Characterization and transcriptional profiles of *Engraulis encrasicolus* GnRH forms

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Abstract

The European anchovy *Engraulis encrasicolus*, a member of the Clupeiformes order, holds a great biological and economical importance. In the past, this species was mostly investigated with the aim of assessing its reproductive biology, trophic ecology, population dynamics and the relations existing with the physical environment. At present days, though, an almost complete lack of information afflicts its neuroendocrinology and reproductive physiology. The hypothalamic–pituitary–gonadal (HPG) axis at its highest levels was herein investigated. In this study, the gonadotropin-releasing hormone (GnRH), a neuropeptide underlying many reproduction-related processes, the most critical of which is the stimulation of gonadotropin synthesis and secretion from the pituitary gland, was cloned. Three forms (salmon GnRH, chicken-II GnRH and the species-specific type) were characterized in their full-length open-reading frames and, in accordance with other Clupeiformes species, the distinctive one was found to be the herring-type GnRH. We qualitatively and semiquantitatively evaluated the localizations of expressions and the temporal transcription patterns of the three GnRH forms in male and female specimens throughout their reproductive cycle as well as described their phylogeny with regard to teleost GnRH lineages, and, specifically, to other Clupeiformes species.

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Introduction

The hypothalamic–pituitary–gonadal (HPG) axis is the principal endocrine reproductive axis in vertebrates (Abraham 2004). It ensures the development, maturation and release of gametes and allows a successful reproductive process (Baba *et al.* 1971, Matsuo *et al.* 1971, Schally *et al.* 1971b). One of its main characters is the gonadotropin-releasing hormone (GnRH), a hypothalamic neuropeptide involved at the earliest stages of the reproductive process. Hypophysiotropic form of the neurohormone is released from the hypothalamus to the pituitary through the mammalian hypothalamic–hypophyseal blood portal or via direct neuronal innervations of the fish pituitary stalk as both environmental and physiological cues are integrated and transduced. The processed GnRH decapeptide regulates the synthesis and secretion of the follicle-stimulating hormone and luteinizing hormone (Schally *et al.* 1971a) from the anterior part of the pituitary gland – adenohypophysis (Kim *et al.* 1995) – by stimulating the expression of the glycoprotein hormone α (GP α) and β -subunits of the gonadotropins (Yaron *et al.* 2003), therefore initiating steroidogenesis and gametogenesis (Millar 2005, Kah *et al.* 2007).

Throughout evolutionary times, the *GnRH* gene underwent duplication events followed by subsequent nucleotide substitutions. All species investigated to date express more than one *GnRH* variant in their brain (e.g. Fernald & White 1999, Adams *et al.* 2002); specifically, primitive teleosts possess two different GnRH forms, whereas more recent species have three (Dubois *et al.* 2001). Differences pertain to the controlled processes, the brain and pituitary concentrations, and the anatomical brain localizations (Holland *et al.* 1998, Carolsfeld *et al.* 2000).

To date, 30 GnRH isoforms were characterized in vertebrate and invertebrate species (Roch *et al.* 2011), and only eight in teleosts (Adams *et al.* 2002). The decapeptides always display characteristics that have been maintained for more than 500 million years of evolution (King & Millar 1980): residues 1–4 at the NH₂ terminus (pGlu-His-Trp-Ser) and 9–10 at the COOH terminus (Pro-Gly-NH₂) are fully conserved and fundamental for ligand recognition, binding and activation of cognate receptors (Guilgur *et al.* 2007); positions 5, 7 and 8 are the most variable, whereas the molecule bends around position 6, typically a Gly. This is usually substituted by D-amino acids in the

construction of agonist analogues designed to display a higher stability and a decreased metabolic clearance (Millar 2005).

Based on phylogenetic analyses, Fernald and White (1999) classified three groups of GnRHs. GnRH1 lineage includes variants with proven hypophysiotropic functions. Among these are the mammalian – m – (Amoss *et al.* 1971), dogfish – dg – (Lovejoy *et al.* 1992), catfish – cf – (Bogerd *et al.* 1994), seabream – sb – (Powell *et al.* 1994), medaka – md – (Okubo *et al.* 2000) and herring – hr – (Carolsfeld *et al.* 2000) GnRHs. The second group comprises the most ancient form identified to date in Gnathostoma, which is as well the most conserved across all vertebrate species (Powell *et al.* 1994, Chen & Fernald 2008), the chicken-II GnRH (cGnRH-II) (Miyamoto *et al.* 1984). It was proposed to possess a neuromodulatory role affecting sexual behaviour rather than stimulating gonadotropin release (Maney *et al.* 1997, Volkoff & Peter 1999). At last, clade 3 comprehends a GnRH variant named salmon – s – (Sherwood *et al.* 1983), so far described only in teleosts. Its presence is not constant and its roles depend on the overall number of GnRH isoforms coexisting in a given species: in those displaying two GnRH isoforms, for example sockeye salmon *Oncorhynchus nerka* (Parhar *et al.* 1995), goldfish *Carassius auratus* (Lin & Peter 1997), zebrafish *Danio rerio* (Steven *et al.* 2003) and some catfish (Dubois *et al.* 2001), sGnRH exerts a hypophysiotropic function; if a three GnRH system is presented, sGnRH has developed specific functions related to sexual behaviour, including olfaction and spawning migration (Wirsig-Wiechmann 2001), even though further information is ever increasingly being collected.

The European anchovy *Engraulis encrasicolus* is the only Mediterranean representative of the Engraulidae family and a member of the Clupeiformes order, a basal teleost group. It holds an elevated importance, both biologically and economically, as it contributes to transfer energy from the basal levels of the food chain and represents one of the main targets of European fishing fleets. Despite the critical roles played by the GnRH system in the HPG axis functioning and, therefore, reproductive physiology, no study has ever investigated the endocrine regulation of the reproductive processes in this species. In our laboratory, we filled such gap of knowledge by (i) cloning the three *GnRH* variants and characterizing their deduced peptides, (ii) qualitatively evaluating the localization of their mRNA expression, (iii) semiquantitatively assessing mRNA abundance patterns during the entire reproductive cycle and (iv) depicting their evolutionary relationships with other teleost GnRH isoforms. A particular emphasis was placed on the Clupeiformes order, which we have contributed to broaden in this sense.

