Trond Moxness Kortner

The Role of Androgens on Previtellogenic Oocyte Growth in Atlantic cod (*Gadus morhua*):

Identification and Patterns of Differentially Expressed Genes in Relation to Stereological Evaluations

Thesis for the degree philosophiae doctor

Trondheim, May 2008

Norwegian University of Science and Technology
Faculty of Natural Sciences and Technology
Department of Biology

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PREFACE AND ACKNOWLEDGEMENTS

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Trondheim, April 2008

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SUMMARY

The internal signals that regulate fecundity are generally not well described. Overt developmental and/or functional changes are controlled at the molecular and cellular levels of biological organization. Therefore, changes in gene expression profile as a result of hormonal exposure and the subsequent molecular processes that lead to developmental, functional and physiological changes may be used as quantitative molecular markers for cellular, physiological, developmental and reproductive effects in an organism. Additionally, the identification of hormonal responsive genes and their expression patterns as a result of hormonal influence are of vital importance in order to shed light on the underlying mechanisms of these fundamental processes of reproduction.

Despite the fact that hormonal effects on the growth of previtellogenic oocytes might involve several cascades of physiological events, observations in fish and mammals point to a pivotal role of androgens. The objectives of the present work implied the identification and sequencing of a number of androgen responsive genes in the previtellogenic Atlantic cod (*Gadus morhua*) ovary using the analytical power of suppressive subtractive hybridization (SSH). Subtracted clones were arrayed, preserved and applied in gene expression profiling.

The present study demonstrates, for the first time, that androgens (particularly the non-aromatizable androgen, 11-ketotestosterone; 11-KT) can induce ovarian development in immature female Atlantic cod, adding further evidence to our hypothesis that androgens are involved in the regulation of previtellogenic oocyte growth and development in this species. In general, both 11-KT and testosterone (T) are capable of inducing oocyte growth, with the former hormone being the strongest modulator. In addition, we also presented the identification, sequencing and the expression patterns of a number of androgen responsive transcripts in the previtellogenic cod ovary. A targeted cod array (CodArray) consisting of subtracted clones with unique expression patterns that were either up or down regulated, after *in vitro* androgen exposure of previtellogenic ovarian tissues was constructed. The parallel expression patterns of candidate genes involved in steroidogenesis and oocyte growth control, add further information on how androgenic action may produce advances in previtellogenic oocyte growth. We believe the novel roles of androgens as promoters of ovarian growth and development presented in this study will be beneficial for the aquaculture industry and for breeding of new captive and endangered species. It can also form the basis for the development of useful models in fisheries management. Differentially expressed genes may form an integral link with quantifiable environmental variables (*e.g.* food availability and growth rates). In general, our findings form the basis for generating more hypotheses for further detailed studies and for deduction of evolutionary relatedness in androgen control of

early oocyte growth in vertebrates. Therefore, these findings may have significant economic benefit for predictive ecological models, particularly with respect to the noticeable and worldwide declining cod stock.

LIST OF PAPERS

The thesis is based on the following papers that will be referred to in the text by their Roman numerals:

I. Kortner TM, Rocha E, Silva P, Castro LFC and Arukwe A (2008). Genomic Approach in Evaluating the Role of Androgens on the Growth of Atlantic cod (*Gadus morhua*) Previtellogenic Oocytes. *Comp Biochem Physiol D, doi: 10.1016/j.cbd.2008.04.001*

II. Kortner TM, Rocha E and Arukwe A. Early growth of Atlantic cod previtellogenic oocytes and zona radiata-related genes are modulated in vitro by 11-ketotestosterone and testosterone. (*submitted*)

III. Kortner TM, Rocha E and Arukwe A. Previtellogenic oocyte growth and transcriptional changes of steroidogenic enzyme genes in immature female Atlantic cod (*Gadus morhua L.*) after exposure to the androgens 11-ketotestosterone and testosterone. (*submitted*)

IV. Kortner TM and Arukwe A (2007). Effects of 17α-methyltestosterone exposure on steroidogenesis and cyclin-B mRNA expression in previtellogenic oocytes of Atlantic cod (*Gadus morhua*). *Comp Biochem Physiol C Toxicol Pharmacol* 146(4):569-580.

V. Kortner TM and Arukwe A (2007). The xenoestrogen, 4-nonylphenol, impaired steroidogenesis in previtellogenic oocyte culture of Atlantic cod (*Gadus morhua*) by targeting the StAR protein and P450scc expressions. *Gen Comp Endocrinol* 150(3):419-29.

ABBREVIATIONS

2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
3β-HSD	3β-hydroxysteroid dehydrogenase
11β-HSD	11β-hydroxysteroid dehydrogenase
11-KT	11-ketotestosterone
17,20β-P	17,20β-dihydroxy-4-pregnen-3-one
20β-HSD	20β-hydroxysteroid dehydrogenase
20β-S	17,20β,21-trihydroxy-4-pregnen-3-one
APE	alkylphenol polyethoxylate
AR	androgen receptor
ARE	androgen responsive element
CNS	central nervous system
Ct	cycle threshold
CYP17	cytochrome P450 17-hydroxylase / C_{17-20}lyase
CYP19	cytochrome P450 aromatase
DDT	1,1,1-trichloro-2,2'*bis*(*p*-chlorophenyl)ethane
DHEA	dehydroepiandrosterone
DHT	5α-dihydrotestosterone
E2	estradiol-17β
EE2	ethynylestradiol
EDC	endocrine-disrupting chemical
EIA	enzyme immunoassay
ER	estrogen receptor
EST	expressed sequence tag
FSH	follicle-stimulating hormone
FSHR	FSH receptor
GnRH	gonadotropin-releasing hormone
GSI	gonadosomatic index
GtH	gonadotropin
GV	germinal vesicle
GVBD	germinal vesicle breakdown
HPGL axis	hypothalamus-pituitary-gonadal-liver axis
HRE	hormone responsive element

hsp90	heat shock protein 90
IGF-1	insulin-like growth factor-1
LH	lutenizing hormone
ME2	methylestradiol
MIH	maturation-inducing hormone
MOA	mode-of-action
MPF	maturation-promoting factor
MS	mass spectrometry
MT	17α-methyltestosterone
NP	nonylphenol
OGFr	opioid growth factor receptor
P45011β	cytochrome P450 11β-hydroxylase
P450*arom*	cytochrome P450 aromatase
P450*scc*	cytochrome P450 side chain cleavage
PCOS	polycystic ovary syndrome
PR	progestogen receptor
PRE	progesterone response element
qPCR	quantitative real-time polymerase chain reaction
SSH	suppressive subtractive hybridization
StAR	steroidogenic acute regulatory protein
T	testosterone
TBT	tributyltin
Vtg	vitellogenin
VtgR	vtg receptor
ZP	zona pellucida
ZR	zona radiata

INTRODUCTION

Teleost fish represent the most extensive group of vertebrates, comprising over 29,000 species. Teleosts display a diverse variety of reproductive strategies, based on energy requirement, mating behavior, gamete structures, and the specificity of recognition molecules on the surface of sperm and eggs. Despite this, reproductive development in all species is a continuous process throughout ontogeny that is controlled by several hormonal and biochemical pathways and second messengers. Therefore, reproductive events are susceptible to endogenous substances at all stages of the life-cycle, including early development, gametogenesis, fertilization, embryonic development, sex differentiation, oogenesis or spermatogenesis, final maturation, ovulation or spermiation, and spawning. Thus, the sensitivity to a particular hormone or other endogenous biomolecules will vary depending on the stage of reproductive development. In teleosts, environmental changes, such as photoperiod and water temperature provide signals that are received by the central nervous system. These signals lead to oocyte growth and maturation that are regulated by pituitary gonadotropins and ovarian sex steroids.

Gametogenesis

Gametogenesis in oviparous vertebrates is a fundamental aspect of reproduction, resulting in the development and ovulation of yolky eggs that provide necessary nutrients for embryonic development. Oocytes in all teleost fish undergo the same basic growth pattern: oogenesis, primary oocyte growth, cortical alveolus stage, vitellogenesis, maturation and ovulation (Figure 1, Tyler and Sumpter 1996). Ovarian development in most teleost fish has been classified as synchronous or asynchronous, depending on the growth pattern of the oocytes at a particular time (Scott 1987). Thus, asynchronous batch spawners (e.g. the Atlantic cod) have ovaries in which eggs are recruited from a heterogenous population of developing oocytes and are subsequently ovulated in several batches during each spawning season (Scott 1987). The fish ovary consists of numerous ovigerous folds extending from the ovarian wall towards the centre of the ovary. The oogonia are formed by mitosis in the vicinity of the luminal epithelium, and are recognized as small nests of diploidic, mitotic cells dispersed within the ovary connective tissue (Grier 2000). Oogenesis commences when oogonia are transformed into primary (previtellogenic) oocytes arrested in meiotic prophase I, and each oocyte is generally surrounded by a covering layer comprising somatic granulosa and theca cells separated by a basement lamina, an inner sublayer and an outer epithelium layer (Kjesbu and Kryvi 1989). The inner acellular enveloping layer has traditionally been referred to as the zona radiata, zona pellucida or chorion vitelline envelope (Tyler and Sumpter 1996), and the

term zona radiata (ZR) will be used in the present work (Arukwe and Goksøyr 2003). During oocyte development, the zona pellucida proteins (ZPs) are deposited at this position. These ZP proteins are synthesized by the liver under estrogenic induction at almost the same time as vitellogenin (Vtg) (Celius and Walther 1998). However, recent data have demonstrated that ZP proteins may originate from the oocyte itself, at least in some teleost species (Chang et al. 1997; Chang et al. 1996). The granulosa cells secrete the basement lamina and are involved (together with thecal cells) in sexual steroidogenesis during oocyte growth and maturation (Nagahama et al. 1995).

Previtellogenesis

During the previtellogenic phase (i.e. preceding the entry of yolk precursors into the oocyte), the oocytes of a typical teleost fish may increase at least 10-fold in diameter (from about 10-15 μm to over 150 μm). An extensive synthesis of RNA, mRNA coding for Vtg receptor (VtgR) and accumulation of mitochondria, polyribosomes and lipids are all characteristics of previtellogenesis (Mommsen and Walsh 1988). In addition, an increase in nucleoli, due to a significant amplification of nucleolar organizer genes is observed (Le Menn et al. 2007). Distinct cytoplasmic structures, referred to as cortical alveoli, can be observed in the advanced previtellogenic oocytes of most teleost fish. The contents of the cortical alveoli (glycoproteins and accociated enzymes) serve to harden the zona radiata after ovulation and prevent polyspermy (Kitajima et al. 1994). Lipid droplets often appear in oocytes during the cortical alveolus stage. At the terminal end of the previtellogenic growth phase, the oocyte contains all the molecules and organelles required for subsequent endocytic and exocytic activities during vitellogenesis (Le Menn et al. 2007).

Vitellogenesis

Vitellogenesis is the synthesis and subsequent uptake of Vtg in oocytes and is the main event responsible for the enormous growth of the oocyte in most teleost, resulting in a 50- to 100-fold increase in the GSI (gonadosomatic index = gonad weight/(body weight – gonad weight) x 100) (Tyler and Sumpter 1996). Oocytes generally need to reach a certain size threshold before they enter vitellogenesis and uptake of Vtg is initiated. Vtg is a bulky (MW; 250-600 kDa) and complex calcium-binding phospholipoglycoprotein, synthesized in liver in response to circulating estrogens (Arukwe and Goksøyr 2003 and references therein). After its hepatic synthesis, Vtg is secreted into the bloodstream and transported to the ovary, where it is taken up by growing oocytes through receptor-mediated endocytosis (Mommsen and Walsh 1988;

Tyler and Sumpter 1996). These specific oocyte Vtg receptors are clustered in clathrin-coated pits.

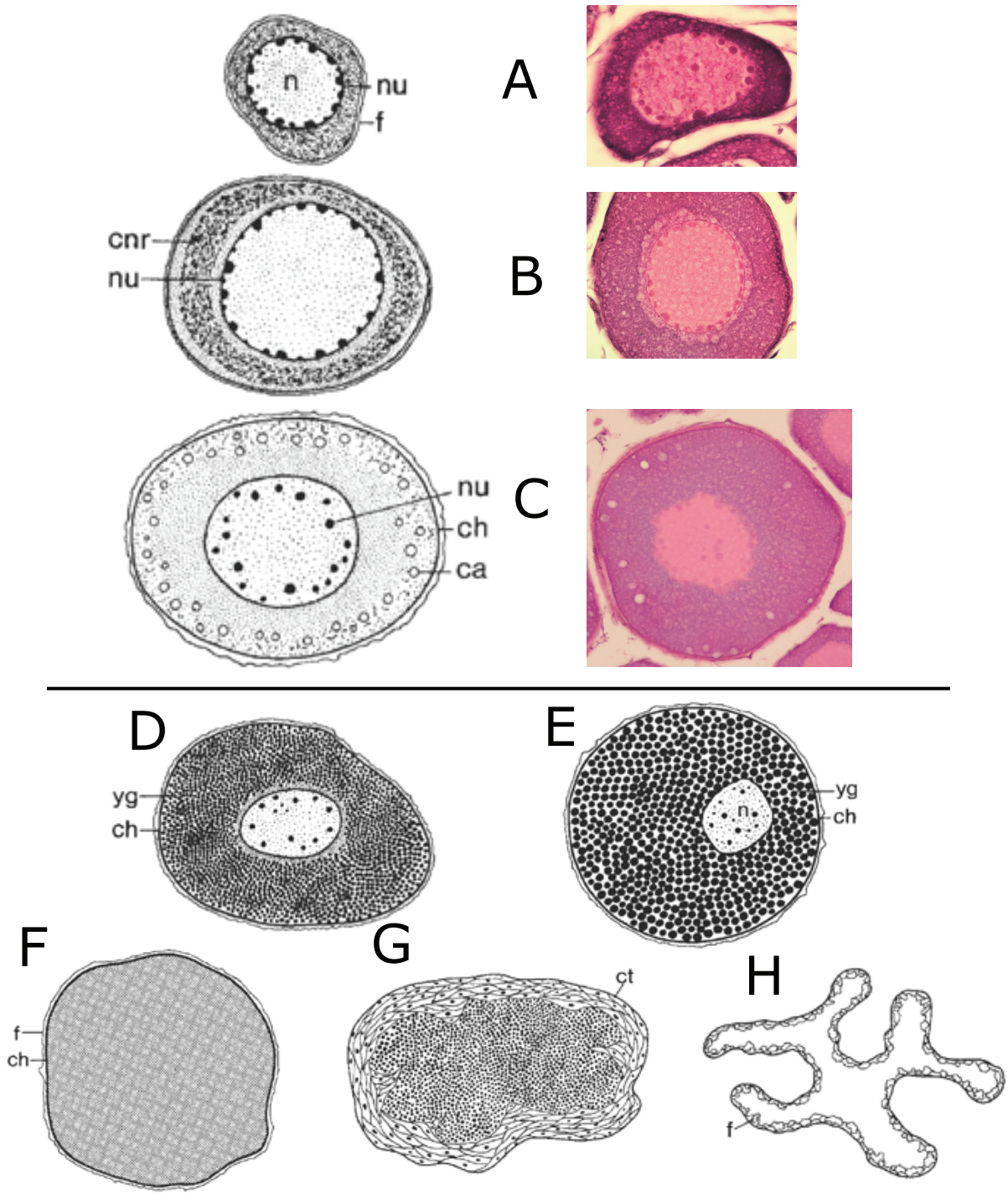


Figure 1. Schematic drawings and micrographs of histological characteristics in cod oogenesis. (A) perinuclear stage oocyte with large circular nucleus and peripheral nucleoli. (B) Circumnuclear ring stage, nucleus still with attached nucleoli. (C) Oocyte with cortical alveoli, thin chorion, nucleus with detached nucleoli. (D)-(E) Vitellogenic oocyte with yolk granules. (F)-(G) Hydrated egg. (H) Postovulatory follicle. Ca; cortical alveoli, ch; chorion, cnr; circumnuclear ring, ct; connective tissue, f; follicle, n; nucleus, nu; nucleolus, yg; yolk granules. Modified from Tomkiewicz et al. (2003).

After entering the oocyte, Vtg is transformed into lipovitellins and phosvitins by golgian lysosomes (Carnevali et al. 1999). Together, these yolk proteins constitute the energy sources and the building substrates for early development of the embryo (Tyler and Sumpter 1996) and can be observed as yolk granules in the oocyte cytoplasm (Kjesbu and Kryvi 1989). Also, the cortical alveoli become less spherical and begin to acquire their final location at the oocyte cortex, and the ratio nucleus/oocyte volume and the total number of nucleoli are reduced relative to previtellogenic growth phase. As for the surrounding events, the ZR becomes further developed, and can be observed as a striated, two-layered structure (Oppen-Berntsen 1990). As the oocyte approaches the end of vitellogenesis, the outer ZR layer becomes porous, and the follicle cells show a cuboidal morphology (Kjesbu and Kryvi 1989). The oocyte is now prepared for the next phase of oogenesis, namely, the final maturation.

Final maturation

During the final maturation phase, which transforms the oocyte into a large yolky haploid egg ready for ovulation and fertilization, the oocyte resumes meiosis. This event is accompanied by a number of maturational processes in the nucleus and cytoplasm of the oocyte (Caussanel and Breuzet 1977; Nagahama et al. 1993). These include chromosome condensation, the migration of the oocyte nucleus (germinal vesicle, GV) to the animal pole with the subsequent germinal vesicle breakdown (GVBD) and formation of the first polar body (Nagahama et al. 1993 and references therein). Finally, meiosis is once again arrested, this time in metaphase II. The egg is now mature, and can undergo embryonic development if fertilized.

Endocrine regulation of oocyte growth and maturation

Generally, there is limited information available about the mechanisms that control oocyte growth and development in teleosts and other animal species. In contrast, a vast number of earlier studies have addressed ovarian physiology in teleost fish, using classical biochemical and histological tools. For example, oogenesis in Atlantic cod has been thoroughly investigated (Dahle et al. 2003; Kjesbu and Kryvi 1989; Kjesbu et al. 1996). In other words, while the developmental events, and in particular the vitellogenic process, occurring in a growing oocyte have been well described, essentially nothing is known about the underlying, fundamental molecular mechanisms that promote and regulate these events, especially in oocytes at previtellogenic growth phase. Given that female fecundity and egg size seem to be largely determined during previtellogenesis in rainbow trout and salmon (Bromage et al. 1992; Campbell et al. 2006; MacKenzie et al. 1998) this still represents a strong gap in our knowledge of fish endocrine and reproductive physiology. Nevertheless, the endocrine

regulation of vitellogenesis and oocyte maturation has been subject to systematic investigation, and is outlined in the following sections.

Hormonal regulation of oocyte growth

Oocyte growth and development is mainly regulated by the endocrine system (Nagahama 1994). According to established theories, environmental factors (e.g. photoperiod and water temperature) provide necessary cues that are perceived by the central nervous system (CNS) that initiate the oocyte developmental processes. In response, gonadotropin-releasing hormone (GnRH) is secreted from hypothalamus, which in turn will stimulate the release of gonadotropins (GtHs) from the pituitary (Figure 2). It has been demonstrated that fish pituitaries, like those of other vertebrates, secrete two GtH species, that were recently designated as follicle-stimulating hormone (FSH) and lutenizing hormone (LH). While FSH is mainly involved in the vitellogenic process, LH plays a role in final oocyte maturation and ovulation (Arukwe and Goksøyr 2003; Nagahama 1994). In accordance, plasma levels of FSH show an extended increase during vitellogenesis, while plasma levels of LH remain low throughout vitellogenesis and are elevated dramatically during spawning. It is well established that the vitellogenic process is regulated by estradiol-17β (E2) synthesized by the ovary under the direct control of GtHs in a process generally regarded as the hypothalamus-pituitary-gonadal-liver (HPGL) axis (Senthilkumaran et al. 2004 and references therein). To outline the production of different steroid hormones, a two-cell model has been proposed by Nagahama (1997). According to this model, circulating GtHs (probably FSH) stimulate the outer thecal cells by a receptor-mediated adenylate cyclase-cAMP cascade. The thecal cells respond by producing and secreting androgens (testosterone; T), which diffuse into granulosa cells, where they are converted into E2 (Figure 3). E2 will subsequently stimulate the hepatic production of Vtg, which is incorporated in growing oocytes as outlined above. GtH secretion is regulated through a feedback mechanism by E2 and T (Peter and Yu 1997). Several other feedback mechanisms, long loops stimulating the CNS as well as short feedback loops within the ovary itself, seem to operate in the entire process (Young et al. 2005 and references therein).

Hormonal regulation of oocyte maturation

The endocrine control of oocyte maturation has also been thoroughly studied in fish, and it is well established that oocyte maturation in fish is regulated by three main hormonal mediators, namely GtH, maturation-inducing hormone (MIH) and maturation-promoting factor (MPF)

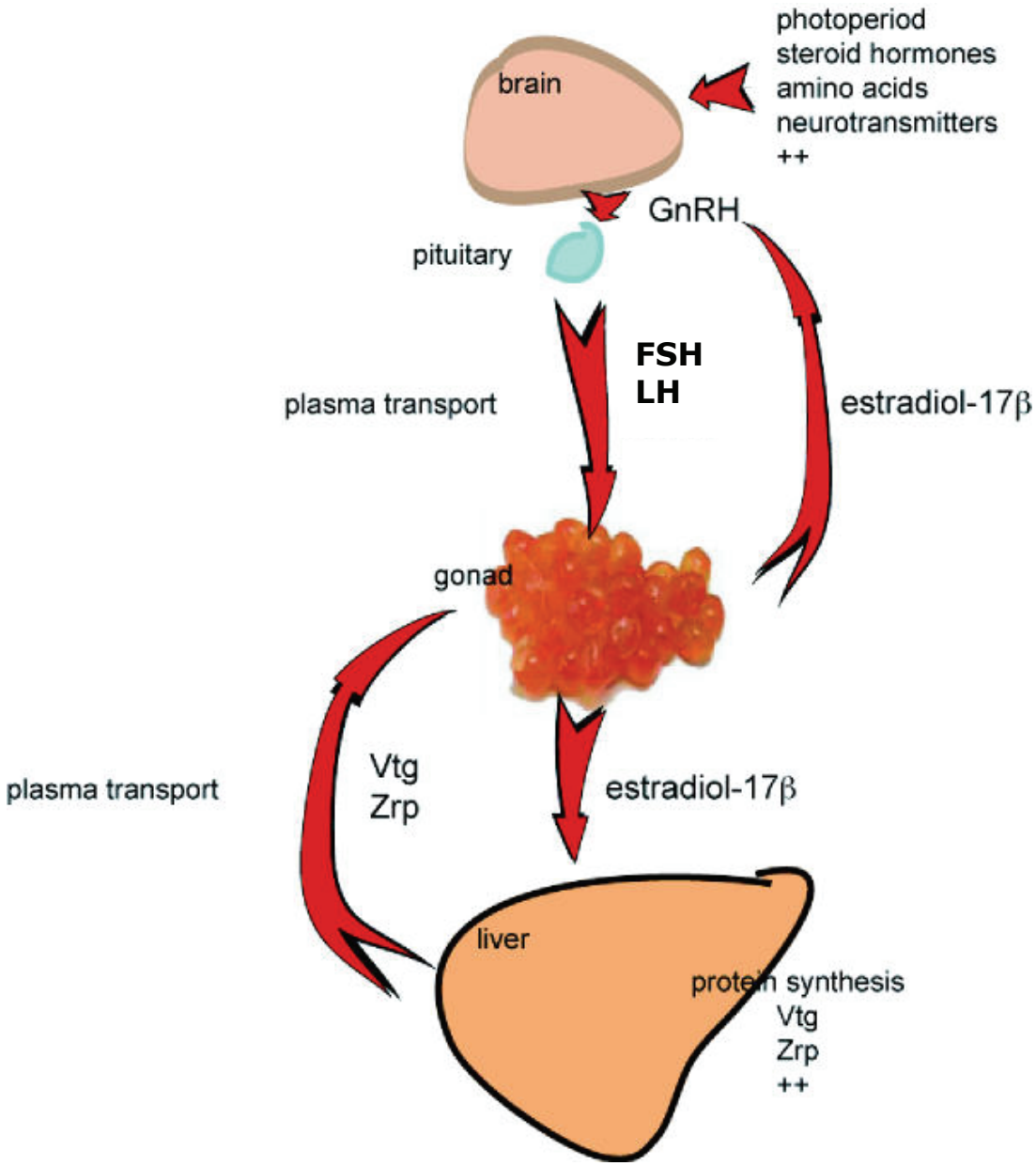


Figure 2. The coordinated action of the hypothalamus-pituitary-gonadal-liver axis during oogenic protein synthesis in female teleosts. The HPGL axis is thought to be regulated mainly through the negative feedback mechanism by estradiol-17β. Abbreviations: GnRH; gonadotropin-releasing hormone, FSH; follicle-stimulating hormone, LH; lutenizing hormone, Vtg; vitellogenin, Zrp; zona radiata protein. Illustration modified from Arukwe and Goksøyr (2003).

(Nagahama 1994; Nagahama 1997). In accordance with the hormonal control of oocyte growth, GtH (probably LH) secreted from the pituitary stimulates the production of MIH in the granulosa cells (Figure 3). Two compounds have been identified as naturally occurring MIHs in teleost fish, namely the C21 steroids 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) (Suwa and Yamashita 2007). Similarly, it

has been demonstrated that 17,20β-P levels are very low during vitellogenesis and sharply increase in mature and ovulated females (Young et al. 1983). The MIH in cod has not been identified yet (Kjesbu et al. 1996). MIH produced in the granulosa cells will subsequently initiate the formation of MPF by interacting with a membrane-bound receptor on the oocyte membrane surface (Zhu et al. 2003a; Zhu et al. 2003b). The MIH signal received on the oocyte surface is transduced to the cytoplasm by a G-protein-coupled reaction, finally resulting in the formation and activation of MPF. It has been demonstrated that MPF exhibits a unique molecular structure as a complex of cdc2 and Cyclin-B (Suwa and Yamashita 2007). MPF will subsequently induce oocyte maturation events such as chromosome condensation and GVBD.

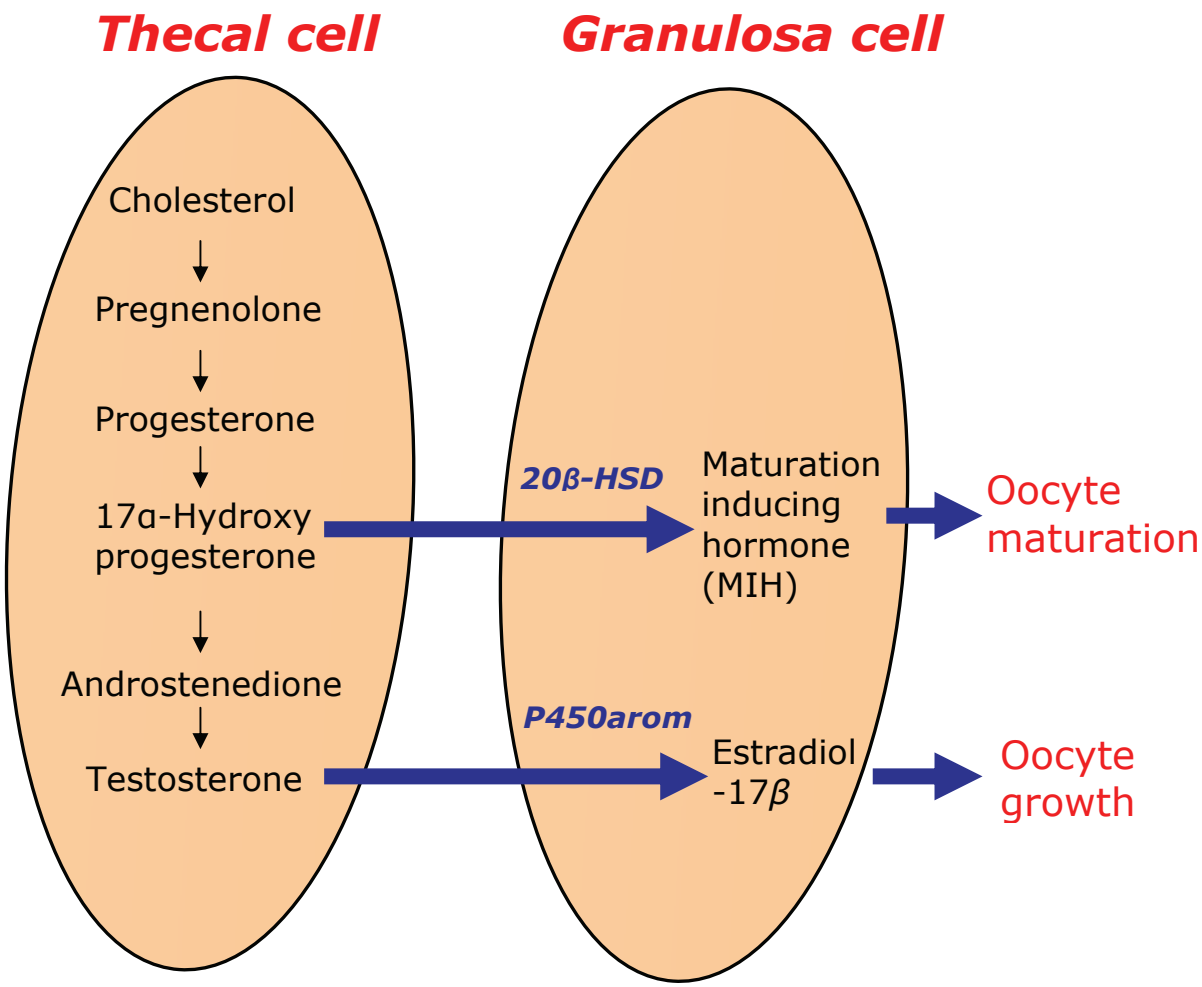


Figure 3. Two-cell type model. Schematic representation of shift in steroidogenesis in ovarian follicles. Modified from Senthilkumaran et al. (2004).

Gonadal steroidogenesis

Integral aspects of fish reproduction are regulated at the molecular level by sex steroid hormones, and teleost fish produce a number of bioactive gonadal steroids, including androgens, estrogens and progestogens. Steroid hormones are known to be involved in a number of physiological functions such as sexual differentiation, ion and carbohydrate homeostasis, adaptation to stress, immune system functioning and reproduction (Dean and Sanders 1996). The synthesis and regulation of steroid hormones involve an array of enzymes and potential biochemical pathways (Figure 4). Nevertheless, all steroid hormones have cholesterol as a common precursor, and they have also the same initial enzymatic step, the cleavage of a six-carbon side chain by cytochrome P450 side chain cleavage (P450*scc*), an enzyme situated in the inner mitochondrial membrane. In general, cellular cholesterol deposited in the outer mitochondrial membrane, lipid droplets or plasma membranes of steroidogenic cells must be delivered to the inner mitochondrial membrane before further enzymatic conversions can take place. The delivery of cholesterol to P450*scc* is meditated by the steroidogenic acute regulatory (StAR) protein, and this process serves as the rate-limiting step in steroidogenesis (Stocco 2000; Stocco 2001; Stocco et al. 2005). This step is rate-limiting because the hydrophobic cholesterol cannot traverse the aqueous intermembrane space of the mitochondria and reach P450*scc* rapidly enough by simple diffusion to support acute synthesis (Stocco 2001). The StAR protein is rapidly synthesized as a 37 kDa precursor phosphoprotein in response to the tropic hormone activation of cAMP protein kinase A intracellular signalling pathways (Artemenko et al. 2001; Clark et al. 1994; Stocco 2000). This cytosolic precursor protein is short-lived and is cleaved to the more long-life 30 kDa form which is the effective mediator of cholesterol transfer (Artemenko et al. 2001).

Pregnenolone, in turn, serves as a substrate for cytochrome P450 17-hydroxylase / C_{17-20}lyase (CYP17). As stated above, E2 is produced during oocyte growth while 17,20β-P is produced during oocyte maturation. Thus, a distinct shift from E2 to 17,20β-P occurs in ovarian follicles immediately prior to oocyte maturation (Senthilkumaran et al. 2004). In fish, the regulation of CYP17 may be a key mechanism in controlling this shift in the steroidogenic pathway from the production of androgens/estrogens to progestogens in the prematurational phase (Senthilkumaran et al. 2004; Young et al. 2005). Other studies have suggested that 20β-hydroxysteroid dehydrogenase (20β-HSD), the enzyme responsible for the subsequent conversion of 17α-hydroxyprogesterone to functional MIH functions as a key enzyme to initiate maturational events (Senthilkumaran et al. 2002, Figure 4).

The lyase activity of CYP17 gives rise to the androgens dehydroepiandrosterone (DHEA) or androstenedione, which in turn can be metabolized into the main C-19 androgenic steroids testosterone (T) and 11-ketotestosterone (11-KT, see Figure 4). Traditionally, androgens have been associated with male development, stimulating spermatogenesis as well as secondary male sexual characters. However, significant amounts of androgens such as T and 11-KT circulate in the blood of most female teleost fish (Borg 1994; Lokman et al. 2002). More specifically, 11-KT is generally believed to be the most potent or active male-specific androgen in teleost fish, but has been found at elevated levels in some female teleosts (Leatherland et al. 1982; Slater et al. 1994). It is known that aromatizable (T) and non-aromatizable (11-KT) androgens may have strikingly different effects (Borg 1994), and this has led to speculations whether androgens are produced solely as precursors during estrogen synthesis or if they represent true ovarian hormones (see section on study outline and objectives). Indeed, it has been demonstrated that androgens can induce vitellogenic responses in teleost fish (Hori et al. 1979; Le Menn et al. 1980; Mori et al. 1998; Peyon et al. 1997). Furthermore, recent studies have suggested the potential role of 11-KT as a promoter of oocyte development in previtellogenic ovaries (Lokman et al. 2007; Rohr et al. 2001). Whether these responses are mediated by androgens to the estrogen receptor rather than through androgen receptors themselves as observed in *Gobius niger* (Le Menn et al. 1980) remains to be elucidated.

The third group of sex steroids, the estrogens, are synthesized from the androgenic precursors via an aromatization step mediated by cytochrome P450 aromatase (CYP19 or P450*arom,* see Figure 4). Thus, P450*arom* is considered the rate-limiting enzyme responsible for regulating local and systemic estrogen levels. Several types of estrogens are naturally present in most teleost fish (Cheshenko et al. 2008). However, E2 is the major estrogen in female teleosts (Arukwe and Goksøyr 2003), and has traditionally been associated with female reproductive and developmental processes. By far, the best documented estrogen-dependent phenomenon in fish is the hepatic synthesis of Vtg (vitellogenesis) during oocyte growth (see section on hormonal regulation of oocyte growth). However, recent studies indicate that estrogens may play an important role in male reproduction by simulating proliferation of gonial stem cells (Miura et al. 1999).

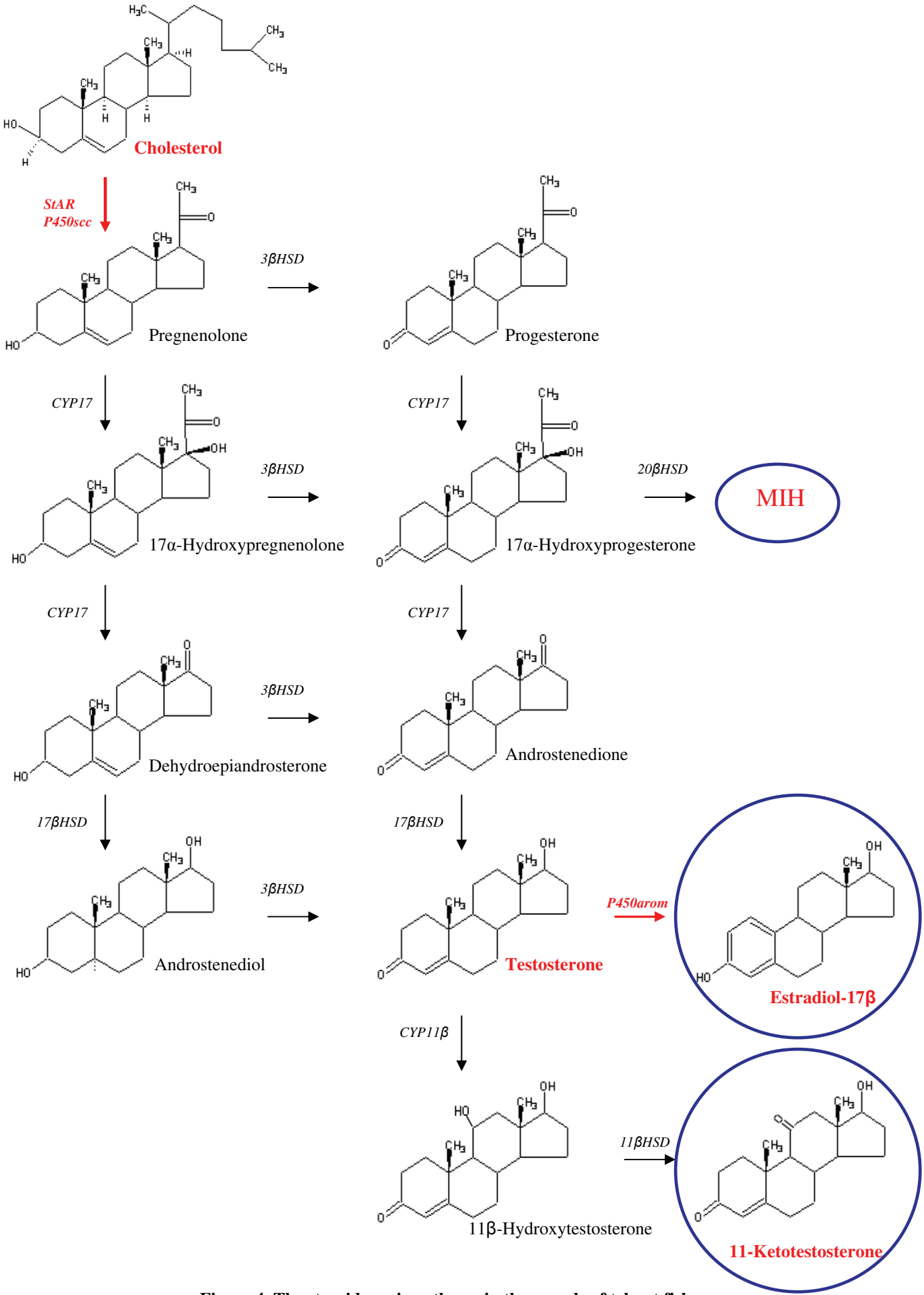


Figure 4. The steroidogenic pathway in the gonads of teleost fish.

Steroid hormone mode-of-action (MOA)

After synthesis, steroid hormones are not normally stored in the cell cytoplasm, but they diffuse out of the cell and act by binding to specific receptors. Since steroid hormones exert their biological effects only in the free and not in the bound form, plasma steroid-binding proteins may serve to buffer free steroid concentrations in conditions of high steroid turnover, thus obviating time consuming *de novo* synthesis (Mommsen and Walsh 1988). The gonad is the main site of sex steroid hormone synthesis as well as a major target for sex hormones. In general, sex hormones mediate their actions via two main mechanisms; the classic, genomic actions involving binding to and activation of specific intracellular nuclear receptors, as well as rapid, nongenomic actions initiated at the cell surface through membrane receptors (Figure 5, Aranda and Pascual 2001; Cheshenko et al. 2008; Falkenstein et al. 2000). The genomic actions of steroid hormones involve the modulation of gene transcription and protein synthesis, and are usually slow, taking hours to days to complete. In contrast, nongenomic actions of steroid hormones are mediated via receptors on the cell surface, and cellular modulations are generally rapid, activating intracellular signalling pathways within seconds (Thomas et al. 2007).

Classical or genomic steroid action

Nuclear receptors are grouped into a large superfamily and are thought to be evolutionarily derived from a common ancestor (Aranda and Pascual 2001). These receptors function as ligand-dependent transcription factors and regulate gene expression by binding to palindromic hormone responsive elements (HREs) at the DNA, usually located in the regulatory region upstream of receptor controlled genes (Aranda and Pascual 2001; Sabo-Attwood et al. 2004). Sex steroids regulate the expression of a battery of genes in a network-like manner, and initiate complex events involved in most aspects of vertebrate development (Falkenstein et al. 2000 and references therein). It is believed that the lipophilic steroid hormones enter their target cells by simple diffusion, although an active transmembrane transport has been suggested (Allera and Wildt 1992). In their unbound state, most sex steroid receptors are located in the nucleus at equilibrium, and are associated with a complex of chaperone proteins that include heat shock protein 90 (hsp90) (Defranco 2000; Pratt and Toft 1997). Upon ligand binding, the receptor will dissociate from the chaperone complex, dimerize with another receptor and subsequently bind to the HRE. Steroid receptors almost exclusively bind as homodimers to the HRE, however, receptors can also bind as heterodimers or monomers (Aranda and Pascual 2001). The recruitment of transcriptional co-factors and RNA polymerase initiate the mRNA transcription (Matthews and Gustafsson 2003). Finally, mRNA

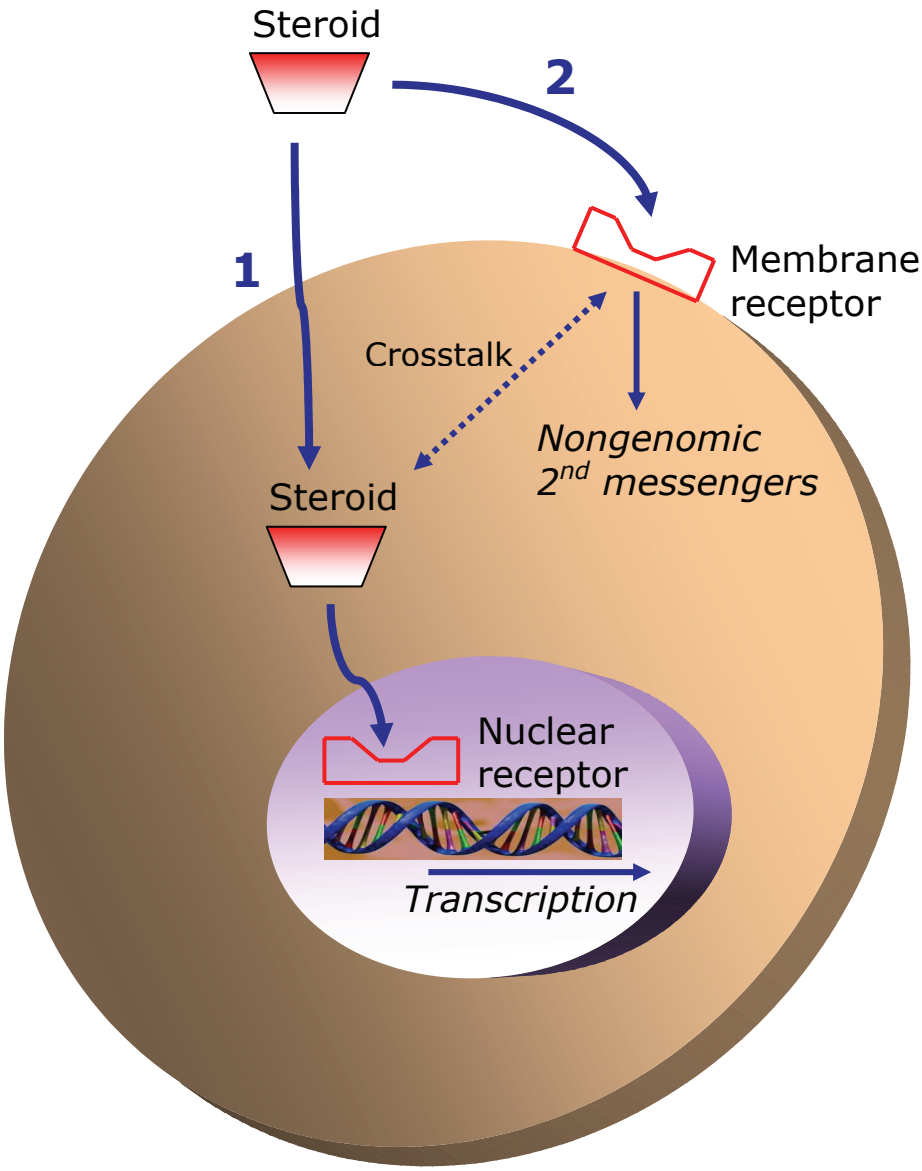


Figure 5. Nuclear (1) and membrane (2) signaling by sex steroids. (1)After ligand binding, the receptor regulates transcription by binding to hormone responsive elements usually located in the regulatory region of target genes. (2) Alternatively, steroids can exert their effects on the cell via non-genomic second messenger cascades initiated at the cell surface.

is translated into polypeptides and modified into active proteins. The classical example of E2-stimulated oogenic protein synthesis described earlier is presented in figure 6. Although most previous studies have focused on transcriptional activation by binding of sex steroid nuclear receptors to positive HREs, nuclear receptor can also repress gene expression in a ligand-dependent manner (Aranda and Pascual 2001). Nuclear receptors can also modulate gene expression without binding to a HRE. This process involves positive or negative interactions

with other transcription factors, a mechanism generally referred to as transcriptional cross-talk (Gottlicher et al. 1998).

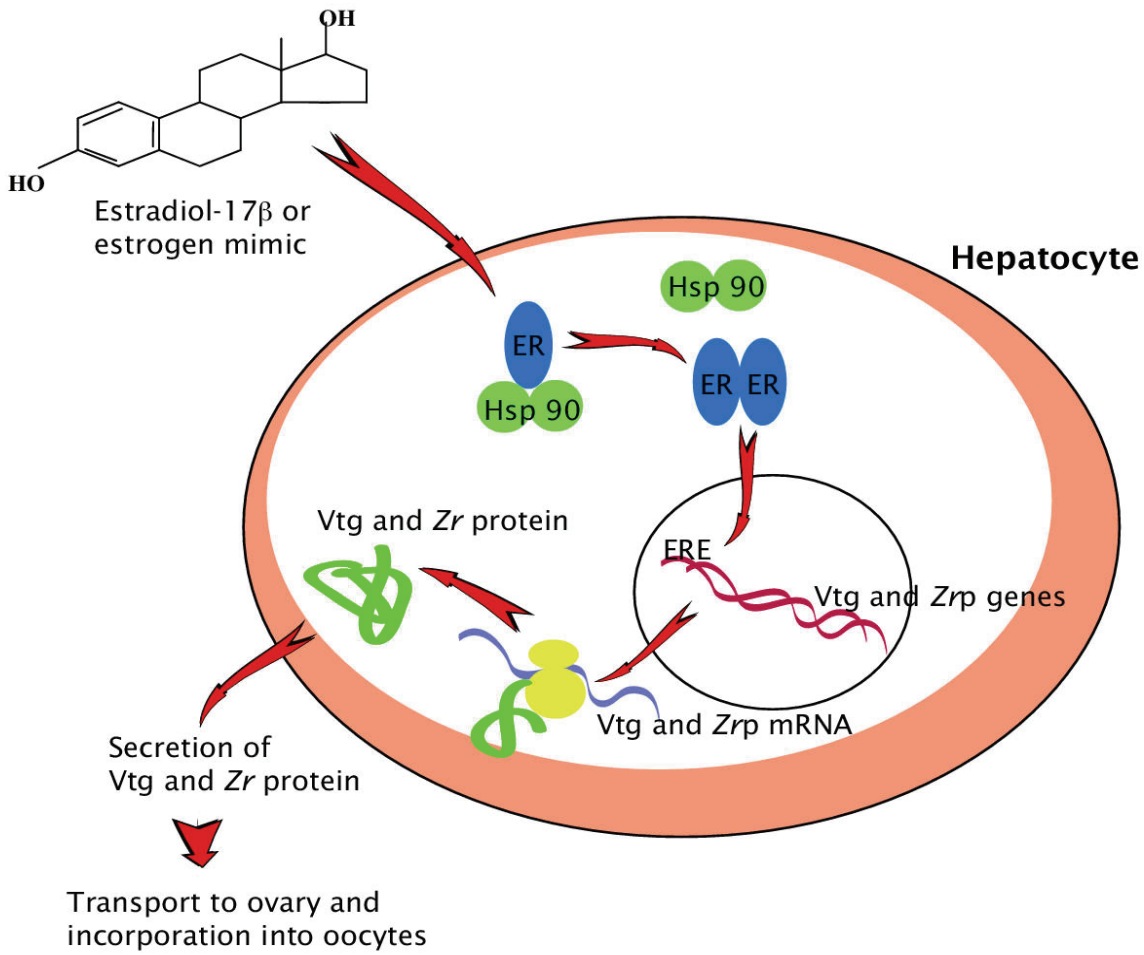


Figure 6. Estrogen stimulated oogenic protein synthesis. Estradiol-17β or an estrogen mimic bind to ER. This results in dissociation of ER from hsp-90 chaperones, receptor dimerization and activation of gene expression after binding to ERE. Figure from Arukwe and Goksøyr (2003).

Genomic effects of E2 are mediated by estrogen receptors (ERs). ERs have been cloned and characterized in several teleost species, and three ER isotypes have been identified; ERα, ERβa (previously called ERγ) and ERβb (Greytak and Callard 2007; Hawkins and Thomas 2004; Hawkins et al. 2000; Menuet et al. 2002; Sabo-Attwood et al. 2004). All these ERs bind estrogens with high affinity. In several teleost species, including Atlantic croaker, largemouth bass, fathead minnow and seabream, ERα and ERβb are primarily expressed in the liver,

whereas ERβa is highly expressed in the ovary (Filby and Tyler 2005; Hawkins et al. 2000; Pinto et al. 2006; Sabo-Attwood et al. 2004). In accordance, ERβa seems to play an integral role in the development and function of the teleost ovary, whereas the above mentioned hepatic Vtg synthesis is mainly mediated via ERα (Thomas et al. 2007). Hormonal regulation of ERs is controlled primarily by estrogens themselves (Thomas et al. 2007).

Androgens mediate their actions by binding to specific androgen receptors (ARs). In most vertebrates, only one functional nuclear AR has been identified, whereas two AR isoforms (ARα and ARβ) have been identified in some teleost fish (Ikeuchi et al. 1999; Takeo and Yamashita 1999; Todo et al. 1999; Touhata et al. 1999). The two AR isotypes seem to differ in steroid binding affinities between species, but have generally high affinity for 11-KT, T or 5α-dihydrotestosterone (DHT). Interestingly, the identification of nuclear ARs in the ovary of Atlantic croaker and kelp bass (Sperry and Thomas 1999a; Sperry and Thomas 1999b; Sperry and Thomas 2000) suggests that androgens may exert direct hormonal actions in the teleost ovary. Recently, a nuclear androgen receptor activated by 11-KT was characterized (Olsson et al. 2005). Little is known concerning the physiological roles of steroids in regulating nuclear AR expression. In contrast to ERs, that are auto-regulated by estrogens, ARs are not generally up-regulated by androgens, and no androgen responsive elements (AREs) have been identified in the promoter or 5'-flanking region of cloned ARs (Olsson et al. 2005).

As stated above, the two natural MIHs identified in teleost fish are the progestogens 17,20β-P and 20β-S. The teleost nuclear progestogen receptors (PRs) form homo- or heterodimers after binding to progestogens, and act as transcription factors that bind to progesterone response elements (PREs) on the promoter regions of progesterone responsive genes (Thomas et al. 2007). Although not conclusive, recent data indicates that progestogens are acting via the nuclear PRs to initiate ovulation of maturing oocytes (Thomas et al. 2007).

Non-classical or non-genomic steroid action

In recent years, it has become generally accepted that many actions of sex steroid hormones are not mediated via the classical, genomic mechanisms, but the identities of most steroid membrane receptors remain unknown (Watson 2003). Nongenomic effects of sex steroids on cellular function usually involve conventional second messenger cascades, such as phospholipase C, phosphoinositide turnover, intracellular pH, free intracellular calcium and protein kinase C (Falkenstein et al. 2000 and references therein). In contrast to genomic steroid action, nongenomic steroid effects are in general insensitive to inhibitors of gene

transcription and protein synthesis. One of the best characterized nongenomic steroid events initiated at the cell surface is the induction of oocyte meiotic maturation by MIHs (see section on hormonal regulation of oocyte maturation). In addition, similar cell surface-initiated actions of both androgens and estrogens have been described recently in fish ovaries (Braun and Thomas 2003; Braun and Thomas 2004; Loomis and Thomas 1999; Loomis and Thomas 2000). However, most functions of these receptors remain unclear, and more comprehensive studies will be required in order to gain insight in their physiological functions in teleosts.

Interaction with hormone mimics

A large number of anthropogenic chemicals released into the environment may interfere and disrupt endocrine homeostasis in humans and animals (Colborn and Clement 1992). Furthermore, it is widely recognized that certain anthropogenic chemicals may cause hormonal imbalance with potential consequences for fecundity and reproduction (Arukwe 2001; Gale et al. 2004). For example, masculinizing effects in zebrafish (McAllister and Kime 2003) and imposex, the development of male sexual characteristics in female neogastrodpod molluscs (Blaber 1970) have been correlated with exposure to tributyltin (TBT). In contrast, feminized responses were observed in seabird embryos exposed to 1,1,1-trichloro-2,2*'bis*(*p*-chlorophenyl)ethane (DDT) (Fry and Toone 1981). Elsewhere, alterations in steroid hormone levels and abnormal morphology of male and female gonads were observed in juvenile alligators exposed to organocloride contaminants (Guillette and Gunderson 2001). An endocrine-disrupting chemical (EDC) is defined as 'an exogenous substance or mixture that alter function(s) of the endocrine system and consequently produces adverse health effects in an intact organism, or its progeny, or (sub)populations' (Goksøyr 2006). While the exact mode of action for endocrine modulators is not fully understood, it is believed that EDCs can affect endocrine processes via three general mechanisms: 1) agonistic/antagonistic effects (hormone mimics), 2) disruption of production, transport, metabolism or secretion of natural occurring hormones and 3) disruption of production and/or function of hormone receptors (Goksøyr et al. 2003; Rotchell and Ostrander 2003).

