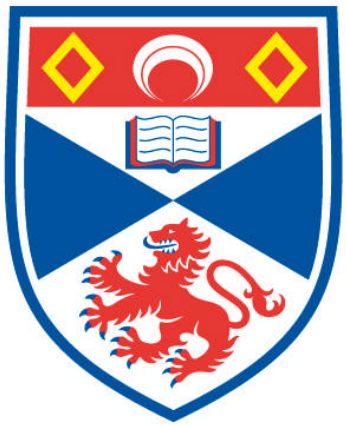
MATERNAL EFFECTS ON OOCYTE QUALITY IN FARMED ATLANTIC HALIBUT (HIPPOGLOSSUS HIPPOGLOSSUS L.)

Maren Mommens

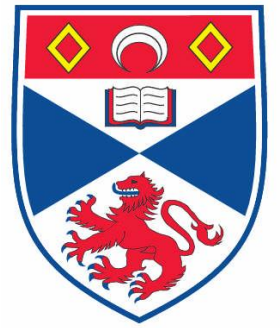
A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



2012

Maternal effects on oocyte quality in farmed Atlantic halibut (*Hippoglossus hippoglossus* L.)

Maren Mommens



This thesis is submitted in partial fulfilment for the degree of PhD at the University of St Andrews

September 2011

This thesis is dedicated to my parents.

Acknowledgements

First of all, I would like to express my gratitude to my supervisors Professor Ian A. Johnston, Professor Igor Babiak and Dr. Jorge M.O. Fernandes for their invaluable support, advice and constructive feedback. My thanks go to Risør Fisk AS and especially Kjell E. Naas and Yngve Attramadal for their cooperation and help during sampling. The microarray collaboration with Dr. Knut Erik Tollefsen from the Norwegian Institute for Water Research (NIVA) was much appreciated and I would like to thank You Song for his excellent training in microarray preparation.

The members of the Fish Muscle Research Group at the University of St. Andrews introduced me to molecular biology and I would especially like to thank Dr. Hung-Tai Lee and Dr. Sitheswarab Nainamalai for help and support in the lab. Dr. Neil Bower arrived at the same time as me in St.Andrews and together with his family we shared our Scotland experience. Special thanks go to Dr. Lara Meischke for including me in her group of friends which contributed to many memorable experiences during my stay.

I would like to thank all members of the Reproductive Biology Group at the University of Nordland for their support and encouragement during my PhD. Special thanks goes to Dr. Sylvie L. Bolla for invaluable help during sampling and sharing her experience and enthusiasm on Atlantic halibut production and fish nutrition with me. Carlos F.C. Lanes and Teshome Bizuayehu contributed to part of this study with samples and help during lab work. I would like to thank Professor Christel Solberg and Dr. Ørjan Hagen from the Seafood Quality Research Group for their contribution to the reference gene analysis. Thanks go to Bjørnar Eggen and Tormod Skålsvik from the Research Station at the University of Nordland for their help during Atlantic halibut stripping. Friends and colleagues at the Faculty of

Bioscience and Aquaculture at the University of Nordland helped out by hiring me during the last part of my PhD to make sure that I could finish.

During my PhD I was lucky to get to know many lovely people from all over the world, making this time not only a scientific, but also a cultural experience. Their friendships and never ending encouragements are much appreciated.

Finally, my greatest gratitude goes to my parents and family for their love, encouragement and support.

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Declaration

I, Maren Mommens, hereby certify that this thesis, which is approximately 45,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

I was admitted as a part-time research student in October 2006 and as a candidate for the degree of PhD in October 2006; the higher study for which this is a record was carried out in the University of St Andrews between 2006 and 2011.

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List of abbreviations

18k Gold	*18K hypothetical goldfish protein*
18S rRNA	*18S ribosomal RNA*
8CS	8-cell stage
16CS	16-cell stage
25EP	25% epiboly
50EP	50% epiboly
20-HETE	20-hydroxyeicosatetraenoic acid
10SS	10-somoite stage
30SS	30-somite stage
µg	Micro gram
µm	Micrometer
µM	Micro molar
ϱ	Spearman's Rho
A	Adenosine
AA	Amino acid
Actb	*β-Actin*
ANOVA	Analysis of variance
AP	Advanced photoperiod
ARA	Arachidonic acid (20:4n6)
Bah	Betaine aldehyde dehydrogenase
BL	Blastula
BLAST	Basic Local Alignement Tool
BTB	Bric-a-brack, Tramtrack and Broad-complex
Buc	*Bucky ball*
BP	Biological processes
bp	Base pair
Bsd	*Blistered*
cAMP	Cyclic adenosine monophosphate
CC	Cellular component
cDNA	Complementary DNA
Cea	*Cellular atoll*
Cei	*Cellular island*
Chk1	*Checkpoint 1*
CL	Cluster
cm	Centimeter
CRL	Cullin-RING ubiquitin ligases
cRNA	Complimentary RNA
CUL2	Cullin 2
Cy3	Cyanine 3
cyp2n	*Cytochrome p450*
cycB	*Cyclin B*
dbEST	Expressed Sequence Tags database
DGLA	Dihomo-γ-linolenic acid (20:3n6)
DHA	Decosahexaenonic acid (20:5n3)
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPA	Docosapentaenoic acid (22:5n3)
E	PCR efficiency (E)

eef2	*Eucaryotic translation elongation factor 2*
eef1a2bp	*Eukaryotic translation elongation factor 1A2 binding protein,*
EET	Epoxyeicosatrienoic acids
EFA	Essential fatty acid
EP	Epiboly
EPA	Eicosapentaenoic acid (20:5n3)
ESA	Eicosatetraenoic acid (20:4n3)
ESCL	Embryonic stem cell lines
EST	Expressed sequence tag
E	E-value
FA	Fatty acid
FAA	Free amino acid
FAME	Fatty acid methyl esters
fau	*40S ribosomal protein S30*
FAO	Food and Agriculture Organization of the United Nations
FDR	False discovery rate
FF	First-feeding
FSH	Follicle-stimulating hormone
FSH-R	Follicle-stimulating hormone-receptor
g	Gram
gapdh	*Glyceraldehyde-3-phosphate dehydrogenase*
GBP	British Pound
GEPAS	Gene Expression Pattern Suit
gDNA	Genomic DNA
GnRHa	Gonadotrophin-releasing hormone agonist
GO	Gene Ontology
GR	Germ ring
GTP	Guanosine triphosphate
Gv	Germinal vesicle
h	Hours
H	High
HB	High blastula
HIRRV	Hirame Rhabdovirus
hpf	Hours past fertilisation
HSP	Highest scoring pair
HT	Hatched larvae
hr6a	*Ubiquitin carrier protein*
IEA	Inferred by Electronic Annotation
IFN	Interferon
IHNV	Infectious Haematopoietic Necrosis Virus
IPN	Infectious pancreatic necrosis
irf7	*interferon regulatory factor 7*
KEGG	Kyoto Encyclopedia of Genes and Genomes
k	Kilo
kg	Kilogram
kop	*Askopos*
l	Liters
L	Low
LA	Linnoleic acid (18:2n6)
LH	Luteinizing hormone

LH-R	Luteinizing hormone receptor
lhβ	*Luteinizing hormone β-subunit*
LNA	Linolenic acid (18:3n3)
LS	Larval stage
lux	*Luciferase*
M	Million
M	Gene expression stability value
mM	Millimolar
m	Metres
m^3	Qubic metres
Mb	Mega bases
MBT	Mid-blastula transition
MF	Molecular function
MHC	Major histocompatibility complex
mhc1dab	*mhc class i antigen alpha chain*
mhc2dab	*mhc class ii antigen alpha chain*
min	Minute
ml	Milliliters
miRNA	Micro RNA
MRF	Myogenic regulating factor
mRNA	Messenger RNA
ms4a8a	*Membrane-spanning 4-domains subfamily a member 8a*
MUFA	Monounsaturated fatty acid
MyHC	Myosin Heavy Chain
MyLC2	Myosin Light Chain 2
MyoD	Myogenic determination factor
myod1a	*Myogenic determination factor subunit 1a*
myod1b	*Myogenic determination factor subunit 1b*
myod2	*Myogenic determination factor 2*
MZT	Maternal-zygotic transition
N	Number
NCBI	National Centre for Biotechnology Information
NERC-EGTDC	Natural Environment Research Council-Environmental Genomics Thematic Programme Data Centre
NL	Neutral lipids
nm	Nanometer
NP	Natural photoperiod
nr	non-redundant
OA	oleic acid (18:1n9)
ORF	Open reading frame
PA	Palmitic acid (16:00)
pd-1l	*Programmed cell death 1 ligand 1*
PDPK1	3-phosphoinositide-dependent protein kinase-1
pg	Pico gram
phb1	*Prohibitin 1*
phb2	*Prohibitin 2*
PINV	Infectious pancreatic necrosis virus
PL	Polar lipids
POA	Palmitoleic acid (16:1n7)
ppt	Parts per thousand

psmb9	*Proteasome subunit beta type-9 precursor*
psq	*pipsueak*
PUFA	Polyunsaturated fatty acid
Pwg	*Pollywog*
qC	quantification cycles
qPCR	Quantitative real-time PCR
QTL	Quantitative trait loci
r	Pearson's correlation coefficient
RIN	RNA integrity number
RNA	Ribonucleic acid
rnf213	*Ring finger protein 213*
rpm	Rounds per minute
Rsa I	*Restriction endonuclease I*
SAM	S-adenosyl methionine
SD	Standard deviation
Sec	Seconds
sGnRH	Salmon gonadotropin-releasing hormone
SSH	Suppressive subtractive hybridisation
SSR	Single sequence repeats
stmn2	*Stathmin 2*
Stx4	*Syntaxin 4*
t	Tonn
T	Total
Tdrd5	*Tudor 5 protein*
tRNA	Transfer RNA
Tubb2	*β2-Tubulin*
UFA	Unsaturated fatty acids
UK	United Kingdom
UPGMA	Un-weighted pair-group method with arithmetic averages
UTR	Untranslated Region
VHSV	Viral Hemorrhagic Septicemia Virus
WPGMA	Weighted pair group method
XPO1	Exportin-1
zDazl.	Deleted in azoospermia-like

Thesis abstract

Atlantic halibut (*Hippoglossus hippoglossus*) oocyte quality is highly variable and one of the major bottlenecks during fry-production for on-growth in commercial Atlantic halibut farming. In this study, the effect of maternally derived oocyte constituents (i.e. yolk components and mRNAs) on oocyte quality (i.e fertilisation, embryonic hatching and normal blastomere symmetry) in farmed Atlantic halibut has been investigated.

Atlantic halibut embryos and larvae depend on nutritional yolk components until larval first feeding. The importance of yolk n-3 fatty acids for oocyte quality was confirmed. However, highest positive correlations with oocyte quality were found for the less studied fatty acids dihomo-γ-linolenic acid (DGLA, 20:3n6) and docosapentaenoic acid (DHA, 20:5n3) that are known to compete with two of the most abundant fatty acids, arachidonic acid (ARA, 20:4n6) and docosahexaenoic acid (DPA, 22:5n3), respectively during fatty acid metabolism. High methionine and aspartic concentrations, amino acids essential to eukaryotic protein synthesis, were found to influence oocyte quality positively while no significant correlations were found between oocyte folate concentrations and oocyte quality.

Before activation of zygotic transcription, maternal mRNAs control cell divisions and embryonic patterning. Due to the limited available genomic information on Atlantic halibut maternal transcripts, an expressed sequence tag (EST) maternal library containing 2,341high quality ESTs was created by suppressive subtractive hybridization (SSH). The maternal library constitutes an EST pool to identify suitable Atlantic halibut reference genes and identify differentially expressed maternal genes in high and low quality Atlantic halibut oocytes.

To perform reliable quantification of gene expression by qPCR, stable reference genes have to be used to normalize target gene expression. *Tubb2*/*Actb* and *Tbb2*/*Fau* were identified as the

best two-gene normalization factors during Atlantic halibut embryonic and larval development. Either of these normalization factors can be used for future developmental gene expression studies in Atlantic halibut. *Tubb2*/*Actb* was further used as reference gene during this study.

Poor embryonic hatching success was found to not be correlated with a general decrease in oocyte maternal transcript abundance but with low transcript levels of specific maternal transcripts by qPCR. The majority of genes showed either no or very minor correlations between their transcript levels and oocyte quality parameters (Fertilisation: 13-93 %, embryonic hatching: 1-94 %). However, maternal transcript levels of three genes, most likely involved in nuclear protein and mRNA transport, growth factor regulation, and embryonic patterning, correlated with oocyte quality.

Further, a new Atlantic halibut 4x44k oligonucleotide microarray was constructed and used to identify 192 strictly maternal genes during Atlantic halibut embryonic development and 20 differentially expressed genes between high and low quality oocytes, involved in immune response, metabolism, RNA transcription, protein degradation, cell signalling and the cytoskeleton. Microarray validation confirmed its suitability for future gene expression studies during Atlantic halibut embryonic development.

The identified maternal genes in this study can serve as a pool for future in-depth studies of embryonic gene expression to advance the knowledge of important developmental processes such as germ cell development, growth and immune response in Atlantic halibut. Some of these may serve as possible markers for Atlantic halibut oocyte quality due to their high expression differences between high and low quality oocytes. Future nutritional studies on Atlantic halibut broodstock should focus on the identified yolk constituents acting positively on oocyte quality.

1 General Introduction

1.1 Atlantic halibut

Atlantic halibut (*Hippoglossus hippoglossus*) belongs to the subfamily Pleuronectinae within the family Pleuronectiformes and is the largest known flatfish. It is a cold-water species and its natural habitat is mainly in the Northern Atlantic Ocean. In western parts of the North Atlantic its range is from the waters outside Virginia and New Jersey up to Greenland. On the European side its habitat spreads from the Bay of Biscay up to the Barents Sea. Atlantic halibut can reach a size over 300 kg and live for 50 years and more. Juvenile Atlantic halibut (> 30cm) feed mainly on crustaceans but as they grow bigger switch gradually to other fish species such as Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), and redfish (*Sebastes marinus*). Halibut males reach considerably smaller size than females seldom exceeding 50 kg (Haug, 1990). Little is known about the reproductive pattern of wild Atlantic halibut but it seems to be under the control of a circannual (seasonal) endogenous mechanism (Björnsson et al., 1998 ; Migaud et al., 2010). Atlantic halibut gametogenesis is initiated by increasing day length and spawning takes place between December to April at 300-700 m depth where temperature and salinity are stable within narrow ranges of 5-7 °C and 34.5-34.9 ppt, respectively (Kjorsvik et al., 1987). Total female annual fecundity varies from 0.5 to 7 million oocytes, depending on age and size of the female. Oocytes are released within approximately one month in several batches in intervals of 3-4 days (Haug & Gulliksen, 1988 ; Norberg et al., 1991). Males produce sperm throughout the whole spawning season (Haug, 1990 ; Babiak et al., 2006).

1.1.1 Atlantic halibut farming and its challenges

Atlantic halibut is farmed in Canada, Scotland, Norway and Iceland and achieves high marked prices (e.g ~8 GBP kg^{-1}in 2010, www.intrafish.com). European production peaked in 2007 but has decreased and flattened out since then (Food and Agriculture Organization of the United Nations: http://www.fao.org/fishery/statistics/en, Figure 1.1).

Seasonally independent oocyte and juvenile production has been achieved for Atlantic halibut by photoperiod manipulation (Næss et al., 2001 ; Björnsson et al., 1998). Although natural spawning may occur in captivity, the usual method entails hand-stripping as collection of gametes by gentle pressuring the broodstock abdomen, followed by artificial fertilisation (Mangor-Jensen et al., 1998). Eggs are normally reared in 250 l upwelling incubators at 5-7 °C and 32-34 ppt (Harboe et al., 1998). Atlantic halibut eggs are buoyant and daylight can trigger increased water permeability of the chorion leading to a reduction in volume and buoyancy. Therefore, eggs are routinely incubated in darkness (Mangor-Jensen & Waiwood, 1995). At 6 ºC, larvae hatch after approximately 16 days (Rollefsen, 1934). Compared to other marine teleosts, the duration of the yolk-sac stage, that is the time from hatching until start of exogenous feeding, is relatively long in Atlantic halibut, taking from 30 to 50 days, depending on temperature (Pittman et al., 1990a). Due to the length of the yolk-sac stage, specialized incubators for larval rearing have been developed. They are typically reared in cylindrical tanks with conical bottom up to 6 m high, so-called upwelling silos. Temperature is typically kept at 5 to 6 °C, but often gradually increased towards the onset of feeding (Harboe & Adoff, 2005). Yolk-sac larvae are relatively sensitive to changes in salinity, as their normal condition is to be neutrally buoyant.

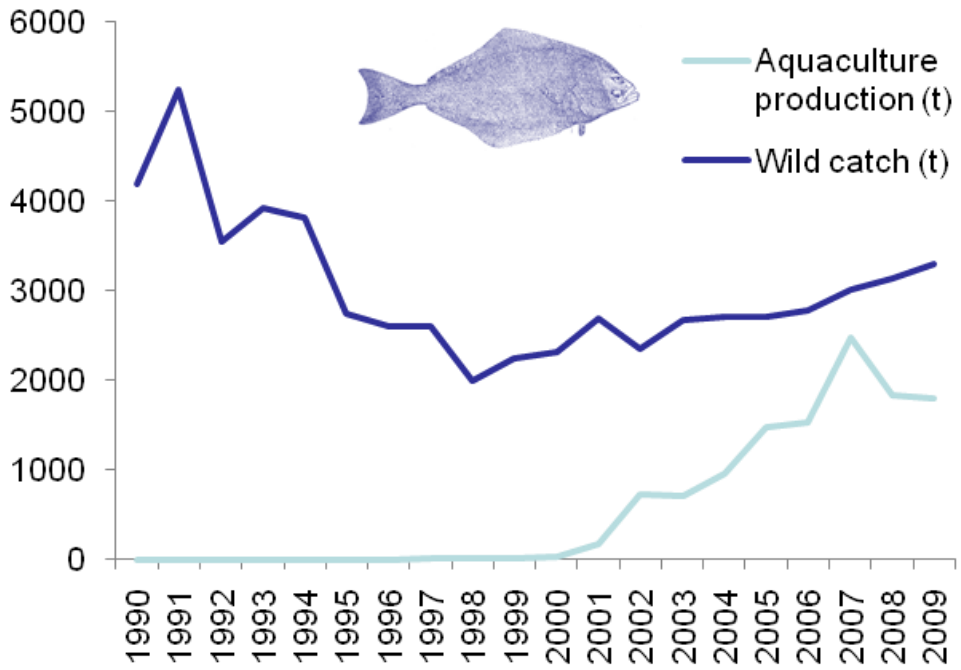

Figure 2.1 European wild catch and aquaculture production of Atlantic halibut.
Wild catch and aquaculture production in tons (t) from 1990 to 2008 (Food and Agriculture Organization of the United Nations: http://www.fao.org/fishery/statistics/en).

Common practice is therefore to alter inlet water salinity if necessary (Mangor-Jensen et al., 1998). Intensive start-feeding of halibut is based on *Artemia* and lasts up to two month (Olsen et al., 1999 ; Hamre et al., 2002). Larvae are usually weaned onto a formulated diet after completion of metamorphosis, at approximately 0.25 g (Rosenlund et al., 1997).

Commercial Atlantic halibut farming strives with challenges in several production stages. Atlantic halibut is stenothermal and broodstock kept at temperatures above 6 °C during the reproductive season produce oocytes of reduced quantities and quality (Brown et al., 2006). However, temperature control for broodstock demands high energy costs and is not always used. Atlantic halibut stripping is labor intensive due to the large size of the broodstock, typically 30-100 kg. Oocyte quality decreases 4-6 h after ovulation which requires careful monitoring of individual females for indication of ovulation (Norberg et al., 1991 ; Bromage et al., 1994). However, in commercial production, females are routinely stripped every 3-4 days independent of their ovulation rhythm (Norberg et al., 1991). Early embryonic mortality in commercially farmed marine teleosts is generally high and Atlantic halibut hatching can be as low as 1 % (Kjorsvik et al., 1990 ; Norberg et al., 1991). Larvae reared under suboptimal conditions develop jaw deformities and yolk-sac oedema (Pittman et al., 1989 ; Lein et al., 1997a ; Lein et al., 1997b). Other types of common malformations include incomplete eye migration during metamorphosis, malpigmentation, and skeletal deformities leading to increased mortality or fry of poor commercial value (Lewis & Lall, 2006 ; Lewis et al., 2004 ; Sæle et al., 2006 ; Hamre et al., 2007 ; Lewis-McCrea & Lall, 2007). Relatively slow growth of Atlantic halibut and its sex-depended dimorphism are the main obstacles in Atlantic halibut production during the on-growing phase (Hendry et al., 2002 ; Foss et al., 2009 ; Imsland et al., 2009).

1.1.2 Atlantic halibut embryonic development

1.1.2.1 Morphology

First characterizations of wild caught embryos and larvae were made by Rollefsen in 1934 (Rollefsen, 1934). Atlantic halibut oocytes are pelagic with no oil globule present. The unfertilized oocyte possesses a soft wrinkled chorion which hardens considerably during first hours after fertilisation. Mean thickness of the chorion is 9.1 µm and small and regular pores are evenly distributed in it. The chorion consists of 18 concentric lamellae (Lønning et al., 1982). The oocyte diameter varies between 3.1- 3.5 mm (Blaxter et al., 1983 ; Haug & Gulliksen, 1988). At 6 °C water temperature, the first germinal disc cleavage takes place after 6 h and subsequent divisions at intervals of ~ 3 h. Gastrulation starts after approximately 4 days at the same temperature (Rollefsen, 1934) (Figure 1.2). First somites are formed between half and complete epiboly and at blastopore closure 8-11 somites are visible (Galloway et al., 2006). The blastopore has a characteristic oblong shape and during the later part of organogenesis the developing embryo is characteristically bent (Lønning et al., 1982 ; Blaxter et al., 1983). Recruitment of new somites continues up to 52 somites at hatching (Andersen et al., 2009). During hatching, the larvae emerge from the egg by dividing the chorion into two well-defined parts (Helvik et al., 1991). At hatching, the larva is at a primitive ontogenetic stage (Pittman et al., 1990b). Eyes are not pigmented and a foetal eye gap may still be visible. The neural retina consists of a pseudo-stratified layer of undifferentiated neuroblastic cells (Kvenseth et al., 1996). The gut is straight and without a lumen. The mouth is not open and there is no stomodeum. The heart is a primitive tube and only the anterior part of the kidney is present. The kidney consists of two parallel tubuli located dorsally between the intestine anlagen and the notochord. No liver, thymus or spleen is developed (Patel et al., 2009b). At hatching there is one pair of external branchial pits and no pectoral fins (Pittman et al.,

1990b). Atlantic halibut larval development has been categorized into different stages from hatching to metamorphosis (Stage 1-4) (Pittman et al., 1990a) and through metamorphosis (Stage 5-9) (Sæle et al., 2004).

1.1.2.2 Egg composition and embryonic metabolism

The proximate Atlantic halibut oocyte composition (% of dry mass) is 55 % yolk protein, 18 % free amino acid (FAA), 15 % lipids, 8 % ions, and 0.5 % glycogen (Finn & Fyhn, 2010). In Atlantic halibut embryos, the dominant portion of energy metabolism switches from lipid/carbohydrate based to FAA as hatching approaches. Subsequently, metabolism switches to yolk protein-bound amino acids (AA), before larvae start exogenous feeding (Finn et al., 1991 ; Finn et al., 1995 ; Zhu et al., 2003).

Oocyte lipid profiles have been studied in egg from wild caught females (Falk-Petersen et al., 1986), between first-time and repeated spawners (Daniel et al., 1993 ; Evans et al., 1996) and through the natural spawning season (Parrish et al., 1993). Of total lipids, polar lipids (PL) represent between 62-80 % and neutral lipids (NL) between 24-29 %. PLs are predominantly phosohatidylcholine (44-62 %), phosphatidylethanolamine (7 %), and sphingomyelin (2 %) while NLs are dominated by triacylglycerols (8-19 %) and cholesterol (7-10 %). The most abundant FAs are palmitic acid (PA, 16:00), oleic acid (OA, 18:1n9), eicosapentaenoic acid (EPA, 20:5n3), and docosahexaenoic acid (DHA, 20:5n3) (Falk-Petersen et al., 1986 ; Bruce et al., 1993). Like in many other marine teleosts, it is assumed that Atlantic halibut has only a reduced ability of converting short-chain polyunsaturated FAs (PUFAs) to long-chain PUFAs, due to limited Δ^5 and Δ^6 desaturase and elongase activity (Tocher, 2003). Hence, in addition to linoleic acid (LA, 18:2n6) and linolenic acid (LNA, 18:3n3), the long-chain PUFAs arachidonic acid (20:4n6, ARA), eicosapentaenoic acid (12.5 %, EPA, 20:5n3), and docosahexaenoic acid (31.2 %, DHA, 22:6n3) are considered essential FAs (EFAs) for

Atlantic halibut. Concentrations of total lipid, triacylglycerols, and sterols were found to be lower in first-time spawners and DHA and ARA significantly higher in repeated spawners (Evans et al., 1996). Cholesterol concentration was found to be significantly higher in oocytes that did not show any signs of successful fertilisation 12 h after fertilisation (Bruce et al., 1993).

The oocyte FAA pool is derived from hydrolysis of yolk proteins during final oocyte maturation and results in an osmotic water influx and rapid oocyte volume increase (Finn et al., 1991 ; Finn et al., 2002 ; Zhu et al., 2003). Serine, alanine, leucine, and lysine have been identified as the most abundant FAAs in Atlantic halibut oocytes and alanine, leucine, glutamine, and lysine as being the most abundant yolk protein-bound AAs in hatched larvae (3.9 dph, Finn et al., 1995 ; Evans et al., 1996 ; Finn et al., 2002). Evans et al. studied FAA concentration in oocytes from first-time and repeated spawners but did not find any significant differences (Evans et al., 1996).

Folate metabolism and its relation to fertilisation success has been studied by Mæland et al. (Mæland et al., 2003). Their data suggests a need for folate for metabolic and growth purposes during embryogenesis of approximately 2 µg g^{-1} embryonic weight gain.

1.1.2.3 Gene regulation of Atlantic halibut oogenesis and embryonic development

The pituitary gonadotropic hormones: follicle-stimulating hormone (FSH) and luteinizing hormone (LH), play pivotal roles in vertebrate reproduction by regulating cell differentiation, proliferation and steroidogenesis in gonadal tissues (Richards, 1994). They exert their hormonal actions on the target cells by binding to the G protein-coupled receptors FSH-R and LH-R. In Atlantic halibut females, a sequential gonadotropic activation of ovarian follicle growth and maturation was found to be regulated by modulating the temporal expression of

fsh-r and *lh-r* in the follicle membrane. Transcripts of LH-β-subunit (*lhβ*) were found to be expressed in ovulated oocytes (Kobayashi & Takeda, 2008).

Studies of gene regulation in Atlantic halibut embryonic development have focused on muscle development focusing on myogenic regulating factors (MRFs): *Myoblast determination factor 1* and *2* (*myod1* and *myod2*) and *myogenin* (*myog*), and genes coding the structural muscle proteins: *Myosin light chain 2* (*mylc2*) and myosin heavy chain (*myhc*). *Myod1*, *myod2* and *myhc* follows the cranial-to-caudal somite formation in Atlantic halibut embryos (Galloway et al., 2006). *Myod1a*, and the alternatively spliced *myod1b*, transcripts are expressed in 4-8 cell stage embryos. *Myod2* is not expressed prior to gastrulation. First somites are formed between half and complete epiboly and *myod1a*, *myod1b* and *myod2* transcript expression increases together with an abrupt *myog* expression increase at the 20-somite stage. A second peak in *myod1* expression around the 40-somite stage coincides with the *myog* expression peak. At this time, *myog* expression is required for myoblast differentiation to myotubes and subsequently to mature muscle cells (Rescan, 2001). Gene expression of the MRFs decreases from about the 45-somite stage and corresponds to the time of first movement and the activation of *myhc* expression followed by the synthesis of contractile myofilaments before final somite formation (Andersen et al., 2009).

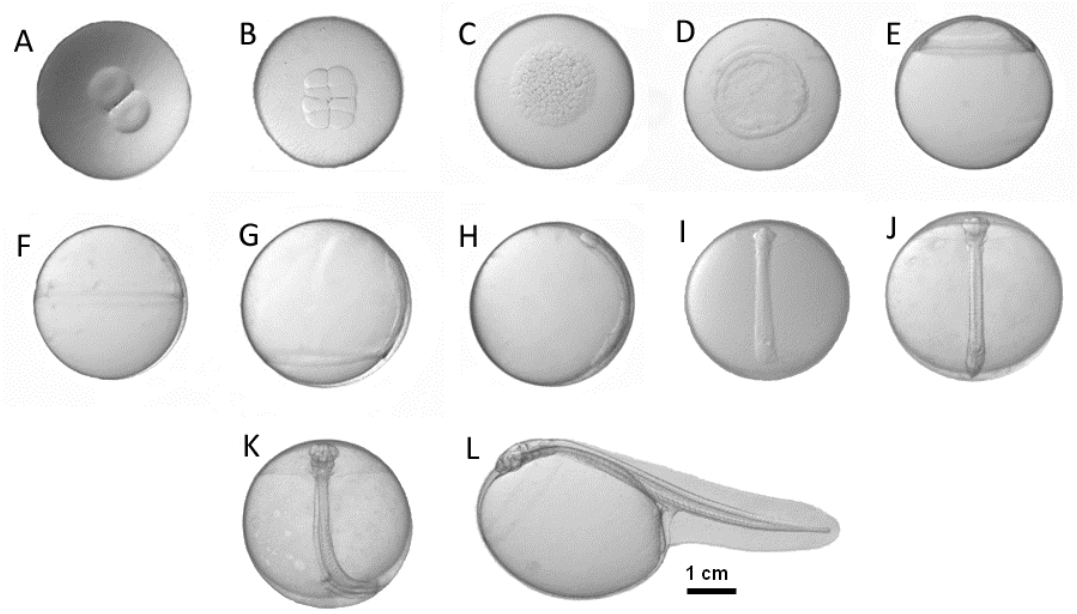

Figure 2.2 Atlantic halibut embryonic development.
A: 2 cells (2C), 6 hours past fertilisation (hpf), B: 8 cell (8C), 8 hpf; C: 128-cells, 26.5 hpf; D: Germ-ring (GR), 82 hpf; E: 25 % Epiboly (25EP), 96 hpf; F: 50 % Epiboly (50EP), 117 hpf; G: 75 % Epiboly, 125 hpf; H: 100 % Epiboly, 142 hpf; I: 20 somites, 194 hpf; J: 30 somites, 250 hpf, K: 40 somites, 315 hpf; L: Hatching, 340 hpf. A – D and I -K: Dorsal view, E – H and L: Ventral view. Development at 6.2 -6.4 ºC. Scale bare 1cm. Photographs were taken under a Stemi SV 11 stereoscope (Carl Zeiss Vision, City, Norway) with an AxioCam HRc camera (Carl Zeiss Vision, City, Norway). Stages based on Rollefsen (1934).

