

Lipid Class and Fatty Acid Composition of the Protozoan Parasite of Oysters, *Perkinsus marinus* Cultivated in Two Different Media

PHILIPPE SOUDANT¹ and FU-LIN E. CHU

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

ABSTRACT. The meront stage of the oyster protozoan parasite, *Perkinsus marinus*, cultivated in two media with different fatty acid profiles was analyzed for its fatty acid and lipid class composition. The composition of fatty acids in the prezoosporangium stage of the parasite as well as that of the host oyster were investigated. Although the lipid class composition of meronts was dominated by phospholipids and triacylglycerol, there was no triacylglycerol detected in either culture medium. Despite the difference in fatty acid composition of the two media, the fatty acid composition of meronts in each medium was dominated by 14:0, 16:0, 18:0, 18:1(n-9), 20:1(n-9), 18:2(n-6) and 20:4(n-6), a profile that differed from its host. The quantities of total lipids and fatty acids in meronts increased as the number of meronts increased and far exceeded the initial amounts in the media and in the initial cell inoculum. The meronts harvested 25 d post-inoculation, had about 3 to 6 times higher total lipids and 4 to 13 times higher fatty acids than the amounts contained in the media. The fatty acid profiles of both prezoosporangia and oysters resembled each other and consisted primarily of 16:0, 20:4(n-6), 20:5(n-3), 22:2Δ7,15, and 22:6(n-3). These results indicate that during meront proliferation, the parasite synthesizes certain fatty acids and lipid classes. For development from meront to prezoosporangium, the parasite may rely on its host for lipid resources.

Key Words. Bivalve, fatty acids, host lipids, lipid synthesis, marine parasite, meront, parasite culture, parasitic protozoan, *Perkinsus marinus*, prezoosporangium.

THE protozoan, *Perkinsus marinus*, is presently the most prevalent parasite of the eastern oyster *Crassostrea virginica* in mid-Atlantic waters. Since the 1950s it has caused severe oyster mortality on the East Coast of the United States. The disease caused by *P. marinus* is infectious and can be transmitted from infected to uninfected oysters (see review by Chu 1996). Four life stages, meront, prezoosporangium (hypnosporangia), zoosporangium, and biflagellated zoospore have been identified and described (Perkins 1966, 1988). Three life stages, meront, prezoosporangium, and biflagellated zoospore are infective (Chu 1996). The uninucleate meront is also called a “trophozoite” (Perkins 1996). Meronts, which are the primary agents for disease transmission (Chu 1996; Perkins 1988), are often found in the phagosomes of hemocytes, and in infected oyster tissues, both intercellularly and intracellularly. Recent technical advances in culturing *in vitro* the meront stage in defined media (Gauthier and Vasta 1993; Kleinschuster and Swink 1993; La Peyre, Faisal, and Burreson 1993) make it possible to characterize this life stage biochemically and physiologically. Prezoosporangia, which develop from meronts, are sometimes observed in moribund and dead oyster tissues. When tissue-associated meronts are placed in fluid thioglycollate medium (FTM) for 4 to 5 d, they develop into prezoosporangia. After incubating thioglycollate-cultured prezoosporangia in estuarine or sea water (20–22 ppt) for 5 to 6 d, zoosporulation (production of biflagellated zoospores) usually occurs.

Lipids play a vital role in long-term survival and in the completion of the life cycle of endogenous parasites (Furlong 1991; Vial and Ancelin 1998). Generally, parasitic protozoa require an exogenous source of essential lipids for growth, differentiation, and life cycle completion. While some parasitic proto-

zoans have a fatty acid composition reflecting that of their hosts or the cultivation medium (Dixon and Williamson 1970), some parasites are capable of modifying exogenous fatty acids; thus, they may produce fatty acid profiles which are distinct from that of their hosts and the culture media. For example, trypansomatids can desaturate and elongate saturated fatty acids from exogenous sources (Dixon, Ginger, and Williamson 1971; Haughan and Goad 1991; Holz 1985; Korn, Greenblatt, and Lees 1965; Lindmark et al. 1991; Meyer and Holz 1966). *Giardia lamblia* is able to desaturate oleic acid to linoleic and linolenic acids (Ellis et al. 1996). It is believed that *Cryptosporidium parvum* converts oleate to linoleate (Mitschler, Welti, and Upton 1994). *Plasmodium falciparum*, to some extent, can alter exogenous fatty acids via desaturation, elongation, and decarboxylation and has a fatty acid composition different from its host (Wunderlich et al. 1991; Vial and Ancelin 1998).

Both meront and prezoosporangium stages of *Perkinsus marinus* are characterized by an abundance of refractile bodies, i.e. lipid droplets, which are, presumably, lipid/fatty acid reservoirs serving as energy sources for proliferation, development, and completion of the parasitic life cycle. Although its host, the oyster, is an ecologically and economically important aquatic species, almost nothing is known about the lipid and fatty acid composition of *Perkinsus*. Our previous study (Chu et al. 2000) demonstrated that both meront and prezoosporangium stages of *P. marinus* incorporated and modified fluorescent lipid analogs from culture media. The objective of the present study was to characterize the fatty acid and lipid class composition of *P. marinus* meronts cultivated in two different media. The fatty acid composition of prezoosporangia and the host oyster were also analyzed.

MATERIALS AND METHODS

In vitro meront cultures. *Perkinsus marinus* meronts were grown in two different culture media: 1) Medium 1 (M1)—a modified DMEM:HAM’s F-12 medium (GIBCO BRL, Gaithersburg, MD) containing 5% (v/v) fetal bovine serum (FBS) (Gauthier and Vasta 1993), and 2) Medium 2 (M2)—a medium described by La Peyre, Faisal, and Burreson (1993). Lipids in these two media were from different sources. The lipids of M1 were derived from the FBS component of the medium. Lipid analyses conducted on M1 revealed a concentration of 58.9 ± 6.0 μg lipid/ml comprised of 46.2 μg steryl esters/ml and 13.9 μg phospholipids/ml. Neither triacylglycerol nor cholesterol were detected in this medium by the methods used. Medium M2 contained no bovine fetal serum, although it did contain

Corresponding Author: F.-L. E. Chu—Telephone number: 1-804-684-7349; FAX number: 1-804-684-7186; E-mail: chu@vims.edu

Abbreviations: CHE, cholesteryl ester; CHO, cholesterol; DAG, diacylglycerol; FA, fatty alcohol; FAME, fatty acid methyl ester; FFA, Free fatty acids; FTM, fluid thioglycollate medium; GLC, gas liquid chromatography; MAG, monoacylglycerol; M1, Medium 1; M2, Medium 2; MS, mass spectrometry; MUFAs, monounsaturated fatty acids; PC, Phosphatidylcholine; PL, phospholipid; PM1, *Perkinsus marinus* cells cultured in Medium 1; PM2, *Perkinsus marinus* cells cultured in Medium 2; PUFAs, polyunsaturated fatty acids; TAG, triacylglycerol; TL, total lipid; TLC, thin layer chromatography; YRW, York River water.

¹ Current address: UMR 6539, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Technopole Brest Iroise, Place Nicolas Copernic, 29280 Plouzané, France.

Table 1. Cell numbers (million of cells/ml) of *Perkinsus marinus*, triacylglycerol (TAG), phospholipids (PL), and total lipid (TL) contents (μg lipid class/ 10^6 cells) in meronts cultivated in Media (M1 & M2) and harvested 1–25 days post-inoculation. Values designated by different superscripted letters are statistically significant from one another ($p \leq 0.05$).

	Medium 1										Medium 2							
	Day 1		Day 6		Day 8		Day 11		Day 15		Day 25		Day 11		Day 15		Day 25	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Cell number ($10^6/\text{ml}$)	0.9 ^a	0.0	30.7 ^b	1.5	42.4 ^c	4.3	61.8 ^d	1.0	79.8 ^d	8.5	87.9 ^d	18.9	0.8 ^a	0.1	3.5 ^b	1.4	5.7 ^c	1.1
μg TAG/ 10^6 cells	—	—	0.9 ^a	0.0	1.4 ^b	0.2	1.8 ^c	0.2	2.2 ^d	0.2	3.0 ^e	0.3	3.5	1.4	4.2	1.4	5.4	0.6
μg PL/ 10^6 cells	3.9 ^a	0.8	1.5 ^b	0.1	1.2 ^c	0.1	1.0 ^{cd}	0.0	0.8 ^d	0.1	0.8 ^d	0.2	1.6	0.1	2.1	0.5	1.5	0.3
μg TL/ 10^6 cells	3.9 ^a	0.8	2.9 ^b	0.2	2.9 ^b	0.2	3.0 ^b	0.2	3.1 ^b	0.2	3.9 ^a	0.3	5.1	1.5	6.3	1.7	6.9	0.3

amino acid mixtures; the added 1% (v/v) of lipid concentrate (100 \times GIBCO) in M2 contained 450 $\mu\text{g}/\text{ml}$ of cholesterol, 1,000 $\mu\text{g}/\text{ml}$ of cod liver oil, and 200 $\mu\text{g}/\text{ml}$ α -tocopherol-acetate. Based on the amount of lipid added to the medium, the lipid concentration of M2 was estimated as 14.5 μg lipid/ml. Because of the low lipid content, no lipid class analysis was conducted for this medium.

Meronts were cultivated in 10-ml aliquots of media in T-25 tissue culture flasks and incubated at 28 °C ($n = 18$ for each medium). The initial concentration of meronts was 10^6 meronts/ml in M1 and 0.4×10^6 meronts/ml in M2. *Perkinsus marinus* meronts cultured in M1 (PM1) and M2 (PM2) were maintained for 25 d.

Meront cultures ($n = 3$ flasks) in M1 and M2 meront cultures ($n = 3$ flasks) were sampled at 6, 8, 11, 15, 25 d and at 1, 6, 11, 15, 25 d post-inoculation, respectively. At each sampling, the number of meronts in each flask was counted (expressed as 10^6 cells/ml), and their sizes measured under a light microscope (Olympus BX40). Meronts were harvested via centrifugation (800 g for 20 min) and the supernatant (medium) saved. Meront pellets were washed with 0.22 μm -filtered York River water (YRW, 18–20 ppt) and freeze-dried. Both freeze-dried meronts and media were stored at -20 °C for later lipid analysis.

