



# In vitro effects of temperature and salinity on fatty acid synthesis in the oyster protozoan parasite *Perkinsus marinus*

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## Abstract

The effects of temperature and salinity on fatty acid synthetic activities in the oyster protozoan parasite, *Perkinsus marinus*, were tested in vitro at 10, 18 and 28 °C in a salinity of 28 psu and 14, 20 and 28 psu at a temperature of 28 °C using <sup>13</sup>C sodium acetate as a substrate. Salinity treatments exhibited few treatment effects, but temperature significantly affected cell proliferation, fatty acid content and fatty acid synthesis rates. Fatty acid synthesis rates increased approximately two-fold for every 10 °C increase in temperature; however, the predominant fatty acid synthesized differed between treatments. At 10 °C, the synthesis rate for 18:1(n-9) was not significantly different from the 18 °C treatment and weight percent of 18:1(n-9) was higher at 10 than 18 and 28 °C. In contrast, the synthesis rate for 20:4(n-6) was over five times lower at 10 than at 18 and 28 °C, and the percent fatty acid content of 20:4(n-6) was over two-fold lower at 10 than at 18 and 28 °C. Results suggest that further elongation and desaturation of 18:1(n-9) to 20 carbon polyunsaturated fatty acids may be inhibited at low temperatures. These findings may be relevant to field observations that disease progression and virulence of this parasite are correlated to high water temperatures.

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**Keywords:** Fatty acid synthesis; Oyster; Parasite; *Perkinsus marinus*; Protozoan; Salinity; Temperature

## 1. Introduction

*Perkinsus marinus*, a causative agent of Dermo disease, is a protozoan parasite that has severely impacted populations of its host the Eastern oyster, *Crassostrea virginica*, from

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southern New England through the Gulf of Mexico. There are four life stages of *P. marinus* that have been described: meront (trophozoite), prezoosporangium (hypnospor), zoosporangium and biflagellated zoospore (Perkins, 1966, 1988). The meront, prezoosporangia and biflagellated zoospore stages can infect oysters via transmission through the water column (Chu, 1996). The meront stage can be found both intracellularly and extracellularly in infected oysters and is thought to be the primary life stage of this parasite (Perkins, 1988; Chu, 1996).

Although *P. marinus* was originally described as a member of the Phylum Apicomplexa based on structural morphology (Perkins, 1976; Levine, 1988), recent genetic analyses suggest that it is more closely related to the dinoflagellates (Goggin and Barker, 1993; Siddall et al., 1997). *P. marinus* meront stage can be cultured axenically in both undefined (Gauthier and Vasta, 1993; Klienschuster and Swink, 1993; La Peyre et al., 1993) and defined media (Gauthier et al., 1995; La Peyre and Faisal, 1997). Cultured meronts have been shown to be capable of infecting oysters (La Peyre et al., 1993) and being sufficiently virulent to cause host mortality (Gauthier and Vasta, 1993).

A key characteristic of the epizootiology of *P. marinus* is the influence of environmental conditions on disease progression. Under conditions of cool water temperatures and/or low salinities disease prevalence and infection intensities remain low. High temperatures and salinities are associated with parasite proliferation and disease outbreaks, both in nature and under laboratory conditions (Andrews, 1988; Andrews and Ray, 1988; Chu et al., 1993; Chu and La Peyre, 1993; Burreson and Ragone-Calvo, 1996; Chu, 1996). In vitro studies have shown that axenic cultures of *P. marinus* proliferate rapidly at temperatures between 20 and 30 °C and salinities over 10 psu (Perkins, 1966; Chu and Greene, 1989; Dungan and Hamilton, 1995; Gauthier and Vasta, 1995). Currently, the relative importance of these environmental conditions on parasite growth versus host immune response is not known.

One factor that may potentially regulate cell replication and disease progression of *P. marinus* is lipid synthesis and acquisition. Cell proliferation requires the formation of new membrane and lipids are a major component of cell membranes. Thus, a reduced ability to acquire or synthesize new lipids may inhibit cell proliferation and stop disease progression. Previous studies have shown that meront stage of *P. marinus* grown in vitro can both acquire and modify lipids from their environment and synthesize fatty acids de novo (Chu et al., 2000, 2002; Soudant and Chu, 2001). When grown axenically in culture containing cod liver oil, which is rich in (n-3) fatty acids, as a lipid source the fatty acid composition of *P. marinus* is a mosaic of media-derived and de novo synthesized fatty acids (Soudant and Chu, 2001). Investigations of the fatty acid synthesis capabilities of *P. marinus* using <sup>13</sup>C acetate as a substrate have revealed that this organism is able to synthesize a range of saturated, (n-9) monounsaturated and (n-6) polyunsaturated fatty acids, but not (n-3) fatty acids (Chu et al., 2002). One of the major fatty acids synthesized by *P. marinus* is arachidonic acid 20:4(n-6), an eicosanoid precursor that is converted into prostaglandins by the human protozoan parasites *Plasmodium falciparum* and *Trypanosoma brucei* (Kubata et al., 1998, 2000). These compounds have been implicated as virulence factors in both *P. falciparum* and *T. brucei* (Kubata et al., 1998, 2000), but have not yet been detected in *P. marinus*.

The purpose of this study was to determine whether fatty acid synthesis by *P. marinus* may contribute to the observations of increased growth and virulence of the parasite at high temperature and high salinity. The effects of these environmental parameters on total fatty acid synthesis rates and the types of fatty acids produced were tested using in vitro cultures of *P. marinus*.

## 2. Materials and methods

### 2.1. Experiments

Experiments were conducted to test the fatty acid synthesis rate in meronts acclimated/incubated at different temperatures (10, 18 or 28 °C) and salinities (14, 20 or 28 psu). All acclimations and incubations were conducted in media instead of seawater. The authors have observed high mortality rates (>90%) when *P. marinus* cells are transferred directly from media into seawater. The temperature and salinity experiments were not conducted at the same time. Different cultures were used for inoculation.

