GREAT LAKES LAKE TROUT EARLY MORTALITY SYNDROME (EMS): CONTAMINANTS, THIAMIN STATUS AND THEIR POSSIBLE INTERACTION

A Dissertation presented to the Faculty of the Graduate School University of Missouri-Columbia

In Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy

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MAY 2006
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GREAT LAKES LAKE TROUT EARLY MORTALITY SYNDROME (EMS): CONTAMINANTS, THIAMIN STATUS, AND THEIR POSSIBLE INTERACTION

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A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worthy of acceptance.

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Interactions between thiamin and dioxin-like compounds in fish early life stages: An Introduction

Salmonid populations in the Great Lakes experienced a decline in the early twentieth century, presumably due to overexploitation by commercial fishermen combined with the introduction of exotic parasites such as the sea lamprey (*Petromyzon marinus*). In the 1960s intensive rehabilitation and stocking programs were implemented, and populations once again became abundant. Beginning in the early 1970s, however, reproductive problems, in some cases involving total recruitment failure, appeared in salmonid populations of the Great Lakes. Sexually mature adults appear capable of reproducing, yet their young do not survive. The syndrome affecting the salmonid offspring has had various names, including "blue-sac", "drop-out", and recently "early mortality syndrome". Several hypotheses regarding the etiology of early mortality syndrome (EMS) in the Great Lakes salmonid populations have been proposed, including thiamin deficiency (Fitzsimons 1995), lack of suitable spawning grounds (Manny et al. 1995), bacterial or viral disease, poor stock quality and adverse effects of persistent, hydrophobic contaminants such as PCDDs, PCDFs, and PCBs, collectively termed the planar halogenated aromatic hydrocarbons (PHHs) (Wilson and Tillitt 1996; Willford et al. 1981; Mac 1988; Walker and Peterson 1990). A syndrome similar to EMS is seen in certain species (i.e. herring *Clupea harengus*, cod *Gadus morhua*, Atlantic salmon *Salmo salar*) in the Baltic Sea (Jensen et al. 1972; Olsson et al. 1972; Tarhanen et al. 1989; Westernhagen et al. 1988), and in Atlantic salmon in New York’s Finger Lakes (Fisher et al. 1995a; Fisher et al. 1995b; Fisher et al. 1998).

Current research suggests that EMS in Great Lakes salmonid populations may be related to nutrient deficiencies in diet of adult females (Fitzsimons 1999; Brown et al. 1998). This hypothesis suggests that changes in food web structure have caused an increase in prey containing thiaminase (i.e., alewife, rainbow smelt) which inactivates thiamin, resulting in thiamin deficiency. The clinical signs of EMS include several behavioral and physical alterations including abnormal swimming, anorexia, lethargy, skeletal malformations and hemorrhaging followed soon after by death. Low thiamin levels in lake trout egg are highly correlated with EMS.
mortality, and fry affected with EMS have been given thiamin to reduce symptoms (Fitzsimons 1995c; Fitzsimons et al. 2001). It appears likely that this nutritional deficiency is a major contributor to the lack of recruitment in Great Lakes salmonid populations such as lake trout.

Research on the mechanism for this phenomenon is still in its early stages. Although strong evidence supports the theory that EMS is attributable to thiamin deficiency in eggs and fry, several inconsistencies make it difficult to accept thiamin deficiency as the sole cause of EMS. For example, EMS accounted for less than 5% of Lake Ontario lake trout mortality in the early 1980s, although thiaminase-containing alewife (Alosa pseudoharengus) comprised the majority of their diet (Symula et al. 1990). Also, the relative abundance of alewife in the Lake Michigan prey base over the last thirty years does not necessarily coincide with trends in EMS (Wells 1985). This information indicates that other factors are likely involved in Great Lakes EMS. Finally, although EMS mortality is highly correlated with low thiamin in eggs, there is a great degree of variability in this relationship. Egg batches with low thiamin can survive with very little evidence of EMS; also, thiamin levels in eggs from feral females widely vary, indicating that at least some offspring could potentially survive past the critical first-feeding stage when EMS typically strikes. If spawned eggs eventually hatch and the fry survive through swim-up, then the question of what causes mortality in these individuals remains unresolved.