Isolation and purification of prezoosporangia. Prezoosporangia, which developed from FTM-cultivated meronts, were isolated based on the method of Chu and Greene (1989). Briefly, tissues from oysters heavily infected with *P. marinus* were cleaned with 70% ethanol then rinsed with 0.22 μm -filtered YRW. The clean infected tissues and associated meronts were incubated in FTM with antibiotics (0.8 mg penicillin and 0.8 mg streptomycin per ml of medium) at 25–28 °C for 5 d. Prezoosporangia were then isolated and purified through a series of centrifugation and washing steps in 0.22 μm -filtered YRW. Isolated prezoosporangia were frozen at -20 °C until lipid extraction. Fluid thioglycolate medium was made by dissolving 29.3 g thioglycollate powder in 1 liter YRW. This was autoclaved before the addition of antibiotics. Lipid and fatty acid analyses on freshly prepared FTM revealed that this medium contained limited amounts of lipid and fatty acids. The level of lipid was not detectable when analyzed using thin layer chromatograph coupled with flame-ionization detector (TLC/FID, Iatroscan TH-10, MK-III analyzer, Iatron Laboratories, Tokyo, Japan). Results of gas liquid chromatography (GLC) analysis on fatty acids showed that the total fatty acid content in the medium was 90 ng fatty acids/ml and 20:4(n-6) comprised less than 2% of total fatty acids (1.55 ng/ml). No lipid and fatty acids analyses were conducted on FTM after incubation with the parasite.

Oysters. Oysters ($n = 10$) obtained from Damariscotta River, Maine, were acclimated in raw YRW for 14 d at 18–20 °C and then examined for possible *P. marinus* infection. *Perkinsus marinus* had not infected any of the acclimated oysters. Five of

these oysters were randomly selected and their whole body tissues were freeze-dried and stored at -20 °C for later analysis of fatty acid composition.

Lipid analysis. Total lipids were extracted from in vitro-cultured meronts, freshly isolated prezoosporangia, and oyster tissues with chloroform-methanol-water (1:2:1, v/v/v) according to the procedure described by Bligh and Dyer (1959). Because of low lipid contents of harvested meront pellets, no lipid analysis was conducted on M2 culture until 11 d post-inoculation. Moreover, due to low lipid content of M2, only M1 was analyzed for lipid class composition. Total lipids from M1 were extracted by addition of chloroform and methanol. Lipid contents and lipid class composition of meront pellets from M1 (1–25 d post-inoculation) and M2 (11–25 d post-inoculation), and meront-free M1 (1–25 d post-inoculation) were analyzed with TLC/FID using Iatroscan TH-10, MK-III analyzer (Chu and Ozkizilcik 1995). Briefly, after activation for 30 min at 110 °C, silica gel rods were spotted, with lipid samples (1–10 $\mu\text{l}/\text{sample}$) using a Hamilton syringe. Silica gel rods were then developed using a solvent mixture containing hexane:diethyl ether:formic acid (85:15:0.04, v/v/v). Following development, silica gel rods were analyzed in the Iatroscan analyzer. Operating conditions were 2,000 ml/min air flow, 0.73 kg/cm^3 hydrogen pressure, and a scan speed of 3.1 mm/sec. Lipid classes were identified by comparison with the co-chromatographed lipid standards. Lipid standards, cholesteryl ester (CHE), free fatty acids (FFA), triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), cholesterol (CHO), fatty alcohol (FA), and phosphatidylcholine (PC) were obtained from Sigma. Peak area integrations were performed by computer analysis (T DataScan, RSS Inc., Bemis, TN). To determine response factor, standard curves (1, 2, 3, 5, and 10 μg) were constructed for each lipid class standard. An internal standard (stearyl alcohol) was added to the meronts, prezoosporangia, and oyster tissue samples (1 mg per 50 mg DW) during lipid extraction. The quantity of each lipid class was determined by comparison with the internal standard peak and corrected with the response factor. Results are expressed as μg total lipid (TL), total phospholipids (PL), and total TAG per ml of meront culture (i.e. TL, PL or TAG contained in medium-free meront pellet of 1-ml culture); and μg TL, PL, and TAG per million of meronts.

Separation of polar and neutral lipids. To determine the fatty acid distribution in polar and neutral lipids, total lipids were fractionated into polar and neutral lipids and analyzed for fatty acids. Separation of polar and neutral lipids was achieved using micro-column liquid chromatography as described by Marty et al. (1992). Total lipids from meront pellets harvested from M 1 at 25 d post-inoculation were evaporated to dryness, redissolved in 0.5 ml chloroform-methanol (98:2), and loaded on a silica gel micro-column (10 \times 12 mm). Neutral and polar lipids were eluted using 13 ml chloroform-methanol (98:2) followed by 13 ml of methanol. Polar and neutral lipid fractions

were collected in tapering glass test tubes and stored at -20°C until fatty acid analysis.

Fatty acid analysis. Fatty acid composition and contents of meronts, prezoosporangia, oyster tissues, and culture media were analyzed using GLC. Total lipids were transesterified in 8-ml Wheaton vials containing $20\ \mu\text{g}$ internal standard (23:0), with 10% BF_3 (w/w) in methanol for 15 min at $95\text{--}100^{\circ}\text{C}$ (Metcalf and Schmitz 1961). After cooling, the fatty acid methyl esters (FAME) were extracted with carbon disulfide (Marty et al. 1992). The organic phase was evaporated, and redissolved in hexane. Separation of FAME was carried out on a GLC (Varian 3300) equipped with a flame ionization detector, using a DBWAX capillary column (J & W, $25\ \text{m} \times 0.32\ \text{mm}$; $0.2\ \mu\text{m}$ film thickness). The column was temperature-programmed from $60\text{--}150^{\circ}\text{C}$ at $30^{\circ}\text{C}/\text{min}$ and $150\text{--}220^{\circ}\text{C}$ at $2^{\circ}\text{C}/\text{min}$, 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 FAMES was based on the comparison of their retention times with those of authentic standards and confirmed by gas liquid chromatography-mass spectrometry (GC-MS). Non-methylene interrupted (NMI) polyunsaturated fatty acids (PUFAs) 20:2 Δ 5,11, 22:2 Δ 7,13, and 22:2 Δ 7,15 were designated 20:2NMI, 22:2NMI1, and 22:2NMI2, respectively. Results are expressed as μg fatty acid/ml of culture or weight percent fatty acid composition.

Statistical analysis. Results are expressed as mean and standard deviation (S.D.) A Statistica[®] computer package was used for data analysis. Differences between sampling dates were tested using one factor ANOVA. The Student-Neumanns-Keul's multiple comparison test was used to compare means when ANOVA was significant. Percentage data were $\text{Arcsin}(\sqrt{x})$ transformed prior to analysis. Differences were considered statistically significant if $p < 0.05$.

RESULTS

Growth, cell number and size distribution of in vitro *Perkinsus marinus* meront culture. M1 meronts proliferated faster than M2 meronts (Table 1; Fig. 1A, B). By 11 d post-inoculation, mean meront numbers in M1 had increased about 98-fold from 0.9×10^6 to 87.9×10^6 cells/ml. However, there was no significant change in meront numbers between 11 and 25 d post-inoculation. In M2, meronts took 25 d to increase 14-fold from 0.4×10^6 to 5.7×10^6 cells/ml. There was a significant increase in cell number between d 11 and d 25 post-inoculation. Sizes of meronts ranged from $1\ \mu\text{m}$ to $> 20\ \mu\text{m}$. Meronts of $3\text{--}5\ \mu\text{m}$ and $6\text{--}10\ \mu\text{m}$ were the major size subpopulations in both PM1 (31% and 55% respectively, at d 1) and PM2 (59% and 39% respectively, at d 1) cultures. The proportion of $3\text{--}5\ \mu\text{m}$ meronts in PM1 increased over time from 31% at d 1 to 86% at d 25, significantly between d 1 (31%) to d 6 (77%). Concomitantly, the proportion of meronts $> 6\ \mu\text{m}$ decreased between d 1 and d 25, from 55% to 13% for $6\text{--}10\ \mu\text{m}$ meronts, from 11% to 3.4% for $11\text{--}15\ \mu\text{m}$ meronts, and from 2.6% to 1% for $16\text{--}20\ \mu\text{m}$ meronts. Similar to meronts cultured in M1, size proportions changed with time in M2. The proportion of $3\text{--}5\ \mu\text{m}$ meronts decreased significantly from d 1 (59%) to d 6 (22%), while $6\text{--}10\ \mu\text{m}$ meronts increased from 39% to 68% ($p = 0.064$). In d 15 and d 25, proportions of these two size groups were similar (45–54% for $3\text{--}5\ \mu\text{m}$ and 44–52% for $6\text{--}10\ \mu\text{m}$).

Lipid content and lipid class composition of culture medium (M1). The total lipid concentration in M1 decreased gradually with time. Significant decreases occurred from 8–25 d post-inoculation (Fig. 1A). This medium contained only steryl esters and phospholipids (Fig. 2A, C). Phospholipids in M1

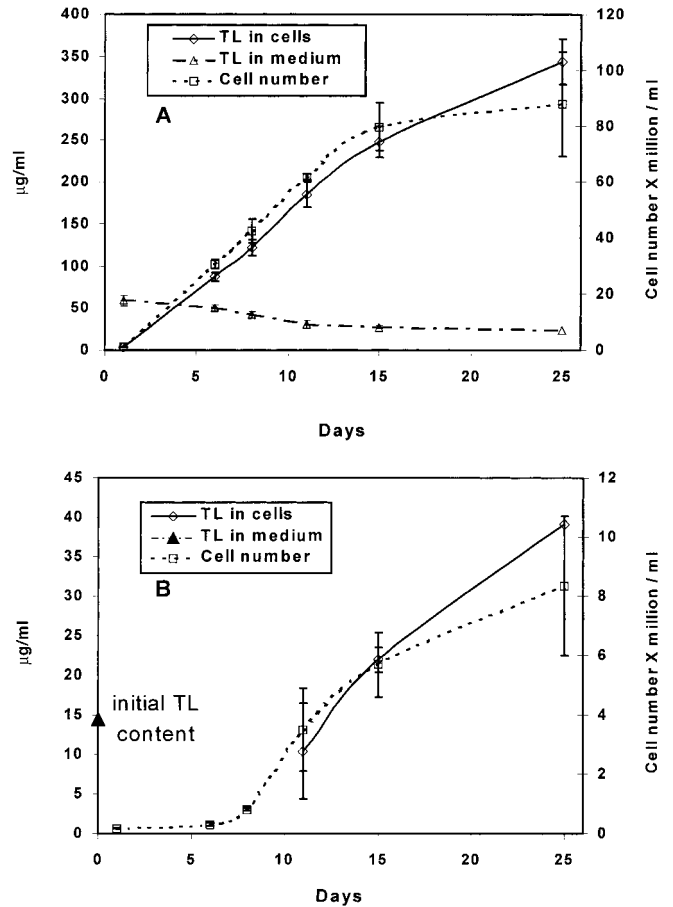


Fig. 1. Changes in the cell numbers (cell number $\times 10^6/\text{ml}$ culture) of *Perkinsus marinus*, total lipid (TL) contents in cell pellets (μg lipid/ml of culture), and total lipid content in media (μg lipid/ml of medium) in Medium 1 (A) and Medium 2 (B), 1–25 d post-inoculation. (Mean, S.D., $n = 3$). The initial lipid concentration of Medium 2 is $14.5\ \mu\text{g}/\text{ml}$ of medium. The total lipid content in Medium 2 was not analyzed during cultivation.

were almost completely depleted after 25 d post-inoculation (Fig. 2A), whereas only half (50.6%) of the steryl esters were consumed (Fig. 2C).

