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Key genes and regulators associated with sexual differentiation and gonad development in Atlantic cod (*Gadus morhua* L.)

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Tromsø, January 2012
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Abstract

Atlantic cod (*Gadus morhua* L.) is a promising cold-water aquaculture species, but early sexual maturation is a major problem in today's cod farming. Teleosts display a wide variety of sex determination and sex differentiation mechanisms, ranging from genetic to environmental factors and the sexual plasticity in gonads and brain possibly reflects on a combination of these. From a practical point of view it is crucial to identify the essential biological mechanisms to develop new methods of controlling fish reproduction in aquaculture. In this thesis, several approaches were taken in order to gain basic knowledge of factors involved in sexual differentiation in Atlantic cod. The Doublesex and mab-3 related transcription factor 1 (Dmrt1) which is involved in testis development in a wide variety of vertebrates was characterised. Expression analyses showed that *dmrt1* was exclusively expressed in cod gonads and the expression was significantly higher in males compared to females. The *dmrt* gene family consists of four additional genes in cod. In order to further clarify the evolution of the gene family members in fish, the chromosomal synteny of *dmrt* flanking genes in different chordate lineages was examined. A novel understanding of the gene duplications of *dmrts* was presented as the duplicated *dmrt2a* and *dmrt2b* was probably not a result of the fish specific whole-genome duplication. Gene expression analyses during early life and the reproductive season was conducted for a selection of genes known to be involved in sexual differentiation. The examined genes included all five *dmrt*s, two SRY-related high mobility group (HMG) containing box gene 9 (*sox9*) paralogs and two cytochrome P450, family 19, subfamily A, polypeptide 1 (c*yp19a1*) paralogs in addition to a single anti-Müllerian hormone (*amh*) gene. The differential expression of the *dmrt* genes during embryogenesis suggested distinct functions in the developing cod. In the adult cod there were considerable differences in the sex-related expression of the *dmrt* genes in gonads and brain compared to other teleosts, except for the male-biased gonad expression of *dmrt1*. The bimodal relationship between *amh* and *cyp19a1a* in developing cod embryos may suggest an essential role in sexual differentiation. During the reproductive season, the upregulation of ovarian expression of *cyp19a1* was strongly correlated with plasma estradiol levels. Gonad expression of *sox9a* was restricted to males, while expression of *sox9b* was male-biased. This is in agreement with the proposed role of the Sox9 paralogs in testicular differentiation. In addition, *sox9b*

signals in the hindbrain and branchial arches of hatched larvae agree with the chondrogenic activity of Sox9 reported in tetrapods. Altogether, the different expression patterns of the examined genes in Atlantic cod embryos, larvae and adults suggest distinct functions for all the genes examined. The findings indicated that Cyp19a1a may be an important factor in female development while it was clearly demonstrated that Dmrt1 has an important role in male development of cod.

Sammendrag (Norwegian abstract)

Atlanterhavstorsk (*Gadus morhua* L.) er en lovende art i akvakultur, men tidlig kjønnsmodning er et stort problem i dagens torskeoppdrett. Det er stor variasjon i mekanismene som regulerer kjønnsbestemmelse og kjønnsutvikling hos beinfisk. Disse spenner seg fra genetiske til miljøbestemte faktorer og plastisiteten i både gonader og hjerne kan gjenspeile en kombinasjon av faktorene. For å utvikle nye metoder til å kontrollere reproduksjon og kjønnsmodning i akvakultur er det viktig å først kartlegge hvilke biologiske mekanismer som er involvert. Denne avhandlingen bidrar til økt basiskunnskap om faktorer involvert i kjønnsmodning hos torsk. Transkripsjonsfaktoren Dmrt1 (Doublesex and mab-3 related transcription factor 1) som er involvert i testisutviklingen hos mange vertebrater ble karakterisert i torsk. Ekspresjonsanalyser viste at *dmrt1* ble utelukkende uttrykt i gonader med et signifikant høyere uttrykk hos hannfisk i forhold til hunnfisk. *Dmrt1* tilhører *dmrt* genfamilien som hos torsk også består av *dmrt2a, dmrt3, dmrt4* og *dmrt5*. For og videre undersøke evolusjonen av denne genfamilien ble syntenien av dmrt flankerende gener analysert i forskjellige ryggstrengsdyr. Det presenteres en ny forståelse av dupliseringen av *dmrt* genene da paralogene *dmrt2a og dmrt2b* antakelig ikke var en del av den fiskespesifikke genomdupliseringen. Ekspresjonsanalyser på tidlige livsstadier og gjennom gytesesongen ble gjort for alle fem *dmrt* genene samt *sox9* og *cyp19a1* paralogene i tillegg til amh. Uttrykket av *dmrt* genene varierte gjennom embryogenesen noe som kan ha sammenheng med spesifikke funksjoner relatert til torskens utvikling. I voksen torsk var det forskjeller i kjønnsrelatert uttrykk både i gonader og hjerne sammenliknet med annen beinfisk, med unntak av *dmrt1* som ser ut til å være hannspesifikk uansett art. Uttrykket av *cyp19a1* og *amh* var bimodalt fordelt i torskeembryoer, noe som peker mot en mulig rolle i kjønnsutvikling. Gjennom gytesesongen var uttrykket av *cyp19a1a* i ovarier sterkt korrelert til mengden plasma-østradiol i blodet. Gonade uttrykket av *sox9a* var hannspesifikt og uttrykket av *sox9b* var noe større i hann fisk sammenlignet med hunnfisk, spesielt ved gyting. Dette samsvarer med *sox9* paralogenes rolle i testisutviklingen. Forskjellene i ekspresjonen av alle genene i torskeembryoer, larver og voksen fisk viser til distinkte funksjoner. Mens funnene indikerer at Cyp19a1a muligens er en viktig faktor i hunntorskens kjønnsmodning, slås det fast at Dmrt1 helt klart spiller en meget viktig rolle i kjønnsmodning av hanntorsk.

List of papers

The thesis is based on these following papers. In the text they are referred to by their roman numerals.

Paper I:

Johnsen H., Seppola M., Torgersen J.S., Delghandi M., Andersen Ø. (2010)

Sexually dimorphic expression of *dmrt1* in immature and mature Atlantic cod (*Gadus morhua* L.). Comparative Biochemistry and Physiology, Part B Biochemistry and Molecular Biology 156; 197-205.

Paper II:

Johnsen H., Andersen Ø. (2012)

Differential expression of five *dmrt* genes identified in the Atlantic cod genome. The fish-specific *dmrt2b* diverged from *dmrt2a* before the fish whole-genome duplication.

Accepted in Gene.

Paper III:

Johnsen H., Tveiten H., Torgersen J.S., Andersen Ø.

Sexually dimorphic expression of *amh* and paralogs of *sox9* and *cyp19a* in Atlantic cod (*Gadus morhua* L.). Manuscript prepared for submission to Molecular Reproduction and Development.

1. Introduction

1.1 Some aspects of Atlantic cod farming

Atlantic cod (*Gadus morhua L.*) is a popular food fish that belongs to the family Gadidae of the superorder *Paracanthopterygii*. It is naturally distributed along the continental shelf of a large part of the North Atlantic Ocean, including the Baltic Sea, the North Sea and the Barents Sea. The Northeast Arctic cod (NEAC), also called the Arcto-Norwegian stock is the largest population of Atlantic cod in the world. This migrating stock often referred to as "skrei" are substantially different from non-migrating Norwegian coastal cod (NCC). Besides differences in otolith structure and vertebrae numbers (Loken and Pedersen, 1996; Nordeide and Pettersen, 1998; Sarvas and Fevolden, 2005b) there are genetic differences between the stocks, especially in the nuclear DNA marker pantophysin (*Pan*I) (Fevolden and Pogson, 1997; Sarvas and Fevolden, 2005b; Sarvas and Fevolden, 2005a). Cod has been one of the most important species for fisheries in the North Atlantic Ocean both from an economic and a socioeconomic point of view (Kurlansky, 1998; Rosenlund and Skretting, 2006). However, due to seasonal variations in catches and the decline of certain cod stocks, the future supply may be less than the market demands (Cook *et al.*, 1997; Hutchings, 2000). Farming of Atlantic cod is therefore an attractive solution to covering the world's demand for fresh white fish independent of seasonal variations (Tilseth, 1990).

Early sexual maturation is considered a major problem in Atlantic cod aquaculture (Dahle *et al.*, 2003; Karlsen *et al.*, 2006; Rosenlund and Skretting, 2006; Taranger *et al.*, 2010) as well as problems related to disease and mortality. Under normal farming conditions almost 100% of cod mature by two years of age (Svasand *et al.*, 1996; Karlsen *et al.*, 2006). In comparison the average age when wild NCC and NEAC attain 50% maturity is 5.7 and 6.9 years, respectively (Berg and Albert, 2003). During sexual maturation, the fish undergo a decrease in body weight (Karlsen *et al.*, 1995; Kjesbu *et al.*, 2006) due to energy allocation for gonad development drained from liver and muscle (Kjesbu *et al.*, 1991; Karlsen *et al.*, 2006). At the same time the filet water content increases, hence reducing market value (Trippel *et al.*, 2008). Mortality in

female cod at spawning is considered a welfare problem (Taranger *et al.*, 2010) and spawning in sea cages might lead to unwanted genetic impact on wild cod (Jorstad *et al.*, 2008).

1.2 Several means to one goal

There have been attempts to slow down sexual maturation in Atlantic cod by starvation (Karlsen *et al.*, 1995), photoperiod manipulations (Karlsen *et al.*, 2006; Taranger *et al.*, 2006; Almeida *et al.*, 2009), selective breeding (Kolstad *et al.*, 2006) and triploidy (Peruzzi *et al.*, 2007; Trippel *et al.*, 2008). Experiments with continuous light have delayed gonad development from two/three to five months (Taranger *et al.*, 2006; Trippel *et al.*, 2008) and up to eight months (Karlsen *et al.*, 2006). The introduction of triploidy can accomplish reduced or impaired gonad development (Peruzzi *et al.*, 2007; Trippel *et al.*, 2008; Piferrer *et al.*, 2009). The effect of triploidy on gonad suppression tends to be greater for females than males. In a number of species triploid males are able to produce functional spermatozoa and spawn (Piferrer *et al.*, 2009), including Atlantic cod (Peruzzi *et al.*, 2009; Feindel *et al.*, 2010). The use of triploidy does not solve the negative effects of sexual maturation in males in particular as they undergo hormonal change and the same negative effects of sexual maturation known in diploids (Piferrer *et al.*, 2009). An ongoing research project at Nofima aims to produce sterile cod by knockout of primordial germ cells (PGCs) using injections with antisense morpholinos, which has been successfully achieved in zebrafish (Slanchev *et al.*, 2005) and in cod (unpublished results). Another method undertaken by the same project is to knockdown the formation of PGCs by vaccination of the mother, hence producing gonadless progeny.

Gaining basic knowledge of sexual determination and differentiation is crucial to expanding our knowledge of the evolution of the mechanisms involved. From a practical point of view it is imperative to understand the biological functions to develop new methods of controlling fish reproduction in aquaculture (Devlin and Nagahama, 2002; Piferrer and Guiguen, 2008). Therefore, it is central to study sexual differentiation in Atlantic cod and map key genes and regulators that are part of the process, both in early life and during the reproductive season.

1.3 Early life, gonad differentiation and puberty in Atlantic cod

Atlantic cod is a gonochoristic fish species, meaning that it maintains the same sex throughout its lifespan. The mechanisms of basic development in teleosts are similar, but there are differences in respect to timing of the developmental events (Falk-Petersen, 2005).

