THE EFFECTS OF LOW SALINITY ON LIPID COMPOSITION IN THE GILLS OF GREY MULLET *LIZA RAMADA*

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ملخص

تأثير انخفاض ملوحة الماء على التركيبة الدهنية لخياشيم فراخ البوري: قمنا بنقل فراخ البوري المتأقلمة في مياه البحر إلى المياه العذبة طيلة 24 ساعة. شملت دراستنا خصائص الدم والتركيبة الدهنية لخياشيم فراخ البوري.

هذا النقل المباشر إلى المياه العذبة أدى إلى انخفاض هام لأوسمولاليتي والإيماتوكريت وعدد الكريات الحمراء.

أثبتت النتائج المتحصل عليها أن نسبة الأحماض الدهنية أحادية عدم التشبع قد ارتفعت وخاصة منها نسبة الحامض الدهني الألوبيكي. لاحظنا أيضا انخفاض في نسبة الأحماض الدهنية الغير المشبعة وخاصة منها C18:4n−3, C20: 5n−3, C22: 5n−3 and C22: 6n−3. بالنسبة لعائلة الأحماض الدهنية المشبعة من فصيلة أوميقا6 فإن الأسيد لنولييكي نجده بنسبة مرتفعة مع انخفاض نسبة الحامض الأراشيدونيكي.

الكلمات المفاتيح: التركيبة الدهنية; الخياشيم; التأقلم في المياه العذبة; *Liza ramada*

RESUME

Effets de la basse salinité du milieu sur la composition en acides gras des branchies du Muge : Liza ramada : Les alevins de *Liza ramada* adaptés à l'eau de mer (EM, 35ppt) ont subi au laboratoire un stress halin par transfert direct de l'eau de mer à l'eau douce (ED, 0,5 ppt) pendant 24 heures. Les aspects hématologiques ainsi que la composition lipidique du tissu branchial des alevins ont été suivis. Le transfert à la basse salinité s'est accompagné d'une diminution significative de l'osmolalité du plasma, du taux d'hématocrite et du nombre des globules rouges.

Le suivi de l'évolution des lipides des branchies des alevins en fonction de la salinité du milieu, montre une augmentation des acides gras monoinsaturés, particulièrement l'acide oleique C18 :1(n-9) à la basse salinité. Une réduction des pourcentages des acides gras polyinsaturés de la famille (n-3) : C18 :4(n-3), C20 : 5(n-3), C22 : 5(n-3) et C22 : 6 (n-3). L'acide linoleique C18:2 (n-6) présente des pourcentages élevés à l'eau douce alors que l'acide arachidonique révèle des pourcentages réduits.

Mots clés : *Liza ramada* ; osmoregulation; branchies ; composition en acide gras ; acclimatation à l'eau douce

ABSTRACT

Thin–lipped grey mullet (*Liza ramada*) adapted to sea water (SW, 35ppt) were submitted to abrupt osmotic stress by transferring the specimens to Freshwater (FW, 0.5 ppt) for 24 hours. The adaptation capabilities of *Liza ramada* were evaluated using parameters such as osmolality, blood hematocrit and red blood cells**.** Variations of fatty acid composition in the gills of the thin lipped grey mullet *liza ramada* subjected to direct exposure to freshwater were determined. The plasma osmolality, blood hematocrit and red blood cells decreased within the first 24 h. There were quantitative and qualitative differences between the fatty acids composition of *liza ramada* gill in response to the media salinity. The monounsaturated fatty acids (MUFA), particularly oleic acid C18:1(n-9) increase at lower salinity. The polyunsaturated fatty acids (PUFA) decreased with decreasing salinity due primarily to changes in n-3 PUFA especially C18:4(n-3), C20: 5(n-3), C22: 5(n-3) and C22: 6(n-3).Higher linoleic acid C18:2(n-6) content observed in fresh water fish was accompanied by a significant decrease of arachidonic acid C20:4(n-6).

Keywords: *Liza ramada*; osmoregulation; gills; Fatty acids composition; freshwater acclimation

INTRODUCTION

Successful salinity acclimation may require a metabolic reorganization to meet the increased energetic demands associated with the exposure to the new environmental salinity. Euryhaline fish showed several metabolic changes and spent large amounts of energy, particularly in osmoregulatory

(gills, intestine and kidney) and metabolic (liver) organs, to compensate for these salinity changes (Susana Sangiao-Alvarellos *et al*., 2005).

