Lipids
September 2005; 40 : 931–939

Techniques for delivery of arachidonic acid to Pacific oyster, Crassostrea gigas, spat: a preliminary investigation

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Abstract: The present study tested two techniques for dietary supplementation of *Crassostrea gigas* spat with PUFA, such as arachidonic acid (AA). The first technique consisted of a preliminary enrichment and growth of an algal concentrate (T-ISO, Isochrysis sp.) with AA dissolved in an ethanol solution, the whole culture then being fed to the spat. This enrichment increased the AA weight percentage in T-ISO neutral and polar lipids from 0.6 to 22.4% and from 0.4 to 6.8%, respectively. The second delivery technique was direct addition separately of free AA dissolved in ethanol solution and algal concentrate (T-ISO + AA) to the spat-rearing tank. To test the efficiency of these delivery techniques, oyster spat were supplemented with AA-enriched T-ISO, T-ISO + AA, and T-ISO alone. The possible biological impacts of these dietary treatments were assessed by measuring growth, condition index, and TAG content of oyster spat. Dry weight and condition index of spat fed AA-enriched T-ISO decreased by 24 and 49%, respectively, after 26 d of feeding; basically, TAG content declined 88% after 34 d of conditioning. When AA was added directly to seawater, spat growth and condition index were comparable with those of oysters fed T-ISO alone. AA incorporation in oyster tissues was assessed by analysis of the FA compositions in both neutral and polar lipid fractions. After 34 d, AA content in neutral lipids reached 7 and 11.7% in the spat fed, respectively, AA-enriched T-ISO and T-ISO + AA, as compared with 1.1% in spat fed only T-ISO. AA incorporation was greater in polar lipids than in neutral lipids, reaching 7.8 and 12.5% in spat fed AA-enriched T-ISO and T-ISO + AA, respectively. A direct addition of PUFA along with the food supply represents an effective and promising means to supplement PUFA to oyster spat.

Keywords: Dietary, Crassostrea gigas, Spat, Arachidonic acid, Food supply

ABSTRACT

The present study tested two techniques for dietary supplementation of *Crassostrea gigas* spat with polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA). The first technique consisted of a preliminary enrichment of an algal concentrate (T-ISO, *Isochrysis* sp.) with arachidonic acid dissolved in an ethanol solution, then fed to the spat. This enrichment increased the AA weight percentage in T-ISO neutral and polar lipids from 0.6 to 22.4% and from 0.4 to 6.8%, respectively. The second delivery technique was direct addition separately of free AA dissolved in ethanol solution and algal concentrate (T-ISO + AA) to the spat rearing tank. To test the efficiency of these delivery techniques, oyster spat were fed AA-enriched T-ISO, T-ISO + AA, and T-ISO alone. The possible biological impacts of these dietary treatments was assessed by measuring growth, condition index, and triacylglycerol content of oyster spat. Dry weight and condition index of spat fed AA-enriched T-ISO decreased by 24% and 49%, respectively, after 26 days of feeding; whereas, triacylglycerol content declined 88% after 34 days of conditioning. When AA was added directly to seawater, spat growth and condition index were comparable to those of oysters fed T-ISO alone. AA incorporation in oyster tissues was assessed by analysis of the fatty acid compositions in both neutral and polar lipid fractions. After 34 days, AA content in neutral lipids reached 7 and 11.7 % in the spat fed, respectively, AA-enriched T-ISO and T-ISO + AA, as compared to 1.1% in spat fed only T-ISO. AA incorporation was greater in polar lipids than in neutral lipids, reaching 7.8 and 12.5% in spat fed AA- enriched T-ISO and T-ISO + AA, respectively. In conclusion, a direct addition of PUFA along with the food supply represents an effective and promising means to supplement PUFA to oyster spat.

In bivalve hatcheries and nurseries, live microalgae are traditionally used as the primary food source. The development of artificial diets, whose nutritional composition can be precisely manipulated, would facilitate studies of bivalve nutrition. The use of artificial diets for bivalve aquaculture has been reviewed by Robert and Trintignac (1) and Knauer and Southgate (2). Microcapsules of the cross–linked, protein-walled type were used to examine aspects of protein (3,4) and carbohydrate (5) metabolism in bivalves. Further efforts were devoted to lipid nutrition, as it was established that bivalves have limited ability to synthesize essential PUFAs (6-9), such as docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), and cholesterol (10-12). Lipids were first successfully supplied to bivalves using gelatin-acacia microcapsules (GAM) (13-16) and lipid microspheres (17-19). Emulsions were subsequently demonstrated to be an alternative way to deliver essential fatty acids (20-23) and sterols (24). Liposomes have been also used to deliver essential lipids and hydrosoluble compounds to oysters (25-28). Nutritional values of these artificial diets were generally tested as a supplement to deficient microalgae. Encapsulation of PUFA in GAM to supplement algal diets lacking essential PUFA, such as EPA or DHA, improved growth of *C. gigas* spat (15,29-31). GAM-containing microalgal lipid was also shown to successfully support the metamorphosis of *C. virginica* larvae (16). Supplementation of *Dunaliella tertiolecta* (which lacks both EPA and DHA) with an EPA- and DHA-rich emulsion improved the reproductive output of *C. gigas* broodstock, as measured by hatching rate (21). These biological effects resulting from modifications in dietary lipid composition were

generally accompanied by changes in fatty acid composition of the oysters themselves (21,30,31).

