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Identification and stable expression of vitellogenin receptor through vitellogenesis in the European eel

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*In teleosts, vitellogenin (**Vtg**) is a phospholipoglycoprotein synthesized by the liver, released into the blood circulation and incorporated into the oocytes* via *endocytosis mediated by the Vtg receptor (**VTGR**) to form the yolk granules. The VTGR is crucial for oocyte growth in egg-laying animals but is also present in non-oviparous vertebrates, such as human. The VTGR belongs to the low-density lipoprotein receptor superfamily (**LDLR**) and is also named very-low-density lipoprotein receptor (**VLDLR**). In this study, we identified and phylogenetically positioned the VTGR of a basal teleost, the European eel,* Anguilla anguilla*. We developed quantitative real-time PCR (**qRT-PCR**) and investigated the tissue distribution of* vtgr *transcripts. We compared by qRT-PCR the ovarian expression levels of* vtgr *in juvenile yellow eels and pre-pubertal silver eels. We also analyzed the regulation of ovarian* vtgr *expression throughout vitellogenesis in experimentally matured eels. The Vtg plasma level was measured by homologous ELISA experimental maturation. Our* in silico *search and phylogenetical analysis revealed a single* vtgr *in the European eel, orthologous to other vertebrate* vtgr*. The qRT-PCR studies revealed that* vtgr *is mainly expressed in the ovary and also detected in various other tissues such as brain, pituitary, gill, fat, heart, and testis, suggesting some extra-ovarian functions of VTGR. We showed that* vtgr *is expressed in ovaries of juvenile yellow eels with no higher expression in pre-pubertal silver eels nor in experimentally matured eels. This suggests that* vtgr *transcription already occurs during early pre-vitellogenesis of immature eels and is not further activated in vitellogenic oocytes. European eel Vtg plasma level increased throughout experimental maturation in agreement with previous studies. Taken together, these results suggest that* vtgr *transcript levels may not be a limiting step for the uptake of Vtg by the oocyte in the European eel.*

Keywords: *Anguilla anguilla*, teleost, lipoprotein receptor 8, oogenesis, reproduction

Implications

This study brings new insight about the involvement of the vitellogenin system on the control of eel reproduction in captivity. The understanding of the mechanisms that control eel reproduction is crucial to improve gamete quality and to succeed in closing the eel life cycle in captivity. Eel aquaculture worldwide still relies on wild glass eels. Achieving a commercial production of glass eels is imperative to reduce the pressure on the wild population and to preserve the wild stock.

Introduction

Due to its unique life cycle, the European eel (*Anguilla anguilla* L., 1758) is a particularly interesting model for the investigation of the regulatory mechanisms of reproductive physiology. The European eel is a catadromous species, with a complex life cycle. The planktonic eel larvae are transported to the coastal waters of Europe and Northern Africa. They undergo their growth period in continental waters to become yellow eels, prior to physiological and morphological changes called silvering into the silver eel stage. Silvering is a puberty-related event that marks the beginning of the reproductive oceanic migration (Dufour *et al.*, 2003). European silver eels are still sexually immature when they start their reproductive migration. In captivity, long-term hormonal treatments (fish pituitary extracts for females; human chorionic gonadotropin (**hCG**) for males) are required to induce sexual maturation in silver eels, but after these treatments, the egg quality and the survival rate of hatched larvae are still low (Pérez *et al.*, 2000 and 2012).

From an evolutionary point of view, *Anguilla* species are members of the elopomorpha superorder, a diverse group of predominantly marine teleost fishes comprising about 1000 species (Chen *et al.*, 2014). Due their phylogenetical position,

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branching at the base of the teleosts, studies on elopomorpha such as eels may provide insights into ancestral regulatory functions in teleosts, the largest group of vertebrates (Henkel *et al.*, 2012a).

Teleost eggs contain a substantial yolk mass, whose constituents are subsequently used by the embryos and larvae during early development. Large portion of the yolk mass is derived from the phospholipoglycoprotein vitellogenin (**Vtg**) which is synthesized by the liver, released to the blood circulation, and incorporated into the oocytes *via* Vtg-receptor (**VTGR**)-mediated endocytosis. Once in the oocyte, Vtg is processed into yolk proteins that serve as a nutrient source for developing fish embryos and larvae.

The synthesis of Vtg in hepatocytes has been extensively studied and is well demonstrated to be under the control of 17β-estradiol (**E2**) through estrogen receptors (Lafont *et al.*, 2016). In oviparous vertebrates, it has been established that VTGR mediates the entrance of Vtg into oocytes, though in birds and amphibians this receptor may also play a role in incorporation into the oocytes of very-low-density lipoprotein (**VLDL**) (Prat *et al.*, 1998). This notion has been adopted for fish (Hiramatsu *et al.*, 2015). The VTGR belongs to the low-density lipoprotein receptor (**LDLR**) superfamily, and has been also named very-low-density lipoprotein receptor (**VLDLR**), or lipoprotein receptor 8 (**LR8**), due to the presence of eight ligand-binding repeats (Bujo *et al.*, 1994; Hiramatsu *et al.*, 2013). Members of the LDLR family bind various ligands and are involved in lipid metabolism in both vertebrates and invertebrates. In vertebrates, VTGR has been described in non-oviparous species such as in mouse, *Mus musculus*, or in human, *Homo sapiens* (Ali *et al.*, 2012). In teleosts, the VTGR has been previously reported in various species including, for example, rainbow trout, *Oncorhynchus mykiss* (Prat *et al.*, 1998), white perch, *Morone americana* (Hiramatsu *et al.*, 2013), cutthroat trout, *Oncorhynchus clarkii* (Mizuta *et al.*, 2013) or in eels. In short-finned eels (*Anguilla australis)*, no changes were observed in *vtgr* expression between yellow and silver eel. In the European eel (*Anguilla anguilla)*, a decreased *vtgr* expression between yellow and silver eels has been demonstrated, suggesting that VTGR is recycled to the oocyte surface during vitellogenic oocyte growth (Jéhannet *et al.*, 2019).

In the present study, we characterized a single *vtgr* gene in the European eel and performed a phylogenetic analysis. We developed specific quantitative real-time PCR (**qRT-PCR**) for eel *vtgr* in order to study its transcript tissue distribution and ovarian profiles during the maturation process. As the oogenesis is blocked until the experimental maturation in captivity, knowledge on the regulatory mechanisms involved in the vitellogenesis may improve the control of eel reproduction in captivity.

Material and methods

Identification of eel vitellogenin receptor sequence

The *vtgr* sequences of vertebrate species were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) databases. The TBLASTN algorithm of the CLC DNA Workbench software (CLC bio, Aarhus, Denmark) was used to retrieve the genomic sequence coding for *vtgr* from Japanese eel genome (Henkel *et al.*, 2012b). Japanese eel *vtgr* sequence was then used to retrieve the genomic sequence coding for vtgr from European eel genome (Henkel *et al.*, 2012a). The exons and splicing junctions were predicted using the empirical nucleotidic splicing signatures, that is, intron begins with 'GT' and ends with 'AG'.

