

The vitamin D receptor and its ligand 1 α ,25-dihydroxyvitamin D₃ in Atlantic salmon (*Salmo salar*)

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Abstract

Seaward migration of *Salmo salar* is preceded by preparatory physiological adaptations (parr–smolt transformation) to allow for a switch from freshwater (FW) to seawater (SW), which also means a switch in ambient calcium from hypocalcemic (<1 mM Ca²⁺) to the plasma (~1.25 mM Ca²⁺) and to strongly hypercalcemic (8–12 mM Ca²⁺). Uptake, storage (skeleton, scales) and excretion of calcium need careful regulation. In fish, the vitamin D endocrine system plays a rather enigmatic role in calcium physiology. Here, we give direct evidence for calcitriol involvement in SW migration. We report the full sequence of the nuclear vitamin D receptor (sVDR0) and two alternatively spliced variants resulting from intron retention (sVDR1 and sVDR2). In FW parr, SW adapting smolts, and in SW adults, plasma

concentrations of 25(OH)D₃ and 24,25(OH)₂D₃ did not change significantly. Plasma calcitriol concentrations were lowest in FW parr, doubled during smoltification and remained elevated in SW adults. Increased calcitriol coincided with a twofold decrease in sVDR mRNA levels in gill, intestine, and kidney of FW smolts and SW adults, when compared with parr. Clearly, there was a negative feedback and dynamic response of the vitamin D endocrine system during parr–smolt transformation. The onset of these dynamic changes in FW parr warrants a further search for the endocrines that initiate these changes. We speculate that the vitamin D system plays a crucial role in calcium and phosphorus handling in Atlantic salmon.

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Introduction

The vitamin D endocrine system in vertebrates is a major regulator of calcium and phosphate homeostasis (Norman *et al.* 2002). Current research focuses on the two vitamin D metabolites 24R,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃) and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃; calcitriol), both of which are hydroxylation products of 25-hydroxyvitamin D₃ (25(OH)D₃). Calcitriol, considered to be the most potent vitamin D metabolite, exerts its genomic actions via the vitamin D receptor (VDR), a member of the nuclear receptor superfamily. The VDR shows great sequence and structural resemblance with the subfamily that includes peroxisome proliferator activator receptor, thyroid hormone, and retinoic acid receptors (DeLuca & Zierold 1998, Issa *et al.* 1998, Jones *et al.* 1998). Low serum levels of calcitriol cause type I rickets; the absence of the VDR results in type II rickets (Yagishita *et al.* 2001, Pettifor 2004). Several genes involved in control of calcium uptake are regulated by vitamin D, e.g. synthesis of calbindin D_{9K}, involved in active intestinal calcium transport, is under strict control of calcitriol (Darwish & DeLuca 1992, Bronner & Pansu 1998). Reabsorption of Ca in the kidney is partly dependent on calcitriol and the VDR. Another

important function of the vitamin D endocrine system is regulation of phosphorus uptake. Phosphorus absorption in mammals is mainly regulated by 1,25(OH)₂D₃. Low-P_i diet, via stimulation of the activity of the renal 25-hydroxyvitamin-D₃-1 α -hydroxylase (1 α OHase), leads to an increase in the plasma level of calcitriol (Brown *et al.* 1999).

The vitamin D system appears to function in a similar fashion in fish. Indeed, all three major vitamin D metabolites are found in fish (Graff *et al.* 1999, Suzuki *et al.* 2000, Larsson *et al.* 2003) and the VDR has been sequenced in Japanese flounder (Suzuki *et al.* 2000), zebrafish (NM130919), and common carp (AJ784084). In European eel, *Anguilla anguilla*, calcitriol administration *in vivo* increased serum phosphate concentration (MacIntyre *et al.* 1976), and in American eel, *Anguilla rostrata*, it increased intestinal calcium absorption (Fenwick *et al.* 1984). This is interesting because in contrast to mammals, fish are not particularly dependent upon food for their calcium. The major fraction of calcium required by the fish is taken up directly from the water via the gills (Verboost *et al.* 1994) and the uptake rate is largely independent of the water calcium concentration. Dietary calcium load may decrease branchial uptake to avoid overload and to ensure the required net influx for homeostasis and growth. In addition,

vitamin D is involved in phosphorus metabolism. Vielma & Lall (1998a) hypothesized about a negative relation between phosphorus concentration in the diet and 25(OH)D₃ content in the liver of Atlantic salmon. A hyperphosphatemic effect of vitamin D has been described in eel, *A. anguilla* (Fenwick *et al.* 1984), catfish, *Clarias batrachus* (Swarup *et al.* 1984), and carp, *Cyprinus carpio* (Swarup *et al.* 1991).

Mineral absorption (including that of calcium and phosphorus) requires regulated (primary or secondary) active transport processes. In fish, the antihypercalcemic stannio-calcin (Wagner *et al.* 1998), the hypercalcemic calciotropes prolactin (PRL), and parathyroid hormone-related protein (PTHrP) dominate the regulation of calcium handling (Abbink *et al.* 2006); little is known about the presumed hypercalcemic role of calcitriol in fish. Seawater (SW) fish such as sea bream do express PTHrP, which was shown to exert hypercalcemic actions (Guerreiro *et al.* 2001, Abbink *et al.* 2006). The control of mineral homeostasis may be different in marine and freshwater (FW) environments (Brown *et al.* 1991, Larsson *et al.* 1995).

During smoltification, many physiological changes take place induced by various hormones. One of the most prominent changes during smoltification is the increase in levels of growth hormone (GH; Ágústsson *et al.* 2001, 2003). GH has an important role in stimulating growth and improving hypo-osmoregulatory ability, which is accomplished through a multitude of mechanisms. One of the most important mechanism is the increase in branchial Na⁺/K⁺-ATPase activity to provide the fish with SW-type chloride cells (McCormick 1996); isoform switch may be a second phenomenon in this adaptation. Ágústsson *et al.* (2003) measured an increase of both GH and PRL mRNA expression during smoltification. After SW transfer, GH mRNA increased; PRL mRNA expression decreased steeply. GH levels increase even further in SW, facilitating a period of fast growth for Atlantic salmon. This period of fast growth demands the supply of many nutrients, especially calcium and phosphorus for skeletal growth.

In this report, we have evaluated plasma vitamin D metabolite levels and quantified Atlantic salmon VDR (sVDR) expression in the three main tissues (gills, intestine, and kidney) involved in calcium and/or phosphorus homeostasis in Atlantic salmon, *Salmo salar*. We quantified plasma vitamin D metabolites and nuclear VDR mRNA levels in FW salmon, SW salmon, and intermediate stages during parr-smolt transformation.

Materials and Methods

Fish

FW-adapted parr and SW-adapted adult Atlantic salmon (NLA strain, Aqua Gen AS, Trondheim, Sør Trøndelag, Norway) were obtained from Matre Aquaculture Research Centre (Institute of Marine Research, Bergen, Norway). Fish

were anesthetized using 0.3 ml/l benzocaine, sampled for blood and subsequently killed by a blow to the head. Tissue was immediately sampled as described below. Fish were not fed 24 h before sampling.

Smolts (Bolaks strain, Tomre Fiskeanlegg AS, Eikelandso- sen, Norway) were provided by the Institute of Marine Research, Bergen, Norway. Smolts were sampled prior to SW exposure, 7 days after transfer to a mixture of 50% FW and 50% SW, and 7 days after transfer to 100% SW. Fish were anesthetized, killed, and sampled similar to parr and adult.

