Molecular physiology of a teleost oocyte aquaporin: Evolution, regulation, and role during oocyte hydration

Fisiología molecular de una acuaporina ovocitaria de teleósteos: Evolución, regulación y papel durante la hidratación del oocito

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Als meus pares, al meu germà i a tu Aure,
gràcies per tot

«Més lluny, heu d'anar més lluny...
més lluny del demà que ara ja s'acosta.
I quan creieu que arribeu, sapigueu trobar noves sendes.»
Itaca
(Kavafis - Carles Riba - Lluís Llach)

«Els ànecs salvatges volaven
arran de la lluïssor de les llacunes immòbils com un mirall
i s'arraulien al si de les jonqueres.
El far de Buda alçava el punt rutilant sobre la plana,
mostrant-lo adés a la mar, adés a la muntanya.
I tota la nit seguia el mateix joc infatigable...»
Nocturn a les Terres de l'Ebre
(Sebastià Juan Arbó)

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Prologue

This thesis was carried out at the Group of Comparative Molecular Physiology of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA), under the Program of Aquaculture, during the period 2008-2012. The objective of this thesis was to begin to dissect the molecular mechanisms involved in the regulation of the teleost-specific aquaporin-1ab (*aqp1ab*) gene (formely termed Aqp1o or Aqp1b) in the oocyte of marine teleosts, and to provide functional evidence for its role during oocyte hydration. To this aim, we employed a variety of molecular and cellular approaches using two model organisms, the gilthead seabream (*Sparus aurata*) and the Atlantic halibut (*Hippoglossus hippoglossus*). The results obtained have elucidated the evolutionary origin of *aqp1ab* in teleosts, and have uncovered for the first time the mechanisms for its precise transcriptional activation during oogenesis. In addition, we have demonstrated the essential and conserved physiological role of Aqp1ab during the hydration of oocytes undergoing meiosis resumption in marine teleosts. These findings thus uncover the importance of the *aqp1ab* gene for the production of viable eggs in marine teleosts, and contribute with a new molecular biomarker for the evaluation of egg quality in aquaculture species.

The thesis presented here is structured in four chapters that correspond to four different scientific papers:

Chapter I: Zapater C, Chauvigné F, Norberg B, Finn RN, Cerdà J. 2011. **Dual neofunctionalization of a rapidly evolving aquaporin-1 paralog resulted in constrained and relaxed traits controlling channel function during meiosis resumption in teleosts.** *Molecular Biology and Evolution* 28:3151-3169.

Chapter II: Zapater C, Chauvigné F, Scott AP, Gómez A, Katsiadaki I, Cerdà J. 2012. **Piscine follicle-stimulating hormone triggers progestin production in gilthead seabream primary ovarian follicles.** *Biology of Reproduction* 87(5):111, 1-13.

Chapter III: Zapater C, Chauvigné F, Fernández-Gómez B, Finn RN, Cerdà J. 2013. **Alternative splicing of the nuclear progestin receptor in a perciform teleost generates novel mechanisms of dominant-negative transcriptional regulation.** *General and Comparative Endocrinology* 182:24-40.

Chapter IV: Zapater C, Chauvigné F, Tingaud-Sequeira A, Finn RN, Cerdà J. 2013. **Primary oocyte transcriptional activation of *aqp1ab* by the nuclear progestin receptor determines the pelagic egg phenotype of marine teleosts.** *Developmental Biology* (in press; doi: 10.1016/j.ydbio.2013.03.001)

During the realization of this thesis, two reviews focused on the role of aquaporins during fish gametogenesis and embryogenesis, one of them reporting preliminary data of the major findings presented in this thesis, have been published:

Chauvigné F, Zapater C, Cerdà J. 2011. Role of aquaporins during teleost gametogenesis and early embryogenesis. ***Frontiers in Physiology*** 2:66.

Cerdà J, Zapater C, Chauvigné F, Finn RN. 2013. Water homeostasis in the fish oocyte: new insights into the role and molecular regulation of a teleost-specific aquaporin. ***Fish Physiology and Biochemistry*** 39:19-27.

The results derived from the present thesis have been presented at the following scientific meetings: 9th International Congress on the Biology of Fish, Barcelona, Spain (July 2010); 9th International Symposium of Reproductive Physiology of Fish, Cochin, India (August 2011,); 7th International Symposium on Fish Endocrinology, Buenos Aires, Argentina (September 2012); and Global Questions on Advanced Biology, Societat Catalana de Biologia, Barcelona, Spain (July 2012).

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Abbreviations

AGI, DL-aminoglutethimide
AQP/Aqp, aquaporin
AQP1o, aquaporin-1 of the ovary
A4, androstenedione
BUS, B-upstream segment
β-Gal, β-galactosidase
βME, β-mercaptoethanol
C, cortisol
cAMP, cyclic AMP
Cbr1, 20β-hydroxysteroid dehydrogenase/carbonyl reductase-like
Cga, gonadotropin α subunit
ChIP, chromatin immunoprecipitation
Ct, cycle threshold
Cyp17a1, cytochrome P450c17 polypeptide I
Cyp17a2, cytochrome P450c17 polypeptide II
Cyp19a1, cytochrome P450 ovarian aromatase
DBD, DNA binding domain
DIG, digoxigenin
ECL, enhanced chemiluminescence
EH, early hydrating oocyte
ELISA, enzyme-linked immunosorbent assay
ER, estrogen receptor
E2, 17β-estradiol
FAA, free amino acid
FSH/Fsh, follicle-stimulating hormone
Fshra, Fsh receptor isoform a
GSI, gonadosomatic index
H, hydrated oocyte
hCG, human chorionic gonadotropin
HRE, hormone responsive element
HRP, horseradish peroxidase
HSD, hydroxysteroid dehydrogenase
IGF-I/II, insulin growth factor I or II
IgG, immunoglobulin G
Indo, indomethacin
LBD, ligand binding domain
LH/Lh, luteinizing hormone
Lhcgrba, Lh receptor isoform ba
LvH, lipovitellin heavy chain
LvL, lipovitellin light chain
MBS, modified Barth's culture medium
MH, mid-hydrated oocyte
MPF, maturation-promoting factor
mPGR/mPgr, membrane progestin receptor
ORF, open reading frame
P_f, osmotic water permeability
PFA, paraformaldehyde
PGC, primordial germ cell
PGR/Pgr, nuclear progestin receptor
PGR-A/B, mammalian PGR isoform A or B
Pgr_tv1, nuclear Pgr transcript variant 1
Pgr_tv2, nuclear Pgr transcript variant 2
Pgr_tv3, nuclear Pgr transcript variant 3
Pgr_tv4, nuclear Pgr transcript variant 4
PhB, phenylbutazone
PNGase, N-glycosidase F
PRE, progestin responsive element
PV, postvitellogenic non-hydrated oocyte
P4, progesterone
P450scc, cytochrome P450scc (Cyp11a1)
qRT-PCR, real-time quantitative RT-PCR
qPCR, real-time quantitative PCR
RACE, rapid amplification of cDNA ends
rFsh, recombinant follicle-stimulating hormone
RIA, radioimmunoassay
RT-PCR, reverse transcriptase polymerase chain reaction
SDS-PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SOX/Sox, SRY-related high mobility group (HMG)-box
Star, steroidogenic acute regulatory protein
T, testosterone
TSS, transcription start site
UTR, untranslated region
Vg/Vtg, vitellogenin
WGD, whole genome duplication

3β-Hsd, 3β hydroxysteroid dehydrogenase
17β-Hsd, 17β hydroxysteroid dehydrogenase
17-P, 17α-hydroxyprogesterone
17,20β-P, 17α,20β-dihydroxy-4-pregnen-3-one
17,20β,21-P, 17α,20β,21-trihydroxy-4-pregnen-3-one
17,21-P, 17,21-dihydroxy-4-pregnen-3,20-dione

INTRODUCTION

In teleosts, oogenesis is a highly regulated process that includes different stages of development, from differentiation and proliferation of primordial germ cells (PGCs) and subsequent oocyte growth, to the formation of viable mature egg ready for fertilization (Lubzens et al. 2010). This process includes many structural and functional changes that ultimately result in the formation of a viable egg. However, the molecular mechanisms involved, athough starting to be uncovered, remain in most cases largely unknown. Most studies have mainly focused on the hormonal regulation of secondary growth and meiosis resumption ('oocyte maturation') while the molecular regulation of early oocyte growth is poorly understood. In this short introduction, I will summarize the current knowledge on the major processes during teleost oogenesis.

1. Stages During Oogenesis in Teleosts

In teleosts, oogenesis can be divided into six periods according to the extend of oocyte growth: oogonia proliferation, primary oocyte growth, cortical alveolus stage, vitellogenesis, maturation and ovulation (Lubzens et al. 2010). The PGCs are differentiated into oogonia which proliferate by mitosis. Oogonia enter into meiosis to form primary oocytes, and these start to connect with somatic cells (pre-follicle cells) for the formation of the ovarian follicle. During the primary growth stage, meiosis is arrested at the diplotene stage (prophase of the first meiotic division), and it remains arrested until the end of the vitellogenic (growth) period. During this period, the oocyte increases considerably in size due to cortical alveoli synthesis, vitellogenin incorporation and yolk protein accumulation. However, during oocyte maturation, the hydration of the oocyte is responsible for almost all the final size of the egg, which is ready to be ovulated and fertilized (Cerdà et al. 2007). The main physiological mechanisms during oocyte growth and maturation in teleosts are summarized in Fig. 1.

1.1. Differentiation of PGCs

The PGCs generate haploid reproductive cells or gametes which are responsible for the development of a new organism transmitting genetic information from one generation to the next. Small numbers of PGCs are produced shortly after fertilization of the egg and during the early stage of embryogenesis, and then migrate from their site of origin towards the position of the gonads where they differentiate into gametes (Wylie 2000; Starz-Gaiano and Lehmann 2001). The PGCs show very cytosolic features as "nuage", which is an aggregation of RNAs and pro-

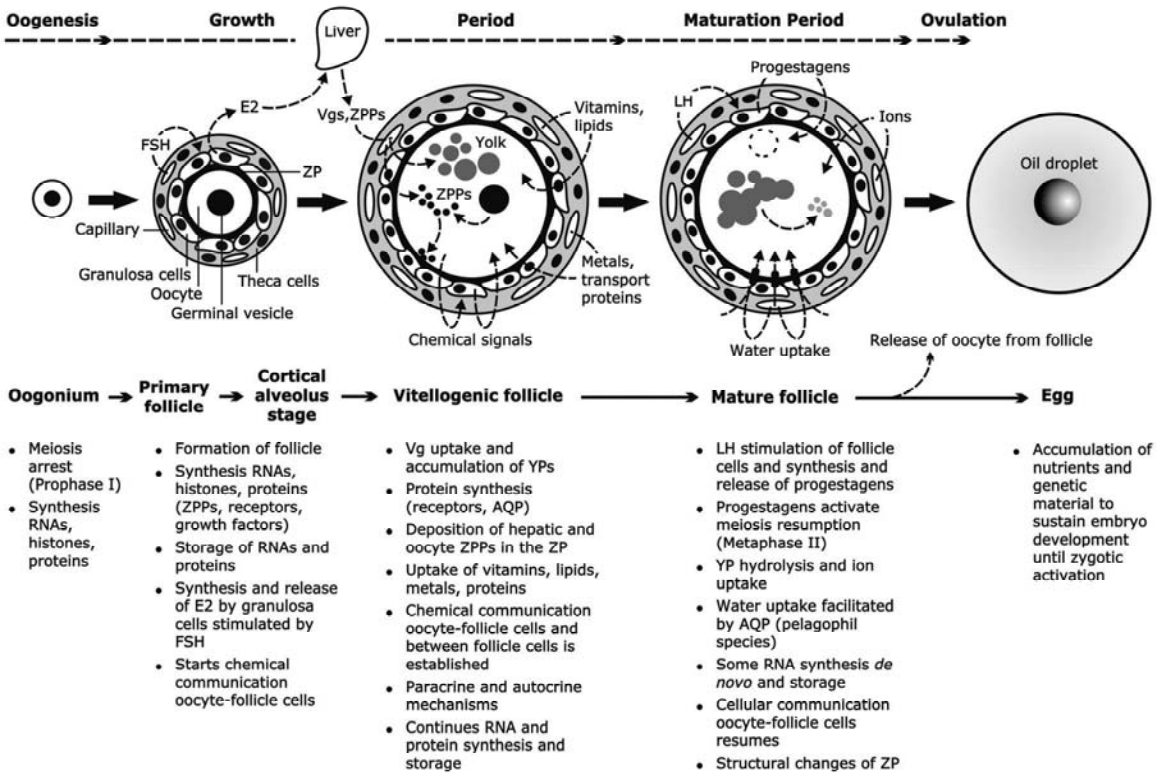

Fig. 1. Schematic diagram of the main molecular mechanisms during oocyte growth and maturation in fish. AQP, aquaporin; E2, 17β-estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone; Vgs, vitellogenins; ZP, zona pellucida; ZPPs, zona pellucida proteins. Taken from Cerdà et al. (2008).

teins associated with mitochondria. The nuage are found also in oogonia, oocytes, spermatogonia, spermatocytes and spermatids, and has been documented in different animal phyla (Eddy 1975). These observations indicate that probably the nuage has an important role in germ line development and may be necessary for differentiation and determination of the PGCs (Williamson and Lehmann 1996).

In fishes, the morphology, function and migration of PGCs are similar to that in other organisms (Gevers et al. 1992; Timmermans 1996). During migration in zebrafish (*Danio rerio*) and Japanese eel (*Anguilla japonica*), the PGCs expressing the chemokine receptor 4b (*cxcr4b*) are guided to the gonad by recognizing the chemokine Sdf-1a expressed by neighbouring somatic cells (Knaut et al. 2003; Knaut and Schier 2008; Saito et al. 2011). The PGCs respond to some signals from somatic cells but have to ignore other in order to remain undifferentiated until they reach the gonad. In zebrafish, the maintenance of undifferentiated PGCs during migration requires the function of nanos-1 as observed in *Drosophila melanogaster* and *Caenorhabditis elegans* (Raz 2003). Immediately, after colonization of the gonad, sexual

differentiation of PGCs into male or female gametes starts with proliferation by mitosis prior to meiotic division.

In mammals, sex determination depends on the chromosomal constitution of the organism and is controlled by the Y-linked SRY gene which up regulates the transcription of *sox9* in the supporting cells of the XY gonad (Swain and Lovell-Badge 1999; Capel 2000). In teleosts, the environmental mechanisms are as important as genetic factors in sex determination, however in some species like the zebrafish or the European eel (*Anguilla anguilla*) no differences have been identified between the chromosomes of the two sexes indicating that probably polygenetic or environmental signals are more implied in sex determination (Traut et al. 2001). In teleost species, the timing of the second germ cell proliferation is advanced in females relative to males and it is even repressed in medaka (*Oryzias latipes*) males (Strüssmann and Nakamura 2002; Kobayashi et al. 2004), suggesting that the first expansion of the PGCs population in females may be the important sex determining factor, enabled by chromosomal sex-determining genes in some species (Lewis et al, 2008). In zebrafish and medaka, ablation of the germ cells population results in the formation of a male with the expression of all the testicular genes (Siegfried and Nüsslein-Volhard 2008; Kurokawa et al. 2007), suggesting that PGCs are essential for the development of females but is dispensable for the development of males. Interactions between PGCs and somatic cells are important for gonad formation and, usually, testicular development occurs later than ovarian development. In males, the number of germ cells does not change after gonadal colonization of the PGCs and meiosis starts between 50 and 70 dph (Kobayashi et al. 2000). In females, the PGCs becomes oogonia through different structural changes, each oogonia proliferates by mitotic division to form oogonial nests in association with pre-follicle (granulosa) cells (Lubzens et al. 2010).

In medaka, male development requires the expression of DM-domain gene on the Y chromosome, DMY/dmrt1bY (Matsuda et al. 2002; Nanda et al. 2002) similar to that observed in mammals. However, the Sox9 orthologue in medaka, Sox9b, is not involved in male development but in the maintenance of PGCs, and the co-orthologue, Sox9a, is expressed in oocytes of the adult ovary (Yokoi et al. 2002; Nakamura et al. 2012). In zebrafish and air-breathing catfish (*Clarias gariepinus*), two mammalian Sox9 orthologues were found; Sox9b is expressed in pre-vitellogenic oocytes, and Sox9a is expressed in different adult tissues (Chiang et al. 2001; Raghuveer and Senthilkumaran 2010), suggesting that Sox9 orthologues have different function in higher and lower vertebrates. In the mouse, Sox3 was determined to be required for gonadal function but not in sex determination in both males and females (Weiss

et al. 2003). In teleosts, aromatase (Cyp19a1a; see below) also plays an important role in sex determination. It is known that suppression of Cyp19a1a is associated to masculinization suggesting that Cyp19a1a promotes ovarian differentiation and inhibits testicular differentiation (Guiguen et al. 2010).

1.2. Oogonia Proliferation and Meiosis Activation and Arrest

During oogonia proliferation, each oogonium multiplies by mitotic divisions which results in large sets of cysts containing oocytes connected by intercellular bridges (Fig. 2). The cysts become delimited by a monolayer of somatic granulosa cells that secrete a basement lamina, separating it from the ovarian stroma cells and forming the cell nests. The transition from oogonia to a primary oocyte is also characterized by the initiation of the first meiotic division before leaving the oogonial nest (Lubzens et al. 2010). During meiosis, the germ line cysts breakdown or disappear during late pachytene when each of oocyte is wrapped by pre-follicle cells and become individualized. Somatic cells in the stroma interact with pre-follicles cells and they rest upon the basement lamina forming a monolayer to then differentiate into theca cells that becomes associated with blood vessels (Mazzoni et al. 2010). The oocytes and the surrounding granulosa cells, basement lamina and theca somatic layer, differentiated into an internal and external theca, constitutes the ovarian follicle. Oocytes progress through the stages of prophase I of meiosis and arrest in the diplotene stage.

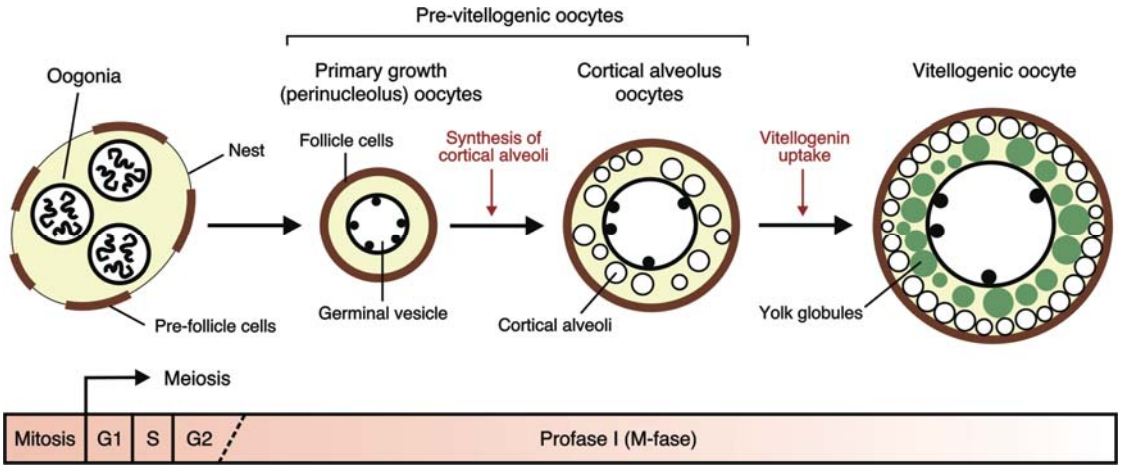

Fig. 2. Schematic representation of the stages from oogonia to vitellogenesis, and the progression of meiosis, during teleost oogenesis.

1.3. Primary Growth and Folliculogenesis

The primary growth (perinucleolus) stage is characterized by a high accumulation of cytoplasmatic RNA in the oocyte, the increase of nuclear and cytoplasmatic volume, the formation of the acellular vitelline envelope between the oocyte and the granulosa cells, and the development of the Balbiani body from nuage, which is enlarged and dispersed throughout the ooplasma as endoplasmatic reticulum and Golgi develop. Folliculogenesis is linked with primary growth and involves the formation of the ovarian follicle by the development of the follicle layers (granulosa and theca) that surround the oocyte (Grier 2012). Follicle cells and oocytes are separated from the germinal epithelium by a basemement membrane that also forms the boundary between the epithelial and stromal compartments (Grier 2000). During this stage, the establishment of microvillar contacts between the oocyte and granulosa cells also takes place (Cerdà et al. 1999; Grier 2012).

During the primary growth stage, the transcription of a large number of genes required during later stages of oogenesis and early embryogenesis occurs. These transcripts are called maternal mRNAs and are crucial for correct embryo development. In amphibians and teleosts, most of the mRNAs present in fully-grown oocytes are already present in the later stages of the primary growth phase (Wallace and Selman 1990; Pelegrí 2003). Some of these genes, such as those involved in zona pelucida formation, maintain their high expression during oocyte development but are virtually undetectable after oocyte maturation (Wessel et al. 2000). Others genes are crucial for embryo development due to its involvement in axis formation, specification of somatic tissue lineages and germline, and activation of the embryonic genome. These genes are accumulated until the maternal to zygotic transition when the zygote genome is activated (Pelegrí 2003). Maternal genes may be used as molecular markers of egg quality because perturbation on these genes results in a dramatic decrease of the survival of the resulting embryo (Mtango et al. 2008).

1.4. Transition into Secondary Growth

The transition from perinuclear stage to the cortical alveolus stage, when the oocyte is preparing to begin vitellogenesis, includes several changes in the oocyte at the structural and hormonal levels. Glycoproteins are synthesized endogenously in large amounts and are incorporated into the cortical alveoli which grow in size. Cortical alveoli are first distributed in all the cytoplasm but during vitellogenesis are displaced to the periphery of the oocyte as yolk accu-

mulates centripetally. At fertilization, the cortical reaction takes place which involves the release of the contents of cortical alveoli into the perivitelline space between oocyte and the vitelline envelope to prevent polyspermy and entry of pathogens (Tyler and Sumpter 1996; Patiño and Sullivan 2002). During the previtellogenic growth, lipid deposition into the oocyte is also initiated which also contributes to the increase in size of the oocyte during this stage (Selman and Wallace 1989). It has been hypothesized that very low density lipoproteins (VLDL), which binds to their receptors and are hydrolyzed into free fatty acids, are the origin of these lipid droplets (Patiño and Sullivan 2002).

1.5. Vitellogenesis

Vitellogenesis is characterized by the incorporation of vitellogenin (Vtg) proteins, lipids and vitamins by the oocyte and the cleavage of Vtgs into yolk proteins (Lubzens et al. 2010). The uptake of lipids, Vtgs and vitamins from the plasma produce a huge increase in the size of the ovarian follicle and the oocyte becomes competent for fertilization. At the same time, the full development of the vitelline envelope surrounding the oocyte takes place (Le Menn et al. 2007), and oocyte and granulosa cells establish intercellular communication through different cell-cell interaction structures (i.e. gap junctions and others) (Cerdà et al. 1999; Le Menn et al. 2007).

The Vtgs are phosphoglycolipoproteins, members of the large lipid transfer protein superfamily (Babin et al. 1999), that are found in all the female oviparous vertebrate and invertebrate species and play an important role as a source of nutrients during early embryo development. A complete Vtg molecule is composed of different domains that corresponds to the different yolk proteins, a heavy chain lipovitellin (LvH), a phosvitin (Pv), a light chain lipovitellin (LvL) and the β' component. There are three major types of Vtgs that are differently processed into yolk proteins, classified as VtgAa, VtgAb, and VtgC, which lacks the phosvitin domain (Finn 2007a,b). The Vtgs are synthesised by the liver in response to estradiol-17β (E2) produced by follicle cells associated to the oocyte (see below) and are post-translationally glycosylated and phosphorylated in the endoplasmic reticulum and Golgi complex (Finn 2007a,b). The Vtgs enter the ovarian follicle through the capillaries in the theca layer and passes through the intercellular spaces of the granulosa layer and through the pore canals of the vitelline envelope until reaching the oocyte surface. The Vtgs are specifically incorporated by the oocyte by receptor-mediated endocytosis through the VLDL receptor precursor (VLDLR), also named in oviparous animals the Vtg receptor (VtgR) (Babin et al. 1999).

Internalization occurs in the coated pits that are invaginated into the cytoplasm and form the coated vesicles that fuse with lysosomes-like multivesicular bodies. Proteolytic cleavage of vitellogenin and subsequent processing into yolk proteins occurs in the multivesicular bodies, and yolk proteins are subsequently stored in yolk globules or platelets in the cytoplasm (Wallace and Selman 1990). Cleavage of Vtgs into yolk proteins is carried out by different lysosomal enzymes such as cathepsin D and cathepsin B (Carnevali et al. 1999a,b; Raldúa et al. 2006; Carnevali et al. 2006).

During vitellogenesis, lipids and vitamins are also incorporated by the oocyte as they are required for embryonic development (Lubzens et al. 2010). Vitamin E serves as an antioxidant and vitamin A is involved in gene transcription regulation. Vitamin A and E are recruited from peripheral tissues and they move to the oocyte by lipoprotein mediated transport (Palace and Werner 2006). The Vtgs can transport vitamin A in the form of retinal (Irie and Seki 2002) and vitamin E as α-tocopherol (Lie et al. 1994). However retinol-binding protein (RBP) and tocopherol binding protein may also be involved (Lubzens et al. 2010). In rainbow trout (*Onchorynchus mykiss*), vitamin A, as retinol or retinyl-ester, bound to Rbp4 in plasma, enters to the oocyte via the Stra6 receptor and Rbp1 mediated cellular metabolization to retinyl-ester (Levi et al. 2008). The lipids accumulated by the oocyte are mostly originated from plasma Vtgs and VLDLs. Neutral lipids from lipolysis of triacylglycerols VLDL and from the esterification of fatty acids are stored in lipid globules.

1.6. Oocyte Maturation and Hydration

When oocytes reach the fully-grown stage they undergo meiosis resumption in response to the hormone signal. During maturation, oocyte meiosis progresses to metaphase of the second meiotic division, and oocytes undergo profound morphological changes, such as the condensation of chromosomes and breakdown of the germinal vesicle (GVBD), which is initially located in the centre of the oocyte and migrate to the animal pole to disintegrate. In oviparous marine teleosts, these nuclear processes are associated with the fusion of yolk globules to form a central mass of fluid yolk, the disassembly of the crystalline structures within the globules, and the cytoplasmic enlargement due to a massive water uptake or hydration (Cerdà et al. 2007). This mechanism is associatd with the accumulation in the oocyte of osmolytes, such as inorganic ions (K^+, Cl^-, PO_4^{3-} or Na^+) and free amino acids (FAAs) resulting from the selective hydrolysis of yolk proteins stored in the oocyte (Cerdà et al. 2007; Finn and Kristoffersen 2007; Finn 2007a,b; Kolarevic et al. 2008; Kristoffersen and Finn 2008; Finn and Fyhn 2010). The

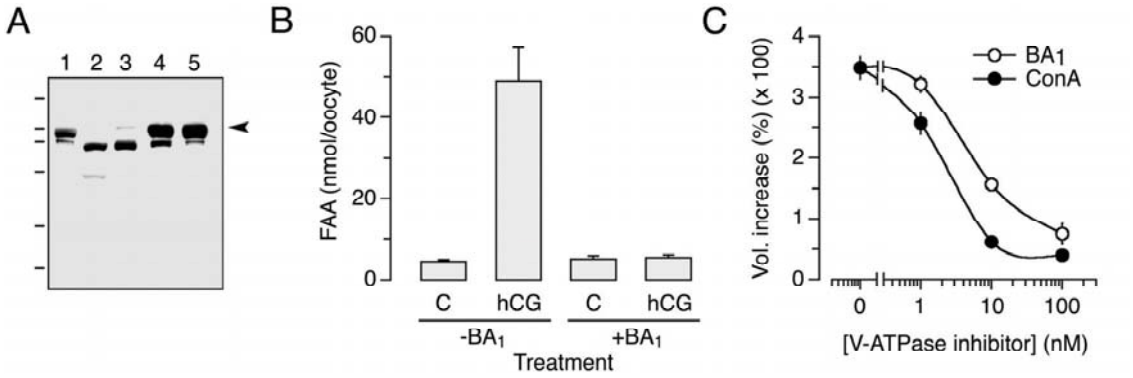

Fig. 3. Inhibition of yolk proteolysis, generation of FAAs, and oocyte hydration in pelagophil teleosts. A, Western blot of gilthead seabream (*Sparus aurata*) yolk proteins of control (lane 1) and progestin(MIS)-stimulated ovarian follicles in the absence (lane 2) or the presence of 1, 10, and 100 nM bafilomycin A1 (BA1), an inhibitor of the vacuolar ATPase (V-ATPase) (lanes 3, 4, and 5, respectively), using a sea bream Vg antisera. Note that the degradation of a yolk protein of ~100 kDa, possibly lipovitellin, is inhibited by treatment with BA1. The position (bars) of molecular weight markers is indicated on the left (from top to bottom: 200, 116, 97, 66, 45, and 29 kDa). B, Effect of BA1 on the generation of FAAs during hCG-induced oocyte maturation in the black sea bass (*Centropristis striata*). C, The treatment of follicles with BA1 and concanamycin (ConA), another V-ATPase inhibitor, prevents the hydration of sea bream oocytes. Taken from Cerdà et al. (2007).

pre-ovulatory process of oocyte hydration of marine teleosts is unique among vertebrates, and is vital for the development of viable embryos as it assures a water reservoir when eggs are released into the hyperosmotic seawater. The water reservoir also contributes to the positive buoyancy of eggs and early embryos in seawater facilitating oxygen exchange and their dispersal in the ocean (Fyhn et al. 1999; Finn and Kristoffersen 2007).

1.6.1. Proteolysis of Yolk Proteins

In teleosts, following incorporation of Vtg into growing oocytes and subsequent cleavage into yolk proteins there is an additional proteolytic event of yolk proteins into FAAs associated with the extent of oocyte hydration during maturation (Wallace and Selman 1985; Greeley et al. 1986) (Fig. 3). In teleosts spawning buoyant eggs in seawater (pelagophil), the LvH of the VtgAa paralogue is disassembled and extensively degraded to FAAs. In the VtgAb paralogue, the LvH is nicked in the C-sheet, whereas a small part of the C-terminal end undergoes proteolysis to FAA, together with the phosvitin, β' component, and much of the LvL of VtgAb (Matsubara et al. 1995, 1999; Reith et al. 2001; Selman et al. 2001; Finn and Kristoffersen 2007; Finn 2007a). Thus, each VtgAa and VtgAb paralogue contributes approximately equal amounts of FAAs to the organic osmolyte pool of the hydrating oocyte during maturation. In teleosts spawning benthic eggs (bentophil), maturation-associated hydrolysis of yolk proteins in much

limited and only a slight increase in FAAs is observed (Wallace et al. 1992; LaFleur et al. 2005; Raldúa et al. 2006).

The major degradative enzymes of yolk proteins in teleost oocytes are cysteine proteases, such as cathepsin L, cathepsin B and cathepsin D (Murakami et al. 1990; Sire et al. 1994; Carnevali et al. 1999b, 2001; Kestemont et al. 1999; Hiramatsu et al. 2002; LaFleur et al. 2005; Raldúa et al. 2006). In the gilthead seabream (*Sparus aurata*), cathepsin L has higher activity during mid-late vitellogenesis while cathepsin B and D have more activity in early vitellogenic oocytes. In addition, isolated cathpesin L was able to cleave purified Lv in vitro, suggesting that cathepsin L is the major protease involved in the proteolysis of Lv in vivo during oocyte maturation (Carnevali et al. 1999a). However, there are evidences that cathepsin B is involved in cathepsin L activation since in zebrafish inhibition of both cathepsin was observe with an specific inhibitor of cathepsin B (Carnevali et al. 2006). In killifish (*Fundulus heteroclitus*), cathepsin L activity decrease during oocyte maturation *in vivo* and *in vitro*, whereas cathepsin B increases during oocyte maturation coinciding with the time of degradation of Lv, suggesting that cathepsin B is most likely the protease involved (LaFleur et al. 2005; Raldúa et al. 2006). Activation of cathepsins and subsequent yolk proteolysis, in both, pelagophil and bentophil species, seems to be related with V-ATPase-mediated acidification of yolk globules (Selman et al. 2001; Raldúa et al. 2006).

1.6.2. Role of Inorganic Ions

Inorganic ions also have an important role as osmotic effectors during oocyte hydration. However, this role differs between bentophil and pelagophil teleosts. In pelagophil teleosts, whereas the FAAs contribute to most of all the oocyte osmolality, the inorganic ions (K^+, Cl^-, Pi and NH_4^+) make up the balance (Selman et al. 2001; Finn et al. 2002a). In bentophil teleosts, FAAs appear to have a minor role in oocyte hydration whereas inorganic ions, such as K^+ and Na^+, are the major osmotic effectors (Greeley et al. 1991; Wallace et al. 1992; Chen et al. 2003). The mechanisms for ion uptake into the oocyte are not well known, although in pelagophil teleosts it has been suggested a possible role of the Na^+,K^+-ATPase in the active transport of K^+ and Na^+ into the oocyte (LaFleur and Thomas 1991; Chen et al. 2003). However, in bentophil teleosts Na^+,K^+-ATPase has apparently no role in ion uptake during oocyte hydration (Wallace et al. 1992), and may be other channels could be involved in this process (Bulling et al. 2000). In killifish, it was suggested that yolk proteolysis could be involved in K^+ accumulation into the oocyte by increasing new K^+-binding sites in the oocyte cytoplasm

(Raldúa et al. 2006) which may diffuses into the oocyte through connecting gap junctions (Cerdà et al. 1993).

1.7. Ovulation

Ovulation is defined as the release of the mature oocyte from the ovarian follicle. During the ovulatory process, the metaphase II oocyte is separated from the follicular layer, the follicle wall is broken and the oocyte is release to the ovarian cavity through the rupture site. Thus, before ovulation, several mechanical and ultrastructural aspects change in the ovarian follicle, such as the disruption of the microvillar connections between the granulosa cells and the oocyte and the opening of the follicle wall (Lubzens et al. 2010). This process requires contraction of the microvilli connected to the granulosa cells and subsequent disruption of gap junctions and other cell-cell adhesion structures (York et al. 1993; Cerdà et al. 1999). Mechanical action of the oocyte on the surrounding follicular layers may also be involved in ovulation.

2. Reproductive Hormones

2.1. Gonadotropins

The gonadotropins belong to the glycoprotein hormone family, which includes the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), the placental derived chorionic gonadotropin (CG), and the thyroid stimulation hormone (TSH) (Pierce and Parsons 1981). The four glycoproteins are related in structure, each consisting in two different subunits, a common α-subunit highly conserved between species and a specific β-subunit that dictates hormonal specificity (Gray 1988; Pierce 1971). Carbohydrate moieties and association of both subunits are necessary for full expression and biological activity (Ryan et al. 1987). In teleosts, the first gonadotropin hormone was isolated from carp and salmon pituitaries (Burzawa-Gerard 1971; Donaldson et al. 1972). Currently, the existence of two different pituitary gonadotropins in teleosts, Fsh and Lh, is well established, which are analogous to their mammalian counterparts FSH and LH (Levavi-Sivan et al. 2010).

The Fsh and Lh are heterodimers composed of two subunits encoded by distinct genes: a hormone-specific β subunit (Fshβ or Lhβ), which confers specific physiological functions, and the common glycoprotein α subunit (Cga), shared also by other members of the glycoprotein hormone family. The two subunits are bound non-covalently and stimulate their receptors as α/β-subunit heterodimers, since the individual subunits do not display significant

bioactivity (Pierce and Parsons 1981). The primary structure of the fish Cga subunit contains two potential sites for N-glycosilation and ten conserved cysteines that form five intramolecular disulfide bridges. Both, Fshβ or Lhβ, contain one potential N-glycosilation site involved in the biosynthesis and regulation of the hormones (Ulloa-Aguirre et al. 1999; Swanson et al. 2003) and a typical twelve highly conserved cysteines residues which form cross-linked disulphide bonds with the Cga, and thus they are needed for proper folding of the protein and receptor interaction (Hearn and Gomme 2000). This structure is conserved in Lhβ subunits, but not in Fshβ which, in fish, has the less conserved structure and primary sequence (Levavi-Sivan et al. 2010). The number and location of the cysteines are important for the formation of the seatbelt and the tertiary structure of the subunit, essential for receptor binding (Lapthorn et al. 1994).

The spatial and temporal pattern of secretion and expression of Fsh and Lh suggest that these hormones are produced in distinct cell-types and are differentially synthesized during the reproductive cycle of teleosts. The control of Fsh and Lh synthesis and secretion is complex and involves the interplay between the gonads, pituitary and hypothalamus (Levavi-Sivan et al. 2010). Both gonadotropins act on the ovaries and testes and, in turn, gonadal steroids and peptides act at the hypothalamus and/or pituitary to regulate either positively or negatively gonadotropin synthesis and secretion. The hypothalamus of teleosts exerts its regulation on gonadotropin release via several neurohormones, such as gonadotropin-releasing hormone (GnRH), dopamine, gamma-amino butyric acid (GABA), pituitary adenylate cyclase activating peptide (PACAP), norepinephrine, neuropeptide Y (NPY), serotonin and kisspeptin, and also via factors related to growth and metabolism (Levavi-Sivan et al. 2010). Transduction of the GnRH signal, leading to the increased expression of *fshb* is mainly done through the cAMP–protein kinase A (PKA) pathway, whereas that of *lhb* is mediated through the protein kinse C (PKC) and MAPK cascades (Yaron et al. 2001). In goldfish (*Caraussius auratus*), activin stimulates *fshb* but suppresses *lhb* expression and its effects can be reversed by follistatin (Yuen and Ge 2004). Follistatin is up regulated by gonadal steroids and also by activin, which in turn is inhibited by follistatin, suggesting that there is a negative feedback to control the activity of activin which is constantly expressed during the reproductive cycle (Cheng et al. 2007). Although, there is more information about the regulation of gonadotropins during the reproductive cycle of teleosts, more research is needed to better understand these mechanisms and confirm them in other fish species.

In salmonids, the plasma levels of Fsh are elevated during early stages of gametogenesis and decrease during oocyte maturation, whereas those of Lh are maintained throughout the cycle to peak only at spawning (Suzuki et al. 1988; Swanson et al. 1991). Data in other teleosts confirm in general these observations (Aizen et al. 2007; Molés et al. 2012; Shimizu et al. 2012), suggesting that Fsh may have a major role during gonad growth whereas Lh may act primarily during oocyte maturation and ovulation. In the gilthead seabream, however, expression of *fshb*, *lhb* and *cga* is not only found in the pituitary but also in the ovary (Wong and Zohar. 2004).

2.1.2. Gonadotropin Receptors

The gonadotropin hormones elicit their biological responses after interaction with their cognate receptors on the surface of target cells, presumably by stimulation of cAMP synthesis with subsequent effects on the activity of various protein kinases. In many teleosts, the cDNAs encoding two distinct gonadotropin receptors, Fshra and Lhcgrba/Lhcgrbb, as it occurs in mammals, have been identified (Levavi-Sivan et al. 2010; Chauvigne et al. 2010). These G protein-coupled receptors consist of a large extracellular domain, characterized by multiple leucine-rich repeats (LRRs) that are critical for specific hormone binding, a seven-transmembrane domain that is the most conserved part, and a short intracellular C-terminus involved in G protein coupling and binding of different intracellular signaling molecules (Fig. 4). In mammals, each gonadotropin has highly specific affinity for its receptor, but in teleosts, the Fshra and Lhcgrba or Lhcgrbb can be cross-activated by their ligands and, therefore, receptor specificity is yet unclear (Levavi-Sivan et al. 2010).

In the teleost ovary, the gonadotropin receptors are expressed in the granulosa and theca cells surrounding the oocyte where they regulate the steroidogenic pathways (see below). The expression levels of both receptors differ throughout oocyte development and have different expression patterns at the follicular level (Levavi-Sivan et al. 2010). In addition, analysis of the gonadal expression profiles of the teleost gonadotropin genes during the reproductive cycle suggests that their receptors are also involved in the regulation of critical periods during oogenesis. In different teleost species, *fshra* gene expression is detectable in the immature ovary and then increases during vitellogenesis and decrease once the oocytes have reached the fully grown stage. In contrast, *lhcgrba* or *lhcgrbb* expression increase during late vitellogenesis suggesting its role in the induction of oocyte maturation and ovulation (Kumar

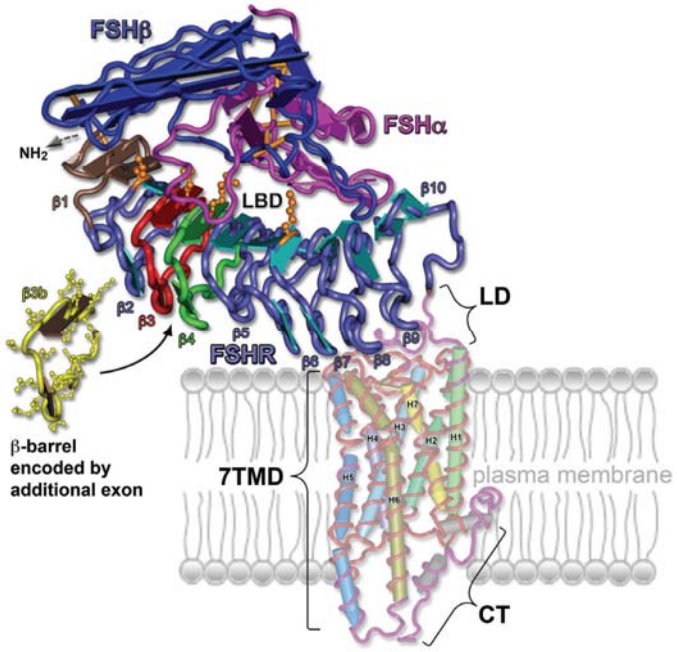

Fig. 4. Three-dimensional render of the Senegalese sole Fshra LBD mapped to the crystallographically resolved structures of human FSHR and squid rhodopsin. The first b-sheet of each leucine-rich repeat in the LBD is numbered 1–10. The a (magenta) and b (blue) chains of human FSH are shown to highlight the b-sheet barrels (b3 red and b4 green) at the interacting domain. Side chains of residues implicated with binding specificity in the human molecule are shown as orange ball and stick renders. The acanthomorph-specific b3b barrel (yellow) localizes between b3 and b4. The FSHR render terminates at the linker domain (LD) extending down to the rhodopsin G-coupled membrane-anchoring complex (7TMD). The transmembrane helices are numbered 1–7 toward the C-terminal domain (CT). Taken from Chauvigné et al. (2010).

et al. 2001a,b; Kwok et al. 2005; Kobayashi et al. 2008; Rocha et al. 2009; Chauvigne et al. 2010; Kazeto et al. 2010; Kitano et al. 2011).

2.2. Steroid Receptors

Steroid hormones, such as androgens, estrogens and progestins, play important roles in the regulation of a large number of physiological processes through interaction with the corresponding receptors. In teleosts, unlike in mammals, the major progestins involved in reproduction are 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and 17α,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P) (Thomas et al. 2007; Nagahama and Yamashita 2008). Typically, steroid receptors are intracellular (i.e. cytoplasmatic) and initiate signal transduction for steroid hormones which lead to changes in gene expression over a time period of hours to days. However, recent studies indicate that some steroid actions involve novel steroid receptors located at the plasma membrane, called membrane steroid receptors, which activate sig-

nal transduction pathways interacting with G proteins and cytoplasmatic kinase signalling molecules (Mani et al. 2012).

2.2.1. Nuclear Steroid Receptors

Nuclear steroid receptors mediate classical genomic actions through the binding to hormone response elements on the promoter region of target genes modifying the transcriptional rates (Thomas 2008). The nuclear receptor family can be divided into four classes based on their dimerization and DNA-binding properties. Class I receptors include the known steroid hormone receptors that are located in the cytoplasm and function as ligand induced homodimers that bind to DNA half-sites organized as inverted repeats. Class II receptors are retained in the nucleus, heterodimerize with retinoid X receptor (RXR), and characteristically bind to direct repeats, although some also bind to symmetrical repeats. Class III receptors bind primarily to DNA direct repeats as homodimers. Class IV receptors typically bind to extended core sites as monomers. Most of the orphan receptors fall into class III and IV categories (Mangelsdorf et al. 1995). The nuclear receptor family can also be divided in different subfamilies categorized according sequence homology. The nuclear steroid receptors are included in the nuclear receptor subfamily 3 (NR3), consisting of three groups: group A, that includes two receptors for estrogens (ERα and ERβ); group B, that includes two estrogens-related orphan receptors (ERRα/ERR1 and ERRβ/ERR2); and group C, that includes glucocorticoid receptors (GR), mineralocorticoid receptors (MR), progestin receptors (PGR), and androgen receptors (AR) (Beato et al. 2000).

The members of the nuclear receptor superfamily share a common protein structure, consisting of a variable N-terminal (A/B) region responsible in some cases for transcriptional regulation bearing the ligand-independent transcriptional activation domain AF-1, a highly conserved DNA binding domain (DBD) which interacts with the hormone response elements (HREs) on the DNA and control the target gene(s), a hinge (D) region that controls the traslocation of the receptor into the nucleus, and the moderately conserved ligand binding domain (LBD) responsible for hormone recognition and specificity of the physiological response, which contains the ligand-dependent AF-2 core transactivation domain (Mangelsdorf et al. 1995; Beato et al. 2000). The nuclear steroid receptors are either located in the cytosol and move to the cell nucleus upon activation, or spend their life in the nucleus waiting for the steroid hormone to enter and activate them. The traffic into the nucleus is dependant of the nuclear localization signals found between the DBD and D region, which in most cases is covered

up by heat shock proteins (HSPs) which bind the receptor until the hormone is present. Upon binding by the hormone the receptor undergoes a conformational change, the HSP come off, and the complex ligand-receptor enters to the nucleus where bind to the HRE of gene promoters and recruit co-regulators to modify the rates of transcription (Tsai and O'Malley 1994; Beato et al. 2000).

The majority of promoters have multiple transcription stars sites (TSSs) within the promoter region indicating different modes of transcription initiation that can be regulated in a tissue-specific manner (Carninci et al. 2006; Kawaji et al. 2006). Transcription is controlled by multiple transcription factors interacting with a variety of *cis*-regulatory elements (CREs), the core promoter that is the target of the pre-initiation complex composed of the basal transcription factors and DNA dependent RNA Polymerase II, proximal and distal promoter regions which are the targets of sequence specific transcription factors, and distal-acting enhancers or silencers. The additional CREs located on proximal and distal promoter regions act independently controlling the transcription initiation rate at the core promoter (Levine and Tjian 2003). The TATA-box was described as the core promoter element indispensable for the recruitment of the basal transcription machinery and selection of the TTS (Lifton et al. 1978; Breathnach and Chambon 1981; Orphanides et al. 1996). However, analyses of different promoters in a wide variety of organisms have shown that TATA-box is not a general feature of core promoters (Basehoar et al. 2004; Kutach and Kadonaga 2000; Ohler et al. 2002; Juven-Gershon and Kadonaga 2009; Trinklein et al. 2003; FitzGerald et al. 2004; Ferg 2008).

The activity of the transcription factors can be modulated by several mechanisms including cofactor binding, interaction with other transcription factors, and covalent modifications (e.g. phosphorylation) of the transcription factor (Vicent et al. 2006). Also, the transcription factor binding sites on the DNA have to be accessible, which is facilitated by DNA, ATP-dependent nucleosome remodelers and histone modifying enzymes (Beato and Vicent 2012). In humans, PGR-mediated transcriptional activation needs a rapid activation of Erk that leads to the phosphorylation of the PGR and the activation of mitogen- and stress-activated protein kinase 1 (Msk1) (Vincent et al. 2010). The receptor complexes are recruited to the target promoter containing the progestin responsive element (PRE) where modify the protruding core histone tails and the linker (Vicent et al. 2006). In the case of the ERs, only the recruitment of co-activator complexes is sufficient for gene activation, such as histone acetyl transferases, histone methyltransferases or ATP-dependent remodelling activites (Metivier et al. 2003; Shang et al. 2000). These modifications, for both the PGR and ER, allow acces to the

specialized ATP-dependent remodelling complexes to displace the linker histones and the repressing complexes (Vincent et al. 2004). After completion of the initial chromatin remodelling steps, the complexes containing mediator and RNA-polymerase with the different basal transcription factors are recruited to the core promoter and transcription starts (Vincent et al. 2010).

In mammals, there are two subtypes of ERs, ERα and ERβ, which are very similar at the amino acid level but show different tissue expression patterns, ligand binding affinities, and functional properties (Mosselman et al. 1996; Kuiper et al. 1997; Harris 2007). In the case of the nuclear PGR, there are two different isoforms, PGR-A and PGR-B, generated by differential estrogen sensitive promoter usage (Kastner et al. 1990). The PGR-B is longer than the isoform A due to an extension of the A/B region termed the B-upstream segment (BUS) that contains the AF-3 additional transactivation domain (Sartorius et al. 1994). The two PGR isoforms are functionally different, PGR-B is usually a stronger transcriptional activator than PGR-A whereas PGR-A is a cell and promoter-specific dominant repressor of PGR-B (Tung et al. 1993; Vegeto et al. 1993). Another additional N-terminally truncated isoform lacking the A/B region and a full DBD, termed PGR-C, has been identified, that is capable of forming heterodimers and modulating the activity of PGR-A and PGR-B (Wei et al. 1990, 1996; Condon et al. 2006). A number of additional Pgr mRNA splice variants are identified in endometrial tissue, testis and breast cancer cells (Cork et al. 2008). However the physiological significance of most of these isoforms remains to be established (Richer et al. 1998; Hisatomi et al. 2003; Marshburn et al. 2005; Samalecos and Gellersen 2008).

In teleosts, there are two different ER subtypes, Erα and Erβ, as in mammals (Kuiper et al. 1996). However, there are two forms of the Erβ subtype, the ErβI (formerly ERγ; see Hawkins et al. 2000; Hawkins and Thomas 2004) and the ErβII, which possibly arose from a duplication of an ancestral *erb* gene early in the teleost lineage following the split between tetrapods and teleosts (Menuet et al. 2002; Haukins et al. 2005). Different *er* transcript variants have been identified in teleosts showing truncations of the N-terminus, internal truncations or extensions, and variable 5’ or 3’ untranslated regions (UTRs) (Pakdel et al. 2000; Patiño et al. 2000; Caviola et al. 2007; Tan et al. 1995; Pinto et al. 2005, 2012). However, the role of the different Er subtypes and splice variants during teleost reproduction is not well understood. There is some controversy among different species on the role of Erβ. In goldfish, the Erα is more important for the induction of vitellogenesis than the Erβ, whereas in rainbow trout experiments suggest that production of vitellogenin is mainly regulated by Erβ (Nelson and

Habibi 2010; Leaños-Castañeda and Van der Kraak 2007). However, in both species the Erβ may be involved in Erα regulation.

The piscine nuclear Pgr has only been cloned and characterized in few species of Chondrichthyes, including the little skate (*Leucoraja erinacea*) (Bridgham et al. 2006), and some basal orders of teleosts belonging to Anguilliformes, Cypriniformes, Salmoniformes and Gadiformes, such as the Japanese eel, zebrafish, Atlantic salmon (*Salmo salar*), and Atlantic cod (*Gadus morhua*), respectively (Todo et al. 2000; Hanna et al. 2010; Chen et al. 2010, 2011, 2012). In addition, an ancestral Pgr-like receptor with a putative ligand-binding preference for 15α- hydroxyprogesterone has been identified in the sea lamprey (*Petromyzon marinus*) (Thornton, 2001; Bryan et al. 2008). In the Japanese eel, there are two related *pgr* genes, *pgr1* and *pgr2*, encoding proteins 55,2% identical at the amino acid level (Ikeuchi et al. 2002). In salmon, zebrafish and cod only one gene encoding a single Pgr has been found (Hanna et al. 2010; Chen et al. 2010, 2011, 2012). Unlike the Er, Pgr splice variants have not been identified in teleosts.

2.2.2. Membrane Steroid Receptors

Membrane-associated steroid receptors mediate non-genomic responses involving the rapid activation of G proteins and intracellular second messenger pathways (Thomas 2012). In teleosts, three novel steroid membrane receptors, which are intermediaries of estrogen, androgen and progestin actions, have been identified (Loomis and Thomas 2000; Zhu et al. 2003a,b; Brawn and Thomas 2004; Thomas et al. 2005; Thomas et al. 2006). Membrane progestin receptors (mPgrs) found in teleosts and other vertebrates are separated into three subtypes, mPgrα, mPgrβ and mPgrγ, on the basis of sequence identity and phylogenetic analysis (Zhu et al. 2003a,b; Tokumoto et al. 2012).

Most of the membrane steroid receptors have seven transmembrane domains and are coupled to G proteins which are characteristics of the G protein-coupled receptor (GPCR) superfamily. However, membrane progestin receptors belong to the progestin and adipoQ receptors family (PAQR) with no structural and sequence homology with GPCRs (Tang et al. 2005; Thomas et al. 2007). Nevertheless, the mPgrα and mPgrβ are coupled to an inhibitory G protein which activation causes the down-regulation of adenylyl cyclase activity with subsequent decrease in cAMP production, and possibly up-regulates PI3K/Akt, MAPkinase and p38MAPkinase pathways (Karteris et al. 2006; Hanna et al. 2006; Dressing et al. 2010; Tho-

mas et al. 2007). The signal transduction pathway for mPgrγ is however unknown (Thomas 2012).

In vertebrates, only one membrane estrogen receptor (GPR30) mediating rapid cell signaling has been identified with conserved estrogen binding and signalling function from fish to mammals, suggesting that this receptor arose early in vertebrate evolution and is involved in important physiological functions (Revankar et al. 2005; Thomas et al. 2010). The GPR30 acts through activating stimulatory G protein resulting in an increase of adenylyl cyclase activity and cAMP production, and also by releasing epidermal growth factor (EGF)-related ligands (Filardo et al. 2002; Filardo and Thomas 2005). Recent studies reveal that the GPR30 is involved in the development and progression of different cancers and in the regulation of different physiological functions (Filardo et al. 2006; Albanito et al. 2007; Wang et al. 2008, 2009; Xu et al. 2009; Noel et al., 2009).

In distinct reproductive tissues, differential expression pattern between the three mPgr subtypes has been observed (Zhu et al. 2003a,b; Tokumoto et al. 2012), suggesting different functions of the mPgrs in vertebrate cells and tissues. Recently, it has been suggested that there is a reciprocal regulation between the GPR30 and mPgrs in zebrafish oocytes, where E2 causes a rapid down-regulation of mPgrα and activates GPR30 while 17,20β-P causes an opposite effect (Pang and Thomas 2010). In the female rat brain, E2 also increases the expression of the mPRβ (Zuloaga et al. 2012). In addition, in Atlantic croaker (*Micropogonias undulatus*), mPRα is up-regulated *in vitro* after gonadotropin treatment (Tubbs et al. 2010), and in souther flounder (*Paralichtys lethostigma*), insulin growth factor I (IGF-I) induced an up-regulation of mPgrα mRNA and protein through Pik3 and Mapk signal transduction pathways (Picha et al. 2012).

3. Hormonal and Molecular Control of Teleost Oogenesis

3.1. Oogonia Proliferation and Meiosis Activation

The control of germ cell proliferation and meiotic activation and arrest are poorly understood in teleosts. In higher vertebrates, oogonia proliferation is regulated by gonadotropins, particularly by FSH, as well as by estrogens and progestins (Yamazaki 1965; Tokarz 1978; Arrau et al. 1983; Angelova and Jordanov 1986; Mendez et al. 2003). Numerous growth factors, such as activin A, stem cell factor (SCF), IGFs, as well as retinoids, may also be involved in this process (Livera et al. 2000; Lyrakou et al. 2002; Martins da Silva et al. 2004). In mammals, the retinoic

acid induces *stra8* expression, the meiotic initiation molecule in both sexes (Anderson et al. 2008). The gene *dazl*, is also important in meiosis regulation as it is involved in the control of the expression of the synaptonemal complex protein 3 (SYCP3) and in the response of pre-meiotic germ cells to retinoic acid (Edson et al. 2009).

In teleosts, recent investigations suggest that sex steroids may be involved in the control of meiosis activation and arrest. In the Japanese eel, 17,20β-P is highly accumulated in early spermatogenesis and induces the expression of meiosis specific markers such as Spo11 and Dmc1, as well as the formation of synaptonemal complexes, suggesting that it is an essential factor for the initiation of meiosis (Miura et al. 2006). Experiments in the Japanese huchen (*Hucho perry*) and common carp (*Cyprinus carpio*) suggest that the progression of germ cells through early oogenesis also involves E2, which acts directly on oogonial proliferation (Miura et al. 2007). In Nile tilapia (*Oreochromis niloticus*), the onset of meiosis has also been linked to the expression of IGF-I in somatic cells and oocytes (Berishvili et al. 2006). A novel member of the transforming growth factor-beta super family, the gonadal soma-derived factor (GSDF), enhances primordial germ cells and spermatogonia proliferation in rainbow trout (*Oncorhynchus mykiss*), and probably can have a similar role in oogonia proliferation (Sawatari et al. 2007).

3.2. Oocyte Growth and Folliculogenesis

3.2.1. Role of Fsh during Oocyte Growth

In most teleosts studied to date, the plasma levels of Fsh are high during the period of oocyte growth and decline with the beginning of oocyte maturation (Suzuki et al. 1988; Swanson et al. 1991; Aizen et al. 2007; Molés et al. 2012; Shimizu et al. 2012). In addition, in many species, the *fhsra* gene is expressed in theca and/or granulosa cells associated to pre- and early vitellogenic oocytes, and its expression level increase as the pre-vitellogenic oocytes enter into vitellogenesis, peaks at mid-vitellogenesis, and decreases once the oocytes have reached the fully-grown stage (Kumar et al. 2001a,b; Kuok et al. 2005; Kobayashi et al. 2008; Rocha et al. 2007, 2009; Chauvigné et al. 2010; Kaseto et al. 2010). These observations thus suggest that Fsh is most likely the major gonadotropin involved in oocyte growth in teleosts. However, in ricefield eels (*Monopterus alba*), Lhβ was localized in the pituitary when primary growth period starts and increases as the oocyte develops, suggesting a role of Lh during the primary growth stage (Wu et al. 2012). Primary growth of oocytes until arrest at the late perinucleolar

or very early cortical alveoli stage can proceed in hypophysectomized fish, and therefore prior to the cortical alveoli stage, follicles may be able to develop in the absence of pituitary gonadotropins (Khoo 1979; Billard 1992). However, in the gilthead seabream, Fshβ and Lhβ transcripts and protein products are found in primary and secondary oocytes (Wong and Zohar 2004), suggesting that gonadotropins of ovarian origin would be still able to regulate the primary growth stage although the fish have been hypophysectomized (Lubzens et al. 2010).

In mammals, the granulosa and theca cells secrete different paracrine factors such as inhibin, activin and growth factors that are involved in regulation of oocyte growth (Buccione 1990; Gilchrist et al. 2004). In turn, the oocye produces different growth factors involved on folliculogenesis, such as the growth and differentiation factor 9 (GDF9) and bone morphogenetic factor 15 (BMP15), which stimulate granulosa cell mitosis and restrict differentiation (Juengel et al. 2004; Moore and Shimasaki 2005). Pituitary gonadotropinds seem to be involved in these process, where FSH modulates the expression of BMP15 *in vitro* while has no effect on GDF9 (Thomas et al. 2005; Guéripel et al. 2006).

In the zebrafish, different Bmp ligands are expressed in the oocyte while its receptors are express in follicle layers, suggesting that follicle cells control oocyte development, and at the same time, the oocyte may regulate the follicular layer function (Li and Ge 2011). In teleosts, Gdf9 and Bmp15 are also expressed in primary growth oocytes when granulosa cells starts to proliferate, suggesting a possible role of these factors during the development of primary ovarian follicles (Baron et al. 2005; Halm et al. 2008). In zebrafish, treatment of ovarian fragments *in vitro* with human chorionic gonadotropin (hCG) produces a decrease in *gdf9* expression levels (Liu and Ge 2007), and in European seabass (*Dicentrarchus labrax*), *bmp15* levels increase during the transition to vitellogenesis, whereas *gdf9* transcripts are high during pre-vitellogenesis and decrease during the progression to vitellogenesis (García-Lopez et al. 2011). These results suggest that in teleosts pituitary gonadotropins may have a role in the regulation of Bmp15 and Gdf9 in the primary growth oocyte.

During the transition into the secondary growth stage, there are also changes in the plasma levels of different hormones. In salmon, gene expression of the intraovarian growth hormone (GH)-IGF system and somatolactin decrease, suggesting that these genes may be more important during the primary oocyte growth (Campbell et al. 2006). Plasma levels of Fsh and E2, and ovarian steroidogenic acute regulatory protein (*star*) mRNA, increase during the accumulation of cortical alveoli in oocytes, and are associated to an elevation of Igf1 in plasma (Campbell et al. 2006). In European seabass, elevated transcript levels of *igf1* and igf2

mRNAs in the ovary are also found at this stage, which may be involved in lipid accumulation (García-Lopez et al. 2011). The increase in Fsh levels in plasma and of the *fshra* expression in granulosa cells, and subsequent increase in aromatase (Cyp19a1a) and E2 production (see also below), are associated with the accumulation of cortical alveoli in the oocyte (Kwok et al. 2005). A recent *in vitro* study in the coho salmon (*Oncorhynchus kisutch*) shows that the predominant role of androgens is the promotion of growth of late perinucleolar-stage follicles, while E2 stimulates both the growth and accumulation of cortical alveoli in early cortical alveolus-stage follicles (Forsgren and Young 2012). The transcript levels of the anti-Müllerian hormone (*amh*) are also up-regulated in granulosa cells during the transition from the perinuclear to the cortical alveolus stage in several teleosts (Rodriguez-Marí et al. 2005; Luckenbach et al. 2008; García-Lopez et al. 2011). However, despite these changes in different hormones during the secondary oocyte growth of teleosts the specific molecular mechanisms involved are yet not well understood.

3.2.2. Steroidogenic Pathways

Sex steroid hormones are synthesized by the follicle cells from cholesterol with the aid of a variety of steroidogenic enzymes including P450 enzymes and hydroxysteroid dehydrogenases (HSDs) under the control of gonadotropins (Young et al. 2004). During oocyte growth the major steroid produced is E2, which promotes the synthesis of hepatic Vtgs that will be incorporated into the oocyte, whereas during oocyte maturation and ovulation the main steroids synthesized are progestins (Nagahama and Yamashita 2008). Both Fsh and Lh can stimulate the production of E2 during vitellogenesis but only Lh seems to stimulate progestin production (Suzuki et al. 1988; Van Der Kraak et al. 1992; Singh and Thomas 1993; Planas et al. 2000; Rocha et al. 2009; Molés et al. 2008; Aizen et al. 2012). In the red seabream (*Pagrus major*), however, Lh, but not Fsh induces E2 production in vitellogenic ovarian follicles through stimulation of *cyp19a1* gene expression and activity (Gen et al. 2001; Kagawa et al. 2003).

Studies in salmonids have proposed a two-cell type model for the production of steroid hormones under gonadotropin regulation (Nagahama 1994). In this model (Fig. 5), cholesterol is converted to pregnenolone by the P450 enzyme cholesterol side chain cleavage (P450scc or Cyp11a) in the inner membrane of the mitochondria of theca cells (Takahashi et al. 1993). This step involves the import of cholesterol into the mitochondria and is regulated by Star which in turn is up-regulated by gonadotropins (Stocco 2001; Kusakabe et al. 2002; Ings and

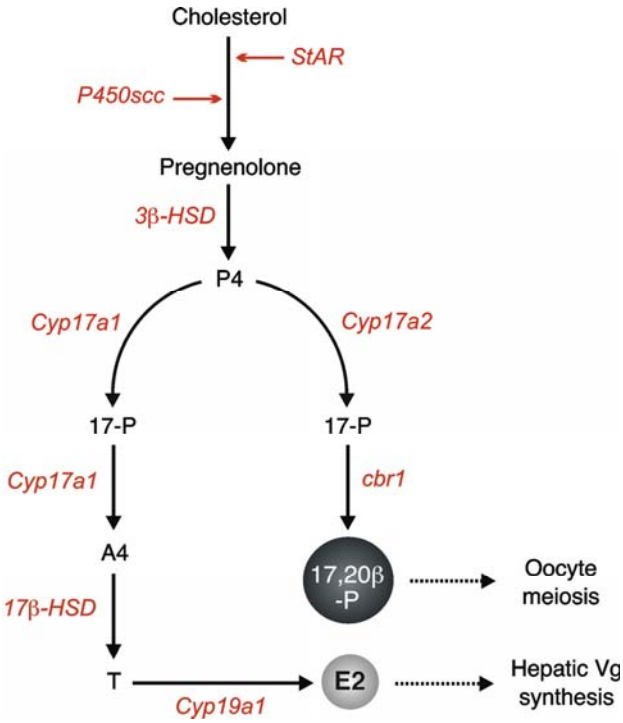

Fig. 5. Schematic diagram of the steroidogenic pathways in theca and granulosa cells of teleosts for the production of estrogens and progestins. P4, progesterone; 17-P, 17α-hydroxyprogesterone; A4, androstenedione; T, testosterone; E2, estradiol-17β; 17,20β-P, 17α,20β-dihydroxy-4-pregnen-3-one.

Van Der Kraak 2006; Nuñez and Evans 2007). After the synthesis of pregnenolone and its conversion to progesterone (P4) by the 3β-Hsd there are two main pathways for androgen/estrogen or progestogen synthesis (Young et al. 2004; Nagahama and Yamashita 2008; Lubzens et al. 2010). The cytochrome P450c17-I (Cyp17a1), which has 17α-hydroxylase and 17,20-lyase enzymatic activities, is involved in the production of both E2 and 17,20β-P. For the production of E2, the Cyp17a1 transforms P4 to 17α-hydroxiprogesterone (17-P) and then to androstenedione (A4), which is converted to testosterone (T) by the 17β-Hsd. The T crosses the basal membrane and is then aromatized to E2 in granulosa cells by the Cyp19a1. The ability to produce E2 is therefore regulated by the level of gene expression and activity of these enzymes, which are controlled presumably by Fsh through its cognate receptor located in the theca and granulosa cells. The salmonid two-cell type model is however not applicable to all teleosts, as reported for the killifish (Petrino et al. 1989) or the Japanese eel (Ijiri et al. 2006).

3.3. Induction of Oocyte Maturation by Lh

The process of meiotic resumption and associated cytoplasmic changes is regulated by the Lh which stimulates the synthesis of the maturation-inducing progestins, which, in turn, activate the maturation-promoting factor (MPF) in the oocyte to resume meiosis (Nagahama and Ya-

mashita 2008). Other compounds can also regulate the process of oocyte maturation in an endocrine or paracrine fashion, such as melatonin (Maitra et al. 2012), TNFα (Crespo et al. 2012), or activin-A, TGFβ1, BMP15 and Igf3 (Tan et al. 2009; Li et al. 2011).

A number of studies in different species have demonstrated that 17,20β-P or 17,20β,21-P are the most potent inducers of oocyte maturation in teleosts (Jalabert 1976; Sundararaj and Goswami 1977; Young et al. 1982; Goetz 1983; Nagahama et al. 1983; Thomas 1994). The two-cell type model has also been proposed for the production of progestins (Fig. 5). This model suggests that during oocyte maturation 17-P is accumulated in theca cells which then difusses into the granulosa cells where is converted to 17,20β-P by the 20β-hydroxysteroid dehydrogenase (HSD)/carbonyl reductase-like (Cbr1) enzyme (Nagahama and Yamashita 2008). In some species, 17-P is converted to 17,21-dihydroxy-pregn-4-ene-3,20-dione (11-deoxycortisol) to the alternative maturation-inducing steroid 17,20β,21-P (Jeng et al. 2012).

3.3.1. The Steroidogenic Shift

During late vitellogenesis and prior to oocyte maturation there is a steroidogenic shift from the production of E2 to the production of 17,20β-P (Nagahama and Yamashita 2008). During these steroidogenic shift the synthesis of the precursors change from T for E2 production to 17-P for 17,20β-P or 17,20β,21-P production, and the enzymes involved in the final conversion also change from Cyp19a1 to Cbr1. These changes may be regulated by gonadotropins, since Fsh stimulates the production of T and Cyp19a1 activity for the production of E2, whereas Lh enhances the production of 17-P and the activity of the Cbr1 (Nagahama and Yamashita 2008). Probably, the surge of Lh induces the steroidogenic shift from E2 to progestin in the granulosa cells at the time of oocyte maturation.

Recent studies have uncovered the molecular basis of the steroidogenic shift in teleosts. This is related to the discovery of a second Cyp17a enzyme in teleosts, the P450c17-II (Cyp17a2), which has only 17α-hydroxylase activity (Zhou et al. 2007a). The expression of the Cyp17a1 is elevated during vitellogenesis and decreases before oocyte maturation, whereas the expression of Cyp17a2 peaks during late vitellogenesis, coinciding with the production of progestins, suggesting that there is a shift in the synthesis of these two enzymes from oocyte growth to maturation (Zhou et al. 2007b). In addition, just before oocyte maturation and in post-ovulatory follicles Cbr1 activity is induced by Lh in a manner dependent on the transcription and translation of other genes, such as *star*; *hsd3b* and *cyp19a1*, during late vitellogenesis (Nagahama 1997; Tanaka et al. 2002; Senthilkumaran et al. 2002; Yoshiura et al.

2003; Nakamura et al. 2005; Nakamoto et al. 2012). Therefore, these changes in gene expression and enzyme activity would result in the accumulation of 17-P in theca or granulosa cells and the production of 17,20β-P (Fig. 5). The transcriptional regulation of the *cyp17a1* and *cyp17a2* genes is mediated by two different transcription factors, Ad4BP/SF-1 that acts on the *cyp17a1* gene through a single promoter site and on the *cyp17a2* gene through two distinct promoter sites, and the Foxl2 that enhances the effect of the Ad4BP/SF-1 on *cyp17a2* transcriptional activation (Zhou et al. 2007b).

3.3.2. The mPgr and Downstream Pathways

Evidence in teleosts suggests that the mPgrα and mPgrβ are involved in oocyte maturation stimulated by progestins in response to Lh (Zhu et al. 2003a,b; Tokumoto et al. 2012). The mPgrα is expressed on the oocyte plasma membrane and treatment with gonadotropins increases its expression during the initial phase of oocyte maturation (Zhu et al. 2003a,b; Hanna and Zhu 2009). Such up-regulation of mPgrα is associated with the development of maturational competence, and microinjection of oocytes with a morpholino antisense oligonucleotide to mPgrα inhibits GVBD (Zhu et al. 2003a; Tokumoto et al, 2006). In the zebrafish, inhibition of mPgrβ also using antisense oligonucleotides suggests that this receptor subtype is also involved in oocyte maturation (Hanna and Zhu 2009, 2011; Tubbs et al. 2010). However, overexpression of both subtypes in oocytes indicates that only mPgrα accelerates the maturation of oocytes.

As discussed above, the Lh surge induces the production of progestins that bind to the mPgr and activates the MPF to resume meiosis. Meiosis resumption is associated with a decrease of the cAMP levels in the oocyte which produces an inactivation of cAMP-dependent protein kinases presumably involved in the activation of the MPF (Jalabert and Finet 1986; Nagahama and Yamashita 2008). In different fish species, data suggest that such decrease in cAMP is mediated through activation of a pertussis toxin-sensitive inhibitory G protein directly coupled to the mPgr which produces a down-regulation of adenylyl cyclase activity (Yoshikuni and Nagahama 1994; Pace and Thomas 2005). There is evidence that mPgrα is coupled to G protein (Thomas et al. 2007) and also that pertussis toxin blocks progestin induction of oocyte maturation (Pace and Thomas 2005). In addition, in cultured cells expressing recombinant mPgrα and exposed to progestin there is a decrease in cAMP production which is inhibited by a pertussis toxin pre-treatment (Zhu et al. 2003a; Pace and Thomas 2005). These observations suggest that mPgrα, and probably mPgrβ, are coupled to an inhibitory G protein

that is activated by progestins resulting in a decrease of cAMP levels and subsequent activation of meiosis resumption.

3.4. Molecular Basis of Oocyte Hydration

During oocyte maturation, the Lh surge and subsequent synthesis of progestins and activation of the MPF in the oocyte, also induces the intraoocytic accumulation of osmolytes, such as inorganic ions and FAAs, for oocyte hydration (see above). It was therefore assumed that oocyte hydration occurred passively by simple diffusion of water through the plasma membrane. However, the recent discovery of the role of a teleost-specific molecular water-channel, termed aquaporin-1b (Aqp1b), during the process of oocyte hydration in pelagophil teleosts (Fabra et al. 2005, 2006; Cerdà 2009), suggests that this process is in fact a highly controlled process based upon the interplay between osmolyte accumulation and water uptake through Aqp1b.

3.4.1. Structure, Classification and Evolution of Teleost Aquaporins

Aquaporins belong to a superfamily of integral membrane proteins that specifically transport water and other non-charged small solutes across biological membranes along an osmotic gradient (King et al. 2004). These membrane proteins have been identified in virtually every living organism, from prokaryota to Eukaryota, indicating that this family of proteins is involved in fluid homeostasis in diverse biological processes throughout the natural world (King et al. 2004). Aquaporins consists of six transmembrane domains connected by five loops (A-E) with their N- and C-terminus located intracellularly (Fig. 6). The deduced sequence of aquaporins reveal a topology of two tandem repeats, oriented at 180º with respect to each other, containing the signature asparagine–proline–alanine (NPA) motif in loops B and E, which is the hallmark of the major intrinsic proteins (MIP) family to which aquaporins belong. Loops B and E and their correct folding are important for water pore formation and the Cys^{189} proximal to the NPA motif in the loop E is responsible for the inhibition of AQP water permeability by submillimolar concentrations of Hg^{2+} (Agre et al. 2002).

Based on structural and permeability properties, the aquaporins are divided into four subgroups: the water-selective channels (AQP0, -1, -2, -4, and -5), the aquaglyceroporins that are also permeable to small solutes such as urea and glycerol (AQP3, -7, -9, and -10), the unorthodox aquaporins (AQP11 and -12), and the AQP8 water and urea channel (King et al. 2004; Tingaud-Sequeira et al. 2010; Cerdà and Finn 2010; Finn and Cerdà 2011). In Eutheria,

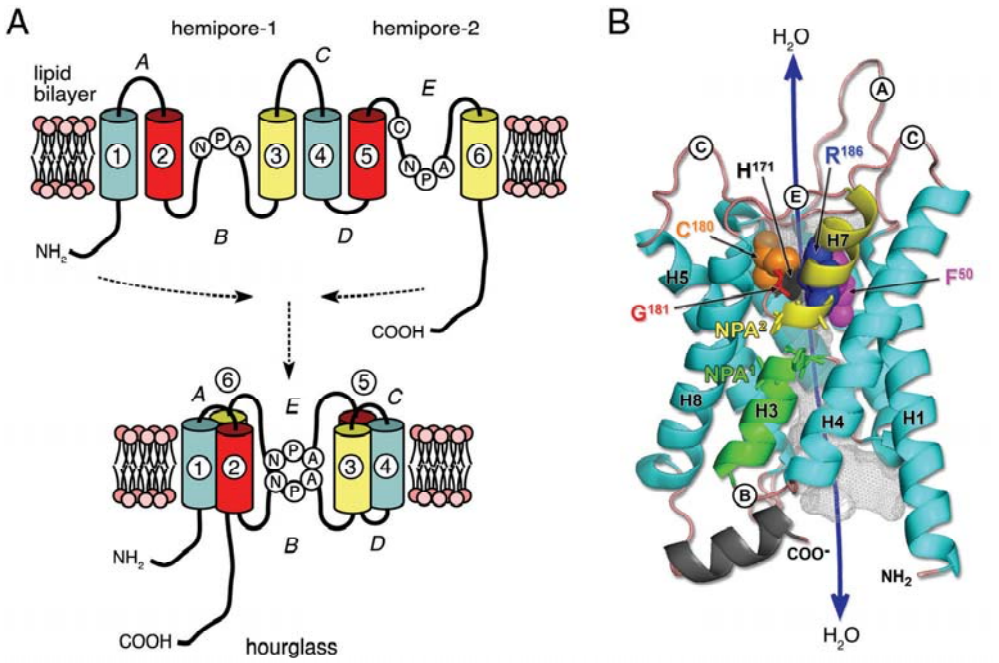

Fig. 6. Topology and structure of the prototypical AQP1. A, 'Hourglass' model for AQP1 topology in which arrangement of loops B and E with highly conserved NPA motifs forms a single aqueous pathway through the AQP1 subunit. Modified from Jung et al. (1994). B, Three-dimensional reconstruction of zebrafish Aqp1a showing the paralog-specific ar/R residues (spacefill). Amino acid positions are numbered according to the zebrafish paralog. The central channels are indicated by double-ended arrows mapped through surface renders of the culled cavities using MacPymol. The model is are based upon the structure masks of bovine (*Bos Taurus*) AQP1 (1J4N, Sui et al. 2001). Modified from Cerdà and Finn (2010).

thirteen aquaporins (AQP0-12) are known, whereas in Prototheria and Amphibia an additional aquaglyceroporin that is closely related to AQP3 and -9, termed AQPxlo, has been identified (Virkki et al. 2002; Cerdà and Finn 2010; Finn and Cerdà 2011). In ostariophysan teleosts such as the zebrafish initial studies of the aquaporin superfamily identified seventeen genes encoding proteins structurally and functionally related to the tetrapod counterparts, *aqp0a*, *-0b*, *-1a*, *-1b*, *-3a*, *-3b*, *-4*, *-7*, *-8aa*, *-8ab*, *-8b*, *-9a*, *-9b*, *-10a*, *-10b*, *-11b* and *-12*, and a putative pseudogene that displays hybrid exons similar to tetrapod AQP5 and -1 (Tingaud- Sequeira et al. 2010; Cerdà and Finn 2010). More recently, a second *aqp8b* gene has been identified by the ensemble genome consortium, bringing the total paralog count to nineteen genes in zebrafish (Finn and Cerdà 2011). The majority of teleosts aquaporins have originated from the ancient whole genome duplication (WGD); however the *aqp8* and *aqp1* genes remained equivocal despite dichotomous and trichotomous clustering in relation to the human orthologs. The duplication history and putative neofunctionalization of the teleost *aqp8aa*, *-8ab*, and *-8b* paralogs has been explained on the basis of a combination of WGD and tandem repli-

cation (Cerdà and Finn 2010), while the *aqp1* paralogs were suggested to have arisen via tandem duplication due to their juxtaposition in teleost genomes (Tingaud-Sequeira et al. 2008).

3.4.2. The Aqp1b Subfamily and its Role During Oocyte Hydration

The Aqp1b (previously named Aqp1o) is a teleost-specific ortholog of the mammalian AQP1 water chanel which function during oocyte hydration was initially identified in the pelagophil teleost gilthead seabream (Fabra et al. 2005). The Aqp1b has also been implicated in the

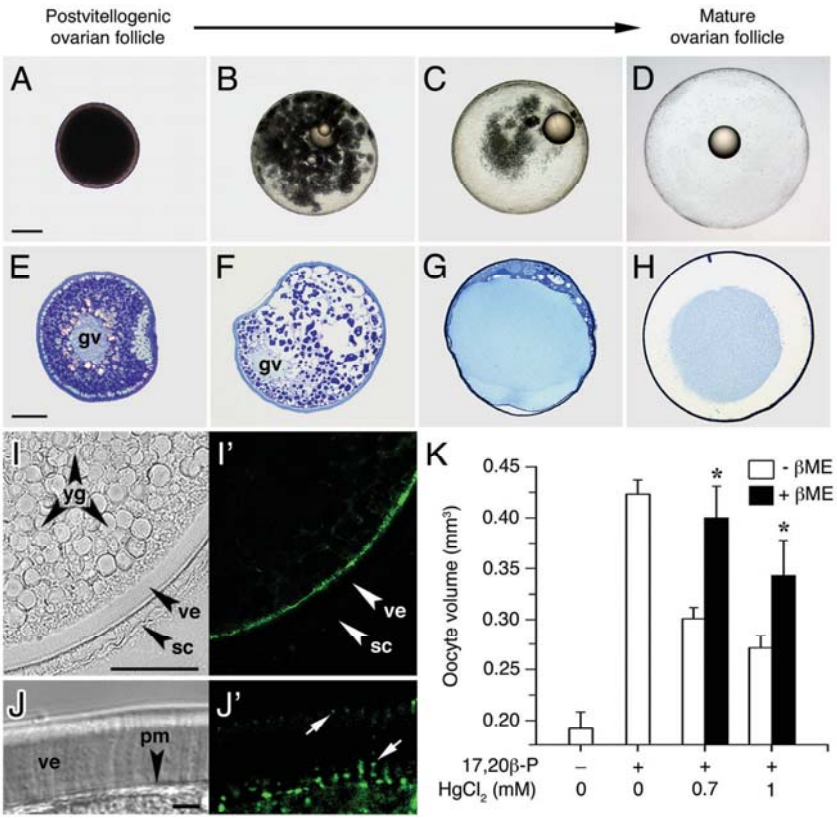

Fig. 7. Oocyte hydration in the pelagophil gilthead seabream and role of aquaporin-1b (Aqp1b). A-D, Photomicrographs of seabream ovarian follicles undergoing oocyte maturation and hydration showing the sequential changes in the morphology of oocytes. E-H, Histological sections of the stages shown in (A-D), stained with methylene blue to show yolk components, in which the migration (F) and breakdown of the germinal vesicle (gv), and the process of yolk globule fusion (F-H) can be observed. I, J, Brightfield (I, J) and immunofluorescence (I', J') images of Aqp1b immunolocalization in postvitellogenic oocytes (I), and in the microvilli of oocytes undergoing hydration (J). The arrow and arrowheads indicate the plasma membrane and the microvilli, respectively. yb, yolk globules; ve, vitelline envelope; sc, somatic cells; pm, plasma membrane. (K) Mercury inhibition of oocyte hydration *in vitro* induced with the progestin 17,20β-P and recovery by 5 mM βME. Values are means ± SEM. The asterisks denote statistically significant differences (P <0·05.) between oocytes treated or not with βME. Scale bars, 200 μm (A-H), 50 μm (I) and 5 μm (J). Taken from Cerdà (1999).

process of oocyte hydration of other marine and catadromous teleosts producing pelagic eggs where high ovarian *aqp1b* expression is found (Tingaud-Sequeira et al. 2008; Sun et al. 2010; Kagawa et al. 2011). Interestingly, *aqp1b* transcripts are also highly accumulated in the ovary of some freshwater species, such as the stinging catfish (*Heteropneustes fossilis*), in which oocytes partially hydrate during meiotic maturation although benthic eggs are produced (Chaube et al. 2011). Phylogenetic analyses reveal that Aqp1b was originated during evolution early in the teleost lineage possibly by tandem duplication of an ancestral AQP1-like gene and further structural divergence of the C-terminus, suggesting that Aqp1b is encoded by a gene unique to teleosts that represents a neofunctionalized water channel (Tingaud-Sequeira et al. 2008).

The role of Aqp1b mediating water influx into the oocyte during oocyte hydration in marine teleosts was investigated using immunocytochemical and functional studies (Fabra et al. 2005, 2006) (Fig. 7). The Aqp1b protein product is found in the oocyte but not in the surrounding follicle cells. The Aqp1b is apparently synthesized in cortical alveolus oocytes and localized in the cytoplasm, whereas it is transported towards the oocyte cortex throughout the growth period (Fabra et al. 2006). In post-vitellogenic oocytes, Aqp1b is completely localized within a thin layer just below the oocyte plasma membrane. During oocyte maturation, shortly after GVBD and before complete hydrolysis of the yolk proteins and maximum K^+ accumulation is reached, Aqp1b is transiently translocated into the oocyte plasma membrane (Fabra et al. 2006). In the Japanese eel, localization of the Aqp1b during oocyte development is similar to that in the seabream, however, in fully-grown oocytes the localization of Aqp1b just below the plasma membrane is not apparent, and may be this reflects a different mechanism of oocyte hydration in this teleost (Kagawa et al. 2011).

The specific subcellular localization of Aqp1b correlating with oocyte hydration in the seabream provided indirect evidence of the physiological role of this channel during oocyte hydration (Fig. 7). This conclusion was subsequently supported by the observation that the swelling of seabream oocytes during meiotic maturation is blocked by mercury and tetraethylammonium, known inhibitors of aquaporin permeability that are effective at blocking Aqp1b-mediated water transport in *Xenopus laevis* oocytes (Fabra et al. 2005, 2006; Tingaud-Sequeira et al. 2008; Kagawa et al. 2009). However, it is well known that these inorganic compounds can also affect K^+ channels and other ion transport proteins, which may play a role for inorganic osmolyte accumulation during oocyte hydration (Cerdà et al. 2007; Finn and Fyhn 2010). Therefore, these findings strongly suggest that Aqp1b is involved in water uptake dur-

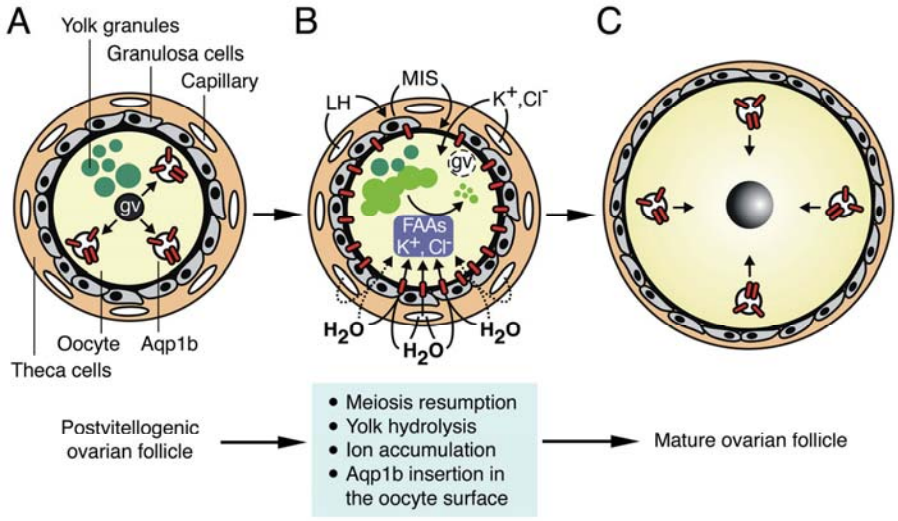

Fig. 8. Proposed model of the main physiological processes during oocyte maturation and hydration in The gilthead seabream. A, During vitellogenesis, the Aqp1b is synthesized and transported within vesicles towards the oocyte plasma membrane. B, At the maturation stage, the granulosa cells are stimulated by LH to produce the maturation-inducing steroid (MIS), i.e. progestins, which will induce meiosis resumption of the oocyte. During this process, the germinal vesicle (gv) migrates towards the animal pole and eventually breaks down, yolk globules fuse with one another and yolk protein hydrolysis is activated, whereas inorganic ions (K^+, Cl^-) are accumulated in the oocyte. At this time, Aqp1b is further translocated into the oocyte plasma membrane, where it can mediate water transport (solid lines) driven by the increase in yolk protein-derived FAAs and of other small-molecular-mass osmotic effectors. Additionally, water influx into the oocyte can occur by simple diffusion through the follicular membranes (dotted lines). C, When yolk proteolysis is completed, Aqp1b-containing vesicles are retrieved from the oocyte plasma membrane. As a result, a highly hydrated oocyte is produced. Taken from Cerdà (1999).

ing fish oocyte hydration (Fig. 8). However, direct evidence for the role of Aqp1b during oocyte hydration is still lacking.

3.4.3. Regulation of Aqp1b During Oocyte Growth and Maturation

Immunocytochemical studies using Aqp1b specific antibodies in the gilthead seabream and Japanese eel have not detected Aqp1b polypeptides in the oocyte until the cortical alveolus or early vitellogenesis, suggesting that Aqp1b is synthesized *de novo* by the oocyte at the initiation of vitellogenesis from a pre-existing mRNA pool (Fabra et al. 2006; Kagawa et al. 2011). These observations suggest that Aqp1b may be highly regulated at the transcriptional and translational level during early oocyte growth. During vitellogenesis and oocyte maturation, low *aqp1b* transcriptional activity in oocytes is observed (Fabra et al. 2006; Kagawa et al. 2011), suggesting that at these stages the major regulatory pathways occur at the post-

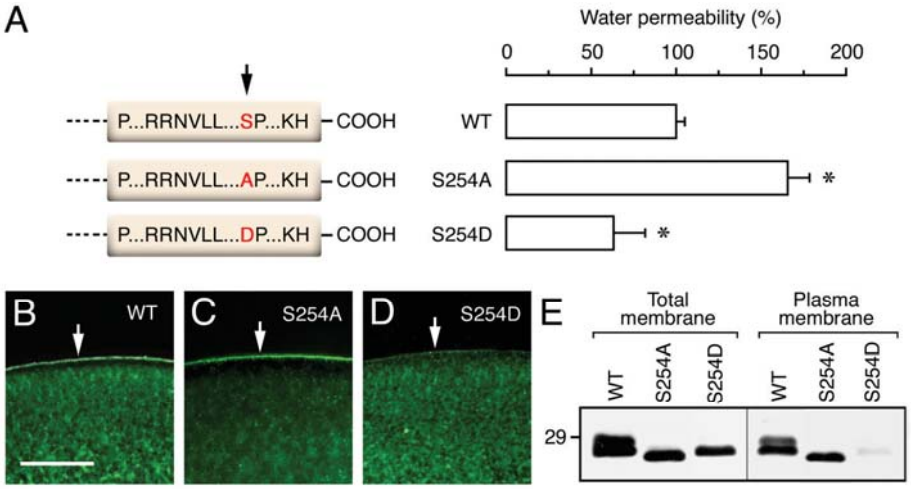

Fig. 9. Role of the C-terminus Pro-directed kinase motif for the intracellular trafficking of gilthead seabream Aqp1b in *Xenopus laevis* oocytes. A, Water permeability of oocytes expressing wild-type (WT) Aqp1b or Aqp1b mutated at the Ser residue of the Pro-directed kinase motif (Aqp1b-S254A and Aqp1b-S254D). Permeability (mean ± SEM) is expressed in % related to oocytes injected with WT Aqp1b. Asterisks denote statistically significant differences ($P<0{\cdot}05$) with respect to oocytes expressing Aqp1b WT. B-D, Localization of WT or mutant Aqp1b in oocytes (arrows indicate the plasma membrane). E, Immunoblots of total and plasma membrane equivalents of oocytes expressing WT or mutant Aqp1b. The apparent molecular mass of a 29-kDa marker is indicated on the left. Note that the Aqp1b-S254A mutant prevented phosphorylation (upper band), and increased protein translocation into the oocyte plasma membrane and water permeability, whereas the Aqp1b-S254D mutant, which mimics constitutively phosphorylated state of Aqp1b, was predominantly located in intracellular vesicles. Taken from Cerdà (1999).

translational level, resulting in the controlled trafficking of Aqp1b-containing vesicles in the oocyte.

Functional expression analyses of seabream Aqp1b in *X. laevis* oocytes revealed that is less functional than its paralog Aqp1a due to partial retention in the cytoplasm (Tingaud-Sequeira et al. 2008). Phylogenetic analyses has indicate that the most divergent region between the amino acid sequence of teleost Aqp1a and Aqp1b is the C-terminus (Tingaud-Sequeira et al. 2008; Finn and Cerdà 2011), which in mammalian AQP2 and amphibian AQPa2 is known to play a role in the intracellular trafficking of the proteins (Deen et al. 2000; Hasegawa et al. 2005). However, although the C-terminus is divergent among teleosts, retains specific motifs that may regulate vesicular trafficking, and can therefore be involved in the control of Aqp1b translocation into the oocyte plasma membrane during oocyte growth and/or maturation (Tingaud-Sequeira et al. 2008; Chaube et al. 2011). These mechanisms were investigated using chimeric constructs of seabream Aqp1a and Aqp1b as well as site-directed mutagenesis (Fig. 9), and the results showed that phosphorylation of Ser^{254}, a consensus site for a Pro-directed kinase that is conserved in most telost Aqp1b, mediates Aqp1b recycling in

X. laevis oocytes (Tingaud-Sequeira et al. 2008). Therefore, it is possible that Pro-directed kinases such as the p38 MAPK, which is involved in oocyte maturation in vertebrates (Perdiguero and Nebreda 2004), might play a role for the regulation of Aqp1b translocation in seabream oocytes. A di-Leu motif, a sorting and internalization signal common in transmembrane proteins (Bonifacino and Traub 2003), is also conserved among teleost Aqp1b, and its mutation induces the mis-folding of the protein inhibiting its translocation into the plasma membrane (Tingaud-Sequeira et al. 2008). These observations thus suggest that the di-Leu sorting motif may also be implicated in the control of Aqp1b expression on the oocyte cell surface.

The regulatory pathways for the control Aqp1b trafficking may be highly divergent among teleosts. In the stinging catfish, for instance, intracellular cAMP drives the sorting of Aqp1b to the plasma membrane of *X. laevis* oocytes though phophorylation of the Ser^{277} (Chaube et al. 2011). This mechanism is thus different to that reported for the gilthead seabream Aqp1b where phosphorylation of the Ser^{254} drives internalization of the protein (Tingaud-Sequeira et al. 2008). The catfish Aqp1b shows therefore functional similarities with amphibian AQP-h2, and Japanese quail and mammalian AQP2 which trafficking is regulated by vasotocin/vasopressin through a cAMP-dependent mechanism (Yang et al. 2004; Fenton et al. 2008; Hasegawa et al. 2003). Interestingly, catfish oocyte hydration is stimulated by vasotocin (Singh and Joy 2010), and then it is possible that in this species a vasotocin-triggered cAMP surge can produce an increased translocation of Aqp1b into the oocyte plasma membrane (Chaube et al. 2011).

3.5. Regulation of Ovulation

In teleosts, the process of ovulation follows the meiotic maturation and hydration of the oocyte and is regulated by Lh and progestins. However, unlike meiosis resumption, ovulation requires progestin-dependent transcriptional activation suggesting that the nuclear Pgr is involved in this process (Pinter and Thomas 1999; Patiño et al. 2003). In Atlantic croaker, arachidonic acid (AA) and its cyclooxygenase (COX) metabolites, as well as the PKC, are suggested to be part of the transduction pathway leading to ovulation (Patiño et al. 2003). Prostaglandin E (PGE) and F (PGF) are known to be involved in ovulation in different fish species (Hsu and Goetz 1992; Lister and Van der Kraak 2008; Fujimori et al. 2011), and in yellow perch (*Perca flavescens*) their synthesis appears to be stimulated by 17,20β-P (Goetz, 1997). However, the control of prostaglandin synthesis by steroids may be complicated and involve an interaction between extra-follicular and follicular tissue (Goetz and Garczynski 1997). In

zebrafish, hCG treatment increases the levels of 17,20β-P and preserve the decline in PGF2α levels (Lister and Van der Kraak 2009). The hCG also increases the expression of cytosolic phospholipase A2 (*cpla2*) and *cox-2* (also termed *ptgs2*) involved in mobilization and metabolism of AA (Lister and Van der Kraak 2009). However, other genes involved in the metabolism of AA, such as *ptgs1* and *ptgsl*, are also down regulated, suggesting that regulation of prostaglandin synthesis possibly involves different endogenous factors that are still unknown (Lister and Van der Kraak 2009).

In addition to Lh and steroids, other hormones have been implied in the ovulatory process, such as vasotocin which induces the production of progestins and subsequent interaction with their receptor (Singh and Joy 2011). In brook trout (*Salvelinus fontinalis*), in *vitro* effects of recombinant tumor necrosis factor alpha (TNFα) in preovulatory ovarian follicles suggest that TNFα mediates follicle weakening and ovarian rupture through stimulation of cell apoptosis (Crespo et al. 2010). High expression of genes involved in proteolysis, inflammation, coagulation, vasodilatation, and angiogenesis have been also observed during ovulation (Bobe et al. 2006, 2009), suggesting that this process is and inflammatory-like reaction as observed in mammals (Espey 1994).

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OBJECTIVES

Previous works have discovered the role of the teleost-specific Aqp1b during oocyte hydration in pelagophil teleosts. However, direct experimental evidence for the function of Aqp1b mediating water uptake in oocytes is still lacking. In addition, initial reports in the gilthead seabream and Japanese eel have shown that *aqp1b* expression is initiated in pre-vitellogenic oocytes, whereas the synthesis of the corresponding polypeptides does not occur until the onset of vitellogenesis, and subsequently during oocyte growth the protein is translocated to the oocyte plasma membrane (see Introduction). These data therefore suggest that the *aqp1b* gene might be regulated at the transcriptional, translational and post-translational level in oocytes, but the molecular mechanisms involved remain unknown. Since the Aqp1b may play a major role during the maturation of oocytes in pelagophil teleosts, the elucidation of these mechanisms is essential to understand the molecular basis of egg formation in teleosts.

In this context, the main objective of this thesis was to provide functional evidence of the essential role of Aqp1b during oocyte hydration in fish, and begin to dissect the molecular mechanisms involved in the transcriptional regulation of *aqp1b* in the oocyte of marine teleosts. Using a combination of cellular and molecular approaches, the following objectives were addressed:

i. To investigate the origin of *aqp1b* in teleosts through extensive phylogenetic and syntenic analyses, and to provide experimental evidence of the role of Aqp1b during oocyte hydration in an advanced teleost, such as the Atlantic halibut (*Hippoglossus hippoglossus*), using specific molecular approaches (**Chapter I**).

ii. To investigate the production of steroid hormones, particularly of progestins, in primary growth ovarian follicles of the gilthead seabream, and its possible gonadotropic regulation, as potential regulators of *aqp1b* transcription and/or translation in pre-vitellogenic primary growth oocytes (**Chapter II**).

iii. To characterize at the molecular and functional level the gilthead seabream nuclear progestin receptor (Pgr), and potential splice variants, and investigate its cellular localization and hormonal regulation in primary growth ovaries (**Chapter III**).

iv. To isolate the promoter region of the gilthead seabream *aqp1b* gene and characterize some of the proximal *cis*-regulatory elements present, and investigate *in vivo* and *in vitro* the role of the nuclear Pgr and other transcription factors in the transcriptional regulation of *aqp1b* in primary growth oocytes (**Chapter IV**).

IMPACT FACTOR

FACTOR DE IMPACTO

El Dr. **Joan Cerdà Luque**, director de la tesis doctoral titulada "*Fisiología Molecular de una Acuaporina Ovocitaria de Teleósteos: Evolución, Regulación y Papel Durante la Hidratación del Oocito*", realizada por **Cinta Zapater Cardona**, certifica la veracidad del factor de impacto y la implicación del doctorando en cada uno de los artículos científicos que se incluyen en la presente tesis doctoral.

Artículo 1 (Chapter I)

Título: **Dual neofunctionalization of a rapidly evolving aquaporin-1 paralog resulted in constrained and relaxed traits controlling channel function during meiosis resumption in teleosts**

Autores: Cinta Zapater, François Chauvigné, Birgitta Norberg, Roderick Nigel Finn, Joan Cerdà

Ref. revista: *Molecular Biology and Evolution* 28(11):3151–3169 (2011)

Factor de impacto: 5,550 (cuartil 1, Q1)

Participación: Cinta Zapater ha realizado la totalidad del trabajo experimental, incluyendo parte de los estudios de traslocación de la acuaporina-1ab en oocitos de *Xenopus laevis* y pez cebra, así como el análisis de los resultados. Asimismo, ha realizado parte de los análisis filogenéticos.

Artículo 2 (Chapter II)

Título: **Piscine follicle-stimulating hormone triggers progestin production in gilthead seabream primary ovarian follicles**

Autores: Cinta Zapater, François Chauvigné, Alexander P. Scott, Ana Gómez, Ioanna Katsiadaki, and Joan Cerdà

Ref. revista: *Biology of Reproduction* 87(5):111 (2012)

Factor de impacto: 4,009 (cuartil 1, Q1)

Participación: Cinta Zapater ha participado en el diseño experimental y ha llevado a cabo todos los experimentos, exceptuando los estudios de hibridación *in situ*, y el análisis de los resultados. Asimismo ha realizado la mayor parte de los análisis filogenéticos y ha redactado la primera versión del manuscrito.

Artículo 3 (Chapter III)

Título: **Alternative splicing of the nuclear progestin receptor in a perciform teleost generates novel mechanisms of dominant-negative transcriptional regulation**

Autores: Cinta Zapater, François Chauvigné, Beatriz Fernández-Gómez, Roderick Nigel Finn, Joan Cerdà

Ref. revista: *General and Comparative Endocrinology* 182:24-40 (2013)

Factor de impacto: 3,267 (cuartil 2, Q2)

Participación: Cinta Zapater ha participado en el diseño de los experimentos, ha generado y analizado la mayor parte de los resultados, y ha redactado la primera versión del trabajo.

Artículo 4 (Chapter IV)

Título: **Primary oocyte transcriptional activation of *aqp1ab* by the nuclear progestin receptor determines the pelagic egg phenotype of marine teleosts**

Autores: Cinta Zapater, François Chauvigné, Angèle Tingaud-Sequeira, Roderick Nigel Finn, Joan Cerdà

Ref. revista: *Developmental Biology*, in press (doi: 10.1016/j.ydbio.2013.03.001) (2013)

Factor de impacto: 4,069 (cuartil 1, Q1)

Participación: Cinta Zapater ha participado en el diseño de los experimentos, y ha generado y analizado todos los resultados, con excepción de los experimentos de hibridación *in situ* e inmunofluorescencia. Asimismo, ha redactado la primera versión del trabajo.

PUBLICATIONS

Chapter I

Dual Neofunctionalization of a Rapidly Evolving Aquaporin-1 Paralog Resulted in Constrained and Relaxed Traits Controlling Channel Function During Meiosis Resumption in Teleosts

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Abstract

The pre-ovulatory hydration of marine teleost oocytes is a unique process among vertebrates. The hydration mechanism is most pronounced in modern acanthomorph teleosts that spawn pelagic (floating) eggs, however the molecular pathway for water influx remains poorly understood. Recently, we revealed that whole genome duplication (WGD) resulted in teleosts harbouring the largest repertoire of molecular water channels in the vertebrate lineage, and that a duplicated aquaporin-1 paralog is implicated in the oocyte hydration process. However, the origin and function of the aquaporin-1 paralogs remains equivocal. By integrating the molecular phylogeny with synteny and structural analyses we show here that the teleost *aqp1aa* and *-1ab* paralogs (previously annotated as *aqp1a* and *-1b*, respectively) arose by tandem duplication rather than WGD, and that the Aqp1ab C-terminus is the most rapidly evolving subdomain within the vertebrate aquaporin superfamily. The functional role of Aqp1ab was investigated in Atlantic halibut, a marine acanthomorph teleost that spawns one of the largest pelagic eggs known. We demonstrate that Aqp1ab is required for full hydration of oocytes undergoing meiotic maturation. We further show that the rapid structural divergence of the C-terminal regulatory domain causes *ex vivo* loss of function of halibut Aqp1ab when expressed in amphibian oocytes, but not in zebrafish or native oocytes. However, by using chimeric constructs of halibut Aqp1aa and -1ab, and antisera specifically raised against the C-terminus of Aqp1ab, we found that this cytoplasmic domain regulates *in vivo* trafficking to the microvillar portion of the oocyte plasma membrane when intra-oocytic osmotic pressure is at a maximum. Interestingly, by co-injecting polyA$^+$ mRNA from post-vitellogenic halibut follicles, *ex vivo* intracellular trafficking of Aqp1ab is rescued in amphibian oocytes. These data reveal that the physiological role of Aqp1ab during meiosis resumption is conserved in teleosts, but the remarkable degeneracy of the cytoplasmic domain has resulted in alternative regulation of the trafficking mechanism.

