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Effect of Unialgal Diets on the Composition of Fatty Acids and Sterols in Juvenile Ark Shell *Tegillarca granosa* Linnaeus

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ABSTRACT: This study has investigated the effects of six different unialgal diets (*Chaetoceros calcitrans*, *Platymonas helgolandica*, *Chlorella* sp., *Isochrysis galbana*, *Nannochloropsis oculata*, and *Pavlova viridis*) on the composition of fatty acids and sterols in juvenile ark shell *Tegillarca granosa* Linnaeus. The best feeding effects on the growth of shellfish were found in *C. calcitrans*, followed by *I. galbana* and *P. viridis*, whereas *Chlorella* sp. and *N. oculata* exhibited relatively poor effects. The fatty acid and sterol compositions in the six microalgae and the juvenile ark shell after feeding were analyzed, and 39 fatty acids and 18 sterols were identified. Although the results demonstrate a close correlation between the sterol compositions in algal species and juvenile ark shell, a similar correlation was not observed between fatty acids. In the juvenile ark shell fed microalgae, the ratio of total saturated fatty acids (SFA) rapidly decreases, whereas the proportion of total polyunsaturated fatty acids (PUFAs) increases considerably. The abundances of AA, EPA, and DHA increase most significantly in shellfish with better growth (fed *C. calcitrans*, *I. galbana*, and *P. viridis*). The number of sterol species is reduced, but the total sterol content in groups fed corresponding microalgae increases, and abundant plant sterols, instead of cholesterol, are accumulated in juvenile ark shell fed appropriate microalgae *I. galbana* and *P. viridis*. Therefore, to be more conducive to human health, *I. galbana* and *P. viridis*, of the six experimental microalgae, are recommended for artificial ark shell culture.

KEYWORDS: *microalgae, Tegillarca granosa, fatty acid, sterol, juvenile ark shell*

INTRODUCTION

Fatty acids, the important parts of lipids, are essential nutrients for human growth and development. Studies indicated that omega-3 and -6 long-chain polyunsaturated fatty acids (PUFAs) are critical for infant and childhood brain development.[1,2] Some fatty acids are also implicated in a variety of intracellular signal transduction processes[3,4] and closely related with the pathogenesis of certain diseases.[5−8] Sterols, the key membrane component of all eukaryotic organisms, are essential lipid nutrients for human beings. Among them, plant sterols play an important role in controlling membrane fluidity and permeability in plant cells, functions identical to those of cholesterol in mammalian cells.[9,10] Plant sterols and plant stanol esters have been shown to reduce plasma lipid levels,[11] inhibit cholesterol absorption,[12] and lower serum LDL-cholesterol[13] and to be anti-inflammatory[14] and are associated with the occurrence of atheromatous arterial disease.[15,16]

Shellfish is rich in essential fatty acids and sterols, especially PUFAs and plant sterols.[17−19] Studies demonstrated that some shellfishes appear to be effective as hypolipidemic diets for normolipidemic men.[20−22] Microalgae are used as the main diet in artificial rearing of juvenile shellfish. Many eukaryotes, including nematodes and marine invertebrates, have lost their ability to synthesize sterols de novo and have to take in sterols from the diet.[23] Similarly, shellfishes have little ability to biosynthesize and biotransform sterols;[24,25] therefore, sterol nutrition from food supply of microalgae becomes very important to shellfish.[26,27] Because the lipid compositions in microalgae vary among species,[28−31] different microalgae may differentially affect the composition of fatty acids and sterols in shellfish.

Although there are extensive studies of the effects of microalgal nutrient on the growth of shellfish in artificial rearing,[26,32−35] special research focusing on the influence of microalgae on the composition of fatty acids and sterols in shellfish has not been reported. Ark shell *Tegillarca gransa* Linnaeus is one of the most important aquacultured economic shellfish species in the coastal area of southeastern China, which has a delicious taste and high nutrition, as well as wide adaptation to temperature and salinity. However, little information is available on the lipid composition of this shellfish species.

The more commonly used microalgae in bivalve hatcheries are species of the following genera: *Isochrysis* (Chrysophyceae); *Pavlova* (Prymnesiophyceae); *Chaetoceros*, *Phaeodactylum*, *Skeletonema*, *Thalassiosira* (Bacillariophyceae); *Dunaliella*, *Platymonas*, *Chlorella*, *Tetraselmis* (Chlorophyceae); and *Nannochloropsis* (Eustigmatophyceae).[32−35] Among them, *Chaetoceros calcitrans*, *Platymonas helgolandica*, *Chlorella* sp., *Isochrysis galbana*, *Nannochloropsis oculata*, and *Pavlova viridis* are most commonly used in mollusc hatcheries in China. Therefore, this study has investigated the changes of fatty acids and sterols in *T. gransa* Linnaeus fed the six unialgal diets. We try to understand the influence of microalgal species on the lipid composition in juvenile shellfish and to provide effective nutrient data for the screening of appropriate feed in artificial rearing of the shellfish.

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MATERIALS AND METHODS

Culture of Microalgae. Microalgal species, including *C. calcitrans* NMBguh003-1 (Bacillariophyceae), *P. helgolandica* NMBluh011, *Chlorella* sp. NMBluh015-2 (Chlorophyceae), *I. galbana* NMBjih021-2 (Chrysophyceae), *N. oculata* NMBluh014 (Eustigmatophyceae), and *P. viridis* NMBjih024 (Prymnesiophyceae), were selected from the microalgal culture laboratory at Ningbo University. Culture medium comprised filtered (0.45 μm) and autoclaved seawater (salinity 25−26‰) enriched with NMB3 medium (KNO_3 100 mg/L, KH_2PO_4 10 mg/L, Fe-citrate·$5H_2O$ 3 mg/L, VB_1 6 μg/L, VB_{12} 0.05 μg/L). Sodium metasilicate (20 mg/L) was added as a silica source for culture of the diatom *C. calcitrans*. The microalgae were grown in 2500 mL flasks at 20 ± 2 °C under continuous illumination provided by cool white fluorescent tubes, at the intensity of 160−180 μmol photons/m^2/s, for about 1 week. In exponential phase, the microalgae were transferred into a 10 L photobioreactor, and air with 2% CO_2 was supplied to support the massive growth. The microalgae in exponential growing phase were harvested and used to feed the juvenile ark shell. Some of the microalgal samples were centrifuged (15000 rpm) and preserved at −20 °C under nitrogen for further analysis. Each microalga was cultured in triplicate.

