Cloning of endothelin-1 (ET-1) from European sea bass (Dicentrarchus labrax) and its gene expression analysis in larvae with retinoic acid-induced malformations

F. Geay, Maria J. Darias, E. Santigosa, E. Desbruyères, P. Quazuguel, Jose-Luis Zambonino-Infante, C.L. Cahu, David Mazurais

▶ **To cite this version:**

F. Geay, Maria J. Darias, E. Santigosa, E. Desbruyères, P. Quazuguel, et al.. Cloning of endothelin-1 (ET-1) from European sea bass (Dicentrarchus labrax) and its gene expression analysis in larvae with retinoic acid-induced malformations. Aquaculture, 2009, 287 (1-2), pp.169-173. 10.1016/j.aquaculture.2008.10.011 . hal-04479485

HAL Id: hal-04479485
https://hal.univ-brest.fr/hal-04479485

Submitted on 27 Feb 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Please note that this is an author-produced PDF of an article accepted for publication following peer review. The definitive publisher-authenticated version is available on the publisher Web site

Aquaculture
February 2009, Volume 287, Issues 1-2, Pages 169-173
http://dx.doi.org/10.1016/j.aquaculture.2008.10.011
© 2009 Elsevier B.V. All rights reserved.

Archimer
Archive Institutionnelle de l'Ifremer
http://www.ifremer.fr/docelec/

Cloning of endothelin-1 (ET-1) from European sea bass (*Dicentrarchus labrax*) and its gene expression analysis in larvae with retinoic acid-induced malformations

F. Geay[a], M.J. Darias[a], E. Santigosa[a], E. Desbruyères[a], P. Quazuguel[a], J.L. Zambonino-Infante[a], C.L. Cahu[a] and D. Mazurais[a, *]

[a] Ifremer Marine Fish Nutrition Team, Nutrition Aquaculture and Genomics Research Unit, UMR 1067, Ifremer, Technopole Brest-Iroise, BP 70, 29280 Plouzané, France

*: Corresponding author : D. Mazurais, Tel.: +33 2 98 22 44 87; fax: +33 2 98 22 43 66, email address : David.Mazurais@ifremer.fr

Abstract:

It is known in vertebrates that endothelin-1 (ET-1) plays a key role in morphogenesis whose expression level greatly influences the development of craniofacial malformations. In the present study, the complete cDNA fragment encoding a precursor of endothelin-1, the preproendothelin-1 (PPET1), was cloned by RACE-PCR from European sea bass. The cDNA encoded a 199 amino acid polypeptide that was composed of the "mature" and "big" ET-1. Relative ET-1 expression levels were investigated in European sea bass larvae fed microparticulate diets containing the standard amount (0.08 g retinol/kg diet: group N) or an excess of retinoic acid which induces skeletal malformations (0.5 g retinol/kg diet: group RA). Real-time reverse transcription polymerase chain reaction analysis revealed that PPET1 mRNA levels were sensitively reduced in the RA group during early development (days 10 and 15 post hatching). Regulation of ET-1 gene expression in larvae fed the teratogenic level of vitamin A confirmed the involvement of ET-1 in the molecular mechanism involved in craniofacial deformities. These results suggest that the expression level of ET-1 may be used as a precocious molecular marker to predict malformations during European sea bass development.

Keywords: Endothelin-1; Gene expression; Morphogenesis; Retinoic acid; European sea bass

