PHYSIOLOGICAL AND MOLECULAR MECHANISMS OF OOCYTE HYDRATION IN AN EVOLUTIONARY OLD TELEOST, ATLANTIC HERRING (*Clupea harengus*)

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Contents

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List of abbreviations

3D	three dimensional
AQP	aquaporin
B	benthic
CatB	cathepsin B
CatD	cathepsin D
CatL	cathepsin L
CT	C-terminal coding region
CV	cleavage variant
ER	endoplasmic reticulum
FW	freshwater
FAA	free amino acid
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
GSI	gonadosomatic index
GVBD	germinal vesicle breakdown
LH	luteinizing hormone
Lv	lipovitellin
LvH	lipovitellin heavy chain
LvHc	LvH C-terminal part
LvHn	LvH N-terminal part
LvL	lipovitellin light chain
LvL-MCS	LvL maturational cleavage site
MIS	maturation inducing steroid
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mya	million years ago
ntDNA	nuclear DNA
ooc	oocyte
ooc LvL-CS	oocyte LvL cleavage site
OV egg	ovulated egg
P	pelagic
PGC	primordial germ cell
Pv	phosvitin
R2	second round of WGD
R3	third round of WGD
SW	seawater
Vtg	vitellogenin (protein)
vtg	vitellogenin (transcript/gene)
Vtgr	vitellogenin receptor
Vwfd	von Willebrand factor type D domain
WGD	whole genome duplication
Yp	yolk protein
Zp	zona protein
β'	beta-component

Abstract

This work investigated physiological and molecular mechanisms of oocyte hydration in Atlantic herring (*Clupea harengus*). Both inorganic and organic solutes were found to be important solutes involved in the hydration process. Inorganic ions represented the dominant osmolytes (71%) while a small pool of free amino acids (FAA) contributed 29% to egg osmolality (Paper I). To understand the origin of the pool of FAA that appear during this period, the molecular processing of vitellogenin (Vtg) and its derivative yolk proteins (Yp) were studied (Paper II). The findings were integrated into a wider context of teleost evolution and biodiversity by examining the phylogenetic relationships of teleost Vtgs in relation to vertebrate whole genome duplications (WGD) and the oceanic radiation of teleosts (Paper III).

Atlantic herring is a marine teleost belonging to the Clupeiformes that spawns benthic eggs (benthophil). It is a representative of one of the first teleost orders to reinvade the oceans during the early Cretaceous. Oocyte hydration in this species is modest, rising from a relative water content of 59% in post-Vtg oocytes to 70-72% in ovulated eggs (OV egg). The major inorganic and organic osmolytes responsible for driving oocyte hydration are Cl^-, K^+, P_i, and a small pool of FAA, respectively. K^+ (autumn and spring spawners) and P_i (spring) maintain their concentrations in the ovulated eggs, while other measured ions, Cl^-, Na^+, NH_4^+, and Mg^{2+} are significantly diluted during the hydration process. In contrast, the concentration of FAA increased during this phase. Due to the low water content of these eggs the relatively small increase in FAA from 1.5% up to 3.3% of dry mass contributed 29% to the calculated ovoplasmic osmolality.

To determine the origin of the FAA pool in Atlantic herring, a hepatically expressed *vtg* transcript was cloned and the deduced Vtg product examined in relation to the deposited oocyte and egg Yps. Unlike all teleosts studied to date, only a single complete type *vtg* transcript (*chvtgAa*) was found to be expressed. Genomic analyses of exon-intron structures conserved between *vtgs* of Atlantic herring and zebrafish show that at least two *vtg* genes exist in the genome, but that Atlantic herring has only a single form of *vtg*.

Partial degradation of Yps in the OV egg showed that moderate proteolytic processing takes place during oocyte hydration. The phosvitin domain, the smallest yet reported for teleosts, and an N-terminal fragment of the lipovitellin light chain are suggested to be the precursors of the FAA pool. Molecular evidence for the presence of the C-terminal coding region of Vtg in the Yp pool is also presented for the first time in any teleost. Conserved maturational-cleavage sites were identified by N-terminal micro-sequencing and the 3D homology of Yp products of Atlantic herring were verified by mapping to the crystallographically resolved structure of lamprey lipovitellin. Multiple methods of phylogenetic analyses of the *vtg* cDNA and Vtg amino acid sequences placed Atlantic herring at the basal root of the Clupeocephala, in full congruence with current understanding of teleost phylogeny.

Integration of phylogenetic analyses of all currently available vertebrate *vtg* sequences (expressed and genomic) with the environmental history of fishes, the three rounds of WGD, and the end-point of lipovitellin heavy chain degradation led to the proposal of a new vertebrate *vtg* gene nomenclature. These combined data further led to the proposal that the neo-functionalisation of duplicated *vtg* genes was a key event in the evolution and success of the teleosts in the oceanic environment.

Aims of the thesis

The overall aim of this thesis was to investigate how a primitive marine teleost solved the physiological challenge of spawning its free-living eggs in the hyper-osmotic condition of seawater. Previous studies have shown that many marine teleosts spawn highly hydrated pelagic eggs (pelagophils), and that the influx of water occurs during the final stages of oocyte maturation mainly due to an increase in the oocyte pool of free amino acids (FAA). Available data on marine benthophil species, however, show that oocyte hydration is comparatively modest with inorganic ions and the amino acid analogue taurine being implicated in the hydration process. To gain insight as to the early origins of the oocyte hydration mechanisms in teleosts with benthic eggs, Paper I examined the simultaneous changes in multiple ions and organic osmolytes during oocyte hydration in the Atlantic herring, an evolutionary old teleost with benthic eggs.

Having determined in Paper I that a small pool of FAA contributes significantly to oocyte hydration also in Atlantic herring, Paper II aimed at identifying the precursor-product relationships between the parent Vtg molecule, the deposited Yps and the origin of the FAA pool in this species. To achieve this, a full-length *vtg* was isolated and cloned from vitellogenic livers and the precursor-products relationships were studied by mapping the Yps to the parent Vtg molecule and by 3D modelling. In order to understand the significance of the *vtg* transcript in this species, a molecular phylogeny-based approach was adopted.

To further investigate the origin and significance of the yolk proteolytic mechanisms involved in oocyte hydration of marine teleosts, Paper III examined the molecular phylogeny of all currently available (late 2006) vertebrate *vtg* genes (expressed transcripts and genomic variants). The aims were to gain insight into when multiple forms of *vtg* genes arose during teleost phylogeny, and what role WGD played in this process. All available vertebrate *vtg* sequences were subjected to parsimony, maximum likelihood, Bayesian, molecular clock and Ka/Ks analyses in the context of teleost evolution and biodiversity.

List of publications

This thesis is based upon the following papers which are referred to in the text by their roman numerals:

Paper I.

Kristoffersen, B. A. and Finn, R. N. (2008). "Major osmolyte changes during oocyte hydration of a clupeocephalan marine benthophil: Atlantic herring (*Clupea harengus*)." Marine Biology (2008) DOI: 10.1007/s00227-008-0961-8 (In press).

Paper II.

Kristoffersen, B. A., Nerland, A., Nilsen, F., Kolarevic, J. and Finn, R. N. (2008). "Genomic and proteomic analyses reveal a single form of vitellogenin in the basal clupeocephalan Atlantic herring, with partial degradation of derivative yolk proteins during oocyte hydration." Mol Biol Evol (Ms to be submitted).

Paper III.

Finn, R. N. and Kristoffersen, B. A. (2007). "Vertebrate Vitellogenin Gene Duplication in Relation to the "3R Hypothesis": Correlation to the Pelagic Egg and the Oceanic Radiation of Teleosts." PLoS ONE 2(1): e169. doi:10.1371/journal.pone.0000169.

Introduction and general discussion

Today fish can be found almost wherever there is water, from the coldest of conditions in the Arctic and Antarctic to tropical warm waters and hot thermal springs, in the deepest seas to mountain lakes, as well as in freshwater and highly saline waters. They also come in all colours and shapes, and some are small and others immense (Nelson 2006). The present thesis takes into consideration some of the reproductive adaptations this multitude of ~ 28,000 species of fish (Balon 1975; Nelson 2006) have adopted. A starting point in this investigation is to look at teleost origins and some of the consequences of the evolutionary paths they have taken.