Culture of Ark Shell Larvae. Larvae of *T. granosa* Linnaeus were obtained from Xingda Hatchery at Yueqing City, Zhejiang, China. They were cultured in a pool (length 8.5 × width 3.5 × depth 1.0 m) with continuous aeration after settlement. The seawater (salinity 20 ‰) was filtered by 1 m thick sand (diameter < 1 mm). Fresh sea-mud dried at 200 °C and filtered by nylon cribrose silk (75 μm) was spread onto the pool bottom at about 1−2 mm thickness. Larvae were fed a certain amount of mixed microalgae (*C. calcitrans*, *I. galbana*, *P. viridis*, *P. helgolandica*, and some other species indeterminata microalgae); usually, microalgal cell at a concentration of 80−100 cells/μL is sufficient to cover the demand of shellfish at a density of 100−120 ind/cm^2. After 10 days of culture at natural temperature (26−32.5 °C), the postlarvae juveniles, with an average size of 0.786 ± 0.045 × 0.875 ± 0.065 mm (shell width × shell length), were put into clean seawater and starved for 12 h prior to subsequent culture experiment to empty the stomach and avoid the effect of residual diets.

Culture of Ark Shell Postlarvae Juveniles. The above-mentioned same seawater and sea-mud were added into plastic boxes (length 20 × width 15 × depth 6 cm), and about 0.6 g wet weight juveniles was sprinkled at a density of 20−25 ind/cm^2. In these boxes, unialgal feed was given every day at the concentration of 100−120 cells/μL except for *P. helgolandica* (15−20 cells/μL). The water and mud were renewed every third day, and at least 30 juveniles were sampled randomly to calculate the survival rate based on the observed numbers of living and dead shells under a TS100 microscope (Nikon Co., Japan). The shell length and width were measured respectively under the same microscope equipped with a microscale ruler. The shell width was measured as the axis running perpendicular to the hinge line, and the length was measured as the longest line running perpendicular to the axis. At the start and end of the 18 days of culture under natural temperature (25.2−35.5 °C), shellfishes were collected after starvation to empty the stomach in clean water for 24 h, sealed under N_2, and stored at −20 °C for further lipid analysis. Each experimental group was triplicated.

Lipid Analysis by GC-MS. Totals of 0.05 g of lyophilized microalgal sample or 0.1 g of lyophilized juvenile ark shell sample were extracted with $CHCl_3/CH_3OH/H_2O$ (1:2:0.8, v/v/v) according to a modified Bligh and Dyer method[36] to obtain the total lipid. The ark shell samples were crushed in vitreous milling before extraction. Fatty acid and sterol analyses were performed according to the method described by Ichihara et al.[37] and Mansour et al.[38] with slight modifications. Briefly, 0.2 mL of toluene, 1.5 mL of methanol, and 0.3 mL of an 8% (w/v) solution of HCl in methanol/water (85:15, v/v) were added sequentially to the lipid sample. This solution (2 mL) was heated at 100 °C for 1 h. Fatty acid methyl esters (FAME) and sterols were extracted into hexane/chloroform (4:1 v/v) and stored under nitrogen at −20 °C. Immediately before gas chromatography (GC) and mass spectrometry (MS) analysis, the transmethylated lipid extract was treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 30 min at 80 °C to convert any lipids containing free hydroxyl groups to their trimethylsilyl ethers.

GC analysis was carried out using an SPB-50 fused silica capillary column, 30 m × 0.25 mm × 0.25 μm (Supelco, Bellefonts, PA, USA), installed in a QP2010 gas chromatograph−mass specterometer equipped with an autosampler (AOC-20) and a FID from Shimadzu Co. (Japan). The temperature of the injector was 250 °C. High-purity helium was used as the carrier gas with the column flow rate at 0.81 mL/min and the precolumn pressure at 73.0 kPa. After injection, the oven temperature was kept at 150 °C for 3.5 min, then increased at a rate of 20 °C/min to 200 °C, kept for 5 min, then increased at a rate of 5 °C/min to the final temperature of 280 °C, and kept at 280 °C for 30 min. The solvent cutoff time was set at 3.5 min. The injection volume was 1 μL with a split ratio of 10:1. The mass spectrometer operated in electron compact mode with electron energy at 70 eV. The ion source temperature was set at 200 °C, and the interface temperature was 250 °C. The mass spectrometer scanned from m/z 50 to 600.

Identification and Quantitative Analysis of Fatty Acids and Sterols. Identification and quantitative analysis of fatty acids and sterols were performed according to the method described by Mansour et al.[38] Briefly, FAME were quantified by adding *n*-nonadecanoic acid methyl ester (19:0 FAME) in chloroform as an internal standard and identified by relative retention times plus database search in commercial mass spectral databases, NIST98-147 and Wiley7. Sterols as TMSi-ethers and steroidal ketones were quantified using epicoprostanol as the internal standard and identified on the basis of relative retention times and comparison with published mass spectral data.[30,31,39−44] The percentage of each fatty acid and sterol was calculated and normalized using square peaks.

Statistical Analysis. Significant differences in biological and biochemical measurements among feeding conditions were detected by one-way ANOVA and Newman−Keuls tests ($P < 0.05$). Analyses were performed using the Statistics software package (SPSS 12.0).

