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Temporal expression of hepatic *estrogen receptor 1*, *vitellogenin1* and *vitellogenin2* in European silver eels

Arjan P. Palstra [a,b,1], Denhi Schnabel [a,c,1], Maaike C. Nieveen [a], Herman P. Spaink [a], Guido E.E.J.M. van den Thillart [a,*]

[a] Molecular Cell Biology, Institute of Biology, Leiden University (IBL), Einsteinweg 55, 2333 CC Leiden, The Netherlands
[b] Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain
[c] Departamento Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología/UNAM, Av. Universidad #2001, Col. Chamilpa C.P. 62210, Cuernavaca, Morelos, Mexico

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ABSTRACT

Because European silver eels have never been caught during or after their 6000-km reproductive migration to the Sargasso Sea, all existing knowledge on their sexual maturation comes from hormonal stimulation. Silver eels that start their oceanic migration are still immature with pre-vitellogenic oocytes. Hence we assumed that vitellogenesis should start with the expression of the estrogen receptor in the liver before the circulating 17β-estradiol (E2) can have any effect. In this study we followed the hepatic vitellogenesis upon 4 weekly injections with carp pituitary extracts (CPE). New molecular primers for the expression of the *estrogen receptor 1* (*esr1*), *vitellogenin1* (*vtg1*) and *vitellogenin2* (*vtg2*) in the liver were developed. Sequences of *vtg2* and *esr1* were not previously described in *Anguilla anguilla*. All eels showed weekly increase of the eye size and pectoral fin length, which are signs of early maturation. The same occurred with the gonadosomatic index, the oocyte stage and diameter, and number of deposited fat droplets. Early vitellogenesis appeared as a 3-step process (1) E2-levels and *esr1* expression were significantly increased already after one injection, (2) *vtg1* and *vtg2* expression were significantly increased after one and two injections, respectively, and (3) *vtg1* and *vtg2* expression increased further after three and four injections. Then also plasma calcium (corresponds with plasma vitellogenin) increased and yolk globuli appeared in the oocytes. These results show that *esr1* is the first of the three genes examined that is expressed during the onset of hepatic vitellogenesis. Furthermore, ovarian vitellogenesis (appearance of yolk globuli in oocytes) occurs 1–2 weeks later than the onset of hepatic vitellogenesis.

1. Introduction

The three most well-known eel species (European eel, *Anguilla anguilla*; American eel, *Anguilla rostrata* and Japanese eel, *Anguilla japonica)* are semelparous and migrate thousands of kilometers to their oceanic spawning grounds. The European eel swims 6000-km from the estuaries and freshwater rivers and lakes to the Sargasso Sea to reproduce. The females spend 8 to over 50 years (mean 12 years) as yellow eels on continental waters to grow and to build up the required fat reserves to fuel their trip and to deposit fats in the oocytes. The large and fatty females with sufficient fat stores (Larsson et al., 1990; Svedäng and Wickström, 1997) cease feeding and start their reproductive migration. They show numerous morphological and physiological changes in preparation of their oceanic journey ('silvering') and leave as silver eels. Knowledge on natural maturation and reproduction of European eels is absent. The silver eels venture into the ocean in autumn, still in a pre-pubertal condition, with less than 2% relative gonad mass. Laboratory experiments show that after artificial maturation female eels reach gonad masses of 40–60% of their body mass (Palstra et al., 2005). However, nobody ever observed maturing silver eels during oceanic migration nor caught them in the act of spawning.

Before leaving the continent as silver eels, the yellow eels are characterized by a severely depressed lipid mobilization (Palstra et al., 2009) and blockage of maturation. Pre-pubertal blockage of maturation is due to a deficient GnRH stimulation and a simultaneous inhibition of pituitary FSH and LH synthesis and release by dopamine (Dufour et al., 1988; Vidal et al., 2004). Oocytes of yellow and silver European eels are in a pre-vitellogenic stage (Versonnen et al., 2004) and have to be stimulated by gonadotropins. Like in salmonids, development until yolk incorporation is characterized by accumulation of cortical alveoli and fat droplets (Campbell et al., 2006). Information on vitellogenesis has increased substantially in recent years but little pro-

* Corresponding author. Fax: +31 71 5274900.
E-mail addresses: a.p.palstra@biology.leidenuniv.nl (A.P. Palstra), g.van.den.thillart@biology.leidenuniv.nl (G.E.E.J.M. van den Thillart).
[1] These authors contributed equally to this work.

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gress has been made in understanding pre-vitellogenic development (Pătino and Sullivan, 2002). Only recently, mechanistic studies on ovarian previtellogenesis have been published on salmonids (Campbell et al., 2006) and eel (*Anguilla australis*; Lokman et al., 2007).

The existing knowledge on eel maturation comes from the artificial induction of maturation by hormonal injections with (carp or salmon) pituitary extract in females. This approach has been used by many researchers in reproduction studies on Japanese eel (Yamamoto and Yamauchi, 1974; Yamauchi et al., 1976), American eel (Sorensen and Winn, 1984), New Zealand eels (*Anguilla dieffenbachii* and *A. australis*; Todd, 1981), and European eel (Boëtius and Boëtius, 1980). Current protocols are still based on the use of pituitary extracts although reproductive success is very limited. Larvae of *A. dieffenbachii*, *A. australis* (Lokman and Young, 2000) and *A. anguilla* (Bezdenezhnykh et al., 1983; Bezdenezhnykh and Prokhorcik, 1984; Pedersen, 2004; Palstra et al., 2005) were obtained by pituitary extract injections but they showed, however, obstructed or delayed hatching, abnormal morphology and died within a few days after hatching prior to exogenous feeding. In contrast, Japanese researchers succeeded in raising larvae up to exogenous feeding and even in producing glass eels from *A. japonica* treated with pituitary extracts (Kagawa, 2003; Kagawa et al., 2005) but they acknowledge that the yield was low and most larvae developed abnormally. Adachi et al. (2003) observed variations in yolk accumulation, egg membrane formation, oocyte maturation and plasma hormone levels. Sato et al. (2003) mentioned variable fertility and hatchability of eggs and also low survival of *A. japonica* larvae. These reproduction problems might be due to alterations in vitellogenesis induced by the weekly hormone injections.

In this study we focused on the onset of liver vitellogenesis, which we assumed should start with the expression of the estrogen receptor in hepatocytes. As European yellow eels have not started vitellogenesis yet (Versonnen et al., 2004; van Ginneken et al., 2007a; Palstra et al., 2009) and cannot be used for artificial reproduction (Palstra and van den Thillart, 2009), it is likely that the estrogen receptor has not been adequately activated. Thus we hypothesized that the combination of circulating estradiol and the presence of the Esr1 in the liver of silver eels has to precede the production of vitellogenin. We studied the effects of 4 weekly injections with carp pituitary extract (CPE) on migratory female silver eels. The maturation response and especially the vitellogenic response of liver and oocytes, was determined by measuring morphometric and histological maturation parameters, blood plasma estradiol (E2) and calcium (Ca), and the expression of genes involved in vitellogenesis in the liver.

2. Methods

2.1. Silver eels

Wild migratory silver eels ($n = 47$) were caught in Lake Grevelingen (The Netherlands), near the sluices at the North Sea side (at 32‰, 12 °C), at the end of October of 2005. Larger eels (>70 cm in length and 800 g body weight) were selected from the catch. They were transported to the laboratory in a 1:1 eel:water ratio in plastic bags filled up with oxygen in 60 l tanks. Silver eels were randomly divided over the experimental groups; 10 eels were immediately measured and dissected as controls, another 10 eels were measured and dissected as rest group after 4 weeks at the end of the experiment. For hormonal stimulation, 27 experimental eels were tagged with small passive implantable glass encapsulated transponders (TROVAN, EID Aalten BV, Aalten, The Netherlands) that were subcutaneously injected at the base of the dorsal fin.