1. Introduction

A significant number of craniofacial abnormalities has been reported in both wild-caught and hatchery reared fish larvae (Boglione et al, 2001). Similar incidence and spectrum of skeletal deformities have been experimentally induced in sea bass (*Dicentrarchus labrax*) larvae fed high vitamin A level (Villeneuve et al., 2006).
Thus, the retinoic acid-induced craniofacial malformations appear as a convenient model to study the molecular mechanisms responsible for craniofacial abnormalities observed in reared fishes. In order to better understand cellular and molecular events implicated in fish craniofacial malformations and to develop potential molecular markers of abnormal development, it is now of great interest to develop molecular tools allowing the investigation of key actors involved in the developmental pathways.
In vertebrates, it is believed that the three members of the endothelin family (ET-1, ET-2, ET-3) are produced in a variety of tissues, where they act as modulators of vasomotor tone, cell proliferation, and hormone production (reviewed by Levin, 1995). Interestingly, it is also admitted that ET-1 affects cranial neural crest-derived structures including the branchial arches and great vessels during vetebrates development (Kurihara et al., 1999). Thus, it has been shown in mice and zebrafish that ET-1 deficiency induced morphological abnormalities of the branchial and pharyngeal arches-derived craniofacial tissues by regulating effectors such as Heart And Neural crest Derivatives-expressed protein 2 (HAND2) and Msh homeobox 1-like protein (msx1) (Kurihara et al., 1994; Thomas et al., 1998; Miller et al., 2000; Clouthier and Schilling, 2004). In addition, ET-1 has been reported to be involved in retinoic acid-induced craniofacial abnormalities through the regulation of its expression level (Zhang et al., 2006). These findings indicate that modification of ET-1 gene expression in the developing organism could perturb the delicate craniofacial development and reveal the great interest to investigate ET-1 gene expression to tackle molecular pathways implicated in craniofacial malformation in reared fish.
The bioactive mature ET-1 is a 21 amino-acid peptide that is generated from a precursor, the preproendothelin-1 (PPET1) through the activity of processing endopeptidases, including dibasic amino acid endopeptidase that cleaves PPET1 to form “Big” ET-1 (Yanagisawa et al., 1988) and endothelin converting enzyme (ECE-1 and/or ECE2) that converts “Big” ET-1 to “mature ET-1” (Shimada et al., 1994). PPET1 cDNA was cloned in different organisms including fish species such as *Danio rerio* , *Oncorhynchus keta* and *Fundulus heteroclitus* (Miller et al., 2000; Wang et al., 2005; Hyndman and Evans, 2007). Sequence analyses revealed that even if PPET1 deduced-amino acid sequences exhibited low identity score (around 30%) between vertebrates species, bioactive ET-1 sequences are well conserved (>80% of identity) suggesting lower rate of mutational acceptance in vertebrates ET-1 sequences and a high conservation of functionality of ET-1 during animal evolution (Wang et al., 2006; Hyndman and Evans, 2007).
In the present study, the complete cDNA of sea bass PPET1 was cloned by RACE-PCR. The sequence obtained allowed us to design specific primers in order to investigate effects of teratogenic concentration of dietary retinoic acid on ET-1 gene expression in sea bass larvae using of real time PCR.

2. Materials and Methods

Animals and diets

All procedures concerning the animals and their handling were conducted in compliance with the Guide for the Care and Use of Laboratory Animals. The study was performed under the licence no. 29.021 by the French Department of Veterinary Services (*Direction Départementale des Services Vétérinaires*) to conduct experimental protocols and samplings on fish.

Two experimental groups (N for “Normal” and RA for “Retinoic Acid enriched”) of sea bass larvae were reared at 20°C and fed, from 6 to 38 days post hatching (dph), on microparticulate diets incorporating standard (0.08g retinol/kg diet) or an excess of retinoic acid (0.5g retinol/kg diet), respectively. The compositions of the two diets, described in table 1, were very similar to those previously used that had supported good growth and survival in European sea bass larvae (Villeneuve et al ., 2005). For the two groups, triplicates of pooled larvae (n=100) were collected at 7, 10 and 15 dph. The incidence of all types of craniofacial malformations (head deformities) was determined at 37 dph by analysing 50 individuals after alizarin red and alcian blue staining (Mazurais et al., 2008). Differences in the deformity incidence between the two nutritional treatments were tested by the G-test (Sokal and Rohlf, 1981). A complete description of different types of craniofacial deformities has been previously described by Villeneuve et al. (2004).

Molecular cloning

Total RNA used for RACE PCR was extracted from sea bass larvae sampled at 20 dph. Based on conserved amino acid sequence in salmon and zebrafish PPET1, forward primers (P1 and P2) described in table 2 were designed for 3’RACE-PCR to obtain a sea bass PPET1 ortholog. Reverse primers called P3, P4, P5 (table 2) were next designed from 3’RACE sequence obtained to perform 5’ RACE PCR. 3’ and 5’ RACE PCR were performed using 5’/3’ RACE Kit (Roche, USA) following the manufacturer’s instructions. All RACE products were T-A cloned and sequenced by Millegen company (France). Sequence results were assembled and translated using BioEdit version 7.0.9.0 (Hall, 1999). Mature ET-1 sequences from major vertebrate clade were aligned using this same software.