Key words: Aquaporin, Oocyte, Meiosis, Hydration, Yolk, Neofunctionalization

1. Introduction

Meiosis resumption is an ubiquitous developmental feature of vertebrate oocytes, in which the germinal vesicle matures in preparation for fertilization (Eppig et al. 2004; Philpott and Yew 2008; Lessman 2009). In oviparous marine teleosts, however, meiosis resumption is synchronized with the cytoplasmic enlargement of the oocyte as a result of a massive water uptake (Fulton 1898). This process of oocyte hydration evolved exclusively in the teleost lineage and is thus unique among vertebrates. It is found in ancient elopomorph, clupeimorph and modern acanthomorph teleosts and represents a pre-adaptation to the oceanic environment since it assures a water reservoir when single-celled eggs are released into the hyperosmotic seawater (Fyhn et al. 1999; Finn and Kristoffersen 2007). The degree of hydration, however, appears to vary according to the system of intracellular osmolyte generation. Available evidence suggests that species that spawn benthic (sinking) eggs predominantly accumulate inorganic ions such as K^+, Cl^-, PO_4^{3-} or Na^+ (Greeley et al. 1991; Cerdà et al. 2007; Kristoffersen and Finn 2008), while those that spawn pelagic (floating) eggs further activate lysosomal proteases (cathepsins) that differentially degrade vitellogenin (Vtg)-derived yolk proteins to generate an additional organic osmolyte pool of free amino acids (FAA) (Cerdà et al. 2007; Finn and Fyhn 2010). The innate stimuli that activate these processes are C_{21} steroids (progestins) synthesized by the granulosa cells in response to a surge of luteinizing hormone secreted by the pituitary (Thomas et al. 2007; Nagahama and Yamashita, 2008). This endocrine cascade initiates meiosis resumption, cytoplasmic maturation, and the accumulation of osmolytes for oocyte hydration (Cerdà et al., 2007; Thomas et al. 2007; Nagahama and Yamashita, 2008). The interesting aspect is the positive selection of these latter mechanisms in modern acanthomorph teleosts. Regardless of systematic affinities or ecological niche, the majority of extant marine acanthomorph teleosts depolymerise VtgAa-type yolk proteins and spawn pelagic eggs (Cerdà et al. 2007; Finn and Kristoffersen 2007; Finn 2007ab; Kolarevic et al. 2008). Consequently, the phenotypic nature of the pelagic egg, which facilitates oxygen exchange and dispersal of the early embryos in the ocean, has been associated with the remarkable radiation of teleosts during the Eocene (Fyhn et al. 1999; Finn and Kristoffersen 2007).

For many years it was thought that the pathway for water entry into the maturing teleost oocyte was benign, occurring via bulk flow across the plasma membrane. However, the short temporal phase of the hydration process suggested that a specific mechanism must have co-evolved to facilitate the controlled movement of water into the oocyte. In line with this hypothesis, we recently identified a teleost-specific ortholog of the mammalian aquaporin-1

water channel (Fabra et al. 2005). This novel aquaporin (Aqp1ab, previously named Aqp1o or Aqp1b) is the channel most likely involved in oocyte hydration (Fabra et al. 2005, 2006; Tingaud-Sequeira et al. 2008). In the perciform teleost gilthead seabream (*Sparus aurata*), which produces pelagic eggs, Aqp1ab is synthesized in early oocytes and transported towards the oocyte cortex throughout the growth period (Fabra et al. 2006). Aqp1ab has also been implicated in oocyte hydration of other species, where high ovarian *aqp1ab* expression is found in marine and catadromous teleosts producing pelagic eggs (Tingaud-Sequeira et al. 2008; Sun et al. 2009, 2010). Interestingly, *aqp1ab* transcripts are also highly accumulated in the ovary of some freshwater species, such as the stinging catfish (*Heteropneustes fossilis*), in which oocytes partially hydrate during meiotic maturation although benthic eggs are produced (Chaube et al. 2011).

In addition to circumstantial evidence (i.e., specific subcellular localization of Aqp1ab in the oocyte correlating with the hydration process), the physiological role of Aqp1ab is supported by the observation that the swelling of oocytes undergoing meiosis resumption is completely or partially blocked by aquaporin inhibitors such as mercury and tetraethylammonium (Fabra et al. 2005, 2006; Kagawa et al. 2009). However, it is known that these compounds can also affect K^+ channels and other ion transport proteins (Armstrong 1990; Jacoby et al. 1999), which may play a role for inorganic osmolyte accumulation in the oocyte (Cerdà et al. 2007; Kristoffersen and Finn 2008). Therefore, direct evidence for the role of Aqp1ab during fish oocyte hydration is still lacking. In addition, while the majority of teleost aquaporins appear to have arisen as a consequence of whole genome duplication (WGD) (Tingaud-Sequeira et al. 2010), the *aqp1aa* and *-1ab* genes were suggested to be the result of tandem duplication (Tingaud-Sequeira et al. 2008). To address these issues, we selected the Atlantic halibut (*Hippoglossus hippoglossus*) as an experimental model, since it is a marine acanthomorph teleost that reproduces at low temperature and spawns one of the largest pelagic eggs known. We isolated two novel aquaporin-1 transcripts and examined the functional role of Aqp1ab during meiosis resumption using *ex vivo* and *in vivo* approaches. To determine the duplication history of the teleost *aqp1aa* and *-1ab* genes, we re-examined the molecular phylogeny of the transcripts and deduced proteins in relation to 26 vertebrate orders. These data revealed that tetrapod *AQP1* and teleost *aqp1aa* orthologs have experienced purifying selection within each clade, while the teleost *aqp1ab* orthologs displayed a paralogous subclustering topology. To determine whether a given subcluster could represent the product of WGD, an extended synteny analysis was performed for selected tetrapod and teleost genomes.

2. Materials and Methods

2.1. Animals and Sample Collection

Adult and juvenile Atlantic halibut were kept in circular tanks of 7 m in diameter at 5-6ºC in filtered seawater (35‰ salinity) at the Austevoll Research Station (Institute of Marine Research, Norway). Tissue samples were dissected from sacrificed juvenile fish, frozen in liquid nitrogen and stored at -80ºC until analysis. Ovarian biopsies were taken weekly from adult females that had reached stable ovulatory rhythms, in connection with regular checking of maturity status of broodstock (Norberg et al. 1991; Finn et al. 2002). Biopsies containing ovarian follicles at different developmental stage and unfertilized eggs were collected from adult females by using 4-mm diameter silicone tubes introduced in the ovipore and attached to 10 ml syringes. Ovarian follicles were immediately placed in 75% Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Madrid, Spain) and 100 µg gentamicin/ml, pH 7.5 (Selman and Wallace 1986), and classified into different stages depending on the extent of oocyte hydration: follicles containing postvitellogenic, non hydrated oocytes (PV), follicles with early hydrating oocytes (EH), follicles with mid-hydrated oocytes (MH), follicles with hydrated oocytes (H), and ovulated oocytes or unfertilized eggs (Egg). Some of the PV and EH ovarian follicles were used for microinjection and *in vitro* incubations; the other follicles were frozen in liquid nitrogen and stored at -80ºC. Adult zebrafish were obtained from a local pet store and maintained as described by Westerfield (1995). Procedures related to the care and use of fish followed the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction.

2.2. Cloning and Sequencing of Atlantic halibut Aquaporin-1aa and -1ab

Full-length Atlantic halibut aquaporin-1a (*hhaqp1aa*) and -1b (*hhaqp1ab*) cDNAs were isolated by RT-PCR. Total RNA was extracted from the intestine using the RNAeasy Minikit (Qiagen GmbH, Hilden, Germany), and 10 µg were reverse transcribed using 0.5 µg oligo-$(dT)_{17}$, 1 mM dNTPs, 40 IU RNAse inhibitor (Roche, Barcelona, Spain), and 10 IU SuperScript II (Invitrogen, Barcelona, Spain), for 1.5 h at 42ºC. A partial cDNA encoding *hhaqp1aa* was isolated by RT-PCR using degenerate oligonucleotide primers (F = 5'-GACCAGGARRTSAAGGTG-3' and R = 5'-CACATVGGCCCSACCCAG-3') designed to regions close to the two highly conserved Asn-Pro-Ala (NPA) motifs of teleost Aqp1aa (Tingaud-Sequeira et

al. 2008). The sequence obtained was used to design specific primers to isolate the 5' and 3' ends by RACE kits (Invitrogen). For the 5' end, the two reverse primers were 5'-AGCAACTGGGCCACAATGTA-3' and 5'-CGCTGATGTGGCCTAAACTC-3', and the 3' end the forward primers were 5'-AACCTTCCAGCTGGTGCTGT-3', and 5'-GTCCGGCTTTGATCCTGAAC-3'. The full-length *hhaqp1aa* cDNA was finally amplified with the 3' RACE kit (Invitrogen) using a forward (5'-TTTAGAGAGGACCAGCTCAAACC-3') and AUAP primers and a high fidelity polymerase (Easy-A™ High-Fidelity PCR Cloning Enzyme; Stratagene, Cultek SLU, Madrid, Spain). The products were cloned into the pGEM-T Easy vector (Promega Biotech Iberica SL, Madrid, Spain) and sequenced with BigDye Terminator version 3.1 in DNA analyzer ABI PRISM 377 (Applied Biosystems, Madrid, Spain). For the cloning of the *hhaqp1ab* cDNA, 10 μg of total RNA extracted from H ovarian follicles were used for reverse transcription as indicated above. The 3' RACE kit was used to amplify the full-length cDNA using a specific forward primer designed from an available expressed sequence tag (EST; GenBank accession no. EB039821), 5'-GTTTAAACAGACTCCCCAAAAC-3', and the AUAP primer. The PCR products were cloned and sequenced as above. The nucleotide sequences of full-length *hhaqp1aa* and *-1ab* cDNAs were submitted to the DDBJ/EMBL/GenBank database under the accession numbers HQ185294 and HQ185295, respectively.

2.3. Phylogenetic Analyses

Molecular phylogenies of the cloned aquaporin transcripts and deduced proteins were analyzed via Bayesian (Mr Bayes v3.1.2) and Maximum likelihood protocols as described previously (Finn and Kristoffersen 2007). Aqp1 orthologs and ESTs (Table 1) were obtained from public databases (Ensembl and GenBank) and multiple sequence alignments constructed using the T-coffee suite of alignment tools (Notredame et al. 2000). Each amino acid alignment was converted to a codon alignment (nucleotide triplets) via PAL2NAL (Suyama et al. 2006) and then manually adjusted to correct for errors using MacVector (MacVector Inc, Cambridge, UK). For Bayesian analyses the following models were tested for the codon alignments: nucmodel = 4by4 with nst = 2 or codon with nst = 6; rates = gamma and invgamma, respectively; and for amino acid alignments: aamodel = mixed. For all alignments, MCMC algorithms were run with 1 and 5 million generations using 3 heated chains and 1 cold chain. All runs were examined for convergence using Tracer version 1.5 (www.beast.bio.ed.ac.uk/Tracer), and majority rule consensus trees summarized with a burnin of 3,500.

Table 1. List of GenBank or ensembl accession numbers of AQP1-like sequences used in the study

Orthologue	Species	Common name	Acc#	Class
AQP1	*Homo sapiens*	Human	ENSP00000311165	Mammalia
AQP1	*Mus musculus*	Mouse	ENSMUSP00000004774	Mammalia
AQP1	*Canis familiaris*	Dog	ENSCAFG00000003102	Mammalia
AQP1	*Dasypus novemcinctus*	9-Banded armadillo	ENSDNOP00000008167	Mammalia
AQP1	*Monodelphis domestica*	Opossum	ENSMODP00000026101	Mammalia
AQP1	*Ornithorhynchus anatinus*	Platypus	ENSOANP00000020359	Mammalia
AQP1	*Anas platyrhynchos*	Mallard duck	ENSAPLP00000011568	Aves
AQP1	*Gallus gallus*	Chicken	ENSGALP00000008350	Aves
AQP1	*Meleagris gallopavo*	Turkey	ENSMGAP00000001252	Aves
AQP1	*Coturnix coturnix*	Common quail	AAU07832	Aves
AQP1	*Passer domesticus*	House sparrow	AAV65290	Aves
AQP1	*Taeniopygia guttata*	Zebra finch	ENSTGUP00000000351	Aves
AQP1	*Anolis carolinensis*	Anole lizard	ENSACAP00000005702	Lepidosauria
AQP1	*Bufo marinus*	Marine toad	AAC69693	Amphibia
AQP1	*Hyla chrysoscelis*	Southern gray treefrog	ABC98208	Amphibia
AQP1	*Hyla japonica*	Japanese treefrog	BAC07470	Amphibia
AQP1	*Protopterus annectens*	West African lungfish	BAI48049	Dipnoi
Aqp1aa	*Takifugu rubripes*	Torafugu	ENSTRUP00000034715	Teleostei
Aqp1aa	*Tetraodon nigroviridis*	Green-spotted pufferfish	ENSTNIP00000011311	Teleostei
Aqp1aa	*Hippoglossus hippoglossus*	Atlantic halibut	HQ185294	Teleostei
Aqp1aa	*Paralichthys olivaceus*	Japanese flounder	FE042295	Teleostei
Aqp1aa	*Solea senegalensis*	Senegalese sole	ABK20157	Teleostei
Aqp1aa	*Cynoglossus semilaevis*	Half-smooth tongue sole	ADG21868	Teleostei
Aqp1aa	*Perca flavescens*	Yellow perch	GO570422	Teleostei
Aqp1aa	*Sparus aurata*	Gilthead seabream	AAV34610	Teleostei
Aqp1aa	*Centropristis striata*	Black seabass	AAV34607	Teleostei
Aqp1aa	*Dicentrarchus labrax*	European seabass	ABI95464	Teleostei
Aqp1aa	*Acanthopagrus schlegelii*	Black porgy	ABO38816	Teleostei
Aqp1aa	*Oreochromis niloticus*	Nile tilapia	GR621845	Teleostei
Aqp1aa	*Miichthys miiuy*	Mi-iuy croaker	GW669182	Teleostei
Aqp1aa	*Dissostichus mawsoni*	Atlantic toothfish	FE196516	Teleostei
Aqp1aa	*Fundulus heteroclitus*	Common mummichog	ACI49538	Teleostei
Aqp1aa	*Gasterosteus aculeatus*	3-spined stickleback	ENSGACP00000022980	Teleostei
Aqp1aa	*Sebastes rastrelliger*	Grass rockfish	EW976757	Teleostei
Aqp1aa	*Oryzias latipes*	Medaka	ENSORLP00000022052	Teleostei
Aqp1aa	*Gadus morhua*	Atlantic cod	GmE100215i29183	Teleostei
Aqp1aa	*Gadus morhua*	Atlantic cod	GO386431	Teleostei
Aqp1aa	*Osmerus mordax*	Raibow smelt	ACO09149	Teleostei
Aqp1aa1	*Oncorhynchus mykiss*	Rainbow trout	CA378544	Teleostei
Aqp1aa2	*Oncorhynchus mykiss*	Rainbow trout	CA369507	Teleostei
Aqp1aa1	*Salmo salar*	Atlantic salmon	ACI66426	Teleostei
Aqp1aa2	*Salmo salar*	Atlantic salmon	ACI67627	Teleostei

(continued on next page)

Table 1. *continued*

Orthologue	Species	Common name	Acc#	Class
Aqp1aa	*Esox lucius*	Northern pike	ACO13816	Teleostei
Aqp1aa	*Danio rerio*	Zebrafish	AY626937	Teleostei
Aqp1aa	*Danio rerio*	Zebrafish	ENSDARG00000023713	Teleostei
Aqp1aa	*Pimephales promelas*	Fathead minnow	DT351671	Teleostei
Aqp1aa	*Anguilla anguilla*	European eel	CAD92027	Teleostei
Aqp1aa1	*Anguilla japonica*	Japanese eel	BAC82110	Teleostei
Aqp1aa2	*Anguilla japonica*	Japanese eel	BAC82109	Teleostei
Aqp1ab	*Takifugu rubripes*	Torafugu	ENSTRUP00000034674	Teleostei
Aqp1ab	*Tetraodon nigroviridis*	Green-spotted pufferfish	ENSTNIP00000001573	Teleostei
Aqp1ab	*Hippoglossus hippoglossus*	Atlantic halibut	HQ185295	Teleostei
Aqp1ab	*Solea senegalensis*	Senegalese sole	AAV34612	Teleostei
Aqp1ab	*Cynoglossus semilaevis*	Half-smooth tongue sole	ADG21867	Teleostei
Aqp1ab	*Sparus aurata*	Gilthead seabream	EF011740	Teleostei
Aqp1ab	*Thunnus thynnus*	Northern bluefin tuna	EG630605	Teleostei
Aqp1ab	*Oreochromis niloticus*	Nile tilapia	GR703528	Teleostei
Aqp1ab	*Gasterosteus aculeatus*	3-spined stickleback	ENSGACP00000022968	Teleostei
Aqp1ab	*Salmo salar*	Atlantic salmon	ACI33306	Teleostei
Aqp1ab	*Oncorhynchus nerka*	Sockeye salmon	EV383061	Teleostei
Aqp1ab	*Danio rerio*	Zebrafish	EU327345	Teleostei
Aqp1ab	*Danio rerio*	Zebrafish	ENSDARG00000091027	Teleostei
Aqp1ab	*Ictalurus punctatus*	Channel catfish	CK418363	Teleostei
Aqp1ab	*Heteropneustes fossilis*	Stinging catfish	HM051492	Teleostei
Aqp1ab	*Anguilla anguilla*	European eel	ABM26906	Teleostei
Aqp5/1b	*Danio rerio*	Zebrafish	ENSDARG00000038202	Teleostei

To validate the inferred tree topologies, syntenic analyses were performed for selected teleost and tetrapod genomes using ensembl v62 and Genomicus v62.01 as described previously (Chauvigné et al. 2010). Three-dimensional models of HhAqp1aa and -1ab were reconstructed using ModWeb (www.modbase.compbio.ucsf.edu) to evaluate the putative folding of the C-terminal domains, while overall tertiary structure was compared to crystallographic data available for human (1H6I) and bull (1J4N) AQP1. Predicted phosphorylation sites were searched using the online NetPhos and NetPhosK servers (www.cbs.dtu.dk) according to Blom et al. (1999, 2004).

2.4. Real-time Quantitative PCR

The expression pattern of *hhaqp1aa* and *-1ab* in different adult tissues of Atlantic halibut, as well as in hydrating ovarian follicles and unfertilized eggs, was determined by quantit-

ative real-time PCR (qPCR). Total RNA was extracted with the RNAeasy Minikit (Qiagen), treated with DNase I using RNase-Free DNase kit (Qiagen), and 0.5 µg reverse transcribed as indicated above. Real-time quantitative PCR (qPCR) amplifications were performed using SYBR Green qPCR master mix (Applied Biosystems) on 2 µl of diluted (1:10) cDNA as described previously (Chauvigné et al. 2010). Primers representing the 3'-end of each aquaporin open reading frame were used to amplify products for *hhaqp1aa* (262 bp using F = 5'-GTCCGGCTTTGATCCTGAAC-3' with R = 5'-CAGGAGTATGGACACGTGCAG-3') and *hhaqp1ab* (174 bp using F = 5'-CCGGCGGTTATACTGGAGTC-3' with R = 5'-AGTGTCAGTGTCCGCTGAGG-3'), and cycle numbers were normalized to 18S ribosomal RNA (149 bp using F = 5'-GAATTGACGGAAGGGCACCACCAG-3' with R = 5'-ACTAAGAACGGCCATGCACCACCAC-3') or β-actin (643 bp using F = 5'-ACATGGAGAAGATCTGGC-3' with R = 5'- GCGTACAGGTCCTTACG-GA-3'). The sequences were amplified in duplicate for each sample on 384-well plates using the ABI PRISM 7900HT sequence detection system (Applied Biosystems). The relative transcript level was calculated by using a standard curve generated for each primer pair from 10-fold serial dilutions of a pool of first-stranded cDNA template from ovary samples. All calibration curves exhibited correlation coefficients higher than 0.98, and the corresponding qPCR efficiencies were greater than 99%. Changes in *hhaqp1ab* expression in hydrating ovarian follicles were determined as fold change with respect the PV follicles using the $2^{-\Delta\Delta Ct}$ method (Livak et al. 2001).

2.5. Production of HhAqp1b Polyclonal Antiserum

Antiserum was raised in rabbits against a synthetic peptide corresponding to amino acid residues 255 to 267 (C-terminus) of HhAqp1ab with the predicted initiation codon (methionine, ATG) designated as residue 1 (Agrisera AB, Sweden). Affinity purification was accomplished against the synthetic peptide immobilized using UltraLinkTM Iodoacetyl resin (Pierce, Rockford, IL, USA) according to manufacturer instructions. Bound IgG was eluted with acidic 100 mM glycine (pH 2.5) into fourfold excess 1 M Tris (pH 9), and precipitated using saturated ammonium sulfate solution. The pellet was resuspended in sterile PBS (pH 7.4) and buffer exchanged to PBS (pH 7.4) using PD-10 Desalting columns (Amersham Biosciences Ab, Uppsala, Sweden).

Table 2. N- and C-terminal amino acid sequences of HhAqp1aa and -1ab wild-type (WT) and chimeric proteins

Construct	Amino acid sequence
	1 12 226 261
HhAqp1aa	MKEIKSKDFWRA...PKYDDFPERMKVLVSGPVGDYDVNGGNETTNVEMTSK
HhAqp1aa-Nab	MSDIKTWVFWRA...PKYDDFPERMKVLVSGPVGDYDVNGGNETTNVEMTSK
HhAqp1aa-Cab	MKEIKSKDFWRA...PRDEPFSEKTRGLFCIRSSADTDTLEPLLDDVEDVKWSKSSAEV
HhAqp1aa-Nab-Cab	MSDIKTWVFWRA...PRDEPFSEKTRGLFCIRSSADTDTLEPLLDDVEDVKWSKSSAEV
	1 6 224 267
HhAqp1ab	MSDIKTWVFWRA...PRDEPFSEKTRGLFCIRSSADTDTLEPLLDDVEDVKWSKSSAEV
HhAqp1ab-Naa	MKEIKSKDFWRA...PRDEPFSEKTRGLFCIRSSADTDTLEPLLDDVEDVKWSKSSAEV
HhAqp1ab-Caa	MSDIKTWVFWRA...PKYDDFPERMKVLVSGPVGDYDVNGGNETTNVEMTSK
HhAqp1ab-Naa-Caa	MKEIKSKDFWRA...PKYDDFPERMKVLVSGPVGDYDVNGGNETTNVEMTSK

The N- and C-terminus regions of HhAqp1a and -1b were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and SOSUI engine v. 1.11 (http://bp.nuap.nagoya-u.ac.jp/sosui/).

2.6. Expression Constructs

Wild-type *hhaqp1aa* and *-1ab* full-length cDNAs were cloned into the *Eco*RV/*Spe*I sites of the oocyte expression vector pT7Ts (Deen et al. 1994). The cDNAs encoding *hhaqp1aa* and *-1ab* chimeras, where the N- and C-terminus were interchanged, were synthesized by Mr Gene GmbH (Germany) and subcloned into the pT7Ts vector (Table 2). Sequence analysis of the clones in pT7Ts was carried out to confirm that the desired chimeras were produced.

2.7. Functional Expression in Fish and Amphibian Oocytes

Complementary RNAs (cRNAs) were synthesized as described (Deen et al. 1994) and microinjected *in vivo* into PV oocytes of Atlantic halibut, and *ex vivo* into oocytes of the African clawed frog (*Xenopus laevis*) and zebrafish (*Danio rerio*).

2.7.1. African clawed frog

Isolation, defolliculation and injection of *X. laevis* stage V-VI oocytes were performed essentially as described previously (Deen et al. 1994). Oocytes were injected with 50 nl of water alone (controls) or containing 1 or 25 ng of *hhaqp1aa* and *-1ab* cRNA, respectively. In some experiments, oocytes were injected with 25 nl water with or without 50 ng of polyA$^+$ mRNA

purified from PV and EH halibut ovarian follicles, or with 25 nl of water containing 25 ng of *hhaqp1ab* cRNA with or without 50 ng mRNA. Subsequently, *hhaqp1ab* and mRNA-injected oocytes were injected again with PBS alone or containing 100, 250 or 500 ng of affinity-purified HhAqp1ab antiserum or rabbit IgG (Sigma-Aldrich; controls) in a volume of 25 nl. The polyA+ mRNA was purified from total RNA using the Oligotex mRNA Mini Kit (Qiagen). The osmotic water permeability (P_f) was measured from the time course of oocyte swelling in a standard assay 48 h after injections. Oocytes were transferred from 200 mOsm modified Barth's culture medium (MBS; 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM Hepes, 0.82 mM $MgSO_4$, pH 7.5) to 20 mOsm MBS at room temperature. Oocyte swelling was followed by video microscopy using serial images at 2 s intervals during the first 20 s period, and P_f values were calculated from the time-course changes in relative oocyte volume as described (Deen et al. 1994). To examine the effect of mercury on P_f, injected oocytes were incubated in MBS containing 0.3 mM $HgCl_2$ for 15 min before and during the swelling assays. The reversibility of the mercury inhibition was determined by rinsing the same oocytes three times with fresh medium, and further incubation with 5 mM β-mercaptoethanol for 15 min prior to the swelling assays.

2.7.2. Atlantic halibut

For *in vivo* expression of HhAqp1aa and -1ab in PV Atlantic halibut oocytes (1.8 mm diameter), the selection, injection and defolliculation of oocytes, as well as the swelling assays, were performed at 4-6ºC. Healthy ovarian follicles were selected from the ovarian biopsies, and after equilibration for approximately 4 h in L-15 medium, follicle-enclosed oocytes were injected with 50 nl of water or *hhaqp1aa* and *-1ab* cRNAs as indicated above. After 24 h in fresh L-15 medium, oocytes were partially defolliculated using watchmaker forceps, and subjected to the swelling assays 24 h later. For the determination of the P_f values, oocytes were transferred to 10-fold diluted L-15 medium (30 mOsm), and the diameter of the oocytes was measured under a stereomicroscope at 5 s intervals during the first 50 s period. The effect of mercury on the P_f and its reversibility with β-mercaptoethanol was tested as in *X. laevis* oocytes.

2.7.3. Zebrafish

Females were sedated by immersion for approximately 15 min in 100 ppm phenoxyethanol, sacrificed by decapitation, and ovaries immediately placed in modified Cortland's culture medium without calcium and magnesium (0.124 M NaCl, 0.006 M $NaHCO_3$, 0.003 M Na_2HPO_4,

0.005 M KCl, 5 g Hepes, 0.03 g penicillin, 0.05 g streptomycin, pH 7.8). Ovarian follicles containing fully-grown oocytes (0.5-0.6 mm diameter) were manually dissected from the ovary, transferred into fresh culture medium, and after equilibration for 2-4 h at 26ºC, were injected with 5 nl of water or containing 9 ng of *hhaqp1aa* or *-1ab* cRNA. Follicles were then incubated for 12-16 h at 26ºC. The P_f measurements were performed as above but under hypertonic conditions using Cortland's with 150 mM sucrose (400 mOsm), since oocytes became slightly translucent after exposure to 10-fold diluted Cortland's. Treatment of follicles with mercury and β-mercaptoethanol was carried as above.

2.8. Inhibition of Atlantic halibut Oocyte Hydration with $HgCl_2$ and HhAqp1ab Antiserum

To test the effect of mercury on oocyte hydration, EH ovarian follicles were separated from the ovarian biopsies and kept in L-15 medium at 4ºC. After 2 h of equilibration, follicles were exposed to increasing doses of $HgCl_2$ (0.25, 0.5 or 1 mM) for 30 min, washed with fresh medium, and subsequently treated or not with 5 mM β-mercaptoethanol for another 30 min. The effect of the HhAqp1ab antiserum was studied by injecting EH follicles with PBS vehicle or with increasing doses of HhAqp1ab antibody or rabbit IgG (100, 200, 250 or 500 ng/oocyte) in a volume of 50 nl. Additionally, a fraction of the follicles injected with 200 ng of anti-HhAqp1ab antibody were co-injected with 50 nl of water containing 25 ng of *hhaqp1aa* or *-1ab* cRNA. In all cases, follicles were transferred to fresh L-15 medium and incubated at 4-5ºC for 48-72 h to resume meiotic maturation and hydration *in vitro*. The diameter of the ovarian follicles was measured before and after the incubation period. Follicle samples from all the treatments were frozen at -80ºC for further analyses.

2.9. SDS-PAGE of Oocyte Yolk Proteins

Halibut ovarian follicles with oocytes at different stages during meiotic maturation and hydration were mechanically homogenized in 1 x Laemmli sample buffer (Laemmli 1970) and denatured at 95°C for 5 min. A volume corresponding to 0.02 oocyte equivalents was loaded in each lane for yolk protein profile determination. SDS-PAGE was performed in 12% acrylamide minigels (7 x 3 x 10 cm) and electrophoresed at constant voltage (130 V) for 1.5 h. After fixation in 12.5% trichloroacetic acid during 1 h at room temperature, the gel was stained overnight in 0.2% PlusOne Coomassie Blue PhastGel R-350 (GE Healthcare, Barcelona, Spain) in 30% methanol and 10% acetic acid solution. The gel was progressively destained in 25% methanol and 7% acetic acid solution until the bands were clearly revealed.

2.10. Membrane Isolation and Immunoblotting

X. laevis oocytes, and Atlantic halibut ovarian follicles and adult tissues, were homogenized in HbA buffer [20 mM Tris pH 7.4, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 1 mM EDTA, 80 mM sucrose, and cocktail of protease inhibitors (Mini EDTA-free, Roche)], and centrifuged twice at 200 x *g* for 5 min at 4ºC. Total membranes were isolated by a final 20 min centrifugation at 13000 x *g* at 4ºC and resuspended in 1 x Laemmli sample buffer. Alternatively, membranes were solubilized in PBS (pH 7.5) containing 0.5% SDS and protease inhibitors, and protein concentration was measured using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories Inc, Madrid, Spain). Plasma membranes were isolated from *X. laevis* oocytes as described (Kamsteeg and Deen 2001). Whole homogenates were prepared by homogenizing the ovarian follicles directly in 1 x Laemmli sample buffer.

For immunoblotting, one volume of protein extract equivalent to 0.5-5 oocytes or ovarian follicles, or 30-60 µg protein for tissues, was denatured at 95ºC for 10 min, and subjected to 12% SDS-PAGE. After electrophoresis, the proteins were transferred onto nitrocellulose membranes (GE Healthcare) with Tris-glycine-methanol buffer (190 mM glycine, 250 mM Tris pH 8.6, and 20% methanol). The membranes were blocked for 1 h at room temperature in TBST (20 mM Tris, 140 mM NaCl, 0.1% Tween, pH 7.6) with 5% non-fat milk powder, and subsequently incubated with the HhAqp1ab antiserum (1:500) overnight at 4ºC. Control membranes were incubated with the HhAqp1ab antiserum pre-adsorbed with a 30-fold molar excess of the immunizing peptide. Bound antibodies were detected with 1:2000-diluted goat anti-rabbit IgG (Rockland, Tebu-bio, Barcelona, Spain) coupled to horseradish peroxidase (HRP), and proteins were visualized by using enhanced chemiluminescence (ECL; Picomax, Rockland). In some experiments, membranes were incubated only with goat anti-rabbit IgG-HRP (1:2000), or with the HhAqp1ab antiserum labelled with HRP (1:100) using the Lightning-Link™ HRP Conjugation kit (Innova Biosciences, Antibody Bcn S.L, Barcelona, Spain), before ECL revelation. Finally, to investigate the possible glycosylation of HhAqp1ab, total membrane homogenates were treated with 500 U of N-Glycosidase F (PNGase F; New England Biolabs, Izasa, Barcelona, Spain) or 80 KU of Endo-α-N-Acetylgalactosaminidase (O-Glycosidase; New England Biolabs) following the manufacturer instructions prior to electrophoresis. Zebrafish aquaporin-3b (Tingaud-Sequeira et al. 2010) and fetuin (Sigma-Aldrich) were used as a positive controls for PNGase F and O-Glycosidase digestion, respectively.

2.11. Immunofluorescence Microscopy

Immunofluorescence localization was performed on paraffin embedded ovarian follicles and oocytes fixed with 4% paraformaldehyde (PFA) overnight at 4ºC. Sections of approximately 6 μm in thickness were blocked with 5% goat serum in PBST (0.1% BSA, 0.1% Tween in PBS) for 1 h, and incubated with anti-HhAqp1ab antibody (1:50 or 1:100) diluted in PBST with 1% goat serum overnight at 4°C. After washing with PBS, sections were incubated with FITC anti-rabbit secondary antibodies (Sigma-Aldrich; 1:300) diluted in PBS for 1 h, washed again with PBS, and incubated 3 min with 1:3000-diluted 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Controls were incubated with pre-adsorbed HhAqp1ab antiserum as described above. Sections were mounted with Fluoromount™ aqueous mounting medium (Sigma-Aldrich), and immunofluorescence was observed and documented with a Zeiss imager.z1 microscope (Carl Zeiss MicroImaging, S.L., Barcelona, Spain).

2.12. Immunoelectron Microscopy

Atlantic halibut PV, EH and LH ovarian follicles collected from the biopsies were fixed in 4% PFA with or without 0.05% glutaraldehyde overnight at 4ºC, transferred to 2% PFA, and processed for immunoelectron microscopy as described previously (Fabra et al. 2006). The sections were blocked in 0.1 M PBS (pH 7.4) with 1% goat serum and 20 mM glycine for 30 min at room temperature, and subsequently incubated with the same buffer containing HhAqp1ab antisera (1:50) overnight at 4°C. After washing, the sections were incubated with protein A-coupled to gold particles (15 nm) in 1% goat serum, 20 mM glycine in PBS for 1 h at room temperature, washed, and contrasted with 2% uranil acetate for 15 min and lead citrate for 5 min. The images were documented and photographed by using a JEOL EM 1010 electron microscope (JEOL, Izasa, Barcelona, Spain).

2.13. Statistical Analysis

Data are the mean $\pm$ SEM and were statistically analyzed using one-way ANOVA followed by Tukey's pairwise comparison with a 95% confidence interval, or by the Student's *t*-test comparing experimental sets against control only. Percentage data that fell between the ranges of 0-20% or 80-100% were square root transformed prior to statistical analyses. Criteria for significant differences were at $p < 0.05$ for all comparisons.

3. Results

3.1. The Duality of Aquaporin-1 Water Channels in Teleosts

By using available EST sequence information and degenerate oligonucleotide primers, full-length cDNAs encoding *hhaqp1aa* and *-1ab* were isolated, confirming the expression of two Aqp1-like channels in Atlantic halibut as reported for other teleosts (Tingaud-Sequeira et al. 2008, 2010). Their open reading frames were 54.1% identical at the amino acid level, with 42.9% and 17.4% identity between the N- and C-terminal cytoplasmic sequences, respectively. Both HhAqp1aa and -1ab deduced amino acid sequences showed the six transmembrane helices and the two Asn-Pro-Ala (NPA) motifs which are typical features of the members of the aquaporin superfamily (Fig. 1). In addition, both sequences had the residues forming the aromatic residue/arginine (ar/R) constriction region that are conserved in water-selective aquaporins (Phe^{50}, His^{172} and Arg^{187}, and Phe^{50}, His^{171} and Arg^{186}, in HhAqp1aa and -1ab, respectively), as well as a Cys residue close to the second NPA motif (Cys^{181} and Cys^{180}, in HhAqp1aa and -1ab, respectively), the site potentially responsible for the blockage of the water pore by mercury (Preston et al. 1993; Hirano et al. 2010).

The molecular phylogeny of the full length cloned transcripts and deduced amino acids was examined in relation to 26 vertebrate orders. All methods of inference revealed three sister clusters with robust separation of sarcopterygian (lungfish and tetrapod) AQP1 from the teleost Aqp1aa and -1ab orthologs (Fig. 2). Addition of the zebrafish Aqp5/1b, apparently a pseudogene (Tingaud-Sequeira et al. 2010), produced some trees where it clustered basal to African lungfish Aqp1, but more often as an ancestral node to the teleost Aqp1aa and -1ab sequences. This putative pseudogene is therefore drawn as a polytomy between the sarcopterygian and actinopterygian orthologs. Higher resolution was achieved from the codon alignments, while amino acid alignments generated some polytomies (Bayesian posterior probabilities $< 50\%$) within the perciform Aqp1aa subclusters. The tetrapod AQP1 and teleost Aqp1aa sequences clustered primarily according to phylogenetic rank wherein HhAqp1aa clustered with other pleuronectiform Aqp1aa orthologs. Such topologies imply minimal evolution of the encoded genes, a notion supported by the comparatively short branch lengths and higher identity values to human AQP1. Although teleost Aqp1aa proteins show greater amino acid substitution (42%) compared to human AQP1, within Teleostei, the Aqp1aa primary structures are well conserved (86%) (Table 3). By contrast the teleost Aqp1ab sequences did not cluster according to phylogenetic rank, but separated into three paralogous subclusters.

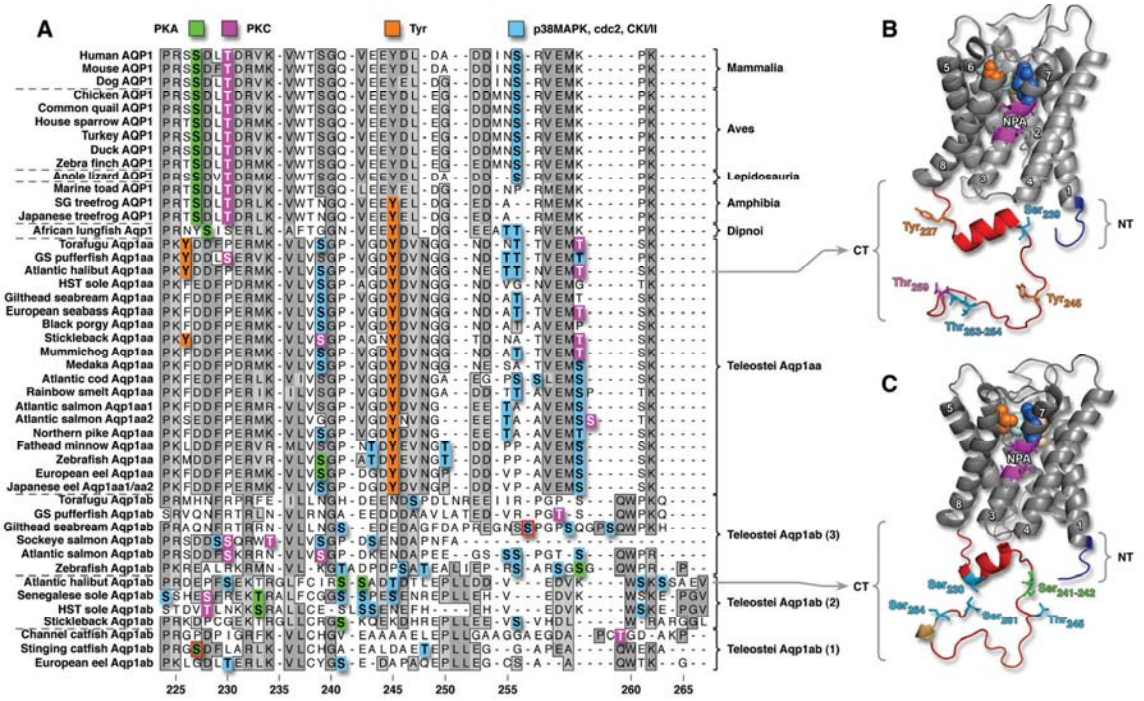


Fig 1. Structural features of vertebrate aquaporin-1 orthologs. (A) Alignment of C-terminal domains illustrating known and putative phosphorylation sites. In gilthead seabream and stinging catfish Aqp1ab phosphorylation of Ser^{254} and Ser^{227}, respectively outlined with red boxes, have been shown to play opposite roles in intracellular trafficking (Tingaud-Sequeira et al., 2008; Chaube et al., 2011). Highly conserved residues are boxed and shaded in dark grey. Residues with similar chemical properties are shaded in light grey. Residue numbers below the alignment are annotated for HhAqp1ab. (B and C) Three-dimensional models of HhAqp1aa (B) and HhAqp1ab (C) illustrating the conserved transmembrane helices (1, 2, 4, 5, 6 and 8) and the two intramembranous hemi-helices (3 and 7) that bear the conserved Asn-Pro-Ala (NPA) motifs. Residues associated with the ar/R constriction are rendered as spacefill: Phe^{50} (pale blue), $His^{172/171}$ (wheat), $Arg^{187/186}$ (Blue), and the putative mercury-sensitive residues Cys^{181}/180 (orange). C-termini are rendered as cartoons in red with stick renders of the pre-dicted phosphorylation sites highlighted in (A).

HhAqp1ab is placed together with similar pleuronectiform and acanthomorph Aqp1ab sequences within subcluster 2, but is distinct from other acanthomorph Aqp1ab orthologs in subcluster 3. The divergent topology of the Aqp1ab sequences is also reflected in the separate clustering of the ostariophysan zebrafish in subcluster 3 compared to the ostariophysan catfishes in subcluster 1. Bayesian branch length values for both the codon and amino acid runs were on average 3.5 times longer for the teleost Aqp1ab sequences compared to the Aqp1aa orthologs and suggest that the Aqp1ab genes are rapidly diverging within the teleost crown clade. Inspection of the C-terminal domains revealed that significantly greater amino acid substitution has occurred within the Aqp1ab cytoplasmic region compared to either Aqp1aa, dipnoan Aqp1 or tetrapod AQP1 (Table 3 and Fig. 1).

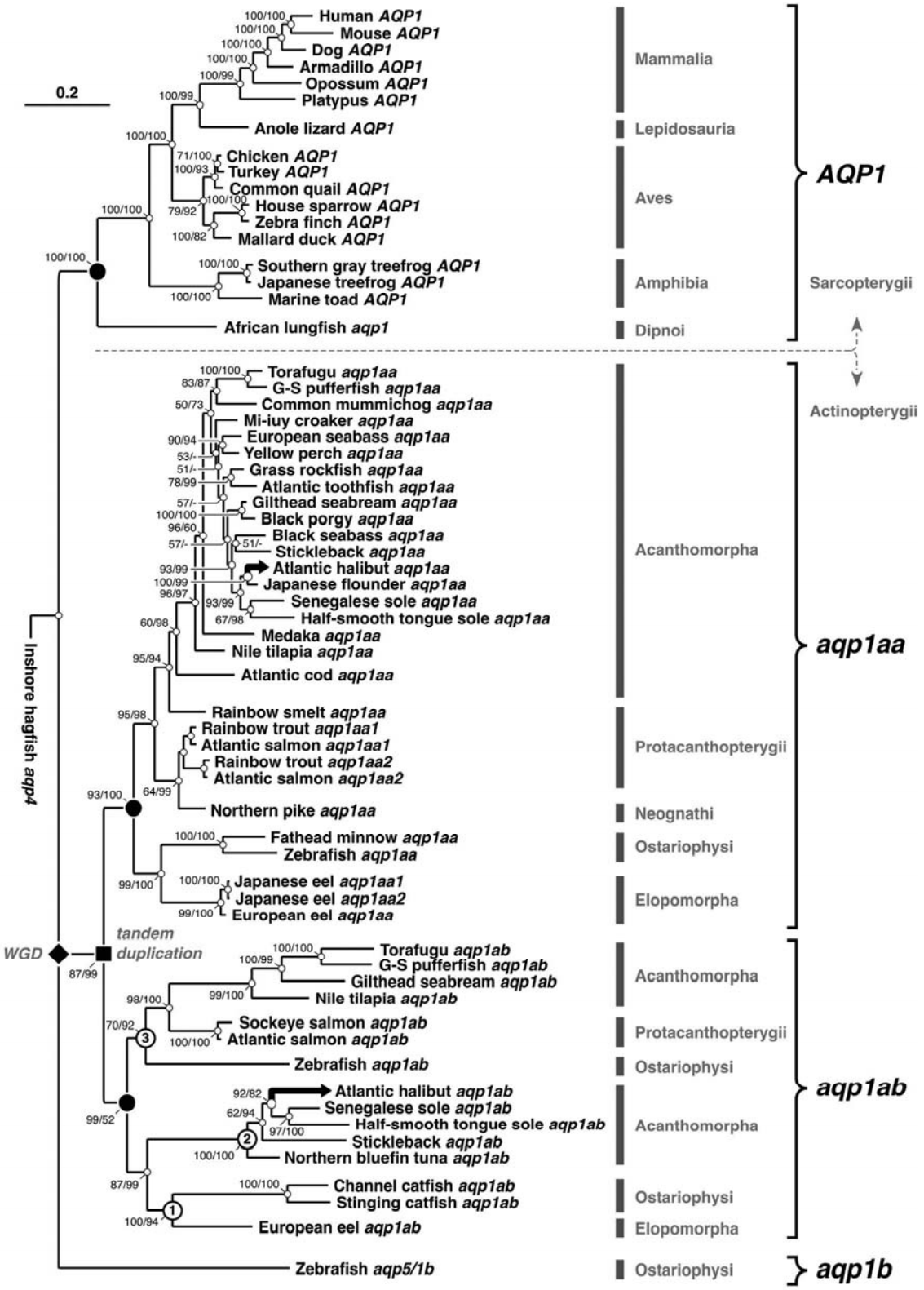

0.2
Human AQP1
Mouse AQP1
Dog AQP1
Armadillo AQP1
Opossum AQP1
Platypus AQP1
Anole lizard AQP1
Chicken AQP1
Turkey AQP1
Common quail AQP1
House sparrow AQP1
Zebra finch AQP1
Mallard duck AQP1
Southern gray treefrog AQP1
Japanese treefrog AQP1
Marine toad AQP1
African lungfish aqp1
Mammalia
Lepidosauria
Aves
Amphibia
Dipnoi
AQP1
Sarcopterygii
Actinopterygii
Torafugu aqp1aa
G-S pufferfish aqp1aa
Common mummichog aqp1aa
Mi-iuy croaker aqp1aa
European seabass aqp1aa
Yellow perch aqp1aa
Grass rockfish aqp1aa
Atlantic toothfish aqp1aa
Gilthead seabream aqp1aa
Black porgy aqp1aa
Black seabass aqp1aa
Stickleback aqp1aa
Atlantic halibut aqp1aa
Japanese flounder aqp1aa
Senegalese sole aqp1aa
Half-smooth tongue sole aqp1aa
Medaka aqp1aa
Nile tilapia aqp1aa
Atlantic cod aqp1aa
Rainbow smelt aqp1aa
Rainbow trout aqp1aa1
Atlantic salmon aqp1aa1
Rainbow trout aqp1aa2
Atlantic salmon aqp1aa2
Northern pike aqp1aa
Fathead minnow aqp1aa
Zebrafish aqp1aa
Japanese eel aqp1aa1
Japanese eel aqp1aa2
European eel aqp1aa
Acanthomorpha
Protacanthopterygii
Neognathi
Ostariophysi
Elopomorpha
aqp1aa
Inshore hagfish aqp4
WGD
tandem duplication
Torafugu aqp1ab
G-S pufferfish aqp1ab
Gilthead seabream aqp1ab
Nile tilapia aqp1ab
Sockeye salmon aqp1ab
Atlantic salmon aqp1ab
Zebrafish aqp1ab
Atlantic halibut aqp1ab
Senegalese sole aqp1ab
Half-smooth tongue sole aqp1ab
Stickleback aqp1ab
Northern bluefin tuna aqp1ab
Channel catfish aqp1ab
Stinging catfish aqp1ab
European eel aqp1ab
Acanthomorpha
Protacanthopterygii
Ostariophysi
Acanthomorpha
Ostariophysi
Elopomorpha
aqp1ab
Zebrafish aqp5/1b
Ostariophysi
aqp1b

Table 3. Group-wise amino acid (AA) sequence identity values (%) of vertebrate aquaporin-1 proteins. Analyses were conducted for full-length alignments, truncated alignments without the C-terminal domain and only the C-terminal domain. *N* represents the number of taxa included in each comparison. Postscripted numbers in parentheses refer to a given Aqp1ab subcluster shown in Fig. 2. Data are presented as means ± standard deviation

Group	Cluster	*N*	Full-length	Full-length - C terminus	C terminus
AA identity *vs* Human AQP1			(300)	(246)	(54)
Tetrapoda	AQP1	12	83 ± 6.0 [a]	83 ± 5.8 [a]	87 ± 7.7 [a]
Dipnoi	Aqp1	1	64	66	54
Teleostei	Aqp1aa	20	58 ± 1.12 [b]	62 ± 1.1 [b]	39 ± 3.1 [b]
	Aqp1ab (3)	6	53 ± 2.2 [c]	58 ± 2.6 [c]	24 ± 4.2 [c]
	Aqp1ab (2)	4	48 ± 0.2 [d]	56 ± 0.4 [c]	11 ± 3.0 [d]
	Aqp1ab (1)	3	50 ± 2.2 [cd]	56 ± 2.0 [c]	20 ± 5.0 [c]
AA identity *vs* HhAqp1aa			(292)	(238)	(54)
Teleostei	Aqp1aa	19	86 ± 4.8 [a]	87 ± 4.5 [a]	79 ± 8.8 [a]
	Aqp1ab (3)	6	63 ± 4.8 [b]	70 ± 4.5 [b]	23 ± 5.6 [b]
	Aqp1ab (2)	4	55 ± 1.4 [c]	64 ± 2.2 [c]	12 ± 4.0 [c]
	Aqp1ab (1)	3	59 ± 3.9 [bc]	67 ± 3.5 [bc]	22 ± 6.1 [bc]

Parenthetic numbers indicate the length of the alignments, including gaps. Significant differences are analyzed by ANOVA and indicated by non-equivalent superscripts.

To test whether the C-terminal domains were responsible for the novel subclustering of the teleost Aqp1ab orthologs, separate Bayesian and maximum likelihood analyses were run on truncated alignments lacking the N- and C-termini. These new trees (data not shown) reflected the topology of the full length analyses, and reveal that significantly greater amino acid substitution occurs throughout the primary structures of the teleost Aqp1ab orthologs. Despite this divergence, three dimensional models of the halibut aquaporin paralogs indicate that the transmembrane helical topology is well conserved, whereas the C-termini are rapidly evolving even in closely related species (Fig. 1).

An extended synteny analysis confirmed that teleost *aqp1aa* and *-1ab* genes are tandemly arranged in orthologous regions upstream of THO complex subunit 1 (*thoc1*) and

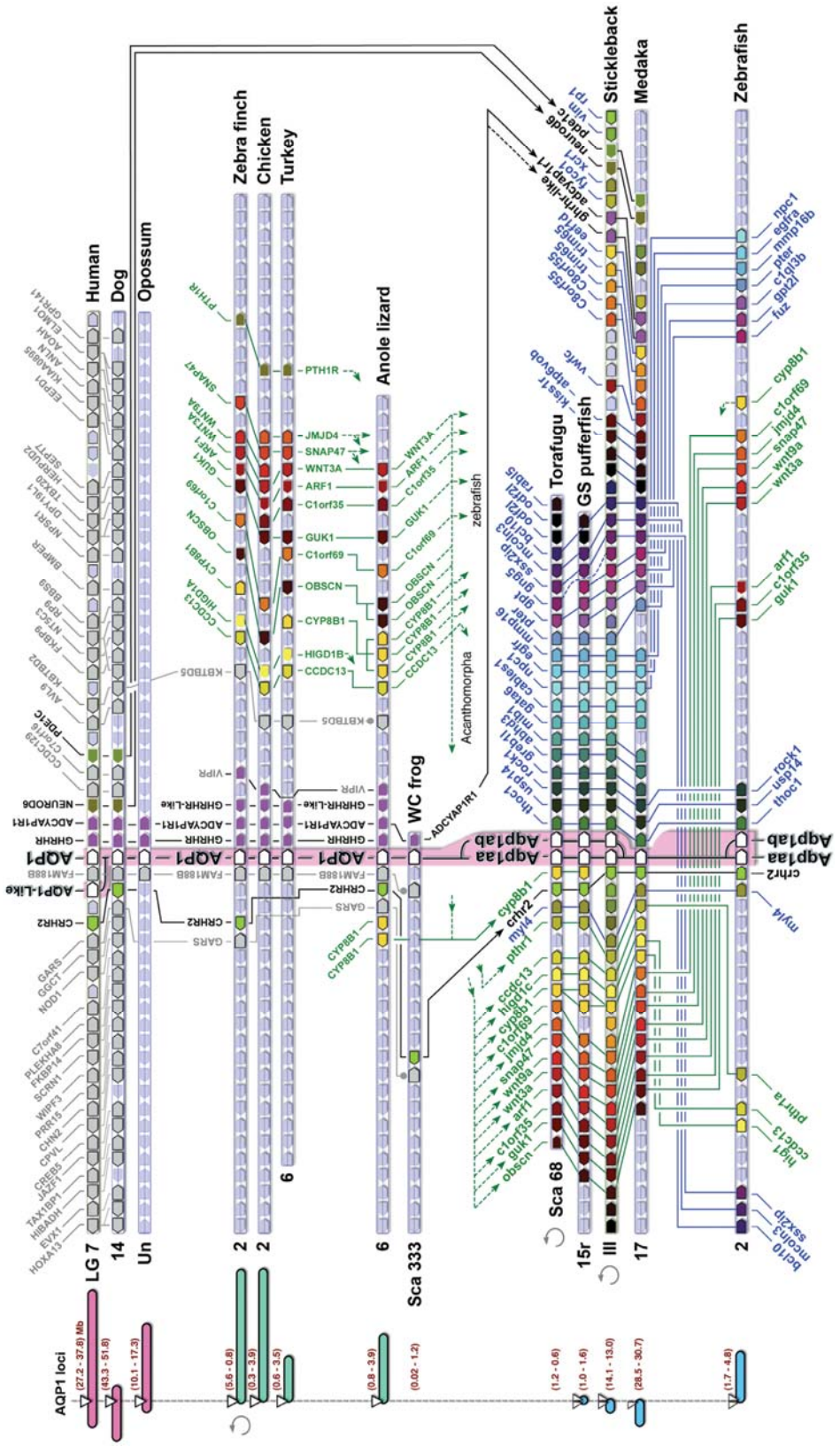
Fig 2. Maximum likelihood tree of orthologous vertebrate aquaporin-1 codons rooted with inshore hagfish aquaporin 4. The Atlantic halibut sequences cloned in the present study are indicated by thick black arrows. Three sister clusters (sarcopterygian AQP1, and teleost *aqp1aa* and *aqp1ab*) are indicated by black circles at the relevant nodes, while three novel subclusters (1-3) are indicated for the *aqp1ab* orthologs. The teleost-specific whole genome duplication (WGD) event is indicated by a black triangle, while tandem duplication is indicated by a black square. Statistical values at each node represent Bayesian posterior probabilities resulting from 5 million MCMC generations of full-length codon/amino acid alignments. Polytomies in the protein trees are annotated with (-). Scale bar represents the rate of nucleotide substitution per site

downstream of corticotropin releasing hormone receptor 2 (*crhr2*) (Fig. 3). The genomic regions are well conserved amongst the acanthomorph species (torafugu, green-spotted pufferfish, stickleback and medaka), while the flanking genes have experienced greater rearrangement in zebrafish. Interestingly the syntenic data show that the teleost *aqp1aa*, *-1ab* and flanking gene loci share greater homology to the genomic regions in the anole lizard and birds. By contrast mammals have only retained syntenic loci for the closely linked G-protein coupled receptors (*CRHR2, GHRHR, ADCYAP1R1*), the neurogenic differentiation factor (*NEUROD6*), and the calmodulin-dependent phsophodiesterase (*PDE1C*). Within Eutheria, however, gene synteny flanking the AQP1 locus is in fact highly conserved. Consequently greater rearrangement of these genomic regions appears to have occurred in the mammalian lineage.

Taking into consideration the tandem arrangement of the teleost *aqp1aa* and *-1ab* genes, the highly conserved synteny within the acanthomorph genomes, and the close phylogenetic clustering of the halibut aquaporins with stickleback *aqp1aa* and *-1ab*, it seems likely that these aquaporin paralogs are tandem duplicates. This suggests that the WGD product (i.e. the true *aqp1b* gene) is lost in the Acanthomorpha. To confirm this notion, we examined the syntenic locus of the zebrafish *aqp5/1b* pseudogene. It is found at the 21.6 Mb locus on LG 3 between integrin alpha 2 (*ita2b*) and corticotropin releasing hormone receptor 1 (*crhr1*), but is also linked to the homeobox ba (*hoxba*) cluster and *aqp8b*. This genomic region is not conserved in the acanthomorph genomes, but is partially conserved in the anole lizard on LG 6 (63-64 Mb locus), the orthologon that retains AQP1 at 1.9 Mb. The acanthomorph *aqp8b* orthologs have, however, remained linked to the *hoxba* clusters on the respective LGs, while the *crhr1* genes are found on the sister paralogons linked to the *hoxbb* clusters and the *aqp8aa* and *-8ab* tandem duplicates. Based upon this evidence we conclude that the functional teleost *aqp1* paralogs (previously named *aqp1a* and *-1b*) are indeed tandem duplicates, and we therefore renamed these genes *aqp1aa* and *-1ab*, while the zebrafish pseudogene was renamed *aqp5/1b* to match the *aqp8* terminology.

3.2. HhAqp1aa and -1ab Are Differentially Expressed

hhaqp1aa and *-1ab* transcript abundance in different organs and tissues of adult Atlantic halibut was determined by qPCR (Fig. 4A). *hhaqp1aa* mRNA was detected ubiquitously at comparable levels in all the tissues analyzed, with less expression in the esophagus, stomach, pyloric caeca, spleen and lens. In contrast, *hhaqp1ab* transcripts were found predominantly in the ovary, followed by testis, ureter, head kidney, rectum and eye, although transcripts could


Human
Dog
Opossum
Zebra finch
Chicken
Turkey
Anole lizard
WC frog
Acanthomorpha
zebrafish
Torafugu
GS pufferfish
Stickleback
Medaka
Zebrafish
AQP1
AQP1-Like
Aqp1aa
Aqp1ab
AQP1 loci
LG 7
14
Un
2
2
6
6
Sca 333
Sca 68
15r
III
17
2

also be detected at much lower levels in most of the other organs. Accumulation of *hhaqp1ab* in the ovary, however, was ~50-fold higher than in testis and ureter, ~600-fold higher than in head kidney, rectum and eye, and >1000-fold higher than in the rest of tissues analyzed.

Antibodies generated against the HhAqp1ab C-terminus specifically identified the protein in total membrane extracts from the ovary, testis, ureter, head kidney, rectum and eye (Fig. 4B). However, the amount of HhAqp1ab protein in the different tissues deduced from Western blotting analysis apparently did not correlate well with the amount of mRNA determined by qPCR in some cases. In addition, multiple HhAqp1ab immunoreactive bands with different molecular masses were observed in different tissues. In the ovary and testis, which differentially express *hhaqp1ab* by ~30-fold, the HhAqp1ab antiserum predominantly detected a ~27 kDa protein of similar intensity, which is near the HhAqp1ab predicted molecular mass (~28.4 kDa) (Fig. 4B). However, other polypeptides of ~50 kDa and ~66 kDa were also detected (Fig. 4B). In extracts from the head kidney, ureter and eye, the 27-kD protein band was not detected, but different immunoreactive polypeptides with molecular masses from ~39 to ~80 kDa, appearing as smeared or more defined bands, were observed. The 27-kD protein, and the other polypeptides of higher molecular mass, were no longer detected after pre-incubation of the HhAqp1ab antiserum with large amounts of the immunizing peptide (Fig. 4B, right panel), indicating that the high molecular weight bands correspond to post-translational modifications of HhAqp1ab. Such modifications do not appear to involve glycosylation because they were not sensitive to PNGase F or O-Glycosidase digestion (data not shown), but most likely represent HhAqp1ab oligomers that are stable under denaturing and reducing conditions, presumably due to the high hydrophobicity of the protein (Van Hoek et al. 1995). The formation of these complexes seems to be more prominent in the ureter.

Fig. 3. Genomic synteny of vertebrate aquaporin-1 orthologs. Linkage groups (LG) are drawn to scale on the left, illustrating the loci of the conserved aquaporin-1 genes shown in the main panel. Red numbers in parentheses represent the length of the analyzed regions in megabases (Mb), and show that mammalian regions are more expanded (7.2-10.3 Mb) compared to birds (3.6-4.8 Mb) and Teleostei (0.6-3.1 Mb). Gene coding direction is indicated by the pointed end of each symbol. A circular arrow to the left of a given region indicates that it is flipped. Syntenic loci that are conserved between tetrapods and teleosts (e.g. *CRHR2*, *AQP1*, *GHRHR*, *ADCYAP1R1*, *NEUROD6*, *PDE1C*) are annotated in black with black linker lines. Closely linked gene loci that are conserved between mammals, birds, the anole lizard and the Western-clawed frog are annotated in grey with grey linker lines. Gene loci that are syntenic between birds, the anole lizard, the Western-clawed frog and teleosts are annotated in green with green linker lines, while syntenic genes specific to the teleost regions are annotated in blue with blue linker lines. Pale blue symbols without linker lines represent non-syntenic genes.

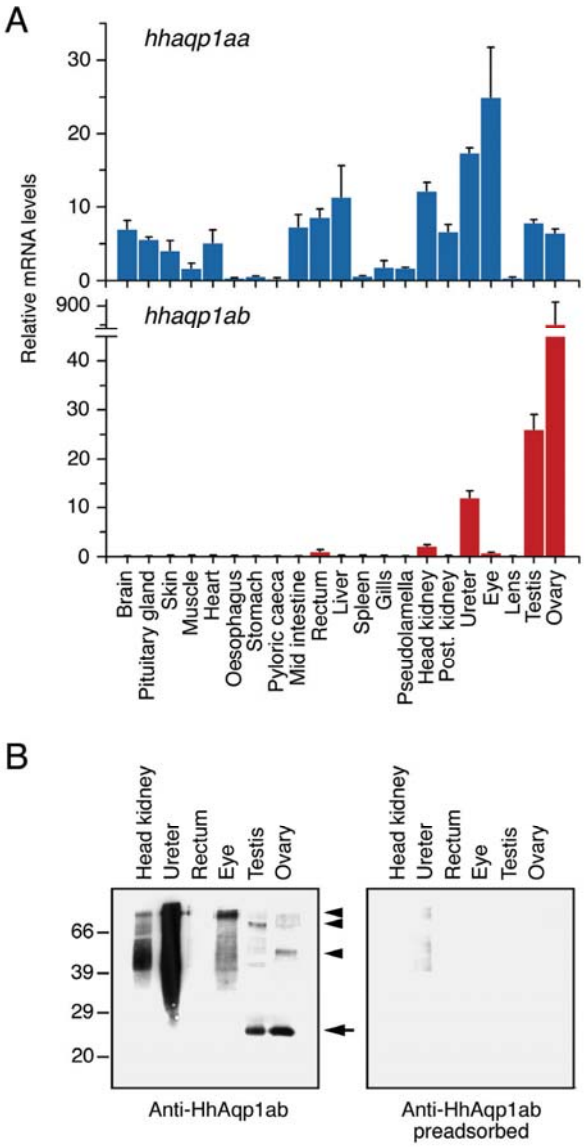


Fig. 4. *hhaqp1aa* and *-1ab* are differentially expressed in Atlantic halibut adult tissues. (A) Tissue distribution of *hhaqp1aa* and *-1ab* in halibut determined by qPCR and using *b-actin* as reference gene. Data are means ± SEM (n = 3 fish). (B) Immunoblot of total membranes (30 µg, and 15 µg for ureter) for HhAqp1ab from selected tissues of halibut. A duplicated membrane (right) was incubated with the HhAqp1ab antisera pre-absorbed with the synthetic peptide used for immunisation. The arrow indicates HhAqp1ab monomers, whereas the arrowheads point to possible post-translational modifications of HhAqp1ab, which were not affected by PNGase F or O-Glycosidase digestion (data not shown). Molecular mass markers (kDa) are on the left.

3.3. HhAqp1ab is Functional Only When Expressed in Halibut Oocytes

To determine whether *hhaqp1aa* and *-1ab* cDNAs encoded functional water channels, *X. laevis* oocytes were injected *ex vivo* with *hhaqp1aa* or *-1ab* cRNAs or with water as negative controls (Fig. 5A-E). Oocytes expressing HhAqp1aa proteins showed a ~22-fold increase in P_f with respect to the control oocytes after an osmotic challenge, which was inhibited by 83% with mercury and partially reversed with β-mercaptoethanol, an observation consistent with the presence of Cys^{181} in HhAqp1aa (Fig. 5B). In contrast, *X. laevis* oocytes expressing HhAqp1ab did not swell significantly more than the controls (Fig. 5B), indicating that this

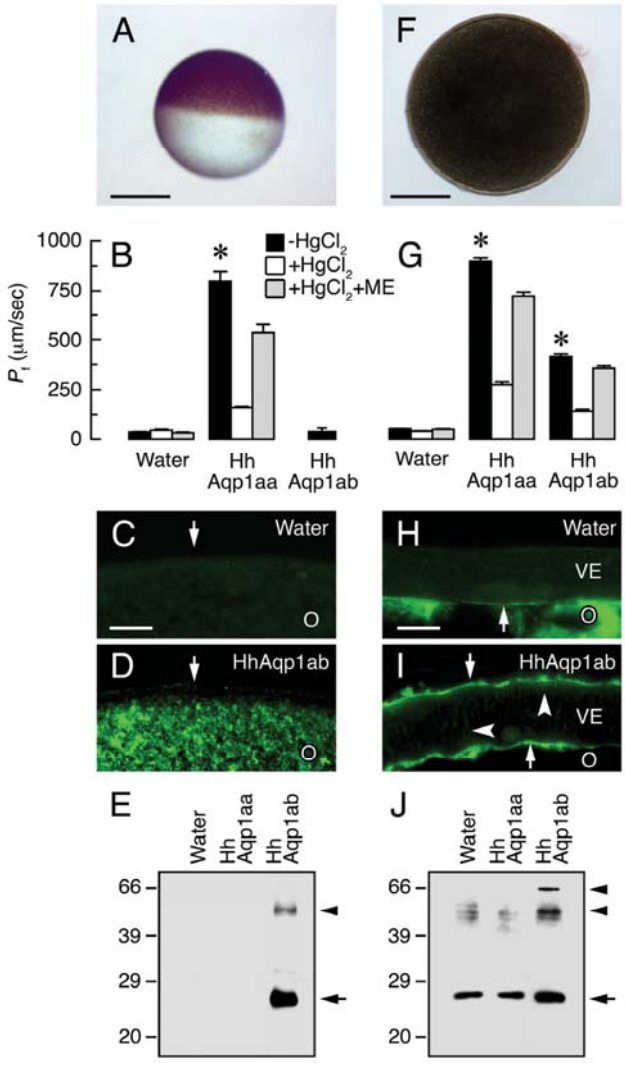


Fig. 5. HhAqp1ab, but not HhAqp1aa, is functional only when expressed in vivo in Atlantic halibut oocytes. (A, F) Photomicrographs of a *X. laevis* stage V oocyte (A) and of a halibut PV ovarian follicle (F). (B, G) Osmotic water permeability ($P_f \pm$ SEM of 3-4 independent experiments; n = 40-45 oocytes) of *X. laevis* (B) and halibut (G) oocytes injected with water (control) or with 1 or 25 ng cRNA encoding HhAqp1aa and -1ab, respectively, and treated with or without 0.3 mM $HgCl_2$ and 5 mM β-mercaptoethanol (ME). An asterisk indicates a significant increase in P_f compared to control and $HgCl_2$-treated oocytes ($P <$ 0.01). (C, D, H, I) The HhAqp1ab is retained in the ooplasm (O) of *X. laevis* oocytes (C, D) but translocated into the plasma membrane (arrows) when expressed in halibut oocytes (H, I). Paraffin sections of water- and HhAqp1ab-injected oocytes probed with the HhAqp1ab antiserum followed by FITC anti-rabbit IgG. Arrowheads point to the microvilli crossing the vitelline envelope (VE). *Scale bars*: 25 µm (C, D); 10 µm (H, I). (E, J) Immunoblots of total membranes of *X. laevis* (E; 0.5 oocyte equiva-lent/lane) and halibut (J; 2 oocyte equivalent/lane) using the HhAqp1ab antiserum of oocytes that were used for the P_f measurements. The arrows indicate HhAqp1ab monomers, whereas the arrowheads points to possible post-translational modifications of HhAqp1ab. Molecular mass markers (kDa) are on the left.

paralog was not functional. However, when HhAqp1aa and -1ab were expressed *in vivo* in PV halibut oocytes (Fig. 5F), both paralogs were functional resulting in ~17- and ~8-fold increase in P_f, respectively, compared to control oocytes (Fig. 5G). As observed in the *ex vivo* experiments, mercury inhibited HhAqp1aa- and -1ab-mediated water transport by ~75%, the inhibition being partially recovered with β-mercaptoethanol (Fig. 5G).

Immunofluorescence microscopy revealed that HhAqp1ab polypeptides were retained in the cytoplasm of *X. laevis* oocytes (Fig. 5, C and D), explaining the functional failure of HhAqp1ab, whereas in halibut oocytes the protein was readily targeted to the plasma membrane (Fig. 5, H and I). Immunoblotting analysis of total membrane protein extracts from *X.*

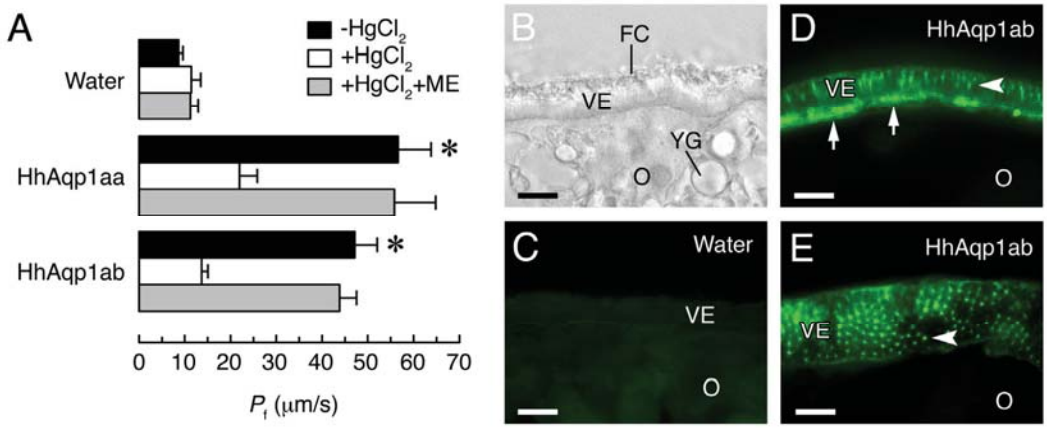


Fig. 6. Functional expression of HhAqp1aa and -1ab in zebrafish oocytes. (A) Osmotic water permeability ($P_f \pm$ SEM of 3 independent experiments; $n = 30$-35 oocytes) of ovarian follicles containing fully-grown oocytes injected with 9 ng of *hhaqp1aa* or *-1ab* cRNA, and treated or not with 0.3 mM $HgCl_2$ and 5 mM β-mercaptoethanol (ME). (B) Phase contrast photomicrograph of a fully-grown ovarian follicle where the follicle cells (FC), vitelline envelope (VE), ooplasm (O) and yolk globules can be observed. (C-E) Immunofluorescence microscopy on paraffin sections from oocytes injected with water (C) or expressing HhAqp1ab (D and E) probed with the HhAqp1ab antiserum followed by FITC anti-rabbit IgG. Note that HhAqp1ab is constitutively translocated into the oocyte plasma membrane (D), and a strong staining is observed in the microvilli crossing the VE (E). *Bars*, 10 μm.

laevis and halibut ovarian follicles expressing HhAqp1ab indicated the translation of HhAqp1ab monomers and the formation of ~50-kDa complexes in both oocytes. However, functional expression of HhAqp1ab and the formation of the 66-kDa complex was only detected *in vivo* in the halibut oocytes (Fig. 5, E and J). Interestingly, when HhAqp1ab was expressed in zebrafish oocytes, the protein was highly accumulated at the plasma membrane, and in this system both HhAqp1aa and -1ab elicited a similar ~6-fold increase in P_f after oocytes were exposed to a hyperosmotic medium (Fig. 6).

To investigate whether the functional failure of HhAqp1ab in *X. laevis* oocytes was due to the expression system used, HhAqp1b was expressed in amphibian oocytes that were injected with polyA$^+$ mRNA purified from PV Atlantic halibut ovarian follicles (Fig. 7). Injection of polyA$^+$ mRNA alone into oocytes resulted in a significant but moderate increase of P_f with respect to control oocytes and those expressing HhAqp1ab alone (1.6-fold), but when halibut polyA$^+$ mRNA was co-expressed with HhAqp1ab a synergistic effect was observed resulting in a marked increase (7.5-fold) of the P_f (Fig. 7A). Exposure of polyA$^+$ mRNA plus HhAqp1ab-expressing oocytes to mercury inhibited water permeability by 75%, and the inhibition was almost fully recovered with β-mercaptoethanol. Accordingly, immunolabeling of oocytes demonstrated that HhAqp1ab was translocated to the oocyte plasma membrane exclusively when it was co-expressed with polyA$^+$ mRNA from halibut ovarian follicles (Fig. 7, B and C).

Western blotting after SDS-PAGE under reducing (Fig. 7D) and non-reducing (data not shown) conditions indicated that the pattern of post-translational modifications of HhAqp1ab in oocytes was identical regardless of the expression of polyA$^+$ mRNA from ovarian follicles, but the 66-kDa HhAqp1ab complexes, previously observed in halibut oocytes, were only detected in the plasma membrane fraction (Fig. 7D). These results thus indicated that *ex vivo* injection of polyA$^+$ mRNA from halibut follicles into *X. laevis* oocytes was able to reproduce the *in vivo* intracellular mechanisms for the insertion of HhAqp1ab into the plasma membrane. Injection of the HhAqp1ab antiserum into oocytes expressing mRNA and HhAqp1ab inhibited oocyte swelling in a dose-dependent manner up to 93% (Fig. 7E), confirming that most of the water transport measured in these oocytes was mediated by HhAqp1ab. In accordance with the inhibition of oocyte P_f, the HhAqp1ab antiserum reduced the presence of the protein at the plasma membrane while its abundance in the total membrane fraction remained unchanged (Fig. 7F). These observations suggest that the antibody most likely affects the turnover of HhAqp1ab in the oocyte plasma membrane.

In some teleosts, the intracellular trafficking of Aqp1ab is regulated by specific domains at the C-terminus of the protein (Tingaud-Sequeira et al. 2008; Chaube et al. 2011). Therefore, we next investigated the presence of similar domains in HhAqp1ab that might interact with factors encoded by mRNAs expressed in Atlantic halibut ovarian follicles. For these experiments, cDNAs encoding chimeric HhAqp1aa and -1ab proteins in which the N- and/or C-terminal domains were exchanged (HhAqp1aa-Nab, -Cab, -Nab-Cab, and HhAqp1ab-Naa, -Caa, -Naa-Caa) were synthesized *in vitro*. The chimeras and wild-type (WT) *hhaqp1aa* and *-1ab* were injected into oocytes, with or without polyA$^+$ mRNA from halibut ovarian follicles, and the P_f monitored (Fig. 7G). Oocytes expressing HhAqp1aa-WT or -Nab showed the same P_f regardless of the presence of polyA$^+$ mRNA, although the HhAqp1aa-Nab oocytes significantly reduced swelling by 19% when compared with oocytes expressing HhAqp1aa-WT. In contrast, the permeability of HhAqp1aa-Cab and -Nab-Cab oocytes was strongly reduced (96%) in the absence of polyA$^+$ mRNA, but the reduction was only 51% when these chimeras were co-expressed with polyA$^+$ mRNA from halibut follicles. As observed earlier, oocytes expressing HhAqp1ab showed a high increase in P_f in the presence of polyA$^+$ mRNA, but the substitution of the N-terminus of HhAqp1ab by that of HhAqp1aa had no effect. However, when the C-terminus of HhAqp1ab was exchanged with that of HhAqp1aa, or both N- and C-termini were substituted, a 2.6-fold increase of P_f was observed, which was not affected by the expression

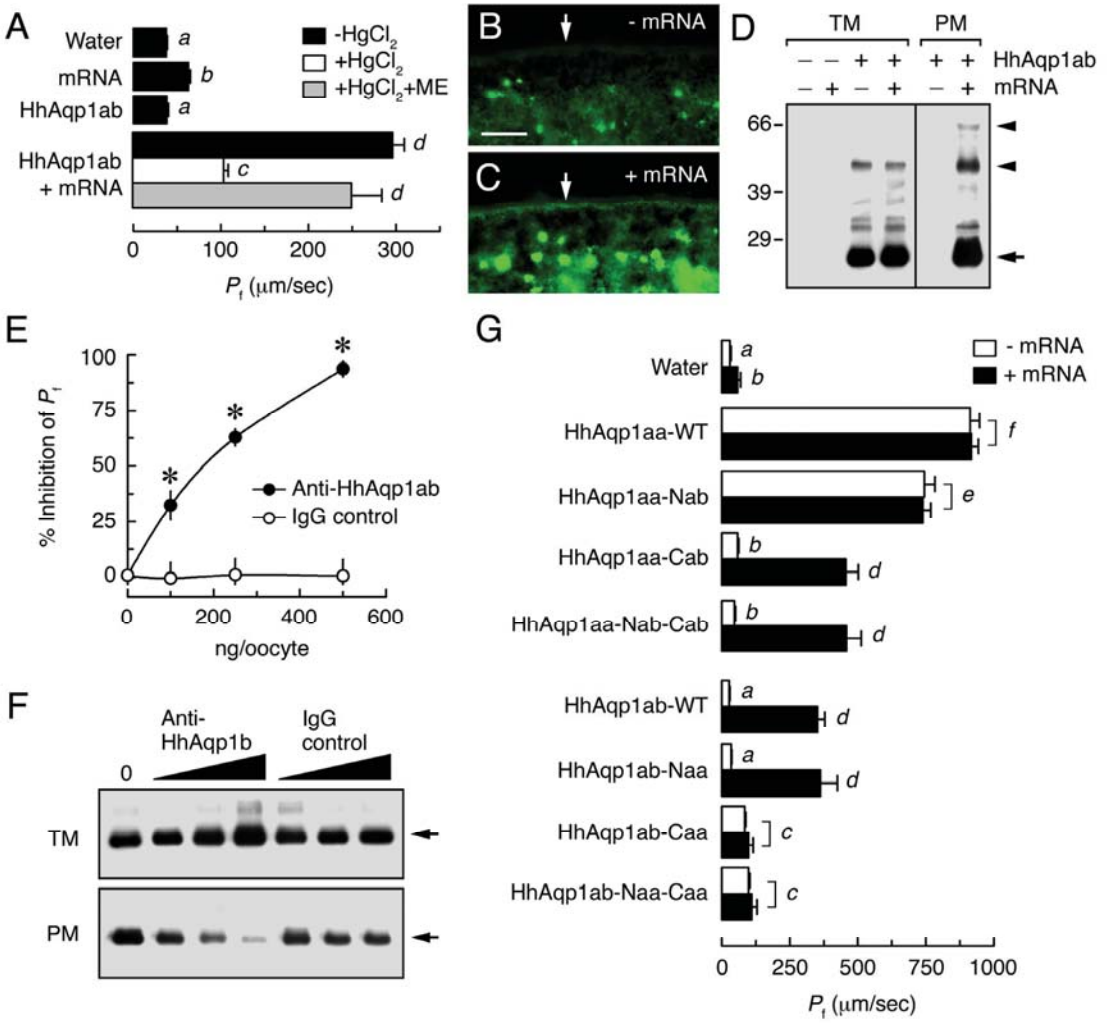


Fig. 7. *Ex vivo* translocation of HhAqp1ab requires its C-terminus and polyA$^+$ mRNA from halibut oocytes. (A) P_f (mean ± SEM of 4 independent experiments; n = 30-45 oocytes) of *X. laevis* oocytes injected with water, polyA$^+$ mRNA purified from halibut PV ovarian follicles, HhAqp1ab alone, or polyA$^+$ mRNA plus HhAqp1ab. Oocytes injected with polyA$^+$ mRNA and HhAqp1ab were treated with mercury and β-mercaptoethanol as in Fig. 3. Values with a different superscript are significantly different (ANOVA, $P < 0.05$). (B-C) HhAqp1ab is translocated into the plasma membrane in the presence of polyA$^+$ mRNA. The arrows point to the plasma membrane. *Bars*, 15 μm. (D) Immunoblots of total membranes (TM; 0.5 oocyte equivalent/lane) or purified plasma membranes (PM; 3 oocyte equivalent/lane), using the HhAqp1ab antiserum, of *X. laevis* oocytes used in A. HhAqp1ab monomers and potential post-translational modifications are indicated as in Fig. 3. Molecular mass markers (kDa) are on the left. (E) Percentage inhibition of P_f (mean ± SEM of 2 independent experiments; n = 30-32 oocytes) of *X. laevis* oocytes expressing HhAqp1ab in the presence of polyA$^+$ mRNA and injected with increasing amounts of the HhAqp1ab antiserum or rabbit IgG (controls). The asterisks indicate significant differences (Student t-test, $P < 0.01$) at each antibody concentration. (F) Immunoblot of total and plasma membranes of oocytes showed in E. (G) P_f (mean ± SEM of 3 independent experiments; n = 40-50 oocytes) of oocytes expressing cRNA encoding wild-type (WT) HhAqp1aa or -1ab, or chimeric proteins in which the N- and C-terminus were exchanged, in the presence or absence of polyA$^+$ mRNA from halibut follicles. Values with different superscript are significantly different (ANOVA, $P < 0.05$).

of halibut polyA+ mRNA in the oocytes. These results therefore suggest that the halibut polyA+ mRNA-derived mechanisms controlling the function of HhAqp1ab in oocytes primarily rely on the C-terminus of the protein.

3.4. Endogenous HhAqp1ab is Up-Regulated During Oocyte Hydration and Translocated into the Oocyte Plasma Membrane

To investigate the regulation of endogenous HhAqp1ab during *in vivo* oocyte hydration in Atlantic halibut, we determined changes in *hhaqp1ab* gene expression and protein synthesis in PV, EH, MH, and H ovarian follicles, as well as in unfertilized eggs, collected from reproductively active females. As it has been previously reported (Finn et al. 2002), halibut ovarian follicles undergoing meiotic maturation swell by a factor of ~4-fold, coincident with the fusion

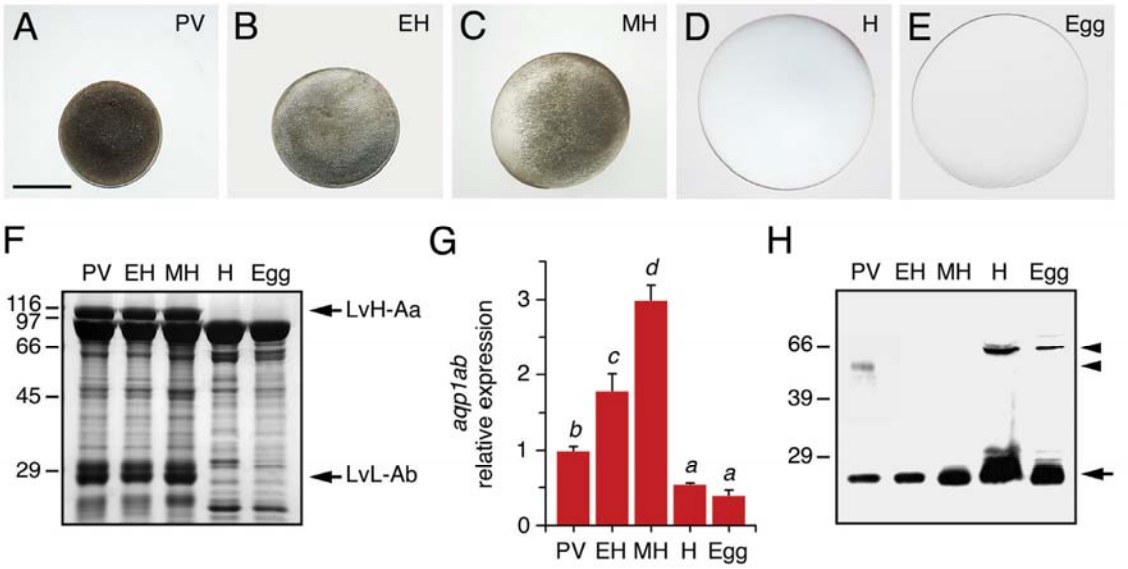


Fig. 8. *hhaqp1ab* and its protein product are sequentially up-regulated during Atlantic halibut oocyte hydration. (A-E) Photomicrographs of isolated Atlantic halibut ovarian follicles undergoing oocyte hydration *in vivo*. PV, postvitellogenic follicle; EH, early hydrating follicle; MH, mid-hydrated follicle; H, hydrated follicle; Egg, unfertilized egg. *Scale bar*: 1 mm. (F) Changes of major yolk proteins of halibut follicles (0.02 follicle equivalents/lane) during hydration resolved by 12% SDS-PAGE and further staining with Coomassie blue R-350. The arrows indicate proteolysis of VtgAa lipovitellin heavy chain and VtgAb lipovitellin light chain (LvH-Aa and LvL-Ab, respectively) during oocyte hydration. Molecular mass markers (kDa) are on the left. (G) *hhaqp1ab* expression levels in halibut ovarian follicles during oocyte hydration as determined by qPCR. Transcript levels of *18s* were used as reference, and relative transcript levels (mean ± SEM; $n = 3$ cDNAs from a pool from 3-4 females) were calculated as fold change with respect the PV follicles. Values with different superscript are significantly different (ANOVA, $P < 0.05$). (H) Representative HhAqp1ab immunoblot of total membranes from halibut ovarian follicles (5 follicle equivalents/lane). The arrow indicates HhAqp1ab monomers, whereas the arrowheads points to possible post-translational modifications of HhAqp1ab. Molecular mass markers (kDa) are on the left.

of yolk globules and progressive 'clearing' of the oocyte cytoplasm (Fig. 8A-E; see also Fig. 9D-F) as a result of alterations in the ultrastructure of crystalline inclusions of yolk globules (Selman et al. 2001). This process renders a ~45% increase in water content in the oocyte (Finn et al. 2002). SDS-PAGE and Coomassie blue staining of Vtg-derived oocyte yolk proteins in extracts from follicle-enclosed oocytes confirmed the complete hydrolysis of the lipovitellin heavy chain of VtgAa (LvH-Aa), and the partial degradation of the lipovitellin light chain of VtgAb (LvL-Ab), during oocyte maturation and hydration (Fig. 8F, arrows) described previously (Finn 2007a). Such massive hydrolysis contributes to most of the organic osmolyte pool of FAA that drives water uptake (Finn 2007a).

The qPCR analyses showed that follicular *hhaqp1ab* transcript levels steadily increase from PV to MH ovarian follicles (3.0 $\pm$ 0.19 units in MH, relative to PV follicles) (Fig. 8G). However, in H follicles and eggs *hhaqp1ab* mRNA was largely degraded (0.55 $\pm$ 0.01 and 0.40 $\pm$ 0.07 units in H and eggs, respectively, relative to PV follicles). The amount of HhAqp1ab monomer protein in the follicle determined by Western blotting notably followed that of its mRNA, progressively increasing from PV to H follicles (Fig. 8H, arrow). Interestingly, the ~50-kDa post-translational modification of HhAqp1ab was noted only in PV follicles, whereas the 66-kDa complexes were detected exclusively in H follicles and eggs, being much more prominent in H follicles (Fig. 8H, arrowheads).

Immunofluorescence and immunoelectron microscopy was subsequently carried out to investigate the subcellular localization of HhAqp1ab in PV, EH, MH and H follicles (Fig. 9). In PV follicles, HhAqp1ab fluorescence and immunogold labeling was found exclusively within vesicles spread in the oocyte cytoplasm between the yolk globules (Fig. 9, A, D, G and M). As hydration commences in EH follicles, HhAqp1ab was detected in the oocyte cytoplasm as well as in the most cortical region of the oocyte just below the plasma membrane (Fig. 9, B, E and H). When oocytes reached a more advanced stage of hydration in MH follicles, HhAqp1ab was localized exclusively within a thin region below the oocyte plasma membrane (Fig. 9, C, F and I). Immunogold analysis of H follicles containing almost or fully hydrated oocytes, revealed the presence of HhAqp1ab along the oocyte microvilli that traverse the vitelline envelope (Fig. 9N-P). Altogether, these findings indicate that the sequential up-regulation of HhAqp1ab during meiotic maturation and oocyte hydration in Atlantic halibut is followed by the translocation of the protein to the oocyte plasma membrane, where it forms non-reducible oligomeric complexes of ~66 kDa. These findings confirm the previous observations using *X. laevis* oocytes co-expressing *hhaqp1ab* cRNA and polyA$^+$ mRNA from halibut ovarian follicles.

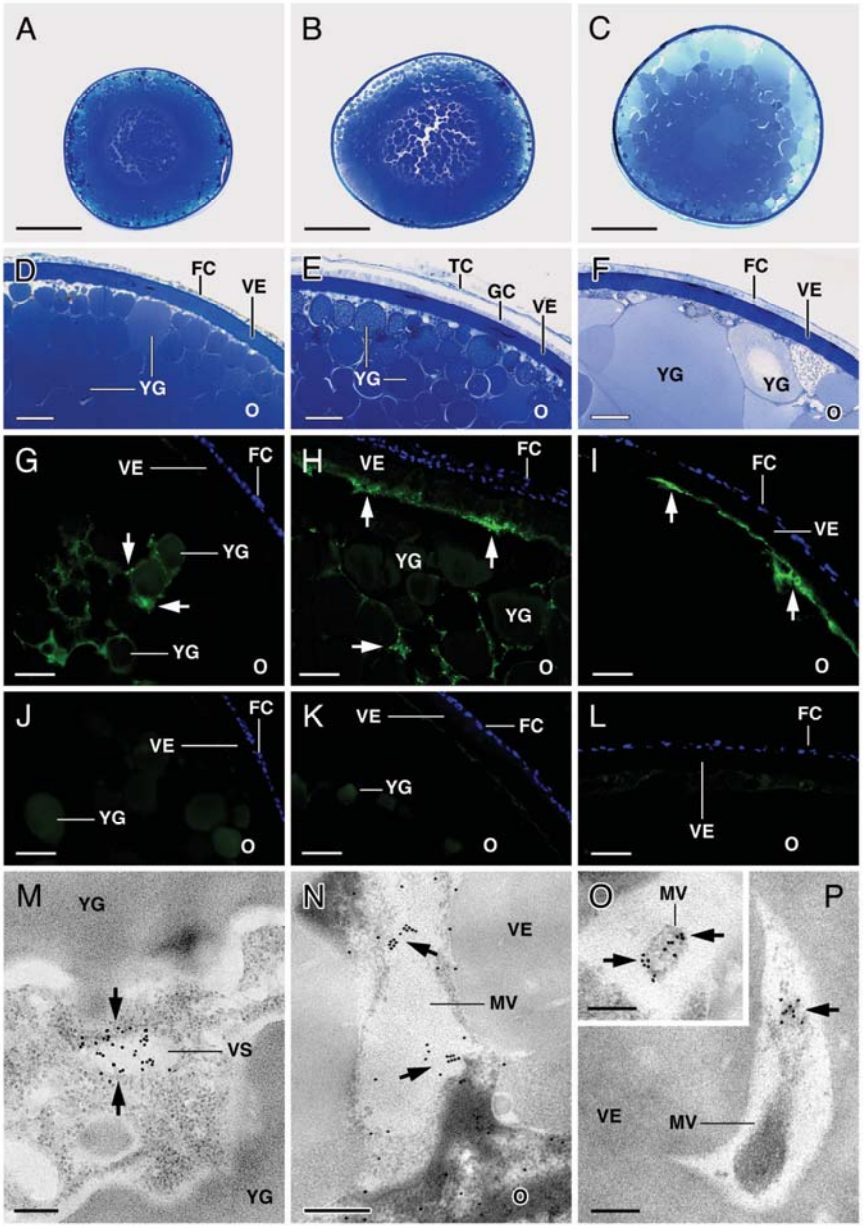


Fig. 9. HhAqp1ab is translocated to the microvillar membranes of Atlantic halibut oocytes during hydration. (A-F) Plastic sections of PV (A, D), EH (B, E) and MH (C, F) ovarian follicles stained with methylene blue (which stains yolk) showing the process of yolk globule fusion and disassembly in the oocyte during hydration. Scale bars: 1 mm (A-C); 50 µm (D-F). (G-L) HhAqp1ab immunofluorescence microscopy on the same stage ovarian follicles revealed that HhAqp1ab (arrows) is translocated from the ooplasm towards the periphery of the oocyte during hydration. Sections were counterstained with DAPI. Control sections (J-L) incubated with preabsorbed HhAqp1ab antiserum. Scale bars: 50 µm. (M-P) Immunoelectron microscopy micrographs of PV and H ovarian follicles using the HhAqp1ab antiserum. In PV follicles (M), immunogold particles (arrows) are localized exclusively within vesicles spread in the ooplasm, whereas in H follicles (N-P) HhAqp1ab gold particles become apparent in the microvilli extending from the oocyte through the vitelline envelope. *Scale bars*: 0.25 µm (M, N); 0.20 µm (O, P). FC, follicle cells; MV, microvilli; TC, theca cells; VE, vitelline envelope; VS, vesicle; YG, yolk globule; O, ooplasm.

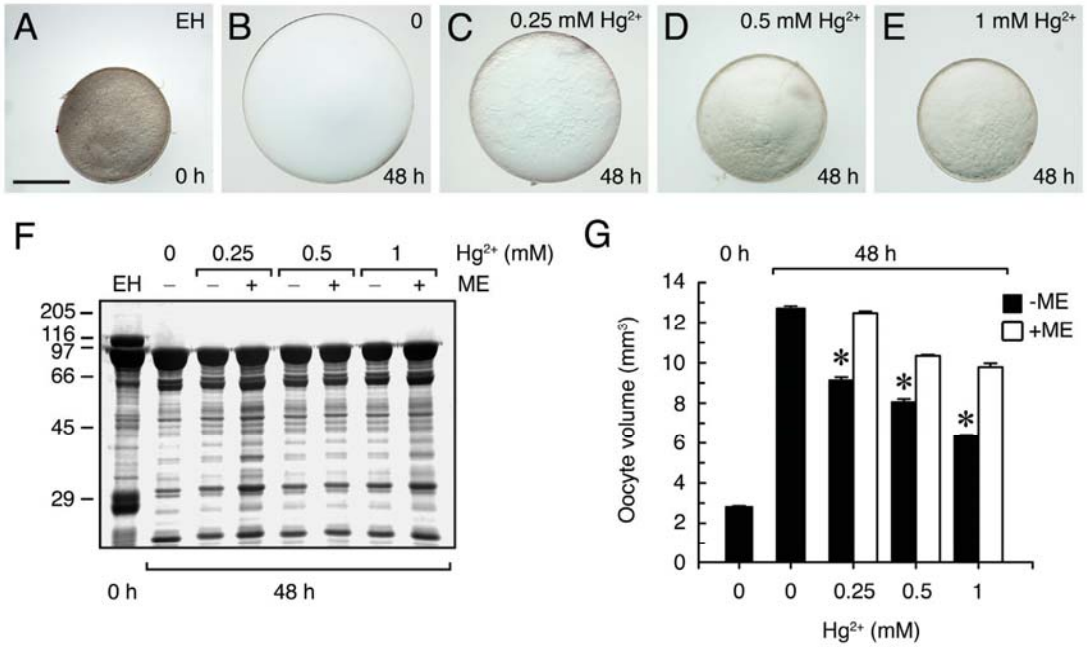


Fig. 10. Atlantic halibut oocyte hydration *in vitro* is inhibited by exposure of follicle-enclosed oocytes to $HgCl_2$. (A-E) Photomicrographs of EH ovarian follicles before (A) and after 48 h without $HgCl_2$ treatment (B) or treated with 0.25 (C), 0.5 (D) or 1 mM (E) $HgCl_2$ for 30 min. *Scale bar*: 1 mm. (F) SDS-PAGE of yolk protein extracts from ovarian follicles before and after treatment with $HgCl_2$ and ME. Molecular mass markers (kDa) are on the left. (G) Effect of $HgCl_2$ on oocyte hydration (mean ± SEM of three independent experiments; $n = 40$-45 follicles) in follicles that were exposed to mercury alone, or to mercury and subsequently to 5 mM β-mercaptoethanol (ME) for another 30 min. The percentage inhibition of oocyte hydration by $HgCl_2$ was 28.1 ± 1.3%, 36.6 ± 1.2%, and 50.2 ± 0.5% for 0.25, 0.5 and 1 mM $HgCl_2$ (asterisks denote significant differences at $P < 0.01$). The effect of low doses of $HgCl_2$ was completely reversed with ME, but only partially reversed with 0.5 and 1 mM $HgCl_2$.

3.5. Mercury Causes a Decrease in the Hydration of Halibut Follicle-Enclosed Oocytes

To investigate the role of HhAqp1ab during oocyte hydration, we first employed $HgCl_2$, since *ex vivo* experiments showed that mercury was effective at inhibiting HhAqp1ab-mediated water transport. In these experiments, halibut EH follicles that had already initiated meiosis resumption and oocyte hydration *in vivo* were briefly exposed to increasing doses of $HgCl_2$ and subsequently treated with or without β-mercaptoethanol (Fig. 10). Treatment of follicles with mercury did not affect oocyte maturation or the hydrolysis of yolk proteins as indicated by SDS-PAGE and Coomassie blue staining (Fig. 10A-F). However, follicles matured in the presence of 0.25, 0.5 or 1 mM $HgCl_2$ showed diminished oocyte hydration compared to untreated controls (Fig. 10, A-E and G). Hydration inhibition was dose-dependent, and could be fully reversed with β-mercaptoethanol when follicles were exposed 0.25 mM $HgCl_2$, but only partially reversed with higher doses of mercury (Fig. 10G).

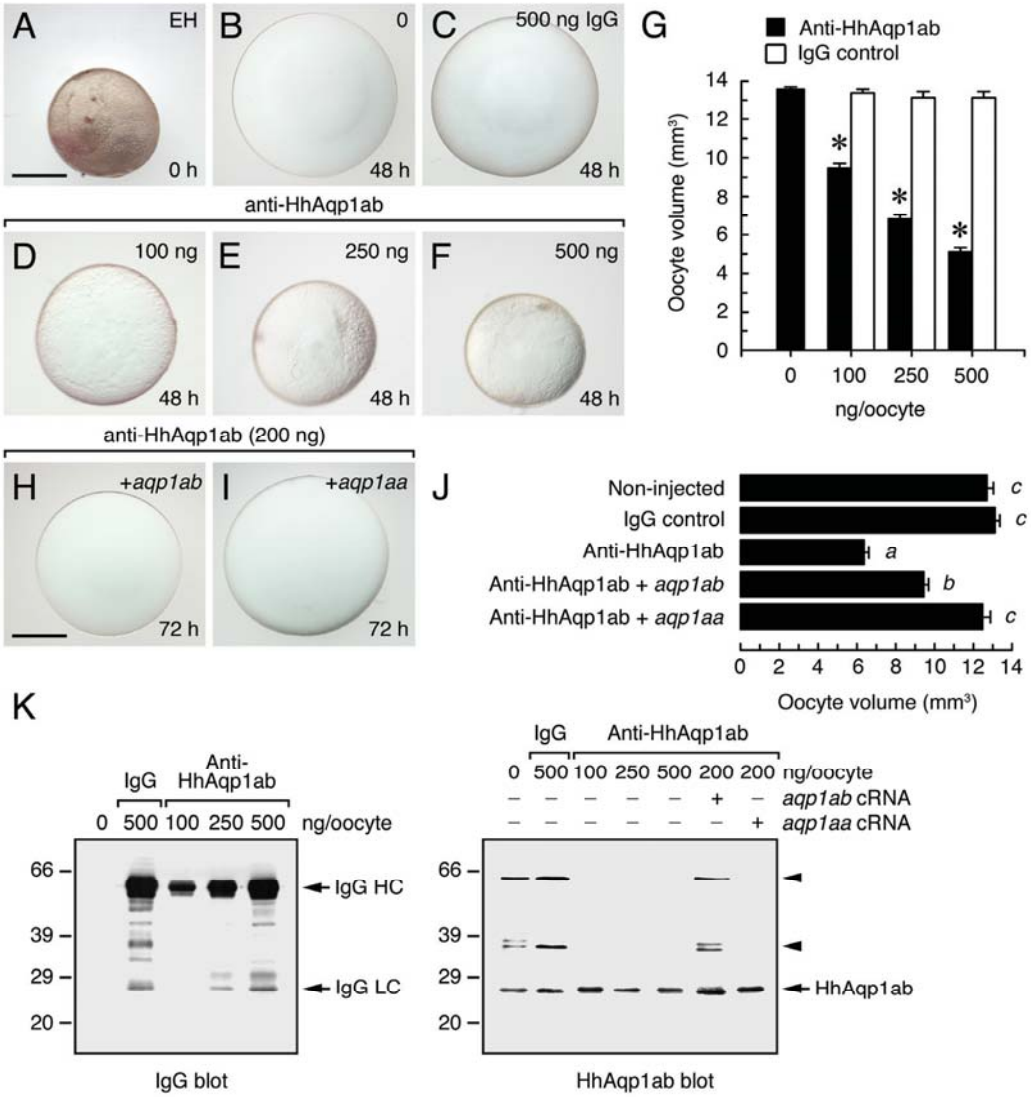


Fig. 11. Inhibition of oocyte hydration *in vitro* by injection of follicle-enclosed oocytes with the HhAqp1ab antiserum, and recovery by over-expression of HhAqp1aa. (A-F) Photomicrographs of Atlantic halibut EH ovarian follicles before (*A*) and after 48 h of injection with PBS (B), 500 ng rabbit IgG (C), or 100 (D), 250 (E) or 500 ng (F) of HhAqp1ab antiserum. *Scale bar*: 1 mm. (G) Inhibition of oocyte hydration (mean ± SEM of three independent experiments; $n = 45$ follicles) with 100, 250 and 500 ng of HhAqp1ab antiserum by 27.5 ± 2.2%, 46.5 ± 1.6%, and 60.5 ± 1.4%, respectively (asterisks denote significant differences at $P < 0.01$). (H, I) Photomicrographs of halibut follicles injected with 200 ng HhAqp1ab antiserum and 25 ng *hhaqp1aa* or *-1ab* cRNA after 72 h of culture. Scale bar: 1 mm. (J) Inhibition of oocyte hydration (mean ± SEM of four independent experiments; $n = 60$ follicles) with injection of 200 ng of rabbit IgG or HhAqp1ab antiserum, and partial or full recovery by co-injection with 25 ng of *hhaqp1ab* and *-1aa* cRNA, respectively. Values with different superscript are significantly different (ANOVA, $P < 0.01$). (K) Representative immunoblots for rabbit IgG (left) or HhAqp1ab (right) of whole homogenates or total membrane extracts, respectively (5 follicle equivalents/lane), from follicles injected with IgG or HhAqpa1b antiserum, and over-expressing or not *hhaqp1ab* or *-1aa* cRNAs. Membranes were probed only with HRP anti-rabbit IgG or with the HhAqp1ab antiserum labelled with HRP. Arrows indicate the heavy (HC) and light (LC) chains of IgG, and the HhAqp1ab monomers, whereas the arrowheads indicate potential post-translational modifications of HhAqp1ab. Molecular mass markers (kDa) are on the left.

3.6. Inhibition of Oocyte Hydration with the HhAqp1ab Antibody is Rescued by Over-Expression of HhAqp1aa

Since it is known that mercury can affect other membrane proteins in addition to aquaporins, we investigated whether specific inhibition of HhAqp1ab was responsible for blocking oocyte hydration. For these experiments, we used the HhAqp1ab antiserum because this antibody was able to inhibit HhAqp1ab-mediated water transport in *X. laevis* oocytes. Follicle-enclosed EH oocytes were thus injected with the anti-HhAqp1ab antibody or rabbit IgG as a control, and subsequently placed in culture medium for 48 h to resume oocyte maturation and hydration *in vitro* (Fig. 11). Injection of the HhAqp1ab antibody did not affect maturational proteolysis of yolk proteins (data not shown), but did inhibit oocyte hydration in a dose-dependent manner (Fig. 11A-F). Relative to controls injected with PBS vehicle, hydration was inhibited by 27%, 46% and 60%, in oocytes injected with 100, 250 and 500 ng/oocyte of HhAqp1ab antiserum, respectively, whereas injection of IgG was ineffective (Fig. 11G). To demonstrate that the reduction of oocyte hydration was caused by the specific inhibition of HhAqp1ab, oocytes injected with the anti-HhAqp1ab antiserum were co-injected with *hhaqp1aa* or *-1ab* cRNA and cultured up to 72 h. Over-expression of exogenous *hhaqp1ab* in oocytes partially recovered the inhibition of oocyte hydration caused by the antibody, whereas the expression of *hhaqp1aa*, the protein product of which is not recognized by the antibody (Fig. 11E), was more effective and fully rescued the hydration of the oocytes (Fig. 11H-J).