2.2. Treatment

The group of 27 eels received weekly intraperitoneal CPE injections at a dose of 20 mg/kg according to the method described before (Palstra et al., 2005), referred to as the CPE group. The CPE group was kept in a 1700-l recirculation system with artificial seawater of 18 °C. To prevent bacterial infections, eels were exposed weekly for 1.5 h to the antibiotic Flumiquin (Flumix, Eurovet, Bladel, The Netherlands, 50 mgl^{-1} for 1–2 h) in 200-l water in a separate tank.

2.3. Measurements and sampling

Each week, 5 or 6 eels were measured and sampled to analyze the treatment effects. Morphometric parameters included body length (BL), body weight (BW), eye diameter horizontal (EDh), eye diameter vertical (EDv) and pectoral fin length (PFL). The following indices were calculated according to the formulae below: condition factor (*K*), eye index (EI) and pectoral fin length index (PFLI).

1. Condition factor (K) = $100 * BW\ BL^{-3}$
   BW: body weight (g), BL: body length (cm).
2. Eye index (EI) = $100 * (((EDh + EDv) * 0.25)^2\ \pi * (10 * BL)^{-1})$
   EDh: eye diameter horizontal (mm), EDv: eye diameter vertical (mm).
3. Pectoral fin length index (PFLI) = $100 * PFL\ BL^{-1}$
   PFL: pectoral fin length (cm).
4. The silver index (SI) was calculated according to Durif et al. (2005).

Blood samples were taken from the caudal vein with heparin-flushed (10.000 IU ml^{-1}) 1 ml syringes, which were immediately placed on ice. The blood was centrifuged for 5 min at 14,000 rpm and blood plasma was stored at −80 °C. Liver and gonads were dissected and weighed. The following indices were calculated according to the formulae below: the gonadosomatic index (GSI) and the hepatosomatic index (HSI).

5. Gonadosomatic index (GSI) = $(GW\ BW^{-1}) * 100\%$
   GW: gonad weight (g), BW: body weight (g).
6. Hepatosomatic index (HSI) = $(LW\ BW^{-1}) * 100\%$
   LW: liver weight (g).

Liver tissue was stored in RNAlater (Ambion) at −20 °C for later analysis. Gonad tissue from a standardized rostral location was stored in Bouin solution at room temperature.

2.4. Blood measurements

As vitellogenic indicators in the blood plasma, 17β-estradiol (E2) and calcium (Ca) were chosen. E2 was measured by ELISA (human kit 55030, Human Gesellschaft fur Biochemica und Diagnostica mbH, Wiesbaden, Germany). Levels of plasma calcium were measured by a colorimetric test (human kit 10011, Human Gesellschaft fur Biochemica und Diagnostica mbH, Wiesbaden, Germany) and indicated circulating vitellogenin (Vtg). Ca was chosen as indirect measure of plasma Vtg in eels as previously reported by Versonnen et al. (2004) based on the significant positive correlation between Ca and Vtg that has been demonstrated for rainbow trout by Verslycke et al. (2002).

2.5. Histology

To remove the Bouin fixative, the gonads were washed in 0.1 M phosphate buffer and 70% ethanol until the solution became transparent. After dehydrating through an accumulating alcohol series the samples were embedded in air-free Technovit 7100 (Kulzer

Histo-Technik) in Peel-A-way molds (Polysciences Inc.) covered with a layer of air-free paraffin oil during polymerization to exclude oxygen (de Jonge et al., 2005). Sections of 10 μm thick were cut using a sledge microtome (Jung Polycut E). Three sections were put on a slide and five slides per sample were made and stained with Mayers Haematoxylin–Eosin. The oocytes were studied visually under the microscope (Nikon Eclipse E400) and overview pictures were taken (Nikon Coolpix 4500). For each section, 10 oocytes were randomly selected that had been cut through the nucleus. For each oocyte, the developmental stage (OS) was determined. The diameter of the oocytes (OD) was determined using UTHSCSA Image Tool 2.0. The number of fat droplets occurring in stage 3 oocytes (lipid droplet stage/cortical alveolus stage); (Adachi et al., 2003; Wallace and Selman, 1981; Tyler and Sumpter, 1996; Palstra et al., 2007) were counted. In fact, what we call fat droplets are the empty lipid vesicles from which the lipids have been extracted by washing in ethanol.

2.6. Oligonucleotides

Following Filby and Tyler (2005), to standardize Esr subtype nomenclature, the official protein and gene designations for the zebrafish have been adopted here according to the Official Zebrafish Nomenclature Guidelines; http://zfin.org.

In *A. japonica*, Todo et al. (1996) cloned the sequence of the *estrogen receptor 2* (*esr2*; previously known as the *estrogen receptor β*) and found that *esr2* expression was elevated during artificially induced maturation, suggesting a role for Esr2 in hepatic vitellogenesis in eel species. However, in zebrafish (Menuet et al., 2004) and largemouth bass (Sabo-Attwood et al., 2004) liver, the Estrogen receptor 1 (Esr1; previously known as the Estrogen receptor α) was found to be highly inducible by E2 in contrast to the Esr2. Since nothing is known about the expression of the *esr1* during hepatic vitellogenesis of eel species, we focused on its expression in this study.

Oligonucleotides were designed to measure the expression levels of *β actin*, the *estrogen receptor 1* (*esr1*), *vitellogenin1* (*vtg1*) and *vitellogenin2* (*vtg2*). They were designed from the reported sequences of *A. japonica* for *vitellogenin1* (*vtg1*) (GenBank AY423445), *vitellogenin2* (*vtg2*) (GenBank AY423444), and 'housekeeping gene' *β actin* (GenBank AB074846). Kazeto et al. (2006) showed that the *β actin* transcript was amplified uniformly over eight different tissues at various stages of induced maturation in Japanese eel. The *estrogen receptor 1* (*esr1*) had not previously been reported in eel and for that purpose we used the sequences from *Salmo salar* (GenBank X89959) and *Oncorhynchus mykiss* (GenBank AJ242740).

2.7. RNA isolation

Total RNA was isolated from the liver of all samples kept in RNAlater using TRIZOL (Invitrogen). Traces of DNA were removed by incubation with DNAse-I (Ambion). In two steps, the DNAse treated RNA was transcribed to cDNA (HT Biotechnology LTD.).

2.8. RT-PCR

A specific RT-PCR was performed using RNA extracts from liver from artificially matured *A. anguilla* (Palstra et al., 2005). RT-PCR was performed using the Superscript II one step RT-PCR system with platinum Taq (Invitrogen), run on the Biometra T1 Thermocycler (Westburg). Reactions were performed with 100 ng of total RNA using 25 pmol of the upper and lower primers. Reverse transcription was performed at 50 °C for 30 min. PCR conditions were 40 cycles of denaturation at 94 °C for 20 s, annealing temperature at 55 °C for all of the genes tested and extension at 72 °C for 1 min followed by a final extension step at 72 °C for 10 min. The PCR products were cloned in pCRII-TOPO vector (Invitrogen), digested with restriction enzymes to identify the correct direction and sequenced (Base Clear lab services) to verify that the final products correspond to the genes of interest by aligning them to genes previously described using the VectorNTI program.

2.9. Sequence alignments and phylogenetic analysis

Global and local sequence alignments of the estrogen sequences formerly known as alpha, beta and gamma were made using the program BLAST and pBLAST and manually adjusted to select only the domain amplified. As described by Salas-Vidal et al. (2005), briefly; the dendrogram of Esr sequences and the phylogenetic tree were analyzed and constructed by two methods. The neighbor-joining method was performed using ClustalX 1.81 (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). ClustalX analysis was done with default settings. Bootstrap sampling was reiterated 1000 times. For the matrix table "Gonnet" was used. For pair-wise alignments the gap opening penalty was set to 35 and the gap extension penalty was set to 0.75. For multiple alignments the gap opening penalty was set to 15 and the gap extension penalty 10–0.30, and divergent sequences alignment was delayed 30%. Trees were drawn using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Nucleotide alignment was done in the Vector NTI program (Invitrogen) with the sequences retrieved from the BLAST results. The sequences selected were those that gave an *E* value of 1e−95 to 6e−48, and a maximal identity from 84% to 75%. The portion of the sequence was edited in order to align only the partial amplification of esr1 from *A. anguilla*.

