

**Teleost reproduction:
Aspects of Arctic char (*Salvelinus
alpinus*) oocyte growth and maturation.**

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ABSTRACT

In all vertebrate species, reproduction is a hormonally controlled process, important for growth and maturation of gonads and germ cells. Production of functional germ cells is of utmost importance to secure the survival of a species. Fish comprises 50% of the known vertebrates and are found in aquatic habitats all over the world. Even though fish have evolved a wide variety of morphological and physiological characteristics, due to large differences in the living environment, the growth and maturation of germ cells follows the same pattern in all species. In this thesis the focus has been directed on oocyte growth and development in Arctic char (*Salvelinus alpinus*), and if stress might inflict disturbances on the reproductive systems.

All sexually mature female egg laying vertebrates produces yolky eggs surrounded by an eggshell. Production of yolk and egg shell is under estrogenic control and it is known that production of egg components can be induced in male and juvenile fish by estrogenic substances. Many manmade chemicals have been found to interfere with hormonally controlled processes. Therefore production of the egg yolk precursor, vitellogenin (VTG), and the egg shell components, vitelline envelope proteins (VEP), have been used as biomarkers for estrogenic effect. Exposure to endocrine disrupting substances (EDS) does not only give rise to hormonal effects on the organism, but in addition it also gives rise to an increase in stress hormone, cortisol (F), levels.

It is evident that a wide variety of substances may affect Arctic char oocyte growth and maturation. VTG and VEP production is found to be under dose dependent estrogenic control, but the production was directly affected by F. Under natural condition it has been found that F increases towards ovulation. Even though both VTG and VEP is under estrogenic control, these studies showed that stress lead to a decrease of VTG while the VEP production increased. These effects was only observed on protein levels indicating that a post transcriptional down regulation of VTG production is mediated by F in Arctic char.

In order for an egg to become fertilizable, it must undergo a maturation phase. This maturation phase is primarily induced by gonadotropins, which in turn induce the production of species specific maturation inducing substances (MIS). To investigate oocyte development in Arctic char a characterization of its MIS receptor was made. The MIS receptor is localized on the oocyte surface and displays a single class of high affinity and low capacity binding

sites. The binding moieties displays association and dissociation kinetics typical of steroid membrane receptors.

Even though high specificity for Arctic char MIS was observed, it was found that some EDS bind to the Arctic char oocyte membrane receptor. This suggest that certain EDS might affect oocyte maturation and thereby might alter the reproductive success. Furthermore, it was found that F did not bind to the MIS receptor in Arctic char. It is therefore suggested that oocytes are more sensitive to stress during the growth phase than during maturation.

ABBREVIATIONS

17,20 β -P	17 α ,20 β -dihydroxy-4-pregnen-3-one
20 β -HSD	20 β -hydroxysteroid dehydrogenase
20 β -S	17 α ,20 β ,21-trihydroxy-4-pregnen-3-one
ACTH	adrenocorticotrophic hormone
AP-1	activator protein 1
ARE	androgen response element
DDD	1,1-dichloro-2,2-bis[p-chlorophenyl]ethane
DDT	1,1,1-trichloro-2,2-bis[p-chlorophenyl]ethane
E2	17- β -estradiol
EDS	endocrine disrupting substance
ER	estrogen receptor
F	cortisol
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
GR	glucocorticoid receptor
HSP	heat shock protein
LDL-R	low-density lipoprotein receptor
LH	luteinizing hormone
MIS	maturation inducing steroid
MT	metallothionein
PCB	polychlorinated biphenyl
SBP	steroid binding protein
T ₄	tetraiodothyroxin
VEP	vitelline envelope protein
VTG	vitellogenin
VTG-R	vitellogenin receptor
ZP	zona pellucida

PAPERS IN THIS THESIS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV).

- I. **Gerpe, M., Kling, P., Berg, A.H., Olsson, P-E.** (2000). Arctic char (*Salvelinus alpinus*) metallothionein: cDNA sequence, expression, and tissue-specific inhibition of cadmium-mediated metallothionein induction by 17 β -estradiol, 4-OH-PCB 30, and PCB 104. *Environ. Tox. Chem.* 19(3):638-645.
- II. **Berg, A.H., Olsson, P-E.** 17 β -estradiol induced vitellogenesis is inhibited by cortisol at the post-transcriptional level in Arctic char (*Salvelinus alpinus*). Manuscript.
- III. **Berg, A.H., Westerlund, L., Olsson, P-E.** (2003). Regulation of Arctic char (*Salvelinus alpinus*) egg shell proteins and vitellogenin during reproduction and in response to 17 β -estradiol. *Gen. Comp. Endocrin.* In Press.
- IV. **Berg, A.H., Thomas, P., Olsson, P-E.** Characterization of the 17,20 β -dihydroxy-4-pregnen-3-one membrane receptor in Arctic char (*Salvelinus alpinus*) ovaries and its upregulation during gonadotropin induction of oocyte maturation. Manuscript.

INTRODUCTION

Water is a necessity for all life on earth. With 71 % of the earth surface covered by water there is a multitude of habitats harbouring a large number of different organisms. Fish comprises more than 50% of the known vertebrate species on earth, making it the largest vertebrate group. Due to large differences in salinity, temperature, pH, nutrition and light periods of the habitats, different fish species have evolved a wide variety of morphological and physiological characteristics. While fresh water cover only 1% of the surface, 40% of the fish species are found in fresh water systems while 0.5% of the species are considered anadromous (migrates between fresh and salt water habitats). The remaining fish species are found in salt water habitats.