Expression

Real time-PCR: For the two treatments (N and RA), total RNA were prepared from pool of 100 larvae at 7, 10 and 15 dph. Triplicates were collected for each date. Total RNA was extracted using TRIzol and reverse-transcribed in duplicate (iScript cDNA Synthesis Kit; Bio-Rad Laboratories, Hercules, CA). These duplicates were then pooled. Real time PCR analyses for PPET1 expression were performed as previously described (Mazurais et al., 2008). Primers (P6-9) used for PPET1 and EF1 (housekeeping gene) amplifications are described in table2. Relative expression were treated using $\Delta\Delta$Ct method implemented by BioRad IQ5 software.
Statistics: Results are expressed as means ± SD. Statistical differences (P <0.05) in relative gene expression between N and RA groups were analysed by randomisation tests (Pfaffl et al., 2002) using REST software (http://www.gene-quantification.info/).

3. Results

Molecular cloning

The full-length sea bass PPET1 cDNA sequence was obtained by combining sequences from the 5’ and 3’RACE PCR (GenBank accession no. **EU807743**). Figure 1 showed the resultant full length nucleotide sequence and the deduced amino acid sequence. The full cDNA included an open reading frame of 597 bp encoding a 199 amino acid polypeptide. In order to annotate the deduced amino acid sequence, it was p-blasted on GenBank databases. Blast request (summarized in table 3) showed that the fragment belongs to the endothelin-1 family. On the 199 amino acid polypeptide sequence the regions corresponding to the “mature” and “big” ET-1 were observed (Figure 1). The sequence contained the two

dibasic cleavage sites for furin (K^{44}-$R^{45}$and R^{66}-R^{67}) and the W^{66}-V^{67} cleavage site for ECE. The "mature" sea bass ET-1 domain exhibited more than 80 % of identity with vertebrates orthologs (Figure 2).

Effect of high dietary retinoic acid level

Deformities: Dierary retinol had a significant effect on the development of deformities in sea bass ($p<0.05$). Indeed, 60 % of fish from RA group exhibited skeletal malformations at 37 dph while 28 % of fish from N group displayed deformities (table 4). Most of deformities were observed at the head level. The types of the malformations detected are similar to those previously described by Villeneuve et al. (2004), affecting mainly the neurocranium and splanchnocranium. Figure 3 represents pugheadness frequently observed.
PPET-1 expression: The effects of diet on the normalized expression of PPET-1 gene was determined from pools of whole larvae at 7, 10 and 15 dph. Statistical analysis indicated that PPET-1 transcript levels were significantly higher at 10 (2.36 fold) and 15 (2.04 fold) dph in N group compared to RA group (Figure 4).

4. Discussion

In the present study, we obtained the full length sea bass PPET1 cDNA using 3' and 5'RACE PCR. The deduced amino acid sequence from the cDNA fragment was identified as a PPET1 based upon homology to PPET1 sequence of other vertebrates including fish species (*Danio rerio*, *Oncorhyncus keta* and *Fundulus heteroclitus*). The amino acid sequence possessed the characteristic features of endothelin 1 gene family: cleavage sites for furin and ECE giving rise to "big" and "mature" ET-1 respectively. Moreover, alignment of the sea bass "mature" ET-1 with that of other vertebrates confirmed that the active domain of the protein is well conserved (minimum identity of 80%) throughout different species. Sea bass PPET1 is the first ET-1 precursor sequence identified in perciforme family. The literature described ET-1 as a key actor of the morphogenesis of the neural crest-derived structures whose expression levels greatly influence the development of craniofacial malformation (Miller et al., 2000; Clouthier and Schilling, 2004). ET-1 thus represents an interesting candidate gene in order to better decipher the molecular mechanisms implied in the genesis of the head deformities and to predict malformations encountered in reared fish.
In this context, we have designed primers based on this PPET1 cDNA sequence in order to compare PPET1 transcript levels in sea bass larvae fed normal (0.08g retinol/kg diet) or teratogenic level (0.5g retinol/kg diet) of retinoic acid. It is well known that hypervitaminosis A induces craniofacial abnormalities in vertebrates in general (Morriss-Kay G, 1993; Young et al., 2000) and particularly in fish (*Danio rerio*: Ellies et al., 1997; *Fundulus heteroclitus*: Vandersea et al., 1998). In sea bass, we had previously shown that a diet containing such excess of retinoic acid induced craniofacial malformations when distributed during early development of larvae (Villeneuve et al., 2005, 2006). The malformation induction by retinal excess was confirmed in the present study. In consequence, the retinoic acid-induced craniofacial malformation model represent a good model to point at molecular actors and markers for craniofacial abnormalities observed in reared fishes. In the present work, we demonstrated that in case of hypervitaminosis A, sea bass larvae exhibited lower level of PPET1 messengers in comparison to fish fed normal diet. It should be noted that the difference in PPET1 mRNA level is detected only from day 10 post hatching. Absence of difference at 7 dph, corresponding to day 1 after the first feeding, is probably related to the low level of food intake occurring during the first two days after mouth opening. The lower level of PPET1 mRNA in larvae fed excess retinoic acid is associated to high level of head deformities (60%) encountered in this group at 37 dph. These data are in good agreement with recent findings revealing that down-regulation of ET-1 expression level is involved in retinoic acid-induced craniofacial malformation in mouse and gnathosomes (Zhang et al.,

