Resolving the complexity of vitellogenins and their receptors in the tetraploid Atlantic salmon (*Salmo salar*) - Ancient origin of the phosvitinless VtgC in chondrichthyean fishes

Øivind Andersen[1,2*], Chunxia Xu[2], Gerrit Timmerhaus[1], Katrine Hånes Kirste[1], Ingun Næve[3], Maren Mommens[3], Helge Tveiten[4]

[1]Nofima AS, P.O. Box 5010, NO-1435 Ås, Norway

[2]Department of Animal and Aquaculture Sciences, Norwegian University of Life Sciences, Norway

[3]Aqua Gen AS, Trondheim, Norway

[4]Nofima AS, NO-9291Tromsø, Norway

Corresponding author

Øivind Andersen
Nofima AS, P.O. Box 5010,
Osloveien 1, NO-1435 Ås, Norway
E-mail: oivind.andersen@nofima.no
Tel: +4793060248

Abbreviations: Vtg; vitellogenin, VtgR; vitellogenin receptor, LLTP; large lipid transfer protein, LvH; heavy lipoprotein, LvL; light lipoprotein, VLDLR; very low density lipoprotein receptor; LDLR; low density lipoprotein receptor, LR8; eight ligand binding repeats, Lrp13; LDLR-related protein 13, ERE; estrogen responsive element, GSI, gonado-somatic index.

Grant sponsor: The Research Council of Norway, grant number: 221648/030.

Running title: Salmon vitellogenins and receptors

Abstract

Egg yolk proteins are mainly derived from vitellogenin (Vtg) serving as essential nutrients during early development in oviparous organisms. Vertebrate Vtgs are predominantly synthesized in the liver of the maturing females and are internalized by binding to specific oocyte receptors (VtgR). Here we clarify the evolutionary history of the vertebrate Vtgs, including the teleost VtgC lacking phosvitin, and investigate the repertoire of Vtgs and VtgRs in the tetraploid Atlantic salmon. Conserved synteny of the *vtg* genes in elephant fish (*Callorhinchus milii*) strongly indicates that the *vtg* gene cluster was present in the ancestor of tetrapods and ray-finned fish. The shortened phosvitin in the VtgC ortholog of this chondrichthyean fish may represent the result of early truncation events that eventually allowed the total disappearance of phosvitin in teleost VtgC. In contrast, the tandem duplicated VtgCs identified in spotted gar (*Lepisosteus oculatus*) both contain the phosvitin domain. The Atlantic salmon genome harbors four *vtg* genes encoding the complete VtgAsa1, phosvitinless VtgC and truncated VtgAsb proteins, while *vtgAsa2* is a pseudogene. The three *vtg* genes were mainly expressed in the liver of the maturing females, and the *vtgAsa1* transcript predominated prior to spawning. The ovarian expression of *vtgr1* and *vtgr2* was dominated by the splice variant lacking the *O*-linked sugar domain. The strongly increased *vtgAsa1* expression during vitellogenesis contrasted with the peak levels of *vtgr1* and *vtgr2* in the previtellogenic oocytes that gradually decreased. Recycling of the oocyte VtgRs is probably not sufficient to maintain the receptor number during vitellogenesis.

Key words: Vitellogenesis, *Salmo salar*, phosvitin, VtgC, elephant fish

Introduction

In oviparous species the maternal supply of vitellogenins (Vtg) are the main source of egg yolk nutrients during early development. Vertebrate Vtgs are preferentially synthesized in the liver and are transported *via* the blood to the growing oocytes to be selectively internalized by receptor-mediated endocytosis (Opresko and Wiley, 1987; Dierks-ventling, 1978; Mouchel et al., 1996; Prat et al., 1998; Dominguez et al., 2012). The Vtg phosholipoproteins are members of the Large Lipid Transfer Protein (LLTP) superfamily, and the three Vtg forms synthesized by jawed vertebrates (Gnathostomes) are processed into the heavy and light lipovitellins (LvH, LvL) and phosvitin in the developing oocyte (Hiramatsu et al., 2002; Amano et al., 2007a; Finn and Kristoffersen, 2007; Finn, 2007; Reading et al., 2009; Yilmaz et al., 2016). The Vtgs of spiny-rayed fish (Acanthomorphs) are made up of the VtgAa and VtgAb forms and the shorter VtgC variant, which lacks the phosvitin domain and the C-terminal β' and CT domains (Hiramatsu et al., 2002; Sawaguchi et al., 2005, 2006; Amano et al. 2007b). Although yolk proteins are mainly used as nutrients for the developing embryo, some marine teleosts cleave VtgAa before spawning to generate a pool of free amino acids used to aid in oocyte hydration of buoyant pelagic eggs (Greeley et al., 1986; Fyhn et al., 1999; Finn et al., 2002). The function of the serine-rich phosvitin is unclear, but this highly phosphorylated domain might be involved in carrying calcium and phosphate required for embryonic bone formation (Wahli, 1988; Hiramatsu et al., 2006). Phylogenetic analyses of vertebrate Vtgs suggested that the phosvitinless VtgC is a neo-functional product of the second whole-genome duplication (Finn and Kristoffersen, 2007; Prowse and Byrne, 2012). The presence of phosvitin in the Vtgs of invertebrates such as the mosquito, agnathan fishes, and the Indonesian coelacanth (*Latimeria menadoensis*) (Sharrock et al., 1992; Chen et al., 1997; Canapa et al. 2012; Nishimiya et al., 2014) indicates that the domain was lost after the divergence of ray-finned fishes (Actinopterygians) and lobe-finned fishes (Sarcopterygians).

The vertebrate *vtg* genes are co-localized in a conserved syntenic region in both teleost fish and oviparous tetrapods suggesting that the *vtg* cluster was already present in the last common ancestor about 450 million years ago (Babin, 2008; Finn et al., 2009; Braasch and Salzburger, 2009). The teleost-specific, or third, whole-genome duplication event was followed by the loss of multiple paralogs that possibly included one of the duplicated *vtg* clusters, while lineage-specific tandem duplications have increased the repertoire of Vtgs (Wang et al., 2000; Babin, 2008; Finn et al., 2009). Similarly, paralog loss and tandem gene duplications in the tetraploid salmonid genome resulted in substantial variation in the number of the salmonid *vtgAsa* and *vtgAsb* genes (Trichet et al., 2000; Buisine et al., 2002). The genus *Oncorhynchus* exhibits a tandem array of highly similar *vtgAsa* genes, while *vtgAsb* has probably been lost in rainbow trout. In contrast, a truncated VtgAsb has been reported in various salmonids, while *vtgAsa* was suggested to be a pseudogene in Atlantic salmon (Trichet et al., 2000; Buisine et al., 2002). However, expression of a *vtgAsa* form was induced in estrogen-stimulated males of Atlantic salmon (Yadetie et al., 1999). The salmonid eggs are among the largest shed by any broadcast spawning teleost, and the multiplicity of salmonid Vtgs was suggested to compensate for the large amounts of yolk (Finn and Kristoffersen, 2007). Substantial amounts of Vtg are synthesized in the liver of the maturing females, which possess plasma Vtg levels above 35 mg/ml during vitellogenesis, and accumulated Vtg comprised about 17 % of the ovary in a landlocked strain of Atlantic salmon (So et al., 1985; King and Pankhurst, 2003).

Whereas a single oocyte receptor seems to mediate uptake of Vtgs in tetrapods, multiple Vtg receptor (VtgRs) have been reported in teleosts (Stifano et al. 1990a,b; Hiramatsu et al., 2015). Six discrete ovarian proteins binding Vtgs were detected by ligand labelling in cutthroat trout (*Oncorhynchus clarkii*), while white perch (*Morone americana*) and rainbow trout (*O. mykiss*) were found to exhibit four VtgRs (Tyler and Lubberink, 1996; Reading et al., 2011, 2014;

Mushirobira et al., 2015). The very low density lipoprotein receptor (VLDLR) is characterized by eight ligand binding repeats (LR8) and is coded by a single *vldlr*, or *vtgr*, gene in oviparous vertebrates, except for the two distinct *vtgr* genes reported in parallel studies of rainbow trout (Prat et al., 1998; Davail et al., 1998). Two splice variants of VtgR are widely distributed in teleosts and tetrapods, and the ovarian form lacking the *O*-linked sugar domain is probably responsible for the Vtg uptake (Bujo et al., 1995; Okabayashi et al., 1996; Prat et al., 1998; Mizuta et al., 2013). In addition to the classical LR8 type, the LDLR-related protein 13 named Lrp13 has been shown to bind the cutthroat trout VtgAs and the VtgAa in perch and tilapia, and Lrp13 has been implicated as an important mediator of yolk deposition in other oviparous vertebrates (Reading et al., 2011; Hiramatsu et al., 2015; Mushirobira et al., 2015). In this study we addressed some of the remaining issues pertaining to the evolutionary history of the Vtgs and VtgRs in vertebrates and the origin and possible ancestral role of the phosvitinless VtgC. Further, we elucidated the hepatic synthesis and ovarian uptake of Vtg in Atlantic salmon by quantifying the expression of the *vtg* and *vtgr* genes in maturing females during the annual reproductive cycle.