Fish evolution

In the vertebrate lineage the split between the Sarcopterygii (lobe-finned fish) and Actinopterygii (ray-finned fish) took place ~450 million years ago (mya) (Maissey 1996; Inoue *et al.* 2003; Gardiner *et al.* 2005; Hurley *et al.* 2007) (Figure 1). Within the Actinopterygii the topology among the basal clades; polypterids (bichirs and reedfish), chondrosteans (sturgeons and paddlefish), lepisosteids (garpike), amiids (bowfin) and teleosts, has been under recent discussion (Inoue *et al.* 2003, 2004, 2005; Hurley *et al.* 2007). Also the divergence date estimates between the clades are still under evaluation, with discrepancies between fossil records and molecular time estimates using either mitochondrial DNA (mtDNA) or nuclear DNA (ntDNA) (Inoue *et al.* 2003, 2005; Hurley *et al.* 2005, 2007) (Figure 1). However, although there remain some issues on the topology amongst the older Actinopterygii, as well as how some species of families within larger orders such as the Ostariophysi are related (Briggs 2005), the major divisions as shown in Figure 1 are widely accepted.

Also the origin of marine teleosts has been a focus of discussion (Griffith 1987, 1991; Fyhn *et al.* 1999) and is still not a resolved case (paper III). The “ancient fish clades”, (polypterids, chondrosteans, lepisosteids and amiids) at the root of the Actinopterygii only contain extant members that are exclusively freshwater-species. Some sturgeons have anadromic behaviour but all are obligate freshwater spawners. Of the oldest teleosts the Osteoglossomorpha (bony tongues) all - both extinct and extant – were and are freshwater species (Kumazawa and Nishida 2000; Nelson 2006). Marine teleosts

first appear among the Elopomorpha (e.g. eels and tarpons) and later among the Clupeocephala, mainly among the Clupeiformes (Maissey 1996; Nelson 2006). However, it is the appearance of the largest actinopterygian clade, the Acanthomorpha

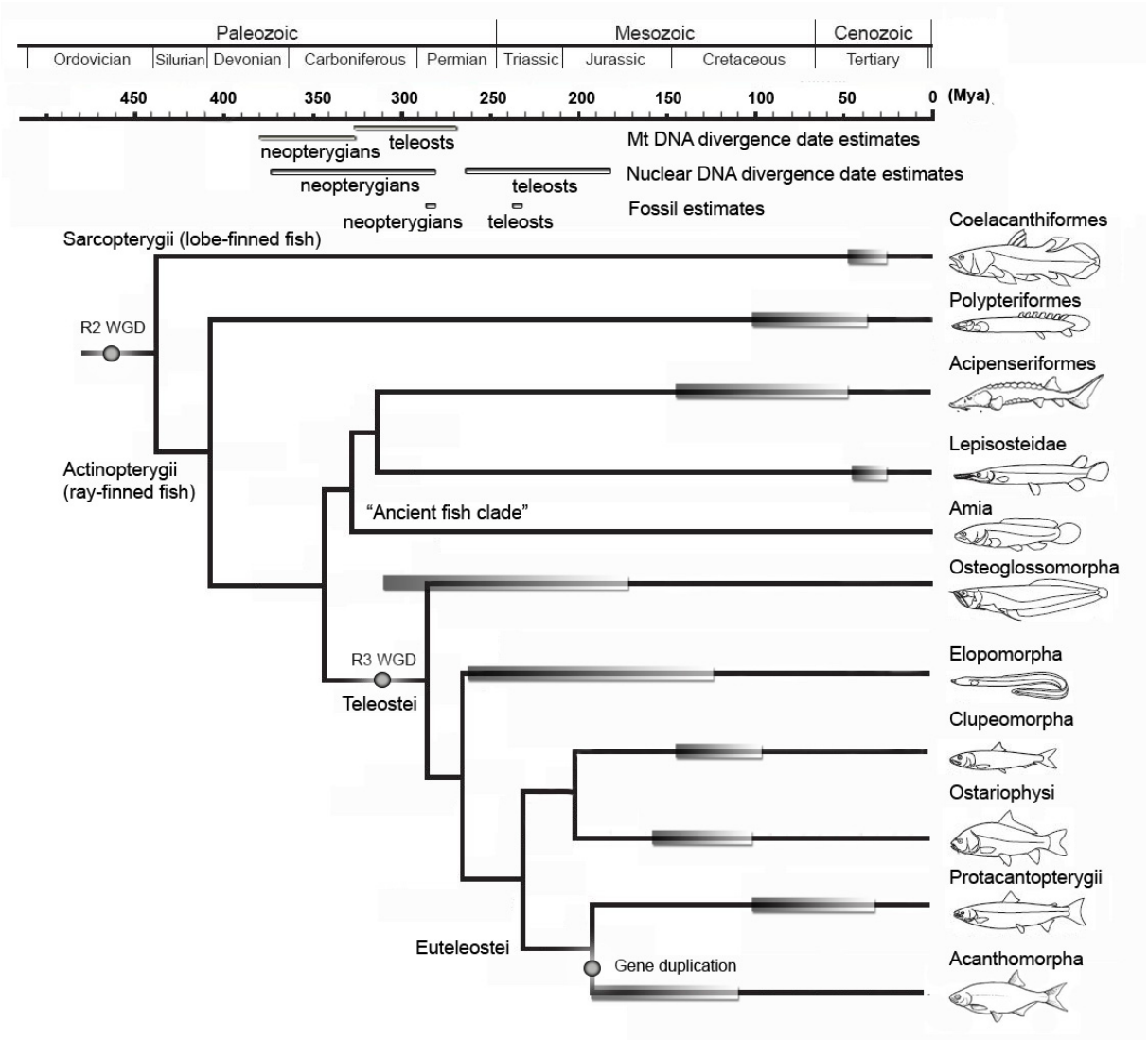


Figure 1 Phylogeny among actinopterygian fishes including divergence and clade speciation time estimates. Modified from Inoue *et al.* 2005 and Hurley *et al.* 2007. Current understanding of divergence date estimates based upon fossils, mitochondrial DNA (mtDNA) and nuclear DNA (ntDNA) phylogenetic analyses are noted beneath the geological timescale, showing the variance in divergence time estimates (Hurley *et al.* 2007). The cladogram shows the organisation of the major fish clades using shaded rectangles to represent mtDNA interval divergence estimates of analysed species within each major clade (95% credibility). Estimated divergence intervals between these major clades is given as ± 30 mya (Inoue *et al.* 2005). The second whole genome duplication (R2 WGD) and teleost-specific whole genome duplication (R3 WGD) and Acanthomorpha *vtg* gene duplication events are annotated (Paper III).

in the late Cretaceous to early Eocene (Maissey 1996; Nelson 2006) that heralds the spectacular rise of the multitude of marine teleosts. The Paracanthopterygii are comprised of >1,300 species of which 99% are marine, and the Acanthopterygii have diversified into a staggering ~15,000 species of which 76% are marine (Nelson 2006; see supplementary Fig. S1, paper III).

A little considered problem in teleost phylogeny concerns the environment (freshwater or seawater) in which they evolved. At some time during teleost evolution the ancestor of the extant marine teleosts had not only to adapt to the hyper-osmotic seawater environment, but also had to evolve the ability to spawn physiologically viable eggs into seawater. Since marine teleost eggs have been shown to have the same hypo-osmotic condition; ~350 mOsmolar, as the parental body fluids (Watanabe and Kuo 1986; Finn *et al.* 2002a), but lack the adult osmoregulatory machinery to compensate for passive water loss and ion uptake, the physiological challenge was greatest for this stage of the life cycle.

