Endocrine Disruptors:

Mechanisms and Impacts

Matt Vijayan

Alice Hontela

Don M^{ac}Kinlay

International Congress on the Biology of Fish
University of British Columbia, Vancouver, CANADA

Endocrine Disruptors:

Mechanisms and Impacts

SYMPOSIUM PROCEEDINGS

Matt Vijayan

Alice Hontela

Don M^{ac}Kinlay

International Congress on the Biology of Fish
University of British Columbia, Vancouver, CANADA

International Standard Book Number (ISBN) 1-894337-28-X

Notice

This publication is made up of a combination of extended abstracts and full papers, submitted by the authors without peer review. The papers in this volume should not be cited as primary literature. The Physiology Section of the American Fisheries Society offers this compilation of papers in the interests of information exchange only, and makes no claim as to the validity of the conclusions or recommendations presented in the papers.

For copies of these Symposium Proceedings, or the other 50 Proceedings in the Congress series, contact:

Don MacKinlay, SEP DFO, 555 West Hastings St.,
Vancouver BC V6B 5G3 Canada
Phone: 604-666-3520 Fax 604-666-6894
E-mail: mackinlayd@pac.dfo-mpo.gc.ca

Website: www.fishbiologycongress.org

CONGRESS ACKNOWLEDGEMENTS

This Symposium is part of the International Congress on the Biology of Fish, held at the University of British Columbia in Vancouver B.C., Canada on July 22-25, 2002.

The sponsors included:

- Fisheries and Oceans Canada (DFO)
- US Department of Agriculture
- US Geological Service
- University of British Columbia Fisheries Centre
- National Research Council Institute for Marine Biosciences
- Vancouver Aquarium Marine Science Centre

The main organizers of the Congress, on behalf of the Physiology Section of the American Fisheries Society, were Don MacKinlay of DFO (overall chair, local arrangements, program and proceedings) and Rosemary Pura of UBC Conferences and Accommodation (facility arrangements, registration and housing). Thanks to Karin Howard for assistance with Proceedings editing and word-processing; to Anne Martin for assistance with the web pages; and to Cammi MacKinlay for assistance with social events.

I would like to extend a sincere 'thank you' to the many organizers and contributors who took the time to prepare a written submission for these proceedings. Your efforts are very much appreciated.

Don MacKinlay
Congress Chair

TABLE OF CONTENTS

Mechanisms and effects of endocrine disruption: are they ecologically relevant?
Van der Kraak, Glen *1*

Development and characterization of CDNA arrays for evaluating endocrine disruption in fish
Denslow, N.D. et al. *5*

Identification of endocrine disruptor effects on the frog tadpole transcriptome
Helbing, Caren *11*

Evaluating the Effects of Endocrine Disrupting Compounds on the early life stages of rainbow trout using cDNA Microarrays
Booth, H.L., Bruno, Joy B. *13*

Gene expression profiles of largemouth bass exposed to nonylphenol and ICI 182,780
Sabo-Attwood, T. et al. *17*

Endocrine disruption in rainbow trout hepatocytes: A cDNA microarray study
Wiseman, Steve and Matt Vijayan *29*

Application of fluorescent indicator dyes to monitor hepatocyte viability in studies on gene expression
Schirmer, Kristin *31*

Characterization through proteomics of the gill derived rainbow trout cell line, RTgill-W1 grown under varying osmotic conditions
Lee, L.E.J., M.E. Kalbarczyk, M. Lamb *35*

Yolk thyroid and steroid hormone metabolism and the consequences on embryonic development: potential sites of action of environmental endocrine disruptors
Leatherland, John, Rakpong Petkam *39,*

Pharmaceuticals as EDCs - the metabolic impact of gemfibrozil in goldfish
Moon, T., Mimeault, Caroline, C.D. Metcalfe, ... *45*

Effect of endocrine disrupting comnpounds on walleye near the metro sewage treatment plant, St. Paul, MN
Jacqueline Conner, et al *49*

Modeling population-level responses of Atlantic croaker to endocrine disrupting chemicals using linked simulation models and laboratory studies
Murphy, CA, KA Rose, SL Diamond, ... *53*

Environmental contaminants in white sturgeon: is endocrine disruption occurring in the Columbia River?
Feist, Grant *59*

Affect of AROCLOR 1254 on endocrine physiology in the channel catfish
Shepherd, Brian, K. Drennon, B. Brammell and A. Elsku *63*

The effect of AROCLOR 1254 on smolt development and subsequent seawater performance and maturation on anadromous Arctic charr
Aas-Hansen, O. and A. Maule *69*

Dietary exposture to PCB126 and B(a)P interferes with the stress response of teleost fish at the pituitary level of the HPI-axis
Quabius, ES, JA Brown , DT Nolan *73*

Disruption of hypothalamo-pituitary-adrenal axis in teleost fish:toxicology and comparative physiology meet
Hontela, Alice and G. LeBlond *79*

Effect of several nonylphenol products on in vitro vitellogenin synthesis in tilapia hepatocytes
Kim, B.-H., Takemura, A. *83*

Effects of Endocrine Disrupting Compounds present in Fraser Valley Agricultural Runoff on Early Life Stages of Rainbow Trout
Bruno, J.B., Booth, H.L. *87*

A proteomic approach in the development of biomarkers of neoplasia in fish using rainbow trout liver and hepatoma derived cell lines
Lee, L.E.J. et al. *93*

Molecular cloning and analysis of a salmon serum C-type lectin
Richards, Robert C. *97*

Development of a real-time quantitative PCR (TAQMAN) assay to assess the effects of endocrine disrupting chemicals on sheepshead minnow (C*yprinodon variegatus*) growth
Iris Knoebl, Nancy D. Denslow, Leah D. Gillis, Leroy C. Folmar…. 101

MECHANISMS AND EFFECTS OF ENDOCRINE DISRUPTION IN FISH: ARE THEY ECOLOGICALLY RELEVANT?

G. Van Der Kraak and A. Lister
[1] Department of Zoology, University of Guelph,
Guelph, ON, N1G 2W1

EXTENDED ABSTRACT ONLY- DO NOT CITE

Introduction

Considerable progress has been made in understanding the mechanisms by which environmental compounds may act to disrupt normal endocrine functioning in fish. And while compounds that interact with endogenous hormone receptors (i.e., the estrogen receptor) and their biological effects still dominate the literature, other potential EDC mechanisms continue to be investigated. In selected cases, EDC mechanisms have been correlated to, and in some cases causally linked to effects observed in feral fish populations, including compromised growth and reproduction, altered development, and abnormal behaviour. Some specific actions of EDCs in fish involve alterations in hormone biosynthesis, metabolism, bioavailability, or transport. Hormone action may be modulated by EDCs that compete with endogenous hormones for specific, high affinity receptors or EDCs may affect down-stream signalling events (Fairbrother et al., 1999; WHO/IPCS, 2002). The most popular examples of EDC-mediated effects in fish are, ultimately, the result of changes to reproductive, adrenal (interrenal), or thyroid physiology. Studies have demonstrated a wide range of effects from the molecular to the whole animal level. Yet, there is a lack of evidence to suggest that many of the effects are ecologically relevant in terms of their impact on population fitness. From a mechanistic perspective, this paper briefly discusses a few of the popularized effects of EDCs such as the induction of vitellogenesis, altered steroid biosynthesis, and compromised stress responses in fish.

Induction of Vitellogenesis by Estrogenic Compounds

Vitellogenin (vtg) induction in juvenile or male fish in the field has become one of the most notable and convincing biological responses of fish linked to exposures

to estrogenic compounds. Numerous studies indicate that several different estrogenic compounds in the complex effluent may be responsible, particularly natural (17□-estradiol (E_2) and estrone) and synthetic estrogens (ethinylestradiol) or industrial chemicals (e.g., nonylphenol). As estrogens are the only known stimuli for the production of vtg, the presence of vtg in male or immature fish is a clear indication of exposure to estrogenic chemicals in the environment. Production of vtg requires the binding of estrogenic compounds to cytosolic estrogen receptors in the liver, followed by their translocation to the nucleus where they stimulate transcriptional activity. Some implications of elevated levels of plasma vtg at the individual level have been examined (e.g., retarded testicular growth, kidney damage, reduction of metabolic expenditure on growth and spermatogenesis, reduced E2 levels), but there is no evidence to suggest that EDC-induced vtg production adversely affects population fitness (Kime, 1998; WHO/IPCS, 2002). Perhaps other adverse effects, such as testicular abnormalities, ovotestis or hermaphroditism, observed in feral fish exposed to estrogenic compounds have more potential than vtg induction to translate into effects at the level of the population.

Reproductive Abnormalities Induced by Pulp and Paper Mill Effluent

Reproductive abnormalities observed in fish residing downstream of pulp mills vary depending on the location and the species examined. In Canada, studies found that fish exposed to bleached kraft mill effluent (BKME) had delayed sexual maturation and reduced expression of secondary sexual characteristics, gonad size, and sex steroid levels. The mechanisms behind these alterations are not well understood and the active compounds in BKME have not been conclusively identified. The mechanism responsible for the demasculinizing effect may involve the concomitant decrease in plasma androgen levels, which are the result of BKME actions at multiple locations in the hypothalamus-pituitary-gonad axis (e.g., decreased pituitary gonadotropin levels, inhibition of steroidogenic enzymes within the gonad). Conversely, masculinizing effects (i.e., development of male-like gonopodium) of BKME have been observed in female mosquitofish in streams below mills in the United States. These effluents were found to contain degraded phytosterols that may act agonistically at the androgen receptor. Although this case clearly demonstrates that BKME is an EDC, there is little evidence to suggest that BKME affects the health or fitness of exposed populations. In fact, one study found that the viability of eggs and sperm and the viability of developing larvae of exposed suckers were normal (Fairbrother et al., 1999; WHO/IPCS, 2002).

Compromised Stress Response by Exposure to Heavy Metals

In fish, stress results in the activation of the hypothalamus-pituitary-interrenal (HPI) axis, culminating in increased plasma cortisol concentrations. Field studies suggest that environmental contaminants may chronically stress fish resulting in a compromised HPI response. Yellow perch and northern pike from sites contaminated with PAHs, PCBs, and heavy metals were unable to produce cortisol in response to acute handling stress and their ACTH-producing cells were atrophied. The atrophy was speculated to be caused by prolonged hyperactivity of these cells. Recent studies on brown trout exposed to metals had comparable levels of cortisol compared with control fish; however, the metal-exposed fish were found to be hypersecreting ACTH and CRH to maintain baseline levels of cortisol. Similar studies demonstrated that fish chronically exposed to metals had a compromised stress response in acute stress trials. While chronic stress can result in elevated glucocorticoids and affect growth, reproduction, and immune responses, more research is necessary to determine the impact of EDC-induced compromised stress responses on the health of feral fish populations (WHO/IPCS, 2002).

Summary

Determining whether the effects of EDCs are relevant to population fitness is complicated because many factors can adversely impact growth, reproduction, and survival. Food availability, disease state, competition, or habitat loss may impinge directly on many of the endocrine measures and physiological endpoints that are used to evaluate fish for the effects of EDCs. Understanding the mechanisms by which EDCs alter endocrine function in fish may provide researchers with a clearer view of the risks that the contaminants pose to fitness and health of feral fish populations, but there are numerous challenges inherent to this task. At present, there is uncertainty in how EDCs effects at the individual level may translate into effects at the level of the population. Further implementation of fish-based screening assays for endocrine-active compounds, as well as an increased emphasis on partial/full-life cycle exposure studies of fish to EDCs will aid in our understanding. It may be possible to better understand the risks posed by EDCs, particularly during critical periods of development, if more research efforts were directed at understanding basic endocrinological processes in a variety of fish species.

References

Fairbrother A, Ankley G, Birnbaum L, Bradbury S, Francis B, Gray LE, Hinton D,

Johnson L, Peterson R, and G Van Der Kraak. 1999. Reproductive and Developmental Toxicology of Contaminants in Oviparous Animals *In* Reproductive and Developmental Effects of Contaminants in Oviparous Vertebrates. Eds. R Di Giulio and D Tillitt. SETAC Press, Pensacola, FL. p 283-361.

Kime D. 1998. Disruption of liver function *In* Endocrine Disruption in Fish. Kluwer Academic Publishers, MA. p.233-246.

WHO/IPCS (World Health Organization/International Petroleum Chemical Society). 2002. Global Assessment of the State-of-the-Science of Endocrine Disruptors. Eds. T. Damstra, S. Barlow, A. Bergman, R. Kavlock, G. Van Der Kraak. WHO/IPCS/EDC/02.2 World Health Organization, Geneva, Switzerland. http://ehp.niehs.nih.gov/who/

DEVELOPMENT AND CHARACTERIZATION OF CDNA ARRAYS

FOR EVALUATING ENDOCRINE DISRUPTION IN FISH

N.D. Denslow
Department of Biochemistry and Molecular Biology and Biotechnology Program, University of Florida, PO Box 100156 HC, Gainesville, FL 32610
P: 352-392-9665
F: 352-392-4441
Denslow@biotech.ufl.edu

P.M. Larkin
Biotechnology Program
University of Florida, Gainesville, FL 32610

T. Sabo-Attwood
Interdisciplinary Graduate Program in Pharmacology and Therapeutics
University of Florida, Gainesville, FL 32610

M. Hemmer
USEPA, NHEERL, Gulf Ecology Division, Gulf Breeze, FL 32561

L. Folmar
USEPA, NHEERL, Gulf Ecology Division, Gulf Breeze, FL 32561

EXTENDED ABSTRACT ONLY – DO NOT CITE

A variety of anthropogenic compounds are able to interact with the endocrine system of vertebrate species, by binding to steroid hormone receptors and altering normal gene expression patterns (Nimrod and Benson 1996). We are developing molecular-based approaches to measure the induction of gene expression in sheepshead minnows (*Cyprinodon variegatus*) and largemouth bass (*Micropterus salmoides*) exposed to estradiol or to hormonally active agents in the environment (Denslow et al., 2001a, 2001b). Sheepshead minnows (SHM) are fractional spawners inhabiting saline estuarine environments while largemouth bass (LMB) predominantly reside in fresh water and are synchronized annual spawners. Each model presents advantages for understanding the impacts of environmental contaminants.

For SHM we have characterized 30 genes, which are either regulated or constitutively expressed after treatment with known estrogenic substances (Larkin et al., 2002a). Using a cDNA macroarray containing these genes, we observed a similar genetic profile with 17α-estradiol (E_2), 17α-ethynyl estradiol (EE_2), diethylstilbestrol (DES), and methoxychlor (MXC). The genetic signature of fish treated with para-nonylphenol (pNP) was identical to fish treated with E_2, EE_2, DES, and MXC except for the additional up-regulation of ubiquitin-conjugating enzyme 9. Endosulfan (ES) produced results that resembled the gene expression patterns of untreated control fish with the exception of the up-regulation of ER α and the down-regulation of 3-hydroxy-3-methylglutaryl CoA reductase. The estrogen responsive cDNA macroarray we have developed can detect dose-dependent changes in gene expression patterns in fish treated with EE_2.

In the case of LMB we have determined expression profiles for 132 genes, some of which are estrogen responsive (Larkin et al., 2001b). We have tested the arrays with fish treated with estradiol (E_2), or with two hormonally active agents: p-nonylphenol (pNP) and 1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene (*p*, *p*'-DDE) using gene array technology. The results of these experiments show that male LMB exposed to E_2 and pNP had similar, but not identical genetic signatures for the genes examined, some of which are known to be estrogen-responsive genes. We also show that exposure of male LMB to *p*, *p*'-DDE results in an increase in the expression of 30% of the estrogen-responsive genes on the array, including those encoding 3 vitellogenin and two zona radiata proteins. In female fish exposed to *p*, *p*'-DDE, on the other hand, the expression of all the estrogen responsive genes was decreased as well as a number of other genes.

In summary, the results of these experiments show that gene arrays will be useful to begin to map gene activation pathways in response to exposure to endocrine disrupting compounds in fish. In general, both strong (E_2, DES, and EE_2) and weak (pNP, methoxychlor) estrogens appear to up-regulate the same set of genes, although there may be a few genes that are regulated differentially providing genetic signatures for specific compounds, perhaps due to their effects on gene transcription through pathways that are independent of the ER. *p,p'* DDE, an anti-androgen induces different gene expression pathways in males and females; increasing estrogen responsive genes in males and decreasing them in females. These results suggest that gene arrays have the potential to map sex-specific gene activation pathways in response to exposure to hormonally active

compounds. Finally, preliminary experiments suggest that the measured response by array is correlated with dose, enabling this approach to be used in a semi quantitative manner.

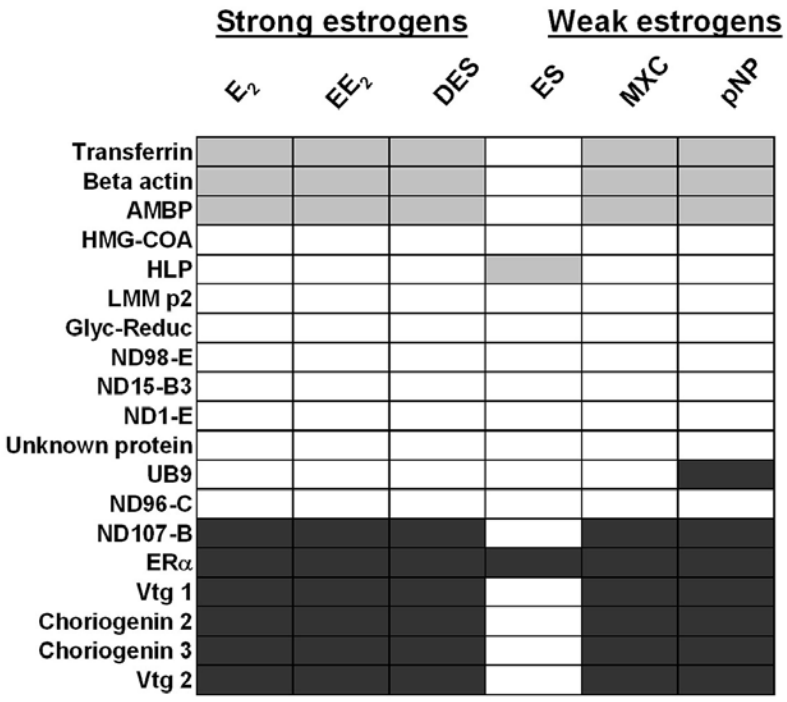

Fig. 1. Summary of genes that are increasing or decreasing more than two fold in sheepshead minnows treated with E_2, EE_2, DES, ES, MXC, or pNP. Black boxes indicate up-regulated genes; whereas, gray boxes indicate down-regulated genes. AMBP is alpha 1-microglobulin/bikunin precursor protein, HMG-COA is 3-hydroxy-3-methylglutaryl CoA reductase, HLP is hepatic lipase precursor, LMM p2 is low molecular mass protein 2, Glyc-Reduc is glycosylate reductase, UB9 is ubiquitin-conjugating enzyme 9, ERα is estrogen receptor alpha, and Vtg is vitellogenin.

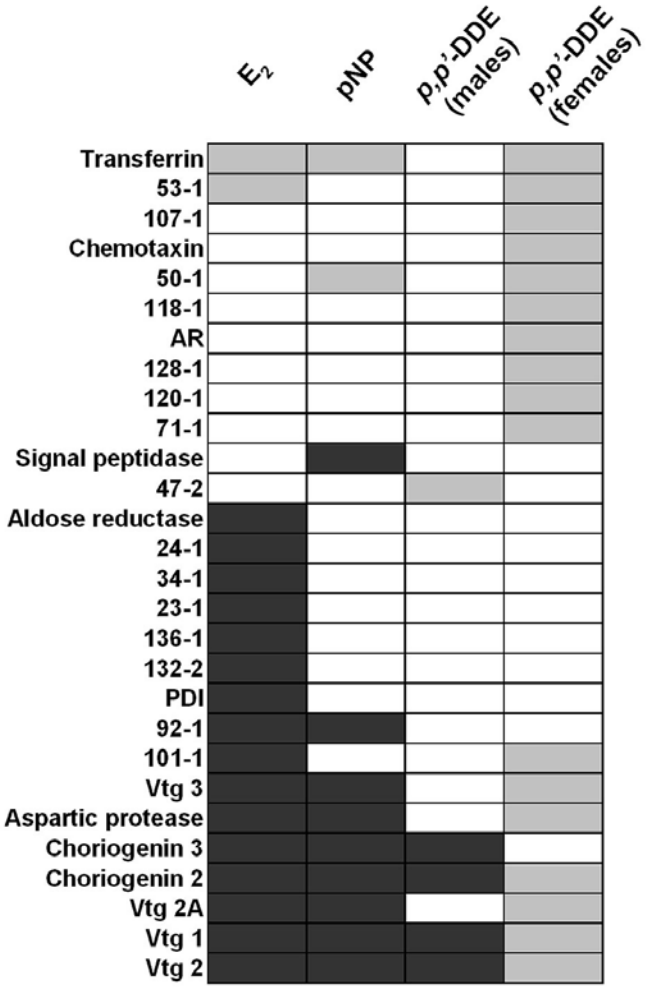

Fig. 2. Summary of genes that are increased or decreased by more than two fold in largemouth bass treated with E_2, pNP, and *p,p*'DDE. Black boxes indicate

up-regulated genes; whereas, gray boxes indicate down-regulated genes. AR is androgen receptor, PDI is protein disulfide isomerase and Vtg is vitellogenin.