Lipid content and lipid class composition of meronts cultivated in M1 and M2. In both M1 and M2 cultures, total lipid (TL), phospholipid (PL), and triacylglycerol (TAG) contents increased as the meront number increased (Fig. 1A, B; 2A, B; 2E, F). The lipid content of M1 meronts ($344\ \mu\text{g}$ lipid/ml of culture) and M2 meronts ($39\ \mu\text{g}$ lipid/ml of culture) (Fig. 1A, B) in 25-day-old cultures exceeded the initial amounts of lipids in the media ~ 6 -fold and ~ 3 -fold, respectively. Total lipids per cell (TL/ 10^6 cells) in PM1 from d 6–15 were significantly lower than TL/ 10^6 cells at d 1 and d 25 (Table 1). Total lipids per cell (TL/ 10^6 cells) in PM2 did not change significantly over time (Table 1).

Meronts cultured in M1 contained steryl esters (SE), TAG, sterols (ST), and PLs. Phospholipid (PL, a structural component) and TAG (an energy reserve component) are major lipid components of meronts cultured in this medium. Phospholipid was the only lipid class detected in meronts at one day post-inoculation (Fig. 2A). This component increased significantly from d 1–6 post-inoculation and slowly from d 8–25 post-inoculation and paralleled the increase in cell number of *P. mar-*

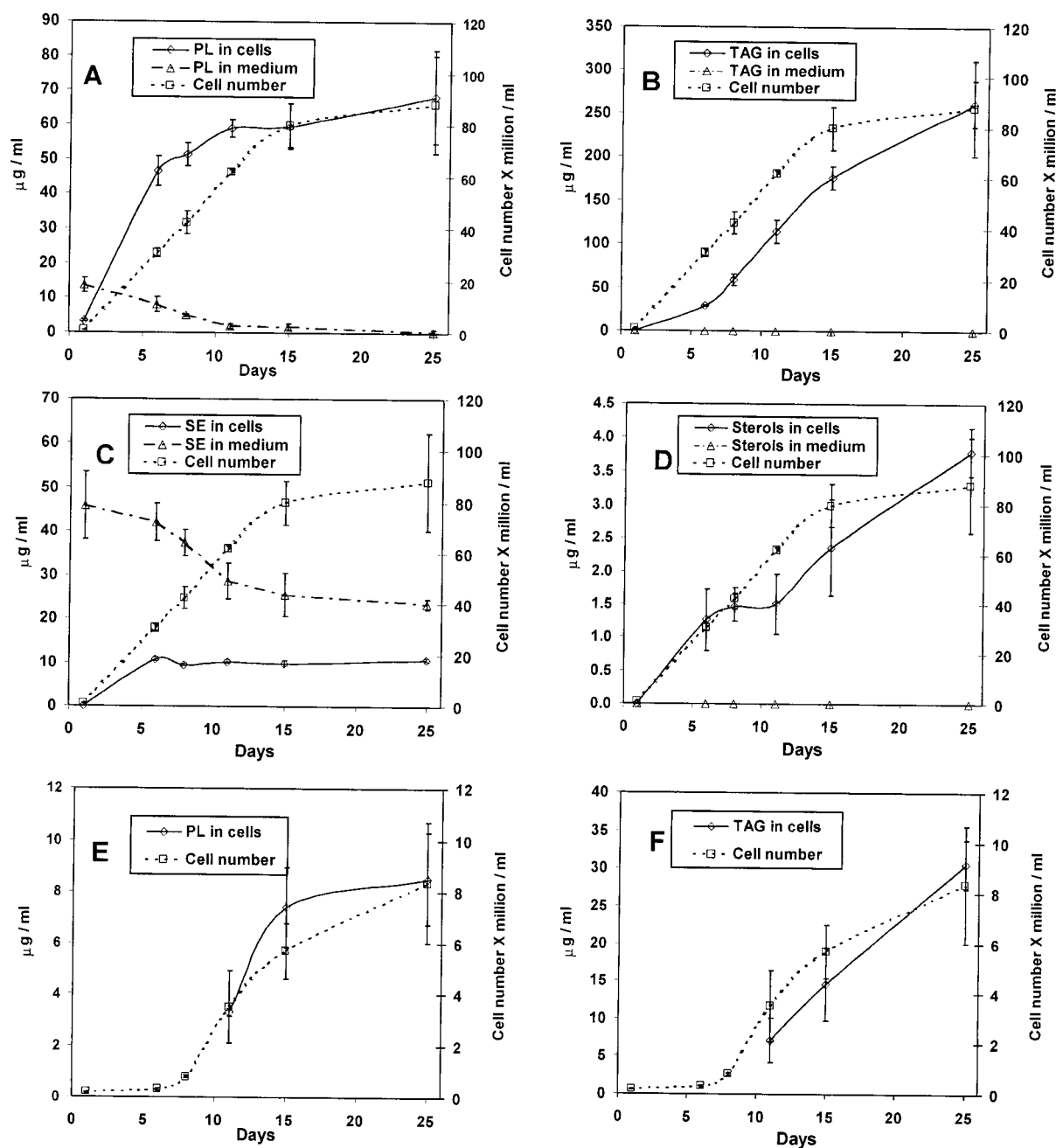


Fig. 2. Changes in the cell number (cell number $\times 10^6$ /ml) of *Perkinsus marinus*, phospholipid (A), triacylglycerol (B), steryl ester (C) and sterols (D) contents in cell pellets (μg lipid/ml of culture) from Medium 1, and in the medium (μg lipid/ml of medium); changes in the cell number (cell number $\times 10^6$ /ml) of *Perkinsus marinus*, phospholipid (E), triacylglycerol (F), contents in cell pellets (μg lipid/ml of culture) from Medium 2. Mean, S.D., $n = 3$.

inus. However, PL content per cell (PL/ 10^6 cells) decreased gradually and significantly from d 1–11 and then remained the same (Table 1). The level of total TAG (Fig. 2B) and TAG per cell (TAG/ 10^6 cells, Table 1), continued to augment significantly from d 1 post-inoculation to d 25 post-inoculation. While PL (100–53% of the total lipids) was the dominant component from 1–6 d post-inoculation in meronts cultivated in M1, TAG (> 75.9% of the total lipid) was prevalent from 11–25 d post-inoculation. Also, no sterols or steryl esters were detected in meronts at d 1 post-inoculation. Both were found in meronts

after 6 d post-inoculation: 9.4–10.8 μg steryl esters and 1.3–3.8 μg sterols/ml of meront culture (Fig. 2 C, D).

Neither sterols nor steryl esters were detected in meronts cultivated in M2 by TLC/FID analysis. However, the increases of PL and TAG in meronts exhibited trends similar to M1 (Fig. 2E, F). Total PL increased from d 11–15 post-inoculation and this level was maintained at 25 d post-inoculation. In contrast, TL and TAG contents continued to climb from d 11–25 post-inoculation. However, no significant changes occurred in PL, TAG, and TL contents per cell from 11–15 d post-inoculation (Table 1).

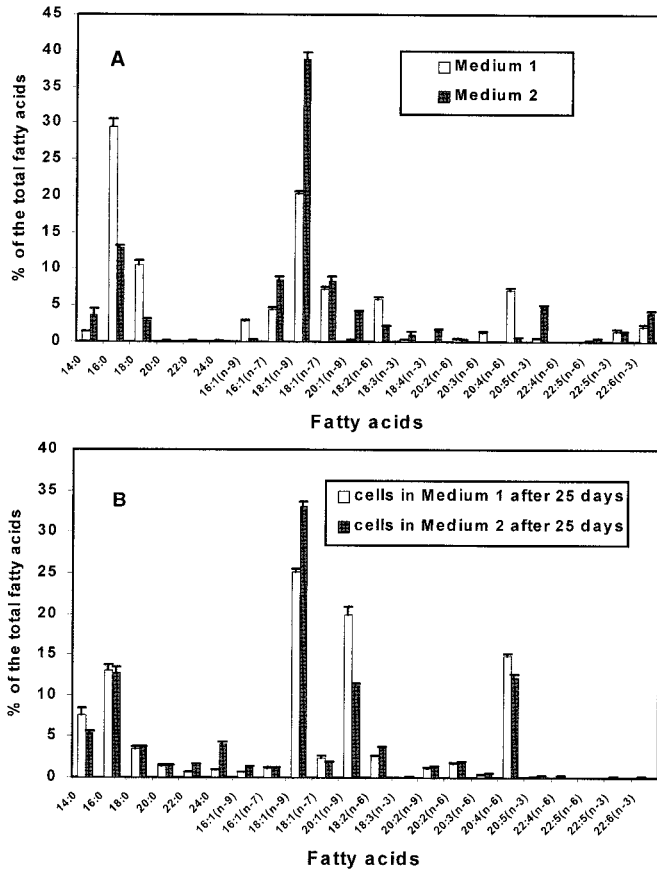


Fig. 3. Fatty acid profiles of Medium 1 and Medium 2 (A) and the meronts of *Perkinsus marinus* (B) cultivated in these media and harvested 25 d post-inoculation. Results are expressed in percentage of the total fatty acid methyl esters. Mean, S.D., $n = 3$.

Fatty acid composition of culture media and meronts.

