Limnol. Oceanogr., 52(5), 2007, 1910–1918

Confirmation of a new food chain utilizing geothermal energy: Unusual fatty acids of a deep-sea bivalve, *Calyptogena phaseoliformis*

Hiroaki Saito[1] *and Kazufumi Osako*[2]
National Research Institute of Fisheries Science, 2-12-4 Fuku-ura, Kanazawa-ku, Yokohama 236-8648, Japan

Abstract

Neutral and polar lipids of the cold-seep clam *Calyptogena phaseoliformis* were determined to assess the trophic relationship at hydrothermal vents. The clam obtained many of its lipids from the chemosynthetic microorganisms within the bacteriocytes on its gill filaments. The major fatty acids in the triacylglycerols and phospholipids in the clam consisted only of the *n*-4 family polyunsaturated fatty acids (PUFA) plus saturated and *n*-7 monounsaturated fatty acids and were completely lacking *n*-3 PUFA, such as docosahexaenoic acid (22:6*n*-3) and icosapentaenoic acid (20:5*n*-3). Such unique fatty acid composition markedly differs from those reported for other marine animals, which depend on organic matter derived from surface-layer phytoplankton. The relationship between *C. phaseoliformis* and its symbiotic bacteria is documented and revealed a closed novel food chain, independent of the photosynthetic food chain. The fluidity of the fatty acids in the animal's membrane are maintained by adaptation of the exogenous *n*-4 family PUFA. These unusual but dominant fatty acids are assimilated from the symbiotic chemosynthetic bacteria, which use only geothermal energy and minerals from the cold-seep vents.

All marine animals contain *n*-3 polyunsaturated fatty acids (PUFA), which originate from both phytoplankton and any symbiotic microorganisms depending on solar energy (Ackman 1989). In particular, marine animals contain high levels of long-chain *n*-3 PUFA, such as docosahexaenoic acid ([DHA] 22:6*n*-3) and icosapentaenoic acid ([EPA] 20:5*n*-3), in their depot and tissue lipids (Ackman 1989; Tocher 2003). Similarly, *n*-6 PUFA, such as linoleic acid (18:2*n*-6) and arachidonic acid (20:4*n*-6), are generally important for terrestrial animals. Either *n*-3 or *n*-6 PUFA usually dominate in marine or terrestrial animals, in particular in their cell membrane phospholipids.

In the deep sea, all nutritional constituents are provided by the fall of organic matter from the surface (the detrital food chain) and vertical migrations of zooplankton and micronekton (the grazing food chain) (Raymont 1983). Not only do all marine animals in the upper water layers gain nutrition through the marine grazing food chain, but also benthic animals involved in the detrital food chain on or near the ocean bottom in some communities are indirectly supported by surface phytoplankton (Ackman 1989).

The deep-sea Vesicomyidae clams of the genus *Calyptogena*, which have degenerate alimentary canals and house symbiotic chemosynthetic bacteria within the bacteriocytes on their gill filaments, have been observed on many cold-seep sites of the bathyal to hadal zones (Sibuet and Olu 1998). The cold-seep bivalves, including Vesicomyids, thrive in cold seawater (2–5°C) in the deep sea and are conspicuous and abundant members of the deep-sea biological communities supported by sulfide-oxidizing chemoautotrophic bacteria (Felbeck et al. 1981; Rau 1981). They assimilate the products and energy from these endosymbionts and completely depend on the chemosynthetic symbionts and their products (Childress and Fisher 1992; Nelson and Fisher 1995). The chemosynthetic bacteria essential for these communities must use geothermal energy and nutrients originating from the vents and be independent of food sources originating from photosynthesis.

To determine the trophic relationships at the cold seep of the abyssal clam *Calyptogena phaseoliformis* (Métivier et al. 1986), we sampled hydrothermal vents in the Japan Trench (at nearly 6,500 m), and measured both its neutral and polar lipids and fatty acids.

Materials and methods

Materials—Specimens of bivalvia *C. phaseoliformis* collected during a survey by the submersible *Shinkai 6500* belonging to the Japan Marine Science and Technology

[1] Corresponding author: (hiroakis@affrc.go.jp).
[2] Present address: Tokyo University of Marine Science and Technology, Konan, Minato-Ku, Tokyo, 108-8477, Japan.

Acknowledgments

The authors thank Robert G. Ackman, Dalhousie University in Halifax, for his excellent advice concerning lipid analysis and biosyntheses of the animals, and for his scholarly comments during the writing of this manuscript. The authors thank Yanic Marty, West Bretagne University in Brest, and Mutsumi Sugita, Shiga University in Japan, for their valuable discussions of the lipid biosynthesis of marine bivalves and Mikiko Tanaka and Akihito Takashima for their skilled technical assistance. We thank the captains and crews of the research vessel *Yokosuka* and the submersible *Shinkai 6500* operation teams for their cooperation and enthusiasm throughout the work and acknowledge the Japan Marine Science and Technology Center for providing us with an opportunity to use their submersible. The manuscript was written with comments and assistance from Robert G. Ackman, Dalhousie University in Halifax. We thank two anonymous reviewers for constructive feedback.

This work was supported in part by the research projects "Development of New Technology for Treatment and Local Recycling of Biomass" from the Ministry of Agriculture, Forestry, and Fisheries of Japan.

Table 1. The biological measurements of *C. phaseoliformis* with relevant data.*

Date	Locality	Sampling depth (m)	Temperature† (°C)	Number†	Length (cm)	Height (cm)	Weight (g)
07 June 1997	40°07′N, 144°11′E	6,291–6,394	1.7–1.8	9	11.6±0.3	3.0±0.1	39.7±4.0

* Results are expressed as mean ± standard error (n= 9).
† Temperature is the seawater temperatures of the sampling depths. Number is the number of replicate specimens.

Center at a depth of 6,367 m at latitude 40°07′N and longitude 144°11′E, in the Japan Trench of the northern Pacific Ocean, are described in detail in Table 1 and shown in Figs. 1 and 2. A total of 39 specimens (mean shell length: 12.7 ± 0.2 cm; mean shell height: 3.2 ± 0.0 cm; mean weight: 59.7 ± 2.8 g) were collected from two different colonies near to each other, and out of them nine randomly selected specimens were considered for lipid analysis.