The synthetic chemical nonylphenol (NP) is a degradation product of alkylphenol polyethoxylates (APEs) that are widely used as raw materials for active surface agents (anionic surface active agent, non-ionic surface active agents), ethyl cellulose stabilizers, oil soluble phenyl resins and esters. It is also used as processed articles for detergents, oil varnishes, rubber auxiliaries and vulcanization accelerators, antioxidants and corrosion inhibitors for petroleum products and sludge generation inhibitors for petroleum (Khim et al.

2001). The fish gonad is a known target organ for endocrine disrupters, and it is well established that NP can act as an estrogen mimic and modulate steroid pathways and reproductive responses like zonagenesis and vitellogenesis (Arukwe et al. 2000; Arukwe et al. 1997; Folmar et al. 2002) and ER levels (Yadetie et al. 1999). Recent studies have demonstrated that alkylphenols can influence the reproductive system in female Atlantic cod, with possible negative effects for the overall reproductive fitness (Meier et al. 2007). Because of its physicochemical properties, NP has enhanced resistance towards biodegradation, thus it has the ability to bioaccumulate in aquatic organisms. However, the mechanisms of actions of this xenoestrogen are still largely unknown, and effects of NP on the basal internal signals that regulate early ovarian growth prior to vitellogenesis (previtellogenesis), fecundity, reproduction and maturation are generally not well described.

Furthermore, pharmaceuticals are ubiquitous pollutants in the aquatic environment where their potential effects on non-target species like fish have only recently become subject of systematic investigations. Available scientific evidence indicates that the reproductive system, including its associated endocrine and neural controls, may be susceptible to alterations by occupational, pharmaceutical or environmental exposures to a variety of chemical and physical agents (DeRosa et al. 1998; Singleton and Khan 2003). The synthetic androgen, 17α-methyltestosterone (MT) is widely applied in aquaculture to control sex determination and induce sex-reversal of genetic females to phenotypic males (Hunter and Donaldson 1983; Kitano et al. 2000; Papoulias et al. 2000). Nevertheless, the specific role or effect of MT remains to be resolved, and factors such as dose, timing and duration of MT treatment can influence the effects. Previous studies addressing the effects of MT treatment in female fish have mainly focused on monitoring sex reversal and proportions of intersex in fish (Kanamori et al. 2006; Kitano et al. 2000; Orn et al. 2003; Zhang et al. 2006). However, MT is generally considered to be aromatizable when administered at high concentrations (Borg 1994; Nakamura 1975; Piferrer et al. 1993). In accordance, previous *in vivo* studies have demonstrated that high doses of MT can induce development of phenotypic female fish, a phenomenon which is referred to as a 'paradoxical feminisation' (Fenske and Segner 2004; Orn et al. 2003; Rinchard et al. 1999; Zerulla et al. 2002). The paradoxical feminization shift in sex ratio suggests an increased aromatase activity with subsequent conversion of MT to methylestradiol (ME2), but the extent and magnitude of MT effects on sex-reversal depend on a number of biotic and abiotic factors including age, size, developmental stage, ambient temperature, nutritional factors, exposure duration, method and season (Higgs et al. 1982).

Genomic approaches for understanding fish reproductive endocrinology

Most developmental events are controlled at the molecular and cellular levels of biological organization. Therefore, gene expression profiling as a result of hormonal exposure and the subsequent molecular processes that lead to developmental and physiological changes may be used as quantitative molecular markers for cellular, physiological, developmental and reproductive effects in an organism. The identification of hormonal responsive genes and their expression patterns as a result of hormonal influence are of vital importance in order to shed light on the underlying mechanisms of these fundamental processes of fish reproduction. The molecular mechanisms of fish reproductive biology and endocrinology have only the recent years become an object for systematic investigation. Recently, many molecular players of fish oogenesis have been identified through large-scale genomic studies (Govoroun et al. 2006; Knoll-Gellida et al. 2006; Rise et al. 2004; von Schalburg et al. 2005; Zeng and Gong 2002). Most focus has so far been on genes involved in sex differentiation and final maturation (Baron et al. 2005; Baron et al. 2008; Baron et al. 2007; Bobe et al. 2006; Kanamori 2000; Vizziano et al. 2007; von Schalburg et al. 2005). These comprehensive surveys have produced a large number of expressed sequence tags (ESTs) from teleost fish, making it possible to profile fish mRNA expression during for example, oocyte growth and determine the roles or effects of specific genes on oocyte developmental events. As previously stated, research on fish oogenesis has so far focused on the vitellogenic process and the events taking place during final maturation. On the contrary, processes during previtellogenic oocyte growth remain largely unexplored (Patino and Sullivan 2002; Tyler and Sumpter 1996). For example, the endocrine and/or intraovarian factors that regulate previtellogenic oocyte growth have not been described in any detail (Luckenbach et al. 2008). Given that much of the mRNA present in full-grown oocytes seems to be produced during previtellogenic growth (Wallace and Selman 1990), and that female fecundity and egg size seems to be largely determined during previtellogenesis in rainbow trout and salmon (Bromage et al. 1992; Campbell et al. 2006; MacKenzie et al. 1998) this still represents a strong gap in our knowledge of fish endocrine and reproductive physiology.

Recently, the molecular basis of previtellogenic oocyte growth and development have received more attention, and a number of differential expressed ovarian genes during previtellogenic oocyte growth in teleost fish have been identified (Campbell et al. 2006; Luckenbach et al. 2008). Useful tools for high throughput analysis of tissue- and stage-specific expression of genes include suppressive subtractive hybridization (SSH), generation of cDNA libraries and microarray analyses. In addition, quantitative (real-time) polymerase

chain reaction (qPCR) analyses are widely employed to obtain molecular signatures that can be more accurate than those of other high throughput methods, such as microarrays (Baron et al. 2005). Taken together, the data generated from large-scale gene expression profiling may serve as a basis for future hypothesis-driven research and provide insights regarding gene networks and pathways involved in reproductive and endocrine events. It may also be useful in order to identify molecular mechanisms and pathways disrupted by environmental toxicants.

The Atlantic cod as a model species

The Atlantic cod (*Gadus morhua*) is widely distributed across the continental shelf regions of the North Atlantic, and several important cod stocks are of great economic and social importance. In Norwegian waters, the Norwegian-Arctic stock, which spends most of its life in the Barents Sea, is the most important. There are also a large number of more or less well-defined local stocks along the coast of Norway. Other important cod stocks are found in areas off Iceland, Greenland and Canada and in the North Sea and the Baltic. These stocks have all suffered from extreme fishing pressure in recent years, and several of them are showing clear signs of overexploitation. Things have gone particularly hard with the Northern cod stock on the Grand Banks off the east coast of Canada (Hannesson 1996). Until recently, no signs of recovery for this stock have been shown even after ten years during which fishing has been banned, following the collapse of the stock in the early 90s. With poor catches as a result of reduced stocks in Norwegian waters as well, total catches of Atlantic cod have displayed a worrying downward trend during the past 10 - 15 years. The cod is well known and a popular species which has a large economic and market value world-wide. Interest in the intensive production of cod has increased dramatically over the past couple of years due to reduced supply from wild fisheries, high market price and relative suitability of cod for culture (Brown et al. 2003; Moksness et al. 2004). Commercial rearing of cod is rapidly increasing, and the Norwegian aquaculture production of cod was 5.500 tons in 2005, while in 2006 the production increased to about 10.000 tons (Karlsen et al. 2005). The potential for cod farming is therefore regarded as extremely high on a global basis. Cod also enjoy a number of natural advantages as a potentially important cultivated species; short egg and larval stages, rapid growth, good feed utilization, suitable behaviour (‘tame’), and, as far as we know, good health. Cod also appear to adapt well to traditional sea-cages, even to the extent that aquaculture technology developed for salmon can easily be adapted to cod. Most of the challenges offered by cod farming generally concern nutrition; selection and testing of optimal fish feed (start feeding, cannibalism in the young fish phase, lipid deposition in the liver and premature sexual maturation). We can expect to encounter challenges on the health side, related to fish

feed and to bacteria, viruses and parasites. On this note, understanding the physiology and genetics of cod will be of vital importance when meeting these challenges in establishing a sustainable population both for fisheries and in aquaculture.

STUDY OUTLINE AND OBJECTIVES

Despite the concept of life-history strategies, the internal signals that regulate fecundity are not known. Subject to fecundity and fertility in fish and mammals, respectively, the overall objective of this study was to study the role/effect of androgens on the growth of previtellogenic oocytes through the characterization of androgen-responsive ovarian genes. This will increase our understanding on the hormonal control of previtellogenic oocyte growth in teleosts. The objective implied the sequencing of a number of ovarian previtellogenic genes in Atlantic cod (*Gadus morhua*). mRNA was isolated from previtellogenic ovarian tissues before and after *in vitro* exposure to androgens (11-KT and T). The mRNA was transcribed into cDNA and libraries constructed in plasmid vectors. Subtracted clones were arrayed, preserved and applied in gene expression profiling. Despite the fact that hormonal effects on the growth of previtellogenic oocytes might involve several cascades of physiological events, observations in fish and mammals point to a pivotal role of androgens and might be linked to nutritional factors involving other growth factors (growth hormone and insulin-like growth factor-1, IGF-1). This might also increase our understanding on the role of growth rate on fecundity. Organ-specific steroid hormone synthesis occurs in the absence of functional hypothalamus-pituitary-gonadal-axis. Except for the evidence that androgens increase oocyte diameter and modify IGF-1 and IGF-1-receptor mRNA abundance, the underlying molecular mechanism(s) involved in possible gene regulation resulting in the growth of previtellogenic oocytes has not been investigated. The detailed understanding of these "within" ovary processes that determine fecundity will help in the future on the potential manipulation of fecundity in teleosts. The potential manipulation of factors that control fecundity will be beneficial for the aquaculture industry and for breeding of new captive and endangered species. Furthermore, studies on the early follicular growth in fish will provide an understanding on the role of androgens in the control of fecundity through modification of gene expression and evaluation of the evolutionary conservation of the control mechanisms of early follicular growth in vertebrates. New molecular techniques provide valuable tools for this kind of investigation and formed an integral aspect of these studies, and we have used the analytical power of suppressive subtractive hybridization (SSH) to open a possible revealing window of understanding into the functional aspects of early oocyte development in organisms that control fecundity. The overall objective of this study was to investigate the roles/effects of androgens (11-KT, T and MT) on the growth of previtellogenic oocytes through quantitative histological analyses and the characterization of androgen-responsive ovarian genes. These objectives are outlined in the following two hypotheses:

Hypothesis #1: Previtellogenic ovarian oocytes show differential gene expression profiles and histological modulations after *in vitro* and *in vivo* androgen exposure, indicating hormonal control of early follicular and oocyte growth.

Hypothesis #2: Differentially expressed ovarian gene products provide evidence of at least some of the biochemical and physiological processes occurring during previtellogenesis in teleosts and can be used to deduce evolutionary relatedness of the control of follicular growth in vertebrates.

METHODOLOGICAL CONSIDERATIONS

In vitro floating agarose method

The use of *in vitro* systems may have several major advantages. These include the ability to directly study mechanistic events, to control the environment for testing specific hypotheses, and that multiple experiments can be performed to validate the initial observations (Shankland et al. 2007). For example, possible indirect mechanisms of hormones through feedback on the brain and/or pituitary can be omitted if employing an *in vitro* organ culture system. In the field of fish reproduction, *in vitro* approaches have proven to be valuable model systems for evaluating hormonal control on spermatogenesis in eels (Miura et al. 2005; Miura et al. 2002; Miura et al. 1991). Additionally, they can be a strong tool for assessment of specific toxicity mechanisms and/or for screening a large number of chemicals (such as agrochemicals, pharmaceuticals or environmental contaminants) (Janosek et al. 2006).

The application of *in vivo* studies are complicated by the variation in genetic background between individuals, as well as ethical questions concerning animal research since a large number of individuals are required to perform statistically valuable experiments (Ge et al. 2003). Regardless, no *in vitro* technique will ever have the complexity of the whole animal system, since *in vitro* systems lack the essential metabolic competence of a living organism, and *in vitro* assays may miss certain chemical effects caused by metabolites or toxicological responses caused by interference with xenobiotic metabolism. While relevance increases as concentrations used *in vitro* approach those that occur in the organism when similar effects are observed, concentration differences might occur for a number of reasons, making extrapolation to *in vivo* events difficult. Therefore, it would be unwise, within certain limits, to disregard data because they were obtained at higher concentrations than those expected in live animals (Tiffany-Castiglioni et al. 1999). Thus, concentration- and time-response evaluations are especially important when evaluating effects *in vitro* in relation to those seen *in vivo* (Tiffany-Castiglioni et al. 1999).

In the present work, we have employed an *in vitro* organ culture technique that is based on the floating-agarose method (Miura et al. 1991; Nader et al. 1999). We have used this method in our laboratory to culture several organs for different toxicological, physiological and endocrinological purposes with successful and reproducible results (**Paper I, II, IV, V** and Vang et al. 2007). In the experimental setup, 1% agarose cylinders are placed in 6-well culture dishes and soaked with culture medium. A nitrocellulose membrane is placed on top of the agarose cylinder, and the organ of interest is excised from the animal, cut into small pieces

and placed on the nitrocellulose membrane. In this way, the tissue pieces are not submerged, but they are still exposed to the chemicals in the medium. Antibiotics may potentially interfere and affect tissue gene expression patterns, and were therefore omitted. Hence, preparation and cultivation of tissue must be performed under strict sterile conditions.

Suppressive subtractive hybridization (SSH) and microarray (CodArray) analysis

SSH is a technique that enables researchers to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other (Diatchenko et al. 1996). Although there are several different methods, the basic theory behind subtraction is simple. First, both mRNA populations are converted into cDNA: i.e. the cDNA that contains specific (differentially expressed) transcripts as 'tester,' and the reference cDNA as 'driver.' Tester and driver cDNAs are hybridized, and the hybrid sequences are then removed (Figure 7). Consequently, the remaining unhybridized cDNAs represent genes that are expressed in the tester, but are absent from the driver mRNA. In the present study, we generated a targeted cDNA library by performing SSH with ovary tissue from juvenile Atlantic cod exposed *in vitro* separately to 11-KT and T (1, 50 and 100 μM) and used against untreated samples (solvent control samples). Because the SSH technique favours the enrichment of high abundance transcripts and is therefore very susceptible to a high false-positive rate (Ji et al. 2002; Larkin et al. 2003), we performed the hybridization in both forward (up-regulated) and reverse (down-regulated) directions to maximize the detection and identification of androgen responsive genes. All clones were PCR amplified and verified by agarose gel electrophoresis. After sequencing and BLAST identification, genes were annotated and downloaded to EST GenBank under the title 'CodArray SSH cDNA library'. Secondly, a targeted gene array (CodArray) was developed based on apparent true-positive differentially expressed genes. Therefore, our targeted gonadal CodArray represents a unique suite of differentially expressed genes that were either up- or down-regulated in response to 11-KT and T exposure.

The EST approach was first demonstrated in the human genome project (Adams et al. 1991), and has proven to be powerful in massive cloning of cDNAs as well as in large scale characterization of cDNA sequences for deciphering genome sequence (Zeng and Gong 2002). In accordance, several research groups have employed SSH, cDNA library generation and microarray analyses in order to identify genes involved in oocytes growth and development (Baron et al. 2005; Luckenbach et al. 2008; von Schalburg et al. 2005).

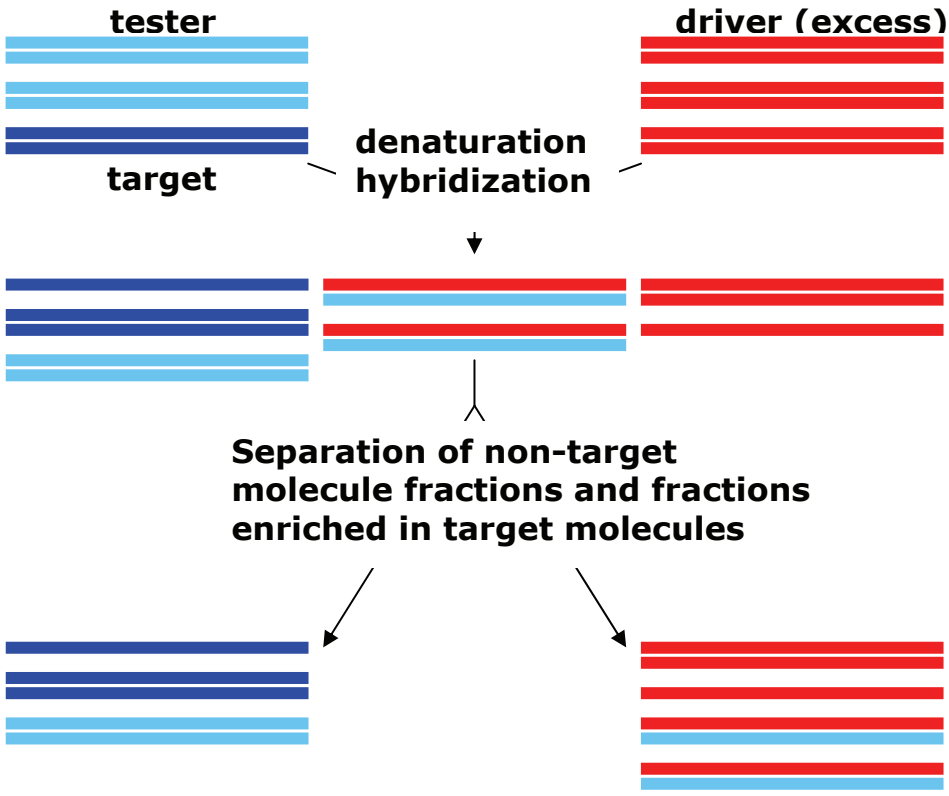


Figure 7. Scheme of the SSH method. The basic principle of subtractive hybridizations is to reverse transcribe two different mRNA populations, heat denature the cDNA pools, and then hybridize together the two samples. The cDNAs that remain un-hybridized, which represent differentially regulated mRNAs, can be PCR amplified, cloned and subsequently sequenced. Subtractive hybridizations can be performed in both directions in order to obtain both up and down-regulated genes within a specific tissue of interest.

Quantitative real-time PCR

The most popular method for quantifying individual gene expression is quantitative real-time polymerase reaction (qPCR). The method is sensitive, capable of high throughput and relatively easy to perform. In addition, expression profiles obtained by qPCR may be more accurate than those of other high-throughput methods, such as microarrays (Baron et al. 2005). For the sake of accuracy and precision, it is necessary to collect quantitative data at a point in which every sample is in the exponential phase of amplification (since it is only in this phase that amplification is extremely reproducible). Analysis of reactions during exponential phase at a given cycle number should theoretically provide several orders of magnitude of dynamic range. Rare targets will probably be below the limit of detection, while abundant targets will

be past the exponential phase. In practice, a dynamic range of 2-3 logs can be quantified. Nevertheless, it is vital to minimize variability and maximize reproducibility by quality-assessing every component of the qPCR assay (Nolan et al. 2006). For example, the selected primer sequences must be specific for the gene of interest in the organism being studied. Also, aiming for as short an amplicon as possible (60-150 bp) is important to ensure efficient denaturation during thermal cycling (Nolan et al. 2006).

Normalization of qPCR data

In order to obtain reliable expression patterns, it is important that qPCR data are normalized with a proper internal control. In the present study, total cDNA for the qPCR reactions were generated from 1 µg total RNA using a combination of random hexamer and poly-T_{18} primers. The expression of individual gene targets was analyzed and every DNA amplification reaction contained controls lacking cDNA template to determine the specificity of target cDNA amplification. Briefly, cycle threshold (*Ct*) values obtained were converted into mRNA copy number using standard plots of *Ct* versus log copy number. Standard plots for each target sequence were generated using known amounts of plasmid containing the amplicon of interest. The criterion for using plasmid standards for normalization is based on equal amplification efficiency with unknown samples, and this is checked prior to extrapolating unknown samples to the standard curve. Data obtained from triplicate runs for individual target cDNA amplification were averaged and expressed as ng/µg of initial total RNA used for reverse transcriptase (cDNA) reaction and thereafter transformed as percentage of control. This absolute quantification method is a well-validated procedure in our laboratory, as we do not use the so-called housekeeping genes because of their variable expression patterns both in our laboratory (Arukwe 2006) and elsewhere (Bustin and Nolan 2004; Mittelholzer et al. 2007; Steele et al. 2002). By employing this method for normalization of qPCR data, it is essential to assess RNA integrity as well as quantity. In the present study, only high quality RNA with A260/A280 ratio above 1.8 and intact ribosomal 28S and 18S RNA bands was used for cDNA synthesis.

Histology and stereological techniques

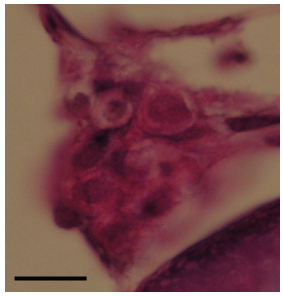
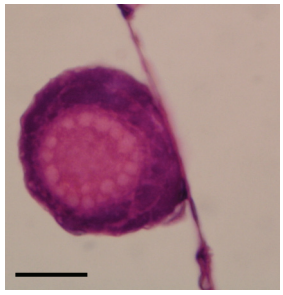
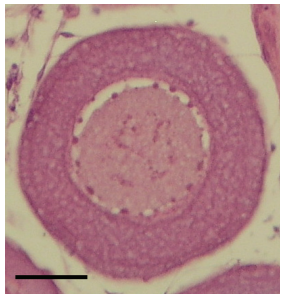
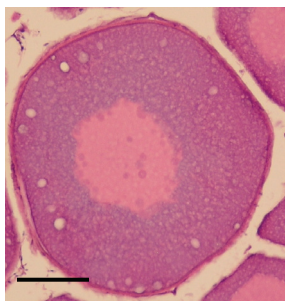
In order to compose a histological growth staging system for oocytes in the previtellogenic cod ovary, we generated a classification system based on previous studies of oocyte development in tilapia (Rocha and Rocha 2006) and cod (Kjesbu and Kryvi 1989, Table 1). The reason for doing this, although oogenesis in Atlantic cod has been thoroughly described previously (Kjesbu and Kryvi 1989), was the requirement of strict, accurate definitions of

oocytes stages in the previtellogenic cod ovary. Furthermore, when examining the tissues, the specific stage of every single oocyte in the field of vision should be possible to define. Thus, morphological details of the nucleus were unsuitable as oocyte stage markers, as far from all oocytes were sectioned through the nucleus. Also, oocytes of the same developmental stage can be quite different in size (Tyler and Sumpter 1996), making size-specific staging suboptimal.

Stereology – a quantitative histological analysis

In the present study we wanted to provide a precise quantification of the fractions of oocytes in different developmental stages in the previtellogenic cod ovary. The stereological method of point-counting described by Freere and Weibel (1967) was employed in order to estimate the relative area of the ovary occupied by oocytes of a certain developmental stage. In the present study, these relative areas are expressed as proportional volume fractions (%), as reviewed by Coward and Bromage (2002). All stereological methods are based upon a simple principle known as 'Delesse Principle' (Delesse 1847), which demonstrates that a random histological section can be quantitatively representative of the total composition of the original unsectioned material (Coward and Bromage 2002). Stereological techniques rely heavily upon the analysed tissue sections and fields being independent from another (Coward and Bromage 2002). In accordance, all stereological studies require representative sampling of the organ and generation of adequate sectioning of the structures to be analyzed (Mandarim-de-Lacerda 2003). The sampling and arbitrary sectioning of the (naturally roundish) oocytes granted the isotropy requirements (Mandarim-de-Lacerda 2003). In addition, the oocyte distribution throughout the ovary must be homogenous. Previous studies have shown that oocyte size distribution is homogenous in cod ovaries (Kjesbu and Holm 1994). Finally, is essential to obtain a total of at least 300 recorded grid points per tissue piece, recorded from a number of independent sections, which is generally accepted in order to establish an estimate precision of about 5% (Weibel 1979).

Table 1. Classification of developmental stages of Atlantic cod previtellogenic oocytes.

Previtellogenic oocyte developmental stage	Main cellular characteristics	Histological images
Proliferating oogonia	Small nests of mitotic cells dispersed within the connective tissue. Oogonia are small and circular, with a clear cytoplasm. Bar=10 µm.	
Initial primary growth	Primary oocytes had one large, usually peripheral nucleus. The oocyte is still attached to the connective tissue. The cytoplasm is strongly basophilic due to the high RNA concentration. Bar=10 µm.	
Primary previtellogenic growth	Primary oocytes showed one large, centrally located nucleus with several basophilic nucleolei at the periphery. The cytoplasm stains also basophilic. Follicular cells are scarce and undifferentiated. Bar=15 µm.	
Advanced previtellogenic growth	The oocyte becomes a larger cell. The cytoplasm has more vesicles and becomes less basophilic due to a decreased RNA concentration. The follicular cells are appearing and begin differentiating. Bar=30 µm.	

GENERAL DISCUSSION

SSH and targeted CodArray

As described in **Paper I**, a custom cDNA library containing clones of transcripts from immature Atlantic cod ovarian tissue exposed to a mixture of androgens (11-KT and T) was generated. Secondly, a targeted gene array (CodArray) was developed based on apparent true-positive differentially expressed genes. Therefore, our targeted ovarian CodArray represents a unique suite of differentially expressed genes that were either up- or down-regulated in response to androgen exposure. When genomic data for an organism is not available, such as the Atlantic cod, the SSH method can be a powerful tool for generating cDNA libraries containing DNA sequences of interest. The use of microarrays to screen cDNA clones generated by SSH also allows the identification of previously unknown genes -a finding that is not possible using conventional arrays of previously cloned genes (Yang et al. 1999). However, SSH usually generates a number of redundant clones in the subtracted amplicons, and several genes suspected to be involved in the transcriptional regulation of oocyte growth and development were not represented after performing SSH in the present study. The reason for this is probably the low expression levels of these genes in cod ovarian tissue. In accordance, it is well known that SSH favours the enrichment of high abundance transcripts (Ji et al. 2002; Pan et al. 2006), which may result in over- or under-representation of certain amplicons in the SSH cDNA library. Therefore, transcripts from some genes were amplified by PCR using specific primers from conserved regions of the respective genes based on sequence information in NCBI GenBank. The PCR products were cloned into *Escherichia coli* plasmids and subsequently added to the array. Sequenced clones were analyzed using Blastx against the NCBI nonredundant (nr) protein database and Blastn against the NCBI nucleotide (nt) database. The chosen e-value cut-off was 10^{-5} for blast searches. Finally, clones were annotated and downloaded to the GenBank EST database under the title CodArray SSH cDNA library. 150 expressed sequences from this library were grouped into 54 UniGene entries (putative genes), and there were 23 contigs of two or greater (groups of clones probably representing overlapping regions) and 31 singletons. Although the present study generated few sequences compared with other surveys, the number of gene profiles generated was already too high to make a one by one analysis suitable. Therefore, we performed an initial microarray screening with subsequent qPCR analyses of genes suspected to be involved in previtellogenic oocyte growth and development. After annotation of the sequenced clones, we found that the major part of the amplicons generated by SSH were of oocyte origin, whereas less abundant transcripts derived from ovarian follicle cells are not likely to be identified by the SSH method employed in the present study. In compliance, this

has been observed earlier, and it has been proposed that construction of cDNA libraries enriched for extra-oocyte components such as the follicle wall will provide a better generation of transcripts from these ovarian components (Goetz et al. 2006; Luckenbach et al. 2008).

In the CodArray experimental design, 36 microarrays were used for each exposure (11-KT and T) and co-hybridizations were performed according to a loop design, in which all biological replicates appeared in more than one array causing the arrays to not be statistically independent (Figure 8). cDNA microarray techniques have some disadvantages compared to more tedious techniques such as qPCR. For example, they are limited in the detection of genes with low expression (Rondeau et al. 2005), and clones from less abundant mRNAs may fall within the 'noise' of the hybridization signals (Yang et al. 1999). In addition, cDNA insert size may be a factor in reliably identifying differentially expressed genes, and short SSH clones from the 5[prime]-end of cDNAs are less likely to give reliable hybridization signals than full-length cDNAs (Yang et al. 1999). In the present study, microarray screening failed to identify some genes (e.g. StAR and P450*scc*) that were shown to be differentially expressed after androgen treatment by qPCR (**Paper I**), and validation of array values by qPCR demonstrated differences in expression profiles for the two methods in some instances. These differences might be due to the mentioned limitations of microarray techniques. To conclude, we believe the microarray approach should be considered a first screening, and qPCR probably produced more accurate molecular signatures, at least in the *in vitro* study (**Paper I**).

Tissue culture conditions

One of the underlying aims of the present study was to develop a sensitive *in vitro* and mechanistic organ culture system for studies on hormonal impacts on biological systems. *In vitro* techniques have considerable appeal for endocrinological and physiological (also toxicological) studies, and a major advantage in using *in vitro* organ culture systems is the consistency and reproducibility of the results that can be obtained using a homogeneous group of experimental and sample materials. Additionally, *in vitro* systems have ethical and economic benefits. Nevertheless, *in vitro* systems will always imply a compromise. If the aim is to mimic normal physiology, it is essential that the system applied represents the real *in vivo* situation, at least for the endpoint(s) of interest, in order to function as a reliable model system. In accordance, the *in vitro* floating agarose method used to investigate the effects of androgens (**Paper I, II and IV**) and NP (**Paper V**) was compared with the *in vivo* situation, using immature female cod (**Paper III**). The details of these comparative studies are outlined below.

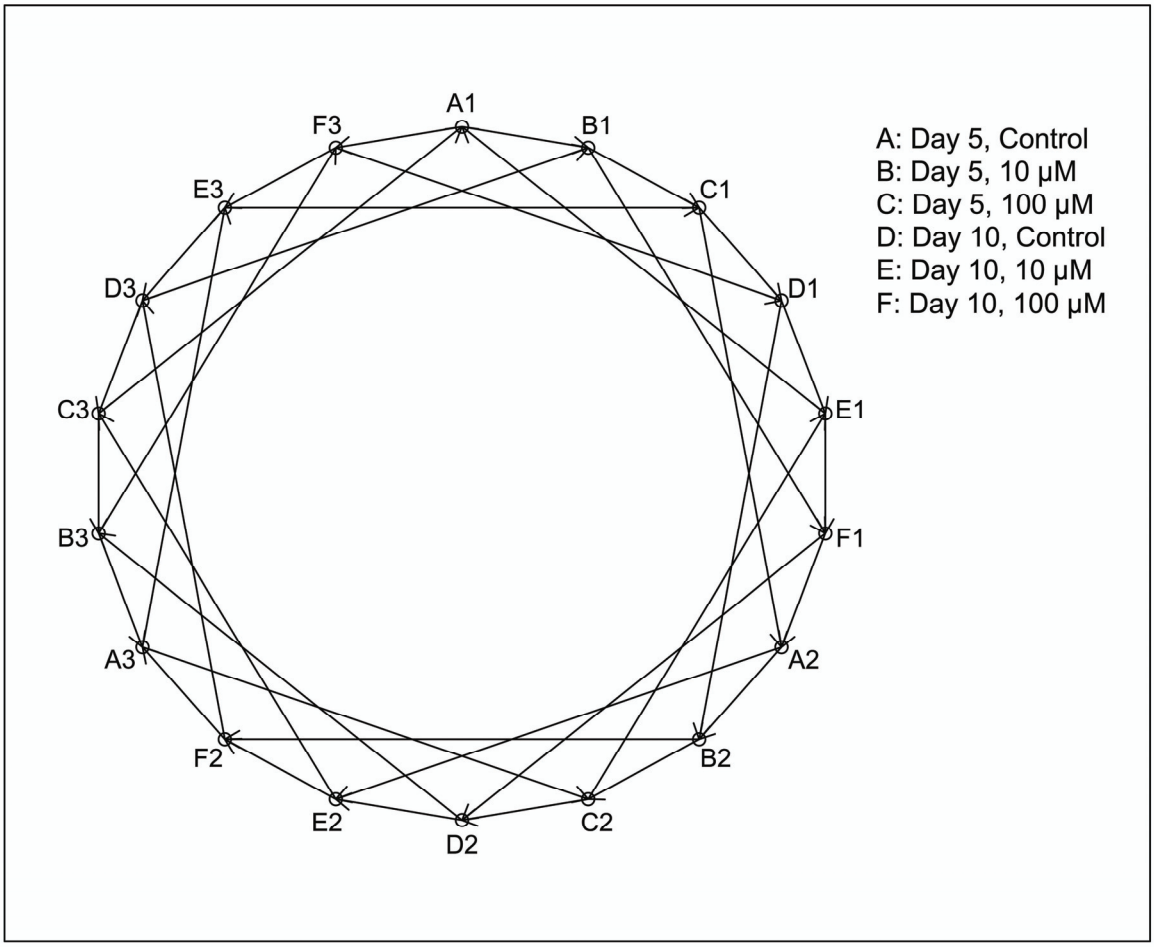


Figure 8. Array design used for evaluating the concentration- and time-dependent effects of the androgens, 11-ketotestosterone (11-KT) and testosterone (T) on previtellogenic cod oocytes. The letters A-F represent the experimental conditions and the numbers 1-3 identify different biological replicates. Each arrow in the figure indicates an array that was run co-hybridizing the sample at the arrow's head (labeled with Cy5 dye) and the sample at the arrow's tail (labeled with Cy3 dye).

Androgen concentrations

The nominal 11-KT and T concentrations (1-1000 µM) used in the present *in vitro* studies (**Paper I and II)** are several orders of magnitude higher than physiological androgen levels reported in fish. Plasma levels of 11-KT in the female fish used for the *in vitro* gonadal cultivation (**Paper I, II, IV and V**) were 1.0 ± 0.5 ng/ml. Prior to performing these studies, there were no studies that had measured physiological androgen levels in previtellogenic stages of Atlantic cod known to us, although a few studies had measured variable T levels in cod during later stages of oocyte development (Dahle et al. 2003; Kjesbu et al. 1996). In a study by Lokman and co-workers (Lokman et al. 2002), the presence of both 11-KT and T were demonstrated in females for a number of teleost species that were generally at low

concentrations (<1 ng/ml). The closest relative to the Atlantic cod, namely the red cod (*Pseudophysics bachus*) displayed a mean female 11-KT level of 0.20 ± 0.02 ng/ml (n=5). In planning and designing the present study, we hypothesized that only a fraction of the given androgen concentrations will be accessible to the oocytes using the *in vitro* floating agarose method after considering biological factors such as bioavailability and bioconcentration. This hypothesis was supported by enzyme immunoassay (EIA) analyses of 11-KT concentration in tissue cultures used in the present study (**Paper I and II,** Figure 9).

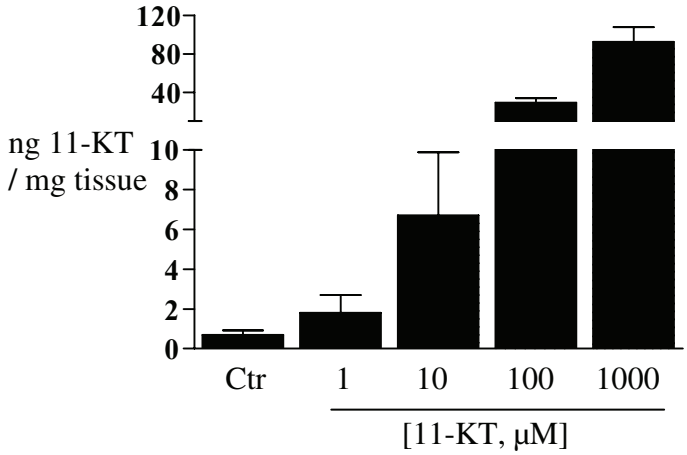


Figure 9. 11-ketotestosterone (11-KT) levels in previtellogenic oocyte cultures of Atlantic cod incubated for 24 h with different 11-KT concentrations. 11-KT levels were determined using enzyme immunoassay method. Data are given as mean value and expressed as ng/mg tissue (wet weight) of *n*=3 ± standard error of the mean (SEM).

Solvent control tissues displayed a mean 11-KT concentration of 0.68 ng/mg tissue (wet weight), whereas mean 11-KT concentration increased 3, 10, 30 and 100-fold when exposed to 11-KT at the nominal concentrations of 1, 10, 100 and 1000 µM, respectively. These measurements were conducted 24 h post-exposure, and steroid concentrations probably decreased rapidly during the next 4 exposure days, before fresh media with 11-KT was added to the cultures (i.e. at day 5). A rapid decrease in plasma steroid hormone levels has been reported previously during *in vivo* steroid administration to fathead minnow (Korte et al. 2000), muskellunge (Rinchard et al. 1999) and rainbow trout (Pakdel et al. 1991). These findings were concomitant with our own *in vivo* observations (**Paper III**), showing a rapid clearance of plasma 11-KT and T levels that paralleled control levels at 21 days after intraperitoneal injection with 5 mg/kg at 7-days interval. In conclusion, we estimate that the lower androgen concentrations used in our *in vitro* studies (1 and 10 µM) represent

physiological relevant concentrations, whereas the higher concentrations (100 and 1000 μM) probably represent an extreme *in vivo* situation. Indeed, if the highest androgen concentrations used create extra-physiological androgen levels in the tissue, results may reflect pharmacological and not biological relevant effects on previtellogenic oocyte development. Extra-physiological androgen concentrations could also artificially induce effects due to androgen metabolites. However, given the previous mentioned rapid clearance of 11-KT and T *in vivo*, it seems unlikely that cultivated tissue was exposed to pharmacological doses of androgens, at least not for a long time interval.

Morphological studies

All the ovarian tissues examined in the present study (both *in vitro* and *in vivo*) displayed a normal histology and were generally healthy, as compared to general teleost ovarian morphology and earlier observations in Atlantic cod (Burton et al. 1997; Kjesbu and Kryvi 1989). Nevertheless, ovarian morphology after *in vitro* cultivation (**Paper I, II, IV and V**) had some differences in comparison with the *in vivo* experiment (**Paper III**). Firstly, the volume densities of atretic oocytes in cultivated ovarian tissue were generally 3-5%, with no significant differences between control and androgen exposed tissues. However, no oocyte atresia was observed in the *in vivo* experiment. A significant increase in volume fractions of atretic oocytes were observed in tissue cultivated for 5 and 10 days, as compared to tissue cultivated for 24 h. Although it has been shown that atresia occurs in oocytes of all developmental classes, very little is known about atresia during early stages of oocyte development (Tyler and Sumpter 1996). The elevated levels of atresia after *in vitro* cultivation could point to possible suboptimal conditions for the tissue, and should be further investigated. In order to further elucidate this phenomenon, we compared the absolute abundance of transcripts of some genes highly expressed in the previtellogenic cod ovary (e.g. cyclin-B and Zona pellucida related genes) after cultivation for 1, 5, 10 and 20 days. Whereas tissues cultivated for 20 days generally showed decreased mRNA levels compared to the earlier time points, no specific patterns could be observed for tissue incubated for 1, 5 and 10 days. Thus, we suggest that ovarian tissue may be kept healthy and in good condition in culture for at least 10 days using the floating agarose method described in the present study. For prolonged incubations, one should carefully monitor RNA integrity and abundance, as well as performing histological evaluations in order to assess the general tissue condition, prior to overall analysis.

Secondly, oocyte areas with a high density of spermatozoa were observed in *in vitro* cultivated ovarian tissue at both day 5 and day 10 of exposure for tissues exposed to 11-KT and T. The appearance of spermatozoa in cultivated ovarian tissue is puzzling, and spermatozoa were not observed *in vivo* for any of the androgen doses administered. The possibility that sex reversal was occurring in cultivated ovarian tissue exposed to androgens should not be excluded. If sex reversal was occurring within 5 days of *in vitro* androgen exposure, one would think that less mature stages of male germ cells (i.e. spermatogonia and spermatocytes) could be observed at earlier time points, at least in some sections (Almeida et al. 2008). However, it should also be noted that not all androgen exposed ovary tissue samples examined contained spermatozoa. Previous studies addressing the effects of 11-KT on previtellogenic ovarian growth have demonstrated the presence of male germ cells in androgen treated female fish *in vivo* (Rohr et al. 2001) but not *in vitro* (Lokman et al. 2007).

Fish used for *in vitro* cultivation (**Paper I, II, IV and V**) displayed mean plasma 11-KT levels of 1 ±0.5 ng/ml, whereas fish used for the *in vivo* experiment (**Paper III**) had plasma 11-KT levels of 0.06 ±0.01 ng/ml. This corresponds to fish weight (150-350 g for fish used for *in vitro* cultivation and 56 ±11 g for fish used in the *in vivo* study) and oocyte developmental stages. Ovaries used for *in vitro* cultivation contained oocytes of advanced previtellogenic growth (see Table 1), whereas previtellogenic primary growth was the most advanced oocyte stage observed in ovaries of fish used in the *in vivo* study. When comparing the present *in vitro and in vivo* observations, one must keep in mind that the fish were in slightly different developmental stages.

Sex steroid levels

The present study adds novel information on the topic of sex steroid plasma levels in female fish, demonstrating for the first time that significant amounts of T, and more interestingly, 11-KT, circulate in the blood of immature female Atlantic cod. As stated above, fish used for *in vitro* cultivation (**Paper I, II, IV and V**) displayed mean plasma 11-KT levels of 1.0 ±0.5 ng/ml, whereas fish used for the *in vivo* experiment (**Paper III**) had plasma 11-KT levels of 0.06 ±0.01 ng/ml. The difference correlates directly to the size of the fish, suggesting that sex steroid levels are elevated with increasing body size. Furthermore, plasma E2 and T levels in fish used for the *in vivo* experiment (**Paper III**) were 0.40 ±0.07 and 0.73 ±0.35 ng/ml, respectively. The observation that E2 levels seem to be lower than those of T in immature female cod is interesting, since previous studies have shown that the opposite is true for

mature and spawning female cod (Dahle et al. 2003; Kjesbu et al. 1996; Norberg et al. 2004). Furthermore, previous studies showed that plasma levels of T did not differ during the developmental stages, but E2 increased steadily with ovarian development (Dahle et al. 2003). This is in contrast to what is generally found in salmonids, and may be a common feature in marine batch-spawners (Norberg et al. 2004). The increased plasma levels of E2 with increased ovarian development are usually explained by the role of E2 as the main promoter of vitellogenesis (see section on vitellogenesis in the introduction). As previously outlined, androgens (e.g. T) serves as precursors for E2 synthesis. The present study adds novel information on the roles of androgens in the previtellogenic cod ovary, suggesting androgen control of oocyte growth and development (see section on previtellogenic oocyte growth and development).

The present study demonstrates data suggesting aromatization of MT at high concentrations (**Paper IV**), adding further evidence to the phenomenon 'paradoxical feminization' described earlier (Fenske and Segner 2004; Orn et al. 2003; Rinchard et al. 1999; Zerulla et al. 2002) and showing that this phenomenon also can take place *in vitro*. Furthermore, we present data showing modulated E2 and 11-KT levels after *in vitro* exposure to a hormone mimic (NP; **Paper V**) that may be correlated with interference of the steroidogenic pathway (see section on modulation of steroidogenic pathway). However, it should be emphasized that the absolute values of the sex steroid measured in these two separate studies (expressed as pg/ml) are not comparable with the other *in vitro* studies (**Paper I and II**) or with steroid plasma levels, as they are not related to tissue weight. Instead, they should be interpreted as semi-quantitative analyses.

All sex steroids were measured using the EIA method. In this regard, it is vital to address the specificity of the different EIA kits employed, as most hormone EIAs show significant cross-reactivity to other steroids. The specificities of the EIA kits employed in the present study are shown in Table 2. Special attention should be paid to the cross-reactivity of MT on the T immunoassay (4.7%, personal communication with manufacturer). Accordingly, T levels measured in ovarian tissue after *in vitro* MT exposure (**Paper IV**) may potentially be incorrect, displaying artificial high T levels due to MT reactivity in the assay. However, T levels in control tissue were not significantly lower than levels in tissue treated with 1 and 10 μM MT (**Paper IV**). This further indicates a rapid steroid clearance in the tissue, as discussed earlier.

Table 2. The specificities of the EIA kits employed in the present study.

EIA kit	11-KT	T	MT	E2
11-KT	100%	<0.01%	n/a	n/a
T	2.2%	100%	~4.7%	<0.01%
E2	n/a	0.01%	n/a	100%

* personal communication with manufacturer

Previtellogenic oocyte growth and development

One of the major findings of the present study was the obvious morphological changes in previtellogenic ovarian tissue after androgen treatment. In general, this is the first study to show that androgens (i.e. 11-KT and T) can promote the growth and development of Atlantic cod previtellogenic oocytes. Furthermore, the phenomenon of androgen induction of oocyte growth was demonstrated both *in vitro* (**Paper I and II**) and in vivo (**Paper III**). More specifically, 11-KT treatment resulted in significantly higher volume fractions of more developed oocytes compared to solvent control fish both in the *in vitro* and *in vivo* experiments. Treatment with T displayed a moderate morphological alteration in gonadal tissues, at least *in vivo* (**Paper III**), where no differences in volume fractions of more developed oocytes between T treated and solvent control fish were registered. Nevertheless, significantly higher fractions of initial primary oocytes and oogonia could be observed after T exposure. Thus, we speculate that the possible promoting effects of T on oocyte growth were suppressed or postponed as compared to treatment with 11-KT. In accordance, 11-KT was found to exert more pronounced effects than T on the growth and development of cod previtellogenic oocytes *in vitro* (**Paper I and II**).

These observations are in compliance with previous studies in other species that have addressed the effects of 11-KT on previtellogenic oocyte growth in teleost fish. In these studies, it was shown that 11-KT promoted the generation of qualitatively larger gonads with more advanced oocytes in immature female eel, and significantly increased previtellogenic oocyte diameters by nearly 10% *in vitro* (Lokman et al. 2007; Rohr et al. 2001). Taken together, these observations suggest a general positive effect of the non-aromatizable androgen 11-KT on the growth and development of previtellogenic oocytes, at least in the teleost species so far studied. The present study adds novel information on the topic of

endocrine regulation of previtellogenic oocyte growth, suggesting that androgens can exert direct regulatory effects on oocyte growth in the previtellogenic cod ovary. As stated earlier, it has been demonstrated that androgens can induce vitellogenic responses in teleost fish (Hori et al. 1979; Le Menn et al. 1980; Mori et al. 1998; Peyon et al. 1997). In addition, the identification of nuclear ARs in the ovary of Atlantic croaker and kelp bass (Sperry and Thomas 1999a; Sperry and Thomas 1999b; Sperry and Thomas 2000) suggests that androgens may exert direct hormonal actions in the teleost ovary. Recently, cytochrome P45011β-hydroxylase (P45011β), an enzyme involved in 11-KT production (see Figure 4), was localized in the ovary of the honeycomb grouper (*Epinephelus merra*) (Alam et al. 2005), adding further information on the role of androgens in female fish.

The possible physiological functions of androgens in the ovary have been investigated also in mammals. In relation to small preantral follicles in mammals, which resemble fish previtellogenic oocytes, given that growth progresses slowly occurring both before and after puberty (Hsueh et al. 2000) and appearing to be mostly GtH-independent (Smitz and Cortvrindt 2002), androgens have been implicated in mammalian oogenesis (McGee 2000). For example, growing preantral and small antral follicles were significantly increased in number, and granulosa and thecal cell proliferation increased in androgen treated rhesus monkey (Vendola et al. 1998). Similarly, testosterone-treated women or those suffering from androgen excess (polycystic ovary syndrome; PCOS) showed increased number of growing follicles (Vercellini et al. 1993). A possible role for androgens in the ovary has also been observed in the mouse, where it was shown that female AR knock-out mice had reduced follicle number and average litter size (Shiina et al. 2006; Yeh et al. 2002). Elsewhere, using an *in vitro* culture system, preantral mice follicles developed faster in the presence of the non-aromatizable androgen DHT (Murray et al. 1998). Similarly, androgens significantly increased the diameter of *in vitro* cultured follicles of immature mice (Wang et al. 2001). Direct androgen effects on ovarian follicles are very likely since AR mRNA abundance in rhesus monkey was highest in preantral to small antral follicles (Weil et al. 1998). Androgens also modify the intra-ovarian gene expression in the rhesus monkey, as demonstrated by increased mRNA abundance of insulin-like growth factor-1 (IGF-1) and IGF-1-receptor (Vendola et al. 1999) in follicles up to early antral stage. Elsewhere, androgen treatment significantly increased granulosa cell FSH receptor (FSHR) mRNA levels (Weil et al. 1999). Nevertheless, the molecular mechanisms of androgen actions in the ovary remain mostly unknown.

In contrast, a few earlier mammalian studies have reported negative effects of androgens on ovarian growth and development. For example, excess androgens levels have been associated with the induction of follicular atresia during murine oocyte maturation (Anderiesz and Trounson 1995). Elsewhere, ovarian androgens have been correlated with inhibition of follicular development (Billig et al. 1993; Farookhi 1985; Jia et al. 1985). From these conflicting reports, it is still largely unclear what effects androgens have on oocyte development. However, it seems likely that androgens do have a functional role during early ovarian development in vertebrates that needs to be further elucidated.

Modulation of the steroidogenic pathway

The rate-limiting step in steroidogenesis is the movement of cholesterol across the mitochondrial membrane by the StAR protein and the subsequent conversion to pregnenolone by the P450*scc* enzyme (see section on steroidogenesis in the introduction). In mammals, StAR and P450*scc* are rapidly synthesized in response to acute tropic hormone stimulation and cAMP. In general, agents that increase steroid biosynthesis also increase StAR mRNA expression (Stocco 2001). However, current knowledge about StAR is mostly based on mammalian studies, whereas the relationship between StAR gene expression and steroid production in fish is not very well described (Hagen et al. 2006; Stocco et al. 2005). In recent years, StAR and P450*scc* have been localized in most steroidogenic organs or tissues of teleost fish, and StAR cDNA has been cloned from a number of teleosts, including Atlantic cod (Goetz et al. 2004), Atlantic croaker (Nunez and Evans 2007), Rainbow trout (Kusakabe et al. 2002) and eel (Li et al. 2003). Interestingly, StAR expression data suggest that the molecular mechanisms of StAR synthesis and regulation in fish may differ from what has been previously observed in mammals (Goetz et al. 2004). Accordingly, these are still subject to continued investigation both in our laboratory and elsewhere.

Effects of sex steroids or their mimics through non-receptor or non-genomic mechanisms that involve regulatory proteins are generally not well studied. Nevertheless, key enzymes involved in steroidogenesis are increasingly being considered as important targets for EDCs (Sanderson 2006), and have become subject for systematic investigation. For instance, it was recently shown that NP induced transcriptional changes of StAR, P450*scc* and the biotransformation enzymes CYP1A1 and CYP3A in Atlantic salmon brain (Arukwe 2005). In another study, NP modulated Atlantic salmon brain and liver P450*arom* isoforms (Meucci and Arukwe 2006). In addition, the synthetic pharmaceutical estrogen, ethynylestradiol (EE2) altered gene transcripts of StAR, P450*scc*, aromatase and CYP11β-hydroxylase in brain, head

kidney and ovary of Atlanic salmon (Lyssimachou and Arukwe 2007; Lyssimachou et al. 2006; Vang et al. 2007). The present study demonstrates that that the synthetic pharmaceutical androgen MT and the estrogen mimic NP modulated StAR and P450*scc* expression in previtellogenic cod gonadal tissue (**Paper IV** and **V**, respectively). This suggests that the experimental samples were experiencing impaired acute steroidogenesis. To further evaluate these transcriptional modulations and investigate the possible correlation between mRNA transcription and protein/enzyme expression, we used histological samples of MT and NP exposed previtellogenic ovarian tissue applied with immunohistochemical detection of StAR and P450*scc* protein expression. We demonstrated the localization of these proteins mainly in developing follicular cells. However, no differences in staining intensities were registered, as the immunohistochemical is mainly a qualitative analytic method. Newly synthesized StAR is often present in low levels (Artemenko et al. 2001) and may not be easily detected by the qualitative immunohistochemical approach. More interesting is the reduced levels of E2 and 11-KT after NP exposure and the observation that 11-KT levels seemed to correlate to StAR mRNA expression (**Paper V**). This suggests that NP can modulate steroidogenesis by targeting the StAR protein. Taken together, we suggest that NP and MT may exert non-receptor mediated effects in the previtellogenic cod ovary, and these effects may have potential consequences for the vitellogenic process and general fecundity. Given the important role of StAR and P450*scc* in the steroiodogenic pathway, they may prove to be effective molecular and cellular targets for pollutants and useful biomarkers to evaluate endocrine system function in fish species.

The gene expressions of StAR, P450scc and 20β-HSD after *in vivo* androgen exposure **(Paper III)** show an increasing pattern after androgen exposure. Furthermore, expression patterns of StAR and P450*scc* were fairly similar. This is in accordance with a previous study by Geslin and Auperin (2004), where a positive correlation between the expression profiles of StAR and P450*scc* was observed, suggesting a similar mechanism of transcriptional regulations for these two genes in teleosts. In accordance with our studies, earlier studies on StAR and P450*scc* gene expression (Campbell et al. 2006; Ijiri et al. 2006; Nakamura et al. 2005; Vizziano et al. 2007), indicate that transcription rates are modestly increased during oocyte growth, indicating exposure to sex hormones (androgens and/or estrogens) or their mimics. In contrast, other studies have shown no alterations (Nunez and Evans 2007) or even decreased levels (Ings and Van Der Kraak 2006) of StAR transcripts after sex hormone exposure or during ovarian development. Nevertheless, StAR and P450*scc* ovarian mRNA levels are low in most

teleost species, and indicate a delicate regulation of steroidogenesis in teleost fish that needs further attention.

