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Highlights

- A single *gnih* paralog has been conserved after the teleost-specific whole genome duplication

- The *gnih* gene encodes three GnIH peptides in a basal teleost, the European eel.
- *Gnih* mRNA is mostly expressed in the diencephalon in the eel.
- Eel GnIH peptides inhibit the expression of gonadotropin subunits *lhβ*, *fshβ*, *gpα* and of GnRH receptor, *gnrh-r2*, by eel primary cultures of pituitary cells.

Abstract

Since its discovery in birds, gonadotropin-inhibitory hormone (GnIH) has triggered investigation in the other groups of vertebrates. In the present study, we have identified a single *gnih* gene in the European eel (*Anguilla anguilla*), a representative species of a basal group of teleosts (Elopomorphs). We have also retrieved a single *gnih* gene in Osteoglossomorphs, as well as in more recently emerged teleosts, Clupeocephala. Phylogeny and synteny analyses allowed us to infer that one of the two *gnih* paralogs emerged from the teleost-specific whole genome duplication (TWGD or 3R), would have been lost shortly after the 3R, before the emergence of the basal groups of teleosts. This led to the presence of a single *gnih* in extant teleosts as in other vertebrates. Two *gnih* paralogs were still found in some teleost species, such as in salmonids, but resulting from the additional whole genome duplication that specifically occurred in this lineage (4R). Eel *gnih* was mostly expressed in the diencephalon part of the brain, as analyzed by quantitative real-time PCR. Cloning of eel *gnih* cDNA confirmed that the sequence of the GnIH precursor encoding three putative mature GnIH peptides (aaGnIH-1, aaGnIH-2 and aaGnIH-3), which were synthesized and tested for their direct effects on eel pituitary cells *in vitro*. Eel GnIH peptides inhibited the expression of gonadotropin subunits (*lhβ, fshβ,* and common *a*-subunit) as well as of GnRH receptor (*gnrh-r2*), with no effect on *tshβ* and *gh* expression. The inhibitory effect of GnIH peptides on gonadotropic function in a basal teleost is in agreement with an ancestral inhibitory role of GnIH in the neuroendocrine control of reproduction in vertebrates.

1. Introduction

The primary factor involved in the brain control of gonadotropin synthesis and secretion in vertebrates is the stimulatory decapeptide gonadotropin-releasing hormone (GnRH). Even if dopamine was reported to be a key inhibitor of gonadotropins in several teleost groups [for reviews: (Dufour et al., 2010; Peter et al., 1986)], no major peptidic inhibitory factor was identified in vertebrates until 2000. At that time, Tsutsui and collaborators described, from quail brain, the isolation and characterization of a novel peptide, having a RFamide (RFa) motif at the C-terminal extremity and inhibiting gonadotropin release from cultured quail anterior pituitaries, which they named gonadotropin-inhibitory hormone (GnIH) (Tsutsui et al., 2000). *Gnih* gene (also called *npvf* gene) homologs have been identified in non-avian vertebrates, including mammals, amphibians, fish and agnathans [for reviews: (Muñoz-Cueto et al., 2017; Ogawa and Parhar, 2014; Osugi et al., 2014b; Tsutsui et al., 2018)]. Multiple peptides are encoded by the *gnih* gene: two in mammals (RFRP-1 and RFRP-3), three in birds and reptiles (GnIH and two GnIH-related peptides, GnIH-RP1 and GnIH-RP2), four in amphibians (fGRP, fGRP-RP-1, fGRP-RP-2 and fGRP-RP-3 in the bullfrog, *Rana catesbeiana* and LPXRFa-1, -2, -3, -4 in the newt, *Cynops pyrrhogaster*) and two or three peptides in teleosts (LPXRFa or GnIHs) [for reviews: (Muñoz-Cueto et al., 2017; Ogawa and Parhar, 2014; Osugi et al., 2014b; Tsutsui et al., 2018; Ubuka and Parhar, 2018)].

A gene encoding three PQRF-peptides was identified in amphioxus (*Branchiostoma floridae*), representative of cephalochordates, the most basal group of chordates (Osugi et al., 2014a; Putnam et al., 2008). Phylogeny and synteny analyses indicate that this gene may represent the common ancestor to both *gnih* and *npff* (neuropeptide FF) genes, which would have arisen through the whole genome duplications that occurred in early vertebrates [(Osugi et al., 2014a); for reviews (Osugi et al., 2015; Tsutsui et al., 2018)].

In vitro studies of the direct pituitary effect of GnIH on gonadotropins have been performed in different vertebrate groups and gave contradictory results. While in birds, the direct inhibitory effect of GnIH on basal gonadotropin release, firstly reported in quail (Tsutsui et al., 2000), has been confirmed in chicken (Ciccone et al., 2004; Maddineni et al., 2008), in other vertebrates, the situation is different. In various mammals, RFRP-3 was only able to inhibit GnRH-induced, but not basal, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion and their expression by primary cultures of pituitary cells [ewe: (Clarke et al., 2008; Sari et al., 2009); bovine: (Kadokawa et al., 2009); pig: (Li et al., 2013)]. In rat, an inhibitory effect of RFRP-3 was observed *in vitro* on both basal and GnRH-stimulated LH secretion (Pineda et al., 2010), while other studies reported either no effect (Anderson et al., 2009) or only inhibition of GnRH-stimulated LH release (Murakami et al., 2008). In the Syrian hamster *(Mesocricetus auratus)*, RFRP-3 had no effect *in vitro* on either basal nor stimulated LH release (Ancel et al., 2012). It can be noticed that *in vivo* studies showed a rapid inhibitory effect of GnIH/RFRP-3 on plasma LH levels in hamster (Kriegsfeld et al., 2006) as well as in rat (Rizwan et al., 2009), that was likely to be directly on the pituitary given the time-frame of the effect. In amphibians, up to now, fGRP and its related peptides have been shown to exhibit *in vitro* a growth hormone (GH)-releasing activity, with no action on gonadotropin release (Koda et al., 2002; Ukena et al., 2003). In fish, the situation is even more complex. Besides the "classical" inhibitory action of GnIH like in birds, a stimulatory effect of GnIH on LH release *in vitro* was also reported in some teleost species [sockeye salmon, *Oncorhynchus nerka* (Amano et al., 2006); grass puffer, *Takifugu niphobles* (Shahjahan et al., 2011); tilapia, *Oreochromis niloticus* (Biran et al., 2014); catla, *Catla catla* (Kumar et al., 2019); for reviews: (Muñoz-Cueto et al., 2017; Ubuka and Parhar, 2018)]. In the goldfish *Carassius auratus*, using primary cultures of pituitary cells, Qi and collaborators have recently reported an inhibition of GnRH-stimulated *lhβ* and

fshβ expression, with no effect on basal gonadotropin expression (Qi et al., 2013), while other studies reported either stimulatory, inhibitory or no effect on both basal and stimulated LH (release and expression) depending on the gonadal stage (Moussavi et al., 2013, 2012).

In the European eel (*Anguilla anguilla*), a member of elopomorphs, the most ancient group of teleosts (Chen et al., 2014; Henkel et al., 2012a), we have recently described an unexpected inhibitory effect of kisspeptin on *lhβ* expression by primary cultures of pituitary cells (Pasquier et al., 2018, 2011). This latter result raised the question whether, in the eel, GnIH could possess an inhibitory effect on gonadotropins or a "paradoxal" stimulatory effect (as in sockeye salmon, grass puffer, tilapia and catla) to be opposed to the unexpected inhibitory effect of kisspeptin.

In the present study, we have identified a single *gnih* gene in the European eel genome encoding three putative GnIH peptides. Our evolutionary study by phylogeny and synteny questioned the impact of the teleost-specific whole genome duplication (TGWD or 3R) on *gnih* paralog number in teleosts. We have investigated *gnih* tissue distribution in the European eel. We have also synthetised the predicted European eel GnIH mature peptides (*A. anguilla*, aa; aaGnIH-1, aaGnIH-2 and aaGnIH-3) and tested their effects on pituitary hormone and receptor expressions using primary cultures of eel pituitary cells.

2. Materials and Methods

2.1. Animals and tissue sampling

Freshwater female European eels (*A. anguilla*) were at the prepubertal "silver" stage, which corresponds to the last continental phase of the eel life cycle, preceding the oceanic reproductive migration. Cloning, tissue distribution and primary cultures were performed using female silver eels purchased from Gebr. Dil import-export BV (Akersloot, The

Netherlands) and transferred to MNHN, France. Animals were anesthetized by cooling and then killed by decapitation under the supervision of authorized person (KR; N°R-75UPMC-F1-08) according to the protocol approved by Cuvier Ethic Committee France (N°68-027).

Various tissues were collected for investigating the distribution of *gnih* expression (n=8 eels for each tissue). The following tissues were sampled, stored in RNAlater (Ambion Inc, Austin, TX, USA) and kept frozen at –20°C until RNA extraction: brain, pituitary, eye, gill, liver, kidney, intestine, spleen, muscle, adipose tissue (fat), and ovary. The brain was further dissected into six parts as previously described (Pasquier et al., 2018): olfactory bulbs, telencephalon, diencephalon, mesencephalon, *cerebellum* and *medulla oblongata*. For cloning, only the di-/mes-encephalon was dissected and used. For primary cultures, pituitaries were collected in culture medium just prior to dispersion (30 to 40 eels per cell culture).