The information regarding the impact of environmental contaminants such as PHHs on Great Lakes salmonid populations is likewise controversial. PHHs are a class of highly toxic environmental contaminants which are often found in complex mixtures (Safe 1990). Some characteristics which make PHHs environmentally significant are their lipophilic nature and bioaccumulation potential, relative environmental stability, and their toxicity to aquatic organisms. PHHs are known to be toxic to fish early life stages (Helder 1980; Helder 1981; Walker et al. 1992; Guiney 1996). It has been suggested that the mechanism for the adverse effects due to these contaminants involves bioaccumulation of the contaminants in sexually mature adult females, and the direct deposition of these lipophilic contaminants into the eggs prior to spawning (Guiney et al. 1997). Fitzsimons (1995a) reviewed the literature regarding the affects of contaminants on early life stage mortality of lake trout in the Great Lakes and concluded that
hatching mortality, blue-sac, and swim-up syndrome mortality were probably not the sole result of contaminant etiology because concentrations have been too low since the 1970s to fully account for the observed increases in these effects. Furthermore, in Lake Ontario lake trout fry there was no significant relationship between EMS mortality and measures of organic chemicals in tissues (Fitzsimons et al. 1995b). Other studies have reached similar conclusions (Williams and Giesy 1992). However, these studies attempted to correlate mortality with contaminant concentrations. Recent evidence suggests that sublethal contaminant effects such as gross lesions (i.e. mild edema, hemorrhage) and behavioral effects may be better indicators of contaminant impacts (Wilson and Tillitt 1996; Fisher et al. 1994; Carvalho et al. 2004). Although contaminant concentrations may not be significantly correlated with mortality in the laboratory, correlations with sublethal effects which may have adverse effects on growth and survival must be further investigated. Although current levels of dioxin-like compounds are likely not great enough to explain the reproductive disorders in Great Lakes salmonids (Cook et al. 2003; Wright and Tillitt 1999), it is possible that current concentrations of dioxin-like compounds may be contributing to fry mortality via sublethal adverse effects.

The most likely scenario for the EMS etiology is that a variety of factors including nutrition, contaminant exposure, spawning conditions, egg quality, and physical changes in the rearing environment combine to produce salmonid early life stage mortality. Thiamin is apparently a major factor, but the extent that the other stressors contribute to EMS has not been demonstrated. Investigations into the role of oxidative stress in TCDD-toxicity (Cantrell et al. 1996), and the potential role of thiamin in pathways in which oxidative stress may occur raise the possibility that the low levels of dioxin-like compounds in certain Great Lakes lake trout populations may play a role in thiamin-related EMS mortality.

Model for the Interaction of Dioxin-like Compounds and Thiamin

Adult female salmonids in the Great Lakes accumulate dioxin-like compounds via exposure to contaminated prey, water and sediments. Prior to spawning, these lipophilic contaminants are directly deposited into the eggs. Concurrently, adult salmonids may be feeding
on a diet which is either thiamin deficient, or contains high quantities of thiaminase-containing prey. Although the degree of thiamin deficiency is not necessarily great enough to directly affect the overall health of adult fish, females may be spawning eggs which are deficient in thiamin. Consequently, Great Lakes salmonid eggs may start out with two major hindrances to healthy growth: some concentration of dioxin-like compounds and low thiamin levels.

In the wild, either of these two major stressors acting alone may not have a significant adverse effect on egg and fry health, but in combination the impact may be much greater (i.e., synergistic). In addition to these factors, poor female health, inadequate spawning substrate, and changes in the physical environment may affect the eggs. Even very low concentrations of dioxin-like compounds can produce sublethal pathological and behavioral lesions in exposed salmonid fry. These adverse effects may affect the ability of fry to survive in the wild. Thiamin deficiency likewise has been shown to produce adverse behavioral effects. The overall impact of both of these stressors in combination could be sufficient to produce both overt mortality and a decrease in egg and fry health.

