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Gene expression of the vitellogenins and the receptors in Atlantic salmon (*Salmo salar*) during the annual reproductive cycle

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Abstract

Atlantic salmon belongs to the primitive group of protacanthomorph teleost and has two *Vtg* genes (*VtgAsa* and *VtgAsb)* and two *VtgR* genes (*VtgR1* and *VtgR2)*. The focus of our research was to characterize predicted salmon Vtgs and VtgRs by alignment analysis, and to evaluate annual changes in gene expression in four tissues (liver, brain, heart and ovary) during reproductive cycle by qPCR quantification procedure. The annual changes in gonad-somatic index and plasma estrogen concentration were also presented to establish the relationship with Vtgs and VtgRs during reproducing cycle. qPCR revealed that *VtgAsa* and *VtgAsb* were predominantly expressed in liver, while weak expression was detected in ovary, brain and heart of female fish. *Vtg* increased significantly during previtellogenesis, and kept on highest level in vitellogenesis period. *VtgR* genes were predominantly expressed in ovary but limited in extra-ovarian tissue, and *VtgR2* was undetectable in liver tissue. Both *VtgR1* and *VtgR2* were expressed at highest levels in previtellogenetic, and then gradually decreased with the oocyte growth and E2 accumulation.

Key words: Atlantic salmon, vitellogenin (*Vtg*), vitellogenin receptor (*VtgR*), qPCR, estrogen

Abbreviations

aa: Amino acid
Apo: Apolipoprotein
BLAST: Basic Local Alignment Search Tool
C: Cytoplasmic domain
Ct: C-terminal coding region
E2: 17β-estradiol
EF1a: Elongation factor 1 *alpha*
EGF: Epidermal growth factor-precursor
ER: Estrogen receptor
ERE: Estrogen response elements
FAA: Free amino acids
GSI: Gonad-somatic index
LBDs: Ligand-binding domains
LD: Light density
LDLR: Low density lipoprotein receptor
VLDLR: Very low density lipoprotein receptor
LR7: Seven ligand binding domain
LR8: Eight ligand binding domain
LR8-: Lacking O-linked sugar domain
LR8+: O-linked sugar domain
Lv: Lipovitellin
LvH: Heavy chain lipovitellin
LvL: Light chain lipovitellin
NCBI: National Center for Biotechnology Information
Pv: Phosvitin
PAMPs: Pathogen associated molecular patterns
qPCR: Quantitative real-time reverse transcription-polymerase chain reaction
RT-PCR: Reverse transcription polymerase chain reaction
T: Transmembrane domain
Vtg: Vitellogenin
Vtg: Vitellogenin
VtgR: Vitellogenin receptor
Yps: Yolk proteins
WGD: Whole genome duplication
β′c: β′-component

Contents

1. INTRODUCTION

1.1 Fish vitellogenins (Vtgs)

1.1.1 Functional roles of fish Vtgs

The vitellogenins (Vtgs) are large apolipoproteins which are the precursor of egg-yolk proteins (Yps) of egg-laying (oviparous) species. Fish Vtgs are synthesized and secreted mainly by the liver-under the 17β-estradiol (E2)-control, transported via bloodstream and absorbed by vitellogenin receptors (VtgRs) on the surface of growing oocytes (Postlethwait and Kaschnits, 1978; Dierks-ventling, 1978; Mouchel *et al*., 1996; Prat *et al.*, 1998; Dominguez *et al*., 2012). Ovarian expression of Vtg has been demonstrated in the cyprinid species zebrafish (*Danio rerio*), white cloud mountain minnow (*Tanichthys albonubes*) and Korean rose bitterling (*Rhodeus uyekii*) (Wang *et al.* 2005; Wang *et al.* 2010; Kong *et al*. 2014). Following the receptor-mediated endocytosis, Vtgs are cleaved into Yps which provide essential nutrients for the developing embryo (Prat *et al*., 1998; Avarre *et al*., 2007). Sequence analysis demonstrated that Vtg and apolipoprotein B (apoB) are homologous, and belong to the large lipid transfer proteins, so lipids can bind to the Vtgs and be carried into oocytes (Li *et al*., 2003). Vtgs decide the egg character (pelagic or benthic) via differently degrading Vtg into amino acid to adjust the osmosis inside the oocyte to present egg floating feature (Fin and Kristoffersen, 2007). The Vtgs also play an important role in defeating virus and bacterium in immune system by recognizing pathogen-associated molecular patterns (PAMPs), binding to the surface of macrophages, and promoting macrophage phagocytosis (Li *et al,* 2008; Zhang *et al.,* 2015). Vtgs are closely related to fish gonadal maturation, so the sexual development can be predicted by measuring its concentration in the blood (Baumann *et al.,* 2013; Chatakondi and Kelly, 2013).

1.1.2 Structure of fish Vtgs

Fish Vtg mostly is large phospholipoglycoproteins dimers of 300-500 kDa composed of two identical subunits (Utarabhand, and Bunlipatanon, 1996). Molecular and biochemical characterization of Vtgs have been studied in various teleost fishes, including walking catfish (*Clarias gariepinus*) (Panprommin *et al.,* 2008), zebrafish (Wang *et al.,* 2000), white perch (*Morone americana*) (Hiramatsu *et al*, 2004; Reading *et al*, 2009; Reading *et al*, 2011), European sea bass (*Dicentrarchus labrax*) (Yilmaz *et al*., 2015; Yilmaz *et al*, 2016), rainbow trout

(*Oncorhynchus mykiss*) (Mouchel *et al*., 1996; Prat *et al*., 1998; Davail *et al*., 1998; and Bouter *et al*., 2010) and masu salmon (*Oncorhynchus masou)* (Fujita *et al*., 2005). Fish Vtg proteins are usually encoded by multigene families containing several genes which are tandemly arranged. The molecular size differs from species to species, but it has conserved function and shares a common general structure (Buisine, Trichet and Wolff 2002; Panprommin *et al.*, 2008). The teleost Vtg is composed of signal peptide, lipovitellin (Lv: consisting of heavy chain [LvH] and light chain [LvL]), phosvitin (Pv), β′-component (β′c), and C-terminal coding region (Ct) , and its subdomain structure is a pentapartite NH^2-(LvH-Pv-LvL- β′-CT)-COO^- (Fig. 1) when signal peptide is removed (Fin and Kristoffersen, 2007; Finn, 2007; Yilmaz *et al*., 2016; Ghosh *et al*., 2016). Lv is the largest part of Vtgs which is an apoprotein transporting amount of phospholipids into oocyte (Yilmaz *et al*., 2015). The LvH plays an important role in VtgR binding, and the receptor binding motif HLTKTKDL, ILLTKTRDL, ILLTKTKDM and TITQVVDVS, located in LvH, are verified in tilapia (Li *et al.*, 2003), VtgAa, VtgAb and VtgC of white perch, respectively (Reading *et al*., 2009). While the Pv is the smallest part, consisting of abundant phosphorylated serine, which increases Vtg solubility and strengthens its structure stability. Cysteine-rich C-terminal domains (β′c and Ct) was reported to protect Vtgs and YPs from early inappropriate proteolysis, and help the Vtg dimer, containing symmetric binding sites, recognize the VtgR and then bind it. (Finn, 2007; Yilmaz *et al*., 2015).

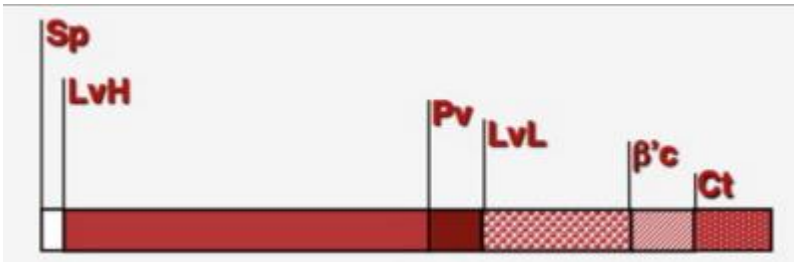


Figure. 1: General structure of fish vitellogenin (retrieved from Yilmaz *et al.,* 2015)

Finn and his colleagues (2009) proposed a model that the spiny rayed teleost (Acanthomorpha) Vtgs could be divided into VtgC and Vtg A-type. The VtgA-types are complete pentapartite proteins, while VtgC is lacking in phosvitins and C-terminal domains and the subdomain structure is (NH^2-[LvH-LvL]-COO^-). The two types of coding genes were separated during the second round of whole genome duplication (WGD) >450 million years ago. Subsequently, the *VtgA*-type genes duplicated and generated paralogous *VtgAa* and *VtgAb* genes

due to chromosomal arrangements followed by lineage-specific gene duplications in the third round of WGD in teleost. The phylogenetic model has been confirmed by Reading *et al*. (2009). The molecular mass of three VtgAa, VtgAb, and VtgC monomers of white perch are ~180, ~180, and ~140 kDa respectively (Reading *et al*., 2008).

During oocyte maturation, heavy chain lipoprotein (LvH) from VtgAa (LvHAa), generates a large pool of free amino acid (FAA) under the catheptic proteolysis, and changes the oocyte osmotic gradient which promotes oocyte hydration, resulting in appropriately floating eggs. LvH from VtgAb is only partially proteolyzed thus contributes to minimum oocyte hydration. Compared to VtgAa and VtgAb, VtgC contributes to limited FAA and is selectively utilized as a nutrient source for late-stage larvae (Reading *et al*; Finn *et al*, 2009). As a result, various ratios of VtgAa:VtgAb:VtgC in oocyte decide the pelagic or benthic egg (Farrell, 2011).

1.1.3 Estrogen induced Vtg gene expression

The steroid hormone E2 is the primary female sex hormone which stimulates *Vtg* expression during vitellogenesis (Bowman *et al.,* 2002). Estrogens are synthesized under the regulation of the hypothalamic-pituitary-gonad axis, diffuse into the cell and bind to estrogen receptors (ERs). Thereafter, the activated ERs form homo- or hetero dimers which are transferred to the cell nucleus bind to specific estrogen response elements (ERE) in the promoter region of estrogen-responsive DNA sequences. (Polzonetti-Magni *et al*., 2004; Gruber *et al.,* 2004). ERs have the strongest affinity for the 15 bp palindromic sequence that consist of two 6 bp inverted repeats inserted with 3 bp spacer. The nucleotide alteration in each ERE half-site and the various spacer size can affect the binding affinity of ERs (Klinge, 2001; Gruber *et al*., 2004). The sequence **aggtca**nnn**tgacct** is designated as the consensus sequence, while **gggg**c**a**nnn**t**a**acct** (the underlines represent mutation from consensus ERE) was detected as the putative functional ERE in rainbow trout *Vtg* (Bouter *et al.,* 2010). With the gradual increase of E2 in plasma during vitellogenesis, ovarian weight and *Vtg* level increased consistently, reaching the peak in the pre-spawning (Ghosh *et al.,* 2016). E2 is specific to maturing females and not normally detected in males and juveniles, whereas exogenous estrogen injection can induce the *Vtg* expression. Therefore numerous studies presented that *Vtg* is a useful biomarker of xenoestrogen exposure in male oviparous vertebrates to monitor environmental estrogenic pollution (Utarabhand and Bunlipatanon, 1996; Booth and Skene, 2006; Levi *et al*., 2009)

1.2 Fish Vtg receptors (VtgR)

1.2.1 Structure of fish VtgR

VtgR localizes in coated pits on the surface of oocytes, binds the Vtgs and transports them into oocytes (Schneider, 1996; Prat *et al.*, 1998; Hiramatsu *et al*., 2003; Hiramatsu *et al*., 2004). The molecular characterization and expression of VtgR have been studied in rainbow trout (Davail *et al*., 1998; Prat *et al*., 1998), cutthroat trout (*Oncorhynchus clarki*) (Mizuta *et al*., 2013), white perch (Hiramatsu *et al*., 2004; Reading *et al.*, 2011), and tilapia (*Oreochromis aureus*) (Li *et al*., 2003). These studies showed that VtgR belongs to the low density lipoprotein receptor family (LDLR) which has the five common characters; i) cysteine-rich ligand-binding domain (LBDs); ii) epidermal growth factor-precursor (EGF) repeats with six cysteines containing each; iii) O-linked sugar, commonly with 5 consensus tetrapeptide motifs; iv) a single transmembrane domain (T), anchoring in the plasma membrane receptor; and v) a census peptide in the cytoplasmic domain (C) (Fig. 2).

Figure. 2: General structure of the vitellogenin receptor comprising the ligand binding domain (LBD), epidermal growth factor-like (EGF), propeller domains (YWTD), transmembrane (T) domain, and cytoplasmic (C) domain (retrieved from Dominguez *et al*., 2012).

