Effect of salinity on the biosynthesis of n-3 long-chain polyunsaturated fatty acids in silverside *Chirostoma estor*.

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Abstract

The genus *Chirostoma* (silversides) belongs to the family Atherinopsidae, which contains around 150 species, most of which are marine. However, Mexican silverside (*Chirostoma estor*) is one of the few representatives of freshwater atherinopsids and is only found in some lakes of the Mexican Central Plateau. However, studies have shown that *C. estor* has improved survival, growth and development when cultured in water conditions with increased salinity. In addition, *C. estor* displays an unusual fatty acid composition for a freshwater fish with high docosahexaenoic acid (DHA) : eicosapentaenoic acid (EPA) ratios. Freshwater and marine fish species display very different essential fatty acid metabolism and requirements and so the present study investigated long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis to determine the capacity of *C. estor* for endogenous production of EPA and DHA, and the effect that salinity has on these pathways. Briefly, *C. estor* were maintained at three salinities (0, 5 and 15 ppt) and the metabolism of ^{14}C-labelled 18:3n-3 determined in isolated hepatocyte and enterocyte cells. The results showed that *C. estor* has the capacity for endogenous biosynthesis of LC-PUFA from 18-carbon fatty acid precursors, but that the pathway was essentially only active in saline conditions with virtually no activity in cells isolated from fish grown in freshwater. The activity of the LC-PUFA biosynthesis pathway was also higher in cells isolated from fish at 15 ppt compared to fish at 5 ppt, The pathway was around 5-fold higher in hepatocytes compared to enterocytes, although the majority of 18:3n-3 was converted to 18:4n-3 and 20:4n-3 in hepatocytes whereas the proportions of 18:3n-3 converted to EPA and DHA were higher in enterocytes. The data were consistent with the hypothesis

that conversion of EPA to DHA could contribute, at least in part, to the generally high DHA:EPA ratios observed in the tissue lipids of *C. estor*.

Introduction

Mexican silverside (*Chirostoma estor,* also reported as *Menidia estor*) from lake Pátzcuaro is one of the most valued freshwater fish in Mexico. The species, locally known as “pez blanco”, has greatly influenced the cultural environment and economy of the native people of the region. The species is now endangered because of a range of factors including over-fishing, environmental degradation of the lake and introduction of exotic species (Martínez-Palacios et al. 2008). However, silverside is a species with high potential for aquaculture commanding a good price in regional markets ($40-80 USD kg^{-1}) (Martínez-Palacios et al. 2008). Recently, there have been efforts to preserve the species through aquaculture techniques (Martínez-Palacios et al. 2002, 2003, 2004, 2006, 2007). The genus *Chirostoma* (silversides) belongs to the family Atherinopsidae, which contains around 150 species, most of which are marine. However, *C. estor* is one of the few representatives of freshwater atherinopsids and is only found in some freshwater lakes of the Mexican Central Plateau. Therefore, although silverside is a freshwater species, it shares many characteristics in common with marine Atherinopsids because of their common ancestry (Barbour 1973). Thus, aquaculture of Mexican silverside *C. estor* involves the transfer of the fish to different salinities over the whole cycle of production from incubation of the eggs to juvenile development. Specifically, silverside has better growth and survival when cultured in saline conditions (Martínez-Palacios et al. 2004).

. *C. estor* is also considered to be a carnivorous species and so their essential fatty acid (EFA) requirements were expected to be more similar to that of a marine carnivorous species than that of a typical freshwater fish (Martínez-Palacios et al.

2008). It has been reported that most freshwater fish studied, in contrast to the marine species studied, have the ability to elongate and desaturate 18-carbon polyunsaturated fatty acids (PUFA) (18:2n-6, linoleic acid/LOA and 18:3n-3 α-linolenic acid/ALA) to long-chain PUFA (LC-PUFA) of 20 carbons (20:4n-6, arachidonic acid/ARA and 20:5n-3, eicosapentanoic acid/EPA) and 22 carbons (22:6n-3, docosahexaenoic acid/DHA) (Tocher 2010). Thus, it is generally assumed that LOA and ALA can satisfy EFA requirements for freshwater species but ARA, EPA and DHA are the required EFA for marine species (Sargent et al. 1995a). However, the feeding habits of fish may also be determinants of precise EFA requirements: carnivorous fish obtain the biologically active LC-PUFA directly from their diet and consequently they now have only a low ability to desaturate and elongate 18-carbon fatty acids whereas herbivorous fish have higher levels of C_{18} PUFA and lower LC-PUFA in their diet and so have retained the ability to convert C_{18} PUFA to LC-PUFA (Sargent et al. 1999). If the assertion that *C. estor* may have characteristics of a marine species is correct, then ARA, EPA and DHA would need to be included in the diet to satisfy their nutritional requirements.

There is scarce information of the lipid and fatty acid compositions and metabolism in *C. estor* (Palacios et al. 2007). Wild fish contained high levels of DHA (20 - 32% of total fatty acids) but surprisingly low levels of EPA (1 - 3%) in contrast with the fatty acid profile found in samples of zooplankton, its natural diet (12% DHA, 13% EPA) (Martínez-Palacios et al. 2003). There are two possible explanations for these findings; firstly, that *C. estor* selectively accumulates DHA preferentially over other fatty acids such as EPA depending on its own physiological requirements, or secondly, that this species has the capacity to convert EPA and/or other n-3 series fatty acids to DHA (Tocher 2003). The second explanation is also supported by the

98 presence of DHA in larvae fed rotifers with a low DHA / high ALA composition
99 (Martínez-Palacios et al. 2006).

