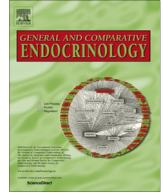




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## Leptin receptor gene in the European sea bass (*Dicentrarchus labrax*): Cloning, phylogeny, tissue distribution and neuroanatomical organization



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## ABSTRACT

In this study, we report the cloning of three transcripts for leptin receptor in the European sea bass, a marine teleost of economic interest. The two shortest variants, generated by different splice sites, encode all functional extracellular and intracellular domains but missed the transmembrane domain. The resulting proteins are therefore potential soluble binding proteins for leptin. The longest transcript (3605 bp), termed *sblepr*, includes all the essential domains for binding and transduction of the signal. Thus, it is proposed as the ortholog for the human *LEPR* gene, the main responsible for leptin signaling. Phylogenetic analysis shows the *sblepr* clustered within the teleost leptin receptor group in 100% of the bootstrap replicates. The neuroanatomical localization of *sblepr* expressing cells has been assessed by *in situ* hybridization in brains of sea bass of both sexes during their first sexual maturation. At histological level, the distribution pattern of *sblepr* expressing cells in the brain shows no clear differences regarding sex or reproductive season. Transcripts of the *sblepr* have a widespread distribution throughout the forebrain and midbrain until the caudal portion of the hypothalamus. A high hybridization signal is detected in the telencephalon, preoptic area, medial basal and caudal hypothalamus and in the pituitary gland. In a more caudal region, *sblepr* expressing cells are identified in the longitudinal torus. The expression pattern observed for *sblepr* suggests that in sea bass, leptin is very likely to be involved in the control of food intake, energy reserves and reproduction.

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**Abbreviations:** Cc, corpus of the cerebellum; CE, cerebellum; CP, central posterior thalamic nucleus; Dlp, lateral posterior part of the dorsal telencephalic area; Vv, ventralnuclei of the ventral telencephalon; Vc, central nuclei of the ventral telencephalon; Dm2, subdivision 2 of the medial dorsal telencephalic area; Dm3, subdivision 3 of the medial dorsal telencephalic area; Dp, posterior portion of the dorsal telencephalon; E, entopeduncular nucleus; HAco, habenular commissure; I, intermediate thalamic nucleus; IL, inferior lobe of the hypothalamus; MaoT, marginal optic tract; NAPv, anterior periventricular nucleus; NAT, anterior tuberal nucleus; NDLL, lateral part of the diffuse nucleus; NDLLm, medial part of the diffuse nucleus of the inferior lobe; NGT, tertiary gustatory nucleus; NH, habenular nucleus; NHd, dorsal habenular nucleus; NHv, ventral habenular nucleus; NLT, lateral tuberal nucleus; NLTi, inferior part of the lateral tuberal nucleus; NLTm, medial part of the lateral tuberal nucleus; NLTv, ventral part of the lateral tuberal nucleus; nMLF, nucleus of the medial longitudinal fasciculus; NPC, central pretectal nucleus; NPGa, anterior pregglomerular nucleus; NPGm, medial pregglomerular nucleus; PI, pineal gland; NPOav, anteroventral part of the parvocellular preoptic nucleus; NPOpc, parvocellular part of paraventricular organ; nPVO, nucleus of the paraventricular organ; NRL, nucleus of the lateral recess; NRLd, dorsal part of the nucleus of the lateral recess; NRLl, lateral part of the nucleus of the lateral recess; NRLv, ventral part of the nucleus of the lateral recess; NRP, nucleus of the posterior recess; NSV, nucleus of the saccus vasculosus; OB, olfactory bulbs; OpN, optic nerve; OT, optic tectum; P, pituitary; PMgc, gigantocellular part of the magnocellular preoptic nucleus; POA, preoptic area; PPD, dorsal periventricular pretectal nucleus; PPv, ventral periventricular pretectal nucleus; PSp, parvocellular superficial pretectal nucleus; PVO, paraventricular organ; SV, saccus vasculosus; TEG, tegmentum; TEL, telencephalon; TLa, nucleus of the torus lateralis; TLo, torus longitudinalis; TPp, periventricular nucleus of the posterior tuberculum; VcE, valvula of the cerebellum; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; VOT, ventral optic tract.

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## 1. Introduction

Leptin is a member of the class-I helical cytokine which was firstly discovered in mouse as the anti-obese hormone. In mammals, is mainly synthesized by the adipose tissue and its signaling pathways act on the brain to adjust the amount of body fat deposits according to the available energy (Wang et al., 2004). Nowadays, several evidences reported in literature on mammals confirm that besides the major role of leptin in energy homeostasis and metabolism this hormone exerts a plethora of different functions, such as reproduction, immune and stress responses, bone formation and angiogenesis (Allison and Myers, 2014; Park and Ahima, 2014).

Over the last 10 years, there has been an increasing interest in the molecular characterization and function of leptin in non-mammalian vertebrates, and in particular in fish. In this later group, due to genome duplication events, up to four leptin genes have been identified in some species such as Atlantic salmon (Angotzi et al., 2013). However only two genes have been described in more evolved species such as tilapia and medaka (Shpilman et al., 2014; Gorissen et al., 2009). Fish sequences generally show low amino acid identity with human leptin form (13–25%) (Gorissen and Flik, 2014). The other common feature among fish leptins is that the distinct isoforms present in the same species usually display differential tissue expression patterns suggesting a potential diversification of physiological functions (Angotzi et al., 2013; Gorissen et al., 2009; Kurokawa and Murashita, 2009). Although the major research focus so far has been the role of leptin in feeding and energy metabolism, research into the contribution of the leptin system in sexual maturation is gaining strength. Leptin stimulates release of *luteinizing hormone (Lh)* and somatolactin in European sea bass and both gonadotropins in rainbow trout (Peyon et al., 2001, 2003; Weil et al., 2003) and the main sexual steroids directly affect the hepatic expression of *lepa1* and *lepa2* in Atlantic salmon (Trombley et al., 2015). An increase in *lep* genes expression has also been associated with sexual maturation in Atlantic salmon male (Trombley et al., 2014) and Arctic charr (Frøiland et al., 2010). Nevertheless a direct involvement of leptin in this process has still to be demonstrated. The physiological actions of this cytokine are mediated by the leptin receptor (Lepr), a membrane spanning receptor of the class-I cytokine receptor family (Tartaglia, 1997). In mammals, six splice variants of the *Lepr* have been characterized of which only the long form encodes all intracellular domain protein motifs necessary for signaling. In the central nervous system of mammals it is the long form the responsible for leptin signal transduction. In fact, the long form of *Lepr* is mainly expressed in the hypothalamic arcuate nucleus, being particularly linked to two different types of neurons. Leptin directly acts on the orexigenic neuropeptide Y and agouti-related peptide neurons (by inhibition of protein expression) and, on the other hand, it also binds on the anorexigenic proopiomelanocortin and the cocaine and amphetamine cells (stimulating the transcription) (Broberger et al., 1998). Beside the long form of the leptin receptor, five shorter isoforms generated by alternative splicing events, have been characterized in mammals, each one showing distinct signaling capacities due to different truncations of the intracellular segment (Lee et al., 1996; Wang et al., 1996). Truncated receptor isoforms show much wider tissue distribution compared to the long form, mostly localized into the central nervous system (CNS).