In teleosts, gonadal steroids are known to modulate both the synthesis and release of gonadotropins by the pituitary and influence several brain functions that are apparently responsible for gender-specific differences in the regulation of hypothalamus-pituitary-gonadal (HPG) axis. Although the StAR and P450*scc* are the main proteins involved in the early steroidogenic pathway, other proteins such as P450*arom*, CYP17, 3β- 11β- and 20β-hydroxysteroid dehydrogenase (HSD) (Miller 1988) are also key enzymes in gonadal steroidogenesis. For examples, the P450*arom* is a crucial steroidogenic enzyme catalyzing the final step in the conversion of androgens to estrogens (Callard et al. 2001; Kishida and Callard 2001) in vertebrates. 20β-HSD is the key steroidogenic enzyme in the production of MIH (Tanaka et al. 2002) (see section on steroidogenesis in the introduction), and was recently cloned from Atlantic cod ovary (Mittelholzer et al. 2007). We have demonstrated the presence of high levels of 20β-HSD transcripts in immature cod ovary, which is composed entirely of oogonia and previtellogenic oocytes (**Paper III**). However, the functional enzyme is not thought to be required in high quantities until oocytes reach final maturation. The increased 20β-HSD mRNA levels after androgen exposure suggest an androgen-mediated increase in the transcription of 20β-HSD mRNA, presumably in response to oocyte developmental requirements.

The experiments described in this thesis also aimed at gaining further insight into the processes that regulate P450*arom,* the rate-limiting enzyme in estrogen production. A vast number of earlier studies have measured P450*arom* mRNA expression in teleosts (for a review, see (Cheshenko et al. 2008). Most of these studies have addressed P450*aromA* (the isoform being dominantly expressed in the ovary) expression profiles with regard to sex differentiation or possible sex reversal, and it is believed that P450*aromA* plays an essential role in the regulation of sexual differentiation and the female reproductive cycle in teleosts (Cheshenko et al. 2008). However, the functional significance of P450*aromA* expression at early developmental stages of teleost oocytes is not well understood, and the effects of sex hormone or their mimics on P450*aromA* expression seem to differ depending on developmental stage, cellular context and species, probably being controlled by different promoters and second messenger pathways (Cheshenko et al. 2008). The present study revealed that transcript levels of P450*aromA* were only modestly affected by 11-KT, T

(**Paper III**) and MT (**Paper IV**). Additionally, generally low transcript levels of P450*aromA* were detected in the previtellogenic cod ovary.

Modulation of genes associated with *zona radiata*

The egg envelope, which is of critical importance for optimal oocyte growth and sperm-egg interaction, consists of a family of related zona pellucida glycoproteins (ZPs) with a conserved ZP domain (Conner and Hughes 2003). Very recent studies have demonstrated that teleost species have at least three isoforms of ZP proteins, ZP1 (ZPB), ZP3 (ZPC) and ZPX (Modig et al. 2006; Modig et al. 2007). However, the ZP nomenclature is confusing, and this complicates comparison between mammalian and fish ZP genes, as well as between different teleost species (Arukwe and Goksøyr 2003). The site of ZP protein synthesis has been a debated issue for years. Earlier studies suggested that some teleost species, such as rainbow trout (Oppen-Berntsen et al. 1992a), Atlantic cod (Oppen-Berntsen et al. 1992b), Atlantic salmon (Oppen-Berntsen et al. 1999), winter flounder (Lyons et al. 1993) and gilthead seabream (Del Giacco et al. 1998) produce ZP proteins exclusively in their liver. However, recent studies have demonstrated ZP mRNA transcripts in the ovary of a number of teleost species, including Atlantic cod (Goetz et al. 2006; Govoroun et al. 2006; Hyllner et al. 2001; Kanamori 2000; Luckenbach et al. 2008; Modig et al. 2006; Zeng and Gong 2002). It has also been demonstrated that some species, such as zebrafish and carp, express ZP proteins exclusively in the ovaries (Chang et al. 1997; Wang and Gong 1999), and the ovary is probably the only site for ZP biosynthesis in these species. However, ZP protein synthesis can probably take place both in liver and ovary for many teleost species. The functional significance of producing ZP proteins in two different sites is not known (Modig et al. 2006).

In the present study, we demonstrate high levels of ZP transcripts in the previtellogenic Atlantic cod ovary (Paper **I, II** and **III**) using qPCR and *in situ* hybridization. However, data obtained after *in vitro* (**Paper I** and **II**) and *in vivo* (**Paper III**) androgen exposure showed conflicting expression profiles of ZP genes, which can generally be described as increased after androgen treatment (*in vitro*) or 11-KT insensitive and decreased after T administration (*in vivo*). Several other studies have demonstrated abundant levels of ZP transcripts in teleost oocytes during previtellogenic growth, displaying higher ZP mRNA levels in the more undeveloped stages of oocyte growth (Chang et al. 1997; Luckenbach et al. 2008; Zeng and Gong 2002). Elsewhere, while E2 regulation of ZP synthesis is a well known phenomenon, androgens have been suggested to regulate ZPs in fish (Miura et al. 1998) and birds (Pan et al. 2001). In terms of environmental toxicology, ZP proteins have also been suggested as an

alternative indicator for estrogenic EDC exposure (Arukwe et al. 1997; Arukwe et al. 2002; Arukwe et al. 2001). Although not conclusive, the present study suggests that androgens can modulate the transcriptional profile of ZP genes in the previtellogenic cod ovary, possibly in correlation with development of the oocyte envelope.

Effects on genes involved in cell cycle and growth control

Final oocyte maturation is triggered by the MPF induced on the oocyte surface by MIHs that are secreted from ovarian granulosa cells (see section on endocrine regulation of oocyte growth and maturation in the introduction). MPF comprises two components; the catalytic subunit cdc2 and the regulatory subunit cyclin-B (Kondo et al. 1997; Morgan 1995). Cyclin-B transcripts have been demonstrated in immature oocytes of goldfish (Nagahama 1997) and zebrafish (Kondo et al. 2001), but they are not translated until later, when the oocyte reach the maturation phase, and earlier reports have demonstrated that MIH stimulates the translation of cyclin-B mRNA in immature oocytes (Hirai et al. 1992; Kondo et al. 2001). Other studies have suggested that the accumulation of cyclin-B in oocytes is involved in the regulation of the early embryonic cell cycle (Aegerter et al. 2004), and that expression of cyclin-B in previtellogenic oocytes indicates somatic follicle cells undergoing mitotic divisions (von Schalburg et al. 2005). The present study demonstrates high levels of cyclin-B mRNA in the previtellogenic cod ovary. Furthermore, we show that natural occurring androgens (**Paper I** and **II**), as well as EDCs (MT; **Paper IV** and NP; **Paper V**) are able to affect cyclin-B transcription levels. Thus, gene expression patterns of cyclin-B may be used as sensitive markers for early oocyte growth and developmental competence in teleost fish (Aegerter et al. 2004).

Androgen treatment was associated with a general increase in opioid growth factor receptor (OGFr) mRNA levels (**Paper III**). The OGFr is generally related to tissue development and cellular renewal, and was recently identified in Atlantic salmon skin (Matejusova et al. 2006). Our findings are interesting since it demonstrates the expression of OGFr mRNA in previtellogenic Atlantic cod ovary. The increase in OGFr mRNA and the related oocyte development after androgen exposure suggests a possible important role of OGFr in oocyte growth in this species that needs further attention.

CONCLUDING REMARKS

Emerging technologies that include genomics and proteomics are revolutionizing the science of reproductive physiology and endocrinology among other scientific disciplines. As a result, researchers have genuine possibilities to fully describe and understand the molecular basis and interactions in organisms that form may contribute to functional and developmental processes. In these contexts, the present study demonstrates, for the first time, that androgens may induce early ovarian development in immature female Atlantic cod. Our findings provided further evidence to our hypothesis that androgens are involved in the regulation of previtellogenic oocyte growth and development in this species. In general, both 11-KT and T are capable of inducing oocyte growth, with the former hormone being the strongest modulator. Additionally, the present study provides the identification, sequencing and the expression patterns of a number of androgen responsive gene transcripts in the previtellogenic cod ovary. A targeted cod microarray (CodArray), consisting of SSH clones with unique expression patterns that were either up or down regulated after *in vitro* androgen exposure of previtellogenic ovarian tissue was developed. The consistent expression patterns of candidate genes involved in steroidogenesis and oocyte growth control adds further information on how androgenic action may cause advances in previtellogenic oocyte growth. Although not conclusive, the demonstration of previtellogenic oocyte growth and development after androgen exposure and the related differential expression of a number of androgen responsive genes reveal novel roles of androgens on the growth and development of previtellogenic oocytes, indicating androgen control of early follicular and oocyte growth in the Atlantic cod ovary. In general, our findings may form the basis for generating more hypotheses for further detailed studies and for deduction of evolutionary relatedness in androgen control of early oocyte growth in vertebrates.

The Atlantic cod is a popular species with large economic value and the farming potential on a global basis is therefore regarded as extremely high. Despite its high economic significance, the Atlantic cod is not a well-studied species from an endocrinological standpoint. Thus, the novel roles of androgens as promoters of ovarian growth and development shown in these experiments will be beneficial for the aquaculture industry and for breeding of new captive and endangered species. It can also form the basis for the development of useful models in fisheries management. Differentially expressed genes may form an integral link with quantifiable environmental variables that may affect fecundity (*e.g.* temperature, salinity, food availability and growth rates). Furthermore, we believe the present study will have a

significant economic benefit for predictive ecological models, particularly with respect to the noticeable and worldwide declining cod stock.

Future perspectives

Although this thesis produced new information concerning the hormonal mechanisms that may regulate early oocyte growth and development in fish, extensive research effort is needed in order to fully understand this aspect of reproductive physiology. Whether the androgen-induced ovarian growth demonstrated in this study is an exception, rather than a rule among different vertebrate species remains to be elucidated. The next step to answering this question is probably to understand the function of ARs in the ovary, including the following specific approaches for further studies:

What receptor does 11-KT bind to?

Are ARs found in both germ cells and follicle cells?

What are the promoter regions of the ARs observed in the teleost ovary?

What are the expression patterns of ARs throughout the reproductive cycle?

Recent proteomics technologies have also given the possibilities to study the functional outcome of expressed genes (i.e. proteins) in developing oocytes. By employing these analytical methods, comprehensive surveys of the protein content in cells and/or organs at particular developmental stage can be undertaken. In addition, they give the possibility to monitor the changes in the relative amount of proteins of interest during reproductive events. Finally, patterns of protein post-translational modifications can be followed. Proteomics technologies include one or two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) with subsequent mass spectrometry (MS) analysis. MS analyses can be particular useful for less studied organisms such as the Atlantic cod, as they generate accurate measurements of protein masses and /or their peptide sequences after enzymatic digestion with trypsin. The characterization of androgen responsive proteins in immature female teleosts will probably offer more explanations on how androgens mediate their ovarian actions, especially when evaluated in relation to transcriptional patterns and sex steroid levels. We have initiated this part of the research, but it is yet to be concluded.

REFERENCES

Adams MD, Kelley JM, Gocayne JD, Dubnick M, Polymeropoulos MH, Xiao H, Merril CR, Wu A, Olde B, Moreno RF and others. 1991. Complementary DNA sequencing: expressed sequence tags and human genome project. Science 252(5013):1651-6.

Aegerter S, Jalabert B, Bobe J. 2004. Messenger RNA stockpile of cyclin B, insulin-like growth factor I, insulin-like growth factor II, insulin-like growth factor receptor Ib, and p53 in the rainbow trout oocyte in relation with developmental competence. Mol Reprod Dev 67(2):127-35.

Alam MA, Komuro H, Bhandari RK, Nakamura S, Soyano K, Nakamura M. 2005. Immunohistochemical evidence identifying the site of androgen production in the ovary of the protogynous grouper Epinephelus merra. Cell Tissue Res 320(2):323-9.

Allera A, Wildt L. 1992. Glucocorticoid-recognizing and -effector sites in rat liver plasma membrane. Kinetics of corticosterone uptake by isolated membrane vesicles--II. Comparative influx and efflux. J Steroid Biochem Mol Biol 42(7):757-71.

Almeida FF, Kristoffersen C, Taranger GL, Schulz RW. 2008. Spermatogenesis in Atlantic cod (Gadus morhua): a novel model of cystic germ cell development. Biol Reprod 78(1):27-34.

Anderiesz C, Trounson AO. 1995. The effect of testosterone on the maturation and developmental capacity of murine oocytes in vitro. Hum Reprod 10(9):2377-81.

Aranda A, Pascual A. 2001. Nuclear hormone receptors and gene expression. Physiol Rev 81(3):1269-304.

Artemenko IP, Zhao D, Hales DB, Hales KH, Jefcoate CR. 2001. Mitochondrial processing of newly synthesized steroidogenic acute regulatory protein (StAR), but not total StAR, mediates cholesterol transfer to cytochrome P450 side chain cleavage enzyme in adrenal cells. J Biol Chem 276(49):46583-96.

Arukwe A. 2001. Cellular and molecular responses to endocrine-modulators and the impact on fish reproduction. Mar Pollut Bull 42(8):643-55.

Arukwe A. 2005. Modulation of brain steroidogenesis by affecting transcriptional changes of steroidogenic acute regulatory (StAR) protein and cholesterol side chain cleavage (P450scc) in juvenile Atlantic salmon (Salmo salar) is a novel aspect of nonylphenol toxicity. Environ Sci Technol 39(24):9791-8.

Arukwe A. 2006. Toxicological housekeeping genes: do they really keep the house? Environ Sci Technol 40(24):7944-9.

Arukwe A, Celius T, Walther BT, Goksøyr A. 2000. Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic salmon (Salmo salar). Aquatic Toxicol 49(3):159-170.

Arukwe A, Goksøyr A. 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comp Hepatol 2(1):4.

Arukwe A, Knudsen FR, Goksøyr A. 1997. Fish zona radiata (eggshell) protein: a sensitive biomarker for environmental estrogens. Environ Health Perspect 105(4):418-22.

Arukwe A, Kullman SW, Berg K, Goksøyr A, Hinton DE. 2002. Molecular cloning of rainbow trout (Oncorhynchus mykiss) eggshell zona radiata protein complementary DNA: mRNA expression in 17beta-estradiol- and nonylphenol-treated fish. Comp Biochem Physiol B Biochem Mol Biol 132(2):315-26.

Arukwe A, Kullman SW, Hinton DE. 2001. Differential biomarker gene and protein expressions in nonylphenol and estradiol-17beta treated juvenile rainbow trout (Oncorhynchus mykiss). Comp Biochem Physiol C Toxicol Pharmacol 129(1):1-10.

Baron D, Houlgatte R, Fostier A, Guiguen Y. 2005. Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol Reprod 73(5):959-66.

Baron D, Houlgatte R, Fostier A, Guiguen Y. 2008. Expression profiling of candidate genes during ovary-to-testis trans-differentiation in rainbow trout masculinized by androgens. Gen Comp Endocrinol.

Baron D, Montfort J, Houlgatte R, Fostier A, Guiguen Y. 2007. Androgen-induced masculinization in rainbow trout results in a marked dysregulation of early gonadal gene expression profiles. BMC Genomics 8:357.

Billig H, Furuta I, Hsueh AJ. 1993. Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. Endocrinology 133(5):2204-12.

Blaber SJM. 1970. The occurrence of a penis-like outgrowth behind the right tentacle in spent females of *Nucella lapillus* (L.). Proceedings of the Malacological Society of London 39:231-233.

Bobe J, Montfort J, Nguyen T, Fostier A. 2006. Identification of new participants in the rainbow trout (Oncorhynchus mykiss) oocyte maturation and ovulation processes using cDNA microarrays. Reprod Biol Endocrinol 4:39.

Borg B. 1994. Androgens in teleosts fishes. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 109:219-245.

Braun AM, Thomas P. 2003. Androgens inhibit estradiol-17beta synthesis in Atlantic croaker (Micropogonias undulatus) ovaries by a nongenomic mechanism initiated at the cell surface. Biol Reprod 69(5):1642-50.

Braun AM, Thomas P. 2004. Biochemical characterization of a membrane androgen receptor in the ovary of the atlantic croaker (Micropogonias undulatus). Biol Reprod 71(1):146-55.

Bromage N, Jones J, Randall C, Thrush M, Davies B, Springate J, Duston J, Barker G. 1992. Broodstock management, fecundity, egg quality and the timing of egg production in the rainbow trout (*Oncorhynchus mykiss*). Aquaculture 100:141-166.

Brown JA, Minkoffb G, Puvanendrana V. 2003. Larviculture of Atlantic cod (*Gadus morhua*): progress, protocols and problems Aquaculture 227:357-372.

Burton MPM, Penney RM, Biddiscombe S. 1997. Time course of gametogenesis in Northwest Atlantic cod (*Gadus morhua*). Canadian Journal of Fisheries and Aquatic Sciences 54(suppl. 1):122-131.

Bustin SA, Nolan T. 2004. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech 15(3):155-66.

Callard GV, Tchoudakova AV, Kishida M, Wood E. 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. J Steroid Biochem Mol Biol 79(1-5):305-14.

Campbell B, Dickey J, Beckman B, Young G, Pierce A, Fukada H, Swanson P. 2006. Previtellogenic oocyte growth in salmon: relationships among body growth, plasma insulin-like growth factor-1, estradiol-17beta, follicle-stimulating hormone and expression of ovarian genes for insulin-like growth factors, steroidogenic-acute regulatory protein and receptors for gonadotropins, growth hormone, and somatolactin. Biol Reprod 75(1):34-44.

Carnevali O, Carletta R, Cambi A, Vita A, Bromage N. 1999. Yolk formation and degradation during oocyte maturation in seabream Sparus aurata: involvement of two lysosomal proteinases. Biol Reprod 60(1):140-6.

Caussanel C, Breuzet M. 1977. [Hormonal control of the ovarian cycle in Labidura riparia (author's transl)]. Ann Endocrinol (Paris) 38(6):403-4.

Celius T, Walther BT. 1998. Oogenesis in Atlantic salmon (Salmo salar L.) occurs by zonagenesis preceding vitellogenesis in vivo and in vitro. J Endocrinol 158(2):259-66.

Chang YS, Hsu CC, Wang SC, Tsao CC, Huang FL. 1997. Molecular cloning, structural analysis, and expression of carp ZP2 gene. Mol Reprod Dev 46(3):258-67.

Chang YS, Wang SC, Tsao CC, Huang FL. 1996. Molecular cloning, structural analysis, and expression of carp ZP3 gene. Mol Reprod Dev 44(3):295-304.

Cheshenko K, Pakdel F, Segner H, Kah O, Eggen RI. 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. Gen Comp Endocrinol 155(1):31-62.

Clark BJ, Wells J, King SR, Stocco DM. 1994. The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). J Biol Chem 269(45):28314-22.

Colborn T, Clement C. 1992. Chemically-Induced Alterations in Sexual and Functional Development: The wildlife/human Connection. Advances in modern environmental toxicology.

Conner SJ, Hughes DC. 2003. Analysis of fish ZP1/ZPB homologous genes--evidence for both genome duplication and species-specific amplification models of evolution. Reproduction 126(3):347-52.

Coward K, Bromage NR. 2002. Stereological point-counting; an accurate method for assessing ovarian function in tilapia. Aquaculture 212:383-401.

Dahle R, Taranger GL, Karlsen O, Kjesbu OS, Norberg B. 2003. Gonadal development and associated changes in liver size and sexual steroids during the reproductive cycle of captive male and female Atlantic cod (Gadus morhua L.). Comp Biochem Physiol A Mol Integr Physiol 136(3):641-53.

Dean DM, Sanders MM. 1996. Ten years after: reclassification of steroid-responsive genes. Mol Endocrinol 10(12):1489-95.

Defranco DB. 2000. Role of molecular chaperones in subnuclear trafficking of glucocorticoid receptors. Kidney Int 57(4):1241-9.

Del Giacco L, Vanoni C, Bonsignorio D, Duga S, Mosconi G, Santucci A, Cotelli F. 1998. Identification and spatial distribution of the mRNA encoding the gp49 component of the gilthead sea bream, Sparus aurata, egg envelope. Mol Reprod Dev 49(1):58-69.

Delesse MA. 1847. Procédé méchanique pour déterminer la composition des roches. C. R. Acad. Sci. (Paris) 25:544.

DeRosa C, Richter P, Pohl H, Jones DE. 1998. Environmental exposures that affect the endocrine system: public health implications. J Toxicol Environ Health B Crit Rev 1(1):3-26.

Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED and others. 1996. Suppression subtractive

hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci U S A 93(12):6025-30.

Falkenstein E, Tillmann HC, Christ M, Feuring M, Wehling M. 2000. Multiple actions of steroid hormones--a focus on rapid, nongenomic effects. Pharmacol Rev 52(4):513-56.

Farookhi R. 1985. Effects of aromatizable and nonaromatizable androgen treatments on luteinizing hormone receptors and ovulation induction in immature rats. Biol Reprod 33(2):363-9.

Fenske M, Segner H. 2004. Aromatase modulation alters gonadal differentiation in developing zebrafish (Danio rerio). Aquat Toxicol 67(2):105-26.

Filby AL, Tyler CR. 2005. Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (Pimephales promelas). Biol Reprod 73(4):648-62.

Folmar LC, Hemmer MJ, Denslow ND, Kroll K, Chen J, Cheek A, Richman H, Meredith H, Grau EG. 2002. A comparison of the estrogenic potencies of estradiol, ethynylestradiol, diethylstilbestrol, nonylphenol and methoxychlor in vivo and in vitro. Aquat Toxicol 60(1-2):101-10.

Freere RH, Weibel ER. 1967. Stereological techniques in microscopy. J R Microsc. Soc. 87:25-34.

Fry DM, Toone CK. 1981. DDT-induced feminization of gull embryos. Science 213(4510):922-4.

Gale WL, Patino R, Maule AG. 2004. Interaction of xenobiotics with estrogen receptors alpha and beta and a putative plasma sex hormone-binding globulin from channel catfish (Ictalurus punctatus). Gen Comp Endocrinol 136(3):338-45.

Ge H, Walhout AJ, Vidal M. 2003. Integrating 'omic' information: a bridge between genomics and systems biology. Trends Genet 19(10):551-60.

Geslin M, Auperin B. 2004. Relationship between changes in mRNAs of the genes encoding steroidogenic acute regulatory protein and P450 cholesterol side chain cleavage in head kidney and plasma levels of cortisol in response to different kinds of acute stress in the rainbow trout (Oncorhynchus mykiss). Gen Comp Endocrinol 135(1):70-80.

Goetz FW, McCauley LA, Goetz GW, Norberg B. 2006. Using global genome approaches to adress problems in cod mariculture. ICES Journal of Marine Science 63:393-399.

Goetz FW, Norberg B, McCauley LA, Iliev DB. 2004. Characterization of the cod (Gadus morhua) steroidogenic acute regulatory protein (StAR) sheds light on StAR gene structure in fish. Comp Biochem Physiol B Biochem Mol Biol 137(3):351-62.

Goksøyr A. 2006. Endocrine disruptors in the marine environment: mechanisms of toxicity and their influence on reproductive processes in fish. J Toxicol Environ Health A 69(1-2):175-84.

Goksøyr A, Arukwe A, Larsson J, Cajaraville MP, Hauser L, Nilsen BM, Lowe D, Matthiesen P. 2003. Molecular/cellular processes and the impact on reproduction. In: Lawrence AJ, Hemingway KL, editors. Effects of pollution on fish: Molecular effects and population responses: Blackwell Science. p 179-220.

Gottlicher M, Heck S, Herrlich P. 1998. Transcriptional cross-talk, the second mode of steroid hormone receptor action. J Mol Med 76(7):480-9.

Govoroun M, Le Gac F, Guiguen Y. 2006. Generation of a large scale repertoire of Expressed Sequence Tags (ESTs) from normalised rainbow trout cDNA libraries. BMC Genomics 7:196.

Greytak SR, Callard GV. 2007. Cloning of three estrogen receptors (ER) from killifish (Fundulus heteroclitus): differences in populations from polluted and reference environments. Gen Comp Endocrinol 150(1):174-88.

Grier H. 2000. Ovarian germinal epithelium and folliculogenesis in the common snook, Centropomus undecimalis (Teleostei: centropomidae). J Morphol 243(3):265-81.

Guillette LJ, Jr., Gunderson MP. 2001. Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. Reproduction 122(6):857-64.

Hagen IJ, Kusakabe M, Young G. 2006. Effects of ACTH and cAMP on steroidogenic acute regulatory protein and P450 11beta-hydroxylase messenger RNAs in rainbow trout interrenal cells: relationship with in vitro cortisol production. Gen Comp Endocrinol 145(3):254-62.

Hannesson R. 1996. Fisheries mismanagement: The case of the North Atlantic cod. Oxford: Blackwell Scientific Publications.

Hawkins MB, Thomas P. 2004. The unusual binding properties of the third distinct teleost estrogen receptor subtype ERbetaa are accompanied by highly conserved amino acid changes in the ligand binding domain. Endocrinology 145(6):2968-77.

Hawkins MB, Thornton JW, Crews D, Skipper JK, Dotte A, Thomas P. 2000. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc Natl Acad Sci U S A 97(20):10751-6.

Higgs DA, Fagerlund UHM, Eales JG, McBride JR. 1982. Application of thyroid and steroid hormones as anabolic agents in fish culture. comp Biochem Physiol B Biochem Mol Biol 73B:143-176.

Hirai T, Yamashita M, Yoshikuni M, Lou YH, Nagahama Y. 1992. Cyclin B in fish oocytes: its cDNA and amino acid sequences, appearance during maturation, and induction of p34cdc2 activation. Mol Reprod Dev 33(2):131-40.

Hori SH, Kodama T, Tanahashi K. 1979. Induction of vitellogenin synthesis in goldfish by massive doses of androgens. Gen Comp Endocrinol 37(3):306-20.

Hsueh AJ, McGee EA, Hayashi M, Hsu SY. 2000. Hormonal regulation of early follicle development in the rat ovary. Mol Cell Endocrinol 163(1-2):95-100.

Hunter GA, Donaldson EM. 1983. Hormonal sex control and its application to fish culture. In: Hoar WS, Randall DJ, Donaldson EM, editors. Fish physiology. New York: Academic Press. p 223-303.

Hyllner SJ, Westerlund L, Olsson PE, Schopen A. 2001. Cloning of rainbow trout egg envelope proteins: members of a unique group of structural proteins. Biol Reprod 64(3):805-11.

Ijiri S, Takei N, Kazeto Y, Todo T, Adachi S, Yamauchi K. 2006. Changes in localization of cytochrome P450 cholesterol side-chain cleavage (P450scc) in Japanese eel testis and ovary during gonadal development. Gen Comp Endocrinol 145(1):75-83.

Ikeuchi T, Todo T, Kobayashi T, Nagahama Y. 1999. cDNA cloning of a novel androgen receptor subtype. J Biol Chem 274(36):25205-9.

Ings JS, Van Der Kraak GJ. 2006. Characterization of the mRNA expression of StAR and steroidogenic enzymes in zebrafish ovarian follicles. Mol Reprod Dev 73(8):943-54.

Janosek J, Hilscherova K, Blaha L, Holoubek I. 2006. Environmental xenobiotics and nuclear receptors--interactions, effects and in vitro assessment. Toxicol In Vitro 20(1):18-37.

Ji W, Wright MB, Cai L, Flament A, Lindpaintner K. 2002. Efficacy of SSH PCR in isolating differentially expressed genes. BMC Genomics 3(1):12.

Jia XC, Kessel B, Welsh TH, Jr., Hsueh AJ. 1985. Androgen inhibition of follicle-stimulating hormone-stimulated luteinizing hormone receptor formation in cultured rat granulosa cells. Endocrinology 117(1):13-22.

Kanamori A. 2000. Systematic identification of genes expressed during early oogenesis in medaka. Mol Reprod Dev 55(1):31-6.

Kanamori A, Yamamura A, Koshiba S, Lee JS, Orlando EF, Hori H. 2006. Methyltestosterone efficiently induces male development in the self-fertilizing hermaphrodite fish, Kryptolebias marmoratus. Genesis 44(10):495-503.

Karlsen Ø, Mangor-Jensen A, van der Meeren T, Taranger GL. 2005. Yngel og matfiskproduksjon av torsk. Fisken og havet, særnr 2 (in norwegian).

Khim JS, Lee KT, Kannan K, Villeneuve DL, Giesy JP, Koh CH. 2001. Trace organic contaminants in sediment and water from Ulsan Bay and its vicinity, Korea. Arch Environ Contam Toxicol 40(2):141-50.

Kishida M, Callard GV. 2001. Distinct cytochrome P450 aromatase isoforms in zebrafish (Danio rerio) brain and ovary are differentially programmed and estrogen regulated during early development. Endocrinology 142(2):740-50.

Kitajima K, Kuroyanagi H, Inoue S, Ye J, Troy FA, 2nd, Inoue Y. 1994. Discovery of a new type of sialidase, "KDNase," which specifically hydrolyzes deaminoneuraminyl (3-deoxy-D-glycero-D-galacto-2-nonulosonic acid) but not N-acylneuraminyl linkages. J Biol Chem 269(34):21415-9.

Kitano T, Takamune K, Nagahama Y, Abe SI. 2000. Aromatase inhibitor and 17alpha-methyltestosterone cause sex-reversal from genetical females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (Paralichthys olivaceus). Mol Reprod Dev 56(1):1-5.

Kjesbu OS, Holm JC. 1994. Oocyte recruitment in first-time spawning Atlantic cod (*Gadus morhua*) in relation to feeding regime. Canadian Journal of Fisheries and Aquatic Sciences 51:1893-1898.

Kjesbu OS, Kryvi H. 1989. Oogenesis in cod, *Gadus morhua L.*, studied by light and electron microscopy. J Fish Biol 34(735-746).

Kjesbu OS, Kryvi H, Norberg B. 1996. Oocyte size and structure in relation to blood plasma steroid hormones in individually monitored, spawning Atlantic cod. J Fish Biol 49:1197-1215.

Knoll-Gellida A, Andre M, Gattegno T, Forgue J, Admon A, Babin PJ. 2006. Molecular phenotype of zebrafish ovarian follicle by serial analysis of gene expression and proteomic profiling, and comparison with the transcriptomes of other animals. BMC Genomics 7:46.

Kondo T, Kotani T, Yamashita M. 2001. Dispersion of cyclin B mRNA aggregation is coupled with translational activation of the mRNA during zebrafish oocyte maturation. Dev Biol 229(2):421-31.

Kondo T, Yanagawa T, Yoshida N, Yamashita M. 1997. Introduction of cyclin B induces activation of the maturation-promoting factor and breakdown of germinal vesicle in growing zebrafish oocytes unresponsive to the maturation-inducing hormone. Dev Biol 190(1):142-52.

Korte JJ, Kahl MD, Jensen KM, Pasha MS, Parks LG, LeBlank GA, Ankley GT. 2000. Fathead minnow vitellogenin: complementary DNA sequence and messenger RNA

and protein expression after 17B-estradiol treatment. Environ Toxicol Chem 19(4):972-981.

Kusakabe M, Todo T, McQuillan HJ, Goetz FW, Young G. 2002. Characterization and expression of steroidogenic acute regulatory protein and MLN64 cDNAs in trout. Endocrinology 143(6):2062-70.

Larkin P, Knoebl I, Denslow ND. 2003. Differential gene expression analysis in fish exposed to endocrine disrupting compounds. Comp Biochem Physiol B Biochem Mol Biol 136(2):149-61.

Le Menn F, Cerda J, Babin PJ. 2007. Ultrastructural aspects of the ontogeny and differentiation of ray-finned fish ovarian follicles. In: Babin PJ, Cerda J, Lubzens E, editors. The Fish Oocyte - from basic studies to biotechnological applications: Springer. p 1-37.

Le Menn F, Rochefort H, Garcia M. 1980. Effect of androgen mediated by the estrogen receptor of fish liver: vitellogenin accumulation. Steroids 35(3):315-28.

Leatherland JF, Copeland P, Sumpter JP, Sonstegard RA. 1982. Hormonal control of gonadal maturation and development of secondary sexual characteristics in coho salmon, Oncorhynchus kisutch, from lakes Ontario, Erie, and Michigan. Gen Comp Endocrinol 48(2):196-204.

Li YY, Inoue K, Takei Y. 2003. Steroidogenic acute regulatory protein in eels: cDNA cloning and effects of ACTH and seawater transfer on its mRNA expression. Zoolog Sci 20(2):211-9.

Lokman PM, George KA, Divers SL, Algie M, Young G. 2007. 11-Ketotestosterone and IGF-I increase the size of previtellogenic oocytes from shortfinned eel, *Anguilla australis*, in vitro. Reproduction 133(5):955-67.

Lokman PM, Harris B, Kusakabe M, Kime DE, Schulz RW, Adachi S, Young G. 2002. 11-Oxygenated androgens in female teleosts: prevalence, abundance, and life history implications. Gen Comp Endocrinol 129(1):1-12.

Loomis AK, Thomas P. 1999. Binding characteristics of estrogen receptor (ER) in Atlantic croaker (Micropogonias undulatus) testis: different affinity for estrogens and xenobiotics from that of hepatic ER. Biol Reprod 61(1):51-60.

Loomis AK, Thomas P. 2000. Effects of estrogens and xenoestrogens on androgen production by Atlantic croaker testes in vitro: evidence for a nongenomic action mediated by an estrogen membrane receptor. Biol Reprod 62(4):995-1004.

Luckenbach JA, Iliev DB, Goetz FW, Swanson P. 2008. Identification of differentially expressed ovarian genes during primary and early secondary oocyte growth in coho salmon, Oncorhynchus kisutch. Reprod Biol Endocrinol 6(1):2.

Lyons CE, Payette KL, Price JL, Huang RC. 1993. Expression and structural analysis of a teleost homolog of a mammalian zona pellucida gene. J Biol Chem 268(28):21351-8.

Lyssimachou A, Arukwe A. 2007. Alteration of brain and interrenal StAR protein, P450scc, and Cyp11beta mRNA levels in atlantic salmon after nominal waterborne exposure to the synthetic pharmaceutical estrogen ethynylestradiol. J Toxicol Environ Health A 70(7):606-13.

Lyssimachou A, Jenssen BM, Arukwe A. 2006. Brain cytochrome P450 aromatase gene isoforms and activity levels in atlantic salmon after waterborne exposure to nominal environmental concentrations of the pharmaceutical ethynylestradiol and antifoulant tributyltin. Toxicol Sci 91(1):82-92.

MacKenzie DS, VanPutte CM, Leiner KA. 1998. Nutrient regulation of endocrine function in fish Aquaculture 161(1-4):3-25.

Mandarim-de-Lacerda CA. 2003. Stereological tools in biomedical research. An Acad Bras Cienc 75(4):469-86.

Matejusova I, Felix B, Sorsa-Leslie T, Gilbey J, Noble LR, Jones CS, Cunningham CO. 2006. Gene expression profiles of some immune relevant genes from skin of susceptible and responding Atlantic salmon (Salmo salar L.) infected with Gyrodactylus salaris (Monogenea) revealed by suppressive subtractive hybridisation. Int J Parasitol 36(10-11):1175-83.

Matthews J, Gustafsson JA. 2003. Estrogen signaling: a subtle balance between ER alpha and ER beta. Mol Interv 3(5):281-92.

McAllister BG, Kime DE. 2003. Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (Danio rerio). Aquat Toxicol 65(3):309-16.

McGee EA. 2000. The regulation of apoptosis in preantral ovarian follicles. Biol Signals Recept 9(2):81-6.

Meier S, Andersen TE, Norberg B, Thorsen A, Taranger GL, Kjesbu OS, Dale R, Morton HC, Klungsoyr J, Svardal A. 2007. Effects of alkylphenols on the reproductive system of Atlantic cod (Gadus morhua). Aquat Toxicol 81(2):207-18.

Menuet A, Pellegrini E, Anglade I, Blaise O, Laudet V, Kah O, Pakdel F. 2002. Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biol Reprod 66(6):1881-92.

Meucci V, Arukwe A. 2006. Transcriptional modulation of brain and hepatic estrogen receptor and P450arom isotypes in juvenile Atlantic salmon (Salmo salar) after waterborne exposure to the xenoestrogen, 4-nonylphenol. Aquat Toxicol 77(2):167-77.

Miller WL. 1988. Molecular biology of steroid hormone synthesis. Endocr Rev 9(3):295-318.

Mittelholzer C, Andersson E, Consten D, Hirai T, Nagahama Y, Norberg B. 2007. 20 beta-hydroxysteroid dehydrogenase and CYP19A1 are differentially expressed during maturation in Atlantic cod (Gadus morhua). J Mol Endocrinol 39(4):319-28.

Miura C, Takahashi N, Michino F, Miura T. 2005. The effect of para-nonylphenol on Japanese eel (Anguilla japonica) spermatogenesis in vitro. Aquat Toxicol 71(2):133-41.

Miura T, Ando N, Miura C, Yamauchi K. 2002. Comparative studies between in vivo and in vitro spermatogenesis of Japanese eel (Anguilla japonica). Zoolog Sci 19(3):321-9.

Miura T, Kudo N, Miura C, Yamauchi K, Nagahama Y. 1998. Two testicular cDNA clones suppressed by gonadotropin stimulation exhibit ZP2- and ZP3-like structures in Japanese eel. Mol Reprod Dev 51(3):235-42.

Miura T, Miura C, Ohta T, Nader MR, Todo T, Yamauchi K. 1999. Estradiol-17beta stimulates the renewal of spermatogonial stem cells in males. Biochem Biophys Res Commun 264(1):230-4.

Miura T, Yamauchi K, Takahashi H, Nagahama Y. 1991. Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (Anguilla japonica). Proc Natl Acad Sci U S A 88(13):5774-8.

Modig C, Modesto T, Canario A, Cerda J, von Hofsten J, Olsson PE. 2006. Molecular characterization and expression pattern of zona pellucida proteins in gilthead seabream (Sparus aurata). Biol Reprod 75(5):717-25.

Modig C, Westerlund L, Olsson PE. 2007. Oocyte Zona Pellucida proteins. In: Babin PJ, Cerda J, Lubzens E, editors. The Fish Oocyte: fronm basic studies to biotechnological applications: Springer. p 113-139.

Moksness E, Kjørsvik E, Olsen Y. 2004. Culture of Coldwater Marine Fish Oxford: Blackwell publishing Ltd.

Mommsen PT, Walsh LP. 1988. Vitellogenesis and oocyte assembly. In: Hoar WS, Randall DJ, Donaldson EM, editors. Fish Physiology. New York: Academic Press. p 347-406.

Morgan DO. 1995. Principles of CDK regulation. Nature 374(6518):131-4.

Mori T, Matsumoto H, Yokota H. 1998. Androgen-induced vitellogenin gene expression in primary cultures of rainbow trout hepatocytes. J Steroid Biochem Mol Biol 67(2):133-41.

Murray AA, Gosden RG, Allison V, Spears N. 1998. Effect of androgens on the development of mouse follicles growing in vitro. J Reprod Fertil 113(1):27-33.

Nader MR, Miura T, Ando N, Miura C, Yamauchi K. 1999. Recombinant human insulin-like growth factor I stimulates all stages of 11-ketotestosterone-induced spermatogenesis in the Japanese eel, Anguilla japonica, in vitro. Biol Reprod 61(4):944-7.

Nagahama Y. 1994. Endocrine regulation of gametogenesis in fish. Int J Dev Biol 38(2):217-29.

Nagahama Y. 1997. 17 alpha,20 beta-dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: mechanisms of synthesis and action. Steroids 62(1):190-6.

Nagahama Y, Yoshikuni M, Yamashita M, Sakai N, Tanaka M. 1993. Molecular endocrinology of oocyte growth and maturation in fish. Fish Physiology and Biochemistry 11(1-6):3-14.

Nagahama Y, Yoshikuni M, Yamashita M, Tokumoto T, Katsu Y. 1995. Regulation of oocyte growth and maturation in fish. Curr Top Dev Biol 30:103-45.

Nakamura I, Evans JC, Kusakabe M, Nagahama Y, Young G. 2005. Changes in steroidogenic enzyme and steroidogenic acute regulatory protein messenger RNAs in ovarian follicles during ovarian development of rainbow trout (Oncorhynchus mykiss). Gen Comp Endocrinol 144(3):224-31.

Nakamura M. 1975. Dosage-dependent changes in the effect of oral administration on gonadal differentiation in Tilapia mossambica. Bull. Fac. Fish. Hokkkaido Univ 26:99-108.

Nolan T, Hands RE, Bustin SA. 2006. Quantification of mRNA using real-time RT-PCR. Nat Protoc 1(3):1559-82.

Norberg B, Brown CL, Halldorsson O, Stensland K, Bjørnsson BT. 2004. Photoperiod regulates the timing of sexual maturation, spawning, sex setroid and thyroid hormone profiles in the Atlantic cod (*Gadus morhua*). Aquaculture 229:451-467.

Nunez BS, Evans AN. 2007. Hormonal regulation of the steroidogenic acute regulatory protein (StAR) in gonadal tissues of the Atlantic croaker (Micropogonias undulatus). Gen Comp Endocrinol 150(3):495-504.

Olsson PE, Berg AH, von Hofsten J, Grahn B, Hellqvist A, Larsson A, Karlsson J, Modig C, Borg B, Thomas P. 2005. Molecular cloning and characterization of a nuclear androgen receptor activated by 11-ketotestosterone. Reprod Biol Endocrinol 3:37.

Oppen-Berntsen DO. 1990. Oogenesis and hatching in teleostean fishes with special reference to eggshell proteins: Dr. Scient. Thesis. University of Bergen, Norway.

Oppen-Berntsen DO, Arukwe A, Yadetie F, Lorens JB, Male R. 1999. Salmon Eggshell Protein Expression: A Marker for Environmental Estrogens. Mar Biotechnol (NY) 1(3):252-260.

Oppen-Berntsen DO, Gram-Jensen E, Walther BT. 1992a. Zona radiata proteins are synthesized by rainbow trout (Oncorhynchus mykiss) hepatocytes in response to oestradiol-17 beta. J Endocrinol 135(2):293-302.

Oppen-Berntsen DO, Hyllner SJ, Haux C, Helvik JV, Walther BT. 1992b. Eggshell zona radiata-proteins from cod (Gadus morhua): extra-ovarian origin and induction by estradiol-17 beta. Int J Dev Biol 36(2):247-54.

Orn S, Holbech H, Madsen TH, Norrgren L, Petersen GI. 2003. Gonad development and vitellogenin production in zebrafish (Danio rerio) exposed to ethinylestradiol and methyltestosterone. Aquat Toxicol 65(4):397-411.

Pakdel F, Feon S, Le Gac F, Le Menn F, Valotaire Y. 1991. In vivo estrogen induction of hepatic estrogen receptor mRNA and correlation with vitellogenin mRNA in rainbow trout. Mol Cell Endocrinol 75(3):205-12.

Pan J, Sasanami T, Kono Y, Matsuda T, Mori M. 2001. Effects of testosterone on production of perivitelline membrane glycoprotein ZPC by granulosa cells of Japanese quail (Coturnix japonica). Biol Reprod 64(1):310-6.

Pan YS, Lee YS, Lee YL, Lee WC, Hsieh SY. 2006. Differentially profiling the low-expression transcriptomes of human hepatoma using a novel SSH/microarray approach. BMC Genomics 7:131.

Papoulias DM, Noltie DB, Tillitt DE. 2000. Effects of methyl testosterone exposure on sexual differentiation in medaka, Oryzias latipes. Mar Environ Res 50(1-5):181-4.

Patino R, Sullivan CV. 2002. Ovarian follicle growth, maturation and ovulation in teleost fish. Fish Physiology and Biochemistry 26:57-70.

Peter RE, Yu KL. 1997. Neuroendocrine regulation of ovulation in fishes: basic and applied aspects. Rev. Fish Biol. Fisheries 7:173-197.

Peyon P, Baloche S, Burzawa-Gerard E. 1997. Investigation into the possible role of androgens in the induction of hepatic vitellogenesis in the European eel: in vivo and in vitro studies. Fish Physiology and Biochemistry 16(2):107-118.

Piferrer F, Baker IJ, Donaldson EM. 1993. Effects of natural, synthetic, aromatizable, and nonaromatizable androgens in inducing male sex differentiation in genotypic female chinook salmon (Oncorhynchus tshawytscha). Gen Comp Endocrinol 91(1):59-65.

Pinto PI, Passos AL, Martins Deborah MPRS, Canario AV. 2006. Characterization of estrogen receptor betab in sea bream (Sparus auratus): phylogeny, ligand-binding, and comparative analysis of expression. Gen Comp Endocrinol 145(2):197-207.

Pratt WB, Toft DO. 1997. Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev 18(3):306-60.

Rinchard J, Dabrowski K, Garcia-Abiado MA, Ottobre J. 1999. Uptake and depletion of plasma 17alpha-methyltestosterone during induction of masculinization in muskellunge, Esox masquinongy: effect on plasma steroids and sex reversal. Steroids 64(8):518-25.

Rise ML, von Schalburg KR, Brown GD, Mawer MA, Devlin RH, Kuipers N, Busby M, Beetz-Sargent M, Alberto R, Gibbs AR and others. 2004. Development and application of a salmonid EST database and cDNA microarray: data mining and interspecific hybridization characteristics. Genome Res 14(3):478-90.

Rocha MJ, Rocha E. 2006. Morphofunctional Aspects of Reproduction from Synchronous to Asynchronous Fishes - An Overview. In: Reinecke M, Zaccone G, Kapoor BG, editors. Fish Endocrinology. p 571-624.

Rohr DH, Lokman PM, Davie PS, Young G. 2001. 11-Ketotestosterone induces silvering-related changes in immature female short-finned eels, *Anguilla australis*. Comp Biochem Physiol A Mol Integr Physiol 130(4):701-14.

Rondeau G, McClelland M, Nguyen T, Risques R, Wang Y, Judex M, Cho AH, Welsh J. 2005. Enhanced microarray performance using low complexity representations of the transcriptome. Nucleic Acids Res 33(11):e100.

Rotchell JM, Ostrander GK. 2003. Molecular markers of endocrine disruption in aquatic organisms. J Toxicol Environ Health B Crit Rev 6(5):453-96.

Sabo-Attwood T, Kroll KJ, Denslow ND. 2004. Differential expression of largemouth bass (Micropterus salmoides) estrogen receptor isotypes alpha, beta, and gamma by estradiol. Mol Cell Endocrinol 218(1-2):107-18.

Sanderson JT. 2006. The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. Toxicol Sci 94(1):3-21.

Scott AP. 1987. Reproductive endocrinology of fish. In: Chester Jones C, Ingleton PM, Phillips JC, editors. Fundamentals of Comparative Vertebrate Endocrinology. New York: Plenum Press. p 223-256.

Senthilkumaran B, Sudhakumari CC, Chang XT, Kobayashi T, Oba Y, Guan G, Yoshiura Y, Yoshikuni M, Nagahama Y. 2002. Ovarian carbonyl reductase-like 20beta-hydroxysteroid dehydrogenase shows distinct surge in messenger RNA expression

during natural and gonadotropin-induced meiotic maturation in nile tilapia. Biol Reprod 67(4):1080-6.

Senthilkumaran B, Yoshikuni M, Nagahama Y. 2004. A shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. Mol Cell Endocrinol 215(1-2):11-8.

Shankland SJ, Pippin JW, Reiser J, Mundel P. 2007. Podocytes in culture: past, present, and future. Kidney Int 72(1):26-36.

Shiina H, Matsumoto T, Sato T, Igarashi K, Miyamoto J, Takemasa S, Sakari M, Takada I, Nakamura T, Metzger D and others. 2006. Premature ovarian failure in androgen receptor-deficient mice. Proc Natl Acad Sci U S A 103(1):224-9.

Singleton DW, Khan SA. 2003. Xenoestrogen exposure and mechanisms of endocrine disruption. Front Biosci 8:s110-8.

Slater CH, Schreck CB, Swanson P. 1994. Plasma profiles of the sex steroids and gonadotropins in maturing female spring chinook salmon (*Oncorhynchus tshawytscha*). Comp Biochem Physiol A 109:167-175.

Smitz JE, Cortvrindt RG. 2002. The earliest stages of folliculogenesis in vitro. Reproduction 123(2):185-202.

Sperry TS, Thomas P. 1999a. Characterization of two nuclear androgen receptors in Atlantic croaker: comparison of their biochemical properties and binding specificities. Endocrinology 140(4):1602-11.

Sperry TS, Thomas P. 1999b. Identification of two nuclear androgen receptors in kelp bass (Paralabrax clathratus) and their binding affinities for xenobiotics: comparison with Atlantic croaker (Micropogonias undulatus) androgen receptors. Biol Reprod 61(4):1152-61.

Sperry TS, Thomas P. 2000. Androgen binding profiles of two distinct nuclear androgen receptors in Atlantic croaker (Micropogonias undulatus). J Steroid Biochem Mol Biol 73(3-4):93-103.

Steele BK, Meyers C, Ozbun MA. 2002. Variable expression of some "housekeeping" genes during human keratinocyte differentiation. Anal Biochem 307(2):341-347.

Stocco DM. 2000. The role of the StAR protein in steroidogenesis: challenges for the future. J Endocrinol 164(3):247-53.

Stocco DM. 2001. StAR protein and the regulation of steroid hormone biosynthesis. Annu Rev Physiol 63:193-213.

Stocco DM, Wang X, Jo Y, Manna PR. 2005. Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. Mol Endocrinol 19(11):2647-59.

Suwa K, Yamashita M. 2007. Regulatory mechanisms of oocyte maturation and ovulation. In: Babin PJ, Cerda J, Lubzens E, editors. The Fish Oocyte: from basic studies to biotechnological applications: Springer. p 323-347.

Takeo J, Yamashita S. 1999. Two distinct isoforms of cDNA encoding rainbow trout androgen receptors. J Biol Chem 274(9):5674-80.

Tanaka M, Nakajin S, Kobayashi D, Fukada S, Guan G, Todo T, Senthilkumaran B, Nagahama Y. 2002. Teleost ovarian carbonyl reductase-like 20beta-hydroxysteroid dehydrogenase: potential role in the production of maturation-inducing hormone during final oocyte maturation. Biol Reprod 66(5):1498-504.

Thomas P, Tubbs C, Berg H, Dressing G. 2007. Sex steroid hormone receptors in fish ovaries. In: Babin PJ, Cerda J, Lubzens E, editors. The Fish Oocyte: from basic studies to biotechnological applications: Springer. p 203-233.

Tiffany-Castiglioni E, Ehrich M, Dees L, Costa LG, Kodavanti PR, Lasley SM, Oortgiesen M, Durham HD. 1999. Bridging the gap between in vitro and in vivo models for neurotoxicology. Toxicol Sci 51(2):178-83.

Todo T, Ikeuchi T, Kobayashi T, Nagahama Y. 1999. Fish androgen receptor: cDNA cloning, steroid activation of transcription in transfected mammalian cells, and tissue mRNA levels. Biochem Biophys Res Commun 254(2):378-83.

Tomkiewicz J, Tybjerg L, Jespersen Å. 2003. Micro- and macroscopic characteristics to stage gonadal maturation of female Baltic cod. J Fish Biol 62:253-275.

Touhata K, Kinoshita M, Tokuda Y, Toyohara H, Sakaguchi M, Yokoyama Y, Yamashita S. 1999. Sequence and expression of a cDNA encoding the red seabream androgen receptor. Biochim Biophys Acta 1450(3):481-5.

Tyler CR, Sumpter JP. 1996. Oocyte growth and development in teleost. Rev. Fish Biol. Fisheries 6:287-318.

Vang SH, Kortner TM, Arukwe A. 2007. Steroidogenic acute regulatory (StAR) protein and cholesterol side-chain cleavage (P450scc) as molecular and cellular targets for 17alpha-ethynylestradiol in salmon previtellogenic oocytes. Chem Res Toxicol 20(12):1811-9.

Vendola K, Zhou J, Wang J, Famuyiwa OA, Bievre M, Bondy CA. 1999. Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary. Biol Reprod 61(2):353-7.

Vendola KA, Zhou J, Adesanya OO, Weil SJ, Bondy CA. 1998. Androgens stimulate early stages of follicular growth in the primate ovary. J Clin Invest 101(12):2622-9.

Vercellini P, Parazzini F, Bolis G, Carinelli S, Dindelli M, Vendola N, Luchini L, Crosignani PG. 1993. Endometriosis and ovarian cancer. Am J Obstet Gynecol 169(1):181-2.

Vizziano D, Randuineau G, Baron D, Cauty C, Guiguen Y. 2007. Characterization of early molecular sex differentiation in rainbow trout, Oncorhynchus mykiss. Dev Dyn 236(8):2198-206.

von Schalburg KR, Rise ML, Brown GD, Davidson WS, Koop BF. 2005. A comprehensive survey of the genes involved in maturation and development of the rainbow trout ovary. Biol Reprod 72(3):687-99.

Wallace RA, Selman K. 1990. Ultrastructural aspects of oogenesis and oocyte growth in fish and amphibians. J Electron Microsc Tech 16(3):175-201.

Wang H, Andoh K, Hagiwara H, Xiaowei L, Kikuchi N, Abe Y, Yamada K, Fatima R, Mizunuma H. 2001. Effect of adrenal and ovarian androgens on type 4 follicles unresponsive to FSH in immature mice. Endocrinology 142(11):4930-6.

