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Influence of highly unsaturated fatty acids on the responses of white shrimp (*Litopenaeus vannamei*) postlarvae to low salinity

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Abstract

Salinity stress tests are commonly applied in shrimp hatcheries to estimate the quality of postlarvae (PL) to be used during growout. Higher larval survival during culture and to a salinity stress test in both fish and crustaceans have been reported when specimens were offered a diet containing high levels of highly unsaturated fatty acids (HUFA). However, it is not clear if increased survival is a result of better overall physiological condition resulting from the diet or a specific effect of HUFA on osmoregulatory mechanisms. This study analyzed if HUFA-rich diets could modify the fatty acid composition of membranes in gills, and if this change in composition could affect the activity of the Na^+/K^+ ATPase pump and carbonic anhydrase in relation to changes in salinity. One-day-old postlarvae (PL1) pooled from different spawns were fed for 20 days with *Artemia* sp. nauplii enriched with three levels of HUFA: low, medium and high. At PL20, survivals during culture and to salinity stress test (tap water for 30 min) were evaluated. Also at this stage, Na^+/K^+-ATPase and carbonic anhydrase activity, morphometric variables, and fatty acid composition in the hepatopancreas and gills were measured after they were submitted to a salinity challenge in dilute seawater (10 ppt) for 3 h. No significant differences were observed in survival rates during culture, but survival to a salinity stress test was higher and gill area was larger in PL20 fed the *Artemia* sp. nauplii enriched with medium HUFA levels, probably as a result of an increased 22:6n-3 content and higher 22:6n-3/20:5n-3 ratio in this diet and in the tissues of the organisms fed this diet. Na^+/K^+-ATPase specific activity was significantly higher in posterior gills, while the specific activity of the carbonic anhydrase was higher in anterior gills. Enzymatic activities increased significantly in PL20 submitted to a salinity challenge, and HUFA levels in the diet affected both. The proportion of fatty acids in hepatopancreas and gills were

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significantly affected not only by diet, but also by exposure to dilute media. This effect is discussed in relation to an increase in gill surface and changes in fatty acid composition in the phospholipids present in gill membranes, which can modify the permeability and the activity of the Na^+/K^+-ATPase pump. The beneficial effect of HUFA supplementation in the diet on survival to salinity stress test is partially related to modification of fatty acid composition of gills and to a larger gill area, which in turn enhances osmoregulatory mechanisms, namely Na^+/K^+-ATPase and carbonic anhydrase activities.

Keywords: Carbonic anhydrase; DHA; EPA; HUFA; Lipid; PUFA; Shrimp; Stress test

1. Introduction

Salinity stress tests are commonly applied in hatcheries to estimate the qualities of postlarvae (PL) to be used during growout. The PL that have a higher survival are considered to be healthier or of better quality (Tackaert et al., 1989; AQUACOP et al., 1991; Duran-Gómez et al., 1991; Rees et al., 1994; Samocha et al., 1998). The quality or survival of PL can be modified by nutrition and culture conditions. Several studies report a higher larval survival to a salinity stress test in PL offered a diet containing high levels of HUFA (Tackaert et al., 1992; Rees et al., 1994; Kontara et al., 1997). However, it is not clear if this increase is a result of a better overall physiological condition as a result of enhanced nutritional status, to a specific effect of the HUFA on osmoregulatory mechanisms, or a combination of both.

The increased resistance of PL fed HUFA-rich diets to a salinity stress could be a result of an increased response of the osmoregulatory mechanisms, particularly the activity of the Na^+/K^+-ATPase pump present in the gills (Tocher et al., 1995). This is based on the hypothesis that dietary HUFA can affect tissue fatty acid composition, especially in gills, although no studies have analyzed this in shrimp PL. In rats, variations in the proportion of HUFA or saturated fatty acids of the cellular membranes affected the Na^+/K^+-ATPase activity (Poon et al., 1981). Free fatty acids may affect the activity of the Na^+/K^+-ATPase pump, as was reported for *Artemia salina* (Morohashi et al., 1991). In addition, membrane lipid composition can directly affect the permeability of the gills (Shinitzky and Barenholz, 1978; Poon et al., 1981; Di Costanzo et al., 1983; Leray et al., 1984; Chapelle and Zwingelstein, 1984; Mantel, 1985; Tocher et al., 1995; Abi-ayad et al., 1997).

This study analyzed on the effect of supplementing HUFA in the diet on tissue fatty acid composition and on physiological responses to low salinity exposure.

2. Material and methods

2.1. Experimental design

PL1 pooled from different spawns were donated by Acuacultores de la Paz (APSA, S.A. de C.V., La Paz, Mexico), and reared in the CIBNOR in 100-l tanks at a density of

30 PL/L, 28 °C, and 35 ppt. From PL1 to PL10, shrimp were fed Brine Shrimp Flakes (Salt Creek, Salt Lake City, UT, USA), with a minimum 53% protein and 8.2% lipid content and a maximum 7.3% ash and 9.4% fiber. In addition, PL were fed during a 20-day trial with *Artemia* sp. nauplii (INVE, Premium Quality, Grantsville, UT, USA) enriched with ICES Standard Reference Emulsions (ICES, 1994), containing three levels of highly unsaturated fatty acids (HUFA): low HUFA content (0/-/c as coded by ICES), medium HUFA content (30/0.6/c), and high HUFA content (50/0.6/c) and three replicates per treatment (nine tanks in total). After disinfecting cysts in sodium hypoclorite, hatching of commercial *Artemia* sp. cysts was accomplished by incubating the culture for 24 h in seawater with vigorous aeration, and separating and washing the hatched nauplii. *Artemia* sp. nauplii were then enriched with 0.3 g/l of emulsion twice per day at 12-h intervals (Leger et al., 1987). At PL10, in addition to enriched *Artemia* sp. nauplii, PLs were offered commercial pellets (Promotora Industrial Acuasistemas, PIASA, S.A. de C.V., La Paz, B.C.S., México) with a 36.7% protein, 12.7% lipids, 8.3% ash, and 1% fiber content. At this age, the density per tank was corrected for mortality, so that all tanks were adjusted to a final density of 4.5 PL per liter. At PL20, 50 larvae per replicate, with three replicates per tank (27 total buckets), were transferred directly to tap water (≤ 3 ppt) for 30 min in 1-l buckets and then returned to the culture salinity (35 ppt), as described in Palacios et al. (1999). Additionally, 30 PL20 per tank (nine total tanks) were sampled to determine growth and gill area.