100 Salinity has been shown to affect lipid and fatty acid composition in salmonids
101 although many effects occurred in advance of seawater transfer during parr-smolt
102 transformation (Bendiksen et al. 2003; Peng et al. 2003). It was also shown that the
103 activity of the LC-PUFA synthesis pathway was regulated by environmental cues in
104 Atlantic salmon (*Salmo salar*) and peaked around seawater transfer and was
105 considerably lower during the seawater phase (Bell et al. 1997; Tocher et al. 2000).
106 These changes in activity reflected changes in the expression of fatty acyl
107 desaturase genes in freshwater and seawater phases (Zheng et al. 2005). There are
108 also several studies reporting fatty acid compositions in fish reared at different
109 salinities (Cordier et al. 2002; Kheriji et al. 2003; Haliloglu et al. 2004; Martinez-
110 Alvarez et al 2005; Dantagnan et al. 2007; Navarro et al. 2009; Xu et al. 2010; Hunt
111 et al., 2011). Results are not consistent with increased associated with both reduced
112 (Ciordier et al. 2002; Kheriji et al. 2003) and increased (Xu et al. 2010; Hunt et al.
113 2011) levels of LC-PUFA including EPA and DHA. In contrast, the effects of salinity
114 on lipid and fatty acid biochemistry and metabolism have been little studied in non-
115 salmonid fish although the modulation of Δ6 fatty acyl desaturase in teleosts was
116 recently reviewed, with the effects of salinity again being variable (Vagner and
117 Santigosa 2011). However, the expression of Δ6 desaturase was higher in liver of
118 rabbitfish (*Siganus canaliculatus*) and red sea bream (*Pagrus major*) reared at lower
119 salinity (10-15 ppt) compared to fish reared at higher salinity (32-33 ppt) (Li et al
120 2008; Sarker et al 2011).

121 The present study aims to investigate the two issues of salinity preference and
122 LC-PUFA metabolism in *C.estor* to determine if there is a relationship between them.

The basic hypothesis investigated was that LC-PUFA synthesis in *C. estor* will be influenced by ambient salinity, and that the improved performance at increased salinity will be related to this interaction between salinity and LC-PUFA biosynthesis. The specific objectives were to determine the pathways and activities of LC-PUFA biosynthesis in *C. estor* in order to elucidate potential mechanisms underpinning its uncommon fatty acid profile and if the pathway reflects its marine ancestry, and to determine if salinity affects the biosynthesis of LC-PUFA in a way that can explain the apparent preference of *C. estor* for saline conditions (Martínez-Palacios et al. 2004).

Materials and methods

Experimental fish

Forty-five juvenile silverside (*Chirostoma estor*) of average initial weight around 50g were obtained from a research production plant (UMSNH, Michoacan, Mexico). Fish were maintained in glass-fiber tanks of 40cm high x 60cm diameter and 100L capacity with constant aeration and temperature control (25± 0.4^{o}C). All the experimental units were maintained in a 12:12 dark:light photoperiod. All fish were fed a standard commercial pellet feed (see Palacios et al. 2007), and every three days tanks were siphoned and 30% of the water was renewed in order to maintain high water quality with dissolved oxygen, nitrites, nitrates, pH, and total ammonia monitored at 3-day intervals. The experimental design consisted of three salinity treatments: freshwater (0 ppt; i.e < 0.05), 5 ppt and 15 ppt of salinity, each in triplicate with 5 fish per tank (15 per treatment). Different salinities were obtained by using artificial seawater (Instant Ocean Synthetic sea salt, Aquarium Systems) and

UV filtered ground water. Fish were fed a diet consisting of *Artemia franciscana* and a commercial feed (1:3) four times per day over 15 days prior to experimentation.

Preparation of isolated hepatocytes and enterocytes

With some modifications, the method for the preparation of isolated hepatocytes and enterocytes established for salmonids was followed (Bell et al. 1997; Tocher et al. 1997, 2002). Briefly, six fish from each salinity treatment (two per tank) were sacrificed with an overdose of benzocaine (50-60mg L^{-1}) to minimize stress (Ross et al. 2007) and the livers and intestinal tracts dissected immediately. The livers and intestines of two fish (i.e. per tank) were pooled for each sample so that there were 3 liver and 3 intestinal samples per treatment. The gall bladder was removed carefully from the liver, the main blood vessels trimmed, and the liver perfused via the hepatic vein with solution A (calcium and magnesium-free Hanks balanced salt solution (HBSS) containing 10 mM HEPES) to clear blood from the tissue. The liver was chopped finely and about 0.5 g was taken and incubated with shaking in 20 ml of solution A containing 0.1% (w/v) collagenase in a temperature controlled incubator at 25 ^{o}C for 45 min. Digested liver tissue was filtered through 100 µm nylon gauze and the cells collected by centrifugation at 300 x g for 2 min. The cell pellet was washed with 20 ml of solution A containing 1% w/v fatty acid-free bovine serum albumin (FAF-BSA) and re-centrifuged. The washing was repeated with a further 20 ml of solution A without FAF-BSA. The hepatocytes were resuspended in 10 ml of Medium 199 containing 10 mM HEPES. One hundred µl of cell suspension was mixed with 400 µl of the vital stain, Trypan Blue, and hepatocytes counted and viability assessed using a haemocytometer.

With relatively minor modification, the above method was used to isolate enterocyte-enriched preparations from *C. estor* intestine as described previously for caecal enterocytes from salmon (Fonseca-Madrigal et al. 2006). Briefly, entire intestinal tracts were dissected, cleaned of adhering adipose tissue, and luminal contents rinsed away with solution A before being chopped finely and incubated with 0.1% (w/v) collagenase as above. The digested intestinal tissue was filtered through 100 µm nylon gauze and the cells collected, washed, resuspended in medium (as above), and viability checked as for hepatocytes. The enriched enterocyte preparation was predominantly enterocytes although some secretory cells were also present.

Viability of both isolated cell preparations was > 95% at isolation and decreased by less than 5% over the period of the incubation. One hundred µl of the hepatocyte and enterocyte suspensions were retained for protein determination according to the method of Lowry et al. (1951) after incubation with 0.4 ml of 0.25% (w/v) SDS/1M NaOH for 45 min at 60 ^{o}C.

