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Additional Information

Molecular characterization of three GnRH receptor paralogs in the European eel, *Anguilla anguilla*: Tissue-distribution and changes in transcript abundance during artificially induced sexual development.

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Abstract

Gonadotropin-releasing hormone receptor (GnRH-R) activation stimulates synthesis and release of gonadotropins in the vertebrate pituitary and also mediates other processes both in the brain and in peripheral tissues. To better understand the differential function of multiple GnRH-R paralogs, three GnRH-R genes (*gnrhr1a, 1b, and 2*) were isolated and characterized in the European eel. All three *gnrhr* genes were expressed in the brain and pituitary of pre-pubertal eels, and also in several peripheral tissues, notably gills and kidneys. During hormonally induced sexual maturation, pituitary expression of *gnrhr1a* (female) and *gnrhr2* (male and female) was up-regulated in parallel with gonad development. In the brain, a clear regulation during maturation was seen only for *gnrhr2* in the midbrain, with highest levels recorded during early vitellogenesis. These data suggest that GnRH-R2 is the likely hypophysiotropic GnRH-R in male eel, while both GnRH-R1a and GnRH-R2 seems to play this role in female eels.

Keywords: gene expression, gonad maturation, brain, qPCR, screening, sequence

1.Introduction

Gonadotropin-releasing hormone (GnRH) plays a central role in the neuroendocrine control of the reproductive process in vertebrates, notably by stimulating synthesis and release of pituitary gonadotropins (Weltzien et al 2004). Teleost fish express two or three different forms (GnRH1-3), with only two forms having been detected in early evolved teleosts such as Elopomorphs: GnRH1 and GnRH2 (King et al., 1990; Okubo et al., 1999a). Similar to what has been shown in mammals (Chieffi et al., 1991; Hsueh and Schaeffer, 1985), extra-pituitary sites of GnRH actions have been detected in a number of reproductive and non-reproductive organs of different eel species such as the ovary, testis, brain, liver, and kidney (Dufour et al., 1993; Okubo et al., 1999a,b).

The actions of GnRH are mediated through binding to membrane receptors (GnRH-R) belonging to the rhodopsin family of G-protein coupled receptors. GnRH-Rs are highly conserved through evolution, as demonstrated by the cloning of a functional *gnrhr* from invertebrates, including the octopus (Kanda et al., 2006) and identification of functional receptors from the tunicate, also including receptor heterodimerization as an additional level of regulation (Kusakabe et al., 2003; Sakai et al., 2010; 2012; Tello et al., 2005). The presence of several forms of GnRH in a single species, acting in multiple tissues, is often associated with the existence of different receptor subtypes. Indeed, there is increasing evidence for the presence of at least two GnRH-R types, Type I and Type II, in most vertebrate classes (Hildahl et al 2011b; Kah et al., 2007; Millar et al., 2001; Okubo et al., 2001; Wang et al., 2001). The first *Gnrhr* was cloned from the mouse αT3 gonadotrope cell line (Reinhart et al., 1992; Tsutsumi et al., 1992) and the first teleost *gnrhr* was cloned from African catfish (*Clarias gariepinus*) pituitary (Tensen et al., 1997). Subsequently, many teleost GnRHRs have been identified (Hildahl et al 2011b; Kah et al., 2007; Levavi-Sivan and Avitan, 2005; Lethimonier et al., 2004; Weltzien et al., 2004). Some species have been shown to encode up to five *gnrhr*s in their genome, as is the case for each of two pufferfish (*Fugu ruripes* and *Tetraodon nigroviridis*; Ikemoto and Park, 2005) and the European seabass (*Dicentrarchus labrax*; Moncaut et al., 2005). The cell/tissue distribution, regulation and function of the various GnRH-Rs are still not clear. Characterization of the spatial and temporal GnRH-R variant gene expression is therefore critical

to improve our understanding of the physiological consequences of GnRH stimulation. To date, most studies on GnRH-R have focused on the brain and pituitary (Hapgood et al., 2005; Jodo et al., 2003; Millar et al., 2004; Moncaut et al., 2005; Okubo et al., 2001, 2003). For example, in tilapia (*Astatotilapia burtoni*; Flanagan et al., 2007) and European seabass (González-Martínez et al., 2004), the Type II GnRH-R2a was proposed as the GnRH receptor involved in the control of gonadotropic function. GnRH-Rs also have been identified in peripheral tissues. In human ovary, *Gnrhr* expression was found in luteal granulosa cells (Cheng et al., 2002), and some studies suggest that the GnRH system is involved in the control of follicle atresia in the ovary (Kang et al., 2003). *gnrhr* transcripts in kidney and gill have been reported in several teleost species (Ikemoto and Park, 2005; Lin et al., 2010; Robison et al., 2001; Tello et al., 2008) demonstrating the possible relation between GnRH and osmoregulation. In spotted green pufferfish (*T. nigroviridis*) two of the five isolated GnRH-R forms were not detected in the pituitary (Ikemoto and Park, 2005), while the four functional GnRH-Rs isolated from adult zebrafish showed different tissue distribution (Tello et al., 2008). These data suggest the different receptors to have different functional roles.

In vivo and *in vitro* experiments have shown that gonadal steroids participate in the regulation of GnRH-R transcripts. For instance, estradiol-17β (E2) showed a positive effect on *gnrhr* expression in pre-spawning black porgy (*Acanthopagrus schlegeli*; Lin et al., 2010) and vitellogenic female tilapia (Levavi-Sivan et al., 2006). In tilapia, a GnRH3 analog stimulated pituitary *gnrhr* transcripts *in vivo* and *in vitro* (Levavi-Sivan et al., 2004), whereas in grouper GnRH2 and GnRH3 inhibited while GnRH1 stimulated pituitary *gnrhr* transcript expression (Hsieh et al., 2007). However, more studies are needed to clarify the regulation and functional significance of the individual GnRH-R forms.

European eel is an important aquaculture species, and belonging to the early evolved Elopomorpha, knowledge of its GnRH systems may provide valuable information about the classification and function of GnRH-Rs in later evolving teleost species. The current work reports for the first time three *gnrhr* genes in an eel species and characterizes their brain and pituitary expression during artificial maturation, providing valuable information regarding the functional significance of each receptor form.

2. Materials and Methods

2.1 Fish maintenance, hormonal treatment and sampling

2.1.1 Maturation of male European eel

One hundred and fifty pre-pubertal male eels (124.1±12.6 g body weight; 39.9±0.21 cm body length) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were transported to the Aquaculture Laboratory at the Universitat Politècnica de València. For one week, the fish were gradually acclimatized to seawater (salinity 37±0.3 ‰). Simultaneously, the water temperature was changed from environmental conditions to 20 ºC. After one week, fish were distributed in equal numbers in three 500 L fibreglass tanks equipped with separate recirculation systems.

Males were hormonally treated for the induction of maturation and spermiation with weekly intraperitoneal injections of human chorionic gonadotropin (hCG) for 13 weeks (1.5 IU g/fish; Angelini Farma-Lepori, Barcelona, Spain), as previously described by Pérez et al. (2000). From the beginning of the experiment and once per week, 6 males were anaesthetised and sacrificed by decapitation to obtain pituitary and gonad samples that were stored in 0.5 ml of RNAlater (Ambion Inc, Huntingdon, UK) at -20 ºC until RNA extraction.

2.1.2 Maturation of female European eel

Forty-four wild pre-pubertal female eels at the silver stage (body weight 744.7±22.2 g; body length 72.2±0.65 cm) caught by local fishermen in the Albufera Lagoon during their migration to the sea were transported to the Aquaculture Laboratory at the Universitat Politècnica de València. Fish were acclimatised for two weeks from fresh water to sea water (salinity 37.0±0.3 ‰) after which water temperature was progressively changed from environmental temperature to 18 ºC, a temperature that was maintained during the rest of the experiment. The fish were distributed in equal numbers in two 500 L fibreglass tanks equipped with separate recirculation systems.

Females were treated with weekly intraperitoneal injections of carp pituitary extract (CPE, Catvis Ltd, Netherlands) for 12 weeks (20 mg/kg body weight). The CPE was diluted in 0.9 g/l NaCl solution (0.9 g/l), centrifuged (1260 g, 10 min) and the supernatant stored at -20 ºC until use one to four weeks later.

Groups of 6 female eels were anaesthetised and sacrificed by decapitation at 0, 4, 8 and 12 weeks of treatment, in addition to six animals newly obtained from the Albufera Lagoon (fresh water conditions). These animals served to observe a possible seawater acclimation effect. Upon sampling, the brain, pituitary and gonad were removed and stored in 0.5 ml of RNAlater (Ambion) at -20 ºC until extraction. The brain was divided into three parts: olfactory bulb, telencephalon and di-and mesencephalon as previously reported by Weltzien et al. (2005).

2.2 Histology processing

For both experiments, after fixation in 10% formalin buffered at pH 7.4, ovaries and testes were dehydrated in ethanol and embedded in paraffin for histological staging. Sections of 5 µm thickness were made with a Shandom Hypercut manual microtome and stained with haematoxylin and eosin. Slides were observed with a Nikon Eclipse E400 microscope, and pictures were taken with a Nikon DS-5M camera attached to the microscope.

Stages of spermatogenesis were determined according to the most advanced germ cell type present and to their relative abundance (Huertas et al., 2006; Peñaranda et al., 2010; Utoh et al., 2004; Fig. 4.1). Stage 1 was characterized by the presence of spermatogonia type A and/or B, stage 2 by the presence of spermatogonia and spermatocytes, at stage 3 spermatids appeared in the testis tissue, and at stages 4, 5 and 6 spermatozoa appeared inside the lumen of lobules. The difference between stages 4-6 was the relative abundance of sperm in the lobule lumen or the different proportions between the volume of lumen filled with sperm and the volume of the whole lobule. Stage 4 was characterized by small lumens, which comprised 10-45% of the whole lobules. Indeed, in some testes parts of the lobules were filled with spermatozoa, while other lobules contained only spermatids. Stage 5 was characterized by an increase in the quantity and proportion of spermatozoa in the gonad, comprising 50-70% of the total volume of the lobule. The lobule wall showed all the germinal cell types: spermatogonia, spermatocytes and spermatids, which did not appear inside the lumen. Stage 6 was characterized by a reduction in the germinal cells other than the spermatozoa with a proportion between spermatozoa and whole lobules between 75-95%. In this stage elongated lumens were observed that were often fused in long wide spaces filled with spermatozoa.

The ovarian development observed during the 12 weeks of treatment was classified in 3 stages, according to previous works (Kayaba et al., 2001; Pérez et al., 2011; Selman and Wallace, 1989; Fig. 4.2): Previtellogenic (PV) - in perinucleolar stage with none or few lipid droplets or in the lipid droplet stage but without yolk vesicles.

Early vitellogenic (EV) - small yolk vesicles restricted to the periphery of the oocyte.

Vitellogenic (V) - including medium and late vitellogenesis. Medium vitellogenesis is characterized by abundant yolk vesicles in the cytoplasm from the membrane to the nucleus and a lower proportion of yolk vesicles than lipid droplets. Late vitellogenesis is described by enlarged yolk vesicles and higher proportion of lipid droplets than yolk vesicles.

