APPLICATION FOR FUNDING - RESEARCH COUNCIL OF NORWAY

PROSJEKT TITTEL:Climate change, emerging pollutants and reproduction dysfunction in fish: Linking climate change with pollution and biological consequences.

Application Number: ES455969 Project Number: 196442
The application was received - 4.6.2009

APPLICANT

Project Owner

Project Owner NTNU, NT-Fakultet, Institute Biology, Department Biology

Project administrator

Eivin Røskaft , Instituttleder
Confirmation 4 The application has been approved by the Project Owner

Project manager

Augustine Arukwe

PROJECT INFO

Primary and secondary objectives of the project

The goal of this research is to elucidate the combined impacts of quantifiable measures of climate change and emerging contaminants (FOCs) on lipid peroxidation and endocrine signaling of in Atlantic Cod.

Hypothesis: We will test the hypothesis that the primary indicators of climate change (temperature and CO2 or hypoxia) will potentiate the effects of FOCs leading to endocrine disruption, developmental and reproductive dysfunction with possible consequences for the wild Atlantic cod populations. The study will therefore evaluate a holistic integration of FOCs sources and transport (i.e. industrial sources and long distance atmospheric transportation), effects of climate change and biological consequences of their interactions.

Secondary objective: Provide an integrated and quantifiable evidence on the effects and interactions between climate change and emerging pollutants on organisms and biological processes.

Project summary

Global warming is the increase in the average temperature of the earth's near-surface air and oceans since the mid-twentieth century and its projected continuation. Current models predict a global warming of about 1.4 - 5.8° C from between 1990 to 2100. Human activities that contribute to climate change include the burning of fossil fuels and agriculture and land-use changes, like deforestation. These cause emissions or net increases of carbon dioxide (CO2), the main gas responsible for climate change, as well as other 'greenhouse' gases. Climate change is often viewed as a phenomenon that will develop in the coming century, but its effects are already being seen. The two most discussed and prominent effects of climate change are increased temperature and carbon dioxide (CO2). In aquatic systems, oxygen levels, or dissolved and biologically available oxygen, are water temperature dependent where warmer water produces lower partial pressure of oxygen or pO2. High levels of aquatic CO2 concentrations (hypercapnia) lead to a lower pH and can have effects on animal physiology such as a decrease in protein biosynthesis rates. Hypercapnia and temperature dependent changes in pO2 can lead to a lack of oxygen or hypoxic stress in aquatic vertebrate organisms. Because of the detrimental effects of decreased oxygen tension, organisms have developed a programmed response to this condition. Thus, a complex series of mechanisms associating temperature, CO2 and perflourinated pollutants with steroidogenesis or estrogen signaling have been proposed. The implications of these types of associations can only be elucidated through systematic investigation and cod provides excellent model.

Supplementary info from applicant

Application type Researcher project
Other relevant programmes/activities/projects

Marine Ecosystems (Delprogram - Marine økosystemer)
Discipline(s)/ specialist field Ecotoxicology, Molecular Toxicology. Analytical chemistry
If applying for additional funding, specify project number
No related applications been submitted to the Research Council and/or any other public funding

Project period; from 20100301 to 20130331

Main activities and milestones in the project period

In vivo experiment - Part 1: 1 – 4 kvartal 2010
In vivo experiment - Part 2: 1 – 3 kvartal 2011
In vitro experiment – 3.kvartal 2011 – 2 kvartal 2012
In vitro, summary and publications - 3 – kvartal 2012 - 1.kvartal 2013

Dissemination of project results

Results obtained from this project will be presented in national and international conferences. It will also be given as media reports, popular science and also be used as teaching material in the forurensningsfag studiet at the Biology Department, NTNU and elsewhere. The results will also be published in peer-reviewed national and international scientific journals especially those directed towards aquatic toxicologists, fisheries biologists, environmental health officers and policy makers, geologists, climate researchers and aquatic ecologists. Any additional new cDNA clones and their sequences will be uploaded to the GenBank at NCBI, and made available to the national and global research community.

Budget /Cost plan (in NOK 1000)

Specification
The requested amount cover 12 months guest researcher stipend for Dr. Lynn and salary of doctoral fellow in 2010, 6 months guest researcher stipend for Dr. Lynn in 2011 and salary for doctoral fellow in 2011 and salary for doctoral fellow in 2012. For all project years, the budget also includes 35% of prof. Arukwes time used in the project and paid by NTNU. The budget also includes analytical reagents, travels and cost of proteome, metabolites and cellular analysis with partners.

Cost code

Funding plan

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Person for whom a fellowship/position is being sought

Martine H. Gjernes , Doctoral fellowship for perioden 01.03.10 – 01.04.13
Scott Lynn , Visiting researcher fellowship 010310 - 310111 (31.12.11)

Documentation for calculation of overseas fellowships and visiting researcher fellowships

Allocations sought from the Research Council

Partners (institution/company) collaborating as consortium participants

1
Institution/ company Environment Canada
Contact person Dr. Robert Letcher
2
Institution/ company University of Porto, Portugal
Contact person Prof. Eduardo Rocha
3
Institution/ company University of Bergen, Norway
Contact person Prof. Anders Goksøyr
4
Institution/ company Michigan State university, USA
Contact person Dr. Scott Lynn

Curriculum vitae (CV) with list of publications
Filename Lynn-CV.pdf
Reference ES446535_002_1_CV_20090602
Filename CV-Martine.pdf
Reference ES446535_002_2_CV_20090602
Filename CV-Arukwe.pdf
Reference ES446535_002_3_CV_20090602
Filename CV NFR AG09.pdf
Reference ES446535_002_4_CV_20090602

Referees
Filename Experts.pdf
Reference ES446535_005_1_Fageksperter_20090603
Recommendation and invitation (overseas fellowships)
Confirmation from partner(s)
Filename Letter-Letcher.pdf
Reference ES446535_008_1_AktiveSamarbeidspartnere_20090602
Filename Letter-Goksoyr.pdf
Reference ES446535_008_2_AktiveSamarbeidspartnere_20090602
Filename Letter-Rocha.pdf
Reference ES446535_008_3_AktiveSamarbeidspartnere_20090603
Other items
Filename Fulbright fellowship.pdf
Reference ES446535_010_1_Annet_20090602
Filename Cover letter.pdf
Reference ES446535_010_2_Annet_20090602