### 2.2. Fatty acid synthesis rate in meronts at 10, 18 or 28 °C

*P. marinus* meront cells from a 7-day-old culture were counted in a hemocytometer and inoculated into fresh medium (585 mmol/kg, 20 psu) (La Peyre et al., 1993) at a concentration of  $10^6$  cells/ml and cultured at 10, 18 or 28 °C for 7 days to acclimate the cultures. This media contains cod liver oil, cholesterol and  $\alpha$  tocopherol at concentrations of 10, 4.5 and 2 mg/l, respectively. The cells were then harvested and used to inoculate 10 ml of fresh media containing either 6 mM Na-1, 2  $^{13}$ C-acetate (Cambridge Isotope Laboratories, Andover, MA) or 6 mM unlabeled Na acetate ( $n=3$ ) at a density of  $10^6$  cells/ml. These new cultures were then incubated at their corresponding acclimation temperature for 3 days before harvesting for fatty acid and protein analyses.

### 2.3. Fatty acid synthesis rate in meronts at 14, 20 or 28 psu

The objective of the salinity experiment was to test the differences in the synthesis rates for fatty acids in *P. marinus* meronts that have been repeatedly subcultured under salinity conditions (i.e., 10, 14 and 28 psu) that are conducive to cell viability and proliferation. Salinities of 10 psu or below were not tested due to the concern of cell viability at low salinity (Burreson et al., 1994; Burreson and Ragone-Calvo, 1996). If cultures were tested under salinity conditions that inhibited cell viability then any observed reduction in fatty acid synthesis rates may be due simply to a reduction in viable cell biomass and not attributable to a physiological response to low salinity.

*P. marinus* meronts were first cultured in different salinity media to be used in the experiment. *P. marinus* meront cells from a 7-day-old culture (585 mmol/kg, 20 psu) (La Peyre et al., 1993) were inoculated into fresh media (La Peyre et al., 1993) each adjusted to three different salinities by the addition of artificial sea salts (Sigma, St Louis, MO) to the media. Inoculated cultures contained  $10^6$  cells/ml. The osmolarities of the media were 375

mmol/kg for the low salinity culture, 585 mmol/kg for the moderate salinity culture and 842 mmol/kg for the high salinity culture. These values correspond to approximately 14, 20 and 28 psu, respectively. After 7 days of acclimation at 28 °C, cells from each treatment were harvested and used to inoculate 10-ml culture flasks with the same media type and salinity containing either 6 mM Na-1, 2 <sup>13</sup>C-acetate (Cambridge Isotope Laboratories, Andover, MA) or 6 mM unlabeled Na acetate ( $n=3$ ). Cell samples from culture flasks for each of the three salinity treatments used for inoculation were counted by hemocytometer and the size of the inoculums used were adjusted accordingly to contain 10<sup>7</sup> cells (10<sup>6</sup> cells/ml). Inoculated culture flasks were incubated at 28 °C for 3 days then harvested for fatty acid and protein analyses.

#### 2.4. Analytical methods

Cell pellets were lyophilized and weighed prior to lipid and protein analyses. Protein contents were determined by the method of [Lowry et al. \(1951\)](#). Total lipids were extracted from cell pellets according to the method of [Bligh and Dyer \(1959\)](#).

Extracted lipids were transesterified in 8-ml Wheaton vials containing 20 µg internal standard (23:0), with 10% BF<sub>3</sub> (w/w) in methanol for 15 min at 95–100 °C ([Metcalf and Schmitz, 1961](#)). The fatty acid methyl esters (FAME) were extracted with carbon disulfide ([Marty et al., 1992](#)), which was evaporated under a stream of nitrogen. Samples were then redissolved in hexane for GC/FID and GC/MS analysis.

Samples were initially analyzed on a Varian model 3300 gas chromatograph (Varian Analytical Instruments, Sunnydale, CA) 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, Folsom, CA). The column was temperature programmed from 60 to 150 °C at 30 °C/min and 150 to 220 °C at 2 °C/min, injector and detector temperatures were 230 and 250 °C, respectively; the flow rates of compressed air and hydrogen were 300 and 30 ml/min. Helium was used as the carrier gas (1.5 ml/min). Identification of FAME 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.

To detect incorporation of stable isotope precursors, FAME were further analyzed qualitatively and quantitatively by mass spectroscopy with a Varian 3400 gas chromatograph equipped with a Varian Saturn 4D GC/MS/MS detector. Methane was used as the reagent gas for positive chemical ionization (CI). The same column used for GC/FID analysis of the FAME samples (J&W DB-WAX, 25 m × 0.32 mm; 0.2 µm film thickness) was used for GC/MS analysis. Carrier gas (helium) flow rate was 1 ml/min. Injection port temperature was 230 °C and the interface was 250 °C. The column was temperature programmed from an initial temperature of 60 °C for a 4-min hold, followed by a 30 °C/min increase to 150 °C and 2 °C/min to 220 °C. Data was collected and processed using Varian Saturn GC/MS software version 5.2. FAME were identified by retention time relative to known standards, fragmentation pattern and mass of the molecular ion. FAME containing <sup>13</sup>C derived from acetate were quantified using standard curves constructed for each FAME standard and ratio with internal standard (23:0). The standard curves were created using four concentrations of each FAME comparable to the concentration of the

sample, along with a fixed amount (20  $\mu\text{g}$ ) of the C23:0 internal standard. The molecular ions in spectra of each FAME were used to quantify masses containing exogenous  $^{13}\text{C}$  relative to the native molecule. Samples from cultures containing non-labeled sodium acetate were also analyzed in an identical manner as negative controls for  $^{13}\text{C}$  incorporation. *P. marinus* meront cultures are not generally synchronous and typically contain a wide range of cell sizes (3–10  $\mu\text{m}$ , Soudant and Chu, 2001). Changes in the distribution of different size classes of cells in cultures differing in age and/or other environmental parameters make cell numbers a rather poor measure of culture biomass. For this reason, fatty acid synthesis rates were expressed as per protein mass rather than cell number. Data were expressed as nanograms of fatty acids containing stable isotope per milligram protein per hour.