2.3 Molecular cloning of eel *gnrhr* cDNAs

In the current work, the nomenclature for GnRH-Rs follows the classification performed by Hildahl et al. (2011b). Total RNA was isolated from RNAlater preserved tissues as described by Hildahl et al. (2011a). First-strand cDNA was synthesized from 2 µg of total RNA using random hexamer primers and superscript III reverse transcriptase (Invitrogen). Design and theoretical evaluation of PCR primers were performed using the Primer3 shareware (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3) and Vector NTI software (Invitrogen; Table 1), respectively. *aa-gnrhr1b* PCR primers for initial fragment cloning were designed from the corresponding Japanese eel sequence (GenBank accession number AB041327). *aa-gnrhr2* primers were selected from a partial sequence of the corresponding receptor from Japanese eel kindly provided by Dr. Kataaki Okubo (University of Tokyo). PCR amplification was performed in an ABI GeneAmpTM system 2700 thermo cycler. The reaction mixture of 25 µl contained 1x PCR buffer (Invitrogen), 200 µM dNTPs (Invitrogen), 0.1 IU of Taq DNA polymerase (Invitrogen), 500 nM of each primer and 1 µl of cDNA template. The first PCR amplification was run as follows: denaturation at 94 ºC for 3 min, followed by 40 cycles of 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s, and finally an extension step at 72 ºC for 10 min. PCR products were visualized in 2% agarose gel stained with ethidium bromide and bands of expected size were purified using Qiaquick Gel Extraction kit (Qiagen) and ligated into the pGEM-T easy vector (Promega, WI, USA). Cloning was performed in competent *E. coli* JM109 cells (Promega). Positive

colonies were isolated and plasmids extracted by Qiagen Plasmid Mini Kit (Qiagen). Plasmids with insert were sequenced using an ABI 3730 DNA analyzer (Applied Biosystems, University of Oslo sequencing service, Oslo, Norway).

The obtained partial sequences were used to design gene specific primers for use with SMART RACE PCR (Clontech, CA, USA) to identify the 5' and 3' end sequences. Following RACE PCR and gel electrophoresis, a single band was isolated, cloned and sequenced as described above. Full-length sequences were confirmed by comparing with the results recently obtained using the massive parallel sequencing of the eel genome (Henkel et al., 2012). This analysis revealed an additional third *gnrhr* gene, which was identified as the putative *aa-gnrhr1a* based on sequence alignment and phylogenetic analysis. A fragment of this gene was then cloned and sequenced as described above. The three aa-gnrhr cDNA sequences were submitted to GenBank and subsequently assigned the following accession numbers: JX567769.1 (*aa-gnrhr1a*), JX567770.1 (*aa-gnrhr1b*), JX567771.1 (*aa-gnrhr2*).

2.4 Phylogenetic analysis

The phylogenic analysis was carried out according to Hildahl et al. (2011b). Briefly, the deduced amino acid sequences from available teleost *gnrhr* genes in addition to at least one representative species from the other vertebrate classes – *Homo sapiens*, *Macaca mulatta, Macaca radiate, Mus musculus* (mammalia); *Gallus gallus* (aves); *Eublepharis macularius* (reptilia); *Rana ridibundus*, *Xenopus laevis* (amphibia) - were aligned, and PHYML analysis was carried out using the Seaview 4.2.12 molecular phylogeny software package (Gouy et al., 2010). The aligned sequences spanned the first transmembrane domain to the end of the seventh transmembrane domain and partial sequences were omitted to assure good alignment. The model of protein evolution was determined using ProTest v1.2.7 software (Abascal et al 2005; Drummond and Strimmer 2001; Guindon and Gascuel 2003). Octopus were used as an outgroup, and the robustness of the obtained tree was analyzed by bootstrap analysis using 500 data set replicates. The alignment file was also used to identify conserved residues and variant specific motifs.

2.5 Tissue-Specific Expression of the Eel *gnrhr*s

Three pre-pubertal silver male and three pre-pubertal silver female eels with average body weights of 118.0±6.47 and 710.7±52.61 g, respectively, were sacrificed. RNA was extracted using the methodology explained above from nine different tissues: gonad, liver, pectoral fin, anterior and posterior kidney, heart, gill, pituitary, and brain. The brain was divided into five parts: olfactory bulbs, telencephalon, di- and mesencephalon, cerebellum, and medulla oblongata as previously reported by Weltzien et al. (2005). Total RNA was treated with DNase I (Turbo DNA-free, Ambion) at 37 °C for 30 min. cDNA was prepared from 0.5 µg total RNA using superscript III reverse transcriptase (Invitrogen) and random hexamer primers according to standard protocol. The expression of *aa-gnrhr* transcripts in the various tissues was analyzed by qPCR.

2.6 Measurement of gene expression by real-time quantitative RT-PCR (qPCR)

2.6.1 Primer design

Acidic ribosomal phosphoprotein P0, ARP (Table 1; Aroua et al., 2007; Peñaranda et al., 2010; Weltzien et al., 2005) was used as reference gene in the quantitative real-time reverse trancriptase polymerase chain reaction (qPCR), because its mRNA expression has been shown to be stable also during experimental treatment (Weltzien et al., 2005). The Primer3 program (http:// frodo.wi.mit.edu/cgi-bin/primer3/primer3) was used to design specific primers to quantify the *aa-gnrhr* gene expression (Table 1). To avoid detection of genomic DNA (gDNA), primers were designed to span an exon-exon boundary or for one of the primers to anneal to an exon-exon boundary. All primers were tested on gDNA and RNA to confirm that they would not amplify potentially contaminating gDNA. The specificity was confirmed by melting curve analysis, gel electrophoresis, and by sequencing of the qPCR products. Furthermore, the possible cross-reactivity between *aa-gnrhr* assays was tested by qPCR using *aa-gnrhr1a*, *1b*, or *2* clones as template.

2.6.2 SYBR Green assay (qPCR)

In order to measure changes in gene expression of *aa-gnrhr*s, qPCR assays were developed and expression analyses performed using a Light Cycler 480 system with SYBR Green I sequence-unspecific detection (Roche, Meylan, France). After an initial activation of *Taq* polymerase at 95 ºC for 10 min, 42 cycles of PCR were performed using the Light Cycler with the following cycling conditions: 95 ºC for 10 s, 60 ºC for

10 s and 72 ºC for 7 s for *aa-gnrhr1a* and *aa-gnrhr1b*; and 95 ºC for 10 s, 61 ºC for 7 s and 72 ºC for 4 s for *aa-gnrhr2*. To evaluate assay specificity, melting curve analysis was performed directly following PCR by slowly (0.1 ºC/s) increasing the temperature from 68 to 95 ºC with a continuous registration of changes in fluorescent emission intensity.

The total volume for every PCR reaction was 10 µl using diluted (1:10) cDNA template (3 µl), forward and reverse primers (250 nM each) and SYBR Green Master Mix (5 µl). Relative standard curves were used to assess PCR efficiency for *aa-gnrhr*s and ARP and to decide which dilutions to use for the unknown samples (Table 1). The efficiency adjusted normalized starting concentration was determined by an efficiency adjusted delta-delta Cq method (Weltzien et al., 2005). The target gene expression quantification was expressed relative to reference gene expression (ARP; Weltzien et al., 2005). Target and reference genes in unknown samples were run in duplicate PCR reactions, and a cDNA pool from various samples was included in each run as a calibrator. Non-template controls (cDNA was replaced by water) for each primer pair were run on all plates.

2.7 Statistical analysis

The data were subjected to analysis of variance (One-way ANOVA). A Student-Newman-Keuls test was used for the comparison between means at a 0.05 significance level ($p<0.05$). The results are presented as means ± standard error of means (SEM). All statistical procedures were run using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).

3. Results

3.1 Cloning and identification

We have isolated three GnRH-R cDNAs in European eel, two Type IA and one Type IIB receptor. These were named according to the nomenclature presented by Hildahl et al (2011b), whereby the two Type IA receptors were given a putative 1a and 1b designation (*aa-gnrhr1a* and *aa-gnrhr1b*, GenBank accession numbers JX567769.1 and JX567770.1, respectively) based on distinct conserved motifs in the third transmembrane domain. The sub-type of the Type IIB gene (*aa-gnrhr2*, GenBank accession number

JX567771.1) could not be determined by conserved sequence or phylogenetic analysis. The full-length sequences were 1158 bp (*r1a*), 1086 bp (*r1b*) and 966 bp (*r2*). All three sequences contain conserved sequences with other vertebrate species (Hildahl et al 2011b), including Type-specific micro-domains in TM3 and a tri-peptide domain in the third extracellular region. The putative *aa-gnrhr1a* has AAFIL and DRHRAI micro-domains, whereas the putative *aa-gnrhr1b* has SAFIL and DRHHAI. These domains were identified as GAFVT and DRQSAI for *aa-gnrhr2*. In addition both Type IA variants possess the PEY tri-peptide and *aa-gnrhr2* has a SHS sequence similar to other teleost receptors (Fig 1).

3.2 Phylogenetic analysis

The phylogenetic tree presented in the current study (Fig 2) supports the division of GnRH-Rs into two Types, I and II, each of which have subforms A and B, giving a total of four clades; IA, IB, IIA and IIB, as presented in Hildahl (2011b). Teleost fish group into two main clades, IA and IIB, distinct from the mammalian Type IB and tetrapod Type IIA clades. The European eel receptors associated with teleost fish Type I and Type II clades, but sub-types could not be determined from this analysis. *aa-gnrhr1b* grouped with the previously identified Japanese eel *gnrhr*, but these receptors branched from the main teleost Type I clade, prior to the distinction of the *gnrhr1a* and *gnrhr1b* clades. Similarly, *aa-gnrhr2* formed a mono-phylogenetic clade with the teleost Type II *gnrhr*s, but it branched from the teleost sub-type clades prior to their branching. The putative *aa-gnrhr1a* associated with the teleost *gnrhr1a* clade, but the bootstrap analysis did not irrefutably support this distinction.

3.3 Tissue specific expression of eel *gnrhr* transcripts

qPCR analysis with *SYBR Green* was utilized to characterize the tissue-specific expression pattern of the eel *aa-gnrhr* transcripts in five parts of the dissected brain, in addition to the pituitary, gonad, liver, pectoral fin, anterior and posterior kidney, heart, and gill from pre-pubertal silver eel. Differential expression was found between the sexes (Fig. 3B and 3C). *aa-gnrhr1b* transcripts were detected in all tissues except in the male liver, fin and anterior/posterior kidney (Fig. 3B). In most of the tissues, females showed higher *aa-gnrhr1b* expression compared to males with the highest levels being observed in the olfactory bulbs, di- and mesencephalon, and cerebellum.

As for *aa-gnrhr1b*, differential expression between sexes was found for *aa-gnrhr2* with no transcripts detected in the female pectoral fin or male cerebellum (Fig. 3C). Gonads, olfactory bulbs, telencephalon, di- and mesencephalon, and medulla oblongata were the tissues that showed highest transcript levels in both sexes. *aa-gnrhr2* transcripts were observed neither in females nor in males in the following tissues: liver, anterior and posterior kidney, heart, and gill .

Unlike *aa-gnrhr1b* and *aa-gnrhr2*, *aa-gnrhr1a* did not show differences between sexes (Fig. 3A). The highest transcript levels were found in the gonads and kidney, while no *aa-gnrhr1a* transcripts were detected in the following tissues: liver, fin, heart and gill.

3.4 Gene expression through gonad development

3.4.1 Male gonad development

Changes in gonad development during hormonal treatment in males are shown in Fig. 4-1A to F, and Fig. 5A. Stage 1 was present only during the first 2 weeks of treatment, while stage 2 was observed in males from week 1 to 3. The percentage of males with stage 3 gonads was very low during week 3 and 4, probably because this is a short-time stage in the cell development process. For this reason, stages 3 and 4 were analyzed together (stage 3/4). In week 7, males with maximally developed gonads (stage 6) were observed for the first time, and thereafter during the rest of the experiment.