PROSJEKTET

***Project title*:**
Climate change, emerging pollutants and reproduction dysfunction in fish: Linking quantifiable measures of climate change with pollution and biological consequences

***Project participants**:*
Prof. Augustine Arukwe: Dept of Biology, NTNU Norway (project leader)
Prof. Anders Goksøyr: Dept. of Molecular Biology, University of Bergen, Norway
Prof. Eduardo Rocha: University of Porto, Portugal
Dr. Robert Letcher: Environment Canada, Ottawa, Canada
Dr. Trond Kortner: Dept of Biology, NTNU Norway
Dr. Anne Skjetne Mortensen: Dept of Biology, NTNU Norway
Msc. Martine H. Gjernes (responsible doctoral fellow)
Dr. Scott Lynn, Michigan State University East Lansing USA (visiting fellow)

Relevance to the proposal call
Data and knowledge on the impact of emerging pollutants (such as FOCs) and their interactions with climate factors that are put in a holistic and integrated scientific approach are being requested by “The Ocean and Coastal Areas Program”. Similar knowledge is also highly needed for both the political and societal concerns on the impact of climate change on biological systems. While the purpose of this work is to gain understanding into mechanisms associated with climate change and FOCs activated disruption of steroidogenesis leading to the disruption of reproduction and development, there are sincere applications and implications in the field of environmental science and ecology. This proposal attempts to tie the direct effects of climate change to physiological endpoints in steroid biosynthesis pathways, reproduction and development. In regions where environmental pollution (FOCs,

PCBs, dioxin, etc.) is particularly severe, reproductive impairment via a disruption of steroidogenesis not only threatens the individual survival [1], but also threatens reproductive continuity and recruitment. Testosterone and estrogen are the key reproductive steroid hormones and most, if not all, of the physiological changes associated with sexual maturation and reproduction are derived from their effects. A disruption of steroidogenesis in fish would result in a loss of reproductive timing and ability, which has population level and even ecological effects [2]. Climate change is a growing global dilemma and this research will attempt to understand the role that the effects of climate change will have on the physiological and ecological health of an important sentinel species.

***Project summary**:*
Global warming is the increase in the average temperature of the earth's near-surface air and oceans since the midtwentieth century and its projected continuation. Current models predict a global warming of about 1.4 - 5.8° C from between 1990 to 2100. Human activities that contribute to climate change include the burning of fossil fuels and agriculture and land-use changes, like deforestation. These cause emissions or net increases of carbon dioxide (CO2), the main gas responsible for climate change, as well as other 'greenhouse' gases. Climate change is often viewed as a phenomenon that will develop in the coming century, but its effects are already being seen. The two most discussed and prominent effects of climate change are increased temperature and carbon dioxide (CO2). In aquatic systems, oxygen levels, or dissolved and biologically available oxygen, are water temperature dependent where warmer water produces lower partial pressure of oxygen or pO2. High levels of aquatic CO2 concentrations (hypercapnia) lead to a lower pH and can have effects on animal physiology such as a decrease in protein biosynthesis rates. Hypercapnia and temperature dependent changes in pO2 can lead to a lack of oxygen or hypoxic stress in aquatic vertebrate organisms. Because of the detrimental effects of decreased oxygen tension, organisms have developed a programmed response to this condition. Thus, a complex series of mechanisms associating temperature, CO2 and organochlorine ollutants with developmental defects, steroidogenesis or estrogen signaling have been proposed. Until now, no such comprehensive study has been performed to understand the combined effects of climate change and contaminants on biological processes. The implications of these types of associations can only be elucidated through systematic investigations and cod provides excellent model.

Relevance of the proposal to the present call to Strategic institutional significance
NTNU has identified six thematic interdisciplinary strategic research areas in Energy and petroleum, resources and environment; Globalization; Information and communication technology; Medical technology; Marine and maritime research; Material technology. Under these thematic areas, functional genomics is an integral aspect of the medical technology and information and communication technology as ecotoxicology is for the marine and environment, energy and petroleum areas. Therefore, our plan is to define our ambitions with regard to the type of solutions that we want to provide and translate these into a plan for competences needed to fulfill this ambition, especially with regard to basic and societal sciences, and to enact upon it. It is therefore on these notes and for strategic reasons, that Prof. Arukwe applied for a Fulbright research scholar/lecturer grant to bring on board a highly qualified fellow (Dr. Scott Lynn) to our groups (the Marine research and cotoxicology), to provide exchange of ideas and increased research collaboration, and become a perfect “tool” that connects several of these thematic research areas at NTNU, namely: Energy and petroleum, resources and environment, Medical technology; Marine and maritime research; Material technology and the different required knowledge areas of the present call. The Fulbright fellowship was awarded to Dr. Lynn (see enclosure) who be arriving NTNU in January 2010 for 6 months and will participate in establishing the new research agenda described in the present proposal and contribute in teaching/mentoring of young scientists with the ecotoxicology groups. Thus, we are seeking with this proposal to extend Dr. Lynn’s stay for another 18 months and funding for a doctoral fellow (Miss Martine H.Gjernes – that

will finish her Msc thesis in the autumn) and work alongside Dr. Lynn to build an environment for understanding the effect/role of climate change on chemically-mediated reproductive dysfunction is fish.