Acknowledgements

This study was funded by contract # OD-5378-NTGX from the USEPA, grant number P42 ES 07375 from the National Institute of Environmental Health Sciences, NIH with funds from EPA, and the Interdisciplinary Center for Biotechnology Research, University of Florida. The contents are solely the responsibility of the authors and do not represent the official views of the NIEHS, NIH or the EPA.

References

Denslow, ND, C.J. Bowman, R.J. Ferguson, H.S. Lee, M.J. Hemmer, and L.C. Folmar. 2001a. Induction of gene expression in sheepshead minnows (*Cyprinodon variegatus*) treated with 17 beta-estradiol, diethylstilbesterol, or ethinylestradiol: The use of mRNA fingerprints as an indicator of gene regulation. Gen Comp Endocrinol 121: 250-260

Denslow, ND, H.S. Lee, C.J. Bowman, M.J. Hemmer, and L.C. Folmar. 2001b. Multiple responses in gene expression in fish treated with estrogen. Comp Biochem Physiol B Biochem Mol Biol 129: 277-282.

Larkin, P., L.C. Folmar, M.J. Hemmer, A.J. Poston, and N.D. Denslow. 2002a. Expression profiling of estrogenic compounds using a sheepshead minnow cDNA macroarray, submitted.

Larkin, P., T. Sabo-Attwood, J.Kelso, and N.D. Denslow, 2002b.Gene expression analysis of largemouth bass exposed to estradiol, nonylphenol, and *p*, *p*'-DDE, submitted.

Nimrod, A.C., and W.H. Benson. 1996. Environmental effects of alkylphenol ethoxylates. Crit Rev Toxicol 26: 335-364.

IDENTIFICATION OF ENDOCRINE DISRUPTOR EFFECTS ON THE FROG TADPOLE TRANSCRIPTOME

Caren C. Helbing
Dept. of Microbiology and Biochemistry
University of Victoria, Victoria, BC, Canada V8W 3P6
e-mail: chelbing@uvic.ca

EXTENDED ABSTRACT ONLY – DO NOT CITE

The thyroid hormones (THs), thyroxine (T_4) and 3,5,3'-triiodothyronine (T_3), are critical for normal growth, development and homeostasis in vertebrates. This is particularly evident in the absolute requirement of THs for frog tadpole metamorphosis. Tadpole metamorphosis is a rapid, complex postembryonic process in which the tadpole transforms into a juvenile frog. Virtually every tissue is a target for TH action and the dramatic structural and functional changes of larval tissues during metamorphosis have been studied extensively. The major mechanism of TH action involves hormone binding to nuclear TH receptors resulting in tissue-specific activation/repression of gene transcription. A change in tissue-specific gene expression precedes overt morphological change and can be used as a means to identify disruption of TH signaling pathways.

Relatively little is known about the effects and identities of endocrine disrupting chemicals (EDCs) that target TH action, particularly at the cellular level. The preemergent herbicide acetochlor [2-chloro-*N*-(ethoxy-methyl)-*N*-(2-ethyl-6-methylphenyl) acetamide] is a persistent organic pollutant that can be detected in shallow ground water one year after field application and there is evidence that it can act as an EDC. The presented work addresses the hypothesis that acetochlor may disrupt T_3-dependent gene expression programs during metamorphosis of the Pipid frog, *Xenopus laevis*. In order to do this, we developed a 420-gene cDNA microarray derived from known frog sequences and use this array to determine the effects of acetochlor on precocious metamorphosis. We show that T_3-induced metamorphosis is accelerated upon acute exposure to an environmentally relevant level of acetochlor. The morphological changes

observed are preceded by alterations in the tadpole tail transcriptome and the nature of these profiles suggest a novel mechanism of action for acetochlor.

Acknowledgements

This study was funded by the National Science and Engineering Research Council (NSERC).

EVALUATING THE EFFECTS OF ENDOCRINE DISRUPTING COMPOUNDS ON EARLY LIFE STAGE RAINBOW TROUT USING cDNA MICROARRAYS

Heather L. Booth
Environmental Toxicology, Pacific Environmental Science Centre
2645 Dollarton Highway, North Vancouver, BC, Canada V7H 1B1
phone: 604-924-2542, fax: 604-924-2554
e-mail: heather.booth@ec.gc.ca

Joy B. Bruno and Graham C. van Aggelen
Environmental Toxicology
Pacific Environmental Science Centre, North Vancouver, BC, Canada V7H 1B1
e-mail: joy.bruno@ec.gc.ca and graham.vanaggelen@ec.gc.ca

Matt M. Vijayan
Dept. of Biology
University of Waterloo, Waterloo, Ontario, Canada N2L 3G1
e-mail: mvijayan@sciborg.uwaterloo.ca

Thomas P. Mommsen and Caren C. Helbing
Dept. of Microbiology and Biochemistry
University of Victoria, Victoria, BC, Canada V8W 3P6
e-mail: tpmom@uvic.ca and chelbing@uvic.ca

Kris R. von Schalburg
Dept. of Biology
University of Victoria, Victoria, BC, Canada V8W 3P6
e-mail: krvs@uvic.ca

EXTENDED ABSTRACT ONLY – DO NOT CITE

Studies with fishes (Tyler et al., 1998; Harries et al., 1997), reptiles, birds and mammals suggest that exposure to endocrine disrupting compounds (EDCs) will result in alterations in development and reproduction by disrupting the hormonal

system. There are hundreds of compounds that act through endocrine disruption in a variety of ways: some EDCs mimic endogenous steroid hormones, such as estradiols or androgens, some antagonize the functions of endogenous hormones (for example anti-androgens), some mimic or alter hormonal substrates, and some EDCs change the synthesis and metabolism of endogenous hormones. In adult fish, hormone-dependent alterations in performance are often transient and tend to return to pre-exposure levels once the compound is withdrawn. In stark contrast, if the early life stages of fish are subjected to untimely exposures to hormone mimicking compounds, it may cause irreversible activation or depression of particular genes. As a result, parameters not normally controlled by a given hormone might be affected throughout life, thereby compromising the normal response to the hormone en route through early life stages with repercussions on development, growth, reproduction, behaviour, and metabolic fitness.

In a study using Environment Canada's standardized early life stage test method (Egg-Alevin-Fry), rainbow trout gametes, obtained from a local hatchery, were dry fertilized and hardened for 30 minutes in the solution of interest, and then were distributed into incubation chambers suspended in 6 L of solution. The embryos were allowed to develop through to the fry stage (~ 57 days) while being exposed to well water (negative control), acetone (carrier control), 17-beta-estradiol at 250 ug/L (positive control) and nonylphenol (a known estrogen mimicking compound) at 50, 100, and 250 ug/L. Nonylphenol altered the rate of development of eggs and alevins, affected the hatching ability of eggs, and changed the behaviour and physiology of larvae. Nonylphenol exposure to 100 ug/L and 250 ug/L was lethal at 49 days and 33 days respectively. Clearly, developing trout larvae responded sensitively to estradiol and nonylphenol. For adult fish and juveniles, vitellogenesis and testis development have been the primary indicators for investigators determining the effects of exposure to estrogen mimicking compounds released into the environment (Kime, 1998). However, rate of development, hatching success, hatching mass, skin integrity, skin colouration, behavioural changes, and substantially altered programmes of gene transcription are also important consequences of exposures to high concentrations of estradiol and, more importantly, to relatively small concentrations of nonylphenol. In fact, concentrations of nonylphenol that failed to affect vitellogenesis and testis morphology in adult platyfish (Kinnberg et al., 2000) exerted pronounced effects in the developing trout. We postulate that these effects would decrease the survival fitness and performance of the fish, ranging from molecular to behavioural levels. Interestingly, chronic exposure (three months) to similar concentrations of nonylphenol exerted

pronounced effects in medaka, skewing the sex ratio in favour of females while also increasing the occurrence of testes-ova in males (Gray and Metcalfe, 1997).

Analysis of the gene expression changes induced by estradiol and nonylphenol will occur using a recently developed rainbow trout cDNA microarray containing 150 genes. This rainbow trout cDNA microarray will provide a valuable tool for gene expression analysis in aquatic toxicology research by broadening the accessibility of information to be gathered about gene expression changes caused by EDCs. Design and development of the microarray occurred over 2 years culminating in printing on Corning glass slides in April 2002. Each of the 150 cDNA pieces incorporated on the array is approximately 500 base pairs in length and was specifically included because of known gene function. Exposures of early life stage rainbow trout to pure EDCs and effluents containing multiple EDCs (such as sewage treatment plants, pulp and paper mills and agriculture) performed over the last few years will be analyzed using the rainbow trout microarrays to promote identification of sub-lethal, gene-altering effects of EDCs in point source environmental toxicology.

Acknowledgements

This study was funded by the Georgia Basin Ecosystem Initiative (GBEI), National Science and Engineering Research Council (NSERC), and the Strategy for Technological Advances in Genomics in the Environment (STAGE). We would like to thank Michelle Linssen for her technical expertise.

References

Gray, M.A. and C.D. Metcalfe. 1997. Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to p-nonylphenol. Environ. Toxicol. Chem. 16: 1082-1086

Harries, J.E., D.A. Sheahan, S. Jobling, P. Matthiessen, P. Neall, J.P. Sumpter, T. Taylor, N. Zaman. 1997. Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. Environ. Toxicol. Chem. 16: 534-552

Kime, D.E. (1998) *Endocrine Disruption in Fish*. Boston : Kluwer Academic Publishers.

Kinnberg, K., B. Korsgaard, P. Bjerregaard, A. Jespersen. 2000. Effects of nonylphenol and 17-beta-estradiol on vitellogenin synthesis and testis morphology in male platyfish *Xiphophorus maculatus*. J. Exp. Biol. 203: 171-181

Tyler, C.R., S. Jobling, J.P. Sumpter. 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit. Rev. Toxicol. 28: 319-361

GENE EXPRESSION PROFILES OF LARGEMOUTH BASS EXPOSED TO NONYLPHENOL AND ICI 182,780

T Sabo-Attwood
Interdisciplinary Graduate Program in Pharmacology and Therapeutics
University of Florida
PO Box 100267
Gainesville, FL 32610
sabo@ufl.edu

PM Larkin[1], J Kelso[1], and ND Denslow[1,2]
[1]Interdisciplinary Center for Biotechnology Research, [2]Department of Biochemistry and Molecular Biology
University of Florida
PO Box 100156
Gainesville, FL 32610
pmlarkin@ufl.edu, jkelso@ufl.edu, denslow@biotech.ufl.edu

Abstract

The goal of this study was to observe changes in gene expression in largemouth bass following exposure to 4- nonylphenol (4-NP) and ICI 182,780 (ICI) using real-time PCR (RT-PCR) and macroarrays. The expression levels of three estrogen receptor (ER) isotypes alpha, beta, and gamma, and vitellogenin (Vtg) I as measured by RT- PCR show that following exposure to 4-NP only ER alpha and Vtg 1were significantly induced. The addition of ICI to 4-NP repressed the induction of ER alpha by 4-NP but surprisingly had no effect on Vtg I mRNA levels. By macroarray, 4 Vtgs, 2 choriogenins, aspartic protease, signal peptidase and one unidentified clone were up-regulated while two genes including transferrin and clone 50-1 were down-regulated by 4-NP. Co-exposure to 4-NP and ICI resulted in a repressed response for aspartic protease. As seen for Vtg I by RT-PCR, the up-regulation of the Vtgs and choriogenins and down-regulation of transferrin by 4-NP was not altered by ICI. An additional set of genes that were not up or down-regulated by 4-NP were depressed by ICI. It is possible that differences in message stability or the composition of response elements in gene promoters play a role in changes in gene expression observed with the ICI treatment.

Introduction

Several chemicals that are found in the environment, termed hormonally active agents (HAA), may be linked to a variety of adverse biological effects in wildlife and humans (Carlsen et al., 1993; Guillette, 1994). One synthetic chemical found in the environment that functions as weak estrogen is 4-nonylphenol (4-NP). 4-NP primarily acts by binding to the estrogen receptor (ER) and subsequently inducing transcription of downstream genes, including the vitellogenins and choriogenins (Celius et al., 1999). However, there is evidence that 4-NP also has other mechanisms of action (Masuyama et al., 2000).

The ER signaling cascade has become increasingly complex with the discovery of multiple ER isotypes namely ER alpha and ER beta in both mammals and fish (Kuiper et al., 1996; Tchoudakova et al., 1999). Additionally, a third isotype termed ER gamma has recently been shown to exist in the teleost fish, Atlantic Croaker (Hawkins et al., 2000). The specific role that each ER subtype plays in the regulation of genes has not been determined. Studies have revealed that estrogen and estrogen mimicking compounds differentially bind and activate the various ER isotypes *in vitro*, however results vary between species including fish and mammals (Gutendorf and Westendorf, 2001; Kuiper et al., 1998; Matthews et al., 2000).

To fully understand the impact of HAA including 4-NP, effects at the molecular level need to be characterized in animals, particularly those that are exposed to these compounds in the environment, including fish. The goal of this study was to measure gene expression in largemouth bass (*Micropterous salmoides*) (LMB) following exposure to 4-NP. In particular, a real-time PCR assay was developed to measure the change in expression of three LMB ER isotypes alpha, beta, gamma, and the ER regulated gene Vtg 1. We also tested the effects of the co-administration of 4-NP with the antiestrogen ICI 182,780 (ICI) on the expression of the ERs and Vtg 1. In addition, we used a gene macroarray, developed in our laboratory, to characterize the specific expression profile of other estrogen responsive genes following exposure to 4-NP and 4-NP in conjunction with ICI.

Materials and methods

Experimental Design and Sample Collection: Adult male LMB were purchased from American Sports Fish Hatchery (Montgomery, Alabama) and maintained

in fiberglass tanks at the University of Florida Aquatic Toxicology Facility as previously described (Larkin in review 2002, Bowman in review 2002). Each fish was injected intraperitoneally (IP) with either 50 mg/kg 4-NP (Fluka # 74430), the combination of 50 mg/kg 4-NP and 1.0 mg/kg ICI (Tocris Cookson Inc.), or vehicle, which consisted of ethanol and dimethylsulfoxide (DMSO, Sigma #5879). Fish were euthanized by submersion in a water bath containing 50-100 ppm MS-22 48 hours post injection and sacrificed by a sharp blow to the head followed by cervical transaction. The livers were excised, immediately flash frozen in liquid nitrogen, and stored at –80°C until RNA was isolated.

RNA Isolation: Isolation of total RNA from liver tissue was performed with the RNA Stat-60 reagent (Tel-test) as described previously (Sabo-Attwood et al., in review). For all RNA samples, the quantity and quality of total RNA was assessed by spectrophotometric readings at 260 nm and by electrophoresis through a 1% formaldahyde agarose gel stained with ethidium bromide.

***Real-Time PCR*:** Real time PCR was performed using reagents and a 5700 thermocycler purchased from Applied Biosystems. Primers and probes for the ER subtypes and Vtg 1 have been described previously (Sabo-Attwood et al., in review). Each real time PCR reaction consisted of 0.01 – 0.2 ug of reverse transcribed total RNA as template. To generate a standard curve, varying amounts of plasmid containing the specific cDNA inserts for each gene were used as template in the PCR reactions. For each gene, a 6 point standard curve encompassing a 1 x 10^6 fold range of approximately 25 – 2.5 x 10^6 copies of cDNA was constructed. Each sample was run in duplicate and normalized 18s rRNA. Both the intra-assay and inter-assay variability never exceeded 10%. Statistical differences between the treatments were determined by one way analysis of variance with Dunnets post-hoc analysis.

Amplification of cDNA to be spotted on the macro arrays: The macroarrays were prepared and printed as previously described (Larkin et al., in review). Briefly, the 132 LMB clones were PCR amplified, purified, and concentrated. Following denaturation, 20 X SSC (3M NaCl, 0.3M sodium citrate, pH 7.0) containing 0.01 mM bromophenol blue was added to the samples to yield a final concentration of 100 ng/μL cDNA template. The PCR products were robotically spotted (Biomek 2000, Beckman Coulter, Fullerton, CA) in duplicate onto neutral nylon membranes (Fisher scientific) using 100 nL pins. Membranes were UV cross-linked and stored under vacuum at room temperature until hybridization.

Labeling of RNA, hybridization, and detection: Radiolabeled probes were generated by random primer labeling of DNase treated (DNA-*free*, Ambion, Austin, TX) total RNA from LMB livers with [α-^{33}P] dATP (Strip-EZ RT, Ambion). The blots were hybridized with ultraArray hybridization buffer (Ambion) as previously described (Larkin et al., in review). The membranes were exposed to a phosphor screen (Molecular Dynamics, Piscataway, NJ) and quantitatively evaluated using a Typhoon 8600 imaging system (Molecular Dynamics). For each cDNA clone, the general background of each membrane was subtracted from the average value of the duplicate spots. The values were normalized to the average value of 12 cDNA clones specific to ribosomal genes. (Larkin et al., 2002). Genes were not included for analysis that had values less than the background value for two out of the three replicates and/or fluctuated more then two fold when aliquots of the same RNA were hybridized to blots printed at the beginning, middle, and the end of the array printing process.

Results

Measurement of ER and Vtg 1 mRNA by real-time PCR: Real-time PCR is a sensitive assay that can be used to quantitate expression levels of genes. Using this technology we designed assays to quantitate the expression of 4 genes, ERs alpha, beta, and gamma, and Vtg 1 in LMB following exposure to 4-NP and 4-NP/ICI. Using primers and probes specific to each gene we were able to differentiate between the ER isotypes with no cross reactivity (Sabo-Atwood et al., in review). Exposure of LMB to a single injection (IP) of 4-NP (50 mg/kg) significantly increased ER alpha by 80 fold ($P < 0.05$) after 48 hours when compared to controls (Figure. 1). During the same time frame, the levels of both ER beta and ER gamma decreased approximately 1.3 fold and 2.6 fold respectively, however these changes were not statistically significant from controls. When the LMB were exposed to a combination of 4-NP (50 mg/kg) and the anti-estrogen ICI (1.0 mg/kg), the levels of ER alpha increased only 4 fold over controls ($P < 0.08$), suggesting that the anti-estrogen had interfered with the activation process (Figure 1). As with the 4-NP treatment, the expression of ER beta and gamma decreased (1.9 fold) but the values did not differ significantly from controls.

Since the Vtg gene is an E_2 responsive gene that is under transcriptional control by ERs in the liver, we also determined the expression levels of Vtg 1 by real-time PCR. Exposure to 4-NP increased message levels by approximately 40 fold over controls ($P < 0.05$), however, this induction was not repressed by the addition of ICI (Figure 2).

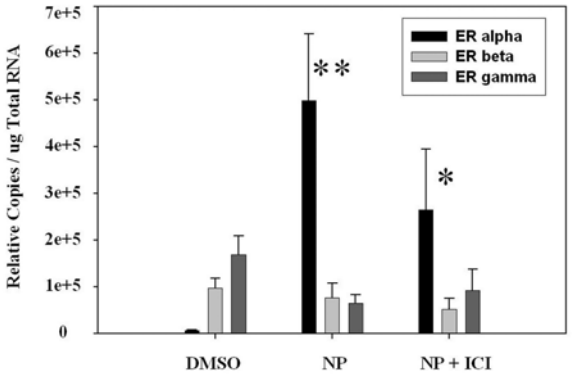

Figure 1 shows the expression of ERs alpha, beta, and gamma measured by real-time PCR. Each bar represents the mean and standard error (n = 7). Statistically significant differences between treatments and controls are marked with astericks. $P < 0.05$ (**), $P < 0.1$ (*).

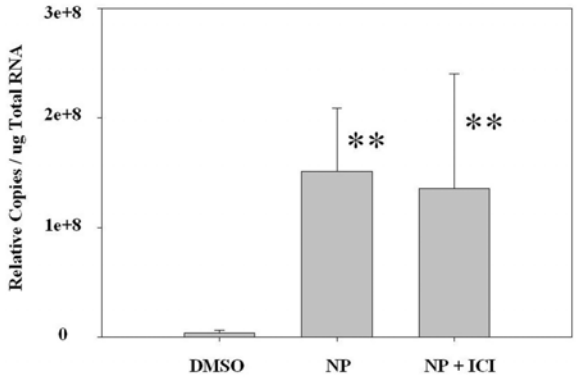

Figure 2 illustrates the expression of VTG I measured by real-time PCR. Each bar represents the mean and standard error (n = 7). Statistically significant differences between treatments and controls is marked with an asterick. $P < 0.05$ (**).

LMB gene array analysis: In order to further characterize the effects of 4-NP alone or in conjunction with ICI on hepatic gene regulation in LMB, we examined the expression of 132 genes, many of which are estrogen responsive, by gene arrays. Total hepatic RNA isolated from control and exposed fish was radiolabeled and hybridized to the membranes. Of the 132 genes on the array, only genes that changed by at least 3 standard deviations from the mean of the 12 ribosomal genes that were used to normalize the data are included in Figure 3. These include several that are up of down-regulated by more than 2 fold, a conservative cutoff generally used for array interpretation. Figure 3 shows the fold induction of each gene over controls for both the 4-NP and 4-NP/ICI treatments.

In the 4-NP treated fish, 9 genes were up-regulated 2 fold or greater including 4 Vtgs, choriogenin 2, choriogenin 3, aspartic protease, signal peptidase, and one unidentified clone designated 92-1. Two genes were found to be down-regulated by 4-NP including transferrin and clone 50-1. In the case of the mixture of 4-NP and ICI, the expression of one gene, aspartic protease, was reduced. In addition, the expression levels of 4 Vtgs, 2 choriogenins, and transferrin were not affected at all, but instead appeared to be expressed to the same levels as with 4-NP alone.