Phylogenetic analysis

Phylogenetic analysis was performed on VTGR amino acid sequences of five sarcopterygians. The human, representative of mammalians; the anole lizard (*Anolis carolinensis*) and the chicken (*Gallus gallus*) representative of sauropsids; the frog (*Xenopus tropicalis*) representative of amphibians; the coelacanth (*Latimeria chalumnae*), a representative of early sarcopterygians; 1 non-teleost actinopterygian the spotted gar (*Lepisosteus oculatus)* and 11 teleost species; the European and Japanese eels, members of an early group of teleosts (elopomorphs), and 9 members of other teleost groups, whose amino-acid sequences were retrieved from NCBI (www.ncbi.nlm.nih.gov) or Ensembl (http://www.ensembl.org/index.html) databases, first aligned using ClustalW (Thompson *et al.*, 1994) and then manually adjusted (Supplementary Figure S1).

The Jones, Taylor and Thornton (**JTT**) protein substitution matrix of the resulting alignment was determined using ProtTest software (Abascal *et al.*, 2005). Phylogenetic analysis of the resulting alignment was performed using the maximum likelihood method (RaxML software Stamatakis, 2014; www.phylo.org), with 1000 bootstrap replicates. Human, frog, *Xenopus tropicalis*, and tilapia LDL receptor-related protein 8 (**LRP8**) were used as out-group.

The RNA samples for vitellogenin receptor transcript tissue distribution

Tissue distribution of *vtgr* transcripts was performed using the total RNA samples previously obtained (Pasquier *et al.*, 2012) and stored at −80°C in a RNA storage solution (Ambion Inc., Austin, TX, USA.). Total RNA samples of eight female silver eels were used for the following tissues (Pasquier *et al.*, 2012): ovary, brain parts (olfactory bulb, telencephalon, di- and mes-encephalon, cerebellum and medulla oblongata), pituitary, eye, liver, gill, spleen, intestine, fat, muscle, heart and kidney. Total RNA samples of testis from four male silver eels were also used (Pasquier *et al.*, 2012).

Ovarian samples for comparison of vitellogenin receptor transcript levels between yellow and silver stages

To compare the ovarian *vtgr* expression between yellow and silver stages, we used samples of ovaries stored in RNAlater (Ambion Inc.) at −20°C, which had been previously collected from wild yellow and silver female eels caught by Prof. E. Feunteun and co-workers, MNHN, Dinard in the river Fremur, France. Ovarian samples from eight female yellow

eels (body weight 84.9 ± 15.5 g; gonadosomatic index (**GSI** = (gonad weight/total body weight) × 100 (Pankhurst, 1982); 0.29 ± 0.01) and from eight female silver eels (body weight 252.8 ± 16.9 g, GSI 2.27 ± 0.19) were used. Total RNA was extracted using Trizol reagent according to the manufacturer's instructions.

Induction of female eel artificial sexual maturation
Fifty-four wild female silver European eels (mean body weight 847 ± 28 g) were caught in the Albufera Lagoon (Valencia; East Coast of Spain) by local anglers and transferred to the Aquaculture Laboratory in the Universitat Politècnica de València. They were randomly distributed and kept in freshwater at 18°C, in two 1500 l tanks and were gradually acclimated (over 10 days) to seawater (37 ± 0.3‰ of salinity) and moved to three 500-l tanks equipped with separated recirculation systems, thermostats and coolers (to maintain 18°C temperature) and covered to maintain a constant shadow and reduce fish stress.

Groups of 6–8 eels were sacrificed by overanesthesia with benzocaine and decapitated during the first 24 h upon arrival to serve as initial freshwater controls (C), and in seawater conditions (1 week after seawater acclimation; W0), and each 4 weeks through the hormonal treatment until week 12 (W4, W8 and W12).

The hormonal treatment consisted of weekly intra-peritoneal injections of carp pituitary extract (**CPE**; Catvis, Ltd., the Netherlands) at a dose of 20 mg/kg to induce the sexual maturation.

Blood was sampled from the caudal vasculature and centrifuged (3000 rpm, 15 min), and blood plasma was stored at −80°C until immunoassay analyses to determine Vtg plasma levels). Ovaries were sampled and weighted for GSI determination; for histological analysis, ovarian pieces were fixed in 10% formalin buffered to pH 7.4 with phosphate buffer; ovarian pieces were also stored in RNAlater (Ambion-Inc., Austin, TX, USA) and kept frozen at −20°C until RNA extraction.

For all tissues, total RNA was isolated using Trizol reagent (Life Technologies, Inc., Carlsbad, CA) and RNA concentration, 280/260 and 280/230 ratios were evaluated by using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain). The RNA was treated with DNase I of NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. First-strand complementary DNA (**cDNA**) was synthesized from 500 ng of total RNA, using a qScript cDNA synthesis kit (Quanta Bioscience, MD, USA) with a mix of random hexamer and oligo (dT) primers.

Oogenesis stages during experimental maturation
To determine the maturation stage of the ovary in response to the experimental maturation, formalin-fixed samples were dehydrated in ethanol, embedded in paraffin and cut into 5 to 10 μm thick sections with a Shandon Hypercut manual microtome (Shandon, Southern Products Ltd., England). The slides were stained with hematoxylin and eosin and observed through a Nikon Eclipse E-400 microscope equipped with a Nikon DS-5M camera (Tokyo, Japan).

Immunoenzymatic assay of eel vitellogenin
The Vtg plasma levels were assayed using a homologous ELISA previously developed for the European eel (Mazzeo *et al.*, 2014).

Quantitative real-time PCR
Primers. European eel *vtgr*-specific qPCR primers were designed based on European eel coding sequences using Primer3 Software (Whitehead Institute/Massachusetts Institute of Technology, Boston, MA, USA). Primers were designed on two different exons, in order to avoid amplification of potential genomic contamination; primers were tested on genomic DNA and RNA to confirm that only cDNA was amplified. The following primers were used Vtgr fw: GCTCATAGACCGCAAGACC, Vtgr rv: GCCTTACACACGCCAGAAGT (Table 1).

Acidic ribosomal phosphoprotein P0 (**ARP**) was used as a reference gene as previously used for eel ovary (Morini *et al.*, 2015). The previously designed primers were ARP fw: GTG CCA GCT CAG AAC ACTG; ARP rv: ACA TCG CTC AAG ACT TCA ATG G) (Weltzien *et al.*, 2005) (Table 1).

Primers were purchased from Integrate DNA Technology, Inc. (IDT, Coralville, IA).