To investigate the incorporation efficiency of microencapsulated lipids, different strategies/techniques have been applied: radio-labeled ^{14}C lipids (31), natural lipid biomarkers (24), and deuterated lipids (32). In most case, the incorporation of micro-encapsulated essential lipids was found to be low. Thus, it appeared important to develop more effective techniques of PUFA delivery that could be easy to apply in hatchery.
Recently, a method to supplement freshwater filter feeders with a single, free-fatty-acids was developed, using the microalgal cell itself as a transfer vehicle; this allowed successfully supplementation of *Daphnia galatea* with individual essential PUFAs (33). Also, feeding rotifers with DHA-enriched *Euglena gracilis* resulted in an increase in DHA in neutral and polar lipids of rotifers, a feed commonly used for fish larvae rearing (34,35).

The present study was conducted to test the efficiency of individual PUFA delivery to *C. gigas* spat with 1) an artificially AA- enriched T-ISO culture, and 2) a direct supply of AA into the tank, along with addition to T-ISO culture. AA has been selected as the PUFA model because its proportion is generally low in oyster spat, which makes incorporation easier to monitor. The biological impacts of the experimental dietary treatments were assessed by measuring the growth and the condition index of spat. Triacylglycerol (TAG) content of spat was used to assess the physiological status, as it is an indicator of energy reserves. Incorporation and assimilation of arachidonic acid was determined by analyzing the fatty acid composition of neutral and polar lipids of the spat.

MATERIALS AND METHODS

Arachidonic acid enrichment of T-ISO.

T-ISO (*Isochrysis* sp. clone Tahiti) was cultivated continuously in exponential phase in a 6-L glass container filled with 1µm-filtered, steam-sterilized, 35 $^{0}/_{00}$ sea water enriched with Conway medium (36) at 1 mL per litre of seawater. The algal culture was maintained at 20±1°C with continuous irradiance from cool-white fluorescence lights (378 µEins/m^2/s) and aeration (air/CO_2, 98.5-1.5%, 500 mL.min^{-1}). Cultures were supplied continuously with fresh medium by a peristaltic pump (30% renewal per day). T-ISO cells were harvested by collecting them in a secondary glass container using a glass pipe welded to the top of the primary glass container containing the algal culture. The AA enrichment was performed on the collected cells. The concentration of the collected cells ranged from 2.3 10^7 to 2.7 10^7 cells/mL.

The microalgal-enrichment technique was modified from (33). Arachidonic acid (AA) from Sigma-Aldrich (St Quentin Fallavier, France) was dissolved in absolute ethanol at 2.5 mg/mL. The harvested algae was first diluted to 5 x 10^6 cells/mL in 1500 mL, and then the AA solution (3 mL) was added directly to the algal culture to obtain a ratio of 1µg AA for 10^6 cells of T-ISO. To determine the optimal time of contact, the fatty acid compositions of T-ISO and AA-enriched T-ISO after 0.1, 1, 2 and 4 hours incubation were analyzed twice in triplicates.

For lipid extraction, samples (5-15 ml of culture) were filtered onto GF/F glass fiber filters (0.7µm), previously muffled at 450°C. Filters were then rinsed with 15 mL of seawater and transferred into a glass vial containing 6 mL of a mixture of $CHCl_3$/MeOH (2:1,by vol). Samples were then stored under nitrogen at –20°C prior to lipid-class and fatty-acid analyses.

Spat culture.

Crassostrea gigas spat were provided by a French commercial hatchery (SATMAR). Spat weighed approximately 50 mg upon arrival; they were acclimated at 18°C for 14 days with T-ISO as a mono-specific diet prior to the dietary experiment. Two hundred spat for each experimental group were maintained in a closed system, using a 20 L tank equipped with airlifts and filled with 1µm-filtered seawater. The tanks and animals were cleaned daily. T-ISO feeding rations, expressed in equivalent algae dry weight, were provided daily to spat at 0.75 % of spat wet weight (including shell). The same algal ration was applied for the entire duration of the dietary experiment (34 days). A peristaltic pump was adjusted to distribute the algal ration over a 4 hour period twice a day.