The goal of this research is to investigate the relationship between dioxin-like contaminants and thiamin levels in the early life stages of lake trout in order to further the current knowledge about EMS and lake trout reproductive dysfunction. With this in mind, several objectives were used to guide the research, including 1) to characterize the effects of a complex chemical extract of Lake Michigan lake trout on developing lake trout embryos in terms of TCDD-equivalents in the lake trout tissue in order to establish the presence or absence of lethal and/or sublethal adverse effects in embryos at environmentally relevant PHH concentrations, and 2) to characterization of the interaction between contaminants and nutrition in fish early life stages using dioxin-like compounds and thiamin as the contaminant and nutritional elements, and 3) to identify the effect of dioxin-like compounds/thiamin interactions on sublethal behavioral endpoints.
A series of seven chapters documents the outcome of the overall investigation:

**Chapter 1.** “The Embryotoxicity of a Complex Chemical Extract of Lake Michigan Lake Trout, 2,3,7,8-TCDD, or 2,3,7,8-TCDF to Developing Lake Trout” describes the sublethal effects of environmentally relevant concentrations of PHHs in Great Lakes lake trout fry.

**Chapter 2.** “Thiamin Supplementation Ameliorates 2,3,7,8-TCDD Toxicity in Early Life Stage Rainbow Trout and Japanese Medaka” describes the relationship between TCDD toxicity and thiamin supplementation in two species of fish.

**Chapter 3.** “Comparison of Prehatch C-Start Responses in Rainbow Trout and Lake Trout Embryos Using a Tactile Stimulus Test” describes a new assay for testing c-start responses in rainbow trout and lake trout embryos, and establishes a basis for comparison for future tests using this assay.

**Chapter 4.** “2,3,7,8-TCDD Exposure Decreases Embryo C-start Response in Rainbow Trout and Lake Trout” describes the effects of sublethal doses of TCDD on a key behavior in fish embryos.

**Chapter 5.** “Feeding Efficiency in Young Lake Trout and Rainbow Trout Injected as Eggs with 2,3,7,8-TCDD” describes the effects of TCDD on early feeding behaviors in lake trout and rainbow trout.

**Chapter 6.** “Thiamin and 2,3,7,8-TCDD Interact to Cause Sublethal Adverse Behavioral Effects in Rainbow Trout and Lake Trout Embryos and Fry” describes the relationship between thiamin and possible contaminant exposure in hatchery-reared and feral lake trout embryos and fry.

**Chapter 7.** “Can Lake Trout Reproduction in the Lower Great Lakes Ever Become Self-sustaining Again?” describes the overall conclusions from the entire body of this research.
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Walker, M.K. and R.E. Peterson (1990) Potencies of polychlorinated dibenzop-dioxin, dibenzofuran, and biphenyl congeners, relative to 2,3,7,8-tetrachlorodibenzo-p-dioxin, for producing early life stage mortality in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 21, 219-238.


Chapter 1: The Embryotoxicity of a Complex Chemical Extract of Lake Michigan Lake Trout, 2,3,7,8-TCDD, or 2,3,7,8-TCDF to Developing Lake Trout

