

## Estimation of the Tissue Distribution of mRNA Transcripts for Desaturase and Elongase Enzymes in *Channa striata* (Bloch, 1793) Fingerlings using PCR Technique

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**Abstract.** The variations in the capacity of fish species to biosynthesize the n-3 highly polyunsaturated fatty acids (HUFA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids which are crucial to the health of higher vertebrates has been well documented. HUFA biosynthesis has been reported to involve enzyme-mediated fatty acyl desaturation and elongation processes. In previous studies, various authors have cloned, characterized and identified different genes for fatty acyl desaturase and elongase (FAD and FAE, respectively) enzymes from fish species such as Atlantic salmon and zebra fish, and have also shown the up and down regulation of the activity of these enzymes in response to fluctuations in dietary HUFA. In this paper, we report on the tissue distribution in the expression of genes (mRNA) for FAD and FAE enzymes in *Channa striata* (Bloch, 1793) fingerlings, to evaluate the tissues of the fish which show the highest activity of these enzymes. For this purpose, mRNA from 8 different tissues of the fish (maintained on a commercial diet) were isolated and analyzed, using conventional PCR technique. The tissue distribution of the genes was estimated to be significantly ( $P < 0.05$ ) higher for both FAD and FAE enzymes in the liver and brain of *C. striata* than in the other 6 tissues measured (muscle, ovary, testis, gill and skin) respectively. The implication of this observation with respect to the use of vegetable oils to substitute fish oil in diets for freshwater fish species is discussed accordingly.

**Keywords:** *Channa striata*, PCR, Desaturase, Elongase, HUFA, mRNA expression

### 1. Introduction

Increasingly in recent times, the fear expressed by researchers predicting that the supply of fish oil (FO), the commonly added oil in fish feeds, would be unable to meet global demands in the near future has become more intense (FAO, 2007), as many vegetable oils (VO) are now suggested as good alternatives to FO in Aquaculture. On the other hand, increasing concern also continues to trail the health benefits to humans following the consumption of fish grown with feeds formulated with VO (Bell et al., 2002; Robin et al. 2003; Torstensen et al. 2005; Tocher et al. 2006). Nonetheless, the only practical, sustainable alternative to FO is VO, rich in C18 PUFA but which lack the n-3 HUFA abundant in FO (Sargent et al. 2002) that are crucial for the normal development of higher vertebrates (Simopoulos 1989, 1991). As a result of feeding VO therefore, tissue fatty acid (FA) compositions of fish fed VO show the characteristic feature of increased C18 PUFA levels and reduced n-3 HUFA content and this has been revealed to lower their nutritional value to the

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human consumer (Bell et al. 2001). To increase tissue contents of n-3 HUFA, some fish species have been reported to possess the ability for the bio-conversion of dietary C18 PUFA precursors to HUFA. Consequently, Torcher (2003) highlighted that since most freshwater fish were capable of this bio-conversion, the activity of the enzymes necessary for this biosynthetic process must be expressed.

$\Delta 5$  and  $\Delta 6$  fatty acyl desaturases (FAD) and elongases (FAE) are critical enzymes in the pathways for the biosynthesis of HUFA. In recent years, significant progress has been made in characterizing FAD involved in HUFA synthesis (Tocher et al. 1998). Authors have reported the isolation of a cDNA of zebrafish (*Danio rerio*, GenBank accession No AF309556), which showed high similarity to the mammalian  $\Delta 6$  desaturase genes (Hastings et al. 2001). FAD cDNAs were also isolated and cloned from rainbow trout (*Oncorhynchus mykiss*, GenBank accession No 56 AF301910) (Seiliez et al. 2001) and gilthead seabream (*Sparus aurata*, GenBank accession No AY055749) (Seiliez et al. 2003). Overall, the primary hypothesis is that the understanding of the molecular basis of HUFA biosynthesis and its regulation in fish will enable researchers to optimize the activity of the pathway, to ensure the efficient and effective use of VO in aquaculture, while safeguarding the nutritional quality of reared fish for the consumer.

Consistent with this hypothesis therefore, different tissues of *C. striata* fingerlings (maintained on a commercial diet) were screened using molecular tools, to evaluate the potential distribution of the genes (mRNA) for the FAD and FAE enzymes, where the primers used in this screening were designed according to the published zebra fish FAD and FAE genes, respectively.

