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Research paper

# Phylogeny and expression patterns of two apolipoprotein E genes in the flatfish Senegalese sole

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ABSTRACT

The apolipoprotein E (ApoE) is a key component of several lipoproteins involved in lipid homeostasis. In this study, two cDNA sequences encoding ApoE (referred to as *apoEa* and *apoEb*) were characterized in the flatfish *Solea senegalensis*. The predicted peptides contained conserved structural blocks related with their capacity for lipid binding and lipoprotein receptor interaction. At genomic level, both genes contained five exons and four introns and they were organized into two tandem arrays with apoA-IV gene copies. The phylogenetic analysis clearly separated them into two well-supported clusters that matched with their organization in the genome of teleosts. Whole-mount *in situ* hybridization located the *apoEa* signal in the yolk syncytial layer (YSL) of lecithotrophic larval stages (0 dph) and in the anterior intestine of exotrophic larvae and benthic fish. In the case of *apoEb*, hybridization signals were located in the YSL, tail bud, eyes and mouth at 0 dph and in the otic vesicle, hindbrain, eyes, pharynx, mouth, heart and intestine at 1 dph. In exotrophic larvae, *apoEb* was ubiquitously expressed in several tissues such as taste buds, brain, mouth, nostril, gills, intestine, liver and around the neuromasts and eyes. Quantification of mRNA levels in pools of whole larvae confirmed distinct expression patterns with a significant reduction of *apoEa* and an increase of *apoEb* mRNA levels throughout larval development. Moreover, only *apoEa* transcripts increased in response to food supply suggesting that this paralog mostly participates in the absorption and transport of dietary lipids and the *apoEb* in the redistribution of endogenous lipids as well as in neural tissue regeneration.

## 1. Introduction

The apolipoprotein E (ApoE) is an exchangeable apolipoprotein principally expressed in the liver although it can also be detected in several other tissues in mammals (Getz and Reardon, 2009). This apolipoprotein plays a major role in lipid homeostasis and is associated with very low density lipoproteins (VLDL), chylomicrons remnants and a subset of high-density lipoproteins (HDL) (Mahley and Rall, 2000; Getz and Reardon, 2009). ApoE facilitates hepatic secretion of VLDL and mediates the uptake and degradation of lipoproteins in the liver by specifically binding to receptors of the low-density lipoprotein (LDL) family. Moreover, ApoE promotes the cholesterol efflux by interacting with the ATP Binding Cassette Subfamily A Member 1 (ABCA1) to generate nascent HDL particles (Arai et al., 1999; Vedhachalam et al., 2007). In addition to this role in lipid mobilization, the ApoE exerts a potent anti-atherogenic action stimulating cholesterol efflux from macrophages in the atherosclerotic lesion, preventing platelets aggregation and regulating associated immune and inflammatory responses (Mahley, 1988; Greenow et al., 2005; Getz and Reardon, 2009).

Along with this role in lipid mobilization through the plasma (*endocrine-like function*), the apoE has also been associated with a *paracrine-like function* in the central nervous system (CNS). This organ possesses its own local cholesterol and lipid transport and recycling system based on the production HDL-sized lipoproteins containing ApoE in the glial cells (Ladu et al., 2000; DeMattos et al., 2001; Gong et al., 2002). This local metabolism is essential for neural cell growth and integrity of cell membranes and the ApoE has been specifically related with the regeneration of injured peripheral nerves and some neurological diseases (Ignatius et al., 1986; Hauser et al., 2011; Melemedjian et al., 2013). In addition to this function in the CNS, the ApoE modulates immune-