Immunoblotting analyses using anti-IgG and HhAqp1ab antibody directly coupled to HRP confirmed that increasing doses of the antibody were effectively injected into the halibut oocytes. Further, the inhibition of oocyte hydration by the HhAqp1ab antiserum correlated with the disappearance of the 66-kDa HhAqp1ab complex, whereas the HhAqp1ab monomer was detected regardless of the treatment of oocytes (Fig. 11K). In extracts from follicles injected with the HhAqp1ab antiserum and over-expressing HhAqp1ab the 66-kDa band could be detected again, suggesting that, as observed in *X. laevis* oocytes, the HhAqp1ab antibody most likely affected the turnover of HhAqp1ab in the plasma membrane of halibut oocytes.

These results support the conclusion that water transport through the tandemly duplicated HhAqp1ab paralog is required for full hydration of Atlantic halibut oocytes. Antibody-induced retention of HhAqp1ab inhibits this hydration, while yolk proteolysis and meiosis resumption in the oocyte is not affected.

4. Discussion

It is now well established that teleosts experienced a specific round of WGD at the root of the crown clade (Amores et al. 1998; Jaillon et al. 2004; Volff 2005; Crow et al. 2006). Indeed our previous analysis of the aquaporin superfamily in zebrafish revealed that WGD endowed teleosts with the largest repertoire of aquaporins in the vertebrate lineage (Tingaud-Sequeira et al. 2010). By re-examining the molecular phylogeny of the vertebrate aquaporin-1 subfamily, the present data reveal that tetrapod *AQP1* and teleost *aqp1aa* orthologs cluster according to phylogenetic rank and display significantly shorter branch lengths compared to the teleost *aqp1ab* orthologs. We show for the first time, however, that the latter *aqp1ab* transcripts and proteins separate into three sister clusters. If subcluster 1 and 2 are collated and compared to subcluster 3, the within cluster topologies also follow phylogenetic rank, with acanthomorph and ostariophysan sequences represented twice. Such paralogous topologies usually indicate the existence of two genes. To investigate this possibility, we conducted an extended synteny analysis of the vertebrate aquaporin-1 loci.

These data reveal that while synteny is highly conserved within the acanthomorph teleosts (medaka, stickleback, and the pufferfishes), it is less conserved when compared to zebrafish, a feature we have noted for other gene families (Finn et al. 2009; Cerdà and Finn 2010; Chauvigné et al. 2010). Nevertheless, the teleost-specific aquaporin paralogs (*aqp1aa* and *-1ab*) are all tandemly arranged between *crhr2* and *thoc1*. The medaka is the only exception, which as noted previously (Tingaud-Sequeira et al. 2008), appears to have lost the *aqp1ab* paralog. An interesting aspect of this analysis is the more conserved gene contiguity between zebrafish and the Sauria, and the absence of syntenic conservation in the mammalian lineage. While it is known that teleost genomes have experienced WGD and subsequent rearrangements (Kasahara et al. 2007; Nakatani et al. 2007; Muffato and Roest-Crollius 2008), the higher conservation of synteny between teleostean, amphibian, reptilian and avian lineages suggests that the regional loss of synteny occurred within Mammalia. Within Eutheria, including the Primates (chimpanzee, gorilla, orangutan, macaque and marmoset), Glires (mouse, rat and rabbit), Lausiatheria (Cow and horse) and Afrotheria (elephant) synteny is highly conserved as indicated between humans and dogs. However, it is broken at the *THOC1* locus, in which the syntenic cassette of blue-annotated genes observed downstream of the *aqp1aa-aqp1ab-thoc1* loci in Teleostei is found on separate chromosomes (e.g. LG 18 in humans and LG 7 in dogs), an arrangement also true for all eutherian mammals. The same cassette of genes has also been rearranged in birds, but in zebra finch and the chicken they have re-

mained linked on the same chromosomes (e.g. LG 2 in the zebra finch and chicken, respectively) and are thus not contiguous. In the turkey, and the anole lizard however, these genes have apparently relocated to LG 3 and LG 4, respectively. We conclude from this analysis that the aquaporin-1 loci are encoded in complex regions of vertebrate genomes that have undergone lineage-specific intra- and inter-chromosomal shuffling.

Importantly, the syntenic data show that the aquaporin-1 subfamily in teleosts reflects the *aqp8* system, in which WGD together with tandem duplication gave rise to *aqp8aa*, *aqp8ab* and *aqp8b* (Tingaud-Sequeira et al. 2010; Cerdà and Finn 2010). In the case of the teleost *aqp1* paralogs, *aqp1aa* and *-1ab* represent the tandem duplicates, while the WGD product (i.e. the true *aqp1b*) is probably lost in many species, but appears to exist in the zebrafish genome as the fused *aqp5/1b* pseudogene (Tingaud-Sequeira et al. 2010; Cerdà and Finn 2010). The co-clustering of *hhaqp1aa* and *-1ab* together with stickleback *aqp1aa* and *-1ab*, respectively, further implies that tandem duplication of an ancestral *aqp1* gene provided the likely basis for the adaptive evolution of the *aqp1ab* paralog.

An interesting aspect of this finding is the co-evolution of the organic osmolyte system that drives oocyte hydration in marine teleosts. Both the yolk precursor gene (*vtgaa*), which is highly expressed in marine teleost oocytes, and ultimately leads to the liberation of FAA in the maturing oocyte, and the water channel (*aqp1ab*) responsible for mediating water influx in a co-ordinated developmental process, appear to have arisen through tandem duplication rather than WGD (Finn et al. 2009; Finn and Fyhn 2010; Cerdà and Finn 2010). The subsequent neofunctionalization of the proteins reveals that gene duplication *per se* is an important foundation of novel cellular pathways that alter the phenotype. In the latter instance, it is the rise of the pelagic egg, which we suggest had broad implications for the oceanic radiation of teleosts (Finn and Kristoffersen 2007; Kristoffersen et al. 2009).

Tandem duplication is not unique to the teleost *aqp1aa* and *-1ab* orthologs. In humans, our synteny analysis reveals a putative *AQP1*-like paralog at the 30.9 Mb locus on LG 7, two genes upstream of the conserved *AQP1* locus. Similarly, *AQP7* and *AQP12* appear to have tandemly duplicated in some Primates, while in teleosts, the *Aqp8* genes have also tandemly duplicated to give rise to *aqp8aa* and *aqp8ab* in addition to the WGD product, *aqp8b* (Tingaud-Sequeira et al. 2010; Cerdà and Finn 2010). For teleosts, however, it is the tandemly duplicated *aqp8aa* and *aqp1ab* genes that have neofunctionalized. We show here through molecular phylogeny and functional assays that the C-terminus of Aqp1ab is the most rapidly evolving aquaporin subdomain in the vertebrate lineage. Within each group, tetrapod AQP1 and

teleost Aqp1aa have undergone similar rates of amino acid substitution (13-17%), while the duplicated Aqp1ab paralog has experienced significantly greater amino acid substitution, particularly within the C-terminal domain. Given that Tetrapoda and Teleostei evolved over similar timescales (~390 million years), the present data provide an eminent example of primary structural divergence in the aftermath of gene duplication.

Neofunctionalization of the *aqp1ab* paralog is further indicated by the more restricted tissue expression pattern. Within Tetrapoda, *AQP1* is expressed in a wide range of tissues (King et al. 2004; Takata et al. 2004), a semi-ubiquitous pattern also noted for *hhaqp1aa* and other teleost *aqp1aa* transcripts (Cerdà and Finn 2010). By contrast *hhaqp1ab* is highly expressed in the ovary, but can also be found at lower levels in the testis, kidney and ureter, observations that further concur with data for other teleosts (Cerdà and Finn 2010; Sun et al. 2010; Tipsmark et al. 2010). The qPCR data for *hhaqp1ab*, however, show that a specific upregulation occurs during meiosis resumption, but ceases at the H oocyte stage. These data correlate very well with the transient hyperosmolality of the halibut ooplasm in relation to the ovarian fluid, due in part to ion accumulation and yolk proteolysis (Finn et al. 2002). The current data further show that increased accumulation of *hhaqp1ab* transcripts occurs during the maximal phase of yolk proteolysis. The subsequent *in vivo* increase of the HhAqp1ab protein in hydrating/hydrated oocytes, and its localization in the microvillar portion of the plasma membrane, further explain the ensuing increase in oocyte size associated with water influx and consequently the decrease in intra-oocytic osmolality (Finn et al. 2002).

As expected from previous studies in teleosts (Fabra et al. 2005; Tingaud-Sequeira et al. 2008; Kagawa et al. 2009; Tingaud-Sequeira et al. 2010), and the structural features of HhAqp1ab, $HgCl_2$ was able to reversibly block HhAqp1ab permeability when expressed in both homologous and heterologous oocytes, as well as to reduce the hydration normally associated with the meiotic maturation of Atlantic halibut oocytes. However, because mercury is not a specific aquaporin blocker, and may affect ion channels involved in the oocyte hydration mechanism (Cerdà et al. 2007; Cerdà 2009; Finn and Fyhn 2010), we tested for a more specific inhibition of HhAqp1ab function by using the HhAqp1ab antisera raised against the C-terminus of the protein. This antisera proved effective at blocking the translocation of HhAqp1ab into the oocyte plasma membrane. Microinjection of the antibody in halibut oocytes resulted in a dose-dependant inhibition of oocyte hydration, while the process of yolk hydrolysis and meiotic maturation were not affected. The immunological inhibition could be fully reversed by the artificial expression of HhAqp1aa, which is functional in halibut oocytes,

but is not recognized by the antibody. Consequently, the decrease of hydration of halibut oocytes can be directly related to the loss of function of HhAqp1ab. These findings, together with the gene expression and cellular localization data, therefore provide for the first time functional evidence of the essential role of Aqp1ab-mediated water transport during the hydration of fish oocytes undergoing meiotic maturation.

Despite the high level of amino acid substitution within the teleost Aqp1ab orthologs, its role during oocyte hydration is most likely conserved in marine species (Tingaud-Sequeira et al. 2008; Cerdà 2009). Part of the explanation underlying this phenomenon may be due to functional constraints in the endocrine pathways that control nuclear and cytoplasmic maturation in addition to oocyte hydration. One such example is a report showing convergent evolution of genes encoding the luteinizing hormone receptor (*lhcgrb*) in Teleostei (Chauvigné et al. 2010). Nevertheless, HhAqp1ab seems to be an unusually highly specialized aquaporin since we found that this protein did not traffic to the oocyte plasma membrane unless it is expressed in native or piscine oocytes. As a molecular phylogenetic member of the Aqp1ab subcluster (2), HhAqp1ab is thus unlike the closely related Senegalese sole (*Solea senegalensis*) Aqp1ab or the more distantly related gilthead seabream ortholog in subcluster (3), or stinging catfish and European eel Aqp1ab channels in subcluster (1), that are functional in heterologous systems such as amphibian oocytes (Tingaud-Sequeira et al. 2008; Chaube et al. 2011). This is surprising given that, to our knowledge, AQP12 orthologs are the only vertebrate aquaporins that are not targeted to the plasma membrane when expressed in *X. laevis* oocytes, a feature that has been explained by the intracellular localization of this aquaporin in pancreatic acinar cells (Itoh et al. 2005; Ohta et al. 2009). The *ex vivo* functional failure of HhAqp1ab in *X. laevis* oocytes could not be alleviated by exposure of oocytes to known signal transduction molecules such as cAMP or cGMP (data not shown), that are known to control trafficking of catfish Aqp1ab as well as of tetrapod AQP2 and AQP-h2 (Hasegawa et al. 2003; Nedvetsky et al. 2009; Chaube et al. 2011). While these results suggest that phosphorylation might not play a role in the plasma membrane localization of HhAqp1ab, its function during HhAqp1ab trafficking can not be ruled out due to the highly divergent nature of the putative phosphorylation sites within the teleost Aqp1ab C-termini (see supplementary fig. S1, Supplementary Material online). Indeed, phosphorylation of Ser^{254} has been shown to mediate Aqp1ab recycling in the gilthead seabream (Tingaud-Sequeira et al. 2008). Interestingly, however, by co-expressing HhAqp1ab cRNAs with $polyA^+$ mRNA purified from PV Atlantic halibut ovarian follicles in *X. laevis* oocytes, it was possible to rescue the *ex vivo* membrane trafficking

of HhAqp1ab. These observations suggest that HhAqp1ab and halibut oocytes may have co-evolved lineage-specific, protein-based mechanisms for the intracellular transport of HhAqp1ab during meiotic maturation. In support of this hypothesis is the observation that HhAqp1ab was fully functional when expressed in oocytes of a distantly related ostariophysan teleost such as the zebrafish.

The precise nature of the polyA$^+$ mRNA-derived mechanism controlling HhAqp1ab plasma membrane localization in oocytes is yet unknown. Our present data reveal however that the HhAqp1ab C-terminus is likely involved, as indicated by functional experiments using wild-type HhAqp1aa and -1ab and chimeric constructs. This finding reinforces the role of the C-terminus in the rapid neofunctionalization of Aqp1ab among teleosts, but the specific function of this domain in the trafficking mechanism of HhAqp1ab remains intriguing. In addition to phosphorylation, C-terminus-mediated interactions with cytoskeletal components, sorting vesicles and lysosomal trafficking regulators (Kamsteeg et al. 2007; Nedvetsky et al. 2009; Moeller et al. 2010), as well as proper folding of the proteins in the endoplasmic reticulum (ER) (van Balkom et al. 2002; Pitonzo and Skach 2006), can control the intracellular transport of aquaporins. In the present study, post-translational modifications of HhAqp1ab resulting from the co-expression of polyA$^+$ mRNA from Atlantic halibut oocytes were not evident by Western blotting analysis of total membrane extracts from *X. laevis* oocytes under reducing or non-reducing conditions. This suggests that HhAqp1ab was properly folded and not retained in the ER even in the absence of polyA$^+$ mRNA from halibut oocytes. Under such conditions, i.e. retention in the ER, aquaporins are usually glycosylated (Deen et al. 1995) and our data indicate that HhAqp1ab is not glycosylated. However, translocation of functional HhAqp1ab into the oocyte plasma membrane was associated with the formation of a 66-kDa HhAqp1ab complex in both native Atlantic halibut oocytes undergoing hydration as well as in *X. laevis* oocytes co-expressing HhAqp1ab and polyA$^+$ mRNA. These complexes most likely represent strong HhAqp1ab oligomers that do not dissociate under denaturing and reducing conditions, as found for the *Escherichia coli* AqpZ (Borgnia et al. 1999) and some plant aquaporins (Ohshima et al. 2001; Casado-Vela et al. 2010). Therefore, whether the enzymatic machinery for the formation of these complexes is only present in teleost oocytes, and/or is a highly specific sorting mechanism of Aqp1ab-containing vesicles that is absent in *X. laevis* oocytes, remains to be investigated.

In conclusion, by using Atlantic halibut as a model species with large oocytes and a reproductive strategy adapted to low temperatures, we provide direct functional evidence for

the essential and conserved physiological role of the tandemly duplicated Aqp1ab paralog during meiosis resumption in marine teleosts. The data show that the rapid divergence of the C-terminal domain of HhAqp1ab results in *ex vivo* loss of function in amphibian oocytes, which can be rescued by injection of polyA$^+$ mRNA from native Atlantic halibut oocytes. These findings thus reveal the dual nature of neofunctionalization of the teleost Aqp1ab water channels where selection pressure has favoured oocyte hydration, but has been relaxed with regard to the specific cellular mechanisms controlling aquaporin trafficking.

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Chapter II

Piscine Follicle-Stimulating Hormone Triggers Progestin Production in Gilthead Seabream Primary Ovarian Follicles

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Abstract

Ovarian growth (vitellogenesis) in most lower vertebrates is mediated by estradiol-17β (E2) secreted by the follicles in response to the follicle-stimulating hormone (Fsh), whereas oocyte maturation and ovulation is mediated by progestins, such as 17α,20β-dihydroxypregn-4-en-3-one (17,20β-P), produced in response to the luteinizing hormone (Lh). In teleosts, the follicular synthesis of 17,20β-P at the time of maturation is primarily due to the up-regulation of P450c17-II (Cyp17a2) and 20β-hydroxysteroid dehydrogenase (Cbr1) enzymes. Here, we show that follicular cells associated with primary growth (previtellogenic) oocytes of the gilthead seabream also express *cyp17a2* and *cbr1*, in addition to P450c17-I (*cyp17a1*) and aromatase (*cyp19a1*), enzymes required for E2 synthesis. Ovaries containing only oogonia and early primary ovarian follicles had a 60-fold higher concentration of 17,20β-P than ovaries in the succeeding stages; and had a higher expression of *cbr1* and the Fsh receptor (*fshra*). Stimulation of explants of primary follicles *in vitro* with recombinant piscine Fsh (rFsh), which specifically activates the seabream Fshra, promoted a rapid accumulation of 17,20β-P, the synthesis being sustained by an external supply of 17α-hydroxyprogesterone. In the presence of Cbr1 inhibitors, rFsh-mediated 17,20β-P production was reduced, with a concomitant increase in testosterone and E2 synthesis. In primary explants, rFsh up-regulated *cyp17a2* and *cbr1* transcription, and simultaneously down-regulated the *cyp17a1* and *cyp19a1* steady-state mRNA levels, within 24 h. In contrast, in explants containing vitellogenic follicles, rFsh had no effect on *cyp17a2* and *cbr1* expression, but increased that of *cyp17a1* and *cyp19a1*. These data suggest a functional Fshra-activated Cyp17a2/Cbr1 steroidogenic pathway in gilthead seabream primary ovarian follicles triggering the production of 17,20β-P.

Key words: Teleost, Progestin, FSH receptor, Cbr1, Cyp17a, Cyp19a1, Oogenesis

1. Introduction

In mammals, ovarian folliculogenesis is under follicle-stimulating hormone (FSH) regulation and luteinizing hormone (LH)-dependent synthesis of androgens as the substrate for the production of estrogens, whereas a preovulatory surge of LH triggers the synthesis of progesterone, which plays critical roles for the induction of ovulation, endometrial receptivity and successful establishment of pregnancy (Drummond 2006). In teleosts, ovarian estrogens are the main steroid hormones controlling oocyte growth (vitellogenesis), whereas either one or other of two progestins, 17α,20β-dihydroxypregn-4-en-3-one (17,20β-P) and 17α,20β,21-trihydroxypregn-4-en-3-one (17,20β,21-P), are involved in the induction of oocyte meiosis resumption (oocyte maturation) and, in some species, also ovulation (Nagahama and Yamashita 2008; Lubzens et al. 2010). In the testis of many, but not all species that have been examined, 17,20β-P also appears to be involved in the final stages of sperm production (Scott et al. 2010).

Studies on salmonid teleosts suggest that during the vitellogenic period Fsh induces estradiol-17β (E2) in the follicular cells surrounding the oocyte through the activation of the Fsh receptor (Fshr) (Kagawa et al. 1982; Nagahama et al. 1991; Yan et al. 1992; Tyler et al. 1997; Santos et al. 2001; Senthilkumaran et al. 2004; Montserrat et al. 2004; Nakamura et al. 2005). This mechanism is likely mediated by the stimulation of transcription and enzyme activity of cytochrome P450c17-I (Cyp17a1) in theca cells (Planas et al. 2000; Swanson et al. 1989; Wright and Zhao 1988) and of P450 ovarian aromatase (Cyp19a1) in granulosa cells (Kagawa et al. 1982; Montserrat et al. 2004). The Cyp17a1 enzyme has a dual function. It has 17α-hydroxylase activity that converts progesterone to 17α-hydroxyprogesterone (17-P), and also 17,20-lyase activity that cleaves the C20,21 side chain of 17-P to convert it into androstenedione (Kazeto et al. 2000a; Zhou et al. 2007). The androstenedione is the immediate precursor of testosterone (T) that in turn is transformed by Cyp19a1 in the granulosa cells to E2. In the Japanese eel (*Anguilla japonica*) ovary, however, a steroidogenic pathway for production of E2 from androstenedione via estrone may exist (Kazeto et al. 2000b). When the oocytes are fully grown, an Lh surge in plasma and an increased expression of the Lh/choriogonadotropin receptor (Lhcgr) in ovarian follicles induces the steroidogenic shift from E2 to progestin production in granulosa cells (Nagahama and Yamashita 2008; Lubzens et al. 2010). In salmonids and possibly in other teleosts, this process appears to be controlled by the down-regulation of Cyp17a1 and Cyp19a1 expression, and the up-regulation firstly of a cytochrome P450c17-II isoform (Cyp17a2) which has 17α-hydroxylase activity only, and secondly of a 20β-

hydroxysteroid dehydrogenase/carbonyl reductase-like (Cbr1) enzyme that is responsible for the conversion of 17-P to 17,20β-P (Chang et al. 1997; Kazeto et al. 2001; Tanaka et al. 2002; Senthilkumaran et al. 2002; Senthilkumaran et al. 2004; Nakamura et al. 2005; Zhou et al. 2007), or in some species, of 17,21-dihydroxy-pregn-4-ene-3,20-dione (11-deoxycortisol) to the alternative maturation-inducing steroid, 17,20β,21-P (Jeng et al. 2012). In rainbow trout (*Oncorhynchus mykiss*), however, a prominent increase in plasma Fsh during ovulation has also been reported (Prat et al. 1996; Breton et al. 1998; Santos et al. 2001), although the functional significance of the Fsh surge remains to be determined. It has been speculated that at this ovarian stage Fsh may potentiate the ovulatory action of Lh, or have a function in initiating the next cycle of ovarian growth (Prat et al. 1996). In coho salmon (*O. kisutch*), Fsh can stimulate, albeit with lower potency than Lh, the production of 17,20β-P by intact ovarian follicles undergoing oocyte maturation and containing mature oocytes (Planas et al. 2000), which might mediate the above actions.

The type of gonadotropic control of ovarian steroidogenesis in salmonids, as well as the two-cell type model for the production of steroid hormones, may not be conserved in other teleosts. For instance, in the red seabream (*Pagrus major*), Lh, but not Fsh, induces E2 production in vitellogenic ovarian follicles through stimulation of Cyp19a1 activity and gene expression (Gen et al. 2001; Kagawa et al. 2003). In other species, such as the common carp (*Cyprinus carpio*), some recombinant piscine Fsh can promote the secretion of 17,20β-P by postvitellogenic follicles (Aizen et al. 2012), although this effect could be produced by promiscuous activation of the Lhcgr by Fsh as it occurs in the amago salmon (*O. masou*) (Levavi-Sivan et al. 2010). The salmonid two-cell type model is clearly not applicable to killifish (*Fundulus heteroclitus*) (Petrino et al. 1989), or the Japanese eel (i.e. Ijiri et al. 2006), and in both species the steroidogenic shift is also not likely to be the mechanism for 17,20β-P production during oocyte maturation (Young et al. 2005). Taken together, these studies suggest diverse mechanisms of the actions of gonadotropins in teleost ovaries, and more detailed studies using homologous hormones and separated follicular layers are probably needed before a general model can be depicted.

An exclusive role of progestins in the final sexual maturation stages of teleosts has also recently been called into question by a number of studies that show clear peaks of plasma 17,20β-P in male and female fish at stages of the reproductive cycle other than oocyte maturation or spermiation (Miura et al., 2007; Scott et al. 2010; Amer et al. 2001; Antonopoulou et al. 2011), and, in one case, the accumulation of 17,20β-P in the gonad of immature fish after go-

nadotropin treatment *in vivo* (Miura and Miura 2011). In regard to other possible roles, 17,20β-P has been shown to increase the levels of meiosis-specific markers in oogonia and spermatogonia in vitro, indicating that 17,20β-P might have a role in stimulating the entry of germ cells into meiosis (Miura et al. 2006, 2007). In support of this hypothesis is the observation that Japanese eel testicular germ cells can produce 17,20β-P in response to 11-ketotestosterone (Miura et al. 2006), and the finding of nuclear progestin receptors, able to specifically bind to 17,20β-P, in germ cells of the testis of Japanese eel (Todo et al. 2000; Ikeuchi et al. 2002; Miura et al. 2006) and in spermatogonia and early spermatocytes of the zebrafish (*Danio rerio*) (Hanna et al. 2010). However, in Atlantic salmon (*Salmo salar*), although nuclear progestin receptors are present in immature testes, they are expressed only in the Sertoli cells surrounding early (pre-meiotic stage) spermatogonia (Chen et al. 2011).

In early ovaries of teleosts, some evidence for the presence of the Cyp17a2/Cbr1 steroidogenic pathway has been reported. Thus, ovarian expression of cyp17a2 has been detected at the time of activation of oocyte meiosis in the Nile tilapia (*Oreochromis niloticus*) (Zhou et al. 2007). In Atlantic cod (*Gadus morhua*), high cbr1 transcript levels have been found in ovaries entirely composed of oogonia and previtellogenic oocytes (Kortner et al. 2009), and in the North African catfish (*Clarias gariepinus*) Cbr1 immunoreactivity has been demonstrated in previtellogenic follicles (Sreenivasulu and Senthilkumaran 2009). In addition, nuclear progestin receptors are present in the nucleus (germinal vesicle) of zebrafish primary growth (previtellogenic) oocytes and surrounding follicle cells (Hanna et al. 2010). However, despite these data, the cellular source of 17,20β-P in teleost previtellogenic ovaries, and its potential gonadotropic control, are unclear. In the present study, using the gilthead seabream (*Sparus aurata*) as an experimental model, we initially investigated whether ovaries at the primary growth stage express functional Cbr1 and other steroidogenic enzymes, and can synthesize 17,20β-P *in vivo*. We then looked at whether there were any concomitant changes in the expression of gonadotropin receptors at this stage, and evaluated their specificity to recombinant piscine gonadotropins. On the basis of these results, we carried out some *in vitro* studies to determine whether recombinant Fsh was able to stimulate the production of 17,20β-P in primary ovarian explants.

2. Materials and Methods

2.1. Animals

Gilthead seabream 1 and 2 years old were obtained from a commercial farm and maintained in the laboratory under natural conditions of temperature and photoperiod as described previously (Fabra et al. 2006). Females (1313 $\pm$ 125 gr, mean $\pm$ SEM) were collected throughout the year, and the stage of ovarian development was determined by histological examination of the gonads. The percentage of the different ovarian follicles in the ovary was scored as previously described (Tingaud-Sequeira et al. 2009). The gilthead seabream is a protandrous hermaphrodite with a group-synchronous ovary in which follicles of all sizes up through vitellogenesis are present during the reproductive period, and populations of follicles are periodically recruited into maturation from a population of oocytes in late vitellogenic stages. Therefore, the ovarian stages were defined based on the most advanced follicle present in the ovary (Fig. 1). Fish showing ambisexual gonads were not used. At all sampling times, fish were sedated with 500 ppm of phenoxyethanol and killed by decapitation. The fish were weighed before removal of the ovary in order to determine the gonadosomatic index (GSI; ovary weight/fish weight x 100). Blood plasma and gonad samples were taken from each fish, frozen in liquid nitrogen and stored at -80°C. Additional pieces of the gonad were processed for histology or *in vitro* culture. Procedures relating to the care and use of animals were approved by the Ethics Committee from Institut de Recerca i Tecnologia Agroalimentàries (IRTA, Spain) in accordance with the European Union regulations.

2.2. cDNA Cloning of Seabream *cbr1* and P450c17 Isoforms

The full-length *cbr1* cDNA was isolated from total RNA of seabream primary ovaries by using 3' and 5' rapid amplification of cDNA ends (RACE) kits (Life Technologies Corp., Carlsbad, CA) as previously described (Zapater at al. 2011). The oligonucleotide primers were designed from an available seabream expressed sequence tag (EST) (GenBank accession no. AM967775) (Table 1). Full-length *cbr1* cDNA was amplified with a specific forward primer and the AUAP primer (3'-RACE; Life Technologies Corp.) using the Easy-A™ High-Fidelity PCR cloning enzyme (Agilent Technologies, Santa Clara CA). The products were cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI) for sequencing. Partial cDNAs encoding seabream P450c17-I (Cyp17a1) and P450c17-II (Cyp17a2) carrying the 3'UTR were isolated from total RNA of the ovary by degenerate primers designed based on conserved regions of

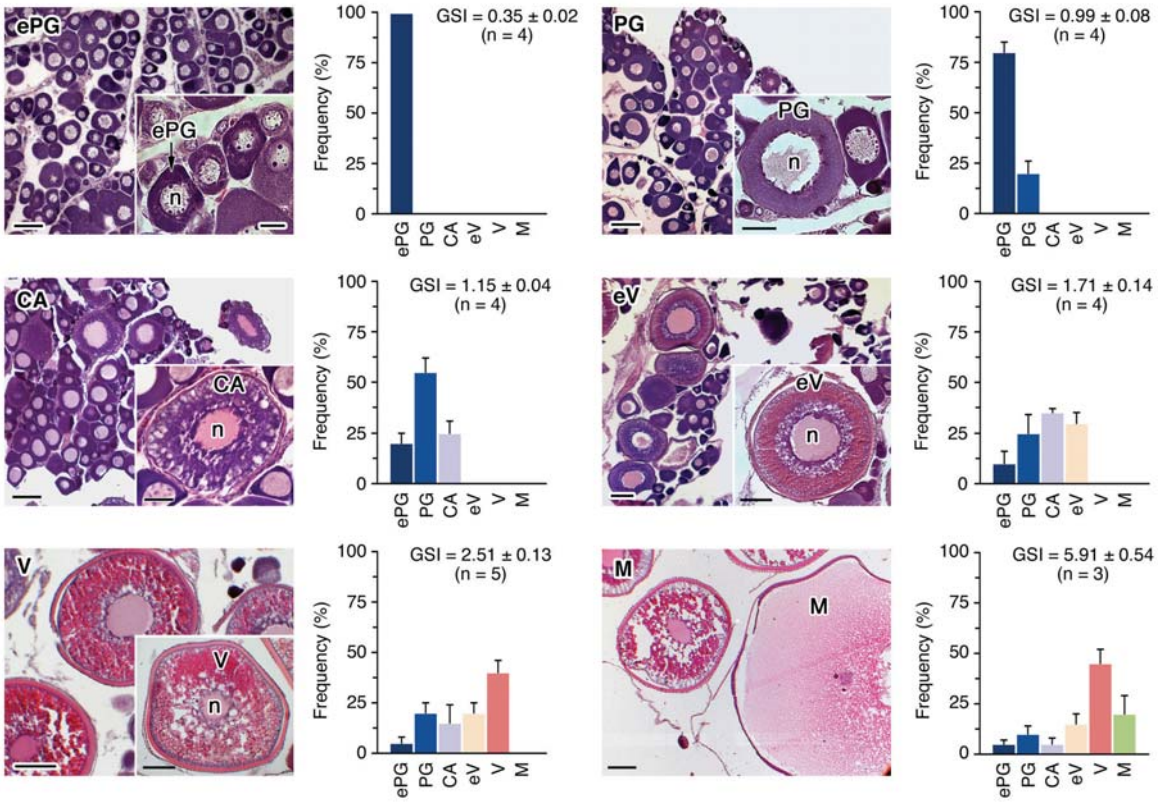

Fig. 1. Representative photomicrographs of histological sections of gilthead seabream ovaries at different developmental stage stained with hematoxylin-eosin, and frequency of ovarian follicles in the ovary at each stage (right panels). The ovarian stages were defined based on the most advanced follicle present as follows: ePG, early primary growth (i.e. perinucleolus) stage in which most oocytes, surrounded by follicle cells, show the proliferation of nucleoli within the nucleus (germinal vesicle) and subsequent movement to its periphery; PG, primary growth stage where some oocytes start to show all nucleoli attached to the internal edge of an enlarged germinal vesicle, and a higher diameter. Both at ePG and PG stages, oocyte meiosis is arrested at the diplotene of the first meiotic division; CA, cortical alveolus stage where oocytes showing nascent cortical alveoli within the ooplasm start to appear; eV, early vitellogenesis stage during which a subpopulation of oocytes initiates vitellogenin incorporation from the bloodstream and their cytoplasm starts to be filled with yolk granules (red) where vitellogenin-derived yolk proteins are stored; V, vitellogenic stage which shows a higher frequency of vitellogenic oocytes of different size; M, maturation stage during which a population of follicle-enclosed oocytes at late stages of vitellogenesis is further recruited into maturation. In these oocytes, the germinal vesicle migrates towards the animal pole and yolk granules fuse with one another eventually forming a large mass of yolk (right oocyte). Data on the frequency of ovarian follicles and gonadosomatic index (GSI) are the mean ± SEM (number of females for each stage is indicated). Note that the GSI increases significantly as vitellogenesis and maturation occurs. Scale bars: 100 μm (ePG, PG, CA, eV, V and M, and eV inset), 50 μm (PG, CA and V insets), 20 μm (ePG inset).

teleost sequences followed by 3'-RACE (Table 1). The nucleotide sequences of gilthead seabream *cbr1*, *cyp17a1* and *cyp17a2* were deposited in the GenBank database under accession numbers JN811567, JQ714032 and JQ714033, respectively.

Table 1. Primer sequences used for the cloning of gilthead seabream steroidogenic enzymes and gonadotropin receptors and qPCR.

Transcript	Sequence 5'-3' (forward or reverse)	Accesssion #	Purpose
cbr1	AGGTCCACTCACCTCTGCAC (F)	AM967775	3'-RACE
cbr1	AAGCAGTTCCAAGGAGACGTTTA (F)	AM967775	3'-RACE
cbr1	AGTCTTGGACACTCCATAC (R)	AM967775	5'-RACE
cbr1	CCGTGTCAGCCATTTTGAAT (R)	AM967775	5'-RACE
cbr1	TTCACGTCGTTGATGTCCAG (R)	AM967775	5'-RACE
cbr1	AGGTCCACTCACCTCTGCAC (F)	JN811567	Full-length amplification
cbr1	GCAACAAGGGCACCGGTCTGGCCATC (F)	JN811567	Site-directed mutagenesis
cbr1	GATGGCCAGACCGGTGCCCTTGTTGC (R)	JN811567	Site-directed mutagenesis
cbr1	CAGGGGAACACTAACATGTGC (F)	JN811567	qPCR
cbr1	CATCAGCGATTAGCCACCAA (R)	JN811567	qPCR
cyp17a1	CAGCCARGGMATHGTGGAYAC (F)	—	Amplification of fragment
cyp17a1	ARGAAGAGRAARAKCTCCAT (R)	—	Amplification of fragment
cyp17a1	ACAGCCTGGTGGACATCTTC (F)	JQ714032	3'-RACE
cyp17a1	CCATGTGCAGAGAGACTTGC (F)	JQ714032	3'-RACE
cyp17a1	AGTTTGGTGTGGTCCTCCAG (F)	JQ714032	qPCR
cyp17a1	TGATGATCCACCCACATGAT (R)	JQ714032	qPCR
cyp17a2	TACAACRAYGGVATYGTGSA (F)	—	Amplification of fragment
cyp17a2	CCACATGTTSACCARAACMCG (R)	—	Amplification of fragment
cyp17a2	GAGGCCTGGTGGACATTTAC (F)	JQ714033	3'-RACE
cyp17a2	TGAAGGAGTGTATCACCGTCAG (F)	JQ714033	3'-RACE
cyp17a2	AAGTTCAATGAAGAGCCGAGAG (F)	JQ714033	qPCR
cyp17a2	GCTTGGGAAAGGCTGCTATT (R)	JQ714033	qPCR
fshra	GCATGCACGTAAACACAGACA (F)	AY587262	Full-length amplification
fshra	CTGGGGACAGAAACGAGACC (R)	AY587262	Full-length amplification, qPCR
fshra	CAATGCACTGAGTCCGGATG (F)	AY587262	qPCR
lhcgrba	TTTGAGTGAGGGCCACATATC (F)	AY587261	Full-length amplification
lhcgrba	TCCAGCATGAGTCATTTTTCC (R)	AY587261	Full-length amplification
lhcgrba	AGGGGGAGCTCACCTGAAAT (F)	AY587261	qPCR
lhcgrba	TTCCTCCAAATCCAGCATGA (R)	AY587261	qPCR
cyp19a	TCAGCAGGAGGCAGAATCTC (F)	AF399824	qPCR
cyp19a	CTGTTTGAATGCATCGGTACAC (R)	AF399824	qPCR
18s	GAATTGACGGAAGGGCACCACCAG (F)	AY993930	qPCR
18s	ACTAAGAACGGCCATGCACCACCAC (R)	AY993930	qPCR

2.3. Analysis of Enzymatic Activity of Seabream Cbr1

Seabream *cbr1* was cloned into the *Eco*RI/*Xba*I sites of the expression vector pcDNA3 (Life Technologies Corp.) with the addition of an epitope FLAG® tag (DYKDDDDK) in the C-

terminus of the encoded protein before the stop codon by PCR. A point mutation was introduced into the seabream Cbr1 to replace Iso^{15} by a Thr by using the QuikChange® II Site-Directed Mutagenesis Kits (Life Technologies Corp.) on the pcDNA3-Cbr1-FLAG plasmid (Table 1). Selected clones were sequenced to confirm that only the desired constructs were synthesized. Characterization of Cbr1 was carried out in human embryonic kidney 293T (HEK293T) cells grown in 24-well plates containing 1 ml of DMEM with 10% (v/v) fetal bovine serum. The cells were cultured at 37ºC in 5% CO_2 during 16-20 h, and then transfected using Lipofectamin (Life Technologies Corp.) with 700 ng of empty pcDNA3 vector, pcDNA3-Cbr1-FLAG or pcDNA3-Cbr1-I15T-FLAG, and 100 ng of β-Galactosidase (β-Gal) plasmid (Promega Corp.) to normalize transfection efficiency. After 16 h incubation, the medium was replaced with DMEM without serum and 17-P (10-100 ng) (Sigma-Aldrich, St. Louis, MO) or 0.1% ethanol vehicle (control) were added to the cells. In other experiments, cells transfected with pcDNA3-Cbr1-FLAG were exposed to 1, 10 or 100 μM of the carbonyl reductase inhibitors indomethacin (Indo) and phenylbutazone (PhB) (Sigma-Aldrich) 1 h before the addition of 100 ng 17-P. In both cases, the culture medium from each well was separated after 24 h of steroid addition by centrifugation at 2000 x g. The cells were lysed with 100 μl of Reporter Lysis Buffer (Promega Corp.) and β-Gal activity was measured by colorimetric detection using nitrophenyl βD-galactopyranoside (Sigma-Aldrich) substrate. Production of 17,20β-P was determined in 150-fold diluted culture medium by enzyme-linked immunosorbent assay (EIA) using commercial antibodies and reagents (Cayman Chemical Company, Ann Arbor, MI). Cross-reactivity of the 17,20β-P antibody against 17-P was 0.4% (data not shown). Three independent trials in triplicate were carried out for each experiment, and results were normalized to β-Gal activity. To verify the same level of translation of the pcDNA3-Cbr1-FLAG and -I15T-FLAG plasmids in HEK293T cells, Western blotting using anti-FLAG® M2 monoclonal antibody (Sigma-Aldrich) was carried out as described (Zapater et al. 2011).

2.4. Phylogenetic analyses

Teleost Cbr1, 17β-hydroxysteroid dehydrogenase (Hsd17b), 3β-hydroxysteroid dehydrogenase (Hsd3b), 17α-hydroxylase/17,20 lyase polypeptide I (Cyp17a1) and steroid 17α-hydroxylase polypeptide II (Cyp17a2) orthologs were retrieved from public databases (GenBank and ensembl v68; Table 2). The deduced amino acid sequences were aligned using MAFFT v.6.857 with the algorithm E-INS-I (Katoh and Toh, 2008). An additional more stringent alignment was constructed by removing ambiguously aligned sites using Gblocks as well

Table 2. List of teleost sequences and accession numbers used in the study

Protein	Species	Accession #
Cbr1	*Sparus aurata*	JN811567
Cbr1	*Tetraodon nigroviridis*	ENSTNIP00000012764
Cbr1	*Oryzias latipes*	NP_001131060
Cbr1a	*Danio rerio*	NP_919387
Cbr1b	*Danio rerio*	ENSDARP00000024104
Cbr1	*Clarias gariepinus*	ACM91728
Cbr1	*Anguilla japonica*	AAL16062
Cbr1	*Salmo salar*	ACI70003
Cbr1	*Oreochromis niloticus*	AAL65409
Cbr1-type A	*Oncorhynchus mykiss*	NP_001118068
Cbr1-type B	*Oncorhynchus mykiss*	AAD20991
Cbr1	*Plecoglossus altivelis*	BAB92960
Cbr1	*Solea senegalensis*	ACK99046
Cbr1	*Gadus morhua*	ABD62879
Cbr1	*Gasterosteus aculeatus*	ENSGACP00000015024
Cbr1	*Anoplopoma fimbria*	ACQ58769
Hsd17b1	*Oreochromis niloticus*	AAV74182
Hsd17b1	*Oryzias latipes*	ABP98810
Hsd17b3	*Danio rerio*	AAS58451
Hsd17b8	*Oreochromis niloticus*	AAV74184
Hsd17b12/Hsd20b type 2	*Oreochromis niloticus*	AAV74183
Hsd17b/Hsd20b type 2	*Sparus aurata*	AM975449
Hsd17b12a	*Tetraodon nigroviridis*	ENSTNIP00000012474
Hsd17b12b	*Tetraodon nigroviridis*	ENSTNIP00000013291
Hsd17b	*Solea senegalensis*	ACL01375
Hsd17b12-B	*Salmo salar*	NP_001135118
Hsd17b12-B	*Danio rerio*	NP_955907
Hsd3b	*Oryzias latipes*	NP_001131037
Hsd3b	*Solea senegalensis*	ACN89887
Hsd3b1	*Tetraodon nigroviridis*	ENSTNIP00000018739
Hsd3b	*Osmerus mordax*	ACO09586
Hsd3b	*Oncorhynchus mykiss*	AAB31733
Hsd3b1	*Danio rerio*	NP_997962
Hsd3b	*Ictalurus punctatus*	AAC16547
Hsd3b1	*Oreochromis niloticus*	ACJ24593
Hsd3b2	*Oreochromis niloticus*	ACJ24595
Cyp17a1	*Sparus aurata*	JQ714032
Cyp17a1	*Takifugu rubripes*	ABU54399

(continued on next page)

Table 2. *continued*

Protein	Species	Accession #
Cyp17a1	*Oryzias latipes*	BAA13252
Cyp17a1	*Anguilla japonica*	AAR88432
Cyp17a1	*Oncorhynchus mykiss*	CAA46675
Cyp17a1	*Danio rerio*	NP_997971
Cyp17a1	*Oreochromis niloticus*	BAF75924
Cyp17a1	*Gasterosteus aculeatus*	ABU54400
Cyp17a1	*Sebastes schlegelii*	ADV59774
Cyp17a1	*Verasper moseri*	ACE79385
Cyp17a1	*Paralichthys olivaceus*	ACN72759
Cyp17a1	*Acanthopagrus schlegelii*	AAW62972
Cyp17a1	*Lateolabrax japonicus*	AEL31248
Cyp17a1	*Pimephales promelas*	CAC38768
Cyp17a1	*Ictalurus punctatus*	NP_001187242
Cyp17a2	*Sparus aurata*	JQ714033
Cyp17a2	*Takifugu rubripes*	ABU54402
Cyp17a2	*Oryzias latipes*	ABQ96161
Cyp17a2	*Gasterosteus aculeatus*	ABU54403
Cyp17a2	*Oreochromis niloticus*	ABQ96160
Cyp17a2	*Danio rerio*	ABU54401
Cyp17a2	*Sebastes schlegelii*	AEJ33653
Cyp17a2	*Paralichthys olivaceus*	ACM47730
Cyp17a2	*Verasper moseri*	ACI95233
Cyp17a2	*Cynoglossus semilaevis*	ACE75879
Cyp1c1	*Anguilla japonica*	AAR15082

as by visual examination (Castresana 2000). Phylogenies were constructed using Maximum likelihood (ML), as implemented in RAxML v.7.2.8 (Stamatakis et al. 2008) with BLOSUM62 amino acid substitution matrix, with 1000 bootstraps. Bayesian analyses of Cyp17a1 and Cyp17a2 sequences was carried out as previously described (Chauvigné et al. 2010), using Japanese eel (*Anguilla japonica*) cytochrome P4501C1 (Cyp1c1) as outgroup. The trees generated were visualized and edited with the software FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

2.5. *In Situ* Hybridization

Pieces of primary growth and vitellogenic ovaries were fixed in 4% paraformaldehyde for 16-20 h at 4ºC to determine the sites of expression of *cyp17a1*, *cyp17a2*, *cyp19a1* and *cbr1*

by *in situ* hybridization (ISH) as described previously (Cerdà et al. 2008). DIG-labeled sense and antisense riboprobes were synthesized with SP6 and T7 RNA polymerases using the DIG RNA labelling Kit (Roche Applied Science, Mannheim, Germany). The probes were as follows: for *cyp17a1*, nucleotides 165-1114; for *cyp17a2*, nucleotides 95-1360; for *cyp19a1*, nucleotides 1106-1788 (GenBank accession number AF399824); and for *cbr1*, full-length cDNA excluding the polyA$^+$. Post-hybridization washing steps were 2x SSC with 50% formamide for 30 min at 50ºC, two washes in 2x SSC at 42ºC or 50ºC for 15 min, and one wash in 0.5x SSC or 0.2x SSC at 42ºC or 50ºC, respectively, for 15 min.

2.6. Gonadotropins and Functional Analysis of Seabream Gonadotropin Receptors

Full-length cDNAs encoding gilthead seabream FSH and LH/choriogonadotropin receptors, Fshra and Lhcgrba following the nomenclature proposed by Chauvigné et al. 2010 (Genbank accession numbers AY587262 and AY587261, respectively), were amplified from total RNA of the ovary with specific primers (Table 1), cloned into the *Eco*RV/*Xba*I sites of the pcDNA3 expression vector, and verified by sequencing as described above. The receptor cDNA constructs were transiently expressed in HEK293T cells. The cells were grown in DMEM suplemented with penicillin/streptomycin, 2 mM glutamine and 10% fetal bovine serum (Life Technologies Corp.), and were incubated at 37ºC in 5% CO_2. Receptor activation was measured using a reporter gene assay following the protocol described by Andersson et al. 2009. Cells were transfected with 300 ng of each receptor or empty pcDNA3, 100 ng of cAMP-responsive reporter gene plasmid (pCRE-luc; Agilent Technologies), and 100 ng of β-Gal plasmid. After 48 h, cells were incubated for 6 h with different concentrations of forskolin (Tocris Bioscience, Minneapolis, MN) as positive control, human chorionic gonadotropin (hCG; Sigma-Aldrich), or European seabass (*Dicentrarchus labrax*) single-chain recombinant FSH and LH (rFsh and rLh, respectively) in serum-free DMEM medium. Seabass rFsh and rLh were produced as described previously (Molés et al. 2011). The cells were lysed with 100 µl of Reporter Lysis Buffer (Promega Corp.) and luciferase and β-Gal activities were measured by luminescence or colorimetric detection, respectively, using reconstituted Luciferin (Biothema, Sweden) or o-nitrophenyl βD-galactopyranoside substrates. The seabream receptors did not show signs of constitutive activity in HEK293T cells since expression of the receptors did not increase basal (i.e. non-stimulated) luciferase activity.

2.7. *In Vitro* Incubations of Ovarian Explants

Ovaries at the primary growth stage (female GSI of 0.88 ± 0.08), containing only oogonia and primary ovarian follicles not showing nascent cortical alveoli, were placed in Petri dishes with 75% Leivovitz L-15 culture medium with L-glutamine (Sigma-Aldrich) and with 100 µg/ml gentamicine at pH 7.5. Ovaries were manually dissected into small fragments (~100 mg) and placed in 24-well plastic tissue culture dishes containing 1 ml of fresh culture medium. Explants were stimulated in triplicate with recombinant, single-chain European seabass *(Dicentrarchus labrax*) Fsh (rFsh; 1-100 ng/ml), in the presence or absence of 17-P (10 or 100 ng/ml). The carbonyl reductase inhibitors were added 1 h prior to the addition of the hormones at 100 µM. Ovarian fragments were cultured at 18ºC in a temperature-controlled incubator up to 72 h. Every 24 h, tissues and culture medium were harvested, frozen in liquid nitrogen and stored at -80°C. In some experiments, explants at the vitellogenic stage (female GSI of 3.02 ± 0.21), containing a significant amount of fully-grown vitellogenic ovarian follicles and lower amounts of preceding stages, but not oocytes showing signs of maturation (Fig. 1), were incubated with rFsh for only 24 h. For incubations of both primary and vitellogenic explants, one single female was used for each experiment, and 3 to 6 separate experiments were carried out for each type of incubation.

2.8. Real-Time Quantitative PCR

Quantification of *cyp17a1*, *cyp17a2*, *cyp19a1* and *cbr1* transcripts in whole ovaries at different developmental stages (Fig. 1) was performed by real-time quantitative PCR (qRT-PCR) using SYBR Green qPCR master mix (Life Technologies Corp.) as described previously (Zapater et al. 2011). Transcript levels of seabream *fshra* and *lhcgrba* were also determined. The specific primers used are listed in Table 1. The relative transcript levels were calculated by using a standard curve generated for each primer pair from 10-fold serial dilutions of a pool of first-stranded cDNA template from ovary samples. Standard curves represented the cycle threshold (Ct) value as a function of the logarithm of the number of copies generated, defined arbitrarily as one copy for the most diluted standard. The amount of *18s* ribosomal transcripts, which did not change significantly during ovarian development (data not shown), was used to express the results as the number of copies of each target gene divided by the number of copies of the normalizing gene. All calibration curves exhibited correlation coefficients higher than 0.98, and the corresponding qRT-PCR efficiencies were greater than 99%.

The comparative Ct method was used for relative quantification of transcript levels in ovarian explants incubated *in vitro* with rFsh for 24 and 72 h. Primers were as above and the quantification was also normalized to *18s* expression which did not change significantly over culture time (data not shown). Fold-changes in the relative mRNA expression with respect to the control group not exposed to rFsh at 24 h were determined using the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

2.9. Steroid RIA

Steroids were extracted from plasma (100 µl) with 4 ml of diethyl ether. After the tubes were allowed to settle, the lower aqueous phase was frozen by dipping each tube into liquid nitrogen, while the organic phase was poured into a separate tube and evaporated to dryness with a stream of nitrogen at 45ºC. For E2 determination, plasma (25 µl) was extracted with 1 ml ethyl acetate that was also evaporated under nitrogen. Ovarian tissue (~100 mg) was extracted as described by Ketata et al. (2007). It was homogenized in 500 µl of molecular grade water (Sigma-Aldrich) and sonicated three times for 30 sec. To enhance the extraction of steroids from binding proteins, 400 µl of HCl at 25 mM was added to the homogenates and the samples were incubated for 15 min at 40°C, then 1.25 ml of 0.07 M Na_2HPO_4 pH 7.4 was added for neutralization. Steroids were extracted with 7 ml of dichloromethane, and after centrifugation at 2,500 x g for 10 min, the organic phase was poured into a separate tube, and evaporated to dryness under nitrogen at 45ºC. To rule out the possibility that the presence of yolk proteins in ovarian samples might affect the extraction efficiency of 17,20β-P, a duplicate sample from each reproductive stage was mixed with 12,000 dpm of 17,20β-P radiolabel and taken through the extraction procedure. There was very little difference in extraction efficiency (80 $\pm$ 1 %; mean $\pm$ SEM) for all ovarian stages. Tissue concentrations of steroids shown have not been corrected for this extraction efficiency. The dried extracts from plasma and ovary were solubilised in 1 ml of assay buffer and measured by radioimmunoassay (RIA) as described by Scott et al. (1984). Duplicate aliquots (100 µl) were assayed in the specific RIAs. In all the assays, the standard concentrations ranged from 500 to 1.95 pg per tube. The extracts were assayed for T, E2, 17,20β-P and 17,20β,21-P depending on each experiment. The specificity of the antibodies and more detailed information of the RIAs have been published elsewhere (Scott et al. 1984; Scott and Sorensen 1994).

2.10. Statistical Analysis

Data are the mean ± SEM and were statistically analyzed using one- or two-way analysis of variance (ANOVA), after log-transformation of the data when necessary, followed by Tukey's pairwise comparison test. Criteria for significant differences were at $P < 0.05$.

3. Results

3.1. Cloning and Functional Characterization of Seabream Cbr1

As a first step to investigate the potential sources of 17,20β-P production during early ovarian development in seabream, a full-length cDNA encoding Cbr1 was isolated from ovaries at the primary growth stage. The seabream *cbr1* cDNA was 1312 bp long and encoded a protein of 275 amino acids with a predicted molecular mass of 30 kDa. Alignment of the deduced amino acid sequence of the seabream Cbr1 cDNA with orthologous sequences from other teleosts showed relatively high identity (75-85%), and revealed the presence of the two characteristic domains for the carbonyl reductase family of enzymes, the Rossmann fold coenzyme binding consensus identified as GlyXXXGlyXGly near the N terminus, and the TyrXXXLys motif crucial for catalytic activity (Fig. 2). Accordingly, maximum likelihood phylogenetic analysis indicated that seabream Cbr1 clustered together with the Cbr1 from other teleosts, and separately from the 3β- and 17β-hydroxysteroid dehydrogenase enzymes (Fig. 3).

In rainbow trout, the Iso^{15} residue within the Rossman fold domain of Cbr1 plays an important role in enzyme binding of cofactor NADPH (Guan et al. 2000). Since seabream Cbr1 shows the same conserved residue (Fig. 4A), we investigated the enzymatic activity of seabream wild-type Cbr1 and Cbr1-I15T mutant by transient transfection of the corresponding cDNAs into HEK293T cells. Both Flag-tagged constructs were expressed at approximately the same levels in HEK293T cells as indicated by Western blotting employing an anti-Flag antibody (Fig. 4B). Conversion of 17-P to 17,20β-P by HEK293T cells after 24 h of precursor addition increased with the expression of wild-type Cbr1 but not with Cbr1-I15T, which released the same 17,20β-P levels as the control cells transfected with empty pcDNA3 vector, possibly due to endogenous 20β-HSD activity (Fig. 4C). The levels of conversion of 17-P to 17,20β-P were relatively low (10-20%) which could be related to the fact that overexpression of seabream Cbr1 in HEK293T cells was toxic as indicated by the lower levels of β-Gal activity of Cbr1-transfected cells when compared to that of control cells (data not shown). The conversion of

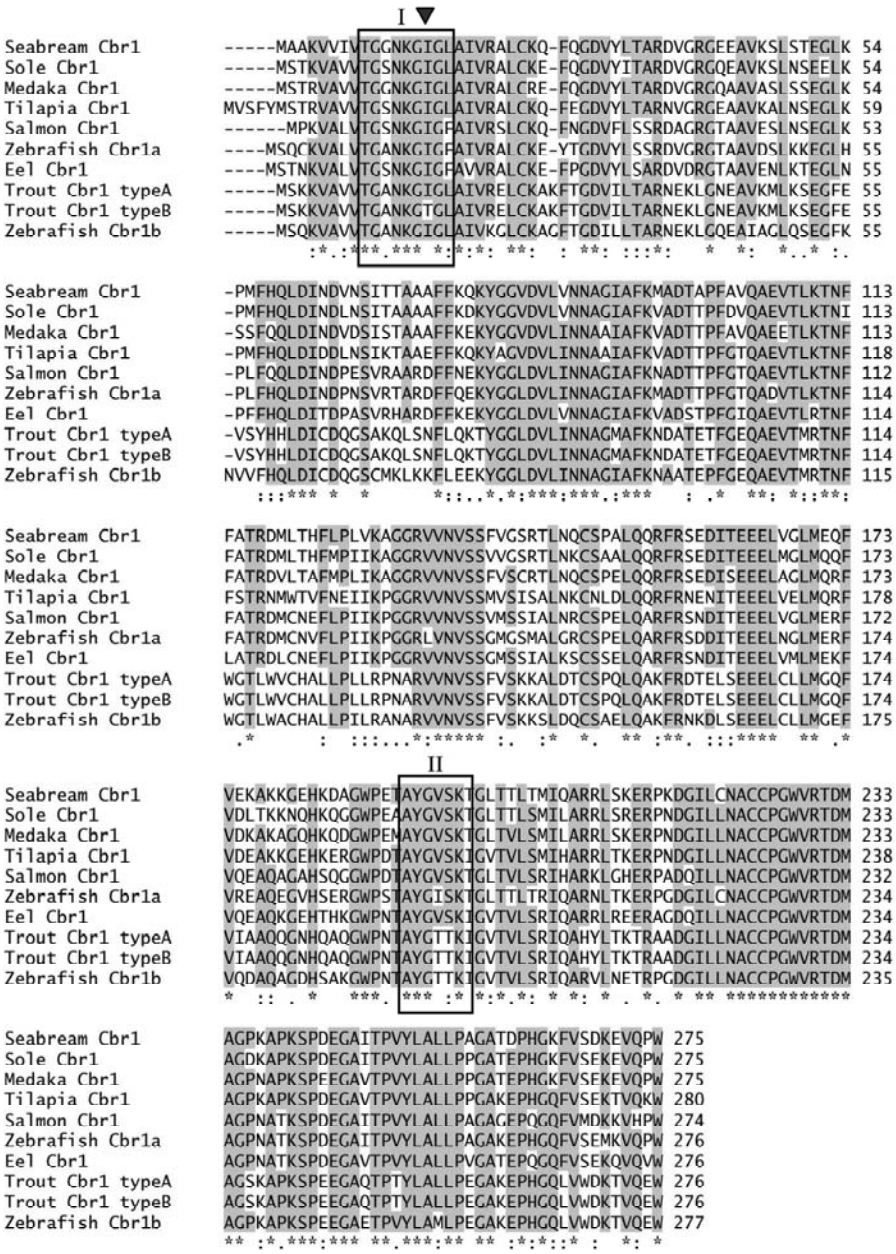

Fig. 2. ClustalW multiple amino acid alignment of gilthead seabream Cbr1 with other teleost counterparts. Gaps, introduced to optimize the alignment, are shown with dashes. Residues conserved in most of the sequences are shaded in grey. Fully conserved amino acids are indicated with an asterisk, whereas conserved and semi-conserved amino acid substitutions are indicated by a double or single dot, respectively. The conserved domains are shown (I, Rossmann fold; II, Active site). The arrowhead indicates the Iso[15] critical for coenzyme (NADPH) binding.

17-P to 17,20β-P by wild-type Cbr1 was significantly reduced in the presence of increasing doses of Indo or PhB (Fig. 4D), compounds that inhibit carbonyl reductase activity of North African catfish recombinant Cbr1 (Sreenivasulu and Senthilkumaran 2009). These results

therefore confirmed that seabream primary growth ovaries express functional Cbr1 able to catalyze the conversion of 17-P to 17,20β-P.

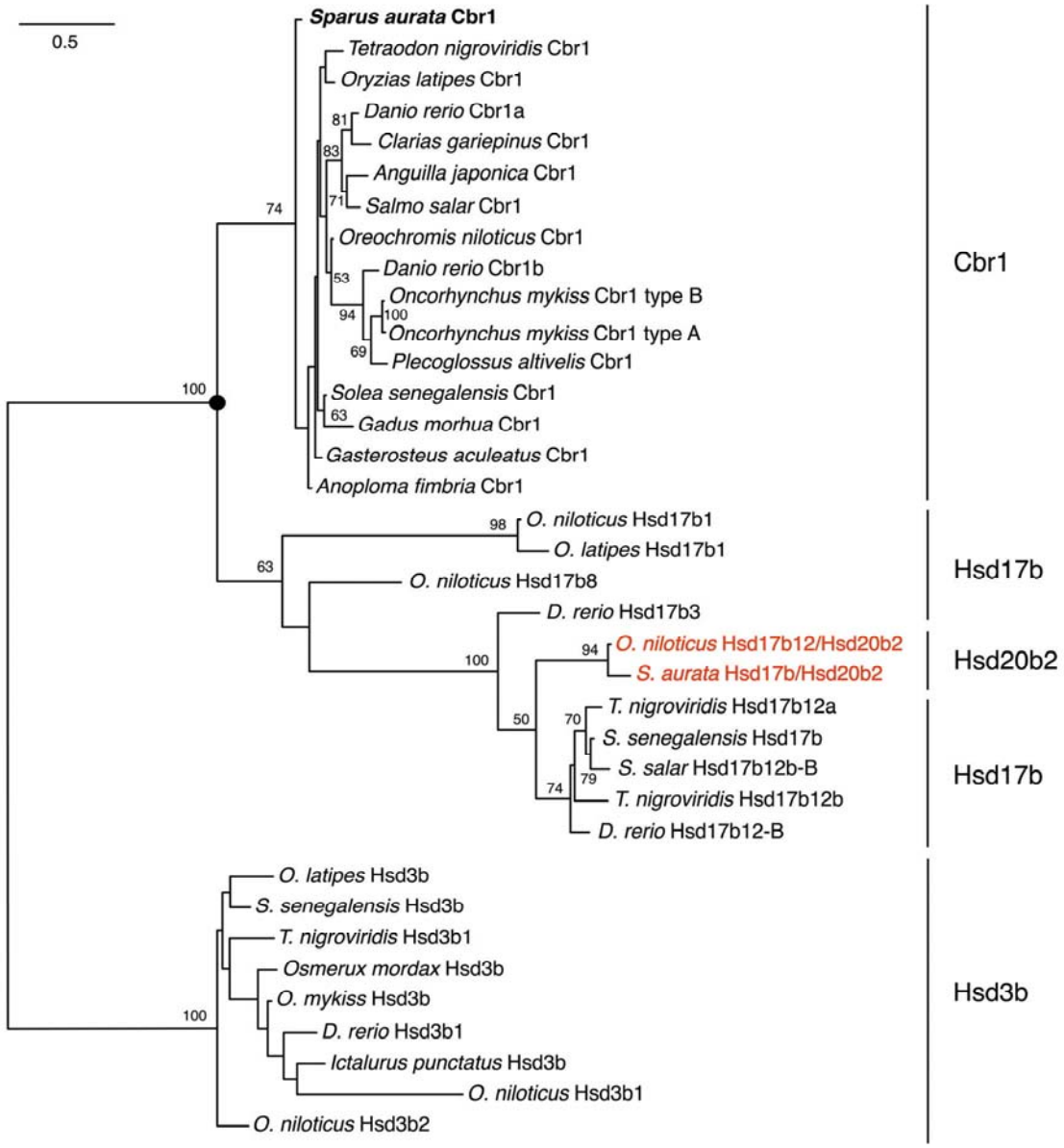

Fig. 3. Maximum likelihood (ML) phylogenetic tree of aligned deduced amino acid sequences of teleost 20β-hydoxysteroid dehydrogenase/carbonyl reductase (Cbr1), 17β-hydroxysteroid dehydrogenase (Hsd17b), and 3β-hydroxysteroid dehydrogenase (Hsd3b). Percentage bootstrap values >50 (100 replicates) are shown at each node. The scale bar calibrates the branch lengths, which indicate the number of amino acid substitutions per site. Note that *O.mossambicus* Hsd17b12 and *S. aurata* Hsd17b should be annotated as Hsd20b type 2, a novel subfamily of HSDs that participate in cortisol catabolism and has apparently no role in teleost reproduction (Tokarz et al. 2012).

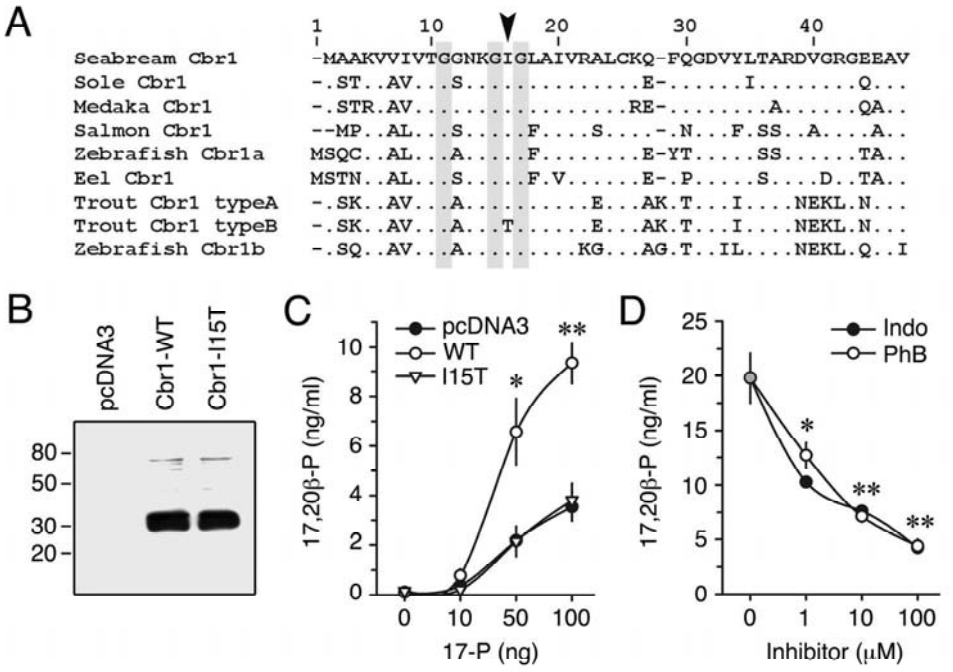

Fig. 4. Functional characterization of gilthead seabream Cbr1. (A) Sequence alignment of teleost Cbr1 at the N terminus. Three Gly residues of the Rossmann fold (GXXXGXG) are highlighted in orange, whereas the Iso residue at position 15, critical for coenzyme (NADPH) binding, is indicated with a red arrowhead. (B) Western blotting of HEK293T cells transiently transfected with empty pcDNA3 vector or with the seabream wild-type (WT) Cbr1 or the Cbr1-I15T mutant using an anti-FLAG antibody. (C) Production of 17,20β-P after incubation of HEK293T cells transiently transfected with the constructs above with increasing doses of 17-P for 24 h. (D) Inhibition of 17,20β-P production with 100 ng 17-P by the carbonyl reductase inhibitors indomethacin (Indo) and phenylbutazone (PhB). In C and D, values (mean ± SEM) represent compiled data from three separate experiments, each with three replicates per condition. *, $P < 0.05$; **, $P < 0.001$, compared with the other constructs (C) or in the absence of inhibitors (D) (Tukey's pairwise comparison test).

3.2. Cloning of Two Types of Cyp17a in the Seabream

Since it has been suggested that the differential expression of Cyp17a1 and Cyp17a2 controls the steroidogenic shift towards 17,20β-P production during teleost oocyte maturation, we cloned the orthologous transcripts in the seabream. By using degenerate primers and 3' RACE, partial cDNAs encoding peptides with high homology to teleost Cyp17a1 and Cyp17a2 were isolated. Bayesian phylogenetic analysis using the seabream partial deduced amino acid sequences and teleost Cyp17a1 and Cyp17a2 orthologs confirmed that each of the cloned sequences clustered separately into each of the Cyp17a1 and Cyp17a2 teleost clades (Fig. 5). The tree thus clearly demonstrated that the seabream Cyp17a1 and Cyp17a2 orthologs were isolated.

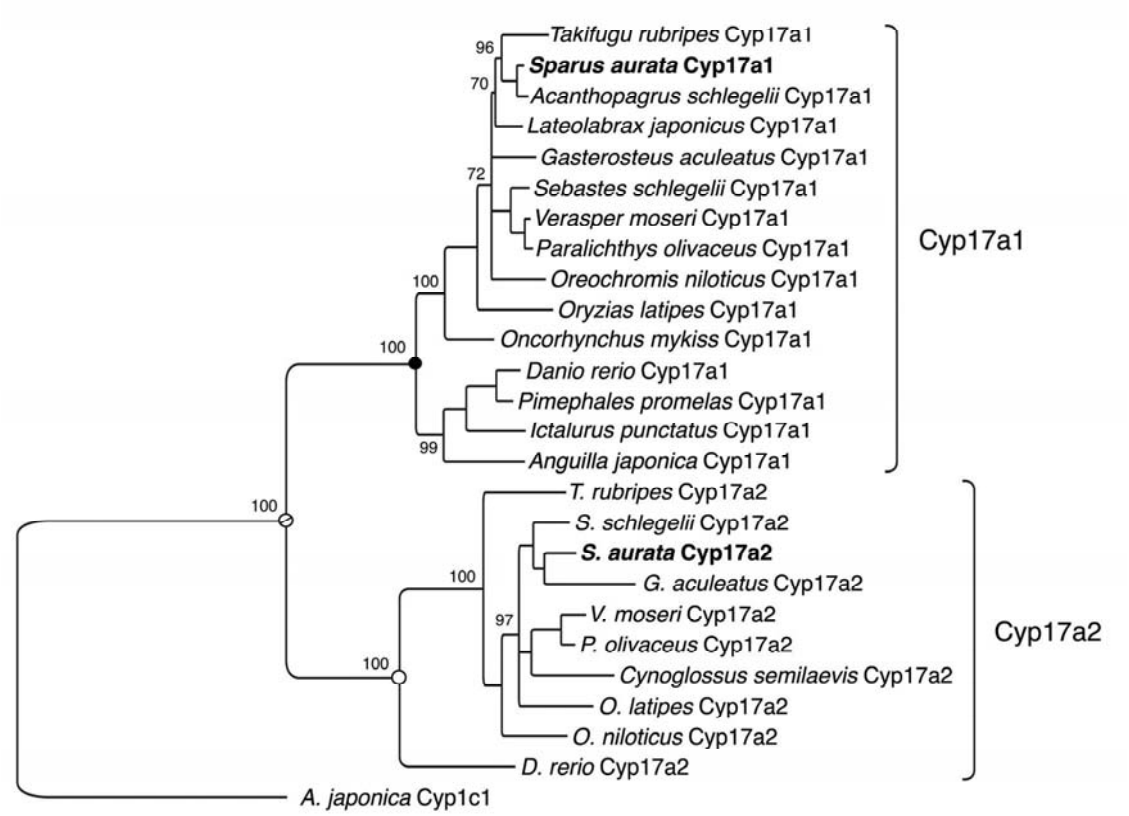

Fig. 5. Bayesian majority rule consensus tree for the amino acid alignment of teleost steroid 17α-hydroxylase/17,20 lyase polypeptide I (cyp17a1) and steroid 17α-hydroxylase (cyp17a2). Japanese eel (*Anguilla japonica*) cytochrome P4501C1 (Cyp1c1) was used as outgroup. Bayesian posterior probabilities are shown at each node.

3.3. Expression Pattern of Steroidogenic Enzymes and Gonadotropin Receptors during Seabream Ovarian Development

The changes in *cyp17a1*, *cyp17a2* and *cbr1* transcript levels during ovarian development were further analyzed by qRT-PCR (Fig. 6). The expression levels of *cyp19a1* and gonadotropin receptors, *fshra* and *lhcgrba*, were also determined using available sequence information. The highest *fshra* expression was detected in ovaries at an early primary growth stage and then progressively decreased up to the vitellogenic stage (Fig. 6A). During ovarian maturation, a further increase in *fshra* expression was noted but reached lower levels compared to the early primary growth stage. In contrast, the expression of *lhcgrba* remained low until the maturation phase, when the levels of *lhcgrba* markedly increased (Fig. 6B). Both *cyp17a1* and *cyp17a2* transcript levels were low at the early primary growth stage and progressively increased throughout ovarian development, with a more prominent accumulation of mRNAs

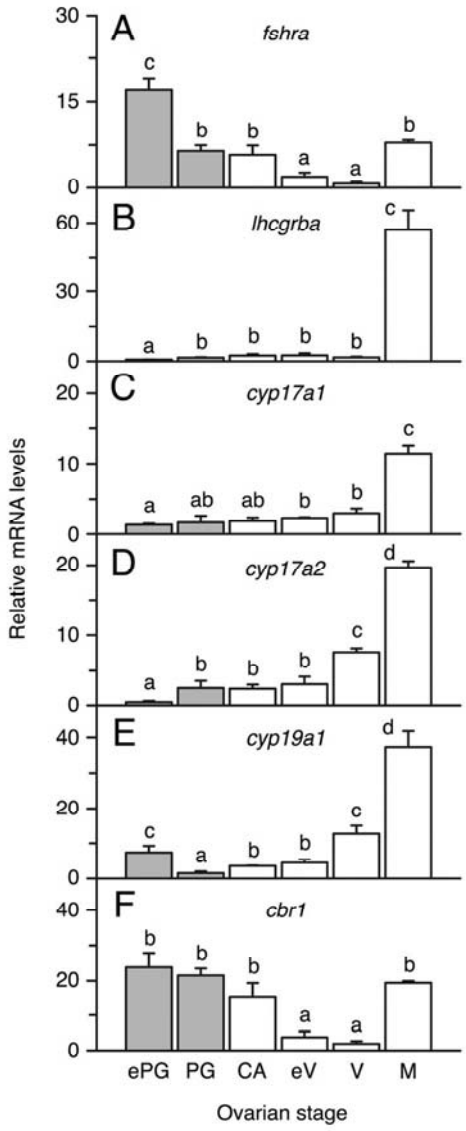

Fig. 6. Temporal expression of *fshra*, *lhcgrba*, *cyp17a1*, *cyp17a2*, *cyp19a1* and *cbr1* transcripts in the seabream ovary determined by qRT-PCR. Data represent relative mean expression values of the different transcripts, normalized to *18s* ribosomal RNA expression, in ovaries at different developmental stage: ePG, early primary oocyte growth; PG, primary oocyte growth; CA, cortical alveolus; eV, early vitellogenesis; V, vitellogenic; M, maturation (see Supplemental Fig. S1 for details). Bars in grey indicate the PG stages. Data are the mean ± SEM ($n = 4$ for ePG, PG, CA and eV; $n = 5$ for V; and $n = 3$ for M), and bars with different superscript indicate values statistically significant (Tukey's pairwise comparison test; $P < 0.05$).

during the maturation stage (Fig. 6, C and D). The *cyp17a2* mRNAs however increased earlier than those of *cyp17a1* during late vitellogenesis. The expression of *cyp19a1* was relatively high at the early primary growth stage, abruptly decreased in more advanced primary growth ovaries, and progressively increased again during vitellogenesis (Fig. 6E). A further increase in *cyp19a1* expression occurred during maturation as noted for the other transcripts. Interestingly, the ovarian *cbr1* transcript levels remained elevated from the early primary growth stage until the cortical alveolus stage, then clearly decreased during vitellogenesis, and increased again during maturation to reach levels similar to those at the primary growth stages (Fig. 6F).

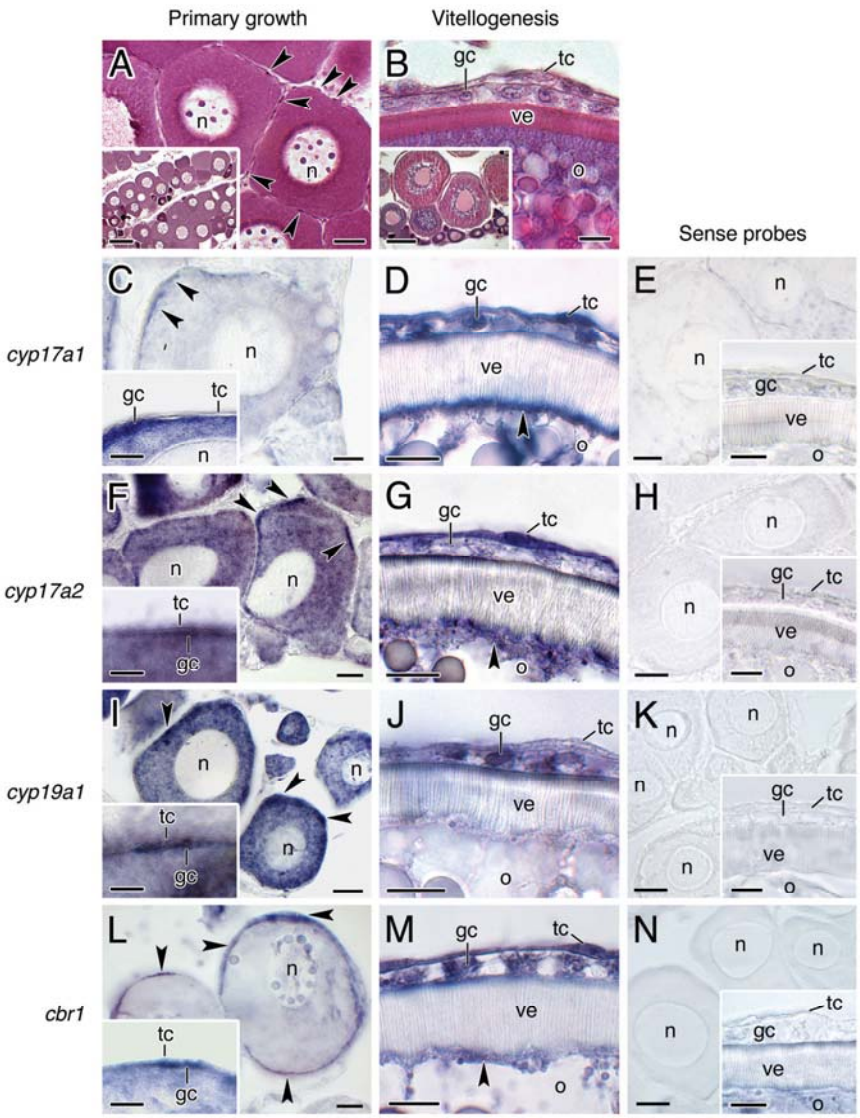

Fig. 7. Expression of *cyp17a1*, *cyp17a2*, *cyp19a1* and *cbr1* in seabream primary growth and vitellogenic ovaries detected by ISH. (A-B) Representative histological sections of primary (A) and vitellogenic (B) ovaries stained with hematoxilin and eosin. In A, arrowheads indicate follicle cells. B shows a detail of the follicular cells (theca and granulosa cells) surrounding a vitellogenic oocyte. (C-N) Representative sections probed with antisense (C-D, F-G, I-J, L-M) or sense (E, H, K, N) riboprobes for *cyp17a1*, *cyp17a2*, *cyp19a1* and *cbr1*, as indicated on the left. The photomicrographs correspond to two females, either at the primary growth (C, F, I, L, E, H, K and N) or at vitellogenic stage (D, G, J, M, and insets in E, H, K and N). The photomicrographs show ovarian follicles at the same stage within the primary growth and vitellogenic phases. Positive signals are colored dark-blue to purple. At the primary growth stage, *cyp17a1*, *cyp17a2*, *cyp19a1* and *cbr1* (C, F, I, and L) transcripts were expressed in flat primordial granulosa cells surrounding the oocyte (insets in C, F, I and L), whereas a weak staining in primordial theca cells was only observed for *cyp17a2* and *cbr1* (F and L insets). The cytoplasm of primary oocytes was also strongly stained for *cyp17a2* and *cyp19a1* probes (F and I). In vitellogenic ovarian follicles, *cyp17a1* and *cbr1* were strongly expressed in both theca and granulosa cells (D and M, respectively), *cyp17a2* in theca cells and weakly in granulosa cells (G), and *cyp19a1* was prominent in granulosa cells but absent in theca cells (J). All transcripts, except *cyp19a1*, were also detected in the ooplasm of vitellogenic oocytes (arrowheads). Sense probes were negative (E, H, K, N). n, nucleus; tc, theca cells; gc, granulosa cells; ve, vitelline envelope; o, oocyte. *Scale bars*, 8 μm (insets in C, F, I and L), 20 μm (A, B, E inset, F, H inset, I, K inset, L, N inset), 25 μm (C-E, G, H, J, K, M, N), 100 μm (A inset, B inset).

3.4. Localization of Steroidogenic Enzymes in the Seabream Ovary by ISH

To determine the ovarian cellular sites of expression of *cbr1* and other steroidogenic enzymes, ISH was performed on histological sections of ovaries at the primary growth and vitellogenic stages (Fig. 7). In primary ovarian follicles, primordial follicle cells (theca and granulosa) appear as two very thin layers of flat cells adjacent to the oocyte (Fig. 7A). Primordial granulosa cells were positive for *cyp17a1*, *cyp17a2*, *cyp19a1* and *cbr1* expression, whereas in theca cells only *cyp17a2* and *cbr1* were weakly expressed (Fig. 7, C, F, I and L). Transcripts of *cyp17a2* and *cyp19a1* were also strongly detected in the ooplasm of primary oocytes (Fig. 7, F and I). In vitellogenic ovarian follicles, follicle cells are fully differentiated; granulosa cells acquire a cuboidal shape whereas theca cells enlarge and elongate (Fig. 7B). In these follicles, expression of *cyp17a1* and *cbr1* was strong in both theca and granulosa cells, and the presence of these transcripts in oocytes was also evident (Fig. 7, D and M). By contrast, *cyp17a2* expression was more prominent in theca cells than in granulosa cells, and was also detected in the oocyte cytoplasm (Fig. 7G), whereas specific *cyp19a1* staining was only noted in granulosa cells (Fig. 7J). For all transcripts, no extrafollicular expression (i.e. interstitial tissue) was noted in the ovarian stages examined. Control sections incubated with sense probes were negative (Fig. 7, E, H, K and N).

3.5. Plasma and Ovarian Levels of E2 and Progestins during the Seabream Reproductive Cycle

The previous data indicated that all steroidogenic enzymes investigated, including *cbr1*, were expressed in follicle cells associated with primary oocytes, and, as expected, in vitellogenic ovarian follicles. This pattern of expression *in vivo* suggested that primary growth follicles are probably able to synthesize both estrogens and progestins. To explore this hypothesis, the changes in E2 and 17,20β-P both in plasma and in the ovary of females showing different stages of ovarian follicle development were determined (Fig. 8). Since 17,20β,21-P has been previously detected in the plasma of the gilthead seabream, in addition to 17,20β-P (Gothilf et al. 1997; Meiri et al. 2002), we also determined the ovarian and circulating levels of this steroid. The plasma levels of E2 progressively increased in females from the primary growth up to the vitellogenic stage, whereas the level of E2 in the ovary remained unchanged (Fig. 8, A and B). At the oocyte maturation stage, however, there was a 10- to 12-fold increase in the concentration of E2 in both plasma and ovary. Plasma levels of 17,20β-P and 1720β,21-P showed a U pattern with elevated concentrations being found at the first and last stages and relatively low concentrations (especially for 17,20β-P) being found in the intervening stages

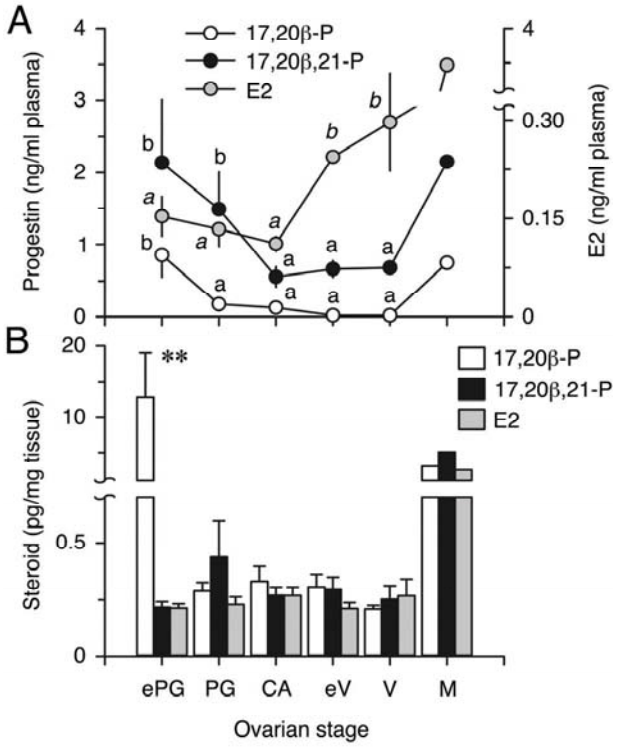

Fig. 8. Levels of 17,20β-P, 17,20β,21-P and E2 in plasma (A) and ovary (B) of seabream 1-year-old females showing different stage of ovarian development. Data are the mean ± SEM ($n = 4$ females; M stage $n = 2$ females). Statistical analysis was performed with the data from the ePG up to the V stages, excluding the M stage. In A, data for each steroid with different superscript are statistically significant (Tukey's pairwise comparison test; $P < 0.05$). In B, **, $P < 0.001$ (Tukey's pairwise comparison test between ePG stage and the other stages).

(Fig. 8A). All three steroids (i.e. 17,20β-P, 17,20β,21-P and E2) had very similar tissue concentrations to each other at every stage apart from the early primary growth stage, at which 17,20β-P was 60-fold higher than E2 or 17,20β,21-P (Fig. 8B).

3.6. Effect of rFsh on Steroid Production by Primary Growth Ovaries In Vitro

To examine whether 17,20β-P production by seabream primary ovarian follicles is regulated by gonadotropins, we first determined the responsiveness of the seabream gonadotropin receptors to European seabass rFsh and rLh. For this, seabream Fshra and Lhcgrba cDNAs were expressed in HEK293T cells and tested for ligand-induced cAMP production using increasing doses of rFsh, rLh and hCG (Fig. 9). These experiments indicated that seabream Fshra was activated by seabass rFsh, but also by rLh and hCG with an EC_{50} 1.5- and 2.8-fold higher, respectively, compared to rFsh. In contrast, the seabream Lhcgrba was specific for rLh and hCG. The receptors did not show signs of constitutive activity since expression of the receptors did not increase basal (i.e. non-stimulated) cell activity. These results therefore demonstrate that seabass rFsh could be used to specifically activate the seabream Fshra in further experiments.

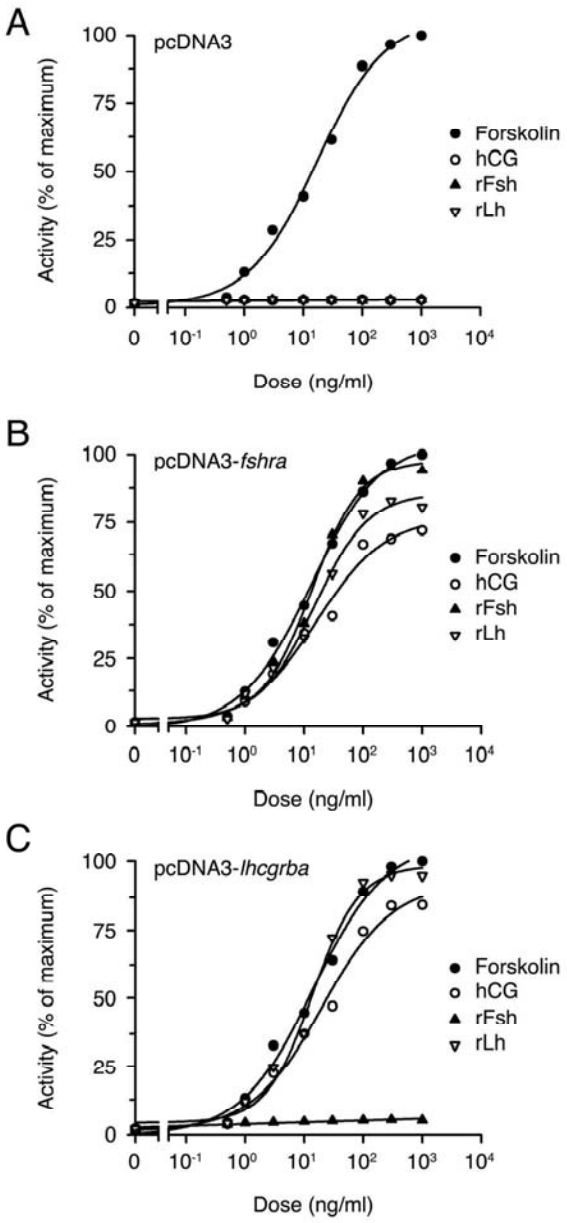

Fig. 9. Effect of seabass recombinant gonadotropins (rFsh and rLh), hCG, or forskolin, on HEK293T cells transiently transfected with reporter gene constructs alone (pcDNA3; A) or in combination with either seabream Fshra (pcDNA3-Fshra; B) or Lhcgrba (pcDNA3-Lhcgrba; C) expression plasmids. Hormone-induced cAMP production was indirectly quantified by measuring the luciferase activity from the reporter vector. The responses were normalized to the maximal activity induced by forskolin, which was set at 100%. Data are from one representative experiment and represent means of duplicates.

Changes in the synthesis of 17,20β-P, T and E2 by ovarian explants at the primary growth stage incubated with rFsh *in vitro* were subsequently determined (Fig. 10). The steroid levels were measured only in the ovarian tissue because the levels in the culture medium were in most cases below the detection limits (data not shown). Incubation with 10 and 100 ng/ml rFsh induced an increase in the ovarian content of 17,20β-P and T at 24 h, whereas the concentrations of these steroids in the explants were no different 48 or 72 h after rFsh stimulation (Fig. 10, A and B). In contrast, the ovarian concentration of E2 did not change until 48 h,

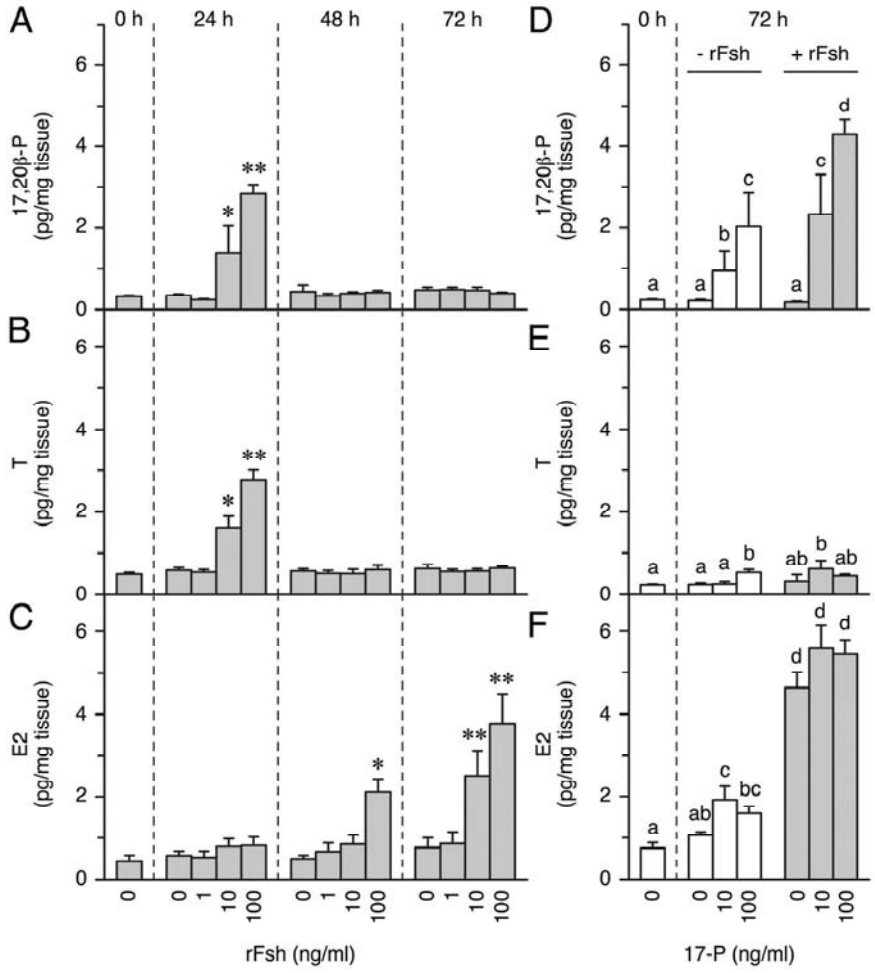

Fig. 10. Effect of piscine recombinant Fsh (rFsh) on steroid production by seabream primary ovarian explants, containing only oogonia and primary ovarian follicles, *in vitro*. (A) Time-course production of 17,20β-P, T and E2 in explants stimulated with increasing doses of seabass rFsh. (B) Production of 17,20β-P, T and E2 by explants incubated with different concentrations of 17-P (10 or 100 ng/ml), and in the presence (grey bars) or absence (white bars) of 100 ng/ml rFsh, after 72 h. In each panel, data (mean ± SEM, $n = 3$ independent experiments) with asteriks (A) or with different superscript (B) are statistically significant (Tukey's pairwise comparison test; $P < 0.05$).

where a significant increase was noted with 100 ng/ml rFsh, whereas at 72 h the rFsh doses of 10 and 100 ng/ml were both effective at stimulating E2 production (Fig. 10C).

To test if the short-term rFsh-induced production of 17,20β-P could be due to a limited concentration of endogenous 17-P precursor, explants were incubated with 100 ng/ml rFsh in the presence of 10 or 100 ng/ml 17-P for 72 h. When the explants were incubated with 17-P, the ovary content of 17,20β-P increased in a 17-P dose-dependant manner, and this stimulation was enhanced in the presence of rFsh (Fig. 10D). The addition of exogenous 17-P, however, stimulated only slightly the production of T regardless of the presence of rFsh (Fig. 10E).

In contrast, the synthesis of E2 was enhanced slightly by 17-P alone, whereas when 17-P was combined with rFsh the production of E2 was clearly increased (Fig. 10F). However, the ovarian content of E2 induced by rFsh was similar regardless of increasing doses of exogenous 17-P, suggesting that 17-P itself had very little effect on E2 synthesis (Fig. 10F).

To confirm the role of Cbr1 in the rFsh-mediated induction of 17,20β-P synthesis, explants were incubated with 17-P and rFsh, in the presence or absence of the Cbr1 inhibitors Indo and PhB, for 72 h (Fig. 11). Both compounds significantly reduced 17-P-induced 17,20β-P production with or without rFsh (Fig. 11A). When the inhibitors were present, the synthesis of T and E2 was significantly enhanced, and in the case of E2, the stimulatory effect was more prominent in the presence of 17-P and rFsh (Fig. 11, B and C).

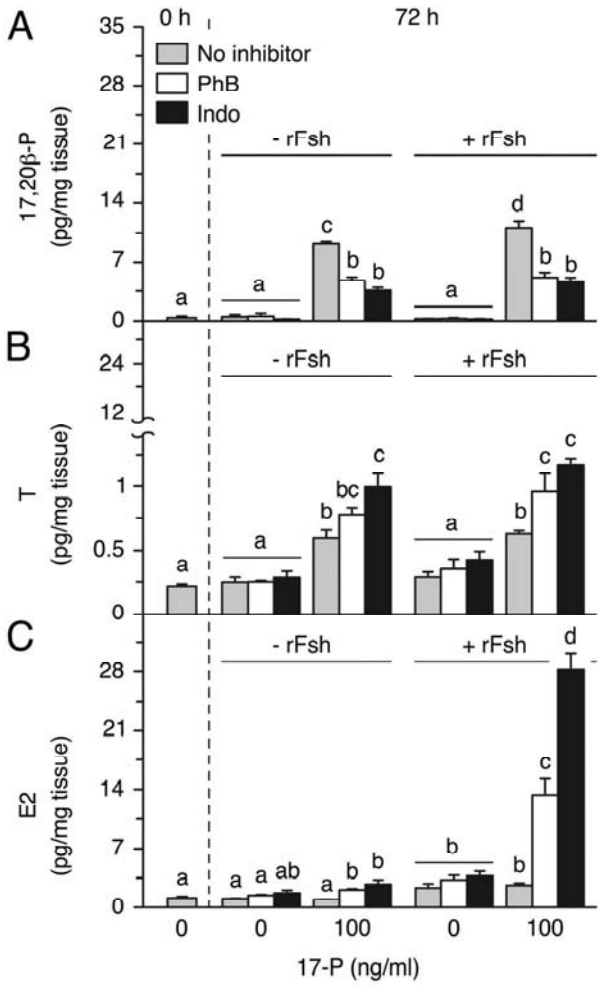

Fig. 11. Effect of carbonyl reductase inhibitors on rFsh-induced steroid production by seabream primary ovarian explants *in vitro*. Changes in the content of 17,20β-P, T and E2 at 72 h in ovarian explants treated with 100 ng/ml rFsh in the presence or absence of 100 ng/ml 17-P, and indomethacin (Indo) or phenylbutazone (PhB). Data (mean ± SEM, $n = 3$ separate experiments) with different superscript are statistically significant (Tukey's pairwise comparison test; $P < 0.05$).

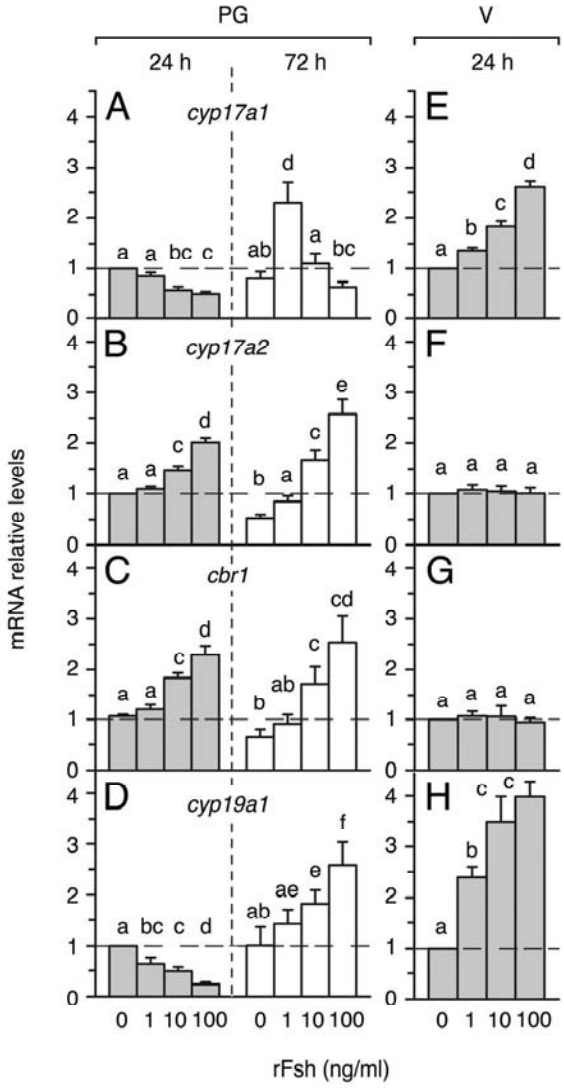

Fig. 12. *In vitro* effect of rFsh on *cyp17a1*, *cyp17a2*, *cyp19a1* and *cbr1* expression in seabream primary growth (PG) and vitellogenic (V) ovarian explants. Ovarian transcript levels were determined by qRT-PCR and normalized to *18s* ribosomal RNA expression after 24 and/or 72 h of stimulation with increasing doses of rFsh as indicated. Data are the mean ± SEM ($n = 6$ separate experiments for A-D; and n = 3 separate experiments for E-H). In each panel, bars with different superscript are statistically significant (Tukey's pairwise comparison test; $P < 0.05$).

3.7. rFsh Trancriptional Regulation of Steroidogenic Enzymes in Primary Growth and Vitellogenic Ovaries In Vitro

To investigate the molecular mechanisms underlying the action of rFsh on 17,20β-P synthesis in primary growth ovarian follicles, the expression levels of *cyp17a1*, *cyp17a2*, *cyp19a1* and *cbr1* were determined by qRT-PCR at 24 and 72 h after rFsh stimulation of primary ovarian explants *in vitro* (Fig. 12). When primary explants were stimulated with rFsh, the steady-state mRNA levels of *cyp17a1* and *cyp19a1* decreased at 24 h (Fig. 12, A and D). In contrast, expression of *cyp17a2* and *cbr1* in the explants significantly increased over time after rFsh stimulation (Fig. 12, B and C). At 72 h, however, the *cyp17a1* transcript levels

changed inversely with the rFsh dose, and those of *cyp19a1* were enhanced in a dose-response manner (Fig. 12, A and D).

Since in some teleosts it has been shown that Fsh can stimulate aromatase transcription and E2 production in vitellogenic ovarian follicles within an 18-h incubation period (i.e. Montserrat et al. 2004), we tested whether rFsh could affect the transcription of *cyp17a1* and *cyp19a1,* and also of *cyp17a2* and *cbr1*, in seabream vitellogenic ovarian explants after 24 h. These experiments confirmed that rFsh induced an increase of both *cyp17a1* and *cyp19a1* transcript levels in a dose-dependent manner (Fig. 12, E and H). However, within the same incubation period in the presence of rFsh, the expression levels of *cyp17a2* and *cbr1* remained unchanged (Fig. 12, F and G). Longer times of incubation of vitellogenic explants with rFsh could not be tested because, under the conditions employed, some vitellogenic follicles present in the explants, unlike the primary follicles, showed some signs of atresia (data not shown).

4. Discussion

In most teleosts, it is well established that Fsh controls follicular estrogen production in the ovary, whereas Lh triggers the synthesis of 17,20β-P (or in some species, 17,20β,21-P), which induces oocyte maturation (Nagahama and Yamashita 2008; Lubzens et al. 2010). The present study provides evidence for the production of 17,20β-P by gilthead seabream primary ovarian follicles in response to pìscine Fsh stimulation. The mechanism for the biosynthesis of 17,20β-P at this follicular stage is likely based on the up-regulation of the same steroidogenic pathways as those that have been reported to be stimulated by Lh in vitellogenic ovarian follicles of some other teleosts at the time of oocyte maturation (Senthilkumaran et al. 2004; Zhou et al. 2007; Nagahama and Yamashita 2008). These findings thus reinforce the notion, previously advanced by Miura and colleagues (Miura et al. 2006, 2007), that progestins also play a role during early ovarian development in teleosts.

The present ISH experiments demonstrated that in the seabream the key steroidogenic enzymes involved in both estrogen (i.e *cyp17a1* and *cyp19a1*) and progestin (i.e. *cyp17a2* and *cbr1*) synthesis are expressed in primordial granulosa cells associated with primary oocytes. The expression of Cbr1 in seabream previtellogenic follicles thus agrees with previous reports on other teleosts, such as the Atlantic cod (Kortner et al. 2009) and North African catfish (Sreenivasulu and Senthilkumaran 2009). In primordial theca cells, however, we only observed a weak expression of *cyp17a2* and *cbr1*. In contrast, in seabream vitellogenic follicles,

cyp17a1, *cyp17a2* and *cbr1* were expressed in both theca and granulosa cells, whereas *cyp19a1* transcripts were found only in granulosa cells, as noted in some other teleosts (Nagahama and Yamashita 2008; Lubzens et al. 2010). However, we also found *cyp17a2* and *cyp19a1* transcripts in the cytoplasm of seabream primary oocytes, whereas in the ooplasm of vitellogenic oocytes we detected *cyp17a1*, *cyp17a2*, as well as *cbr1* mRNAs. This latter observation is not completely surprising because, in some teleosts, mitochondrial and microsomal 20β-hydroxysteroid dehydrogenase activities have been detected in ovulated oocytes devoid of follicle cells (Kazeto et al. 2001). Also, *cyp17a1* and *cyp19a1* have been shown to be expressed and active in the cytoplasm of trout and amphibian vitellogenic oocytes (Yang et al. 2003; Gohin et al. 2011a,b).

During the reproductive cycle of the seabream, the changes in the plasma levels of estrogen and progestins were in agreement with previous reports on this species (Gothilf et al. 1997; Meiri et al. 2002; Wong et al. 2006). In general, the changes in the ovarian expression of steroidogenic enzymes followed a similar trend with the changes in steroid production. However, there were some differences with respect to that reported for other teleosts (Chang et al. 1997; Senthilkumaran et al. 2004; Nakamura et al. 2005; Zhou et al. 2007), such as the higher increase in *cyp17a1* and *cyp19a1* expression in the seabream ovary at the maturation stage. Although the levels of the corresponding proteins and enzyme activities at each ovarian stage were not determined, these differences may be caused by the group-synchronous nature of the seabream ovary and therefore the presence of vitellogenic follicles not recruited into maturation in ovaries at the maturation stage (Fig. 1). Also, in the case of *cyp19a1*, an additional confounding effect to interpret the expression data may be the absence of transcripts in the cytoplasm of vitellogenic oocytes, which can induce a dilution effect of the target mRNA in follicle cells due to the increased volume of these oocytes. Therefore, isolation of follicle cells, or the use of follicle-cell enriched fractions (Luckenbach et al. 2008), are possibly necessary to determine developmental changes in the expression of steroidogenic genes in follicle cells.