Lipid extraction and the analysis of lipid classes—Each soft part of the randomly selected five specimens was immersed in a reagent mixture containing chloroform and methanol (2 : 1, vol/vol) immediately after measuring the biological data and briefly rinsing in distilled water on the research vessel *Yokosuka.* The remaining four specimens were instantly frozen at −80°C on the research vessel, brought to our laboratory and preserved at the same temperature for 2 months. Thereafter, the frozen specimens were thawed, and each soft part was immersed in the reagent mixture as described above for the lipid extraction. The lipids of nine selected specimens were individually analyzed. Briefly, each sample was homogenized in the same reagent mixture as described above, and the crude total lipid of each homogenized sample was extracted according to the Folch procedure (Folch et al. 1957).

The extracted crude total lipids were separated into classes on silicic acid columns (Kieselgel 60, 70–230 mesh, Merck and Co.), and a quantitative analysis of the lipid constituents was performed using gravimetric analysis of fractions collected from column chromatography (Saito and Kotani 2000). The first eluate (dichloromethane/*n*-hexane, 2 : 3, v/v: first fraction) was collected as the steryl ester (SE), wax ester (WE), and diacyl glyceryl ether (GE) fraction. Then the following: dichloromethane (second fraction) eluted the triacylglycerols (TAG), dichloromethane/ether (35 : 1, v/v: third fraction) eluted the sterols (ST), dichloromethane/ether (9 : 1, v/v: forth fraction) eluted the diacylglycerols, dichloromethane/methanol (9 : 1, v/v: fifth fraction) eluted the free fatty acids, dichloromethane/methanol (1 : 5, v/v: sixth fraction) eluted the phosphatidylethanolamine (PE), dichloromethane/methanol (1 : 20,v/v: seventh fraction) eluted the ceramide aminoethyl phosphonate with other minor phospholipids, and dichloromethane/methanol (1 : 50, v/v: eighth fraction) eluted phosphatidylcholine (PC), respectively.

The lipid classes from each lipid fraction were identified by comparison of the retention (*Rf*) values of standards using plate thin layer chromatography (Merck and Co.,

Fig. 1. Photograph of *Calyptogena phaseoliformis* sampled at hydrothermal vents at a depth of 6,367 m and latitude 40°07′N and longitude 144°11′E in the Japan Trench of the northern Pacific Ocean on 07 June 1997. Symbiotic chemosynthetic bacteria are housed within the bacteriocytes on their gill, which are well developed. Scale is shown in centimeters.

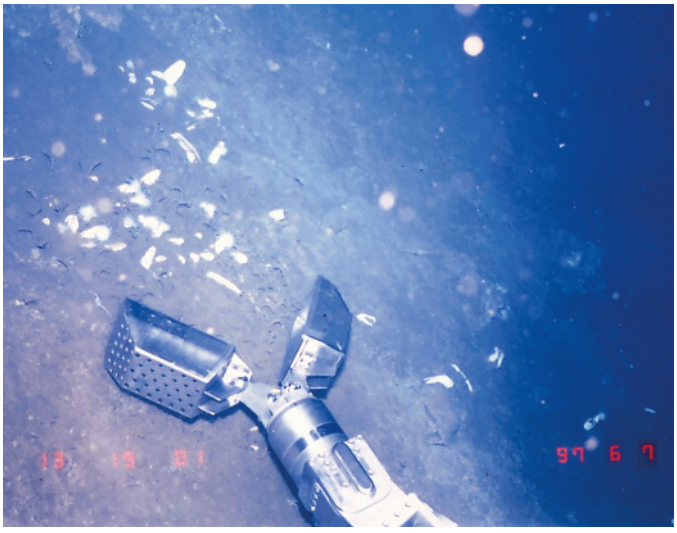

Fig. 2. Photograph of the sampling site of *Calyptogena phaseoliformis* with the manipulator of the submersible *Shinkai 6500* of the Japan Marine Science and Technology Center. The size of the scoop shovels at the clamp of the manipulator is 20.4 cm in length, 12.2 cm in width, and 9.0 cm in height.

Kieselgel 60; thickness of silica gel: 0.25 mm) for analysis. All sample lipids were dried under argon at room temperature and stored at −40°C.

Nuclear magnetic resonance spectrometry and the determination of lipid classes—Spectra were recorded on a GSX-270 nuclear magnetic resonance (NMR) spectrometer (JEOL) in the pulsed Fourier transform mode at 270 MHz in deuterochloroform solution using tetramethylsilane as an internal standard (Saito and Kotani 2000).

Some fractions often contained several classes; for instance, the first fraction contained WE, SE, and GE. The molar ratios of WE, GE, and SE were determined by the quantitative analysis of NMR results using characteristic peaks. In NMR, the amount of WE was obtained by the total amount of the combined integrations of the triplet peaks (from 3.90 ppm to 4.20 ppm) as the two protons at the ester alcohol; the amount of GE was obtained by that of the singlet peak (3.50–3.80 ppm) as the two ether protons linked with glycerol carbons, and the amount of SE was obtained by that of multiplet peak (4.30–4.80 ppm) as one proton at the carbon linked esterized C-3 alcohol of the ST. The actual ratios of the WE, SE, and GE in the first fraction were determined as the respective integration divided by the sum of total integrations of the three combined peaks from 3.50 ppm to 4.80 ppm. The actual weight of each class was obtained by calculating the ratio and multiplying by the total weight of the first fraction. Similarly, the third fraction sometimes contained TAG and ST, which have two characteristic peaks: for TAG (3.90–4.40 ppm) an octet-like peak for four protons and for ST (3.40–3.60 ppm) a multiplet peak for one proton. The actual respective weight of TAG and ST in the third fraction was obtained by calculating the integration of divided respective peak and multiplying the total weight of the third fraction (Saito 2004).

The preparation of methyl esters and gas-liquid chromatography of the esters—Individual components of TAG, PE, and PC fractions were converted into fatty acid methyl esters by direct transesterification with methanol containing 1% of concentrated hydrochloric acid under reflux for 1.5 h. The methyl esters were purified using silica gel column chromatography by elution with dichloromethane/*n*-hexane (2 : 1, v/v) (Saito and Kotani 2000).

The compositions of the fatty acid methyl esters were determined by gas liquid chromatography. Analysis was performed on Shimadzu GC-8A (Shimadzu Seisakusho) and HP-5890 series II (Hewlett Packard, Yokogawa Electric Corporation) gas chromatographs equipped with an Omegawax-250 fused silica capillary column (30 m × 0.25 mm inside diameter; 0.25 μm film, Supelco Japan). The temperatures of the injector and the column were held at 230°C and 215°C, respectively, and the split ratio was 1 : 76. Helium was used as the carrier gas at a constant inlet rate of 0.7 mL min^{-1}. In the fatty acid composition of PE, significant levels of dimethylacetals (DMA), such as DMA 18:0, were included (Table 2). The theoretical values of the fatty acid composition were obtained by deleting the DMA from the TFA of PE. After this treatment, we described both the PE and calculated PE so as to provide a more correctable form in Table 2.