The teleost VtgR typically has a single form with the size of ~100 kDa and belongs to the very low density lipoprotein receptor (VLDLR) branch of the LDLR superfamily (Daivail *et al*., 1998). The VtgR is ~91 kDa in rainbow trout (review: Davail *et al.*, 1998), about 95-105 kDa in cutthroat (Mizuta *et al*., 2013), and 100 kDa in coho salmon (*Oncorhynchus kisutch)* (Stifani *et al*., 1990). The VLDLR has eight ligand binding domains (LR8) which differs from LDLR that only has seven ligand binding domains (LR7). Based on the presence or absence of the O-linked sugar domain, the LR8 is characterized as two forms, the form lacking the O-linked sugar domain (LR8-) and the form that contained the O-linked sugar domain (LR8+). The LR8- was mainly expressed in ovary, whereas the LR8+ was highly expressed in somatic tissues in rainbow trout and cutthroat trout (Prat *et al*., 1998; Mizuta *et al*., 2013).

1.2.2 Ligand binding to VtgR

The ligand-binding domains control the interaction between receptor and lipoproteins (Review: Dominguez *et al.*, 2012). Different forms of lipoprotein show different affinity to various LBDs, for example, LBD 5 is important for binding apolipoprotein E (apoE), LBD 2-7 cooperatively bind apoB, and the first three LBDs were reported to interact with Vtgs in tilapia [Russell *et al*. (1989), as cite in Li *et al.*, 2003]. Additionally, different forms of Vtg bind different forms of VtgR (Review: Yilmaz *et al*., 2015). Reading *et al.* (2011) purified three types of white perch vitellogenin (VtgAa, VtgAb and VtgC), and ligand blotting revealed three forms of VtgR, a large receptor (>212 kDa) that bound only to VtgAa, two smaller receptors (~116 and ~110.5 kDa) bound to VtgAb, and the VtgC did not specifically bind to ovarian membrane proteins in either assay. However, the Vtg-VtgR interactions are still unclear.

1.2.3 Tissue expression of fish VtgR

Davail and his college (1998) used the full-length trout *VtgR4* cDNA as probe in Northern blots, and showed a specific transcripts of ~3.9 kb in ovary, but not in heart and muscle, which are the major sites expressing VLDLR in mammals. Li *et al*. (2003) cloned and characterized two forms of tilapia *VtgR*, and found that *VtgR* was specifically expressed in ovarian tissues by Northern analysis, but reverse transcription polymerase chain reaction (RT-PCR) showed that the trace levels of expression of VtgR or homologous LDLR existed in other somatic tissues. Hiramatsu *et al*. (2004) and Mizuta *et al.* (2013) used real-time reverse transcription-polymerase chain reaction (RT-qPCR) to measure *VtgR* expression in white perch and cutthroat trout. The results revealed that ovary was the main site for *VtgR* mRNA expression and the highest expression emerged during previtellogenesis and decreased with oocyte growth.

1.3 Sexual maturation and spawning in Atlantic salmon

Atlantic salmon (*salmon salar*) is an anadromous species, and it means they migrates between the ocean and river during their different life stages (Liu *et al.*, 2011). After spawning in freshwater in the autumn, the eggs are hatched over winter and emerge in spring as fry. The growing parr often spend 2-5 years in freshwater and then undergo physiological and behavioral

changes, a process called smoltification, which prepares them for ocean life (Linnaeus, 1758; Hansen and Quinn, 1998). After spending1, 2 or more years at sea until sexual maturity, they return to their freshwater rivers to spawn. However, for farmed Atlantic salmon, the whole farming production cycle is about 3 years, and the total freshwater production cycle takes approximately 10-16 months which is at least one year shorter than wild salmon (Harvest, 2015).

1.4 Tetraploid genome in salmonids

Salmonids belong to the protacanthopterygii that is the most primitive group of teleost (Davidson *et al.*, 2010). They are classified into nine genera which have more than 92% similar DNA according to the Nelson (2006). The common ancestor of salmonids allegedly experienced all chromosomes duplication event about 90 million years ago (Macqueen and Johnston, 2014). Buisine, Trichet and Wolff (2002) presented that most salmonids, except *Oncorhynchus* genus, inherited two *Vtg* clusters, *VtgA and VtgB,* from their common tetraploid ancestor, and *Oncorchynchus* species have only cluster *VtgA* and lost the *VtgB* cluster.

2. MATERIAL AND METHODS

2.1 Materials

Chemicals	**Producer**
PureLink™ RNA Mini Kit	Thermo Fisher Scientific, USA
70% EtoH (ethanol), 30%DEPC (Dietyl-Poly Carbonate 0.1%)	
5 PRIME Isol-RNA Lysis Reagent (5P)	USA
Chloroform	VWR International, PA, USA
PureLink™ DNase For Use with PureLink™ Kits	Thermo Fisher Scientific, USA
TaqMan® Reverse Transcription Reagents	Thermo Fisher Scientific, USA
LightCycler®480 SYBR Green I Mater	Roche Applied Science, Germany
AmpliTaq Gold with Gene Amp	Applied Biosystem
SYBR Safe DNA gel stain	Invitrogen by life technology, USA
Equipment	
Precellys 24 Lysis and homogenization	Birtin Technology, France
Centrifuge 5424 and 5415R eppendorf	VWR
LightCycler®480	Roche Diagnostics Gmbh, Germany
Avanti™ J-30 I Centrifuge	Beckman Coulter, USA
Veriti 96 Well Thermal Cycler	Applied Biosystem
NanoDrop 1000 Spectrophotometer	BMG LAVTECH, Ortenberg, Germany
Bio-Rad electrophoresis apparatus	Bio-Rad, China

2.2 Methods

2.2.1 Experimental fish and samples

The Fish examined in this study were reared by the AquaGen salmon breeding company at Kyrksæterøra/ Hemne, Sør-Trøndelag, Norway. Start-feeding of the hatched larvae was initiated in February 2012, and the one-year smolt were transferred to the sea cage in May 2013. The fish were treated with artificial light (LD 24:0) from March 2015 to promote sexual maturation, then transferred into indoor freshwater tank ($60m^3$) in May 2015, and reared at the photoperiod of LD 8:16 until spawning in September and early November 2015. The temperature was recorded regularly in 3 and 6 m depth sea cage and indoor tanks (Appendix 1).

In order to analyze annual changes in gene expression of Vtgs and VtgR, ovary, liver, brain and heart were collected monthly from five females and two males from Sep 2014 to Sep 2015. Plasma estrogen levels were measured in blood sampled from females. The fish weight and length were registered after they were anaesthetized using Metacain and sacrificed by cutting gill arteries. Blood samples were collected from the caudal vein, mixed well, centrifuged at 500rpm for 10 minutes in 4°C and kept on ice until transfered to the -80°C freezer in Vessøra. The gonads were weighed for calculating the gonad-somatic index (GSI; gonad weight /body weight). The dissected tissues were kept immediately in RNA-later for extraction of total RNA.

2.2.2 RNA isolation and measurement

The total RNA from salmon liver, ovary, brain and heart tissue was isolated using PureLink™ RNA Mini Kit. This kit provides a safe, simple, reliable, and rapid column-based method for isolating high-quality total RNA. Approximately 20mg tissue was added into 800 µl lysis buffer, using Precyllis 24 with 2×20 seconds for homogenizing at 5500 rpm. After 2 minute centrifuging at 12000×g, 600 µl supernatant was mixed well with 700µl 70% EtOH. Then the mixture was transferred to the filter tube and centrifuged at 12000×g for 30 second for RNA attaching. 350 µl wash buffer I was added to the filter tube and centrifuged again. The RNA attached filter was treated by PureLink™ DNase kit which provides rapid and efficient removal of DNA from RNA. 80 µl DNase solution was added, incubating at room temperature for 15-20 minute. Thereafter, the RNA was washed by 350µl wash buffer I once and 500 µl wash buffer II

twice at the same centrifuging speed of 12000×g for 30 second. After washing, the tube was centrifuged empty in 12000×g for 1 minute to remove the redundant reagent. 60 μl RNase free water was added to the filter tube and incubated at room temperature for 1 minute. Finally, RNA was eluted by centrifugation at 12000×g for 2 minute. The RNA samples were kept at -70℃ until analysis.

Besides the PureLink™ RNA Mini Kit, Isol-RNA Lysis Reagent (5P) was needed to optimize RNA purification from tissues with high fat content like ovary. 50-100mg tissue was homogenized in 1ml Isol-RNA Lysis Reagent. After 5 minute incubation in room temperature, 200μl Chloroform was added into the tube and mixed well. Thereafter, the sample was incubated in room temperature for 2-3minute and then the homogenate is separated into aqueous and organic phases by centrifugation at 12000×g for 15 minute in 4℃. 500 μl aqueous and equal volume of PureLink™ RNA lysis buffer were mixed well with 500 μl 96% EtOH. The rest steps about binding, washing and eluting were as same as the above.

NanoDrop 1000 Spectrophotometer was used to measure the RNA concentration and purity. 260/280 ratio of sample is used to assess the purity of DNA and RNA. 260/230 ratio is a parameter of nucleic acid purity. All RNA samples in our experiments had 260/280 ratios between 1.8 ~2.3 which means high quality RNA.

2.2.3 cDNA synthesis and quantitative real-time RT-PCR (qPCR)

TaqMan® Reverse Transcription Reagents was used to perform the reverse transcription of RNA to cDNA. Mixture was made first as table1, and then was added into 96 well plate with 3.7 μl RNA (40 ng/ μl). The plate was centrifuged at AvantiTM J-30 I Centrifuge at speed of 755 ×g for 1 minute. After then, the cDNA synthesis was run in Veriti 96 Well Thermal Cycler machine under the setting showed in table1. Finally, the cDNA was stored at -20℃ for qPCR running.

Quantitative real-time PCR (qPCR) has proven to be a powerful tool to quantify gene expression (Livak and Schnittgen, 2001; Schnittgen and Livak, 2008). It is a well-established procedure for converting the continuous collection of fluorescent signal from polymerase chain reaction into a numerical value for each sample (Dorak, 2007a). LightCycler® 480 SYBR Green I Master, a one-component hot start reaction mix which contains FastStart Taq DNA Polymerase

and detected fluorescent, was used as a DNA double-strand-specific dye, which provides convenient, sensitive, specific and quantitative PCR. In addition of SYBR Green, the qPCR reaction requires template cDNA and gene-specific primers. Elongation factor 1 *alpha* (*EF1 a*) was chosen as the internal control gene because of its stable expression (Mizuta, *et al*, 2013). The target gene primer sequences were designed as table2.

Table 1: cDNA mixture preparing and running procedure setting

Reagens		***1x (µl)***
10xRT-buffer		1.0
MgCl2 (25 mM)		2.2
dNTP (2,5 mM)		2.0
oligo-dT		0.3
hexamer		0.3
RNase inhib		0.3
RT		0.2
Totalt mix		6.3
RNA + h2o (40 ng/ul)		3.7
Veriti 96 Well Thermal Cycler		
cDNA-program:	25 ℃	10 min
	48 ℃	60 min
	95 ℃	5 min

Table 2: Sequence of primes

Reference gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
EF1a	CACCACCGGCCATCTGATCTACAA	TCAGCAGCCTCCTTCTCGAACTTC
Target gene		
Vtg Asa	TGAAGGACTTTGGTCTGGCTTACACA	CTGCTGGCACTCTACACACTTC
Vtg Asb	TCAAAGAGTTTGGTCTGGCTTACATG	CTGCTGGCACTCAACACATTTC
Vtg R1	TCTGTAACGGGGAGGATGAC	ACAGGAGGATGGAGCACATT
Vtg R2	TCCCGCAACTTTGTGTGTAA	GCGTTGCCACATTGAAACT

The reaction mix for qPCR consisted of 6 µl diluted (1:10) cDNA, 0.5 µl forward primer, 0.5 µl reverse primer and 5 µl SYBR Green I Master. All samples were analyzed in triples. The qPCR was proceeded in LightCycler®480 machine at the running condition as showed in table3.

PCR amplification efficiency can be calculated by running a template dilution series. All samples were collected to make a gene pool. The gene pool were diluted by 5 series of 1:1, 1:2,

1:4, 1:8, 1:16 and then used as template for 5 primers. After running of PCR, the efficiency of different primers were worked out by the LightCycler®480.

Table 3: qPCR running program setting

Setup			
Detection Format	**Block Type**	**Reaction Volume**	
SYBR Green	384	12 µl	
Programs			
Program Name	**Cycles**	**Analysis Mode**	
Pre-Incubation	1	None	
Amplification	45	Quantification	
Melting Curve	1	Melting Curve	
Cooling	1	None	
Temperature Targets			
Target (°C)	**Acquisition Mode**	**Hold (hh:mm:ss)**	**Ramp Rate (°C/s)**
Pre-Incubation			
95	None	00:05:00	4.4
Amplification			
95	None	00:00:15	4.4
60	None	00:00:15	2.2
72	Single	00:00:15	4.4
Melting Curve			
95	None	00:00:15	4.4
65	None	00:01:00	2.2
97	Continue	–	(0.11)
Cooling			
40	None	00:00:10	1.5

2.2.4 RT-PCR tissue expression of VtgR1 splice variants

The PCR reaction mixture contained 15 µl dH2O, 2.5 µl 10×PCR Buffer (contains 15mM $Mgcl_2$), 1 µl dNTP, 1.5 µl AmpliTaq Gold Polymerace, 0.5 µl of each forward and reverse primer (Table 4), and 4 µl cDNA in a total volume of 25 µl. The PCR was run for 40 cycles followed the conditions: 95℃ for 30s, 60℃ for 30s, and 72℃ for 30s. Thereafter, 4 µl PCR products and 1 µl loading dye was subjected to electrophoresis through a 1.5% agarose gel in 1× TBE buffer at 80V with 100 bp DNA marker.