Three dietary treatments were tested: 1) T-ISO, 2) AA-enriched T-ISO (after one hour of incubation of algae and AA together), and 3) T-ISO and AA added directly into the tank (T-ISO + AA). For the latter treatment, AA was added into the tank at the same final concentration as the AA-enriched T-ISO treatment (1µg AA per 10^6 cells of T-ISO). After 11, 18, 26 and 34 days, oyster spat were sampled randomly, rinsed, and weighed. Dry meat and shell weights were measured on 20 individual oysters. Condition index was then calculated as CI = (dry weight meat/dry weight shell) x 100 (37). Three pools of ten individuals were also sampled after 18, 26 and 34 days of treatment for lipid analysis. Animals removed for lipid analyses were starved for 48h to avoid artifacts of non-assimilated fatty acids from micro-algae and supplemented AA, which could still be present in the digestive tract at the time of the sampling.

Lipid class analysis.

Total lipids were extracted according to Folch et al (38) and stored at -20°C under nitrogen until use. Neutral lipid classes were analyzed using HPTLC (high performance thin layer chromatography) CAMAG system (CAMAG Automatic TLC sampler 4 and scanner 3). A preliminary run was carried out to remove possible impurities using hexane/diethyl ether (1:1) then the plates were activated for 30 min at 120°C. Lipid samples (0.5 to 5 µg) were directly spotted on the plate by the CAMAG automatic sampler. A double development was performed using two solvent systems, first (hexane/diethyl ether/ acetic acid, 20:5:0.5, by vol) and second (hexane/diethyl ether, 97:3, by vol). Lipid classes appeared as black spots after dipping plates in a cupric sulfate phosphoric acid solution and heating for 20 min at 160°C (charring). Black spots were quantified by Wincats software (CAMAG), based upon a plate scanning at 370 nm.

The different lipid classes were identified according to authentic standards. To quantify the oyster lipid classes, a standard mixture was run on each HPTLC plate. This

standard mixture was prepared in proportions similar to those found in oyster neutral lipids: 1.1 µg.mL^{-1} menhaden oil for TAG, 0.33 µg.mL^{-1} cholesterol, 59.1 µg.mL^{-1} C18:1 alcohol, 38.8 µg.mL^{-1} cholesterol oleate and 26.7 µg.mL^{-1} oleic acid. Results were expressed as µg of each identified neutral lipid class per oyster.

Fatty acid analysis.

Total lipid extracts were evaporated to dryness under vacuum and were recovered with three washings of 500 µl each of $CHCl_3$/MeOH (98:2, by vol). The neutral and polar lipids were separated on a silica gel microcolumn (30 mm x 5 mm, Kieselgel Merck, 70-230 mesh) using $CHCl_3$/MeOH (98:2, by vol) and MeOH, respectively. The fractions were collected in tapering vials containing 2.3 µg of C23:0 (internal standard) and trans-esterified with 12% BF_3 (w/w) in methanol for 15 min, at 100 °C (39). After cooling, the fatty acid methyl esters (FAMEs) were extracted with hexane. Separation of FAMEs was carried out on a GC (HP 6890), equipped with a flame ionization detector, an on-column injector, and a J&W DBWAX capillary column (30 m x 0.25 mm; 0.25 µm film thickness). The column was temperature- programmed from 60 to 150 °C at 30 °C/min and 150 to 220 °C at 2°C/min. Hydrogen was used as the carrier gas at 2.0 mL.min^{-1}. Identification of FAMEs was based on comparison of retention times with those of authentic standards. Non-methylene interrupted (NMI) polyunsaturated fatty acids (PUFAs) 22:2Δ7,13 and 22:2Δ7,15 were respectively designated 22:2i and 22:2j. Fatty-acid peaks were integrated and analysed using HP chemstation software. Results were expressed as µg fatty acid/oyster or as weight-percent fatty acid composition for each fraction.

RESULTS

Fatty acid composition of spat fed with T-ISO.

After 14 days feeding with T-ISO for acclimation, several fatty acids increased in both neutral and polar lipids of spat. 18:1n-9, 18:2n-6, 18:3n-3, and 22:6n-3 weight percentages increased; whereas, the 20:5n-3 weight percentage decreased (Table 1).

Table 1: Fatty acid composition of neutral and polar lipids *C.gigas* spat prior and after 14 days of acclimation with T-ISO diet. Results are expressed as weight percentage of total fatty acids and as total µg per oyster of neutral or polar lipids (Mean and S.D, n=3).