Blood and tissue collection

Immediately after anesthesia blood was collected from the caudal vessels using heparized syringes fitted with 24-gauge needles; the blood was kept on ice. After centrifugation at 1811 g for 15 min at 4 °C, the plasma thus obtained was collected and stored at -80 °C until analysis for 25(OH)D₃, 1 α ,25(OH)₂D₃, and 24R,25(OH)₂D₃, as described by Aksnes (1980).

The fish were killed by spinal transection and gill, gut (the section posterior to the caecae) and kidney tissue were collected and stored in RNAlater (Ambion, Foster City, CA, USA). A mid-piece of the left second gill arch of the fish was taken. The gut was flushed with saline to remove food and then stored in RNAlater. The peritoneal cavity was opened and the kidney exposed; a piece from the left side was sampled. Sampling of the parr and adult fish was carried out at about 2-h travel distance from the institute. To standardize the procedure, all samples were stored 24 h at 4 °C in RNAlater before they were frozen at -80 °C until RNA extraction.

Cloning and sequencing

To obtain the nucleotide sequence of the sVDR, two oligonucleotide primers, SSfw and SSrv (Table 1) were designed based on data of two teleosts, the zebrafish (*Brachydanio rerio*) and Japanese flounder (*Paralichthys olivaceus*). A piece of the mid-intestine was homogenized in TRIzol reagent (Invitrogen) and RNA isolated according to the manufacturer's instructions. One microgram RNA was reverse-transcribed with 300 ng random primers, 10 nmol dNTPs, 200 nmol, 10 U RNase inhibitor, and 200 U RT Superscript II (Gibco-BRL) for 50 min at 37 °C. PCR with the SSfw and SSrv primers on the cDNA yielded a partial sVDR sequence. The 5' and 3' ends of the sequence were obtained by rapid amplification of cDNA ends (RACE)-PCR (GeneRacer, Invitrogen) according to the manufacturer's protocol, including the use of nested PCR. Gene-specific primers used for the RACE-PCR are found in Table 1.

PCR products were ligated into a pCR4-TOPO plasmid vector and transferred to chemically competent TOP10 *Escherichia coli* cells (TOPO TA cloning kit, Invitrogen). After selection on LB-kanamycin agar, plasmids of transformed cells were isolated (miniprep; Bio-Rad). Sequencing was performed by the dideoxynucleotide procedure (Sanger *et al.* 1977) using the ABI Prism BigDye

Table 1 Primer sequences

Name	Sequence (5' → 3')
SSfw	AAG GCA ACT GGC TTT CAC TTC AA
SSrv	CAA AGC CGA TGA CTT TTT GGA
RACESSfw	GAT GTA CCA GGA CAG TGC TGG C
RACESSfwn	AGA CTC CAG TGA GGA CGA TGG C
RACESSrv	GCA GGT GAA ACT GGC CTT ACG C
RACESSrvn	TTG CAG CCC TCA CAG GTC ATG G
QVDRfw	CAG GTC ATC GCC TCA TTG GT
QVDRrv	CTG AGG AGG CAT CTG ACA GAG A
QEL1afw	CCC CTC CAG GAC GTT TAC AAA
QEL1arv	CAC ACG GCC CAC AGG TAC A
Q18Sfw	CCC CGT AAT TGG AAT GAG TAC ACT TTT
Q18Srv	ACG CTA TTG GAG CTG GAA TTA CC
OSVDRF	CCC ACC ACA AGA CCT ATG AT
OSVDRR	CGC AGT ACT TGA AGT CAG GA

Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The Netherlands) on the ABI Prism 310 automated sequencer (Applied Biosystems).

Phylogenetic analysis

Sequences of available vertebrate sVDR receptors were retrieved from the NCBI site (www.ncbi.nih.gov), using the GenBank database. Multiple-sequence alignment was carried out with ClustalW at the EMBL website (www.ebi.ac.uk/clustalw/) from the European Bioinformatics Institute. Phylogenetic and molecular evolutionary analyses were conducted using molecular evolutionary genetics analyses (MEGA) version 3.1 (Kumar *et al.* 2004) and a phylogenetic tree was constructed on the basis of amino acid difference (*p*-distance) with a neighbour-joining method with 1000 bootstrap replications.

Tissue distribution

Salmon from the NLA strain (Aqua Gen AS), smoltified 6 months prior to sampling, were used for the tissue distribution analysis of sVDR. Fresh samples of pyloric caecae, midgut, hindgut, head kidney, kidney, spleen, liver, ovary, muscle, heart, and brain were homogenized using MagNA Lyser Green Beads (Roche) with Trizol (Invitrogen). Skin, gill, and vertebra samples were flash-frozen in liquid N₂ and homogenized in a mortar prior to Trizol extraction. Total RNA was purified using Trizol extraction and subjected to DNase treatment (DNA-free, Ambion). RNA quantity and quality (A_{260/280}) was assessed using a NanoDrop ND100 spectrophotometer (Saveen Biotech, Wilmington, DE, USA) and by gel electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA and PCR products were prepared using the OneStep RT-PCR kit (Qiagen) with gene-specific primers for sVDR (OSVDRF and OSVDRR, Table 1) and 18S (AJ427629). One microgram RNA was used in all reactions. Thermal cycling

conditions were 30 min at 50 °C (RT), 15 min at 95 °C, 35 cycles with 40 s at 94 °C, 1 min at 55 °C, 1 min at 72 °C, and a final elongation step with 10 min at 72 °C. PCR products were separated on a 2% agarose gel at 80 V (0.16 A) for 1 h using ethidium bromide as a marker.

Real-time quantitative PCR

Expression levels of sVDR mRNA were assessed by the use of quantitative RT-PCR. Subsequent to Trizol extraction, RNA was DNase treated to remove possible traces of genomic DNA using the DNA-free kit (Ambion). Briefly, RNA was diluted with 0.1 volume DNase buffer and incubated with two units rDNase I for 30 min at 37 °C. To inactivate DNase, 0.1 volume of DNase Inactivation reagent was added and the sample incubated for 2 min at room temperature. RNA quantity and quality (A_{260/280}) were assessed using an Eppendorf Biophotometer. The RNA was reverse-transcribed using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems). Briefly, solutions were (final concentration): MgCl₂ (5 mM), PCR buffer II (1× volume), dNTPs (1 mM each), RNase Inhibitor (1 U/μl), random hexamers (18S) or oligo d(T)₁₆ primers (2.5 μM), and MuLV Reverse Transcriptase (2.5 U/μl) in a total volume of 20 μl. After an initial step of 10-min incubation at 25 °C, reverse transcription was performed for 60 min at 48 °C followed by an inactivation step for 5 min at 95 °C and a decrease to 4 °C. For quantitative PCR analysis, 5 μl of five times diluted RT-mix was used as template in 25 μl amplification mixture, containing 12.5 μl SYBR Green Master Mix (Applied Biosystems) and 3 μl each primer (final concentration 600 nM). Primers were designed using Primer Express 2.0. The sequences of the primer sets used in the quantitative PCR for sVDR (QVDRfw and QVDRrv) and the housekeeping genes 18S (Q18Sfw and Q18Srv) and elongation factor 1-a (QEL1fw and QEL1rv) are found in Table 1. All primer sets had an efficiency > 98% and all products showed a single melting transition. After an initial step at 95 °C for 10 min, a real-time quantitative PCR of 40 cycles was performed (GeneAmp 5700, Applied Biosystems), each cycle consisting of 15-s denaturation at 95 °C and 1-min annealing and extension at 60 °C. Cycle threshold values were determined and expression was quantified using the relative expression software tool (REST) program (Pfaffl *et al.* 2002). All samples were assayed in triplicate. Both housekeeping genes gave similar results. Results are presented here only relative to 18S expression.