Genes which were reduced by the mixture and that exhibited as least a 2 fold change in expression when compared to 4-NP alone are marked with an asterisk. These genes included aspartic protease, protein disulfide isomerase, integral membrane protein, methionine sulfoxide reductase, ER gamma, glucocorticoid receptor, aldose reductase, ER beta, FK506 binding protein, and 21 unidentified clones. All of these genes except for clone 53-1 were down-regulated by the addition of ICI to 4-NP.

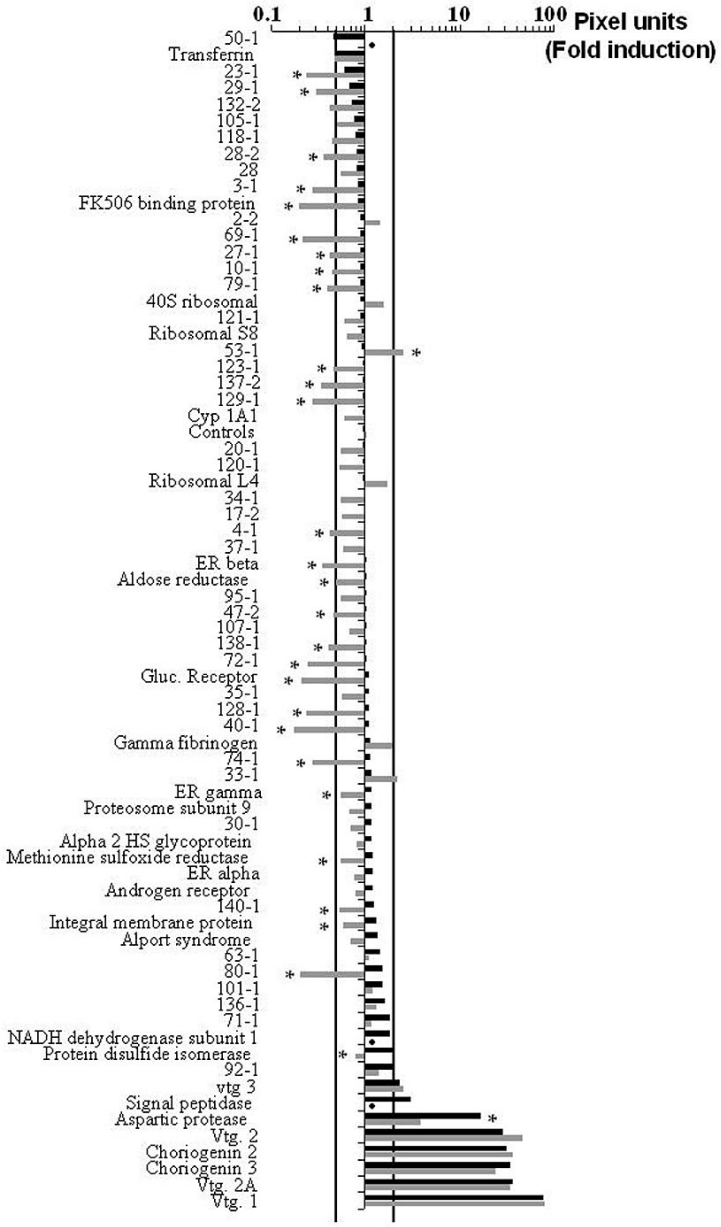
0.1
1
10
100
Pixel units
(Fold induction)
50-1
Transferrin
23-1
29-1
132-2
105-1
118-1
28-2
28
3-1
FK506 binding protein
2-2
69-1
27-1
10-1
79-1
40S ribosomal
121-1
Ribosomal S8
53-1
123-1
137-2
129-1
Cyp 1A1
Controls
20-1
120-1
Ribosomal L4
34-1
17-2
4-1
37-1
ER beta
Aldose reductase
95-1
47-2
107-1
138-1
72-1
Gluc. Receptor
35-1
128-1
40-1
Gamma fibrinogen
74-1
33-1
ER gamma
Proteosome subunit 9
30-1
Alpha 2 HS glycoprotein
Methionine sulfoxide reductase
ER alpha
Androgen receptor
140-1
Integral membrane protein
Alport syndrome
63-1
80-1
101-1
136-1
71-1
NADH dehydrogenase subunit 1
Protein disulfide isomerase
92-1
vtg 3
Signal peptidase
Aspartic protease
Vtg. 2
Choriogenin 2
Choriogenin 3
Vtg. 2A
Vtg. 1

Figure 3 shows gene expression profiles from control, 4-NP, and 4-NP/ICI reated male fish. The mean intensity values for each of the cDNA clones for 4-NP (black bar) or 4-NP/ICI (gray bar) divided by the mean intensity values of the respective cDNA clones from control fish. Genes that changed by more then two fold for the 4-NP treatment compared to the 4-NP/ICI treatment are indicated by an asterick. The black dots in panel indicate genes that were not used in the analysis (see methods section).

Discussion

The goal of this study was to observe changes in gene expression following exposure to the weak estrogen mimic 4-NP and the anti-estrogen ICI using real-time PCR and macroarrays. We picked 4 genes to monitor by real-time PCR, ERs alpha, beta, gamma, and Vtg 1. Real-time PCR is a sensitive and specific assay that allows for relative quantitation of gene expression levels with a limit of detection of 25 copies. More importantly, the ER isotypes share a great deal of sequence identity specifically in the DNA and ligand binding regions, but we were able to distinguish between the ER subtypes using this assay (Sabo-Attwood et al., in review).

Macroarrays allow researchers to simultaneously monitor the expression patterns of multiple genes from the same sample. Although not as quantitative and specific as real-time PCR, the macroarrays are an appropriate tool for rapidly screening large numbers of genes. We have constructed a LMB specific gene array containing 132 clones and have previously shown that the inter-assay variability of these arrays was minimal (Larkin et al., in review).

To determine the effect of 4-NP on the expression levels of ERs alpha, beta, and gamma *in vivo*, we measured the relative copies of each by real-time PCR. The data in Figure 1 shows only ER alpha was significantly induced, approximately by 80 fold compared to controls. The expression levels of ER beta and gamma did not significantly change. This result suggests that the three ERs differ in their regulation by estrogenic compounds. Apparently in LMB, only ER alpha is inducible by 4-NP.

We have previously shown that during the reproductive cycle of female LMB, as E_2 synthesis increases there is a strong correlation between the induction of vitellogenin and ER alpha mRNA (Sabo-Attwood et al., in review). Since ER alpha expression increased following exposure to 4-NP, we wanted to determine

if the mRNA expression levels of one vitellogenin, Vtg 1 also increased. Figure 2 shows there was a significant ($p < .05$) induction of Vtg 1 mRNA approximately 40 fold compared to controls following exposure to 4-NP. While the fold induction level is less than that for ER alpha, it actually is 3 orders of magnitude greater in copy number. This difference in induction was reported previously for LMB (Bowman et al., in review) and rainbow trout (Flouriot et al., 1996). The difference in expression response for these two genes may be due to the nature of the response elements in the promoters of these genes.

ICI has been used in numerous mammalian models but has not been well characterized in fish. Since the ICI compound is an anti-estrogen in mammalian systems we had expected that it would diminish the response observed by 4-NP for genes that were regulated via the ER pathway. Figure 1 shows that the combination of 4-NP and ICI induced ER alpha expression over controls but not to the same extent when compared to 4-NP alone. In fact, the response was approximately half that seen with the 4-NP treatment. Although the difference between ER alpha expression by the 4-NP and 4-NP/ICI treatments is not statistically significant ($p< 0.16$) the data suggests that ICI repressed the induction of ER alpha by 4-NP. This was the expected result for an anti-estrogen. The expression of ER beta and gamma did not differ significantly from controls for any of the treatments.

Since ICI diminished the induction of ER alpha we expected the expression of Vtg 1 to be repressed as well. Surprisingly, the induction of Vtg 1 message by 4-NP was not affected by the ICI treatment (Figure 2). It is possible that since we are measuring steady state levels of the mRNAs only at 48 hours by the real-time PCR assay, we missed the inhibition by ICI. Consistent with this hypothesis is the suggestion that ER alpha mRNA half-life is shorter than that of Vtg (Bowman et al., in review).

In order to determine the expression profile of multiple genes following exposure to 4-NP we hybridized radiolabeled RNA from exposed and control fish livers onto a macroarray. All of the genes that were up-regulated by 4-NP except for signal peptidase have been shown previously to be induced by E_2 (2.5 mg/kg) by this method (Larkin et al., in review). The 4 Vtg's, 2 choriogenins, and aspartic protease were up-regulated while transferrin and clone 50-1were down-regulated (Figure 3). This indicates 4-NP is acting at least in part as an estrogen mimic.

To determine the effect of the anti-estrogen ICI on gene expression, we determined the expression pattern of the same 132 genes following exposure to the combination of 4-NP and ICI by macroarray. The induction of one gene, aspartic protease was decreased by 75% by ICI, similar to the pattern observed with ER alpha by real-time PCR.

Surprisingly, a number of genes on the array including the Vtgs and choriogenins which have been well characterized as E_2 responsive genes were not affected by ICI. As shown in Figure 3, the 4 Vtgs and both choriogenins were induced to the same extent as in the 4-NP treated fish. This correlates to the data seen by real-time PCR which showed no difference in Vtg 1 induction of mRNA between the two treatments. Also, transferrin which has been shown previously by our lab to be down-regulated by exposure to E_2 in LMB and sheepshead minnows was also down regulated by 4-NP. However, like the Vtgs and choriogenins, the expression of this gene was not affected by the ICI compound. Why the induction of certain genes such as the Vtgs and choriogenins was not affected whereas the up-regulation other genes such as aspartic protease were repressed is not known. It is possible that differences in message stability or the composition of response elements in gene promoters may play a role.

There was also a set of genes that were not up or down-regulated by 4-NP, but were down-regulated by ICI. The expression of these genes were altered by at least 2 fold by ICI compared to administration of 4-NP alone. These include protein disulfide isomerase (PDI), integral membrane protein, methionine sulfoxide reductase, ER gamma, glucocorticoid receptor, aldose reductase, FK506 binding protein, and 20 unidentified clones. Both PDI and aldose reductase were shown to be upregulated in LMB by E_2 (2.5 mg/kg) previously (Larkin et al., in review). Since 4-NP is a weak estrogen the capacity of this compound to induce certain genes may be limited. The fact that ICI depressed genes that were induced by E_2 suggests that they are regulated in part by ERs. Only one gene, clone 53-1 was up-regulated by ICI. Since the effects of ICI in fish *in vivo* have not been well characterized it is difficult to speculate on the precise mechanisms involved in this genetic profile. Additional experiments are being conducted to further characterize the effects of anti-estrogens on gene expression in fish.

The change in expression levels for the ERs on the array does not exactly correspond to the levels measured by real-time PCR. Compared to genes such as the Vtgs and choriogenins, the levels of expression of the ERs is low. We have

previously shown that more inter-assay variability exists in genes with low expression compared to genes that are highly expressed. Also, since the ER cDNA clones on the array contain domains with high sequence identity between the isotypes, it is possible that cross-reactivity plays a role in the differential expression levels observed between the real-time PCR and macroarray analysis. We are confident that the real-time PCR assay specifically amplifies each ER with no cross-reactivity between isotypes.

Overall our study indicates that 4-NP acts as an estrogen mimic based on the induction of known E_2 responsive genes such as the Vtgs and choriogenins by both real-time PCR and macroarray analysis. Using real-time PCR we found that only ER alpha mRNA, not ER beta or gamma is significantly induced by 4-NP. The expression of some genes following exposure to the ICI compound was as expected which included the reduction in ER alpha and aspartic protease mRNAs as measured by real-time PCR and macroarray respectively. Interestingly the antiestrogen ICI did not affect the induction of other E_2 and 4-NP responsive genes including the Vtgs and choriogenins.

Acknowledgements: This publication was made possible by grant number P42 ES 07375 from the National Institute of Environmental Health Sciences, NIH with funds from EPA. The contents are solely the responsibility of the authors and do not represent the official views of the NIEHS, NIH, or the EPA. The authors wish to acknowledge expert technical help from Kevin Kroll, and the donation of several cDNA clones and assistance in construction of the arrays from Dr. Evan Gallagher, Jannet Kocerha, Chris J Tzikas, University of Florida, and Dr. Chris Bowman, CIIT, Research Triangle Park.

References

Carlsen, E., Giwercman, A., and Skakkebaek, N. E. 1993. Declining sperm counts and increasing incidence of testicular cancer and other gonadal disorders: is there a connection? Ir. Med. J. 86: 85-86.

Celius, T., Haugen, T. B., Grotmol, T., and Walther, B. T. 1999. A sensitive zonagenetic assay for rapid *in vitro* assessment of estrogenic potency of xenobiotics and mycotoxins. Environ. Health Perspect. 107: 63-68.

Flouriot, G., Pakdel, F., and Valotaire, Y. 1996. Transcriptional and post-transcriptional regulation of rainbow trout estrogen receptor and vitellogenin gene expression. Mol. Cell Endocrinol. 124: 173-183.

Guillette, L. J. 1994. Endocrine-disrupting environmental contaminants and reproduction: lessons from the study of wildlife. Women's health today: Perspectives on current research and clinical practice., D. R. Popkin and L. J. Peddle, eds., Parthenon Publ. Group, New York, 201-207.

Gutendorf, B., and Westendorf, J. 2001. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology. 166: 79-89.

Hawkins, M. B., Thornton, J. W., Crews, D., Skipper, J. K., Dotte, A., and Thomas, P. 2000. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc. Natl. Acad. Sci. U S A. 97: 10751-10756.

Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. A. 1996. Cloning of a novel receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. U S A. 93: 5925-5930.

Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J., Safe, S. H., van der Saag, P. T., van der Burg, B., and Gustafsson, J. A. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology. 139: 4252-4263.

Masuyama, H., Hiramatsu, Y., Kunitomi, M., Kudo, T., and MacDonald, P. N. 2000. Endocrine disrupting chemicals, phthalic acid and nonylphenol, activate pregnane X receptor-mediated transcription. Mol. Endocrinol. 14: 421-428.

Matthews, J., Celius, T., Halgren, R., and Zacharewski, T. 2000. Differential estrogen receptor binding of estrogenic substances: a species comparison. J. Steroid Biochem. Mol. Biol. 74: 223-234.

Tchoudakova, A., Pathak, P., and Callard, G. V. 1999. Molecular cloning of an estrogen receptor beta subtype from the goldfish, *Carassius auratus*. Gen. Comp.Endocrinol. 113: 388-400.

ENDOCRINE DISRUPTION IN RAINBOW TROUT

HEAPTOCYTES: A cDNA MICROARRAY STUDY

Steve Wiseman
and Mathilakath M. Vijayan
Department of Biology, University of Waterloo,
Waterloo, Ontario, Canada N2L 3G1
Tel: (519) 888 4567 x 2035, Fax: (519) 746 0614,
E-mail: mvijayan@sciborg.uwaterloo.ca

EXTENDED ABSTRACT ONLY – DO NOT CITE

cDNA microarrays are an orderly arrangement of "target" cDNA material immobilized onto a substrate, normally coated glass microscope slides. Different fluorophores are used to label cDNA prepared from either total RNA or messenger RNA, representing control and experimental conditions. The fluorescently labeled cDNA's from two treatments are mixed together and this "probe" is hybridized to "target" cDNA on the array. The labeled messenger sequence quantitatively anneals to "target" cDNA sequences and the differences in the expression of individual genes between the two treatments can be detected and quantified using a DNA chip reader. The ability to simultaneously monitor the expression of thousands of genes makes cDNA microarrays an extremely powerful tool in physiology and environmental toxicology.

In collaboration with Drs. Tom Mommsen, Graham van Aggelen and Caren Helbing, we have developed a rainbow trout-specific cDNA microarray incorporating a battery of 160 genes involved in cellular physiology, including growth, immune responses, endocrine system, metabolism and reproduction. Primers were designed to obtain unique 450-550 bp gene fragments and were immobilized on the array. This highly specific microarray was used to examine gene expression changes in rainbow trout exposed to a variety of contaminants, including endocrine disruptors.

In this study our objective was to investigate whether the trout-specific cDNA microarray can be used as a tool to examine global gene expression changes associated with endocrine disrupting compounds in rainbow trout. To this end,

we used hepatocytes in primary culture as a model system to characterize the utility of the microarray, especially because the *in vitro* system allowed for parallel treatment comparisons, thereby reducing inter-fish variability inherent with *in vivo* studies. For this study, we examined changes in gene expression associated with exposure of trout hepatocytes to two know xenoestrogens, nonylphenol, and beta-sitosterol. Estradiol was used as a positive control in order to compare and contrast the gene expression profiles associated with these xenoestrogens. The findings presented here highlight the power of a cDNA microarray approach for detecting endocrine disruptors in the aquatic environment.

Acknowledgements

This study was funded by the Natural Sciences and Engineering Research Council (NSERC), Canada, Research and Strategic grants. We thank Ms. Roula Raptis and Ms. Jana Malhotra for their assistance with this study.

APPLICATION OF FLUORESCENT INDICATOR DYES TO MONITOR HEPATOCYTE VIABILITY IN STUDIES ON GENE EXPRESSION

Kristin Schirmer
Junior Research Group - Molecular Animal Cell Toxicology
UFZ Centre for Environmental Research Leipzig-Halle
Permoserstr. 15, 04318 Leipzig, Germany
Phone: ++49-341-235-2699
Fax: ++49-341-235-2401
e-mail: Kristin.Schirmer@uoe.ufz.de

Anja Schreer
Junior Research Group - Molecular Animal Cell Toxicology
UFZ Centre for Environmental Research Leipzig-Halle
Permoserstr. 15, 04318 Leipzig, Germany
Phone: ++49-341-235-2318
Fax: ++49-341-235-2401
e-mail: Anja.Schreer@uoe.ufz.de

Summary

We have adopted the application of two fluorescent indicator dyes to studying the viability of primary rainbow trout hepatocytes. The two fluorescent dyes, alamar Blue, which indicates metabolic activity of a cell, and CFDA-AM (5-carboxyfluorescein diacetate acetoxymethyl ester), which is an indirect measure of cell membrane integrity, can be monitored conveniently in multi-well plates without extraction. Application of the two dyes to hepatocyte cultures exposed to varying concentrations of estradiol revealed a dose-dependent decrease in cell viability which appeared to occur unrelated to gene expression. Monitoring the viability of primary cells in this way will add important information to understanding results obtained in studies on gene expression, which can be performed on parallel or, possibly, the same cultures immediately upon fluorescent measurement.

Introduction

Primary fish hepatocyte cultures are indispensable tools for advancing understanding of fish liver physiology and toxicology (Pesonen & Andersson, 1997; Segner, 1998). One important advantage over the use of

whole fish is that they allow the role of selected parameters to be investigated in a defined culture system. This is particularly useful for studying gene expression where subtle changes may be important but difficult to detect if the experimental system is less defined.

In order to study gene expression, various doses of the variable to be investigated are generally applied in order to decipher the dose at which gene expression arises and to identify whether a dose-response relationship occurs. Changes in gene expression at a certain dose may eventually lead to changes at the cellular level but as well, changes at the cellular level might occur independent of changes to gene expression or even alter them. Thus, it would be useful to deploy a method that allows cell viability to be monitored immediately prior to extracting RNA for studying gene expression. To develop such a method was the incentive for initiating the work presented here.

Materials and Methods

Primary cultures of rainbow trout hepatocytes: Cells were isolated from rainbow trout weighing 150-230 g using a two-step collagenase perfusion technique as discussed by Segner (1998). Cells were plated at a density of $1x10^6$ cells per 600 µL per well serum- and phenol red-free L-15 culture medium in matrigel-coated 24-well Primaria plates (Falcon).

Exposure to 17β-estradiol: Estradiol (Sigma) was dissolved in ethanol and diluted 1:100 in culture wells do obtain the desired final concentrations when dosing for the first time. On each subsequent day, a half-medium change was performed and the appropriate amount of estradiol re-added to account for the loss due to the change. With this dosing procedure, the final content of ethanol per well was 1% (v/v).

Cell viability assays: Cell viability was assessed using a combination of two fluorescent indicator dyes, alamar Blue™ (BioSource) and CFDA-AM (Molecular Probes). The procedure followed the detailed description outline in Schirmer et al. (1997) with minor modifications.

Results and Discussion

Viability of rainbow trout hepatocytes was monitored directly in tissue culture wells without having to sacrifice the cells. Both the alamar Blue and the CFDA-AM assay were capable of individually assessing the general health of the cells but together, allowed for a more differentiated response analysis.

As indicated by alamar Blue but not CFDA-AM, ethanol by itself yielded a slight decrease in the metabolic activity of the cells when compared to the blank without ethanol (Fig. 1). Previously, another indicator of metabolic activity, MTT, was shown to sensitively detect mitochondrial impairment caused by ethanol (Mikami et al., 1997). As indicated by both alamar Blue and CFDA-AM, estradiol led to a dose-dependent impairment of cell viability but with alamar Blue, impairment could be detected more sensitively (Fig. 1). The toxicity caused by estradiol did not appear to be due to a specific regulation of genes as it occurred rapidly and did not change over the course of the experiment.

Overall, the assays applied here have the potential to yield information essential to the understanding of gene expression and cellular function.

References

Mikami, K., Haseba, T., Ohno, Y. 1997. Ethanol induces transient arrest of cell division (G2+M block) followed by G0/G1 block: dose effects of short- and longer-term ethanol exposure on cell cycle and cell functions. Alcohol & Alcoholism 32:145-152.