Materials and methods

Sample collection

Male and female *Engraulis encrasicolus* specimens at different sexual maturity stages were sampled for brain and gonad tissues during the 2015 MEDIAS research cruises (Leonori *et al.* 2011, MEDIAS 2012), carried out in the FAO geographical sub areas 17 ('Northern and Central Adriatic Sea') and 18 ('Southern Adriatic Sea') by the acoustic research group of the CNR-ISMAR of Ancona (Leonori *et al.* 2012). Samples were taken within the Data Collection Framework Regulation (Commission Regulation (EC) No. 665/2008 of the 14 July 2008). No animal experimentation was performed in this study; therefore, ethics approval was not necessary, in accordance with the Italian legislation.

Sexual maturity stages were classified according to the MEDITS working group's Instruction Manual (2012); 1: 'Immature'; 2c: 'Maturing'; 3: 'Mature/Spawner'; 4a: 'Spent'; 4b: 'Resting'. Tissues were maintained in RNA_{later} until processing.

RNA extraction and cDNA synthesis

Total RNA was extracted with RNAzol® RT reagent (Sigma-Aldrich®, R4533) following the manufacturer's instructions and eluted in RNase-free water. Concentration was determined with the Nanophotometer TM P-Class (Implem GmbH, P330). Integrity was verified by gel red staining of 28S and 18S ribosomal RNA bands on a 1% agarose gel. cDNA was retro-transcribed from 3 µg RNA using the Tetro Reverse Transcriptase cDNA synthesis kit (Bioline, BIO-65050). Nucleic acids were kept at –20°C until use.

Cloning, sequencing and ORF extension

GnRH sequences of *Engraulis japonicus* (GenBank accession numbers JX406273, JX406274 and JX406275), a sibling species of *E. encrasicolus* distributed from the Java Sea to the Sea of Japan, were used as templates for primer design with the web-based Primer3 software (Untergasser *et al.* 2012). Initial amplification was obtained with primers presented in Table 1. A 3-min denaturation at 94°C, a 35-cycle amplification consisting of denaturation, annealing and extension for 30s each at 94°C, 60°C and 72°C, respectively, and a last 10-min extension step at 72°C were set as protocol. PCR products were visualized on a 2% agarose gel stained with GelRed (Diatech Pharmacogenetics, 41003). Amplification yielded single-banded products of the expected size, which were excised, purified with the MinElute Gel Extraction Kit (Qiagen, 28604) and cloned into the pGEM-T vector system (Promega, A3600). DNA was sequenced using a T7 sequenase version 2.0 DNA sequencing kit (GE Life Sciences, 70770) and a dye-terminator kit (Perkin-Elmer, US82005) on an ABI PRISM DNA sequencer (Perkin-Elmer).

Results obtained from the Sanger sequencing were submitted to the BLAST suite (Madden, 2003 – <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Table 1 Oligonucleotide sequences employed for amplification of the three *E. encrasicolus* GnRH isoforms and reference genes through conventional and real-time q-PCRs.

	Sequence (5'–3')		Annealing temperature (°C)
	Forward	Reverse	
hrGnRH	CAAGACCTCTCCAGCCACTG	GCTGATCTCTGGTGCTACT	60
cGnRH-II	CAGGAGGCAAGAGGGATGTG	TCGACCACTGACGCTCTCTA	60
sGnRH	AACGCAGCATTGGAGGAGAA	GTGTGCGTGATCAGTCAAG	60
β-actin	CGTGACATCAAGGAGAAGCTGTGC	CAGACTCATCGTACTCCTGCTTGC	60
EF-α	GAGACAGCAAGAACGACCCA	AGAACTGCAGGCGATGTGA	60

The full-length open-reading frames of the three GnRH isoforms were obtained by conventional and/or RACE PCRs. RACE cDNA from brain and gonad tissues was synthesized with the FirstChoice® RLM-RACE Kit (Life Technologies, AM1700M) as per the manufacturer's instructions. 3' RACE outer/inner primer and adapter sequences are presented in Table 2. GnRH1_F/GnRH2_F were employed in combination with 3' outer/inner to extend *gnrh1* and *gnrh2* respectively.

Completion of the coding sequence at the 5' end was achieved by means of conventional amplification. Oligonucleotide sequences designed on the partial *Engraulis encrasicolus* *gnrh* isoforms are presented in Table 2.

Protein prediction, identification and multiple alignments

The primary structures of the encoded proteins were predicted and characterized by web-based bioinformatics tools such as ExPASy Translate (<http://web.expasy.org/translate/>), ORF Finder (<http://www.ncbi.nlm.nih.gov/projects/gorf/>), the BLAST suite (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), SignalP (Petersen et al. 2011 – <http://www.cbs.dtu.dk/services/SignalP/>) and SMART (Letunic et al. 2015 – <http://smart.embl-heidelberg.de/>).

Full-length prepro-GnRH open-reading frames were aligned with Clustal Omega v. 1.2.1 (Sievers et al. 2011 – <http://www.ebi.ac.uk/Tools/msa/clustalo/>) from the Multiple Sequence Alignment tools provided by the EBI website.

GnRH decapeptides of the same teleost species were aligned within each GnRH class with MUSCLE v. 3.8

(Edgar 2004 – <http://www.ebi.ac.uk/Tools/msa/muscle/>). The printing and shading of the alignment files were obtained with BOXSHADE v. 3.21 (http://www.ch.embnet.org/software/BOX_form.html), with symbols representing a consensus threshold of 0.5.

RT-PCR

RT-PCR amplification of GnRH isoforms in both sexes was performed in a MyCycler™ Thermal Cycler (Bio-Rad, 170-9703). The reaction consisted in 16 µL of 2x concentrated Platinum® Blue PCR SuperMix (Thermo Fisher Scientific, 12580015), 2 µL of 10 µM specific forward and reverse primer (Table 1) and 2 µL of cDNA synthesized from brain and gonad tissues sampled from M/F 2c, diluted 1:10. The thermal protocol was iterated for 35 cycles and the annealing temperature was set at 60°C.

Real-time qPCR

The three GnRH isoforms were relatively quantified in both brain and gonad tissues of *Engraulis encrasicolus* males and females. Real-time qPCRs with the SYBR green method were performed in an iQ5 iCycler thermal cycler (Bio-Rad, 179-8891). Amplification conditions were optimized after several trials at different times and temperature. Annealing temperature was set at 60°C in all cases. The observed single peak in the dissociation (melt) curve at the end of the amplification cycle indicated the absence of primer–dimer formation and the achievement of primer specificity. Two housekeeping genes (*ef-α* and *β-actin*) served as a reference for relative mRNA quantification. Oligonucleotide sequences are presented in Table 2.