The Arctic char (*Salvelinus alpinus*) is a salmonid species that was classified in 1758 by the Swedish taxonomist Linnaeus. It has the most northerly distribution of any freshwater fish and has a circumpolar distribution in Arctic freshwater systems (Johnsson, 1980). The Arctic char displays two life strategies, anadromous Arctic char, which migrate between salt and fresh water during growth and spawning, and the resident form, which remain in fresh water throughout the lifecycle (Nordeng, 1983; Berg & Berg, 1993). The Arctic char is a fish of great commercial value in countries in the northern hemisphere mainly due to its ability to grow at low temperatures (Berg & Berg, 1989), but the species also displays high sensitivity to environmental change such as climate change, eutrophication, acidification, discharge of toxic chemicals and introduction of other species in the ecosystem.

The natural spawning period for the Arctic char is in October/November and during this time, the female produces between 500 to 7000 large (4-5mm) and sticky eggs (Johnsson, 1980). Functional reproduction is of outmost importance for species survival. Depending on habitat large variations in reproductive strategies are present in fish.

TELEOST REPRODUCTION:

Reproduction involves hormonally regulated processes, such as growth and maturation of gonads and germ cells. For the survival of a species, the production of functional germ cells

(eggs and sperm) that can participate in fertilization are an important event. Thus, the reproductive tissues must be produced during embryonic development, and growth and maturation of germ cells must function after sexual maturation of the organism. Fish have evolved many reproductive strategies, and there are viviparous (internal fertilization, the embryo develop inside the mother which provides the growing embryo with nutrition), ovoviviparous (internal fertilization, mothers lay yolky eggs which are retained in the oviduct until hatching) and oviparous (external fertilization, embryos develop outside the mother) species. In the oviparous and ovoviviparous species all nutrients are contained within the egg. This includes proteins and lipids, but also vitamins and minerals necessary for embryo to survival and development until the feeding stage.

The pattern of oocyte growth and maturation is independent of the reproductive strategy of the teleost species (Fig 1). The development of the oocyte is halted in the first prophase of mitosis and remains inactive until growth and maturation takes place.

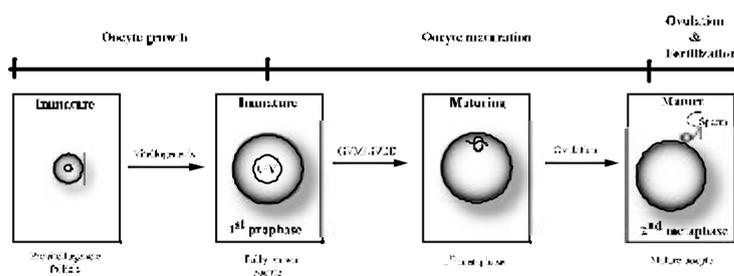


Fig. 1 Schematic description of the different stages in teleost oocyte growth and maturation.

The onset of the growth of the oocytes is induced by an external signal such as light intensity (photoperiod), feeding, social factors and water temperature (Fahien and Sower, 1990), triggering the hypothalamic centre to produce and release gonadotropins (Feist and Schreck, 1996). The hypothalamic – pituitary – gonadal – axis is outlined in Fig. 2. In fish two types of gonadotropins, FSH and LH, has been identified (Idler and Ng, 1979; Idler and Ng, 1983; Planas *et al.*, 2000). FSH function as the primary signal-transducer for the onset of oocyte growth, while LH is involved in the control of oocyte maturation. FSH controls the oocyte growth by stimulating the follicle cells enclosing the primary oocyte to increase in size

and number and to produce 17 β -estradiol (E2), the most potent estrogen in teleost fish. Estrogens are a family of female sex hormones, which controls reproduction, development, morphological differentiation, growth and metabolism. The production of E2 is accomplished by a two-cell type model (Fig.3) (Kagawa *et al.*, 1982; Nagahama, 1983; Yaron, 1995). Thecal cells produce testosterone from cholesterol, and the produced testosterone then diffuses into the granulosa cells where it is converted into E2 by the enzyme aromatase (Kawaga *et al.*, 1982). The produced E2 is secreted into the circulation where approximately 95 % of the E2 binds to serum steroid binding proteins (SBP) or albumins that protects the E2 from degradation before entering the target cell (Petra *et al.*,1991). While bound to SBP, E2 is considered inactive. The E2 is transported to the target cells where free E2 exert nongenomic actions by membrane receptors (Thomas, 2000) or enters the cells by facilitated diffusion and is retained by high affinity binding to a specific estrogen receptor (ER). Inactivated ER is bound to heat shock protein, HSP90, and localised to the cellular cytoplasm (Pratt and Toft, 1997). When E2 binds to the ER, the HSP90 complex dissociates, and the E2-ER is translocated into the nucleus.

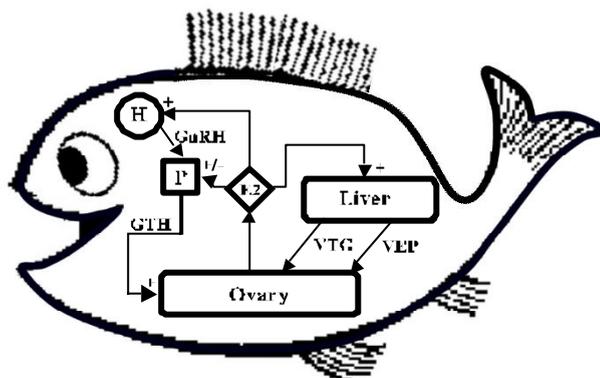


Fig 2. Schematic description of endocrine control of egg growth in teleost fish. The hypothalamus (H) produces and releases gonadotropin releasing hormone (GnRH) which act on the pituitary (P) to induce release of gonadotropins (GTHs). The GTHs are transported to the ovary where they induce the growth and proliferation of the granulosa cells. The growing granulosa cells start to produce 17- β -estradiol (E2) which is released to the blood. E2 has a positive feedback on both H and P but also induce the hepatic production of vitellogenin (VTG) and egg shell proteins (VEP). The VTG and VEPs are transported from the hepatocyte to the growing oocyte where the VTG are taken up and cleaved for yolk formation and the VEPs are used for egg shell assembly.