2.2. RNA extraction and cDNA synthesis

Total RNA from tissue samples was extracted using Trizol reagent (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's recommendations. Samples were homogenized by sonication a few seconds in Trizol. Following extraction, samples were submitted to a deoxyribonuclease I (Roche, Meylan, France) treatment. First strand cDNA was synthetized using 400 ng (tissue distribution) or 1 µg (cloning) of total RNA and a SuperScript III First Strand cDNA Synthesis Kit (Invitrogen), according to the manufacturer's recommendations. cDNAs were stored at –20°C until PCR and qPCR procedures.

2.3. *In silico* prediction of European eel *gnih* gene and identification of vertebrate *gnih (npvf)* genes

Gnih gene was identified in the European eel genome assembly [GCA_000695075.1; (Henkel et al., 2012b)] using TBLASTN search on CLC Workbench 6 (Qiagen, Vento, The Netherlands).

Gnih (npvf) gene sequences were sought in different vertebrates with a focus on teleost representatives. Gene sequences were retrieved from GenBank genomic database by an extensive Blast search using related species GnIH (NPVF) amino-acid sequences as query using CLC-Main Workbench browser. The annotation of non-annotated *gnih (npvf)* genes were performed either manually using the Blast alignment of the genomic sequence carrying the *gnih (npvf)* with protein sequences of related species *gnih (npvf)* or using the WebAUGUSTUS gene prediction server (http://bioinf.uni-greifswald.de/augustus/) with the *Danio rerio* parameters. All the sequence references as well as the genome assembly information are given in Supplementary Table 1. The nomenclature followed the recommended nomenclature in fish: the gene symbol is written in small letter in italic and the protein in capitals.

2.4. Cloning of eel *gnih* cDNA

Specific cloning primer sets were designed, based on European eel *gnih* genomic sequence using Primer3 web browser (Supplementary Table 2). Three primer sets were designed 150-300 bp apart from the coding sequence while the fourth one flanked the coding sequence and was used for the nested PCR (Supplementary Table 2). All primers were purchased from Eurofins (Ebersberg, Germany).

PCR reactions were performed on a MyCycler Thermal Cycler (Bio-Rad, Marne-la-Coquette, France) in a final volume of 50 µl using GoTaq mix (Promega, Charbonnières, France) on brain cDNA. First PCR amplifications were carried out using the 3 first primer sets with the following thermal conditions after an initial step of 94°C for 2 min, 45 cycles at

94°C for 30 s, 56°C for 30 s and 72°C for 70 s and a final extension at 72°C for 5 min. The resulting PCR products were amplified in a nested PCR using the primer set 4 (F4R4) with higher stringent thermal conditions: an initial step of 94°C for 2 min, 45 cycles at 94°C for 30 s, annealing temperature for 30 s, decreasing every 5 cycles from 60°C to 57.5°C, 72°C for 70s, and a final extension at 72°C for 5 min. Amplified products were separated on agarose gel and purified using QIAquick gel extraction kit (Qiagen). Purified PCR fragments were sequenced after subcloning into a TOPO cloning vector (Invitrogen), by the sequencing service of GATC Biotech (Constance, Germany).

2.5. Phylogeny analysis

Multiple sequence alignments of amino-acid sequence of GnIH (NPVF) preproproteins were performed using the progressive alignment algorithm available on CLC workbench and further manually edited. The tree topology was inferred using Maximum likelihood algorithm using PhyML 3.0 and with JTT as substitution model on SeaView 4.64 (Gouy et al., 2010). Strength of branch nodes was first evaluated by aLRT test and then by boostrap using 100 replicates. The consensus tree was plotted using the R package tree (Yu et al., 2017). The GnIH (LPXRF) of lamprey (*Petromyzon marinus*) and the PQRF of amphioxus were used to root the tree.

2.6. Synteny analysis

The genomic region carrying the *gnih*/*npvf* of the spotted gar (*Lepisosteus oculatus*), as an actinopterygian non-teleostean, was considered as reference region before the 3R duplication. The 3R duplicates of the *gnih*/*npvf* flanking genes were sought in the genome of teleost representatives, focusing on the European eel (*A. anguilla*) and the Japanese eel (*Anguilla japonica*), and other basal teleost species, an osteoglossomopha, the arowana

(*Scleropages formosus*) and an ostariophysi, the zebrafish (*Danio rerio*). The 3R-paralogous neighbouring genes were mapped by blast search and by using gene prediction lists of the genomic region identified as being the corresponding 3R-ohnologous regions. For the eel, the genes of the two genomic 3R-ohnologous regions were predicted using Augustus prediction server. References and locations of the genes of the synteny analysis are given in the Supplementary Table 3.

2.7. Sequence predictions, synthesis and 3D structure predictions of eel GnIH peptides

Mature peptides were predicted from the eel GnIH precursor using NeuroPred web browser [http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py, (Southey et al., 2006)]. Signal peptide of GnIH precursors were predicted using Signal 4 (Petersen et al., 2011) on CLC workbench.

Predicted European eel GnIH peptides [aaGnIH-1 (19 aa), aaGnIH-2 (20 aa) and aaGnIH-3 (32 aa); Table 1] were synthesized as previously described (Gutierrez-Pascual et al., 2009; Pasquier et al., 2018).

Structure predictions of eel GnIH peptides were modelled using the I-TASSER server, an automated protein-modeling server (Yang et al., 2014). Only models with the C-score between 2 and -5 were considered. Visualization of the structures was performed using the Jmol software v2.1 (Jmol: an open-source Java viewer for chemical structures in 3D. http://www.jmol.org/).

2.8. Primary culture of eel pituitary cells

2.8.1. Dispersion and culture

Dispersion and primary culture of pituitary cells were performed using an enzymatic and mechanical method as previously described (Montero et al., 1996). Briefly, pituitaries

were cut into slices and incubated at 25°C in a solution of porcine type II trypsin (Sigma-Aldrich, Saint-Quentin Fallavier, France). After 1 h, the trypsin solution was replaced by a solution of DNase (Sigma-Aldrich) and soya bean trypsin inhibitor (Sigma-Aldrich) for 10min. Pituitary slices were then washed with calcium-free phosphate-buffered saline (PBS) (Gibco, Thermo Fisher scientific, Villebon-sur-Yvette, France) and mechanically dispersed by repeated passages through a plastic transfer pipette (Falcon, Thermo Fisher scientific, Villebon-sur-Yvette, France). After estimating the number of viable cells by Trypan Blue coloration (Sigma-Aldrich), cells were plated on 96-well plates (60,000 cells/well) pre-coated with poly-L-lysine (Sigma-Aldrich). Cultures were performed in serum-free culture medium (Medium 199 with Earle's salt and sodium bicarbonate buffer, 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml fungizone; Gibco) at 18°C under 3% CO_2 and saturated humidity.

2.8.2. *In vitro* treatments

GnIH treatments were started 24 h after the beginning of culture to allow cell attachment (Day 0). Replicates of 5 wells for control and each treated group were used. Eel GnIH peptides (sequences are given in Table 1) stock solutions (10^{-4} M) were prepared in ultrapure water and stored at –20°C. Stock solutions were diluted in culture medium just before addition to the culture wells. Culture medium was changed and treatment added to the cells on Day 0, Day 3, Day 7 and Day 10. Cultures were stopped on Day 10. The effects of treatments were tested in 3 to 6 independent experiments performed on different cell preparations from different batches of fish. Figures display the pooled results of these different experiments.

2.8.3. Cell RNA extraction and cDNA synthesis

Total RNA was directly extracted in wells using the Cell-to-cDNA II Kit (Ambion Inc.) according to the manufacturer's recommendations. Cells were washed with PBS (Gibco) and lysed with Cell Lysis II Buffer (80 µl/well). The lysates were digested with RNase-free

DNase I (Roche). Four µl of RNA solution of each samples was then reverse transcribed with a SuperScript III First Strand cDNA Synthesis Kit (Invitrogen). The samples obtained were stored at –20°C until qPCR.

2.9. Quantitative real-time PCR (qPCR)

2.9.1. Primers

2.9.1.1. Design of primers for eel *gnih*

Eel *gnih* specific primers (Supplementary Table 2) were designed based on the European eel *gnih* sequence obtained in this study, using the Primer3 Software (Whitehead Institute/Massachusetts Institute of Technology, Boston, MA). Amplicon size was 125 bp. Forward and reverse primers were located in different exons to prevent amplification of genomic DNA. To optimize the assay, different annealing temperature were tested according to the Tm of primers. To check their specificity, amplification products were sequenced at GATC biotech.