Phylogenetic analyses

Phylogeny of the three gonadotropin-releasing hormone classes was inferred by analyzing 45 GnRH sequences belonging to overall 22 teleost species, chosen independently on their evolutionary history, at both DNA and protein levels. As for DNA, a codon-based alignment was achieved with the MUSCLE algorithm (Edgar 2004) at the default parameters. The model that best described the substitution pattern was screened with the model selection (ML) analysis. Based on the lowest Bayesian Information Criterion (BIC) score, which equalled 6481.572, the maximum likelihood phylogenetic tree was constructed employing the Kimura 2-parameter (Kimura 1980) matrix-based model. The non-uniformity of evolutionary rates among sites was modelled by a discrete Gamma distribution (five categories (+G, parameter = 0.7635)).

Table 2 Primer sequences used for extension of *E. encrasicolus* GnRH open-reading frame.

	Sequence (5'–3')
3' RACE outer primer	GCGAGCACAGAATTAATACGACT
3' RACE inner primer	CGCGGATCCGAATTAATACGACTCACTATAGG
3' RACE adapter	GCGAGCACAGAATTAATACGACTCACTATAGGT12VN
GnRH1_F	CAAGACCTCTCCAGCCACTG
GnRH1_F1	CAAGTGATGGAGGGTCTCCC
GnRH1_R1	CATTGCTCTGCTCATCAGGTG
GnRH2_F	CAGGAGGCAAGAGGGATGTG
GnRH2_F1	ATACAGCTCCAGTGATGGCG
GnRH2_R1	CGTCAGGGCTTCCAACAGG
GnRH2_R2	GGTAGCTGCACTCTCTGC
GnRH3_F1	GAAAAAGGGATGGAGCAGGG
GnRH3_R1	GGCCTTCGACCTCTTCTCAC
GnRH3_R2	GGCCGTGAGAGGAATGAGAG

As for peptides, alignment and best substitution model were performed and screened with the same above-described methods. The lowest BIC score was 4991.046 and the model used for maximum likelihood tree construction was the Jones–Taylor–Thornton one (Jones *et al.* 1992). In addition to a discrete Gamma distribution among sites (five categories (+G, parameter = 7.8178)), a certain fraction of sites was considered as evolutionarily invariable (+I).

The bootstrap method, with the number of bootstrap replication set at 1000, was used to test the inferred phylogeny (Felsenstein 1985).

Phylogenetic analyses were performed with the MEGA suite v. 6 (Tamura *et al.* 2013), according to Hall (2013).

Statistical analysis

The differences among GnRHs' relative mRNA abundances at all sexual maturity stages were assessed for statistical significance by one-way analysis of variance (ANOVA). Data were normalized with $\ln(x+1)$ in order to homogenize the variance and fulfil conditions for applying a parametric test. The *post hoc* Tukey test was used for testing multiple comparisons with a confidence interval of 95% ($P < 0.05$). Statistical analyses were performed with GraphPad Prism v. 6.

Results

Engraulis encrasicolus GnRH isoforms

The full-length coding sequences of the three GnRHs of *Engraulis encrasicolus*, namely hrGnRH (a species-specific form belonging to the GnRH1 class), cGnRH-II (GnRH2) and sGnRH (GnRH3), were cloned. They were deposited into the NCBI's GenBank with the accession numbers KU323664, KU323665 and KU323666 respectively.

The partial GnRH1 cDNA consisted of 434 nt and contained a full-length open-reading frame of 267 nt. The encoded protein was 88-aa long and included a predicted signal peptide of 22 residues (Fig. 1A). Its deduced amino acid sequence was pGlu-His-Trp-Ser-His-Gly-Leu-Ser-Pro-Gly-NH₂. Nucleotide alignment analysis yielded very few results, indicating the low identity with other species-specific GnRH sequences. BLASTn similarity rates to *Engraulis japonicus* and *Alosa sapidissima* forms accounted for 95% and 70% (data not shown), whereas amino acid identity was 93.18% and 55.81% (Table 3) respectively. A complete conservation among GnRH decapeptides was shown by a MUSCLE alignment and a BOXSHADE shading (Fig. 2A).

As for GnRH2, we successfully obtained its cDNA, full-length at the 3' end. It consisted of 624 nt, with a full-length open-reading frame of 261 nt encoding for an 86-aa protein. The predicted signal peptide was 24 residues long. A BLASTn search resulted in a 98% and 85% identity with the *Engraulis japonicus* and *Clupea harengus* GnRH2 forms (data not shown), whereas the average amino acidic similarity to other teleosts'

A hrGnRH

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atgaggagtaaggggccctcggtgtgtctgctgttactgacgtgctcctgcagtcg
M R S K G A L V C L L L V T A A V L Q C
ctgtctcagcactggtcacatggcctgagtcggagggaagaggtgactctccc
S S Q H W S H G L S P G S R R E A D S F
accgaatccaaagtgtgagggtctcccccgagaggagggcgctgtgttgagtcac
T E S Q V M E G L P P R G G A R C W S D
accagagaggcagccaatcaggaaaggccagcacactggaacagctgtgtccacctgatg
T R E A A N Q E R P S T L E Q L V H L M
agcagagcaaatgaagtgtttagctgagacacccaggaggctccataatcactacaatg
S R A N E V Y D
tggtttacattattgtttatgatagaaatgttcactgaaagtcaccactgcttttgctc
aaacaattattaataatgagcctgttcaataatacatgatctcctgtatgaaataaagaat
atcttgctcttc
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B cGnRH-II