1.3.1 Early life and gonad differentiation in Atlantic cod

The stages of embryonic development and timing of organ differentiation in Atlantic cod have been extensively described by Hall *et al*. (2004) and Gorodilov *et al*. (2008) and will only be briefly summarised here (Figure 1). Cod have small, pelagic eggs and spawn in several batches throughout the spawning season (Falk-Petersen, 2005). The following periods include fertilisation, zygote, cleavage, blastula, gastrula and segmentation when new somites are added in a linear fashion as a function of time until just before hatching. There is no distinct pharyngula period in cod. Hatching occurs relatively synchronous and is commenced after the last somites are completed and most body organs are developed (Hall *et al.*, 2004). The yolk sac period (eleutheroemryonic period) is the last stage of embryonic development (Morrison, 1993) and the hatchling is upside down. As the size of the yolk sac diminishes the embryos reorientate to the adult position (Hall *et al.*, 2004). The intake of exogenous feed starts about day 5 or 6 and the fish becomes dependent on external food around day 9 (Morrison, 1993). The larval period is initiated by the first exogenous feeding. Metamorphosis is the gradual process in which larval morphology and anatomy is matured into the juvenile structure and function. The juvenile period is reached at 20-30 mm length when the fins are fully developed (Falk-Petersen, 2005). The adult period starts with the onset of gonad maturation (Jobling, 1995).

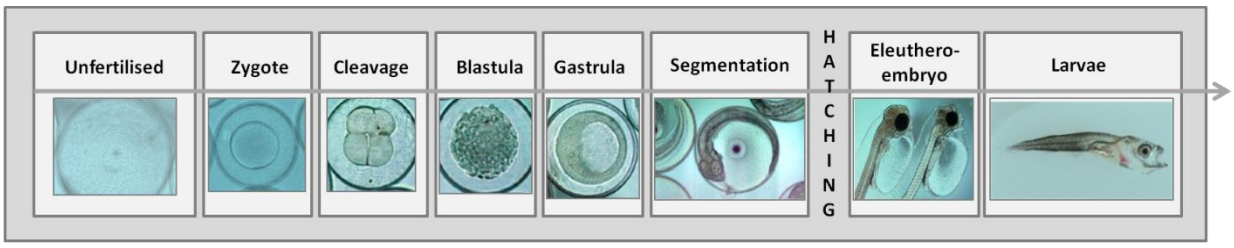

Figure 1: Timeline of early life history of Atlantic cod from fertilisation until the larvae stage. Each stage is named according to Hall *et al.* (2004) and Gorodilov *et al.* (2008).The photos from unfertilised to hatchlings were kindly provided by Saskia Mennen, while the larvae photo was taken by Frank Gregersen.

During vertebrate embryogenesis the bipotential gonad develops into either testis or ovary (Brennan and Capel, 2004). All gametes originate from PGCs (Wylie, 1999), which are highly specialised cells that migrate to the region of the presumptive gonad early in embryogenesis (Devlin and Nagahama, 2002). Histological findings in Atlantic cod have revealed that the pronephric ducts, the precursors of kidney, bladder and reproductive organs are visible at the 30-somites stage (Hall *et al.*, 2004). A few germinal cells forming the gonad were visible on the ventral side of the pronephric ducts during the yolk sac stage (Morrison, 1993). In 18 mm total length (TL) (84 days post hatch (dph)) Atlantic cod larvae gonads were long and threadlike with a few visible PGCs (Chiasson *et al.*, 2008). At 19 mm TL (90 dph) gonads were larger, pear shaped with an increased number of PGCs. Gonad differentiation occurred between 27 and 35 mm TL (102-112 dph). At 35 mm TL gonads could be divided into two groups based on morphology. There are however indications that gonad differentiation in Atlantic cod might start prior to 16 mm TL (Haugen *et al.*, 2011).

1.3.2 Puberty in Atlantic cod

The key component in regulating vertebrate puberty is the brain-pituitary-gonad axis (Weltzien *et al.*, 2004). The onset of vitellogenesis and spermiogenesis in cod starts between September-November depending on water temperature (Kjesbu, 1994; Almeida *et al.*, 2008) and continues for approximately six months. The structure of fish gonads is comparable to that of other vertebrates, with a mixture of germ cells and associated somatic support cells (Devlin and Nagahama, 2002) summarised in figure 2. In females, the ovaries are paired structures located in the posterior part of the body cavity and are made up of germ cells, oogonia, oocytes, granulosa cells and theca cells. Steroidgenesis takes place in the latter two cell types. The stage of maturity can be described by the size distribution of oocytes (Kjesbu *et al.*, 1991; Dahle *et al.*, 2003).

In males, the paired testes consist of several lobes around a central collecting duct. In each lobe, spermatogenesis occurs in a gradient of development, with undifferentiated spermatogonia at the periphery and the mature spermatids close to the collecting duct. Spermatogenesis involves initial proliferation of spermatogonia by 11 mitotic divisions and maturation occurs in a wave that progresses through each growing lobe (Almeida *et al.*, 2008). The sertoli cells main function

is to support germ cell survival, development and physiological function, while Leydig cells are involved in the synthesis of androgens (Schulz *et al.*, 2010).

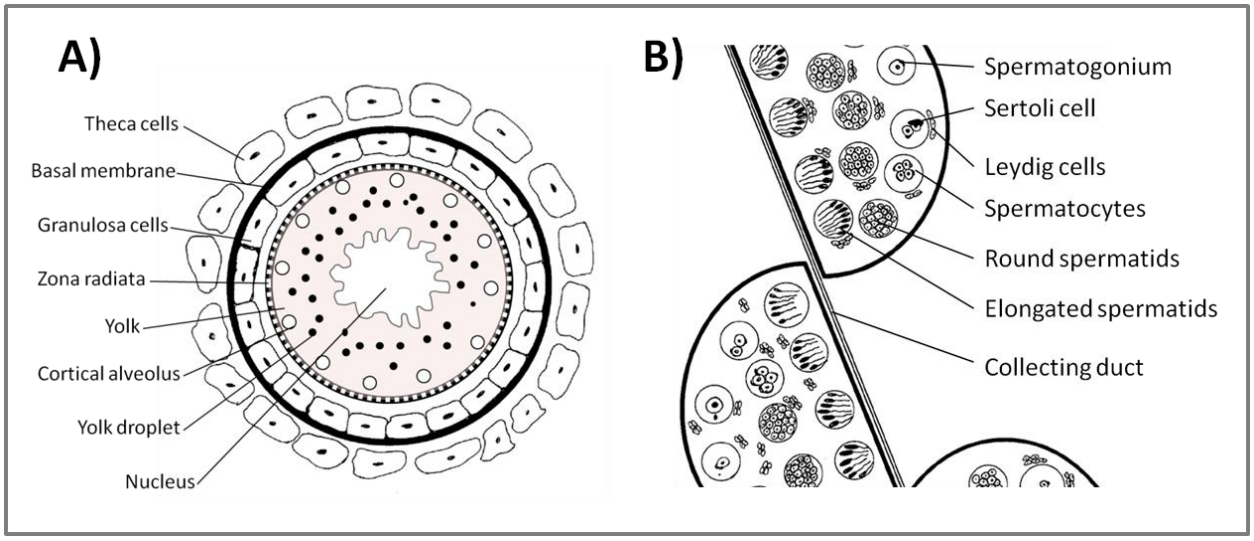

Figure 2: Schematic drawing of A) oocyte and B) testis lobes of Atlantic cod. Figure A is modified from Andersen (1992) and shows the primary oocyte surrounded by the follicle consisting of granulosa and theca cells. Figure B portrays the wave of maturation that happens through each growing lobe in cod as described in Almeida *et al.* (2008). Drawings by Hanne Johnsen.

Figure 3 illustrates the enormous growth of the cod gonads through the reproductive season. The immature ovary and testes (3A and B) are very small compared to maturing ovary and testis (3C and D). Although the maturing fish in images C and D were not ready to spawn, the gonads filled much of the abdominal cavity. Atlantic cod are "broadcast" spawners releasing large amounts of eggs without providing parental care, while spawning involves a complex range of behaviours in both sexes which includes courtship and sound (Hutchings *et al.*, 1999; Rowe and Hutchings, 2006).

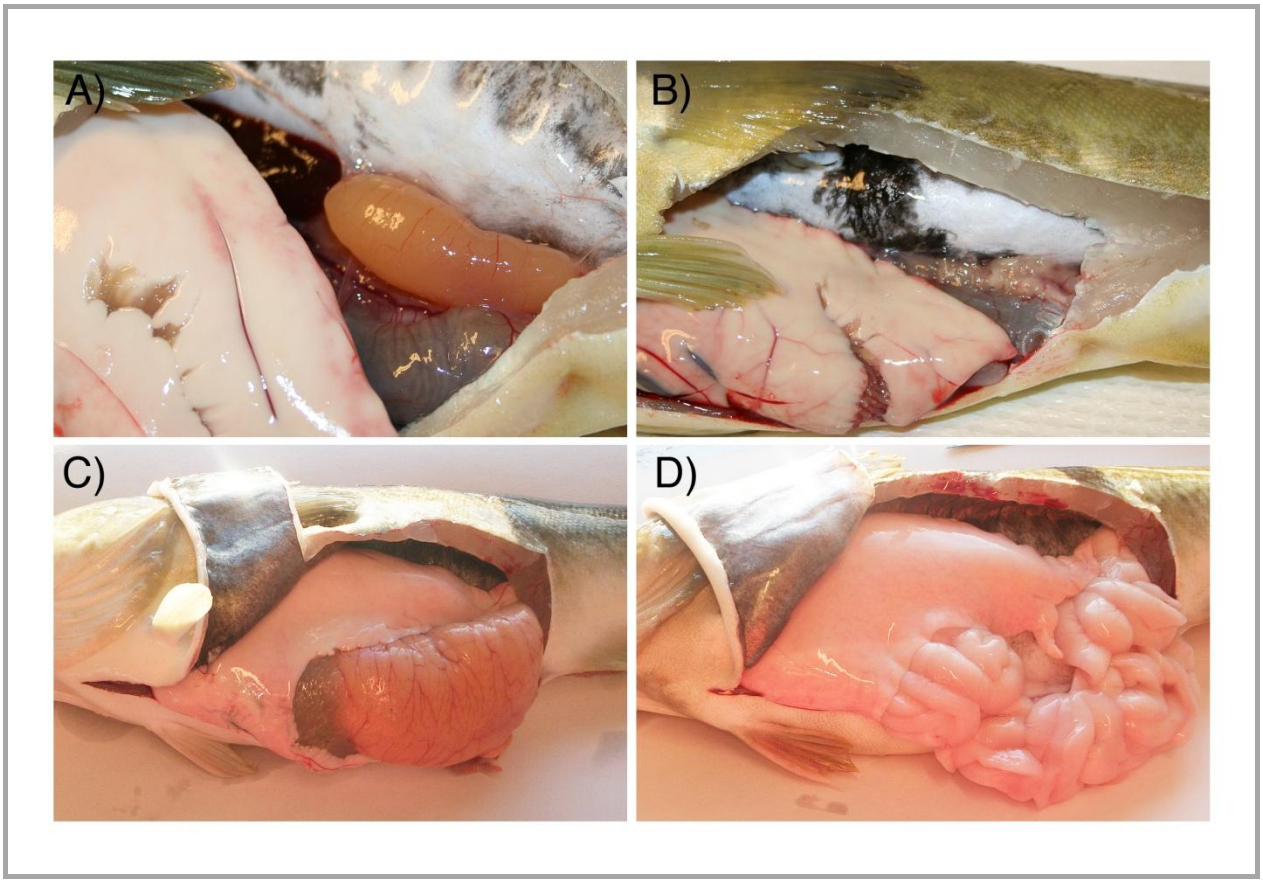

Figure 3: Pictures of immature A) ovary and B) testes of ~500g cod captured in September and of maturing C) ovary and D) testis of ~1 kg cod captured in February. Photos by Hanne Johnsen.