Statistical analysis

Statistical analysis of sVDR mRNA expression was analyzed using the REST program (Pfaffl *et al.* 2002). Vitamin D metabolite data are expressed as means ± standard deviation (s.d.); differences among groups were assessed by ANOVA. Significance of differences was assessed by parametric (Student's *t*-test) or non-parametric (Mann-Whitney *U*-test), tests where appropriate and *P* < 0.05 was taken as fiducial limit.

Table 2 Serum concentrations of the vitamin D metabolites 25(OH)D₃, 24,25(OH)₂D₃, and 1,25(OH)₂D₃ in Atlantic salmon parr, adult, FW smolt, 50% SW smolt, and SW smolt. Values are the average of 8 fish ± s.d.

Group	25(OH)D ₃ (nM)	24,25(OH) ₂ D ₃ (nM)	1,25(OH) ₂ D ₃ (nM)
Parr	4.1 ± 3.87	0.9 ± 0.56	0.46 ± 0.22*
FW smolt	5.1 ± 7.59	2.0 ± 0.88	1.19 ± 0.45 [†]
50% SW smolt	1.4 ± 1.46	7.1 ± 9.02	4.2 ± 1.00 [‡]
SW smolt	1.8 ± 3.10	1.5 ± 2.83	0.98 ± 0.43 [†]
Adult	5.8 ± 5.56	0.6 ± 0.31	0.96 ± 0.27 [†]

Significant differences ($P=0.05$) are indicated with *, †, or ‡. FW, freshwater; SW, seawater.

Results

Plasma concentrations of vitamin D metabolites

Plasma levels of 1,25(OH)₂D₃ (Table 2) increase significantly ($P<0.05$) in FW smolt (1.19 ± 0.45 nM) when compared with FW parr (0.46 ± 0.22 nM). The highest plasma levels of 1,25(OH)₂D₃ (4.20 ± 1.00 nM) were found in the transition phase, when the fish were in 50% FW/50% SW, which is

about nine times higher than the value for parr. Plasma levels of 1,25(OH)₂D₃ decrease again after transfer to 100% SW (0.98 ± 0.43 nM) and stay at this level in adult fish (0.96 ± 0.27 nM), a value two times higher than that observed in parr. No significant changes in plasma concentrations of either 25(OH)D₃ or 24,25(OH)₂D₃ were found between any of the groups, although significant variations in values between individual fish within the same group occurred.

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acaagacagacattactgcacctgaggggttgaagaataagagagccacctaaaggagtgttaggcagaggagagaggagaataactcg 90
ttttctcgacaccttggggattttgtctggaactgtattttctaattcagaggaaatggatccacagaccctctctgagtcactcagt 180
1  M E T M A V S T S V V G P D E F D R N A P R I C G V C G D K
gatggagacctgctgtgagcacgtctgtggtgggctgtgagtttgaccgcaacgcccgcgcctctgtggcgtgtggcgacaa 270
31  A T G F H F N A M T C E G C K G F F R R S M K R K A S F T C
ggccacagcgttccaacttcaacgacctgacctgtgagggtgcaaggcttctctcagcgcagcatgaagcgttaaggccagtttcaactg 360
61  P F N G S C T I T K D N R R H C Q A C R L K R C V D I G M M
ccccttcaacggcagctgtacctaccacaggaacaaccggcggcactgccaggcctgcccgcctcaaacgctgctggacatcggcatgat 450
91  K E F I L T D E E V Q R K K D L I Q K R K E E E A A R E A L
gaaggagtccatcctgacagatgaggaggtgacaggaagaaggacctgatccagaagaggaaggaggaaggcggcggcggaggcctt 540
121 R P R L N D E Q S Q V I A S L V E A H H K T Y D D S Y S D F
gagaactaggctgaacgacgagcagagccaggctcctcctctggtggagccaccacaagacctatgtagctcctactctgactt 630
151 S R F R P P V R E G P V T R S A S R A A S L H S L S D A S S
ctcacgcttcaggccccagtgccagagggcccagtaaacgcagtgccagctcgtgcagcctccctcactctctgtcagatgctcctc 720
181 D S F N H S P E S V D T K L M N F S S L L M M Y Q D S A G S
agactccttcaaccactcaccagcgtctgtggacaccaggctgatgaacttcagcagctctgttgatgatgtaccaggacagtgctggcag 810
211 P D S S E D D G S K L S M L P H L A D L V S Y S I Q K V I G
cccagactccagtgaggacgatgctccaaactctctatgctgcccacactggtgacctgacagctacagcatccagaaggtcactcgg 900
241 F A K M I P G F R G L T A E D Q T A L L K S S A I E I I M L
ctctcgctaagatgatctctggtctcaggggctgactgctgaggaccagaccgctgctgaaatccagtgccatcgagatcatcatgct 990
271 R S N Q S F N P E D M S W S C G G G P D F K Y C V N D V T K
gcttccaaccagctccttcaaccagaggacatgtcctggagctgtggtggtgctcctgacttcaagtactcgtcaccagcgtcaccaa 1080
301 A G H T L D L L E P L V K F Q V G L K K L K L H E E E H V L
gggggtcacactctggacctgttgagccactggtgaagttccaggtgggctgaagaaactcaagctccatgaggaggaacacgtgct 1170
331 F M A I C L L S P D R P G V Q D H A K I E V P Q D R L S E V
gttcactggccatctgctgtctctcagcctcagacctgggtgacagatcacgagcttcccagacctctgctggagggt 1260
361 L Q A Y I R V N H P G G R L L Y A R M I Q K L A D L R S L N
gctccaggcctacatcagagtgaaacccccagggagcggctgctctatgccaggatgatccagaagctggccgacctgcccagcctcaa 1350
391 E E H S K Q Y R S L S F Q P E H S M Q L T P L V L E V F G S
cgaggagcactccaagcagtaaccgctcgtctcctccagccagagcacagcatgcagctcaccocgctggtgctggagggttttggggag 1440
421 E V S *
cgaggtgtcatagcagccctctgaccocctcccactgagggaccocctagatggcgttgagatgctggatggaggattcagcagtggaag 1530
ggatattgaggggatagccgaatggacctgatgtcacactgtcaagaaggttttgggggttaattaggggatgtgagtaaaagaga 1620
Ggagtgaggggtttgataaggttgaatttccactgataaaaaaaaaaaaaaaaaaaaaa 1684

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Figure 1 Full-length nucleotide and deduced amino acid sequence of the Atlantic salmon canonical vitamin D receptor (sVDR0) cDNA. The start codon is underlined, the stop codon is indicated by an asterisk (*). The first nucleotide of an exon, based on the zebrafish genome is boxed in gray. EMBL accession number AJ780914.