### 2.5. Data analysis

Statistical differences between treatments were determined by one-way analysis of variance (ANOVA). When treatment effects were found to be significant at the  $p < 0.05$  level individual comparisons were conducted using Tukey's test. Percentage data were arcsine transformed prior to statistical analysis.

## 3. Results

### 3.1. Growth and fatty acid synthesis rates

Cell proliferation and fatty acid synthesis rates of *P. marinus* cultures differed significantly among temperature treatments (Table 1). Cell numbers in the 10, 18 and 28 °C cultures increased from  $10^6/\text{ml}$  to  $1.32 \pm 0.21$ ,  $5.35 \pm 1.26$  and  $14.57 \pm 0.90 \times 10^6/\text{ml}$ , respectively (Table 1). Similarly, cell proliferation, as measured by total protein, increased with increasing temperature from  $0.21 \pm 0.06$  mg protein/ml culture at 10 °C to  $0.80 \pm 0.22$  and  $1.11 \pm 0.13$  mg protein/ml culture at 18 and 28 °C, respectively. The fatty acid content normalized to protein was significantly higher ( $p < 0.05$ ) at 28 °C ( $20.72 \pm 3.70$   $\mu\text{g}$  fatty acids/mg protein) relative to the 18 °C treatment ( $11.97 \pm 2.91$   $\mu\text{g}$  fatty acids/mg protein) (Table 1). However, the mean fatty acid content per unit protein of the 10 °C treatment was not significantly different from those of the 18 and 28 °C treatment. Similarly, the fatty acid synthesis rates increased with increasing culture

Table 1  
Growth, lipid content and fatty acid synthesis rates for *P. marinus* cultures at 10, 18 and 28 °C

Culture parameter	10 °C	18 °C	28 °C
Culture density ( $10^6$ cells/ml)	$1.32 \pm 0.21^a$	$5.35 \pm 1.26^b$	$14.57 \pm 0.90^c$
mg protein/ml culture	$0.21 \pm 0.06^a$	$0.80 \pm 0.22^b$	$1.11 \pm 0.13^b$
$\mu\text{g}$ fatty acids/mg protein	$16.06 \pm 0.83^{ab}$	$11.97 \pm 2.91^a$	$20.72 \pm 3.70^b$
ng fatty acids synthesized/mg protein/h	$24.36 \pm 3.88^a$	$60.78 \pm 15.74^a$	$144.45 \pm 23.01^b$

Cultures were inoculated at  $10^6$  cells/ml and harvested 72 h later. All values are mean  $\pm$  standard deviation ( $n = 3$ ). Different letters denote treatments that are significantly different at the  $p < 0.05$  level.

temperature (Table 1). The 28 °C treatment fatty acid synthesis rate ( $144.45 \pm 23.01$  ng fatty acid synthesized/mg protein/h) was significantly higher than in the 18 and 10 °C treatments ( $60.78 \pm 15.74$  and  $24.36 \pm 3.88$  ng fatty acid synthesized/mg protein/h, respectively). In contrast to the results from the temperature treatments, salinity had no significant effect on growth, fatty acid content and fatty acid synthesis rates of *P. marinus* (Table 2). Although the 28 °C treatment from the temperature experiment and the 20 psu treatment from the salinity experiment were maintained under identical temperature and salinity conditions the 20 psu salinity treatment had a much higher biomass at harvest than did the 28 °C temperature treatment (Tables 1 and 2). These differences are probably attributable to the fact that different cultures were used for the salinity and temperature experiments. *P. marinus* meront cultures are not generally synchronous and typically contain a wide range of cell sizes (3–10 µm, Soudant and Chu, 2001). The cultures used for the salinity and temperature experiments may have differed in average cell size and/or prevalence of tomonts.

### 3.2. Fatty acid compositions

*P. marinus* cultures incubated at different temperatures exhibited differences in their fatty acid compositions (Table 3). The 10 °C treatment differed from both the 18 and 28 °C treatments by having a significantly higher percentage of monounsaturated fatty acids (MUFA) ( $56.73 \pm 2.48\%$  at 10 °C vs.  $38.79 \pm 0.14\%$  and  $40.06 \pm 0.34\%$ , for the 18 and 28 °C treatments, respectively) (Table 3). Levels of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) were both significantly lower in the 10 °C treatment ( $18.44 \pm 1.70\%$  for SFA and  $21.65 \pm 1.40\%$  for PUFA) than in the 18 °C treatment ( $29.89 \pm 0.27\%$  and  $30.65 \pm 0.15\%$  for SFA and PUFA, respectively) and 28 °C treatment ( $27.94 \pm 0.27\%$  and  $30.01 \pm 0.32\%$  for SFA and PUFA, respectively). The relatively high levels of MUFA in the 10 °C treatment was largely attributable to increased levels of 18:1(n-9) ( $38.98 \pm 1.35\%$ ) compared to the 18 °C ( $25.58 \pm 0.19\%$ ) and 28 °C ( $25.53 \pm 0.24\%$ ) (Table 3). The predominant saturated fatty acids 14:0, 16:0 and 18:0 were all at lower abundance in the 10 °C treatment than at 18 and 28 °C (Table 3). The major difference between treatments in the PUFA was the two-fold higher levels of 20:4(n-6) in the 18 and 28 °C treatments ( $18.89 \pm 0.06\%$  and  $19.85 \pm 0.39\%$ , respectively) relative to the 10 °C treatment ( $9.65 \pm 0.88\%$ ) (Table 3).

