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Fatty acid composition in *Mytilus galloprovincialis* organs: trophic interactions, sexual differences and differential anatomical distribution

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ABSTRACT: The fatty acid (FA) profiles in neutral lipids (NLs) and polar lipids (PLs) of the gills, digestive gland and mantle of female and male mussels *Mytilus galloprovincialis* (cultured in Ría de Arousa, Galicia, NW Spain, and studied during an annual seasonal cycle) reflected dietary, sexual and functional differences. Seasonal differences arose from variations in the trophic environment, with diatom FA biomarkers dominating during the spring bloom, biomarkers from dinoflagellate-rich diets dominating during winter mixing, and a combination of both sources apparent during the summer and autumn transitory periods. The influence of diet was more pronounced in the NL (energetic reserves) than in the PL (structural membrane components) FA signatures of all organs, although dietary changes were mirrored more clearly in the digestive gland. Differences between males and females were restricted to the mantle and probably resulted from gonadal ripeness during spring. The NL and PL FA profile of the mantle was characterized by the abundance of 14:0, 16:0 and 16:1n-7 in females and predominance of 18:0, 18:3n-3 and 22:6n-3 in males, suggesting that each gender has distinct energetic and structural requirements during gametogenesis. Lipids containing alkenyl ethers or non-methylene interrupted (NMI) FAs were mostly found in the PLs of the gills and the mantle, supporting their role as structural and functional components in the membranes of organs directly exposed to physicochemical fluctuations. Competition between NMI and polyunsaturated fatty acids (PUFAs) was only evident in the mantle, suggesting that deficiencies in PUFAs might provoke de novo biosynthesis of NMI to satisfy reproductive demands.

KEY WORDS: Mussels · Neutral and polar lipids · Non-methylene interrupted fatty acids · Biomarkers · Sexual variation · Bivalve organs

INTRODUCTION

The structure of the plankton community in coastal upwelling ecosystems is highly dynamic (Figueiras et al. 2002, Froján et al. 2014). The Galician Rías (NW Spain) are among these dynamic ecosystems, where from March to October, northerly winds cause coastal upwelling surges of cold nutrient-rich water that induce blooms of diatoms (Álvarez-Salgado et al. 2002). During the rest of the year, southerly winds induce downwelling episodes of warmer nutrient-poor waters, causing changes in the composition of the plankton community towards a dominance of dinoflagellates (Álvarez-Salgado et al. 2008, Froján et al. 2014). Diatoms and dinoflagellates contain a characteristic suite of fatty acids (FAs) that can be used as biomarkers to discern the trophic relationships between primary producers and bivalves (Kelly

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& Scheibling 2012, Parrish 2013). This range of biomarkers includes 20:5n-3 (eicosapentaenoic acid, EPA) and certain C16 FAs, which are very abundant in most diatoms, while 22:6n-3 (docosahexaenoic acid, DHA) and certain C18 FAs are the main dinoflagellate biomarkers (Sargent et al. 1988, Budge et al. 2001, Dalsgaard et al. 2003, Shin et al. 2008). Previous studies in a Galician Ría (Irisarri et al. 2014a), the Venezuelan Caribbean Sea (Freites et al. 2010) and the German North Sea (Pogoda et al. 2013) analyzed the total lipid (TL) FA composition of bivalves, and found enhancements in diatom-derived FAs during the spring bloom, while dinoflagellate biomarkers predominated during autumn and winter downwelling. Mussels have developed a series of feeding and biochemical strategies (Fernández-Reiriz et al. 1996, Freites et al. 2002a, Irisarri et al. 2014b) to cope with rapid changes in their feeding environment, which explains the ecological success and great production of mussels in the Galician Rías (Pérez-Camacho et al. 2013).

Lipids constitute the densest form of energy that can be transferred from primary producers to higher trophic levels in marine ecosystems (Parrish 2013). In bivalves, lipids comprise about 10% of dry weight (Pirini et al. 2007, Pogoda et al. 2013). Neutral lipids (NLs) are the most abundant and include triglycerides, alkenyldiacylglycerides, wax esters, hydrocarbons, free FAs and sterols (Freites et al. 2002c, Hanus et al. 2009). Polar lipids (PLs) include glycolipids and mainly phospholipids (Freites et al. 2002c, Ventrella et al. 2008, Pogoda et al. 2013). Fatty aldehydes, derived from alkenylacyl glycerophospholipids, or 'plasmalogens,' are prominent components of the PLs in bivalves and are analyzed using their dimethyl acetal derivatives (DMA) (Joseph 1989, Kraffe et al. 2004, Hanus et al. 2009).

Several studies have reported the FA composition of the bulk tissues of bivalves (Fernández-Reiriz et al. 1996, Freites et al. 2002 a,b, Pirini et al. 2007, Pogoda et al. 2013). However, the FA composition of bivalves is tissue-specific, and the metabolism of NL and PL FAs differs with the physiological role of each organ (Klingensmith 1982, Turunen & Pekkarinen 1990, Caers et al. 1999, 2003, Kraffe et al. 2004, Shin et al. 2008, Ezgeta-Balić et al. 2012). PLs predominate in the gills (86–95% of TLs), with particularly high levels of non-methylene-interrupted (NMI) FAs (Delaporte et al. 2005, Ventrella et al. 2008). NMIs, both dienoic (NMID) and trienoic (NMIT), have unusual double bonds with more than 1 methylene group between ethylenic bonds (Zhukova 1991). In contrast, Martínez-Pita et al. (2012b) found that NLs predominated in the digestive gland (62–65% dry weight) and mantle (55–59% dry weight) of the mussel *Mytilus galloprovincialis* and decreased to sustain gametogenesis.

The lipid composition of tissues also differs markedly between males and females, as marine bivalves in temperate latitudes exhibit cyclic changes in reproductive stages as a consequence of the seasonality of environmental conditions (Soudant et al. 1996, Pérez-Camacho et al. 2003, Fernández-Reiriz et al. 2007). NLs accumulate in eggs and early larval stages, while PLs are synthesized to build gamete membranes (Labarta et al. 1999, Narváez et al. 2008). The gonad and mantle of ripe females are richer in TLs compared to the equivalent male tissues (Soudant et al. 1996, Pérez-Camacho et al. 2003, Miller et al. 2014). Accordingly, variations in lipid classes are more pronounced in female than male clams conditioned at different temperatures (Fernández-Reiriz et al. 2007). Triglyceride boosts provide energy in mature female gonads, while high levels of phospholipids might be necessary for the spermatozoal membrane (Soudant et al. 1996, Martínez-Pita et al. 2012a,b). Under nutritive stress, females preferentially catabolize glycogen and proteins before lipids, presumably to save lipids for oocyte development (Pérez-Camacho et al. 2003, Albentosa et al. 2007).