Nevertheless, we found that the high content of 17,20β-P in early primary growth ovaries was concomitant with an elevated *cbr1* expression. Interestingly, despite the very large number of studies on sex steroids in teleosts, very few reports have measured steroids in tissue (as opposed to plasma) extracts. Unlike the protein hormones of the pituitary gland, steroids are not stored in intracellular vesicles after their synthesis (Thomson 1998). It is assumed that most of the steroid would diffuse out of the cells as soon as it is synthesized and there would thus be little point in measuring tissue concentrations. However, it was only by

extracting the ovarian tissue that we were able to clearly establish that the elevated *cbr1* ovarian expression was matched by elevated 17,20β-P concentrations. Although plasma concentrations of 17,20β-P were slightly elevated in the same fish, they were little different from the plasma concentrations of E2 or 17,20β,21-P. In a previous study on Senegalese sole (*Solea senegalensis*), 17,20β-P was also easier to measure in extracts of the testis than in the plasma (Agulleiro et al. 2007), and similarly in the male sea lamprey (*Petromyzon marinus*) the putative androgen in this species, androstenedione, could only be measured in testis extracts and not in the plasma (Bryan et al. 2007). In the case of the sea lamprey, this phenomenon was attributed to the presence of a protein in the testis with a high binding capacity (as well as affinity) for androstenedione. The presence of binding activity for 17,20β-P in seabream primary oocytes might explain the present results, although this is presently no more than speculation. It is of interest, though, that specific binding activity for 17,20β-P has been demonstrated in mature testes of the North Sea plaice (*Pleuronectes platessa*) (Mugnier et al. 2000). If steroid binding proteins are the reason for the tendency of 17,20β-P to accumulate in the seabream gonad tissue, it will be interesting to know whether the binding activity is due to the presence of progestin receptor(s) or sex steroid binding globulins, both of which would have the ability to retain 17,20β-P in the ovary rather than allowing it to diffuse away.

The high content in 17,20β-P of seabream primary growth ovaries was coincident with a high ovarian expression of *fshra* but not of *lhcgrba*. Although the actual protein levels of both receptors in primary ovaries could not be determined, this first suggested to us that it might be Fshra that controls 17,20β-P synthesis at this stage. To investigate this hypothesis, we used available recombinant gonadotropins from another perciform teleost, i.e. the European seabass. The characterization of the response of seabream Fshra and Lhcgrba to seabass rFsh and rLh indicated, as found for some other teleosts (Levavi-Sivan et al. 2010; Chauvigné et al. 2012), that the Fshra could be activated by rFsh and rLh, whereas the Lhcgrba was rLh specific. The high promiscuity of the seabream Fshra to seabass rLh (which has to be confirmed using homologous hormones), obviated the use of rLh to discern the involvement of Fshra or Lhcgrba in 17,20β-P synthesis in primary ovarian explants *in vitro*. Therefore, we decided to test only rFsh, which is Fshra specific since it does not activate the seabream Lhcgrba even at pharmacological doses. These experiments showed that rFsh stimulated 17,20β-P production, which was sustained over the period of culture by the addition of 17-P precursor, whereas T and E2 production were relatively unaffected by 17-P. The rFsh-mediated synthesis of 17,20β-P over E2 was found to correlate with the up-regulation of

cyp17a2 and *cbr1* transcription, and the decrease of *cyp17a1* and *cyp19a1* mRNA levels. Therefore, these data strongly suggest the role of the Fshra in the activation of the Cyp17a2/Cbr1 pathway in seabream primary ovarian follicles. The mechanism of Fshra action is similar to the proposed action of Lh, likely via the Lhcgrba or Lhcgrbb, in postvitellogenic ovarian follicles of other teleosts at the time of induction of oocyte maturation and ovulation (Nagahama and Yamashita 2008; Lubzens et al. 2010; Senthilkumaran et al. 2004; Zhou et al. 2007; Tanaka et al. 2002). The specific ligand involved in this mechanism *in vivo* (i.e. whether it is Fsh or Lh), as well as its origin (either in the pituitary or secreted from developing oocytes; Wong and Zohar 2004), is however unclear from the present experiments, since the seabream Fshra can also be activated by rLh. Although tools for measuring of pituitary and circulating Fsh levels are unavailable for the gilthead seabream, previous studies have shown that the plasma levels of Lh in immature females with previtellogenic ovaries are much lower (~1 ng/ml) than those at maturation (~17 ng/ml) (Holland et al. 1998), which may point to the involvement of Fsh rather than Lh in the gonadotropic control of steroidogenesis in primary ovaries. However, without information on the levels of circulating Fsh during the reproductive cycle of the seabream, and detailed characterization of the response of seabream Fshra to homologous hormones, this conclusion may be premature.

In our *in vitro* experiments, the stimulation of primary explants with rFsh also induced a retarded increase in *cyp19a1* expression, which coincided with an increase in the concentration of E2 in the tissue. Surprisingly though, at the same time, the expression of *cyp17a1* was negatively correlated with the rFsh dose. To reconcile these and previous observations, including the prominent expression of *cyp19a1* in the cytoplasm of primary oocytes, we propose a model that summarizes our current hypothesis for the Fshra-mediated regulation of steroidogenic pathways in seabream primary ovarian follicles (Fig. 13). This model involves the activation of the Fshra in primordial follicle cells by Fsh (or Lh) which rapidly drives the up-regulation of *cyp17a2* and *cbr1* transcription, and the down-regulation of *cyp17a1* and *cyp19a1*, resulting in an enhanced production of 17,20β-P. The increase in T synthesis that we noted in our studies is probably due to induction of *hsd17b* expression, as suggested to occur in zebrafish vitellogenic ovarian follicles incubated with hCG (Ings and Van der Kraak 2006) or in FSH-stimulated human granulosa-luteal cells (Whitehead and Lacey 2003). This would trigger the rapid transformation of androstenedione (as formed by Cyp17a1) to T, which might then diffuse slowly into the oocytes where it may be aromatized to E2 by Cyp19a1 (Gohin et al. 2011a, b). The relatively late production of E2 after rFsh stimulation *in vitro* may

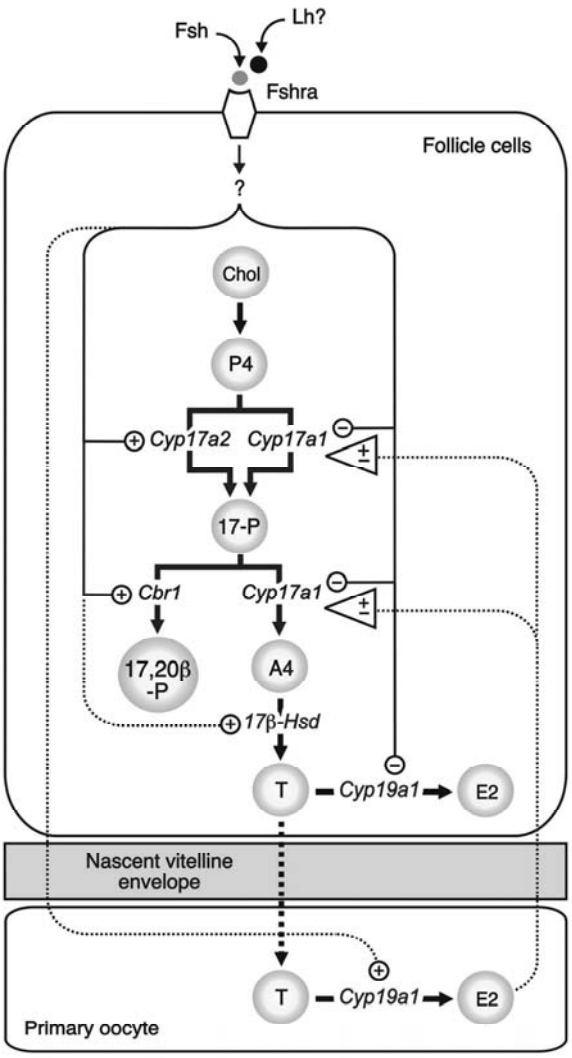

Fig. 13. Proposed model for the Fshra-mediated regulation of steroidogenesis in seabream primary growth ovarian follicles. Dashed lines represent potential pathways that need furher supporting evidence. Positive symbol indicates up-regulation, whereas negative symbol indicates down-regulation. The triangle with plus and minus indicates a possible negative feedback mechanism.

reflect the fact that *cyp19a1* transcription is initially downregulated, but then subsequently upregulated - perhaps stimulated by second messengers (i.e. cAMP) originating in follicle cells and translocated into the oocyte via heterologous gap junctions between granulosa cells and the oocyte (i.e. Cerdà et al. 1993; Selman et al. 1993). The E2 produced in the oocyte may in turn exert a negative feedback mechanism on follicular *cyp17a1* expression, since it is known that estrogen can regulate the steady-state mRNA levels of *cyp17a* in trout (Govoroun et al. 2001) and rat testis (Sakaue et al. 2002). Such potential dialog between primary oocytes and follicle cells to control steroid synthesis, similar to that suggested to occur in amphibian vitellogenic follicles (Sretarugsa and Wallace 1997; Yang et al. 2003) remains however to be demonstrated.

In complete contrast to the situation in primary ovarian explants, we noted that rFsh had no effect on *cyp17a2* and *cbr1* expression in vitellogenic ovarian explants within a 24-h period. Instead, only *cyp17a1* and *cyp19a1* rapidly increased in a dose-dependent manner, likely favouring estrogen synthesis. Although in the gilthead seabream it is not known whether Lh can be more effective than Fsh for E2 production in vitellogenic ovarian follicles, as it occurs in the protogynous red seabream (Gen et al. 2001; Kagawa et al. 2003), our observations would agree with the Fsh and cAMP activation of *cyp19a1* expression in vitellogenic follicles, leading to E2 production, reported in some other teleosts (Tan et al. 1986; Nakamura et al. 2003; Montserrat et al. 2004; Kamei et al. 2006). In addition, while it is not known if rFsh was able to stimulate *cyp17a2* and *cbr1* expression in vitellogenic explants in the long term (i.e. 72 h), these data suggest a potential switch in Fshra signalling in follicle cells during folliculogenesis from a short-term inhibition of *cyp17a1* and *cyp19a1* expression in primary follicles to its rapid activation in vitellogenic follicles. In mammals, putative oocyte-derived paracrine factors, such as bone morphogenetic proteins, have been suggested to modulate FSH-induced steroidogenesis in primary granulosa cells (Miyoshi et al. 2007). Therefore, whether this potential change in Fshra signalling in seabream vitellogenic follicles is induced by Lh, by changes in the Fshra and Lhcgrba expression levels, or by oocyte-derived factors, is unknown.

The physiological function of 17,20β-P in seabream primary ovarian follicles is as yet unknown. Recently, it has been suggested that 17,20β-P is the trigger of meiosis in teleost germ cells (Miura et al. 2006, 2007; Miura and Miura 2011). In the present study, rFsh stimulated 17,20β-P production in ovarian explants containing exclusively oogonia and primary growth follicles in which oocyte meiosis is already arrested in diplotene of the first meiotic division for the remainder of oocyte development (Selman et al. 1993). Therefore, it is unclear whether the 17,20β-P produced at this ovarian stage of the seabream is related to the activation of meiosis. In the zebrafish (Hanna et al. 2010), as well as in the gilthead seabream (Zapater et al. 2013, Chapter III), nuclear progestin receptors are persistent in the nucleus of late primary growth oocytes. In the mammalian ovary and endometrium, it is well known that the nuclear progesterone receptor is able to activate or suppress the transcription of a high number of genes, including those involved in primordial follicle assembly (Nilsson et al. 2006). Therefore, it is tempting to speculate that 17,20β-P may regulate gene transcription in early ovarian follicles of teleosts, perhaps allowing the storage in oocytes of mRNAs critical for later oogenesis and early embryogenesis, and/or the expression of other genes required for follicle formation. Such genes may include cell-adhesion molecules, connexins, vitellogenin receptor

isoforms, zona pellucida glycoproteins, cathepsins, CDC-like kinases or cyclins (Cerdà et al. 1999; Luckenbach et al. 2008; Tingaud-Sequeira et al. 2009; Yamamoto et al. 2011;), as well as maternal transcripts essential for the establishment of the dorsoventral axis in early embryos (Langdon and Mullins 2011).

In conclusion, our findings provide the first evidence in teleosts to our knowledge for progestin production in primary growth ovarian follicles in response to the Fshra activation. This mechanism appears to involve the up-regulation of the same steroidogenic enzymes (*cyp17a2* and *cbr1*) that have been described to be activated during the induction of oocyte maturation and ovulation by Lh in most teleosts. The present data thus challenge the established view that the Cyp17a2/Cbr1 steroidogenic pathway is specific to teleost oocyte maturation. However, seabream primary ovaries also express low levels of *lhcgrba*, which could be biologically relevant. In the present study, the specific role of this receptor could not be evaluated because the piscine rLh employed was not Lhcgrba specific. Therefore, an additional role of the Lhcgrba in the control of steroidogenesis in seabream early ovarian follicles can not be ruled out and should be investigated in the future.

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Chapter III

Alternative Splicing of the Nuclear Progestin Receptor in a Perciform Teleost Generates Novel Mechanisms of Dominant-Negative Transcriptional Regulation

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Abstract

In mammals, downstream function of the nuclear progestin receptor (PGR) can be differentially regulated in each target tissue by altering the expression levels of PGR mRNA variants. Such PGR isoforms have also been identified in birds and reptiles, but not in non-amniote vertebrates. Based upon extensive phylogenetic, syntenic and functional analyses, here we show that higher orders of Teleostei retain a single pgr gene, and that four different pgr transcript variants of the extant gene are expressed in the ovary of an evolutionary advanced perciform teleost, the gilthead seabream (*Sparus aurata*). Three of the isoforms (*pgr_tv2*, *pgr_tv3* and *pgr_tv4*) arise from alternative pre-mRNA splicing resulting in different N-terminally truncated receptors, whereas one isoform (*pgr_tv1*) is a deletion variant. Seabream wild-type Pgr shows the highest transactivational response to native euteleostean progestins, 17α,20β-dihydroxy-4-pregnen-3-one and 17α,20β,21-trihydroxy-4-pregnen-3-one, whereas the Pgr_tv3 and Pgr_tv4 isoforms independently regulate novel nuclear and cytosolic mechanisms of dominant-negative repression of Pgr-mediated transcription. In the seabream ovary, the wild-type Pgr protein is localized in oogonia, in the nuclei of primary (previtellogenic) oocytes, as well as in follicular (granulosa) cells and the oocyte cytoplasm of early and late vitellogenic ovarian follicles. Expression of wild-type *pgr*, *pgr_tv3* and *pgr_tv4* was the highest in seabream primary ovaries, while expression of both inhibitory receptor isoforms, but not of pgr, decreased during vitellogenesis. Stimulation of primary ovarian explants *in vitro* with recombinant piscine follicle-stimulating hormone and estrogen differentially regulated the temporal expression of *pgr*, *pgr_tv3* and *pgr_tv4*. These findings suggest that, as in mammals, ovarian progestin responsiveness in the seabream, particularly during early oogenesis, may be regulated through alternative splicing of the nuclear *pgr* mRNA. Thus, the dominant-negative mechanism of PGR transcriptional regulation likely evolved prior to the separation of Actinopterygii (ray-finned fishes) from Sarcopterygii (lobe-finned fishes).

Key words: Teleost, Nuclear progestin receptor, Ovary, Isoform, Dominant-negative inhibition, FSH, Estrogen

1. Introduction

Progestins play important roles in vertebrate reproduction through their ligand-activation of specific cognate receptors. As for other steroid receptors, two main classes of receptor have been reported for progestins, belonging either to the nuclear receptor superfamily or to the membrane-associated receptor family (Falkenstein et al. 2000; Aranda and Pascual 2001; Thomas 2012). The tetrapod nuclear progestin receptor (PGR) is a progesterone (P4)-activated transcription factor (Aranda and Pascual 2001; Wang et al. 2004; Katsu et al. 2008), which binds to progesterone response elements (PRE) on the promoter regions of genes resulting in changes in gene transcription (Hill et al. 2012). Teleost Pgrs, however, have higher ligand affinities for hydroxylated progestins, such as 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) or 17α,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P) (Pinter and Thomas, 1995, 1997; Todo et al. 2000; Ikeuchi et al. 2002; Hanna et al. 2010; Chen et al. 2010, 2011, 2012), which are the major progestins involved in teleost gametogenesis (Nagahama and Yamashita 2008; Scott et al. 2010). In contrast, membrane-associated Pgrs are seven transmembrane G-protein coupled receptors that mediate rapid non-genomic actions of progestins through the activation of intracellular second messenger pathways (Thomas 2012).

The primary structure of the nuclear PGR is characterized by a variable N-terminus or A/B region, bearing the ligand-independent transcriptional activation domains AF-1 and AF-3, a highly conserved DNA binding domain (DBD), the hinge or D region responsible for the nuclear localization signal, and a moderately conserved ligand binding domain (LBD) towards the C terminus, which contains the ligand-dependent AF-2 core transactivation domain and mediates receptor homo- and heterodimerization (Aranda and Pascual 2001; Cork et al. 2008; Hill et al. 2012). In mammals and birds, it is well established that the PGR is expressed as two major isoforms, PGR-A and PGR-B, which arise from the same gene by utilization of two estrogen sensitive promoters (Conneely et al. 1987; Kastner et al. 1990; Li and O'Malley 2003). The two receptor isoforms are identical in the C-terminal LBD and DBD and most of the N-terminal domain except for an extension of 164 amino acids unique to PGR-B, termed the B-upstream segment (BUS). For most target genes, PGR-B is a stronger transcriptional activator than PGR-A, while PGR-A can act as a dominant repressor of PGR-B and other nuclear receptors (Tung et al. 1993; Vegeto et al. 1993). Selective gene ablation studies in mice and expression profiling data indicate that PGR-A and PGR-B regulate different gene subsets, with PGR-A being indispensable for ovarian and uterine functions, and PGR-B for mammary gland development (Mulac-Jericevic and Conneely 2004; Jacobsen and Horwitz 2012). Another additional

N-terminally truncated isoform lacking the A/B region and a full DBD, termed PGR-C, has been identified, that is capable of forming heterodimers and modulating the activity of PGR-A and PGR-B *in vitro* (Wei et al. 1990, 1997; Condon et al. 2006). Studies on breast cancer cells, endometrial tissue, and testis have further led to the identification of a number of additional PGR mRNA splice variants and new leader exons (i.e. PGR-M, PGR-S, PGR-T, PGR-i45, PGRΔ2, PGRΔ2+3) by cDNA library screening and reverse transcription (RT)-PCR (Richer et al. 1998; Hisatomi et al. 2003; Marshburn et al. 2005; Cork et al. 2008; Springwald et al. 2010). It has been thus suggested that tissue-specific variations in the expression levels of PGR splice variants may regulate the downstream physiological functions of the mammalian PGR (Cork et al. 2008). However, it has been found that antibodies raised against full-length PGR are not capable of specifically detecting N-terminally truncated isoforms (Samalecos and Gellersen 2008; Pang and Thomas 2011), and that PGR-T, PGR-S, and PGR-M cDNAs fail to yield detectable translation products (Samalecos et al. 2008). Therefore, the physiological significance of some of the mammalian PGR isoforms described to date remains unclear.

In other non-mammalian vertebrates, the PGR-A, PGR-B and PGR-C isoforms have only been characterized in reptiles (Custodia-Lora and Callard 2002; Katsu et al. 2008). In amphibians, such as *Xenopus laevis*, two different *pgr* cDNAs have been isolated (Tian et al. 2000), but each encodes a PGR polypeptide that shows overlapping, non-identical (<80%) A/B regions. Since *X. laevis* is a tetraploid organism (Hughes and Hughes 1993), these isolated cDNAs likely represent the products of duplicated genes rather than different PGR variants. Piscine Pgrs have also been identified in Chondrichthyes, including the little skate (*Leucoraja erinacea*) (Bridgham et al. 2006) and some basal orders of teleosts belonging to Anguilliformes, Cypriniformes, Salmoniformes and Gadiformes, such as the Japanese eel (*Anguilla japonica*), zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*), and Atlantic cod (*Gadus morhua*), respectively (Todo et al. 2000; Ikeuchi et al. 2002; Hanna et al. 2010; Chen et al. 2010, 2011, 2012). In addition, an ancestral Pgr-like receptor with a putative ligand-binding preference for 15α-hydroxyprogesterone (Bryan et al. 2008) has been identified in the sea lamprey (*Petromyzon marinus*) (Thornton 2001). With the exception of *X. laevis* and the Japanese eel, in which two related *pgr* genes, *pgr1* and *pgr2*, encoding proteins 55.2% identical at the amino acid sequence level, have also been described (Todo et al. 2000; Ikeuchi et al. 2002), only a single *pgr* gene has been reported in the majority of vertebrates studied to date (Hanna et al. 2010; Baker and Uh 2012). Considering that it is now well established that a fish-specific whole genome duplication occurred after separation of the Teleostei from the

Neopterygii (Amores et al. 2011), it remains unclear whether duplicated *pgr* genes exist throughout the teleost crown-clade.

The expression of nuclear *pgr* transcript variants arising from alternative promoter usage or mRNA splicing has also not been reported for any vertebrate other than mammals, birds and reptiles, suggesting that such variants could be limited to the Amniota. In contrast, the existence of nuclear estrogen receptor isoforms in both sarcopterygian and actinopterygian vertebrates is well established (Pakdel et al. 2000; Patiño et al. 2000; Wu et al. 2003; Caviola et al. 2007; Iwabuchi et al. 2008; Pinto et al. 2012), although in most cases their structure and function remain to be elucidated. It is therefore unknown whether the regulation of Pgr function by Pgr isoforms is an amniote-specific mechanism or may be conserved in distantly related actinopterygian vertebrates. In the present work, we aimed to clarify the multiplicity of the teleost *nr3c* subfamily of nuclear receptors, and to identify and functionally characterize mRNA splice variants of the nuclear *pgr* in the gilthead seabream (*Sparus aurata*). Through extensive phylogenetic and syntenic analyses, we show that a single *pgr* gene exists in higher orders of Teleostei, and that four *pgr* isoforms are expressed in the ovary of an evolutionary advanced perciform teleost. Two of these isoforms are differentially expressed in the ovary in response to gonadotropin and estrogen stimulation, and can generate distinct mechanisms of dominant-negative repression of Pgr-mediated transcription.

2. Materials and Methods

2.1. Animals

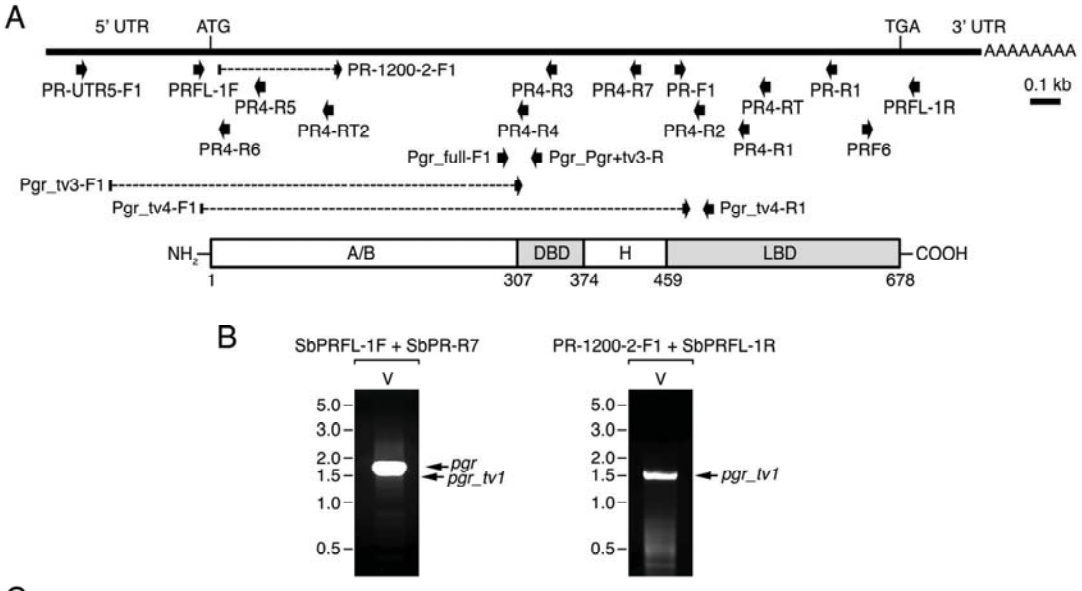
Adult gilthead seabream were obtained from a commercial farm and maintained as described previously (Zapater et al. 2012). Females were sacrificed at times that corresponded to different ovarian stages determined by histological examination of the gonads as previously described (Zapater et al. 2012). At all sampling times, fish were sedated with 500 ppm of phenoxyethanol and killed by decapitation. The gonadosomatic index (GSI) was determined according to the formula: gonad weight (g)/fish weight (g) x 100. Ovarian samples were taken from each fish, processed for immunocytochemistry, or frozen in liquid nitrogen and stored at -80°C. Additional pieces of the gonad were processed for *in vitro* culture. Procedures relating to the care and use of animals were approved by the Ethics Committee from Institut de Recerca i Tecnologia Agroalimentàries (IRTA, Spain) in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

2.2. Cloning of Seabream *pgr* cDNA and Splice Variants

Total RNA was extracted from the ovary using the RNeasy mini kit (Qiagen) and treated with DNase using the RNase-Free DNase kit (Qiagen) following the manufacturer's instructions. An aliquot of 10 µg of total RNA was reverse transcribed using 20 IU of SuperScript II RT (Life Technologies Corp.), 0.5 µM oligo(dT)$_{12-18}$, 40 IU of RNase out and 1 mM dNTPs for 1.5 h at 42°C. The PCR was carried out with 1 µl of the RT reaction in a volume of 50 µl containing 5 µl of PCR buffer plus Mg^{2+}, 0.2 mM dNTPs, 1 µM of each degenerated forward and reverse oligonucleotide primers, and 1 IU of Taq polymerase (Roche). The sequence of the primers is indicated in Fig. 1. Reactions were amplified using one cycle of 95°C for 5 min, then 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by a final 7-min elongation at 72°C. The products were cloned into the pGEM-T Easy vector (Promega) and sequenced by BigDye Terminator Version 3.1 cycle sequencing on ABI PRISM 377 DNA analyzer (Applied Biosystems). The 5' and 3' ends of the *pgr* cDNA were amplified by three and one consecutive rounds, respectively, of rapid amplification of cDNA ends (RACE; Life Technologies Corp.) using specific primers (Fig. 1). The full-length cDNA was finally amplified using a high fidelity polymerase (Easy-A™ High-Fidelity PCR Cloning Enzyme; Stratagene) and sequenced. To clone the full-length cDNAs of the different transcript variants of the seabream *pgr* (*pgr_tv2*, *pgr_tv3* and *pgr_tv4*), 5 µg of total RNA from the ovary was reverse transcribed as described above and the cDNAs ampified using oligonucleotide primers located in the 5' and 3' UTR of the full-length *pgr* (Fig. 1). The PCR products were cloned into the pGEM-T Easy vector and sequenced as described. The full-length nucleotide sequence of *pgr_tv1* was assembled *in silico* using overlapping partial cDNAs (Fig. 1B). The nucleotide sequences of the seabream *pgr* and its splice variants were submitted to GenBank under accession numbers JQ692980 (*pgr*), JQ804918 (*pgr_tv1*), JQ804919 (*pgr_tv2*), JQ804920 (*pgr_tv3*), and JQ804921(*pgr_tv4*).

2.3. Phylogenetic and Syntenic Analyses

Vertebrate *nr3c* orthologs were retrieved from public databases (GenBank and ensembl v68, see Annex 1 for accession numbers) and the deduced amino acid sequences aligned with the default t-coffee v9.01 (Notredame et al. 2000) and L-INS-I MAFFT v6.903b (Katoh and Toh 2008) algorithms. These core alignments were compared and converted to codon alignments using Pal2Nal (Suyama et al. 2006) and subsequently manually adjusted to account for errors as described previously (Finn and Kristoffersen 2007; Chauvigné et al. 2010). The interim



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Oligo name	Sequence	Purpose
PR-F1	5'-TGGTCMAARTCTYTNCCAGGKTTTC-3'	Amplification of fragment, 3'-RACE
PR-R1	5'-TCCATSAGYTTGGTSAGRTGGTAGA-3'	Amplification of fragment
SbPR4-RT	5'-CTGTGTTGAGGAGAATTATG-3'	5'-RACE
SbPR4-R1	5'-CAGGCAGAGGTCGTAAATCG-3'	5'-RACE
SbPR4-R2	5'-TGGATGAGGGTCATCTGGTC-3'	5'-RACE
SbPR4-R3	5'-GCCCAGCGCAGAGATAACTA-3'	5'-RACE
SbPR4-R4	5'-CGTCACCACAGATCATGCAC-3'	5'-RACE
SbPR4-RT2	5'-TTCTCATCTTTGATGAACTTA-3'	5'-RACE
SbPR4-R5	5'-GCTTGAATTCAGACCGTGGA-3'	5'-RACE
SbPR4-R6	5'-GCTTCGATCAAGTCGCTCAC-3'	5'-RACE
SbPR-R7	5'- GTACACGACCTCCGGTTCAA-3'	Amplification 5' of *pgr_tv1*
SbPRFL-1F	5'-AAGCCACGCACTCTCTGCT-3'	Amplification full-length of *pgr*
SbPRFL-1R	5'-TCCAGTCACAGGACGTCTCC-3'	Amplification *pgr* and transcript variants full-length cDNAs, qPCR total *pgr*
PR-1200-2-F1	5'- CGAAGCTGGCTTTGATCCGTC-3'	Amplification 3' of *pgr_tv1*
SbPR-F6	5'-TGTCTGACCACCTTCATCCA-3'	qPCR total *pgr*
SbPR-UTR5-F1	5'-CCACCCGAACAAGGAAATAC-3'	Amplification *pgr* and transcript variants full-length cDNAs
Pgr_full-F1	5'-CCATCACCCTTCTCTGCATT-3'	qPCR for *pgr*
Pgr_Pgr+tv3-R	5'-ACTATGGTGGCCTTCGACTG-3'	qPCR for *pgr* and *pgr_tv3*
Pgr_tv3-F1	5'-AATATGCAAGTGTGCCACCT-3'	qPCR for *pgr_tv3*
Pgr_tv4-F1	5'-TGCTGTGCGCATCTCCTGCT-3'	qPCR for *pgr_tv4*
Pgr_tv4-R1	5'-CTGGATGAGGGTCATCTGGT-3'	qPCR for *pgr_tv4*

Fig. 1. (A) Schematic drawing of the gilthead seabream full-length *pgr* mRNA showing the location of the oligonucleotide primers used for cloning, RT-PCR and qRT-PCR. Primers flanking splicing sites are indicated with dotted lines. The structural regions of the encoded protein (below), A/B region, DNA binding domain (DBD), cellular localization signal or hinge domain (H), and ligand binding domain (LBD), are boxed. (B) Representative gel images of RT-PCR on vitellogenic ovaries using the primers indicated above each gel to clone partial cDNA of the *pgr_tv1*. (C) Primer sequences used in cloning and qRT-PCR.

Bayesian analyses were performed to establish the putative phylogeny, and the adjusted amino acid alignment re-arranged hierarchically to match the preliminary tree topology. To establish whether gene duplications were specific to the teleost crown-clade, this latter alignment was then used to identify Nr3c protein fragments via the tblastn algorithm (Altschul et al. 1990) in the NCBI curated genomes (www.ncbi.nlm.nih.gov) of the chondrichthyan ghost shark (*Callorhinchus milii*), basal Sarcopterygii including the coelacanth (*Latimeria chalumnae*) and Chinese softshell turtle (*Pelodiscus sinensis*), the basal actinopterygian spotted gar (*Lepisosteus oculatus*), and the teleosts Atlantic salmon (*Salmo salar*) and European seabass (*Dicentrarchus labrax*). Contiguous nucleotide sequences were retrieved and trimmed to match each protein fragment, and finally concatenated to construct a putative cDNA for each gene. Deduced amino acid sequences from the putative cDNAs were manually incorporated into a final alignment, which was converted to a codon alignment using Pal2Nal. After removal of the poorly conserved N-termini, molecular phylogenies of the concatenated DBD, hinge and LBD domains of vertebrate *nr3c* codon and amino acid alignments were inferred using Bayesian (Mr Bayes v3.2.0 [Ronquist and Huelsenbeck 2003]; with 10 million generations) and maximum likelihood (ML: PAUP v4b10-x86-macosx [Swafford 2002]) methods as described previously (Finn and Kristoffersen 2007; Applebaum et al. 2012; Zapater et al. 2011). To test for alternative topologies, a ML analysis of the amino acid alignment was performed using the quartet-puzzling algorithm implemented in Tree-Puzzle 5.2 (Schmidt et al. 2002) and maximum parsimony and neighbour joining methods with 1000 bootstraps via PAUP. The trees were rooted using the yellow fever mosquito (*Aedes aegypti*) Nr2f ortholog sevenup (Svp) as the outgroup. To verify the topology of the Pgr orthologs, an additional molecular phylogeny of the full-length Pgr amino acid sequences was constructed using ML as implemented in RAxML v.7.2.8 (Stamakis et al. 2008) with the BLOSUM62 amino acid substitution matrix. This latter tree was rooted with the putative Pgr (steroid receptor, SR) of the sea lamprey (*Petromyzon marinus*). All trees generated were visualized and edited with either the Archeopteryx v0.972 (www.phylosoft.org/archaeopteryx/), Geneious® Pro v5.6.5 (Biomatters Ltd) or FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) software packages. To evaluate whether duplicated *pgr* genes existed in Teleostei, syntenic analyses of the available genomic loci were compared to selected tetrapod loci using the human *PGR* as the search gene, and the alignment view of Genomicus v68.01 (www.dyogen.ens.fr/genomicus-68.01).

2.4. Antibody Production

A polyclonal antiserum was raised in rabbits against a synthetic peptide corresponding to amino acid residues 170-183 (A/B region) of seabream Pgr with the predicted initiation codon (methionine, ATG) designated as residue 1 (Agrisera AB, Sweden). The antiserum was affinity purified against the synthetic peptide as described previously (Zapater et al. 2011).

2.5. Transactivation Assays for Seabream Pgr and Transcript Variants

Human embryonic kidney (HEK) 293T cells were used to express the seabream Pgr, Pgr_tv2, Pgr_tv3 and Pgr_tv4 proteins. Transactivation assays were performed as described previously (Chen et al. 2010) with some modifications. Cells were seeded in 24 well plate (~4 x 10^5 cells per well) in Dulbecco modified Eagle medium (DMEM; Life Technologies Corp.) supplemented with 10% v/v fetal bovine serum (FBS), 260 U/ml of penicillin and streptomycin and 2 mM L-glutamine, at 37°C in a 5% CO_2 incubator. After 24 h, the cells were transfected using a standard calcium phosphate precipitation method with 100 ng of *pgr*, *pgr_tv2*, *pgr_tv3* or *pgr_tv4* expression plasmids or 100 ng of empty pcDNA3 (control cells), 50 ng of a mouse mammary tumor virus (MMTV) luciferase reporter construct (either pGL3-MMTV-Luc [Chen et al. 2010] or MMTV-LTR-Luc [Beato et al. 1989; Tung et al. 1993]), and 50 ng of β-Galactosidase (β-Gal) plasmid (Promega Corp.) to normalize transfection efficiency. To test the effect of the co-expression of Pgr and each of the transcript variants, cells were transfected with 100 ng of the *pgr* expression plasmid and different amounts of the *pgr_tv2*, *pgr_tv3* or *pgr_tv4* plasmids (5.3, 11.1, 33 or 100 ng). Different amounts of the empty vector pcDNA3 were cotransfected to use equal amounts of total DNA in the individual transfections. After 24 h, the medium was replaced by DMEM/F-12 without phenol red (Life Technologies Corp.), supplemented with 5% v/v charcoal-stripped FBS, 260 U/ml of penicillin and streptomycin, containing different steroids with final concentrations ranging between 10^{-10} M and 10^{-5} M (n = 3 per condition tested). The steroids tested (purchased from Sigma-Aldrich) were: 17,20β-P, 17,20β,21-P, P4, 17α-hydroxyprogesterone (17-P), 17β-estradiol (E2), 17,21-dihydroxypregn-4-ene-3,20-dione (17,21-P), testosterone (T) and cortisol (C). Final concentration of the ethanol vehicle was 0.1%. In other experiments, cells were stimulated with 10 μM 17,20β-P in the presence or absence of 10 μM of the mammalian PGR antagonist RU486 (Sigma-Aldrich). After incubation at 37°C for 24-36 h, the cells were harvested in Reporter Lysis buffer (Promega), incubated at -80°C for 2-3 h, vortexed, centrifugated at maximum speed during 1 min, and the supernatant collected. Luciferase activity was determined by add-

ing 150 µl of reconstituted Luciferin (BioThema) to 20 µl of cell lysates in a 96-well plate, and luminescence was measured with a Orion II microplate luminometer (Titertek-Berthold). Luciferase activity was normalized to β-Gal activity measured by colorimetric detection using nitrophenyl βD-galactopyranoside (Sigma-Aldrich) substrate. Reporter activity was expressed as the ratio of normalized luciferase activity to that of control cells. Each treatment was tested in at least three independent experiments using cells from different transfections.

2.6. *In vitro* Culture of Ovarian Explants

Subsamples of ovaries at the primary growth stage, containing only oogonia and primary ovarian follicles not showing nascent cortical alveoli, were placed in Petri dishes with 75% Leivovitz L-15 culture medium with L-glutamine (Sigma-Aldrich) and with 100 µg/ml gentamicine at pH 7.5. Ovaries were manually dissected into small fragments and placed in 24-well plastic tissue culture dishes containing 1 ml of fresh culture medium. For each experiment, explants from one female were incubated in triplicate with European seabass (*Dicentrarchus labrax*) single-chain recombinant follicle-stimulating hormone (rFsh; 1-100 ng/ml) or with E2 (10 or 100 ng/ml). The methods for the production of seabass rFsh and recombinant luteinizing hormone (rLh), and their characterization on seabream gonadotropin receptors, have been published elsewhere (Zapater et al. 2012). Explants were cultured at 18ºC in a temperature-controlled incubator up to 72 h. Every 24 h, ovarian fragments were harvested, frozen in liquid nitrogen and stored at -80°C until analysis. In some experiments, the E2 was replaced daily in the medium.

2.7. Gene Expression Analyses

The levels of total *pgr* transcripts, as well as of *pgr*, *pgr_tv3* and *pgr_tv4*, were determined by real-time quantitative RT-PCR (qRT-PCR) using SYBR green qPCR master mix (Life Technologies Corp.) as previously described (Zapater et al. 2012). For total *pgr*, primers flanked the stop codon of the seabream *pgr* cDNA. To specifically amplify the *pgr*, *pgr_tv3* or *pgr_tv4* transcript variants, forward primers in the A/B region (*pgr*) or across the splicing sites (*pgr_tv3* and *pgr_tv4*) and reverse primers in the retained domains were employed (Supplementary Fig. S1, A and C). In all cases, the reference gene was *18s* ribosomal RNA, for which the expression levels did not change significantly between experimental samples (data not shown), using the forward and reverse primers 5'-GAATTGACGGAAGGGCACCACCAG-3' and 5'-ACTAAGAACGGCCATGCACCACCAC-3', respectively. The relative total *pgr* transcript

level in different tissues of adult fish and in ovaries at different developmental stages during the reproductive cycle was calculated by using a standard curve generated for each primer pair from 10-fold serial dilutions of a pool of first-stranded cDNA template from ovary samples. All calibration curves exhibited correlation coefficients higher than 0.98, and the corresponding qRT-PCR efficiencies were greater than 99%. The changes in expression levels of *pgr*, *pgr_tv3* and *pgr_tv4* transcript variants in primary ovarian explants treated with hormones for 24 or 72 h *in vitro* were determined as fold change with respect the 24 h non-treated controls using the $2^{-\Delta\Delta Ct}$ method as previously described (Zapater et al. 2012).

The relative abundance of the *pgr_tv2*, *pgr_tv3* and *pgr_tv4* transcript variants with respect to that of the *pgr* in ovaries at different developmental stages was estimated by standard RT-PCR on 5 µg total RNA. For this analysis, forward and reverse primers were in the 5' and 3' UTR regions of the *pgr* cDNA, respectively, that were conserved in all the variants (Fig. 1, A and C). This approach successfully amplified the *pgr*, *pgr_tv2*, *pgr_tv3* and *pgr_tv4* transcript variants in one reaction. The PCR products were electrophoresed in 1% agarose gels and the intensity of bands corresponding to each transcript variant was determined by densitometry using the Quantity-One software (Bio-Rad Laboratories).

2.8. *In situ* Hybridization

For *in situ* hybridization, subsamples of ovaries were fixed in 4% paraformaldehyde (PFA) for 16-20 h at 4°C, then dehydrated and embedded in Paraplast (Sigma-Aldrich). *In situ* hybridization on 7-µm sections was carried out with digoxigenin-alkaline phosphatase (DIG-AP) incorporated cRNA probes as previously described (Zapater et al. 2012). DIG-AP riboprobes were synthesized with T3 and T7 RNA polymerases from the *pgr* cDNA using the DIG RNA Labeling Kit (Roche). Two different probes were synthesized, one corresponding to nucleotides 1429 to 2331 and other corresponding to nucleotides -474 to 201. The DIG-labeled probes were detected as previously described (Zapater et al. 2012), where the resulting dark blue to purple color indicated localization of the transcripts. Sections were examined and photographed with a Zeiss imager.z1 microscope (Carl Zeiss MicroImaging, S.L).

2.9. Immunofluorescence Microscopy

HEK293T cells were transfected with Flag-tagged or untagged seabream Pgr, and equimolar amounts of Flag-tagged Pgr_tv3 or Pgr_tv4, using lipofectamine (Life Technologies Corp.). Cells were grown on coverslips in 12-well plates in medium DMEM/F-12 (1:1) sup-

plemented with 5% v/v charcoal-stripped FBS and 2.6% penicillin/streptomicin for 30h, and subsequently exposed to 0.1 μM of 17,20β-P or ethanol for 1 h. After ethanol or steroid stimulation, cells were fixed with 4% PFA for 15 min at room temperature. The coverslips were washed with PBS, blocked with 3% non-fat milk powder in PBS, and incubated with 1:1000 of anti-FLAG antibody and anti-Pgr antiserum in PBS overnight at 4°C. After washing with PBS, sections were incubated with goat anti-mouse TRITC (Sigma-Aldrich; 1:500) or sheep anti-rabbit FITC (Sigma-Aldrich; 1:500) secondary antibodies in PBS for 1 h at room temperature. Cells were washed again with PBS, and incubated for 3 min with 4',6-diamidino-2-phenylindole (1:3000; DAPI; Sigma-Aldrich). Sections were mounted with Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich), and immunofluorescence was observed and documented as above.

2.10. Western Blot Analysis

Ovarian fragments at different developmental stages and adult tissues (testis and gills), or HEK293T transfected cells, were homogenized in 50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 1 mM PMSF, and protease inhibitors (EDTA-free Protease Inhibitor Cocktail Tablets, Roche), and centrifuged at 14000 x g for 10 min at 4ºC. One aliquot of the supernatant was removed to determine the protein concentration with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories Inc.), and the rest mixed with 4 x Laemmli sample buffer. For immunoblotting, 15 or 30 μg of total protein were denatured at 95ºC for 10 min, subjected to 8.5% SDS-PAGE, and transferred onto nitrocellulose membranes as described previously (Zapater et al. 2011). Membranes were incubated with anti-seabream Pgr antiserum (1:600) or anti-FLAG® M2 monoclonal antibody (1:400; Sigma-Aldrich) overnight at 4 ºC. Control membranes were incubated with the Pgr antiserum pre-adsorbed with a 30-fold molar excess of the immunizing peptide. Bound antibodies were detected with 1:2000-diluted goat anti-rabbit IgG or anti-mouse IgG (Rockland) coupled to horseradish peroxidase (HRP), and proteins were visualized by using enhanced chemiluminescence (Pico-max, Rockland). For semi-quantitative determination of Pgr abundance in primary ovarian explants after hormone treatment *in vitro*, triplicate protein samples were loaded in duplicate membranes and incubated with either anti-Pgr antiserum or anti-alpha-tubulin rabbit antibody (0.3 μg/ml; Abcam ab18251). The corresponding reactive bands were analyzed by densitometry as indicated above.

2.11. Immunocytochemistry

For immunohistochemistry, ovarian fragments were fixed with 4% PFA for 6 h at room temperature. Sections of approximately 6 µm in thickness were blocked with 5% goat serum 0.1% BSA in PBST (0.1% Tween in PBS) for 1 h, and incubated with the anti-seabream Pgr antibody (1:400) in PBST with 1% goat serum and 0.1% BSA overnight at 4°C. After washing with PBST, sections were incubated with a secondary anti-rabbit IgG HRP-coupled antibody (Rockland) for 2 h, and specific staining was revealed with diaminobenzidine (DAB). Sections were counterstained with hematoxylin, dehydrated, and mounted in Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich). The images were documented and photographed as described above.

2.12. Statistical Analyses

Data are the mean ± SEM and were statistically analyzed using one- or two-way analysis of variance (ANOVA), or by Kruskal-Wallis one way ANOVA on Ranks, followed by Tukey's pairwise comparison. P value < 0.05 was considered statistically significant.

3. Results

3.1. Isolation of Seabream Nuclear *pgr* cDNA and Phylogenetic Analysis of *nrc3* Genes

A full-length cDNA encoding the seabream nuclear Pgr was isolated from the ovary by RT-PCR followed by 5' and 3' RACE (Fig. 2). The cDNA was 2785 bp long with an open reading frame (ORF) of 2037 bp, and encoded a protein of 678 amino acids with a calculated molecular mass of 75.3 kDa. The deduced amino acid sequence of the seabream Pgr showed the four characteristic subdomains for the nuclear receptor superfamily (A/B region, DBD, hinge and LBD), and shared high sequence homology with that of other perciform teleost Pgrs (74-85% amino acid identity).

The extended phylogenetic analysis of the seabream Pgr in relation to 267 chordate *nr3c* sequences revealed distinct subclusters for the *nr3c1*, *-2*, *-3* and *-4* transcripts and proteins with robust separation (≥99% Bayesian posterior probabilities) of actinopterygian and sarcopterygian sequences (Fig. 3A; see Annex 2 for the annotated trees). Each method of phylogenetic inference showed dual paralogous subclusters of the teleost *nr3c1* (glucocorticoid receptors, *gra* and *grb*) and *nr3c4* (androgen receptors, *ara* and *arb*) in relation to the chondrichthyan (sharks and rays), basal actinoptergygian (sturgeons, Chondrostei and spotted

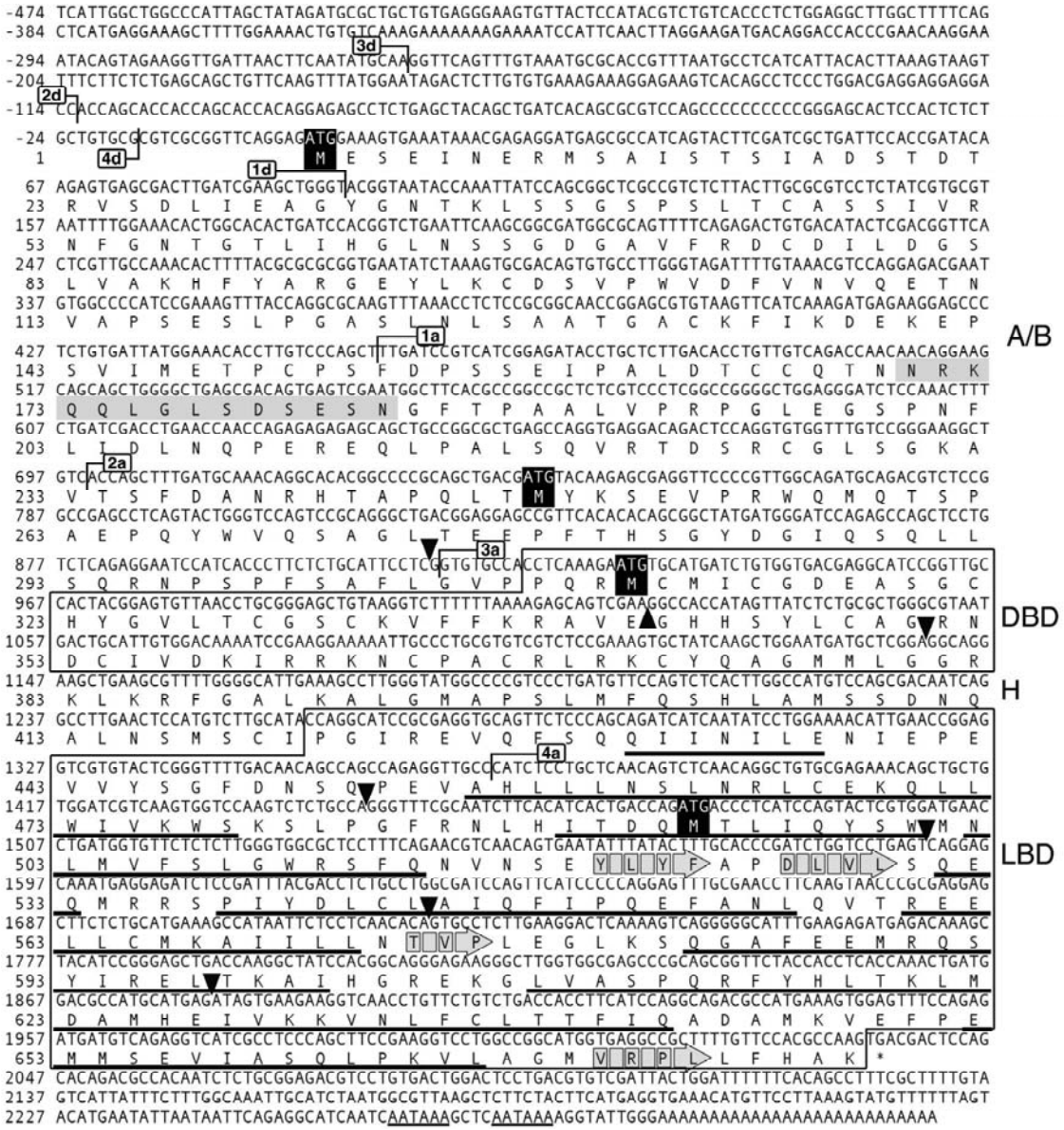
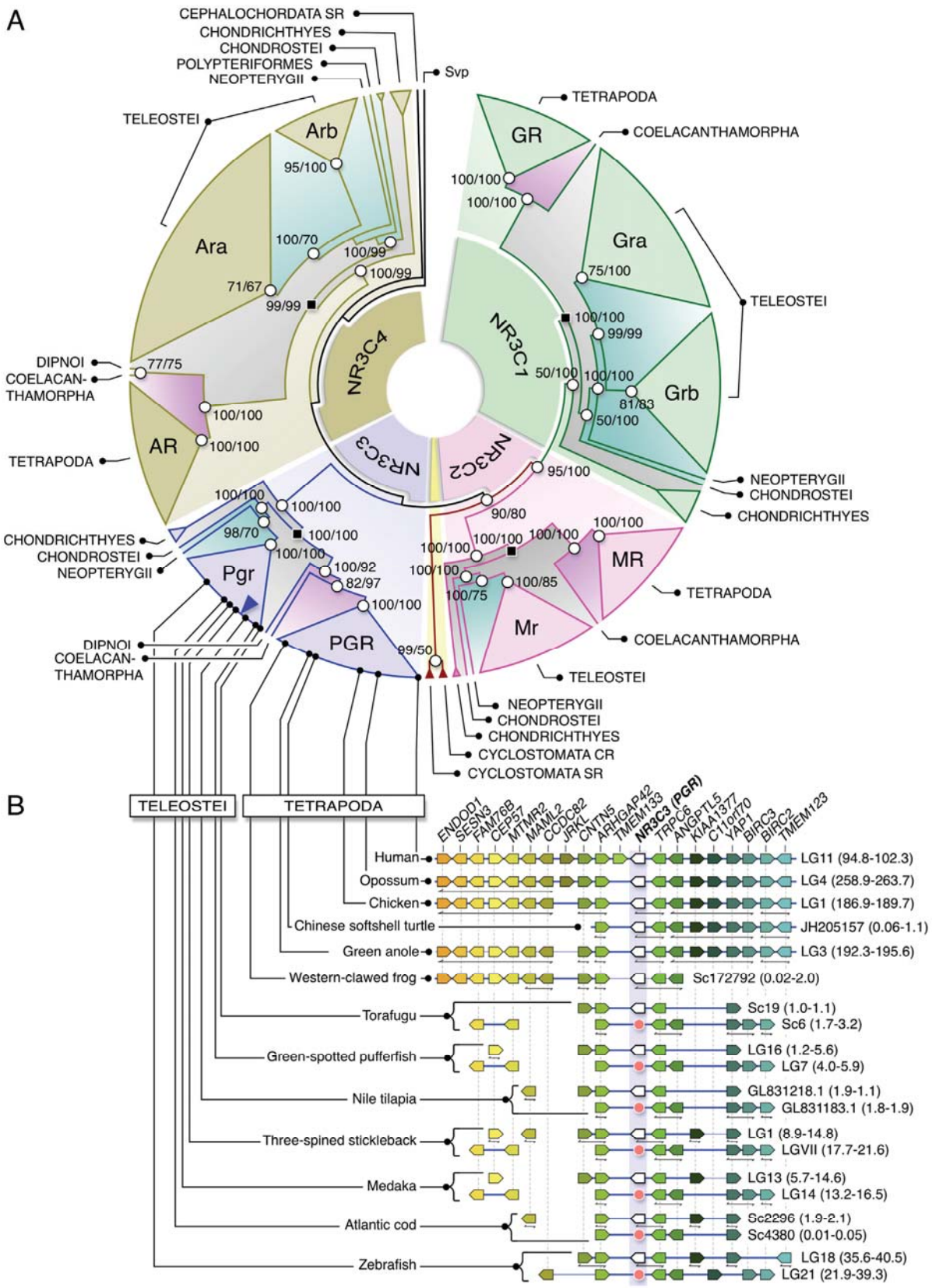


Fig. 2. Nucleotide sequence of seabream *pgr* full-length cDNA, and deduced amino acid sequence of the Pgr protein. The four splice donor sites (1d-4d) and splice acceptor sites (1a-4a) indicated on the nucleotide sequence, give rise to a deletion *pgr_tv1*, and N-terminus truncated *pgr_tv2*, *pgr_tv3* and *pgr_tv4* splice variants. Exon boundaries, based on the zebrafish *pgr* gene (ensembl accession number ENSDARG00000035966), are indicated with black arrowheads. The four putative ATG start codons (for Pgr/Pgr_tv1, Pgr_tv2, Pgr_tv3 and Pgr_tv4) are boxed in black, whereas the A/B region, DNA binding domain (DBD), hinge (H) and ligand binding domain (LBD) of the full Pgr polypeptide are marked with open boxes on the right. The amino acid sequence used for immunogen synthesis and antibody production is highlighted in grey, whereas the alpha helices (H1-H12) are underlined, and the beta sheets are shown with grey arrows. In the 3' UTR, two potential polyadenylation signal sites are underlined.

gar, Neopterygii) and all sarcopterygian orthologs. In contrast, with the exception of the Japanese eel *pgr-1* and *pgr-2*, only single orthologs of the *nr3c2* (mineralocorticoid, *mr*) and *nr3c3*

(*pgr*) genes were identified in all vertebrates examined, including Teleostei. The ancestral steroid receptors (SR) including the putative sea lamprey *pgr*, and corticosteroid receptors (CR) present in Cyclostomata (hagfishes and lampreys) clustered as sister branches below the *nr3c2* subfamily in all nucleotide analyses. The SR subcluster did, however, separate as a polytomy basal to the *nr3c3* and *nr3c4* clusters in the amino acid analyses. Within Teleostei, the gilthead seabream *pgr* transcript and Pgr protein clustered together with other perciform species of Cichlidae (the Nile tilapia, *Oreochromis niloticus* and Burton´s mouthbrooder, *Haplochromis burtoni*) and the gasterosteiform three-spined stickleback (*Gasterosteus aculeatus*). Phylogenetic analysis of the full-length Pgr amino acid sequences confirmed this relationship (data not shown). Further evidence that a single *pgr* gene exists in Teleostei was obtained from the synteny analyses (Fig. 3B). These latter data revealed high conservation of 17 tetrapod orthologs that flank the human *PGR*. Between 10-15 of these flanking genes are present in teleost genomes located in two paralogous linkage groups (LG). In all genomes examined, however, the *pgr* gene is closely linked to the transient receptor potential cation channel (*TRPC6*), which in Teleostei is present as a duplicated gene on the separate LGs. The partially conserved synteny of the paralogous teleost regions suggests that a second *pgr* that should have resulted from the teleost-specific whole genome duplication is likely lost from this locus.

Comparison of the seabream Pgr primary structure with that of other teleosts and human PGR-B revealed high sequence identity in the DBDs and LBDs between teleosts and human (89-97% and 63-84%, respectively), whereas the A/B regions are highly divergent (6-35% identity) (Fig. 4). As noted for other teleosts (Chen et al. 2010, 2011; Hanna et al. 2010), the seabream Pgr lacks the typical Pro-rich motif in the N-terminus of the human PGR-B, which is responsible for the interaction with the c-Src family of tyrosine kinases (Boonyaratanakornkit et al. 2001). In the seabream Pgr, most of the residues suggested to be critical for progestin binding in the LBD were conserved with respect to human PGR-B (Fig. 4). The exceptions were Met^{801}, Leu^{887} and Tyr^{890} (human PGR-B numbering) in helices 7 and 10/11 that were substituted by Iso^{546}, and Val^{632} and Phe^{635}, respectively, in the seabream Pgr. In contrast, zebrafish and Atlantic salmon Pgr, and Japanese eel Pgr-2, show only one amino acid substitution in the LBD (Val by Leu in position 887). The residues forming the P box (GSCKV) and D box (AGRND) regions in the DBD, involved in discrimination of core DNA recognition motifs and receptor dimerization (Aranda and Pascual 2001), were fully conserved in the seabream Pgr.


A
CEPHALOCHORDATA SR
CHONDRICHTHYES
CHONDROSTEI
POLYPTERIFORMES
NEOPTERYGII
Svp
TELEOSTEI
Arb
95/100
Ara
100/70
100/99
100/99
71/67
99/99
TETRAPODA
GR
COELACANTHAMORPHA
100/100
100/100
Gra
75/100
TELEOSTEI
NR3C1
100/100
99/99
50/100
100/100
Grb
81/83
50/100
NR3C4
NR3C3
NR3C2
DIPNOI
COELACAN-
THAMORPHA
77/75
100/100
AR
100/100
TETRAPODA
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98/70
100/100
CHONDRICHTHYES
CHONDROSTEI
NEOPTERYGII
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Pgr
100/92
82/97
100/100
90/80
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100/100
100/100
100/100
MR
100/75
100/85
TETRAPODA
Mr
COELACANTHAMORPHA
DIPNOI
COELACAN-
THAMORPHA
PGR
99/50
TELEOSTEI
NEOPTERYGII
CHONDROSTEI
CHONDRICHTHYES
CYCLOSTOMATA CR
CYCLOSTOMATA SR
B
TELEOSTEI
TETRAPODA
ENDOD1
SESN3
FAM76B
CEP57
MTMR2
MAML2
CCDC82
JRKL
CNTN5
ARHGAP42
TMEM133
NR3C3 (PGR)
TRPC6
ANGPTL5
KIAA1377
C11orf70
YAP1
BIRC3
BIRC2
TMEM123
Human
LG11 (94.8-102.3)
Opossum
LG4 (258.9-263.7)
Chicken
LG1 (186.9-189.7)
Chinese softshell turtle
JH205157 (0.06-1.1)
Green anole
LG3 (192.3-195.6)
Western-clawed frog
Sc172792 (0.02-2.0)
Torafugu
Sc19 (1.0-1.1)
Sc6 (1.7-3.2)
Green-spotted pufferfish
LG16 (1.2-5.6)
LG7 (4.0-5.9)
Nile tilapia
GL831218.1 (1.9-1.1)
GL831183.1 (1.8-1.9)
Three-spined stickleback
LG1 (8.9-14.8)
LGVII (17.7-21.6)
Medaka
LG13 (5.7-14.6)
LG14 (13.2-16.5)
Atlantic cod
Sc2296 (1.9-2.1)
Sc4380 (0.01-0.05)
Zebrafish
LG18 (35.6-40.5)
LG21 (21.9-39.3)

3.2. Identification of Seabream *pgr* Splice Variants

During the amplification of the full-length *pgr* cDNA from seabream ovaries using forward and reverse oligonucleotide primers located in the 5' and 3' UTRs, respectively, we identified four *pgr* transcript variants designated as *pgr_tv2*, *pgr_tv3* and *pgr_tv4*, which were 1584, 1223 and 1013 bp long, respectively (Fig. 5A). An additional isoform 2030 bp long, termed *pgr_tv1*, was detected during the 5' RACE experiments, but its full-length cDNA could not be cloned using the 5' and 3' UTR primers. However, ovarian expression of *pgr_tv1* was confirmed by using primers flanking one of the splicing sites and within the ORF of the *pgr* transcript (Fig. 1B), and its sequence was assembled *in silico* using overlapping PCR products. Alignment of the nucleotide sequences of all *pgr* transcripts against other teleost *pgr* genes revealed that each transcript most likely arose from alternative splicing within the first exon (*pgr_tv1*), or between the 5' UTR and exon 1, exon 2 or exon 4 for *pgr_tv2*, *pgr_tv3* and *pgr_tv4*, respectively (Fig. 5B). Alignment of the deduced amino acid sequence of the four transcript variants considering the first ATG in frame as the start codon, indicated that *pgr_tv1* encodes a protein of 62.6 kDa with an internal in-frame deletion of 121 amino acids in the A/B region, whereas the *pgr_tv2*, *pgr_tv3* and *pgr_tv4* variants represented different N-terminal truncations with calculated molecular masses of 49.1, 41.9 and 21.6 kDa, respectively (Fig. 5B). The deduced Pgr_tv2 polypeptide lacks approximately half of the A/B region, whereas the Pgr_tv3 lacks the entire A/B region. The *pgr_tv4* isoform encodes the shortest polypeptide, bearing only a C-terminal fragment of the LBD that nevertheless includes all ligand-binding amino acid residues (Fig. 4 and Fig. 5B).

Fig. 3. Annotated Bayesian majority rule consensus trees of (A) the chordate *nr3c* codon alignment and (B) Nr3c amino acid alignment of the DBD, hinge and LBD domains. The trees are rooted using yellow fever mosquito sevenup (Svp) as the outgroup. Both data sets resolve the Nr3c nuclear receptor subfamily into the four major clades: Nr3c1 (glucocorticoid receptors), Nr3c2 (mineralocorticoid receptors), Nr3c3 (progestin receptors) and Nr3c4 (androgen receptors), in which Teleostei retain duplicate glucocorticoid and androgen receptors, but single mineralocorticoid and progestin receptors. Grey shading indicates the position of ancestral chondrichthyan receptors, blue shading the position of ancestral actinopterygian receptors, and purple shading the position of ancestral sarcopterygian receptors. Bayesian posterior probabilities derived from 10 million mcmc generations and a burnin of 3500 are shown at each node. Accession numbers are given in Annex 1.

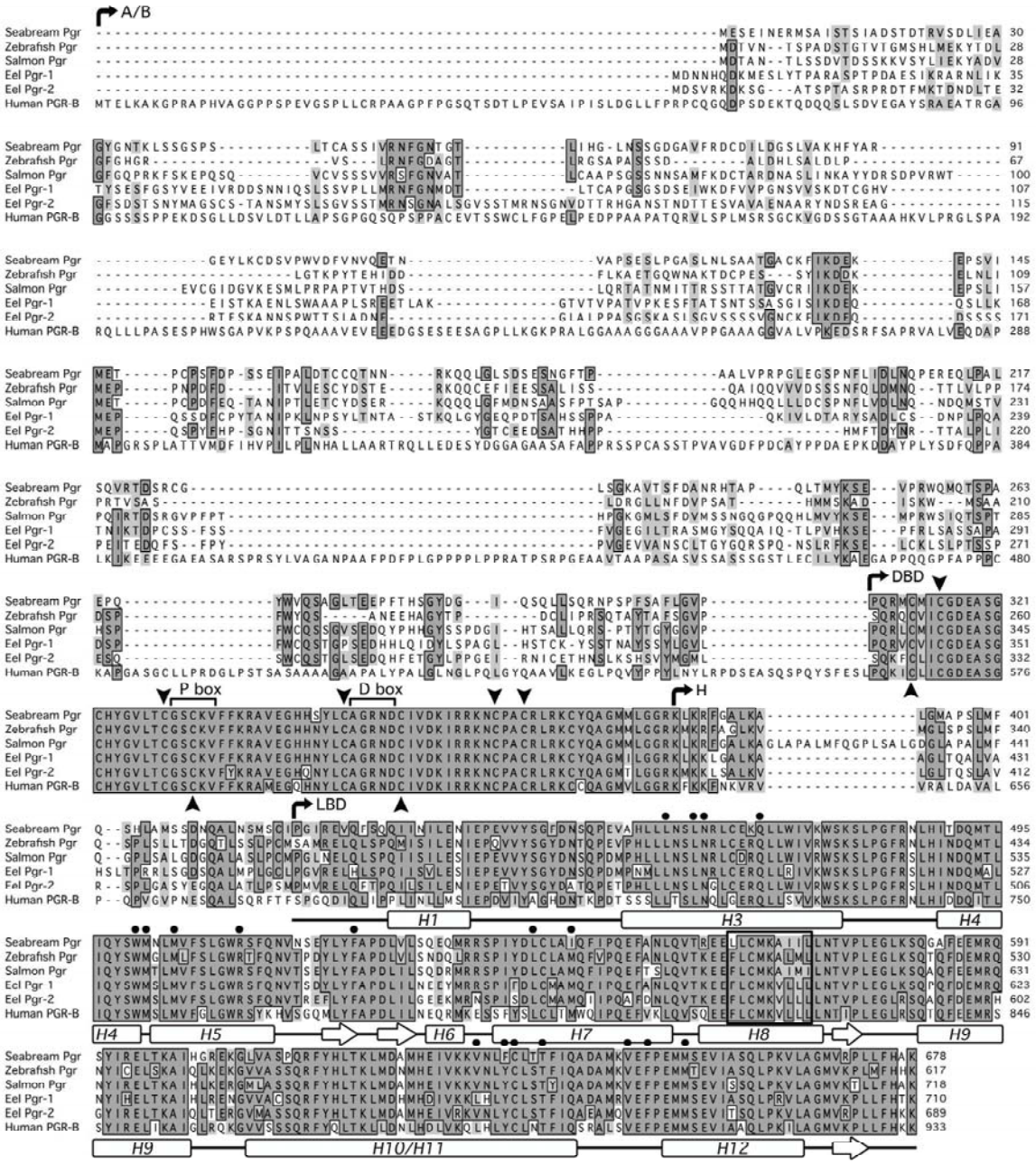


Fig. 4. Amino acid sequence aligment of the gilthead seabream nuclear Pgr with that of other representative teleosts. See Supplementary Table S1 for accession numbers. Subdomains are indicated with black arrows as: A/B region; DBD, DNA binding domain; H, cellular localization signal or hinge domain; and LBD, ligand binding domain. Fully conserved residues are boxed in dark grey, while conserved residues with similar chemical properties are shaded in light grey. Gaps, introduced to optimize the alignment, are shown with dashes. The putative membrane localization-like motif (FXCXXXXLL), which in seabream Pgr is LXCXXXXIL, is boxed, whereas the P box (GSCKV) and D box (AGRND) conserved regions, involved in discrimination of core DNA recognition motifs and receptor dimerization, respectively, are indicated in brackets. Arrowheads and dots above the sequences indicate putative zinc fingers and progestin interacting amino acids, respectively. Alpha helices (H1-H12) and beta sheets are shown below the sequences with open boxes and horizontal arrows, respectively.

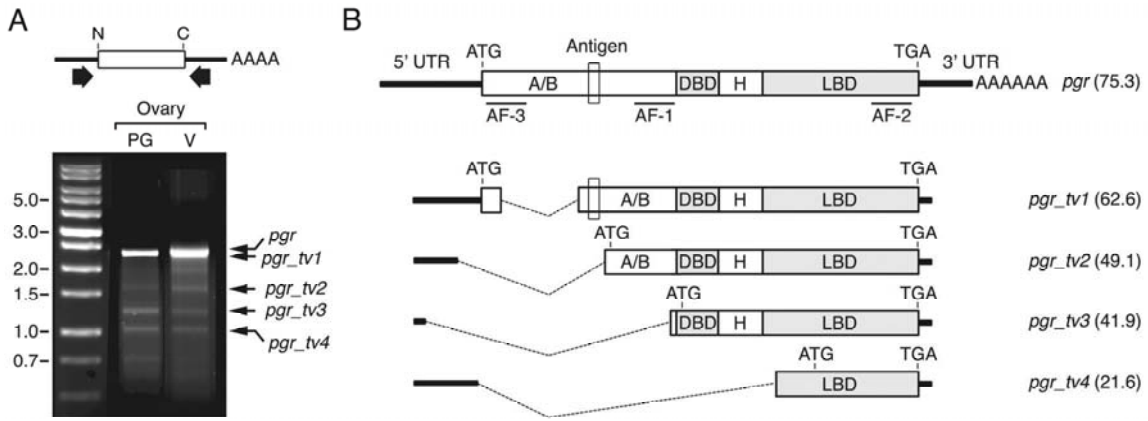


Fig. 5. Structure and alternative splicing of seabream *pgr*: (A) Representative gel image of RT-PCR analysis using primers flanking the full-length *pgr* cDNA (upper diagram) of ovaries at the primary growth (PG) and vitellogenic (V) stages showing amplified bands corresponding to the *pgr* and the four splice variants, *pgr_tv1*, *pgr_tv2*, *pgr_tv3* and *pgr_tv4*. (B) Diagram of the structure of seabream full-length *pgr* mRNA and splice variants showing the sequences encoding the functional domains boxed: A/B region, DNA binding domain (DBD), hinge (H) and ligand binding domain (LBD). Based on the mammalian PGR (Cork et al., 2008), the putative ligand-independent transcriptional activation domains AF-1 and AF-3, as well as the ligand-dependent AF-2 core transactivation domain, in the seabream Pgr deduced polypeptide are shown with dotted lines. The nucleotide sequence of the *pgr_tv1* was assembled *in silico* based on overlapping PCR products (see Supplementary Fig. S1). Alternative splicing within the first exon gives rise to one *pgr* isoform with an internal deletion in the A/B region (*pgr_tv1*), whereas splicing between the 5' UTR and exon 1, exon 2 or exon 4 results in three different N-terminally truncated *pgr* isoforms (*pgr_tv2*, *pgr_tv3* and *pgr_tv4*, respectively). The predicted molecular masses (kDa) of the polypeptides deduced from the different *pgr* variants are given to the right in parentheses.

3.3. Transcriptional Activity of Seabream Pgr

The transcriptional properties of seabream Pgr were studied using luciferase assays in HEK293T cells, which do not display endogenous PGR activity (Chen et al. 2010). The expression vector for Pgr was transfected into these cells together with a vector containing a luciferase gene driven by the MMTV promoter, which contains several PGR binding sites. Luciferase activity was measured after stimulation of cells with increasing concentrations of different steroid hormones. A clear dose-response on Pgr-mediated activation of the MMTV promoter was observed with 17,20β-P and 17,20β,21-P (EC_{50} of 26.4 and 28.9 nM, respectively), whereas P4 and 17-P were much less efficient (EC_{50} of 0.6 and 1.5 μM, respectively) (Fig. 6A). Other steroids, such as 17,21-P, T and C were completely ineffective at activating Pgr-mediated transcription of luciferase up to a dose of 10 μM. However, E2 was apparently able to partially stimulate the transcriptional activity of Pgr although it was much less potent than 17,20β-P or 17,20β,21-P. When a fixed dose of 1 μM was assayed, 17,20β-P and 17,20β,21-P were the most potent inducers of luciferase activity (49- and 45-fold above controls, respectively), and this

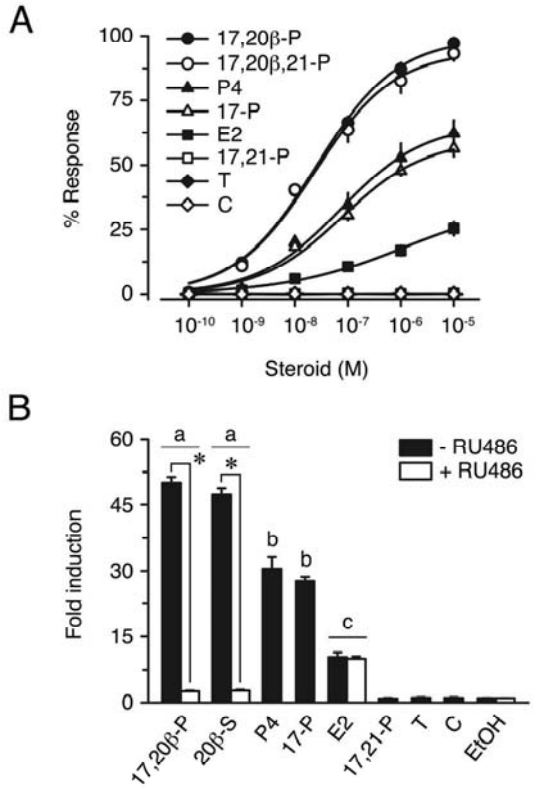


Fig. 6. Transcriptional activity of seabream nuclear Pgr determined using luciferase reporter assays in HEK293T cells. (A) Ligand-induced transactivation of the seabream Pgr after incubation of cells with increasing doses of various progestins and other steroid hormones. Percentage of response values are given relative to the maximal amount of luciferase activity for each condition. The same results were obtained using pGL3-MMTV-Luc or MMTV-LTR-Luc reporter plasmids. (B) Fold induction in luciferase activity of Pgr transfected cells exposed to 1 μM of the different steroids in the presence or absence of 10 μM of the nuclear PGR antagonist RU486. Data are expressed as the ratio of steroid:ethanol control (EtOH). Bars with different supercript or with and asterisk are significantly different ($P < 0.05$ and $P < 0.01$, respectively).

activation was completely abolished with 10 μM of the PGR inhibitor RU486 (Fig. 6B). In contrast, the slight stimulatory effect of E2 on luciferase activity (8-fold above controls) was not affected by RU486 (Fig. 6B), suggesting that the activation of the MMTV promoter by E2 was not mediated by the Pgr but possibly through non-genomic mechanisms.

3.4. Dominant-Negative Activity of Seabream Pgr_tv3 and Pgr_tv4

The transactivation activity of Pgr_tv2, Pgr_tv3 and Pgr_tv4, and their ability to act as dominant-negative inhibitors of Pgr-mediated transcription, was subsequently investigated. For this purpose, HEK293T cells were transiently transfected with *pgr*, *pgr_tv2*, *pgr_tv3* or *pgr_tv4* expression vectors alone, or co-transfected with *pgr* and equal amounts of *pgr_tv2*, *pgr_tv3* or *pgr_tv4*. In both cases, luciferase activity was measured after addition of 10 μM 17,20β-P. The *pgr_tv1* isoform was not tested because its full-length mRNA could not be isolated *in vivo* as previously indicated. The Pgr_tv2 isoform showed the same transcriptional activation efficiency as the full Pgr, whereas the Pgr_tv3 and Pgr_tv4 isoforms elicited significant reductions in transcriptional activity, reaching ~56% and ~15% of the response induced by Pgr, respectively (Fig. 7A). Co-expression of Pgr and Pgr_tv2 did not affect Pgr-induced transcription, whereas equimolar concentrations of Pgr_tv3 or Pgr_tv4 inhibited Pgr-

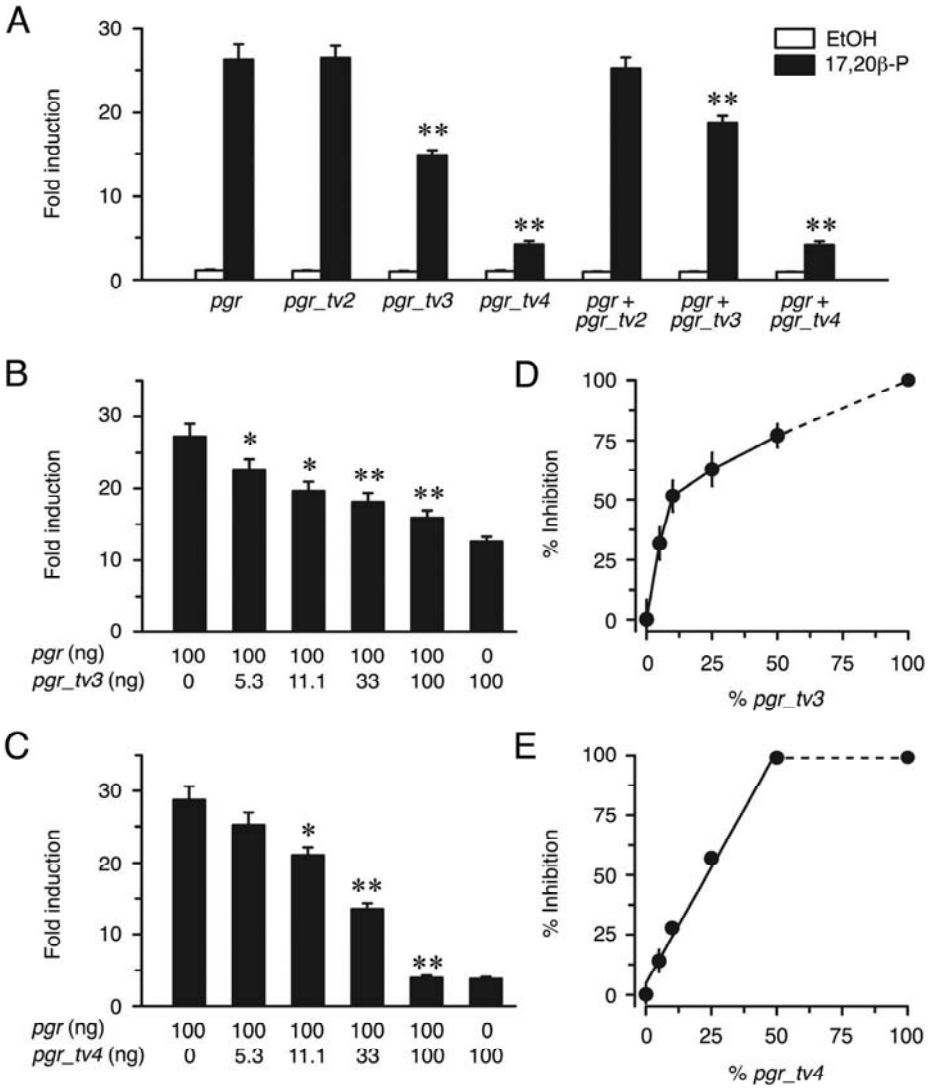


Fig. 7. The dominant-negative activity of seabream Pgr_tv3 and Pgr_tv4. (A) Luciferase activity of cells transfected with Pgr, Pgr_tv2, Pgr_tv3 and Pgr_tv4 alone or in combination (50% of each) after addition of ethanol (EtOH) or 10 μM 17,20β-P. Different amounts of the empty expression vector were cotransfected to use equal amounts of total DNA in the individual transfections. Equal amounts of transfected Pgr_tv3 and Pgr_tv4 expression vectors resulted in a decrease in transcriptional activity of 65 ± 7% and 100%, respectively. (B-C) Transcriptional activity of Pgr and Pgr_tv3 (B), or Pgr and Pgr_tv4 (C), in the presence of 10 μM 17,20β-P after transfection of different Pgr and Pgr_tv3, or Pgr and Pgr_tv4, expression vector amounts. (D-E) Correlation of the percentage inhibition of luciferase activity with the relative amount of Pgr_tv3 (D) or Pgr_tv4 (E) co-expressed with Pgr. In all panels, data represent the mean ± SEM of three independent experiments, with duplicates or triplicates for each condition. The asterisks indicate a significant difference ($P < 0.01$) with respect the values elicited by the expression of *pgr* alone..

mediated luciferase activity by 65 ± 7% and 100%, respectively (Fig. 7A). To investigate how the Pgr_tv3 and Pgr_tv4 isoforms mediate such transcriptional repression, cells were transiently cotransfected with Pgr and increasing amounts of Pgr_tv3 or Pgr_tv4 (Fig. 7B and C).

The results showed that transcriptional activity of Pgr was inhibited in a dose-response manner by either Pgr_tv3 or Pgr_tv4, indicating that the dominant-negative regulation of transcription is not due to nonspecific squelching effects. However, calculation of the percent inhibition of Pgr-mediated transcription in the presence of Pgr_tv3 revealed that the degree of inhibition is biphasic. Low amounts of Pgr_tv3 cDNA (5% and 10% with respect that of Pgr) rapidly inhibit transcription, whereas higher amounts result in a reduced rate of inhibition (Fig. 7D). In contrast, inhibition of Pgr-mediated transcription in the presence of Pgr_tv4 was less efficient compared to that of Pgr_tv3 at low ratios of Pgr_tv4 to Pgr, and unlike the biphasic inhibitory nature of Pgr_tv3, Pgr_tv4-mediated inhibition remained linear with increasing ratios (Fig. 7E).

To study the subcellular localization of Pgr, Pgr_tv3 and Pgr_tv4, expression plasmids encoding these isoforms tagged with a Flag at the C terminus were constructed and transfected into HEK293T cells. Immunofluorescence of transfected cells using an anti-Flag antibody indicated that Pgr, Pgr_tv3 and Pgr_tv4 were localized in the cytoplasm in the absence of ligand (Fig. 8A). After administration of 0.1 μM 17,20β-P, Pgr and Pgr_tv3 completely translocated to the nucleus, whereas the Pgr_tv4 isoform remained in the cytoplasm (Fig. 5A). Western blot on cell lysates of the same cells confirmed that the Pgr and Pgr_tv3 fusion proteins were correctly expressed (Fig. 8B), as judged from the molecular masses of the reactive bands, of ~75 and ~40 kDa, respectively. These bands closely match the *in silico* calculated values of Pgr and Pgr_tv3 (75.3 and 41.9 kDa, respectively; Fig. 5B). However, in extracts from cells transfected with Pgr_tv4 alone two reactive bands of ~20 and ~22 kDa were observed (Fig. 8B), around the calculated molecular mass of this isoform (21.6 kDa; Fig. 5B). None of these bands were affected by treatment with alkaline phosphatase (data not shown), suggesting the likely presence of two alternative start codons in the *pgr_tv4* mRNA when expressed in HEK293T cells. No differences in the Pgr, Pgr_tv3 or Pgr_tv4 reactive bands were observed between cells exposed to ethanol or 17,20β-P (Fig. 8B).

The pattern of inhibition and subcellular localization of Pgr_tv3 and Pgr_tv4 in HEK293T cells suggested that the two isoforms exert different inhibitory mechanisms on Pgr-mediated transcription. To investigate changes in the subcellular localization of Pgr in the presence of Pgr_tv3 and Pgr_tv4, we prepared a specific antibody against a synthetic peptide corresponding to a short region of the A/B region of seabream Pgr (Fig. 5B). Double immunofluorescence experiments using the Flag and Pgr antibodies were then carried out on cells expressing Pgr and equimolar concentrations of Flag-tagged Pgr_tv3 or Pgr_tv4. The co-

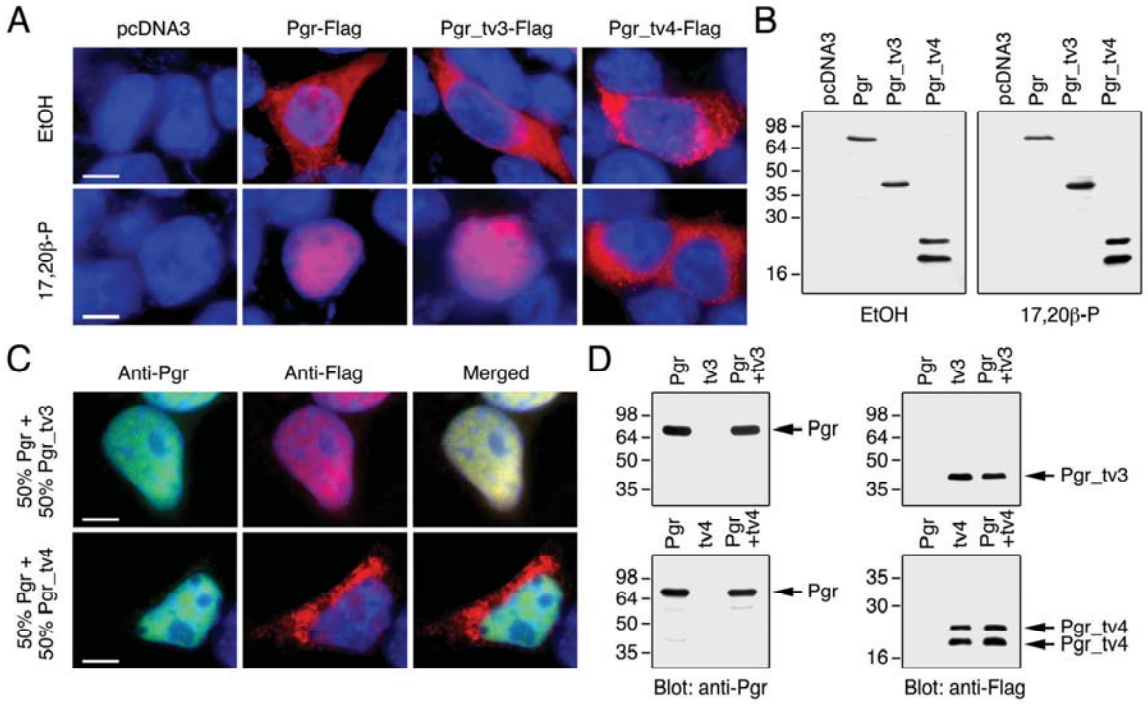


Fig. 8. Subcellular localization of the seabream nuclear Pgr and splice variants in HEK293T cells and Western blot analyses. (A) Immunofluorescence of cells transfected with pcDNA3 vector alone, or Flag-tagged Pgr, Pgr_tv3 or Pgr_tv4 and exposed to ethanol (EtOH) or 0.1 μM 17,20β-P using an anti-Flag antibody. Scale bar, 5 μm. (B) Western blots of the same cells using the Flag antibody. Molecular mass markers (kDa) are on the left. (C) Double immunofluorescence of cells cotransfected with equimolar concentrations of untagged Pgr and Pgr_tv3 or Pgr_tv4 as indicated, and exposed to 17,20β-P using the specific seabream Pgr antiserum and Flag antibodies. Scale bar, 5 μm. (D) Western blots of the same cells probed with the seabream Pgr or Flag antibodies as indicated. The arrows point to receptor monomer. Molecular mass markers (kDa) are on the left.

expression of Pgr with Pgr_tv3 did not prevent the translation or nuclear localization of either receptor form in response to 17,20β-P stimulation (Fig. 8, C and D). Likewise, co-expression of Pgr with Pgr_tv4 did not affect the synthesis or nuclear import of the full Pgr, and the expression of the Pgr did not affect the cytoplasmic localization of the Pgr_tv4 after progestin addition (Fig. 8, C and D). In both cases, the Pgr or Flag antibodies were not able to co-immunoprecipitate the Pgr with the Pgr_tv3 or Pgr_tv4 isoforms (data not shown).

3.5. Expression of *pgr* and Splice Forms During Ovarian Development *in vivo*

To determine the pattern of expression of the full *pgr* and the different splice variants in the seabream *in vivo*, we first evaluated the levels of total *pgr* transcripts in adult tissues by qRT-PCR using primers flanking the C terminus common to all transcript variants. *pgr* transcripts were detected in the ovary, testis, intestine, brain, liver, muscle and gills (Fig. 9). The

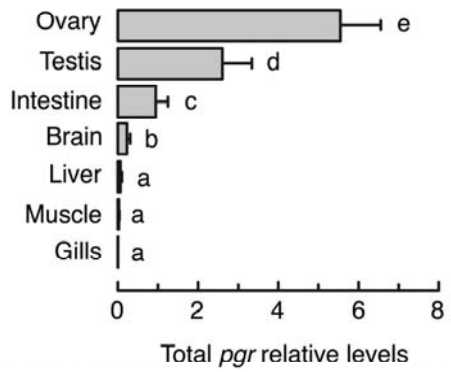


Fig. 9. Tissue distribution of total *pgr* transcripts (mean ± SEM; $n = 3$ fish) in adult gilthead seabream determined by qRT-PCR using the *18s* ribosomal mRNA as reference gene. Data with different superscript are significantly different ($P < 0.05$).

transcript levels were highest in the ovary followed by the testis, intestine and brain. Some *pgr* expression was also noted in the liver, muscle and gills but at almost undetectable levels.

Changes in the expression of total *pgr* mRNAs, and specifically of the wild-type *pgr*, were further determined in ovaries at different stages of development. Since the gilthead seabream shows a group-synchronous ovary, in which ovarian follicles at different developmental stages and increasing size progressively appear in the ovary as sexual maturation proceeds, ovarian stages were defined based on the most advanced follicle present in the ovary (Zapater et al. 2012). High levels of total *pgr* transcripts were found in early primary growth (previtellogenic) ovaries, which contain only oogonia and primary follicles with perinucleolar-stage oocytes. However, expression levels were markedly reduced in slightly more advanced primary ovaries, and progressively decreased with the appearance of cortical alveoli and vitellogenic oocytes (Fig. 10A). The decrease in the expression levels was thus negatively correlated with the progressive increase in the GSI (Fig. 10A). In contrast, the levels of the wild-type *pgr* transcripts remained similar from the primary growth up to the vitellogenic stage (Fig. 10A). A further marked accumulation of total and wild-type *pgr* transcripts in the ovary was observed during the maturation stage, i.e. when a subpopulation of post-vitellogenic follicles resumed meiosis (Fig. 10A).

Using oligonucleotide primers in the 5' and 3' UTRs, able to amplify the *pgr*, *pgr_tv2, pgr_tv3 and pgr_tv4* isoforms in one PCR reaction (Fig. 5A), and subsequent band densitometry of the electrophoresed PCR products, it was possible to estimate the abundance of each transcript variant with respect to that of the full *pgr* during ovarian development. The highest accumulation of the three transcript variants was observed at the early primary growth stage, reaching ~40% for *pgr_tv2* and *pgr_tv3*, and ~24% for *pgr_tv4* (Fig. 10B). However, the relative abundance of all three variants decreased throughout ovarian growth, but a small in-

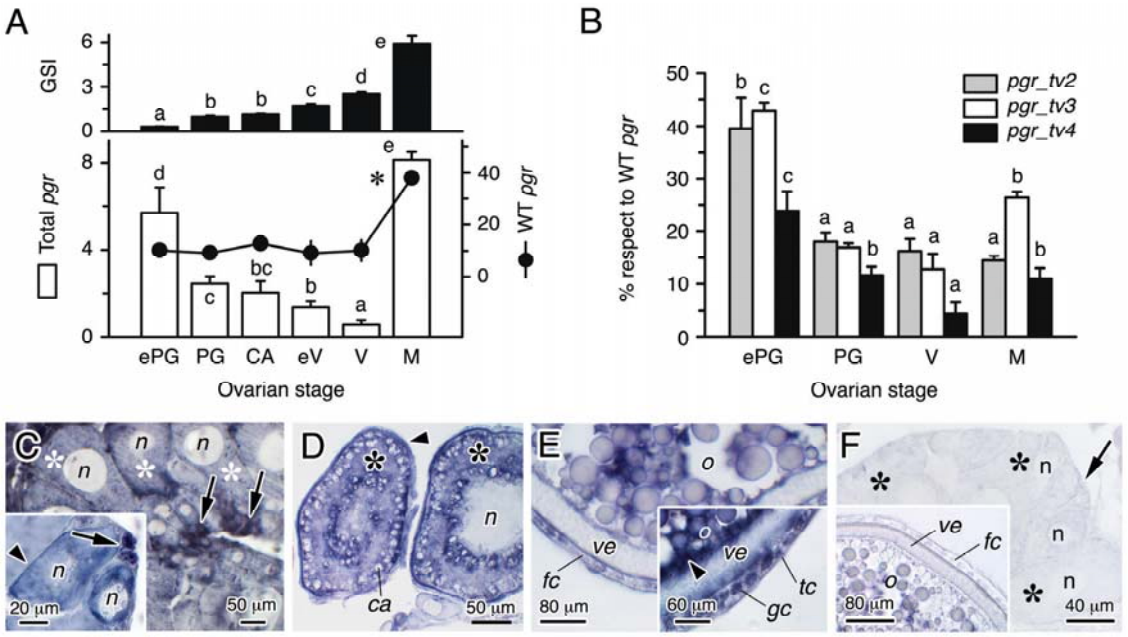


Fig. 10. Pattern of mRNA expression of the seabream nuclear *pgr* and splice variants during ovarian development. (A) Changes (mean ± SEM; n = 4-5 females) in the expression of total *pgr* transcripts (bars) and wild-type (WT) *pgr* (line) in the whole ovary during development determined by qRT-PCR. Data represent relative mean expression, normalized to *18s* ribosomal RNA expression, in ovaries at different developmental stages: ePG, early primary growth; PG, primary growth; CA, cortical alveolus; eV, early vitellogenic; V, vitellogenic; M, maturation. The panel above shows the gonadosomatic index (GSI) of the same females at each stage. Bars with different superscript are significantly different ($P <$ 0.05). The asterisk indicates significant differences ($P <$ 0.05) between the M stage *vs*. the preceeding stages. (B) Relative abundance (mean ± SEM; n = 4 females) of *pgr_tv2, pgr_tv3* and *pgr_tv4* transcripts with respect the WT *pgr* variant determined by densitometric quantification of the corresponding band intensities in RT-PCR gels as showed in Fig. 2A. For each transcript variant, bars with different superscript are significantly different ($P <$ 0.01). (C-F) Cellular localization of *pgr* transcripts in the seabream ovary by *in situ hybridization*. Paraffin sections of ovaries at the PG (C, C inset, and F) CA (D) or V (E and inset) stages were hybridized with antisense DIG-labeled riboprobes corresponding to nucleotides 1429 to 2331 of the *pgr* cDNA. The hybridization signal is colored dark-blue to purple. (C and inset) Oogonia (arrows) and ePG oocytes (asterisks) showing *pgr* staining in the ooplasm. (C inset) The arrowhead points to follicle cell surrounding the oocyte. (D) CA follicles with *pgr* staining in the cytoplasm of oocytes and in follicle cells (arrowhead). (E and inset) *pgr* staining in the cytoplasm of V oocytes (arrowhead) and in granulosa cells. (F and inset) Control sections incubated with sense probes in which oogonia and PG oocytes are indicated with arrows and asterisks, respectively, as in panel C. Identical results were obtained with probes corresponding to the 5' UTR and N-terminus of the *pgr* cDNA (not shown). o, oocyte; n, nucleus; ca, cortical alveoli; gc, granulosa cells; tc, theca cells, ve, vitelline envelope.

crease in the amount of *pgr_tv3* and *pgr_tv4*, but not of *pgr_tv2*, was detected during the maturation stage.

The cellular sites of *pgr* expression in the seabream ovary were determined by *in situ* hybridization employing DIG-labeled riboprobes corresponding to part of the LBD and complete 3' UTR of the full *pgr* cDNA, and thus capable of detecting all transcript isoforms. Consis-

tent with the results obtained by qRT-PCR, strong *pgr* expression was detected in oogonia, and in the ooplasm of primary oocytes and surrounding follicle cells (Fig. 10C and inset). In follicles at the cortical alveolus stage, *pgr* staining in the ooplasm and follicle cells remained visible (Fig. 10D). By the vitellogenic stage, the staining of *pgr* transcripts in the oocyte cytoplasm appeared to be more intense just below the oocyte plasma membrane (Fig. 10E), whereas *pgr* expression in the follicular layer was restricted to the granulosa cells (Fig. 10E inset). Control sections incubated with sense probes were negative (Fig. 10F and inset). Identical results were obtained with wild-type *pgr*-specific riboprobes corresponding to the 5' UTR and N-terminus of the full *pgr* cDNA (data not shown).

3.6. Immunodetection of Pgr in the Seabream Ovary

Immunoblotting of protein extracts from the ovary, testis and gills using the seabream Pgr specific antiserum, and an anti-alpha tubulin antibody, confirmed the presence of a reactive band of ~75 kDa corresponding to the wild-type Pgr in different ovarian developmental stages and in the testis, but not in the gills (Fig. 11A). However, another band was also detected in the ovaries and testes with a molecular mass of ~62 kDa. This band matches the predicted molecular mass of the Pgr_tv1 isoform (62.6 kDa) that retains the amino acid sequence of the A/B region used for antibody production (Fig. 5B). In ovaries at the cortical alveolus and vitellogenic stages, additional high-molecular weight bands, of ~85, ~100, ~125 and ~145 kDa, were noted (Fig. 11A). In protein extracts from isolated vitellogenic follicles, some of these high-molecular weight polypeptides persisted (Fig. 11A, right panel), suggesting that they most likely did not originate in primary growth stage ovaries. These polypeptides possibly correspond to oligomers or posttranslational modifications of the Pgr and/or Pgr_tv1 isoforms. None of the polypeptide bands detected in the ovaries and testes were observed when the Pgr antibody was preabsorbed with the synthetic peptide used for immunization (Fig. 11B), confirming the specificity of the reactions.

Immunohistochemical localization of Pgr in the seabream ovary showed strong immunostaining using the specific Pgr antibody in the cytoplasm and nucleus of oogonia, as well as in the nuclei of primary oocytes and primordial granuloa cells adjacent to the oocyte (Fig. 11, C and D). At this stage, the very thin theca layer superficial to the granulosa cells was not significantly stained (Fig. 11D). In ovarian follicles at the cortical alveolus stage, immunoreaction in the oocyte nucleus and granulosa cells persisted, whereas staining in the oocyte cytoplasm was strong below the plasma membrane (Fig. 11E). In vitellogenic follicles, the oocyte nucleus

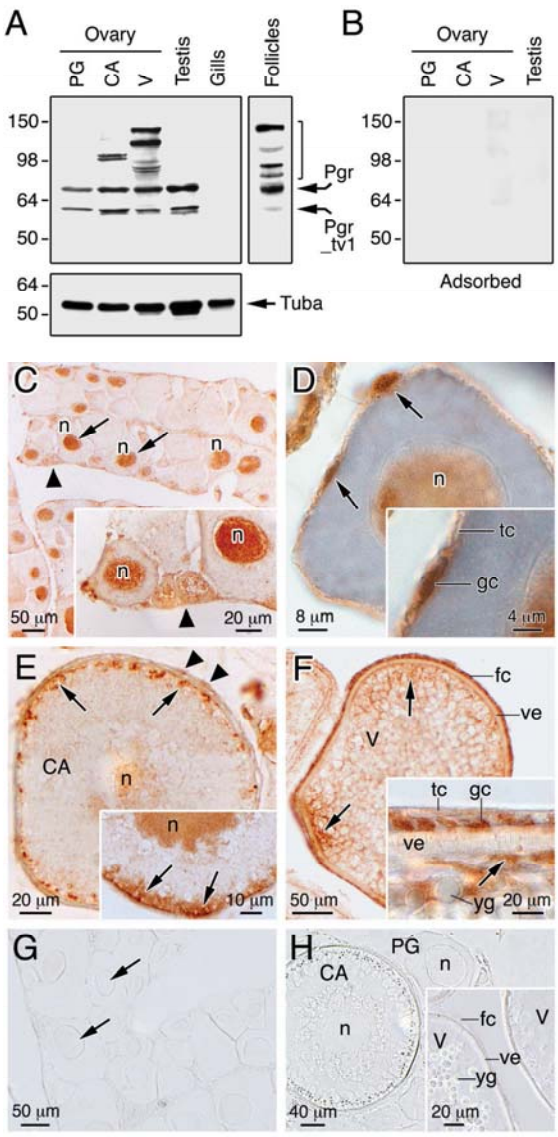


Fig. 11. (A) Immunoblotting of protein extracts from seabream ovaries at different developmental stages, primary growth (PG), cortical alveolus (CA) and vitellogenic (V) stages, testis and gills, using the seabream Pgr antiserum. Approximately, the same amount of protein (30 µg) was loaded in each lane. A replicated membrane was probed with the anti-alpha-tubulin antibody (lower panel). Western blot of protein extracts from isolated vitellogenic follicles are also shown (right panel). (B) Duplicate membrane shown in A incubated with the Pgr antibody pre-absorbed with the synthetic peptide used for immunisation. The arrows point to Pgr and putative Pgr_tv1 monomers, whereas the bracket indicate potential oligomers or post-translational modifications of the Pgr and/or Pgr_tv1 isoforms. Molecular mass markers (kDa) are on the left. (C-H) Pgr immunostaining images of representative paraffin sections of seabream ovaries. (C, D) Photomicrographs of primary growth ovaries, where the Pgr is localized in the cytoplasm and nucleus of oogonia (C inset, arrowhead) and in the nucleus (germinal vesicle) of primary oocytes. Primordial granulosa cells adjacent to the oocyte were also positive whereas theca cells were not significantly stained (D and inset; section counterstained with hematoxylin). (E) Positive reaction in the cytoplasm of cortical alveolus oocytes below the plasma membrane (arrows), and in associated granulosa cells (arrowheads). (F) Diffuse Pgr staining in the cytoplasm of vitellogenic oocytes close to the plasma membrane (arrows), as well as in the nuclei of surrounding granulosa cells whereas theca cells were negative (inset). (G, H) Control sections of primary growth (G) or vitellogenic ovaries (H) incubated with preabsorbed Pgr antiserum. PG, primary growth oocyte; CA, cortical alveoli oocyte; V, vitellogenic oocyte; n, nucleus; fc, follicle cells; tc, theca cells; gc, granulosa cells; ve, vitelline envelope.

was no longer stained, and the staining in the oocyte cytoplasm became more diffuse but remained close to the plasma membrane (Fig. 11F). In these follicles, the nuclei of granulosa cells were strongly stained while theca cells were clearly negative (Fig. 11F inset). Control sections of all developmental stages incubated with the Pgr antibody pre-adsorbed with the peptide did not show positive reactions (Fig. 11, G and H).

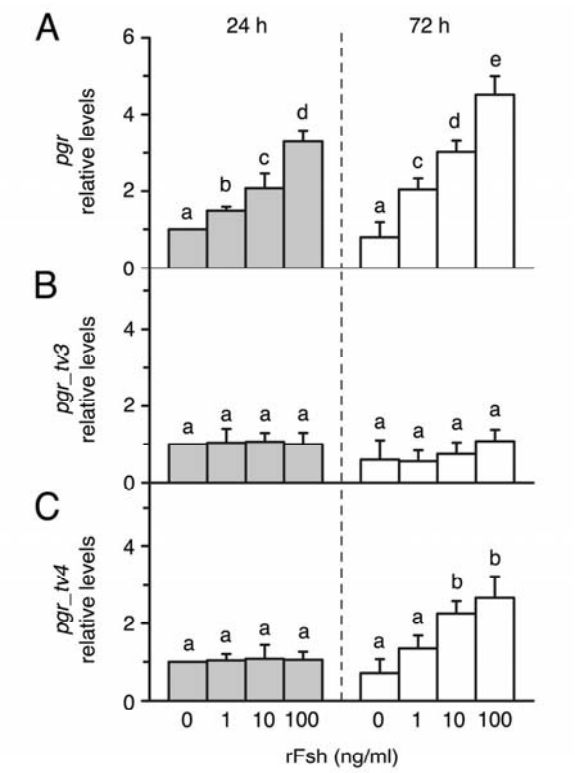
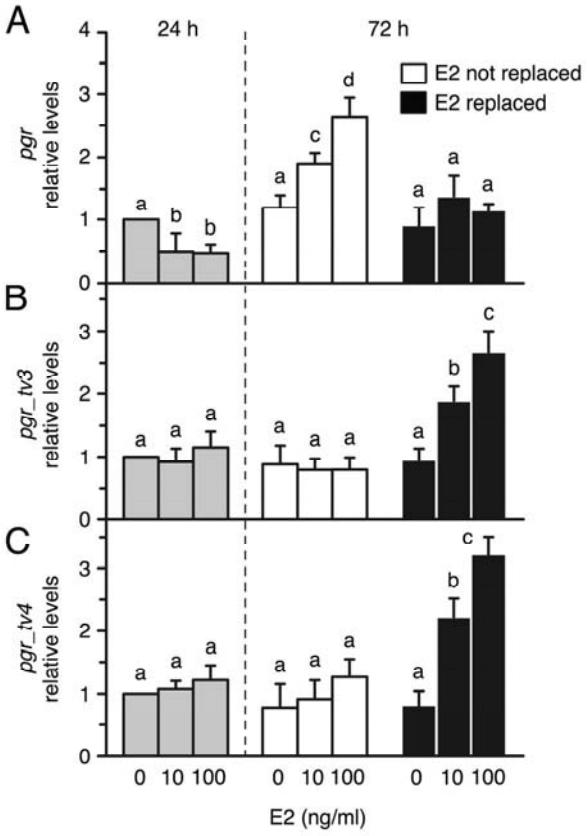


Fig. 12. *In vitro* effect of seabass recombinant follicle-stimulating hormone (rFsh) on *pgr*, *pgr_tv3* and *pgr_tv4* transcript levels in primary ovarian explants after 24h (A) or 72 h (B) of gonadotropin addition. Data (mean ± SEM; $n = 4$ separate experiments) were determined by qRT-PCR, using isoform-specific primers, and normalized to *18s* ribosomal mRNA expression. Bars with different superscript for each isoform at each time period are significantly different from the 24 h control group ($P < 0.05$).