1.4 Sexual determination and differentiation

Sexual determination can be defined as the genetic or environmental process that determines the sex of an individual, while sexual differentiation refers to the development of the gonad after the determination of sex (Hayes, 1998; Sandra and Norma, 2010). Different from the conserved morphological changes, sex determination mechanisms vary considerably in the different vertebrate groups and even between closely related species. All sexual determination mechanisms that have been reported in vertebrates are also present in fish, however the processes of sexual determination and differentiation in fish are highly plastic and can be influenced by environmental factors such as temperature, pH and social interactions (Devlin and Nagahama, 2002). In temperate areas the main environmental factors controlling reproduction of fish are photoperiod and/ or temperature (Taranger *et al.*, 2010). In gonochoristic fish species one can

observe all possible kinds of genetic sex determination, from the uncomplicated male- female heterogametes in some cases with the influence of autosomal genes to more complicated systems that involve several loci in the absence of sex chromosomes or involving several pairs of sex chromosomes (Devlin and Nagahama, 2002; Sandra and Norma, 2010).

Some of the key genes and regulators of sexual differentiation and gonad maturation have been the main focus of this thesis and these will be further described in the following section.

1.4.1 Key regulators of sexual determination and differentiation

Only two genes of vertebrate sex determination have been identified to date, the non-homologous mammalian *Sry* (Sex determining region Y) and the medaka (*Oryzias latipes*) *dmrt1* (Doublesex/Mab3-related transcription factor 1) duplication, *dmy* distinctive to the Y chromosome (Koopman, 2001; Matsuda *et al.*, 2002; Nanda *et al.*, 2002; Siegfried, 2010). In birds, the homogametic males carry the ZZ pair of sex chromosomes while the heterogametic female carries the ZW pair. There is evidence that the Z linked *Dmrt1* is the master regulator required for testis development in chicken (Smith *et al.*, 2009; Siegfried, 2010). An hypothesis concerning sexual determination and differentiation is that genes at the top of the hierarchy such as *Sry*, have become involved in this process relatively recently and are not conserved, whereas at least some of the downstream genes have been present for much longer and are well conserved in a wide variety of species (Marin and Baker, 1998; Herpin and Schartl, 2011).

The Doublesex/ Mab3-related transcription factor (*dmrt*) gene family

Although teleost fishes portray a wide variety of sexual determination and differentiation systems, the core of the cascade is well conserved in fishes. The transcription factor Dmrt1 is considered a downstream regulator in testis differentiation across vertebrates. Its expression seems to be crucial for the development of the male phenotype, demonstrated by male-biased expression of *dmrt1* in all vertebrate groups. It has been described in a wide variety of metazoans (Zhu *et al.*, 2000; Hodgkin, 2002), including teleosts where it has been described in several species (Smith *et al.*, 1999a; Guan *et al.*, 2000; Kettlewell *et al.*, 2000; Marchand *et al.*, 2000; Nanda *et al.*, 2002; Pask *et al.*, 2003; Veith *et al.*, 2006), including Atlantic cod (Paper I). The

dmrt1 gene encodes a protein containing a DM domain that is highly conserved across phyla (Raymond *et al.*, 1998; Raymond *et al.*, 2000; Hodgkin, 2002). Genes encoding the DM domain have been described in fruitfly (*Drosophila melanogaster*) as *dsx* (*doublesex*) and in roundworm (*Caenorhabditis elegans*) as *mab-3* (Baker and Wolfner, 1988; Raymond *et al.*, 1998). The DM domain contains a characteristic double zinc finger motif for DNA binding, that unlike classic zinc fingers binds to the minor groove of DNA rather than the major groove (Zhu *et al.*, 2000; Murphy *et al.*, 2007). Putative downstream targets of DM family members are largely unexplored (Hodgkin, 2002; Hong *et al.*, 2007; Murphy *et al.*, 2010; Herpin and Schartl, 2011). However, recent findings suggested that mammalian Dmrt1 is a bifunctional transcriptional regulator meaning it can activate some genes while repressing others. Also auto- and cross regulation has been indicated as Dmrt1 can bind to its own promoter as well as that of Dmrt3, Dmrt4, Dmrt5, Dmrt7 and Dmrt8 (Murphy *et al.*, 2010). This regulation might be conserved in other vertebrates due to negative auto- and cross regulation of Dmy by Dmrt1 in medaka (Herpin *et al.*, 2010). In mammals *dmrt1* has been established as a key regulator of spermatogonial development since it was found to determine whether male germ cells undergo mitosis or meiosis (Matson *et al.*, 2010).

In vertebrates, the following DM family genes have been identified so far; *dmrt1*, *dmrt2a/terra*, *dmrt2b*, *dmrt3*(*A3*), *dmrt4*(*A1*), *dmrt5*(*A2*), *dmrt6*(*B1*), *dmrt7*(*C2*) and *dmrt8*(*C1*). The latter two found only in mammals (Hong *et al.*, 2007), *dmrt6*(*B1*) only in tetrapods (Veith *et al.*, 2006) and *dmrt2b* found only in teleosts. Human *dmrt1* is clustered with *dmrt2* and *dmrt3* on the distal 9p, which is deleted in patients suffering from 46, XY sex reversal (Ottolenghi *et al*., 2000; Barbaro *et al*., 2009). Although not clear which genes or genetic mechanisms are involved in the impaired gonad development, the major candidate genes are *dmrt1* and *dmrt3* (Barbaro *et al.*, 2009).

The *dmrt* gene family may be part of other developmental processes since the pattern of expression is not exclusive to the gonads (Hong *et al.*, 2007). The first evidence that DM family members had extragonadal roles was indicated by the identification of the expression pattern of *terra*, homologue to *dmrt2a*, in the presomitic mesoderm and the developing somites in zebrafish (Meng *et al.*, 1999; Hong *et al.*, 2007). Five of the vertebrate *dmrt* genes have been identified in extragonadal tissues, such as the central nervous system (*dmrt3*, *dmrt4*, *dmrt5* and *dmrt6* in mouse, chicken, frog and fish), nasal placodes (*dmrt3* in mouse and chicken, *dmrt4* in frog and

platyfish and *dmrt5* in platyfish) and in somites (*dmrt2/terra* in mouse, chicken and fish and *dmrt3* in chicken) reviewed in Hong *et al.* (2007).

SRY-related high mobility group (HMG) containing box gene 9 (*sox9*)

The SRY-related high mobility group (HMG) containing box (*sox*) family of transcription factors is involved in numerous developmental processes including sexual differentiation (Piferrer and Guiguen, 2008) and Sox9 has been connected to male sexual development across vertebrates (Siegfried, 2010). It has been identified in several teleosts and two *sox9* paralogs have been described in some species including zebrafish, stickleback, fugu, medaka, rainbow trout and rice field eel (Chiang *et al.*, 2001; Cresko *et al.*, 2003; Zhou *et al.*, 2003; Koopman *et al.*, 2004; Nakamoto *et al.*, 2005; Vizziano *et al.*, 2007). The Sox9 transcription factor contains a conserved HMG-box domain consisting of three α-helices and connecting loops that bind in the DNA minor groove (Weiss, 2001) and show 50% identity to the HMG box of Sry (Prior and Walter, 1996). In mammals, expression of *sox9* in the absence of *sry* is enough to ensure male development, however, in non-mammalian vertebrates, the role of *sox9* is less clear (Siegfried, 2010). In adult zebrafish, the expression of *sox9a* was detected in testis, brain, kidney and muscle and *sox9b* only in ovary (Chiang *et al.*, 2001; Rodriguez-Mari *et al.*, 2005). In mammals, *sox9* has been found to be a direct target of *sry* and to upregulate the expression of *amh* (Sekido and Lovell-Badge, 2009; Sekido, 2010).

Anti-Müllerian hormone (*amh*)

Anti-Müllerian hormone (Amh), also known as Müllerian inhibiting substance (Mis) or Müllerian inhibiting hormone (Mih), is a member of the transforming growth factor β (Tgf-β) superfamily (Josso *et al.*, 2001; Piferrer and Guiguen, 2008; Sandra and Norma, 2010; Siegfried, 2010). In mammals, the main role of Amh is the regression of Müllerian ducts (Vigier *et al.*, 1989; Josso *et al.*, 2001; Sandra and Norma, 2010; Josso, 2011) that in females differentiate to fallopian tubules and uterus (Munsterberg and Lovell-Badge, 1991; Rodriguez-Mari *et al.*, 2005). It is expressed in testes by Sertoli cells from the time of differentiation until puberty and in ovaries by granulosa cells from birth until menopause (Josso *et al.*, 2001; Josso, 2011). Fish lack Müllerian ducts, but still show Sertoli cell expression of *amh* (Siegfried, 2010), indicating

that *amh* might be important for other aspects of gonad development and function (von Hofsten and Olsson, 2005). In mammals, Amh is a direct target of Sox9 (De Santa Barbara *et al.*, 1998; Brennan and Capel, 2004) and it inhibits the expression of aromatase (*cyp19a1a*) (Rouiller-Fabre *et al.*, 1998). A similar pattern of regulation has been described in zebrafish (Rodriguez-Mari *et al.*, 2005), while the expression of *amh* precedes that of *sox9* in chicken and alligator (Smith *et al.*, 1999b; Western *et al.*, 1999). In trout, expression of *amh* is significantly repressed by testosterone (T) and 11-keto testosterone (11-KT) (Schulz *et al.*, 2010).

Cytochrome P450, family 19, subfamily A, polypeptide 1 (*cyp19a1*)

The major products of all vertebrate gonads are sex steroids. The activity of the enzymes that synthesise steroids is of great importance for the regulation of the type and quantity of steroid hormones (Piferrer and Guiguen, 2008). The gene *cyp19a1* encodes the enzyme cytochrome P450 aromatase (P450arom), which is a key factor in converting androgens to estrogens (Simpson *et al.*, 1994; Payne and Hales, 2004; Guiguen *et al.*, 2010; Sandra and Norma, 2010), hence controlling the balance of the sex steroids and is therefore of great importance to sexual differentiation (Piferrer and Guiguen, 2008; Siegfried, 2010). In tetrapods the gene is present as a single copy, however teleost fish have two *cyp19a1* paralogues, *cyp19a1a* mainly expressed in the female gonads and *cyp19a1b* predominantly expressed in the brain (Chang *et al.*, 2005; Barney *et al.*, 2008; Patil and Gunasekera, 2008; Piferrer and Guiguen, 2008; Siegfried, 2010). Sex steroids are primarily produced in the gonads (Schulz *et al.*, 2010). In fish the sex steroid 17β-estradiol (E2) is known to induce and maintain ovarian development and the levels are much higher in females compared to males. Testis development is largely regulated by 11-ketotestosterone (11KT) (Sandra and Norma, 2010). In rare minnow, pejerrey and zebrafish exposure to estrogen resulted in cessation of male gonad development and sex reversal correlated with pronounced decrease in *dmrt1* expression (Schulz *et al.*, 2007; Fernandino *et al.*, 2008; Zhang *et al.*, 2008). In tilapia Dmrt1 suppresses the female pathway by repressing aromatase gene transcription and thus estrogen production (Wang *et al.*, 2010). Combined, there may be a feedback loop between *dmrt1*, *cyp19a1a* and by implication the estrogen/androgen balance (Herpin and Schartl, 2011).

Another transcription factor associated with sexual determination and differentiation is Foxl2 (forkhead box L2), a member of the winged helix/forkhead group (Piferrer and Guiguen, 2008). It is the earliest known sex dimorphic marker of ovarian differentiation in vertebrates (Wang *et al.*, 2004). Fish have two paralogues of *foxL2* genes (Baron *et al.*, 2005; Sandra and Norma, 2010). Its function is closely linked to upregulating the expression of aromatase (*cyp19a1*) (Wang *et al.*, 2007) as it has been characterised as an upstream regulator of the *cyp19a1* promoter (Guiguen *et al.*, 2010). In mammals FoxL2 binds directly to TESCO, the enhancer element of *sox9*, resulting in reduced *sox9* expression in ovaries (Uhlenhaut *et al.*, 2009; Siegfried, 2010). In other words, FoxL2 and Sox9 have opposing roles in establishing and maintaining the various female and male gonadal cell types respectively (Uhlenhaut *et al.*, 2009). The mechanisms of sex determination and sex differentiation in fish are briefly summarised in figure 4.