The fluctuations in the composition of the diet and the reproductive cycle are among the main factors that affect the FA signature in bivalves (Freites et al. 2002a,b, 2010, Ventrella et al. 2008, 2013, Ezgeta-Balić et al. 2012, Hurtado et al. 2012, Irisarri et al. 2014a). Even if bivalves have some capability to modulate the synthesis of FAs independently of their diet (Ventrella et al. 2013, Aranda-Burgos et al. 2014), their capacity to biosynthesize de novo polyunsaturated FAs (PUFAs) is limited (Albentosa et al. 1996, Pirini et al. 2007, Fernández-Reiriz et al. 2011), so they must acquire most PUFAs from filtering phytoplankton, which can contain 50% of their weight as n-3 PUFAs (Sargent et al. 1988).

In this study, we investigated variations in NL and PL FA composition in 3 different tissues (i.e. gills, digestive gland and mantle) of female and male *M. galloprovincialis* during an annual seasonal cycle. We addressed 3 main hypotheses: (H_1) The NL and PL FA profiles of mussel tissues vary seasonally with the natural diet; (H_2) NL and PL FA signatures differ between females and males owing to different energetic and structural requirements during gamete development; and (H_3) the anatomical distribution of NL and PL FAs depends on the physiological role of each organ.

MATERIALS AND METHODS

Sample collection

Adult mussels *Mytilus galloprovincialis* (50–60 mm shell length) were obtained from a commercial mussel raft in the Ría Arousa (Galicia, NW Spain). Ría Arousa harbors the largest number of floating mussel rafts of the Galician Rías, with 2404 culture units that occupy 17% of the total surface of the embayment. Mussels were picked from the suspended ropes in a sufficient numbers to guarantee 5 female and 5 male replicate samples to assess any possible sexual differences in FA composition. Bivalves were sampled during winter (January), spring (March), summer (July) and autumn (October) of the year 2003, to study the seasonal differences in FA composition. Mussels were opened by cutting the posterior adductor muscle and the gills; the digestive gland and mantle were dissected from the rest of the tissues to study the premise that mussels have a different FA composition depending on the tissue. Mussels were sexed after microscopic examination of a small sample of the mantle (n = 10 per season). Organs were stored on ice and transferred to the laboratory for analyses. Samples were frozen at −50°C until lyophilization with a freeze-dryer (Ilshin Lab). The dry samples were stored at −20°C for a maximum of 15 d until further homogenization with an ultrasonic Branson Sonifier (250/450 USA).

Analytical methods

Lipids were extracted according to the method of Bligh & Dyer (1959) as modified by Fernández-Reiriz et al. (1989) as follows. Lipids were extracted with chloroform:methanol (1:2), and after centrifugation, the precipitate was extracted again with chloroform:methanol (2:1). Both supernatants were purified with chloroform:methanol:water (8:4:3) (Folch et al. 1957). The solvents contained 0.05% butylated hydroxytoluene as an antioxidant. TLs were colorimetrically quantified following the method described by Marsh & Weinstein (1966) with a tripalmitin standard (Sigma Aldrich). TLs were separated into NL and PL by a modification of the solid phase extraction procedure described by Kaluzny et al. (1985). Briefly, aliquots of lipid extracts containing 0.5 mg TLs were evaporated to dryness under a gentle stream of N_2, redissolved in 1 ml of chloroform and loaded onto 360 mg aminopropyl solid-phase extraction cartridges (Sep-Pak, Waters) that had been previously pre-washed with hexane. NLs were then eluted with 3 ml of chloroform/2-propanol (2:1) and PLs (including free FAs in this fraction) with 2 ml of methanol. The procedure was checked for purity of each lipid fraction employing lipid standards or alternative mussel extracts by thin layer chromatography as described by Freites et al. (2002c).

Each lipid fraction was evaporated to dryness, the residue was dissolved in 0.4 ml toluene, and the FAs and aldehydes from the TLs were converted to FA methyl esters (FAMEs) and DMA, respectively, with 1 ml of 1.5% sulfuric acid in methanol, according to Christie (1982). FAMEs were injected in a gas chromatograph (Perkin-Elmer, 8500) equipped with a flame ionization detector and a 30 m × 0.25 mm (0.20 µm phase thickness) silica capillary column (Supelco, SP-2330). Nitrogen was used as the carrier gas at a pressure of 0.069 Pa. The injector was programmed at 275°C and operated in solvent elimination mode (Medina et al. 1994). The column temperature increased from 140°C to 210°C at a rate of 1°C min^{-1}. FAMEs and DMA were identified by comparison and/or co-injection with analog samples characterized by GC/MS of both FAMEs and DMA or 4,4-dimethyloxazoline derivatives (Garrido & Medina 1994, 2002). Nonadecanoic acid was used as an internal standard and a response factor was determined for each FA for quantitative analysis employing quantitative FAME mixtures (Larodan). For those acids with no standard available (i.e. NMI), the response factor of the most similar FAME (in terms of C atoms and number of double bonds) was used. Results for each component were expressed as the relative percentage (%) of the total FAME or DMA content ± standard deviation.

Data analyses

We used multivariate ANOVA to test for differences in (1) seasonal variations in the NL and PL FA profile of 3 different organs (gills, digestive gland, mantle) (H_1), (2) NL and PL FA signatures between female and male tissues (H_2) and (3) differences in abundance of NL and PL FAs depending on the tissue (H_3). The data on the relative FA percentage of the samples was logarithmically (log [x + 1]) transformed and converted into a Bray-Curtis similarity matrix to start the multivariate analyses. First, we performed a 2-way ANOSIM to test whether samples within NL or PL classes clustered by sex or season. A non-metric multidimensional scaling (NMDS) plot was performed for the visualization of the

ANOSIM clusters. The separation of the seasonal or sexual groups was inferred from the R and p values of ANOSIM and the stress (Euclidean distance) of the NMDS ordination plot. R = 1, R = 0.5 and R = 0 indicate a perfect, satisfactory and poor separation of the clusters, respectively (Clarke & Gorley 2006). Stress levels <0.05, <0.1 and <0.2 correspond to an excellent, good and potentially useful representation of the data in the 2 dimensional plot, respectively (Clarke & Gorley 2006); samples with similar FA signatures clustered together in the plot. SIMPER was performed to calculate the average dissimilarity between group samples (i.e. seasons and sex) for each tissue and to identify the top 6 FAs contributing to within-group dissimilarity (Kelly & Scheibling 2012). Multivariate analyses were performed using PRIMER v.6.0 software (Clarke & Gorley 2006).

Differences in the principal NL and PL FA classes were tested using a 2-way ANOVA with sex and season as independent variables. ANOVA was performed for the specific FA that contributed to the highest sexual and seasonal differences identified by the SIMPER analyses. Assumptions of normality and homoscedasticity were checked with Shapiro-Wilk and Levene tests prior to the ANOVA. A ranked ANOVA (non-parametric) was performed when data did not fit these assumptions. ANOVA was followed by Tukey's HSD post hoc test. Univariate statistical analyses were computed with STATISTICA 7.0 software (StatSoft) on log-untransformed percentage values.