Medium M1 contained only minor amounts of n-3 series PUFAs (Fig. 3A). Palmitic acid 16:0, 18:0, 18:1(n-9), 18:1(n-7), 18:2(n-6) and 20:4(n-6) were the major fatty acids in this

medium. Concentrations of total saturated, monounsaturated, and polyunsaturated fatty acids decreased with time in medium that cultivated *P. marinus* (Table 2). The concentration of total fatty acid (TFA) in the medium decreased from 22 $\mu\text{g}/\text{ml}$ of medium to 6 $\mu\text{g}/\text{ml}$ of medium 25 d post-inoculation. It is interesting to note that 20:1(n-9) was not detected in this medium in the beginning, but it appeared in the medium at 11, 15, and 25 d post-inoculation; its concentration in the medium was 0.21 $\mu\text{g}/\text{ml}$ of medium at 25 d post-inoculation.

Medium M2 contained fatty acids such as 16:0, 16:1(n-7), 18:1(n-9), 18:1(n-7), 20:5(n-3), and 22:6(n-3), but was deficient in (n-6) C20–22 fatty acids (Fig. 3A; Table 3). Similar to M1, TFA content diminished in the medium that cultivated *P. marinus*, from 7.7 $\mu\text{g}/\text{ml}$ of medium at d 1 post-inoculation to 0.6 $\mu\text{g}/\text{ml}$ of medium at 25 d post-inoculation (Table 3). The polyunsaturated fatty acids (PUFAs), 20:5(n-3), 22:5(n-3), and 22:6(n-3), disappeared almost completely by d 6 post-inoculation. Decreases of saturated and monounsaturated fatty acids (MUFAs) also occurred from d 1–25.

Despite the difference in fatty acid profiles of M1 and M2 (Fig. 3A) and deficiency of long chain PUFAs of (n-6) series in M2, *P. marinus* meronts cultivated in these two media shared a similar fatty acid profile (Fig. 3B). The major fatty acids were 14:0, 16:0, 18:0, 18:1(n-9), 20:1(n-9), 18:2(n-6), and 20:4(n-6). The most prevalent PUFAs were 18:2(n-6) and 20:4(n-6). The latter accounted for 70.3% of the total PUFAs of meronts in PM1 and 58.7% in PM2 at 25 d post-inoculation. As cell number increased, in contrast to the depletion of total fatty acid contents in the two culture media, these fatty acids along with the other minor components increased with time. In particular, the concentrations of 14:0, 16:0, 18:0, 18:1(n-9), 20:1(n-9), 18:2(n-6), and 20:4(n-6) increased to 21.9, 37.8, 10.3, 72.6, 57.7, 7.7, and 43.1 $\mu\text{g}/\text{ml}$ in PM1 25 d post-inoculation (Table 4), and to 1.53, 3.64, 1.06, 9.45, 3.19, 1.05, and 3.51 $\mu\text{g}/\text{ml}$ in PM2 25 d post-inoculation (Table 5). Also, the amount of 20:5(n-3), 22:5(n-3), and 22:6(n-3) increased to 0.21, 0.21, and 0.35 $\mu\text{g}/\text{ml}$ in PM1 (Table 4). Some of the fatty acids (e.g. 16:0, 18:1(n-9), 20:1(n-9), 18:2(n-6), 20:2(n-6), 20:4(n-6)), continued to increase after proliferation had plateaued. Specifically, the amounts of 18:1(n-9), 20:1(n-9), 18:2(n-6), 20:4(n-6) (the major MUFAs and PUFAs) in meronts harvested at d 25 were many times higher than their respective amounts present in both

Table 2. Fatty acid composition of culture Medium 1, 1–25 days post-inoculation with *Perkinsus marinus* (meronts). Results are expressed in μg fatty acid/ml of medium (Mean, S.D., $n = 3$). Only major fatty acid components are shown. Fatty acids with values <1.0 $\mu\text{g}/\text{ml}$ at day 1 are not included.

Fatty acid ^a $\mu\text{g}/\text{ml}$ of medium	Day 1		Day 6		Day 8		Day 11		Day 15		Day 25	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
16:0	6.49	1.34	4.96	0.15	4.69	0.87	2.81	0.17	2.02	0.32	1.57	0.03
18:0	2.31	0.36	1.55	0.03	1.55	0.30	0.82	0.04	0.56	0.11	0.40	0.01
18:1(n-9)	4.45	0.81	3.56	0.03	3.04	0.50	2.11	0.09	1.63	0.22	1.25	0.00
18:1(n-7)	1.58	0.29	1.27	0.06	1.14	0.14	0.67	0.01	0.51	0.06	0.43	0.04
18:2(n-6)	1.28	0.30	1.07	0.04	0.88	0.17	0.62	0.02	0.48	0.04	0.38	0.01
20:4(n-6)	1.57	0.25	1.23	0.01	1.06	0.04	0.74	0.05	0.61	0.09	0.46	0.02
TO.SAT. ^b	9.55	1.80	7.18	0.25	7.07	1.43	4.17	0.25	3.09	0.62	2.35	0.07
TO.MONO. ^b	7.95	1.53	6.30	0.12	5.52	0.95	3.85	0.11	3.11	0.44	2.42	0.08
TO.POLY.(n-6) ^b	3.29	0.61	2.64	0.04	2.40	0.24	1.56	0.04	1.29	0.05	0.92	0.03
TO.POLY.(n-3) ^b	0.95	0.05	0.63	0.01	0.47	0.04	0.28	0.01	0.21	0.04	0.15	0.00
TOTAL ^b	21.97	4.02	16.96	0.12	16.00	1.71	10.01	0.35	7.79	1.12	5.93	0.05

^a The 14:0, 15:0, 17:0, 20:0, 22:0, 24:0, 16:1(n-9), 16:1(n-5), 20:1(n-9), 20:1(n-7), 22:1(n-9), 24:1(n-9), 18:3(n-3), 18:3(n-6), 20:2(n-6), 20:3(n-6), 20:4(n-3), 20:5(n-3), 22:5(n-6), and 22:5(n-3) were detected but <1.0 $\mu\text{g}/\text{ml}$ at day 1.

^b TO.SAT: Total saturated fatty acids; TO.MONO: Total monounsaturated fatty acids; TO.POLY.(n-6): Total (n-6) polyunsaturated fatty acids; TO.POLY.(n-3): Total (n-3) polyunsaturated fatty acids; TOTAL: Sum of TO.SAT + TO.MONO + TO.POLY + total branched fatty acids + total dimethyl acetals.

Table 3. Fatty acid composition of culture Medium 2, 1–25 days post-inoculation with *Perkinsus marinus* (meronts). Results are expressed in $\mu\text{g/ml}$ of medium (Mean, S.D., $n = 3$). Only major fatty acid components are shown. Fatty acids with values $<0.2 \mu\text{g/ml}$ at day 1 are not included. ND = non-detected.

Fatty acid ^a $\mu\text{g/ml}$ of medium	Day 1		Day 6		Day 11		Day 15		Day 25	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
16:0	0.99	0.14	0.91	0.00	0.20	0.06	0.11	0.03	0.10	0.01
16:1(n-7)	0.65	0.12	0.54	0.02	0.08	0.01	0.08	0.01	0.06	0.02
18:1(n-9)	2.97	0.41	2.00	0.04	0.38	0.12	0.20	0.03	0.11	0.02
18:1(n-7)	0.64	0.12	0.44	0.02	0.07	0.02	0.05	0.01	0.02	0.01
20:5(n-3)	0.37	0.07	0.06	0.01	0.01	0.00	0.02	0.00	0.01	0.00
22:6(n-3)	0.30	0.04	0.04	0.00	0.01	0.00	0.01	0.00	ND	—
TO.SAT ^b	1.54	0.25	1.46	0.01	0.36	0.12	0.26	0.05	0.20	0.01
TO.MONO ^b	4.80	0.72	3.44	0.00	0.71	0.17	0.43	0.04	0.26	0.06
TO.POLY.(n-6) ^b	0.25	0.04	0.15	0.00	0.03	0.01	0.05	0.01	0.04	0.01
TO.POLY.(n-3) ^b	1.03	0.15	0.23	0.02	0.03	0.01	0.03	0.00	0.01	0.00
TOTAL ^b	7.65	1.15	5.33	0.05	1.15	0.31	0.79	0.08	0.52	0.06

^a The 14:0, 15:0, 17:0, 18:0, 16:1(n-9), 16:1(n-5), 20:1(n-9), 22:1(n-9), 24:1(n-9), 18:2(n-6), 18:3(n-3), 20:2(n-6), 20:4(n-6), 20:4(n-3), 22:5(n-6), 22:5(n-3) were detected but $<0.2 \mu\text{g/ml}$ at day 1.

^b TO.SAT: Total saturated fatty acids; TO.MONO: Total monounsaturated fatty acids; TO.POLY.(n-6): Total (n-6) polyunsaturated fatty acids; TO.POLY.(n-3): Total (n-3) polyunsaturated fatty acids; TOTAL: Sum of TO.SAT + TO.MONO. + TO.POLY + total branched fatty acids + total dimethyl acetals.

Table 4. Fatty acid composition of *Perkinsus marinus* (meronts) cell pellets harvested from Medium 1, 1–25 days post-inoculation. Results are expressed in $\mu\text{g/ml}$ of culture (Mean, S.D., $n = 3$). Only major fatty acid components are shown. Fatty acids with values $<0.2 \mu\text{g/ml}$ at day 25 are not included.