2.9.1.2. Other gene primers

Gene specific primers were previously designed based on the nucleotide sequence of the European eel *gpα* (Quérat et al., 1990a), *fshβ* (Schmitz et al., 2005), *lhβ* (Quérat et al., 1990b), *gh* (Gong et al., 2002), thyrotropin-*β* subunit [*tshβ*; (Maugars et al., 2014)] GnRH-type 2 receptor [*gnrh-r2*; (Peñaranda et al., 2013)] and *β-actin* (Aroua et al., 2007) cDNA, the latter being used as reference gene. European eel possesses, as other teleosts, a dual TSH system [European eel (Maugars et al., 2014); Atlantic salmon *Salmo salar* (Fleming et al., 2019)]: a "classical" *tshβ* (named *tshβ1 or tshβa*), which is exclusively expressed in the pituitary (and that was assayed in this study) and a second *tshβ* (named *tshβ3 or tshβb)* with low expression in pituitary and high expression in ovary (Maugars et al., 2014). European eel possesses three GnRH receptors (GnRH-R1a, GnRH-R1b and GnRH-R2) and GnRH-R2 is

the only one whose expression increases during female maturation (Peñaranda et al., 2013). *Gnrh-r1a* and *gnrh-r1b* expressions were below the threshold of detection in primary cultures of pituitary cells (Campo et al., 2018; Pasquier et al., 2018), and were thus not assayed in this study.

The qPCR primers are listed in Supplementary Table 2. They were all purchased from Eurofins.

2.9.2. Quantitative PCR assays

Quantitative PCR assays were performed using the LightCycler® System (Roche, Ltd. Basel, Switzerland) with SYBR Green I sequence-unspecific detection as previously described (Campo et al., 2018; Pasquier et al., 2018, 2011). The qPCRs were prepared with 4 µl of diluted cDNA template, 2 µl of PCR grade water, 2 µl of SYBR Green master mix and 1 µl of each forward and reverse primer (500 nM each at final concentration). The protocol was an initial step of polymerase activation for 10 min at 95ºC; then 41 cycles (*β-actin*, *gh, gpα, lhβ*, *fshβ and tshβ*) of 10 s at 95ºC for denaturing, 5 s at 60ºC for annealing, 10 s at 72ºC for primer extension and a single final extension step of 5 min at 72°C. For *gnih*, the protocol was an initial step of polymerase activation for 10 min at 95ºC; 41 cycles of 15 s at 95ºC, 5 s at 60ºC, 10 s at 72ºC and a single final extension step of 5 min at 83ºC. For *gnrh-r2*, the protocol was an initial step of polymerase activation for 10 min at 95ºC; 42 cycles of 10 s at 95ºC, 7 s at 61ºC, 4 s at 72ºC and a single final extension step of 5 min at 72ºC. Each program ended with a melting curve analysis by slowly increasing the temperature (0.01°C/s) up to 95°C with a continuous registration of changes in fluorescent emission intensity. Serial dilutions of cDNA pools of either brain (*gnih* tissue distribution) or pituitary cells (cell culture experiments) were used as a standard curve. One chosen dilution was also included in each run as a calibrator. Each qPCR run contained a non-template control (cDNA was substituted

by water) for each primer pairs to confirm that reagents were not contaminated. The efficiency of all primers was tested, and the specificity of each reaction was assessed by melting curve analysis to ensure the presence of only one product. Each sample was analyzed in duplicate by qPCR. Normalisation of data was performed using total RNA content for the tissue distribution samples, and using *β-actin* mRNA level for cell culture samples.

2.10. Statistical analysis

Results are given as mean ± SEM. Means were compared by one-way ANOVA Tukey's multiple comparison test using Instat (GraphPad Software Inc., San Diego, Calif., USA).

3. Results

3.1. Prediction of European eel *gnih* gene

A single *gnih (npvf)* gene was found in European eel genome [on the scaffold AZBK01S009502.1 of the Anguilla_anguilla_v1_09_nov_10 genome assembly and on the scaffold 1747 of the Racon- and Pilon-corrected candidate assembly; (Jansen et al., 2017)]. A single *gnih* gene was also identified in the American eel *Anguilla rostrata* (assembly ASM160608v1) and the Japanese eel *A. japonica* (on the scaffold BEWY01082450 of the Ajp_01 assembly) genomes. The *gnih* (*npvf*) gene found in Japanese eel genome corresponded to the *gnih* (*npvf*) nucleic sequence (LC163968).

3.2. Cloning of cDNA

Using European eel specific *gnih* primers, PCRs performed on brain cDNAs led to the identification of a 654 bp GnIH transcript sequence (GenBank: MN022784) corresponding

the full-length coding sequence (CDS). Once translated, the cloned GnIH CDS gave a 217-aa GnIH precursor (Supplementary Figure 1).

The comparison of *gnih* mRNA to the European eel genome allowed to reannotate the *GnIH* genomic sequence (Supplementary Figure 2) and showed that the transcript is encoded by 3 exons. Exon 1 (141 bp) encoded for N-terminal region (52aa) including the signal peptide in N-terminal position. Exon 2 (410 bp) encoded for the 3 GnIH peptides (137 aa). The cloned sequence was identical to the corresponding sequence in the European eel genome.

3.3. Identification of *gnih* (*npvf)* genes and phylogeny analysis

We have investigated the *gnih* (*npvf*) in genome assemblies, recently released, of key teleost species including representatives of two basal goups, Elopomorphs, with the Japanese eel (*A. japonica*) and the European eel (*A. anguilla*), and Osteoglossomorphs, with the arowana (*S. formosus*), the arapaima (*Arapaima gigas*), and the *Paramormyrops kingsleyae*. A single *gnih* gene was found in the genomes of the basal teleosts, elopomorphs and osteoglossomorphs, as well as in the genomes of the other teleost species examined, except for the salmonid species where two *gnih* genes were identifed (Supplementary Figure 3). In the Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) two *gnih* genes were found on two different chromosomes (Ssa14 and Ssa27 in salmon genome assembly ICSASG_v2 and on the Chr14 and Chr18 in trout genome assembly Omyk_1.0), likely as a result of the salmonid-specific genome duplication (4R).

In the attempt to identify *gnih* (*npvf*) genes in representatives of all vertebrate classes, we have also searched for *gnih* in chondrichthyans, using new released genomes: in bamboo shark (*Chiloscyllium punctatum*, Cpunctatum v1_0), cloudy catshark (*Scyliorhinus torazame*, Storazame_v1.0) and whale shark (*Rhincodon typus*, ASM164234v2). No *gnih* (*npvf*) was

found in these three chondrichtyans. In contrast, in these three species, blast search returned a gene encoding NPFF, the sister gene of *gnih* (*npvf*) in vertebrates.

The phylogeny analysis was conducted on 33 GnIH (NPVF) preproproteins from 31 osteichtyan species including 20 teleosts, and using the preproGnIH (LPXRF) of lamprey and the preproPQRF of amphioxus as outgroup (Figure 1). All osteichtyan preproGnIH (preproNPVF) sequences clustered into a single clade. Coelacanth (*Latimeria chalumnae*) preproGnIH sequence was at the basis of the other sarcopterygian sequences, in agreement with its phylogenetical position. All teleost preproGnIH sequences formed a single clade. The position of the preproGnIH sequence of a non-teleost actinopterygian, at the basis of the sarcopterygian clade rather than at the basis of the teleost clade was unexpected, and may reflect a specific divergence of teleost sequences. Among the teleost clade, the elopomorph European eel and Japanese eel preproGnIH sequences clustered together within a clade grouping also the three osteoglossomorph species. The sequences from the other teleosts grouped in another clade, with a tree typology in agreement with their phylogenetic positions. The duplicated GnIH of salmonids, rainbow trout and Atlantic salmon, were illustrated. Each of the duplicated salmonid preproGnIH clustered with one of the duplicated trout preproGnIH, reflecting the common 4R-salmonid origin of the duplicated paralogs. The single preproGnIH of the pike, *Esox lucius*, representative of the esociforms, sister group of salmonids but which has not undergone the 4R, was at the basis of the salmonid duplicated sequences, in agreement with the phylogeny.

3.4. Synteny analysis

We have retrieved only a single *gnih* (*npvf*) gene in basal teleosts, elopomorphs and osteoglossomorphs, as in the other groups of teleosts, more recently emerged (clupeocephala). In order to elucidate the question of the fate of the *gnih* duplicated paralogs after the teleost

3R, and of the orthology between the single *gnih* of basal teleosts and the single *gnih* (*npvf*) of clupeocephala, we have performed a synteny analysis (Figure 2). The *gnih* (*npvf*) neighbouring genes were studied in the non-teleost actinopterygian, the spotted gar, as a reference and in representative teleosts, including basal species such as the eels and the arowana (Figure 2), and clupeocephala such as the zebrafish, pike, and tilapia. The synteny analysis confirmed the duplication of the *gnih* genomic region, in agreement with the 3R. *Gnih* neighbouring genes in spotted gar such as *mpp6*, *cbx3*, *snx10*, *hox9* were duplicated and conserved on each 3R-duplicated genomic region in all teleosts studied in the synteny analysis. In contrast, a single 3R-paralog was conserved for *gnih*, as well as for some neighbouring genes, such as *pde11a*, *nfe2l3* and *skap2* in all teleost species studied. Remarkably, for all teleosts studied, the single *gnih* paralog was found within the same 3R-duplicated genomic region, reflecting the orthology of the conserved single *gnih* paralog of basal teleosts and clupeocephala. The teleost *gnih* genomic region is indeed characterized by the presence of the single conserved *nfe2l3* paralog, in adjacent position to *gnih,* and the loss of *pde11a* and *skap2* paralogs, which have been conserved only by the other 3R-duplicated genomic region.