Abstract

Planar halogenated hydrocarbons (PHHs) are known to be present in the Great Lakes ecosystem in sufficient concentrations to potentially cause adverse effects on reproduction in certain species of fish. Previously, we demonstrated the toxicity of a PHH mixture to newly fertilized rainbow trout eggs. The current study investigates the embryotoxicity of the same mixture on developing lake trout embryos. This study had four objectives: 1) to determine the accuracy of predictions about lake trout egg 2,3,7,8-TCDD equivalent concentrations (TEQ) made from the previous studies on rainbow trout; 2) to determine the relative potency (REP) of 2,3,7,8-TCDF in early life stage lake trout; 3) to examine the chemical composition of the Lake Michigan lake trout extract in relation to an additive model of toxicity; and 4) to estimate the hazard these chemicals may represent to Great Lakes lake trout populations. The extract was made from whole adult lake trout collected from Lake Michigan in 1988. Graded doses of the final extract were injected into eggs of hatchery reared lake trout. The doses used for the injections were quantified as TEQs based on the concentrations of dioxins, furans and non-ortho-PCBs in the extract, and as equivalent amounts found in the eggs of the original lake trout (eggEQ). The LD50 of 2,3,7,8-TCDD in lake trout was 81 pg/g egg. Additionally, we determined the LD50 for 2,3,7,8-TCDF in lake trout to be 2.5 ng/g egg. Total TEQs in the Lake Michigan lake trout sample were 14.7 pg TEQ/g. The extract of the Lake Michigan lake trout was embryotoxic to lake trout embryos with an LD50 value of 7 eggEQ (4-11, 95% F.L.). The LD50 of the extract in terms of TEQs was 103 TEQs/g of lake trout egg. The estimated lowest observable adverse effect levels (LOAEL) for sublethal responses were: 29 pg TEQ/g for craniofacial anomalies, 15 pg TEQ/g for yolksac edema, and 2 pg TEQ/g for hemorrhage in the lake trout embryos. The mixture of chemicals present in lake trout from Lake Michigan acted to cause embryotoxicity in an approximately additive fashion. The composition of the extract was portioned equally on a TEQ basis among non-ortho-substituted PCBs, dioxins, and furans. This study confirms our earlier conclusions that PHHs in lake trout from Lake Michigan are above a threshold for adverse effects on mortality, but that these compounds may have implications for the lack of recruitment in certain Great Lakes lake trout populations.

Introduction

Lake trout (Salvelinus namaycush) were historically native to the lower Great Lakes. However, the lake trout fishery in the Great Lakes is currently hatchery-stocked: no natural reproduction occurs in Lake Michigan and few signs of natural reproduction are seen in northern Lake Huron and Lake Ontario (Marsden et al. 1988; Holey et al. 1995; Hansen et al. 1995; Manny et al. 1995). The exact cause of the lack of recruitment observed in Great Lakes lake trout populations is not known at this time; however, it is known that lake trout are particularly sensitive to the effects of planar halogenated hydrocarbons (Walker et al. 1991; Walker et al. 1994).
The chemical mixture that exists in Lake Michigan lake trout is embryotoxic to salmonids (Wright and Tillitt 1999). Graded doses of the extract from Lake Michigan lake trout injected into newly fertilized eggs of rainbow trout (Oncorhynchus mykiss) caused dose-related mortality in the embryos. The symptoms of toxicity in the developing rainbow trout embryos, (including yolksac edema, hemorrhage, and craniofacial deformities) also increased with dose of the extract. These symptoms, in addition to other gross pathological lesions (i.e., pericardial edema, altered cardiovascular function, and arrested development), are characteristic of 2,3,7,8-TCDD toxicity in early life stage salmonids (Walker et al. 1994; Zabel et al. 1995b; Hornung et al. 1999). The embryotoxicity and gross pathologies of different PHH compounds are similar across salmonid species, and suggest a common mechanism of action mediated by the aryl hydrocarbon receptor (AhR) and the induction of cytochrome P4501As (CYP1As) (Peterson 1993; Hornung et al 1996; Zabel et al. 1995b). In early life stage lake trout, CYP1A induction in the vasculature endothelium may be associated with changes in the vascular system, leading to the characteristic dioxin-like symptoms of yolksac, pericardial, and meningial edema (Guiney et al. 1997).

The mixture of PHHs in Lake Michigan lake trout tissue followed an additive model of toxicity with respect to rainbow trout early life stage mortality (Wright and Tillitt 1999). 2,3,7,8-TCDD equivalent concentrations (TEQs) derived from relative potency values (REPs) and toxic equivalency factors (TEFs) of the various PHH congener were used to make predictions about the potential for PHHs to cause effects in lake trout, based on the relative sensitivity differences between rainbow trout and lake trout (Walker and Peterson 1990). The predictions suggested that the existing concentrations of PHHs in Lake Michigan lake trout are below the concentration necessary to cause overt mortality. Lake trout eggs from several locations in Lake Ontario and Lake Superior do, in fact, have total TEQs below the 2,3,7,8-TCDD no observable adverse effect level (NOAEL) for sac fry mortality (Guiney et al. 1996). However, the TEQs in lake trout eggs may be above the threshold for the sublethal effects of yolksac edema and hemorrhaging (Wright and Tillitt 1999).