Another set of PL20 from each tank was transferred to dilute seawater (10 ppt) for 3 h in 4-l buckets to assess Na^+/K^+-ATPase and carbonic anhydrase activity. Control groups were manipulated similarly, but transference was done to water at a salinity of 35 ppt, similar to the salinity of the culture tanks. After 3 h, approximately 30 PL20 (± 100 mg), in triplicate samples from each tank (27 total buckets), were collected on absorbent paper and frozen at -70 °C. The anterior and posterior gills and hepatopancreas were dissected and processed separately.

2.2. Enzymatic and morphological analyses

The anterior and posterior gills were homogenized in 0.25 M sucrose, 6 mM EDTA, and 50 mM Tris buffer at pH 7.2, and the ATPase activity associated with the Na^+/K^+-ATPase pump was quantified in the resulting homogenate by determination of inorganic phosphate (Pi) released by the activity of the ATPase, as described by Holliday (1985). This was done by comparing phosphate content after incubation in two media: One medium was composed by 167 mM NaCl, 50 mM KCl, 30 mM Tris buffer at pH 7.2, 50 mM $MgSO_4$ and 25 mM ATP. In the second medium, the composition was similar, but ouabain was added at a final concentration of 1.67 mM, and NaCl, which had a final concentration of 217 mM, substituted KCl from the first medium. Pi content in the two media was measured at 750 nm (Spectronic Genesys 2) by reduction of ammonium molybdate to phosphomolybdate (Petersen, 1978) against a standard of HNa_2PO_4. The difference in Pi concentration between the media represents the Pi liberation by Na^+/K^+-ATPase activity, which is expressed in μmol liberated per hour. Total proteins were determined by the Coomassie blue method (Bradford, 1976), against a standard of bovine serum albumin.

Carbonic anhydrase activity was quantified based on its esterase activity, which was estimated by hydrolysis of the ester *p*-nitrophenyl acetate (*p*-NPA) to nitrophenol (Armstrong et al., 1966). An aliquot of the same gill homogenate that was used for ATPase analysis was incubated with *p*-NPA, and absorbance was read at 348 nm (Spectronic Genesys 2) to quantify release of nitrophenol, based on a millimolar extinction coefficient of 5 (Armstrong et al., 1966). The activity of carbonic anhydrase was expressed in mmol of *p*-NPA hydrolyzed per mg protein of tissue per min.

The anterior and posterior gills of the PL fixed in formaldehyde were dissected after removing the carapace, using a dissection microscope (3.5 ×). We considered the anterior gills as the anterior and posterior arthrobranchs and pleurobranch located in the soma, corresponding to the third pair of maxilliped and to the soma of the first and second pair of pereopods (Dall et al., 1990). The gill area of the anterior and posterior gills of PL was assessed using an image analyzer (Image-Pro). To achieve this, each anterior arthrobranch from each shrimp was dissected and displayed along its axis, with the paired branches extended along its length, and photographed using a microscope (40 ×). Only one pair of branches with its axis and filaments was used to determine the area for each gill.

Approximately 30 mg of gills of PL20 from each 4-l bucket (27 total buckets) was pooled and lipids were extracted with chloroform/methanol (2:1) according to Bligh and Dyer (1959), and neutral and polar lipid fractions were separated and fatty acid analyzed as described in Palacios et al. (2001).

Data are presented as mean ± standard error (S.E.). Two-factor ANOVA analysis ($P < 0.05$) was applied to analyze the differences in survival and biochemical variables, using three HUFA treatment levels (low, medium, high HUFA content) as one independent variable, and two salinity treatment levels (diluted seawater/10 ppt vs. control or standard seawater/35 ppt) as the second variable. Percentage data were transformed to arcsine values for statistical analysis.

3. Results

The fatty acid composition (%) of ICES emulsions and of *Artemia* sp. nauplii enriched with these oils during 24 h are shown in Table 1. Although the levels of HUFA in the emulsion are in accordance to their ranking from low to high HUFA levels, the final concentration of HUFA in enriched *Artemia* is very similar between the medium and the high HUFA levels. In addition, it can be observed that both in the emulsions and in the enriched *Artemia*, the DHA levels and the DHA/EPA ratio are higher for the medium HUFA than for the high HUFA levels.

Survival during culture, shown in Table 2, was not significantly different among the three HUFA treatments. Survival and gill area of PL20 submitted to the salinity stress test were both significantly higher for PL fed *Artemia* sp. nauplii enriched with medium HUFA levels. There were no differences in total body or gill weight or length of PL20 fed nauplii enriched with different HUFA levels (Table 2). The hepatopancreas of PL20 fed high-HUFA-enriched nauplii were larger than PL20 fed low-HUFA-enriched nauplii.

Table 1
Major fatty acid composition (%) of emulsions with low, medium and high HUFA levels, and pooled samples of *Artemia* sp. nauplii after a 24-h enrichment with these emulsions

	ICES emulsions			Enriched *Artemia* sp. nauplii		
	Low	Medium	High	Low	Medium	High
14:0	6.1	0.8	0.6	4.7	1.7	0.4
16:0	13.7	12.7	8.8	16.9	16.7	11.5
18:0	5.5	4.7	4.9	7.6	6.3	8.3
16:1n-9	0.9	0.2	0.5	0.7	0.6	1.7
16:1n-7	3.5	5.2	3.6	3.0	2.4	2.1
18:1n-9	34.8	27.9	25.3	31.5	13.1	24.1
18:1n-7	8.8	11.1	8.8	6.4	11.4	7.2
20:1n-*x*	0.8	0.7	2.0	0.9	2.5	1.9
18:2n-6	6.7	4.5	3.9	7.8	4.4	4.0
18:3n-3	13.0	13.1	13.9	11.7	5.4	9.5
20:4n-6	1.8	1.3	1.6	1.6	1.9	1.4
20:5n-3	2.1	5.7	14.6	4.2	18.0	15.6
22:6n-3	0.1	9.3	7.7	1.5	14.3	9.3
Σ Saturated	25.8	18.8	14.9	29.4	25.0	20.2
Σ MUFA	48.8	45.1	40.3	42.4	30.0	37.0
Σ PUFA	25.4	36.1	44.9	28.2	45.0	42.9
Σ HUFA	5.8	18.5	27.1	8.7	35.3	29.4
n-3/n-6	1.8	4.4	5.9	2.0	6.7	6.7
DHA/EPA	0.02	1.65	0.53	0.36	0.80	0.59
PUI	126	192	230	135	243	227

MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids (all fatty acids with double bonds); HUFA = highly polyunsaturated fatty acids (fatty acids with four or more double bonds); 20:1n-*x* is the sum of 20:1n-11 + 20:1n-9 + 20:1n-7; PUI = polyunsaturated index; DHA = 22:6n-3 and EPA = 20:5n-3.