2006; Vieux-Rochas et al., 2007). This data suggests that PPET-1 expression level could predict deformities incidence induced by retinol as well as by other factors influencing the specific molecular pathways implying endothelin. However, we have to keep in mind that other biological processes (such as ossification) may contribute also to cranial malformation and we cannot presume that PPET1 expression could predict perturbation of the related pathways.

Although our experiment did not address the precise molecular and cellular mechanisms involved in the teratogenic effects of vitamin A excess in sea bass, we can suggest that retinoic acid could act through a RAR-dependent mechanism on ET-1 expression in sea bass jaw to modify skeletal formation. In line with this hypothesis, we have already shown that RAR alpha gene was expressed in the jaw during the early development of sea bass larvae (Villeneuve et al., 2004) and data obtained in zebrafish indicated expression of ET-1 in craniofacial structures (Miller et al., 2000). Unfortunately, our attempts to detect ET-1 gene expression in larvae by means of in situ hybridization failed until now probably due to low expression level of messenger.

In conclusion, the cloning of the sea bass PPET1 cDNA constitutes a molecular tools of great interest to better understand molecular pathways involved in craniofacial development and to predict malformations in a model of economical and ecological interest.

Acknowledgements

The authors thank Hervé Le Delliou for the feeds analyses. M. J. Darias and E Santigosa were supported by postdoctoral fellowships from the Fundación Ramón Areces (Spain) and Alfonso Martin Escudero (Spain), respectively.

References

Boglione, C., Gagliardi, F., Scardi, M., Cataudella, S., 2001. Skeletal descriptors and quality assessment in larvae and post-larvae of wild-caught and hatchery-reared gilthead sea bream (*Sparus aurata* L. 1758). Aquaculture 192, 1-22.

Clouthier, D.E., Schilling, T.F., 2004. Understanding endothelin-1 function during craniofacial development in the mouse and zebrafish. Birth Defects Res C Embryo Today 72, 190-199.

Ellies, D.L., Langille, R.M., Martin, C.C., Akimenko, M.A., Ekker, M., 1997. Specific craniofacial cartilage dysmorphogenesis coincides with a loss of dlx gene expression in retinoic acid-treated zebrafish embryos. Mechanisms of Development 61, 23-36.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41, 95-98.

Hyndman, K.A., Evans, D.H., 2007. Endothelin and endothelin converting enzyme-1 in the fish gill: evolutionary and physiological perspectives. J. Exp. Biol. 210, 4286-4297.

Kurihara, H., Kurihara, Y., Oda, H., Maemura, K., Suzuki, H., Kodama, T., Nagai, R., Ishikawa, T., Yazaki, Y., 1994. Role of endothelin-1 in mouse embryogenesis. Circulation 90, 293-293.

Kurihara, H., Kurihara, Y., Nagai, R., Yazaki, Y. 1999. Endothelin and neural crest development. Cell. Mol. Biol. 45, 639-651.