Given this background, the present study addresses four objectives: 1) to determine the accuracy of predictions about lake trout egg TEQs made from the previous studies of rainbow.
trout; 2) to determine the REP for 2,3,7,8-tetrachlorodibenzo-furan (2,3,7,8-TCDF) in early life stage lake trout; 3) to examine the chemical composition of the Lake Michigan lake trout extract in relation to an additive model of toxicity; and 4) to estimate the hazard these chemicals may represent to Great Lakes lake trout populations.

**Experimental Methods**

*Extraction and Clean-up of lake trout tissue*

Lake trout collected in 1988 from Lake Michigan near Sheboygan, WI, served as the source of the complex environmental mixture used for the extraction. The extraction and cleanup procedures of the lake trout were described previously (Wright and Tillitt 1999) and followed the methods of Meadows et al. (1993). Briefly, the lake trout tissue (whole fish) was ground and mixed with 4X Na₂SO₄ (sodium sulfate) and extracted in large, 4-cm i.d. glass extraction columns with methylene chloride (CH₂Cl₂). The extracted lipid was dialyzed against 80:20 hexane:CH₂Cl₂ utilizing polyethylene membranes. The sample was then subjected to a twostep reactive/destructive column cleanup on sulfuric acid-impregnated silica gel and potassium silicate. The final sample was then subjected to high performance gel permeation chromatography (HP/GPC). Dosing solutions were prepared by evaporation of the methylene chloride from the extract, and dissolution of the chemical residues into triolein as a carrier solvent for dosing. Triolein is the major neutral lipid of fish and serves as a useful carrier solvent for injections because doses do not adversely affect growth, development, or mortality of the eggs and fry (Walker et al. 1996; Wright and Tillitt 1999).

**Analytical Methods**

Triplicate aliquots (25 g) of the Lake Michigan lake trout sample were prepared for analysis according to the methods of Feltz et al. (1995) and analyzed as in Peterman et al. (1996). Each sample was spiked with 5 ng of ¹³C-labeled non-o-PCBs (#77, 126, and 169) and 50-500 pg of ¹³C-labeled PCDDs or PCDFs and column-extracted with CH₂Cl₂. All extracts were then treated by a two-stage reactive cleanup and were further purified using HP/GPC. PCDDs, PCDFs, and PCBs were separated on PX-21 activated carbon dispersed on C₁₈ HPLC packing material and analyzed as previously described (Feltz et al. 1995). The analytes were then
separated by HPLC, isolating four fractions: fraction 1, bulk and di-ortho-PCB congeners; fraction 2, mono-ortho-PCB congeners; fraction 3, non-ortho-PCB congeners; and fraction 4, PCDDs/PCDFs. Mono-ortho-PCB congeners were determined by GC/ECD, while non-o-PCBs, PCDDs and PCDFs were determined by gas chromatography/high resolution mass spectrometry (GC/HRMS) (Peterman et al. 1996). Dioxin toxic-equivalents (TEQs) in the samples were calculated with the relative potency factors (REPs) developed for early life stage mortality in rainbow trout where available, or with TEFs from the PLHC-1 or H4IIE bioassays (Safe 1990; Walker and Peterson 1990; Zabel et al. 1995; Tillitt and Cantrell 1992; Tillitt unpublished).

Injection of Lake trout eggs

Lake trout eggs, combined from 3 females, were air shipped at ~4°C from the Iron River National Fish Hatchery and arrived fertilized (pooled milt from 3 males). Eggs were slowly warmed to within one degree of the incubator water temperature (10°C ± 1) before placement in pre-formed agarose plates in preparation for injection. The injection volume delivered to each egg (~50 nl, or 0.1% of egg volume) was quantified by measuring the size of a triolein droplet which forms at the tip of the needle during injection. Injections were conducted with glass micropipettes, a regulated gas pressure system, and a digital control device (Walker et al. 1996).