## **2. Materials and Methods**

### **2.1. Fish and Collection of Tissue Samples**

*Channa striatus* (Bloch 1793) fingerlings (ranging in weight between 2 to 5 g) were maintained in a rectangular, 4000 L capacity fibreglass tank on commercial catfish, post-larval crumbled pellets (Post Larva 1 crumbles; Gold Coin Specialties Sdn Bhd, Johor, Malaysia) containing a minimum of 300 and 100 g/kg crude protein and lipid, respectively for 2 weeks prior to sampling.

Fish were randomly selected, killed by a sharp blow to the head and immediately dissected using sterilized dissecting knives and forceps for the removal of 8 different tissues, in triplicate (liver, brain, muscle, kidney, skin, intestine, ovary and testis). Tissue samples were separately stored in 2 mL of RNA later® and preserved at -80 °C until used for total RNA extraction within 3 days.

### **2.2. Total RNA Extraction from Tissues**

Total RNA was extracted from approximately 0.05 to 0.1 g of samples from each replicate pool of tissues (where appropriate), using TRI Reagent® (Molecular Research Center, USA) based on the manufacturer's specifications as follows; all tissues were homogenized in 1 mL of TRI Reagent® (the skin and muscle were flash-frozen with liquid nitrogen before homogenizing). The homogenates were centrifuged at 12,000 g for 10 min at 4 °C to remove the insoluble material in the tissues. The supernatant was transferred to new, sterile 1.5 mL micro-centrifuge tube, into which 0.2 mL chloroform was added and subsequently shaken vigorously for 15 sec. The mixture was allowed to stand at room temperature for 15 min, after which it was centrifuged again at 12,000 g for 15 min at 4 °C. From 3 layers formed (upper clear aqueous phase, an intermediate phase and a lower red, phenol-chloroform phase), the upper aqueous phase was transferred to a new, sterile 1.5 mL micro-centrifuge tube and precipitated by the addition of 0.5 mL Isopropanol. The sample was subsequently kept at -20 °C for 10-30 min (overnight for some samples), followed by centrifugation at 12,000 g for 8 min at 4 °C. The supernatant was discarded, while RNA pellet formed was mixed with 75% ethanol to wash away any salts. This was centrifuged again at 7,500 g for 5 min at 4 °C. Ethanol was discarded carefully and the RNA pellet was air-dried at room temperature for 5-10 min. Finally, the RNA pellet was dissolved in appropriate quantity of RNase-free water and incubated on a water bath for 10 min at 55 °C. Isolated total RNA was stored at -80 °C until used.

### **2.3. DNase Treatment of Total RNA**

The subsequent DNase treatment of the total RNA extracted with RQ1 RNase-free DNase (Promega, USA) was carried out, to eliminate all traces of DNA contamination in the RNA samples. The reaction was

performed in a 10  $\mu$ L mixture, containing 1  $\mu$ g total RNA, 1  $\mu$ L of RQ1 RNase-free DNase, 1  $\mu$ L of 10X buffer (Promega, USA) and distilled water. The mixture was incubated at 37  $^{\circ}$ C for 30 min, followed by 65  $^{\circ}$ C for 10 min. Two micrograms of DNase-treated total RNA was reverse transcribed into first-stand cDNA at 42  $^{\circ}$ C for 1 h in a total volume of 20  $\mu$ L containing 1  $\mu$ L XM-MLV RT reaction buffer (Promega, USA), 25 ng of Random primers, 0.5 mM of each dNTP, 24 u of recombinant RNasin $^{\circ}$  Ribonuclease Inhibitor and 200 U of M-MLV Reverse Transcriptase (Promega, USA). PCR was then performed to amplify a partial cDNA fragment of snakehead desaturase, elongase and  $\beta$ -Actin (as housekeeping gene), using primers from Primer3 online program (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/>). The primers were designed according to published zebra fish desaturase (Genbank accession number: AF309556), zebra fish elongase (Genbank accession number: AF532782) and zebra fish  $\beta$ -actin (Genbank accession number: AF057040). The sequences of forward and reverse oligonucleotide primers used and their amplicon sizes, to further verify the specificity of the primers used in PCR analysis, are as shown in Table 1.