Levin, E.R., 1995. Mechanisms of disease - endothelins. New England Journal of Medicine 333, 356-363.

Mazurais, D., Darias, M.J., Gouillou-Coustans, M.F., Le Gall, M.M., Huelvan, C., Desbruyeres, E., Quazuguel, P., Cahu, C., Zambonino-Infante, J.L., 2008. Dietary vitamin mix levels influence the ossification process in European sea bass (*Dicentrarchus labrax*) larvae. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 294, R520-R527.

Miller, C.T., Schilling, T.F., Lee, K.H., Parker, J., Kimmel, C.B., 2000. sucker encodes a zebrafish Endothelin-1 required for ventral pharyngeal arch development. Development 127, 3815-3828.

Morriss-kay, G., 1993. Retinoic acid and craniofacial development - molecules and morphogenesis. Bioessays 15, 9-15.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research 30, e36.

Shimada, K., Takahashi, M., Tanzawa, K., 1994. Cloning and functional expression of endothelin-converting enzyme from rat endothelial cells. J. Biol. Chem. 269,18275 -18278.

Thomas, T., Kurihara, H., Yamagishi, H., Kurihara, Y., Yazaki, Y., Olson, E.N., Srivastava, D., 1998. A signaling cascade involving endothelin-1, dHAND and Msx1 regulates development of neural-crest-derived branchial arch mesenchyme. Development 125, 3005-3014.

Vandersea, M.W., McCarthy, R.A., Fleming, P., Smith, D., 1998. Exogenous retinoic acid during gastrulation induces cartilaginous and other craniofacial defects in *Fundulus heteroclitus*. Biological Bulletin 194, 281-296.

Vieux-Rochas, M., Coen, L., Sato, T., Kurihara, Y., Gitton, Y., Barbieri, O., Le Blay, K., Merlo, G., Ekker, M., Kurihara, H., Janvier, P., Levi, G., 2007. Molecular dynamics of retinoic acid-induced craniofacial malformations: implications for the origin of gnathostome jaws. PLoS ONE 2, e510.

Villeneuve, L., Gisbert, E., Cahu, C.L., Le Gall, M.M., Zambonino-Infante, J.L., 2004. Expression and localization of some retinoid receptors during European sea bass (*Dicentrarchus labrax*) larvae development. Aquaculture 242, 537-551.

Villeneuve, L., Gisbert, E., Delliou, H.L., Cahu, C.L., Zambonino-Infante, J.L., 2005. Dietary levels of all-trans retinol affect retinoid nuclear receptor expression and skeletal development in European sea bass larvae. British Journal of Nutrition 93, 791-801.

Villeneuve, L.A.N., Gisbert, E., Moriceau, J., Cahu, C.L., Zambonino-Infante, J.L., 2006. Intake of high levels of vitamin A and polyunsaturated fatty acids during different developmental periods modifies the expression of morphogenesis genes in European sea bass (*Dicentrarchus labrax*). British Journal of Nutrition 95, 677-687.
Wang, H.Y., Quan, J.X., Kotake-Nara, E., Uchide, T., Andoh, T., Saida, K., 2006. cDNA cloning and sequence analysis of preproendothelin-1 (PPET1) from salmon, *Oncorhynchus keta*. Experimental Biology and Medicine 231, 709-712.
Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., Masaki, T., 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332, 411-415.
Young, D.L., Schneider, R.A., Hu, D., Helms, J.A., 2000. Genetic and teratogenic approaches to craniofacial development. Critical Reviews in Oral Biology & Medicine 11, 304-317.
Zhang, Z.F., Xu, Y.J., Li, L., Han, J., Zheng, L.P., Liu, P., Li, Y., 2006. Prevention of retinoic acid-induced early craniofacial abnormalities by folinic acid and expression of endothelin-1/dHAND in the branchial arches in mouse. British Journal of Nutrition 96, 418-425.