1.2 Oocyte quality in teleosts

Oocytes are the female haploid reproductive cells prior to fertilisation by spermatozoa. Fertilisation results in a diploid zygote developing into a multi-cellular embryo. Good-quality teleost oocytes can be defined as those developing into embryos exhibiting low mortalities at fertilisation and hatching, and normal developing larvae with high survival. Teleost oocyte quality can be affected by a variety of factors (Kjorsvik et al., 1990 ; Brooks et al., 1997 ; Bobe & Labbe, 2009). Factors such as broodstock nutrition, endocrine control, and stress through handling affect oocyte quality indirectly. After ovulation and/or stripping, oocyte quality is not longer under parental influence but under the control of extrinsic factors (e.g water quality) and intrinsic factors of the oocyte itself (e.g yolk constituents, hormones, mRNAs, Figure 1.3).

1.2.1 Broodstock husbandry

The effect of broodstock nutrition on oocyte quality is one of the most studied reproductive factors in aquaculture today (Izquierdo et al., 2001). In many teleosts a positive relation between female size and fecundity, oocyte size, and offspring size has been found (Kamler, 2008). Studies on the specific effects of broodstock nutrition on oocyte quality are limited and sometimes contradicting (Bobe & Labbe, 2009). In Atlantic halibut, only Mazorra et al. (2003) have performed a broodstock nutrition experiment testing the influence of arachidonic acid (ARA) in broodstock diet on oocyte quality (Mazorra et al., 2003). High ARA (1.8 %) levels resulted in significantly higher fertilisation rates, blastomere morphology scores and embryonic hatching rates compared to low ARA levels (0.4 %) (Mazorra et al., 2003).

Broodstock holding temperatures can affect gamete quantity and quality (Devauchelle et al., 1988 ; Tveiten & Johnsen, 1999 ; Anguis & Cañavate, 2005). In Atlantic halibut, high holding temperature during vitellogenesis can cause a delay in spawning and reduction of

oocyte quality (Brown et al., 2006). Endocrine regulation and photoperiod treatment are used to ensure constant gamete production outside the natural spawning period (Migaud et al., 2010)). Gonadotrophin-releasing hormone agonist (GnRHa) implants have been shown to stimulate spermiation but did not affect fertilisation rates in Atlantic halibut males (Vermeirssen et al., 2004). Photoperiod treatment of Atlantic halibut broodstock is successfully used to ensure constant gamete production, but does decrease gamete quality compared to natural spawning (Næss et al., 1996 ; Björnsson et al., 1998 ; Babiak et al., 2006). Hand-stripping is commonly used for species that do not release their ovulated oocytes spontaneously when reared in captivity and can be stressful for broodstock fish, resulting in low oocyte quality (Brooks et al., 1997). Hand-stripping also increases the risk for over-ripening, also called post-ovulatory aging (POA), in teleost species where ovulation rhythms are difficult to estimate. POA reduces the fertilisation ability of oocytes and their subsequent development into normal embryos (McEvoy, 1984 ; Kjorsvik et al., 1990 ; Bromage et al., 1994 ; Lahnsteiner, 2000).

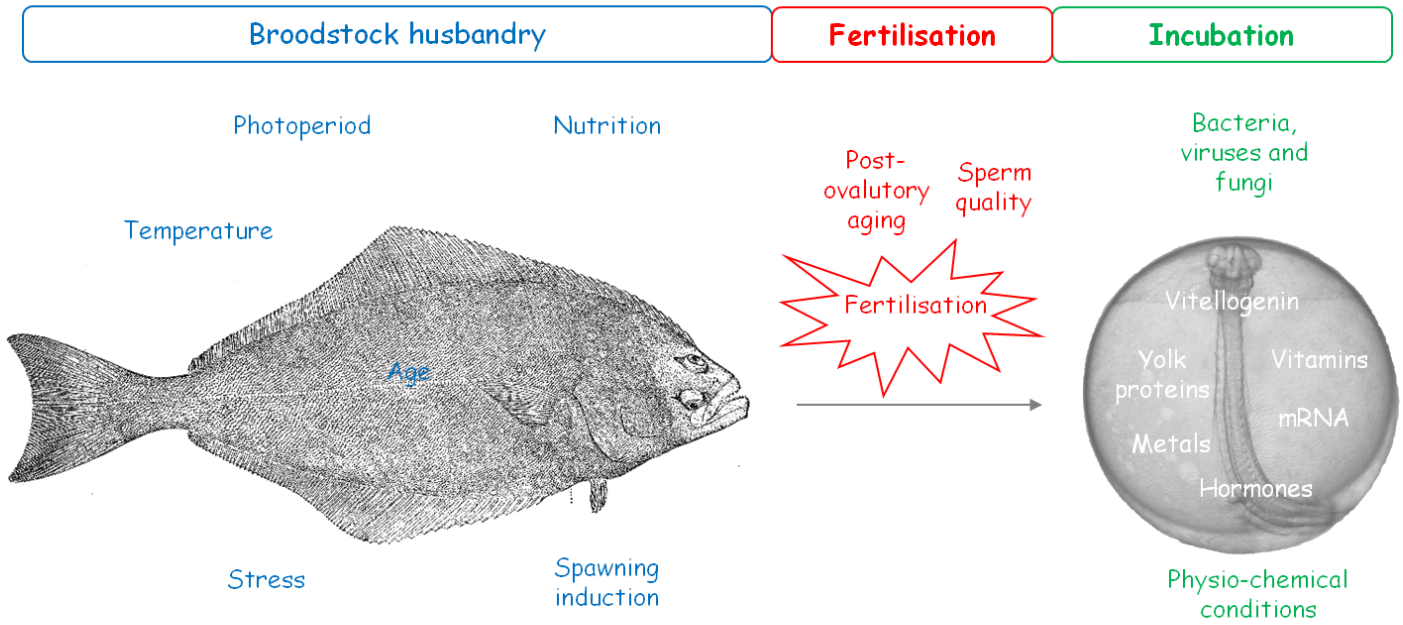

Figure 2.3 Factors influencing fish oocyte quality in aquaculture.

1.2.2 Oocyte fertilisation and incubation

Successful fertilisation requires both oocytes and spermatozoans of high quality. In Atlantic halibut, spermatozoa concentration increases in a linear-like mode from the beginning of the spawning season in February towards the end of the spawning season in May. The concentration increase correlates with a decrease in spermatozoa motility and fertilisation ability. Because of the increasing spermatozoa quality reduction, an asynchrony can occur between spermatozoa and oocyte production towards the end of the reproductive season when high quality oocytes are still produced by females (Babiak et al., 2006). To overcome this problem, Atlantic halibut spermatozoa have also been cryopreserved successfully and larval survival did not differ significantly between larvae produced with cryopreserved spermatozoa and freshly collected spermatozoa (Babiak et al., 2008).

Husbandry practices need to provide a healthy environment during egg incubation. Water quality control is important to control potential pathogens (viruses, bacteria and fungi) that adhend and colonize teleost eggs within hours after fertilisation (Brock & Bullis, 2001). Water flow-rates, temperature, salinity, and pH have to be species-specific optimised and standardised protocols have been established for Atlantic halibut (Mangor-Jensen et al., 1998 ; Olsen et al., 1999).

1.2.3 Markers for oocyte quality

Estimation of oocyte quality before or just after fertilisation is important to avoid costly and unnecessary incubation of low quality oocytes. Accurate methods to identifying poor quality oocytes are therefore of high importance to hatcheries. In salmonids, lipid droplet distribution has been tested as a marker for oocyte quality but has resulted in

inconsistent results (Mansour et al., 2007 ; Ciereszko et al., 2009). Low pH values of ovarian fluid have been associated with reduced oocyte quality due to POA in turbot (Fauvel et al., 1993). The symmetry of the first visible cells after fertilisation has been considered as a useful predictive tool for oocyte quality assessment in commercially farmed marine teleosts (Kjorsvik et al., 1990 ; Brooks et al., 1997). After fertilisation, a series of mitotic cell divisions takes place that divides the cytoplasm into numerous cells called blastomers. In teleosts, the large oocyte yolk volume restricts cleavage to a small area of cytoplasm at the animal pole, so-called discoidal meroblastic cleavage. Many marine teleosts, including Atlantic halibut, produce non-pigmented eggs where blastomers are easily visible. Therefore it has been possible to routinely use early blastomere symmetry at the 8-16 cell stage for oocyte quality assessment during Atlantic halibut farming (Shields et al., 1997). During studies in zebrafish (*Danio rerio*), turbot (*Scophthalmus maximus*), haddock, and Atlantic halibut a positive correlation between early blastomere symmetry and high oocyte quality characteristics like high embryonic hatching and larval survival rates were found (Strehlow et al., 1994 ; Shields et al., 1997 ; Kjorsvik et al., 2003 ; Rideout et al., 2004). Studies on yellowtail flounder (*Limanda ferruginea*) and Atlantic cod support these findings but state that blastomere symmetry corrections may occur during later divisions (Vallin & Nissling, 1998 ; Avery & Brown, 2005 ; Avery et al., 2009). All of these studies exclusively look at the correlation between blastomere symmetry and oocyte quality without explaining the underlying reasons which may be related to biochemistry and/or genetics (Kjorsvik et al., 1990 ; Brooks et al., 1997).

1.3 Gene regulation of embryonic development

1.3.1 Maternal mRNAs

Maternally synthesized mRNAs and proteins control virtually all aspects of early embryonic development. Loaded into oocytes during oogenesis they implement basic biosynthetic processes, direct first mitotic divisions and specify initial cell fate and patterning (Dworkin & Dworkin-Rastl, 1990). When the zygotic genome becomes activated, the embryo begins to utilize products derived from its own genome. This change from maternal to zygotic transcription is called maternal-zygotic transition (MZT, Figure 1.4).

The genes necessary to generate maternally synthesized mRNAs and proteins are so-called maternal genes. They have been classified as strictly maternal or maternal-zygotic genes (Pelegri, 2003). Strictly maternal genes are expressed only during oogenesis and early embryonic development before the MZT. Maternal-zygotic genes are expressed both before and after the MZT. Disruption of strictly maternal gene expression, for example due to mutations, can be fatal for embryonic development. Maternal genes have been extensively studied using classical genetics in invertebrate model organisms like the common fruit fly (*Drosophila melanogaster*), nematodes (*Caenorhabditis elegans*), and in vertebrate species such as African clawed frog (*Xenopus laevis*), zebrafish, and mouse (*Mus musculus*) (Kemphues et al., 1988 ; St.Johnston & Nüsslein-Volhard, 1992 ; Dosch & Niehrs, 2000 ; Roy & Matzuk, 2006). A precise temporal and spatial control of maternal gene expression is important during early embryonic development. Translation of mRNAs during early development can be potentially controlled by polyadenylation dependent activation, localization

dependent activation and by regulated repression (Seydoux, 1996). During polyadenylation dependent activation the dormant form of maternal mRNAs with short poly (A) tails (20-40 adenosines) are elongated to several hundreds of adenosines (Hake & Richter, 1997).

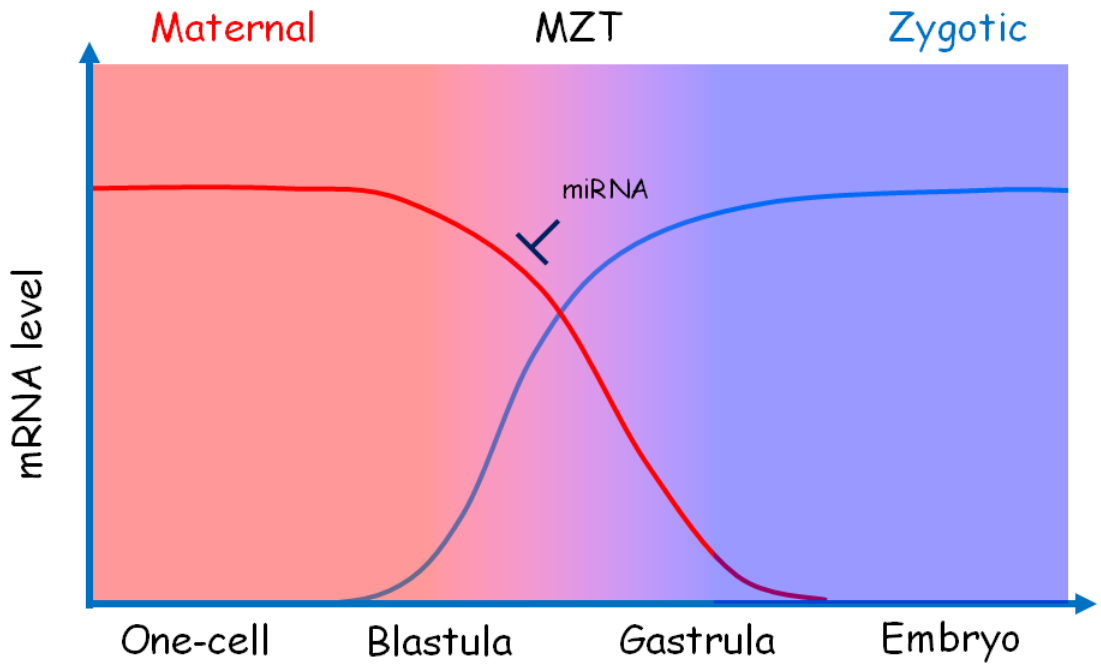

Figure 1.4 Schematic representation of maternal-zygotic transition (MTZ).

This poly (A) lengthening can stabilize and activate mRNAs for translation where as poly (A) removal can trigger degradation and translation repression.

In zebrafish, early detectable effects of maternal genes have been found during oogenesis, egg activation, fertilisation, and cytokinesis (Dosch et al., 2004). During later embryonic development they have been found to affect cell fate determination, morphogenesis, and cell viability (Pelegri, 2003). For example, in teleosts, the establishment of the animal-vegetal polarity is made through the formation of the Balbani body at the vegetal pole in stage I oocytes (Abrams & Mullins, 2009). In absences of strictly maternal *bucky ball* transcripts (*Buc*) the Balbani body is not form (Marlow & Mullins, 2008 ; Abrams & Mullins, 2009). *Buc* is also involved in the positioning of several mRNAs that control primordial germ cell differentiation and other mRNAs (Howley & Ho, 2000 ; Abrams & Mullins, 2009). Mutation of the strictly maternal genes *cellular island* (*Cei*) and *cellular atoll* (*Cea*) affects early cleavage division in zebrafish (Yabe et al., 2007 ; Yabe et al., 2009). Deficiencies of maternal-zygotic genes such as *blistered* (*Bsd*) and *pollywog* (*Pwg*) can lead to the disruption of the organization of the body plan during embryogenesis (Wagner et al., 2004).

As the embryo continues to develop, the zygotic genome is activated during the MZT. Some major features characterize the MZT, including loss or decay of mRNA molecules of maternal origin, activation of transcription of the zygotic genome, developmental arrest in the presence of transcriptional inhibitors, and marked qualitative changes in protein synthetic (Telford et al., 1990). The MZT has been most extensively studied in model species such as California purple sea urchin (*Strongylocentrotus purpuratus*), *C. elegans*, *D. melanogaster*, *X. leavis*, zebrafish, and

mouse. Throughout these different groups of metazoans, the MZT takes place at different developmental stages (Tadros & Lipshitz, 2009). Experiments conducted with transcription inhibitors in the two cyprinids, zebrafish and carp (*Cyprinus carpio*), and in salmonid rainbow trout (*Oncorhynchus mykiss*) implied a species-specific timing of activation of transcription of zygotic mRNA. In cyprinids it takes place during the blastula stage and in rainbow trout later, at the start of epiboly (Baumann & Sander, 1984 ; Stroband et al., 1992 ; Nagler, 2000 ; O'Boyle et al., 2007). The MZT consists of two steps. First, a subset of maternal mRNAs is degraded, followed by the start of zygotic transcription. However, pre-MZT accumulation of some zygotic transcripts has been found in zebrafish (Mathavan et al., 2005). The degradation of maternal mRNAs is regulated by both maternally encoded proteins and zygotically encoded proteins, and microRNAs (miRNAs). With the start of zygotic transcription, zygotically encoded proteins and miRNAs provide a positive feedback which enhances the efficiency of maternal mRNA degradation. miRNAs are short non-coding RNAs (20-22 bp) that negatively control the target mRNAs by binding to the 3´untranslated region (UTR). In zebrafish, the predominantly expressed miR430 family facilitates deadenylation and clearance of maternal mRNAs (Figure 1.4; Giraldez et al., 2006).

1.4 Teleosts genomic resources

1.4.1 Introduction to teleosts genomes

Teleosts are the largest group of vertebrates and comprise ~23,600 species. Teleost genomes vary widely in size, from 0.39 pg to > 5 pg of DNA per haploid cell, with a modal value of ~ 1 pg (equivalent to ~ 1000 Mb) (Smith & Gregory, 2009). Most of the large genomes (> 2 pg) are polyploid. Among the vertebrates, polyploidization is common only in fishes, amphibians, and reptiles. In teleosts, polyploidization has occurred independently in cyprinids (carps), cyprinodontiformes (live bearers), catostomids (suckers), and salmonids (Taylor et al., 2001). Teleost genomes seem to be more 'plastic' in comparison with other vertebrate genomes because genetic changes, such as polyploidization, gene duplication, gain of spliceosomal introns and speciation, are more frequent in fishes (Venkatesh, 2003). The study of the teleost genomes has been closely connected to the sequencing of the human genome and the need to identify the sequences structure and function. The work on teleost genomes was intensified with the start of the Human Genome Project in 1990 when the main species studied for genome information at that time where invertebrates like *C. elegans* and *D. melanogaster*. Compared to these invertebrates, teleosts share many similarities in developmental pathways, organ systems, and physiological mechanisms with humans (Clark, 2003). The two main teleosts investigated during the early 1990s were the zebrafish, the marine Tiger puffer (*Takifugu rubripes*), and its freshwater relative, the Green spotted puffer (*Tetradon nigrovirdis*). The zebrafish is a popular tropical aquarium fish with short generation time (about three month), large egg batches all year round, easy maintenance, and external development of a transparent embryo. Large-

scale mutagenesis screens in zebrafish filled a gaping hole in vertebrate developmental biology (Haffter et al., 1996 ; Stainier et al., 1996). Until then, the ability to study genes via their mutant phenotypes on large scales had only been possible in *D. melanogaster* and *C. elegans*. *T. nigrovirdis* has the smallest known vertebrate genome with 350 Mb, closely followed by *T. rubripes* with 380 Mb. Their small genome size, approximately eight times smaller than the human or mouse genome made them useful model vertebrate genomes (Brenner et al., 1993 ; Crnogorac-Jurcevic et al., 1997).

Today there are 34 entries for teleosts in the Genome Project database of the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nhi.gov). With the increase in worldwide aquaculture production genomic work on commercially farmed teleosts has increased (Canario et al., 2008). While the genomes of model teleosts have been sequenced totally or to the draft level, the genetic data available on commercial interesting teleosts consists mainly of expressed sequence tags (ESTs; Table 1.1). ESTs are short, single pass cDNA sequences generated from randomly selected library clones and are a quick and easy way to generate data from any species (Clark, 2003). ESTs also provide the raw data for probe design of oligonucleotide microarrays. Zebrafish is the seventh species with the highest number of entries in the dbEST database (1.5 M, release 100209) after human (8.3 M), mouse (4.8 M), maize (2.0 M), cattle (1.6 M), pig (1.5 M) and thale cress (1.5 M) (http://www.ncbi.nlm.nih.gov/dbEST). For the twenty most valuable farmed teleosts, genomic information is manly available for salmoniformes and perciformes (Table 1.2.).

There are 22,864 Atlantic halibut ESTs currently available at the EST database dbEST (Table 1.2). The first Atlantic halibut EST libraries resulting in 1,072 ESTs were constructed from liver, kidney, and spleen to investigate the immune response of

vaccination against *Vibrio anguillarum* and *Aeromonas salmonicida* (Park et al., 2005). In addition, six EST libraries (51,117 ESTs) were constructed from liver, kidney, spleen, peripheral blood, and thymus from Atlantic halibut injected with nodavirus, infectious pancreatic necrosis virus (PINV), or vibriosis vaccine at various time points (Patel et al., 2009a). The highest number of Atlantic halibut ESTs (12,675 ESTs) were created during the Pleurogene project, a Canadian and Spanish collaboration, focusing on the genomics of two flatfishes, Atlantic halibut and Senegal sole (*Solea senegalensis*) (http://pleurogene.ca/index.php). Libraries were constructed from five different larval stages (hatched, mouth-opening, midway to metamorphosis, and post-metamorphosis) and eight different tissues (testis, ovary, liver, head kidney, spleen, skin, gill, and intestine) (Douglas et al., 2007). Over 4,000 ESTs were obtained from Atlantic halibut two cell stage embryos, 1 day-old yolk-sac larvae, and fast skeletal muscle of juveniles (Bai et al., 2007).

Table 2.1 Overview of the 20 teleost species with most expressed sequence tags (ESTs). ESTs are sorted by the number of ESTs available (N) and their relative percentage of total number (% T; dbEST database: http://www.ncbi.nlm.nih.gov/projects/dbEST/, release 100209, October 2, 2009). Species with fully sequenced genomes are written in bold.

		ESTs	
Common name	**Latin name**	**N**	**% T**
Zebrafish	*Danio rerio*	1,481,930	32.2
Japanese medaka	*Orytrzias latipes*	616,739	13.4
Atlantic salmon	*Salmo salar*	494,152	10.7
Channel catfish	*Ictalurus punctatus*	354,434	7.7
Rainbow trout	*Oncorhynchus mykiss*	287,923	6.3
Three-spined stickleback	*Gasterosteus aculeatus*	276,992	6.0
Fathead minnow	*Pimephales promelas*	249,941	5.4
Atlantic cod	*Gadus morhua*	206,507	4.5
Catfish	*Ictalurus furcatus*	139,475	3.0
Nile tilapia	*Oreochromis niloticus*	177,222	2.1
Common mummichog	*Fundulus heteroclitus*	74,755	1.6
Gilthead seabream	*Sparus aurata*	67,232	1.5
European seabass	*Dicentrarchus labrax*	54,200	1.2
Rainbow smelt	*Osmerus mordax*	36,028	0.8
Common carp	*Cyprinus carpio*	32,046	0.7
Japanese pufferfish	*Takifugu rubripes*	26,069	0.6
Inshore hagfish	*Eptatretus burgeri*	23,884	0.5
European perch	*Perca flavescens*	21,968	0.5
Atlantic halibut	*Hippoglossus hippoglossus*	22,834	0.5
Roach	*Rutilus rutilus*	18,470	0.4
Guppy	*Poecilia reticulata*	16,215	0.4

Table 2.2 Overview of number of sequence tags (ESTs) available on dbEST database for the twenty most valuable aquaculture teleost species.
Values are given in million $ and relative percentage of total value (%T). ESTs are given in number of ESTs (N) and relative percentage of total number (%T, Food and Agriculture Organization of the United Nations (FAO), http://www.fao.org/fishery/statistics/en, release 100209, October 2, 2009).

Common name	**Latin name**	**Value (M)**	**Value %T**	**N**	**% T**
Atlantic salmon	*Salmo salar*	7.5	19.4	494,152	50.1
Silver carp	*Hypophthalmichthys molitrix*	3.5	9.2	0	0.0
Grass carp	*Ctenopharyngodon idellus*	3.5	9.0	640	0.1
Common carp	*Cyprinus carpio*	3.1	7.9	32,046	3.3
Catla	*Catla catla*	3.0	7.6	1	0.0
Nile tilapia	*Oreochromis niloticus*	2.6	6.7	177,222	0.0
Rainbow trout	*Oncorhynchus mykiss*	2.6	6.6	287,795	29.2
Bighead carp	*Hypophthalmichthys nobilis*	2.1	5.5	0	0.0
Crucian carp	*Carassius carassius*	1.6	4.1	0	0.0
Mandarin fish	*Siniperca chuatsi*	1.5	3.9	32	0.0
Japanese amberjack	*Seriola quinqueradiata*	1.3	3.4	1,381	0.1
Japanese eel	*Anguilla japonica*	1.1	2.8	198	0.0
Milkfish	*Chanos chanos*	0.8	2.0	0	0.0
Gilthead seabream	*Sparus aurata*	0.7	1.8	67,232	6.8
Flathead grey mullet	*Mugil cephalus*	0.6	1.7	5	0.0
Olive flounder	*Paralichthys olivaceus*	0.5	1.4	9,983	1.0
Silver seabream	*Pagrus auratus*	0.5	1.2	0	0.0
Coho salmon	*Oncorhynchus kisutch*	0.5	1.2	2,325	0.2
European seabass	*Dicentrarchus labrax*	0.4	1.1	54,200	5.5
Japanese seabass	*Lateolabrax japonicas*	0.3	0.8	75	0.0

1.4.2 Applications for commercial farming

Teleosts represent a major worldwide source for food. Aquaculture, is the fastest growing food-producing sector, accounts for nearly 50 % of the world's food fish (FAO). Some of today's challenges in aquaculture are to ensure a stable supply of fry for on-growth in marine species, to reduce production time by increasing growth rates, to overcome feed limitations by looking for alternative feed sources such as plants, to avoid uncontrolled reproduction of farmed species with wild populations, and to improve stress tolerance and disease resistance. Increasingly, genomic research and biotechnology is used to deal with these challenges to improve aquaculture production and to ensure a stable and increasing production.

A range of genetic tools have been applied to commercially farmed teleost. Selective breeding programs where individual and family performance is assessed for a range of commercial traits (e.g. growth, sexual maturation, body conformation, and disease resistance) have lead to increased gains between generations in for example catfish, common carp, rainbow trout, Atlantic cod, Atlantic salmon (*Salmo salar*), and Nile tilapia (*Oreochromis niloticus*, Eknath et al., 2007 ; Weber & Silverstein, 2007 ; Robinson & Hayes, 2008 ; Antonello et al., 2009 ; Wachirachaikarn et al., 2009 ; Garber et al., 2010 ; Nielsen et al., 2010).

Chromosome set manipulations like triploidy leads to sterility and are used in the production of larger rainbow trout, common carp, and channel catfish (*Ictalurus punctatus*) (Dunham, 2004). Triploids can also be used to avoid interbreeding between escaped farmed strains and wild populations (Cnaani & Levavi-Sivan, 2009). Gynogenesis has been induced in some fish species to obtain mono-sex offspring

(Komen & Thorgaard, 2007). Techniques to produce all-male strains has been established in common carp and Nile tilapia (Mair et al., 1997 ; Bongers et al., 1999 ; Ezaz et al., 2004 ; Müller-Belecke & Hörstgen-Schwark, 2007) while all-females strains are preferred in flatfishes, for example turbot and Atlantic halibut (Piferrer et al., 2004 ; Cal et al., 2006 ; Tvedt et al., 2006).

Gene transfer technologies have mainly been used to improve growth performance. Growth hormone transgenic lines have been developed for Atlantic salmon (Du et al., 1992 ; Cook et al., 2000), rainbow trout (Devlin et al., 2001), Nile tilapia (Maclean et al., 2002), channel catfish (Dunham et al., 1992), and common carp (Fu et al., 2007). Transgenic lines with increased disease resistance and sterility are under development (Dunham, 2009).

Teleost ESTs have been found to be a rich source of genetic markers called single sequence repeats (SSR) loci, also called microsatellites (Coulibaly et al., 2005 ; Ju et al., 2005). SSRs consist of a variable number of short sequence repeats (2-6 nucleotides). In aquaculture, SSRs are efficiently used for individual identification, paternity analysis, and relatedness estimation in the management of hatchery bloodstocks (Chistiakov et al., 2006). In Atlantic halibut SSRs have been used for population studies of wild and farmed fish, for ploidy determination studies during gynogensis experiments and construction of a genetic linkage map (McGowan & Keith, 1999 ; Coughlan et al., 2000 ; Jackson et al., 2003 ; Reid et al., 2005 ; Ding et al., 2009). Genetic linkage maps provide a likely position of its known genes and/or genetic markers relative to each other in terms of relative distances (recombination frequency) between them. Genetic linkage maps are prepared to scan for quantitative trait loci (QTL). QTLs refer to phenotypes, such as growth or disease resistance, that are

inherited in various degrees and can be credited to the interactions between two or more genes and their environment (Ohtsuka et al., 1999). In Atlantic salmon, a QTL has been identified for infectious pancreatic necrosis (IPN) resistance and has been incorporated into a commercial Atlantic salmon breeding program (Moen et al., 2009) After the creation of a genetic linkage map in Atlantic halibut, the identification of markers for several traits, including body weight, length, width, myotome height, pigmentation, and eye migration, has been used in a Canadian breeding program (Reid et al., 2007) (http://pleurogene.ca).

The availability of numerous ESTs and the corresponding cDNA libraries has enabled a rapid production of microarrays in a number of commercial teleosts, but mainly salmonids (Douglas, 2006 ; Canario et al., 2008). Microarray technology, through simultaneous analysis of the expression of thousands of genes, allows the identification of candidate genes involved in the function of multiple physiological, morphological, and behavioural traits of interests. In salmonids, gene microarray studies have been used to study important topics in aquaculture such as immune response, nutrition, growth, smoltification, and reproduction (Jordal et al., 2005 ; Gahr et al., 2008 ; Leaver et al., 2008 ; Von Schalburg et al., 2008 ; Bobe & Labbe, 2009 ; Seear et al., 2009). For Atlantic halibut, a first generation oligonucleotide microarray (50-mer) comprising 9,277 genes has been designed during the Pleurogene project (http://pleurogene.ca) (Douglas et al., 2008). This microarray has been used to study differential gene expression between five larval stages, from hatching to post-metamorphosis, during a weaning experiment of larvae to microencapsulated diet and to study the effect of replacing fish meal with soybean meal in diets for juveniles (Aluru & Vijayan, 2009 ; Murray et al., 2009 ; Murray et al., 2010).

1.4.3 Molecular markers for oocyte quality

Molecular markers can be defined as Type I or actual genes of known function and Type 2 or anonymous DNA segments like microsatellites. While Type 2 markers have been successfully applied to improve aquaculture production as described above, the use of Type 1 markers is still rare. Potential molecular markers for oocyte quality have been identified in rainbow trout. Oocytes with high maternal mRNA levels of prohibitin 2 (*phb2*) experienced low developmental potential, i.e percentage of normal alevins at yolk-sac resorption (Bonnet et al., 2007). Salmonids can retain their ovulated oocytes in the body cavity for several days. Depending on the female, this leads to a decrease in oocyte quality due to over-ripening (post-ovulatory aging, POA) (Aegerter and Jalabert, 2004). A number of maternal mRNAs have been identified to be differentially expressed in high and low quality rainbow trout oocytes, induced by POA (Aegerter et al., 2005). In marine teleosts no potential molecular markers for oocyte quality have been identified. Maternal transcripts in commercially farmed marine teleosts have not been studied previously, even so high embryonic mortalities are often observed within several days after fertilisation, around the MZT (Blaxter et al., 1983 ; Kimmel, 1989). Reliable molecular quality markers could increase hatchery production efficiency and provide a potential tool to improve broodstock husbandry.