Pesonen, M. and Andersson, T.B. 1997. Fish primary hepatocyte cultures: an important model for xenobiotic metabolism and toxicity studies. Aquatic Toxicol. 37: 253-267.

Schirmer, K., Chan, A.G.J., Greenberg, B.M., Dixon, D.G. and Bols, N.C. 1997. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. Toxicol. *In Vitro* 11, 107-119.

Segner, H. 1998. Isolation and primary culture of teleost hepatocytes. Comp. Biochem. Physiol. Part A 120: 71-81.

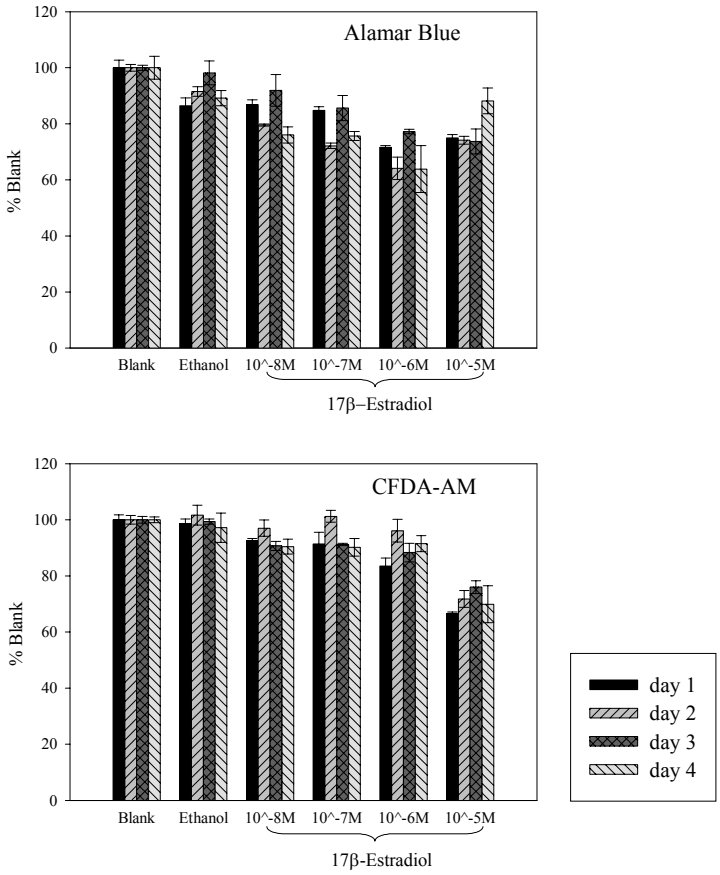

Fig. 1 Viability of primary rainbow trout hepatocytes upon exposure to 17-β Estradiol as assessed with the alamar Blue and the CFDA-AM indicator dyes. Fluorescence was measured as arbitrary units and expressed as a % of the readings in blank wells, which contained cells but neither ethanol nor estradiol. Each bar represents the mean of three culture wells with vertical lines indicating the standard deviation.

CHARACTERIZATION THROUGH PROTEOMICS OF THE GILL DERIVED RAINBOW TROUT CELL LINE, RTGILL-W1 GROWN UNDER VARYING OSMOTIC CONDITIONS

L.E.J. Lee
Department of Biology
Wilfrid Laurier University
Waterloo, ON, Canada, N2L 3C5
Tel (519) 884-1970 x 2252, Fax (519) 746-0677
e-mail: llee@wlu.ca

M.E. Kalbarczyk[1], M. P. Lamb[1] and N.C. Bols[2]
[1]Department of Biology, Wilfrid Laurier University, Waterloo, ON, Canada
[2]Department of Biology, University of Waterloo, Waterloo, ON, Canada

EXTENDED ABSTRACT ONLY – DO NOT CITE

Quantitative and qualitative measurements of protein changes in response to biological perturbations such as disease, hormonal or drug treatments and response to toxicants can be readily evaluated using proteomic analysis. Observation of changes at the protein level, unlike genomics, allows for functional analysis of cells and organisms, and the determination of mechanisms of action at the cellular level. However, the complexity and variability of organismal responses make it difficult to evaluate whole organism proteomes. In vitro assays have been instrumental in environmental toxicology and fish cell lines have been crucial in screening and elucidating mechanisms of action of various aquatic pollutants. Thus a proteomics approach to evaluate changes in fish cell lines in response to environmental contaminants has been initiated.

One of the first sites of action of aquatic pollutants in fish is at the gill surfaces and evaluation of chemical effects using gill cells in culture have been difficult because of the confounding effects of culture media and serum additives that may bind toxicants. In the present study, the RTgill-W1 cell line (Bols et al. 1994), was tested for growth under varying water concentrations and changes in their proteome under varying osmotic conditions were evaluated using 2D gel electrophoresis. RTgill-W1, which is an epithelioid cell line derived from gill explants of rainbow trout and is available from the American Type Tissue Collection (CRL2523), were maintained for over 6 months in tissue culture

inserts with water on the apical surface and culture media in the basolateral surfaces. Moreover, cells were adapted to grow under hypoosmotic and hyperosmotic conditions and were passaged several times. Hypoosmotic conditions included growth in 25% media: 75% water, 50% media:50% water, while hyperosmotic conditions included growth in 75% media:25% seawater, 50% media:50% sea water. Although growth rates were significantly reduced under the varying osmotic conditions, cells could be maintained for prolonged time periods especially in the 50:50 mixtures. Analysis of 2D gels of control, hypoosmotic (50% water), and hyperosmotic (50% seawater) cells showed slight differences in protein profiles which is currently under analysis (Fig.1).

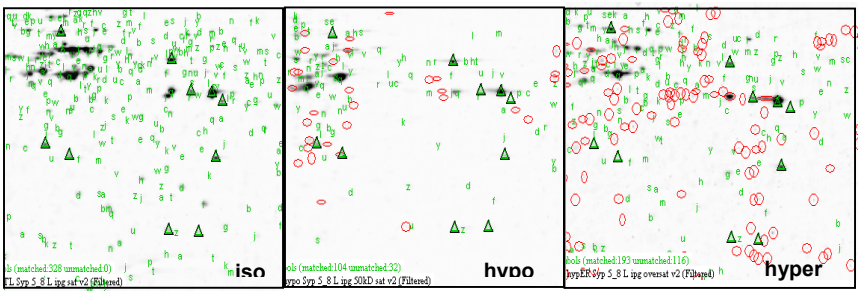

Fig.1 Protein profile of RTgill-W1 grown in varying osmotic states separated by IEF 5-8 in the first dimension and by 12.5% SDS-PAGE in the second dimension. Proteins were visualized by Sypro Ruby staining. Circles indicate protein spots not present in control isosmotic cells. Triangles indicate landmarking protein spots.

Morphologically, RTgill-W1 in hyper- or hypo-osmotic media were more epithelial than in the iso-osmotic control media (Fig.2). Granulated cells were observed in the hyper- and hypo-osmotically grown cells, but this was more predominant in the hyperosmotic medium. Tests are currently being performed to assess whether the granular cells are chloride-like cells. The above findings make RTgill-W1 a readily available and useful model for physiological and toxicological studies of fish gills. Aquatic contaminants could be directly added to the cells and comparisons in protein profiles could be made and changing protein patterns could then be further analysed to elucidate mechanisms of toxicant action.

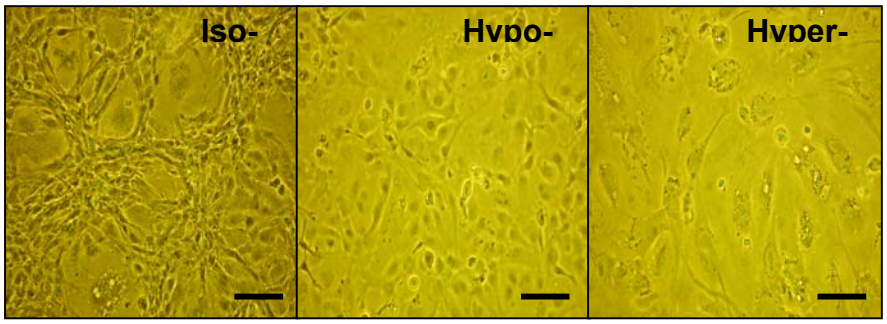

Fig. 2. Morphology of RTgill-W1 grown in varying osmotic states. Phase contrast micrographs taken at 100x magnification. Bar=100µm.

References

Bols, N. C., A. Barlian, M. Chirino-Trejo, S. J. Caldwell, P. Goegan and L. E. J. Lee (1994) Development of a cell line from primary cultures of rainbow trout, *Oncorhynchus mykiss* (Walbaum), gills. J. Fish Diseases 17: 601-611

Acknowledgements

We thank CFI for providing the infrastructure and NSERC for the operating grants. We also thank M. Allen for 2D gel analysis.

YOLK THYROID AND STEROID HORMONE METABOLISM AND THE CONSEQUENCES ON EMBRYONIC DEVELOPMENT: POTENTIAL SITES OF ACTION OF ENVIRONMENTAL ENDOCRINE DISRUPTORS

John Leatherland
Department of Biomendical Sciences, University of Guelph,
Guelph, ON N1G 2W1, Canada;
phone 519 824 4120, fax 519 767 1450; e-mail jleather@ovc.uoguelph.ca

Rakpong Petkam
Department of Biomedical Sciences, University of Guelph, Guelph, Canada

Jason Raine
Department of Biomedical Sciences, University of Guelph, Guelph, Canada

Colin Cameron
Department of Biomedical Sciences, University of Guelph, Guelph, Canada

EXTENDED ABSTRACT ONLY - DO NOT CITE

Introduction

There is increasing evidence to show that some toxicological factors act as hormone mimics, or negatively impact hormone function in vertebrates (reviewed in Guillette and Crain, 2000), with these so-called environmental endocrine disrupting chemicals (EDCs) affecting various aspects of developmental and/or reproductive function.

Because of the chemical diversity of known EDCs, their mode of action is pluralistic, with some EDCs affecting aryl hydrocarbon receptors (AHR), some impairing hormone transport in the blood, and some interacting with hormone receptors. Although the molecular mechanisms of EDC action has been the focus of recent studies, there needs to be a continued balance between molecular events and the down-stream cellular and whole organism responses. Given the extremely complex cellular signaling events, and the remarkable information redundancy in cell systems, specific molecular changes brought about by a specific EDC may not necessarily translate into a biological response. The effects of EDCs on embyronic development is a case in point; there are well-characterized examples of a developmental delay between exposure of an embryo to a specific insult and the defined/diagnosed downstream biological response (Colborn et al., 1993; Crews et al., 2000; McLachlan, 2001).

Oocytes and Embryos of Oviparous Species in EDC Toxicology Research: the Roles of Yolk Hormones

Embryos of aquatic ectotherms are particularly appropriate for this "paradigm shift" in toxicology research. They are available in large numbers for experimental work, and the markers for their early developmental biology are beginning to be defined. Equally interesting, however, is the role played by the oocyte factors in the early developmental biology of oviparous species that produce very yolky eggs. The composition of the cytoplasmic milieu of the oocyte, and thus the factors affecting pronucleus, zygotic nucleus and early embryo nucleus activities, is determined by the physiological condition of the female. We know from studies of mammalian oocytes that early gene expression is significantly affected by cytoplasmic influences and epigenetic factors (Jenuwein and Allis, 2001). Thus, it must be assumed that a similar relationship exists between the zygotic and embryonic nuclei of oviparous ectotherms, such as fish. The functions of the many factors are still largely unknown, and the factors that influence oocyte viability are controversial. In addition to these naturally occurring molecules, the lipid-rich oocytes are a preferential partitioning tissue for lipophilic organochlorine compounds, some of which are suspect EDCs. If these compounds interfere with hormone metabolism, they may have profound effects on tissue- and gender-specific developmentally-programmed patterns of gene expression, giving rise to developmental abnormalities.

In this paper, we examine the influences of the yolk steroid and thyroid hormones of salmonid eggs on early developmental events. We are particularly interested in the capacity of the embryos to metabolize and excrete these hormones to maintain

its own steroid and thyroid hormone milieu. Figure 1 illustrates the potential sites of actions of toxicological agents on thyroid and steroid hormone economy of the ovarian follicle and early developmental stage embryo. We summarize several studies of ovarian and embryo endocrine physiology, that form the basis of ongoing toxicological studies.

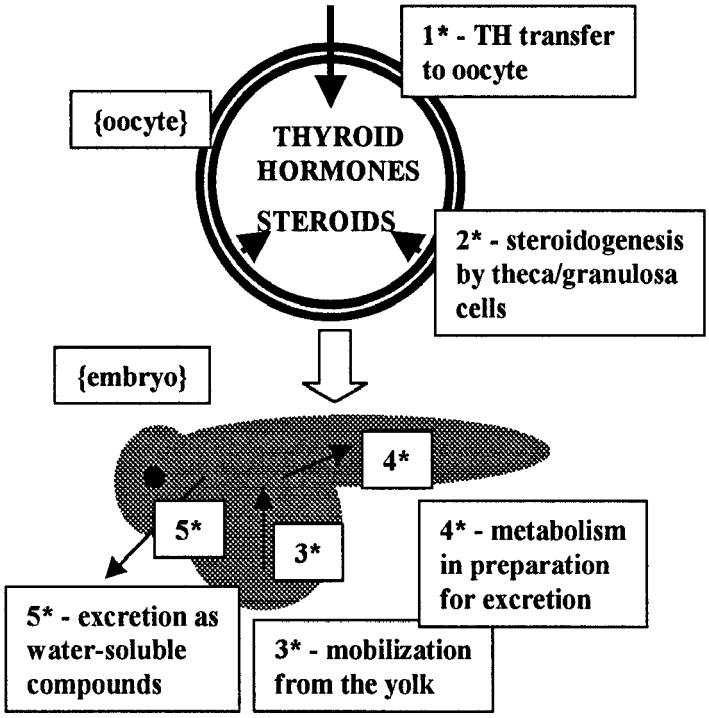

Figure 1. Diagrammatic representation of potential sites of action of xenobiotic interactions with the thyroid and steroid hormone economy and homeostasis of the maturing oocyte and early developmental stage embryo of a salmonid fish. These include: - 1* the transfer of thyroid hormone (TH) to the ooplasm, 2* steroid production by the theca and granulosa cells of the ovarian follicle, 3* mobilization of hormone products from the yolk, 4* the metabolism of thyroid and steroid hormones, and 5* the production of water-soluble excretory products for excretion

via the gills (and possibly the kidney).

Rationale for the Studies

The research focuses on the roles of yolk steroid and thyroid hormones, and to a lesser extent of GH, on early developmental events in salmonid fishes to address the questions: i) are these hormones essential for early development, or do they pose a potential hazard for the early embryo?, and ii) is normal embryo development influenced if hormone metabolism and clearance is compromised, as might be brought about by the presence of environmental contaminants in the yolk?

Experimental Work

The paper will describe studies at that we have made of several stages of hormone incorporation into yolk and hormone clearance from yolk, and preliminary reports on the actions of some suspect EDCs on these events.

Ovarian follicles

We have focused on factors that influence the trafficking of thyroid hormones between the plasma of the female, or the ovarian fluid in the body cavity of the female and the ooplasm, and the thecal/granulosal cells and the oocyte. Using an in vitro ovarian follicle model, and HPLC separation of steroid products, we have also examined the influence of many factors that impact on the steroidogenic pathways of theca and granulosa cells. In addition, we report on the actions of putative EDCs, and of AHR modulators, such as β-naphthoflavone, on steroidogenic pathways of the theca/granulosa cells.

Developing embryos

We report on the clearance of thyroid hormones (TH) from the developing embryos, and on physiological responses when TH levels are manipulated experimentally. We will also report on the actions of DDT and its metabolites on the ability of embryos to metabolize and clear TH. In additional studies, we are exploring the effects of experimentally-altered TH content of the yolk of oocytes and gene expression in embryos (with a particular interest in genes coding for the production of TH receptors).

We also report on a) the ability of developing salmonid embryos to metabolize, and conjugate a range of steroids, by specific (e.g., pregnenolone) or more general

steroidogenic pathways, and b) the influence of DDT and metabolites on the the ability of the embryos to metabolize, conjugate and produce water-soluble steroid forms that can be excreted.

References

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

Crews, D., E. Willingham and J.K. Skipper. 2000. Endocrine disruptors: present issues, future developments. Quart. Rev. Biol. 75: 243-260.

Guillette, L. and D.A. Crain. 2000. Environmental Endocrine Disruptors: An Evolutionary Perspective. New York: Taylor & Francis. 355p.

Jenuwein, T. and C.D. Allis. 2001. Translating the hisone code. Science 293: 1074-1079.

McLachlan, J.A. 2001. Environmental signalling: what embryos and evolution teach us about endocrine disrupting chemicals. Endocrine Rev. 22: 319-341.

PHARMACEUTICALS AS EDCS - THE METABOLIC IMPACT

OF GEMFIBROZIL IN GOLDFISH

T.W. Moon
Department of Biology, University of Ottawa
Ottawa, ON K1N 6N5 Canada
Tel: (613)562-5800 x 6002 Fax: (613)562-5486
email: tmoon@science.uottawa.ca

C. Mimeault[1], V.L. Trudeau[1] and C.D. Metcalfe[2]
[1]Department of Biology, University of Ottawa
[2]Environmental and Resource Studies, Trent University
Peterborough, ON K9J 7B4 Canada cmetcalfe@trentu.ca

EXTENDED ABSTRACT ONLY- DO NOT CITE

Pharmaceuticals are reported in the aquatic environment at concentrations that can exceed $\mu g \bullet L^{-1}$ post sewage treatment plant (Kolpin et al., 2002). Pharmaceuticals are designed to be bioactive, yet most of the literature available is limited to their occurrence rather than to their fate or effects on non-target organisms. This study was designed to test the bioactivity of a lipid regulator, gemfibrozil in the goldfish, *Carassius auratus*, a widely used model species in endocrine studies. This drug is reported in Canadian sewage treatment effluents at concentrations exceeding 2 $\eta g \bullet L^{-1}$ (Metcalfe et al., 2002).

Gemfibrozil (GEM) is a lipid and cholesterol lowering fibrate drug that acts as a peroxisomal proliferator (PP), increasing cell peroxisome numbers and size through the activation of a nuclear receptor called the peroxisomal proliferator-activated receptor (PPAR) (Gonzalez et al., 1998). PPARs activate genes containing a PPAR responsive element, including those that code for many aspects of lipid catabolism (Kersten et al., 2000). Acyl-CoA oxidase (ACO), the first enzyme of the peroxisomal β-oxidation pathway, is one such enzyme that leads to hydrogen peroxide (H_2O_2) production and the potential for cellular oxidative stress. There are significant species differences in the sensitivities towards these fibrate drugs, with rodents hyper-responsive compared with humans. These differences may relate to the level of PPAR present (Gonzalez et al., 1998). Fibrate drugs induce peroxisomes in the trout less effectively than the rodent (Yang et al., 1990), but GEM does inhibit sex steroid production in the goldfish (G. Van Der Kraak, pers. commun.).

This study tests the hypothesis that GEM induces changes in lipid catabolism by acting as a PP in the goldfish. The objectives of this study are to examine the impact of GEM on 1) plasma lipid and carbohydrate levels, 2) tissue antioxidants and lipid peroxidation, and 3) induction of hepatic PPAR isoforms. Goldfish received intraperitoneal injections of dimethyl sulfoxide (DMSO; control) or 10 $\mu g \bullet g^{-1}$ (low dose, LDG) or 100 $\mu g \bullet g^{-1}$ (high dose, HDG; equivalent to a human therapeutic dose) GEM (in DMSO carrier) every other day for 8 days. Fish were sacrificed 48 h after the last dose, followed by blood sampling and tissue harvesting. Plasma metabolites, tissue enzymes and antioxidants were assayed using routine methods. PPARα/γ cDNAs were cloned and sequenced based on other PPAR sequences available in GenBank.

Blood glucose levels significantly increased at both dose levels of GEM (DMSO, 0.4 ± 0.08; LDG, 0.8 ± 0.06 $mg \bullet mL^{-1}$) while blood triacylglycerides decreased only in the HDG-treated group (DMSO, 3.3 ± 0.3; HDG, 1.9 ± 0.24 $mg \bullet mL^{-1}$); no changes were noted in cholesterol or protein levels. Hepatic ACO activities increased ~ 60-fold when comparing the saline and LDG-treatments (0.014 ± 0.01 to 2.4 ± 0.8 $mU \bullet mg^{-1}$ protein), while the HDG treatment was not significant. A number of tissue antioxidant enzymes were measured including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and glutathione reductase (GR). Only hepatic GPx significantly increased with HDG while all other enzymes were unaffected in the liver. Contrary to these stable values, hepatic total glutathione (GSH-eq) significantly increased at both GEM concentrations (DMSO, 0.2 ± 0.06; LDG, 1.6 ± 0.35 $\mu mol \bullet g^{-1}$) in the absence of changes in thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation. Thus, there was no indication of oxidative stress in the liver, as the antioxidants effectively overcame any change in H_2O_2 generated by the increased ACO activities. However, significant decreases in GPx, GST and GSH-eq were noted in the hearts of HDG-treated goldfish. Changes also occurred in interrenal tissue SOD activities.

Gemfibrozil increased PPARα transcripts by 1.6-fold in the liver without changes in PPARγ; GEM is a known PPARα ligand. In additional, serum cortisol values tended to decrease especially with HDG treatment. More interesting was the significant negative slope between cortisol level and body weight in the DMSO-treated goldfish and the positive slope for both the LDG- and HDG-treated fish. This dissociation between cortisol and body mass needs further study.