Gene expression in male pituitary was analyzed using 6 animals every two weeks of treatment. *aa-gnrhr1a* and *aa-gnrhr1b* gene expression was stable during gonad development, showing no significant differences neither during the weeks of treatment nor at the different stages of development (Fig. 6AD). *aa-gnrhr2* expression, on the other hand, was elevated compared to fresh water-sampled animals after only two weeks of treatment (Fig. 6E), maintaining this progressive increase along the experiment up to the 10th week of treatment before a non-significant decrease at week 12. When analyzing the data by the stages of gonad development instead of weeks of treatment, *aa-gnrhr2* provided similar results (Fig. 6F), displaying a progressive increase along spermatogenesis and becoming significantly different in the last stages of development (stage 5 and 6).

3.4.2 Female gonad development

The experiment was arrested at 12 weeks of treatment, before ovulation was reached, due to a lack of animals to continue the experiment. Therefore the gonad development was divided in three stages: pre-vitellogenic (PV), early vitellogenic (EV) and vitellogenic (V) stage (Fig. 4-2A to D). At 4 weeks of treatment, 83.3% of the females had reached early vitellogenesis (Fig. 5B). After 8 weeks of treatment, similar proportions of the three stages (33%) were observed, while at 12 weeks 83.3% of the animals had reached the vitellogenic stage.

aa-gnrhr1a expression in the olfactory bulbs decreased from fresh water to seawater samples, remaining low for the remainder of the experiment (Fig. 7A). Similar results were observed in pituitary samples (Fig. 7D). In telencephalon and di-and mesencephalon, no differences were found during the treatment (Fig. 7B and 7C). *aa-gnrhr1b* showed a significant increase at 12 weeks of treatment in the pituitary (Fig. 7H), whereas no differences were observed in the different parts of the brain at any week (Fig. 7E, 7F and 7G). *aa-gnrhr2* did not change in the olfactory bulbs (Fig. 7I), whereas a higher expression was observed at 4 weeks of treatment in the telencephalon and di-and mesencephalon (Fig. 7J and7K) and a significant increase at 12 weeks in the pituitary (Fig. 7L).

Similar profiles were observed when the gene expression was related to stages of ovarian development. *aa-gnrhr1a* expression levels in the pituitary and different parts of the brain did not change significantly during the different stages of development (Fig. 8A-D). At the vitellogenic stage, a significant increase was shown for *aa-gnrhr1b* in the pituitary (Fig. 8H), but no differences were found in the brain (Fig. 8E-G). Finally, *aa-gnrhr2* showed higher expression in the di- and mesencephalon at the beginning of vitellogenesis (Fig. 8K). Furthermore, its expression increased at the vitellogenic stage in the pituitary (8L). No differences were observed in olfactory bulbs and telencephalon for *aa-gnrhr2* transcripts (Fig. 8I and 8J).

4. Discussion

4.1 Phylogenetic analysis

The phylogenetic analysis clearly distinguishes the *aa-gnrhr*s as Type IA (*aa-gnrhr1a* and *aa-gnrhr1b*) and

Type IIB (*aa-gnrhr2*) receptors. The branching of the putative *aa-gnrhr1b* and *aa-gnrhr2* prior to the separation of the subtype specific clades reflects the eel's position as an early evolvedteleost, although distinct subtypes are expected in eel based on the proposed three rounds of whole genome duplication (WGD) in teleost fish (Kah et al., 2007; Meyer and Van de Peer, 2005;). Analysis of Hox gene clusters from the recent European eel genome sequencing supports the notion that the teleost specific WGD occurred before the branching of the Anguilliformes (Henkel et al., 2012). The putative distinction of the eel Type I *gnrhr*s based on conserved motifs further supports the branching of these sub-types following a third round of WGD, although lack of a clear phylogenetic distinction of these receptors reflects the low overall conservation of *gnrhr* genes. The teleost Type II *gnrhr*s are more highly conserved with no distinct functional motifs currently identified (Hildahl et al., 2011b), and at present the significance of subtype proliferation is poorly understood. The different teleost Type II *gnrhr* variants are differentially regulated in multiple species (Hildahl et al., 2011b; Ikemoto and Park, 2005; Moncaut et al., 2005) and *gnrhr2a* appears to be the most likely hypophysiotrophic receptor (Flanagan et al., 2007; Hildahl et al 2011b; Lin et al., 2010). Further information concerning the functional regulation of the Type II *gnrhr*s should help clarify their molecular evolution.

4.2 Tissue specific expression of *aa-gnrhr* transcripts

Differential expression profiles were observed for the three *aa-gnrhr* genes in the different sexes and tissues, similar to other teleosts (Robison et al., 2001; Yu et al., 1998). This emphasizes the complex regulation of the different receptor variants, making it difficult to speak about their function in general. The widespread tissue distribution also supports the involvement of GnRH in diverse physiological processes.

All *aa-gnrhr* transcripts were detected in all parts of the brain (except *aa-gnrhr2* in male cerebellum), and also in the pituitary in both sexes. This suggests that these receptors have a functional significance throughout the central nervous system, probably related to neuromodulation, in addition to processes like reproductive behavior (Yamamoto et al., 1997), but possibly other as yet unknown functions. The pituitary showed the lowest mean values for *aa-gnrhr1b* and *aa-gnrhr*2 (*aa-gnrhr1a* did not display significant differences between brain and pituitary). This is in contrast to the Japanese immature eel wherein the

highest expression level of *gnrhr1b* was observed in the pituitary (Okubo et al., 2000). Results similar to those obtained in the current work were found in adult Atlantic cod brain (*Gadus morhua*). *gm-gnrhr1b* was classified as receptor IA and *gm-gnrhr2a*, *gm-gnrhr2b* and *gm-gnrhr2c* as receptor IIB, and most of them (not *r2a*) showed higher expression in the brain compared to the pituitary (Hildahl et al., 2011b).

The expression of *gnrhr*s in gonads has been reported in other vertebrate species, including both fish (Lethimonier et al., 2004; Weltzien et al., 2004) and mammals (Hapgood et al., 2005; Ramakrishnappa et al., 2005). GnRH was found to induce apoptosis during the late stages of spermatogenesis in the testis of mature goldfish (*Carassius auratus*; Andreu-Vieyra et al., 2005). Supporting this observation, *gnrhr* expression was detected in ovarian and testicular tissue from recrudescent goldfish (Yu et al., 1998). In addition, testis from territorial dominant Burton´s mouthbreeder (*Haplochromis burtoni*; Robison et al., 2001) showed much higher *gnrhr* mRNA levels compared to ovarian tissue. The expression of *aa-gnrhr* in ovary and testis of European eel further suggests that these receptors are involved in regulating GnRH functions in the gonads, but it should be emphasized that the fish used for tissue distribution were all immature (silver stage).

Analysis of the tissue distribution of *aa-gnrhr* mRNA showed that the action of GnRH is not limited to the brain-pituitary-gonad axis (Levavi-Sivan and Avitan, 2005). *aa-gnrhr1a* and *aa-gnrhr1b* had a wider distribution among non-reproductive tissues than *aa-gnrhr2*, the latter which was more restricted to the central nervous system and gonadal tissue. Comparable results were detected in sexually mature European sea bass, with a broad distribution for *gnrhr*s belonging to receptor type IA, while the *gnrhr*s type IIB was more restricted to the central nervous system (Moncaut et al., 2005). The observed presence of *aa-gnrhr*s in gill and kidney in the current study is in line with *gnrhr* expression in gill and kidney reported in other teleost species (Ikemoto and Park, 2005; Lin et al., 2010; Robison et al., 2001; Tello et al., 2008), and indicates a possible function of GnRH in osmoregulation. .

4.3 Gene expression through gonad development

The increase in pituitary *aa-gnrhr2* expression during testis development suggests this as the primary Gnrhr mediating GnRH's reproductive function in male eels. This is in line with the increasing pituitary levels of

GnRH1 peptide, considered as the hypophysiotropic form in the European eel, following induced maturation (Dufour et al., 1993), but not with the corresponding brain *gnrh1* transcript levels that remained stable throughout testis development (Peñaranda et al., 2010).

Analysis of *gnrhr* gene expression in females provides further insight into the function of these receptors during oogenesis and ovulation, including their expression in pituitary and brain. In tilapia the receptor classified as receptor 1 (Type II *gnrhr2a*) was proposed to be the GnRH receptor involved in the control of gonadotropic function, while the receptor classified as receptor 2 (Type I *aa-gnrhr1b*) could play a role in modulating sensory inputs and body growth (Chen and Fernald, 2006; Levavi-Sivan et al., 2006 Parhar et al., 2002, 2005). Similarly, Type II *gnrhr2a* in pejerrey (*Odontesthes bonaerensis*) increased during gonad maturation in brain, pituitary and gonad, and variations were not observed for Type I *gnrhr1b* (Guilgur et al., 2009). Also, in the Atlantic cod, *gnrhr2a* is considered the main hypophysiotropic receptor (Hildahl et al 2011b; 2013). Similar results were obtained in the current work, with pituitary *aa-gnrhr2* transcript levels increasing with gonad development both in male and female eels.

aa-gnrhr1a and *1b* pituitary transcript levels remained stable during gonad development, except at the vitellogenic stage for *aa-gnrhr1b* in female pituitary. The increased levels of *aa-gnrhr1b* during vitellogenesis may be related to regulation of gonadotropins, but may also participate in regulating other pituitary hormones like growth hormone (Melamed et al., 1998; Onuma et al., 2005; Peter and Marchant, 1995; Yaron et al., 2003).

The expression of *aa-gnrhr1a* was not correlated with gonad development, but a significant decrease was observed from freshwater to seawater condition in female eels, again indicating that *aa-gnrhr1a* may be involved in osmoregulation. A positive effect of GnRH on prolactin release (hormone with osmoregulatory functions) was observed both *in vitro* (Tipsmark et al., 2005; Weber et al., 1997) and *in vivo* (Seale et al., 2002) in adult tilapia (Manzon, 2002). Freshwater control samples were not collected from male eels, as a consequence we cannot say whether the observed decrease in *aa-gnrhr1a* expression is common to both sexes, but a potential role for GnRH in eel freshwater osmoregulation will be interesting to investigate further in the future.

Pituitary *aa-gnrhr2* gene expression increased during gonadal development in both sex, suggesting a possible role during gonad maturation. In European silver eel *in vivo* experiments, E2 induced an increase in pituitary *lhb* expression (Schmitz et al., 2005), but this effect was not observed *in vitro* in pituitary cell culture from the silver eel stage (Aroua et al., 2007). These results suggest that E2 action *in vivo* is indirect, possibly via its stimulatory effect on Gnrh1 (Aroua et al., 2009). Higher E2 plasma levels were observed at the EV stage compared to PV stage in female eels reared at 20 ºC (Pérez et al., 2011), which could be responsible for the *aa-gnrhr2* increase observed at this stage in the di-mesencephalon, where Gnrh1-positive neurons are located in the eel (Montero et al., 1994). Supporting this hypothesis, Weltzien et al. (2006) reported that in prepubertal silver female eels, E2 treatment resulted in decreased gene expression of tyrosine hydroxylase (*th*) specifically in di- and mesencephalon. *th* expression reflects central dopaminergic activity (Goldstein et al., 1992), and dopamine is considered responsible for the inhibitory control of gonadotrope activity in many teleost species including the eel (Dufour et al., 2005; Aroua et al., 2009; Peter et al., 1986).