Table 4: Primers setting sequences

Gene	**Forward primer (5'-3')**	**Reverse primer (Vtg R1_2) (5'-3')**
Vtg R1 X1	GCAGACCAGCTCCCTCAAA	GCCACATCAAGTAGCCTCCT
Vtg R1 X2	GCAGACCAGCGACCACAG	
Vtg R1 X3	TGCAGACCAGAAGCCAACA	

2.2.5 Phylogenetic and alignments study

Atlantic salmon Vtgs and VtgRs amino acid sequences in this study were retrieved from National Center for Biotechnology Information (NCBI), and other teleost Vtg and VtgR sequences were identified by Basic Local Alignment Search Tool (BLAST) (Appendix 2). Thereafter, the web phylogeny.fr (http://phylogeny.lirmm.fr/phylo_cgi/simple_phylogeny.cgi) was used to present the phylogenetic tree for Vtgs and VtgRs respectively (Dereeper *et al.*, 2008). The alignments were constructed in the web phylogeny.fr (http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=muscle) combined with NCBI (Edgar, 2004).

The molecular weight of the salmon proteins were calculated using the Compute pI/Mw tool at http://web.expasy.org/compute_pi/.

2.2.6 Data analysis method

The log2 Pfaffl values method were more reliable than the ΔΔCt method to quantify the gene expression. The ΔΔCt method is based on the assumption of theoretical 100% amplification efficiency of PCR, and that the internal control gene and target genes have similar PCR efficiency (Livak and Schnittgen, 2001). However, the log2 Pfaffl was the improvement of ΔΔCt method which accounted for actual PCR efficiency. Pfaffl values were calculated in the Microsoft Excel using the equation of Pfaffl values= $(1+E_{Ref})^{\Delta Ct\ Ref\ (Ct\ sample\ -Ct\ Mean\ of\ control\ group)}$/ $(1+E_{Target})^{\Delta Ct\ Target\ (Ct\ sample\ -Ct\ Mean\ of\ control\ group)}$. Ct values were defined as the number of cycles required for the fluorescent signal to cross the threshold (Dorak, 2007b). The Ct values of target and reference genes were exported from the LightCycler®480 and all the Ct >38 were excluded, because it indicated a weak reaction and the amounts of target genes were minimum.

The final datum were analyzed by One-way analysis of variance (ANOVA) followed by Tukey-Kramer Honestly Significant Difference (TukeyHSD) ($P<0.05$ means significant difference) and presented in means ± standard errors by using the R-studio software.

3. RESULTS

3.1 Sequence alignment and phylogenetic analysis of salmon Vtgs and VtgRs

Two *Vtgs* were identified in the Atlantic salmon genome, named *VtgAsa* and *VtgAsb*. Salmon *VtgAsa* (NCBI access No. AGKD03028439.1) consisted of 34 exons and codes for a protein of 1659 amino acids (aa) with a calculated molecular weight of 182662 Da. The alignment of salmon VtgAsa with white perch VtgAb showed that the general structure were similar and were composed of full linear YP (LvH, Pv, LvL, β′-c, and C-terminus) (Fig. 3). The salmon *VtgAsb* (NCBI access No. AGKD03007768.1) consisted of only 10 exons and coded for protein of 459 aa with a molecular weight of 50683 Da. This truncated protein was lacking the LvL, Pv and C-terminus, but also the CGLC motif and potential O- / N-linked glycosylation sites. Nonetheless, both VtgAsa and VtgAsb contained the conserved short motif IHLTKSKDL and VHLTKTKDL respectively, necessary for the transfer into the oocytes.

Imperfect palindromic ERE motifs were identified in the promoter of salmon *VtgAsa* and *VtgAsb* by searching for the consensus **aggtca**nnn**tgacct** in the upstream genomic sequences. Both promoters have the same putative ERE sequence **tgg<u>a</u>ca**t**tga<u>t</u>ct** in addition to the **aggtca**agc**tgacc<u>a</u>** and **gggg<u>c</u>a**ggt**<u>t</u>aacct** motif in *VtgAsa* and **agg<u>c</u>cg**ggt**<u>t</u>aacct** in *VtgAsb*, which were located 186 bp (*VtgAsa*) and 197 bp (*VtgAsb*) upstream of the translation start signal (ATG), respectively (Fig. 5).

The Atlantic salmon genome was shown to harbor two *VtgR* genes named *VtgR1* and *VtgR2* (NCBI access No. XM_014138209.1 and XM_014128995.1). The predicted salmon VtgR1 of 873 aa (96343 Da) and VtgR2 of 842 aa (93180 Da) showed highly similar structure with European seabass VtgR with eight cysteine-rich LBDs, three EGFs, five YWTDs, one TM and one CD, and VtgR1 had O-linked sugar domain (Fig. 4).

Phylogenetic tree of Vtgs and VtgR were constructed based on amino acid sequences. The Vtg tree comprises the five clusters of VtgC, VtgAb, VtgAa, salmonid and carp (Fig. 6 A). The VtgAsa and VtgAsb of Atlantic salmon resided in different branch of the salmonid cluster with the VtgAsa closer to rainbow trout and white spotted Arctic Char (*Salvelinus leucomaenis).*

The salmon VtgR1 and VtgR2 were found in different clusters, and VtgR1 was in the same branch as cutthroat trout and rainbow trout (Fig. 6 B).

```
                    Signal peptide | LvH
WP VtgAb      1     MRVLVLAFTVALAAANHINFAPEFAAGKTYVYKYEALIMGGLPEEGLGRAGVKVLSKVLINAASPNTFIVKLVDPEIFEY  80
SS VtgAsa     1     MRAVVLALTLALVASQSVNFAPEFAASKTYVYKYEALVLGGLPEEGLARAGVKIISKVLISAVAENTYLLKLVNPEIFEY  80
SS VtgAsb     1     MKAVVFALTLALIASQHVNFAPAFEASKAYVYKYEALLLGGLPDEGLSRAGIKVISKVLIGAIAPTIYMLKLVDPKIFEY  80
                    *..:*:*:*:** *:: :**** * *.*:********::****:***.***:*::*****.* : . :::***:*:****

WP VtgAb     81     SGIWPKDAFVPATKLTSALAAQLLTPIKFEYTNGVVGRVFAPAGVSVTVLNIYRGILNIFQLNIKKTQNVYELQEPGAQG  160
SS VtgAsa    81     SGVWPKDPFVPAAKLTSALAAQFSIPIKFEYARGVVGKVFAPTAVSETVLNVHRGILNILQLNIKKTQNVYELQEAGAQG  160
SS VtgAsb    81     SGVWSKDPFVPAAKLTSALAAQLLTPIKFEYANGVVGKVFAPTGVSETVLNIHRGILNILQLNIKNTQNVSEIQEVGAQG  160
                    **:*.**.****:*********:  ******:.****.****:.** ****::******:*****:**** *:** ****


WP VtgAb     161    VCKTHYVISEDAKADRILLTKTKDMNQCQERIIKDIGLAYTEKCVECEARGKILKGAAAFNYIMKPTATGALLLEATATE  240
SS VtgAsa    161    VCKTHYVISEDTKAERIHLTKSKDLNNCQERIMKDFGLAYTEKCVECQQRQKTLMGAAAYNYIMKPAASGALIMKATVTE  240
SS VtgAsb    161    VCKTHLVISEDAKTGHVHLTKTKDLNHCQEKIIKEFGLAYMEKCVECQQRGKNLRGAAAYNYIMKPAATGAIIMEDTVTE  240
                    ***** *****:*: .: ***:**:*:***.*:*::**** ******: * * * ****:******:*:**:::: *.**

WP VtgAb     241    LIQFSPFNILNGAAQMEAKQILTFLEIEKTPVVPIRADYLHRGSLQYEFGSELLQTPIQLLKISNAEAQIVEVLNHLVTF  320
SS VtgAsa    241    LHQFTPLNEMTGAAQMEAKQMLTFVEIKNAPIILSDANYDRRGSIRYEFATEILQIPIQLLKISNAHAQAVKILNRLVTY  320
SS VtgAsb    241    LHQFSPFNEMNGAAHMEAKQTLAFIEIKKTPVEPIKEEYLHCGSIQYQFATEILQTPIQLLRISNASAQIVEILNHLVEY  320
                    * **:*:* :.***:***** *:*:**:::*:     :* . **:.*:*.:*:** *****.**** ** *::**.** :

WP VtgAb     321    NAAKVHEDAPLKFIELIQLLRVARYESIEALWTQFKARPDYRHWVMNAVPAIGTHVALRFLKDKFLAAELTIAEAAQALL  400
SS VtgAsa    321    DTAQVHDDAPLKFMQFIQLLRMASSETIEAIWAEFKDKPAYRHWILDAVPSIGSRVAVRFIKEKFLAGDITIFETAQALV  400
SS VtgAsb    321    NVAKVHVDAPLKFIQLVQLMRVASYESIKAIWNQFKAKPAFRYWILDAIPAIGTPVTLKFIKEELVDGHITIAEAAPALV  400
                    :.*:** ******::::**:*:*  *:*:*:* :** .* :*:*:::*:*:**: *::.*:*:::: . :** *:* **:

                                                                N
WP VtgAb     401    ASVHMVTADLEAIKIVADLAMNNKIQENPVLREIVMLGYGTLVAKFCTENPTCPAELVKPIHELAVQAAAEGEIEELVVA  480
SS VtgAsa    401    AAVHMVAADLETVKLAESLAFNHKIQTHPVLREIAMLGYGTMVSKYCVENPNCNPELVKPIHERAVEAVANSKFEELSMV  480
SS VtgAsb    401    AAVHMVTADVETVKLFETLAFNHKIPVNRVLHEVAMLGYGTRISKYCAAYPTCHADFLK----------------------  459
                    *:****:**:*::*:   **:*:**  : **.*:.****** ::*:*.  *.* .:::*

WP VtgAb     481    LKVLGNAGHPASLKTIMKLLPGFGSAAAGLPLRVHIDAVLALRNIAKKEPKMIQEIAVQLFMDKALHPELRMVVAIVLFE  560
SS VtgAsa    481    LKVLGNAGHPASIKPITKLLPVFGTAAAALPLRVQADAVLALRNIAKREPRMVQEVAVQLFMDKALHPELRMLACIVLFE  560
SS VtgAsb           ---------------------------------------------------------------------------------

WP VtgAb     561    TKLPMGLLTTLADALLKEKNLQVASFVYSYMKAMTKNTAPDFASVAAACNVAVKILSPKFDRMSYRFSRALYLDAYHNPW  640
SS VtgAsa    561    TKPPMGLVTTFANILKTEENLQVASFTYSHMKSLTRSTAPDFASVAAACNVAVKMLSTKFRRLSCHFSKAIHLDAYYSPL  640
SS VtgAsb           ---------------------------------------------------------------------------------

WP VtgAb     641    MMGAAASAFYINDAATVLPRAIVAKARTYLAGAYADVLELGVRTEGVQEALLKIHEAPENTERITKMRQVMKALSEWRAN  720
SS VtgAsa    641    RIGAAASAFYINNAATIFPRTVVAKARTYFAGAAADVLEVGVRTEGIQEALLKIPTVTENVDRITKMKRVIKALSDWRSL  720
SS VtgAsb           ---------------------------------------------------------------------------------
                                                                                                    O
WP VtgAb     721    PLSQPLASVYVKFFGQEIAFANIDKAIVDQIIELASGPAIHTYGRRVLDALLSGFAVHYAKPMLVAEVRRILPTVVGLPM  800
SS VtgAsa    721    TTRKPLASIYVKFFGQEVAFANIDKPIIDQALQLANSPSARALGKNALKALLAGATFQYAKPLLAAEVRRIFPTAVGLPM  800
```