1.5 Objectives of the present study

The overall objective of this study is to study the effect of maternal factors on Atlantic halibut oocyte quality. The specific objectives are:

-To increase the genomic information in Atlantic halibut by creating a maternal EST library and identifying strictly maternal genes by studying gene expression during embryonic development.

-To select suitable Atlantic halibut reference genes for gene expression studies during embryonic and larval development.

-To study the relation between maternal mRNA levels and Atlantic halibut oocyte quality.

-To study the relation between oocyte fatty acid, amino acid, and folate concentrations and Atlantic halibut oocyte quality.

-To create a new Atlantic halibut microarray to identify maternal genes and analyse differential gene expression during embryonic development and between high and low quality oocytes.

2 Construction of a maternal EST library by suppressive subtractive hybridisation (SSH) in Atlantic halibut

2.1 Abstract

The commercial production of Atlantic halibut suffers from a major bottleneck during juvenile production for on-growing. Early embryonic development is under the control of maternally provided transcripts and proteins and has been studied in only few commercially important teleosts. The present study aimed to create an Atlantic halibut maternal expressed sequence tag (EST) library by suppressive subtractive hybridisation (SSH) to increase the information of maternal genes during early Atlantic halibut embryonic development. Gene expression of selected genes was screened during early embryonic development to identify strictly maternal transcripts as potential molecular markers for Atlantic halibut oocyte quality. A maternal EST library containing 2,341 high quality sequences was constructed. Putative genes consisted of 73.5 % unknown genes. The expressions of twenty-one selected genes were measured by qPCR from fertilisation to the 10-somite stage. Transcripts of three strictly maternal genes were identified as they were only detectable before the start of gastrulation: *askopos* (*Kop*), *si:dkey-30j22.9* (Tudor family member), and *tudor 5 protein* (*Tdrd5*). They are candidate genes to be tested for their potential as molecular markers for Atlantic halibut oocyte quality.

2.2 Introduction

Genomic technologies are expected to improve aquaculture production and solve present bottlenecks and problems. Studies so far have concentrated on the teleost immune system, control of growth, and reproduction (De-Santis & Jerry, 2007 ; Cerdà et al., 2008 ; Dios et al., 2008). One of the major bottlenecks in commercial farming of marine teleosts is variable qualities and quantities during fry-production for on-growing. Variable oocyte quality, high larval mortality, and body malformations during larval development reduce production efficiency and keeps production costs high. Little attention has been paid to the molecular and intracellular events that occur during gametogenesis and early embryonic development in teleosts (Bobe & Labbe, 2009). Early embryonic development is controlled by maternal factors, mRNAs and proteins, produced during oogenesis by the female and stored in the mature oocyte. Loaded into the oocyte during oogenesis, maternal mRNAs implement basic biosynthetic processes, direct first mitotic divisions and specify initial cell fate and patterning (Dworkin & Dworkin-Rastl, 1990). The teleost embryo starts to utilize products derived from its own genome during the maternal-zygotic transition (MZT), when the zygotic genome becomes activated. The MZT is a progressive process and some maternal-zygotic genes are expressed both before and after the MZT (Mathavan *et al*. 2005). Maternal-zygotic and finally zygotic gene expression regulates the later parts of axis formation and organogenesis during fish embryonic development (Schier et al 2001).

Experiments conducted with transcription inhibitors in two cyprinids, zebrafish (*Danio rerio*) and common carp (*Cyprinus carpio*), and in the salmonid rainbow trout (*Oncorhynchus mykiss*) implied a species-specific timing of zygotic genome activation. In the cyprinids, the MZT was found to take place during the blastula stage while in

rainbow trout the MZT took place later, at the start of epiboly (Baumann & Sander, 1984 ; Stroband et al., 1992 ; Nagler, 2000 ; O'Boyle et al., 2007). In Atlantic halibut the exact timing of the MTZ has not been previously estimated. Genomic information of maternal genes in commercially farmed teleosts has only been available for rainbow trout (Aegerter et al., 2004 ; Aegerter et al., 2005 ; Bonnet et al., 2007) but has recently been extended to Atlantic halibut (Bai et al., 2007).

Atlantic halibut is considered a valuable species for cold water marine fish farming, but current production suffers from typical problems during fry production as described above (Chapter 1.1.1) (Kjorsvik et al., 1990 ; Olsen et al., 1999 ; Hamre et al., 2007). Genomic studies of early developmental stages of Atlantic halibut have concentrated on embryonic muscle development and larval development and metamorphosis (Galloway et al., 2006 ; Douglas et al., 2007). Recently, an unbiased 2-cell EST library has been created providing first information about maternal transcripts in Atlantic halibut (Bai et al., 2007)

The aim of this study was to increase the genomic information on maternal gene expression in Atlantic halibut. A maternal EST library was created by suppressive subtractive hybridisation (SSH) between 8-cell stage embryos (8C) and embryos at the 10-somite stage (10SS). Genes with known functions during embryonic development and random genes were selected from the EST library. Their relative gene expression was screened during early embryonic development to identify strictly maternal transcripts.

2.3 Material and Methods

2.3.1 Fish husbandry and sample collection

From four females, high quality oocyte batches were collected at a commercial Atlantic halibut farm (Risørfisk AS, Risør, Norway) in 2007. All oocytes were fertilised *in vitro* with pooled sperm from two random males. The female broodstock consisted of fish between 30-40 kg, fed EWOS Premix (EWOS, Bergen, Norway) and kept under natural photoperiod conditions. Eggs were incubated in large scale in 280 l incubators at salinity between 33-35 ‰ and temperature between 6.2 -6.4 ºC. Normally developing embryos ($n = 100$) were collected at the following stages based on the embryonic staging of Atlantic halibut after Rollefsen (1934, Figure 1.2): 8cell-stage (8C), 8 hours post fertilisation (hpf); 16-cell stage (16CS), 12 hpf; blastula (BL), 45 hpf; germ ring (GR), 82 hpf; 25 % epiboly (25EP) 96 hpf; 50 % epiboly (50EP), 117 hpf; and 10 somite stage (10SS), 142 hpf. Samples were wrapped in tinfoil and snap-frozen in liquid nitrogen.

2.3.2 RNA extraction and cDNA synthesis

Total RNA for all samples were extracted according to the Tri reagent method (Sigma, St-Louise, MO USA) using QIAazol (Qiagen, Nydalen, Sweden). Total RNA was treated with the gDNA wipe-out buffer supplied with the QuantiTect reverse transcription kit (Qiagen) to remove traces of genomic DNA contamination. RNA concentration was quantified using the Nanodrop spectrophotometer (Nanodrop Technologies/Saven Werner, Kristiansand, Norway).

Blunt-ended cDNA fragments for the subtraction were produced with a SMART PCR cDNA synthesis kit (Clontech, Saint-Germain-en-Laye, France) and digestion with *Rsa* I. The digested cDNA was then purified with a QIAquick PCR Purification kit (Quiagen) and quantified using Nanodrop.

2.3.3 Suppressive subtractive hybridisation

A forward subtractive library between 8-cell stage (maternal) and 10 somite stage (zygotic) embryos was created by suppressive subtractive hybridization (SSH) using the PCR-select cDNA subtraction kit (Clontech, Figure 2.1) (Fernandes et al., 2005). Blunt-ended cDNA fragments from 8C were used as a tester while the fragments from 10SS were used as a driver. The ligation step was optimized for the halibut samples using an Atlantic halibut specific primer for *β2-tubulin* (*Tubb2*) (Fwd: TACAATGAGGCTTCAGGTGG, Rev: TCCCTCTGTGTAGTGACCCTTG) using an annealing temperature of 65 ºC and amplifying a product size of 134 bp. The subtracted PCR product for the 8C embryo was cloned with the TOPO TA Cloning Kit (Invitrogen, Paisley, UK) and random clones were picked for sequencing. Insert checks were carried out by PCR with 1 µl of colony template mixed with 20 µl of reaction mix (dNTPs, 2 mM), PCR buffer (10 x), T3 primer short (10 µM, 5' ATTAACCCTCACTAAAG 3'), T7 primer short (10 µM, 5' AATACGACTCACTATAG 3'), Taq DNA Polymerase (GE Healthcare, Nydalen, Norway) and MilliQ water. The PCR involved an initial denaturation step at 96 °C for 2.5 min followed by 36 amplification cycles: 96 °C for 20 sec., 48 °C for 30 sec, and 72 °C for 1 min with a final extension at 72 °C for 5 min. 5’ end sequencing PCR sequencing reactions with T3 primer (5’ AATTAACCCTCACTAAAGGG 3’) were performed using the ABI prism Big Dye

Terminator Sequencing Kit (PE Applied Biosystems, USA) added BetterBase (1:5; Web Scientific, Crewe, UK). The sequencing reaction comprised an initial denaturation at 96 °C for 1 min and 25 cycles at 96 °C for 10 sec and 60 °C for 3 min and DNA was send for sequencing at the Oxford University sequencing facility with an ABI 3700 capillary sequencer (PE Applied Biosystems, USA).

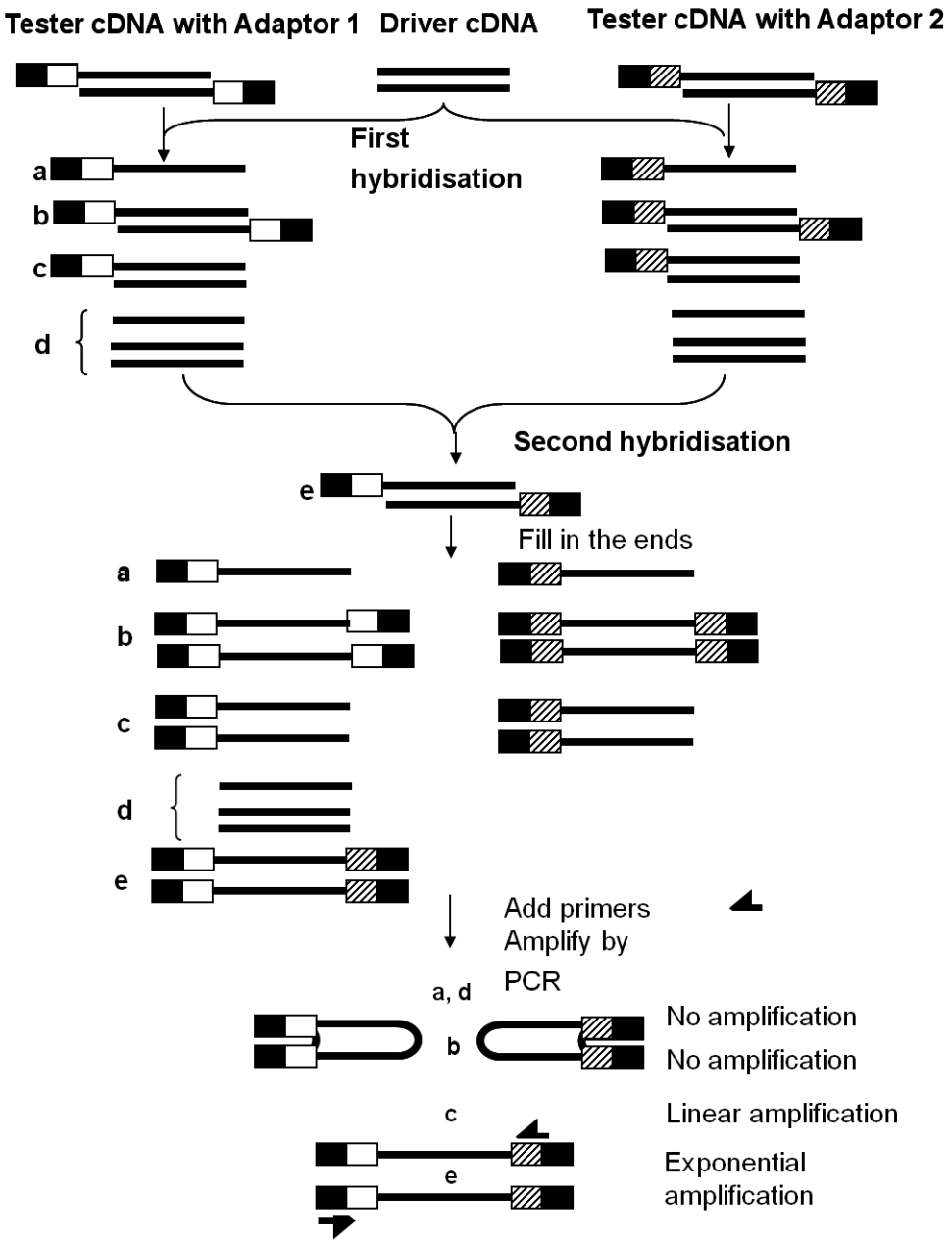

Figure 2.1 Principle of suppressive subtractive hybridisation (SSH).
Based on the principles of Clontech PCR-Selected cDNA Subtraction kit (Clontech).

2.3.4 Sequence processing and bioinformatics analysis

The raw sequence trace data were processed by the EST analysis pipeline developed by the Natural Environment Research Council-Environmental Genomics Thematic Programme Data Centre (NERC-EGTDC; University of Edinburgh, UK; Figure 2.2). The electrophoregrams were first analyzed by trace2dbEST (accessible through http://envgen.nox.ac.uk/est.html) which processes raw sequencing chromatograph trace files from EST projects into quality-checked sequences. High quality sequences required > 150 high quality bases, based on signal strength, peak shape and peak local environment (Ewing et al., 1998). Sequences were submitted to dbEST (http://www.ncbi/nlm/nih.gov/dbEST) jointly with their BLAST-based preliminary annotation. PartiGene (Parkinson et al., 2004) was then used to cluster the sequences and contig assembly. The non-redundant clusters were submitted to BLASTX similarity searches against the non-redundant (nr) protein database at the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The EST annotation tool Blast2GO (Gotz et al., 2008) was employed for gene ontology (Gene Ontology, 2004), enzyme code annotation and pathway mapping with the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa et al., 2008). Non-annotated cluster were translated using EMBOSS Transeq (http://www.ebi.ac.uk/Tools/emboss/transeq/) and searched for conserved domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

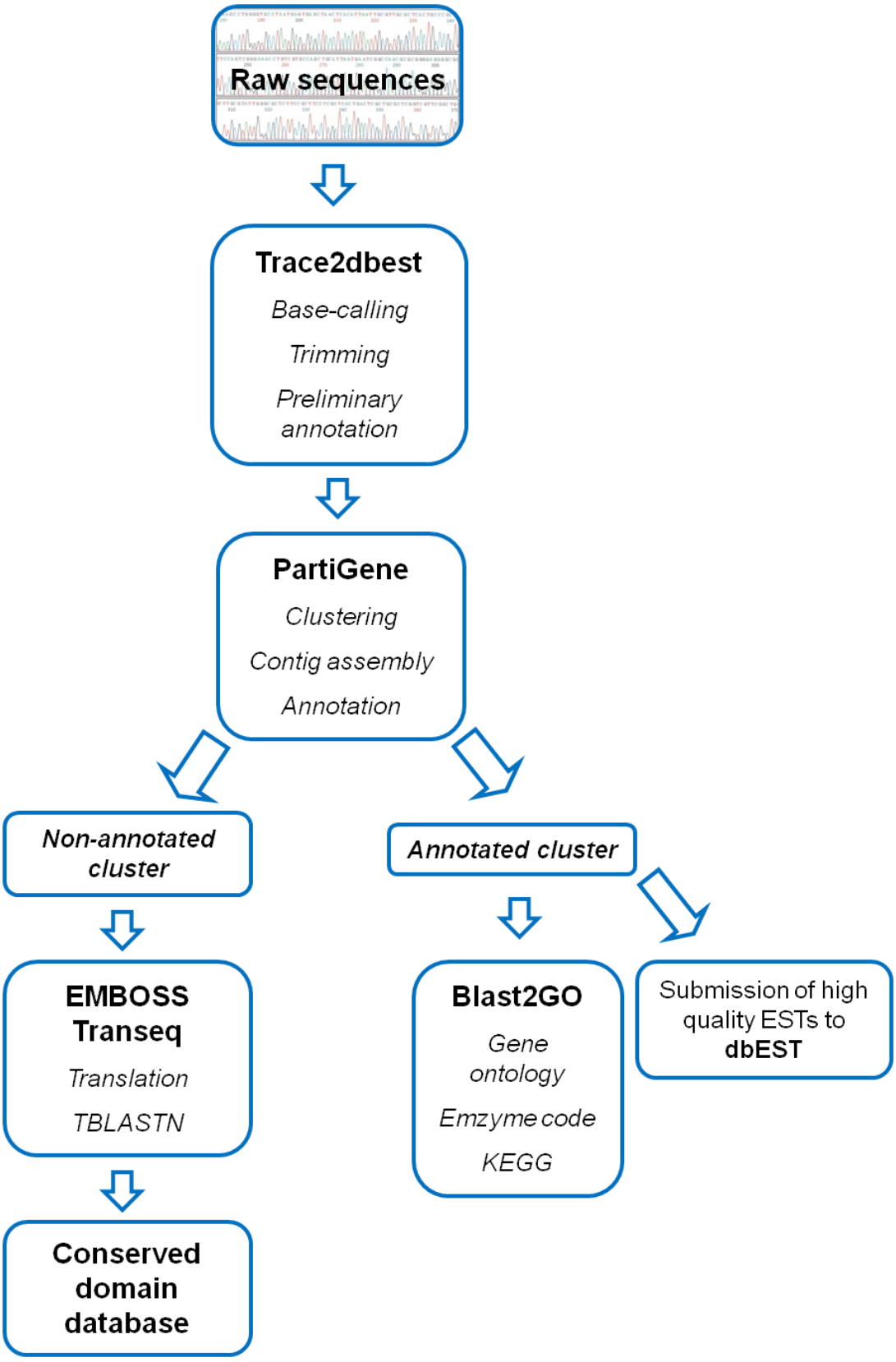

Figure 2.2 Overview of sequence processing and bioinformatics analysis.

2.3.5 Relative gene expression by quantitative real-time PCR (qPCR)

Twenty-one genes from the maternal cDNA library were chosen for screening during embryonic development based on their known function during embryonic development according to literature. Ten of them with significant BLASTX results (Table 2.1) and eleven with identified conserved domains (Table 2.2). Whenever possible, primers were designed across the most conserved splice junctions. All gene specific primers crossed at least one intron/exon border containing both donor and acceptor sites, in order to avoid amplification of any contaminating genomic DNA. Primer pairs for qPCR amplification were designed manually and screened for hairpins, homo- and cross-dimers using *Netprimer* (http://www.premierbiosoft.com/netprimer; Table 2.3 and 2.4). To confirm that the right product was amplified, a quantitative real-time PCR (qPCR) was performed on pooled cDNA for each primer pair. The different products were sequenced directly for additional verification. Each sample was checked for genomic DNA contamination by running a qPCR with RNA treated with gDNA wipe-out buffer (Qiagen). Gene amplifications by qPCR were performed with a LightCycler® 480 thermocycler (Roche, Basel, Switzerland). Each 10 µl reaction in a 96-well plate comprised 4 µl of 70 x diluted cDNA template, 1 µl of each primer pair at 5 µM and 5 µl of QuantiTect SYBR Green containing ROX as reference dye (Qiagen). After an initial denaturation step of 15 min at 95 ºC, 45 cycles of amplification were performed according to the following thermal cycles: denaturation for 15 s at 94 ºC, annealing for 20 s at 60 ºC and extension for 20 s at 72 ºC. Fluorescence data were acquired during this last step. A dissociation protocol with a gradient from 65 to 97 ºC was used to investigate the specificity of the qPCR reaction and the presence of primer dimers. All samples were run in duplicate along with minus reverse transcriptase, no template and a

positive plate control. Five-point standard curves of a 5-fold dilution series (1:2-1:16) from pooled cDNA were used for PCR efficiency calculation. To assess suitable reference genes for the qPCR studies the known reference genes *elongation factor 2* (*Eef2*), *β-Actin* (*Actb*), and *tubb2* were tested (Fernandes et al., 2008; Table 2.5). Because of their stable quantification cycles across the seven embryonic stages the three genes HHC01138, HHC1517, and HHC00353 were added to the list of known reference genes to test their potential as reference genes. GeNorm (Vandesompele et al., 2002) was used to assess the most suitable reference genes. Primers for qPCR were designed as described above for the genes of interest (Table 2.5). HHC01517 and HHC00353 were selected as the most suitable reference genes (Figure 2.3).

2.3.6 Data analysis and statistics

Clustering was performed according to the single-linkage method and weighted pair group method (WPGMA) using Pearson's correlation coefficient (r) as distance measurement using Gene Expression Pattern Suit 4.0 (GEPAS, http://www.gepas.org). The data were $\log_2$ transformed and standardized against the first stage, 8C. Statistical analysis was done using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). A significant difference in gene expression during embryonic development was analyzed by one-way ANOVA ($p < 0.05$). When significant differences were identified, a supplementary Tukey's post-hoc test was performed to investigate differences between developmental stages ($p < 0.05$).

Table 2.1 List of selected genes, with significant BLASTX results, used for gene expression quantification of maternal library from Atlantic halibut.
The name, symbol, BLASTX result with species, and E-value, GenBank accession number, and function of each gene are shown.

Gene name (***Gene symbol***)	**BLASTX results (*Species*)**	**E-value**	**Accession number**	**Function**	**References**
Askopos (*Kop*)	Askopos (*D. rerio*)	6e-14	Q5YCX2	Primordial germ cells	Blaser et al., 2005
Si:dkey-30j22.9	*Si:dkey-30j22.9* (*D. rerio*)	6e-37	XM_688932.3	Uncharacterized	Travis Thomson, 2004
Betaine aldehyde dehydrogenase (*Bah*)	Betaine aldehyde dehydrogenase (*O.latipes*)	2e-32	NM_001104848.1	Metabolism	Wang et al., 2007
Checkpoint 1(*Chk1*)	CHK1 checkpoint homolog (*X.tropicalis*)	3e-17	CR848200.2	Mitosis	Kappas et al., 2000
Prohibitin 2 (*Phb2*)	Prohibitin-2 (*S.salar*)	5e-30	NM_001141404.1	Transcription regulation	Bonnet et al., 2007
Syntaxin 4 (*Stx4*)	Syntaxin 4 (*L. japonicus*)	1e-38	EF513752.1	Transport	Wyman et al., 2003
18K hypothetical goldfish protein (*18k Gold*)	Hypothetical 18K protein goldfish mitochondrion (*O. australis*)	2e-17	JC1348	Uncharacterized	Tingaud-Sequeira et al., 2009
Ubiquitin carrier protein (*Hr6a*)	Predicted similar to ubiquitin-conjugating enzyme HR6A (*O. anatinus*)	1e-31	XM_001511341.1	DNA damage	Roest et al., 2004
Tudor 5 protein (*Tdrd5*)	Tdrd5 protein (*D. rerio*)	1e-12	BC134985.1	Primordial germ cells and abdominal segmentation	Boswell & Mahowald, 1985 ; Travis Thomson, 2004

Table 2.2 List of selected genes, with conserved domain hits, used for gene expression quantification of maternal library from Atlantic halibut.
The name, symbol, conserved domain search results, and E-value, and function of each gene are shown.

Gene name	Conserved domain search results	E-value	Function	References
HHC00057	Cullin	7e-04	Cell division	Maniatis, 1999
HHC00068	Elongin subunit A	2e-10	Transcription regulation	Gerber et al., 2004
HHC00130	Stathmin family	9e-16	Cytoskeleton	Koppel et al., 1999
HHC00222	SET domain	1e-12	Methyltransferase	Sun et al., 2008
HHC00255	Phosphoinositide-dependent kinase 1	5e-50	Cytokinesis	Belham et al., 1999
HHC00309	JmjC domain	2e-28	Histone modification	Takeuchi et al., 2006
HHC00334	CDC45-like protein	4e-60	DNA replication	Yoshida et al., 2001
HHC01010	Dynamin family	1e-05	GTPases	Seugnet et al., 1997
HHC01032	SET domain.	2e-21	Methyltransferase	Sun et al., 2008
HHC01194	Geminin	2e-10	DNA replication	Kroll, 2007
HHC01310	Tetratricopeptide repeat domain	2e-4	Protein binding	Schlegel et al., 2007

Table 2.3 Primer information of selected genes, with significant BLASTX results.
Name, forward and reverse sequences, size, efficiency (%) ,and R^2 are shown.

Name	Forward	Reverse	Size (bp)	E (%)	R^2
Kop	TCTGGTAGTTCCTGCGTGTGAG	GCTCTTCAACCTCATCACCCA	55	104	0.998
Sidkey-30j22.9	GCAAGGTGTCACTCAAGGCAC	GTACTTCAGACCTGTGGAGGGTT	95	102	0.999
Bah	GTATCCACCAAACGGCACTTC	GCAGGTACTCAGGCGAGCC	50	104	0.999
Chk1	GGCAGGTACTCATTCCAATTACAG	GAAACGGCTACCACATCCAAG	83	100	0.998
Phb22	GGAAGGACTACGACGAGCGAG	GGGACACCTGTGCTCTCTGTG	69	99	0.999
Stx4	GATGATGAAAATGAGGACAAAGC	CCCATCCTCCTCTGACTTCTTG	252	100	0.998
18k Gold	AGTTACTTCTTCTCCCGCAAGC	GATCCAACATCGAGGTCGTAAAC	122	93	0.999
Hr6a	TATGTTTGGACATCCTACAGAATCG	CGGACTGTTGGGATTTGGTTC	58	98	0.999
Tdrd5	CTGTCACTCTGAGGGCTTTATCC	TCTGCTGGATGTGGCTCCTC	88	100	0.997
HHC01032	CCGCATTGATGACTTTGATGTG	CTGGACTCATAGTGGCTAATTCACC	143	99	0.996

Table 2.4 Primer information of selected genes, with conserved domain hits.
Name, forward and reverse sequences, size, efficiency (%), and R^2 are shown.

Name	Forward	Reverse	Size (bp)	E (%)	R^2
HHC00057	CAGGTCGTCTGTTTTGCCATTC	CATAAAGAAGGTGGAAGCCAGG	146	92	0.999
HHC00068	ACATCTCCTCCCACGATTCA	TTGAGGAGTGCAACCCAATC	114	98	0.998
HHC00130	GGAGGGATCTTTGGTTTCTTTG	CAACAAGGAGAACCGCACAG	67	99	0.998
HHC00222	TACTGTGTAGATGCCACGAAAGAG	CCGTTGATGTCGTGGAGTTTG	96	97	0.999
HHC00255	ATAATACATCCCAAAGCCCAGAG	CACAATAAGGGGATAATACACAGAGA	198	104	0.999
HHC00309	GGACGGGGAGATTAGAGTCATC	GAGCCCAAGTCCTGGTATGCC	93	105	0.998
HHC00334	CCATGAGGTAGCAGTAGAGGAAGG	GCTGGTATTGTCCTGGCGAAG	115	95	0.997
HHC01010	GAAGAGAGGAAGAACATAAAGACGG	CATCCCTGAGTAGAGCACACTTG	149	93	0.999
HHC01015	CCACTAGAAGTGTGTGCAAGATC	CGTTTCCAGGTTTTTTGAATCC	85	97	0.998
HHC01194	ACTAAGACCCAGCCAGCAGAAG	GGTGGAGGGAGGAGTTTCTTTG	179	99	0.999
HHC01310	TATGAGGAAGCGGTGGTTTG	GAGCCTGCCCAACCTTATCAT	75	98	0.998

Table 2.5 Primer information of reference genes.
Name, GenBank accession number, forward and reverse sequence, size, efficiency (%), and R^2 are shown for each reference gene.

Gene name (Abbrevation)	**Accession number**	**Forward**	**Reverse**	**Size (bp)**	**E (%)**	**R^2**	**REferences**
CP2 transcription factor (HHC01138)	NP_989715	CAGTCCTGGCGACCGATGT	CAAGATGGAGATTCGCAACTGT	76	99	0.998	(Acloque et al., 2004)
Exportin 1(HHC00353)	CAAE00000000.1	CGAGGTACTCTCCACTCTCATTCTC	AACCTCAGTTTTTATCCAGGTTCAC	81	98	0.999	(Callanan et al., 2000)
BTB/POZ protein (HHC01517)	NM_001099229	AGCAGGTTCTCCATGTTGAGTG	CTATTTCAAAGCCATGTTCACAGG	143	95	0.998	(Smith et al., 2006)
β2-Tubulin (*Tubb2*)	DT805564	CTACAATGAGGCTTCAGGTGG	TCCCTCTGTGTAGTGACCCTTG	134	96	0.998	(McCurley & Callard, 2008)
β-Actin (*Actb*)	EB103323	GAGAAGATGACTCAGATCATGTTCG	CCAGCCAGGTCCAGACGG	154	91	0.999	(Van Nes et al., 2005)
Elongation factor 2 (*Eef2*)	EB173938	ATGGAGTCATTTGGTTTCACAGC	GAGACCCTTGCGTTTGCG	121	94	0.999	(McCurley & Callard, 2008)

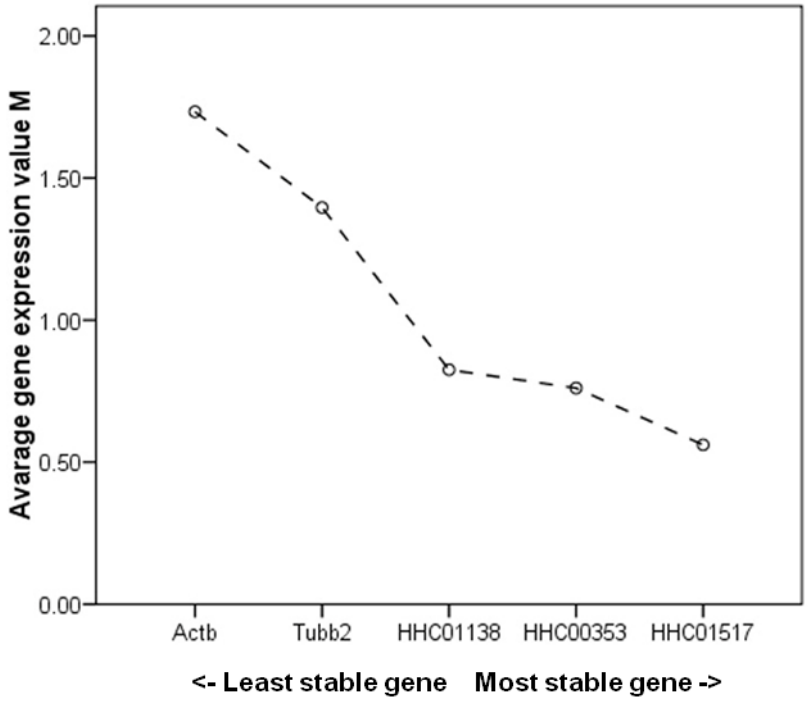

Figure 2.3 Reference gene stability values throughout early Atlantic halibut embryonic development.
Average expression stability values were calculated by geNorm. Expression stability of the reference genes is inversely correlated to their stability index.