Wang H, Gong Z. 1999. Characterization of two zebrafish cDNA clones encoding egg envelope proteins ZP2 and ZP3. Biochim Biophys Acta 1446(1-2):156-60.

Watson CS. 2003. The identities of membrane steroid receptors: Kluwer academic publishers.

Weibel ER. 1979. Stereological Methods, Practical Methods for Biological Morphometry. London, UK: Academic Press.

Weil S, Vendola K, Zhou J, Bondy CA. 1999. Androgen and follicle-stimulating hormone interactions in primate ovarian follicle development. J Clin Endocrinol Metab 84(8):2951-6.

Weil SJ, Vendola K, Zhou J, Adesanya OO, Wang J, Okafor J, Bondy CA. 1998. Androgen receptor gene expression in the primate ovary: cellular localization, regulation, and functional correlations. J Clin Endocrinol Metab 83(7):2479-85.

Yadetie F, Arukwe A, Goksøyr A, Male R. 1999. Induction of hepatic estrogen receptor in juvenile Atlantic salmon in vivo by the environmental estrogen, 4-nonylphenol. Sci Total Environ 233(1-3):201-10.

Yang GP, Ross DT, Kuang WW, Brown PO, Weigel RJ. 1999. Combining SSH and cDNA microarrays for rapid identification of differentially expressed genes. Nucleic Acids Res 27(6):1517-23.

Yeh S, Tsai MY, Xu Q, Mu XM, Lardy H, Huang KE, Lin H, Yeh SD, Altuwaijri S, Zhou X and others. 2002. Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. Proc Natl Acad Sci U S A 99(21):13498-503.

Young G, Crim LW, Kagawa H, Kambegawa A, Nagahama Y. 1983. Plasma 17 alpha, 20 beta-dihydroxy-4-pregnen-3-one levels during sexual maturation of amago salmon (Oncorhynchus rhodurus): correlation with plasma gonadotropin and in vitro production by ovarian follicles. Gen Comp Endocrinol 51(1):96-105.

Young G, Kusakabe M, Nakamura I. 2005. Gonadal Steroidogenesis in Teleost Fish. In: Melamed P, Sherwood N, editors. Molecular Aspects of Fish And Marine Biology - Hormones and their Receptors in Fish Reproduction: World Scientific. p 155-223.

Zeng S, Gong Z. 2002. Expressed sequence tag analysis of expression profiles of zebrafish testis and ovary. Gene 294(1-2):45-53.

Zerulla M, Lange R, Steger-Hartmann T, Panter G, Hutchinson T, Dietrich DR. 2002. Morphological sex reversal upon short-term exposure to endocrine modulators in juvenile fathead minnow (Pimephales promelas). Toxicol Lett 131(1-2):51-63.

Zhang W, Zhang Y, Zhang L, Zhao H, Li X, Huang H, Lin H. 2006. The mRNA expression of P450 aromatase, gonadotropin beta-subunits and FTZ-F1 in the orange-spotted grouper (Epinephelus Coioides) during 17alpha-methyltestosterone-induced precocious sex change. Mol Reprod Dev.

Zhu Y, Bond J, Thomas P. 2003a. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc Natl Acad Sci U S A 100(5):2237-42.

Zhu Y, Rice CD, Pang Y, Pace M, Thomas P. 2003b. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc Natl Acad Sci U S A 100(5):2231-6.

ERRATA

Paper V.

Previtellogenic oocytes had a diameter of approximately 100 µm. In accordance, bar in Fig. 4 and Fig. 5 equals 30 µm.

In Table 1, the correct primer pair sequences shall be:

Target Gene	Primer sequence*	
	Forward	Reverse
StAR	CAACGTCAAGCAGGTCAAGA	GCATCGGGCTTCAACACTAT
P450*scc*	AACAACTACTTCCGCAGCCT	CGGTAGAACAATGAGCTGGA
Cyclin-B	CGGGAGATGGAGATGACTGT	TCTCGTAGTCCACCATGCAG

* sequences are given in the 5’ – 3’ order

PAPER I

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Genomic approach in evaluating the role of androgens on the growth of Atlantic cod (*Gadus morhua*) previtellogenic oocytes

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ABSTRACT

Previous studies have suggested that androgens may play an integral role in early oocyte development in fish. This study evaluated the effects of androgens (11-ketotestosterone: 11-KT and testosterone: T) on gene expression patterns and growth of cod previtellogenic oocytes. cDNA libraries of androgen-responsive genes were generated using suppressive subtractive hybridization (SSH) of clones containing differentially expressed genes in oocytes separately exposed to different concentrations of 11-KT and T, in addition to a solvent control. Secondly, a targeted microarray was developed based on differentially expressed genes. In the experimental setup, tissue was cultured *in vitro* with different concentrations of 11-KT and T (0, 10 and 100 μM). The array analyses showed 0.5–3.5-fold significant alterations in transcript levels for a number of genes. Real-time PCR and in -situ hybridization were also used to analyze the changes in expression for selected genes. Quantitative histological analyses showed a consistent stereological validation of oocyte growth and development after exposure to androgens. The present study reveals novel roles of androgens on the development of previtellogenic oocytes, suggesting androgen control of early oocyte growth in cod. The strong effects of 11-KT on oocyte growth support our hypothesis that non-aromatizable androgens may exert direct hormonal effects in previtellogenic oocytes, with possible consequences for overt fecundity.

1. Introduction

Prior to vitellogenesis (previtellogenesis), androgens are suggested to play an integral role in the regulation of oocyte growth and development. Preliminary evidence supporting these assumptions are derived from the induction of oocyte growth in androgen exposed immature female eels (Rohr et al., 2001). It has been demonstrated that 11-ketotestosterone (11-KT, the active male-specific androgen in teleosts) induces growth (increase in oocyte diameter) and development of previtellogenic eel oocytes both *in vitro* and *in vivo* (Lokman et al., 2007; Rohr et al., 2001). Generally, androgens appear to play a pivotal role in stimulating the growth of small ovarian follicles in vertebrates, at least in mammals and fish (Rohr et al., 2001; Weil et al., 1999, 1998). In mammals, androgens also modify the intra-ovarian gene expression, as demonstrated by increased mRNA abundance of insulin-like growth factor-1 (IGF-1) and IGF-1-receptor (Vendola et al., 1999) in follicles up to early antral stage. The role of androgens in previtellogenic oocytes is supported by the observation that the early ovarian growth of species-specific critical stage progressed in hypophysectomized freshwater turtle, *Chrysemys picta*, indicating that gonadotrophins were not necessary (Ho et al., 1982). Furthermore, Bieniarz and Kime (1986) were unable to demonstrate specific binding of radio labeled GtH (^{125}I-GtH) to previtellogenic common carp (*Cyprinus carpio*) ovaries. Except for the evidence that androgens increase oocyte diameter and modify growth factor mRNA abundance, the underlying molecular mechanism(s) involved in possible regulation of genes whose functional products modulate the growth of previtellogenic oocytes has not been investigated.

Oviparous vertebrates lay yolky eggs that provide necessary nutrients for embryonic development. Oogenesis is an integral aspect of reproduction that comprises a multi-step process resulting in egg laying and can be divided into previtellogenesis, vitellogenesis and final oocyte maturation (Tyler and Sumpter, 1996). The fish ovary consists of numerous ovigerous folds extending from the ovarian wall towards the centre of the ovary. The oogonia are formed by mitosis in the vicinity of the luminal epithelium, and are recognized as small nests of mitotic cells dispersed within the ovary connective tissue. Oogenesis commences when oogonia are transformed into previtellogenic oocytes, and each oocyte is simultaneously surrounded by a follicular cell envelope (Kjesbu and Kryvi, 1989). Final maturation transforms small primary oocytes in meiotic prophase into large yolky haploid eggs ready for ovulation and fertilization (Caussanel and Breuzet, 1977). Oogenesis in Atlantic cod has previously been investigated (Dahle et al., 2003; Kjesbu and Kryvi, 1989; Kjesbu et al., 1996) and these studies mainly focused on the vitellogenic process.

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Thus, what is known today about previtellogenesis in cod (and teleosts in general) is in sharp contrast with the knowledge regarding the processes of vitellogenesis and final oocyte maturation (Tyler and Sumpter, 1996). The endocrine system plays a major role in controlling oogenesis in oviparous vertebrates. Gonadotropins secreted from the pituitary gland finally stimulate the ovarian production of steroids hormones (estrogens), which in turn mediate the vitellogenic process. Final oocyte maturation is mediated by progesterone-derivatives (Chan et al., 1978). During the previtellogenic phase, the oocytes of a typical teleost fish may increase at least 10-fold in diameter (from about 10–15 μm to over 150 μM). An extensive synthesis of RNA, mRNA coding for vitellogenin (Vtg)-receptor, accumulation of mitochondria, polyribosomes, lipids and cortical alveoli are all characteristics of previtellogenesis (Mommsen and Walsh, 1988). In addition, the formation of the zona radiata and ovarian follicle are integral aspects of early oocyte growth (Abraham et al., 1984). Despite the fact that these changes are integral and critical processes in oogenesis, essentially nothing is known about their endocrine and/or physiological control in the early stages. Given that fecundity seems to be largely determined during previtellogenesis in response to nutritional cues in rainbow trout (Bromage et al., 1992; MacKenzie et al., 1998), this still represents a strong gap in our knowledge of fish endocrine and reproductive physiology.

Like in other vertebrates, testosterone (T) is present in female teleost fish. In addition, the non-aromatizable androgen 11-ketotestosterone (11-KT) is usually present, and generally believed to be the most potent androgen in teleosts (Borg 1994; Lokman et al., 2002). Despite that 11-KT is generally believed to be the active male-specific androgen in teleosts, it has been found at elevated levels in some female teleosts (Leatherland et al., 1982; Slater et al., 1994). However, aromatizable (T) and non-aromatizable (11-KT) androgens may have strikingly different effects (Borg 1994). Androgens mediate their actions by binding to specific receptors belonging to the steroid hormone receptor super-family. Androgen receptor (AR) isoforms have been identified in several teleosts (Ikeuchi et al., 1999; Takeo and Yamashita, 1999; Todo et al., 1999; Touhata et al., 1999). ARs are usually located in the nucleus, and have normally high binding affinities for either T or 5α-dihydrotestosterone (DHT) (Sperry and Thomas, 1999a,b, 2000). Recently, a nuclear androgen receptor activated by 11-KT was characterized (Olsson et al., 2005). Despite the significance of these findings for fish reproductive physiology, the above mentioned studies did not describe exactly the roles or effects of androgens in the fish ovary. We believed that the identification of androgen-responsive genes and the characterization of specific gene expression patterns due to androgen stimulation of fish oocytes will provide a better understanding of the internal signals that control fecundity in teleosts.

Therefore, we have used the analytical power of suppressive subtractive hybridization (SSH) with subsequent development of a targeted cDNA gene array (CodArray) to open a possible revealing window in understanding the functional aspects of androgen effects on oocyte growth and development, and we present the identification of a number of androgen-responsive genes in the previtellogenic cod ovary. SSH, sequencing of cod androgen-responsive previtellogenic ovarian genes and expression profiling studies represent a potentially important scientific and practical approach for developing an efficient hormonal control of fecundity in the aquaculture industry (especially for new species) and fishery stock management.

2. Materials and methods

2.1. Chemicals and reagents

Testosterone (T) was purchased from Fluka chemika-biochemika (Buchs, Switzerland) and 11-ketotestosterone (11-KT) was kindly provided by Dr Bente Nilsen (Biosense Laboratories AS, Norway). Trizol reagent for RNA purification, TA cloning kit, Leibovitz L-15 medium and Superscript III cDNA synthesis kit were purchased from Gibco-Invitrogen life technologies (Carlsbad, CA, USA). Bovine serum albumin (BSA) and *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (Hepes) were purchased from Sigma Chemical (St. Louis, MO, USA). Iscript cDNA Synthesis Kit and iTaq™Sybr® Green supermix with ROX were purchased from Bio-Rad laboratories (Hercules, CA, USA) and Generuler™ 100 bp DNA ladder and dNTPs from Fermentas GmbH (Germany). RNA later, sodium salt citrate (SSC) and sodium dodecyl sulfate (SDS) washing solutions were purchased from Ambion (Austin, TX, USA), Oligotex mRNA kit from Qiagen (Valencia, CA, USA), MultiScreen-PCR96 filter plates from Millipore (Billerica, MA, USA) and 3DNA array 350 microarray kit from Genisphere (Hatfield, PA, USA). Amicon YM30 columns were purchased from Millipore (Volketswil, Switzerland). 11-KT enzyme immunoassay (EIA) kit (Cat. No. 582751) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals were of the highest commercially available grade.

2.2. Animals and floating agarose in vitro culture technique

Juvenile, previtellogenic female Atlantic cod (*Gadus morhua*, Gadidae), 150–350 g body mass were purchased from Akvaforsk Genetic Centre (Sunndalsøra, Norway) and kept in circulating water at 10 °C and 12:12 h light:dark photoperiod. The organ culture technique employed was based on the *in vitro* agarose floating method, which has successfully been used previously in our laboratory for related studies on cod and Atlantic salmon gonadal tissues (Kortner and Arukwe, 2007a,b; Vang et al., in press). Briefly, juvenile female cod were anesthetized, sacrificed and washed in 70% ethanol. Ovaries were removed, cut into small pieces (1 × 1 × 1 mm) and grown in 6-well dishes on a floating agarose substrate covered with a nitrocellulose membrane in basal culture media. The basal culture medium consisted of Leibovitz L-15 medium supplemented with 0.1 mM L-glutamate, 0.1 mM L-aspartate, 1.7 mM L-proline, 0.5% BSA, and 10 mM HEPES (pH 7.4). The gonadal tissue was cultured randomly in triplicates (n=3) for 5 and 10 days with different concentrations of testosterone and 11-KT (0 (control), 10 and 100 μM) in a humidified incubator at 10 °C. The control group received ethanol (carrier vehicle for the androgens) and the final concentration of ethanol in all exposure groups was 0.3% (v/v). The medium was changed every 5 days after sampling at days 5 and 10 of exposure. After cultivation, tissues for RNA purification were homogenized directly in Trizol reagent and stored at −80 °C until further processing. Tissues for histological analyses were placed in tissue cassettes with a nylon mesh and fixed in 4% paraformaldehyde.

2.3. Production of substracted cDNA library

We generated a targeted cDNA library by performing suppressive subtractive hybridization (SSH) with ovary tissue from juvenile Atlantic cod exposed separately to 11-KT and T (1, 50 and 100 μM) and used against untreated samples (solvent control samples). Tissues were incubated *in vitro* as described above, with cultivation for 7 and 14 days. After 7 and 14 days of exposure, samples were collected and transferred to RNA later. The total RNA was isolated using phenol: chloroform, and messenger RNA (mRNA) was purified from total RNA using an Oligotex mRNA kit and used for subtractive hybridization. The SSH experiment was performed in the forward and reverse directions to obtain up-regulated and down-regulated genes, respectively. Subtractive hybridizations were constructed using the Clontech (Palo Alto, CA, USA) SSH kit following the manufacturer's protocol. The SSH was performed by EcoArray Inc. (Alachua, FL, USA) under contract. Sequenced clones were analyzed using Blastx against the NCBI nonredundant (nr) protein database and Blastn against the NCBI nucleotide (nt) database. The chosen e-value cut-off was 10^{-5} for blast searches. Finally, clones were annotated and downloaded to the NCBI GenBank EST database under the title CodArray SSH cDNA library.

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2.4. Array spotting

The cod gonadal cDNA array (CodArray) was constructed using clones with expression patterns that were either up- or down-regulated in the subtracted library. However, SSH generated a number of redundant clones in the subtracted amplicons, and several genes suspected to be involved in the transcriptional regulation of oocyte growth and development were not represented after performing SSH in the present study. Therefore, transcripts for some genes (e.g. steroidogenic acute regulatory protein (StAR), cytochrome *P450*-mediated side-chain cleavage (P450*scc*) and cyclin-B) were amplified by PCR using specific primers from conserved regions of the respective genes based on sequence information in NCBI GenBank. The PCR products were cloned into *Escherichia coli* plasmids and subsequently added to the array. All clones were PCR amplified, verified by agarose gel electrophoresis and then purified using MultiScreen-PCR96 Filter Plates. Clones were diluted in 50% DMSO buffer and spotted onto Corning Ultragaps glass slides (humidity: 50%, temperature: 18 °C) and cross-linked by UV radiation (800 mJ). The printing service was provided by the Norwegian Microarray Consortium (NMC) at the National Technology Platform supported by the functional genomics programme (FUGE) of the Norwegian Research Council (NFR).

2.5. RNA purification and cDNA synthesis

Total RNA was purified from tissues homogenized in Trizol reagent according to established procedures. Quantification, purity and RNA integrity were evaluated by absorbance at 260 and 280 nm using a NanoDrop® ND-1000 UV–Visible Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis. High quality RNA with A260/A280 ratio above 1.8 and intact ribosomal 28S and 18S RNA bands was used for cDNA synthesis. Total cDNA for the real-time polymerase chain reaction (PCR) were generated from 1 μg total RNA from all samples using a mixture of random and poly-T primers from iScript cDNA synthesis kit as described by the manufacturer (Bio-Rad). Prior to quantification each cDNA sample was diluted 1:6.

2.6. Probe labelling and hybridization

Hybridization of the array was performed using Cy5/Cy3-labeled cDNA probes. cDNA probes were generated using 5 μg total RNA from the respective exposure conditions in triplicates ($n=3$) using Superscript III cDNA synthesis kit as described by the manufacturer (Invitrogen) and 1 μL Cy5 or Cy3 RT primers, 1 μL dNTPs, 2 μL of 0.1 M DTT and 1 μL of RNAse inhibitor using 3DNA Array 350 microarray kit (Genisphere) for each reaction. cDNA probes were generated by incubating samples for 1 h at 50 °C. After incubation, the RT reaction was terminated by adding 0.5 M NaOH/50 mM EDTA, and neutralized in Tris buffer. Probes generated for the two different exposure conditions were mixed and concentrated using YM30 columns. Each cDNA probe mix was diluted in water and hybridization buffer to a total volume of 60 μL according to the manufactures' protocol. cDNA probe hybridization was performed overnight at 60 °C using a Advalytix SlideBooster SB800 (Advalytix AG, Brunnthal, Germany). After cDNA probe hybridization, slides were washed in 2x SSC/0.1% SDS at 55 °C for 10 min, then washed (3×5 min) in 1x SSC at RT and finally washed (2×5 min) in 0.1x SSC at RT. Thereafter, hybridization of 3DNA Array 350 Capture Reagent was performed for 2.5 h before slide wash as described above and drying by compressed air. Finally, slides were dipped in a stabilization and drying solution (Agilent Technologies, CA, USA) for 30 s and scanned using an Agilent G2505B micro array scanner.

2.7. Array experimental design and statistical analysis

For both treatments in the experiment (T and 11-KT) microarrays were used to study gene expression levels at two different time points (5 and 10 days) and three different concentrations (0 (solvent control), 10 and 100 μM), six conditions in total. For each condition three biological replicates were available. To compare the experimental conditions, 36 arrays were used for each treatment (11-KT and T) and co-hybridizations were performed according to a loop design, in which all biological replicates appeared in more than one array causing the arrays not to be statistically independent. The images from the scanned arrays were processed using the GenePix Pro 5.1 software. The data were then filtered to remove spots that had been flagged 'Absent', 'Not Found' or 'Bad'. Spots that had more than 20% saturated pixels, or had a mean foreground intensity less than 1.5 times the local median background intensity were also removed. No background subtraction was performed. The data from each array were log-transformed and normalized using the global loess method (Yang et al., 2001). The arrays were then scaled to having the same median absolute deviation. The reason for using a global instead of printtip loess approach was the sparse information available for each subarray. For making statistical inference on differentially regulated genes the limma package (Smyth, 2004) was used. The limma approach is based on fitting a linear model to the expression data for each probe on a microarray. To adjust for the fact that the same biological replicate was used on more than one array a model was fitted with a coefficient for each replicate. From the model, contrasts could then be extracted between replicates from different experimental conditions. For each pair of experimental conditions hypothesis tests were performed to test if the expression level of the genes had changed. From the resulting *p*-values, *q*-values (Storey, 2002) were calculated. The *q*-value for a gene is the expected proportion of false positives one will get when calling that gene significant. For a gene to be considered significantly differentially expressed between two conditions its *q*-value was required to be lower than 0.05, in effect controlling the false discovery rate (FDR) (Benjamini and Hochberg, 1995) for each comparison at a 0.05 level.

2.8. Database submission

The array data were prepared according to the "minimum information about microarray experiment" (MIAME) recommendations, and deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) with accession number (to be provided later).

2.9. Primer optimization, cloning and sequencing

PCR primers for amplification of 106–246 bp gene specific PCR products were designed from conserved regions of the studied genes. The primer sequences, their amplicon size and the optimal annealing temperatures are shown in Table 1. Prior to PCR reactions, all primer pairs were used in titration reactions in order to determine optimal primer pair concentrations and their optimal annealing temperatures. All chosen primer pair concentrations used at the selected annealing temperatures gave a single band for the expected amplicon size in all reactions.

2.10. Quantitative (real-time) PCR

The expression of individual gene targets was analyzed using the Mx3000P REAL-TIME PCR SYSTEM (Stratagene, La Jolla, CA, USA). Every 25-μL DNA amplification reaction contained 12.5 μL of iTAQ™YBR® Green Supermix with ROX (Bio-Rad), 5 μL of diluted cDNA and 200 nM of each forward and reverse primers. The 3-step real-time PCR program included an enzyme activation step at 95 °C (5 min) and 40 cycles of 95 °C (30 s), 52–60 °C for 30 s, depending on the primers used (see Table 1) and 72 °C (30 s). Controls lacking cDNA template were included to determine the specificity of target cDNA amplification. Cycle threshold (Ct) values obtained were converted into mRNA copy number using standard plots of Ct versus log copy number. Standard plots for each target sequence were generated using

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Table 1
Primer pair sequences, amplicon size and annealing temperature conditions for gene transcripts analyzed by real-time PCR

Target gene	Primer sequence[a]		Amplicon size (bp)	Annealing temp. (°C)	Genbank accession no.
	Forward	Reverse			
ZPA	CACATCAGCTCACCCTCAGA	GACGCCCTACTAGTCCCACA	106	60	ES880740
Cyclin-B	CGGGAGATGGAGATGACTGT	TCTCGTAGTCCACCATGCAG	150	58	CO542315
HSP90β	CAGGAACCAGGTAGGCAGAG	CCAAAACCGGACCTAATCAA	144	55	ES880664
StAR	CAACGTCAAGCAGGTCAAGA	GCATCGGGCTTCAACACTAT	246	52	AY291434
P450*scc*	AACAACTACTTCCGCAGCCT	CGGTAGAACAATGAGCTGGA	170	55	AY706102

[a] Sequences are given in the 5′–3′ orientation.

known amounts of plasmid containing the amplicon of interest. The criterion for using the standard curve is based on equal amplification efficiency with unknown samples, and this was checked prior to extrapolating unknown samples to the standard curve. Data obtained from triplicate runs for individual target cDNA amplification were averaged and expressed as ng/μg of initial total RNA used for reverse transcriptase (cDNA) reaction and thereafter transformed as percentage of control. This absolute quantification method is a well-validated procedure in our laboratory, as we do not use the so-called housekeeping genes because of their parallel modulation pattern with experimental samples both in our laboratory (Arukwe, 2006) and elsewhere (Steele et al., 2002).

2.11. *In situ* hybridization

In situ hybridization procedures were performed according to standard protocols (Morel and Cavalier, 2001). Briefly, the orientation of insert relative to the T7 or SP6 RNA promoter binding site was determined, using the promoter at the 3′ end of the insert to get an antisense probe. A PCR analysis with primers flanking/including promotor binding sites generated 1 μg of DNA. *In vitro* transcription with SP6 or T7 RNA polymerase was performed with a DIG RNA labelling kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. The riboprobe was purified after DNaseI digestion with lithium chloride precipitation. Thereafter, the DIG-labelled riboprobe was dissolve in DEPC-water to get a concentration of about 0.1 μg/μL. One microliter of riboprobe was then run in an agarose gel to check riboprobe generation and then stored at −80 °C until used. When running slides, controls were made both by omitting the antisense probe and using a sense probe, getting no signal for both conditions.

2.12. Previtellogenic oocyte histological and stereological analyses

Cultured cod previtellogenic gonadal tissues were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 24 h. After fixation, the tissues were washed twice in 70% ethanol, dehydrated in a graded series of ethanol baths using an automatic tissue processor (Leica Microsystems, Nussloch, Germany), cleared in Tissue-Clear®, embedded in paraffin blocks, sectioned at 4 μM and stained with Mayer's haematoxylin and eosin. Tissues were examined using a workstation made with a microscope (Olympus BX-50, Tokyo, Japan) equipped with a complete rage of infinity-corrected objectives and a matching condenser, a microcator (Heidenhain MT-12, Traumrent, Germany) to control the movements in *z*-axis (accuracy 0.5 μM), a motorized stage (Prior, Fulbourm, UK) for stepwise displacement in *x*–*y* axis (accuracy 1 μM), and a CCD video camera (Sony, Tokyo, Japan) connected to a PC monitor (Sony). The whole system was controlled by the software Olympus CAST-Grid (version 1.5).

A classification system for previtellogenic cod oocytes was developed based on previous studies of oocyte development in tilapia (Rocha and Rocha, 2006) and cod (Kjesbu and Kryvi, 1989) and is presented in Table 2. For the stereological evaluation, tissue slides were analysed under the 20× objective lens. Volume fractions of the different stages of oocytes were calculated by stereological point-counting (Freere and Weibel, 1967). According to size and frequency of each oocyte stage, a particular two-lattice point grid was used; 1:4 and 1:6. The interpoint distances (in μm) for the sets were approximately: 77 (horizontal), 53 (vertical), for the 1:4 grid; and 51 (horizontal), 35 (vertical), for the 1:6 grid. The 1:4 point set of points was used to count hits over the reference space (ovary) *versus* oocytes of primary growth, whereas the 1:6 set of points was used to count hits over the reference space (ovary) *versus* all other oocyte classes and spermatozoa (when present). The first field of vision was selected randomly and thereafter, fields were systematically sampled by stepwise movements of the stage in *x*- and *y*-directions ($step_{x,y}$=600 μM). The grid points directly overlying structures were recorded. This procedure was repeated on a number of systematically chosen fields within tissue pieces exposed to different concentrations of 11-KT and T (n=3), in order to obtain a total of at least 300 recorded grid points per tissue piece, which is generally accepted in order to establish a variance of about 5% (Weibel, 1979). The same individual (T.M.K.) performed all the measurements and countings. The proportional volume fraction (V_V) (calculated as percentage) occupied by each stage of oocyte development was then estimated, according to the general formula: V_V (structure, reference)=V_V (s, r)=100×[P(s)]/[R×P(r)], in which V_V

Table 2
Classification of developmental stages of Atlantic cod previtellogenic oocytes

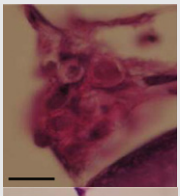
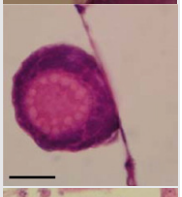
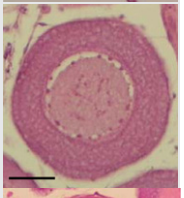
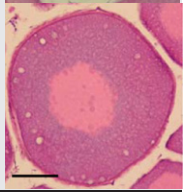
Previtellogenic oocyte developmental stage	Main cellular characteristics	Histological images
Proliferating oogonia	Small nests of mitotic cells dispersed within the connective tissue. Oogonia are small and circular, with a clear cytoplasm. Bar=10 μM.	
Initial primary growth	Primary oocytes had one large, usually peripheral nucleus. The oocyte is still attached to the connective tissue. The cytoplasm is strongly basophilic due to the high RNA concentration. Bar=10 μM.	
Primary previtellogenic growth	Primary oocytes showed one large, centrally located nucleus with several basophilic nuclei at the periphery. The cytoplasm stains also basophilic. Follicular cells are scarce and undifferentiated. Bar=15 μM.	
Advanced previtellogenic growth	The oocyte becomes a larger cell. The cytoplasm has more vesicles and becomes less basophilic due to a decreased RNA concentration. The follicular cells are appearing and begin differentiating. Bar=30 μM.	

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Table 3
Genes whose expression patterns were either up- or down-regulated by exposure of previtellogenic cod oocyte cultures to 11-ketotestosterone (11-KT) and testosterone (T), using targeted cod cDNA array (CodArray) analysis

10 μM 11-KT exposure at day 10 compared with 10 μM 11-KT exposure at day 5.	Log. ratio
Putative gene identity	
tRNA pseudouridine synthase A	0.95
H/ACA ribonucleoprotein complex subunit 2	0.69
NifU-like N-terminal domain containing protein	0.56
Cytochrome c oxidase polypeptide VIc precursor	0.52
Heat shock protein HSP 90-beta	−0.46

100 μM 11-KT exposure at day 10 compared with 100 μM 11-KT exposure at day 5.	Log. Ratio
Putative gene identity	
H/ACA ribonucleoprotein complex subunit 2	1.26
tRNA pseudouridine synthase A	0.78
PHD finger-like domain protein 5A	0.72
Cytochrome c oxidase polypeptide VIc precursor	0.57
CDKN2A interacting protein	0.62
Zona pellucida-like protein X1	0.60
40 S ribosomal protein S27	0.43
Cyclin-B	0.40
Sentrin-related protein	0.37
Apoptosis-inducing TAF9-like domain 1	−0.35
Heat shock protein HSP 90-beta	−0.43
40 S ribosomal protein S11	−0.53
Zona pellucida protein X2	−0.78
Egg envelope glycoprotein	−0.80
ZPA domain containing protein	−0.74

100 μM 11-KT exposure at day 10 compared with control at day 10.	Log. Ratio
Putative gene identity	
H/ACA ribonucleoprotein complex subunit 2	0.70
Zona pellucida-like protein X1	0.68
Cyclin-B	0.59
PHD finger-like domain protein 5A	0.49

10 μM T exposure at day 5 compared with control at day 5.	Log. Ratio
Putative gene identity	
ZPA domain containing protein	0.35
Zona pellucida protein X2	0.32
PHD finger-like domain protein 5A	−0.33

100 μM T exposure at day 5 compared with control at day 5.	Log. Ratio
Putative gene identity	
FK506-binding protein 3	0.29
Cytochrome c oxidase polypeptide VIc precursor	0.28
Zona pellucida protein X2	0.26
Heat shock protein HSP 90-beta	−0.32
PHD finger-like domain protein 5A	−0.37

100 μM T exposure at day 5 compared with 10 μM T exposure at day 5.	Log. Ratio
Putative gene identity	
Heat shock protein HSP 90-beta	−0.38
ZPA domain containing protein	−0.48
Heat shock protein HSP 70	−0.52
Zona pellucida protein X2	−0.54

10 μM T exposure at day 10 compared with 10 μM T exposure at day 5.	Log. ratio
Putative gene identity	
H/ACA ribonucleoprotein complex subunit 2	−0.32
Zona pellucida protein X2	−0.38

10 μM T exposure at day 10 compared with control at day 10.	Log. ratio
Putative gene identity	
ZPA domain containing protein	−0.33
Heat shock protein HSP 70	0.77
ZPA domain containing protein	0.65
Heat shock protein HSP 90-beta	0.54
Zinc finger CCHC domain containing protein 10	0.44
Ribonucleoside-diphosphate reductase M2	0.26
Cytochrome c oxidase polypeptide VIc precursor	−0.26
THAP domain containing protein 4	−0.28
Sentrin-related protein	−0.36

Each data point is derived from three biological replicates ($n=3$) and log2-transformed. Some data points are mean values of several redundant clones (see supporting information). All values represented are significantly different ($q<0.05$).

(s, r) is the percentage of the total volume of a reference space occupied by one "particle" within that space, $P(s)$ is the number of points over the "particles" of interest, R is the ratio of fine to coarse points (4 or 6), and $P(r)$ is the total number of points falling over the reference space.

2.13. Steroid hormone analysis

11-ketotestosterone concentrations were measured in gonad tissues cultured for 24 h using enzyme immunoassay (EIA) kits from Cayman Chemical Company (Ann Arbor, MI, USA). Tissues were thawed on ice, homogenized in 1:4 volume of 0.1 M Na-phosphate buffer and centrifuged at 14,000× g for 15 min. The supernatants were purified by extraction using organic solvent to prevent the interference of lipids and proteins in the analysis. Briefly, the supernatant was mixed with 4 ml diethyl ether using a vortex mixer. After phase separation, the aqueous portion was frozen in an ethanol/dry ice bath. The lipophilic phase was decanted into a clean tube, and the ether phase was evaporated by heating to 30 °C. The dry extract was reconstituted in 300 μL EIA buffer by vortexing. Enzyme immunoassay was run according to the manufacturer's protocol. Data were quantified against a standard curve that was linearized using a logit transformation of B/B0 (bound sample/maximum bound).

2.14. Statistical analysis

Statistics was performed with GraphPad Prism, version 2.1 (GraphPad Software Inc. 1996). Significant differences between control and exposure groups were performed using one-way ANOVA after testing for normality and variance homogeneity. Statistical differences between exposure groups were analyzed using the Tukey's Multiple Comparison Test. The level of significance was set at $p \leq 0.05$.

3. Results

3.1. SSH and array gene expression pattern

150 expressed sequences from the subtracted cDNA library were grouped into 54 UniGene entries (putative genes), and there were 23 contigs of two or greater (groups of clones probably representing overlapping regions) and 31 singletons. Table 3 shows a summary of genes whose expression patterns were significantly modulated after exposure to different concentrations of 11-KT and T in the array analysis. For complete lists of all genes, including unknown and redundant sequences, size of queries, Blast e-values and accession numbers, see the Supplemental data. The array analyses showed that exposure of previtellogenic oocytes to androgens produced differential gene expression patterns, showing 0.5–3.5-fold significant alterations of transcript levels from genes mainly of oocyte origin, as well as a number of transcripts whose function in fish are yet to be identified (see Supplemental data). Treatment with androgens produced modulations in mRNA levels of some zona radiata associated genes. Additionally, 11-

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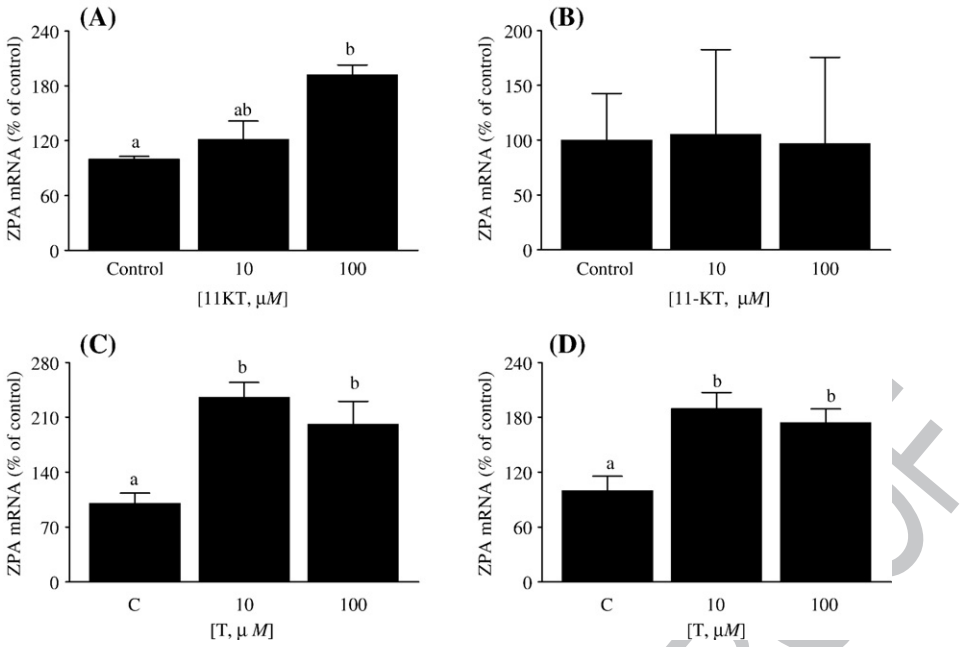

Fig. 1. Expression of ZPA domain containing protein (ZPA) mRNA levels in cultured previtellogenic oocytes of Atlantic cod exposed to 11-KT and T at 0 (control), 10 and 100 μM, and sampled at days 5 and 10 of exposure. Panels A and B, represent 11-KT exposure at days 5 and 10, respectively, while Panels C and D, represent T exposure at days 5 and 10 respectively. Data are given as mean expressed as percentage (%) of control (n=3)±standard error of the mean (SEM). Different letters denote exposure groups that are significantly different (p<0.05).

KT treatment was associated with an induced transcription of cyclin-B. The array data indicated that previtellogenic oocytes exposed to the highest 11-KT concentration (100 μM) experienced the strongest modulations of gene transcripts on the CodArray. Significant alterations of transcript levels for individual genes were usually observed in samples exposed both to the low (10) and high (100 μM) androgen concentrations. Furthermore, the array data demonstrate that gene expression patterns varied within a given exposure and/or time of exposure.

3.2. Real-time PCR (qPCR) analyses of selected transcripts

On the basis of previous studies and the results obtained from the array analysis, we selected five genes for quantitative analysis using real-time PCR with gene specific primer pairs; ZPA domain containing protein (ZPA), cyclin-B, heat shock protein 90β (Hsp90β), StAR and P450*scc*. The primary criterion for selecting these genes for real-time PCR validation was because they belong to our targeted study objectives, namely growth and development of previtellogenic oocytes and because they showed modulated expression patterns on the array analysis or in previous studies on our laboratory.

At day 5 of exposure, the expression of ZPA after 11-KT and T exposure showed an apparent concentration-dependent increase in mRNA expression (Fig. 1A and C, respectively). At day 10 of exposure, a different pattern of expression was observed for tissues exposed to 11-KT (Fig. 1B), showing no differences, either within the exposure groups or compared with the solvent control. On the contrary, exposure to T

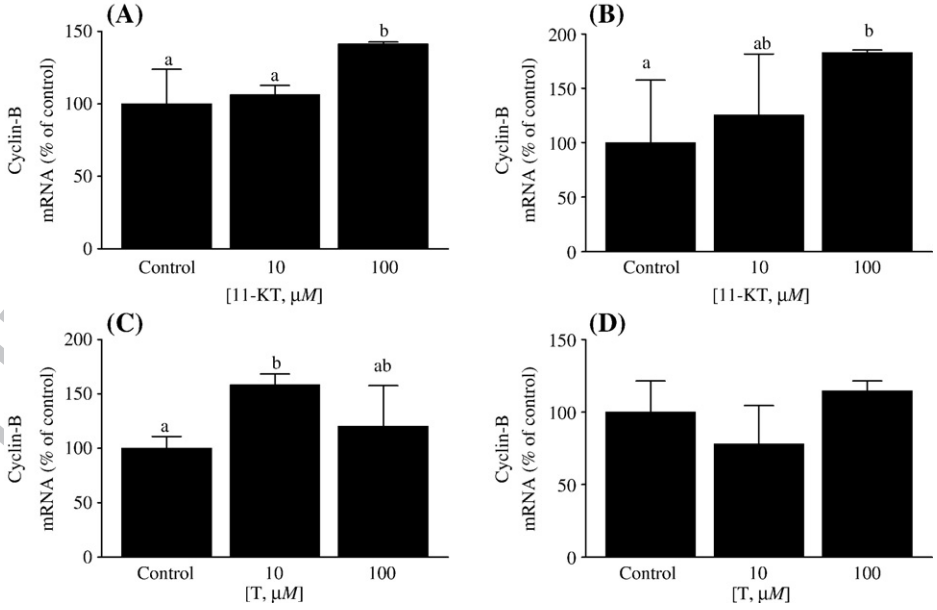

Fig. 2. Expression of cyclin-B mRNA levels in cultured previtellogenic oocytes of Atlantic cod exposed to 11-KT and T at 0 (control), 10 and 100 μM, and sampled at days 5 and 10 of exposure. Panels A and B, represent 11-KT exposure at days 5 and 10, respectively, while Panels C and D, represent T exposure at days 5 and 10 respectively. Data are given as mean expressed as percentage (%) of control (n=3)±standard error of the mean (SEM). Different letters denote exposure groups that are significantly different (p<0.05).

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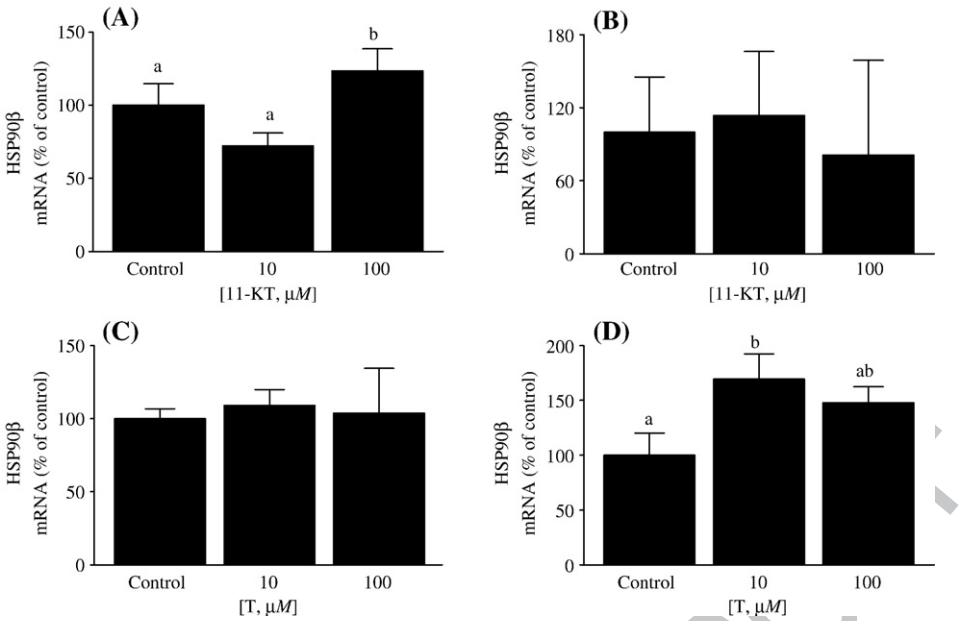

Fig. 3. Modulation of heat shock protein 90β (Hsp90β) mRNA levels in cultured previtellogenic oocytes of Atlantic cod exposed to 11-KT and T at 0 (control), 10 and 100 μM, and sampled at days 5 and 10 of exposure. Panels A and B, represent 11-KT exposure at days 5 and 10, respectively, while Panels C and D, represent T exposure at days 5 and 10 respectively. Data are given as mean expressed as percentage (%) of control ($n=3$) ± standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p<0.05$).

concentrations produced a respective 2-fold increase in ZPA expression at day 10 of exposure, compared to the solvent control (Fig. 1D).

The expression of cyclin-B in cod previtellogenic oocytes was modulated after exposure to different concentrations of 11-KT in a concentration-specific manner at days 5 and 10 of exposure (Fig. 2A and B, respectively). A general pattern of increase in cyclin-B mRNA expression was observed, reaching a 1.5- and 2-fold increase for groups exposed to the highest 11-KT concentration (100 μM) compared to the control at days 5 and 10, respectively. At day 5 of exposure, the expression pattern of cyclin-B in tissues exposed to T showed a 1.5-fold increase in the group exposed to 10 μM. No differences in expression of cyclin-B between groups exposed to T were observed at day 10 of exposure (Fig. 2D).

The expression of Hsp90β in cod previtellogenic oocytes was modulated after exposure to different concentrations of 11-KT and T at days 5 and 10 of exposure (Fig. 3). At day 5, 100 μM 11-KT produced a minor, but significant increase in Hsp90β expression (Fig. 3A). On the contrary, no significant differences between 11-KT exposure groups and solvent control were observed at day 10 of exposure (Fig. 3B). Tissues incubated with T showed no significant differences at day 5 of exposure, compared to the solvent control (Fig. 3C) and an apparent T concentration-dependent effect was observed at day 10 of exposure (Fig. 3D).

Quantification of StAR mRNA showed expression profiles for tissues exposed to 11-KT and T that were time- and concentration-specific responses. At day 5 of exposure, a 0.5-fold significant increase in StAR mRNA was observed for samples exposed to the highest 11-KT

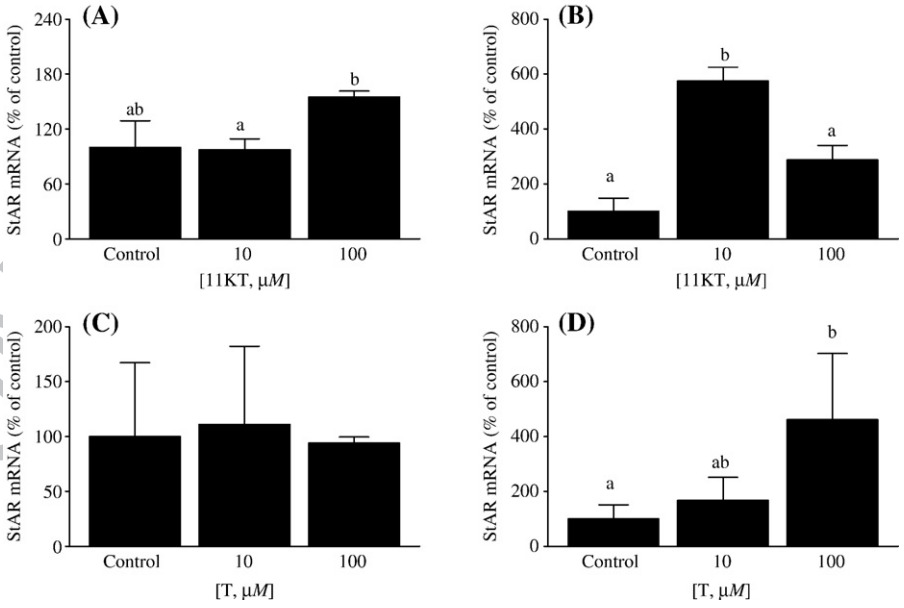

Fig. 4. Expression of StAR mRNA levels in cultured previtellogenic oocytes of Atlantic cod exposed to 11-KT and T at 0 (control), 10 and 100 μM, and sampled at days 5 and 10 of exposure. Panels A and B, represent 11-KT exposure at days 5 and 10, respectively, while Panels C and D, represent T exposure at days 5 and 10 respectively. Data are given as mean expressed as percentage (%) of control ($n=3$) ± standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p<0.05$).

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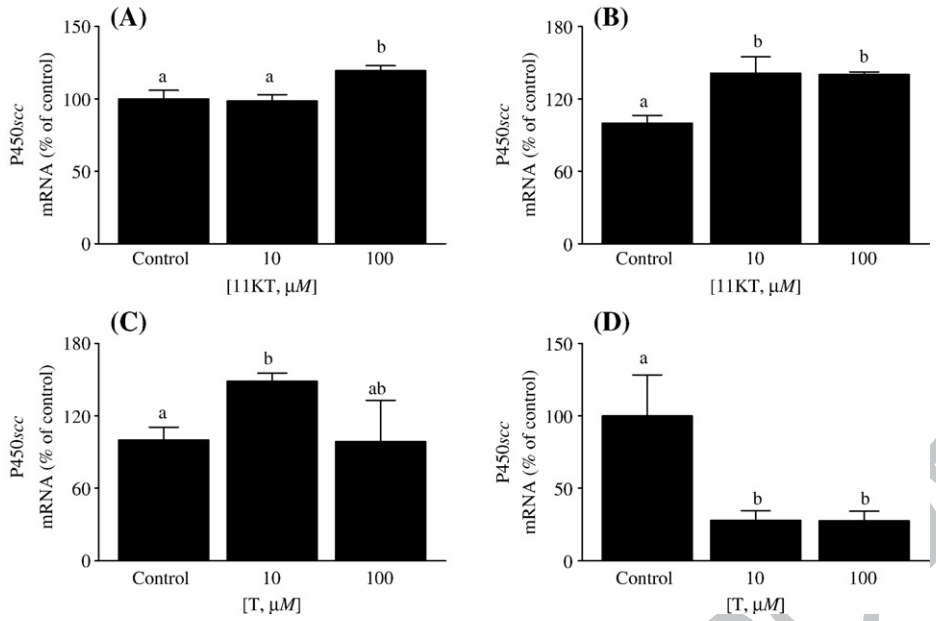

Fig. 5. Modulation of P450*scc* mRNA levels in cultured previtellogenic oocytes of Atlantic cod exposed to 11-KT and T at 0 (control), 10 and 100 μM, and sampled at days 5 and 10 of exposure. Panels A and B, represent 11-KT exposure at days 5 and 10, respectively, while Panels C and D, represent T exposure at days 5 and 10 respectively. Data are given as mean expressed as percentage (%) of control ($n=3$)±standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p<0.05$).

concentration (100 μM, Fig. 4A) whereas 11-KT displayed a concentration-specific modulation of StAR mRNA levels at 10 days cultivation (Fig. 4B) with 10 μM 11-KT producing the only significant increase compared the solvent control. No differences in StAR expression were observed for tissues exposed to T at day 5 (Fig. 4C). At day 10 of exposure, treatment with T produced a concentration-specific modulation of StAR levels, displaying a 4-fold increase for samples exposed to 100 μM, compared to control (Fig. 4D).

For P450*scc*, we observed a 0.2-fold significant increase of P450*scc* expression in tissues exposed to 100 μM 11-KT (Fig. 5A). A similar expression pattern was observed at day 10 of exposure, showing significant increase of P450*scc* mRNA levels for samples exposed to 10 and 100 μM 11-KT (Fig. 5B). At day 5 of exposure, an apparent concentration-dependent effect was observed, showing significant increase for samples exposed to 10 μM T, compared to control (Fig. 5C). Interestingly, tissues exposed to T produced a consistent decrease of P450*scc* expression at day 10 of exposure, showing 3-fold decreases for samples exposed to both T concentrations, compared to the control (Fig. 5D).

3.3. Histology and stereological analysis of cod oocyte growth and development

All the gonadal tissues examined displayed normal histology. The volume densities (%) of the different previtellogenic oocyte developmental stages in relation to total gonadal tissue volume in pieces exposed to different concentrations of 11-KT and T are presented in Tables 4 and 5, respectively. In addition, the volume fractions of oocytes of advanced previtellogenic growth are presented in Fig. 6. At day 5 of exposure, tissues exposed to 11-KT showed significantly higher volume fractions of oocytes in advanced growth phase as compared to the solvent control (Table 4 and Fig. 6A). A 2-fold increase in advanced oocyte volume densities was observed for tissues exposed to 10 μM 11-KT, whereas a 1.5-fold increase was observed for the group exposed to 100 μM 11-KT at day 5. Induced development was also observed, in a dose-dependent manner, in tissues exposed to T at day 5, where a 2- and 3.5-fold increase in volume fractions of oocytes in advanced previtellogenic growth phase was observed for tissues exposed to 10 and 100 μM T, respectively (Table 5 and Fig. 6C). Ovary areas with high density of spermatozoa were observed at day 5 of exposure for tissues exposed to both concentrations of 11-KT (10 and 100 μM) and the highest concentrations of T (100 μM). The volume densities of atretic oocytes in all exposure groups (both 11-KT and T) were generally 3–5%, showing no significant differences between control and androgen exposed tissues. Furthermore, the volume densities of oocytes in primary growth phase were by far the most abundant in all tissues, showing volume fractions between 28 and 50%. No significant differences in primary oocyte volume densities were observed between control and androgen exposed tissues.

At day 10 of exposure, tissues exposed to 11-KT (10 and 100 μM) showed significantly higher volume fractions of oocytes in advanced previtellogenic growth phase compared to the solvent control (Table 4

Table 4
Volume densities (%) of previtellogenic oocyte developmental stages in relation to total gonadal tissue volume ($n=3$) in tissues exposed to 11-ketotestosterone (11-KT)

11-ketotestosterone (μM)	Day 5			Day 10		
	Control	10	100	Control	10	100
Previtellogenic oocyte developmental stage						
1st growth	42±6	51±6	38±13	34±4	33±4	31±6
Advanced growth	1.2±1.3	2.8±1.2	1.8±0.9	4.9±1.8	12.8±5.3	13.6±5.2
1st atresia	5.0±1.3	4.2±1.2	3.2±1	1.8±0.4	1.2±0.9	1.0±0.3
2nd atresia	–	0.3±0.4	0.6±0.8	3.7±2.1	5.3±3.5	5.7±3
Spermatozoa	–	0.4±1	1.3±1	–	1.4±1.5	1.5±1.7

Data are expressed as mean±standard deviation.