While fish express two or more leptin genes, only one orthologous of the *Lepr* gene has been identified in fish genomes likely binding all the leptin isoforms (Gorissen et al., 2009; Prokop et al., 2012). Several *lepr* splice variants have been isolated in different fish species but, like the case of mammals, only the long form seems to contain all the protein domains required for intra-

cellular signal transcription (ligand binding domain, transmembrane domain, JAK and STAT docking sites) (Gong et al., 2013; Rønnestad et al., 2010). Most studies on fish focused so far on the molecular characterization, phylogenetic analysis and tissue expression pattern of *lepr*, nevertheless little is known about the physiology and functions of this receptor. Notably, the detailed neuroanatomical localization of *lepr* expressing cells in distinct brain regions is still lacking in fish, with the exception of the pioneer work on embryos and adults of zebrafish (Liu et al., 2010).

In the current study we report the cloning and the exhaustive characterization of the *lepr* in the European sea bass (*Dicentrarchus labrax*). This teleost is a marine perciform of great economic interest that shows a clear interaction between growth, feeding and reproduction (Carrillo et al. 2015). But much remains to be done to understand the possible neuroendocrine role of leptin in this species. With the aim of contributing to this understanding, in the present work we isolated sea bass *lepr* gene and two alternatively-spliced isoforms and studied *lepr* tissue distribution pattern as well as its neuroanatomical localization.

## 2. Material and methods

### 2.1. Animals and tissue collection

Male and female European sea bass (*D. labrax*) of 2 and 3 years of age, respectively, were raised under natural photoperiod and temperature conditions at the facilities of the Instituto de Acuicultura de Torre de la Sal (Spain) (40° NL). They were sampled at two different periods during their first sexual maturation: in September (non breeding; GSI:  $0.08 \pm 0.01$  and  $0.61 \pm 0.15$  males and females, respectively) and February (breeding; GSI:  $4.01 \pm 0.19$  and  $5.46 \pm 0.93$  males and females, respectively). The first sampling was performed during the onset of spermatogenesis in males (stage II) and previtellogenesis in females, and the second one at spermiation in males (stage V) and ovulation/maturation in females. Gonadal development was classified by light microscopy, following previously established criteria (Asturiano et al., 2000; Begtashi et al., 2004). Animals were sacrificed in accordance with the Spanish (Royal Decree Act 53/2013) and European (2010/63EU) legislations concerning the protection of animals used for experimentation. The protocol used to sacrifice the animals was approved by the Welfare Committee of the IATS (Number of Register 09-0201) supervised by the Ministry of Rural and Marine Environment.

### 2.2. RNA purification and cDNA synthesis

Total RNA was extracted from the brain of adult fish using the Maxwell<sup>®</sup> 16 LEV simply RNA Tissue Kit (Promega Corp.) on a Maxwell<sup>®</sup> 16 Instrument (Promega Corp.) following the manufacturer's protocol. This kit includes an individual DNaseI treatment step in order to discard genomic DNA contamination. Total RNA integrity was assessed by electrophoresis in agarose gel and concentration determined by NanoDrop 2000c spectrophotometer reading (Thermo Scientific). cDNA was synthesized from 2 µg of total RNA using random hexamer primers (2.5 ng/µl), dNTPs (0.5 mM of each dNTP) and the SuperScript<sup>™</sup> III Reverse Transcriptase (200U Invitrogen, Carlsbad, CA, USA) in 20 µl reactions. Reverse transcription was performed at 50 °C for 50 min. Reaction was terminated by heating at 70 °C for 15 min. The RNA used for cDNA synthesis came from pooled tissues of male and female adult sea bass ( $n = 2$  animals/tissue) sampled during the pre-spawning season (October).

### 2.3. Cloning of sea bass *lepr* cDNA and its alternatively-spliced transcripts

The putative coding sequence for sea bass long form leptin receptor (*sblepr*) was identified through TBLASTN search on the sea bass genome database (Tine et al., 2014). The amino acid sequence of stickleback (*Gasterosteus aculeatus*) *lepr* (Protein ID ENSGACP0000009534.1), taken from Ensembl (<http://www.ensembl.org>) was used as query. Using brain cDNA as template, three fragments corresponding to the extracellular, intracellular domains and the stop codon region of the *sblepr* were PCR amplified using the *Phusion* High-Fidelity DNA Polymerase (Thermo Scientific™) and primer pairs sbsLepr1-sbsLepr2, sbsLepr3-sbsLepr4 and sbsLepr5-sbsLepr6 (Supplementary Table 1), respectively. The following conditions were used: initial denaturation at 98 °C for 30 s followed by 30 cycles at 98 °C for 10 s, annealing temperature at 50 °C for 30 s and an extension time based on the polymerase processivity (corresponding to 15–30 s/kb of target). To obtain the full *sblepr* coding sequence, equal amount of each purified fragment (2.5 ng/μl) was used as template in an overlapping PCR reaction using *Phusion* High-Fidelity DNA Polymerase and the primer pairs sbsLepr1-RvsbsLepr6. Improved specificity was achieved by using touchdown PCR. The PCR conditions were an initial denaturation step at 98 °C for 30 s followed by 30 cycles at 98 °C for 10 s, an annealing temperature span starting from 66 °C to 62 °C for 30 s and an extension time based on the polymerase processivity (corresponding to 15–30 s/Kb of target). The final step was a single extension cycle at 72 °C for 7 min. The generated PCR product was gel purified and modified using an A-tailing procedure in order to be ligated into the pGEM-T Easy Vector (Promega Corporation, Madison, WI, USA) according to the manufacturer's instruction. Using a sea bass cDNA brain library as template, a PCR was performed where the specific reverse primer sbsLepr7 (Supplementary Table 1), annealing to the extracellular domain of the *sblepr*, was used in combination with the universal primer FwdSKL which anneals to the multiple cloning site of the UNIZAP XR vector, thus amplifying a fragment containing the 5' region of the *sblepr* cDNA. The resulting PCR product was inserted into the pGEM-T Easy vector using the same strategy described above.

Most of the alternatively-spliced *Lepr* transcripts described in the literature are the result of exon removal, particularly exons coding for the intracellular domain. We therefore amplified by PCR the exons coding for the transmembrane and intracellular domains using primers pair sbsExon11 and sbsExon15 (Supplementary Table 1) placed in exon 11 and exon 15 respectively. All PCR products were loaded on agarose gels, separated by electrophoresis, purified and sequenced.

### 2.4. Sequence analysis and phylogeny

The Sequencher DNA Sequence Analysis software (Gene Code Corporation) was used to assemble sequence contigs. Ig-like C2-type domain and fibronectin type 3 domains were identified through HMMER searches against the Pfam (Punta et al., 2012) database using the predicted amino acid sequence from sea bass *sblepr* as query. The presence and location of a signal peptide cleavage site was predicted using the SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>). Amino acid sequences were aligned with the CLUSTALW multiple alignment software (Thompson et al., 1994) using default parameters. Phylogenetic analysis was performed with the Molecular Evolutionary Genetics Analysis (MEGA 5) software (Tamura et al., 2011). The phylogenetic tree was constructed with the neighbor-joining method and 1000 bootstrap permutations. All amino acid sequences used for alignments

and phylogenetic analysis were extracted from GenBank (<http://www.ncbi.nlm.nih.gov/genbank>) and Ensembl (<http://www.ensembl.org>).