Fig. 1 depicts the Na^+/K^+-ATPase activity in anterior and posterior gills of PL20 fed diets with different HUFA levels and exposed to reduced salinity (10 ppt). The Na^+/K^+-ATPase activity increased significantly in PL20 transferred to hypo-osmotic media,

Table 2
Survival during culture (PL10 to PL20), to a salinity stress test applied at PL20 (0 ppt), and morphological variables of PL20 fed *Artemia* sp. nauplii enriched with emulsions with low, medium and high HUFA levels, and exposed to tap water during 3 h

Survival during culture	Low	Medium	High	*P*
PL10–PL20 (%)	85.4 ± 7.2	66.3 ± 2.9	78.1 ± 3.9	N.S.
Survival to stress test (%)	56.2 ± 6.3b	69.0 ± 2.9a	53.7 ± 3.0b	0.05
Total weight (mg)	26.2 ± 1.2	28.8 ± 1.2	28.6 ± 1.2	N.S.
Total length (cm)	1.70 ± 0.02	1.74 ± 0.03	1.75 ± 0.03	N.S.
HP weight (mg)	3.35 ± 0.17b	3.91 ± 0.17b	4.03 ± 0.20a	0.03
Gill weight (mg)	3.05 ± 0.13	3.42 ± 0.11	3.37 ± 0.09	N.S.
Gill area (μm^2/mm)	118 ± 11b	156 ± 8.2a	112 ± 7.2b	0.003

Data were analyzed by unifactorial ANOVA, with HUFA levels (*H* = low, medium and high HUFA levels) as the main factor. Results are reported as mean ± S.E. Means were compared with a Newman–Keuls post hoc test, and means not sharing the same superscript are significantly different. *N* = 3 pooled samples of ± 50 PL each.
HP = hepatopancreas; N.S. = not significantly different.

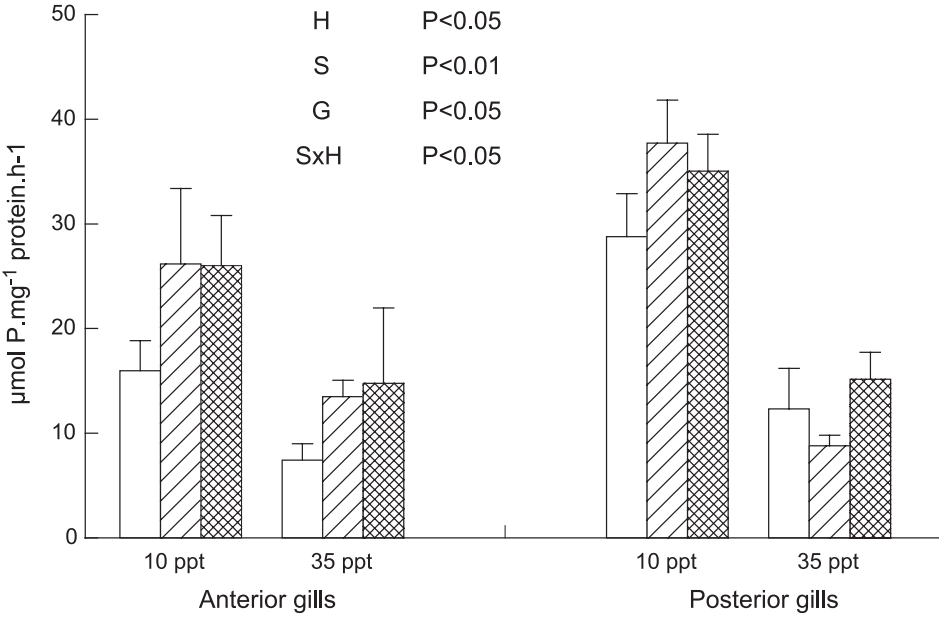

Fig. 1. Na^+/K^+-ATPase specific activity (µmol Pi/mg protein/h) in relation to a salinity challenge in gills of PL20 fed *Artemia* sp. nauplii enriched with low, medium and high HUFA levels. Data were analyzed with a trifactorial ANOVA, considering gills (G = anterior vs. posterior), salinity (S = 35 ppt or control vs. 10 ppt) and HUFA levels (H = low—white bars, medium—hatched bars, and high HUFA—double hatched bars) as main factors. Results are presented as mean ± S.E. N = 3 pooled samples of ± 30 PL each.

regardless of diet (salinity main effect, $P < 0.001$). This effect was observed in both anterior (from 8.9 at 35 ppt to 26.4 at 10 ppt) and posterior gills (from 12.6 at 35 ppt to 35.8 at 10 ppt), but a significant main effect of gill position was also obtained ($P < 0.01$), with higher values in posterior gills. A significant main effect of diet ($P < 0.05$) and interaction between diet and salinity exposure ($P < 0.05$) were also found for the Na^+/K^+-ATPase specific activity, indicating that the influence of salinity depends on HUFA levels in the diet. Indeed, regardless of gill position, the increase in Na^+/K^+-ATPase activity was significantly lower in low-HUFA-enriched diet (from 10.6 to 22.4) than in the medium- (from 11.3 to 34.4) and high- (from 10.5 to 33.6) HUFA-enriched diets.

The specific activity of carbonic anhydrase is depicted in Fig. 2. It was significantly higher in anterior gills (mean value: 0.46 in anterior gills, 0.23 in posterior gills; $P < 0.01$), and increased in PL20 in hypo-osmotic media (mean value: 0.38 at 10 ppt; 0.31 at 35 ppt; $P < 0.05$). The specific activity of the carbonic anhydrase was significantly affected by the diet offered to the PL; it increased in relation to HUFA enrichment (mean value: 0.29 at low; 0.36 at medium; 0.39 at high HUFA; $P < 0.05$).