Previous studies have identified oocyte hydration as a plausible mechanism that pre-adapts the newly spawned egg to solitary existence in the saline environment (Fyhn *et al.* 1999; Finn *et al.* 2002a, b; Finn 2007a). However, no study has properly examined the osmolyte changes during oocyte hydration in more primitive teleosts. Atlantic herring (*Clupea harengus*) is a member of the order Clupeiformes belonging to the subdivision Clupeocephala that also contains the sister group Ostariophysi. Unlike the Ostariophysi (~8000 species in 68 families), which are almost exclusively freshwater fishes, the Clupeiformes mostly contain marine species (364 species in 5 families) (Saitoh 2003; Nelson 2006; Saitoh *et al.* 2006). Some 150 fossil species of Clupeiformes have been described, with marine forms dating back to the early Cretaceous (Maissey 1996; Poyato-Ariza *et al.* 2000; Peng *et al.* 2006). Members of the Clupeiformes are thus an intriguing model for studying the physiological and molecular mechanisms of oocyte hydration, since they were among the earliest of extant fish groups to invade the oceans. The availability of Atlantic herring made it the chosen model, since it spawns during the spring and autumn season in the coastal waters near Bergen, Norway.

Oogenesis

Oogenesis is here used as a term to describe the developmental stages from primordial germ cells (PGCs) until ovulated (OV) eggs ready to be fertilised. The strategies employed by teleosts during this process varies from viviparity where the egg is fertilised internally and develops inside the body of the mother to emerge (be born) as a free living individual; to ovoviviparity where the egg is fertilised internally and may develop inside the mother until or near hatching whereupon it is laid to develop further in the external aquatic environment; to oviparity where the egg is freely broadcast, fertilised externally and the enclosed embryo derives all nourishment from the yolk. Oviparity is the main form of reproduction amongst teleosts.

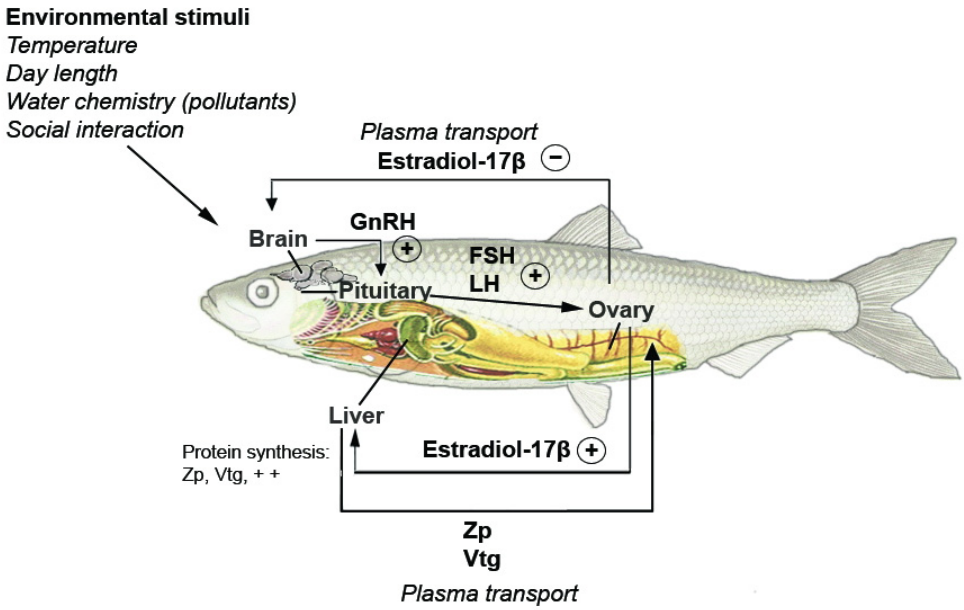
Oogenesis can be split into six major steps from the early PGCs to the fully formed OV eggs: 1) Germ line segregation and PGC formation; 2) sex differentiation where PGCs transform into oogonia; 3) onset of meiosis when oogonia transform into oocytes; 4) growth of oocytes by inclusion of cytoplasmic components such as cortical alveoli, vitellogenin (Vtg) and extracellular zona proteins (Zp) while under meiotic arrest (diplotene); 5) oocyte maturation with germinal vesicle breakdown (GVBD) that coincides with oocyte hydration in marine teleosts; and finally, 6) ovulation with expulsion of the ovum from its follicle (Wallace and Selman 1981; Selman and Wallace 1989; Patiño and Sullivan 2002; Patiño *et al.* 2003; Jalabert 2005).

Oocyte growth

Oocyte growth in teleost fish has been recognised as taking three basic strategies. Synchronous ovaries where all oocytes develop as a single group and are spawned simultaneously, e.g. semelparous anadromous fish such as Pacific salmonids (*Oncorhynchus* genera) (Murua and Saborido-Rey 2003). Group-synchronous ovaries where at least two developmental stages of oocytes are present, typically a synchronous population of larger oocytes (“clutch”) and a heterogeneous population of smaller oocytes acting as recruitment for the clutch. This is by far the most common reproductive strategy in teleosts and includes batch-spawning fishes like Atlantic cod (e.g. Mårstøl *et al.* 1993) and Atlantic halibut (Finn *et al*. 2002a) with several egg

batches during the spawning season, and Atlantic and Pacific herring, *Clupea pallasi*, with single clutches of spawned eggs (e.g. Gamble *et al*. 1985; Koya *et al*. 2003). Lastly, asynchronous ovaries where oocytes of all stages are present without any developmental stage dominating, e.g. killifish (also called common mummichog), *Fundulus heteroclitus*, and medaka, *Oryzias latipes* (Wallace and Selman 1981; Jalabert 2005). The combined effect of the migratory behaviour of Atlantic herring which arrives at the spawning ground in a mature state shortly before spawning, and its reproductive strategy with a single egg clutch spawned, necessitated the sampling of a large number of wild females by local field work for the present studies in order to obtain both vitellogenic oocytes and OV eggs.

Despite the variety of reproductive strategies teleost oogenesis is a regulated process (Rosenfeld *et al*. 2007). Environmental cues trigger the central nervous system (Fig. 2) causing the release of gonadotropin-releasing hormones (GnRH) from the hypothalamus (Samosa *et al*. 2002; Sherwood and Adams 2005) that acts upon the pituitary gland causing release of follicle-stimulating hormones (FSH) and luteinizing



Figur 2 Schematic illustration of the regulation of vitellogenesis in a female Atlantic herring (*Clupea harengus*). Environmental cues trigger the hypothalamo-pituitary-gonad-hepatic-gonad (HPGHG) axis resulting in hepatic synthesis of Vtg and Zp and their ovarian uptake by the oocyte. GnRH; gonadotropin-releasing hormone, LH; luteinising hormone, FSH; follicle-stimulating hormone, Zp; zona proteins, Vtg; vitellogenin. Modified from (Arukwe and Goksøyr 2003)

hormones (LH). Primarily FSH but also LH induce ovarian estradiol production that in turn induces hepatic production of Vtg and Zp (Nagahama 1994; Mosconi *et al.* 2002; Okuzawa 2002; Arukwe and Goksøyr 2003). The respective roles of FSH and LH have been found to vary between species, however besides ovarian estradiol induction they are also associated with developmental expression of the Vtg receptor (Vtgr) for Vtg uptake into the oocyte (Okuzawa 2002; Patiño and Sullivan 2002; Jalabert 2005; Amano, M *et al.* 2007; Babin *et al.* 2007).