The E2-ER complex has been shown to bind as a dimer to estrogen responsive elements (EREs), located upstream or within estrogen responsive genes (Pratt and Toft, 1997), initiating activation of previously silent genes and enhanced or decreased transcription of other genes.

Estrogen stimulation has also been shown to induce changes in hepatic morphology, such as proliferation of the rough endoplasmatic reticulum and golgi apparatus. E2 has been found to induce the production of the egg yolk precursor vitellogenin (VTG) (Campbell and Idler, 1980; Chen, 1983; Mommsen and Walsh, 1988) and the vitelline envelope proteins (VEP) (Hyllner *et al.*, 1991) but it also stimulate the production and release of FSH and LH (fig.2). Female fish livers contain high levels of ER, which allows the production of VTG and VEP following estrogen stimulation.

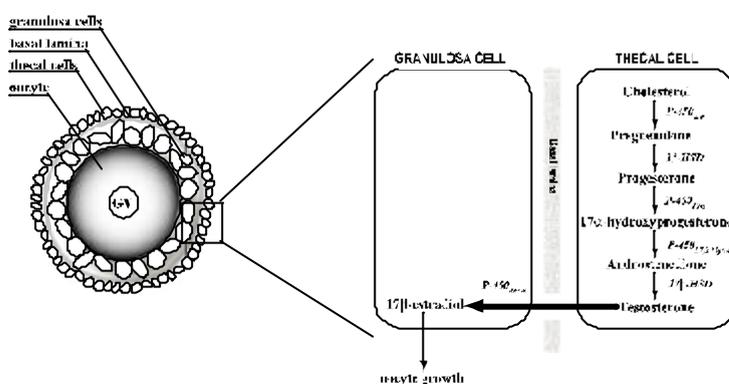


Fig. 3 Description of the two cell system producing E2 in mature female Arctic char. FSH is released from the pituitary and induces thecal and granulosa cell growth and proliferation. FSH displays different effects on the two cell types. In the thecal cells FSH induces the production of testosterone while in the granulosa cells FSH upregulates aromatase, the enzyme which converts testosterone into E2.

OOCYTE GROWTH

During development all oocytes becomes surrounded by a follicle cell layer. In order to produce a functional egg the teleost oocytes enter a growth phase prior to oocyte maturation. The growth is due to a large accumulation of egg yolk mainly dependent upon the production and release of E2 from the follicular cells and defined as vitellogenesis (E2 controlled synthesis and uptake of VTG). During growth phase E2 also induces production of VEP necessary for egg shell assembly.

Vitellogenin

In 1935 a specific phosphoprotein was discovered in the plasma from egg laying hens (Laskowski, 1935). The plasma protein called serum vitellin, was found to be present in the plasma only in sexually mature females, but the production of the protein was also found to be inducible in roosters by exposing them to 17 β -estradiol. The protein is suggested to function as a casein which binds and transport calcium into the growing oocyte (Shjeide, 1985). Further studies of the protein classified it as the major proteinacious precursor of yolk in hens. Due to this finding the female specific plasma protein was renamed vitello genin. The name vitellogenin was a term, used for all plasma egg yolk precursors in insects (Pan *et al.*, 1969) that became adapted as a name for all yolk precursors in all egg laying species. It was found that the teleost female specific plasma protein had its origin in hepatocytes (Plack and Frazer, 1971, Aida *et al.*, 1973a) and in 1973 the first teleost VTG was described from the ayu (*Plecoglossus altivelis*) as a female specific plasma protein produced under 17 - β -estradiol control in the liver of sexual mature individuals (Aida *et al.*,1973b).

The teleost VTG is a phospholipoglycoprotein and has been characterized in a wide variety of teleost species such as rainbow trout (*Oncorhynchus mykiss*) (Chen *et al.*, 1983; Norberg and Haux, 1985; Mouchel *et al.*, 1996), brown trout (*Salmo trutta*) (Norberg and Haux, 1985; Sherry *et al.*, 1999), turbot (*Scophthalmus maximus*) (Silversand and Haux, 1989), wolfish (*Anarchichas lupus*) (Silversand *et al.*, 1993), white sturgeon (*Acipenser transmontanus*) (Bidwell and Carlsson, 1995), gilthead seabream (*Sparus aurata*) (Mosconi *et al.*, 1998), fathead minnow (*Pimephales promelas*) (Parks *et al.*, 1999), cod (*Gadus morhua*), and Arctic char (*Salvelinus alpinus*) (Johnsen *et al.*, 1999). This yolk precursor is produced under estrogen control in the liver of sexually mature females and is transported from the liver as a

dimer via the blood to the oocytes (Copeland *et al.*, 1986). The growing oocytes takes up VTG by receptor mediated endocytosis (Byrne *et al.*, 1989; Shibata *et al.*, 1993) and proteases in the cell cleave VTG into the smaller yolk units lipovitelin, phosvitin (Ng and Idler 1983; Carnevali *et al.*, 1999) and phosvettes (Matsubara *et al.*, 1999), which function as nutrition for growing embryos (Wahli *et al.*, 1981). Vitellogenesis is crucial for normal embryo development and disruption of this system results in starvation of the embryos and hepatic damage in the adults (Folmar *et al.*, 2001)

VTG is also thought to function as a metal-ion transporter, since it has been found that the protein, as well as its metabolites, contains zinc, copper (Montorzi *et al.*, 1994; Montorzi *et al.*, 1995) and magnesium (Falchuk and Montorzi, 2001). Presence of metal-ions is crucial for a functional maturation of the oocyte and embryonic development since it is necessary in protein, lipid and carbohydrate metabolism (Vallee and Falchuk, 1993; Falchuk, 1998) and certain metal ions are necessary for correct folding and stability of metalloproteins. It has also been suggested that VTG possesses steroid binding capacities allowing maternal steroids necessary for embryonic development to be transported into the growing oocyte (Reis *et al.*, 2000).