SYBR green assay. For all the samples, qPCR of eel *vtgr* messenger RNAs (mRNAs) were performed using the model 7500 unit (Applied Biosystems, Foster City, CA, USA) with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fischer Scientific, Waltham, USA). The total volume for PCR was 20 μl, performed with 5 μl of 1/20 diluted DNA template, forward and reverse primers (250 nM each), and SYBR Green/ROX Master Mix (12 μl). The qPCRs were performed as follows: after an initial activation of Taq polymerase at 95°C for 10 min, 40 PCR cycles were performed at the following cycling conditions: 95°C for 10 s and 60°C for 30 s. Each qPCR run contained a non-template control (cDNA was substituted by water) to confirm that reagents were not contaminated. The efficiency was 1.92 for *vtgr* and 2.05 for *arp*. The specificity of each reaction was assessed by melting curve analysis to ensure the presence of only one product. Individual tissue samples were then analyzed in duplicate by qPCR. Serial dilutions of cDNA pool of ovary tissues were run in duplicate and used as a common standard curve.

Table 1 *Quantitative PCR primer sequences for the reference gene acidic ribosomal phosphoprotein P0 (ARP) and the vitellogenin receptor (Vtgr) of the European eel*

Name	Sequence (5′ to 3′)	Orientation	Length/ efficiency
ARP fw	GTG CCA GCT CAG AAC ACG	Forward	107
ARP rv	ACA TCG CTC AAG ACT TCA ATG G	Reverse	$E = 105\%$
Vtgr fw	GCTCATAGACCGCAAGACC	Forward	147
Vtgr rv	GCCTTACACACGCCAGAAGT	Reverse	$E = 92\%$

One of these dilutions was included in each run of the corresponding gene as a calibrator. Normalization of data was performed using total RNA levels for tissue distribution or ARP mRNA levels for comparing *vtgr* expression between ovarian stages.

Statistical analysis. Results are presented as mean ± SEM. Statistical analyses were performed to compare *vtgr* transcript levels and also Vtg plasma levels. Data were first checked for normality and homogeneity of variance of was checked by Bartlett. Due to the heteroscedasticity of variance and the small number of samples, means were compared by the non-parametric Mann–Whitney *U* test for the statistical analysis between yellow and silver stages and by the non-parametric Kruskal–Wallis for the statistical analysis during experimental maturation. Differences were considered significant when $P < 0.05$. All statistical procedures were performed using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).

Results

Characterization and phylogenetic analyses of the eel vitellogenin receptor

The search in European and Japanese eel genomes (Henkel *et al*., 2012a and 2012b) revealed a single eel *vtgr* gene orthologous to the other vertebrate *vtgr*.

Phylogenetic analysis of amino acid VTGR sequences from 17 osteichthyes species of key phylogenetical positions was performed (Figure 1). Phylogenetic analysis revealed that actinopterygian and sarcopterygian VTGR clustered in two monophyletic groups. The VTGR sequence of an actinistian, the coelacanth, branched at the basis of the sarcopterygian clade, in agreement with the phylogenetical position of this species. Similarly, the VTGR sequence of the non-teleost actinopterygian, a holostean, the spotted gar, branched at the basis of the actinopterygian clade. Both Japanese and European eel VTGR sequences branched at the basis of the other teleost sequences, in agreement with the phylogenetical basal position of elopomorpha among teleosts.

Tissue distribution of vitellogenin receptor transcripts

The *Vtgr* mRNA expression was compared in various tissues of eight female silver eels (Figure 2). The ovary was the predominant site of expression of *vtgr* mRNA. The *Vtgr* expression was also found in all brain parts (olfactory bulb, telencephalon, mes-/di-encephalon, cerebellum and medulla oblongata) as well as in the pituitary, and in some peripheral tissues such as gill, fat and heart it being between 35- and 13-fold lower than in ovaries. Very low expression of *vtgr* was detected in other tissues such as eye, liver, spleen, intestine, muscle, and kidney (from 160-fold in the muscle to 3000-fold lower in liver than in ovaries). The *Vtgr* mRNA was also measured in the testis of male silver eels, showing a low but detectable expression, being about 200-fold lower than in ovaries of female silver eels.

Ovarian vitellogenin receptor mRNA expression in yellow and silver eels and during induced maturation

The CPE treatment induced vitellogenesis. The stages observed among control and treated eels were the pre-vitellogenic stage (**PV**, showing peri-nucleolar and lipid droplet stages); early vitellogenic oocytes (**EV**, with small yolk globules restricted to the periphery of the oocyte), mid-vitellogenic oocytes (**MV**, with oocytes having abundant yolk vesicles) and late vitellogenic oocytes (**LV**, showing more abundant yolk vesicles than lipid droplets) (Figure 3).

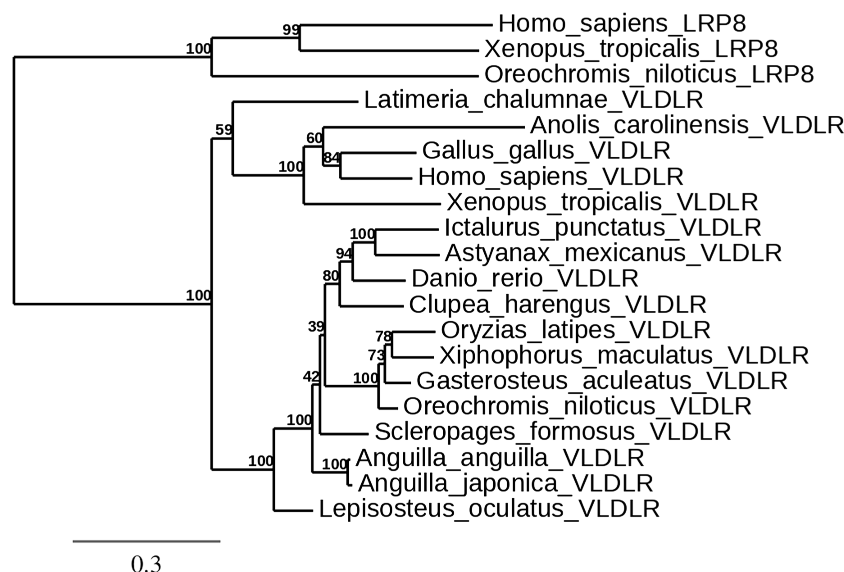

Figure 1 Consensus phylogenetic tree of vertebrate vitellogenin receptor (VTGR/VLDLR). The phylogenetic tree was constructed based on the amino acid sequences of VTGR using the maximum likelihood method with 1000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%). The LDL receptor-related protein 8 (LRP8) was used as the out-group. For references of sequences see Table 1, for sequence alignment see Supplementary Figure S1. VLDLR = very-low-density lipoprotein; LDL = low-density lipoprotein.