Although ionoregulation in fish is mediated by a group of structures including the gastrointestinal epithelium and kidney. The gill is the major site in the balance of ion movement between diffusional gains or losses (Evans, 1993).

The gills are probably the organs that consume most energy during osmoregulation since they must ensure isosmotic regulation of intracellular fluid and also anisosmotic regulation of extra cellular fluid (Sangiao-Alvarellos *et al*., 2003).

The mullets possess osmoregulation abilities which appear early during the developpement (Norldlie *et al.*, 1982) and allow them to maintain elevated growth rates also under hypo-osmotic conditions (Cardona and Castello-Orvay, 1997).

Stocking the impounded waters with mullets fry could be done in most North African regions and in most coastal countries with arid climate where management of fresh water resources implies the utilisation of reservoirs (Ben khemis *et al.*, 2006).

In Tunisia, fry of this species are widely exploited in stoking the dam reservoirs. So annually, millions of mullet fry (8 millions during the season 2005-2006) are transferred from coastal areas to inland water and artificial lakes. In addition, extensive mullet farming contributes to satisfy the increased local market's demand (El Cafsi *et al*., 2003). Among, five species of Mugilidie in Tunisia, *Liza ramada* euryhaline and eurytherme has a better growth in dam lakes. Additionally, the thin lipped grey mullet *Liza ramada* are commercially the most important and suitable for culture because of their fast growth compared with *Liza aurata* and *Liza saliens* (Hotos and Vlahos, 1998)

During the transfer of fry from sea to freshwater, fish are exposed to some environmental variations temperature, dissolved oxygen and especially salinity, which may lead to physiological perturbations negatively affecting growth and physiological quality of the future adult fish.

Many studies have analysed the effects of environmental salinity and there are few studies available on gill fatty acid composition of *Liza ramada*. Electron micrographs reveal moderate amounts of lipid deposits in fish (Morgan and Tovell, 1973), the tissue may possess a surprising capacity to synthesise lipids (Hansen *et al.*, 1999, 2002). Schmid and Barden, 1965 concluded that the lipid was likely an important factor in preventing body fluid dilution by acting as a barrier to the inward flux of water. It seems reasonable to suggest that gill surface lipids could likewise be involved in a tightening of the gill membrane against both an inward flux of water and a passive ion loss to a hypoosmotic ambient medium.

The present work's objective examines the possible variations of plasma osmolality, hematocrit and fatty acid composition in the gills of juveniles mullet *Liza ramada* subjected to sudden acclimation to freshwater.

MATERIALS AND METHODS

1 *Fish and maintenance*

Immature mullet (*Liza ramada*) were collected around the Khelij entrance into the Tunis gulf. *Liza ramada* originating from the same population (same area, capture, period and size) had a total length of 8.5 ± 0.5 cm and weighed between 4.8±0.7 mg were transferred to the laboratory at faculty of Sciences (Tunis, Tunisia) . They were acclimated to seawater (35ppt-1113 mOsm/kg) in 400 l aquaria in closed circuit, where the water recirculated through mechanical and biological filters. Fish were fed daily with commercial food (for European sea bass, Valencia, Spain) at 1% of body mass and were fasted for 24 h before sampling. During the experiments, the photoperiod was maintained at 14 h light: 10 dark and temperature was held between 18-20°C. The water salinity was checked every day and corrected when it was necessary. No mortality was observed during the experiments.

2 *Acclimation protocol*

To determine the salinity tolerance and osmoregulation capabilities of the juvenile mullet, two types of acclimation trials were conducted. First, fish were transferred from initial salinity (8-15ppt (the Khelij entrance)) to seawater (35ppt) in 50 l aquaria during an initial acclimation period (30 days). Fish were then transferred directly into freshwater (0.5ppt) and seawater (35ppt) during a period of 24 hours. The salinity of appropriate experimental tanks was reduced to 0.5 ppt via the addition of tap water (aerated in 1000 –l holding tanks for 2-3 days prior to addition) which was fully aerated and temperature regulated.

3 *Sampling*

For both experiments, after 24 hours, twelve fish were captured carefully with net, killed by immersion in liquid nitrogen, weighed and sampled.

Blood was obtained in ammonium-heparinised syringes from the ventral aorta. Gill arches were removed and quickly stored at – 30 °C until determination of fatty acid composition.

4 *Analytical techniques*

Hematocrit values (Hc, %) was determined by centrifuging (2600*g*, 5min) the blood for 10 min in a microhematocrit centrifuge and measuring in on microcapillary reader. Red blood cells were counted under the light microscope using a Neubauer

haemocytometer after blood dilution with phosphate-buffered saline.