The fatty acid composition (%) in the hepatopancreas of PL20 fed a diet enriched with HUFA and transferred from 35 to 10 ppt is presented in Table 3. The proportion of fatty acids in hepatopancreas was, in general, similar between the PL20 fed a diet with

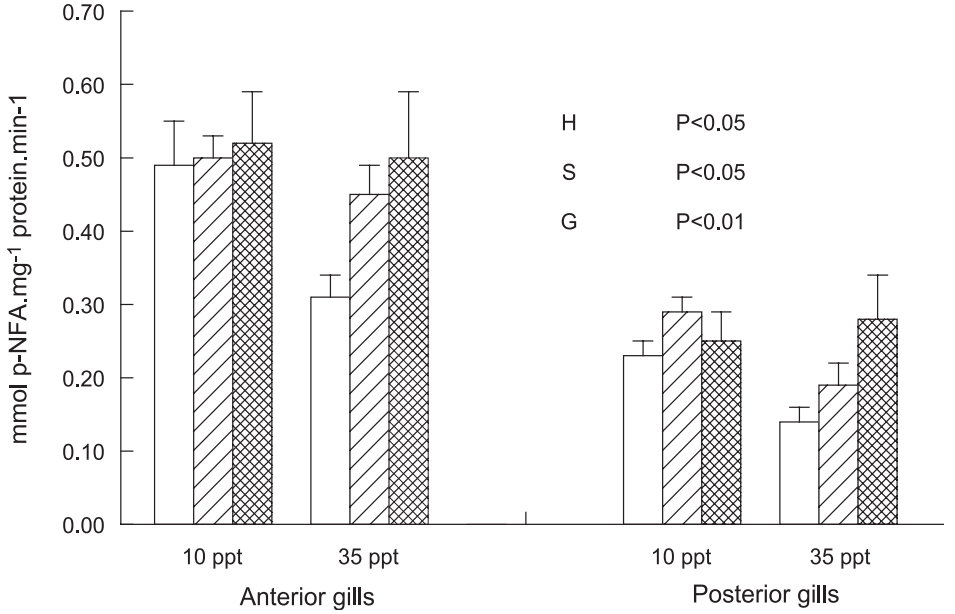

Fig. 2. Carbonic anhydrase specific activity (mmol *p*-NFA/mg protein/min) in relation to a salinity challenge in gills of PL20 fed *Artemia* sp. nauplii enriched with low, medium and high HUFA levels. Data were analyzed with a trifactorial ANOVA, considering gills (G = anterior vs. posterior), salinity (S = 35 ppt or control vs. 10 ppt) and HUFA levels (H = low—white bars, medium—hatched bars, and high HUFA—double hatched bars) as main factors. The results of the trifactorial ANOVA are shown directly in the figure (no significant interaction were found), and data are presented as mean ± S.E. N = 3 pooled samples of ± 30 PL each.

medium and high HUFA levels. HUFA content in hepatopancreas was significantly higher in PL20 fed a diet with medium or high HUFA levels, in comparison with the low HUFA diet (mean value: 22.6 for low; 30.8 for medium; and 32.5 for high HUFA; $P < 0.01$). A similar effect was observed for the saturation index (mean value: 188 for low; 224 for medium; and 232 for high HUFA; $P < 0.01$), and the proportion of 22:6n-3 (mean value: 6.5 for low; 14.8 for medium; 12.9 for high HUFA; $P < 0.01$). However, the proportion of 20:5n-3 was significantly higher in PL20 fed the diet with high HUFA compared to PL20 fed the diets with low and medium HUFA levels (mean value: 11.6 for low; 11.3 for medium; and 15.6 for high HUFA; $P < 0.05$). These differences resulted in a significantly higher ratio of DHA/EPA in PL20 fed the diet with medium HUFA levels (mean value: 0.55 for low; 1.49 for medium; 0.85 for high HUFA; $P < 0.01$). The 20:4n-6 was significantly lower in PL20 fed with high HUFA levels in comparison to PL20 fed with low and medium HUFA levels (mean value: 4.0 for low; 4.6 for medium; 3.3 for high HUFA; $P < 0.05$), and 18:2n-6 was significantly higher in the diet with low HUFA levels (mean value: 7.7 for low; 5.9 for medium; 5.3 for high HUFA; $P < 0.01$). Thus, the ratio between the n-3 and n-6 fatty acids was significantly different among the three diets (mean value: 2.2 for low; 3.1 for medium; 4.2 for high; $P < 0.01$). All the proportions of fatty acids differed between neutral and polar fraction in the hepatopancreas, which

Table 3
Major fatty acid composition (%) in the polar and neutral fraction of the hepatopancreas of PL20 fed *Artemia* sp. enriched with low, medium and high HUFA contents, and submitted to a salinity challenge