Table 2

Growth, lipid content and fatty acid synthesis rates for *P. marinus* cultures at 14, 20 and 28 psu

Culture parameter	Low	Moderate	High
	14 psu	20 psu	28 psu
	(375 mmol/kg)	(585 mmol/kg)	(842 mmol/kg)
mg protein/ml culture	$0.45 \pm 0.03$	$0.47 \pm 0.01$	$0.47 \pm 0.01$
µg fatty acids/mg protein	$41.00 \pm 5.40$	$39.91 \pm 0.36$	$38.69 \pm 3.60$
ng fatty acids synthesized/mg protein/h	$285.38 \pm 42.03$	$264.81 \pm 8.75$	$261.55 \pm 21.75$

All values are mean  $\pm$  standard deviation ( $n=3$ ). Different letters denote treatments that are significantly different at the  $p < 0.05$  level.

Table 3  
Weight % fatty acid composition of *P. marinus* cultures grown at 10, 18 and 28 °C

FAME	10 °C	18 °C	28 °C
14:0	0.75 ± 0.10 <sup>a</sup>	5.04 ± 0.27 <sup>b</sup>	5.54 ± 0.11 <sup>b</sup>
15:0	0.40 ± 0.04 <sup>b</sup>	0.82 ± 0.01 <sup>c</sup>	0.24 ± 0.01 <sup>a</sup>
16:0	8.81 ± 0.49 <sup>a</sup>	12.68 ± 0.12 <sup>c</sup>	11.60 ± 0.27 <sup>b</sup>
17:0	0.49 ± 0.06 <sup>b</sup>	0.63 ± 0.01 <sup>b</sup>	0.14 ± 0.12 <sup>a</sup>
18:0	3.30 ± 0.57 <sup>a</sup>	5.23 ± 0.18 <sup>b</sup>	4.75 ± 0.08 <sup>b</sup>
20:0	0.88 ± 0.19 <sup>a</sup>	1.51 ± 0.04 <sup>b</sup>	1.07 ± 0.01 <sup>a</sup>
22:0	0.59 ± 0.12	–	–
24:0	3.02 ± 1.85	3.98 ± 0.10	4.75 ± 0.20
16:1(n – 9)	0.80 ± 0.05 <sup>b</sup>	0.68 ± 0.03 <sup>a</sup>	0.90 ± 0.07 <sup>b</sup>
16:1(n – 7)	2.67 ± 0.15 <sup>a</sup>	3.25 ± 0.01 <sup>b</sup>	2.48 ± 0.03 <sup>a</sup>
18:1(n – 9)	38.98 ± 1.35 <sup>b</sup>	25.58 ± 0.19 <sup>a</sup>	25.53 ± 0.24 <sup>a</sup>
18:1(n – 7)	4.26 ± 0.17 <sup>c</sup>	3.74 ± 0.06 <sup>b</sup>	2.83 ± 0.04 <sup>a</sup>
20:1(n – 11)	1.18 ± 0.09 <sup>b</sup>	1.04 ± 0.03 <sup>b</sup>	0.25 ± 0.22 <sup>a</sup>
20:1(n – 9)	5.71 ± 0.35 <sup>b</sup>	3.17 ± 0.05 <sup>a</sup>	5.90 ± 0.14 <sup>b</sup>
20:1(n – 7)	–	–	–
22:1(n – 11)	0.90 ± 0.10 <sup>b</sup>	0.91 ± 0.11 <sup>b</sup>	0.69 ± 0.01 <sup>a</sup>
22:1(n – 9)	1.53 ± 0.42 <sup>b</sup>	0.43 ± 0.02 <sup>a</sup>	1.49 ± 0.14 <sup>b</sup>
18:2(n – 6)	2.01 ± 0.08 <sup>a</sup>	5.13 ± 0.08 <sup>c</sup>	3.81 ± 0.03 <sup>b</sup>
20:2(n – 9)	1.32 ± 0.25	1.53 ± 0.06	1.16 ± 0.13
20:2(n – 6)	2.36 ± 0.22	2.04 ± 0.04	1.82 ± 0.67
22:2(n – 6)	–	–	–
20:3(n – 6)	0.21 ± 0.00 <sup>a</sup>	1.08 ± 0.02 <sup>c</sup>	0.64 ± 0.01 <sup>b</sup>
20:4(n – 6)	9.65 ± 0.88 <sup>a</sup>	18.89 ± 0.06 <sup>b</sup>	19.85 ± 0.39 <sup>b</sup>
20:5(n – 3)	2.65 ± 0.39 <sup>c</sup>	1.27 ± 0.02 <sup>a</sup>	1.70 ± 0.03 <sup>b</sup>
22:5(n – 3)	0.96 ± 1.66	0.28 ± 0.07	0.22 ± 0.19
22:6(n – 3)	1.25 ± 1.01	0.43 ± 0.05	0.82 ± 0.01
Total SFA	18.44 ± 1.70 <sup>a</sup>	29.89 ± 0.27 <sup>b</sup>	27.94 ± 0.27 <sup>b</sup>
Total MUFA	56.73 ± 2.48 <sup>b</sup>	38.79 ± 0.14 <sup>a</sup>	40.06 ± 0.34 <sup>a</sup>
Total PUFA	21.65 ± 1.40 <sup>a</sup>	30.65 ± 0.15 <sup>b</sup>	30.01 ± 0.32 <sup>b</sup>

All values are mean ± standard deviation ( $n=3$ ). Different letters denote significant differences at the  $p < 0.05$  level. SFA=saturated fatty acids, MUFA=monounsaturated fatty acids, PUFA=polyunsaturated fatty acids.