2.10. Quantitative RT-PCR

Quantitative RT-PCR was performed using the qPCR MasterMix for SYBR® Green I (Eurogenetec). The conditions in the Chromo 4™ Detector (Bio-Rad laboratories) used were 95 °C during 10 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing temperature at 55 °C for 30 s and extension at 72 °C for 40 s and the reading of the plate in each cycle, followed by a final step at 65 °C for 10 min. Melting analysis was performed to show the specificity of the reaction. To show that only one final product was amplified, a melting curve from 65 °C to 95 °C holding during 5 s each 0.5 °C, was generated. The data were analyzed using the opticon monitor 3 program (Bio-Rad laboratories).

Standard curves for each specific gene *β actin*, *esr1*, *vtg1* and *vtg2* were generated for the C_t *values* vs. the logarithmic total DNA from the plasmids containing each of the specific genes. Standard curves were created for each well plate. R^2-values were for all standard curves >0.98. The equations of the standard curves were used to calculate the corresponding amount of DNA for the mean of each duplo-measurement for each sample. This amount was normalized to the expression level of the housekeeping gene *β actin* (between *x%* and 100%). All samples were measured in duplo for each of the genes of choice. Per gene of choice, two well-plates with samples were subjected to quantitative RT-PCR, each containing a standard curve plus (1) control group and rest group and (2) CPE treated groups.

2.11. Statistics

Normality of the data and homogeneity of variances were checked by Kolmogorov–Smirnov tests. Paired observations before and after treatments of morphometric parameters were tested with Student *t*-tests with one-tailed probabilities or, for indices, with Wilcoxon tests. With a univariate general linear model (GLM), analysis of covariance (ANCOVA) with one-tailed probabilities was performed on log transformed unpaired observations in search for group effects in changes of parameters with BW (for GW, LW, OD, number fat droplets) as a cofactor. In case of occur-

rence of significant group effects, ANOVA with a post hoc Bonferroni test was performed to specify the effects between particular groups. Significant shifting of oocyte stage was tested with a Mann–Whitney *U*-test. One-tailed Spearman correlation tests were performed to test GSI vs. *esr1*, *vtg1* and *vtg2*. All statistical tests were performed in SPSS 12.0 for Windows. All results were expressed as means ± SE. *P*-values $\leqslant 0.05$ were considered to indicate statistically significant differences.

2.12. Ethics

Experiments complied with the current laws of the Netherlands and were approved by the animal experimental committee.

3. Results

3.1. RT-PCR and alignment of sequences

The expression of the genes for *esr1*, *vtg1* and *vtg2* was found in the liver of mature female eels. As a positive control for all the samples *β actin* was amplified. Analysis of the sequences showed that we have indeed amplified the genes of interest with the oligonucleotides shown in Table 1.

The *β actin* PCR product of *A. anguilla* was aligned with *β actin* mRNA of other species (see Supplemental data file 1) and showed high similarity with *A. japonica* and *Dicentrarchus labrax* sequences. *β actin* has recently been partially cloned for *A. anguilla* (Pierron et al., 2007; Aubry et al., 2007).

The *vtg1* PCR product of *A. anguilla* was aligned with mRNA of *vtg1* of other species (see Supplemental data file 2) and showed high similarity with *A. japonica* and *Conger myriaster*. *Vtg1* was cloned partially for *A. anguilla* in this study and the sequence was deposited in GenBank as EU073127. Recently, a partial sequence of vtg1 of *A. anguilla* was deposited in GenBank as AM076793.

Two genes were not previously described in *A. anguilla*: *vtg2* and *esr1*. The *vtg2* PCR product of *A. anguilla* was aligned with mRNA of *vtg2* of *A. japonica* and *C. auratus* (see Supplemental data file 3). The partial sequence of *vtg2* of *A. anguilla* was deposited in GenBank as EU073128.

Table 1
Sequence of the oligonucleotides used for *esr1*, *vtg1*, *vtg2* and *β actin*, their GenBank accession number and PCR product size.

Target gene	GenBank	Forward sequence	Reverse sequence	PCR product size
esr1	EU073125	GTCGAGGACAAAGCCATCAT	CCGATCATCAGCACCTCCAG	318
vtg1	EU073127	AAGCAACCCACCTTGATACG	TCAAGACACGCTTCATCCTG	241
vtg2	EU073128	CGAGGATGCTCCCCTAAAGT	CCCCCTCAGCTGTGGTAATA	220
β actin		CTCCCTGGAGAAGAGCTACG	GGAGTTGAAGGTGGTCTCGT	153

