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Dietary Enhancement of Selected Fatty Acid Biosynthesis in the Digestive Gland of *Mytilus galloprovincialis* Lmk.

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ABSTRACT: The fatty acid composition of the digestive gland from the mussel *Mytilus galloprovincialis* subjected to three different dietary regimens for 30 days was analyzed. Samples were collected at the beginning and end of the trial to obtain a comprehensive picture of fatty acid dynamics. Group A was unfed; group B received a diet consisting of 100% *Thalassiosira weissflogii* and, thus, similar to natural food; and group C received a diet consisting of 100% wheat germ conferring a 18:2ω-6 abundance. Results indicate that fatty acid composition of lipid and phospholipid classes was affected by dietary treatments. However, adult mussel homeostatic skills minimized effects, and thus, only wheat germ diet deeply modified the fatty acid composition. Furthermore, in group C, the occurrence of the non-methylene-interrupted trienoic fatty acids was indicative of *de novo* fatty acid synthesis presumably because of active fatty acid elongation and Δ5 desaturation system, also supported by the general ω-3 polyunsaturated fatty acid decrease.

KEYWORDS: *Mytilus galloprovincialis, digestive gland, fatty acid composition, NMI fatty acids*

INTRODUCTION

Bivalve lipid metabolism has been the subject of a number of studies, largely because of the important position held by this group in marine food chains.[1] Indeed, these filter-feeding animals feed abundantly on phytoplankton, which, therefore, plays a role in the accumulation of lipids formed during primary production. It is well-known that bivalve fatty acids are highly unsaturated, mainly because of the ω-3 long-chain polyunsaturated fatty acids (PUFAs) as 20:5ω-3 and 22:6ω-3, well-known as effective factors in human health and nutrition.[2] As other marine animals, bivalves have a very limited or no capability to synthesize ω-3 and ω-6 long-chain PUFAs by elongation and desaturation of the precursors 18:2ω-6 and 18:3ω-3 for the absence of Δ6 desaturase. As filter-feeders, bivalves feed upon phytoplankton, which constitutes the major source of linoleic acid (18:2ω-6), linolenic acid (18:3ω-3), and C_{20} and C_{22} PUFAs,[3,4] which affect survival, growth, and reproduction.[5,6]

Mollusk lipids contain, in addition to or in replacement of the typical marine animal fatty acids, non-methylene-interrupted (NMI) dienoic and trienoic fatty acids, having unusual unsaturation features, because they possess double bonds with more than one methylene group between ethylenic bonds.[1,7,8] Some of these fatty acids, more precisely 20:2Δ5,11 and 20:2Δ5,13, were demonstrated to be *de novo* synthesized in bivalves by elongation and Δ5 desaturation of 18:1ω-9 and 16:1ω-7, which are very abundant in phytoplankton, especially in diatoms.[9] A following elongation step converts them into 22:2Δ7,13 and 22:2Δ7,15, respectively.

Recent reports on the nutraceutical anti-inflammatory potential of *Mytilus edulis* fatty acids have been related to the presence of NMI fatty acids, which can act as an antimetabolite to arachidonic acid.[10] The preferential incorporation in polar lipids, sometimes in competition with PUFA ω-3, and the selective distribution among different tissues and organs, which favors those exposed to the external environment, suggest a structural and functional role in biological membranes.[7]

Recently, in *Mytilus galloprovincialis*, the two NMI trienoic (NMIT) fatty acids, 20:3Δ5,11,14 and 22:3Δ7,13,16, were identified and suggested that they can be synthesized in mollusks by Δ5 desaturation of 20:2ω-6 to produce 20:3Δ5,11,14, followed by a chain-elongation step to 22:3Δ7,13,16.[11] Our results on *M. galloprovincialis* fed a wheat germ diet with a very high content of 18:2ω-6 documented an unusual level of the two NMIT derivatives, 20:3Δ5,11,14 and 22:3Δ7,13,16, in the total soft body.[7] The ability to biosynthesize NMIT fatty acids can represent a great adaptive mechanism for organisms incapable of biosynthesizing the essential long-chain PUFA 20:4ω-6, 20:5ω-3, and 22:6ω-3, from the precursors 18:2ω-6 and 18:3ω-3, and also exposed to fluctuating temperatures, which requires membrane fluidity adaptations.

Previous studies[7] suggested a relationship between the NMI dienoic (NMID) fatty acid level in bivalve tissues and the fatty acid composition of the diet. Because molluscan lipid studies frequently involve the entire organism, only few reports on the tissue distribution of fatty acids are available.[12] In mussels, as in all bivalves, the digestive gland plays a central role in metabolism through intracellular digestion of food particles and in nutrient distribution to reproductive tissues during gamete maturation.[13] In terms of the bivalve energy budget, the digestive gland may be considered as a lipid storage organ. Thus, any changes in the lipid composition produced by diet are first displayed here.[14]

A deep knowledge of the link between the ingested food and fatty acid pattern of mussels can also help to trace the origin of

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the product,[15] to evaluate the possibility of substituting a natural diet with waste material, and to predict or favor the nutritional value and/or nutraceutical potential for consumers.[10,16] Because some fatty acids are conservatively transferred through the food web, they can also provide trophic information based on the fatty acid marker concept.[17−19]

In the present study, we aimed at investigating further the effect of dietary fatty acids on the lipid content and fatty acid composition, especially NMID fatty acids, in lipid and phospholipid classes of the digestive gland of *M. galloprovincialis*. To this purpose, the effect of deeply different dietary treatments was explored by feeding mussels wheat germ or a monoalgal diet based on the diatom *Thalassiosira weissflogii* to compare retention and modification of PUFA ω-3 and ω-6 as well as synthesis and/or modification of NMI fatty acids. Additionally, the impact of starvation was studied to obtain a comprehensive picture of the fatty acid dynamics in starved and fed animals and to gather information about the relative importance of different fatty acids.

MATERIALS AND METHODS

Experimental Design, Diets, and Animal Sampling. Adult *M. galloprovincialis* of 4.5−5.0 cm shell length, obtained from an Italian commercial hatchery (Copralmo, Cesenatico, Italy) in early autumn, were transported to the laboratory and subjected to a 7 day period of acclimatization to laboratory conditions (*t*, 19.0 °C; salinity, 3.0%; dissolved oxygen, 7.0 mg of O_2 L^{-1}; pH, 8.2). The acclimation period was devised to purge all animals in filtered seawater for 1 week prior to using them in feeding experiments.

Before the start of the experimental dietary regimen, 100 mussels were used for initial sampling. Remaining mussels were randomly split into 6 groups of 110 specimens for duplicate exposure to the three experimental dietary regimens for 30 days. Group A was unfed. Group B received a diet consisting exclusively of *T. weissflogii* as marine microalgae concentrates, Instant Algae, supplied by Reed Mariculture. Group C was fed a diet consisting of 100% wheat germ (Food for All, Italy), which was prepared via the method by Albentosa et al.[20] as follows: The wheat germ was first ground in a food blender and then sieved. Particles passing through a 60 μm sieve were gathered and stored in a freezer until the feeding trials. Every day, the daily feeding ration of wheat germ was thawed and suspended in seawater with a blender.