Assay of hepatocyte and enterocyte fatty acyl desaturation/elongation activities

Six ml of each hepatocyte or enterocyte suspension were dispensed into 25 cm^2 tissue culture flasks and incubated at 20^{o}C for 2h with 0.3 µCi (~ 1 µM) [1-^{14}C]18:3n-3 or [1-^{14}C]20:5n-3, added as complexes with FAF-BSA in phosphate buffered saline as described previously (Ghioni et al. 1997). After incubation, the cell suspensions were transferred to glass conical test tubes and centrifuged at 500 x g for 2 min. The supernatants were discarded and the cell pellets washed with 5 ml of ice-cold HBSS/FAF-BSA. The supernatant was carefully discarded and total lipid extracted

198 from the cell pellets using ice-cold chloroform/methanol (2:1, v/v) containing 0.01%
199 (w/v) BHT as described in detail previously (Tocher and Harvie 1988). Fatty acid
200 methyl esters (FAME) were prepared from total lipid by acid-catalyzed
201 transesterification using 2 ml of 1% H_2SO_4 in methanol plus 1 ml toluene as
202 described by Christie (1993), and FAME extracted and purified as described
203 previously (Tocher and Harvie 1988). The methyl esters were redissolved in 100 µl
204 isohexane containing 0.01% BHT and applied as 2.5 cm streaks to TLC plates
205 impregnated by spraying with 2 g silver nitrate in 20 ml acetonitrile and pre-activated
206 at 110^{o}C for 30 min. Plates were fully developed in toluene/acetonitrile (95:5, v/v)
207 (Wilson and Sargent 1992) and autoradiography performed with Kodak MR2 film for
208 6 days at room temperature. Areas of silica containing individual PUFA were scraped
209 into scintillation mini-vials containing 2.5 ml of scintillation fluid (Ultima Gold, Perkin
210 Elmer, Monterrey, Mexico) and radioactivity determined in a scintillation ß-counter
211 (Beckman LS Analyzer, Beckman Coulter de Mexico SA, Mexico City).
212
213 *Statistical analysis*
214
215 All the data are presented as means ± SD (n = 3) and all statistical analyses were
216 performed using S-Plus 2000 Professional Release 2 (MathSoft, Inc., Cambridge,
217 MA, USA). The effects of salinity on LC-PUFA synthesis was analyzed by one-way
218 ANOVA followed, where appropriate, by Tukey's post-test to determine significant
219 differences between individual treatments (Zar 1999).
220
221 *Materials*

[1-^{14}C]18:3n-3 and [1-^{14}C]20:5n-3 (50-55 mCi/mmol) were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). HBSS, Medium 199, HEPES buffer, collagenase (type IV), FAF-BSA, BHT, silver nitrate and all solvents (HPLC grade) were obtained from Sigma Chemical Co. (St. Louis, MO, USA.). Thin-layer chromatography (TLC) plates, precoated with silica gel 60 (without fluorescent indicator) were obtained from Merck (Whitehouse Station, NJ, USA).

Results

Desaturation and elongation of ALA, [1-^{14}C]18:3n-3

Irrespective of tissue, activity of the LC-PUFA synthesis pathway from 18:3n-3 was very low in fish maintained in freshwater. Increasing salinity resulted in significantly increased LC-PUFA synthesis in both hepatocytes and enterocytes as measured by the recovery of radioactivity in the summed desaturated products (18:4, 20:4, 20:5, 22:5 and 22:6) of [1-^{14}C]18:3n-3 (Fig.1). In both cell types, the rate of LC-PUFA synthesis was highest in fish cultured at 15 ppt salinity, with rates of 0.41 ± 0.10 and 0.09 ± 0.04 pmol/h/mg protein in hepatocytes and enterocytes, respectively. These values were 50- and 5-fold higher in hepatocytes and enterocytes, respectively, than the activity observed in fish in freshwater. In both tissues, LC-PUFA synthesis at the 5 ppt salinity was intermediate between the activities in freshwater and 15 ppt salinity with values of 0.13 ± 0.01 and 0.06 ± 0.01 pmol/h/mg protein in hepatoctes and enterocytes, respectively. The LC-PUFA synthesis activity was 2.3- and 4.6-fold higher in hepatocytes than in enterocytes at 5 and 15 ppt, respectively (Fig.1). The rank order for recovery of radioactivity in desaturated products of 18:3n-3 was 18:4

>20:4 >22:6 >22:5 > 20:5 in hepatocytes (Fig. 2) whereas in enterocytes it was 20:4 >20:5 > 22:6 > 22:5 > 18:4 (Fig 3). In hepatocytes, recovery of radioactivity in DHA exceeded that recovered in EPA, with the recovery of radioactivity in EPA and DHA combined amounting to around 25% of the total radioactivity recovered (Fig. 2). In contrast around 50% of total radioactivity recovered in enterocytes was as EPA and DHA combined. Furthermore, in enterocytes, the recovery of radioactivity in EPA increased, and that in DHA decreased, with increasing salinity (Fig. 3).

Desaturation and elongation of EPA, [1-^{14}C]20:5n-3

As with [1-^{14}C]18:3n-3, desaturation/elongation activity towards EPA in tissues from fish maintained in freshwater was very low and increasing salinity significantly increased desaturation/elongation activity in both hepatocytes and enterocytes as measured by the recovery of radioactivity in the summed products (22:5 and 22:6) of [1-^{14}C]20:5n-3 metabolism (Fig. 4). However, in contrast to LC-PUFA synthesis from [1-^{14}C]18:3n-3, the activity in hepatocytes was similar in fish at both 5 and 15 ppt salinity with values of 0.37 ± 0.16 and 0.36 ± 0.15 pmol/h/mg protein, respectively. In enterocytes, highest activity was obtained in fish at 5 ppt salinity, with a value of 0.13 ± 0.01 pmol/h/mg protein compared to 0.05 ± 0.02 in fish reared at 15 ppt (Fig. 4). Similar to the data obtained with [1-^{14}C]18:3n-3, the LC-PUFA synthesis activity from [1-^{14}C]20:5n-3 was 2.9- and 7.0-fold higher in hepatocytes than in enterocytes from fish reared at 5 ppt and 15 ppt, respectively (Fig.4). There was also a significant difference in the products of [1-^{14}C]20:5n-3 metabolism between hepatocytes and enterocytes irrespective of treatment. The rank order for recovery of radioactivity in products of 20:5n-3 metabolism was 22:6 >22:5 in hepatocytes, with the recovery of