*Abbreviations:* ai, anterior intestine; b, brain; ABCA1, ATP Binding Cassette Subfamily A Member 1; BCIP, bromo-chloro-indolyl-phosphate; CNS, central nervous system; DMSO, dimehtylsulfoxide; dph, days post-hatch; e, eye; HDL, high-density lipoproteins; hb, hindbrain; h, heart; in, intestine; LDL, low-density lipoprotein; lv, liver; MAB, Malic acid buffer; MABTr, MAB + 0.1% TritonX-100; m, mouth; NBT, nitro blue tetrazolium; nm, neuromasts; n, nostril; ov, otic vesicle; PFA, paraformaldehyde; ph, pharynx; pi, posterior intestine; sk, skin; tb, taste buds; tlb, tail bud; VLDL, very low density lipoprotein; ysl, yolk syncytial layer; WISH, for whole-mount *in situ* hybridization

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mediate responses (Jofre-Monseny et al., 2008), and the susceptibility to bacterial and viral infections (Itzhaki et al., 1997; Roselaar and Daugherty, 1998; de Bont et al., 1999).

Although ApoE has been extensively studied in mammals due to its involvement in cardiovascular and neurodegenerative diseases, much less information is available in fish. Two paralogs encoding ApoE (referred to as *apoEa* and *apoEb*) were previously identified in teleosts. These genes appeared tandemly arranged with *apoA-IV* genes (Kondo et al., 2005; Otis et al., 2015; Roman-Padilla et al., 2016a). Expression analysis indicated a subfunctionalization of these two paralogs, with the *apoEa* mostly expressed in digestive organs and the *apoEb* ubiquitously distributed in a species-specific way (Kondo et al., 2005; Kim et al., 2009; Otis et al., 2015). In sole, a previous transcriptomic study in premetamorphic larvae identified the *apoEa* as differentially expressed in response to dietary triacylglycerol (TAG) levels (Hachero-Cruzado et al., 2014; Bonacic et al., 2016; Roman-Padilla et al., 2017). Moreover, *apoEb* mRNA abundance was significantly different in the upper olfactory rosette of wild and cultivated males (Fatsini et al., 2016). However, a full characterization of *apoE* is required to identify sole-specific expression patterns and properly interpret expression profiles in high-throughput genomic studies for a better understanding of lipid management mechanisms with impact in the aquaculture of this species.

In this study, we: i) Identified the genomic structure of both *apoE* paralogs in Senegalese sole; ii) Characterized the spatio-temporal expression patterns of *apoE* paralogs during larval development and in juvenile tissues; iii) Evaluated the transcriptional response of both *apoE* genes in response to feeding. The results obtained are important to establish the specific role of each paralog in lipid metabolism and homeostasis.

## 2. Material and methods

### 2.1. Fish samples

All procedures were authorized by the Bioethics and Animal Welfare Committee of IFAPA and given the registration number 06-11-15-337 by the National authorities for regulation of animal care and experimentation.

Samples of juvenile (n = 3) liver, spleen, brain, gills, intestine, head kidney, heart, skeletal muscle, and skin used in this work were obtained in a previously unrelated study (Armesto et al., 2014). To study the expression patterns in pre-metamorphic and metamorphic larvae, larval samples were taken from a previous trial of our group that investigated the expression patterns of *apoA-I* and *apoA-IV* genes (Roman-Padilla et al., 2016a; Roman-Padilla et al., 2016b). In sole, the lecithotrophic larval period spans from hatch (day 0) to 3 days post-hatch (dph) when larvae open the mouth for first feeding. The metamorphic transformation normally occurs from day 12 to 18 dph and depending on the eye position, larvae can be classified from S1 (at the beginning of eye migration) to S4 (at the end of eye migration) (Fernandez-Diaz et al., 2001; Manchado et al., 2008). In this study, a group of larvae (in triplicate tanks) were fasted until 5 dph whereas a second group was fed from 3 dph under a standard co-feeding protocol using rotifer (*Brachionus plicatilis*) and *Artemia* metanauplii enriched with microalgae (*Tisochrysis lutea*, *T-iso*) until the end of metamorphosis. Larvae were sampled before mouth opening (0 and 1 dph), at 3 and at 5 dph in both experimental groups and at 9 and 21 dph in the second fed group. For qPCR analysis, larvae were anesthetized in MS-222, washed in DEPC water, frozen in liquid nitrogen, and stored at − 80 °C until use. In addition, some larvae were fixed overnight in 4% paraformaldehyde (PFA) at 4 °C, dehydrated in methanol and stored at − 20 °C until used for whole-mount *in situ* hybridization (WISH) as previously described (Campinho et al., 2015; Roman-Padilla et al., 2016a; Roman-Padilla et al., 2016b).