Each group was held in a 150 L aquarium of filtered natural seawater and kept in trays 5 cm suspended from the bottom. Recirculation systems were used at a flow of 1.0 L/min. The water was partly renewed every 10 days. The water temperature, salinity, dissolved oxygen, and pH were monitored regularly and were maintained at 19 ± 1 °C, 2.9−3.1%, 7.0 mg of O_2 L^{-1}, and pH 8.2, respectively. Each aquarium was aerated to keep food in suspension.

Preliminary trials were carried out to evaluate the sedimentation rates in the experimental tanks of both the wheat germ particles and the diatom *T. weissflogii*. While the microalgal diet remained in suspension for a long time (after 6 h, the number of cells amounted to 15 × 10^6/L), wheat germ flour showed very high sedimentation rates (tank water was practically transparent to spectrophotometrical control after 1−2 h). Such a different sedimentation tendency was considered in calculating the daily feeding ration. Therefore, the ration of group B was decreased from 1 to 0.6% of the mussel live weight, and the ration of group C was increased from 1.0 to 1.2% of the mussel live weight, to compensate for sedimentation losses. Because the total lipid content (Table 1) was different in the two diets (27.4 and 13.5% of dry weight in B and C, respectively), a different daily feeding ration to B and C groups also ensures a similar amount of lipids. Animals were fed once a day; at the same time, the recirculation system was stopped for 6 h.

Samplings were carried out at the initial point (0) and after 30 days of dietary conditioning. For each sampling and dietary treatment, 4 pools of 10 mussels were used. Sampled mollusks were killed by breaking the adductor muscles, and their digestive gland was immediately dissected from each animal. Crystalline style was removed, and samples were stored in liquid nitrogen until analyzed.

Table 1. Content of Total Lipids, Neutral Lipids, Polar Lipids, and Phospholipids of *T. weissflogii* (Diet B) and Wheat Germ Flour (Diet C)[a]

	total lipids (% dry weight)	neutral lipids (% TL)	polar lipids (% TL)	phospholipids (% TL)
T. weissflogii (diet B)	27.4 ± 0.6	22.0 ± 1.8	78.0 ± 3.2	53.7 ± 2.0
wheat germ flour (diet C)	13.5 ± 0.2	78.2 ± 5.3	21.8 ± 1.1	19.5 ± 1.0

[a]Values are means of four determinations ± SD.

Lipid Analyses. Lipid analyses were carried out on the whole digestive gland. Total lipids (TL) were extracted with 2:1 (v/v) chloroform/methanol, containing 0.1% (w/v) butylated hydroxytoluene (BHT) as an antioxidant, by Folch's method[21] and evaluated gravimetrically after complete evaporation of the solvent. Phospholipids (PL) were quantitatively evaluated by colorimetric determination of phosphorus[22] according to the method by Bartlett,[23] as modified by Marinetti.[24] Lipid extracts were separated into neutral lipids (NL) and polar lipids (PoL) according to Marks et al.[25] The three different lipid extracts, TL, NL, and PoL, were submitted to direct transmethylation with a 14% (v/v) BF_3 methanolic solution according to Morrison and Smith.[26] Individual PL classes were separated by one dimension thin-layer chromatography (TLC) on a 20 cm × 20 cm × 0.25 mm silica gel 60 plate (Merck KgaA, Darmstadt, Germany), which was developed in methylacetate/*n*-propanol/chloroform/methanol/0.25% (w/v) KCl solution (25:35:20:10:10, v/v/v/v/v).[27] The obtained PL classes, phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylethanolamine (PE), were directly transmethylated with 2 M NaOH in methanol according to Christoferson and Glass.[28] Fatty acid methyl esters (FAMEs) were analyzed on a Varian 3380 gas chromatograph equipped with a fused silica capillary column, DB-23 J&W Scientific (30 m × 0.25 mm), and flame ionization detector at 300 °C. The carrier gas was nitrogen at a flow rate of 1.2 mL min^{-1}. The oven temperature was set in programmed mode from 150 to 230 °C at 5 °C min^{-1} and final isoterm. Data were processed using a Varian Star Chromatography Workstation. Because there is no absolute method for identifying fatty acids by gas liquid chromatography (GLC), a combination of identification procedures was applied to the FAME mixtures under study. On the basis of equivalent chain length (ECL), results of catalytic hydrogenation, separation of fatty acids of different unsaturation degrees by 20% $AgNO_3$ TLC, and comparison to known standards, tentative identification was possible for the majority of fatty acids. Results are expressed as a percentage of total fatty acids (w/w).

The location of double bonds in NMID fatty acids was assessed by analyzing the characteristic fragmentation pattern of the 2-alkenyl-4,4-dimethyloxazoline derivatives[29] obtained on a mass spectrometer, Fisons MD 800, 70 eV.

Calculations and Statistics. The following indices of the fatty acid profile of lipid and PL classes were calculated: total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), total polyunsaturated fatty acids (PUFA), and the unsaturation index (UI), a parameter employed to evaluate the fatty acid unsaturation degree, calculated according to the formula: [% monoenoic + 2(% dienoic) + 3(% trienoic), ..., etc./% total SFA]. All results are presented as the mean ± standard deviation (SD) of four determinations on different pools. Statistical analyses were performed using the software program SIGMASTAT. To compare the differences among all dietary treatments and with the initial conditions (I), all data were submitted to analysis of variation (ANOVA) with a significance level of $p < 0.05$, followed by Dunnett's test and Student−Newman−Keuls' test as required. Before statistical analyses, data were checked for normal

distribution and variance homogeneity. Whenever these assumptions were violated, before statistical analyses, data were transformed (arcsin of the square root) to ensure normality (percentage data) or $\log_{10}$ transformed to ensure homogeneity of variances. In most cases, after these transformations, both assumptions were satisfied. Data are presented in figures and tables as untransformed percentage values.

RESULTS

Dietary Fatty Acid Composition. The mussels were fed diets that deeply differed in lipid class (Table 1) and fatty acid composition (Table 2). Between lipid classes, polar lipids were the main component of diet B, composed of 100% of *T. weissflogii*, with the PL component amounting to 53.7% (as a percentage of TL). As previously reported,[7] the major fatty acids of the diet B were 16:0, 16:1ω-7, 16:3ω-4, and 20:5ω-3, the typical diatom fatty acids. In diet C, composed of 100% wheat germ flour, neutral lipids clearly prevailed (78% TL) between lipid classes as well as 18:2ω-6 (60.3%) between fatty acids. Also, 16:0, 18:1ω-9, and 18:3ω-3 were present in appreciable amounts, with the latter also being the most long-chain and unsaturated fatty acid in wheat germ flour.