Results

Conserved synteny of fish *vtgs* and *vtgrs*

The tetraploid Atlantic salmon genome was found to contain four *vtg* genes named *vtgAsa1*, *vtgAsa2*, *vtgAsb* and *vtgC*, which are positioned on the homeologous chromosomes Ssa10 and Ssa23 (Figure 1). Salmon *vtgAsb* is flanked by the *ctbs* and *ssx2ip* genes, while *vtgC* and the duplicated *vtgAsa1* and *vtgAsa2* are neighbor genes of *adgrl2* and *adgrl4*, respectively. Similarly, the latter genes are positioned adjacent to the *vtgC* orthologs in the other species examined, and *ctbs* and *ssx2ip* are flanking the tandem repeated *vtgABs* or *vtgAa* and *vtgAb*. Conserved synteny of the *vtg* genes was also found in the African coelacanth (*Latimeria*

chalumnae), but the *vtgC* ortholog and the *vtgABII-vtgABIII* duplicates are mapped to two unassembled scaffolds. The spotted gar (*Lepisosteus oculatus*) genome harbors two tandem repeated *vtgC* genes named *vtgC1* and *vtgC2*, which both contain the single exon encoding the serine repeats of the phosvitin domain. Intriguingly, the *vtgC* identified in the holocephalian elephant fish (*Callorhinchus milii*) codes for a Vtg possessing the shortened phosvitin sequence SSDSSSASSSSQESS (pos. 1116-1130). This apparently represents a truncation of this hypervariable region, which have been further modified in teleosts, resulting in the complete loss of phosvitin together with the C-terminal domains.

The fish *vtgr* genes also showed conservation of synteny between the cartilaginous, lobe-finned and ray-finned fish species examined (Fig. 1). In Atlantic salmon, the duplicated *vtgr* genes designated *vtgr1* and *vtgr2* are mapped to the homeologous chromosomes Ssa13 and Ssa01. The salmon *vtgr* duplicates are flanked by paralogs of *sh3bp2* and *kcnv2a*, which are also neighbor genes of the single *vtgr* in three-spined stickleback (*Gasterosteus aculeatus*) and spotted gar. The single coelacanth *vtgr* is flanked by *smarca2* and *kcnv2*, which in elephant fish are flanking the *vtgr1* duplicate and the low density lipoprotein receptors *lrp1* and *lrp2*, while the *vtgr2* paralog is positioned on a separate scaffold. The predicted elephant fish VtgR1 and VtgR2 share less than 50% sequence identity compared to 91% identity between the two salmon VtgR paralogs.

Single complete salmon Vtg and multiple Vtgr splice variants

Salmon *vtgAsa1* consists of 34 exons and codes for a complete protein of 1659 amino acids (aa) with a calculated molecular weight (Mw) of 182,662 Da (Supplemental Figure S1). The truncated VtgAsb protein of 459 aa (Mw 50,683 Da) comprising the N-terminal signal peptide and the LvH region was predicted from the open reading frame within exons 1-9 using the

conventional gt-ag intron splice sites. The salmon *vtgC* gene consists of 27 exons and codes for an incomplete protein of 1281 aa (Mw 142,950 Da) lacking phosvitin and the C-terminal β' and CT domains. We searched for palindromic estrogen responsive elements (ERE) with the consensus aggtcannntgacct sequence in the putative promoter regions of the salmon *vtg* genes and identified imperfect ERE motifs within a region of 690 bp (*vtgAsa1*), 213 bp (*vtgAsb*) and 197 bp (*vtgC*) upstream of the ATG translational start site (Supplemental Figure S2). The proximal ERE of salmon *vtgAsa1* was shown to be identical to the functional ERE reported in the rainbow trout *vtgAsa* promoter (Bouter et al., 2010).

Salmon *vtgr1* and *vtgr2* were shown to be alternatively spliced in the exon coding for the *O*-linked sugar domain. The *vtgr1* gene consists of 20 exons coding for the complete receptor of 873 aa (X1 variant, Mw 96343 Da), while the short X3 variant of 852 aa is lacking the sugar domain, which is partially deleted in the X2 variant of 859 aa (Supplemental Figure S3). Salmon *vtgr2* contains 19 exons coding for a complete receptor of 863 aa (X1 variant, 95346 Da), while the short X2 variant of 842 aa is missing the *O*-linked sugar domain. We examined the tissue expression of the splice variants of salmon *vtgr1* and *vtgr2* by performing qPCR on cDNAs from early vitellogenic ovary, liver, brain and heart (Fig. 2). Both receptors expressed the short variant lacking the sugar domain at high levels in the ovary compared to the brain and heart. The complete *vtgr2* was expressed at low levels in ovary, brain and heart, while the expression of the complete *vtgr1* was limited to the brain. Only the short *vtgr1* variant was expressed at significant levels in the liver.

Expression of salmon *vtgs* and *vtgrs* during maturation

Salmon females and males were kept together in seawater net pens from May 2013 to May 2015 and were then transferred to indoor freshwater tanks until spawning in Sep - Oct 2015.

GSI levels in females gradually increased from <1% in Sep 2014 to above 20% in Aug 2015 (Fig. 3), while mean body weight increased from 5.75 kg to 10.33 kg. Plasma E_2 was maintained at low levels (< 5 ng/ml) until June 2015, but then strongly increased to peak levels of 27 ng/ml in Aug 2015, while highly variable E_2 levels were measured in Sep 2015 at the time of ovulation.

We quantified the expression of the functional salmon *vtg* and *vtgr* genes in the liver, ovary and brain of maturing females during the previtellogenic, vitellogenic and post-vitellogenic stages. Salmon *vtgAsa1, vtgAsb* and *vtgC* were mainly expressed in the liver, and the levels strongly increased at early vitellogenesis (Fig. 4). The expression of *vtgAsa1* and *vtgC* were similar at the vitellogenic stage, but the levels of *vtgAsa1* peaked at about 40 and 1000 times higher levels than *vtgC* and *vtgAsb*, respectively, prior to spawning. The three genes were expressed at much lower levels in the ovary and brain, although the ovarian expression of *vtgAsa1* increased significantly during maturation. The ovarian levels of the *vtgC* and *vtgAsb* transcripts were relatively stable compared to the variable levels measured in the female brain.

Contrasting with the very low expression of the three salmon *vtg* genes at the previtellogenic stage, both *vtgr1* and *vtgr2* were abundantly expressed in the previtellogenic ovary (Fig. 5). Then the expression of the two paralogs gradually decreased during vitellogenesis and the lowest levels were measured concomitant with the peaked levels of plasma E_2 in Aug 2015. The low and stable mRNA levels in the brain consisted mainly of the *vtgr1* transcript, while both genes were expressed at very low levels in the liver (data not shown).

Discussion

Multiplicity of Vtg ligands and receptors have been reported in various teleost species, but the number of functional genes and their concerted expression profiles have been largely unknown, particularly in salmonids. This study resolved the repertoire of *vtg* and *vtgr* genes in Atlantic salmon, which was shown to express only one complete Vtg protein, but two genetic distinct receptors of the LR8 type. The mapping of the salmon *vtgAsa* and *vtgAsb* together with the *vtgr1* and *vtgr2* paralogs to the homeologous chromosomes ascertains the tetraploid origin of the gene pairs in salmonids. Lineage-specific tandem duplications have resulted in multiple *vtg* genes in teleosts, including Northern pike (*Esox lucius*) representing the closest phylogenetic order of the tetraploid salmonids (Supplemental Figure S4). Contrasting with non-salmonids, only one complete Vtg has to our knowledge been documented in salmonids, although we noted the reports on two similar *vtgAsa* genes coding for the C-terminal region in rainbow trout and Arctic char (*Salvelinus alpinus*) (Le Guellec et al., 1988; Ren et al., 1996; Berg et al., 2004). A single complete Vtg protein was characterized in rainbow trout (Banoub et al., 2003) that does not support the suggested transcription of the adjacent *vtg2* gene (Mouchel et al., 1997), which is probably orthologous to the *vtgAsa2* pseudogene in Atlantic salmon (Buisine et al., 2002; this study). The intact salmon *vtgAsa1* was found to be identical to the partial *vtg* sequence obtained from E_2 stimulated males (Yadetie et al., 1999), and the calculated molecular weight of the complete protein is comparable to the 187,335 Da obtained by *de novo* sequencing the single Vtg isolated from Atlantic salmon (Banoub et al., 2004). Truncated Vtgs have been predicted from exons 1-7 of the multiple *vtgAsa* genes in rainbow trout and from *vtgAsb* genes in other salmonids (Buisine et al. 2002), but it is unknown whether they are dimerized and internalized for storage in the oocyte, and eventually used by the developing embryo. Although the N-terminal region of tilapia Vtg was shown to interact with Vtgr *in vitro* (Li et al., 2003), the folding and dimerization of Vtgs probably involve the Cys-rich C-terminal region (Mouchel et

198 al., 1996). Several studies have documented that Vtgs have been co-opted for other purposes
199 than reproduction, and serum Vtg in Atlantic salmon was shown to neutralize infectivity of
200 infectious pancreatic necrosis virus (Garcia et al., 2010). The function of lipovitellin and
201 phosvitin domains of fish Vtgs as novel players in maternal immunity (Li et al., 2008; Sun and
202 Chang, 2015) suggests that the LvH domain of the truncated salmonid Vtgs may play a role as
203 immune competent molecules, similar to what is seen in the mammalian ortholog of Vtgs, the
204 von Willebrand factor (Kreuz, 2008). Expression of the salmon *vtg* and *vtgr* genes in the brain
205 may relate to signal transduction or general lipid metabolism in the nervous system as suggested
206 in the cutthroat trout (Mizuta et al., 2013).
207
208 Appropriate composition of the accumulated Vtgs in the growing oocyte is probably essential
209 for the viability of the embryo and newly hatched larvae. The ratios of the yolk Vtg subtypes
210 differ substantially among teleosts that utilize different reproduction strategies and early life
211 histories (Finn et al., 2002; Hiramatsu et al., 2015; Williams et al., 2015). The VtgAa, VtgAb,
212 and VtgC types were shown to contribute substantially to the pool of yolk protein in ratios of
213 1.4:1.4:1 in striped bass (*Morone saxatilis*) spawning nearly neutrally buoyant eggs in
214 freshwater (Williams et al., 2015), while the marine goldsinny wrasse (*Ctenolabrus rupestris*)
215 ovulates floating eggs whose yolk is almost entirely comprised of yolk proteins derived from
216 VtgAa (Kolarevic et al., 2008). Salmonids spawn in freshwater and the large benthic eggs
217 contain small amounts of VtgC when compared to the complete VtgAsa. Sakhalin taimen
218 (*Hucho perryi*) showed VtgAsa:VtgC ratios of ~22:1 in serum and vitellogenic yolk (Amano et
219 al., 2010), while >100 times higher serum levels of VtgAs compared to VtgC was measured in
220 cutthroat trout at late vitellogenesis (Mushiroba et al., 2013). Correspondingly, the ovarian
221 expression of *vtgAsa* in Atlantic salmon and cutthroat trout peaked before spawning at about

40 and 140 times higher levels, respectively, than the *vtgC* levels (Mushiroba et al., 2013; this study).