	Neutral fraction			Polar fraction			*H*	*F*	*H* × *F*
	Low	Medium	High	Low	Medium	High			
14:0	2.5 ± 0.5	0.8 ± 0.1	1.1 ± 0.3	0.8 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.000	0.000	N.S.
16:0	12.7 ± 2.5	14.0 ± 0.3	12.1 ± 0.8	16.4 ± 1.8	18.0 ± 1.8	15.7 ± 1.4	N.S.	0.007	N.S.
18:0	9.0 ± 0.9	7.1 ± 0.6	7.4 ± 0.6	10.1 ± 0.9	10.3 ± 0.6	10.0 ± 0.2	N.S.	0.000	N.S.
16:1n-9	1.1 ± 0.3	1.2 ± 0.4	1.3 ± 0.2	0.8 ± 0.4	0.8 ± 0.3	0.7 ± 0.3	N.S.	0.050	N.S.
16:1n-7	4.5 ± 3.0	2.1 ± 0.2	1.8 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	N.S.	0.050	N.S.
18:1n-9	29.4 ± 1.6	25.4 ± 1.4	25.0 ± 1.3	18.8 ± 1.0	14.5 ± 0.9	15.5 ± 0.6	0.002	0.000	N.S.
18:1n-7	6.9 ± 0.3	7.3 ± 0.4	7.3 ± 0.2	5.7 ± 0.4	5.9 ± 0.6	6.1 ± 0.3	N.S.	0.001	N.S.
20:1n-*x*	3.0 ± 0.3	2.9 ± 0.3	4.2 ± 0.3	1.8 ± 0.3	2.2 ± 0.6	1.8 ± 0.1	N.S.	0.000	N.S.
18:2n-6	7.5 ± 1.0	7.3 ± 0.2	6.2 ± 1.1	7.9 ± 0.5	4.8 ± 0.4	4.3 ± 0.5	0.003	0.020	N.S.
18:3n-3	6.6 ± 1.1	6.3 ± 1.1	7.4 ± 0.3	6.9 ± 0.6	4.3 ± 0.4	4.4 ± 0.2	N.S.	0.011	N.S.
20:4n-6	3.0 ± 0.3	4.3 ± 0.5	2.8 ± 0.4	5.0 ± 0.4	4.9 ± 0.5	3.7 ± 0.3	0.010	0.002	N.S.
20:5n-3	8.0 ± 0.9	7.3 ± 0.9	11.2 ± 1.1	15.3 ± 1.9	14.7 ± 1.3	20.1 ± 1.3	0.002	0.000	N.S.
22:6n-3	4.5 ± 0.8	13.0 ± 0.3	10.3 ± 0.5	8.5 ± 1.5	16.5 ± 1.8	15.5 ± 1.4	0.000	0.000	N.S.
Σ Saturated	25.0 ± 2.4	22.7 ± 1.2	21.5 ± 0.5	28.0 ± 2.6	29.7 ± 2.4	26.6 ± 1.7	N.S.	0.004	N.S.
Σ MUFA	44.9 ± 2.9	38.9 ± 1.6	38.8 ± 1.3	27.9 ± 1.4	24.5 ± 1.6	25.1 ± 1.1	0.023	0.000	N.S.
Σ PUFA	30.1 ± 0.9	38.4 ± 1.4	38.8 ± 1.3	44.1 ± 3.6	45.8 ± 3.0	48.4 ± 2.7	0.033	0.000	N.S.
Σ HUFA	15.9 ± 2.0	24.9 ± 1.3	25.3 ± 2.1	29.4 ± 3.5	36.7 ± 3.1	39.7 ± 3.0	0.002	0.000	N.S.
n-3/n-6	1.9 ± 0.4	2.3 ± 0.2	3.4 ± 0.6	2.4 ± 0.2	3.7 ± 0.3	4.9 ± 0.3	0.000	0.001	N.S.
DHA/EPA	0.5 ± 0.1	1.9 ± 0.2	0.9 ± 0.1	0.6 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	0.000	0.000	0.001
PUI	161 ± 5.4	205 ± 5.3	208 ± 7.5	215 ± 17	242 ± 16	257 ± 14	0.003	0.000	N.S.

Data were initially analyzed by trifactorial ANOVA, considering HUFA levels (*H* = low, medium and high HUFA contents), salinity challenge (*S* = 10 ppt and control) and fraction (*F* = neutral and polar) as main factors. No significant differences were obtained as a result of the salinity challenge; so only the results of the other two variables are presented, with the ANOVA results and interactions in the last three columns. Only major fatty acids (>1%) are shown. Data were transformed to arcsine for analyses, but only untransformed results are shown as mean ± S.E. *N* = 3 pooled samples of ± 30 PL each.
See Table 1 for abbreviations. N.S. = not significantly different.

resulted in a higher proportion of total saturated (mean value: 28.2 in the polar vs. 23.1 in the neutral fraction; $P < 0.01$) and polyunsaturated fatty acids in the polar fraction (mean value: 46.1 in the polar vs. 35.8 in the neutral fraction; $P < 0.01$), and a lower proportion of total monounsaturated in this fraction (mean value: 25.7 in the polar vs. 41.2 in the neutral fraction; $P < 0.01$).

The amount of fatty acid in the neutral fraction in gills of PL20 fed different levels of HUFA and exposed to dilute media is shown in Table 4. The 22:6n-3 was significantly lower in the PL20 fed nauplii with low HUFA levels (mean value of DHA: 9.5 for low; 17.6 for medium; 17.0% for high HUFA; $P < 0.01$), and was higher in the PL20 exposed to dilute media (mean value: 12.6 in the control group; 16.6 for 10 ppt; $P < 0.05$). The 20:5n-3 was significantly higher in PL20 transferred to dilute media (mean value: 10.9 in the control group; 14.3% for 10 ppt; $P < 0.05$), but no differences were observed in relation to the HUFA levels in the diet. No significant differences were observed in the DHA/EPA ratio. Total saturated fatty acids were higher in PL20 fed nauplii with low HUFA levels (mean value: 30.6 for low; 28.9 for medium; 24.9% for high HUFA levels; $P < 0.05$), and

Table 4
Major fatty acid composition (%) in the neutral fraction of gills of PL20 fed *Artemia* sp. enriched with low, medium and high HUFA levels, and submitted to a salinity challenge

	35 ppt (control)			10 ppt			*H*	*S*	*H* × *S*
	Low	Medium	High	Low	Medium	High			
14:0	2.5 ± 0.7	1.9 ± 0.3	1.4 ± 0.3	2.3 ± 0.6	1.0 ± 0.1	1.8 ± 0.5	N.S.	N.S.	N.S.
16:0	17.6 ± 1.5	15.9 ± 0.5	13.1 ± 0.7	13.1 ± 1.3	12.6 ± 0.5	11.8 ± 1.0	N.S.	0.006	N.S.
18:0	13.4 ± 1.6	10.9 ± 0.1	9.6 ± 0.9	9.0 ± 2.1	11.1 ± 0.1	9.6 ± 0.9	N.S.	N.S.	N.S.
16:1n-9	3.0 ± 0.4	1.9 ± 0.1	1.2 ± 0.1	3.3 ± 0.4	1.3 ± 0.1	2.0 ± 0.3	0.000	N.S.	N.S.
16:1n-7	2.1 ± 0.4	2.2 ± 0.1	1.5 ± 0.2	1.0 ± 0.2	0.9 ± 0.1	1.4 ± 0.4	N.S.	0.008	N.S.
18:1n-9	19.6 ± 2.6	17.5 ± 0.5	17.8 ± 2.4	16.4 ± 1.8	13.5 ± 0.5	13.1 ± 0.4	N.S.	0.042	N.S.
18:1n-7	4.8 ± 0.9	4.6 ± 0.7	4.9 ± 0.8	5.5 ± 0.7	5.3 ± 0.7	4.9 ± 0.4	N.S.	N.S.	N.S.
20:1n-*x*	3.3 ± 0.8	1.9 ± 1.0	4.1 ± 1.1	4.6 ± 1.3	2.5 ± 1.0	2.1 ± 0.4	N.S.	N.S.	N.S.
18:2n-6	5.8 ± 1.0	4.1 ± 0.5	4.3 ± 0.9	6.5 ± 0.5	4.8 ± 0.5	4.5 ± 0.4	0.050	N.S.	N.S.
18:3n-3	4.7 ± 0.8	3.9 ± 0.3	5.4 ± 0.7	5.7 ± 0.4	4.1 ± 0.3	4.9 ± 0.3	N.S.	N.S.	N.S.
20:4n-6	3.7 ± 0.8	6.6 ± 0.3	4.1 ± 0.6	4.2 ± 0.3	5.6 ± 0.3	5.6 ± 1.5	0.005	N.S.	N.S.
20:5n-3	8.8 ± 2.1	10.5 ± 0.9	13.5 ± 2.0	14.0 ± 0.5	13.6 ± 0.9	15.2 ± 1.9	N.S.	0.050	N.S.
22:6n-3	8.9 ± 1.3	14.0 ± 2.2	15.5 ± 2.5	10.2 ± 0.7	21.2 ± 2.2	18.4 ± 1.8	0.000	0.017	N.S.
Σ Saturated	35.0 ± 2.3	31.4 ± 1.0	26.2 ± 1.9	26.2 ± 2.0	26.3 ± 1.0	23.6 ± 1.6	0.034	0.004	N.S.
Σ MUFA	32.8 ± 2.3	28.1 ± 2.1	29.4 ± 3.3	30.6 ± 1.7	23.5 ± 2.1	23.5 ± 0.7	0.034	0.038	N.S.
Σ PUFA	32.2 ± 3.9	40.2 ± 3.1	44.4 ± 3.3	43.1 ± 2.6	50.2 ± 3.1	53.8 ± 2.1	0.006	0.001	N.S.
Σ HUFA	21.7 ± 3.6	32.5 ± 3.9	34.6 ± 4.5	30.9 ± 1.9	41.3 ± 3.9	43.5 ± 2.7	0.002	0.004	N.S.
n-3/n-6	2.4 ± 0.1	2.8 ± 0.4	4.4 ± 0.7	3.0 ± 0.1	3.9 ± 0.4	4.4 ± 0.6	0.001	N.S.	N.S.
DHA/EPA	1.5 ± 0.5	1.5 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	1.6 ± 0.1	1.3 ± 0.2	N.S.	N.S.	N.S.
PUI	172 ± 17	217 ± 18	239 ± 19	221 ± 10	267 ± 18	277 ± 11	0.001	0.001	N.S.