2.4 Results

2.4.1 Characterization of EST library

An EST library was constructed by suppressive subtractive hybridization, subtracting cDNA from 8C embryos against the cDNA from 10SS embryos. A total of 4,592 clones were randomly picked and sequenced from their 5' end. After screening for vector and *E. coli* sequences, only ESTs longer than 150 bp were chosen for further analysis. The analysis resulted in 2,341 high quality EST sequences with an average length of 344 bp that were submitted to the EST database dbEST (http://www.ncbi/nlm/nih.gov/dbEST, GeneBank accession numbers FK701051-FK703391) together with their BLAST-based preliminary annotations. Grouping the EST sequences into non-redundant clusters with PartiGene resulted in a total of 1,064 putative gene clusters. The overall redundancy for the maternal library was 2.7, with 77 % of the putative genes being represented by only one EST. Subjecting non-redundant clusters to BLASTX similarity searches against the non-redundant (nr) protein database at NCBI resulted in significant matches for 26.5 % of the clusters (Table 2.6). In addition, 28 % of the cluster had matches against unnamed and hypothetical protein products. The largest gene clusters with significant hits corresponded to structural proteins or metabolic enzymes such as mitochondrial genes encoding cytochrome *b* and cytochrome oxidase subunits and the nuclear genes encoding myosin heavy chain. The remaining 45.5 % of the assembled clusters did not have significant matches against the nr protein database. Annotation in Blast2GO against the Gene Ontology (GO) database resulted in 699 clusters being annotated with a total of 3,956 GO terms at a mean GO level of 5.07. Furthermore, 261 enzyme codes were mapped to 196 sequences. The annotated sequences were grouped into different

classes of ontology according to the GO terms, as shown in Figure 2.4. Most of the genes involved in biological processes were part of metabolic and cellular processes. Half of the annotated genes were classified as genes with the function of binding, followed by the function of catalytic activity. Searching against the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways resulted in the annotation of 169 of the clusters representing 83 different pathways (http://www.genome.jp/kegg/pathway.html). The thirteen most represented pathways are shown in Table 2.7.

Table 2.6 Most commonly abundant EST clones of maternal EST library.
Name, number of sequences, number of clusters and transcript abundance are given for each clone.

Gene name	**Number of sequences**	**Number of clusters**	**Transcript abundance (%)**
Unknown genes			
Genes with no significant hits	1064	395	45.5
Unnamed protein products *T. nigrovirdis*	508	218	21.7
Hypothetical proteins *D. rerio*	134	62	5.7
Hypothetical proteins others (*Gallus*, *Oryzia*, *Xenopus*, *Mus*, *Homo*)	14	10	0.6
Mitochondrial genes			
Cytochrome b	83	3	3.5
Mitochondrial hypothetical 18K protein-goldfish	63	63	2.7
Cytochrome c oxidase subunit I	21	11	0.9
Cytochrome c oxidase subunit II	11	5	0.5
Cytochrome oxidase subunit III	8	3	0.3
Nuclear genes			
Myosin heavy chain	38	10	1.6
skeletal muscle fast troponin T	19	5	0.8
Creatine kinase	19	7	0.8
Parvalbumin	18	2	0.8
Tropomyosin alpha chain	11	2	0.5
Septin 7	10	1	0.4
Caprin family member 2	9	3	0.4
Odorant receptor	9	2	0.4
Skeletal muscle alpha actin 1	8	4	0.3
RNA binding protein with multiple splicing 2	8	2	0.3
Cyclin A2	8	1	0.3

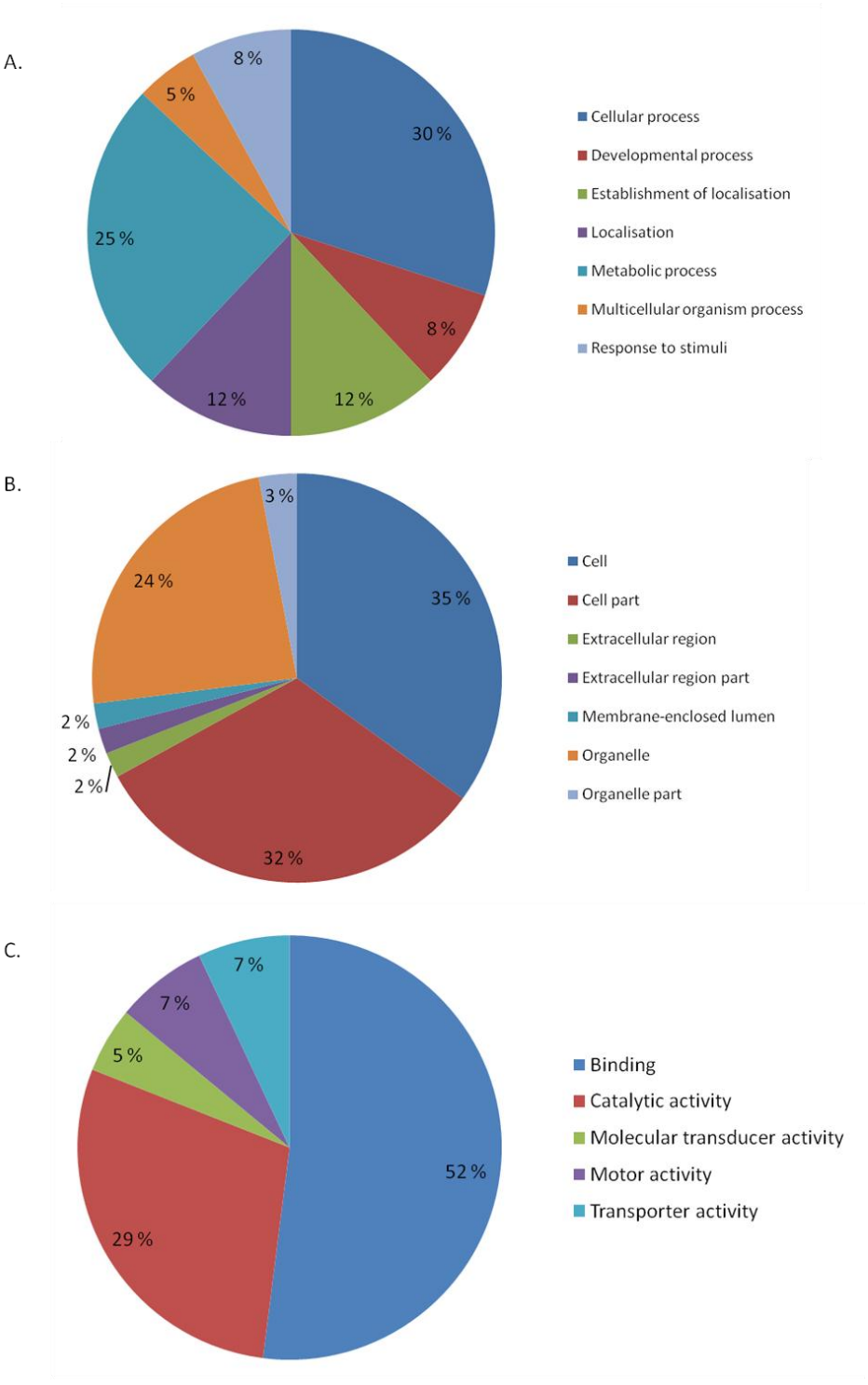

Figure 2.4 Gene classification based on Gene Ontology (GO).
A) Biological process, B) Cellular component, and C) Molecular function

Table 2.7 The most commonly represented KEGG pathways of maternal sequences. Name, number of clusters, and transcript abundance are given for each pathway.

Pathway	**Number of clusters**	**Transcript abundance (%)**
Urea cycle and metabolism of amino groups	8	4.7
Glycan structures	6	3.6
Purine metabolism	6	3.6
Glycolysis/Gluconeogenesis	5	3.0
Drug metabolism-other enzymes	5	3.0
Pyruvate metabolism	5	3.0
Pyrimidine metabolism	4	2.4
Lysine degradation	4	2.4
Tryptophan metabolism	4	2.4
Butanoate metabolism	4	2.4
Pentose phosphate pathway	4	2.4
Carbon fixation	4	2.4
Beta-Alanine metabolism	4	2.4

2.4.2 Screening of relative gene expression during embryonic development

Twenty-one genes were selected from the library for screening of their relative expression during early development, 14 novel genes and 7 genes with documented roles in early development (Table 2.1 and 2.2). Relative gene expression was screened during early embryonic development from 8C to 10SS by quantitative real-time PCR (qPCR). Class discovery analysis resulted in two main clusters containing 15 and 6 genes (Figure 2.5). The relative expressions of the 15 genes, grouped together in the largest cluster, changed significantly during embryonic development ($p < 0.05$). Inside this cluster, the four genes *askopos* (*Kop*), *si:dkey-30j22.9*, *tudor 5 protein* (*Tdrd5*), and HHC00130 (Stathmin family member) were sorted into a sub cluster showing very low to zero expression during the later stages of development. This was confirmed by the significant change in relative expression among the developmental stages (*Kop*: $F_{6,28} = 189.9$, $p < 0.001$; *si:dkey-30j22.9*: $F_{6,28} = 28.9$, $p < 0.001$; *Tdrd5*: $F_{6,28} = 74.5$, $p < 0.001$; HHC00130: $F_{6,28} = 62.1$, $p < 0.001$) as shown in Figure 2.6; B, I, and L. For these four maternal genes, their relative expression decreased significantly to a very low or zero level of expression between the blastula stage (BL) and the germ ring stage (GR). A similar significant drop between these two stages was found for six other genes in this cluster (HHC00068: $F_{6,28} = 4.0$, $p < 0.001$; HHC00309: $F_{6,28} = 8.4$, $p < 0.001$; HHC00334: $F_{6,28} = 3.0$, $p < 0.02$; HHC01010: $F_{6,28} = 1.7$, $p < 0.001$; HHC01032: $F_{6,28} = 13.5$, $p < 0.001$; HHC01310: $F_{6,28} = 14.8$, $p < 0.001$; Figure 2.6 B; K, O, P, Q, S, and U) though these did not decrease to similar low or zero relative expression during later stages. The remaining five genes of this larger cluster did not show any significant difference in gene expression between the blastula and germ ring stage.

The smaller cluster contained genes showing an opposite expression pattern (Figure 2.5). In this group of six genes, lower relative expression during early developmental stages was observed in comparison to the later stages. The expression of the three maternal-zygotic genes *prohibitin 2* (*Phb2*), HHC00057 (orthologue of cullin), and HHC00255 (orthologue of phosphoinositide-dependent kinase 1; Figure 2.6 B; E, J, and N) increased significantly from 8C stage to 10SS stage (*Phb2*: $F_{6,28} = 9.0$, $p < 0.001$; HHC00057: $F_{6,28} = 7.8$, $p < 0.001$; HHC00255: $F_{6,28} = 4.0$, $p < 0.005$). Two of the genes in this cluster did not show any significant differences in relative gene expression during early embryonic development (Figure 2.6 B; *Ubiquitin carrier protein* (*Hr6a*), H and HHC00222, M). Gene expression between the five batches of Atlantic halibut oocytes that were analyzed was found to be significantly different ($p < 0.05$) for all genes except from *Hr6a* and HHC00222.

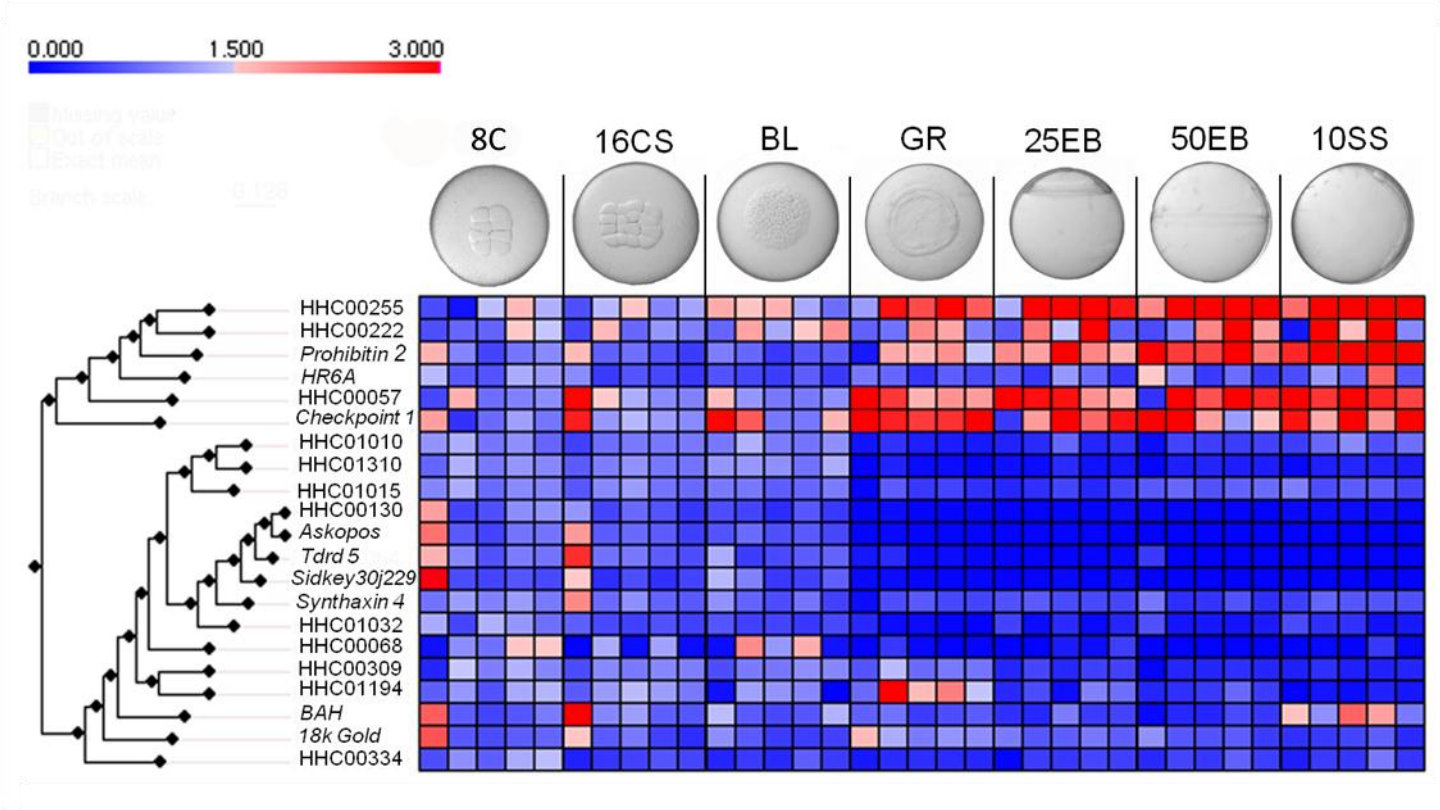

Figure 2.5 Clustering of genes, according to their relative gene expression during early embryonic development.

Genes were clustered using the single-linkage method with Pearson correlation coefficient as distance measurement. The developmental stages were 8-cell stage (8C), 8 hours past fertilisation (hpf); 16 cell stage (16CS), 12 hpf; blastula (BL), 45 hpf; germ ring (GR), 82 hpf; 25 % epiboly (25EP) 96 hpf; 50 % epiboly (50EP), 117 hpf; and 10 somite stage (10SS), 142 hpf (n = 5). Data was standardized against the first stage, 8C. Colour bar indicates relative gene expression in relation to 8C. Red colour shows up-regulation and blue colour down-regulation in relation to 8C. Reference genes are not included.

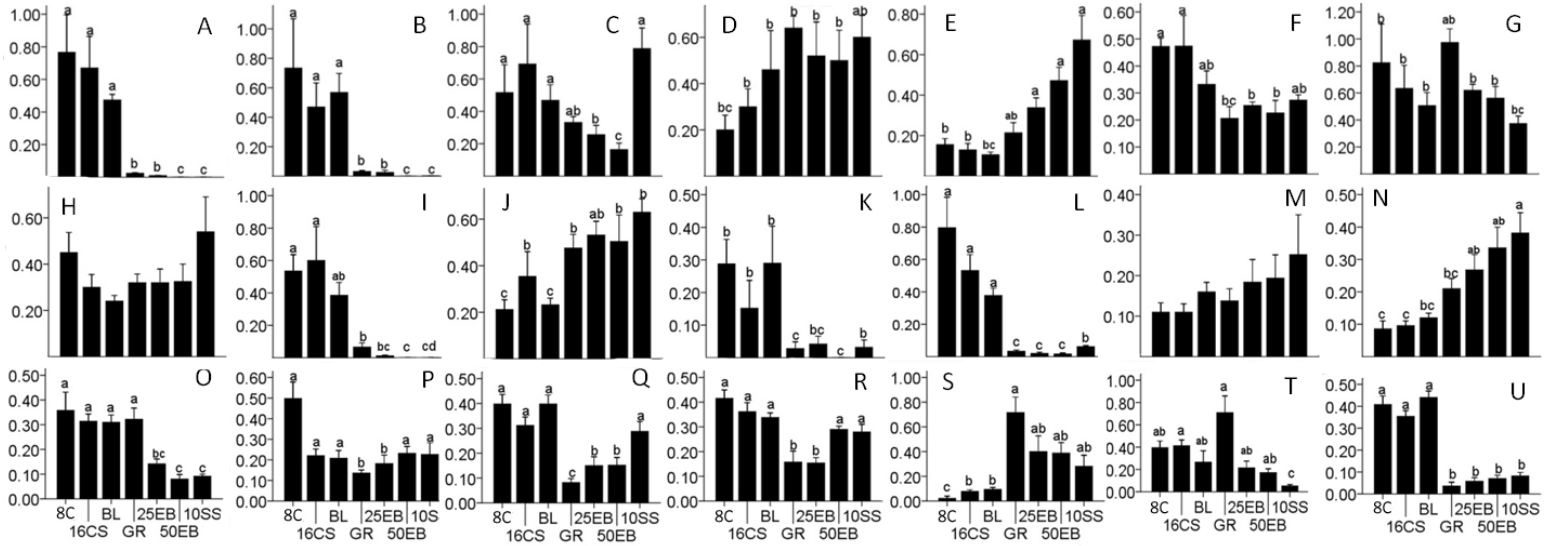

Figure 2.6 Relative gene expressions of selected Atlantic halibut genes from 8C to 10SS stage.
Expression pattern of A: *Askopos*, B: *Si:dkey-30j22.9*, C: *BAH*, D: *Checkpoint 1*, E: *Prohibitin 2*, F: *Syntaxin 4*, G: *18k hypothetical goldfish protein*, H:*HR6A*, I: *Tudor 5 protein*, J: HHC00057, K: HHC00068, L: HHC00130, M: HHC00222,N: HHC00255, O: HHC00309, P: HHC00334,Q: HHC01010,R: HHC01015, S: HHC01032, T: HHC01194, U: HHC01310. Error bars indicate the standard deviation (*n*=5). The developmental stages were 8-cell stage (8C), 8 hours past fertilisation (hpf); 16 cell stage (16CS), 12 hpf; blastula (BL), 45 hpf; germ ring (GR), 82 hpf; 25 % epiboly (25EP) 96 hpf; 50 % epiboly (50EP), 117 hpf; and 10 somite stage (10SS), 142 hpf (*n* = 5).

2.5 Discussion

2.5.1 Characterization of the EST library

1,419 maternal ESTs were previously reported in Atlantic halibut from sequencing an unbiased cDNA library obtained from 2-cell stage embryos (Bai et al., 2007). In the present study we used suppressive subtractive hybridization (SSH) to subtract the transcripts expressed both before and after the switch from maternal to zygotic expression. The analysis resulted in a library containing 2,341EST sequences. Due to the relatively short size of the ESTs, the default cut-off < 10-3, recommended by the software PartiGene, was chosen for the BLASTX analysis (Parkinson et al., 2004). This decreased the stringency of the search, decreasing the possibility that significant matches would be overlooked. The low redundancy of the library of 2.7 suggests that the SSH worked efficiently since 77 % of the putative genes were singletons containing only one EST, representing rare mRNAs. However, the largest gene clusters encoded for common genes such as cytochrome *b* and cytochrome oxidase subunits (Table 2.6). Although SSH greatly enriches for differentially expressed genes the subtracted sample will still contain some cDNAs that correspond to mRNAs common to both the tracer and driver samples, depending somewhat on the quality of RNA purification and the performance of the particular subtraction (Lukyanov et al., 2007). However, it mainly arises when very few mRNA species are differentially expressed in tracer and driver. The number of strictly maternal genes is generally lower compared to maternal-zygotic genes (Mathavan et al., 2005). In general, fewer differentially expressed mRNAs and less quantitative difference in expression lead to higher background – even if one obtains a good enrichment for differentially expressed cDNAs.

Annotated genes in the previous study contained almost twice as many genes classified as involved in metabolic processes compared to our library. In addition, genes involved in developmental processes were almost absent representing only 0.1 % of the total genes compared to this studie's library with 8 % (Figure 2.4) (Bai et al., 2007)

2.5.2 Screening of relative gene expression through embryonic development

Among the 21 selected genes only three showed strictly maternal expression patterns (Figure 2.6). Only annotated genes with known functions during embryonic development were selected, not all of them known to be strictly maternal in other organisms. Choosing randomly picked genes from the EST library may have increased the number of strictly maternal genes. The three genes: *Kop*, *si:dkey-30j22.9*, and *Tdrd5* showed an expression pattern typical for strictly maternal genes (Figure 2.6; A, B, and I). By stage 25 % epiboly (25EB), their expression was no longer detectable anymore, possibly due to the degradation of their transcripts. This could indicate that the MZT in Atlantic halibut takes place between the BL and GR stage. *Kop* mRNA is continuously expressed in the zebrafish primordial germ cells (PGCs) during migration towards the putative gonads (Blaser et al., 2005). *Si:dkey-30j22.9* and *Tdrd5* encode proteins containing several Tudor domains. Tudor domains were identified as common protein motifs found in the *D. melanogaster* Tud protein which plays a dual role in abdomen development and germ cell formation (Boswell & Mahowald, 1985 ; Travis Thomson, 2004). *Si:dkey-30j22.9* encodes an uncharacterized protein found in the zebrafish containing 6 tudor domains. *Tdrd5* has been found to be expressed exclusively in mouse testis, implying that expression of this gene is restricted to the male germ line throughout development to adulthood (Smith et al., 2004). It is unknown how *Kop*, *si:dkey-30j22.9*, and *Tdrd5* may influence embryonic development.

Three genes *Phb2,* HHC00057, and HHC00225 were significantly up-regulated during the later embryonic stages, representing maternal-zygotic genes (Figure 2.6 B; E, J, and N). *Phb2,* together with *prohibitin 1* (*Phb1*), codes for highly conserved proteins in eukaryotic cells that are present in multiple cellular compartments. In rainbow trout, *Phb2* mRNA abundance was found to correlate negatively with developmental success (Bonnet et al., 2007). HHC00057 codes for a Cullin protein orthologue which are RING H2 finger proteins that are part of a protein complex which forms the largest known class of ubiquitin ligases, the cullin-RING ubiquitin ligases (CRLs). In zebrafish, CUL2 has been found to be required for normal embryonic development and vasculogenesis (Maeda et al., 2008). HHC00255 codes for an orthologue of 3-phosphoinositide-dependent protein kinase-1 (PDPK1) which mediates the cellular effect of insulin and growth factors by activating a group of kinases (Belham et al., 1999 ; Mora et al., 2004). It also plays a role in cell cycle resumption during oocyte maturation in starfish (Hiraoka et al., 2004). Lawlor et al. showed that PDPK1-deficient mice embryos displayed multiple abnormalities including lack of somites; forebrain and neural crest derived tissues and died after a few days. Mice embryos with reduced PDPK1 activity were 40-50 % smaller than normal animals. The volume of a number of PDPK1-deficient cells was reduced by 35-60 %, but not their cell number, nuclear size or proliferation (Lawlor et al., 2002). How PDPK1 influences teleost embryonic development has not been studied.

The genomic information created in this study provides the base for future studies on maternal genes expression in Atlantic halibut. The identified strictly maternal and maternal-zygotic genes will be tested for their potential as molecular markers for

Atlantic halibut oocyte quality by correlating their transcript abundance to fertilisation and embryonic hatching rates.

Picked only a small number of EST, are they respective? It is expected that the number of true maternal EST is small compared to maternal-zygotic genes. Makes SSH challengeing.

3 Selection of suitable reference genes for quantitative real-time (qPCR) studies of Atlantic halibut development

3.1 Abstract

Gene expression studies are an essential tool to identify the factors that control normal development and growth in teleost embryos and larvae. High embryonic mortality and larval malformations are common during juvenile production for on-growth in aquaculture. Quantitative real-time PCR (qPCR) is the most accurate method of quantifying gene expression, provided that suitable endogenous controls are used to normalize the data. To date, no reference genes have been validated for developmental gene expression studies in Atlantic halibut. In this study the expression profiles of 6 commonly used reference genes were determined (*Actb*, *Eef2*, *Fau*, *Gapdh*, *Tubb2*, and *18S rRNA*) in 6 embryonic and 5 larval stages of Atlantic halibut development. There were significant changes in expression levels throughout development, which stress the importance and complexity of finding appropriate reference genes. The three software applications (*BestKeeper*, *geNorm*, and *NormFinder*) used to evaluate the stability of potential reference genes produced comparable results. *Tubb2* and *Actb* were the most stable genes across the different developmental stages, whereas *18S rRNA* and *Gapdh* were the most variable genes and thus inappropriate to use as reference genes. According to *geNorm* and *NormFinder*, the best two-gene normalization factors corresponded to the geometric average of *Tubb2*/*Actb* and *Tbb2*/*Fau*, respectively. Either of these normalization factors can be used for future developmental gene expression studies in Atlantic halibut.

3.2 Introduction

Gene expression studies are an essential tool to identify the factors that control normal early development and its complex molecular pathways. Microarrays and quantitative real-time PCR (qPCR) are the methods of choice for quantification of gene expression. In particular, qPCR is commonly used in human diagnostics and expression studies in various biological systems (Bustin et al., 2005). The accuracy of qPCR results depends on several factors, including the RNA integrity of the starting material, enzyme and primer performance, reference gene used, and method chosen for data analysis (Pfaffl & Hageleit, 2001 ; Bustin & Nolan, 2004 ; Skern et al., 2005). Relative quantification of gene expression by qPCR is based on the expression ratio of a target gene *versus* a reference gene (Pfaffl, 2001). A vast number of reference genes have been proposed as references for gene expression analysis (Warrington et al., 2000 ; Olsvik et al., 2005 ; Ingerslev et al., 2006 ; Tang et al., 2007). Reference genes are typically genes that are transcribed at a relatively constant level across various conditions, such as developmental stage or tissue type, and their expression is assumed to be unaffected by experimental parameters. It is unlikely that an ideal universal reference gene exists. Many studies have used reference genes without proper validation of their presumed stability of expression, even though transcript levels of reference genes can vary considerably. For example, two of the most commonly used reference genes in qPCR studies, *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) and *β-actin* (*Actb*), have been found to vary in expression by 8-fold and 7- to 22-fold, respectively, during a survey of 535 human reference genes (Warrington et al., 2000). The use of a single reference gene has been shown to lead to erroneous normalization of up to 3- and 6-fold in expression studies in various human tissues (Vandesompele et al., 2002). Hence, the

use of only one reference gene for normalization of gene expression studies should not be considered sufficient (Bustin et al., 2005). The choice of suitable reference genes for normalization of qPCR data during development is by no means trivial, since they must be relatively unaffected by marked changes in transcriptional activity, particularly before and after the start of zygotic transcription. Nevertheless, this task can be achieved by computational methods recently developed to assess the expression stability of candidate reference genes, namely *geNorm* (Vandesompele et al., 2002), *NormFinder* (Andersen et al., 2004), and *BestKeeper* (Pfaffl et al., 2004).

The Atlantic halibut is considered a valuable candidate for commercial marine cold water fish farming but the current production outcome suffers from a major bottleneck during larval production. During this early production stage, the major problems are high embryonic and larval mortality (Pittman et al., 1990a) as well as body malformations during larval development and metamorphosis (Lewis & Lall, 2006 ; Sæle et al., 2006). The most serious disorder during larval development is termed "gaping" and it is associated with the locking of the jaw cartilage. Hence, larvae are not able to close their mouth and cannot eat, which leads inevitably to death by starvation. The amount of "gapers" in a batch increases at suboptimal rearing temperatures (Lein et al., 1997a) and salinity conditions (Lein et al., 1997b). Gaping has also been correlated with mechanical stress and bacterial invasion (Morrison & Macdonald, 1995) in the rearing tanks. Other skeletal malformations during metamorphosis include scoliosis in the vertebral column (Lewis & Lall, 2006) and arrested eye migration (Sæle et al., 2004). Juveniles with skeletal malformations can represent up to 30% of a total production batch and are routinely discarded during fry production. Despite their significant economical importance, the molecular basis of such abnormalities is not

known. Gene expression studies, particularly qPCR, are essential to improve the understanding of this as well as other fundamental issues regarding aquaculture species. In fact, some recent studies have been conducted to investigate gene expression changes during development of Atlantic halibut, albeit using a single reference gene. *Actb* has been used as the reference gene in expression studies on aromatases (Van Nes et al., 2005), estrogen receptors (Van Nes & Andersen, 2006), and myogenic regulation factors (Galloway et al., 2006) in Atlantic halibut embryos and larvae. In spite of being a suitable reference gene in cattle (Robinson et al., 2007) and zebrafish (Tang et al., 2007), *Actb* transcript levels have been shown to vary as a response to biochemical stimuli, as well as during growth and differentiation in other mammalian systems (Ruan & Lai, 2007). *18S ribosomal RNA* (*18S rRNA*) has been used during expression studies of *insulin growth factor receptors* (Hildahl et al., 2007a) and *type I keratin* genes (Campinho et al., 2007) in Atlantic halibut. The use of *18S rRNA* should be considered carefully in studies where the target genes are expected to show low expression levels. In cattle, *18S rRNA* has been considered unsuitable because of the vast difference in abundance between rRNA and target genes (Robinson et al., 2007), which results in different amplification kinetics that may generate misleading quantification data (Bustin et al., 2005).

The aim of this work was to identify optimal reference genes for qPCR studies of early embryonic and larval development in Atlantic halibut. No systematic survey of appropriate qPCR references for such developmental stages has been conducted to date in any aquaculture species. The selected genes comprised some 'classical' reference genes used as qPCR references (*Actb*, *18S rRNA*, and *Gapdh*) and three genes that have recently been acknowledged as potential reference genes for qPCR: *40S ribosomal*

protein S30 (*Fau*), *eukaryotic translation elongation factor 2* (*Eef2*), and *β2-tubulin* (*Tubb2*). The identification of the most suitable genes for normalization of qPCR data will be an essential tool for future developmental gene expression studies in Atlantic halibut.