### 2.5. Quantitative real-time PCR (qPCR)

The tissue expression patterns of *sblepr* in different tissues were quantified by qPCR of the cDNA on a Mastercycler® *realplex2* (Eppendorf AG, Hamburg, Germany) in ABgene's96-well optical plates (Thermo Fisher Scientific, Inc., Epsom, UK). All samples were run in duplicate. Primers set sbsLepr9-sbsLepr10 (105 pb amplicon size) for *sblepr* qPCR assay (Supplementary Table 1) was designed using the Primer3 software (<http://simgene.com/Primer3>). Calibration curves were generated with serial dilutions of cDNA. The efficiencies of PCR reaction was 0.97. For each 20 μl of PCR, optimized amounts of primers (0.4 μM) were mixed with 1 μl of RT reaction in 1× PyroTaqEvaGreen qPCRmix Plus (no ROX; CultiK Molecular Bioline). PyroTaq is activated by a 15 min incubation step at 95 °C and subsequently qPCR cycling conditions were an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing-extension at 60 °C for 1 min. A last melt curve analysis cycle was included to verify the existence of a single qPCR product and ensure the absence of primer-dimer artifacts. The captured data were analyzed by the *realplex* software (version 2.2). Expression of the 18S ribosomal RNA (18S) gene (Felip et al., 2008) (specific primer pairs sbs18S-sbs18S in Supplementary Table 1) in 1:50 diluted cDNA samples was used as a reference gene for data normalization. The  $\Delta\Delta C_t$  method was used to determine the relative mRNA expression (Livak and Schmittgen, 2001). Expression of *sbslepr* in testis and ovary are plotted separately. Data from somatic tissues expression in both female and male samples were treated collectively and are plotted as the median value. Data are represented as the relative fold increase respect to the lowest mean expression according to Tinoco et al. (2012).

### 2.6. In situ hybridization

Males and females ( $n = 2-4$  per sex and season) were anesthetised in 0.1% phenoxy ethanol (Merck Schuchardt OHG, Hohenbrunn, Germany) and then transcardially perfused using a peristaltic pump with 50 ml of 0.65% NaCl and a fixative solution (4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4, and 5% acid picric). Tissues were collected, fixed overnight in the same fixative solution at room temperature, dehydrated, embedded in paraffin and cut transversally in series at 6 μm-stick. All sections were mounted onto poly-L-Lysine-coated slides and keep at 4 °C.

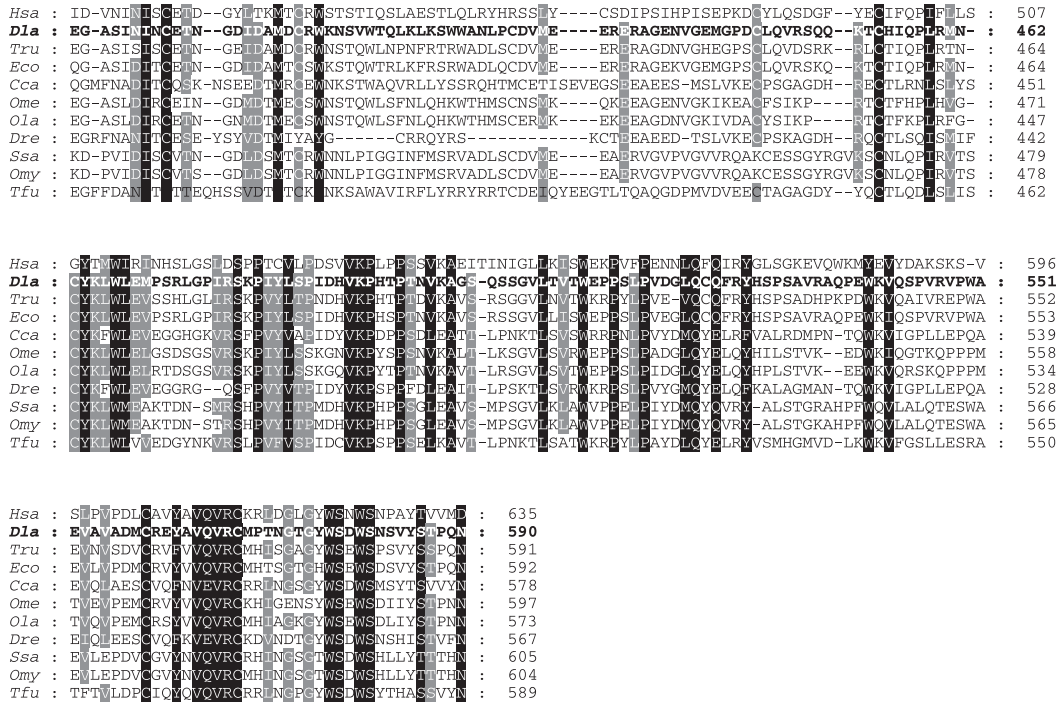
*In situ* hybridization (ISH) for *sblepr* was performed as described previously (Escobar et al., 2013a) with slight modifications. A 803 pb fragment of *sblepr* cDNA (nucleotide 644-1447) cloned into the pGEM-T Easy Vector (Promega Corporation, Madison, WI, USA) using the primer pairs sbsLepr3-sbsLepr8 was used for the synthesis of digoxigenin-labeled cRNA probes. The plasmid was linearized with *SacII* or *Sall* restrictions enzymes and antisense or sense RNA probes were generated using SP6 or T7 RNA polymerases (Promega, Madison, WI) by *in vitro* transcription according to the DIG RNA labeling MIX manufacturer's instructions (Roche Applied Science, Indianapolis, IN).

## 3. Results

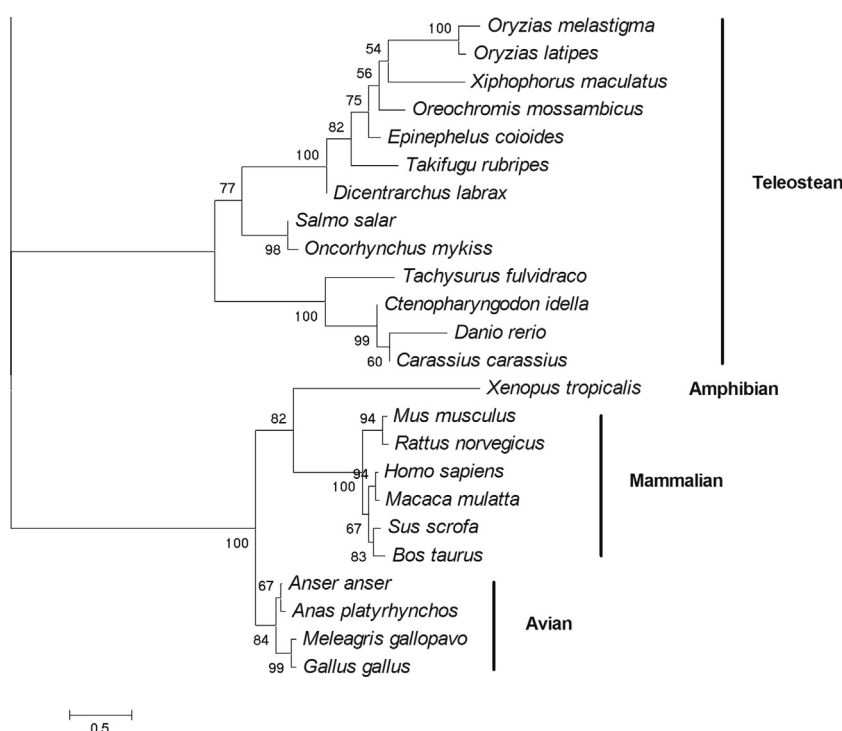
### 3.1. Cloning and phylogeny of sea bass leptin receptor transcripts

Three transcripts of sea bass leptin receptor were cloned in this study. The longest cDNA is the only transcript that includes all the





**Fig. 2.** Amino acid sequence alignment of the leptin binding domain of human and fish Lepr proteins. Sequences were aligned using ClustalW. Identical amino acids are highlighted in black and similar (80%) in grey. Species abbreviations and GenBank/RefSeq/Ensembl accession numbers are: *Hsa*, *Homo sapiens* (AAA93015) ***Dla***, ***Dicentrarchus labrax*** (KF918755); *Tru*, *Takifugu rubripes* (BAG67079); *Eco*, *Epinephelus coioides* (AFU55262); *Cca*, *Carassius carassius* (ADZ75460); *Ome*, *Oryzias melastigma* (ABC86922); *Ola*, *Oryzias latipes* (BAH24203); *Dre*, *Danio rerio* (XP\_005165935); *Ssa*, *Salmo salar* (BAI23197); *Omy*, *Oncorhynchus mykiss* (AGC55253); *Tfu*, *Tachysurus fulvidraco* (AFO67946). The numbers on the right refer to amino acid position on the complete sequences.