Following the transformation of oogonia into oocytes, meiosis is halted at the diplotene stage of chromosomal development (Prophase I), and the previtellogenic stage of the oocyte growth starts. At this stage oocyte ribosomal RNA and heterogeneous RNA production is highly active and much of the full-grown oocyte mRNA seems to be produced during this stage (Wallace and Selman 1990; Patiño and Sullivan 2002; Suwa and Yamashita 2007). Levels of trout mRNA for Vtgr and cathepsin D (CatD) peak during this stage and then decline during the following vitellogenic stage of oocyte growth (Brooks *et al.* 1997; Prat *et al.* 1998). The early expression of Vtgr mRNA and its subsequent decline in expression during growth has been suggested to imply that endocytosed Vtgr is recycled to the oocyte plasma membrane assisting in further Vtg uptake during vitellogenic growth (Patiño and Sullivan 2002). In teleosts the oocyte grows within a follicle, an ovarian structure that is highly similar in most fishes. It consists of an enclosing tri-layer of inner granulosa cells, and an outer thecal layer that is separated from the granulosa layer by a basal membrane (Wallace and Selman 1981; Jalabert 2005). The thecal and granulosa layers co-produce steroids in response to differential expression of the FSH and LH receptors and are responsible for production of estradiol and maturation-inducing steroid (MIS), during vitellogenesis and maturation, respectively (Patiño and Sullivan 2002). The extracellular eggshell (chorion) forms beneath the granulosa layer constructed from Zp (Oppen-Berntsen *et al.* 1992; Hyllner *et al.* 2001).

During vitellogenic growth of the oocytes, Vtg synthesised in the liver is post-translationally glycosylated and phosphorylated in the endoplasmic reticulum (ER) and Golgi complex prior to secretion into the circulating plasma as homodimeric

lipoprotein complexes. The dimeric Vtg complex binds to Vtgr anchored in the oocyte plasma membrane and is internalised via clathrin-mediated endocytosis (Wallace and Selman 1990; Conner and Schmidt 2003; Babin *et al*. 2007). The internalised vesicles are then sorted to early endosomes. The primary cleavage event occurs following the activation of ATP-dependent V-class proton pumps that acidify the vesicles (Busson-Mabillot 1984; Fagotto 1995; Selman *et al.* 2001; Raldúa 2005) thereby activating CatD which cleaves Vtg into its constituent Yps (Hiramatsu and Hara 1997; Carnevali *et al.* 1999b, 2006, 2008; Hiramatsu 2002; Fabra and Cerdà 2004). In vertebrates Vtg is typically cleaved into lipovitellin (Lv), phosvitin (Pv), and beta-component (β´) (Romano 2004; Hiramatsu *et al.* 2005). In a recent review on vertebrate yolk complexes this classic understanding is shown to be even more complex (Finn 2007b).

Primary cleavage event and multiplicity of *vtg* genes

Vtg processing during the primary cleavage event in teleosts is confounded by the multiplicity of Vtg forms expressed in teleosts. Amongst the older lineages only single forms of complete-type Vtg have been documented in the Chondrostei (Bidwell and Carlson 1995; Zhang *et al.* 2005) and Hyperoartia (Sharrock *et al.* 1992; Anderson *et al.* 1998). In the Elopomorpha one form has been reported in the conger eel (Mikawa *et al.* 2006), and two forms in the Japanese eel (three genes: *vtgAe1-3*) (Okumura *et al.* 2002; Wang and Lou 2006). Within the Ostariophysi up to three forms (VtgAo1, VtgAo2, and VtgC) are recognised (Wang *et al.* 2000, 2005; Miracle *et al.* 2006; Kang *et al.* 2007). One of these forms (VtgAo1) is a truncated Vtg lacking the von Willebrand factor type D domain (Vwfd), and is the major form that is expressed in zebrafish three fold higher than that of the other two (Wang *et al*. 2005). This truncated form has only been found among the Ostariophysi. VtgAo2 is a complete type, and the third form (VtgC) is a phosvitinless form lacking both the Pv and the Vwfd domains. Salmonidae members have also two forms of *vtg* (VtgAsa and VtgAsb) and with the exception of *Oncorhynchus* genera multiple copies of both forms are known (Mouchel *et al.* 1996; 1997; Trichet *et al.* 2000; Buisine *et al.* 2002). Acanthomorph teleosts contain the largest numbers of examined species to date, and here up to three forms of Vtg (VtgAa, VtgAb, and VtgC) have been found (Ding *et al.*

1989; LaFleur *et al.* 1995, 2005; Matsubara *et al.* 1999; Reith *et al.* 2001; Hiramatsu *et al.* 2003; Matsumoto *et al.* 2003; Ohkubo *et al.* 2004; Amano, H. *et al.* 2007; Davis *et al.* 2007; Finn 2007a; Kolarevic *et al*. 2008; Sawaguchi *et al.* 2007). In this thesis the new *vtg* gene nomenclature of Finn and Kristoffersen (2007) is used (see paper III).

Within the Acanthopterygii Vtg-derived Yp conjugates have been corroborated by either N-terminal sequencing or mass-spectrometry in six species for the primary cleavage event (see Finn, 2007b). In Atlantic halibut the VtgAa paralogue is found to have LvH, Pv-LvL, LvL and a β′ in the oocyte, whereas the VtgAb paralogue has LvHn, LvHc-Pv, LvHc, Pv-LvL, LvL and a β′ Yp conjugates in the oocyte (Finn 2007a). Here "n" and "c" refer to whether the segment of the conjugate is of the N-terminal or the C-terminal part of the protein, respectively. Barfin flounder, a species that was known to only have two forms of Vtg (Matsubara *et al.* 1999) has recently been shown to also have the VtgC form (Sawaguchi *et al.* 2007). The VtgAa-derived Yp conjugates (previously reported as VtgA) are similar to those in Atlantic halibut with the reported addition of a CT domain (Matsubara *et al.* 2003). In the VtgAb paralogue (previously reported as VtgB) there is a minor LvH domain and a major shorter N-terminal LvHn domain. Pv is found as a Pv-LvL conjugate in this species, and there are separate LvL, β′, and CT domains (Matsubara *et al.* 1999, 2003). Japanese common goby has a full length VtgAa form (previously reported as Vg-530) and a VtgC form (previously reported as Vg-320). Here a LvH-Pv-LvL conjugate and a β′ are found in the oocyte of the VtgAa form, and the VtgC form is not processed and remains as a LvH-LvL conjugate (Ohkubo *et al.* 2003, 2004). Similar findings showing the complexities of Vtg-derived Yp products exists for common mummichog (LaFleur *et al.* 2005), mosquitofish (Sawaguchi *et al.* 2005a, b), red sea bream (Sawaguchi *et al.* 2006), haddock (Reith *et al.* 2001), and silver lamprey (Anderson *et al.* 1998). A recent review of these findings is given by Finn (2007b).

Oocyte maturation

A LH surge triggers the resumption of the first meiotic division by stimulating production of MIS. MIS is a C21 progestin steroid that unlike typical steroids acting through intracellular receptors acts on cell-surface receptors. MIS is found as 17α,20β-dihydroxy-4-pregnen-3one or 17,20β,21-trihydroxy-4-pregnen-3one depending on the species (Jalabert 2005; Carnevali *et al.* 2006; Suwa and Yamashita 2007). During this final stage of maturation, additional proteolysis of Vtg is observed. This second proteolysis is unique to marine teleosts (Byrne *et al.* 1989), and was for the first time observed in the killifish, an estuarine fish laying demersal (benthic) eggs (Wallace and Selman 1985). Vtg proteolysis depends not only upon what types of eggs are spawned; e.g. benthic or pelagic, but also on the differential expression of the form of *vtg* genes (Matsubara *et al.* 1999; Reith *et al.* 2001; LaFleur *et al.* 2005; Sawaguchi *et al.* 2006; Finn 2007a; Kolarevic et al. 2008). Proteolysis with additional cleavage of Yps at this