3.3 Material and Methods

3.3.1 Fish husbandry and sample collection

The Atlantic halibut used in this experiment were sampled between May 18th and July 23rd at Risørfisk AS hatchery (Risør, Norway). Oocytes were obtained by stripping the females and fertilised *in vitro* with pooled sperm from two males. Eggs and larvae were reared at 6.2 ± 0.1 °C (range). The developmental stages examined were the following: two-cell (2C), high blastula (HB), germ ring (GR), 10-somite (10SS), 30-somite (30SS), 50-somite (50SS), hatching/ larval stage 1 (LS1), larval stage 2 (LS2), larval stage 3 (LS3), larval stage 4 (LS4), and first feeding (FF; Figure 3.1). A total of 5 batches were followed from fertilisation to first feeding and approximately 50 embryos and larvae at each developmental stage were sampled from each of the batches. Samples were wrapped in tinfoil and snap-frozen in liquid nitrogen until analysis.

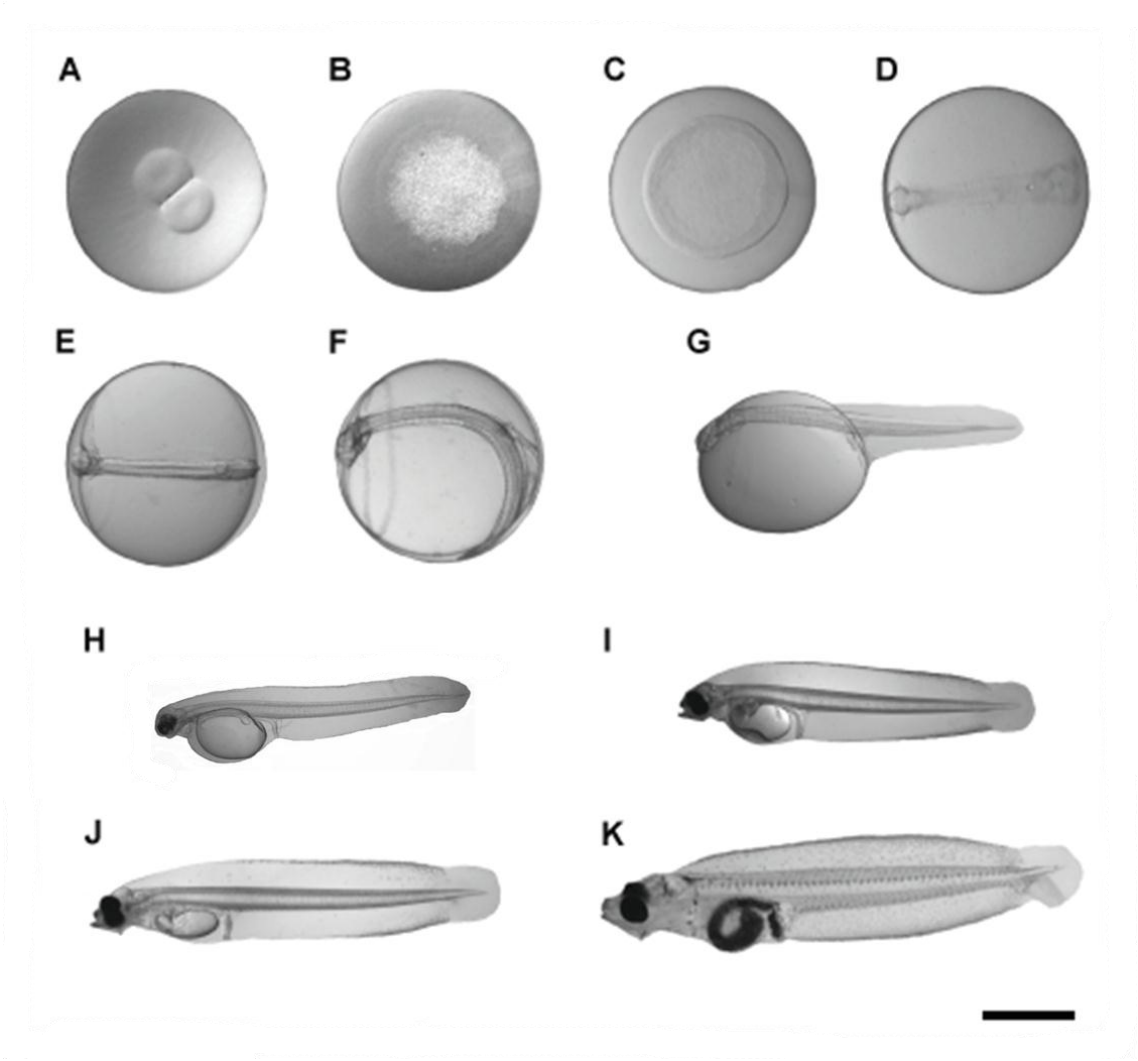

Figure 3.1 Overview of Atlantic halibut developmental stages.
Embryonic stages (A-F) were 2-cell stage (A) at 2 degreedays post-fertilisation (ddpf), high blastula (B) during blastoderm formation at 10 ddpf, germ ring (C) visible at start of gastrulation at 20 ddpf, tail bud/ 10 somite-stage (D) at 30 ddpf, 30-somite stage (E) at 50 ddpf and end of segmentation (F), with the Kupffer's vesicle present at 65 ddpf. Photographs were taken under a Stemi SV 11 stereoscope (Carl Zeiss Vision, City, Norway) with an AxioCam HRc camera (Carl Zeiss Vision, City, Norway). Scale bar represents 5mm. Larval stages (G-K), defined according to Pittman et al. (Pittman et al. 1990), were the following: larval stage 1 (G) at 0 degreedays post-hatching (ddph), larval stage 2 (H) at 130 ddph, larval stage 3 (I) at 160 ddph, larval stage 4 (J) at 200 ddph, and onset of first feeding (K) at 220 ddph. Images were acquired with an Olympus SZXIZ stereoscope coupled to a ColourView IIIu camera (Olympus, Oslo, Norway). Anterior is left. Scale bar corresponds to 1 mm (A-H) or 2 mm (I-K).

3.3.2 RNA extraction and cDNA synthesis

Circa 100 mg of Atlantic halibut embryos (10-15 eggs) or larvae (4-5) were placed into a Lysing Matrix D tube (QBiogene/Medinor, Oslo, Norway) containing QIAzol (Qiagen, Nydalen, Sweden) and homogenized for 40 s at 6000 rpm using the MagNA Lyser instrument (Roche, Mannheim, Germany), according to the Tri reagent method (Sigma, Oslo, Norway). Total RNA was treated with the gDNA wipeout buffer supplied with the QuantiTect reverse transcription kit (Qiagen) to remove traces of genomic DNA contamination. Assessment of RNA quality was performed on a 1.2 % (w/v) agarose gel containing SYBR safe DNA gel stain (Invitrogen/VWR, Tromsø, Norway) and photographed with the Gel logic 200 imaging system (Kodiac/Perderson & Sønn, Oslo, Norway). RNA samples were then quantified using a Nanodrop spectrophotometer (Nanodrop Technologies/Saven Werner, Kristiansand, Norway). All samples had absorbance ratios 260/280 nm greater than 1.9, indicative of high purity RNA. cDNA was synthesized with the QuantiTect reverse transcription kit (Qiagen) using a 96 well PCR from Techne (Barloworld Ltd, Stone, USA). The resulting single-stranded cDNA products were quantified using the Nanodrop spectrophotometer and then diluted 50-fold with deionized water, prior to using them as templates for the qPCR reactions.

3.3.3 Primer design

The primers for *Gapdh*, *Actb*, *Eef2*, *Fau*, and *Tubb2* were based on high-quality expressed sequence tags (ESTs) derived from three Atlantic halibut cDNA libraries (Bai et al., 2007). BLAST similarity searches at the *National Center for Biotechnology Information* (NCBI, http://www.ncbi.nlm.nih.gov/BLAST) were performed to confirm

the identity of the genes. It is noteworthy that the halibut *Actb* gene used in this study is the orthologue of zebrafish *β-actin 1* (also known as *bactin1*), which is located on chromosome 1 of the zebrafish genome. The genomic sequences of orthologous genes from *D, rerio*, *G. aculeatus*, *O. latipes*, *T. rubripes,* and *T. nigroviridis* were retrieved from *Ensembl* (http://www.ensembl.org). Intron/exon borders within the Atlantic halibut EST sequences were then predicted using the *Spidey* software (http://www.ncbi.nlm.nih.gov/spidey) by aligning the halibut ESTs with the corresponding genomic sequences from the other teleost species. Whenever possible, primers were designed across the most conserved splice junctions. All gene specific primers crossed at last one intron/exon border containing both donor and acceptor sites, in order to avoid amplification of any contaminating genomic DNA. Primer pairs for qPCR amplification were designed manually and screened for hairpins, homo- and cross-dimers using *Netprimer* (http://www.premierbiosoft.com/netprimer). The *18S rRNA* primers from Atlantic halibut have been recently reported (Hildahl et al., 2007b). The primer sequences used to amplify all reference genes are listed on Table 3.1, as are the corresponding amplicon sizes and PCR efficiencies.

Table 3.1: Reference gene and primer information.
The name, symbol, GenBank accession number and function of each reference gene are shown.

Gene name	Accession	Function	Fwd sequence (5'→3')	Rev sequence (5'→3')	Size (bp)	E (%)	R^2
18S Ribosomal RNA (*18S rRNA*)	N/A[1]	Small ribosomal subunit	GCATGCCGGAGTCTCGTT	TGCATGGCCGTTCTTAGTTG	140[2]	96.5	0.999
β-Actin (*Actb*)	EB103323	Cytoskeleton	GAGAAGATGACTCAGATCATGTTCG	CCAGCCAGGTCCAGACGG	154	89.0	0.999
Eukaryotic translation elongation factor 2 (*Eef2*)	EB173938	Transport	ATGGAGTCATTTGGTTTCACAGC	GAGACCCTTGCGTTTGCG	121	91.5	0.999
40S Ribosomal protein S30 (*Fau)*	EB102997	Small ribosomal subunit	GACACCCAAGGTTGAAAAGCAG	GGCATTGAAGCATTTAGGAGTTG	149	89.6	0.999
Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)	DN794823	Glycolysis	GCAAGGTCATCCCCGAGC	TGTTTTCATAGCTGGCAGGTTTC	122	91.5	0.999
β2-Tubulin (*Tubb2*)	DT805564	Cytoskeleton	CTACAATGAGGCTTCAGGTGG	TCCCTCTGTGTAGTGACCCTTG	134	93.9	0.998

[1]The 18S rRNA sequence from Atlantic halibut is not publicly available.

[2]Estimated by agarose gel electrophoresis

3.3.4 Quantitative real-time PCR (qPCR)

Gene amplifications by qPCR were performed with a LightCycler® 480 thermocycler (Roche, Oslo, Norway). Each 10 µl reaction in a 96-well plate comprised 4 µl of 50x diluted cDNA template, 1 µl of each primer pair at 5 µM and 5 µl of QuantiTect SYBR Green containing ROX as reference dye (Qiagen). Plates were sealed with adhesive optical film (Roche) and, after an initial denaturation step of 15 min at 95 ºC, 45 cycles of amplification were performed according to the following thermocycling profile: denaturation for 15 s at 94 ºC, annealing for 20 s at 60 ºC and extension for 20 s at 72 ºC. Fluorescence data were acquired during this last step. A dissociation protocol with a gradient from 65 to 97 ºC was used to investigate the specificity of the qPCR reaction and the presence of primer dimers. Gene expression levels were recorded as C_T values that corresponded to the number of cycles at which the fluorescence signal can be detected above a threshold value, arbitrarily set to 0.3. The C_T value is therefore inversely correlated to the initial amount of DNA present in the PCR reaction. All samples were run in duplicate and minus reverse transcriptase and no template controls were included in all plates, along with a positive plate control. Five-point standard curves of a 5-fold dilution series (1:1-1:625) from pooled cDNA were used for PCR efficiency calculation. The PCR efficiency (*E*) is given by the equation $E = (10^{-\frac{1}{m}} - 1) \times 100$ (Radonic et al., 2004), where *m* is the slope of linear regression model fitted over log-transformed data of the input cDNA concentrations *versus* C_T values.

3.3.5 Statistical analysis

Differences in expression levels of *Gapdh*, *Actb*, *Eef2*, *Fau*, *Tubb2*, and *18S rRNA* with developmental stage were examined by one-way ANOVA with Holm-Sidak post-hoc tests. When the data did not meet the normality and/or equal variance requirements, a Kruskal-Wallis one-way ANOVA on ranks with a Dunn's test for post-hoc comparisons was performed instead. The *SigmaStat* statistical package (Systat software, London, UK) was used for all analyses. Significance levels were set at $p < 0.05$.

3.3.6 Evaluation of expression stability

Evaluation of expression stability was performed using three independent statistical applications: *geNorm* (Vandesompele et al., 2002), *NormFinder* (Andersen et al., 2004), and *BestKeeper* (Pfaffl et al., 2004). *GeNorm* (Vandesompele et al., 2002) is a *Microsoft Excel* application that determines the expression stability of reference genes based on overall pairwise comparisons between them. In brief, the principle behind this algorithm is that expression ratio of two ideal reference genes is identical in all samples, regardless of the experimental conditions or cell type. The software calculates a stability value (*M*), which is inversely correlated to gene expression stability, and ranks the reference genes accordingly. Stepwise exclusion of the gene with the highest stability index, assuming that the genes are not co-regulated, results in a combination of two reference genes that have the most stable expression across the tested samples. The identification of stable reference genes with *BestKeeper* (Pfaffl et al., 2004) is based on pair wise comparisons of raw cycle threshold (C_T) values of each gene. The genes showing least variation are incorporated into a *BestKeeper* index, which can be used in very much the same way as a single

reference gene in normalization of gene expression. Stable reference genes show a strong correlation with the *BestKeeper* index. *NormFinder* (Andersen et al., 2004) is also an application for *Excel* but, unlike *GeNorm* and *BestKeeper*, it uses a model-based approach to determine the optimal reference genes. In this mathematical model a separate analysis of the sample subgroups and estimation of both intra- and inter-group variation in expression levels are included into the calculation of a gene stability value. *Normfinder* also calculates the best combination of two genes for a two-gene normalization factor and its corresponding stability value.

3.4 Results

3.4.1 Developmental expression profiles of candidate reference genes

Analysis of the raw expression levels across all eleven developmental stages identified some variation amongst candidate reference genes (Figure 3.2). *Tubb2* showed the lowest global variability, whereas *Gapdh* had the highest variation in expression levels. Except for *18S rRNA*, which was highly expressed (Mean C_T = 10.09), the other candidate reference genes were expressed at moderate levels, with mean C_T values of 18.61, 19.02, 19.51, 20.16, and 22.61 for *Fau*, *Actb*, *Eef2*, *Tubb2*, and *Gapdh*, respectively (Figure 3.2). All genes were expressed throughout the different embryonic and larval stages, but with different expression patterns (Figure 3.3). Apart from *18S rRNA* ($p > 0.05$), all other candidate reference genes showed significant overall changes in expression with developmental stage ($p < 0.05$). Despite its relatively stable expression throughout development, *18S rRNA* had the highest intra-group variation (Figure 3.3). Expression profiles were generally characterized by low transcript levels (i.e., higher C_T values) at the 2-cell and high blastula stages, gradually increasing levels during gastrulation (GR and 10SS) and reaching a plateau by the end of somitogenesis. From hatching until first feeding, expression remained relatively stable.

3.4.2 Analysis of expression stability

The *BestKeeper* descriptive statistics, based on the raw C_T values for each gene, ranked the candidate reference genes in the following preliminary order, from most to least stable: *Tubb2* > *18S rRNA* > *Eef2* > *Fau* > *Actb* > *Gapdh* (Table 3.2). Generally, the six reference genes correlated well with each other. Particularly strong inter-gene correlations ($p > 0.05$) were found for *Eef2*/*Gapdh* ($r = 0.855$), *Eef2*/*Fau*

(r = 0.921), *Actb*/*Tubb2* (r = 0.903), and *Gapdh*/*Fau* (r = 0.925). The high Pearson's coefficients of determination indicated that these gene pairs had very similar overall expression patterns. The best correlations with the *BestKeeper* index were observed for *Tubb2* (r = 0.950, p = 0.001) and *Actb* (r = 0.937, p = 0.001), thus identifying these two genes as the most reliable references for normalization. On the other hand, *18S rRNA* was found to be the least suitable gene (r = 0.614, p = 0.001). In summary, the *BestKeeper* pairwise analysis sorted the candidate reference genes in the following series of decreasing stability: *Tubb2* > *Actb* > *Gapdh* > *Eef2* > *Fau* > *18S rRNA*.

The stability of gene expression over the developmental time course was also assessed by *geNorm* (Table 3.3). There were differences in the relative stability of gene expression between developmental stages, but *Tubb2* and *Actb* were classified as the best candidates, since they were the most stable genes in 6 (GR, 10SS, 30SS, 50SS, LS1, and LS2) and 4 (2C, HB, LS4, and FF) of the 11 stages, respectively (Table 3.3). *Fau* was rated the worst gene in 5 stages (2-cell, high blastula, 50-somite and larval stages 3 and 4), whereas *Gapdh* and *18S rRNA* were classified as the least stable genes in two stages each (germ ring and 30-somite, and 10-somite and larval stage 1, respectively, Table 3.3). When the data from all stages were combined and analyzed simultaneously, the candidate reference genes were ordered from the most to least stable, as follows: *Tubb2* > *Actb* > *Eef2* > *Fau* > *18S rRNA* > *Gapdh*. Pairwise comparisons performed by *geNorm* to identify the finest combination of genes for calculation of a normalization factor revealed that *Tubb2* and *Actb* were the best pair, with a joint stability value of M = 1.17 (Figure 3.4).

According to *NormFinder*, *Tubb2*, and *Gapdh* were found to be the most and least stable reference genes, with stability indices of 0.633 and 1.667, respectively. The

overall ranking of the candidate reference genes was *Tubb2* > *Eef2* > *Actb* > *Fau* > *18S rRNA* > *Gapdh*. The most stable genes were found to be *Tubb2* (germ ring, 10- and 50-somite and larval stage 1) and *Actb* (2-cell, high blastula and first feeding). The genes that showed the greatest variability were *18S rRNA* (10-somite, larval stages 1 and 3) and *Fau* (2-cell, high blastula, 50-somite and larval stage 4) (Table 3.4). Nevertheless, with a stability index of 0.467, *Fau* and *Tubb2* were selected as the best combination of genes for a two-gene normalization factor (Figure 3.5). The *NormFinder* analysis of individual developmental stages was broadly similar to the corresponding *geNorm* output (Table 3.3 and 3.4).

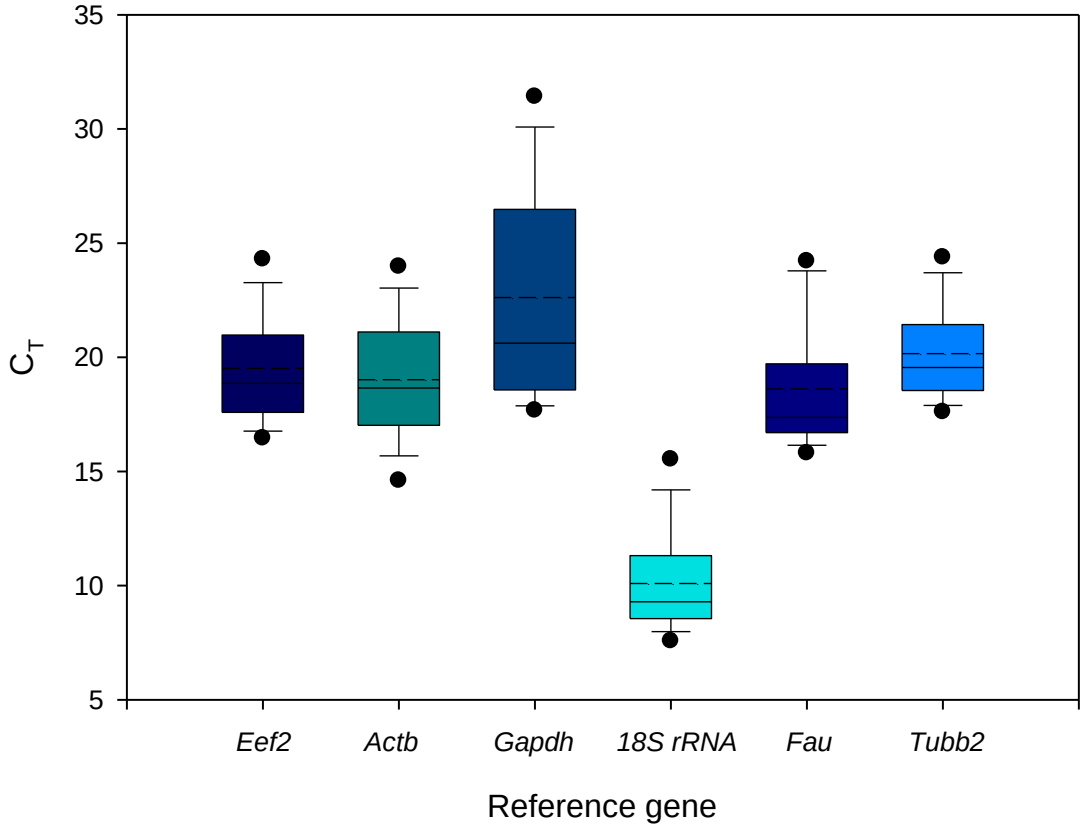

Figure 3.2 Overall expression patterns of potential reference genes during early development of Atlantic halibut.
The raw cycle threshold (C_T) qPCR data of each reference gene in all samples (n = 55) are represented in a box-and-whisker diagram. Mean values are represented by a dashed line. The 5th and 95th percentiles are indicated by the dots below and above each box, respectively.

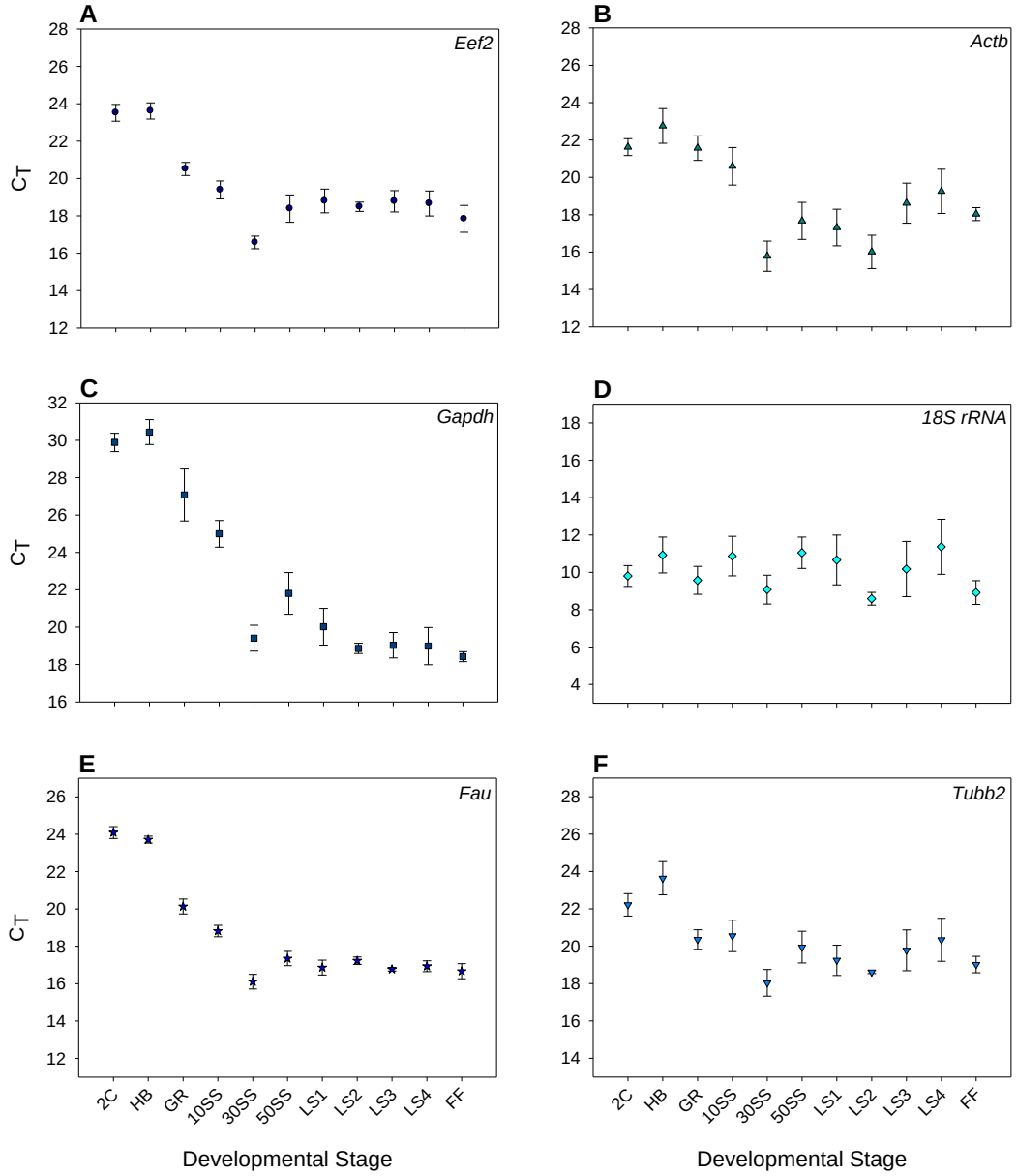

Figure 3.3 Individual developmental expression profiles of potential reference genes. Transcript levels of *Eef2* (A), *Actb* (B), Gapdh (C), *18S rRNA* (D), *Fau* (E), and *Tubb2* (F) during early development of Atlantic halibut were determined by qPCR. Data are shown as raw cycle threshold (C_T) values and represented as mean ± S.E. (n = 5). Abbreviations: 2-cell (2C), high blastula (HB), germ ring (GR), tail bud/ 10-somite (10SS), 30-somite (30SS), 50-somite (SS), larval stage 1 (LS1), larval stage 2 (LS2), larval stage 3 (LS3), larval stage 4 (LS4), and first feeding (FF).

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Table 3.2 Inter-gene relations and correlations between the reference genes and the *BestKeeper* index.
Pairwise correlation analyses were performed based on the cycle threshold values of the six reference genes. Pearson's correlation coefficients (r) are shown. The higher the correlation coefficient, the more stable reference gene. Correlations below the significance threshold ($p > 0.05$) are indicated by an asterisk. The two most significant correlations of reference genes versus the BestKeeper index are highlighted.

	Eef2	*Actb*	*Gapdh*	*18S rRNA*	*Fau*	*Tubb2*
Actb	0.757	-	-	-	-	-
Gapdh	0.855	0.786	-	-	-	-
18S rRNA	0.243*	0.633	0.281*	-	-	-
Fau	0.921	0.697	0.925	0.090*	-	-
Tubb2	0.763	0.903	0.742	0.769	0.664	-
BestKeeper	0.868	0.937	0.900	0.614	0.827	0.950

Table 3.3 Indices of expression stability of six reference genes during early development of Atlantic halibut determined with the *geNorm*.
The stability values are inversely correlated to gene expression stability. The most and least stable reference genes are shaded blue and yellow, respectively. The developmental stages are as follows: 2-cell (2C), high blastula (HB), germ ring (GR), tail bud/ 10-somite (10SS), 30-somite (30SS), 50-somite (SS), larval stage 1 (LS1), larval stage 2 (LS2), larval stage 3 (LS3), larval stage 4 (LS4), and first feeding (FF).

	Eef2	*Actb*	*Gapdh*	*18S*	*Fau*	*Tubb2*
2C	0.620	0.607	0.962	0.787	1.002	0.754
HB	1.120	0.911	1.257	1.043	1.290	0.925
GR	1.449	1.207	2.264	1.310	1.514	1.071
10SS	1.098	1.115	1.137	1.534	1.320	1.026
30SS	1.139	1.089	1.369	1.097	1.055	1.043
50SS	0.920	1.140	1.386	0.991	1.465	0.855
LS1	2.069	1.562	1.735	2.345	1.975	1.458
LS2	0.961	1.850	1.108	1.049	0.877	0.846
LS3	1.787	1.402	1.362	2.216	2.249	1.435
LS4	1.709	1.256	1.315	1.884	1.969	1.311
FF	1.426	0.687	0.737	1.396	0.895	0.775

Table 3.4 Indices of expression stability of six reference genes during early development of Atlantic halibut.
Stability data for reference gene expression were determined with *NormFinder* applications. The stability values are inversely correlated to gene expression stability. The most and least stable reference genes are shaded blue and yellow, respectively. The developmental stages are as follows: 2-cell (2C), high blastula (HB), germ ring (GR), tail bud/ 10-somite (10SS), 30-somite (30SS), 50-somite (SS), larval stage 1 (LS1), larval stage 2 (LS2), larval stage 3 (LS3), larval stage 4 (LS4), and first feeding (FF).

	Eef2	*Actb*	*Gapdh*	*18S*	*Fau*
2C	0.099	0.047	0.581	0.477	0.620
HB	0.539	0.386	0.672	0.609	0.767
GR	0.776	0.446	1.470	0.575	0.814
10SS	0.543	0.475	0.521	0.992	0.804
30SS	0.610	0.651	0.833	0.634	0.486
50SS	0.273	0.531	0.816	0.463	0.891
LS1	1.261	0.559	0.613	1.539	1.157
LS2	0.381	1.236	0.625	0.397	0.026
LS3	0.914	0.360	0.290	1.436	1.431
LS4	0.960	0.321	0.200	1.226	1.248
FF	0.953	0.076	0.760	0.934	0.343

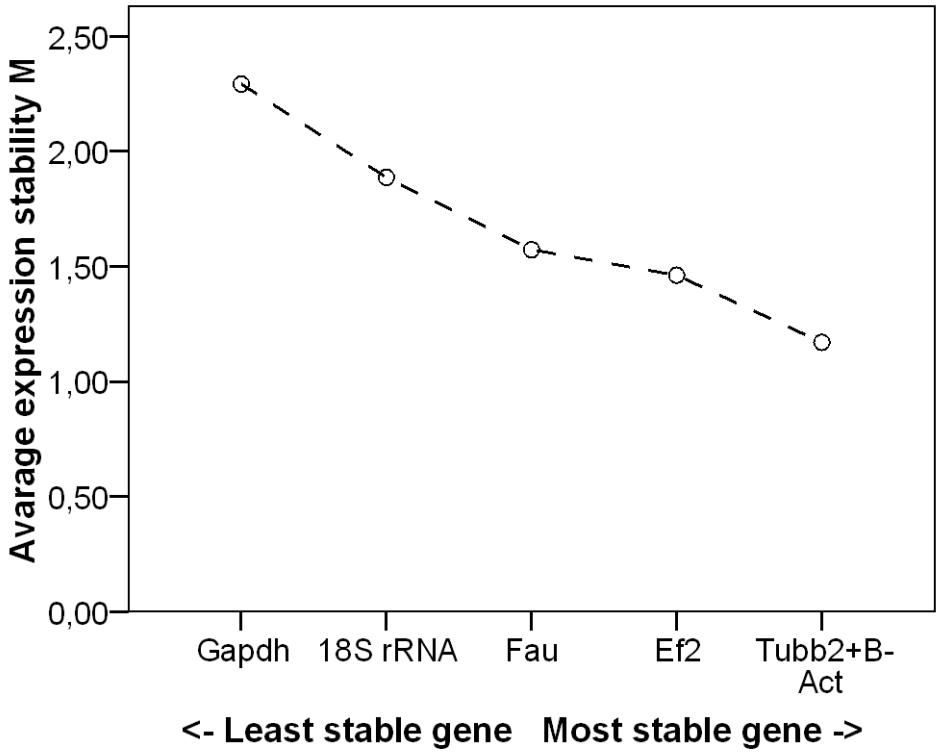

Figure 3.4 Ranking of reference genes according to their expression stability during Atlantic halibut development.
Average expression stability values calculated by *geNorm*.