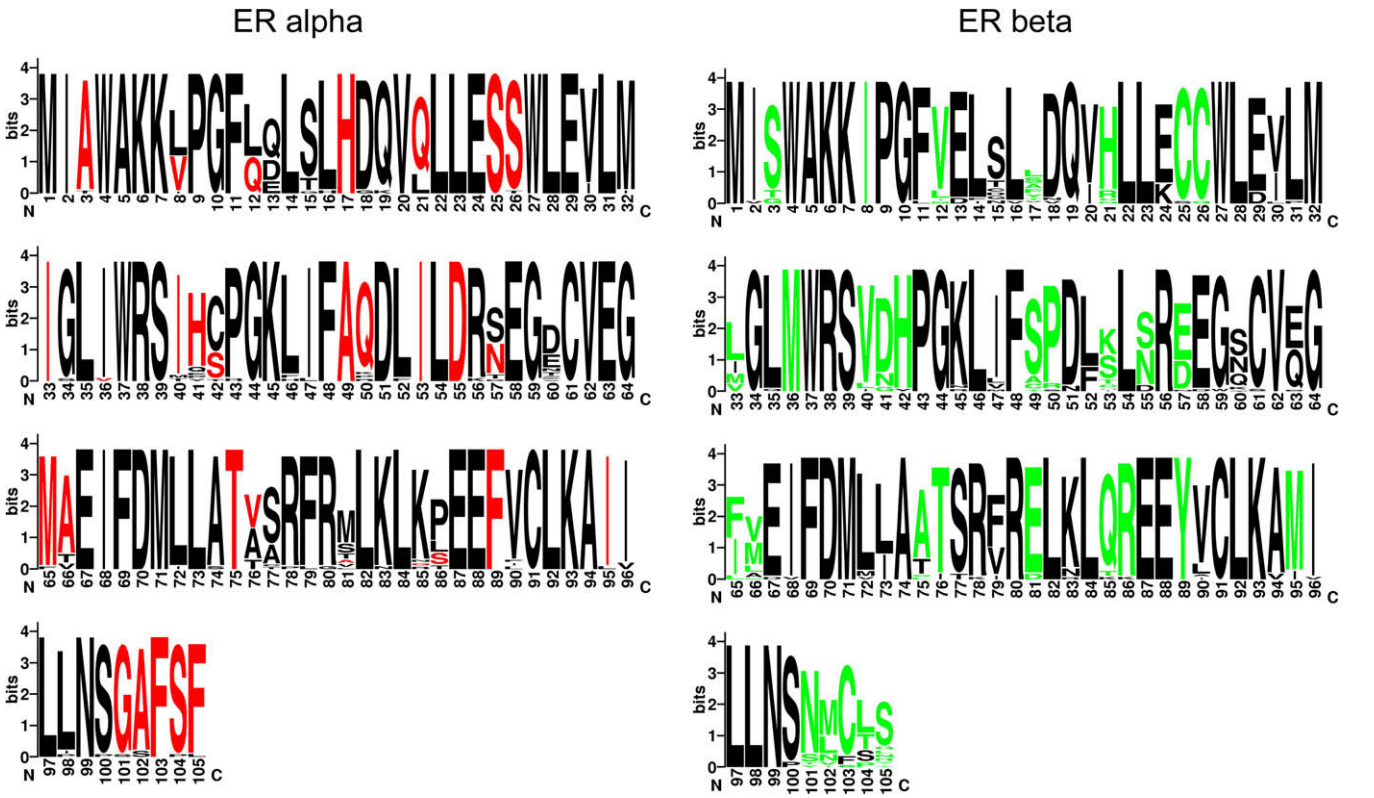

Fig. 1. Comparison of the amplified domain of the Estrogen receptor 1 (Esr1), or ER alpha, with estrogen receptor 2 (Esr2), or ER beta, in teleosts through LOGO representation of conserved amino acids common to all teleost Esr1 and Esr2 proteins. Aligned blocks of conserved ligand-binding domain sequences present in Esr proteins were used as input to the LOGO web server at http://weblogo.berkeley.edu/ (Crooks et al., 2004) to obtain the representation of the relative frequency of each amino acid in the motifs. Note that many positions are highly conserved, thus they have the maximum possible frequency value of 100%. Colour code representation: conserved amino acids between Ers1 and Esr2 are shown in black. Red letters show the conserved amino acids present in Esr1 from *Anguilla anguilla*, the size of the letter represents the conservation between the Esr1 proteins described for teleosts. Green letters represent the different amino acids present only in Esr2 proteins. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

3.2. Sequence alignment and phylogeny of esr1

Moreover, *esr1* was not previously identified in any eel species. It is the first time that a partial sequence of eel *esr1* is obtained. The amplified product was sequenced and aligned to verify that it corresponded to the *esr1* of *A. anguilla*. The results demonstrated that it corresponds to the well-conserved carboxyl terminal ligand domain (compare Fig. 1a and b). The amplified product was analyzed with BLAST and all the sequences retrieved from Esr1 and Esr2 were posteriorly aligned, and the region amplified was identified as part of the ligand domain. Furthermore only the region that corresponded to the same 105 amino acids from every Esr1 and Esr2 was aligned to identify the relative frequency of each amino acid in the protein. The comparison between Esr1 and Esr2 showed that

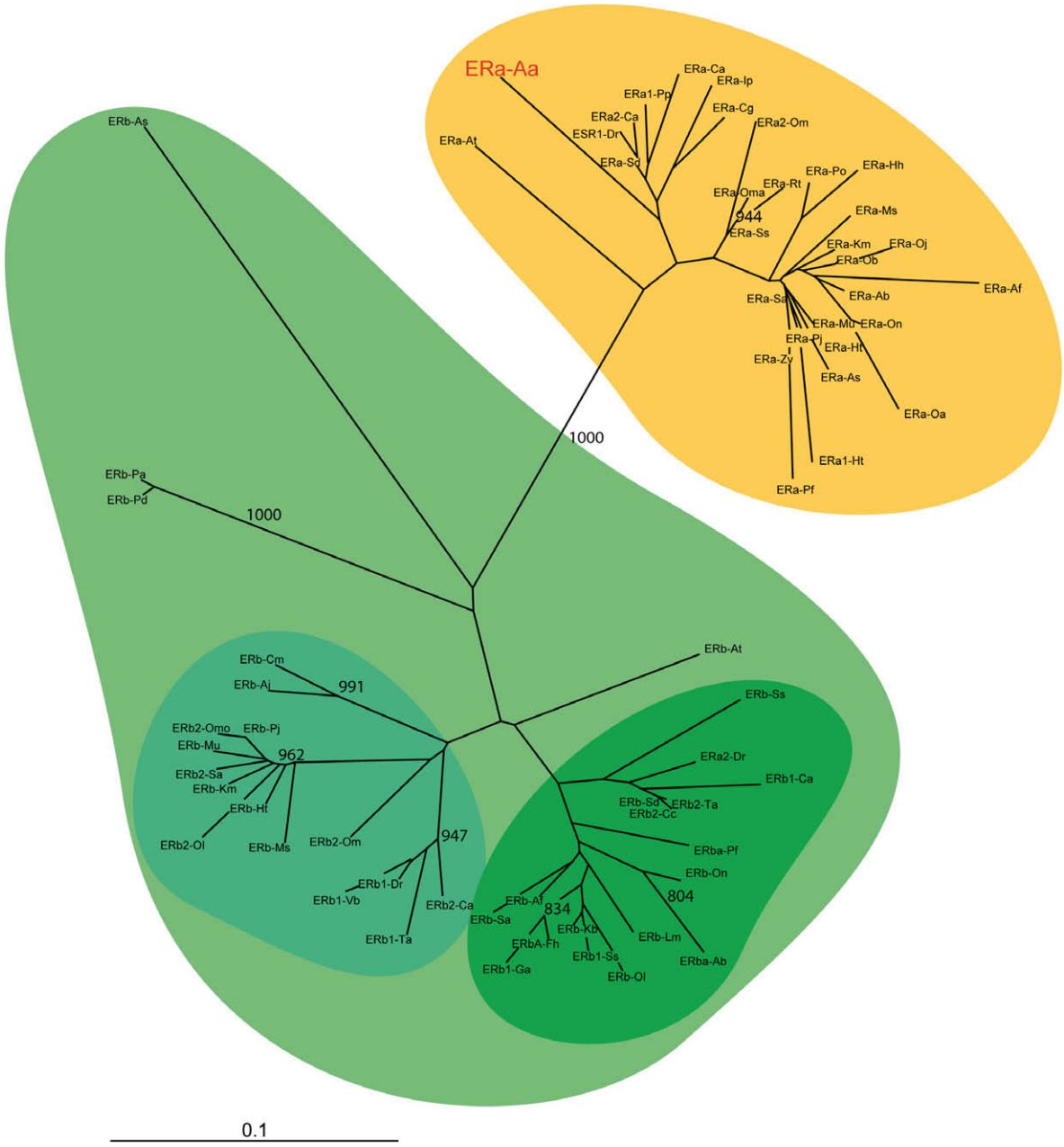

Fig. 2. Unrooted phylogenetic tree of fish estrogen receptors. The tree was constructed by neighbor-joining analysis based on an alignment of the amino acid sequences of the ligand-binding domain of estrogen receptors in fish. The numbers indicate the occurrence of nodes during bootstrap analysis with 1000 reiterations. Only values above 800 are shown. ERα (Estrogen receptor 1 or alpha plus subtypes) in yellow, ERβ (Estrogen receptor 2 or beta plus subtypes) in green (nomenclature of receptor subtypes as applied by the authors); Aa, *Anguilla anguilla*; Ab, *Astatotilapia burtoni*; Af, *Acanthogobius flavimanus*; Aj, *Anguilla japonica*; As, *Acipenser schrenckii* (Amur sturgeon); At, *Atractosteus tropicus* (tropical gar); Ca, *Carassius auratus* (goldfish); Cc, *Cyprinus carpio* (common carp); Cg, *Clarias gariepinus* (North African catfish); Cm, *Conger myriaster* (whitespotted conger); Dr, *Danio rerio* (Zebrafish); Fh, *Fundulus heteroclitus* (mummichog); Ga, *Gambusia affinis* (western mosquitofish); Hh, *Hippoglossus hippoglossus* (Atlantic halibut); Ht, *Halichoeres tenuispinis* (Chinese wrasse); Ip, *Ictalurus punctatus*, (channel catfish); Kb and km, *Kryptolebias marmoratus* (mangrove rivulus); Lm, *Lepomis macrochirus* (bluegill); Ms, *Micropterus salmoides* (largemouth bass); Mu, *Micropogonias undulatus* (Atlantic croaker); Oa, *Oreochromis aureus*; Ob, *Odontesthes bonariensis* (pejerrey); Oj, *Oryzias javanicus*; Ol, *Oryzias latipes* (Japanese medaka); Om, *Oncorhynchus mykiss* (rainbow trout); Oma, *Oncorhynchus masou* (Cherry salmon); Omo, *Oreochromis mossambicus* (Mozambique tilapia); On, *Oreochromis niloticus* (Nile tilapia); Pa, *Protopterus annectens*; Pd, *Protopterus dolloi*; Pf, *Perca flavescens* (yellow perch); Pj, *Pseudolabrus japonicus*; Po, *Paralichthys olivaceus* (bastard halibut); Pp, *Pimephales promelas*; Sa, *Sparus aurata* (gilthead seabream); Sd, *Spinibarbus denticulatus*; Ss, *Salmo salar* (Atlantic salmon); Ta, *Tanichthys albonubes*; Vb, *Varicorhinus barbatulus*; Zv, *Zoarces viviparus*. Some of the sequences were not drawn because they were 100% identical and overlapped in the figure, but they were used for the analysis (data not shown). (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