The science and innovative aspects

Climate change: Any long-term significant change in the average weather experienced by a given region is generally referred as climate change. These changes can be caused by dynamic processes on earth, external forces including variations in sunlight intensity, and most recently by human activities. Global warming is the increase in the average temperature of the earth's near-surface air and oceans since the mid-twentieth century and its projected continuation. Current models predict a global warming of about 1.4 - 5.8° C from between 1990 to 2100. Human activities that contribute to climate change include in particular the burning of fossil fuels, agriculture and land-use changes like deforestation. These cause emissions of carbon dioxide (CO_2), the main gas responsible for climate change, as well as other 'greenhouse' gases. Climate change is often viewed as a phenomenon that will develop in the coming century, but its effects are already being seen. The two most discussed and prominent effects of climate change are increased temperature [3-5] and carbon dioxide (CO_2) [3, 5, 6]. In aquatic systems, oxygen levels, or dissolved and biologically available oxygen, are water temperature dependent where warmer water produces lower partial pressure of oxygen or pO_2. High levels of aquatic CO_2 concentrations (hypercapnia) lead to a lower pH and can have effects on animal physiology such as a decrease in rates of protein biosysthesis [7]. Hypercapnia and temperature dependent changes in pO_2 can lead to a lack of oxygen or hypoxic stress in aquatic vertebrate organisms. Hypoxia is defined as a state in which the level of oxygen drops below normal in cells or tissues. Because of the detrimental effects of decreased oxygen tension, organisms have developed a programmed response to this condition [8]. For example, in response to cellular hypoxia, tissues adapt to consume less oxygen by shifting ATP production from mitochondrial fatty acid beta-oxidation to glycolysis. The transcriptional activation of glucose transporters and glycolytic enzymes by hypoxia is mediated by hypoxia-inducible factor 1 (HIF-1).

Perfluorooctanoic acid (PFOA) and perfluorodecanoic acid (PFDA) - are ubiquitous environmental contaminants and have been detected globally in wildlife and humans. By being neither fat- nor water-soluble, perfluorinated compounds represent a very unique chemistry with not well-understood toxicological properties and clearly the presence of different length (perfluorinated) carbon chains and functional groups are likely to influence toxicity. Fluorinated organochemicals (FOCs) are among the halogenated organochemicals that have been less studied intensively with regard to their ecotoxicological properties, compared with chlorinated and brominated organics. Nonetheless, these compounds are being produced for decades and have a broad application spectrum in industry and household as surfactants, lubricants, adhesives, refrigerants, fire retardants, propellants, agrochemicals and medicines [9, 10]. One class of FOCs, the perfluorinated sulfonates is used as catalysts and surfactants.Perfluorooctanyl sulfonate (PFOS) is one of these compounds with important applications as wetting and foaming agent and as precursor of other surfactants and pesticides [10]. FOCs are *peroxisome proliferators*, and accumulate in the liver where they inhibit glutathione peroxidase, a selenoprotein essential for thyroid hormone conversion. They also cause cancer in the liver and it was reported recently that PFOS is occurring worldwide in wildlife tissues at relatively high concentrations in top predators (e.g. 3680 ng/g wet liver weight in mink) by Giesy and Kannan [11]. Even in remote areas, PFOS is present in detectable concentrations in a great diversity of organisms ([12-16]. Since fish-eating animals have higher PFOS burdens than their prey, it was suggested that PFOS has a tendency to bioaccumulate to higher trophic levels in the food chain. PFOS levels in tuna fish from the Northern Pacific were lower than the limit of quantification, while tuna liver from the Mediterranean contained 21-87 ng PFOS/g ww. In livers from tuna fish taken in the North Pacific the levels were below the detection limit of 7 ng/g [17]. The levels

were clearly higher in populated and industrialized area than in remote locations. In a later paper [12, 13], PFOS levels in blood from tuna and swordfish from the Mediterranean Sea were reported. Levels ranged 27-52 (mean 40) ng/mL and 4-14 (mean: 7.2) ng/mL, respectively [18]. The same is also true for all the perfluorochemicals in Atlantic salmon. In several fish spscies, very high PFOS concentrations have been found in liver and these include, plaice *Pleuronectes platessa* (7760 ng/g ww) and feral gibel carp *Carassius auratus* (9031 ng/g ww) in Belgium [19]. High concentrations of PFOS have also been reported from liver tissue of tilapia Oreochromis niloticus (1100 ng/g ww) and mullet bile *Mugil incilis* (up to 3673 ⌈g/L) [20]. The widespread distribution of PFOS in the environment continues to raise concern. The ecotoxicity of FOCs has only been demonstrated in few studies and mainly concern acute toxicity to aquatic organism. PFOS is moderately acute toxic and slightly chronically toxic to aquatic organisms. There seems to be large species difference in the biological response, because PFOS was three orders of magnitude more toxic to the aquatic midge *Chironomus tentans* than to most other aquatic organisms. The scarce database indicates a need for further studies. In a recent study by Inoue et al (2004), FOCs concentrations were detected in maternal and cord blood samples. Pregnant women 17–37 years of age were enrolled as subjects, revealing a high correlation between PFOS concentrations in maternal and cord blood (r_2 = 0.876). However, the authors did not find any significant correlations between PFOS concentration in maternal and cord blood samples and age bracket, birth weight, or levels of thyroid-stimulating hormone or free thyroxine, although these variables were elevated in the study subjects. Their study revealed that human fetuses in Japan might be exposed to relatively high levels of FOCs. *In Norway FOCs has been shown to occur widely in freshwater system, although with low levels concentrations compared to other industrialized nations (NILU, 2004)*. Recent media reports in Norway showed a rampant detection in pregnant women in the North.

Hypoxic Stress

Hypoxia Inducible Factors (HIFs), which include HIF1 ⟨, HIF2⟨ and HIF3 ⟨ along with their nuclear dimers, are transcription factors that control the cellular response to decreases in oxygen availability [21]. HIFs are members of the PAS (Per, ARNT, SIM) superfamily of proteins, which is characterized by the presence of a PAS domain that controls dimerization [22, 23] and they also contain a basic-Helix-Loop-Helix domain (bHLH) in their minoterminus which acts as a point of contact for DNA. HIF1⟨, HIF2⟨, and HIF3⟨ are primarily cytoplasmic and under normal oxygen tension conditions that are quickly degraded by ubiquitin pathway. Under hypoxic stress however, the cytoplasmic HIFs are stabilized and signaled to translocate to the nucleus where they are free to form dimers with Aryl-hydrocarbon Receptor Nuclear Translocator (Arnt) and Arnt2 [24, 25]. These heterodimeric pairs bind genomic DNA at sites called Hypoxia Response Elements (HREs) to initiate transcriptional responses in gene expression [23].