**Fig. 3.** Phylogenetic tree of Lepr. Tree was generated using an alignment of complete protein sequences. Bootstrap values, number of times each branching was obtained, are indicated as a percentage out of 1000 bootstrap iterations. GenBank/RefSeq/Ensembl accession numbers of the sequences are: *Oryzias melastigma*, ABC86922; *Oryzias latipes*, BAH24203; *Xiphophorus maculatus*, ENSXMAP00000002254; *Oreochromis mossambicus*, AGT28753; *Epinephelus coioides*, AFU55262; *Takifugu rubripes*, BAG67079; *Dicentrarchus labrax*, KF918755; *Salmo salar*, BAI23197; *Oncorhynchus mykiss*, AGC55253; *Tachysurus fulvidraco*, AFO67946; *Ctenopharyngodon idella*, AFU35431; *Danio rerio*, XP\_005165935; *Carassius carassius*, ADZ75460; *Xenopus (Silurana) tropicalis*, ABD63000; *Mus musculus*, AAC52408; *Rattus norvegicus*, AAC52587; *Homo sapiens*, AAA93015; *Macaca mulatta*, AAF34683; *Sus scrofa*, AAC61766; *Bos taurus*, ENSBTAP00000007764; *Anser anser*, AEE61372; *Anas platyrhynchos*, ENSAPL00000015829; *Meleagris gallopavo*, ENSMGAP00000012466; *Gallus gallus*, AAF31355.

Sea bass lepr exon 15'- 94bp  
 1810 aggtcgcagtgccggacatgtgccgggagatgctgggtgcaagtacgctgcatgccactaatggcactggctactggagcagctggagcaactcgggtga  
 V A V A D M C R E Y A V Q V R C M P T N G T G Y W S D W S N S V Y

1910 ctccacacctcagaacagcagagcctacagagagacccctgacgctacatgatgctgatgatcatctccttccctccatcgtcctgctttgtcaccctg  
 S T P Q N S R A T R G D P A A Y M M L M I I S F L S I V L F V T L

2010gtcctctcccaaacagatgaaaaagtttatgtggaaggatgtgcccaacctaacaagtgtcctgggccaaggactagacttcaaaaaggctgaca  
 V L S Q N Q M K K F M W K D V P N P N K C S W A K G L D F K K A D T

2110 cctttgatcagctgttccgacctccagaggcctcccacccctggcgatgctcctcctcgtcgagaacatttccaaagctgcatagtggaaggttga  
 F D Q L F R P P E G L P P W P M L L P S E N I S K V V I V D K V D

Sea bass lepr exon 15'- 254bp  
 1810 aggtcgcagtgccggacatgtgccgggagatgctgggtgcaagtacgctgcatgccactaatggcactggctactggagcagctggagcaactcgggtga  
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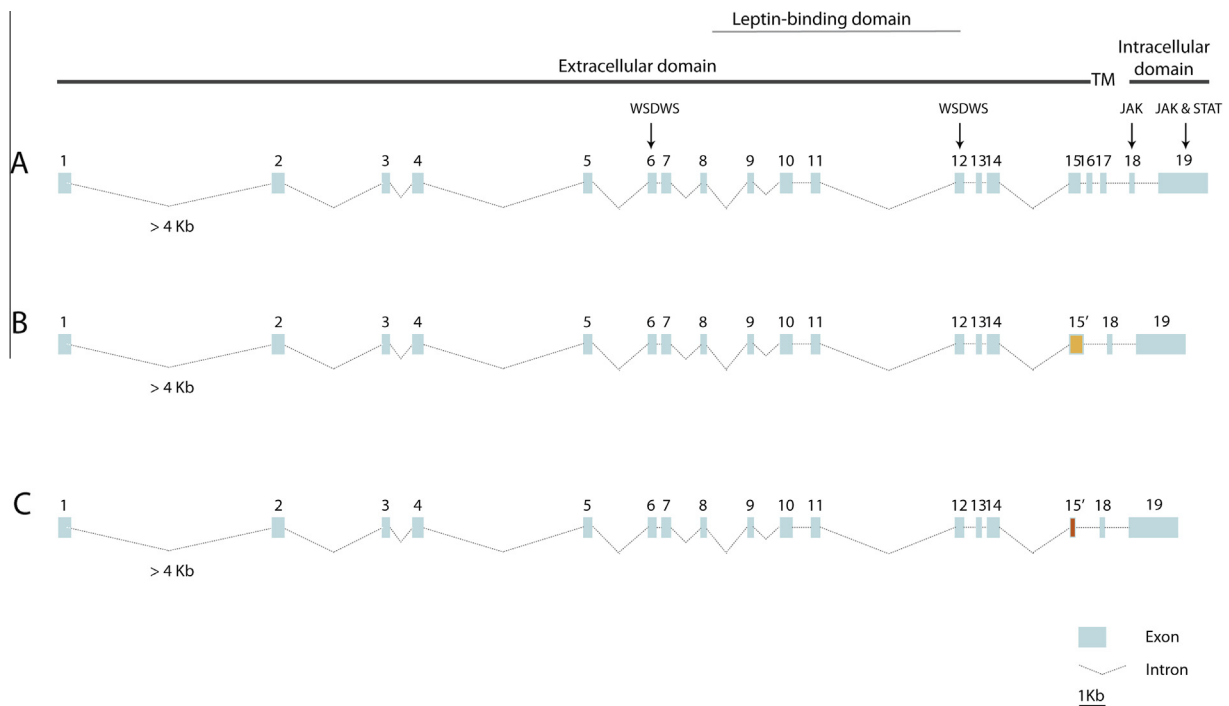
1910 ctccacacctcagaacagcagaggtacaaatggagaaggggagcagatttacaccacaggtatgctccagttgctggaatgttatatcagttccatattca  
 S T P Q N S R G T M E K G S R F T P Q V C S S C V N V I S V P Y S

2010 ccattgaaagtgtttaagggtagacacagtgccagctactttatgtgtttatattataggctgatgtgtattttccaaactgttacagcactagaggag  
 P L K C L R V T Q C Q Y F N C V I N Y R S D V Y F P T V T A T R G D

2110 acctgacgctacatgatgctgatgatcatctcctcctcctccatcgtcctgctgtttgtcaccctggctcctcctcccaaacagatgaaaaagtttatgtg  
 P A A Y M M L M I I S F L S I V L F V T L V L S Q N Q M K K F M W

2210 gaaggatgtgcccaaccctaacaagtgtcctcctgggccaaggactagacttcaaaaaggctgacacctttgatcagctgttccgacctccagagggcctc  
 K D V P N P N K C S W A K G L D F K K A D T F D Q L F R P P E G L

**Fig. 4.** Nucleotide and deduced amino acid sequence of the two *sblepr* cDNA isoforms. Numbers on the left refer to position of the nucleotide residues. The alternative exon 15, exon 15', short (15'-94 bp) and large (15'-254 bp) versions is double underlined.