	Spat prior acclimation				Spat after acclimation			
	Neutral lipids		Polar lipids		Neutral lipids		Polar lipids	
	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
14:0	6.0	0.2	2.2	0.1	10.7	0.5	3.1	0.2
16:0	9.4	0.2	13.2	0.5	9.1	0.1	10.9	0.6
18:0	2.3	0.14	4.3	0.1	1.3	0.1	2.8	0.2
16:1n-7	7.6	0.1	2.5	0.1	3.6	0.1	1.5	0.2
18:1n-9	**2.9**	**0.4**	**1.3**	**0.3**	**20.4**	**0.4**	**4.4**	**0.2**
18:1n-7	10.5	0.2	7.1	0.4	4.9	0.3	5.2	0.3
20:1n-9	0.0	0.1	0.1	0.1	0.9	0.0	1.3	0.0
20:1n-7	2.7	0.0	4.4	0.3	2.4	0.2	4.1	0.2
18:2n-6	**0.9**	**0.1**	**0.6**	**0.1**	**10.0**	**0.2**	**3.8**	**0.2**
18:3n-3	**0.4**	**0.0**	**0.3**	**0.0**	**5.7**	**0.2**	**1.9**	**0.1**
18:4n-3	3.4	0.1	1.5	0.1	5.4	0.1	1.7	0.1
20:2n-6	0.1	0.0	0.1	0.0	0.5	0.0	0.5	0.1
20:3n-6	0.1	0.0	0.1	0.0	0.2	0.0	0.1	0.0
20:4n-6	0.5	0.0	1.1	0.1	0.5	0.0	1.5	0.5
20:4n-3	0.2	0.0	0.1	0.0	0.2	0.0	0.2	0.0
20:5n-3	**24.6**	**0.5**	**21.2**	**1.2**	**3.2**	**0.4**	**8.7**	**0.3**
22:2i	0.1	0.0	1.4	0.1	0.3	0.0	1.2	0.1
22:2j	1.3	0.1	0.2	0.0	1.3	0.2	5.1	0.2
22:4n-6	0.0	0.0	5.5	0.3	0.1	0.0	0.2	0.0
22:5n-6	0.1	0.0	0.1	0.0	1.5	0.0	2.5	0.1
22:5n-3	0.3	0.0	0.3	0.0	0.2	0.0	0.7	0.0
22:6n-3	**3.0**	**0.3**	**8.6**	**0.3**	**9.4**	**0.9**	**15.3**	**0.3**
Total FA (µg/oyster)	57,1	4,1	16,9	7,6	47,3	3,1	12.3	3.8

AA enrichment of T-ISO. (Table 2).

Fatty acid compositions of T-ISO and T-ISO incubated with AA for 0.1,1,2 and 4 hours were analysed in this experiment. Results for neutral lipids and polar lipids are reported in Table 2. The mean AA percentages in oysters fed T-ISO were 0.6% and 0.4% in neutral and polar lipids, respectively. Immediately after the addition of AA to the algae (i.e after the lipid extraction procedure), a significant increase in AA percentage in the microalgae was observed in both neutral (22.4%) and polar (3.4 %) lipids. Then, after 4 hours incubation, the AA weight percentage decreased from 22.4% to 16.7% in the neutral lipids, but it increased from 3.4% to 6.8% in polar lipids. Concomitantly, the 22:6n-3 weight percentage decreased from 3.9% to 2.4% in neutral lipids, and from 9.5% to 2.3% in polar lipids. As a consequence, the n-3/n-6 ratio declined from 2.9 to 0.5 in neutral lipids and from 3.1 to 0.9 in polar lipids after 4 hours of AA incubation.

Table 2 : Fatty acid (FA) composition of neutral and polar lipids of T-ISO and T- ISO incubated 0.1, 1, 2 and 4 hours with arachidonic acid at a ratio of 1 µg per 10^6 cells. Results are expressed as percentage of total fatty acids and as total ng fatty acids per algal cell in neutral and polar lipids (Mean and S.D, n=6). Different letters denote significant differences between conditions (ANOVA, $p < 0.05$).

	T-ISO		Arachidonic acid enriched T-ISO							
Incubation time	0 hours		0.1 hours		1 hour		2 hours		4 hours	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Neutral lipid										
20:4n-6	0.6^{a}	0.2	22.4^{b}	5.0	19.2^{bc}	4.1	18.3^{bc}	4.7	16.7^{c}	5.1
20:5n-3	0.4^{a}	0.2	0.2^{ab}	0.1	0.2^{b}	0.1	0.2^{ab}	0.1	0.2^{ab}	0.0
22:6n-3	3.9^{a}	0.9	2.6^{b}	0.9	2.6^{b}	0.5	2.5^{b}	0.6	2.4^{b}	0.4
n-3/n-6	2.9^{a}	0.5	0.5^{b}	0.2	0.5^{b}	0.1	0.6^{b}	0.1	0.5^{b}	0.0
Total FA (ng/cell)	1.2^{ab}	0.3	1.5^{ab}	0.3	1.2^{a}	0.3	1.6^{b}	0.4	1.3^{ab}	0.3
Polar lipid										
20:4n-6	0.4^{a}	0.2	3.4^{b}	0.90	6.2^{c}	1.2	6.0^{c}	2.1	6.8^{c}	1.1
20:5n-3	0.8^{a}	0.3	0.7^{ab}	0.1	0.5^{bc}	0.1	0.4^{bc}	0.1	0.2^{c}	0.0
22:6n-3	9.5^{a}	3.3	5.2^{b}	1.4	3.7^{bc}	0.3	3.0^{bc}	0.5	2.3^{c}	0.3
n-3/n-6	3.1^{a}	0.8	2.1^{b}	0.1	1.5^{c}	0.2	1.4^{c}	0.1	0.9^{c}	0.2
Total FA (ng/cell)	1.2^{a}	0.2	1.3^{a}	0.7	0.7^{b}	0.1	0.6^{b}	0.2	0.5^{b}	0.1

Growth and performance of spat under different dietary treatments (Table 3).