Table 2. Fatty Acid Composition[a] of the Diets Used in the Present Trial (wt %)

fatty acid	*T. weissflogii*	wheat germ flour
14:0	4.3 ± 0.3	0.1 ± 0.0
15:0	1.5 ± 0.1	0.1 ± 0.0
16:0	17.8 ± 0.8	17.7 ± 0.2
16:1ω-7	14.3 ± 0.1	0.2 ± 0.0
16:2ω-7	1.2 ± 0.0	*b*
16:2ω-4	4.9 ± 0.2	*b*
16:3ω-4	12.5 ± 0.4	*b*
18:0	0.7 ± 0.1	0.4 ± 0.1
18:1ω-9	0.9 ± 0.0	11.9 ± 0.2
18:1ω-7	0.6 ± 0.0	0.6 ± 0.1
18:2ω-6	1.8 ± 0.0	60.3 ± 0.1
18:2ω-3	0.5 ± 0.1	*b*
18:3ω-3	0.6 ± 0.0	8.8 ± 0.1
18:4ω-3	2.0 ± 0.0	*b*
20:4ω-6	1.7 ± 0.2	*b*
20:4ω-3	0.4 ± 0.1	*b*
20:5ω-3	28.1 ± 0.7	*b*
22:5ω-6	0.5 ± 0.0	*b*
22:6ω-3	5.8 ± 0.2	*b*
SFA	24.3 ± 1.3	18.2 ± 0.1
MUFA	15.8 ± 0.1	12.7 ± 0.1
PUFA	59.9 ± 1.4	69.1 ± 0.0
I	2.6 ± 0.1	1.6 ± 0.0
I × 100/SFA	10.8 ± 0.9	8.8 ± 0.1

[a]Expressed as a percentage of total fatty acids. Values are means of two determinations ± SD. [b]Levels < 0.1%.

Lipid Class in the Digestive Gland. The different dietary regimens produced significantly different values of total lipid and PL contents in the three groups. The initial total lipid content (I) was unaffected by the algal diet but significantly decreased in the unfed mussels and significantly increased in mussels fed wheat germ flour (Table 3). Also, PL levels were significantly different in all groups at the end of the trial with respect to initial sampling. Values increased in unfed (A) and microalgal fed (B) groups but decreased in the C group. When PL were expressed as a percentage of wet weight, all changes disappeared.

Fatty Acid Composition of Total Lipids, Polar Lipids, and Neutral Lipids of the Digestive Gland. Dietary conditioning affected in a different way total lipid fatty acids of the three dietary groups (Table 4); while initial basal features of group I were approximately saved in groups A and B, fatty acids of group C were deeply affected by the wheat germ flour diet. The main significant and specific features that distinguished fatty acids of group C from the initial values were the following: (a) a drastic drop of essential long-chain PUFA 20:5ω-3 (from 14.1% of group I to 3.4%) and 22:6ω-3 (from 25.9 to 6.2%); (b) a slighter decrease in 20:4ω-6 (from 4.0 to 1.8%) and in total SFA (from 25.2 to 17.0); (c) 18:1ω-9 increased from 2.0 to 9.6%; 18:2ω-6 increased from 1.7 to 42.9%; and 18:3ω-3 increased from 1.9 to 5.6%; (d) the total PUFA ω-3 level was reduced by 65%, while the total PUFA ω-6 level increased substantially because of the very abundant presence of 18:2ω-6; and (e) while the NMID fatty acids, 20:2Δ5,11, 20:2Δ5,13, and 22:2Δ7,15, decreased, the NMIT fatty acids were not different from the initial value. The observed changes did not affect the UI probably because of the generous increase in 18:2ω-6, which counterbalances the parallel decrease in ω-3 unsaturated fatty acids. All of the described distinguishing aspects of group I from group C were shared by groups A and B.

The same fatty acid change pattern caused by diet C in total lipids was mirrored in polar lipid and neutral lipid fatty acids, with the peculiarity that these changes were attenuated in polar lipids and magnified in neutral lipids.

Fatty Acid Composition of PL Classes. The fatty acid pattern of PL classes was clearly influenced by dietary conditioning, and thus, after 30 days of treatment, a high number of fatty acids were found significantly changed (ANOVA; $p < 0.05$). We focused on the following selected fatty acids: (1) 22:6ω-3 and 20:5ω-3 given their metabolic significance and well-known to be crucial for bivalve development and membrane functioning; (2) 18:2ω-6, 20:2ω-6, and 20:4ω-6 given the close connection with diet C composition; (3) NMID and NMIT fatty acids, cumulatively defined as non-methylene-interrupted fatty acids (NMIFA), for the peculiarity of molecular structures and possible relationships with dietary precursors; and (4) finally, we offer a general evaluation on the observed response to the

Table 3. Total Lipid and PL Contents of the Digestive Gland at the Initial Sampling (I) and End of the Feeding Trial[a]

	I	A	B	C
total lipids (% wet weight)	3.4 ± 0.2	2.8 ± 0.2 b[b]	3.2 ± 0.1 c	5.2 ± 0.1 a[b]
phospholipids (% TL)	30.8 ± 0.1	38.9 ± 2.0 a[b]	36.2 ± 4.1 a[b]	20.2 ± 1.0 b[b]
phospholipids (% wet weight)	1.0 ± 0.1	1.1 ± 0.1 a	1.1 ± 0.1 a	1.0 ± 0.1 a

[a]A, unfed group; B, group fed *T. weissflogii*; C, group fed wheat germ flour. Values are means of four determinations ± SD. Data in one row followed by a different letter are significantly different (ANOVA; Student−Newman−Keuls' method; $p < 0.05$). [b]These values are significantly different from the I value (ANOVA; Dunnett's method; $p < 0.05$).

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Table 4. Fatty Acid Composition (wt % of Total Fatty Acids) of Total Lipids, Neutral Lipids, and Polar Lipids at the Initial Sampling (I) and End of the Feeding Trial[a]