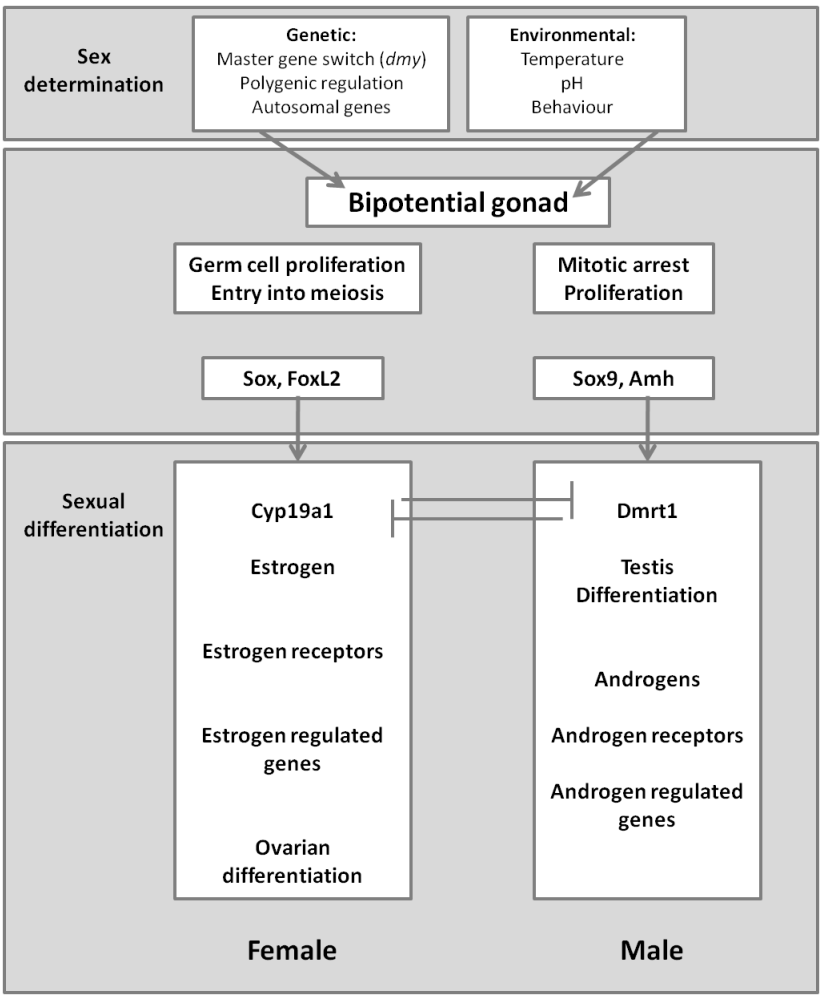

Figure 4: Diagram of sex determination and differentiation in fish.

The figure does not represent any particular species, but presents a consensus on some of the current knowledge. Both Cyp19a1 and Dmrt1 occupy a central position in sex differentiation in fish and may antagonise each other. The time frames of female and male development portrayed in this figure are independent and should not be compared. Modified from Piferrer and Guiguen (2008).

1.5 The evolution of duplicated genes

Identifying highly conserved chromosomal regions is an important tool in tracing complex ancient evolutionary processes inaccessible by other approaches. This approach has demonstrated that great numbers of cellular processes are shared across immense phylogenetic distances. The phenomenon of gene duplication was, in 1970, suggested to be one of the main mechanisms driving the evolution of vertebrates (Ohno, 1970; Ohno, 1999; Zhang, 2003). It was later indicated that the vertebrate genome had undergone two events of whole-genome duplications (WGDs) preceding the divergence of vertebrates into tetrapods and teleosts, often referred to as the 2R hypothesis or the 1-2-4 rule (Meyer and Schartl, 1999; Ohno, 1999; Wolfe, 2001; Hokamp *et al.*, 2003; Robinson-Rechavi *et al.*, 2004). In 1993 Lars G. Lundin published evidence of chromosome duplication with four sets of paralogous chromosomal regions in man and mouse (Lundin, 1993) and the Hox-gene cluster later provided molecular genetic evidence for the 2R hypothesis with one cluster in amphioxus and four paralogous clusters in tetrapods (Garcia-Fernandez and Holland, 1994; Holland *et al.*, 1994). Although the 2R hypothesis has been the subject of controversy (Hughes, 1999; Hughes *et al.*, 2001; Pennisi, 2001), evidence has been accumulating that supports the hypothesis (Spring, 1997; Abi-Rached *et al.*, 2002; Larhammar *et al.*, 2002; Lundin *et al.*, 2003; Dehal and Boore, 2005; Meyer and Van de Peer, 2005) and finally conclusive evidence for two rounds of genome duplication on the stem line of jawed vertebrates (Putnam *et al.*, 2008).

The first round (1R) of WGD has been suggested to predate the Cambrian explosion (Meyer and Schartl, 1999). This probably took place prior to the divergence of jawless fish while the second WGD may have occurred after the jawless fish diverged (Ohno, 1999; Panopoulou and Poustka, 2005). It is unclear exactly when the WGDs occurred relative to the diversification of jawless fish, as there have been debates over the monophyly of cyclostomes (hagfish and lampreys) (Takezaki *et al.*, 2003) as well as the timing relative to the cyclostomes-gnathostome split (Kuraku, 2008; Kuraku *et al.*, 2009). Several lines of evidence suggest that a third whole-genome duplication (3R) took place in the stem lineage of teleosts after the split from tetrapods, estimated to have taken place ~350 million years ago (Meyer and Schartl, 1999; Taylor *et al.*, 2003; Meyer and Van de Peer, 2005). In salmonids and cyprinids a fourth genome duplication event has probably occurred (Allendorf and Thorgaard, 1984; Meyer and Schartl, 1999).

Although the genome duplications have increased the number of genes, this do not necessarily mean that fish possess eight times as many genes as invertebrates. Gene silencing is known to be frequent after gene and genome duplications (Wagner, 1998; Lynch and Conery, 2003), meaning that many duplicated genes are lost during evolution. With respect to phenotypes, there is probably no good reason for fish to have more genes than mammals. It has however been suggested that the complexity in the genomic architecture of fish has permitted them to adapt quickly in response of changing regimes (Wittbrodt *et al.*, 1998) and this may serve as an explanation of their evolutionary success and diversity (Meyer and Schartl, 1999).

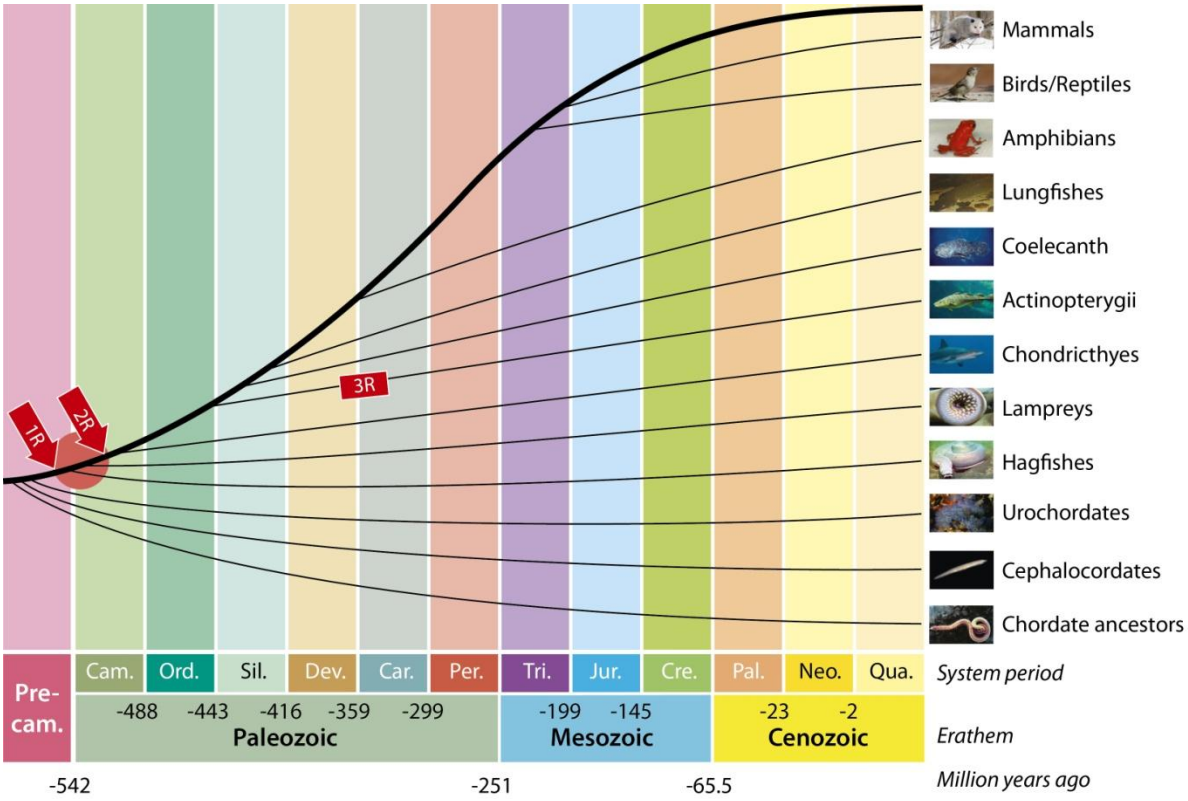

Figure 5: The evolution of vertebrates seen in context of the proposed whole genome duplications. The Erathem, system periods and million years are listed after the International commission on Stratigraphy (http://www.stratigraphy.org). The branchings of the subphyla are drawn in accordance with evolutionary timescales (Kumar and Hedges, 1998; Clark, 2002; Blair Hedges and Kumar, 2003). The assumed time points of 1R/2R are shown by red arrows and the underlying red spot marks the area debated by Kuraku *et al*. (2009). The putative timepoint of the fish specific WGD (3R) is indicated with a red box on the *Actinopterygiian* lineage. The figure was modified from Larhammar *et al.* (2009) and (http://www.biologycorner.com/resources/vertebrates_evolution.jpg), by Hanne Johnsen and Oddvar Dahl.

2. Aim of the thesis

The general purpose was to clarify several aspects of sexual determination, differentiation and gonad maturation in Atlantic cod. It was considered important to gain basic knowledge of several genes involved in early life sexual differentiation and gonad development through the reproductive season.

The major aims were:

1) Molecular characterisation of Atlantic cod Dmrt1 with emphasis on expression differences between the sexes (Papers I and II).

2) Examination of the origin and divergence of the Atlantic cod Dmrt members by phylogeny and synteny analyses (Paper II).

3) Study the expression of key genes involved in sexual differentiation and gonad development in Atlantic cod during early life and during the reproductive season (Papers II and III).

3. General summary

Paper I

Sexually dimorphic expression of *dmrt1* in immature and mature Atlantic cod (*Gadus morhua* L.)

A key factor implicated in testes development across vertebrates the Doublesex and mab-3 related transcription factor 1 (Dmrt1) was mapped in Paper I for the first time in a species of the superorder *Parachantopterygii*. The predicted cod Dmrt1 of 310 amino acids contained a highly conserved DM domain, including six Cys residues probably involved in the formation of a double zinc-finger motif for DNA binding. The tissue expression analysis revealed that *dmrt1* was exclusively expressed in gonads and sexually dimorphic expression of *dmrt1* was evident by using qPCR with significantly higher expression in males compared to females. The signal was localised to the germ cells in both genders by *in situ* hybridisation. Although significantly less expressed in the ovary, Dmrt1 might also play a role in oogenesis. Southern blot analysis revealed several DM domain-containing genes in the cod genome, but no sex-linked polymorphism was shown.

Working with Paper I it was evident that more time points were needed to further understand gene expression in early life as well as during the reproductive season in a species that undergoes annual spawning.

Paper II

Differential expression of five *dmrt* genes identified in the Atlantic cod genome. The fish-specific *dmrt2b* diverged from *dmrt2a* before the fish whole-genome duplication.