Blood osmolality was determined with duplicate 10µl sample using a micro-osmometer. Distilled water (0 mosm/kg) and a sea water (300 mosm /kgH_2O) calibration solution were used as reference.

Extraction of total lipid from gill filaments was performed by the method of Folch *et al*. (1957) (chloroform/ methanol; 2:1, vol/vol).

Fatty acid from total lipids was transformed into their corresponding methyl esters as described by Cecchi et al. (1985).Transmethylation was made by the addition of 2ml of hexane,0.5 ml of 3% sodium methylate, a known amount of methyl heptadecanoate acid (C17:0) as the internal standard, 0.2 ml of 1N H2SO4 and 1.5 ml of 10% sodium chloride. The superior phase that contains fatty acid methyl esters was aspired and the solvent volume reduced in a stream of nitrogen, prior to analysis.

The fatty acid methyl esters were analyzed on a HP 6890 gas chromatograph (Agilent Palo Alto, CA, USA) equipped with a flame ionization detector (FID). The esters were separated on a RT-2560 capillary column (100m length, 0.25mm i.d., 0.20mm film thickness). The oven temperature was kept at 170 °C for 2 min, followed by a 3 °C/min ramp to 240 °C and finally held there for an additional 15 min period. Nitrogen was used as carrier gas at a flow rate of 1.2 ml/min. The injector and detector temperatures were maintained at 225 °C. Methyl esters were identified with the aid of authentic standard mixtures (sigma).Peak areas were calculated automatically using a logiciel HP Chemstation (Rev .A. 0401).

The different fatty acids in mullet were obtained by comparing the retention times of the fatty acids under study and those of a mixture of methyl esters (SUPELCO PUFA-3)

The fatty acids quantification is based on an internal standard (heptadecanoate acid (C17:0)) not present in our samples. Peaks comprising less than 0.1% of the total area were not considered. Biochemical results were expressed as percentage of the total organ dry weight.

2.3 *Statistics*

All results are expressed as means ± SEM. Data were analysed by one way ANOVA, and when ever a significant effect was indicated ($p<0.05$), the Duncan test was used to compare treatment means. Statistical analyses were carried out using the software STATISTICA6.0.

RESULTS

1 *Osmoregulation*

No change in behaviour or activity levels was observed in the fish during the course of the trial. The juvenile mullet were hyperosmotic in SW while in FW were hyposmotic. These observations were supported by the significant variations in blood parameters. Mean values for the haematological parameters observed throughout the experience are summarized in table I.

Table I: Osmololality, hematocrit and Red blood cells in thin lipped grey mullet (*Liza ramada*) at different water salinity (0.5, 35 ppt) during hypo osmotic exposure.

	Salinity	
	35ppt (SW)	0.5ppt (FW)
Osmolality (mosm/Kg)	341 ± 75.33*	234 ± 37*
HC (%)	25 ± 5.01*	15.37 ± 4.77*
RBC (10^6 / mm^3)	1.09 ± 0.40*	0.48 ± 0.42*

Each value is the mean ± S.E.M of n=6 per group.

In 100% SW, the maintainable level of plasma osmolality was 341±75.34 mosm/Kg respectively. This value has significantly changed at 24 h after SW adapted fish were transferred to FW. Their blood osmolality decreased significantly (234± 37.01mosm/ Kg).

Regarding haematology hematocrit and erythrocyte number, they show important variations and similar responses to in decreased salinity.

Hematocrit values ranged from 25 ± 5. 01% to 15. 37 ± 4. 77% throughout the study. Hematocrit levels differ among treatment groups until 24 h, at which time the low-salinity group had an hematocrit level significantly lower than the seawater group. RBC values decreased significantly with passage at low salinity from1.09 ± 0.4010^6 to 0.48 ± 0.42 10^6 / mm^3.

2 Fatty acid of gills

Water Salinity has significantly affected the fatty acids composition of the mullet gills as demonstrated in the Table II.