**Fig. 5.** Drawing of the gene structure of the coding region of long-form *sblepr* and the two amplified shorter isoforms, *sblepr*<sub>11</sub> and *sblepr*<sub>12</sub>. (A) The long form *sblepr* includes an extracellular domain containing a leptin-binding domain and other conserved features (e.g. twoWSXWS motifs). The extracellular domain is followed by a transmembrane domain (TM) and an intracellular domain that includes two JAK2 binding motif boxes and a STAT-binding motif box. (B) The *sblepr*<sub>11</sub> has an alternative exon 15, the exon 15' that in this isoform is 254 bp long and exons 16 and 17 are missing. (C) The isoform *sblepr*<sub>12</sub> has a shorter (94 bp) version of the alternative exon 15' and exons 16 and 17 are missing. In both isoforms the TM, encoded by exon 17, is missing. The annotation assembly from the sea bass genome database (Tine et al., 2014) was used to retrieve information regarding introns sequence.

domains for binding and transduction of the signal and therefore was considered to be the canonical- or long-form of the sea bass leptin receptor, that we named *sblepr*. The amplified cDNA (GenBank Accession No. KF918755) is 3605 bp long and has an open reading frame (ORF) that codes for a protein of 1149 amino acids (Fig. 1). The first 26 residues are predicted to be the putative signal peptide. Pfam searches and sequence alignment analysis allowed the identification of all functionally important domains conserved among vertebrate Leprs. These include two fibronectin type 3 domains, an Ig-like C2-type domain, a pair of repeated trypto-

phan/serine motifs (WSXWS) in the extracellular domain, two JAK2-binding motif boxes and a STAT-binding domain in the intracellular domain (Fig. 1). Amino acids 384–590 in the extracellular domain are predicted to form the leptin-binding domain of sbLepr (Figs. 1 and 5). The sbLepr protein has the highest identity to leptin receptor of other fish species (78–34%) and is only 23% identical to the human LEPR (Fig. 2).

The evolutionary relationship of the *sblepr* to other fish, amphibian, and mammalian and avian leptin receptors was inferred by performing a phylogenetic analysis by the neighbor-

joining method. The topology of the resulting tree (Fig. 3) shows the *sbLepr* clustered within the teleost leptin receptor group in 100% of the bootstrap replicates.

Using a PCR strategy, two shorter sea bass *lepr* variants were amplified. They were termed isoform 1 and 2 (*lepr<sub>11</sub>* and *lepr<sub>12</sub>*), respectively. Generation of these transcripts is based on the usage of different splice sites. Both isoforms encode all the functional domains in the extracellular and intracellular domains but they lack a transmembrane domain and thus the produced proteins should bind leptin and be mostly soluble (soluble binding proteins for leptin; LepBPs) (Fig. 5). Apart from having fewer exons (exons 16 and 17 are missing), the nucleotide sequence of exon 15 is changed due to an alternative splicing junction (Figs. 4 and 5). The difference between both isoforms concerns the length of this exon that is 254 bp and 94 bp long in *lepr<sub>11</sub>* and *lepr<sub>12</sub>*, respectively (Fig. 4).

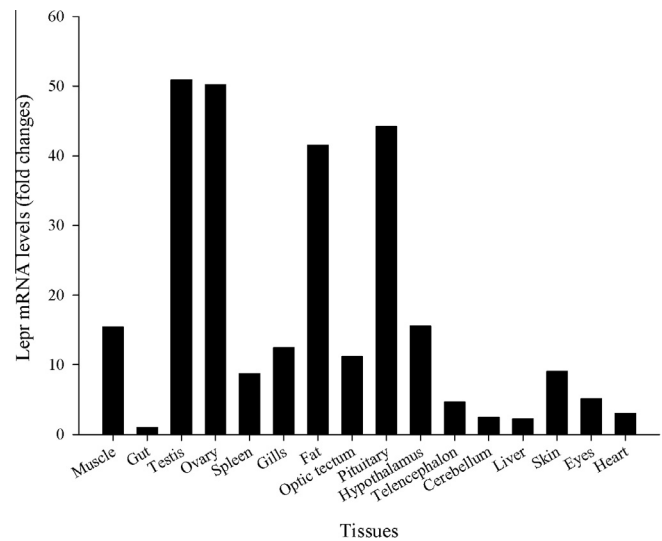
### 3.2. Study of *sblepr* tissue distribution

The highest expression levels are observed in the gonads (both testis and ovary), the pituitary gland and the adipose tissue (Fig. 6). The expression of *sblepr* is relatively high also in the muscle and brain, notably elevated in the hypothalamus, followed by the gills, spleen and skin (Fig. 6).

### 3.3. Neuroanatomical localization of *sblepr* expressing cells in sea bass brain and pituitary

Neither an evident sexual dimorphism, nor obvious differences with reproductive seasons are observed regarding the localization of *sblepr* expressing cells in the brain and pituitary of European sea bass during their first sexual maturation. Leptin receptor expressing cells are widespread into the forebrain and midbrain of adult male and female sea bass until the caudal portion of the hypothalamus. A major site of expression of *sblepr* is detected at the level of the telencephalon. Within the periventricular region of the telencephalon, *sblepr* expressing cells are observed in the subpallial region, where many scattered tiny cells appear to completely cover the dorsal (Vd), ventral (Vv) and post commissural (Vp) parts (Figs. 7B,C,E, 8A). In the pallium, a conspicuous number of *sblepr* containing cells are remarkably detected in the pars dorsalis (Dd) (Figs. 7B and C, 8D), in the pars lateralis dorsal (Dld) (Figs. 7C and D, 8D), in the subdivision 2, 3 and 4 of the pars medialis (Dm2, Dm3, Dm4), in the pars lateralis ventralis (subdivision 2, Dlv2) and in the nucleus taenia (NT) (Figs. 7C–E, 8B–D). They appear as dense clusters of small positive cells corresponding to the structures of the telencephalon.

*Sblepr* expressing cells are very evident at the level of the preoptic area (POA) (Figs. 7D, 8E) in distinct regions including the parvocellular preoptic nucleus in both its anteroventral and parvocellular components (NPOav, NPOpc) that represent major sites of expression. Also the posterior gigantocellular part of the magnocellular preoptic nucleus (PMgc) (Figs. 7G, 8F) exhibits *sblepr* mRNA expression under the form of fluorescent fur surrounding the very large nuclei characteristic of the magnocellular cells (Fig. 8F). A string of *sblepr* mRNA is detected in cells located along the boundary of the dorsal habenula (NHd) (Figs. 7G, 8G). In a more caudal region, *sblepr* mRNA expression is observed in the tuberal region, notably into the dorsal (NLTd) (Figs. 7H, 8H), ventral (NLTV) (Figs. 7H and I, 8L and M) and the medial zones (NLTm) (Figs. 7H, 8K and L) parts. Fig. 8K shows the medial part of the lateral tuberal nucleus, that is placed just above the pituitary stalk, as well as a portion of the pituitary gland (Pit), where *sblepr* expressing cells are detected (Figs. 7H and I, 8I–K). Lastly, *sblepr* expressing cells are identified in the posterior recess nucleus (NRP), as shown in Figs. 7J and 8P. Positive cells are observed at



**Fig. 6.** Tissue distribution of the leptin receptor (*sblepr*) in sea bass. Data represents the relative fold increase calculated from qPCR using  $\Delta\Delta$  Ct method after standardization with 18S gene. The fold increase is respect to the lowest mean expression value (gut) that was set as one. Testis and ovary are depicted separately.

the level of the lateral recess starting from its opening, in the ventral part (NRLv), to its more caudal regions (Figs. 7I and J, 8M, O). High expression level of *sblepr* mRNA is evident into the longitudinal torus (TL0) (Figs. 7J, 8N) and the cerebellar valvula (VCe) including both the granular (G) and molecular (M) layers (Figs. 7H–J, 8N).