Quantitation of individual components was performed by means of a Shimadzu Model C-R3A (Shimadzu Seisakusho) and HP ChemStation System (A. 06 revision, Yokogawa HP) electronic integrators.

The preparation of 4,4-dimethyloxazoline derivatives and analysis of 4,4-dimethyloxazoline by gas chromatography–mass spectrometry—The 4,4-dimethyloxazoline (DMOX) derivatives were prepared by adding an excess amount of 2-amino-2-methyl propanol (1 mL) to a small amount of the fatty acid methyl esters (10.0 mg) in a test tube under an argon atmosphere (Saito 2004). The mixture was heated at 180°C for 48 h. The reaction mixture was cooled and poured onto saturated brine and extracted with *n*-hexane (three times). The pooled *n*-hexane extract was washed with saturated brine, and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure, and the samples were again dissolved in *n*-hexane for analysis by gas chromatography–mass spectrometry (MS).

Analysis of the DMOX derivatives was performed on a HP G1800C GCD Series II (Hewlett Packard, Yokogawa Electric) gas chromatograph mass spectrometer equipped with the same capillary for determining the respective fatty acids with the HP WS (HP Kayak XA, G1701BA version, PC workstations). The temperatures of the injector and the column were held at 230°C and 215°C, respectively. The split ratio was 1 : 75, and the ionization voltage was 70 eV. Helium was used as the carrier gas at a constant inlet rate of 0.7 mL min^{-1}.

Fatty acid methyl esters were identified using (1) marine lipid methyl esters as standards (Omegawax test mixture No. 4-8476, Supelco Japan) and (2) by comparison of mass spectral data obtained by gas chromatography–mass spectrometry.

Structural elucidation of the novel n-*4 family PUFA in* C. phaseoliformis *lipids*—The MS-charts of a major fatty acid in *C. phaseoliformis* are representatively displayed in Fig. 3. In Fig. 3, MS peaks of a DMOX derivative of 20:4*n*-1,4,7,15 are M$^+$-357, 342, 330, 316, 302, 290, 276, 262, 250, 236, 222, 208, 194, 180, 166, 153, 136, 126, 113 (base peak), and three pairs of the peaks M-342 and M-330, M-302 and M-290, and M-262 and M-250 are respectively reflected by three double bonds, Δ-19 (*n*-1), Δ-16 (*n*-4) and Δ-13 (*n*-7); a pair of the peaks M-153 and M-136 shows a Δ-5 (*n*-15) double bond. In the MS-chart of the DMOX derivative of 20:4*n*-1,4,7,15, another MS peak of the DMOX derivatives is proposed to be observed: i.e., M$^+$-357, 342, 328, 316, 302, 290, 276, 262, 250, 236, 222, 208, 194, 180, 166, 153, 136, 126, 113 (base peak). A set of the four MS peaks M-328, M-316, M-302, and M-290 might be considered to reflect the Δ-18 (*n*-2) and Δ-16 (*n*-4) conjugated double bonds. With the other two pairs of the M-262 and M-250 and the M-153 and M-136 reflecting Δ-13 (*n*-7) and Δ-5 (*n*-15) double bonds, the whole set of these peaks seems as if from a conjugated dienoic fatty acid 20:4*n*-2,4,7,15. This is the same as the authentic *n*-1 PUFA, such as 18:1*n*-1,4 and 18:4*n*-1,4,7,10, which could be

Table 2. Fatty acid composition of *C. phaseoliformis.**†

	TAG Mean SE	PE‡ Mean SE	Calculated PE Mean SE	PC Mean SE
Total saturated	7.9±0.3	7.6±0.4	8.1±0.4	12.8±0.7
14:0	1.0±0.1	1.0±0.1	1.1±0.1	1.0±0.1
15:0	1.0±0.1	0.8±0.1	0.9±0.1	0.6±0.1
16:0	4.4±0.3	4.4±0.3	4.7±0.3	10.4±0.6
17:0	0.4±0.1	0.3±0.0	0.3±0.0	0.4±0.1
18:0	0.9±0.1	0.9±0.0	1.0±0.0	0.4±0.1
Total monoenoic§	80.6±0.5	40.3±1.2	42.7±1.2	34.9±1.3
15:1*n*-6	0.5±0.1	0.3±0.0	0.3±0.0	0.0±0.0
16:1*n*-7	44.9±0.9	25.2±1.3	26.8±1.4	18.9±1.0
16:1*n*-5	0.4±0.0	0.2±0.0	0.2±0.0	0.8±0.1
17:1*n*-8	0.1±0.0	0.1±0.0	0.1±0.0	0.3±0.0
17:1*n*-6	0.4±0.1	0.2±0.0	0.2±0.0	0.1±0.0
18:1*n*-13	0.5±0.0	0.6±0.0	0.6±0.0	1.3±0.1
18:1*n*-9(8)	0.2±0.1	0.0±0.0	0.0±0.0	0.1±0.0
18:1*n*-7(6)	13.9±0.3	2.0±0.1	2.1±0.1	5.0±0.2
18:1*n*-5	0.3±0.1	0.1±0.0	0.1±0.0	0.6±0.0
19:1*n*-7	2.4±0.3	0.6±0.0	0.7±0.0	1.1±0.1
19:1*n*-6	0.4±0.1	0.0±0.0	0.0±0.0	0.0±0.0
20:1*n*-13	1.7±0.1	6.1±0.6	6.4±0.5	2.7±0.2
20:1*n*-9(8)	0.7±0.2	0.8±0.0	0.9±0.0	0.6±0.0
20:1*n*-7(6)	13.1±0.5	3.5±0.2	3.7±0.2	2.8±0.3
21:1*n*-7	0.6±0.1	0.2±0.0	0.2±0.0	0.3±0.0
Total NMIDs‖	6.6±0.2	27.0±1.5	28.4±1.3	18.5±0.8
19:2*n*-7,14	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0
20:2*n*-9(8),15	0.3±0.1	1.3±0.1	1.4±0.1	2.3±0.1
20:2*n*-7(6),15	4.2±0.2	17.4±1.0	18.3±0.9	11.5±0.7
20:2*n*-5,15	0.2±0.0	0.4±0.0	0.4±0.0	0.3±0.0
21:2*n*-7,16	0.8±0.1	5.6±0.3	5.9±0.3	2.6±0.1
21:2*n*-5,16	0.2±0.0	0.2±0.1	0.2±0.1	0.2±0.0
22:2*n*-9(8),15	0.0±0.0	0.4±0.0	0.4±0.0	0.4±0.0
22:2*n*-7(6),15	0.8±0.1	1.4±0.1	1.5±0.1	0.8±0.1
Total polyenoic	4.3±0.3	18.1±0.9	19.2±1.0	32.0±1.9
18:2*n*-4,7	0.1±0.0	0.2±0.0	0.2±0.0	0.6±0.1
20:2*n*-4,7	0.0±0.0	0.2±0.0	0.2±0.1	0.2±0.0
20:3*n*-4,7,15	1.0±0.1	3.9±0.2	4.2±0.2	9.9±0.6
20:4*n*-1,4,7,15	2.0±0.2	9.0±0.6	9.6±0.7	15.9±1.1
21:3*n*-4,7,16	0.3±0.0	3.6±0.2	3.8±0.2	3.4±0.2
21:4*n*-7,10,13,16	0.0±0.0	0.2±0.0	0.2±0.0	0.1±0.0
21:4*n*-4,7,10,16	0.4±0.0	0.1±0.0	0.1±0.0	0.1±0.0
21:4*n*-1,4,7,16	0.0±0.0	0.2±0.0	0.2±0.0	0.2±0.0
22:3*n*-7,10,15	0.0±0.0	0.1±0.0	0.1±0.0	0.4±0.0
22:3*n*-4,7,15	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0
22:4*n*-4,7,10,15	0.0±0.0	0.0±0.0	0.0±0.0	0.2±0.0
22:4*n*-1,4,7,15	0.3±0.0	0.3±0.0	0.3±0.0	0.7±0.1
Total *n*-7 fatty acids	85.2±0.3	74.2±0.8	78.5±0.6	75.3±0.7
Total fatty acids	99.4±0.1	93.0±1.0	98.3±0.2	98.1±0.1
DMA 20:1		5.2±0.9		
DMA 22:1		0.2±0.0		
Total DMA‡		5.4±0.9		