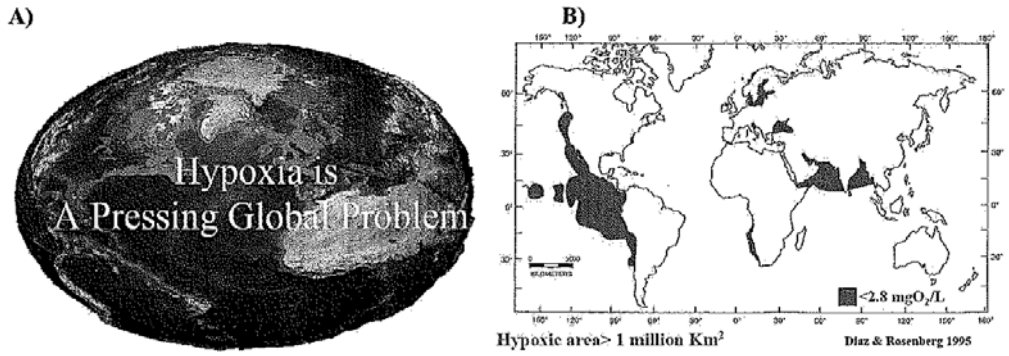

Figure 1: Hypoxia is defined as shortage of oxygen (O2) to <2 ml/L or <2.8 mg/L and is known as a pressing global problem (A). Figure 1B shows significant portion of world freshwater and marine ecosystem affected by hypoxia, including areas in central Norway and the Baltic sea (adapted from [26]*).*

Figure 1: Hypoxia is defined as shortage of oxygen (O2) to <2 ml/L or <2.8 mg/L and is known as a pressing global problem (A). Figure 1B shows significant portion of world freshwater and marine ecosystem affected by hypoxia, including areas in central Norway and the Baltic sea (adapted from [26]*).*

Hypoxia and AhR interaction

Several studies have recently shown an interaction between the AhR and hypoxia signaling systems in vertebrate cells. The experiments of Khan *et al* [27] indicate that hypoxia rapidly induces proteins that inhibit AhR responsiveness. Prasch et al [28] also found that hypoxia significantly reduced constitutive CYP1A1 and AhR mRNA expression in a HIF2〈 dependent manner in human lung endothelial cells. Inversely, Seifert *et al.* [29] found that TCDD treatment reduces the hypoxic dependent stabilization and subsequent transcriptional ability of HIF1〈 in a human hepatoma cell line. Lee *et al.* [30] identified 33 genes which exhibited an interaction between AhR activation and hypoxic response in a human hepatoma cell line using rtPCR and microarray studies. Their results suggest that AhR-HIF1 cross-talk cannot be explained completely by competition for Arnt and most likely involves other cofactors, response elements, and promoter context. These results taken together imply that there is considerable interaction between the hypoxia and dioxin response pathways in vertebrate systems.

Estrogen Receptor and Steroidogenesis

The steroid hormones are a key component of the vertebrate endocrine system and are comprised of three separate groups known as the mineralocorticoids (aldosterone), glucocorticoids (cortisol) and sex steroids (estrogen, testosterone and11-ketotestosterone). The sex steroids, testosterone and estrogen (E_2), are preferentially produced in males and females, respectively, and are primarily synthesized in the gonads (testis and ovary). In addition, 11- ketotestosterone is produced in both male and females gonads where it plays significant roles in spermatogenesis and development of previtellogenin oocyte, respectively. Androgens and converted to estrogens by the aromatase enzyme coded by the cyp19 gene and these sex steroids play key roles in sexual differentiation, maturation and reproduction [31] by binding to their respective transcriptional receptors, estrogen (ER) and androgen (AR). Ligand bound ER produces a transcriptional response in target genes by binding to Estrogen Response Elements (EREs) in genomic DNA to control expression levels. Endocrine disruption is the process by which a chemical insult or some other stress disrupts the endocrine system's normal pattern. Alteration of aromatase CYP19 expression and/or activity, either upregulation or downregulation, may lead to diverse disturbances of the above mentioned processes. Prediction of multiple transcriptional regulatory elements in the promoters of teleost cyp19 genes suggests the possibility for several EDC classes to affect

cyp19 expression on the transcriptional level. These sites include cAMP responsive elements, a steroidogenic factor 1/adrenal 4 binding protein site, an estrogen-responsive element (ERE), half-EREs, dioxin-responsive elements, and elements related to diverse other nuclear receptors (peroxisome proliferator activated receptor, retinoid X receptor, retinoic acid receptor). Certain compounds including phytoestrogens, xenoestrogens, FOCs, fungicides and organotins may modulate aromatase CYP19 activity on the post-transcriptional level. Diverse EDCs may affect the expression and/or activity of aromatase cyp19 genes through a variety of mechanisms, many of which need further characterization in order to improve the prediction of risks posed by a contaminated environment to teleost fish population. In regards to sex hormones, this can occur in a variety of ways ranging from chemicals that mimic steroids, environmental estrogens are the most common, to ones that interfere with steroidogenesis (the natural production of steroids). Cholesterol is the primary substrate in steroid biosynthesis and its transport from the outer to the inner mitochondrial membrane represents a rate-limiting step in steroidogenesis [32]. The steroidogenic acute regulatory (StAR) protein, an intracellular cholesterol transport protein, has been shown to play a key role in steroidogenesis and is therefore a target for endocrine disruptors [33].

Peroxisome proliferator activated receptors (PPARs)

Xenobiotic and drugs can produce alteration in target gene expression patterns through activation of nuclear receptors and other transcription factors including aryl hyrdrocarbon receptor (AhR), constitutive androstane receptors (CAR), pregnane-X receptor (PXR), PPAR-⟨, and NF-E2–related factor (Nrf2). The PPARs belong to the group of nuclear receptor proteins that function as transcription factors regulating the expression of genes. PPARs play essential roles in the regulation of cellular differentiation, development, and metabolism (carbohydrate, lipid, and protein) of higher organisms where they show isoform tissue expression patterns [34, 35]. PPAR⟨ and PPAR© are the molecular targets of a number of marketed drugs, e.g. the fibrates. The synthetic chemical perfluorooctanoic acid activates PPAR⟨ while the synthetic perfluorononanoic acid activates both PPAR⟨ and PPAR© [35] (Mortensen et al. In prerp). Drugs, such as thiazolidinediones or TZDs act by binding to PPARs (specifically PPAR©). Normal PPAR ligands include free fatty acids (FFAs) and eicosanoids. When activated, the receptor migrates to the DNA, activating transcription of a number of specific genes. Recently in our laboratory, we showed that tributltin (TBT), PFOS and PFOA produced *in vivo* and *in vitro* modulations of organspecific patterns (i.e. in the brain, kidney, gonad and liver) of PPAR signaling, oxidative stress, estrogenic responses and lipid peroxidation in the Atlantic salmon and also affect salmon larval development (Mortensen et al, 2009a,b,c; Kortner et al. 2009; Pavlikova et al. All in prep).