## 4. Discussion

The major goal of the present study was to map for the first time, *sblepr* expression in the brain of a marine teleost as a tool for future studies on the role of leptin in the neuroendocrine function of teleosts. Accordingly we have cloned and analysed the sequence of three leptin receptor transcripts in the European sea bass, an economically valued marine perciform species. The longest cDNA sequence (*sblepr*) has been proposed as the European sea bass ortholog of the mammalian long form of leptin receptor (LEPR or Obrb), the major responsible for leptin signaling. Thus, this isoform has been further characterized by performing phylogenetic analysis, tissue distribution studies and a fine neuroanatomical localization of *sblepr* expressing cells in brain and pituitary of adult fish.

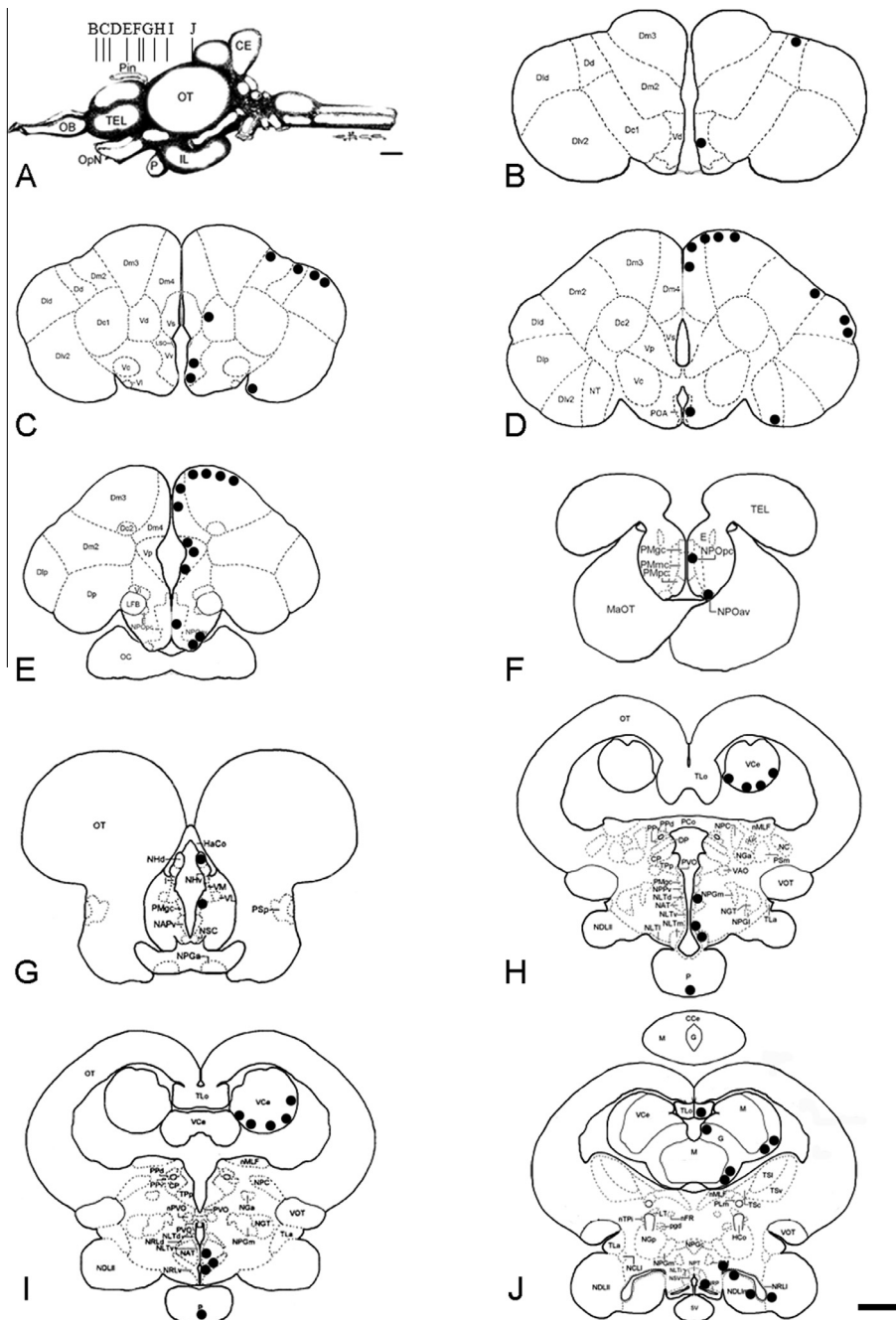
Sequence analysis shows that the predicted *sbLepr* is only 23% identical to the human LEPR but stretches of high sequence identity can be recognized and they correspond to functionally important protein domains that are known to be essential for ligand binding and signal transduction. These conserved structural constraints could partially explain the enhanced release of Lh from dispersed pituitary cells obtained from male sea bass after treatment with mouse recombinant leptin (Peyon et al., 2001). On the other hand, phylogenetic analysis indicate that *sblepr* clusters with all teleost orthologs so far isolated, being closely evolutionarily related to other perciform.

In fish, multiple alternatively spliced *lepr* transcripts have been found (Cao et al., 2011; Gong et al., 2013; Rønnestad et al., 2010). They generate shorter isoforms of the receptor and at least some of them act as leptin binding proteins in the blood (Gong et al., 2013). In rainbow trout, fasting resulted in temporal and spatial changes in gene expression and plasma levels of the leptin receptor isoforms (Gong et al., 2013). These data suggest the existence of a complex regulatory mechanism of leptin action in salmonids

where soluble leptin receptor isoforms could be involved in modulating the endocrine and/or paracrine output of leptin in target tissues. Both *sblepr* transcript isoforms isolated in this study have their transmembrane domains missing, while maintaining the extracellular domain, suggesting that they could produce soluble proteins able to bind leptin. They differ from the reported fish leptin receptor shorter isoforms in that they conserve their capability of JAK/STAT signaling. The contribution of European sea bass leptin receptor isoforms to the regulation of the leptin system in this teleost could be a challenging promising future research area.