RESULTS

NL and PL FA profiles in gills: variations with season and sex (H_1 and H_2)

We detected 34 different FAs in the NLs and PLs of the gills (see Table S1 in the Supplement, available at www.int-res.com/articles/suppl/m528p221_supp.pdf). Gill NLs were most enriched in 16:0, EPA and 16:1n-7, while the PLs of the gills presented 16:0, DMA 18:0 and EPA among the most abundant components (Figs. 1 & 2). PUFAs and saturated FAs (SAFAs) were more abundant than monounsaturated FAs (MUFAs) in both lipid fractions (Figs. 1 & 2). DMA and NMI showed more than 2-fold greater levels in PLs than in NLs of the gills (ANOVA, $p < 0.05$, Fig. 2).

The results from the SIMPER analysis and the NMDS plot showed that the composition of the gills differed significantly throughout the seasons (Fig. 3, Table 1). Gill FA profiles in summer and autumn were characterized by significantly higher levels of diatom markers in both lipid fractions, while the diatom biomarker EPA showed significantly higher levels in spring in both lipid fractions (Fig. 4). Gill NLs and PLs showed significantly greater levels of the dinoflagellate-derived biomarker DHA in winter, summer and autumn (Fig. 4). Neutral and polar SAFAs and MUFAs were significantly higher in autumn–summer, while PUFAs prevailed in spring (ANOVA, $p < 0.001$). Polar NMIs in the gills were significantly higher in spring than in winter (ANOVA, $p < 0.05$; Table S1).

Gill FA signatures showed a high degree of similarity in female and male mussels, which was displayed by the overlap of the FA clusters plotted in the NMDS (Fig. 3). SIMPER confirmed that average sexual differences were small (Table 1), and FAs contributing to this dissimilarity showed statistically similar levels in both sexes (ANOVA, $p > 0.05$, Fig. 1).

NL and PL FA profiles in the digestive gland: variations with season and sex (H_1 and H_2)

We detected 35 different FAs in the NLs and PLs of the digestive gland. FAs 20:5n-3, 16:0 and 16:1n-7 were particularly high in the NLs of the digestive gland, while the most prevalent components in the PLs were 16:0, 20:5n-3 and DMA 18:0 (Table S2 in the Supplement; Figs. 1 & 2). PUFAs and SAFAs were followed by MUFAs in both lipid fractions (Figs. 1 & 2). DMA and NMI in PLs doubled the proportions observed for the NLs of the digestive gland (ANOVA, $p < 0.05$, Fig. 2).

The NL and PL FA profiles of the digestive gland formed 3 well-defined seasonal clusters in the NMDS plot: winter, spring and summer–autumn (Fig. 3). SIMPER indicated that differences in winter were attributable to significantly higher levels of the dinoflagellate marker DHA, while the diatom markers 14:0 and EPA were significantly higher in spring in both lipid fractions (ANOVA, $p < 0.001$, Figs. 1 & 4). Diatom and dinoflagellate markers were present in significant proportions in the NLs and PLs of the digestive gland during summer–autumn (Fig. 4). SAFAs and MUFAs in both NLs and PLs were higher in summer than winter, whereas PUFAs showed the opposite pattern (ANOVA, $p < 0.001$).

ANOSIM and the NMDS plot showed few sexual differences in the composition of the digestive gland (Fig. 3). The average sexual dissimilarity was small (SIMPER, Table 1), and FAs contributing to this dif-

ference showed similar levels in the NLs and PLs of female and male mussels (ANOVA, $p > 0.05$, Fig. 3).

FA profiles of NLs and PLs in the mantle: variations with season and sex (H_1 and H_2)

We detected 35 different FAs in the NLs and PLs of the mantle (Table S3 in the Supplement). The NLs of the mantle were characterized by high proportions of 16:0, 20:5n-3 and 16:1n-7, while the main components of PLs of the mantle were 16:0, 20:5n-3 and DMA 18:0. The PLs and NLs of the mantle had greater amounts of PUFAs than SAFAs and MUFAs (Fig. 2). DMA and NMI showed higher proportions in the PLs than in the NLs of the mantle (ANOVA, $p < 0.05$, Fig. 2).

The NMDS plot and ANOSIM results revealed 3 distinct seasonal groupings for the FA signatures of both lipid fractions: winter, spring and summer–autumn (Fig. 3). SIMPER indicated that the FAs influencing this seasonal dissimilarity in both lipid fractions were: greater levels of the dinoflagellate marker DHA and 18:4n-3 in winter, significantly higher levels of the diatom-associated markers 14:0 and EPA in spring, and high levels of diatom and dinoflagellate markers in summer–autumn (Figs. 1 & 4, ANOVA, $p < 0.001$). Neutral SAFAs and MUFAs in the mantle were highest in spring–summer than winter, while PUFAs showed an opposite storage pattern (ANOVA, $p < 0.001$). DMA and NMI peaked in spring and summer,