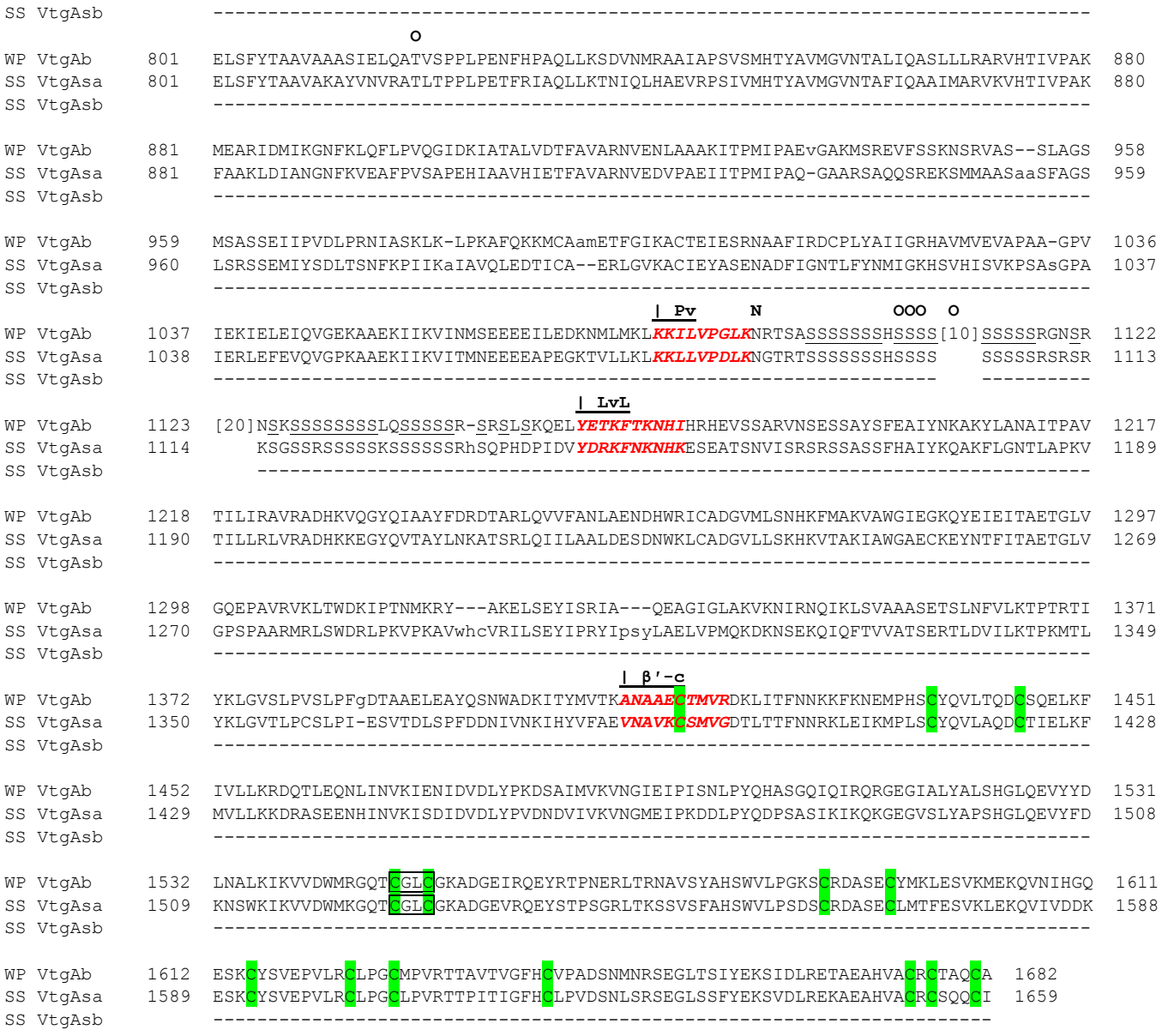
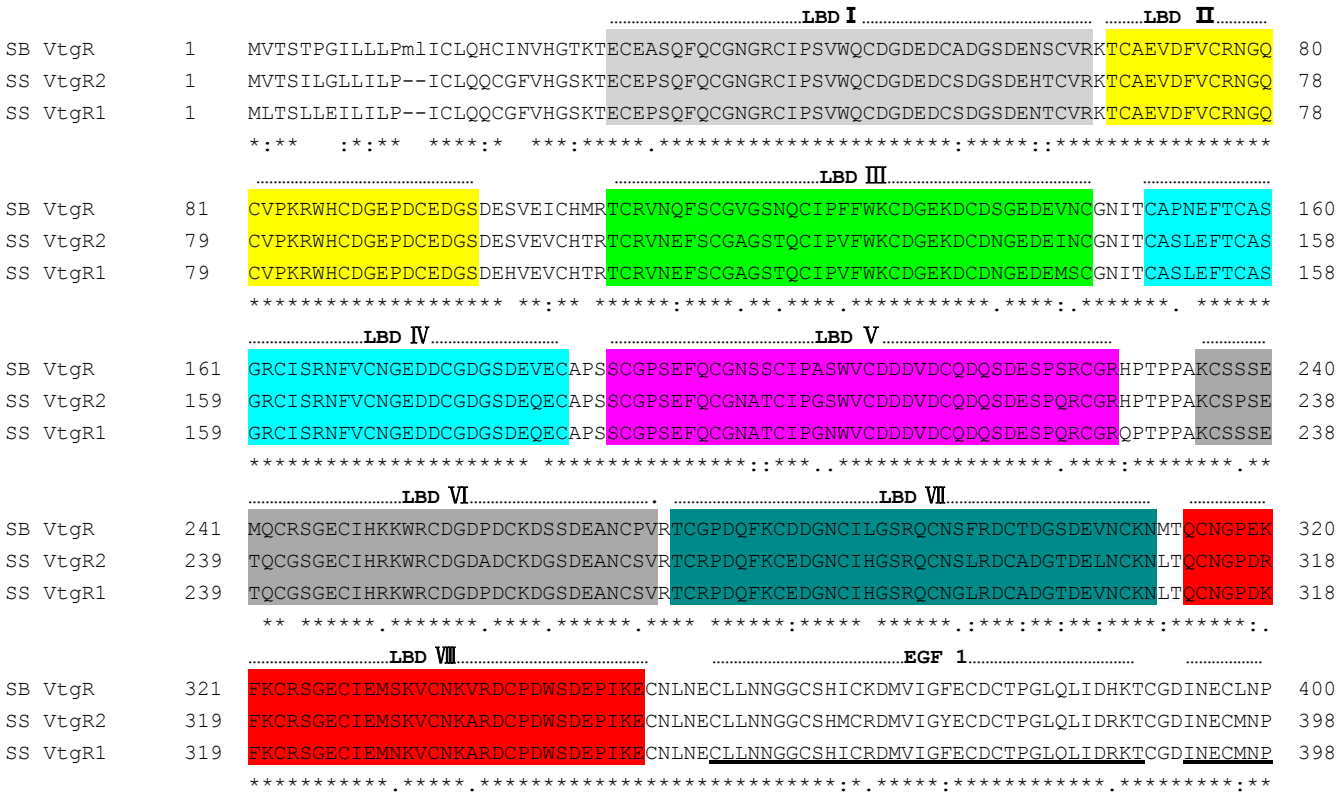

SS VtgAsb
O
WP VtgAb 801 ELSFYTAAVAAASIELQATVSPPLPENFHPAQLLKSDVNMRAAIAPSVSMHTYAVMGVNTALIQASLLLRARVHTIVPAK 880
SS VtgAsa 801 ELSFYTAAVAKAYVNVRATLTPPLPETFRIAQLLKTNIQLHAEVRPSIVMHTYAVMGVNTAFIQAAIMARVKVHTIVPAK 880
SS VtgAsb
WP VtgAb 881 MEARIDMIKGNFKLQFLPVQGIDKIATALVDTFAVARNVENLAAAKITPMIPAEvGAKMSREVFSSKNSRVAS--SLAGS 958
SS VtgAsa 881 FAAKLDIANGNFKVEAFPVSAPEHIAAVHIETFAVARNVEDVPAEIITPMIPAQ-GAARSAQQSREKSMMAASaaSFAGS 959
SS VtgAsb
WP VtgAb 959 MSASSEIIPVDLPRNIASKLK-LPKAFQKKMCAamETFGIKACTEIESRNAAFIRDCPLYAIIGRHAVMVEVAPAA-GPV 1036
SS VtgAsa 960 LSRSSEMIYSDLTSNFKPIIKaIAVQLEDTICA--ERLGVKACIEYASENADFIGNTLFYNMIGKHSVHISVKPSAsGPA 1037
SS VtgAsb
| Pv N OOO O
WP VtgAb 1037 IEKIELEIQVGEKAAEKIIKVINMSEEEEILEDKNMLMKLKKILVPGLKNRTSASSSSSSSHSSSS[10]SSSSSRGNSR 1122
SS VtgAsa 1038 IERLEFEVQVGPKAAEKIIKVITMNEEEEAPEGKTVLLKLKKLLVPDLKNGTRTSSSSSSSHSSSS SSSSSRSRSR 1113
SS VtgAsb
| LvL
WP VtgAb 1123 [20]NSKSSSSSSSSLQSSSSSR-SRSLSKQELYETKFTKNHIHRHEVSSARVNSESSAYSFEAIYNKAKYLANAITPAV 1217
SS VtgAsa 1114 KSGSSRSSSSSKSSSSSSRhSQPHDPIDVYDRKFNKNHKESEATSNVISRSRSSASSFHAIYKQAKFLGNTLAPKV 1189
SS VtgAsb
WP VtgAb 1218 TILIRAVRADHKVQGYQIAAYFDRDTARLQVVFANLAENDHWRICADGVMLSNHKFMAKVAWGIEGKQYEIEITAETGLV 1297
SS VtgAsa 1190 TILLRLVRADHKKEGYQVTAYLNKATSRLQIILAALDESDNWKLCADGVLLSKHKVTAKIAWGAECKEYNTFITAETGLV 1269
SS VtgAsb
WP VtgAb 1298 GQEPAVRVKLTWDKIPTNMKRY---AKELSEYISRIA---QEAGIGLAKVKNIRNQIKLSVAAASETSLNFVLKTPTRTI 1371
SS VtgAsa 1270 GPSPAARMRLSWDRLPKVPKAVwhcVRILSEYIPRYIpsyLAELVPMQKDKNSEKQIQFTVVATSERTLDVILKTPKMTL 1349
SS VtgAsb
| β'-c
WP VtgAb 1372 YKLGVSLPVSLPFgDTAAELEAYQSNWADKITYMVTKANAAECTMVRDKLITFNNKKFKNEMPHSCYQVLTQDCSQELKF 1451
SS VtgAsa 1350 YKLGVTLPCSLPI-ESVTDLSPFDDNIVNKIHYVFAEVNAVKCSMVGDTLTTFNNRKLEIKMPLSCYQVLAQDCTIELKF 1428
SS VtgAsb
WP VtgAb 1452 IVLLKRDQTLEQNLINVKIENIDVDLYPKDSAIMVKVNGIEIPISNLPYQHASGQIQIRQRGEGIALYALSHGLQEVYYD 1531
SS VtgAsa 1429 MVLLKKDRASEENHINVKISDIDVDLYPVDNDVIVKVNGMEIPKDDLPYQDPSASIKIKQKGEGVSLYAPSHGLQEVYFD 1508
SS VtgAsb
WP VtgAb 1532 LNALKIKVVDWMRGQTCGLCGKADGEIRQEYRTPNERLTRNAVSYAHSWVLPGKSCRDASECYMKLESVKMEKQVNIHGQ 1611
SS VtgAsa 1509 KNSWKIKVVDWMKGQTCGLCGKADGEVRQEYSTPSGRLTKSSVSFAHSWVLPSDSCRDASECLMTFESVKLEKQVIVDDK 1588
SS VtgAsb
WP VtgAb 1612 ESKCYSVEPVLRCLPGCMPVRTTAVTVGFHCVPADSNMNRSEGLTSIYEKSIDLRETAEAHVACRCTAQCA 1682
SS VtgAsa 1589 ESKCYSVEPVLRCLPGCLPVRTTPITIGFHCLPVDSNLSRSEGLSSFYEKSVDLREKAEAHVACRCSQQCI 1659
SS VtgAsb

Figure. 3: Alignment of deduced amino acid sequences of white perch (*Morone americana)* VtgAb (WP) and Atlantic salmon (*Salmo salar*, SS) VtgAsa and VtgAsb. Residue positions were indicated by the numbers on the left and right. Signal peptide was highlight in blue. Internal peptide sequences were indicated in red italic boldface type. Black italic letters set in boldface type indicated biochemically determined amino acid sequences of perch Vtg-derived polypeptides. N-terminal sequences of white perch YPs were indicated by a vertical line above the perch Vtg sequences labeled with the name of LvH, Pv, LvL, and β'-c. The locations of cysteine residued at the C-terminus of Vtg were highlight in green. The conserved CGLC motif was boxed. The (O) and (N) above the sequence indicated potential O- and N-linked glycosylation sites in white perch Vtg sequences, respectively. Potential phosphorylation sites in the Pv domain were underlined. The putative Vtg receptor-binding region was highlight in red for perch and yellow for salmon. Dashes indicated gaps inserted for optimal alignment. Asterisks or dot under each pair of aligned amino acids indicated identity or similarity, respectively.