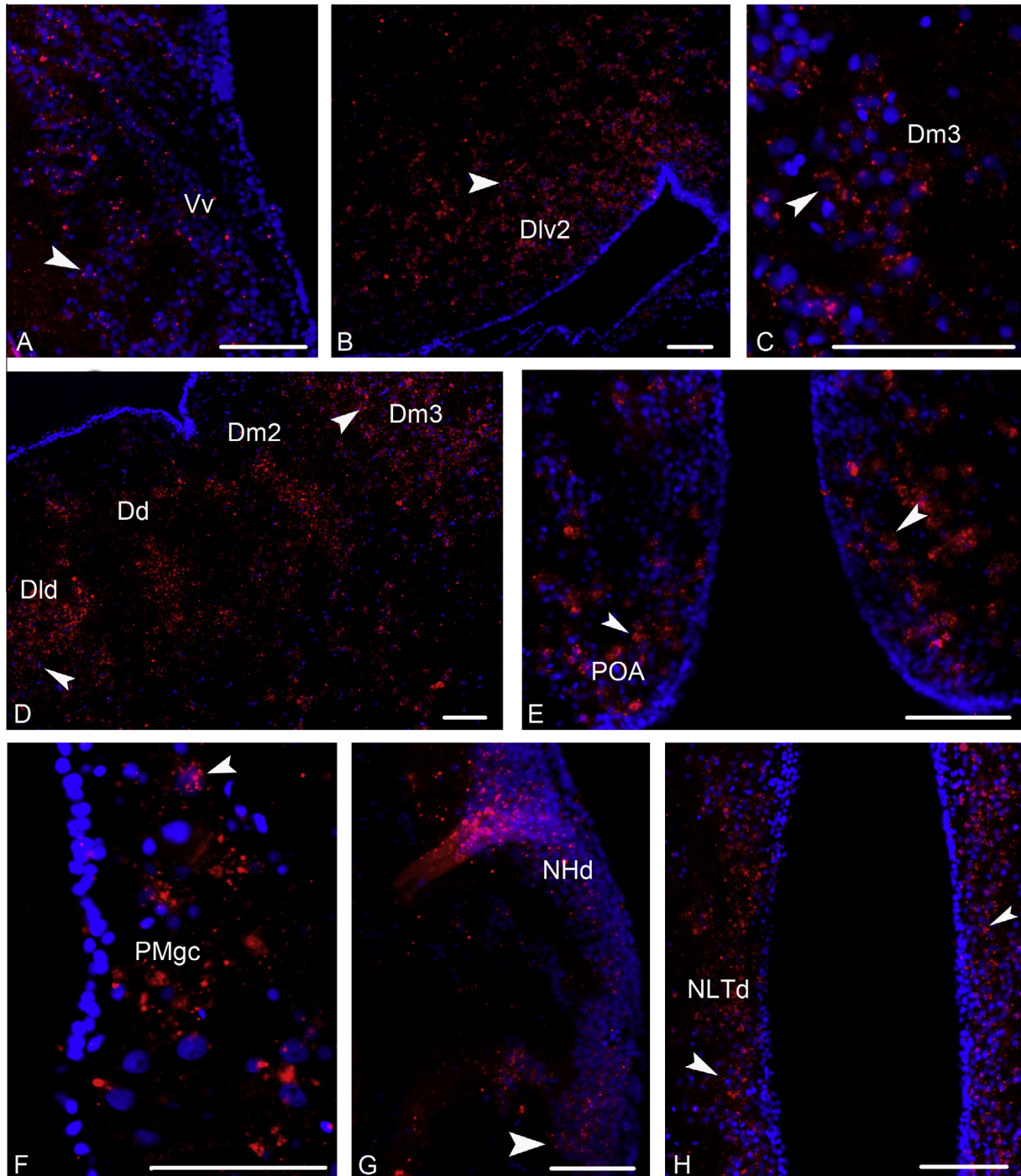
Analysis of the distribution of the long form of *lepr* in the CNS of European sea bass by *in situ* hybridization has not revealed any

obvious change according to sex or reproductive season (gonadal resting and mature stages). These results do not exclude the existence of sexual dimorphism in *sblepr* expression in European sea bass CNS, or the absence of differences regarding gonadal stage. To confirm such result, a quantitative study of the *in situ* hybridization signal should be performed. Further investigation is required to better characterize the contribution of *LepR* in European sea bass reproduction. The specific localization of *sblepr* expressing cells reported in our work is a foundation for this kind of studies. In all animals, *sblepr* expressing cells were widely observed in the fore- and hindbrain including the most caudal nuclei of the hypothalamus.



**Fig. 7.** Schematic drawings of transverse sections taken from the brain atlas of the European sea bass (Cerdá-Reverter et al., 2001a,b) summarizing a representative distribution of *sblepr* expressing neurons in female (Fe) and male (Ma) European sea bass during the breeding (Br) and non-breeding (NBr) seasons. (A) Sideview of the sea bass brain indicating the levels of the transverse sections. (B–J) Black dots indicate the location of *sblepr* expressing *sblepr* cells. Scale bar = 1 mm.





**Fig. 8.** *In situ* hybridization of *sblepr* on transverse sections. Cells expressing *sblepr* (arrowheads) in the ventral (Vv) nuclei of the ventral telencephalonVd (A). Cells expressing *sblepr* (arrowheads) in the pars lateralis ventralis (subdivision 2, Div2), in the subdivision 2, 3 and 4 of the pars medialis (Dm2, Dm3, Dm4), the telencephalic dorsal area in the pars dorsalis (Dd) and in the pars lateralis dorsal (Dld) (B–D). Numerous cells expressing *sblepr* (arrowheads) were located at the level of the preoptic area (POA) (E) and also the more posterior gigantocellular part of the magnocellular preoptic nucleus (PMgc) (F). Cells expressing *sblepr* (arrowheads) along the boundary of the dorsal habenula (NHd) (G). Numerous cells expressing *sblepr* (arrowheads) were located at the dorsal part of the lateral tuberal nucleus (NLTd) (H) along the third ventricle of the hypothalamus. Notable expression of *sblepr* in the pituitary (I–J), the lateral tuberal zone, the ventral (NLTv) and the medial (NLTm) parts (K–M). High expression of *sblepr* is evident in the longitudinal torus (TLo) (N) and at the level of the lateral recess starting from its opening, in the ventral part (NRLv), until its lateral region (NRLl), defining the boundary of these recesses (M, O). Cells expressing *sblepr* (arrowheads) in the posterior recess nucleus (NRP) (P). A (NBr–Fe); B–D (Br–Ma); E (NBr–Ma); F (NBr–Fe); G–H (NBr–Fe); I–J (Br–Fe); K–L (Br–Ma) and N–P (NBr–Ma). Bar = 25  $\mu$ m.

Despite of the low sequence homology between fish and mammalian leptin receptor forms, the overall distribution of the main expression sites of *lepr* in the brain of European sea bass and rat, mouse or human is comparable. For instance, among the regions showing an intense *in situ* hybridization signal in mouse and rat

brains are the arcuate, ventromedial, paraventricular nuclei of the hypothalamus, the hippocampus and the medial habenula (Elmquist et al., 1998; Mercer et al., 1996a,b). The homologous nuclei in fish are the lateral and anterior tuberal nuclei, the parvocellular preoptic nucleus of the hypothalamus, the medial dorsal