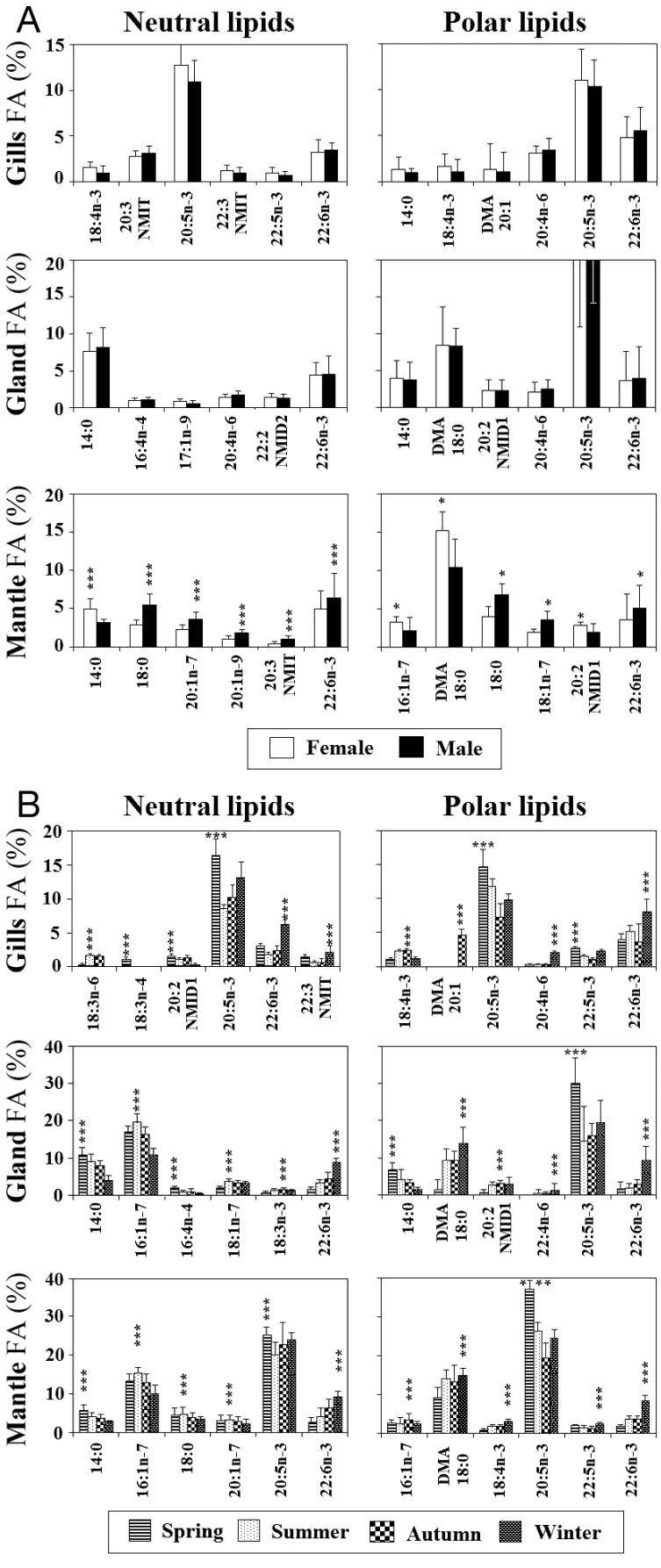

Fig. 1. Principal neutral lipid and polar lipid fatty acids (FAs) that contributed to the sexual and seasonal dissimilarities observed for the gills, digestive gland and mantle of mussels *Mytilus galloprovincialis*. (A) Mean ± SD obtained for each sex after 4 seasonal samplings (n = 20); (B) mean ± SD obtained for each season (n = 10). FAs that contributed to sexual and seasonal differences were identified by SIMPER analysis. Inter-sexual and inter-seasonal statistical differences are represented with ***$p < 0.001$, *$p < 0.05$ (2-way ANOVA). 20:2NMID1 = 20:2Δ5,11; 22:2NMID2 = 22:2Δ7,15; 20:3NMIT = 20:3Δ5,11,14; 22:3NMIT = 22:3Δ7,13,16; DMA: dimethyl acetal derivative FA

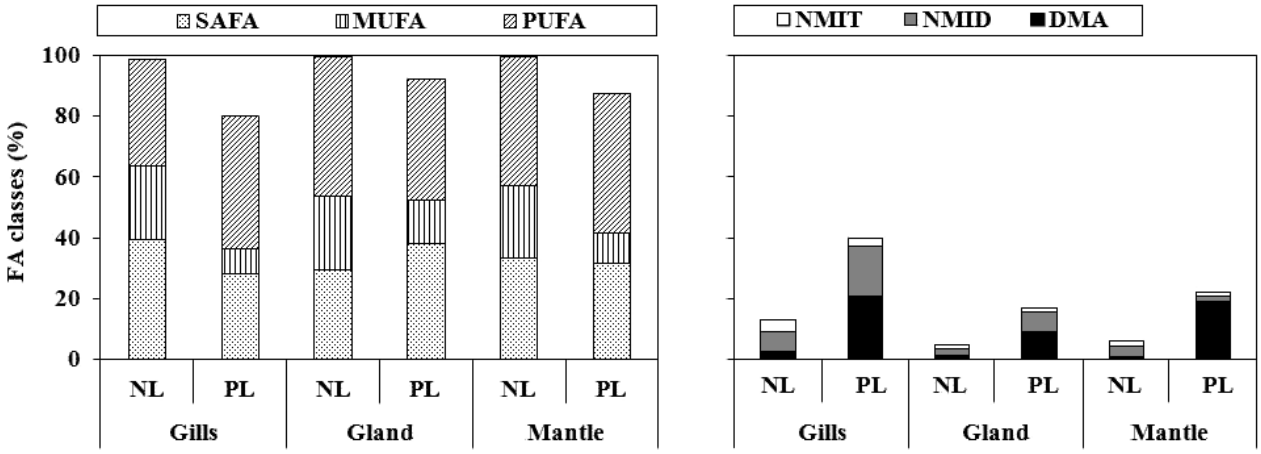

Fig. 2. Comparison of the average proportions of the principal neutral lipid (NL) and polar lipid (PL) fatty acid (FA) classes present in the gills, digestive gland, and mantle of female and male mussels *Mytilus galloprovincialis* during a complete seasonal cycle. SAFA: saturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA; NMID: dieonic non-methylene-interrupted FA; NMIT: trienoic non-methylene-interrupted FA; DMA: dimethyl acetal derivative FA