### 2.2. Sequence and phylogenetic analysis

Some *apoE* encoding sequences described in Hachero-Cruzado et al. (2014) as well as those identified in the databases SoleaDB*v*3 and *v*4.1 (Benzekri et al., 2014) were downloaded, assembled using Seqman*v*8.1 (Lasergene, DNASTAR), edited using EditSeq and aligned with other teleost sequences using the clustalW method implemented in MegAlign*v*8.1 program (Lasergene, DNASTAR).

For phylogenetic analysis, a set of sequences encoding for ApoE, ApoA-I and ApoA-IV in vertebrates were retrieved from GenBank/EMBL/DDBJ and ENSEMBL (www.ensembl.org) databases (Accession numbers are shown in Supplementary file 1). Maximum likelihood (ML) phylogenetic analysis was carried out using ProtTestv3.2 (Darriba et al., 2011) and the PHYLIP package (Felsenstein, 1989). The best-fit model for sequence evolution was JTT + I + G + F. The PHYLIP package was then employed to estimate the bootstrap values using SEQBOOT (100 replicates) and the data were analyzed using the software PHYML. The consensus phylogenetic tree was then obtained (CONSENSE). Trees were drawn using Figtree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). Sequence names and accession numbers used in the phylogeny are indicated in Supplementary file 1. Putative signal peptide was identified using SignalIP (http://www.cbs.dtu.dk/services/SignalP/). Genomic sequences were retrieved from GeneBank (accession numbers LC056058 and LC056059) (Roman-Padilla et al., 2016a) and assembled with cDNA sequences using Seqman*v*8.1.

### 2.3. ISH and WISH analysis

*In situ* hybridization (ISH) and WISH methods were those previously optimized for sole (Campinho et al., 2015; Roman-Padilla et al., 2016a). To synthetize probes, a fragment of each *apoE* paralog was PCR amplified using targeted gene-specific primers (Table 1). Paralog-specific primers were designed using Oligo *v*6.89 software (Medprobe). PCR reactions for probe amplification were carried out using cDNA of a larval pool as template. PCR products were cloned in TOPO-TA vector and identity confirmed by sequencing using a BigDye® Terminator v3.1

**Table 1**
Primers used to amplify the probes for ISH and WISH and those required to quantify gene expression of *apoE* paralogs. The amplicon sizes are indicated.

| Technique | Primers | | Fragment size (bp) |
|---|---|---|---|
| | Primer pair name | Sequence | |
| Probes ISH/WISH | SseapoEa_3 | GATGTTTGCAGTTATCTTCGCTCTGG | 626 |
| | SseapoEa_4 | GGCGAAGCTCTCTGTCAAGATGCCAAAG | |
| | SseapoEb_1 | GCTGATGTCTCTGTAAGTCGCTGGGAGGA | 950 |
| | SseapoEb_2 | TGATTAAATGAGGAGAAAGGCTGTCGCTGT | |
| qPCR | SseapoEaqF | CCCGTACACGCAGGAGGCCGCAAA | 97 |
| | SseapoEaqR | CATCTGCTCCCGGGCCTCCTCCAT | |
| | SseapoEbqF | GCTGACCCCCTTGACCGAAACCTCCAC | 100 |
| | SseapoEbqR | GCGATCCTTGGCATCCAGCATGTCCTT | |