	total lipids				neutral lipids				polar lipids			
fatty acid	I	A	B	C	I	A	B	C	I	A	B	C
14:0	2.4 ± 0.2	1.1 ± 0.2 a[b]	1.3 ± 0.1 a[b]	0.4 ± 0.1 b[b]	3.7 ± 0.1	1.8 ± 0.1 c[b]	2.2 ± 0.1 a[b]	0.4 ± 0.1 b[b]	1.2 ± 0.1[c]	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
15:0	0.6 ± 0.1	0.6 ± 0.1 a	0.7 ± 0.1 a	0.3 ± 0.1 b[b]	0.7 ± 0.1	2.0 ± 0.1 a[b]	2.3 ± 0.1 a[b]	0.7 ± 0.2 b	0.6 ± 0.1	0.6 ± 0.1 a	0.5 ± 0.1 a	0.4 ± 0.1 a
16:0	15.8 ± 0.9	15.1 ± 1.0 a	16.0 ± 0.4 a	13.3 ± 0.4 b[b]	17.9 ± 1.1	17.4 ± 1.3 a	17.7 ± 0.8 a	13.1 ± 0.4 b[b]	15.4 ± 0.1	14.2 ± 0.9 a	14.6 ± 0.8 a	15.0 ± 0.4 a
17:0 iso	0.6 ± 0.1	0.8 ± 0.1 a	0.8 ± 0.1 a	0.7 ± 0.1 a	0.6 ± 0.1	0.9 ± 0.1 a[b]	0.9 ± 0.1 a[b]	0.5 ± 0.1 b	0.6 ± 0.1	0.9 ± 0.1 a[b]	0.9 ± 0.1 a[b]	1.0 ± 0.1 a[b]
17:0	2.0 ± 0.1[c]	2.3 ± 0.1	2.3 ± 0.1	0.8 ± 0.1	1.8 ± 0.1	1.3 ± 0.1 a[b]	1.3 ± 0.1 a[b]	0.3 ± 0.1 b[b]	2.9 ± 0.1	3.1 ± 0.2 a	2.9 ± 0.1 a	2.1 ± 0.1 b[b]
18:0	3.8 ± 0.1	4.0 ± 0.2 a	3.6 ± 0.2 c	1.6 ± 0.1 b[b]	4.5 ± 0.2	4.0 ± 0.4 a	4.0 ± 0.4 a	1.5 ± 0.1 b[b]	4.9 ± 0.1	5.2 ± 0.4 a	4.3 ± 0.2 b[b]	3.9 ± 0.2 b[b]
total SFA	25.2 ± 0.7	24.0 ± 0.9 a	24.6 ± 0.5 a	17.0 ± 0.3 b[b]	29.2 ± 1.0	27.4 ± 1.2 a	28.3 ± 0.8 a	16.5 ± 0.4 b[b]	25.5 ± 0.1	24.7 ± 1.2 a	23.9 ± 0.8 a	22.8 ± 0.5 a[b]
16:1ω-7	3.3 ± 0.3	2.7 ± 0.5 c	3.8 ± 0.1 a	1.2 ± 0.1 b[b]	5.0 ± 0.3	3.7 ± 0.3 c[b]	6.0 ± 0.4 a[b]	1.5 ± 0.1 b[b]	1.1 ± 0.1[c]	0.9 ± 0.2 c	1.4 ± 0.1 a	0.6 ± 0.1 b
18:1ω-9	2.0 ± 0.1	1.8 ± 0.1 b	1.7 ± 0.1 b[b]	9.6 ± 0.5 a[b]	2.4 ± 0.1	1.9 ± 0.1 b[b]	1.9 ± 0.3 b[b]	10.5 ± 0.3 a[b]	0.8 ± 0.1	0.8 ± 0.1 b	0.7 ± 0.1 b	3.6 ± 0.1 a[b]
18:1ω-7	2.3 ± 0.1	2.1 ± 0.1 c	2.7 ± 0.1 a[b]	1.9 ± 0.1 b[b]	2.6 ± 0.1	3.1 ± 0.2 a[b]	3.3 ± 0.3 a[b]	1.8 ± 0.1 b[b]	1.8 ± 0.2	1.3 ± 0.1 b[b]	1.8 ± 0.1 a	1.4 ± 0.1 b[b]
20:1ω-11	2.2 ± 0.1	2.3 ± 0.1 a	2.0 ± 0.1 c	0.7 ± 0.1 b[b]	1.5 ± 0.1	0.9 ± 0.1 a[b]	0.8 ± 0.2 a[b]	0.3 ± 0.1 b[b]	4.6 ± 0.3	3.9 ± 0.1[b]	3.9 ± 0.2[b]	2.8 ± 0.2[b]
20:1ω-9	2.4 ± 0.1	2.9 ± 0.1 a[b]	2.5 ± 0.1 c	2.0 ± 0.1 b[b]	2.4 ± 0.1	3.1 ± 0.4 a[b]	2.5 ± 0.2 c	1.9 ± 0.2 b[b]	3.0 ± 0.2	4.0 ± 0.5 a[b]	3.8 ± 0.1 a[b]	4.3 ± 0.2 a[b]
20:1ω-7	0.7 ± 0.1	1.0 ± 0.1 a[b]	0.9 ± 0.1 a	0.2 ± 0.1 b[b]	1.0 ± 0.1	1.0 ± 0.1 a	1.0 ± 0.1 a	0.3 ± 0.1 b[b]	0.6 ± 0.1	1.3 ± 0.2 a[b]	1.0 ± 0.1 c[b]	0.5 ± 0.1 b
total MUFA	12.8 ± 0.5	12.8 ± 0.5 b	13.6 ± 0.2 c	15.7 ± 0.4 a[b]	14.9 ± 0.2	13.7 ± 0.2 b[b]	15.5 ± 0.5 a	16.2 ± 0.3 a[b]	12.0 ± 0.6	12.2 ± 0.2 b	12.6 ± 0.2 b	13.2 ± 0.1 a[b]
20:2Δ5,11	3.2 ± 0.1	4.2 ± 0.3 a[b]	3.5 ± 0.1 c	1.8 ± 0.2 b[b]	1.4 ± 0.1	1.9 ± 0.1 a[b]	1.3 ± 0.1 c	0.8 ± 0.1 b[b]	5.5 ± 0.1	8.8 ± 0.4 a[b]	6.9 ± 0.5 b[b]	7.0 ± 0.1 b[b]
20:2Δ5,13	0.8 ± 0.2	0.8 ± 0.1 a	0.5 ± 0.1 c	0.2 ± 0.1 b[b]	0.3 ± 0.1	0.9 ± 0.1 a[b]	0.4 ± 0.1 b	0.2 ± 0.1 b	0.5 ± 0.1	1.1 ± 0.2 a[b]	0.8 ± 0.3 b[b]	0.6 ± 0.1 b
22:2Δ7,13	0.5 ± 0.1	0.6 ± 0.1 a	0.5 ± 0.1 a	0.3 ± 0.1 b	0.4 ± 0.1	0.3 ± 0.1 a	0.2 ± 0.1 a	0.2 ± 0.1 a	0.6 ± 0.1	0.7 ± 0.1 ab	0.6 ± 0.1 b	0.9 ± 0.1 a[b]
22:2Δ7,15	2.9 ± 0.2	3.7 ± 0.4 a[b]	3.4 ± 0.1 a[b]	1.1 ± 0.1 b[b]	1.7 ± 0.1	2.5 ± 0.1 a[b]	1.8 ± 0.2 c	0.6 ± 0.1 b[b]	4.5 ± 0.2	5.3 ± 0.1 a[b]	5.2 ± 0.1 a[b]	3.8 ± 0.1 b[b]