To elucidate the function and evolution of the Dmrt family members in fish, we investigated the expression patterns of five *dmrt* genes in Atlantic cod and examined the chromosomal synteny of *dmrt* flanking genes in different chordate lineages. The developing cod embryos displayed conserved sequential expression of *dmrt4*, *dmrt2a*, *dmrt5*, *dmrt3* and *dmrt1*. Differential expression in the larval head and abdominal segment was consistent with the spatial patterns reported in other teleost species. In contrast, teleosts showed substantial differences in the sex-related expression of the *dmrt* genes in the adult gonads and brain, except for the conserved

male-biased gonad expression of *dmrt1*. Synteny analyses lead to the understanding that the *dmrt2* duplication probably took place prior to the fish specific whole-genome duplication. While the functions of *dmrt2a* and *dmrt2b* associated with somitogenesis diverged in the fish lineage, *dmrt2b* became lost in the tetrapod lineage and probably also in Atlantic cod.

Paper III

Sexually dimorphic expression of *amh* and paralogs of *sox9* and *cyp19a1* in Atlantic cod (*Gadus morhua* L.).

Teleosts exhibit a wide variety of sex determination and sex differentiation mechanisms. The sexual plasticity in gonad and brain development of most species probably involves a combination of genetic and environmental factors. We examined the expression patterns of *amh* and the *sox9* and *cyp19a1* paralogs in Atlantic cod throughout embryogenesis and reproductive season. Both *sox9a* and *sox9b* transcripts were identified shortly after fertilisation, and the *sox9b* signal in the branchial arches and hindbrain of the hatched larvae agrees with the chondrogenic activity of tetrapod Sox9. The bimodal relationship between the levels of *amh* and *cyp19a1a* in the cod embryos may suggests a role in sexual differentiation. During the reproductive season, the ovarian expression of *cyp19a1a* was strongly correlated with the levels of plasma estradiol. In addition, the expression of both *cyp19a1* paralogs in cod testes indicates a conserved role of testicular estrogen in spermatogenesis. The abundant expression of *cyp19a1a* and *cyp19a1b* in the larval and adult brain is compatible with the intense neurogenesis that occurs throughout the life of teleosts. The gonad expression of *sox9a* was restricted to males, while male-biased expression of *sox9b* was shown in the mature gonads, in agreement with the proposed role of the Sox9 paralogs in testicular differentiation.

4. Discussion

4.1 Some general aspects

One reason why the understanding of sexual differentiation in Atlantic cod is complicated arises from the inability to distinguish between the sexes morphologically and genetically at an early time point. By using histology it is possible to differentiate between the sexes of Atlantic cod of 35 mm TL (16 wph) (Chiasson *et al.,* 2008). There are however indications that gonad differentiation might start prior to 16 mm TL as the appropriate time window for masculinisation appeared to start at 12 mm TL (Haugen *et al.*, 2011).

The lack of distinct sex chromosomes reported in Atlantic cod (Ghigliotti *et al.*, 2011) makes the use of genotyping in sex determination difficult. However, the successful production of 100% females with sperm from sex-reversed cod females provides evidence for female homogamety in Atlantic cod (Haugen *et al.*, 2011). Similarly, most experimental data point towards the lack of sex chromosomes in zebrafish (Orban *et al.*, 2009), while all-female populations have been successfully produced (Tong *et al.,* 2010). However, female heterogamety was suggested in zebrafish due to an inability to form all females in the F1 generation.

The background for the selection of time points used for early life gene expression analyses in paper II and III were based on the fact that histological changes must have been preceded by molecular changes. As an appropriate protocol to create a monosex-stock was lacking at the time of sampling, it was decided to use a natural mixed-sex group which had recently (to sampling) been described in zebrafish by Jorgensen *et al.* (2008).

Taken together Atlantic cod may possess a multigenetic sex determination system that is highly plastic in that sex can be altered via external factors such as hormone treatment. To control the onset of puberty and/ or create a sterile population the underlying molecular mechanisms need to be further understood and this thesis fills in some of the gaps in the knowledge of genes involved in sexual differentiation and gonad development in Atlantic cod.

The results of this thesis are discussed in detail in Papers I-III, but some selected and updated topics will be discussed further in the following sections.

4.2 Dmrts in Atlantic cod (Paper I and II)

The full length Atlantic cod *dmrt1* cDNA was cloned and sequenced in Paper I. The *dmrt1* sequence of 1980 nts corresponded to a protein coding region of 933 bps flanked upstream by a 5`UTR of 87 bps and downstream by a 3`UTR of 960 bps. Three alternative polyA signals were identified in the 3`UTR, which may mean that three different transcripts can be produced by polyadenylation, two of which were identified in Paper I. Alternative splicing increases the transcriptome diversity and has been suggested to play a major role in genome evolution allowing new exons to evolve with less constraint (Boue *et al.*, 2003). Alternative splicing of the 3´end of *dmrt1* has been reported in several species, including rice field eel, mouse, chicken, zebrafish and Indian mugger (Guo *et al.*, 2005; Huang *et al.*, 2005; Lu *et al.*, 2007; Zhao *et al.*, 2007; Anand *et al.*, 2008). In all the reported species the transcripts were primarily expressed in testes and the non-spliced *dmrt1* transcript was predominantly expressed. It is rare that alternative splicing consistently occurs at the 3´end of a gene and it has been suggested that this may also be one of the characteristics of *dmrt1* conserved across phyla (Zhao *et al.*, 2007). The nine g/atgc repeats identified in the 3´UTR of Atlantic cod *dmrt1* strongly resembles the *dmrt1* 3´UTR *cis*-regulatory motif (CTGCTGCAGGT-consensus) well conserved from ecdysozoans to mammals (Herpin *et al.*, 2009). Intriguingly, a male- and female- associated allele was detected within the *dmrt1* 3`UTR *cis*-regulatory element in zebrafish and it was predicted that the female associated allele would yield lower *dmrt1* expression relative to the male allele (Bradley *et al.*, 2011). Although well described in many species, knowledge of how Dmrt1 functions as a transcription factor and its target genes is limited. However, some information has surfaced in recent years. In mammals, Dmrt1 was revealed as a bifunctional transcriptional regulator (Murphy *et al.*, 2010). A large number of promoter proximal regions were bound by Dmrt1 in the developing mouse testis. Auto- and cross regulation was indicated as it bound to its own promoter and the promoter of six other *dmrt* genes. It has been suggested that Dmrt1 in *Xenopus* directly represses the expression of *foxL2* and/ or *cyp19a1* (Yoshimoto *et al.*, 2010). In mice deletion of *dmrt1* in both fetal and adult males lead to increased expression of *foxL2* (Matson *et al.*, 2011). The target genes of Dmrt1 in Atlantic cod are candidates for further investigation and may clarify several questions related to sex determination and differentiation in this species.

Southern blot analyses were conducted to search for sequence polymorphism and additional DM domain genes. The existence of additional DM genes were suggested and *in silico* analyses of the

draft cod genome sequence (Star *et al.*, 2011) identified four *dmrt*s in addition to *dmrt1*. Regrettably, the nomenclature in Paper I is not identical with that used in Paper II. In Paper I the additional Dmrts were named Dmrt2a, Dmrt2b, Dmrt3 and Dmrt4 based on BLAST searches. When working with the sequences for Paper II it was clear that the sequence initially named Dmrt2a was in fact Dmrt5 and the one first named Dmrt2b was actually Dmrt2a. This is a good example of why BLAST search alone is insufficient when working with unknown sequences. Further searches of the cod genome sequence gave no support for a fish-specific Dmrt2b in the Atlantic cod genome. The nomenclature of the *dmrt* gene family may be subject to confusion as the synonymous names appear in some publications and GenBank annotations, while lack in others. In this thesis it was decided to use the nomenclature described in Volff *et al*. (2003), Hong *et al*. (2007) and also in a recent review by Herpin and Schartl (2011). However, when appropriate the synonymous names are given in parenthesis to avoid misunderstandings. The names in question are *dmrt3* (A3), *dmrt4* (A1), *dmrt5* (A2), *dmrt6* (B1), *dmrt7* (C2) and *dmrt8* (C1). All five cod Dmrt proteins display the characteristic well conserved DM domain (Paper II). The putative putative nuclear localization signal KGHKR adjacent to the intertwined CCHC and HCCC zinc binding sites are well conserved across the examined species and between the different Dmrt sequences. However, each of the Dmrt members appears to have a specific signature of conserved residues in the DM domain of the different Dmrt sequences.

In Paper I a phylogenetic tree was constructed using Dmrt1 sequences from a selection of teleosts, amphibians, mammals and a single bird. The clusters of tetrapods and teleosts segregated with high bootstrap confidence values. Within the *A*ctinopterygiian species the branches in general clustered according to the established phylogeny as noted by the superorders on the figure. Within the superorder *Acanthoperygii* the order *Beloniformes* to which teleosts of the genus *Oryzias* belong to, segregated with 100 percent bootstrap confidence value. Also the *Tetradontiformes* to which fugu and *Tetradon* belong separated with high bootstrap value. Many of the nodes segregated with low bootstrap confidence value, such as Atlantic cod and rainbow trout. When a new tree was constructed using the method described in Paper II and all branches with bootstrap confidence values less than 70 percent were collapsed the result was the following tree portrayed in figure 6. The tree followed the general topology as described in Paper I. Tetrapods and teleosts segregated with high bootstrap confidence values. Within the various superorders, the orders *Beliniformes*, *Tetradontiformes* and *Siluriformes* segregated with 100%

bootstrap confidence values. One of the reasons why the node harboring the Atlantic cod Dmrt1 showed low bootstrap values may simply be due to the lack of available Dmrt1 sequences from other *Gadiformes* and *Parachantopterygian* species. All the Drosophila sequences were outgroups to the Dmrt1 sequences from tetrapods and teleosts.

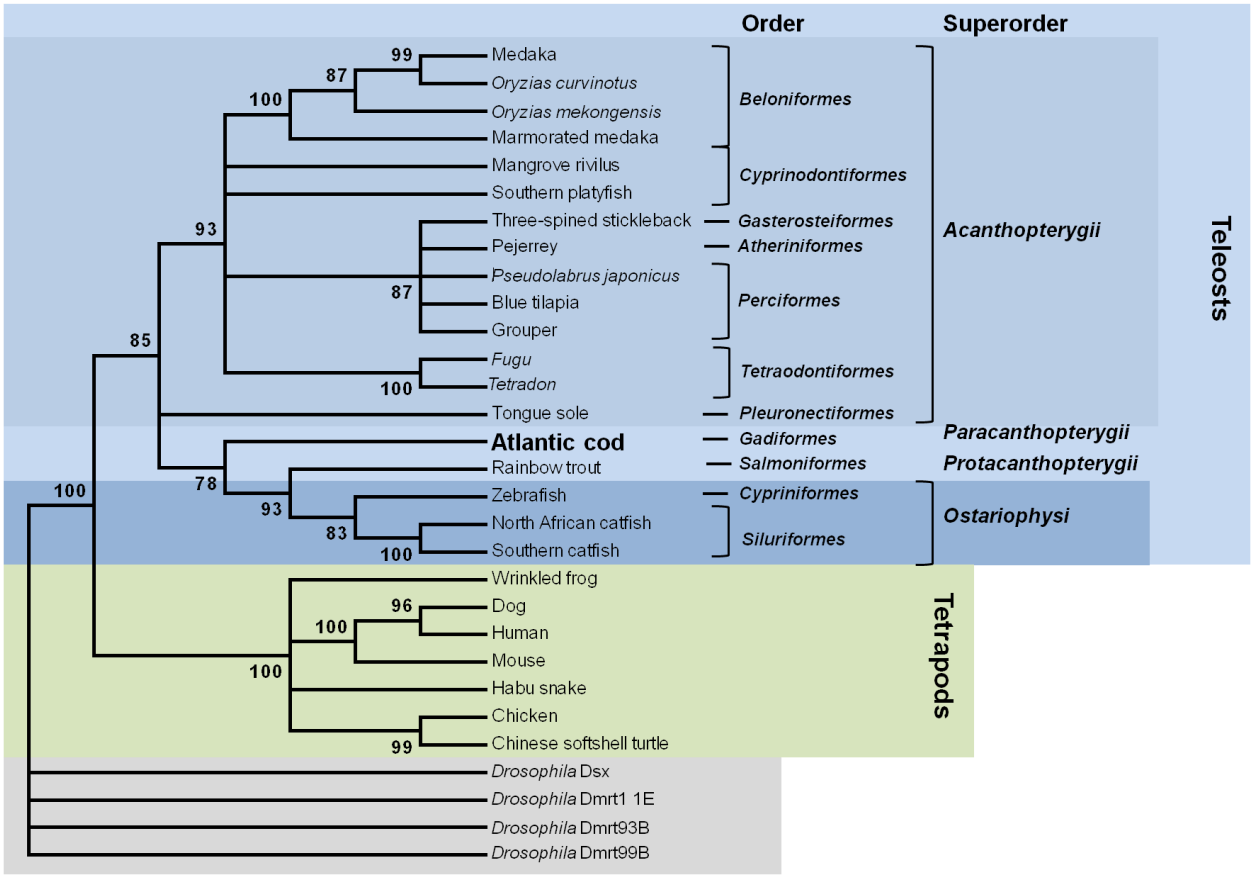

Figure 6: Phylogenetic tree showing the relationship between Dmrt1 sequences found in teleosts, tetrapods and *Drosophila*. The tree was constructed in Mega5 as described in Paper II. GeneBank accession numbers for teleosts and tetrapods can be found in figure legends 2 and 3 in Paper I while the GenBank accession numbers for the *Drosophila* sequences can be found in figure legend 2 in Paper II.