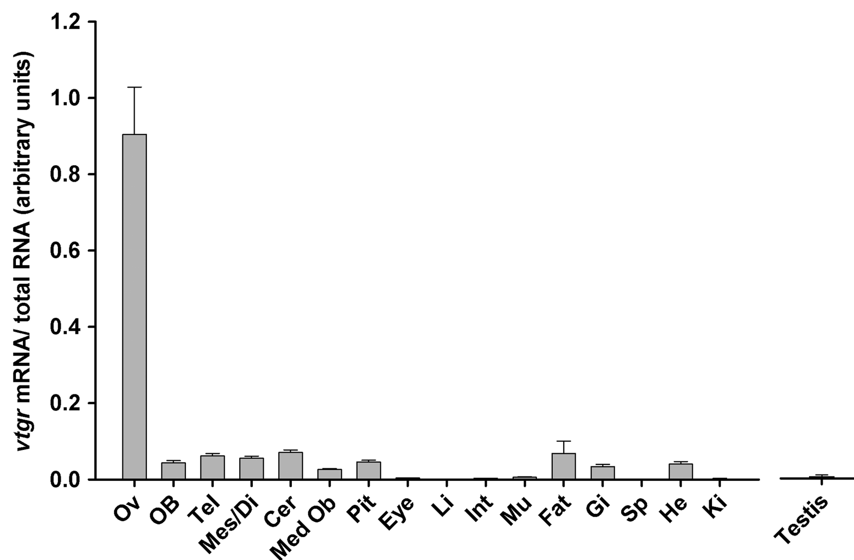

Figure 2 Tissue distribution of vitellogenin receptor mRNA in silver European eel, as analyzed by qPCR. Data are normalized to the amount of total RNA. Values are means ± SEM; $n = 8$ female silver eels for OB = olfactory bulb; Tel = telencephalon; Mes/Di = mes/diencephalon; Pit = pituitary; Cer = cerebellum; Med Ob = medulla oblongata; Ov = ovary; Gi = gill; Li = liver; Sp = spleen; Int = intestine; Mu = muscle; He = heart; Ki = kidney; $n = 4$ male silver eels for testis; mRNA = messenger RNA; qPCR = quantitative PCR. Data are normalized to total RNA.

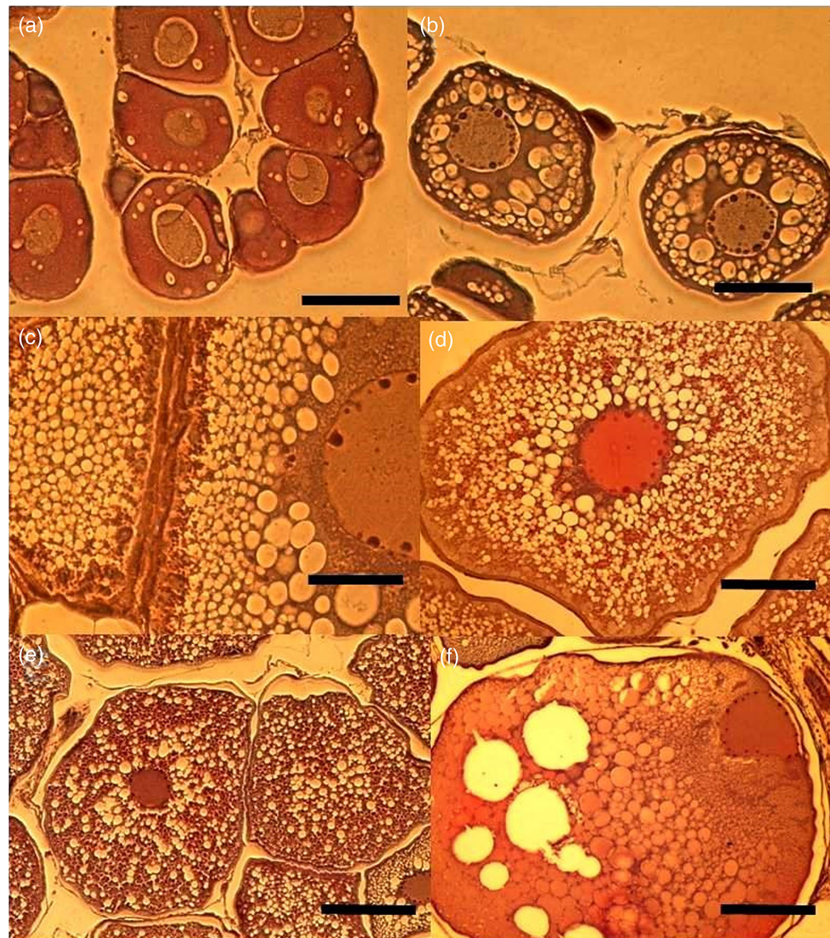

Figure 3 (colour online) Ovarian developmental stages in the European eel. (a) Pre-vitellogenic, perinucleolar stage; (b) pre-vitellogenic, lipid droplet stage; (c) early vitellogenic stage; (d) mid-vitellogenic stage; (e) late vitellogenic stage; (f) nuclear migration stage. Scale Bar: a, b, d: 100 μm; c: 50 μm; e, f: 200 μm.

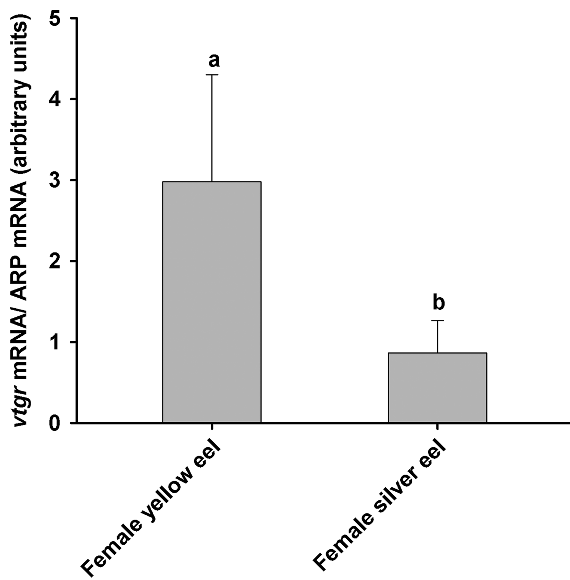

Figure 4 Vitellogenin receptor (vtgr) mRNA expression in the ovary of yellow and silver female European eels, as measured by quantitative PCR (qPCR). Data are normalized to eel acidic ribosomal phosphoprotein P0 (ARP) and results are shown as means ± SEM ($n = 8$ yellow eels; $n = 10$ silver eels). Different letters indicate significant differences ($P < 0.05$).