Two separate dose-response experiments were conducted with the Lake Michigan lake trout extract, with the number of eggs injected per dose averaging 50 and 75 in the two trials respectively. Doses, measured as egg-equivalents (eggEQ), were based on gram tissue/gram egg values normalized to the lipid content of lake trout eggs. It is assumed that lipophilic compounds such as PHHs will be concentrated in the lipid material of an organism. The lipid correction factor is used because the tissue of whole adult lake trout is comprised of ~ 15% lipid, whereas lake trout egg tissue contains approximately 3% lipid (Herbert and Keenleyside 1995; Mac et al. 1985). The doses used were control (triolein), 0.02, 0.10, 0.20, 1.0, 2.0, 4.0, 10.0, and 20.0 eggEQ; or in terms of TEQs, the doses were 0.3, 1.5, 3, 15, 29, 59, 147, and 294 pg TEQ/g wet weight.

In addition, two dose-response experiments were conducted with 2,3,7,8-TCDD (0, 10, 20, 40, 60, 80, and 100 pg/g), with from 50 to 58 eggs per dose being injected in the first
experiment and from 33 to 38 eggs per dose being injected in the second experiment. A single
dose-response experiment with 54 eggs per dose was conducted using 2,3,7,8-TCDF doses of 0,
0.1, 0.4, 1, 2, 4, and 8 ng/g.

All eggs were reared in a vertical-flow trout incubator with water temperature of 10°C ± 1.
Mortality was monitored daily, and in the Lake Michigan extract experiments the occurrence of
three gross pathological lesions (hemorrhage, yolk sac edema, and craniofacial deformities) was
quantified for all hatched fry at 900 degree days.

Statistical Analysis

Our mortality data and gross pathological lesions were analyzed by probit analysis,
corrected for control responses (SAS 1988). Probit models were tested for goodness-of-fit (p
values <0.05). The no observable adverse effect level (NOAEL) and the lowest observable
adverse effect level (LOAEL) for the mortality data and pathological lesion data were analyzed
using Dunnett's test (p < 0.05) to compare the treatment and control groups (SAS 1988).

Results

Contaminant Exposure Assessment

The concentrations of PCDDs, PCDFs, and non-o-PCBs were determined in the Lake
Michigan lake trout tissue (Table 1). The concentration of 1,2,3,7,8-PCDD was the greatest of
the PCDDs (except for OCDD) and had the greatest contribution to the 2,3,7,8-TCDD-equivalents
(TEQs). The PCDF congener 2,3,7,8-TCDF had the greatest concentration, while 2,3,4,7,8-
PeCDF had the greatest contribution to the TEQs among PCDF congeners. The concentrations
of the planar PCB congeners were 59-2600 pg/g. PCB congener 3,3',4,4'-TCB (#77) had the
greatest concentration of the non-ortho-substituted PCBs (2600 pg/g), while 3,3',4,4',5-PCB
(#126) resulted in the greatest TEQ contribution among any of the measured PHHs.
Concentrations of mono-o-chlorosubstituted PCB congeners were approximately 5-75 ng/g in the
tissue of the lake trout used for extraction. Total TEQs based on the concentrations of dioxins,
furans and non-o-chlorosubstituted PCBs in the lake trout sample were 14.7 pg TEQ/g (Table 1).
Table 1. Total TCDD-equivalent concentrations (TEQs) of PCDDs, PCDFs, and Non-ortho-PCBs in Lake Michigan lake trout tissue, calculated from [pg/g, wet weight] in tissues and relative potency factors (REPs). Lake trout were collected from Lake Michigan near Sheboygan, WI in 1988.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>REP</th>
<th>[pg/g] in Tissue</th>
<th>TEQ</th>
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<tr>
<td><strong>Dioxins</strong></td>
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<td></td>
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<tr>
<td><strong>Non-ortho-PCBs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># 81</td>
<td>0.00056&lt;sup&gt;b&lt;/sup&gt;</td>
<td>291</td>
<td>0.16</td>
</tr>
<tr>
<td># 77</td>
<td>0.00016&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2,600</td>
<td>0.4</td>
</tr>
<tr>
<td># 126</td>
<td>0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>883</td>
<td>4.4</td>
</tr>
<tr>
<td># 169</td>
<td>0.000041&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.3</td>
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</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td></td>
<td></td>
<td>5.0</td>
</tr>
<tr>
<td><strong>Total TEQ</strong></td>
<td></td>
<td></td>
<td>14.7</td>
</tr>
</tbody>
</table>