A large number of VTG-genes have been isolated and characterised from several oviparous species. The VTG-genes belong to a small gene family, with variable gene numbers depending on species (Wahli *et al.*, 1981; Wang *et al.*, 2000; Trichet *et al.*, 2000). VTG among oviparous species are highly conserved (Wahli, 1988; Chen *et al.*, 1997; Babin *et al.*, 1999) and gives rise to multiple forms of the VTG protein, which have been found to have different roles during the oocyte maturation and the embryonic development (Carnevali *et al.*, 1999; Matsubara *et al.*, 1999; Reith *et al.*, 2001).

VTG is transported across the oolemma into the growing oocyte by membrane bound VTG receptors (VTG-R). VTG-R has been cloned and characterised from a wide variety of oviparous species such as birds (Yusko *et al.*, 1981), amphibians (Opresko *et al.*, 1987; Stifani *et al.*, 1990a), fish (Stifani *et al.*, 1990b; Tyler and Lancaster, 1993; Nuñez Rodrigues *et al.*, 1996) and invertebrates (Hafer and Ferenz, 1994). All VTG-R belong to the same family, the low-density lipoprotein receptor (LDL-R) superfamily and evolutionary studies show that the function of the VTG-R has evolved from a common ancestor (Davail *et al.*, 1998).

The encoded VTG-R protein contains: A) a ligand binding domain with an octameric cysteine rich repeat at the N-terminus; B) an epidermal growth factor precursor homology domain; C) a single transmembrane domain securing the protein to the plasma membrane; D) a short

cytoplasmatic domain with the consensus, Phe-Asp-Asn-Pro-Val-Tyr, which has been found to mediate internalisation of the receptor via coated pits (Schneider, 1996). Recent findings show that the VTG-R are multifunctional and bind a wide variety of ligands such as very low density lipoproteins (VLDL) (Stifani *et al.*, 1990c; Schneider and Nimpf, 1993), riboflavin-binding proteins (Mac Lachlan *et al.*, 1993) and α_2 -macroglobulins (Schneider and Nimpf, 1993). Uptake of VTG by the growing oocyte is up to 25 times faster than uptake of other plasma proteins, indicating that it occurs through receptor mediated endocytosis. It has also been shown that the incorporation of VTG may be altered due to interference with hormones such as insulin, T₄ (Shibata *et al.*, 1993) and FHS (Tyler *et al.*, 1991).

Shortly after VTG binds to the receptor, the VTG-VTG-R complex is incorporated into the oocyte by invagination and budding of the plasma membrane. In order for the oocytes to control the transmembrane traffic, invagination can only take place when the VTG-Rs are present on certain areas of the cell membrane, so called coated pits. These coated pits are areas (about 2% of the surface area) with high electron density and a high frequency of a “coating” protein, clathrin (Goldstein *et al.*, 1985). When the coated vesicles enters the cells they are decoated and fuse with other vesicles at the endosomal storage area (Geuze *et al.*, 1983). A decrease of the pH leads to dissociation of VTG and VTG-R and the VTG will be cleaved into the smaller yolk proteins (Turkewitz *et al.*, 1988) while the VTG-R is reused (Bu and Schwartz, 1994).

Vitelline envelope proteins

All vertebrate species produce an egg surrounded by an acellular envelope. The protective envelope enclosing the egg has different functions such as uptake of nutrients, functional buoyancy (Podolsky, 2002), protection of the growing oocyte, species specific sperm binding, guidance of the sperm to the micropyle (Dumont and Brummet, 1980) and does also possess bactericidal properties (Kudo and Inoue, 1989). It therefore is of great importance that the eggshell is assembled the right way. A correctly assembled eggshell is also vital for functional hatching of the embryo.

At the onset of growth the oocyte is enclosed in a single layer of granulosa cells. The gonadotropins released at this stage will induce the granulosa cells to increase in size and numbers and form a thick cell layer. At the same time the mesenchyme cells of the ovary

differentiate into thecal cells forming a thick outer cell-layer separated from the granulosa cells by a basal lamina. During the growth phase the oolemma will form microvillies, which extend between the plasma membrane and the granulosa cells. In most teleosts it has been shown that the E2 produced by the granulosa cells will stimulate hepatocytes to produce VEP, necessary for production of a functional eggshell. In some species such as carp (*Cyprinus carpio*) (Chang *et al.*, 1996), goldfish (*Carassius auratus*) (Chang *et al.*, 1997) and zebrafish (*Danio rerio*) (Wang and Gong, 1999) the eggshell components are primarily produced in the ovary. VEPs that are synthesized in liver, are transported via the circulation to the ovaries. The assembly of the eggshell is believed to be initiated at the base of the microvilli. This process start prior to vitellogenesis, and the vitelline envelope continues to assemble during the whole oocyte growth phase.

The teleost vitelline envelope is a species-specific structure consisting of two to four structural proteins with masses ranging between 47 and 129 kDa (Hamazaki *et al.*, 1985; Oppen-Berntsen *et al.*, 1990; Hyllner and Haux, 1992). The vitelline envelope components all share a common motif called the Zona Pellucida (ZP) domain (Bork and Sander, 1992). The glucoproteins containing this ZP domain has been found to form the ZP envelope that encloses the mammalian oocyte (Greve and Wassarman, 1985) In this process ZP 2 and ZP3 are "building blocks" and ZP1 is considered as a "crosslinker" between these (Wassarman, 1988). In salmonids three different VEPs have been identified, VEP α , VEP β and VEP γ (Hyllner, 1994). The VEP α and VEP β are expressed only in hepatocytes while VEP γ is expressed in both liver and ovary of the maturing Arctic char (Westerlund *et al.*, 2001). The three VEPs are more sensitive to E2 than VTG (Celius and Walther, 1998a), are expressed prior to vitellogenesis (Hyllner *et al.*, 1994; Celius and Walther, 1998b) and are expressed in juvenile fish (Westerlund *et al.*, 2001).