The phosvitinless VtgC remains as an unprocessed LvH-LvL conjugate in the oocyte yolk and probably functions as nutrition in late-stage larvae (Finn, 2007; Finn and Kristoffersen, 2007; Reading et al., 2009). However, the ovarian uptake of VtgC is largely unknown and no ovarian lipoprotein receptor was found to bind white perch VtgC, while the cutthroat trout VtgC was shown to bind to an unidentified oocyte receptor (Reading et al., 2011; Hiramatsu et al., 2015). Dephosphorylation of chicken and mosquito Vtgs reduced their uptake by oocytes indicating that the phosphorylated phosvitin may play a role in Vtg receptor recognition (Miller et al., 1982; Dhadialla et al., 1992; Chen et al., 1997). The identification of two *vtgr* genes in the elephant fish possessing a VtgC with a shortened phosvitin could shed light on possible co-evolution of receptor-ligand pairs in vertebrates (Li et al., 2003). The tandem duplication of *vtgC* seen in spotted gar might have occurred before lepisosteids separated from teleosts and was followed by neofunctionization of a phosvitinless VtgC paralog in the latter group. The loss of serine repeats probably occurred by removal of the phosvitin coding exon concomitant with the loss of the C-terminal domains.

The striking difference between the serine-rich Vtgs of tetrapods and the invertebrates Vtgs lacking the phosphate-and calcium-carrying polyserine tracts led Wahli (1988) to cautiously speculate that this could be related to skeleton formation in the developing vertebrates. We consistently traced the loss of phosvitin back to chondrichthyean fishes by the identification of a very short serine sequence in the VtgC ortholog of elephant fish, and relative low serine content was reported in the phosvitin domain of a complete Vtg in the catshark (*Scyliorhinus torazame*) (Yamane et al., 2013). By losing its phosvitin domain, the VtgC may have lost its

ability to contribute to bone formation, but still functional in transporting other nutrients for storage in the oocyte, or in contributing an immune function. It should be noted that the absence of bone in the endoskeleton of the elephant fish was consistently associated with the lack of genes encoding secreted calcium-binding phosphoproteins (Venkatesh et al., 2014). The accumulation of partially and completely processed yolk components in chondrichthyean fishes might be related to their different reproductive modes, including placental species in which yolk metabolites and yolk granules are made available to the developing embryo by different means (Hamlett, 1989; Dulvy and Reynolds, 1997).

The expression of two genetically distinct VtgRs in Atlantic salmon agrees with the identification of two highly similar *vtgr* genes in rainbow trout (Prat et al., 1998; Davail et al., 1998), and Western blot analysis of cutthroat trout VtgR revealed a broad band of 95-105 kDa that was suggested to represent two similar sized receptors (Mizuta et al., 2013). Contrasting with the increased hepatic expression of salmon *vtgAsa1* during maturation, the ovarian expression of the *vtgr* paralogs peaked at the previtellogenic stage and gradually decreased concomitant with the increased plasma E_2 levels and ovarian growth. Accordingly, activated estrogen receptors have been shown to stimulate *vtg* transcription and to stabilize *vtg* transcripts (Brock and Shapiro, 1983; Flouriot et al., 1996; Bouter et al. 2010), but may also repress *vtgr* transcriptional activity as reported in largemouth bass (*Micropterus samonides*) (Dominguez et al., 2014). The decreased ovarian expression of the salmon *vtgrs* during vitellogenesis seems to contradict the 100-fold increase in number of receptors per oocyte in rainbow trout (Lancaster and Tyler, 1994; Rodriguez et al., 1996). The apparent discrepancy is probably not explained by the recycling of the oocyte receptors, because recycled proteins are generally degraded more rapidly than those confined to the cell surface (Hare and Taylor, 1991). The stability of the VtgR proteins is unknown, but is likely comparable to the turnover rates of the LDLR, which

was reported to degrade in macrophages and fibroblasts with $t_{1/2}$ of ~2 h and 12-13 h, respectively, at 4 °C (Yoshimura et al., 1988; Hare, 1990). Additionally, the two oocyte VtgRs are dominated by the splice variants lacking the *O*-linked sugar domain, which was shown to hinder proteolytic cleavage of the extracellular domain in mammalian LDLRs (Kozarsky et al., 1988; Magrané et al., 1999). Knowledge about the stability of the *vtgr* transcripts is lacking, but mRNA stabilization through AU-rich elements as reported for *ldlr* mRNAs (Li et al., 2009; Adachi et al., 2014) is probably insufficient to maintain the oocyte receptors for an extended period. Further studies are therefore needed to clarify the molecular mechanisms underlying the coordinated increase in the hepatic synthesis and ovarian uptake of Vtgs during vitellogenesis.

Materials and methods

Identification of fish *vtg* and *vtgr* genes

Fish *vtg* and *vtgr* sequences were retrieved from phylogenetic distant species representing cartilaginous, lobe-finned and ray-finned fishes by searching the gene databases at http://www.ncbi.nlm.nih.gov and http://www.ensembl.org (release 87) (Aken et al., 2016). Accession numbers are given in Supplemental Table S1. The identity of unannotated genes were determined by BLAST analysis (Altschul et al., 1997) with the sequences against known orthologs and by examination of the flanking genes for conserved synteny. Molecular weight of the predicted salmon proteins was calculated using the Compute Mw tool (Gasteiger et al., 2005).

Experimental fish and tissue sampling

Atlantic salmon females and males were reared by the AquaGen breeding company. The hatched larvae were start-fed in Feb 2012, and the one-year old smolts were transferred to sea-water net-pens in the Hemne fjord (63 °N, 9 °E) in May 2013. The fish were kept at natural temperature and photoperiod during seawater phase, except for the artificial light (LD 24:0)

during Jan - May 2014 to avoid early maturation in males (Leclercq et al., 2011), and from Mar 2015 until freshwater transfer to accelerate sexual maturation to promote sexual maturation (Taranger et al., 1999). In May 2015 the fish were transferred into indoor freshwater tank (60 m^3) and reared at 16 °C and at short day photoperiod (LD 8:16). The temperature was gradually decreased to 7 °C during nine days in mid Aug 2015 to induce final maturation and spawning that occurred in Sep - early Nov 2015. Temperature was recorded regularly at 3 m and 6 m depth in the seawater net-pens and in the freshwater tanks (Supplemental Figure S5). During seawater phase the fish were fed according to appetite with Ewos Opal 120 until one year before ovulation, when they were fed with Ewos Opal Breed. The fish were not fed after transfer to freshwater.

Ovary, liver and brain were sampled from five females once a month during Sep 2014 - Sep 2015, while heart was dissected from three females in Sep-Oct 2014. The fish were sacrificed with an overdose of tricaine methanesulphonate (200 mg/L, Pharmaq, Norway) according to suppliers instructions, followed by spinal transection. Body and ovary weights were registered for calculating gonado-somatic index (GSI). Dissected samples from ovary, liver, brain and heart were immediately added RNA*later* (Sigma) and stored at -20°C before extraction of total RNA. Blood was drawn from the caudal vein using heparinized vacuum tubes, and plasma was collected after centrifugation at 500 rpm for 10 min at 4°C. The plasma was kept on ice for 1-5 hr and stored at -80°C freezer until the measurement of E_2 titer using ELISA kit from Cayman chemical (Ann Arbor, MI, USA) (Næve et al., unpublished).

RNA extraction and qPCR

Total RNA was extracted from the salmon tissues using PureLink® RNA Mini Kit (Thermo Fisher Scientific), by adding 20 mg tissue to 800 µL lysis buffer according to manufacturer's instructions. DNA was removed using On-column PureLink ® DNase (Thermo Fisher Scientific). RNA purification was optimized from the fat-rich ovary by homogenizing 50-100

mg tissue in 1 mL Isol-RNA Lysis Reagent (5 Prime, Careforde). RNA quantity and quality were measured using a 1000-ND Nanodrop spectrophotometer, and the RNA was stored at -70°C. cDNA was synthesized by adding 150 ng RNA into a 10 µL reaction using TaqMan® Reverse Transcription Reagents (Applied Biosystems) and stored at -20°C. The relative expression levels of the *vtg* and *vtgr* genes identified in Atlantic salmon were determined by quantitative real-time PCR (qPCR) with elongation factor 1α (*ef1α)* as reference gene (Mushirobira et al., 2015). The *ef1α* gene was evaluated as the most stable reference gene among 6 different reference genes tested for 8 distinct tissues in the Atlantic salmon (Olsvik et al., 2005), and *ef1α* transcript levels were not different across various ovarian stages when mRNA prepared from the ovaries of rainbow trout was used as template (Luckenbach et al., 2008). Specific primers for the Atlantic salmon genes and splice variants were designed using the Primer3 software (Koressaar and Remm, 2007; Untergasser et al., 2012) (Supplemental Table S2). A two-fold standard dilution of pooled cDNAs was set up for each primer set to determine the amplification efficiency. Non-specific contamination in the qPCR reaction was ruled out by including controls without template and melting curve analysis was performed to verify the measurement of a single specific product. SDS 2.3 software (Applied Biosystems) was used to collect all data that was thereafter analyzed using RQ manager 1.2 (Applied Biosystems). The qPCR was run in triplicates on a LightCycler®480 using LightCycler® 480 SYBR Green I Master (Roche) in a total volume of 12 µL containing 6 µL diluted (1:10) cDNA, 5 µL SYBR Green I Master, and 0.5 µL of 10 µM forward and reverse primers. The cycling profile was 5 min at 95°C, followed by 45 cycles of 95°C for 15 s, 60°C for 15s and 72°C for 15 s. Relative gene expression during the annual reproductive cycle was quantified by the log2 Pfaffl method using the equation of Pfaffl values (Livak and Schnittgen, 2001). Tissue expression of the *vtgr* splice variants was evaluated using the cycle threshold (Ct) values. The final data were analyzed by One-way analysis of variance (ANOVA) followed by Tukey-

Kramer Honestly Significant Difference (TukeyHSD) and presented as means ± standard error of the mean (SEM) using the R software package (R Core Team 2016).