RESULTS AND DISCUSSION

Growth of Juvenile Ark Shell on Different Unialgal Feeds. The juvenile ark shell *T. granosa* fed *C. calcitrans* showed the highest growth rate followed by *I. galbana*, *P. viridis*, and *P. helgolandica*, whereas the lowest growth rates were observed with *Chlorella* sp. and *N. oculata* (Figure 1). The growth differences became significant after rearing for 9 days (n

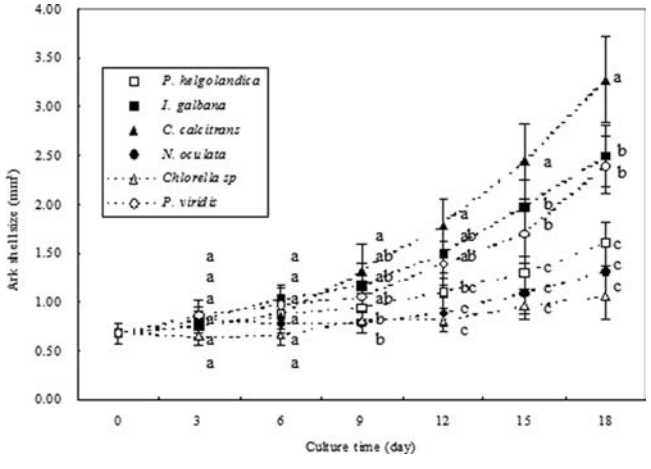

Figure 1. Growth of postlarvae juvenile ark shell *Tegillarca granosa* Linnaeus fed six unialgal diets over 18 days. The ark shell size is expressed as shell length × shell width (mm^2); the average data are obtained from 10 random samples ($n = 3$); the legend for each unialgal species is shown in the figure. Values within the same group sharing a common letter are not significantly different ($P > 0.05$).

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= 3, P = 0.025), although no significant differences were observed in the first 6 days (n = 3, P = 0.063). After 18 days of culture, the six different unialgal feeds are divided into three categories, that is, *C. calcitrans* as best feed, *I. galbana* and *P. viridis* as good feed, and *P. helgolandica*, *N. oculata*,, and *Chlorella* sp. as poor feed, with obvious statistical significance (n = 3, P < 0.001). Moreover, it has also been observed that the higher the shellfish growth, the greater the survival rate (about 95% for *C. calcitrans*, >90% for *I. galbana* and *P. viridis*, about 50% for *P. helgolandica*, and <20% for *Chlorella* sp. and *N. oculata*).

Fatty Acid Composition of Microalgae. A total of 36 fatty acid species were identified from the 6 microalgal samples and are presented in Table 1. Total fatty acid contents of microalgae ranged from 33.4 mg/g dry weight (*P. helgolandica*) to 59.3 mg/g dry weight (*I. galbana*). The percentage of SFAs in total fatty acids was highest in *N. oculata* (31.7%) and lowest in *P. viridis* (24.0%), and the main SFAs found in most microalgae were 16:0 and 14:0.

The abundance of monounsaturated fatty acid (MUFA) was high in *C. calcitrans* (>40%), with 16:1(n-7) as the dominant species (>35% of total fatty acids); it was also high in *Chlorella* sp. (35.1%), dominated by 18:1(n-9) at 30%. On the other hand, the abundance of MUFA in *I. galbana*, *P. viridis*, and *P. helgolandica* was lower (about 20% of total) and composed mostly of monounsaturated 16:1 and 18:1.

The high abundance of PUFA was found in *I. galbana*, *P. viridis*, and *P. helgolandica* up to about 55% of total fatty acids. The main PUFAs in *I. galbana* are polyunsaturated C18 and DHA, and DHA constitutes 9.7% of total fatty acids, the highest among all six unialgal species. The main PUFAs in *P. viridis* are EPA, polyunsaturated C16 and C18, with little AA and DHA (2.7 and 2.2% of total fatty acids, respectively). EPA in *P. viridis* constitutes 22.6% of the total fatty acids and is highest among all microalgal species. PUFAs in *P. helgolandica* were dominated by 16:4(n-3) and 18:3(n-3) (12.7 and 20.4% of total fatty acids, respectively), which were all the highest compared to those in other microalgal species. Besides, *P. helgolandica* contained 6.2% EPA and little AA (1.2%). DHA was not detected in this species. The main PUFAs in *C. calcitrans* mainly consisted of AA (11.8% of total, the highest compared to those in other species) and EPA (7.5% of total), whereas the DHA proportion was low (0.6% of total). For the other two microalgae, DHA was absent, and the PUFAs were mostly polyunsaturated C16 and C18. Besides, some EPA and AA existed in *N. oculata* (10.0 and 13.4% of total fatty acids, respectively) and were found lowest in *Chlorella* sp.

Effect of Unialgal Diets on the Composition of Fatty Acids in Juvenile Ark Shell. A total of 31 fatty acids were identified from juvenile ark shell samples fed the six unialgal diets (Table 2). The major fatty acids in juvenile ark shell at the start of experiment were SFA (up to 69.0% of total), MUFA (16.0%), and PUFA (14.5%), as shown in Table 2. After 18 days of culture, the proportion of SFA was reduced obviously but remained at about 40%, whereas the proportions of MUFA and PUFA increased to about 25 and 30%, respectively. Total SFA, MUFA, and PUFA in juvenile ark shell fed different unialgal diets showed no obvious correlation with the lipid composition in corresponding microalgae.