The relative contribution of PCDDs, PCDFs, and non-o-chlorosubstituted PCBs were nearly equivalent, each of these classes contributed approximately 5 pg TEQ/g of lake trout.

*Embryotoxicity of 2,3,7,8-TCDD and Lake Michigan lake trout extract*

2,3,7,8-TCDD was lethal to lake trout embryos, with an LD50 of 81 pg/g (76-101, 95% C.L.) with a NOAEL of 20 pg/g and a lowest observable adverse effect level (LOAEL) of 40 pg/g based on mortality (Figure 1). Characteristic dioxin-like symptoms such as yolksac edema and hemorrhage were present in the lake trout eggs and fry injected with 2,3,7,8-TCDD. The extract of the Lake Michigan lake trout was embryotoxic to lake trout, based on elevated mortality from fertilization through swim-up, with an LD50 value based on the probit model of 7 eggEQ (4-11, 95% C.L.) (Figure 2). Mortality increased in a dose-dependent fashion and was significantly elevated over sham-injected controls at a dose of 2 eggEQ. Gross lesions characteristic of exposure to PHHs were present, and also increased in a dose-related manner in the exposed embryos (Figure 3).

The effective dose in which 50% of the organisms exhibited hemorrhaging (ED50), was 0.4 eggEQ and the LOAEL was 0.1 eggEQ (Table 2). Subcutaneous edema of the yolksac increased in a dose-related manner with an ED50 of 3.0 eggEQ and a LOAEL of 1.0 eggEQ (Table 2). Craniofacial deformities, including domed skull, foreshortened maxillae, and deformed jaw structures, also increased in a dose-dependent manner, but were less sensitive biological markers of toxicity as compared to hemorrhaging or yolksac edema. The ED50 and LOAEL estimates for craniofacial deformities were 7.4 eggEQ and 2 eggEQ, respectively (Table 2).