Ethics statement

In accordance to Norwegian and European legislation related to animal research, formal approval of the experimental protocol by the Norwegian Animal Research Authority (NARA) is not required because the experimental conditions are practices undertaken for the purpose of recognized animal husbandry. Such practices are exempted from the European convention on the protection of animals used for scientific purposes (2010/63/EU), cf. article 5d and do not require approval by the Norwegian ethics board according to the Norwegian regulation on animal experimentation, § 2, 5a, d "non-experimental husbandry (agriculture or aquaculture)" and "procedures in normal/common breeding and husbandry".

Acknowledgements

We are very grateful to two anonymous referees for the extensive and very helpful reviews of our paper.

Author's Contributions

Ø.A. and H.T. designed the study. I.N. and M.M. provided the tissue samples, and C.X. and K.H.K. performed the laboratory analyses. G.T. analyzed the data, and Ø.A. wrote the manuscript with contributions from all authors.

Conflict of interests

The authors declare no conflict of interest.

References

Adachi S, Homoto M, Tanaka R, Hioki Y, Murakami H, Suga H, Matsumoto M, Nakayama KI, Hatta T, Iemura S, Natsume T. 2014. ZFP36L1 and ZFP36L2 control LDLR mRNA stability via the ERK-RSK pathway. Nucl Acids Res 42:10037-10049.

Aken BL, Ayling S, Barrell D, Clarke L, Curwen V, Fairley S, Banet JF, Billis K, Girón CG, Hourlier T, Howe K, Kähäri A, Kokocinski F, Martin FJ, Murphy DN, Nag R, Ruffier M, Schuster M, Tang YA, Vogel JH, White S, Zadissa A, Flicek P, Searle SMJ. 2016. The Ensembl gene annotation system. Database 2016, baw093

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 25:3389-3402.

Amano H, Fujita T, Hiramatsu N, Sawaguchi S, Matsubara T, Sullivan CV, Hara A. 2007b Purification of multiple vitellogenins in grey mullet (*Mugil cephalus*). Mar Biol 152:1215-1225.

Amano H, Fujita T, Hiramatsu N, Shimizu M, Sawaguchi S, Matsubara T, Kagawa H, Nagae M, Sullivan CV, Hara A. 2007a. Egg yolk proteins in gray mullet (*Mugil cephalus*): purification and classification of multiple lipovitellins and other vitellogenin-derived yolk proteins and molecular cloning of the parent vitellogenin genes. J Exp Zool A Ecol Genet Physiol 307:324-341.

Amano H, Mochizuki M, Fujita T, Hiramatsu N, Todo T, Hara A. 2010. Purification and characterization of a novel incomplete-type vitellogenin protein (VgC) in Sakhalin taimen (*Hucho perryi*). Comp Biochem Physiol A 157:41–48.

Babin PJ. 2008. Conservation of a vitellogenin gene cluster in oviparous vertebrates and identification of its traces in the platypus genome. Gene 413:76-82.

398 Banoub J, Cohen A, Mansour A, Thibault P. 2004. Characterization and *de novo* sequencing of
399 Atlantic salmon vitellogenin protein by electrospray tandem and matrix-assisted laser
400 desorption/ionization mass spectrometry. Eur J Mass Spectrom 10:121-134.

401 Banoub J, Thibault P, Mansour A, Cohen A, Heeley DH, Lackman D. 2003. Characterisation
402 of the intact rainbow trout vitellogenin protein and analysis of its derived tryptic and
403 cyanogen bromide peptides by matrix-assisted laser desorption/ionization time-of-flight-
404 mass spectrometry and electrospray ionisation quadrupole/time-of-flight mass
405 spectrometry. Eur J Mass Spectrom 9:509-524.

406 Berg H, Modig C, Olsson PE. 2004. 17beta-estradiol induced vitellogenesis is inhibited by
407 cortisol at the post-transcriptional level in Arctic char (*Salvelinus alpinus*). Reprod Biol
408 Endocrinol 2:62.

409 Buisine N, Trichet V, Wolff J. 2002. Complex evolution of vitellogenin genes in salmonid
410 fishes. Mol Genet Genomics 268:535-542.

411 Bouter A, Buisine N, Le Grand A, Mouchel N, Chesnel F, Le Goff C, Le tillyV, Wolff J, Sire
412 O. 2010. Control of vitellogenin genes expression by sequences derived from transposable
413 elements in rainbow trout. Biochim Biophys Acta 1799:546–554.

414 Bujo H, Lindstedt KA, Hermann M, Mola Dalmau L, Nimpf J, Schneider WJ. 1995. Chicken
415 oocytes and somatic cells express different splice variants of a multifunctional receptor. J
416 Biol Chem 270: 23546–23551.

417 Braasch I, Salzburger W. 2009 *In ovo omnia*: diversification by duplication in fish and other
418 vertebrates. J Biol 8:25.

419 Brock ML, Shapiro DJ. 1983. Estrogen stabilizes vitellogenin mRNA against cytoplasmic
420 degradation. Cell 34:207-214.

421 Canapa A, Olmo E, Forconi M, Pallavicini A, Makapedua MD, Biscotti MA, Barucca M. 2012.
422 Composition and phylogenetic analysis of vitellogenin coding sequences in the Indonesian
423 coelacanth *Latimeria menadoensis.* J Exp Zool B Mol Dev Evol 318:404-416.

424 Chen JS, Sappington TW, Raikhel AS. 1997. Extensive sequence conservation among insect,
425 nematode, and vertebrate vitellogenins reveals ancient common ancestry. Mol Evol
426 44:440–451.

427 Davail B, Pakdel F, Bujo H, Perazzolo LM, Waclawek, Schneider WJ, Le Menn. 1998.
428 Evolution of oogenesis: the receptor for vitellogenin from the rainbow trout. J Lipid Res
429 39:1929–1937.

430 Dhadialla TS, Hays AR, Raikel AS. 1992. Characterization of the solubilized mosquito
431 vitellogenin receptor. Insect Biochem Mol Biol 22:803-816.

432 Dierks-ventling C. 1978. Vitellogenin synthesis in isolated hepatocytes. FEBS Lett 92:109-113.

433 Dominguez GA, Bisesi JH Jr, Kroll KJ, Denslow ND, Sabo-Attwood T. 2014. Control of
434 transcriptional repression of the vitellogenin receptor gene in largemouth bass (*Micropterus*
435 *salmoides*) by select estrogen receptors isotypes. Toxicol Sci 141:423–431.

436 Dominguez GA, Quattro J M, Denslow ND, Kroll KJ, Prucha MS, Porak WF, Grier HJ, Sabo-
437 Attwood TL. 2012. Identification and transcriptional modulation of the largemouth bass,
438 *Micropterus salmoides*, vitellogenin receptor during oocyte development by insulin and sex
439 steroids. Biol Reprod 87, 67:1-12.

440 Dulvy NK, Reynods JD. 1997. Evolutionary transitions among egg-laying, live-bearing and
441 maternal inputs in sharks and rays. Proc R Soc Lond B 264:1309-1315.

442 Finn RN. 2007. Vertebrate yolk complexes and the functional implications of phosvitins and
443 other subdomains in vitellogenins. Biol Reprod 76:926-935.

444 Finn RN, Kolarevic J, Kongshaug H, Nilsen F. 2009. Evolution and differential expression of
445 a vertebrate vitellogenin gene cluster. BMC Evol Biol 9:2.

446 Finn RN, Kristoffersen BA. 2007. Vertebrate vitellogenin gene duplication in relation to the
447 "3R hypothesis": Correlation to the pelagic egg and the oceanic radiation of teleosts. PLoS
448 ONE 2:e169.

449 Finn RN, Warmboldt M, Fyhn HJ. 2002. Differential processing of yolk proteins during oocyte
450 hydration in marine fishes (Labridae) that spawn benthic and pelagic eggs. Mar Ecol Progr
451 Ser 237:217-226.

452 Flouriot G, Pakdel F, Valotaire Y. 1996. Transcriptional and post-transcriptional regulation of
453 rainbow trout estrogen receptor and vitellogenin gene expression. Mol Cell Endocrinol
454 124:173-83.

455 Fyhn HJ, Finn RN, Reith M, Norberg B. 1999. Yolk protein hydrolysis and oocyte free amino
456 acids as key features in the adaptive evolution of teleost fishes to seawater. Sarsia 84:451-
457 456.