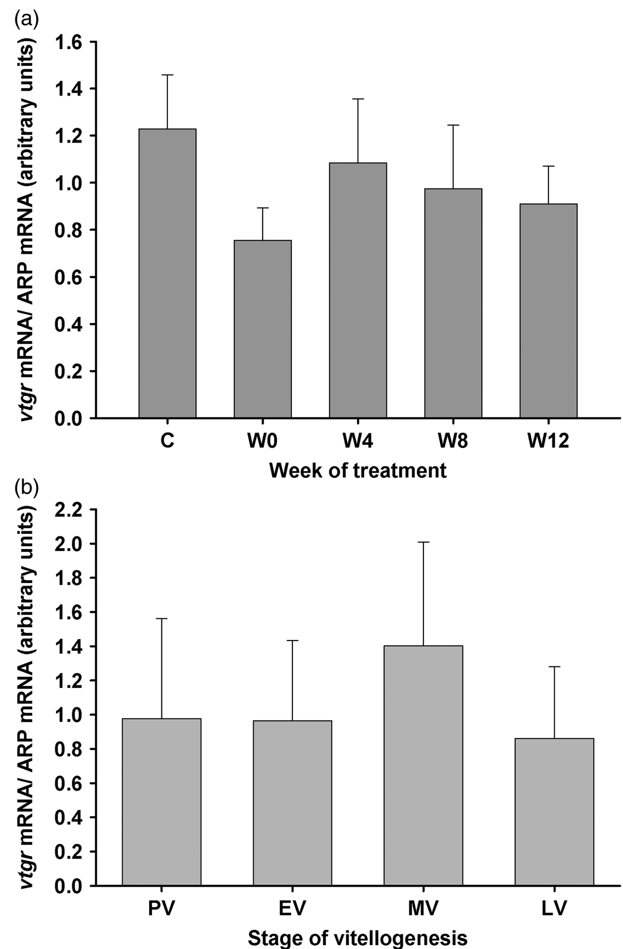

Figure 5 Vitellogenin receptor (vtgr) mRNA expression in the ovary of female European eels during experimental maturation, as measured by quantitative PCR (qPCR). (a) Ovarian vitellogenin receptor mRNA levels in each experimental group ($n = 5$ to 7 eels/group). C: freshwater control; W0: seawater control (week 0); W4: week 4 of carp pituitary extract (CPE) treatment; W8: week 8 of CPE treatment; W12: week 12 of CPE treatment. (b) Ovarian vitellogenin receptor mRNA levels according to oocyte stages. PV = pre-vitellogenic stage; EV = early vitellogenic stage; MV = mid-vitellogenic stage; LV = late vitellogenic stage. See main text for description of gonad developmental stages ($n = 4$ to 16 eels/stage). Data are normalized to eel acidic ribosomal phosphoprotein P0 (ARP) and results are shown as means ± SEM. No significant differences between groups were observed.

The *Vtgr* mRNA was already expressed in the ovary of juvenile yellow eels (GSI 0.29 ± 0.01; primary oocytes) and its relative expression decreased in silver eels (GSI 2.27 ± 0.19; PV or EV) (Figure 4).

Regarding the *vtgr* mRNA expression during the oogenesis induced by hormonal treatment, *vtgr* expression appeared relatively stable. No significant variations were observed between the experimental groups from initial freshwater controls (GSI 1.28 ± 0.20), seawater controls (GSI 0.83 ± 0.11), to 4 weeks (GSI 2.19 ± 0.34), 8 weeks (GSI 4.52 ± 1.05) and 12 weeks (GSI 18.21 ± 3.65) of hormonal treatment (Figure 5a). When analyzed by oocyte stage from PV (GSI 1.04 ± 0.10), EV (GSI 3.01 ± 0.32), MV (GSI 6.60 ± 1.47) to LV (GSI 17.26 ± 1.42), and no significant variation in *vtgr* relative expression was observed (Figure 5b).

Vitellogenin plasma levels in female European eel during induced maturation

Plasma Vtg levels increased progressively during the experimental maturation from PV to LV stage, that is, it was 650-, 1 222.5- and 3 125-fold higher, respectively, in EV, MV and LV vitellogenesis compared to the PV stage (Figure 6, $P < 0.05$).

Discussion

A single vitellogenin receptor gene in the eel

In teleosts, as in other oviparous vertebrates, Vtg is synthesized by the liver, released into the blood circulation and incorporated into the oocytes. In the European eel, two *Vtg* genes were previously described (Palstra *et al.*, 2010). The Vtg endocytosis in the oocyte is mediated by VTGR. Our *in silico* analysis revealed the presence of a single *vtgr* gene in the European eel as in most other vertebrates.

The presence of a single *vtgr* gene in the eel and other teleost species investigated as well as in non-teleost actinopterygian and in sarcopterygians, reveals no impact of the teleost-specific whole genome duplication (**TWGD** also named 3R for third whole genome duplication) on teleost *vtgr* gene number. This suggests that one of the two *vtgr* paralog would have been lost early after the TWGD in the teleost lineage. Two paralogs (*vtgr1* and *vtgr2)* were identified in the rainbow trout (*Oncorhynchus mykiss*; Prat *et al.*, 1998) and Atlantic salmon (*Salmo salar*; Andersen *et al.*, 2017), which likely result from the additional genome duplication event (4R) that occurred specifically in the salmonid lineage. Surprisingly, two *vtgr* paralogs were also reported in a chondrichthyan, the elephant shark (*Callorhinchus milii*)

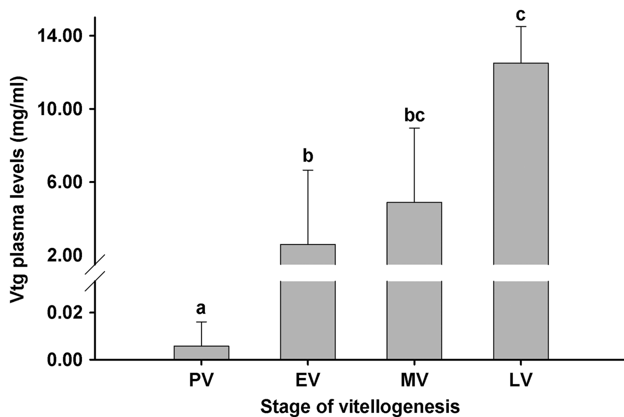

Figure 6 Vitellogenin (Vtg) levels in blood plasma in female European eel during experimental maturation, as measured by ELISA. Results are presented according to the oocyte stages (see legend of Figure 4). PV = pre-vitellogenic stage; EV = early vitellogenic stage; MV = mid-vitellogenic stage; LV = late vitellogenic stage. Results are shown as means ± SEM ($n = 4$ to 17 eels/stage). Different letters indicate significant differences ($P < 0.05$).

(Andersen *et al.*, 2017), the origin of which would deserve further investigation.

Phylogeny analysis placed eel VTGR on the basis of the teleost VTGR clade, in agreement with the basal position of elopomorphs among teleosts. All teleost VTGR sequences, including the eel VTGR, clustered into a monophyletic clade supporting the early loss of the other 3R-paralog, before the emergence of the elopomorphs.