Data were analyzed by bifactorial ANOVA, considering HUFA levels (*H* = low, medium and high HUFA levels) and salinity challenge (*S* = 10 ppt and control) as main factors. The results of the ANOVA are presented in the last three columns. Only major fatty acids (>1%) are shown. Data were transformed to arcsine for analyses, but only untransformed results are shown as mean ± S.E. *N* = 3 pooled samples of ± 30 PL each.
See Table 1 for abbreviations. N.S. = not significantly different.

were lower in PL20 exposed to low salinity (mean value: 30.9 in the control group; 25.4% for 10 ppt; $P < 0.01$). A similar trend was observed for the total monounsaturated fatty acids in relation to diet (mean value: 31.7 for low; 25.8 for medium; 26.5% for high HUFA levels; $P < 0.05$), and salinity (mean value: 30.1 in the control group, and 25.9% at 10 ppt; $P < 0.05$). The polyunsaturated fatty acids were lower in the PL20 fed the low-HUFA-enriched nauplii (mean value: 37.7 for low; 45.3 for medium; and 48.6% for high HUFA; $P < 0.01$), and were higher in the PL20 transferred to 10 ppt (mean value: 39.0 in the control group; 48.7% at 10 ppt; $P < 0.01$). Similarly, the saturation index was lower in the low-HUFA-enriched diet (mean value: 196 for low; 242 for medium; 258 for high HUFA levels; $P < 0.01$), and higher in PL20 exposed to decreased salinity (mean value: 209 in the control group; and 254 at 10 ppt; $P < 0.01$). No significant interactions were found between salinity challenge and HUFA levels in the diet.

In the polar fraction, fatty acid content in gills was mostly affected by the diet (Table 5). The most relevant results to be mentioned in relation to diet are the 22:6n-3 levels (mean values: 8.0 for low; 16.8 for medium; 14.8% for high HUFA levels; $P < 0.01$) and the DHA/EPA ratio (mean values: 0.80 for low; 1.35 for medium; 0.95 for high HUFA levels; $P < 0.01$). In addition, only the 22:6n-3 (mean value: 14.8 in the control group; 11.6 at 10

Table 5
Major fatty acid composition (%) in polar fraction of gills of PL20 fed *Artemia* sp. enriched with low, medium and high HUFA levels, and submitted to a salinity challenge