458 Garcia J, Munro ES, Monte MM, Fourrier MCS, Whitelaw J, Smail DA, Ellis AE. 2010.
459 Atlantic salmon (*Salmo salar* L.) serum vitellogenin neutralizes infectivity of infectious
460 pancreatic necrosis virus (IPNV). Fish Shellfish Immunol 29:293-297.

461 Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A. 2005.
462 Protein Identification and Analysis Tools on the ExPASy Server. In: John M. Walker (ed):
463 The Proteomics Protocols Handbook, Humana Press, pp. 571-607

464 Greeley MS Jr, Calder DR, Wallace RA. 1986. Changes in teleost yolk proteins during oocyte
465 maturation: correlation of yolk proteolysis with oocyte hydration. Comp Biochem Physiol
466 84B:1–9

467 Hamlett WC. 1989. Evolution and morphogenesis of the placenta in sharks. J Exp Zool 252
468 Suppl S2:35–52.

469 Hare JF. 1990. Compartmentation and turnover of the low density lipoprotein receptor in skin
470 fibroblasts. J Biol Chem 265:21758-21763.

471 Hare JF, Taylor K. 1991. Mechanisms of plasma membrane protein degradation: Recycling
472 proteins are degraded more rapidly than those confined to the cell surface. Proc Natl Acad
473 Sci USA 88:5902-5906.

474 Hiramatsu N, Ichikawa N, Fukada H, Fujita T, Sullivan CV. 2002. Identification and
475 characterization of proteases involved in specific proteolysis of vitellogenin and yolk
476 proteins in salmonids. Comp Physiol Biol 292:11–25.

477 Hiramatsu N, Matsubara T, Fujita T, Sullivan CV, Hara A. 2006. Multiple piscine vitellogenins:
478 biomarkers of fish exposure to estrogenic endocrine disruptors in aquatic environments.
479 Mar Biol 149:35–47

480 Hiramatsu N, Todo T, Sullivan CV, Schilling J, Reading BJ, Matsubara T, Ryu YW, Mizuta H,
481 Luo W, Nishimiya O, Wu M, Mushirobira Y, Yilmaz O, Hara A. 2015. Ovarian yolk
482 formation in fishes: Molecular mechanisms underlying formation of lipid droplets and
483 vitellogenin-derived yolk proteins. Gen Comp Endocrinol 221:9-15.

484 Kolarevic J, Nerland A, Nilsen F, Finn RN. 2008. Goldsinny wrasse (*Ctenolabrus rupestris*) is
485 an extreme vtgAa-type pelagophil teleost. Mol Reprod Dev 75:1011–1020.

486 Koressaar T, Remm M. 2007. Enhancements and modifications of primer design program
487 Primer3. Bioinformatics 23:1289-1291.

488 Kozarsky K, Kingsley D, Krieger M. 1988. Use of a mutant cell line to study the kinetics and
489 function of O-linked glycosylation of low density lipoprotein receptors. Proc Natl Acad Sci
490 USA 85:4335-4339.

King HR, Pankhurst, NW. 2003. Ovarian growth and plasma sex steroid and vitellogenin profiles during vitellogenesis in Tasmanian female Atlantic salmon (*Salmo salar*). Aquaculture 219:797-813.

Kreuz W. 2008. The role of VWF for the success of immune tolerance induction. Thromb Res 122:S7-S12.

Lancaster PM, Tyler CR. 1994. Developmental expression and modulation of the vitellogenin receptor in ovarian follicles of the rainbow trout, *Oncorhynchus mykiss*. Gen Comp Endocrinol 269:458–466.

Leclercq E, Taylor JF, Sprague M, Migaud H. 2011. The potential of alternative lighting-systems to suppress pre-harvest sexual maturation of 1+ Atlantic salmon (*Salmo salar*) post-smolts reared in commercial sea-cages. Aquacult Engineering 44:35-47.

Le Guellec K, Lawless M, Valotaire Y, Kress M, Tenniswood M. 1988. Vitellogenin gene expression in male rainbow trout (*Salmo gairdneri*). Gen Comp Endocrinol 71:359-371.

Li H, Chen W, Zhou Y, Abidi P, Sharpe O, Robinson WH, Kraemer FB, Liu J. 2009. Identification of mRNA binding proteins that regulate the stability of LDL receptor mRNA through AU-rich elements. J Lipid Res 50:820-831.

Li A, Sadasivam M, Ding JL. 2003. Receptor-ligand interaction between vitellogenin receptor (VtgR) and vitellogenin (Vtg), implications on low density lipoprotein receptor and apolipoprotein B/E. J Biol Chem 278:2799-2806.

Li Z, Zhang S, Liu Q. 2008. Vitellogenin functions as a multivalent pattern recognition receptor with an opsonic activity. PLoS ONE 3:e1940.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real time quantitative PCR and the 2 $-\Delta\Delta$Ct method. Methods 25:402-408.

514 Luckenbach JA, Iliev DB, Goetz FW, Swanson P. 2008. Identification of differentially
515 expressed ovarian genes during primary and early secondary oocyte growth in coho salmon,
516 *Oncorhynchus kisutch*. Reprod Biol Endocrinol 6:2.

517 Magrané J, Casaroli-Marano RP, Reina M, Gåfvels M, Vilaró S. 1999. The role of O-linked
518 sugars in determining the very low density lipoprotein receptor stability or release from the
519 cell. FEBS Lett 451:56-62.

520 Miller MS, Benore-Parsons M, White HB. 1992. Dephosphorylation of chicken riboflavin-
521 binding protein and phosvitin decreases their uptake by oocytes. J Biol Chem 257:6818-
522 6824.

523 Mizuta H, Luo W, Ito Y, Mushirobira Y, Todo T, Hara A, Reading BJ, Sullivan CV, Hiramatsu
524 N. 2013. Ovarian expression and localization of a vitellogenin receptor with eight ligand
525 binding repeats in the cutthroat trout (*Oncorhynchus clarki*). Comp Biochem Physiol B
526 166:81-90.

527 Mouchel N, Trichet V, Naimi BY, Le Pennec JP, Wolff J. 1997. Structure of a fish
528 (*Oncorhynchus mykiss*) vitellogenin gene and its evolutionary implications. Gene 197:147-
529 152.

530 Mouchel N, Trichet V, Betz A, Le Pennec JP, Wolff J. 1996. Characterization of vitellogenin
531 from rainbow trout (*Oncorhynchus mykiss*). Gene 174:59-64.

532 Mushirobira Y, Mizuta H, Luo W, Morita Y, Sawaguchi S, Matsubara T, Hiramatsu N, Todo
533 T, Hara A. 2013. Changes in levels of dual vitellogenin transcripts and proteins in cutthroat
534 trout *Oncorhynchus clarki* during ovarian development. Nippon Susian Gakkaishi 79:175-
535 189.

536 Mushirobira Y, Mizuta H, Luo W, Todo T, Hara A, Reading BJ, Sullivan CV, Hiramatsu N.
537 2015. Molecular cloning and partial characterization of a low-density lipoprotein receptor-

related protein 13 (Lrp13) involved in vitellogenin uptake in the cutthroat trout (*Oncorhynchus clarki*). Mol Reprod Develop 82:986–1000.

Nishimiya O, Kunihiro Y, Hiramatsu N, Inagawa H, Todo T, Hara A. 2014. Biochemical and immunochemical characterization of two discrete vitellogenin proteins and their derived lipovitellins in the inshore hagfish (*Eptatretus burgeri*). Zoolog Sci 31:251-257.

Okabayashi K, Shoji H, Nakamura T, Hashimoto O, Asashima M, Sugino H. 1996. cDNA cloning and expression of the *Xenopus laevis* vitellogenin receptor. Biochem Biophys Res Commun 224:406–413.

Olsvik PA, Lie KK, Jordal AEO, Nilsen TO, Hordvik I. 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol Biol 6:21

Opresko LK, Wiley HS. 1987. Receptor-mediated endocytosis in *Xenopus* oocytes. I. Characterization of the vitellogenin receptor system. J Biol Chem 262:4109-4115.

Prat F, Coward K, Sumpter JP, Tyler CR. 1998. Molecular characterization and expression of two ovarian lipoprotein receptors in the rainbow trout, *Oncorhynchus mykiss*. Biol Reprod 58:1146-1153.

Prowse TAA, Byrne M. 2012. Evolution of yolk protein genes in the Echinodermata. Evol Develop 14:139-151.

R Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Reading BJ, Hiramatsu N, Sawaguchi S, Matsubara T, Hara A, Lively MO, Sullivan CV. 2009. Conserved and variant molecular and functional features of multiple egg yolk precursor proteins (vitellogenins) in white perch (*Morone americana*) and other teleosts. Mar Biotechnol 11:169-187.

561 Reading BJ, Hiramatsu N, Sullivan CV. 2011. Disparate binding of three types of vitellogenin
562 to multiple forms of vitellogenin receptor in white perch. Biol Reprod 84:392-399.

563 Reading BJ, Hiramatsu N, Schilling J, Molloy KT, Glassbrook N, Mizuta H, Luo W, Baltzegar
564 DA, Williams VN, Todo T, Hara A, Sullivan CV. 2014. Lrp13 is a novel vertebrate
565 lipoprotein receptor that binds vitellogenins in teleost fishes. J Lipid Res 55:2287-2295.

566 Ren L, Lewis SK, Lech JJ. 1996. Effects of estrogen and nonylphenol on the post-
567 transcriptional regulation of vitellogenin gene expression. Chemico-Biological Interactions
568 100:67-76.

569 Rodriguez JN, Bon E, Le Menn F. 1996. Vitellogenin receptors during vitellogenesis in the
570 rainbow trout *Oncorhynchus mykiss*. J Exp Zool 274:163-170.