Hypoxia, FOCs, PPAR and reproductive dysfunction

It has been demonstrated that hypoxia may produce major impairment of key reproductive processes in fish by modulating specific hormones, neurotransmitters and receptors that are integral aspects of the Hypothalamus- Pituitary-Gonad-axis (HPG-axis) as well as selected genes controlling steroidogensis and vitellogenesis in fish (see above). The potency of hypoxia induced endocrine disruption is higher than many known EDCs. For example, in zebrafish, hypoxia has been shown to down-regulate aromatase and alter the ratio of testosterone to estradiol during early sex development, leading to a male biased F1 generation [26, 36]. Hypoxia has been shown to cause malformation during development in many fish species, and embryos in some species may undergo complete developmental arrest under anoxia. In zebrafish embryos, blastomeres were arrested during the S and G2 phases of the cell cycle under anoxia. Fish embryos developed under hypoxia lost their normal synchronization, and abnormalities in spinal and vascular development are commonly observed. Laboratory studies have shown that hypoxia caused endocrine disruption through decreasing levels in the brain of serotonin, a chemical important for brain function. The decrease in serotonin was caused by a decrease in an enzyme that plays a role in the

serotonin synthesis pathway. The first clear evidence that a wild population of estuarine fish has experienced reproductive impairment through hypoxia was recently provided [37].

Acyl coenzyme A (acyl-CoA) or steroid acyltransferases catalyses the conversion of steroid to an inactive non-polar form that is subsequently retained in lipoidal tissues of an organisms and thus prevents excretion. Particularly, they catalyses the conjugation of steroids with the fatty acid moiety from acyl-CoAs forming a steroid fatty acid ester [38] and it has been suggested that their expression in rodents is controlled by PPAR〈 [39, 40]. All steroid hormone groups (gluccoorticoids, mineralocoorticoids and sex steroids) can be estrified with fatty acids and steroid fatty acid esters occur in the hydrophobic, lipid fractions of several tissues, such as fat deposits, but also in the brain, ovaries (including follicular fluids), placenta, uterus and mammary glands [41, 42]. Systemic circulation of steroidal fatty acid esters takes by binding to lipoprotein particles (LDL and HDL) in the plasma, although plasma levels of these are very low [43]. The role of PPAR〈 in the esterification of steroids have received great attention, as a result of the understanding that fatty acid estrogens esters increases estrogenic potency, compared with the parent compound and could act as long-lived steroids [41, 44]. For example, mammalian studies have shown that E2 and estriol (E3) are converted to their fatty acid esters derivatives, and these estrogens does not bind to the ER, but require enzymatic hydrolysis by esterases to liberate the bioactive hormone to exert their actions. For E2-fatty acyl esters, injection of immature rodents with E2-stereate activated the HP-ovarian axis and enhanced puberty [45]. Similarly, endogenous testosterone fatty acid esters were shown to increase the potency and duration of action, compared to testosterone [46]. Available evidence suggests that acyl-CoA – steroid acyltransferases are not steroid specific, as testosterone was found to be both substrate and inhibitor of the esterification of estradiol [47]. Thus, the disruption of these processes by FOCs and subsequent effects of climate change (temperature and hypoxia) through PPAR modulation may have significant consequences for fish reproduction.

The physiological role of AhR is yet to be fully characterized. The high degree of AhR conservation and promiscuity suggests an important fundamental role in cellular physiology [48-50]. The involvement of AhR in cell cycle regulation provided evidence on the influence of AhR ligands on cell proliferation, differentiation, and apoptosis, but the molecular mechanism by which the AhR affects the cell cycle is not known. A number of endogenous ligands have been reported to activate AhR [51]. In addition, the activation of un-liganded AhR by phosphorylation has been suggested [52]. The conversion of l-tryptophan to indole-3 pyruvate in mouse tissue extracts followed by the spontaneous reaction of indole-3-pyruvate with water produces a large number of compounds acting as AhR agonists and indicated a physiological role of AhR in the metabolism of endogenously generated compounds [53]. AhR double knockout (AhR null (-/-)) mice have been used to determine the physiological role of AhR, and studies showed that the AhR is required for vascular development regulation in liver and other organs [54] establishing a role of AhR in development and physiological homeostasis. In Baltic salmon embryo suffering from the M74 syndrome, a relationship between reduced DNA-binding activity of HIF- 1〈 and subsequent down-regulation of its target gene, namely the vascular endothelial growth factor (VEGF) was observed [55]. Impaired vascular development and abnormal haemorrhages are observed in mammalian embryos when HIF-1〈 function, Arnt or VEGF are modulated. Since aquatic hypoxia commonly occurs over thousands of square kilometers in freshwater and marine aquatic systems worldwide, understanding the effects of quantifiable measures of climate change (temperature and CO2) will imply that endocrine disruption and reproductive impairment in fish may be a widespread environmental problem and represent quantifiable measures of climate change. Recent study suggests that when persistent coastal hypoxia occurs, there is a potential long-term threat to fish populations and fishery resources. With worldwide increases in hypoxia possibly due to climate change, it's something we must be concerned about, because so many people rely on fishing for their livelihood [56].

Project Proposal

The goal of this research is to elucidate the combined impacts of quantifiable measures of climate change and emerging contaminants (FOCs) on lipid peroxidation and endocrine signaling in Atlantic Cod.

Overall Hypothesis: We will test the hypothesis that the primary indicators of climate change (temperature and CO_2 or hypoxia) will potentiate the effects of FOCs leading to endocrine disruption, developmental and reproductive dysfunction with possible consequences for the wild Atlantic cod populations. The study will therefore evaluate a holistic integration of FOCs sources and transport (i.e. industrial sources and long distance atmospheric transportation), effects of climate change and biological consequences of their interactions.