In conclusion, three *aa-gnrhr* mRNAs have been isolated and characterized from European eel. Phylogenetic and sequence analysis revealed two Type IA *gnrhr*s (putative *aa-gnrhr1a* and *aa-gnrhr1b*) and one Type IIB *gnrhr* (*aa-gnrhr2*). This is the first report of two Type I GnRH receptor subtypes in eels, supporting the notion that the teleost specific WGD occurred prior to the branching of the Elopomorpha. *aa-gnrhr2* is the most likely candidate to mediate gonadotropic function of GnRH in male European eel, while both the putative *aa-gnrhr1b* and *aa-gnrhr2* seem to play this role in female eel oogenesis.

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Yu, K.L., He, M.L., Chik, C.C., Lin, X.W., Chang, J.P., Peter, R.E., 1998. mRNA expression of gonadotropin-releasing hormones (GnRHs) and GnRH receptor in goldfish. Gen. Comp. Endocrinol. 112, 303-311.

Table 1. Primer sequence for RT-PCR to identify gene fragments, 5' and 3' RACE PCR and qPCR.

Name	Sequence (5'-3')	Orientation	Usage
aa-gnrhr1a	TGG TCA TGA GTT GCT GCT ACA	Forward	qPCR[1] (82 bp)
	AGA CAC CCC TCT CCG TCT TT	Reverse	qPCR[2] (E=1.862)
aa-gnrhr1b	AGA CGG AGG AAT TGG AGG AGG A	Reverse	RACE 5' PCR
	AAA CCA GCA GCA TGC ACA CCT TCA G	Forward	RACE 3' PCR
	TCG TCA CGC TCT ACG TTG TC	Forward	qPCR[1] (107 bp)
	AGG CAG GAC TCT CCA CCT TT	Reverse	qPCR[2] (E=2.037)
aa-gnrhr2	GCG CTT GCG CAG GTG GAT GGT GAA C	Reverse	RACE 5' PCR
	GGA AGA ACC AGT ACC ACA GGC CCA GCA GGT AG	Reverse	Nested RACE 5' PCR
	CTC CAA ACG CAT GAC CAA AGG GAA GGC AT	Forward	RACE 3' PCR
	CTT CCA AGG AAG TGC ATC TCC GC	Forward	Nested RACE 3' PCR
	TCA CCT TCT CCT GCC TCT TC	Forward	qPCR[1] (105 bp)
	TTG GAA GAT GCC TTC CCT TT	Reverse	qPCR[2] (E=1.876)
aa-arp	GTG CCA GCT CAG AAC ACG	Forward	qPCR[1] (107 bp)
	ACA TCG CTC AAG ACT TCA ATG G	Reverse	qPCR[2] (E=2.142)

[1] qPCR amplicon length is given in parenthesis following the forward qPCR primers.
[2] qPCR efficiency for each primer pair is given in parenthesis following the reverse qPCR primer

Figure Captions

Figure 1. Conserved sequences among vertebrate GnRH-R variants. Alignment of representative fish (dl, tn, aa) Type I and Type II GnRH-R variants, non-mammalian tetrapod Type I (rr) and Type II (rr) GnRH-R and human Type I GnRH-R, spanning transmembrane regions 1 – 7. Transmembrane regions are boxed in grey. Additional conserved amino acid residues involved in control of GnRH-R expression and ligand binding (green and yellow), micro-domains in transmembrane region 2 (pink), and a tri-peptide sequence in extracellular domain 3 (blue) are identified. Percent conservation between amino acid sequences is shown at the bottom of the alignment in pink. Receptor variant names as given in GenBank are given following the species name and the Type distinctions according to Hildahl et al 2011b are given in parenthesis.

Figure 2. Phylogenetic tree. Phylogenetic tree showing the relationship among vertebrate GnRH-R amino acid sequences spanning transmembrane domain 1 to transmembrane domain 7. The tree was generated by maximum likelihood analysis in Seaview 4 software using an evolutionary replacement model generated by ProtTest. Bootstrap analysis was performed on 500 trees and the values greater than or equal to 50% are provided on the branch nodes. The three European eel GnRH-R variants (accession numbers: JX567769.1, JX567770.1, JX567771.1) are organized in two separate clades with non-mammalian Type IA and Teleost Type IIB receptors. Note the receptor subtypes could not be determined from this analysis and were further determined by conserved sequence analysis. The sequences were as follows with the NCBI accession number and given variant indicator where available in parenthesis (ni = not indicated): A. burtoni (ni, AAK29745; I, AAU89433); A. japonica (ni, BAB11961); A. schlegelii (I, AAV71128); C. auratus.(A, AAD20001; B, AAD20002); C. gariepinus (ni, CAA66128; 2, AAM95605); C. lalia (2–1, BAE87050; 1–2, BAE87049; 1–1, BAE87048); D. labrax (1a, CAE54804; 1b, CAE54806; 2a,CAD11992; 2b partial sequence, CAE54807; 2c, CAE54805); D. rerio (1, NP001138452; 2, NP001138451; 4, NP001091663; 3, NP001170921); E. coioides (I, ABF93210); E. macularius (2, BAD11150); F heteroclitus (A, BAG12379; B, AB471799); G. aculeatus (clone CH213-16009); G. gallus (ni, NP001012627;2, NP989984); G. morhua (1b, ADD92008; 2a, ADD92009; 2b, ADD92010; 2c, ADD92011); H. sapiens (1, NP000397); K

marmoratus (ni, ABK88381); M. musculus (ni, NP034453); M. mulatta (II, AAK52745); M. radiate (I, AAK52745); M. saxatilis (ni, AAF28464); M. undulatus (2a, ABB97085); O. bonariensis (1b, ABI75337; 2a, ABI75336); O. latipes (1, BAB70504; 3, BAC97833; 2, BAB70503); O. aureusniloticus (I, AAQ88391; III, AAQ88392); O. mykiss (1, CAB93351); O. niloticus (I, BAC77240; II, BAC77241); O. vulgaris (ni, BAE66647); P. olivaceus (ni, AAY28982); R. ridibundus (1, AAP15162; 2, AAP15163; 3, AAP15164); S. dumerili (ni, CAB65407); T. nigroviridis (2–1, BAE45700; 2–2, BAE45702; 1–1, BAE45694; 1–2, BAE45696; 1–3, BAE45698); X. laevis (I, NP001079176; II, AAK49334). Species Types are identified as defined in described in Hildahl et al (2011b).

Figure 3. Quantitative gene expression of three GnRH receptors variants in various tissues from immature eel, males (gray) and females (dark gray). N = 3 for all tissues. Tissues in which genes were not detected are labeled nd (not detectable). Tissue abbreviations are G = gonad, L = liver, F= fin, AK= anterior kidney, PK= posterior kidney, H= heart, Gi= gill, OB= olfactory bulb, T= telencephalon, DM= di-mesencephalon, C= cerebellum, M= medulla oblongata, and P= pituitary.

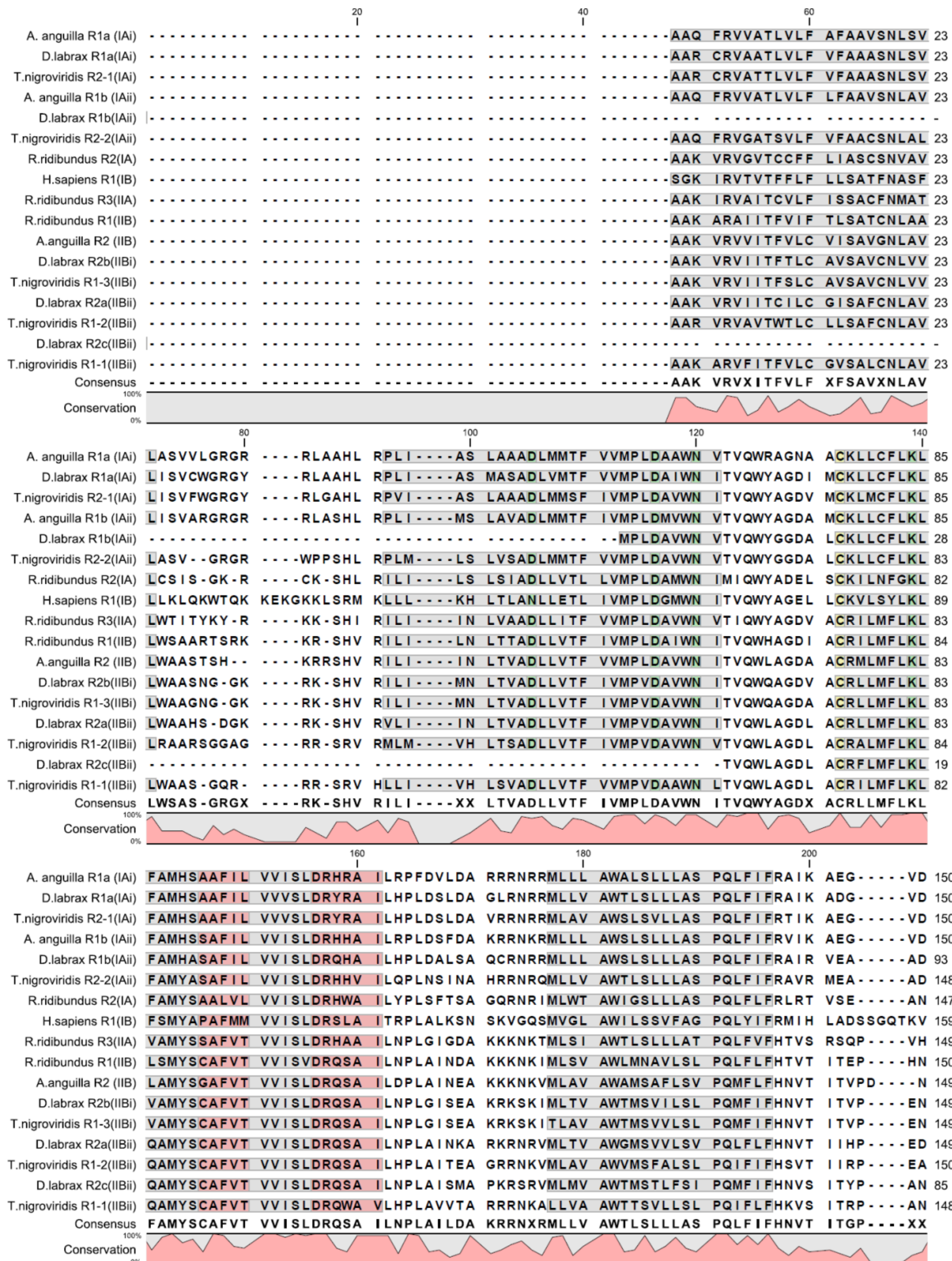
Figure 4. Photomicrographs of histological sections for the different stages found during treatment. 1) Sections for the different males stages found during treatment: 1.A) Testis at stage 1. 1.B) Testis at stage 2. 1.C) Testis at stage 3. 1.D) Testis at stage 4. 1.E) Testis at stage 5. 1.F) Testis at stage 6. SPG, spermatogonia; SPC, spermatocyte; SPD, spermatid; SPZ, spermatozoa. 2) Sections of oocytes at different times during hormonal treatment. 2.A) Previtellogenic stage. 2.B) Early vitellogenic stage 2C and 2D) Mid and Late Vitellogenic stage. Scale bar, 100 μm

Figure 5. Percentage of the different stages of gonad development during the weeks of treatment. A) Percentage of stages (1-6) gonad during the treatment in male eels. B) Percentage of the different stages of ovarian development at 0, 4, 8 and 12 weeks of CPE treatment, and fresh water conditions (c). Previtellogenic stage (black); early vitellogenic stage (gray); vitellogenic stage (dark gray).