total NMID	7.3 ± 0.5	9.2 ± 0.6 a[b]	7.9 ± 0.2 c	3.5 ± 0.4 b[b]	3.8 ± 0.2	5.5 ± 0.3 a[b]	3.8 ± 0.3 c	1.7 ± 0.1 b[b]	11.1 ± 0.3	15.9 ± 0.6 a[b]	13.6 ± 0.5 c[b]	12.3 ± 0.3 b[b]
20:3Δ5,11,14	0.4 ± 0.1	0.5 ± 0.1 a	0.4 ± 0.1 a	0.6 ± 0.1 a	0.3 ± 0.1	0.4 ± 0.1 a	0.3 ± 0.1 a	0.5 ± 0.1 a	0.4 ± 0.1	0.6 ± 0.1 b	0.5 ± 0.1 b	1.2 ± 0.1 a[b]
22:3Δ7,13,16	1.1 ± 0.1	1.3 ± 0.1 a[b]	1.3 ± 0.1 a[b]	0.8 ± 0.1 b[b]	0.9 ± 0.1	1.3 ± 0.1 a	1.2 ± 0.1 a[b]	0.7 ± 0.1 b	1.4 ± 0.1	1.5 ± 0.1 b	1.6 ± 0.1 b	2.3 ± 0.1 a[b]
total NMIT	1.4 ± 0.1	1.7 ± 0.1 a[b]	1.7 ± 0.1 a[b]	1.4 ± 0.2 b	1.2 ± 0.1	1.7 ± 0.2 a[b]	1.5 ± 0.1 a[b]	1.2 ± 0.1 b	1.8 ± 0.1	2.1 ± 0.1 b[b]	2.1 ± 0.2 b[b]	3.5 ± 0.1 a[b]
18:2ω-6	1.7 ± 0.2	1.6 ± 0.2 b	1.7 ± 0.1 b	42.9 ± 1.4 a[b]	2.2 ± 0.1	1.9 ± 0.1 b[b]	2.1 ± 0.1 c	46.9 ± 0.7 a[b]	0.9 ± 0.1	0.7 ± 0.1 b	0.9 ± 0.1 b	17.2 ± 0.5 a[b]
20:2ω-6	0.5 ± 0.1	0.5 ± 0.1 b	0.6 ± 0.1 b	1.1 ± 0.1 a[b]	0.5 ± 0.1	0.6 ± 0.1 b	0.5 ± 0.1 b	1.1 ± 0.1 a[b]	0.4 ± 0.1	0.4 ± 0.1 b	0.4 ± 0.1 b	1.3 ± 0.1 a[b]
20:4ω-6	4.0 ± 0.3	5.3 ± 0.6 a[b]	5.2 ± 0.2 a[b]	1.8 ± 0.2 b[b]	2.3 ± 0.3	4.3 ± 0.7 a[b]	3.6 ± 0.6 a[b]	1.3 ± 0.1 b[b]	6.8 ± 0.1	8.5 ± 0.7 a[b]	8.5 ± 0.1 a[b]	5.9 ± 0.2 b[b]
22:4ω-6	0.5 ± 0.1	0.6 ± 0.1 a	0.5 ± 0.1 a	0.3 ± 0.1 a	0.3 ± 0.1	0.5 ± 0.1 a	0.5 ± 0.1 a	0.2 ± 0.1 b	0.7 ± 0.1	0.9 ± 0.1 a	0.8 ± 0.1 a	0.6 ± 0.1 b
total ω-6 PUFA	6.8 ± 0.1	8.1 ± 0.5 b[b]	8.1 ± 0.2 b[b]	46.2 ± 1.3 a[b]	5.3 ± 0.3	7.3 ± 0.7 b[b]	6.8 ± 0.5 b[b]	49.5 ± 0.8 a[b]	8.9 ± 0.1	10.4 ± 0.6 b[b]	10.7 ± 0.1 b[b]	25.0 ± 0.5 a[b]
18:3ω-3	1.9 ± 0.2	1.3 ± 0.1 c[b]	1.1 ± 0.1 b[b]	5.6 ± 0.1 a[b]	1.8 ± 0.1	1.5 ± 0.1 c[b]	1.3 ± 0.1 b[b]	6.3 ± 0.1 a[b]	0.5 ± 0.1	0.7 ± 0.1 b[b]	1.1 ± 0.1 c[b]	2.1 ± 0.1 a[b]
18:4ω-3	1.9 ± 0.1	1.4 ± 0.1 a[b]	1.4 ± 0.1 a[b]	0.3 ± 0.1 b[b]	2.5 ± 0.1	1.6 ± 0.1 a[b]	1.7 ± 0.2 a[b]	0.3 ± 0.1 b[b]	1.6 ± 0.1	1.2 ± 0.1 a[b]	1.2 ± 0.2 a[b]	0.8 ± 0.1 b[b]
20:5ω-3	14.1 ± 0.3	13.1 ± 0.6 c[b]	15.2 ± 0.4 a[b]	3.4 ± 0.4 b[b]	13.1 ± 0.2	12.8 ± 0.6 c	14.8 ± 0.8 a[b]	2.6 ± 0.2 b[b]	14.0 ± 0.2	12.4 ± 0.5 c[b]	14.7 ± 0.3 a[b]	7.8 ± 0.3 b[b]
22:4ω-3	1.1 ± 0.1	1.2 ± 0.1 a	1.1 ± 0.1 a	0.4 ± 0.1 b[b]	0.9 ± 0.1	1.0 ± 0.1 a	1.0 ± 0.1 a	0.3 ± 0.1 b[b]	1.3 ± 0.1	1.3 ± 0.1 a	1.3 ± 0.1 a	1.0 ± 0.1 b[b]
22:5ω-3	1.3 ± 0.1	1.5 ± 0.1 a	1.4 ± 0.1 a	0.4 ± 0.1 b[b]	1.2 ± 0.1	1.3 ± 0.2 a	1.2 ± 0.2 a	0.4 ± 0.1 b[b]	1.3 ± 0.1	1.4 ± 0.1 a	1.3 ± 0.2 a	0.9 ± 0.1 b[b]
22:6ω-3	25.9 ± 0.3	25.7 ± 0.5 a	23.9 ± 0.4 a	6.2 ± 1.1 b[b]	26.0 ± 0.4	26.1 ± 1.0 a	24.2 ± 1.1 a	5.2 ± 0.6 b[b]	21.9 ± 0.3	17.6 ± 0.2 a[b]	17.5 ± 0.8 a[b]	10.5 ± 0.4 b[b]
total ω-3 PUFA	46.3 ± 0.5	44.2 ± 0.3 a[b]	44.1 ± 0.6 a[b]	16.3 ± 1.5 b[b]	45.6 ± 0.8	44.4 ± 0.6 a	44.1 ± 1.3 a	15.0 ± 1.0 b[b]	40.7 ± 0.4	34.7 ± 0.8 c[b]	37.2 ± 1.4 a[b]	23.2 ± 0.7 b[b]
total PUFA	61.9 ± 1.3	63.2 ± 0.9 b	61.8 ± 0.6 b	67.3 ± 0.7 a[b]	55.9 ± 1.2	58.9 ± 1.2 c[b]	56.2 ± 0.9 b	67.3 ± 0.4 a[b]	62.5 ± 0.7	63.1 ± 1.3 a	63.6 ± 0.8 a	63.9 ± 0.5 a
UI[d]	12.1 ± 0.5	12.8 ± 0.6 a	12.3 ± 0.4 a	11.7 ± 0.6 a	9.9 ± 0.5	10.8 ± 0.7 ab	10.2 ± 0.4 b	11.6 ± 0.3 a[b]	11.5 ± 0.2	11.3 ± 0.7 a	12.0 ± 0.7 a	10.2 ± 0.4 b