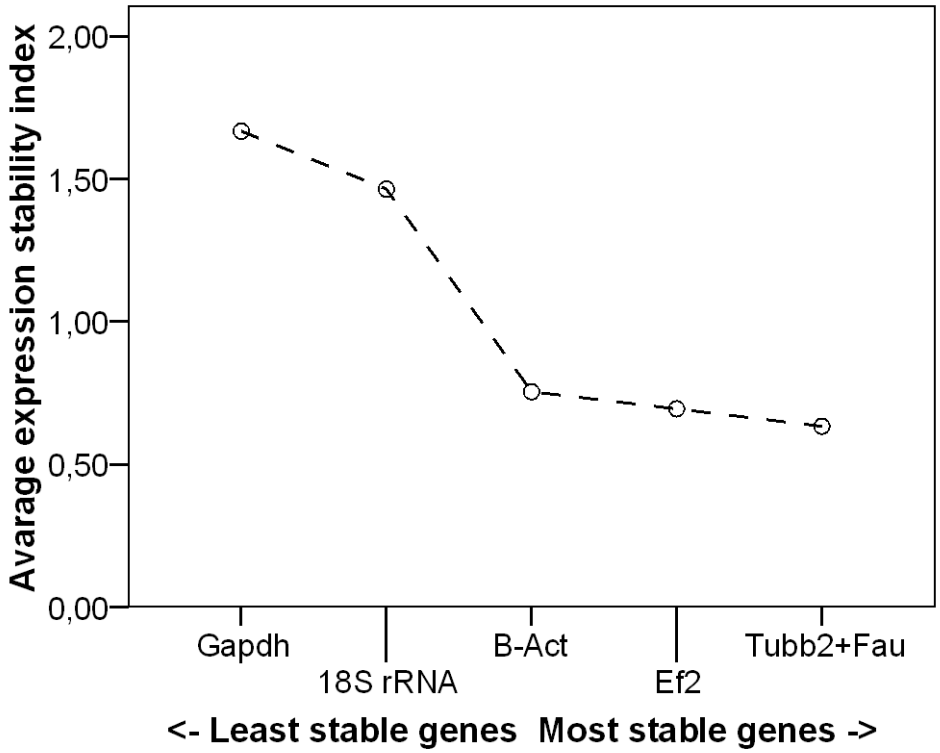

Figure 3.5 Ranking of reference genes according to their expression stability during Atlantic halibut development.
Average expression stability values calculated by *NormFinder*.

3.5 Discussion

Apart from *18S rRNA*, all other candidate reference genes showed significant overall changes in expression throughout the developmental stages (Figure 3.3, D). The observed expression profiles, characterized by lower transcript levels slowly increasing until gastrulation and reaching a plateau by the end of somitogenesis, are likely to correspond to activation of zygotic transcription. This transition from the maternal to the zygotic transcript is known as the midblastula transition (MBT) in zebrafish (Kane & Kimmel, 1993). Since transcriptional activity varies dramatically during development, it is evident that a straightforward statistical analysis of raw C_T values is not suitable to select the best reference genes for normalization of these qPCR data. The largest increase in expression levels throughout development was detected for *Gapdh*. This regulation of *Gapdh* during Atlantic halibut embryogenesis contrasts sharply to the pattern observed in zebrafish. *Gapdh* expression in zebrafish embryos is negligible during gastrulation and somatogenesis, increasing only during the pharyngula period and at hatching (Tang et al., 2007).

The qPCR data set was analyzed with *BestKeeper* (Pfaffl et al., 2004), *geNorm* (Vandesompele et al., 2002), and *Normfinder* (Andersen et al., 2004) since there is no single accepted method to examine gene expression stability. In addition, different statistical methods can potentially yield discrepant results. Validating the reference genes using several applications and identifying differences and similarities between the outputs of alternative software, makes the consensus conclusions more reliable.

BestKeeper defined *18S rRNA* to be the least suitable gene ($r = 0.614$, $p = 0.001$), despite showing a regular mean expression level throughout development (Figure 3.3, D). This apparent discrepancy is due to the different expression pattern

of *18S rRNA* when compared to the other potential reference genes, since the *BestKeeper* (Pfaffl et al., 2004) and *geNorm* (Vandesompele et al., 2002) algorithms assume that the expression ratio of two ideal reference genes is identical in all samples.

There was a statistically significant correlation between *Gapdh* and the *BestKeeper* index ($r = 0.900$, Table 3.2), since *Gapdh* expression levels correlated well with all other reference genes except *18S rRNA*. This result is inconsistent with the *geNorm* or *NormFinder* conclusions. The latter identified *Gapdh* as the least suitable reference gene based on its unstable expression pattern during embryonic development and large intra-group variation, respectively. *Gapdh* seems to be inappropriate for normalization of these qPCR data, in accordance to the marked changes observed in *Gapdh* transcript levels during Atlantic halibut development (Figure 3.3, C). The instability of *Gapdh* has been reported in numerous other systems, including Atlantic salmon smoltification (Olsvik et al., 2005) and zebrafish embryonic development (Tang et al., 2007). The differential regulation of *Gapdh* is probably connected to its direct role in S phase-dependent histone H2B transcription (Zheng et al., 2003). *18S rRNA* was also classified as one of the worst candidate reference genes for developmental studies in Atlantic halibut, despite showing a relatively low variation across all samples (Figure 3.3, D). Since rRNA molecules are the major constituents of total RNA, it is unsurprising that they correlate well with the total RNA mass. However, the use of rRNA as endogenous controls for qPCR has been criticized (Vandesompele et al., 2002), since they do not always represent the mRNA fraction (Solanas et al., 2001) and their high abundance makes it difficult to accurately subtract the baseline value for qPCR data analysis.

The use of a normalization factor based on the geometric average of the best reference genes is generally recommended, since the variation in the average of multiple genes tends to be smaller than the variation in individual genes (Vandesompele et al., 2002 ; Andersen et al., 2004). According to *geNorm*, the best gene pair for calculation of a normalization factor is *Tubb2*/*Actb* (Figure 3.4), whereas *NormFinder* indicates that *Tubb2*/*Fau* are the best genes for computation of the two-gene normalization factor (Figure 3.5). *Fau,* which codes for the ribosomal protein S30 fused to a ubiquitin-like protein (Kas et al., 1992), shows an overall stability similar to *Actb* and less intra-group variation (Figure 3.3, E). As a consequence, the geometric mean of *Tubb2* and *Fau* might be more appropriate, since these two genes belong to distinct functional classes (Table 3.1) and are thus less likely to be co-regulated. *geNorm* tends to select for genes with most similar expression profiles across the whole data set (Vandesompele et al., 2002), making this pairwise method sensitive to co-regulation of reference genes (Andersen et al., 2004).

The general consensus from *BestKeeper*, *geNorm*, and *NormFinder* is that the most adequate reference gene for developmental studies in Atlantic halibut is *Tubb2*, an isoform of the *β2-Tubulin* subunit, which is a major constituent of microtubule polymers (Cleveland & Sullivan, 1985). Microtubule arrays are vital to many developmental processes, including the epibolic movements that occur during gastrulation (Solnica-Krezel & Driever, 1994) and the cohesion of post-cytokinesis blastomeres in zebrafish (Jesuthasan, 1998). *BestKeeper* and *geNorm* analyses classified *Actb* as being the second most stable reference gene in this study. This result is in agreement with a previous report (Tang et al., 2007), which showed that *Actb* is one of the most suitable reference genes for qPCR expression studies during

zebrafish development. However, this paper by Tang *et al.* (2007) did not examine the potential of *tubulin* as a reference gene, since the primers used were not specific enough.

4 Maternal transcripts in Atlantic halibut during embryonic development

4.1 Abstract

A stable production of high quality oocytes is one of the major bottlenecks in present marine teleost aquaculture production. Several markers to estimate oocyte quality during hatchery procedure have been suggested with varying success. Recently, the focus has changed to identify molecular markers to optimise oocyte quality and broodstock husbandry. Atlantic halibut females are routinely hand-striped leading to variable oocyte quality due to post-ovulatory aging. The aim of this study was to relate maternal gene expression to oocyte quality and identify potential markers for oocyte quality in Atlantic halibut. Relative gene expressions of 18 genes at the 8-cell stage were correlated with oocyte quality parameters. The majority of genes showed either no or very minor correlations between their transcript levels and oocyte quality parameters. However, transcript abundances from two uncharacterized genes correlated positively with embryonic hatching success ($r > 0.50$, HHC00353: $r = 0.58$, $p < 0.01$; HHC01517: $r = 0.56$, $p < 0.01$). Transcript abundance from HHC00255 correlated negatively with normal blastomere percentage ($r = -0.62$, $p < 0.05$). Poor embryonic hatching success was not correlated with a general decrease in transcript abundance from maternal genes but with low transcript levels of some specific genes

4.2 Introduction

High quality teleost oocytes can be defined as those that exhibit high fertilisation rates develop into normal embryos experiencing low mortality and high hatching rates. A stable production of high quality oocytes is one of the major bottlenecks in marine aquaculture today. Easy detectable markers for oocyte quality could increase the efficiency of juvenile production by preventing incubation of low quality oocytes. Some of the suggested markers, which are easily applicable on hatchery location, are lipid drop distribution, physical and chemical parameters of the ovarian or coelomic fluid and blastomere symmetry. The distribution of lipid droplets has been suggested as a marker of oocyte quality for brown trout (*Salmo trutta*), Arctic char (*Salvelinus alpinus*), sharpsnout seabream (*Diplodus puntazzo*), common dentex (*Dentex dentex*), and gilthead seabream (*Sparus aurata*; Lahnsteiner & Patarnello, 2005 ; Mansour et al., 2007 ; Lahnsteiner et al., 2008 ; Mansour et al., 2008) . But inconsistent results between the lipid droplet distribution and oocyte quality were found in rainbow trout (*Oncorhynchus mykiss,* Ciereszko et al., 2009). Low pH values of ovarian or coelomic fluid have been associated with reduced oocyte quality due to post-ovulatory aging in turbot (*Scophthalmus maximus*) and rainbow trout (Fauvel et al., 1993 ; Lahnsteiner, 2000 ; Aegerter et al., 2004). Blastomere symmetry at early stages of cleavages has been considered as a useful predictive tool for oocyte quality assessment in aquaculture of marine teleosts (Kjorsvik et al., 1990 ; Brooks et al., 1997). In haddock (*Melanogrammus aeglefinus*), turbot, and Atlantic halibut, a positive correlation between early blastomere symmetry and high oocyte quality characteristics like high embryonic hatching and survival rates were found (Strehlow et al., 1994 ; Kjorsvik et al., 2003 ; Shields et al., 1997 ; Rideout et al., 2004). Studies on yellowtail flounder (*Limanda ferruginea*) and Atlantic cod support

these findings but state that blastomere symmetry corrections during later divisions may occur, improving embryonic hatching and survival rates (Avery & Brown, 2005 ; Avery et al., 2009 ; Vallin & Nissling, 1998). All of these studies look exclusively at the correlation between blastomere symmetry and oocyte quality without explaining the underlying biochemical or genetic mechanisms (Kjorsvik et al., 1990 ; Brooks et al., 1997).

By using newly available genomic and proteomic tools, relations between levels of maternal mRNAs and proteins and oocyte quality have been identified in commercially farmed teleosts (Aegerter et al., 2005 ; Bonnet et al., 2007 ; Crespel et al., 2008). Early embryonic development is controlled by maternal factors, mRNAs and proteins, produced during oogenesis by the female and stored in the oocyte. These factors become activated upon fertilisation and initiate processes crucial to first mitotic divisions, specify initial cell fate, and embryonic patterning (Pelegri, 2003 ; Schier, 2007). This includes the specification of somatic tissue lineages (Pelegri, 2003) and the germline (Raz, 2002). In zebrafish (*Danio rerio*) for example, mutations in strictly maternal genes affect cell adhesion, pronuclear fusion, and spindle formation during early cleavage and the induction of the dorsal organizer (Abdelilah et al., 1994 ; Pelegri et al., 1999 ; Kelly et al., 2000 ; Dekens et al., 2003). .

Atlantic halibut is considered a valuable species for cold water marine fish farming, but current production suffers from a bottleneck during fry production for on-growing. The major problems during the early production stage include high embryonic and larval mortality and body malformations during larval development and after metamorphosis (Kjorsvik et al., 1990 ; Olsen et al., 1999 ; Hamre et al., 2007). Stripping is routinely used in Atlantic halibut juvenile production. Stripping

can lead to variations in oocyte ripeness and consequently, variable oocyte quality (Bromage et al., 1992). Early cell symmetry at the 8-16 cell stage is routinely used in Atlantic halibut farming for oocyte quality assessment (Shields et al., 1997). The information of maternal mRNAs in Atlantic halibut has been restricted to an EST library created from the 2-cell stage embryos (Bai et al., 2007).

The purpose of this study was to relate maternal mRNA levels to oocyte quality in Atlantic halibut and identify potential markers for oocyte quality. Nine previously identified genes (Chapter 2), with three different patterns of relative gene expression during early embryonic development (strictly maternal, maternal-zygotic, and constant), were selected to study differences in their correlation with oocyte quality. To test, if the specific selection of genes influences their correlation of gene expression with oocyte quality, nine separate random genes were chosen from the maternal EST library in addition for the same analysis.

4.1.1 Fish husbandry and sample collection

Oocytes samples were collected from Atlantic halibut females at two different locations in Norway. Four batches were sampled from a commercial farm (Risørfisk AS, Risør, Norway) in 2007 and 25 batches at Bodø University College, Bodø, Norway in 2006, 2008, and 2009 (Table 4.1). All oocytes were fertilised *in vitro* with pooled sperm from two random males. At 8-cell stage, samples (n = 100) from each batch were wrapped in tinfoil and snap-frozen in liquid nitrogen until further analysis. In 2006, some females were kept under natural photoperiod conditions and fed herring for human consumption (winter herring) stuffed with Fish Breed-M (1:1, INVE Aquaculture NV, Dendermonde, Belgium). Other females, kept under photoperiod advanced of approximately 1 month, were fed Fish Breed-M. Eight batches were incubated in large scale incubators (250 l). Relative fertilisation (%) was estimated by incubating egg samples in triplicates (approximately 100 oocytes per replicate) in Petri dishes at temperature between 5.0 -5.4 ºC overnight to estimate relative fertilisation (%) at the 8-cell stage. Relative embryonic hatching (%) was estimated by daily volumetric measurements of dead embryos from incubators from fertilisation until hatching. In 2008 and 2009, all females were kept under natural photoperiod and fed Fish Breed-M. Seventeen batches were incubated in Petri dishes, at 5.5 ± 0.5 °C in 33 ‰ filtered seawater, added Penicillin-Streptomycin-Neomycin solution (5000 Units Penicillin, 5 mg Streptomycin, and 10 mg Neomycin per ml, Sigma, St. Louise, Mo, USA). Relative fertilisation was estimated at 8-cell stage. The water in the Petri dishes was changed after GR stage. To estimate relative hatching, dead embryos were counted and removed every second day until hatching. For 14 batches, blastomere symmetry was estimated at the

8-cell stage (n = 39). Regular blastomere cleavage and abnormalities in blastomeres were estimated according to Shields *et al*. (1997, Figure 4.1).

4.1.2 RNA extraction and cDNA synthesis

Total RNA for all samples were extracted according to the Tri reagent method (Sigma, St-Louise, MO USA) using QIAazol (Qiagen, Nydalen, Sweden). Total RNA was treated with the gDNA wipeout buffer supplied with the QuantiTect reverse transcription kit (Qiagen) to remove traces of genomic DNA contamination. RNA concentration was quantified using the Nanodrop spectrophotometer (Nanodrop Technologies/Saven Werner, Kristiansand, Norway). All samples had absorbance ratios 260/280 nm greater than 1.9, indicative of high purity RNA. cDNA was synthesized with the QuantiTect reverse transcription kit (Qiagen).

4.1.3 Relative gene expression by quantitative-real time PCR (qPCR)

Nine previously identified genes (Chapter 2) with three different patterns of relative gene expression, strictly maternal, maternal-zygotic, and constant, were selected to test if their gene expression was related to Atlantic halibut oocyte quality (Table 4.2).

Their gene expression was estimated in twenty-nine Atlantic halibut batches at the 8-cell stage. In addition, 9 separate random genes from the maternal EST library were chosen for the same analysis (Table 4.3). Whenever possible, primers were designed across the most conserved splice junctions. All gene specific primers crossed at least one intron/exon border containing both donor and acceptor sites, in order to avoid amplification of any contaminating genomic DNA. Primer pairs for qPCR amplification were designed manually and screened for hairpins, homo- and cross-dimers using *Netprimer* (http://www.premierbiosoft.com/netprimer/; (Table 4.4). To

confirm that the right product was amplified, a qPCR was performed on pooled cDNA for each primer pair. The different products were sequenced directly for additional verification. Each sample was checked for genomic DNA contamination by running a qPCR with RNA treated with gDNA whipeout buffer (Qiagen). Gene amplifications by qPCR were performed with a LightCycler® 480 thermocycler (Roche, Basel, Switzerland). Each 10 µl reaction in a 96-well plate comprised 4 µl of 70 x diluted cDNA template, 1 µl of each primer pair at 5 µM and 5 µl of QuantiTect SYBR Green containing ROX as reference dye (Qiagen). After an initial denaturation step of 15 min at 95 ºC, 45 cycles of amplification were performed according to the following thermal cycles: denaturation for 15 s at 94 ºC, annealing for 20 s at 60 ºC and extension for 20 s at 72 ºC. Fluorescence data were acquired during this last step. A dissociation protocol with a gradient from 65 to 97 ºC was used to investigate the specificity of the qPCR reaction and the presence of primer dimers. All samples were run in duplicate along with minus reverse transcriptase, no template and a positive plate controls. Five-point standard curves of a 5-fold dilution series (1:2-1:16) from pooled cDNA were used for PCR efficiency calculation. To assess suitable reference genes for the qPCR studies the known reference genes *elongation factor 2* (*Eef2*), *β2-tubulin* (*Tubb2*), *β-Actin* (*Actb*), HHC01138, HHC1517, and HHC00353 were tested as described in Chapter 2.2.5. *Tubb2* and *Actb* were selected for normalisation (Figure 4.2). According to GeNorm these genes were rather stable and, therefore, expression profiles were normalized assuming a similar quantity of total RNA for all studied stages. Nevertheless, there might be some variation in total RNA levels per embryo during early development.

4.1.4 Data analysis and Statistics

Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Pearson's correlation coefficient was estimated between gene expression and egg batch performance parameters. Fertilisation, embryonic hatching, and blastomere symmetry percentages were *arc sin square roots* transformed before correlation analysis (Zar, 1999). Statistical significance was established at $p < 0.05$.

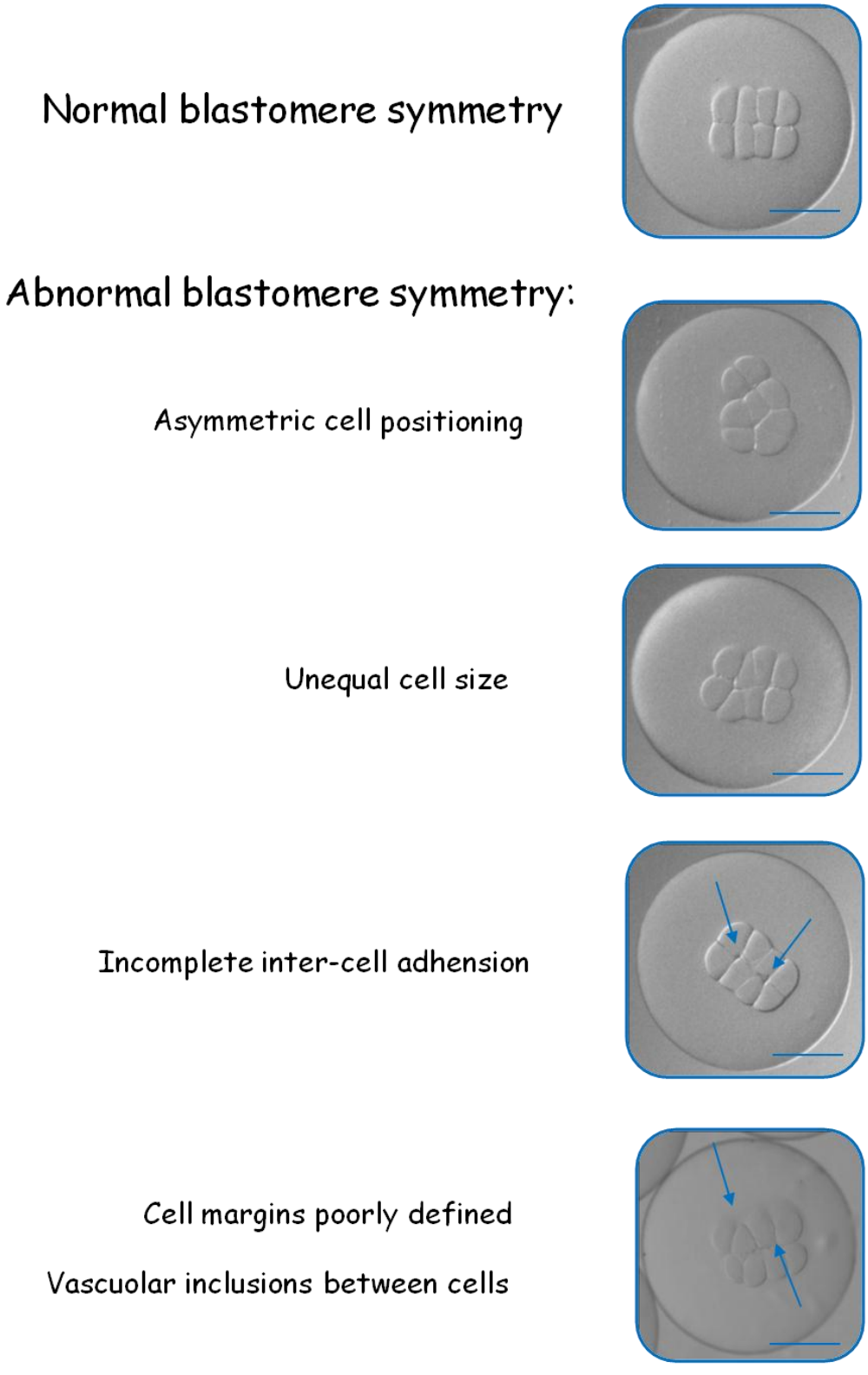

Figure 4.1 Blastomere morphology in 8-cell stage Atlantic halibut embryos. Scale bare: 1mm, adapted after Shields *et al*. (Shields et al., 1997)

Table 4.1 Sample overview.
Samples were collected from fifteen female Atlantic halibut at two locations, Bodø University College (1) and Risørfisk AS (2). For each female, name and weight are given. For each batch, sample year, batch number, incubation method: Small-scale in Petri-dishes (S) or large-scale in 280 l incubators (L) are given. For each batch incubated in small-scale, fertilisation rate (% ± SD, *n* = 3), embryonic hatching rate (% ± SD, *n* = 3), and rate of symmetric blastomeres (% ± SD, *n* = 30) are given. n.a stands for blastomere symmetry not evaluated.

Female	**Location**	**Weight**	**Sample year**	**Batch nr.**	**Incubation**	**Fertilisation (%) ± SD**	**Hatching (%) ± SD**	**Symmetry (%) ± SD**
VF1	1	37	2008	1	S	91 ± 1	88 ± 1	n.a
				2		93 ± 1	86 ± 3	n.a
				3		92 ± 1	37 ± 9	n.a
				4		38 ± 3	26 ± 3	n.a
G39R	1	39	2008	1	S	49 ± 1	20 ± 2	n.a
				2		60 ± 8	36 ± 6	n.a
				3		35 ± 2	23 ± 9	n.a
				4		26 ± 5	15 ± 4	n.a
G39S	1	80	2008	1	S	89 ± 3	43 ± 7	n.a
				2		13 ± 1	1 ± 1	n.a
G32	1	45	2008	1	S	25 ± 2	20 ± 1	n.a

Table continues on next page

Table 4.1 continued

Female	Location	Weight	Sample year	Batch nr.	Incubation	Fertilisation (%) ± SD	Hatching (%) ± SD	Symmetry (%) ± SD
G8	1	83	2008	1	S	84 ± 3	28 ± 4	n.a
R1	2	30-40	2007	1	L	92 ± 2	92	n.a
R2	2		2007	1	L	93 ± 1	90	n.a
R3	2		2007	1	L	68 ± 2	78	n.a
R4	2		2007	1	L	90 ± 3	94	n.a
G39R	1	39	2006	1	L	49 ± 1	33	33 ± 0
Y46	1	67	2006	1	L	90 ± 3	81	57 ± 1
				2	L	90 ± 3	82	33 ± 0
Y32	1	36	2006	1	L	20 ± 2	9	17 ± 0
Y7	1	32	2006	1	L	43 ± 1	31	7 ± 0
O17	1	96	2006	1	L	74 ± 2	63	7 ± 0
				2	L	96 ± 5	89	37 ± 0
Y30	1	95	2006	1	L	43 ± 4	35	3 ± 0
Y46	1		2009	1	S	80 ± 1	45 ± 1	63 ± 0

Table continues on next page

Table 4.1 continued

Female	**Location**	**Weight**	**Sample year**	**Batch nr.**	**Incubation**	**Fertilisation (%) ± SD**	**Hatching (%) ± SD**	**Symmetry (%) ± SD**
G39S	1	80	2009	1	S	68 ± 1	24 ± 2	37 ± 0
G8	1	83	2009	1	S	69 ± 2	45 ± 1	30 ± 0
Y30	1	95	2009	1	S	86 ± 1	48 ± 2	13 ± 0
Y44	1	36	2009	1	S	84 ± 1	36 ± 1	40 ± 0

Table 4.2 List of selected genes used for quantification of gene expression in 29 Atlantic halibut oocyte batches.
The genes have been selected form an initial screening of a maternal EST library. Gene name, BLASTX results, gene accession nr, E-value from BLASTX search, and function.

Name	**Accession**	**E-value**	**Function**
Askopos	Q5YCX2	6e-14	Primordial germ cells
si:dkey-30j22.9	XM_688932.3	6e-37	Uncharacterized protein
Tudor 5 protein	BC134985.1	1e-12	Primordial germ cells and normal abdominal segmentation
Prohibitin 2	NM_001141404.1	5e-30	Transcription regulation
HHC00057		7e-04	Cell division
HHC00130		9e-16	Cytoskeleton
HHC00255		5e-50	Cytokinesis
HHC00353	XP_870795	1e-11	Transport
HHC01517	NM_001099229	8e-14	Protein-protein interaction

Table 4.3 List of random genes used for quantification of gene expression in 29 Atlantic halibut oocyte batches.
The genes have been randomly selected form the same maternal EST library as genes in table 4.1. Gene name, BLASTX results, gene accession nr, E-value from BLASTX search, and function are given.

Name	**BLASTX results (Species)**	**Accession**	**E-value**	**Function**
HHC00005	profilin 2 like protein (*Danio rerio*)	CAN88191	3e-06	Cytoskeleton
HHC00036	unnamed protein product (*Tetraodon nigroviridis*)	CAF92632	7.2	Uncharacterized
HHC00106	creatine kinase 1 (*Paralichthys olivaceus*)	ABU42561	2e-102	Metabolism
HHC00223	RNA binding protein with multiple splicing 2 (*Danio rerio*)	NP_956553	1e-59	RNA binding
HHC00189	similar to Lamina-associated polypeptide 2 isoform alpha (*Danio rerio*)	XP_001921942	3e-16	Cell division
HHC01032	unnamed protein product (*Tetraodon nigroviridis*)	CAG12058	5e-53	Uncharacterized
HHC01306	SH3-domain GRB2-like endophilin B2 (*Danio rerio*)	CAM15470	1e-73	Apoptosis
HHC01385	caprin family member 2 (*Danio rerio*)	NP_001013291	4e-20	RNA translation
HHC01481	hypothetical protein LOC561007 (*Danio rerio*)	NP_001122176	8e-70	Uncharacterized

Table 4.4 Primer information of selected genes.
For each reference gene, primer sequences, amplicon sizes, reaction efficiencies (*E*), and Pearson's coefficients of determination (R^2) are shown.