In Paper II, the phylogeny analyses of Dmrt1, Dmrt2 (Dmrt2a and Dmrt2b), Dmrt3, Dmrt4 and Dmrt5 showed that the different Dmrts clustered off with high bootstrap confidence values. The expected topology was in general followed, with one exception. Xenopus Dmrt2 separated with low bootstrap values, and did not cluster with the tetrapod clade as would be expected. Interestingly, the clusters harbouring Dmrt4 (A1) and Dmrt5 (A2) branched off together with 99% bootstrap confidence value and then separated to the Dmrt4 cluster and Dmrt5 cluster with

74% and 100% bootstrap confidence values respectively. The topology raises the question whether this may be due to a duplication event. All the Drosophila sequences as well as Amphioxus Dmrt-like and human Dmrt6 (B1), Dmrt7 (C2) and Dmrt8 (C1) were outgroups to the Dmrt2 (a and b), Dmrt1, Dmrt3, Dmrt4 and Dmrt5 clusters.

To further understand the evolution of the Dmrts, synteny analyses were performed searching for orthologous genes in the genome of diverse teleosts, tetrapods and Amphioxus. The *dmrt2a, dmrt3 and dmrt1* are closely linked in the genome of Atlantic cod, three-spined stickleback, medaka, pufferfish and zebrafish. The gene cluster shared conserved synteny with human chromosomes 4q and 9p. Duplicates of several flanking genes were found to be closely linked in a paralogous region lacking the *dmrt2a-dmrt3-dmrt1* cluster in the teleosts examined. The fish-specific *dmrt2b* gene was also found to be closely linked to genes sharing conserved synteny between the teleosts examined. The *dmrt2b* flanking genes in teleosts shared highly conserved synteny with human 1p34.2-q31.1, which also harbours *dmrt5* and *dmrt6*. Many of the syntenic genes were identified in two paralogous regions mapped to separate chromosomes or scaffolds in the examined teleosts, whereas *dmrt2b* could not be found in the cod genome. The location of the paralogous regions on different linkage groups in the teleost genomes examined (except for the large zebrafish chromosome 5 which might represent a chromosomal fusion as discussed in Paper II), suggests that they originated from the fish-specific whole genome duplication event, whereas the duplication of *dmrt2* must have occurred at an earlier stage in vertebrate evolution. Our findings conflict with the hypothesis that *dmrt2a* and *dmrt2b* in the teleost lineage was a result of the fish-specific whole genome duplication as described by (Zhou *et al.*, 2008). In the research by Zhou *et al*. (2008), there was low sequence homology between the linked paralogs which suggests that the duplication possibly occurred prior to separation of fish from tetrapods. In addition the reported synteny between *dmrt2a* and *dmrt2b* in stickleback and two pufferfish species (fugu and *Tetradon*) was only linked to one duplicated gene named "similar to ankyrin repeat domain 15". This designation is in fact synonymous to *kank1* which is linked to *dmrt2a*, while *kank4* synonymous to ankyrin repeat domain 38 is linked to the fish specific *dmrt2b* and tetrapod *dmrt6* as shown in Paper II.

On human chromosome 9p, *ELAVL2* is linked to *DMRT4* and to the *DMRT1-DMRT3-DMRT2* cluster. Similarly, in teleosts *elavl2* is linked to *dmrt4* except from zebrafish which apparently

lack *dmrt4*. Teleosts seem to have a conserved region containing *cdkn2c*, *faf1* and *elavl4* closely linked to *dmrt5*, concomitant with the same region flanking *DMRT5* (*A2*) and *DMRT6* (*B1*) on human chromosome 1p. In stickleback and probably in medaka this region is located on the same chromosome as *dmrt2b*. In fugu *elavl4* is mapped to s135, while *dmrt5*, *faf1* and *cdkn2c* are mapped to s122. As both regions occupy one of the distal regions of the scaffolds one can only speculate if maybe these two scaffolds should have been linked. Zebrafish harbours *dmrt5* and the well conserved flanking genes on chromosome 8, meaning that *dmrt5* and *dmrt2b* are not on the same chromosome in this species.

The fish specific *dmrt2b* is flanked by *serpb1*, *kank4* and *lrp8*, which are also linked to *dmrt6* and *dmrt5* on human chromosome 1. Combined with the conserved synteny of the *dmrt5* flanking genes in teleosts, especially with regards to the location of *dmrt5* to stickleback VIII there are indications for an ancestral *dmrt2b-dmrt5-dmrt6* cluster. After the separation of tetrapods and teleosts, *dmrt6* and dmrt2b were probably subsequently lost in the teleost and tetrapod lineages respectively. Althoug, *dmrt2b* was identified in pufferfish (fugu), the expression of *dmrt2b* mRNA was not detected in any larval or adult tissue (Yamaguchi *et al.*, 2006) which suggests that *dmrt2b* is in fact a pseudogene in pufferfish while it was probably lost in Atlantic cod. When searching www.ensembl.org for *dmrtB1* (*dmrt6*) one do get one hit in *Tetradon* (ENSTNIG00000019534), further blast search did however reveal that this sequence is not related to *dmrt6*. Surprisingly, neither of the *dmrt1-dmrt2a-dmrt3*, nor *dmrt2b* and *dmrt5* genes seem to have a surviving duplicate after the teleost-specific whole genome duplication with one exception, the duplication of the *dmrt2a/2-dmrt3-dmrt1* cluster in medaka resulting in the Y-linked *dmrt1bY* and *dmrt3p* pseudogene (Kondo et al. 2006).

In zebrafish, *dmrt4* appears to be lost, and in chicken neither *dmrt4* nor *dmrt5* could be identified. If this is due to the sequences actually being lost, or are they simply not found yet one can speculate. What we do know is that *dmrt2b* was lost in tetrapods and *dmrt6* in teleosts and the evolutionary consequences of the lineage-specific loss or inactivation of specific *dmrt* genes has probably involved acquisition or modification of the subfunctions of others.

4.3 Sox9, Cyp19a1 and Amh in Atlantic cod (Paper III)

The search for the *sox9, cyp19a1* and *amh* genes was done using BLAST search of the draft cod genome database. The search for *sox9* resulted in two duplicated copies named *sox9a* and *sox9b* that both consisted of three exons encoding a predicted Sox9a and Sox9b of 505 and 508 aa, respectively. The Atlantic cod Sox9 paralogs share a well conserved HMG domain with the highly conserved nuclear localisation signal (NLS) motifs, Bipartite NLS (KRPMNAFMVWAQAARRK) and Basic cluster (BC) NLS (QPRRRKS) (Südbeck and Scherer, 1997). Also, Atlantic cod Sox9a and Sox9b contain the well conserved NES (nuclear export signal) motif (ELSKTLGKLWRLLNE) (Gasca *et al.*, 2002), summarised in figure 7. Studies from mammals have shown that cytoplasmic Sox9 protein is present in undifferentiated gonads of both sexes, but in male gonad it becomes nuclear at the onset of testis differentiation (Morais da Silva *et al.*, 1996; De Santa Barbara *et al.*, 2000), probably due to the functions of the NLS and NES motifs.

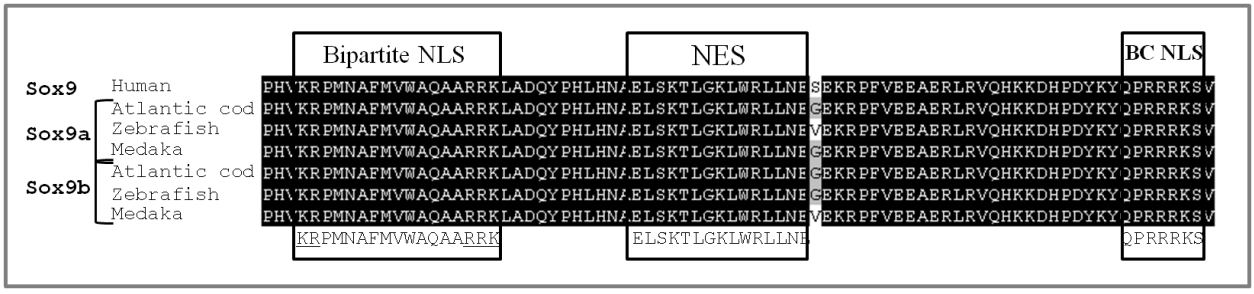

Figure 7: Alignment of the HMG domain of human Sox9 (NP_000337), Atlantic cod Sox9a (JN802288), zebrafish Sox9a (NP_571718), medaka Sox9a (AAX62152), Atlantic cod Sox9b (JN802289), zebrafish Sox9b (AAH67133) and medaka Sox9b (AAX62151). Both NLS motifs and the NES motif are shown with consensus sequences. The bipartite NLS motif is underlined and bacic cluster NLS is abbreviated BC NLS.

The phylogeny analyses conducted in Paper III settled the two Atlantic cod Sox9 paralogs as co-orthologs of a single tetrapod Sox9. Transcription factors of the Sox family arose at the beginning of multicellularity in animals indicating that that their ability to regulate the expression of extracellular matrix, cell adhesion and signaling molecules may have been very important in the evolution of metazoans (Guth and Wegner, 2008). The sox family consists of nine groups. Most Sox groups identified in mammals have a single corresponding sequence in invertebrate model organisms, suggesting a duplication and divergence mechanism has operated during the evolution of vertebrates (Koopman *et al.*, 2004). The transcription factor Sox9

belongs to the *soxE* gene family. A duplication event must have occurred early in the evolution of the SoxE group as invertebrates and early chordates probably have only one *soxE* gene (Hui Yong Loh and Russell, 2000) while the jawless hagfish and lampreys possess at least two and three SoxE genes respectively (McCauley and Bronner-Fraser, 2006; Ota *et al.*, 2007). In all jawed vertebrates the *soxE* family probably consists of three *sox* genes named *sox8*, *sox9* and *sox10* (Bowles *et al.*, 2000). *Fugu* have a duplication of each of the genes in the *soxE* group (Koopman *et al.*, 2004), while several fish have duplicate copies of *sox9* (Paper III). This supports the theory of the fish specific whole genome duplication.