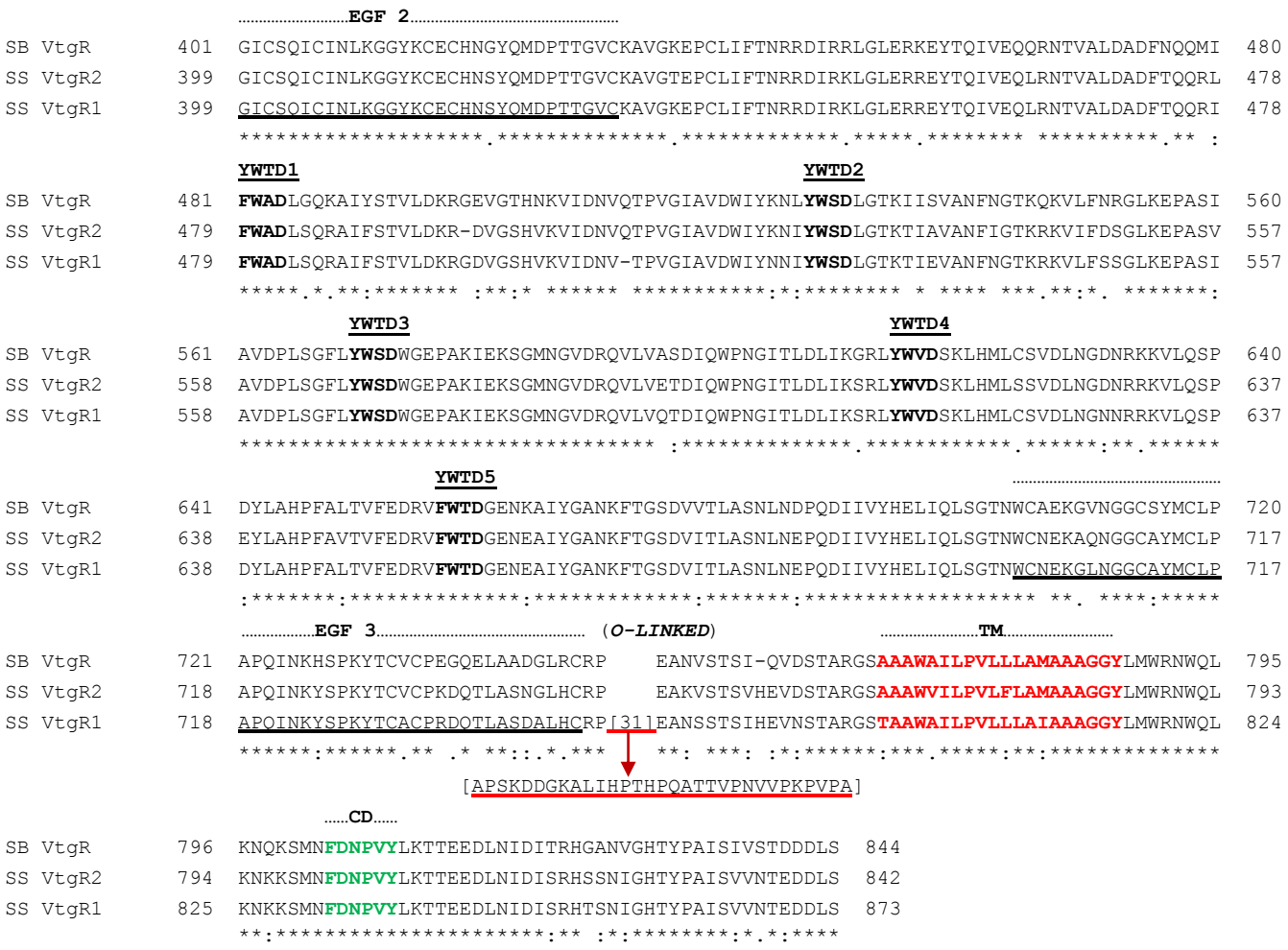


Figure. 4: Alignment of amino acids for European seabass, *Dicentrarchus labrax* (SB) VtgR, and two Atlantic salmon, *Salmo salar* (SS) VtgR1 and VtgR2. The O-Linked domain was underlined in red in SS VtgR1. Eight ligand-binding domains (LBDs) were highlight in different colors, three epidermal growth factor-like domains (EGF 1,2, and 3)were underlined in black, five YWTD propeller domains were in bold, transmembrane (TM) was in red bold, and cytoplasmic domain (CD)was in green bold. The number in the bracket meant extra amino sequence. Dashes indicated gaps inserted for optimal alignment. Asterisks or dot under each pair of aligned amino acids indicated identity or similarity, respectively.

(A)

attgaacaacaaacctgttgtaggcctgcatattcagtgtcattgactgacaagctggcatagtcttatctat**aggtca**agc**tgacca**ttgttgccacatggttggttcatctgaataa
caaatacttgttcaaggacttcaatagcgttgaggactagcttggaccaatggctgatccatgttcaagtgccaaaactcgtcgggttttgacattactagtattctaatttatcacat
tctctaacatattagaattgatcataaatgtactgatccaaacctagacaatatattttatttagcattgtgttgcatcattatttaggcaccaactgcctacttatctctactgggtt
ctacttctaaagtacaagagcctttgtgtacatttttaattagtggttagataaatagagaaaaataactaatgtagtatttaaagaaaaatgaaggtcatagtatacataacttgttg
gataaaacaccatgtgattctccagacaattttcattaaagaactgacttaga**tggaca**t**tgatct**gtaaagggctaaatggcagt**ggggca**ggt**taacct**aacctatgggtgtatgtg
gccacctcagaactagtgataacccactcaacaggctttattatgagcacatactgatctcaacttatgtaacgttagttaattatatattttgttacgattgttttgtcgacagaaaa
ttgctttaaaaggcagtctgggactgttgttcctcacatcacactggcc**ATG**

(B)

taggt**tggacattgatct**ttcaacacagagaagcagatatgaa**aggccgggttaacct**aaccttatgagtttgtgatctctctcatcctgtttctcagtcagattgtctaatttctac
aatctgagccaaatctaaaaaaaactacttggcaatgctgtccatttgttaatgatatatttttgccacaggcattatcagcaaaagtttagctttaaaaggctgcctgggactgtttt
cctcacatccaactgacc**ATG**

Figure. 5: Several potential estrogen responsive elements (EREs) in the promoter of salmon in *VtgAsa* **(A)** and *VtgAsb* **(B)**. The potential ERE sequences were highlight in bold yellow and the same putative ERE of *VtgAsa* and *Asb* were boxed in dotted line. The functional ERE motifs that similar to rainbow trout pERE2 were boxed in solid line respectively. The nucleotide alteration in each half-site of the ERE palindrome were underlined compared to the consensus ERE sequence. Transcriptional start signal (ATG) was in bold green.

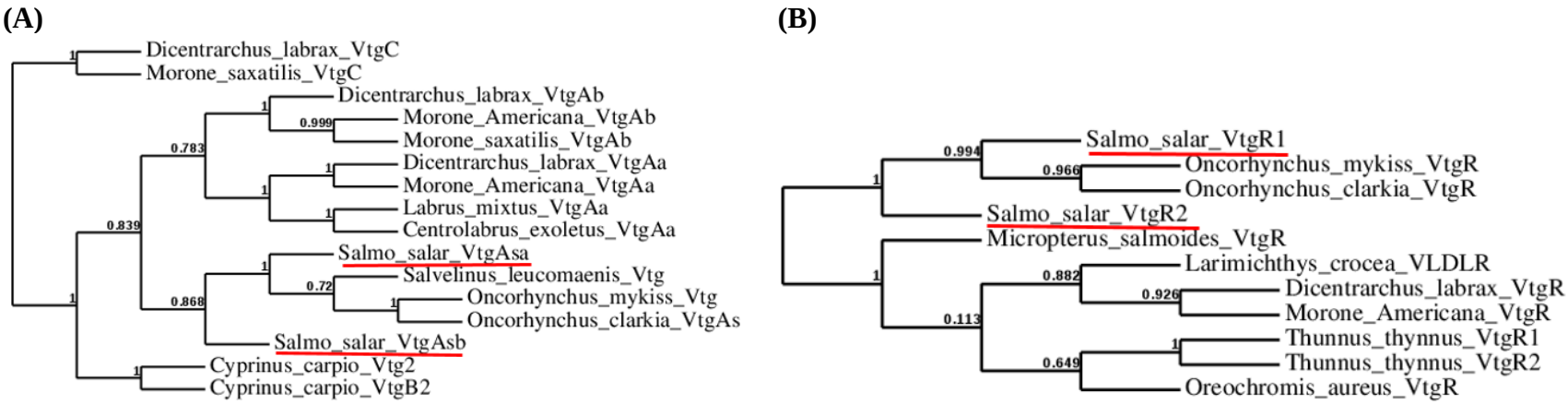


Figure. 6: Phylogenetic tree of fish Vtgs (A) and VtgRs (B) based on the amino acid sequences. Numbers at branch nodes represented branch support value, as the numbers approach to 1, the support got stronger.

(A)

Forward primer sequence →

```
VtgR1 X1        CCGCGAGACCAGACGCTCGCCTCAGACGCCCTTCACTGCAGACCAGCTCCCTCAAAGGATGATGGGAAAGCTCTAATACA
VtgR1 X2        CCGCGAGACCAGACGCTCGCCTCAGACGCCCTTCACTGCAGACCA----------------------------------
VtgR1 X3        CCGCGAGACCAGACGCTCGCCTCAGACGCCCTTCACTGCAGACCA----------------------------------
                *********************************************

VtgR1 X1        TCCCACTCACCCCCAAGCGACCACAGTGCCAAATGTTGTCCCCAAACCTGTCCCTGCTGAAGCCAACAGCAGTACATCCA
VtgR1 X2        ----------------GCGACCACAGTGCCAAATGTTGTCCCCAAACCTGTCCCTGCTGAAGCCAACAGCAGTACATCCA
VtgR1 X3        ----------------------------------------------------------GAAGCCAACAGCAGTACATCCA
                                                                          **********************

VtgR1 X1        TCCATGAGGTGAACTCCACAGCTAGAGGATCTACAGCTGCCTGGGCAATCCTCCCTGTGTTGTTGCTGGCCATTGCTGCA
VtgR1 X2        TCCATGAGGTGAACTCCACAGCTAGAGGATCTACAGCTGCCTGGGCAATCCTCCCTGTGTTGTTGCTGGCCATTGCTGCA
VtgR1 X3        TCCATGAGGTGAACTCCACAGCTAGAGGATCTACAGCTGCCTGGGCAATCCTCCCTGTGTTGTTGCTGGCCATTGCTGCA
                ********************************************************************************
```

← *Reverse primer sequence*

```
VtgR1 X1        GCAGGAGGCTACTTGATGTGGCGTAACTGGCAGCTGAAGAATAAAAAGAGCATGAACTTC
VtgR1 X2        GCAGGAGGCTACTTGATGTGGCGTAACTGGCAGCTGAAGAATAAAAAGAGCATGAACTTC
VtgR1 X3        GCAGGAGGCTACTTGATGTGGCGTAACTGGCAGCTGAAGAATAAAAAGAGCATGAACTTC
                ************************************************************
```

(B)

```
                                 YWTD5                                             ........................
VtgR1_X1     644  AHPFALTVFEDRVFWTDGENEAIYGANKFTGSDVITLASNLNEPQDIIVYHELIQLSGTNWCNEKGLNGGCAYMCLPAPQ  723
VtgR1_X2     644  AHPFALTVFEDRVFWTDGENEAIYGANKFTGSDVITLASNLNEPQDIIVYHELIQLSGTNWCNEKGLNGGCAYMCLPAPQ  723
VtgR1_X3     644  AHPFALTVFEDRVFWTDGENEAIYGANKFTGSDVITLASNLNEPQDIIVYHELIQLSGTNWCNEKGLNGGCAYMCLPAPQ  723
                  ....... EGF 3 ............     .......... (O-LINKED) ............             .....
VtgR1_X1     724  INKYSPKYTCACPRDQTLASDALHCRPAPSKDDGKALIHPTHPQATTVPNVVPKPVPAEANSSTSIHEVNSTARGSTAAW  803
VtgR1_X2     724  INKYSPKYTCACPRDQTLASDALHCRP                 ATTVPNVVPKPVPAEANSSTSIHEVNSTARGSTAAW  786
VtgR1_X3     724  INKYSPKYTCACPRDQTLASDALHCRP                 --------------EANSSTSIHEVNSTARGSTAAW  772
                  ***************************                               **********************
                  .....TM..........                 ...CD...
VtgR1_X1     804  AILPVLLLAIAAAGGYLMWRNWQLKNKKSMNFDNPVYLKTTEEDLNIDISRHTSNIGHTYPAISVVNTEDDLS  873
VtgR1_X2     787  AILPVLLLAIAAAGGYLMWRNWQLKNKKSMNFDNPVYLKTTEEDLNIDISRHTSNIGHTYPAISVVNTEDDLS  856
VtgR1_X3     773  AILPVLLLAIAAAGGYLMWRNWQLKNKKSMNFDNPVYLKTTEEDLNIDISRHTSNIGHTYPAISVVNTEDDLS  842
                  *************************************************************************
```

Figure. 7: Sequence alignment of three VtgR1 splice variance at the nucleotide level (A) and amino acid level (B). Three forward primer sets designed as *VtgR1 X1*, *VtgR1 X2* and *VtgR1 X3* with different highlight color in gray, green and blue respectively (A).The reverse primer sequences were the same and highlighted in yellow. (B) Epidermal growth factor-like domains EGF 3 was underlined in black, O-Linked domain was underlined in red, YWTD 5 propeller domains was in bold, transmembrane (TM) was in red bold, and cytoplasmic domain (CD) was in green bold. Asterisks or dot under each pair of aligned amino acids indicated identity or similarity, respectively.