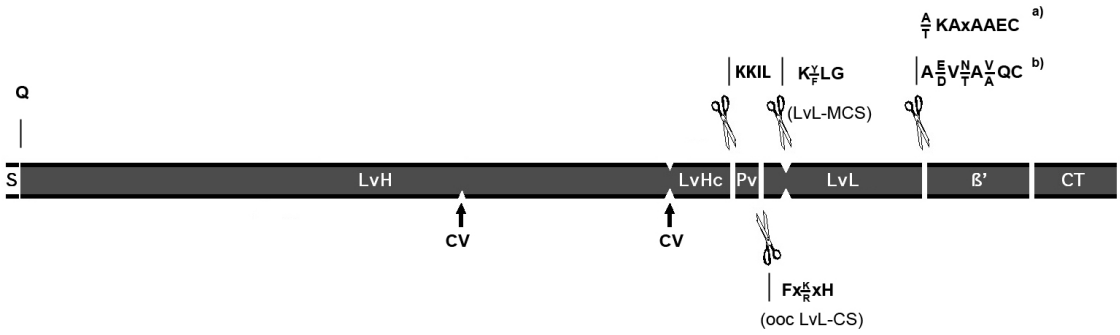


Figure 3. Linear scale representation of a full length complete-type teleost vitellogenin (Vtg) showing the sub-domain structure and consensus sequences of conserved cleavage sites (modified from Finn 2007b). The sub-domain map and consensus sequences were obtained from an alignment of vertebrate vitellogenins (Paper III). Most of the cleavage sites are recognised by cathepsin D, however the light chain lipovitellin (LvL) also has a maturational cleavage site (LvL-MCS) recognised by cathepsin B or cathepsin L in addition to its oocyte LvL cleavage site (ooc LvL-CS) (Finn 2007a, b). The consensus N-terminus of the mature heavy chain lipovitellin (LvH) after the signal peptide (S) is a pyroglutamate (Q). The LvH also contains two sites for minor cleavage variants (CV) that are found in silver lamprey (Anderson *et al.* 1998), common mummichog (LaFleur *et al.* 2005), Atlantic halibut (Finn 2007a), and Atlantic herring (Paper II). For the beta-component (β´) one consensus cleavage sequence is found for acanthomorph teleosts; a), and one less conserved for all older teleosts; b). Consensus amino acid sequences of conserved cleavage sites are shown in bold capital letters, non-conserved amino acids are noted with a small x, and for site variants with few substitutions, the two most common amino acids are shown.

stage is mediated through activation of other cathepsins; e.g. CatL in sea bream (Carnevali *et al.* 1999a) or CatB in barfin flounder (Matsubara *et al.* 2003). A current model of Vtg processing at the Yp level and conserved cleavage/nick sites is shown in Figure 3. Freshwater species can show electrophoretic band-shifts for the Yps during oocyte hydration like in zebrafish (Dosch *et al.* 2004), but without the production of a FAA pool.

Hydration and yolk protein processing during final maturation.

Prior to ovulation and expulsion from the follicle, teleost oocytes go through a hydration process that is unique in the vertebrate lineage. This is seen as a volume increase due to uptake of water and is driven by a transient intra-oocytic osmotic potential caused either by inorganic ions or FAA derived from the maturational proteolysis of Vtg (Wright and Fyhn 2001; Finn *et al.* 2002a; Cerdà *et al.* 2007; Finn 2007a). Water influx into the maturing oocytes is mediated by specialised aquaporins (AQP) that are temporarily inserted into the oocyte plasma membrane during oocyte maturation (Fabra *et al.* 2005, 2006; Cerdà *et al.* 2007). Changes in oocyte volume ranges from slight in freshwater (Craik and Harvey 1984; Greeley *et al.* 1986; Milla *et al.* 2006) and euryhaline species (Craik and Harvey 1986; Greeley *et al.* 1986), to several fold in marine species (Craik and Harvey 1986; Greeley *et al.* 1986; Cerdà *et al.* 1996; Thorsen and Fyhn 1996; Finn *et al.* 2002a, b). The degree of hydration also varies according to whether the species spawns pelagic eggs (pelagophils) or benthic, non-buoyant eggs (benthophils). The latter typically has a 1.0 – 3.0 fold volume increase, while pelagophils can have a 3.1 – 8.4 fold increase (Cerdà *et al.* 2007). The relative water content of pelagophils reaches levels of >90%, and the process is mainly driven by the secondary proteolysis of VtgAa-derived LvH resulting in a build-up of an osmolytically active FAA pool (Matsubara *et al.* 1999; Reith *et al.* 2001; Finn *et al.* 2002a, b; Sawaguchi *et al.* 2006; Finn 2007a; Kolarevic et al. 2008).

Not only the LvH domain is subjected to proteolysis in pelagophils, but also Pv and β´ derived from both the VtgAa and the VtgAb forms may be extensively degraded (Finn 2007a). However, the major source of FAA is the VtgAa LvH (Matsubara *et al.* 1999;

Finn 2007a; Kolarevic *et al*. 2008). In benthophils extensive proteolysis is not seen, but limited proteolysis has been reported in some species that express VtgAa-type Yps. Common mummichog, corkwing (*Crenilabrus melops*), and ayu (*Plecoglossus altivelis*) have all been shown to have a limited yolk proteolysis resulting in a small pool of FAA (Greeley *et al.* 1986; McPherson *et al.* 1989; Finn *et al.* 2002b; Chen *et al.* 2003; LaFleur *et al.* 2005; Raldúa *et al.* 2006). In other marine benthophils, such as ballan wrasse (*Labrus bergylta*), and cuckoo wrasse (*Labrus mixtus*) no such depolymerisation of yolk proteins appears to take place (Finn *et al.* 2002b). Benthophil freshwater spawners may have some maturational cleavage/nicking but they essentially do not degrade they Vtg Yps to FAA; e.g. rainbow trout (*Oncorhynchus mykiss*) (Tyler 1993; Milla *et al.* 2006), zebrafish (*Danio rerio*) (Dosch *et al.* 2004), white sturgeon (*Acipenser transmontanus*) (Bidwell and Carlson 1995), hybrid sturgeon; bester (*Huso huso x Acipenser ruthenus*), (Hiramatsu *et al.* 2002), and silver lamprey (*Ichthyomyzon unicuspis*) (Anderson *et al.* 1998).

Osmotic effectors during hydration in pelagophils

Although FAA are now acknowledged as a major osmolyte driving the water influx during oocyte hydration in the pelagic egg (see reviews by Wright and Fyhn 2001; Cerdà *et al*. 2007), the amount of FAA cannot account for the total osmotic potential needed to hydrate the oocyte. In the pelagophil Atlantic halibut (*Hippoglossus hippoglossus*) it was shown that while FAA; derived primarily from differential proteolysis of Vtg-derived Yps, contributed approximately 50% to the oocyte osmolality, inorganic ions (Cl^-, K^+, P_i, NH_4^+) made up the balance (Finn *et al.* 2002a, ; Finn 2007a). The major inorganic osmolytes involved in the hydration process, however, appear to vary according to species. In Atlantic halibut and grey mullet (*Mugil cephalus*) Cl^- is the major inorganic osmolyte, while in plaice (*Pleuronectes platessa*) (Craik and Harvey 1984; Thorsen and Fyhn 1991), lemon sole (*Microstomus kitt*) (Thorsen and Fyhn 1991), Atlantic croaker (*Micropogonias undulates*), and spotted seatrout (*Cynoscion nebulosus*) (Lafleur and Thomas 1991), black sea bass (*Centopristes striata*) (Selman *et al.* 2001) and gilthead seabream (*Sparus aurata*)

(Fabra *et al.* 2006) K^+ is argued to be the major inorganic osmolyte involved in hydration. Further studies are needed to clarify these issues.

Osmotic effectors during hydration in benthophils

In marine benthophil teleosts with reported maturational proteolysis, the increase in FAA during the maturational hydration is given as 2.9-3.8 fold as compared to the pelagophil teleosts with levels of 9.1-15 fold. Also, taurine; an amino acid analogue not used for protein synthesis but implicated in cell volume regulation (Lang *et al.* 1998), is reported as the most abundant amino acid with values up to 74% of the total FAA pool (Greeley *et al.* 1991; Thorsen *et al.* 1993; Thorsen and Fyhn 1996; Wright and Fyhn 2001; Finn *et al.* 2002b) as compared to pelagophils where taurine only constitutes 2-4% of the total FAA pool (Thorsen *et al.* 1993; Thorsen and Fyhn 1996; Finn *et al.* 2002b). As stated above, the eggs of benthophils are less hydrated than those of pelagophils, and as Vtg-derived Yp proteolysis is either limited or absent, ions appear to be the major osmolytes. In such species, the hydration process appears to be driven by the differential movement of inorganic ions across the plasma membrane. In common mummichog K^+; as seen in many pelagophils, is regarded as the major inorganic osmolyte with Na^+ playing a lesser role in the hydration process (Greeley *et al.* 1991; Selman *et al.* 2001). Similar data with the dominance of K^+ and Cl^- were found previously for developing eggs of Atlantic herring (Hølleland and Fyhn 1986). In contrast Na^+ is the dominant ion in ayu (Chen *et al.* 2003). One important note is the absence of data on other inorganic osmolytes in benthophils, such as Cl^-, Mg^{2+}, HCO_3-, and P_i as well as nitrogen waste compounds such as NH_4^+, as these could also be involved in the hydration process as seen in pelagophils (Cerdà *et al.* 2007).