there are several amino acids conserved but there is a characteristic signature that corresponds to either the Esr1 or Esr2 proteins (shown in red or green), interestingly the region that we were able to amplify has such signature characteristic in Esr1 proteins (red) and not present in Esr2 (green).

Phylogenetic analysis by the neighbor-joining method (Fig. 2) produced consistent phylogenetic relationships among the different Esr1 proteins in teleosts related with the partial sequence that we have amplified (Fig. 2). Even more when nucleotide sequences from Esr1 from *A. anguilla* were aligned, only Esr1 from different teleosts were retrieved and the analysis of the aligned sequences gave a 96.2% of similarity and a identity of 43.6% (see Supplemental data file 4). From this analysis we can conclude that we have amplified a part of the ligand-binding domain of the Esr1 in *A. anguilla*, different from the previously reported sequence of the Esr in *A. japonica* (Todo et al., 1996). The partial sequence of eel *esr1* was deposited in GenBank as EU073125.

Table 2
Morphometric parameters (av ± SE.) of (a) eels that were sampled at the start as control group and eels that were sampled after 4 weeks resting, (b) eels that were sampled at the start and after 1 weekly carp pituitary (CPE) – injection, (c) eels that were sampled at the start and after 2 weekly CPE – injections, (d) eels that were sampled at the start and after 3 weekly CPE – injections, and (e) eels that were sampled at the start and after 4 weekly CPE – injections. BL, body length; BW, body weight; *K*, condition factor; EI, eye index; PFLI, pectoral fin length index; SI, silver index. Significant changes ($P < 0.05$) are indicated in bold.

(a)		
	Control	4-wks rest
n	10	10
BL (cm)	85 ± 2	83 ± 1
BW (g)	1295 ± 101	1181 ± 47
K	0.210 ± 0.009	0.209 ± 0.004
EI	10.6 ± 0.4	10.8 ± 0.5
PFLI	4.47 ± 0.08	4.77 ± 0.12
Is	4	4
(b)		
	Start	1-wk CPE
n	6	6
BL (cm)	84 ± 2	84 ± 2
BW (g)	1259 ± 96	**1284 ± 98**
K	0.216 ± 0.009	**0.220 ± 0.009**
EI	11.0 ± 0.24	11.4 ± 0.5
PFLI	4.64 ± 0.13	4.65 ± 0.13
Is	4	4
(c)		
	Start	2-wks CPE
n	5	5
BL (cm)	85 ± 2	85 ± 2
BW (g)	1259 ± 111	**1284 ± 112**
K	0.204 ± 0.006	**0.208 ± 0.007**
EI	12.1 ± 0.5	12.6 ± 0.4
PFLI	4.66 ± 0.07	4.69 ± 0.12
Is	4	4
(d)		
	Start	3-wks CPE
n	5	5
BL (cm)	86 ± 2	86 ± 2
BW (g)	1275 ± 83	**1297 ± 86**
K	0.202 ± 0.011	**0.205 ± 0.011**
EI	10.5 ± 0.4	**11.6 ± 0.3**
PFLI	4.76 ± 0.13	4.83 ± 0.12
Is	4	4
(e)		
	Start	4-wks CPE
n	6	6
BL (cm)	84 ± 2	84 ± 2
BW (g)	1206 ± 87	**1231 ± 89**
K	0.205 ± 0.013	**0.209 ± 0.013**
EI	11.5 ± 0.3	**13.7 ± 0.38**
PFLI	4.60 ± 0.09	**4.72 ± 0.11**
Is	4	4

3.3. Quantitative RT-PCR

For each of the analyzed genes, only one product was generated which corroborated with the RT-PCR results. The melting curve for the *β actin* products (see Supplemental data file 5) showed that only one product was generated during the reaction with a melting temperature of 88 °C. The overlay of the melting temperatures for *esr1*, *vtg1* and *vtg2* were found at, respectively, 89, 85, and 84 °C (see Supplemental data file 4), all indicating single gene products. Results were verified before used on RNA samples of experimental eels.

3.4. Experimental eels

Eels at the start of the experiment (control and CPE groups: $n = 37$) measured 84 ± 1 cm and weighed 1243 ± 40 g ($K = 0.209 \pm 0.024$). All eels were silver with EIs of 11.1 ± 0.2 (range 8.8–13.2) and actively migrant (36 in stage IV, 1 in stage V). Eels from the different groups did not show significant differences in BL and BW. During the experiments, some eels developed wounds on the top of the head, these eels died ($n = 2$) or were removed ($n = 3$).

3.5. Morphometry

Eels in the CPE group showed an immediate increase in weight (about 2%) lasting over all weeks (Table 2). The EI had significantly

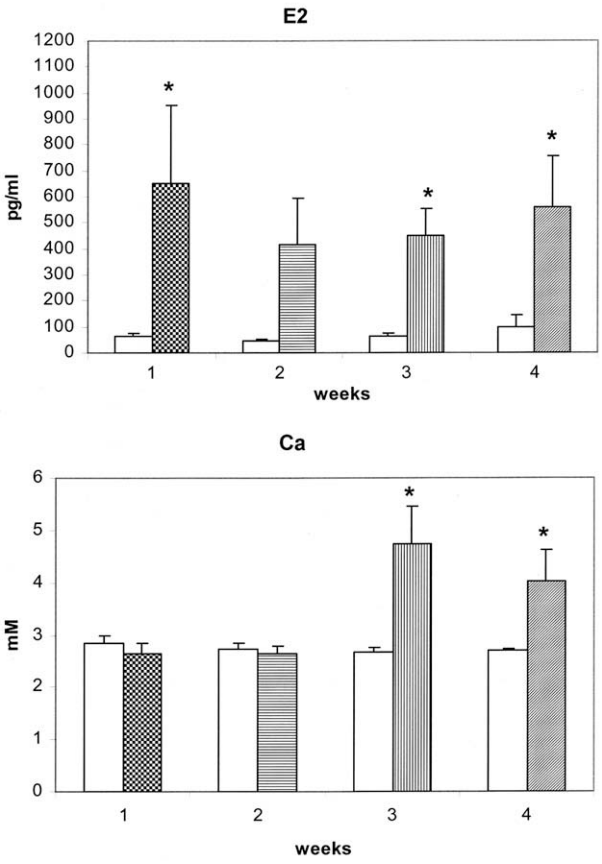

Fig. 3. Changes in plasma 17β-estradiol (E2) and calcium (Ca) (av ± SE) after weekly carp pituitary extract (CPE) – injections. (a) E2 values in eels before (white bars) and after 1 up to 4 weekly CPE injections (1 wk $n = 6$, 2 wks $n = 5$, 3 wks $n = 5$, 4 wks $n = 6$ eels) and (b) Ca values. Significant changes ($P < 0.05$) are indicated by *.

increased (>10%) after 3 weeks of injections ($P < 0.001$) (Table 2). After 4 weeks, this difference had almost doubled. Eels from the rest group showed no significant difference in EI with respect to eels from the control group, indicating that there was neither a time-effect nor an interaction-effect. The PFLI showed a similar increase as the EI, significant for eels of the CPE group after 4 weeks (>2.5%; $P < 0.05$; Table 2). These differences had no consequences for the SI.