3.5. Tissue distribution of European eel *gnih* mRNA

Specific qPCR protocol was developed for eel *gnih* and applied to the analysis of the tissue distribution of its transcripts (Figure 4). *Gnih* mRNA was specifically expressed in the diencephalon part of the brain. Its expression was close to the limit of detection in the other parts of the brain (olfactory bulbs, telencephalon, mesencephalon, *cerebellum* and *medulla oblongata*), as well as in pituitary and peripheral tissues (Figure 3).

3.6. Prediction and three-dimensional structure of European eel mature GnIH peptides

399 The identification of the potential N-terminal and C-terminal cleavage sites led to the
400 prediction of three mature peptides (aaGnIH-1, aaGnIH-2 and aaGnIH-3) (Table 1). Two of
401 them present the conserved domain of LPXRF peptides, with the 19-amino-acid aaGnIH-1
402 ending by the LPLRF motif, and the 32-amino acid aaGnIH-3 ending by the LPQRF motif. In
403 contrast, aaGnIH-2, a 20-amino-acid peptide, exhibited a LTTRF C-terminal extremity. All
404 the three predicted peptides were amidated due to the presence of a GR site downstream their
405 position in the GnIH precursor.

406 GnIH-1 peptide sequence was the same in the three *Anguilla* species investigated (*A.*
407 *anguilla*, *A. rostrata* and *A. japonica*). GnIH-2 and -3 sequences were also identical in *A.*
408 *rostrata* and *A. anguilla*, but in *A. japonica*, GnIH-2 peptide differed by two amino-acids
409 (Ala-Gln instead of Tyr-Arg in positions 7-8) and GnIH-3 peptide by one amino-acid (Ile
410 instead of Met in position 18). A third amino-acid (Ser instead of Pro in position 3) differed in
411 *A. japonica* cloned cDNA (BAV18007.1) but not in the genomic sequence (Table 1;
412 Supplementary Figure 3).

413 The three-dimensional (3D) secondary structures of the three European eel GnIH
414 peptides were predicted using the I-TASSER server (Figure 4). The 3 peptides had a similar
415 C-terminal coiled region, and they presented an internal α-helix with some differences in the
416 size and location. For aaGnIH-1, the helix size was 7 amino acids (residues 8 to 14) and 8
417 amino acids for aaGnIH-3 (residues 12 to 19). Their helixes were in the central part of the
418 peptide and their N- and C-terminal extremities were close spatially, or even nested for
419 aaGnIH-3. On the other hand, aaGnIH-2 had a longer helix (11 residues from 2 to 12) that
420 started very close to the N-terminal extremity of the peptide and had an extended overall
421 conformation.

422

3.7. *In vitro* effects of synthesized eel GnIH peptides on the expression of pituitary hormones by primary cultures of eel pituitary cells

The three predicted European eel mature GnIH peptides (aaGnIH-1, -2 and -3) were synthetized and their effects tested on 10-day primary cultures of eel pituitary cells for 10 days, as previously performed with eel kisspeptin and tachykinin peptides (Campo et al., 2018; Pasquier et al., 2018).

3.7.1. Dose-dependent effect of eel GnIH peptides on *lhβ* mRNA levels

The effects of various concentrations of aaGnIH-1, aaGnIH-2 and aaGnIH-3 (from 10^{-10} to 10^{-6} M) were tested over 10 days of culture (Figure 5A). None of the three eel GnIH peptides had significant effect on *lhβ* mRNA levels at 10^{-10} M. In contrast, at 10^{-8} and 10^{-6} M, they all significantly and dose-dependently inhibited *lhβ* mRNA levels in comparison to controls (aaGnIH-1: $P < 0.001$ at both doses; aaGnIH-2: $P<0.05$ at 10^{-8} M and $P<0.001$ at 10^{-6} M; aaGnIH-3: $P < 0.001$ at both doses). None of the three eel GnIH at any dose tested affected the mRNA levels of *β-actin* (Figure 5B).

3.7.2. Effects of eel GnIH peptides on other pituitary hormone and receptor mRNA levels

To test whether the inhibitory effect of eel GnIHs was specific to *lhβ* expression, the effect of 10^{-6} M aaGnIH-1, aaGnIH-2 and aaGnIH-3 was investigated on other pituitary hormones (Figure 6) over 10 days. As in the experiment above, all three eel GnIH peptides significantly inhibited *lhβ* mRNA levels (54, 33 and 53% of inhibition, respectively, $P < 0.001$). Eel GnIH-2 and -3 peptides slightly but significantly inhibited *fshβ* mRNA levels (17 and 11 % of inhibition, respectively; $P<0.05$). All three aaGnIH peptides also inhibited common α glycoprotein subunit (*gpα*) mRNA levels (31, 18 and 33% of inhibition, respectively; $P < 0.001$ for aaGnIH-1 and -3; $P<0.05$ for aaGnIH-2). Eel GnIH peptides also inhibited pituitary GnRH receptor, *gnrh-r2*, mRNA levels (50, 40 and 38% of inhibition,

448 respectively; P < 0.001). In contrast, the peptides had no effect on other pituitary hormone,
449 *tshβ* and *gh,* mRNA levels (Figure 6).
450
451 **4. Discussion**
452
453 **4.1. Characterization of European eel *GnIH***
454 A single *gnih* gene was identified in the genome of the European eel, one
455 representative of elopomorphs, as in other teleosts and in tetrapods. Using NeuroPred
456 software, three mature peptides of 19 (aaGnIH-1), 20 (aaGnIH-2) and 32 (aaGnIH-3) amino-
457 acids were predicted in the European eel. A single *gnih* gene encoding three mature peptides
458 was also found in other *Anguilla* species. Three putative peptides are also encoded in the
459 GnIH precursor of an osteoglossomorph (arowana), some cypriniformes (goldfish, common
460 carp *Cyprinus carpio*, zebrafish, catla, red piranha *Pygocentrus nattereri*, golden-line barbel
461 *Sinocyclocheilus grahami* and *Sinocyclocheilus rhinocerous*), a siluriforme, the catfish
462 (*Ictalurus* punctatus); a salmoniforme, (Atlantic salmon) and some percomorpha (orange-
463 spotted grouper *Epinephelus coioides*, Senegalese sole *Solea senegalensis*, Japanese flounder,
464 *Cichlasoma dimerus,* tilapia, medaka *Oryzias latipes* and, bluehead wrasse *Thalassoma*
465 *bifasciatum*) [for reviews: (Di Yorio et al., 2019; Muñoz-Cueto et al., 2017)], as in most
466 tetrapods [birds and reptiles; for reviews: (Ogawa and Parhar, 2014; Osugi et al., 2014b;
467 Tsutsui et al., 2018)]. In some other teleosts, belonging to the superorder Acanthopterygi,
468 only two peptides are predicted from GnIH precursor [for reviews: (Di Yorio et al., 2019;
469 Muñoz-Cueto et al., 2017)]. Thus, in teleosts, only late-branching evolved species exhibit two
470 encoded peptides, suggesting a loss of one peptide in the course of teleost evolution (Di Yorio
471 et al., 2019). The existence of only two peptides in the GnIH precursor is also observed in
472 mammals [for reviews: (Ogawa and Parhar, 2014; Osugi et al., 2014b; Tsutsui et al., 2018)].

The multiple sequence alignment of the GnIH preproproteins highlighted that the spotted gar, a non-teleostean actinopterygian, presents three putative functional LPXRFa peptides and the stigmate of a fourth at the C-terminal extremity, matching with the fGRP-RP-3-like of the frogs and the GRP-RP-2-like of the lizards and the GnIH-RP-2 of the chicken (Supplementary Figure 3). In coelacanth, five LPXRFa peptides could be predicted, the four first peptides correspond to the four peptides predicted in frog and in lizard, while the fifth found at the C-terminal extremity appeared to be specific of the coelacanth.