Extra-ovarian vitellogenin receptor expression in European eel

The qPCR analysis of *vtgr* transcript tissue distribution showed the highest expression in the ovary, in agreement with the major role of VTGR in oogenesis in the eel as in all oviparous vertebrates. Our study also revealed moderate *vtgr* expression in various somatic tissues such as brain, pituitary, gill, fat and heart. A few studies also reported extra-ovarian expression of *vtgr* in some other teleosts: in heart and brain of cutthroat trout (Mizuta *et al.*, 2013); in heart, liver and brain of Atlantic salmon (Andersen *et al.*, 2017) and in liver and muscle of white perch (*Morone americana*) (Hiramatsu *et al.*, 2013). In oviparous tetrapods, contrasting results were reported in amphibians and birds. In frog, high *vtgr* expression was found in heart (Okabayashi *et al.*, 1996), while in chicken *vtgr* expression is restricted to the oocyte where it is involved in VLDL and Vtg uptake into the oocyte (Bujo *et al.*, 1994). In non-oviparous mammals, *vtgr* is predominantly expressed in the heart, brain and adipose tissues (Takahashi *et al.*, 1992), which is similar to the extra-ovarian expression of *vtgr* found in the eel. This suggests that extra-ovarian functions may represent ancient properties of VTGR largely conserved throughout vertebrate evolution.

Various studies in mammals proposed a signaling role of VTGR in brain and its implication in neurodevelopment. This is consistent with our finding of the expression *vtgr* in different parts of the eel brain (olfactory bulbs, telecephalon, mes- and di-encephalon, cerebellum and medulla oblongata). In human, VTGR expression has been shown in several brain cell types during early development, and VTGR may function as signal transducer for the reelin, a secreted glycoprotein that regulates neuronal positioning in laminated structures of the developing brain (Herz and Bock, 2002). Consistent with this, VTGR-knockout mice exhibit neurodevelopmental defects in laminated structures of the central nervous system (Trommsdorff *et al.*, 1999). Genetic studies of human VTGR mutations strongly support the role of VTGR in neuronal function (Ali *et al.*, 2012).

Recently, Nader *et al.* (2018) described a novel function for VTGR, as chaperone-trafficking function for membrane progesterone receptor beta (**mPRβ**) in frog. The authors showed that the VTGR is required for the transit of mPRβ from the endoplasmic reticulum to the Golgi. Such a mechanism may be hypothesized in other vertebrates, including in the eel, in which we previously described five mPRs (mPRα, mPRAL1, mPRAL2, mPRγ and mPRδ) with a large tissue distribution (Morini *et al.*, 2017).

We found *vtgr* expression in eel testis, as also reported in mammals (Takahashi *et al.*, 1992) and amphibians (Okabayashi *et al.*, 1996). Low levels of *vtgr* transcripts have been detected in the testis of a non-teleost actinopterygian, the white sturgeon (*Acipenser transmontanus*), and in some teleosts, zebrafish (*Danio rerio*), and tilapia (*Oreochromis mossambicus*) (Bidwell and Carlson, 1995). It was hypothesized that *vtgr* may be involved in nutrient transport, uptake of hormones, vitamins, and other biomolecules for white sturgeon spermatocytes (Bidwell and Carlson, 1995).

Early expression of vitellogenin receptor during oogenesis in the eel

In the European eel, we showed that *vtgr* is already expressed in the ovary of the juvenile yellow eel and no further increase in the relative expression was observed in the pre-pubertal silver eel (Jéhannet *et al.*, 2019) nor throughout the experimental maturation. These observations suggest that *vtgr* transcription already occurs during early PV of immature fish and is not further activated in vitellogenic oocytes. Similarly, no increase in *vtgr* mRNA expression associated with oocyte growth has been shown in white perch, largemouth bass (*Micropterus salmoides)*, cutthroat trout and Atlantic salmon, with *vtgr* highly expressed in ovaries during PV and gradually decreased during vitellogenesis (Hiramatsu *et al.*, 2013; Mizuta *et al.*, 2013; Andersen *et al.*, 2017). This suggests a similar expression pattern for all teleosts including the eels.

Vitellogenin receptor mRNA expression is not a limiting step for oocyte incorporation of vitellogenin in the eel

Previous studies in European eel led to the conclusion that immature ovaries were not able to incorporate Vtg (Dufour *et al.*, 1988). Treating the pre-pubertal female silver eel with E2 stimulates Vtg synthesis and release by the liver leading to high Vtg plasma levels but does not induce Vtg incorporation in yolk granules in the oocyte. Treatment with exogenous

gonadotropin (Burzawa-Gerard and Dumas-Vidal, 1991) or stimulation of endogenous gonadotropin release (Dufour *et al.*, 1988) is necessary to induce Vtg incorporation into the oocyte. In the rainbow trout, unilateral ovariectomy during EV lead to an increase in plasma FSH which may mediate vitellogenic development (Tyler et al., 1997). Remarkably, however, in the present study we demonstrated that *vtgr* transcripts were already expressed in immature yellow and silver eels. Furthermore, European eel Vtg plasma level increased throughout experimental maturation, while ovarian *vtgr* expression remained relatively stable. These results suggest that oocyte *Vtgr* expression may not be a limiting step for the uptake of Vtg by the oocyte in the European eel. They also indicate that ovarian *vtgr* expression is not under the regulation of pituitary gonadotropins.

Nonetheless, our analyses are based on *vtgr* gene expression and data about the regulation of the VTGR protein are lacking. In the cutthroat trout, VTGR proteins seem to be synthesized and stored in the ooplasm before the onset of vitellogenesis. During the vitellogenesis, the Vtg-VTGR complex is internalized and VTGR is recycled back to the surface of the oolemma (Mizuta *et al.*, 2013). Further analyses of VTGR protein levels are needed to clarify the molecular mechanisms underlying the ovarian uptake of Vtg during vitellogenesis in the eel.

Other potential key actors in vitellogenin incorporation in the eel

Among the LDLR family members, other possible lipoprotein receptors are involved in the endocytosis of lipoproteins and thus in fish ovarian growth and yolk formation process. Both very low-density lipoprotein (**VLDL**) and low-density lipoprotein (**LDL**) have been considered to be candidate transporters of neutral lipids as triacylglycerides and cholesterol into the oocytes (Hiramatsu *et al.*, 2013). The LDLR has been extensively studied for its ligand-binding properties in mammals and may be involved in binding and endocytosis of LDL in chicken (Hummel *et al.*, 2003). In the short-finned eel (*Anguilla australis*), the authors suggested that LDLR is a major player implicated in the accumulation of ovarian fatty acid (Damsteegt *et al.*, 2015). Furthermore, according to Reading *et al.* (2014), the LDLR-related protein 13 (named Lrp13), also member of the LDLR superfamily, appears to function as a VTGR and may be an important mediator of yolk formation in fish. In addition, Lrp13 was shown to bind the Vtg in perch and tilapia and was implicated as an important mediator of yolk deposition in other oviparous vertebrates (Hiramatsu *et al.*, 2015; Andersen *et al.*, 2017). Regarding the LR7-type LDLR, functional similarities with the classical VTGR in terms of ovarian yolk formation *via* receptor-mediated endocytosis of lipoproteins was found in the cutthroat trout (Mizuta *et al.*, 2013). In the European eel, new candidates such as LDLR, LDLR-related protein 13 or LR7-type LDLR may also be involved and have to be explored.