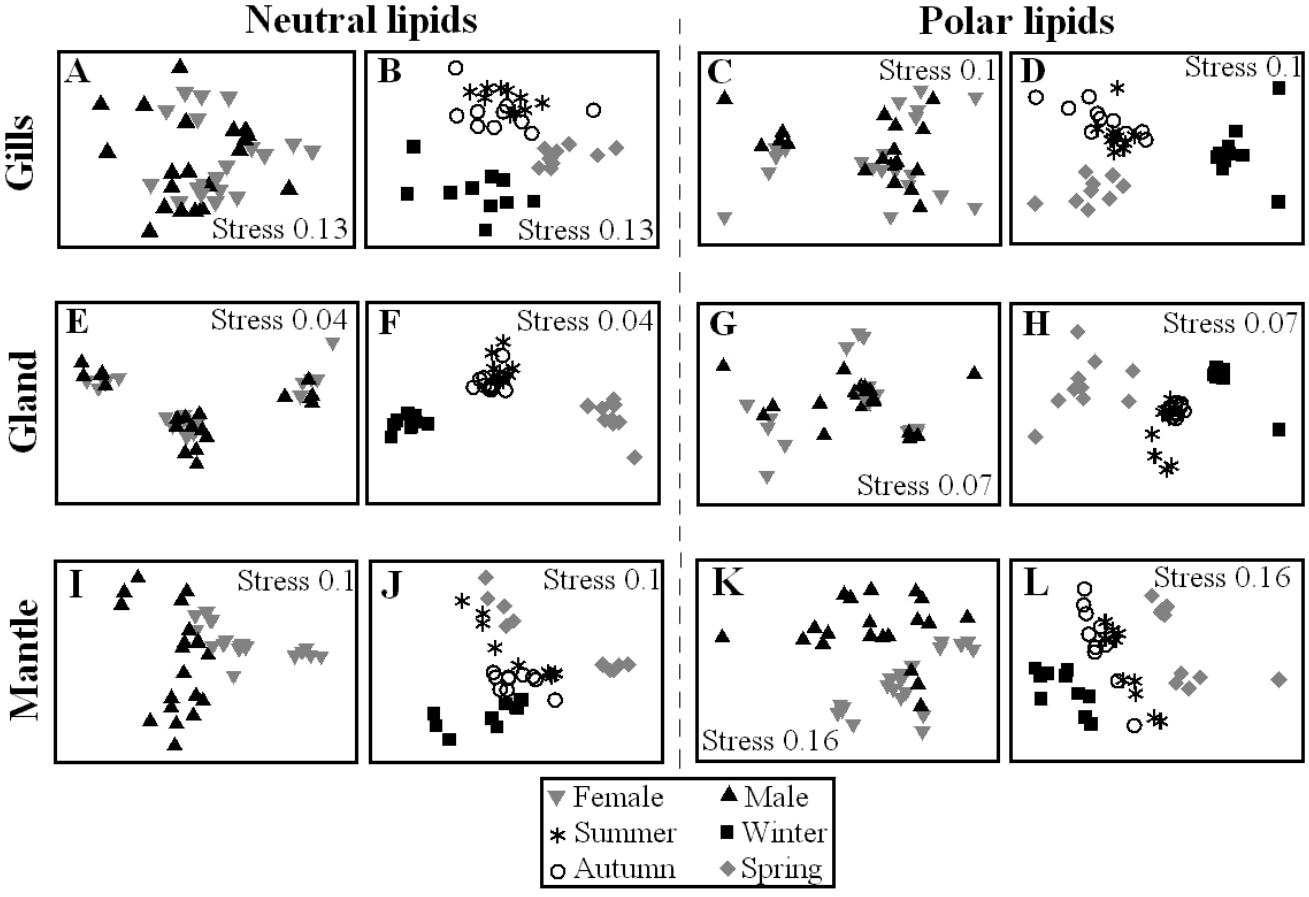

Fig. 3. Non-metric multi-dimensional scale plots (NMDS) indicating the spatial segregation found among the neutral and polar lipid fatty acid composition of the gills, digestive gland, and mantle of (A, E, I, C, G, K) female and (B, F, J, D, H, L) male *Mytilus galloprovincialis* and the seasonal variations in composition of the 3 tissues. Stress levels <0.05, <0.1 and <0.2 correspond to an excellent, good, and potentially useful representation, respectively

Table 1. Dissimilarity percentages and global R values obtained for sex and seasonal comparisons of neutral and polar lipid fatty acid (FA) signatures of gills, digestive gland and mantle of mussels *Mytilus galloprovincialis*. Dissimilarity percentages were calculated with SIMPER analyses to assess which FAs contributed to gender and seasonal differences. Global R and significance values (*p < 0.05, **p < 0.01, ***p < 0.001; ns: not significant) were obtained with 2-way ANOSIM. R = 1, R = 0.5, and R = 0 indicate a perfect, satisfactory and poor separation of samples, respectively

	Gills		Gland		Mantle	
	Neutral	Polar	Neutral	Polar	Neutral	Polar
Sex						
Female vs. male	10.41	11.18	10.26	10.93	16.73	14.28
Global R value	0.62*	0.1 ns	0.2 ns	0.3*	0.35***	0.4***
Seasons						
Summer vs. Autumn	10.52	8.22	4.75	9.19	9.06	10.39
Summer vs. Winter	17.32	14.83	11.58	18.97	12.61	13.22
Summer vs. Spring	16.61	9.81	12.94	14.89	9.89	13.44
Autumn vs. Winter	15.54	15.51	9.99	21.65	12.44	13.63
Autumn vs. Spring	14.69	10.88	13.86	21.66	13.74	15.60
Winter vs. Spring	17.44	16.36	20.98	29.28	15.40	17.61
Global R value	0.7***	0.81***	0.91***	0.7***	0.43***	0.6***

respectively. SAFAs, MUFAs, DMA and NMI in PLs were significantly higher in autumn–winter, whereas PUFAs peaked in spring (ANOVA, $p < 0.001$).

The FA signatures of NLs and PLs of the mantle of female and male mussels formed 2 well-separated clusters in the NMDS plot (Fig. 3). This was further highlighted by the high dissimilarity detected between the NLs and PLs of both sexes (SIMPER, Table 1). The mantle of female mussels showed statistically higher proportions of 14:0, 16:0 and 16:1n-7 in the NLs and higher proportions of 16:1n-7, DMA18:0 and 20:2Δ5,11 in the PLs (ANOVA, $p < 0.05$, Fig. 1). On the other hand, the mantle of male mussels was characterized by greater