3.6. Blood plasma parameters

Plasma E2 significantly increased almost 10-fold in the CPE group already after 1 week (Fig. 3a) and showed equally significantly elevated levels after 3 and 4 weeks. No changes occurred in the rest group in comparison with the controls. Ca increased in the CPE group in week 3 (76%, $P < 0.05$) and week 4 (49%, $P < 0.05$; Fig. 3b). No changes were found in the rest group in comparison with the controls.

3.7. Internal parameters

The GSI increased in the CPE group already after 1 week (46%, $P < 0.001$; Fig. 4a) and subsequently rose with every week (week 2: 76%, week 3: 136%, week 4: 199%). The GSI of resting eels remained similar to values of the controls. No significant changes in HSI were found (Fig. 4b).

3.8. Oocyte histology

Of the eels in the control group, 60% contained solely oocytes in stage 3: the lipid vesicle stage, and 40% of the eels still contained stage 2 oocytes without any lipid vesicles (Adachi et al., 2003; Wallace and Selman, 1981; Tyler and Sumpter, 1996; Palstra et al., 2007). The average stage was 2.87 ± 0.19. These oocytes had a diameter of 201 ± 8 μm and contained 140 ± 20 fat droplets (Fig. 5). After one CPE injection, the oocyte diameter and the number of fat droplets non-significantly (ns) increased (Fig. 5). After two injections, these characteristics increased further and were significant ($P < 0.05$; Fig. 5). After three injections, all eels contained oocytes with yolk globuli, 30–80% of the oocytes contained yolk globuli and represented stage 4; vitellogenesis (Figs. 5 and 6). This caused an increase in the average stage ($P < 0.01$) and also the number of fat droplets increased ($P < 0.01$). After four injections, the oocyte stage ($P < 0.01$) and diameter ($P < 0.05$) further increased but the number of fat droplets decreased ($P < 0.01$; Fig. 5), probably resulting from fusion of the fat droplets. The amount of lipids stored between oocytes decreased over the weeks and was almost absent after 4 weeks. Resting eels did not show development and even regression since the oocyte diameter ($P < 0.01$) and the number of fat droplets ($P < 0.01$) decreased.

3.9. Expression of esr1, vtg1 and vtg2

Because no significant differences in GSI occurred between the control and rest groups (Fig. 4), only the rested eels and CPE-treated

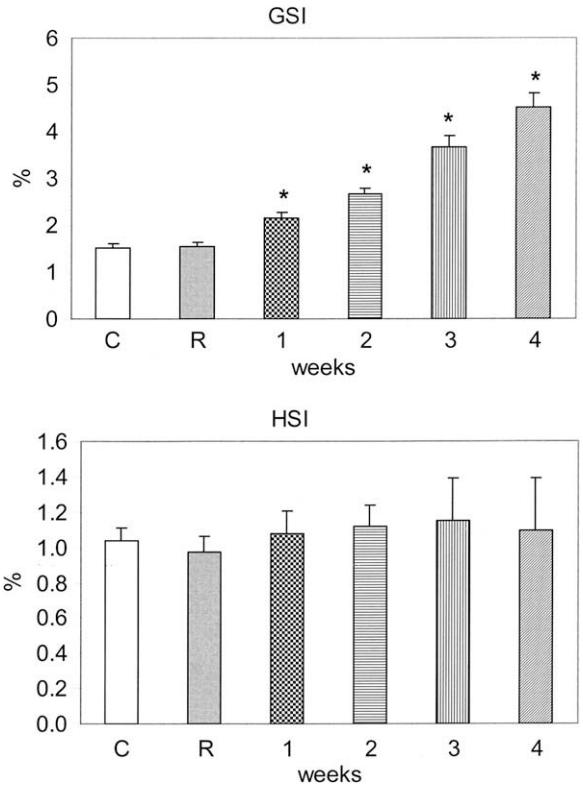

Fig. 4. Changes in gonadosomatic index (GSI) and hepatosomatic index (HSI) (av ± SE) after weekly carp pituitary extract (CPE) – injections. (a) GSI values in eels of the control group (C, $n = 10$), the rest group (R, $n = 10$) and after 1 up to 4 weekly CPE injections (1 wk $n = 6$, 2 wks $n = 5$, 3 wks $n = 5$, 4 wks $n = 6$ eels) and (b) HSI values. Significant changes ($P < 0.05$) are indicated by *.

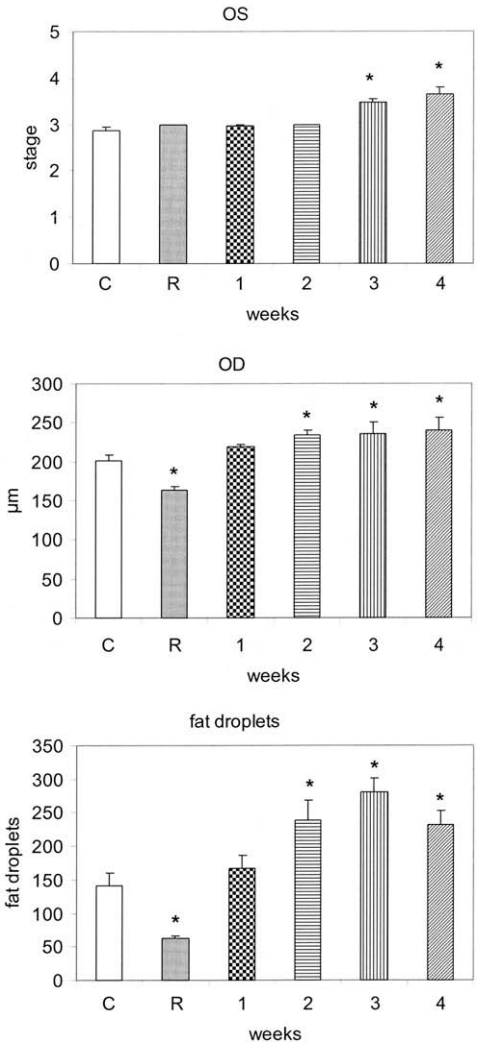

Fig. 5. Changes in oocyte characteristics (av ± SE) after weekly carp pituitary extract (CPE) – injections. (a) The oocyte stage (OS) in eels of the control group (C, $n = 10$), the rest group (R, $n = 10$) and after 1 up to 4 weekly CPE injections (1 wk $n = 6$, 2 wks $n = 5$, 3 wks $n = 5$, 4 wks $n = 6$ eels), (b) the oocyte diameter (OD) and (c) the number of fat droplets. Significant changes ($P < 0.05$) are indicated by *.