Comparison of the composition of fatty acids between juvenile ark shell and its unialgal feed indicated that certain fatty acids with lower proportion in microalgae, such as 16:1(n-9), 16:1(n-5), 17:2(n-6), 17:2(n-5), 18:2(n-3), and 18:5(n-3), were not detected in the juveniles, whereas other fatty acids, such as 20:2(n-6), 20:3(n-6), and 21:0, not found in microalgae were detected in corresponding shellfish. The main SFAs in the ark shell were 14:0, 16:0, 17:0, and 18:0; the main MUFAs in the ark shell were monounsaturated C16, C18, and C20; and the main PUFAs were polyunsaturated C18, C20, and C22. AA, EPA, and DHA were also detected in all ark shell samples.

Among all kinds of main fatty acids in microalgae, only 14:0 can be reflected perfectly in all of the fed shellfish: the higher levels in *C. calcitrans*, *I. galbana*, and *P. viridis* (6.8−14.1%) resulted in the higher ratios of this fatty acid in corresponding shellfish (5.5−8.1%); consistently, the lower levels in the other three microalgae (0.8%-4.1%) resulted in its lower ratios in

Table 1. Fatty Acid Composition of Six Microalgae Used in the Present Trials[a]

fatty acid	*C. calcitrans*	*I. galbana*	*P. viridis*	*P. helgolandica*	*N. oculata*	*Chlorella* sp.
12:00	0.1	–[b]	0.1	–	0.1	0.1
13:00	–	–	–	–	0.2	–
14:00	14.1	13.7	6.8	0.8	4.4	1.4
14:1(n-3)	0.5	0.1	–	–	0.1	0.1
15:00	0.7	0.3	0.2	0.9	0.4	0.1
16:00	8.4	10.2	16.8	23.1	24.8	21.1
16:1(n-7)	37.9	6.4	12.7	–	15.4	2.3
16:2(n-6)	4.9	–	0.5	–	0.3	1.4
16:1(n-9)	–	–	1.3	0.8	1.1	–
16:1(n-5)	1.3	–	–	1.8	–	–
16:3(n-6)	–	–	1.2	2.2	1.0	–
16:2(n-4)	1.9	0.6	0.7	–	0.9	0.1
16:3(n-4)	–	–	0.5	–	–	–
16:3(n-3)	5.0	–	5.3	–	–	5.8
16:4(n-3)	–	–	0.2	12.7	2.4	–
17:00	–	–	–	–	0.4	0.1
17:2(n-6)	–	–	–	–	0.4	–
17:2(n-5)	–	–	–	–	0.5	–
18:00	1.0	–	–	1.6	1.4	–
18:1(n-9)	0.5	11.3	4.0	6.0	8.4	30.0
18:1(n-7)	0.8	1.6	2.0	7.9	2.3	2.5
18:2(n-6)	1.2	16.8	4.1	8.6	5.9	13.9
18:2(n-3)	–	–	–	–	0.5	0.1
18:3(n-6)	–	2.6	1.1	0.8	3.4	–
18:3(n-3)	0.5	9.3	6.4	20.4	1.0	16.7
18:4(n-3)	–	12.9	6.2	2.0	–	–
18:5(n-3)	–	1.6	–	–	–	–
20:00	–	–	–	–	–	3.1
20:1(n-9)	–	–	0.8	3.0	0.8	0.2
20:4(n-6)	11.8	0.3	2.7	1.2	10.0	0.1
20:5(n-3)	7.5	0.7	22.6	6.2	13.4	0.2
22:5(n-6)	–	1.6	1.7	–	–	–
22:6(n-3)	0.6	9.7	2.2	–	–	–
24:00:00	0.6	–	–	–	–	0.2
25:00:00	–	–	–	–	–	0.1
26:00:00	–	–	–	–	–	0.1
others	0.7	0.4	–	–	0.5	0.4
SFA	24.7	24.2	24	26.4	31.7	26.2
MUFA	41.2	19.4	20.8	19.5	28.1	35.1
PUFA	33.4	56.0	55.2	54.1	40.2	38.3
TFA[c] (mg/g)	38.6	59.3	42.9	33.4	35.4	37.9

[a]Expressed as percentage of total fatty acids. [b]Levels <0.1%; the same expression is used in Tables 2, 4, and 5. [c]TFA, total fatty acids in microalgae (mg/g dry microalgae).

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Table 2. Fatty Acid Composition of Juvenile *T. granosa* Fed Corresponding Microalgae[a]