The salinity treatments also exhibited differences in fatty acid compositions, but the relationship between salinity level and fatty acid modulation was rather ambiguous (Table 4). The moderate salinity treatment had a lower percentage of SFA ( $28.89 \pm 0.29\%$ ) than either the low salinity or high salinity treatments ( $32.83 \pm 0.26\%$  and  $33.10 \pm 0.59\%$ , respectively) (Table 4). The lower levels of SFA in the moderate salinity treatment were attributable to significantly lower levels of 14:0, 16:0, 18:0 and 20:0 relative to the low and high salinity treatments. Levels of monounsaturates increased significantly with increasing salinity from  $37.23 \pm 0.06\%$  at low salinity to  $41.01 \pm 0.29\%$  at the moderate salinity and  $44.10 \pm 0.43\%$  at the high salinity (Table 4). Conversely, the levels of polyunsaturates were significantly lower in the high salinity treatment ( $20.75 \pm 0.81\%$ ) relative to both the low and moderate salinity treatments ( $28.20 \pm 0.30\%$  and  $28.09 \pm 1.08\%$ , respectively) (Table 4). The largest differences between treatments in the percentages of monounsaturates were the higher levels of 16:1(n – 7) and 20:1(n – 9) in the high salinity treatment. Differences in the polyunsaturate composition were

Table 4

Weight % fatty acid composition of *P. marinus* cultures grown at 14, 20 and 28 psu

FAME	Low	Moderate	High
	14 psu	20 psu	28 psu
	(375 mmol/kg)	(585 mmol/kg)	(842 mmol/kg)
14:0	6.90 ± 0.20 <sup>b</sup>	5.80 ± 0.27 <sup>a</sup>	7.82 ± 0.72 <sup>b</sup>
15:0	0.15 ± 0.13	0.25 ± 0.01	0.30 ± 0.01
16:0	13.71 ± 0.05 <sup>b</sup>	12.76 ± 0.13 <sup>a</sup>	14.55 ± 0.03 <sup>c</sup>
17:0	–	–	–
18:0	7.04 ± 0.13 <sup>b</sup>	5.22 ± 0.45 <sup>a</sup>	5.89 ± 0.13 <sup>a</sup>
20:0	1.45 ± 0.02 <sup>b</sup>	1.22 ± 0.14 <sup>a</sup>	1.27 ± 0.03 <sup>ab</sup>
22:0	–	–	–
24:0	3.57 ± 0.22	3.64 ± 0.43	3.27 ± 0.05
16:1(n – 9)	0.51 ± 0.01 <sup>a</sup>	0.91 ± 0.02 <sup>c</sup>	0.65 ± 0.02 <sup>b</sup>
16:1(n – 7)	1.67 ± 0.03 <sup>a</sup>	2.66 ± 0.07 <sup>b</sup>	4.00 ± 0.05 <sup>c</sup>
18:1(n – 9)	24.60 ± 0.10 <sup>a</sup>	25.46 ± 0.22 <sup>b</sup>	25.28 ± 0.32 <sup>b</sup>
18:1(n – 7)	1.90 ± 0.00 <sup>a</sup>	3.16 ± 0.08 <sup>c</sup>	2.86 ± 0.02 <sup>b</sup>
20:1(n – 11)	0.35 ± 0.02 <sup>b</sup>	0.36 ± 0.02 <sup>b</sup>	0.26 ± 0.04 <sup>a</sup>
20:1(n – 9)	6.22 ± 0.03 <sup>a</sup>	6.32 ± 0.38 <sup>a</sup>	8.74 ± 0.16 <sup>b</sup>
20:1(n – 7)	–	–	0.05 ± 0.09
22:1(n – 11)	0.96 ± 0.03 <sup>b</sup>	0.76 ± 0.11 <sup>a</sup>	0.74 ± 0.02 <sup>a</sup>
22:1(n – 9)	1.03 ± 0.03 <sup>a</sup>	1.38 ± 0.13 <sup>b</sup>	1.51 ± 0.04 <sup>b</sup>
18:2(n – 6)	4.87 ± 0.05 <sup>b</sup>	4.26 ± 0.45 <sup>ab</sup>	3.85 ± 0.05 <sup>a</sup>
20:2(n – 6)	1.32 ± 0.15 <sup>a</sup>	1.78 ± 0.25 <sup>b</sup>	1.64 ± 0.07 <sup>ab</sup>
20:2(n – 9)	1.32 ± 0.02 <sup>b</sup>	1.17 ± 0.09 <sup>a</sup>	1.17 ± 0.03 <sup>a</sup>
22:2(n – 6)	–	–	0.31 ± 0.53
20:3(n – 6)	0.85 ± 0.01 <sup>b</sup>	0.68 ± 0.02 <sup>a</sup>	0.66 ± 0.01 <sup>a</sup>
20:4(n – 6)	19.84 ± 0.20 <sup>b</sup>	19.40 ± 0.54 <sup>b</sup>	13.12 ± 0.16 <sup>a</sup>
20:5(n – 3)	–	0.61 ± 0.09	–
22:5(n – 3)	–	–	–
22:6(n – 3)	–	0.18 ± 0.05	–
Total SFA	32.83 ± 0.26 <sup>b</sup>	28.89 ± 0.74 <sup>a</sup>	33.10 ± 0.59 <sup>b</sup>
Total MUFA	37.23 ± 0.06 <sup>a</sup>	41.01 ± 0.29 <sup>b</sup>	44.10 ± 0.43 <sup>c</sup>
Total PUFA	28.20 ± 0.30 <sup>b</sup>	28.09 ± 1.08 <sup>b</sup>	20.75 ± 0.81 <sup>a</sup>

All values are mean ± standard deviation ( $n=3$ ). Different letters denote significant differences at the  $p < 0.05$  level. SFA=saturated fatty acids, MUFA=monounsaturated fatty acids, PUFA=polyunsaturated fatty acids.

primarily due to significantly lower levels of 20:4(n – 6) in the high salinity treatment ( $13.12 \pm 0.16\%$ ) relative to the low ( $19.84 \pm 0.20\%$ ) and moderate ( $19.40 \pm 0.54\%$ ) salinity treatments.