Tables

Table 1. Composition of the experimental compound diets

Ingredients (g/kg Diet)	N	RA
Fish meal*	555	555
Hydrolysed fish meal (CPSP)*	140	140
Soyabean lecithin*	175	175
Marine lecithin*	0	0
Vitamin mixture (without RA) **	80	80
Mineral mixture***	40	40
Betaine	10	10
Retinol (all-trans)	0,08	0,5

* All dietary ingredients were commercially obtained: fish meal (La Lorientaise, Lorient, France), hydrolysed fish meal (CPSP, Soluble Fish Protein Concentrate, Sopropêche, Boulogne sur mer, France), Soyabean lecithin (Ets louis François, Saint Maur des Fossés, France).
** Composition/kilogram of the vitamin mixture: choline concentrate 50%, 200 g; vitamin E (500 UI/g) 10 g; vitamin D3 (500,000 UI/g) 0.50 g; vitamin B3 1 g, vitamin B5 2 g; vitamin B1 100 mg; vitamin B2 0.4 g; vitamin B6 300 mg; vitamin C 20 g; vitamin B9 100 mg; vitamin concentrate B12 (1 g/kg), 1 g; biotin, 1 g; vitamin K3 1 g; meso-inositol 30 g; cellulose, 732 1 g.
*** Composition/kilogram of the mineral mixture: 90 g KCl, 40 mg KIO_3, 500 g $CaHPO_4$ $2H_2O$, 40 g NaCl, 3 g $CuSO_4$ $5H_2O$,4 g $ZnSO_4$ $7H_2O$, 20 mg $CoSO_4$ 7H2O, 20 g $FeSO_4$ $7H_2O$, 3 g $MnSO_4$ H_2O, 215 g $CaCO_3$, 124 g $MgSO_4$ $7H_2O$, and 1 g NaF.

Table 2. Sequences of primers used in the present study

Primer name	Purposes	Abbreviation	Sequence (5′–3′)
PPET1-P1	3’RACE	P1	CGCAACNTTCCTRGACAA
PPET1-P2	3’RACE	P2	GGAGTGCGTCTACTTCTGCCA
PPET1-P3	5’RACE	P3	GCTGTCTCACCCTCCACTTC
PPET1-P4	5’RACE	P4	CAGAAGTTTCGGCATGTGTT
PPET1-P5	5’RACE	P5	GCCCTGTCCGTAGGAGACCA
PPET1-P6	qPCR	P6	ACACCTGAGCGCGTGGTCT
PPET1-P7	qPCR	P7	TTCGGCATGTGTTGTCGTT
EF1-P1	qPCR	P8	GCTTCGAGGAAATCACCAAG
EF1-P2	qPCR	P9	CAACCTTCCATCCCTTGAAC

Table 3. Best hits on GenBank databases obtained after p-blast of the deduced 199 amino acids sequence

species	Annotation	reference	score (bits)	E-Value
Fundulus heteroclitus	ET-1A	ABS42948.1	188	$1e^{-46}$
Oncorhynchus keta	ET-1	BAF30875.1	185	$1e^{-45}$
Danio rerio	ET-1	NP_571594.1	174	$2e^{-42}$

Table 4. Occurrence of deformities in Normal (N) and Retinoic Acid (RA) groups. Different letters indicate significant differences between groups ($p<0.05$).

Group	Total deformities	Cranial deformities	Column deformities
N (n=50)	28% (a)	28% (a)	0%
RA (n=50)	60% (b)	58% (b)	4%