radioactivity in DHA increasing with salinity with percentages of 57%, 65% and 78% at 0, 5 and 15 ppt, respectively (Fig. 5). In enterocytes, the recovery of radioactivity in 22:5 exceeded the recovery in 22:6, with approximately 25%, 10% and 37% of radioactivity recovered in DHA at 0, 5 and 15 ppt salinity, respectively (Fig 6).

Discussion

The primary objectives of the present work were to establish the extent and activity of the LC-PUFA synthesis pathway in enterocytes and hepatocytes of *C. estor* and, furthermore, to determine whether these activities were influenced by salinity. The results demonstrated that both hepatocytes and enterocytes of *C. estor* displayed physiologically relevant activities of LC-PUFA synthesis from ALA, particularly in saline conditions. Thus, the values in hepatocytes and enterocytes from *C. estor* at 15 ppt salinity (0.41 and 0.09 pmol/h/mg protein, respectively) were lower than those obtained in Atlantic salmon hepatocytes and enterocytes (0.9 and 1.2 pmol.h/mg protein, respectively) (Zheng et al 2005), but higher than those obtained in hepatocytes from the marine teleost Atlantic cod (*Gadus morhua*) (0.02 pmol/h/mg protein) and similar to values from cod enterocytes (0.15 pmol/h/mg protein) (Tocher et al. 2006). In addition, interest in the LC-PUFA synthesis pathway in *C. estor* is partly due in its tissue fatty acid composition that, unusually for a freshwater species, shows a very high DHA:EPA ratio (Martínez-Palacios et al. 2006). In marine and freshwater fish tissue DHA:EPA ratios are most commonly in the range of 1:1 to 2:1 although ratios lower than this are also found in some species, particularly in Southern oceans (Sargent et al. 1989). In contrast, *C. estor* has a fatty acid profile with a DHA:EPA ratio that can vary from 10:1 to 20:1 (Martínez-Palacios et al. 2006).

297 This is generally unusual, even in marine fish, and very uncommon in freshwater
298 species (Ackman 1980). A few marine species show high DHA:EPA ratios, most
299 notable tuna species that can display ratios between 4 and 11, depending upon
300 tissue and species (Tocher 2003). In tuna the high tissue DHA:EPA ratios appear to
301 be due to generally higher DHA levels combined with relatively low EPA levels
302 (Tocher 2003). However, in *C. estor,* it appears that the main cause of the high
303 DHA:EPA ratios is the latter factor, that is, relatively low EPA levels in tissues, rather
304 than exceptionally high DHA (Martínez-Palacios et al. 2006). The present study has
305 confirmed that enterocytes and, especially, hepatocytes of *C. estor* demonstrated
306 significant DHA synthesis from EPA, particularly in saline conditions. Therefore, the
307 data are consistent with the hypothesis that conversion of EPA to DHA, particularly in
308 the liver, but also in the intestine, could contribute, at least in part, to the generally
309 high DHA:EPA ratios observed in the tissue lipids of *C. estor* (Martínez-Palacios et
310 al. 2006).
311 Previously, intestine and pyloric caeca were shown to be tissue sites of substantial
312 LC-PUFA biosynthesis in salmonids (Atlantic salmon and trout) (Fonseca-Madrigal et
313 al. 2005, 2006), and this is why the capacity of enterocytes in *C. estor* for LC-PUFA
314 production was also investigated in the present study. The present study has
315 demonstrated that intestine in *C. estor* had the capability for LC-PUFA biosynthesis
316 but at significantly lower level than liver. The lower capacity of intestine for LC-PUFA
317 biosynthesis in *C. estor* in comparison to salmonids could be related to the feeding
318 habits of the species as *C. estor* is a zooplanktivorous fish with a short intestinal tract
319 and agastric digestive system (1:0.7 size of fish:size of intestine) with no pyloric
320 caeca (Martínez-Palacios et al. 2006). This is, of course, completely different to the
321 digestive tract of carnivorous species such as salmonids, which have a considerably

longer digestive system including a stomach and multiple caeca (Olsen and Ringø 1997). The results therefore suggest that the enterocytes in the much smaller intestinal tract in *C. estor* and planktonivorous fish in general may not express the same range of activities for processing absorbed nutrients as carnivorous fish species, and may be focused more on the digestive and absorption roles. For example, the activity of the LC-PUFA biosynthesis pathway is an order of magnitude lower in enterocytes from *C. estor* compared to enterocytes from Atlantic salmon (Zheng et al. 2005). Hepatocytes from *C. estor* showed much higher LC-PUFA synthesis activity, which is expected due to the liver generally being the most important organ in fatty acid and lipid metabolism in most fish species (Henderson 1996; Grum et al. 2002; Tocher 2003; Fonseca-Madrigal et al. 2005, 2006).