### 3.3. Synthesis rates for individual fatty acids

Incorporation of  $^{13}\text{C}$  from acetate was detected in 14:0, 16:0, 18:0, 20:0, 24:0, 18:1(n – 9), 20:1(n – 9), 18:2(n – 6), 20:2(n – 6) and 20:4(n – 6) (Tables 5 and 6). In the temperature treatments synthesis rates for all fatty acids with detectable incorporation were significantly higher at 28 °C relative to 10 °C (Table 5). Synthesis rates for the 18 °C treatment were between those of the 10 and 28 °C treatments for all fatty acids except 20:1(n – 9), which did not have detectable incorporation in the 18 °C treatment (Table 5).

Table 5

Rate of fatty acid synthesis of *P. marinus* cultures at 10, 18 and 28 °C

Fatty acid	10 °C	18 °C	28 °C
14:0	0.59 ± 0.30 <sup>a</sup>	5.69 ± 1.42 <sup>b</sup>	10.61 ± 1.30 <sup>c</sup>
16:0	2.86 ± 0.16 <sup>a</sup>	14.78 ± 3.29 <sup>b</sup>	24.91 ± 3.69 <sup>c</sup>
18:0	3.88 ± 0.89 <sup>a</sup>	6.99 ± 1.52 <sup>a</sup>	11.97 ± 2.39 <sup>b</sup>
20:0	–	–	1.22 ± 2.11
24:0	–	–	9.90 ± 2.51
18:1(n – 9)	12.08 ± 1.48 <sup>a</sup>	13.68 ± 3.09 <sup>a</sup>	38.23 ± 4.63 <sup>b</sup>
20:1(n – 9)	1.97 ± 0.43 <sup>a</sup>	–	9.02 ± 0.40 <sup>b</sup>
18:2(n – 6)	–	–	4.71 ± 1.41
20:2(n – 6)	–	–	0.92 ± 1.59
20:4(n – 6)	2.98 ± 5.17 <sup>a</sup>	19.64 ± 7.17 <sup>b</sup>	32.96 ± 5.89 <sup>b</sup>
Total SFA	7.33 ± 0.91 <sup>a</sup>	27.47 ± 6.19 <sup>b</sup>	58.61 ± 11.88 <sup>c</sup>
Total MUFA	14.05 ± 1.07 <sup>a</sup>	13.68 ± 3.09 <sup>a</sup>	47.25 ± 4.95 <sup>b</sup>
Total PUFA	2.98 ± 5.17 <sup>a</sup>	19.64 ± 7.17 <sup>a</sup>	38.59 ± 7.66 <sup>b</sup>

Rates of the synthesis of fatty acids containing <sup>13</sup>C from the sodium acetate substrate are expressed as ng fatty acid/mg protein/h (*n* = 3). Different letters denote significant differences between treatments (*p* < 0.05). SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.

At 10 °C, the highest synthesis rate was for 18:1(n – 9) (12.08 ± 1.48 ng/mg protein/h) with the next highest synthesis rate for 18:0 (3.88 ± 0.89 ng/mg protein/h), which is presumably the precursor to 18:1(n – 9). In the 18 °C treatment, the highest rate of synthesis was detected in 20:4(n – 6) (19.64 ± 7.17 ng/mg protein/h) followed by 16:0 and 18:1(n – 9) (14.78 ± 3.29 and 13.68 ± 3.09 ng/mg protein/h, respectively). In the 28 °C treatment, the highest synthesis rates were found in 18:1(n – 9) and 20:4(n – 6) (38.23 ± 4.36 and 32.96 ± 5.89 ng/mg protein/h, respectively).

Table 6

Rate of fatty acid synthesis of *P. marinus* cultures at 14, 20 and 28 psu

Fatty acid	Low	Moderate	High
	14 psu (375 mmol/kg)	20 psu (585 mmol/kg)	28 psu (842 mmol/kg)
14:0	27.13 ± 2.69	2.92 ± 1.24	29.13 ± 5.05
16:0	59.72 ± 7.09	53.75 ± 2.41	60.46 ± 4.84
18:0	34.14 ± 4.48 <sup>b</sup>	25.58 ± 2.44 <sup>a</sup>	27.76 ± 2.02 <sup>ab</sup>
20:0	nd	nd	nd
24:0	15.34 ± 2.65	15.74 ± 1.94	13.56 ± 0.69
18:1(n – 9)	70.73 ± 10.04	65.61 ± 2.56	63.51 ± 5.29
20:1(n – 9)	12.25 ± 10.73	17.93 ± 0.81	23.79 ± 1.84
18:2(n – 6)	nd	nd	nd
20:2(n – 6)	nd	nd	nd
20:4(n – 6)	66.07 ± 10.01 <sup>b</sup>	63.27 ± 3.92 <sup>b</sup>	43.35 ± 2.47 <sup>a</sup>
Total SFA	136.33 ± 16.30	117.99 ± 4.70	130.90 ± 12.47
Total MUFA	82.98 ± 17.56	83.55 ± 3.32	87.30 ± 7.09
Total PUFA	66.07 ± 10.01 <sup>b</sup>	63.27 ± 3.92 <sup>b</sup>	43.35 ± 2.47 <sup>a</sup>

Rates of the synthesis of fatty acids containing <sup>13</sup>C from the sodium acetate substrate are expressed as ng fatty acid/mg protein/h (*n* = 3). Different letters denote significant differences between treatments (*p* < 0.05). SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.

In the salinity effect experiment, there were only two significant differences in fatty acid synthesis rates between treatments (Table 6). The low salinity treatment had a significantly higher synthesis rate for 18:0 relative to the moderate salinity treatment ( $34.14 \pm 4.48$  and  $25.58 \pm 2.44$  ng/mg protein/h, respectively) with the high salinity treatment exhibiting a synthesis rate intermediate between the two (Table 6). The other difference in synthesis rate between treatments was for 20:4(n – 6). The synthesis rate of this fatty acid in the high salinity treatment was significantly lower ( $43.35 \pm 2.47$  ng/mg protein/h) relative to the moderate and low salinity treatments ( $63.27 \pm 3.92$  and  $66.07 \pm 10.01$  ng/mg protein/h, respectively) (Table 6). The latter two salinity treatments had similar 20:4(n – 6) synthesis rates.