Figures

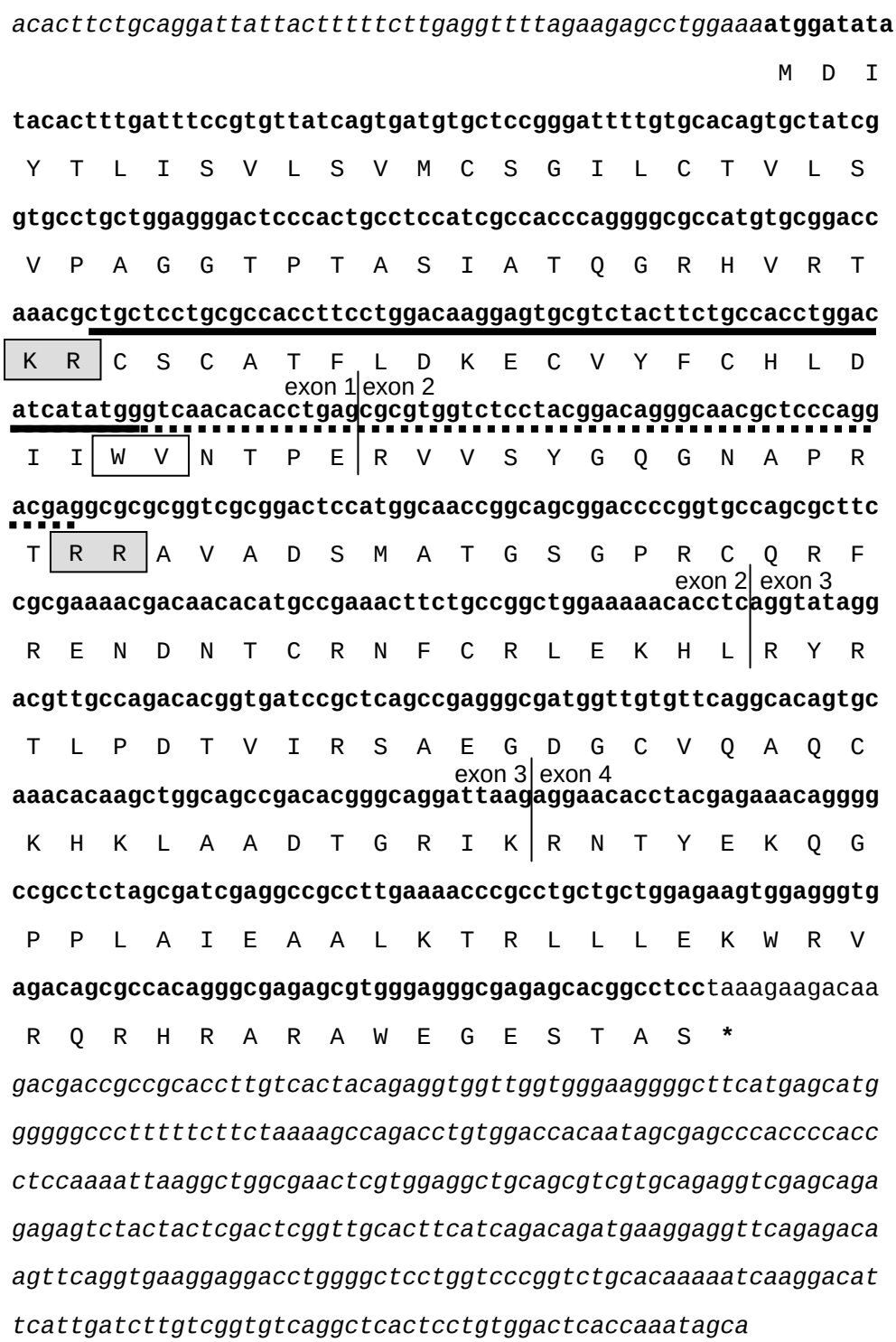

Figure 1: Nucleotide and deduced amino acid sequences of sea bass (*Dicentrarchus labrax*) PPET1. Open reading frame and non-coding regions are indicated in bold and italic letters, respectively. The stop codon is shown by an asterisk. The sequence corresponding to the "mature" and "big" ET1 are underlined by solid and dashed lines, respectively. The cleavage sites for furin, present at the beginning and the end of the "big" ET-1, are grey-boxed. The cleavage site for ECE is white-boxed. Putative exon/intron boundaries predicted from the gene structure of zebrafish PPET1 are shown by thin bars.

	Mature ET-	Identity (%)
ET-1 [Dicentrarchus labrax]	CSCATFLDKECVYFCHLDIIW	-
ET-1 [Fundulus heteroclitus	CSCATFLDKECVYFCHLDIIW	10
ET-1 [Danio rerio]	CSCASFLDKECVYFCHLDIIW	0
ET-1 [Oncorhynchus keta]	CSCATFLDKECVYFCHLDIIW	95
ET-1 [Xenopus laevis]	CSCSSLMDKECVYFCHLDIIW	10
ET-1 [Gallus gallus]	CSCSSLLDEECVYFCHLDIIW	0
ET-1 [Homo sapiens]	CSCSSLMDKECVYFCHLDIIW	80
		85
		80

Figure 2: Alignment of vertebrate “mature” ET-1 sequences. Identical amino acid are highlighted in grey. The percentages of identity for each ET-1 domain compared to sea bass mature ET-1 are indicated on the right column. GenBank accession numbers: ET-1 [*Fundulus heteroclitus*] ABS42948; ET-1 [*Danio rerio*] NP_571594; ET-1 [*Oncorhynchus keta*] BAF30875; ET-1 [*Xenopus laevis*] NP_001090567; ET-1 [*Gallus gallus*]XP_418943; ET-1 [*Homo sapiens*] NP_001946.