Workpackage 1 (WP1). In vivo experiment: Hypothesis: quantifiable measures of climate change and FOCs will produce effects on fatty acid □□-oxidation, biotransformation and be endocrine-disruptive in cod with quantifiable consequences for overt reproduction.

Group of male and female adult Atlantic cod will be split evenly into separate treatment groups and exposed to different treatment regimes involving temperature, CO_2, PFOS and PFOA. This study will be multi-factorial with three temperatures (4, 8 and 12° C), two CO_2 levels (ambient and 10 pCO_2) and three PFOS and PFOA concentrations (5 - 20 mg/L). A second experiment will be performed in parallel using the similar group of cod and concentrations of PFOS and PFOA in 0.8 or 5.8 mg O2/L. After 3, 7 and14 days, fish will be humanly sacrificed and brain, liver and gonad tissue will be harvested. Cobalt is a transition metal that replaces iron in heme proteins. In contrast to iron, cobalt does not bind oxygen when incorporated into protoporphyrin [57]. Previous studies have suggested a putative oxygen sensor in the cell membrane that contains a heme protein [57]. When iron is replaced by cobalt, this oxygen sensor signals to the cell that there is a state of oxygen deficiency despite the normal oxygen levels. A third experiment will also be performed in parallel using a similar group of fish in experiment 1 and 2 above using a systemic hypoxia model generated using cobalt chloride (COCl2) treatment. The CoCl2 concentrations will be added in the ambient water (0, 5 and 20 mM solution) and exposed for a total of 14 days in the presence and absence of the PFOS and PFOA concentrations. It has been previously shown that COCl2 produces systemic hypoxia in rats [58, 59]. After 3, 7 and14 days, fish will be humanly sacrificed and brain, liver and gonad tissue will be harvested.

In all experimental setups, the mRNA expression and activity of medium-chain fatty acid dehydrogenase (MCAD), fatty acid binding protein (L-FABP), PPARs, carnitine palmitoyl-transferase I (CPT-I), uncoupling protein 2 (UCP-2), Bcl-2, peroxisomal fatty acyl-CoA oxidase (ACOX), very long-chain acyl-CoA dehydrogenase (VLCAD), long-chain acyl-CoA dehydrogenase (LCAD) will be analyzed using qPCR. In addition, genes involved in mitochondrial respiratory chain and ATP synthesis, including cytochrome c oxidase subunit I (COXI), NADH dehydrogenase subunit I (NDI) and ATP synthase F0 subunit 6 (ATPo6) will also be analyzed. These analyses will demonstrate that turbulence of fatty acid ®-oxidation and oxidative stress responses may be involved in FOCsinduced tissue toxicity. The effects on xenobiotic and steroidogenic pathways will be determined using immunochemical, molecular and cellular techniques (PPAR-isoforms, StAR, AhR-variants), HIF-isoforms, ER〈,ER®, CYP1A1, cyp19-isoforms, P450scc, 3®-HSD). We recently developed a targeted cDNA array in cod [60] and the project manager is a member of the national fish functional genomics (Genofisk) steering group – making sequences for the transcripts targeted in this study easily available through cod genomics database. We have ptimized real-time PCR assays for several of the mentioned variables and several other genes in the other pathways, and recently produced antibodies against synthetic peptides for the StAR protein, P450scc, aromatase, PPAR〈, ER and AhR. CYP1A1 analysis and other proteomic analysis for differential protein expression patterns will be performed with Prof. Anders Goksøyr at the molecular biology department of University of Bergen. Tissue distribution and metabolite

formation of FOCs will be performed in collaboration with Dr. Robert Letcher of Environment Canada, Ottawa. Histopathology and cellular localization of proteins and mRNA will be performed with Prof. Eduardo Rocha of University of Porto, Portugal. Enzyme activities of different substrates (aromatase, EROD, BROD, MROD and PROD) in the biotransformation pathway will also be measured. In addition, we will analyze physiological parameters (hormones levels) in plasma of exposed individuals using enzyme immunoassay (EIA) methods. We are involved in several ongoing projects with all the partners mentioned in this project.

<u>WP2. In vivo experiment:</u> Hypothesis: quantifiable measures of climate change and FOCs will produce effects on fatty acid □-oxidation, biotransformation and be endocrine-disruptive in cod eggs and larvae with possible consequences for overt development and survival.

In larval experiments, cod eggs will be placed in the larval tanks a few days before hatching (5-7 °C). Larvae will be reared at Sealab NTNU (100 larvae per litre; salinity 34 ppt; increase to 12 °C at time of feeding; continuous light), according to our live feed production and larval rearing standard conditions for cod [61]. The fertilized eggs will be exposed continuously to different concentrations of PFOS and PFOA (4 - 12 mg/L), under different treatment regimes involving temperature and CO_2. This study will be multi-factorial with three temperatures (4, 8 and 12°C), CO_2 levels (ambient and 10 pCO_2) and three PFOS and PFOA concentrations (4 - 12 mg/L), separately. A second experiment will be performed in parallel using the similar group of eggs and concentrations of PFOS and PFOA in 0.8 and 5.8 mg O2/L. The entire studies will last for about 3 months and is designed to cover pre-hatch and post-hatch periods (including yolk-sac stages) and early development. For calculation of larval growth, larval samples will routinely be taken at hatching, at the time for the first offer of exogenous food, during change of feed types and at the termination of the experiments. Eggs and larvae from all experiments will be sampled for studies of development, functional histology and biochemical and molecular analyses. In addition, hatching success (%- age of hatched eggs in relation to the total) will be recorded. Thereafter, potential behavioral changes will be recorded on a daily basis, and samples will be collected at weekly intervals where a sub-group will be fixed for histopathology and immunohistochemical evaluations, protein, enzymes and gene expression analysis. In addition to mRNA transcript mentioned in part 1, VEGF, organ volume, apoptosis and vascular development indexes and developmental deformities will be evaluated by stereology in collaboration with Dr. Rocha. Thyroid-, growth- and sex steroid hormone levels will be evaluated using enzyme immunoassay (EIA) methods.