Studies on the development of *C. estor* aquaculture showed that this species displays improved survival, growth and development when cultured in water conditions with increased salinity (Martinez-Palacios et al. 2004). Generally, egg fertilization and incubation as well as many physiological processes including lipid metabolism are dependent on, or influenced by, salinity (Bœuf and Payan 2001). For example, changes in the fatty acid composition of tissue lipids associated with changes in salinity have been reported previously in a number of fish species including guppy (*Poecilia reticulata*) (Daikoku et al. 1982), milkfish (*Chanos chanos*) (Borlogan and Benítez 1992) and turbot (*Psetta maxima*) (Tocher et al. 1994, 1995). These adaptations in response to salinity include altered proportions of total phospholipids and individual phospholipid classes, as well as changes in fatty acid composition including levels of LC-PUFA and n-3/n-6 PUFA ratio. However, the data are variable depending upon species and whether low or high salinity is the actual challenge for that species. For instance, in marine fish, reduced salinity increased

347 percentages of DHA and ARA in mullet (*Mugil cephalus*) (Kheriji et al. 2003), but
348 reduced proportions of EPA and DHA in Japanese sea bass (*Lateolabrax japonicus*)
349 and European sea bass (*Dicentrarchus labrax*) (Xu et al. 2010; Hunt et al. 2011).
350 In the present study, there was a clear relationship between the salinity of the water
351 the fish were maintained and synthesis of LC-PUFA, independent of cell type, with
352 higher LC-PUFA synthesis activity in cells of fish cultured in water with higher salinity
353 compared to fish cultured in freshwater. However, it is perhaps more appropriate and
354 noteworthy to highlight the fact that the activity of the LC-PUFA synthesis pathway
355 was very low in freshwater. This was actually the most unusual feature of the
356 pathway in *C. estor*, rather the activities observed at higher salinity. Clearly, there
357 was very little activity in freshwater and this was largely unprecedented as all the
358 freshwater fish species examined to date have generally shown appreciable LC-
359 PUFA synthesis activity (Tocher 2010), such that 18:3n-3 and/or 18:2n-6 can satisfy
360 their essential fatty acid requirements (NRC 2011).
361 The adaptation processes in response to a saline environment are primarily a series
362 of physiological changes involved osmoregulation, the regulation of ion balances
363 between the external medium and the corporal fluids (Morgan 1997; Laiz-Carrión et
364 al. 2004). Many of adaptations depend upon membrane processes and so changes
365 in lipid and, especially, fatty acid metabolism can be linked to the capacity of the fish
366 to adapt to salinity through changes in lipid and fatty acid compositions of
367 membranes that, in turn, affect membrane-associated proteins (receptors, enzymes
368 etc). Therefore, the influence of salinity on LC-PUFA production may be related to
369 the osmoregulatory response required for adaptation to higher salinity. The effects of
370 salinity on fatty acid compositions have been investigated (Tocher et al. 1994, 1995)
371 and the effects of salinity on LC-PUFA synthesis in hepatocytes have been indirectly

372 investigated in studies on the process of smoltification in Atlantic salmon (Bell et al.
373 1997; Tocher et al. 2000, 2002). In a trial investigating LC-PUFA synthesis in both
374 hepatocytes and enterocytes in farmed salmon, a peak of LC-PUFA production
375 occurred around the time the fish were transferred from freshwater to seawater, with
376 synthetic activity declining rapidly in the seawater phase to minimum levels (Tocher
377 et al. 2002). Although the effects of salinity on lipid and fatty acid biochemistry and
378 metabolism have been little studied in non-salmonid fish, the expression of Δ6 fatty
379 acyl desaturase in liver of the marine teleosts, rabbitfish and red sea bream, was
380 higher in fish maintained at low salinity compared to fish reared at high salinity (Li et
381 al 2008; Sarker et al 2011).This association between salinity and LC-PUFA
382 biosynthesis observed in fish was one of the factors underpinning the hypothesis
383 tested in the present study and the specific objectives were developed in this context.
384 However, the precise links between salinity changes, and LC-PUFA synthesis fatty
385 acid composition in fish including *C. estor* require further investigation.
386 Irrespective of the precise mechanistic links, the results presented, showing very low
387 levels of activity in fish reared in freshwater and increased capacity for LC-PUFA
388 synthesis essentially in hepatocytes as salinity increased may be related with the fact
389 that this species displays better growth performance and development when cultured
390 in saline water. At the most simplistic level, increased capability for endogenous
391 synthesis of the biologically and physiologically essential LC-PUFA would be
392 potentially beneficial to the fish in comparison to the situation in freshwater where the
393 pathways appear almost totally suppressed. Therefore, it is tempting to speculate
394 that the differing activity of the LC-PUFA synthesis pathway is an underpinning factor
395 on the effect of salinity on growth performance of *C. estor*. However, it is not so clear
396 how the effect of salinity on the activity of LC-PUFA synthesis pathway in *C. estor*

relates to current knowledge of LC-PUFA and environmental salinity or to discuss of the possible marine origin of this species. Marine species generally have a reduced ability to produce LC-PUFA compared to freshwater species (Tocher 2010). This has been explained as a possible evolutionary adaptation to the generally higher levels of DHA in the marine environment (Sargent et al. 1995b), and so marine species have had less evolutionary pressure to retain the ability to endogenously produce LC-PUFA; in contrast, freshwater food webs are generally characterized by lower levels of DHA (Sargent et al. 1995b) and so evolutionary pressure for endogenous production of LC-PUFA has been retained in freshwater species (Tocher 2010). Therefore, this hypothesis would suggests it would be more advantageous for *C. estor* to have higher LC-PUFA biosynthesis in freshwater where the supply of EPA and, especially, DHA would likely be lower.