OOCYTE MATURATION

When the oocyte is fully grown it is arrested in a late G2 stage of the first meiosis until the maturation phase is initiated. The germinal vesicle (GV) in an immature oocyte is localized in the center of the oocyte. The GV contains maternal RNA, ribosomal RNA and specific proteins such as nucleoplasmin, a protein involved in pronucleus formation during fertilization. The initial sign of maturation onset is a migration of the GV towards the animal

pole of the oocyte. When the GV reached the animal pole the membrane surrounding the GV breaks down, during a process known as germinal vesicle breakdown (GVBD) and the content in the GV is spread into the surrounding cytoplasm. At this stage the oocyte continues its development through meiosis I, the chromosomes are aligned for meiosis II and are arrested at this stage until fertilization. Onset of the maturation phase is mediated by a plasma membrane mechanism that triggers a signal transduction cascade resulting in oocyte maturation. This stimuli may be hormonal or mechanical (fertilization) and the stimulus responsible for maturation onset varies among species (Young *et al.*, 1986).

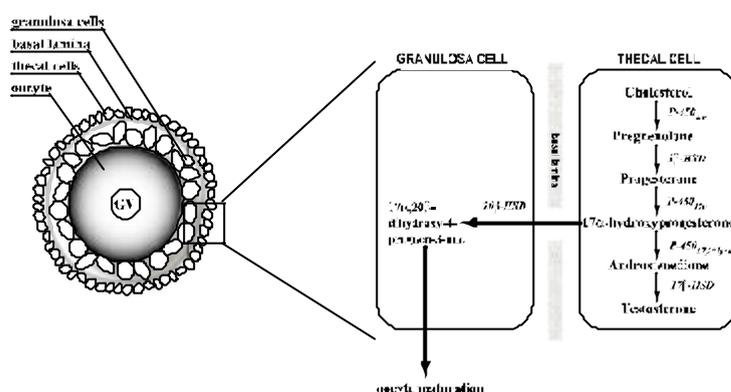


Fig. 4 Schematic description of the two cell production of the salmonid MIS, 17,20 β -P. FSH released by the pituitary stimulates the thecal cells to produce testosterone from cholesterol. When the pituitary starts the release of LH the testosterone precursor 17 α -hydroxyprogesterone is transported from the thecal cells to the granulosa cells. In the granulosa cells the LH induces production of the enzyme 20 β -HSD which converts 17 α -hydroxyprogesterone into the MIS 17,20 β -P which in turn induces oocyte maturation.

In teleost fish the luteinizing hormone (LH) primarily induces oocyte maturation by an increase of 17 α -hydroxyprogesterone production by the thecal cells and 20 β -hydroxysteroid dehydrogenase (20 β -HSD) synthesis in the granulosa cells (Fig.4.) (Young *et al.*, 1982, Nagahama, 1994). The produced 17 α -hydroxyprogesterone is transported from the thecal cells into the granulosa cells where the 20 β -HSD converts it into a maturation-inducing steroid (MIS) (Young *et al.*, 1986; Nagahama *et al.* 1993; Nagahama *et al.*, 1987; Patiño *et al.* 2001).

In teleost, two different forms of MIS has been found. These are the C21 steroids $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ($17,20\beta$ -P) (Nagahama and Adachi, 1985) in salmonid species and $17\alpha,20\beta,21$ -trihydroxy-4-pregnen-3-one (20β -S) (Trant *et al.*, 1986; Trant and Thomas, 1989) for sciaenids. MIS is released by the granulose cells, binds to specific G-protein coupled receptors localized on the oocyte membrane surface (Gallo *et al.*, 1995; Oba *et al.*, 1997) and activate a cytoplasmatic signal transduction factor. This factor, which is called the maturation-promoting factor (MPF), was discovered in *Xenopus* (Lohka *et al.*, 1988) and was identified as a phosphorylated CDC-2 kinase – Cyclin B complex witch induces the onset of GVBD (Fig. 5). Following GVBD the oocyte is fully matured and ovulation and fertilization may occur.

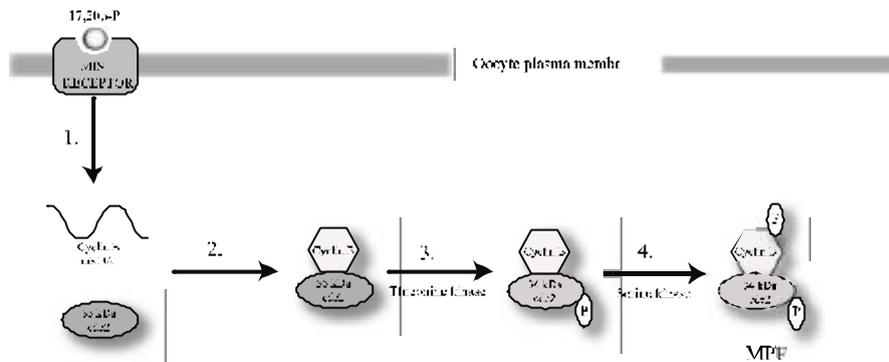


Fig.5 Description of the maturation induction in teleosts. In the growing oocyte the MIS binds to its membrane receptor initiating transcription of the cyclin B mRNA (1.). The produced Cyclin B forms a complex with cdc2 (2.) and is phosphorylated (3 & 4) in order to form the MPF. Initiation of the oocyte maturation is thereafter promoted by MPF.