Table 5
Volume densities (%) of previtellogenic oocyte developmental stages in relation to total gonadal tissue volume ($n=3$) in tissues exposed to testosterone (T)

Testosterone (μM)	Day 5			Day 10		
	Control	10	100	Control	10	100
Previtellogenic oocyte developmental stage						
1st growth	33±6	31±6	33±6	46±2	31±9	37±10
Advanced growth	1.7±0.9	3.5±1.8	5.8±1.7	6.3±1.5	14.4±2.4	11.4±2.0
1st atresia	3.6±0.8	4.4±1.5	3.3±0.9	2.1±0.6	1.8±0.6	1.5±0.5
2nd atresia	–	0.2±0.4	1.0±0.6	1.2±1	2.4±2.6	4.5±2.3
Spermatozoa	–	–	0.1±0.2	–	0.5±0.6	0.6±0.4

Data are expressed as mean±standard deviation

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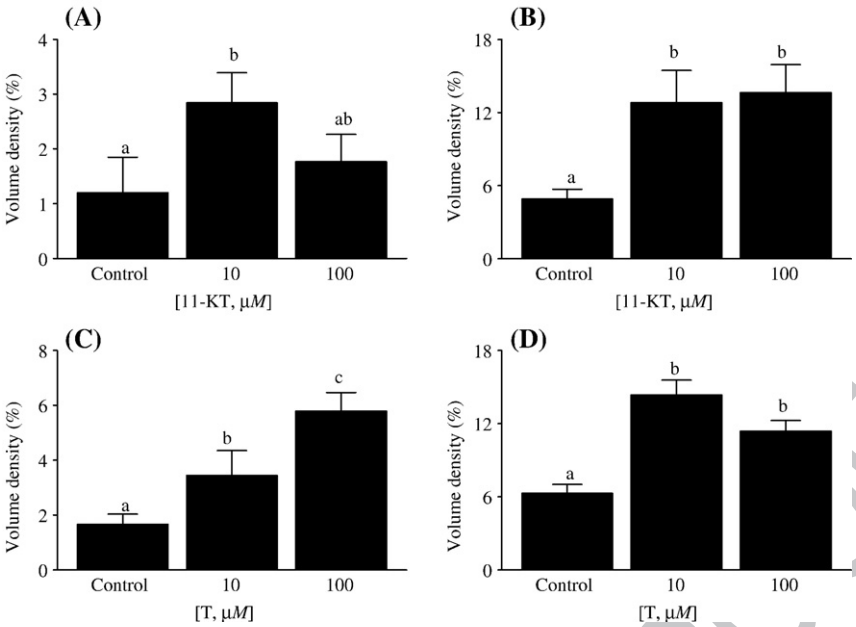

Fig. 6. Volume densities (%) of previtellogenic oocytes of advanced growth in cultured previtellogenic oocytes of Atlantic cod exposed to 11-ketotestosterone (11-KT) and testosterone (T) at 0 (control), 10 and 100 μM. Oocytes were sampled at days 5 (A; 11-KT, C; T) and 10 (B; 11-KT, D; T) of exposure. Data are given as mean expressed as percentage (%) of control (n=3)±standard error of the mean (SEM). Different letters denote exposure groups that are significantly different (p<0.05).

and Fig. 6B), displaying a 2.6- and 2.8-fold increase in advanced oocyte volume densities for tissues exposed to 10 and 100 μM 11-KT, respectively. The same pattern is also true for tissues exposed to T (Table 5 and Fig. 6D) showing a 2.3- and 1.8-fold increase in volume fractions of previtellogenic oocytes in advanced growth phase in tissues exposed to 10 and 100 μM T, respectively. Ovary areas with high density of spermatozoa were observed at day 10 of exposure for tissues exposed to all concentrations of 11-KT and T, with minor, but significantly higher volume fractions observed in tissues exposed to 11-KT as compared to tissues exposed to T. The volume densities of atretic oocytes showed no significant differences between control and androgen exposed tissues. However, a general shift from primary to secondary atretic oocytes was observed at day 10 of exposure, compared to day 5 of exposure.

3.4. In situ hybridization

A summary of cellular localization of mRNA levels using in situ hybridization of ZPA and zona pellucida protein X2 (ZPX2) RNA probes on tissues exposed to 11-KT and T are presented in Table 6 and some representative micrographs are shown in Fig. 7. At 5 days of exposure, a clear positive probe signal was revealed only in the control sections for both treatments (11-KT and T). For both treatments (11-KT; Fig. 7B and T; Fig. 8C), ZPA was highly expressed at 10 days of exposure as compared to control (Fig. 7A). In all cases, the signal was stronger for samples exposed to T than samples exposed to 11-KT. In positive slides, the sections were generally characterized by smaller oocytes showing high levels of ZPA mRNA expression. Larger oocytes showed relatively lower expression levels. The expression of ZPX2 was similar to ZPA expression (Table 6, micrographs not shown).

Table 6
Summary of in situ RNA probe expression

	11-KT				T			
	Control		100 μM		Control		100 μM	
	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10
ZPA	+	+	+/−	++	++	+++	+/−	++++
ZPX2	+	+	+/−	++	++	+++	+/−	++++

++++ Very strong signal; +++ strong signal; ++ moderate signal; + weak signal; +/− weak or no signal.

3.5. 11-KT tissue levels

In order to estimate androgen uptake by gonadal tissue cultures, we measured 11-KT concentrations in samples exposed to 0 (control), 10 and 100 μM 11-KT for 24 h (n=3). Solvent control tissues displayed a mean 11-KT concentration of 0.68 ng/mg tissue (Fig. 8). When compared with control samples, mean tissue 11-KT levels increased 10 and 30-fold after exposure to 10 and 100 μM 11-KT, respectively.

4. Discussion

Prior to overt morphological or developmental changes, the first interaction between a hormone and tissue occurs at the molecular and cellular levels of biological organization. Therefore, changes in gene expression as a result of hormonal exposure and the subsequent molecular processes that lead to developmental and physiological changes may be used as quantitative markers for cellular, physiological, developmental and reproductive effects in an organism. Herein, we show that gene expression patterns and the growth of cod previtellogenic gonadal tissues were modulated after exposure to androgens. Both 11-KT and T were capable of inducing previtellogenic oocyte growth and development, with 11-KT being the strongest modulator.

4.1. Evaluation of SSH and CodArray data

When genomic data for an organism is limited, such as the Atlantic cod, the SSH method can be a powerful tool for generating cDNA libraries containing DNA sequences of interest. The use of microarrays to screen cDNA clones generated by SSH also allows the identification of previously unknown genes—a finding that is not possible using conventional arrays of previously cloned genes (Yang et al., 1999). However, SSH usually generates a number of redundant clones in the subtracted sequences, and several genes suspected to be involved in the transcriptional regulation of oocyte growth and development

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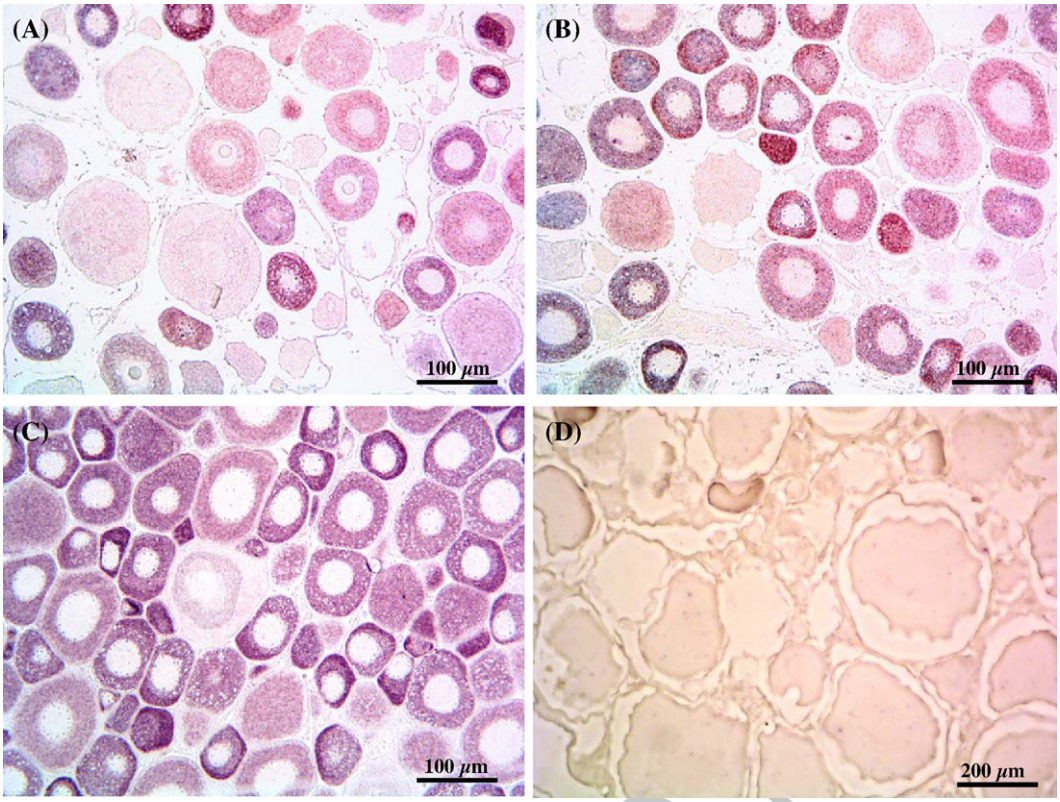

Fig. 7. Sections of cultured previtellogenic oocytes of Atlantic cod exposed to 11-ketotestosterone (11-KT) and testosterone (T) at 0 (control) and 100 μM in situ hybridized to ZPA domain containing protein (ZPA) RNA probes. A positive signal was obtained for control sections (A), samples exposed to 100 μM 11-KT (B) showed a stronger signal as compared to control, whereas an even stronger positive reaction was observed in samples exposed to 100 μM T (C). Negative control incubated with the sense probe (D) showed no signal. Note that Fig. 9 D has a 2× magnification compared with 9A, B and C, to visualize oocytes due to lack of signal.

were not represented after performing SSH in the present study. The reason for this is probably the low expression levels of these genes in cod ovarian tissue. In accordance, it is well known that SSH favours the enrichment of high abundance transcripts (Ji et al., 2002; Pan et al., 2006), which may result in over- or under-representation of certain transcripts in the subtracted library. Therefore, transcripts from some genes were amplified by PCR using specific primers from conserved regions of the respective genes based on sequence information in NCBI GenBank. The PCR products were cloned into *E. coli* plasmids, sequenced and subsequently added to the array.

Although the present study generated few sequences compared with other surveys, the number of gene profiles generated was already too high for suitable one by one analysis. Therefore, we performed an initial array screening with subsequent qPCR analyses of genes suspected to be involved in previtellogenic oocyte growth and development. After annotation of the sequenced clones, we found that the major part of the amplicons generated by SSH were of oocyte origin, whereas less abundant transcripts derived from ovarian follicle cells were likely not identified by the SSH method. In compliance, this has been observed earlier, and it has been proposed that construction of cDNA libraries enriched for extra-oocyte components such as the follicle wall will provide a better generation of transcripts from these ovarian components (Goetz et al., 2006; Luckenbach et al., 2008).

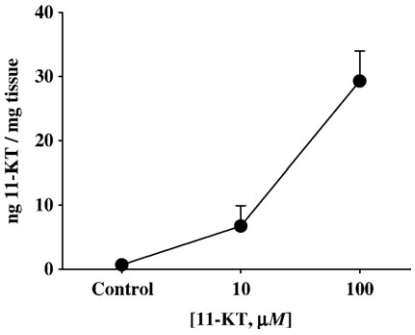

Fig. 8. 11-ketotestosterone (11-KT) levels in previtellogenic oocyte cultures of Atlantic cod incubated for 24 h with different 11-KT concentrations. 11-KT levels were determined using enzyme immunoassay method. Data are given as mean value and expressed as ng/mg tissue (wet weight) of $n=3\pm$standard error of the mean (SEM).

cDNA microarray techniques have some disadvantages compared to more tedious techniques such as qPCR. For example, they are limited in the detection of genes with low expression (Rondeau et al., 2005) and clones from less abundant mRNAs may fall within the 'noise' level of the hybridization signals (Yang et al., 1999). In addition, cDNA insert size may be a factor in reliably identifying differentially expressed genes, and short subtracted clones from the 5[prime]-end of cDNAs are less likely to give reliable hybridization signals than full-length cDNAs (Yang et al., 1999). In the present study, array screening failed to identify some genes (e.g. StAR and P450*scc*) that were shown to be differentially expressed after androgen treatment by qPCR analysis. Additionally, validation of array values by qPCR demonstrated differences in expression profiles for the two methods in some instances. These differences might be due to the mentioned limitations of microarray techniques. To conclude, we believe the microarray approach should be considered a first screening, and qPCR probably produced more accurate molecular signatures, at least in the present study.

4.2. Modulation of genes associated with the zona radiata

The present study demonstrated high levels of ZP transcripts in the previtellogenic Atlantic cod ovary using qPCR and in situ hybridization

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analyses. Although some data show conflicting patterns, a general increased mRNA expression after androgen treatment was observed. In vertebrates, the egg envelope, which is of great importance for the sperm–egg interaction, consists of a family of related ZP proteins with a conserved ZP domain. Very recent studies have demonstrated that teleost species have at least three isoforms of ZP proteins, ZP1 (ZPB), ZP3 (ZPC) and ZPX (Modig et al., 2006; Modig et al., 2007). However, the ZP nomenclature is confusing, and this complicates comparison between mammalian and fish ZP genes, as well as between different teleost species (Arukwe and Goksoyr, 2003). Several other studies have demonstrated abundant levels of ZP transcripts in teleost oocytes during previtellogenic growth, displaying higher ZP mRNA levels in the more undeveloped stages of oocyte growth (Chang et al., 1997; Luckenbach et al., 2008; Zeng and Gong, 2002). Elsewhere, while E2 regulation of ZP synthesis is a well known phenomenon, androgens have been suggested to regulate ZPs in fish (Miura et al., 1998) and birds (Pan et al., 2001). Although not conclusive, the present study suggests that androgens can modulate the transcriptional profile of ZP genes in the previtellogenic cod ovary, possibly in correlation with development of the oocyte envelope.

4.3. Effects on cyclin-B mRNA levels

Final oocyte maturation is triggered by the maturation-promoting factor (MPF), and MPF comprises two components; the catalytic subunit cdc2 and the regulatory subunit cyclin-B (Kondo et al., 1997; Morgan, 1995). Cyclin-B transcripts have been demonstrated in immature oocytes of goldfish (Nagahama, 1997) and zebrafish (Kondo et al., 2001) but they are not translated until later, when the oocyte reach the maturation phase. Other studies have suggested that the accumulation of cyclin-B transcripts in oocytes is involved in the regulation of the early embryonic cell cycle (Aegerter et al., 2004), and that expression of cyclin-B in previtellogenic oocytes indicates somatic follicle cells undergoing mitotic divisions (von Schalburg et al., 2005). The present study demonstrates high levels of cyclin-B mRNA in the previtellogenic cod ovary, and we show that natural occurring androgens are able to increase cyclin-B transcription levels. Gene expression patterns of cyclin-B have been suggested as markers for early oocyte growth and developmental competence in teleost fish (Aegerter et al., 2004), but more studies assessing the regulation of cyclin-B transcription levels in specific oocyte developmental stages are needed in order to support this suggestion.

4.4. Modulation of heat shock protein 90β expression

Heat shock proteins are a family of highly conserved intracellular proteins found in all organisms (Lindquist and Craig, 1988). Despite the fact that heat shock proteins are primitive and act to protect the cell against the cytotoxic consequences of protein degradation, a number of heat shock genes are expressed at high levels during normal cell growth (Morimoto et al., 1994). The expression of heat shock genes in teleost embryonic development have been investigated (Krone et al., 2003), as well as heat shock protein expression during oogenesis, where it has been shown that growing oocytes spontaneously express high levels of heat shock protein 70 and 90 (Neuer et al., 1999). Extensive molecular characterization and biochemical studies have demonstrated that vertebrate members of Hsp90 family play a post-translational regulatory role within the cell by interacting with several important cellular signalling molecules and transcription factors, such as steroid receptors, and modulating their activities (Krone et al., 1997). A recent large-scale genomic study describing expression profiles of zebrafish testis and ovary demonstrated that Hsp90 was the most abundant transcript in testis, whereas Hsp90 showed a much lower expression level in the ovary (Zeng and Gong, 2002). In contrast, this study demonstrated that Hsp90β was among the most abundant transcripts in the previtellogenic cod ovary, and 11-KT and T produced increased Hsp90β mRNA levels, albeit showing time-dependent expression patterns. Thus, it seems likely that Hsp90β plays an important role in oocyte development, at least in Atlantic cod.

4.5. Modulation of StAR and P450scc mRNA levels

The rate-limiting step in steroidogenesis is the movement of cholesterol across the mitochondrial membrane by the StAR protein and the subsequent conversion to pregnenolone by the P450*scc* enzyme (Stocco, 2001). In mammals, StAR and P450*scc* are rapidly synthesized in response to acute tropic hormone stimulation and cAMP, and in general, agents that increase steroid biosynthesis also increase StAR mRNA expression (Stocco, 2001). However, current knowledge about StAR is mostly based on mammalian studies, whereas the relationship between StAR gene expression and steroid production in fish is not very well described (Hagen et al., 2006; Stocco et al., 2005). Interestingly, StAR expression data suggest that the molecular mechanisms of StAR synthesis and regulation in fish may differ from what has been previously observed in mammals (Goetz et al., 2004). The gene expressions of StAR presented in the present study show more than a 4-fold increase in StAR mRNA compared to control at day 10 of exposure for samples exposed to 10 μM 11-KT and 100 μM T. Additionally, we observed differential expression patterns of StAR and P450*scc*. For example, samples exposed to T showed a general increase in StAR mRNA levels at day 10 of exposure, whereas a consistent decrease in P450*scc* mRNA expression was observed at the same time interval. These findings are in contrast with a previous study by Geslin and Auperin (2004), where a positive correlation between the expression profiles of StAR and P450*scc* was observed, suggesting a similar mechanism of transcriptional regulations for these two genes in teleosts. The different effects of 11-KT and T are generally explained by the possible aromatization of T to E2, that will subsequently reduce cellular levels of T (Lokman et al., 1998). However, a previous *in vitro* study by Mori et al. (2000) demonstrated an almost ignorable conversion of T to E2 in primary cultures of immature male rainbow trout hepatocytes. In addition, HPLC studies showed that T is metabolized mainly into testosterone-glucuronide and androstenedione in these cells (Cravedi et al., 1996). While our study demonstrates that 11-KT and T gave rise to different expression patterns of transcripts directly involved in steroidogenesis, additional studies are needed to determine the precise role of aromatizable and non-aromatizable androgens in the regulation of ovarian steroidogenic genes and proteins in teleosts.

4.6. Histology and stereological analysis of the growth and development of cod oocytes

The volume fractions occupied by oocytes of different developmental classes in the previtellogenic cod ovary were modulated after exposure to androgens. In general, both 11-KT and T produced previtellogenic oocyte growth and development, with 11-KT comparatively being the strongest modulator. Relevant to small preantral follicles in mammals, that resemble fish previtellogenic oocytes (given that growth progresses slowly occurring both before and after puberty (Hsueh et al., 2000) and appearing to be mostly gonadotrophin-independent), androgens has been implicated in mammalian oogenesis (McGee, 2000). For example, growing preantral and small antral follicles were significantly increased in number, and granulosa and thecal cell proliferation increased in androgen treated rhesus monkey (Vendola et al., 1998). Similarly, testosterone-treated women or those suffering from androgen excess (polycystic ovarian syndrome, androgen-producing tumors) showed increased number of growing follicles (Vercellini et al., 1993). Some of these effects may be indirect via surrounding stromal tissue (Vendola et al., 1999). Direct androgen effects on ovarian follicles are very likely since androgen receptor (AR) mRNA abundance in rhesus monkey was highest in preantral to small

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antral follicles (Weil et al., 1998). Androgens also modify the intra-ovarian gene expression in the rhesus monkey, as demonstrated by increased mRNA abundance of insulin-like growth factor-1 (IGF-1) and IGF-1-receptor (Vendola et al., 1999) in follicles up to early antral stage. Except for the evidence that androgens increase oocyte "diameter" and modify growth factor mRNA abundance, the underlying molecular mechanism(s) involved in possible gene regulation resulting in the growth of previtellogenic oocytes has not been investigated, and have been addressed in the present study.

The present study shows a consistent stereological validation of oocyte growth and development after exposure to androgens. Tissues exposed to 11-KT and T showed significantly higher volume fractions of previtellogenic oocytes of advanced growth as compared to the solvent control. A second stereological approach was employed in the present study in order to investigate the mean volume of previtellogenic oocytes of different developmental classes. This method is referred to as the Point Sampled Intercept (PSI), and allows an unbiased estimation of the mean volume and variability of particles of arbitrary shape (Gundersen and Jensen, 1985). PSI analysis of cod previtellogenic ovary tissues exposed to 11-KT and T showed similar patterns as observed by the previous discussed method of volume fractions, demonstrating an apparent increase in oocyte volume after androgen exposure (data not shown). All stereological studies require representative sampling of the organ and generation of adequate sectioning of the structures to be analyzed (Mandarim-de-Lacerda, 2003). The sampling and arbitrary sectioning of the (naturally roundish) oocytes granted the isotropy requirements (Mandarim-de-Lacerda, 2003). Also, previous studies have shown that oocyte size distribution is homogenous in cod ovaries (Kjesbu and Holm, 1994). Finally, all the gonadal tissues examined in the present study displayed a normal histology. The volume densities of atretic oocytes were generally 3–5%, showing no significant differences between control and androgen exposed tissues. Although it has been shown that atresia occurs in oocyte of all developmental classes, very little is known about atresia during early stages of oocyte development (Tyler and Sumpter, 1996). Oocyte areas with a high density of spermatozoa were observed at both day 5 and day 10 of exposure for tissues exposed to 11-KT and T, with higher volume fractions observed in tissues exposed to 11-KT compared to tissues exposed to T. The appearance of spermatozoa in cultivated ovarian tissue is puzzling, and the possibility that sex reversal was occurring in cultivated ovarian tissue exposed to androgens should not be excluded. If sex reversal was occurring within 5 days of *in vitro* androgen exposure, one would think that less mature stages of male germ cells (i.e. spermatogonia and spermatocytes) could be observed at earlier time points, at least in some sections. However, it should also be noted that not all androgen exposed ovary tissue samples examined contained spermatozoa. Previous studies addressing the effects of 11-KT on previtellogenic ovarian growth have demonstrated the presence of male germ cells in androgen treated female fish *in vivo* (Rohr et al., 2001) but not *in vitro* (Lokman et al., 2007).

4.7. Evaluation of in vitro floating agarose method

From an endocrine and reproductive physiology point of view, these findings represent an interesting aspect of the role of androgens on previtellogenic oocyte growth in teleosts. However, we do acknowledge that the nominal 11-KT and T concentrations (10 and 100 μM) used in the present study are several orders of magnitude higher than physiological androgen levels reported in fish. In the present study, plasma levels of 11-KT in the female fish used for the *in vitro* gonadal incubation was 1±0.5 ng/ml of $n=4$ individuals. To our knowledge, there are no previous studies that have measured physiological androgen levels in previtellogenic stages of cod, although a few studies have measured variable T levels in cod during later stages of oocyte development (Dahle et al., 2003; Kjesbu et al., 1996). In a study by Lokman et al. (2002), it was demonstrated that the presence of both 11-KT and T in females for a number of teleost species were generally at low concentrations (<1 ng/ml). In planning and designing this study, we hypothesized that only a fraction of the given androgen concentrations will be accessible to the oocytes using the *in vitro* floating agarose method after considering biological factors such as bioavailability and bioconcentration. This hypothesis was supported by EIA analyses of 11-KT concentration in tissue cultures used in the present study (see Fig. 8). Solvent control tissues displayed a mean 11-KT concentration of 0.68 ng/mg tissue (wet weight), whereas mean 11-KT concentration increased 10 and 30-fold when exposed to 11-KT at the nominal concentrations of 10 and 100 μM, respectively. These measurements were conducted 24 h post-exposure, and steroid concentrations probably decreased rapidly during the next 4 exposure days, before fresh media with 11-KT was added to the cultures (i.e. at day 5). A rapid decrease in plasma steroid hormone levels has been reported previously during *in vivo* steroid injection of fathead minnow (Korte et al., 2000; Pakdel et al., 1991). Thus, we estimate that the low androgen concentration used in the present study (10 μM) represents a physiological relevant concentration, whereas the high concentration (100 μM) probably represents an extreme *in vivo* situation.

Furthermore, in a separate study using 1 and 1000 μM 11-KT and T (i.e. one magnitude lower and higher of the concentrations used in the present study), we found very modest differences in the studied variables (Kortner et al. submitted). In addition, quantitative histological analyses showed a consistent stereological validation of oocyte growth after exposure to androgens both when exposed to 10 and 100 μM (this study) or 1 and 1000 μM (Kortner et al. submitted). The modest differences between androgen concentrations observed in our studies may be explained by the fact that steroid hormone effects on cellular processes are regulated by auto-regulatory mechanisms and functional alterations will occur when threshold levels are reached irrespective of administered concentration. This assumption is further supported by the fact that histological analyses of all tissues did not show any toxicity related differences between control and 11-KT or T exposed samples when exposed to 1, 10, 100 or 1000 μM. In accordance, previous *in vitro* studies have used comparable androgen concentrations used in the present study (Braun and Thomas, 2003; Mori et al., 1998). Nevertheless, an *in vitro* system lacks the absolute metabolic competence of an intact organism and our finding does not directly reflect an *in vivo* condition. However, the findings in the present study represent some novel aspects of the roles of androgens on the early growth of previtellogenic oocytes that recently has been validated *in vivo* in our laboratory using physiological androgen levels (Kortner et al. in preparation).

5. Conclusions

The present study provides the identification, sequencing and the expression patterns of a number of androgen-responsive genes in the previtellogenic cod ovary. We present the first targeted cod array (CodArray), consisting of clones with unique expression patterns that were either up- or down-regulated after androgen exposure using an *in vitro* previtellogenic oocyte culture technique with subsequent SSH analysis. Quantitative real-time polymerase chain reactions demonstrated the changes in expression for selected genes that are believed to be involved in the growth and development of previtellogenic oocytes in the Atlantic cod. Furthermore, we show that androgens promote oocyte growth and development in cod previtellogenic gonadal tissues. We present a consistent stereological validation of oocyte growth and development after exposure to androgens. In general, both 11-KT and T are capable of inducing previtellogenic oocyte growth and development, with the former hormone being the strongest modulator. Although not conclusive, the demonstration of previtellogenic oocyte growth and development after androgen exposure and the related differential expression of a number of androgen-responsive genes reveal novel roles of androgens concerning

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the maturation and development of previtellogenic oocytes, indicating androgen control of early follicular and oocyte growth in the cod ovary.

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Appendix A. Supplemental data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbd.2008.04.001.

References

Abraham, M., Hilge, V., Lison, S., Tibika, H., 1984. The cellular envelope of oocytes in teleosts. Cell Tissue Res. 235, 403–410.

Aegerter, S., Jalabert, B., Bobe, J., 2004. Messenger RNA stockpile of cyclin B, insulin-like growth factor I, insulin-like growth factor II, insulin-like growth factor receptor Ib, and p53 in the rainbow trout oocyte in relation with developmental competence. Mol. Reprod. Dev. 67, 127–135.

Arukwe, A., 2006. Toxicological housekeeping genes: do they really keep the house? Environ. Sci. Technol. 40, 7944–7949.

Arukwe, A., Goksoyr, A., 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: Oogenetic, population, and evolutionary implications of endocrine disruption. Comp. Hepatol. 2 (1), 4.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J. R. Stat. Soc., Ser. B 47, 289–300.

Bieniarz, K., Kime, D.E., 1986. Autoradiographic localization of gonadotrophin receptors in ovaries of the common carp, *Cyprinus carpio* L. Gen. Comp. Endocrinol. 64, 151–156.

Borg, B., 1994. Androgens in teleost fishes. Comp. Biochem Physiol., C 109, 219–245.

Braun, A.M., Thomas, P., 2003. Androgens inhibit estradiol-17beta synthesis in Atlantic croaker (*Micropogonias undulatus*) ovaries by a nongenomic mechanism initiated at the cell surface. Biol. Reprod. 69, 1642–1650.

Bromage, N., Jones, J., Randall, C., Thrush, M., Davies, B., Springate, J., Duston, J., Barker, G., 1992. Broodstock management, fecundity, egg quality and the timing of egg production in the rainbow trout (*Oncorhynchus mykiss*). Aquaculture 100, 141–166.

Caussanel, C., Breuzet, M., 1977. [Hormonal control of the ovarian cycle in *Labidura riparia* (author's transl)]. Ann. Endocrinol. (Paris) 38, 403–404.

Chan, L., Means, A.R., O'Malley, B.W., 1978. Steroid hormone regulation of specific gene expression. Vitam. Horm. 36, 259–295.

Chang, Y.S., Hsu, C.C., Wang, S.C., Tsao, C.C., Huang, F.L., 1997. Molecular cloning, structural analysis, and expression of carp ZP2 gene. Mol. Reprod. Dev. 46, 258–267.

Cravedi, J.P., Paris, A., Monod, G., Devaux, A., Flouriot, G., Valotaire, Y., 1996. Maintenance of cytochrome *P450* content and phase I and phase II enzyme activities in trout hepatocytes cultured as spheroidal aggregates. Comp. Biochem Physiol., C 113, 241–246.

Dahle, R., Taranger, G.L., Karlsen, O., Kjesbu, O.S., Norberg, B., 2003. Gonadal development and associated changes in liver size and sexual steroids during the reproductive cycle of captive male and female Atlantic cod (*Gadus morhua* L.). Comp. Biochem Physiol., A 136, 641–653.

Freere, R.H., Weibel, E.R., 1967. Stereological techniques in microscopy. J. R. Microsc. Soc. 87, 25–34.

Geslin, M., Auperin, B., 2004. Relationship between changes in mRNAs of the genes encoding steroidogenic acute regulatory protein and P450 cholesterol side chain cleavage in head kidney and plasma levels of cortisol in response to different kinds of acute stress in the rainbow trout (Oncorhynchus mykiss). Gen. Comp. Endocrinol. 135, 70–80.

Goetz, F.W., Norberg, B., McCauley, L.A., Iliev, D.B., 2004. Characterization of the cod (*Gadus morhua*) steroidogenic acute regulatory protein (StAR) sheds light on StAR gene structure in fish. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 137, 351–362.

Goetz, F.W., McCauley, L.A., Goetz, G.W., Norberg, B., 2006. Using global genome approaches to address problems in cod mariculture. ICES J. Mar. Sci. 63, 393–399.

Gundersen, H.J., Jensen, E.B., 1985. Stereological estimation of the volume-weighted mean volume of arbitrary particles observed on random sections. J. Microsc. 138, 127–142.

Hagen, I.J., Kusakabe, M., Young, G., 2006. Effects of ACTH and cAMP on steroidogenic acute regulatory protein and P450 11beta-hydroxylase messenger RNAs in rainbow trout interrenal cells: Relationship with *in vitro* cortisol production. Gen. Comp. Endocrinol. 145, 254–262.

Ho, S.M., Taylor, S., Callard, I.P., 1982. Effect of hypophysectomy and growth hormone on estrogen-induced vitellogenesis in the freshwater turtle, *Chrysemys picta*. Gen. Comp. Endocrinol. 48, 254–260.

Hsueh, A.J., McGee, E.A., Hayashi, M., Hsu, S.Y., 2000. Hormonal regulation of early follicle development in the rat ovary. Mol. Cell. Endocrinol. 163, 95–100.

Ikeuchi, T., Todo, T., Kobayashi, T., Nagahama, Y., 1999. cDNA cloning of a novel androgen receptor subtype. J. Biol. Chem. 274, 25205–25209.

Ji, W., Wright, M.B., Cai, L., Flament, A., Lindpaintner, K., 2002. Efficacy of SSH PCR in isolating differentially expressed genes. BMC Genomics 3 (1), 12.

Kjesbu, O.S., Kryvi, H., 1989. Oogenesis in cod, *Gadus morhua* L., studied by light and electron microscopy. J. Fish. Biol. 34, 735–746.

Kjesbu, O.S., Holm, J.C., 1994. Oocyte recruitment in first-time spawning Atlantic cod (*Gadus morhua*) in relation to feeding regime. Can. J. Fish Aquat. Sci. 51, 1893–1898.

Kjesbu, O.S., Kryvi, H., Norberg, B., 1996. Oocyte size and structure in relation to blood plasma steroid hormones in individually monitored, spawning Atlantic cod. J. Fish. Biol. 49, 1197–1215.

Kondo, T., Yanagawa, T., Yoshida, N., Yamashita, M., 1997. Introduction of cyclin B induces activation of the maturation-promoting factor and breakdown of germinal vesicle in growing zebrafish oocytes unresponsive to the maturation-inducing hormone. Dev. Biol. 190, 142–152.

Kondo, T., Kotani, T., Yamashita, M., 2001. Dispersion of cyclin B mRNA aggregation is coupled with translational activation of the mRNA during zebrafish oocyte maturation. Dev. Biol. 229, 421–431.

Korte, J.J., Kahl, M.D., Jensen, K.M., Pasha, M.S., Parks, L.G., LeBlank, G.A., Ankley, G.T., 2000. Fathead minnow vitellogenin: complementary DNA sequence and messenger RNA and protein expression after 17B-estradiol treatment. Environ. Toxicol. Chem. 19, 972–981.

Kortner, T.M., Arukwe, A., 2007a. Effects of 17alpha-methyltestosterone exposure on steroidogenesis and cyclin-B mRNA expression in previtellogenic oocytes of Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 146, 569–580.

Kortner, T.M., Arukwe, A., 2007b. The xenoestrogen, 4-nonylphenol, impaired steroidogenesis in previtellogenic oocyte culture of Atlantic cod (*Gadus morhua*) by targeting the StAR protein and P450scc expressions. Gen. Comp. Endocrinol. 150, 419–429.

Krone, P.H., Sass, J.B., Lele, Z., 1997. Heat shock protein gene expression during embryonic development of the zebrafish. Cell. Mol. Life Sci. 53, 122–129.

Krone, P.H., Evans, T.G., Blechinger, S.R., 2003. Heat shock gene expression and function during zebrafish embryogenesis. Semin. Cell Dev. Biol. 14, 267–274.

Leatherland, J.F., Copeland, P., Sumpter, J.P., Sonstegard, R.A., 1982. Hormonal control of gonadal maturation and development of secondary sexual characteristics in coho salmon, *Oncorhynchus kisutch*, from lakes Ontario, Erie, and Michigan. Gen. Comp. Endocrinol. 48, 196–204.

Lindquist, S., Craig, E.A., 1988. The heat-shock proteins. Annu. Rev. Genet. 22, 631–677.

Lokman, P.M., Vermeulen, G.J., Lambert, J.G.D., Young, G., 1998. Gonad histology and plasma steriod profiles in wild New Zealand freshwater eels (*Anguilla dieffenbachii* and *A. australis*) before and at the onset of the natural spawning migration. I. Females. Fish Physiol. Biochem. 19, 325–338.

Lokman, P.M., Harris, B., Kusakabe, M., Kime, D.E., Schulz, R.W., Adachi, S., Young, G., 2002. 11-Oxygenated androgens in female teleosts: prevalence, abundance, and life history implications. Gen. Comp. Endocrinol. 129, 1–12.

Lokman, P.M., George, K.A., Divers, S.L., Algie, M., Young, G., 2007. 11-Ketotestosterone and IGF-I increase the size of previtellogenic oocytes from shortfinned eel, *Anguilla australis*, *in vitro*. Reproduction 133, 955–967.

Luckenbach, J.A., Iliev, D.B., Goetz, F.W., Swanson, P., 2008. Identification of differentially expressed ovarian genes during primary and early secondary oocyte growth in coho salmon, *Oncorhynchus kisutch*. Reprod. Biol. Endocrinol. 6 (1), 2.

MacKenzie, D.S., VanPutte, C.M., Leiner, K.A., 1998. Nutrient regulation of endocrine function in fish. Aquaculture 161, 3–25.

Mandarim-de-Lacerda, C.A., 2003. Stereological tools in biomedical research. An. Acad. Bras. Cienc. 75, 469–486.

McGee, E.A., 2000. The regulation of apoptosis in preantral ovarian follicles. Biol. Signals Recept. 9, 81–86.

Miura, T., Kudo, N., Miura, C., Yamauchi, K., Nagahama, Y., 1998. Two testicular cDNA clones suppressed by gonadotropin stimulation exhibit ZP2- and ZP3-like structures in Japanese eel. Mol. Reprod. Dev. 51, 235–242.

Modig, C., Modesto, T., Canario, A., Cerda, J., von Hofsten, J., Olsson, P.E., 2006. Molecular characterization and expression pattern of zona pellucida proteins in gilthead seabream (*Sparus aurata*). Biol. Reprod. 75, 717–725.

Modig, C., Westerlund, L., Olsson, P.E., 2007. Oocyte Zona pellucida proteins. In: Babin, P.J., Cerda, J., Lubzens, E. (Eds.), The Fish Oocyte: From Basic Studies to Biotechnological Applications. Springer, pp. 113–139.

Mommsen, T.P., Walsh, P.J., 1988. Vitellogenesis and oocyte assembly. Fish Physiol. 12A, 347–406.

Morel, G., Cavalier, A., 2001. In Situ Hybridization in Light Microscopy. CRC Press LLC, USA.

Morgan, D.O., 1995. Principles of CDK regulation. Nature 374 (6518), 131–134.

Mori, T., Matsumoto, H., Yokota, H., 1998. Androgen-induced vitellogenin gene expression in primary cultures of rainbow trout hepatocytes. J. Steroid Biochem. Mol. Biol. 67, 133–141.

Mori, T., Sumiya, S., Yokota, H., 2000. Electrostatic interactions of androgens and progesterone derivatives with rainbow trout estrogen receptor. J. Steroid Biochem. Mol. Biol. 75, 129–137.

Morimoto, R.I., Tissieres, A., Georgopoulos, C., 1994. The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor Laboratory Press, New York.

Nagahama, Y., 1997. 17 alpha,20 beta-dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: mechanisms of synthesis and action. Steroids 62, 190–196.

Neuer, A., Spandorfer, S.D., Giraldo, P., Jeremias, J., Dieterle, S., Korneeva, I., Liu, H.C., Rosenwaks, Z., Witkin, S.S., 1999. Heat shock protein expression during gametogenesis and embryogenesis. Infect. Dis. Obstet. Gynecol. 7, 10–16.

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Olsson, P.E., Berg, A.H., von Hofsten, J., Grahn, B., Hellqvist, A., Larsson, A., Karlsson, J., Modig, C., Borg, B., Thomas, P., 2005. Molecular cloning and characterization of a nuclear androgen receptor activated by 11-ketotestosterone. Reprod. Biol. Endocrinol. 3, 37.

Pakdel, F., Feon, S., Le Gac, F., Le Menn, F., Valotaire, Y., 1991. In vivo estrogen induction of hepatic estrogen receptor mRNA and correlation with vitellogenin mRNA in rainbow trout. Mol. Cell Endocrinol. 75, 205–212.

Pan, J., Sasanami, T., Kono, Y., Matsuda, T., Mori, M., 2001. Effects of testosterone on production of perivitelline membrane glycoprotein ZPC by granulosa cells of Japanese quail (*Coturnix japonica*). Biol. Reprod. 64, 310–316.

Pan, Y.S., Lee, Y.S., Lee, Y.L., Lee, W.C., Hsieh, S.Y., 2006. Differentially profiling the low-expression transcriptomes of human hepatoma using a novel SSH/microarray approach. BMC Genomics 7, 131.

Rocha, M.J., Rocha, E., 2006. Morphofunctional aspects of reproduction from synchronous to asynchronous fishes—an overview. In: Reinecke, M., Zaccone, G., Kapoor, B.G. (Eds.), Fish Endocrinol. 571–624.

Rohr, D.H., Lokman, P.M., Davie, P.S., Young, G., 2001. 11-Ketotestosterone induces silvering-related changes in immature female short-finned eels, *Anguilla australis*. Comp. Biochem. Physiol., A 130, 701–714.

Rondeau, G., McClelland, M., Nguyen, T., Risques, R., Wang, Y., Judex, M., Cho, A.H., Welsh, J., 2005. Enhanced microarray performance using low complexity representations of the transcriptome. Nucleic Acids Res. 33 (11), e100.

Slater, C.H., Schreck, C.B., Swanson, P., 1994. Plasma profiles of the sex steroids and gonadotropins in maturing female spring chinook salmon (*Oncorhynchus tshawytscha*). Comp. Biochem. Physiol., A. 109, 167–175.

Smyth, G.K., 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3 Article3.

Sperry, T.S., Thomas, P., 1999a. Characterization of two nuclear androgen receptors in Atlantic croaker: Comparison of their biochemical properties and binding specificities. Endocrinology 140, 1602–1611.

Sperry, T.S., Thomas, P., 1999b. Identification of two nuclear androgen receptors in kelp bass (*Paralabrax clathratus*) and their binding affinities for xenobiotics: Comparison with Atlantic croaker (*Micropogonias undulatus*) androgen receptors. Biol. Reprod. 61, 1152–1161.

Sperry, T.S., Thomas, P., 2000. Androgen binding profiles of two distinct nuclear androgen receptors in Atlantic croaker (*Micropogonias undulatus*). J. Steroid Biochem. Mol. Biol. 73, 93–103.

Steele, B.K., Meyers, C., Ozbun, M.A., 2002. Variable expression of some "housekeeping" genes during human keratinocyte differentiation. Anal. Biochem. 307, 341–347.

Stocco, D.M., 2001. StAR protein and the regulation of steroid hormone biosynthesis. Annu. Rev. Physiol. 63, 193–213.

Stocco, D.M., Wang, X., Jo, Y., Manna, P.R., 2005. Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. Mol. Endocrinol. 19, 2647–2659.

Storey, J.D., 2002. A direct approach to false discovery rates. J. R. Stat. Soc., Ser. B 64, 479–498.

Takeo, J., Yamashita, S., 1999. Two distinct isoforms of cDNA encoding rainbow trout androgen receptors. J. Biol. Chem. 274, 5674–5680.

Todo, T., Ikeuchi, T., Kobayashi, T., Nagahama, Y., 1999. Fish androgen receptor: cDNA cloning, steroid activation of transcription in transfected mammalian cells, and tissue mRNA levels. Biochem. Biophys. Res. Commun. 254, 378–383.

Touhata, K., Kinoshita, M., Tokuda, Y., Toyohara, H., Sakaguchi, M., Yokoyama, Y., Yamashita, S., 1999. Sequence and expression of a cDNA encoding the red seabream androgen receptor. Biochim. Biophys. Acta 1450, 481–485.

Tyler, C.R., Sumpter, J.P., 1996. Oocyte growth and development in teleost. Rev. Fish Biol. Fish 6, 287–318.

Vang, S.H., Kortner, T.M., Arukwe, A., . in press. Steroidogenic acute regulatory (StAR) protein and cholesterol side-chain cleavage (P450scc) as molecular and cellular targets for 17-ethynylestradiol in salmon previtellogenic oocytes. Chem Res Toxicol.

Vendola, K.A., Zhou, J., Adesanya, O.O., Weil, S.J., Bondy, C.A., 1998. Androgens stimulate early stages of follicular growth in the primate ovary. J. Clin. Invest. 101, 2622–2629.

Vendola, K., Zhou, J., Wang, J., Famuyiwa, O.A., Bievre, M., Bondy, C.A., 1999. Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary. Biol. Reprod. 61, 353–357.

Vercellini, P., Parazzini, F., Bolis, G., Carinelli, S., Dindelli, M., Vendola, N., Luchini, L., Crosignani, P.G., 1993. Endometriosis and ovarian cancer. Am. J. Obstet. Gynecol. 169, 181–182.

von Schalburg, K.R., Rise, M.L., Brown, G.D., Davidson, W.S., Koop, B.F., 2005. A comprehensive survey of the genes involved in maturation and development of the rainbow trout ovary. Biol. Reprod. 72, 687–699.

Weibel, E.R., 1979. Stereological Methods, Practical Methods for Biological Morphometry. Academic Press, London, UK.

Weil, S.J., Vendola, K., Zhou, J., Adesanya, O.O., Wang, J., Okafor, J., Bondy, C.A., 1998. Androgen receptor gene expression in the primate ovary: cellular localization, regulation, and functional correlations. J. Clin. Endocrinol. Metab. 83, 2479–2485.

Weil, S., Vendola, K., Zhou, J., Bondy, C.A., 1999. Androgen and follicle-stimulating hormone interactions in primate ovarian follicle development. J. Clin. Endocrinol. Metab. 84, 2951–2956.

Yang, G.P., Ross, D.T., Kuang, W.W., Brown, P.O., Weigel, R.J., 1999. Combining SSH and cDNA microarrays for rapid identification of differentially expressed genes. Nucleic Acids Res. 27, 1517–1523.

Yang, Y.H., Dudoit, S., Luu, P., Speed, T.P., 2001. Normalization for cDNA microarray data. In: Bittner, M.L., Chen, Y., Dorsel, A.N., Dougherty, E.R. (Eds.), Microarrays: Optical Technologies and Informatics, pp. 141–152.

Zeng, S., Gong, Z., 2002. Expressed sequence tag analysis of expression profiles of zebrafish testis and ovary. Gene 294, 45–53.

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SUPPLEMENTAL DATA

Genes whose expression patterns were either up- or down-regulated by exposure to 11-ketotestosterone (11-KT) or testosterone (T) at 10 and 100 µM for 5 and 10 days incubation. Each data point is derived from three biological replicates (n=3) and log2-transformed. All values represented are significantly different ($q < 0.05$).

Table 1. 10 µM 11-KT exposure at day 10 compared with 10 µM 11-KT exposure at day 5.

Genbank no.	Putative gene identity	Log. ratio	Size of query (bp)	E-value	Species	Accession no.
ES880759	Unknown	1.10	379			
ES880761	Unknown	1.04	361			
ES880763	Unknown	0.99	379			
ES880747	tRNA pseudouridine synthase A	0.95	280	5e-10	*Danio rerio*	NM_001008603
ES880644	Unknown	0.94	292			
ES880773	Unknown	0.86	127			
ES880774	Unknown	0.79	358			
ES880641	H/ACA ribonucleoprotein complex subunit 2	0.69	297	2e-05	*Canis familiaris*	XP_531874
ES880770	Unknown	0.68	379			
ES880785	Unknown	0.65	423			
ES880698	Unknown	0.57	440			
ES880749	Unknown	0.57	503			
ES880642	NifU-like N-terminal domain containing protein	0.56	420	1e-35	*Danio rerio*	CAE50167
ES880673	Cytochrome c oxidase polypeptide VIc precursor	0.52	215	6e-06	*Thunnus obesus*	AAQ14272
ES880730	Unknown	0.43	461			
ES880704	Heat shock protein HSP 90-beta	-0.46	542	2e-60	*Paralichthys olivaceus*	AAO92751
ES880741	Unknown	-0.52	536			

ES880713	Unknown	-0.61	521			
ES880708	Unknown	-0.66	574			

Table 2. 100 µM 11-KT exposure at day 10 compared with 10 µM 11-KT exposure at day 10.

Genbank no.	Putative gene identity	Log. ratio	Size of query (bp)	E-value	Species	Accession no.
ES880759	Unknown	0.65	379			

Table 3. 100 µM 11-KT exposure at day 10 compared with 100 µM 11-KT exposure at day 5.

Genbank no.	Putative gene identity	Log. ratio	Size of query (bp)	E-value	Species	Accession no.
ES880759	Unknown	1.85	379			
ES880644	Unknown	1.58	292			
ES880763	Unknown	1.39	379			
ES880761	Unknown	1.36	361			
ES880773	Unknown	1.36	127			
ES880641	H/ACA ribonucleoprotein complex subunit 2	1.26	297	2e-05	*Canis familiaris*	XP_531874
ES880770	Unknown	1.25	379			

ES880774	Unknown	1.16	358			
ES880749	Unknown	1.04	503			
ES880698	Unknown	0.99	440			
ES880747	tRNA pseudouridine synthase A	0.78	280	5e-10	*Danio rerio*	NM_001008603
ES880640	PHD finger-like domain protein 5A	0.76	473	1e-47	*Danio rerio*	NP_775373
ES880785	Unknown	0.73	423			
ES880643	Unknown	0.70	175			
ES880628	PHD finger-like domain protein 5A	0.65	340	1e-47	*Danio rerio*	NP_775373
ES880690	Cytochrome c oxidase polypeptide VIc precursor	0.65	215	2e-05	*Thunnus obesus*	AAQ14272
ES880680	Cytochrome c oxidase polypeptide VIc precursor	0.64	215	6e-06	*Thunnus obesus*	AAQ14272
ES880719	CDKN2A interacting protein	0.62	475	8e-14	*Bos taurus*	NP_001039577
ES880703	Cytochrome c oxidase polypeptide VIc precursor	0.62	239	4e-06	*Thunnus obesus*	AAQ14272
ES880781	Zona pellucida-like protein X1	0.60	276	7e-13	*Sparus aurata*	AAY21008
ES880732	Unknown	0.57	389			
ES880673	Cytochrome c oxidase polypeptide VIc precursor	0.48	215	6e-06	*Thunnus obesus*	AAQ14272
ES880676	Cytochrome c oxidase polypeptide VIc precursor	0.47	215	2e-06	*Thunnus obesus*	AAQ14272
ES880730	Unknown	0.46	461			
ES880729	Unknown	0.45	442			
ES880697	40S ribosomal protein S27	0.43	384	7e-08	*Epinephelus coioides*	AAM27204
ES880764	Unknown	0.42	484			
CO542315	CyclinB	0.40	684	1e-67	*Carassius auratus gibelio*	AAF82779
ES880649	Unknown	0.38	260			
ES880651	Sentrin-related protein	0.37	464	7e-07	*Tetraodon nigroviridis*	CAG10351

ES880760	Unknown	0.36	503			
ES880757	apoptosis-inducing TAF9-like domain 1	-0.35	534	2e-24	*Mus musculus*	NP_081539
ES880705	Unknown	-0.36	88			
ES880704	Heat shock protein HSP 90-beta	-0.43	542	2e-60	*Paralichthys olivaceus*	AAO92751
ES880728	Unknown	-0.51	321			
ES880743	Unknown	-0.51	425			
ES880750	40S ribosomal protein S11	-0.53	460	5e-58	*Salmo salar*	AAG22824
ES880710	Unknown	-0.56	480			
ES880740	ZPA domain containing protein	-0.56	535	4e-23	*Danio rerio*	NP_997883
ES880742	Unknown	-0.57	536			
ES880717	Unknown	-0.57	478			
ES880741	Unknown	-0.62	536			
ES880713	Unknown	-0.64	521			
ES880718	Zona pellucida protein X2	-0.66	581	3e-16	*Oryzias latipes*	AAN31186
ES880709	Unknown	-0.70	563			
ES880629	Unknown	-0.70	358			
ES880733	Zona pellucida protein X2	-0.70	578	6e-59	*Oryzias latipes*	AAN31186
ES880627	Egg envelope glycoprotein	-0.74	500	3e-13	*Danio rerio*	XP_001335392
ES880721	Zona pellucida protein X2	-0.75	554	2e-49	*Oryzias latipes*	AAN31186
ES880630	Egg envelope glycoprotein	-0.86	519	1e-18	*Danio rerio*	XP_001335392
ES880722	ZPA domain containing protein	-0.91	275	7e-15	*Oryzias latipes*	NP_001098216
ES880738	Zona pellucida protein X2	-1.02	415	3e-16	*Oryzias latipes*	AAN31186

Table 4. 100 µM 11-KT exposure at day 10 compared with control at day 10.

Genbank no.	Putative gene identity	Log. ratio	Size of query (bp)	E-value	Species	Accession no.
ES880759	Unknown	1.05	379			
ES880644	Unknown	0.87	292			
ES880763	Unknown	0.79	379			
ES880773	Unknown	0.75	127			
ES880774	Unknown	0.71	358			
ES880749	Unknown	0.71	503			
ES880641	H/ACA ribonucleoprotein complex subunit 2	0.70	297	2e-05	*Canis familiaris*	XP_531874
ES880781	Zona pellucida-like protein X1	0.68	276	7e-13	*Sparus aurata*	AAY21008
ES880761	Unknown	0.68	361			
ES880698	Unknown	0.61	440			
CO542315	CyclinB	0.59	684	1e-67	*Carassius auratus gibelio*	AAF82779
ES880770	Unknown	0.57	379			
ES880640	PHD finger-like domain protein 5A	0.49	473	1e-47	*Danio rerio*	NP_775373
ES880717	Unknown	-0.59	478			
ES880629	Unknown	-0.59	358			

Table 5. 10 µM T exposure at day 5 compared with control at day 5.

Genbank no.	Putative gene identity	Log. ratio	Size of query (bp)	E-value	Species	Accession no.
ES880731	ZPA domain containing protein	0.43	577	2e-62	*Oryzias latipes*	NP_001098216
ES880721	Zona pellucida protein X2	0.37	554	2e-49	*Oryzias latipes*	AAN31186
ES880751	ZPA domain containing protein	0.35	579	7e-62	*Oryzias latipes*	NP_001098216
ES880738	Zona pellucida protein X2	0.34	415	3e-16	*Oryzias latipes*	AAN31186
ES880709	Unknown	0.34	563			
ES880713	Unknown	0.33	521			
ES880742	Unknown	0.31	536			
ES880779	Zona pellucida protein X2	0.26	563	5e-53	*Oryzias latipes*	AAN31186
ES880722	ZPA domain containing protein	0.26	275	7e-15	*Oryzias latipes*	NP_001098216
ES880633	Unknown	-0.29	53			
ES880628	PHD finger-like domain protein 5A	-0.33	340	1e-47	*Danio rerio*	NP_775373
ES880761	Unknown	-0.38	361			
ES880649	Unknown	-0.38	260			
ES880643	Unknown	-0.38	175			
ES880770	Unknown	-0.40	379			
ES880654	Unknown	-0.41	362			
ES880759	Unknown	-0.41	379			
ES880760	Unknown	-0.44	503			
ES880773	Unknown	-0.54	127			

ES880644	Unknown	-0.65	292			

Table 6. 100 µM T exposure at day 5 compared with control at day 5.