3.2 Salmon VtgR1 splice variants and RT-PCR tissue expression

The salmon *VtgR1* consists of three transcripts, *X1*, *X2* and *X3* (NCBI access No. XM_014138209.1, XM_014138210.1, and XM_014138211.1, repectively) coding for three receptors differing in the putative O-linked sugar domain (Fig. 7). Tissue specific expression of the different transcript was examed by RT-PCR using specific primer sets flanking the alternative splice site.

cDNA from ovary, brian, liver and heart was used as RT-PCR templates, and three bands were amplified corresponding to the expected sizes of 225, 174 and 133 bp (Fig. 8). *VtgR1 X1* was observed in ovary and heart tissue, *VtgR1 X2* also detected in brain, and *VtgR1 X3* was observed in all four tissues, ovary, liver, brain and heart.

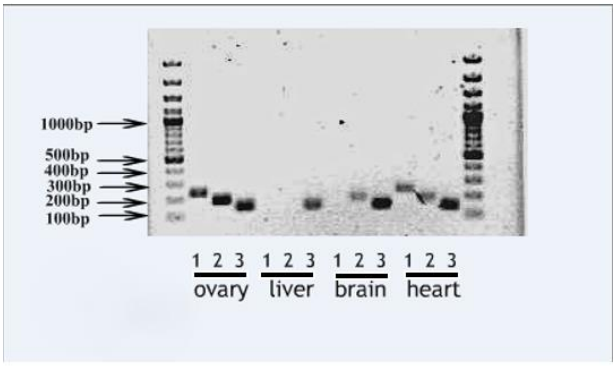


Figure. 8: RT-PCR screening of various tissues to detect splice variants of Atlantic salmon *VtgR1*. cDNA pool from ovary, brian, liver and heart were used as RT-PCR templates. Three primer sets (1-3) designed as *VtgR1 X1, VtgR1 X2* and *VtgR1 X3* respectively (see Fig. 7 for primer sequences). The DNA marker was start from 100bp.

3.3 Salmon Vtgs and VtgRs expression during the reproductive cycle

The expression of the salmon genes were quantified by real-time qPCR in adult females and males sampled from August 2014 until spawning in September 2015. Both *Vtgs* and *VtgRs* were dramatically expressed in females compare to males (Fig. 9 A). *VtgAsa* and *VtgAsb* were predominantly expressed in liver, but were also detected in ovary, heart and brain (Fig. 9 B). *VtgR1* was mainly expressed in ovary, but also in brain, liver and heart, while the *VtgR2* was dominantly expressed in ovary and was not detectable in liver tissue.

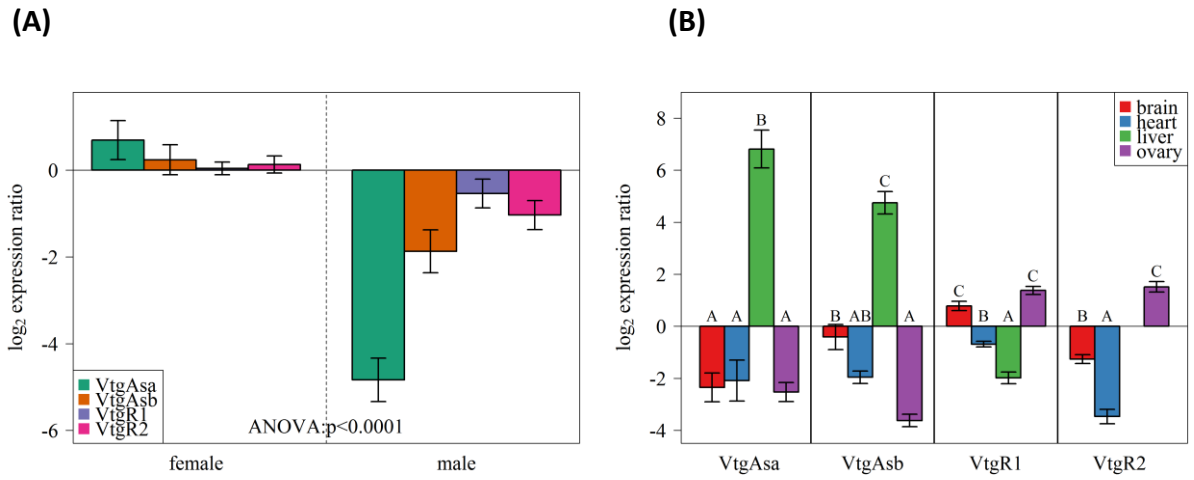


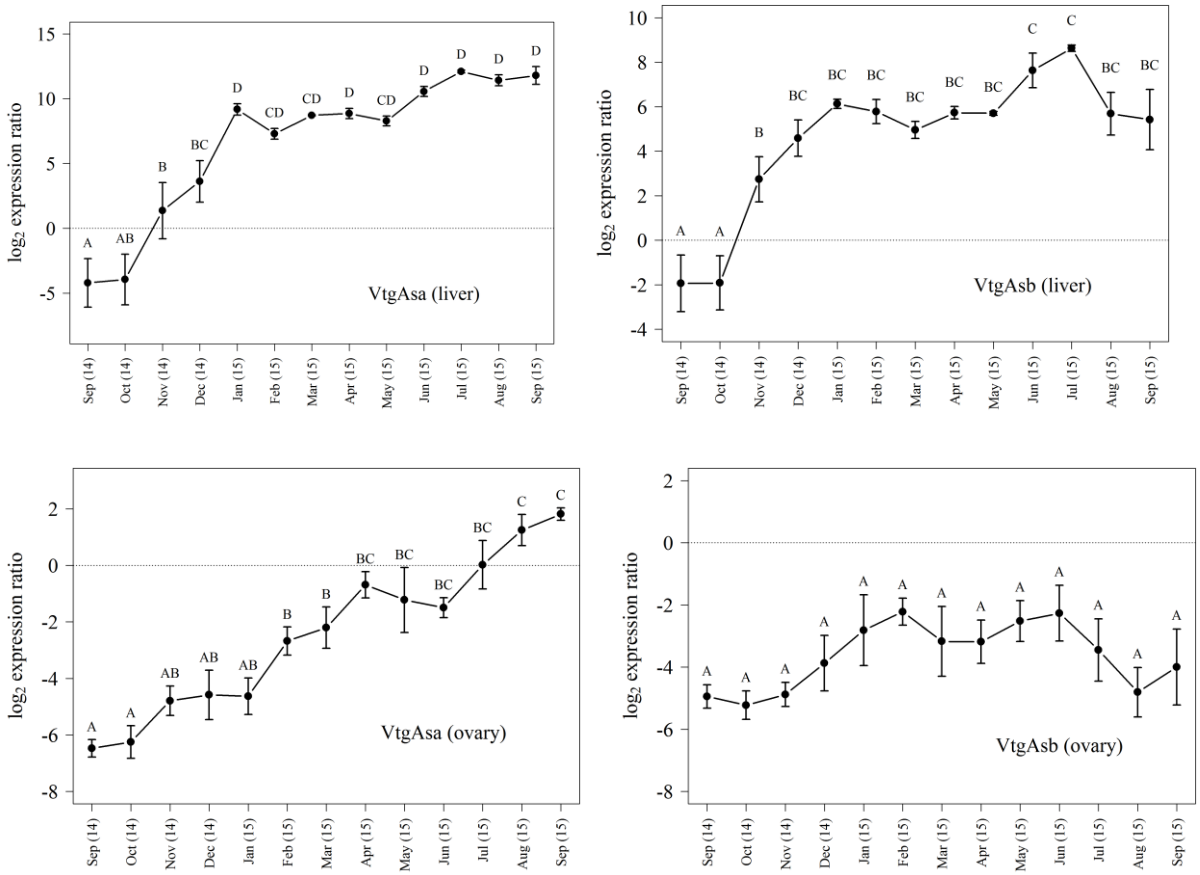
Figure. 9: The overview of the *Vtg* and *VtgR* genes relative expression in sex **(A)** and female tissues **(B).** The ANOVA P<0.0001 in (A) meant significantly different between males and females. The data in (B) were presented in mean ± standard error. Different letters denoted that the values were significantly different according to the ANOVA analysis (P<0.05). The VtgR2 was undetectable in liver tissue.

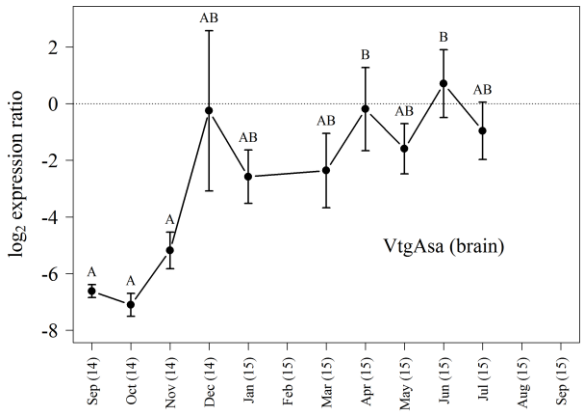
VtgAsa gene expression in liver increased significantly from October to January, then generally kept on the high expression until spawning. *VtgAsb* showed similar trend as *VtgAsa*, but reached the peak in June and decreased sharply after July 2015. In addition, the annual gene expression of *VtgAsa* in ovary and brain were general similar as in liver, whereas *VtgAsb* was stable in ovary and brain through the reproduction cycle (Fig. 10 A). Conversely, *VtgR1* and *VtgR2* genetic expressions in ovary remained at the maximal levels before May, thereafter decreased dramatically, reached minimum level in August 2015 and then went up again. The trends of *VtgR1* and *VtgR2* gene expression in brain were undiversified, whereas *VtgR1* had significantly higher expression than *VtgR2* (Fig. 10 B).

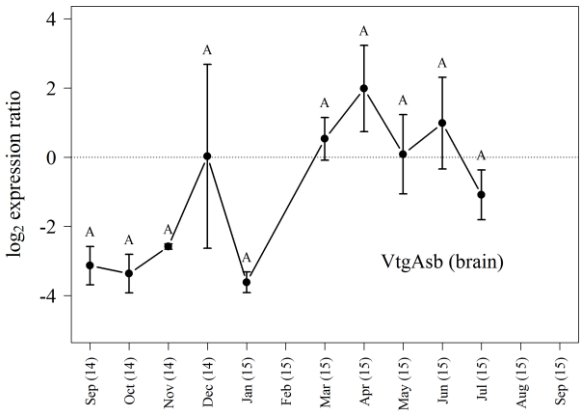
Detectable GSI levels were observed after December 2014 and remained stable from December through February 2014. Then GSI gradually arose from March 2015, reached to the top in August 2015, and kept on highest value until spawning. E2 concentration in blood was maintained at very low levels less than 5 ng/ ml until June 2015. Subsequently, it increased dramatically and peaked in August 2015, and then declined rapidly in post-vitellogenesis period in September 2015 (Fig. 10 C).

In general, when *VtgR* genes maintained at the highest levels in previtellogenesis stage, the *Vtg* genes increased significantly. Conversely, when the *Vtg* genes reached and kept on highest levels in vitellogenesis stage, the *VtgR* genes decreased dramatically. Moreover, GSI and estradiol concentration in plasma had opposite changing trend from *VtgR* genes during vitellogenesis stage. When GSI and E2 concentration peaked in August, the *VtgR* genes reached to lowest level simultaneously.

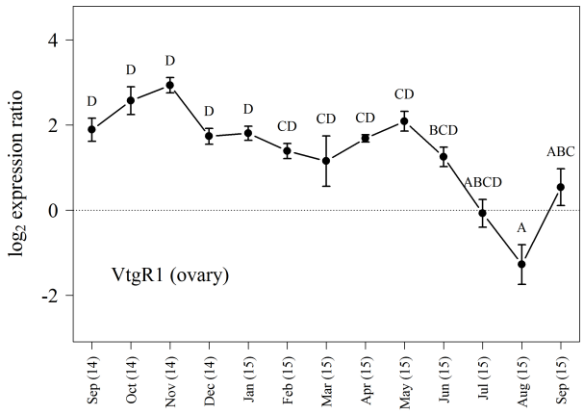
(A)

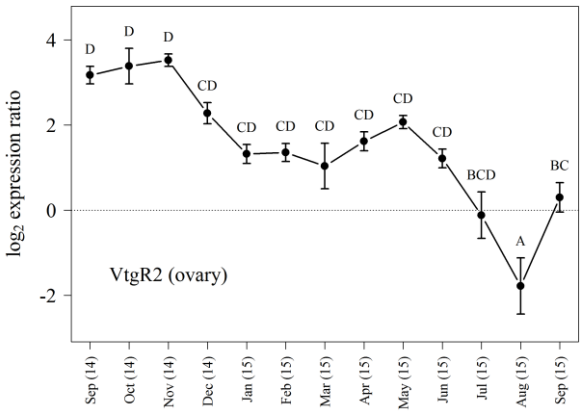


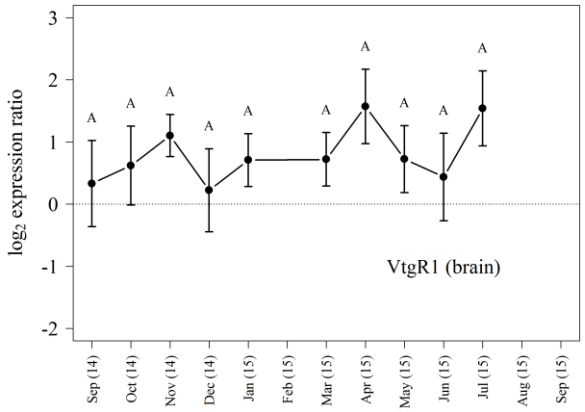

log2 expression ratio
VtgAsa (brain)
Sep (14)
Oct (14)
Nov (14)
Dec (14)
Jan (15)
Feb (15)
Mar (15)
Apr (15)
May (15)
Jun (15)
Jul (15)
Aug (15)
Sep (15)