This study clearly indicates that GEM is bioactive in goldfish at the concentration used and that a low dose may be more effective than a high dose for some parameters tested. Apparent disruptions in both carbohydrate and lipid metabolism, and some imbalance in tissue oxidative status were observed. Whether this acute exposure is reflective of a more chronic impact of GEM is presently being investigated by using environmentally relevant exposures.

References

Gonzalez, F.J., Peters, J.M. and Cattley, R.C. 1998. Mechanism of action of the nongenotoxic peroxisome proliferators: Role of the peroxisome proliferator-activated receptor α. J. Natl. Cancer Inst. **90**: 1702-1709.

Kersten, S., Desvergne, B. and Wahil, W. 2000. Roles of PPARs in health and disease. Nature **405**: 421-424.

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

Metcalfe, C.D., Koenig, B.G., Ternes, T.A. and Hirsch, R. 2002. Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. Environ. Sci. Technol., in press.

Yang, J.-H., Kostecki, P.T., Calabrese, E.J. and Baldwin, L.A. 1990. Induction of peroxisome proliferation in rainbow trout exposed to ciprofibrate. Tox. Appl. Pharmacol. **104**: 476-482.

Acknowledgements

This study was supported by grants from the Toxic Substances Research Initiative and NSERC of Canada Research and Strategic Grants to the authors.

EFFECTS OF ENDOCRINE DISRUPTING COMPOUNDS ON WALLEYE, *STIZOSTEDION VITREUM*, NEAR THE METRO SEWAGE TREATMENT PLANT, SAINT PAUL, MINNESOTA

Jacquelyn N. Connor
Department of Fisheries, Wildlife and Conservation Biology
University of Minnesota
1980 Folwell Avenue, St. Paul, MN 55108-6124
jnc@fw.umn.edu

Jim T. Levitt
Minnesota Department of Natural Resources
1200 Warner Road, St. Paul, MN 55106
Jim.Levitt@dnr.state.mn.us

Heiko L. Schoenfuss
Department of Biological Sciences, St. Cloud State University
720 Fourth Avenue South, St. Cloud, MN 56301
hschoenfuss@stcloudstate.edu

Ira R. Adelman
Department of Fisheries, Wildlife and Conservation Biology
University of Minnesota
1980 Folwell Avenue, St. Paul, MN 55108-6124
ira@fw.umn.edu

EXTENDED ABSTRACT ONLY – DO NOT CITE

Recent studies in the United Kingdom and the United States have reported the presence of estrogenic compounds, natural and synthetic, in treated domestic and industrial sewage effluent released from sewage treatment plants (STPs) (Desbrow et al., 1998, Ternes et al., 1999). These compounds may reduce the reproductive potential of exposed fish. Evidence of exposure to estrogenic compounds has been verified by the presence of elevated levels of vitellogenin (VTG), a female egg yolk protein precursor, in male fish. Male fish, although capable of synthesizing VTG, typically only do so when exposed to chemicals with estrogenic properties. VTG induction in male fish has been linked to the

presence of estrogenic chemicals in sewage effluent in the United Kingdom (Desbrow et al., 1998) and is a bio-indicator of the presence of estrogenic chemicals in the aquatic environment (Sumpter and Jobling, 1995).

This research is attempting to determine if estrogenic compounds in the Minneapolis and St. Paul Metropolitan STP effluent are having a significant effect on the reproductive potential of male and female walleye (*Stizostedion vitreum*) exposed to the effluent. It is currently unclear whether the presence of vitellogenin (VTG) in male fish is only a biomarker for exposure to estrogenic compounds or if it indicates an impact on fish reproductive health. Numerous studies have indicated that estrogenic compounds may reduce the reproductive potential of fish populations in a number of ways including: disrupting of normal hormonal activities, causing the development of intersexed gonads, affecting sexual differentiation, and causing decreased gonad size relative to unexposed populations (Purdom et al., 1994, Jobling et al., 1998).

Results

In the present study, we investigated the effects of the Metro STP effluent on the reproductive potential of walleye by evaluating VTG induction, steroid hormone levels, gonadosomatic index (GSI), sex ratio, reproductive condition, and histopathology. Impacts on reproduction of walleye is of particular concern because this species is the major sport fish sought by anglers in Minnesota and throughout much of the United States, has considerable economic importance, and is recognized as the "Minnesota state fish," giving it high public visibility.

In 2000, 2001, and 2002 walleye were collected from the Metro STP effluent channel and a reference site 20 km upstream prior to and during the spawning season. In 2000 and 2001, exposure of the walleye to estrogenic compounds was confirmed by the presence of VTG in males. Male walleye appeared to be extremely sensitive to VTG induction in contrast to other species reported in the literature. Male walleye collected from the Metro STP effluent channel had decreased gonad size, no expressible milt, elevated levels of serum estradiol, and one case of intersexed testes. Upstream males had low VTG concentrations and expressible milt. Female walleye were found with atretic ovaries at the Metro STP and the reference site. Table one summarizes gonadal abnormalities among fish from the 2000 and 2001 field seasons. Preliminary data from 2002 collections show similar trends. No males collected from the Metro STP expressed milt; 12 out of 13 collected from the reference site expressed milt.

VTG and hormone levels and gonadal histopathology have not yet been analyzed on the fish collected in 2002.

Table 1. Summary of fish collected during the 2000 and 2001 field season and frequency of gonadal abnormalities at the Metro sewage treatment plant (STP) site and a reference site.

	Reference 2000	Metro STP 2000	Metro STP 2001
Number of Males (Intersex)	5 (0)	2 (0)	4 (1)
Number of Females (Atretic)	4 (2)	14 (9)	6 (0)
Immature Females	0	4	6
Totals	9	20	16

During the 2001 field season, we tagged 68 walleye and returned them to the effluent channel over 12 sampling dates between November 15, 2000 and March 26, 2001. Only five were recaptured, suggesting that the walleye did not spend the entire winter in the effluent channel. This suggests that walleyes collected from the Metro STP effluent channel for VTG, hormone, and histological evaluation were exposed to the effluent, but not necessarily resident in the channel.

The combination of the presence of high levels of VTG in males, a highly skewed sex ratio biased towards females, lack of milt production in males, and frequent gonadal abnormalities in both sexes suggests a likely exposure of walleye in the effluent channel to endocrine disrupting chemicals.

Acknowledgements

The authors gratefully acknowledge funding from the Metropolitan Council of Environmental Services and the Minnesota Water Resource Center. We thank Dr. Robert Polta and Garry Durland of the Metro STP for logistical assistance, Dr. Richard Leino and Dr. John Kettinger of the University of Minnesota for their assistance in the histological procedures and examination and thanks to Dr. Nancy Denslow for conducting the VTG analysis.

References

Desbrow, C., E. J. Routledge, G. C. Brighty, J. P. Sumpter, and M. Waldock. 1998. Identification of estrogenic chemicals in STW effluent. I. Chemical fractionation and in vitro biological screening. Environmental Science and Technology. 32: 1549-1558.

Jobling, S., M. Nolan, C. R. Tyler, G. Brighty, and J.P. Sumpter. 1998. Widespread sexual disruption in wild fish. Environmental Science and Technology. 32: 2498-2506.

Purdom, C. E., P. A. Hardiman, V. J. Bye, N. C. Eno, C. R. Tyler, and J. P. Sumpter. 1994. Estrogenic effects of effluents from sewage treatment works. Chemistry and Ecology. 8: 275-285.

Sumpter, J. P. and S. Jobling. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environmental Health Perspectives. 103 (Suppl. 7): 173-178.

Ternes, T. A., M. Stumpf, J. Mueller, K. Haberer, R.-D. Wilken, and M. Servos. 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants – I. investigations in Germany, Canada and Brazil. The Science of the Total Environment. 225: 81-90.

MODELING POPULATION-LEVEL RESPONSES OF ATLANTIC CROAKER TO ENDOCRINE DISRUPTING CHEMICALS USING LINKED SIMULATION MODELS AND LABORATORY STUDIES

Cheryl A. Murphy
Department of Oceanography and Coastal Sciences
Louisiana State University
Baton Rouge, LA 70803
Tel: 225-578-5970
Fax: 225-578-6513
email: cmurph4@lsu.edu

Kenneth A. Rose
Department of Oceanography and Coastal Sciences
Coastal Fisheries Institute
Louisiana State University
Baton Rouge, LA

Sandra L. Diamond
Department of Biology
Texas Tech University
Lubbock, TX

Lee A. Fuiman
Marine Science Institute
University of Texas
Port Aransas, TX

Peter Thomas
Marine Science Institute
University of Texas
Port Aransas, TX

EXTENDED ABSTRACT ONLY - DO NOT CITE

Introduction

The influx of man-made chemicals into the environment since the beginning of the Industrial Revolution has produced many unexplained detrimental effects on wildlife populations (Colborn *et al*., 1993). Moreover, chemicals that result in endocrine disruption are receiving increasing attention. However, endocrine disruption as a cause of population decline in fish has been difficult to ascertain because most of the adverse effects on individuals are subtle or sublethal, and are small relative to other sources of population variation. Since endocrine disrupting chemicals (EDCs) were first recognized, numerous biomarkers have been measured that demonstrate abnormalities in fish exposed to industrial and domestic wastes (e.g. vitellogenin production in male fish). These biomarkers appear to correlate with decreased fertility, but to date, few studies directly link adverse effects of EDCs to fish population response. We use a combination of laboratory studies, nonlinear statistical analysis, and individual-based and matrix projection modeling to link lethal and sublethal effects of endocrine disrupting chemicals to fish population dynamics (Fig. 1). We demonstrate our approach using the effects of PCBs on Atlantic croaker (*Micropogonias undulatus*).

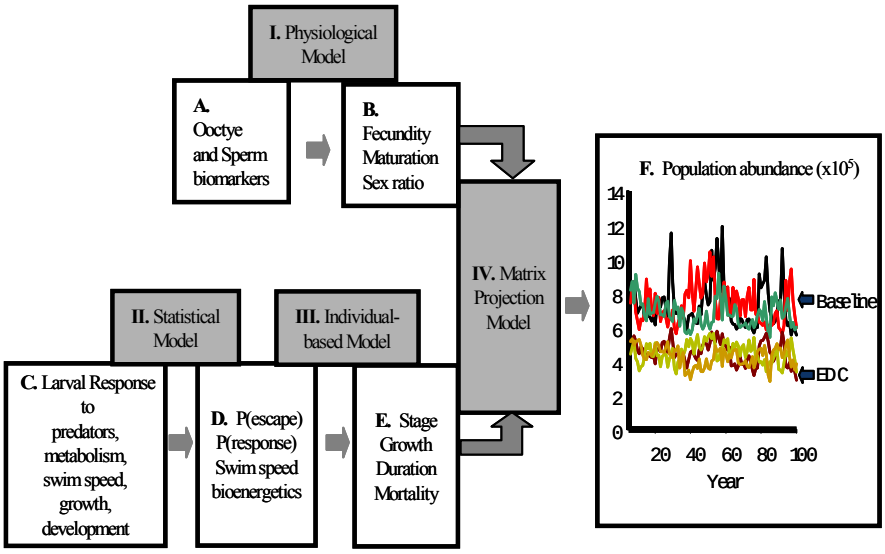

Figure 1. Toxicological endpoints measured in the laboratory linked to four models that culminate in a projection of Atlantic croaker population abundance over time.

Model I -Physiological Model

We conducted laboratory toxicity experiments on Atlantic croaker to compare commonly measured biomarkers (e.g., changes in steroid levels, estrogen receptor concentrations, and vitellogenin production (Fig 1A)) between control and PCB-exposed fish. In conjunction with these biomarkers, we also measured fecundity, oocyte maturation, fertilization success, and hatching success (Fig 1B). We used regression tree methods and neural network analysis to quantify the relationships between the biomarkers and the number of viable oocytes produced by PCB-exposed and control fish. We then use the changes in the number of viable oocytes (fecundity) as an input to the matrix projection model to simulate PCB exposure (Fig 1 IV).

Model II - Statistical Model

We performed behavior experiments using larval croaker spawned from control and PCB-exposed adult females. We measured response that included routine swimming speed and the timing and speed of evasive responses to artificial predators (using visual and vibrational stimuli). These behavioral responses for control and PCB-exposed larvae (Fig 1C) were then used with a statistical model developed by Fuiman,*et al.* (in prep) to determine how changes in swimming speed and predator avoidance behavior due to parental PCB exposure translate into the probability of escaping a real fish predator (Fig 1D).

Model III -Individual-Based Model

We used an individual-based model of a larval fish cohort that simulates larval encounter and capture of zooplankton prey (resulting in larval growth) and encounters and capture by individual invertebrate and fish predators (resulting in larval mortality). Swimming speed and probability of escaping a real predator (from the statistical model Fig 1D) are inputs to the individual-based model. We use the individual-based model to predict larval stage duration and survival in control and PCB-exposed larvae (Fig 1E). The changes in stage duration and survival due to PCB exposure are then inputted into the matrix projection model.

Model IV – Matrix Projection Model

We modified an existing life table developed for Atlantic croaker (Diamond *et*

al., 1999) to create a baseline matrix population model that uses a stage within age for YOY, and annual age classes for age-1 and older. The model simulates biannual spawning and three spatial boxes (YOY in North Carolina estuaries; YOY in Virginia estuaries; adults in the mid-Atlantic Bight). Model estimation and corroboration was based on data collected from fishery-independent surveys and published literature. Density dependence and stochasticity due to environmental variation were also incorporated. The matrix model was used to simulate the population dynamics under various combinations of feasible PCB exposures, other stressors, and natural variation (Fig 1F).

Simulations: Scenario and Results

To illustrate our approach, we simulated a croaker population for 100 years in which exposure to PCB was assumed to occur in North Carolina estuaries every year. We further assumed that PCB burdens in adult female spawners were completely eliminated with first spawn. Therefore, PCB effects were imposed on age-1 fecundity of individuals spawned in North Carolina, and imposed on the swimming speed and predator escape ability of the oceanic larvae spawned by these age-1 fish. Simulation results indicated a small, but consistent, reduction in the long-term average population abundance compared to baseline (unexposed) conditions. However, the reduction was masked by interannual fluctuations due to density-dependence and environmental variability.

Our analysis at this time is preliminary and demonstrates that it is possible to scale the sublethal tissue- and organism-level effects of EDCs to population-level responses. We emphasize that this analysis establishes proof of principle rather then predicting PCB effects on croaker. We are presently refining our analyses to enable us to make more accurate predictions of likely field effects.

References

Colborn, T., F.S. Saalvom, and A.M. Soto. 1993. Developmental effects of endocrine_disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* 101:378-384

Diamond, S. L., Crowder, L. B., and L. G. Cowell. 1999. Catch and bycatch: the qualitative effects of fisheries on population vital rates of Atlantic croaker. *Trans. Am. Fish. Soc*. 128: 1085-1105.

Acknowledgements

This work was funded by US EPA Science to Achieve Results (STAR) program.

ENVIRONMENTAL CONTAMINANTS IN WHITE STURGEON: IS ENDOCRINE DISRUPTION OCCURRING IN THE COLUMBIA RIVER?

Grant W. Feist
Oregon Cooperative Fish and Wildlife Research Unit,
Department of Fisheries and Wildlife
Oregon State University, Corvallis, OR 97331, USA
ph 541, 737-2463, fax 541, 737-3590
feistg@onid.orst.edu

Molly A.H. Webb and Carl B. Schreck
Oregon Cooperative Fish and Wildlife Research Unit,
Department of Fisheries and Wildlife
Oregon State University, Corvallis, OR 97331, USA

Martin S. Fitzpatrick and Eugene P. Foster
Oregon Department of Environmental Quality, Portland, OR 97204, USA

Deke T. Gundersen
Environmental Science Program, Pacific University,
Forest Grove, OR 97116, USA

Alec G. Maule
Biological Resources Division, US Geological Survey, Columbia River
Research Laboratory, Cook, WA 98605, USA

EXTENDED ABSTRACT ONLY - DO NOT CITE

The lower Columbia River supports one of the most productive white sturgeon, *Acipenser transmontanus*, fisheries in North America. Fish trapped behind the dams of the hydroelectric system however, have reduced reproductive success when compared to animals in the free-flowing portion of the river. Reduced reproductive fitness of fish in these impounded sections of the river has been attributed to habitat, flow and temperature but environmental toxicants could also be playing a role. The long-lived, late-maturing and benthic lifestyle of sturgeon make them particularly susceptible to the actions of persistent bioaccumulating pollutants. We are presenting the preliminary findings of an

ongoing study examining possible effects of environmental toxicants on sturgeon reproductive physiology.

Fish were collected from the free-flowing portion of the Columbia River in the estuary and pools above Bonneville, The Dalles and John Day dams. Condition factor (CF) and gonadosomatic index (GSI) were determined for each fish and blood samples were collected for analysis of plasma estradiol, testosterone (T), 11-ketotestosterone (KT) and vitellogenin (Vg). Livers and gonads were examined histologically and analyzed for 19 chlorinated pesticides and 26 PCB congeners. Livers were also analyzed for mercury content.

Some males and immature females showed elevated vitellogenin levels in The Dalles and John Day pools indicating an exposure to some type of xenoestrogen. Mercury was present in all livers examined at relatively high concentrations. Most of the pesticides and PCBs analyzed were detected in at least some of the samples. Metabolites of DDT (DDE and DDD) were consistently found in fish at relatively high levels. A negative correlation was found between CF and levels of DDE, DDD, total PCBs and mercury. Some gonadal abnormalities were observed, including inter-sex fish. In males, a negative correlation was found between plasma androgens and DDE content of livers (Figure 1). Male

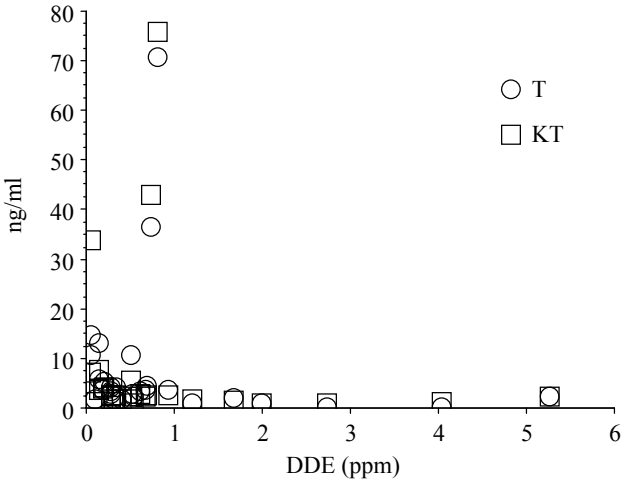

Figure 1. Plasma testosterone (T) or 11-ketotestosterone (KT) versus liver p-p'-DDE (DDE) in parts per million (ppm) for Columbia River male white sturgeon. Reciprocal-Y regression: p=0.0005 for T and 0.0452 for KT

fish also showed a negative correlation between GSI and DDE content of livers. Fish residing in the reservoir behind the oldest of the dams examined (Bonneville) had the highest contaminant loads (Figure 2).

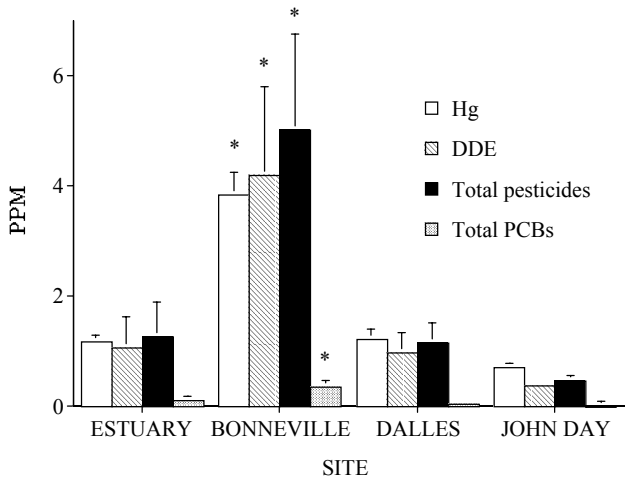

Figure 2. Concentrations in parts per million (ppm) of mercury (Hg), p-p'-DDE (DDE), total pesticides and total PCBs in livers of Columbia River white sturgeon from 4 locations. Histograms represent mean + S.E.M. "*" denotes statistically different from other locations (ANOVA; Bonferroni post-hoc test, $p<0.05$).

Elevated levels of VG seen in some fish were not correlated with any of the chlorinated pesticides or PCB congeners that were analyzed. This suggests that some other xenoestrogen(s) not being monitored by this study may be causing these feminizing effects. Tissue concentrations of DDE and PCBs found in this study were similar to those seen previously by our laboratory in Columbia River white sturgeon. (Foster *et al*., 2001a). The negative correlation between plasma androgens and DDE content of livers in males has also been documented by our laboratory in a previous sampling year (Foster *et al*., 2001b). We have preliminary evidence that the mechanism by which DDE reduces plasma androgens involves one of the liver cytochrome P450 isozymes. Liver content of p,p-DDE is positively correlated with the putative cytochrome P450 3A. This enzyme is responsible for metabolizing androgens. Thus, DDE may be

decreasing plasma androgens in males by enhancing steroid metabolization and excretion.

Fish with the highest tissue toxicant loads were residing in the reservoir of the oldest dam suggesting that these compounds are accumulating behind dams over time. These fish also had the lowest CF and highest incidence of gonadal abnormalities. Our results indicate that exposure to environmental contaminants may be affecting both growth and reproductive physiology of sturgeon in some areas of the Columbia River.