	diet													
	0[b]		*C. calcitrans,*		*I. galbana*		*P. viridis*		*P. helgolandica*		*N. oculata*		*Chlorella* sp.	
fatty acid	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
12:0	0.3	0.1	0.1	0.1b	0.1	0.1b	0.1	0.1b	0.1	0.1b	0.7	0.2a	0.3	0.1b
13:0	0.4	0.2	0.1	0.1b	0.1	0.1b	0.1	0.0b	0.2	0.1b	0.4	0.1a	0.1	0.2b
14:0	2.4	0.0	5.7	0.5b	8.1	1.1a	5.5	0.2b	1.1	0.1d	2.1	0.3c	2.8	0.4c
14:1(n-3)	–	–	–	–	0.3	0.2	0.3	0.1	–	–	0.6	0.2	0.8	0.1
15:0	0.7	0.0	0.5	0.1c	0.6	0.0bc	0.6	0.2bc	0.8	0.1b	0.8	0.1b	1.4	0.3a
16:0	40.8	1.2	21.4	1.1c	21.1	0.9c	22.8	1.1c	21.4	0.9c	29.7	0.6a	25.2	1.1b
16:1(n-7)	1.0	0.2	15.0	0.9a	3.4	0.5c	10.4	1.2b	1.7	0.2d	1.5	0.3d	2.1	0.3d
16:2(n-6)	–	–	1.7	0.2b	0.3	0.0c	0.2	0.1c	0.3	0.2c	2.8	0.4a	1.7	0.1b
16:3(n-6)	–	–	1.6	0.5	–	–	–	–	0.6	0.4	–	–	–	–
16:2(n-4)	–	–	–	–	–	–	–	–	1.8	0.5	–	–	–	–
16:3(n-4)	–	–	0.4	0.2	0.3	0.2	–	–	–	–	–	–	–	–
17:0	6.3	0.7	1.8	0.2c	2.4	0.3b	2.8	0.3b	3.8	0.4a	1.0	0.2d	0.7	0.3d
18:0	18.1	0.5	11.2	0.5b	8.8	0.4c	14.4	0.7a	12.5	0.8b	9.0	0.5c	8.2	0.7c
18:1(n-9)	7.8	0.5	2.5	0.3d	19.5	0.9a	2.0	0.3d	10.8	0.5c	17.4	0.5b	17.7	0.8b
18:1(n-7)	3.7	0.4	6.8	0.7a	2.0	0.2d	5.5	0.6a	3.5	0.3c	4.2	0.4c	3.2	0.6c
18:2(n-6)	4.4	0.3	2.7	0.3d	5.6	0.7c	2.3	0.4d	10.2	0.6b	17.9	1.3a	17.4	1.5a
18:3(n-6)	–	–	0.3	0.1	0.3	0.2	0.2	0.0	–	–	–	–	–	–
18:3(n-3)	1.0	0.2	0.7	0.1d	3.8	0.3c	0.6	0.1d	9.1	0.4a	6.0	0.7b	5.6	0.6b
18:4(n-3)	–	–	0.4	0.1	5.0	0.4	1.0	0.2	–	–	–	–	–	–
20:0	–	–	–	–	–	–	–	–	–	–	–	–	3.0	0.5
20:1(n-9)	3.5	0.0	3.2	0.5b	4.2	0.4b	3.8	0.6b	8.8	0.4a	3.8	0.1b	1.7	0.4c
20:2(n-6)	3.2	0.3	6.4	0.8a	2.0	0.3c	5.4	1.0b	0.7	0.1d	0.6	0.1d	1.0	0.2d
20:3(n-6)	1.6	0.2	0.5	0.1b	0.4	0.1b	0.7	0.3b	5.9	0.4a	0.5	0.2b	0.5	0.1b
20:4(n-6)	0.5	0.1	6.1	0.7a	1.5	0.4c	5.1	0.4b	0.6	0.2d	0.4	0.0d	0.6	0.2d
20:5(n-3)	1.0	0.2	7.5	0.6a	1.0	0.1c	2.9	0.3b	3.0	0.1b	0.3	0.0d	0.5	0.2cd
21:0	–	–	0.2	0.1	1.1	0.2	0.4	0.1	–	–	–	–	–	–
22:5(n-6)	2.0	0.0	–	–	2.0	0.1	7.9	0.4	0.8	0.2	–	–	–	–
22:6(n-3)	0.8	0.2	2.4	0.5c	6.2	0.3a	4.4	0.1b	2.4	0.0c	0.4	0.1d	0.8	0.1d
24:0	–	–	–	–	–	–	–	–	–	–	–	–	1.1	0.1
25:0	–	–	–	–	–	–	–	–	–	–	–	–	0.8	0.3
26:0	–	–	–	–	–	–	–	–	–	–	–	–	2.1	0.1
others	0.5	–	1.2	0.4	–	–	0.6	0.2	–	–	–	–	0.6	0.3
SFA	69.0	1.4	40.8	1.4b	42.3	1.3b	46.6	1.7a	39.8	1.8b	43.7	1.4ab	45.7	1.6a
MUFA	16.0	0.9	27.3	0.7ab	29.4	1.4a	22.1	0.8c	24.7	0.9b	27.4	1.2ab	25.5	0.9b
PUFA	14.5	0.7	30.7	1.4b	28.3	1.3b	30.7	1.7b	35.5	1.8a	28.9	0.9b	28.2	1.4b

[a]Expressed as percentage of the total fatty acids; mean, SD = standard deviation; $n = 3$. Values within the same row sharing a common letter are not significantly different ($P > 0.05$). [b]Diet 0 indicates the initial stage of the present trials. The same expression is used in Table 5.

corresponding shellfish (0.8–4.4%). Other major SFAs (15:0, 16:0, 17:0, and 18:0) revealed similar respective proportions in shellfish fed different unialgal diets, although some fatty acids (17:0 and 18:0) had not been detected in most microalgae.

Most of the main MUFAs and PUFAs, such as 16:1(n-7), 18:1(n-9), 18:2(n-6), 18:3(n-3), 18:4(n-3), 20:4(n-6), and 22:6(n-3), in unialgal diets could be partially reflected in experimental shellfish with better growth (fed *C. calcitrans*, *I. galbana*,, and *P. viridis*) but not in those that grew poorly (fed *P. helgolandica*, *N. oculata*, and *Chlorella* sp.). For example, among *C. calcitrans*, *I. galbana*, and *P. viridis*, higher levels of 16:1(n-7) (37.9%) and 20:4(n-6) (11.8%) in *C. calcitrans* resulted in higher ratios of these fatty acids in shellfish (15.0 and 6.1%, respectively); the abundance of DHA in *I. galbana* (9.7%) resulted in a higher proportion of this fatty acid in shellfish (6.2%), too. On the other hand, among *P. helgolandica*, *N. oculata*, and *Chlorella* sp., the higher levels of 16:1(n-7) (15.4%) and 20:4(n-6) (10.0%) in *N. oculata* did not result in higher ratios of these fatty acids in the shellfish (1.5 and 0.4%, respectively).

Omega-3 and -6 long-chain PUFAs were found in all ark shell samples (Table 2). The abundances of AA, EPA, and DHA, which are essential nutrients for human health, most significantly increased in shellfish with better growth (fed *C. calcitrans*, *I. galbana*, and *P. viridis*; Table 2), whereas the accumulation was not obvious in those with poor growth (fed *P. helgolandica*, *N. oculata*, and *Chlorella* sp.).