Genbank no.	Putative gene identity	Log. ratio	Size of query (bp)	E-value	Species	Accession no.
ES880768	FK506-binding protein 3	0.29	517	1e-47	*Danio rerio*	NP_001004519
ES880690	Cytochrome c oxidase polypeptide VIc precursor	0.28	215	2e-05	*Thunnus obesus*	AAQ14272
ES880721	Zona pellucida protein X2	0.27	554	2e-49	*Oryzias latipes*	AAN31186
ES880694	Unknown	0.27	508			
ES880742	Unknown	0.26	536			
ES880718	Zona pellucida protein X2	0.24	581	3e-16	*Oryzias latipes*	AAN31186
ES880724	Unknown	-0.25	526			
ES880669	Heat shock protein HSP 90-beta	-0.32	502	2e-53	*Paralichthys olivaceus*	AAO92751
ES880773	Unknown	-0.32	127			
ES880644	Unknown	-0.34	292			
ES880628	PHD finger-like domain protein 5A	-0.35	340	1e-47	*Danio rerio*	NP_775373
ES880640	PHD finger-like domain protein 5A	-0.38	473	1e-47	*Danio rerio*	NP_775373
ES880682	Unknown	-0.44	92			

Table 7. 100 µM T exposure at day 5 compared with 10 µM T exposure at day 5.

Genbank no.	Putative gene identity	Log. ratio	Size of query (bp)	E-value	Species	Accession no.
ES880649	Unknown	0.45	260			
ES880774	Unknown	0.31	358			
ES880644	Unknown	0.31	292			
ES880770	Unknown	0.28	379			
ES880785	Unknown	0.23	423			
ES880667	Heat shock protein HSP 90-beta	-0.28	465	5e-46	*Paralichthys olivaceus*	AAO92751
ES880664	Heat shock protein HSP 90-beta	-0.33	561	6e-59	*Paralichthys olivaceus*	AAO92751
ES880700	Heat shock protein HSP 90-beta	-0.37	381	6e-30	*Lepomis macrochirus*	BAF57908
ES880691	Heat shock protein HSP 90-beta	-0.38	470	1e-47	*Paralichthys olivaceus*	AAO92751
ES880709	Unknown	-0.38	563			
ES880734	ZPA domain containing protein	-0.39	578	2e-62	*Oryzias latipes*	NP_001098216
ES880711	ZPA domain containing protein	-0.40	576	4e-63	*Oryzias latipes*	NP_001098216
ES880720	Unknown	-0.42	500			
ES880710	Unknown	-0.43	480			
ES880670	Heat shock protein HSP 90-beta	-0.44	431	3e-40	*Paralichthys olivaceus*	AAO92751
ES880669	Heat shock protein HSP 90-beta	-0.46	502	2e-53	*Paralichthys olivaceus*	AAO92751
ES880682	Unknown	-0.51	92			

AJ632154	Heat shock protein HSP 70	-0.52	1176	0	*Salmo salar*	CAG14941
ES880738	Zona pellucida protein X2	-0.54	415	3e-16	*Oryzias latipes*	AAN31186
ES880751	ZPA domain containing protein	-0.54	579	7e-62	*Oryzias latipes*	NP_001098216
ES880731	ZPA domain containing protein	-0.61	577	2e-62	*Oryzias latipes*	NP_001098216

Table 8. 10 µM T exposure at day 10 compared with 10 µM T exposure at day 5.

Genbank no.	Putative gene identity	Log. ratio	Size of query (bp)	E-value	Species	Accession no.
ES880773	Unknown	0.52	127			
ES880733	Zona pellucida protein X2	-0.30	578	6e-59	*Oryzias latipes*	AAN31186
ES880718	Zona pellucida protein X2	-0.31	581	3e-16	*Oryzias latipes*	AAN31186
ES880641	H/ACA ribonucleoprotein complex subunit 2	-0.32	297	2e-05	*Canis familiaris*	XP_531874
ES880741	Unknown	-0.33	536			
ES880742	Unknown	-0.33	536			
ES880699	Unknown	-0.35	312			
ES880721	Zona pellucida protein X2	-0.54	554	2e-49	*Oryzias latipes*	AAN31186

Table 9. 10 µM T exposure at day 10 compared with control at day 10.

Genbank no.	Putative gene identity	Log. ratio	Size of query (bp)	E-value	Species	Accession no.
ES880644	Unknown	0.42	292			
ES880759	Unknown	0.37	379			
ES880751	ZPA domain containing protein	-0.33	579	7e-62	*Oryzias latipes*	NP_001098216

Table 10. 100 µM T exposure at day 10 compared with 10 µM T exposure at day 10.

Genbank no.	Putative gene identity	Log. ratio	Size of query (bp)	E-value	Species	Accession no.
ES880773	Unknown	-0.35	127			
ES880759	Unknown	-0.44	379			

Table 11. 100 µM T exposure at day 10 compared with 100 µM T exposure at day 5.

Genbank no.	Putative gene identity	Log. ratio	Size of query (bp)	E-value	Species	Accession no.
ES880731	ZPA domain containing protein	0.84	577	2e-62	*Oryzias latipes*	NP_001098216
ES880751	ZPA domain containing protein	0.81	579	7e-62	*Oryzias latipes*	NP_001098216
AJ632154	Heat shock protein HSP 70	0.77	1176	0	*Salmo salar*	CAG14941

ES880738	Zona pellucida protein X2	0.77	415	3e-16	*Oryzias latipes*	AAN31186
ES880682	Unknown	0.73	92			
ES880669	Heat shock protein HSP 90-beta	0.72	502	2e-53	*Paralichthys olivaceus*	AAO92751
ES880664	Heat shock protein HSP 90-beta	0.67	561	6e-59	*Paralichthys olivaceus*	AAO92751
ES880691	Heat shock protein HSP 90-beta	0.65	470	1e-47	*Paralichthys olivaceus*	AAO92751
ES880684	Heat shock protein HSP 90-beta	0.60	466	5e-47	*Paralichthys olivaceus*	AAO92751
ES880710	Unknown	0.60	480			
ES880670	Heat shock protein HSP 90-beta	0.56	431	3e-40	*Paralichthys olivaceus*	AAO92751
ES880700	Heat shock protein HSP 90-beta	0.56	381	6e-30	*Lepomis macrochirus*	BAF57908
ES880734	ZPA domain containing protein	0.55	578	2e-62	*Oryzias latipes*	NP_001098216
ES880671	Heat shock protein HSP 90-beta	0.53	465	5e-42	*Dicentrarchus labrax*	AAQ95586
ES880667	Heat shock protein HSP 90-beta	0.51	465	5e-46	*Paralichthys olivaceus*	AAO92751
ES880709	Unknown	0.49	563			
ES880666	Heat shock protein HSP 90-beta	0.48	429	2e-40	*Paralichthys olivaceus*	AAO92751
ES880768	FK506-binding protein 3	0.45	517	1e-47	*Danio rerio*	NP_001004519
ES880748	Zinc finger CCHC domain containing protein 10	0.44	469	5e-23	*Gallus gallus*	XP_414654
ES880711	ZPA domain containing protein	0.40	576	4e-63	*Oryzias latipes*	NP_001098216
ES880720	Unknown	0.39	500			
ES880692	Heat shock protein HSP 90-beta	0.34	407	3e-35	*Paralichthys olivaceus*	AAO92751
ES880678	Heat shock protein HSP 90-beta	0.30	432	1e-41	*Oncorhynchus mykiss*	BAD90024
ES880708	Unknown	0.27	574			
ES880707	Ribonucleoside-diphosphate reductase M2	0.26	402	1e-19	*Danio rerio*	AAH75746
ES880724	Unknown	0.25	526			

ES880717	Unknown	0.21	478			
ES880713	Unknown	0.20	521			
ES880718	Zona pellucida protein X2	-0.20	581	3e-16	*Oryzias latipes*	AAN31186
ES880694	Unknown	-0.20	508			
ES880742	Unknown	-0.23	536			
ES880756	FK506-binding protein 3	-0.24	527	1e-45	*Danio rerio*	NP_001004519
ES880673	Cytochrome c oxidase polypeptide VIc precursor	-0.24	215	6e-06	*Thunnus obesus*	AAQ14272
ES880780	Unknown	-0.25	492			
ES880764	Unknown	-0.25	484			
ES880776	FK506-binding protein 3	-0.25	532	1e-47	*Danio rerio*	NP_001004519
ES880703	Cytochrome c oxidase polypeptide VIc precursor	-0.27	239	4e-06	*Thunnus obesus*	AAQ14272
ES880782	THAP domain-containing protein 4	-0.28	584	2e-50	*Danio rerio*	CAQ13268
ES880674	Unknown	-0.28	450			
ES880761	Unknown	-0.28	361			
ES880758	FK506-binding protein 3	-0.29	533	4e-50	*Danio rerio*	NP_001004519
ES880763	Unknown	-0.29	379			
ES880783	FK506-binding protein 3	-0.30	436	7e-31	*Danio rerio*	NP_001004519
ES880768	FK506-binding protein 3	-0.30	517	1e-47	*Danio rerio*	NP_001004519
ES880785	Unknown	-0.31	423			
ES880741	Unknown	-0.32	536			
ES880774	Unknown	-0.35	358			
ES880651	Sentrin-related protein	-0.36	464	7e-07	Tetraodon nigroviridis	CAG10351
ES880649	Unknown	-0.37	260			

ES880643	Unknown	-0.37	175
ES880644	Unknown	-0.38	292
ES880759	Unknown	-0.38	379
ES880699	Unknown	-0.40	312
ES880770	Unknown	-0.40	379

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Effects of 17α-methyltestosterone exposure on steroidogenesis and cyclin-B mRNA expression in previtellogenic oocytes of Atlantic cod (*Gadus morhua*)

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Abstract

Steroid hormone (estrogens and androgens) synthesis and regulation involve a large number of enzymes and potential biochemical pathways. In the context of these biochemical pathways, it is believed that the true rate-limiting step in acute steroid production is the movement of cholesterol across the mitochondrial membrane by the steroidogenic acute regulatory (StAR) protein and the subsequent conversion to pregnenolone by cytochrome P450-mediated side-chain cleavage (P450*scc*) enzyme. Oocyte development is a complex process that is triggered by the maturation-promoting factor (MPF) involving cyclin-B as a regulatory factor. In the present study, we evaluated the endocrine effects of 17α-methyltestosterone (MT) on steroidogenic pathways of Atlantic cod (*Gadus morhua*), using an *in vitro* previtellogenic oocyte culture technique that is based on an agarose floating method. Tissue was cultured in a humidified incubator at 10 °C for 1, 5, 10 and 20 days with different concentrations of the synthetic androgen MT (0 (control), 1, 10, 100 and 1000 μM) dissolved in ethanol (0.3%). Gene expressions for StAR, P450*scc*, aromatase-α (P450*aromA*) and cyclin-B were detected using validated real-time PCR with specific primer pairs. Cellular localization of the StAR protein and P450*scc* were performed using the immunohistochemical technique with antisera prepared against synthetic peptide for both proteins. Steroid hormones (estradiol-17β: E2 and testosterone: T) levels were estimated using enzyme immunoassay. Our data showed significant concentration-specific increase (at day 1 and 5) and decrease (at day 10 and 20) of the StAR mRNA expression after exposure to MT. P450*scc* expression showed a MT concentration-specific decrease during the exposure periods and cyclin-B mRNA expression was decreased in MT concentration-dependent manner at days 10 and 20 (reaching almost total inhibition after exposure to 1000 μM MT). MT exposure produced variable effects on the P450*aromA* mRNA expression that can be described as concentration-specific increase (day 1) and decrease (days 5 and 10). Cellular localization of the StAR protein and P450*scc* demonstrated their expression mainly in ovarian follicular cells. MT produced an apparent concentration-and time-dependent increase of E2 and T levels. Thus, the present study reveals some novel effects of pharmaceutical endocrine disruptor on the development of previtellogenic oocytes in cod. The impaired steroidogenesis and hormonal imbalance reported in the present study may have potential consequences for the vitellogenic process and overt fecundity in teleosts.

Keywords: Steroidogenesis; Cyclin-B; Previtellogenic oocytes; Methyltestosterone; Atlantic cod

1. Introduction

Pharmaceuticals are ubiquitous pollutants in the aquatic environment where their potential effects on non-target species like fish has only recently become subject of systematic investigations. Available scientific evidence indicate that the reproductive system, including its associated endocrine and neural controls, may be susceptible to alterations by occupational, pharmaceutical or environmental exposures to a variety of chemical and physical agents (Singleton and Khan, 2003); (DeRosa et al., 1998). The synthetic androgen, 17α-methyltestosterone (MT) is widely applied in aquaculture to control sex determination and induce sex-reversal of genetic females to phenotypic males (Hunter and Donaldson, 1983; Kitano et al., 2000; Papoulias et al., 2000). Nevertheless, the specific role or effect of MT remains to be resolved, and factors such as dose, timing and duration of MT treatment can influence the effects. It has been shown that high MT concentrations and/or prolonged exposure times, may induce phenotypic female characteristics (Rinchard et al., 1999; Orn et al., 2003). At the transcript level, a suppression of P450*aromA* gene expression after MT treatment

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has been reported in several studies (Kitano et al., 1999; Fenske and Segner, 2004). Furthermore, in several teleost species such as common carp (*Cyprinus carpio*; (Nagy et al., 1981), rainbow trout (*Oncorhynchus mykiss*; (Ostrowski and Garling, 1987) and tilapia (*Oreochromis mossambicus*; (Kuwaye et al., 1993), the anabolic effects of MT on growth have been demonstrated. Previous studies addressing the effects of MT treatment in female fish have mainly focused on monitoring sex reversal and proportions of intersex in fish (Kitano et al., 2000; Orn et al., 2003; Kanamori et al., 2006).

The synthesis and regulation of steroid hormones involve a large number of enzymes and potential biochemical pathways. In the context of these biochemical pathways, it is believed that the true rate-limiting step in acute steroid production is the movement of cholesterol across the mitochondrial membrane by the steroidogenic acute regulatory (StAR) protein, and the subsequent conversion to pregnenolone by cytochrome P450-mediated side-chain cleavage enzyme (P450*scc*) (Stocco 1997; Stocco 2001a). The StAR protein and P450*scc* have been localized in most steroidogenic organs or tissues, including cod ovary (Kortner and Arukwe, 2007), and are rapidly synthesized in response to acute tropic hormone stimulation. Cytochrome P450 aromatase (P450*arom*) is another main enzyme involved in steroidogenesis, responsible for the conversion of a large range of androgens into estrogens (Simpson et al., 1994). It has been shown that teleost fish have two distinct mRNA isoforms of P450*arom*. P450*aromA* is predominantly expressed in the ovary and is involved in sex differentiation and oocyte growth, whereas P450*aromB* is highly expressed in brain (Callard et al., 2001; Kishida and Callard, 2001). Estrogens (17β-estradiol) and androgens (testosterone and 11-ketotestosterone) are known to be involved in a number of physiological functions such as sexual differentiation, ion and carbohydrate homeostasis, adaptation to stress, immune system functioning and reproduction (Dean and Sanders, 1996).

Oocyte maturation is a complex process that is triggered by the maturation-promoting factor (MPF) induced on the oocyte surface by the maturation-inducing hormone (MIH) that is secreted from follicle cells. During this process, cell division cycle 2 (cdc2) functions as a catalytic subunit while cyclin-B functions as a regulatory unit whose activity is controlled by inhibitory phosphorylation of cdc2 on threonine 14/tyrosine 15 (T14/Y15) (Morgan 1995; Kondo et al., 1997). Thus, the accumulation of cyclin-B in oocytes controls the timing of early embryonic cell cycle (Aegerter et al., 2004). It has been shown that natural occurring steroids as well as endocrine-disrupting chemicals (EDCs) are able to affect cyclin-B synthesis (Kudo et al., 2004; Tokumoto et al., 2004; Tokumoto et al., 2005; Kortner and Arukwe, 2007). Thus, gene expression patterns of cyclin-B may be used as a marker for oocyte developmental competence.

The present study was designed to evaluate the effects of MT on gene expression patterns, whose functional products may regulate steroidogenesis and modulate development of previtellogenic oocytes. In addition, we investigated the effects of MT on steroid hormone levels in previtellogenic oocytes. These were performed using an *in vitro* organ culture technique that was based on an agarose floating method and Atlantic cod (*Gadus morhua*) as a model species. The Atlantic cod is a popular species with large economic value and the farming potential on a global basis is therefore regarded as extremely high. Despite its high economic significance, the Atlantic cod is not a well-studied species neither from an endocrinological or toxicological standpoint. We hypothesize that exposure of previtellogenic oocytes to MT will produce differential gene expression and hormone patterns, whose functional products may modulate steroidogenesis with significant effect on early oocyte growth and development. After thorough validation, these responses may be prognostic, diagnostic and indicative of the effects of pharmaceuticals and chemical endocrine disruptors on the growth and development of previtellogenic oocyte in teleosts with potential implication for overt fecundity.

2. Materials and methods

2.1. Chemicals and reagents

17α-Methyltestosterone was purchased from Fluka Chemika-Biochemika (Buchs, Switzerland). Trizol reagent for RNA purification, TA cloning kit and Leibovitz L-15 medium were purchased from Gibco-Invitrogen life technologies (Carlsbad, CA, USA). Bovine serum albumin (BSA) and *N*-[2-hydroxyethyl]piperazine-*N*′*e*-[2-ethanesulfonic acid] (Hepes) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Iscript cDNA synthesis kit and iTaq™Sybr® Green supermix with ROX were purchased from Bio-Rad laboratories (Hercules, CA, USA). Generuler™ 100 bp DNA ladder and dNTPs were purchased from Fermentas GmbH (Germany). Superpicture™ polymer detection kit (Cat. No. 87-9263) was purchased from Zymed (San Francisco, CA, USA), and Tissue-clear® and Tissue-mount was purchased from Sakura Finetek Europe (Zoeterwoude, The Netherlands). Testosterone and estradiol-17β enzyme immuno-assay (EIA) kits (Cat. No. 582701 and 582251) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals were of the highest commercially available grade.

2.2. Animals and floating agarose in vitro culture technique

Juvenile female Atlantic cod, 150–350 g body weight were purchased from Akvaforsk genetic center (Sunndalsøra, Norway) and kept in circulating water at 10 °C and a 12 h light:12 h dark photoperiod. The *in vitro* organ culture techniques employed were based on the agarose floating method (Nader et al., 1999; Kortner and Arukwe, 2007). Briefly, juvenile female cod were anesthetized, sacrificed and washed in 70% ethanol. Ovaries were removed, cut into small pieces (1 × 1 × 1 mm) and grown in 6-well dishes on a floating agarose substrate covered with a nitrocellulose membrane in basal culture media. The basal culture medium consisted of Leibovitz L-15 medium supplemented with 0.1 mM L-glutamic acid, 0.1 mM L-aspartic acid, 1.7 mM L-proline, 0.5% BSA, and 10 mM HEPES (pH 7.4). The gonadal tissue was cultured randomly in triplicates ($n=3$) for 1, 5, 10 and 20 days with different concentrations of methyltestosterone (0 (control), 1, 10, 100 and 1000 μM) in a humidified incubator at 10 °C. The control

group received ethanol (carrier vehicle for MT) and the final concentration of ethanol in all exposure groups never exceeded 0.3% (v/v). The medium was changed every 5 days after sampling at day 5, 10 and 20 of exposure. After cultivation, tissues for RNA purification were homogenized directly in Trizol reagent and stored at −80 °C until further processing. Tissues for immunohistochemical analyses were placed in tissue cassettes with a nylon mesh and fixed in 4% paraformaldehyde, whereas the tissues for steroid analyses were snap-frozen in liquid nitrogen and thereafter stored at −80 °C until further processing.

2.3. RNA purification and cDNA synthesis

Total RNA was purified from tissues homogenized in Trizol reagent according to established procedures (Chirgwin et al., 1979; Chomczynski and Sacchi, 1987), and RNA concentrations were determined using a NanoDrop® ND-1000 UV-Visible Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total cDNA for the real-time polymerase chain reaction (PCR) were generated from 1 μg total RNA from all samples using a mixture of random and poly-T primers from iScript cDNA synthesis kit as described by the manufacturer (Bio-Rad).

2.4. The StAR protein and P450scc antibody production and purification

Polyclonal antibodies for the StAR protein and P450*scc* were produced by immunisation of rabbits with synthetic peptides for both proteins (Eurogentec, Searing, Belgium). The peptide sequences were; (StAR) H2N-MPE QRG VVR AEN GPT C-CONH2 and (P450*scc*) H2N-CLL KNG EDW RSN RVI L-CONH2 with respective molecular weights of 1743.97 and 1916.2 (kiloDalton, kDa), and respective isoelectric point (pI) of 6.45 and 9.45. Rabbits were immunized once a week with the synthetic peptides coupled to Keyhole limpet hemocyanin (KLH)- *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS). Rabbits were bled after the fourth boost. The resulting sera were purified using Hitrap rProtein A affinity column (Amersham, Uppsala, Sweden). Briefly, serum was diluted 1:4 in binding buffer (20 mM sodium phosphate, pH 7.0) after filtration through a 0.45 μm filter. The column was washed with 5 × column volume of 20% ethanol and binding buffer. Thereafter, the diluted serum was applied with a flow rate of 1 mL/min. The column was then washed with 5 × column volume of binding buffer, and the purified antibody was eluted with 0.1 M sodium citrate buffer (pH 3.5) into tubes containing 80 μl of 1 M Tris–HCl (pH 9.0). Protein concentrations were determined using a NanoDrop® ND-1000 UV-Visible Spectrophotometer.

2.5. Primer optimization, cloning and sequencing

PCR primers for amplification of 150–246 bp gene-specific PCR-products were designed from conserved regions of the studied genes. The primer sequences, their amplicon size and the optimal annealing temperatures are shown in Table 1. Prior to PCR reactions, all primer pairs were used in titration reactions in order to determine optimal primer pair concentrations and their optimal annealing temperatures. All chosen primer pair concentrations used at the selected annealing temperatures gave a single band pattern for the expected amplicon size in all reactions. PCR products from the genes to be investigated were cloned into pCRII vector in INVαF′ *E. coli* (Invitrogen). Each plasmid was sequenced using ABI-prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Department of Biology, NTNU, Norway. Sequences were confirmed using NCBI nucleotide BLAST software (http://www.ncbi.nlm.nih.gov/BLAST).

2.6. Quantitative (real-time) PCR

Quantitative (real-time) PCR was used for evaluating gene expression profiles. For each treatment, the expression of individual gene targets was analyzed using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA). The following concentrations of forward and reverse primers were used for each 25-μL real-time PCR reaction: StAR, P450*scc*, cyclin-B and P450*aromA* at 200 nM each. Each 25-μl DNA amplification reaction contained 12.5 μL 2 × SYBR Green mix (Stratagene) with ROX (reference dye), and 1 μL cDNA. The real-time PCR program included an enzyme activation step at 95 °C (3 min) and 40 cycles of 95 °C (30 s), 52 °C (StAR), 55 °C (P450*scc*), 58 °C (cyclin-B), 60 °C (P450*arom*) (30 s), and 72 °C (30 s). Controls lacking cDNA template were included to determine the specificity of target cDNA amplification. Cycle threshold (*Ct*) values obtained were converted into copy number using standard plots of *Ct* versus log copy number. Standard plots for each target sequence were generated using known amounts of plasmid containing the amplicon of interest and only the concentration of the amplicon was used in this calculation. The criterion for using the standard curve is based on equal amplification efficiency with unknown samples,

Table 1
Primer pair sequence, amplicon size and annealing temperature conditions for genes used for real time PCR

Target Gene	Primer sequence[a]				
	Forward	Reverse	Amplicon size (bp)	Annealing temp. (°C)	Accesion number
StAR	CAACGTCAAGCAGGTCAAGA	GCATCGGGCTTCAACACTAT	246	52	AY291434
P450*scc*	AACAACTACTTCCGCAGCCT	CGGTAGAACAATGAGCTGGA	170	55	AY706102
P450*aromA*	GAGGAGACGCTCATCCTCAG	TAGCTGCGTGTCTTCTTCCA	167	60	AJ555405
Cyclin-B	CGGGAGATGGAGATGACTGT	TCTCGTAGTCCACCATGCAG	150	58	CO541727

[a]Sequences are given in the 5′–3′ order.

and this is usually checked prior to extrapolating unknown samples to the standard curve. Data obtained from triplicate runs for individual target cDNA amplification were averaged and expressed as ng/μl of initial total RNA used for reverse transcriptase (cDNA) reaction and thereafter transformed as percentage of control. This absolute quantification method is a well-validated procedure in our laboratory, as we do not use the so-called housekeeping genes because of their parallel modulation pattern with experimental samples both in our laboratory (Arukwe 2006) and elsewhere (Steele et al., 2002).

2.7. Previtellogenic oocyte histology and immunohistochemical analysis

Cultured previtellogenic gonadal tissues were fixed in 4% paraformaldehyde in 0.1 mM sodium phosphate buffer (pH 7.4) for 24 h. After fixation, the tissues were washed twice in 70% ethanol, dehydrated in a graded series of ethanol baths using an automatic tissue processor (Leica Microsystems, Nussloch, Germany), cleared in Tissue-Clear®, embedded in paraffin blocks and sectioned at 5 μm. Immunohistochemical analysis was preformed using SuperPicTure™ polymer detection kit (Zymed, Cat. number 87-9263) according to the manufacturer's protocol. In brief, sections were deparaffinized, rehydrated in graded series of ethanol and incubated for 1 h with rabbit polyclonal antibody against the StAR protein and P450*scc* diluted 1:200. After subsequent washing in PBS, sections were incubated with a horseradish peroxidase (HRP) polymer-conjugated anti-rabbit secondary antibody for 10 min. Colour visualizations were performed by incubation with 3,3′-diaminobenzidine (DAB) chromogen for 5 min, and hematoxylin was used as a counterstain. Control stains were performed by omission of the primary antibody. Sections were mounted using Tissue-Mount, and relative intensity and localization of the StAR protein and P450*scc* staining were evaluated using light microscopy.

2.8. Steroid hormone analysis

Estradiol-17β and testosterone concentrations were measured in cultured gonad tissue using enzyme immunoassay (EIA) kits from Cayman Chemical Company (Ann Arbor, MI, USA). Tissues cultured for 1, 5, 10 and 20 days were used for steroid hormone determinations. Tissues stored at −80 °C were thawed on ice, homogenized in 1:4 volume of 0.1 M Na-phosphate buffer and centrifuged at 14,000 *g* for 15 min. The supernatants were purified by extraction using organic solvent to prevent the interference of lipids and proteins in the analysis. Briefly, the supernatant was mixed with 4 mL diethyl ether using a vortex mixer. After phase separation, the aqueous portion was frozen in an ethanol/dry ice bath. The lipophilic phase was decanted into a clean tube, and the ether phase was evaporated by heating to 30 °C. The dry extract was reconstituted in 300 μL EIA buffer by vortexing. Enzyme immunoassays were run as recommended by Cayman with a development time of 75 min (estradiol-17β) and 60 min (testosterone) and the cross-reactivity of E2 and T antibodies with other steroid hormones are negligible. Data were quantified against a standard curve that was linearized using a logit transformation of B/B0 (bound sample/maximum bound).

2.9. Statistical analyses

Statistical analysis was performed with GraphPad Prism, version 2.1 (GraphPad Software Inc. 1996). Significant differences between control and exposure groups were performed using One-way ANOVA. Statistical differences between exposure groups were analyzed using the Tukey's Multiple Comparison Test. The level of significance was set at $p=0.05$ unless otherwise stated.

3. Results

3.1. Modulation of StAR, P450scc and P450aromA expressions

The expression of StAR in cod previtellogenic oocytes was modulated after exposure to different concentrations of MT in a time-specific manner (Fig. 1). At days 1 and 5 of exposure, MT produced an apparent concentration-dependent increase of StAR mRNA expression (Fig. 1A and B, respectively), showing a respective significant 3-fold and 2.5-fold increase after exposure to 100 and 1000 μM MT at day 1, and a 4.5-fold and 2-fold significant increase after exposure to 100 and 1000 μM MT at day 5 compared to the solvent control. On the contrary, StAR showed a minor, non-significant concentration-specific decrease at 10 and 20 days of exposure, compared to control (Fig. 1C and D, respectively). The expression of P450*scc* in cod previtellogenic oocytes was modulated after exposure to different concentrations of MT in a time-specific manner (Fig. 2). No differences between MT exposure groups were observed at day 1 of exposure (Fig. 2A). At day 5 of exposure, MT exposure caused significant reduction of P450*scc* for all MT concentrations compared to the solvent control (Fig. 2B). At 10 d of exposure (Fig. 2C), MT exposure produced variable effects on the P450*scc* mRNA expression that can be described as concentration-specific increases (1 and 100 μM) and an almost total inhibition (10 and 1000 μM). A minor, but non-significant decrease after MT exposure was observed at 20 days of exposure (Fig. 2D). The expression of P450*aromA* in cod previtellogenic oocytes was modulated after exposure to different concentrations of MT in a time-specific manner (Fig. 3). At 1 day of exposure (Fig. 3A), MT exposure produced a concentration-dependent induction of P450*aromA* mRNA expression. On the contrary, a general decrease was observed for high MT concentrations compared to the solvent control at 5 and 10 days of exposure (Fig. 3B and C, respectively). No significant differences between exposure groups were observed at day 20 of exposure, albeit showing an apparent increasing trend (Fig. 3D).

3.2. Modulation of cyclin-B expression

The expression of cyclin-B in cod previtellogenic oocyte was modulated after exposure to different concentrations of MT in a time-specific manner (Fig. 4). At day 1 of exposure, MT produced a minor, but non-significant decrease of cyclin-B transcription (Fig. 4A). In contrast, cyclin-B showed a small increase after

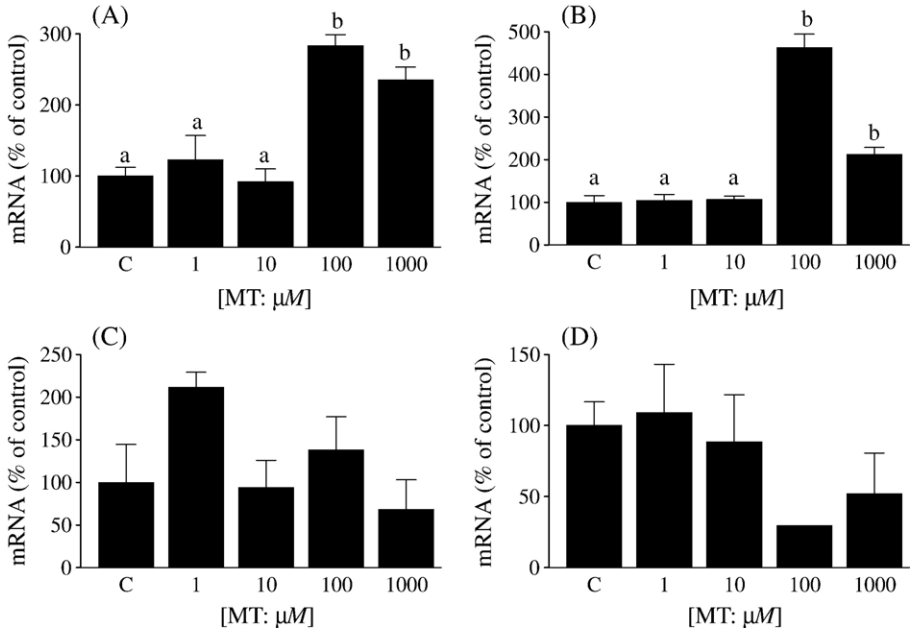

Fig. 1. Expression of StAR mRNA levels in cultured previtellogenic oocytes of Atlantic cod exposed to methyltestosterone (MT) at 0 (control), 1, 10, 100 and 1000 μM. Previtellogenic oocytes were sampled at day 1 (A), 5 (B), 10 (C) and 20 (D) of exposure. Messenger RNA (mRNA) levels were quantified using real-time PCR with specific primer pairs. Data are given as mean expressed as percentage (%) of control ($n=3$)±standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p<0.05$), analyzed using ANOVA followed by Tukey's multiple comparison test.

exposure to 1000 μM of MT at day 5, compared to control (Fig. 4B). The expression patterns of cyclin-B at day 10 and 20 of exposure showed an increase at 1 μM and thereafter, an MT concentration-specific decrease was observed (Fig. 4C and D, respectively). The MT-mediated decrease in cyclin-B mRNA expression reached an almost total inhibition after exposure to highest MT concentration (1000 μM) at day 10 and 20 of exposure.

3.3. Histology and immunohistochemical analysis of the StAR protein and P450scc

The cellular localization of the StAR protein and P450*scc* in cultured previtellogenic oocytes of cod sampled at day 10 of exposure is shown in Fig. 5A, B, C and D, E, F respectively. Using rabbit polyclonal antibodies prepared against synthetic

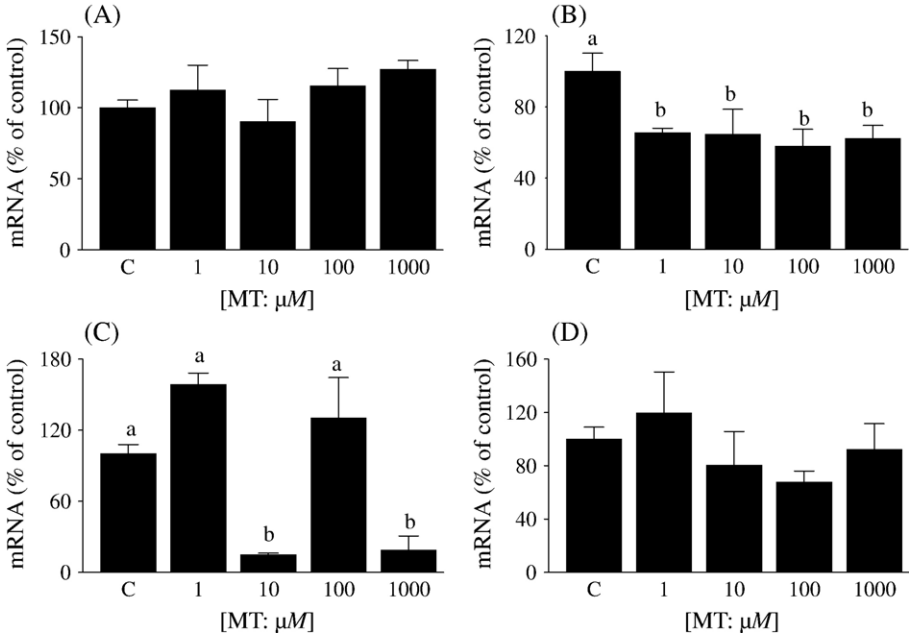

Fig. 2. Expression of P450*scc* mRNA levels in cultured previtellogenic oocytes of Atlantic cod exposed to methyltestosterone (MT) at 0 (control), 1, 10, 100 and 1000 μM. Previtellogenic oocytes were sampled at day 1 (A), 5 (B), 10 (C) and 20 (D) of exposure. Messenger RNA (mRNA) levels were quantified using real-time PCR with specific primer pairs. Data are given as mean expressed as percentage (%) of control ($n=3$)±standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p<0.05$), analyzed using ANOVA followed by Tukey's multiple comparison test.

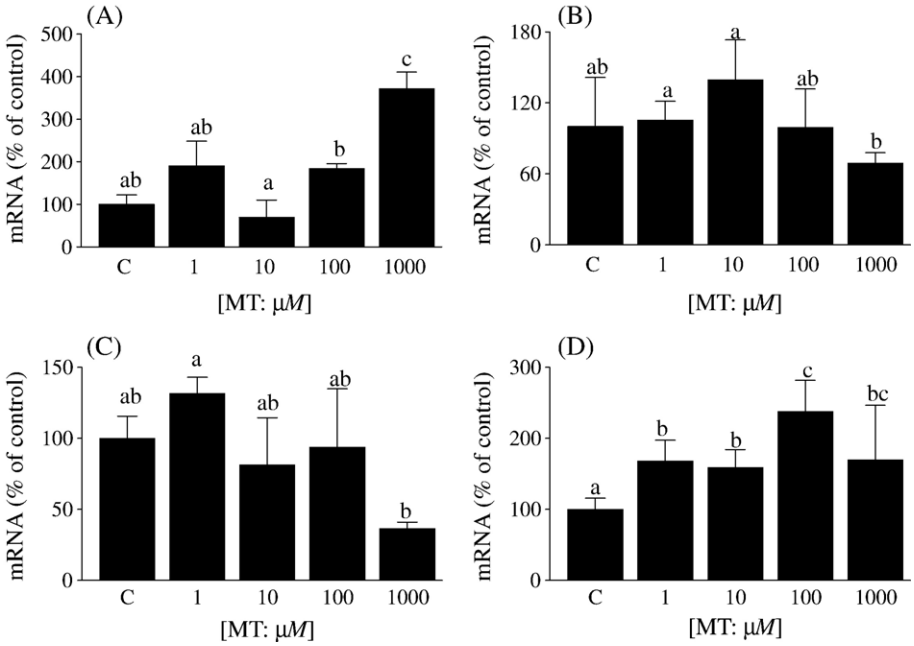

Fig. 3. Expression of P450*aromA* mRNA levels in cultured previtellogenic oocytes of Atlantic cod exposed to methyltestosterone (MT) at 0 (control), 1, 10, 100 and 1000 μM. Previtellogenic oocytes were sampled at day 1 (A), 5 (B), 10 (C) and 20 (D) of exposure. Messenger RNA (mRNA) levels were quantified using real-time PCR with specific primer pairs. Data are given as mean expressed as percentage (%) of control ($n=3$)±standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p<0.05$), analyzed using ANOVA followed by Tukey's multiple comparison test.

peptide sequences, the cellular localization of StAR and P450*scc* were mainly observed in follicular cells of the oocyte boundary layer in both control and exposed tissues. The relative intensity of both proteins was generally very low and did not show significant differences between control and MT exposure groups. In addition, no significant differences in staining intensity between day 5 and 10 were observed (data not shown). Evaluation of oocyte morphology and size revealed that previtellogenic oocytes had a diameter of approximately 100 μm and there were no significant differences between

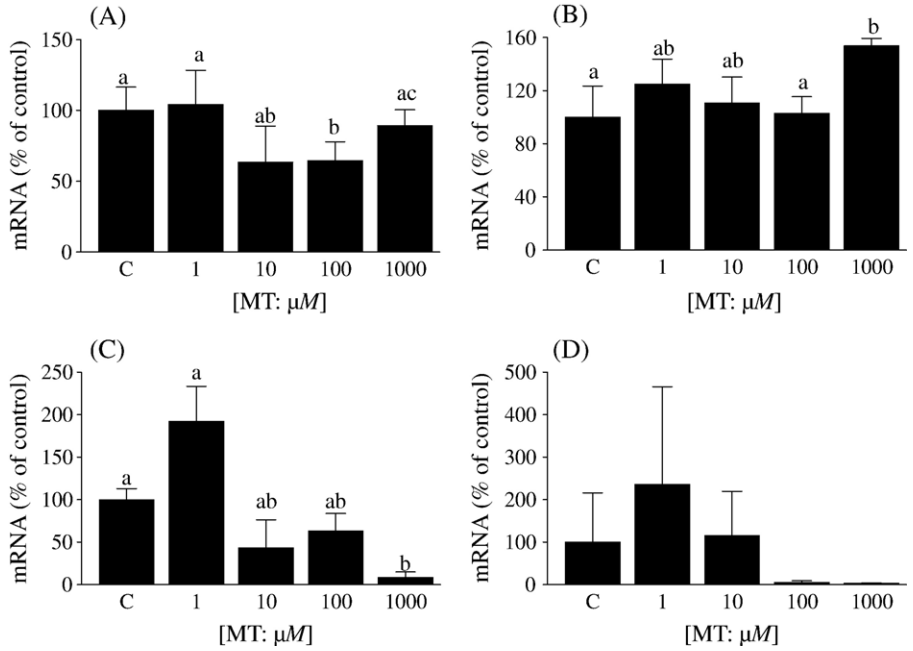

Fig. 4. Expression of cyclin-B mRNA levels in cultured previtellogenic oocytes of Atlantic cod exposed to different concentrations of methyltestosterone (0 (control), 1, 10, 100 and 1000 μM) and sampled at day 1 (A), 5 (B), 10 (C) and 20 (D) of exposure. Messenger RNA (mRNA) levels were quantified using real-time PCR with specific primer pairs. Data are given as mean expressed as percentage (%) of control ($n=3$)±standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p<0.05$), analyzed using ANOVA followed by Tukey's multiple comparison test.

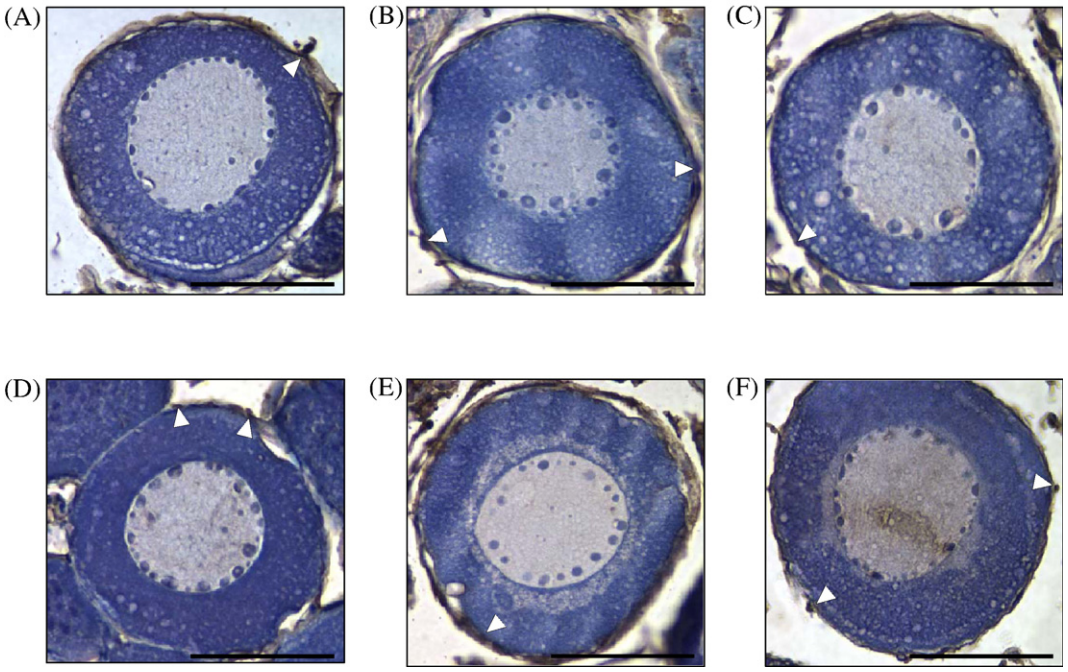

Fig. 5. Cellular localization of the StAR protein (A: control, B: 10 μM, C: 100 μM) and P450*scc* (D: control, E: 10 μM, F: 100 μM) in cultured previtellogenic oocytes of Atlantic cod exposed to different concentrations of MT, and sampled at day 10 of exposure. The figures are representative histological and immunohistochemical sections, as there were not significant differences between the exposure groups or to control. Arrows show positive StAR and P450*scc* staining in follicular cells of the oocyte boundary. Bar equals 50 μm.

control and MT-exposed samples. All sections examined displayed a normal histology.

3.4. Modulation of E2 and T levels

Estradiol-17β (E2) and testosterone (T) concentrations were measured in previtellogenic gonad tissue cultures exposed to MT for 1, 5, 10 and 20 days. Testosterone levels at 1, 5, 10 and 20 days of exposure to MT concentrations is shown in Fig. 6A, B, C and D, respectively. MT produced a small, but significant concentration-dependent increase of T levels, reaching 800 pg/mL in tissues exposed to the highest MT concentration (1000 μM). In general, T levels in the four different sampling days showed apparent similar patterns. E2 levels at 1, 5, 10 and

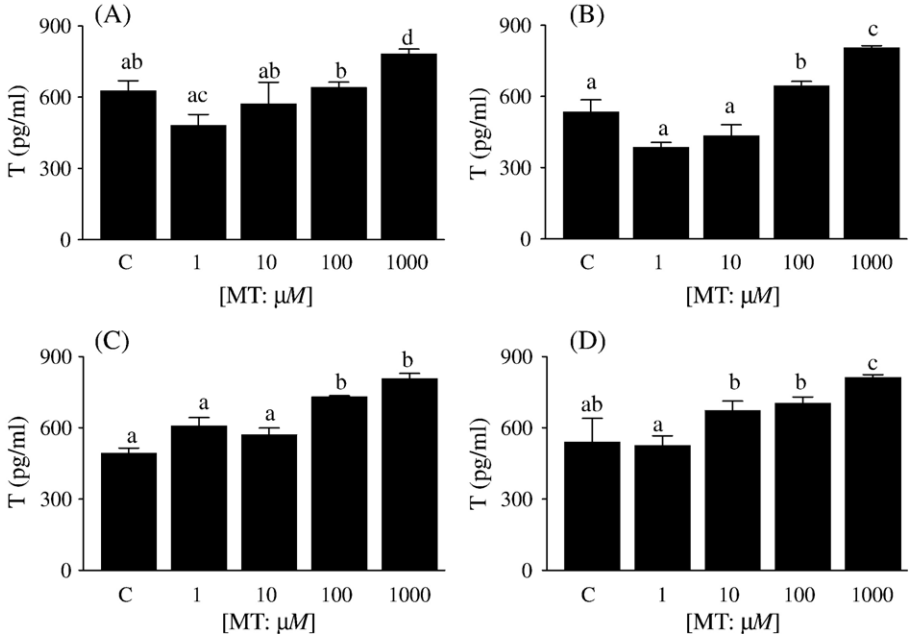

Fig. 6. Testosterone (T) levels at day 1, 5, 10 and 20 (A, B, C and D, respectively) in cultured previtellogenic oocytes of Atlantic cod exposed to different concentrations of MT. Steroid hormone levels were determined using enzyme immunoassay method. Data are given as mean value and expressed as pg/mL of $n=3\pm$standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p<0.05$), analyzed using ANOVA followed by Tukey's multiple comparison test.

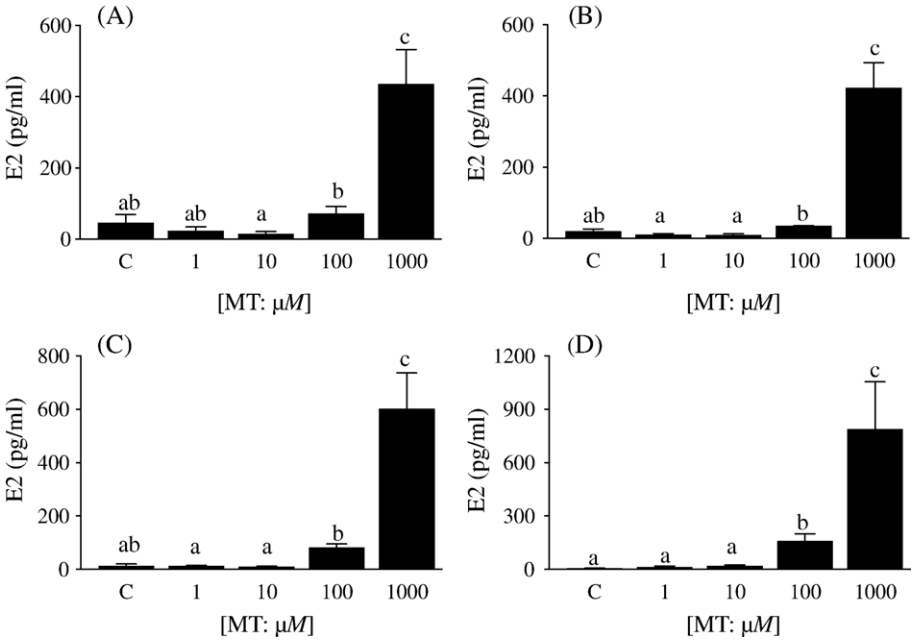

Fig. 7. Estradiol-17β levels (E2) at day 1, 5 10 and 20 (A, B, C and D, respectively) in cultured previtellogenic oocytes of Atlantic cod exposed to different concentrations of MT. Steroid hormone levels were determined using enzyme immunoassay method. Data are given as mean value and expressed as pg/mL of $n=3\pm$ standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p<0.05$), analyzed using ANOVA followed by Tukey's multiple comparison test.

20 days of exposure to MT concentrations are shown in Fig. 7A, B, C and D, respectively. MT produced an apparent concentration-and time-dependent increase of E2 levels. In general, exposures to low concentrations of MT (1 and 10 μM) showed slightly decreased E2 levels at all four sampling days. In contrast, exposures to the higher concentrations of MT (100 and 1000 μM) produced significant increases in E2 levels, reaching a 150-fold increase compared to the solvent control for the highest MT exposure (1000 μM) at 20 days of exposure.

4. Discussion

Despite its enormous economic importance for Norway and on the global basis, the Atlantic cod is not a well-studied species from an endocrinological or toxicological standpoint. Therefore, the present study was performed in order to develop an oocyte culture system to determine the effects of the synthetic pharmaceutical androgen, MT, on early ovarian growth by quantifying the gene expression patterns of the protein (StAR) and enzymes (P450*scc* and *P450aromA*) responsible for critical steps in ovarian steroidogenesis. In addition, the effects of MT on oocyte growth and development were examined by quantifying the expression pattern of cyclin-B. We show that MT modulates StAR, P450*scc,* P450*aromA* and cyclin-B gene expressions and that these effects were dependent on time of exposure and MT concentration. Interestingly, a apparent direct relationship between the MT-induced decreases of StAR, P450*scc,* P450*aromA* and cyclin-B at day 10 of exposure were demonstrated. Thus, the present study reveals some novel aspects of MT, a pharmaceutical and potent endocrine modulator known to be present in the aquatic environment at biologically active concentrations, on development and oocyte growth in teleosts.

4.1. Effects on StAR, P450scc, P450arom mRNA and steroid hormone levels

The present study demonstrates that both StAR, P450*scc* and P450*aromA* were modulated by MT exposures in a time-specific manner. The expression levels of these genes were affected at all exposure times. However, day 10 of exposure produced a unique, but consistent pattern of MT effect. At this exposure time, a general decrease in transcript levels for StAR, P450*scc* and P450*aromA* were observed in cultured cod previtellogenic oocytes. This particular pattern of MT modulation of StAR, P450*scc* and P450*aromA* genes suggests a time-and concentration-dependent control mechanism for in vitro oocyte regulation of steroidogenesis. Furthermore, changes in transcript levels are not always good surrogate for changes in protein levels. Using immunohistochemical analysis, we found minor differences in staining intensity in the StAR protein and P450*scc* after exposure to MT. Early mammalian studies showed that the StAR protein is a 30 kDa protein that is first synthesized in the cytosol as 37 kDa precursor in response to the activation of cAMP protein kinase-A intracellular signaling pathways (Artemenko et al., 2001). Newly synthesized StAR was recently shown as the effective mediator of cholesterol transfer protein and this is often present in low levels with very high estimated effectivity in the excess of 400 cholesterol molecules for each StAR molecule (Artemenko et al., 2001; Kusakabe et al., 2002). In addition, experiments in Y-1 and primary adrenal cells have established that basal StAR mRNA is sufficient for maximum cAMP-stimulated cholesterol metabolism providing that newly synthesized p37 StAR precursor is phosphorylated, transferred to the matrix and proteolytically cleaved to pp30 (Jefcoate et al., 2000). Therefore, the subtle changes in the StAR protein may

not be easily detected by the qualitative immunohistochemical approach, as could be expected using transcript analysis. Nevertheless, these observations are in compliance with previous *in vivo* studies, showing a decrease of P450*aromA* mRNA levels in gonads after MT treatment (Kitano et al., 2000; Fenske and Segner, 2004). Since the expression of P450*aromA* mRNA showed an MT concentration-dependent increase at day 1 of exposure, it suggests a bi-phasic mode MT effect on the aromatase pathway of previtellogenic cod oocytes. Recently, we demonstrated a general decrease in StAR and P450*scc* mRNA levels after exposure to the xenoestrogen. 4-nonylphenol in cultured cod previtellogenic oocytes (Kortner and Arukwe, 2007). Thus, we suggest a general gonadal feedback control mechanism with similar biological properties after exposure to sex steroid hormones or xenoestrogens. Regardless of organ or tissue, the delivery of cholesterol from the outer mitochondrial membrane by the StAR protein to the inner mitochondrial membrane, where P450*scc* resides, is the ultimate rate-limiting step in steroid hormone biosynthesis (Stocco 2000; 2001b). Modulation of this process will eventually affect testicular, ovarian and adrenocortical functions in exposed individuals. Therefore, the StAR protein plays a critical role in the regulation of hormonally induced acute steroid production by stimulating cholesterol transfer through hydrophobic tunnel structures formed within its molecule (Stocco 2000; 2001b). Thus, the impaired steroidogenesis and hormonal imbalance reported in the present study may have potential consequences for the vitellogenic process and overt fecundity in teleosts.