Although the data obtained to date with over 30 species, generally support this hypothesis linking LC-PUFA levels and, especially, DHA levels in the different food webs to evolutionary pressure for endogenous production of LC-PUFA, there are several potential confounding factors including precise feeding habit of different species (herbivorous vs. carnivorous/piscivorous) as well as phylogenetic issues. For instance, defining fish species simply as marine or freshwater is often not ideal considering the large number of euryhaline and diadromous species. Furthermore, the effect of feeding habit can also be generalized with the ability for endogenously LC-PUFA biosynthesis being retained in herbivorous fish, but not in omnivorous, carnivorous, or piscivorous fish. As alluded to above, most fish species studied to date could fit either of these generalizations (environment or feeding habit). However, recent studies have contributed directly to this debate. A feeding study with rabbitfish, *Siganus canaliculatus*, which consumes benthic algae and seagrasses and is thus a

422 rare example of a herbivorous marine fish, suggested that it was able to
423 biosynthesize EPA and DHA (Li et al. 2008). Very recently, it was shown that
424 rabbitfish possess all the fatty acyl desaturase activities required for endogenous
425 synthesis of LC-PUFA (Li et al. 2010). These data suggest that trophic level and/or
426 feeding habit are indeed important factors associated with or determining a species'
427 ability for endogenous LC-PUFA synthesis.
428 The genus *Chirostoma* (silversides also known as *Menidia*) belongs to the family
429 Atherinopsidae, which contains around 150 species, most of which are marine. Thus,
430 *C. estor* is among the few representatives of totally freshwater atherinopsids and,
431 although it is only found in some lakes of the Mexican Central Plateau, it shares
432 common ancestry with marine Atherinopsids (Barbour 1973). However, the
433 evolutionary pathway for *C. estor* is not entirely clear (Barbour 1973), although
434 relationships determined by classical phylogeny can give clues to evolutionary history
435 (Nelson 2006). Significant advances in determining the molecular mechanisms of LC-
436 PUFA biosynthesis in fish have been made in the last decade with the cloning and
437 functional characterization of fatty acyl desaturases and elongases from many fish
438 including freshwater, diadromous and marine species (Tocher 2010). Phylogenetic
439 analyses of the desaturase and elongase sequences have revealed some insights
440 into the possible evolutionary history of LC-PUFA biosynthesis in fish species
441 (Hastings et al. 2001; Zheng et al. 2004, 2009; Morais et al. 2009). The phylogenetic
442 sequence analysis generally reflected classical phylogeny, and grouped fish
443 desaturases in three distinct clusters (Leaver et al 2008). The Ostariophysi (common
444 carp and zebrafish), the Salmoniformes (trout and salmon), and the Acanthopterygia
445 (tilapia, sea bream, turbot, stickleback and medaka), with the cod
446 (Paracanthopterygii) branching from the Acanthopterygia line. However, many

447 questions still remain (Leaver et al. 2008; Li et al. 2010; Monroig et al. 2010) and *C.*
448 *estor* represent an interesting species to study in this respect. The interesting
449 ancestry, pattern of LC-PUFA biosynthesis activity and the effects of salinity, which
450 conflicts with the existing paradigm, make *C. estor* a choice candidate for molecular
451 studies with the isolation, cloning and characterization of fatty acyl desaturases and
452 elongases being important goals for future studies.
453 The results of the present study have provided data that contribute to our
454 understanding of the unusual fatty acid profile found in tissues of *C. estor* indicating
455 that it could be explained, at least partly, by endogenous metabolic activity resulting
456 in elongation and desaturation of EPA to DHA. Moreover, with respect to the well-
457 known beneficial effect of n-3 LC-PUFA on human health, it is noteworthy that the
458 results demonstrate that the increased ambient salinity used as part of the
459 management of this species in aquaculture farming should positively affect the
460 nutritional quality of the flesh in terms of fatty acid composition. However, a complete
461 understanding of fatty acid metabolism in *C. estor* requires further more extensive
462 analysis to determine the potential roles of selective *β*-oxidation, acylation and
463 incorporation of fatty acids into lipid classes, and lipid and fatty acid transport
464 between tissues.
465

466 **Acknowledgements**

467

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470 MAR from Spanish MEC.

471

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668
669

669
670 Legends to Figures:

671

672 FIG. 1. Total LC-PUFA biosynthesis (desaturation/elongation) activity in hepatocytes
673 and enterocytes of *C. estor* cultured at different salinities. Results are means ± S.D.
674 (n= 3) and represent the rate of conversion (pmol.h^{-1}.mg $protein^{-1}$) of [1-^{14}C]18:3n-3
675 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3,
676 20:5n-3, 22:5n-3 and 22:6n-3). Columns assigned to a specific cell type with different
677 superscript letters are significantly different as determined by one-way ANOVA
678 followed by the Tukey post test ($P < 0.05$).

679

680 FIG. 2. Individual fatty acid products of the desaturation and elongation of [1-
681 ^{14}C]18:3n-3 in *C. estor* hepatocytes. Results are means ± S.D. (n = 3) and represent
682 the rate of production (pmol.h^{-1}.mg $protein^{-1}$) of individual fatty acids as determined
683 by the recovery of radioactivity in each fatty acid fraction. Columns referring to a
684 specific fatty acid having different superscript letters are significantly different as
685 determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$).

686

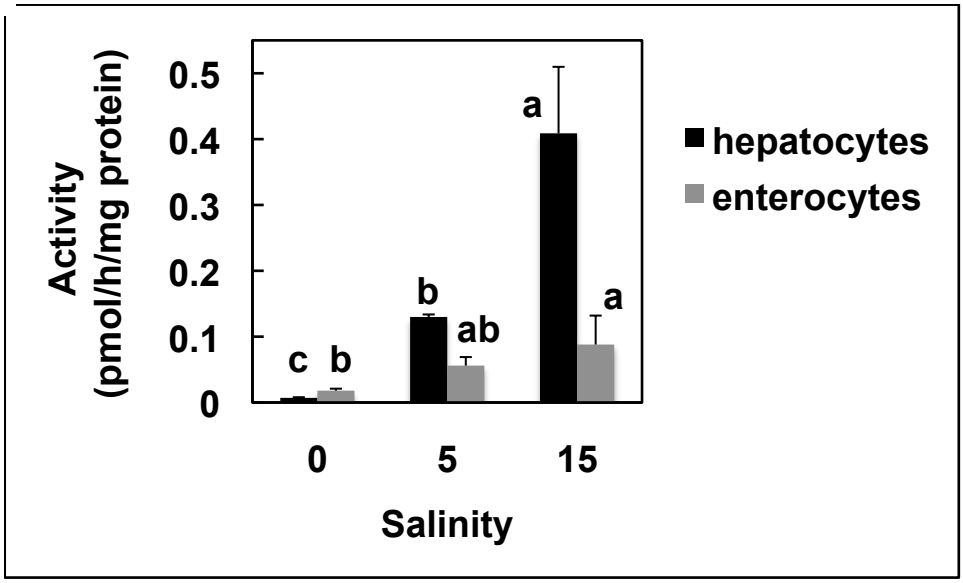
687 FIG. 3. Individual fatty acid products of the desaturation and elongation of [1-
688 ^{14}C]18:3n-3 in *C. estor* enterocytes. Results are means ± S.D. (n = 3) and represent
689 the rate of production (pmol.h^{-1}.mg $protein^{-1}$) of individual fatty acids as determined
690 by the recovery of radioactivity in each fatty acid fraction. Columns referring to a
691 specific fatty acid having different superscript letters are significantly different as
692 determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$).