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atggcggtgtgggtacaggtgggtcctgctggcagcgtgctcctgttcctgggtgtagag
M A C G Y R W V L L A A V L L F L G V E
ctgtctggtgctcagcactggtctcaggtatgtaccaggaggcaagaggatgtggac
L S G A Q H W S H G W Y P G S R R D V D
accttcaactcagcacaggtctcggaggagatgaagtgtgtgaggcaggagagtgacgc
T F N S A Q V S E E I K L C E A G E C S
tacctgctggcccgaggaggaaacctactcaagagcatcctgttggaagccctgacgaga
Y L R P Q R R N L L K S I L L E A L T R
gaattccagaggaggagtgacctgctggctgggctgagccatttccatgtgtgga
E F Q R R K
gcgtcagtggtcgagtgatctctagtctttgtccattgtttgtgtgttcagtcgaaga
ggcccatgtgactgggtgctgcttggatataatgacctgtgcaaaatctgtgacagt
gtgatgaatgccaaacaaacgcgcatttttcccttcaaatgtttgtgtgacatca
gaatcgtgtcatgcacatgcatcatgcaaccatttagcagtaataatagctgtgtgcaa
cgggatctgctgcgcagtggttgcataatcttctaccattattaaacacatcatgtgtga
tctgaaaaa
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C sGnRH

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atggagcagggcaggttggtgtgtgctgcccgtgttgccgtgtaaggagtggtg
M E Q G R L V L L P V L A C A C K E C V
tgtagcactggtctgacggctggctacggggcgcaaacgcagcatttgaggagaaactg
Q Q H W S Y G W L P G S R R S I G G E L
gaggctgattcaggtatgatgatgatgagactctcattcctctcagggccgaaaaa
E A D F R M M D D G D T L I P L T A E K
ttacaatccaatgatgtatcattgataacattgaggagaatgctgtgagaagaggtcga
L Q S N D A I I D N I E E N A V R R G R
aggccattacgaagagaattgctgactgatgcacgcacac
R P L R R E L L D
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Signal peptide
GnRH decapeptide
Cleavage site
GAP (GnRH Associated Peptide)

Figure 1 cDNA sequence and deduced primary structures of hrGnRH (GnRH1) (A), cGnRH-II (GnRH2) (B) and sGnRH (GnRH3) (C). The initial methionine is highlighted in green colour, whereas the stop codon is indicated by an asterisk highlighted in red colour. Conserved regions such as signal peptides, GnRH decapeptides, Gly-Lys-Arg cleavage sites and GnRH-associated peptides are shown according to the figure legend.

cGnRH-II was 67.45%, with *E. japonicus* showing the highest degree of 100% (Table 3 and Fig. 2B).

Cloning of the GnRH3 cDNA yielded a partial sequence of 281 nt containing a full-length open-reading frame of 270 nt. The encoded protein was 89 residues long and 21 aa composed the signal peptide. The degree of similarity to *E. japonicus* sGnRH (GnRH3) at DNA and protein levels was 97% (data not shown) and 94.38% (Table 3) respectively. Homology with other teleosts' sGnRH ranged from 42.05% (*C. auratus*) to 45.05% (*D. rerio*) (Table 3). The decapeptide had a completely conserved primary structure among several analyzed teleosts (Fig. 2C).

Apart from the signal peptide at the N-terminus and the typical GnRH decapeptide (highlighted in light grey colour and boxed respectively), the primary structures of

Table 3 Degree of identity among several teleost prepro-GnRH isoforms assessed at the full-length ORF level, expressed as percentages. The analyses was conducted with Clustal Omega multiple sequence alignment tool (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Refer to caption of Fig. 2 for information regarding GenBank accession numbers of the protein entries used for similarity assessment. Comparisons between *Engraulis* spp. are underlined.

GnRH form (species)	% of AA identity		
	<i>E. encrasicolus</i>		
	hrGnRH	cGnRH-II	sGnRH
hrGnRH (<i>E. japonicus</i>)	93.18	25.32	21.33
hrGnRH (<i>E. encrasicolus</i>)	/	25.32	20.00
wfGnRH (<i>C. clupeaformis</i>)	28.74	25.30	28.21
mdGnRH (<i>O. latipes</i>)	25.88	18.75	20.78
sbGnRH (<i>S. aurata</i>)	26.44	23.46	24.36
pjGnRH (<i>M. albus</i>)	30.68	20.48	20.00
sbGnRH (<i>D. labrax</i>)	28.41	21.69	23.75
sbGnRH (<i>M. undulatus</i>)	23.53	22.78	27.27
sbGnRH (<i>M. cephalus</i>)	33.72	21.95	24.05
sbGnRH (<i>H. burtoni</i>)	26.14	25.00	22.89
sbGnRH (<i>O. niloticus</i>)	26.14	23.81	22.89
cfGnRH (<i>C. gariepinus</i>)	37.33	35.06	19.44
hrGnRH (<i>A. sapidissima</i>)	55.81	29.87	21.33
cGnRH-II (<i>E. japonicus</i>)	25.32	100.00	27.27
cGnRH-II (<i>E. encrasicolus</i>)	25.32	/	27.27
cGnRH-II (<i>C. auratus</i>)	25.32	56.98	28.57
cGnRH-II (<i>C. gariepinus</i>)	25.32	62.79	29.87
cGnRH-II (<i>A. japonica</i>)	28.75	65.12	34.62
cGnRH-II (<i>O. mykiss</i>)	30.38	61.73	28.57
cGnRH-II (<i>O. latipes</i>)	29.73	67.50	29.33
cGnRH-II (<i>M. cephalus</i>)	29.11	65.88	27.63
cGnRH-II (<i>H. burtoni</i>)	29.11	65.88	27.63
cGnRH-II (<i>O. niloticus</i>)	29.11	65.88	27.63
cGnRH-II (<i>S. aurata</i>)	30.38	64.71	28.95
cGnRH-II (<i>M. saxatilis</i>)	26.58	67.06	28.95
cGnRH-II (<i>D. labrax</i>)	27.85	65.88	28.95
sGnRH (<i>E. japonicus</i>)	21.33	28.57	94.38
sGnRH (<i>E. encrasicolus</i>)	20.00	27.27	/
sGnRH (<i>P. major</i>)	25.61	29.49	44.44
sGnRH (<i>S. aurata</i>)	25.61	29.49	44.44
sGnRH (<i>D. labrax</i>)	24.39	30.77	44.44
sGnRH (<i>H. burtoni</i>)	23.17	32.05	43.21
sGnRH (<i>O. latipes</i>)	26.83	30.77	44.44
sGnRH (<i>O. masou</i>)	20.51	28.57	45.21
sGnRH (<i>D. rerio</i>)	22.50	27.85	45.45
sGnRH (<i>C. auratus</i>)	21.25	31.65	42.05
sGnRH (<i>C. nebulosus</i>)	24.39	30.77	44.44
sGnRH (<i>C. carpio</i>)	22.50	27.85	43.18
sGnRH (<i>M. cephalus</i>)	25.61	29.49	42.68
sGnRH (<i>O. niloticus</i>)	24.69	33.33	44.30

the three isoforms displayed conserved domains, such as the Gly-Lys-Arg (GKR) cleavage site and a 56-, 52- and 58-aa long GnRH-associated peptide (GAP) at the C-terminus, respectively, highlighted in dark grey colour and underlined (Fig. 1).