Membrane-associated components may also be involved in the endocytosis of Vtg. Mizuta *et al.* (2013) proposed a model for Vtg uptake mediated by VTGR in salmonids, where the Vtg-VTGR complex is internalized through clathrin-coated pits. Clathrin seems to be a good candidate as *cltc-a1* and *vtgr* transcript levels were significantly correlated in cutthroat trout (Mizuta *et al.*, 2017). Other molecules may be involved in the endocytosis of Vtg, as tight junction (**TJ**) which regulates the passage of ions and small molecules between cells, and adherent junctions, physically linked to the TJ. In zebrafish, according to Clelland and Kelly (2010), exogenous E2 had little effect on TJ machinery in PV follicles but reduced the abundance of two transcripts of claudin orthologs in mid- to late-stage vitellogenic follicles. They proposed the possibility that E2 may alter TJ integrity, thereby regulating the access of Vtg to the oocyte and the follicle development (Clelland and Kelly, 2010). In the European eel, up-regulation of Vtg under estradiol treatment was observed in eel *in vivo* (Burzawa-Gérard and Dumas-Vidal, 1991) as well as *in vitro* by hepatocyte primary cultures (Lafont *et al.*, 2016). Nevertheless, ovaries from E2-treated eels are not able to incorporate Vtg (Dufour *et al.*, 1988), and CPE treatment is necessary to induce ovarian vitellogenesis (Mazzeo *et al.*, 2014). We suggest that the gonadotropic treatment modulates the expression of TJ proteins, which may represent a limiting factor for the incorporation of Vtg in the oocytes, through a stable expression of VTGR. This regulatory pathway deserves further investigation.

In conclusion, we characterized the European eel single *vtgr* and investigated the regulation of its transcript levels in the ovary between yellow and silver stages as well as during hormonal induction of vitellogenesis by gonadotropic treatment. Interestingly, *vtgr* mRNA was already expressed at the PV stage in yellow eel, and its expression did not increase at silvering nor during induced vitellogenesis, whereas Vtg plasma level increases through oocyte development. This suggests that ovarian vitellogenesis is not mediated through increased levels of *vtgr* expression, so that the *vtgr* expression may not be a limiting step for the uptake of Vtg by the oocyte in the European eel. This study also revealed extra-ovarian expression of *vtgr*, such as in the brain or in the testis, which may reflect ancestral functions of VTGR, independent of vitellogenesis, and conserved among oviparous and non-oviparous vertebrates.

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Declaration of interest

The authors declare no conflict of interests.

Ethics statement

Eels were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 2010/63/EU). In order to comply with the '3R' ethical recommendation (reduce, refine, replace) for animal experimentation and especially for endangered species such as the European eel, we made sure to use previous samples where possible, in order to reduce the number of eels to be sacrificed.

The experimental maturation study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 on protection of animals used for scientific purposes. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Universitat Politècnica de València (UPV) (Permit Number: 2014/VSC/PEA/00147). All efforts were made to minimize animal suffering and stress.

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary material

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References

Abascal F, Zardoya R and Posada D 2005. ProtTest: selection of best-fit models of protein evolution. Bioinformatics 21, 2104–2105.

Ali BR, Silhavy JL, Gleeson MJ, Gleeson JG and Al-Gazali L 2012. A missense founder mutation in VLDLR is associated with Dysequilibrium Syndrome without quadrupedal locomotion. BMC Medical Genetics 13, 80.

Andersen Ø, Xu C, Timmerhaus G, Kirste KH, Næve I, Mommens M and Tveiten H 2017. Resolving the complexity of vitellogenins and their receptors in the tetraploid Atlantic salmon (*Salmo salar*): ancient origin of the phosvitin-less VtgC in chondrichthyean fishes. Molecular Reproduction and Development 84, 1191–1202.

Bidwell CA and Carlson DM 1995. Characterization of vitellogenin from white sturgeon, *Acipenser transmontanus*. Journal of Molecular Evolution 41, 104–112.

Bujo H, Hermann M, Kaderli MO, Jacobsen L, Sugawara S, Nimpf J, Yamamoto T and Schneider WJ 1994. Chicken oocyte growth is mediated by an eight ligand binding repeat member of the LDL receptor family. The EMBO Journal 13, 5165–5175.

Burzawa-Gerard E and Dumas-Vidal A 1991. Effects of 17 beta-estradiol and carp gonadotropin on vitellogenesis in normal and hypophysectomized European silver female eel (*Anguilla anguilla* L.) employing a homologous radioimmunoassay for vitellogenin. General and Comparative Endocrinology 84, 264–276.

Chen JN, López JA, Lavoué S, Miya M and Chen WJ 2014. Phylogeny of the Elopomorpha (Teleostei): evidence from six nuclear and mitochondrial markers. Molecular Phylogenetics and Evolution 70, 152–161.

Clelland ES and Kelly SP 2010. Tight junction proteins in zebrafish ovarian follicles: stage specific mRNA abundance and response to 17b-estradiol, human chorionic gonadotropin, and maturation inducing hormone. General and Comparative Endocrinology 168, 388–400.

Damsteegt EL, Mizuta H, Hiramatsu N and Lokman PM 2015. How do eggs get fat? Insights into ovarian fatty acid accumulation in the shortfinned eel, *Anguilla australis*. General and Comparative Endocrinology 221, 94–100.

Dufour S, Burzawa-Gerard E, Le Belle N, Sbaihi M and Vidal B 2003. Reproductive endocrinology of the European eel, *Anguilla anguilla*. In Eel biology (eds. K Aida, K Tsukamoto and K Yamauchi), pp 373–383. Springer-Verlag, Tokyo, Japan.

Dufour S, Lopez E, Le Menn F, Le Belle N, Baloche S and Fontaine YA 1988. Stimulation of gonadotropin release and of ovarian development, by the administration of a gonadoliberin agonist and of dopamine antagonists, in female silver eel pretreated with estradiol. General and Comparative Endocrinology 70, 20–30.

Henkel CV, Burgerhout E, de Wijze DL, Dirks RP, Minegishi Y, Jansen HJ, Spaink HP, Dufour S, Weltzien FA, Tsukamoto K and van den Thillart GE 2012a. Primitive duplicate Hox clusters in the European eel's genome. PLoS ONE 7, e32231.

Henkel CV, Dirks RP, de Wijze DL, Minegishi Y, Aoyama J, Jansen HJ, Turner B, Knudsen B, Bundgaard M, Hvam KL, Boetzer M, Pirovano W, Weltzien FA, Dufour S, Tsukamoto K, Spaink HP and van den Thillart GE 2012b. First draft genome sequence of the Japanese eel, *Anguilla japonica*. Gene 511, 195–201.

Herz J and Bock HH 2002. Lipoprotein receptors in the nervous system. Annual Review of Biochemistry 71, 405–434.