Growth and performance parameters, as measured by dry weight and condition index of spat fed T-ISO, AA-enriched T-ISO, T-ISO + AA were monitored for 34 days. For the spat fed T-ISO, dry weight increased steadily until 26 days. The CI was maintained between 4.6 and 5.1 from 11 to 26 days. But, after 34 days, dry weight dropped to 2.7 mg and CI to 4.1. The AA-enriched T-ISO dietary treatment clearly impaired spat growth and condition. Mean dry weight of the spat fed AA-enriched T-ISO was significantly lower than that of spat fed T-ISO from 18 to 34 days of conditioning. Similarly, condition index was lowest in spat fed AA enriched T-ISO at all sampling dates. In contrast, when AA was added directly to the tank, no such negative effects were observed. Some slight reductions (statistically significant) in dry weight and condition index were observed in this treatment, compared to T-ISO alone after 26 days of dietary conditioning. However, these differences were not seen after 34 days.

Table 3: Impact of dietary conditioning [T-ISO alone, arachidonic acid enriched T-ISO (AA-enriched T-ISO), and T-ISO and arachidonic acid added directly into the tank (T-ISO + AA)] on the dry weight (mg/oyster) and condition index of *C. gigas* spat (Mean and S.D., n=20). Different letters denote significant differences between conditions (ANOVA, $p < 0.05$).

Diets	T-ISO		AA-enriched T-ISO		T-ISO + AA	
Dry weight	Mean	S.D	Mean	S.D	Mean	S.D
T0	2.1	0.5				
11 days	2.3 [a]	0.8	1.8 [a]	0.8	2.2 [a]	1.1
18 days	3.1 [a]	1.4	1.8 [b]	0.8	2.8 [a]	1.1
26 days	4.1 [a]	2.3	1.8 [b]	0.5	2.9 [c]	0.8
34 days	2.7 [a]	0.7	1.6 [b]	0.4	2.6 [a]	1.2
Condition index						
T0	5.6	1.5				
11 days	4.6 [a]	1.1	3.6 [b]	0.8	4.0 [b]	0.7
18 days	4.6 [a]	1.1	2.8 [b]	0.6	4.1 [a]	1.4
26 days	5.1 [a]	0.9	2.6 [b]	0.7	3.6 [c]	0.9
34 days	4.1 [a]	0.8	2.7 [b]	0.4	4.0 [a]	1.3

Lipid class composition of spat under different dietary treatments (Table 4).

HPTLC analysis identified and quantified five lipid classes in the neutral lipids of oyster spat: steryl ester (SE), TAG, free sterol (FS), free alcohol (FAO) and FFA. As SE and FAO were found in small amounts, occasionally below the detection threshold, only the results obtained for the TAG, FS and FFA are presented here. TAG was the major storage lipid class detected in spat. The TAG, FS and FFA contents in spat at the beginning of the experiment (after the acclimation period) were, respectively, 65.4, 18.2 and 1.6 µg/oyster. The TAG content increased from 65.4 µg/oyster to 148.9 µg/oyster in spat fed T-ISO, and to 130.6 µg/oyster in spat fed T-ISO + AA. There was no significant difference between these two dietary treatments during the experiment. In contrast, feeding spat with AA-enriched T-ISO resulted in a drastic decrease of TAG content after 18 days, which dropped below 22 µg/oyster at the end of the experiment.
The FFA content (Table 4) ranged from 0.3 to 1.9 µg/oyster, regardless of treatment or sampling time, and maximal percentages in neutral lipids were 1.7, 3.5 and 1.4% in spat fed T-ISO, AA-enriched T-ISO, and T-ISO + AA, respectively.

Table 4: Impact of dietary conditioning [T-ISO alone, arachidonic acid enriched T-ISO (AA-enriched T-ISO), and T-ISO and arachidonic acid added directly into the tank (T-ISO + AA)] on the triacylglycerol, free fatty acid and sterol contents of *C. gigas* spat (Mean and S.D, n=3). Results are expressed in µg of lipid class/oyster. Different letters denote significant differences between conditions (ANOVA, $p < 0.05$).