571 Sawaguchi S, Kagawa H, Ohkubo N, Hiramatsu N, Sullivan CV, Matsubara T. 2006.
572 Molecular characterization of three forms of vitellogenin and their yolk protein products
573 during oocyte growth and maturation in red seabream (*Pagrus major*), a marine teleost
574 spawning pelagic eggs. Mol Reprod Dev 73:719-736.

575 Sawaguchi S, Koya Y, Yoshizaki N, Ohkubo N, Andoh T, Hiramatsu N, Sullivan CV, Hara A,
576 Matsubara T. 2005. Multiple vitellogenins (Vgs) in mosquitofish (*Gambusia affinis*):
577 identification and characterization of three functional Vg genes and their circulating and
578 yolk protein products. Biol Reprod 72:1045-1060.

579 Sharrock WJ, Rosenwasser TA, Gould J, Knott J, Hussey D, Gordon JI, Banaszak L. 1992.
580 Sequence of lamprey vitellogenin. Implications for the lipovitellin crystal structure. J Mol
581 Biol 226:903-907.

582 So YP, Idler DR, Hwang SJ. 1985. Plasma vitellogenin in landlocked Atlantic salmon (*Salmo*
583 *salar* Ouananiche): Isolation, homologous radioimmunoassay and immunological cross-
584 reactivity with vitellogenin from other teleosts. Comp Biochem Physiol B 81:63-71.

585 Stifano S, Barber DL, Nimpf J, Schneider WJ. 1990a. A single chicken oocyte plasma
586 membrane protein mediates uptake of very low density lipoprotein and vitellogenin. Proc
587 Natl Acad Sci USA 87:1955-1959.

588 Stifano S, Nimpf J, Schneider WJ. 1990b. Vitellogenesis in *Xenopus laevis* and chicken:
589 Cognate ligands and oocyte receptors. J Biol Chem 265: 882-888.

590 Sun C, Zhang S. 2015. Immune-relevant and antioxidant activities of vitellogenin and yolk
591 proteins in fish. Nutrients 7:8818–8829.

592 Taranger GL, Haux C, Hansen T, Stefansson SO, Björnsson, BT, Walther BT, Kryvi H. 1999.
593 Mechanisms underlying photoperiodic effects on age at sexual maturity in Atlantic salmon,
594 *Salmo salar*. Aquaculture 177:47-60.

595 Trichet V, Buisine N, Mouchel N, Mora P, Penda AM, Le Pennec JP, Wolff J. 2000. Genomic
596 analysis of the vitellogenin locus in rainbow trout (*Oncorhynchus mykiss*) reveals a complex
597 history of gene amplification and retroposon activity. Mol Gen Genet 263:828-837.

598 Tyler CR, Lubberink K. 1996. Identification of four ovarian receptor proteins that bind
599 vitellogenin but not other homologous plasma lipoproteins in the rainbow trout,
600 *Oncorhynchus mykiss* J Comp Physiol B 166:11-20.

601 Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Roze SG. 2012.
602 Primer3 – new capabilities and interfaces. Nucl Acids Res 40:e115.

603 Venkatesh B, Lee AP, Ravi V, Maurya AK, Lian MM, Swann JB, Ohta Y et al. 2014. Elephant
604 shark genome provides unique insights into gnathostome evolution. Nature 505:174-179.

605 Wahli W. 1988. Evolution and expression of vitellogenin genes. Trends Genet 4:227-232.

606 Wang H, Yan T, Tan JTT, Gong Z. 2000. A zebrafish vitellogenin gene (*vtg3*) encodes a novel
607 vitellogenin without a phosvitin domain and may represent a primitive vertebrate
608 vitellogenin gene. Gene 256:303-310.

609 Williams VN, Reading BJ, Amano H, Hiramatsu N, Schilling J, Salger SA, Islam Williams T,
610 Gross K, Sullivan CV. 2014. Proportional accumulation of yolk proteins derived from
611 multiple vitellogenins is precisely regulated during vitellogenesis in striped bass (*Morone*
612 *saxatilis*). J Exp Zool 321A:301–315.

613 Yadetie F, Arukwe A, Goksøyr A, Male R. 1999. Induction of hepatic estrogen receptor in
614 juvenile Atlantic salmon *in vivo* by the environmental estrogen, 4-nonylphenol. Sci Total
615 Environ 233:201-210.

616 Yilmaz O, Prat F, Ibanez AJ, Koksoy S, Amano H, Sullivan CV. 2016. Multiple vitellogenins
617 and product yolk proteins in European sea bass (*Dicentrarchus labrax*): Molecular
618 characterization, quantification in plasma, liver and ovary, and maturational proteolysis.
619 Comp Biochem Physiol B 194-195:71-86.

620 Yoshimura A, Seguchi T, Yoshida T, Shite S, Waki M, Kuwano M. 1988. Novel feature of
621 metabolism of low density lipoprotein receptor in a mouse macrophage-like cell line,
622 J774.1. J Biol Chem 263:11935-11942.

623 Yamane K, Yagai T, Nishimiya O, Sugawara R, Amano H, Fujita T, Hiramatsu N, Todo T,
624 Matsubara T, Hara A. 2013. Characterization of vitellogenin and its derived yolk proteins
625 in cloudy catshark (*Scyliorhinus torazame*). Fish Physiol Biochem 39:373–390.

626

627 # Figure legends

628

629 **Figure 1**. Synteny analysis of the *vtg* (left column) and *vtgr* genes (right column) identified
630 in Atlantic salmon, three-spined stickleback, spotted gar, African coelacanth and elephant
631 fish. Homologous genes are shown in same color. Linkage group and scaffold identity are
632 included, and vertical bar indicates intervening genes. Gene IDs and chromosomal positions
633 are given in Supplemental Table S1.

634

635 **Figure 2**. Tissue expression of the splice variants of Atlantic salmon *vtgr1* and *vtgr2* measured
636 by qPCR. cDNAs from each tissue were pooled from two females sampled in Sep-Oct 2014.
637 Expression levels are denoted by the cycle threshold (Ct) values meaning that high expression
638 gives low Ct value. The y-axis is reversed for better visualization of the values given as mean
639 and error bars for SEM. Samples without detectable expression were marked as ”not detected”
640 (ND).

641

642 **Figure 3**. **A**. Plasma E_2 titer, and **B**. Gonado-somatic index (GSI, ●) in Atlantic salmon females
643 during pre-vitellogenesis (I), vitellogenesis (II) and post-vitellogenesis (III). The stages were
644 determined by histological examination of ovarian samples (Næve et al., unpublished). Plasma
645 E_2 levels are presented as mean ± SEM, except for the single values at Dec 2014 and Jan 2015.
646 Different letters denote significant differences tested by ANOVA analysis (P<0.05).

647

648 **Figure 4**. Relative expression levels of the Atlantic salmon *vtgAsa1* (black), *vtgAsb* (blue)
649 and *vtgC* (red) in female liver (**A**), ovary (**B**) and brain (**C**) during an annual reproductive
650 cycle. No brain was sampled in Feb 2015 and prior to spawning. *ef-1α* was used as reference
651 gene. Values are presented as mean ± SEM (n=5-7). ANOVA p-values for the three genes are

652 shown in the plots. Time points not sharing a letter were significantly different from each
653 other.
654
655 **Figure 5**. Relative expression levels of the Atlantic salmon *vtgr1* and *vtgr2* in female ovary
656 (**A**) and brain (**B**) during an annual reproductive cycle. *ef-1α* was used as reference gene.
657 Values are presented as mean ± SEM (n=5-7). ANOVA p-values for the two genes are
658 included. Gene expression in brain was not measured in Aug-Sep 2015. Time points not
659 sharing a letter were significantly different from each other.
660

Legends for Supplemental materials

Supplemental Figure S1. Sequence alignment of Atlantic salmon VtgAsa1, VtgAsb and VtgC. The heavy and light lipovitellin (LvH, LvH), phosvitin (Pv), β' and CT domains are indicated.

Supplemental Figure S2. Putative estrogen responsive elements (EREs) in the promoter region of Atlantic salmon v*tgAsa1, vtgAsb and vtgC*. Imperfect ERE half sites are shown in bold and translational start site is underlined.

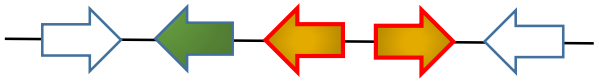
Supplemental Figure S3. Alignment of Atlantic salmon VtgR1 and VtgR2. The following domains are highlighted: Eight ligand-binding domains (LBDs) shaded, three epidermal growth factor-like domains (EGF) in bold italics, consensus YWTD motifs in bold, *O*-linked sugar domain in underlined bold letters, transmembrane domain (TM) and cytoplasmic domain (CD) underlined. Dashes are inserted for optimal alignment. The *O*-linked sugar domain in the complete receptor variants is lacking in the short splice variants and is partially deteled in the middle variant of VtgR1.

Supplemental Figure S4. Water temperature during seawater and freshwater phases of the experimental period.

Supplemental Figure S5. Chromosomal positions of the Northern pike (*Esox lucius*) *vtg* genes identified by searching at NCBI.