ENDOCRINE DISRUPTION

As discussed above, the mechanisms controlling oocyte growth and maturation are complex and can easily be disturbed by exogenous factors such as endocrine disrupting substances (EDS). In teleost the focus on EDS has been the identification of biomarkers for reproductive disturbances, while less effort has been made to determine the physiological mechanisms. Since both VTG and VEP are under estrogenic control, only females produce these proteins during sexual maturation. However, both VEP and VTG can be induced in both male and juvenile fish by exposure to estrogen or to estrogen agonists (Thomas and Smith, 1993; White *et al.*, 1994; Jobling *et al.*, 1995; Arukwe *et al.*, 1997). Due to this both systems have been used as biomarkers of estrogenic activity. It has been shown that certain substances bind to the ER and mediate signals leading to expression of estrogen-regulated genes. The number of substances with estrogenic effect present in the environment is constantly increasing. Substances such as DDT, dioxins, alkylphenol polyethoxylates, some polychlorinated biphenyls (PCB:s), phthalate esters e.t.c. displays estrogenic/ antiestrogenic activity (Thomas and Smith, 1993; Jobling *et al.*, 1995; Sumpter and Jobling, 1995; Donohoe and Curtis, 1996). Many of these xenobiotics are lipophilic, and therefore easily bioaccumulated. Xenoestrogens do not bind to the serum steroid binding proteins or albumins, indicating that these substances may induce stronger estrogenic effect compared to E2, when present at the same concentration in the circulation. It has been shown that VEP regulation differs from VTG regulation (Celius and Walther, 1998a.; Celius and Walther, 1998b; Westerlund *et al.*, 2001). Thus, other factors beside E2 may influence the expression patterns of these genes. As substances may interact in an additive, synergistic, potentiating or antagonistic fashion it is important to understand the interactions of endogenous compound on reproductive processes.

In 1987 it was determined that the expression of metalloproteins called metallothioneins (MT) were regulated during the reproductive processes (Olsson *et al.*, 1987). MTs are a family of ubiquitous, highly conserved, cytosolic, heavy-metal binding proteins with a large number of cystein residues (Kägi, 1993). Cellular production of MT is inducible by a wide variety of physical and chemical stimulies such as metals, hormones, interferones and UV light (Milles *et al.*, 2000) Due to the metal-binding abilities of MT, the proteins primary function has been indicated to be heavy-metal detoxification and regulation of trace-metal homeostasis. In rainbow trout it has been shown that the hepatic expression of MT increases towards ovulation (Olsson *et al.*, 1990). Down regulation of VTG expression coincided with a

rise of hepatic MT expression during the reproductive season of rainbow trout (Olsson *et al.*, 1985). In rainbow trout it has been suggested that MT is regulated by the presence of free zinc during sexual maturation (Olsson *et al.*, 1987). Other non-essential heavy metals such as cadmium (Cd) inhibit E2 induced VTG synthesis in rainbow trout (Olsson *et al.*, 1995). It has been found that Cd induces stress responses in animals but more studies are needed to understand how stress inducers interact with the endocrine system.

AIMS

Industrial development has led to the release of a multitude of anthropogenic substances in the environment. Many of these substances have been shown to alter or impair reproductive processes by direct or indirect interactions with the endocrine system. Since there are important variations in the reproductive signalling mechanisms between fish species, it is important that reproduction is studied in a number of species.

In this thesis the emphasis is on oocyte growth, maturation and the interactions between different substances on endocrine parameters in the salmonid species Arctic char (*Salvelinus alpinus*).

Specific aims

- It has been shown that estrogen can affect the heavy metal detoxification system involving up-regulation of MT. Is it possible that estrogen analogues affect MT in a similar way?
- The heavy metal, Cd, has been shown to induce physiological stress and interfere with reproduction in many organisms. Is it possible that other physiological stressors affect the E2 linked reproductive systems?
- Does the major “stress” steroid, cortisol (F), have a direct effect on vitellogenesis?
- Since VEP expression differs from the VTG expression in teleosts, is this reflected by differences in stress response?
- Oocyte maturation is a complex process that may be influenced by many factors. How is oocyte maturation in Arctic char regulated and can exogenous factors influence final oocyte maturation?

RESULTS AND DISCUSSION

It is known that many manmade chemicals, present in the environment, can affect and alter the functions of the reproductive systems in different organisms. Therefore it is of great importance to investigate and characterize the reproductive systems and also study different parameters that might affect these. In this thesis systems involved in the Arctic char oocyte growth and maturation have been characterized. While it has been shown that the yolk and egg-shell proteins are under estrogen control in oviparous teleosts it is also of great importance to investigate if the systems can be affected by any other substance, as this would have implications on the usefulness of VTG and VEP as biomarkers.

It has been shown that teleost VTG production is not only regulated by circulating E2 but that other factors also affect the vitellogenesis. In 1987 it was established that during the reproductive season in rainbow trout a decrease of circulating VTG coincided with an increase of hepatic MT (Olsson *et al.*, 1987). The study presented in paper (I) was undertaken to investigate if there was correlations between MT expression and EDS in Arctic char. It was found that the MT inducing heavy metal Cd, induced expression of MT mRNA in both liver and kidney. Coadministration of Cd and E2 reduced the hepatic MT mRNA levels when compared with Cd exposed animals, indicating that E2 regulated hepatic processes are involved in reduction of hepatic MT mRNA expression. Reduced hepatic MT levels were also seen when coexposing animals with Cd and the EDSs, 2,2',4,6,6'-pentachloro-biphenyl (PCB#104) and 2',4',6',-trichloro-4-biphenylol (4-OH-PCB#30). This inhibition of MT suggest that EDS may increase Cd toxicity in wildlife.

Beside upregulation by heavy metals, MT is also upregulated by free radicals through AP1/ARE elements (Angel *et al.*, 1991; Kling and Olsson, 2000) and F (Hyllner *et al.*, 1989). Cd is a strong inducer of physiological stress responses. Stress activates the hypothalamus-pituitary-interrenal axis, characterised by a release of adrenocorticotrophic hormone (ACTH) into the bloodstream and a secretion of F from the interrenal cells in the kidney. It has been found that a many types of stress may affect the reproductive processes (de Montalembert *et al.*, 1978; Campbell *et al.*, 1992). The second study (II) was performed to investigate if vitellogenesis was under strict estrogenic control in Arctic char or if VTG was affected by introducing a stress factor, i.e. cortisol. The study showed that Arctic char VTG is under estrogenic control and that E2 induced expression of VTG in a dose dependent manner.