Figure 6. Effect of treatment on *aa-gnrhr* expression in eel male pituitary. *aa-gnrhr1a* (black), *aa-gnrhr1b* (gray) and *aa-gnrhr2* (dark grey) expression at different weeks of treatment (A,C,E) and in relation to the stage of testis development (B,D,F). Different superscript letters means significant differences ($p<0.05$), n = 5-6.

Figure 7. Effect of treatment on *aa-gnrhr* expression at 0, 4, 8 and 12 weeks of CPE treatment, and fresh water conditions (c) in female eel brains and pituitaries. *aa-gnrhr1a* (gray), *aa-gnrhr1b* (dark grey) and *aa-gnrhr2* (black) expression at different weeks of treatment in olfactory bulb (A,E,I), telencephalon (B,F,J), di-mesencephalon (C,G,K), and pituitary (D,H,L). Different superscript letters means significant differences ($p<0.05$), n = 5-6.

Figure 8. Effect of treatment on *aa-gnrhr* expression in relation with the stage of ovarian development in eel female brain: previtellogenic stage (PV); early vitellogenic stage (EV); vitellogenic stage (V). *aa-gnrhr1a* (gray), *aa-gnrhr1b* (dark grey) and *aa-gnrhr2* (black) expression at different stages in olfactory bulb (A,E,I), telencephalon (B,F,J), di-mesencephalon (C,G,K) and pituitary (D,H,L). Different superscript letters means significant differences ($p<0.05$), n = 5-6.


20
40
60
A. anguilla R1a (IAi) ------AAQ FRVVATLVLF AFAAVSNLSV 23
D.labrax R1a(IAi) ------AAR CRVAATLVLF VFAAASNLSV 23
T.nigroviridis R2-1(IAi) ------AAR CRVATTLVLF VFAAASNLSV 23
A. anguilla R1b (IAii) ------AAQ FRVVATLVLF LFAAVSNLAV 23
D.labrax R1b(IAii) - -
T.nigroviridis R2-2(IAii) ------AAQ FRVGATSVLF VFAACSNLAL 23
R.ridibundus R2(IA) ------AAK VRVGVTCCFF LIASCSNVAV 23
H.sapiens R1(IB) ------SGK IRVTVTFFLF LLSATFNASF 23
R.ridibundus R3(IIA) ------AAK IRVAITCVLF ISSACFNMAT 23
R.ridibundus R1(IIB) ------AAK ARAIITFVIF TLSATCNLAA 23
A.anguilla R2 (IIB) ------AAK VRVVITFVLC VISAVGNLAV 23
D.labrax R2b(IIBi) ------AAK VRVIITFTLC AVSAVCNLVV 23
T.nigroviridis R1-3(IIBi) ------AAK VRVIITFSLC AVSAVCNLVV 23
D.labrax R2a(IIBii) ------AAK VRVIITCILC GISAFCNLAV 23
T.nigroviridis R1-2(IIBii) ------AAR VRVAVTWTLC LLSAFCNLAV 23
D.labrax R2c(IIBii) - -
T.nigroviridis R1-1(IIBii) ------AAK ARVFITFVLC GVSALCNLAV 23
Consensus ------AAK VRVXITFVLF XFSAVXNLAV
Conservation
100%
0%
80
100
120
140
A. anguilla R1a (IAi) LASVVLGRGR ----RLAAHL RPLI----AS LAAADLMMTF VVMPLDAAWN VTVQWRAGNA ACKLLCFLKL 85
D.labrax R1a(IAi) LISVCWGRGY ----RLAAHL RPLI----AS MASADLVMTF VVMPLDAIWN ITVQWYAGDI MCKLLCFLKL 85
T.nigroviridis R2-1(IAi) LISVFWGRGY ----RLGAHL RPVI----AS LAAADLMMSF IVMPLDAVWN ITVQWYAGDV MCKLMCFLKL 85
A. anguilla R1b (IAii) LISVARGRGR ----RLASHL RPLI----MS LAVADLMMTF IVMPLDMVWN VTVQWYAGDA MCKLLCFLKL 85
D.labrax R1b(IAii) --MPLDAVWN VTVQWYGGDA LCKLLCFLKL 28
T.nigroviridis R2-2(IAii) LASV--GRGR ----WPPSHL RPLM----LS LVSADLMMTF VVMPLDAVWN VTVQWYGGDA LCKLLCFLKL 83
R.ridibundus R2(IA) LCSIS-GK-R ----CK-SHL RILI----LS LSIADLLVTL LVMPLDAMWN IMIQWYADEL SCKILNFGKL 82
H.sapiens R1(IB) LLKLQKWTQK KEKGKKLSRM KLLL----KH LTLANLLETL IVMPLDGMWN ITVQWYAGEL LCKVLSYLKL 89
R.ridibundus R3(IIA) LWTITYKY-R ----KK-SHI RILI----IN LVAADLLITF VVMPLDAVWN VTIQWYAGDV ACRILMFLKL 83
R.ridibundus R1(IIB) LWSAARTSRK ----KR-SHV RILI----LN LTTADLLVTF IVMPLDAIWN ITVQWHAGDI ACRILMFLKL 84
A.anguilla R2 (IIB) LWAASTSH-- ----KRRSHV RILI----IN LTVADLLVTF VVMPLDAVWN ITVQWLAGDA ACRMLMFLKL 83
D.labrax R2b(IIBi) LWAASNG-GK ----RK-SHV RILI----MN LTVADLLVTF IVMPVDAVWN ITVQWQAGDV ACRLLMFLKL 83
T.nigroviridis R1-3(IIBi) LWAAGNG-GK ----RK-SHV RILI----MN LTVADLLVTF IVMPVDAVWN ITVQWQAGDA ACRLLMFLKL 83
D.labrax R2a(IIBii) LWAAHS-DGK ----RK-SHV RVLI----IN LTVADLLVTF IVMPVDAVWN ITVQWLAGDL ACRLLMFLKL 83
T.nigroviridis R1-2(IIBii) LRAARSGGAG ----RR-SRV RMLM----VH LTSADLLVTF IVMPVDAVWN VTVQWLAGDL ACRALMFLKL 84
D.labrax R2c(IIBii) -TVQWLAGDL ACRFLMFLKL 19
T.nigroviridis R1-1(IIBii) LWAAS-GQR- ----RR-SRV HLLI----VH LSVADLLVTF VVMPVDAAWN LTVQWLAGDL ACRILMFLKL 82
Consensus LWSAS-GRGX ----RK-SHV RILI----XX LTVADLLVTF IVMPLDAVWN ITVQWYAGDX ACRLLMFLKL
Conservation
100%
0%
160
180
200
A. anguilla R1a (IAi) FAMHSAAFIL VVISLDRHRA ILRPFDVLDA RRRNRRMLLL AWALSLLLAS PQLFIFRAIK AEG-----VD 150
D.labrax R1a(IAi) FAMHSAAFIL VVVSLDRYRA ILHPLDSLDA GLRNRRMLLV AWTLSLLLAS PQLFIFRAIK ADG-----VD 150
T.nigroviridis R2-1(IAi) FAMHSAAFIL VVVSLDRYRA ILHPLDSLDA VRRNRRMLAV AWSLSVLLAS PQLFIFRTIK AEG-----VD 150
A. anguilla R1b (IAii) FAMHSSAFIL VVISLDRHHA ILRPLDSFDA KRRNKRMLLL AWSLSLLLAS PQLFIFRVIK AEG-----VD 150
D.labrax R1b(IAii) FAMHASAFIL VVISLDRQHA ILHPLDALSA QCRNRRMLLL AWSLSLLLAS PQLFIFRAIR VEA-----AD 93
T.nigroviridis R2-2(IAii) FAMYASAFIL VVISLDRHHV ILQPLNSINA HRRNRQMLLV AWTLSLLLAS PQLFIFRAVR MEA-----AD 148
R.ridibundus R2(IA) FAMYSAALVL VVISLDRHWA ILYPLSFTSA GQRNRIMLWT AWIGSLLLAS PQLFLFRLRT VSE-----AN 147
H.sapiens R1(IB) FSMYAPAFMM VVISLDRSLA ITRPLALKSN SKVGQSMVGL AWILSSVFAG PQLYIFRMIH LADSSGQTKV 159
R.ridibundus R3(IIA) VAMYSSAFVT VVISLDRHAA ILNPLGIGDA KKKNKTMLSI AWTLSLLLAT PQLFVFHTVS RSQP----VH 149
R.ridibundus R1(IIB) LSMYSCAFVT VVISVDRQSA ILNPLAINDA KKKNKIMLSV AWLMNAVLSL PQLFLFHTVT ITEP----HN 150
A.anguilla R2 (IIB) LAMYSGAFVT VVISLDRQSA ILDPLAINEA KKKNKVMLAV AWAMSAFLSV PQMFLFHNVT ITVPD----N 149
D.labrax R2b(IIBi) VAMYSCAFVT VVISLDRQSA ILNPLGISEA KRKSKIMLTV AWTMSVILSL PQMFIFHNVT ITVP----EN 149
T.nigroviridis R1-3(IIBi) VAMYSCAFVT VVISLDRQSA ILNPLGISEA KRKSKITLAV AWTMSVVLSL PQMFIFHNVT ITVP----EN 149
D.labrax R2a(IIBii) QAMYSCAFVT VVISLDRQSA ILNPLAINKA RKRNRVMLTV AWGMSVVLSV PQLFLFHNVT IIHP----ED 149
T.nigroviridis R1-2(IIBii) QAMYSCAFVT VVISLDRQSA ILHPLAITEA GRRNKVMLAV AWVMSFALSL PQIFIFHSVT IIRP----EA 150
D.labrax R2c(IIBii) QAMYSCAFVT VVISLDRQSA ILNPLAISMA PKRSRVMLMV AWTMSTLFSI PQMFIFHNVS ITYP----AN 85
T.nigroviridis R1-1(IIBii) QAMYSCAFVT VVISLDRQWA VLHPLAVVTA RRRNKALLVA AWTTSVLLSL PQIFLFHKVS ITRP----AN 148
Consensus FAMYSCAFVT VVISLDRQSA ILNPLAILDA KRRNXRMLLV AWTLSLLLAS PQLFIFHNVT ITGP----XX
Conservation
100%
0%

```
                                   220                      240                      260                      280
A. anguilla R1a (IAi)          FTQCVTHGSF QKRWQETVYN MFHFVTLYVV PLLVMSCCYT RILMEISRQM RKSKDGEGC- ---------L 210