The search for *cyp19a1* genes in the cod genome also resulted in two duplicated copies designated *cyp19a1a* and *cyp19a1b*, both containing nine exons encoding putitative proteins of 536 and 508 aa respectively. The phylogeny analyses in Paper III placed teleost Cyp19a1a and Cyp19a1b seem to be co-orthologs of the single Cyp19a1 in tetrapods. The partial sequence of a single *amh* gene was identified in the cod genome and the deduced 320 aa is encoded by the putative exons 4-7. According to the phylogenetic analyses conducted in Paper III, the single copy of Amh in teleosts separated from the tetrapods forming two separated branches. The search for *foxL2* in the cod genome resulted in a short partial sequence. However, all attempts to make viable qPCR assays using *foxL2* primers were, for unknown reasons, unsuccessful and the gene was eliminated from further studies.

4.4 Gene expression in early life, juvenile and adult Atlantic cod (Papers I, II and III)

The gene expression analyses in Paper II and III were conducted using the same material sampled from cod throughout early life and during the reproductive season. The results will be collectively discussed in the following sections. Although the main focus of this thesis is sexual differentiation and gonad development, some of the genes have additional functions related to other developmental processes. Some of these are also included in the discussion. The gene expression during early life of Atlantic cod will be discussed in the order expression was detected as shown in figure 8.

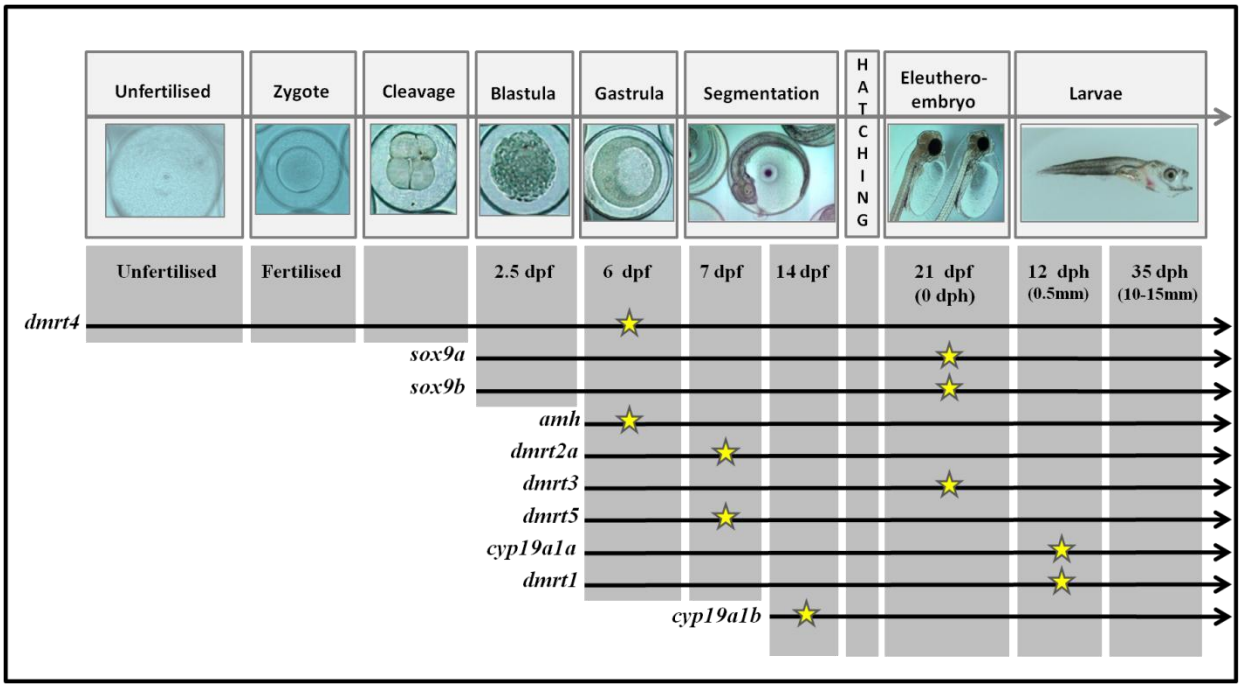

Figure 8: Schematic overview on the onset of and the peak in expression of the genes examined during early life of Atlantic cod (Papers II and III). Each star corresponds to the observed peak in expression for the given gene. The pictures do not correspond to the exact timing of sampling, but they do illustrate the corresponding developmental period. The photos from unfertilised to hatchlings were kindly provided by Saskia Mennen, while the larvae photo was taken by Frank Gregersen.

4.4.1 Gene expression during early life (Papers II and III)

The presence of *dmrt4* transcripts in unfertilised eggs probably resulted from maternal transfer of mRNA and was supported by the peak in *dmrt4* expression quantified at late gastrulation and decline thereafter. Consistently, maternal transfer and early onset expression of *dmrt4* was described in medaka (Kondo *et al.*, 2002; Winkler *et al.*, 2004). Conversely, in olive flounder and blue tilapia different patterns of *dmrt4* expression were detected (Wen *et al.*, 2009; Cao *et al.*, 2010) suggesting divergent functions for Dmrt4 during early development in different species. At 5 wph *dmrt4* was predominantly expressed in head of cod larvae which agrees with observations in the developing olfactory placodes of medaka, platyfish and flounder (Winkler *et al.*, 2004; Veith *et al.*, 2006; Wen *et al.*, 2009).

The SoxE family in which Sox9 belongs to is central in regulating formation, maintenance and survival of neural crest cells (Haldin and LaBonne, 2010) a population of multipotent cells

unique to the vertebrate embryo (Huang and Saint-Jeannet, 2004). In embryos of mouse and chicken Sox9 has a central role in induction and maintenance of neural stem cells (Scott *et al.*, 2010). In cod, early onset expression of *sox9a* and *sox9b* was evident during the blastula period at 2.5 dpf. The expression of *sox9a* peaked significantly at hatching, whereas there were no significant variations in the expression of *sox9b* during embryogenesis. A significant decrease of both *sox* paralogs was seen after hatching and in 35 dph old larvae (10-15 mm) expression of *sox9a* and *sox9b* was predominant in head. In newly hatched cod larvae, the expression of *sox9b* was principally in the branchial arches, with signals also present in hindbrain. During stickleback, zebrafish and medaka embryogenesis similar patterns of expression of *sox9a* and *sox9b* were detected (Yokoi *et al.*, 2002; Klüver *et al.*, 2005). In tetrapods, Sox9 has a well known function in chondrogenesis (Bi *et al.*, 1999; Kypriotou *et al.*, 2003; Akiyama, 2008; Ohgo *et al.*, 2010). In adult Atlantic cod the expression of *sox9a* and *sox9b* was significantly higher in gills (including branchial arches) compared to gonads. Taken together with the predominant expression of *sox9b* in the branchial arches of newly hatched larva, this indicates a role for the *sox9* paralogs in chondrogenic activity in Atlantic cod similar to that in tetrapods.

The *sox9a* and *sox9b* transcripts detected prior to the onset of *amh* expression may suggest that one or both of the *sox9* paralogs are involved in upregulating the expression of *amh* that in turn may downregulate the expression of *cyp19a1*, as described in mammals and zebrafish (Rodriguez-Mari *et al.*, 2005; Siegfried, 2010). Although variable, the expression of *amh* in cod peaked at late gastrulation followed by a reduction in expression. When comparing the expression of *amh* with that of *cyp19a1* in cod embryos and hatchlings, one could see a bimodal pattern of distribution that may indicate a role in sexual differentiation.

The expression of cod *dmrt2a* peaked at the onset of somitogenesis. Consistently, *dmrt2*/*terra* is suggested to play a specific role in early somitogenesis in mice and zebrafish (Meng *et al.*, 1999). In zebrafish, *terra* is identified as a coordinator of left-right asymmetry and presomitic mesoderm bilateral symmetry (Saúde *et al.*, 2005). In cod larvae at 35 dph, *dmrt2a* transcripts were detected in head and abdomen. Similarly, the expression of *dmrt2a* was detected in the developing head of platyfish and medaka (Winkler *et al.*, 2004; Veith *et al.*, 2006), conversely zebrafish *terra* was not expressed in the developing head during embryogenesis (Meng *et al.*, 1999). The expression of *dmrt5* in whole embryos of zebrafish peaked during somitogenesis (Guo *et al.*, 2004) much like the observed expression in Atlantic cod. Transcripts encoding *dmrt5*

were exclusive to the head of 35 dph old cod larvae. In zebrafish and platyfish, the expression of *dmrt5* was detected in the developing brain (Guo *et al.*, 2004; Veith *et al.*, 2006). The expression of *dmrt3* peaked at hatching and transcripts were predominantly expressed in the head of 35 dph larvae. A similar pattern of expression was shown in medaka where the *dmrt3* transcript was restricted to the neural tube of the developing embryo (Winkler *et al.*, 2004). This suggests a role for both *dmrt5* and *dmrt3* in the developing central nervous system of Atlantic cod.

The first transcripts of *cyp19a1a* in cod were detected at late gastrulation and peaked at 12 dph. Similarly, the expression of *dmrt1* in cod was low in embryos with a significant peak at 12 dph. This corresponds to expression in rainbow trout where *dmrt1* and *cyp19a1a* peaked simultaneously at hatching (Hale *et al.*, 2011). In mouse and chicken embryos *dmrt1* is expressed in the genital ridge (Raymond *et al.*, 1999). However, embryonic transcripts of *dmrt1* were neither detected in medaka nor in platyfish (Winkler *et al.*, 2004; Veith *et al.*, 2006). Of the genes examined in the developing cod, c*yp19a1b* was expressed latest. The expression of *cyp19a1b* which is often referred to as brain-aromatase was restricted to the head in the 35 dph cod larvae. In North African catfish, *cyp19a1b* transcripts were detected in female brains from 45 dph and in male brains from 100 dph (Rasheeda *et al.*, 2010). The peak in *cyp19a1b* expression at late somitogenesis in Atlantic cod may be connected to the completion of the brain patterning at the 30-somite stage described by Hall *et al.* (2004).

Conventional histology analyses have shown that the precursors of reproductive organs are visible in cod embryos at the 30-somites stage (Hall *et al.*, 2004). During the yolk sac stage a few germinal cells were visible ventral to the pronephric ducts in the posterior part of the body (Morrison, 1993). Although the sex of Atlantic cod larvae could not be distinguished before (35 mm TL) 16 wph (Chiasson *et al.*, 2008), there are indications that gonad differentiation might start prior to 16 mm TL (Haugen *et al.*, 2011). Hence, the dimorphic expression of *amh* and *cyp19a1a* during embryogenesis of Atlantic cod also supports that the first molecular evidence of sexual differentiation may be detectable at earlier time points. Further, the significant peak in *dmrt1* expression at 12 dph (5 mm TL) may be relevant with respect to sexual differentiation. Similarly in rainbow trout recent findings demonstrated a peak in *dmrt1* expression at hatching higher in males than females (Hale *et al.*, 2011). The use of histology provides a good visual indicator on the main events of sexual differentiation as described in Hall *et al.* (2004), Chiasson

et al. (2008) and Gorodilov *et al.* (2008). However, when visible it has already happened on the molecular level, as recently demonstrated in rainbow trout where sexual dimorphism was confirmed during embryogenesis by using microarray, thus demonstrating that transcriptional differences are present before the completion of gonadogenesis (Hale *et al.*, 2011).

Defining molecular indicators of sexual differentiation is therefore important to trace sexual differentiation at early time points. In the 35 dph old cod larvae the expression of *dmrt1* was restricted to the abdomen with prominent individual variations suggesting a role in sexual differentiation.

4.4.2 Gene expression in juvenile and adult Atlantic cod (Papers I, II and III)

Expression of *dmrt1* was restricted to the gonads of Atlantic cod and the expression was significantly higher in males compared to females throughout the reproductive season (Papers I and II), concomitant with the male biased expression of *dmrt1* reported from several other teleosts as reviewed in Herpin and Schartl (2011). Atlantic cod males showed significantly higher expression of *dmrt1* at the juvenile stage compared to the adult stage and *in situ* hybridisation confirmed the expression in males by abundant staining in immature, maturing and adult male gonads (Paper I). The expression was restricted to the spermatogonia and absent from sertoli cells, similar to the pattern of expression seen in zebrafish and catfish (Guo *et al.*, 2005; Raghuveer and Senthilkumaran, 2009). This may reflect the cystic structures maturing in a wave through the testicular lobes of Atlantic cod as reported by Almeida *et al.* (2008). In female cod, the expression of *dmrt1* was constrained to the germ cells as reported in zebrafish (Guo *et al.*, 2005).