	35 ppt (control)			10 ppt			*H*	*S*	*H* × *S*
	Low	Medium	High	Low	Medium	High			
14:0	1.6 ± 0.6	0.4 ± 0.2	0.5 ± 0.1	1.6 ± 0.6	0.5 ± 0.1	0.7 ± 0.3	0.014	N.S.	N.S.
16:0	20.2 ± 0.8	17.5 ± 1.1	17.6 ± 0.8	18.5 ± 1.4	19.1 ± 1.4	19.3 ± 1.1	N.S.	N.S.	N.S.
18:0	10.2 ± 0.7	11.4 ± 0.4	11.5 ± 0.1	8.7 ± 1.1	10.4 ± 0.5	8.2 ± 1.7	N.S.	0.005	N.S.
16:1n-9	1.8 ± 0.7	0.8 ± 0.2	0.7 ± 0.1	1.6 ± 0.5	1.4 ± 0.3	0.9 ± 0.3	N.S.	N.S.	N.S.
16:1n-7	1.2 ± 0.2	1.1 ± 0.2	0.9 ± 0.2	1.6 ± 0.6	1.5 ± 0.6	1.4 ± 0.2	N.S.	N.S.	N.S.
18:1n-9	20.6 ± 2.1	14.4 ± 0.4	14.8 ± 0.4	16.9 ± 1.7	15.0 ± 0.7	15.9 ± 0.3	0.005	N.S.	N.S.
18:1n-7	5.1 ± 1.0	5.8 ± 0.4	6.0 ± 0.4	5.5 ± 0.5	6.3 ± 0.5	6.7 ± 0.3	N.S.	N.S.	N.S.
20:1n-*x*	3.0 ± 0.3	2.4 ± 1.3	2.2 ± 0.7	5.4 ± 1.8	3.3 ± 0.7	4.1 ± 1.2	N.S.	N.S.	N.S.
18:2n-6	6.1 ± 0.9	4.2 ± 0.2	4.2 ± 0.2	6.6 ± 0.7	4.6 ± 0.1	4.3 ± 0.2	0.001	N.S.	N.S.
18:3n-3	4.2 ± 0.6	3.4 ± 0.5	3.8 ± 0.3	5.8 ± 0.2	4.3 ± 0.1	4.6 ± 0.3	0.050	0.008	N.S.
20:4n-6	4.0 ± 0.2	4.8 ± 0.4	3.2 ± 0.5	4.7 ± 0.5	4.1 ± 0.7	3.3 ± 0.3	0.019	N.S.	N.S.
20:5n-3	11.1 ± 1.6	13.3 ± 1.3	17.2 ± 1.2	11.7 ± 1.5	11.2 ± 1.3	14.8 ± 1.5	0.008	N.S.	N.S.
22:6n-3	8.6 ± 1.0	19.1 ± 1.9	16.6 ± 1.9	7.4 ± 0.6	14.4 ± 2.5	13.0 ± 1.2	0.000	0.032	N.S.
Σ Saturated	33.0 ± 1.5	30.3 ± 1.3	30.2 ± 1.2	31.6 ± 2.2	31.9 ± 1.6	29.9 ± 1.7	N.S.	N.S.	N.S.
Σ MUFA	31.7 ± 2.0	24.6 ± 1.8	24.5 ± 1.7	31.0 ± 0.9	27.5 ± 1.8	29.0 ± 1.8	0.019	N.S.	N.S.
Σ PUFA	35.2 ± 3.4	45.0 ± 3.1	45.2 ± 2.8	37.4 ± 2.3	40.6 ± 3.3	41.1 ± 2.5	0.050	N.S.	N.S.
Σ HUFA	24.9 ± 2.5	37.4 ± 3.3	37.1 ± 3.0	25.0 ± 1.6	31.7 ± 3.4	32.1 ± 2.8	0.006	N.S.	N.S.
n-3/n-6	2.4 ± 0.2	4.1 ± 0.4	5.2 ± 0.7	2.2 ± 0.2	3.5 ± 0.2	4.4 ± 0.5	0.000	N.S.	N.S.
DHA/EPA	0.9 ± 0.2	1.4 ± 0.1	1.0 ± 0.1	0.7 ± 0.2	1.3 ± 0.1	0.9 ± 0.1	0.000	N.S.	N.S.
PUI	189 ± 13	245 ± 16	244 ± 15	189 ± 8.9	218 ± 17	221 ± 13	0.001	N.S.	N.S.

Data were analyzed by bifactorial ANOVA, considering HUFA levels (*H* = low, medium and high HUFA contents) and salinity challenge (*S* = 10 ppt and control) as main factors. The results of the ANOVA are presented in the last three columns. Only major fatty acids (>1%) are shown. Data were transformed to arcsine for analyses, but only untransformed results are shown as mean ± S.E. *N* = 3 pooled samples of ± 30 PL each.
See Table 1 for abbreviations. N.S. = not significantly different.

ppt; $P < 0.05$) and 18:0 (mean value: 11.0 in the control group; 9.1% at 10 ppt; $P < 0.01$) decreased when exposed to low salinity, while the 18:3n-3 increased (mean value: 3.8 in the control group; 4.9% at 10 ppt; $P < 0.01$). No significant interactions were found between salinity challenge and HUFA levels in the diet.

4. Discussion

The Na^+/K^+-ATPase specific activity increased in PL20 submitted to a 10-ppt salinity challenge of 3 h (Fig. 1). The activity of the Na^+/K^+-ATPase pump was higher in posterior gills, where most of the osmoregulatory activity takes place (Holliday, 1985; Siebers et al., 1985; Thuet et al., 1988; Dickson et al., 1991; Péqueux, 1995). Carbonic anhydrase also increased in response to exposure to low salinity, in accordance to its role in producing H^+ to serve as a counterion during the active uptake of Na^+ (Thuet et al., 1988; Henry, 1996). In contrast to the Na^+/K^+-ATPase pump, carbonic anhydrase was higher in anterior gills, reflecting its predominant role in respiration (Henry and Cameron, 1983) that is independent of osmoregulation, and takes place principally in anterior gills, which are more ramified.

The proportions of fatty acids in gills were also affected by the salinity challenge, although it only lasted 3 h. In the neutral fraction, the decrease in several saturated and monounsaturated fatty acids, together with an increase in polyunsaturated fatty acids following the exposure to dilute media probably indicate a selective fatty acid oxidation to obtain energy. Thus, polyunsaturated fatty acids from the acylglyceride reserves are spared while the saturated and monounsaturated fatty acids are metabolized. It should be noted that we are reporting percentages, and thus increased proportions of HUFA in response to low salinity could be explained by the decrease in another type of fatty acids.

Changes in the membrane fatty acid composition in relation to salinity have been widely studied. Chapelle et al. (1977) found no differences in the insaturation state of fatty acids in the gills of the crab *Eriocheir sinensis* acclimated to freshwater for 14 days. Transfer from freshwater to seawater produced an increase in 22:6n-3 in the intestine brush border membrane in trout after 1 day (Leray et al., 1984). Morris et al. (1982) reported that in amphipods acclimated several weeks to dilute seawater, there was an increase in saturated fatty acids of phospholipids in gills and a decrease in apparent permeability. The substitution of fatty acids in the phospholipids of membranes would require several dehydrase and desaturation enzymes that are probably controlled by hormones or divalent ions (Leray et al., 1984). Morris et al. (1982) concluded that sudden changes of salinity are too rapid to be accountable for substitution of membrane components. However, in the present work, a 3-h salinity challenge was enough to affect the proportion of some fatty acids in the polar fraction. A possible explanation, taking into account the effect of salinity on the proportions of fatty acids present in the neutral fraction of gills that was discussed above, could be the result not only of the selective oxidation rate, but also to the transfer of fatty acids between polar and neutral lipids. Only the proportion of 22:6n-3 was affected in both fractions in response to low salinity, with an increase in neutral fraction and a decrease in polar fraction, as supported by a significant interaction between salinity and the lipid fraction (mean value: at 35 ppt, 12.8 in the neutral and 14.8 in the polar fraction; at 10 ppt, 16.6 in the neutral and 11.6 in the polar fraction; $S \times F = P < 0.01$, not shown in the results). Thus, when the organisms are exposed to a lower salinity, at least this fatty acid decreases in proportion in the polar fraction and increases in the neutral fraction, even if the exposure is of a short duration. Thuet et al. (1988) observed decreased permeability in lobsters exposed to dilute seawater, and this decrease occurred within 3 h of transfer. Thus, postlarvae exposed to a low-salinity challenge could modify the proportion of certain fatty acids present in the membranes as a first step to decrease permeability.