RT-PCR

A qualitative assessment of GnRH mRNA expressions was performed in brain and gonad tissues of stage 2c ('Maturing') female and male of *E. encrasicolus* (Fig. 3). After 35 cycles, three bands of distinct expected sizes were evident on a 2% agarose gel. Products were

sequenced to confirm their identity of GnRH-specific amplicons. The three *gnrhs* were always transcribed, although not all isoforms were expressed with the same extent and they were found being more faintly expressed in males than in females. Male *cGnRH-II* appeared more evident in the brain than the gonad. Female *sGnRH* transcriptions, although more noticeable than in males, had different band intensities between tissues and the one at the central level was more marked than the gonad's. Two housekeeping genes were included, namely *ef-a* and *b-actin*. All precautionary measures were taken to avoid contamination of the samples. A negative control was included in the analyses and indicated as no template control in Fig. 3.

Altogether, these results demonstrate that the European anchovy possesses three distinctive GnRH forms that can be subjected to modulated tissue transcription at the 'Maturing' sexual developmental stage.

Real-time qPCRs

Relative mRNA abundances of herring-, chicken-II- and salmon-type GnRHs were investigated semiquantitatively throughout the reproductive cycle of female (Fig. 4) and male (Fig. 5) *Engraulis encrasicolus* specimens in brain and gonad tissues. *GnRH* types were always expressed in both tissues. Brain *hrGnRH* transcription displayed similar trends in both sexes (Figs 4A and 5A). Statistically significant variations in mRNA abundances occurred at stages 2c, 3 and 4a ('Maturing', 'Mature/Spawner' and 'Spent'). Transcription of *sGnRH* in female brain (Fig. 4E) followed a similar pattern, which was supported by statistical significance at the reproductively active stages. The highest transcription values were found at the spawning phase.

Ovaries and testicles had different *hrGnRH* mRNA levels, and it was not possible to describe a general trend (Figs 4B and 5B). The formers had a statistically significant 2.5-fold higher abundance of type 1 *GnRH* in the maturing phase and lower non-significant levels among F3, F4a and F4b stages. The latters had the lowest *hrGnRH* transcription value at spawning, whereas mRNA levels were similar at the remaining stages.

Despite with different extent, female gonad and male brain had comparable *cGnRH-II* transcription trends (Figs 4D and 5C), which significantly increased from maturing to spawning individuals and declined in fish that had reached the resting stage. *sGnRH* and *cGnRH-II* in female gonad (Figs 4D and 4F) were transcribed similarly.

Gene expression of *cGnRH-II* in female brain (Fig. 4C) significantly increased from immature to reproductively active fish and remained at comparable levels throughout the reproductive cycle, as indicated by the lack of statistically significant differences.

Figure 2 Multiple alignments of teleost GnRH1 (A), GnRH2 (B) and GnRH3 (C) decapeptides. Identical, conservative and unrelated residues are shaded in black, grey and white colour respectively. GenBank accession numbers are as follows: **GnRH1**: *C. clupei* AAP57221.1, *O. latipes* BAB16303.1, *S. aurata* AAA75469.1, *M. albus* AAW51121.1, *D. labrax* AAF62898.1, *M. undulatus* AAQ16501.2, *M. cephalus* AAQ83269.1, *H. burtoni* AAC59691.1, *O. niloticus* BAC56849.1, *C. gariepinus* CAA54971.1, *A. sapidissima* AAN04492.1, *E. japonicus* AFO70217.1, *E. encrasicolus* KU323664.1. **GnRH2**: *E. japonicus* AFO70218.1, *E. encrasicolus* KU323665.1, *C. auratus* AAC59858.1, *C. gariepinus* CAA54969.1, *A. japonica* BAA82609.1, *O. mykiss* AAB82559.1, *O. latipes* BAB16300.1, *M. cephalus* AAQ83270.1, *H. burtoni* AAA74993.1, *O. niloticus* BAC56850.1, *S. aurata* AAA75447.1, *M. saxatilis* AAD03816.1, *D. labrax* AAF62900.1. **GnRH3**: *P. major* BAA05104.1, *S. aurata* AAA98845.1, *D. labrax* AAF62899.1, *H. burtoni* AAB20243.1, *O. latipes* BAB16302.1, *O. masou* BAA01740.1, *D. rerio* CAC18539.1, *C. auratus* BAB18904.1, *C. nebulosus* AAV74403.1, *C. carpio* AAO39752.1, *M. cephalus* AAQ83268.1, *P. major* BAA05104.1, *E. japonicus* AFO70219.1, *O. niloticus* XP_013126782.1, *E. encrasicolus* KU323666.1.

Phylogeny

The evolutionary relationships among the GnRH forms of *E. encrasicolus* and those of other teleost species were inferred by the maximum likelihood method based on the JTT matrix-based model. Only the tree constructed from prepro-GnRH peptide sequences is reported, as a good correspondence between it and the one built from codon-based DNA multiple alignments was found. **Figure 6** shows the tree with the highest log likelihood (−2144.1972); it is drawn to scale, with branch lengths measured in the number of substitutions per site and the scale bar of 0.2, indicating the estimated evolutionary distance units. High bootstrap values supported the statistical significance of the tree's conformation.

Teleost GnRHs were clustered into three different branches and marked as GnRH1, GnRH2 and GnRH3,

Figure 3 Qualitative tissue distribution analyses of GnRH isoforms assessed in the brain and gonads of stage 2c ('Maturing') female and male *Engraulis encrasicolus* after 35 cycles of RT-PCR amplification. *ef-α* and *β-actin* were used as reference genes.

Figure 4 Semiquantitative measurement of mRNA abundance of the three GnRH types throughout the reproductive cycle of female *Engraulis encrasicolus* in the brain and gonad. The calculation was made over two reference genes, *ef-α* and *β-actin*. Letters represent statistical significance ($P < 0.05$) between two consecutive sexual maturation stages, as indicated by the one-way ANOVA and the *post hoc* Tukey test.