European eel peptides, aaGnIH-1 and -3, possessed the conserved C-terminal LPXRF motif (LPLRFamide and LPQRFamide, respectively), followed by an amidation site (Gly-Arg) common to most vertebrate GnIHs [for review: (Osugi et al., 2014b)]. In contrast, aaGnIH-2 possessed another RFamide signature, LTRRFamide, which is, to the best of our knowledge, unique among vertebrates/teleosts. The presence of a RFamide motif different from LPXRF at the C-terminal extremity of a predicted GnIH is also observed in some other teleosts. For instance, the signature MPXRF (X=M or Q) is found in all or some of the predicted GnIH peptides from takifugu/tiger puffer *Takifugu rubripes*, tetraodon *Tetraodon nigroviridis* and stickleback *Gasterosteus aculeatus* (Zhang et al., 2010), grass puffer (Shahjahan et al., 2011), medaka (Osugi et al., 2014b), tilapia (Biran et al., 2014), orange-spotted grouper (Wang et al., 2015), half-smooth tongue sole *Cynoglossus semilaevis* (Wang et al., 2018), Senegalese sole (Aliaga-Guerrero et al., 2018), European sea bass *Dicentrarchus labrax* (Paullada-Salmerón et al., 2016a), *Cichlasoma dimerus* (Di Yorio et al., 2016) [for reviews : (Di Yorio et al., 2019; Muñoz-Cueto et al., 2017)]. Thus, Munoz-Cueto and collaborators chose to name these teleost peptides LPXRF-like peptides (Muñoz-Cueto et al., 2017). Such MPXRFa motif (X=L) is also observed in a bird (Pekin drake, *Anas platyrhynchos domestica*) for GnIH-RP1 (Fraley et al., 2013). In the red-eared slider turtle (*Trachemys scripta elegans*), in addition to three LPXRF (X=L or Q) peptides, another

RFamide (LLHRFamide) sequence is observed in the precursor between GnIH and GnIH-RP-2, leading to four potential RF-peptides (Ukena et al., 2016). In lamprey, the C-terminal sequence of LPXRFa-1 and -2 is QPQRFa, while LPXRFa-2-like peptide ends with RPQRFa (Osugi et al., 2012). All the above-mentioned peptides end by RFa, but in some teleosts, one of the predicted GnIH peptides is not an RF-amide: HQVRYa in grass and tiger puffer (Shahjahan et al., 2011) or LPQRLa in grouper (Wang et al., 2015). In the coelacanth, GnIH-2 also ends by LPLRLa (Muñoz-Cueto et al., 2017). The human GnIH precursor, in addition to RFRP-1 (-LPLRFamide) and RFRP-3 (-LPQRFamide), also encodes a predicted RFRP-like peptide with a C-terminus LPLRSamide motif (Ubuka et al., 2009). In tilapia, the third putative RFa-related peptides (RFRP) encoded by GnIH precursor lacks an amidation site (Biran et al., 2014). Further studies need to be done to investigate whether these non-conventional GnIH peptides, present in some vertebrates, are produced by the brain and possess physiological effects.

4.2. Expression of *gnih* mRNA in the diencephalon part of the brain

The tissue distribution of *gnih* transcripts, which was analysed in the present study in females at silver stage, may differ in males or in females at different physiological stage. qPCR data showed a high and specific expression of *gnih* in the diencephalon part of the eel brain, in agreement with previous data in other vertebrates (for review: Ogawa and Parhar, 2014). The intense presence of GnIH neurons in the diencephalic *nucleus posterioris periventricularis* (NPPv) was found to be a common pattern in teleosts [for reviews: (Di Yorio et al., 2019; Muñoz-Cueto et al., 2017)]. Projections of GnIH fibers to the pituitary have been reported in various teleost species, providing the anatomical basis for the neuroendocrine role of GnIH [for review: (Di Yorio et al., 2019)]. However, in some teleosts, no GnIH pituitary innervation was observed [Indian major carp, *Labeo rohita* (Biswas et al.,

2015); *Cichlasoma dimerus* (Di Yorio et al., 2016)]. Immunocytochemical studies of GnIH neurons in the eel would allow to assess the pituitary innervation, as well as possible brain projections linked to potential additional extra-pituitary functions of GnIH.

In the eel, *gnih* transcript levels were very low, at the limit of detection threshold, in other parts of the brain than diencephalon. This differs from some other neuropeptides such as kisspeptins (Pasquier et al., 2018) and tachykinins (Campo et al., 2018), which are more widely expressed in the eel brain. In some teleosts, such as Indian major carp (Biswas et al., 2015) and European sea bass (Paullada-Salmerón et al., 2016a) GnIH-immunoreactive (-ir) cells and *gnih* expression are observed in various brain regions, while in some other teleosts, GnIH-ir cells were restricted to NPPv [sockeye salmon (Amano et al., 2006); tilapia (Ogawa et al., 2016); for reviews: (Di Yorio et al., 2019; Muñoz-Cueto et al., 2017)]. This suggests variations among teleost species in the potential roles of GnIH as brain neuromediator.

In some teleosts, *gnih* expression has also been reported in peripheral tissues, in particular in gonads [for reviews: (Muñoz-Cueto et al., 2017; Ogawa and Parhar, 2014)]. Such a gonadal expression has also been reported in birds, mammals and primates, suggesting a role in steroid synthesis and gamete maturation [for review: (McGuire and Bentley, 2010)]. In the prepubertal female silver eel, the expression of GnIH was very low, at the limit of detection threshold in the ovary as well as in the other peripheral tissues investigated.

4.3. Direct pituitary inhibitory effect of eel GnIH peptides on gonadotropic axis

We have shown that the predicted mature eel GnIH peptides possess a significant inhibitory effect on gonadotropin subunits, *lhβ*, *fshβ*, *gpα*, and GnRH receptor, *gnrh-r2*, mRNA expression by primary cultures of pituitary cells from prepubertal silver eels. Whether these decreases in mRNA expression involved a reduction in the relative number of gonadotrophs in the culture could deserve some future investigations. It should be also

548 noticed that these results have been obtained in females at silver stage, and may differ in
549 males or in females at a different physiological stage. Our study focused on transcript levels
550 and did not investigate gonadotropin release, as gonadotropin levels in the culture medium of
551 silver eel pituitary cells are too low to be detected (Montero et al., 1996).

552 Inhibitory or stimulatory effects of GnIH peptides on *lhβ* expression *in vitro* have been
553 reported in some other teleosts. Some experiments were also performed on primary cultures
554 of pituitary cells. *In vitro* experiments in the goldfish show a seasonal variation of GnIH
555 effect on *lhβ* expression by pituitary cells. Indeed, gfGnIH-3 (gfLPXRFa-3 or gGnIH)
556 suppresses *lhβ* mRNA levels at early recrudescence and prespawning but elevated *lhβ* at mid-
557 recrudescence (Moussavi et al., 2012). In contrast, Qi and collaborators reported no effect of
558 gfGnIH-3 (GnIH-III) on basal gonadotropin synthesis, but an inhibition of GnRH-stimulated
559 *lhβ* synthesis by primary culture of pituitary cells from female goldfish in the late vitellogenic
560 stage (Qi et al., 2013). In their study, GnIH-2 (GnIH-II) has no effect neither on basal nor on
561 stimulated *lhβ* expression (Qi et al., 2013). Various effects of GnIH were also reported in
562 other teleosts. In grass puffer, goldfish (gf) LPXRFa stimulates *lhβ* mRNA expression in
563 cultured pituitary cells [only gfLPXRFa-1 was tested because of its high sequence similarity
564 to grass puffer LPXRFa-1 (Shahjahan et al., 2011)]. Others *in vitro* studies used whole
565 (explants) or diced pituitaries, which could not discriminate between direct effects on
566 pituitary cells or indirect effects on neurosecretory fibers innervating the pituitary cells.
567 Indeed, in teleosts, differently from tetrapods, hypophysiotropic nerve endings directly
568 innervate the adenohypophysis [for review: (Zohar et al., 2010)]. In common carp, GnIH-3
569 (GnIH-III) decreases *lhβ* mRNA expression by diced pituitaries (Peng et al., 2016). In
570 zebrafish, LPXRFa-3 reduces *lhβ* mRNA levels by adult male pituitary explants; the two
571 other peptides encoded in the zebrafish LPXRFa precursor were not tested (Spicer et al.,
572 2017). In *Astyanax altiparanae* males, zebrafish GnIH-3 attenuates the cGnRH2-stimulatory

573 action on *lhβ* mRNA expression by pituitary explants, without any effect on basal *lhβ*
574 expression level (Branco et al., 2019). In contrast, Wang and collaborators describe a
575 stimulation of basal *lhβ* mRNAs by LPXRFa-1 by whole pituitary culture in half-smooth
576 tongue sole (Wang et al., 2019b). In chicken *Gallus gallus*, GnIH treatment of diced pituitary
577 glands from adult cockerels has no effect on *lhβ* mRNAs (Ciccone et al., 2004).

578 In European eel, aaGnIH-2 and -3, but not aaGnIH-1, inhibited *fshβ* expression, while
579 they all inhibited *lhβ* expression. This may implicate that different GnIH receptors [for now,
580 up to 3 in teleosts (Wang et al., 2019a)] are present on pituitary *lhβ*- and *fshβ*- expressing
581 cells, which should be further investigate in our model. In goldfish, Qi and collaborators
582 report no effect on basal gonadotropin synthesis, but an inhibition of GnRH-stimulated *fshβ*
583 synthesis by pituitary cells from female goldfish in the late vitellogenic stage treated with
584 gfGnIH-III (Qi et al., 2013). Moussavi and collaborators have already demonstrated that
585 gfLPXRFa-3 (gGnIH) consistently attenuates basal and GnRH-induced *fshβ* mRNA levels in
586 a dose-dependent manner in mid and late gonadal recrudescent goldfish (Moussavi et al.,
587 2013, 2012) In common carp, GnIH-3 (GnIH-III) decreases *fshβ* mRNA expression at
588 100 μM, but not at 10 μM (Peng et al., 2016). In *Astyanax altiparanae* males, zebrafish
589 GnIH-3 attenuates the cGnRH2 stimulatory action on *fshβ* mRNA expression by pituitary
590 explants, without any effect on basal *fshβ* expression level (Branco et al., 2019). In cultured
591 pituitary cells of grass puffer, it has been reported a stimulatory effect of gfLPXRFa-1 on *fshβ*
592 mRNA levels (Shahjahan et al., 2011). Finally, in chicken, GnIH depresses *fshβ* mRNAs
593 (Ciccone et al., 2004).