<u>WP3. In vitro experiment:</u> Hypothesis: quantifiable measures of climate change and FOCs will produce effects on in vitro fatty acid □-oxidation, biotransformation and be endocrine-disruptive in cod and these effects will be partially comparable to effects observed in vivo.

Collagenase perfusion, isolation and culture of salmon hepatocytes will be performed by a two-step perfusion technique with modifications as previously described [62]. The cell suspension will be filtered through a 150 ⌈M nylon monofilament and centrifuged at 50 x g for 5 min. Cells will be washed three times with serum-free medium and finally resuspended in complete medium. Following collagenase perfusion and isolation of hepatocytes, viability of cells will be determined by the trypan blue exclusion and cytotoxicity assay methods. A cell viability value of > 90% is an optimal criterion for further use of these cells in our laboratory. Cells will thereafter be plated on a 35 mm TPP Tissue Culture Plates (Techno Plastic Products AG, Switzerland) at the recommended density for monolayer cells of 5 x 10_6 cells in 3 ml DMEM medium (without phenol red) containing 2.5% (v/v) FBS, 0.3 g/L glutamine, and 1% (v/v) penicillin-streptomycin-neomycin solution. The cells were cultured at different temperature regimes in a sterile incubator in the presence and absence of additional, but different O_2/CO_2 regimes for 48 hr prior to chemical exposure. Hepatocytes will be exposed PFOS and PFOA (2–50 mg/L). This in vitro have previously been shown to have effects in salmon hepatocytes [63] In all exposure conditions, media and hepatocytes will be harvested

at 6, 12 and 24 h after exposure and analyzed according to methods described above. ***Please note that an MSc student will be attached to each sub-goal of this proposal.***

Justification for PFOS and PFOA concentrations: Mammalian and teleost studies have shown high LC50 values, suggesting that acutely exposed animals can tolerate quite high levels of PFOS. In rats orally administered PFOS, the LD50 was found to be 200 mg/kg [64]. In fish, LC50 values range from 7.2 to 22 mg/L [65]. LC50 has been reported in the range of 7.8 to 22 mg/L in rainbow trout [64]. Recently, it was shown that PFOS and PFOA were able to produce oxidative stress and induce apoptosis with involvement of caspases in primary culture of tilapia hepatocytes. In rat hepatoma cells treated with 2–50mg PFOS/L for 96 h, genes encoding enzymes involved in fatty acid metabolism, cytochrome P450s, or genes involved in hormone regulation were differentially expressed ([66]. One of the major pathways affected by PFOS exposure was peroxisomal fatty acid _-oxidation. In liver of chicken exposed to 0.02–0.1 mg PFOS/L for 4 weeks numerous genes were differentially expressed, many of them encoding enzymes related to transport of electrons and oxygen or genes involved in the metabolism of lipids and fatty acids [67]. Given that FOCs has a high adherence to solid surfaces, we estimate that a fraction of the given concentrations will be available to the fish and that will be environmentally relevant. However, the actual exposure concentration will be determined in collaboration with Dr. Letcher (see below).

Atlantic Cod as 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 have all suffered from extreme fishing pressure in recent years, and several of them are showing clear signs of over-exploitation. The Northern cod stock on the Grand Banks off the east coast of Canada (Hannesson, R. 1996. Fisheries mismanagement: The case of the North Atlantic cod. Blackwell Scientific Publications, Oxford, pp. 1-160) has suffered severe fishing pressure. Until recently, this stock has not shown any signs of recovery, even after ten years of fishing ban, 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 worldwide. 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 [68, 69]. 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 [70]. The potential for cod farming is therefore regarded as extremely high on a global basis. In Midt-Norge, NTNU and SINTEF researchers have a national and international profile on the nutritional and environmental aspects of cod larval growth and survival. Cod also enjoy a number of natural advantages as a potentially important cultivated species that include - short egg and larval stages, rapid growth, good feed utilization, suitable behavior (‘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. Cod has also a developmental and reproductive sensitivity towards temperature and salinity. 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). In the future, we can expect to encounter challenges on the health side, related to fish feed and to bacteria, viruses and parasites and climate change. 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 quaculture

[71]. Increasing ocean temperatures (and changes in salinity) since the mid-1980's have already been shown to have a negative impact on the survival of young cod by modifying the plankton ecosystem [72] and smaller prey fish [73]. Cell culture results are interesting and informative, but they do not account for the complex physiological interactions associated with vertebrate organisms and therefore live animal experiences are necessary.

The experiments of Seifert *et al.* [74] indicate that organisms exposed to AhR ligands will have a reduced capacity to respond to hypoxic stress, but hypoxic stress in turn reduces the AhR mediated cross-talk with ER [75]. Also, hypoxia and dioxin recruitment of Arnt leads to a reduction in ER transcriptional activity [76]. Recently studies indicate that ERs in hypothalamic neurons are in part responsible for temperature regulation [77]. All of these results show a complex series of mechanisms associating temperature, CO_2 and organochlorine pollutants to steroidogenesis or estrogen signaling. These types of associations can only be elucidated with a live vertebrate model and the importance of Atlantic cod makes it the perfect choice. Therefore, integrating our recent findings with quantifiable climate change parameters (namely temperature and CO2 or hypoxia) will provide a mechanistic understanding of the interactions between climate change and contaminants on organisms and ecosystems.