Figure 5 Semiquantitative measurement of mRNA abundance of the three GnRH types throughout the reproductive cycle of male *Engraulis encrasicolus* in the brain and gonad. The calculation was made over two reference genes, *ef- α* and *β -actin*. Letters represent statistical significance ($P < 0.05$) between two consecutive sexual maturation stages, as indicated by the one-way ANOVA and the *post hoc* Tukey test.

grouping all species-specific GnRHs, all cGnRH-II sequences and all salmon-type GnRHs respectively. *E. encrasicolus* had a GnRH representative in each subgroup. Its forms always strongly correlated with the homologs of the Japanese anchovy, as represented by the bootstrap values of 100 in all the three cases.

The clade clustering salmon-type GnRHs appeared to be more equally distributed than the other two, as five evolutionary-distant orders (Perciformes, Mugiliformes, Salmoniformes, Beloniformes and Cypriniformes) were enclosed in only two 79% bootstrapped subgroups. The former four were included in only one branch. GnRH3-wise, Clupeiformes' *Engraulis* spp. shared a common ancestor with other taxa but were shown to have undergone a more ancient speciation event and therefore occupied a detached position.

As for the species-specific form, *E. encrasicolus*, *E. japonicus* and *A. sapidissima* (three Clupeiformes species) were closely related. Interestingly, herring-type GnRH seemed to have a common shared ancestor with the euryhaline Anguilliformes *A. japonica* and the freshwater Salmoniformes *C. clupeaformis*.

Figure 6 Rooted phylogenetic tree built with Mega v.6 using 45 teleost prepro-GnRH sequences. Relative branch lengths indicate the evolutionary rates of the lineages. Bootstrap values are reported at nodes. Accession numbers of the GenBank protein entries used for inferring the evolutionary history are reported as follows:

GnRH1 clade: cfGnRH *C. gariepinus* CAA54970.1, cfGnRH *C. gariepinus* CAA54971.1, hrGnRH *E. japonicus* AFO70217.1, hrGnRH *E. encrasicolus* KU323664, hrGnRH *A. sapidissima* AAN04492.1, mGnRH *A. japonica* BAA82608.1, wfGnRH *C. clupeaformis* AAP57221.1, mdGnRH *O. latipes* BAB16303.1, sbGnRH *M. undulates* AAQ16501.2, sbGnRH *S. aurata* AAA75469.1, sbGnRH *D. labrax* AAF62898.1, sbGnRH *H. burtoni* AAC59691.1, sbGnRH *O. niloticus* BAC56849.1, pjGnRH *M. albus* AAW51121.1, sbGnRH *M. cephalus* AAQ83269.1. **GnRH2 clade:** cGnRH-II *C. auratus* AAC59858.1, cGnRH-II *C. gariepinus* CAA54969.1, cGnRH-II *E. japonicus* AFO70218.1, cGnRH-II *E. encrasicolus* KU323665, cGnRH-II *A. japonica* BAA82609.1, cGnRH-II *O. mykiss* AAB82559.1, cGnRH-II *O. latipes* BAB16300.1, cGnRH-II *S. aurata* AAA75447.1, cGnRH-II *H. burtoni* AAA74993.1, cGnRH-II *O. niloticus* BAC56850.1, cGnRH-II *M. cephalus* AAQ83270.1, cGnRH-II *M. saxatilis* AAD03816.1, cGnRH-II *D. labrax* AAF62900.1. **GnRH3 clade:** sGnRH *S. aurata* AAA98845.1, sGnRH *P. major* BAA05104.1, sGnRH *C. nebulosus* AAV74403.1, sGnRH *M. cephalus* AAQ83268.1, sGnRH *D. labrax* AAF62899.1, sGnRH *O. masou* BAA01740.1, sGnRH *O. latipes* BAB16302.1, sGnRH *H. burtoni* AAB20243.1, sGnRH *O. niloticus* XP_013126782.1, sGnRH *D. rerio* CAC18539.1, sGnRH *D. rerio* NP_878307.2, sGnRH *C. auratus* BAB18904.1, sGnRH *C. carpio* AAO39752.1, sGnRH *E. japonicus* AFO70219.1, sGnRH *E. encrasicolus* KU323666. **Out-group:** GnRH-I *P. marinus* AAF78455.1, GnRH-III *P. marinus* AAL12249.1. GnRH types of *E. encrasicolus* are underlined.

Two candidates, GnRH-I and GnRH-III, belonging to *P. marinus* (a jawless species of the Agnatha superclass), grouped outside of the three clusters and were chosen as tree root accordingly to the out-group method.

Discussion

Investigations carried out so far on the European anchovy *Engraulis encrasicolus* (e.g. Millán 1999, Allain *et al.* 2007, Schismenou *et al.* 2012, Basilone *et al.* 2013, Giannoulaki *et al.* 2013) have mostly focused on understanding the relationship of the species with the physical environment as well as assessing pure biological parameters rather than taking into consideration its neuroendocrinology. Except for one previous contribution (Miccoli *et al.* 2016), such species lack of a physiological perspective and, at present days, the hypothalamic–hypophyseal–gonadal (HPG) axis is completely uncharacterized.

In this study, the components of the GnRH system of *E. encrasicolus* were described for the first time. With no genomic resource available, we initially hypothesized the scenario of this species to be consistent with those of other Clupeiformes members, specifically the herring *Clupea harengus pallasii* (Carolsfeld *et al.* 2000), the shad *Alosa sapidissima* (Abraham 2004) and the Japanese anchovy *Engraulis japonicus* (Sukhan *et al.* 2013).

We cloned three GnRH forms -hr, c-II and s- (Figs 1 and 2), demonstrating that in the European anchovy – as in the large majority of modern teleosts – multiple variants regulate additional processes other than stimulating the release of the two gonadotropins (Dubois *et al.* 2001, Zohar *et al.* 2010). We were always successful in obtaining the full-length open-reading frames and therefore the complete prepro-GnRHs (Fig. 1). Despite the mature decapeptide is generated by a post-translational processing of the GnRH precursor (Wąlikowska & Polkowska 2009), the current view on reproductive neuroendocrine mechanisms could be broadened by characterizing the regions external to the GnRH decapeptide, as these were found to possess biological activeness (Millar *et al.* 1986).