594 In a representative of the most ancient lineage of vertebrates, the lamprey,
595 intraperitoneal injections of GnIH (LPXRFa-2) stimulates gonadotropin β expression (Osugi
596 et al., 2012).

In the European eel, we have shown an inhibitory effect of all three aaGnIH peptides on *gpα* expression *in vitro*. Such an effect was also reported in *vitro* in chicken (Ciccone et al., 2004), as well as in some teleosts [zebrafish (Spicer et al., 2017); tongue sole (Wang et al., 2019b)].

We have also reported an inhibitory effect of eel GnIH peptides on the expression of *gnrh-r2* expression *in vitro*, as recently shown with kisspeptin (Pasquier et al., 2018) and tachykinin (Campo et al., 2018) peptides. In the sea bass pituitary, GnRHR-II-1a is the main GnRH receptor and co-localizes with all LH- and some FSH-producing cells (González-Martínez et al., 2004; Moncaut et al., 2005). Consistently, it has been shown that intracerebroventricular (icv) injection of sbGnIH-2 significantly reduces the expression of pituitary *gnrhr-II-1a* (Paullada-Salmerón et al., 2016b). In contrast, the second sea bass GnIH peptide, sbGnIH-1, does not induce any changes in pituitary *gnrhr-II-1a* expression (Paullada-Salmerón et al., 2016b). In the sea bass brain, the main GnRH receptor is GnRHR-II-2b and its expression is not affected by icv injection of sbGnIH-1 or sbGnIH-2 (Paullada-Salmerón et al., 2016b). Our study in the European eel is the first one in vertebrates showing a direct pituitary inhibitory effect of GnIH on GnRH receptor expression.

While in the eel, GnIH has an inhibitory effect on gonadotropin expression *in vitro*, no effect has been demonstrated on *gh* mRNA levels. However, a predominant stimulatory effect of GnIH is observed in other teleosts *in vitro* [goldfish (Moussavi et al., 2014); grass puffer (Shahjahan et al., 2016); tongue sole (Wang et al., 2019b)] except, in the European sea bass, in which *in vivo* experiment have shown that icv injection of sbGnIH-2 (but not sbGnIH-1) induces a decrease in *gh* mRNA levels (Paullada-Salmerón et al., 2016b). To the best of our knowledge, only one study on *gh* expression is available in mammals. In ovines, RFRP-3 has no effect *in vitro* on the expression of *gh* [primary culture of sheep pituitary cells: (Sari et al., 2009)].

In conclusion regarding the neuroendocrine effect of GnIH in the eel, all the 3 peptides encoded by the eel *gnih* gene are able to exert an inhibitory action on the expression of pituitary genes involved in the pituitary control of reproduction (*lhβ, fshβ, gpα* and *gnrh-r2)*. These results demonstrate that GnIH peptides may exert a dual inhibitory action on the gonadotropic function in the eel: they inhibit the expression of gonadotropin subunits (*lhβ, fshβ, gpα* subunits) and also may reduce the responsiveness of gonadotropic cells to GnRH via the inhibition of the expression of GnRH receptors. In addition to dopamine (Jolly et al., 2016; Vidal et al., 2004) and some other neuropeptides such as kisspeptins (Pasquier et al., 2018) and tachykinins (Campo et al., 2018), GnIH may thus represent one of the brain actors involved in the prepubertal blockade of eel sexual maturation. This inhibition is specific, as we have observed no effect on the expression of other pituitary hormones, *gh* and *tshβ*. The common inhibitory effect of the 3 eel GnIH peptides maybe related to the relative similarity of their C-terminal coil 3D structure and sequences (LPLRFamide, LTTRFamide and LPQRFamide). This is the first teleost species in which all three existing peptides have a similar inhibitory effect on pituitary hormone expression *in vitro*. Further studies should also investigate their possible synergistic effects. Our results in a basal teleost are in agreement with previous data demonstrating an inhibitory role in reproduction of GnIH in ancestral vertebrates, which would have been conserved in mammals, birds and most teleosts, whereas a change to a stimulatory role would have occurred in some teleost and agnathans (Tsutsui et al., 2018). Our finding of the inhibitory effect of GnIH in the eel opens new research avenues to investigate the *in vivo* effects of GnIH peptides and GnIH receptor antagonists in this species of basic and applied relevances.

4.2. Evolutionary scenario of GnIH in fish

Previous studies have inferred that vertebrate *gnih* (*npvf*) originates from an ancestral

PQRF-encoding gene present at least in a chordate ancestor. One of the two rounds of whole genome duplications (1R/2R) that occurred early in the vertebrate lineage would have given rise to *gnih* and its sister gene *npff* (for review: Tsutsui et al., 2018). Up to four *gnih (npvf)/npff* paralogs could have thus been expected in vertebrates from the 1R and 2R, while only two paralogs (a single *gnih* and a single *npff*) are retrieved in agnathans (sea lamprey) as well as in basal sarcopterygians (coelacanth) and basal actinopterygians (spotted gar). This suggests an early loss of two *gnih (npvf)/npff* paralogs shortly after 1R/2R.

In the present study, we have identified a *npff* gene but did not retrieve any *gnih* gene in the recently released chondrichytan genomes. This suggests the loss of *gnih* in this lineage, which deserves further investigations.

Previous studies in various teleosts reported a single *gnih* gene. Considering that a third round of whole genome duplication (3R) specifically occurred at the basis of the teleost lineage, two *gnih* paralogs could have been expected. We addressed this question in the present study, with a special focus on basal teleosts.

Recently, new genome assemblies have been released including representative species of the most basal groups of teleosts, elopomorphs (*Anguilla* species), osteoglossomorphs (*e.g.* arowana and arapaima), and of relatively basal groups among clupeocephala, clupeiforms (herring, *Clupea harangus*, and pilchard, *Sardina pilchardus*), gymnotiforms (electric eel, *Electrophorus electricus*). Previous investigations on the evolution of various genes have shown that teleost basal species have often retained more paralogous genes, whatever their origin (from 1R/2R or 3R), than more recently emerged teleosts (*e.g.* kisspeptin receptors: Pasquier et al., 2012; leptin receptors: (Morini et al., 2015); progesterone receptors: (Morini et al., 2017)).

Taking advantage of the new genomes available we have traced the evolutionary scenario of *gnih* (*npvf*) in teleosts. Our search in genomic databases has revealed only a single

672 *gnih* gene in representative species of the most basal groups of teleosts, elopomorphs and
673 osteoglossomorphs, as well as in representatives of basal clupeocephala group, clupeiforms,
674 and of more recent groups of clupeocephala. This suggests the conservation of only a single
675 3R-duplicated *gnih* paralog in all extant teleosts. The synteny analysis indicated the orthology
676 of the single conserved *gnih* paralog among elopomorphs, osteoglossomorphs and
677 clupeocephala. This allows us to infer that the loss of one of the two 3R-duplicated *gnih*
678 paralogs occurred shortly after the 3R, before the emergence of the most basal groups
679 elopomorphs and osteoglossomorphs, leading to the presence of a single *gnih* in the whole
680 teleost lineage. Accordingly, the possible variations in *gnih* physiological functions among
681 teleosts cannot be related to the conservation of one *versus* the other of the 3R-paralogs, since
682 the same single *gnih* 3R-paralog has been conserved in all teleosts.
683 We retrieved two *gnih* genes in salmonids, such as Atlantic salmon and rainbow trout,
684 but the phylogeny analysis showed that the duplicated genes clustered in a same clade with
685 the pike *gnih* branching at the basal position. Salmonids are known to have undergone a 4th
686 genomic duplication (salmonid 4R). The presence of two paralogs in salmonids while a single
687 gene was found in pike, member of the esoxiforme group that have diverged from salmonids
688 before the 4R suggests that the salmonid duplicated *gnih* derived from the 4R.
689
690 In conclusion, we identified in the eel a single *gnih* gene, expressed in the
691 diencephalon and encoding three putative GnIH peptides. The three eel GnIH peptides were
692 synthesized and shown to inhibit the expression of gonadotropin subunits and GnRH receptor
693 by eel pituitary cells. This inhibitory action of GnIH in a basal teleost is in agreement with the
694 hypothesis of an ancestral inhibitory role of GnIH in the neuroendocrine control of
695 reproduction in vertebrates. Phylogeny and synteny analyses supported an early loss of one of

696 the 3R-paralog of GnIH leading to the presence of a single GnIH in the teleost lineage as in
697 other vertebrates.
698
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Figure Legends

Figure 1: Consensus tree of vertebrate GnIH (NPVF) preproprotein sequences.

The tree was inferred by Maximum Likelyhood using PhyML and rooted using the lamprey (*P. marinus*) LPXRF and amphioxus (*B. japonicum*) PQRFa precursor sequences. Bootstrap values from 100 replicates are indicated for each node. The species name is followed by the chromosome or scaffold number carrying the GnIH. The sequence references are given in the Supplementary Table 1.

Figure 2: Conserved synteny of the *gnih (npvf)* gene in actinopterygians and impact of the teleost-specific whole genome duplication.