The present work was based on the hypothesis that the fatty acid composition in gills could be modified by the diet, and that changes in the fatty acid composition would affect the osmoregulatory mechanisms, and thus the survival to a salinity stress test. Previous studies on dietary supplementation of HUFA clearly showed that HUFA content in PL is directly increased with the level of HUFA included in the diet (Rees et al., 1994; Kontara et al., 1995; Naessens et al., 1995; Wouters et al., 1997). In addition, Mourente and Rodríguez (1997) also found that a diet rich in 22:6n-3 could increase the concentration of certain phospholipids in the postlarvae. In shrimp PL, the incorporation of HUFA into different tissues in relation to different HUFA levels in the diet has not been analyzed. We did observe changes in the fatty acids proportions in the hepatopancreas in relation to HUFA enrichment of *Artemia* nauplii. It should be noted that although some variations in

the fatty acid content could be a result of the ongoing digestion of the enriched *Artemia* nauplii still present in the digestive system of the PL20, food was removed 3 h before the salinity challenge, and including the 3 h of the salinity challenge per se, at least 6 h would have elapsed since last fed and when the PL were sampled, which should have been enough time to digest the enriched nauplii. Therefore, differences in the fatty acid composition of the hepatopancreas are probably a result of differences at the structural/reserve level. In addition to changes in the hepatopancreas, gills were also affected by the HUFA-content of the diet, and thus a significant result of this work is that the proportions of HUFA in both neutral and polar lipids of gills are increased in relation to HUFA enrichment.

In relation to HUFA beneficial effects on shrimp PL culture, we did not observe any influence of HUFA supplementation on survival during culture or final size of PL, which agrees with some studies (Mourente and Rodríguez, 1997; Wouters et al., 1997), while others reported an increase in survival (Rees et al., 1994), size (Wouters et al., 1997) or both (Kanazawa et al., 1985; Kontara et al., 1997). Some discrepancies could be explained by the particular age of PL used: studies analyzing different phases of PL development showed a beneficial effects of HUFA on survival or growth in more advanced stages of PL (Kontara et al., 1997; Wouters et al., 1997). Although HUFA levels in the diet did not significantly affect survival during culture and growth, we did found that survival to a salinity stress test was higher for PL20 fed the medium HUFA levels. Previous works obtained contradictory results, with sometimes a higher survival to low salinity stress when PL were fed high HUFA levels (Tackaert et al., 1992; Rees et al., 1994; Kontara et al., 1997; Chim et al., 2001), but other studies failed to demonstrate such an influence (Naessens et al., 1995; Wouters et al., 1997). Some discrepancies could be explained again by the particular age of PL: in older PL, the effect of HUFA supplementation is less clear than in younger organisms (Tackaert et al., 1992; Rees et al., 1994; Bonilla, 2001). Postlarvae have an increased osmoregulation capacity at certain stages of development (Charmantier et al., 1988; Rosas et al., 1999), which coincide with their migration to estuaries. In organisms of the same age, an increased resistance of PL fed HUFA-rich diets could be a result of an increased response of some osmoregulatory mechanisms associated with changes in lipids. The effect of fatty acids on membrane permeability has been discussed above, but in addition to permeability of gills, the fatty acids present in the membrane can directly modulate other osmoregulatory mechanisms, i.e. Na^+/K^+-ATPase pump present in gills (Towle, 1981). Poon et al. (1981) substituted fatty acids from the phospholipids present in the cellular membranes of rat tumor lymphocytes and found that the activity of the Na^+/K^+-ATPase was decreased by saturated fatty acids and increased by HUFA. We did observe an interaction in the activity of the Na^+/K^+-ATPase pump between salinity and HUFA levels in the diet. Interestingly, the basal activity of Na^+/K^+-ATPase at 35 ppt was similar between PL20 fed different HUFA levels, but when PL20 were submitted to a 3-h salinity challenge, the activity increased more in PL20 fed the medium- and high-HUFA-enriched diets. The activity of carbonic anhydrase was increased in relation to the HUFA levels in the diet at the control salinity, although the activity of this enzyme at 10 ppt was similar for the three diets. Enhancement of Na^+/K^+-ATPase and carbonic anhydrase activities by HUFA supplementation could explain a higher survival to salinity stress test in groups fed medium HUFA levels enriched diets. However, we cannot

explain on the same terms why the group fed high levels of HUFA did not have a higher tolerance to reduced salinity exposure than the group fed low HUFA levels. It has been suggested that too high levels of HUFA could be detrimental (Rees et al., 1994). On the other hand, several authors have pointed out that not only total HUFA must be considered in the diet of fish and crustacean larvae, but that special attention should be given to the specific 22:6n-3 levels and the resulting DHA/EPA ratio (Watanabe, 1993; Mourente and Rodríguez, 1997; Sargent et al., 1997; Wouters et al., 1997). In agreement, the highest 22:6n-3 levels and DHA/EPA ratio were found in *Artemia* nauplii enriched with the medium HUFA levels, which was also reflected in PL gill and hepatopancreas. In any case, neither explanation is related to Na^+/K^+-ATPase specific activity, thus survival to a salinity stress test cannot be fully explained by a lower efficiency of osmoregulatory mechanisms in terms of enzymatic activities.

Other mechanisms that can be affected by the diet, and which can modulate the osmoregulatory capacity should be considered. Postlarvae fed a rich HUFA diet were observed to have a more ramified structure in gills (Rees et al., 1994). In our study, the gill surface was larger in the PL20 fed the medium HUFA diet. A larger gill surface can increase the surface of ion transport and the number of Na^+/K^+-ATPase pumps that are available to exchange ions. A diet with adequate fatty acid composition, or in this case, fatty acid levels, i.e. the medium HUFA diet, seem to produce the best results in survival to a stress test, possibly by promoting an increase in the synthesis of new membranes in gills that would result in an increase in surface or ramifications, and can incorporate the most suitable fatty acid composition to counteract the effect of salinity changes that modify either permeability or functional enzymes (Na^+/K^+-ATPase pump). It is concluded that the beneficial effect of HUFA supplementation in the diet on survival to salinity stress test is partially related to modification of fatty acid composition of gills and to a larger gill area, which in turn enhances osmoregulatory mechanisms, namely Na^+/K^+-ATPase and carbonic anhydrase activities.

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