Synopsis of paper I-III

To further understand the evolution of mechanisms of oocyte hydration in marine species, a primitive marine benthophil, Atlantic herring was chosen as model in the present study. Earlier work had been done to describe these processes in several marine species (Thorsen *et al.* 1993; Matsubara *et al.* 1999; Selman *et al.* 2001; Finn *et al.* 2002a, b; Finn 2007a), but no primitive benthophil teleost had yet been thoroughly described. With the exception of the Elopomorpha, herrings were among the first teleosts to reinvade the oceans during the early Cretaceous.

In **paper I** the major inorganic and organic osmolytes responsible for hydrating the oocytes during meiotic maturation in autumn- and spring spawning stocks were studied. The inorganic ions; Cl^-, K^+, and P_i, were shown to be the dominant osmolytes participating in the maturational influx of water in this species. Intriguingly, a small pool of FAA also appeared during meiotic maturation, which, due to the relatively low water content of the OV egg, contributed 29% to the total ovoplasmic osmolarity. Despite the OV eggs of spring-spawning herring being up to twice the size of the autumn-spawning stock the GSI (27 ± 3%) and degree of hydration (70 – 72% water) were equivalent. Normalising the data with respect to dry mass revealed that the physiological mechanisms underlying the maturational influx of water were the same for both types of egg. Only K^+ and P_i (spring) remained undiluted in the OV eggs, while all other ions, including Cl^-, Na^+, NH_4^+, and Mg^{2+} were significantly diluted. In contrast, the concentration of FAA increased during the hydration process. Although Cl^- showed the greatest increase (Δ19.3 nmol • ind^{-1}) in the autumn-sampled eggs, its dilution during the hydration process could be explained by the doubling of FAA content from 1.5 – 3.1% of dry mass. This small increase in FAA content contributed 29% to the calculated ovoplasmic osmolarity (autumn eggs: 376 mOsmol • kg^{-1} oocyte water; spring eggs: 379 mOsmol • kg^{-1} oocyte water). The total sum of charged solutes in both egg types were close to electroneutrality at a pH of 5-6 indicating that no charged osmolytes was overlooked. If the composition of osmolytes in the egg of a benthic and a pelagic species is compared (Fig. 4) the reduced importance of the FAA pool for the driving force of oocyte hydration in the benthophil compared to the

pelagophil is clearly evident. Paper I discusses the differential movement of the inorganic and organic osmolytes that underlie oocyte hydration in Atlantic herring in relation to current models of transmembrane ion flux. In light of the recent discovery of the almost omnipresence of AQP (Agre *et al.* 2002) as well as the role of AQP1o in the oocyte hydration of gilthead seabream (*Sparus aurata*) (Fabra *et al.* 2006), a future study of AQP involvement in Atlantic herring oocyte hydration would further increase the understanding of hydration in this basal teleost. Indeed, based upon recent findings (Fabra *et al.* 2006; Cerdà *et al.* 2007; Finn 2007a) it has been suggested that teleost oocyte hydration is a highly conserved mechanism where the interplay between protein hydrolysis and ion influx creates an osmotic gradient, while the SaAQP1o facilitates the water influx

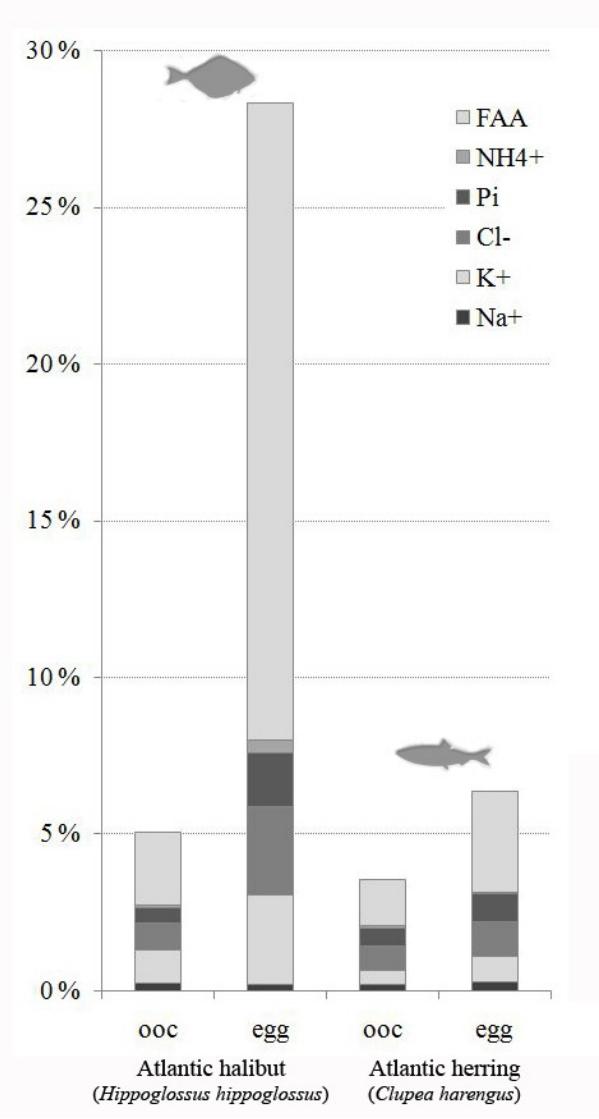


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Figure 4. The major osmolytes responsible for driving oocyte hydration during meiotic maturation in marine teleosts, presented as percentage of oocyte (ooc) or ovulated egg (egg) dry mass values. Data for the pelagophil Atlantic halibut (*Hippoglossus hippoglossus*) is modified from (Finn *et al.* 2002a) and data for the benthophil Atlantic herring (*Clupea harengus*) is from Paper I.

Paper II was based on the findings in Paper I that an increase in the pool of FAA plays a significant role in hydration of Atlantic herring oocytes. This is a novel observation and reminiscent of the more dramatic mechanism in pelagophils. To determine whether yolk proteolysis also represents the underlying mechanism in this basal benthophil, hepatically expressed *vtg* was isolated and cloned in order to establish the precursor-product relationships to deposited oocyte and egg Yps. Unlike

other teleosts studied to date, however, only a single *vtg* transcript (*chvtgAc*) was found to be expressed in the liver. Specific use of primers designed from conserved transcript regions of acanthomorph forms of *vtg* transcripts (*vtgAb* and *vtgC*) did not reveal multiple forms. To verify that only a single form of *vtg* exists in the genome of Atlantic herring, genomic DNA was extracted using primers designed from conserved exons flanking heterogeneous introns in zebrafish *vtgAo1* and *vtgAo2* genes. The data show that that at least two closely related gene variants exist in the genome, but that only a single form of complete-type *vtg* is expressed in Atlantic herring. The putative amino acid sequence showed the herring Vtg to conform to the pentapartite NH_2-(LvH-Pv-LvL-ß´-CT)-COO^- structure of complete teleost Vtgs. Multiple phylogenetic analyses consistently clustered the *chvtgAa* transcript and ChvtgAa protein as the basal sister group to the Ostariophysi in full congruence with the Clupeocephalan rank (Fig. 5b). These analyses were run with all available teleost sequences and found to be fully consistent with results presented in Paper III. Three-dimensional modelling of ChvtgAa against the 3D structure of lamprey lipovitellin (Anderson *et al.* 1998; Thompson and Banaszak 2002) revealed that the modelled tertiary structure of Atlantic herring Vtg was highly conserved despite its relatively low sequence identity and similarity (Fig. 5c). N-terminal microsequencing of oocyte and egg Yps proved to be difficult for several bands excised from SDS-PAGE gels, so mass spectrometry; q-TRAP and q-TOF, and western blotting was performed to facilitate identification of the Yps. Positive identification of SDS-PAGE band against the deduced ChvtgAc primary structure showed that some proteolytic processing occurs during oocyte hydration. The data indicate that the Pv domain, the smallest yet reported for teleosts, and an N-terminal fragment of the LvL contribute to the FAA pool. Data that further support the notion that these regions are the source of the FAA pool are the significant increase in free P_i and free serine in the OV egg (Paper I).