* Data are mean±SE for several samples (n=17–18). TAG, triacylglycerols; PE, phosphatidylethanolamine; PC, phosphatidylcholine; NMIDs, non-methylene interrupted dienoic fatty acids; DMA, dimethylacetals.

† The major fatty acids were selected if at least one mean datum was detected at a level of 0.2% or more of the total fatty acids.

‡ The fatty acids of PE contained significant levels of alkenyl glyceryl ether type PE, and DMA were derived from the PE by the condition of esterification.

§ The NMIDs 20:2*n*-7,15 and 20:2*n*-9,15 contained significant levels of 20:2*n*-6,15 and 20:2*n*-8,15, respectively. The NMIDs 22:2*n*-7,15 and 22:2*n*-9,15 contained significant levels of 22:2*n*-6,15 and 22:2*n*-8,15, respectively.

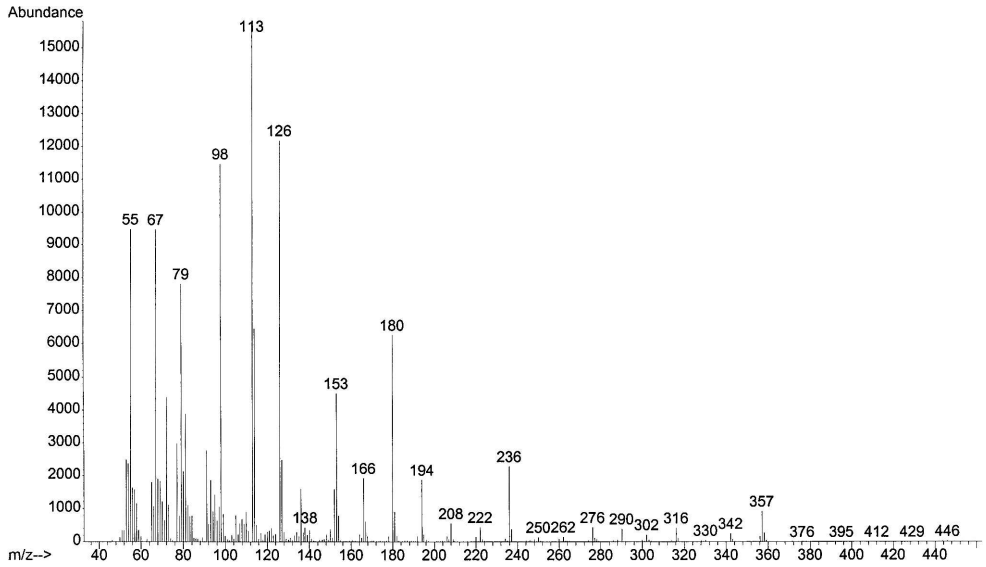

Fig. 3. MS peaks of a DMOX derivative of 20:4*n*-1,4,7,15 are M^+-357, 342, 330, 316, 302, 290, 276, 262, 250, 236, 222, 208, 194, 180, 166, 153, 136, 126, 113 (base peak). Three pairs of the peaks M-342 and M-330, M-302 and M-290, and M-262 and M-250 are respectively reflected by three double bonds, Δ-19 (*n*-1), Δ-16 (*n*-4) and Δ-13 (*n*-7), and a pair of the peaks M-153 and M-136 shows a Δ-5 (*n*-15) double bond.

confused with conjugated 18:2*n*-1,4 and 18:4*n*-2,4,7,10, in the Christie home page (http://www.lipidlibrary.co.uk/). We definitely know that the peak of the 20:4*n*-1,4,7,15 in the chromatogram does not actually contain that of 20:4*n*-2,4,7,15, because of the large difference in the retention time between two fatty acids.

A typical example of the series of other characteristic *n*-4 family fatty acids in *C. phaseoliformis* is provided in Web Appendix 1 (http://www.aslo.org/lo/toc/vol_52/issue_5/1910a1.pdf).

Results and discussion

Lipid content and classes of C. phaseoliformis—The lipid content of *C. phaseoliformis* was relatively high (2.7%±0.3%; Table 3) in comparison with those of other marine bivalves (approximate range 0.5–2.0%) (Soudant et al. 1999; Saito 2004). Therefore the environmental nutritional levels of the hydrothermal vent must be sufficiently high for the maintenance of animal life with the aid of symbiotic bacteria, despite the extremely cold and high-pressure conditions (Luts et al. 1994).