GnRH1 of *E. encrasicolus* was coherent with the herring-type GnRH (Carolsfeld *et al.* 2000). hrGnRH shares a high decapeptide identity with the medaka (Okubo *et al.* 2000), seabream (Powell *et al.* 1994) and catfish (Bogerd *et al.* 1994) GnRH types. Compared with md and sb, the only dissimilar amino acids are at position 5 (H vs F and Y respectively), whereas divergence between hr and cf occurs at residue 8 (S vs N) (Fig. 2A). In relation to mGnRH, positions 5 and 8 differ. The situation we highlighted by BOXSHADE alignment is in line with that described by Gothilf *et al.* (1995) and Okubo *et al.* (2000). Considering the currently available information on the order and our results, we speculate that hr is the standard Clupeiformes-specific GnRH type.

A typical vertebrate GnRH structure AA-wise (Cheng & Leung 2005, Kah *et al.* 2007) was confirmed in all the three cases. The protein organization comprises a 21–24 residue long leucine-rich signal peptide, a GnRH decapeptide, a GKR processing site and a 52–58 aa-long GnRH-associated peptide (GAP). The functional roles of the majority of the peptide regions are established, except for GAP. Most authors agree that it ensures the precursor's proper folding and assists in the gonadotropin release (Millar *et al.* 1986, Yu *et al.* 1988). In mice, mutations on exon 3 and 4 encoding for most of the GAP region prevented GnRH secretion, ultimately leading to sexual immaturity (Seeburg *et al.* 1987). This region seems to have diverged the most both within and between species (Lin *et al.* 1998). GAPs of prepro-GnRH2 and three of seabass *Dicentrarchus labrax* share an average inter-species identity of 93% while that of prepro-GnRH1 ranges between 35 and 57%, suggesting that the species-specific gene variant underwent a much faster evolution (Zohar *et al.* 2010). In other terms, we presented a similar result by investigating amino acid identity among several teleost prepro-GnRHs species (Table 3). The most divergent class was indeed found to be GnRH1, whereas the one sharing the highest similarity was the GnRH2, further outlining the strong but differential evolutionary pressure that acted on discrete GnRH forms.

Through immunohistochemistry, *in situ* hybridization and transgenesis analyses, a general pattern of GnRH types' localization was previously documented (Okubo & Nagahama 2008, Zohar *et al.* 2010). The reproductively relevant GnRH1 neurons predominantly distribute in the ventral forebrain, mainly in the pituitary-innervating preoptic area, and in the hypothalamus; the conserved cGnRH-II is located in the midbrain tegmentum; if the third teleost-specific sGnRH is present, it is found at the terminal nerve ganglion (Gothilf *et al.* 1996). However, an overlapping distribution of GnRH1 and 3 in the olfactory bulbs was described in the seabass (González-Martínez *et al.* 2001), lake whitefish (Vickers *et al.* 2004), medaka (Okubo *et al.* 2006), Atlantic croaker (Mohamed *et al.* 2005), American cichlid (Pandolfi *et al.* 2005) pejerrey (Guilgur *et al.* 2007) and Japanese anchovy (Sukhan *et al.* 2013). Even though our study was not aimed at and did not describe the spatial localization of any GnRH neurons in the European anchovy, because of the cited evidences and particularly the one referring to the Japanese sibling, we speculate that *E. encrasicolus* displays a similar situation. However, an *ad hoc* study is necessary to demonstrate this hypothesis.

The multiple tasks of such a widely distributed GnRH system were first inferred and later demonstrated by evidences based on anatomical distributions occupied. Although cGnRH-II could stimulate gonadotropin release *in vitro* (Bosma *et al.* 2000), its capability *in vivo* was extremely low, likely because its neurons do not

project into the pituitary (Amano *et al.* 2002) but are instead localized in areas associated with reproductive behaviour (Millar 2005) and enter the spinal cord (Carolsfeld *et al.* 2000). Confirmation was achieved when sexual arousal in rodents (e.g. Rissman *et al.* 1997) and energy coordination in the musk shrew (Temple *et al.* 2003) were demonstrated to depend on it. GnRH2 is also involved in (i) the release of melatonin (Servili *et al.* 2010) and growth hormone (Trudeau 1997) as well as regulation of prolactin (Weber *et al.* 1997) and somatolactin (Kakizawa *et al.* 1997); (ii) the control of isotocin/vasotocin electrical activities (Saito *et al.* 2003) and synaptic transmission (Kinoshita *et al.* 2007); and (iii) autocrine/paracrine processes (Millar 2005). As neurons localized in the terminal nerve ganglion are able to integrate olfaction and sight, GnRH3 was proposed to serve neuromodulatory roles (Sukhan *et al.* 2013), particularly referred to sexual behaviour (Yamamoto *et al.* 1997, Ogawa *et al.* 2006) and environmental stimuli (Kudo *et al.* 1996). In Actinopterygii, first (~400 MYA), and true bony fish (Teleostei), then (~145 MYA), such an expanded GnRH system represented an evolutionary advantage, as a refined control of reproduction was likely one of the factors that increased offspring survival, especially in batch-spawning species that release their gametes in schools (Carolsfeld *et al.* 1996), ultimately facilitating fish evolutionary radiation and their phenotypic diversification (Nelson 1994). As a possible direct result, nowadays bony fish embrace more than 20,000 species, equalling approximately one third of all vertebrates (Yaron *et al.* 2003).

A number of papers about teleost and other vertebrate groups such as mammals and reptiles (Uzbekova *et al.* 2001, Ikemoto & Park 2003) report peripheral GnRH mRNA expression in the gonads, eyes, olfactory epithelium and gills. In those tissues, the system was suggested to take part in additional functions related to reproduction (autocrine/paracrine roles, chemosensory processes and osmoregulation respectively) (Guilgur *et al.* 2007). Considering these data, we employed stage 2c male and female specimens to perform a qualitative analysis (Fig. 3). *hr*, *c-II* and *sGnRH* types were transcribed at both central and peripheral levels even though mRNA abundances were not always comparable. Likely, such distributions confirm the involvement of the different GnRH forms into processes related to the detection of environmental stimuli (e.g. photoperiod, lunar cycle and temperature), which are perceived and integrated at a central level in order to trigger the reproductive process at the most favourable period of the year. Our findings represent the first indication of the functional specialization to which GnRH types of *E. encrasicolus* were subjected. Retrieving evidences about the presence of the GnRH pathway in the gonads of further teleost species could contribute to reinforce the hypothesis proposed by Powell *et al.* (1996), who suggested a direct gonadal

regulation by the GnRH system after several forms isolated from tunicates' neural tissue were shown able to activate the gonads.