D.labrax R1a(IAi)              FTQCVTHGSF QYLWQETAYN MFHFVTLYVF PLLVMTFCYT RILAKIN--G QMHKTKD--G E------HCL 210
T.nigroviridis R2-1(IAi)       FTQCVTHGSF LHLWQETTYN MFHFVTLYIF PLLVMIFCYT RIFTKIN--G QIHENKD--N E------SCL 210
A. anguilla R1b (IAii)         FTQCVTHGSF QQRWQETVYN MFLFVTLYVV PLLVMSLCYT RILIEINHQI HKSKGGESC- ---------L 210
D.labrax R1b(IAii)             FTQCVTHGSF HHRWQETVYN MFHFTTLYVF PLLVMSCCYS RILLHIH--L QHLRDKA--G A------SYL 153
T.nigroviridis R2-2(IAii)      FTQCATHGSF SRRWQETLYN MFHFITLYVV PLLVMSCCYS RILLHIH--L QHLRDKA--G E------SYL 208
R.ridibundus R2(IA)            FTQCATHGSF PQRWQKTTYN MFTFCTLFVT PLSVMIVCYT RILWEIG--K QMNH-K---N E--------L 203
H.sapiens R1(IB)               FSQCVTHCSF SQWWHQAFYN FFTFSCLFII PLFIMLICNA KIIFTLT--R VLHQDP---H E-------LQ 217
R.ridibundus R3(IIA)           FVQCATLGSF KAHWLETLYN MFTFCCFFLL PLLIMVFCYG RILLEIS--R KMKKAEASSR E------VNL 211
R.ridibundus R1(IIB)           FTQCTTRGSF QQHWQETVYN MVSFVCLFLL PLLIMIFCYS RILLEIS--K RMSKGTLSSK E------VYL 212
A.anguilla R2 (IIB)            FTQCSTRGSF TEHWQETVYN MFTFSCLFLL PLAIMISCYT RILMEIS--K RMTKGKASSK E------VHL 211
D.labrax R2b(IIBi)             FTQCTTHGSF VQHWQETLYN MFTFVCLFLL PLVIMIFCYT RILIEIS--S RMARNNMLSK D------VHL 211
T.nigroviridis R1-3(IIBi)      FTQCTTHGSF VQHWQETLYN MFTFACLFLL PLIIMIFCYT RILVEIS--S RMARNNLLSR D------VHL 211
D.labrax R2a(IIBii)            FTQCTTRGSF VTHWHETAYN MFTFSCLFLL PLVIMITCYT RIFCEIS--K RMKKDNLPSN E------VHL 211
T.nigroviridis R1-2(IIBii)     FTQCTTRGSF PTRWHETAYN MFTFSCLFLL PLVVMVTCYT RILVEIS--K RV-ENNSSSN Q------VCL 211
D.labrax R2c(IIBii)            FTQCTTRGSF VTHWQETAYN MFTFSCLFLL PL-------- ---------- ---------- ---------- 117
T.nigroviridis R1-1(IIBii)     FTQCTTRGSF SSRWQETAYN LLTFACLFLL PLALMVTCYT RIFLHIS--R RLARRRPSSG E------PDL 210
Consensus                      FTQCTTHGSF XQXWQETXYN MFTFVCLFLL PLLVMIFCYT RILLEIS--K RMXKDKA-SX E------VHL
100%
Conservation
0%

                                              300                      320                      340
A. anguilla R1a (IAi)          RRSGTDTIPK ARMKTLKMTI IIVVSFVVCW TPYYLLGIWY WFHPQMLHVT --PEYVHHVF FVFGNLNTCC 278
D.labrax R1a(IAi)              RRSGTDMIPK ARMKTLKMTI VIVSSFVICW TPYYLLGIWY WFQPAMIKHT --PEYVHHIL FVFGNLNTCC 278
T.nigroviridis R2-1(IAi)       RRSGGDVIPK ARMKTLKMSV VIVSSFVICW TPYYLLGIWY WFHPAMIQHT --PEYVHHML FVFGNLNTCC 278
A. anguilla R1b (IAii)         RRSGTDMIPK ARMKTLKMTI IIVVSFVVCW TPYYLLGIWY WFHPQMIQVT --PEYVNHIL FVFGNLNTCC 278
D.labrax R1b(IAii)             RRSGTDIIPK ARMKTLKMTV VIVLSFVVCW TPYYLLGIWY WFQPDMLHVT --PEYVHHAV FVFGNLNTCC 221
T.nigroviridis R2-2(IAii)      RRSGTDIIPK ARLKTLRMTV VIVLSFVVCW TPYYLLGIWY WFQPDMLRVT --PEYVHHVL FVFGNLNTCC 276
R.ridibundus R2(IA)            ARSKNDLISK ARLKTLKMTL VIVISFMVCW TPYYLLGIWY WFQPEMIHLT --PEYVHHSL FLFGLLHTCT 271
H.sapiens R1(IB)               LNQSKNNIPR ARLKTLKMTV AFATSFTVCW TPYYVLGIWY WFDPEMLNRL --SDPVNHFF FLFAFLNPCF 285
R.ridibundus R3(IIA)           RRSY-NNIPR ARMRTFKMSL VIVLTFIVCW TPYYLLGIWY WFSPEMLTSR RVPPSLSHIP FLFGLFNTCL 280
R.ridibundus R1(IIB)           RCSK-NNIPK ARMRTLKVSI VIVSSFIICW TPYFFLGLWY WFYPEIMKEK -IPQSTTHIL FIFGLVNACL 280
A.anguilla R2 (IIB)            RRSK-NNIPK ARMRALKMSI VIVVSFIVCW TPYYLLGLWY WFFPEDLEET -VSHSLTHML FIFGLFNACL 279
D.labrax R2b(IIBi)             RRSH-NNIPK ARVRTLKMSI VIVTSFIICW TPYYLLGLWY WLFPEKMEET -VSHSLTHML FIFGLFNACL 279
T.nigroviridis R1-3(IIBi)      RRSH-NNIPK ARMRTLKMSI VIVTSFIVCW TPYYLLGLWY WLFPEKMEET -VSHSLTHML FIFGLFNACL 279
D.labrax R2a(IIBii)            RRSK-NNIPR ARMRTLKMGI VIVSSFIVCW TPYYLLGLWY WFFPDDLEGK -VSHSLTHIL FIFGLVNACL 279
T.nigroviridis R1-2(IIBii)     RRSR-NNIPR ARMRTLKMSI VIVASFIICW TPYYLLGLWY WFFPEDLEGK -VSHAFTHIL FIFGLLNACL 279
D.labrax R2c(IIBii)            ---------- ---------- ---------- ---------- ---------- ---------- ---------- 117
T.nigroviridis R1-1(IIBii)     RRSR-DNIPR ARRRTLKMSI VIVLCFVLCW GPYYLLGLWY WFFPEHLEGK -VSHSLTHIL FIFGLFNTCA 278
Consensus                      RRSG-XNIPK ARMXTLKMSI VIVXSFXVCW TPYYLLGIWY WFFPEMLEXT --PEXVTHIL FIFGLLNTCL
100%
Conservation
0%

                                   360                      380                      400                      420
A. anguilla R1a (IAi)          DPIIYGLYTP SFR------- ---------- ---------- ---------- ---------- ---------- 291
D.labrax R1a(IAi)              DPVIYGFYTP SFR------- ---------- ---------- ---------- ---------- ---------- 291
T.nigroviridis R2-1(IAi)       DPIIYSFYTP SFR------- ---------- ---------- ---------- ---------- ---------- 291
A. anguilla R1b (IAii)         DPVIYGLYTP SFR------- ---------- ---------- ---------- ---------- ---------- 291
D.labrax R1b(IAii)             DPVIYGFYTP SFR------- ---------- ---------- ---------- ---------- ---------- 234
T.nigroviridis R2-2(IAii)      DPLVYGFYTP SFR------- ---------- ---------- ---------- ---------- ---------- 289
R.ridibundus R2(IA)            DPLVYGLYTP SFK------- ---------- ---------- ---------- ---------- ---------- 284
H.sapiens R1(IB)               DPLIYG-YF- SL-------- ---------- ---------- ---------- ---------- ---------- 295
R.ridibundus R3(IIA)           DPIIYGLFTI HFR------- ---------- ---------- ---------- ---------- ---------- 293
R.ridibundus R1(IIB)           DPITYGLFTI HFR------- ---------- ---------- ---------- ---------- ---------- 293
A.anguilla R2 (IIB)            DPITYGLFTI HLR------- ---------- ---------- ---------- ---------- ---------- 292
D.labrax R2b(IIBi)             DPITYGLFTI HLH------- ---------- ---------- ---------- ---------- ---------- 292
T.nigroviridis R1-3(IIBi)      DPITYGLFTV RLH------- ---------- ---------- ---------- ---------- ---------- 292
D.labrax R2a(IIBii)            DPVIYGLFTI HFR------- ---------- ---------- ---------- ---------- ---------- 292
T.nigroviridis R1-2(IIBii)     DPLIYGLFTI RFR------- ---------- ---------- ---------- ---------- ---------- 292
D.labrax R2c(IIBii)            ---------- ---------- ---------- ---------- ---------- ---------- ---------- 117
T.nigroviridis R1-1(IIBii)     DPLVYGLFTV RFR------- ---------- ---------- ---------- ---------- ---------- 291
Consensus                      DPIIYGLXTP SFR------- ---------- ---------- ---------- ---------- ----------
100%
Conservation
0%
```