Fatty acid ^a $\mu\text{g/ml}$ of culture	Day 1		Day 6		Day 8		Day 11		Day 15		Day 25	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
12:0	0.01	0.00	0.19	0.04	0.50	0.01	0.61	0.18	1.46	0.24	1.82	0.16
14:0	0.20	0.01	3.60	0.83	7.53	0.46	9.53	1.76	17.77	1.18	21.90	1.63
16:0	0.48	0.02	8.13	1.55	16.34	1.14	21.21	2.81	33.25	1.95	37.82	0.92
18:0	0.21	0.01	4.26	0.94	8.92	0.14	13.53	1.83	21.36	0.57	10.26	1.00
20:0	0.03	0.00	0.80	0.14	1.56	0.09	2.45	0.28	3.89	0.15	3.93	0.47
22:0	0.02	0.00	0.43	0.05	0.78	0.02	1.19	0.10	1.83	0.03	2.12	0.12
24:0	0.03	0.00	0.60	0.06	0.96	0.03	1.34	0.08	1.91	0.05	2.86	0.18
16:1(n-9)	0.02	0.01	0.27	0.05	0.68	0.04	0.79	0.08	1.25	0.09	1.83	0.16
16:1(n-7)	0.05	0.00	0.68	0.05	1.20	0.07	1.27	0.12	1.77	0.27	3.27	0.19
18:1(n-9)	0.46	0.03	10.38	1.53	16.61	0.79	22.83	2.52	45.27	4.00	72.59	2.06
18:1(n-7)	0.08	0.01	1.45	0.30	2.02	0.05	2.73	0.42	4.46	0.47	6.97	0.63
20:1(n-9)	0.16	0.02	6.91	1.20	15.72	0.30	25.39	2.90	37.77	2.02	57.74	4.38
20:1(n-7)	0.01	0.01	0.37	0.13	0.74	0.26	0.90	0.10	1.49	0.27	2.37	0.95
22:1(n-9)	0.01	0.00	0.09	0.01	0.21	0.02	0.34	0.03	0.46	0.07	0.72	0.07
18:2(n-6)	0.08	0.04	1.21	0.13	1.78	0.12	2.04	0.15	3.22	0.25	7.65	0.18
18:3(n-3)	0.01	0.01	0.04	0.01	0.00	0.00	0.05	0.04	0.10	0.02	0.15	0.03
20:2(n-9)	0.18	0.16	1.22	0.28	1.93	0.07	2.52	0.25	3.07	0.26	3.36	0.46
20:2(n-6)	0.07	0.01	1.66	0.24	2.26	0.08	2.87	0.28	3.23	0.06	5.01	0.16
20:3(n-6)	0.03	0.01	0.31	0.02	0.47	0.02	0.66	0.06	0.83	0.03	1.03	0.04
20:4(n-6)	0.27	0.02	10.46	1.27	16.96	0.51	23.77	1.29	28.82	1.19	43.10	1.80
20:5(n-3)	0.02	0.01	0.08	0.01	0.10	0.02	0.12	0.00	0.15	0.02	0.21	0.01
22:4(n-6)	0.00	0.00	0.21	0.11	0.21	0.08	0.35	0.05	0.51	0.01	0.55	0.10
22:5(n-3)	0.03	0.00	0.12	0.02	0.16	0.02	0.19	0.04	0.21	0.01	0.21	0.05
22:6(n-3)	0.04	0.00	0.19	0.02	0.21	0.01	0.25	0.03	0.29	0.02	0.35	0.05
TO.SAT ^b	1.01	0.04	18.10	3.59	36.74	1.46	50.05	6.71	81.74	3.52	81.03	1.07
TO.MONO ^b	0.81	0.06	20.22	3.22	37.34	0.39	54.54	6.24	92.88	2.40	146.22	6.80
TO.POLY.(n-6) ^b	0.45	0.05	13.84	1.67	21.69	0.58	29.69	1.66	36.62	1.19	57.33	1.97
TO.POLY.(n-3) ^b	0.10	0.01	0.44	0.05	0.48	0.04	0.61	0.10	0.76	0.00	0.92	0.05
TOTAL ^b	2.57	0.31	53.82	8.80	98.31	2.04	137.49	14.39	215.38	1.82	289.00	8.24

^a The 15:0, 17:0, 16:1(n-5) and 17:1(n-8) were detected but $<0.2 \mu\text{g/ml}$ at day 25.

^b TO.SAT: Total saturated fatty acids; TO.MONO: Total monounsaturated fatty acids; TO.POLY: Total polyunsaturated fatty acids; TO.(n-6): Total (n-6) polyunsaturated fatty acids; TO.POLY.(n-3): Total (n-3) polyunsaturated fatty acids; TOTAL: Sum of TO.SAT + TO.MONO. + TO.POLY + total branched fatty acids + total dimethyl acetals.

Table 5. Fatty acid composition of *Perkinsus marinus* (meronts) cell pellets harvested from Medium 2, 1–25 days post-inoculation. Results are expressed in $\mu\text{g/ml}$ of culture (Mean, S.D., $n = 3$). Only major fatty acid components are shown. Fatty acids with values $<0.1 \mu\text{g/ml}$ at day 25 are not included. ND = non-detected.

Fatty acid ^a $\mu\text{g/ml}$ of culture	Day 1		Day 6		Day 11		Day 15		Day 25	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
14:0	0.08	0.00	0.10	0.01	0.18	0.02	0.91	0.08	1.53	0.13
16:0	0.06	0.01	0.12	0.00	0.36	0.08	1.74	0.15	3.64	0.33
18:0	0.02	0.00	0.03	0.00	0.08	0.02	0.92	0.05	1.06	0.03
20:0	ND	—	ND	—	0.01	0.01	0.26	0.03	0.40	0.01
22:0	ND	—	ND	—	0.01	0.00	0.19	0.03	0.47	0.02
24:0	0.02	0.00	0.02	0.00	0.05	0.01	0.27	0.01	1.18	0.08
16:1(n-9)	ND	—	0.02	0.00	0.05	0.01	0.08	0.01	0.36	0.06
16:1(n-7)	0.03	0.00	0.06	0.00	0.16	0.02	0.22	0.05	0.31	0.06
18:1(n-9)	0.28	0.02	0.47	0.04	0.97	0.11	4.24	0.18	9.45	0.37
18:1(n-7)	0.03	0.00	0.07	0.01	0.20	0.02	0.35	0.04	0.56	0.02
20:1(n-9)	0.02	0.00	0.03	0.00	0.10	0.02	1.12	0.15	3.19	0.12
22:1(n-9)	0.01	0.00	0.01	0.00	0.01	0.00	0.02	0.01	0.06	0.02
18:2(n-6)	0.01	0.00	0.03	0.00	0.13	0.02	0.55	0.03	1.05	0.09
18:3(n-3)	ND	—	ND	—	0.01	0.00	0.02	0.00	0.04	0.00
20:2(n-9)	0.02	0.00	0.02	0.01	0.04	0.01	0.19	0.02	0.37	0.02
20:2(n-6)	0.01	0.00	0.01	0.00	0.05	0.01	0.15	0.04	0.58	0.04
20:3(n-6)	0.00	0.00	0.00	0.00	0.01	0.00	0.09	0.01	0.16	0.02
20:4(n-6)	0.04	0.00	0.05	0.00	0.21	0.07	1.77	0.18	3.51	0.27
20:5(n-3)	0.01	0.00	0.02	0.00	0.05	0.01	0.06	0.02	0.06	0.01
TO.SAT. ^b	0.20	0.02	0.29	0.02	0.75	0.15	4.57	0.22	8.54	0.54
TO.MONO. ^b	0.38	0.03	0.67	0.05	1.54	0.16	6.13	0.31	14.02	0.61
TO.POLY.(n-6) ^b	0.07	0.01	0.11	0.02	0.46	0.12	2.56	0.19	5.29	0.40
TO.POLY.(n-3) ^b	0.02	0.01	0.03	0.01	0.09	0.01	0.08	0.02	0.11	0.01
TOTAL ^b	0.73	0.06	1.17	0.03	2.94	0.44	13.54	0.51	28.56	1.61

^a The 12:0, 15:0, 17:0, 16:1(n-5), 17:1(n-8), 22:5(n-6), 22:5(n-3) and 22:6(n-3) were detected but $<0.1 \mu\text{g/ml}$ at day 25.

^b TO.SAT: Total saturated fatty acids; TO.MONO: Total monounsaturated fatty acids; TO.POLY: Total polyunsaturated fatty acids; TO.(n-6): Total (n-6) polyunsaturated fatty acids; TO.POLY.(n-3): Total (n-3) polyunsaturated fatty acids; TOTAL: Sum of TO.SAT + TO.MONO. + TO.POLY + total branched fatty acids + total dimethyl acetals.

media at d 1 (Fig. 4A–H). Moreover, the quantity of total fatty acids in meronts harvested from M1 and M2 at 25 d was significantly higher than the initial amounts contained in cell inoculum plus media (PM1: 289 μg fatty acid/ml meronts at d 25 vs. 24.5 μg fatty acid/ml meronts + medium at d 1; PM2: 28.6 μg fatty acid/ml meronts at d 25 vs. 8.4 μg fatty acid/ml meronts + medium at d 1) and 13 and 4 times higher than the amounts present in M1 and M2, respectively.

Fatty acid composition of polar and neutral lipids of meronts cultured in M1. Fatty acid composition analysis performed on polar and neutral lipids of meronts (25 d post-inoculation) from M1 revealed differences in fatty acid distribution between polar and neutral lipids (Fig. 5). Among the major fatty acids, polar lipids had higher proportions of 20:4(n-6), 18:1(n-9), and 16:0 than neutral lipids; 18:0 and 20:1(n-9) were present in higher proportions in neutral than in polar lipids.

Fatty acid profiles of prezoosporangia and oyster. Generally, *P. marinus* prezoosporangia had a fatty acid profile similar, qualitatively and quantitatively, to its host, *C. virginica* (Fig. 6). Both had high-weight percentage of n-3 family PUFAs, particularly 20:5(n-3) and 22:6(n-3), a characteristic of marine organisms. However, the prezoosporangia contained significantly higher percentage of 22:6(n-3) and a lower percentage of 20:2 NMI, 22: 2NMI1 and 22:2 NMI2 than its host.

DISCUSSION

This paper represents the first report of lipid class and fatty acid composition of in vitro cultured *Perkinsus marinus* meronts. Lipids are important membrane components and a source of energy. The medium with higher lipid content (M1) appeared to sustain better growth of *P. marinus* in terms of proliferation.

Scavenging of lipids from the host environment for membrane synthesis during proliferation, growth, and development to the next stage and for energy is probably a common phenomenon in a parasite's life. Incorporation of phospholipids, fatty acids, cholesterol, and lysophospholipids from culture media and/or the host has been reported in several parasitic protozoa (Brouwers et al. 1997; Lujan, Mowatt, and Nash 1996; Redman et al. 1997; Stevens et al. 1997; Vial and Ancelin 1998) and the nematode *Schistosoma mansoni* (Furlong et al. 1995). The depletion of lipids and fatty acids in culture media with the increase of *P. marinus* meronts indicated that *P. marinus* effectively assimilated exogenous lipids and used them for proliferation. Moreover, *P. marinus* meronts preferentially incorporated and used phospholipids over steryl esters for membrane synthesis during proliferation. Our recent studies incubating meronts with fluorescent lipid analogs (Chu et al. 1999; Chu et al. 2000) also demonstrated that *P. marinus* was able to liberate fluorescent-labeled acyl chains from phospholipids, but not from cholesteryl esters, and incorporate them into other lipids. *Perkinsus marinus* meronts cultured in M1 took up almost all of the 20:5(n-3), 22:5(n-3), and 22:6(n-3) present in the medium and incorporated them into their lipids.