[a]A, unfed group; B, group fed *T. weissflogii*; and C, group fed wheat germ flour. Values are means ± SD; $n = 4$. Different letters in a row indicate significant differences among dietary treatments (ANOVA; Student–Newman–Keuls' method; $p < 0.05$). [b]These values are significantly different from the corresponding initial sampling value (ANOVA; Dunnett's method; $p < 0.05$). [c]These I values as well as data without a letter are excluded from statistical analysis because they cause violations against the assumption of homogeneity of variances. [d]UI = unsaturation index.

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experimental conditions of total ω-3 and ω-6 PUFA, NMIFA, MUFA, and SFA in PL classes. Thus, in the following sections, we will offer details on the specific hypothesized incorporation or synthesis of the above listed main groups of selected fatty acids according to dietary regimens.

Effect of the Dietary Regimen on 20:5ω-3 and 22:6ω-3 Distribution in PL Classes. Starvation and microalgal diet (Figure 1) nearly unaffected the 20:5ω-3 level of the considered

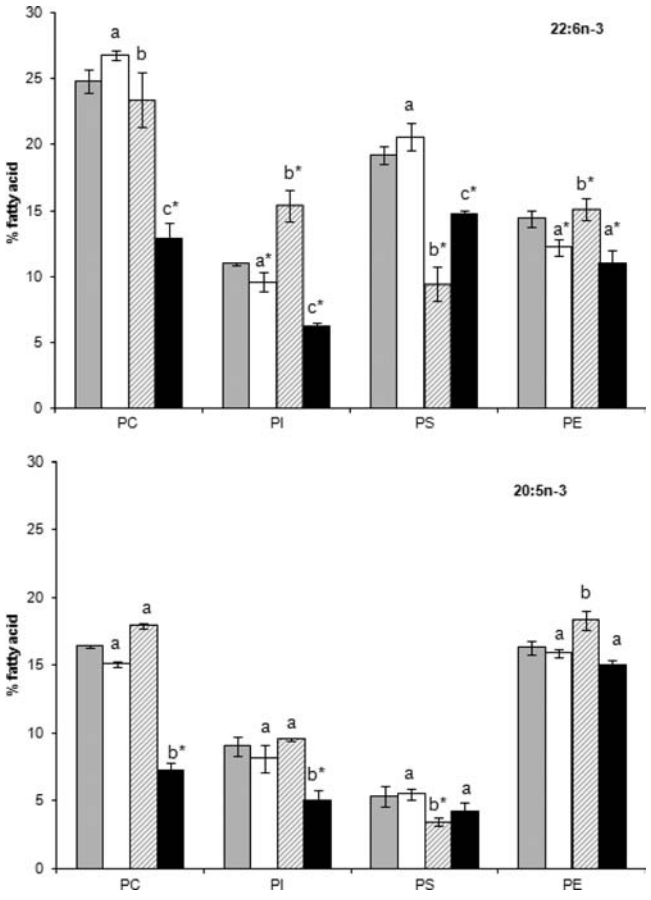

Figure 1. Distribution of 20:5ω-3 and 22:6ω-3 fatty acids in phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylethanolamine (PE) of the digestive gland. I, gray bars; A, white bars; B, hatched bars; and C, black bars. Values are means ± SD; n = 4. Values followed by an asterisk are significantly different from the corresponding initial time value (I) (ANOVA; Dunnett's method; $p < 0.05$). Different letters indicate significant differences between dietary treatments (ANOVA; Student–Newman–Keuls' method; $p < 0.05$).

PL classes; only PS and PE percentages of group B were significantly different from A and C groups. The 20:5ω-3 level of PS was also lower than the initial value. On the contrary, the same two dietary regimens were more effective in modulating 22:6ω-3 distribution; apart from levels of 22:6ω-3 in PC, differences among the two dietary groups and/or the initial value were almost always present.

Considerable changes were observed in group C, where percentages of both the long-chain PUFA 20:5ω-3 and 22:6ω-3 were constantly and significantly lower with respect to initial values in almost all of the PL classes considered, excluding 20:5ω-3 in PE and PS apparently unaffected by the C diet.

Effect of the Dietary Regimen on 18:2ω-6, 20:2ω-6, and 20:4ω-6 Distribution in PL Classes. Initial proportions of 18:2ω-6 and 20:2ω-6 were generally maintained in all of the PL classes of groups A and B and conversely extraordinary increased in group C with respect to both initial values and those of groups A and B (Figure 2). Although 20:2ω-6 was not

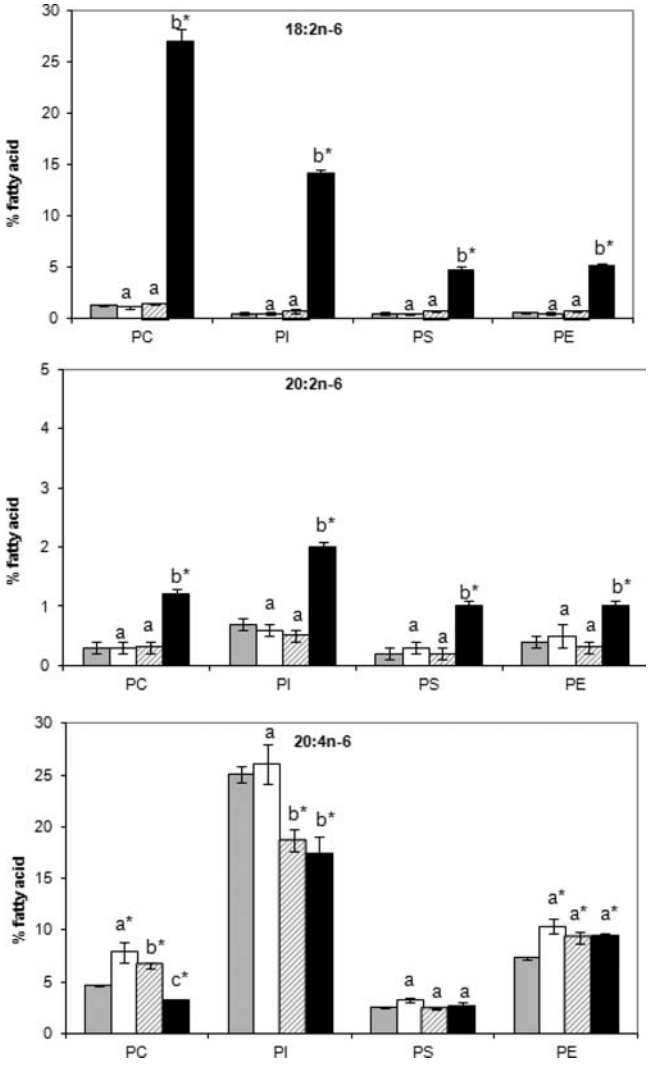

Figure 2. Distribution of 18:2ω-6, 20:2ω-6, and 20:4ω-6 fatty acids in phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylethanolamine (PE) of the digestive gland. I, gray bars; A, white bars; B, hatched bars; and C, black bars. Values are means ± SD; n = 4. Values followed by an asterisk are significantly different from the corresponding initial time value (I) (ANOVA; Dunnett's method; $p < 0.05$). Different letters indicate significant differences between dietary treatments (ANOVA; Student–Newman–Keuls' method; $p < 0.05$).