Comparing of the major classes of fatty acids (saturated, monounsaturated, polyunsaturated), our results revealed, that after 24h in freshwater, there were quantitative and qualitative differences between the fatty acids composition of *liza ramada* gill in response to the water salinity. Palmitic acid was the predominant saturated fatty acid in both types of fish .The monounsaturated fatty acids (MUFA), particularly oleic acid C18:1(n-9) increase at lower salinity. The polyunsaturated fatty acids (PUFA) decreased with decreasing salinity due primarily to changes in n-3 PUFA especially C18:4(n-3), C20:

5(n-3), C22: 5(n-3) and C22: 6(n-3)**.** Linoleic acid C18:2(n-6) content was higher in fresh water fish and was accompanied by a significant increase of arachidonic acid C20:4(n-6).Finally, total n-3 values did not change significantly with lower environmental salinity whereas total n-6 showed a significant increase.

The salinity of the water seems to cause an important change in the fatty acid pattern.

Table II: Gill-fatty acid (%total gill lipids) of mullet *liza ramada* at different salinity (0.5, 35ppt) during hypoosmotic exposure.

	Salinity (ppt)	
	35 (SW)	0.5 (FW)
C14 :0	4.63 ±0.16	5.21±0.21
C16 :0	15.63±0.75	16.54±0.27
C18 :0	4.84±0.71	4.16±0.24
SFA	**25.91±0.67**	**24.79± 0.20**
C16:1(n-7)	7.04±0.72	6.49±0.34
C18:1(n-9)	17.45±0.14	5. 35±2.04
C18:1(n-7)	3.37±0.45	3.95±0.71
C20:1(n-9)	4.95±2.07	15.71±1.59
22:1(n-11)	2.77±2.47	1.62±0.87
MUFA	**34.81±1.38**	**32.03±0.78**
C18:2(n-6)	6.36±0.82	26.24±0.73
C20:4(n-6)	5.33±0.65	1.07±0.18
PUFA (n-6)	**11.69**±0.73	**27.31**± 0.97
C18:3(n-3)	4.56±0.05	3.23±0.09
C18:4(n-3)	5.74±0.91	1.30±0.08
C20:5(n-3)	5.55±0.11	3.37±0.76
C22:5(n-3)	3.29±0.39	1.97±0.13
C22: 6(n-3)	9.18±0.23	6.23±0.02
PUFA (n-3)	**28. 32±1.59**	**16.10 ±0.76**
n-3/n-6	**2.43 ± 0.22**	**0.65±0.01**

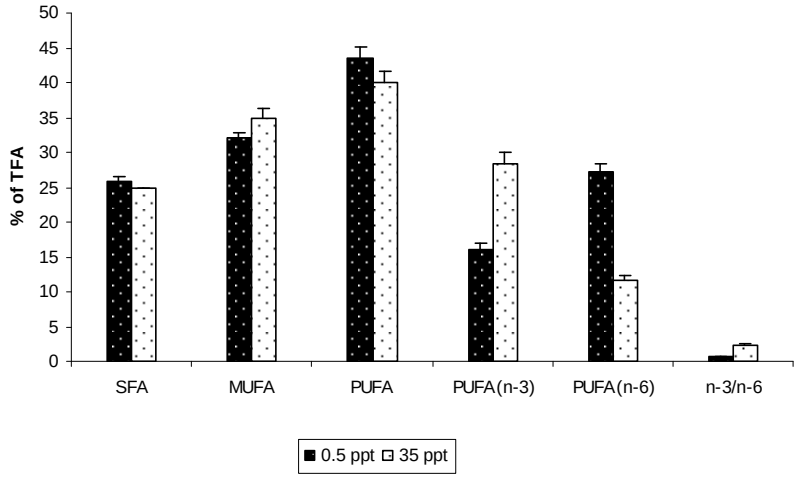

Fig .1. The distribution of fatty acids (% total gill lipids) into saturated, monounsaturated, and polyunsaturated fatty acids mullet gill.