3.7. Gonadotropin and Estrogen Regulation of Pgr Expression in Primary Ovaries *in vitro*

The previous experiments demonstrated that seabream primary ovarian follicles express high levels of the full *pgr* transcript, as well as of transcripts encoding the two dominant-negative isoforms *pgr_tv3* and *pgr_tv4*. To investigate if the transcription of the three isoforms could be hormonally regulated at this ovarian stage, primary explants were incubated *in vitro* with European seabass rFsh, and the expression level of each isoform determined by qRT-PCR using isoform-specific oligonucleotide primers (Fig. 12). When ovarian explants were stimulated with rFsh (1-100 ng/ml), a dose-dependent increase in *pgr* transcript levels was observed at 24 and 72 h, the effect being slightly more potent 72 h after hormone addition (Fig. 12A). In contrast, the levels of *pgr_tv3* and *pgr_tv4* did not change at 24 h (Fig. 12B and C), whereas only those of *pgr_tv4* started to increase in a dose-dependent manner at 72 h, but to a lesser extent than those of *pgr* (Fig. 12C).

In a subsequent set of *in vitro* experiments, the effect of E2 (10 and 100 ng/ml) on the temporal expression of the *pgr* isoforms in primary ovarian explants was investigated (Fig. 13). Since E2 can be metabolized relatively rapidly in the tissues, the effect of daily replacement of the hormone in the culture medium was also tested. At 24 h, *pgr* transcript levels



splice variants (GCE, 182:24, 2013)

Fig. 13. *In vitro* effect of 17β-estradiol (E2) on *pgr*, *pgr_tv3* and *pgr_tv4* transcript levels in primary ovarian explants after 24 h (A) or 72 h of hormone addition, without replacement of E2 (B) or with daily replacement of E2 (C). Data (mean ± SEM; $n = 4$ separate experiments) were determined by qRT-PCR as in Fig. 8. Bars with different superscript for each transcript are significantly different from the 24 h control group ($P < 0.05$).

were significantly down-regulated in the presence of E2, whereas at 72 h *pgr* expression was stimulated in a dose-dependent manner, but only when E2 was not replaced daily (Fig. 13A). In contrast, the expression levels of *pgr_tv3* and *pgr_tv4* were unaffected by E2 at 24 h or at 72 h without E2 replacement, but intriguingly, the transcript levels of both isoforms, particularly of *pgr_tv4*, were significantly up-regulated at 72 h when the E2 was replaced (Fig. 13B and C).

Western blot analyses, using the seabream Pgr specific antibody confirmed the stimulatory effect of rFsh on Pgr expression (Fig. 14). Thus, rFsh elevated the Pgr protein levels, and also of the putative Pgr_tv1 isoform, in primary explants cultured *in vitro* after 72 h of treatment (Fig. 14A). Conversely, as noted in the transcript expression experiments, E2 treatment significantly reduced the Pgr and Pgr_tv1 protein levels at 24 h (Fig. 14B), but resulted in increased levels at 72 h when E2 was not replaced in the culture medium (Fig. 14C).

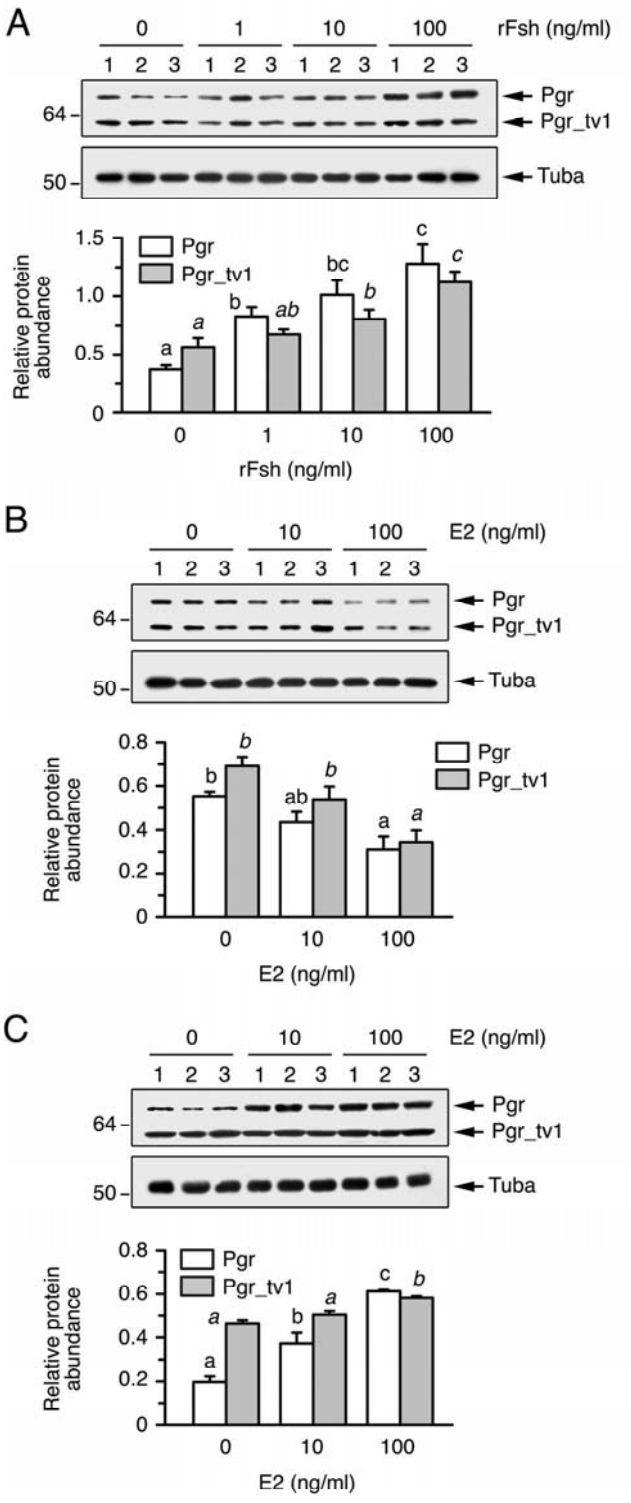


Fig. 14. *In vitro* effect of seabass rFsh and E2 on Pgr and putative Pgr_tv1 relative protein abundance in primary ovarian explants. The effects of rFsh at 72 h (A), and of E2 at 24 h (B) and at 72 h without E2 replacement (C) are shown. Western blots of protein extracts from ovarian explants (15 or 30 μg) from a representative experiment, in which each treatment was in triplicate (lanes 1, 2 and 3), are shown above each panel. Duplicate membranes were probed with the seabream anti-Pgr antiserum or with the anti-alpha-tubulin (Tuba) antibody. Molecular mass markers (kDa) are on the left. The relative abundance of the Pgr and putative Pgr_tv1 polypeptides (mean ± SEM; $n = 3$ separate experiments) was determined by densitometric analysis of the reactive bands. In each graph, data for Pgr and Pgr_tv1 with different superscript are statistically significant (Tukey's pairwise comparison test; $P < 0.05$).

4. Discussion

In mammals, it is established that nuclear steroid hormone receptors, including the PGR, are expressed in normal and tumorigenic tissues as multiple isoforms arising from alternative promoter usage and alternative exon splicing (Cork et al. 2008; Springwald et al. 2010; Dehm and Tindall 2011). However, studies in several non-amniote vertebrates, including amphibians and teleosts, have not reported different isoforms of the Pgr (Tian et al. 2000; Todo et al. 2000; Thornton 2001; Ikeuchi et al. 2002; Wang et al. 2004; Katsu et al. 2008; Chen et al. 2010, 2011, 2012; Hanna et al. 2010). The present study provides the first evidence for the loss of a paralogous nuclear *pgr* ortholog in the genomes of higher orders of Teleostei, and the expression of multiple *pgr* isoforms in the actinopterygian lineage. Although in the seabream the translation of these isoforms *in vivo* remains to be demonstrated, our data suggest that ovarian expression of N-terminally truncated isoforms can potentially act as dominant-negative repressors of wild-type Pgr function.

As reported in basal teleosts, the seabream full-length *pgr* transcript encodes a protein with a typical nuclear steroid receptor structure showing high homology in the amino acid sequences of the DBD and LBD with other vertebrate PGRs. Sequence and phylogenetic analyses indicated, as expected, that the seabream Pgr was more similar to that of other perciform teleosts, whereas it was more distantly related with the Pgr of salmonids, cyprinids or anguillids. The seabream LBD shows three amino acid substitutions in the positions suggested to be important for P4 binding in human PGR-B (Williams and Sigler 1998) in the segment corresponding to α-helices 7 and 10/11, thus two more than in zebrafish and Atlantic salmon Pgrs, and Japanese eel Pgr-2 (Ikeuchi et al. 2002; Chen et al. 2010, 2011; Hanna et al. 2010). The two additional amino acid substitutions in the seabream LBD seem not to affect the highest affinity of the receptor for 17 and 20 hydroxyl groups of progestins (i.e. 17,20β-P and 17,20β,21-P) in transactivation assays using MMTV-luciferase reporters, as shown for other teleost nuclear Pgrs (Todo et al. 2000; Ikeuchi et al. 2002; Chen et al. 2010, 2011; Hanna et al. 2010). The divergence in other residues of α-helix 3 through α-helix 5 in fish and mammals, including the replacement of Gly^{722} (human PGR-B) by a Cys residue in α-helix 3, which is conserved in the seabream and other teleost Pgrs and seems to interact with Met^{759} (human PGR-B), may be relevant for differences in the responses of fish and mammalian PGRs to hydroxylated progestins (Baker and Uh 2012).

By using an RT-PCR approach we were able to isolate complete mRNAs of three transcript variants of the *pgr* gene from the seabream ovary. Transactivation assays showed that

each of these isoforms (*pgr_tv2*, *pgr_tv3* and *pgr_tv4*) encoded functional receptors. Analysis of the seabream *pgr* mRNA using available information on the genomic organization of the *pgr* gene in teleosts suggest that each isoform is generated by alternative pre-mRNA splicing rather than by alternate initiation of translation. Thus, splicing between the 5' UTR and exon 1, exon 2 or exon 4, respectively, of the seabream *pgr* gene leads to mRNAs encoding N-terminally truncated Pgr isoforms lacking approximately half of the A/B region (Pgr_tv2), the full A/B region and a small portion of the DBD (Pgr_tv3), or bearing only part of the LBD (Pgr_tv4). A fourth variant, *pgr_tv1*, could only be identified by PCR using internal primers, and apparently originates from a splicing site within exon 1, causing an in-frame deletion of 121 amino acids in the A/B region. The transactivation activity of seabream Pgr_tv2 was not significantly different to that of the full Pgr, suggesting that the transactivation domains AF-1 and AF-2 common to many steroid receptors (Aranda and Pascual 2001) are conserved in the seabream Pgr and Pgr_tv2. The Pgr_tv3 isoform, however, showed ~44% less efficient transactivation activity compared to the Pgr, which is consistent with the lack of AF-1 and AF-3 but intact DBD and LBD in this isoform. The subcellular translocalization of Pgr_tv3 in HEK293T cells from the cytoplasm into the nucleus in response to 17,20β-P is also consistent with the presence of an intact nuclear localization signal (hinge region) in this isoform. Intriguingly, however, when the Pgr and Pgr_tv3 isoforms were co-expressed, the Pgr_tv3 exerted a biphasic, concentration-dependent dominant-negative inhibition on Pgr-mediated transcription. At low ratios of Pgr-tv3 to Pgr, Pgr-mediated transcription is rapidly inhibited, but at higher concentrations of Pgr-tv3 (≥ 15%), this inhibition is more attenuated. The biphasic pattern of inhibition is possibly related to the ability of Pgr_tv3 to translocate into the nucleus, dimerise and compete with Pgr for PRE and/or common nuclear co-factors, in a similar fashion to mammalian PGR-A or PGR-C (Vegeto et al 1993; Wei et al. 1997). Immunoprecipitation experiments failed to demonstrate the formation of Pgr and Pgr_tv3 heterodimers and therefore whether Pgr_tv3 has the ability to heterodimerise with Pgr affecting its transactivation ability, as suggested for human PGR-C (Wei et al. 1997), remains to be determined.

The seabream Pgr_tv4 represents a novel naturally occurring dominant-negative Pgr isoform, which to our knowledge has not been described previously in any vertebrate. The Pgr_tv4 isoform only bears part of the LBD, including a presumptive AF-2 transactivational domain towards the C-terminus, and it is thus slightly different to mammalian PGR-S and PGR-T (Hirata et al. 2000, 2002a,b). These latter isoforms are expressed in the testis and retain different intronic sequences preceding exon 4, resulting in mRNAs that lack the A/B region

and DBD but contain the hinge sequence. The seabream Pgr_tv4 also seems to be different to the PGR-M mRNA discovered in the T47D breast cancer cell line, which encodes a protein with a 5' signal sequence and complete hinge region, LBD and dimerisation domain (Saner et al. 2003). Transactivation assays showed, however, that the seabream Pgr_tv4 elicited an ~4-fold increase in luciferase activity, suggesting that this isoform can promote some transcriptional activity despite lacking the DBD and nuclear localization signal. Since Pgr_tv4 likely lacks the ability to enter the nucleus and bind DNA, these observations suggest that Pgr_tv4 may be able to activate transcription through non-genomic mechanisms, as suggested for mammalian PGR-S, PGR-T and PGR-M (Cork et al. 2008). These latter mammalian PGR isoforms are suggested to be secreted or be membrane bound (Cork et al. 2008). However, our data show that the seabream Pgr_tv4 appears to be predominantly cytoplasmatic when expressed in HEK293T cells. Therefore, the strong dominant-negative inhibition of Pgr_tv4 on Pgr-mediated transcription in the presence of 17,20β-P is likely mediated by a different mechanism than that of the Pgr_tv3 isoform. Since co-expression of Pgr with Pgr_tv4 did not prevent the translocation of Pgr into the nucleus in the presence of an excess of the ligand (10 μM), or the cytoplasmic localization of the Pgr_tv4, the inhibitory mechanism, which is complete at a ratio of 0.5 Pgr_tv4:Pgr, apparently does not involve ligand competition. Rather, the mechanism may involve higher Pgr_tv4 affinity for limiting concentrations of some other cytoplasmic factor(s), such as some kinases (Aranda and Pascual 2001), required for Pgr DNA binding and/or transactivation, but not for Pgr trafficking. This however remains a speculation and further research is needed to clarify the inhibitory mechanism of the seabream Pgr_tv4 isoform.

In teleosts, including the gilthead seabream, Pgr has a a more restrictive expression pattern compared to mammals (Graham and Clarke 1997), but, as in other vertebrates (Graham and Clarke 1997; Wang et al. 2004; Camacho-Arroyo et al. 2007), is highly expressed in the brain and reproductive organs (Chen et al. 2010, 2011; Hanna et al. 2010; Ikeuchi et al. 2002). Recent studies on the zebrafish have shown that, as in mammals (Brinton et al. 2008), the Pgr is widely expressed in diverse brain regions, particularly in radial glial cells and more weakly in neurons (Diotel et al. 2011). In contrast to reports on basal teleosts (Chen et al. 2010, 2011; Hanna et al. 2010), we detected significant expression of seabream *pgr* transcripts in the intestine although the specific cell sites of expression and the presence of different transcript variants were not determined. Nevertheless, these observations agree with the constitutive expression of the PGR in the gastrointestinal tract of reptiles (Custodia-Lora and Callard

2002), in the smooth muscle layer of the small intestine of mouse and chicken (Salomaa at al. 1989; Uotinen et al. 1999), as well as in specialized interstitial cells of the human fetal gut wall, i.e. interstitial cells of Cajal, involved in the modulation of gastrointestinal tract motility (Inoue et al. 2001).

During ovarian development, we observed that the expression of total *pgr* transcripts was higher during early stages of folliculogenesis and oocyte development, as well as during the oocyte maturation stage when progestins are well known to be involved in oocyte meiosis resumption and ovulation (Thomas et al. 2007; Nagahama and Yamashita 2008). The progressive decrease in the expression of total *pgr* during ovarian growth (i.e. from a late primary growth to the vitellogenic stage) was apparently only due to the reduced expression of the *pgr_tv2*, *pgr_tv3* and *pgr_tv4* isoforms during this period, since the levels of the wild-type *pgr* remained unchanged. This is supported by the *in situ* hybridization data which did not show major changes in the expression of the wild-type *pgr* in the ovary, and with the Western blotting experiments, which, when normalized to alpha-tubulin, indicated a slight increase in the relative amount of wild-type Pgr protein levels as vitellogenesis proceeds. *In situ* hybridization and immunolocalization experiments also confirmed the expression of wild-type Pgr mRNA and protein in follicle (granulosa) cells of primary, cortical alveolus and fully-grown vitellogenic ovarian follicles of the seabream as reported for zebrafish (Hanna et al. 2010). The expression of the Pgr in granulosa cells of vitellogenic follicles would be consistent with a role of this receptor during the ovulation process, as described for other teleosts (Thomas et al. 2007) and mammals (Conneely et al. 2003). We also found strong expression of the *pgr* mRNA in the ooplasm, and of the Pgr protein in the cytoplasm and nucleus, of oogonia presumptively entering meiosis. These observations agree with a recently suggested role of Pgr in progestin-mediated initiation of oogonial meiosis in teleosts (Miura et al. 2006, 2007). However, in primary oocytes, which are arrested in the diplotene phase of the first meiotic division (Selman et al. 1993), the mRNA was restricted to the cytoplasm and the protein product to the nucleus. This specific pattern of expression suggests that the processing of the *pgr* mRNA into protein in the cytoplasm of primary oocytes and further progestin-mediated activation and translocation of the Pgr back into the nucleus is tightly regulated. In a recent study, we found that seabream primary ovarian follicles produce 17,20β-P *in vivo*, as well as in response to seabass rFsh stimulation *in vitro* through the up-regulation of the P450c17-II/20β-hydroxysteroid dehydrogenase pathway in granulosa cells (Zapater et al. 2012). Taken together, these findings may suggest an additional role of 17,20β-P-activated Pgr in the regu-

lation of gene expression in early post-meiotic oocytes prior to entering into the vitellogenic phase. Such a mechanism, however, has yet to be demonstrated in the gilthead seabream or any other teleost.

A novel finding of the present work, which differs from zebrafish oocytes (Hanna et al. 2010), is the peripheral cytoplasmic localization of the nuclear Pgr in a region close to the plasma membrane in seabream cortical alveolus and vitellogenic oocytes. Nuclear Pgrs, as other steroid receptors, exist predominantly in the nucleus and mediate gene transcription, according to their classical role as transcription factors, but a separate pool of functional receptors are localized in the plasma membrane and can activate non-genomic signaling pathways upon ligand-binding (Lösel and Wehling 2003). A palmitoylation motif, FXCXXXX(L/I)L, within the LBD of nuclear steroid receptors has been shown to be crucial for membrane translocation (Pedram et al. 2007). In *X. laevis* oocytes, it has been suggested that in response to P4, the membrane-bound fraction of the nuclear PGR triggers the resumption of the cell cycle in meiosis-arrested oocytes (Bayaa et al. 2000; Tian et al. 2000; Martínez et al. 2006, 2007). Further studies in *X. laevis* and teleosts have disputed this view suggesting that G-protein coupled, transmembrane Pgrs are the major mediators of oocyte maturation in lower vertebrates (Zhu et al. 2003; Josefsberg-Ben-Yehoshua et al. 2007; Hanna and Zhu 2011; Thomas 2012; Tokumoto et al. 2012). However, it has also been found that overexpression of the zebrafish nuclear Pgr in both native and immature oocytes of *X. laevis* accelerates progestin-induced oocyte maturation (Hanna and Zhu 2011), as it occurs when *X. laevis* oocytes are microinjected with homologous *pgr-1* transcripts (Bayaa et al. 2000; Tian et al. 2000). These data suggest that the nuclear Pgr may still be involved in meiosis resumption perhaps through interactions with membrane Pgrs (Hanna and Zhu 2011). Overexpression of the seabream nuclear Pgr in *X. laevis* oocytes also acceletrates P4-induced oocyte maturation (data not shown), and interestingly, the cytoplasmic localization of the Pgr in seabream vitellogenic oocytes coincides with the detection of potential posttranslational modifications of the protein in vitellogenic follicles. The seabream Pgr shows a conserved palmitoylation motif in the LBD (LXCXXXXIL), where only the first Phe is substituted by another hydrophobic residue (Leu), unlike in the zebrafish Pgr where the Iso/Leu or Leu/Leu combination at positions +5/6 relative to Cys, essential for membrane localization (Pedram et al. 2007), is disturbed by Met/Leu (Hanna et al. 2010). Although it remains unknown whether the post-translational modifications of the seabream Pgr occur in the oocyte (and not in granulosa cells), and are related to palmitoylation and membrane localization, our observations would be consistent

with a role of the nuclear Pgr, in addition to membrane Pgrs, in the regulation of oocyte maturation in seabream. Further research is needed to investigate this hypothesis.

In humans and rodents, the PGR-A and PGR-B isoforms, as well as different splice variants (i.e. PGRΔ3, PGRΔ6), are co-expressed in the ovary, with relatively higher levels of PGR-A *vs*. PGR-B expression (Ilenchuk and Walters 1987; Schneider et al. 1991; Misao et al. 1998; Conneely et al. 2003). Different isoform expression titres are also apparent in the seabream, where the *pgr_tv2*, *pgr_tv3* and *pg_tv4* mRNA variants are co-expressed with wild-type *pgr* throughout ovarian development, but in general with higher isoform ratios to full-length *pgr* during early and late oogenesis. For example, the ratio of both dominant-negative *pgr_tv3* and *pgr_tv4* isoforms to *pgr* in the ovary were the highest, ~43% and ~25%, respectively, during the early primary growth stage. Although in the present work we could not determine the level of translation of these isoforms *in vivo*, such transcript levels of *pgr_tv3* and *pgr_tv4* are possibly insufficient for complete inhibition of Pgr-mediated transcription (based on the pattern of inhibition of Pgr_tv3 and Pgr_tv4 in HEK293T cells), but can potentially modulate the transcriptional function of the Pgr. This mechanism would resemble the regulation of PGR-C and other splice forms on PGR-B or PGR-A function suggested to occur in human breast cancer cells and the ovary, respectively (Richer et al. 2002; Condon et al. 2006; Cork et al. 2008). In reproductive tissues, PGR-A and PGR-B control a different subset of genes (Richer et al. 2002; Mulac-Jericevic and Conneely 2004), and hence over-expression of PGR-A protein compared to PGR-B, which is common in breast cancer cells (Graham et al. 1995, 2005), can change progestin responsiveness of cells (Graham et al. 2005). We cannot rule out a similar mechanism in the seabream since it remains to be investigated whether Pgr, Pgr_tv2 and Pg_tv3 can regulate distinct subsets of progestin-dependent target genes in the ovary.

The expression of wild-type Pgr in seabream ovarian follicles at the primary growth stage, together with an elevated ratio of *pgr_tv3* and *pgr_tv4* transcripts, prompted us to investigate the potential endocrine regulation of these isoforms in primary ovaries cultured *in vitro*. Initial experiments were carried out using European seabass rFsh as the gonadotropin source, since seabass rFsh can regulate steroidogenesis *in vitro* in seabream primary ovaries (Zapater et al. 2012). In these trials, we observed that rFsh up-regulated the wild-type *pgr* mRNA and protein levels over time, thus in a similar fashion to that described in mural granulosa cells of mammalian pre-ovulatory follicles where FSH and luteinizing hormone (LH) control PGR-A and PGR-B transcription through a cAMP-mediated pathway (Natraj and Richards 1993; Park-Sarge and Mayo 1994; Clemens et al. 1998). The short-term (24 h) rFsh-mediated

up-regulation of *pgr* expression in seabream primary ovarian explants coincides with the stimulation of 17,20β-P production but not of E2 (Zapater et al. 2012). These observations imply that rFsh, acting most likely via the Fsh receptor (Zapater et al. 2012), simultaneously promotes the synthesis of Pgr and its ligand in primary ovaries, and that this relatively rapid effect is likely estrogen independent as it occurs in mammalian granulosa cells (Clemens et al. 1998). The positive gonadotropic regulation of wild-type Pgr expression may agree with the increased levels of the corresponding polypeptides that we observed in the seabream ovary during vitellogenesis *in vivo*, a period that in most teleosts reflects high levels of circulating Fsh (Levavi-Sivan et al. 2010). Interestingly, however, the expression of the inhibitory *pgr_tv4* isoform in primary explants was only increased after 72 h of rFsh stimulation, when E2 levels are up-regulated in the explants (Zapater et al. 2012), suggesting that *pgr_tv4* expression might be primarily under estrogenic control.

The response of primary explants upon direct E2 stimulation *in vitro* were however more intriguing since short-term treatments resulted in down-regulation of both *pgr* mRNA and protein levels, whereas long-term (72 h) treatments elicited a different effect depending upon whether E2 was replenished in the culture medium. Thus, when E2 was not replaced the expression of Pgr was up-regulated, whereas when the E2 was replaced (i.e. the elevated E2 concentrations were maintained over time) this stimulatory effect was prevented. Conversely, the *pgr_tv3* and *pgr_tv4* transcript levels were highly increased only when E2 was replaced. In the mammalian ovary and brain, E2 increases the expression of PGRs (Graham and Clarke 1997: Micevych and Sinchak 2008), and in zebrafish it has recently been shown that E2 also up-regulates Pgr expression in neurons and radial glial cells (Diotel et al. 2011). In human breast cancer cells, however, E2 can differentially regulate the expression of PGR-A and PGR-B (Vegeto et al. 1993; Graham et al. 1995). Our data suggest that in seabream primary growth-stage ovaries E2 may induce a rapid down-regulation of Pgr expression, perhaps by non-genomic mechanisms, but elicit a latent up-regulation of Pgr expression possibly through a negative feedback mechanism. When the ovarian E2 levels remain high, transcription of *pgr_tv3*, and particularly of *pg_tv4*, are also activated, which may be then available for the modulation of progestin function in the ovary. In any event, the present observations indicate a complex interplay between the endocrine signals exerted by gonadotropin and E2 and the transcriptional regulation of Pgr isoforms in the seabream ovary. Further studies should therefore investigate the cellular sites where these regulatory pathways may take place, and

establish how the Pgr_tv3 and Pgr_tv4 protein levels and potential post-translational modifications control the activity of these receptors.

In summary, we show for the first time that higher orders of Teleostei have likely lost the second nuclear *pgr* gene that should have arisen from the fish-specific whole genome duplication, and express multiple functional isoforms of the extant receptor. Each isoform arises from alternative 5´or exon splicing. Two of the isoforms, Pgr_tv3 and Pgr_tv4, can act as dominant-negative repressors of wild-type Pgr-mediated transcription through nuclear and cytoplasmic mechanisms, respectively. The inhibitory Pgr isoforms are co-expressed at the mRNA level with wild-type Pgr in the ovary, where their expression is differentially regulated *in vivo* during development as well as after gonadotropin and estrogen stimulation *in vitro*. Although the translation of each isoform *in vivo* remains to be unequivocally demonstrated in future studies, the present findings suggest that Pgr-mediated progestin functions in the ovary of advanced teleosts may be regulated through the differential expression of Pgr splice variants. It is thus reasonable to conclude that alternative splicing of the *pgr* mRNA is likely a conserved mechanism that evolved prior to the separation of Actinopterygii from Sarcopterygii to modulate progestin responsiveness.

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Chapter IV

Primary Oocyte Transcriptional Activation of *aqp1ab* by the Nuclear Progestin Receptor Determines the Pelagic Egg Phenotype of Marine Teleosts

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Abstract

In marine teleosts, the *aqp1ab* water channel plays a vital role in the development of the pelagic egg phenotype. However, the developmental control of *aqp1ab* activation during oogenesis remains to be established. Here, we report the isolation of the 5'-flanking region of the teleost gilthead seabream *aqp1ab* gene, in which we identify conserved *cis*-regulatory elements for the binding of the nuclear progestin receptor (Pgr) and members of the Sox family of transcription factors. Subcellular localization studies indicated that the Pgr, as well as *sox3* and *-8b* transcripts, are co-expressed in seabream oogonia, whereas in meiosis-arrested primary growth (pre-vitellogenic) oocytes, when *aqp1ab* mRNA and protein are first synthesized, the Pgr appears to be completely translocated from the ooplasm into the nucleus. By contrast, *sox9b* is highly expressed in more advanced oocytes, coinciding with a strong depletion of *aqp1ab* transcripts in the oocyte. Functional characterization of wild-type and mutated *aqp1ab* promoter constructs, using mammalian cells and *Xenopus laevis* oocytes, demonstrated that *aqp1ab* transcription is initiated by the Pgr, which is activated by the progestin 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P), the natural ligand of the seabream Pgr. *In vitro* incubation of seabream primary ovarian explants with the follicle-stimulating hormone or 17,20β-P confirmed that progestin-activated Pgr enhanced Aqp1ab synthesis via the *aqp1ab* promoter. However, transactivation assays in heterologous systems showed that Sox transcription factors can potentially modulate this mechanism. These data uncover the existence of an endocrine pathway involved in the early activation of a water channel necessary for egg formation in marine teleosts.

Keywords: Aqp1ab, Promoter, Oocyte, Progestin, Pgr, Sox factors

1. Introduction

Prior to zygotic activation, early development of vertebrates is controlled by maternal factors that are synthesized and stored during oogenesis. Studies in different model organisms have shown that these maternal factors, primarily mRNAs, play important roles during early pattern formation of the embryo (Heasman 2006; Langdon and Mullins 2011; Sengupta and Boag 2012). Much less is known, however, concerning the molecular regulation of other maternal factors, which play specific roles during the final stages of egg formation.

In oviparous marine teleosts, such as the gilthead seabrem (*Sparus aurata*), one maternal factor involved in the formation of a competent egg was identified as a molecular water channel controlling the pre-ovulatory hydration of the oocyte (Fabra et al. 2005). Studies in modern and ancient lineages have revealed that this factor is a tandemly arranged teleost-specific water channel termed Aqp1ab, that is synthesized during early oogenesis, transported to the oocyte cortex during the growth (vitellogenic) period, and temporally inserted in the oocyte plasma membrane during meiosis resumption (Fabra et al. 2006; Kagawa et al. 2009; Zapater et al. 2011). Due to a concomitant hydrolysis of yolk proteins and increase of organic and inorganic osmolytes (Cerdà et al. 2007; Finn 2007; Finn and Kristoffersen 2007; Kristoffersen et al. 2009) a massive uptake of water mediated by Aqp1ab occurs, which together with the accumulation of lipids, confers the pelagic (buoyant) phenotype of most marine teleost eggs (Fulton 1898; Finn and Kristoffersen 2007). The process of oocyte hydration in marine teleosts is essential for the survival of the embryos in the ocean since it assures a water reservoir prior to the development of osmoregulatory organs, and facilitates egg dispersal (Fyhn et al. 1999; Finn and Kristoffersen 2007; Cerdà 2009). Indeed, it has been shown that specific immunological inhibition of Aqp1ab in oocytes undergoing meiotic maturation blocks the hydration mechanism (Zapater et al., 2011), resulting in sinking eggs that are unable to develop into embryos (Carnevali et al. 2001). Thus, the pelagic phenotype of marine teleost eggs represents a crucial developmental adaptation that has been associated with the successful radiation of this vertebrate group in the oceanic environment (Finn and Kristoffersen 2007).

Studies of marine teleosts that produce highly or moderately hydrated eggs have revealed an usually high accumulation of *aqp1ab* transcripts in the ovary (Fabra et al. 2006; Tingaud-Sequeira et al. 2008; Singh and Joy 2010; Chaube et al. 2011; Kagawa et al. 2011; Zapater et al. 2011). In contrast, in freshwater teleosts that produce non-hydrated eggs, such as the zebrafish (*Danio rerio*), this mechanism does not occur (Tingaud-Sequeira et al. 2008,

2010), which coincides with the fact that in these species yolk proteolysis and oocyte hydration during meiosis resumption is either absent, or is very minor (Selman et al. 1993; Finn and Fyhn 2010). However, as for tetrapod aquaporins that are expressed in the oocyte, such as AQP3, -7, -9, or AQPxlo (Edashige et al. 2000; Ford et al. 2000; Schreiber et al. 2000; Virkki et al. 2002; Jin et al. 2011), the ovarian regulation of the *aqp1ab* gene is not yet known. The expression of *aqp1ab* in fish oocytes thus offers an opportune experimental system to investigate the transcriptional regulation of aquaporins in vertebrate germ cells. In addition, because the *aqp1ab* gene plays a crucial role during the later stages of egg formation, when developmental regulation may differ from genes involved in the control of maternal RNAs required for early embryogenesis, this model may represent a paradigm for molecular pathways controlling the early expression of genes specifically needed for oocyte development.

To begin to dissect the molecular mechanisms involved in the transcriptional regulation of *aqp1ab* in marine teleost oocytes, we isolated and functionally characterized the promoter of the gilthead seabream *aqp1ab* gene. After cellular localization studies and *in vitro* experiments with ovarian explants, we found that *aqp1ab* transcription and translation are precisely initiated in meiosis-arrested primary growth (pre-vitellogenic) oocytes by the nuclear progestin receptor (Pgr), which is activated by the progestin 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) synthesized by granulosa cells in response to the follicle-stimulating hormone. These data thus reveal a novel endocrine pathway for the tight developmental regulation of *aqp1ab* during oogenesis of a non-mammalian vertebrate.

2. Materials and Methods

2.1. Animals

Adult gilthead seabream were obtained and maintained in the laboratory as previously described (Fabra et al. 2006). Females were collected and sacrificed at different times throughout the natural reproductive cycle. At all sampling times, the gonadosomatic index (GSI) was determined [gonad weight (g)/fish weight (g) x 100] and gonad samples were taken from each fish, frozen in liquid nitrogen and stored at -80°C. Three different pieces of the gonad were processed for *in situ* hybridization, immunofluorescence or *in vitro* culture. Additional gonad samples were fixed in modified Bouin solution (75% picric acid and 25% formalin) to determine the stage of ovarian development by histological analysis as described by Zapater et al. (2012). Procedures relating to the care and sacrifice of animals were approved

by the Ethics Committee from Institut de Recerca i Tecnologia Agroalimentàries (IRTA, Spain) in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

2.2. Cell Culture

The human breast cancer cell line MCF-7 was kindly provided by Dr. Miguel Beato (Centre de Regulació Genòmica and Universitat Pompeu Fabra, Barcelona, Spain). MCF-7 cells were maintained at 37°C in an atmosphere of air/CO_2 [95:5 (v/v)] in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Life Technologies Corp., Carlsbad, CA) supplemented with 10% v/v fetal bovine serum (FBS; Invitrogen), 260 U/ml of penicillin and streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen). Human embryonic kidney cells 293T (HEK293T) were grown under the same conditions.

2.3. Isolation of the Gilthead Seabream *aqp1ab* Promoter

The genomic sequence covering the intergenic cassette between seabream *aqp1aa* and *aqp1ab* loci (~10 kb) was amplified by PCR on liver-extracted genomic DNA using the Expand Long Template PCR system 3 (Roche, Basel, Switzerland) and a forward gene specific primer in the C-terminus of *aqp1aa* (GenBank accession number AY626939; 5'-CGACGTTAACGGAGGCAATG-3') and a reverse primer in the N-terminus of *aqp1ab* (GenBank accession number AY626938; 5'-CCGATGATGGCGGTCAAAC-3'). The PCR product was purified and partially sequenced by BigDye Terminator Version 3.1 cycle sequencing on ABI PRISM 377 DNA analyzer (Applied Biosystems, Life Technologies Corp.) using the *aqp1ab* reverse primer. Using this genomic sequence new primers were designed to sequence up to 1.7 kb upstream of the *aqp1ab* ATG codon. Forward and *aqp1ab* reverse primers were finally employed to amplify a 1.672 kb fragment using a high fidelity polymerase (Easy-A™ High-Fidelity PCR Cloning Enzyme; Agilent Life Sciences, Santa Clara, CA). This fragment showed 100% sequence identity to an overlapping portion from the 5'-end of the *aqp1ab* cDNA, and was cloned into the pGEM-T Easy vector (Promega, Madison, WI), sequenced on both strands, and used for further *in silico* and functional analyses. The nucleotide sequence was submitted to GenBank under accession number KC107825.

2.4. Promoter Constructs and Site-Directed Mutagenesis

The -1.672-kb DNA fragment of the 5' flanking region of the seabream *aqp1ab* gene was subcloned into the *Bgl*II and *Sma*I sites of the luciferase reporter vector pGL3-basic (Promega). Shorter deletion constructs (-1005, -661, -509, -398, and -255 bp) were synthesized by PCR from the pGL3-*aqp1ab*-prom parent construct using different forward primers and a common reverse primer (5'-GCAGATCTCTCCGCGTCGAATGTGTTCG-3'). The forward primers were 5'-GCGCCCGGGGCTTTACACTACTTCAAAGTG-3', 5'-GCGCCCGGGGCTATAAATCC GGCTTGCGTG-3', 5'-CGCCCGGGGATCATTGGGTACGTATTGC-3', and 5'-CGCCCGGGGGGCAGG ACCGATGAGCGGC-3', for the -1005, -661, -509 and -398 bp deletions, respectively. The -255-bp deletion was obtained by digestion of the 1.672-kb fragment with *Xho*I and *Bgl*II. Mutations were introduced into the putative PRE and SOX binding sites of the 1.672-kb or 661-bp wild type fragments using the QuikChange Site-Directed Mutagenesis Kit (Agilent Life Sciences). For the PRE (5'-TGTTCT-3') and SOX (5'-(A/T/G)(T/A)CAATG-3') sites, the sequences were respectively converted to 5'-AAAAAA-3' or 5'-(A/T/G)(T/A)AAAAG-3' (nucleotides substituted are underlined). All constructs were sequenced to verify the desired deletion or mutation, and to check that no mutations were introduced into the luciferase open reading frame.

2.5. RNA Ligase-Mediated Rapid Amplification of 5' cDNA Ends (5' RLM-RACE)

5' RLM-RACE (Maruyama and Sugano 1994; Schaefer 1995; Volloch et al. 1994) was performed using the GeneRacer kit (Invitrogen, Life Technologies Corp.). In short, the total RNA from primary ovaries was extracted using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany) and treated with DNase using the RNase-Free DNase kit (Qiagen) following the manufacturer's instructions. The RNA was dephosphorylated using calf intestinal phosphatase, and then decapped using tobacco acid pyrophosphatase to target full-length messenger RNAs. An RNA oligonucleotide was then ligated to the full-length decapped mRNAs and reverse transcription was performed at 50ºC using an *aqp1ab* cDNA-specific primer (5'-ACTGCAGGATTAAAGTGTG-3') and SuperScript™ III RT (Invitrogen). PCR was done to amplify the resultant cDNAs using the GeneRacer 5' primer and a primer consisting of bases in the N-terminus of the *aqp1ab* cDNA (5'-CTCTGAACCAGAGTGGCGATA-3'). Nested PCR was then done to eliminate the possibility of artifacts using the GeneRacer 5' nested primer and *aqp1ab* primers (5'-GACCTTCAGCTCCTGAGCAA-3'). The RACE nested PCR products were cloned into

the pCR®4-TOPO® vector using the TOPO TA Cloning kit (Invitrogen) and sequenced as above.

2.6. Cloning of Gilthead Seabream Sox cDNAs

Total RNA (10 µg), extracted from the ovary was reverse transcribed using 20 IU of SuperScript II RT (Invitrogen), 0.5 µM oligo(dT)12–18, 40 IU of RNase out and 1 mM dNTPs for 1.5 h at 42°C. The PCR was carried out with 1 µl of the RT reaction in a volume of 50 µl containing 5 µl of PCR buffer plus Mg^{2+}, 0.2 mM dNTPs, 1 µM of each degenerate forward and reverse oligonucleotide primers (5'-ATGAAYGCNTTYATGGTNTGG-3' and 5'-GGNCGRTAYTTR TARTCNGG-3', respectively), and 1 IU of Taq polymerase (Roche). Reactions were amplified using one cycle of 95°C for 5 min, then 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final 7-min elongation at 72°C. The products were cloned into the pGEM-T Easy vector and sequenced. The 5' and 3' ends of three *sox* cDNAs were amplified by one or two consecutive rounds of 5' and 3' RACE (Invitrogen) using specific primers. The full-length cDNAs were finally amplified using the Easy-A™ High-Fidelity PCR Cloning Enzyme and sequenced. The nucleotide sequences of the seabream *sox* cDNAs were submitted to GenBank under accession numbers JX508598 (*sox3*), JX508600 (*sox8b*) and JX508599 (*sox9b*).

2.7. Sequence and Phylogenetic Analyses

The DNA sequence of the 5' flanking region of seabream *aqp1ab* was analyzed with the Matinspector [www.genomatix.de; (Cartharius et al. 2005)], Transcription Element Search [TESS; www.cbil.upenn.edu/cgi-bin/tess; (Schug and Overton 1997)], and Alibaba2.1 [www.gene-regulation.com/pub/programs/alibaba2/index.html; (Grabe 2002)] software resources for the presence of putative binding sites for transcription factors. The 5' genomic regions of teleost *aqp1ab* were retrieved from available Ensembl v70 genomes, including the green-spotted pufferfish (*Tetraodon nigroviridis*), Nile tilapia (*Oreochromis niloticus*), three-spined stickleback (*Gasterosteus aculeatus*), and zebrafish. The absence of the *aqp1ab* gene previously reported for medaka (*Oryzias latipes*) (Tingaud-Sequeira et al. 2008) was confirmed here for the southern platyfish (*Xiphophorus maculatus*), a related atherinomorph teleost. In addition to the ensembl data, the intergenic region of the Atlantic salmon (*Salmo salar*) *aqp1aa* and *aqp1ab* genes was retrieved from the National Center for Biotechnology Information (NCBI) whole-genome shotgun contigs database (www.ncbi.nlm.nih.gov). Each intergenic region was analyzed as above.

For phylogenetic analyses of Sox transcription factors, the deduced amino acid sequences of the seabream *sox3*, *sox8b* and *sox9b* nucleotides were aligned (t-coffee and MAFFT) in relation to 116 vertebrate orthologs, converted to codon alignments using Pal2Nal (Suyama et al. 2006) and analyzed via Bayesian (Mr Bayes v3.2 with 2 million MCMC generations) and maximum likelihood (PAUP v4b10-x86-macosx) protocols as described previously (Finn and Kristoffersen 2007; Kristoffersen et al. 2009; Tingaud-Sequeira et al. 2010; Applebaum et al. 2012).

2.8. Transient Transfection and Reporter Assays in Cultured Cells

MCF-7 and HEK293T cells were plated in 24-well plates as described above and maintained until cells reached 90-95% confluence. For HEK293T cells, transient transfections were performed in the same medium using a standard calcium phosphate precipitation method. For MCF-7 cells, transfections were done in serum-free DMEM with 260 U/ml of penicillin and streptomycin and 2 mM L-glutamine using Fugene (Promega) according to the manufacturer's instructions. HEK293T cells were co-transfected with 75 ng of wild type or mutated pGL3-*aqp1ab*-prom luciferase reporter plasmid, 75 ng of the Pgr, Sox3, Sox8b or Sox 9b expression vectors [cloned into the *Eco*RI and *Xba*I sites of the pcDNA3 vector (Invitrogen)], and 50 ng of β-Galactosidase (β-Gal) plasmid (Promega), as indicated for each experiment. To test the effect of the coexpression of Pgr and Sox factors different amounts of the empty vector pcDNA3 were cotransfected to use equal amounts of total DNA in the individual transfections. Control cells were transfected with the same amounts of pGL3 and pcDNA3 empty vectors and β-Gal. MCF-7 cells were co-transfected only with the pGL3-*aqp1ab*-prom and β-Gal plasmids. After 24 h, for both HEK293T and MCF-7 cells, the medium was replaced by transactivation assay medium, DMEM/F12 without phenol red (Invitrogen) supplemented with 5% v/v charcoal-stripped FBS (Invitrogen), 260 U/ml of penicillin and streptomycin and 2 mM L-glutamine, containing different steroids (purchased from Sigma-Aldrich). MCF-7 cells, expressing endogenous PGR, were exposed to progesterone (P4; 1-100 ng/ml), whereas HEK293T cells expressing the seabream Pgr were stimulated with 17,20β-P, 17α,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P), 17β-estradiol (E2), or testosterone (T) (from 10^{-10} M to 10^{-5} M). In all cases, the final concentration of the ethanol vehicle was 0.1% (v/v), and the steroids were tested in triplicate. In other experiments, HEK293T cells were stimulated with 0.1 μM 17,20β-P in the presence or absence of 10 μM of the mammalian PGR antagonist RU486 (Sigma-Aldrich). After incubation at 37°C for 24-36 h, the cells were harvested in Reporter Lysis buf-

fer (Promega), incubated at -80°C for 2-3 h, vortexed, centrifuged at maximum speed during 1 min, and the supernatant collected. Luciferase activity was determined using the Luciferase Assay System (Promega) on 20 µl of cell lysates in a 96-well plate, and luminescence was measured with a Orion II microplate luminometer (Titertek-Berthold). Luciferase activity was normalized to β-Gal activity measured by colorimetric detection using nitrophenyl β-D-galactopyranoside (Sigma-Aldrich) substrate. Reporter activity was expressed as the ratio of normalized luciferase activity to that of control cells.

2.9. Microinjection and Luciferase Assays in *Xenopus laevis* Oocytes

Luciferase assays in *X. laevis* oocytes were carried out as previously described (Tian et al. 2000; Li et al. 2009) with some modifications. The seabream Pgr, Sox3, Sox8b and Sox9b cDNAs were subcloned into the *Eco*RV and *Spe*I sites of the pT7Ts expression construct (Deen et al. 1994), and capped RNAs (cRNAs) were synthesized as previously described (Zapater et al. 2011). The isolation, defolliculation, and injection of *X. laevis* oocytes were done as described previously (Deen et al. 1994). Stage IV oocytes were injected with 25 nl of water containing 5 ng of the β-Gal plasmid and 5 ng of wild-type or mutated pGL3-*aqp1ab*-prom luciferasa reporter construct, or empty pGL3 (controls), and incubated in modified Barth's medium (MBS) for 24 h at 18ºC. Oocytes were then injected again with water (controls) or 5 ng of Pgr and/or Sox3, Sox8b or Sox 9b cRNAs in a volume of 25 nl and incubated in MBS at 18ºC for 24 h. After 24 h, oocytes were exposed to 100 ng/ml 17,20β-P or 0.1 % (v/v) ethanol vehicle for 6 h at 18ºC, and subsequently oocytes were homogenized in 200 µl of reporter lysis buffer (Promega). The homogenate was centrifuged at 14,000 x g for 15 min at 4ºC, and the aqueous supernatant collected to measure luciferase activity as described above. Reporter activity was expressed as the ratio of normalized luciferase activity to β-Gal to that of control oocytes.

2.10. *In situ* Hybridization

Ovarian pieces at different developmental stages were fixed in 4% paraformaldehyde (PFA) for 16-20 h at 4ºC to determine the sites of expression of *aqp1ab*, *sox3*, *sox8b* and *sox9b* by *in situ* hybridization according to previously described protocols (Zapater et al. 2012). DIG-labelled sense and antisense riboprobes were synthesized with SP6 and T7 RNA polymerases using the DIG RNA labelling Kit (Roche). The probes were as follows: for *aqp1ab*, nucleotides 700-948; for *sox3*, nucleotides 301-950; for *sox8b*, nucleotides 1145-1551; and for *sox9b*, nucleotides 1301-1664. Post-hybridization washing steps were 2x SSC with 50%

formamide for 30 min at 50ºC, two washes in 2x SSC at 42ºC or 50ºC for 15 min, and one wash in 0.5x SSC or 0.2x SSC at 42ºC or 50ºC, respectively, for 15 min. Sections were examined and photographed with a Zeiss imager.z1 microscope (Carl Zeiss MicroImaging, S.L, Oberkochen, Germany).

2.11. Immunofluorescence Microscopy

Ovarian samples were fixed with 4% PFA for 6 h at room temperature and subsequently dehydrated and embedded in Paraplast (Sigma-Aldrich). Sections of ~6 µm in thickness were blocked with 5% goat serum, 0.1% BSA in PBST (0.1% Tween in PBS) for 1 h, and incubated (1:400) with affinity-purified antibodies against gilthead seabream Pgr (Zapater et al. 2013) or Aqp1ab (Fabra et al. 2005) in PBST with 1% goat serum and 0.1% BSA overnight at 4°C. For Aqp1ab antigen retrieval, sections were treated with PBS containing 0.1% SDS for 10 min at room temperature before blocking. After the incubation with the primary antibodies, sections were washed with PBST and incubated with secondary anti-rabbit IgG FITC- or Cy3-coupled antibodies (Sigma-Aldrich) for 2 h at room temperature. Sections were counterstained with 4',6-diamidino-2-phenylindole (1:3000; DAPI; Sigma-Aldrich) for 3 min and mounted with Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich). Images were documented as above.

2.12. *In vitro* Incubation of Ovarian Explants

Ovaries containing only oogonia and primary growth stage follicles, were placed in Petri dishes with 75% Leivovitz L-15 culture medium with L-glutamine (Sigma-Aldrich) and 100 µg/ml gentamicine at pH 7.5. Ovaries were manually dissected into small fragments (~100 mg) and placed in 24-well plastic tissue culture dishes containing 1 ml of fresh culture medium. For each experiment, explants from one female were incubated in triplicate with recombinant, single-chain European seabass (*Dicentrarchus labrax*) follicle-stimulating hormone (rFsh; 1-100 ng/ml), produced as previously reported (Zapater et al. 2012), or with 17,20β-P (10 or 100 ng/ml). Control groups were treated with an equivalent volume of the cell culture medium used for rFsh production or ethanol. In some experiments, explants were preincubated with 100 µM of the steroidogenesis inhibitor DL-aminoglutethimide (AGI), the carbonyl reductase inhibitor indomethacin (Indo; Sreenivasulu and Senthilkumaran, 2009; Zapater et al., 2012), or the Pgr antagonist RU486, for 1 h prior to the addition of the hor-

mones. Explants were cultured at 18ºC in a temperature-controlled incubator up to 72 h. Every 24 h, tissues were harvested, frozen in liquid nitrogen and stored at -80°C until analysis.

2.13. Real-time Quantitative Reverse Transcription PCR (qRT-PCR)

Quantification of *aqp1ab* transcripts in whole ovaries at different developmental stages was performed by qRT-PCR using SYBR green qPCR master mix (Life Technologies Corp.) as previously described (Chauvigne et al. 2010). Specific *aqp1ab* forward and reverse primers were 5'-GCGACGGAGTGATGTCAAAGG-3' and 5'-AGATAAGAGCCGCCGCTATGC-3', respectively. Cycle numbers were normalized to *18s* ribosomal RNA, for which expression levels did not change significantly between experimental samples (data not shown), using the forward and reverse primers 5'-GAATTGACGGAAGGGCACCACCAG-3' and 5'-ACTAAGAACGGCCATGCACCAC CAC-3', respectively. The relative transcript level was calculated by using a standard curve generated for each primer pair from 10-fold serial dilutions of a pool of first-stranded cDNA template from ovary samples. All calibration curves exhibited correlation coefficients higher than 0.98, and the corresponding qRT-PCR efficiencies were greater than 99%.

The comparative Ct method was used for relative quantification of the *aqp1ab* transcript levels in ovarian explants after hormone treatment *in vitro*. Primers were as above and the quantification was also normalized to *18s*. Fold-changes in the relative mRNA expression with respect the 24 h controls were determined using the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

2.14. Immunoblotting

Ovarian pieces were homogenized in cold lysis buffer [50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 0.5% Na deoxycholate, 0.5% SDS, 10 mM DTT, 10 mM NaF, 0.5 mM Na_3VO_4 and protease inhibitors (EDTA-free Protease Inhibitor Cocktail Tablets, Roche)], and centrifuged at 14,000 x *g* for 10 min at 4°C. One aliquot of the supernatant was removed to determine the protein concentration with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories Inc., Hercules, CA), and the rest mixed with 4 x Laemmli sample buffer. For immunoblotting, 30 µg of total protein were denatured at 95°C for 10 min, subjected to 8.5% SDS-PAGE, and transferred onto nitrocellulose membranes as previously described (Zapater et al. 2011). The membranes were blocked with 5% non-fat milk powder in TBST for 1 h at room temperature and subsequently incubated overnight at 4 ºC with the seabream Aqp1ab antiserum (1:500). Bound antibodies were detected with 1:2000-diluted goat anti-rabbit IgG

coupled to horseradish peroxidase (Rockland, Gilbertsville, PA), and proteins were visualized by using enhanced chemiluminescence (Picomax, Rockland). For semi-quantitative determination of Aqp1ab abundance in primary ovarian explants after hormone treatment *in vitro*, triplicate protein samples were transferred to duplicate membranes and incubated with either anti-Aqp1ab antiserum or anti-alpha-tubulin rabbit antibody (0.3 µg/ml; abcam ab18251). The corresponding reactive bands were analyzed by densitometry using the Quantity-One software (Bio-Rad Laboratories Inc.).

2.15. Chromatin Immunoprecipitation (ChIP) Assays

Frozen ovarian explants previously incubated *in vitro* with ethanol or 100 ng/ml 17,20β-P for 72 h, in the presence or absence of RU486, were cross-linked by homogenization in 5 ml of buffer A (60 mM KCl, 15 mM NaCl, 15 mM HEPES pH 7.6, 60 mM $MgCl_2$, 0.5% Triton X-100, 0.5 mM DTT, and protease inhibitors) containing 1.5% formaldehyde for 15 min at room temperature with rocking. The cross-linking reaction was quenched with 225 mM glycine for 5 min. The homogenate was centrifuged at 4,000 x *g* for 5 min at 4°C, and the pellet washed twice in buffer A and once in lysis buffer (140 mM NaCl, 15 mM HEPES pH7.6, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.5 mM DTT, 0.1% Na deoxycholate and protease inhibitors). The samples were then lysed in lysis buffer supplemented with 0.1% SDS and 0.5% Triton X-100 and sonicated with three 15-sec pulses at 30% amplitude on ice using a Digital Sonifier® S-250D (Branson Ultraschall, Dietzenbach, Germany). The sonicated chromatin was spun for 15 min at 14,000 x *g* to pellet celluar debris, and the supernatant was diluted 1:1 (v/v) with the ChIP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1 and 167 mM NaCl). Ten percent of the volume of the supernatant was removed and kept as "input". The remaining supernatant was pre-cleared by adding activated G protein magnetic beads (Merck Millipore, Billerica, MA) and salmon sperm DNA (Sigma-Aldrich) and incubated for 30 min at 4ºC with rocking. The precleared chromatin was divided into two equal aliquots and each aliquot incubated overnight at 4°C with 10 µg of the seabream Pgr specific antibody or normal rabbit IgG. Next, activated G protein magnetic beads and salmon DNA sperm were added to the extract and incubated for 1 h at 4ºC. The beads were washed at 4ºC while rocking with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 2 mM Tris-HCl pH 8.1, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 2 mM Tris-HCl pH 8.1, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% Triton X-100, 1% Na deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), and TE buffer pH 8.0. After the washes, the chroma-

tin was eluted from the beads with the elution buffer (1% SDS and 0.1M $NaHCO_3$), and both immunoprecipitated products and input samples reverse cross-linked by adding 200 mM NaCl at 65°C overnight. Subsequently, samples were digested with 20 µg proteinase K (Sigma-Aldrich) and purified with phenol/chloroform. Immunoprecipitated and input DNA from each replicate were analyzed in triplicate by real-time quantitative PCR (qPCR) using specific primers flanking the putative PRE(a) (forward 5'-TTTGCCAGTGTTGCACTCAT-3', and reverse 5'-TCTGGGCACCAACAGAGAAT-3') and PRE(b) (forward 5'-GGTGGCAGCTCTCCTGTATT-3', and reverse 5'-CAGGCTACGTCCTTGACCAT-3') in the seabream *aqp1ab* promoter. For PRE(a) and PRE(b) primers, the DNA was diluted 10- and 50-fold, respectively, and the PCR amplification protocol was the same as for qRT-PCR. For quantification, the calculated chromatin concentration (determined with the ΔΔCt method) obtained with the anti-Pgr antibody was normalized with the input chromatin specific for each condition since the level of background signal (IgG) with both primer sets was undetectable for all experimental conditions (not shown).

2.16. Statistical Analysis

All reporter and ChIP experiments, and *in vitro* incubations, were repeated between three and four times (as indicated in the figure legends), with all treatments performed in triplicate. The data represent means (± SEM) of independent experiments carried out on cells from different transfections or on different batches of oocytes, or peformed on single females, such that *n* equals the number of experiments/females in each case. Data were statistically analyzed using one- or two-way analysis of variance (ANOVA), or by Kruskal-Wallis one way ANOVA on Ranks, followed by Tukey's pairwise comparison. Significance was assessed relative to $P < 0.05$.

3. Results

3.1. *aqp1ab* is Transcribed and Translated in Primary Growth Oocytes *In Vivo*

We intitially re-examined the developmental regulation of *aqp1ab* gene expression during gilthead seabream oogenesis by performing *in situ* hybridization and immunofluorescence microscopy (Fig. 1). The seabream shows a group-synchronous ovary in which ovarian follicles containing oocytes from previtellogenesis up to the maturation stage can be sequentially observed in a single ovary during the reproductive season (Fabra et al. 2006). Thus, histological sections of each stage (oogonia, previtellogenic, primary growth [i.e perinucleolus stage],

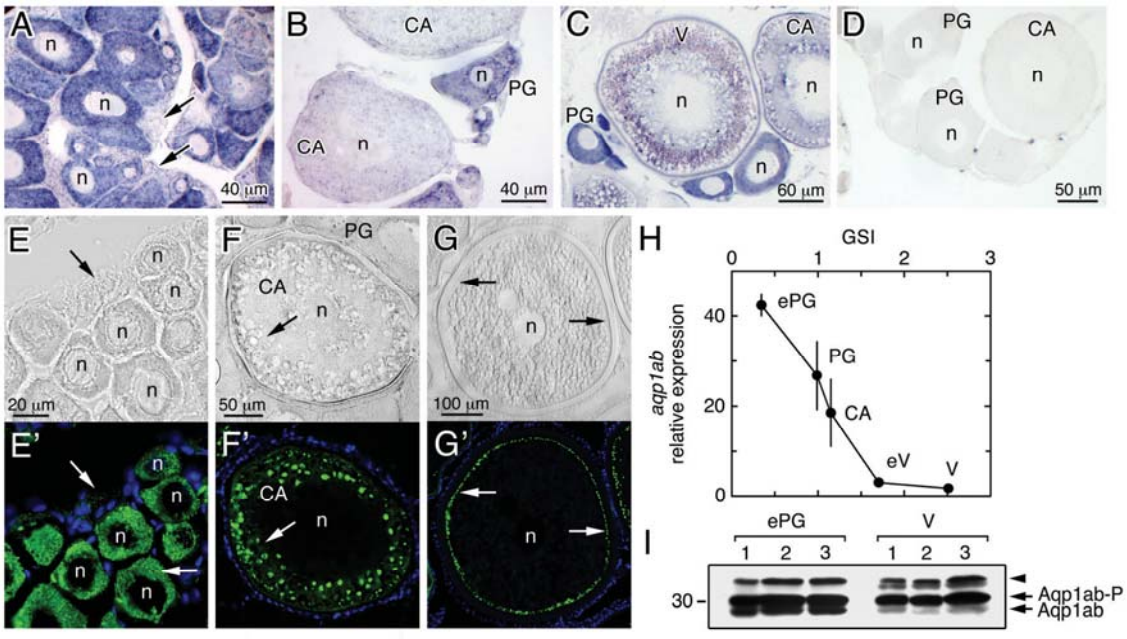


Fig. 1. Representative (n = 3 females) photomicrographs of *aqp1ab in situ* hybridization (A-D), and Aqp1ab bright field (E-G) and immunofluorescence microscopy images (E'-G'), on paraffin sections of ovaries at the primary growth (A and E) or vitellogenic stages (B-D and F-G). For immunofluorescence, sections were counterstained with DAPI (blue color) for visualization of cell nuclei. Transcripts for *aqp1ab* are accumulated in the cytoplasm of primary growth oocytes, whereas oogonia (arrows) are negative (A). The expression of *aqp1ab* in cortical alveolus and vitellogenic oocytes was hardly detectable (B and C). Control sections incubated with sense probes were negative (D). Antigen retrieval treatment with 0.1% SDS revealed that Aqp1ab polypeptides colocalize with the mRNA in the cytoplasm of primary growth oocytes (E and E'). In cortical alveolus (F) and vitellogenic oocytes (G), the protein is translocated towards the oocyte plasma membrane (arrows in F' and G'). Note that in these oocytes, Aqp1ab can be detected without SDS treatment, whereas in primary growth oocytes (E' and F') SDS is necessary. Follicle cells surrounding primary growth or vitellogenic oocytes did not express Aqp1ab. n, nucleus; PG, primary growth oocyte; CA, cortical alveolus oocyte; V, vitellogenic oocyte. H, Quantitative changes in *aqp1ab* expression determined by qRT-PCR of ovarian samples in relation to the gonadosomatic index (GSI) of females and oogenic stage of development. Data represent relative mean expression (± SEM; n = 4-5 females), normalized to *18s* ribosomal RNA, in ovaries at early or more developed primary growth stages (ePG and PG, respectively), in both cases containing exclusively oogonia and primary growth oocytes, or at the cortical alveolus (CA), early vitellogenic (eV), and vitellogenic (V) stages. I, Representative Aqp1ab immunoblot of protein extracts from ovaries at the ePG and V stages from three females. The arrows indicate dephosphorylated (Aqp1ab) and phosphorylated (Aqp1ab-P) aquaporin monomers (Tingaud-Sequeira et al., 2008), whereas the arrowhead points to other post-translational modifications of Aqp1ab or potential Aqp1ab dimers. Molecular mass marker (kDa) is on the left.

cortical alveoli, and vitellogenic oocytes), allowed us to determine the cellular sites of *aqp1ab* mRNA and Aqp1ab protein expression during oocyte development *in vivo*.

Transcripts of *aqp1ab* were first noted by *in situ* hybridization in the cytoplasm of primary growth oocytes, whereas no signal was observed in oogonia (Fig. 1A). At later stages of oocyte development, i.e. cortical alveoli and vitellogenic stages, *aqp1ab* expression in the oop-

lasm was very low (Fig. 1, B and C). Control sections incubated with sense probes were negative (Fig. 1D). Immunofluorescence microscopy using a seabream Aqp1ab affinity-purified antibody (Fabra et al., 2005) and antigen retrieval treatments (i.e. 0.1% SDS) revealed the presence of Aqp1ab polypeptides spread in the cytoplasm of primary oocytes (Fig. 1, E and E'), in cytoplasmic vesicles located in the peripheral ooplasm of cortical alveoli oocytes (Figs. 1, F and F'), and within a thin layer below the oocyte plasma membrane of advanced vitellogenic oocytes (Fig. 1, G and G'). These data thus confirm that seabream oogonia do not express *aqp1ab* mRNA or protein product, but show for the first time that both molecules are initially expressed and co-localized in primary growth oocytes.

Determination of *aqp1ab* transcript levels in different ovarian stages by qRT-PCR revealed an inverse relationship to the GSI, where the highest levels are accumulated in ovaries at an early primary growth stage, but decline to background levels by the early vitellogenic phase (Fig. 1H). Such a strong reduction of the *aqp1ab* transcript levels during ovarian growth is possibly due to the associated decrease in the frequency of primary follicles in the ovary (up to ~85%) (Zapater et al., 2012). Immunoblotting experiments confirmed the immunocytochemical data showing no change in the Aqp1ab expression titre between the early primary growth and vitellogenic stages (Fig. 1I), although the proportion of dephosphorylated *vs*. phosphorylated Aqp1ab (Tingaud-Sequeira et al., 2008) seems to be lower in vitellogenic ovaries (Fig. 1I, arrows). Additional Aqp1ab reactive bands of ~50 kDa were also detected in both ovarian stages (Fig. 1I, arrowhead) which may correspond to glycosylated forms of Aqp1ab or Aqp1ab dimers. These data thus suggest that there is a short temporal phase of *aqp1ab* gene transcription in the primary growth oocytes, and that the resultant transcripts are immediately translated prior to their depletion at the onset of vitellogenesis. The *de novo* pool of Aqp1ab proteins in the primary growth oocytes may be post-translationally modified (phosphorylated), transported and stored in the peripheral ooplasm during subsequent stages of oocyte development.

3.2. Isolation of the Gilthead Seabream *aqp1ab* Promoter and Mapping of the Transcription Initiation Sites

Since the genome sequence of the gilthead seabream is not available, the first step to investigate the transcriptional regulation of *aqp1ab* was to isolate its promoter region from purified genomic DNA. In teleosts, the *aqp1aa* and *aqp1ab* loci are tandemly arranged in the genome (Tingaud-Sequeira et al. 2008; Zapater et al. 2011), and therefore the *aqp1ab* 5'

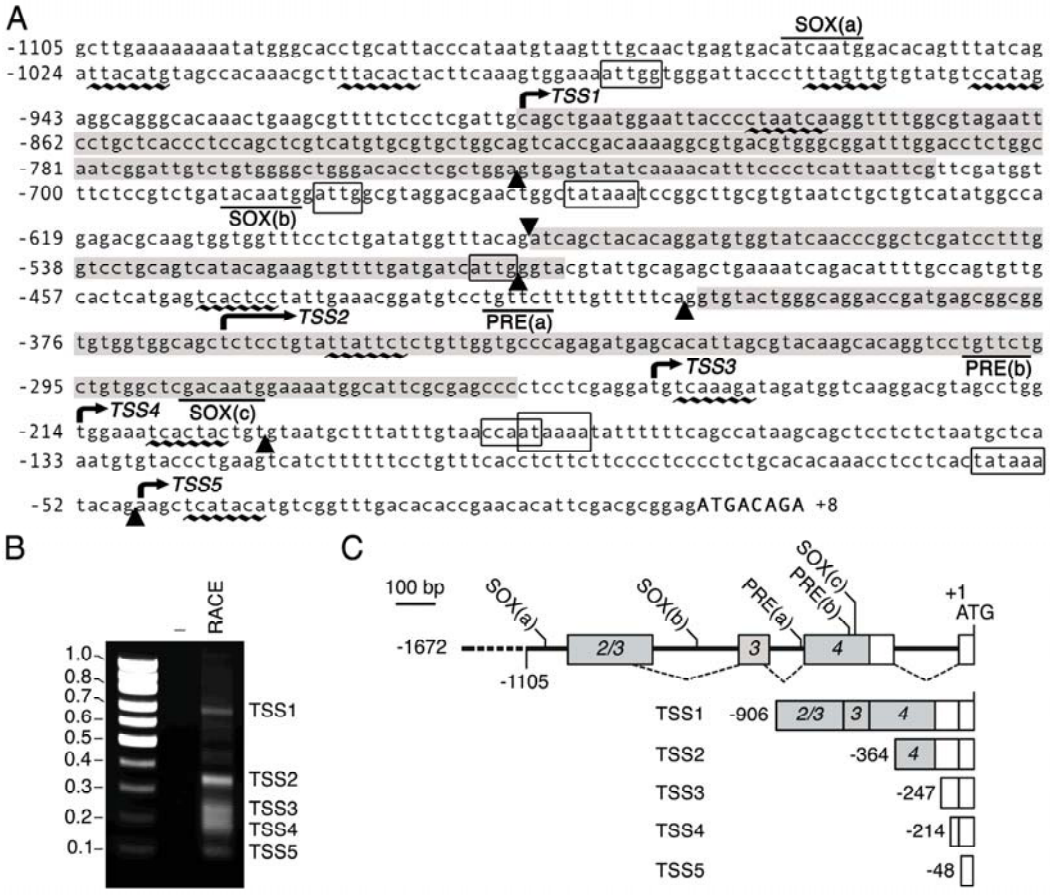


Fig. 2. Nucleotide sequence of the gilthead seabream *aqp1ab* proximal promoter and mapping of the transcription initiation sites (TSS) by RLM-RACE. A, The proximal 1.10-kb nucleotide sequence flanking the 5' of the *aqp1ab* gene is shown (see also Supplemental Fig. S1). Numbers on the left indicate distance from position +1 which has been assigned to the first nucleotide of the translation start codon ATG. The TSSs determined by RLM-RACE (see B below) are indicated by right-angled arrows, whereas the splicing sites for mRNA synthesis are indicated by arrowheads (see B below). The three genomic regions upstream of the *aqp1ab* gene that show sequence similarity to exons 2/3, 3 and 4, respectively, of the *aqp1ab* gene are highlighted in grey. *In silico* identified TATA and CCAAT boxes in the *aqp1ab* promoter are boxed, lnr and lnr-like sequences are underlined with wavy lines, and core sequences for progestin responsive elements (PREs) and SOX binding sites are underlined with straight lines. B, Agarose gel electrophoresis of nested PCR reaction products from the RLM-RACE procedure using RNA isolated from seabream primary ovaries. Controls (minus) omitted the cDNA in the PCR reaction. Molecular size markers (kb) are indicated on the left. On the right, the major PCR products are marked. C, Schematic representation of the duplicated seabream *aqp1ab* pseudogene upstream of the translation initiation codon showing the putative exons (boxed in grey), the PRE and SOX binding sites, and the structure of different mRNAs originating from five major TSS (TSS1-TSS5). The TSSs were identified by cloning and sequencing the RLM-RACE products shown in B. The grey boxes indicate sequences of the mRNA 5' UTR showing similarity with *aqp1ab* exons 2/3, 3 and 4.

flanking region was amplified by PCR using specific primers designed to the C- and N- termini of the previously cloned (Fabra et al. 2005) gilthead seabream *aqp1aa* and *aqp1ab* cDNAs, respectively. Further PCR using genomic specific primers identified a product of 1,770 bp (Fig.

2A), showing an identical match to 38 bp in the 5' UTR of the *aqp1ab* cDNA, and to 98 bp coding for the N-terminus of the deduced protein (Fabra et al. 2005), confirming that this genomic sequence was directly upstream of the *aqp1ab* gene. Analysis of the nucleotide sequence of the *aqp1ab* most proximal 5' flanking region (1,672 bp) revealed three domains (nucleotides -906 to -706, -581 to -498, and -405 to -259, considering position +1 the first nucleotide of the translation start codon ATG) with similarity to exons 2/3 (59% identity), 3 (71% identity) and 4 (40% identity), respectively, of the *aqp1ab* gene (Fig. 2A), suggesting the presence of traces in the genome of a duplicated *aqp1ab* pseudogene.

The transcription start sites (TSSs) of the seabream *aqp1ab* gene were subsequently determined by 5' RLM-RACE using RNA isolated from primary ovaries. Five different bands were obtained after the 5' RACE nested PCR reaction with estimated sizes of ~630, 320, 260, 180 and 100 bp, with the 320-bp band being the most discrete and intense (Fig. 2B). Cloning and sequencing of these PCR products revealed five different DNA sequences, each preceded by a sequence corresponding to the RNA oligonucleotide adapter and thus representing authentic cap sites. These sites map to -906, -364, -247, -214, and -48 nucleotides upstream of the *aqp1ab* start codon (Fig. 2C), indicating the presence of alternative TSSs. Interestingly, alignment of the 5' UTR nucleotide sequence of the five different mRNAs with that of the 5' flanking genomic region of *aqp1ab* indicated the existence of mRNA splicing at sites close to the putative exons of the *aqp1ab* pseudogene (Fig. 2A).

The full 1.6-kb 5' flanking genomic sequence of the seabream *aqp1ab* gene was analysed for putative *cis*-acting regulatory sequences using the Matinspector and TESS software resources. These analyses revealed the presence of consensus sequences for core promoter elements important for the interaction with the basal transcription machinery, such as several TATA and CCAAT boxes and Sp1, AP1, E-box and initiator (lnr) elements (Fig. 2A and Table 1). The sequence also contained many motifs that could be bound by caudal-related homeobox (CDX), Krüppel-like (KLF), Pre-B cell leukemia (PBX) and NKX-homeodomain transcription factors involved in gut and kidney development and disease, and three potential cAMP-responsive element binding protein (CREB) sites (Table 1). In addition, the proximal promoter region of seabream *aqp1ab* contained putative binding sites for the nuclear PGR and for members of the SOX family of transcription factors, both known to be expressed in the oocyte of teleosts (Kanda et al. 1998; Rodríguez-Marí et al. 2005; Hanna et al. 2010, Zapater et al. 2013). Two progestin-responsive elements (PRE), termed PRE(a) and PRE(b), were respectively identified between nucleotides -423 to -416 and -302 to -297, based upon the consen-

sus core sequence TGTTCT (Yin et al., 2012), and three SOX binding sites, termed SOX(a), SOX(b) and SOX(c), were respectively identified between nucleotides -1046 to -1040, -688 to -682 and -286 to -280 based upon the consensus sequence A/T/G)(A/T)CAATG (Mertin et al. 1999) (Fig. 2A). Both PRE and SOX *cis*-elements were also detected in the *aqp1ab* 5' flanking region of different teleosts, regardless of whether they reproduce in seawater or freshwater and spawn pelagic or benthic eggs (Fig. 3).

Table 1. Putative *cis*-acting regulatory sequences in the gilthead seabream *aqp1ab* promoter [1]

Family	Description [2]	Core sequence [3]	Position(s) [4]
TATA	CPE for basal transcription	ATAAA (-)	-1526; -1158
		TATAA (+)	-1443
		TATAAAA (-)	-1402
		TATAAA (+)	-1189; -662; -61
		TATAAAA (+)	-181
Sp1	Sp1 general activator recognition site	GGGG (+)	-1315
		GGTG (+)	-1310
		CCTCCA (-)	-853
		GGGGCTGGGA (+)	-766
		ACTCCT (-)	-445
		CCTCCT (-)	-259
AP1	Activator protein 1	ATGAGTCAC (-)	-452
		CATGAGTCACT (-)	-451
E-box		CATATGGC (+)	-629
CAAT	CCAAT box	CCACCAATT (-)	-986
		CCAAT (-)	-551
		CCAAT (+)	-184
lnr, lnr-like	CPE initiator sequences	TTACATG (+)	-1023
		TTACACT (+)	-1002
		TTAGTTG (+)	-963
		CCATAGA (+)	-949
		CTAATCA (+)	-887
		TCACTCC (+)	-447
		TTATTCT (+)	-355
		TCAAAGA (+)	-245
		TCACTAC (+)	-208
		TCATACA (+)	-43
PRE	Progestin responsive element	TGTTCT (+)	-434; -313
SOX	Sox binding site	CAAT (+)	-1050; -692; -290
TCF/LEF-1	Wnt signal transduction pathway	ATCAATG (+)	-1051
		TCAAAG (+)	-998; -251
		ACCAAAG (-)	-548
CREB	cAMP-responsive element binding protein	TGACATCA (-)	-1263
		TGACAT (+)	-1056
		TGACG (+)	-817
CDX2	Cdx-2 caudal-related intestinal factor	AGTTTTCTGAC (-)	-1481
		CTTTTTATTCC (-)	-1192
		GGATTTATAGC (-)	-665
		TATTTTATTGG (-)	-184
KKLF	Kidney-enriched kruppel-like factor (KLF15)	GGGGTG (+)	-1318
		GGGGAG (-)	-90

(continued on next page)

Table 1. *continued*

Family	Description [2]	Core sequence [3]	Position(s) [4]
GKLF	Gut-enriched Krueppel-like factor (KLF4)	AAAAATTAAGGG (+)	-1326
		AAAAAATATGGG (+)	-1099
		ACAACTAAAGGG (-)	-967
		AAAGG (-)	-549
BKLF	Basic Krueppel-like factor (KLF3)	GGGTG (+)	-1313
PBX-HOX	PBX-HOX binding site	GTGATATAT (-)	-1666
		TTGATTTAT (+)	-1591
		ATGATTTTT (-)	-1512
		GATTTAT (-)	-663
		GATAGAT (+)	-245
		ATGCTTTAT (+)	-198
PBX1-MEIS1	Binding site for a Pbx1/Meis1 hetero-dimer	TGATGTCAT (+)	-1257
NFKAPPAB	NF-kappaB (NFKB1)	GGGATTTTTC (-)	-1416
		GGGATTACCC (+)	-976
PAX	Pax bindig site	ATTGAATCATGATTAAAG (+)	-1381
		CACTCATG (+)	-461; -457
		AGTCACTCCTATTGAAAC (+)	-452
		GCCACAGCAGAACAGGACC (-)	-312
GATA	GATA TF binding site	GATAA (+)	-1575
		GATAA (-)	-1397
		GAT (-)	-1172
		GATA (-)	-1035
		GATA (+)	-694
NKX	Hmx3/Nkx5-1 or Nkx-2.5/Csx homeo-domain TFs	TTAA (-)	-1636
		AAATGTTT (+)	-1559
		AAACATTT (-)	-1554
		AATAATT (+)	-1457
		TAAGTA (+)	-1443
		TTAA (+)	-1324
		TAAGTT (+)	-1071
		AAACGCTT (+)	-1015
		AAGCGTTT (-)	-1010
		AAGCGTTT (+)	-932
		AAACGCTT (-)	-927
		AAACATTT (+)	-738
		AAATGTTT (-)	-733
		AAGTG (+)	-618
		AAACGGAT (+)	-441
HIF1	Hypoxia induced factor-1 binding site	ACGT (-)	-806
PARF	PAR-type chicken Vtg promoter-binding protein	GTAA (+)	-1067

[1] The seabream *aqp1ab* nucleotide sequence was analyzed with the Matinspector and TESS softwares. Only relevant sites with a good matrix similarity (≥0.8) are shown.
[2] Abbreviations: CPE, core promoter element; TF, transcription factor; Vtg, vitellogenin.
[3] Plus or minus indicate that the site is on the forward or reverse strand, respectively.
[4] Position -1 was assigned to the first nucleotide 5' to the ATG first codon.

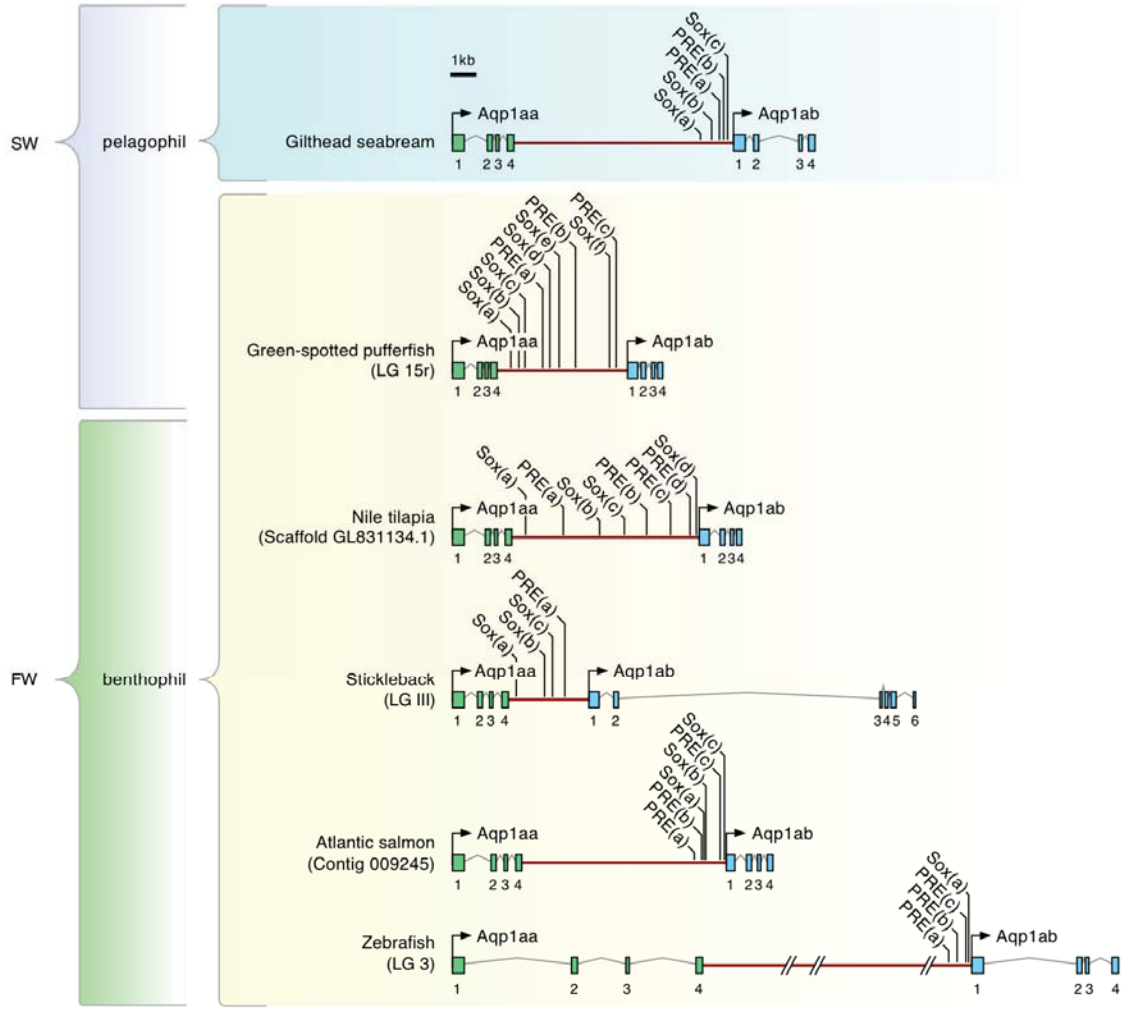


Fig. 3. Genomic organization of teleost *aqp1aa* and *aqp1ab* paralogs (drawn to scale) showing the position of consensus sequences for progestin response elements (PREs) and sex determining region Y-box (SOX) binding sites in the promoter regions of teleost *aqp1ab*. Data are arranged according to phylogeny, and whether the species spawns pelagic (pelagophil) or benthic (benthophil) eggs in seawater (SW) or freshwater (FW). Exon numbers are indicated below each gene. Only the putative proximal (~1 kb) *cis*-regulatory elements are shown for Atlantic salmon and zebrafish. Double diagonal lines indicate polyN breaks in the intergenic region of zebrafish. See materials and methods in the main text for details of response element identification.

3.3. Progestin Receptor and Sox Transcription Factors are Differentially Expressed in Seabream Oogonia and Primary Growth Oocytes

In recent studies, we have found that the nuclear Pgr and its ligand 17,20β-P are expressed and synthesized, respectively, in primary ovaries of the gilthead seabream (Zapater et al. 2012, 2013). To investigate the relationship between Pgr and Aqp1ab expression during

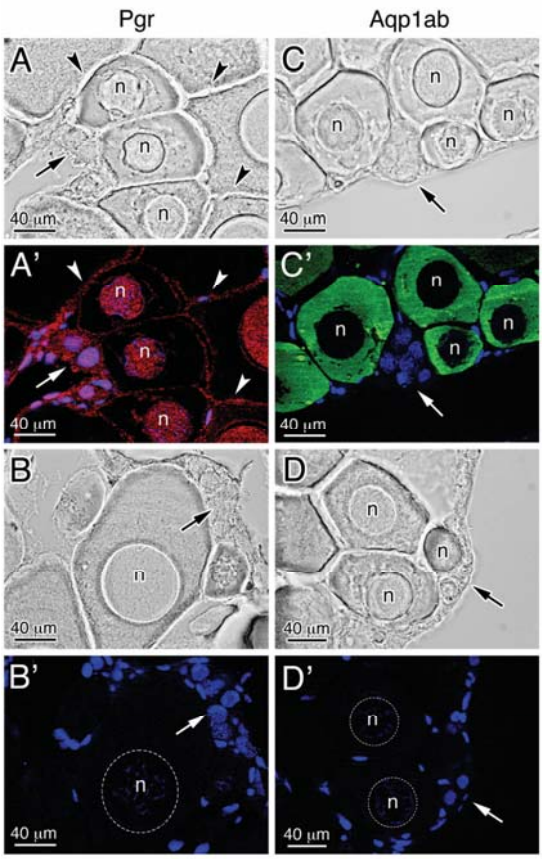
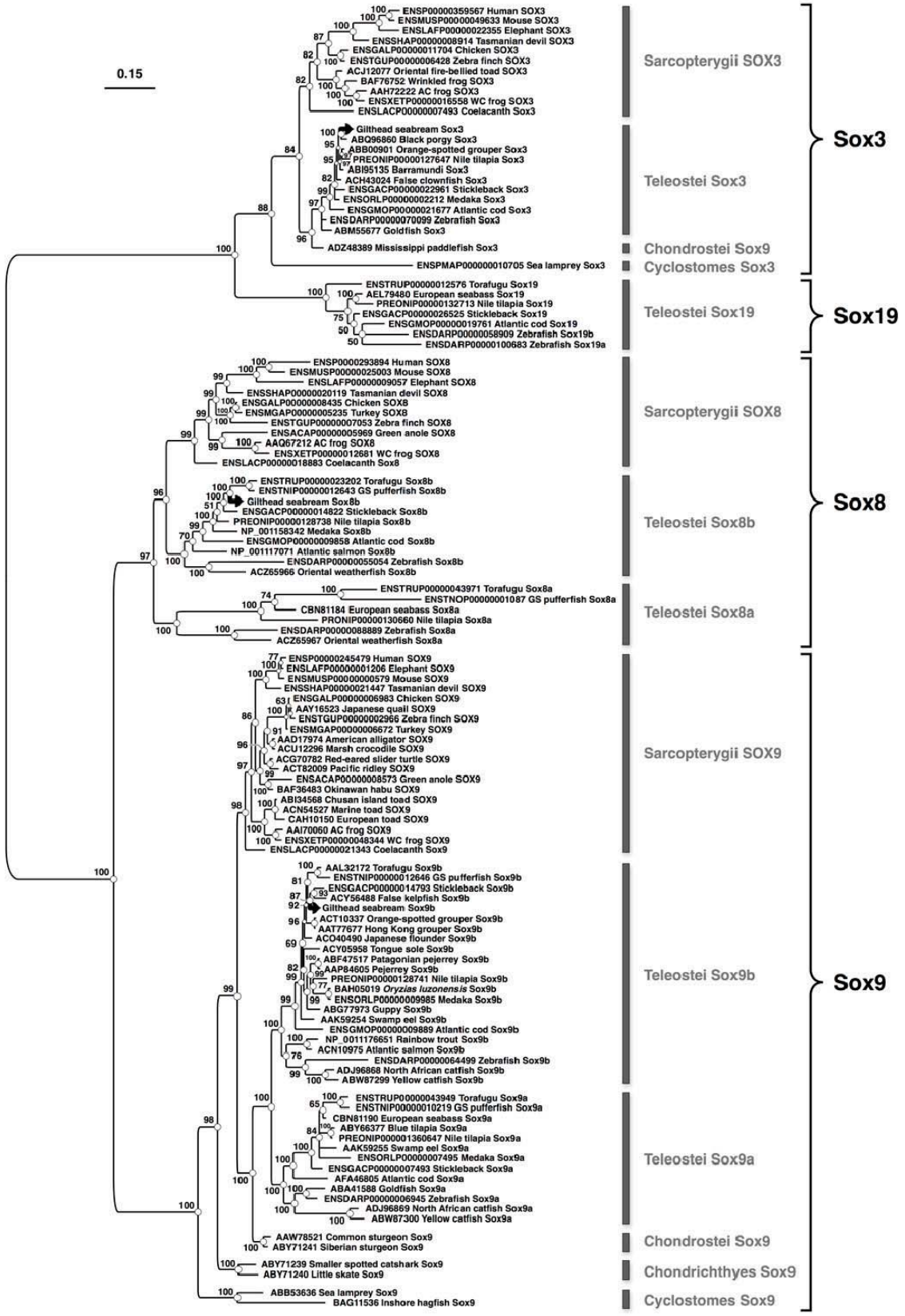


Fig. 4. Differential subcellular localization of the nuclear Pgr and Aqp1ab in oogonia and primary growth oocytes of the gilthead seabream. Representative bright field (A-D) and epifluorescence (A'-D') microscopy images of Pgr (A and A') and Aqp1ab (C and C') polypeptides on paraffin sections (n = 3 females). Separate immunofluorescence experiments using the Pgr or Aqp1ab antisera were carried out on sections from the same ovary at the primary growth stage. Sections were counterstained with DAPI. Oogonia express the Pgr both in the nucleus (n) and cytoplasm (arrows in A and A'), whereas Aqp1ab polypeptides were not detected at this stage (arrows in C and C'). In meiosis-arrested primary growth oocytes, the Pgr was localized almost exclusively in the nucleus (A and A'), as well as in granulosa cells surrounding oocytes (arrowheads in A and A'), while Aqp1ab appears spread in the cytoplasm C and C'). Control sections incubated with the antisera preadsorbed with the synthetic peptides used for immunization were negative (B and B', and D and D').

the transition from oogonia to primary oocytes, we carried out immunofluorescence microscopy on sections from primary growth ovaries employing the Aqp1ab specific antiserum as well as an affinity-purified antibody against the seabream Pgr (Zapater et al. 2013) (Fig. 4). The results indicated that the Pgr is expressed in the cytoplasm and nucleus of oogonia, whereas in primary growth oocytes, Pgr is almost exclusively localized in the nucleus as well as in the associated follicle (granulosa) cells (Fig. 4, A and A'). The latter nuclear localization of the Pgr in primary growth oocytes temporally coincided with the first expression and synthesis of Aqp1ab in the cytoplasm (Fig. 4, C and C'), suggesting that Pgr could transcriptionally activate *aqp1ab*.

Fig. 5. Bayesian majority rule consensus tree of the amino acid alignment of gilthead seabream Sox3, Sox8b and Sox9b transcription factors in relation to 116 vertebrate orthologs. The tree is mid-point rooted with posterior probabilities shown at each node. Scale bar indicates the rate of expected amino acid substitution per site. Accession numbers are annotated with the taxa.


0.15
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ENSMUSP00000049633 Mouse SOX3
ENSLAFP00000022355 Elephant SOX3
ENSSHAP00000008914 Tasmanian devil SOX3
ENSGALP00000011704 Chicken SOX3
ENSTGUP00000006428 Zebra finch SOX3
ACJ12077 Oriental fire-bellied toad SOX3
BAF76752 Wrinkled frog SOX3
AAH72222 AC frog SOX3
ENSXETP00000016558 WC frog SOX3
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Sarcopterygii SOX3
Gilthead seabream Sox3
ABQ96860 Black porgy Sox3
ABB00901 Orange-spotted grouper Sox3
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ABI95135 Barramundi Sox3
ACH43024 False clownfish Sox3
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ENSORLP00000002212 Medaka Sox3
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ABM55677 Goldfish Sox3
Teleostei Sox3
ADZ48389 Mississippi paddlefish Sox3
Chondrostei Sox9
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Cyclostomes Sox3
Sox3
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AEL79480 European seabass Sox19
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ENSLACP00000018883 Coelacanth Sox8
Sarcopterygii SOX8
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Gilthead seabream Sox8b
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BAF36483 Okinawan habu SOX9
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Gilthead seabream Sox9b
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ABF47517 Patagonian pejerrey Sox9b
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ABG77973 Guppy Sox9b
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ACN10975 Atlantic salmon Sox9b
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ADJ96868 North African catfish Sox9b
ABW87299 Yellow catfish Sox9b
Teleostei Sox9b
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ABA41588 Goldfish Sox9a
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ABW87300 Yellow catfish Sox9a
Teleostei Sox9a
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ABY71241 Siberian sturgeon Sox9
Chondrostei Sox9
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Sox9

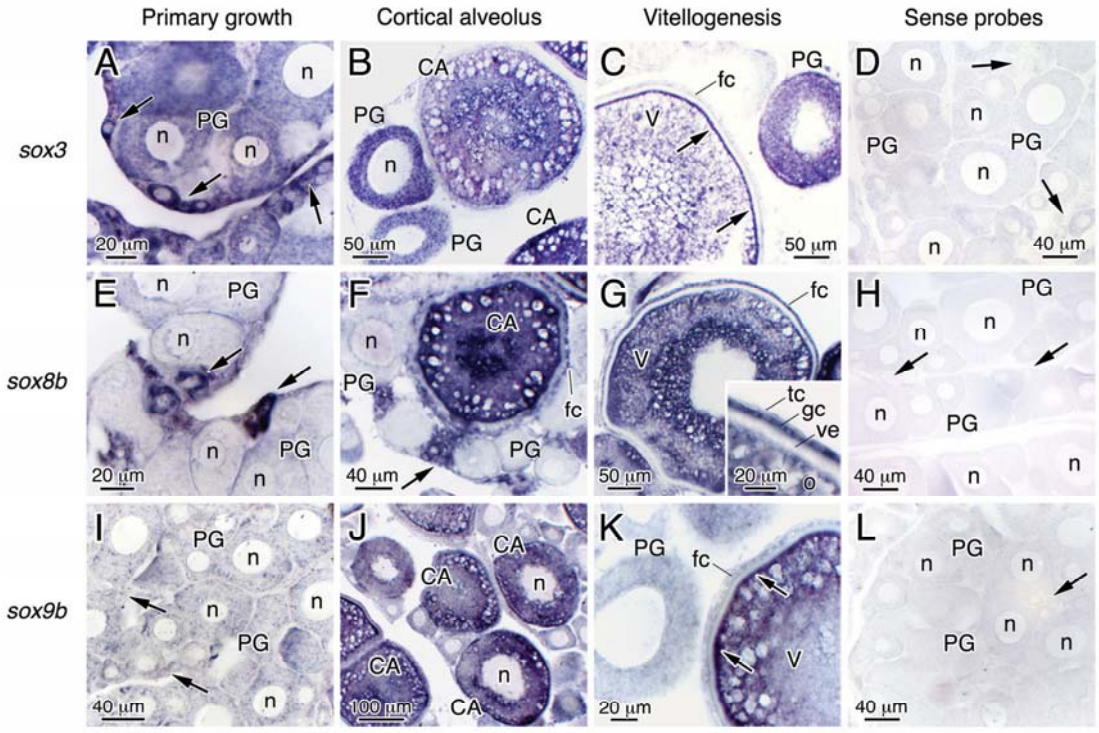

Fig. 6. Gilthead seabream *sox* transcription factors are differentially expressed during oogenesis. Representative *in situ* hybridization for *sox3*, *sox8b* and *sox9b* on paraffin sections of the same primary ovary ($n = 3$ females). Transcripts for *sox3* were highly expressed in the cytoplasm of oogonia (arrows) and primary growth oocytes (A). Ooplasm *sox3* expression persisted in cortical alveolus (B) and vitellogenic (C) oocytes. In contrast, *sox8b* was detected only in the cytoplasm of oogonia and early primary growth oocytes (arrows in E and F), whereas more developed primary growth oocytes were negative. In cortical alveolus (F) and vitellogenic oocytes (G), *sox8b* transcripts were highly expressed in the cytoplasm, as well as in follicle (granulosa) cells surrounding vitellogenic oocytes (G and inset). *sox9b* expression was not observed in oogonia (arrows in I) and transcripts were only detected very weakly in primary growth oocytes, whereas they were strongly expressed in the cytoplasm of cortical alveolus (J) and vitellogenic (K) oocytes. Follicle cells associated to early vitellogenic oocytes were also weakly labelled with *sox9b* probes (K). Control sections incubated with sense probes were negative (D, H, and L; oogonia are indicated by arrows). n, nucleus; o, oocyte; PG, primary growth oocytes; CA, cortical alveolus oocytes, V, vitellogenic oocytes; ve, vitelline envelope; fc, follicle cells; gc, granulosa cells; tc, theca cells.

To explore the role of Sox transcription factors in the regulation of *aqp1ab*, we first employed RT-PCR and 5' and 3' RACE, using ovarian RNA and degenerate primers designed within the Sox factor HMG-box sequence, to isolate seabream *sox* paralogs expressed in the seabream ovary. This approach resulted in the cloning of three different full-length cDNAs with deduced amino acid sequence homologies to Sox proteins. Bayesian analyses of the isolated nucleotide and deduced amino acid sequences in relation to a broad selection of vertebrate *sox* orthologs revealed duplicated *sox8* and *sox9* genes and a single *sox3* gene in teleosts (Fig. 5). Based upon the co-clustering of the seabream *sox* transcripts and proteins with the zebra-

fish, and the Ensembl v70 nomenclature for the zebrafish *sox* orthologs (http://www.ensembl.org), we classified the seabream sequences as *sox3*, *sox8b* and *sox9b*.

To investigate the cellular sites of *sox3*, *sox8b* and *sox9b* in the seabream ovary *in situ* hybridization was carried out on sections of ovaries at the primary growth, cortical alveoli and vitellogenic stages (Fig. 6). For *sox3*, strong expression was observed in the cytoplasm of oogonia, and of primary, cortical alveoli and vitellogenic oocytes – the labelling in the ooplasm of vitellogenic oocytes was mostly concentrated close to the plasma membrane (Fig. 6A-C). Expression of *sox8b* was strongly detected in the ooplasm of oogonia but not in primary growth oocytes, and subsequently observed in the oocyte cytoplasm as well as in granulosa cells of cortical alveoli and vitellogenic ovarian follicles (Fig. 6E-G). In contrast to *sox3* and *sox8b*, oogonia did not express *sox9b*, and primary growth oocytes showed a very weak *sox9b* staining in the cytoplasm, whereas ooplasmic *sox9b* expression was prominent in cortical alveoli and early vitellogenic oocytes (Fig. 6I-K). At this later stage, a low level of *sox9b* expression was also observed in granulosa cells (Fig. 6K). For the three *sox* transcripts, control sections incubated with sense probes were negative (Fig. 6, D, H and L). These findings thus demonstrated that *sox3*, *sox8b* and *sox9b* are differentially expressed during seabream early oogonesis, and that their oocytic expression precedes or co-localizes with that of *aqp1ab*.

3.4. Progestins Specifically Stimulate *aqp1ab* Promoter Activity in Both Somatic and Germinal Cells Via Proximal PREs

The functional significance of the putative PRE sequences identified in the proximal promoter of the seabream *aqp1ab* gene was first investigated in human breast cancer MCF-7 cells, which express high levels of endogenous estrogen receptor, but also the PGR and the glucocorticoid receptor. MCF-7 cells were transiently transfected with the full *aqp1ab* promoter construct (-1672/-1) designed to drive a luciferase reporter in the pGL3-basic vector. Transfected MCF-7 cells showed constitutive *aqp1ab* promoter activity (~40-fold increase with respect control cells), which was stimulated in a dose-dependent manner (up to ~140-fold) with the external addition of progesterone (P4; 1-100 ng/ml) but not with E2 (1-100 ng/ml) (Fig. 7A). Preincubation of cells with 10 μM of the PGR antagonist RU486 inhibited both constitutive and P4-stimulated *aqp1ab* promoter activity (Fig. 7B), suggesting the involvement of the PGR. Further transient transfection of MCF-7 cells with full-length (-1.672/-1) and four 5' deletion (-1005/-1, -509-1, -398/-1, and -255/-1) constructs, in the presence or absence of P4, indicated that shorter constructs retained full activity as long as the two PREs

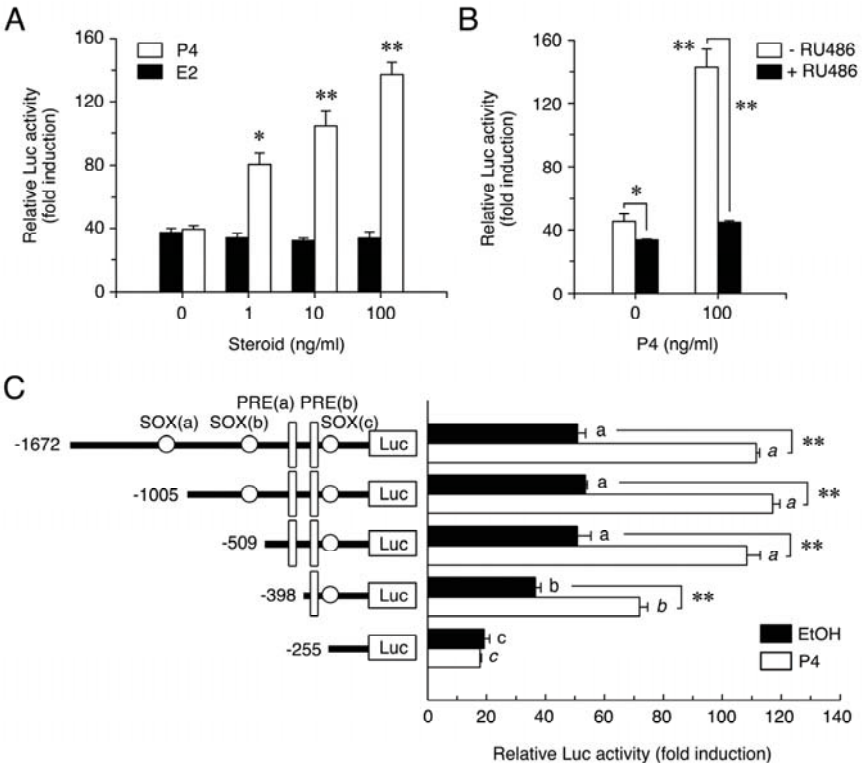

Fig. 7. Activation of the seabream *aqp1ab* promoter in MCF7 cells. A, Reporter activity of cells transiently transfected with the seabream *aqp1ab* promoter reporter construct and treated with either ethanol vehicle (EtOH), progesterone (P4) or 17β-estradiol (E2). B, Cells were treated as in A in the presence of absence of the PGR antagonist RU486 (10 μM). In A and B, data show the relative reporter (luciferase, Luc) activity from four independent experiments (mean ± SEM, n = 3), and were analyzed by ANOVA followed by Tukey's test. The asterisks denote significant differences (*, $P < 0.05$; **, $P < 0.01$) *vs*. ethanol-treated or indicated treatments. C, MCF-7 cells were transiently transfected with wild type seabream *aqp1ab* promoter or shorter deletion constructs, as indicated on the left, and treated with ethanol (EtOH) or P4. Data on reporter activity (mean ± SEM; n = 3) were analyzed by two-way ANOVA followed by the Tukey's test. Bars from EtOH- or P4-treated groups with different superscript are significantly ($P < 0.05$) different. **, $P < 0.01$ *vs*. indicated treatments.

were intact, whereas the relative activity was reduced by ~30% when the most distal PRE(a) was removed. When both PRE sequences were eliminated constitutive activity decreased further by ~60%, and P4-induced luciferase activity was completely abolished (Fig. 7C). This suggested that the two PRE sequences were equally involved in constitutive and progestin-induced promoter activity.