Project -TEAM

Prof. Augustine Arukwe, Drs. Anne S. Mortensen and Trond M. Kortner: Prof. Arukwe is the group leader for Molecular and Cellular Toxicology/Physiology Research Group at the biology department of NTNU. The group comprises today of Drs Mortensen and Kortner that are recent products of the group, 2 PhD and 9 MSc fellows and a senior engineer (Marianne D. Hansen 50%). An integral emphasis of our research has been on the studies of functional and developmental alterations of wildlife caused by exposure to environmental stressors and during physiological changes. In addition and in collaboration with the aquaculture groups at NTNU, we are studying the hormonal and environmental control of oocyte development and ontogeny of digestive development in Atlantic cod (Gadus morhua) and Atlantic salmon (*Salmo salar*). The development of physiological genomics/proteomics response biomarkers in freshwater and marine species is one aspect of the research. A major and pioneering focus has been on the development and validation of targeted gene chips for use as sensitive screens for environmental and endocrine stress indicating chemically-induced differential gene expressions via receptor-mediated process and for use in the studies hormonal control of reproductive processes and larvae-microbial interactions. Our immediate and future research focus is to consolidate on past research gains within the past years (resulting on the average, 15 publications/year since 2006) by developing our research group from within and outside (bringing international expertise) on larval development, reproductive and general health and the influence of chemical substances.
Methodology: Our laboratory uses appropriate techniques in our investigations. We routinely employ both molecular and cellular methods. Methods in more routine use include: real-time polymerase chain reaction (realtime PCR), molecular gene cloning, sequencing and characterization, immunochemical and immunohistochemical assays, nucleic acid hybridization both in situ and in isolated samples, organ perfusion and in vitro primary cell culture (of liver cells), whole animal evaluation (in vivo), use of chemiluminescence and radio-isotope labeling for cell synthetic estimates, cell fractionation and enzyme purification, antibody production, enzyme activity studies, and other methods in molecular gene expression (for studies of chemically-induced gene expression).

Dr. Scott G. Lynn This project is well suited to my skill set as I have considerable molecular experience along with fish handling and dissecting experience from my PhD research. My Ph.D. dissertation title is “Cloning, characterization and expression of key endocrine genes associated with estrogen stimulated sexually-dimorphic growth in yellow perch (*Perca flavescens*)”. This research focused on seasonal, developmental and estrogen induced changes in expression of key endocrine genes associated with growth or sexual maturation

in the yellow perch. Our work in yellow perch has shown that liver estrogen receptor expression shows significant sex-specific differences during development and in response to season.
In November 2006, I began a postdoctoral fellowship at Michigan State University through the NIEHS training grant to the Center for Integrative Toxicology. At the beginning of this post-doc, I worked on a project generating plasmid constructs and stable cell lines for the NIH grant "Environmental, microbial and mammalian biomolecular responses to AhR ligands". In September 2007, I began to work exclusively on a project investigating the role of HIF1 in mitochondrial function.
This funding opportunity will provide me with a level of toxicological and biochemical training that will compliment my existing knowledge and is a natural extension of my career and mentoring of younger fellows in Prof. Arukwe's laboratory. My overall career goal is to become a toxicology professor at a nationally recognized university performing research, teaching, and mentoring younger scientists. The results generated from this work should be of a caliber for publication in international peer-reviewed journals and could very likely lead to future and further collaborations by Dr. Arukwe, his research group, the involved partners and myself.

Prof. Eduardo Rocha: Prof. Eduardo Rocha is a Professor at the University of Porto, Portugal, and group leader at both the Laboratory of Histology and Embryology, of the ICBAS, and the Laboratory of Cellular, Molecular, and Analytical Studies, of the CIIMAR, where most of the team work is currently focused on both field and experimental toxicology, and on structural and physiological fundamental aspects of relevance for a proper understanding of toxicant effects, namely in fish. Works have been done in liver, gonads, gills and kidney. At this precise moment the research group integrates involves the leader plus 10 PhD collaborators, including 3 full-time post-docs. Also, the team integrates 6 PhD and 2 MSc students, supervised or co-supervised by the Group Leader, plus 2 young project grantees. Three full-time technicians are also engaged in the research team. Two technicians will be engaged soon for a professional training period. The group expertise covers cell biology and histology techniques, being an international reference in quantitative (stereological) approaches in microscopy, also molecular tools, biochemistry and analytical chemistry. Current work deals with endocrine modeling and disruption of members of the nuclear receptor superfamily, with toxicopathology (in several organs, at histological and ultrastructural levels), toxicogenomics and finally with chemical toxicology, validating new methods and conducting wide field surveys targeting an increasing range of endocrine disruptors compounds, with emphasis on estrogenic compounds.

Dr. Robert Letcher: Dr. Letcher is a chemical toxicologist of high international reputation at the NWRC, Environment Canada, Carleton University (Ottawa, Ontario, Canada). A fundamental aspect of research initiatives in my laboratory is to investigate the involvement and importance of metabolic processes on the fate and bioactivation of fate of organohalogen contaminants in exposed biota and their ecosystems. With respect to bioactivation, impacts on endocrine (estrogenic, thyroidogenic) functions are of particular interest. Research carried out in my group and with collaborators has investigated the immunological and catalytic characterization of Phase I and II enzyme systems (e.g., cytochrome P450 monooxygenases (CYPs)) in relation to organohalogen contaminants, in various vertebrate marine and freshwater organisms (e.g., polar bear, beluga whales, seals and fish) and recently conatiminant dynamics under changing climate. Furthermore, I lead a research group/facility for the ultratrace analysis/determination of legacy and emerging chemicals of environmental concern. Among those contaminants are xeno-hormones such as EE2 and polyfluoroalkyl compounds (PFCs) such as the persistent and bioaccumulative PFOS. My group has methods based on liquid chromatography-mass spectrometry (LC-MS) in place to determine ultratrace levels of these substances in wildlife tissues.

Prof. Ander Goksøyr: Anders Goksøyr, dr. scient. professor, expertise in molecular toxicology, aquatic toxicology, biomarker research, endocrine disruption, biotransformation, nuclear receptors. Prof. Goksøyr has ong experience in project management, incl. national and international projects. Published 115 papers in peer- reviewed international scientific journals, editorial board member of international journals (Aquatic Toxicology, Marine Environmental Research Comparative Hepatology), reviewer for journals and science funding bodies in many countries.

Budget:

** The requested budget will cover salary for 3 years Martine Gjernes (PhD fellow), visiting fellow Dr, Scott Lynn and include costs for consumables (also for the 3 Msc students). The experiments described in this project will constitute the researcher training projects of graduate students, who will carry out all of their experimental work on the samples collected from the project and supervised by Dr. Arukwe and the partners. The fellow and students will also have the opportunity to interact with other researchers at the Biology Dept., NTNU and external partners. These researchers will therefore act as co-supervisors for the graduate students. ----------*

APPENDIX

References

CURRICULUM VITAE

- Scott G. Lynn

- Martine Hron Gjernes

- Anders Goksøyr

- Augustine Arukwe