Although parasitic protozoans generally require exogenous sources of essential fatty acids to support their growth and life cycle completion, some of them are capable of modifying exogenous lipids and fatty acids to some extent (Brouwers et al. 1997; Ellis et al. 1996; Redman et al. 1997; Vial and Ancelin 1998). Results of the present study indicate that lipid metabolism in cultured *P. marinus* meronts extends beyond the modification of exogenous lipids and fatty acids. Although studies

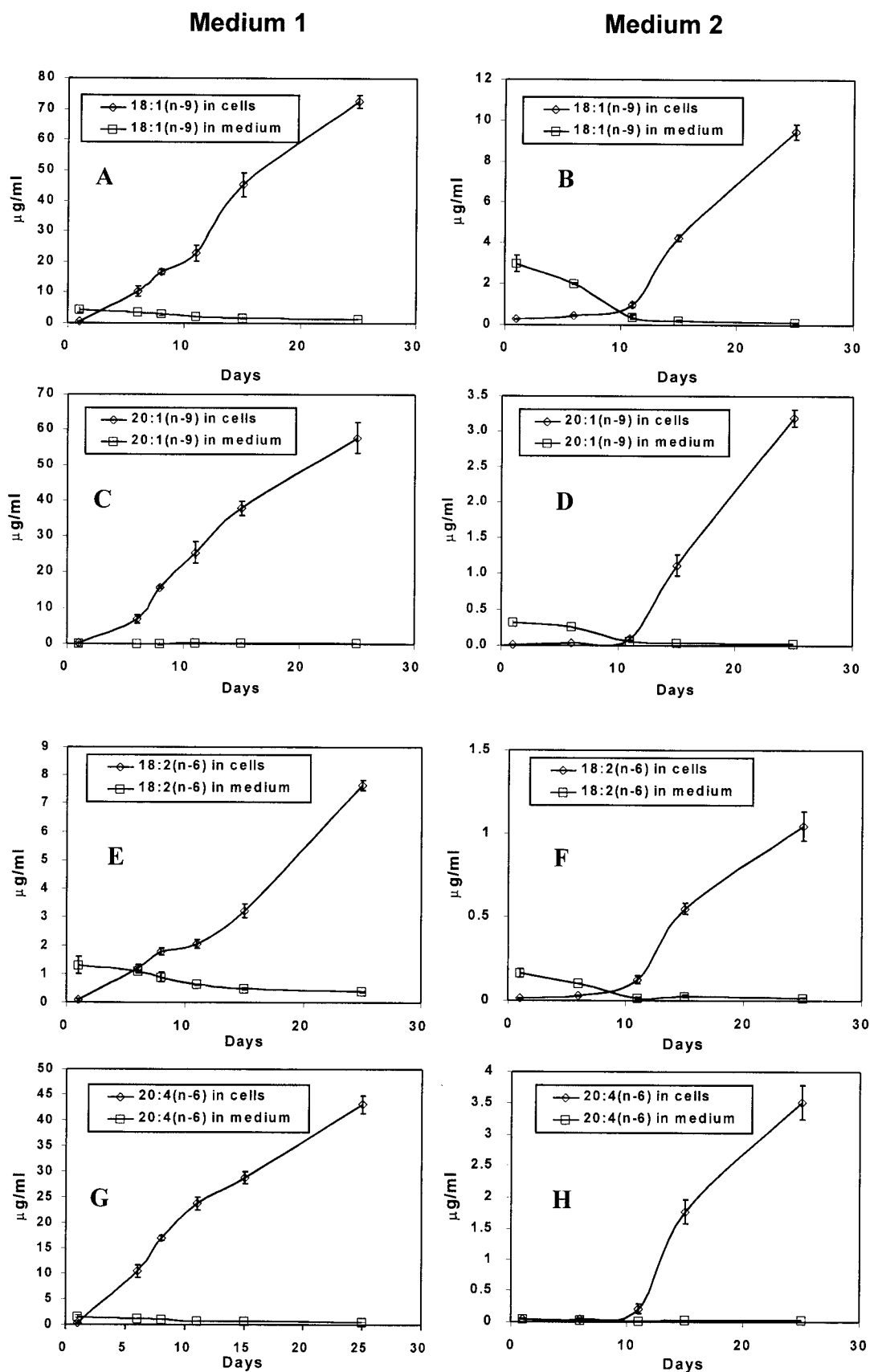


Fig. 4. A-H: Changes in the concentrations of 18:1(n-9), 20:1(n-9), 18:2(n-6) and 20:4(n-6) in pellets of *Perkinsus marinus* meronts (µg fatty acids/ml of culture), and in Medium 1 and 2 (µg fatty acids/ml of medium), 1-25 d post-inoculation. Mean, S.D., n = 3.

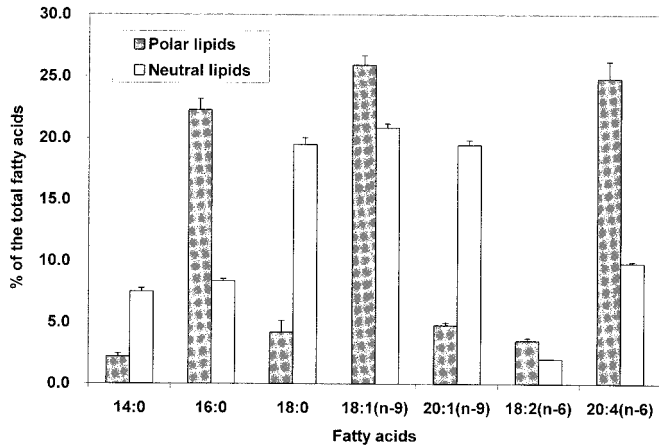


Fig. 5. Fatty acid composition of polar and neutral lipids of meronts of *Perkinsus marinus* harvested from Medium 1, 25 d post-inoculation. Results are expressed in percentage of the total fatty acid methyl esters. Mean, S.D., n = 3.

are needed using radio- or stable isotope-labeled precursors to determine the synthetic pathways involved, the qualitative differences in lipids and fatty acids between the culture media and *P. marinus* meronts, and the significant increase in amounts of lipids and fatty acids found in *P. marinus* meronts compared to the contents contained in the initial medium plus inoculum, indicate that in vitro cultured meronts of this parasite are capable of synthesizing de novo lipids and fatty acids. Most of the increased amounts must be derived from de novo synthesis by the parasite.

In vitro cultured meronts appear able to synthesize phospholipids and TAG de novo. The increased total lipid contents in meront cultures were reflections of increased cell numbers (membrane synthesis) and accumulation of TAG in meronts as energy reserves since there was no significant change in TL/10⁶ cells during proliferation. The decrease of PL/10⁶ cells during log growth phase (1–11 d post-inoculation) in PM1 was probably attributable to the increase in proportion of small-sized meronts. The continuous increase of the total TAG and TAG per 10⁶ cells during and after proliferation suggests an important energy reserve role of this component, although the increase of TAG/10⁶ cells in PM2 was not statistically significant. Triacylglycerols are the most common forms of energy storage in plants and in animals (Gurr and Harwood 1991). Triacylglycerols are also thought to be used by protozoan parasites, such as trypanosomatids and *Plasmodium* sp., as energy depots and as storage of fatty acid groups (Haughan and Goad 1991; Vial and Ancelin 1992). However, *G. lamblia*, a primitive protozoan parasite, also appeared able to store its energy and fatty acid in SE (Haughan and Goad 1991; Kaneda and Goutsu 1988). Cultivated trichomonads contained similar amounts of SE and TAG (Lindmark et al. 1991). Sterols do not appear to be a requirement for membrane synthesis during proliferation at the meront stage, since no sterol was provided in either medium and it was not detected in meronts cultivated in M2.

The predominance of 18:2(n-6), 20:2(n-6), and 20:4(n-6) in the PUFA profiles of meronts cultured in both media revealed characteristics similar to terrestrial animals. This differs from the fatty acid profile of its host, *C. virginica*. Like other bivalves, the PUFA profile of oysters was usually predominated by 20:5(n-3), 22:6(n-3), and 22:2NMI2. Thus, in vitro cultured *P. marinus* meronts may possess a system to synthesize all required fatty acids. This system may include fatty acid synthe-

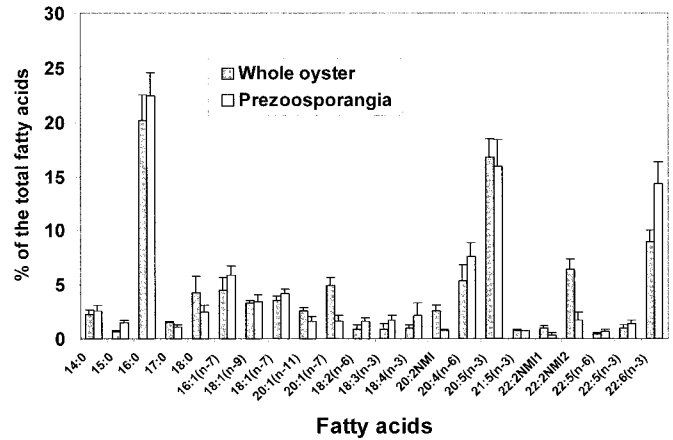


Fig. 6. Fatty acid composition of prezoosporangia of *Perkinsus marinus* and of its host oyster *Crassostrea virginica*. Results are expressed in percentage of the total fatty acid methyl esters. Mean, S.D., n = 3.

tase, desaturase, and elongase activities that permit the parasite to synthesize saturated, monounsaturated, and polyunsaturated fatty acids.

There are two types of fatty acid biosynthesis. Saturated fatty acids synthesis in *P. marinus* meronts probably, as in most animals, takes place in the cytosol through the Type I fatty acid synthetase (Gurr and Harwood 1991) using acetyl-CoA as the 'primer' molecule activated within the mitochondria. The ultrastructural study of *P. marinus* meronts (Perkins 1996) revealed the presence of numerous cytoplasmic mitochondria. Type II fatty acid synthesis is common in bacteria but is confined to the plastids in plants and algae. Waller et al. (1998) identified several fatty acid biosynthetic genes encoding enzymes in the apicomplexan plastids of *P. falciparum* and *Toxoplasma gondii*. They hypothesized that a Type II fatty acid biosynthetic pathway is present in apicoplasts of *P. falciparum* and *T. gondii*. Saturated fatty acids such as 12:0 and 14:0 are typical chloroplast fatty acids in microalgae and plants (Gurr and Harwood 1991; Pohl 1982). It is uncertain whether a Type II fatty acid synthetic pathway exists in *P. marinus* although the parasite has much high levels of 14:0. A non-photosynthetic plastid has been identified in the biflagellated zoospores of *P. marinus* (Perkins 1976). There are no reports of finding this organelle in meronts or prezoosporangia. The ability to synthesize saturated fatty acids de novo was found to be either limited or absent in most parasitic protozoa studied (Haughan and Goad 1991; Jarroll et al. 1981; Lindmark et al. 1991; Sherman 1979; Vial et al. 1990), with the exception of *Trypanosoma cruzi*, which incorporated C¹⁴ acetate into saturated (16:0 and 18:0), monounsaturated (18:1), and diunsaturated (18:2) fatty acids (de Lema and Aeberhard 1986).