Table 1: Oligo-nucleotide primers used in the conventional PCR analysis

| Gene           | Amplicon | Sense 5'-3'           | Anti sense 5'-3'       |
|----------------|----------|-----------------------|------------------------|
| Desaturase     | 336 nt   | CATCACGCTAAACCCAACA   | GGGAGGACCAATGAAGAAGAA  |
| Elongase       | 474 nt   | CCGTATCTGTGGTGAAGAAG  | AGTTTGAGAAGAGCAGGATGAG |
| $\beta$ -Actin | 159 nt   | CCGTGACATCAAGGAGAAGCT | TCGTGGATACCGCAAG ATTCC |

## 2.4. Conventional PCR for the Expression of Desaturase, Elongase and $\beta$ -Actin Genes

Conventional PCR was performed on different tissues, to quantify the relative expression of mRNA of desaturase and elongase enzymes in each tissue. In this step, 3  $\mu$ L of first strand cDNA was added in a reaction mixture of 25  $\mu$ L, containing Green Go Taq $^{\circ}$  Flexi buffer, 2.0 mM MgCl $_2$ , 0.2 mM of each dNTP, 0.4  $\mu$ M of each primer and 1 U of Go Taq $^{\circ}$  DNA polymerase (Promega, USA). The PCR program used was an initial denaturation at 94  $^{\circ}$ C for 1 min; cycle amplification at 56  $^{\circ}$ C for 30 s (obtained from annealing temperature optimization carried out at between 50 and 62  $^{\circ}$ C for 30 s at each temperature, to verify the optimal annealing temperature) and 72  $^{\circ}$ C for 30 s with a final extension step at 72  $^{\circ}$ C for 10 min. The PCR products were separated by agarose gel electrophoresis containing 1% ethidium bromide, which were validated by running series of PCR reactions at different cycles to determine the number of cycles which generated maximal products. mRNA bands for the desaturase and elongase enzymes were visualized digitally by using a CCDBIO 16SC imaging system (Hitachi Genetic Systems/MiraiBio, Alameda, CA) and quantitated by densitometry (GENE TOOLS and GENE SNAP software, Hitachi Genetic Systems/MiraiBio), to calculate the ratio of the copy numbers of transcripts in each tissue relative to that for the reference ( $\beta$ -actin) genes in the same tissues. The PCR assays were validated by running series of PCR reactions to determine number of cycles which generate half-maximal PCR reaction and were verified to be 32 cycles for desaturase and  $\beta$ -Actin and 33 cycles for the elongase genes accordingly.

## 2.5. Statistical Analysis

For tissue mRNA expression profiles, results were expressed as mean normalised values ( $\pm$  SD) corresponding to the ratio between the copy numbers of the FAD and FAE transcripts and the copy numbers of the reference gene,  $\beta$ -Actin mRNA in the same tissues. A single factor analysis of variance (ANOVA) was carried out at a significance level of 0.05, followed by Duncan's Multiple Range post hoc Test, with SPSS statistical analysis software.

## 3. Results

### 3.1. Expression of Desaturase and Elongase Genes in Different Tissues of *C. Striata* Fingerlings

The agarose gel chromatogram showing the PCR products of mRNA bands for desaturase and elongase enzymes in the 8 tissues of *C. striata* studied are shown in Figure 1, whereas the normalized values (mean  $\pm$  SD) corresponding to the copy numbers of the FAD and FAE transcripts in the liver, testis, brain, ovary, kidney, muscle, intestine and skin of *C. striata* fingerlings are as also shown in Figure 2 accordingly.

The sequenced PCR product revealed that FAE (167 bp) and FAD (159 bp) derived from PCR analysis were 100% identical to the published zebra fish elongase (Genbank accession number: AF532782) and desaturase (Genbank accession number: AF309556), respectively, and further confirmed the specificity of the primers used in the PCR analysis.

From the results, expression of mRNA for FAD enzymes was similar in the liver, brain and ovary (with more recorded in the liver) and the expression was significantly ( $P < 0.05$ ) higher than in other tissues tested. This was followed by testis, then the intestine, whereas mRNA for FAD expression was recorded to be significantly the lowest ( $P < 0.05$ ) in the skin. No expressions of mRNA for FAD were detectable in both the kidney and muscle tissues, respectively.