Hiramatsu N, Luo W, Reading BJ, Sullivan CV, Mizuta H, Ryu YW, Nishimiya O, Todo T and Hara A 2013. Ovarian lipoprotein receptors in teleosts. Fish Physiology and Biochemistry 39, 29–32.

Hiramatsu N, Todo T, Sullivan CV, Schilling J, Reading BJ, Matsubara T, Ryu YW, Mizuta H, Luo W, Nishimiya O, Wuf M, Mushirobira Y, Yilmaz O and Hara A 2015. Ovarian yolk formation in fishes: molecular mechanisms underlying formation of lipid droplets and vitellogenin-derived yolk proteins. General and Comparative Endocrinology 221, 9–15.

Hummel S, Lynn EG, Osanger A, Hirayama S, Nimpf J and Schneider WJ 2003. Molecular characterization of the first avian LDL receptor: role in sterol metabolism of ovarian follicular cells. Journal of Lipid Research 44, 1633–1642.

Jéhannet P, Kruijt L, Damsteegt EL, Swinkels W, Heinsbroek LTN, Lokman PM and Palstra AP. A mechanistic model for studying the initiation of anguillid vitellogenesis by comparing the European eel (*Anguilla anguilla*) and the shortfinned eel (*A. australis*). General and Comparative Endocrinology (in press). https://doi.org/10.1016/j.ygcen.2019.02.018

Lafont AG, Rousseau K, Tomkiewicz J and Dufour S 2016. Three nuclear and two membrane estrogen receptors in basal teleosts, *Anguilla sp.*: identification, evolutionary history and differential expression regulation. General and Comparative Endocrinology 235, 177–191.

Mazzeo I, Peñaranda DS, Gallego V, Baloche S, Nourizadeh-Lillabadi R, Tveiten H, Dufour S, Asturiano JF, Weltzien FA and Pérez L 2014. Temperature modulates the progression of vitellogenesis in the European eel. Aquaculture 434, 38–47.

Mizuta H, Luo W, Ito Y, Mushirobira Y, Todo T, Hara A, Reading BJ, Sullivan CV and Hiramatsu N 2013. Ovarian expression and localization of a vitellogenin receptor with eight ligand binding repeats in the cutthroat trout (*Oncorhynchus clarki*). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 166, 81–90.

Mizuta H, Mushirobira Y, Nagata J, Todo T, Hara A, Reading BJ, Sullivan CV and Hiramatsu N 2017. Ovarian expression and localization of clathrin (Cltc) components in cutthroat trout, *Oncorhynchus clarki*: evidence for Cltc involvement in endocytosis of vitellogenin during oocyte growth. Comparative Biochemistry and Physiology, Part A: Molecular and Integrative Physiology 212, 24–34.

Morini M, Peñaranda DS, Vílchez MC, Gallego V, Nourizadeh-Lillabadi R, Asturiano JF, Weltzien FA, Pérez L 2015. Transcript levels of the soluble sperm factor protein phospholipase C zeta 1 (PLCζ1) increase through induced spermatogenesis in European eel. Comparative Biochemistry and Physiology Part A 187, 168–176.

Morini M, Peñaranda DS, Vílchez MC, Gallego V, Nourizadeh-Lillabadi R, Lafont AG, Dufour S, Asturiano JF, Weltzien FA and Pérez L 2017. Nuclear and membrane progestin receptors in the European eel: characterization and expression in vivo through spermatogenesis. Comparative Biochemistry and Physiology Part A 207, 79–92.

Nader N, Dib M, Courjaret R, Hodeify R, Machaca R, Graumann J and Machaca K 2018. The VLDL receptor regulates membrane progesterone receptor trafficking and non-genomic signaling. Journal of Cell Science 131, jcs212522.

Okabayashi K, Shoji H, Nakamura T, Hashimoto O, Asashima M and Sugino H, 1996. cDNA cloning and expression of the *Xenopus laevis* vitellogenin receptor. Biochemical and Biophysical Research Communications 224, 406–413.

Palstra AP and van den Thillart GE 2010. Swimming physiology of European silver eels (*Anguilla anguilla* L.): energetic costs and effects on sexual maturation and reproduction. Fish Physiology and Biochemistry 36, 297–322.

Pankhurst NW 1982. Relation of visual changes to the onset of sexual maturation in the European eel *Anguilla anguilla* (L.). Journal of Fish Biology 21, 127–140.

Pasquier J, Lafont A-G, Jeng S-R, Morini M, Dirks R, van den Thillart G, Tomkievicz J, Tostivint H, Chang C-F, Rousseau K and Dufour S 2012. Multiple kisspeptin receptors in early osteichthyans provide new insights into the evolution of this receptor family. PLoS ONE 7, e48931.

Pérez L, Asturiano JF, Tomás A, Zegrari S, Barrera R, Espinós JF, Navarro JC and Jover M 2000. Induction of maturation and spermiation in the male European eel: assessment of sperm quality throughout treatment. Journal of Fish Biology 57, 1488–1504.

Pérez L, Vílchez MC, Gallego V, Mazzeo I, Peñaranda DS, Weltzien FA, Dufour S and Asturiano JF 2012. Trying to reproduce the European eel (Anguilla anguilla) under captivity: experiments with females. In Proceeding of the Domestication in Finfish Aquaculture, October 2012, Olsztyn, Poland, pp. 11–15.

Prat F, Coward K, Sumpter J and Tyler C 1998. Molecular characterization and expression of two ovarian lipoprotein receptor in the rainbow trout, *Oncorhynchus mykiss*. Biology of Reproduction 58, 1146–1153.

Reading BJ, Hiramatsu N, Schilling J, Molloy KT, Glassbrook N, Mizuta H, Luo W, Baltzegar DA, Williams VN, Todo T, Hara A and Sullivan CV 2014. Lrp13 is a novel vertebrate lipoprotein receptor that binds vitellogenins in teleost fishes. Journal of Lipid Research 55, 2287–2295.

Stamatakis A 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313.

Takahashi S, Kawarabayashi Y, Nakai T, Sakai J and Yamamoto T 1992. Rabbit very low density lipoprotein receptor: a low density lipoprotein receptor-like protein with distinct ligand specificity. Proceedings of the National Academy of Sciences of the United States of America. 89, 9252–9256.

Thompson JD, Higgins DG and Gibson TJ 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22, 4673–4680.

Trommsdorff M, Gotthardt M, Hiesberger T, Shelton J, Stockinger W, Nimpf J, Hammer RE, Richardson JA and Herz J 1999. Reeler/disable-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. Cell 97, 689–701.

Tyler CR, Pottinger TG, Coward K, Prat F, Beresford N, Maddix S, 1997. Salmonid follicle-stimulating hormone (gth i) mediates vitellogenic development of oocytes in the rainbow trout, *Oncorhynchus mykiss*. Biology of Reproduction 57, 1238–1244.

Weltzien FA, Pasqualini C, Vernier P and Dufour S 2005. A quantitative real-time RT-PCR assay for European eel tyrosine hydroxylase. General and Comparative Endocrinology 142, 134–142.