Name	**Forward**	**Reverse**	**Size (bp)**	***E* (%)**	R^2
Askopos	TCTGGTAGTTCCTGCGTGTGAG	GCTCTTCAACCTCATCACCCA	55	104	0.998
Sidkey-30j22.9	GCAAGGTGTCACTCAAGGCAC	GTACTTCAGACCTGTGGAGGGTT	95	102	0.999
Betaine aldehyde dehydrogenase	GTATCCACCAAACGGCACTTC	GCAGGTACTCAGGCGAGCC	50	104	0.999
Checkpoint 1	GGCAGGTACTCATTCCAATTACAG	GAAACGGCTACCACATCCAAG	83	100	0.998
Prohibitin 2	GGAAGGACTACGACGAGCGAG	GGGACACCTGTGCTCTCTGTG	69	99	0.999
Synthaxin 4	GATGATGAAAATGAGGACAAAGC	CCCATCCTCCTCTGACTTCTTG	252	100	0.998
18K hypothetical goldfish protein	AGTTACTTCTTCTCCCGCAAGC	GATCCAACATCGAGGTCGTAAAC	122	93	0.999
HR6A	TATGTTTGGACATCCTACAGAATCG	CGGACTGTTGGGATTTGGTTC	58	98	0.999
Tudor 5 protein	CTGTCACTCTGAGGGCTTTATCC	TCTGCTGGATGTGGCTCCTC	88	100	0.997
HHC00057	CAGGTCGTCTGTTTTGCCATTC	CATAAAGAAGGTGGAAGCCAGG	146	92	0.999
HHC00068	ACATCTCCTCCCACGATTCA	TTGAGGAGTGCAACCCAATC	114	98	0.998
HHC00130	GGAGGGATCTTTGGTTTCTTTG	CAACAAGGAGAACCGCACAG	67	99	0.998
HHC00222	TACTGTGTAGATGCCACGAAAGAG	CCGTTGATGTCGTGGAGTTTG	96	97	0.999

Table 4.4 continues on next page

Table 4.4 continued

Name	Forward	Reverse	Size (bp)	*E* (%)	R^2
HHC00255	ATAATACATCCCAAAGCCCAGAG	CACAATAAGGGGATAATACACAGAGA	198	104	0.999
HHC00309	GGACGGGGAGATTAGAGTCATC	GAGCCCAAGTCCTGGTATGCC	93	105	0.998
HHC00334	CCATGAGGTAGCAGTAGAGGAAGG	GCTGGTATTGTCCTGGCGAAG	115	95	0.997
HHC01010	GAAGAGAGGAAGAACATAAAGACGG	CATCCCTGAGTAGAGCACACTTG	149	93	0.999
HHC01015	CCACTAGAAGTGTGTGCAAGATC	CGTTTCCAGGTTTTTTGAATCC	85	97	0.998
HHC01032	CCGCATTGATGACTTTGATGTG	CTGGACTCATAGTGGCTAATTCACC	143	99	0.996
HHC01194	ACTAAGACCCAGCCAGCAGAAG	GGTGGAGGGAGGAGTTTCTTTG	179	99	0.999
HHC01310	TATGAGGAAGCGGTGGTTTG	GAGCCTGCCCAACCTTATCAT	75	98	0.998
HHC00005	CCGAGCGGAGGGATTTAGC	CAGCCATCAGGTTGTCCACG	229	99	0.998
HHC00036	TAAACGGCTCTGTTGTCCCAT	GTACACGCTGTCGCTTCCAG	284	92	0.999
HHC00106	AGGCATCTGGCACAATGAGAAC	CTCTCTACTTCTGGGCAGGGAT	462	89	0.999
HHC00189	GGGTGGCTTCATCTCACTTC	GTTTGAACCCGTGTCGGAG	313	95	0.998
HHC00233	AACAGCCTGTCGGGTTTGTAAC	GATGCTGGGATCAGTGCTGC	211	92	0.997
HHC01306	TCGGCTTTTTCCACTTCCTC	GGTGGGGGAATGTGAGAGAAG	262	98	0.999
HHC01032	CTATGATGGCAAGGGTATCGG	TTAGGGCTTACTAGCGAACGGG	297	99	0.998
HHC01385	GAACTACCAGAGGAGAGGGCAATC	CTTACCCAAAGACCCCATCCTG	127	98	0.997
HHC01481	GGGACTCATCTCGTGGGTCT	AGCAGTATTACTTGACCTCGCC	125	98	0.999

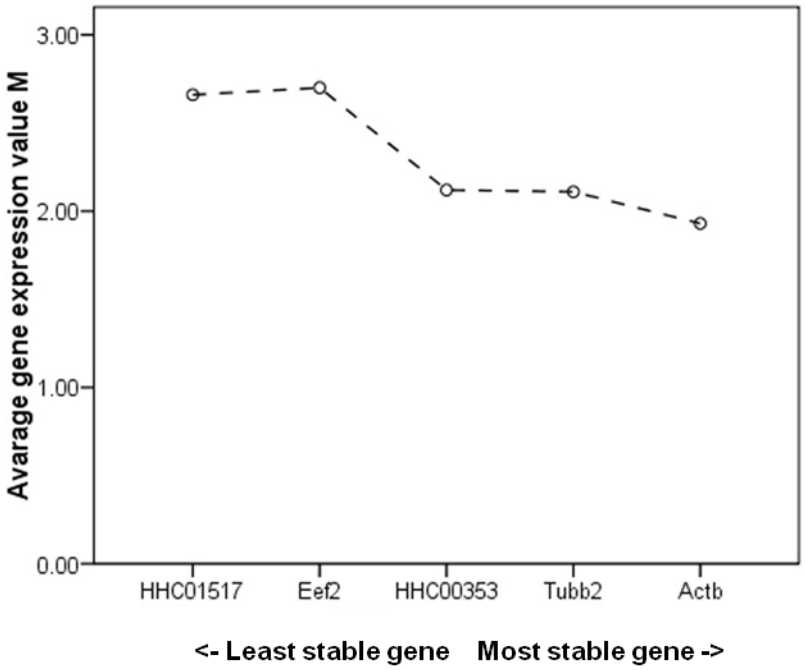

Figure 4.2 Reference gene stability values.
Ranking of reference genes according to their expression in twenty-nine batches of Atlantic halibut oocytes. Average expression stability values were calculated by *geNorm*. Expression stability of the reference genes is inversely correlated to their stability index.

4.2 Results

The relative gene expression of 18 genes was measured in 29 different batches of 8-cell stage Atlantic halibut embryos (Table 4.1). No significant correlations between gene expression and fertilisation rates were found. The expression of seven genes (39 % of total) correlated positively with embryonic hatching rates. Expressions of the two uncharacterized genes HHC00353 and HHC01517 correlated stronger with embryonic hatching ($r > 0.5$, HHC00353: $r = 0.58$, $p < 0.01$ and HHC01517: $r = 0.56$, $p < 0.01$; Figure .4.3, A and B), compared to the other five genes ($r \leq 0.5$, *kop*: $r = 0.38$, $p < 0.05$; *si:dkey-30j22.9*: $r = 0.50$, $p < 0.05$; HHC0057: $r = 0.41$, $p < 0.05$; HHC00130: $r = 0.43$, $p < 0.05$; HHC00255: $r = 0.41$, $p < 0.05$). The expression of HHC00255 negatively correlated with the percentage of symmetric blastomeres ($r = -0.62$, $p < 0.05$; Figure 4.3, C).

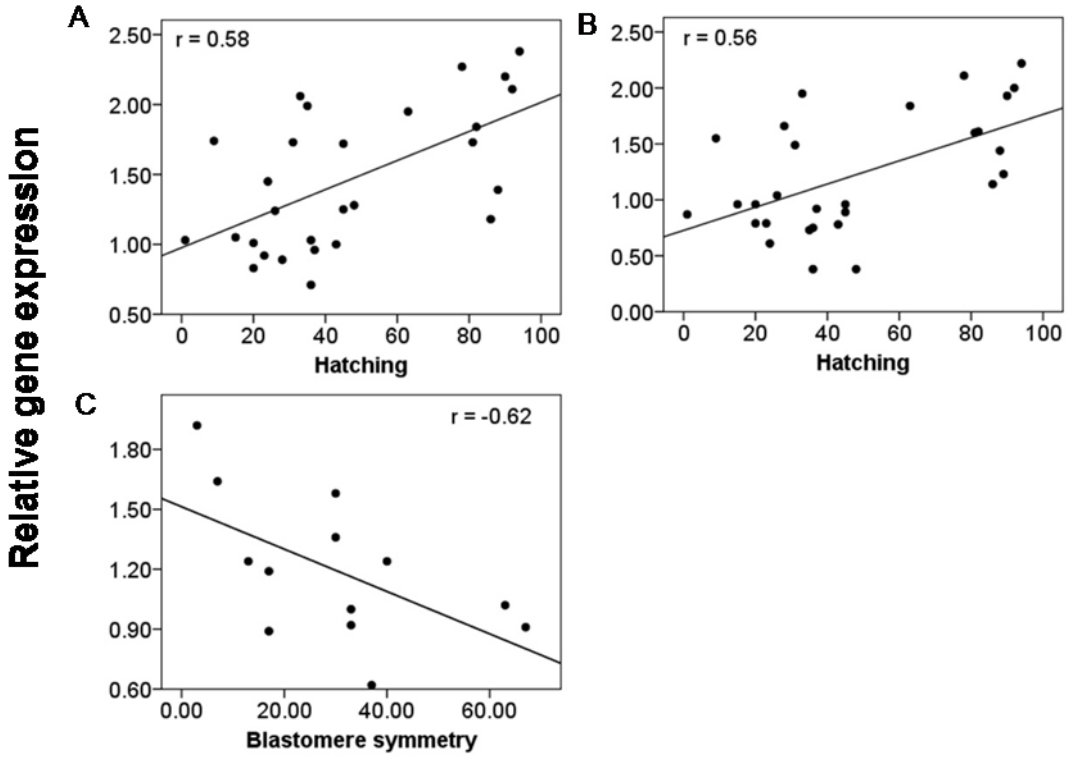

Figure 4.3 Correlations between gene expression and Atlantic halibut oocyte quality ($r \geq 0.5$).
Gene expression in relation to embryonic hatching (%): (A) HHC353, (B) HHC01517, (C) HHC00255, (n = 29). The correlation coefficient (r) is given for each regression line in each plot.

4.3 Discussion

In this study maternal transcript levels were related to embryonic hatching success and normal blastomere symmetry. Acquiring a standardized experimental set-up has been challenging due to different broodstock husbandry and incubation methods at the two locations, as well as the change from industrial to experimental incubation at the facilities at the University of Nordland. Hence the final heterogeneous final material consisted of oocyte batches from different breeders, held under different feeding conditions and photoperiods. Despite the oocyte heterogeneity significant correlations between transcript levels and oocyte quality could be identified. The two uncharacterized genes HHC00353 and HHC01517 correlated stronger with embryonic hatching ($r > 0.5$, $p < 0.01$; Figure 4.3, A and B), compared to five other genes, *kop*, *si:dkey-30j22.9*, HHC00057, HHC00130, and HHC00255 which showed statistically significant but very minor correlations. The expression of HHC00255 negatively correlated with the percentage of symmetric blastomeres ($r > -0.5$, $p < 0.05$; Figure 4.3 C).

HHC00353 codes for an orthologue of an exportin 1-like protein. It is a member of the importin β superfamily of nuclear transport receptors. Exportin 1 (XPO1) is a major receptor for the export of proteins and RNAs out of the nucleus. XPO1 is also implicated in various steps during mitosis (Hutten & Kehlenbach, 2007). In *X. laevis* inhibitions of XPO1 activity was leading to a developmental arrest during neurulation (Callanan et al., 2000). The role of XPO1 in embryonic development in teleosts has not been studied. HHC01517 encodes a protein with Bric-a-brack, Tramtrack and Broad-complex (BTB) domains. In *D. melanogaster*, the maternally expressed gene *pipsueak*

(*psq*) codes for a BTB domain protein (PsqA) which is required for correct abdominal segmentation in embryos but it is unknown if it plays a similar role during embryogenesis in teleosts (Siegel et al., 1993). The relation of *kop*, s*i:dkey-30j22.9*, and HHC00057 to embryonic development has been described in Chapter 2. HHC00130 encodes for an orthologue to the stathmin protein family. In zebrafish, the temporal and spatial expression of two orthologues of *stathmin 2* (*stmn2*) has been described (Burzynski et al., 2009), although it is not known whether *stmn2* has a regulatory role during embryonic development. Expression of HHC00255 was found to be positively correlated to embryonic hatching percentage and negatively correlated with normal blastomere symmetry. This is in contrast to earlier findings during embryonic development in mice, where a reduced expression of PDPK1 was found to influence cell size (Lawlor et al., 2002). However, abnormal blastomere symmetry is defined not only as cells of unequal size, but also by asymmetric cell positioning, incomplete inter-cell adhesion, poorly defined cell margins and vascular inclusions between cells (Shields et al., 1997). HHC00255 high expression levels could be an indicator for suboptimal regulation of pathways involved in growth and/or cell division during early cell division. In several marine teleost species, early blastomere symmetry has been found to correlate with high embryonic hatching and survival rates (Strehlow et al., 1994 ; Kjorsvik et al., 1990 ; Rideout et al., 2004). In contrast, others argued that high embryonic hatching and survival rates are maintained through cell symmetry corrections in consecutive developmental stages (Vallin & Nissling, 1998 ; Avery & Brown, 2005 ; Avery et al., 2009). None of these studies have investigated the molecular mechanisms that regulate early cell divisions. In this study we have, for the first time, found a correlation between gene expression and blastomere symmetry. With 67% being the

highest percentage of oocytes with normal blastomere symmetry, symmetry was generally low in this study. Even though Shields *et al*. (1997) established a score system for blastomere symmetry, the method suffers from its subjectivity. It is unclear if the estimated low blastomere symmetry in this study were true or due to a too strict estimation of symmetry. In this study, only correlations between general abnormal blastomere symmetry was analysed. Distinguishing between different types of abnormal symmetry (i.e asymmetric cell positioning, unequal cell size and incomplete inter-cell adhension; Shields *et al*., 1997) could have provided this study with additional information about gene expression and early cell divisions. *Phb2* has earlier been shown to be differentially expressed in oocytes with low and high developmental potential in rainbow trout (Bonnet et al., 2007). In the present study, expression levels of *phb2* did differ significantly in oocytes with low and high embryonic hatching rates and did not correlate with embryonic hatching success.

Poor embryonic hatching success was not correlated with a general decrease in transcript abundance, but with low transcript levels for specific genes. Similar gene specific relations have been found by Aegerter *et al*. (2003) studying post-ovulatory aging in rainbow trout. Out of the seven studied genes, three genes were found to be down-regulated and four to be up-regulated in oocytes with low larval survival compared to oocytes with high larval survival. In the same study, similar variations in gene expression were found in post-ovulatory oocytes. The mechanisms inducing differential maternal transcript abundances in teleost oocytes are unknown. A possible explanation could be a reduced incorporation of specific maternal mRNAs into the oocyte during oogenesis.

This chapter has been embargoed at the request of the author, in accordance with University regulations.

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7 General discussion

7.1 Atlantic halibut genomic resources

Techniques for the evaluation of gene expression have progressed over the last two decades from methods developed for the analysis of single protein-coding genes (e.g. Northern blotting, semi-quantitative and quantitative reverse-transcription PCR) to techniques focusing on identifying total gene expression (Transcriptome) (e.g. Subtractive hybridisation, sequencing of expressed sequence tags (ESTs), serial analysis of gene expression (SAGE), microarrays, 454-sequencing and lately high through-put deep-sequencing (RNA-seq)). Good quality gene expression studies often rely on established genomic databases which are still rare among commercially farmed teleosts compared to mammals and model organisms (e.g. humans, mice, *Drosophila*, *Xenopus*, and zebrafish). Information on maternal genes in commercially teleosts oocytes has been restricted to rainbow trout (*Oncorhynchus mykiss*) although EST libraries have been created from ovarian tissues for several commercially farmed species (Rexroad et al., 2003 ; Aegerter et al., 2004 ; Aegerter et al., 2005 ; Von Schalburg et al., 2005 ; Govoroun et al., 2006 ; Bonnet et al., 2007 ; Luckenbach et al., 2008). These studies have the disadvantage of including transcripts that may arise from follicle cells (granulosa or theca cells) or interstitial cells and are therefore not oocyte-specific and strictly maternal. In Chapter 2 a maternal SSH library has been created that resulted in 2,341 high quality ESTs increasing the number of maternal Atlantic halibut ESTs available at the NCBI dbEST database by 64 %. In contrast to previous, unbiased sequencing of maternal transcripts in Atlantic halibut embryos, SSH has the advantage that maternal-zygotic transcripts are subtracted and suppressed and strictly maternal

transcripts are amplified (Bai et al., 2007). The maternal EST database produced will serve as a resource for future in-depth studies of maternal gene expression and was used to provide sequence information for gene expression studies in Chapter 3, 4 and 6.

qPCR is considered an accurate, sensitive and fast quantification method of gene expression with multiple applications (i.e. qantification of miRNA, siRNA, SNP, copy number variant, and single cell quantification (Elbashir et al., 2002 ; Benes & Castoldi, 2010 ; D'Haene et al., 2010 ; Erali & Wittwer, 2010 ; Ståhlberg & Bengtsson, 2010). In addition, qPCR is routinely used to validate microarray expression data and has also been used to confirm next generation sequencing results (Bettencourt et al., 2010 ; Raha et al., 2010 ; Aanes et al., 2011 ; Roberts et al., 2011 ; Zhang et al., 2011). To perform reliable gene expression quantification by qPCR, stable reference genes have to be used to normalize target gene expression. To identify one universal reference gene has been unsuccessful and it is recommended to validate several possible reference genes for each new experimental setup (Thellin et al., 2009). In previous Atlantic halibut gene expression studies, single reference genes commonly used in humans and model species, (Actb and 18SrRNA) have been used (Van Nes et al., 2005 ; Galloway et al., 2006 ; Van Nes & Andersen, 2006 ; Hildahl et al., 2007a). In Chapter 3 a systematic survey of commonly used reference genes during embryonic and larvae developmental stages was performed, not previously carried out in a commercially farmed teleost. *Tubb2* and *actb* were identified as the most stable gene-pair across embryonic and larval stages. However, separate testes of reference genes were performed in Chapter 2 and 3, following the general conclusion from chapter 3 that experiment- specific tests of reference genes should be performed for each new study. Since then, suitable reference genes for gene expression studies during embryonic and

larval development have been identified in several commercially farmed teleost (i.e. European seabass (*Dicentrarchus labrax*), Atlantic cod (*Gadus morhua*) and Senegalese sole (*Solea senegalensis*) (Infante et al., 2008 ; Mitter et al., 2009 ; Sæle et al., 2009 ; Skjærven et al., 2011). An additional evaluation of reference genes in twice as many Atlantic halibut embryonic stages as used in the present study ranked *ef1a1* and *ubce* as best reference genes before activation of zygotic transcription (Øvergård et al., 2010) (Øvergård et al., 2010). This emphasizes the need for experiment-specific evaluation of reference genes. The normalization of embryonic gene expression is challenging due to a large increase in cell number and mRNA synthesis in addition to a complex mRNA degradation and activation pattern across the maternal-zygotic transition (MZT). Therefore, an exogenous reference gene (e.g Luciferase) should also be considered, as used in Chapter 6.

In Chapter 4 expression levels were quantified for 30 maternal transcripts in Atlantic halibut 8C embryos by qPCR and revealed that poor embryonic hatching success was not related with a general decrease in maternal transcript abundance, but with low transcript levels of specific genes. As a next step, a 4x44k custom made Atlantic halibut (*Oncorhynchus mykiss*) oligonucleotide microarray was created based on the increased number of maternal EST created in Chapter 2. In contrast to qPCR, microarrays can be used for genome wide studies of gene expression and microarray technology can be expanded to include alternative splicing, SNP detection, array comparative hybridisation genomics (aCGH), differential methylation hybridisation (DMH), and chromatin immunoprecipitation on microarrays (ChIP-on-chip, Mockler & Ecker, 2005). Microarray technology has been successfully used to identify markers for oocyte quality during *in vitro* fertilisation (IVF) in humans and mammals (Gasca et al.,

2007 ; Leoni et al., 2007 ; Bettegowda et al., 2008 ; Thelie et al., 2009 ; Hamel et al., 2010). In rainbow trout, microarray-based differential gene expression in high and low quality oocytes has been performed previously, but not in a commercially farmed marine teleost like Atlantic halibut (Bonnet et al., 2007). The new Atlantic halibut microarray was successfully used to screen maternal gene expression, and to identify differential gene expression in low and high quality oocytes. It has proven to be suitable for future analysis of Atlantic halibut embryonic gene expression which is likely to advance our understanding of important developmental processes such as germ cell development, growth and immune response.

7.2 Maternal effects on oocyte quality

In sexual reproduction, the offspring phenotype (e.g morphology, biochemical and physiological properties, and behavior) is the result of the parental genotype and phenotype (Maestripieri & Mateo, 2009). The maternal phenotype (e.g size, age, and nutritional status) is traditionally considered to have a stronger effect on the offspring phenotype compared to paternal phenotype due to the large difference in cytoplasmic contribution in their respective gametes (e.g a human ovum is 85,000 times the volume of a spermatozoa). Contained within the oocytes cytoplasm are maternal factors (i.e. mitochondria, hormones, antibodies, proteins, and mRNA transcripts) which regulate early embryonic development in the zygote after fertilisation and prior the activation of the zygotic genome. While mammalian offspring are provided with nutrients through the placenta during gestation, oviparous animals (all birds, most fishes, amphibians and reptiles) provide their offspring with nutrients through the incorporation of yolk into their oocytes during oogenesis. In this study the maternal effect of yolk sac constituents

(i.e. fatty acids, amino acids and folate) and maternal mRNAs on Atlantic halibut oocyte quality (i.e. fertilisation normal blastomere symmetry, and embryonic hatching rates) was studied.

Broodstock nutrition can influence yolk sac composition and is the most extensively studied maternal effect on oocyte quality in aquaculture (Izquierdo et al., 2001 ; Watanabe & Vassallo-Agius, 2003 ; Bobe & Labbe, 2009). However, to determine exact nutritional requirements for broodstock of different teleost species is time-consuming and costly. In the absence of specific broodstock feed, diets are generally chosen randomly and range from frozen fish to commercial diets. Since teleosts are a largely heterogeneous group regarding habitat and physiological adaptations, generalizing nutritional requirements between different species can be misleading (Bone & Moore, 2008). Atlantic halibut broodstock usually consists of a small number of large-sized females (30-90 kg) that requires high manpower for gamete stripping. Previous Atlantic halibut broodstock nutritional experiments, trying to establish relations between oocyte yolk components and oocyte quality, have typically been performed on a low number of females (1-8 individuals) and few nutritional components (i.e. total lipid, fatty acids or folate (Bruce et al., 1993 ; Daniel et al., 1993 ; Parrish et al., 1993 ; Parrish et al., 1994 ; Evans et al., 1996 ; Mazorra et al., 2003). In Chapter 5 a comprehensive analysis of Atlantic halibut oocyte composition in relation to oocyte quality including amino acids (AA), fatty acids (FA) and folate was performed. Oocyte concentrations of total n-3 FAs, eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3) correlated positively with fertilisation and embryonic hatching rates, which is in agreement with the established knowledge of FA requirements in marine teleosts (Tocher, 2010). In contrast to

previous studies, the highest positive correlations between FA concentrations and fertilisation and embryonic hatching rates were not found for the most commonly studied PUFAs: EPA, DHA, or arachidonic acid (ARA, 20:4n6), but for dihomo-γ-linolenic acid (DGLA, 20:3n6) and docosapentaenoic acid (DPA, 22:5n3). DGLA and DPA are known competitors to ARA and DHA respectively during FA metabolism (Willis, 1981 ; Rubin & Laposata, 1991 ; Stark et al., 2007 ; Schmitz & Ecker, 2008). Dietary ARA and DHA requirements are usually considered well-covered through marine broodstock feed and it remains unclear how DGLA and DPA contributes to higher fertilisation and embryonic hatching rates in Atlantic halibut oocytes (Tocher, 2010). Methionine and aspartic acid are essential to eukaryotic protein synthesis and their oocyte concentrations were found to correlate positively with fertilisation and embryonic hatching rates respectively (Neidle & Dunlop, 1990 ; Hashimoto et al., 1995 ; Wu, 2009). Folate deficiencies are known to lead to major embryonic deformities in higher vertebrates and reduced growth in teleosts (Cowey & Woodward, 1993 ; Duncan et al., 1993 ; Tamura & Picciano, 2006 ; Gray & Ross, 2009). In the present study, no significant correlations between oocyte folate concentrations and oocyte quality were found.

In contrast to the yolk nutritional components, the possible role of non-yolky cytoplasmic components such as hormones, antibodies, structural and regulatory proteins and mRNAs have received far less attention in farmed teleosts. Nevertheless, maternal mRNAs are essential for early embryonic development before the start of zygotic transcription. They control important aspects of early embryonic development like the first mitotic divisions, specification of initial cell fate and embryonic patterning (Dworkin & Dworkin-Rastl, 1990). The influence of maternal mRNAs on oocyte

quality has been of especial interest in bovine (Mourot et al., 2006 ; Patel et al., 2007 ; Wrenzycki et al., 2007 ; Lee et al., 2009 ; Zhang et al., 2010) and human studies (Gasca et al., 2007 ; Anderson et al., 2009 ; Haouzi & Hamamah, 2009 ; Hamel et al., 2010) in connection with in vitro maturation (IVM) and in vitro fertilisation (IVF). Among commercially farmed teleosts, the relation between maternal mRNAs and oocyte quality have been only been studied in rainbow trout previously (Aegerter et al., 2003 ; Aegerter et al., 2004 ; Aegerter et al., 2005 ; Bonnet et al., 2007). In the present study the relation between maternal transcript abundance and oocyte quality was explored in a commercially farmed marine teleost. In total, 23 significant differentially expressed maternal transcripts were identified between low and high quality oocytes, most of them known to be involved in cytoskeleton dynamics, immune response, metabolism, RNA transcription, protein degradation, and cell signalling. By qPCR, significant correlations between an exportin 1-like protein orthologue (HHC00353) and Atlantic halibut oocyte quality was identified (Chapter 4). Exportin 1 has recently been related to ovine oocyte quality (Powell et al., 2010). None of the identified maternal transcripts have been related to teleost oocyte quality and only *mhc2dab* transcripts had been found in teleost oocytes previously (Li et al., 2010).

It is uncertain what generated the differential maternal gene expression in Atlantic halibut oocytes of varying quality in this study. In general, mRNA synthesis can be regulated pre-transcriptional (i.e gene silencing through histone modification, DNA methylation, and non-coding RNA), during transcription (i.e. activation and modification of DNA transcription factors, co-activators, and co-repressors) and after transcription (i.e polyadenylation, deadenylation, and miRNAs, Liu et al., 2009 ; Cheng & Blumenthal, 2010 ; Malecová & Morris, 2010). Alternative splicing of pre-mRNA

can result in different mRNAs that can be translated into different protein isoforms and translation of mRNA transcripts can vary in efficiency and accuracy due to variations in mRNA codons and the tRNA pool (Black, 2003 ; Gingold & Pilpel, 2011). Oocytes exhibit particular post-transcriptional regulatory mechanisms that control maternal mRNA stability and translation from oogenesis to the start of zygotic transcription. Polyadenylation of maternal mRNAs during oocyte maturation usually protects mRNAs from degradation and activates translation (Hake & Richter, 1997). In contrast, regulatory RNA or protein mediated deadenylation triggers mRNA degradation and translation repression to allow normal embryonic development after MZT (Tadros & Lipshitz, 2009). In *Xenopus tropicalis*, oocyte post-ovulatory aging (POA) induced a general decrease in maternal gene expression and a female-specific shortening of maternal mRNAs by deadenylation in oocytes developing into embryos experiencing high malformation and mortality rates (Kosubek et al., 2010). In contrast, POA induced both a decrease and increase in specific maternal transcripts in rainbow trout oocytes (Aegerter et al., 2005). In the present study, timing of Atlantic halibut female hand-stripping was timed according to their individual ovulation rhythms and POA was not considered a significant factor influencing Atlantic halibut oocyte quality (Chapter 4 and 6). Hence, low Atlantic halibut maternal transcript levels may be due to a lack or sub-optimal polyadenylation during oocyte maturation leading to poor transcript translation and/or degradation resulting in low oocyte quality. In zebrafish, maternal mRNA degradation has been identified to be induced by miR-430 family during mid-blastula stage by binding to target sites in the 3' cis untranslated region (UTR). In absence of miR-430s, maternal mRNAs accumulate and are thought to interfere with embryonic morphogenesis (Giraldez et al., 2006). Recently, a new maternal mRNA

degradation pattern has been identified in zebrafish leading to mRNA degradation from 1-cell to 16-cell stage (Aanes et al., 2011). High transcript levels of specific maternal mRNAs in low quality Atlantic halibut oocytes may be the result of impaired degradation during the first cell divisions, leading to transcript accumulation interfering with embryonic development.

7.3 Concluding remarks and future perspectives

The sample material in the present study consists of oocytes collected from Atlantic halibut females kept at two different locations (Risør Fisk AS and University of Nordland). Samples were collected over several years, from broodstock, held under natural and advanced photoperiod regime and fed with two different feeds (Winter herring and Fish-Breed M). First in 2008, both broodstock groups at the University of Nordland were routinely fed with one feed (Fish-breed M). The overall low number of samples is due to the time-extensive procedure of detecting female ovulation timing and hand-stripping of females. Oocytes were incubated using both industrial-size (large-scale) and experimental-size (Petri-dish) approaches, depending on the availability of manpower and equipment during different years. Survival data is restricted to hatching success due to technical restraints, i.e routinely pooling of batches after hatching during industrial production and limitations in man-power. A better experimental design comparing two defined broodstock groups would have benefitted the outcome of this study. However, despite the large heterogeneity of the sampling material significant correlation between gene expression and nutritional component concentrations with hatching survival were found.

In this thesis, genomic tools (i.e. maternal EST database, qPCR reference genes, and microarray) were produced that are applicable for future gene expression studies during Atlantic halibut embryonic and larval development to increase knowledge about Atlantic halibut reproduction, metabolism, immune response and growth (Chapter 3,4 and 6).

In this study an influences of yolk constituents and maternal mRNAs on Atlantic halibut oocyte quality has been shown. The importance of n-3 FAs for oocyte quality in Atlantic halibut was supported but less studied FAs (e.g. DGLA, DPA, OA, and POA) should not be neglected during future nutritional studies on Atlantic halibut broodstock. Together with methionine and aspartic acid they could act as possible indicators to improve Atlantic halibut broodstock nutrition and oocyte quality (Chapter 5). The differentially expressed maternal mRNAs in high and low quality oocytes are possible markers for Atlantic oocyte quality and could be useful for marker-assisted selection during selective Atlantic halibut breeding (Chapter 4 and 6). Their exact function during embryonic development need to be further characterized through functional studies (e.g.in situ hybridisation, loss-of-function analysis, and functional annotation) and will increase our knowledge of how Atlantic halibut embryonic development is regulated through these transcripts. Performing comparative polyadenylation and 3'UTR assays could reveal how their transcript levels are regulated (Kosubek et al., 2010 ; Aanes et al., 2011).

Oocyte maturation and developmental competence acquisition remains poorly understood, especially in non-mammalian vertebrates. In addition, follicular recruitment in teleosts is highly variable in comparison to mammals, complicating the understandability of underlying processes (Jalabert, 2005). Recently, essential roles for

other maternal oocyte constituents (i.e. mitochondria, nucleolus and imprinted control regions (ICRs)) have been identified during mammalian embryonic development and could most likely influence teleost oocyte quality (Bourc'his & Proudhon, 2008 ; Ogushi et al., 2008 ; Schulz et al., 2010 ; Wai et al., 2010).

8 Publications

I. **Mommens M**, Fernandes JMO, Bizuayehu TT, Bolla SL, Johnston IA and I Babiak. 2010. Maternal mRNAs as molecular markers for egg quality in Atlantic halibut (*Hippoglossus hippoglossus* L.). BMC Research Notes 3:138.