References

Foster, E.P., Fitzpatrick, M.S., Feist, G.W., Schreck, C.B. and Yates, J. (2001a). Gonad organochlorine concentrations and plasma sex steroid levels in white sturgeon (*Acipenser transmontanus*) from the Columbia River, USA. Bull. Environ. Contam. Toxicol. 67:239-245.

Foster, E.P., Fitzpatrick, M.S., Feist, G.W., Schreck, C.B., Yates, J., Spitsbergen, J.M. and Heidel, J. (2001b). Altered reproductive physiology, EROD induction, reduced condition factor, and the occurrence of organochlorine pollutants in white sturgeon (*Acipenser transmontanus*) from the Columbia River. Arch. Environ. Contam. Toxicol. 41(2):182-191.

EFFECTS OF AROCLOR 1254 ON ENDOCRINE PHYSIOLOGY

IN THE CHANNEL CATFISH (*ICTALURUS PUNCTATUS*)

Brian S. Shepherd*,
Katherine Drennon, Ben F. Brammell and Adria A. Elskus
Department of Biology, University of Kentucky, 101 Morgan Building,
Lexington, KY 40506-0225 U.S.A.
*Phone: (859) 323-1791; Fax (859) 257-1717; E-mail: bsshep2@uky.edu

EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

The channel catfish, *Ictalurus punctatus*, has a wide distribution in North America. As an obligate benthic species, catfish are frequently exposed to pollutants. Concern exists that these pollutants may affect individual traits and consequently population stability. Recent studies have demonstrated that many pollutants, called endocrine disrupting chemicals (EDCs), can negatively effect how teleosts adapt to changes in their environment (e.g., salinity) (Fairchild *et al.*, 1999; Madsen *et al.*, 1997). These findings underscore the necessity for understanding how EDC exposure influences individual traits, particularly endocrine physiology.

To examine this, a better understanding of the roles that the pituitary hormones, growth hormone (GH) and prolactin (PRL), play in the environmental physiology of the channel catfish are needed. To address this, we have established a method to measure blood levels of GH in channel catfish. We report, herein, the effects of polychlorinated biphenyls (Aroclor 1254) on plasma GH levels in catfish. Our aim is to understand how pituitary hormones influence adaptive responses of catfish to EDC(s) and, in turn, how EDCs influence endocrine physiology.

Methods

Assay Development:

For the GH ELISA, a 96-well plate was coated with 250ng/ml catfish GH in carbonate buffer, with additional wells for non-specific binding (NSB), and was incubated overnight at 4°C without shaking. Wells were then emptied and blocked (without shaking) with PBST-NGS (5%) (Normal Goat Serum) overnight at 4°C.

Hormone standards and plasma samples were serially diluted in PBST-BSA (1%) and mixed 1:1 and 1:4, respectively, with the primary antibody diluted 1:10,000 in PBST-NGS (2%). Controls to determine maximum and non-specific binding were also prepared. Tubes were incubated at 37°C for ninety minutes, at which time 100µl of each reaction was pipetted into the 96-well plate and incubated at 37°C for ninety minutes. The plate was then washed, coated with secondary antibody conjugate (GAR-HRP: goat anti-rabbit horseradish peroxidase) diluted 1:5,000 in PBST-NGS (2%) and incubated at 37°C for ninety minutes. Wells were washed, developed (10 minutes) by the addition of o-phenylenediamine dihydrochloride and H_2O_2 (substrate) and read at 450 nm using a Molecular Devices plate reader.

In Vivo Studies:

Animals were held in dechlorinated fresh water. Anesthetized animals were given i.p. injections of vehicle (corn oil) or vehicle containing estradiol (5 mg/kg) or the PCB mixture Aroclor 1254 at 1, 10 and 100 mg/kg. Seven days after injection, the animals were sampled for plasma and other tissues for analytical work.

Results

Using optimized conditions (Ab dilution of 1:10,000 and coating antigen of 250ng/well), we have determined the best standard concentration range, for our ELISA, to be 0.58 ng/ml to 150 ng/ml (Figure 1).

To demonstrate GH-Ab specificity, dilutions of pituitary homogenates and purified hormones from catfish (PRL) and other teleosts were tested. Catfish pituitary homogenate dilutions were parallel to our standard curves, whereas pituitary homogenates or purified hormones (data not shown) from other teleosts were not. A 1:4 plasma dilution was found to be within the linear range of the plasma binding curve and was used for all assays.

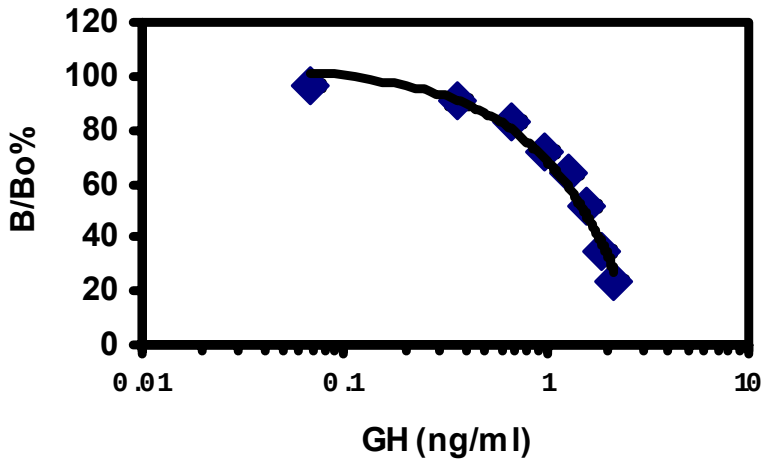

Figure 1: Standard curve of native channel catfish growth hormone (GH) in a competitive ELISA. The ratio of the quantity bound (B) to the maximum binding (Bo) (zero or lowest standard) is determined by optical density (O.D.) at 450 nm and is shown as a percentage for GH standards ranging from 1.17 ng/ml to 150 ng/ml. All values are means + SEM

To assess whether the PCB mixture, Aroclor 1254, exerted any estrogen-like actions in the channel catfish, animals were injected with oil implants containing estradiol or Aroclor 1254 at different doses. Estradiol significantly ($P < 0.05$) elevated plasma GH levels above control values (Figure 2). While mean GH levels in the Aroclor-treated groups were lower than control values, only the 10 mg/kg dose group was significantly ($P < 0.05$) lower than the control group.

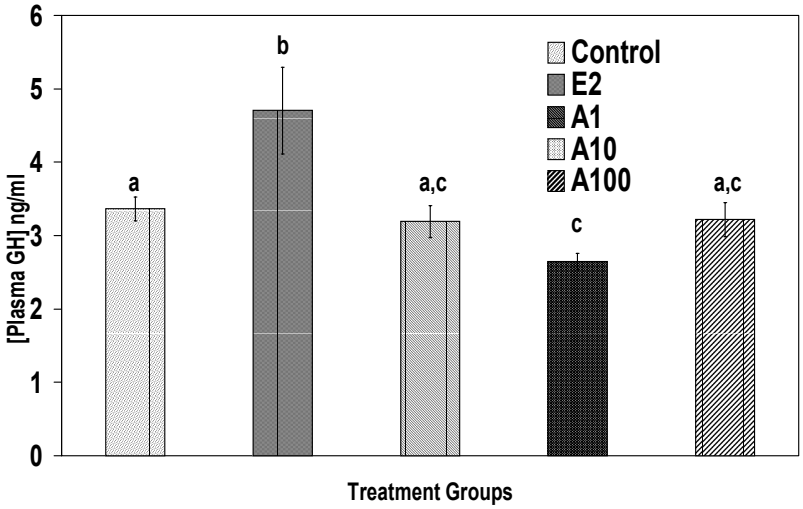

Figure 2: Effects of estradiol and Aroclor 1254 (PCB) injection on plasma GH levels in channel catfish. Plasma GH levels were determined using a homologous ELISA. All values are means ± SEM. E_2= 5 mg/kg Estradiol, A_1=1 mg/kg Aroclor, A_{10}=10 mg/kg Aroclor, A_{100}=100 mg/kg Aroclor.

Discussion

We have developed an ELISA for the measurement of GH levels in channel catfish, thus enabling investigations into how environmental stressors alter levels of this hormone in this species.

Our results show that estrogen stimulates GH secretion in the channel catfish which is consistent with published findings in goldfish (Zou *et al.*, 1997).

Recent studies have reported that EDCs can influence pituitary CYP1A1 and -1A2 expression suggesting that pituitary P450 expression may influence pituitary physiology. In support of this, Cravedi and co-workers (Cravedi *et al.*, 1995) demonstrated that GH treatment reduced induction of hepatic xenobiotic metabolizing enzymes in trout, suggesting the presence of an intricate regulatory relationship between the pituitary and hepatic physiology in response to EDC exposure. Therefore, it would seem reasonable to propose that a reduction in plasma GH, following exposure to a xenobiotic, has adaptive significance in that such a reduction would facilitate xenobiotic metabolism by P450 enzymes in the liver. Additional work is underway to examine the effects of Aroclor 1254 on other metabolic and endocrine sectors.

Acknowledgements

We thank Drs. Hirano, Kawauchi, Moriyama, Silverstein and Small for their assistance with hormone purification. This work was supported by grants from the USDA/NRICGP (97-352206-5094 and 2002-35206-11629) and the USGS/KWRRI (01HQGR0133) to B.S.S and EPA-STAR (GOK20395) to A.A.E. The views expressed herein are those of the authors and do not necessarily reflect the views of the U.S. Government.

References

Cravedi, J.P., Paris, A., Perdu-Durand, E. and Prunet, P. (1995) Influence of growth hormone on hepatic mixed function oxidase and transferase of rainbow trout. *Fish Physiol. Biochem.*, 14, 259-266.

Fairchild, W.L., Swansburg, E.O., Arsenault, J.T. and Brown, S.B. (1999) Does an association between pesticide use and subsequent declines in catch of Atlantic salmon (*Salmo salar*) represent a case of endocrine disruption. *Environ. Health Perspect.*, 107, 349-358.

Madsen, S.S., Mathiesen, A.B. and Korsgaard, B. (1997) Effects of 17 beta-estradiol and 4-nonylphenol on smoltification and vitellogenesis in Atlantic salmon (*Salmo salar)*. *Fish Physiol. Biochem.*, 17, 303-312.

Zou, J.J., Trudeau, V.L., Cui, Z., Brechin, J., Mackenzie, K., Zhu, Z., Houlihan, D.F. and Peter, R.E. (1997) Estradiol stimulates growth hormone production in female goldfish. *Gen. Comp. Endocrinol.*, 106, 102-112.

THE EFFECT OF AROCLOR 1254 ON SMOLT DEVELOPMENT AND SUBSEQUENT SEAWATER PERFORMANCE AND MATURATION IN ANADROMOUS ARCTIC CHARR

Øyvind Aas-Hansen and Even Jørgensen
Norwegian Institute for Nature Research, the Polar Environmental Centre,
N-9296 Tromsø, Norway

Alec G. Maule
USGS-BRD, WFRC, Columbia River Research Laboratory,
5501A Cook-Underwood Rd., Cook, WA 98605 USA

Mathilakath M. Vijayan
Department of Biology, University of Waterloo,
Waterloo, Ontario, Canada N2L 3G1

.

EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

The winter residence in freshwater of the anadromous Arctic charr (*Salvelinus alpinus*) is associated with low food intake and lipid mobilization, and the charr are emaciated at the time of seaward migration in the spring (Jørgensen et al., 1997). Previous experiments with anadromous charr have shown that lipophilic pollutants such as polychlorinated biphenyls (PCBs) are redistributed from ”insensitive” fat stores to sensitive tissues like the brain and liver in late winter/early spring. This redistribution corresponds with much stronger biomarker responses (CYP1A induction) in spring (lean fish) than in autumn (fat fish) in these fish (Jørgensen et al., 2002). Hence, the fish appear to be extra sensitive to these pollutants, especially during the period of smoltification, which pre-adapts the fish for the summer seawater residence. Our study investigated the sensitivity of the smoltification process toward lipophilic, persistent pollutants such as PCB, which are found in alarming concentrations in Arctic wildlife.

Material and methods

In the present experiment wild, anadromous Arctic charr were captured on ascendence. They were individually tagged and transferred to indoor tanks in which they were held at natural freshwater temperatures and light conditions, without being fed, until the following spring. In November, the fish were randomly divided into three groups. The fish in 2 groups were given either 1 or 100 mg PCB (Aroclor 1254)/kg fish. These groups are hereafter termed the high dose (HD) and low dose (LD) groups, whereas the fish in the third group were uncontaminated controls (C). The PCB (dissolved in fish oil) was given as one single dose, administrated orally by force feeding, whereas the controls were given fish oil only.

At four time points from February until the fish had smolted in the beginnig of June, 15 fish from each group were sampled for blood for analyses of plasma osmolality and chloride concentrations, and levels of growth hormone (GH), insulin-like growth factor (IgF), thyroid hormones (T3/T4) and cortisol. At the same time point, fish were subjected to a 72-h seawater (33 ppt) challenge test for determining their hypoosmoregulatory capacity.

At the time when the charr had smoltified, the remaining 92 fish (n = 30, 31 and 31 fish from the C, LD and HD groups, respectively) were transferred to one tank with full-strength seawater (natural temperature, 5-10^{o} C), where they were held for 2 months. During this period they were fed in excess. In the beginning of August, they were again transferred to freshwater where they were held, without being fed, until thefish were fully mature in October. Growth and survival during the seawater period, and the proportion of the fish that matured, were recorded in each treatment.

Results

A strongly improved hypoosmoregulatory capacity was seen in the uncontaminated controls in June as compared to February. In June, plasma osmolality (353.8 ± 8.3 mOsm) and chloride concentrations (164.1 ± 3.9 mmol/L) after 72 h in seawater of control fish were comparable to the levels that has been seen in wild anadromous charr when exposed to seawater at the time of their seawater entry in the beginning of June (Aas-Hansen, unpublished results). This indicates that the control fish used in the present experiment were fully pre-adapted for seawater residence at the time when their wild conspecifics descend to the sea. In contaminated fish there were a dose-related reduction in their

hypoosmoregulatory capacity, with plasma osmolality levels being 366.6 ± 7.1 and 394.3 ± 11.3 mOsm in LD and HD fish, respectively, after the 72 h seawater challenge test in June.

Consistent with the abolishment of their hypoosmoregulatory capacity there was an increase in mortality with increasing PCB level during the 2 month seawater residence, with total moralities being 13, 36 and 47 % in the control, LD and HD groups, respectively. Furthermore, the high dose PCB seemed to cause a reduction in the specific growth rate of the surviving fish during the seawater residence, being 0.60 ± 0.02, 0.61 ± 0.05 and 0.42 ± 0.04 %/day for the C, LD and HD fish, respectively. There was no effect of PCB on the proportion of fish in each group that matured.

Discussion

The present experiment is ecologically realistic by the following reasons: 1) We used wild, anadromous charr with a natural body composition; 2) The fish were contaminated in the autumn and held without food throughout winter, consistent with the "natural" contaminant exposure during the summer feeding excursion to the sea, and subsequent fasting throughout the winter residence in freshwater of free-living anadromous charr; 3) The fish were given Aroclor 1254, which has been shown to have a congener composition similar to that found in wild fish in the Arctic (Gundersen et al., 2000) and 4) we measured both the development of seawater tolerance, and subsequent seawater performance in an experimental set-up that mimicked the anadromous life strategy of the Arctic charr.

The results showed that an ecologically realistic contaminant (Aroclor 1254) effected the smolt development and subsequent seawater performance of the Arctic charr. Taken together, the results indicate that a body burden of PCB comparable to the highest levels that has been detected in Arctic charr in the arctic (i.e. 5 mg PCB/kg body wet weight; Skotvold et al., 1998) have consequences for the seawater performance of anadromous charr. Preliminary data on plasma levels of hormones involved in the smoltification process indicate that the PCB related effects on smolt development and quality, were accompanied by PCB-related differences in plasma hormone levels. Effects on smolt development and seawater performance will be discussed in relation to differences in plasma levels of relevant hormones.

References

Gundersen, D.T., Miller, R., Mischler, A., Elpers, K., Mims, S.D., Millar, J.G., Blazer, V., 2000. Biomarker response and health of polychlorinated biphenyl- and chlordane-contaminated paddlefish from the Ohio River Basin, USA. Environ. Toxicol. Chem. 19, 2275-2285.

Jørgensen, E.H., Johansen, S.J.S., Jobling, M., 1997. Seasonal patterns of growth, lipid deposition and lipid depletion in anadromous Arctic charr. J. Fish. Biol. 51, 312-326.

Jørgensen, E.H., Foshaug, H., Andersson, P., Burkow, I.C. and Jobling, M., 2002. PCB toxicokinetics and P450 1A responses in anadromous Arctic charr during winter emaciation. Environ. Toxicol. Chem. In press.

Skotvold, T., Wartena Elleks, M.M., Schlaback, M., 1998. Persistent organic contaminants in Arctic charr (*Salvelinus alpinus*) on Bear Island. Organohalogen Compounds 39, 411-414.

DIETARY EXPOSURE TO PCB 126 AND BENZO(A)PYRENE [B(A)P] INTERFERES WITH THE STRESS RESPONSE OF TELEOST FISH AT THE PITUITARY LEVEL OF THE HPI AXIS

E. S. Quabius
(Universities of Exeter & Aberdeen) University of Aberdeen, Department of Zoology, Tillydrone Avenue, Aberdeen AB24 2TZ, Scotland, UK,
e.s.quabius@abdn.ac.uk

J. A. Brown (University of Exeter), D. T. Nolan, S. E. Wendelaar Bonga
(University of Nijmegen, The Netherlands)

EXTENDED ABSTRACT ONLY - DO NOT CITE

Polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs) are highly toxic pollutants structurally resembling steroid hormones, interfering with synthesis and action of gonadal and adrenocortical steroids (Safe, 1984), and impairing the stress response in fish (Hontela, 1998). The stress response of fish, co-ordinated via the hypothalamus-pituitary-interrenal (HPI) axis is affected by environmental toxicants (Wendelaar Bonga, 1997), but the level(s) at which this occurs are unclear. To further elucidate the effects that organic environmental pollutants have on the stress response in teleost fish, we examined the effects of dietary benzo(a)pyrene [B(a)P] and PCB126 exposure on the stress response of fresh-water and marine fish and subjected the fish to additional procedures known to activate the HPI axis, in particular net confinement (Wendelaar Bonga, 1997) and ACTH injection (Girard, et al.1998).

Tilapia (*Oreochromis mossambicus*) and rainbow trout (*Oncorhynchus mykiss*) were exposed to PCB126 and turbot (*Scophtalmus maximus*) were exposed to Benzo(a)pyrene [B(a)P], via the diet (50 µg * kg bodyweight^{-1}day^{-1}). After 5 or 7 days, blood samples were taken at-rest, to obtain basal plasma levels of the investigated parameters, or after net-confinement, testing the responsiveness to a superimposed stressor. Plasma was analysed for ACTH (trout and tilapia), cortisol and glucose (all species) and free-fatty-acids (turbot). Basal hormone and metabolic-fuel levels were unaffected by either toxicant. Confinement

resulted in significant increases of all parameters. However, ACTH levels after confinement were significantly higher in PCB fed fish, cortisol responses to confinement were unaffected by either toxicant, and impaired hyperglycaemic responses to confinement were only observed after PCB-exposure. Turbot were also injected with ACTH (to mimic an activation of the HPI-axis providing a test for ACTH-responsiveness). This resulted in similarly increased cortisol and metabolic-fuel levels in B(a)P and control fed turbot.

Hereafter fish were treated differently: trout and tilapia were starved for 3 weeks to allow for mobilisation of stored PCBs (Carlson, 1980), and were then sampled at-rest or after confinement. Turbot were fed B(a)P for another 3 weeks, then sampled at-rest, after confinement or after ACTH-injection. Despite the different treatments during the second part of the experiments, the results obtained showed certain levels of similarity: Both starvation after previous PCB exposure and continuous exposure to B(a)P resulted in lower metabolic-fuel levels in fish exposed to contaminated diets, but basal hormone levels were unaffected by either toxicant. Confinement resulted in elevated hormone levels but these were significantly lower in toxicant-treated than in control fish. Injecting turbot with ACTH, resulted in significant increases of plasma hormone and metabolic fuel levels in both groups, indicating that ACTH-responsiveness was similar in control and B(a)P-fed fish.

Resting plasma hormone levels, indicative of a primary stress response and metabolic fuel levels, indicative of secondary stress responses (Wendelaar Bonga, 1997), were not influenced by the treatment with either toxicant, indicating that the fish were not stressed by the toxicant treatment itself. However, both toxicants impaired the ability of the fish to respond to an additional (non-toxic) stressor in the same fashion as control fish and this is in agreement with data observed in feral fish caught from sites contaminated with various chemicals including PCBs and PAHs compared to fish captured from control sites (Hontela, 1998). The higher confinement-induced increases in plasma ACTH levels in PCB fed trout and tilapia, seen after 1 week of exposure, are thought to be compensatory for depressed corticosteroid responses. Those only become obvious after toxicant exposures of longer than 1 week and are also apparent when fish were starved after the initial exposure period, resulting in lower confinement-induced plasma cortisol levels in exposed fish after both continuous toxicant exposure or starvation after initial exposure. The absence of the above mentioned compensatory mechanisms at the end of the additional starvation period (increasing exposure to PCB) together with the unaltered ACTH sensitivity even after 4 weeks of continuous exposure to B(a)P, suggest

that dietary exposure to organic toxicants impairs the co-ordination of the stress response in teleost fish at the pituitary level of the HPI axis.