The lipids of all samples contained medium levels of both neutral (mainly TAG, ST, and free fatty acids) and polar lipids (mainly PE and PC) with significant levels of ceramideaminoethylphosphonate. Although *C. phaseoliformis* is a definitive deep-sea species, the lipid classes were similar to those of shallow-water bivalves (Soudant et al. 1999; Saito 2004). Hence, the lipid classes of various bivalve species, which range from shallow water to deep sea, might not be influenced by changes in the environmental conditions such as temperature and pressure.

Fatty acid composition in depot TAG of the C. phaseoliformis *lipids*—Fatty acids in TAG (>0.2% of the total fatty acids [TFA]) are shown in Table 2. In its depot TAG, five dominant fatty acids (each accounted for more than about 3% of TFA) were detected: 16:0 as the saturate; 16:1*n*-7, 18:1*n*-7, 20:1*n*-7 as the monoenes; 20:2 *n*-7,15 as the non-methylene interrupted dienoic fatty acid (NMID) with significant levels (more than about 1% of TFA) of six other minor fatty acids; 14:0 and 15:0 for saturates; 19:1*n*-7 and 20:1*n*-13 for monoenes; and 20:3*n*-4,7,15 and 20:4*n*-1,4,7,15 for the *n*-4 family PUFA (*n*-1 and *n*-4 PUFA).

Similar to the typical bacterial fatty acids (Fulco 1983), very high levels of total monoene fatty acids (80.6%±0.5%) were observed in its TAG, whereas it had low levels of saturated fatty acids (total saturates 7.9%±0.3%). For

Table 3. The lipid contents and classes of the *Calyptogena phaseoliformis*.

Lipid contents*	WE†	SE†	GE†	TAG†	ST†	DG†	FFA†	PE†	CAEP†‡	PC†
2.7±0.3	0.5±0.2	0.6±0.2	1.6±0.5	36.2±3.8	11.7±2.9	1.9±0.3	15.1±1.3	16.0±2.1	12.7±1.8	3.7±0.7

* Results of mean lipid contents are expressed as weight percent of wet tissues of muscle. Data are mean±standard errors (*n*=9).
† Results are expressed as weight percent of total lipids. Data are mean±standard errors (*n*=9). WE, SE, GE, TAG, ST, DG, FFA, PE, CAEP, and PC are wax esters, steryl esters, diacyl glycerylethers, triacylglycerols, sterols, diacylglycerols, free fatty acids, phosphatidylethanolamine, ceramideaminoethylphosphonate, and phosphatidylcholine, respectively.
‡ The CAEP fraction contained small amounts of other minor phospholipids.

example, palmitoleic acid 16:1*n*-7 (melting point 41°C), which is derived from palmitic acid 16:0 (melting point 63.1°C) by enzymatic action of Δ-9 desaturase, was determined as a major monoene in *C. phaseoliformis* lipid. For adaptation to low temperature, high levels of monoenes are more advantageous than those of saturates (DeLong and Yayanos 1986). Longer *n*-7 monoenes, vaccenic acid 18:1*n*-7 and icosenoic acid 20:1*n*-7, which might be directly derived by C-2 elongations of 16:1*n*-7 as a parent acid (Ackman 1989) or by an exceptional anaerobic pathway of bacteria (Fulco 1983), were also determined as other major monoenes in this clam, whereas the two long-chain saturates stearic acid 18:0 and icosanoic acid 20:0 were detected at only low levels. Two *n*-9 monoenes, such as 18:1*n*-9 and 20:1*n*-9, are usually observed as major monoenes in all other animal lipids, because almost all animals biosynthesize long chain monoenes mainly by derivation from 18:0 with the reaction of the same Δ-9 desaturase by a normal oxygen-dependent pathway (Fulco 1983; Cook 1991). Only some species of sulfur-oxidizing bacteria, such as *Thiobacillus* A2 (Thiele et al. 1984) and *Paracoccus versutus* (Knief et al. 2003), mainly produce 18:1*n*-7 in their lipids. Furthermore, a pale yellow simple substance (sulfur) was collected on the gill surface of *C. phaseoliformis,* and the extracted crude lipids often included significant amounts of sulfur. Both can be obtained by oxidation of hydrogen sulfide plentiful in this environment. The similarity to the *n*-7 monoenes in sulfur-oxidizing bacteria, as well as the significant presence of 18:1*n*-7 and 20:1*n*-7 in the *C. phaseoliformis* lipids, led to the conclusion that this clam may be dependent on the above types of bacterial lipids. As for biosynthesis of the *n*-4 family PUFA in vent animal lipids, two reports consistently support our results: Fang et al. (1993) observed a medium level of 18:3*n*-7,10,13 and a trace level of 22:3*n*-7,11,15 with high levels of monoenes and NMID in an unknown species of cold-seep mussels, and Pond et al. (2002) found a small level of 18:3*n*-7 with significant levels of *n*-3 and *n*-6 PUFA in the vent worm *Ridgea piscesae*.

Fatty acid composition in tissue phospholipids of the C. phaseoliformis *lipids*—With a fatty acid composition similar to that of TAG, the same saturated and monoene fatty acids were determined as dominants (more than about 3% of TFA) in the two tissue phospholipids (PE and PC); 16:0 as a saturate, and 16:1*n*-7, 18:1*n*-7, 20:1*n*-7, and 20:1*n*-13 as monoenes. The PUFA were 20:2*n*-7,15 and 21:2*n*-7,16 as NMIDs, and 20:3*n*-4,7,15, 20:4*n*-1,4,7,15, and 21:3*n*-4,7,16 as non-methylene interrupted (NMI)-PUFA, with significant levels (more than about 1% of TFA) of five fatty acids; 14:0 and 18:0 as saturates, 18:1*n*-13 and 19:1*n*-7 as monoenes, and 22:2*n*-7,15 as NMID are listed in Table 2. In the fatty acid composition of PE, significant levels of DMA, such as DMA 18:0, were often included (Table 2; Kraffe et al. 2004). The theoretical values (calculated PE) of the fatty acid composition were obtained by deleting the DMA from the TFA of PE.

Although there were small differences in fatty acid levels between the TAG and the phospholipids, the sorts of acids in all classes were close to each other; in particular, major fatty acids in all of the lipids belong to the novel *n*-4 family fatty acids (total *n*-4 family fatty acids: 85.2% for TAG, 78.5% for PE, and 75.3% for PC), such as *n*-7 monoenes, *n*-7 NMIDs, and the *n*-4 PUFA (*n*-1 and *n*-4 PUFA). Contrary to the high levels of *n*-7 monoenes (80.6%±0.5%) in the depot TAG, higher levels of the *n*-4 family PUFA (19.2%±1.0% for PE and 32.0%±1.9% for PC) were detected in the tissue phospholipids. The latter might mainly form membrane lipids, except for the noticeable level of monoenes 20:1*n*-13, which is considered to be biosynthesized from 18:1*n*-13 by C-2 elongation, whereas 18:1*n*-13 is derived from 18:0 by the Δ-5 desaturase generally common in bivalves (Zhukova 1991).