Administration of F resulted in elevated VTG protein levels while coinjections of the fish with E2 and F lead to a dose dependent decrease of VTG plasma protein, even though no effect could be seen on hepatic VTG mRNA levels (II). This indicates post-transcriptional regulation of VTG following F exposure. Studies of stress effects on VTG expression has been made earlier, but the results are somewhat ambiguous showing opposite effects. Pelissero *et al.* (1993) and Sundararaj *et al.* (1982) have both shown that F does not effect VTG synthesis at the protein level in catfish (*Heteropneustes fossilis*) and rainbow trout respectively, while Teitsma *et al.* (1998) has shown that F diminish binding of E2 to ER in the liver in rainbow trout. It was suggested that F may influence ER transcription or may destabilize the ER mRNA thereby decreasing the half-life of the ER mRNA. It has also been suggested that ER and the glucocorticoid-receptor (GR) may interact and the expression of estrogen regulated genes may be suppressed by F in the rainbow trout liver (Teitsma *et al.*, 1998). In contrast to this, it has been shown that F itself upregulates the VTG expression *in vivo* in *Xenopus*, due to crosstalk between the ER and the GR (Marilley *et al.*, 1998). It has been established that each MT molecule bind 7 molecules of Zn for proper folding (Furey, *et al.*, 1986). VTG has been suggested to function as a metal transporting protein and metal-ions (Zn^{2+}) are crucial for correct folding of metalloproteins. While metal-ions bind to cysteins in MT ($K_d = 18.2$), Zn binds to histidines in VTG ($K_d = 12.1$) (Auld *et al.*, 1996; Martell and Smith, 1974). Since F induce MT (Hyllner *et al.*, 1989) and E2 induce VTG, the coexposure to F and E2 will induce both hepatic MT and VTG synthesis. Since cysteins bind metal-ions with higher affinity than histidines (Glover *et al.*, 2002), newly produced hepatic MT will bind Zn^{2+} stronger than VTG. This suggests that competition for Zn between MT and VTG may lead to a lack of Zn in VTG with a concomitant degradation of VTG.

With the growing amounts of xenobiotics present in the environment the need for biomarkers of reproductive disturbances has increased. Polyclonal VTG antibodies have been produced for a number of teleost species (Kishida *et al.*, 1992; Buerano *et al.*, 1995; Lomax *et al.*, 1998; Parks *et al.*, 1999; Sherry *et al.*, 1999; Brion *et al.*, 2002; Kordes *et al.*, 2002; Tyler *et al.*, 2002; Hennies *et al.*, 2003). However, the intra species specificity is generally not adequate to allow detection of heterologous VTGs. Due to this species specific antibodies are needed to in order to increase the number of species in which VTG can serve as a biomarker for estrogenicity. However, as Arctic char VTG is affected by F this suggests that exposure to xenobiotics that induce increased levels of stress hormones, may reduce the usefulness of VTG as a biomarker.

The production and assembly of a functional eggshell is an important event during oocyte growth. VEP expression is more responsive to E2 than VTG (Celius and Walther, 1998a) and has also been suggested to be a more sensitive biomarker for estrogenicity. Since stress factors clearly affect VTG expression would it not be plausible to assume that the same effects would be seen on VEP expression?

When studies were made on fish undergoing oocyte growth under natural conditions (III) an increase of F could be seen prior ovulation, a pattern previously reported in rainbow trout (Sundararaj *et al.* 1982) and coho salmon (*Oncorhynchus kisutch*) (Feist, 1990). In Arctic char the rise of F was preceded by an increase of E2. The VTG and VEP levels were correlated to elevated E2 levels during the reproductive season. While F alone had no effect on VEP levels a dose-dependent increase of VEP expression was observed when fish were exposed to E2. Coexposure to E2 and F resulted in a stronger response than E2 alone. These results are in contrast to the observed reduction of VTG (II, III). A possible explanation for this may be that VEPs do not need metal-ions to fold correctly, and should therefore not be affected by the sequestration of Zn by MT. It was also found that F do not affect the VTG or VEP production if administered more than 8 days after E2 exposure. Under natural conditions E2 was found to increase in May and reach its maximum in September while elevated F levels were first observed in September.

During sexual maturation the increase in F coincided with the growth of the ovaries in Arctic char (III). This suggested that F could be involved in upregulation of oocyte components. No correlation was observed between VTG or VEP and F during sexual maturation. However, the E2 levels were 20 fold lower in this study (III) compared to other studies where the fish were not exposed to elevated temperatures (Korsgaard *et al.*, 1986; Mackay and Lazier, 1993; Pawlowski *et al.*, 2000). There may be a temperature effect that interferes with the normal oocyte development in the present study (III). It should be noted that Arctic char, which spawned at high temperature had low embryo survival (Torleif Andersson, National Board of Fisheries Research Station, Kälmarne, Sweden, personal communication). When juvenile Arctic char were exposed to E2 and F, the F exposure resulted in reduced E2 induced plasma VTG levels, while the same coexposure lead to an increase in plasma VEP levels. It is clear that further research is needed to better understand the mechanisms underlying the differences in effects of F on VTG and VEP production.

These findings (I, II, III) indicate that stress responses differ between systems and that this must be considered in reproductive endocrinology. If an Arctic char is exposed to estrogenic compounds, while experiencing a certain level of stress (physical, chemical or mental) this is likely to reduce the circulating VTG. A decreased amount of deposited VTG in the growing oocyte, could lead to a decrease in embryo survival due to starvation of the offspring. At the same time stress increase VEP production resulting in a thicker egg shell. This may not be advantageous as a thicker egg shell decrease the permeability of the vitelline envelope and may also alter the size of the micropyle, resulting in a decreased fecundity. All deleterious effects seen on Arctic char VTG and VEP expression in this thesis has been done by exposing the fish to an acute stress situation and it is important to perform additional studies to determine the effects of natural hormone fluctuations on VTG and VEP. Furthermore it is important that tools that can be used to predict the outcome of a complex exposure situation leading to effects on the offspring or a decrease of fecundity are available.