We next examined the steroid regulation of the seabream *aqp1ab* promoter activity by its native nuclear Pgr. For these experiments, we transiently transfected HEK293T cells, which lack endogenous PGR, with a seabream Pgr expression vector (Zapater et al. 2013) and full *aqp1ab*-luciferase promoter construct. In a previous study, we reported that 17,20β-P and 17,20β, 21-P were the most potent progestins activating Pgr-mediated luciferase activity of

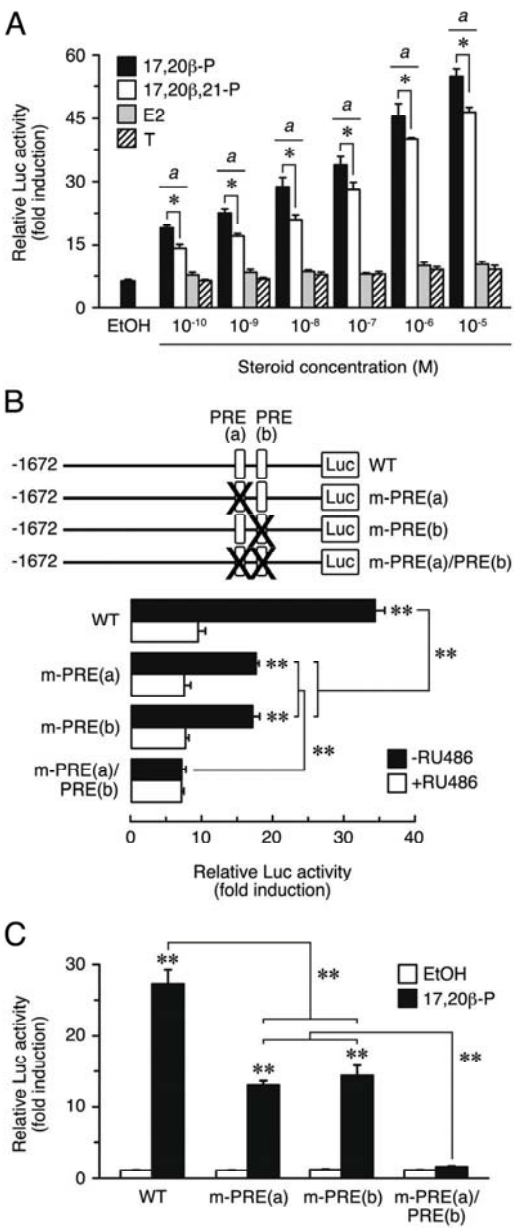

Fig. 8. The two proximal PREs of the seabream *aqp1ab* promoter are equally potent and additive for progestin-specific activation of transcription in somatic and germ cells. A, HEK293T cells were transiently cotransfected with wild type seabream *aqp1ab* promoter reporter construct and the seabream Pgr expression vector and treated with either the ethanol vehicle (EtOH) or the indicated concentrations of different steroids. Data show the relative luciferase (Luc) activity from four independent experiments (mean ± SEM, n = 4), and were analyzed by ANOVA followed by Tukey's test of the significant interaction. The line indicated by *a* is significant ($P < 0.01$) *vs.* vehicle-treated controls, whereas the asterisks denote significant differences between the 17,20β-P- and 17,20β,21-P-treated groups (*, $P < 0.05$; **, $P < 0.01$). B, HEK293T cells were transfected as in A using wild type (WT) or PRE(a) and/or PRE(b) mutated *aqp1ab* promoter [m-PRE(a), m-PRE(b) or m-PRE(a)/PRE(b), as indicated in the diagram], and exposed to 0.1 μM 17,20β-P in the presence or absence of the PGR inhibitor RU486 (10 μM). Data are from three independent experiments (n = 3) and were statistically analyzed as in A. **, $P < 0.01$ *vs.* RU486-treated or indicated treatments. C, Relative Luc activity (mean ± SEM; n = 3 separate experiments) of stage IV *X. laevis* oocytes injected with the same *aqp1ab* promoter constructs as in B and seabream Pgr cRNA, and treated with either ethanol or 100 ng/ml 17,20β-P. **, $P < 0.01$ *vs.* ethanol-treated controls or indicated treatments.

HEK293T transfected with a MMTV promoter (Zapater et al. 2013). Hence, we first tested the efficiency of different doses (10^{-10} to 10^{-5} M) of 17,20β-P and 17,20β, 21-P, as well as of E2 and T, at activating Pgr-mediated *aqp1ab* transcriptional activity. As expected, HEK293T cells showed a lower constitutive *aqp1ab* promoter activity (~7-fold increase with respect control cells) compared to MCF-7 cells, and both 17,20β-P and 17,20β, 21-P stimulated promoter activity in a dose-dependent manner (up to ~50-fold with 10^{-5} M), whereas E2 and T were completely ineffective (Fig. 8A). However, unlike the previous observations using the MMTV pro-

moter, we found that 17,20β-P is a slightly but significantly more potent inducer of luciferase activity than 17,20β, 21-P at all doses tested (Fig. 8A).

In a subsequent series of experiments, the contribution of the two core promoter PREs for Pgr-mediated *aqp1ab* transcription in response to 17,20β-P was investigated. Mutated constructs in either of the two PRE sequences [m-PRE(a) or m-PRE(b)], or in both [m-PRE(a)/PRE(b)], were synthesized by site-directed mutagenesis and separately transfected together with the seabream Pgr expression vector into HEK293T cells, which were then exposed to 0.1 μM 17,20β-P in the presence or absence of RU486. The results showed that each of the m-PRE(a) and m-PRE(b) mutant *aqp1ab* reporters reduced by approximately half the response of the wild-type *aqp1ab* promoter to 17,20β-P (~34-fold increase in reporter activity with respect control cells), whereas the double PRE mutant completely blocked progestin-stimulated promoter activity (Fig. 8B). The wild-type, m-PRE(a) and m-PRE(b) reporter activity was completely inhibited by RU486, while the inhibitor had no effect on the activity of the m-PRE(a)/m-PRE(b) reporter construct suggesting that constitutive activation of the seabream *aqp1ab* in HEK293T cells is not Pgr-dependent. These data thus confirmed previous observations on MCF-7 cells transfected with *aqp1ab* promoter deletions, and strongly suggested that PRE(a) and PRE(b) are *bona fide* cis-regulatory elements of the *aqp1ab* promoter.

Since the *aqp1ab* transcripts are highly accumulated *in vivo* in oocytes of the seabream, the next experiments were aimed at examining whether the Pgr-mediated regulation of the *aqp1ab* promoter via PREs observed in somatic cells was a conserved mechanism in other vertebrate germ cells. For these experiments, we used direct injection of promoter constructs into *X. laevis* stage IV immature oocytes, which presumably have low levels of endogenous PGR since they are not competent to respond to P4 by undergoing meiosis resumption (data not shown). Oocytes were injected with wild-type and mutant PRE *aqp1ab* constructs, together with cRNA encoding the seabream Pgr, and subsequently exposed to 100 ng/ml 17,20β-P. Constitutive seabream *aqp1ab* promoter activity was very low in *X. laevis* oocytes (~2-fold increase in luciferase activity with respect control oocytes injected with empty pGL3) regardless of whether or not the oocytes expressed the seabream Pgr, were exposed to ethanol vehicle alone, or were exposed to either 17,20β-P or ethanol, respectively (Fig. 8C). However, the reporter activity elicited by the wild-type *aqp1ab* construct was strongly stimulated in the presence of 17,20β-P (~27-fold increase with respect control oocytes), whereas as seen in HEK293T cells the m-PRE(a) and m-PRE(b) mutant constructs each induced only half of the response (Fig. 8C). Similarly, the m-PRE(a)/m-PRE(b) mutant construct completely reduced

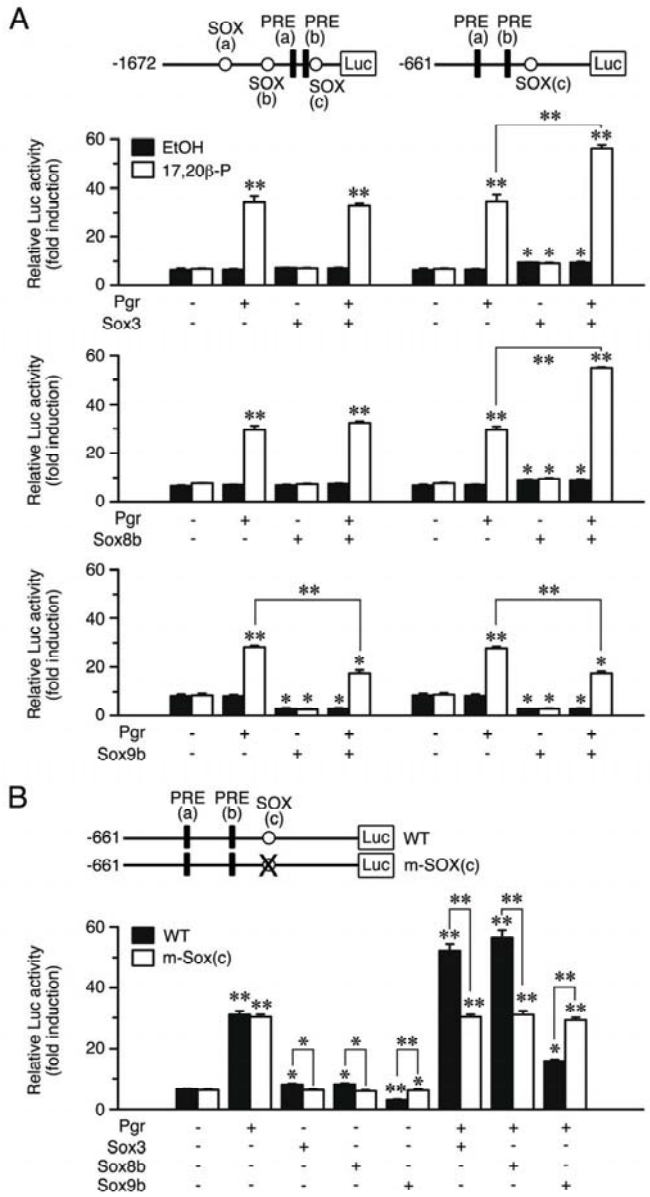

Fig. 9. Sox transcription factors regulate seabream *aqp1ab* promoter activity via distal and proximal SOX binding sites in HEK293T cells. A, Cells were transiently cotransfected with full-length or a -661 deletion fragment of the *aqp1ab* reporter and different combinations of seabream Pgr, Sox3, Sox8b and Sox9b expression vectors as indicated, and treated with either ethanol vehicle or 10 μM 17,20β-P. B, Cells were transfected with the wild-type (WT) -661-deletion construct or with the same construct in which the SOX(c) site was mutated [m-SOX(c)], together with Pgr and Sox expression vectors as above, and treated with 10 μM 17,20β-P. Schematic representations of the regulatory regions present in each promoter construct are indicated above each panel. In both A and B, the data represent mean (± SEM) relative reporter activity of three independent experiments ($n = 3$). Data for the different length reporters were analyzed in separate two-way ANOVAs followed by Tukey's post hoc analysis of the significant interactions. *, $P < 0.05$; **, $P < 0.01$ *vs.* cells transfected with empty pcDNA3 or indicated treatments.

17,20β-P-mediated reported activity to basal levels. Collectively, these results confirmed that 17,20β-P-activated Pgr specifically stimulates seabream *aqp1ab* promoter activity in both somatic and germ cells via two proximal PREs.

3.5. Differential Regulation of Seabream *aqp1ab* Transcription by Sox3, Sox8b and Sox9b

To investigate the action of Sox factors in the regulation of the seabream *aqp1ab* promoter, we first employed HEK293T cells transiently transfected with seabream Sox3, Sox8b or Sox9b expression vectors, and the full 1.6-kb *aqp1ab* promoter construct or a 5' deletion

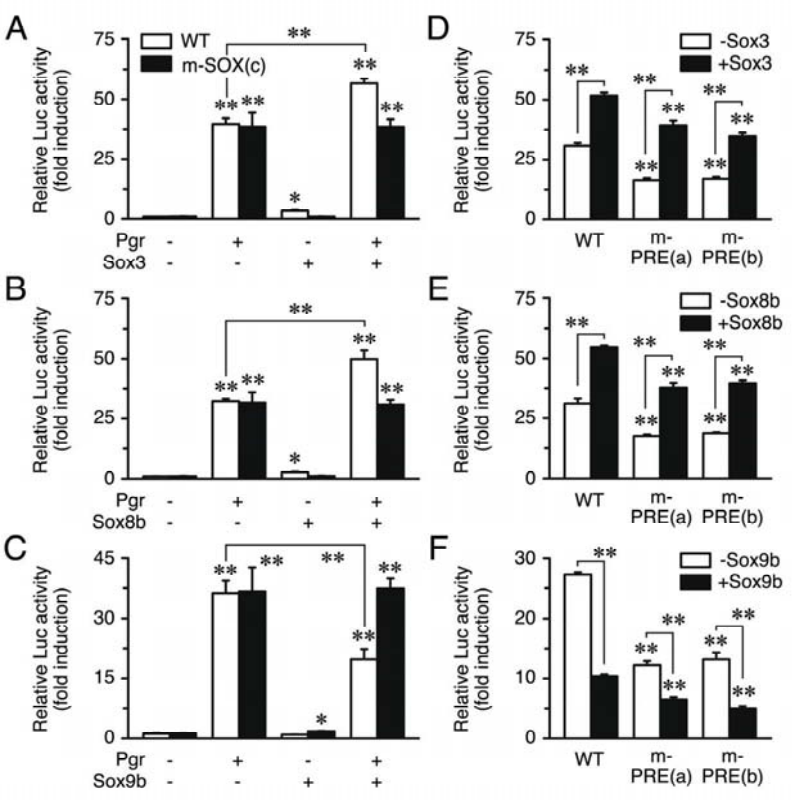

Fig. 10. The proximal SOX binding site of the seabream *aqp1ab* promoter can play synergistic or inhibitory roles on PRE(a)- and PRE(b)-mediated progestin transcriptional activation in *X. laevis* oocytes. A-C, Oocytes were injected with the wild-type (WT) -661-deletion construct of the *aqp1ab* promoter or with the m-SOX(c) mutant construct, and with different combinations of Pgr, Sox3, Sox8b and Sox9b cRNAs as indicated, and treated with 10 μM 17,20β-P. D-F, Oocytes were injected with the WT -661-deletion *aqp1ab* construct or with the same construct in which either of the two PRE sequences were mutated [m-PRE(a) and m-PRE(b), respectively], together with Pgr cRNA with or without cRNAs of Sox3, Sox8b or Sox9b, and treated with 17,20β-P. Data (mean ± SEM) in each panel show relative reporter activity from three independent experiments (n = 3) and were analyzed separately by two-way ANOVA followed by the Tukey's test. In A-C, *, $P < 0.05$; **, $P < 0.01$ *vs*. oocytes injected with promoter constructs alone or indicated treatments. In D-F, *, $P < 0.05$; **, $P < 0.01$ *vs*. oocytes injected with WT *aqp1ab* construct or indicated treatments.

fragment (-661/-1) in which the SOX(a) and SOX(b) sites were removed while both PREs were intact (Fig. 6). In these experiments, we also examined the effect of the co-expression of Sox factors with similar amounts of Pgr in the presence or absence of 17,20β-P. Both Sox3 and Sox8b were transcriptionally inactive in cells transfected with the full *aqp1ab* promoter, whereas they elicited a small but significant reporter activity (~1.3-fold higher than cells expressing the *aqp1ab* promoter alone) in cells transfected with the -661 deletion construct, regardless of the presence of the progestin in the medium (Fig. 9A). Interestingly, when Sox3 and Sox8b were co-expressed together with Pgr a synergic stimulation of the promoter activity of the -661-deletion construct in the presence of 17,20β-P was observed (Fig. 9A). In contrast,

Sox9b expression repressed constitutive and 17,20β-P-mediated reporter activity by ~55% of both the full and shorter *aqp1ab* promoter constructs (Fig. 9A). The stimulatory effect of Sox3 and Sox8b, as well as the repressor effect of Sox9b, on the promoter activity of the -661 *aqp1ab* deletion fragment was lost when cells were transfected with the same construct in which the SOX(c) site was mutated [m-SOX(c)] (Fig. 9B).

To corroborate these observations in germ cells, *X. laevis* oocytes were injected with the same wild-type or m-SOX(c) constructs as above, together with cRNAs encoding Pgr and/or Sox factors (Fig. 10A-C). Identical results to the HEK293T cell experiments were obtained, confirming that the SOX(c) element was needed for the synergistic effect of Sox3 and Sox8b on Pgr-induced *aqp1ab* promoter activity, as well as for the inhibitory role of Sox9b on transcription. By further using *X. laevis* oocytes and the injection of -661-deletion constructs in which either of the two PRE were mutated separately [m-PRE(a) and m-PRE(b) mutant constructs, respectively], we also examined the involvement of the SOX(c) site and the two PRE sequences in the regulation of the *aqp1ab* promoter by Sox3, Sox8b and Sox9b in the presence of the Pgr. These experiments indicated that the Sox3- and Sox8b-activated SOX(c) interacted equally with PRE(a) and PRE(b) to synergize with the Pgr to stimulate *aqp1ab* transcription, and that Sox9b-activated SOX(c) was capable of inhibiting Pgr-promoted *aqp1ab* activity mediated by PRE(a) or PRE(b) (Fig. 10D-F). Collectively, these data suggested that the Sox3- or Sox8b-bound SOX(c) element plays a cooperative role in the activation of the seabream *aqp1ab* promoter by interacting with either of the two PREs, whereas upstream SOX(a) and/or SOX(b) sites down-regulate this effect. By contrast, when SOX(c) is activated by Sox9b, an inhibition of constitutive and Pgr-mediated *aqp1ab* transcription occurs.

3.6. Gonadotropin and Progestin Activate *aqp1ab* Transcription and Translation in Primary Ovarian Explants *In Vitro*

The previous results strongly suggested that 17,20β-P may play a major role in the transcriptional regulation of *aqp1ab* in seabream oocytes. To test this hypothesis in a homologous *ex vivo* system, we incubated seabream primary ovarian explants, containing exclusively oogonia and primary growth oocytes, with gonadotropin or 17,20β-P *in vitro*, and subsequently determined the *aqp1ab* transcript levels by qRT-PCR. The rFsh from another perciform teleost, the European seabass, was used as the gonadotropin source, since seabass rFsh promotes 17,20β-P synthesis *in vitro* by seabream primary ovaries (Zapater et al. 2012). Incubation of the explants with different doses of rFsh (1-100 ng/ml) increased the *aqp1ab* mRNA

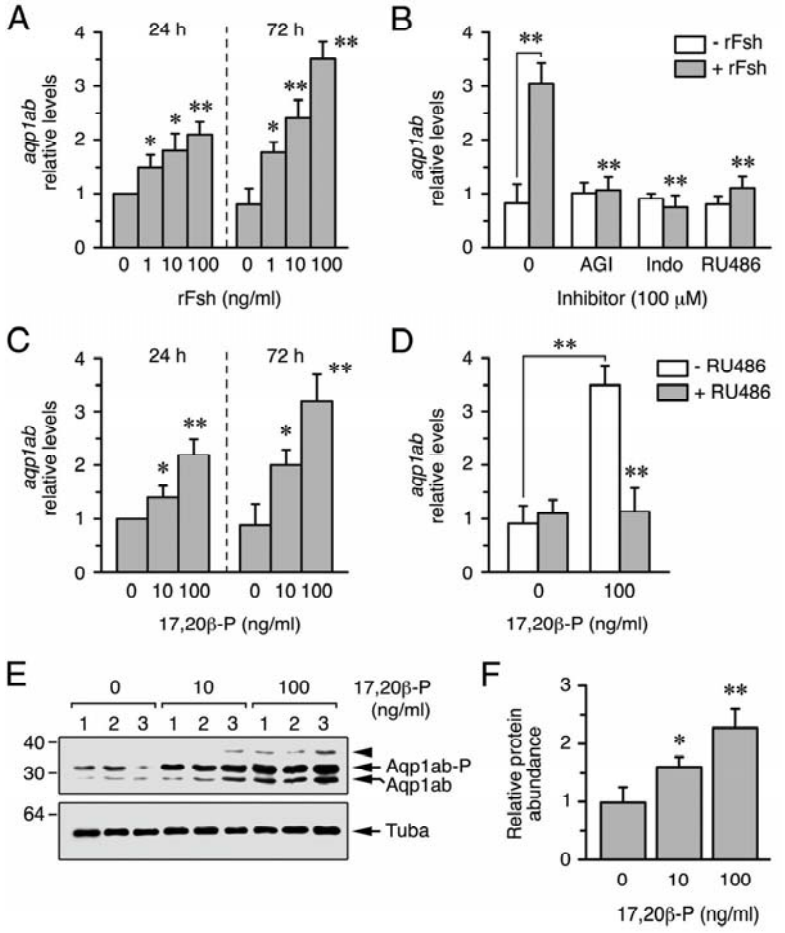

Fig. 11. Gonadotropin (Fsh) and progestin stimulate *aqp1ab* transcription and translation in seabream primary ovarian explants *in vitro*. A, Effect of seabass rFsh on *aqp1ab* transcript levels in explants after 24 h or 72 h of gonadotropin addition. Data (mean ± SEM; n = 4 independent experiments) were determined by qRT-PCR and normalized to *18s* ribosomal mRNA expression. Bars with asterisks are significant (ANOVA; *, $P < 0.05$; **, $P < 0.01$) *vs*. the 24-h control group. B, Inhibition of rFsh (100 ng/ml)-mediated *aqp1ab* expression by 100 μM of steroidogenesis (AGI), carbonyl reductase (Indo) and PGR (RU486) inhibitors at 72 h. Data are means (± SEM) of three separate expriments ($n = 3$) analyzed by two-way ANOVA. **, $P < 0.01$ *vs.* rFsh-treated group without inhibitors or indicated treatments. C, Effect of 17,20β-P on *aqp1ab* expression (mean ± SEM; n = 4) determined and analyzed as in A. D, Inhibition of 17,20β-P-promoted *aqp1ab* expression by 100 μM RU486 at 72 h. Data are from three independent experiments ($n = 3$) and were analyzed as in B. **, $P < 0.01$ *vs.* 17,20β-P-treated group without inhibitor or indicated treatments. E, Representative Aqp1ab immunoblot of protein extracts from explants stimulated in triplicate (lanes 1, 2 and 3) with 17,20β-P for 72 h. Phosphorylated and dephosphorylated Aqp1ab monomers are indicated as in Fig. 1. Duplicate membranes were probed with anti-alpha-tubulin (Tuba) antibody. Molecular mass markers (kDa) are on the left. F, Relative abundance of Aqp1ab-reactive bands (mean ± SEM; n = 3 separate experiments) determined by densitometric analysis. Bars with asterisks are significant (ANOVA; *, $P < 0.05$; **, $P < 0.01$) *vs*. non-treated groups.

levels in a dose-dependent manner after 24 and 72 h of hormone exposure (Fig. 11A). The rFsh-mediated stimulatory effect on *aqp1ab* expression was abolished in the presence of ste-

roidogenesis and carbonyl reductase inhibitors, such as AGI and Indo, respectively, as well as of RU486 (Fig. 11B), suggesting that the gonadotropic regulation of *aqp1ab* transcription may be mediated by rFsh-promoted synthesis of progestins. Incubation of explants with 10 or 100 ng/ml of 17,20β-P confirmed this mechanism since the progestin up-regulated the *aqp1ab* mRNA levels in a dose-dependent manner at 24 and 72 h (Fig. 11C); the stimulation being inhibited with RU486 (Fig. 11D). The effect of 17,20β-P on *aqp1ab* translation was also investigated in the same explants by immunoblotting, which showed that the progestin elevated the levels of Aqp1ab monomer, as well as of post-translationally modified peptides, after 72 h of treatment (Fig. 11, E and F).

3.7. Pgr is Recruited to the *aqp1ab* Promoter by 17,20β-P *In Vitro*

To investigate whether differences in *aqp1ab* promoter occupancy by the Pgr contribute to altered *aqp1ab* expression in primary explants *ex vivo*, quantitative ChIP assays were performed (Fig. 12). Explants were treated with 100 ng/ml 17,20β-P, in the presence or absence of RU486, for 72 h, and chromatin was isolated with an IgG or Pgr antibody and subsequently analyzed by qPCR using specific primers flanking each of the PRE sequences in the seabream proximal *aqp1ab* promoter (Fig. 12A). As shown in Figure 9B, DNA fragments including the two PRE(a) and PRE(b) sequences in the *aqp1ab* proximal promoter region (-470/-334 and -373/-216, respectively) were specifically immunoprecipitated with the anti-Pgr antibody but not with a non-specific IgG. Quantification of the immunoprecipitated chromatin by qPCR indicated that Pgr binding to both PRE(a) and PRE(b) containing DNA regions was enhanced ~5-6-fold by treatment with 17, 20β-P and was completely prevented in the presence of RU486 (Fig. 12D). The increased recruitment of endogenous Pgr was not the result of a 17,20β-P-promoted Pgr synthesis, since immunoblotting analysis (Fig. 12C) showed that the progestin did not increment the polypeptides corresponding to the wild-type Pgr or the Pgr_tv1 alternative splicing isoform (Zapater et al. 2013). These results therefore suggest that 17,20β-P enhances Pgr recruitment to both PRE sequences in the *aqp1ab* promoter, which may contribute to the stimulation of transcriptional activity.

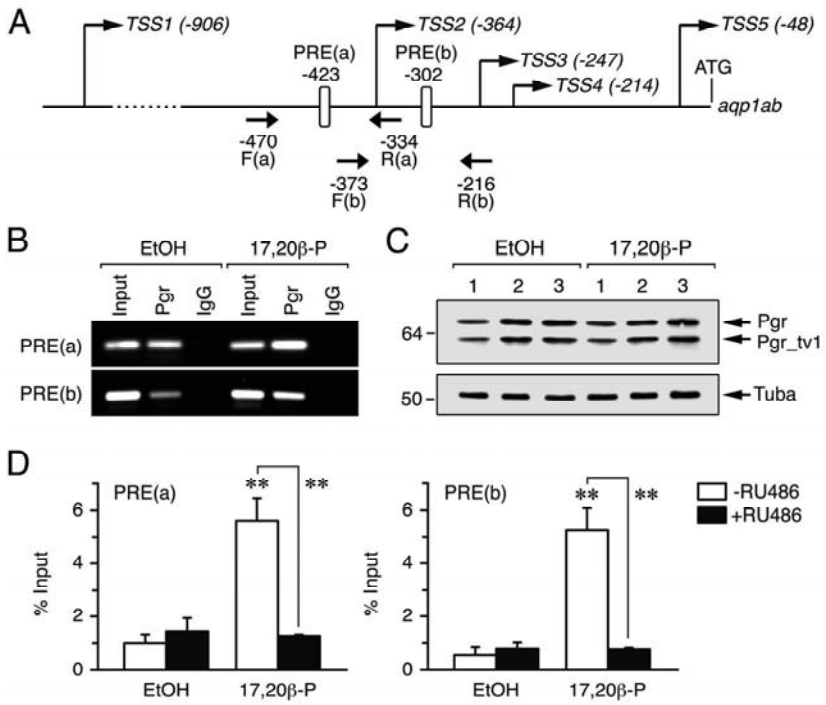

Fig. 12. Recruitment of Pgr to the PREs of the seabream *aqp1ab* promoter by 17,20β-P in primary explants *ex vivo* determined by ChIP-qPCR. A, Diagram of the *aqp1ab* proximal promoter in which the putative PRE sequences (open boxes) and transcription start sites (TSSs) are indicated. The position of the forward (F) and reverse (R) primers used in ChiP-qPCR, specific for the PRE(a) and PRE(b) amplicons, are indicated below with arrows. B, Representative gel electrophoresis of PCR products amplified with each PRE primer set from seabream Pgr antibody- and IgG-immunoprecipitated chromatin in explants exposed to ethanol (EtOH) or 17, 20β-P (100 ng/ml) for 72 h. C, Representative Pgr immunoblot of triplicate (lanes 1, 2 and 3) protein extracts from the groups shown in B. The arrows point to Pgr and a putative Pgr splice variant (Pgr_tv1). Duplicate membranes were probed with anti-alpha-tubulin (Tuba) antibody. Molecular mass markers (kDa) are on the left. D, ChIP-qPCR analysis of the PRE(a) and PRE(b) in the seabream *aqp1ab* proximal promoter after treatment of explants as in B in the presence or absence of 100 μM RU486. Data (± SEM; n = 4 independent experiments) are presented relative to input chromatin alone since the background signal levels (IgG) were undetectable (data not shown). **, $P < 0.01$ *vs.* ethanol-vehicle controls or indicated treatments.

4. Discussion

As a first step toward elucidating the transcriptional mechanisms that underly *aqp1ab* expression in teleost oocytes, we cloned and functionally characterized the 5'-flanking region of the seabream *aqp1ab* gene. As observed in the core promoter of many mammalian aquaporins (Inase et al. 1995; Umenishi and Verkman 1998a,b; Borok et al. 2000), the seabream *aqp1ab* proximal promoter shows several TATA and CCAAT boxes, as well as Sp1, AP1 and E-box elements. Experimental determination of the TSSs by RLM-RACE indicated five different initiation sites in primary oocytes, although the majority of transcripts seem to initiate at po-

sition 364 upstream of the ATG start codon (TSS2). Alternative TSSs are also found in some mammalian aquaporin genes, such as mouse *AQP8* (Calamita et al. 1999) or human *AQP4* (Lu et al. 1996). In the seabream *aqp1ab* 5'-flanking region, the lnr sequences [consensus sequence $YYA_{(+1)}NWYY$ in humans (Bucher 1990), where $A_{(+1)}$ usually designates the TSS], which facilitate binding of regulatory proteins to the core promoter and are important for TSS selection and promoter strength (Smale and Baltimore 1989), are found very close to the experimentally determined *aqp1ab* TSSs. Thus, the $A_{(+1)}$ nucleotide of the lnr sequences were located at 22, 12, 5, 9 and 7 nucleotides donwstream of the TSS1, TSS2, TSS3, TSS4 and TSS5, respectively, determined by RLM-RACE experiments. The TSS1 (-906), TSS2 (-364), TSS3 (-247) and TSS4 (-214) are however respectively positioned 252, 298, 415 and 448 nucleotides downstream of a TATA box (-662), which is much farther than the optimal position (-31 to -30 in relation to the TSS) described in metazoans (Ponjavic et al. 2006). For TSS5, however, two TATA boxes are located 13 and 133 nucleotides upstream of the initiation site. The absence of a proximal TATA box upstream of TSS1-TSS4, and the presence of a GC-rich region particularly upstream of TSS2, suggests that transcription of *aqp1ab* in oocytes may be predominantly TATA-independent. A similar mechanism has been suggested for some mammalian aquaporins, such as the mouse *AQP8* gene (Calamita et al. 1999), the mouse and human *AQP5* (Lee et al. 1996) and the rat *AQP5* in salivary glands (Borok et al. 2000).

The *in silico* analysis of the seabream *aqp1ab* promoter identified a duplicated *aqp1ab* pseudogene and numerous putative *cis*-acting regulatory elements that may serve as targets for sequence-specific enhancer/silencer transcription factors. An interesting aspect of the former observation was that the experimentally determined expression variants approximately utilised the conserved *aqp1ab* splice sites of exons 2-4, supporting the notion that a degraded *aqp1ab* gene exists at this locus. Among the *cis*-acting regulatory elements identified, we found numerous consensus sequences that may be bound by transcription factors involved in gut and/or kidney development in mammals and zebrafish, such as CDX (Flores et al. 2008; Beck and Stringer 2010), Krüppel-like factors (KLF4, KLF15; Mallipattu et al. 2012; Li et al. 2011; Yu et al. 2012), PBX1 (Schnabel et al. 2003), or NFKB1 (Panzer et al. 2009). This observation is consistent with the expression of *aqp1ab* in osmoregulatory organs of teleosts, such as the kidney and rectum (Cerdà and Finn 2010), in addition to the oocyte, and with the constitutive activation of the seabream *aqp1ab* promoter in HEK293T and Caco-2 cells (data not shown). In the teleost kidney and rectum, Aqp1ab has been proposed to maintain water balance by mediating transcellular water transport across epithelia (Cerdà and Finn, 2010).

The seabream *aqp1ab* promoter also contains three potential CREB sites responsive to cAMP that are found in mamalian aquaporin genes including *AQP2* (Yasui et al. 1997; Yu et al. 2009) and *AQP5* (Wang and Zheng 2011), as well as one potentially conserved site for hypoxia inducible factor-1, which can activate murine *AQP1* transcription in endothelial cells in response to hypoxia (Abreu-Rodríguez et al. 2011).

More interestingly, the seabream *aqp1ab* promoter contains two PREs and three putative binding sites for SOX transcription factors, which are also found upstream of the *aqp1ab* gene in different teleosts. *In situ* hybridization and immunolocalization experiments showed that the Pgr and *sox3* and *sox8b* were strongly expressed in seabream oogonia, thus preceding the activation of *aqp1ab* transcription and translation in primary growth oocytes. Moreover, in these latter oocytes the Pgr is almost completey translocated from the cytoplasm into the nucleus, indicating the possible activation of the receptor by its ligand 17,20β-P, which at this stage is produced by granulosa cells in response to Fsh (Zapater et al. 2012). In contrast, *sox9b* appears to be expressed at low levels in primary growth oocytes but its expression level was greatly enhanced in more advanced stages of oocyte development. These findings therefore provided circumstantial evidence for a possible role of Pgr as well as of Sox factors in regulating *aqp1ab* transcription in seabream primary growth oocytes. In this study, we also detected the presence of *sox8b* transcripts in granulosa cells surrounding cortical alveolus and early vitellogenic oocytes. Although *sox8* has not been detected in the granulosa cells of zebrafish (Rodríguez-Marí et al. 2005), this observation in seabream is in line with the finding of *SOX8* expression in mural granulosa cells of mouse preovulatory follicles (Salmon et al. 2005). Interestingly, *sox3* and aromatase (*cyp19a1*) (Zapater et al. 2012) are co-expressed in the ooplasm of seabream primary oocytes, which would be consistent with *sox3* activation of *cyp19a1* transcription as shown in some amphibians (Oshima et al. 2009).

Experimental analysis of the seabream *aqp1ab* promoter in heterologous systems showed that the nuclear Pgr can regulate *aqp1ab* transcription in transactivation assays. Since teleost Aqp1ab is expressed in both somatic and germ cells (Cerdà and Finn 2010), for these assays we employed HEK293T cells as well as *X. laevis* oocytes. Frog oocytes have typically been used in early gene regulation studies because they are transcriptionally very active, show a basal transcriptional machinery distinct from that in somatic cells, and, unlike gilthead seabream oocytes, are very large and easy to collect and microinject in high quantities (Li et al. 2009). Therefore, stage IV *X. laevis* oocytes, although at a more advanced stage than seabream primary growth oocytes, offer a good alternative to investigate *aqp1ab* transcription in

germ cells. The experiments in HEK293T cells and oocytes gave identical results showing that progestin-activated seabream Pgr is capable of activating the intact seabream *aqp1ab* promoter through the two PREs in the 5' flanking region of the *aqp1ab* promoter, and that this activity is ablated by mutations or deletions of these sites. However, because the different heterologous systems employed may have different activating and repressive factors compared to seabream primary growth oocytes, the transcriptional regulation of *aqp1ab* by the Pgr was further investigated *ex vivo* in seabream primary ovarian explants exposed to 17,20β-P in the presence or absence of the Pgr inhibitor RU486. By using direct ChIP assays, qRT-PCR and Western blotting, these experiments confirmed that 17,20β-P enhanced the recruitment of the Pgr to both PREs in the *aqp1ab* promoter, and subsequently activated *aqp1ab* transcription and translation. Because 17,20β-P is synthesized in response to Fsh in seabream primary ovarian follicles (Zapater et al. 2012), we also tested the ability of Fsh to drive *aqp1ab* expression. As expected, Fsh stimulated *aqp1ab* transcription, which was abolished by inhibitors of the P450c17-II (Cyp17a2)/20β-hydroxysteroid dehydrogenase (Cbr1) steroidogenic pathway required for progestin synthesis (Zhou et al. 2007; Zapater et al. 2012). Therefore, these findings are consistent with the notion that *aqp1ab* expression in seabream primary growth oocytes is activated by 17,20β-P produced by the granulosa cells in response to Fsh.

In the mammalian ovary, it is well known that the nuclear PGR regulates a high number of genes including those involved in primordial follicle assembly (Nilsson et al. 2006). In non-mammalian vertebrates, including teleosts, the role of progestins has primarily been associated with meiosis initiation or resumption (Nagahama and Yamashita 2008; Miura et al. 2006, 2007), so it is not known if these steroid hormones also play a role beyond the mediation of cell cycle progression. However, the prevalence of the Pgr immunoreaction in the nuclei of seabream primary growth oocytes (Zapater et al. 2013; present study), as well as in zebrafish (Hanna et al. 2010), suggests that the nuclear Pgr may regulate gene transcription in early ovarian follicles of teleosts. The present study reveals for the first time that this is most likely the case, and that *aqp1ab* is one of the target genes of the Pgr in seabream primary growth oocytes. By combining our present and earlier results, we propose a model that summarizes our current hypothesis for the endocrine regulation of *aqp1ab* transcription in seabream oocytes (Fig. 13). In this model, activation of the Fsh receptor (Fshra) in primordial granulosa cells surrounding primary oocytes rapidly drives the up-regulation of *cyp17a2* and *cbr1* transcription, and the concomitant down-regulation of P450c17-I (*cyp17a1*) and *cyp19a1* responsible for estrogen synthesis, resulting in an enhanced production of 17,20β-P

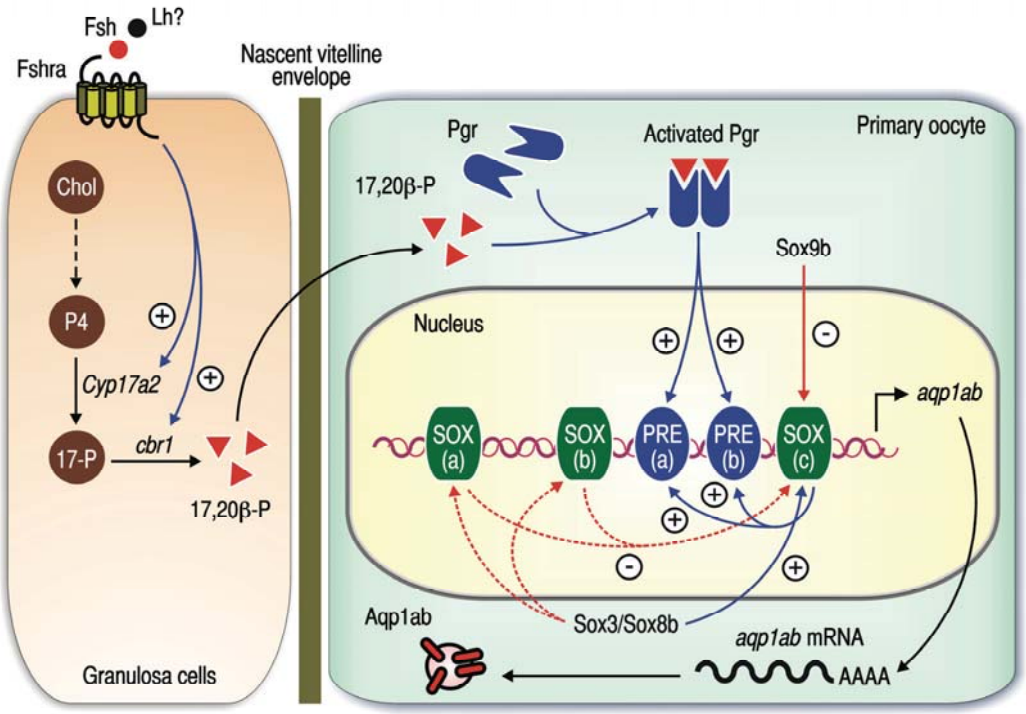

Fig. 13. Proposed model for the endocrine pathway involved in *aqp1ab* transcriptional regulation in gilthead seabream primary growth oocytes. The scheme depicts the Fsh receptor (Fshra)-mediated progestin (17,20β-P) synthesis and subsequent activation of *aqp1ab* transcription through the nuclear Pgr, potentially under positive and negative modulation by Sox transcription factors. At the primary growth stage, activation of the Fshra in granulosa cells triggers 17,20β-P production through the up-regulation of the P450c17-II (Cyp17a2)/20β-hydroxysteroid dehydrogenase (Cbr1) steroidogenic pathway in granulosa cells (Zapater et al., 2012). Binding of 17,20β-P to Pgr activates the receptor which promotes transcription likely through independent interaction with two PREs [PRE(a) and PRE(b)] in the *aqp1ab* proximal promoter. Interaction of Sox3 or Sox8b with a proximal SOX(c) binding site can act synergistically with the PRE(a) or PRE(b) to enhance Pgr-mediated transcription. The distal SOX(a) and/or SOX(b) sites can however down-regulate the synergistic effect of Sox3 or Sox8b through the SOX(c) site (dashed lines). By contrast, Sox9b represses *aqp1ab* transcription only through the SOX(c) binding site. Thus, presumably low levels of expression of Sox9b in primary growth oocytes allow Pgr-driven *aqp1ab* mRNA and protein synthesis, whereas increased Sox9b levels in more advanced oocyte stages may inhibit *aqp1ab* expression.

(Zapater et al., 2012). The subsequent binding of 17,20β-P to the nuclear Pgr in the primary growth oocyte activates the receptor and promotes *aqp1ab* transcription through independent interaction with two *cis*-acting PREs in the *aqp1ab* proximal promoter. Transactivation and ChiP assays showed that both PREs appear to be equally effective at activating *aqp1ab* transcription, and therefore it remains unknown why two PREs evolved in the *aqp1ab* promoter. It may be speculated though that selection pressure has favoured the retention of multiple PREs for the efficient activation and synthesis of gene products such as Aqp1ab, in order that sufficient levels of the channel are available in the fully-grown oocytes for rapid water flux during meiosis resumption.

The proposed endocrine pathway for the developmental regulation of the *aqp1ab* gene during oogenesis may be essential for the production of viable eggs in seabream and other acanthomorph teleost that spawns pelagic eggs, since the *aqp1ab* protein product is required for full hydration of oocytes undergoing meiotic maturation (Zapater et al. 2011). However, *in silico* analysis of the seabream *aqp1ab* promoter also revealed the presence of putative binding sites for transcription factors other than the Pgr that might be of relevance during oocyte development, such as TCF/LEF-1 involved in the Wnt signaling pathway (Harwood et al. 2008; Langdon and Mullins 2011), CREB (Sundaram et al. 2003) or PBX-HOX (Villaescusa et al. 2004). Moreover, we found different TSSs of *aqp1ab* in seabream oocytes suggesting the usage of various transcription initiation sites. Therefore, it is possible that some of these transcription factors may also be involved in the regulation of *aqp1ab* transcription in teleost oocytes. In addition, we have recently shown that four splice variants of the nuclear Pgr are expressed in the seabream primary growth stage ovary, and that two of the isoforms can operate as dominant-negative inhibitors of Pgr-mediated transcription (Zapater et al. 2013). Whether these isoforms are involved in *aqp1ab* transcriptional regulation remains to be determined.

The functional analysis of the seabream *aqp1ab* promoter in heterologous systems also demonstrated that Sox transcription factors can differentially regulate *aqp1ab* transcription, which implies that they could modulate the action of the Pgr (Fig. 13). Although this regulation could not be directly validated *ex vivo* in the present study, our data in both HEK293T cells and *X. laevis* oocytes indicated that Sox3 and Sox8b were able to significantly activate *aqp1ab* transcription, although they were much less efficient than the Pgr. However, by acting through the SOX(c) binding site closely located to the proximal PRE(b), both factors elicited a positive synergistic effect with that of Pgr. Such a mechanism likely involves Sox-mediated conformational changes in the chromatin that exposes the PREs to facilitate Pgr binding (Vicent et al. 2010; Clarke and Graham 2012). In contrast, in both somatic cells and oocytes, Sox9b acting through the same *cis*-regulatory SOX(c) site exerted a more potent transcriptional repression of *aqp1ab* compared to the activation abilities of either Sox3 or Sox8b. This observation is consistent with the decrease of *aqp1ab* mRNA levels in cortical alveoli stage oocytes *in vivo,* which show a pronounced *sox9b* expression. Since at this stage of oogenesis there was also a prominent expression of *sox3* and *sox8b* in the ooplasm, and that the nuclear localization of the Pgr was reduced with respect to that observed in primary growth stage

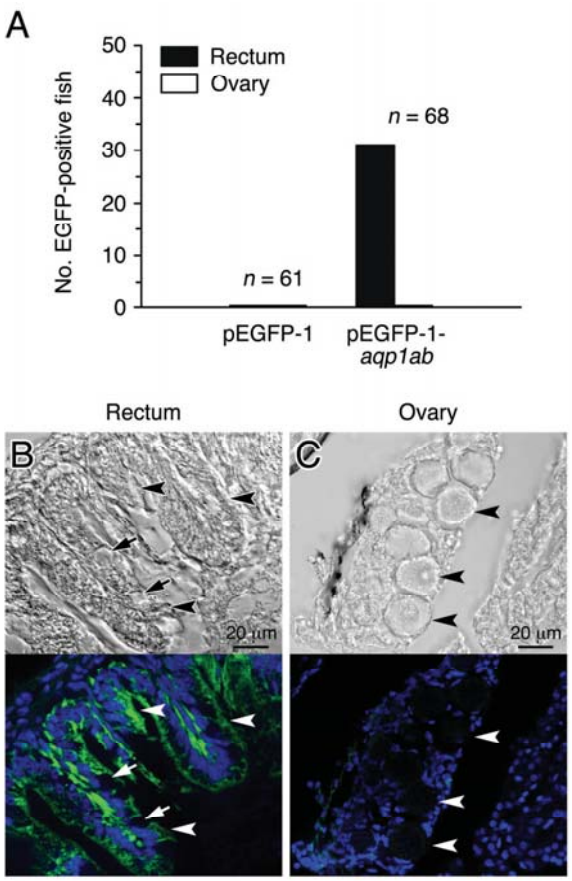

Fig. 14. The 1.672-kb seabream *aqp1ab* promoter directs specific expression of EGFP in the rectum but not in oocytes of the zebrafish. A, The seabream *aqp1ab*-pEGFP-1 construct was linearized with *Stu*I and 250 pg were microinjected into zebrafish embryos at the 1- to 2-cell stage. EGFP fluorescence in the rectum and the ovary was determined by immunofluorescence microscopy on transversal paraffin sections of 35-40 days post fertilization females using an anti-EGFP goat polyclonal antibody (abcam 111258) and a Zeiss imager.z1 microscope (Carl Zeiss MicroImaging, S.L.). The graph shows the number of pEGFP-1- (controls) and *aqp1ab*-pEGFP-1-expressing fish (n = 61 and 68, respectively) showing EGFP staining in the rectum and the ovary (note no EGFP expression in the ovary in both cases). B and C, Representative bright field (upper) and epifluorescence (lower) microscopy images of EGFP staining in the rectum (B) and its absence in the ovary (C)

oocytes (Zapater et al. 2013), is it possible to speculate that rather low *in vivo* levels of Sox9b may be able to inhibit *aqp1ab* expression in seabream cortical alveoli oocytes.

The extensive searching of available genomes revealed that *cis*-acting PREs and SOX sites are also present in the 5' flanking region of *aqp1ab* genes of different teleosts regardless of their reproductive strategy (i.e. production of hydrated pelagic *vs*. non-hydrated benthic eggs). This is illustrated, for instance, in the zebrafish, which produces non-hydrated benthic eggs and in which putative Pgr and Sox responsive elements are found in the 5' proximal genomic region of the *aqp1ab* gene (Fig. 3). Considering that the nuclear Pgr is detected in the nucleus of zebrafish primary growth oocytes (Hanna et al. 2010), one could expect a prominent activation of the *aqp1ab* in these oocytes. However, this mechanism apparently does not occur since in zebrafish ovarian *aqp1ab* transcripts are hardly detected by RT-PCR (Tingaud-Sequeira et al. 2008, 2010). To investigate the underlying mechanism, we carried out a preliminary experiment using transient transgenic zebrafish expressing *aqp1ab* promoter-enhanced green fluorescent protein (EGFP). The results showed that when the *aqp1ab*-EGFP

plasmid was microinjected into zebrafish embryos at 1-2 cell stages, the 1.672- kb *aqp1ab* promoter was able to target EGFP expression specifically to the rectum of 35-40 days post fertilization females (Fig. 14, A and B), which is also consistent with the prominent abundance of Aqp1ab at the apical brush border of rectal enterocytes in the seabream (Raldúa et al. 2008). However, EGFP expression was not detected in the zebrafish oocytes (Fig. 14C). Interestingly, in contrast to the seabream, zebrafish primary growth and cortical alveoli stage oocytes strongly express *sox9b* (Rodríguez-Marí et al. 2005), which represses seabream *aqp1ab* promoter activity. Based on these observations, and in accordance with our proposed model, it can be hypothesized that Sox9b proteins inhibit Pgr-mediated *aqp1ab* transcription in zebrafish oocytes, and perhaps in other teleosts in which *aqp1ab* is not required for the production of viable eggs. However, because mutant SOX *aqp1ab* constructs were not tested in these preliminary experiments, this hypothesis needs to be addressed in future studies, as well as the existence of additional mechanisms that can regulate the action of the Pgr on *aqp1ab* transcription in the ovary of teleosts that produce non-hydrated benthic eggs.

In conclusion, the present work shows the existence of a tight developmental regulation of the seabream maternal factor *aqp1ab*, a functional water channel with an essential role for oocyte hydration during meiosis resumption in marine teleosts. Our data suggest that *aqp1ab* is specifically induced in meiosis-arrested primary growth oocytes by the nuclear Pgr, which is activated by the progestin 17,20β-P produced by granulosa cells in response to a gonadotropic surge. These observations thus reveal that the previously unknown target genes of the nuclear Pgr in marine teleost primary growth oocytes include *aqp1ab*. The Pgr-mediated mechanism can also be potentially modulated by Sox transcription factors involved in gonad differentiation (Lefebvre et al. 2007), and therefore *aqp1ab* may also represent one of the downstream targets of Sox associated with oocyte differentiation in teleosts. In any event, our study provides a mechanistic framework for understanding the developmental pathways directing aquaporin transcriptional regulation in vertebrate oocytes and molecular mechanisms involved in determining the phenotype of the marine teleost pelagic egg.

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OVERALL DISCUSSION

In this thesis, we have investigated the evolutionary origin of the aquaporin-1ab (previously termed AQP1o or Aqp1b) in teleosts (Fabra et al. 2005, 2006; Tingaud-Sequeira et al. 2008, 2010), as well as its molecular regulation and physiological role in the oocyte of two model teleosts, the perciform gilthead seabream (*Sparus aurata*) and the pleuronectiform Atlantic halibut (*Hippoglossus hippolossus*). Our data provide strong evidence for the origin of Aqp1ab by a tandem duplication event of an ancestral AQP1-like water channel, and demonstrate its essential role during the hydration of oocytes undergoing meiosis resumption (oocyte maturation) in marine teleosts. We also show that the *aqp1ab* gene is temporally activated in meiosis-arrested primary growth oocytes of the gilthead seabream, both at the transcriptional and translational level, by the progestin hormone 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P). The data also suggest a dual regulation of *aqp1ab* transcription by 17,20β-P and Sox transcription factors that ultimately allows the storage of high amounts of Aqp1ab protein product in primary growth oocytes in a precise and timely manner before entering into the growth (vitellogenic) phase. During vitellogenesis, Aqp1ab trafficking and insertion in the oocyte surface is regulated by yet unknown mechanisms relying on the Aqp1ab C-terminus, which seem to be highly specialized in evolutionary advanced teleosts. Therefore, the present findings reveal that the Aqp1ab-mediated mechanism of oocyte hydration in marine teleosts is a conserved and highly regulated process both at the transcriptional and post-translational level.

1. Evolutionary Origin of the Teleost Aqp1ab Subfamily of Water Channels

By cloning the Atlantic halibut Aqp1aa and -1ab cDNAs and re-examining the molecular phylogeny of the vertebrate aquaporin-1 subfamily, the present data reveal that tetrapod AQP1 and teleost *aqp1aa* orthologs cluster according to phylogenetic rank and display significantly shorter branch lengths compared to the teleost *aqp1ab* orthologs. We show for the first time, however, that the latter *aqp1ab* transcripts and proteins separate into three sister clusters. If subcluster 1 and 2 are collated and compared to subcluster 3, the within cluster topologies also follow phylogenetic rank, with acanthomorph and ostariophysan sequences represented twice. Such paralogous topologies usually indicate the existence of two genes, which was investigated by an extended synteny analysis of the vertebrate aquaporin-1 loci.

The data revealed that while synteny is highly conserved within the acanthomorph teleosts, it is less conserved when compared to zebrafish, a feature that has been have noted for other gene families (Finn et al. 2009; Cerdà and Finn 2010; Chauvigné et al. 2010). Neverthe-

less, the teleost-specific aquaporin paralogs (*aqp1aa* and *-1ab*) are all tandemly arranged between the genes *crhr2* and *thoc1*. The medaka is the only exception, which as noted previously (Tingaud-Sequeira et al. 2008), appears to have lost the *aqp1ab* paralog. An interesting aspect of this analysis is the more conserved gene contiguity between zebrafish and the Sauria, and the absence of syntenic conservation in the mammalian lineage. While it is known that teleost genomes have experienced a whole genome duplication (WGD) event and subsequent rearrangements (Kasahara et al. 2007; Nakatani et al. 2007; Muffato and Roest-Crollius 2008), the higher conservation of synteny between teleostean, amphibian, reptilian and avian lineages suggests that the regional loss of synteny occurred within Mammalia. Within Eutheria, synteny is highly conserved but it is broken at the *THOC1* locus, in which the syntenic cassette of genes observed downstream of the *aqp1aa-aqp1ab-thoc1* loci in Teleostei is found on separate chromosomes. We concluded from this analysis that the aquaporin-1 loci are encoded in complex regions of vertebrate genomes that have undergone lineage-specific intra- and inter-chromosomal shuffling.

The syntenic data show that the aquaporin-1 subfamily in teleosts reflects the *aqp8* system, in which WGD together with tandem duplication gave rise to different *aqp8* paralogs (Tingaud-Sequeira et al. 2010; Cerdà and Finn 2010). In the case of the teleost *aqp1aa* and *-1ab* paralogs they represent tandem duplicates, while the WGD product (i.e. the true *aqp1b*) is probably lost in many species, but appears to exist in the zebrafish genome as the fused *aqp5/1b* pseudogene (Tingaud-Sequeira et al. 2010; Cerdà and Finn 2010). The co-clustering of the Atlantic halibut *aqp1aa* and *-1ab* together with stickleback (*Gasterosteus aculeatus*) *aqp1aa* and *-1ab*, respectively, further implies that tandem duplication of an ancestral *aqp1* gene provided the likely basis for the adaptive evolution of the *aqp1ab* paralog.

An interesting aspect of this finding is the co-evolution of the organic osmolyte system that drives oocyte hydration in marine teleosts. Both the yolk precursor gene (*vtgaa*), which is highly expressed in marine teleosts and is the major source of osmotically active free amino acids (FAA) in the oocyte, and the water channel (*aqp1ab*) responsible for mediating water influx into the oocyte, appear to have arisen through tandem duplication rather than WGD (Finn et al. 2009; Finn and Fyhn 2010; Cerdà and Finn 2010). The subsequent neofunctionalization of the proteins reveals that gene duplication *per se* is an important foundation of novel cellular pathways that alter the phenotype. In the latter instance, it is the rise of the pelagic egg, which has been suggested to have a broad implications for the oceanic radiation of teleosts (Finn and Kristoffersen 2007; Kristoffersen et al. 2009).

Tandem duplication is however not unique to the teleost *aqp1aa* and *-1ab* orthologs, since in human the synteny analysis revealed a putative AQP1-like paralog at the 30.9 Mb locus on LG 7, two genes upstream of the conserved *AQP1* locus. Similarly, *AQP7* and *-12* appear to have tandemly duplicated in some Primates (Tingaud-Sequeira et al. 2010; Cerdà and Finn 2010). For teleosts, however, it is the tandemly duplicated *aqp1ab* gene that has neofunctionalized, as suggested by the molecular phylogeny and functional assays of the Aqp1ab C-terminus which is the most rapidly evolving aquaporin subdomain in the vertebrate lineage. Within each group, tetrapod AQP1 and teleost Aqp1aa have undergone similar rates of amino acid substitution (13-17%), while the duplicated Aqp1ab paralog has experienced significantly greater amino acid substitution, particularly within the C-terminal domain. Given that Tetrapoda and Teleostei evolved over similar timescales (~390 million years), the present data provide an eminent example of primary structural divergence in the aftermath of gene duplication.

2. Conserved Physiological Role of Aqp1ab During Oocyte Hydration in Marine Teleosts

In previous studies in pelagophil teleosts, such as the gilthead seabream and Japanese eel (*Anguilla japonica*), the physiological role of Aqp1ab during oocyte hydration is supported by circumstantial evidence (i.e., specific subcellular localization of Aqp1ab in the oocyte correlating with the hydration process), and the observation that the swelling of oocytes undergoing meiosis resumption *in vitro* is completely or partially blocked by aquaporin inhibitors such as mercury and tetraethylammonium (Fabra et al. 2005, 2006; Kagawa et al. 2009). However, it is known that these compounds can also affect K^+ channels and other ion transport proteins (Armstrong 1990; Jacoby et al. 1999), which may play a role for inorganic osmolyte accumulation in the oocyte (Cerdà et al. 2007; Kristoffersen and Finn 2008). Therefore, direct evidence for the role of Aqp1ab during fish oocyte hydration is still lacking.

To investigate whether the role of Aqp1ab during oocyte hydration is conserved in more advanced teleosts, we selected the Atlantic halibut as an experimental model. This species is a marine acanthomorph teleost that reproduces at low temperature and spawns one of the largest pelagic eggs known, and thus their large oocytes can be readily manipulated for experimental purposes. In the Atlantic halibut, the *aqp1ab* gene is highly expressed in the ovary as it occurs in other teleosts (Tingaud-Sequeira et al. 2008). However, in this species we found an specific and transient up-regulation of *aqp1ab* transcripts during meiosis resumption, and a subsequent rise of the protein product, which is translocated into the microvillar

portion of the oocyte plasma membrane. Thus, the Atlantic halibut Aqp1ab channel seems to be regulated in the oocyte in a similar fashion to gilthead seabream Aqp1ab (Fabra et al. 2006). The data in halibut correlate very well with the transient hyperosmolality of the ooplasm in relation to the ovarian fluid, due in part to ion accumulation and yolk proteolysis (Finn et al. 2002), and are thus consistent with a role of Aqp1ab mediating water influx and consequent decrease in the intra-oocytic osmolality of Atlantic halibut oocytes (Finn et al. 2002).

As expected from previous studies (Fabra et al. 2005; Tingaud-Sequeira et al. 2008; Kagawa et al. 2009; Tingaud-Sequeira et al. 2010), and the structural features of Atlantic halibut Aqp1ab, $HgCl_2$ was able to reversibly block halibut Aqp1ab permeability when expressed in both homologous and heterologous oocytes, as well as to reduce the hydration normally associated with the meiotic maturation of Atlantic halibut oocytes. However, because mercury is not a specific aquaporin blocker, we tested for a more specific inhibition of Aqp1ab function by using an antisera raised against the C-terminus of halibut Aqp1ab. This antisera proved effective at blocking the translocation of Aqp1ab into the plasma membrane of *Xenopus laevis* oocytes. Microinjection of the antibody into Atlantic halibut follicle-enclosed oocytes undergoing hydration in vivo resulted in a dose-dependant inhibition of oocyte hydration, while the process of yolk hydrolysis and meiotic maturation were not affected. The immunological inhibition could be fully reversed by the artificial expression of the Aqp1aa paralog, which is functional in halibut oocytes, but is not recognized by the antibody. Consequently, the decrease of hydration of halibut oocytes can be directly related to the loss of function of Aqp1ab. These findings, together with the gene expression and cellular localization data, therefore provide for the first time functional evidence of the essential role of Aqp1ab-mediated water transport during the hydration of fish oocytes undergoing meiotic maturation.

3. Transcriptional Regulation of *aqp1ab* in the Gilthead Seabream Oocyte

The studies in the gilthead seabream and Japanese eel, and also in the Atlantic halibut, employing indicate that the *aqp1ab* transcripts are highly accumulated in the ovary, presumably in pre-vitellogenic oocytes, and subsequently the Aqp1ab protein seems to be synthesized and slowly transported to the oocyte cortex during the growth (vitellogenic) period (Fabra et al. 2006; Tingaud-Sequeira et al. 2008; Kagawa et al. 2011). These observations suggest that the *aqp1ab* gene might be regulated at the transcriptional, translational and post-translational level in oocytes, but the molecular mechanisms involved remain unknown. In the gilthead

seabream, we first re-examined the developmental regulation of *aqp1ab* gene expression during oogenesis *in vivo* by performing *in situ* hybridization and immunofluorescence microscopy. The results showed that there is a short temporal phase of *aqp1ab* gene transcription in primary growth (perinucleolus) pre-vitellogenic oocytes, in which meiosis is already arrested, and that the resultant transcripts are in fact immediately translated prior to their depletion at the onset of vitellogenesis. The *de novo* pool of Aqp1ab proteins in the primary growth oocytes may be post-translationally modified (Tingaud-Sequeira et al. 2008), transported and stored in the peripheral ooplasm during subsequent stages of oocyte development. Therefore, in the present thesis we begun to dissect the molecular mechanisms involved in the transcriptional regulation of *aqp1ab* in primary growth oocytes of the gilthead seabream.

3.1. Isolation and Characterization of the Seabream *aqp1ab* Promoter

As a first step toward elucidating the transcriptional mechanisms that underly *aqp1ab* expression in oocytes, we cloned the 5'-flanking region of the seabream *aqp1ab* gene. Analysis of the nucleotide sequence of the *aqp1ab* most proximal 5' flanking region (1,672 bp) revealed three domains (nucleotides -906 to -706, -581 to -498, and -405 to -259, considering position +1 the first nucleotide of the translation start codon ATG) with similarity to exons 2/3 (59% identity), 3 (71% identity) and 4 (40% identity), respectively, of the *aqp1ab* gene, suggesting the presence of traces in the genome of a duplicated *aqp1ab* pseudogene.

As observed in the core promoter of many mammalian aquaporins (Inase et al. 1995; Umenishi and Verkman 1998a,b; Borok et al. 2000), the seabream *aqp1ab* proximal promoter shows consensus sequences for core promoter elements important for the interaction with the basal transcription machinery, such as several TATA and CCAAT boxes, Sp1, AP1, E-box and initiator (lnr) elements, which facilitate binding of regulatory proteins to the core promoter and are important for TSS selection and promoter strength (Smale and Baltimore 1989). Experimental determination of the transcription start sites (TSSs) by RLM-RACE indicated five different initiation sites in primary oocytes, although the majority of transcripts seem to initiate at position 364 upstream of the ATG start codon (TSS2). Alternative TSSs are also found in some mammalian aquaporin genes, such as mouse AQP8 (Calamita et al. 1999) or human AQP4 (Lu et al. 1996). In the seabream *aqp1ab* 5'-flanking region, the lnr sequences are found very close to the experimentally determined *aqp1ab* TSSs. The TSS1 (-906), TSS2 (-364), TSS3 (-247) and TSS4 (-214) are however respectively positioned 252, 298, 415 and 448 nucleotides downstream of a TATA box (-662), which is much further than the optimal

position (-31 to -30 in relation to the TSS) described in metazoans (Ponjavic et al. 2006). For TSS5, however, two TATA boxes are located 13 and 133 nucleotides upstream of the initiation site. The absence of a proximal TATA box upstream of TSS1-TSS4, and the presence of a GC-rich region particularly upstream of TSS2, suggests that transcription of *aqp1ab* in oocytes may be predominantly TATA-independent. A similar mechanism has been suggested for some mammalian aquaporins, such as the mouse *AQP8* gene (Calamita et al. 1999), the mouse and human *AQP5* (Lee et al. 1996) and the rat *AQP5* in salivary glands (Borok et al. 2000).

The *in silico* analysis of the seabream *aqp1ab* promoter identified a duplicated *aqp1ab* pseudogene and numerous putative *cis*-acting regulatory elements that may serve as targets for sequence-specific enhancer/silencer transcription factors. An interesting aspect of the former observation was that the experimentally determined TSSs approximately utilised the conserved *aqp1ab* splice sites of exons 2-4, supporting the notion that a degraded *aqp1ab* gene exists at this locus. Among the *cis*-acting regulatory elements identified, we found numerous consensus sequences that may be bound by transcription factors involved in gut and/or kidney development in mammals and zebrafish, such as CDX (Flores et al. 2008; Beck and Stringer 2010), Krüppel-like factors (KLF4, KLF15; Mallipattu et al. 2012; Li et al. 2011; Yu et al. 2012), PBX1 (Schnabel et al. 2003), or NFKB1 (Panzer et al. 2009). This observation is consistent with the expression of *aqp1ab* in osmoregulatory organs of teleosts, such as the kidney and rectum (Cerdà and Finn 2010), in addition to the oocyte, and with the constitutive activation of the seabream *aqp1ab* promoter in HEK293T and Caco-2 cells (see below). In the teleost kidney and rectum, Aqp1ab has been proposed to play a role to maintain water balance by mediating transcellular water transport across epithelia (Cerdà and Finn 2010). The seabream *aqp1ab* promoter also contains three potential CREB sites responsive to cAMP that are found in mamalian aquaporin genes including *AQP2* (Yasui et al. 1997; Yu et al. 2009) and *AQP5* (Wang and Zheng 2011), as well as one potentially conserved site for hypoxia inducible factor-1, which can activate murine *AQP1* transcription in endothelial cells in response to hypoxia (Abreu-Rodríguez et al. 2011).

3.2. The Nuclear Progestin Receptor and Sox Factors Regulate *aqp1ab* Transcription

Notably, the seabream *aqp1ab* promoter contains two progestin responsive elements [PRE(a) and PRE(b)], which can bind the nuclear progestin receptor (Pgr), and three putative binding sites for SOX transcription factors [SOX(a), SOX(b) and SOX(c)]. All these *cis*-regulatory sites are conserved upstream of the *aqp1ab* gene of different teleosts. It is known that different *sox*

genes, such as *sox3*, *-8*, *-9b*, are expressed in oogonium and early oocytes of non-mammalian vertebrates (Koyano et al. 1997; Rodríguez-Mari et al. 2005; Yao et al. 2007; Raghuveer and Senthilkumaran 2010; Dumond et al. 2011), although in most cases their specific roles during sex determination and/or gonad differentiation remain to be elucidated. In addition, recent studies have demonstrated the expression of both the mRNA and protein product of the nuclear Pgr in primary oocytes of zebrafish (Hanna et al. 2010; Zapater et al. 2012). Therefore, the functional relevance of the Pgr and Sox transcription factors for the transcriptional regulation of the *aqp1ab* in seabream oocytes was first investigated by cloning the corresponding cDNAs and determining their pattern of expression during oogenesis.

3.2.1. Isolation of Seabream Nuclear Pgr and Sox Transcription Factors

By using RT-PCR followed by 5' and 3' RACE, the seabream nuclear Pgr cDNA was isolated, which encodes for a protein with a typical nuclear steroid receptor structure showing high homology in the amino acid sequences of the DNA binding domain (DBD) and ligand binding domain (LBD) with other vertebrate PGRs. The seabream Pgr is similar to that found in other perciform teleosts (Todo et al. 2000; Ikeuchi et al. 2002; Hanna et al. 2010; Chen et al. 2010, 2011, 2012), while it is more distantly related with the Pgr of salmonids, cyprinids or anguillids, and shows some amino acids substitution in the LBD which do not affect the affinity of the receptor for 17 and 20 hydroxyl groups of progestins, 17,20β-P and 17α,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P), in transactivation assays.

Phylogenetic and syntenic analyses provided the first evidence for the loss of a paralogous nuclear *pgr* ortholog in the genomes of higher orders of Teleostei. However, in seabream, in contrast with amphibians and other basal teleosts (Tian et al. 2000; Todo et al. 2000; Thornton 2001; Ikeuchi et al. 2002; Wang et al. 2004; Katsu et al. 2008; Chen et al. 2010, 2011, 2012; Hanna et al. 2010) but similar to that reported in mammals (Conneely et al. 1987; Kastner et al. 1990; Li and O'Malley 2003; Cork et al. 2008), three different transcription variants of the *pgr* gene were found to be expressed in the ovary, which were termed *pgr_tv2*, *pgr_tv3* and *pgr_tv4*. These isoforms encode functional receptors and are generated by alternative pre-mRNA splicing rather than by alternate initiation of translation. Thus, splicing leads to mRNAs encoding N-terminally truncated Pgr isoforms lacking approximately half of the A/B region (Pgr_tv2), the full A/B region and a small portion of the DBD (Pgr_tv3), or bearing only part of the LBD (Pgr_tv4). A fourth isoform (Pgr_tv1) was also identified which apparently originates from a splicing site within exon 1, causing an in-frame deletion of 121 amino acids

in the A/B region. Transactivation assays using mammalian HEK293T cells indicate that the transcriptional activity of the Pgr_tv2 isoform is not significantly different to that of the wild-type Pgr. In contrast, both Pgr_tv3 and Pgr_tv4 variants show less transactivation efficiency than the Pgr, and are associated with nuclear and cytosolic mechanisms, respectively, of dominant-negative repression of Pgr-mediated transcription. The mechanism underlying the dominant-negative inhibition of Pgr_tv3 on Pgr function is likely related to the ability of Pgr_tv3 to translocate into the nucleus, dimerise and compete for PRE and/or common nuclear cofactors, similar to that has been suggested for mammalian PGR-A or PGR-C isoforms (Vegeto et al. 1993; Wei et al. 1996). The seabream Pgr_tv4 variant represents however a novel naturally occurring dominant-negative Pgr isoform, which can promote some transcriptional activity despite lacking the DBD and nuclear localization signal, possibly through non-genomic mechanisms (Cork et al. 2008). The strong dominant-negative inhibition of Pgr_tv4 on Pgr-mediated transcription, in the presence of 17,20β-P, is therefore likely mediated by a different mechanism than that of the Pgr_tv3 isoform, which may involve higher Pgr_tv4 affinity for limiting concentrations of some cytoplasmic factor(s), such as some kinases (Aranda and Pascual 2001), required for Pgr DNA binding and/or transactivation, but not for Pgr trafficking.

To isolate seabream *sox* paralogs expressed in the ovary, we also employed RT-PCR and 5' and 3' RACE, using degenerate primers designed within the Sox factor HMG-box sequence. This approach resulted in the cloning of three different full-length cDNAs with deduced amino acid sequence homologies to Sox proteins. Bayesian analyses of the isolated nucleotide and deduced amino acid sequences in relation to a broad selection of vertebrate sox orthologs revealed duplicated *sox8* and *-9* genes and a single *sox3* gene in teleosts. Based upon the co-clustering of the seabream *sox* transcripts and proteins with the zebrafish, we classified the seabream sequences isolated as *sox3*, *-8b* and *-9b*.

3.2.2. Expression of the Pgr, and Sox3, -8b and -9b During Seabream Oogenesis

The developmental expression of the seabream nuclear *pgr* and *sox3*, *-8b* and *-9b* in relation to that of *aqp1ab* during early oogenesis *in vivo*, determined after *in situ* hybridization and immunolocalization (for the Pgr) experiments, showed that the Pgr and *sox3* and *-8b* transcripts are strongly expressed in oogonia, thus preceding the activation of *aqp1ab* transcription and translation in primary growth oocytes. This specific localization of the Pgr protein both in the cytoplasm and the nucleus of oogonia entering meiosis, would be consistent with

its recently proposed role during progestin-mediated initiation of oogonial meiosis in teleosts (Miura et al. 2006, 2007). However, in primary growth oocytes the Pgr is almost completey translocated from the cytoplasm into the nucleus indicating the likely activation of the receptor by its cognate ligand at this oocyte stage. In contrast, *sox9b* transcripts appear to be expressed at low levels in primary growth stage oocytes but its expression level was greatly enhanced in more advanced stages of oocyte development (i.e. cortical alveolus). These findings therefore provided circumstantial evidence for a possible role of Pgr as well as of Sox factors in regulating *aqp1ab* transcription in seabream primary growth oocytes.

In our studies, we also detected the presence of Pgr in granulosa cells associated with seabream primary growth oocytes, as well as in cortical alveolus and fully-grown vitellogenic ovarian follicles, as reported for zebrafish (Hanna et al. 2010). The expression of the Pgr in granulosa cells of late vitellogenic follicles would be consistent with the known role of this receptor during ovulation (Thomas et al. 2007). However, unlike in zebrafish (Hanna et al. 2010), we found peripheral cytoplasmic localization of the nuclear Pgr in a region close to the plasma membrane in seabream cortical alveolus and vitellogenic oocytes. This pattern of staining, together with the presence of a conserved palmitoylation motif in the LBD of the seabream Pgr, may suggest a role of this receptor, possibly in addition to membrane Pgrs (Zhu et al. 2003; Josefsberg-Ben-Yehoshua et al. 2007; Hanna and Zhu 2011; Thomas 2012; Tokumoto et al. 2012), in the regulation of meiotic maturation of seabream oocytes (Bayaa et al. 2000; Tian et al. 2000; Martínez et al. 2006, 2007). The *sox8b* transcripts are also expressed in granulosa cells surrounding cortical alveolus and early vitellogenic oocytes of the seabream. Although *sox8* has not been detected in the granulosa cells of zebrafish (Rodríguez-Marí et al. 2005), this observation in seabream is in line with the finding of SOX8 expression in mural granulosa cells of mouse preovulatory follicles (Salmon et al. 2005). Interestingly, *sox3* and *cyp19a1* (see below) are co-expressed in the ooplasm of seabream primary oocytes, which would be consistent with *sox3* activation of *cyp19a1* transcription as shown in some amphibians (Oshima et al. 2009).

In humans and rodents, the PGR-A and PGR-B isoforms, as well as different splice variants (i.e. PGRΔ3, PGRΔ6), are co-expressed in the ovary (Ilenchuk and Walters 1987; Schneider et al. 1991; Misao et al. 1998; Conneely et al. 2003). Different isoform expression titres are also apparent in the seabream ovary, where the *pgr_tv2*, *pgr_tv3* and *pg_tv4* mRNA variants are co-expressed with wild-type *pgr* throughout development, but in general with higher isoform ratios to full-length *pgr* during early and late oogenesis. Although in the present work we

could not determine the level of translation of these isoforms *in vivo*, such transcript levels of *pgr_tv3* and *pgr_tv4* are possibly insufficient for complete inhibition of Pgr-mediated transcription (based on the pattern of inhibition of Pgr_tv3 and Pgr_tv4 in HEK293T cells), but can potentially modulate the transcriptional function of the Pgr. This mechanism would resemble the regulation of PGR-C and other splice forms on PGR-B or PGR-A function suggested to occur in human breast cancer cells and the ovary, respectively (Richer et al. 2002; Condon et al. 2006; Cork et al. 2008). In reproductive tissues, PGR-A and PGR-B control a different subset of genes (Richer et al. 2002; Mulac-Jericevic and Conneely 2004), and hence over-expression of PGR-A protein compared to PGR-B, which is common in breast cancer cells (Graham et al. 1995, 2005), can change progestin responsiveness of cells (Graham et al. 2005). Therefore, we cannot rule out a similar mechanism in the seabream ovary since it remains to be investigated whether Pgr, Pgr_tv2 and Pg_tv3 can regulate distinct subsets of progestin-dependent target genes.

3.2.3. The Pgr and Sox factors Differentially Regulate *aqp1ab* transcription

Functional analysis of the seabream *aqp1ab* promoter confirmed that the nuclear Pgr, as well as Sox3, -8b and -9b, differentially regulate *aqp1ab* transcription. Since teleost Aqp1ab is expressed in both somatic and germ cells (Cerdà and Finn 2010), we employed HEK293T cells and *X. laevis* oocytes for transactivation assays. Frog oocytes have typically been used in early gene regulation studies because they are transcriptionally very active, show a basal transcriptional machinery distinct from that in somatic cells, and, unlike gilthead seabream oocytes, are very large and easy to collect and microinject in high quantities (Li et al. 2009). Therefore, stage IV *X. laevis* oocytes, although at a more advanced stage than seabream primary growth oocytes, offer a good alternative to investigate *aqp1ab* transcription in germ cells. The experiments on HEK293T cells and oocytes, using wild-type and mutated *aqp1ab* promoter constructs, gave identical results demonstrating that the Pgr is a major inducer of *aqp1ab* transcription, whereas Sox3 and -8b cooperate with Pgr to activate *aqp1ab*. The stimulatory effect of these Sox factors through the SOX(c) binding site, which is closely located to the proximal PRE(b), was synergistic with that of Pgr suggesting that this mechanism may involve Sox-mediated conformational changes in the chromatin that exposes the PREs to facilitate their binding by the Pgr (Vicent et al. 2010; Clarke and Graham 2012). In contrast, in both somatic cells and oocytes, Sox9b acting through the same cis-regulatory SOX(c) site exerts a more potent transcriptional repression of *aqp1ab* compared to the activation abilities of either Sox3 or

-8b. These findings may explain the developmental decrease of the *aqp1ab* mRNA levels *in vivo* due to the increased expression of *sox9b* in the ooplasm of seabream cortical alveoli stage oocytes. Considering that at this latter stage of oogenesis there is a prominent expression of *sox3* and *-8b* in the ooplasm, and the nuclear localization of the Pgr is reduced with respect to that observed in primary growth stage oocytes, it is likely that rather low levels of Sox9b are able to inhibit *aqp1ab* expression in seabream cortical alveolus oocytes.

The transcriptional regulation of *aqp1ab* by the Pgr was confirmed *ex vivo* by using seabream ovarian explants at the primary growth stage exposed to 17,20β-P in the presence or absence of the Pgr inhibitor RU486, followed by qRT-PCR, Western blot and chromatin immunoprecipitation (ChIP) assays. These experiments demonstrated that 17,20β-P enhanced the recruitment of the Pgr to both PREs in the *aqp1ab* promoter in primary growth oocytes and activated *aqp1ab* transcription and translation.

3.3. The Follicle-Stimulating Hormone Receptor Activates *aqp1ab* Transcription Through Progestin Synthesis

The previous findings strongly suggest that the nuclear Pgr is the major regulator of *aqp1ab* transcription in gilthead seabream primary growth oocytes. To confirm that the Pgr ligand (i.e. 17,20β-P) is produced at this stage, and ellucidate the possible endocrine pathway involved, a series of experiments *in vivo* and *in vitro* were carried out.

As a first approach, the major enzymes involved in 17β-estradiol (E2) and progestin synthesis in teleosts, P450c17-I (Cyp17a1) and aromatase (Cyp19a1), and P450c17-II (Cyp17a2) and 20β-hydroxysteroid dehydrogenase (Cbr1), respectively, were cloned. Subsequently, their pattern of mRNA expression in seabream primary growth and vitellogenic follicles *in vivo* was determined by *in situ* hybridization and qRT-PCR. The experiments demonstrated that all these steroidogenic enzymes are expressed in primordial granulosa cells associated with primary oocytes. The expression of *cbr1* in seabream previtellogenic follicles agrees with previous reports on other teleosts, such as the Atlantic cod (Kortner et al. 2009) and North African catfish (Sreenivasulu and Senthilkumaran 2009). In primordial theca cells, however, we only observed a weak expression of *cyp17a2* and *cbr1*. In contrast, in vitellogenic follicles, *cyp17a1*, *cyp17a2* and *cbr1* are expressed in both theca and granulosa cells, whereas *cyp19a1* transcripts are found only in granulosa cells, as noted in some other teleosts (Nagahama and Yamashita 2008; Lubzens et al. 2010). However, we also found *cyp17a2* and *cyp19a1* transcripts in the cytoplasm of seabream primary oocytes, whereas in the ooplasm of

vitellogenic oocytes we detected *cyp17a1*, *cyp17a2*, as well as *cbr1* mRNAs. This latter observation is not completely surprising because, in some teleosts, mitochondrial and microsomal 20β-hydroxysteroid dehydrogenase activities have been detected in ovulated oocytes devoid of follicle cells (Kazeto et al. 2001). Also, *cyp17a1* and *cyp19a1* have been shown to be expressed and active in the cytoplasm of trout and amphibian vitellogenic oocytes (Yang et al. 2003; Gohin et al. 2011a,b).

The higher expression of *cbr1* in the ovary occurred during the early primary growth stage, and was concomitant with a high ovarian content of 17,20β-P *in vivo* and high expression of the follicle-stimulating hormone receptor (*fshra*) but not of the luteinizing/choriogonadotropin receptor (*lhcgrba*). However, the plasma concentrations of 17,20β-P were slightly elevated in the same fish, and were only little different from the plasma concentrations of E2 or 17,20β,21-P. The presence of binding activity for 17,20β-P in seabream primary oocytes, as it occurs for testicular androstenedione in sea *lamprey (Petromyzon marinus*) (Bryan et al. 2007), might explain these results, although this is presently no more than speculation. It is of interest, though, that specific binding activity for 17,20β-P has been demonstrated in mature testes of the North Sea plaice (*Pleuronectes platessa*) (Mugnier et al. 2000). If steroid binding proteins are the reason for the tendency of 17,20β-P to accumulate in the seabream gonad tissue, it will be interesting to know whether the binding activity is due to the presence of progestin receptor(s) or sex steroid binding globulins, both of which would have the ability to retain 17,20β-P in the ovary rather than allowing it to diffuse away.

The previous observations suggested that it might be Fshra that controls 17,20β-P synthesis at the primary growth stage. To investigate this hypothesis, we used available recombinant gonadotropins from another perciform teleost, i.e. the European seabass, to stuy the effect of gonadotropins on steroid synthesis in primary ovarian explants *in vitro*. The characterization of the response of seabream Fshra and Lhcgrba to seabass recombinant follicle-stimulating hormone (rFsh) and luteinizing hormone (rLh) using HEK293T cells indicated, as found for some other teleosts (Levavi-Sivan et al. 2010; Chauvigné et al. 2012), that the Fshra could be activated by rFsh and rLh, whereas the Lhcgrba was rLh specific. The high promiscuity of the seabream Fshra to seabass rLh (which has to be confirmed using homologous hormones), obviated the use of rLh to discern the involvement of Fshra or Lhcgrba in 17,20β-P synthesis in primary ovarian explants *in vitro*. Therefore, we decided to test only rFsh, which is Fshra specific since it does not activate the seabream Lhcgrba even at pharmacological doses. The experiments *in vitro* showed that rFsh stimulated 17,20β-P production within 24 h,

which was sustained over the period of culture by the addition of 17-P precursor, whereas T and E2 production were relatively unaffected by 17-P. The rFsh-mediated synthesis of 17,20β-P over E2 was found to correlate with the up-regulation of *cyp17a2* and *cbr1* transcription, and the decrease of *cyp17a1* and *cyp19a1* mRNA levels. Therefore, these data strongly suggest the role of the Fshra in the activation of the Cyp17a2/Cbr1 pathway in seabream primary ovarian follicles triggering the production of 17,20β-P. In addition, we found that rFsh also up-regulates wild-type *pgr* mRNA and protein levels over time, thus in a similar fashion to that described in mural granulosa cells of mammalian pre-ovulatory follicles where FSH and LH control PGR-A and PGR-B transcription through a cAMP-mediated pathway (Natraj and Richards 1993; Park-Sarge and Mayo 1994; Clemens et al. 1998). Thus, the short-term (24 h) rFsh-mediated up-regulation of *pgr* expression in seabream primary ovarian explants coincides with the stimulation of 17,20β-P production but not of E2. These observations imply that rFsh, acting most likely via the Fsh receptor, simultaneously promotes the synthesis of Pgr and its ligand in primary ovaries, and that this relatively rapid effect is likely estrogen independent as it occurs in mammalian granulosa cells (Clemens et al. 1998). The 17,20β-P produced at this stage will subsequently activate the *aqp1ab* promoter in primary growth oocytes as indicated above, which is consistent with the inhibition of rFsh-mediated *aqp1ab* transcription in explants cultured in the presence of steroidogenesis and 17,20β-P synthesis inhibitors, such as DL-aminoglutethimide and indomethacin, respectively.

The mechanism of Fshra action on steroidogenesis in seabream primary growth follicles is similar to the proposed action of Lh, likely via the Lhcgrba or Lhcgrbb, in postvitellogenic ovarian follicles of other teleosts at the time of induction of oocyte maturation and ovulation (Nagahama and Yamashita 2008; Lubzens et al. 2010; Senthilkumaran et al. 2004; Zhou et al. 2007; Tanaka et al. 2002). The specific ligand involved in this mechanism *in vivo* (i.e. whether it is Fsh or Lh), as well as its origin (either in the pituitary or secreted from developing oocytes; Wong and Zohar 2004), is however unclear from the present studies, since the seabream Fshra can also be activated by rLh. Although tools for measuring of pituitary and circulating Fsh levels are unavailable for the gilthead seabream, previous studies have shown that the plasma levels of Lh in immature females with previtellogenic ovaries are much lower (~1 ng/ml) than those at maturation (~17 ng/ml) (Holland et al. 1998), which may point to the involvement of Fsh rather than Lh in the gonadotropic control of steroidogenesis in primary ovaries. However, without information on the circulating levels of Fsh during the reproductive

cycle of the seabream, and detailed characterization of the response of seabream Fshra to homologous hormones, this conclusion may be premature.

3.4. A Model for the Molecular Regulation of *aqp1ab* in Seabream Oocytes

In our *in vitro* experiments, the stimulation of primary explants with rFsh also induced a retarded increase in *cyp19a1* expression, which coincided with an increase in the concentration of E2 in the tissue. Surprisingly though, at the same time, the expression of *cyp17a1* was negatively correlated with the rFsh dose. To reconcile these and previous observations, including the prominent expression of *cyp19a1* in the cytoplasm of primary oocytes, we propose a model that summarizes our current hypothesis for the Fshra-mediated regulation of steroidogenic pathways in seabream primary ovarian follicles and further activation of the *aqp1ab* promoter (Fig. 1). This model involves the activation of the Fshra in primordial follicle cells by Fsh (or Lh) which rapidly drives the up-regulation of *cyp17a2* and *cbr1* transcription, and the down-regulation of *cyp17a1* and *cyp19a1*, resulting in an enhanced production of 17,20β-P. The increase in T synthesis that we noted in our studies is probably due to the induction of 17β hydroxysteroid dehydrogenase (*hsd17b*) expression, as suggested to occur in zebrafish vitellogenic ovarian follicles incubated with human chorionic gonadotropin (hCG) (Ings and Van der Kraak 2006) or in FSH-stimulated human granulosa-luteal cells (Whitehead and Lacey 2003). This would trigger the rapid transformation of androstenedione (as formed by Cyp17a1) to T, which might then diffuse slowly into the oocytes where it may be aromatized to E2 by Cyp19a1 (Gohin et al. 2011a, b). The relatively late production of E2 after rFsh stimulation *in vitro* may reflect the fact that *cyp19a1* transcription is initially downregulated, but then subsequently upregulated - perhaps stimulated by second messengers (i.e. cAMP) originating in follicle cells and translocated into the oocyte via heterologous gap junctions between granulosa cells and the oocyte (i.e. Cerdà et al. 1993; Selman et al. 1993). The E2 produced in the oocyte may in turn exert a negative feedback mechanism on follicular *cyp17a1* expression, since it is known that estrogen can regulate the steady-state mRNA levels of *cyp17a* in trout (Govoroun et al. 2001) and rat testis (Sakaue et al. 2002). Such potential dialog between primary oocytes and follicle cells to control steroid synthesis, similar to that suggested to occur in amphibian vitel-logenic follicles (Sretarugsa and Wallace 1997; Yang et al. 2003) remains however to be demonstrated.

The 17,20β-P produced by granulosa cells associated to primary growth oocytes in response to Fshra activation binds subsequently to the nuclear Pgr of oocytes, activates the re-

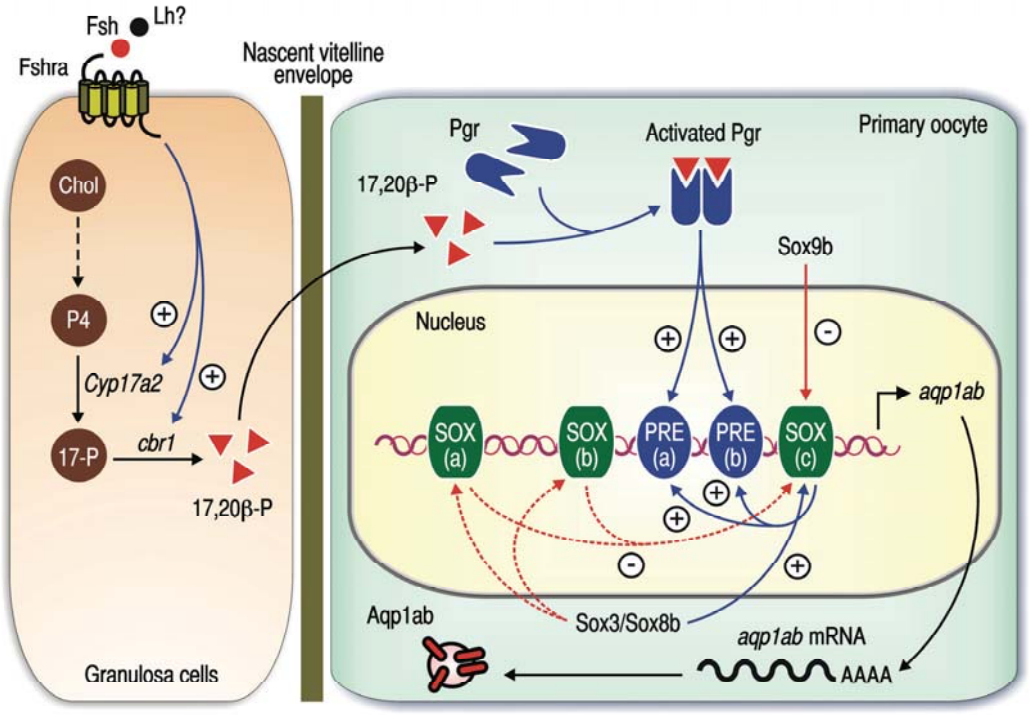

Fig. 1. Proposed model for the endocrine pathway involved in *aqp1ab* transcriptional regulation in gilthead seabream primary growth oocytes. The scheme depicts Fsh receptor (Fshra)-mediated progestin (17,20β-P) synthesis and subsequent activation of *aqp1ab* transcription through the nuclear Pgr under positive and negative modulation by Sox3, -8b and -9b. At the primary growth stage, activation of the Fshra in granulosa cells triggers 17,20β-P production through the up-regulation of the P450c17-II (Cyp17a2)/20β-hydroxysteroid dehydrogenase (Cbr1) steroidogenic pathway in granulosa cells. Binding of 17,20β-P to Pgr activates the receptor which promotes transcription likely through independent interaction with two PREs [PRE(a) and PRE(b)] in the *aqp1ab* proximal promoter. Interaction of Sox3 or -8b with a proximal SOX(c) binding site can act synergistically with the PRE(a) or PRE(b) to enhance Pgr-mediated transcription. The distal SOX(a) and/or SOX(b) sites can however down-regulate the synergistic effect of Sox3 or Sox8b through the SOX(c) site (dashed lines). By contrast, Sox9b represses *aqp1ab* transcription only through the SOX(c) binding site. Thus, presumably low levels of expression of Sox9b in primary growth oocytes allow Pgr-driven *aqp1ab* mRNA and protein synthesis, whereas increased Sox9b levels in more advanced oocyte stages may inhibit *aqp1ab* expression.

ceptor and promotes *aqp1ab* transcription through independent interaction with two *cis*-acting PREs in the *aqp1ab* proximal promoter (Fig. 1). Both PRE(a) and PRE(b) are equally effective at transactivating *aqp1ab* in heterologous somatic and germinal cells, and therefore it remains unknown why two PREs are necessary in the *aqp1ab* promoter. It may be speculated that selection pressure has favoured the retention of multiple PREs for the efficient activation and synthesis of gene products such as Aqp1ab, in order that sufficient levels of the channel are avalailable in the fully-grown oocytes for rapid water flux during meiosis resumption. In any event, interaction of Sox3 or -8b with a proximal SOX(c) binding site synergistical-

ly stimulates the Pgr-mediated *aqp1ab* transcriptional activity. The distal SOX(a) and/or SOX(b) sites can however down-regulate the synergistic stimulation of Sox3 or -8b mediated by the SOX(c) *cis*-element. In contrast, Sox9b represses *aqp1ab* transcription through the SOX(c) site. Hence, low expression levels of Sox9b in primary growth oocytes may allow Pgr-driven *aqp1ab* mRNA and protein synthesis, whereas increased Sox9b levels in more developed oocytes would inhibit *aqp1ab* transcription. In addition to PRE and SOX elements, our *in silico* analysis of the seabream *aqp1ab* promoter revealed the presence of consensus sequences for the binding of transcription factors that may be of relevance during oocyte development, such as TCF/LEF-1 involved in the Wnt signalling pathway (Harwood et al. 2008; Langdon and Mullins 2011), CREB (Sundaram et al. 2003) or PBX-HOX (Villaescusa et al., 2004). Therefore, it is possible that some of these transcription factors participate together with Pgr and Sox in the regulation of *aqp1ab* transcription in seabream oocytes. Indeed, exposure of seabream primary explants to cAMP induces increased *aqp1ab* mRNA levels (data not shown). In addition, we have recently shown that seabream primary growth ovaries express up to four alternative splicing forms of the nuclear Pgr, two of which are associated with nuclear and cytosolic mechanisms of dominant-negative repression of Pgr-mediated transcription. It would be therefore of interest in future studies to elucidate the cellular localization of these inhibitory Pgr isoforms in the seabream primary ovarian follicles (oocyte *vs*. somatic cells), as well as their potential role at regulating *aqp1ab* transcription.

The proposed model elucidates important aspects of the endocrine regulation of the *aqp1ab* gene in a modern acanthomorph teleost that spawns pelagic eggs, which is the most prevalent reproductive strategy evolved by marine teleosts (Finn and Kristoffersen, 2007). However, extensive searching of available genomes revealed that *cis*-acting PREs and SOX sites are also present in the 5' flanking region of *aqp1ab* genes of different teleosts regardless of their reproductive strategy (i.e. production of hydrated pelagic *vs*. non-hydrated benthic eggs). This is illustrated, for instance, in the freshwater zebrafish, which produces non-hydrated benthic eggs and in which Pgr and Sox responsive elements are found in the 5' proximal genomic region of the aqp1ab gene. As in the gilthead seabream, the nuclear Pgr is detected in the nucleus of primary growth oocytes of the zebrafish (Hanna et al. 2010), and hence one could expect a prominent activation of the *aqp1ab* in these oocytes. However, this mechanism apparently does not occur since in the zebrafish ovarian *aqp1ab* transcripts are hardly detected by RT-PCR (Tingaud-Sequeira et al. 2008, 2010). To investigate the underlying mechanism, we carried out a preliminary experiment using transient transgenic zebrafish

expressing *aqp1ab* promoter-enhanced green fluorescent protein (EGFP). The results showed that when the *aqp1ab*-EGFP plasmid was microinjected into zebrafish embryos at 1-2 cell stages, the 1.672- kb aqp1ab promoter was able to target EGFP expression specifically to the rectum of 35-40 days postfertilization females, which is consistent with the prominent abundance of Aqp1ab at the apical brush border of rectal enterocytes in the seabream (Raldúa et al. 2008). However, EGFP expression was not detected in oocytes. Interestingly, in contrast to the seabream, primary growth and cortical alveoli stage oocytes of the zebrafish strongly express *sox9b* (Rodríguez-Marí et al. 2005), which we found here represses seabream *aqp1ab* promoter activity. Based on these observations, and in accordance with our proposed model, it can be speculated that Sox9b proteins or other oocyte factors may inhibit Pgr-mediated *aqp1ab* transcription in zebrafish oocytes and perhaps in other teleosts in which *aqp1ab* is not required for the production of viable eggs. However, because mutant SOX *aqp1ab* constructs were not tested in these preliminary experiments, this hypothesis needs to be addressed in future studies, as well as the existence of additional mechanisms for *aqp1ab* transcriptional regulation in the ovary of teleosts that produce non-hydrated benthic eggs.

4. Post-Translational Regulation of Aqp1ab Trafficking

In this thesis, we have found that despite the high level of amino acid substitution within the teleost Aqp1ab orthologs, its role during oocyte hydration is most likely conserved in marine species. However, the Atlantic halibut Aqp1ab seems to be an unusually highly specialized aquaporin since we found that this protein did not traffic to the oocyte plasma membrane unless it is expressed in native or piscine oocytes. This is surprising given that, to our knowledge, AQP11 and/or -12 orthologs are the only vertebrate aquaporins, including those of teleosts (Tingaud-Sequeira et al. 2010), that are not targeted to the plasma membrane when expressed in *X. laevis* oocytes, a feature that has been explained by the intracellular localization of this aquaporin in pancreatic acinar cells (Itoh et al. 2005; Ohta et al. 2009). The *ex vivo* functional failure of halibut Aqp1ab in *X. laevis* oocytes could not be alleviated by exposure of oocytes to known signal transduction molecules such as cAMP or cGMP (data not shown), that are known to control trafficking of catfish Aqp1ab as well as of tetrapod AQP2 and AQP-h2 (Hasegawa et al. 2003; Nedvetsky et al. 2009; Chaube et al. 2011). While these results suggest that phosphorylation might not play a role in the plasma membrane localization of halibut Aqp1ab its function during Aqp1ab trafficking can not be ruled out due to the highly divergent nature of the putative phosphorylation sites within the teleost Aqp1ab C-termini. Indeed,

phosphorylation of Ser^{254} has been shown to mediate Aqp1ab recycling in the gilthead seabream (Tingaud-Sequeira et al. 2008). Interestingly, however, by co-expressing Atlantic halibut Aqp1ab cRNAs with polyA$^+$ mRNA purified from post-vitellogenic Atlantic halibut ovarian follicles in *X. laevis* oocytes, it was possible to rescue the *ex vivo* membrane trafficking of halibut Aqp1ab. These observations suggest that Aqp1ab and halibut oocytes may have co-evolved lineage-specific, protein-based mechanisms for the intracellular transport of Aqp1ab during meiotic maturation. In support of this hypothesis is the observation that halibut Aqp1ab was fully functional when expressed in oocytes of a distantly related ostariophysan teleost such as the zebrafish.

The precise nature of the polyA$^+$ mRNA-derived mechanism controlling Aqp1ab plasma membrane localization in Atlantic halibut oocytes is yet unknown. Our present data reveal however that the halibut Aqp1ab C-terminus is likely involved, as indicated by functional experiments using wild-type halibut Aqp1aa and -1ab and chimeric constructs. This finding reinforces the role of the C-terminus in the rapid neofunctionalization of Aqp1ab among teleosts previously suggested (Tingaud-Sequeira et al. 2008; Cerdà 2009), but the specific function of this domain in the trafficking mechanism of Atlantic halibut Aqp1ab remains intriguing. In addition to phosphorylation, C-terminus-mediated interactions with cytoskeletal components, sorting vesicles and lysosomal trafficking regulators (Kamsteeg et al. 2007; Nedvetsky et al. 2009; Moeller et al. 2010), as well as proper folding of the proteins in the endoplasmic reticulum (ER) (van Balkom et al. 2002; Pitonzo and Skach 2006), can control the intracellular transport of aquaporins. In the present study, post-translational modifications of halibut Aqp1ab resulting from the co-expression of polyA$^+$ mRNA from Atlantic halibut oocytes were not evident by Western blotting analysis of total membrane extracts from *X. laevis* oocytes under reducing or non-reducing conditions. This suggests that halibut Aqp1ab was properly folded and not retained in the ER even in the absence of polyA$^+$ mRNA from halibut oocytes. Under such conditions, i.e. retention in the ER, aquaporins are usually glycosylated (Deen et al. 1995) and our data indicate that halibut Aqp1ab is not glycosylated. However, translocation of functional halibut Aqp1ab into the oocyte plasma membrane was associated with the formation of a 66-kDa Aqp1ab complex in both native Atlantic halibut oocytes undergoing hydration as well as in *X. laevis* oocytes co-expressing halibut Aqp1ab and polyA$^+$ mRNA. These complexes most likely represent strong Aqp1ab oligomers that do not dissociate under denaturing and reducing conditions, as found for the *Escherichia coli* AqpZ (Borgnia et al. 1999) and some plant aquaporins (Ohshima et al. 2001; Casado-Vela et al. 2010). There-

fore, whether the enzymatic machinery for the formation of these complexes is only present in teleost oocytes, and/or is a highly specific sorting mechanism of Aqp1ab-containing vesicles that is absent in *X. laevis* oocytes, remains to be investigated.

5. References

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CONCLUSIONS

Conclusions

1. The functional *aqp1* paralogs of teleosts (*aqp1aa* and *aqp1ab*) are tandem duplicates where the whole genome duplication product (*aqp1b*) is lost in the Acantomorpha. The C-terminus of the *aqp1ab* protein product is the most rapidly evolving subdomain within the vertebrate aquaporin superfamily, suggesting that this aquaporin has neofunctionalized.

2. Specific immunological inhibition of Aqp1ab in Atlantic halibut (*Hippoglossus hippoglossus*) oocytes, and further rescue of aquaporin function by over-expression of the Aqp1aa paralog, provides direct experimental evidence for the essential role of Aqp1ab during oocyte hydration. These findings confirm previous conclusions in the the gilthead seabream (*Sparus aurata*), and suggest that the physiological function of Aqp1ab during meiosis resumption mediating water uptake into the oocyte is conserved in marine teleosts spawning pelagic (buoyant) eggs.

3. The rapid divergence of the C-terminal domain of the Atlantic halibut Aqp1ab results in *ex vivo* loss of function in amphibian oocytes, which can be rescued by injection of polyA$^+$ mRNA from native post-vitellogenic ovarian follicles. These findings reveal the dual nature of neofunctionalization of the teleost Aqp1ab water channels where selection pressure has favoured oocyte hydration, but has been relaxed with regard to the specific cellular mechanisms controlling aquaporin trafficking.

4. In the gilthead seabream, there is a short temporal phase of *aqp1ab* gene transcription in meiosis-arrested oocytes at the primary growth (perinucleolus) stage, and the resultant transcripts are immediately translated prior to their depletion at the onset of vitellogenesis. The *de novo* pool of Aqp1ab proteins may be post-translationally modified, transported and stored in the peripheral ooplasm during subsequent stages of oocyte development.

5. Isolation of the 5'-flanking region of the seabream *aqp1ab* gene reveals the presence of two conserved *cis*-regulatory progestin responsive elements (PREs) for the putative binding of the nuclear progestin receptor (Pgr), as well as three binding sites for members of the SRY-related high mobility group (HMG)-box (SOX) family of transcription factors.

6. The higher orders of Teleostei retain a single *pgr* gene. However, in the gilthead seabream four different *pgr* transcript variants are expressed in primary growth ovaries, three of them arising from alternative pre-mRNA splicing resulting in different N-terminally truncated receptors. The seabream wild-type Pgr shows the highest transactivation response to native euteleostean progestins, such as 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P), whereas two of the Pgr isoforms independently regulate novel nuclear and cytosolic mechanisms of dominant-negative repression of Pgr-mediated transcription. This suggests that Pgr-mediated progestin functions in the ovary of advanced teleosts may be regulated through the differential expression of Pgr splice variants.

7. Wild-type Pgr and three different Sox transcription factors are differentially expressed during seabream early oogenesis. The Pgr, as well as *sox3* and *-8b* transcripts, are co-expressed in oogonia, whereas in primary growth oocytes the Pgr is completely translocated from the ooplasm into the nucleus. By contrast, *sox9b* is highly expressed in more advanced oocytes (cortical alveolus and vitellogenic), coinciding with a strong depletion of *aqp1ab* transcripts in the oocyte.

8. Transactivation assays in mammalian cells and *Xenopus laevis* oocytes using wild-type and mutated seabream *aqp1ab* promoter constructs, demonstrated that *aqp1ab* transcription is dependent on the Pgr, with Sox3 and -8b acting synergistically, while Sox9b acts as a repressor. Thus, the higher expression of *sox9b* in seabream cortical alveolus oocytes would be consistent with its role as a repressor of *aqp1ab* transcription *in vivo*.

9. The ligand of the Pgr, 17,20β-P, is produced *in vivo* and *in vitro* by granulosa cells associated with primary growth oocytes in response to the activation of the follicle-stimulating hormone receptor (Fshra), which drives the up-regulation of the cytochrome P450c17-II/20β-hydroxysteroid dehydrogenase carbonyl reductase-like steroidogenic pathway for progestin synthesis as well as of the nuclear Pgr. Subsequently, the 17,20β-P activates the Pgr of oocytes, and promotes *aqp1ab* transcription and translation through independent interaction with the two *cis*-acting PREs in the *aqp1ab* proximal promoter.

10. Our data have thus uncovered the endocrine and molecular pathway for the precise transcriptional regulation of *aqp1ab* during seabream oogenesis, in which the nuclear Pgr is likely the major regulator and is modulated by Sox transcription factors.