Similar to the expression of mRNA for FAD enzyme, expression of mRNA for FAE enzyme was also significantly ( $P < 0.05$ ) the highest in the liver and brain, but additionally, in the intestine as well; the expression was not significantly ( $P > 0.05$ ) varied among these 3 tissues, but were the highest noted (with the intestine being numerically higher). These were followed by expression in the Kidney, testis and skin, where the expression of mRNA for FAE enzyme in these tissues was recorded at about half of that noted in the brain, liver and intestine, respectively. Expression of mRNA for FAE enzyme was significantly ( $P < 0.05$ ) the lowest in both ovary and muscle respectively, compared to the 6 others. Therefore, whereas FAD enzyme expression was recorded in 6 tissues (Liver, brain, ovary, testis, intestine and skin), the expression of mRNA for FAE enzyme was recorded in all the 8 tissues tested, although at varying degrees for each of the enzymes.

Generally, expression of mRNA for FAE enzyme was recorded to be between 2-10 fold or more higher than that in genes for FAD enzyme in the tissues where both enzymes were recorded, except in the ovary, where mRNA for FAD enzyme was noticed to be expressed at a higher level than that for the FAE.

#### 4. Discussion

Presently, reasons responsible for the increase in the HUFA biosynthetic activity, as a result of the limited intake of HUFA from the diet remains relatively unclear (Torcher et al. 2003) and was even speculated to be species dependent (Jaya-Ram 2008). Although no evidence is advanced in this paper to correlate the obtained mRNA expression levels with the activities of the enzymes, it has been suggested that one of the most important mechanisms responsible for the increased HUFA biosynthesis during limited dietary HUFA intake could be through the up-regulation in the expression of mRNAs for FAD and FAE enzymes (Bell et al., 2003). To support this suggestion, Torcher (2003) opined that since most freshwater fish and salmonids are capable of producing DHA from 18:3n-3, they must therefore express the activities of the enzymes necessary for that biosynthetic pathway; a phenomenon which has been shown to occur in response to fluctuations in certain dietary nutrients (Sargent et al., 2002). Meanwhile, Brenner (1981) reported that the activity of FAD enzymes is under nutritional regulation in mammals, while Torcher et al. (2003) demonstrated this in fish, consistent with the observations made in different tissues of *C. striata* in the present study.

Observation of significantly ( $P < 0.05$ ) higher expression of mRNA for both FAD and FAE enzymes in the liver and brain of *C. striata* noted in the current study concur with that of Zheng et al. (2009), who also reported the highest FAD and FAE activities in the liver and brain, respectively of *Cobia (Rachycentron canadum)*. To support this observation, Torcher et al. (2006) also demonstrated the highest order of magnitude of desaturase activity in the brain of Cod (*Gadus morhua*, L), with high levels also shown in the liver and kidney, followed by the intestine than in the other tissues monitored. Additionally, Torcher et al. (1996) earlier reported that 10-fold more DHA was produced from EPA than from 18:3n-3 in primary cultures of brain cells from turbot (*Scophthalmus maximus*, L). Similarly, expression of genes for desaturase enzyme was shown to be high in liver and brain of a freshwater species rainbow trout, *Onchorynchus mykiss* (Seiliez et al. 2001), in Atlantic salmon, *Salmo salar* (Zheng et al. 2005) and Gilthead sea bream, *Sparus aurata* (Diez et al. 2007), but very low in kidney and white muscle of rainbow trout and in the intestine of sea bream.

The observation of higher mRNA expression for FAD and FAE enzymes in brain and liver seem to suggest an important role in neural tissues, possibly to guarantee sufficient DHA despite fluctuations in the supply of EPA and DHA in the diet, particularly during crucial periods of development, as suggested by

Sargent et al. (2002). This speculation was confirmed by the conclusions of Mourente (2003), who noted that during larval development, fish demonstrated considerable demand for DHA for neural tissues development. Bell et al. (1995) also reported that the deficiency of dietary DHA resulted in larval Atlantic herring showing impaired ability to capture prey at natural intensities of light, supporting the need for brain cells of fish to show the capacity for DHA production, to guarantee steady supply and avoid shortages from dietary sources.