The increases in levels of C-20 and C-21 NMIDs and the decrease of the monoene levels observed in the PE and PC contrast sharply with the extremely high levels of monoenes in TAG. This is how this clam might accumulate more highly unsaturated fatty acids in its phospholipids, even including C-21 and NMID in quantity. This includes the same Δ-5 desaturase for biosynthesis of NMIDs from monoenes; for example, 20:2*n*-7,15 and 21:2*n*-7,16 might be derived from 20:1*n*-7 and 21:2*n*-7. The same mechanism of using Δ-5 desaturase might be expanded in many cases of synthesizing NMI-PUFA. Thus, 20:3*n*-4,7,15, 20:4*n*-1,4,7,15, 21:3*n*-4,7,16, 21:4*n*-4,7,10,16, and 21:4*n*-1,4,7,16 would be obtained from corresponding unsaturated dienes and trienes, such as 20:3*n*-4,7, 20:4*n*-1,4,7, 21:3*n*-4,7, 21:4*n*-4,7,10, and 21:4*n*-1,4,7. Furthermore, C-22 NMI-PUFA: 22:3*n*-7,10,15, 22:3*n*-4,7,15, 22:4*n*-4,7,10,15, and 22:4*n*-1,4,7,15 were also obtained by C-2 elongations of the former C-20 series NMI- PUFA.

Clearly *C. phaseoliformis* requires sufficient levels of PUFA in its membrane lipids toward maintaining fluidity, either because of the extremely low seawater temperature (1.7°C) of the habitat (Table 1) or for closely packing in membranes. Unlike the various fatty acids such as *n*-3 and *n*-6 PUFA found in the lipids of shallow-water bivalves in normal marine food webs, only the *n*-4 family PUFA were found among all the long chain PUFA in the *C. phaseoliformis* lipids. For their fluidity, this clam might accumulate the *n*-4 PUFA in its phospholipids (PE and PC) and be restricted to using only these *n*-4 PUFA, for instance, 20:3*n*-4,7,15, 20:4*n*-1,4,7,15, and 21:3*n*-4,7,16 as substitutes for the *n*-3 PUFA, and these novel *n*-4 family PUFA might compose its unique lipids.

n-*3 PUFA essentiality for bivalves*—Marine bivalve lipids generally contain *n*-3 PUFA, which originate from phytoplankton. Some reports demonstrate that long-chain *n*-3 PUFA are essential for mollusks (Thompson and Harrison 1992; Budge et al. 2001), and some bivalve lipids at the hydrothermal vents include significant levels of *n*-3 and *n*-6 PUFA (Vesicomyidae clam; *Calyptogena magnifica* [Ben-Mlih et al. 1992], mussels; *Bathymodiolus thermophilus* [Ben-Mlih et al. 1992], *Bathymodiolus* sp. [Phleger et al. 2005], and *Solemya velum* [Conway and Capuzzo 1991], other bivalve species; and *Lucinoma annulata, Parvilucina tenuisculpta, Lucinoma borealis*, and *Thyasira flexuosa* [Fullarton et al. 1995]).

On the contrary, Pond et al. (1998) suggested that a *Bathymodiolus* sp. might not require *n*-3 PUFA as the essential fatty acids. In our previous report (Saito 2004), the seasonal fluctuation of DHA in polar lipids PE and PC of *Pinctada fucata martensii* differed from those of fish species, which have consistently high levels of DHA in their polar lipids. This suggests that mollusks might not have absolute requirements for DHA as an essential fatty acid. In the present study, we determined the structures of novel *n*-1 and *n*-4 PUFAs in detail. The lack of long-chain *n*-3 PUFA in the lipids of *C. phaseoliformis* strongly supports the nonessentiality of *n*-3 PUFA for some clams.

Relationship between the novel n-*4 PUFA of* C. phaseoliformis *and those of the symbiotic bacteria*—Ecologically, Sibuet and Olu (1998) suggested that cold-seep bivalves, including Vesicomyid clams, wholly depend on symbiotic microorganisms and their chemosynthetic products. Rau (1981) reported a non-photosynthetic food chain by the analyses of stable isotopes. The close trophic relationship between host bivalves and their symbionts has been demonstrated by physiological, phylogenetical, and microscopical analyses (Childress and Fisher 1992; Nelson and Fisher 1995; Sibuet and Olu 1998). For instance, the strong dependence of the Vesicomyids, such as *Calyptogena magnifica* and *Calyptogena elongata*, on the endosymbionts is described in detail with physiological observations (Fiala-Médioni and Le Pennec 1988; Le Pennec and Fiala-Medioni 1988). Moreover, phylogenetical coadaptation and cospeciation have been estimated from the close relationship between the host clams and their symbionts (Cary and Giovannoni 1993). Furthermore, Peek et al (1998) suggested chemosynthetic bacterial contribution to the nutrition of cold-seep clams and elucidated a close relationship between the Vesicomyid clams and the symbionts, which produce both of their nutrients. The strong trophic linkage between the clams and the symbionts was proved by biological approaches. In particular, *C. phaseoliformis* also hosts endosymbiotic chemoautotrophic sulfur-oxidizing bacteria. Fiala-Medioni and Le Pennec (1988) reported the details for highly developed gill and completely degenerate digestive glands of *C. phaseoliformis* and the close trophic relationship with the endocellular symbionts.