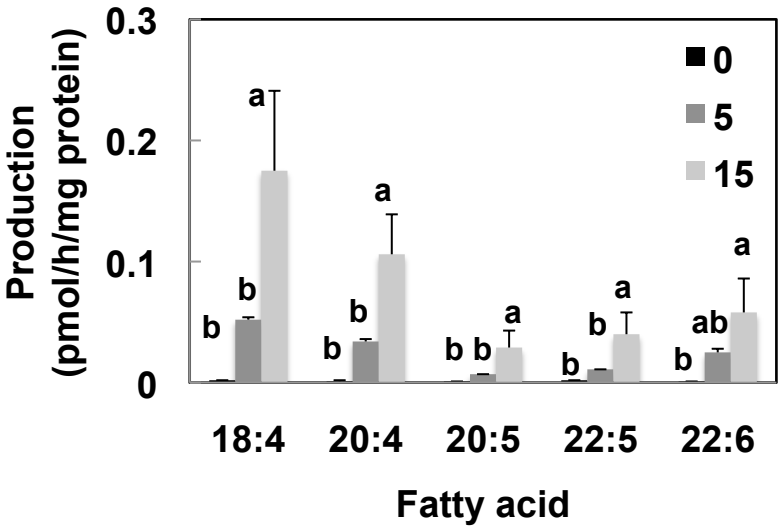
693

FIG. 4. Production of desaturation/elongation products from labeled EPA in hepatocytes and enterocytes of *C. estor* cultured at different salinities. Results are means ± S.D. (n= 3) and represent the rate of conversion (pmol.h^{-1}.mg $protein^{-1}$) of [1-^{14}C] 20:5n-3 to metabolised products (sum of radioactivity recovered as 22:5n-3 and 22:6n-3). Columns representing a specific cell type with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$).

FIG. 5. Individual fatty acid products of the desaturation and elongation of [1-^{14}C] 20:5n-3 in *C. estor* hepatocytes. Results are means ± S.D. (n = 3) and represent the rate of production (pmol.h^{-1}.mg $protein^{-1}$) of individual fatty acids as determined by the recovery of radioactivity in each fatty acid fraction. Columns referring to a specific fatty acid having different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$).

FIG. 6. Individual fatty acid products of the desaturation and elongation of [1-^{14}C] 20:5n-3 in *C. estor* enterocytes. Results are means ± S.D. (n = 3) and represent the rate of production (pmol.h^{-1}.mg $protein^{-1}$) of individual fatty acids as determined by the recovery of radioactivity in each fatty acid fraction. Columns referring to a specific fatty acid having different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$).


Activity
(pmol/h/mg protein)
0.5
0.4
0.3
0.2
0.1
0
c
b
b
ab
a
a
0
5
15
Salinity
hepatocytes
enterocytes