The spotted gar (*L. oculatus*) *gnih* genomic region is used as a reference. This region is duplicated in teleosts after teleost-specific whole genome duplication (TWGD or 3R). The duplicated genomic regions of Japanese eel (*A. japonica*), European eel (*A. anguilla*), arowana (*S. formosus*), zebrafish (*D. rerio*), pike (*E. lucius*) and medaka (*O. latipes*) are illustrated. *Gnih* genes are in blue. 3R *gnih* paralogous genes, which have been lost, are in grey with a red cross. Gene positions are given in Mega base below the genes. Full names and references of *gnih* and neighbouring genes are given in the Supplementary Table 3.

Figure 3: Tissue distribution of eel *gnih* transcripts.

Gnih mRNA levels were analyzed by qPCR in various parts of the brain, in the pituitary and peripheral tissues in silver female European eels. Olfactory bulbs (OB), telencephalon (Tel), diencephalon (Di), mesencephalon (Mes), *cerebellum* (Cer), *medulla oblongata* (MO), pituitary (Pit), eye, gill, liver, kidney, intestine, spleen, muscle, adipose tissue (Fat), and

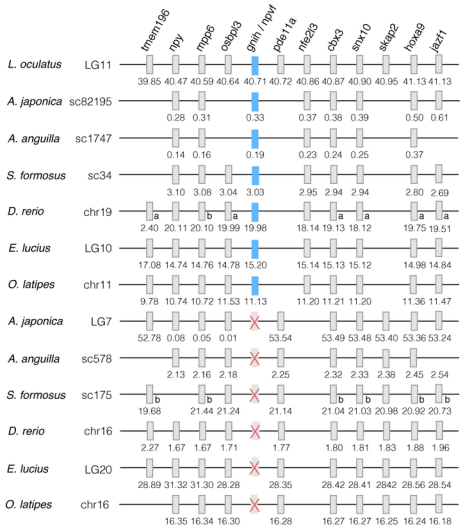
1058 ovary. The relative abundance of *gnih* mRNA was normalised to the amount of total RNA.
1059 Each bar represents mean ± SEM from 8 individuals.
1060
1061 **Figure 4:** Predicted three-dimensional structures of eel GnIH peptides.
1062 The three-dimensional (3D) structures of European eel GnIH-1, -2 and -3 peptides (aaGnIH-1,
1063 aaGnIH-2, aaGnIH-3) were predicted using I-TASSER server. Models with a C-score
1064 betwwen 2 and -5 are presented. They are visualised rainbow-colored, with the N-terminal
1065 end in blue and the C-terminal end in red, showing the central part of the peptides in green.
1066
1067 **Figure 5**: Dose-dependent effects of eel GnIH peptides on *lhβ* expression by primary cultures
1068 of eel pituitary cells.
1069 Pituitary cells from silver female European eels were treated with various concentrations of
1070 the three European eel GnIH peptides (aaGnIH-1, aaGnIH-2, aaGnIH-3) for 10 days. mRNA
1071 levels of luteinising hormone β subunit (*lhβ*) (A) and reference gene *β-actin* (B) were
1072 quantified by qPCR. Data of *lhβ* were normalized against *β-actin*. Each point represents mean
1073 ± SEM of three (GnIH-2) or six (GnIH-1 and -3) independent cell culture experiments with
1074 five well replicates/group/experiment. *, $P < 0.05$; ***, $P<0.001$ *versus* controls, ANOVA.
1075
1076 **Figure 6:** Effects of eel GnIH peptides on the expression of various pituitary hormones and
1077 receptor by primary cultures of eel pituitary cells.
1078 Pituitary cells from silver female European eels were treated with 10^{-6} M of the three
1079 European eel GnIH peptides (aaGnIH-1, aaGnIH-2, aaGnIH-3) for 10 days The mRNA levels
1080 for luteinising hormone β subunit *lhβ* (A), follicle stimulating hormone β subunit *fshβ* (B),
1081 thyrotropin subunit β subunit *tshβ* (C), common glycoprotein hormone α subunit *gpα* (D),
1082 growth hormone *gh* (E) and GnRH receptor *gnrh-r2* (F) were quantified by qPCR. Data were

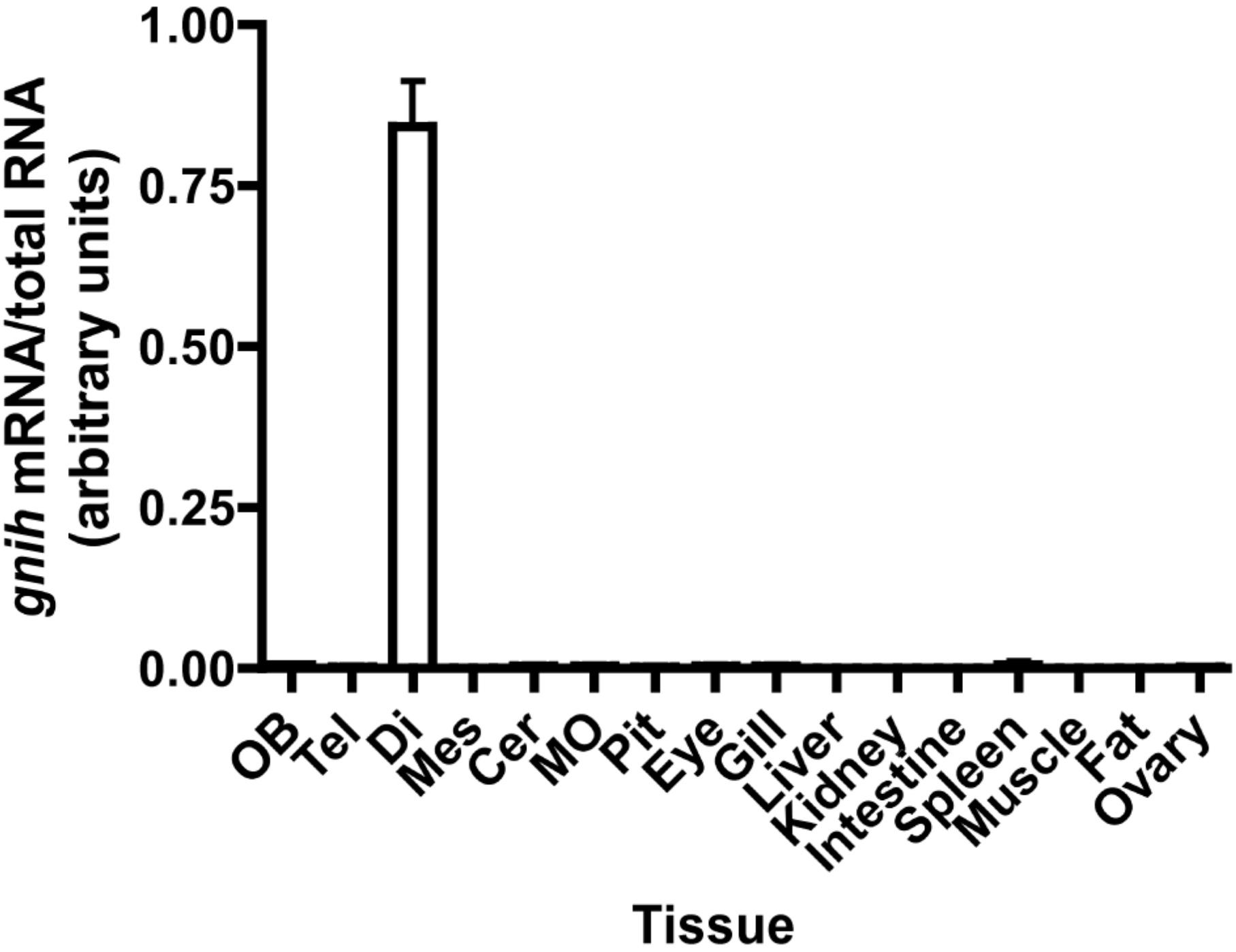
1083 normalized against *β-actin*. Each bar represents mean ± SEM of three (GnIH-2) or six (GnIH-

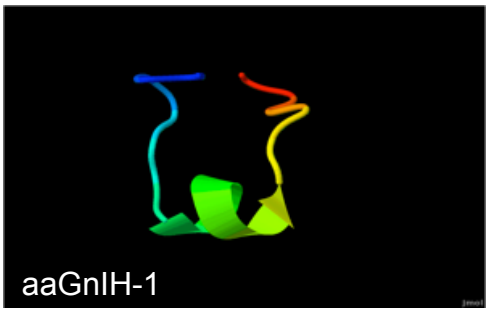
1084 1 and -3) independent cell culture experiments with five well replicates/group/experiment. *,

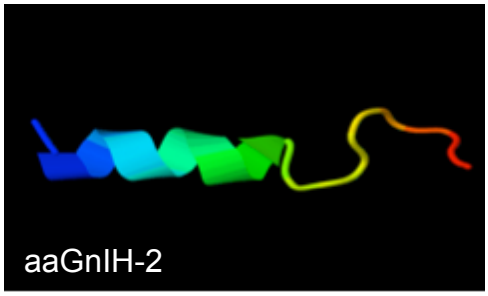
1085 $P < 0.05$; ***, $P<0.001$ *versus* controls, ANOVA.