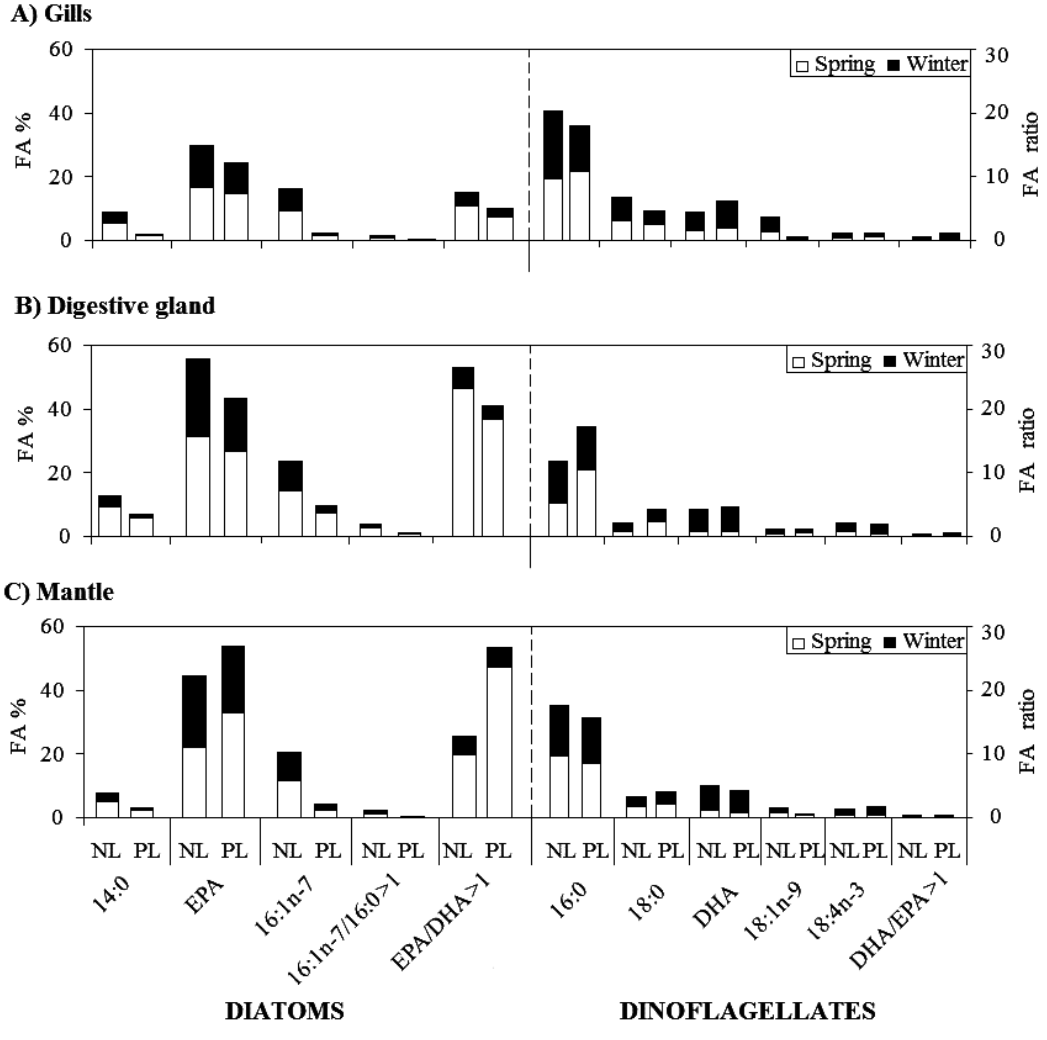

Fig. 4. Abundance of the principal fatty acid (FA) biomarkers (% total FA) and FA ratios indicative of diatom and dinoflagellate dietary inputs to the neutral and polar lipid fractions of: (A) gills, (B) digestive gland and (C) mantle of mussels *Mytilus galloprovincialis* during spring and winter

percentages of 18:0, 20:1n-7, 20:3Δ5,11,14 and DHA in the NLs and by comparatively higher content of PL 18:0, 18:1n-7 and DHA (ANOVA, $p < 0.05$, Fig. 1). The above sexual differences were generally most evident during the typical spawning period in spring (ANOVA, $p < 0.001$).

Anatomical differences in FA composition (H_3)

According to the NMDS plot and ANOSIM results, the FA profile in the NLs of the organs was segregated into 2 groups: gills–gland and mantle (Fig. 5, stress 0.007, Global R: 0.5, $p = 0.001$). The gills proved to be more different from the mantle than the digestive gland (15.3% and 10.3% dissimilarity, respectively, SIMPER). The digestive gland and the mantle also had a very different FA profile (15.4% dissimilarity, SIMPER). These differences mainly stemmed from the comparatively greater levels of 14:0, 16:4n-4 and 17:1n-9 in NLs of the gills and the digestive gland, and from the higher proportions of 20:1n-7, DHA and 22:2Δ7,15 in the mantle (Fig. 5; ANOVA, $p < 0.05$). Nonetheless, dissimilarity between the neutral FAs of the gland and the mantle of males during spring (25%, SIMPER) was greater than that detected between the gland and the mantle of females (13%, SIMPER). With regard to FA classes, the digestive gland had significantly higher levels of PUFAs than the other organs, with remarkably higher levels in spring (Fig. 2; ANOVA, $p < 0.001$). DMA and NMI were consistently higher in the gills with respect to other tissues (Fig. 2; ANOVA, $p < 0.001$).

The PL FA signatures of the 3 organs were distinct from each other (Fig. 5, stress 0.05, global R: 0.5, $p = 0.001$). The gills were 22.8% distinct from the digestive gland and 19.1% different from the mantle, while the gland and the mantle were 17.6% different in their PLs (SIMPER). The gills contained the highest amount of DMA18:0, DHA, 20:2NMIΔ5,11, 20:2NMIΔ5,13 and 20:1n-11, the digestive gland had very high proportions of 16:1n-7, and the mantle showed the highest percentage of EPA, especially during spring (Fig. 5; ANOVA, $p < 0.001$). Unlike in the case of the NLs, dissimilarity between the FAs in PLs of the male digestive gland and the mantle during spring (20%, SIMPER) was almost equal to that observed between the female digestive gland and the mantle (22%, SIMPER). MUFAs showed greater

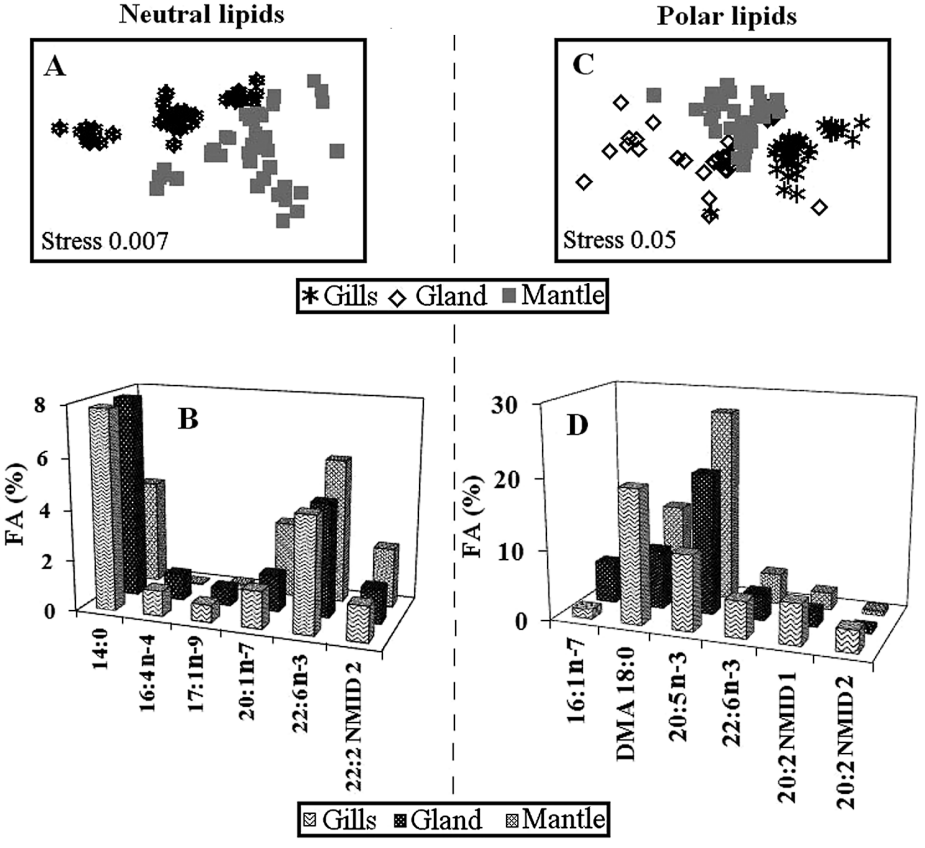

Fig. 5. Non-metric multidimensional scale plots (NMDS) showing the overall spatial segregation of the (A) neutral lipid (NL) and (C) polar lipid (PL) fatty acid (FA) composition of the gills, digestive gland and mantle of mussel *Mytilus galloprovincialis*. Stress levels <0.05 are indicative of an excellent spatial representation of the data. The principal (B) NL and (D) PL FAs that contributed to the observed dissimilarities between the 3 tissues as indicated by SIMPER analysis are shown. 20:2NMID1 = 20:2Δ5,11; 20:2NMID2 = 20:2Δ5,13; 22:2NMID2 = 22:2 Δ7,15

amounts in the digestive gland, whereas PUFAs were generally higher in the mantle (ANOVA, $p < 0.001$; Fig. 2). DMA and NMI were highest in the PLs of the gills (ANOVA, $p < 0.001$; Fig. 2).