Production (pmol/h/mg protein)
0.3
0.2
0.1
0
0
5
15
a
b
b
a
b
b
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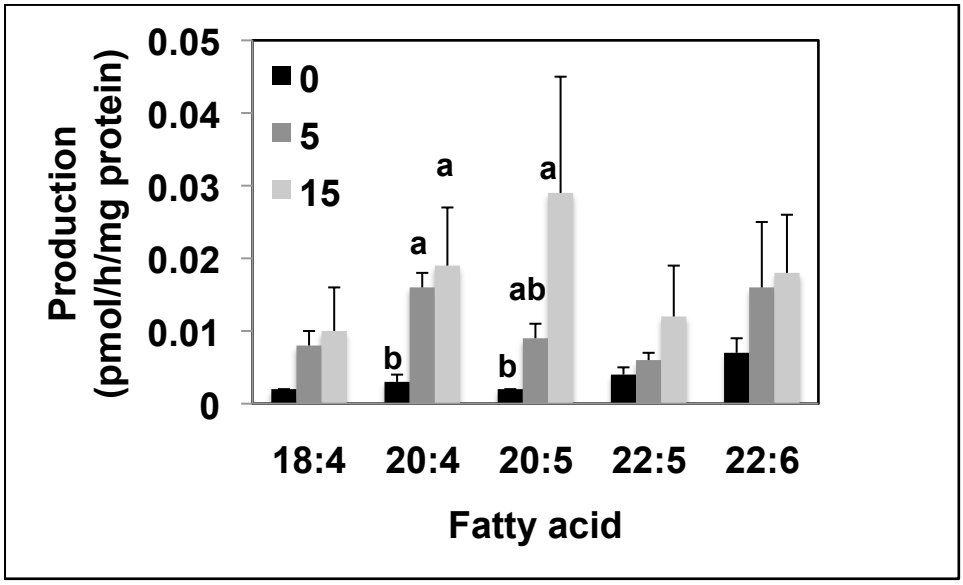
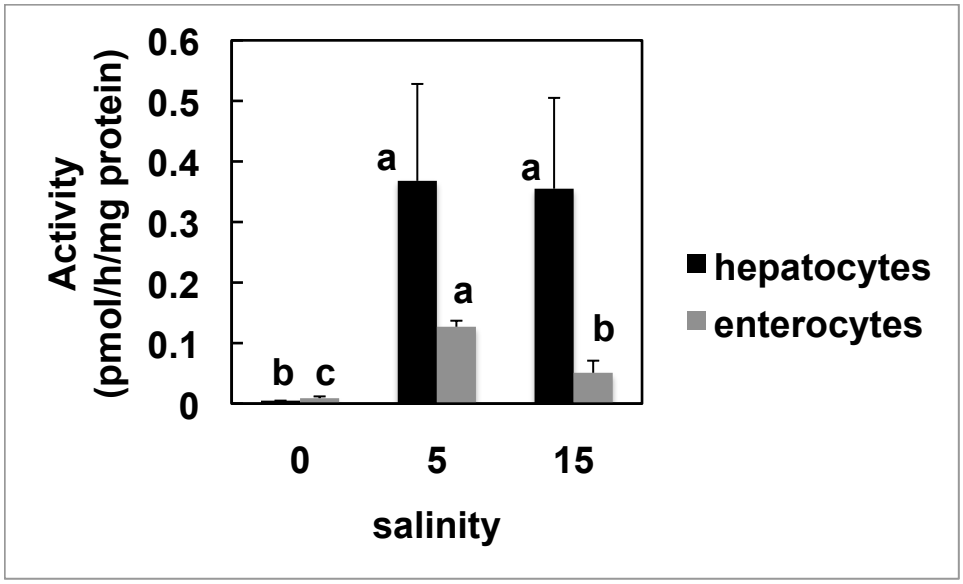
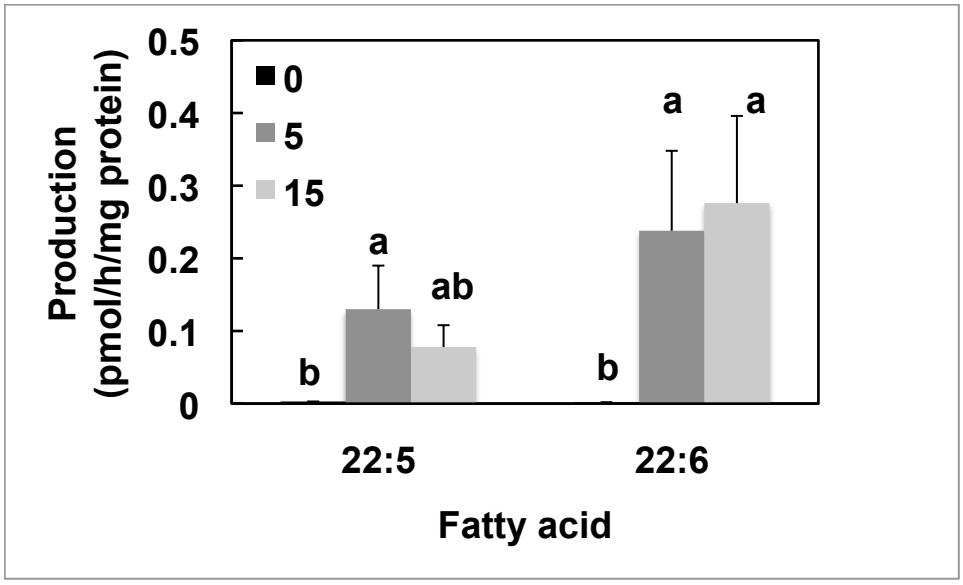
b b
a
b
b
a
b
ab
a
18:4
20:4
20:5
22:5
22:6
Fatty acid


Production (pmol/h/mg protein)
0.05
0.04
0.03
0.02
0.01
0
0
5
15
a
a
a
ab
b
b
18:4
20:4
20:5
22:5
22:6
Fatty acid


Activity
(pmol/h/mg protein)
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
a
b
c
b
0
5
15
salinity
hepatocytes
enterocytes


Production (pmol/h/mg protein)
0.5
0.4
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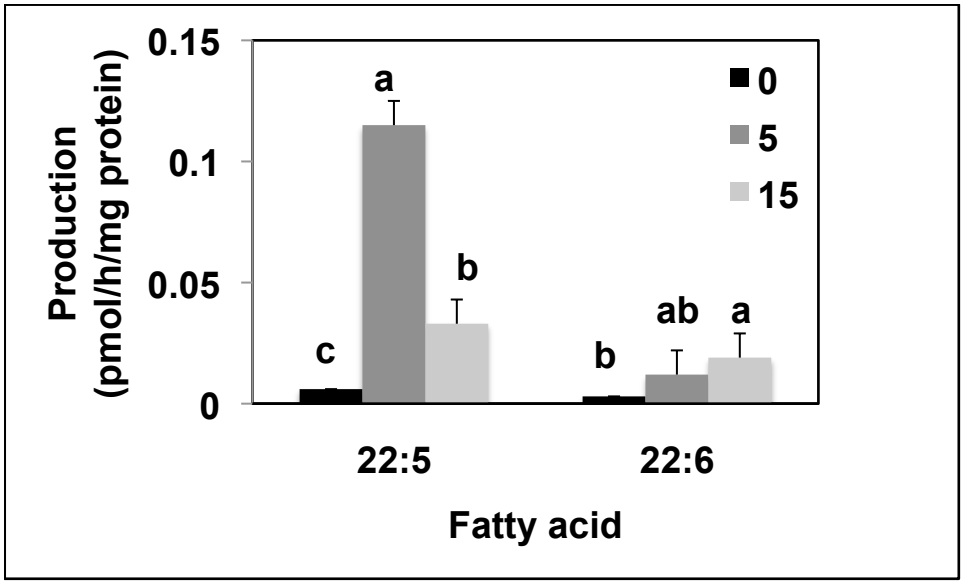
22:5
22:6
Fatty acid


Production (pmol/h/mg protein)
0.15
0.1
0.05
0
a
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ab
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b
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22:5
22:6
Fatty acid