#### 4. Discussion

The Eastern oyster inhabits and propagates in temperate, euryhaline waters. As such they are subject to great changes in the temperature and salinity of their environment on both a daily and seasonal basis. Previous findings have demonstrated that *P. marinus* growth and disease progression are correlated with high water temperatures and salinities (Andrews, 1988; Andrews and Ray, 1988; Chu et al., 1993; Chu and La Peyre, 1993; Burreson and Ragone-Calvo, 1996; Chu, 1996). Of these two factors, temperature has been shown to have the greatest influence on disease progression (Andrews and Ray, 1988; Burreson and Ragone-Calvo, 1996; Chu and Volety, 1997). In Chesapeake Bay, the intensity of infection levels generally starts to rise in June with increasing water temperatures and peaks in August/September when water temperatures approach 30 °C (Burreson and Ragone-Calvo, 1996). Dermo-associated mortality is also high in late summer and peaks when infection intensities are highest (Burreson and Ragone-Calvo, 1996). In vitro cultured *P. marinus* are readily maintained and proliferate at 21–28 °C (Volety, 1995).

Previous studies on the physiology of *P. marinus* have demonstrated that this organism has remarkable lipid biosynthesis capabilities compared to other protozoan parasites (Soudant and Chu, 2001; Chu et al., 2002; Lund and Chu, 2002). In vitro cultured *P. marinus* have been shown to be capable of assimilating and modifying lipids from media and synthesizing both fatty acids and phospholipids de novo (Chu et al., 2000, 2002; Soudant and Chu, 2001; Lund and Chu, 2002). Most protozoan parasites, such as *Giardia lamblia*, have no ability to synthesize fatty acids and must acquire them from their host (Meyer and Holz, 1966; Haughan and Goad, 1991; Ellis et al., 1996; Lujan et al., 1996; Stevens et al., 1997). Others, such as *P. falciparum*, *Trypanosoma cruzi* and *Leishmania donovani*, can synthesize fatty acids de novo, but have limited ability for chain elongation and desaturation (Aeberhard et al., 1981; Jacobs et al., 1982; Surolia and Surolia, 2001).

The fatty acids synthesized by *P. marinus* are highly unusual for a marine organism. The predominant PUFA produced is arachidonic acid, 20:4(n – 6), an eicosanoid precursor (Soudant and Chu, 2001; Chu et al., 2002). This lipid profile is unexpected since (n – 6) fatty acids are typical of “terrestrial” organisms (Gurr and Harwood, 1991). The host oyster has a more typical marine fatty acid profile characterized by an abundance of

PUFAs of the (n – 3) series (>26%) and low levels of 20:4(n – 6) (5%) (Chu and Greaves, 1991; Soudant and Chu, 2001).

The present study also found that 20:4(n – 6) was the predominant PUFA in *P. marinus* at all temperature and salinity treatments (Tables 3 and 4). The percentage of 20:4(n – 6) ranged from a low of 9.6% in the 10 °C treatment to a high of 19.9% in the 28 °C treatment (Table 3). In a separate study of *P. marinus* cultured at 10, 18 and 28 °C 20:4(n – 6) was also the most abundant PUFA, ranging from a low of 3.3% of total fatty acids at 10 °C to a high of 17.2% at 18 °C (Chu et al., 2003). Although these values are somewhat lower than reported here, in both studies 20:4(n – 6) content was significantly lower in 10 °C treatments than at 18 and 28 °C. Interestingly, both the percent content and synthesis rate for 20:4(n – 6) were significantly lower in the highest salinity tested relative to the two lower salinity treatments (Tables 4 and 6).

Results from the current study indicate that not only is proliferation of *P. marinus* positively correlated to temperature, but the fatty acid content is also higher at warmer temperatures when normalized to protein content (Table 1). Previous studies have also shown that the lipid metabolism of *P. marinus* is significantly affected by temperature (Chu et al., 2003). When *P. marinus* cultures were acclimated to 10, 18 and 28 °C and incubated with fluorescently labeled palmitic acid (FL C16:0) and phosphatidylcholine (FL PC) incorporation of the fluorescent lipids into meront cells increased with increasing temperature (Chu et al., 2003). Together, these findings indicate that cell proliferation, endogenous fatty acid synthesis and the uptake of exogenous lipids are all highest at 28 °C, the highest tested temperature. This temperature is comparable to average late summer water temperatures in Chesapeake Bay, when *P. marinus* prevalence and infection intensity in wild oysters is highest (Bureson and Ragone-Calvo, 1996).

Interestingly, the synthesis rate for total fatty acids increases approximately two-fold for every 10 °C increase in temperature (Table 1). The relationship between temperature and rate of a reaction is often expressed as  $Q_{10}$ .  $Q_{10}$  is the rate of a reaction at  $T^{\circ} + 10^{\circ}$  divided by the rate at  $T^{\circ}$ . A  $Q_{10}$  value of around 2.0 is common for many biochemical processes (Hochochka and Somero, 1984). If, in fact, the  $Q_{10}$  for fatty acid synthesis in *P. marinus* is roughly equal to 2, then the data from the present study suggest a lack of ability to increase enzyme titer to boost the fatty acid synthesis rate at cold temperatures.