The high contents of 18:1(n-9) and 16:1(n-7) found in *P. marinus* meronts suggest that a desaturation of 18:0 and 16:0 catalyzed by $\Delta 9$ desaturase has occurred. Protists usually synthesize 18:1(n-9) and 16:1(n-7) by direct oxidative desaturation (Erwin and Bloch 1964; Korn, Greenblatt, and Lees 1965). Desaturation of 18:0 to 18:1(n-9) and the presence of $\Delta 9$ desaturase have been suggested for parasitic protozoans, such as *C. parvum* (Mitschler, Welti, and Upton 1994), *G. lamblia* (Jarroll et al. 1981), and demonstrated in trypanosomatids (de Lema and Aeberhard 1986; Korn, Greenblatt, and Lees 1965; Meyer and Holz 1966) and in free-living protozoa such as amoebae and ciliates (Erwin and Bloch 1964).

The totals of 18:2(n-6) and 20:2(n-6) recorded in *P. marinus* meronts were much greater than the original amounts contained

in the media. This indicates the possibility of desaturation of 18:1(n-9) to 18:2(n-6) using $\Delta 12$ desaturase, and then of elongation of 18:2(n-6) to 20:2(n-6). Presence of $\Delta 12$ or $\Delta 15$ has been proposed for *T. cruzi* (de Lema and Aerberhard 1986). To synthesize PUFA, a new double bond is usually introduced in animals between an existing double bond and the carboxyl group, whereas a new double bond is inserted in plants between an existing double bond and the terminal methyl (Gurr and Harwood 1991). However, some primitive organisms, such as euglenids, dinoflagellates, ciliates, and amoebae have the ability to desaturate in either direction (Avery, Lloyd, and Harwood 1994; Erwin and Bloch 1964; Kaneshiro et al. 1979).

The level of 20:4(n-6) found in cultured *P. marinus* meronts was particularly high (24.9% of the total fatty acid of the polar lipids and 9.9% of the total fatty acid of the neutral lipids), compared to their host and to the amount contained in the growth media. It increased continually throughout the experimental period even after cell numbers had peaked. These results suggest that de novo synthesis of 20:4(n-6) occurred. The production of 20:4(n-6) is generally considered an 'animal' characteristic and usually it requires the γ -linolenic pathway of desaturation. Usage of the γ -linolenic pathway for polyenoic fatty acid synthesis has been described in ciliates, trypanosomatids (Korn, Greenblatt, and Lees 1965), and some phytoplankton species, such as *Porphyridium cruentum* (Rhodophyceae), *Ochromonas danica* (Chrysophyceae), and *Spirula platensis* (Cyanophyceae) (Pohl 1982). A different pathway employing 20:2(n-6) instead of 18:3(n-6) as an intermediate to synthesize 20:4(n-6) has also been described in heterotrophic euglenids (Pohl 1982) and in amoebae (Korn, Greenblatt, and Lees 1965). Because no 18:3(n-6), but significant amounts of 20:2(n-6), were present in *P. marinus* meronts, it is tempting to speculate that *P. marinus* meronts used the latter pathway for 20:4(n-6) synthesis. The continuous increase and high amount of 20:4(n-6) found in polar lipids of meronts harvested at 25 d post-inoculation suggest that this component is one of the essential structural constituents in *P. marinus* meronts. However, it is unclear whether meronts of *P. marinus* in oysters prefer to synthesize 20:4(n-6) as membrane components, rather than using 20:5(n-3) and 22:6(n-3), which are plentiful in the host tissues.

In vitro cultured *P. marinus* also appeared capable of synthesizing long chain fatty acids such as, 20:0, 20:1(n-9), 22:0, 22:1(n-9), 24:0 and 24:1(n-9) via elongation. These long chain fatty acids are probably derived from 18:0 and 18:1(n-9). Such elongation is believed to take place in membranes of the endoplasmic reticulum in animal cells (Gurr and Harwood 1991). The presence of long chain saturated and monounsaturated fatty acids is a typical "animal" characteristic, although substantial amounts of 20:1 (n-9) were reported in some marine Euglenophyceae and Cryptophyceae (Pohl 1982). The metabolic role of 20:1(n-9) in *P. marinus* is unclear. The level of this fatty acid in *P. marinus* is high, especially in the neutral lipids.

Based upon ultrastructural and morphological analyses, *P. marinus* has been placed in the Phylum Apicomplexa (Levine 1978; Perkins 1976). After reexamination of the ultrastructure of this parasite (Vivier 1982) and because of the recent published SSU rRNA and actin gene sequence data, it has been proposed that the phylogenetic position of *P. marinus* is closer to the dinoflagellates than to apicomplexa (Flores, Siddall, and Bureson 1996; Fong et al. 1993; Reece et al. 1997; Siddall et al. 1997). Results of the present study revealed that *P. marinus* shares some similarities in fatty acid synthesis with free-living amoeba and heterotrophic euglenids. The ability of *P. marinus* to synthesize unsaturated and saturated fatty acids is beyond those described for any apicomplexa. However, both phototrophic and heterotrophic dinoflagellates contain little (n-6)

PUFA (< 5%) and biosynthesize and accumulate high levels of (n-3) series PUFA (Harrington et al. 1970). Typical marine dinoflagellate species contain high weight percentages of 18:4(n-3), 18:5(n-3), and 22:6(n-3) (Pohl 1982). Thus, one might speculate that *Perkinsus* and dinoflagellates evolved from a common ancestor, but with different metabolic pathways.

The resemblance of the fatty acid composition of *P. marinus* prezoosporangia to that of its host suggests that they use a different metabolic mode from that seen in meronts. Since FTM was deficient in 20:5(n-3) and 22:6(n-3) (Volety 1995; Volety, Chu, and Ozkizilcik 1995), these components must be derived from the host tissues. Thus, for development from meront to prezoosporangium, the parasite may have to rely on its host for lipid resources.

In summary, in vitro cultured *P. marinus* meronts are capable not only of modifying exogenous lipids but also of synthesizing their own fatty acids and lipids. This is supported by the findings: (1) the total amounts of lipids and fatty acids in meront cultures after 25 d far exceeded the initial contents present in the media and initial cell inoculum; (2) the lipids and fatty acids incorporated by the parasite from the media accounted for only 10.3% and 5.6% to 25.6% respectively, of the total increase in lipids and fatty acids at 25 d post-inoculation; (3) the similarity in fatty acid profiles in meronts cultivated in two media that differed in fatty acid composition; (4) lack of detectable TAG in the media, but tremendous amounts of this component present in the meronts, particularly in those cultivated in M1; (5) the continued increases of phospholipid content and meront number at the time when phospholipids in the media were almost exhausted; and (6) no 20:4(n-6) detectable in M2 and only a small amount of it present in M1, but the absolute weights of this fatty acid and the others increased during the whole growth period. However, to determine the fatty acid and lipid biosynthetic pathways, further metabolic studies are needed using radio- or fluorescent-labeled precursors. Also, *Perkinsus marinus* prezoosporangia appear to have lost their ability to synthesize lipids and fatty acids.

ACKNOWLEDGMENTS

This study was supported by a grant from the Metabolic Biochemistry Program, Molecular and Cellular Bioscience Division, National Science Foundation (Grant # MCB9728284) and a grant from Jeffress Trust Funds, National Bank, Virginia (Grant # J-352). The authors would like to thank Ms. Georgeta Constantin, and Mr. Lee Steider for technical assistance, Mr. Luis A. Cruz-Rodriguez for part of the statistical analysis, and Drs. Robert C. Hale, Ken Webb, and Eric Lund for the constructive review of the first draft of the manuscript. Special thanks go to Ms. Ellen Harvey for GC/MS verification of fatty acid identification. Contribution # 2354 from the Virginia Institute of Marine Science, College of William and Mary.

LITERATURE CITED

- Avery, S. V., Lloyd, D. & Harwood, J. L. 1994. Changes in membrane fatty acid composition and $\Delta 12$ -desaturase activity during growth of *Acanthamoeba castellanii* in batch culture. *J. Eukaryot. Microbiol.*, **41**:396-401.
- Bligh, E. G. & Dyer, W. J. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, **37**:911-917.
- Brouwers, J. F. H. M., Smeenk, I. M. B., van Golde, L. M. G. & Tielsens, A. G. M. 1997. The incorporation, modification and turnover of fatty acids in adult *Schistosoma mansoni*. *Mol. Biochem. Parasitol.*, **88**: 175-185.
- Chu, F.-L. E. 1996. Laboratory investigations of susceptibility, infectivity and transmission of *Perkinsus marinus* in oysters. *J. Shellfish Res.*, **15**:57-66.
- Chu, F.-L. E. & Greene, K. H. 1989. Effect of temperature and salinity on in vitro culture of the oyster pathogen, *Perkinsus marinus* (Apicomplexa: Perkinsea). *J. Invert. Pathol.*, **53**:260-268.