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acaagacagacattactgcaacctgaggggttgaagaataagagagccacctaaaggagtgttaggcagaggagagaggagaataactcg 90
ttttctgcagacctggggattttctctggaactgtattttctaattcagaggaaatggatccacagaccocctctctgagtcactcagt 180
1  M E T M A V S T S V V G P D E F D R N A P R I C G V C G D K
g a t g g a g a c c a t g g t g t g a g c a o g t c t g t g t g g g o c t g a t g a g t t t g a c o g c a a c o g c o c o g c a t c t g t g g o c t g t g g o g a c a a 270
31  A T G F H F N A M T C E G C K G F F R R S M K R K A S F T C
g g c c a c a g g e t t c c a a c t t c a a c g c c a t g a c o t g t g a g g g c t g c a a a g g c t t c t t c a g c g c a g c a t g a a g g t a a g g c a g t t t c a c c t g 360
61  P F N G S C T I T K D N R R H C Q Q A C R L K R C V D I G M M
c c c c t c a a c g g c a g c t g a c c a t c a c c a a g g a c a c c g g c a c c g c a c t g c c a g c c t g c o g g c t c a a a c g c t g c g t g g a c i t g g a c a t c g g c a t g a t 450
91  K E F I L T D E E V Q R K K D L I Q K R K E E E A A R E A L
g a a g g a g t c a t c o t g a c a g a t g a g g a g g t g c a g a g g a a g a a g g a c c t g a t c c a a g a g a g g a a g g a g g a a g a g g c g g c o g g g a g g c c t t 540
121 R P R L N D E Q S Q V I A S L V E A H H K T Y D D S Y S D F
g a g a c c t a g g c t g a a c g a c g a g c a g a g c c a g g t c a t c o g c o t c a t t g g t g g a g g c c c a c c a a g a c c t a t g a t g a c t o c t a c t c t g a c t t 630
151 S R F R P P V R E G P V T R S A S R A A S L H S L S D A S S
c t c a c g c t t c a g g c c c c a g t g c g a g a g g g c c c a g t a a c a c g c a g t g c c a g t c g t g c a g c c t c c o t t c a c t c t c t g t c a g a t g c c t c c t c 720
181 D S F N H S P E S V D T K L M N F S S L L M M Y Q D S A G S
a g a c t c c t t c a a c c a c t c a c a g g t g t g t g g a c a c c a a g c a t g a a c t t c a g c a g t c t g t t g a t g a t g t a c c a a g c a g t g a c g a g g c a g 810
211 P D S S E D D G S K L S M L P H L A D L V S Y S I Q K V I G
c c c a g a c t c c a g t g a g g a c a t g g c t c a a a c t c t c t a t g c t g c c c a c c t g g t g a c c t g g t c a g c t a c a g a t c c a g a a g g t c a t c o g g 900
241 F A K M I P G F R G L T A E D Q T A L L K S S A I E I I M L
c t t c g c t a a g a t g a t t c o t g g c t c o a g g g g c t g a c t g c t g a g g a c c a g a c c c c c t g c t g a a a t c c a g t g c c a t c g a g a t c a t c a t g c t 990
271 R S N Q S F N P E D M S W S C G G G P D F K Y C V N D V T K
g c g t t c c a a c c a g t c o t t c a a c c a g a g g a c a t g t c o t g g a g c t g g t g g t g g t c o t g a c t t c a a g t a c t g c g t c a a c g a c g t c a c c a a 1080
301 G E I H V H T Q Y M Y *
g g t t g a a a t a c a t g t a c a c a c a c a a t a c a t g t a t t a a a c t c c o c t c c g c a t g t t t a a a t t g g c t c t a t a c t c c a g c t t t t t g g t t t t g a 1170
g a t c a a a t g t t t c a t a t a a a g c a a a g g t g a c a t t c t g c a c a g t t a t t t t a g g t a t t t t c a t a t c t g t t t a c c g t t t a g a a a t g g a a g 1260
c a c t a t g t a c a g t g c a t t t a a a a a g t a t t c a g a c t c c g t g a c t t t t c c a c a t t t t g t t a c g t t a t a c t t a t c t a a a a t t g a t t a a a t a 1350
g t t t c o t c a t c a a t c a a c a c a c a c t a c c c c a t a a t a t g a c a a a g c a a a a a c t g t t t t t g g a a a t g t t t g c a a a t g t a t a a t a a t a a a a a 1440
a a a a a a a a a a a a a 1452

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Figure 2 Full-length nucleotide and deduced amino acid sequence of Atlantic salmon vitamin D receptor (sVDR1) cDNA. The start codon is underlined, the stop codon is indicated by an asterisk (*). The place of intron retention is boxed. EMBL accession number AM238619.

Cloning and sequencing of *sVDR*

The full-length 1684 bp cDNA sVDR0 sequence contains an open reading frame encoding a protein of 423 amino acids (Fig. 1; EMBL database: accession number AJ780914). During 3' RACE, two isoforms of the sVDR0, viz. sVDR1 (AM238619, Fig. 2) and sVDR2 (AM238620, Fig. 3), were identified. We compared the sVDR0 sequence with that present in the zebrafish VDR genome and identified eight exons in the sVDR0 (start of each exon is boxed in gray in Fig. 1). In both spliced variants sVDR1 and sVDR2, the first two base pairs differed from the sVDR0 and are 'gt' (boxed in Figs 2 and 3), which is the characteristic signal for the start of an intron. The strongest evidence for intron retention is probably the place of the splicing site, which is exactly in between two exons. We therefore conclude that the isoforms sVDR1 and sVDR2 are both generated from the primary transcript by alternative splicing due to intron retention.

Phylogenetic analysis

The relationship between the salmon canonical sVDR0 and orthologs of other species is presented in a consensus phylogenetic tree (Fig. 5). VDRs are rather conserved in vertebrates (Fig. 4), indicated by relatively high sequence identities in the fish line (*Danio rerio* 89%; *P. olivaceus* type a 90%; type b 87%) and with that of *Homo sapiens* (70%). The proper clustering of the fish VDR sequences (indicated in gray) and separate from amphibia, reptiles, birds, and

mammals is in line with the consensus about evolution of the vertebrates (Fig. 5).

Tissue distribution

To assess the tissue distribution of the sVDR gene expression, we used RT-PCR (Fig. 6). The predicted 470 bp sVDR was found throughout the intestine, head kidney, kidney, pituitary, ovary, muscle, and gill. We could not detect sVDR expression in the spleen, heart, liver, and skin. Attempts to demonstrate sVDR in skeletal tissue failed, which we relate to the difficult extraction of RNA from this tissue. However, in other unpublished studies, we succeeded to obtain sVDR signal from both bone and scales. The midgut RT, which served as control for potential genomic DNA contamination, was negative. The preparation of cDNA was assumed to be, judged on the successful amplification of an 87 bp 18S fragment in all organs.