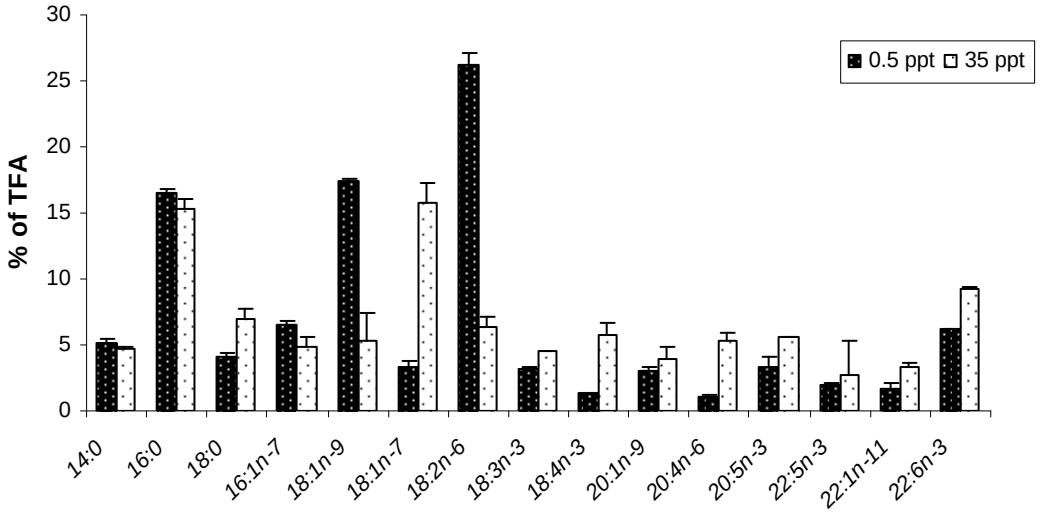

Fig.2. The fatty acid composition of gill from mullet *liza ramada* at different water salinity (0.5, 35ppt)

DISCUSSION

In the present study *Liza ramada* seems able to tolerate direct exposure to an hyposmotic environment of 0.5ppt without mortality. In spite of their adaptation possibility to salinity change, low salinity seems to be a case of halin stress for *liza ramada* juveniles which is a primary marine species**.** This is in according with observations on the osmoregulatory capacity of other species of marine fish such as *Chelon labrosus* (Lasserre *et al.*, 1975); *Mylio macrocephalus* (Woo and Wu, 1982); *Gadus morhua* (Dutil *et al*., 1992); *Hippoglossoides platessoides* (Munro *et al.*, 1994); *Sparus aurata* (Sangiao-Alvarellos et al., 2003, 2005; Laiz-Carrion et al., 2005a,b); *Solea senegalensis* (Arjona et al., 2007).

The results of the present work show that the plasma osmolality, blood hematocrit and Red blood cells decreased within the first 24 h. These observations suggest also that during the first 24 h in FW, the mullet gill water permeability is high.

During the first stage of acclimation, the Hematocrit, and red blood cell decreased (Table I).These changes can be attributed to changes in the water content in the blood, caused by the change in environmental salinity (Plaut, 1998). Thus, in hyper osmotic environment, at the beginning of exposure, the fish would lose water passively, and thereby undergo increases in the blood cell element concentrations. After wards, the compensatory increase in water ingestion would provide a transitory dilution of the blood parameters (Martínez-Álvarez *et al*., 2002).

Salinity osmolality values are significantly lower in dilute media, it is suggested that a significant degree of haemolysis is produced by disruption of blood cells due to the influx of water. Ciccotti *et al*. (1994) found that osmolality could be considered as a good marker of functional adaptation to FW in *Mugil cephalus*: its decreasing trend followed the salinity decrease, although a significant recovery was observed over the 3 weeks period in FW.

Fishes respond in different ways to maintain homeostasis after stress (Wedemeyer *et al*., 1990). Many physiological changes are involved in such a stress response including haematology (Dethloff *et al*., 1999), osmolality (McDonald et Milligan, 1997), hormone release, and energetic metabolism (Barton and Iwama, 1991;Carragher and Rees, 1994)

The ability to withstand salinity variation depends upon osmoregulatory capacity and metabolic reorganization to provide related energetic support. Lipids may play an important role in supplying energy requirements during osmotic stress **(**Jarvis and Ballantyne, 2003**)**.

In teleosts, it has been reported that a change in lipid composition from a freshwater pattern, relatively low in PUFAs, to a marine pattern, relatively rich in long-chain PUFAs, occurs as a response to seawater entry **(**Sheirdan *et al*., 1985, Li and Yamada, 1992**)** and that incorporation of some fatty acids into gills may indicate a specific physiology function for these fatty acids in relation to the capability of osmoregulation (Daikoku *et al.*, 1982, Bell and sargent, 1987).

The effects of the variation water salinity had repercussions on the qualitative plan, which is on the percentages of the various fatty acids. Our results show that the decrease in salinity was followed by an insignificant decrease in the percentage of the SFA and MUFA associated to a significant decrease of the total PUFA and a significant increase of the PUFA (n-6).

When comparing levels of individual fatty acids among groups at each time period, numerous

differences were detected. The decrease in water salinity was followed by an increase in C20:1(n-9) and C18:2(n-6) percentages. These results are in accordance with those of Lovern (1964); Ackman *et al.* (1969); Meister (1971); Yamada et Hayashi (1975), El Cafsi (1998) and Khériji *et al.* (2004).