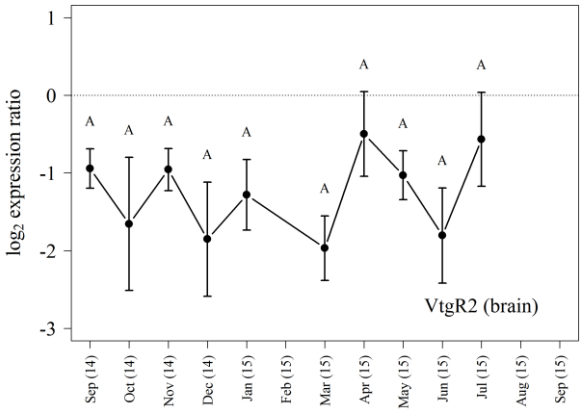

log2 expression ratio
VtgAsb (brain)
Sep (14)
Oct (14)
Nov (14)
Dec (14)
Jan (15)
Feb (15)
Mar (15)
Apr (15)
May (15)
Jun (15)
Jul (15)
Aug (15)
Sep (15)

(B)


log2 expression ratio
VtgR1 (ovary)
Sep (14)
Oct (14)
Nov (14)
Dec (14)
Jan (15)
Feb (15)
Mar (15)
Apr (15)
May (15)
Jun (15)
Jul (15)
Aug (15)
Sep (15)


log2 expression ratio
VtgR2 (ovary)
Sep (14)
Oct (14)
Nov (14)
Dec (14)
Jan (15)
Feb (15)
Mar (15)
Apr (15)
May (15)
Jun (15)
Jul (15)
Aug (15)
Sep (15)


log2 expression ratio
VtgR1 (brain)
Sep (14)
Oct (14)
Nov (14)
Dec (14)
Jan (15)
Feb (15)
Mar (15)
Apr (15)
May (15)
Jun (15)
Jul (15)
Aug (15)
Sep (15)


log2 expression ratio
VtgR2 (brain)
Sep (14)
Oct (14)
Nov (14)
Dec (14)
Jan (15)
Feb (15)
Mar (15)
Apr (15)
May (15)
Jun (15)
Jul (15)
Aug (15)
Sep (15)

(C)

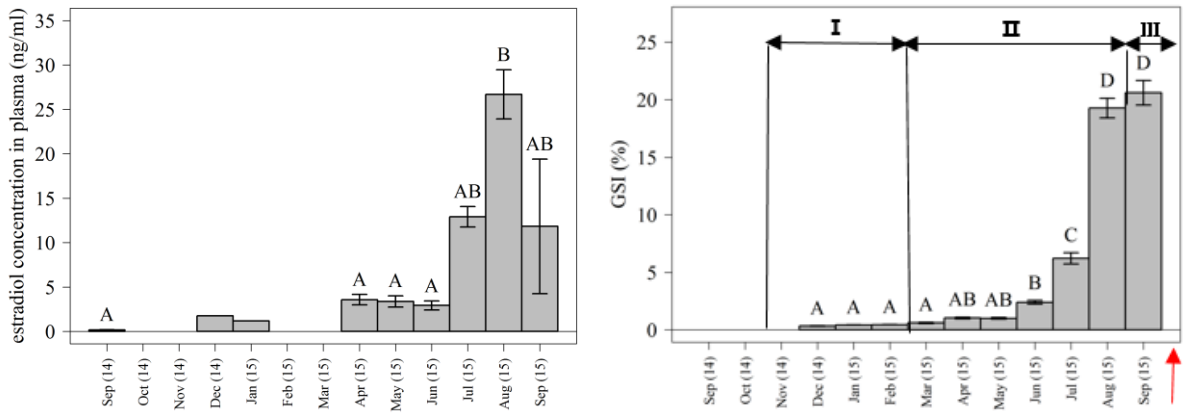


Figure. 10: Annual changes in *Vtg* and *VtgR* genes relative expression, estradiol concentration and GSI in female Atlantic salmon from Sep-2014 to Sep-2015. (A*) VtgAsa* and *VtgAsb* annual changes in liver, ovary and brain. (B) *VtgR1* and *VtgR2* annual changes in ovary and brain. (C) Changes in estradiol (E2) concentration in plasma and GSI (only left gonad was sampled initially). We assumed that Ⅰ is pre-vitellogenesis stage from December 2014 to February 2015, Ⅱ is vitellogenesis stage from March to August, and Ⅲ is post-vitellogenesis period. The Atlantic salmon was spawning during September and October (showed as red arrow). All the data were presented in mean ± standard error. Different letters denoted that the values were significantly different according to the ANOVA analysis (P<0.05). Numbers above the month showed year.

4. DISCUSSION

Atlantic salmon belongs to the primitive group of protacanthomorph teleost and has two Vtgs, designed VtgAsa and VtgAsb, which are dissimilar to VtgAa, VtgAb and VtgC in acanthomorph teleost (Fig. 6 A) (Finn *et al*., 2009; Reading *et al*., 2009; Yilmaz *et al*., 2016). qPCR revealed that *VtgAsa* and *VtgAsb* were predominantly expressed in liver, while weak expression was detected in ovary, brain and heart of female fish (Fig. 9 B). The notion adopts well to most teleost studies showing that *Vtgs* are dominantly expressed in liver, but also in extrahepatic tissues, including heart, brain, ovary, intestine, skin, gill, kidney, muscle and spleen (Wang *et al*., 2005, 2010; Yin *et al*., 2009; Ma *et al*., 2009; Zhong *et al*., 2014). Interesting, cyprinid *Vtg* is mainly expressed in the ovary (*3 Refs, see Intro*). Since Vtgs are apolipoprotein,

they bind lipids to be transported to other tissues, and various studies reported that *Vtgs* were expressed in adipocytes of these tissues (Panprommin *et al*., 2008). Additionally, Vtgs also played an important role in defeating virus and bacterium in immune system (Li *et al*, 2008; Zhang *et al.*, 2015). Overall, the *Vtgs* expression in extrahepatic tissues might be related to a role of immunity and not as yolk precursor.

Interesting, when *VtgAsa* gene expression increased rapidly in liver, it increased accordingly in ovary and brain, but for the *VtgAsb*, the increased expressions in liver was not found in ovary and brain (Fig. 10 A). The deduced amino acid sequences alignment (Fig. 3) revealed that Atlantic salmon VtgAsa had similar full-length pentapartite type proteins as white perch VtgAb (Reading *et al.,* 2009) with the structure NH^2-(LvH-Pv-LvL- β′-CT)-COO^-, while VtgAsb is the truncated protein lacking the (Pv-LvL- β′-CT)-COO^- domain. VtgAsa and VtgAsb contain conserved motif IHLTKSKDL and VHLTKTKDL, respectively, in the LvH domain that is similar to the tilapia and white perch motif receptor binding domain (Li *et al.,* 2003; Reading *et al.,* 2009). The Pv is the smallest part of the Vtgs, consisting of abundant phosphorylated serines, which increase Vtg solubility in the plasma and strengthened its structure stability to prevent early degradation (Reading *et al.,* 2009). C-terminal domains (β′c and Ct) was full of cysteine that might form Vtg dimers, and CGXC motif in β′-CT was suggested to play an important role in processing of disulfide linkages of circulating Vtg prior to oocyte maturation (Reading *et al*., 2009). Consequently, although VtgAsb has receptor binding domain in LvH, the incomplete VtgAsb might be an unstable peptide structure being degraded in the way of transportation, while the VtgAsa is transported to ovary successfully without degradation.

Moreover, *VtgAsa* and *VtgAsb* gene expression increased rapidly in liver though E2 concentration was in low level during pre-vitellogenesis stage. Thereafter, *VtgAsa* and *VtgAsb* kept on maximum expression level despite E2 increased dramatically during vitellogenesis stage. (Fig. 10). Several putative EREs were found in Atlantic salmon *Vtgs* promoters, including one 1 bp spacer putative ERE sequence **tgga**c**a**t**tga**t**ct** in both *VtgAsa* and *VtgAsb*, two 3 bp spacer **aggtca**agc**tgacc**a and **ggggca**ggt**t**a**acct** motifs in *VtgAsa*, and one 3 bp spacer **aggccg**ggt**t**a**acct** in Vtg*Asb* (Fig. 5). The **ggggca**ggt**t**a**acct** motif of *VtgAsa* was same as rainbow trout pERE2 which had been proved to be functional ERE that bound ER, while various ERs had various affinity to different types of ERE (Bouter *et al.,* 2010). Unal *et al.* (2014) demonstrated that there were four ER subtypes in salmonids, differentially regulated by E2, and played different roles in

vitellogenesis. Therefore, VtgAsa and VtgAsb might be differentially regulated by E2 and could also explain that *VtgAsa* and *VtgAsb* had different gene expression according to the E2 changes during the post-vitellogenesis stage (Fig 10 A and C). In addition, *Vtg* genes were also detected in male fish even in very low level, and Wand *et al.* (2010) also found *Vtg* expression in testes of white cloud mountain minnow, suggesting that Vtgs were not simply controlled by E2. Yin *et al.* (2009) and Ma *et al.* (2009) presented that teleost Vtg *in vivo* can be regulated by both estrogen and adrenergic signals.

Atlantic salmon expressed two forms of VtgR genes, named *VtgR1* and *VtgR2*. Real-time RT-PCR (qPCR) revealed that they were predominantly expressed in ovary, but weak in brain, liver and heart, as similarly described in cutthroat trout (Mizuta *et al.*, 2013), and the annual expressions in extra-ovarian tissues were changeless (Fig 9 and 10 B). VtgRs might activate signal conduction of nervous system or regulate lipid metabolism [Trommsdorff *et al.(1999),* as cited in Mizuta *et al.*, 2013], consistent with the expression of the salmon *VtgRs* in the brain and in the heart metabolizing large amount of fatty acid to provide energy for blood circulating (Yin *et al.*, 2009).

Eight LBDs VtgR1 and VtgR2 belonged to the VLDLR (Prat *et al*., 1998), and varied in the splicing of O-linked sugar domain (Fig. 4). The non-O-linked *VtgR2* was dominantly expressed in ovary but was undetectable in liver, while the O-linked *VtgR1* was highly expressed in ovary and brain (Fig. 9 B). Therefore, VtgR1 seems to be an ovarian specific receptor located on the surface of oocytes to accept the Vtgs acting as yolk precursor. In comparison, VtgR2 is probably more common and might accept not only Vtgs but also homologous lipoprotein of importance for metabolism. In order to examine whether the O-linked domain of *VtgR* is associated with tissue expression, we performed a gene expression study of the three reported transcript by designing 3 different forward primers of *VtgR1* to target cDNA flanking sequence which encoded the putative O-linked sugar domain. The *VtgR1 X1* transcript encoded full length of O-linked sugar domain, the *VtgR1 X2* transcript had a truncated sequence that encoded part of the O-linked sugar domain, while the shortest *VtgR1 X3* transcript lacked the O-linked sugar domain (see Fig. 7). *VtgR1 X1* was detected in ovary and heart, whereas *VtgR2 X2* was found in ovary, brain and heart, and *VtgR1 X3* was found in all four tissues. In other words, with the deduction of sequence which encode O-linked amino acids, the traces of band were detected in more somatic tissues (Fig. 8). In contrast, O-linked sugar domain lacking LR8- was shown to be ovarian specific VtgR in rainbow trout,

white perch, and European seabass (Prat *et al.*, 1998; Hiramatsu *et al.*, 2004; Yilmaz *et al.*, 2015). Nonetheless, conventional RT-PCR had limitations to confirm diverse in the degree of VtgR mRNA expression among different tissue types of ovarian development, therefore it is not a strictly quantitative method (Hiramatsu *et al*., 2004).

Both *VtgR1* and *VtgR2* were expressed at highest levels in previtellogeneis stage of oocyte, probably to fully prepare for the uptake of Vtgs (Dominguez *et al.*, 2012). Then the expressions were gradually decreased with the oocyte growth during vitellogenesis (Fig. 10). The whole trend in *VtgR* genes expression was in accordance with other teleost fish (Davail et al., 1998; Perazzolo *et al.*, 1999; Hiramatsu *et al.*, 2004; Agulleiro *et al.*, 2007; Luckenbach *et al.*, 2008; Dominguez *et al.*, 2012; Mizuta *et al*., 2013). Moreover, E2 concentration in plasma had opposite changing trend from *VtgR* genes during vitellogenesis stage. Recent studies revealed that E2 was able to suppress *VtgR* expression in females (Chakraborty *et al*., 2011; Dominguez *et al*., 2012). We hypothesize that because *Vtg* genes were expressed at low levels in liver during the previtellogenesis stage, gonad grew a little, and GSI was not changing visually. Moreover, E2 was less accepted by the E2 receptor which bond to functional ERE located at the *Vtg* DNA sequences, and therefore the E2 inhibition of *VtgR* genes expression in ovary was reduced. However, when vitellogenesis was initiated, Vtgs were synthesized abundantly and promoted rapid growth of oocyte. According to Chakraborty *et al*. (2011) and Dominguez *et al*. (2012), it might be speculated that accumulating E2 levels concomitantly suppressed *VtgR* genes expression.