DISCUSSION

Seasonal variations in the FA signatures of NLs and PLs (H_1)

The NL and PL FA signatures of the mussel organs were grouped into 3 significantly different clusters that corresponded with the main oceanographic periods of the Galician Rías: (1) dinoflagellate predominance during winter mixing, (2) diatom bloom during spring upwelling, (3) small-sized diatoms and some dinoflagellates during the summer and autumn transition periods (Varela et al. 2001, Figueiras et al. 2002, Froján et al. 2014). The NL and PL FA profiles of the mussel organs during spring showed significantly higher levels of diatom-associated biomarkers, suggesting a significant contribution of diatoms to the shellfish diet during this period. In contrast, the NL and PL FA profile of the mussel tissues during winter revealed a trophic shift towards a dinoflagellate-based diet. Diatom FA biomarkers were predominant in the bivalves' organs in summer and autumn, although dinoflagellate markers were also detected but in lower proportions. The succession of diatom and dinoflagellate FA markers observed in mussels from Ría de Arousa (southern Ría) resembled the seasonal fluctuations detected for *Mytilus galloprovincialis* cultured in Ría Ares-Betanzos (northern Ría, Galicia, NW Spain) (Irisarri et al. 2014a). Similarly, the FA profile of the gonad in female scallops *Nodipecten nodosus* was grouped in 2 clusters that reflected the diatom and dinoflagellate dominance during the upwelling and downwelling periods, respectively (Freites et al. 2010). Although NL and PL FA profiles of bivalve organs reflect the temporal shifts in the diet (Kraffe et al. 2004, Pirini et al. 2007, Ventrella et al. 2008, Hurtado et al. 2012, Dudognon et al. 2014), these changes are illustrated more conservatively in the PLs owing to their structural role (Napolitano & Ackman 1993, Delaporte et al. 2005, Fernández-Reiriz et al. 2006, Pirini et al. 2007, Ventrella et al. 2013). A noteworthy result was that the NL and PL FA signatures of the gills were also capable of reflecting dietary shifts (Piretti et al. 1988, Birkely et al. 2003, Delaporte et al. 2005, Ventrella et al. 2008, Dudognon et al. 2014), discarding the hypothesis that gills have a relatively stable lipid composition (Kluytmans et al. 1985). However, dietary-induced changes were more pronounced in the composition of the digestive gland, i.e. the food-processing organ with faster turnover rate, as seen in previous studies (Shin et al. 2008, Irisarri et al. 2014a).

PUFAs followed an opposite seasonal pattern to that of SAFAs and MUFAs in the 3 sampled organs (Piretti et al. 1988, Turunen & Pekkarinen, 1990, Napolitano & Ackman 1993, Pirini et al. 2007, Ezgeta-Balić et al. 2012, Irisarri et al. 2014a). SAFAs and MUFAs were significantly higher during the warmer seasons (summer–autumn), while PUFAs increased in the colder seasons (winter–spring). This was probably because the high degree of unsaturation of PUFAs confers a low melting point to hydrophobic phospholipid tails and maintains membrane fluidity at colder temperatures (Sargent 1976, Hall et al. 2002). The degree of unsaturation also increases in the gills, digestive gland and mantle of bivalves during cold seasons (Turunen & Pekkarinen 1990, Napolitano & Ackman 1993, Parent et al. 2008). The temporal evolution of PUFAs was attributable to changes in EPA and DHA, the 2 major FAs present in this class. The prevalence of EPA and DHA probably originated from the consumption and accumulation of diatom and dinoflagellate markers during spring and winter, respectively (Napolitano & Ackman 1993, Freites et al. 2002a).

Previous studies found a competition between the levels of NMI and n-3 PUFAs, as deficiencies in PUFAs may provoke *de novo* biosynthesis of NMI (Klingensmith 1982, Zakhartsev et al. 1998, Fernández-Reiriz et al. 1998, Caers et al. 1999, Ventrella et al. 2008, 2013, Hurtado et al. 2012). This opposite pattern was confined to the mantle, as an increment in PUFAs during winter–spring was followed by a decrease in NMI. Similarly, this opposite relation was neither observed for the digestive gland nor the adductor muscle of *M. galloprovincialis* (Ezgeta-Balić et al. 2012). The biosynthesis of NMI in this species appeared to be independent of a high dietary input of precursors or a PUFA shortage (Pirini et al. 2007). This tissue-specific modulation could suggest that the competition between PUFAs and NMI is triggered by reproductive demands.

Sexual differences in FA composition among mussel organs (H_2)

In this study, differences between the NL and PL FA signature of female and male mussels were only evident in the mantle tissue. The mantle supports the

development of the oocytes and spermatocytes in the gonads (Zandee et al. 1980, Mathieu & Lubet 1993). Thus, it is not surprising that sexual differences were most evident in spring, when the gonads were probably ripe, since the main mass spawning event in the Galician Rías occurs with the spring bloom (Villalba 1995). Gonad maturation extends throughout late autumn and winter downwelling, when food is scarce and gametes develop at the expense of reserve storage during the summer (Villalba 1995, Figueiras et al. 2002). Our results agreed with previous studies that described high levels of 14:0, 16:0 and 16:1n-7 in the gonads and mantle of mature female bivalves (Kluytmans et al. 1985, Caers et al. 1999, Birkely et al. 2003, Martínez-Pita et al. 2012a,b) and other marine invertebrates like sea urchins (Hughes et al. 2005), with the exception of higher proportions of 16:0 detected in male bivalves on 2 occasions (Caers et al. 1999, Martínez-Pita et al. 2012a). Furthermore, our results were consistent with other surveys that detected higher levels of 18:0, 20:1n-7 and DHA in the gonads and mantle of male bivalves (Caers et al. 1999, 2003, Birkely et al. 2003, Martínez-Pita et al. 2012a,b). However, 18:0 and DHA predominated in female *M. edulis* before spawning (Kluytmans et al. 1985), while 18:1n-7 and 18:3n-3 showed larger pecent values in the gonads of female clams *Argopecten purpuratus*, *Mya truncata* and *Donax trunculus* (Caers et al. 1999, Birkely et al. 2003, Martínez-Pita et al. 2012a). Furthermore, 18:3n-3, EPA and DHA were related to gonadal maturity in female scallops (Freites et al. 2010), whereas 16:1n-7 and DHA peaked in the gonads of ripe male and female oysters *Pteria sterna*, respectively (Hawkyns-Martínez et al. 2014). These contrasting results suggest that sexual differences in FA composition do not always follow a common pattern in bivalves, and the preferential accumulation of individual FAs in each gender might depend upon energetic and structural specificities of gametes in each species. The accumulation of DHA in the PLs of males in this study might be necessary for the synthesis of spermatocyte membranes (Caers et al. 2003). The higher accumulation of DHA in the NLs of the mantle in males compared to females further suggests that DHA could be easily mobilized from the lipid reserves for membrane synthesis or other energy-demanding processes during the latest stages of spermatogenesis in spring. Thus, our results are in agreement with previous studies that reported sexual differences in the FA composition of the gonads and the mantle of marine invertebrates, suggesting that females and males might have distinct energetic and structural requirements during gametogenesis (Kluytmans et al. 1985, Caers et al. 1999, 2003, Birkely et al. 2003, Hughes et al. 2005, Martínez-Pita et al. 2012a,b, Hawkyns-Martínez et al. 2014).