Expression of the sVDR

Due to low expression of sVDR1 and sVDR2 (500–1000 times lower than sVDR0), we were not able to get reliable results on their quantitative expression. We therefore decided to design one primer set over an intron and at the beginning of the sequence to include all three transcripts sVDR0, sVDR1, and sVDR2 in our quantitative analyses. sVDR

```

acaagaacagacattactgacacctgaggggttgaagaataagagagccacctaaaggagtgt taggcagaggagagaggagaatactcg 90
ttttctcgacaccttggggatttctctggaactgtattttctaatccagaggaatggatccacagacccctctctgagtcactcagt 180
1 M E T M A V S T S V V G P D E F D R N A P R I C G V C G D K
gatggagaccatgctctgtgagcagctctgtggtggggcctgatgagttgacccgcaacgcgcgcgcatctgtggcgtctgtggcgacaa 270
31 A T G F H F N A M T C E G C K G F F R R S M K R K A S F T C
ggccacaggcttccacttcaacgccatgacctgtgagggtctgcaaggcttcttcagcgcgacatgaagcgttaaggccagtttccactg 360
61 P F N G S C T I T K D N R R H C Q A C R L K R C V D I G M M
ccccctcaacggcagctgtaccatcaccgaagcaaccggcggcactgccaggcctgccg gctcaaacgctgctggacatggcagat 450
91 K E E I L T D E E V Q R K K D L I Q K R K E E E A A R E A L
gaaggagtccatcctgacagatgaggaggtgacaggaagagacctgatccagaagaggagggaagaggcggcggggagggcctt 540
121 R P R L N D E Q S Q V I A S L V E A H H K T Y D D S Y S D F
gagacctagggtgaacgacgagcagagccaggtcatcgcctcattggtggaggccaccacaagacctatgatgactcctactctgactt 630
151 S R F R P P V R E G P V T R S A S R A A S L H S L S D A S S
ctcacgcttcaggccccagtgcgagagggcccagtaacacgcagtgccagctctgagcgcctccctcactctctgtcagatgctcctc 720
181 D S F N H S P E S V D T K L M N F S S L L M M Y Q D S A G S
agactcctcaaccactcaccaggtctgtggacaccaagctgatgacttccagcagctctgttgatgaccaggacagtgctggcag 810
211 P D S S E D D G S K L S M L P H L A D L V S Y S I Q K V I G
cccagactccagtgaggacgatggctccaaactctctatgctgccccacctggctgacctggtcagctacagcatccagaaggtcatcgg 900
241 F A K M I P G F R L L S C *
cttcgctaagatgattcctggcttccagcttattgtcctgctgaaaggtgaaatcctcctccagtgctgttgggaaggcagatgaaccag 990
gtttcctctaggattttgctctgcttaggtccgttccatttggtttttttcttaaaaaaagactccccagctccttaagtattacaa 1080
gcatgccataacatgatgacaccactatgcttggaaatatgtaatgtgtgtattg gatttggcccaaacataacactttgtaatc 1170
aggcaaaaaagtaattgctttgccacattttgacagcattacgttagtgcttggcaaacaggatgcattgtttggaatatatgta 1260
ttttgacaggtcccttttccactctgttaattaggttagtatttttggtaactacaatgtgttgaaccatcctcagtttttccaat 1350
cccagccatataactttgtaactgttttaagtcaaaaaaaaaaaaaaaaaaaaaa 1405

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Figure 3 Full-length nucleotide and deduced amino acid sequence of Atlantic salmon vitamin D receptor (sVDR2) cDNA. The start codon is underlined, the stop codon is indicated by an asterisk (*). The place of intron retention is boxed. EMBL accession number AM238620.

expression in the kidney (Table 3) is 2.8-fold down-regulated ($P=0.01$) in FW smolt when compared with parr. During SW transfer, the sVDR is up-regulated to levels intermediate between parr and FW smolt. In adult SW fish, the expression of the sVDR is again down-regulated and threefold ($P=0.015$) lower than that in parr, but not significantly different from FW smolt.

sVDR expression in the midgut is 2.2-fold ($P=0.007$) down-regulated in the FW smolt when compared with parr. In 50% SW smolt, the expression of the sVDR increases ($P<0.05$) when compared with FW smolt, to a similar level as seen in parr. In SW fish, the expression declines again and adult fish show a significantly lower sVDR expression ($P<0.05$) than parr and fishes in 50% SW.

The expression of sVDR in the gill of FW smolt is down-regulated 2.4-fold ($P=0.0015$) when compared with parr. During SW transfer, sVDR expression is up-regulated to the same level as in parr. sVDR expression in adult fishes is significantly decreased ($P<0.05$) when compared with parr, 50% SW and SW smolt, but not significantly different from FW smolt.

In Figs 7–9, we plotted calcitriol plasma levels against sVDR mRNA expression in kidney, gill, and intestine

respectively. Each graph shows six individual fish from groups ‘parr’, ‘FW smolt’, and ‘adult’. In all tissues, we see a negative correlation between calcitriol plasma levels and sVDR mRNA expression. Data on parr are clustering separately from FW smolt and adult fish.

Discussion

The demonstration of sVDR in calcium regulatory tissues plus a negative feedback relation between plasma calcitriol levels and sVDR mRNA expression provide firm evidence for a functional vitamin D endocrine system in Atlantic salmon. No correlation between plasma levels of 24,25(OH)₂D₃ or 25(OH)D₃ and sVDR mRNA was found, suggesting that 1,25(OH)₂D₃ is the only ligand for sVDR tested in this study. Three other key observations were made. First, salmon sVDR has two spliced variants due to intron retention. Secondly, plasma calcitriol levels more than doubled in SW fish and coincide with decreasing levels of sVDR expression in gill, intestine, and kidney. Thirdly, vitamin D metabolite levels and sVDR expression in FW smolts are not significantly different to SW adult fish,