In conclusion, our study identified and characterized two *Vtg* genes and two *VtgR* genes in Atlantic salmon and presented reproductive stage-specific expression in four tissues liver, brain, heart and ovary by qPCR quantification procedure. Besides, the annual changes of GSI and plasma estrogen concentration were also presented to examine salmon reproducing. Furthermore, we supposed that E2 was affected by the Vtgs synthesis, and E2 can inhibit the VtgRs production. Nonetheless, we were not sure how different forms of VtgR accept different forms of Vtg (Review: Yilmaz *et al*., 2015). The specific relationship between VtgRs and Vtgs, and potential E2 suppression of *VtgR* gene expression warrant further research.

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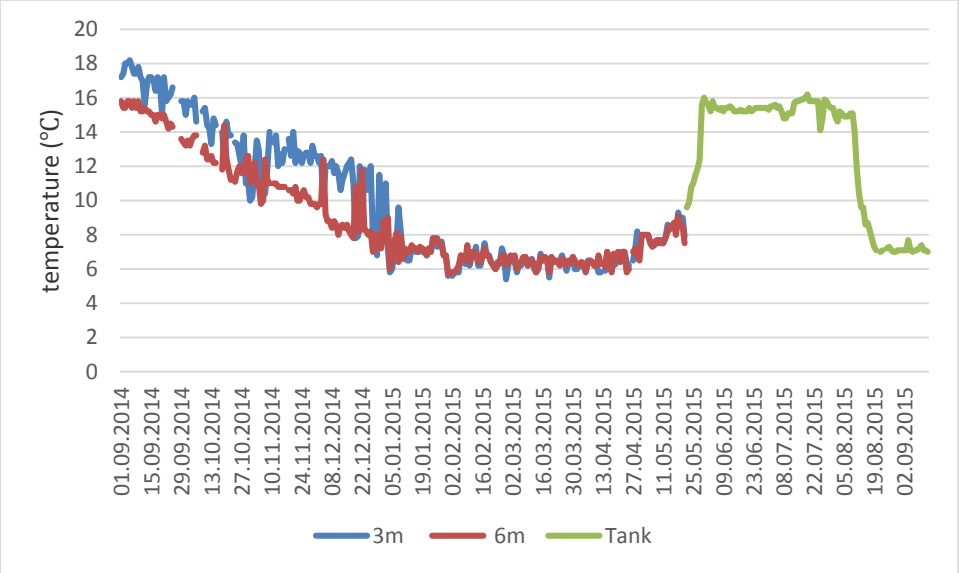
APPENDIX

Appendix 1: Annual changes of temperature in salmon aquaculture

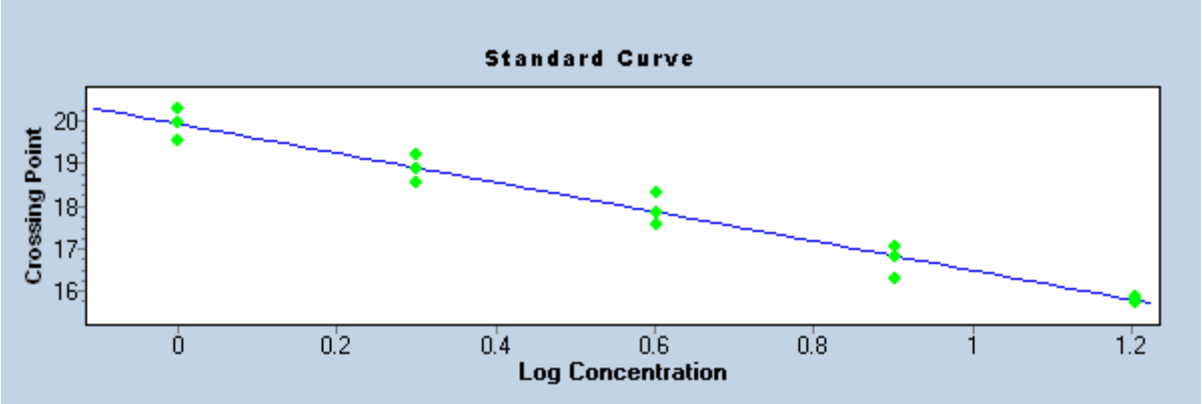


Appendix 2: The accession of different protein

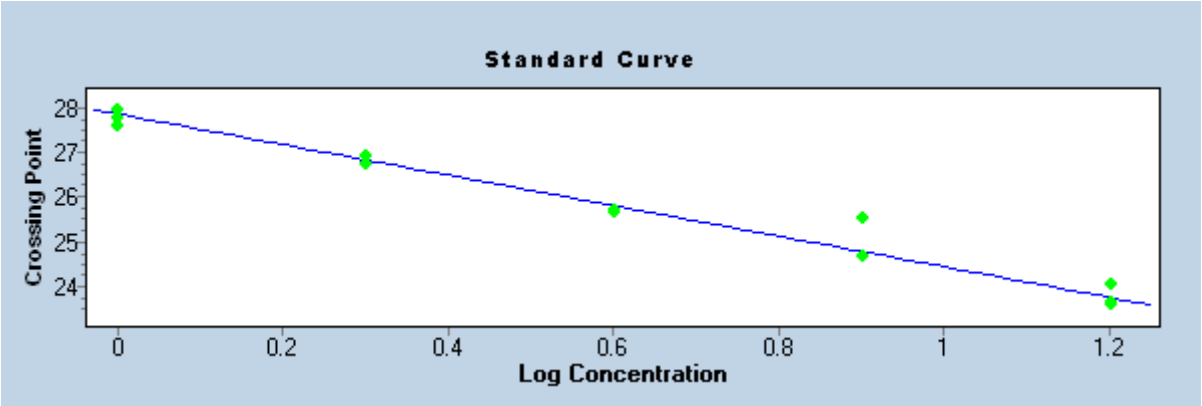
Protein type	Species	Accession
Vtgs	**Salmo salar VtgAsa**	XP_014024135.1
	Salmo salar VtgAsb	AAO72350.1
	Dicentrarchus labrax VtgC	AFA26671.1
	Morone saxatilis VtgC	ADZ57174.1
	Morone saxatilis VtgAb	ADZ57173.1
	Morone Americana VtgAb	AAZ17416.1
	Dicentrarchus labrax VtgAb	AFA26670.1
	Dicentrarchus labrax VtgAa	AFA26669.1
	Centrolabrus exoletus VtgAa	ACK36963.1
	Labrus mixtus VtgAa	ACK36967.1
	Morone Americana VtgAa	AAZ17415.1
	Oncorhynchus clarkia VtgAs	AGQ04606.1
	Oncorhynchus mykiss	Q92093.1
	Salvelinus leucomaenis	BAM22589.1
	Cyprinus carpio VtgB2	BAD51933.1
	Cyprinus carpio Vtg2	BAC07526.1
VtgRs	**Salmo salar VtgR1**	XP_013993684.1
	Salmo salar VtgR2	XP_013984478.1
	Oncorhynchus clarkia VtgR	AHH55319.1
	Oncorhynchus mykiss VtgR	NP_001117847.1
	Thunnus thynnus VtgR1	AEC12211.1
	Dicentrarchus labrax VtgR	CBX54721.1
	Thunnus thynnus VtgR2	AEC12210.1
	Larimichthys crocea VLDLR	KKF31482.1
	Oreochromis aureus VtgR	AAO27569.1
	Micropterus salmoides VtgR	ADO17799.1
	Morone Americana VtgR	AAO92396.1

Appendix 3: Standard curve of Vtg, VtgR and EF1a

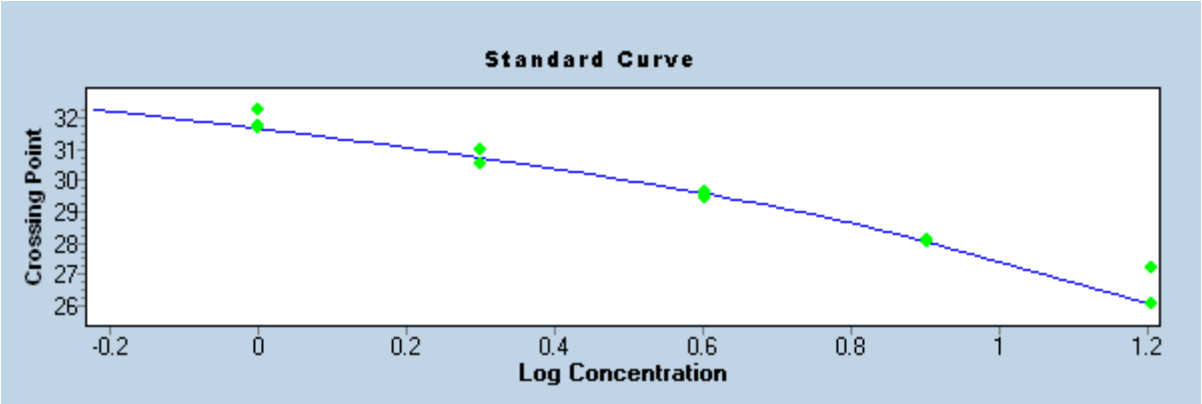
(A) ***VtgAsa, efficiency=0.964***



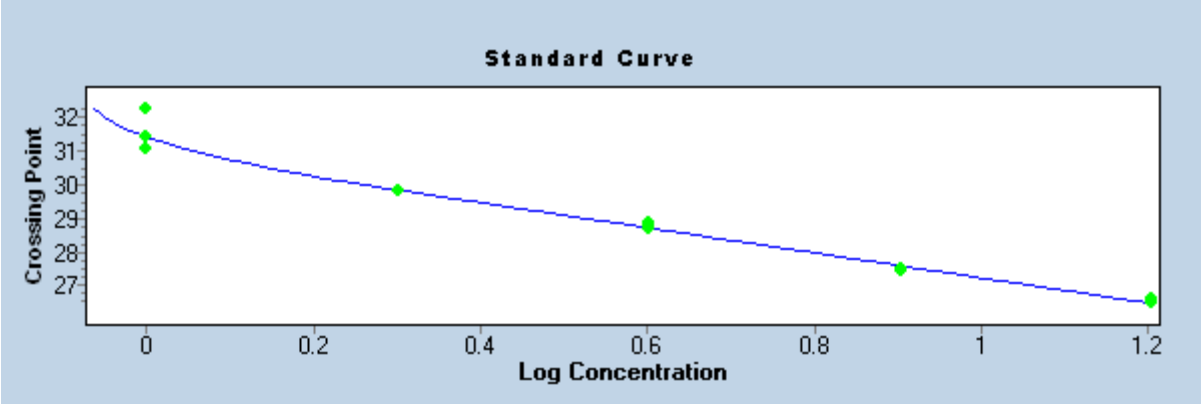
(B) VtgAsb, efficiency=0.955



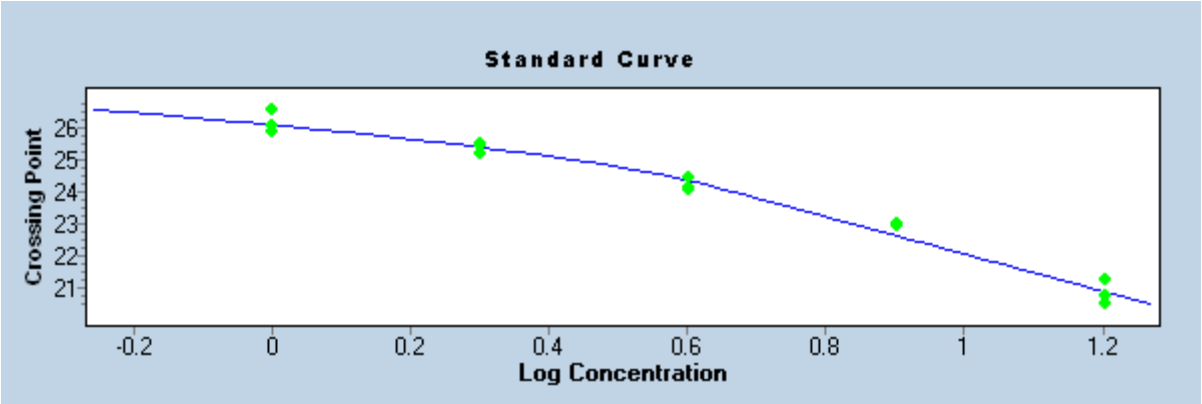
(C) VtgR1, efficiency=0.723

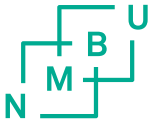


(D) ***VtgR2, efficiency=0.86***



(E) ***EF1a, efficiency=0.744***





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