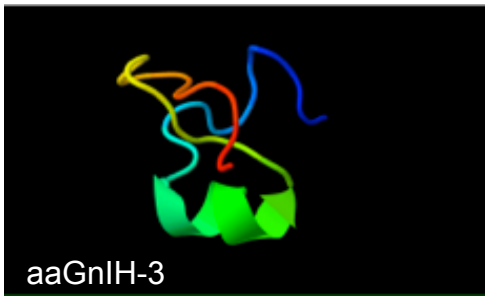

Cynoglossus semilaevis GnIH Chr18
75
61
Dicentrarchus labrax GnIH LG9
Takifugu rubripes RFRP Chr12
89
Oreochromis niloticus GnIH LG22
51
91
Oryzias latipes GnIH Chr11
83
Gadus morhua GnIH LG22 *
Osmerus eperlanus GnIH Sc7180003227904
69
98
Oncorhynchus mykiss GnIHα Chr14
Salmo salar GnIHα Ssa14
61
Oncorhynchus mykiss GnIHβ Chr18
100
79
Salmo salar GnIHβ Ssa27
Esox lucius GnIH LG10
50
Astyanax mexicanus GnIH Sc3131
52
60
Electrophorus electricus GnIH Sc218
91
Ictalarus punctatus GnIH Chr12
61
Danio rerio GnIH Chr19
57
Sardina pilchardus GnIH Sc435
100
Clupea harengus GnIH Sc676
Scleropages formosus GnIH Sc34
89
Arapaima gigas GnIH Sc59
83
Paramormyrops kingsleyae GnIH Sc212
55
Anguilla anguilla GnIH Sc9505
100
Anguilla japonica GnIH Sc82195
Gallus gallus GnIH Chr2
48
Gekko japonicus GnIH Sc904
67
Homo sapiens GnIH Chr7
83
42
Ornithorhynchus anatinus GnIH
99
Nanorana parkeri GnIH Sc4
90
72
Xenopus tropicalis GnIH Chr6
65
Latimeria chalumnae GnIH Sc150 *
Lepisosteus oculatus GnIH LG11
Petromyzon marinus LPXRF
Branchiostoma japonicum PQRF


tmem196
npy
mpp6
osbpl3
gnlh1 / npvf
pde11a
nfe2l3
cbx3
snx10
skap2
hoxa9
jazf1
L. oculatus LG11 39.85 40.47 40.59 40.64 40.71 40.72 40.86 40.87 40.90 40.95 41.13 41.13
A. japonica sc82195 0.28 0.31 0.33 0.37 0.38 0.39 0.50 0.61
A. anguilla sc1747 0.14 0.16 0.19 0.23 0.24 0.25 0.37
S. formosus sc34 3.10 3.08 3.04 3.03 2.95 2.94 2.94 2.80 2.69
D. rerio chr19 a b a a a a a a 2.40 20.11 20.10 19.99 19.98 18.14 19.13 18.12 19.75 19.51
E. lucius LG10 17.08 14.74 14.76 14.78 15.20 15.14 15.13 15.12 14.98 14.84
O. latipes chr11 9.78 10.74 10.72 11.53 11.13 11.20 11.21 11.20 11.36 11.47
TWGD
A. japonica LG7 52.78 0.08 0.05 0.01 53.54 53.49 53.48 53.40 53.36 53.24
A. anguilla sc578 2.13 2.16 2.18 2.25 2.32 2.33 2.38 2.45 2.54
S. formosus sc175 b b b b b b 19.68 21.44 21.24 21.14 21.04 21.03 20.98 20.92 20.73
D. rerio chr16 2.27 1.67 1.67 1.71 1.77 1.80 1.81 1.83 1.88 1.96
E. lucius LG20 28.89 31.32 31.30 28.28 28.35 28.42 28.41 2842 28.56 28.54
O. latipes chr16 16.35 16.34 16.30 16.28 16.27 16.27 16.25 16.24 16.18

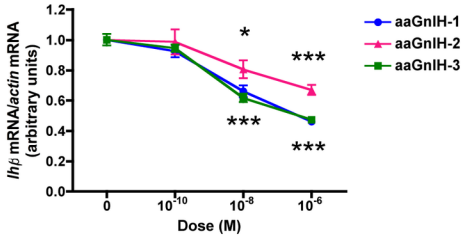

1.00
0.75
0.50
0.25
0.00
gnih mRNA/total RNA
(arbitrary units)
OB
Tel
Di
Mes
Cer
MO
Pit
Eye
Gill
Liver
Kidney
Intestine
Spleen
Muscle
Fat
Ovary
Tissue


aaGnIH-1

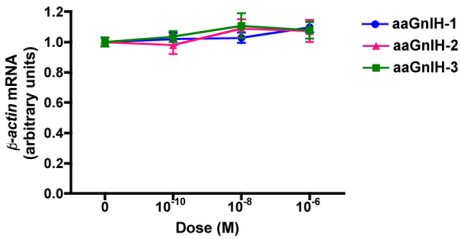

aaGnIH-2


aaGnIH-3

A) *lhβ*



B) *β-actin*



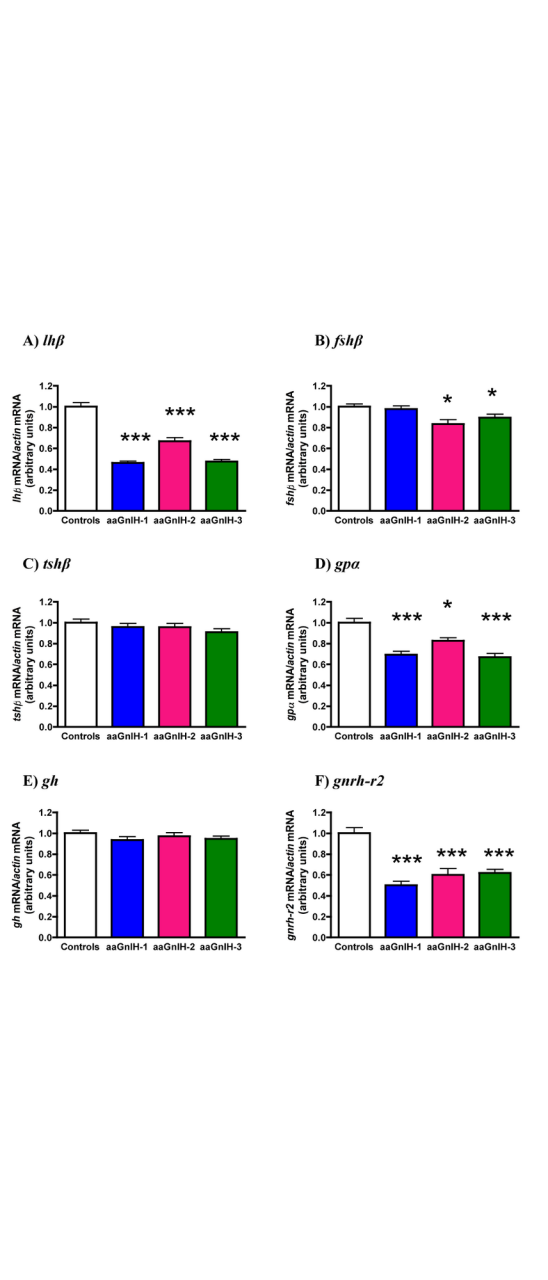

A) lhβ
lhβ mRNA/actin mRNA (arbitrary units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
Controls
aaGnIH-1
aaGnIH-2
aaGnIH-3
B) fshβ
fshβ mRNA/actin mRNA (arbitrary units)
*
*
C) tshβ
tshβ mRNA/actin mRNA (arbitrary units)
D) gpα
gpα mRNA/actin mRNA (arbitrary units)
***
*
***
E) gh
gh mRNA/actin mRNA (arbitrary units)
F) gnrh-r2
gnrh-r2 mRNA/actin mRNA (arbitrary units)
***
***
***

Table 1: Comparison of predicted sequences of GnIH peptides in *Anguilla* species.
Aa : *Anguilla anguilla*; ar: *Anguilla rostrata*; aj: *Anguilla japonica.* Amino-acids differing from *A. anguilla* are highlighted in red. For GnIH-2 of *A. japonica*, the proline in position 3 was substituted by a serine in the cloned cDNA (BAV18007.1) but not in the genomic sequence (scaffold BEWY01082450 of the Ajp_01 assembly).

Peptide	Sequence	Length (aa)
aaGnIH-1	LYPPVAKPALLHAN**LPLRF**-NH_2	19
arGnIH-1	LYPPVAKPALLHAN**LPLRF**-NH_2	
ajGnIH-1	LYPPVAKPALLHAN**LPLRF**-NH_2	
aaGnIH-2	SS P RAATRMLQFPLS**LTRRF**-NH_2	20
arGnIH-2	SS P RAATRMLQFPLS**LTRRF**-NH_2	
ajGnIH-2	SS P/**S** RAA**AQ**MLQFPLS**LTRRF**-NH_2	
aaGnIH-3	SPETDSPIALPCHQCARMGGVASPSAT**LPQRF**-NH_2	32
arGnIH-3	SPETDSPIALPCHQCARMGGVASPSAT**LPQRF**-NH_2	
ajGnIH-3	SPETDSPIALPCHQCAR**I**GGVASPSAT**LPQRF**-NH_2	