With regard to a semiquantitative pattern of mRNA expression, the relative abundance of pituitary *hrGnRH* in the herring was much higher than in any other forms' (Carolsfeld *et al.* 2000). Pituitary *hrGnRH* abundance in the American shad was 4- to 48-fold higher than *sGnRH* and *cGnRH-II* (Abraham 2004). Our data showed that in the European anchovy, *hrGnRH* relative abundances were 11- and 3.6-fold as well as 9.3- and 11-fold higher than *cGnRH-II* and *sGnRH* in stage 3 ('Mature/Spawner') female and male specimens respectively (Figs 4A and 5A). In teleost displaying three GnRH forms, the species-specific variant, which is the major inducer of gonadotropins release from the pituitary, is the most abundant one mRNA-wise (Powell *et al.* 1995, Gothilf *et al.* 1996). Taking this into consideration, we suggest that the herein described herring-type GnRH be indeed the hypophysiotropic form involved in triggering reproduction in *E. encrasicolus*, even though dedicated analyses, possibly at the protein level, might be useful to support our conclusions. In addition, from a pure research point of view, we could define the mRNA expression pattern throughout the reproductive cycle of both sexes, at central and peripheral levels. Ours is the first attempt performed on a Clupeiformes species, as none of the just-mentioned papers reported such an aspect. Considering the similar expression patterns of *hr*- and *sGnRHs* at the central level (Fig. 4A and E), we speculate about their possible evolutionary convergence, which is discussed more in details hereafter. An important information that can be deduced from comparing the three forms of GnRH expressions at both levels is the lack of correlation among brain and gonad mRNA trends, especially in female specimens (Fig. 4). This is likely due to the above-mentioned GnRH involvement in autocrine/paracrine processes (Millar 2005, Ramakrishnappa *et al.* 2005): in rainbow trout, GnRHs were shown to take part in the regulation of gonadal maturation and spawning (Gray *et al.* 2002); in the protandry gilthead seabream, GnRH was found to direct the differentiation of the switching gonad and to modulate androgen levels (Soverchia *et al.* 2007); in the goldfish, the GnRH system was shown to be able to induce or inhibit the apoptotic process, according to the ovarian developmental stages (Andreu-Vieyra *et al.* 2005). We hypothesize that a similar situation is also present in the European anchovy, but further studies are needed to specifically address this point.

The evolution of the GnRH system was inferred by analyzing a set of teleost GnRHs including 45 homologues from 22 species. GnRH1, GnRH2 and GnRH3 clades, in which European anchovy *hrGnRH*, *cGnRH-II* and *sGnRH* were, respectively, allocated, were shown to be monophyletic (Fig. 6). We demonstrated that the European anchovy *hrGnRH* is phylogenetically

close to mGnRH, sbGnRH, pjGnRH and wfGnRH, consistently with the findings of Abraham (2004) and Dubois *et al.* (2002); it grouped with other GnRH1 types known to possess hypophysiotropic functions -m, cf, wf, md, sb and pj-, further confirming its homology with such GnRH lineage.

The three GnRH variants of the *Engraulis* spp. were highly related to each other, as indicated by the similar branch lengths and by the bootstrap statistical test. Their relation with other Clupeiformes species could be assessed only in the case of *Alosa sapidissima* hrGnRH, which was placed in a separate, yet highly related, clade from *Engraulis* spp. despite displaying an identical branch length to that of *E. encrasicolus*. We could not evaluate how the evolutionary history of the American shad's cGnRH-II or sGnRH relates to the European anchovy's, as Abraham (2004) was successful in isolating only the hrGnRH type. The same applies to *Clupea harengus*, as Carolsfeld (2000) characterized only the decapeptide sequences of hr, c-II and sGnRHs (UniProtKB/Swiss-Prot references P81749, P68075 and P69106). As the short length and the conserved primary structure of the GnRH peptides prevent phylogenetic analyses from being effectively accomplished if only the mature protein is considered (Guilgur *et al.* 2007, Kah *et al.* 2007), we did not add them to the dataset to be analyzed.

According to King and Millar (1992), Montero and Dufour (1996) and White and Fernald (1998), the origin of GnRH1 ancestor and GnRH2 lineages appears clear and was caused by a 600 MY-old gene duplication before the divergence of fish from tetrapods. This would explain the presence of at least two GnRH forms in all classes analyzed to date, with chicken-II type being the most conserved (Dubois *et al.* 2002). Despite that consistent data relating lineages 1 and 3 exist (e.g. Dubois *et al.* 2002, Chen & Fernald 2008), less certainty afflicts the timing of their duplication. Two hypothesis were formulated: as for lineages 1 and 2, the first one suggests that they originated before the divergence of fish and tetrapods (White & Fernald 1998, Guilgur *et al.* 2007, Okubo & Nagahama 2008), implying that GnRH3 was either lost or still remains to be described in tetrapods. Conversely, the second one proposes that their duplication occurred within the fish lineage, approximately 320 MYA (Kah *et al.* 2007), inferring their close relationship. The partially shared anatomical brain localization as well as the similar gene expression patterns that we described (Figs 4 and 5) further confirms the picture. Because of recent evidences supporting the existence of a third fish-specific genome duplication (Christoffels *et al.* 2004, Meyer & Van De Peer 2005), the latter hypothesis is favoured. The expected duplicated cGnRH-II form underwent neither specialization nor spatial segregation and was lost because of functional redundancy, as it happens in 50–80% of cases (Lynch & Force 2000).

In conclusion, in this study, we delivered crucial information regarding the HPG axis of the European anchovy at its highest level. We established the GnRH forms possessed by this species and characterized them with bioinformatics, molecular and phylogenetic analyses in view of preliminarily assessing the reproductive-relevant variant, describing their tissue localization as well as their temporal pattern of expression and, at last, providing additional details as to the evolutionary development of the three GnRH lineages. These results lay the groundwork for additional studies aimed at further understanding the reproductive physiology of such an important resource.

Declaration of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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