- Chu, F. L. E. & Ozkizilcik, S. 1995. Lipid and fatty acid composition of striped bass (*Morone saxatilis*) larvae during development. *Comp. Biochem. Physiol.*, **111B**:665–674.
- Chu, F.-L. E., Soudant, P., Volety, A. K. & Huang, Y. 2000. *Perkinsus marinus*: uptake and interconversion of fluorescent lipid analogs in the parasite of the oyster, *Crassostrea virginica*. *Exp. Parasitol.*, **95**: 240–251.
- Chu, F.-L. E., Soudant, P., Huang, Y., Volety, A. K. & Constantin, G. 1999. Uptake, distribution, and bioconversion of fluorescent lipid analogs in the oyster protozoan parasite, *Perkinsus marinus*. *J. Shellfish. Res.*, **18**:318.
- de Lema, M. G. & Aeberhard, E. 1986. Desaturation of fatty acids in *Trypanosoma cruzi*. *Lipids*, **21**:718–720.
- Dixon, H. & Williamson, J. 1970. The lipid composition of blood and culture forms of *Trypanosoma lewisi* and *Trypanosoma rhodesiense* compared with that of their environment. *Comp. Biochem. Physiol.*, **33**:111–128.
- Dixon, H., Ginger, C. D. & Williamson, J. 1971. The lipid metabolism of blood and culture forms of *Trypanosoma lewisi* and *Trypanosoma rhodesiense*. *Comp. Biochem. Physiol.*, **39B**:247–266.
- Ellis, J. E., Wyder, M. A., Jarroll, E. L. & Kaneshiro, E. S. 1996. Changes in lipid composition during in vitro encystation and fatty acid desaturase activity of *Giardia lamblia*. *Mol. Biochem. Parasitol.*, **81**:13–25.
- Erwin, J. & Bloch, K. 1964. Biosynthesis of unsaturated fatty acids in microorganisms. *Science*, **143**:1006–1012.
- Flores, B. S., Siddall, M. E. & Bureson, E. M. 1996. Phylogeny of the haplosporidian (Eukaryota: Avelolata) based on small subunit ribosomal RNA sequence. *J. Parasitol.*, **82**:616–623.
- Fong, D., Rodriguez, R., Koo, K. & Sun, J. 1993. Small subunit ribosomal RNA gene sequence of the oyster parasite *Perkinsus marinus*. *Mol. Mar. Biol. Biotechnol.*, **2**:346–350.
- Furlong, S. T. 1991. Unique roles for lipids in *Schistosoma mansoni*. *Parasitol. Today*, **7**:59–62.
- Furlong, S. T., Thibault, K. S., Morbelli, L. M., Quinn, J. J. & Rogers, R. A. 1995. Uptake and compartmentalization of fluorescent lipid analogs in larval *Schistosoma mansoni*. *J. Lipid Res.*, **36**:1–12.
- Gauthier, J. D. & Vasta, G. R. 1993. Continuous in vitro culture of the eastern oyster parasite *Perkinsus marinus*. *J. Invert. Pathol.*, **62**:321–323.
- Gurr, M. I. & Harwood, J. L. 1991. Lipid Biochemistry, an Introduction. 4th ed., Chapman & Hall, New York.
- Harrington, G. W., Beach, D. H., Dunham, J. E. & Holz Jr., G. G. 1970. The polyunsaturated fatty acids of marine dinoflagellates. *J. Protozool.*, **17**:213–219.
- Haughan, P. A. & Goad, L. J. 1991. Lipid biochemistry of trypanosomatids. In: Coombs, G. and North, M. (ed.), *Biochemical Protozoology*. Taylor & Francis Ltd., London. p. 286–303.
- Holz Jr., G. G. 1985. Lipid of *Leishmanias*. In: Chang, K.-P. and Bray, R.S. (ed.), *Leishmaniasis*. Elsevier, Amsterdam. p. 79–82.
- Jarroll, E. L., Muller, P. J., Meyer, E. A., & Morse, S. A. 1981. Lipid and carbohydrate metabolism of *Giardia lamblia*. *Mol. Biochem. Parasitol.*, **2**:187–196.
- Kaneda, Y. & Goutsu, T. 1988. Lipid analysis of *Giardia lamblia* and its culture medium. *Ann. Trop. Med. Parasitol.*, **82**:83–90.
- Kaneshiro, E. S., Beischel, L. S., Merkel, S. J. & Rhoads, D. E. 1979. The fatty acid composition of *Paramecium aurelia* cells and cilia: changes with culture age. *J. Protozool.*, **26**:147–158.
- Kleinschuster, S. J. & Swink, S. L. 1993. A simple method for the in vitro culture of *Perkinsus marinus*. *Nautilus*, **107**:76–78.
- Korn, E. D., Greenblatt, C. L. & Lees, A. M. 1965. Synthesis of unsaturated fatty acids in the slime mold *Physarum polycephalum* and the zooflagellates *Leishmania tarentolae*, *Trypanosoma lewisi*, and *Crithidia* sp.: a comparative study. *J. Lipid Res.*, **6**:43–50.
- La Peyre, J. F., Faisal, M. & Bureson, E. M. 1993. In vitro propagation of the protozoan *Perkinsus marinus*, a pathogen of the Eastern oyster, *Crassostrea virginica*. *J. Eukaryot. Microbiol.*, **40**:304–310.
- Levine, N. D. 1978. *Perkinsus* gen. n. and other new taxa in the protozoan phylum Apicomplexa. *J. Parasitol.*, **64**:549.
- Lindmark, D. G., Beach, D. H., Singh B. N. & Holz Jr., G. G. 1991. Lipids and lipid metabolism of trichomonads (*Trichomonas foetus* and *Trichomonas vaginalis*). In: Coombs, G. and North, M. (ed.), *Biochemical Protozoology*. Taylor & Francis Ltd., London. p. 329–335.
- Lujan, H. D., Mowatt, M. R. & Nash, T. E. 1996. Lipid requirements and lipid uptake by *Giardia lamblia* trophozoites in culture. *J. Eukaryot. Microbiol.*, **43**:237–242.
- Marty, Y., Delaunay, F., Moal, J. & Samain, J.-F. 1992. Change in the fatty acid composition of *Pecten maximus* (L.). *J. Exp. Mar. Biol. Ecol.*, **163**:221–234.
- Metcalf, L. D. & Schmitz, A. A. 1961. The rapid preparation of fatty acid esters for gas chromatography analysis. *Anal. Chem.*, **33**:363–364.
- Meyer, H. & Holz Jr., G. G. 1966. Biosynthesis of lipids by kinetoplastid flagellates. *J. Biol. Chem.*, **241**:5000–5007.
- Mitschler, R. R., Welti, R. & Upton, S. J. 1994. A comparative study of lipid compositions of *Cryptosporidium parvum* (Apicomplexa) and Madin-Darby Bovine Kidney Cells. *J. Eukaryot. Microbiol.*, **41**:8–12.
- Perkins, F. O. 1966. Life history studies of *Dermocystidium marinum*, an oyster pathogen. Ph. D. Dissertation. Florida State University, Tallahassee, Florida. 273 p. Available on microfilm from Florida State University, Accession Number AAG6609078.
- Perkins, F. O. 1976. Zoospores of the oyster pathogen, *Dermocystidium marinum*. I. Fine structure of the conoid and other sporozoan-like organelles. *J. Parasitol.*, **62**:959–974.
- Perkins, F. O. 1988. Structure of protistan parasites found in bivalve molluscs. *Amer. Fish. Soc. Spec. Publ.*, **18**:93–111.
- Perkins, F. O. 1996. The structure of *Perkinsus marinus* (Mackin, Owen and Collier, 1950) Levine, 1978 with comments on taxonomy and phylogeny of *Perkinsus* spp. *J. Shellfish. Res.*, **15**:67–87.
- Pohl, P. 1982. Lipids and fatty acids of microalgae. In: Zaborovsky, O. R. (ed.), *CRC Handbook of Biosolar Resources*. CRC Press, Boca Raton, Florida **1**:383–405.
- Redman, C. A., Kennington, S., Spathopoulou, T. & Kusel, J. R. 1997. Interconversion of sphingomyelin and ceramide in adult *Schistosoma mansoni*. *Mol. Biochem. Parasitol.*, **90**:145–153.
- Reece, K., Siddall, M. E., Bureson, E. M. & Graves, J. E. 1997. Phylogenetic analysis of *Perkinsus* based on actin gene sequences. *J. Parasitol.*, **83**:417–423.
- Sherman, I. W. 1979. Biochemistry of *Plasmodium* (malaria parasites). *Microbiol. Rev.*, **43**:453–495.
- Siddall, M. E., Reece, K. S., Graves, J. E. & Bureson, E. M. 1997. 'Total evidence' refutes the inclusion of *Perkinsus* species in the phylum Apicomplexa. *Parasitology*, **115**:165–176.
- Stevens, T. L., Gibson, G. R., Adam, R., Maier, J., Allison-Ennis, M. & Das, S. 1997. Uptake and cellular localization of exogenous lipids by *Giardia lamblia*, a primitive eukaryote. *Exp. Parasitol.*, **86**:133–143.
- Vial, H. J. & Ancelin, M.-L. 1992. Malaria lipids, an overview. In: Avila, J. L. & Harris, J. R. (ed.), *Subcellular Biochemistry, Intracellular Parasites*. Plenum Press, New York. **18**:259–306.
- Vial, H. J. & Ancelin, M.-L. 1998. Malaria lipids. In: Sherman, I. W. (ed.), *Malaria: Parasite Biology, Pathogenesis, and Protection*. ASM Press, Washington, D.C. p. 159–175.
- Vial, H. J., Ancelin, M.-L., Philippot, J. R. & Thuet, M. J. 1990. Biosynthesis and dynamics of lipids in *Plasmodium*-infected mature mammalian erythrocytes. *Blood Cells*, **16**:531–555.
- Vivier, E. 1982. Réflexions et suggestions à propos de la systématique des sporozoaires: création d'une classe des Hematozoa. *Protistologica*, **18**:449–457.
- Volety, A. K. 1995. A study of the histozoic oyster parasite, *Perkinsus marinus*: (1) Disease processes in American oysters (*Crassostrea virginica*); (2) Biochemistry of *Perkinsus marinus*. Ph.D. Dissertation. School of Marine Science, College of William and Mary. 208 p.
- Volety, A. K., Chu, F.-L. E. & Ozkizilcik, S. 1995. Biochemical characterization of the oyster parasite, *Perkinsus marinus*: lipid and fatty acid composition. *J. Shellfish. Res.*, **14**:280. Available on microfilm from School of Marine Science, College of William and Mary Accession # AA19530357.
- Waller, R. F., Keeling, P. J., Donald, R. G. K., Striepen, B., Handman, E., Lang-Unnasch, N., Cowman, G. S., Besra, G. S., Roos, D. S. & McFadden, G. I. 1998. Nuclear-encoded proteins target to the plastid in *Toxoplasma gondii* and *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA*, **95**:12352–12357.
- Wunderlich, F., Fiebig, S., Vial, H. & Kleinig, H. 1991. Distinct lipid compositions of parasite and host cell plasma membranes from *Plasmodium chabaudi*-infected erythrocytes. *Mol. Biochem. Parasitol.*, **44**: 271–278.