In mammals, Sox9 plays an essential role in testis determination and cartilage development (Wagner *et al.*, 1994; Healy *et al.*, 1999; Jakob and Lovell-Badge, 2011). The testes specific expression of *sox9a* in the maturing Atlantic cod gonads and the male biased expression of *sox9b* (Paper III) clearly indicated a role in testes maturation. However, expression of *sox9b* was also detected in ovaries of Atlantic cod. Findings from medaka implied a role for *sox9b* in both ovary and testes development (Nakamura *et al.*, 2008; Nakamura *et al.*, 2011), as both sertoli cells and granulosa cells originated from *sox9b* expressing cells. In addition, oogonia at early stages of

oogenesis were surrounded by *sox9b* expressing cells in medaka. Although *sox9* expression is downregulated during ovarian differentiation in tetrapods, the ovarian expression of *sox9b* throughout sexual maturation in diverse teleost species suggests that this paralog has acquired a role in the maturing ovary. A possible reason may be that the mammalian ovaries produce relatively few eggs and the mitotic divisions of germ cells are completed before birth. In species with high fecundity such as fish, especially multiple spawning fish, the number of oocytes is infinite and mitotic divisions of oogonia can produce mature eggs continuously. This was reviewed in Nakamura *et al.* (2011) and while the molecular and cellular mechanisms underlying the continuous production of oocytes needs to be addressed for several species, *sox9b* was suggested an important factor in medaka. Based on its expression pattern *sox9b* may very well have the same function in Atlantic cod, however further studies are needed to confirm.

Male biased expression was evident for *cyp19a1b* and transcripts were restricted to the testes throughout the reproductive season in Atlantic cod (Paper III). The conserved estrogen responsive element (ERE) (Diotel *et al.*, 2010) in the promoter of *cyp19a1b* was not identified in Atlantic cod and may explain why the transcript was absent in cod ovaries. In catfish a faint expression of *cyp19a1b* was detected in testes from 60 dph with no corresponding expression in ovary despite the presence of ERE in the promoter (Rasheeda *et al.*, 2010). However, weak *cyp19a1b* expression was detected in ovaries of catfish at a later time point. In Nile tilapia *cyp19a1b* was present in gonads of male and sex-reversed females as well as there being weak expression in ovaries at different developmental stages (Sudhakumari *et al.*, 2003; Chang *et al.*, 2005; Sudhakumari *et al.*, 2005). To my knowledge, our study (Paper III) is the first to detect gonad expression of *cyp19a1b* exclusive to testes and the functional significance of *cyp19a1b* in male gonads needs further research.

The expression of *cyp19a1a* in cod gonads was female biased until March, while no sexually dimorphic expression was seen at spawning as the expression of *cyp19a1a* in testes increased. Similar findings have been reported in Atlantic croaker (Nunez and Applebaum, 2006) and the highest expression levels of *cyp19a1a* were seen in the developing ovaries compared to spawning, regressing and resting ovaries. The correlation between the expression of *cyp19a1a* and the plasma concentration of E2 was strong for the whole period. However, the most positive correlation was seen from November-February with a corresponding R^2 value of 0.9. This correlation is probably linked to the conserved role of aromatase in converting androgens to

estrogen. Male-biased expression of cod *amh* was detected in the immature gonads in November, but was followed by increased ovarian *amh* expression. This occurred at the same time as increased *cyp19a1a* expression in the maturing ovaries and elevated plasma estrogen levels. These findings do not support Amh as an anti-aromatase factor in adult teleosts gonads and the regulatory mechanisms involved should be further studied in Atlantic cod.

Strong *dmrt2a* expression has been reported in gonads of medaka, platyfish and pufferfish (Brunner *et al.*, 2001; Veith *et al.*, 2006; Yamaguchi *et al.*, 2006) similar to what was detected in Atlantic cod. However, the *dmrt2a* expression in cod proved to be ovary specific throughout the spawning season, clearly giving *dmrt2a* a role in oogenesis. Although the expression of *dmrt4* in Atlantic cod was higher in female gonads during the initial months of maturation, a slight male-biased gonad expression was evident at spawning. Sexually dimorphic male biased *dmrt4* expression was reported in adult Japanese flounder and medaka (Winkler *et al.*, 2004; Wen *et al.*, 2009). The expression of *dmrt4* in tilapia was restricted to the ovaries (Guan *et al.*, 2000; Cao *et al.*, 2010) and expression of *dmrt4* in Fugu was detected in the gonads of both sexes (Yamaguchi *et al.*, 2006). A testes specific pattern of expression of *dmrt3* has been reported in Fugu (Yamaguchi *et al.*, 2006). This is comparable to the expression of *dmrt3* in cod that was restricted to testes except for at spawning, when expression also was detected in ovaries. The gonad expression of Atlantic cod *dmrt5* in both sexes is consistent with the expression of zebrafish *dmrt5* in developing germ cells (Guo *et al.*, 2004). It is evident that singular time points may be insufficient in getting the whole picture when working with genes involved in sexual maturation. The upregulated expression of cod *dmrt3* in both genders and *dmrt4*, *dmrt5* and *sox9b* in males at spawning as well as variable levels of and onset of expression during ontogeny underlines the importance of comparing different species at similar stages during sexual maturation.

One of the striking things with the fish brain is its constant growth. In teleost brain, *cyp19a1b* expression is restricted to radial glial cells (Forlano *et al.*, 2001; Menuet *et al.*, 2005) known as neural progenitors supporting the continuous growth of the adult fish brain. The significant expression of Atlantic cod *cyp19a1a* and *cyp19a1b* in brain may indicate involvement in this intense neurogenesis. Although not dimorphic, the expression of *cyp19a1b* in brain of Atlantic cod was thousand-fold upregulated compared to the expression in testes, clearly indicating a

function as brain-aromatase. Sexual differences in *cyp19a1b* expression were recently reported in the brain of developing catfish and adult medaka (Rasheeda *et al.*, 2010; Okubo *et al.*, 2011) with female brains expressing highest levels of *cyp19a1b*. The difference in expression was however not detected until puberty in medaka (Okubo *et al.*, 2011). Expression of *amh* was seen in gonads of both sexes throughout the reproductive season and a non dimorphic transcript was detected in the brain. Similarly, expression of *amh* was detected in brain of tilapia and European sea bass at the onset of testis differentiation (Halm *et al.*, 2007; Poonlaphdecha *et al.*, 2011). In mammals and in zebrafish *amh* has been described as an inhibitor of *cyp19a1* (Vigier *et al.*, 1989; Rouiller-Fabre *et al.*, 1998; Rodriguez-Mari *et al.*, 2005). Given the high level transcripts of both *cyp19a1* paralogs detected in the cod brain, the concurrent detection of *amh* may be due to a function in the regulation of aromatase in the brain. It is however important to note that Amh may not function as an anti-aromatase factor in adult cod gonads.

Both *sox9a* and *sox9b* were expressed at significantly higher levels in brain compared to gonads in adult Atlantic cod and expression of *sox9* have been detected in the brain of several vertebrates (Pompolo and Harley, 2001; Klüver *et al.*, 2005; Alcock *et al.*, 2009). In humans, mutations in Sox9 cause campomelic dysplasia (CD) (Foster, 1996), a condition associated with brain and skeletal abnormalities as well as XY sex reversal. The high level of *sox9a* and *sox9b* expression in cod brain is probably related to a significant function. Together with the important roles of Sox9 associated with chondrogenesis and neural stem cells these findings conclude that the *sox9* paralogs are probably not suitable candidates for knockdown studies.

In Japanese flounder, *dmrt4* was strongly expressed in the brain (Wen *et al.*, 2009) and in blue tilapia *dmrt4* was expressed in brain of both sexes with a slight male biased expression in hypothalamus (Cao *et al.*, 2010). Interestingly in cod, expression of *dmrt4* was only detected in male brains, clearly indicating a role in male sexual development that needs further study. The expression of *dmrt5* in the brain was significantly higher than that in gonads, however the expression did not appear to be sex linked. Expression of *dmrt5* in brain has been reported in adult platyfish, zebrafish, pufferfish and mouse (Kim *et al.*, 2003; Guo *et al.*, 2004; Veith *et al.*, 2006; Yamaguchi *et al.*, 2006) suggesting a conserved function in the brain. Recent findings identified mouse *dmrt5* as an important regulatory factor of neural fate specification in the ventral midbrain (Gennet *et al.*, 2011).

The combined expression data clearly states Dmrt1 as an important factor related to male development. Among the analysed genes it is probably the best candidate for knockdown studies since its expression seems to be restricted to the gonads. It is however doubtful that knockdown of *dmrt1* will result in sterile cod. The male pathway is probably favoured in the presence of Dmrt1 possibly by suppressing *cyp19a1a* transcription and in turn estrogen production as described in tilapia by Wang *et al.* (2010). Thus knockdown of *dmrt1* may lead to the female pathway as was shown in mice (Raymond *et al.*, 2000) instead of creating sterile fish. This need to be further addressed and may in the future lead to new methods to create monosex-stock without the use of hormone treatment.

5. Concluding remarks

Several aspects of sexual differentiation in Atlantic cod were clarified through this thesis and basic knowledge on some key regulators was obtained. Expression of all five *dmrts*, *sox9* and *cyp19a1* paralogs and *amh* were investigated during early life and in maturing gonads during the reproductive season. Timeline sampling is proved important when working with fish that are annual breeders as well as when working with embryology. All of the examined genes showed distinct patterns of expression that may be linked to specific functions.

- An important factor in male development, Dmrt1 was characterised in cod and may well be one of the most important factor regulating the male pathway in this species.
- Female biased expression of *cyp19a1a* along with the strong correlation between *cyp19a1a* and the levels of plasma estradiol, clearly demonstrate the importance of this factor in the female pathway of cod.
- The Sox9 paralogs are probably involved in chondrogenesis in cod as well as being important factors in testes differentiation. Expression of *sox9b* in ovaries indicated a function connected to oogenesis and may be related to the high fecundity of cod ovaries.
- One or both of the *sox9* paralogs may be the factor regulating the onset of *amh* expression in Atlantic cod as was shown in mammals and zebrafish.
- Expression of *cyp19a1a* and *cyp19a1b* in male gonads indicate a possible role for estradiol in testes maturation and requires further study.
- Detection of *amh* in brain of Atlantic cod may indicate a function in regulating the expression of both *cyp19a1* paralogs.
- Male specific *dmrt4* expression detected in adult cod brain may be important with respect to sexual maturation and warrants further studies.
- The ancient *dmrt2* was duplicated prior to the *Actinopterygiian* split from the vertebrate lineage.
- The duplicated *dmrt2b* may have been lost in the Atlantic cod genome.

6. Future perspective

The studies included in this thesis present one step further in elucidating molecular mechanisms of sexual differentiation in Atlantic cod. However, to fully understand the complexities of sexual differentiation in cod further studies are needed. It may be valuable to perform SNP based analyses of the *dmrt1* 3`UTR as was done in zebrafish revealing male and female specific alleles. As *dmrt1* probably has a key function in the male pathway, it would be interesting to try to identify the target genes in cod by performing ChIP-chip analyses.

The various genes included in this thesis are mapped under normal conditions and the qPCR assays created for these studies may be used further to study the same questions under other conditions. For instance it would be interesting to see how temperature would affect the expression of the key genes involved in sexual differentiation and gonad maturation which is also a hot topic with respect to global warming. Also to study sexual differentiation and gonad maturation in context of pollution and sex steroid antagonists and agonists may be interesting both from a scientific and environmental point of view. As cod embryos devoid of PGCs have been successfully created, it would be exciting to study the expression of the genes included in this thesis on cod that may be sterile.

Since all females have been successfully created in cod, it may also be possible to create all males. If so, expression analyses on genes involved in sexual differentiation should for the future also be done using monosex stocks.

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