References

Safe, S (1984): Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): biochemistry, toxicology and mechanism of action. Crit. Rev. Toxicol. 13, 319-395.

Hontela, A (1998): Interrenal dysfunction in fish from contaminated sites: in vivo and in vitro assessment. Environ. Toxicol. Chem. 17, 44-48.

Wendelaar Bonga, SE (1997): The stress response in fish. Physiol. Rev. 77, 591-625.

Girard,C; Brodeur,JC; Hontela,A (1998): Responsiveness of the interrenal tissue of yellow perch (*Perca flavescens*) from contaminated sites to an ACTH challenge test in vivo. Can. J. Fish. Aquat. Sci. 55, 438-450.

Carlson,GP (1980): Influence of starvation of the induction of xenobiotic metabolism by polychlorinated biphenyls. Life Sci. 27, 1571-1576.

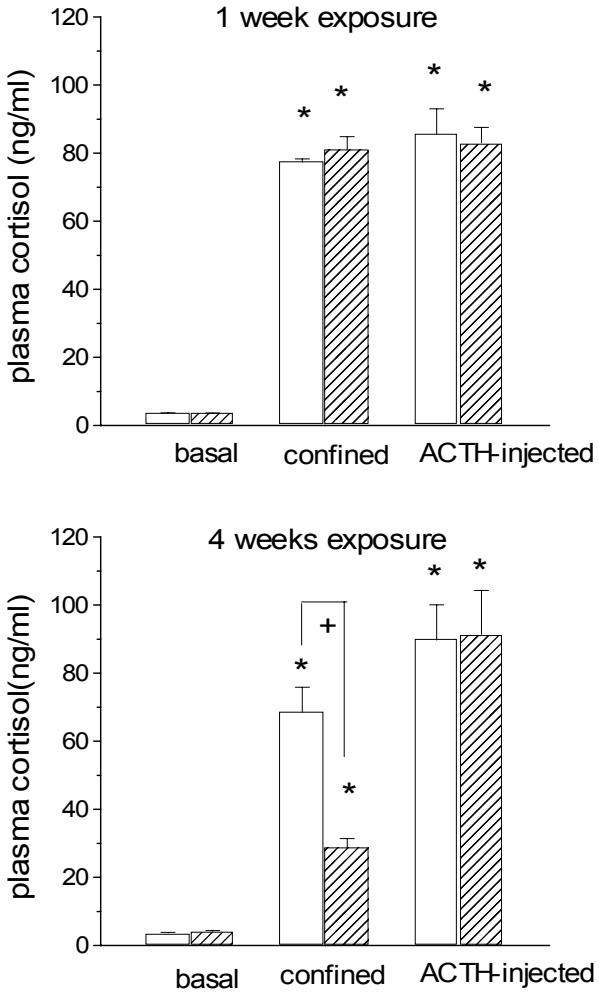

Figure 1: Effect of dietary B(a)P on the primary stress response in turbot. Data for trout and tilapia are published (Quabius et al. 2000) Environmental Toxicology and Chemistry Vol 12 p 2892 - 2899. Open bars represent control fish and hatched bars represent fish receiving the B(a)P containing diet (n=8 +/- SEM). Asterisks indicate significant differences from basal sample, and pluses indicate significant differences due to the B(a)P exposure.

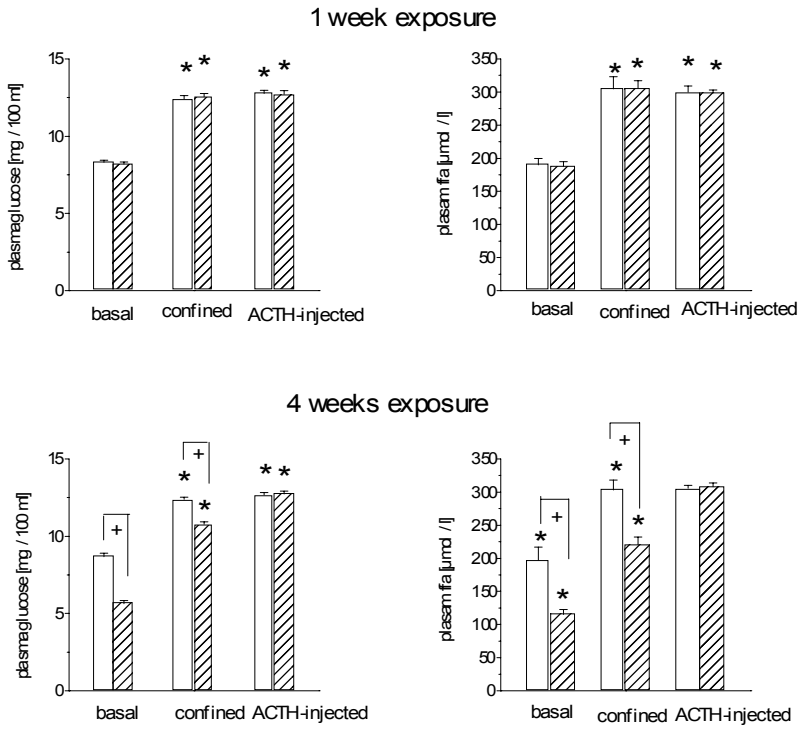

Figure 2: Effect of dietary B(a)P on second7ary stress responses in turbot. Data for trout and tilapia are published (Quabius et al. 2000) Environmental Toxicology and Chemistry Vol 12 p 2892 - 2899. Open bars represent control fish and hatched bars represent fish receiving the B(a)P containing diet (n=8 +/- SEM). Asterisks indicate significant differences from basal sample, and pluses indicate significant differences due to the B(a)P exposure.

DISRUPTION OF THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS IN TELEOST FISH AND AMPHIBIANS : TOXICOLOGY AND COMPARATIVE PHYSIOLOGY MEET

A. Hontela,
Département des Sciences Biologiques, Université du Québec à Montréal,
TOXEN Research Center, Montréal, Québec, Canada,
ph.514-987-3000, xt 6602, FAX 514-987-4647, hontela.alice@uqam.ca;

J. Dorval, B. Goulet, V.S. Leblond,
Département des Sciences Biologiques, Université du Québec à Montréal,
TOXEN Research Center, Montréal, Québec, Canada

EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

There is substantial evidence that some of the chemicals used in industrial processes, pulp and paper production, waste water treatment and agriculture become bioavailable to aquatic species and interfere with their normal endocrine function. Exposures to metals or organic pollutants such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls impair the capacity of fish to secrete cortisol in response to adrenocorticotropic hormone (ACTH) or to a physical confinement (Hontela 1997). To establish mechanism-based links between exposure to environmental pollutants and health effects in wildlife species exposed chronically in the environment, our research group has developed complementary experimental approaches, in the field as well as in the laboratory. Ecotoxicological field studies together with mechanistic toxicological laboratory experiments are used to identify chemicals with the capacity to disrupt the hypothalamo-pituitary-adrenal (HPA) axis, compare the adrenotoxic potential of these chemicals, and elucidate their mechanisms of action (Benguira and Hontela 2000; Benguira et al. 2002; Bisson and Hontela 2002). The objectives of this study were to quantitatively assess the impact of pesticides on adrenal steroidogenesis in fish and amphibians, and to identify the intracellular sites of action.

Materials and methods

Adrenal cells were isolated from rainbow trout, *Oncorhynchus mykiss*, yellow perch, *Perca flavescens*, and two amphibian species, the south african clawed frog, *Xenopus laevis*, and the bullfrog, *Rana catesbeiana*. Cells were incubated *in vitro* in presence of various test chemicals (cadmium and a series of pesticides, edosulfan, diazinon, mancozeb, atrazine, *o,p'*-DDD) and the secretory capacity of the cells was subsequently tested with ACTH, dbcAMP and pregnenolone). The cell viability as well as antioxydant defense capacity (GSH, catalase, lipid peroxydation) were also measured.

Results and discussion

Concentration-dependant effects on cell viability and the secretory capacity of the adrenal cells were observed for all the test pollutants, except for atrazine in *Xenopus* and the trout. The toxicological characteristics of the test chemicals were determined by the LC50 (lethal concentration that kills 50% of the adrenal cells) and EC50 (effective concentration that inhibits cortisol secretion by 50%). Toxicants with a strong capacity to disrupt cortisol secretion without causing cell death (high ratio LC50/EC50) were identified (ex. cadmium) as well as nonspecific toxicants that are higly cytotoxic (ex. diazinon, ration LC50/EC50 about 1). Important differences in sensitivity to the toxicants between rainbow trout, a model teleost (Table 1) and other animal species investigated were also revealed. Disruption of the signalling pathways leading to cortisol as well as an imbalance in the prooxidant-antioxidant balance was observed, in a concentration-dependant pattern, in cells exposed to selected test pesticides. Endosulfan disrupted cortisol synthesis following acute *in vitro* exposures and while catalase activity was elevated at non-cytotoxic doses of endosulfan, activity of GPx was decreased, GST did not change and glutathione (GSH) levels were depleted. An increase in lipid peroxidation (LPO) was also detected. To investigate the effects of pesticides on the signalling pathways, *in vitro* experiments with *o,p'*-DDD and atrazine were completed. Exposure to *o,p'*-DDD induced a dose-dependant loss of the capacity to secrete cortisol. Stimulation with dbcAMP restored cortisol secretion while the actions of forskolin on cortisol secretion and cAMP production were blocked by *o,p'*-DDD but not atrazine. NaF-induced increase in cAMP production was also blocked by *o,p'*-DDD. These results suggest that adenylate cyclase is a target in *o,p'*-DDD mediated disruption of steroidogenesis and that atrazine is not an adrenotoxic chemical in teleost corticosteroidogenic cells. Our results increase the understanding of the mechanisms of action of environmental pollutants

within the HPA axis, and provide a mechanistic link between exposure and physiological effects, both important aspects Environmental Risk Assessment.

References

Benguira S. and A. Hontela. 2000. ACTH- and cAMP-stimulated cortisol secretion in interrenal tissue of rainbow trout (*O. mykiss*) exposed *in vitro* to *o,p'*-DDD, *p,p'*-DDT or *p,p'*-DDD. Env. Toxicol. Chem. 19: 842-847.

Benguira S., Leblond V.S., Weber J.P., and A. Hontela. Loss of capacity to elevate plasma cortisol in rainbow trout (*O. mykiss*) treated with a single injection of *o,p'*-DDD. Env. Toxicol. Chem. 21(8) : 000-000

Bisson M., and A. Hontela. 2002. Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan, and mancozeb in adrenocortical steroidogenic cells of rainbow trout exposed *in vitro*. Toxicol. Appl. Pharmacol. 180 : 110-117.

Hontela A. 1997. Endocrine and physiological responses to xenobiotics in fish : Role of glucocorticosteroid hormones. *Reviews in Toxicology* 1: 1-46. Environmental Toxicology, IOS Press.

Table 1. Adrenotoxicity (LC50, EC50 and LC50/EC50) of chemicals tested in adrenal cells of rainbow trout

Toxicant	Viability LC50 (μM)	ACTH-stimulated cortisol production EC50 (μM)	LC50/EC50
Atrazine	>50000	>50000	—
$CdCl_2$	10800	168	64.29
$ZnCl_2$	22800	355	64.22
Mancozeb	>5000	312	>16.04
Endosulfan	405	38	10.66
CH_3HgCl	1140	116	9.83
$HgCl_2$	199	22.3	8.92
o,p'-DDD	385	130	2.96
Diazinon	305	233	1.31

EFFECT OF SEVERAL NONYLPHENOL PRODUCTS ON *IN VITRO* VITELLOGENIN SYNTHESIS IN TILAPIA HEPATOCYTES

B.-H. Kim
Tropical Biosphere Research Center, University of the Ryukyus, 3422 Sesoko, Motobu, Okinawa 905-0227, Japan; Tel: +81-980-47-6215/Fax: +81-980-47-4919/e-mail: kimvtg@yahoo.co.kr

A. Takemura and M. Nakamura
Tropical Biosphere Research Center, University of the Ryukyus, 3422 Sesoko, Motobu, Okinawa 905-0227, Japan; Tel: +81-980-47-6215/Fax: +81-980-47-4919/e-mail: tilapia@lab.u-ryukyu.ac.jp

EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

Nonylphenol (NP) is a degradation product of a widely-used nonionic surfactant group, alkylphenol phlyethoxylates, and has estrogenic potential (Yadetie et al., 1999). It has been reported that NP treatment induces appearance of a female-specific protein, vitellogenin (VTG) in the blood circulation of certain juvenile or male teleost fishes (Monteverdi and Di Giulio, 1999; Kinnberg et al., 2000). Although it is possible to purchase NP products from several companies for experimental uses, the purity and the mixtures of NP products are different from different companies and even among lots from the same company. Therefore, it is likely that the estrogenic potential of each NP is not constant. The aim of the present study was to compare estrogenic potential of NP from three companies using primary cultures of hepatocytes from tilapia (*Oreochromis mossambicus*). Medium VTG concentration was used as an indicator of estrogenic potential associated with NP.

Materials and Methods

Tilapia (200-300g) were collected using a casting net from rivers and maintained in concrete tanks (2 metric ton capacity) with filtered freshwater and aeration at ambient water temperature at the Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan.

Isolation and primary culture of tilapia hepatocytes were done in accordance with the method of Takemura and Kim (2001). Estradiol-17β (E_2, Sigma, St. Louis, MO), three NP products [(A) Kanto Kagaku, Tokyo Japan; (B) Aldrich, Milwaukee, WI; (C) Wako Pure Chemicals, Tokyo, Japan] and tamoxifen (Sigma) were dissolved in ethanol and added to the culture media after 2 days of pre-culture. Medium VTG was measured by an enzyme-linked immunosorbent assay (Takemura and Kim, 2001).

Results and Discussion

At 3 days after onset of culture without hormone and NP treatments, the hepatocytes conjugated and formed chains, and started to joint together and formed a monolayer. Addition of NP from the companies A/B and C to the medium at 10^{-3} M caused death of cells and delay of cell adhesion, respectively. Death of hepatocytes was not observed in any media to which NP was added at 10^{-4} M. Adhesion of hepatocytes was slower in the media with NP from the companies A and B than the company C. It is considered that high concentration of NP is toxic against the hepatocytes.

Treatments of E_2 at 10^{-7} M and NP at 10^{-4} M from the companies A and B resulted in a significant increase of VTG synthesis, while NP from the company C did not induce VTG synthesis. Treatment of NP from the company B alone induced significant increase of VTG level in the medium of female hepatocytes. However, co-treatment of this NP and tamoxifen reduced VTG synthesis. Tamoxifen is known to be a nonsteroidal antiestrogen and binds strongly to ER. These results show that some NP have estrogenic effect in the primary culture of tilapia hepatocytes and act through ER. A similar effect of NP on VTG induction was reported in primary hepatocyte cultures of channel catfish, *Ictalurus punctatus,* (Monteverdi and Di Giulio, 1999) and rainbow trout, *Oncorhynchus mykiss* (Islinger et al., 2000).

Our results suggest differences in the induction level of VTG among the different NP products. According to a data sheet from the company C, it was ascertained that the NP from the company C had high purity and showed only a single peak by HPLC. Although information on purity of NPs from the companies A and B was not available at present, the estrogenic potential of NP products may perhaps be due to their impurity. Yadetie et al. (1999) induced expression of ER mRNA in the liver of juvenile Atlantic salmon, *Salmo salar*, with 85% pure NP product. In the present study, importance of impurity on estrogenicity could not be assessed, although it may be considered that the estrogenic potential of the NP product is synergistic by

admixtures. Our results suggest that the potential for differences in estrogenic activity may be applicable to other compounds and not just to NP. Consequently, comparison of experimental results from different laboratories may be risky, unless the products used are similar.

References

Islinger M., S. Pawlowski, H. Hollert, A. Volkl and T. Braunbeck. 2000. Measurement of vitellogenin-mRNA expression in primary cultures of rainbow trout hepatocytes in a non-radioactive dot blot/RNAase protection-assay. Sci. Total Environ. 233: 109-122

Kinnberg K., B. Korsgaard, P. Bjerregaard and A. Jespersen. 2000. Effects of nonylphenol and 17β-estradiol on vitellogenin synthesis and testis morphology in male platyfish *Xiphophorus maculatus*. J. Exp. Biol. 203: 171-181

Monteverdi G.H. and R.T. Di Giulio. 1999. An enzyme-linked immunosorbent assay for estrogenicity using primary hepatocyte cultures from the channel catfish (*Ictalurus punctatus*). Arch. Environ. Contam. Toxicol. 37: 62-69

Takemura A. and B.-H. Kim. 2001. Effects of estradiol-17β treatment on *in vivo* and *in vitro* synthesis of two distinct vitellogenins in tilapia. Comp. Biochem. Physiol. 129A: 641-651

Yadetie F., A. Arukwe, A. Goks□yr and A. Male. 1999. Induction of hepatic estrogen receptor in juvenile Atlantic salmon *in vivo* by the environmental estrogen, 4-nonylphenol. Sci. Total Environ. 233: 201-210

Acknowledgements

This study was supported in part by Integrated Research Program for Effects of Endocrine Disruptors on Agriculture, Forestry and Fisheries and Their Action Mechanisms on Domestic Animals and Fishes.

EFFECTS OF ENDOCRINE DISRUPTING COMPOUNDS PRESENT IN FRASER VALLEY AGRICULTRUAL RUNOFF ON EARLY LIFE STAGES OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

J. B. Bruno,
Pacific Environmental Science Center, Environment Canada, 2645 Dollarton Highway, North Vancouver, BC V7H 1B1,
phone 604-924-2518; fax 604-924-2555, email: joy.bruno@ec.gc.ca

G.C. van Aggelen,
Pacific Environmental Science Center, Environment Canada,
North Vancouver, BC;

K.R. von Schalburg,
Department of Biochemistry and Microbiology, University of Victoria,
Victoria, BC;

M.M. Vijayan
Department of Biology, University of Waterloo, Waterloo, ON;

T.P. Mommsen
Department f Biochemistry and Microbiology, University of Victoria,
Victoria, BC

EXTENDED ABSTRACT ONLY - DO NOT CITE

Introduction

Agricultural manure runoff can contain both anthropogenic and natural endocrine disrupting compounds (EDCs). Anthropogenic sources include antibiotics and growth hormones, while natural sources include estrogen hormones and phytoestrogens. Natural sources become problematic when high density livestock or dairy operations such as those found in the Fraser Valley dispose of their manure to land. Given the high rainfall and water table in the Fraser Valley there is a high percentage of runoff generated from the application

of the manure to the fields. At particular risk to the manure runoff is the early life stages of salmonids that are spawned during the high precipitation fall/winter period. In order to determine the effect manure runoff has on the early life stages of fish, laboratory tests with rainbow trout (*Oncorhynchus mykiss*) and agricultural runoff were conducted each fall for three years (1999-2001) and in the winter of the first year.

Materials and Method

Field Procedures. Agricultural plots on Seabird Island established by Agriculture and AgriFood Canada (1995) for Corn trials using Best Management Practices then turned over to grass in 1998 were used in the study. The plots are gently sloped (3-4%) with collection troughs and tanks at the end of each plot. Manure application rates and times coincided with the local agricultural community practices except that guidelines for manuring were complied with. Runoff was collected after each rain event following the final application of manure and transported to the laboratory. For the first two years bovine manure obtained from the University of British Columbia Agricultural Department was used on the plots, the third year hog manure from the local farming community was used.

Laboratory Procedures. Rainbow trout (*Oncorhynchus mykiss*) eggs and milt were obtained from a Department of Fisheries and Oceans (health) certified trout farm. Testing was conducted in accordance with the standardized Environment Canada test method for embryo/alevin/fry (EAF) (Environment Canada, 1998). Negative controls (well/dilution water, field plot runoff and solvent) and positive controls (nonylphenol and estradiol) were included in the study design. During the toxicity tests water quality parameters were measured. At specific time intervals, organisms were removed and preserved for gene expression profiling. Exposure concentrations for the bovine manure rain event diluted runoff were 100%, 50% and 25%. Due to the low dissolved oxygen content of the hog manure runoff (< 0.3 mg/L in 100%) a lower concentration series was required for this manure (25%, 10% and 1%).

Conclusions

Salmonid early life stage bioassays conducted with two out of the three main sources of manure agricultural runoff (cow, hog, chicken) in the Fraser Valley indicate that manure runoff is lethal to rainbow trout, and that hog manure in

particular is extremely toxic to fish. The median effective concentration for nonviable embryos (EC50) for cow manure runoff was 65.4%, 70.7% and 54.2%, for years 1 fall, 1 winter and 2 fall, respectively. The runoff in the 1st year of the fall study with cow manure was most toxic to eggs (56% non-viability in 100% runoff , Figure 1), whereas in the 1st year winter and 2nd year the runoff was most toxic to the alevins. In the winter study all the alevins died within thirty-five days of total exposure to the runoff.