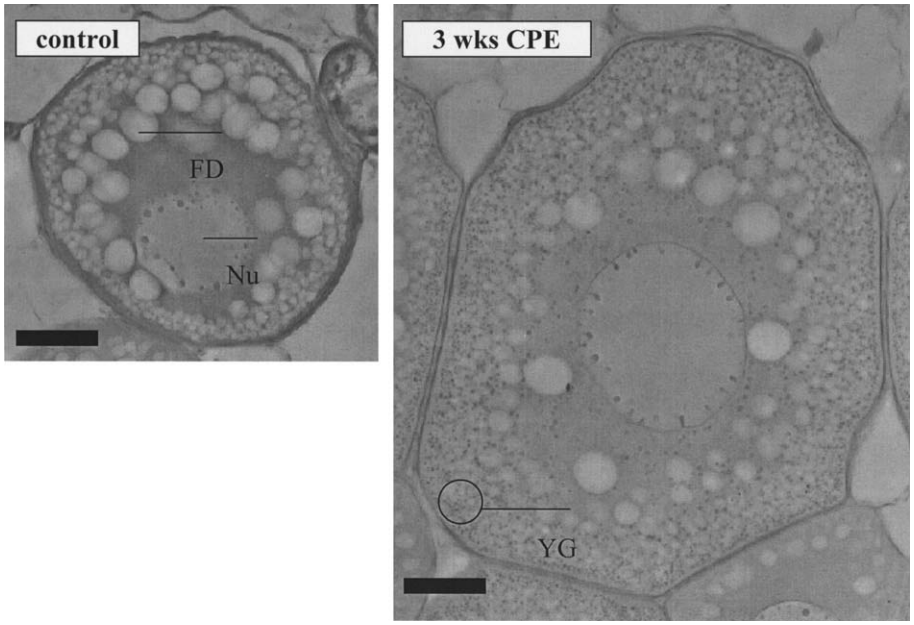

Fig. 6. Oocyte histology of controls and eels that received 3 weekly CPE injections. Nu, nucleus; FD, fat droplet; YG, yolk globuli. The scale bar represents 50 μm.

eels were used for quantitative RT-PCR. Eels from the rest group already showed expression of the *esr1* (Fig. 7a). After one injection, *esr1* expression increased fivefold and a further increase occurred after 3 injections. After 4 injections, levels decreased again to the levels of the controls. Eels of the rest group did not show expression of *vtg1* (Fig. 7b) and *vtg2* (Fig. 7c) but levels increased after one injection. Levels increased further after the 3rd and 4th injection up to levels of >2 ng for *vtg1* and >0.001 ng for *vtg2*.

Expression data of all groups were pooled and *esr1*-, *vtg1*- and *vtg2* expression was plotted vs. GSI as a marker of the maturation status to elucidate the timing of expression (Fig. 8). Expression of the *esr1* was not significantly correlated to GSI and was already apparent at the lowest GSI values of 1. However, expression of *vtg1* and *vtg2*, both significantly correlated to GSI ($P < 0.001$), occurred later at GSI = 2.

4. Discussion

4.1. The role of the Esr1 in eel

For the first time a partial sequence of eel *esr1* was obtained (GenBank EU073125). It was cloned by using oligonucleotides from the reported sequences from *S. salar* (Rogers et al., 2000) and *O. mykiss* (Pakdel et al., 2000), showing that salmonid cDNA primers could be used to detect the gene in eel. The *esr1* has been cloned for several other fish species like gilthead sea bream (Munoz-Cueto et al., 1999; Socorro et al., 2000), Nile tilapia (Chang et al., 1999), channel catfish (Pãtino et al., 2000), Atlantic croaker (Hawkins et al., 2000), rainbow trout (Menuet et al., 2001; Leaños-Castañeda and van der Kraak, 2007), zebra fish (Bardet et al., 2002; Menuet et al., 2002), goldfish (Choi and Habibi, 2003), largemouth bass (Sabo-Attwood et al., 2004), fathead minnow (Filby and Tyler, 2005) and sea bass (Muriach et al., 2008). The eel *esr1* sequence showed high similarity with trout, salmon, halibut and sea bream sequences.

Two subtypes of estrogen receptors are known; Esr1 (previously known as ERα) and Esr2 (previously known as ERβ). Esr2 has more than one form (Menuet et al., 2001, 2002; Nagler et al., 2007): Esr2b (previously known as ERβ1) and Esr2a (previously known as ERγ; Sabo-Attwood et al., 2004). The specific role of each subtype in the induction of Vtg production in fish is, however, still not known. They are thought to mediate different biological effects (Leaños-Castañeda and van der Kraak, 2007). Our study showed that CPE-stimulation increased blood plasma levels of E2. It is probable that this induced the fivefold higher expression of the *esr1*, followed by an increase of Vtg production and release. A significant role for Esr1 in hepatic vitellogenesis is suggested for zebra fish (Menuet et al., 2004) and largemouth bass (Sabo-Attwood et al., 2004). Future studies should investigate the E2 sensitivity of Esr1 and Esr2 and their specific roles during vitellogenesis in the European eel.

4.2. Effects of CPE-treatment on maturation

CPE-treatment induced a progressive increase of the eye diameter (as reported earlier for European eel by Pankhurst (1982)) and pectoral fin length (in contrast to findings for European eel of Durif et al. (2006)), which are signs of silvering and early maturation. The same occurred for the gonadosomatic index (GSI; as reported for European eel by Fontaine et al. (1964)) accompanied by increases of the average oocyte stage, diameter and number of fat droplets (like described by Yamamoto et al. (1974) for Japanese eel). In our study GSI values were significantly different 1 week after the first injection and finally reached an average value of 4.53 ± 0.72 1 week after the 4th injection. Our results are comparable to the values reported by Schmitz et al. (2005) who used silver eels from the River Loire and applied the same dose of carp pituitary extract from the same manufacturer (Catvis, The Netherlands). Thus, the response to CPE-treatment, reflecting the silvering status and sensitivity for maturation, is rather similar for Lake Grevelingen and River Loire silver eels. Our results disagree with the results of Durif et al. (2006) who used the same protocol on silver eels from the River Loire. Differences in gonad weight in eels that Durif et al. (2006) used became significant only after the 4th injection. The difference is likely due to a difference in water temperatures; eels in our study were kept at 18 °C while eels in Durif's study were kept at outdoor temperatures ranging between 8 °C in January to an average 26 °C in June. A similar negative relation between temperature and the speed of maturation has been shown for

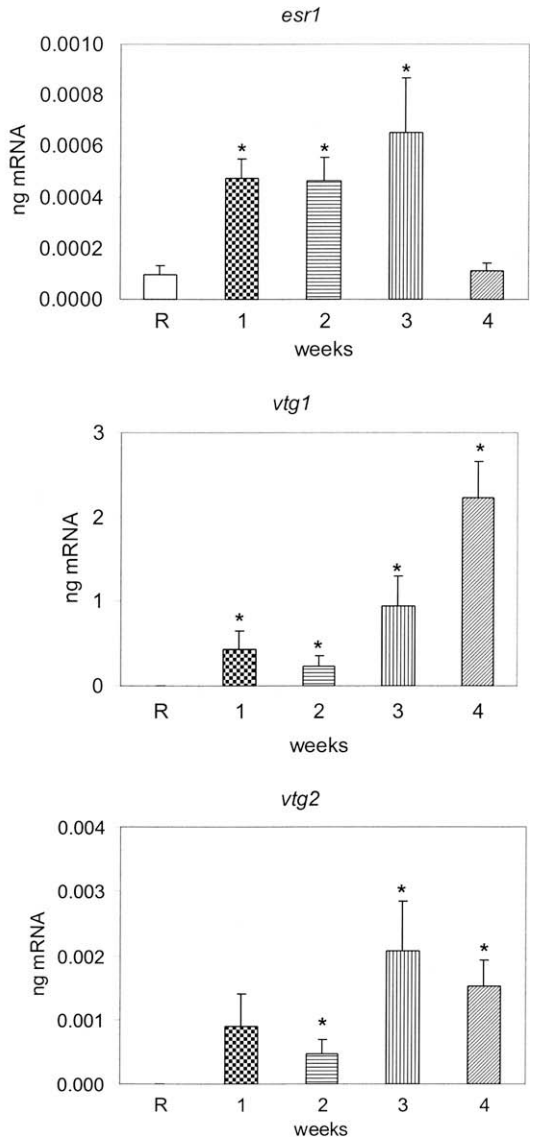

Fig. 7. Expression levels (av ± SE) determined by QRT-PCR of the *estrogen receptor 1* (*esr1*), *vitellogenin1* (*vtg1*) and *vitellogenin2* (*vtg2*). (a) *esr1* expression in eels of the rest group (R, $n = 10$) and after 1 up to 4 weekly CPE injections (1 wk $n = 6$, 2 wks $n = 5$, 3 wks $n = 5$, 4 wks $n = 6$ eels), (b) *vtg1* expression and (c) *vtg2* expression. Significant changes ($P < 0.05$) are indicated by *.