It is known that some marine microalgae species, including cyanobacteria, produce shorter chain *n*-1 and *n*-4 PUFA, such as 16:3*n*-4 and 16:4*n*-1 (Thompson and Harrison 1992; Budge et al. 2001). Ackman (1989) suggested the role of these *n*-1 and *n*-4 PUFA as substitutes for the *n*-3 and *n*-6 PUFA families. Cook (1991) pointed out that the *n*-4 family PUFA such as 20:4*n*-7 derived from 16:1*n*-7 in mammals might have the same role as that of 20:4*n*-6 because of their structural similarity. Although long-chain *n*-3 PUFA, such as DHA, were often found in the cells of deep-sea barophilic prokaryotes with *n*-7 monoenes (DeLong and Yayanos 1986; Yano et al. 1998), the sulfur-oxidizing bacteria might produce monoenes rather than *n*-3 PUFA (*Thiobacillus neopolitanus* [Agata and Vishniac 1973]; *Thiobacillus* A2 [Thiele et al. 1984]; *Paracoccus versutus* and *Starkeya novella* [Knief et al. 2003]; and *Thiomicrospira crunogena* [Conway and Capuzzo 1991]). High levels of the unusual fatty acids in the *C. phaseoliformis* may indicate a complete bacterial contribution to host nutrition, and its symbiotic bacteria might play a vital role for the production of *n*-4 PUFA, as well as the role of PUFA previously reported (DeLong and Yayanos 1986; Vargas et al. 1998; Yano et al. 1998). *C. phaseoliformis* may depend on bacterial lipids as an energy source and as constituents of its plasma membrane lipid, because high levels of *n*-7 monoenes and medium levels of the *n*-4 family PUFA were observed in its depot TAG and tissue phospholipids. Moreover, the presence of the same kind of fatty acids in all classes of *C. phaseoliformis* suggests that enzymatic synthesis and chemical modification by the clam is not very active, and it is more likely to simply accumulate and utilize the symbiotic bacterial fatty acids in its depot and tissue lipids. The limitation of the kinds of fatty acids found suggests that the symbiotic bacteria would produce only *n*-4 family fatty acids through their specific enzymatic systems.

In view of the above findings, it is inferred that the clam exclusively depends on the symbiotic organisms. In addition, compared with the low levels of PUFA in other normal bacterial lipids, the high levels of the *n*-4 PUFA in the clam phospholipids provide further insights that the symbiotic bacteria are the producers of long-chain polyunsaturate lipids that provide adaptation to the extremely high pressure and low seawater temperature (DeLong and Yayanos 1986; Metz et al. 2001), similar to the case of *n*-3 PUFA, such as DHA and EPA produced by the other unique deep-sea barophilic bacteria (DeLong and Yayanos 1986; Yano et al. 1998).

Characteristics of the odd chain fatty acids in the C. phaseoliformis *lipids*—Odd chain fatty acids are generally found as the minor components in bacterial lipids (Fulco 1983) and at trace levels in higher marine animals (Ackman 1989); at most, medium levels of limited kinds of odd chain fatty acids were confirmed in the lipid of the sulfur-using bacteria *Thiobacillus thioparus* (Agata and Vishniac 1973). In contrast with the simple fatty acid compositions of most other bacteria, various kinds of odd chain fatty acids from C-15 to C-21 were detected in the lipid of *C. phaseoliformis*; in particular, a significant amount of many sorts of C-21 chain *n*-4 family PUFA were first determined in its phospholipids. These characteristic long-chain fatty acids might be the novel terminal odd chain acids in the lipids of the clam and the symbiotic bacteria, and the variations of these novel odd chain PUFA also suggest a high diversity of enzymes in the symbiotic bacteria, as described above (Tunnicliffe 1991).

C14 to C17 branched fatty acids are found in some bacterial lipids (marine bacteria, Nichols et al. 1993), as well as *Bacillus* species and Gram-positive bacteria (Fulco 1983; Nichols et al. 1993), thermophiles (Ljungdahl 1979), and sulfur-oxidizing bacteria (Knief et al. 2003) as the dominant components and are present in noticeable levels in other marine animal lipids. The biosynthetic pathway of the symbiotic bacteria in the clam may differ from those of many other normal bacteria, because none of the branched

forms of the shorter saturated fatty acids, such as iso and anteiso fatty acids, were present in the *C. phaseoliformis* lipid.

The marked difference between the fatty acids of the *C. phaseoliformis* and those of other marine bivalve species confirms the existence of an alternative food chain based on bacterial chemosynthetic production in the hydrothermal vent (Felbeck et al. 1981; Rau 1981; Sibuet and Olu 1998).

A unique and high diversity of fatty acids from C12 to C22 was observed in *C. phaseoliformis*; actually, >60 kinds of fatty acids were found and >50 kinds of fatty acids were determined in its lipids, although all of them belong to the novel *n*-4 family PUFA with saturated and monounsaturated fatty acids. Such a highly diversified variety of fatty acids may expand the greater possibility of chemosynthetic bacteria being responsible for the origin of life (Tunnicliffe 1991; Childress and Fisher 1992; Metz et al. 2001).

References

Ackman, R. G. 1989. Fatty acids, p. 103–137. *In* R. G. Ackman [ed.], Marine biogenic lipids, fats, and oils. V. I. CRC Press.

Agata, A. D., and W. Vishniac. 1973. Characterization of *Thiobacillus* species by gas-liquid chromatography of cellular fatty acids. Arch. Mikrobiol. **89:** 257–267.

Ben-Mlih, F., J.-C. Marty, and A. Fiala-Médioni. 1992. Fatty acid composition in deep hydrothermal vent symbiotic bivalves. J. Lipid Res. **33:** 1797–1806.

Budge, S. M., C. C. Parish, and C. H. Mckenzie. 2001. Fatty acid composition of phytoplankton, settling particulate matter and sediments at a sheltered bivalve aquaculture site. Mar. Chem. **76:** 285–303.

Cary, S. C., and S. J. Giovannoni. 1993. Transovarial inheritance of endosymbiotic bacteria in clams inhabiting deep-sea hydrothermal vents and cold seep. Proc. Natl. Acad. Sci. USA. **90:** 5695–5699.

Childress, J. J., and C. R. Fisher. 1992. The biology of hydrothermal vent animals: Physiology, biochemistry, and autotrophic symbioses. Oceanogr. Mar. Biol. Annu. Rev. **30:** 337–441.

Conway, N., and J. M. Capuzzo. 1991. Incorporation and utilization of bacterial lipids in the *Solemya velum* symbiosis. Mar. Biol. **108:** 277–291.

Cook, H. W. 1991. Fatty acid desaturation and chain elongation in Eucaryotes, p. 141–169. *In* D. E. Vance and J. Vance [eds.], Biochemistry of lipids, lipoproteins, and membranes. Elsevier.

Delong, E. F., and A. A. Yayanos. 1986. Biochemical function and ecological significance of novel bacterial lipids in deep-sea Procaryotes. Appl. Environ. Microbiol. **51:** 730–737.

Fang, J., P. A. Comet, J. M. Brooks, and T. L. Wade. 1993. Nonmethylene interrupted fatty acids of hydrocarbon seep mussels: Occurrence and significance. Comp. Biochem. Physiol. **104B:** 287–291.

Felbeck, H., J. J. Childress, and G. N. Somero. 1981. Calvin-Benson cycle and sulphide oxidation enzymes in animal from sulphide-rich habitats. Nature **293:** 291–293.