SUMMARY

INTRODUCCIÓN

En teleósteos la oogénesis es un proceso altamente regulado que incluye diferentes estadios de desarrollo, desde la diferenciación y proliferación de las células germinales primordiales (PGCs) y el subsiguiente crecimiento del ovocito, hasta la formación de un huevo maduro preparado para la fertilización. En esta breve introducción se resumen los conocimientos actuales sobre los principales procesos de la oogénesis de teleósteos.

1. Estadios de la Oogénesis

La oogénesis de teleósteos se puede dividir en seis periodos diferentes según el crecimiento del oocito: proliferación de la oogonia, crecimiento primario, etapa de alveolo cortical, vitelogénesis, maduración meiótica del oocito y ovulación (Lubzens et al. 2010). Las PGCs se diferencian en oogonia las cuales proliferan por mitosis. Las oogonias entran en meiosis para formar los oocitos primarios, los cuales empiezan a conectar con las células somáticas (células pre-foliculares) para la formación del folículo ovárico. Durante la etapa de crecimiento primario, la meiosis se interrumpe en el estado de diploteno (profase de la primera división meiótica), y se mantiene detenida hasta el final de la vitelogénesis. Durante este periodo, el oocito incrementa considerablemente su tamaño debido a la síntesis de los alveolos corticales, la incorporación de vitelogenina y la acumulación de proteínas del vítelo. En peces marinos, la hidratación del oocito durante la maduración es la principal causa del incremento de tamaño del huevo el cual ya está preparado para ser ovulado y fertilizado (Cerdà et al. 2007). Los principales mecanismos fisiológicos durante el crecimiento del oocito y la maduración se resumen en la Fig. 1.

1.1. Diferenciación de las PGCs

Las PGCs generan las células reproductivas haploides o gametos las cuales son producidas al principio de la embriogénesis y más tarde migran hacia la posición de las gónadas donde se diferencian en gametos (Wylie 2000; Starz-Gaiano and Lehmann 2001). Durante la migración, las PGCs responden a señales específicas de las células somáticas (Knaut et al. 2003.; Knaut y Schier 2008; Saito et al. 2011), pero deben que ignorar otras con el fin de permanecer no diferenciadas durante la migración. Inmediatamente, después de la colonización de la gónada,

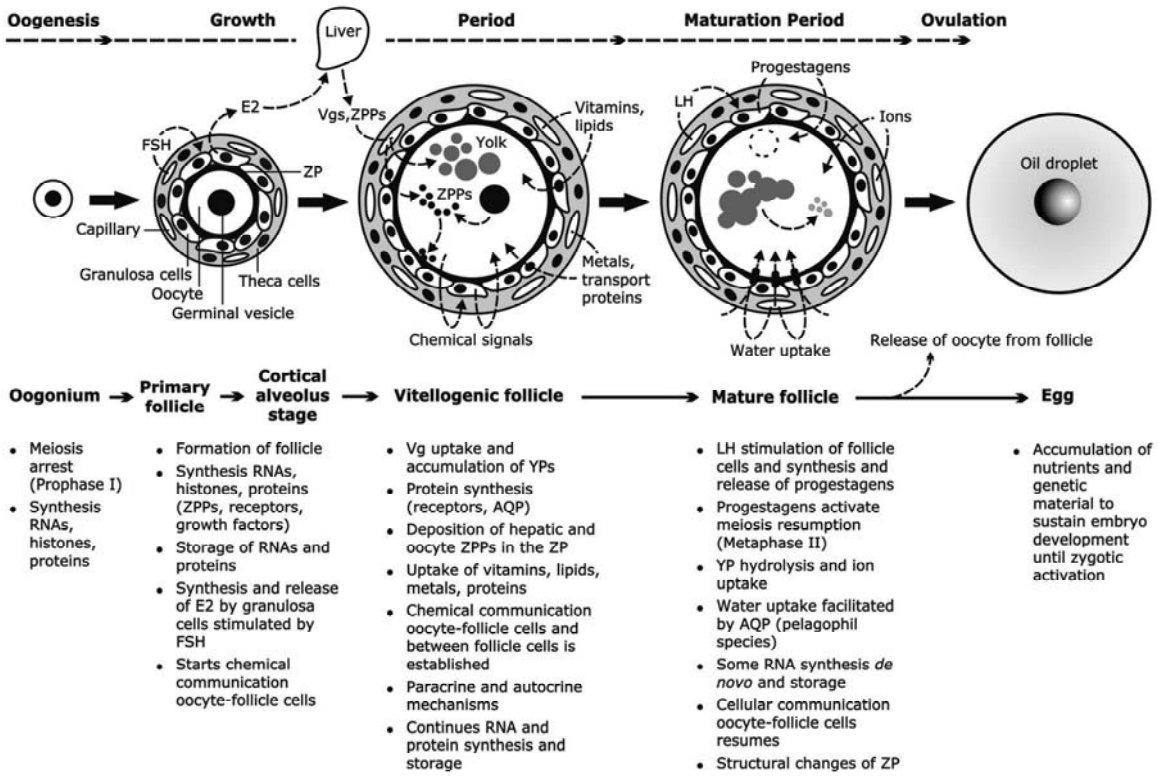

Fig. 1. Representación esquemática de los principales mecanismos moleculares durante el crecimiento y maduración del oocito de peces. AQP, acuaporina, E2, 17β-estradiol, FSH, hormona estimulante del folículo, LH, hormona luteinizante; Vgs, vitellogeninas; ZP, zona pelúcida; ZPPS, proteínas de la zona pelúcida. Reproducido de Cerdà et al. (2008).

empieza la diferenciación sexual de las PGCs en gametos femeninos o masculinos con la proliferación por mitosis seguida de la división meiótica.

En mamíferos, la determinación del sexo depende de la constitución cromosómica del organismo y parece que es controlada por el gen SRY que incrementa la transcripción del factor de transcripción *SOX9* en las células de sostén de la gónada XY (Swain y Lovell-Badge 1999; Capel 2000). En los teleósteos, los mecanismos ambientales son tan importantes en la determinación del sexo como los factores genéticos, sin embargo en algunas especies, como el pez cebra (*Danio rerio*) o la anguila Europea (*Anguilla anguilla*) no se han identificado diferencias entre los cromosomas de los dos sexos lo que indica probablemente que las señales poligénicas o ambientales están más implicadas en la determinación del sexo (Traut et al. 2001). Por el contrario, en medaka (*Oryzias latipes*), el desarrollo masculino requiere la expresión del gen DMY/dmrt1bY similar a lo observado en mamíferos (Matsuda et al. 2002; Nanda et al. 2002). Sin embargo, los genes ortólogos en teleósteos, Sox9b y -9a, parece que no están involucrados en el desarrollo masculino, mostrando diferencias entre los vertebrados

superiores e inferiores (Chiang et al. 2001; Yokoi et al. 2002; Raghuveer y Senthilkumaran 2010; Nakamura et al. 2012). En los teleósteos, la aromatasa (Cyp19a1a, véase más adelante) también juega un papel importante en la determinación del sexo. Así, la supresión de Cyp19a1a se asocia a la masculinización, lo que sugiere que la Cyp19a1a promueve la diferenciación ovárica y inhibe la diferenciación testicular (Guiguen et al. 2010).

1.2. Proliferación de las Oogonias, Activación y Arresto de la Meiosis

Durante la proliferación de las oogonias, cada oogonia se multiplica por divisiones mitóticas y estas quedan conectadas por puentes intercelulares (Fig. 2). Estas estructuras se delimitan por una monocapa de células de la granulosa primordiales que secretan la lámina basal, separándolas de las células del estroma y formando así los nidos de oogonias. La transición de oogonia a oocito primario se caracteriza por el inicio de la primera división meiótica antes de abandonar el nido oogonial (Lubzens et al. 2010). Durante la meiosis, los nidos oogoniales se rompen o desaparecen durante el paquiteno, cuando cada uno de los ovocitos es envuelto por las células pre-foliculares y se individualizan. Las células somáticas del estroma interactúan con las células pre-foliculares y descansan sobre la lámina basal formando una monocapa para después diferenciarse en las células de la teca las cuales se asocian con los vasos sanguíneos (Mazzoni et al. 2010). Los oocitos, las células de la granulosa, la lámina basal y las células de la teca, constituyen el folículo ovárico. Durante esta etapa, los oocitos progresan en la meiosis hasta la profase I y se detienen en la etapa de diploteno.

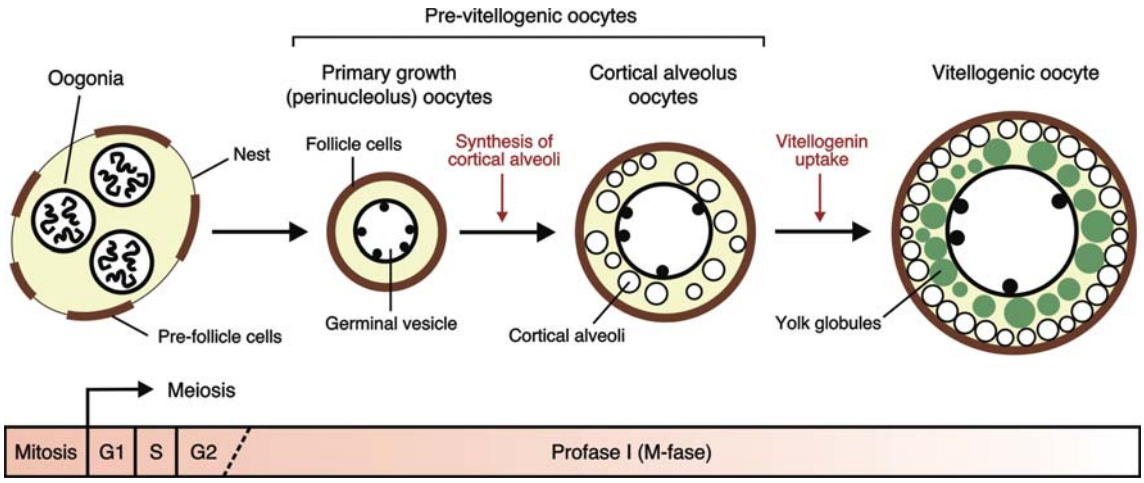

Fig. 2. Representación esquemática de los diferentes estadíos durante el desarrollo ovocitario de peces, desde oogonia hasta vítelogénesis, y de la progresión de la meiosis.

1.3. Crecimiento Primario y Foliculogénesis

El periodo de crecimiento primario del oocito se caracteriza por una alta acumulación de ARN citoplasmático, un aumento del volumen celular, la formación de la envoltura vitelina acelular entre el oocito y las células de la granulosa, y el desarrollo del cuerpo de Balbiani, el cual se amplía y se dispersa por todo el ooplasma al mismo tiempo que se desarrollan el retículo endoplasmático y el aparato de Golgi. La foliculogénesis está vinculada con el crecimiento primario e incluye el desarrollo de las capas foliculares (células de la granulosa y la teca) que rodean el oocito (Grier 2012). Las células foliculares y el oocito se separan del epitelio germinal por una membrana basal que también forma el límite entre los compartimentos epiteliales y el estroma (Grier 2000). Durante esta etapa, se comienzan a producir contactos intercelulares entre el oocito y las células de la granulosas (Cerda et al. 1999; Grier 2012).

Durante la etapa de crecimiento primario, se produce la transcripción de un gran número de genes necesarios en las etapas posteriores de la oogénesis así como durante la embriogénesis temprana. Estos transcritos son llamados ARNm maternos y son cruciales para un correcto desarrollo del embrión. En los anfibios y peces teleósteos, la mayoría de los ARNm presentes en los oocitos que han llegado a su máximo crecimiento ya están presentes en las etapas finales de la fase de crecimiento primario (Wallace y Selman 1990; Pelegrí 2003). Los genes maternos podrían ser empleados como marcadores moleculares de la calidad del huevo porque cualquier perturbación en estos genes produce una dramática disminución de la supervivencia del embrión (Mtango et al. 2008).

1.4. Transición Hacia el Crecimiento Secundario

La transición de la etapa perinuclear a la etapa de alveolo cortical, que es cuando el oocito se prepara para comenzar la vitelogénesis, incluye cambios a nivel estructural y hormonal en el oocito. El cambio más típico es la síntesis de una gran cantidad de glicoproteínas endógenas que se incorporan en los alveolos corticales. Estos alvéolos corticales están inicialmente distribuidos de forma uniforme en el citoplasma, pero durante la vitelogénesis estos se desplazan a la periferia del oocito a medida que el vítelo se acumula de forma centrípeta. Posteriormente durante la fecundación, se lleva a cabo la reacción cortical que implica la liberación del contenido de los alveolos corticales en el espacio perivitelino para prevenir la polispermia y la entrada de agentes patógenos (Tyler y Sumpter 1996; Patiño y Sullivan 2002). Durante el crecimiento previtelogénico, se inicia también la deposición de lípidos en el oocito lo que contribu-

ye al aumento de tamaño de estos durante esta etapa (Selman y Wallace 1989). Se ha sugerido que el origen de estas gotas lipídicas son las lipoproteínas de muy baja densidad (VLDL), las cuales se unen a sus receptores y son hidrolizadas en ácidos grasos libres (Patiño y Sullivan 2002).

1.5. Vítelogénesis

La vítelogénesis se caracteriza por la incorporación de vítelogeninas (Vtgs), lípidos y vitaminas por el oocito, y la escisión de las Vtgs en proteínas del vítelo, lo cual produce un enorme aumento en el tamaño del folículo ovárico y el oocito se hace competente para la fertilización (Lubzens et al. 2010). Al mismo tiempo, se lleva a cabo el desarrollo completo de la envoltura vitelina que rodea el oocito (Le Menn et al. 2007) y se establece la comunicación intercelular entre el oocito y las células de la granulosa a través de diferentes estructuras de interacción célula-célula (uniones gap, uniones adherentes y otros) (Cerdà et al. 1999; Le Menn et al. 2007).

Las Vtgs son fosfoglicoproteínas que se encuentran en todos los vertebrados e invertebrados ovíparos, y juegan un papel importante como fuente de nutrientes durante el desarrollo embrionario temprano (Babin et al. 1999). Una molécula de Vtg completa se compone de una cadena pesada de lipovitelina (LvH), la fosvitina (Pv), una cadena ligera de lipovitelinina (LvL), y el componente β'. Existen al menos tres tipos principales de Vtgs las cuales se procesan en el oocito de forma distinta y se clasifican como VtgAa, VtgAb, y la VtgC, la cual carece del dominio de fosvitina (Finn 2007a,b). Las Vtgs son sintetizadas por el hígado, glicosiladas y fosforiladas en el retículo endoplasmático y el complejo de Golgi, en respuesta a estradiol-17β (E2) producido por las células del folículo ovárico (ver más adelante) (Finn 2007a,b). Las Vtgs entran en el folículo ovárico a través de los capilares de la teca y pasan a través de los espacios intercelulares de la granulosa y a través de los poros de la envoltura vitelina hasta llegar a la superficie de los oocitos. Allí, las Vtgs se incorporan específicamente por endocitosis mediada por receptor a través del precursor del receptor de VLDL (VLDLR), denominado también en animales ovíparos como el receptor de Vtg (Vtgr) (Babin et al. 1999). La escisión proteolítica de las Vtgs y su posterior procesamiento a proteínas del vítelo se produce en los cuerpos multivesiculares por la acción de las enzimas lisosomales como la catepsina D y catepsina B (Carnevali et al. 1999a, b; Raldúa et al. 2006; Carnevali et al. 2006.). Posteriormente las proteínas del vítelo se almacenan en los glóbulos o plaquetas de vítelo del citoplasma (Wallace y Selman 1990).

Durante la vítelogénesis, los lípidos y las vitaminas, también necesarios para el desarrollo embrionario, se incorporan asimismo en el oocito (Lubzens et al. 2010). Las vitaminas A y E son reclutadas en los tejidos periféricos y son transportadas a los oocitos por las lipoproteínas (Palace y Werner 2006), aunque la proteína fijadora de retinol (RBP) y la proteína de unión al tocoferol también parecen estar implicadas (Lubzens et al. 2010). Las Vtgs también pueden transportar vitamina A en forma de retinal (Irie y Seki 2002) y vitamina E como α-tocoferol (Lie et al. 1994).

1.6. Maduración e Hidratación del Oocito

La meiosis se reanuda en la etapa final del crecimiento del oocito en respuesta a señales hormonales. En esta fase, la meiosis en el oocito progresa hasta la metafase II de la segunda división meiótica, y al mismo tiempo los oocitos experimentan profundos cambios morfológicos, tales como la condensación de los cromosomas y la ruptura de la vesícula germinal (GVBD). En teleósteos marinos ovíparos, estos procesos nucleares están asociados con la fusión de los glóbulos de vítelo para formar una masa central de vitelo fluido, la ruptura de las estructuras cristalinas dentro de los glóbulos, y el crecimiento del citoplasma debido a la absorción masiva de agua o hidratación (Cerdà et al. 2007). Este mecanismo esta mediado por la acumulación en el oocito de diversos osmolitos, tales como iones inorgánicos (K^+, Cl^-, PO_4^{3-}, Na^+) y aminoácidos libres (FAAS) resultantes de la hidrólisis selectiva de las proteínas del vítelo almacenadas en el oocito (Cerda et al. 2007; Finn y Kristoffersen 2007; Finn 2007a, b; Kolarevic et al. 2008; Kristoffersen y Finn 2008; Finn y Fyhn 2010). El proceso pre-ovulatorio de hidratación del oocito que se produce en teleósteos marinos es vital para producir embriones viables, ya que este asegura una reserva de agua para cuando los huevos son liberados en el agua de mar hiperosmótica, y contribuye a la flotabilidad positiva de los huevos y embriones en el mar para facilitar el intercambio de oxígeno y su dispersión (Fyhn et al. 1999; Finn y Kristoffersen 2007).

1.6.1. Procesamiento de las Proteínas del Vítelo

En los oocitos de teleósteos, después de la incorporación de Vtgs y su posterior escisión en proteínas del vítelo, existe un mecanismo proteolítico adicional de estas en FAAs que ocurre durante la fase de maduración e hidratación del oocito (Wallace y Selman 1985; Greeley et al. 1986) (Fig. 3). En teleósteos pelagófilos, los cuales producen huevos pelágicos muy hidratados, las VtgAa y VtgAb contribuyen con cantidades aproximadamente iguales de FAAs al

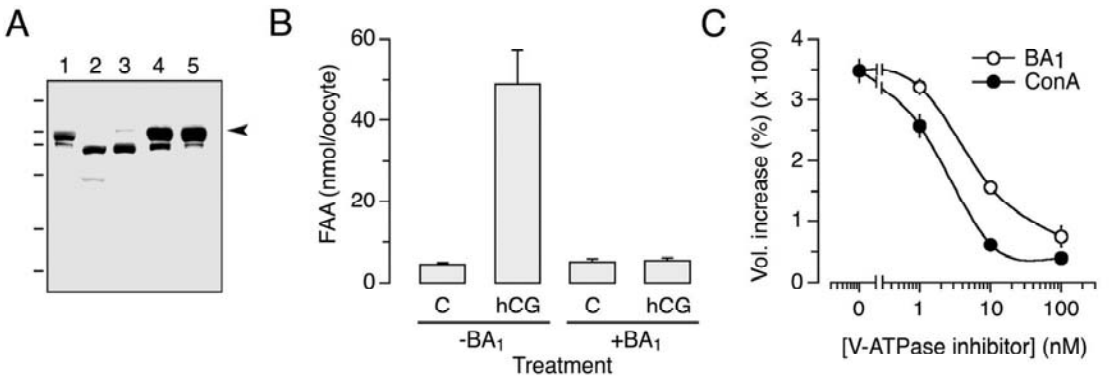

Fig. 3. Inhibición de la proteólisis del vítelo, generación de FAAs, e hidratación del oocito en teleósteos pelagófilos. A, Western blot de las proteínas del vítelo de la dorada de folículos ováricos controles (carril 1) y estimulados con 17,20β-P en ausencia (carril 2) o presencia de 1, 10, y 100 nM de bafilomicina A1 (BA1), inhibidor de la ATPasa vacuolar (carriles 3,4 y 5, respectivamente), usando un anticuerpo contra la Vtg de dorada. Se observa que la degradación de las proteínas del vítelo de ~100 kDa, posiblemente lipovitelina, se inhibe por tratamiento con BA1. La posición (barras) de los marcadores de peso molecular se indica en la izquierda (de arriba a bajo: 200, 116, 97, 66, 45, y 29 kDa). B, El efecto de la BA1 sobre la generación de FAAs durante la maduración del oocito inducida con hCG en *Centropristis striata*. C, El tratamiento de los folículos con BA1 y concanamicina (ConA), otro inhibidor de la ATPasa vacuolar, previene la hidratación de los oocitos de dorada. Reproducido de Cerdà et al. (2007).

conjunto de osmolitos orgánicos del oocito durante la hidratación (Matsubara et al. 1995, 1999; Reith et al. 2001; Selman et al. 2001; Finn y Kristoffersen 2007; Finn 2007a). Por el contrario, en teleósteos bentófilos, emisores de huevos bentónicos poco o nada hidratados, la hidrólisis de las proteínas del vítelo es en gran parte limitada y sólo se observa un ligero aumento de FAAs (Wallace et al. 1992; LaFleur et al. 2005; Raldúa et al. 2006).

Los principales enzimas procesadores de las proteínas del vitelo en teleósteos son cisteína o aspartico proteasas, como la catepsina L, B y D (Murakami et al. 1990; Sire et al. 1994; Carnevali et al. 1999b, 2001; Kestemont et al 1999; Hiramatsu et al. 2002; LaFleur et al. 2005; Raldúa et al. 2006). En la dorada (*Sparus aurata*), se ha sugerido que la catepsina L es la principal proteasa implicada en la proteólisis de Lv *in vivo* durante la maduración del oocito (Carnevali et al. 1999a). Sin embargo, existen evidencias de que la catepsina B puede activar la catepsina L, ya que en el pez cebra se ha observado la inhibición de las dos catepsinas con un bloqueante específico de la catepsina B (Carnevali et al. 2006). En el pez momia (*Fundulus heteroclitus*), por el contrario, es la catepsina B la proteasa más probable involucrada en la degradación de la Lv (LaFleur et al. 2005; Raldúa et al. 2006). La activación de las catepsinas y la posterior proteólisis del vítelo, tanto en especies pelagófilas como en bentófilas, parece estar relacionada con la acidificación de los gránulos de vítelo mediada por una ATPasa vacuolar (Selman et al. 2001; Raldúa et al. 2006).

1.6.2. Papel de los Iones Inorgánicos

Los iones inorgánicos son también importantes efectores osmóticos durante la hidratación del oocito. Sin embargo, este papel es diferente entre teleósteos bentófilos y pelagófilos. En los teleósteos pelagófilos, mientras que los FAAs contribuyen a la mayor parte de toda la osmolalidad de oocitos, los iones inorgánicos (K^+, Cl^-, Pi y $NH4^+$) mantienen el balance (Selman et al. 2001; Finn et al. 2002a.). En los teleósteos bentófilos los iones inorgánicos, como el K^+ y el Na^+, son los principales efectores osmóticos (Greeley et al. 1991; Wallace et al. 1992; Chen et al. 2003). Los mecanismos para la absorción de iones en el oocito no se conocen del todo bien, aunque en teleósteos pelagófilos se ha sugerido un posible papel de la Na^+, K^+-ATPasa en el transporte activo iones (LaFleur y Thomas 1991; Chen et al. 2003). Sin embargo, en teleósteos bentófilos esta ATPasa no juega aparentemente un papel relevante en la absorción de iones durante la hidratación del oocito (Wallace et al. 1992), y por tanto puede ser que otros canales iónicos estén involucrados en este proceso (Bulling et al. 2000).

1.7. Ovulación

La ovulación se define como la liberación del oocito maduro del folículo ovárico. Durante el proceso de ovulación, el ovocito en metafase II se separa de la capa folicular, la pared del folículo se rompe y el oocito es liberado a la cavidad del ovario a través de la zona de rotura. Por lo tanto, antes de la ovulación, varios aspectos mecánicos y ultraestructurales cambian en el folículo ovárico, tales como la interrupción de las conexiones entre las células de la granulosa y el oocito y la apertura de la pared del folículo (Lubzens et al. 2010). Este proceso requiere la contracción de las microvellosidades del oocito y las células de la granulosa, así como la alteración posterior de las uniones gap y otras estructuras de adherencia celular (York et al. 1993; Cerdà et al. 1999). La acción mecánica de los oocitos sobre las capas foliculares circundantes también puede estar involucrada en la ovulación.

2. Hormonas Reproductivas

2.1. Gonadotropinas

Las gonadotropinas pertenecen a la familia de las hormonas glicoproteicas, que incluye las gonadotropinas de la pituitaria, la hormona estimulante del folículo (FSH) y la hormona lutei-

nizante (LH), la gonadotropina coriónica derivada de placenta (CG), y la hormona de estimulación tiroidea (TSH) (Pierce y Parsons 1981). Las cuatro glicoproteínas están formadas por una subunidad α común muy conservada entre las especies y una subunidad β específica que dicta la especificidad hormonal (Gray 1988; Pierce 1971). La asociación de ambas subunidades es necesaria para la correcta actividad biológica de las gonadotropinas (Ryan et al. 1987). En los teleósteos existen dos gonadotropinas, Fsh y Lh, que son análogas a las de mamíferos, FSH y LH (Burzawa-Gerard 1971; Donaldson et al. 1972; Levavi-Sivan et al. 2010). Las dos subunidades de las gonadotropinas están unidas de forma no covalente y estimulan sus receptores como heterodímeros α/β, ya que las subunidades individuales no muestran bioactividad significativa (Pierce y Parsons 1981).

El patrón espacio-temporal de expresión y secreción de la Fsh y la Lh sugiere que estas hormonas se producen en diferentes tipos celulares y se sintetizan de forma diferente durante el ciclo reproductor de teleósteos. El control de la síntesis y secreción de la Fsh y Lh es complejo e implica la interacción entre las gónadas, la hipófisis y el hipotálamo (Levavi-Sivan et al. 2010). Ambas gonadotropinas actúan sobre las gónadas y, a su vez, los esteroides y péptidos gonadales regulan su síntesis y secreción a través del hipotálamo y/o la hipófisis. El hipotálamo ejerce su regulación sobre la liberación de gonadotropina a través de varias neurohormonas, como la hormona liberadora de gonadotropina (GnRH), la dopamina, el ácido gamma-amino butírico (GABA), el péptido activador de la adenilato ciclasa hipofisaria (PACAP), la noradrenalina, el neuropéptido Y (NPY), la serotonina, las kispeptinas, y otros factores relacionados con el crecimiento y el metabolismo (Levavi-Sivan et al. 2010).

En los salmónidos, los niveles de Fsh en plasma son elevados durante las primeras etapas de la gametogénesis y disminuyen durante la maduración de los oocitos, mientras que los de Lh se mantienem bajos durante todo el ciclo y aumentan sólo en el desove (Suzuki et al. 1988; Swanson et al. 1991; Aizen et al. 2007; Molés et al. 2012; Shimizu et al. 2012). Ello sugiere que la Fsh puede tener un papel importante durante el crecimiento de las gónadas, mientras que la Lh puede actuar sobre todo durante la maduración de los oocitos y la ovulación. En la dorada, sin embargo, la expresión de *fshb, lhb* y *cga* no se detecta sólo en la glándula pituitaria sino también en el ovario (Wong y Zohar 2004).

2.1.2. Receptores de Gonadotropinas

Las gonadotropinas ejercen sus efectos biológicos a través de receptores específicos en la superficie de las células diana, los cuales estimulan la síntesis de AMPc con efectos posteriores

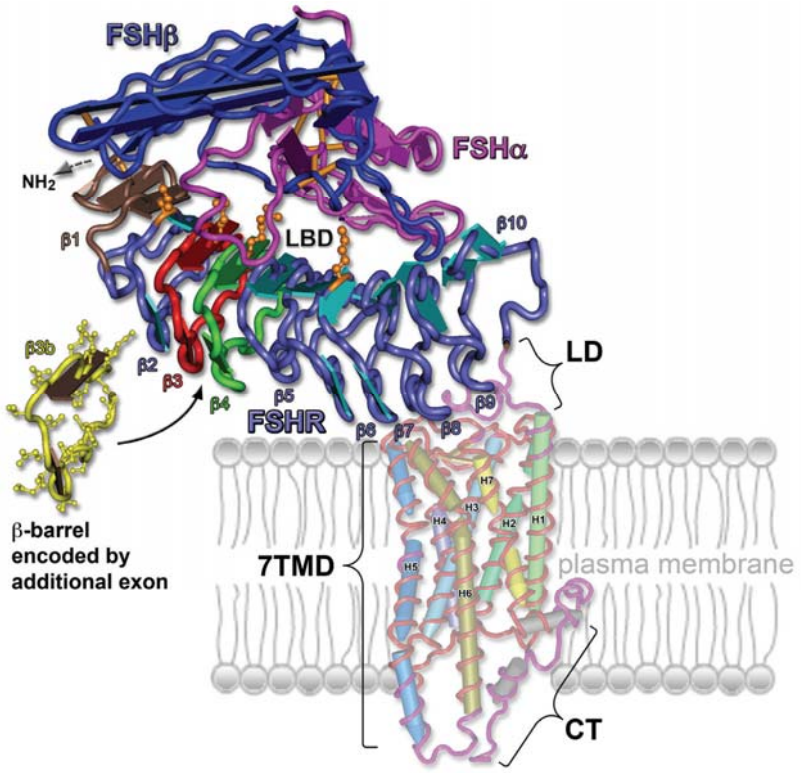

Fig. 4. Representación tridimensional del LBD del receptor de Fsh (Fshra) del lenguado Senegalés mapeado empleando las estructuras resueltas del FSHR humano y la rodopsina de calamar. La primera lamina β de cada repetición rica en leucina en el LBD está numerada 1-10. Se muestran las cadenas a (magenta) y b (azul) de la FSH humana para destacar las cadenas β (b3 rojo y b4 verde) del dominio interactivo. Las cadenas laterales de los residuos implicados con especificidad de unión en la molécula humana se muestran como cilindros anaranjados. La cadena 3b3 (amarillo) especifico de acantomorfos esta localizado entre b3 y b4. El modelo del FSHR termina en el dominio de unión (LD) extendiéndose abajo hacia el complejo de anclaje a la membrana acoplado a la rodopsina G (7TMD). Las hélices transmembrana se numeran del 1 al 7 hasta el dominio C-terminal (CT). Reproducido de Chauvigné et al. (2010).

sobre la actividad de diferentes proteínas quinasas (Levavi-Sivan et al. 2010). En muchos teleósteos, se han identificado los ADNc que codifican el receptor de Fsh (Fshra) y el receptor de Lh (Lhcgrba/Lhcgrbb) (Levavi-Sivan et al. 2010; Chauvigne et al. 2010). Estos receptores acoplados a proteínas G poseen un extenso dominio extracelular, caracterizado por múltiples repeticiones ricas en leucina (LRR) que son críticas para la unión específica con la hormona, siete dominios transmembrana que son la parte más conservada, y un corto C-terminal intracelular implicado en el acoplamiento de las proteínas G y la unión de diferentes moléculas de señalización intracelular (Fig. 4). En los mamíferos, cada gonadotropina tiene una afinidad altamente específica para su receptor, pero en los peces teleósteos, el Fshra y

Lhcgrba/Lhcgrbb pueden ser activados de forma cruzada por sus ligandos y, por lo tanto, la especificidad de los receptores todavía no está clara (Levavi-Sivan et al. 2010).

En el ovario de los teleósteos, los receptores de gonadotropinas se expresan en las células de la granulosa y de la teca que rodean el oocito donde regulan las vías esteroidogénicas (ver más adelante). Los niveles de expresión de ambos receptores difieren durante el desarrollo ovocitario y muestran diferentes patrones de expresión a nivel folicular, sugiriendo que estos participan en la regulación de los periodos críticos durante la oogénesis (Levavi-Sivan et al. 2010). En diferentes especies de teleósteos, la expresión del gen *fshra* es detectable en el ovario inmaduro y luego aumenta durante la vítelogénesis y disminuye una vez que los oocitos han alcanzado la fase de máximo crecimiento. Por el contrario, la expresión de *lhcgrba o lhcgrbb* aumenta durante la vítelogénesis tardía lo que sugiere un papel en la inducción de la maduración y la ovulación (Kumar et al. 2001a,b; Kwok et al. 2005; Kobayashi et al. 2008; Rocha et al. 2009; Chauvigne et al. 2010; Kazeto et al. 2010; Kitano et al. 2011).

2.2. Los Receptores de Esteroides

Las hormonas esteroideas, tales como andrógenos, estrógenos y progestinas, desempeñan papeles importantes en la regulación de un gran número de procesos fisiológicos mediante la interacción con sus receptores correspondientes. En los teleósteos, a diferencia de en mamíferos, las progestinas principales implicadas en la reproducción son 17α, 20β-dihidroxi-4-pregnen-3-ona (17,20 β-P) y 17α,20β,21-trihidroxi-4-pregnen-3-ona-(17,20 β,21-P) (Thomas et al. 2007; Nagahama y Yamashita 2008). Normalmente, los receptores de esteroides son intracelulares (es decir, citoplasmáticos) e inician la transducción de señales que conducen a cambios en la expresión génica durante un período de tiempo desde horas a días. Sin embargo, estudios recientes indican que algunas de las acciones rápidas de los esteroides están mediadas por receptores localizados en la membrana plasmática los cuales activan vías de transducción de señal interaccionando con las proteínas G y moléculas de señalización quinasa citoplasmáticas (Mani et al. 2012).

2.2.1. Receptores de Esteroides Nucleares

Los receptores de esteroides nucleares median acciones genómicas clásicas a través de la unión a elementos de respuesta a hormonas (HRE) de la región promotora de los genes diana modificando las tasas de transcripción (Thomas 2008). Los miembros de la superfamilia de receptores nucleares comparten una estructura proteica común, que consta de una región N-

terminal variable (A/B) responsable en algunos casos de la regulación transcripcional mediante el dominio de activación transcripcional independiente de ligando AF-1, un dominio de unión al ADN altamente conservado (DBD) que interactúa con los HREs y controla el gen(es) diana, una región bisagra (D) que controla la traslocación del receptor en el núcleo, y el dominio de unión a ligando moderadamente conservado (LBD) responsable del reconocimiento de la hormona y la especificidad de la respuesta fisiológica, el cual contiene el dominio AF-2 (Mangelsdorf et al. 1995; Beato et al. 2000). Los receptores de esteroides nucleares están situados en el citoplasma y viajan al núcleo de la célula después de la activación, o bien están situados en el núcleo a la espera de que entre el esteroide y los active. El tráfico en el núcleo es dependiente de las señales de localización nuclear que se encuentran entre el DBD y la región D, que en la mayoría de los casos están cubiertas por proteínas de choque térmico (HSPs) las cuales se unen al receptor hasta que la hormona está presente. Tras la unión de la hormona, el receptor experimenta un cambio conformacional, la HSP se libera, y el complejo ligando-receptor entra al núcleo donde se une a los HRE de las zonas promotoras génicas y recluta co-reguladores para modificar las tasas de transcripción (Tsai y O 'Malley 1994; Beato et al. 2000).

La mayoría de los promotores tienen múltiples sitios de inicio de la transcripción (TSSs) dentro de la región promotora, lo que indica diferentes modos de iniciación de la transcripción que pueden ser regulados de una forma específica en cada tejido (Carninci et al 2006; Kawaji et al. 2006). La transcripción es controlada por varios factores de transcripción que interaccionan con distintos elementos cis-reguladores (CREs), el núcleo del promotor que es el destino del complejo de pre-iniciación compuesto por los factores de transcripción basal y la ADN polimerasa II dependiente de ARN, las regiones promotoras proximal y distal que son reconocidas por los factores de transcripción específicos de secuencia, y los sitios distales potenciadores o silenciadores de la transcripción. Los CREs adicionales situados en las regiones proximal y distal actúan de forma independiente controlando la tasa de iniciación de la transcripción en el núcleo del promotor (Levine y Tjian 2003). La caja TATA fue descrita como el elemento del núcleo del promotor indispensable para el reclutamiento de la maquinaria de transcripción basal y la selección de los TSS (Lifton et al. 1978; Breathnach y Chambon 1981; Orphanides et al. 1996). Sin embargo, análisis de distintas regiones promotoras en una gran variedad de organismos han mostrado que la caja TATA no es una característica general en los promotores (Basehoar et al. 2004; Kutach y Kadonaga 2000; Ohler et al. 2002; Juven-Gershon y Kadonaga 2009; Trinklein et al. 2003; FitzGerald et al. 2004; Ferg 2008).

La actividad de los factores de transcripción puede ser modulada por varios mecanismos incluyendo la unión de cofactores, la interacción con otros factores de transcripción, y las modificaciones covalentes (por ejemplo, fosforilación) de los factores de transcripción (Vicent et al. 2006). Además, los sitios de unión de los factores de transcripción en el ADN se mantienen accesibles mediante remodeladores de nucleosomas dependientes de ATP y enzimas modificadoras de histonas (Beato y Vincent 2012). En humanos, la activación transcripcional mediada por el PGR necesita una rápida activación de Erk que lleva a la fosforilación del receptor y la activación de la proteínas quinasa 1 activada por estrés y mitógenos (MSK1) (Vincent et al. 2010). Los complejos de receptores son reclutados hacia el promotor diana que contiene los elementos de respuesta a progesterona (PRE), donde modifican las colas de las histonas centrales y el enlazador (Vincent et al. 2006). En el caso de los ER, sólo el reclutamiento de complejos co-activadores es suficiente para la activación de genes, tales como histona acetil transferasas, histonas metiltransferasas o actividades remodeladotas dependientes de ATP (Metivier et al. 2003; Shang et al. 2000). Estas modificaciones, tanto para el PGR como para el receptor de estrógenis (ER), permiten el acceso a los complejos de remodelación especializados dependientes de ATP para desplazar las histonas enlazantes y los complejos represores (Vincent et al. 2004). Después de la finalización de la remodelación de cromatina, los complejos que contienen la ARN polimerasa con los diferentes factores de transcripción basal son reclutados hacia el núcleo del promotor y se inicia la transcripción (Vincent et al. 2010).

En los mamíferos hay dos subtipos de ERs, ERα y ERβ, que son muy similares a nivel de aminoácidos pero que muestran diferentes patrones de expresión de tejido, afinidades de unión a ligando y propiedades funcionales (Mosselman et al. 1996; Kuiper et al. 1997, Harris 2007). En el caso del PGR nuclear, hay dos isoformas diferentes, PGR-A y PGR-B, generadas por el uso diferencial del promotor sensible a estrógeno (Kastner et al. 1990). La PGR-B es más larga que la isoforma A debido a una extensión de la región A/B denominada el "segmento B-upstream" (BUS) que contiene el dominio de transactivación adicional AF-3 (Sartorius et al. 1994). Las dos isoformas de PGR son funcionalmente diferentes, la PGR-B es por lo general un fuerte activador transcripcional, mientras que la PGR-A es un represor específico de la PGR-B (Tung et al. 1993; Vegeto et al. 1993). Se ha identificado otra isoforma con el N-terminal truncado que carece de la región A/B y del DBD completo, denominada PGR-C, que es capaz de formar heterodímeros y modular la actividad de PGR-A y PGR-B (Wei et al. 1990, 1996; Condon et al. 2006). En el tejido endometrial, testículo y células de cáncer de mama se han identificado además diferentes variantes del PGR que parecen ser generadas por empal-

me alternativo del ARNm (Cork et al. 2008). Sin embargo, la importancia fisiológica de la mayoría de estas isoformas aún no está clara (Richer et al. 1998; Hisatomi et al. 2003; Marshburn et al. 2005; Samalecos y Gellersen 2008).

En los teleósteos, como en mamíferos, también existen el Erα y Erβ (Kuiper et al. 1996). Sin embargo, se han descrito dos isoformas del subtipo Erβ, la ErβI (anteriormente ERγ; véase Hawkins et al. 2000; Hawkins y Thomas 2004) y la ErβII, que posiblemente surgió de una duplicación de un gen ancestral *erb* temprano en el linaje de los teleósteos (Menuet et al. 2002; Haukins et al. 2005). En teleósteos también se han identificado diferentes variantes de transcripción del *er* que muestran truncamientos del extremo N-terminal, truncamientos o extensiones internas, y variaciones de las regiones 5' o 3' no traducidas (UTRs) (Pakdel et al. 2000; Patiño et al. 2000; Caviola et al. 2007; Tan et al. 1995; Pinto et al. 2005, 2012). Sin embargo, aún no se ha identificado el papel que pueden jugar los diferentes subtipos y variantes transcripcionales del ER durante la reproducción. Existe cierta controversia sobre el papel del Erβ entre diferentes especies de peces, ya que algunos estudios sugieren que el Erα es más importante para la inducción de la vítelogénesis que el Erβ, mientras que otros trabajos apuntan que la producción de la vítelogenina está regulada principalmente por el Erβ (Nelson y Habibi 2010; Leaños Castañeda y Van der Kraak 2007).

Además de mamíferos y anfibios, el PGR nuclear se ha clonado y caracterizado en algunas especies de condictrios, incluyendo el *Leucoraja erinacea* (Bridgham et al. 2006) y en representantes de algunos órdenes de teleósteos pertenecientes a anguiliformes, cipriniformes, salmoniformes y gadiformes, como la anguila Japonesa, el pez cebra, el salmón del Atlántico (*Salmo salar*) y el bacalao del Atlántico (*Gadus morhua*), respectivamente (Todo et al. 2000; Hanna et al. 2010; Chen et al. 2010, 2011, 2012). Además, en la lamprea marina (*Petromyzon marinus*) se ha identificado un receptor de Pgr ancestral con preferencia para la unión con 15α-hydroxyprogesterone (Thornton 2001; Bryan et al. 2008). En la anguila japonesa, hay dos genes relacionados, *pgr1* y *pgr2*, que codifican proteínas 55,2% idénticas a nivel de aminoácidos (Ikeuchi et al. 2002). En salmón, pez cebra y bacalao, sólo se ha encontrado un único gen para el Pgr (Hanna et al. 2010; Chen et al. 2010, 2011, 2012). A diferencia del Er, en teleósteos, no se han identificado variantes de transcripción para el Pgr nuclear.

2.2.2. Receptores de Esteroides de Membrana

Los receptores de membrana de esteroides median respuestas no genómicas que implican la activación rápida de proteínas G y de vías intracelulares de mensajeros secundarios (Thomas

2012). En los teleósteos, se han identificado receptores de esteroides de membrana para estrógenos, andrógenos y progestinas (Loomis y Thomas 2000; Zhu et al. 2003a, b; Brawn y Thomas 2004; Thomas et al. 2005; Thomas et al. 2006). Los receptores de membrana de progestinas (mPgrs) de teleósteos y otros vertebrados se dividen en tres subtipos, mPgrα, mPgrβ y mPgrγ, según la identidad de secuencia y el análisis filogenético (Zhu et al. 2003a, b; Tokumoto et al 2012).

La mayoría de los receptores de membrana de esteroides tienen siete dominios transmembrana y están unidos a proteínas G, características de los receptores acoplados a proteína G (GPCR). Los receptores de membrana de progestina pertenecen a la familia de la receptores progestina y adipoQ (PAQR) sin homología estructural y de secuencia con los GPCRs (Tang et al. 2005; Thomas et al. 2007). El mPgrα y mPgrβ están acoplados a una proteína G inhibidora cuya activación provoca la inhibición de la actividad de la adenilato ciclasa con la consiguiente disminución en la producción de AMPc, y posiblemente incrementa las vías de PI3K/Akt, MAP quinasa y p38 MAP quinasa (Karteris et al. 2006; Hanna et al. 2006; Dressing et al. 2010; Thomas et al. 2007). Las vias de transducción de señal para el mPgrγ todavía se desconocen (Thomas 2012).

En vertebrados, existe un patrón de expresión diferencial entre los tres subtipos de mPgr en distintos tejidos reproductivos lo que sugiere que ejercen diferentes funciones fisiológicas (Zhu et al. 2003a,b; Tokumoto et al. 2012). Recientemente, se ha observado la existencia de una regulación recíproca entre un recepor de membrana de estrógenos (GPR30) y los mPgrs en oocitos de pez cebra, donde el E2 causa una rápida disminución del mPgrα y activa el GPR30, mientras que la 17,20β-P causa un efecto opuesto (Pang y Thomas 2010). Además, en la corvina Atlántica (*Micropogonias undulatus*), el mPRα está regulado *in vitro* por gonadotropinas (Tubbs et al. 2010), y en el lenguado de Florida (*Paralichtys lethostigma*), el factor de crecimiento de insulina tipo I (IGF-I) induce un aumento de ARNm y proteína de mPgrα a través de las vías de transducción de señal Pik3 y MAP quinasa (Picha et al. 2012).

3. Control Hormonal y Molecular de la Oogénesis en Teleósteos

3.1. Proliferación de las Oogonias y Activación de la Meiosis

El control de la proliferación de las células germinales y la activación e interrupción de la meiosis es muy desconocido en teleósteos. En los vertebrados superiores, la proliferación de las oogonias está regulada por gonadotropinas, particularmente por la FSH, así como por

estrógenos y progestágenos (Yamazaki 1965; Tokarz 1978; Arrau et al. 1983; Angelova and Jordanov 1986; Mendez et al. 2003). En los teleósteos, investigaciones recientes sugieren que los esteroides sexuales pueden estar implicados en el control de la activación y la detención de la meiosis. En la anguila Japonesa, la 17,20β-P se acumula al principio de la espermatogénesis e induce la expresión de marcadores específicos de meiosis como el Spo11 y el Dmc1, así como la formación de complejos sinaptonémicos, lo que sugiere que es un factor esencial para la iniciación de la meiosis (Miura et al. 2006). Los experimentos con huchen japonés (*Hucho perry*) y carpa común (*Cyprinus carpio*), sugieren que la progresión de las células germinales a través de la oogénesis temprana también implica el E2, que actúa directamente sobre la proliferación oogonial (Miura et al. 2007). En tilapia (*Oreochromis niloticus*), el inicio de la meiosis también se ha ligado a la expresión del IGF-I en las células somáticas y los oocitos (Berishvili et al. 2006). El factor derivado del soma gonadal (GSDF), mejora las células germinales primordiales y la proliferación de las espermatogonias en la trucha arco iris (*Oncorhynchus mykiss*), y probablemente puede tener un papel similar en la proliferación de las oogonias (Sawatari et al. 2007).

3.2. Crecimiento del Oocito y Foliculogénesis

3.2.1. Papel de la Fsh Durante el Crecimiento Ovocitario

En muchos teleósteos los niveles de Fsh en plasma son elevados durante el período de crecimiento de los oocitos y disminuyen con el inicio de la maduración (Suzuki et al. 1988; Swanson et al. 1991; Aizen et al. 2007; Molés et al. 2012; Shimizu et al. 2012). Además, en muchas especies, el gen *fhsra* se expresa en las células de la teca y de la granulosa asociadas a oocitos pre-vitelogénicos, y su nivel de expresión aumenta cuando los oocitos entran en vitelogénesis y disminuye una vez que los oocitos han llegado a la etapa de máximo crecimiento (Kumar et al. 2001a,b; Kuok et al. 2005; Kobayashi et al. 2008; Rocha et al. 2007, 2009; Chauvigné et al. 2010; Kaseto et al. 2010). Estas observaciones sugieren que la Fsh es probablemente la principal gonadotropina implicada en el crecimiento de los oocitos en teleósteos. Sin embargo, en la anguila de la arroz (*Monopterus alba*), la Lhβ se detecta en la pituitaria al inicio del período de crecimiento primario y aumenta a medida que el oocito se desarrolla, lo que sugiere el papel de la Lh durante la fase de crecimiento primario (Wu et al. 2012).

En los mamíferos, las células de la granulosa y de la teca secretan diferentes factores paracrinos como la inhibina, activina y factores de crecimiento los cuales están implicados en la

regulación del crecimiento de los oocitos (Buccione 1990; Gilchrist et al. 2004). A su vez, el oocito produce otros factores de crecimiento para la foliculogénesis, tales como el factor de crecimiento y diferenciación 9 (GDF9) y el factor morfogenético óseo 15 (BMP15), que estimulan la mitosis y restringen la diferenciación de las células de la granulosa (Juengel et al. 2004; Moore and Shimasaki 2005). Las gonadotropinas pituitarias podrían estar también implicadas en estos procesos (Thomas et al. 2005; Guéripel et al. 2006).

En el oocito del pez cebra se expresan diferentes ligandos Bmp, mientras que sus receptores se encuentran en las capas foliculares, lo que sugiere que las células del folículo controlan el desarrollo de los oocitos, y al mismo tiempo, el oocito puede regular la función de la capa folicular (Li y Ge 2011). En otras especies, el Gdf9 y Bmp15 también se expresan en oocitos en crecimiento primario cuando las células de la granulosa comienzan a proliferar, lo que sugiere un posible papel de estos factores en el desarrollo de los folículos ováricos primarios (Baron et al. 2005; Halm et al. 2008). Asimismo, también se ha sugerido que las gonadotropinas pituitarias pueden tener un papel en la regulación de Bmp15 y Gdf9 en el oocito en crecimiento primario (Liu y Ge 2007; García-López et al. 2011).

Durante la transición a la etapa de crecimiento secundario, también se observan cambios en los niveles plasmáticos de diferentes hormonas. Los niveles plasmáticos de la Fsh y el E2, y del ARNm de *star*, incrementan durante la acumulación de los alveolos corticales en el oocito, y se asocian con una elevación de Igf1 en plasma (AMPcbell et al. 2006). En la lubina, se han encontrado niveles elevados de ARNm de *igf1* y *igf2* durante el mismo estado ovárico, los cuales probablemente tienen un papel en la acumulación lipídica (García-Lopez et al. 2011). El incremento de los niveles de Fsh en plasma y de la expresión de *fshra* en las células de la granulosa, junto con el subsiguiente aumento de la expresión de Cyp19a1a y producción de E2 (ver más adelante), están asociados con la acumulación de alvéolos corticales en el oocito (Kwok et al. 2005). Un estudio reciente en el salmón (*Oncorhynchus kisutch*) muestra que el papel predominante de los andrógenos es la estimulación del crecimiento de los folículos en estadio perinuclear tardío, mientras que el E2 estimula tanto el crecimiento como la acumulación de los alveolos corticales (Forsgren and Young 2012).

3.2.2. Vías Esteroidogénicas

Las hormonas esteroideas sexuales derivan del colesterol y son sintetizadas en las células foliculares por distintos enzimas steroidogénicos, incluyendo las enzimas P450 e hidroxiesteroide deshidrogenasas (HSDs), bajo el control de las gonadotropinas (Young et al. 2004).

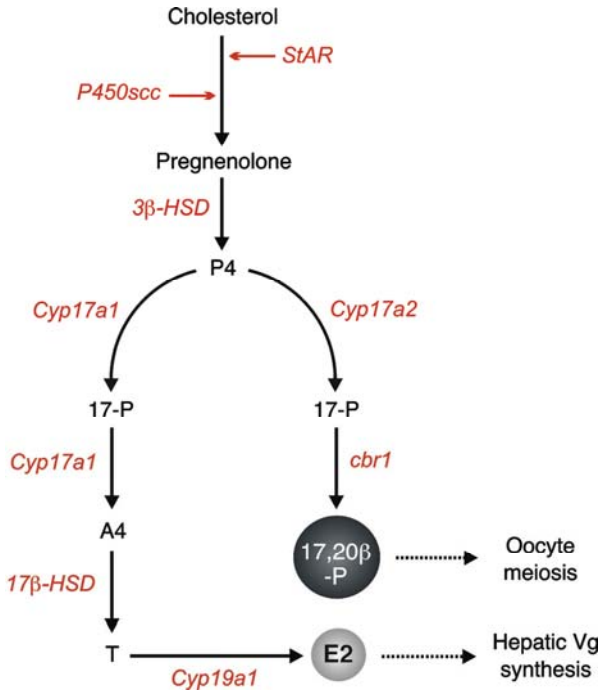

Fig. 5. Diagrama esquemático de la producción de estrógenos y progestágenos y de las vías esteroidogénicas en las células de la teca y de la granulosa de teleósteos. P4, progesterona; 17-P, 17α-hydroxyprogesterona; A4, androstenediona; T, testosterona; E2, estradiol-17β; 17,20β-P, 17α,20β-dihydroxy-4-pregnen-3-ona.

Durante el crecimiento del oocito se produce mayoritariamente E2, el cual promueve la síntesis de Vtgs hepáticas que serán incorporadas en el oocito, mientras que durante la maduración y ovulación del ovocito se producen mayoritariamente las progestinas (Nagahama and Yamashita 2008). Tanto la Fsh como la Lh pueden estimular la producción de E2 durante la vitelogénesis pero sólo la Lh parece estimular la producción de progestinas (Suzuki et al. 1988; Van Der Kraak et al. 1992; Singh y Thomas 1993; Planas et al. 2000; Rocha et al. 2009; Molés et al. 2008; Aizen et al. 2012).

En estudios con salmónidos se ha propuesto el modelo de dos céuluas para la producción de las hormonas esteroideas bajo regulación gonadotrópica (Nagahama 1994). En este modelo (Fig. 5), el enzima P450scc (o Cyp11a) convierte el colesterol en pregnenolona en la membrana interna de las mitocondrias en las células de la teca (Takahashi et al. 1993). Este paso incluye el transporte del colesterol al interior de la mitocondria y está regulado por Star que a su vez está regulada por las gonadotropinas (Stocco 2001; Kusakabe et al. 2002; Ings and Van Der Kraak 2006; Nuñez and Evans 2007). Después de la síntesis de pregnenolona y su conversión a progesterona (P4) por el enzima 3β-Hsd, hay dos vías mayoritarias para la síntesis de andrógeno/estrógeno o para la síntesis de progestágenos (Young et al. 2004; Nagahama and Yamashita 2008; Lubzens et al. 2010). El citocromo P450c17-I (Cyp17a1), que tiene actividad enzimática 17α-hydroxilasa y 17,20-liasa, está implicado en la producción tan-

to de E2 como de 17,20β-P. Para la producción de E2, la Cyp17a1 transforma la P4 en 17α-hidroxiprogesterona (17-P) y después a androstenediona (A4), la cual es convertida a testosterona (T) por la 17β-Hsd. La T cruza la membrana basal y es aromatizada a E2 en las células de la granulosa por el Cyp19a1. La habilidad de producir E2 está por lo tanto regulada a nivel de expresión génica y actividad de estos enzimas, lo cual parece estar controlado por Fsh mediante su receptor especifico en las células de la teca y la granulosa. Sin embargo, el modelo de dos células de salmónidos no es aplicable a otros teleósteos, tal y como se ha observado en medaka (Petrino et al. 1989) o en la anguila Japonesa (Ijiri et al. 2006).

3.3. Inducción de la Maduración del Ovocito por Lh

El proceso de reanudación de la meiosis en teleósteos y los cambios citoplasmáticos asociados están regulados por la Lh la cual estimula la síntesis de progestinas inductoras de la maduración en las celulas de la granulosa (Nagahama y Yamashita 2008). Las progestinas, 17,20β-P o la 17,20β,21-P (Jalabert 1976; Sundararaj and Goswami 1977; Young et al. 1982; Goetz 1983; Nagahama et al. 1983; Thomas 1994), activan el factor promotor de la maduración (MPF) en el oocito el cual promueve la reanudación de la meiosis. El modelo de dos células también se ha propuesto para explicar la producción de progestinas, en el cual se sugiere que durante la maduración de los ovocitos el 17-P se acumula en las células de la teca y luego se difunde hasta las células de la granulosa donde es convertido a 17,20β-P por la enzima Cbr1 (Nagahama y Yamashita 2008). En algunas especies, el 17-P es convertido en 17,21-dihidroxi-pregn-4-ene-3,20-diona (11-deoxicortisol) para producir 17,20β,21-P (Jeng et al. 2012).

3.3.1. El Cambio de las Vías Esteroidogénicas

Durante la vítelogénesis tardía, previamente a la maduración del oocito, hay una alteración de la esteroidogénesis en la cual se pasa de la producción de E2 a la producción de 17,20β-P (Nagahama and Yamashita 2008). Durante este cambio esteroidogénico la síntesis de los precursores cambia de T para la producción de E2 a 17-P para la producción de 17,20β-P o 17,20β,21-P, y los enzimas implicados en la conversión final también se alteran, disminuye la actividad del Cyp19a1 y aumenta la del Cbr1. Estos cambios pueden estar regulados por las gonadotropinas, ya que la Fsh estimula la producción de T y la actividad de Cyp19a1 favoreciendo la producción de E2, mientras que la Lh estimula la producción de 17-P y la actividad del Cbr1 (Nagahama and Yamashita 2008). Probablemente, el pico de Lh que se produce du-

rante la maduración del oocito induce el cambio esteroidogénico de E2 a progestinas en las células de la granulosa.

Estudios recientes han descubierto las bases moleculares del cambio esteroidogénico en teleósteos, lo cual está relacionado con el descubrimiento de un segundo enzima Cyp17a, el P450c17-II (Cyp17a2) que posee solamente actividad enzimática 17α-hydroxylasa (Zhou et al. 2007a). La expresión de Cyp17a1 es elevada durante la vítelogénesis y disminuye justo antes de la maduración del oocito, mientras que la del Cyp17a2 tiene su máximo durante la vítelogénesis tardía, coincidiendo con la producción de progestinas, lo que sugiere que hay un cambio en la síntesis de estos dos enzimas desde el crecimiento del ovocito hasta la maduración (Zhou et al. 2007b). Por lo tanto, estos cambios de expresión génica y actividad enzimática resultan en la acumulación de 17-P en las células de la teca y de la granulosa y en la producción de 17,20β-P (Fig. 5). La regulación transcripcional de los genes *cyp17a1* y *cyp17a2* esta mediada por dos factores de transcripción diferentes, el Ad4BP/SF-1 que actúa en el gen *cyp17a1* mediante un único sitio en el promotor y en el gen *cyp17a2* mediante dos sitios distintos en el promotor, y el Foxl2 que induce el efecto del Ad4BP/SF-1 en la activación transcripcional de *cyp17a2* (Zhou et al. 2007b).

3.3.2. El mPgr y las Vías Señalizadoras

En teleósteos hay evidencias que sugieren que el mPgrα y el mPgrβ, estimulados por las progestinas en respuesta a la Lh, están implicados en la maduración del oocito (Zhu et al. 2003a,b; Tokumoto et al. 2012). El mPgrα se expresa en la membrana plasmática del oocito y el tratamiento con gonadotropinas incrementa su expresión durante la fase inicial de la maduración (Zhu et al. 2003a,b; Hanna and Zhu 2009). Este incremento del mPgrα está asociado con el desarrollo de la competencia maduracional, y la microinyección de los oocitos con un morfolino antisentido del mPgrα inhibe la GVBD (Zhu et al. 2003a; Tokumoto et al, 2006). En el pez cebra, la inhibición del mPgrβ sugiere que esta isoforma también está implicada en la maduración del oocito (Hanna and Zhu 2009, 2011; Tubbs et al. 2010). Sin embargo, la sobre expresión de los dos subtipos en el oocito indica que solo el mPgrα acelera la maduración.

Tal y como ya se ha mencionado, el pico de Lh induce la producción de progestinas las cuales se unen al mPgr y activan el MPF para reanudar la meiosis. La reanudación de la meiosis está asociada con la disminución de los niveles de AMPc en el oocito produciendo la inactivación de las quinasas dependientes de AMPc que parecen mantener el bloqueo del MPF (Jalabert and Finet 1986; Nagahama and Yamashita 2008). Hay evidencias de que el mPgrα está

acoplado a proteína G (Thomas et al. 2007) y también de que la toxina pertussis bloquea la inducción de la maduración por progestinas (Pace and Thomas 2005). Además, en células cultivadas que expresan el mPgrα recombinante y que se han expuesto a progestinas hay una disminución de la producción de AMPc que es inhibida con un pre-tratamiento con la toxina pertussis (Zhu et al. 2003a; Pace and Thomas 2005). Estas observaciones sugieren que el mPgrα, y probablemente el mPgrβ, están acoplados a una proteína G inhibidora que se activa por progestinas resultando en una disminución de los niveles de AMPc y la subsiguiente activación de la reanudación de la meiosis.

3.4. Bases Moleculares de la Hidratación del Oocito

Durante la maduración del oocito, el pico de Lh y la subsiguiente síntesis de progestinas y activación del MPF, también induce la acumulación de osmolitos inorgánicos y orgánicos en el interior del oocito que permiten su hidratación (ver arriba). Hasta hace poco, se creía que la hidratación del oocito ocurría de forma pasiva por simple difusión del agua a través de la membrana plasmática. Sin embargo, el reciente descubrimiento del papel de un canal molecular de agua específico de teleósteos, la acuaporina-1b (Aqp1b), durante el proceso de la hidratación del oocito en teleósteos pelagófilos (Fabra et al. 2005, 2006; Cerdà 2009), sugiere que este proceso se encuentra en realidad muy controlado para permitir la acumulación de osmolitos y la entrada de agua a través de la Aqp1b.

3.4.1. Estructura, Clasificación y Evolución de las Acuaporinas de Teleósteos

Las acuaporinas pertenecen a las superfamilia de proteínas integrales de membrana las cuales específicamente transportan agua y otros solutos no cargados de bajo peso molecular a través de las membranas biológicas a favor de un gradiente osmótico (King et al. 2004). Estas proteínas de membrana se han identificado en todos los organismos vivos, desde procariotas hasta eucariotas, indicando que esta familia de proteínas esta implicada en la homeostasis de diferentes procesos biológicos en todo el mundo natural (King et al. 2004). Las acuaporinas consisten en seis dominios transmembrana conectados por cinco lazos (A-E) con sus N- y C-terminal localizados intracelularmente (Fig. 6). La secuencia deducida de las acuaporinas ha revelado una topología de dos repeticiones en tándem, orientadas 180º con respeto a la otra, que contienen el motivo asparagina-prolina-alanina (NPA) en los lazos B y E, que es la marca de la familia MIP en la que pertenecen las acuaporinas. Los lazos B y E y su correcto plegamiento son importantes para la formación del poro y la Cys^{189} próxima al motivo NPA en el

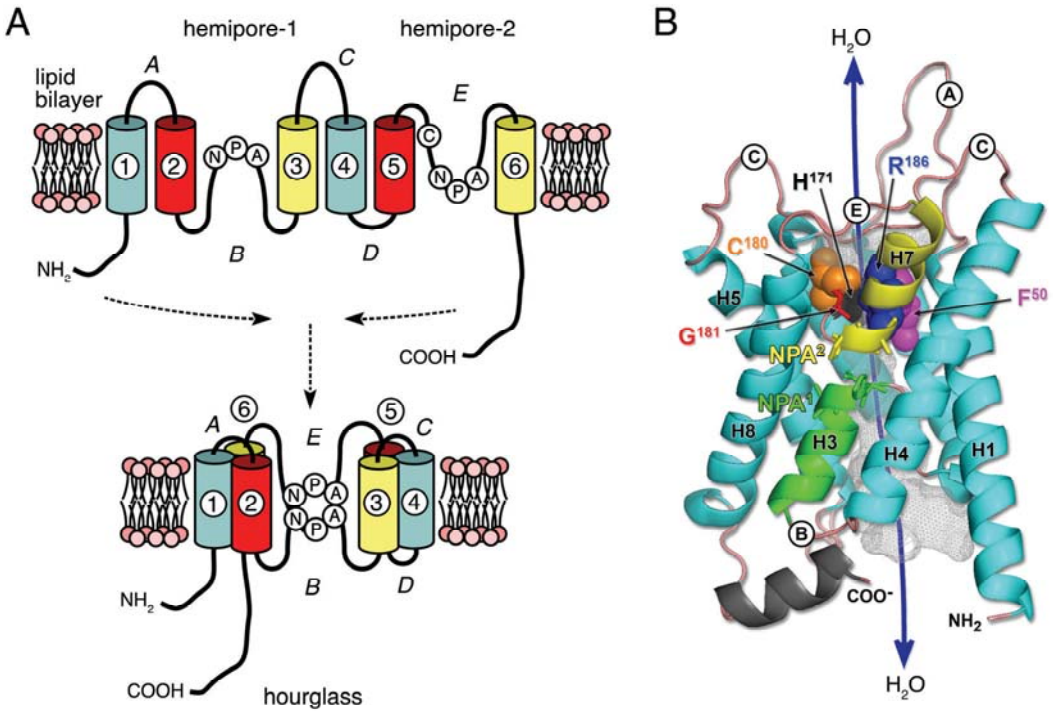

Fig. 6. Topología y estructura del prototipo de la AQP1. A, modelo en "reloj de arena" para la topología de la AQP1 de mamíferos en la cual la disposición de los lazos E y E con los motivos NPA altamente conservados forma una único poro de agua a través de la subunidad de la AQP1. Modificado de Jung et al. (1994). B, Reconstrucción tridimensional de la Aqp1a de pez cebra mostrando los residuos parálogo-específicos ar/R (espacio lleno). La posición de los aminoácidos está numerada según el parálogo del pez cebra. Los canales centrales se indican con flechas de doble extremo mapeadas a través de los resíduos de superficie de las cavidades de desecho usando MacPymol. El modelo esta basado en la estructura de la AQP1 bovina (*Bos Taurus*) (1J4N, Sui et al. 2001). Modificado de Cerdà y Finn (2010).

lazo E es responsable de la inhibición de la permeabilidad al agua de las AQP por concentraciones submilimolares de Hg^{2+} (Agre et al. 2002).

Las acuaporinas se dividen en cuatro grupos según sus propiedades estructurales y su permeabilidad: los canales selectivos al agua (AQP0, -1, -2, -4, y -5), las acuagliceroporinas que son también permeables a pequeños solutos como la urea y el glicerol (AQP3, -7, -9, y -10), las acuaporinas heterodoxas (AQP11 y -12), y la AQP8 permeable a agua y urea (King et al. 2004; Tingaud-Sequeira et al. 2010; Cerdà and Finn 2010; Finn and Cerdà 2011). En euterios, se conocen trece acuaporinas (AQP0-12), mientras que en prototerios y anfibios se ha identificado una acuagliceroporina adicional que esta relacionada con las AQP3 y -9, denominada AQPxlo (Virkki et al. 2002; Cerdà y Finn 2010; Finn y Cerdà 2011). En teleósteos ostarioficios como el pez cebra, estudios iniciales han identificado 17 genes que codifican acuaporinas estructuralmente y funcionalmente relacionadas con las de tetrápodos, *aqp0a*, *-0b*, *-1a*, *-1b*, *-3a*, *-3b*, *-4*, *-7*, *-8aa*, *-8ab*, *-8b*, *-9a*, *-9b*, *-10a*, *-10b*, *-11b* y *-12*, así como un putativo pseudogen con

exones similares a las AQP5 y -1 de tetrápodos (Tingaud-Sequeira et al. 2010; Cerdà y Finn 2010). Recientemente, se ha identificado un segundo gen para la *aqp8b* dando un total de 19 genes parálogos en el pez cebra (Finn y Cerdà 2011). La mayoría de acuaporinas de teleósteos se han originado a partir de una duplicación completa del genoma (WGD) a excepción de los genes *aqp8* y *aqp1*. El historial de duplicación y la putativa neofuncionalización de los parálogos *aqp8aa*, *-8ab*, y *-8b* en teleósteos se ha explicado en base a la combinación de la WGD y de una replicación en tándem (Cerdà y Finn 2010), mientras que se sugiere que los parálogos de la *aqp1* provienen de una duplicación en tándem debido a su yuxtaposición en el genoma de teleósteos (Tingaud-Sequeira et al. 2008).

3.4.2 La Subfamilia de la Aqp1b y su Papel en la Hidratación del Oocito

La Aqp1b (denominada anteriormente Aqp1o) es un ortólogo de la AQP1 de mamíferos especifico de teleósteos que se identificó inicialmente en la dorada, y que juega un papel esencial durante la hidratación del oocito (Fabra et al. 2005). La Aqp1b ha sido también implicada en el proceso de hidratación del oocito de otros teleósteos marinos y catádromos que producen huevos pelágicos (Tingaud-Sequeira et al. 2008; Sun et al. 2010; Kagawa et al. 2011). Los transcritos de la *aqp1b* también se acumulan en grandes cantidades en el ovario de algunas especies de agua dulce, como el bagre (*Heteropneustes fossilis*), en el que los oocitos se hidratan parcialmente durante la maduración meiótica aunque se producen huevos bentónicos (Chaube et al. 2011). Análisis filogenéticos sugieren que el origen de la Aqp1b durante la evolución de los teleósteos es por duplicación en tándem de un gen ancestral de la AQP1 y posterior divergencia estructural del C-terminal (Tingaud-Sequeira et al. 2008).

El papel de la Aqp1b en el transporte de agua durante la hidratación del oocito de teleósteos marinos ha sido investigado usando técnicas immunocitoquímicas y funcionales (Fabra et al. 2005, 2006) (Fig. 7). La Aqp1b se sintetiza aparentemente en los oocitos en estadio de alveolo cortical y se localiza en el citoplasma del oocito pero no en las células foliculares que lo rodean, y es transportada hacia el córtex del oocito durante el periodo de crecimiento (Fabra et al. 2006). En los oocitos post-vitelogénicos, la Aqp1b se localiza debajo de la membrana plasmática. Durante la maduración meiótica, justo después de la GVBD y antes de la completa hidrólisis de las proteínas del vítelo y de la máxima acumulación de K^+, la Aqp1b es transportada transitoriamente a la membrana plasmática del oocito (Fabra et al. 2006). En la anguila Japonesa, la localización de la Aqp1b durante el desarrollo del oocito es similar que en la dorada (Kagawa et al. 2011). Sin embargo, parece que en los oocitos completamente des-

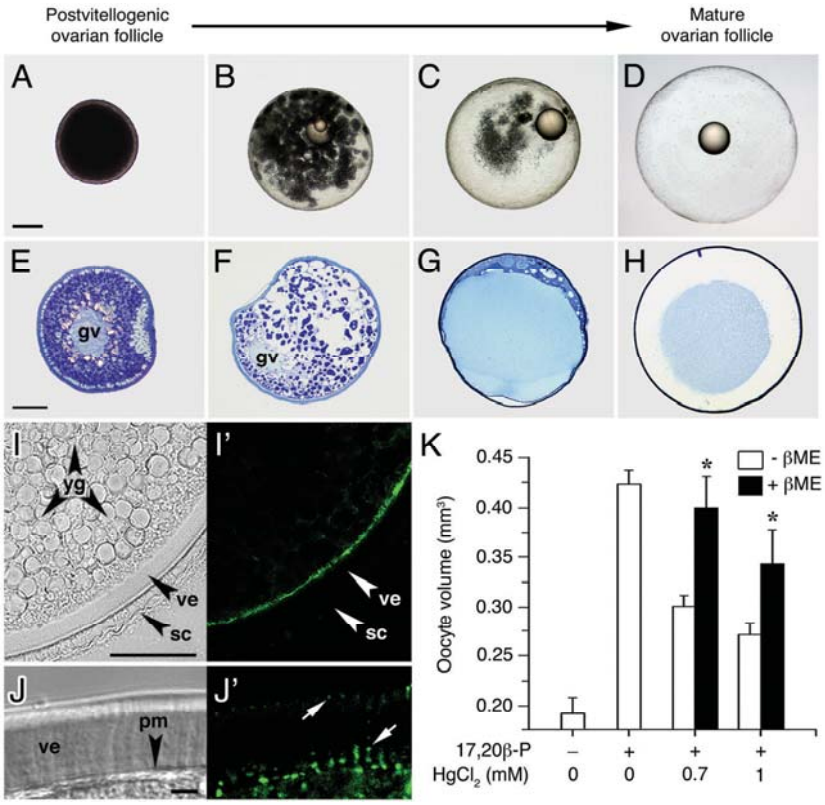

Fig. 7. Hidratación del ooocito de dorada y papel de la acuaporina-1b (Aqp1b). A-D, Microfotografías de folículos ováricos en maduración e hidratación. E-H, Secciones histológicas de los mismos estados en A-D donde se observa la migración (F) y la rotura de la vesícula germinal (gv), y el proceso de fusión de los gránulos del vítelo (F-H). I-J, Imágenes en campo claro (I, J) y de immunofluorescencia (I', J') de la Aqp1b en los ooocitos post-vitelogénicos (I), y en las microvellosidades de los oocitos en hidratación (J). La flecha y las cabezas de flecha indican la membrana plasmática y las microvellosidades, respectivamente. yg, gránulos del vítelo; ve, envoltura vitelina; sc, células somáticas; pm, membrana plasmática. (k) Inhibición con mercurio de la hidratación del oocito inducida *in vitro* con progestina 17,20β-P y recuperación con 5 mM βME. Valores promedio ± SEM. Los asteriscos indican diferencias significativas (P<0·05.) entre oocitos tratados o no con βME. Barras de escala, 200 μm (A-H), 50 μm (I) and 5 μm (I). Reproducido de Cerdà (1999).

arrollados la Aqp1b no se encuentra debajo de la membrana plasmática, lo que puede indicar diferentes mecanismos de hidratación de los oocitos en esta especie (Kagawa et al. 2011).

La correlación positiva entre la localización subcelular de la Aqp1b en el oocito de la dorada con el proceso de hidratación proporciona evidencias indirectas del papel fisiológico de este canal (Fig. 7). Esta conclusión se apoya en la observación de que la hidratación de los oocitos de dorada durante la maduración meiótica se bloquea con mercurio y tetraetilamonio, conocidos inhibidores de la permeabilidad de las acuaporinas que bloquean el transporte de agua mediado por la Aqp1b en oocitos de *Xenopus laevis* (Fabra et al. 2005, 2006; Tingaud-Sequeira et al. 2008; Kagawa et al. 2009). Sin embargo, es conocido que estos compuestos inorgánicos también pueden afectar a canales y transportadores iónicos, los cuales pueden

jugar un papel importante en la hidratación del oocito como se ha mencionado anteriormente (Cerdà et al. 2007; Finn and Fyhn 2010). Por lo tanto, aunque los datos sugieren que la Aqp1b está implicada en la captación de agua durante la hidratación de los oocitos de teleósteos (Fig. 8), se requieren evidencias directas para demostrarlo.

3.4.3. Regulación de la Aqp1b Durante el Crecimiento y la Maduración del Oocito

Tanto en la dorada como en la anguila Japonesa, estudios immunocitoquímicos utilizando anticuerpos específicos para la Aqp1b no detectan la proteína en el oocito hasta el estadio de alveolo cortical o vitelogénesis temprana (Fabra et al. 2006; Kagawa et al. 2011). Esto puede indicar que la Aqp1b se sintetiza *de novo* en el oocito al inicio de la vitelogénesis desde un pool de ARNm preexistente. Por tanto el gen *aqp1ab* podría estar altamente regulado a nivel transcripcional y traduccional al principio de la etapa de crecimientodel oocito. Durante la vítelogénesis y la maduración, se observa una baja actividad transcripcional de *aqp1b* (Fabra et al. 2006; Kagawa et al. 2011), sugiriendo que en estos estadios la mayor vía de regulación

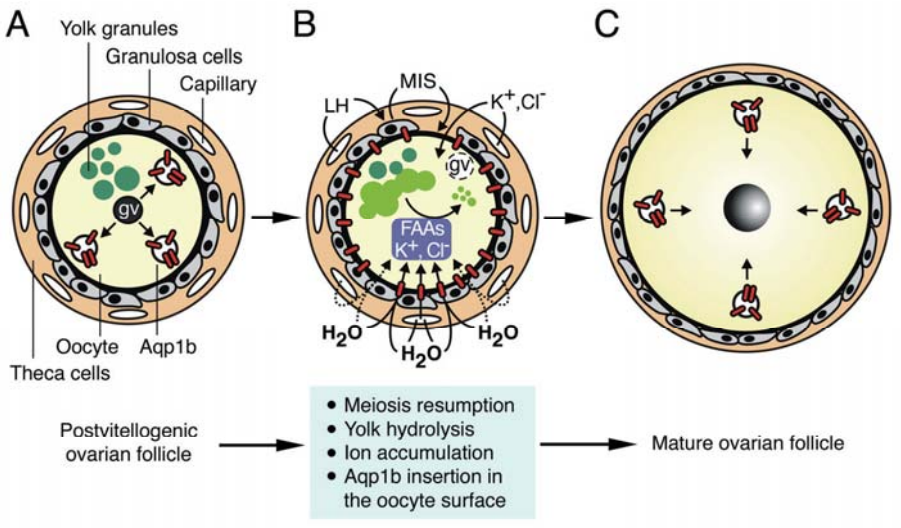

Fig. 8. Modelo propuesto de los principales procesos fisiológicos durante la maduración y la hidratación del oocito de dorada. A, durante la vitelogénesis, la Aqp1b se sintetiza y se transporta hacia la membrana plasmática del oocito. B, En el estadio de maduración, las células de la granulosa son estimuladas por la LH para producir el MIS (17,20β-P), que a su vez induce la reanudación de la meiosis del oocito. Durante este proceso, la vesicular germinal (gv) migra hacia el polo animal y eventualmente se rompe, los gránulos del vítelo se fusionan y se activa la hidrólisis de las proteínas del vitelo, mientras que los iones inorgánicos (K^+, Cl^-) se acumulan en el ovocito. Al mismo tiempo, la Aqp1b se trasloca a la membrana plasmática del oocito, donde facilita el transporte de agua (líneas sólidas). Adicionalmente, el flujo de agua al oocito puede ocurrir por simple difusión a través de las membranas foliculares (líneas punteadas). C, Cuando la proteólisis del vítelo se ha completado, las vesículas que contienen la Aqp1b son internalizadas. Reproducido de Cerdà (1999).

ocurre a nivel post-traduccional para el control del tráfico intracelular hacia la membrana plasmática del oocito de las vesículas de Aqp1b.

Los análisis funcionales de la Aqp1b de dorada en oocitos de *X. laevis* revelan que es menos activa que el parálogo Aqp1a debido a que la Aqp1b se retiene parcialmente en el citoplasma (Tingaud-Sequeira et al. 2008). Análisis filogenéticos han indicado que la región más divergente entre la secuencia de aminoácidos de la Aqp1a y la Aqp1b es el C-terminal (Tingaud-Sequeira et al. 2008; Finn and Cerdà 2011), conocido por tener un papel en el tráfico intracelular de la AQP2 de mamíferos y la AQPa2 de anfibios (Deen et al. 2000; Hasegawa et al. 2005). Sin embargo, aunque el C-terminal es divergente entre los distintos teleósteos, este retiene motivos específicos que pueden regular el trafico vesicular y, por lo tanto, pueden estar implicados en el control de la translocación de la Aqp1b a la membrana plasmática durante el crecimiento y/o la maduración del ovocito (Tingaud-Sequeira et al. 2008; Chaube et al. 2011). Estos mecanismos han sido investigados usando quimeras de Aqp1a y Aqp1b de la dorada así como mediante mutagénesis dirigida, y los resultados muestran que la fosforilación del residuo Ser^{254}, un punto consenso para quinasas dirigidas por prolina que está conservado en la mayoría de Aqp1b en teleósteos, media el reciclaje de la Aqp1b en los oocitos de *X. laevis* (Tingaud-Sequeira et al. 2008). Otra secuencia retenida en el C-terminal de la Aqp1b de teleósteos es el motivo di-Leucina, señal de internalización común en proteínas transmembrana (Bonifacino and Traub 2003). La mutación de esta secuencia induce un error de plegamiento de la proteína, inhibiendo su translocación a la membrana plasmática (Tingaud-Sequeira et al. 2008), lo que sugiere que el dominio di-Leu puede estar también implicado en el control del tráfico intracelular de la Aqp1b.

OBJETIVOS

En trabajos previos se ha descubierto el papel de la Aqp1b específica de teleósteos durante la hidratación de los oocitos en peces pelagófilos. Sin embargo, todavía se carece de evidencias experimentales directas de la función de la Aqp1b en el transporte de agua a los oocitos. Asimismo, los trabajos iniciales realizados en la dorada y la anguila Japonesa muestran que la expresión del gen *aqp1b* se inicia en oocitos pre-vitelogénicos, mientras que la síntesis de los correspondientes polipéptidos no se produce hasta el inicio de la vitelogénesis, y posteriormente, durante la fase de crecimiento ovocitaria, las proteínas se transportan a la membrana plasmática del oocito (véase la Introducción). Estos datos sugieren que el gen *aqp1b* podría estar regulado en los oocitos tanto a nivel transcripcional, traduccional como post-translacional, pero los mecanismos moleculares implicados siguen siendo desconocidos. Ya que la Aqp1b puede jugar un papel importante durante la maduración de los oocitos en teleósteos pelagófilos, la elucidación de estos mecanismos es esencial para entender la base molecular de la formación del huevo de teleósteos.

En este contexto, el principal objetivo de esta tesis ha sido aportar evidencias funcionales del papel esencial de la Aqp1b durante la hidratación del oocito de teleósteos marinos, y empezar a diseccionar los mecanismos moleculares implicados en la regulación transcripcional del gen *aqp1b* en el oocito. Usando una combinación de enfoques moleculares y celulares, los objetivos específicos han sido los siguientes:

1. Investigar el origen de la *aqp1b* en teleósteos mediante un amplio análisis filogenético y sinténico, y proporcionar evidencias experimentales de la función de la Aqp1b durante la hidratación del oocito en un teleósteo evolutivamente avanzado, como el fletán Atlántico (*Hippoglossus hippoglossus*), empleando herramientas moleculares específicas (**Capítulo I**).

2. Investigar la producción de hormonas esteroideas, particularmente de progestinas, en folículos ováricos de dorada en crecimiento primario, así como su posible regulación gonadotrópica, como potenciales reguladores de la transcripción y/o traducción del gen *aqp1b* en oocitos en crecimiento primario (**Capítulo II**).

3. Caracterizar a nivel molecular y funcional el receptor nuclear de progestinas (Pgr) de la dorada, así como sus posibles isoformas, e investigar su localización celular y regulación hormonal en ovarios en crecimiento primario (**Capítulo III**).

4. Aislar la región promotora del gen *aqp1b* de la dorada y caracterizar de modo funcional algunos de los elementos proximales *cis*-reguladores presentes, e investigar *in vitro* e *in vivo* el papel del Pgr nuclear y otros factores de transcripción en la regulación transcripcional del gen *aqp1b* en oocitos en crecimiento primario (**Capítulo IV**).

RESÚMENES DE LOS CAPÍTULOS

Capítulo I

La Doble Neofuncionalización de una Acuaporina-1 de Rápida Evolución Resulta en Mecanismos Conservados y no Conservados para el Control de la Función del Canal Durante la Reanudación de la Meiosis en Teleósteos

La hidratación pre-ovulatoria del oocito de teleósteos marinos es un proceso único entre los vertebrados. El mecanismo de hidratación es más pronunciado en teleósteos modernos acantiuros (de aletas espinosas) que producen huevos pelágicos (flotantes). Sin embargo el mecanismo molecular implicado en el transporte de agua hacia el oocito permanece desconocido. Recientemente, hemos puesto de manifiesto que el proceso de duplicación global del genoma ('Whole genome duplication', WGD) ha proporcionado a los teleósteos el mayor repertorio de canales moleculares de agua (acuaporinas) en el linaje de los vertebrados. Una de estas acuaporinas, parálogo de la acuaporina-1 (AQP1) de mamíferos, está implicada en el proceso de hidratación del oocito. Sin embargo, el origen y la función de los parálogos de la AQP1 de teleósteos sigue siendo ambigua. Mediante integración de la filogenia molecular con análisis sinténicos y estructurales aquí demostramos que los genes parálogos *aqp1aa* y *aqp1ab* de teleósteos (anteriormente denominados *aqp1a* y *-1b*, respectivamente) surgieron por duplicación génica en tándem en lugar de WGD, y que el C-terminal de la Aqp1ab es el subdominio que ha evolucionado más rápidamente dentro de la superfamilia de las acuaporinas de vertebrados. Seguidamente, hemos investigado el papel funcional de la Aqp1ab en el fletán Atlantico, un teleósteo marino acantiuro que genera uno de los mayores huevos pelágicos conocidos, y hemos demostrado que esta acuaporina es esencial para la completa hidratación de los oocitos durante la maduración meiótica. Nuestros datos muestran también que la rápida divergencia estructural del dominio regulador C-terminal de la Aqp1ab del fletán provoca la pérdida de su función *ex vivo* en oocitos de anfibios pero no en oocitos post-vitelogénicos nativos o de pez cebra. Mediante el uso de construcciones quiméricas entre las Aqp1aa y -1ab del fletán, y de un antisuero específico producido contra el C-terminal de la Aqp1ab de esta especie, hemos encontrado que este dominio citoplasmático posiblemente regula el tráfico *in vivo* a la porción microvillar de la membrana plasmática de los oocitos cuando la presión osmótica intra-oocítica es máxima. La co-inyección de ARNm poliA$^+$ extraído de folículos post-

vitelogénicos de fletán rescata *ex vivo* el tráfico intracelular de la Aqp1ab en oocitos de anfibios. Estos datos revelan por tanto que el papel fisiológico de la Aqp1ab en los oocitos durante la maduración meiótica está conservado en los peces teleósteos, pero la notable degeneración del dominio citoplasmático C-terminal se ha traducido posiblemente en mecanismos alternativos para el control del tráfico intracelular.

Capítulo II

La Hormona Folículo Estimulante de Peces Desencadena la Producción de Progestinas en Folículos Ovàricos de Dorada

El crecimiento ovárico (vitelogénesis) en la mayoría de los vertebrados inferiores está mediado por el 17β-estradiol (E2) secretado por los folículos en respuesta a la gonadotropina estimulante del folículo (FSH), mientras que la reanudación de la meiosis en los oocitos (maduración del oocito) y la ovulación están inducidas por progestinas, tales como la 17α,20β-dihidroxipregn-4-en-3-ona (17,20β-P), que se producen en respuesta a la hormona luteinizante (LH). En los teleósteos, la síntesis folicular de 17,20β-P durante la maduración se debe principalmente a la sobreexpresión de los enzimas P450c17-II (Cyp17a2) y 20β-hidroxiesteroide deshidrogenasa (Cbr1). En este trabajo demostramos que las células foliculares asociadas a los oocitos en crecimiento primario (pre-vitelogénicos) de la dorada también expresan *cyp17a2* y *cbr1*, además de P450c17-I (Cyp17a1) y aromatase (Cyp19a1), enzimas necesarias para la síntesis de E2. En ovarios de dorada con sólo oogonias y folículos ováricos primarios la concentración de 17,20β-P es ~60 veces mayor que en ovarios en estados sucesivos (i.e. estado de alveolo cortical y vitelogénico), y se observa una expresión más elevada de *cbr1* y del receptor de FSH (*fshra*). La estimulación de explantes de folículos primarios con FSH recombinante de lubina (rFsh) *in vitro*, la cual activa específicamente el Fshra de dorada, promueve una rápida acumulación de 17,20β-P, cuya síntesis se mantiene en cultivo si se añade de forma externa 17α-hidroxiprogesterona. En presencia de inhibidores de la enzima Cbr1 la producción de 17,20β-P mediada por rFsh se reduce, y aumenta de forma concomitante tanto la síntesis de testosterona como de E2. En los explantes de folículos primarios, la rFsh induce una sobreexpresión de los niveles de *cyp17a2* y *cbr1*, y al mismo tiempo reduce los niveles estables de los ARNs *cyp17a1* y *cyp19a1*, en 24 h. Por el contrario, en explantes que contienen folículos vitelogénicos, la rFSH no tiene efecto sobre la expresión de *cyp17a2* y *cbr1*, pero aumenta la de *cyp17a1* y *cyp19a1*. Estos datos sugieren la presencia de una via esteroideogénica Cyp17a2/Cbr1 activada vía el Fshra en folículos ováricos primarios de la dorada la cual puede desencadenar la producción de 17,20β-P en este estado ovárico.

Capítulo III

El Corte y Empalme Alternativo del Receptor Nuclear de Progestinas en un Teleósteo Perciforme Genera Nuevos Mecanismos de Regulación Transcripcional Dominante Negativa

En los mamíferos, la función integrada del receptor nuclear de progestinas (PGR) puede ser regulada de forma diferente en cada tejido diana mediante la alteración de los niveles de expresión de variaciones del ARNm codificante del PGR. Tales isoformas del PGR se han identificado también en aves y reptiles, pero no en vertebrados no amniotas. En base a extensos estudios filogenéticos, sinténicos y funcionales, en este trabajo mostramos que los órdenes superiores de teleósteos retienen un solo gen *pgr*, y que cuatro variantes diferentes del ARNm de *pgr* se expresan en el ovario de un teleósteo perciforme evolucionado como la dorada (*Sparus aurata*). Tres de las isoformas (*pgr_tv2*, *pgr_tv3* y *pgr_tv4*) surgen del empalme alternativo del *pgr* pre-ARNm, lo que produce tres receptores con el N terminal truncado en diferente extensión, mientras que una isoforma (*pgr_tv1*) es una variante de deleción. El Pgr completo de la dorada muestra el efecto transactivational más elevado en respuesta a progestinas nativas de euteleósteos, como la 17α,20β-dihidroxi-4-pregnen-3-ona y 17α,20β,21-trihidroxi-4-pregnen-3-ona, mientras que las isoformas *Pgr_tv3* y *Pgr_tv4* ejercen de modo independiente nuevos mecanismos nucleares y citosólicos de represión dominante negativa de la transcripción mediada por el Pgr. En el ovario de la dorada, la proteína Pgr se localiza en las oogonias, en el núcleo de oocitos primarios (pre-vitelogénicos), así como en las células foliculares (células de la granulosa) y el citoplasma del oocito en folículos ováricos vitelogénicos. La expresión génica de *pgr*, *pgr_tv3* y *pgr_tv4* es la más alta en ovarios en crecimiento primario, mientras que la expresión de ambas isoformas inhibitorias, pero no del *pgr*, disminuye durante la vitelogénesis. La estimulación de explantes de ovarios primarios con hormona estimulante del folículo recombinante de lubina, así como con estrógeno, *in vitro* regula de forma diferencial la expresión temporal de *pgr*, *pgr_tv3* y *pgr_tv4*. Estos hallazgos sugieren, como posiblemente ocurre en los mamíferos, que la capacidad de respuesta ovárica a las progestina en la dorada, particularmente durante la oogénesis temprana, puede estar regulada a través del corte y empalme alternativo del ARNm del *pgr* nuclear. De este modo, el mecanismo dominante negativo de la regulación transcripcional del PGR probablemente evolucionó antes de la separación de Actinopterigios (peces con aletas espinosas) de Sarcopterigios (peces con aletas lobuladas).

Capítulo IV

La Activación Transcripcional de *aqp1ab* en el Oocito Primario Mediada por el Receptor Nuclear de Progestinas Determina el Fenotipo Pelágico de los Huevos de Teleósteos Marinos

En los teleósteos marinos, el canal de agua *aqp1ab* juega un papel vital en el desarrollo del fenotipo pelágico de los huevos. Sin embargo, el control transcripcional del gen *aqp1ab* durante la oogénesis es totalmente desconocido. En este trabajo, se describe el aislamiento de la región flanqueante 5 ' del gen *aqp1ab* de la dorada, en el cual hemos identificado elementos *cis*-reguladores de unión al receptor nuclear progestinas (Pgr) y a miembros de la familia Sox de factores de transcripción. Estudios de localización subcelular indican que el Pgr, así como los factores Sox3 y -8b, se co-expresan en las oogonias de la dorada. En los oocitos en crecimiento primario (pre-vitelogénesis), en los cuales la meiosis está detenida y ocurre la síntesis inicial de ARNm y proteína de *aqp1ab*, el Pgr es completamente traslocado desde el ooplasma al núcleo. Por el contrario, la expresión de *sox9b* no se detecta de forma significativa hasta el estado de alveolo cortical, coincidiendo con una fuerte disminución de la transcripción de *aqp1ab* en el oocito. La caracterización funcional del promotor de *aqp1ab* en células de mamífero y oocitos de *Xenopus laevis*, empleando tanto construcciones intactas como mutadas, ha demostrado que la transcripción de *aqp1ab* se inicia por el Pgr, el cual se activa por la progestina 17α,20β-dihidroxi-4-pregnen-3-ona (17,20β-P), el ligando natural del Pgr de dorada. La incubación *in vitro* de explantes primarios de ovario de dorada con la hormona estimulante del folículo o con 17,20β-P confirma que el Pgr activado por la progestina induce la síntesis de Aqp1ab a través de la activación del promotor del gen *aqp1ab*. Sin embargo, los ensayos de transactivación en sistemas heterólogos también muestran que los factores de transcripción Sox pueden potencialmente modular este mecanismo. Estos datos revelan la existencia de una vía endocrina implicada en la activación temprana de un canal molecular de agua necesario para la formación de los huevos de teleósteos marinos.

DISCUSIÓN GENERAL

En esta tesis, hemos investigado el origen evolutivo de la acuaporina-1ab (anteriormente denominada AQP1o o Aqp1b) de teleósteos (Fabra et al. 2005, 2006; Tingaud-Sequeira et al. 2008, 2010), así como su regulación molecular y su papel durante la hidratación del oocito. Para ello se han empleado dos modelos de teleósteos perciformes y pleuronectiformes, la dorada (*Sparus aurata*) y el fletán Atlántico (*Hippoglossus hippolossus*), respectivamente. Los datos obtenidos sugieren que la Aqp1ab ha surgido probablemente por duplicación génica en tándem a partir de un canal ancestral tipo AQP1, y esta acuaporina juega un papel esencial durante la hidratación de los oocitos que han reanudado la meiosis (maduración meiótica) en teleósteos marinos. Hemos demostrado que en los oocitos en crecimiento primario, el gen *aqp1ab* se activa temporalmente, tanto a nivel transcripcional como traduccional, por la progestina 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P), la cual se produce en las células de la granulosa en respuesta a gonadotropinas. Los datos también sugieren, no obstante, que la transcripción del gen *aqp1ab* podría estar regulada de forma dual por la 17,20β-P y factores de transcripción Sox. Este mecanismo resulta en la acumulación de grandes cantidades de proteína Aqp1ab de forma precisa en los oocitos en crecimiento primario justo antes de entrar en la fase de crecimiento (vítelogénesis). Durante la fase de vítelogénesis, el tráfico intracelular y la inserción de la Aqp1ab en la membrana plasmática del oocito también parecen estar regulados por mecanismos, aún desconocidos, relacionados con el C-terminal de la Aqp1ab. Este mecanismo podría estar altamente especializado en teleósteos evolutivamente avanzados como los pleuronectiformes. Por lo tanto, los resultados logrados en esta tesis demuestran que el mecanismo de hidratación del oocito mediado por la Aqp1ab en teleósteos marinos es un proceso conservado y altamente regulado tanto a nivel transcripcional como postraduccional.

1. Origen Evolutivo de la Subfamilia de Canales de Agua Aqp1ab de Teleósteos

Mediante la clonación de los cDNAs de la Aqp1aa y -1ab del fletán Atlántico y el examen exhaustivo de la filogenia molecular de la subfamilia de la acuaporina-1 (AQP1) de vertebrados, hemos concluido que la *AQP1* de tetrápodos y los ortólogos *aqp1aa* de teleósteos se agrupan según su rango filogenético y muestran longitudes de rama más cortas en comparación con los ortólogos *aqp1ab*. El análisis sinténico de los genes de vertebrados relacionados con la AQP1 confirma la existencia de dos genes en teleósteos (*aqp1aa* y *-1ab*). No obstante, los da-

tos revelan que mientras la sinténia está altamente conservada entre los teleósteos acantomorfos, esta se muestra menos conservada cuando se compara con el pez cebra (*Danio rerio*), lo cual se ha observado para otras familias de genes (Finn et al. 2009; Cerdà and Finn 2010; Chauvigné et al. 2010). Los parálogos *aqp1aa* and *-1ab* específicos de teleósteos están dispuestos en tándem entre los genes *crhr2* y *thoc1* en todas las especies analizadas. El medaka es la única excepción, tal y como se ha descrito previamente (Tingaud-Sequeira et al. 2008), el cual parece que ha perdido el parálogo *aqp1ab*. Un aspecto interesante de este análisis es la mayor conservación de contigüidad génica entre el pez cebra y los saurios, y la ausencia de conservación sinténica en el linaje de mamíferos. Aunque se sabe que el genoma de teleósteos sufrió una duplicación del genoma entero (WGD) y subsiguientes reordenamientos (Kasahara et al. 2007; Nakatani et al. 2007; Muffato and Roest-Crollius 2008), la elevada conservación sinténica entre los linajes de teleósteos, anfibios, reptiles y aves sugiere que la perdida regional de sinténia ocurrió entre los mamíferos. Entre los euterios, la sinténia está muy conservada pero se pierde en el locus *THOC*, en el cual el casete sinténico de los genes posteriores al loci *aqp1aa-aqp1ab-thoc1* de teleósteos se encuentra en un cromosoma separado. De este análisis hemos concluido que el locus *AQP1* está posiblemente codificado en regiones complejas de los genomas de vertebrados los cuales han sido objeto de reordenamiento intra- e intercromosomal especifico de linaje.

Los datos del análisis sinténico muestran asimismo que la subfamilia de la AQP1 en teleósteos refleja el sistema del gen *aqp8*, en el cual la WGD junto con una duplicación en tándem probablemente dio lugar a diferentes parálogos de la *aqp8* (Tingaud-Sequeira et al. 2010; Cerdà and Finn 2010). Los parálogos *aqp1aa* y *-1ab* de teleósteos representan duplicados en tándem, mientras que el producto de la WGD probablemente desapareció en la mayoría de especies, pero parece mantenerse en el genoma del pez cebra como un pseudogen fusionado aqp5/1b (Tingaud-Sequeira et al. 2010; Cerdà and Finn 2010). La co-agrupación filogenética de los genes *aqp1aa* y *-1ab* del fletán atlántico con los genes del pez espinoso (*Gasterosteus aculeatus*), implica además que la duplicación en tándem del gen ancestral AQP1 sirvió, probablemente, de base para la evolución adaptativa del parálogo *aqp1ab*.

Un aspecto interesante de este descubrimiento es la co-evolución del sistema de osmolitos orgánicos que facilita la hidratación del oocito en teleósteos marinos. Tanto el gen del precursor de vítelo (*vtgaa*), el cual encuentra altamente expresado en teleósteos marinos y es la principal fuente de aminoácidos libres (FAA) osmóticamente activos en el oocito, como el gen *aqp1ab*, responsable de la mediación del flujo de agua hacia el oocito, parecen haber surgido

por duplicación en tándem mas que a partir de la WGD (Finn et al. 2009; Finn and Fyhn 2010; Cerdà and Finn 2010). La subsiguiente neofuncionalización de las proteínas correspondientes revela que la duplicación génica *per se* puede ser una fuente importante de nuevas vías celulares que alteran el fenotipo, como la aparición de los huevos pelágicos que ha facilitado la radiación oceánica de los teleósteos (Finn and Kristoffersen 2007; Kristoffersen et al. 2009).

La duplicación en tándem no es sin embargo única para los ortólogos *aqp1aa* y *-1ab* de teleósteos, ya que en humanos el análisis sinténico revela un putativo parálogo del gen *AQP1* en el locus 30.9 Mb en LG 7, dos genes anteriores al locus *AQP1*. De forma parecida, los genes *AQP7* y *-12* parecen estar duplicados en tándem en algunos primates (Tingaud-Sequeira et al. 2010; Cerdà and Finn 2010). En teleósteos, sin embargo, es el duplicado *aqp1ab* el que se ha neofuncionalizado, como sugiere la filogenia molecular y los ensayos funcionales del C-terminal de la Aqp1ab el cual es el subdominio de las acuaporinas de vertebrados que ha evolucionado más rápidamente. Dentro de cada grupo, la AQP1 de tetrápodos y la Aqp1aa de teleósteos han sido sometidos a tasas similares de sustitución de aminoácidos (13-17%), mientras que el parálogo Aqp1ab ha experimentado una sustitución de aminoácidos significativamente mayor, particularmente en el dominio C-terminal. Teniendo en cuenta que los tetrápodos y los teleósteos han evolucionado a lo largo de peridos de tiempo similares (~390 millones de años), estos datos proporcionan un remarcable ejemplo de la divergencia en la estructura primaria como consecuencia de la duplicación de genes.

2. Papel Fisiológico de la Aqp1ab Durante la Hidratación del Oocito de Teleósteos Marinos

En estudios anteriores en teleósteos pelagófilos, como la dorada y la anguila japonesa (*Anguilla japonica*), el papel fisiológico de la Aqp1ab durante la hidratación del oocito se apoya en evidencias circunstanciales (p. ej., presencia de la Aqp1ab en la membrana plasmática del oocito en el momento de la hidratación), así como en la observación de que el incremento de volumen de los oocitos que están reanudando la meiosis *in vitro* se bloquea total o parcialmente por inhibidores típicos de algunas acuaporinas, como el mercurio o el tetraetilamonio (Fabra et al. 2005, 2006; Kagawa et al. 2009). Sin embargo, es conocido que estos compuestos también afectan a canales y transportadores iónicos (Armstrong 1990; Jacoby et al. 1999), los cuales podrían jugar un papel en la acumulación de osmolitos inorgánicos en el oocito durante la hidratación (Cerdà et al. 2007; Kristoffersen and Finn 2008). Por tanto, no existen todavía evidencias directas del papel de la Aqp1ab durante la hidratación del oocito de peces marinos.

Para investigar si el papel de la Aqp1ab en la hidratación del oocito está conservado en teleósteos marinos evolutivamente avanzados, hemos seleccionado el fletán Atlántico como modelo experimental. Esta especie se reproduce a bajas temperaturas y produce uno de los huevos pelágicos de mayor tamaño conocido, y por lo tanto los oocitos pueden ser manipulados más fácilmente para propósitos experimentales. En el fletán Atlántico, el gen *aqp1ab* se expresa de forma predominante en el ovario como ocurre en otros teleósteos (Tingaud-Sequeira et al. 2008). Sin embargo, en esta especie hemos encontrado una sobre regulación específica y transitoria de este gen durante la reanudación de la meiosis, junto con un aumento posterior de la proteína, la cual se transfiere a las microvellosidades de la membrana plasmática de los oocitos durante el proceso de hidratación. Por tanto, el canal Aqp1ab en el oocito del fletán podría estar regulado de una forma similar que en la dorada (Fabra et al. 2006). Estos datos correlacionan muy bien con la hiperosmolalidad transitoria del oocito del fletán con relación al fluido ovárico durante la maduración, debido en parte a la acumulación de iones y la proteólisis del vítelo (Finn et al. 2002), y son por tanto consistentes con el papel de la Aqp1ab en el transporte de agua.

Tal y como se esperaba a partir de estudios previos (Fabra et al. 2005; Tingaud-Sequeira et al. 2008; Kagawa et al. 2009; Tingaud-Sequeira et al. 2010), y de las características estructurales de la Aqp1ab del fletán, hemos visto que el $HgCl_2$ bloquea de forma reversible la permeabilidad mediada por la Aqp1ab en oocitos homólogos y heterólogos, así como la hidratación de los oocitos nativos durante la maduración meiótica. Sin embargo, debido a que el mercurio no es un inhibidor especifico de la Aqp1ab, hemos utilizado también un anticuerpo específico contra el C-terminal de la Aqp1ab del fletán, el cual inhibe la translocación de la Aqp1ab a la membrana plasmática de los oocitos de *Xenopus laevis*. La microinyección del anticuerpo en oocitos de fletán *in vivo* resulta en una inhibición dosis dependiente de la hidratación, mientras que el proceso de hidrólisis del vítelo y la maduración meiótica no se afectan. La inhibición inmunológica se puede revertir completamente mediante la expresión artificial del parálogo Aqp1aa, que es funcional en oocitos de fletán pero no es reconocido por el anticuerpo. En consecuencia, la reducción en la hidratación de los oocitos de fletán se puede relacionar directamente con la pérdida de la función de la Aqp1ab. Estas observaciones, junto con los datos de expresión génica y localización celular, proporciona por primera vez evidencias funcionales directas del papel esencial de la Aqp1ab en el transporte de agua durante la hidratación del oocito de peces marinos.