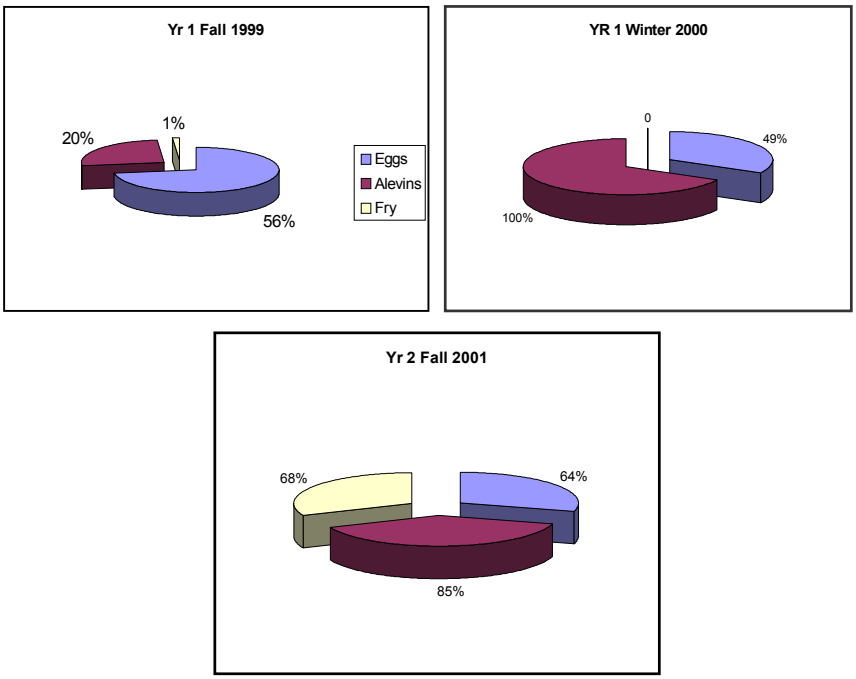

Figure 1. Toxicity of 100% Runoff from cow manured fields on different stages of rainbow trout

Runoff from hog manure was extremely toxic it had an EC50 of 2.7%. Greater than 50% of eggs hatched in the 25% and 10% runoff concentrations however, alevins were dead within a short period of exposure. Toxicity due to sediment contributed roughly the same amount of toxicity as the 50% cow manure runoff concentration,

approximately 36% of the test organisms died due to sediment smothering the eggs and clogging gills. For the hog manure exposures, sediment did not contribute to the toxicity.

Compound	**YR1 Fall 1999 COW**	**YR 1 Winter 2000 COW**	**YR 2 Fall 2000 COW**	**YR 3 Fall 2001 HOG**
Desmosterol	.02	.2	.02	<0.005
Cholesterol	1.0	13.0	.11	.2
Stigmasterol	.4	.4	.1	<0.005
Epicoprostanol	.1	.03	.01	.3
Coprastanol	.2	.03	.01	2.0
Beta-Sitosterol	.7	2.1	.2	.2
Equol	Not available	<0.2	<0.2	.05

Table 1. Concentration of Sterols in Manure Runoff (μg/L)

Table 1 indicates some of the sterols present in manure runoff. These EDC steroids may affect steroid metabolizing systems and alter hormonal balance and disturb normal reproductive function. Desmosterol is the precursor to cholesterol and stigmasterol in plants. Epicoprosterol is the precursor to coprosterol in bacteria, coprosterol is a reduced metabolite of cholesterol produced by bacteria in the intestinal tract of mammals such as hogs. B-sitosterol interferes with the cholesterol production pathway. Equol is an isoflavone found in alfalfa and soy, in animal systems equol inhibits aromatase which normally converts testosterone to estrogen.

The EDC positive controls, estradiol (25-50 ug/L) and nonylphenol (25 ug/L), like the manure runoff decreased hatching success, increased alevin mortalities, and increased non-viability at test end. Physiological effects included estradiol exposed alevins being paler and having poor skin quality, while nonylphenol exposed alevins were darker, smaller and had scoliosis. These spinal deformities enhance predation (Kruzynski and Birtwell, 1994). Dark pigmentation, either

indicating blindness or continuous spreading of melanophores due to chronic stress conditions and / or hormonal imbalance reduces the fish's chance of survival.

Gene analysis of the organisms exposed to estradiol and nonylphenol indicates an increased and accelerated expression of the β-estrogen receptor and vitellogenin compared with the solvent control, implying a loss of the normal regulation of gene control. Exposures with these EDCs indicate they disrupt early development at multiple levels of organization, ultimately to severely compromise the survival of the larvae. This study indicates early life stages of fishes are highly sensitive to agricultural runoff and EDCs which are potentially deleterious, either immediate or delayed.

Acknowledgements

We thank the Tait Bands of Seabird Island and Agriculture and AgriFood Canada for the use of the experimental agricultural plots and Michelle Linssen for excellent technical help. We gratefully acknowledge financial support through the Georgia Basin Environmental Initiative, Natural Sciences and Engineering Research Council and Strategy for Technological Advances in Genomics in the Environment.

References

Environment Canada. 1998. Biological test method: toxicity tests using early life stages of salmonid fish (rainbow trout). Conservation and Protection, Ottawa, ON, Report EPS 1/RM/28 Second Edition.100 p.

Kruzynski, G. M. and I. K. Birtwell. 1994. A predation bioassay to quantify the ecological significance of sublethal responses of juvenile chinook salmon (*Oncorhynchus tshawytscha*) to the antisapstain fungicide TCMTB. Can.J.Fish.Aquat.Sci. 51: 1780-1790

A PROTEOMIC APPROACH IN THE DEVELOPMENT OF BIOMARKERS OF NEOPLASIA IN FISH USING RAINBOW TROUT LIVER AND HEPATOMA DERIVED CELL LINES

L.E.J. Lee
Department of Biology
Wilfrid Laurier University
Waterloo, ON, Canada, N2L 3C5
Tel (519) 884-1970 x 2252, Fax (519) 746-0677
e-mail: llee@wlu.ca

J. E. Mitchell, S. Willfang, M. S. Allen and M. P. Lamb.
Department of Biology, Wilfrid Laurier University, Waterloo, Ontario, Canada.

EXTENDED ABSTRACT ONLY – DO NOT CITE

In humans and several mammalian model systems, tumor markers (biomarkers) have been developed that could facilitate and speed up the detection of tumors with minimal discomfort in the organisms involved. Results from tumor biomarker observations have been applied to develop appropriate treatments as well as to minimize or avoid cancer-causing factors. In fish, despite the recent reports and correlation of increased liver tumors in fish caught in polluted waters, few efforts have been made to develop simple tumor markers and the diagnosis of cancer is only made following death of the organisms and microscopical analysis of affected tissues by expert pathologists. A proteomic approach was initiated to evaluate for changes in protein expression among normal and neoplastic cell lines derived from rainbow trout livers in an attempt to detect biomarkers of transformation. In humans, this approach has led to the development of various tumor biomarkers which have improved cancer screening, diagnosis and treatments. The study of tumors in fish could similarly be enhanced if suitable models and bioindicators became available. Comparison of cellular proteomes using cell lines has facilitated evaluation of changes due to disease or toxicant action. Towards this goal, RTL-W1, a cell line derived from normal trout liver (Lee et al., 1993) and RTH-149, a cell line derived from aflatoxin-induced hepatoma of trout liver (Fryer et al., 1981) (Fig. 1) were evaluated for visible differences in their proteome using 2D gel electrophoresis and computerized image analysis.

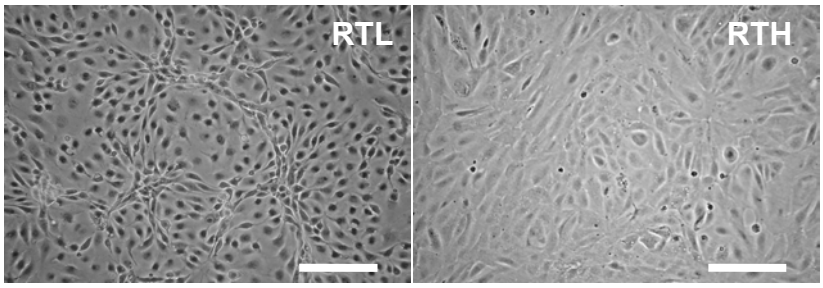

Fig. 1.Phase contrast micrographs of confluent monolayers of trout liver and hepatoma cell lines taken at 100x magnification. Bar= 150 µm.

Crude protein extracts were prepared from whole cell lysates and these were separated by isoelectric focussing followed by molecular weight separation. Over 1000 proteins spots could be discerned in 7cm gels after silver staining for each of the cell lines (Fig. 2).

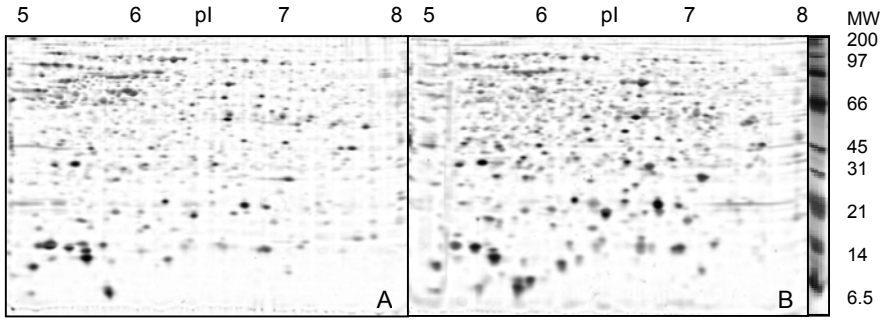

Fig. 2.Comparison of 2D protein profiles (silver stained) of cell extracts from A. RTL-W1, B. RTH-149. First dimension separation was performed with IPG strips linear range of pH 5-8 and second dimension separation was done in 12.5% acrylamide gels

Between 20 to 30% of extracted cellular proteins differed between the two cell lines, whereas, greater than 40% difference was observed between the liver cell lines and trout cell lines derived from tissues other than liver. Among various differing protein spots, a group of proteins in the pI range of 5 to 5.5 and molecular weight of 17kD was noted to differ slightly among the two liver cell lines but that appears to be common to all trout cells. This group of proteins had been postulated to be useful in species identification among differing hake muscle specimens (Pineiro et al., 2001). Nucleoside diphosphate kinase A

(NDKA) is one protein in this group that could be further investigated as this protein has also been shown to be anti-metastatic in rodent cells, and the corresponding spot appear to be reduced in RTH-149. Comparison of protein profiles whose expression is modulated by disease, toxicant exposure or is due to transformation events, could provide insights into the mechanisms of aquatic toxicant action and help develop new biomarkers. The expected significance of this work is far reaching and could have an economic impact in the advancement of the aquaculture and fisheries industry.

References

Lee, L. E. J., J. H. Clemons, D. G. Bechtel, S. J. Caldwell, K. B. Han, M. Pasitschniak-Arts, D. D. Mosser and N. C. Bols (1993) Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. Cell Biol. Toxicol. 9: 279-294

Fryer, L., B. McCain and J. Leong (1981) A cell line derived from Rainbow Trout (*Salmo gairdneri)* Hepatoma. Fish Pathology 15:193-200.

Pineiro, C., J. Vazquez, A. I. Marina, J. Barros-Velazquez and J. M. Gallardo (2001) Characterization and partial sequencing of species-specific sarcoplasmic polypeptides from commercial hake species by mass spectrometry following two-dimensional electrophoresis. Electrophoresis 22: 1545-1552

Acknowledgements

We thank CFI for providing the infrastructure and NSERC for the operating grants.

MOLECULAR CLONING AND ANALYSIS OF A

SALMON SERUM C-TYPE LECTIN

Robert C. Richards[1]
[1]NRC Institute for Marine Biosciences
1411 Oxford St., Halifax NS, B3H 3Z1
Canada

David Hudson[2], Pierre Thibault[3] and K. Vanya Ewart[1]
[2]Department of Biochemistry and Molecular Biology
Dalhousie University, Tupper Medical Building
Halifax NS, B3H 4H7, Canada;
[3]NRC Institute for Biological Sciences
100 Sussex Drive, Ottawa ON, K1A OR6, Canada

EXTENDED ABSTRACT ONLY – DO NOT CITE

Innate immunity involves the direct recognition and destruction of a pathogen without prior exposure. Fish have a relatively variable capacity for antibody-mediated immunity (reviewed elsewhere). Therefore, the role of innate immunity, including humoral events such as complement activation and cellular systems such as phagocytosis by macrophages, is expected to be very important in fish, particularly under conditions in which antibody-mediated immunity might be compromised. Innate immunity hinges on the recognition of non-self cells and this frequently occurs by means of the distinct carbohydrate patterns on their surfaces. In chicken and in several mammals, C-type (Ca^{2+}-dependent) lectins can play this role. For example, the human mannose-binding lectin plays a key role in resistance to common diseases and lectin deficiency is linked to increased susceptibility to infection (Turner and Hamvas, 2000).

To determine whether similar immune-active lectins might be present in fish, mannose-binding proteins were purified from serum of healthy Atlantic salmon (*Salmo salar*) and the most abundant protein was characterised. This multimeric serum lectin was found to bind common salmon pathogens and was shown to opsonise one of these as well (Ewart et al., 1999; Ottinger et al., 1999).

cDNA clones encoding this lectin were sought in order to better study its structure and expression. Internal protein sequence was required in order to

produce oligonucleotide primers for the lectin. Proteolysis followed by mass spectrometry sequencing gave peptide sequences suitable for oligonucleotide design. Since the mammalian and chicken lectins were produced in liver, our initial attempts at cloning the fish lectin focused on that tissue. No clones were isolated. However, using the oligonucleotides, a portion of a gene for the lectin was amplified from genomic DNA using the polymerase chain reaction (PCR). This allowed the design of new PCR primers for lectin expression analysis. Reverse-transcriptase PCR using these primers showed kidney to be the site of synthesis, and not liver. A 641 base pair lectin cDNA was then cloned from kidney and fully sequenced. Screening for this cDNA clone using high-fidelity PCR revealed the presence of several similar cDNAs with minor sequence variations. This implied the presence of a lectin multi-gene family, later confirmed by Southern blotting. The 173-amino-acid sequence of the lectin confirmed its homology with the C-type lectin superfamily. The sequence is most similar to the subfamilies of lectins that bind galactose and related sugars. Furthermore, this lectin lacks the collagen triple helix "stalk" that is characteristic of the immune-active mannose-binding lectins in mammals and birds. In contrast to the galactose-binding lectins, however, the salmon serum lectin contains the Glu-Pro-Asn motif of mannose-binding C-type lectins. Carbohydrate binding-inhibition assays show binding to mannose and related sugars including glucose, N-acetylglucosamine and others. The lectin is secreted into the blood and Western blotting using phage-display antibodies show that it forms a complex multimer in blood plasma, consistent with its opsonizing role.

Studies are underway to determine the mechanism of oligomerisation in this lectin and future work will involve its binding to pathogen and immune cell surface ligands. Further collaborative work is planned to determine whether expression of this protein is responsive to infection in the fish. With this knowledge, it will be feasible to evaluate this protein for use in fish health monitoring and biotechnology for disease prevention.

References

Ewart, K.V., Johnson, S.C. and Ross, N.W. 1999. Identification of a novel pathogen-binding lectin in salmon serum. Comp. Biochem. Physiol. C 123: 53-59.

Ottinger, C.A., Johnson, S.C., Ewart, K.V., Brown L.L., and Ross, N.W. 1999. Enhancement of anti-*Aeromonas salmonicida* activity in Atlantic

salmon (*Salmo salar*) macrophages by a mannose-binding lectin. Comp. Biochem. Physiol. C 123: 9-15.

Turner, M.W. and Hamvas, R.M. 2000. Mannose-binding lectin: structure, function, genetics and disease associations. Rev. Immunogenet. .2: 305-322.

Acknowledgements

This work was supported by NRC IMB and by an NSERC research grant to KVE.

DEVELOPMENT OF A REAL-TIME QUANTITATIVE PCR (TaqMan) ASSAY TO ASSESS THE EFFECTS OF ENDOCRINE DISRUPTING CHEMICALS ON SHEEPSHEAD MINNOW (*Cyprinodon variegatus*) GROWTH

Iris Knoebl
Oregon Cooperative Fisheries Research Unit, Oregon State University/U.S. EPA, Gulf Ecology Division, Sabine Island Drive, Gulf Breeze, FL
850-934-9279 (knoebl.iris@epamail.epa.gov)

Dr. Nancy D. Denslow
Department of Biochemistry and Molecular Biology and Biotechnology Program, University of Florida

Dr. Leah D. Gillis, NRC Postdoctoral Fellow

Dr. Leroy C. Folmar, U.S. EPA, Gulf Ecology Division

EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

The threat of widespread distribution and persistence of endocrine-disrupting contaminants (EDCs) in the environment and the potential for serious effects in human, fish and wildlife populations has warranted a Federal research strategy (National Science and Technology Council report, 1996). Considerable research effort has been given to pollutants that may mimic estrogen (Colborn *et al.*, 1996) little attention has been directed toward other equally important endocrine systems which may also be affected by anthropogenic chemicals.

Increasing evidence also implicates gonadal steroids in somatic growth regulation. Chronic exposures to estrogen have been shown to inhibit somatic growth (Borski *et al.*, 1996; Murphy and Friesen, 1988), while chronic exposure to dihydrotestosterone enhanced growth (Borski *et al.*, 1996) in ovariectomized,

hypophysectomized female rats. Growth regulation by estradiol in rats appears to involve inhibition of growth hormone (GH)-dependent hepatic insulin-like growth factor (IGF)-I gene expression. Estrogens have also been shown to impair growth in several fish species (Bulkley, 1972 ; Donaldson *et al., 1979*) , however possible mechanisms for growth inhibition were not examined.

Some pesticides have also been shown to inhibit growth in fish. For example, chlorpyrifos (Dursban), a commonly used pesticide for mosquito control and garden use, reduced growth in the sheepshead minnow (*Cyprinodon variegatus*) after 28 days of exposure to concentrations as low as < 3.0 ug/L (Cripe *et al.*, 1986). Chlorpyrifos (an organo-phosphate pesticide) inhibits acetylcholinesterase; however the mechanism for growth inhibition in fish has not been established. Chlorpyrifos has been shown to affect hypothalamic GnRH gene expression, cell survival, and neurite outgrowth in vitro. In in vivo experiments, t chlorpyrifoscaused significant alterations in GnRH mRNA levels in female. These findings suggest that chlorpyrifos may act as an EDC (Gore, 2001).

Alterations in somatotropic activity in response to endogenous and xenobiotic chemicals can be evaluated by measuring insulin-like growth factor (IGF) synthesis in response to chemical exposure. Insulin-like growth factors are anabolic peptide hormones, which play a crucial role in growth and development. Hepatic IGF-I synthesis is controlled by growth hormone (GH) and may also be influenced by other hormones (T_3, estradiol). We have developed an assay for to measure IGF-I mRNA in Sheepshead minnow livers by quantitative real-time PCR (TaqMan). TaqMan analysis is a highly sensitive method to measure specific sequences from a small amount of total RNA using a fluorescent probe and specific primer pairs. Measuring induction of IGF-I will allow us to determine whether these measurements have predictive value for growth enhancement or inhibition due to chemical exposure.

We conducted an 18-week aquatic exposure of newly hatched sheepshead minnows (*Cyprinodon variegatus*) to E_2 and chlorpyrifos. The fish were exposed to two measured concentrations of E_2 (21 and 112.5 ng/L) and to three measured concentrations (6.5, 13.3 and 24.3 ug/L) of chlorpyrifos. Fish exposed to the highest dose of E_2 grew larger than controls only during the last week of the experiment. Fish exposed to the lower dose of E_2 were not significantly different from controls. The fish exposed to all doses of chloryprifos had significantly reduced growth in a dose-dependent manner compared to controls. Hepatic IGF-I mRNA levels were measured using TaqMan technology, but no

significant differences were found in hepatic IGF-I mRNA levels in any treatments. These results were in contrast to other research done in our lab in which fish were injected with 5 ug/g body weight E_2 had significantly reduced hepatic IGF-I mRNA up to 12 h after injection (data not shown).

In conclusion, aquatic exposure to environmentally relevant concentrations of E_2 did not affect hepatic IGF-I mRNA levels in the SHM. Aquatic exposure to chlorpyrifos inhibited growth in a dose-dependent manner, but the mechanism of action is still unclear. Hepatic IGF-I mRNA levels in chlorpyrifos treated fish were no different than control fish. The TaqMan method is a useful tool to screen for physiological effects of both anthropogenic chemicals and endogenous hormones on somatic growth.

References

Borski, R. J., W. Tsai, et al. (1996). Regulation of somatic growth and the somatotropic axis by gonadal steroids: Primary effect on insulin-like growth factor I gene expression and secretion. Endocrinology 137: 3253-3259.

Bulkley, R. V. (1972). Diethylstilbestrol in catfish feed. Transactions of the American Fisheries Society 101(3): 537-539.

Colborn T. and C. C. (1992). Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection. Princeton, NJ, Princeton Scientific Publishing Co.

Cripe, G. M., D. J. Hansen, et al. (1986). Effects of diet quantity on sheepshead minnows (*Cyprinodon variegatus*) during early life-stage exposures to chlorpyrifos. Aquatic Toxicology and Environmental Fate: ASTM STP 921. T. M. Poston and R. Purdy. Philadelphia, American Society for Testing and Materials. 9: 450-460.

Donaldson, E. M., U. H. M. Fagerlund, et al. (1979). Hormonal enhancement of growth. Fish Physiology - Bioenergetics and Growth**.** W. S. Hoar, D. J. Randall and J. R. Brett. New York, NY, Academic Press, Inc. VIII: 455-597.

Gore, A.C. (2001). Environmental toxicant effects on neuroendocrine function. Endocrine. 14(2):235-46.

Murphy, L. J. and H. G. Friesen (1988). Differential effects of estrogen and growth hormone on uterine and hepatic insulin-like growth factor I gene expression in the ovariectomized hypohysectomized rat. Endocrinology 122: 325- 332.

Ostrowski, A. C. and J. Garling, D.L. (1986). Dietary androgen-estrogen combinations in growth promotion in fingerling rainbow trout. The Progressive Fish-Culturist 48: 268-272.