Fiala-Médioni, A., and M. Le Pennec. 1988. Structural adaptations in the gill of the Japanese subduction zone bivalves (Vesicomydae) *Calyptogena phaseoliformis* and *Calyptogena laubieri*. Oceanog. Acta **11:** 185–192.

Folch, J., M. Lees, and G. H. Sloan-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226:** 497–509.

Fulco, A. J. 1983. Fatty acid metabolism in bacteria. Prog. Lipid Res. **22:** 133–160.

Fullarton, J. G., P. R. Dando, J. R. Sargent, S. A. J. Southward, and E. C. Southward. 1995. Fatty acids of hydrothermal vent *Ridgeia piscesae* and inshore bivalves containing symbiotic bacteria. J. Mar. Biol. Ass. U. K. **75:** 455–468.

Knief, C., K. Altendorf, and A. Lipski. 2003. Linking autotrophic activity in environmental samples with specific bacterial taxa by detection of 13C-labelled fatty acids. Environ. Microbiol. **5:** 1155–1167.

Kraffe, E., P. Soudant, and Y. Marty. 2004. Fatty acids of serine, ethanolamine, and choline plasmalogens in some marine bivalves. Lipids **39:** 59–66.

Le Pennec, M., and A. Fiala-Medioni. 1988. The role of the digestive tract of *Calyptogena laubieri* and *Calyptogena phaseoliformis*, vesicomyid bivalves of the subduction zones of Japan. Oceanolog. Acta **11:** 193–199.

Ljungdahl, L. G. 1979. Physiology of thermophilic bacteria. Adv. Microb. Physiol. **24:** 149–243.

Luts, R. A., T. M. Shank, D. J. Fornari, R. M. Haymon, M. D. Lilly, K. Von Damm, and D. Desbruyeres. 1994. Rapid growth at deep-sea vents. Nature **371:** 663–664.

Métivier, B., T. Okutani, and S. Ohta. 1986. *Calyptogena phaseoliformis* n. sp., an usual vesicomyid bivalve collected by the submersible *Nautile* from abyssal depth of the Japan and Kuril Trenches. Venus **45:** 161–168.

Metz, J. G., and others. 2001. Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. Science **293:** 290–293.

Nelson, D. C., and C. R. Fisher. 1995. Chemoautotrophic and methanotrophic endosymbiotic bacteria at deep-sea vents and seeps, p. 125–167. *In* D. M. Karl [ed.], The microbiology of deep-sea hydrothermal vents. CRC Press Inc.

Nichols, D. S., P. D. Nichols, and T. A. McMeekin. 1993. Polyunsaturated fatty acids in Antactic bacteria. Ant. Sci. **5:** 149–160.

Peek, A. S., R. M. Feldman, R. A. Lutz, and R. C. Vrijenhoek. 1998. Cospeciation of chemoautotrophic bacteria and deep sea clams. Proc. Natl. Acad. Sci. USA. **95:** 9962–9966.

Phleger, C. F., M. M. Nelson, A. K. Groce, S. C. Cary, K. J. Coyne, and P. D. Nichols. 2005. Lipid composition of deep-sea hydrothermal vent tube worm *Riftia pachyptila*, crabs *Munidopsis subsquamosa* and *Bythograea thermydron*, mussels *Bathymodiolus* sp. and limpets *Lepetodrilus* spp. Comp. Biochem. Physiol., B **141:** 196–210.

Pond, D. W., C. E. Allen, M. V. Bell, C. L. Van Dover, A. E. Fallick, D. R. Dixon, and J. R. Sargent. 2002. Origins of long-chain polyunsaturated fatty acids in the hydrothermal vent worms *Ridgea piscesae* and *Protis hydrothermica*. Mar. Ecol. Prog. Ser. **225:** 219–226.

———, M. V. Bell, D. R. Dixon, A. E. Fallick, M. Segonzac, and J. R. Sargent. 1998. Stable-carbon-isotope composition of fatty acids in hydrothermal vent mussels containing methanotrophic and thiotrophic bacterial endosymbionts. Appl. Environ. Microbiol. **64:** 370–375.

Rau, G. H. 1981. Hydrothermal vent clam and tube worm $^{13}C/^{12}C$: Further evidence of nonphotosynthetic food sources. Science **213:** 338–340.

Raymont, J. E. G. 1983. Vertical migration of zooplankton, p. 489–524. *In* J. E. G. Raymont [ed.], Plankton and productivity in the oceans. V. 2, 2nd ed. Pergamon Press Ltd.

Saito, H. 2004. Lipid and FA composition of the pearl oyster *Pinctada fucata martensii*: Influence of season and maturation. Lipids **39:** 997–1005.

———, and Y. Kotani. 2000. Lipids of five boreal species of the calanoid copepod: Origin of the monoene fats of marine animals at the higher trophic levels in the grazing food chain of subarctic ocean ecosystem. Mar. Chem. **71:** 69–82.

Sibuet, M., and K. Olu. 1998. Biogeography, biodiversity and fluid dependence of deep-sea cold-seep communities at active and passive margins. Deep-Sea Res. II **45:** 517–567.

Soudant, P., K. Van Ryckeghem, Y. Marty, J. Moal, J. F. Samain, and P. Sorgeloos. 1999. Comparison of the lipid class and fatty acid composition between a reproductive cycle in nature and a standard hatchery conditioning of he Pacific oyster *Crassostrea gigas*. Comp. Biochem. Physiol. **123B:** 209–222.

Thiele, O. W., J. Oulevey, and D. H. Hunneman. 1984. Ornithine-containing lipids in *Thiobacillus* A2 and *Achromobacter* sp. Eur. J. Biochem. **139:** 131–135.

Thompson, P. A., and P. J. Harrison. 1992. Effects of monospecific algal diets of varying biochemical composition of the growth and survival of Pacific oyster (*Crassostrea gigas*) larvae. Mar. Biol. **113:** 645–654.

Tocher, D. R. 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Rev. Fish. Sci. **11:** 107–184.

Tunnicliffe, V. 1991. The biology of hydrothermal vents: Ecology and evolution. Oceanogr. Mar. Biol. Annu. Rev. **29:** 319–407.

Yano, Y., A. Nakayama, K. Ishihara, and H. Saito. 1998. Adaptive changes in membrane lipids of barophilic bacteria in response to changes in growth pressure. Appl. Environ. Microbiol. **64:** 479–485.

Zhukova, N. V. 1991. The pathway of the biosynthesis of non-methylene interrupted dienoic fatty acids in molluscs. Comp. Biochem. Physiol. **100B:** 801–804.

Received: 20 March 2006
Accepted: 25 March 2007
Amended: 18 April 2007