II. Fernandes JMO, **Mommens M**, Hagen Ø, Babiak I and C Solberg. 2008. Selection of suitable reference genes for real-time PCR studies of Atlantic halibut development. Comparative Biochemistry and Physiology – Part B: Biochemistry and Molecular Biology 150 (1), pp.23-32.

9 Appendix

Supplementary table A Fatty acid concentration in Atlantic halibut oocytes.
Concentrations are given in (mg g dry weight $^{-1}$).

Female	**G39R**	**G39R**	**G39R**	**G39R**	**G39S**	**G39S**	**G39S**	**G39S**	**G39S**	**G32**	**G8**	**G8**	**Y46**	**Y46**	**Y32**	**Y7**	**O17**	**Y30**	**Y30**	**Y30**	**Y4**
Sample year	**2008**	**2008**	**2008**	**2008**	**2009**	**2009**	**2008**	**2008**	**2006**	**2008**	**2009**	**2008**	**2009**	**2006**	**2006**	**2006**	**2006**	**2009**	**2009**	**2006**	**2009**
Batch nr.	**1**	**2**	**3**	**4**	**1**	**2**	**1**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**2**	**1**	**1**
14:0	2.2	2.2	2.3	2.4	2.5	3.4	3.2	2.2	4.2	2.4	2.4	3.2	3.0	3.6	2.4	2.2	3.7	2.5	2.3	4.0	2.5
16:0	18.6	18.5	19.3	19.5	20.0	21.2	20.6	18.2	20.5	21.0	20.7	20.9	20.7	20.3	19.2	19.1	21.7	19.6	20.7	21.8	19.4
16:1n7 (POA)	4.2	4.2	4.5	4.4	3.7	3.5	3.1	4.1	3.0	3.4	2.8	3.1	3.1	2.5	3.7	2.6	2.4	3.3	2.6	2.7	3.3
18:0	3.5	3.5	3.7	3.7	3.5	3.0	3.3	3.4	3.0	4.3	4.3	3.3	3.2	2.9	4.0	4.4	3.0	4.3	4.1	3.1	4.2
18:1n9 (OA)	9.2	9.1	9.5	9.6	7.7	7.7	8.7	9.0	8.6	9.2	7.5	8.8	7.1	8.0	9.7	8.2	7.9	8.2	8.4	8.3	8.0
18:1n7	2.6	2.6	2.7	2.7	2.7	2.3	2.2	2.5	1.9	2.8	2.7	2.2	2.2	1.8	2.8	2.7	1.9	2.9	2.6	1.9	2.8
18:2n6 (LA)	5.0	5.0	5.2	5.3	4.0	4.2	4.6	4.9	2.8	6.1	4.0	4.7	3.4	2.6	4.9	6.6	2.8	4.0	4.4	3.1	4.0
18:3n6	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.2
18:3n3 (LNA)	0.4	0.4	0.5	0.5	0.4	0.6	0.6	0.5	0.5	0.5	0.4	0.6	0.5	0.5	0.5	0.7	0.6	0.4	0.5	0.6	0.4
18:4n3	0.3	0.3	0.3	0.3	0.4	0.5	0.4	0.4	0.5	0.4	0.2	0.4	0.2	0.5	0.4	0.4	0.5	0.1	0.1	0.3	0.1
20:0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
20:1n9	2.0	2.0	2.0	2.1	1.5	2.2	3.0	1.9	4.9	2.0	1.5	3.0	2.0	4.9	2.3	1.9	5.0	1.5	1.3	5.3	1.5
20:1n7	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
20:2n6	0.7	0.8	0.8	0.8	0.6	0.5	0.6	0.7	0.4	0.8	0.6	0.6	0.4	0.4	0.6	0.9	0.4	0.6	0.6	0.4	0.6
20:3n6 (DGLA)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
20:4n6 (ARA)	1.6	1.6	1.7	1.7	1.7	1.4	1.2	1.6	0.9	1.6	1.5	1.2	1.2	0.8	1.2	1.4	0.8	1.7	1.6	0.9	1.6
20:4n3 (ESA)	0.3	0.3	0.3	0.3	0.3	0.4	0.5	0.3	0.5	0.3	0.4	0.5	0.4	0.4	0.4	0.4	0.5	0.4	0.4	0.5	0.4

Table continues on next page

Supplementary table A continued

Female	**G39R**	**G39R**	**G39R**	**G39R**	**G39S**	**G39S**	**G39S**	**G39S**	**G39S**	**G32**	**G8**	**G8**	**Y46**	**Y46**	**Y32**	**Y7**	**O17**	**Y30**	**Y30**	**Y30**	**Y4**
Sample year	**2008**	**2008**	**2008**	**2008**	**2009**	**2009**	**2008**	**2008**	**2006**	**2008**	**2009**	**2008**	**2009**	**2006**	**2006**	**2006**	**2006**	**2009**	**2009**	**2006**	**2009**
Batch nr.	**1**	**2**	**3**	**4**	**1**	**2**	**1**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**2**	**1**	**1**
20:5n3 (EPA)	10.3	10.3	10.8	11.0	14.1	14.9	11.6	10.1	13.9	11.4	13.8	11.6	12.0	13.5	11.3	12.0	12.9	14.0	14.4	13.7	13.9
22:1n9	0.6	0.6	0.6	0.6	0.6	1.4	1.3	0.6	3.0	0.6	0.5	1.3	0.8	2.5	0.7	0.6	2.6	0.5	0.5	2.8	0.5
22:5n6	0.9	0.9	0.9	0.9	1.0	0.8	0.8	0.8	0.6	1.0	0.9	0.8	0.8	0.8	0.9	0.8	0.6	1.0	0.9	0.7	1.0
22:5n3 (DPA)	1.2	1.2	1.2	1.2	1.4	1.6	1.4	1.2	1.4	1.3	1.7	1.4	1.4	1.4	1.3	1.6	1.6	1.6	1.8	1.6	1.5
22:6n3 (DHA)	28.0	28.0	28.9	29.5	28.9	30.9	30.9	27.5	29.9	32.1	31.4	31.2	31.3	31.8	29.3	28.6	32.2	29.9	30.4	32.7	29.4
24:1n9	0.6	0.6	0.6	0.6	0.6	0.9	0.6	0.6	1.4	0.6	0.6	0.6	0.5	0.8	0.7	0.6	0.6	0.8	0.5	1.0	0.7
Total FA	92.7	92.4	96.4	97.8	96.5	102.1	99.5	91.0	102.0	102.6	98.7	100.1	94.9	99.8	96.6	96.2	99.1	98.0	96.3	106.0	96.6
Saturated	24.4	24.3	25.5	25.7	26.2	27.7	26.7	23.9	27.8	27.8	27.6	27.5	27.0	26.9	25.6	25.7	28.5	26.5	27.2	29.0	26.1
Unsaturated	68.3	68.1	70.9	72.1	70.3	74.4	72.0	67.2	74.7	74.8	71.1	72.5	67.9	73.8	71.0	70.4	73.6	71.5	72.5	77.0	70.4
Monounsaturated	19.4	19.3	20.2	20.3	17.1	18.3	19.2	19.1	23.2	19.0	15.8	19.3	16.0	20.7	20.1	16.8	20.6	17.5	18.6	22.3	17.1
Polyunsaturated	48.9	48.8	50.8	51.8	53.2	56.1	52.9	48.1	51.6	55.8	55.3	53.2	51.9	52.7	50.9	53.7	53.1	54.1	55.3	54.7	53.3
n-3	40.5	40.4	42.0	42.8	45.6	48.9	45.4	39.9	46.6	46.1	47.9	45.7	45.8	48.1	43.1	43.7	48.2	46.5	47.5	49.4	45.8
n-6	8.4	8.4	8.8	9.0	7.7	7.2	7.5	8.2	4.9	9.7	7.4	7.6	6.1	7.3	7.8	10.0	4.8	7.6	7.8	5.3	7.5
n-3/n-6	4.8	4.8	4.8	4.8	6.0	6.8	6.1	4.8	9.5	4.7	6.5	6.0	7.5	9.4	5.5	4.4	10.0	6.1	6.1	9.4	6.1
DHA/EPA	2.7	2.7	2.7	2.7	2.0	2.1	2.7	2.7	2.2	2.8	2.3	2.7	2.6	2.4	2.6	2.4	2.5	2.1	2.1	2.4	2.1
EPA/ARA	6.4	6.5	6.4	6.4	8.2	10.5	9.4	6.5	16.1	7.2	8.9	9.7	9.9	16.9	9.4	8.8	16.7	8.4	9.2	15.8	8.6
LNA/LA	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.1

Supplementary table B Amino acid concentration in Atlantic halibut oocytes.
Concentration is given as (mg g dry weight $^{-1}$).

Female	**G39 R**	**G39 R**	**G39 R**	**G39 R**	**G39 S**	**G39 S**	**G39 S**	**G39 S**	**G39 S**	**G32**	**G8**	**G8**	**Y46**	**Y46**	**Y32**	**Y7**	**O17**	**Y30**	**Y30**	**Y30**	**Y4**
Sample year	**2008**	**2008**	**2008**	**2008**	**2009**	**2009**	**2008**	**2008**	**2006**	**2008**	**2009**	**2008**	**2009**	**2006**	**2006**	**2006**	**2006**	**2009**	**2009**	**2006**	**2009**
Batch nr.	**1**	**2**	**3**	**4**	**1**	**2**	**1**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**2**	**1**	**1**
Aspartic acid	35.2	31.3	33.4	33.5	32.8	32.7	33.1	30.6	34.9	34.4	33.1	35.6	34.6	35.4	30.4	30.1	36.6	34.4	32.7	36.9	35.0
Glutamic acid	64.2	57.5	62.1	63.6	59.9	59.6	61.8	57.7	65.5	62.9	62.3	66.7	62.3	66.1	55.7	59.3	66.1	61.9	58.7	67.4	62.8
Serine	24.2	20.9	22.8	23.5	22.2	22.6	23.6	21.4	24.7	22.7	23.4	25.4	23.3	25.2	21.3	22.6	24.7	23.2	22.2	25.5	23.9
Histidine	15.8	14.2	14.6	15.1	13.9	13.7	14.7	13.3	15.5	15.2	14.2	15.8	13.8	15.4	12.7	13.9	14.9	14.0	12.6	15.4	13.8
Glycine	16.0	14.7	15.9	16.2	14.5	14.2	15.9	14.6	16.5	16.1	15.5	16.8	15.3	16.7	14.6	15.3	14.3	15.8	14.7	18.2	15.3
Threonine	18.8	16.8	18.3	18.6	18.0	20.6	18.5	17.3	19.3	19.0	18.4	19.7	17.8	19.7	17.3	18.0	22.3	18.4	17.5	19.7	17.9
Arginine	23.7	21.3	22.7	23.5	21.8	21.8	22.6	21.1	24.1	23.4	22.8	24.6	22.7	24.0	20.9	21.6	23.7	22.8	21.6	24.1	22.6
Alanine	41.2	36.8	38.0	39.4	34.5	37.4	38.6	35.6	41.6	40.8	39.3	41.2	38.3	40.5	35.8	35.5	39.9	39.0	36.9	41.0	38.4
Taurine	4.9	4.0	3.9	2.9	3.9	4.4	4.4	4.3	4.8	4.3	4.0	4.1	3.8	4.8	4.0	3.0	3.2	3.5	3.8	3.8	4.4
Tyrosine	17.8	16.1	17.5	17.8	16.6	16.3	17.3	16.3	18.2	17.7	17.3	18.7	17.2	18.3	15.6	16.7	18.1	17.2	16.1	18.5	17.2
Cystin	4.0	3.8	4.4	4.3	4.3	3.9	4.2	4.0	4.4	4.3	4.0	4.6	4.3	5.0	3.9	4.2	4.1	4.4	4.1	4.5	4.2
Valine	28.4	26.0	27.1	27.7	26.2	26.3	26.8	25.0	28.6	27.9	27.7	28.8	27.6	28.8	24.7	25.7	28.5	27.6	25.7	28.9	27.4
Methionine	11.6	10.0	10.8	11.2	10.7	10.8	10.9	10.1	12.1	11.6	11.9	12.2	12.0	12.5	9.4	9.9	12.3	11.5	10.5	9.3	11.3
Isoleucine	26.3	24.5	24.7	25.3	24.1	24.0	24.4	22.6	26.3	25.7	25.1	26.5	25.1	25.7	22.9	23.0	26.3	25.3	23.5	26.0	25.5
Tryptophan	5.8	5.5	5.8	5.8	6.0	5.4	5.7	5.6	6.0	5.8	5.6	6.5	5.7	6.0	5.7	5.5	6.3	6.0	5.4	5.8	6.4
Phenylalanine	17.0	15.6	16.9	17.2	16.5	15.8	16.9	15.8	17.6	17.2	16.8	18.4	16.8	17.8	15.5	15.9	18.0	16.9	15.8	17.9	17.1
Leusin	46.4	41.8	42.5	43.9	41.6	41.6	42.6	39.1	46.0	45.0	43.3	46.2	42.9	45.0	39.8	40.4	45.3	43.1	40.9	46.0	43.5
Lysin	34.2	30.3	33.2	34.6	30.3	30.2	33.9	28.8	35.9	29.2	30.3	35.5	29.3	35.1	26.4	28.4	29.8	29.5	26.5	32.1	28.1
Prolin	30.4	27.2	34.3	34.2	31.5	31.5	34.2	31.3	33.9	32.2	33.1	35.9	33.3	36.3	28.2	31.7	34.7	33.2	29.9	37.0	32.1
Total AA	465.2	417.8	448.5	457.7	428.7	432.4	449.5	414.5	475.2	454.8	447.5	482.8	446.0	477.8	404.4	420.4	468.5	447.4	418.6	478.0	446.5

Supplementary table C Biological prosess (BP) gene ontology annotations (GOs) for Atlantic halibut micoroarray probes.
Given are GO level, GO ID number, GO term, sequence number and GO graph score.

Level	GO ID	Term	#Seqs	Graph Score
1	GO:0008150	biological process	4060	5002
2	GO:0009987	cellular process	2928	1676
2	GO:0008152	metabolic process	2799	2542
3	GO:0044238	primary metabolic process	2495	1541
3	GO:0044237	cellular metabolic process	2030	899
2	GO:0065007	biological regulation	1742	984
3	GO:0050789	regulation of biological process	1709	1583
3	GO:0043170	macromolecule metabolic process	1691	868
3	GO:0009058	biosynthetic process	1432	850
4	GO:0044260	cellular macromolecule metabolic process	1334	615
4	GO:0034641	cellular nitrogen compound metabolic process	1176	504
3	GO:0006807	nitrogen compound metabolic process	1176	302
4	GO:0006139	nucleobase, nucleoside, nucleotide metabolic process	1176	840
4	GO:0019538	protein metabolic process	1143	778
2	GO:0051179	localization	902	309
4	GO:0006810	transport	902	859
3	GO:0051234	establishment of localization	902	516
0	GO:0071840	cellular component organization or biogenesis	844	366
2	GO:0016043	cellular component organization	844	609
2	GO:0032502	developmental process	841	764
4	GO:0010467	gene expression	840	530
4	GO:0044249	cellular biosynthetic process	832	311
5	GO:0034645	cellular macromolecule biosynthetic process	832	518
5	GO:0060255	regulation of macromolecule metabolic process	33	12
7	GO:0040029	regulation of gene expression, epigenetic	33	33
3	GO:0008037	cell recognition	16	16
2	GO:0016032	viral reproduction	15	15
2	GO:0051704	multi-organism process	9	3
3	GO:0044419	interspecies interaction between organisms	9	5
4	GO:0044403	symbiosis, encompassing mutualism through parasitism	9	9
3	GO:0007028	cytoplasm organization	3	3
4	GO:0007010	cytoskeleton organization	192	192
3	GO:0065008	regulation of biological quality	185	57
2	GO:0000003	reproduction	178	178
3	GO:0019725	cellular homeostasis	139	139
4	GO:0042592	homeostatic process	139	83
2	GO:0040007	growth	129	113
3	GO:0007154	cell communication	126	99
3	GO:0009719	response to endogenous stimulus	105	105
3	GO:0007610	behavior	90	90
3	GO:0009628	response to abiotic stimulus	84	84
3	GO:0007267	cell-cell signaling	83	83
3	GO:0009607	response to biotic stimulus	71	71
3	GO:0019748	secondary metabolic process	67	67
7	GO:0016049	cell growth	50	50

Table continues on next page

Supplementary table C continued

Level	GO ID	Term	#Seqs	Graph Score
5	GO:0032535	regulation of cellular component size	50	18
4	GO:0090066	regulation of anatomical structure size	50	11
6	GO:0008361	regulation of cell size	50	30
4	GO:0007005	mitochondrion organization	45	45
4	GO:0019222	regulation of metabolic process	33	7
6	GO:0010468	regulation of gene expression	33	20
4	GO:0006519	cellular amino acid and derivative metabolic process	380	380
3	GO:0044281	small molecule metabolic process	380	228
3	GO:0048856	anatomical structure development	353	212
4	GO:0009653	anatomical structure morphogenesis	353	353
3	GO:0007049	cell cycle	351	351
4	GO:0006629	lipid metabolic process	302	302
5	GO:0006811	ion transport	302	302
6	GO:0006412	translation	301	301
5	GO:0045184	establishment of protein localization	280	168
3	GO:0033036	macromolecule localization	280	60
4	GO:0008104	protein localization	280	101
5	GO:0015031	protein transport	280	280
2	GO:0016265	death	268	162
3	GO:0008219	cell death	266	266
2	GO:0008283	cell proliferation	259	259
4	GO:0006091	generation of precursor metabolites and energy	253	253
4	GO:0005975	carbohydrate metabolic process	250	250
6	GO:0006259	DNA metabolic process	220	220
4	GO:0009790	embryo development	220	220
3	GO:0009605	response to external stimulus	207	207
4	GO:0009059	macromolecule biosynthetic process	832	311
2	GO:0023052	signaling	828	219
2	GO:0032501	multicellular organismal process	787	500
3	GO:0007275	multicellular organismal development	787	818
4	GO:0023060	signal transmission	783	470
3	GO:0023046	signaling process	783	282
4	GO:0050794	regulation of cellular process	783	470
5	GO:0007165	signal transduction	783	783
5	GO:0044267	cellular protein metabolic process	762	467
5	GO:0090304	nucleic acid metabolic process	713	470
3	GO:0009056	catabolic process	698	698
2	GO:0050896	response to stimulus	644	621
6	GO:0006350	transcription	563	563
0	GO:0071842	cellular component organization at cellular level	488	259
3	GO:0006996	organelle organization	487	429
4	GO:0043412	macromolecule modification	478	287
3	GO:0006950	response to stress	478	478
6	GO:0006464	protein modification process	478	478
3	GO:0048869	cellular developmental process	406	244
4	GO:0030154	cell differentiation	406	406

Supplementary table D Molecular function (MF) gene ontology annotations (GOs) for Atlantic halibut micoroarray probes.
Given are GO level, GO ID number, GO term, sequence number and GO graph score.

Level	GO ID	Term	#Seqs	Graph Score
1	GO:0003674	molecular function	4391	3801
2	GO:0005488	binding	3507	3447
3	GO:0005515	protein binding	2221	2218
2	GO:0003824	catalytic activity	2122	1422
3	GO:0016787	hydrolase activity	911	732
3	GO:0003676	nucleic acid binding	773	554
3	GO:0000166	nucleotide binding	754	754
3	GO:0016740	transferase activity	636	464
4	GO:0003677	DNA binding	336	336
2	GO:0005215	transporter activity	335	290
2	GO:0005198	structural molecule activity	302	302
4	GO:0003723	RNA binding	289	289
2	GO:0060089	molecular transducer activity	286	127
3	GO:0004871	signal transducer activity	286	211
5	GO:0016301	kinase activity	259	198
4	GO:0016772	transferase activity	259	119
4	GO:0008233	peptidase activity	255	255
2	GO:0030528	transcription regulator activity	222	222
2	GO:0030234	enzyme regulator activity	205	205
4	GO:0008092	cytoskeletal protein binding	200	190
5	GO:0046872	metal ion binding	198	119
4	GO:0043169	cation binding	198	71
3	GO:0043167	ion binding	198	43
6	GO:0005509	calcium ion binding	198	198
4	GO:0004872	receptor activity	195	195
4	GO:0005102	receptor binding	178	178
5	GO:0022838	substrate-specific channel activity	56	34
6	GO:0005216	ion channel activity	56	56
5	GO:0004518	nuclease activity	53	53
5	GO:0042578	phosphoric ester hydrolase activity	50	18
7	GO:0004721	phosphoprotein phosphatase activity	50	50
6	GO:0016791	phosphatase activity	50	30
3	GO:0003682	chromatin binding	42	42
2	GO:0016209	antioxidant activity	25	25
2	GO:0045182	translation regulator activity	10	10
4	GO:0005326	neurotransmitter transporter activity	6	6
3	GO:0019825	oxygen binding	6	6
5	GO:0016773	phosphotransferase activity	156	94
6	GO:0004672	protein kinase activity	156	156
3	GO:0008289	lipid binding	134	134
5	GO:0003779	actin binding	133	133
3	GO:0003700	sequence-specific DNA binding transcription factor	128	128
2	GO:0009055	electron carrier activity	115	115
4	GO:0016788	hydrolase activity, acting on ester bonds	103	43
4	GO:0008135	translation factor activity, nucleic acid binding	87	87
3	GO:0030246	carbohydrate binding	80	80
5	GO:0016818	hydrolase activity	64	14
4	GO:0016817	hydrolase activity, acting on acid anhydrides	64	8

Table continues on next page

Supplementary table D continued

Level	GO ID	Term	#Seqs	Graph Score
8	GO:0003774	motor activity	64	64
6	GO:0016462	pyrophosphatase activity	64	23
7	GO:0017111	nucleoside-triphosphatase activity	64	38
3	GO:0022857	transmembrane transporter activity	62	16
4	GO:0022891	substrate-specific transmembrane transporter activity	56	20
5	GO:0015075	ion transmembrane transporter activity	56	34
5	GO:0015267	channel activity	56	20
3	GO:0022892	substrate-specific transporter activity	56	12
4	GO:0022803	passive transmembrane transporter activity	56	12

Supplementary table E Cellular component (CC) gene ontology annotations (GOs) for Atlantic halibut micoroarray probes.
Given are GO level, GO ID number, GO term, sequence number and GO graph score.

Level	GO ID	Term	#Seqs	Graph Score
1	GO:0005575	cellular component	4090	2170
2	GO:0005623	cell	3914	1779
3	GO:0044464	cell part	3548	982
4	GO:0005622	intracellular	3359	1300
4	GO:0044424	intracellular part	3194	1453
2	GO:0043226	organelle	2822	873
5	GO:0043229	intracellular organelle	2762	1241
5	GO:0005737	cytoplasm	2477	1732
3	GO:0043227	membrane-bounded organelle	2371	891
6	GO:0043231	intracellular membrane-bounded organelle	2371	1485
5	GO:0044444	cytoplasmic part	1692	1303
7	GO:0005634	nucleus	1420	1089
2	GO:0032991	macromolecular complex	1362	788
3	GO:0043234	protein complex	1164	1164
6	GO:0043232	intracellular non-membrane-bounded organelle	943	607
3	GO:0043228	non-membrane-bounded organelle	943	364
4	GO:0044446	intracellular organelle part	737	311
3	GO:0044422	organelle part	737	186
6	GO:0044428	nuclear part	691	481
6	GO:0070013	intracellular organelle lumen	608	245
4	GO:0043233	organelle lumen	608	147
2	GO:0031974	membrane-enclosed lumen	608	88
7	GO:0031981	nuclear lumen	608	409
5	GO:0005886	plasma membrane	543	543
4	GO:0016020	membrane	543	326
7	GO:0005739	mitochondrion	542	542
6	GO:0005829	cytosol	519	519
6	GO:0005654	nucleoplasm	482	482
7	GO:0005856	cytoskeleton	421	409
2	GO:0005576	extracellular region	366	248
7	GO:0005783	endoplasmic reticulum	301	301
7	GO:0005794	Golgi apparatus	266	266
6	GO:0005840	ribosome	248	248
5	GO:0030529	ribonucleoprotein complex	248	149
6	GO:0005730	nucleolus	199	199
3	GO:0044421	extracellular region part	174	93
7	GO:0005694	chromosome	159	155
3	GO:0031982	vesicle	142	51
7	GO:0016023	cytoplasmic membrane-bounded vesicle	142	142
4	GO:0031988	membrane-bounded vesicle	142	85
6	GO:0031410	cytoplasmic vesicle	142	85
4	GO:0005615	extracellular space	110	110
4	GO:0031012	extracellular matrix	75	45
5	GO:0005578	proteinaceous extracellular matrix	75	75
7	GO:0005768	endosome	71	71
7	GO:0005773	vacuole	69	37
4	GO:0031975	envelope	67	24
4	GO:0031967	organelle envelope	66	40
4	GO:0012505	endomembrane system	66	40

Table continues on next page

Supplementary table E continued

Level	GO ID	Term	#Seqs	Graph Score
5	GO:0005635	nuclear envelope	66	66
7	GO:0005815	microtubule organizing center	61	61
6	GO:0044430	cytoskeletal part	61	37
8	GO:0015630	microtubule cytoskeleton	61	37
7	GO:0000323	lytic vacuole	56	34
8	GO:0005764	lysosome	56	56
7	GO:0000228	nuclear chromosome	55	55
7	GO:0005777	peroxisome	38	38
7	GO:0042579	microbody	38	23
6	GO:0005811	lipid particle	13	13
5	GO:0005929	cilium	12	12
4	GO:0042995	cell projection	12	7
7	GO:0009536	plastid	5	5
4	GO:0030312	external encapsulating structure	3	3
5	GO:0030313	cell envelope	1	1
5	GO:0005618	cell wall	1	1

Supplementary table F Uncharacterized maternal genes present in CL6.

Probe name	GenBank Accession	BLASTX hit gene description
HH_Contig566	EB035999	unnamed protein product [Tetraodon nigroviridis]
HH_166850885	FD698679	unnamed protein product [Tetraodon nigroviridis]
HH_Contig2902	FK701210	unnamed protein product [Tetraodon nigroviridis]
HH_Contig310	EB041103	unnamed protein product [Tetraodon nigroviridis]
HH_90603209	EB036650	unnamed protein product [Tetraodon nigroviridis]
HH_Contig569	EB039737	unnamed protein product [Tetraodon nigroviridis]
HH_Contig2540	DT806209	---NA---
HH_Contig1071	EB036838	---NA---
HH_193889242	FK701753	---NA---
HH_90988400	EB103569	---NA---
HH_Contig2923	FK701620	---NA---
HH_Contig2987	FK703367	---NA---
HH_90988799	EB103968	---NA---
HH_Contig2859	FK702420	---NA---
HH_90988503	EB103672	---NA---
HH_75737824	DT805824	---NA---
HH_193889675	FK702270	---NA---
HH_Contig2808	FK701258	---NA---
HH_Contig2806	FK701852	---NA---
HH_166850908	FD698966	---NA---
HH_Contig2567	FK701248	---NA---
HH_Contig3022	FK701593	---NA---
HH_75738215	DT806215	---NA---
HH_Contig3015	FK701942	---NA---
HH_Contig2836	FK702443	---NA---
HH_193889112	FK702534	---NA---
HH_193890171	FK702823	---NA---
HH_75737803	DT805803	---NA---
HH_90603204	EB036645	---NA---
HH_193889004	FK701637	---NA---
HH_193890000	FK703214	---NA---
HH_90988658	EB103827	---NA---
HH_90603288	EB036729	---NA---
HH_Contig96	EB103607	---NA---
HH_193888931	FK701201	---NA---
HH_Contig3098	FK702139	---NA---
HH_193890524	FK703348	---NA---
HH_Contig1168	EB036392	---NA---
HH_Contig2972	FK702959	---NA---
HH_193888687	FK701481	---NA---
HH_Contig117	DN794342	---NA---
HH_193890462	FK702895	---NA---
HH_90599721	EB033162	---NA---
HH_Contig1274	FK702709	---NA---
HH_90988653	EB103822	---NA---
HH_Contig1152	EB036435	---NA---
HH_90988551	EB103720	---NA---
HH_193889791	FK701911	---NA---
HH_90988629	EB103798	---NA---
HH_166850869	FD698663	---NA---

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Supplementary table F continued

Probe name	GenBank Accession	BLASTX hit gene description
HH_Contig3033	DT806219	---NA---
HH_193889799	FK703165	---NA---
HH_193889948	FK702750	---NA---
HH_90988333	EB103502	---NA---
HH_90606597	EB040038	---NA---
HH_166851059	FD698418	---NA---
HH_Contig3049	FK701393	---NA---
HH_90601816	EB035257	---NA---
HH_166850905	FD698963	---NA---
HH_193888699	FK701493	---NA---
HH_EST_193889508	FK702627.1	---NA---
HH_Contig2797	FK702048	---NA---
HH_90988209	EB103378	---NA---
HH_193889740	FK702301	---NA---
HH_90988269	EB103438	---NA---

Supplementary table G: Maternal genes common between Atlantic halibut and zebrafish according to i) Mathavan *et al.* (2005) (Mathavan et al., 2005) and ii) Aanes *et al*. (2011)(Aanes et al., 2011).

i)

Gene name (Abbreviation)	**Accession**
Apoptosis regulatory protein siva (*siva1*)	BM101541
Superoxide dismutase (*sod1*)	NM_131294
Claudin 4 (*cldn4*)	NM_213274
Nuclear distribution gene e homolog 1 (*nde1*)	NM_201307
Cyclin b2 (*cycb2*)	NM_199430
Actin binding protein anillin (*anln*)	AI878452
Rho gtpase activating protein 11a (*arhagap11a*)	NM_001193539
Nuclear autoantigenic sperm protein (*nsap*)	NM_199782
Novel protein lim domain 7 (*lmo7b*)	NM_001128231
Tartrate-resistant acid phosphatase type 5 precursor (*acp5*)	NM_214773
Elongin a (*ela1*)	NM_200121
Zinc finger protein 180 (*znf180*)	AW232088
Solute carrier family 16 (*slc16a13*)	NM_212708
Cue domain containing 2 (*cuedc2*)	NM_001017994
Moloney leukemia virus 10-like homolog (*mov10l*)	EB909625

ii)

Gene name (Abbreviation)	**Accession**
Spindle assembly 6 homolog (sass6)	NM_213438
Chloride intracellular channel 1 (clic1)	NM_212682
Tropomyosin α1 (tpm1)	NM_001102629
Bloom syndrome protein (blm)	XP_701357
Tudor domain containing 7(tdrd7)	NM_001099343
Novel protein lim domain 7 (lmo7b)	NM_001128231
Carbonic anhydrase 7 (ca7)	NM_200813
Zinc finger protein 451 (*znf451*)	CD758949
DNA (cytosine-5)-methyltransferase 1 (nmt1)	NM_131189
Kinesin family member 20a (Kif20a)	BC098606
B-cell translocation gene 3 (btg3)	NM_001007351
Wd repeat domain 83 (Wdr83)	NM_001002429

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