Diets	T-ISO		AA-enriched T-ISO		T-ISO + AA	
Free Fatty acid	Mean	S.D	Mean	S.D	Mean	S.D
T0	1.6	0.4				
18 days	1.3 [a]	0.2	0.3 [b]	0.0	0.6 [c]	0.1
26 days	1.5 [a]	0.2	0.5 [b]	0.1	1.4 [a]	0.3
34 days	1.9 [a]	0.8	0.7 [b]	0.3	1.5 [ab]	0.1
Triacylglycerides						
T0	65.4	8.1				
18 days	114.3 [a]	32.2	21.6 [b]	6.3	85.3 [a]	9.6
26 days	131.2 [a]	28.7	22.0 [b]	3.4	135.2 [a]	29.8
34 days	148.9 [a]	29.8	18.3 [b]	6.1	130.6 [a]	25.5
Sterols						
T0	18.2	2.7				
18 days	22.5	3.2	12.7	2.8	19.6	3.0
26 days	22.9	3.4	13.4	0.8	23.9	6.0
34 days	27.9	1.6	14.2	2.1	23.2	0.4

Fatty acid composition of spat under different dietary treatments.

Proportions of saturated, mono-unsaturated, and poly-unsaturated fatty acids were not affected by the dietary treatments and were stable in both neutral and polar lipids for the entire duration of the experiment (data not shown). After 18 days of conditioning, and when compared to spat fed T-ISO alone, the 20:4n-6 weight-percentage in spat fed the AA-enriched T-ISO diet increased by approximately four-fold in neutral lipids (Table 5) and by two-fold in polar lipids (Table 5). The 20:4n-6 weight percentage increased nine times in neutral lipids (Table 5) and three times in polar lipids (Table 5) in spat fed T-ISO + AA added separately. The 20:4n-6 weight percentage in both polar and neutral lipids of spat AA-supplemented diets continued to increase between 18 and 26 days, and reached a plateau between 26 and 34 days (Table 5). After 26 days of feeding with the T-ISO + AA diet, 20:4n-6 reached approximately 12% of the PUFAs in both neutral and polar lipids. The 22:4n-6 weight- percentage in neutral and polar lipids followed the same pattern as the 20:4n-6 percentage, according to dietary treatment (Table 5). The weight percentage increases in 20:4n-6 and 22:4n-6 observed with AA supplemented diets were partially compensated by a decrease of 22:6n-3 in polar lipids (Table 5). Finally, after 34 days of conditioning, the n-3/n-6 ratio of spat fed T-ISO + AA decreased to 1.1 in both neutral and polar lipids and was much lower than in spat fed T-ISO alone, which had an n-3/n-6 ratio at 2.0 and 2.2 in neutral and polar lipids, respectively.

Table 5: Fatty acid composition of neutral and polar lipids in oyster spat after 18, 26, and 34 days of feeding with T-ISO, arachidonic acid enriched T-ISO (AA-enriched T-ISO) and T-ISO and arachidonic acid added directly into the tank (T-ISO + AA). Results are expressed as percentage of total fatty acids of polar and neutral lipids (Mean and S.D, n=3).