detected in the C diet, it increased other than in all of the lipid classes and also in all of the PL classes of group C. However, the most serious variations in group C involved the content of 18:2ω-6, which increased more than 20 times with respect to the values of all other groups in PC and PI and more than 10 times in PS and PE.

The change pattern in 20:4ω-6 levels was very complex: in PC, the 20:4ω-6 level of the three groups was significantly different among them and with respect to I values; in PI, the observed decrease with respect to group I involved only the

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groups B and C; in PS, no changes were perceivable; and in PE, similar values were exhibited by all experimental groups, all significantly higher than the initial values.

Effect of the Dietary Regimen on NMID and NMIT Fatty Acid Distribution in PL Classes. The results related to NMI fatty acids are shown in Figure 3. NMID fatty acids represented

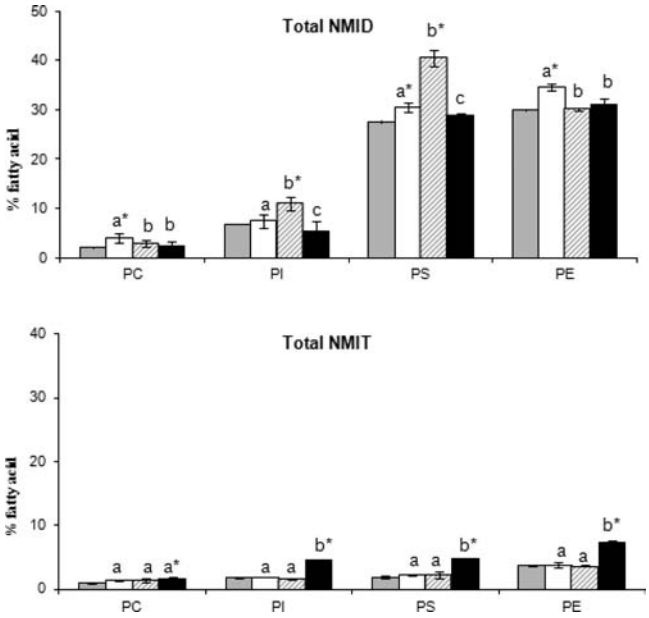

Figure 3. Distribution of total NMID and total NMIT fatty acids in phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylethanolamine (PE) of the digestive gland. I, gray bars; A, white bars; B, hatched bars; and C, black bars. Values are means ± SD; $n = 4$. Values followed by an asterisk are significantly different from the corresponding initial time value (I) (ANOVA; Dunnett's method; $p < 0.05$). Different letters indicate significant differences between dietary treatments (ANOVA; Student–Newman–Keuls' method; $p < 0.05$).

a quantitatively limited fraction of PC and PI, with values never higher than 10%. On the contrary, they accounted for valuable proportions of the total fatty acids in PS and PE, where the most representative were 22:2Δ7,15 and 20:2Δ5,11, respectively, whose initial content amounted to 22% and 18.1%, respectively. At the end of the trial, NMID fatty acid percentages higher than initial values were exhibited in PC, PS, and PE by group A and in PI and PS by group B, while group C was always not significantly different from the initial value.

The NMIT fatty acids, 20:3Δ5,11,14 and 22:3Δ7,13,16, accounted for a small proportion of total fatty acids. While 20:3Δ5,11,14 was preferentially selected in PI, followed by PE, 22:3Δ7,13,16 concentrated in PS and PE. NMIT significantly increased in all PL classes of group C, with values significantly higher with respect to the initial values and also to those of groups A and B.

Effect of the Dietary Regimen on Total SFA, Total MUFA, and Total ω-3 and ω-6 PUFA Distribution in PL Classes. Among PL, PE was the most unsaturated class because of the combination of high total PUFA ω-3 and low MUFA and SFA contents. This feature is shared by all groups. A and B dietary regimens rarely affected total ω-3 and ω-6 levels of the various PL classes, which were only occasionally significantly different from the initial values (Figure 4). On the contrary, diet C deeply affected total ω-3 and ω-6 fatty acid amounts, which were always in all of the PL classes significantly different from the initial values, with an opposite trend. While total PUFA ω-3 levels were always reduced by diet C, PUFA ω-6 values were increased.

Total SFA and MUFA showed an overall trend approximately opposite that of total PUFA and reciprocally almost compensatory (when MUFAs decreased, SFAs remained constant and vice versa).

DISCUSSION

Bivalve fatty acid composition of lipid and PL classes results from different simultaneous factors, such as environmental temperature, physiological state, food availability, and composition. According to a previous paper[7] and to several studies on different bivalve species that have reported that dietary treatments can seriously affect tissue fatty acid composition,[30−34] results indicate that, also in the mussel digestive gland, this statement appears self-evident. The mussel digestive gland is the first part of the body to receive the food and the site of digestion. Thus, changes of fatty acid compositions will be very fast in the digestive gland when the food sources are altered[35] or when variegated conditions, such as nutrition, age, reproduction, and stress, are changing.[36]

Results indicated that mussels were able to consume both of the provided diets. Consumption and assimilation of the food source could be traced using fatty acid composition, which is a very useful method for studying the trophic sources used by marine organisms.[15] Homeostatic skills of adult mussels helped to partially control changes, and thus, while the fatty acid composition of both groups A and B were only for some aspects influenced by dietary regimens, that of the group C was totally and deeply touched by the diet. As a matter of fact, the wheat germ diet (group C), a very severe and non-natural dietary regimen, deeply modified the digestive gland fatty acid composition in all lipid and PL classes examined, thus confirming previous results on the whole soft body.[7] In polar lipids, usually considered more conservative with respect to neutral lipids,[34] the diet C effect was minimized but constantly present. On the contrary, in neutral lipids, more receptive to external inputs and scarcely capable of adequately selecting dietary fatty acids, the wheat germ diet fatty acid features were amplified and the fatty acid profiles of the digestive gland shifted toward those of the correspondent diet.