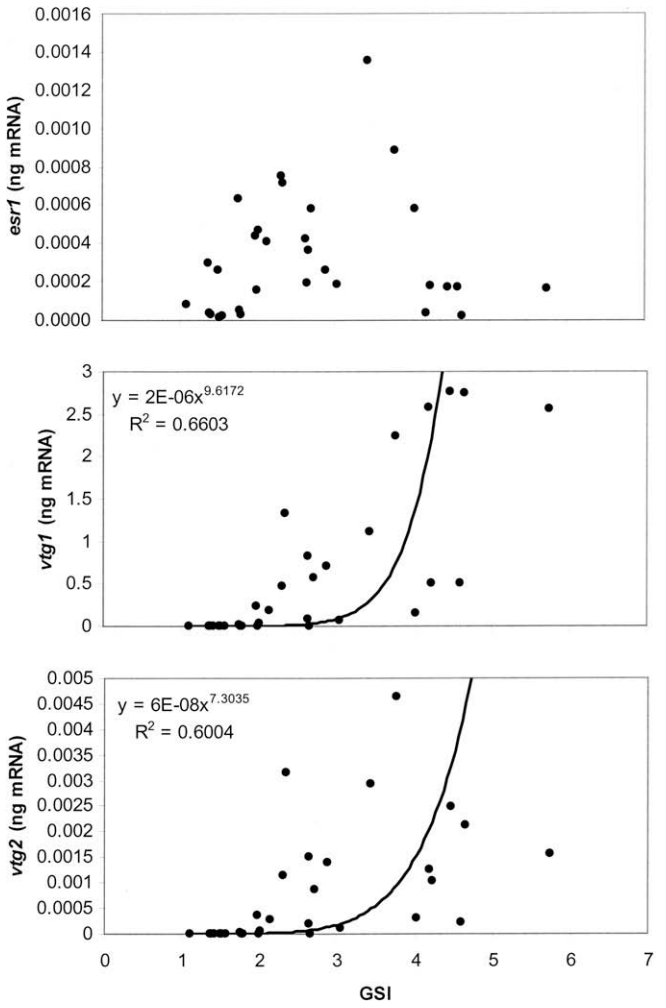

Fig. 8. Timing of *esr1*, *vtg1*- and *vtg2* expression, plotted against maturation parameter GSI (Gonadosomatic index). One-tailed Spearman correlations were significant for GSI vs. *vtg1* ($P < 0.001$) and GSI vs. *vtg2* ($P < 0.001$). Expression of the *esr1* was already apparent at GSI = 1, the expression of *vtg1* and *vtg2* became apparent at GSI = 2.

A. anguilla (Pérez et al., 2008; Palstra and van den Thillart, 2009) as well as for *A. japonica* (Sato et al., 2003, 2006).

4.3. Effects of CPE-treatment on hepatic and ovarian vitellogenesis

Our study showed for the first time that CPE-treatment in European eels stimulated almost instantly hepatic vitellogenesis as evidenced by immediate changes in the expression of its target genes. Levels of 17β-estradiol (E2) and expression of the *esr1* were increased already 1 week after the first CPE injection. As expected, expression of the *esr1* occurred before expression of *vtg1* and *vtg2*. The expression levels of *vtg1* and *vtg2* were increased after one and two injections. Expression of *vtg1* and *vtg2*, and plasma calcium levels further increased after three and four injections while yolk globuli appeared in the oocytes (Fig. 6). Ovarian vitellogenesis thus occurred 1–2 weeks after the start of hepatic vitellogenesis. This indicates that these two processes are under different control. Earlier studies (Olivereau and Olivereau, 1979; Dufour et al., 1983; Burzawa-Gerard and Dumas-Vidal, 1991) have shown that Vtg synthesis and secretion are under control of E2, and that deposition of yolk in the oocytes is under control of gonadotropins.

Durif et al. (2006) found that plasma Vtg levels were already increased after 2 weekly injections, although water temperatures in their study were much lower. Pérez et al. (2008) found that Vtg levels rise at low temperatures of 10 °C, even stronger than at 20 °C. However, Sato et al. (2003) found that for *A. japonica* Vtg levels started to rise after 4 injections in eels kept at 10 °C, while eels at 20 °C showed an immediate rise. Temperature thus has a major influence on the progress of eel vitellogenesis.

Two results are puzzling. Eels that were CPE-injected for 4 weeks showed higher GSIs than eels that were injected for 3 weeks, and plasma E2 and Ca, and *vtg1* and *vtg2* expression were still as high, but the number of fat droplets and the *esr1* expression were lower. Fat droplets might have started fusion at this point thereby lowering their numbers. The lower *esr1* expression is harder to explain. In a study where we measured *esr1* expression in eels that were CPE-injected for 8 weeks (Pérez et al., 2008), resulting, however, in similar GSIs as the eels in this study, *esr1* expression also dropped suggesting that this result is consistent. Several explanations may be appropriate. For instance, the Esr1 may be recycled like suggested for the Vtg receptor which is highly

expressed during previtellogenesis (Perazzolo et al., 1999). Or maybe Esr2 plays a role at the moment that *esr1* expression decreases. Increased plasma growth hormone or prolactin levels may be involved in the facilitation of E2 binding or Vtg may be able to regulate its own synthesis and incorporation (reviewed by Babin et al. (2007)) leading to a lower need of *esr1* expression. The occurrence of fewer fat droplets and the lower esr1 expression may even have a common base. For instance, a decrease in plasma 11-ketotestosterone level may be responsible since androgens have been reported to be involved in Vtg synthesis (reviewed by Babin et al., 2007) and 11-ketotestosterone plays a stimulating role in fat transfer and/or accumulation in oocytes of Japanese eel (Endo et al., 2008). Future investigations on the continuation of vitellogenesis may identify the cause of the drop in *esr1* expression.

4.4. Simultaneous CPE-induced lipid and yolk deposition in the oocytes

During CPE-treatment, fat droplets and yolk globules were found in this study to be deposited at the same time. This corresponds with the results of Adachi et al. (2003) in Japanese eel. This is, however, in contrast to the natural situation where these processes most probably occur sequentially.

As response to swimming, silver eels deposit lipid droplets in the oocytes in large quantities (Palstra et al., 2007). We also observed that swimming stimulates E2 release (van Ginneken et al., 2007b) and growth of the oocytes until 250 μm (Palstra et al., 2007), which marks the onset of vitellogenesis in Japanese eels (Adachi et al., 2003). Indeed, Vtg levels remained low (van Ginneken et al., 2007b; Palstra et al., 2009) and in none of the eels that were subjected to swimming exercise in those investigations, oocytes were found containing yolk globuli (Palstra et al., 2007; van Ginneken et al., 2007b). The separation of both processes by swimming appears to indicate a sequential order of events under natural conditions, quite in contrast to the prompt induction of vitellogenesis by CPE-treatment. This suggests that the instant induction of vitellogenesis by injections of pituitary extract in migrating silver eels does not correspond with natural stimulation of maturation by swimming. The simultaneous occurrence of vitellogenesis and fat deposition in the oocytes may be one of the reasons for the abnormal maturation and low gamete quality in hormone-induced female eel.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2009.09.006.

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