687 **Supplemental Table S1**. Gene ID and genomic location of the *vtg* and *vtgr* genes in the five
688 fish species examined.
689
690 **Supplemental Table S2**. Primer sequences for real-time qPCR of the *vtg* and *vtgr* genes in
691 Atlantic salmon. F-forward, R-reverse.
692

Ssa23

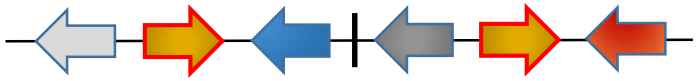


zranb2 adgrl4 vtgAsa2 vtgAsa1 cldn18

Ssa13

dspp sh3bp2 vtgr1 kcnv2aa k0020

Ssa10

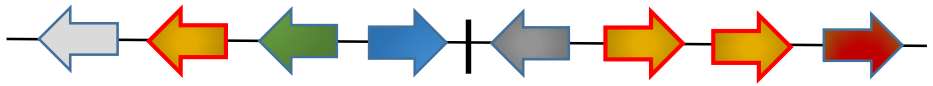


negr1 vtgC adgrl2 ctbs vtgAsb ssx2ip

Ssa01

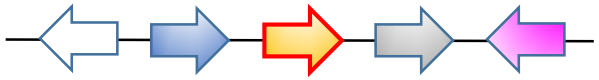
ppp3cc sh3bp2 vtgr2 kcnv2ab k0020

LGVIII



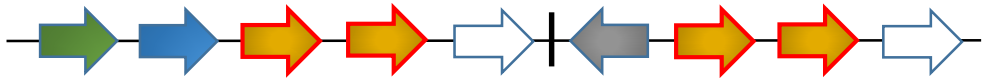
negr1 vtgC adgrl4 adgrl2a ctbs vtgAb vtgAa ssx2ipb

LGXIV



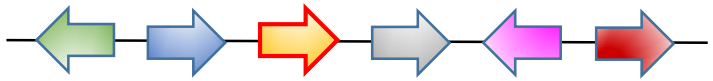
spp1 sh3bp2 vtgr kcnv2a pum3

LG10



adgrl2 adgrl4 vtgCI vtgCII cp4b1 ctbs vtgABIII vtgABII znf692

LG2

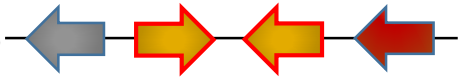


dspp sh3bp2 vtgr kcnv2 pum3 carm1

JH17947

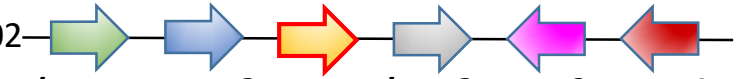
ptgfr vtgAB1 adgrl4

JH126745



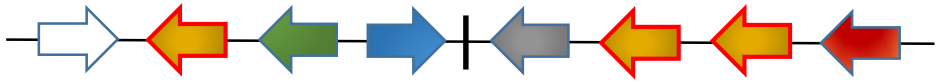
ctbs vtgABIII vtgABII ssx2ip

JH127502

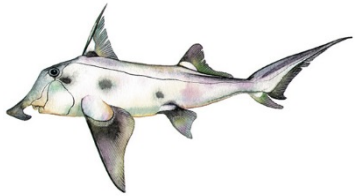


dspp smarca2 vtgr kcnv2 pum3 carm1

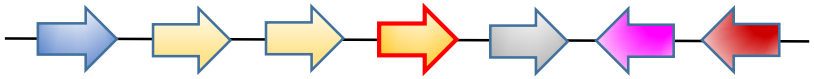
Sc7



ifi441 vtgC adgrl4 adgrl2 ctbs vtgABIII vtgABII ssx2ip

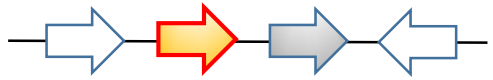


Sc24

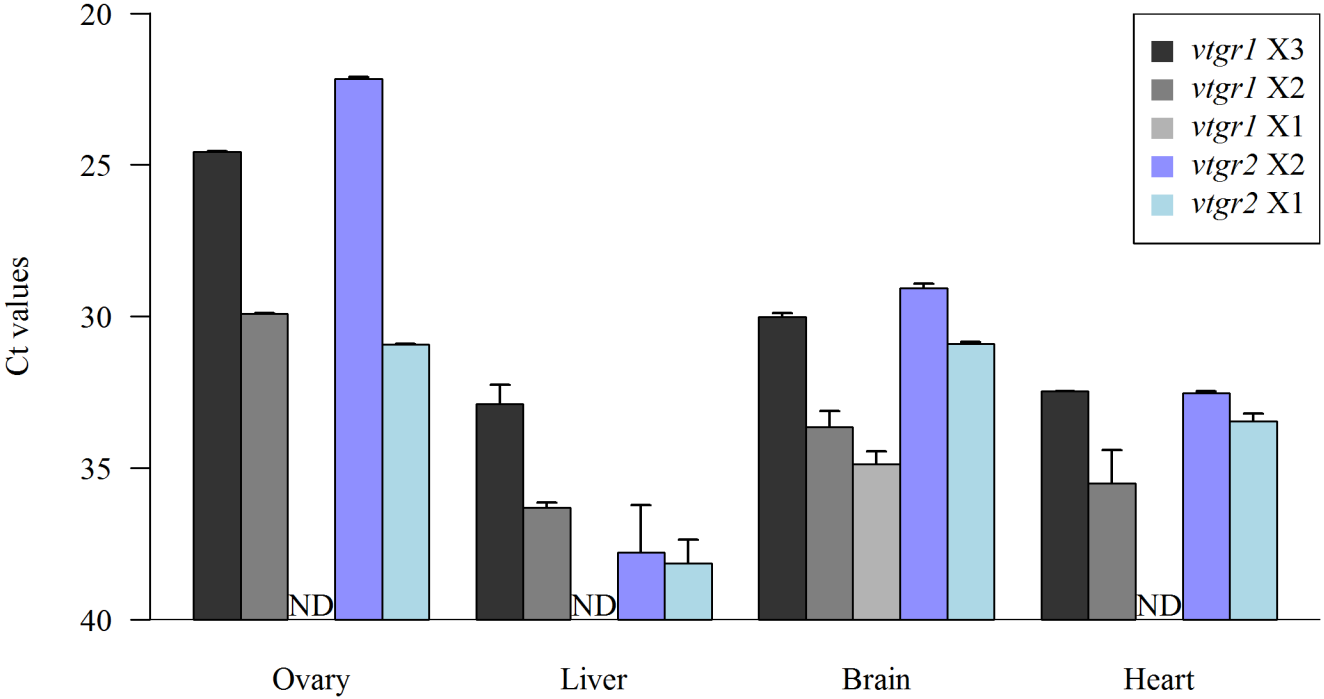


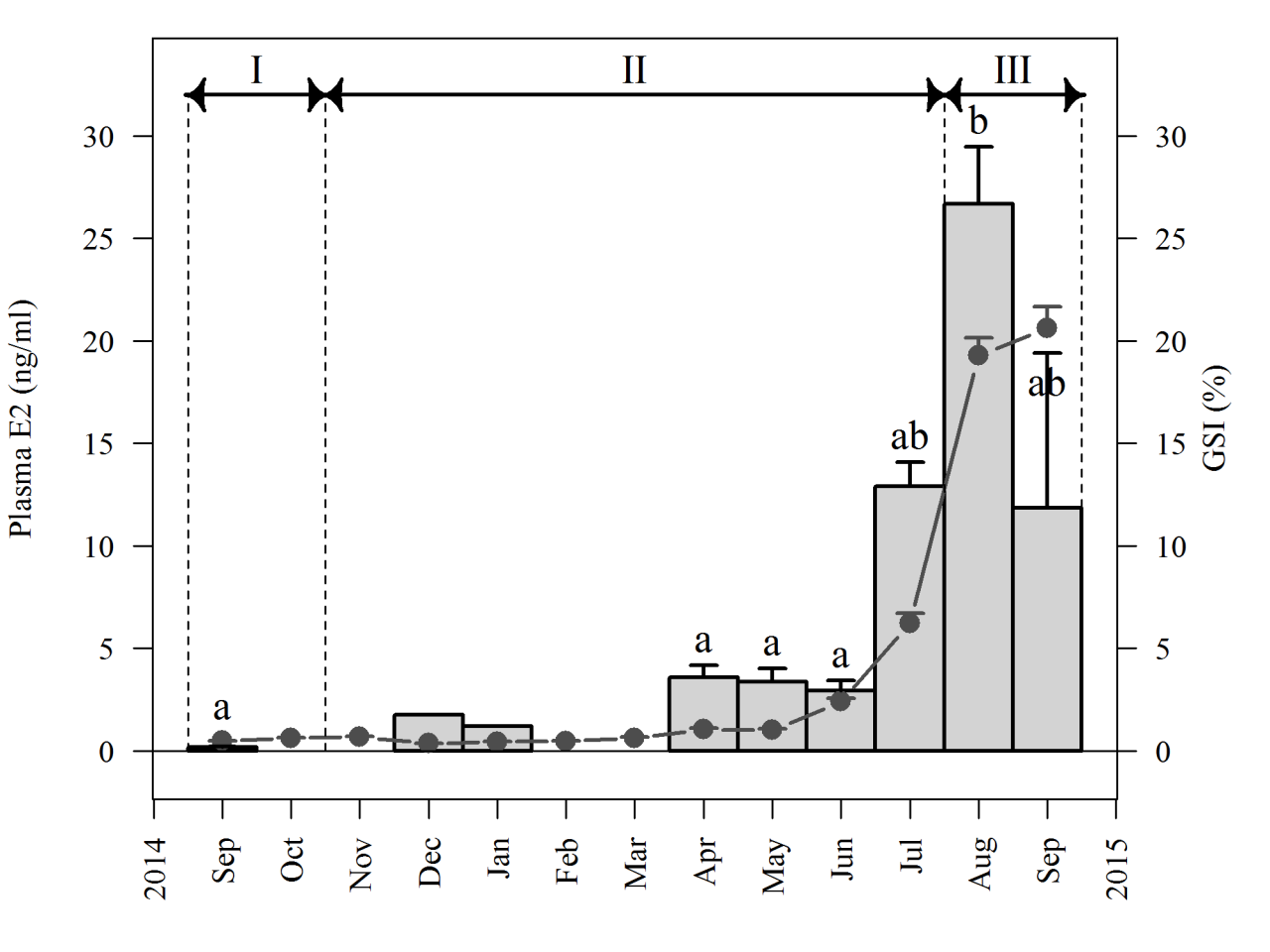
smarca2 lrp2 lrp1 vtgr1 kcnv2a pum3 carm1