Furthermore, the sex steroid hormone (E2 and T) levels were modulated in cod previtellogenic oocytes in an apparent MT concentration-and time-dependent manner. The T levels were generally high, reaching 800 pg/mL in oocyte tissue exposed to the highest MT concentration (1000 μM). Interestingly, the E2 levels were generally very low in solvent control groups and in oocytes exposed to low MT concentrations (1 and 10 μM). However, after exposure to higher concentrations of MT, a strong increase in E2 levels were observed, reaching 800 pg/L in tissues exposed to the 1000 μM MT at day 20 of exposure. MT is a potent androgen receptor agonist and is generally considered to be aromatizable when administered at high concentration (Nakamura 1975; Piferrer et al., 1993; Borg 1994). Previous *in vivo* studies have demonstrated that MT promotes masculinization and development of phenotypic female fish, a phenomenon which is referred to as a "paradoxical feminisation" (Rinchard et al., 1999; Zerulla et al., 2002; Orn et al., 2003; Fenske and Segner, 2004). The extent and magnitude of MT effect on masculinization and sex-reversal depends on a number of biotic and abiotic factors including age, size, developmental stage, ambient temperature, nutritional factors, exposure duration and method and season (Higgs et al., 1982). The paradoxical feminization shift in sex ratio suggests an increased aromatase activity with subsequent conversion of MT to methylestradiol (ME2) (Hornung et al., 2004). These findings are in accordance with our observations showing that MT produced an apparent concentration-dependent increase of P450*aromA* mRNA that is consistent with E2 levels at day 1 of exposure. However, decreased P450*aromA* mRNA levels were observed at days 5 and 10 of exposure, suggesting a negative feedback control mechanism for previtellogenic oocyte regulation of steroidogenesis. Nevertheless, given the relatively high concentration of T in all exposed samples and the strong increase in E2 levels observed in samples exposed to the two highest MT concentrations, suggest the conversion of MT to E2. Therefore, MT seems to be aromatizable at these concentrations *in vitro*.

4.2. Effects on cyclin-B mRNA levels

Oocyte maturation is a complex process that is triggered by the maturation-promoting factor (MPF) and cyclin-B functions as a regulatory subunit (Kondo et al., 1997). The expression of MPF is thought to be a response to stimulation on the oocyte surface by the maturation-inducing hormone (MIH). Previous reports has demonstrated that MIH stimulates the translation of cyclin-B mRNA stored in immature oocytes (Hirai et al., 1992). Furthermore, it is thought that cyclin-B accumulation in oocytes controls the timing of early embryonic cell cycle (Aegerter et al., 2004). Despite the knowledge that full grown oocytes mature in response to MIH through a mechanisms that involves the binding of synthesized cyclin-B protein to pre-existing cdc2 enabling the cdk7-mediated phosphorylation of cdc2 at threonine 161 (T161), it is well known that previtellogenic and vitellogenic oocytes does not respond to MIH stimulation. The failure of growing (previtellogenic and vitellogenic) oocytes to mature after MIH treatment is suggested to be due to their failure to synthesize the cyclin-B protein from the mRNA stored in oocytes (Kondo et al., 1997). It has also been shown that natural occurring steroids as well as endocrine-disrupting chemicals (EDCs) are able to affect cyclin-B synthesis (Kudo et al., 2004; Tokumoto et al., 2004; Tokumoto et al., 2005). In our recent study, we reported the expression of cyclin-B in previtellogenic cod oocytes, whose expression patterns were modulated by the xenoestrogen 4-nonylphenol in a time- and concentration-specific manner (Kortner and Arukwe, 2007). The biphasic expression pattern suggests a time-specific effect of MT on oocyte development. Thus, gene expression of cyclin-B may be used as a sensitive marker for early oocyte growth and developmental competence in teleost species.

In several teleost species such as common carp (*C. carpio*; (Nagy et al., 1981), rainbow trout (*O. mykiss*; (Ostrowski and Garling, 1987) and tilapia (*O. mossambicus*; (Kuwaye et al., 1993), the anabolic effect of MT on growth have been demonstrated. In the present study, the expression pattern of cyclin-B paralleled the expression patterns of StAR, P450*scc* and P450*aromA*, showing decreased mRNA levels at day 10 of exposure. It should be noted that the only two compounds identified as naturally occurring MIH in fish are both progestin C21 steroids, namely 17α, 20β-dihydroxy-4-pregnen-3-one and 17α, 20β, 21-trihydroxy-4-pregnen-3-one (Nagahama and Adachi, 1985; Patino and Thomas, 1990). Therefore, the decreased expressions of StAR, P450*scc* and P450*aromA* may lead to decreased levels of functional MIH that may eventually result in decreased levels of cyclin-B synthesis in cod ovary. Thus, MT and other xenoandrogens may exhibit modulatory effects in the early growth and maturation process of previtellogenic oocytes.

From an endocrinological point of view, it has been suggested that androgens may increase oocyte diameter and modify growth factor receptors (Vendola et al., 1999; Rohr et al., 2001), but the underlying molecular mechanism(s) involved in possible gene regulation in the growth of previtellogenic oocytes is not well understood. Recently, we observed that both aromatizable (T) and non-aromatizable (11-KT) androgens induce oocyte development in the previtellogenic cod ovary, as well as differential gene expression patterns, whose functional products modulate oocyte growth (Kortner et al., submitted). The role of androgens in previtellogenic oocytes is supported by the observation that the early ovarian growth of species-specific critical stage progressed in hypophysectomized freshwater turtle, *Chrysemys picta*, indicating that gonadotropins (GtHs) were not necessary (Ho et al., 1982). Furthermore, Bieniarz and Kime (Bieniarz and Kime, 1986) were unable to demonstrate specific binding of radio labeled GtH (^{125}I-GtH) to previtellogenic common carp *(C. carpio)* ovaries. Recently, Rohr et al., (Rohr et al., 2001) demonstrated that 11-KT (the active male-specific androgen in teleosts) induces an increase in the diameter and development of previtellogenic eel (*Anguilla australis)* oocytes. In mammals, androgens also modify the intra-ovarian gene expression in the rhesus monkey, as demonstrated by increased mRNA abundance of insulin-like growth factor-1 (IGF-1) and IGF-1-receptor (Vendola et al., 1999) in follicles up to early antral stage. Based on the above named studies, androgens appear to play a pivotal role in stimulating the growth of small ovarian follicles in vertebrates, at least in mammals and fish. These observations strongly indicate that StAR, P450*scc* and P450*aromA* may be important targets for androgens involvement in the regulation of fecundity in females. However, there are still important gaps of knowledge concerning androgen-induced effects in females on the whole organism level and this should be studied in more detail. In this regard, the identification and full-length gene cloning of the StAR protein in Atlantic cod (Goetz et al., 2004) represents a promising sign in the understanding the roles and/or effects of androgens in this important species. Furthermore, although physiological (1 and 10 μM) and apparently high and pharmacological (100 and 1000 μM) concentrations of MT were used in the present study and therefore the high concentrations cannot directly be extrapolated to physiological androgens levels, they should rather be seen a an initial mechanistic approach and method development in understanding the role of androgens on early oocyte growth. Thus, the true effect of androgens on early oocyte growth and development should be investigated using physiologically relevant concentrations. This study is currently in progress in our laboratory.

From a toxicological point of view, the cod is a marine species and MT does not represent an environmental issue of reasonable concern in the marine environment, compared to freshwater environment. However, there are very few data on the effect of environmental androgens in fish species, particularly in the ovary and available data has focused mainly on male species. Therefore, the findings in the present study represent novel informations that can be used to deduce the effects of xenoandrogens on oocyte development and overt fecundity. Our results reveal some novel aspects of MT effects on development and oocyte growth in teleosts, suggesting impaired steroidogenesis and hormonal imbalance with potential consequences for the vitellogenic process and overt fecundity. The Atlantic cod is a popular species with large economic value and therefore the aquaculture potential on a global basis is therefore regarded as extremely high. In addition, cod is also the main marine species in Norway for coastal water pollution monitoring (Goksøyr et al., 1994; Martin-Skilton et al., 2006; Sturve et al., 2006). The National Joint Assessment and Monitoring Programme (JAMP, OSPAR) have used this species in fjord monitoring since the beginning of the 1980s (Tromp and Wieriks, 1994). Norwegian offshore oil industry has recently adopted the Atlantic cod as a promising organism to be used in biomonitoring studies, particularly in relation to produced water discharges that may contain both estrogenic and androgenic chemicals. Some studies indicate that alkyl phenols released from offshore platforms along with production water are capable of disturbing the hormonal balance and reproductive capacity of cod (Meier et al., 2002). These studies suggest that increased petroleum release in natural habitat of cod may cause a threat to the species.

5. Conclusions

The present study demonstrates that *in vitro* exposure of previtellogenic cod oocytes to MT modulated the gene expression of the main steroidogenic protein (StAR) and enzymes (P450*scc* and P450*aromA*) and the oocyte maturation regulatory factor, cyclin-B. The consistency in the expression patterns observed in these parameters at day 10 of exposure, suggests a general mode of action for their role in steroidogenesis and early ovarian growth. Thus, these results reveal some novel information of androgen effects on internal signals that regulate fecundity, development and oocyte growth. In addition, our findings suggest that the cultured previtellogenic oocytes are experiencing alterations in key steroidogenic pathways. Most importantly, the floating agarose method shows great potential as a sensitive *in vitro* organ culture system for studies on endocrine disrupting chemicals. Thus, the impaired steroidogenesis and hormonal imbalance reported in this study may have potential consequences for the vitellogenic process and overt fecundity in teleosts. In view of the integral role of steroids in homeostatic process, growth, metabolism, reproduction and development of central nervous system and function, these effects may have serious impact on this endocrine pathway and potentially affect organismal reproductive performance and health. In this regard and given the relative concern about the increasingly higher concentrations of synthetic pharmaceuticals reported in effluents and surface waters from Europe (Desbrow et al., 1998); (Larsson et al., 1999) and North America (Kolpin et al., 2002) pharmaceuticals in the environment represent serious health concern both to humans and wildlife.

Acknowledgements

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References

Aegerter, S., Jalabert, B., Bobe, J., 2004. Messenger RNA stockpile of cyclin B, insulin-like growth factor I, insulin-like growth factor II, insulin-like growth factor receptor Ib, and p53 in the rainbow trout oocyte in relation with developmental competence. Mol. Reprod. Dev. 67, 127–135.

Artemenko, I.P., Zhao, D., Hales, D.B., Hales, K.H., Jefcoate, C.R., 2001. Mitochondrial processing of newly synthesized steroidogenic acute regulatory protein (StAR), but not total StAR, mediates cholesterol transfer to cytochrome P450 side chain cleavage enzyme in adrenal cells. J. Biol. Chem. 276, 46583–46596.

Arukwe, A., 2006. Toxicological housekeeping genes: do they really keep the house? Environ. Sci. Technol. 40, 7944–7949.

Bieniarz, K., Kime, D.E., 1986. Autoradiographic localization of gonadotrophin receptors in ovaries of the common carp, *Cyprinus carpio* L. Gen. Comp. Endocrinol. 64, 151–156.

Borg, B., 1994. Androgens in teleost fishes. Comp Biochem Physiol C 109, 219–245.

Callard, G.V., Tchoudakova, A.V., Kishida, M., Wood, E., 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. J. Steroid Biochem. Mol. Biol. 79, 305–314.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., Rutter, W.J., 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294–5299.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Dean, D.M., Sanders, M.M., 1996. Ten years after: reclassification of steroid-responsive genes. Mol. Endocrinol. 10, 1489–1495.

DeRosa, C., Richter, P., Pohl, H., Jones, D.E., 1998. Environmental exposures that affect the endocrine system: public health implications. J. Toxicol. Environ. Health, Part B Crit. Rev. 1, 3–26.

Desbrow, C., Routledge, E.J., Brighty, G., Sumpter, J.P., Waldock, M.J., 1998. Indentification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitrobiological screening. Environ. Sci. Technol. 32, 1559–1565.

Fenske, M., Segner, H., 2004. Aromatase modulation alters gonadal differentiation in developing zebrafish (Danio rerio). Aquat. Toxicol. 67, 105–126.

Goetz, F.W., Norberg, B., McCauley, L.A., Iliev, D.B., 2004. Characterization of the cod (*Gadus morhua*) steroidogenic acute regulatory protein (StAR) sheds light on StAR gene structure in fish. Comp. Biochem. Physiol. B 137, 351–362.

Goksøyr, A., Beyer, J., Husøy, A.M., Larsen, H.E., Westrheim, K., Wilhelmsen, S., Klungsøyr, J., 1994. Accumulation and effects of aromatic and clorinated hydrocarbons in juvenile Atlantic cod (*Gadus morhua*) caged in a polluted fjord (Sørfjorden, Norway). Aquat. Toxicol. 29, 21–35.

Higgs, D.A., Fagerlund, U.H.M., Eales, J.G., McBride, J.R., 1982. Application of thyroid and steroid hormones as anabolic agents in fish culture. Comp. Biochem. Physiol. B 73, 143–176.

Hirai, T., Yamashita, M., Yoshikuni, M., Lou, Y.H., Nagahama, Y., 1992. Cyclin B in fish oocytes: its cDNA and amino acid sequences, appearance during maturation, and induction of p34cdc2 activation. Mol. Reprod. Dev. 33, 131–140.

Ho, S.M., Taylor, S., Callard, I.P., 1982. Effect of hypophysectomy and growth hormone on estrogen-induced vitellogenesis in the freshwater turtle, Chrysemys picta. Gen. Comp. Endocrinol. 48, 254–260.

Hornung, M.W., Jensen, K.M., Korte, J.J., Kahl, M.D., Durhan, E.J., Denny, J.S., Henry, T.R., Ankley, G.T., 2004. Mechanistic basis for estrogenic effects in fathead minnow (*Pimephales promelas*) following exposure to the androgen 17alpha-methyltestosterone: conversion of 17alpha-methyltestosterone to 17alpha-methylestradiol. Aquat. Toxicol. 66, 15–23.

Hunter, G.A., Donaldson, E.M., 1983. Hormonal sex control and its application to fish culture. Fish Physiol. 9, 223–303.

Jefcoate, C.R., Artemenko, I.P., Zhao, D., 2000. Relationship of StAR expression to mitochondrial cholesterol transfer and metabolism. Endocr. Res. 26, 663–680.

Kanamori, A., Yamamura, A., Koshiba, S., Lee, J.S., Orlando, E.F., Hori, H., 2006. Methyltestosterone efficiently induces male development in the self-fertilizing hermaphrodite fish, *Kryptolebias marmoratus*. Genesis 44, 495–503.

Kishida, M., Callard, G.V., 2001. Distinct cytochrome P450 aromatase isoforms in zebrafish (*Danio rerio*) brain and ovary are differentially programmed and estrogen regulated during early development. Endocrinology 142, 740–750.

Kitano, T., Takamune, K., Kobayashi, T., Nagahama, Y., Abe, S.I., 1999. Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). J. Mol. Endocrinol. 23, 167–176.

Kitano, T., Takamune, K., Nagahama, Y., Abe, S.I., 2000. Aromatase inhibitor and 17alpha-methyltestosterone cause sex-reversal from genetical females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (*Paralichthys olivaceus*). Mol. Reprod. Dev. 56, 1–5.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Kondo, T., Yanagawa, T., Yoshida, N., Yamashita, M., 1997. Introduction of cyclin B induces activation of the maturation-promoting factor and breakdown of germinal vesicle in growing zebrafish oocytes unresponsive to the maturation-inducing hormone. Dev. Biol. 190, 142–152.

Kortner, T.M., Arukwe, A., 2007. The xenoestrogen, 4-nonylphenol, impaired steroidogenesis in previtellogenic oocyte culture of Atlantic cod (*Gadus morhua*) by targeting the StAR protein and P450scc expressions. Gen. Comp. Endocrinol 150, 419–429.

Kudo, C., Wada, K., Masuda, T., Yonemura, T., Shibuya, A., Fujimoto, Y., Nakajima, A., Niwa, H., Kamisaki, Y., 2004. Nonylphenol induces the death of neural stem cells due to activation of the caspase cascade and regulation of the cell cycle. J. Neurochem. 88, 1416–1423.

Kusakabe, M., Todo, T., McQuillan, H.J., Goetz, F.W., Young, G., 2002. Characterization and expression of steroidogenic acute regulatory protein and MLN64 cDNAs in trout. Endocrinology 143, 2062–2070.

Kuwaye, T.T., Okimoto, D.K., Shimoda, S.K., Howerton, R.D., Lin, H.R., Pang, P.K.T., Grau, E.G., 1993. Effect of 17α-methyltestosterone on the growth of the euryhaline tilapia, *Oreochromis mossambicus*, in fresh water and in sea water. Aquaculture 113, 137–152.

Larsson, D.G.J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A.H., Olsson, P.E., Förlin, L., 1999. Ethinyloestradiol — an undesired fish contraceptive. Aquat. Toxicol. 45, 91–97.

Martin-Skilton, R., Thibaut, R., Porte, C., 2006. Endocrine alteration in juvenile cod and turbot exposed to dispersed crude oil and alkylphenols. Aquat Toxicol. 78 (Suppl 1), S57–S64.

Meier, S., Andersen, T.E., Hasselberg, L., Kjesbu, O.S., Klungsøyr, J., Svardal, A., 2002. Hormonal effects of C4-C7 alkylphenols on cod (Gadus morhua), p. 70.

Morgan, D.O., 1995. Principles of CDK regulation. Nature 374, 131–134.

Nader, M.R., Miura, T., Ando, N., Miura, C., Yamauchi, K., 1999. Recombinant human insulin-like growth factor I stimulates all stages of 11-ketotestosterone-induced spermatogenesis in the Japanese eel, *Anguilla japonica*, in vitro. Biol. Reprod. 61, 944–947.

Nagahama, Y., Adachi, S., 1985. Identification of maturation-inducing steroid in a teleost, the amago salmon (*Oncorhynchus rhodurus*). Dev. Biol. 109, 428–435.

Nagy, A., Bercsenyi, M., Csanyi, V., 1981. Sex reversal in carp (*Cyprinus carpio*) by oral adminisration of methyltestosterone. Can J Fish Aquat Sci 38, 725–728.

Nakamura, M., 1975. Dosage-dependent changes in the effect of oral administration on gonadal differentiation in Tilapia mossambica. Bull. Fac. Fish. Hokkkaido Univ 26, 99–108.

Orn, S., Holbech, H., Madsen, T.H., Norrgren, L., Petersen, G.I., 2003. Gonad development and vitellogenin production in zebrafish (*Danio rerio*) exposed to ethinylestradiol and methyltestosterone. Aquat. Toxicol. 65, 397–411.

Ostrowski, A.C., Garling, D.L.J., 1987. Effect of 17alpha-methyltestosterone treatment and withdrawal on growth and dietary protein utilization of juvenile rainbow trout fed practical diets varying in protein level. J. World Aquac. Soc. 18, 71–77.

Papoulias, D.M., Noltie, D.B., Tillitt, D.E., 2000. Effects of methyl testosterone exposure on sexual differentiation in medaka, Oryzias latipes. Mar. Environ. Res. 50, 181–184.

Patino, R., Thomas, P., 1990. Gonadotropin stimulates 17 alpha,20 beta,21-trihydroxy-4-pregnen-3-one production from endogenous substrates in Atlantic croaker ovarian follicles undergoing final maturation in vitro. Gen. Comp. Endocrinol. 78, 474–478.

Piferrer, F., Baker, I.J., Donaldson, E.M., 1993. Effects of natural, synthetic, aromatizable, and nonaromatizable androgens in inducing male sex differentiation in genotypic female chinook salmon (*Oncorhynchus tshawytscha*). Gen. Comp. Endocrinol. 91, 59–65.

Rinchard, J., Dabrowski, K., Garcia-Abiado, M.A., Ottobre, J., 1999. Uptake and depletion of plasma 17alpha-methyltestosterone during induction of masculinization in muskellunge, Esox masquinongy: effect on plasma steroids and sex reversal. Steroids 64, 518–525.

Rohr, D.H., Lokman, P.M., Davie, P.S., Young, G., 2001. 11-Ketotestosterone induces silvering-related changes in immature female short-finned eels, *Anguilla australis*. Comp. Biochem. Physiol. A 130, 701–714.

Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C.R., Michael, M.D., et al., 1994. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr. Rev. 15, 342–355.

Singleton, D.W., Khan, S.A., 2003. Xenoestrogen exposure and mechanisms of endocrine disruption. Front. Biosci. 8, s, 110–118.

Steele, B.K., Meyers, C., Ozbun, M.A., 2002. Variable expression of some "housekeeping" genes during human keratinocyte differentiation. Anal. Biochem. 307, 341–347.

Stocco, D.M., 1997. A StAR search: implications in controlling steroidogenesis. Biol. Reprod. 56, 328–336.

Stocco, D.M., 2000. The role of the StAR protein in steroidogenesis: challenges for the future. J. Endocrinol. 164, 247–253.

Stocco, D.M., 2001a. StAR protein and the regulation of steroid hormone biosynthesis. Annu. Rev. Physiol. 63, 193–213.

Stocco, D.M., 2001b. Tracking the role of a star in the sky of the new millennium. Mol. Endocrinol. 15, 1245–1254.

Sturve, J., Hasselberg, L., Falth, H., Celander, M., Forlin, L., 2006. Effects of North Sea oil and alkylphenols on biomarker responses in juvenile Atlantic cod (*Gadus morhua*). Aquat. Toxicol. 78 (Suppl 1), S73–S78.

Tokumoto, T., Tokumoto, M., Horiguchi, R., Ishikawa, K., Nagahama, Y., 2004. Diethylstilbestrol induces fish oocyte maturation. Proc. Natl. Acad. Sci. U. S. A. 101, 3686–3690.

Tokumoto, T., Tokumoto, M., Nagahama, Y., 2005. Induction and inhibition of oocyte maturation by EDCs in zebrafish. Reprod. Biol. Endocrinol. 3, 69.

Tromp, D., Wieriks, K., 1994. The OSPAR convention: 25 years of North Sea protection. Mar. Pollut. Bull. 29, 622–626.

Vendola, K., Zhou, J., Wang, J., Famuyiwa, O.A., Bievre, M., Bondy, C.A., 1999. Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary. Biol. Reprod. 61, 353–357.

Zerulla, M., Lange, R., Steger-Hartmann, T., Panter, G., Hutchinson, T., Dietrich, D.R., 2002. Morphological sex reversal upon short-term exposure to endocrine modulators in juvenile fathead minnow (*Pimephales promelas*). Toxicol. Lett. 131, 51–63.

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The xenoestrogen, 4-nonylphenol, impaired steroidogenesis in previtellogenic oocyte culture of Atlantic cod (*Gadus morhua*) by targeting the StAR protein and P450*scc* expressions

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Abstract

The steroidogenic acute regulatory (StAR) protein and cytochrome P450-mediated cholesterol side-chain cleavage (P450*scc*) have been localized in most steroidogenic organs and are rapidly synthesized in response to acute tropic hormone stimulation. In this study, we present the development of cod previtellogenic oocyte *in vitro* culture system, histological and molecular methods for evaluating the effects of endocrine disruptors such as nonylphenol (NP) on steroid hormone levels, the StAR protein and P450*scc*. In addition, expression pattern of cyclin-B was studied, because of cyclin B's role as an indicator of oocyte growth in fish. The *in vitro* previtellogenic oocyte culture technique was based on an agarose floating method. Tissue was cultured in a humidified incubator at 10 °C for 4, 7, 14 and 21 d with different concentrations of nonylphenol (0 (control), 1, 10, 50 and 100 μM) dissolved in ethanol (0.3%). Gene expressions were detected using validated real-time polymerase chain reaction (PCR) with specific primers. Immunohistochemistry of the StAR protein and P450*scc* were performed using antisera prepared against synthetic peptide for both proteins. Estradiol-17β (E2) and 11-ketotestosterone (11-KT) tissue levels were estimated using enzyme immunoassay. Our data show that nonylphenol produced a unique and consistent concentration-specific pattern of modulation for the StAR protein, P450*scc* and cyclin-B gene expression at day 14 after exposure. This pattern is generally described as increasing from 0 (control) to 1 and 10 μM, and decreased at 50 and 100 μM. The observed changes in the StAR protein, P450*scc* and cyclin-B levels showed a direct relationship with changes in 11-KT levels at day 14 after exposure. Cellular localization of StAR and P450*scc* were specific to the follicular cells of previtellogenic oocytes, but with no differences in staining intensities. No significant change in oocyte diameter was observed between the exposure groups. Our data reveal some novel aspects of nonylphenol effects on maturation and oocyte growth in teleosts, suggesting impaired steroidogenesis and hormonal imbalance with potential consequences for the vitellogenic process and overt fecundity.

Keywords: Steroidogenesis; StAR; P450*scc*; Cyclin-B; Nonylphenol; Endocrine disruption; Previtellogenic oocytes; Atlantic cod; *In vitro* system

1. Introduction

The synthesis and regulation of steroid hormones, such as estrogens and androgens, involve a vast number of enzymes and potential biochemical pathways. Generally, it is believed that the true rate-limiting step in acute steroid production is the movement of cholesterol across the mitochondrial membrane by the steroidogenic acute regulatory (StAR) protein, and the subsequent conversion to pregnenolone by cytochrome P450-mediated side-chain cleavage enzyme (P450*scc*) (Stocco, 1997, 2001a). The StAR protein and P450*scc* have been localized in most steroidogenic organs or tissues, including the ovaries, and are rapidly synthesized in response to acute tropic hormone stimulation. Estrogens (17β-estradiol) and androgens (testosterone and 11-ketotestosterone) are known to be involved in a number of physiological functions such as sexual differentiation, ion and carbohydrate homeostasis, adaptation to stress, immune system functioning and reproduction (Dean and Sanders, 1996).

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Prior to vitellogenesis (previtellogenesis), androgens may play an integral role in the regulation of oocytes (immature eggs) growth. Preliminary evidence supporting these assumptions are derived from the induction of oocyte growth in *in vitro* androgen exposed immature female eels, probably by gene regulation mechanisms (genomics) or by protein modification mechanisms (proteomics) (Rohr et al., 2001). It was demonstrated that 11-ketotestosterone (11-KT, the active male-specific androgen in teleosts) induces an increase in the diameter and development of previtellogenic eel oocytes (Rohr et al., 2001). Generally, androgens appear to play a pivotal role in stimulating the growth of small ovarian follicles in vertebrates, at least in mammals and fish (Weil et al., 1998, 1999; Rohr et al., 2001). The effects of androgens on follicle development are common, despite the diversity in follicular development in these two species. For example, the addition to the pool of developing oocytes through oogonial proliferation in fish is a lifetime continuous process (Tyler and Sumpter, 1996), whereas oocyte numbers in mammals are fixed at birth (Hsueh et al., 2000). However, several recent studies have shown that oocytes can be formed in adult mouse and humans (Johnson et al., 2004; Bukovsky et al., 2005).

Oocyte maturation is a complex process that is triggered by the maturation-promoting factor (MPF) and during this process, cyclin-B functions as a regulatory factor (Kondo et al., 1997). Thus, the accumulation of cyclin-B in oocytes controls the timing of early embryonic cell cycle (Aegerter et al., 2004). It has been shown that natural occurring steroids as well as endocrine-disrupting chemicals (EDCs) are able to affect cyclin-B synthesis (Kudo et al., 2004; Tokumoto et al., 2004, 2005). Thus, gene expression patterns of cyclin-B may be used as a marker for oocyte developmental competence.

A large number of anthropogenic chemicals released into the environment may disrupt endocrine homeostasis in humans and animals by interfering with developmental processes and endocrine systems (Colborn and Clement, 1992). These anthropogenic chemicals may cause hormonal imbalance with potential consequences for fecundity and reproduction (Gale et al., 2004). A number of structurally diverse environmental chemicals such as atrazine (Danzo, 1997; Hayes et al., 2002), ROUNDUP^{TM} herbicide (Walsh et al., 2000a) and pharmaceuticals such as letrozole (Assikis and Buzdar, 2002, 2004) and ketoconazole (Miranda et al., 1998; Hegelund et al., 2004) are known to modulate enzymes or proteins involved in steroidogenesis. The majority of xenoestrogens and natural estrogens that have endocrine disrupting effects exert their actions through modulation of steroid pathways (Jalabert et al., 2000; Monod et al., 2004). However, the mechanisms of action of these xenoestrogens are not well understood and have not been well investigated with regard to ovarian steroidogenic pathways. Recently, we showed that nonylphenol modulated brain steroidogenic enzymes (StAR, P450*scc* and CYP11β) and the biotransformation enzymes, CYP1A1 and CYP3A (Arukwe, 2005).

Nonylphenol (NP) is a degradation product of alkylphenol polyethoxylates (APEs) that are widely used as raw materials for active surface agents (anionic surface active agent, nonionic surface active agent), ethyl cellulose stabilizers, oil soluble phenyl resins, esters. It is also used as processed articles for detergents, oil varnishes, rubber auxiliaries and vulcanization accelerators, antioxidants and corrosion inhibitors for petroleum products, sludge generation inhibitors for petroleum (Khim et al., 2001). The ovary is a known target organs for endocrine disrupters, and it is well established that NP modulates reproductive responses like zonagenesis and vitellogenesis (Arukwe et al., 1997b, 2000; Folmar et al., 2002). The effects of NP on the basal internal signals that regulate early ovarian growth prior to vitellogenesis (previtellogenesis), fecundity, reproduction and maturation are generally not known. In the present study, we present the development of Atlantic cod previtellogenic oocyte *in vitro* culture system, histological and molecular methods for evaluating the effects of EDCs (such as nonylphenol) on the rate-limiting (StAR and P450*scc*) steroidogenic pathways. In addition, gene expression pattern of cyclin-B was studied, due to cyclin-B's role as an indicator of oocyte growth in juvenile fish. The Atlantic cod is a popular species with large economic value and the farming potential on a global basis is, therefore, regarded as extremely high. In addition, cod is also the main marine fish species for coastal water pollution monitoring (Cole, 1979; Goksøyr et al., 1994; Martin-Skilton et al., 2006; Sturve et al., 2006). Some studies indicate that alkyl phenols released from offshore platforms along with production water may affect the hormonal balance and reproductive capacity of cod (Meier et al., 2002). The main objectives of this study were (1) to develop an *in vitro* model ovarian system for studying the effects of endocrine disrupting chemicals and (2) evaluate the effects of nonylphenol on previtellogenic oocyte growth and gene expression patterns. We hypothesize that exposure of previtellogenic oocytes to different concentrations of nonylphenol will cause differential gene expression patterns, whose functional products modulate steroidogenesis and early oocyte growth. These responses will indicate adverse physiological effects, impaired steroidogenesis with potential consequences for overt fecundity and serve as sensitive and mechanistic markers of effect for EDCs.

2. Materials and methods

2.1. Chemicals and reagents

4-Nonylphenol (NP; 85% of *p*-isomers) was purchased from Fluka chemika-biochemika (Buchs, Switzerland). The impurities in 4-nonylphenol consist mainly of phenol (8–13%), tripropylene (~1%) and 2,4-dinonylphenol (~1%). Trizol reagent for RNA purification, TA cloning kit and Leibovitz L-15 medium were purchased from Gibco-Invitrogen life technologies (Carlsbad, CA, USA). Bovine serum albumin (BSA) and *N*-[2-hydroxyethyl]piperazine-*N* ′-[2-ethanesulfonic acid] (Hepes) were purchased from Sigma Chemical (St. Louis, MO, USA). Iscript cDNA Synthesis Kit and iTaq™Sybr® Green supermix with ROX were purchased from Bio-Rad laboratories (Hercules, CA, USA). Generuler™

100 bp DNA ladder and dNTPs from Fermentas GmbH (Germany), RNA later were purchased from Ambion (Austin, TX, USA). Superpicture™ polymer detection kit (Cat. No. 87-9263) was purchased from Zymed (San Francisco, CA, USA), and Tissue-clear® and Tissue-mount was purchased from Sakura Finetek Europe (Zoeterwoude, The Netherlands). 11-Ketotestosterone and estradiol-17β enzyme immuno-assay (EIA) kits (Cat. Nos. 582251 and 582751) were purchased from Cayman chemical company (Ann Arbor, MI, USA). All other chemicals were of the highest commercially available grade.

2.2. Animals and culture technique

Juvenile female Atlantic cod of 200–250 g body weight were purchased from Akvaforsk genetic center (Sunndalsøra, Norway) and kept in circulating water at 10 °C and a 12 h light:12 h dark photoperiod. The *in vitro* organ culture techniques employed were based on the agarose floating method described by Nader and coworkers (1999). Briefly, juvenile female cod were anesthetized, sacrificed and washed in 70% ethanol. Ovaries were removed, cut into small pieces (1 × 1 × 1 mm) and grown in 6-well dishes on a floating agarose substrate covered with a nitrocellulose membrane in basal culture media. The basal culture medium consisted of Leibovitz L-15 medium supplemented with 0.1 mM L-glutamic acid, 0.1 mM L-aspartic acid, 1.7 mM L-proline, 0.5% BSA and 10 mM Hepes (pH 7.4). Tissue preparation and culture were performed under strict sterile conditions without antibiotics, because antibiotics may have the potential of affecting the gene expression patterns. The gonadal tissue was cultured for 4, 7, 14 and 21 days with different concentrations of NP dissolved in ethanol at 0 (control), 1, 10, 50 and 100 μM in a humidified incubator at 10 °C. The control group received ethanol and the final concentration of ethanol in all exposure groups never exceeded 0.3% (v/v). The medium was changed every 7 days after sampling at day 7, 14 and 21 after exposure. After cultivation, tissues for RNA purification were homogenized directly in Trizol reagent and stored at −80 °C until further processing. Tissues for immunohistochemical analyses were placed in tissue cassettes with a nylon mesh and fixed, whereas the tissues for steroid analyses were flash-frozen in liquid nitrogen and stored at −80 °C until further processing.

2.3. RNA purification and cDNA synthesis

Total RNA was purified from tissues homogenized in Trizol reagent according to established procedures (Chirgwin et al., 1979; Chomczynski and Sacchi, 1987), and RNA concentrations were determined using a NanoDrop® ND-1000 UV–visible Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total cDNA for the real-time polymerase chain reaction (PCR) were generated from 1 μg total RNA in all samples using poly-T primers from iScript cDNA synthesis kit as described by the manufacturer (Bio-Rad).

2.4. StAR and P450scc antibody production and purification

Polyclonal antibodies for the StAR protein and P450*scc* were produced by immunization of rabbits with synthetic peptides for both proteins (Eurogentec, Searing, Belgium). The peptide sequences were; (StAR) H_2N–MPE QRG VVR AEN GPT C–$CONH_2$ and (P450*scc*) H_2N–CLL KNG EDW RSN RVI L–$CONH_2$ with respective molecular weights of 1743.97 and 1916.2 (kilodalton, kDa), and respective isoelectric point (p*I*) of 6.45 and 9.45. Rabbits were immunized once a week with the synthetic peptides and were bled after the fourth boost. The resulting sera were purified using Hitrap rProtein A affinity column (Amersham, Uppsala, Sweden). Briefly, serum was diluted 1:4 in binding buffer (20 mM sodium phosphate, pH 7.0) after filtration through a 0.45 μm filter. The column was washed with 5× column volume of 20% ethanol and binding buffer. Thereafter, the diluted serum was applied with a flow rate of 1 ml/min. The column was then washed with 5× column volume of binding buffer, and the purified antibody was eluted with 0.1 M sodium citrate buffer (pH 3.5) into tubes containing 80 μl of 1 M Tris–HCl (pH 9.0). Protein concentrations were determined using a NanoDrop® ND-1000 UV–visible Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.5. Primer optimization, cloning and sequencing

PCR primers for amplification of 150–246 bp gene-specific PCR-products were designed from conserved regions of the studied genes. The primer sequences, their amplicon size and the optimal annealing temperatures are shown in Table 1. Prior to PCRs, all primer pairs were used in titration reactions in order to determine optimal primer pair concentrations and their optimal annealing temperatures. All chosen primer pair concentrations used at the selected annealing temperatures gave a single band pattern for the expected amplicon size in all reactions. PCR products from the genes to be investigated were cloned into pCRII vector in INVF' *Escherichia coli* (Invitrogen). Each plasmid was sequenced using ABI-prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Department of Biology, NTNU, Norway. Sequences were confirmed using NCBI nucleotide BLAST software (http://www.ncbi.nlm.nih.gov/BLAST).

2.6. Quantitative (real-time) PCR

Quantitative (real-time) PCR was used for evaluating gene expression profiles. For each treatment, the expression of individual gene targets was analyzed using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA). The following concentrations of forward and reverse primers were used for each 25-μl real-time PCR: StAR, P450*scc* and cyclin-B at 600 nM each. Each 25-μl DNA amplification reaction contained 12.5 μl 2× SYBR Green mix (Stratagene), 0.75 μl of 1 mM ROX (reference dye), and 1 μl cDNA. The real-time PCR program included an enzyme activation step at 95 °C (10 min) and 40 cycles of 95 °C (30 s), 52 °C (StAR), 55 °C (P450*scc*), 58 °C (cyclin-B) (1 min) and 72 °C (30 s). Controls lacking cDNA template or *Taq* DNA polymerase were included to determine the specificity of target cDNA amplification. Cycle threshold (*Ct*) values obtained were converted into copy number using standard plots of *Ct* versus log copy number. Standard plots for each target sequence were generated using known amounts of plasmid containing the amplicon of interest. Triplicate data obtained for target cDNA amplification were averaged, normalized to gene copies and expressed as percent of control.

2.7. Histology and immunohistochemistry

Cultured previtellogenic gonadal tissues were fixed in 4% *para*-formaldehyde in 0.1 mM sodium phosphate buffer (pH 7.4) for 24 h. After fixation, the tissues were washed twice in 70% ethanol, dehydrated in a graded series of ethanol baths using an automatic tissue processor (Leica Microsystems, Nussloch, Germany), cleared in Tissue-Clear®, embedded in paraffin blocks and sectioned at 5 μm. Immunohistochemical analysis was

Table 1
Primer pair sequences, amplicon size and annealing temperature conditions for genes of interest used for real-time RT-PCR

Target gene	Primer sequence[a]		Amplicon size (nucleotides)	Annealing temperature (°C)
	Forward	Reverse		
StAR	CAACGTCAAGCAGGTCAAGA	ATAGTGTTGAAGCCCGATGC	246	52
P450*scc*	GATGCAGCTCTTCCTCATCC	AGACGCCCATCATATTGACC	170	55
Cyclin-B	CGGGAGATGGAGATGACTGT	CTGCATGGTGGACTACGAGA	150	58

[a] Sequences are given in the 5′-3′order.

preformed using SuperPicTure™ polymer detection kit (Zymed, Cat. No. 87-9263) according to manufacturer's protocol. In brief, sections were deparaffinized, rehydrated in graded series of ethanol and incubated for 1 h with rabbit polyclonal antibody against the StAR protein and P450*scc* diluted 1:200. After subsequent washing in PBS, sections were incubated with a HRP polymer-conjugated anti-rabbit secondary antibody for 10 min. Colour visualizations were performed by incubation with DAB chromogen for 5 min, and hematoxylin was used as a counterstain. Control stains were performed by omission of the primary antibody. Sections were mounted using Tissue-Mount, and relative intensity and localization of the StAR protein and P450*scc* staining were evaluated using light microscopy.

2.8. Steroid hormone analysis

Estradiol-17β and 11-ketotestosterone concentrations were measured in cultured gonad tissue using enzyme immunoassay (EIA) kits from Cayman Chemical Company (Ann Arbor, MI, USA). Tissues cultured for 7 and 14 days were used for steroid hormone determinations. Tissues stored at −80 °C were thawed on ice, homogenized in 1:4 volume of 0.1 M Na phosphate buffer and centrifuged at 10,000*g* for 15 min. The supernatants were purified by extraction using organic solvent to prevent the interference of lipids and proteins in the analysis. Briefly, the supernatant was mixed with 5 ml diethyl ether using a vortex mixer. After phase separation, the aqueous portion was frozen in an ethanol/dry ice bath. The lipophilic phase was decanted into a clean tube, and the ether phase was evaporated using a vacuum centrifuge. The dry extract was reconstituted in 300 µl EIA buffer by vortexing. Enzyme immunoassays were run as recommended by Cayman with a development time of 75 min. Data were quantified against a standard curve that was linearized using a logit transformation of B/B_0 (bound sample/maximum bound).

2.9. Statistical analyses

Statistical analysis was performed with GraphPad Prism, version 2.1 (GraphPad Software Inc., 1996). Significant differences between control and exposure groups were performed using one-way ANOVA. Statistical differences between exposure groups were analyzed using the Tukey's Multiple Comparison Test. The level of significance was set at $p = 0.05$ unless otherwise stated.

3. Results

3.1. Modulation of StAR protein and P450scc expressions

The expression of the StAR protein in cod previtellogenic oocyte was modulated after exposure to different concentrations of nonylphenol in a time-specific manner (Fig. 1). At day 7 post-exposure, nonylphenol produced an apparent time-dependent increase (albeit not significant; Fig. 1A). On the contrary, the StAR protein showed a 2- and 4-fold increase after exposure to 1 and 10 µM of nonylphenol and thereafter, a 2-fold reduction was observed at 50 and 100 µM at day 14 compared to control (Fig. 1B). A similar pattern of expression was observed at day 21 after exposure, where nonylphenol produced a 4-fold increase at 1 µM, and thereafter a concentration-specific reduction of the StAR protein (Fig. 1C). No differences between exposure groups were observed at day 4 post-exposure (data not shown).

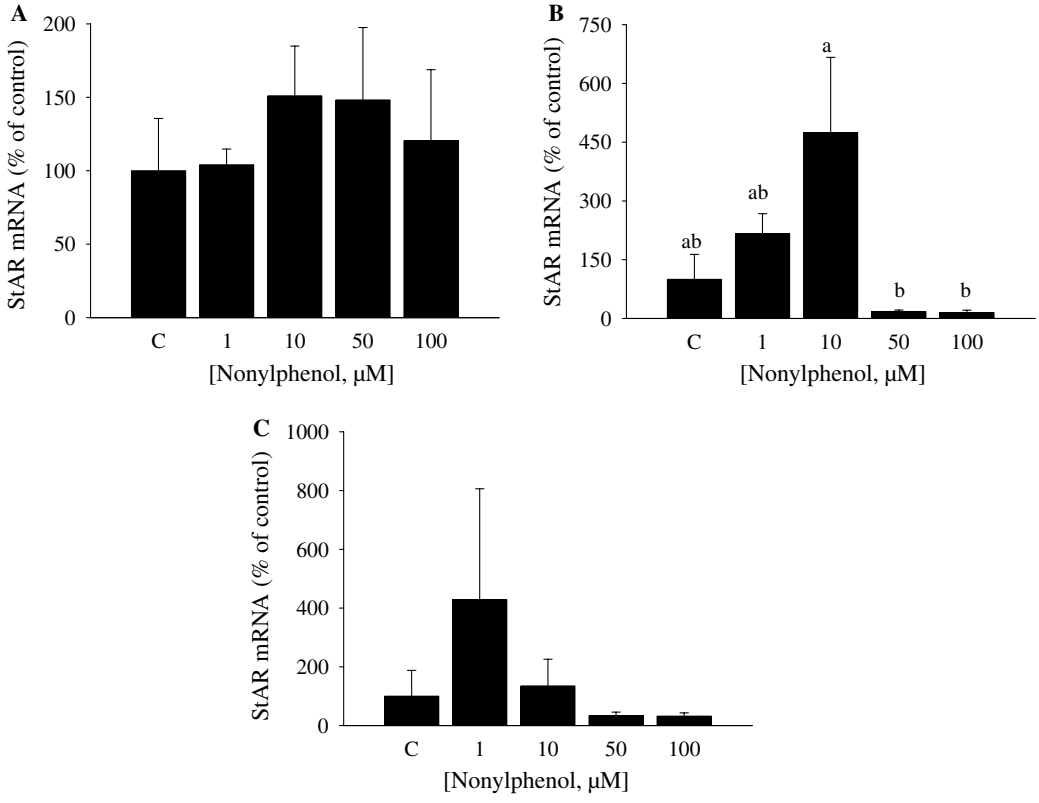

Fig. 1. Expression of the StAR protein mRNA levels in cultured previtellogenic oocytes of Atlantic cod exposed to different concentrations of nonylphenol (0 (control), 1, 10, 50 and 100 µM) and sampled at day 7 (A), 14 (B) and 21 (C) after exposure. Messenger RNA (mRNA) levels were quantified using real-time PCR with specific primer pairs. Data are given as mean expressed as percentage (%) of control ($n = 3$) ± standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p < 0.05$), analyzed using ANOVA followed by Tukey's multiple comparison test.

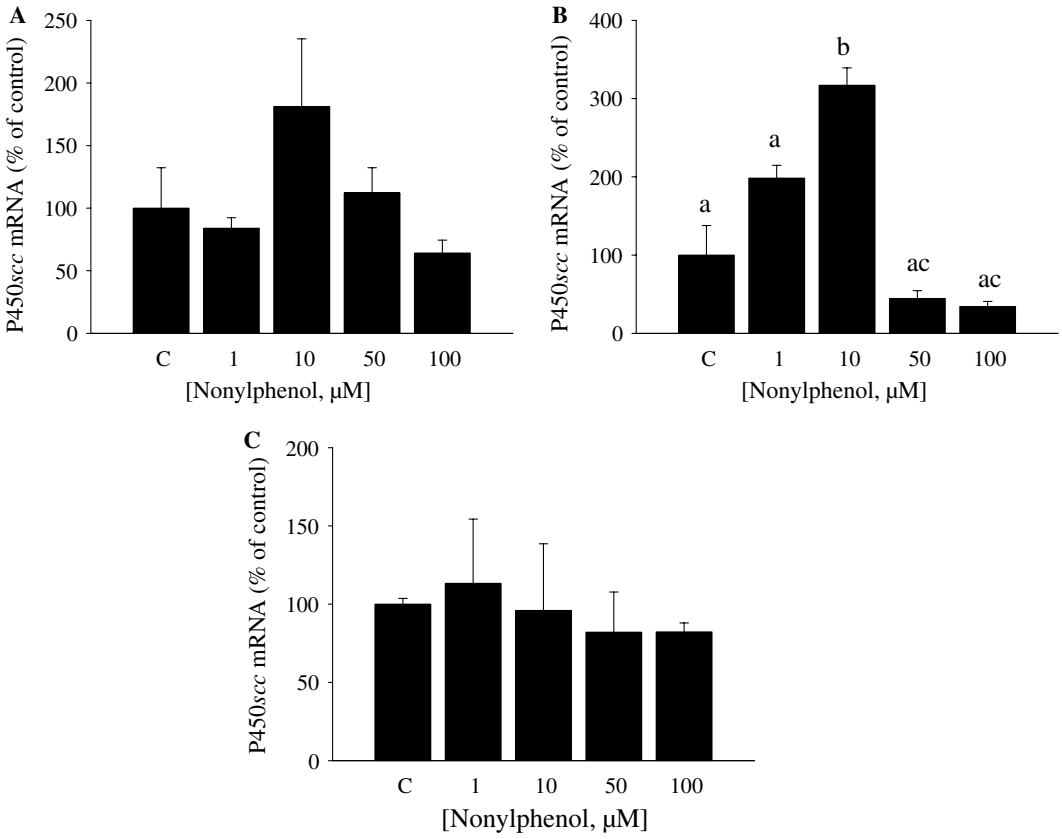

Fig. 2. Expression of P450*scc* mRNA levels in cultured previtellogenic oocytes of Atlantic cod exposed to different concentrations of nonylphenol (0 (control), 1, 10, 50 and 100 μM) and sampled at day 7 (A), 14 (B) and 21 (C) after exposure. Messenger RNA (mRNA) levels were quantified using real-time PCR with specific primer pairs. Data are given as mean expressed as percentage (%) of control ($n = 3$) ± standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p < 0.05$), analyzed using ANOVA followed by Tukey's multiple comparison test.

The expression of P450*scc* in cod previtellogenic oocyte was modulated after exposure to different concentrations of nonylphenol in a time-specific manner (Fig. 2). At day 7 post-exposure (Fig. 2A), a time-dependent increase was observed after exposure to 10 μM of nonylphenol, and thereafter nonylphenol exposure caused a concentration-specific non-significant reduction of P450*scc*. However, P450*scc* mRNA levels showed a 1- and 3-fold increase after exposure to 1 and 10 μM of nonylphenol and thereafter, a 1-fold reduction was observed at 50 and 100 μM at day 14 compared to control (Fig. 2B). A similar pattern of expression was observed at day 21 after exposure, where nonylphenol produced an increase at 1 μM, and thereafter a concentration-specific reduction of P450*scc* (albeit not significant; Fig. 2C). No differences between exposure groups were observed at day 4 post-exposure (data not shown).

3.2. Modulation of cyclin-B expressions

The expression of cyclin-B in cod previtellogenic oocyte was modulated after exposure to different concentrations of nonylphenol in a time-specific manner (Fig. 3). At day 7 post-exposure, nonylphenol produced a minor, but non-significant concentration-specific increase of cyclin-B (Fig. 3A). In contrast, cyclin-B showed a 4-fold increase after exposure to 10 μM of nonylphenol and thereafter, an almost total inhibition of cyclin-B transcription was observed at 50 and 100 μM at day 14, compared to control (Fig. 3B). A similar pattern of expression was observed at day 21, where nonylphenol produced an increase at 1 μM, and thereafter a concentration-specific reduction of cyclin-B was observed at 50 and 100 μM (albeit not significant; Fig. 3C). No differences between exposure groups were observed at day 4 post-exposure (data not shown).

3.3. Immunohistochemical analysis of the StAR protein and P450scc

The cellular localization of the StAR protein and P450*scc* in cultured previtellogenic oocytes of cod exposed to different concentrations of nonylphenol and sampled at day 14 after exposure is shown in Fig. 4 (StAR) and 5 (P450*scc*). Using rabbit polyclonal antibodies prepared against synthetic peptide sequences, the cellular localization of StAR and P450*scc* were mainly observed in follicular cells of oocyte membranes in both control and exposed tissues (Figs. 4 and 5). The relative intensity of both proteins was generally very low and did not show significant differences between control and nonylphenol exposed groups. In addition, no significant differences in staining intensity

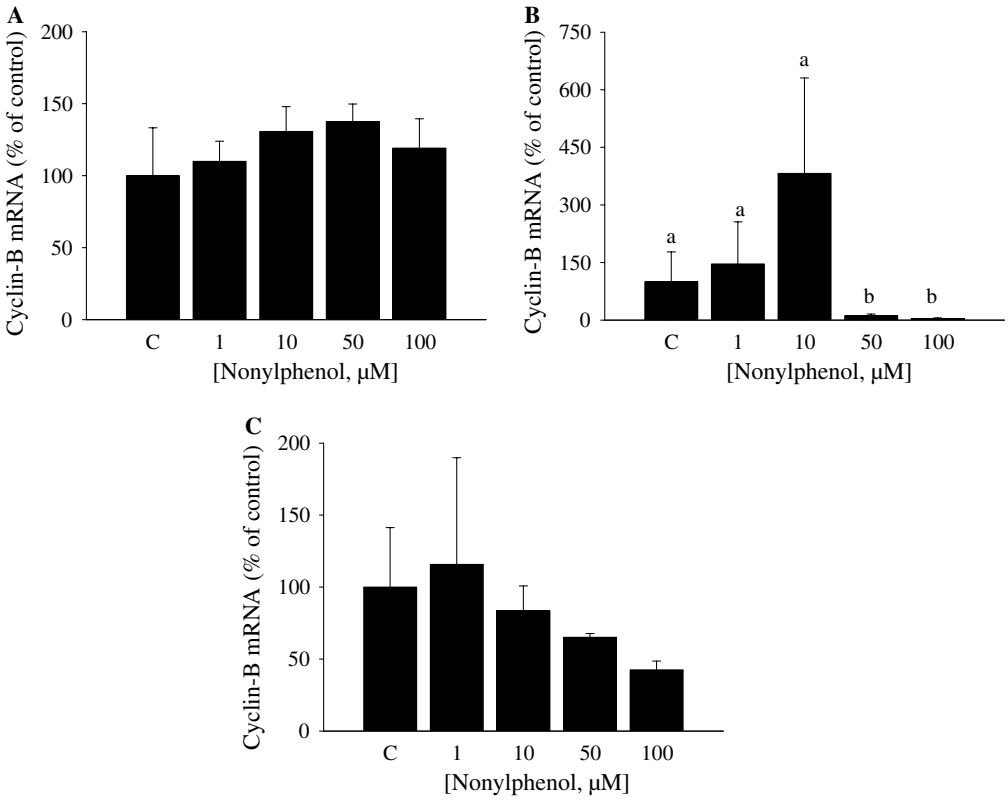

Fig. 3. Expression of cyclin-B mRNA levels in cultured previtellogenic oocytes of Atlantic cod exposed to different concentrations of nonylphenol (0 (control), 1, 10, 50 and 100 μM) and sampled at day 7 (A), 14 (B) and 21 (C) after exposure. Messenger RNA (mRNA) levels were quantified using real-time PCR with specific primer pairs. Data are given as mean expressed as percentage (%) of control ($n = 3$) ± standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p < 0.05$), analyzed using ANOVA followed by Tukey's multiple comparison test.

between day 7 and 14 were observed (data not shown). Evaluation of oocyte morphology and size revealed that previtellogenic oocytes had a diameter of approximately 300 μm. However, the mean size of the previtellogenic oocytes did not show significant differences after nonylphenol exposure.

3.4. Modulation of estradiol-17β and 11-ketotestosterone levels

Estradiol-17β (E2) and 11-ketotestosterone (11-KT) concentrations were measured in gonad tissue culture exposed to different concentrations of nonylphenol for 7 and 14 days. E2 levels after 7 and 14 days exposure to nonylphenol concentrations is shown in Fig. 6A and B, respectively. At day 7 post-exposure, E2 showed a 1-fold induction in the group exposed to 1 μM nonylphenol, compared to solvent control group (Fig. 6A). Thereafter, apparent concentration-specific reductions of E2 levels were observed (Fig. 6A). At day 14 post-exposure, a 1-fold reduction in E2 level was observed in the groups exposed to 50 and 100 μM compared to the solvent control group (Fig. 6B). 11-KT levels in cod previtellogenic oocytes exposed to nonyphenol for 7 and 14 days are shown in Fig. 6C and D, respectively. At day 7 post-exposure, 11-KT showed a 0.6-fold increase in the group exposed to 100 μM nonylphenol, compared to solvent control group (Fig. 6C). In contrast, exposures to 1, 10 and 50 μM produced respective 1-fold reduction of 11-KT levels (Fig. 6C). At day 14 post-exposure, a 2.5-fold increase in 11-KT level was observed in the group exposed to 10 μM nonylphenol (Fig. 6D). In the groups exposed to 50 and 100 μM, respective 0.7- and 0.4-fold reductions in 11-KT levels were observed (Fig. 6D).

