinus* infective cells such as meronts probably rely mainly on water and tidal currents to reach distant host populations. Increased survival time may result in enhanced dispersal distance of infective cells.

That only light infection (Table 3) was noted in oysters in all treatment groups after 9 wk of meront injection is not surprising since cultured meront cells are usually less infective or virulent than wild meront cells isolated from infected oysters (Bushek et al. 1994a, Chintala et al. 2002, Ford et al. 2002). The single infection in the control group probably originated from Maine.

Fatty acid synthetic activity continued, with a slight increase in 18:1(1n-9) and 20:4(n-6) in meronts maintained in lipid-free medium. The decrease of fatty acid content in those meronts maintained in YRW was due to heavy mortality, which resulted in reduction in cell numbers. This suggests that the nutrients in YRW cannot sustain fatty acids and lipid synthesis, thus prohibiting proliferation, although the survivors managed to maintain a fatty acid profile similar to that of the inoculum. The lipid-free medium consists of many small components such as amino acids, vitamins and sugars. In nature, there is dissolved organic matter (DOM) comprised of similar molecules including free amino acids, monosaccharides and short chain, free fatty acids. Although most of these are refractory, there is considerable evidence that many microbes, filter feeders and larval and juvenile fishes consume them for energy, particularly at times of food limitation (Manahan 1983, Feral 1985, Korsgaard 1991, Diaz et al. 1994, Otake et al. 1995, Kragh & Sondergaard 2004). The infectivity shown by meronts maintained in ASW for 7 d was surprising, since ASW contains only inorganic salts. With this in mind, it is conceivable that meront cells can live in the water column for days, if not weeks, until an opportunity for reinfection arises. Hyponospores of *Perkinsus marinus* can tolerate temperatures as low as 4°C for 4 to 7 d (Chu & Greene 1989), and within oyster tissues hyponospores remained viable after freezing (Andrew & Hewatt 1957). Thus, the suggestion that *P. marinus* may survive over the winter outside its host and reinfect oysters the following spring (Mackin 1962) is supportable. However, a time frame of several months outside the host may be too long a period for the parasite to survive without the essential nutrient supply derived from the host environment, especially since DOM availability and concentration are seasonally variable. We found high cell mortality in *P. marinus* meront cell culture at the stationary phase in regular media. Cell viability dropped below 40% in 28 d old cultures and ranged from 11 to 25% between 38 and 47 d old cultures (Soudant et al. 2005). Thus, in addition to low temperature and salinity, nutrient availability could be a limiting factor for *P. marinus* distribution.

The heavy mortality in *Perkinsus marinus* at the stationary phase and beyond may be due to limitation of sterols. Sterols are essential structural components in addition to phospholipids and long chain polyunsaturated fatty acids. Thus far, only parasitic protozoans of the family Trypanosomatidae have been reported to have the ability of synthesizing sterols (Contreras et al. 1997, Lepesheva et al. 2004). Although *P. marinus* has a greater fatty acid synthetic ability than other parasitic protozoans (Chu et al. 2002), it must acquire sterols from its host for survival and membrane synthesis. We have found that meronts proliferate in medium with cholesterol as the only lipid source (unpubl. obs.). *P. marinus* meronts are also incapable of synthesizing n-3 fatty acids (Soudant & Chu 2001, Chu et al. 2002). The increase in n-3 long chain fatty acids such as 20:5(n-3) and 22:6(n-3) in meronts grown in regular medium is due to incorporation and assimilation rather than de novo synthesis.

In summary, the present study has demonstrated that although heavy mortality occurred when *in vitro* cultured meronts were transferred to estuarine water (i.e. YRW), the survivors remained viable for up to 14 d. Despite some mortality in meronts maintained in lipid-free medium, those that survived were able to replicate and their fatty acid synthetic ability continued. Viable meronts that had been kept in ASW for 2, 3 or 7 d were infective. The highest *Perkinsus marinus* prevalence (100%) was in the oysters inoculated with meronts that had been kept in ASW for 7 d. These results suggest that *P. marinus* meront cells can survive for at least 14 d in the water column and that viable meronts remain infective for at least 7 d. However, the present study was conducted using *in vitro* cultured meront cells, which could differ greatly from wild meronts in terms of survival and infectivity. Future studies should be directed at testing the viability and infectivity of wild meronts freshly isolated from infected oyster tissues, and should extend the length of incubation time in water for longer than 7 d.

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