	Initial		18 days						26 days						34 days					
			T-ISO		AA-enriched T-ISO		T-ISO + AA		T-ISO		AA-enriched T-ISO		T-ISO + AA		T-ISO		AA-enriched T-ISO		T-ISO + AA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Neutral Lipid																				
20:4n-6	**0.5**	**0.0**	**0.9**	**0.2**	**4.3**	**0.3**	**9.4**	**1.4**	**1.0**	**0.1**	**6.2**	**0.9**	**12.1**	**0.5**	**1.1**	**0.1**	**7.0**	**0.2**	**11.7**	**0.3**
20:5n-3	3.2	0.4	1.8	0.2	3.3	0.9	2.4	0.4	1.9	0.0	3.7	0.7	2.3	0.0	1.9	0.3	3.3	0.4	2.0	0.1
22:4n-6	0.1	0.0	0.1	0.0	0.2	0.0	0.4	0.0	0.1	0.0	0.3	0.0	0.7	0.3	0.1	0.0	0.4	0.1	0.5	0.0
22:5n-6	1.5	0.0	2.1	0.1	1.5	0.3	2.0	0.3	2.3	0.0	1.7	0.3	2.3	0.1	2.4	0.2	1.7	0.2	2.4	0.0
22:5n-3	0.2	0.0	0.3	0.0	0.3	0.1	0.2	0.0	0.3	0.0	0.4	0.1	0.3	0.0	0.3	0.0	0.4	0.3	0.2	0.0
22:6n-3	9.4	0.9	10.1	0.7	9.3	3.1	9.3	0.6	11.8	0.3	9.2	1.8	9.6	0.1	12.0	0.7	8.5	1.2	9.4	0.2
n-3/n-6	1.9	0.1	2.5	0.1	1.8	0.2	1.3	0.1	2.3	0.1	1.5	0.2	1.1	0.1	2.0	0.1	1.3	0.1	1.1	0.0
Total FA (µg/oyster)	47.3	3.1	57.8	15.5	19.4	3.1	43.4	29.5	116.8	7.2	25.4	0.9	102.3	34.2	109.5	26.8	19.6	6.8	107.8	14.0
Polar lipid																				
20:4n-6	**1.5**	**0.5**	**2.8**	**0.2**	**5.9**	**0.3**	**10.1**	**0.1**	**3.4**	**0.4**	**7.7**	**1.4**	**12.3**	**0.7**	**3.7**	**0.2**	**7.8**	**0.7**	**12.5**	**0.6**
20:5n-3	8.7	0.3	5.6	0.5	7.4	0.4	5.5	0.3	5.0	0.2	6.3	0.5	4.0	0.1	4.8	0.2	5.0	0.3	3.7	0.0
22:4n-6	0.2	0.0	0.3	0.0	0.4	0.0	0.8	0.0	0.3	0.0	0.5	0.0	1.3	0.0	0.3	0.0	0.9	0.1	1.3	0.1
22:5n-6	2.5	0.1	3.9	0.1	2.9	0.1	3.0	0.1	4.1	0.1	2.7	0.2	3.3	0.2	4.5	0.1	3.1	0.2	3.1	0.2
22:5n-3	0.7	0.0	0.7	0.1	0.8	0.0	0.7	0.0	0.6	0.0	0.8	0.0	0.6	0.0	0.6	0.0	0.9	0.1	0.5	0.0
22:6n-3	15.3	0.3	18.2	1.0	16.1	1.5	14.7	0.4	18.3	0.5	15.0	0.8	14.2	0.6	18.8	0.4	15.7	0.8	12.6	0.3
n-3/n-6	3.4	0.2	2.7	0.1	2.2	0.1	1.5	0.0	2.3	0.1	1.9	0.2	1.2	0.1	2.2	0.1	1.9	0.1	1.1	0.1
Total FA (µg/oyster)	12.3	3.8	25.4	2.8	28.6	1.2	33.3	4.5	54.6	7.4	32.0	2.5	31.6	7.0	54.3	4.9	21.2	3.1	30.8	4.6

DISCUSSION

AA algal enrichment. The present study demonstrated that AA was immediately incorporated into both algal neutral and polar lipids. This quick AA association with neutral lipids, rather than polar lipids alone, suggested that most of the AA probably was associated as free fatty acid on the surface of T-ISO cells, and that the main mechanism of this enrichment was probably resulting from an algal coating by adsorption. HPTLC analysis showed that free fatty acids increased in the AA-treated algal samples (15.8%) in comparison with non treated T-ISO (5.5%).Von Elert (33) made similar observations when enriching *Scenedesmus obliquus* and *Stephanodiscus hantzschii* with various unsaturated fatty acids (18:1n-7, 18:3n-3, 20:4n-6, 20:5n-3 and 22:6n-3) and was uncertain whether these PUFAs were only adsorbed on cell surfaces or truly incorporated within the cells.

The rapid increase in AA within polar lipids cannot be explained by the above mechanism, but this percentage increased steadily during incubation and may indicate that some of the adsorbed AA might have been incorporated in membrane phospholipids thereafter by deacylation-acylation processes (40). Our results are in agreement with those obtained with *Chlorella vulgaris* and *Euglena gracilis* PUFA enrichments (34,35). When *E. gracilis* was enriched with 0.5% of 22:6n-3 (DHA) for 24 hours, exogenous DHA was mainly accumulated in neutral lipids and, to a lesser extent, in the three major phospholipids of *E. gracilis:* namely PC, PE and PI. Most likely, because of the large incorporation of AA within the algae, a significant reduction in DHA percentage from 3.9 to 2.4% in neutral lipids and from 9.5 to 2.3% in polar lipids was observed after 4 hours of incubation, as well as a decrease in n-3/n-6 ratio from 2.9 to 0.5 in neutral lipids and from 3.1 to 0.9 in polar lipids. These changes may also affect the dietary quality of the enriched algae as DHA is well established to be an essential PUFA for bivalve growth and reproduction (6-9).

AA absorption by spat.

Ingestion and assimilation of AA were estimated by analyzing the fatty acid composition changes of neutral and polar lipids in spat during the experiment. Oyster spat represent a good model target organism for dietary experiments because the fatty acid compositions of polar lipids, and especially neutral lipids, are rapidly affected by dietary modifications. Fourteen days of feeding acclimation with T-ISO was enough to change the proportions of the PUFAs, such as 20:5n-3 and 22:6n-3, appreciably. The dietary imprint of T-ISO was observed consistently when this was used as a mono-specific diet for various species of bivalves : oysters, clams, and scallops (41- 44). This prior knowledge facilitated the distinction of fatty acid composition modifications atributable to AA supplementation, without confounding effects of the mono-specific diet imprint.