EPA and AA were found in all six microalgae (Table 1), but DHA was detected only in *C. calcitrans*, *I. galbana*, and *P. viridis*. Efficient incorporation of DHA and AA was found in all ark shell, especially in those that showed better growth (fed *C. calcitrans*, *I. galbana*, and *P. viridis*; Table 2). It has been reported that DHA and AA are of importance in shellfish metamorphosis, sexual maturation, reproduction, and growth.[32,33] The selective bioaccumulation of AA could be related to its role as a precursor for eicosanoid production,[45] and the influence of dietary supplementation of arachidonic acid on prostaglandin production and oxidative stress in shellfish was obvious.[46] If these compounds could not be supplied through diets, the alternative approach is to bioconvert

Table 3. Sterols Found in the Six Microalgae and *T. granosa* Larvae Fed Corresponding Microalgae

no.	sterol[a]	systematic name (trivial name)	retention time (min)
1	$C_{27:1}(\Delta^5)$	cholest-5-en-3β-ol (cholesterol)	34.69
2	$C_{28:2}(\Delta^{5,22}$/24Me)	24β-methylcholesta-5,22*E*-dien-3β-ol (brassicasterol)	36.26
3	$C_{28:2}(\Delta^{5,24(28)}$/24Me)	24β-methylcholesta-5,24(28)-dien-3β-ol (24-methylencholesterol)	37.14
4	$C_{28:1}(\Delta^5$/24Me)	24α-methylcholest-5-en-3β-ol (campesterol)	38.07
5	$C_{28:3}(\Delta^{6,8(14),22}$/24Me)	24-methylcholesta-6,8(14),22-trien-3β-ol[b]	38.60
6	$C_{28:1}(\Delta^5$/24Me/3,25diol)	24-methylcholest-5-ene-3β,25β-diol[b]	38.66
7	$C_{29:2}(\Delta^{5,22}$/24Et)	24β-ethylcholesta-5,22-dien-3β-ol (stigmasterol)	39.12
8	$C_{28:0}(5\alpha$/24Me)	24-methyl-5α-cholestan-3β-ol (24-methylcholestanol)[b]	39.99
9	$C_{29:0}(5\alpha$/4,4diMe)	4α,4β-dimethyl-5α-cholestan-3β-ol (4α,4β-dimethylcholestanol)	40.53
10	$C_{29:4}(\Delta^{5,7,9(11),22}$/24Et)	24-ethylcholesta-5,7,9(11),22-tetraen-3β-ol[b]	40.92
11	$C_{29:1}(\Delta^5$/24Et)	24β-ethylcholest-5-en-3β-ol (β-sitosterol)	41.14
12	$C_{30:1}(\Delta^{22}$/4Me,24Et)	4α-methyl-24α-ethylcholest-22-en-3β-ol (4α-methylporiferasterol)	41.89
13	$C_{29:2}(\Delta^{5,24(28)E}$/24Et)	24β-ethylcholesta-5,24(28)*E*-dien-3β-ol (fucosterol)	42.23
14	$C_{29:2}(\Delta^{5,24(28)Z}$/24Et)	24β-ethylcholesta-5,24(28)*Z*-dien-3β-ol (isofucosterol)	43.15
15	$C_{29:1}(\Delta^7$/24Et)	24-ethylcholest-7-en-3β-ol[b]	44.13
16	$C_{27:2}(\Delta^{3,5}$/7one)	cholesta-3,5-dien-7-one	45.48
17	$C_{29:0}(5\alpha$/4α,24diMe/3,4-diol)	4α,24β-dimethylcholstan-3β,4β-diol (methylpavlovol)	48.01
18	$C_{30:0}(5\alpha$/4αMe,24Et/3,4-diol)	4α-methyl-24β-ethylcholstan-3β,4β-diol (ethylpavlovol)	52.77

[a]The nomenclature is $C_{m:n}(\Delta^x/y)$, where m is the total number of carbon atoms and n is the number of double bonds. The sign of x indicates the positions of the double bonds, and y indicates the numbers and positions of the methyl group, ethyl group, and hydroxy. The same expression is used in Tables 4 and 5. [b]The C-24 stereochemistry is not defined.

Table 4. Sterol Composition of the Six Microalgae Used in the Present Trials[a]

sterol	*C. calcitrans*	*I. galbana*	*P. viridis*	*P. helgolandica*	*N. oculata*	*Chlorella* sp.
$C_{27:1}(\Delta^5)$	59.5	–	4.1	–	89.2	1.1
$C_{28:2}(\Delta^{5,22}$/24Me)	1.1	99.7	0.1	–	0.6	–
$C_{28:2}(\Delta^{5,24(28)}$/24Me)	3.8	–	–	–	–	–
$C_{28:1}(\Delta^5$/24Me)	–	–	8.8	80.1	1.3	–
$C_{28:3}(\Delta^{6,8(14),22}$/24Me)	–	–	–	–	–	7.3
$C_{28:1}(\Delta^5$/24Me/3,25diol)	–	–	8.5	9.1	–	–
$C_{29:2}(\Delta^{5,22}$/24Et)	–	–	9.2	–	7	5.5
$C_{28:0}(5\alpha$/24Me)	15.8	–	–	–	–	–
$C_{29:0}(5\alpha$/4,4diMe)	–	–	–	–	–	43.1
$C_{29:4}(\Delta^{5,7,9(11),22}$/24Et)	–	–	–	–	–	12.4
$C_{29:1}(\Delta^5$/24Et)	–	0.3	21	9.3	1.6	12
$C_{30:1}(\Delta^{22}$/4Me,24Et)	–	–	10.5	–	–	–
$C_{29:2}(\Delta^{5,24(28)E}$/24Et)	10.4	–	1.5	–	0.3	3
$C_{29:2}(\Delta^{5,24(28)Z}$/24Et)	–	–	4.1	–	–	–
$C_{29:1}(\Delta^7$/24Et)	–	–	–	–	–	14.3
$C_{27:2}(\Delta^{3,5}$/7one)	7.4	–	–	–	–	–
$C_{29:0}(5\alpha$/4α,24diMe/3,4-diol)	–	–	27.4	–	–	–
$C_{30:0}(5\alpha$/4αMe,24Et/3,4-diol)	–	–	4.8	–	–	–
others	2			2.5	–	1.3
TS[b](mg/g)	2.0	2.5	2.3	1.9	2.3	3.2
TS%[c]	4.9	4.0	5.0	5.3	6.2	7.7