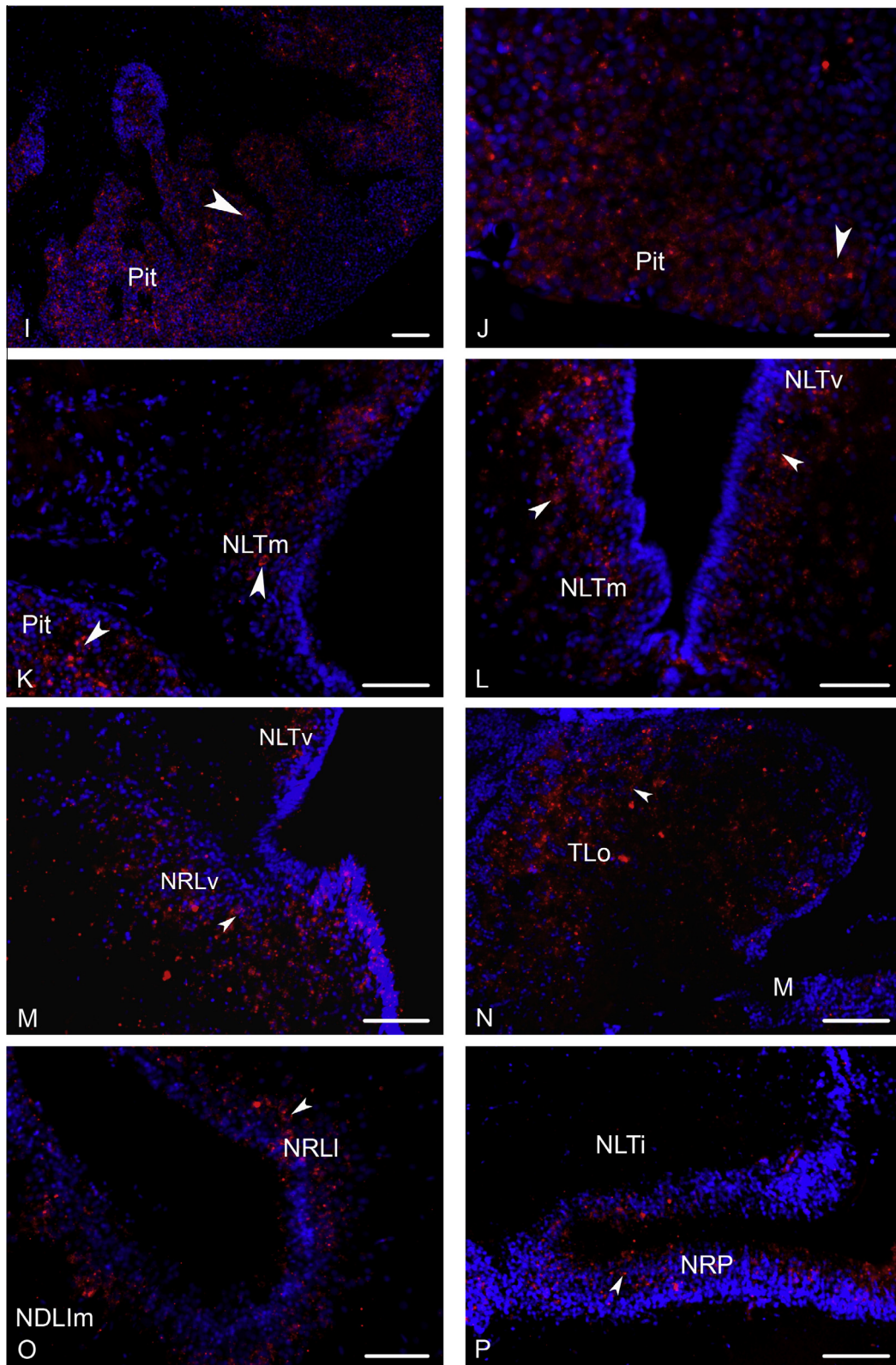


Fig. 8 (continued)

telencephalon, and the dorsal habenula, respectively and in the sea bass brain (this work), they also show an evident expression of *lepr*. Thus, several hypothalamic and extra hypothalamic regions expressing *lepr* are conserved in vertebrates, suggesting that part of the neural pathways implied in the transmission of leptin functions in vertebrates might be similar.

Starting from the anterior brain of European sea bass, *lepr* expressing cells were observed in the telencephalic ventral area (Vd, Vv) and the hypothalamic parvocellular preoptic nucleus, the anteroventral and parvocellular parts (NPOav, NPOpv), being regions that extensively express neuropeptide Y (NPY) in this species (Cerdá-Reverter et al., 2000). Leptin receptor could potentially

be coexpressed in NPY containing cells also in the lateral tuberal nucleus (NLT). This region is the fish homolog for the mammalian arcuate nucleus, major hypothalamic center for the control of food intake, feeding behavior and energy homeostasis in vertebrates. Agouti-related protein 1 (AgRP1) containing neurons have also been reported to be present in the same location in the European sea bass brain (Agulleiro et al., 2014). Thus, although double-labeling studies have not been performed yet, it is likely that NPY neurons of the NLT might express both *agrp1* and *lepr* messengers in European sea bass (Agulleiro et al., 2014; Cerdá-Reverter et al., 2000). Moreover, according to the existing literature, POMC-derived peptides and CART cells are present in the same hypothalamic region in both mammals and fish (Akash et al., 2014; Gautvik et al., 1996; Koylu et al., 1998). All together, these evidences could suggest that there might be an interplay between leptin and the orexigenic NPY/AGRP and the anorexigenic POMC/CART systems.

The populations of *sblepr* expressing cells observed in the lateral and ventral parts of the lateral recess nuclei and in the posterior recess probably contain NPY in European sea bass (Cerdá-Reverter et al., 2000). This means that interaction between leptin signaling and NPY neurons could also be occurring at the level of the more caudal tuberal hypothalamus. There is an extensive literature describing NPY as one of the most powerful orexigenic neuropeptide in teleost (Volkoff et al., 2005), acting on the regulation of food intake and energy metabolism at both hypothalamic and pituitary levels. Moreover, NPY plays a role in the stimulation of gonadotropins and growth hormone from the pituitary. In fact, the administration of NPY, both *in vivo* and *in vitro* (to dispersed pituitary cells) in European sea bass stimulates Lh release. This NPY effect is dependent on the nutritional status of the animal at the moment of the treatment (Cerdá-Reverter et al., 1999; Peyon et al., 2001). From this finding it is clear that NPY acts directly on Lh secreting gonadotrophs, but it cannot be excluded an upstream role of NPY firing from the hypothalamus. In fact, NPY expressing cells are also located at the level of the preoptic area, where gonadotropin releasing hormone immunoreactive cells are well characterized in European sea bass brain (González-Martínez et al., 2002). Our results clearly show the presence of *sblepr* containing cells at both brain (preoptic region) and pituitary levels. This is in line with the hypothesis that NPY is mediating the leptin effect on the modulation of feeding behavior and reproductive functions. Such an assumption has already been proposed by Peyon and collaborators when they observed that a co-treatment with leptin and NPY was more potent in stimulating Lh and somatolactin release in late pubertal sea bass pituitary cells than when leptin or NPY alone (Peyon et al., 2001, 2003).

Also worth of mentioning is the fact that 100% of the somatostatin immunoreactive cells of the mediobasal hypothalamic (nucleus lateralis tuberis) neurons express kisspeptin receptors (Escobar et al., 2013b). Somatostatin inhibits the secretion of growth hormone from pituitary somatotropes in both mammals and teleost (Burgus et al., 1973; Canosa et al., 2005; Fryer et al., 1979; Marchant et al., 1987), leading us to propose possible interactions between the growth/metabolic and the reproductive axis. In fact, in male sea bass fish size and/or energy level are very likely a permissive condition to attain the threshold of hormone synthesis and release needed to achieve its reproductive function (Carrillo et al., 2015).

Altogether these observations reinforce the hypothesis that leptin may regulate the reproductive and somatic growth processes by interacting with several hormonal systems located in the main hypophysiotrophic areas in the sea bass CNS. This is supported by the tissue expression pattern study, showing the highest *sblepr* expression levels in organs connected to the reproductive function (brain, pituitary and gonads) and somatic growth (muscle).

In conclusion, we report on the cloning and sequence analysis of three transcripts for sea bass leptin receptor. Two short variants are proposed to produce soluble proteins able to bind leptin, whereas the long form, termed *sblepr*, is likely the ortholog of human *LEPR* gene.

To the best of our knowledge this work is the first to report the neuroanatomical organization of the long form of leptin receptor expressing cells in a perciform. The CNS localization of *sblepr* suggests a role of leptin signaling in multiple key physiological processes. Notably, the neural pathways controlling feeding intake, energy homeostasis and reproduction express *sblepr*. These functions are tightly linked in most fish species, as well as in European sea bass, since gonad maturation is dependent on the nutritional status. In fact, European sea bass is a seasonal spawner who gradually reduces food intake during the period of gonadal development until reaching minimal values during the spawning season (Zanuy and Carrillo, 1985). All this places leptin among the best candidates to link appetite, metabolic rate, and energy stores with the neuroendocrine control of reproduction at least in European sea bass. Our hypothesis needs to be sustained by further research including, for example, exogenous leptin administration under different nutritional regimes.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ygcen.2016.03.017>.

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