The monounsaturated fatty acid decreases could be related with their importance as energy source, with 18:1(n-9) and 16:1(n-7) acids being the preferred substrates for catabolism **(**Izquierdo, 1996**).**

The low level of C18:2(n-6) is probably a marine fat characteristic as most freshwater fish have fats with 5% or more of this acid (Ackman, 1967; Farkas and Herodek, 1967; Mangold ,1973; Reichwald and Meizies 1973 **)**. Our results demonstrated that after 24 hours in dilute seawater the gills of *Liza ramada* contained large quantities of the linoleic acid C18:2(n-6). (6, 36±0, 73 vs 26, 24±0, 82) and much lower quantities in the seawater samples.

Dantagnan *et al.* (2007) mentioned that the C18:2(n-6) acid content was higher in freshwater fish and its reduction during embryo and larval development was accompanied by a significant increase of C20:4(n-6). This was not observed in embryos or larvae of wild or reared brood stock fish, suggesting certain elongation and desaturation of C18:2(n-6) to form C20:4(n-6) only in fish coming from the freshwater environment.

The PUFA (n-3) decrease during the passage to the low salinity, but it is necessary to indicate especially that only C18:4(n-3), C20: 5(n-3), C22: 5(n-3) and C22: 6(n-3) among all the PUFA (n-3) group that have a highly significant decrease ($p < 0.05$) of their proportions.

Khériji *et al.* (2004) demonstrated that the decrease in the salinity of the media induces the decrease of some polar lipid PUFA such as the C22:6(n-3) and the C20:4(n-3) in freshwater fish where the C22:5(n-3) and the C20:5(n-3) are absent in muscle of *Mugil cephalus* fry.

The lipids of marine fish contain proportionally more PUFAs (especially those of n-3 long chains) and a greater n-3/ n-6 relationship than the lipids of freshwater fish. (Martinez-Alvarez *et al*., 2005).

Several studies have shown that acclimation from FW to SW produce an increase in the amount of polyunsaturated fatty acids (PUFA) in gills of several fish species like rainbow trout (Hansen *et al.*, 1992), masu salmon (Li and Yamada, 1992), Atlantic salmon (Tocher *et al.*, 2000), sea bass (Cordier *et al.*, 2002), and eel (Hansen *et al*., 2002). These changes cause increased fluidity of the membranes at the time of hyperosmotic acclimation. Apart from these changes in composition, only a few studies have assessed changes in lipolytic capacity of gills during osmotic acclimation addressing an increased capacity (Li and Yamada, 1992).

Water salinity has an effect on composition, particularly, the PUFA levels of fish and the ratio n-3/ n-6 is much lower in fish living in a FW than a SW environment (Steffens, 1997).

For instance, we observed that the ratio n-3/ n-6 PUFA can differ considerably between freshwater and marine environments: The n-3/n-6 average ratios are 0.65 and 1.99 for freshwater and marine fish gill, respectively. The n-6 series tends to predominate in fresh water while the n-3 series appears to predominate in saltwater environments (Cowey & Sargent, 1972; Jobling, 1994).

Water permeability could be reduced by changing the fatty acid composition of phospholipids in gills, both at high and low salinity, to decrease water permeability and avoid excessive water gain at low salinities or loss at high salinities (hurtado *et al*., 2007).

Finally the nature and lipid quantities in fish vary according to species and habitat (Ackman and Eaton, 1966; Ackman, 1967). Furthermore, these results suggest a predominant effect of the environment on fatty acid profiles to physiologically prepare the adapting fry to lower salinity conditions, directly affecting electrolyte transport across membranes and indirectly affecting osmoregulation through certain derivatives such as prostaglandins.

The relation of HUFA and osmoregulation is also supported by the higher HUFA content in their issues of marine fish and other animals than their equivalents from freshwater environments (Bell *et al*., 1986; Sargent *et al*., 1990).

CONCLUSION

The present study confirms the capacity of juvenile mullet to tolerate abrupt changes in salinity and the fish osmoregulate well over a wide range of salinities. The results show that environmental salinity has direct effects on the fish lipid metabolism and suggest that variations of gill fatty acids may have a role in osmoregulatory mechanisms. These characteristics make mullet a very good model fish for research in osmoregulation.

Acknowledgments

The authors thank the members of ecophysiology Laboratory in Tunis for their help.

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