Figure 4 Multiple alignment of VDRs of seven different vertebrate species and the sea lamprey. Hyphens indicate gaps that were introduced to achieve maximum identity. Identical amino acids are indicated in black boxes; conservative substitutions are in gray. *, cysteine residues critical for Zinc finger formation; \wedge , amino acids important for heterodimerization with retinoid X receptor (RXR); #, amino acids important for binding 1,25(OH)₂D₃. The different domains of the nuclear receptor are indicated below the sequence. Accession numbers: sea lamprey (*Petromyzon marinus*) VDR, AY249863; bastard halibut (*Paralichthys olivaceus*) VDR type A, AB037674; bastard halibut (*Paralichthys olivaceus*) VDR type B, AB037673; Atlantic salmon (*Salmo salar*) VDR, AJ780914; zebrafish (*Danio rerio*) VDR, NM_130919; xenopus (*Xenopus laevis*) VDR, U91846; chicken (*Gallus gallus*) VDR, NM_205098; and human (*Homo sapiens*) VDR, NM_000376.

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Salmon 1 -----MEIMAVSTSMVGE--DEFDRNAPRI CGVCGDKATGFHFNAMTCEGCKGF
Lamprey 1 -----MMATNMTVSTSNALE--DEFEGGVPKVCGVCGDKATGFHFNAMTCEGCKGF
HalibutA 1 -----MEIMAVSTSMVGE--DEFDRNAPRI CGVCGDKATGFHFNAMTCEGCKGF
HalibutB 1 -----MEPTVSTSSIAS--DEFDRNMPRI CGVCGDKATGFHFNAMTCEGCKGF
Danio 1 MLTENSAVNSGGKSKCEAGACESTVNGDATSIMIMAVSTSATSQ--DEFDRNAPRI CGVCGDKATGFHFNAMTCEGCKGF
Xenopus 1 -----MERMAATTSIADTDMEFDKNVPRI CGVCGDKATGFHFNAMTCEGCKGF
Chicken 1 -----MSELRGSWDEQQSMAYLPDADMEITVPASTSLPPEAGDFDRNVPRI CGVCGDKATGFHFNAMTCEGCKGF
Human 1 -----MEIMAVSTSMVGE--DEFDRNAPRI CGVCGDKATGFHFNAMTCEGCKGF
    
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Salmon 48 FRRSMKRKASFTCPFNGSCITIKDNRHRCQACRLKRCVDIGMMKEFILTDEEVQRKDLIKRKEE---EAREALRPR
Lamprey 51 FRRSMKRKASFTCPFNGSCITIKDNRHRCQACRLKRCVDIGMMKEFILTDEEVQRKDLIKRKEE---DSARFVHTFQ
HalibutA 48 FRRSMKRKASFTCPFNGSCITIKDNRHRCQACRLKRCVDIGMMKEFILTDEEVQRKDLIKRKEE---EAREAMRPR
HalibutB 48 FRRSMKRKASFTCPFNGSCITIKDNRHRCQACRLKRCVDIGMMKEFILTDEEVQRKDLIKRKEE---EAREARPR
Danio 80 FRRSMKRKASFTCPFNGSCITIKDNRHRCQACRLKRCVDIGMMKEFILTDEEVQRKDLIKRKEE---EAREARPR
Xenopus 49 FRRSMKRKASFTCPFNGSCITIKDNRHRCQACRLKRCVDIGMMKEFILTDEEVQRKDLIKRKEE---EAREARPR
Chicken 71 FRRSMKRKASFTCPFNGSCITIKDNRHRCQACRLKRCVDIGMMKEFILTDEEVQRKDLIKRKEE---EAREARPR
Human 48 FRRSMKRKASFTCPFNGSCITIKDNRHRCQACRLKRCVDIGMMKEFILTDEEVQRKDLIKRKEE---EAREARPR
    
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Salmon 124 LNDEQSQVIASLVEAHHKTYDYSYDFSRFRPVPVREGPVT--RSASRAASLHSLSDASSDSFN---HSPESVDTKLNF
Lamprey 127 LLEQERLIATLEAHRKTYDASYSDFSRFRPVPVREGPVT--RSGDGSPECRNATNPFIMSIINSDMELP-----
HalibutA 124 LNDEQSQVIASLVEAHHKTYDYSYDFSRFRPVPVREGPVT--RSASRAASLHSLSDASSDSFN---HSPESVDTKLNF
HalibutB 128 LIDQSQVIASLVEAHHKTYDYSYDFSRFRPVPVREGPVT--RSASRAASLHSLSDASSDSFN---HSPESVDTKLNF
Danio 156 LSEPCQRIATLLEAHHKTYDYSYDFSRFRPVPVREGPVT--RSASRAASLHSLSDASSDSFN---HSPESVDTKLNF
Xenopus 125 LSEPCQRIATLLEAHHKTYDYSYDFSRFRPVPVREGPVT--RSASRAASLHSLSDASSDSFN---HSPESVDTKLNF
Chicken 147 LSEPCQRIATLLEAHHKTYDYSYDFSRFRPVPVREGPVT--RSASRAASLHSLSDASSDSFN---HSPESVDTKLNF
Human 124 LSEPCQRIATLLEAHHKTYDYSYDFSRFRPVPVREGPVT--RSASRAASLHSLSDASSDSFN---HSPESVDTKLNF
    
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Salmon 198 SLLMMYQDSGSSPDSSEEDS--SLSMLPHLADLVYSIQKVIQFQAKMIPGFRDLTAEQIALLKSSAIEVIMLRNSQSF
Lamprey 189 -----KASASGAEAAAE--DELSMLPHLADLVYSIQKVIQFQAKMIPGFRDLTAEQIALLKSSAIEVIMLRNSQSF
HalibutA 197 SLLMMYQDSGSSPDSSEEDS--SLSMLPHLADLVYSIQKVIQFQAKMIPGFRDLTAEQIALLKSSAIEVIMLRNSQSF
HalibutB 201 NLLMMYQDSGSSPDSSEEDS--SSLSMLPHLADLVYSIQKVIQFQAKMIPGFRDLTAEQIALLKSSAIEVIMLRNSQSF
Danio 229 SLLMMYQDSGSSPDSSEEDS--SLSMLPHLADLVYSIQKVIQFQAKMIPGFRDLTAEQIALLKSSAIEVIMLRNSQSF
Xenopus 199 SLLMMYQDSGSSPDSSEEDS--SLSMLPHLADLVYSIQKVIQFQAKMIPGFRDLTAEQIALLKSSAIEVIMLRNSQSF
Chicken 226 SLLMMYQDSGSSPDSSEEDS--SLSMLPHLADLVYSIQKVIQFQAKMIPGFRDLTAEQIALLKSSAIEVIMLRNSQSF
Human 202 SLLMMYQDSGSSPDSSEEDS--SLSMLPHLADLVYSIQKVIQFQAKMIPGFRDLTAEQIALLKSSAIEVIMLRNSQSF
    
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Salmon 277 NEDMSWTCGSNDFKYSVDVTKAGHTLLELLEPLVKFQVGLKKNLHHEEHVLMIAICLLSPDRPGVDHARVEIQDR
Lamprey 259 TMEIDNSWTCGSNDFKYSVDVTKAGHTLLELLEPLVKFQVGLKKNLHHEEHVLMIAICLLSPDRPGVDHARVEIQDR
HalibutA 275 NEDMSWTCGSNDFKYSVDVTKAGHTLLELLEPLVKFQVGLKKNLHHEEHVLMIAICLLSPDRPGVDHARVEIQDR
HalibutB 280 NEDMSWTCGSNDFKYSVDVTKAGHTLLELLEPLVKFQVGLKKNLHHEEHVLMIAICLLSPDRPGVDHARVEIQDR
Danio 308 NEDMSWTCGSNDFKYSVDVTKAGHTLLELLEPLVKFQVGLKKNLHHEEHVLMIAICLLSPDRPGVDHARVEIQDR
Xenopus 275 NEDMSWTCGSNDFKYSVDVTKAGHTLLELLEPLVKFQVGLKKNLHHEEHVLMIAICLLSPDRPGVDHARVEIQDR
Chicken 304 TMEIDNSWTCGSNDFKYSVDVTKAGHTLLELLEPLVKFQVGLKKNLHHEEHVLMIAICLLSPDRPGVDHARVEIQDR
Human 280 TMDMSWTCGSNDFKYSVDVTKAGHTLLELLEPLVKFQVGLKKNLHHEEHVLMIAICLLSPDRPGVDHARVEIQDR
    
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Salmon 357 LSEFLQAYIRCRHPPESRLLYAKMIQKLADLRSLNEEHSKQYRSLSFQPEHSMQLTPLVLEVFGEVSE
Lamprey 338 LSETFLQAYIRCRHPPESRLLYAKMIQKLADLRSLNEEHSKQYRSLSFQPEHSMQLTPLVLEVFGEVSE
HalibutA 354 LSEFLQAYIRCRHPPESRLLYAKMIQKLADLRSLNEEHSKQYRSLSFQPEHSMQLTPLVLEVFGEVSE
HalibutB 359 LSETFLQAYIRCRHPPESRLLYAKMIQKLADLRSLNEEHSKQYRSLSFQPEHSMQLTPLVLEVFGEVSE
Danio 387 LSEFLQAYIRCRHPPESRLLYAKMIQKLADLRSLNEEHSKQYRSLSFQPEHSMQLTPLVLEVFGEVSE
Xenopus 354 LSEFLQAYIRCRHPPESRLLYAKMIQKLADLRSLNEEHSKQYRSLSFQPEHSMQLTPLVLEVFGEVSE
Chicken 383 LSETFLQAYIRCRHPPESRLLYAKMIQKLADLRSLNEEHSKQYRSLSFQPEHSMQLTPLVLEVFGEVSE
Human 359 LSEFLQAYIRCRHPPESRLLYAKMIQKLADLRSLNEEHSKQYRSLSFQPEHSMQLTPLVLEVFGEVSE
    