[a]Expressed as percentage of total sterols; mean. [b]TS, dry weight of total sterols in microalgal (mg/g dry microalgae). [c]TS%, total sterols percentage of total (sterols + fatty acids). The same expression is used in Table 5.

them from other fatty acids, such as 18:3(n-3), 18:2(n-6), and 18:1(n-9), that can be elongated and desaturated in the host animals.[47] We observed that the proportions of 18:1(n-9), 18:2(n-6), and 18:3(n-3) in shellfish with poor growth showed more obvious accumulation than those in shellfish with better growth (Table 2), although the proportions of these fatty acids in corresponding microalgae were not apparently different (Table 1). It seemed that the elongation and desaturation processes in shellfish with poor growth were restricted to 18-carbon fatty acids with unknown mechanisms that might explain the lowered growth and lowered contents of DHA and AA observed in shellfishes fed *P. helgolandica*, *Chlorella* sp., and *N. oculata*.

Although the proportions of EPA were highest in *P. viridis* (22.6%), high in *N. oculata* (13.4%), and low in *C. calcitrans* (7.5%), EPA abundances in experimental shellfish failed to show a proportional incorporation, and the highest proportion was observed in animals fed *C. calcitrans*. As a matter of fact, Thompson et al. also found that the growth rate of larval *C. virginca* was not positively correlated with the EPA content in their algal diet.[35] Sargent et al. argue that a possible negative effect of relatively excessive EPA over DHA in larval fish feeds

Table 5. Sterol Composition of *T. granosa* Larvae Fed Corresponding Microalgae[a]

	diet													
	0		*C. calcitrans*		*I. galbana*		*P. viridis*		*P. helgolandica*		*N. oculata*		*Chlorella* sp.	
sterol	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
$C_{27:1}(\Delta^5)$	62.1	0.4	**89.8**	**0.7b**	**21.0**	**1.1e**	**20.7**	**1.3e**	**32.8**	**3.1d**	**93.2**	**1.0a**	**78.4**	**2.4c**
$C_{28:2}(\Delta^{5,22}/24Me)$	19.6	0.7	**2.6**	**0.2d**	**73.3**	**1.7a**	**8.8**	**1.0b**	**4.5**	**1.2c**	**1.8**	**0.5d**	**9.0**	**0.9b**
$C_{28:1}(\Delta^5/24Me)$	15.0	1.0	**3.4**	**0.4bc**	**3.5**	**0.4bc**	**5.7**	**0.6b**	**58.2**	**2.9a**	**2.3**	**0.3c**	**5.1**	**0.7bc**
$C_{28:1}(\Delta^5/24Me/3,25diol)$	–	–	–	–	–	–	0.4	0.2	–	–	–	–	–	–
$C_{29:2}(\Delta^{5,22}/24Et)$	3.3	0.3	**1.7**	**0.3c**	**1.8**	**0.1c**	**30.8**	**1.6a**	**2.6**	**0.8c**	**1.2**	**0.3c**	**6.3**	**1.2b**
$C_{29:1}(\Delta^5/24Et)$	–	–	–	–	0.4	0.2	22.0	1.1	–	–	1.5	0.6	–	–
$C_{30:1}(\Delta^{22}/4Me,24Et)$	–	–	–	–	–	–	5.8	0.4	–	–	–	–	–	–
$C_{29:2}(\Delta^{5,24(28)E}/24Et)$	–	–	1.9	0.4	–	–	–	–	–	–	–	–	–	–
$C_{29:0}(5\alpha/4\alpha,24diMe/3,4-diol)$	–	–	–	–	–	–	5.6	0.3	–	–	–	–	–	–
$C_{30:0}(5\alpha/4\alpha Me,24E/3,4-diol)$	–	–	–	–	–	–	0.2	0.1	–	–	–	–	–	–
others		–	0.6	0.2	–	–	–	–	1.9	0.6	–	–	1.2	0.2
TS%	23.3	0.6	20.7	1.0	17.3	0.8	27.1	1.1	14.5	4.0	6.5	1.3	4.1	1.9

[a]Expressed as the percentage of total sterols; mean, SD = standard deviation; $n = 3$). Values within the same row sharing a common letter are not significantly different ($P > 0.05$).

may be due to the established role of EPA to competitively suppress the production of eicosanoids from arachidonic acid.[48] It might partly explain why the highest proportion of EPA and the best growth were observed in shellfish fed *C. calcitrans*, which has moderate EPA proportion among the six microalgae.

Sterol Composition of Microalgae. Eighteen sterols were identified in the six microalgae (Table 3) by comparison of the structural characteristics with those in the literature (Table 4).[30,31,39−44]

A methyl or ethyl group was found at position C-24 in most of the 18 sterols except for cholesterol, 4α,4β-dimethylcholestanol, and cholesta-3,5-dien-7-one. In addition, most of these sterols were C_{28} and C_{29} sterols except for cholesterol (C_{27}) and ethylpavlovol (C_{30}), whereas double bonds were mostly positioned at C-5, C-22, and C-24 (Table 3).