To further investigate Arctic char oocytes, the maturation process was studied. It has been demonstrated that oocyte maturation and ovulation is induced in teleost fish by production of maturation inducing substances (MIS), identified as hydroxylated progestins (Scott and Canario 1987; Thomas and Trant, 1989), of the ovarian follicles (Nagahama, 1987). Two C21 steroids, 17,20 β -P and 20 β -S, have been identified as MIS. While 17,20 β -P is the major MIS for salmonids and cyprinids (Scott *et al.*, 1987), 20 β -S has been shown to be the predominant MIS in sciaenids and some other perciform fishes (Thomas, 2000).

The MIS has been shown to induce oocyte maturation in teleosts by binding of the MIS to receptors located on the oocyte plasma membrane (Patiño and Thomas, 1990, Liu and Patiño, 1993; King *et al.*, 1997). The first known nongenomic effect of a steroid was reported in 1964 when it was found that the glucocorticoid aldosterone affects the erythrocytes sodium exchange (Spach *et al.*, 1964; Schmidt *et al.*, 2000). The first identification of a teleost MIS was made in a salmonid fish (Nagahama and Adachi, 1985), but no salmonid MIS receptor have ever been fully characterized in this major teleost group. Upregulation of MIS receptors by gonadotropins during oocyte maturation have been demonstrated in several teleost species (Thomas and Patiño 1991; King *et al.*, 1997) but not much is known about the processes regulation maturation in fish. To investigate the salmonid oocyte maturation process a characterisation of the MIS receptor in Arctic char was made (IV).

A single class of high affinity (K_d , 13.8 \pm 1.1 nM), low capacity (B_{max} -1.6 \pm 0.6 pmol/g ovary) binding sites was identified by saturation and Scatchard analyses. The binding was

classified as a classical steroid receptor-binding moiety since it also had rapid association and dissociation of the steroid to its membrane binding sites as well as high ligand and tissue specificity. An increase in receptor quantity was found when ovary fragments were incubated in the presence of hCG, a substance that has been shown to initiate oocyte maturation (Thomas and Trant, 1989). Following hCG exposure oocytes present in the ovarian fragments was studied at different time points to determine the oocytes ability to undergo GVM and GVBD. It was found that the Arctic char oocytes proceeded into GVM and GVBD after just 12 hours of hCG induction. This finding together with the hCG initiated increase of MIS receptor binding indicates that the characterized receptor has a biological function during oocyte maturation in Arctic char.

By investigating GVBD occurrence and MIS binding to its receptor in the presence of known agonists and antagonist it has been suggested that EDS can bind to the steroid membrane receptors and thereby affect oocyte maturation and successful fertilization (Ghosh and Thomas, 1995; Thomas and Das, 1997; Das and Thomas, 1999; Thomas, 2000).

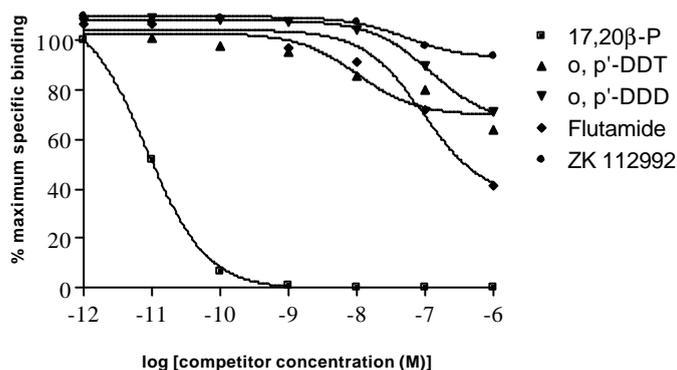


Fig. 6 Competition study of the known EDS, o-p'-DDT (pesticide), o-p'-DDD (pesticide), flutamide (anti androgen) and ZK 112992 (progestins receptor agonist). This indicates that 17,20β-P has a strong affinity to the receptor, but at high concentration the tested EDS are able to displace the Arctic char MIS from its receptor.

The known EDS Kepone and *o-p'*-DDD were found to impair 20β -S binding to spotted seatrout (*Cynoscion nebulosus*) ovarian membranes and also displayed a high MIS receptor binding thereby proving that EDS might act at membrane receptors altering rapid steroid actions (Das and Thomas, 1999).

Investigation of steroid specificity of the Arctic char MIS receptor revealed that receptor binding was specific for $17,20\beta$ -P, but some EDS displayed a minor binding to the MIS receptor (Fig. 6). The relative binding-affinities of all progestogens, steroids and EDS tested were less than 5% of that of $17,20\beta$ -P for the receptor. However, the results indicate that some EDS might bind to the Arctic char MIS receptor and thereby disturb the oocyte maturation process.

CONCLUSIONS

It may be concluded from the present thesis that a wide variety of substances may impair the delicate processes involved in Arctic char oocyte growth and maturation thereby affecting the reproductive success. It was found that commonly used biomarkers such as VTG and VEP were directly affected by the stress hormone, cortisol. An interesting observation was that even though both VTG and VEP is under estrogenic regulation, stress had opposite effect on the two systems leading to a decrease of VTG production while increasing VEP production. The low binding of EDS and high specific binding of 17 β -P to the Arctic char oocyte membrane receptor suggest that oocytes are more sensitive to EDS during the growth phase than during maturation. This does not exclude that some EDS which displays MIS receptor binding might affect oocyte maturation and thereby cause reproductive disturbances. It is important to further investigate how conserved these systems are. If these systems are not highly conserved, specific exposures could lead to extinction of whole populations. In order to conserve the biological diversity it is therefore important to further investigate and characterize processes involved in reproduction and how EDS and stress might effect these in a multitude of species.

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