Nearly all the cloned and functionally characterised elongases from fish so far reported in the literature show sequences and functionality which bear similarity to that of the mammalian ELOVL5 (Hastings et al. 2005; Agaba et al. 2005). Theoretically therefore, only that Elov15-like elongase could be required for the production of DHA from 18:3n-3 in both fresh and marine water fish; suggesting that one elongase is all that would be required for the performance of both the elongations of EPA necessary for the production of DHA in freshwater and marine species.

The genes of the HUFA biosynthetic pathway are also up-regulated in response to the replacement of FO by VO. In Atlantic salmon fed a linseed oil (high 18:3n-3) based diet for 20 weeks, the expression of desaturase and elongase genes was shown to be positively and negatively correlated with 18:3n-3 and n-3 HUFA contents, respectively (Leaver et al. 2008). On the contrary, Seiliez et al. (2003) demonstrated that expression of liver desaturase mRNA in freshwater rainbow trout was not affected significantly by dietary VOs, including linseed oil, rapeseed oil, olive oil and a blend VO. In total, these observations suggest that salmonids in the seawater phase (and freshwater fish in general) are capable of up-regulating HUFA biosynthetic genes and their corresponding activities in response to decrease in HUFA in their diets.

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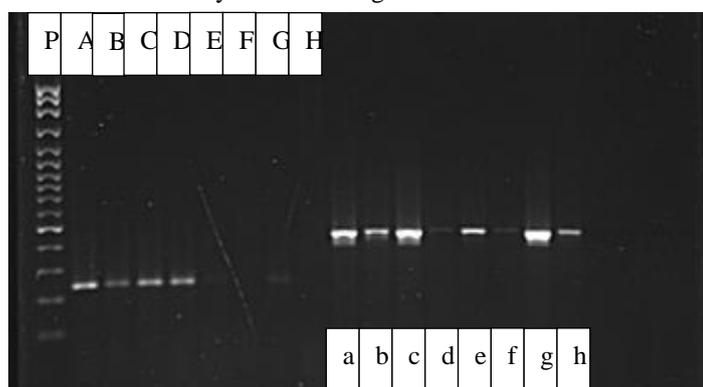


Fig. 1: Agarose gel chromatogram showing PCR products of genes for Desaturase and Elongase enzymes in 8 tissues of *Channa striata* fingerlings; Lane P, DNA molecular marker; Lanes A to H are bands for desaturase genes in liver (A), testis (B), brain (C), ovary (D), kidney (E), muscles (F), intestine (G) and skin (H); Lanes a-h, are bands for Elongase genes in liver (a), testis (b), brain (c), ovary (d), kidney (e), muscles (f), intestine (g) and skin (h).

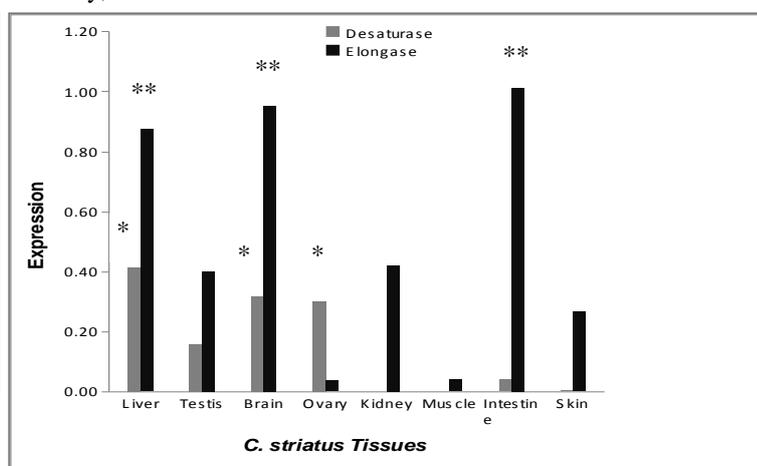


Fig. 2: Relative expression of genes for desaturase and elongase enzymes in 8 different tissues of *C. striatus* fingerlings; Stars (\* or \*\*) on similar colored bars imply significant differences ( $P < 0.05$ )