3. Regulación Transcripcional de la Aqp1ab en Oocitos de Dorada

Los estudios realizados hasta la fecha indican que el ARNm de *aqp1ab* se acumula en grandes cantidades en el ovario, presumiblemente en oocitos pre-vitelogénicos, y posteriormente la proteína Aqp1ab se sintetiza y transporta lentamente hacia la corteza del oocito durante el periodo de crecimiento (vitelogénesis) (Fabra et al. 2006; Tingaud-Sequeira et al. 2008; Kagawa et al. 2011). Estas observaciones sugieren que el gen *aqp1ab* en el oocito podría estar regulado a nivel transcripcional, traduccional y postraduccional, aunque los mecanismos moleculares implicados son desconocidos. En la dorada, hemos reexaminado la regulación de la expresión del gen *aqp1ab* durante la oogénesis *in vivo* mediante hibridación *in situ* y microscopía de inmunofluorescencia. En oocitos en crecimiento primario o pre-vitelogénicos en los que la meiosis está detenida, se ha observado que hay una corta fase de tiempo de transcripción del gen *aqp1ab* y que los transcritos resultantes son inmediatamente traducidos antes del inicio de la vitelogénesis. Este pool de proteínas de Aqp1ab es posiblement modificado postraduccionalmente (Tingaud-Sequeira et al. 2008), transportado y almacenado en la periferia del ooplasma durante los siguientes estadios de desarrollo ovocitario. En este trabajo hemos comenzado a analizar los mecanismos moleculares implicados en la regulación transcripcional del gen *aqp1ab* en los oocitos en crecimiento primario de la dorada.

3.1. Aislamiento y Caracterización del Promotor del gen *aqp1ab* de la Dorada

Como primer paso para elucidar los mecanismos transcripcionales implicados en la expresión del gen *aqp1ab* en oocitos, hemos clonado la región 5' flanqueante del gen *aqp1ab* de la dorada. Los análisis de la secuencia de nucleótidos de la parte proximal de esta región (1,672 pb) revelan tres dominios (nucleótidos -906 a -706, -581 a -498, y -405 a -259, considerando la posición +1 el primer nucleótido del codón de inició de la traducción ATG) con semejanza a los exones 2/3 (59% identidad), 3 (71% identidad) y 4 (40% identidad), respectivamente, del gen de la aqp1ab, lo cual sugirie la presencia de trazas de un pseudogen duplicado de la *aqp1ab* en el genoma de la dorada.

Tal y como se observa en el núcleo del promotor de distintas acuaporinas de mamíferos (Inase et al. 1995; Umenishi and Verkman 1998a,b; Borok et al. 2000), el promotor proximal del gen *aqp1ab* de dorada muestra secuencias consenso de elementos importantes para la interacción basal con la maquinaria de los factores de transcripción, así como distintas secuencias TATA y CCAAT, Sp1, AP1, E-box y elementos iniciadores (lnr), las cuales facilitan la

unión de proteínas reguladoras al núcleo del promotor y son importantes para la selección de los factores de transcripción (Smale and Baltimore 1989). La determinación experimental de los sitios de inicio de la transcripción (TSSs) en oocitos primarios por RLM-RACE indica cinco sitios diferentes de inicio, aunque la mayoria de los transcritos parece iniciarse en la posición 364 anterior al codón de inicio (TSS2). En algunos genes de acuaporinas de mamíferos, como *AQP8* de ratón (Calamita et al. 1999) o AQP4 de humano (Lu et al. 1996) se han encontrado también TSSs alternativos. El análisis de los TSSs en relación a las secuencias TATA y lnr sugieren que la transcripción del gen *aqp1ab* en oocitos de dorada es posiblemente TATA independiente, como se ha propuesto para el gen AQP8 de ratón (Calamita et al. 1999), el gen AQP5 de ratón y humano (Lee et al. 1996), o el gen AQP5 de rata en glándulas salivales (Borok et al. 2000).

El análisis *in silico* del promotor del gen *aqp1ab* de dorada ha revelado la presencia de numerosos elementos *cis*-reguladores conservados los cuales pueden ser reconocidos por factores de transcripción. Entre estos elementos se han encontrado numerosas secuencias de consenso que pueden ser vinculadas con factores de transcripción implicados en el desarrollo del intestino y/o del riñón en mamíferos y pez cebra, como CDX (Flores et al. 2008; Beck and Stringer 2010), factores Krüppel-like (KLF4, KLF15; Mallipattu et al. 2012; Li et al. 2011; Yu et al. 2012), PBX1 (Schnabel et al. 2003), o NFKB1 (Panzer et al. 2009). Esta observación es consistente con la expresión del gen *aqp1ab* en órganos osmoreguladores de teleósteos, como el riñón y el recto (Cerdà and Finn 2010), además de los oocitos, y con la activación constitutiva del promotor *aqp1ab* de dorada en las células HEK293T y Caco-2 (ver más adelante). En el riñón y el recto de teleósteos, la Aqp1ab puede tener un papel en el mantenimiento del balance hídrico mediando el transporte de agua a través del epitelio (Cerdà and Finn 2010). El promotor *aqp1ab* de dorada también contiene tres sitios potenciales para CREB que responden a AMPc y se encuentran en genes de acuaporinas de mamíferos, incluyendo *AQP2* (Yasui et al. 1997; Yu et al. 2009) y *AQP5* (Wang and Zheng 2011), así como un potencial sitio conservado para el factor inducible por hipoxia-1, el cual activa la trascripción del gen AQP1 en respuesta a hipoxia (Abreu-Rodríguez et al. 2011).

3.2. El Receptor Nuclear de Progestinas y Factores Sox Regulan la Transcripción del Gen *aqp1ab*

El promotor del gen *aqp1ab* de dorada contiene dos elementos de respuesta a progestinas [PRE (a) y PRE (b)], los cuales pueden unirse al receptor nuclear de progestinas (PGR), y tres putativos sitios de unión para factores de transcripción SOX [SOX (a), SOX (b) y SOX (c)]. To-

dos estos elementos *cis*-reguladores están conservados en la región 5' del gen *aqp1ab* de distintos teleósteos. Se sabe que diferentes genes *sox*, tales como *sox3*, *-8*, o *-9b*, se expresan en oogonias y oocitos primarios en vertebrados no mamíferos (Koyano et al. 1997; Rodríguez-Mari et al. 2005; Yao et al. 2007; Raghuveer and Senthilkumaran 2010; Dumond et al. 2011), aunque en la mayoría de los casos, aún no se han dilucidado sus funciones específicas durante la determinación del sexo y/o diferenciación de las gónadas. Estudios recientes han demostrado asimismo la expresión del ARNm y de la proteína del Pgr nuclear en oocitos primarios de pez cebra (Hanna et al. 2010). En base a estos datos, hemos investigado la relevancia funcional del Pgr y de factores de transcripción Sox durante la regulación transcripcional del gen *aqp1ab* en oocitos primarios de dorada, en primer lugar mediante la clonación de los cDNAs correspondientes y la determinación de su patrón de expresión durante la oogénesis.

3.2.1. Aislamiento del Pgr Nuclear y Factores de Transcripción Sox de la Dorada

El ADNc completo del Pgr nuclear de dorada se ha aislado utilizando RT-PCR seguido de RACE 5' y 3'. Este ADNc codifica una proteína con la estructura típica de los receptores de esteroides nucleares, y muestra una elevada homologia en la secuencia de aminoácidos del dominio de unión a ADN (DBD) y del dominio de unión a ligando (LBD) con otros PGRs de vertebrados. El Pgr de dorada es similar al que se ha clonado en otros teleósteos perciformes (Todo et al. 2000; Ikeuchi et al. 2002; Hanna et al. 2010; Chen et al. 2010, 2011, 2012), mientras que es un poco más distinto que el Pgr de salmónidos, y muestra la afinidad más elevada, como ocurre en todos los teleósteos, para los grupos de progestinas 17 y 20 hidroxilo, 17,20β-P y 17α,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P).

El análisis filogenético y sinténico han confirmado la presencia de un único gen *pgr* en los genomas de teleósteos, incluyendo la dorada. Sin embargo, en esta especie, a diferencia de los anfibios y otros teleósteos basales (Tian et al. 2000; Todo et al. 2000; Thornton 2001; Ikeuchi et al. 2002; Wang et al. 2004; Katsu et al. 2008; Chen et al. 2010, 2011, 2012; Hanna et al. 2010), pero parecido a lo que se ha descrito en mamíferos (Conneely et al. 1987; Kastner et al. 1990; Li and O'Malley 2003; Cork et al. 2008), hemos encontrado tres variantes distintas del gen *pgr* expresadas en el ovario, las cuales hemos denominado *pgr_tv2*, *pgr_tv3* y *pgr_tv4*. Estas isoformas codifican receptores funcionales y se han generado por empalme alternativo del pre-ARNm en al región 5' no codificante. De este modo, se producen ARNm que codifican isoformas del Pgr truncadas en el N-terminal las cuales carecen aproximadamente de la mitad de la region A/B (Pgr_tv2) o de toda la esta región y una pequeña porción del DBD (Pgr_tv3),

o que solamente tienen una parte del LBD (Pgr_tv4). Hemos identificado también una cuarta isoforma (Pgr_tv1) que aparentemente se origina a partir de un sitio de empalme alternativo dentro del exon 1, causando una deleción de 121 aminoácidos en la región A/B.

Ensayos de transactivación usando células de mamífero HEK293T indican que la actividad transcripcional de la isoforma Pgr_tv2 no es significativamente diferente de la del Pgr natural (completo). Por el contrario, tanto el Pgr_tv3 com el Pgr_tv4 muestran una eficiencia menor de transactivación que el Pgr, y ejercen mecanismos dominante negativos nucleares y citosólicos, respectivamente, de represión de la transcripción mediada por el Pgr. El mecanismo subyacente a la inhibición dominante negativa del Pgr_tv3 está probablemente relacionado con la capacidad de esta isoforma de traslocarse al núcleo, dimerizar y competir por los PRE y/o cofactores nucleares comunes, de modo similar a lo que se ha sugerido para las isoformas PGR-A y el PGR-C de mamíferos (Vegeto et al. 1993; Wei et al. 1996). Sin embargo, el Pgr_tv4 representa una nueva isoforma dominante negativa de origen natural que puede promover la actividad transcripcional, a pesar de carecer del DBD y de la señal de localización nuclear, posiblemente a través de mecanismos no genómicos (Cork et al. 2008). La fuerte inhibición dominante negativa del Pgr_tv4 en presencia de 17,20β-P está probablemente mediada por un mecanismo diferente al de la isoforma Pgr_tv3. Este mecanismo podría estar mediado por una mayor afinidad del Pgr_tv4 a algunos factores citoplasmáticos con concentraciones limitantes, como algunas quinasas (Aranda and Pascual 2001), requeridos para la unión del Pgr al ADN y/o para la transactivación, pero no para el tráfico intracelular del Pgr.

Los ADNc de parálogos de sox con expresión ovárica en la dorada se aislaron también mediante RT-PCR y 5' y 3' RACE, empleando oligonucleótidos degenerados diseñados dentro de la secuencia conservada de la caja HMG de las proteínas tipo Sox. Esto dio lugar a la clonación de tres ADNc completos distintos codificantes de factores Sox. El análisis Bayesiano de las secuencias de nucleótidos y aminoácidos en relación con una amplia selección de ortólogos de *sox* de vertebrados reveló una duplicación para los genes *sox8* y *-9* y un solo gen para el *sox3* en teleósteos. Sobre la base de la co-agrupación filogenética de los transcritos y las proteínas Sox de dorada con los de pez cebra, las secuencias aisladas en dorada se han clasificado como Sox3, -8b y -9b.

3.2.2. Expresión del Pgr, Sox3, Sox8b y Sox9b Durante la Oogénesis de la Dorada

La expresión del *pgr* nuclear, *sox3*, *-8b* y *-9b* de dorada en relación con la de la *aqp1ab* durante la oogénesis temprana *in vivo*, determinada mediante hibridación *in situ* e inmunolocaliza-

ción (para el Pgr y la Aqp1ab), demuestra que el Pgr y, los ARNm de *sox3* y de *-8b* se expresan de forma elevada en las oogonias, de este modo precediendo la activación de la transcripción y traducción del gen *aqp1ab* en oocitos en crecimiento primario. Esta localización específica de la proteína Pgr, tanto en el citoplasma como en el núcleo de las oogonias que inician la meiosis, es coherente con el supuesto papel del Pgr nuclear durante la activación de la meiosis mediada por progestina (Miura et al. 2006, 2007). Sin embargo, en los oocitos en crecimiento primario el Pgr está prácticamente todo translocado al interior del núcleo, lo que indica que en este estadio el receptor está probablemente activado por su ligando específico. En cambio, los transcritos de *sox9b* parecen expresarse a bajos niveles en los oocitos en crecimiento primario siendo el nivel de expresión mucho mayor en estadios más avanzados del desarrollo del oocito (alveolo cortical). Estas observaciones proporcionan por tanro evidencia circunstancial del posible papel del Pgr, así como de los factores Sox, en la regulación de la transcripción del gen *aqp1ab* en oocitos en crecimiento primario de dorada.

En nuestro estudio hemos detectado también la presencia del Pgr en las células de la granulosa asociadas a los oocitos en crecimiento primario, así como en folículos ováricos en estadio de alveolo cortical y vitelogénicos, como se ha documentado previamente en el pez cebra (Hanna et al. 2010). La expresión del Pgr en las células de la granulosa de folículos vitelogénicos tardíos es consistente con el papel de este receptor durante la ovulación (Thomas et al. 2007). Sin embargo, a diferencia de lo encontrado en el pez cebra, en dorada el Pgr nuclear se localiza también en la región cercana a la membrana plasmática de oocitos en estadio de alvéolo cortical y vitelogénicos. Este patrón, junto con la presencia de motivos conservados de palmitoilación en el LBD del Pgr de dorada, podría sugerir el papel del Pgr, posiblemente con los Pgr de membrana (Zhu et al. 2003; Josefsberg-Ben-Yehoshua et al. 2007; Hanna and Zhu 2011; Thomas 2012; Tokumoto et al. 2012), en la regulación de la maduración meiótica (Bayaa et al. 2000; Tian et al. 2000; Martínez et al. 2006, 2007). Los transcritos de *sox8b* también se expresan en las células de la granulosa que rodean a los oocitos en estadio de alveolo cortical y vitelogénicos tempranos. Aunque, el *sox8* no se ha detectado en las células de la granulosa en el pez cebra (Rodríguez-Marí et al. 2005), nuestra observación coincide con la localización de ARNm de *SOX8* en las células de la granulosa en folículos preovulatorios de ratón (Salmon et al. 2005). Otra observación interesante es que el *sox3* y *cyp19a1* (ver más adelante) se co-expresan en el ooplasma de los oocitos de dorada en estadio primario, lo cual es consistente con la activación de la transcripción de *cyp19a1* mediada por *sox3* como se ha visto en algunos anfibios (Oshima et al. 2009).

En humanos y roedores, las isoformas PGR-A y PGR-B, así como otras variantes generadas por empalme alternativo (p. ej. PGRΔ3, PGRΔ6), se coexpresan en el ovario (Ilenchuk and Walters 1987; Schneider et al. 1991; Misao et al. 1998; Conneely et al. 2003). En el ovario de dorada, hemos observado diferentes niveles de expresión de las distintas isoformas; las variantes *pgr_tv2*, *pgr_tv3* y *pg_tv4* están coexpresadas con el *pgr* salvaje durante todo el desarrollo, pero en general muestran ratios más altas con respecto al *pgr* durante los estadios iniciales y finales de la oogénesis. Aunque en este trabajo no hemos podido determinar los niveles de traducción *in vivo* de todas estas isoformas, los niveles de transcripción del *pgr_tv3* y *pgr_tv4* que hemos detectado son posiblemente insuficientes para la completa inhibición de la transcripción mediada por el Pgr (en base al patrón de inhibición del Pgr_tv3 y Pgr_tv4 en células HEK293T), pero podrían ser capaces de modular la función transcripcional del Pgr. Este mecanismo se asemejaría a la regulación de la función del PGR-A o PGR-B mediante el PGR-C y otras isoformas que se ha sugerido ocurre en el ovario o en células de cáncer de mama en humano (Richer et al. 2002; Condon et al. 2006; Cork et al. 2008). En los tejidos reproductivos el PGR-A y el PGR-B controlan diferentes genes (Richer et al. 2002; Mulac-Jericevic and Conneely 2004), y por tanto la sobreexpresión del PGR-A comparado con el PGR-B, común en células de cáncer de mama (Graham et al. 1995, 2005), puede cambiar la respuesta de las células a la progestina (Graham et al. 2005). Por lo tanto, la existencia en el ovario de dorada de un mecanismo similar no se puede descartar, ya que aún no se ha investigado si el Pgr, Pgr_tv2 o Pgr_tv3 pueden regular distintos subconjuntos de genes diana que dependen de progestinas.

3.2.3. El Pgr y Factores Sox Regulan de Forma Distinta la Transcripción del Gen *aqp1ab*

Los análisis funcionales del promotor *aqp1ab* de dorada han confirmado que el Pgr nuclear, así como el Sox3, -8b y 9b, regulan de forma diferente la transcripción del gen *aqp1ab*. Como este gen se expresa tanto en células somáticas como germinales (Cerdà and Finn 2010), para estos ensayos de transactivación hemos empleado tanto células HEK293T como oocitos de *X. laevis*. Los experimentos en células HEK293T y oocitos, empleando construcciones intactas y mutadas del promotor *aqp1ab*, dan resultados idénticos demostrando que el Pgr es un inductor importante de la transcripción del gen *aqp1ab*, mientras que el Sox3 y el -8b cooperan con el Pgr para activarla. El efecto de los factores Sox se realiza a través del sitio de unión SOX(c) y es sinérgico con el Pgr, sugiriendo que este mecanismo implica cambios conformacionales mediados por Sox en la cromatina que exponen los PREs para facilitar su unión con el Pgr (Vi-

cent et al. 2010; Clarke and Graham 2012). En cambio, tanto en células somáticas como en oocitos, el Sox9b ejerce una represión transcripcional del gen *aqp1ab* más potente en comparación con la activación por parte de Sox3 o -8b a través del mismo sitio SOX(c). Estas observaciones podrían explicar la fuerte reducción de los niveles de expression de la *aqp1ab* en oocitos en estadio de alveolo cortical *in vivo* en la dorada ya que estos oocitos expresan niveles más elevados de sox9b. Considerando que en este estadio de la ooogénesis de la dorada existe también una elevada expresión del *sox3* y *-8b* en el ooplasma, y que la localización nuclear del Pgr se reduce respecto a lo observado en el estadio de crecimiento primario, es probable que niveles bajos del Sox9b sean todavía capaces de inhibir la expresión de la *aqp1ab* en oocitos de dorada en estadio de alveolo cortical.

La regulación transcripcional del gen *aqp1ab* por el Pgr se ha confirmado *ex vivo* mediante el uso de explantes de ovario de dorada en fase de crecimiento primario expuestos a 17,20β-P, en presencia o ausencia de un inhibidor del Pgr, RU486, seguido por qRT-PCR, Western blot y ensayos de inmunoprecipitación de cromatina (ChIP). Estos experimentos demuestran que el 17,20β-P aumenta la incorporación del Pgr a los dos PREs del promotor proximal del gen *aqp1ab* en oocitos en crecimiento primario, activando así tanto la transcripción como la síntesis de proteína Aqp1ab.

3.3. El Receptor de la Hormona Folículo Estimulante Activa la Transcripción del Gen *aqp1ab* a Través de la Síntesis de Progestinas

Los hallazgos anteriores sugieren que el Pgr nuclear es el principal regulador de la transcripción del gen aqp1ab en oocitos en crecimiento primario de dorada. Para confirmar que el ligando del Pgr (es decir, la 17,20β-P) se produce efectivamente en esta etapa, y poder elucidar así la posible vía endocrina implicada, se llevaron a cabo una serie de experimentos tanto *in vivo* como *in vitro*.

Como primera tarea, hemos clonado en la dorada los principales enzimas implicados en la síntesis de 17β-estradiol (E2) y progestinas en teleósteos, P450c17-I (Cyp17a1) y aromatasa (Cyp19a1), y P450c17-II (Cyp17a2) y 20β-hidroxiesteroide deshidrogenasa (Cbr1), respectivamente. Empleando estos ADNc determinamos el patrón de expresión de los ARNm correspondientes en folículos en crecimiento primario y vitelogénicos mediante hibridación *in situ* y qRT-PCR. Los experimentos demuestran que todos estos enzimas esteroidogénicos se expresan en las células primordiales de la granulosa asociadas a los oocitos primarios. La expresión del *cbr1* en folículos previtelogénicos de dorada concuerda con informes anteriores en otros

teleósteos, como el bacalao del Atlántico (Kortner et al. 2009) y el bagre norte africano (Sreenivasulu and Senthilkumaran 2009). En las células primordiales de la teca, sin embargo, se observa muy poca expresión de *cyp17a2* y *cbr1*. En cambio, en los folículos vitelogénicos, el *cyp17a1*, *cyp17a2* y *cbr1* se expresan tanto en las células de la teca como de la granulosa, mientras que el *cyp19a1* se expresa sólo en las células de la granulosa, tal y como se ha descrito en otros teleósteos (Nagahama and Yamashita 2008; Lubzens et al. 2010). Sin embargo, en el citoplasma de oocitos primarios de dorada también hemos detectado transcritos de *cyp17a2* y *cyp19a1*, mientras que en el ooplasma de oocitos vitelogénicos se detectan ARNm de *cyp17a1*, *cyp17a2*, así como *cbr1*. Esta última observación no es sorprendente porque en algunos teleósteos se han detectado actividades 20β-hidroxiesteroide deshidrogenasa mitocondriales y microsomales en oocitos desprovisto de células somáticas (Kazeto et al. 2001). Asimismo, ARNm de *cyp17a1* y *cyp19a1* y actividades enzimáticas se detectan en el citoplasma de oocitos vitelogénicos de trucha y anfibios (Yang et al. 2003; Gohin et al. 2011a,b).

La expresión ovárica más elevada de *cbr1* en la dorada se observa durante el estadio de crecimiento primario, y es concomitante con un elevado contenido tisular en 17,20β-P *in vivo* y una elevada expresión del receptor de la hormona folículo estimulante (*fshra*), pero no del receptor de la hormona luteinizante (*lhcgrba*). Sin embargo, en los mismos individuos las concentraciones en plasma de 17,20β-P son ligeramente más altas y sólo un poco diferentes de las concentraciones de E2 o 17,20β,21-P. Estos resultados se podrían explicar por la posible presencia de actividad de unión a 17,20β-P en oocitos primarios de dorada, como ocurre en la lamprea marina (*Petromyzon marinus*) para la androstendiona testicular (Bryan et al. 2007). De hecho, en la platija (*Pleuronectes platessa*) se ha demostrado actividad específica de unión a 17,20β-P en testículos maduros (Mugnier et al. 2000). Si las proteínas de unión de esteroides son la razón de la tendencia del 17,20β-P a acumularse en el tejido gonadal de dorada, será interesante investigar en el futuro si la actividad de unión se debe a la presencia de Pgrs nucleares o de membrana o de globulinas transportadoras de esteroides sexuales, ya que ambos tienen la capacidad de retener 17,20β-P en el ovario evitando su difusión.

Las observaciones anteriores sugieren que el Fshra podría controlar la síntesis de 17,20β-P en ovarios en la etapa de crecimiento primario. Para investigar esta hipótesis, hemos utilizado gonadotropinas recombinantes de otro teleósteo perciforme, la lubina, para estudiar el efecto de las gonadotropinas sobre la síntesis de esteroides in vitro en explantes primarios de ovario. La caracterización de la respuesta del Fshra y del Lhcgrba de dorada a las hormonas recombinantes folículo estimulante (rFSH) y luteinizante (rLH) de lubina utilizando células

HEK293T indica, como se ha observado en otros teleósteos(Levavi-Sivan et al. 2010; Chauvigné et al. 2012), que el Fshra puede ser activado por rFsh y rLh, mientras que el Lhcgrba es específico para rLh. La elevada promiscuidad del Fshra de dorada frente a la rLh de lubina (que debe ser confirmada en el futuro con hormonas homólogas), excluye el uso de la rLh para discernir la implicación de la Fshra o la Lhcgrba en la síntesis de 17,20β-P *in vitro* en explantes de ovario primario. Por tanto, se decidió probar sólo la rFsh, que es específica para el Fshra y no activa el Lhcgrba de dorada incluso a dosis farmacológica. Los experimentos *in vitro* han demostrado que la rFsh estimula la producción de 17,20β-P en las primeras 24 h, la cual se mantiene elevada si se añade el precursor 17-P al medio de cultivo, mientras que la producción de T y de E2 no se ve afectada por el 17-P. La síntesis de 17,20β-P mediada por rFsh por encima de la producción de E2 correlaciona con el aumento de la transcripción de *cyp17a2* y *cbr1* en los explantes y la disminución de los niveles de ARNm de *cyp17a1* y *cyp19a1*. Estos datos sugieren por tanto el papel de la Fshra en la activación de la vía Cyp17a2/Cbr1 en folículos ováricos primarios de dorada para la activacíón de la producción de 17,20β-P. Además, hemos encontradom que la rFsh también aumenta los niveles de ARNm y de proteína del Pgr, tal y como se ha descrito en las células de la granulosa de mamíferos donde la FSH y la LH controlan la transcripción del PGR-A y del PGR-B a través de AMPc (Natraj and Richards 1993; Park-Sarge and Mayo 1994; Clemens et al. 1998). Por lo tanto, el aumento a corto plazo (24 h), de la expresión del *pgr* mediado por rFsh en los explantes de ovario de dorada, coincide con la estimulación de la producción de 17,20β-P pero no de E2. Estas observaciones implican que la rFsh, actuando a través del receptor de Fsh, promueve simultáneamente la síntesis del Pgr y de su ligando en ovarios primarios, y que este es independiente de estrógeno como ocurre en las células de la granulosa de mamíferos (Clemens et al. 1998). El 17,20 β-P producido en esta etapa activará posteriormente el promotor del gen *aqp1ab* en los oocitos en crecimiento primario como se ha indicado anteriormente, lo cual es consistente con la inhibición de la transcripción de *aqp1ab* mediada por rFSH en presencia de inhibidores de la esteroidogénesis y de la síntesis de 17,20β-P, tales como DL-aminoglutetimida y indometacina, respectivamente, en explantes cultivados *in vitro*.

El mecanismo de acción del Fshra sobre la esteroidogénesis en folículos de dorada en crecimiento primario es parecido a la acción propuesta par la Lh, vía el Lhcgrba o el Lhcgrbb, en folículos postvitelogénicos de otros teleósteos durante la inducción de la maduración y la ovulación del oocito (Nagahama and Yamashita 2008; Lubzens et al. 2010; Senthilkumaran et al. 2004; Zhou et al. 2007; Tanaka et al. 2002). No obstante, el ligando específico implicado en

este mecanismo en ovarios primarios de dorada *in vivo* (Fsh o Lh), así como su origen (tanto en la pituitaria como secretado de los oocitos en desarrollo; Wong and Zohar 2004), no está claro a partir de nuestro estudio, ya que el Fshra de dorada puede ser activado también por rLh. Aunque herramientas para la medición de los niveles circulantes y en la pituitaria de Fsh no están disponibles todavía para la dorada, estudios previos han demostrado que los niveles plasmáticos de Lh en hembras con ovarios inmaduros previtelogénicos son mucho más bajos (~ 1 ng/ml) que los que se observan durante la maduración (~ 17 ng/ml) (Holland et al. 1998). Esto puede sugerir la participación de la Fsh, más que la de la Lh, en el control gonadotrófico de la esteroidogénesis en ovarios primarios de dorada. Sin embargo, esta conclusión puede ser prematura sin información disponible sobre los niveles circulantes de Fsh durante el ciclo reproductivo de la dorada, o datos sobre la caracterización detallada de la respuesta del Fshra de dorada a las hormonas homólogas.

3.4. Modelo para la Regulación Molecular del Gen *aqp1ab* en Oocitos de Dorada

En los experimentos *in vitro* realizados en este estudio, la estimulación de los explantes primarios con rFsh induce también un aumento retardado de la expresión de *cyp19a*, que coincide con un aumento de la producción de E2 en el tejido. Al mismo tiempo, la expresión de *cyp17a1* correlaciona negativamente con la dosis de rFsh. Para conciliar estos datos con todas las observaciones previas, incluyendo la prominente expresión de *cyp19a1* en el citoplasma de oocitos primarios, hemos propuesto un modelo que resume nuestra hipótesis actual para la regulación de las vías esteroidogénicas en folículos primarios de dorada mediada por Fshra y la posterior activación del promotor del gen *aqp1ab* (Fig. 1). Este modelo implica en primera instancia la activación del Fshra por Fsh (or Lh) en las células foliculares primordiales, lo cual conduce rápidamente a un aumento de la transcripción de *cyp17a2* y *cbr1*, y a la disminución de la expresión de *cyp17a1* y *cyp19a1*, resultando en un aumento neto de la producción de 17,20β-P. El aumento en la síntesis de T que también se observa en nuestros estudios probablemente es debido a la inducción de la expresión de 17β-hidroxiesteroide deshidrogenasa (*hsd17b*), como se ha descrito en folículos vitelogénicos de pez cebra incubados con la gonadotropina coriónica humana (hCG) (Ings and Van der Kraak 2006), o en células de la granulosa humanas estimuladas con FSH (Whitehead and Lacey 2003). Esto daría lugar a la rápida transformación de la androstenediona (formada por el Cyp17a1) en T, que luego podría difundirse lentamente hacia el interior de los oocitos donde podría ser aromatizada a E2 por el Cyp19a1 (Gohin et al. 2011a, b). La producción tardía de E2 después de la estimulación con

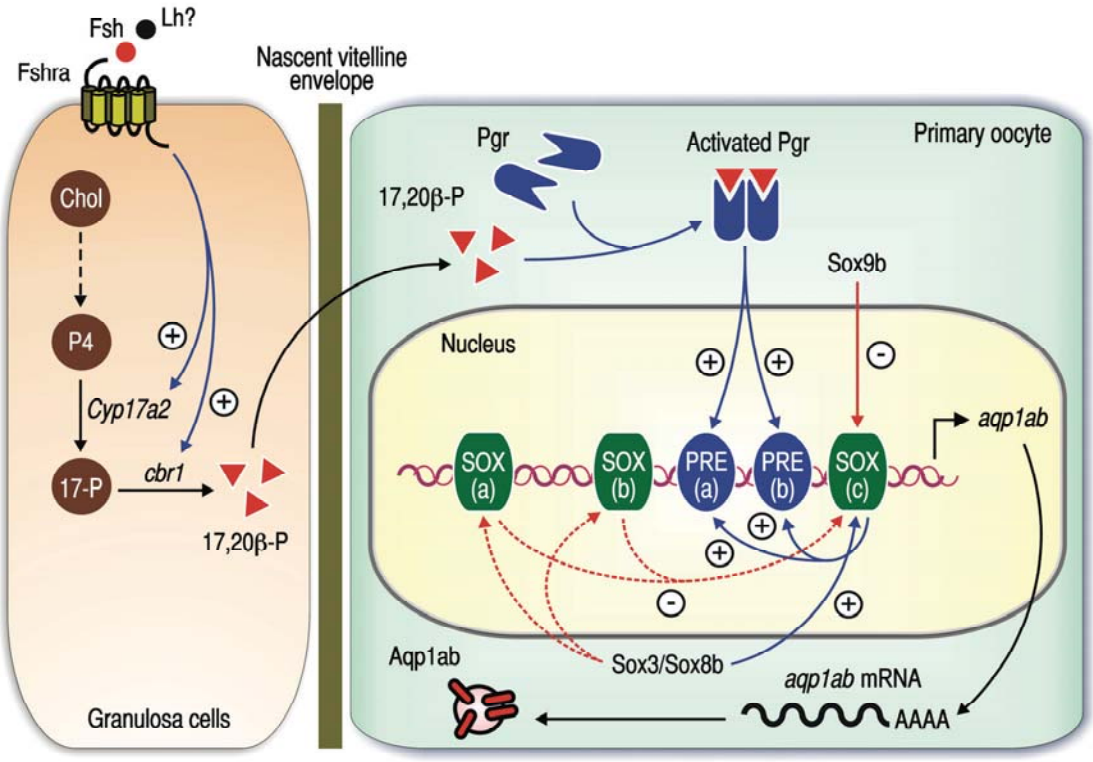

Fig. 1. Modelo propuesto para el control endocrino de la regulación transcripcional del gen *aqp1ab* de dorada en oocitos en crecimiento primario. El modelo indica la síntesis de progestinas (17,20 β-P) mediada por el receptor de la hormona estimulante del folículo (Fshra) y la posterior activación de la transcripción de *aqp1ab* a través del receptor nuclear de progestinas (Pgr) bajo modulación positiva y negativa de Sox3, -8b y -9b. En la etapa de crecimiento primario, la activación del Fshra en las células de la granulosa desencadena la producción de 17,20 β-P a través de la activación transcripcional de la vía esteroidogénica P450c17-II (Cyp17a2)/20β-hidroxiesteroide deshidrogenasa (Cbr1) en estas células. La unión del 17,20 β-P con el Pgr activa el receptor el cual promueve la transcripción de *aqp1ab* probablemente través de la interacción independiente con dos PRE *cis*-reguladores [PRE(a) y PRE(b)] situados en la zona proximal del promotor. La interacción de Sox3 o -8b con la secuencia SOX(c) puede actuar sinérgicamente con el PRE(a) o PRE(b) para aumentar la transcripción mediada por el Pgr. Los elementos SOX(a) y/o SOX(b) distales sin embargo pueden inhibir el efecto sinérgico de Sox3 o -8b a través de SOX(c) (líneas discontinuas). Por el contrario, Sox9b reprime la transcripción de *aqp1ab* exclusivamente a través de SOX(c). Así, niveles presumiblemente bajos de expresión de Sox9b en oocitos en crecimiento primario permiten la síntesis de ARNm y proteína de *aqp1ab* mediada por el Pgr, mientras que el aumento de los niveles de Sox9b en etapas ovocitarias más avanzadas podrían inhibir la expresión *aqp1ab*.

rFsh *in vitro* puede reflejar la disminución inicial de la transcripción de *cyp19a1* y el posterior aumento estimulado por mensajeros secundarios (como el AMPc) originados en las células foliculares y translocados al oocito a través de canales intercelulares (“gap junctions”) entre las células de la granulosa y el oocito (ej. Cerdà et al. 1993; Selman et al. 1993). El E2 producido en el oocito podría entonces ejercer un mecanismo de feedback negativo sobre la expresión folicular de *cyp17a1*, ya que es conocido que el estrógeno puede regular los niveles de

ARNm de *cyp17a* en la trucha (Govoroun et al. 2001) y en testículos de rata (Sakaue et al. 2002). Esta comunicación entre las células foliculares y el oocito primario para controlar la síntesis de esteroides, parecida a la que ocurre en folículos vitelogénicos de anfibio (Sretarugsa and Wallace 1997; Yang et al. 2003) no ha sido demostrada.

En el modelo propuesto, el 17,20β-P producido en las células de la granulosa asociadas a los oocitos primarios en respuesta a la activación del Fshra se une posteriormente al Pgr nuclear de los oocitos, activa el receptor y promueve la transcripción del gen *aqp1ab* a través de interacciones independientes con dos elementos *cis*-reguladores PRE localizados en el promotor proximal del gen *aqp1ab* (Fig. 1). Tanto el PRE(a) como el PRE(b) son igualmente efectivos transactivando el gen *aqp1ab* en células somáticas heterólogas y células germinales, y por tanto no hay explicación para la existencia de dos PREs redundantes en el promotor *aqp1ab*. Es posible especular que la presión selectiva ha favorecido la retención de múltiples PREs para una activación eficiente de productos génicos como la Aqp1ab, con el fin de conseguir suficientes niveles de proteína en los oocitos al final del periodo de crecimiento y así asegurar la rápida captación de agua durante la reanudación de la meiosis. En cualquier caso, la interacción del Sox3 o -8b con un sitio de unión SOX(c) proximal estimula de forma sinérgica la actividad transcripcional del gen *aqp1ab* mediada por el Pgr. Sin embargo, los sitios SOX(a) y/o SOX(b) distales pueden disminuir este efecto sinérgico. Por el contrario, el Sox9b reprime la transcripción del gen *aqp1ab* a través del mismo sitio SOX(c). Por tanto, bajos niveles de expresión del Sox9b en los oocitos en crecimiento primario pueden permitir la síntesis de ARNm y proteína de la Aqp1ab mediada por Pgr, mientras que elevados niveles de Sox9b en oocitos más desarrollados pueden inhibir la transcripción. Además de los elementos PRE y SOX, el análisis *in silico* del promotor de la aqp1ab de dorada ha revelado la presencia de secuencias consenso para la unión de factores de transcripción que pueden ser de relevancia durante el desarrollo del oocito, como el TCF/LEF-1 implicado en la vía de señalización de Wnt (Harwood et al. 2008; Langdon and Mullins 2011), CREB (Sundaram et al. 2003) o PBX-HOX (Villaescusa et al., 2004). Por lo tanto, es posible que algunos de estos factores de transcripción participen junto con el Pgr y el Sox en la regulación de la transcripción del gen *aqp1ab* en oocitos de orada. De hecho, la exposición de explantes de ovario primario de dorada a AMPc induce un aumento de los niveles de ARNm de la *aqp1ab* (datos no mostrados). Asimismo, hemos demostrado en este trabajo que los ovarios en crecimiento primario de dorada expresan hasta cuatro isoformas distintas del Pgr nuclear, dos de las cuales pueden ejercer mecanismos dominante negativos sobre la transcripción de *aqp1ab* mediada por Pgr. Seria

interesante, por tanto, elucidar en futuros estudios la localización celular específica de estas isoformas inhibidoras del Pgr en los folículos primarios de dorada, así como su posible papel en la regulación de la transcripción del gen *aqp1ab*.

El modelo que proponemos puede contribuir a aclarar aspectos importantes de la regulación endocrina del gen *aqp1ab* en un teleósteo moderno acantomorfo que produce huevos pelágicos, que es la estrategia reproductiva más frecuentemente desarrollada por los teleósteos marinos (Finn and Kristoffersen, 2007). Sin embargo, una búsqueda extensiva en los genomas disponibles de teleósteos revela que los elementos *cis*-reguladores PRE y SOX también están presentes en las regiones flanqueantes 5' de los genes *aqp1ab* de distintas especies independientemente de su estrategia reproductiva (producción de huevos pelágicos hidratados o bentónicos no hidratados). Esto se observa, por ejemplo, en el pez cebra, el cual produce huevos bentónicos no hidratados y en el que los elementos reguladores Pgr y Sox se encuentran en la región genómica proximal 5' del gen *aqp1ab*. Como en la dorada, en el pez cebra, el Pgr nuclear se localiza en el núcleo de los oocitos en crecimiento primario (Hanna et al. 2010), y por tanto, en estos oocitos se podría esperar una activación del gen *aqp1ab*. Sin embargo, aparentemente este mecanismo no ocurre ya que en el ovario del pez cebra los transcritos de *aqp1ab* son difícilmente detectables por RT-PCR (Tingaud-Sequeira et al. 2008, 2010). Para investigar el mecanismo subyacente, se han realizado experimentos preliminares usando peces cebra transgénicos transitorios para la proteína verde fluorescente (EGFP) bajo el control del promotor del gen *aqp1ab* de dorada. Los resultados muestran que cuando la construcción *aqp1ab*-EGFP es microinyectada en los embriones en estadio de 1-2 células, el promotor *aqp1ab* de 1.672-kb es capaz de dirigir la expresión de EGFP de forma específica en el recto de las hembras a los 35-40 dpf. Este resultado es consistente con la expresión de aqp1ab en la membrana apical de los enterocitos rectales de dorada (Raldúa et al. 2008). Sin embargo, la expresión de EGFP no se detecta en los oocitos. Cabe destacar, al contrario que en la dorada, que los oocitos en crecimiento primario y alveolo cortical de pez cebra aparentemente expresan elevados niveles de *sox9b* (Rodríguez-Marí et al. 2005), el cual hemos visto es capaz de reprimir la actividad del promotor *aqp1ab*. En base a estas observaciones, y de acuerdo con nuestro modelo, se puede especular que las proteínas Sox9b o otros factores del oocito de pez cebra podrían inhibir la activación del promotor *aqp1ab* de dorada mediada por Pgr, lo cual puede ocurrir en otros teleósteos en los que el gen *aqp1ab* no se requiere para la producción de huevos viables. Sin embargo, debido a que mutaciones en las secuencias reguladoras SOX del promotor aqp1ab no se han ensayado en estos experimentos preliminares, esta hipótesis

necesita confirmarse en el futuro, así como la existencia de mecanismos adicionales para la regulación transcripcional del gen *aqp1ab* en el ovario de teleósteos que producen huevos bentónicos no hidratados.

4. Regulación Posttraduccional del Tráfico Intracelular de la Aqp1ab

En la presente tesis hemos demostrado que a pesar del alto nivel de sustitución aminoacídica que existe entre los ortólogos de la Aqp1ab en teleósteos, su papel durante la hidratación del oocito aparece conservado en las especies marinas que producen huevos pelágicos. Sin embargo, la Aqp1ab del fletán Atlántico parece ser una acuaporina inusualmente especializada ya que esta proteína no se transporta a la membrana plasmática del oocito a menos que sea expresada en oocitos nativos o de otros peces. Este hallazgo es sorprendente dado que, según nuestro conocimiento, los ortólogos de la AQP11 y/o -12 son las únicas acuaporinas de vertebrados que no son transportadas a la membrana plasmática cuando se expresan en oocitos de *X. laevis* (Itoh et al. 2005; Ohta et al. 2009; Tingaud-Sequeira et al. 2010). Este efecto se ha explicado por la localización intracelular de estas acuaporinas en los tejidos nativos, como en las células acinares pancreáticas (Itoh et al. 2005; Ohta et al. 2009). La disfunción *ex vivo* de la Aqp1ab del fletán en oocitos de *X. laevis* no puede solucionarse con la exposición de los oocitos a moléculas de transducción de señal como el AMPc o el GMPc (datos no mostrados), las cuales es conocido están implicadas en el control del tráfico intracelular de la Aqp1ab del bagre así como de las AQP2 y AQP-h2 de tetrápodos (Hasegawa et al. 2003; Nedvetsky et al. 2009; Chaube et al. 2011). Mientras que estos resultados sugieren que la fosforilación podría no tener un papel relevante en el transporte de la Aqp1ab del fletán a la membrana plasmática, no se puede descartar completamente su función durante el tráfico intracelular debido a la naturaleza altamente divergente de los sitios potenciales de fosforilación en los C-terminales de las Aqp1ab de teleósteos. En efecto, en dorada, se ha demostrado que la fosforilación de la Ser^{254} puede mediar el reciclaje de la Aqp1ab en oocitos de *X. laevis* (Tingaud-Sequeira et al. 2008). Sin embargo, hemos visto que si los oocitos de *X. laevis* se co-inyectan con el ARNc de la Aqp1ab del fletán con ARNm $poliA^+$ extraído de folículos posvitelogénicos de fletán, es posible rescatar *ex vivo* el tráfico de la Aqp1ab a la membrana plasmática del oocito. Estas observaciones sugieren que la Aqp1ab y los oocitos del fletán han evolucionado conjuntamente mecanismos específicos de linaje para el transporte intracelular de la Aqp1ab durante la maduración meiótica. Esta hipótesis se apoya en la observación de que la Aqp1ab de fletán es

completamente funcional cuando se expresa en oocitos de un teleósteo ostariofisio lejanamente relacionado como es el pez cebra.

La naturaleza de los mecanismos derivados del ARNm poliA$^+$ que supuestamente controlan la localización de la Aqp1ab del fletán en la membrana plasmática del oocito no han sido identificados de momento. Sin embargo, hemos obtenido datos que revelan que el C-terminal de la Aqp1ab probablemente juega un papel relevante, como indican los experimentos funcionales usando las Aqp1ab y -1aa de fletán intactas y construcciones quiméricas en oocitos de *X. laevis*. Esta observación refuerza nuestra hipótesis de que el C-terminal de la Aqp1ab está implicado en la rápida neofuncionalización de esta acuaporina en teleósteos, como se ha sugerido previamente (Tingaud-Sequeira et al. 2008; Cerdà 2009), si bien la función específica de este dominio en el mecanismo de transporte de la Aqp1ab del fletán sigue siendo desconocido. Es conocido que la fosforilación de resíduos específicos, las interacciones del C-terminal con componentes del citoesqueleto, vesículas de transporte y reguladores lisosomales de tráfico (Kamsteeg et al. 2007; Nedvetsky et al. 2009; Moeller et al. 2010), así como el correcto plegamiento de las proteínas en el retículo endoplasmático (ER) (van Balkom et al. 2002; Pitonzo and Skach 2006), pueden controlar el transporte intracelular de las acuaporinas. En el presente estudio, los experimentos de Western blot bajo condiciones reductoras o no reductoras de extractos de membrana total de oocitos de *X. laevis* que expresan la Aqp1ab del fletán no han puesto en evidencia la existencia de modificaciones postraduccionales de la Aqp1ab como resultado de la co-expresión con el ARNm poliA$^+$ de oocitos de fletán. Esto sugiere que esta acuaporina se procesa correctamente y no se queda retenida en el ER incluso en ausencia del ARNm poliA$^+$. En estas condiciones, como la retención en el ER, las acuaporinas están normalmente glicosiladas (Deen et al. 1995), y nuestros datos indican que la Aqp1ab del fletán disfuncional en oocitos de *X. laevis* no se encuentra glicosilada. Sin embargo, la translocación de la Aqp1ab del fletán a la membrana plasmática del oocito *in vivo* parece estar asociada con la formación de un complejo de ~66-kDa, tanto en oocitos nativos en hidratación como en oocitos de *X. laevis* que co-expresan la Aqp1ab y el ARNm poliA$^+$. Estos complejos podrían ser oligomeros de Aqp1ab que no se disocian incluso bajo fuertes condiciones de desnaturalización o reducción, de un modo similar a lo que se ha descrito para la AqpZ de *Escherichia coli* (Borgnia et al. 1999) y para algunas acuaporinas de plantas (Ohshima et al. 2001; Casado-Vela et al. 2010). Por lo tanto, queda por averiguar si la maquinaria para la formación de estos complejos está sólo presente en los oocitos de teleósteos, y/o si el mecanismo

de transporte de la Aqp1ab en oocitos es específico de teleósteos y está ausente en los oocitos de anfibios y vertebrados superiores.

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CONCLUSIONES

1. Los genes funcionales parálogos *aqp1* de teleósteos (*aqp1aa* y *aqp1ab*) son muy probablemente duplicados génicos en tándem donde el producto de la duplicación completa del genoma (*aqp1b*) se ha perdido en los Acantopterigios. El C terminal de la proteína Aqp1ab es el subdominio que ha evolucionado más rápidamente dentro de la superfamilia de las acuaporinas de vertebrados, lo que sugiere que esta acuaporina se ha neofuncionalizado.

2. La inhibición inmunológica específica de la Aqp1ab en los oocitos del fletán Atlántico (*Hippoglossus hippoglossus*), y el posterior rescate de la función de la acuaporina mediante la sobreexpresión del parálogo Aqp1aa, proporciona una evidencia experimental directa de la función esencial de la Aqp1ab durante la hidratación del oocito. Estos resultados confirman conclusiones previas realizadas en la dorada dorada (*Sparus aurata*), y sugieren que la función fisiológica de la Aqp1ab facilitando el transporte de agua al oocito durante la reanudación de la meiosis se conserva en teleósteos marinos que producen huevos pelágicos (flotantes).

3. La rápida divergencia del dominio C terminal de la Aqp1ab del fletán resulta en la pérdida de la función *ex vivo* de la Aqp1ab en oocitos de anfibios, la cual puede ser rescatada mediante inyección de ARN poliA$^+$ extraído de folículos ováricos nativos post-vitelogénicos. Estos resultados revelan la naturaleza dual de la neofuncionalización de los canales de agua Aqp1ab de teleósteos, donde la presión de selección ha favorecido su función en la hidratación de los oocitos pero se ha relajado con respecto a los mecanismos celulares específicos de control del transporte de la acuaporina.

4. En la dorada, hay una breve fase temporal de transcripción del gen *aqp1ab* en oocitos en la etapa de crecimiento primario (perinucleolar), en los cuales la meiosis está detenida, y los ARNm resultantes se traducen inmediatamente antes de su reducción al inicio de la vitelogénesis. La reserva de proteínas Aqp1ab producida *de novo* puede ser modificada después de la traducción, y los péptidos transportados y almacenados en el ooplasma periférico durante las etapas subsiguientes de desarrollo ovocitario.

5. El aislamiento de la región 5' flanqueante del gen *aqp1ab* de la dorada revela la presencia de dos secuencias *cis*-reguladoras de respuesta a progestinas (PRE), las cuales pueden

mediar la unión al receptor nuclear de progestinas (Pgr), así como tres sitios potenciales de unión para miembros de la familia de factores de transcripción SOX (SRY-related HMG box).

6. Los órdenes superiores de teleósteos retienen un único gen *pgr*. Sin embargo, en ovarios en crecimiento primario de la dorada se expresan cuatro variantes diferentes del ARNm de *pgr*, tres de ellos derivados del empalme alternativo del *pgr* pre-ARNm produciendo tres receptores con el N terminal truncado en diferente extensión. El Pgr completo de la dorada muestra la capacidad de transactivación más elevada en respuesta a progestinas nativas de euteleósteos, tales como la 17α,20β-dihidroxi-4-pregnen-3-ona (17,20β-P), mientras que dos de las isoformas del Pgr regulan de modo independiente nuevos mecanismos nucleares y citosólicos de represión dominante negativa de la transcripción mediada por el Pgr. Esto sugiere que las funciones fisiológicas mediadas por el Pgr en el ovario de teleósteos evolutivamente avanzados pueden ser reguladas a través de la expresión diferencial de distintas isoformas del Pgr.

7. Durante la oogénesis temprana en la dorada, el Pgr y tres factores de transcripción Sox diferentes se expresan de forma distinta. El Pgr, así como los ARNm de *sox3* y *-8b*, se expresan en las oogonias, mientras que en los oocitos en crecimiento primario el Pgr es completamente traslocado desde el ooplasma al núcleo. Por el contrario, el ARNm de *sox9b* se expresa de forma marcada sólo en oocitos más avanzados (en estado de alveolo cortical y vitelogénesis), coincidiendo con una fuerte disminución del ARNm de *aqp1ab* en el oocito.

8. Los ensayos de transactivación en células de mamífero y oocitos de *Xenopus laevis*, utilizando el promotor del gen *aqp1ab* tanto intacto como mutado, ha demostrado que la transcripción de *aqp1ab* depende del Pgr, con el Sox3 y -8b actuando sinérgicamente, mientras que el Sox9b ejerce un efecto represor de la transcripción. Por lo tanto, la mayor expresión de *sox9b* en oocitos de dorada en estado de alveolo cortical sería consistente con la fuerte reducción de la transcripción de *aqp1ab in vivo*.

9. El ligando del Pgr, 17,20β-P, se produce *in vivo* e *in vitro* por las células de la granulosa asociadas a los oocitos en crecimiento primario en respuesta a la activación del receptor de la hormona estimulante del folículo (Fshra). El Fshra estimula la vía esteroideogénica mediada por el citocromo P450c17-II/20β-hidroxiesteroide deshidrogenasa carbonil re-

ductasa para la síntesis de progestinas, así como la producción del Pgr nuclear. Posteriormente, el 17,20β-P activa el Pgr de los oocitos, y promueve la transcripción y la traducción del gen *aqp1ab* a través de la interacción con las dos secuencias PRE *cis*-reguladoras situadas en el promotor proximal del gen *aqp1ab*.

10. Nuestros datos han descubierto por tanto el preciso mecanismo endocrino y molecular para la regulación transcripcional del gen *aqp1ab* durante la oogénesis de la dorada, en el cual el Pgr es probablemente el principal regulador siendo su efecto modulado por factores de transcripción Sox.

ANNEXES

Annex 1: Accession numbers of sequences used for the phylogenetic analyses of *pgr* genes in Chapter III. Data are arranged according to Annex 2

Accession #	Ortholog isoform	Animal	Species	Class/Rank	Order	Family
NR3C1						
ENSP00000231509	GR	Human	*Homo sapiens*	Mammalia	Primates	Hominidae
ENSCAFP00000009468	GR	Dog	*Canis lupus familiaris*	Mammalia	Carnivora	Canidae
ENSECAP00000007606	GR	Horse	*Equus caballus*	Mammalia	Perissodactyla	Equidae
ENSLAFP00000004197	GR	African savanna elephant	*Loxodonta africana*	Mammalia	Proboscidea	Elephantidae
ENSMUSP00000111231	GR	Mouse	*Mus musculus*	Mammalia	Rodentia	Muridae
ENSSHAP00000001942	GR	Tasmanian devil	*Sarcophilus harrisii*	Mammalia	Dasyuromorphia	Dasyuridae
ENSMODP00000012934	GR	Gray short-tailed opossum	*Monodelphis domestica*	Mammalia	Didelphimorphia	Didelphidae
ENSOANP00000009152	GR	Platypus	*Ornithorhynchus anatinus*	Mammalia	Monotremata	Ornithorhynchidae
ENSGALP00000011948	GR	Chicken	*Gallus gallus*	Aves	Galliformes	Phasianidae
ENSMGAP00000009653	GR	Turkey	*Meleagris gallopavo*	Aves	Galliformes	Phasianidae
ENSAPLP00000006970	GR	Duck	*Anas platyrhynchos*	Aves	Anseriformes	Anatidae
ENSTGUP00000000147	GR	Zebra finch	*Taeniopygia guttata*	Aves	Passeriformes	Estrildidae
ENSACAP00000013512	GR	Green anole	*Anolis carolinensis*	Lepidosauria	Squamata	Iguanidae
AFJ50181	GR	Eastern diamonback rattlesnake	*Crotalus adamanteus*	Lepidosauria	Squamata	Viperidae
ENSPSIP00000017259	GR	Chines softshell turtle	*Pelodiscus sinensis*	Sauropsida	Testudines	Trionychidae
X72211	GR	African clawed frog	*Xenopus laevis*	Amphibia	Anura	Pipidae
ENSXETP00000003968	GR	Western clawed frog	*Xenopus (Silurana) tropicalis*	Amphibia	Anura	Pipidae
AEA10368	GR	Wood frog	*Rana sylvatica*	Amphibia	Anura	Pipidae
BAJ61740	GR	Japanes firebelly newt	*Cynops pyrrhogaster*	Amphibia	Caudata	Salamandridae
AFYH01071167	Gr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01071171	Gr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01071176	Gr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01071177	Gr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
ENSLACP00000017638	Gr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
ENSTRUP00000015645	Gra-S	Torafugu	*Takifugu rubripes*	Acanthomorpha	Tetraodontiformes	Tetraodontidae
ENSTRUP00000015645	Gra-L	Torafugu	*Takifugu rubripes*	Acanthomorpha	Tetraodontiformes	Tetraodontidae
ENSTNIP00000021375	Gra-L	Green-spotted pufferfish	*Tetraodon nigroviridis*	Acanthomorpha	Tetraodontiformes	Tetraodontidae
BAJ76679	Gra-L	Senegalese sole	*Solea senegalensis*	Acanthomorpha	Pleuronectiformes	Soleidae
BAA25997	Gra-L	Japanese flounder	*Paralichthys olivaceus*	Acanthomorpha	Pleuronectiformes	Paralichthyidae
AEX56588	Gra-L	European flounder	*Platichthys flesus*	Acanthomorpha	Pleuronectiformes	Pleuronectidae
HM010952	Gra-L	Red drum	*Sciaenops ocellatus*	Acanthomorpha	Perciformes	Sciaenidae
AY549305	Gra-L	European seabass	*Dicentrarchus labrax*	Acanthomorpha	Perciformes	Moronidae
ACF75335	Gra-L	Yellow perch	*Perca fluviatilis*	Acanthomorpha	Perciformes	Percidae
AF263739	Gra-S	Burton's mouth-brooder	*Astatotilapia burtoni*	Acanthomorpha	Perciformes	Cichlidae
AF263740	Gra-S	Burton's mouth-brooder	*Astatotilapia burtoni*	Acanthomorpha	Perciformes	Cichlidae
ABS11150	Gra	Daffodil Cichlid	*Neolamprologus pulcher*	Acanthomorpha	Perciformes	Cichlidae
ENSONIP00000010662	Gra	Nile tilapia	*Oreochromis niloticus*	Acanthomorpha	Perciformes	Cichlidae
ABK59939	Gra	Plainfin midshipman	*Porichthys notatus*	Acanthomorpha	Batrachoidiformes	Batrachoididae
AEA77170	Gra	Gulf toadfish	*Opsanus beta*	Acanthomorpha	Batrachoidiformes	Batrachoididae
ENSGACP00000027401	Gra-S	Three-spined stickleback	*Gasterosteus aculeatus*	Acanthomorpha	Gasterosteiformes	Gasterosteidae
ENSGACP00000027400	Gra-L	Three-spined stickleback	*Gasterosteus aculeatus*	Acanthomorpha	Gasterosteiformes	Gasterosteidae
ENSORLP00000001939	Gra-L	Medaka	*Oryzias latipes*	Acanthomorpha	Beloniformes	Adrianichthyidae
ADZ24979	Gra-L	Indian ricefish	*Oryzias dancena*	Acanthomorpha	Beloniformes	Adrianichthyidae
ENSGMOP00000019143	Gra-L	Atlantic cod	*Gadus morhua*	Acanthomorpha	Gadiformes	Gadidae
CAA90937	Gra-L	Rainbow trout	*Oncorhynchus mykiss*	Protacanthopterygii	Salmoniformes	Salmonidae
ACF75917	Gra-L	Marble trout	*Salmo marmoratus*	Protacanthopterygii	Salmoniformes	Salmonidae
ACS91455	Gra-S	Atlantic salmon	*Salmo salar*	Protacanthopterygii	Salmoniformes	Salmonidae
AY863149	Gra-L	Brown trout	*Salmo trutta*	Protacanthopterygii	Salmoniformes	Salmonidae
AAW56453	Gra-L	Atlantic salmon	*Salmo salar*	Protacanthopterygii	Salmoniformes	Salmonidae
ABQ53595	Gra	Cherry salmon	*Oncorhynchus masou*	Protacanthopterygii	Salmoniformes	Salmonidae
CAI51316	Gra-L	Common carp	*Cyprinus carpio*	Ostariophysi	Cypriniformes	Cyprinidae
ADT91059	Gra	Goldfish	*Carassius auratus*	Ostariophysi	Cypriniformes	Cyprinidae
BAH70337	Gra-L	Japanese eel	*Anguilla japonica*	Elopomorpha	Anguilliformes	Anguillidae
AFK14018	Gra-L	Freshwater butterflyfish	*Pantodon buchholzi*	Osteoglossomorpha	Osteoglossiformes	Pantodontidae
ENSTRUP00000018415	Grb	Torafugu	*Takifugu rubripes*	Acanthomorpha	Tetraodontiformes	Tetraodontidae
ENSTNIP00000011800	Grb	Green-spotted pufferfish	*Tetraodon nigroviridis*	Acanthomorpha	Tetraodontiformes	Tetraodontidae
AAX18925	Grb	Black porgy	*Acanthopagrus schlegelii*	Acanthomorpha	Perciformes	Sparidae
ABF30967	Grb	Gilthead seabream	*Sparus aurata*	Acanthomorpha	Perciformes	Sparidae

AY619996	Grb	European seabass	*Dicentrarchus labrax*	Acanthomorpha	Perciformes	Moronidae
ADE06402	Grb	Cunner	*Tautogolabrus adspersus*	Acanthomorpha	Perciformes	Labridae
ENSGACP00000024074	Grb	Three-spined stickleback	*Gasterosteus aculeatus*	Acanthomorpha	Gasterosteiformes	Gasterosteidae
ABS11149	Grb	Daffodil Cichlid	*Neolamprologus pulcher*	Acanthomorpha	Perciformes	Cichlidae
AF263738	Grb	Burton's mouth-brooder	*Astatotilapia burtoni*	Acanthomorpha	Perciformes	Cichlidae
ADC90323	Grb	Mozambique tilapia	*Oreochromis mossambicus*	Acanthomorpha	Perciformes	Cichlidae
ENSONIP00000022570	Grb	Nile tilapia	*Oreochromis niloticus*	Acanthomorpha	Perciformes	Cichlidae
ENSORLP00000007570	Grb	Medaka	*Oryzias latipes*	Acanthomorpha	Beloniformes	Adrianichthyidae
ADZ24980	Grb	Indian ricefish	*Oryzias dancena*	Acanthomorpha	Beloniformes	Adrianichthyidae
ADX07108	Grb	Pejerrey	*Odontesthes bonariensis*	Acanthomorpha	Atheriniformes	Atherinopsidae
BAK20182	Grb	Clown anemonefish	*Amphiprion ocellaris*	Acanthomorpha	Perciformes	Pomacentridae
ACH68603	Grb	Tongue sole	*Cynoglossus semilaevis*	Acanthomorpha	Pleuronectiformes	Cynoglossidae
BAJ76678	Grb	Senegalese sole	*Solea senegalensis*	Acanthomorpha	Pleuronectiformes	Soleidae
CAI30312	Grb	European flounder	*Platichthys flesus*	Acanthomorpha	Pleuronectiformes	Pleuronectidae
ENSGMOP00000006025	Grb	Atlantic cod	*Gadus morhua*	Acanthomorpha	Gadiformes	Gadidae
AY495372	Grb	Rainbow trout	*Oncorhynchus mykiss*	Protacanthopterygii	Salmoniformes	Salmonidae
AY533141	Grb	Fathead minnow	*Pimephales promelas*	Ostariophysi	Cypriniformes	Cyprinidae
CAI51316	Grb	Common carp	*Cyprinus carpio*	Ostariophysi	Cypriniformes	Cyprinidae
ENSDARP00000054263	Grb1	Zebrafish	*Danio rerio*	Ostariophysi	Cypriniformes	Cyprinidae
AFK14016	Grb-S	Freshwater butterflyfish	*Pantodon buchholzi*	Osteoglossomorpha	Osteoglossiformes	Pantodontidae
AFK14017	Grb-L	Freshwater butterflyfish	*Pantodon buchholzi*	Osteoglossomorpha	Osteoglossiformes	Pantodontidae
AHAT01021403	Gr	Spotted gar	*Lepisosteus oculatus*	Neopterygii	Semionotiformes	Lepisosteidae
AHAT01021404	Gr	Spotted gar	*Lepisosteus oculatus*	Neopterygii	Semionotiformes	Lepisosteidae
AFK14015	Gr	Sterlet	*Acipenser ruthenus*	Chondrostei	Acipenseriformes	Acipenseridae
AEF12278	Gr	Atlantic stingray	*Dasyatis sabina*	Chondrichthyes	Myliobatiformes	Dasyatidae
ABD46744	Gr	Little skate	*Leucoraja erinacea*	Chondrichthyes	Rajiformes	Rajidae
AEF12276	Gr	Brownbanded bambooshark	*Chiloscyllium punctatum*	Chondrichthyes	Orectolobiformes	Hemiscylliidae
AEF12275	Gr	Atlantic sharpnose shark	*Rhizoprionodon terraenovae*	Chondrichthyes	Carcharhiniformes	Carcharhinidae
AEF12277	Gr	Smaller spotted catshark	*Scyliorhinus canicula*	Chondrichthyes	Carcharhiniformes	Scyliorhinidae
AAVX01046756	Gr	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae
NR3C2						
ENSP00000341390	MR	Human	*Homo sapiens*	Mammalia	Primates	Hominidae
ENSECAP00000014151	MR	Horse	*Equus caballus*	Mammalia	Perissodactyla	Equidae
ENSMUSP00000034031	MR	Mouse	*Mus musculus*	Mammalia	Rodentia	Muridae
ENSCAFP00000011580	MR	Dog	*Canis lupus familiaris*	Mammalia	Carnivora	Canidae
ENSLAFP00000017164	MR	African savanna elephant	*Loxodonta africana*	Mammalia	Proboscidea	Elephantidae
AEFK01215506	MR	Tasmanian devil	*Sarcophilus harrisii*	Mammalia	Dasyuromorphia	Dasyuridae
AEFK01215508	MR	Tasmanian devil	*Sarcophilus harrisii*	Mammalia	Dasyuromorphia	Dasyuridae
AEFK01215509	MR	Tasmanian devil	*Sarcophilus harrisii*	Mammalia	Dasyuromorphia	Dasyuridae
ENSSHAP00000006561	MR	Tasmanian devil	*Sarcophilus harrisii*	Mammalia	Dasyuromorphia	Dasyuridae
ENSMODP00000000987	MR	Gray short-tailed opossum	*Monodelphis domestica*	Mammalia	Didelphimorphia	Didelphidae
ENSOANP00000008376	MR	Platypus	*Ornithorhynchus anatinus*	Mammalia	Monotremata	Ornithorhynchidae
ENSACAP00000003608	MR	Green anole	*Anolis carolinensis*	Lepidosauria	Squamata	Iguanidae
ENSPSIP00000007055	MR	Chines softshell turtle	*Pelodiscus sinensis*	Sauropsida	Testudines	Trionychidae
ENSGALP00000016283	MR	Chicken	*Gallus gallus*	Aves	Galliformes	Phasianidae
ENSMGAP00000009653	MR	Turkey	*Meleagris gallopavo*	Aves	Galliformes	Phasianidae
ENSAPLP00000006970	MR	Duck	*Anas platyrhynchos*	Aves	Anseriformes	Anatidae
BAL45929	MR	Budgerigar	*Melopsittacus undulatus*	Aves	Psittaciformes	Psittacidae
ADC79403	MR	House sparrpw	*Passer domesticus*	Aves	Passeriformes	Passeridae
BAK54012	MR	Bengalese finch	*Lonchura striata domestica*	Aves	Passeriformes	Estrildidae
ENSTGUP00000000147	MR	Zebra finch	*Taeniopygia guttata*	Aves	Passeriformes	Estrildidae
BC081082	MR	African clawed frog	*Xenopus laevis*	Amphibia	Anura	Pipidae
ENSXETP00000040897	MR	Western clawed frog	*Xenopus (Silurana) tropicalis*	Amphibia	Anura	Pipidae
AFYH01136255	Mr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01136256	Mr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01136259	Mr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01136261	Mr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01136266	Mr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01136290	Mr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
ENSLACP00000015400	Mr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
ENSTRUP00000037997	Mr	Torafugu	*Takifugu rubripes*	Acanthomorpha	Tetraodontiformes	Tetraodontidae
ENSTNIP00000020882	Mr	Green-spotted pufferfish	*Tetraodon nigroviridis*	Acanthomorpha	Tetraodontiformes	Tetraodontidae
ADF36551	Mr	Orange-spotted grouper	*Epinephelus coioides*	Acanthomorpha	Perciformes	Serranidae
ACR45947	Mr	European perch	*Perca fluviatilis*	Acanthomorpha	Perciformes	Percidae

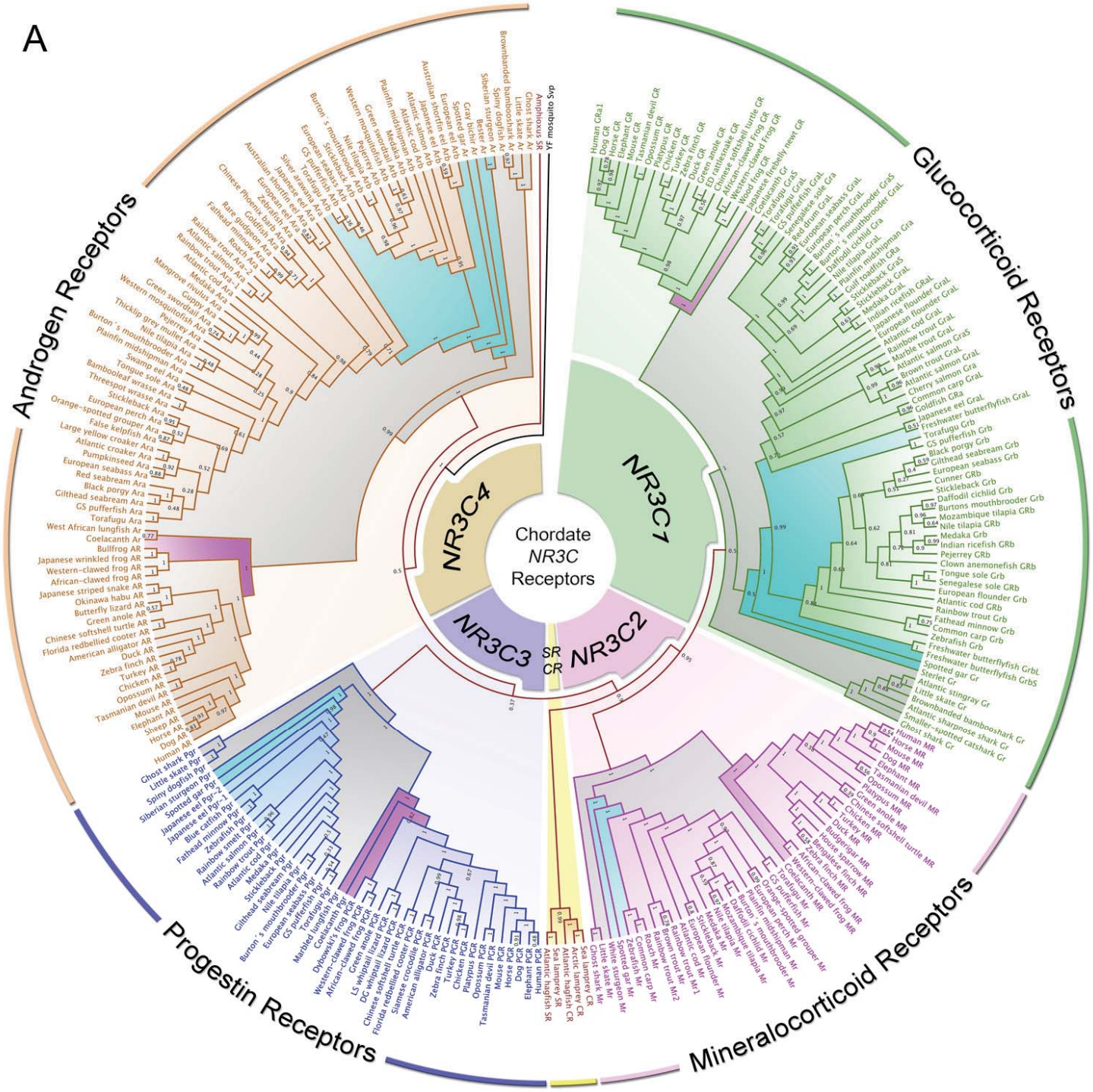
ADC45510	Mr	Plainfin midshipman	*Porichthys notatus*	Acanthomorpha	Batrachoidiformes	Batrachoididae
AF263741	Mr	Burton's mouth-brooder	*Astatotilapia burtoni*	Acanthomorpha	Perciformes	Cichlidae
ABS11148	Mr	Daffodil Cichlid	*Neolamprologus pulcher*	Acanthomorpha	Perciformes	Cichlidae
ADL27418	Mr	Mozambique tilapia	*Oreochromis mossambicus*	Acanthomorpha	Perciformes	Cichlidae
ENSONIP00000012611	Mr	Nile tilapia	*Oreochromis niloticus*	Acanthomorpha	Perciformes	Cichlidae
ENSORLP00000009438	Mr	Medaka	*Oryzias latipes*	Acanthomorpha	Beloniformes	Adrianichthyidae
ENSGACP00000022715	Mr	Three-spined stickleback	*Gasterosteus aculeatus*	Acanthomorpha	Gasterosteiformes	Gasterosteidae
AEX56587	Mr	European flounder	*Platichthys flesus*	Acanthomorpha	Pleuronectiformes	Pleuronectidae
ENSGMOP00000002015	Mr	Atlantic cod	*Gadus morhua*	Acanthomorpha	Gadiformes	Gadidae
NM_001124740	Mr1	Rainbow trout	*Oncorhynchus mykiss*	Protacanthopterygii	Salmoniformes	Salmonidae
AY863149	Mr	Brown trout	*Salmo trutta*	Protacanthopterygii	Salmoniformes	Salmonidae
NM_001124483	Mr2	Rainbow trout	*Oncorhynchus mykiss*	Protacanthopterygii	Salmoniformes	Salmonidae
ACS34897	Mr	Roach	*Rutilus rutilus*	Ostariophysi	Cypriniformes	Cyprinidae
CAH03995	Mr	Common carp	*Cyprinus carpio*	Ostariophysi	Cypriniformes	Cyprinidae
ENSDARP00000053817	Mr	Zebrafish	*Danio rerio*	Ostariophysi	Cypriniformes	Cyprinidae
AFK14018	Mr	Freshwater butterflyfish	*Pantodon buchholzi*	Osteoglossomorpha	Osteoglossiformes	Pantodontidae
AHAT01015409	Mr	Spotted gar	*Lepisosteus oculatus*	Neopterygii	Semionotiformes	Lepisosteidae
AHAT01015410	Mr	Spotted gar	*Lepisosteus oculatus*	Neopterygii	Semionotiformes	Lepisosteidae
AHAT01015411	Mr	Spotted gar	*Lepisosteus oculatus*	Neopterygii	Semionotiformes	Lepisosteidae
AAX18245	Mr	White sturgeon	*Acipenser transmontanus*	Chondrostei	Acipenseriformes	Acipenseridae
AEF12278	Mr	Atlantic stingray	*Dasyatis sabina*	Chondrichthyes	Myliobatiformes	Dasyatidae
ABD46745	Mr	Little skate	*Leucoraja erinacea*	Chondrichthyes	Rajiformes	Rajidae
AAVX01002585	Mr	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae
AAVX01059793	Mr	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae
AAVX01103515	Mr	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae
AAVX01310810	Mr	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae
AAVX01569250	Mr	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae
CR						
AAK20930	CR	Sea lamprey	*Petromyzon marinus*	Hyperoartia	Petromyzontiformes	Petromyzontidae
DC618249	CR	Arctic lamprey	*Lethenteron camtschaticum*	Hyperoartia	Petromyzontiformes	Petromyzontidae
ABD46742	CR	Atlantic hagfish	*Myxine glutinosa*	Hyperotreti	Myxiniformes	Myxinidae
SR						
AAK20931	SR	Sea lamprey	*Petromyzon marinus*	Hyperoartia	Petromyzontiformes	Petromyzontidae
ABD46743	SR	Atlantic hagfish	*Myxine glutinosa*	Hyperotreti	Myxiniformes	Myxinidae
ACB10649	SR	Amphioxus	*Branchiostoma floridae*	Chordata	Cephalochordata	Branchiostomidae
NR3C3						
ENSP00000325120	PGR	Human	*Homo sapiens*	Mammalia	Primates	Hominidae
ENSLAFP00000005438	PGR	African savanna elephant	*Loxodonta africana*	Mammalia	Proboscidea	Elephantidae
ENSCAFP00000005906	PGR	Dog	*Canis lupus familiaris*	Mammalia	Carnivora	Canidae
ENSECAP00000009675	PGR	Horse	*Equus caballus*	Mammalia	Perissodactyla	Equidae
ENSMUSP00000096584	PGR	Mouse	*Mus musculus*	Mammalia	Rodentia	Muridae
ENSSHAP00000019635	PGR	Tasmanian devil	*Sarcophilus harrisii*	Mammalia	Dasyuromorphia	Dasyuridae
ENSMODP00000000609	PGR	Gray short-tailed opossum	*Monodelphis domestica*	Mammalia	Didelphimorphia	Didelphidae
ENSOANP00000016631	PGR	Platypus	*Ornithorhynchus anatinus*	Mammalia	Monotremata	Ornithorhynchidae
ENSGALP00000027736	PGR	Chicken	*Gallus gallus*	Aves	Galliformes	Phasianidae
ENSMGAP00000016105	PGR	Turkey	*Meleagris gallopavo*	Aves	Galliformes	Phasianidae
ENSTGUP00000013165	PGR	Zebra finch	*Taeniopygia guttata*	Aves	Passeriformes	Estrildidae
ENSAPLP00000003406	PGR	Duck	*Anas platyrhynchos*	Aves	Anseriformes	Anatidae
BAD08350	PGR	American alligator	*Alligator mississippiensis*	Archosauria	Crocodylia	Alligatoridae
AAB81722	PGR	Siamese crocodile	*Crocodylus siamensis*	Archosauria	Crocodylia	Crocodylidae
BAF91193	PGR	Florida redbellied cooter	*Pseudemys nelsoni*	Sauropsida	Testudines	Emydidae
ENSPSIP00000015478	PGR	Chines softshell turtle	*Pelodiscus sinensis*	Sauropsida	Testudines	Trionychidae
AAB35740	PGR	Desert-grassland whiptail lizard	*Aspidoscelis uniparens*	Lepidosauria	Squamata	Teiidae
ACJ45777	PGR	Little-striped whiptail lizard	*Aspidoscelis inornata*	Lepidosauria	Squamata	Iguanidae
ENSACAP00000003608	PGR	Green anole	*Anolis carolinensis*	Lepidosauria	Squamata	Iguanidae
AAG42362	PGR	African clawed frog	*Xenopus laevis*	Amphibia	Anura	Pipidae
ENSXETP00000012055	PGR	Western clawed frog	*Xenopus (Silurana) tropicalis*	Amphibia	Anura	Pipidae
AAN63590	PGR	Dybowski's frog	*Rana dybowskii*	Amphibia	Anura	Ranidae
AFYH01098700	Pgr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01098703	Pgr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01098705	Pgr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01098708	Pgr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
ENSLACP00000014940	Pgr	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae

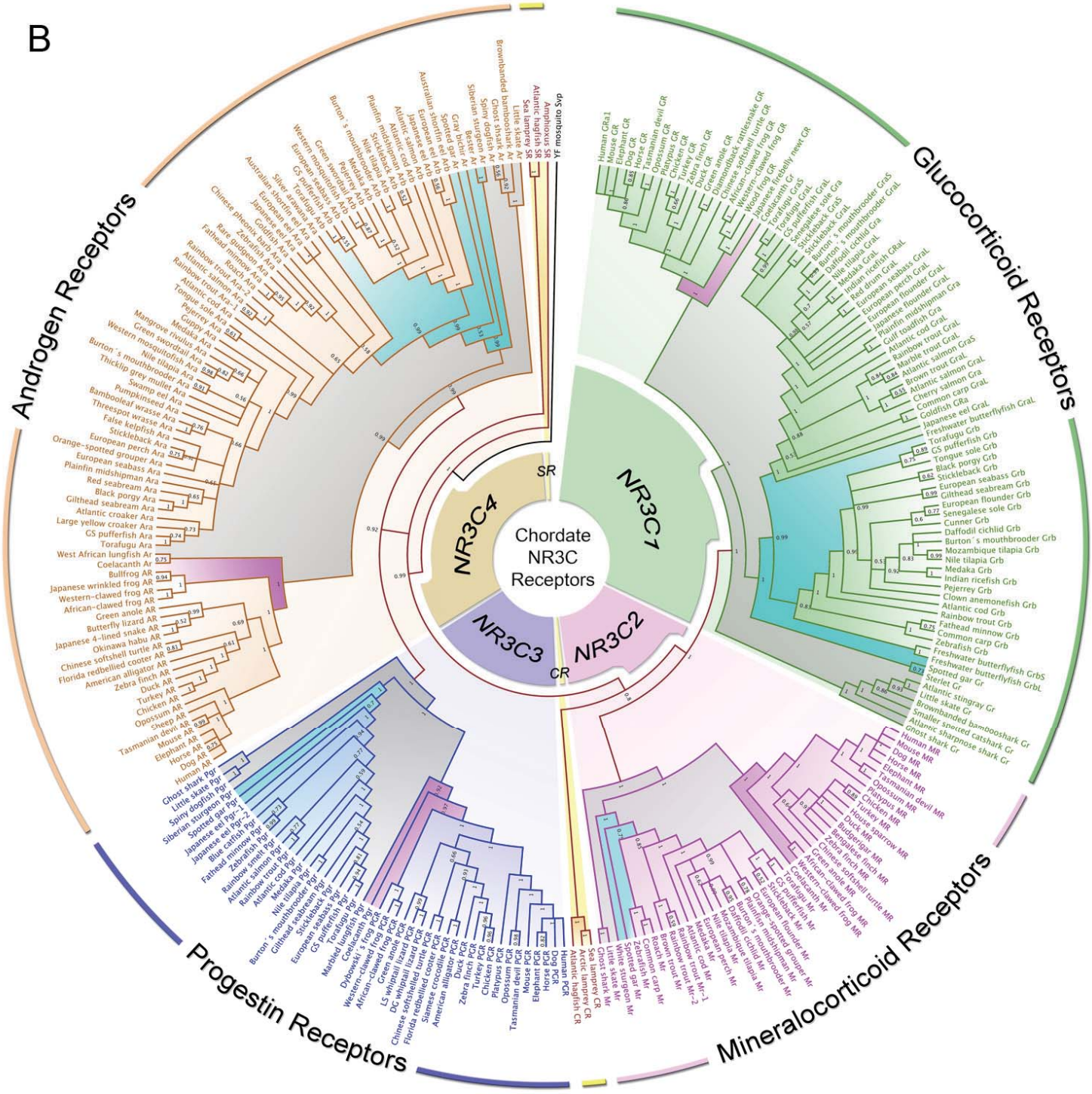
FL668670	Pgr	Marbled lungfish	*Protopterus aethiopicus*	Dipnoi	Lepidosireniformes	Protopteridae
ENSTRUP00000033391	Pgr	Torafugu	*Takifugu rubripes*	Acanthomorpha	Tetraodontiformes	Tetraodontidae
ENSTNIP00000002483	Pgr	Green-spotted pufferfish	*Tetraodon nigroviridis*	Acanthomorpha	Tetraodontiformes	Tetraodontidae
CABK01005626	Pgr	European seabass	*Dicentrarchus labrax*	Acanthomorpha	Perciformes	Moronidae
CABK01022339	Pgr	European seabass	*Dicentrarchus labrax*	Acanthomorpha	Perciformes	Moronidae
ACM51148	Pgr	Burton's mouth-brooder	*Astatotilapia burtoni*	Acanthomorpha	Perciformes	Cichlidae
PREONIP00000115346	Pgr	Nile tilapia	*Oreochromis niloticus*	Acanthomorpha	Perciformes	Cichlidae
JQ692980	Pgr	Gilthead seabream	*Sparus aurata*	Acanthomorpha	Perciformes	Sparidae
ENSGACP00000016086	Pgr	Three-spined stickleback	*Gasterosteus aculeatus*	Acanthomorpha	Gasterosteiformes	Gasterosteidae
NP_001165515	Pgr	Medaka	*Oryzias latipes*	Acanthomorpha	Beloniformes	Adrianichthyidae
ACF21816	Pgr	Atlantic cod	*Gadus morhua*	Acanthomorpha	Gadiformes	Gadidae
CX256633	Pgr	Rainbow trout	*Oncorhynchus mykiss*	Protacanthopterygii	Salmoniformes	Salmonidae
ADK94875	Pgr	Atlantic salmon	*Salmo salar*	Protacanthopterygii	Salmoniformes	Salmonidae
EL533504	Pgr	Rainbow smelt	*Osmerus mordax*	Protacanthopterygii	Osmeriformes	Osmeridae
ENSDARP00000052205	Pgr	Zebrafish	*Danio rerio*	Ostariophysi	Cypriniformes	Cyprinidae
AFM74474	Pgr	Fathead minnow	*Pimephales promelas*	Ostariophysi	Cypriniformes	Cyprinidae
FD142692	Pgr	Blue catfish	*Ictalurus furcatus*	Ostariophysi	Siluriformes	Ictaluridae
BAA89539	Pgr-1	Japanese eel	*Anguilla japonica*	Elopomorpha	Anguilliformes	Anguillidae
BAB85993	Pgr-2	Japanese eel	*Anguilla japonica*	Elopomorpha	Anguilliformes	Anguillidae
AHAT01003092	Pgr	Spotted gar	*Lepisosteus oculatus*	Neopterygii	Semionotiformes	Lepisosteidae
ABF50787	Pgr	Siberian sturgeon	*Acipenser baerii*	Chondrostei	Acipenseriformes	Acipenseridae
ABB53420	Pgr	Spiny dogfish	*Squalus acanthias*	Chondrichthyes	Squaliformes	Squalidae
ABD46747	Pgr	Little skate	*Leucoraja erinacea*	Chondrichthyes	Rajiformes	Rajidae
AAVX01000542	Pgr	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae
AAVX01052307	Pgr	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae
AAVX01062614	Pgr	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae
AAVX01239858	Pgr	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae
AAVX01248136	Pgr	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae
AAVX01278682	Pgr	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae
NR3C4						
ENSP00000363822	AR	Human	*Homo sapiens*	Mammalia	Primates	Hominidae
ENSCAFP00000024499	AR	Dog	*Canis lupus familiaris*	Mammalia	Carnivora	Canidae
ENSECAP00000003261	AR	Horse	*Equus caballus*	Mammalia	Perissodactyla	Equidae
ENSECAP00000008070	AR	Horse	*Equus caballus*	Mammalia	Perissodactyla	Equidae
AAC97958	AR	Sheep	*Ovis aries*	Mammalia	Ruminantia	Bovidae
ENSLAFP00000020342	AR	African savanna elephant	*Loxodonta africana*	Mammalia	Proboscidea	Elephantidae
ENSMUSP00000052648	AR	Mouse	*Mus musculus*	Mammalia	Rodentia	Muridae
ENSSHAP00000019607	AR	Tasmanian devil	*Sarcophilus harrisii*	Mammalia	Dasyuromorphia	Dasyuridae
ENSMODP00000015542	AR	Gray short-tailed opossum	*Monodelphis domestica*	Mammalia	Didelphimorphia	Didelphidae
ENSGALP00000007301	AR	Chicken	*Gallus gallus*	Aves	Galliformes	Phasianidae
ENSMGAP00000011364	AR	Turkey	*Meleagris gallopavo*	Aves	Galliformes	Phasianidae
ENSTGUP00000002888	AR	Zebra finch	*Taeniopygia guttata*	Aves	Passeriformes	Estrildidae
ENSAPLP00000006185	AR	Duck	*Anas platyrhynchos*	Aves	Anseriformes	Anatidae
BAE95686	AR	American alligator	*Alligator mississippiensis*	Archosauria	Crocodylia	Alligatoridae
BAF91192	AR	Florida redbellied cooter	*Pseudemys nelsoni*	Sauropsida	Testudines	Emydidae
ENSPSIP00000011283	AR	Chines softshell turtle	*Pelodiscus sinensis*	Sauropsida	Testudines	Trionychidae
ENSACAP00000009410	AR	Green anole	*Anolis carolinensis*	Lepidosauria	Squamata	Iguanidae
BAI50383	AR	Butterfly lizard	*Leiolepis reevesii rubritaeniata*	Lepidosauria	Squamata	Agamidae
BAJ15431	AR	Okinawa habu	*Trimeresurus flavoviridis*	Lepidosauria	Squamata	Viperidae
BAJ15432	AR	Japanese four-lined ratsnake	*Elaphe quadrivirgata*	Lepidosauria	Squamata	Colubridae
AAI70349	AR	African clawed frog	*Xenopus laevis*	Amphibia	Anura	Pipidae
ENSXETP00000011091	AR	Western clawed frog	*Xenopus (Silurana) tropicalis*	Amphibia	Anura	Pipidae
BAJ41476	AR	Japanese wrinkled frog	*Glandirana rugosa*	Amphibia	Anura	Ranidae
AAP85538	AR	Bullfrog	*Rana catesbeiana*	Amphibia	Anura	Ranidae
AFYH01029665	Ar	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01029666	Ar	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01029667	Ar	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01029669	Ar	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
AFYH01029671	Ar	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
ENSLACP00000017054	Ar	Coelacanth	*Latimeria chalumnae*	Coelacanthimorpha	Coelacanthiformes	Coelacanthidae
ABF50783	Ar	West African lungfish	*Protopterus annectens annectens*	Dipnoi	Lepidosireniformes	Protopteridae
ENSTRUP00000031457	Ara	Torafugu	*Takifugu rubripes*	Acanthomorpha	Tetraodontiformes	Tetraodontidae
ENSTNIP00000014780	Ara	Green-spotted pufferfish	*Tetraodon nigroviridis*	Acanthomorpha	Tetraodontiformes	Tetraodontidae
AEO13404	Ara	Gilthead seabream	*Sparus aurata*	Acanthomorpha	Perciformes	Sparidae

AAO61694	Ara	Black porgy	*Acanthopagrus schlegelii*	Acanthomorpha	Perciformes	Sparidae
BAA33451	Ara	Red seabream	*Pagrus major*	Acanthomorpha	Perciformes	Sparidae
AAT76433	Ara	European seabass	*Dicentrarchus labrax*	Acanthomorpha	Perciformes	Moronidae
ABF50766	Ara	Pumpkinseed	*Lepomis gibbosus*	Acanthomorpha	Perciformes	Centrarchidae
AAU09477	Ara	Atlantic croaker	*Micropogonias undulatus*	Acanthomorpha	Perciformes	Sciaenidae
ADD39720	Ara	large yellow croaker	*Larimichthys crocea*	Acanthomorpha	Perciformes	Sciaenidae
ACH78366	Ara	False kelpfish	*Sebastiscus marmoratus*	Acanthomorpha	Scorpaeniformes	Sebastinae
ADQ43815	Ara	Orange-spotted grouper	*Epinephelus coioides*	Acanthomorpha	Perciformes	Serranidae
ABF50764	Ara	European perch	*Perca fluviatilis*	Acanthomorpha	Perciformes	Percidae
ENSGACP00000026868	Ara	Three-spined stickleback	*Gasterosteus aculeatus*	Acanthomorpha	Gasterosteiformes	Gasterosteidae
AAG48340	Ara	Threespot wrasse	*Halichoeres trimaculatus*	Acanthomorpha	Perciformes	Labridae
ADI24924	Ara	Siebold´s bambooleaf wrasse	*Pseudolabrus sieboldi*	Acanthomorpha	Perciformes	Labridae
ACH68602	Ara	Tongue sole	*Cynoglossus semilaevis*	Acanthomorpha	Pleuronectiformes	Cynoglossidae
ACO40458	Ara	Swamp eel	*Monopterus albus*	Acanthomorpha	Synbranchiformes	Synbranchidae
AAL92878	Ara	Burton's mouth-brooder	*Astatotilapia burtoni*	Acanthomorpha	Perciformes	Cichlidae
ENSONIP00000016176	Ara	Nile tilapia	*Oreochromis niloticus*	Acanthomorpha	Perciformes	Cichlidae
AEI16508	Ara	Thicklip grey mullet	*Chelon labrosus*	Acanthomorpha	Mugiliformes	Mugilidae
ADR30384	Ara	Pejerrey	*Odontesthes bonariensis*	Acanthomorpha	Atheriniformes	Atherinopsidae
BAD81046	Ara	Western mosquitofish	*Gambusia affinis*	Acanthomorpha	Cyprinodontiformes	Poeciliidae
ACS50393	Ara	Green swordtail	*Xiphophorus hellerii*	Acanthomorpha	Cyprinodontiformes	Poeciliidae
ACL79841	Ara	Guppy	*Poecilia reticulata*	Acanthomorpha	Cyprinodontiformes	Poeciliidae
ABC68612	Ara	Mangrove rivulus	*Kryptolebias marmoratus*	Acanthomorpha	Cyprinodontiformes	Rivulidae
ENSORLP00000011941	Ara	Medaka	*Oryzias latipes*	Acanthomorpha	Beloniformes	Adrianichthyidae
AAZ14095	Ara	Plainfin midshipman	*Porichthys notatus*	Acanthomorpha	Batrachoidiformes	Batrachoididae
ENSGMOP00000010954	Ara	Atlantic cod	*Gadus morhua*	Acanthomorpha	Gadiformes	Gadidae
BAA32785	Ara1	Rainbow trout	*Oncorhynchus mykiss*	Protacanthopterygii	Salmoniformes	Salmonidae
AAL29928	Ara	Atlantic salmon	*Salmo salar*	Protacanthopterygii	Salmoniformes	Salmonidae
BAA32784	Ara2	Rainbow trout	*Oncorhynchus mykiss*	Protacanthopterygii	Salmoniformes	Salmonidae
ADD52201	Ara	Roach	*Rutilus rutilus*	Ostariophysi	Cypriniformes	Cyprinidae
AAF88138	Ara	Fathead minnow	*Pimephales promelas*	Ostariophysi	Cypriniformes	Cyprinidae
ADC35724	Ara	Rare gudgeon	*Gobiocypris rarus*	Ostariophysi	Cypriniformes	Cyprinidae
AAM09278	Ara	Goldfish	*Carassius auratus*	Ostariophysi	Cypriniformes	Cyprinidae
ACA96518	Ara	Chinese Phoenix barb	*Spinibarbus denticulatus*	Ostariophysi	Cypriniformes	Cyprinidae
ENSDARP00000088795	Ara	Zebrafish	*Danio rerio*	Ostariophysi	Cypriniformes	Cyprinidae
CBV44425	Ara	European eel	*Anguilla japonica*	Elopomorpha	Anguilliformes	Anguillidae
ADK47209	Ara	Australian shortfin eel	*Anguilla australis*	Elopomorpha	Anguilliformes	Anguillidae
BAA83805	Ara	Japanese eel	*Anguilla japonica*	Elopomorpha	Anguilliformes	Anguillidae
BAI58986	Ara	Silver arawana	*Osteoglossum bicirrhosum*	Osteoglossomorpha	Osteoglossiformes	Osteoglossidae
ENSTRUP00000012893	Arb	Torafugu	*Takifugu rubripes*	Acanthomorpha	Tetraodontiformes	Tetraodontidae
ENSTNIP00000018864	Arb	Green-spotted pufferfish	*Tetraodon nigroviridis*	Acanthomorpha	Tetraodontiformes	Tetraodontidae
ABF50776	Arb	European seabass	*Dicentrarchus labrax*	Acanthomorpha	Perciformes	Moronidae
ENSGACP00000024489	Arb	Three-spined stickleback	*Gasterosteus aculeatus*	Acanthomorpha	Gasterosteiformes	Gasterosteidae
AAD25074	Arb	Burton's mouth-brooder	*Astatotilapia burtoni*	Acanthomorpha	Perciformes	Cichlidae
ENSONIP00000022113	Arb	Nile tilapia	*Oreochromis niloticus*	Acanthomorpha	Perciformes	Cichlidae
ADR30383	Arb	Pejerrey	*Odontesthes bonariensis*	Acanthomorpha	Atheriniformes	Atherinopsidae
BAD81045	Arb	Western mosquitofish	*Gambusia affinis*	Acanthomorpha	Cyprinodontiformes	Poeciliidae
ACS50392	Arb	Green swordtail	*Xiphophorus hellerii*	Acanthomorpha	Cyprinodontiformes	Poeciliidae
ENSORLP00000010323	Arb	Medaka	*Oryzias latipes*	Acanthomorpha	Beloniformes	Adrianichthyidae
AFM22699	Arb	Plainfin midshipman	*Porichthys notatus*	Acanthomorpha	Batrachoidiformes	Batrachoididae
ENSGMOP00000004335	Arb	Atlantic cod	*Gadus morhua*	Acanthomorpha	Gadiformes	Gadidae
AGKD01034183	Arb	Atlantic salmon	*Salmo salar*	Protacanthopterygii	Salmoniformes	Salmonidae
AGKD01152160	Arb	Atlantic salmon	*Salmo salar*	Protacanthopterygii	Salmoniformes	Salmonidae
AGKD01161467	Arb	Atlantic salmon	*Salmo salar*	Protacanthopterygii	Salmoniformes	Salmonidae
BAA75464	Arb	Japanese eel	*Anguilla japonica*	Elopomorpha	Anguilliformes	Anguillidae
ADK47208	Arb	Australian shortfin eel	*Anguilla australis*	Elopomorpha	Anguilliformes	Anguillidae
CBV44424	Arb	European eel	*Anguilla japonica*	Elopomorpha	Anguilliformes	Anguillidae
AHAT01015635	Ar	Spotted gar	*Lepisosteus oculatus*	Neopterygii	Semionotiformes	Lepisosteidae
BAI58985	Ar	Gray bichir	*Polypterus senegalus*	Actinopterygii	Polypteriformes	Polypteridae
BAI49424	Ar	Bester	*Acipenser ruthenus x Huso huso*	Chondrostei	Acipenseriformes	Acipenseridae
ABF50786	Ar	Siberian sturgeon	*Acipenser baerii*	Chondrostei	Acipenseriformes	Acipenseridae
AAP55843	Ar	Spiny dogfish	*Squalus acanthias*	Chondrichthyes	Squaliformes	Squalidae
BAI49423	Ar	Brownbanded bambooshark	*Chiloscyllium punctatum*	Chondrichthyes	Orectolobiformes	Hemiscylliidae
ABW79801	Ar	Little skate	*Leucoraja erinacea*	Chondrichthyes	Rajiformes	Rajidae
AAVX01046756	Ar	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae

AAVX01455731	Ar	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae
AAVX01503296	Ar	Ghost shark	*Callorhinchus milii*	Chondrichthyes	Chimaeriformes	Callorhinchidae

Annex 2: Annotated Bayesian majority rule consensus trees of (A) the chordate *nr3c* codon alignment and (B) Nr3c amino acid alignment of the DBD, hinge and LBD domains. The trees are rooted using yellow fever mosquito sevenup (Svp) as the outgroup. Both data sets resolve the Nr3c nuclear receptor subfamily into the four major clades: Nr3c1 (glucocorticoid receptors), Nr3c2 (mineralocorticoid receptors), Nr3c3 (progestin receptors) and Nr3c4 (androgen receptors), in which Teleostei retain duplicate glucocorticoid and androgen receptors, but single mineralocorticoid and progestin receptors. Grey shading indicates the position of ancestral chondrichthyan receptors, blue shading the position of ancestral actinopterygian receptors, and purple shading the position of ancestral sarcopterygian receptors. Bayesian posterior probabilities derived from 10 million mcmc generations and a burnin of 3500 are shown at each node. Accession numbers are given in Annex 1.


A
Androgen Receptors
Glucocorticoid Receptors
Progestin Receptors
Mineralocorticoid Receptors
Chordate
NR3C
Receptors
NR3C1
NR3C2
NR3C3
NR3C4
SR
CR


B
Androgen Receptors
Glucocorticoid Receptors
Progestin Receptors
Mineralocorticoid Receptors
Chordate
NR3C
Receptors
NR3C4
NR3C1
NR3C3
NR3C2
SR
CR
Vf mosquito Grp
Amphioxus SR
Atlantic hagfish SR
Sea lamprey SR
Little skate Ar
Spiny dogfish Ar
Ghost shark Ar
Brownbanded bamboosharkAr
Siberian sturgeon Ar
Gray bichir Ar
Spotted gar Ar
European eel Arb
Japanese eel Arb
Atlantic salmon Arb
Atlantic cod Arb
Australian shortfin eel Arb
Plainfin midshipman Arb
Stickleback Arb
Nile tilapia Arb
Medaka Arb
Burton´s mouthbrooder Arb
Pejerrey Arb
Western mosquitofish Arb
Green swordtail Arb
European seabass Arb
GS pufferfish Arb
European eel Ara
Japanese eel Ara
Australian shortfin eel Ara
Silver arowana Ara
Chinese phoenix barb Ara
Zebrafish Ara
Rare gudgeon Ara
Roach Ara
Fathead minnow Ara
Rainbow trout Ara-2
Atlantic salmon Ara
Rainbow trout Ara-1
Atlantic cod Ara
Tongue sole Ara
Pejerrey Ara
Guppy Ara
Medaka Ara
Mangrove rivulus Ara
Green swordtail Ara
Western mosquitofish Ara
Nile tilapia Ara
Burton´s mouthbrooder Ara
Thicklip grey mullet Ara
Swamp eel Ara
Pumpkinseed Ara
Bamblooleaf wrasse Ara
Threespot wrasse Ara
False kelpfish Ara
Stickleback Ara
European perch Ara
Orange-spotted grouper Ara
European seabass Ara
Plainfin midshipman Ara
Red seabream Ara
Black porgy Ara
Gilthead seabream Ara
Atlantic croaker Ara
Large yellow croaker Ara
GS pufferfish Ara
Torafugu Ara
West African lungfish Ar
Coelacanth Ar
Bullfrog AR
Japanese wrinkled frog AR
Western-clawed frog AR
African-clawed frog AR
Green anole AR
Butterfly lizard AR
Japanese 4-lined snake AR
Okinawa habu AR
Chinese softshell turtle AR
Florida redbellied cooter AR
American alligator AR
Zebra finch AR
Duck AR
Turkey AR
Chicken AR
Opossum AR
Sheep AR
Tasmanian devil AR
Mouse AR
Elephant AR
Horse AR
Dog AR
Human AR
Ghost shark Pgr
Little skate Pgr
Spiny dogfish Pgr
Siberian sturgeon Pgr
Spotted gar Pgr
Japanese eel Pgr-2
Japanese eel Pgr
Blue catfish Pgr
Zebrafish Pgr
Fathead minnow Pgr
Rainbow smelt Pgr
Zebrafish Pgr
Atlantic salmon Pgr
Rainbow trout Pgr
Atlantic cod Pgr
Medaka Pgr
Nile tilapia Pgr
Burton´s mouthbrooder Pgr
Gilthead seabream Pgr
Stickleback Pgr
European seabass Pgr
GS pufferfish Pgr
Torafugu Pgr
Coelacanth Pgr
Dybowski´s frog PCR
Western-clawed frog PCR
African-clawed frog PCR
Green anole PCR
LS whiptail lizard PCR
DC whiptail lizard PCR
Chinese softshelled turtle PCR
Florida redbellied cooter PCR
Siamese crocodile PCR
American alligator PCR
Duck PCR
Zebra finch PCR
Turkey PCR
Chicken PCR
Platypus PCR
Opossum PCR
Tasmanian devil PCR
Mouse PCR
Elephant PCR
Horse PCR
Dog PCR
Human PCR
Atlantic hagfish CR
Arctic lamprey CR
Sea lamprey CR
Little skate Mr
Ghost shark Mr
White sturgeon Mr
Spotted gar Mr
Zebrafish Mr
Common carp Mr
Roach Mr
Brown trout Mr
Rainbow trout Mr-2
Rainbow trout Mr-1
Atlantic cod Mr
Medaka Mr
European perch Mr
Nile tilapia Mr
Mozambique tilapia Mr
Daffodil cichlid Mr
Burton´s mouthbrooder Mr
Plainfin midshipman Mr
Orange-spotted grouper Mr
European flounder Mr
Stickleback Mr
GS pufferfish Mr
Torafugu Mr
Coelacanth Mr
Western-clawed frog MR
African-clawed frog MR
Green anole MR
Zebra finch MR
Budgerigar MR
Bengalese finch MR
House sparrow MR
Turkey MR
Chicken MR
Platypus MR
Opossum MR
Tasmanian devil MR
Elephant MR
Horse MR
Dog MR
Mouse MR
Human MR
Ghost shark Gr
Smaller sharpnose shark Gr
Atlantic sharpnose shark Gr
Brownbanded bamboosharkGr
Little skate Gr
Atlantic stingray Gr
Sterlet Gr
Spotted gar Gr
Freshwater butterflyfish GrbL
Freshwater butterflyfish GrbS
Fathead minnow Grb
Common carp Grb
Zebrafish Grb
Rainbow trout Grb
Atlantic cod Grb
Clown anemonefish Grb
Pejerrey Grb
Indian ricefish Grb
Medaka Grb
Nile tilapia Grb
Mozambique tilapia Grb
Burton´s mouthbrooder Grb
Cunner Grb
Senegalese sole Grb
European flounder Grb
Gilthead seabream Grb
European seabass Grb
Stickleback Grb
Black porgy Grb
Tongue sole Grb
GS pufferfish Grb
Torafugu Grb
Freshwater butterflyfish GraL
Japanese eel GraL
Goldfish GRa
Cherry salmon GraL
Common carp GraL
Atlantic salmon GraL
Brown trout GraL
Atlantic salmon GraS
Rainbow trout GraL
Marble trout GraL
Atlantic cod GraL
Gulf toadfish Gra
Plainfin midshipman Gra
Japanese flounder GraL
European flounder GraL
European perch GraL
European seabass GraL
Indian ricefish GRaL
Medaka GraL
Nile tilapia GraL
Daffodil cichlid GraL
Burton´s mouthbrooder GraS
Burton´s mouthbrooder GraL
Senegalese sole Gra
Stickleback GraS
Stickleback GraL
GS pufferfish GraL
Torafugu GraS
Coelacanth Gr
Japanese fireblly newt GR
Wood frog GR
Western-clawed frog GR
African-clawed frog GR
Chinese softshell turtle GR
Diamondback ratlesnake GR
Green anole GR
Zebra finch GR
Duck GR
Chicken GR
Turkey GR
Opossum GR
Platypus GR
Tasmanian devil GR
Horse GR
Dog GR
Elephant GR
Mouse GR
Human GRa1