4. Discussion

Despite popular demand from regulatory agencies, bioassays for evaluating the effect of endocrine disruptors have the potential to overlook chemicals that can modulate estrogenic responses through mechanisms that does not involve direct binding to the estrogen receptors (ERs). In addition to their effect as direct agonist to the ERs, the effects of xenoestrogens, such as nonylphenol, are sometimes interpreted as interference with steroidogenesis and with the steroidal regulation of the normal development and function of the male and female reproductive tracts (Colborn et al., 1993; Danzo, 1997). One potentially important mechanism is the impairment of steroidogenic enzyme activity and/or protein expressions. Recently, we showed that nonylphenol produced estrogenic effects via transcriptional modulation of the StAR protein and P450*scc* expres-

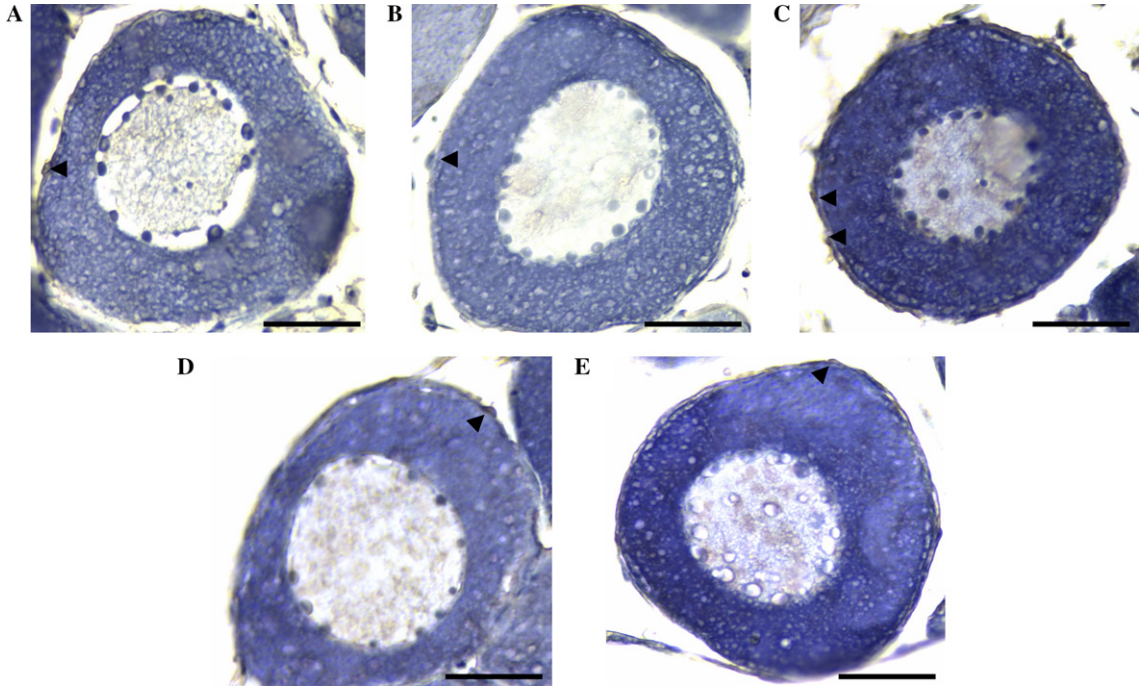

Fig. 4. Cellular localization of the StAR protein in cultured previtellogenic oocytes of Atlantic cod exposed to different concentrations of nonylphenol (A, control; B, 1 μM; C, 10 μM; D, 50 μM; E, 100 μM) and sampled at day 14 after exposure. The figures are representative histological section, as there were not significant differences between the exposure groups. Arrows indicate follicle cells with positive StAR staining. Bar equals 100 μm.

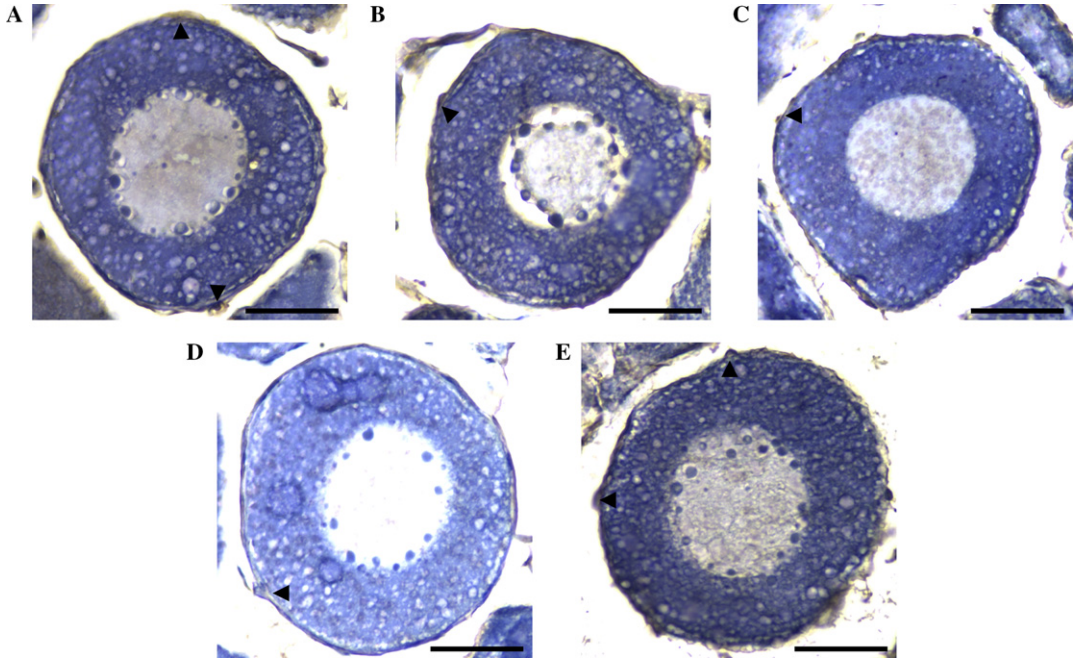

Fig. 5. Cellular localization of P450*scc* in cultured previtellogenic oocytes of Atlantic cod exposed to different concentrations of nonylphenol (A, control; B, 1 μM; C, 10 μM; D, 50 μM; E, 100 μM) and sampled at day 14 after exposure. The figures are representative histological section, as there were not significant differences between the exposure groups. Arrows indicate follicle cells with positive P450*scc* staining. Bar equals 100 μm.

sions in Atlantic salmon (*Salmo salar*) brain (Arukwe, 2005). Because estrogens play significant roles in sex determination and differentiation, the enzymes and proteins involved in steroid hormone synthesis are of critical endocrine significance. Furthermore, the Atlantic cod is not a well-studied species neither from an endocrinological or toxicological standpoint. Indeed, this species is of enormous economic importance both in Norway and internationally. Therefore, the present study was performed in order to develop an oocyte culture system and determine

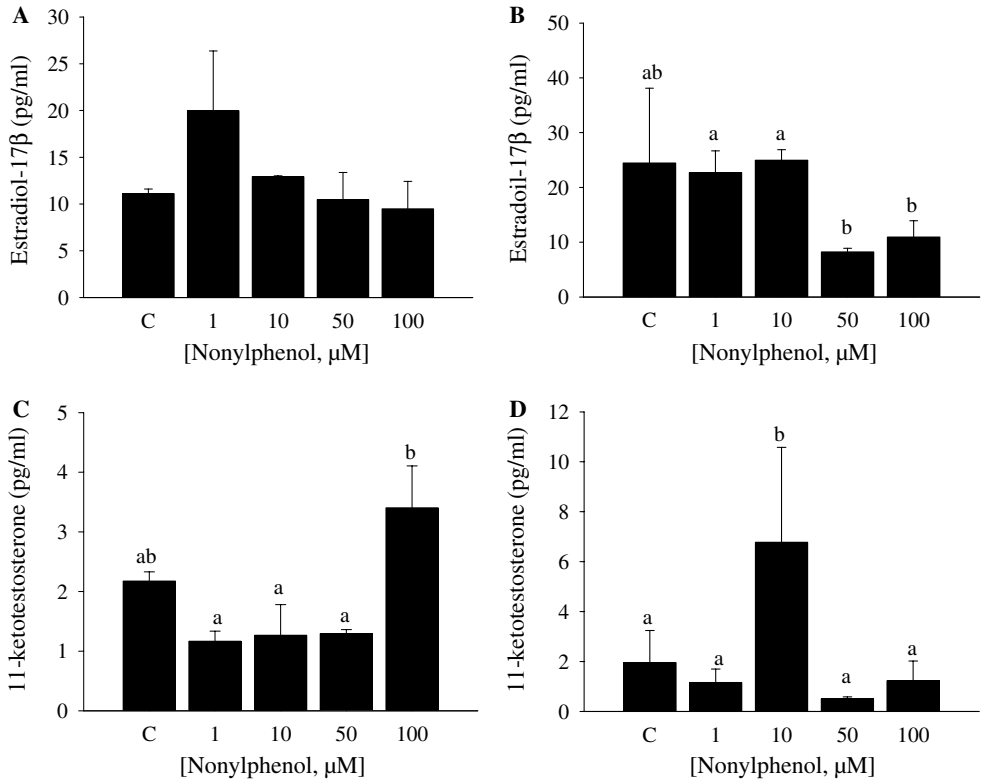

Fig. 6. Estradiol-17β levels (E2) at days 7 and 14 (A and B, respectively) and 11-ketotestosterone (11-KT) levels at days 7 and 14 (C and D, respectively) in cultured previtellogenic oocytes of Atlantic cod exposed to different concentrations of nonylphenol. Steroid hormone levels were determined using enzyme immunoassay method. Data are given as mean value and expressed as pg/ml of $n = 3 \pm$ standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p < 0.05$), analyzed using ANOVA followed by Tukey's multiple comparison test.

the effects of nonylphenol on the expression patterns of protein (StAR) and enzyme (P450*scc*) responsible for the rate-limiting step in steroidogenesis in ovary tissue from previtellogenic Atlantic cod. In addition, the effects of nonylphenol on cyclin-B were studied. We show that nonylphenol modulates the StAR protein, P450*scc* and cyclin-B gene expressions and that these effects were dependent on time and nonylphenol concentration. Interestingly, the nonylphenol-induced decreases of the StAR protein and P450*scc* were consistent with decreased levels of both oocyte E2 and 11-KT levels, showing a direct relationship with 11-KT levels. Thus, the present study reveals some novel aspects of nonylphenol effects on maturation and oocyte growth in teleosts. The impaired steroidogenesis and hormonal imbalance reported in the present study may have potential consequences for the vitellogenic process and overt fecundity in teleost.

4.1. Effects on the StAR protein and P450scc gene expression and steroid hormone levels

The present study shows that both the StAR protein and P450*scc* were modulated by nonylphenol concentrations in a time-specific manner. Although the expression of the StAR protein and P450*scc* were affected at all exposure times, day 14 post-exposure produced a unique pattern of effect. At this exposure time, cultured cod previtellogenic oocytes showed concentration-specific increases at lower concentrations (1 and 10 μM) and decreases at higher concentrations (50 and 100 μM). This unique pattern of nonylphenol modulation of the StAR protein and P450*scc* genes suggests a negative feedback control mechanism for oocyte regulation of steroidogenesis. It should be noted that the nonylphenol mediated modulation of the StAR protein and P450*scc* transcriptional changes at day 14 post-exposure showed a perfect correlation with changes in 11-KT levels at the same time interval and concentrations. Regardless of organ or tissue, the delivery of cholesterol from the outer mitochondrial membrane by the StAR protein to the inner mitochondrial membrane, where P450*scc* resides, is the ultimate rate-limiting step in steroid hormone biosynthetic process. This process may potentially affect testicular, ovarian and adrenocortical functions (Stocco, 2000, 2001b). Therefore, the perfect correlation between the StAR protein and 11-KT is in accordance with the understanding that the StAR protein plays a critical role in the regulation of hormonally induced acute steroid production by stimulating cholesterol transfer through hydrophobic tunnel structures formed within its molecule (Stocco, 2000, 2001b). Furthermore, these findings represent a novel aspect of nonylphenol as an endocrine disrupting chemical.

In this study, steroid hormone (E2 and 11-KT) levels were also evaluated in cod previtellogenic oocytes exposed to different concentration of nonylphenol. Our data show

that exposure to nonylphenol had severe effects on the levels of these naturally occurring steroid hormones in cultured cod previtellogenic oocytes. The most striking observation was the reduced levels of both E2 and 11-KT in oocyte cultures exposed to 50 and 100 μM nonylphenol at day 14 post-exposure. This suggests that nonylphenol modulates steroidogenesis by targeting the StAR protein. These findings are in compliance with previous *in vivo* studies, where a decrease in plasma E2 levels were observed after exposure to nonylphenol in several fish species, including cod (Arukwe et al., 1997a; Harris et al., 2001; Meier et al., 2002). It has been shown that plasma E2 levels are significantly elevated in relation to gonadal development and time of reproduction in female cod (Comeau et al., 2001; Dahle et al., 2003). Thus, our data are in accordance with the observations on the transcriptomic level, suggesting that nonylphenol may postpone the developmental process of previtellogenic oocytes. 11-KT is considered the main androgen in teleosts (Borg, 1994) and the effects of xenoestrogens on 11-KT is not well studied, at least not in cod. Androgens may increase oocyte diameter and modify growth factor receptors (Vendola et al., 1999; Rohr et al., 2001), but the underlying molecular mechanism(s) involved in possible gene regulation in the growth of previtellogenic oocytes has not been investigated and will be discussed below.

From an endocrinological and reproductive physiological standpoint, the direct correlation between the StAR protein, P450*scc*, and 11-KT represent a novel aspect on the role of androgens on the growth of previtellogenic oocytes. Recently, we have observed that aromatizable (testosterone) and non-aromatizable (11-KT and methyltestosterone) androgens cause differential gene expression patterns, whose functional products modulate previtellogenic oocyte growth in cod (Kortner and Arukwe, in preparation). The role of androgens in previtellogenic oocyte is supported by the observation that the early ovarian growth of species-specific critical stage progressed in hypophysectomized freshwater turtle, *Chrysemys picta*, indicating that gonadotropins (GtHs) were not necessary (Ho et al., 1982). Furthermore, Bieniarz and Kime (1986) were unable to demonstrate specific binding of radio labeled GtH (^{125}I-GtH) to previtellogenic common carp (*Cyprinus carpio*) ovaries. Elsewhere, Rohr and coworkers (2001) demonstrated that 11-KT (the active male-specific androgen in teleosts) induces an increase in the diameter and development of previtellogenic eel (*Anguilla australis*) oocytes. In mammals, androgens also modify the intra-ovarian gene expression in the rhesus monkey, as demonstrated by increased mRNA abundance of insulin-like growth factor-1 (IGF-1) and IGF-1-receptor (Vendola et al., 1999) in follicles up to early antral stage. Based on the above named studies, androgens appear to play a pivotal role in stimulating the growth of small ovarian follicles in vertebrates, at least in mammals and fish. Given that this happens despite the diversity in follicular development in these two species, the findings in the present study suggest a generalized effect of nonylphenol in vertebrates, at least with the methods applied in our study. For example, addition to the pool of developing oocytes through oogonial proliferation in fish is a lifetime continuous process (Tyler and Sumpter, 1996), whereas oocyte numbers in mammals are fixed at birth. Therefore, the effects of nonylphenol on early follicular growth in fish will provide an understanding on the role of xenoandrogens in the control of fecundity through modification of gene expression patterns. It may also be used in the evaluation of evolutionary conservation of the control mechanisms for early follicular growth in vertebrates.

Furthermore, the expression patterns in the StAR protein and P450*scc* are closely related. Exposure of previtellogenic oocyte tissue to the highest concentrations of nonylphenol (50 and 100 μM) produced an almost total transcriptional inhibition of the genes. Previously, it has been reported that the pesticides lindane and dimethoate can inhibit steroidogenesis by reducing the StAR protein expression (Walsh et al., 2000b). These observations suggest that other environmental estrogens may also inhibit steroidogenesis by targeting the StAR protein expression and/or other steroidogenic proteins or enzymes (Walsh et al., 2000b). Recently, we reported the modulations of brain steroidogenesis after exposure to nonylphenol and ethynylestradiol (EE2), where alterations of both StAR and P450*scc* mRNA levels were observed (Arukwe, 2005; Lyssimachou and Arukwe, 2006). Taken together, these observations strongly indicate that the StAR protein and P450*scc* may be important targets for environmental pollutants that disrupt steroidogenesis. However, not much is known concerning xenoestrogen-induced effects on steroidogenesis on the whole organism level and this should be studied in more detail. In this regard, the identification and full-length gene cloning of the StAR protein in Atlantic cod (Goetz et al., 2004) represents a promising sign in the understanding of xenoestrogen-induced toxicity of steroidogenesis in this important species.

4.2. Effects on cyclin-B gene expression

Another interesting finding in the present study is the consistency of nonylphenol mediated effects on steroidogenic genes and cyclin-B. Oocyte maturation is a complex process finally triggered by the maturation-promoting factor (MPF), in which cyclin-B functions as a regulatory subunit (Kondo et al., 1997). The expression of MPF is thought to be a response to stimulation on the oocyte surface by the maturation-inducing hormone (MIH). Previous reports has demonstrated that MIH stimulates the translation of cyclin-B mRNA stored in immature oocytes (Hirai et al., 1992). Furthermore, it is thought that cyclin-B accumulation in oocytes controls the timing of early embryonic cell cycle (Aegerter et al., 2004). It has been shown that natural occurring steroids as well as endocrine-disrupting chemicals (EDCs) are able to affect cyclin-B synthesis (Kudo et al., 2004; Tokumoto et al., 2004, 2005). Thus, gene expression of cyclin-B may be used as a sensitive marker of

oocyte developmental competence. The present study demonstrates that previtellogenic cod ovaries expressed cyclin-B, and that the expression is modulated by the xenoestrogen, nonylphenol in a time- and concentration-specific manner. Interestingly, the expression pattern follows the expression patterns observed for the StAR protein and P450*scc*. The only two compounds identified as naturally occurring MIH in fish are C21 steroids, 17α, 20β-dihydroxy-4-pregnen-3-one and 17α, 20 β, 21-trihydroxy-4-pregnen-3-one (Nagahama and Adachi, 1985; Patino and Thomas, 1990). With this in mind, one might speculate that the decreased expressions of the StAR protein and P450*scc* will lead to decreased levels of functional MIH in the cod ovary, and that this eventually will result in decreased levels of cyclin-B. Thus, nonylphenol may exhibit a potent cytotoxicity effect through cell degradation and/or delay in the maturation process. Nevertheless, these assumptions need to be further investigated in a different designed study.

5. Conclusions

The present study shows that *in vitro* exposure of previtellogenic cod ovaries to nonylphenol modulates the expression of the main steroidogenic protein (StAR) and enzyme (P450*scc*) in the rate-limiting step in acute steroid production, as well as the gene expression of cyclin-B. A unique pattern of expression was observed at day 14 post-exposure, where concentration-specific effects were observed. Interesting, the expression patterns for the StAR protein and P450*scc* showed a direct relationship with 11-KT levels. These results reveal some novel aspects of NP effects on maturation and oocyte growth in teleosts, suggesting impaired steroidogenesis and hormonal imbalance with potential consequences for the vitellogenic process. The Atlantic cod is a popular species with large economic value and therefore the aquaculture potential on a global basis is therefore regarded as extremely high. In addition, cod is also the main marine fish species in Norway for coastal water pollution monitoring (Goksøyr et al., 1994; Martin-Skilton et al., 2006; Sturve et al., 2006). The National Joint Assessment and Monitoring Programme (JAMP, OSPAR) have used this species in fjord monitoring since the beginning of the 1980s (Tromp and Wieriks, 1994). Norwegian offshore oil industry have recently adopted the Atlantic cod as a promising organism to be used in biomonitoring studies, particularly in relation to produced water discharges. Some studies indicate that alkyl phenols released from offshore platforms along with production water are capable of disturbing the hormonal balance and reproductive capacity of cod (Meier et al., 2002). These studies suggest that increased petroleum release in natural habitat of cod may cause a threat to the species.

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References

Aegerter, S., Jalabert, B., Bobe, J., 2004. Messenger RNA stockpile of cyclin B, insulin-like growth factor I, insulin-like growth factor II, insulin-like growth factor receptor Ib, and p53 in the rainbow trout oocyte in relation with developmental competence. Mol. Reprod. Dev. 67, 127–135.

Arukwe, A., 2005. Modulation of brain steroidogenesis by affecting transcriptional changes of steroidogenic acute regulatory (StAR) protein and cholesterol side chain cleavage (P450scc) in juvenile Atlantic salmon (Salmo salar) is a novel aspect of nonylphenol toxicity. Environ. Sci. Technol. 39, 9791–9798.

Arukwe, A., Celius, T., Walther, B.T., Goksoyr, A., 2000. Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic salmon (Salmo salar). Aquatic Toxicol. 49, 159–170.

Arukwe, A., Förlin, L., Goksoyr, A., 1997a. Xenobiotic and steroid biotransformation enzymes in Atlantic Salmon (Samlo Salar) liver treated with an estrogenic compound, 4-nonylphenol. Environ. Toxicol. Chem. 16, 2576–2583.

Arukwe, A., Knudsen, F.R., Goksoyr, A., 1997b. Fish zona radiata (eggshell) protein: a sensitive biomarker for environmental estrogens. Environ. Health Perspect. 105, 418–422.

Assikis, V.J., Buzdar, A., 2002. Recent advances in aromatase inhibitor therapy for breast cancer. Semin. Oncol. 29, 120–128.

Assikis, V.J., Simons, J.W., 2004. Novel therapeutic strategies for androgen-independent prostate cancer: an update. Semin. Oncol. 31, 26–32.

Bieniarz, K., Kime, D.E., 1986. Autoradiographic localization of gonadotrophin receptors in ovaries of the common carp, Cyprinus carpio L. Gen. Comp. Endocrinol. 64, 151–156.

Borg, B., 1994. Androgens in teleosts fishes. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 109, 219–245.

Bukovsky, A., Svetlikova, M., Caudle, M.R., 2005. Oogenesis in cultures derived from adult human ovaries. Reprod. Biol. Endocrinol. 3, 17.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., Rutter, W.J., 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294–5299.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Colborn, T., Clement, C. (Eds.), 1992. Chemically-induced alterations in sexual and functional development: the wildlife/human connection. Advances in Modern Environmental Toxicology.

Colborn, T., vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect. 101, 378–384.

Cole, H.A., 1979. Pollution of the sea and its effects. Proc. R. Soc. Lond. B Biol. Sci. 205, 17–30.

Comeau, L.A., Campana, S.E., Chouinard, G.A., Hanson, J.M., 2001. Timing of Atlantic cod Gadus morhua seasonal migrations in relation to serum levels of gonadal and thyroidal hormones. Mar. Ecol. Prog. Ser. 221, 245–253.

Dahle, R., Taranger, G.L., Karlsen, O., Kjesbu, O.S., Norberg, B., 2003. Gonadal development and associated changes in liver size and sexual steroids during the reproductive cycle of captive male and female Atlantic cod (Gadus morhua L.). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 136, 641–653.

Danzo, B.J., 1997. Environmental xenobiotics may disrupt normal endocrine function by interfering with the binding of physiological ligands to steroid receptors and binding proteins. Environ. Health Perspect. 105, 294–301.

Dean, D.M., Sanders, M.M., 1996. Ten years after: reclassification of steroid-responsive genes. Mol. Endocrinol. 10, 1489–1495.

Folmar, L.C., Hemmer, M.J., Denslow, N.D., Kroll, K., Chen, J., Cheek, A., Richman, H., Meredith, H., Grau, E.G., 2002. A comparison of the

estrogenic potencies of estradiol, ethynylestradiol, diethylstilbestrol, nonylphenol and methoxychlor in vivo and in vitro. Aquat. Toxicol. 60, 101–110.

Gale, W.L., Patino, R., Maule, A.G., 2004. Interaction of xenobiotics with estrogen receptors alpha and beta and a putative plasma sex hormone-binding globulin from channel catfish (Ictalurus punctatus). Gen. Comp. Endocrinol. 136, 338–345.

Goetz, F.W., Norberg, B., McCauley, L.A., Iliev, D.B., 2004. Characterization of the cod (Gadus morhua) steroidogenic acute regulatory protein (StAR) sheds light on StAR gene structure in fish. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 137, 351–362.

Goksøyr, A., Beyer, J., Husøy, A.M., Larsen, H.E., Westrheim, K., Wilhelmsen, S., Klungsøyr, J., 1994. Accumulation and effects of aromatic and clorinated hydrocarbons in juvenile Atlantic cod (*Gadus morhua*) caged in a polluted fjord (Sørfjorden, Norway). Aquat. Toxicol. 29, 21–35.

Harris, C.A., Santos, E.M., Janbakhsh, A., Pottinger, T.G., Tyler, C.R., Sumpter, J.P., 2001. Nonylphenol affects gonadotropin levels in the pituitary gland and plasma of female rainbow trout. Environ. Sci. Technol. 35, 2909–2916.

Hayes, T., Haston, K., Tsui, M., Hoang, A., Haeffele, C., Vonk, A., 2002. Herbicides: feminization of male frogs in the wild. Nature 419, 895–896.

Hegelund, T., Ottosson, K., Radinger, M., Tomberg, P., Celander, M.C., 2004. Effects of the antifungal imidazole ketoconazole on CYP1A and CYP3A in rainbow trout and killifish. Environ. Toxicol. Chem. 23, 1326–1334.

Hirai, T., Yamashita, M., Yoshikuni, M., Lou, Y.H., Nagahama, Y., 1992. Cyclin B in fish oocytes: its cDNA and amino acid sequences, appearance during maturation, and induction of p34cdc2 activation. Mol. Reprod. Dev. 33, 131–140.

Ho, S.M., Taylor, S., Callard, I.P., 1982. Effect of hypophysectomy and growth hormone on estrogen-induced vitellogenesis in the freshwater turtle, Chrysemys picta. Gen. Comp. Endocrinol. 48, 254–260.

Hsueh, A.J., McGee, E.A., Hayashi, M., Hsu, S.Y., 2000. Hormonal regulation of early follicle development in the rat ovary. Mol. Cell Endocrinol. 163, 95–100.

Jalabert, B., Baroiller, J., Breton, B., Fostier, A., Le Gac, F., Guiguen, Y., Monod, G., 2000. Main neuro-endocrine, endocrine and paracrine regulations of fish reproduction, and vulnerability to xenobiotics. Ecotoxicology 9, 25–40.

Johnson, J., Canning, J., Kaneko, T., Pru, J.K., Tilly, J.L., 2004. Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature 428, 145–150.

Khim, J.S., Lee, K.T., Kannan, K., Villeneuve, D.L., Giesy, J.P., Koh, C.H., 2001. Trace organic contaminants in sediment and water from Ulsan Bay and its vicinity, Korea. Arch. Environ. Contam. Toxicol. 40, 141–150.

Kondo, T., Yanagawa, T., Yoshida, N., Yamashita, M., 1997. Introduction of cyclin B induces activation of the maturation-promoting factor and breakdown of germinal vesicle in growing zebrafish oocytes unresponsive to the maturation-inducing hormone. Dev. Biol. 190, 142–152.

Kudo, C., Wada, K., Masuda, T., Yonemura, T., Shibuya, A., Fujimoto, Y., Nakajima, A., Niwa, H., Kamisaki, Y., 2004. Nonylphenol induces the death of neural stem cells due to activation of the caspase cascade and regulation of the cell cycle. J. Neurochem. 88, 1416–1423.

Lyssimachou, A., Arukwe, A., in press. Alteration of brain and interrenal StAR protein, P450scc and CYP11β mRNA levels in Atlantic salmon after nominal waterborne exposure to the synthetic pharmaceutical estrogen, ethynylestradiol. J. Toxicol. Environ. Health.

Martin-Skilton, R., Thibaut, R., Porte, C., 2006. Endocrine alteration in juvenile cod and turbot exposed to dispersed crude oil and alkylphenols. Aquat. Toxicol. 78 (Suppl 1), S57–S64.

Meier, S., Andersen, T.E., Hasselberg, L., Kjesbu, O.S., Klungsøyr, J., Svardal, A., 2002. Hormonal effects of C4–C7 alkylphenols on cod (Gadus Morhua), p. 70.

Miranda, C.L., Henderson, M.C., Buhler, D.R., 1998. Evaluation of chemicals as inhibitors of trout cytochrome P450s. Toxicol. Appl. Pharmacol. 148, 237–244.

Monod, G., Rime, H., Bobe, J., Jalabert, B., 2004. Agonistic effect of imidazole and triazole fungicides on in vitro oocyte maturation in rainbow trout (Oncorhynchus mykiss). Mar. Environ. Res. 58, 143–146.

Nader, M.R., Miura, T., Ando, N., Miura, C., Yamauchi, K., 1999. Recombinant human insulin-like growth factor I stimulates all stages of 11-ketotestosterone-induced spermatogenesis in the Japanese eel, Anguilla japonica, in vitro. Biol. Reprod. 61, 944–947.

Nagahama, Y., Adachi, S., 1985. Identification of maturation-inducing steroid in a teleost, the amago salmon (Oncorhynchus rhodurus). Dev. Biol. 109, 428–435.

Patino, R., Thomas, P., 1990. Gonadotropin stimulates 17 alpha,20 beta,21-trihydroxy-4-pregnen-3-one production from endogenous substrates in Atlantic croaker ovarian follicles undergoing final maturation in vitro. Gen. Comp. Endocrinol. 78, 474–478.

Rohr, D.H., Lokman, P.M., Davie, P.S., Young, G., 2001. 11-Ketotestosterone induces silvering-related changes in immature female short-finned eels, Anguilla australis. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 130, 701–714.

Stocco, D.M., 1997. A StAR search: implications in controlling steroidgenesis. Biol. Reprod. 56, 328–336.

Stocco, D.M., 2000. The role of the StAR protein in steroidogenesis: challenges for the future. J. Endocrinol. 164, 247–253.

Stocco, D.M., 2001a. StAR protein and the regulation of steroid hormone biosynthesis. Annu. Rev. Physiol. 63, 193–213.

Stocco, D.M., 2001b. Tracking the role of a star in the sky of the new millennium. Mol. Endocrinol. 15, 1245–1254.

Sturve, J., Hasselberg, L., Falth, H., Celander, M., Forlin, L., 2006. Effects of North Sea oil and alkylphenols on biomarker responses in juvenile Atlantic cod (*Gadus morhua*). Aquat. Toxicol. 78 (Suppl 1), S73–S78.

Tokumoto, T., Tokumoto, M., Horiguchi, R., Ishikawa, K., Nagahama, Y., 2004. Diethylstilbestrol induces fish oocyte maturation. Proc. Natl. Acad. Sci. USA 101, 3686–3690.

Tokumoto, T., Tokumoto, M., Nagahama, Y., 2005. Induction and inhibition of oocyte maturation by EDCs in zebrafish. Reprod. Biol. Endocrinol. 3, 69.

Tromp, D., Wieriks, K., 1994. The OSPAR convention: 25 years of north sea protection. Mar. Pollut. Bull. 29, 622–626.

Tyler, C.R., Sumpter, J.P., 1996. Oocyte growth and development in teleost. Rev. Fish Biol. Fisheries 6, 287–318.

Vendola, K., Zhou, J., Wang, J., Famuyiwa, O.A., Bievre, M., Bondy, C.A., 1999. Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary. Biol. Reprod. 61, 353–357.

Walsh, L.P., McCormick, C., Martin, C., Stocco, D.M., 2000a. Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. Environ. Health Perspect. 108, 769–776.

Walsh, L.P., Webster, D.R., Stocco, D.M., 2000b. Dimethoate inhibits steroidogenesis by disrupting transcription of the steroidogenic acute regulatory (StAR) gene. J. Endocrinol. 167, 253–263.

Weil, S., Vendola, K., Zhou, J., Bondy, C.A., 1999. Androgen and follicle-stimulating hormone interactions in primate ovarian follicle development. J. Clin. Endocrinol. Metab. 84, 2951–2956.

Weil, S.J., Vendola, K., Zhou, J., Adesanya, O.O., Wang, J., Okafor, J., Bondy, C.A., 1998. Androgen receptor gene expression in the primate ovary: cellular localization, regulation, and functional correlations. J. Clin. Endocrinol. Metab. 83, 2479–2485.

Doctoral theses in Biology
Norwegian University of Science and Technology
Department of Biology

Year	Name	Degree	Title
1974	Tor-Henning Iversen	Dr. philos Botany	The roles of statholiths, auxin transport, and auxin metabolism in root gravitropism
1978	Tore Slagsvold	Dr. philos. Zoology	Breeding events of birds in relation to spring temperature and environmental phenology.
1978	Egil Sakshaug	Dr.philos Botany	"The influence of environmental factors on the chemical composition of cultivated and natural populations of marine phytoplankton"
1980	Arnfinn Langeland	Dr. philos. Zoology	Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake.
1980	Helge Reinertsen	Dr. philos Botany	The effect of lake fertilization on the dynamics and stability of a limnetic ecosystem with special reference to the phytoplankton
1982	Gunn Mari Olsen	Dr. scient Botany	Gravitropism in roots of *Pisum sativum* and *Arabidopsis thaliana*
1982	Dag Dolmen	Dr. philos. Zoology	Life aspects of two sympartic species of newts (*Triturus, Amphibia*) in Norway, with special emphasis on their ecological niche segregation.
1984	Eivin Røskaft	Dr. philos. Zoology	Sociobiological studies of the rook *Corvus frugilegus.*
1984	Anne Margrethe Cameron	Dr. scient Botany	Effects of alcohol inhalation on levels of circulating testosterone, follicle stimulating hormone and luteinzing hormone in male mature rats
1984	Asbjørn Magne Nilsen	Dr. scient Botany	Alveolar macrophages from expectorates – Biological monitoring of workers exosed to occupational air pollution. An evaluation of the AM-test
1985	Jarle Mork	Dr. philos. Zoology	Biochemical genetic studies in fish.
1985	John Solem	Dr. philos. Zoology	Taxonomy, distribution and ecology of caddisflies (*Trichoptera*) in the Dovrefjell mountains.
1985	Randi E. Reinertsen	Dr. philos. Zoology	Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds.
1986	Bernt-Erik Sæther	Dr. philos. Zoology	Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach.
1986	Torleif Holthe	Dr. philos. Zoology	Evolution, systematics, nomenclature, and zoogeography in the polychaete orders *Oweniimorpha* and *Terebellomorpha*, with special reference to the Arctic and Scandinavian fauna.
1987	Helene Lampe	Dr. scient. Zoology	The function of bird song in mate attraction and territorial defence, and the importance of song repertoires.
1987	Olav Hogstad	Dr. philos. Zoology	Winter survival strategies of the Willow tit *Parus montanus*.
1987	Jarle Inge Holten	Dr. philos Botany	Autecological investigations along a coust-inland transect at Nord-Møre, Central Norway

1987	Rita Kumar	Dr. scient Botany	Somaclonal variation in plants regenerated from cell cultures of *Nicotiana sanderae* and *Chrysanthemum morifolium*
1987	Bjørn Åge Tømmerås	Dr. scient. Zoology	Olfaction in bark beetle communities: Interspecific interactions in regulation of colonization density, predator - prey relationship and host attraction.
1988	Hans Christian Pedersen	Dr. philos. Zoology	Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care.
1988	Tor G. Heggberget	Dr. philos. Zoology	Reproduction in Atlantic Salmon (*Salmo salar*): Aspects of spawning, incubation, early life history and population structure.
1988	Marianne V. Nielsen	Dr. scient. Zoology	The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (*Mytilus edulis*).
1988	Ole Kristian Berg	Dr. scient. Zoology	The formation of landlocked Atlantic salmon (*Salmo salar* L.).
1989	John W. Jensen	Dr. philos. Zoology	Crustacean plankton and fish during the first decade of the manmade Nesjø reservoir, with special emphasis on the effects of gill nets and salmonid growth.
1989	Helga J. Vivås	Dr. scient. Zoology	Theoretical models of activity pattern and optimal foraging: Predictions for the Moose *Alces alces*.
1989	Reidar Andersen	Dr. scient. Zoology	Interactions between a generalist herbivore, the moose *Alces alces*, and its winter food resources: a study of behavioural variation.
1989	Kurt Ingar Draget	Dr. scient Botany	Alginate gel media for plant tissue culture,
1990	Bengt Finstad	Dr. scient. Zoology	Osmotic and ionic regulation in Atlantic salmon, rainbow trout and Arctic charr: Effect of temperature, salinity and season.
1990	Hege Johannesen	Dr. scient. Zoology	Respiration and temperature regulation in birds with special emphasis on the oxygen extraction by the lung.
1990	Åse Krøkje	Dr. scient Botany	The mutagenic load from air pollution at two work-places with PAH-exposure measured with Ames Salmonella/microsome test
1990	Arne Johan Jensen	Dr. philos. Zoology	Effects of water temperature on early life history, juvenile growth and prespawning migrations of Atlantic salmion (*Salmo salar*) and brown trout (*Salmo trutta*): A summary of studies in Norwegian streams.
1990	Tor Jørgen Almaas	Dr. scient. Zoology	Pheromone reception in moths: Response characteristics of olfactory receptor neurons to intra- and interspecific chemical cues.
1990	Magne Husby	Dr. scient. Zoology	Breeding strategies in birds: Experiments with the Magpie *Pica pica*.
1991	Tor Kvam	Dr. scient. Zoology	Population biology of the European lynx (*Lynx lynx*) in Norway.
1991	Jan Henning L'Abêe Lund	Dr. philos. Zoology	Reproductive biology in freshwater fish, brown trout *Salmo trutta* and roach *Rutilus rutilus* in particular.
1991	Asbjørn Moen	Dr. philos Botany	The plant cover of the boreal uplands of Central Norway. I. Vegetation ecology of Sølendet nature reserve; haymaking fens and birch woodlands
1991	Else Marie Løbersli	Dr. scient Botany	Soil acidification and metal uptake in plants
1991	Trond Nordtug	Dr. scient. Zoology	Reflctometric studies of photomechanical adaptation in superposition eyes of arthropods.

1991	Thyra Solem	Dr. scient Botany	Age, origin and development of blanket mires in Central Norway
1991	Odd Terje Sandlund	Dr. philos. Zoology	The dynamics of habitat use in the salmonid genera *Coregonus* and *Salvelinus*: Ontogenic niche shifts and polymorphism.
1991	Nina Jonsson	Dr. philos.	Aspects of migration and spawning in salmonids.
1991	Atle Bones	Dr. scient Botany	Compartmentation and molecular properties of thioglucoside glucohydrolase (myrosinase)
1992	Torgrim Breiehagen	Dr. scient. Zoology	Mating behaviour and evolutionary aspects of the breeding system of two bird species: the Temminck's stint and the Pied flycatcher.
1992	Anne Kjersti Bakken	Dr. scient Botany	The influence of photoperiod on nitrate assimilation and nitrogen status in timothy (*Phleum pratense* L.)
1992	Tycho Anker-Nilssen	Dr. scient. Zoology	Food supply as a determinant of reproduction and population development in Norwegian Puffins *Fratercula arctica*
1992	Bjørn Munro Jenssen	Dr. philos. Zoology	Thermoregulation in aquatic birds in air and water: With special emphasis on the effects of crude oil, chemically treated oil and cleaning on the thermal balance of ducks.
1992	Arne Vollan Aarset	Dr. philos. Zoology	The ecophysiology of under-ice fauna: Osmotic regulation, low temperature tolerance and metabolism in polar crustaceans.
1993	Geir Slupphaug	Dr. scient Botany	Regulation and expression of uracil-DNA glycosylase and O^6-methylguanine-DNA methyltransferase in mammalian cells
1993	Tor Fredrik Næsje	Dr. scient. Zoology	Habitat shifts in coregonids.
1993	Yngvar Asbjørn Olsen	Dr. scient. Zoology	Cortisol dynamics in Atlantic salmon, *Salmo salar* L.: Basal and stressor-induced variations in plasma levels ans some secondary effects.
1993	Bård Pedersen	Dr. scient Botany	Theoretical studies of life history evolution in modular and clonal organisms
1993	Ole Petter Thangstad	Dr. scient Botany	Molecular studies of myrosinase in Brassicaceae
1993	Thrine L. M. Heggberget	Dr. scient. Zoology	Reproductive strategy and feeding ecology of the Eurasian otter *Lutra lutra*.
1993	Kjetil Bevanger	Dr. scient. Zoology	Avian interactions with utility structures, a biological approach.
1993	Kåre Haugan	Dr. scient Bothany	Mutations in the replication control gene trfA of the broad host-range plasmid RK2
1994	Peder Fiske	Dr. scient. Zoology	Sexual selection in the lekking great snipe (*Gallinago media*): Male mating success and female behaviour at the lek.
1994	Kjell Inge Reitan	Dr. scient Botany	Nutritional effects of algae in first-feeding of marine fish larvae
1994	Nils Røv	Dr. scient. Zoology	Breeding distribution, population status and regulation of breeding numbers in the northeast-Atlantic Great Cormorant *Phalacrocorax carbo carbo*.
1994	Annette-Susanne Hoepfner	Dr. scient Botany	Tissue culture techniques in propagation and breeding of Red Raspberry (*Rubus idaeus* L.)
1994	Inga Elise Bruteig	Dr. scient Bothany	Distribution, ecology and biomonitoring studies of epiphytic lichens on conifers
1994	Geir Johnsen	Dr. scient Botany	Light harvesting and utilization in marine phytoplankton: Species-specific and photoadaptive responses

1994	Morten Bakken	Dr. scient. Zoology	Infanticidal behaviour and reproductive performance in relation to competition capacity among farmed silver fox vixens, *Vulpes vulpes*.
1994	Arne Moksnes	Dr. philos. Zoology	Host adaptations towards brood parasitism by the Cockoo.
1994	Solveig Bakken	Dr. scient Bothany	Growth and nitrogen status in the moss *Dicranum majus* Sm. as influenced by nitrogen supply
1995	Olav Vadstein	Dr. philos Botany	The role of heterotrophic planktonic bacteria in the cycling of phosphorus in lakes: Phosphorus requirement, competitive ability and food web interactions.
1995	Hanne Christensen	Dr. scient. Zoology	Determinants of Otter *Lutra lutra* distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink *Mustela vision*.
1995	Svein Håkon Lorentsen	Dr. scient. Zoology	Reproductive effort in the Antarctic Petrel *Thalassoica antarctica*; the effect of parental body size and condition.
1995	Chris Jørgen Jensen	Dr. scient. Zoology	The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity
1995	Martha Kold Bakkevig	Dr. scient. Zoology	The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport.
1995	Vidar Moen	Dr. scient. Zoology	Distribution patterns and adaptations to light in newly introduced populations of *Mysis relicta* and constraints on Cladoceran and Char populations.
1995	Hans Haavardsholm Blom	Dr. philos Bothany	A revision of the *Schistidium apocarpum* complex in Norway and Sweden.
1996	Jorun Skjærmo	Dr. scient Botany	Microbial ecology of early stages of cultivated marine fish; inpact fish-bacterial interactions on growth and survival of larvae.
1996	Ola Ugedal	Dr. scient. Zoology	Radiocesium turnover in freshwater fishes
1996	Ingibjørg Einarsdottir	Dr. scient. Zoology	Production of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*): A study of some physiological and immunological responses to rearing routines.
1996	Christina M. S. Pereira	Dr. scient. Zoology	Glucose metabolism in salmonids: Dietary effects and hormonal regulation.
1996	Jan Fredrik Børseth	Dr. scient. Zoology	The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics.
1996	Gunnar Henriksen	Dr. scient. Zoology	Status of Grey seal *Halichoerus grypus* and Harbour seal *Phoca vitulina* in the Barents sea region.
1997	Gunvor Øie	Dr. scient Bothany	Eevalution of rotifer *Brachionus plicatilis* quality in early first feeding of turbot *Scophtalmus maximus* L. larvae.
1997	Håkon Holien	Dr. scient Botany	Studies of lichens in spurce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters.
1997	Ole Reitan	Dr. scient. Zoology	Responses of birds to habitat disturbance due to damming.
1997	Jon Arne Grøttum	Dr. scient. Zoology	Physiological effects of reduced water quality on fish in aquaculture.
1997	Per Gustav Thingstad	Dr. scient. Zoology	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher.

1997	Torgeir Nygård	Dr. scient. Zoology	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as Biomonitors.
1997	Signe Nybø	Dr. scient. Zoology	Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway.
1997	Atle Wibe	Dr. scient. Zoology	Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry.
1997	Rolv Lundheim	Dr. scient. Zoology	Adaptive and incidental biological ice nucleators.
1997	Arild Magne Landa	Dr. scient. Zoology	Wolverines in Scandinavia: ecology, sheep depredation and conservation.
1997	Kåre Magne Nielsen	Dr. scient Botany	An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoacetius*.
1997	Jarle Tufto	Dr. scient. Zoology	Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models
1997	Trygve Hesthagen	Dr. philos. Zoology	Population responces of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters
1997	Trygve Sigholt	Dr. philos. Zoology	Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
1997	Jan Østnes	Dr. scient. Zoology	Cold sensation in adult and neonate birds
1998	Seethaledsumy Visvalingam	Dr. scient Botany	Influence of environmental factors on myrosinases and myrosinase-binding proteins.
1998	Thor Harald Ringsby	Dr. scient. Zoology	Variation in space and time: The biology of a House sparrow metapopulation
1998	Erling Johan Solberg	Dr. scient. Zoology	Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment
1998	Sigurd Mjøen Saastad	Dr. scient Botany	Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity.
1998	Bjarte Mortensen	Dr. scient Botany	Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro.
1998	Gunnar Austrheim	Dr. scient Botany	Plant biodiversity and land use in subalpine grasslands. – A conservtaion biological approach.
1998	Bente Gunnveig Berg	Dr. scient. Zoology	Encoding of pheromone information in two related moth species
1999	Kristian Overskaug	Dr. scient. Zoology	Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach
1999	Hans Kristen Stenøien	Dr. scient Bothany	Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)
1999	Trond Arnesen	Dr. scient Botany	Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway.

1999	Ingvar Stenberg	Dr. scient. Zoology	Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos*
1999	Stein Olle Johansen	Dr. scient Botany	A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis.
1999	Trina Falck Galloway	Dr. scient. Zoology	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
1999	Torbjørn Forseth	Dr. scient. Zoology	Bioenergetics in ecological and life history studies of fishes.
1999	Marianne Giæver	Dr. scient. Zoology	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic
1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*.
1999	Ingrid Bysveen Mjølnerød	Dr. scient. Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos. Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient. Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999	Per Terje Smiseth	Dr. scient. Zoology	Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (*Luscinia s. svecica*)
1999	Gunnbjørn Bremset	Dr. scient. Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999	Frode Ødegaard	Dr. scient. Zoology	Host spesificity as parameter in estimates of arhrophod species richness
1999	Sonja Andersen	Dr. scient Bothany	Expressional and functional analyses of human, secretory phospholipase A2
2000	Ingrid Salvesen, I	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000	Ingar Jostein Øien	Dr. scient. Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000	Pavlos Makridis	Dr. scient Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000	Sigbjørn Stokke	Dr. scient. Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)
2000	Odd A. Gulseth	Dr. philos. Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard
2000	Pål A. Olsvik	Dr. scient. Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway

2000	Sigurd Einum	Dr. scient. Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001	Jan Ove Evjemo	Dr. scient. Zoology	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species
2001	Olga Hilmo	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forset systems
2001	Ingebrigt Uglem	Dr. scient. Zoology	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
2001	Bård Gunnar Stokke	Dr. scient. Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002	Ronny Aanes	Dr. scient	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
2002	Mariann Sandsund	Dr. scient. Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002	Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002	Frank Rosell	Dr. scient. Zoology	The function of scent marking in beaver (*Castor fiber*)
2002	Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002	Terje Thun	Dr.philos Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002	Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002	Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002	Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and
2002	Henrik Jensen	Dr. scient Biology	Causes and consequenses of individual variation in fitness-related traits in house sparrows
2003	Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003	Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003	Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003	Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003	Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana
2003	Marit Stranden	Dr.scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)
2003	Kristian Hassel	Dr.scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*
2003	David Alexander Rae	Dr.scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003	Åsa A Borg	Dr.scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective

2003	Eldar Åsgard Bendiksen	Dr.scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt
2004	Torkild Bakken	Dr.scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr.scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar
2004	Tore Brembu	Dr.scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr.scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr.scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*).
2004	Lene Østby	Dr.scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr.scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr.scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004	Børge Moe	Dr.scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr.scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr.scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr.scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period
2005	Tonette Røstelien	PhD Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr.scient Biology	Studies on antifreeze proteins
2005	Eugen G. Sørmo	Dr.scient Biology	Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyrid hormone and vitamin A concentrations.
2005	Christian Westad	Dr.scient Biology	Motor control of the upper trapezius
2005	Lasse Mork Olsen	PhD Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005	Åslaug Viken	PhD Biology	Implications of mate choice for the management of small populations

2005	Ariaya Hymete Sahle Dingle	PhD Biology	Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia
2005	Anders Gravbrøt Finstad	PhD Biology	Salmonid fishes in a changing climate: The winter challenge
2005	Shimane Washington Makabu	PhD Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005	Kjartan Østbye	Dr.scient Biology	The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation
2006	Kari Mette Murvoll	PhD Biology	Levels and effects of persistent organic pollutans (POPs) in seabirds Retinoids and α-tocopherol – potential biomakers of POPs in birds?
2006	Ivar Herfindal	Dr.scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006	Nils Egil Tokle	Phd Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus*
2006	Jan Ove Gjershaug	Dr.philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006	Jon Kristian Skei	Dr.scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006	Johanna Järnegren	PhD Biology	Acesta Oophaga and Acesta Excavata – a study of hidden biodiversity
2006	Bjørn Henrik Hansen	PhD Biology	Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway
2006	Vidar Grøtan	PhD Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006	Jafari R Kideghesho	phD Biology	Wildlife conservation and local land use conflicts in western Serengeti, Corridor Tanzania
2006	Anna Maria Billing	PhD Biology	Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction
2006	Henrik Pärn	PhD Biology	Female ornaments and reproductive biology in the bluethroat
2006	Anders J. Fjellheim	PhD Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006	P. Andreas Svensson	phD Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system
2007	Sindre A. Pedersen	PhD Biology	Metal binding proteins and antifreeze proteins in the beetle *Tenebrio molitor* - a study on possible competition for the semi-essential amino acid cysteine
2007	Kasper Hancke	PhD Biology	Photosynthetic responses as a function of light and temperature: Field and laboratory studies on marine microalgae
2007	Tomas Holmern	PhD Biology	Bushmeat hunting in the western Serengeti: Implications for community-based conservation
2007	Kari Jørgensen	PhD Biology	Functional tracing of gustatory receptor neurons in the CNS and chemosensory learning in the moth *Heliothis virescens*

2007	Stig Ulland	PhD Biology	Functional Characterisation of Olfactory Receptor Neurons in the Cabbage Moth, */Mamestra Brassicae/* L. (Lepidoptera, Noctuidae). Gas Chromatography Linked to Single Cell Recordings and Mass Spectrometry
2007	Snorre Henriksen	PhD Biology	Spatial and temporal variation in herbivore resources at northern latitudes
2007	Roelof Frans May	PhD Biology	Spatial Ecology of Wolverines in Scandinavia
2007	Vedasto Gabriel Ndibalema	PhD Biology	Demographic variation, distribution and habitat use between wildebeest sub-populations in the Serengeti National Park, Tanzania
2007	Julius William Nyahongo	PhD Biology	Depredation of Livestock by wild Carnivores and Illegal Utilization of Natural Resources by Humans in the Western Serengeti, Tanzania
2007	Shombe Ntaraluka Hassan	PhD Biology	Effects of fire on large herbivores and their forage resources in Serengeti, Tanzania
2007	Per-Arvid Wold	PhD Biology	Functional development and response to dietary treatment in larval Atlantic cod *(Gadus morhua* L.) Focus on formulated diets and early weaning
2007	Anne Skjetne Mortensen	PhD Biology	Toxicogenomics of Aryl Hydrocarbon- and Estrogen Receptor Interactions in Fish: Mechanisms and Profiling of Gene Expression Patterns in Chemical Mixture Exposure Scenarios
2008	Brage Bremset Hansen	PhD Biology	The Svalbard reindeer (*Rangifer tarandus platyrhynchus*) and its food base: plant-herbivore interactions in a high-arctic ecosystem
2008	Jiska van Dijk	PhD Biology	Wolverine foraging strategies in a multiple-use landscape
2008	Flora John Magige	PhD Biology	The ecology and behaviour of the Masai Ostrich (Struthio camelus massaicus) in the Serengeti Ecosystem, Tanzania
2008	Bernt Rønning	PhD Biology	Sources of inter- and intra-individual variation in basal metabolic rate in the zebra finch, */Taeniopygia guttata/*
2008	Sølvi Wehn	PhD Biology	Biodiversity dynamics in semi-natural mountain landscapes. - A study of consequences of changed agricultural practices in Eastern Jotunheimen