After 18 days of feeding with AA-enriched T-ISO, a clear increase in the AA weight percentage in spat neutral and polar lipids, up to 4.3 and 5.9 %, respectively, after 18 days was obtained, and a plateau was reached at 7.0% in neutral lipids and 7.8% in polar lipids after 34 days of feeding, respectively seven and two times the values of the T-ISO alone dietary treatment. These results demonstrate the high efficiency of AA incorporation within spat. We considered the possibility that the AA-enriched T-ISO

condition could have been detrimental/toxic for the microalgae, modifying their morphological and/or motility characteristics. Indeed, T-ISO was observed to loose its motility after 4 hours of contact with AA at 0.01 $mg.mL^{-1}$ in the AA-enriched T-ISO condition (P. Miner, pers. comm.). The loss of motility of T-ISO could possibly affect feeding behavior of the spat and, thus, could explain a lower AA incorporation compared to the T-ISO + AA condition.

Indeed, the direct addition of AA to the tank, simultaneously with the microalgae (T-ISO + AA) permitted the highest incorporation of AA in both neutral and polar lipids of spat, reaching approximately 12% in neutral and polar lipids after 26 and 34 days of feeding. This higher incorporation efficiency, compared to the AA-enriched T-ISO feeding, is possibly related to a higher algal ingestion rate or direct AA absorption by spat. Some papers showed that marine invertebrates can absorb dissolved organic matter (45,46). More specifically, bivalves can absorb free amino acids from sea water and use them as a nutritional supplement (47,48). Jaeckle and Manahan (49) demonstrated that addition of 1µM glucose and of a mixture of 16 amino acids enhanced metabolic rate of oyster larvae and concluded that organic chemistry of seawater can affect the growth and metabolism of oyster larvae. However, there is only one paper demonstrating such direct absorption for dissolved lipids, or more specifically for free fatty acids (50).

Biological effects.

The possible biological impacts of these dietary treatments (T-ISO, AA-enriched T-ISO, and T-ISO + AA diets) were assessed by measuring growth, condition index and lipid storage indicators of the conditioned spat.

Results of the present study demonstrated that AA-enriched T-ISO diet had an unexpected but very clear negative effect on spat growth. compared to the T-ISO diet. Specifically, dry weight and condition index of spat fed AA-enriched T-ISO showed respectively a decrease of 24% and 49% after 26 days of feeding, and triacylglyceride content (a storage lipid) was reduced by 88% after 34 days, as compared to spat fed the unsupplemented T-ISO diet.

Thus, one possible explanation for such a biological effect could be that the AA-enriched T-ISO enrichment resulted in drastic morphological and/or biochemical changes in T-ISO cells, making the micro-algae toxic or unsuitable for filtration/ingestion by spat. Moreover, this biological effect is unlikely to be the result of the spat fatty acid composition changes by the AA-enriched T-ISO diet, because the T-ISO +AA diet, which affected more or at least at the same extent the spat fatty acid composition, had similar growth performance and lipid-storage content as the unsupplemented T-ISO diet. Nevertheless, the absence of significant effects of the high AA enrichment (12% in polar and neutral lipids) obtained with the T-ISO +AA diet on spat performance is quite surprising. These modifications of fatty acid composition may, however, have on other, important biological functions, such as defense mechanisms, as previously reported by Delaporte et al. (43) and Sargent et al. (53). Finally, it must be noted that, according to previous field and laboratory experiments, the highest values of AA measured in oyster neutral or polar lipids always remained below a 10% threshold. For instance, AA weight percentage ranged from 0.9 to 2.% in NL and from 1.5% to 4.3% in PL of *C. gigas* natural spat (51). The AA weight percentage reached 6% of total fatty acids of *C.*

virginica gills (in which lipids are mostly in polar lipids) after 56 days of feeding with a micro-algae mixture containing in equal proportions: *Tetraselmis maculata, Thalassiosira weissflogi, Chaetoceros calcitrans* and *Isochrysis galbana* (52). Delaporte et al. (44) measured up to 7.5% of AA in hemocyte polar lipids of *C. gigas* fed for 8 weeks *C. calcitrans,* which contained 2% AA. Thus, the 12% value measured in polar lipid of spat feed the T-ISO +AA diet in our experiment is above values reported previously.

In conclusion, from this preliminary study, it appears that direct addition of AA is a promising technique to assess biological effects of this PUFA on oysters. However, an acceptable concentration for free fatty acid and algae should be determined to prevent alteration of algal mobility and spat feeding behavior. The present study also suggests that other PUFAs could be manipulated by the direct addition to the tank for bivalve nutrition studies.

ACKNOWLEDGMENTS

The authors would like to thank Chris Langdon and Gary Wikfors for the critical review of this manuscript and for editing English language. This research was a part of the MOREST project supported by a grant from IFREMER, the Région Bretagne, Région Normandie, Région Pays de Loire, Région Poitou-Charente and the Conseil Général du Calvados. Contribution N°967 of the IUEM, European Institute for Marine Studies (Brest, France).

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