Findings from a separate study indicating that *P. marinus* at 10 °C have a lower percentage of SFA and a higher percentage of MUFA in comparison to cultures grown at higher temperatures (Chu et al., 2003) are further supported by the results of the present study. The increased MUFA and decreased SFA at 10 °C are consistent with this being a homeoviscous adaptation to maintain membrane fluidity at reduced temperatures (Gurr and Harwood, 1991). The low levels of PUFA observed in the present study are also consistent with the results of the separate study (Chu et al., 2003). The observed changes in MUFA and PUFA levels in response to temperature were primarily due to the increased percentage of 18:1(n – 9) and decreased percentage of 20:4(n – 6) in the 10 °C treatment relative to the 18 and 28 °C treatments (Table 3). It is possible that increasing levels of MUFA and decreasing levels of SFA at low temperatures allow *P. marinus* meronts to increase membrane fluidity without the use of additional PUFA. Similar trends in synthesis rates for 18:1(n – 9) and 20:4(n – 6) (Table 5) suggest that these results are primarily a reflection of endogenous fatty acid synthesis and not selective incorporation of fatty acids from the media. Further research

to determine the localization of synthesized fatty acids within particular lipid classes is still required to fully understand the contribution of different fatty acids to the mechanism of homeoviscous adaptation in *P. marinus*.

The differences in 18:1(n-9) and 20:4(n-6) synthesis at different temperatures suggest a temperature-sensitive component to the desaturase/elongation capabilities of *P. marinus*. The synthesis rates for 18:1(n-9) at 10 and 18 °C are not significantly different ( $12.08 \pm 1.48$  and  $13.68 \pm 3.09$  ng/mg protein/h, respectively), but the synthesis rates for 20:4(n-6) are vastly different ( $2.98 \pm 5.17$  and  $19.64 \pm 7.17$  ng/mg protein/h, respectively). Viewed separately, neither rate is consistent with the total synthesis rates doubling with a roughly 10 °C increase in temperature. However, if the synthesis rates for these two fatty acids are combined by summing the values and comparing the combined rates at 10 and 18 °C (mean rates of 15.1 and 33.3 ng/mg protein/h, respectively), the relationship seen for total synthesis rates is restored. This suggests that flux through the synthesis pathway for fatty acids up to 18:1(n-9) is regulated primarily by environmental temperature while further desaturation is inhibited in the 10 °C treatment.

Other studies have noted temperature-induced changes in desaturase activity in a number of protozoans. It has been hypothesized that observed changes in the ratios of saturated to unsaturated fatty acids of *T. cruzi* transferred from 28 to 37 °C is due to down regulation of  $\Delta 9$  and  $\Delta 12$  desaturase activities (Florin-Christensen et al., 1997). In the free living amoeba, *Acanthamoeba castellanii*, an increase in lipid desaturation was noted when the growth temperature was shifted downwards from 30 to 15 °C resulting in a rapid and marked increase in the synthesis of PUFA (Jones et al., 1991; Avery et al., 1994). Fatty acyl  $\Delta 12$  desaturation in *A. castellanii* was induced when culture temperatures were stepped down from 30 to 15 °C (Jones et al., 1993). These results suggest that temperature plays an important role in the regulation of desaturase activity in protozoans.

The regulation of 20:4(n-6) synthesis in *P. marinus* may be an important factor in determining virulence of the parasite. Arachidonic acid is an important precursor to a wide range of eicosanoids including several prostaglandins, thromboxanes and leukotrienes (Samuelsson et al., 1978; Hammerström, 1983). Although eicosanoids have not yet been reported in *P. marinus*, they have been implicated as virulence factors in several protozoan parasites including *P. falciparum* and *T. brucei* (Kubata et al., 1998, 2000). The function and fate of synthesized arachidonic acid in *P. marinus* clearly requires further investigation.

Unlike the temperature treatments, the salinity treatments did not result in clear treatment effects for fatty acid composition and synthesis rates. Most studies on salinity effects on lipid composition have been conducted using bacteria or yeast. Very little has been published on the effects of salinity on fatty acid composition of marine protozoans. The marine ciliate *Parauronema acutum* grown in culture at NaCl concentrations of 0, 1.5 and 5% exhibited much higher HUFA levels at the intermediate salinity level (Sul and Erwin, 1998). Another study on *Chlorella* found large changes in PUFA content and composition with cultures raised at different temperatures, but little variation in fatty acid composition in cultures raised at salinities ranging from 4 to 30 psu (Teshima et al., 1983). The most interesting observation on salinity effects from the present study is the significantly lower levels of total 20:4(n-6) and synthesized 20:4(n-6) in the high salinity treatment relative to the moderate and low salinity treatments (Tables 4 and 6).

One factor that may help explain the difference between the strong effect of salinity on virulence in vivo and minor effects on fatty acid synthesis in vitro for *P. marinus* is the range of salinities tested. The lowest salinity tested (14 psu, 375 mmol/kg) is still within the range of salinities tolerated by *P. marinus*. In vitro cultured *P. marinus* have been shown to proliferate at salinities ranging from 340 to 1930 mmol/kg (10–60 psu, Dungan and Hamilton, 1995). Thus, the lowest salinity tested in this study may not have been low enough to produce physiologically relevant treatment effects. However, salinities much below 14 psu may affect cell viability (Burreson and Ragone-Calvo, 1996). It is also possible that the reduced pathogenicity of *P. marinus* in vivo under low salinity conditions may be due to factors other than parasite virulence and growth. A laboratory study revealed that temperature was the most important factor, followed by infective parasite concentration and salinity, in determining the susceptibility of oysters to *P. marinus* infection (Chu and Volety, 1997).

## 5. Conclusion

In summary, our results suggest that fatty acid synthesis rates in *P. marinus* are positively correlated to water temperature. Furthermore, synthesis rates for the eicosanoid precursor, arachidonic acid, are suppressed at 10 °C; a temperature at which parasite load decreases over time in infected oysters (Burreson and Ragone-Calvo, 1996). At 10 °C, the primary unsaturated fatty acid synthesized is 18:1(n–9), whereas at 18 and 28 °C arachidonic acid is the primary unsaturate synthesized. Salinity had little effect on fatty acid synthesis other than a decrease in arachidonic acid content and synthesis rate in the highest salinity treatment (28 psu). These findings suggest that it is possible that fatty acid synthesis may be a contributing factor to the pathogenicity of *P. marinus* under conditions of high water temperature, however, further study of the metabolic fate of synthesized arachidonic acid is required to confirm or refute this relationship.

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