Figure 1

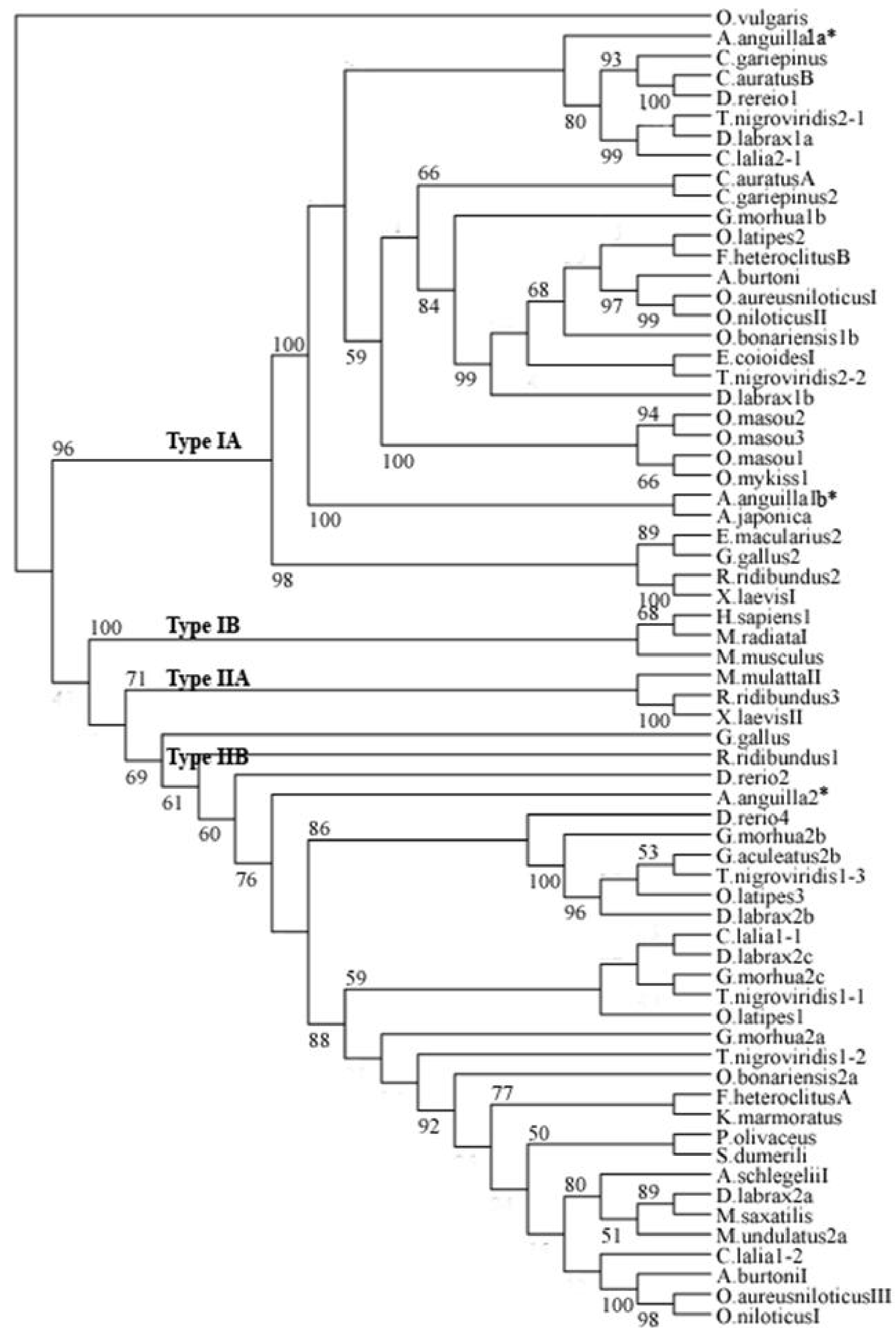


Figure 2

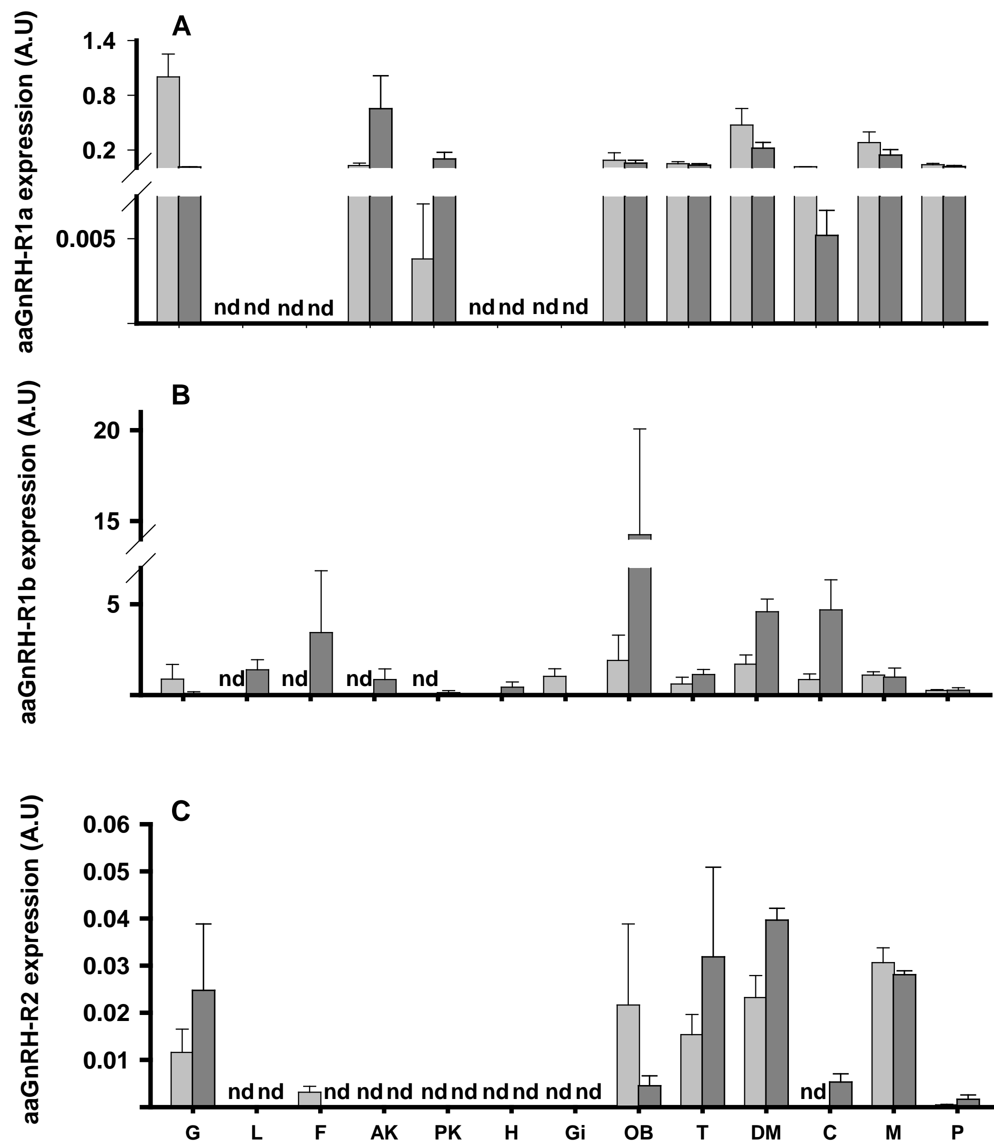


Figure 3

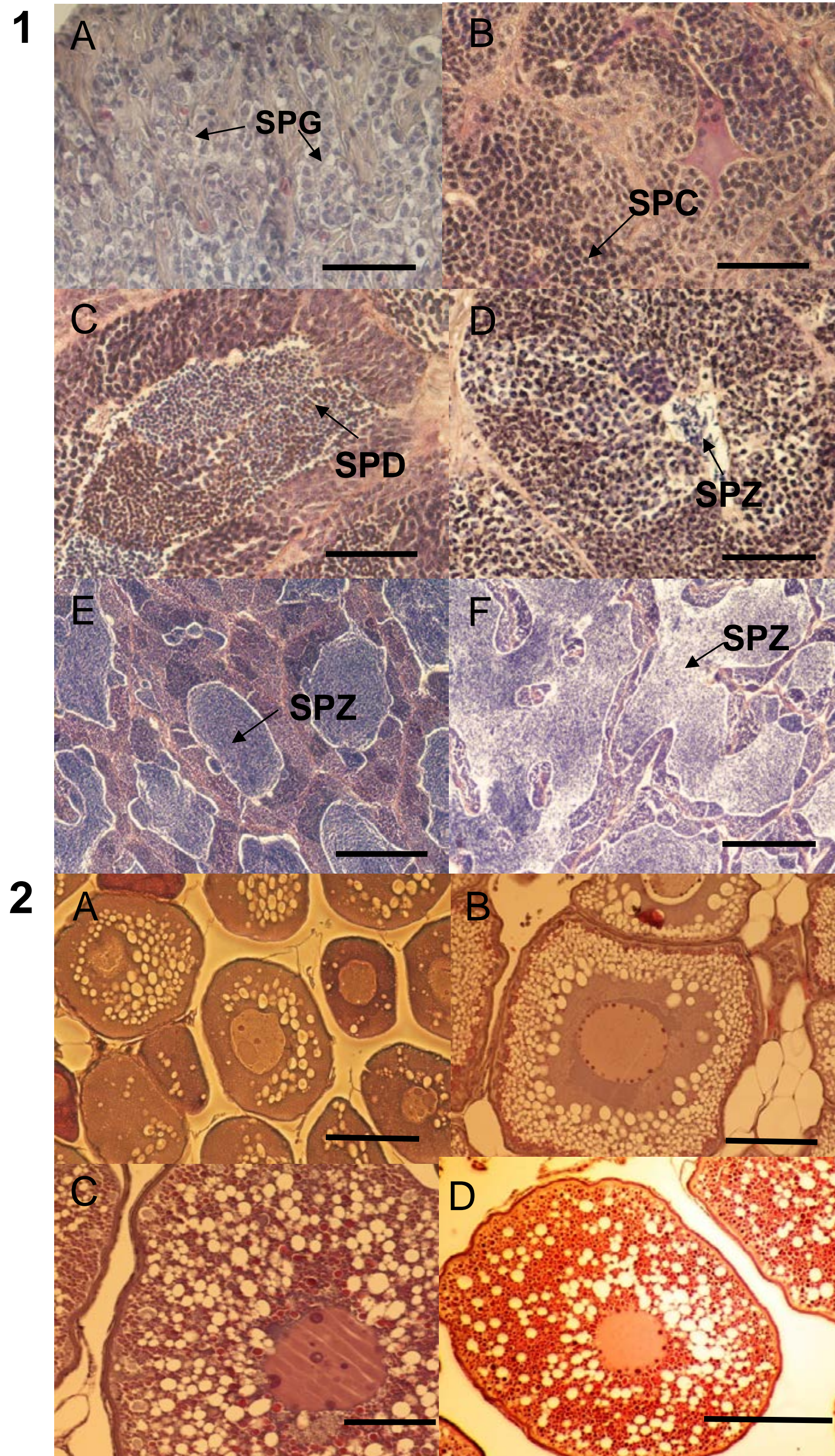


Figure 4