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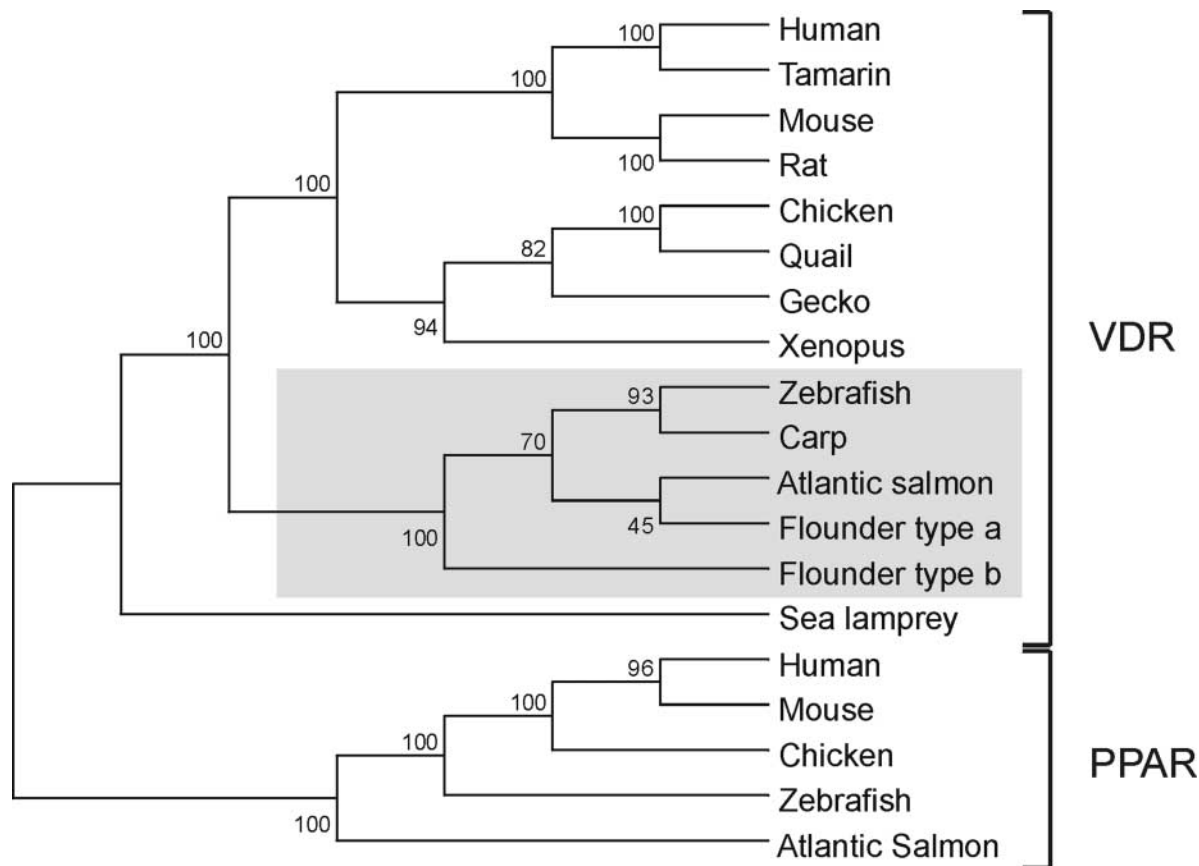


Figure 5 Neighbor-joining tree of VDR receptor amino acid sequences. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. Peroxisome proliferative activated receptor (PPAR) was used as out-group. Accession numbers: sea lamprey (*Petromyzon marinus*) VDR, AY249863; bastard halibut (*Paralichthys olivaceus*) VDR type a, AB037674; bastard halibut (*Paralichthys olivaceus*) VDR type b, AB037673; atlantic salmon (*Salmo salar*) VDR, AJ780914; zebrafish (*Danio rerio*) VDR, NM_130919; common carp (*Cyprinus carpio*) VDR, AJ784084; chicken (*Gallus gallus*) VDR, NM_205098; quail (*Coturnix japonica*), U12641; gecko (*Gekko gecko*) VDR, AY254096; xenopus (*Xenopus laevis*) VDR, U91846; rat (*Rattus norvegicus*) VDR; NM_017058; mouse (*Mus musculus*) VDR, NM_009504; tamarin (*Saguinus oedipus*) VDR, AF354232; human (*Homo sapiens*) VDR, NM_000376; atlantic salmon (*Salmo salar*) PPAR, AJ292963; zebrafish (*Danio rerio*) PPAR, XM_686663; chicken (*Gallus gallus*) PPAR, NM_001001464; mouse (*Mus musculus*) PPAR, NM_011144; and human (*Homo sapiens*) PPAR, NM_001001928.

indicating a calcemic pre-adaptation in FW. We will elaborate on these findings below.

VDRs in salmon

Intron retention has been demonstrated for a mouse VDR once before (Ebihara *et al.* 1996). This is the first report for such a phenomenon outside mammals, in a teleostean VDR. Intron retention of both the sVDR1 (intron 7) and sVDR2 (intron 6) occurs in the E/F domain and as a consequence both receptors are predicted to be impaired in retinoid X receptor (RXR) heterodimerization and ligand binding. The splicing of the murine VDR occurs at intron 8, and also in the E/F domain (Ebihara *et al.* 1996), and it was concluded that homodimer-spliced variants may compete at specific VDR responsive elements and by doing so prevent actions of the

canonical VDR. We can only speculate on a similar role for the two spliced variants in salmon. However, we predict that the vast majority (>99%) of the sVDR mRNA consists of the canonical receptor sVDR0, making this transcript presumably the most important in Atlantic salmon.

However, low mRNA levels do not, by default, exclude a role for the spliced variants and it is important to make some remarks on the nature of these variants. A common feature among the spliced variants known to us is that splicing always occurs in the E/F domain. Indeed, in common carp (*C. carpio*) VDR (AJ784084), we found two alternatively spliced variants that result from intron retention, again in the E/F domain. Cypriniformes (belonging to the Ostariophysi) and Salmoniformes (belonging to the Protacanthopterygi; Nelson 1994) share a common ancestor in the Cretaceous period some 130–140 million years ago. Teleosts and mammals shared a common

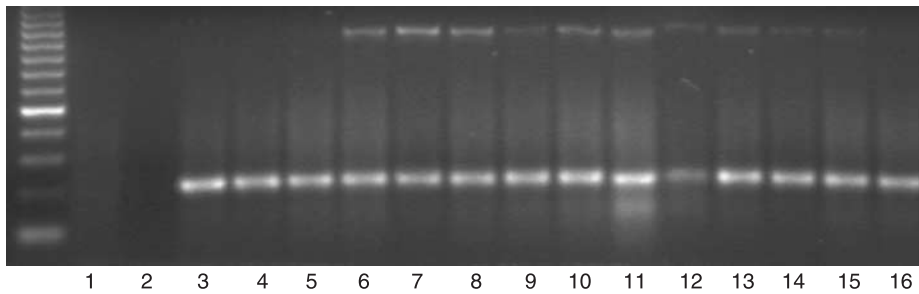


Figure 6 RT-PCR analysis of tissue expression of sVDR, 18S serves as a control. Lane 1, NAC; 2, NTC; 3, spleen; 4, heart; 5, liver; 6, hind intestine; 7, mid intestine; 8, pyloric caecae; 9, head kidney; 10, kidney; 11, ovary; 12, testis; 13, muscle; 14, pituitary; 15, gill; and 16, skin.

ancestor 450 million years ago (Hoegg & Meyer 2005). The 'survival' of spliced variants during a significant time of evolution suggests a functional role for the variants.

A recently proposed conformational ensemble model for VDR activity by Norman (2006) describes that the chirality of calcitriol may translate into either slow classical genomic or more rapid responses previously assigned to a putative membrane receptor. The canonical salmon VDR and splice variant sVDR1 could function along the model proposed by Norman (2006); the splice variant sVDR2 lacks amino acids R274 and S278, two residues that are crucial for ligand stabilization and their absence could impair functionality of this receptor. As both splice variants are impaired in their heterodimerization with the RXR (Ebihara *et al.* 1996), genomic actions seem excluded, and rapid membrane-bound receptor activity remains a possibility. Indeed, it now appears that the VDR in some cells is associated with caveolae in or near the plasma membrane (Huhtakangas *et al.* 2006), which would give an anatomical argument for plasma membrane-associated VDR activity. Clearly, this puzzle is very complex and warrants further research.

From FW to SW: increasing calcitriol and decreasing sVDR

Plasma calcitriol levels more than doubled in SW adult salmon when compared with FW parr. Simultaneously, the mRNA expression of sVDR decreases in gill, intestine, and kidney. Three important considerations concerning the sVDR and its

ligand calcitriol should be advanced before we can further hypothesize about the function of the vitamin D endocrine system in this salmon. First, the affinity of the sVDR to calcitriol is unknown and awaits transactivation studies. Secondly, we have no information on metabolic clearance and secretion rates or distribution space of calcitriol in FW and/or SW Atlantic salmon. Thirdly, both slow genomic responses as well as more rapid responses of calcitriol exist that require different regulation. This means we cannot solely depend on calcitriol and sVDR data to understand the calcitriol system completely.