Distribution of FAs in NLs and PLs, with special reference to NMI and DMA (H_3)

Our results agreed well with other studies that observed comparatively higher levels of plasmalogens (Caers et al. 1999, Kraffe et al. 2004) and NMI (Klingensmith 1982, Zakhartsev et al. 1998, Kraffe et al. 2004, Ventrella et al. 2008) in the PLs of the gills relative to other organs. Plasmalogens may act as scavengers of reactive oxygen species in the membranes of organs directly exposed to physicochemical variations in seawater, such as the gills (Kraffe et al. 2004). NMI are distributed in the inner layer of the cell membrane and are thought to protect against lipid peroxidation processes and microbial lipases (Kraffe et al. 2004, Barnathan 2009). NMI might also provide a structural and functional role to adjust membrane fluidity at low temperatures, due to a lower melting point conferred by the unusual position of the double bonds (Zakhartsev et al. 1998). In this sense, Freites et al. (2002a) observed that mussels *M. galloprovincialis* from the rocky shore of Ría de Arousa had greater levels of NMI than subtidal mussels, as the latter bivalves do not undergo prolonged periods of air exposure over the tidal cycle. Another striking result was that the gills showed almost 2-fold higher levels of PL 20:1n-11. This FA was specifically abundant in the aminoglycerophospholipid plasmalogens of the gills in clams *Ruditapes philippinarum*, suggesting that 20:1n-11 could be functionally equivalent to NMI (Kraffe et al. 2004). Given the generally low levels of 20:1n-11 in the phytoplankton, it is believed that this FA has an endogenous origin after Δ9 desaturation of 20:0 (Kraffe et al. 2004).

The digestive gland resembled the NL FA composition of the gills, although both tissues differed in their PL profile due to the higher levels of DMA and NMI in the gills. Similarly, Ezgeta-Balić et al. (2012) observed low levels of NMI in the TLs in the digestive gland of *M. galloprovincialis*. As in our study, the NL and PL fractions of the digestive gland of clams *Macoma balthica* were also rich in EPA, 16:0 and 16:1n-7, although the abundance of the latter FA varied among each lipid fraction (Turunen & Pekkarinen 1990). Our outcomes agreed with other studies that showed a preferential incorporation of

EPA in the NLs of the digestive gland with respect to the mantle and the gills (Klingensmith 1982, Napolitano & Ackman 1993, Caers et al. 1999). EPA was significantly higher in the NLs of the digestive gland during spring, suggesting a main energetic function prior to spawning (Freites et al. 2002a, Hurtado et al. 2012). Indeed, Miller et al. (2014) observed higher proportions of EPA precursors (18:3n-3 and 18:4n-3) in the digestive gland of female mussels *Perna canaliculus*, which may indicate a higher rate of synthesis in the gland before lipids are transported to the gonads. The digestive gland is responsible for the intracellular digestion of the food, but is also the main lipid storage organ and distributes reserves to the mantle during gametogenesis (Zandee et al. 1980, Napolitano & Ackman 1993, Caers et al. 1999, Cartier et al. 2004, Martínez-Pita et al. 2012a). Thus, it is plausible that the digestive gland acts as an EPA reserve in order to mobilize it to the mantle during gamete maturation. In fact, even if PUFAs are mainly regarded as structural lipids for homeoviscous adaptations, the NLs of the digestive gland could also act as temporary reserves before being transferred to structural lipids (Caers et al. 1999, Pirini et al. 2007).

The composition of the mantle in our study was in agreement with that reported for other bivalves. Klingensmith (1982) reported that the NLs of the mantle of clams *Mercenaria mercenaria* were also characterized by high levels of 20:1n-7 and DHA, although these FAs were not present in the mantle in the highest proportions relative to other body parts. The highest levels of EPA were registered in spring, which could suggest a structural role of EPA prior to the main spawning period. In the present study, the mantle also showed more than double amounts of structural DMA than the digestive gland. Martínez-Pita et al. (2012b) reported higher levels of DMA 18:0 and DHA in the mantle of *Mytilus galloprovincialis* relative to the digestive gland. Caers et al. (1999) and Kraffe et al. (2004) detected higher amounts of plasmalogens in the PL fraction of the gills and the mantle of scallops relative to the digestive gland, an organ which is not directly exposed to external physicochemical stress. Lastly, the greater similarity between the NL FA profile of the female mantle and the gland during spring compared to that observed for male mussels suggested that energetic lipid reserves were being transferred from the digestive gland to the female gonads via the hemolymph during gametogenesis (Napolitano & Ackman 1993, Soudant et al. 1996, Caers et al. 1999, 2003, Birkely et al. 2003, Martínez-Pita et al. 2012b). Similarly, the FA composition of the gonads in female clams had a closer resemblance to the composition of the stomach content than the gonads of males (Birkely et al. 2003).

In conclusion, the present study demonstrated significant differences in the NL and PL FA profiles of mussels depending on the season, sex and tissue. Seasonal variations in the diet were more pronounced in the NL (energetic reserves) than in the PL (structural) FA profile and were mirrored more clearly in the digestive gland. Maximum levels of PUFAs during winter–spring were probably associated with the maintenance of membrane fluidity at lower temperatures. Given that the competition between PUFAs and NMI levels was restricted to the mantle, this mechanism may have been triggered by reproductive demands. Sexual differences were only evident in the mantle during spring and suggested that females and males might have different energetic and structural requirements during gametogenesis. NMI and plasmalogens were preferentially incorporated in the PLs of the gills and the mantle, suggesting that these FAs have a structural and functional role in the membranes of organs exposed to physicochemical fluctuations. The greater similarity between the NL FA profile of the female mantle and the digestive gland during spring suggested that energetic lipid reserves were being transferred from the digestive gland to the female gonads during gametogenesis.

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