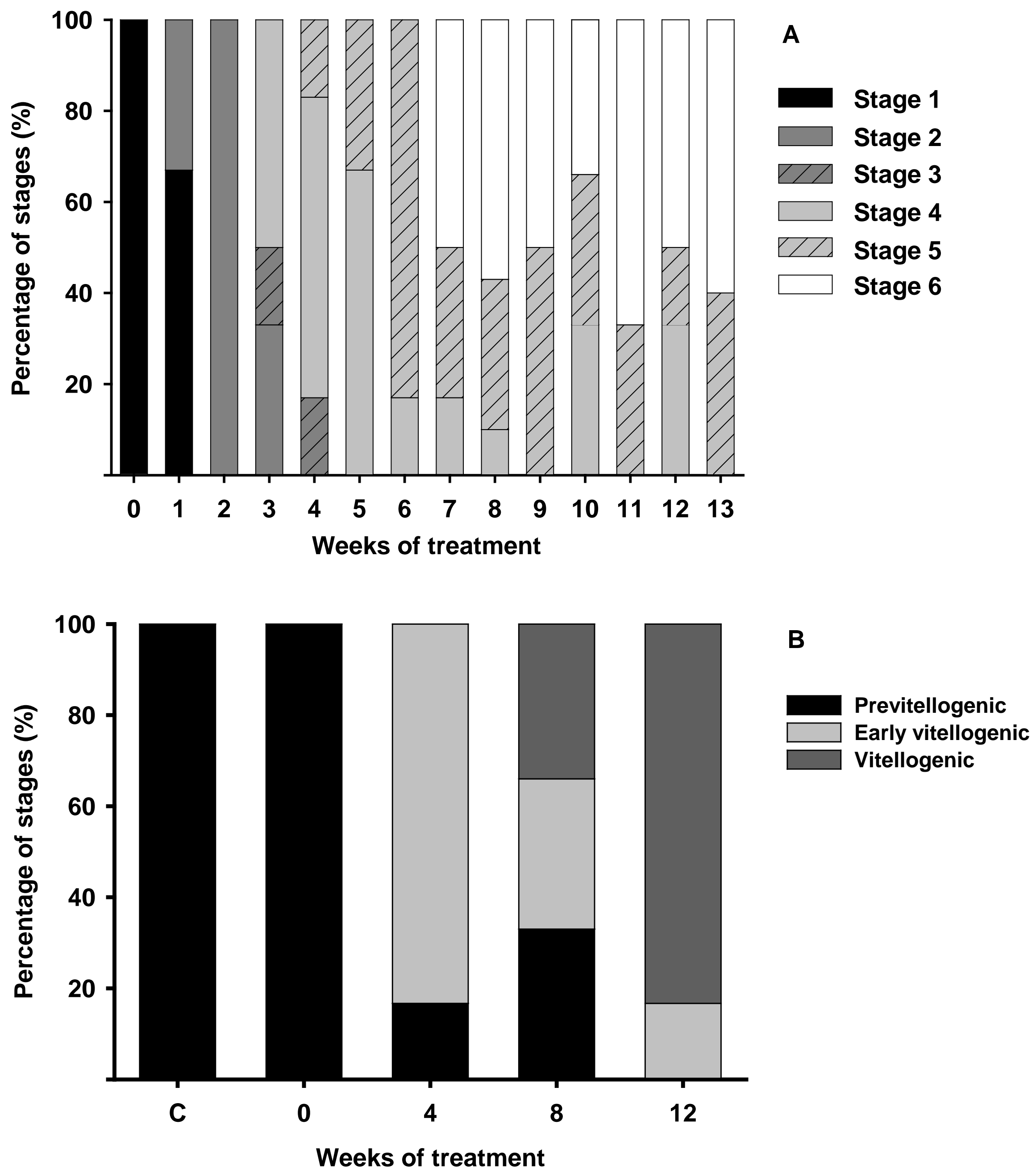


Figure 5

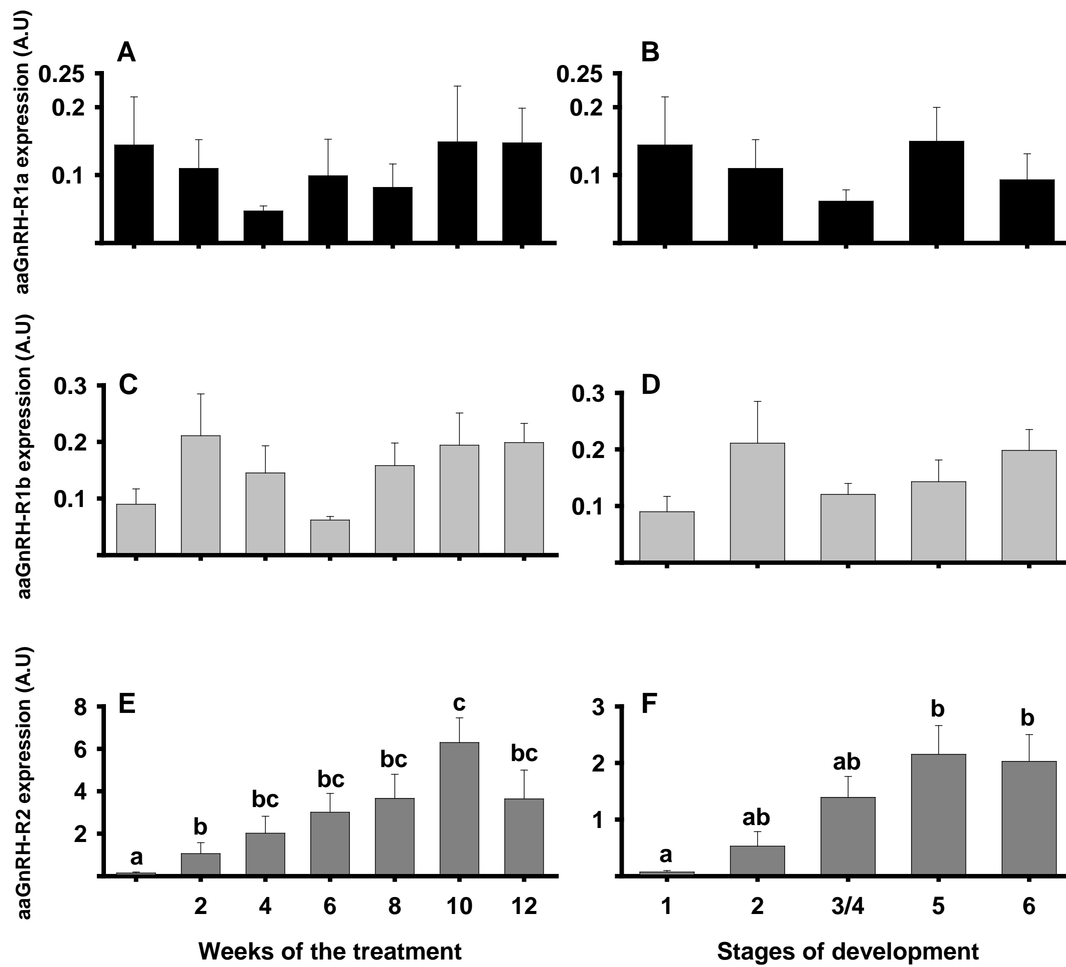


Figure 6

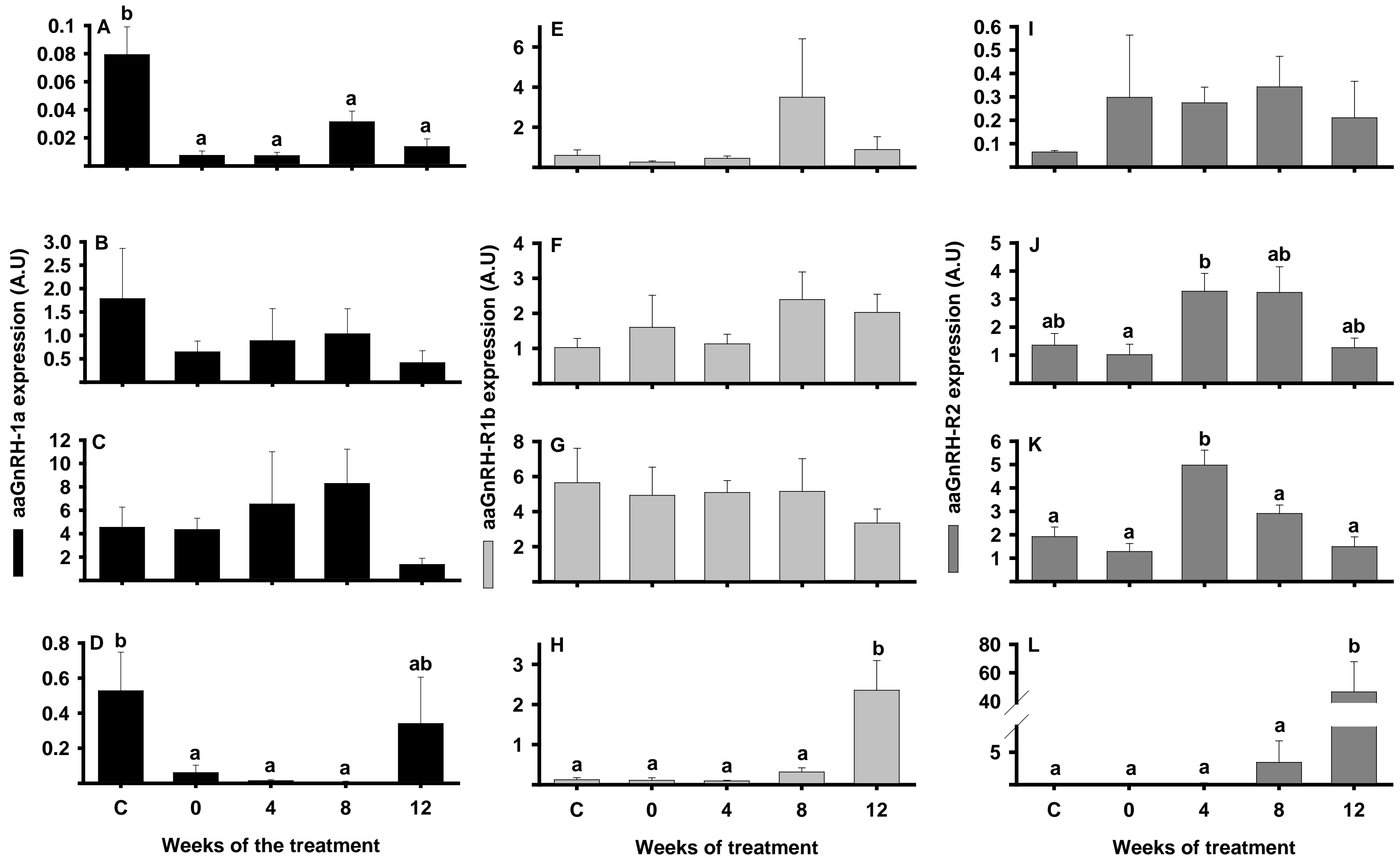


Figure 7

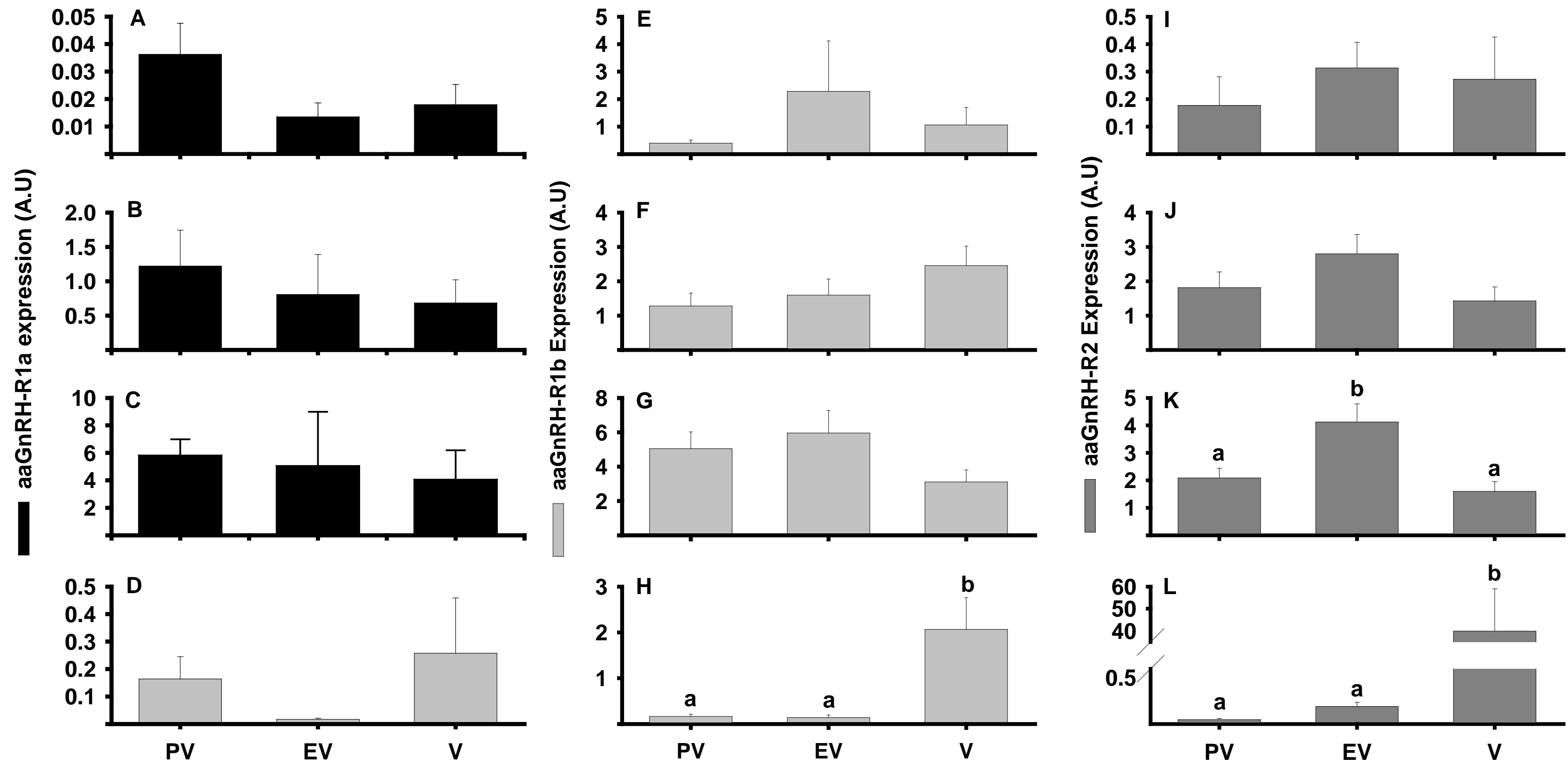


Figure 8