The starvation effect on fatty acid composition was almost coincident with that of diet B, confirming the high skill of adult mussels to face stress conditions and the preference for carbohydrates in dietary emergency.[37] The selective increase in total NMID, NMIT, and total ω-6 PUFA in starved mussels, mirroring the increased level of 20:2Δ5,11, 22:3Δ7,13,16, and 20:4ω-6, respectively, confirms the changes in NMI fatty acid levels in other starved bivalves.[38] In the absence of exogenous PUFA supply, such changes were related to deficiencies of essential fatty acids related to the absence in bivalves of the Δ6 desaturase enzymatic system,[7] which allows for the biosynthesis of long-chain PUFAs. The active presence in bivalves of Δ5 desaturation and elongation systems allows for the *de novo* synthesis of NMID[9] and NMIT fatty acids,[36] which can adequately substitute for long-chain PUFA by ensuring suitable physical features to membrane lipids for optimal molecular mobility and activity of membrane-bound proteins.[38,39] On the other hand, a contribution to the NMI increase because of their relative metabolic inertia with respect to conventional fatty acids cannot be excluded.[38] Barton and Gunstone[40] showed

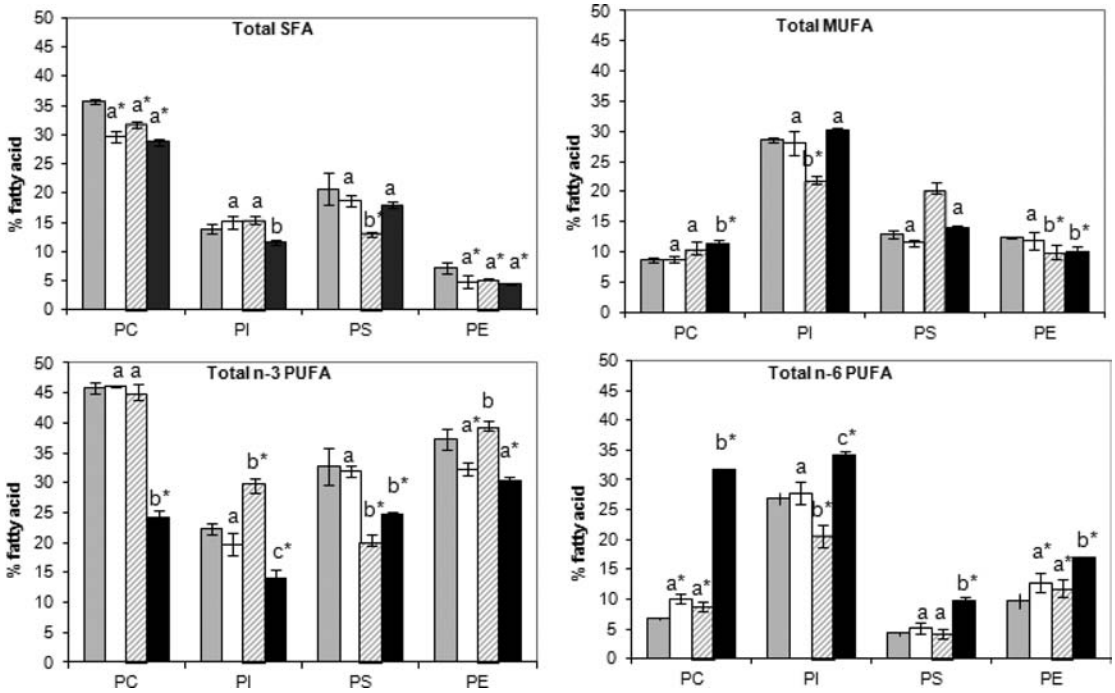

Figure 4. Distribution of total ω-3 PUFA, ω-6 PUFA, MUFA, and SFA in phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylethanolamine (PE) of the digestive gland. I, gray bars; A, white bars; B, hatched bars; and C, black bars. Values are means ± SD; $n = 4$. Values followed by an asterisk are significantly different from the corresponding initial time value (I) (ANOVA; Dunnett's method; $p < 0.05$). Different letters indicate significant differences between dietary treatments (ANOVA; Student−Newman−Keuls' method; $p < 0.05$).

that, among several PUFA isomers, the lowest melting point was related to the more central position of the double bonds. Consequently, synthesis of NMI fatty acids by mollusks could be a means to restore a suitable membrane fluidity to respond to homeoviscosity requirements at low temperatures. The very high level of these compounds in the aminophospholipids PE and PS, which are primarily localized in the inner layer of the membrane and involved in peculiar membrane properties,[41] appears to confirm the suggested important role.

The 20:4ω-6 increase under non-feeding conditions can be a result of selective retention related to the well-known role in the synthesis of the series 2 prostaglandins.[7,30]

Even if mussels can ingest mesozooplankton and also assimilate nutrients from zooplankton in their tissues,[35] they are traditionally assumed to mainly consume phytoplankton. On this basis, the monoalgal diet, similar to natural food, did not substantially influence the fatty acid composition of lipid and PL classes. The very high level of the typical diatom fatty acids, 16:1ω-7, 16:3ω-4, and 20:5ω-3, in the provided diet was not completely transferred in digestive gland fatty acid composition. Possibly, in mussels, as in other bivalves,[14,42] a similar independence from diets of fatty acid composition of tissues could depend upon species-specific metabolic features capable of modulating the accumulation of specific fatty acids when dietary content exceeds animal requirement. These findings suggest that fatty acid composition of the mussel digestive gland is also species-modulated other than diet-oriented. Different metabolic transformations can be involved as chain-elongation,[43] unsaturation, deacylation−reacylation processes to build suitable lipid molecules.

In contrast with all other groups, the fatty acid pattern of the digestive gland in group C appeared clearly shifted toward dietary fatty acids, with the level of 18:2ω-6 even higher with respect that of the whole soft body.[7] This enhanced dietary effect can be related to the double function of the digestive gland as a site for early storage and for the later delivery of metabolic reserves to the different tissues.

Previous reports[6,7] provided evidence of a direct correlation between dietary 18:2ω-6 content and tissue accumulation of NMI fatty acids. The high content of 18:2ω-6 and 18:1ω-9 and the shortage of long-chain PUFA in the diet of group C, all conditions potentially capable of enhancing NMI fatty acid biosynthesis, clearly enhanced NMIT biosynthesis in polar lipids and in all of the PL classes considered, particularly in PI, PS, and PE, without activating NMID fatty acid biosynthesis. This selective increase in NMIT fatty acids, which are absent in the wheat germ diet, confirmed that while mollusks lack Δ6 desaturase to synthesize the traditional ω-3 and ω-6 long-chain PUFA, they have active fatty acid elongation and desaturation systems permitting the *de novo* synthesis of NMIT fatty acid, selectively accumulated in polar lipids.[7] The identification of factors capable of activating NMI biosynthesis can play an important role in increasing the nutraceutical potential of mussel fatty acids, which, as reported in rats, contain biologically significant anti-inflammatory activity without observable adverse side effects.[10]

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Notes

The authors declare no competing financial interest.

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