Hence it is argued that yolk proteolysis and the generation of an organic osmolyte pool of FAA was an adaptive response to spawning in seawater also in teleost groups prior to the rise of Acanthomorpha. However, this mechanism was not evolutionarily successful in terms of biodiversity until gene duplication and neo-functionalisation occurred in the Acanthomorpha.

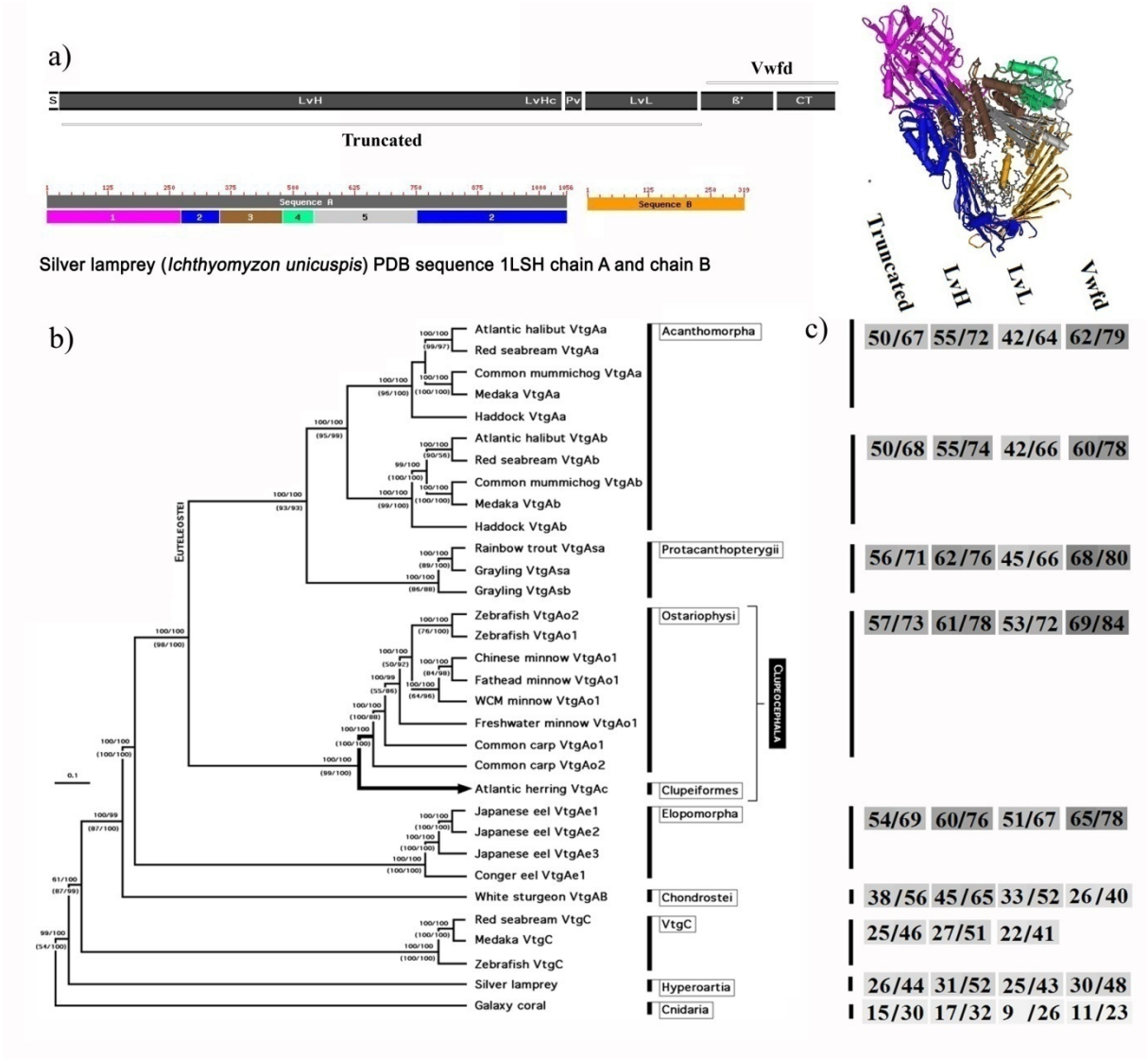

Figure 5. Correlation between 3D structure, inferred Bayesian phylogeny and Atlantic herring (*Clupea harengus*) vitellogenin (Vtg) domain identity and similarity values compared to different forms of Vtg. a) A complete-type Vtg aligned above the lipovitellin sequence from Silver lamprey (*Ichthyomyzon unicuspis*), the only species with a resolved lipovitellin 3D structure. Colours in the silver lamprey sequence match the resolved sub-domain structures. b) Bayesian majority rule consensus tree of Atlantic herring ChvtgAc in relation to other fishes, rooted with galaxy coral. c) The identity and similarity values of Vtg sub-domains in relation to Atlantic herring.

Paper III investigated the phylogenetic relationships of vertebrate Vtgs in order to understand the origin of the teleost Vtg forms with maturational proteolysis of their Yps. Earlier studies had presented phylogenetic trees of several teleost Vtgs (e.g. Byrne *et al*. 1989; Chen et al. 1997; Sappington & Raikel, 1998; Babin *et al*. 1999; Buisine *et al*. 2002; Wang *et al*. 2000, 2005, 2006; Mikawa *et al*. 2006; Miracle *et al*. 2006; Kang *et al*. 2007), however, each study used the older neighbour-joining method for tree construction and not one of the more recent methods of phylogenetic inference that have been developed (Huelsenbeck & Ronquist, 2001; Huelsenbeck *et al*. 2001; Swafford, 2002; Ronquist & Huelsenbeck, 2003). These latter methods, and in particular Bayesian inference, though not without its pitfalls (Huelsenbeck *et al*. 2002), are now considered the premier tools for establishing phylogenetic relations based upon molecular data (Huelsenbeck *et al*. 2001; Holder & Lewis, 2003; Glenner *et al*. 2004). Further, each of these previous studies had only examined a subset of the deduced Vtg proteins. With the discovery of the novel phosvitinless form of *vg3* in zebrafish (Wang *et al*. 2000) it was proposed that this gene (denoted here as *vtgC*) was evolutionarily primitive and more closely related to insect *vtgs* (Wang *et al*. 2000). This view has persisted in the literature (Hiramatsu *et al*. 2006) and recent phylogenetic analysis of Vtgs using the neighbour-joining method (Mikawa *et al*. 2006) maintains this position. Also, since it is now established that the teleost lineage has experienced three rounds of whole genome duplication (WGD: R1, R2, R3) (Amores *et al.* 1998; Crow *et al.* 2006) from early chordate roots, it was considered important (paper III) to attempt to reconcile these events with the proposed tree model and the fossil record.

All available (late 2006) vertebrate *vtg* genes; genomic and expressed sequences, were accessed, and their homology verified using the blast 2 sequence tool (Tatusova and Madden, 1999). Vtgs belong to the super-family of large lipid transfer proteins (LLTP) that includes, among others, apolipoprotein B (ApoB), so in order to reconcile the phylogeny of the teleost Vtgs in relationship to other taxa, ApoB was included as well as cnidarian and molluscan Vtg as outgroups. Although not included in paper III, all invertebrate homologues of the LLTP family were accessed and phylogenetically tested in order to establish the robustness of the vertebrate Vtg tree. Data sets were run

for both complete-type Vtg and corresponding *vtg* data sets, as well as cut down data sets consisting of only conserved regions. All sets, including amino acid and codon alignments, were subjected to parsimony, maximum likelihood, Bayesian, molecular clock and Ka/Ks analyses.