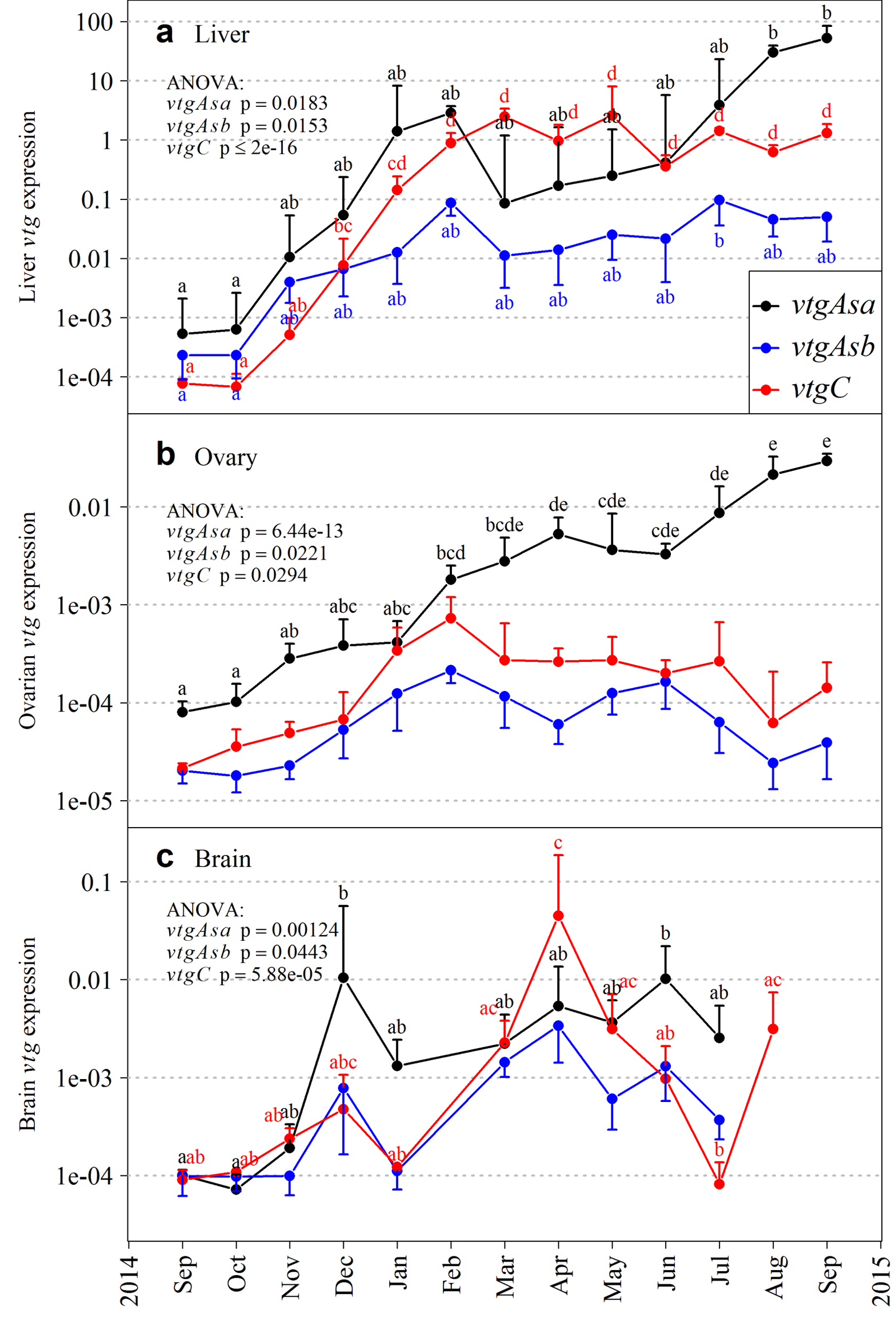
Sc412

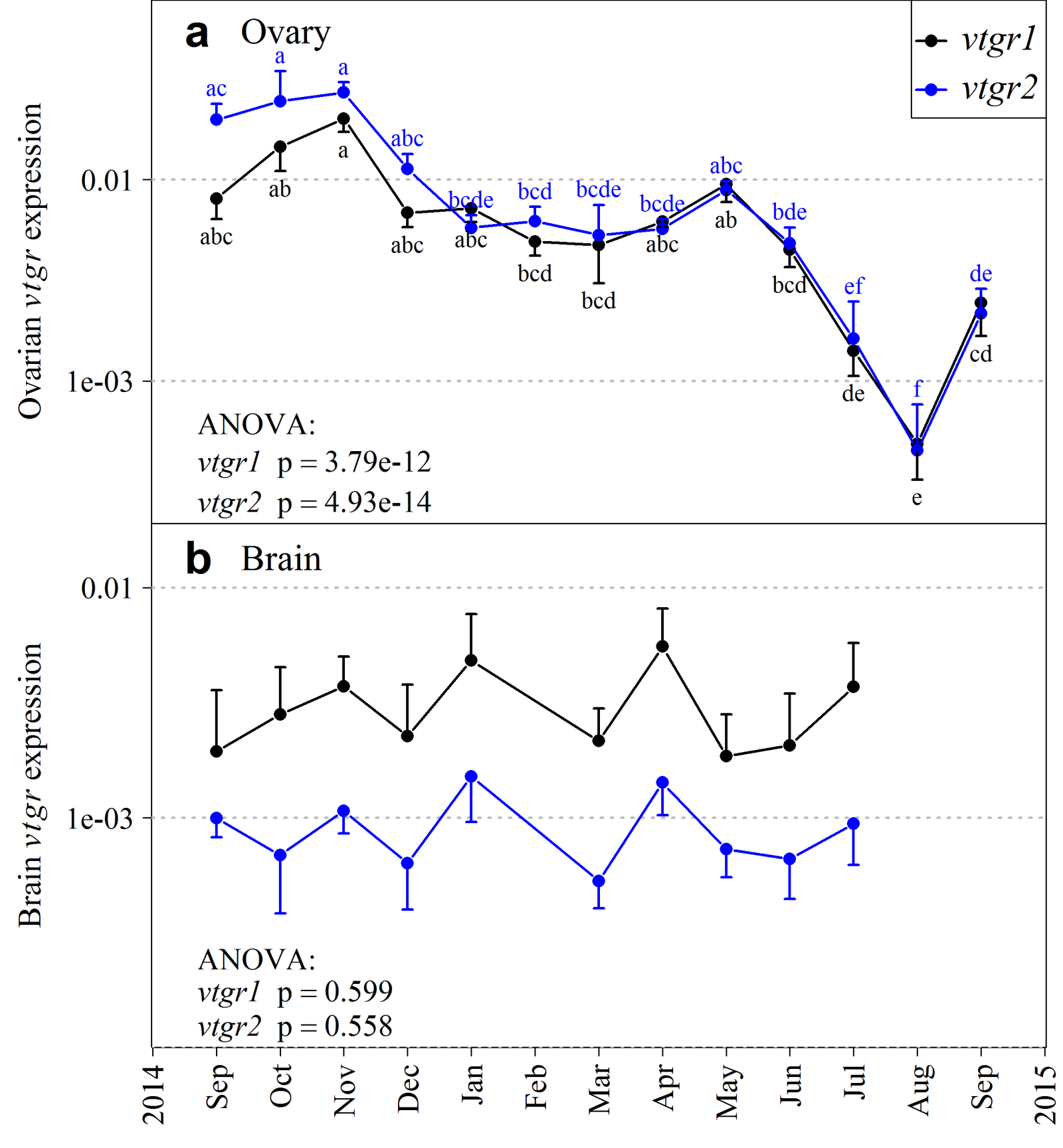


nbufa7 vtgr2 kcnv2b rfx2


20
25
30
35
40
Ct values
ND
ND
ND
Ovary
Liver
Brain
Heart
vtgr1 X3
vtgr1 X2
vtgr1 X1
vtgr2 X2
vtgr2 X1


I
II
III
Plasma E2 (ng/ml)
GSI (%)
0
5
10
15
20
25
30
a
a
a
ab
b
ab
2014
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
2015


a Liver
ANOVA:
vtgAsa p = 0.0183
vtgAsb p = 0.0153
vtgC p ≤ 2e-16
Liver vtg expression
100
10
1
0.1
0.01
1e-03
1e-04
vtgAsa
vtgAsb
vtgC
b Ovary
ANOVA:
vtgAsa p = 6.44e-13
vtgAsb p = 0.0221
vtgC p = 0.0294
Ovarian vtg expression
0.01
1e-03
1e-04
1e-05
c Brain
ANOVA:
vtgAsa p = 0.00124
vtgAsb p = 0.0443
vtgC p = 5.88e-05
Brain vtg expression
0.1
0.01
1e-03
1e-04
2014
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
2015


a Ovary
vtgr1
vtgr2
Ovarian vtgr expression
0.01
1e-03
ac
a
a
abc
abc
ab
a
abc
abc
bcde
bcd
bcd
bcde
bcd
abc
ab
bde
bcd
ef
de
de
f
e
cd
ANOVA:
vtgr1 p = 3.79e-12
vtgr2 p = 4.93e-14
b Brain
Brain vtgr expression
0.01
1e-03
ANOVA:
vtgr1 p = 0.599
vtgr2 p = 0.558
2014
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
2015