As mentioned in the considerations above, rapid responses to calcitriol exist: within minutes after administration, calcitriol increases intestinal calcium transport and activates protein kinase C in FW fish (Nemere *et al.* 2000) and mammals (Boyan *et al.* 1999, Wali *et al.* 2003). Larsson *et al.* (2003) conducted binding studies to enterocyte membrane receptors for calcitriol in rainbow trout (*Oncorhynchus mykiss*), a close relative of salmon. They demonstrated that after acclimation to SW specific binding of calcitriol decreased, which they interpreted as a down-regulation of a putative membrane VDR due to a decreased need for short-term calcitriol-driven calcium transport. The longer-term calcitriol actions via the nuclear VDR have not been studied in salmon before. Increasing plasma calcitriol concentrations do not, by default, result in increased stimulation of vitamin D responsive elements. Studies in humans (Ebeling *et al.* 1992) and rats (Horst *et al.* 1990) have demonstrated that intestinal resistance to calcitriol exists and

Table 3 Vitamin D receptor (sVDR) expression in the gill, intestine, and kidney of parr, FW smolt, 50% SW smolt, SW smolt, and adult Atlantic salmon. Values are the average of 8 fish \pm s.d.

	Kidney	Intestine	Gill
Group			
Parr	100 \pm 39.2 (100)*	128 \pm 61.7 (100)*	146 \pm 52.7 (100)*
FW smolt	41 \pm 28.3 (41) [†]	58 \pm 29.2 (45) [†]	56 \pm 16.6 (39) ^{††}
50%SW smolt	64 \pm 57.9 (64)* [†]	136 \pm 88.3 (107)*	104 \pm 51.4 (71)* [†]
SW smolt	82 \pm 61.8 (82)* [†]	86 \pm 18.1 (67)* [†]	125 \pm 57.8 (86)*
Adult	41 \pm 27.2 (40) [†]	56 \pm 24.5 (44) [†]	49 \pm 14.5 (33) [†]

The sVDR expression in the parr kidney group was set at 100%, other sVDR expressions are relative to parr kidney. Relative changes in sVDR expression within each tissue is indicated within brackets (tissue parr = 100%) and significant differences ($P=0.05$) are indicated with *, †, or ††. FW, freshwater; SW, seawater.

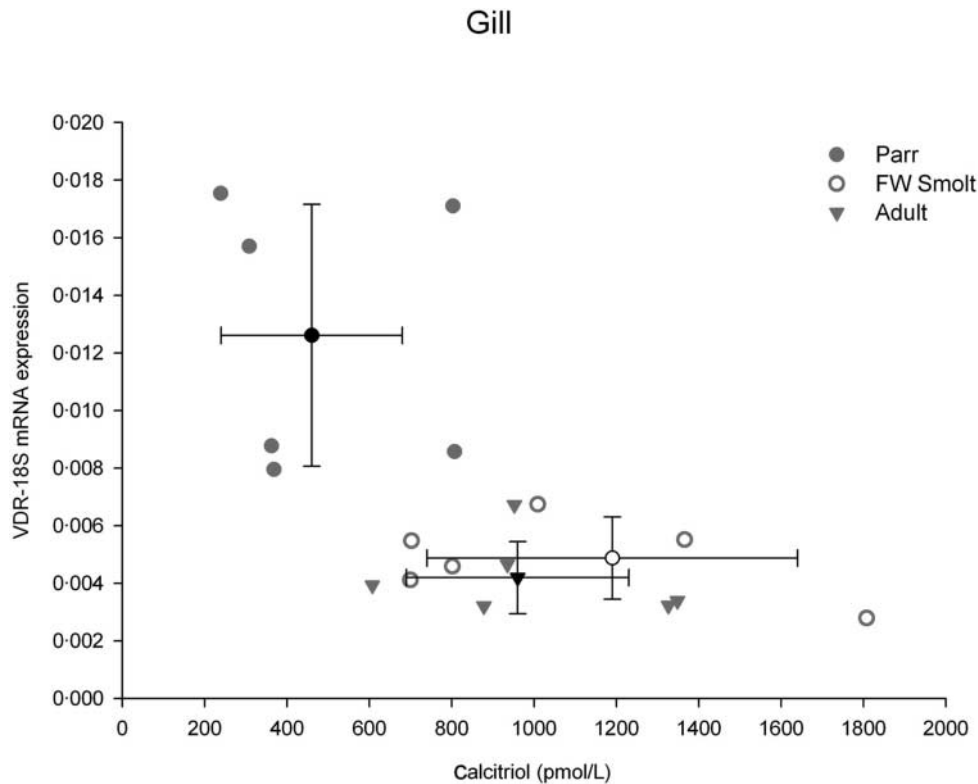


Figure 8 Plasma calcitriol and sVDR expression in the gill of Atlantic salmon (*Salmo salar*) in two fresh-water stages; parr and FW smolt and the salt-water stage adult.

genomic regulation. Moreover, it suggests a (direct) coupling of calcitriol and phosphorus transport mechanisms. Indeed, if this direct coupling exists in Atlantic salmon than the predicted enhanced need for phosphorus during the growth spurt of the salmon at sea would clearly benefit from higher plasma calcitriol levels to guarantee maximum phosphorus uptake and retention within physiological limits. With the differences in calcium and phosphorus availability and uptake routes mentioned above, the picture emerges of a dual uptake route for calcium (gills and intestine) and a single route for phosphorus (intestine). The ion-transporting cells in fish gills (chloride cells; Flik *et al.* 1996) and intestine (enterocytes) thus differ in their make-up for calcium uptake or combined calcium and phosphorus uptake respectively and it is interesting to elucidate the role of the vitamin D endocrine system in these transports.

However, we must realize that unlike mammals that are dependent upon their food for calcium, fish have access to an unlimited amount of calcium via the ambient water. In fish, it is phosphorus availability that is limited, and fast non-genomic responses in phosphorus uptake are therefore highly desirable. Further research is needed to investigate the role of the vitamin D endocrine system in short- and long-term phosphorus uptake in salmon.

Smoltification and vitamin D: pre-adaptation to SW

During smoltification, the fish prepares itself for life at sea and undergoes morphological as well as physiological changes. External stimuli like light trigger the smoltification process, and several hormones involved in the smoltification process (GH, PRL) have been identified (McCormick *et al.* 1987). On the basis of this study calcitriol can be added to the list of hormones involved or taking part in the smoltification process. Compared with parr, calcitriol plasma level and sVDR mRNA expression in FW smolts significantly ($P < 0.05$) increase and decrease respectively these levels are retained in SW adult fish. There is no significant difference ($P > 0.05$) in calcitriol plasma levels and sVDR mRNA levels between FW smolt and SW adult salmon. This indicates that during smoltification, the calcitriol-sVDR axis in FW is changing to the SW status. Plasma calcitriol levels during SW transfer significantly fluctuate. They are highest in 50% SW smolts, about 3–4 times higher than FW or SW smolts. This is in line with observations in rainbow trout (Larsson *et al.* 2003). One day after SW transfer calcitriol plasma levels in trout peaked and decreased with time spent in SW.

Changes in sVDR mRNA level and plasma calcitriol in our study do substantiate a role for the vitamin D endocrine system in the physiology of Atlantic salmon. The observation of sVDR variants that are spliced in one of

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