The six microalgae exhibited differences in sterol species and proportions. The one with richest sterols was *P. viridis*, which contained 11 sterols with methylpavlovol (27.4%) and β-sitosterol (21.0%) as the dominant species, followed by *Chlorella* sp. (9 sterols) containing mainly 4α,4β-dimethylcholestanol (43.1%). Cholesterol was found in *C. calcitrans* and *N. oculata* as the predominant sterol (59.5 and 89.2%, respectively). The sterol composition of *P. helgolandica* consisted mostly of campesterol (80.1%), whereas *I. galbana* was the only species with brassicasterol (99.7%) as almost the sole sterol.

Effect of Unialgal Diets on the Composition of Sterols in Juvenile Ark Shell. It was demonstrated that the lack of PUFA in some microalgae was probably not the only factor responsible for the low feed value;[45] therefore, more and more attention has been paid to the nutritional value of sterols for bivalves.[26,27,32,33,49]

From our results (Tables 4 and 5), sterol structures in juvenile *T. granosa* became simplified in general in comparison with those in corresponding microalgae, consisting mostly of cholesterol, brassicasterol, campesterol, and stigmasterol. After 18 days of culture, only 10 sterol species were found in shellfish fed a unialgal diet, indicating that some algal sterols cannot be assimilated or accumulated effectively by the shellfish. However, the total sterol proportion, expressed as the ratio of total sterols/(total sterols + total fatty acids), increased (Tables 4 and 5), especially in those with better growth (fed *C. calcitrans*, *I. galbana*, and *P. viridis*), demonstrating the ability of ark shell to accumulate sterol content.

By comparison of the composition of sterols in juvenile ark shell with those in corresponding unialgal feed, a close correlation of sterol compositions between algal species and juvenile ark shell was discovered (Tables 4 and 5). We have noted two phenomena. First, most sterols in microalgae, except for cholesterol, brassicasterol, campesterol, and stigmasterol, cannot undergo biological conversion in the ark shell. For example, 4α-methylporiferasterol, methylpavlovol, and ethylpavlovol, which were detected only in *P. viridis*, were also detectable only in ark shell fed *P. viridi*. This result is in accordance with that from Soudant et al.[26] Second, among the four sterols, cholesterol, brassicasterol, campesterol, and stigmasterol, found in all experimental shellfish, the higher the sterol proportion in a dietary microalga, the higher the percentage of this sterol in corresponding ark shell. For example, the higher levels of cholesterol in *C. calcitrans* (59.5%) and *N. oculata* (89.2%) resulted in higher levels of these sterols in corresponding juveniles (89.8 and 93.2%, respectively); similar results were observed with brassicasterol in *I. galbana* (99.7%) and corresponding shellfish (73.3%), as well as with campesterol in *P. helgolandica* and sitosterol in *P. viridis*. These results suggest that the accumulated sterols in ark shell came from dietary microalgae directly, and the proportion of certain sterols in tissue of bivalves can serve as good microalgal diet tracers, which is consistent with the results from Soudant et al.[26,33]

The proportion of cholesterol in juvenile ark shell is highest among those of all sterols, 62.1% at the start of experiment and 21−93% after the feeding. The apparent accumulation of cholesterol is found in the ark shell fed *C. calcitrans*, *Chlorella* sp., and *N. oculata*. Although no cholesterol was found in *P. helgolandica* and *I. galbana*, cholesterol in juvenile ark shell fed the two microalgae increased significantly (Table 5). Tsitsa-Tzardis[29] suggested that cholesterol found in oysters fed microalgae without cholesterol might be derived from dealkylation of dietary phytoplankton sterols.

Soudant et al.[26] suggested the main function of phytosterols in shellfish juveniles to be a structural role in the cell membrane, because they found that the substitution of cholesterol with other algal sterols such as brassicasterol or

campesterol did not appear to cause any physiological damage during spat development. In our experiment, *I. galbana* contained almost solely brassicasterol, but the juvenile grew on it quite well, only slower than on *C. calcitrans*. Among all experimental shellfishes, the highest proportion of brassicasterol (73.3%) was found in the ark shell fed *I. galbana*, suggesting that brassicasterol could substitute cholesterol in its absence and be accumulated in the shellfish.

P. viridis contained little cholesterol and brassicasterol while having a high abundance of 24-ethyl-5-ene-steroid (9.2% stigmasterol and 21.1% β-sitosterol), and the highest proportions of stigmasterol and β-sitosterol (30.8 and 22.0%, respectively) were found in ark shell fed *P. viridis*. Statistically significant correlations of the occurrence of double bonds at position C-5 and/or ethyl group rather than methyl group at position C-24 with the adult growth of oyster *C. virginica* have been reported by Wikfors et al.,[50] and we believe that the ark shell may share a similar mechanism.

On the basis of the results and references discussed above, we concluded that the juvenile ark shell has biotransformation ability to convert other sterols into cholesterol or to substitute the growth promotion role of cholesterol with structurally similar sterols. Feeding on appropriate unialgae such as *I. galbana* and *P. viridis*, resulted in abundant plant sterols, instead of cholesterol, being accumulated in the ark shell. Although the highest proportion of campesterol (58.2%) was found in ark shell fed *P. helgolandica*, it was not a suitable diet for ark shell due to the lower growth rate and total sterol proportion compared to those in shellfishes on *C. calcitrans*, *I. galbana*, and *P. viridis* diets.

In conclusion, the abundances of AA, EPA, and DHA, essential nutrients for human health, increased most significantly in shellfish with better growth (fed *C. calcitrans*, *I. galbana*, and *P. viridis*). Meanwhile, because plant sterols are more beneficial to human health than cholesterol,[11−16] among the six experimental microalgae, *I. galbana* and *P. viridis* are the recommended diets for ark shell, even if the shellfish would grow more quickly when feeding on *C. calcitrans*.

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Notes

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