All of the methods of phylogenetic inference consistently placed VtgC after silver lamprey (Fig. 5b, see also Fig. 1 paper III). This topology was maintained regardless of whether molluscan or cnidarian Vtg was used to root the tree. This also applied when including other members of the LLTP super-family, and phylogenetic trees were consistent with those of Smolenaars *et al*. (2007) in their recent review on LLTP diversity and evolution. VtgC is thus more closely related to vertebrate Vtgs than to insects Vtg as proposed in paper III. Correlating this with whole genome duplication events suggests the split to have taken place shortly after the speciation of the lampreys (>450 mya), and it is proposed that *vtgC* is a product of the 2R WGD event (paper III).

The consistent topology of the consensus Bayesian trees with the majority of the nodes being supported by 100% posterior probability values presented a new problem. Previous reports on teleost Vtgs have classified the cloned *vtg* cDNAs rather haphazardly, without taking into account teleost phylogeny, the paralogous or orthologous nature of the *vtg*, or the consequences of WGD in the teleost lineage. To avoid confusion in future studies yielding novel *vtg* sequences a strict terminology as given in paper III should be followed. This is in accord with recommendations in the guidelines for gene nomenclature (HUGO) in the Human Genome Project (Wain *et al.* 2002).

Within the Acanthomorpha paralogous gene clustering was observed, that further correlated to the pelagic and benthic nature of the eggs. The other *vtg* genes clustered according to taxonomic groups (Protacanthopterygii, Ostariophysi, Elopomorpha, Chondrostei and Sarcopterygii). Hyperoartia were followed by the group of ApoB where orthologous clustering of this LLTP member was observed. Hence, the congruence between the accepted teleost phylogeny (Fig. 1; Nelson 2006), the

paralogous and orthologous clustering of *vtg* (Fig. 5b; also Fig.1, paper III), and WGD events (Fig. 6), lead to the proposal of a new *vtg* gene nomenclature (paper III).

The paralogous clustering of acanthomorph *vtg* into a *vtgAa* and a *vtgAb* form (Fig. 1 Paper III) closely follows the reported proteolysis of Vtg-derived Yps during marine teleost oocyte hydration. In acanthomorph teleosts a differential proteolysis of Vtg-derived Yps is observed in pelagic species, where mainly the VtgAa LvH Yp is degraded to give rise to the FAA pool that drives oocyte hydration (Matsubara *et al*. 1999; Reith *et al*. 2001; Sawaguchi *et al*. 2006; Finn 2007a; Kolarevic *et al*. 2008). For benthic species a partial cleavage and hydrolysis of VtgAa-derived Yps may also be observed (LaFleur *et al*. 2005). However, for both pelagic and benthic species the LvH

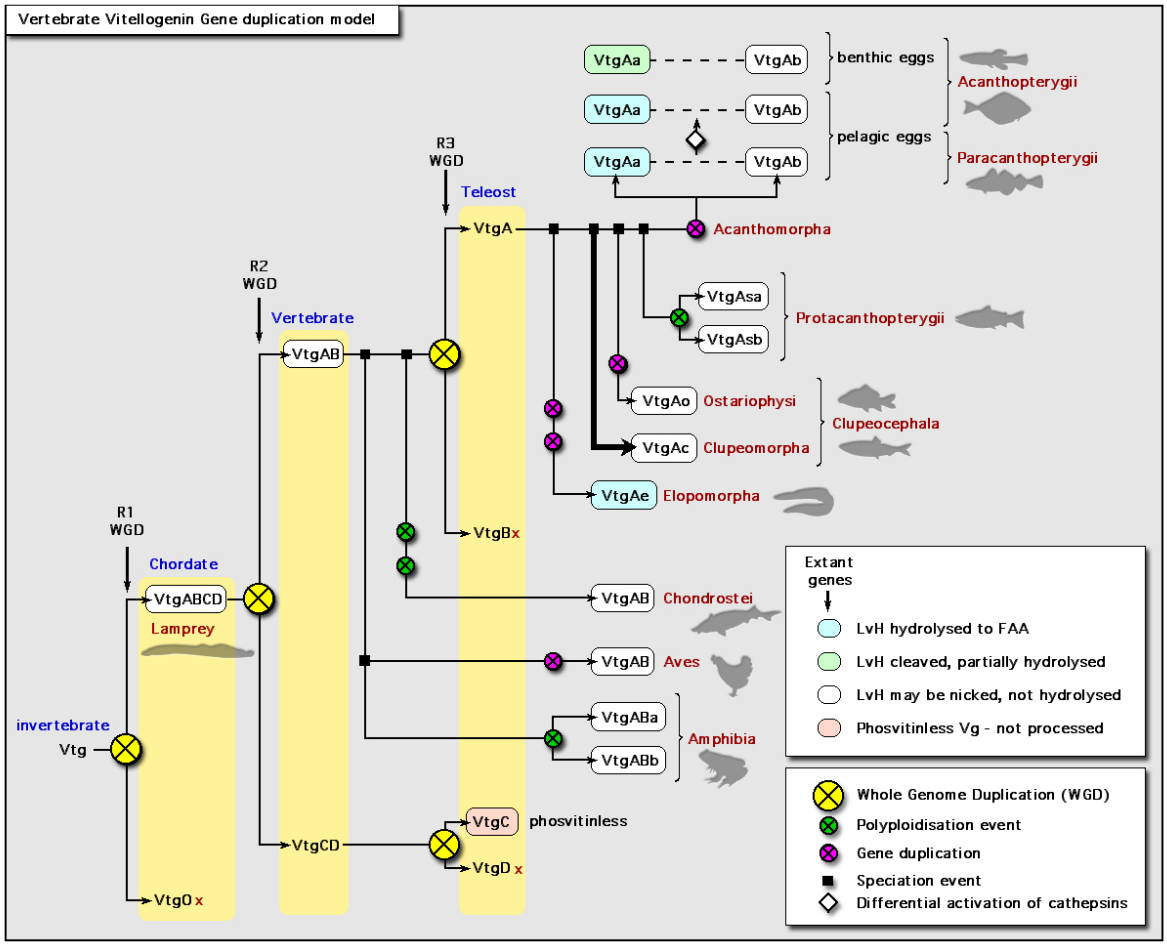

Figure 6. Phylogentic position of Atlantic herring ChvtgAc (paper II) in relation to vertebrate Vtgs (paper III). Classification scheme for the Vtg proteins takes into consideration whole genome duplication events (WGD) and lineage-specific gene duplication events. Classification is based upon clade structure of a consensus rule Bayesian phylogenetic tree and differential proteolysis of the lipovitellin heavy chain

of the VtgAb paralogue may be nicked but not degraded. Following vitellogenesis Yps of both forms (Aa and Ab) will be exposed to the acid cathepsin hydrolases during the oocyte hydration, however it is primarily the *vtgAa* LvH that is degraded suggesting a neo-functionalisation of this form Vtg.

The acanthomorph speciation and radiation occurred during the early Eocene following the Cretaceous-Tertiary boundary extinction event, a time when competition and predation was low (Everhart 2000; Keller 2001). Since R3 took place at the base of the crown group of teleosts (>290 mya) (Crow *et al.* 2006) but the fossil record shows that the acanthomorph "teleost explosion" occurred 55 mya (Maissey 1996), WGD alone cannot explain the oceanic radiation of teleosts. The present work shows that yolk proteolysis was an active process in the primitive Atlantic herring, but that this mechanism did not become evolutionarily successful until duplication of the VtgAa and VtgAb paralogues in the Acanthomorpha. It is argued that neo-functionalisation of the VtgAa paralogue was a key event in the evolution and success of the marine teleosts. This proposed model appropriately matches the fossil record, is fully congruent with teleost phylogeny, and explains the physiology and ecology of the freely broadcast egg.

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