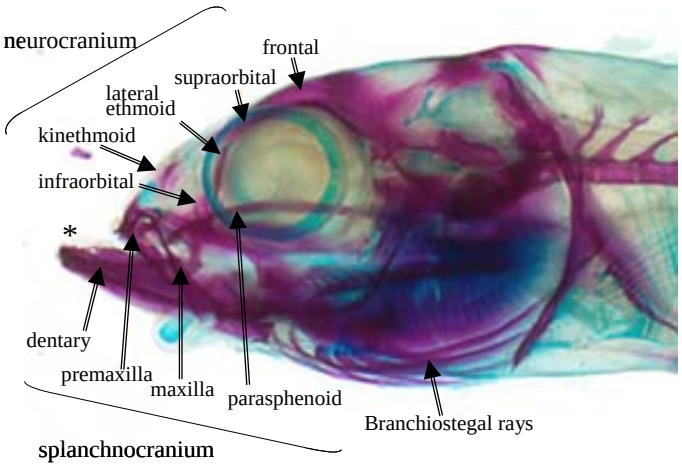

Figure 3: Retinal induced craniofacial malformations in sea bass larvae at 37 dph. Larvae was stained with alizarin red and alcian blue to reveal calcified and cartilage structures respectively. Pugheadness is indicated by an asterisk. Several bones are not visible since they were covered by other structures.

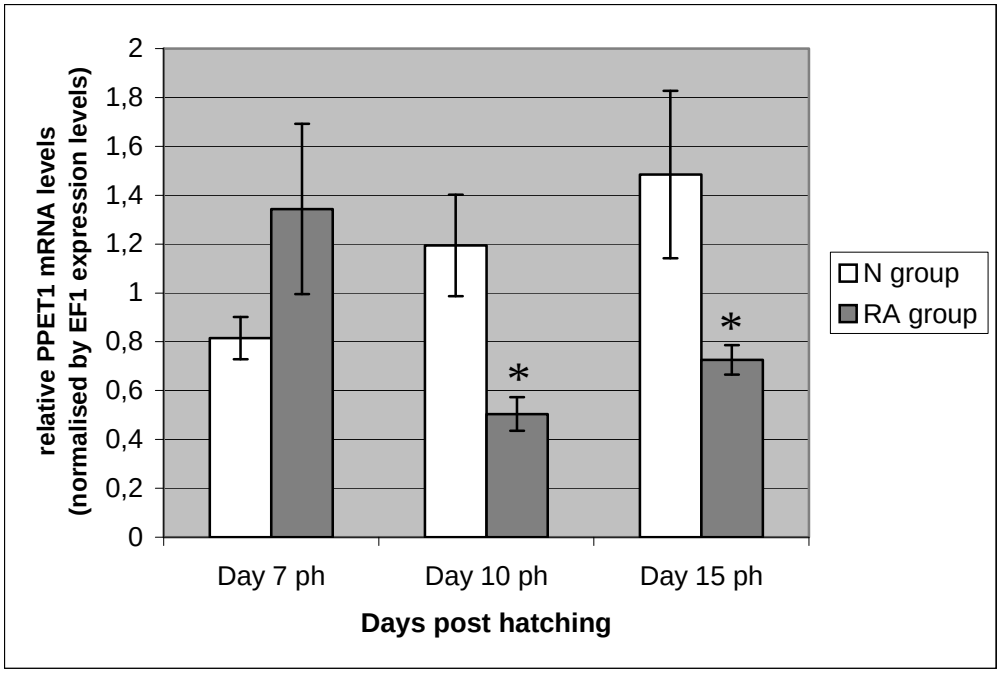

Figure 4: Effects of high dietary retinoic acid level on PPET-1 expression during the early seabass larval development by real time PCR. Data represent the average (± SD) obtained for 3 pools of larvae per group at each date. The stars indicate differential expression between the two groups ($P<0.05$).