*Early life stage toxicity of 2,3,7,8-TCDF in lake trout*

The toxicity of 2,3,7,8-TCDF was observed during the hatching and sac-fry stages of the lake trout (Table 3), and was manifested in a similar manner to that of 2,3,7,8-TCDD. The LD50 for 2,3,7,8-TCDF in lake trout was 2.5 ng/g (1.8-3.1, 95% F.L.), with mortality following a typical dose-response relationship (Figure 4). The relative potency (REP) for 2,3,7,8-TCDF, calculated as \( \frac{LD50_{TCDD}}{LD50_{TCDF}} \), was 0.032.
Figure 1. 2,3,7,8-TCDD dose-response in lake trout sac fry injected as eggs with TCDD doses of 0, 10, 20, 40, 60, 80, and 100 pg/g. Mean and standard deviations are shown from two dose-response experiments with from 33 to 58 eggs per dose. “*” indicates LOAEL (p < 0.05).
Figure 2. Lake Michigan lake trout extract-induced mortality in lake trout early life stages. The lake trout extract injected into newly fertilized lake trout eggs is from Lake Michigan lake trout collected near Sheboygan, WI in 1988. Dashed and solid lines represent the probit model, and 95% confidence limits, respectively. Data points represent actual mortality percent ages from treatment groups from two dose-response experiments, with the number of eggs injected per dose averaging 50 and 75 in the two trials, respectively. The doses used were control (triolein), 0.02, 0.10, 0.20, 1.0, 2.0, 4.0, 10.0, and 20.0 eggEQ.
Figure 3. Incidence of gross lesions at 900 degree days, based on percent occurring in surviving fry, in lake trout injected as eggs with a Lake Michigan lake trout extract. The lake trout extract injected into newly fertilized lake trout eggs is from Lake Michigan lake trout collected near Sheboygan, WI in 1988. Means and standard deviations are shown from two dose-response experiments, with the number of eggs injected per dose averaging 50 and 75 in the two trials, respectively. The doses used were control (triolein), 0.02, 0.10, 0.20, 1.0, 2.0, 4.0, 10.0, and 20.0 eggEQ. "***" indicates LOAEL (p = 0.05).
Table 3. 2,3,7,8-TCDF-induced mortality in three lake trout early life stages (egg, hatch, and fry). Newly fertilized lake trout eggs were injected with doses of 0 (triolein), 0.1, 0.4, 1, 2, 4, and 8 ng/g TCDF. Values represent percent mortality at each stage from a single dose-response experiment with 54 eggs per dose.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Egg</th>
<th>Hatch</th>
<th>Fry</th>
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<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7.1</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
<td>7.7</td>
<td>7.7</td>
</tr>
<tr>
<td>0.4</td>
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<td>1</td>
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<tr>
<td>2</td>
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</tr>
<tr>
<td>4</td>
<td>0</td>
<td>27.7</td>
<td>44.7</td>
</tr>
<tr>
<td>8</td>
<td>2.6</td>
<td>30.8</td>
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</tr>
</tbody>
</table>
Table 4. 2,3,7,8-TCDD LD50 values from lake trout early life stage studies. Mean values and confidence limits (where available) are shown along with the route of exposure to lake trout young.

<table>
<thead>
<tr>
<th>Value (pg/g)</th>
<th>Exposure Route</th>
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<tr>
<td><strong>LD50</strong></td>
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</tr>
<tr>
<td>44 (36-52)</td>
<td>Water Exposure&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>47 (21-65)</td>
<td>Egg Injection&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>53 (41-55)</td>
<td>Water Exposure&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>58 (36-90)</td>
<td>Maternal Transfer&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>65 (60-71)</td>
<td>Water Exposure&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>69 (64-75)</td>
<td>Water Exposure&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>69 (58-80)</td>
<td>Water Exposure&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>74 (70-80)</td>
<td>Egg Injection&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>80 (68-91)</td>
<td>Egg Injection&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>81 (76-101)</td>
<td>Egg Injection&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>85 (36-210)</td>
<td>Water Exposure&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td><strong>LOAEL</strong></td>
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<tr>
<td>40</td>
<td>Water Exposure&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
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<tr>
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<td>Maternal Transfer&lt;sup&gt;c&lt;/sup&gt;</td>
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</tr>
<tr>
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</tr>
<tr>
<td>34 (26-40)</td>
<td>Water Exposure&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>41 (8-47)</td>
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</tr>
<tr>
<td>44</td>
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</tr>
<tr>
<td>45 (31-55)</td>
<td>Water Exposure&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Guiney et al. 1996; <sup>b</sup> Guiney et al. 1997; <sup>c</sup> Walker et al. 1994; <sup>d</sup> Walker et al. 1996; <sup>e</sup> Zabel et al. 1995b; <sup>f</sup> This study
Figure 4. 2,3,7,8-TCDF-induced mortality in lake trout early life stages. Newly fertilized lake trout eggs were injected with doses of 0 (triolein), 0.1, 0.4, 1, 2, 4, and 8 ng/g TCDF. Dashed and solid lines represent the probit model, and 95% confidence limits, respectively. Data points represent actual mortality percentages from treatment groups from a single dose-response experiment with 54 eggs per dose.
Table 2. ED50 and LOAEL values for mortality and gross pathological lesions in lake trout inject as newly fertilized eggs with an extract from Lake Michigan lake trout, with conversions into TCDD equivalent concentrations. The lake trout extract is from Lake Michigan lake trout collected near Sheboygan, WI in 1988. Values are from Probit analysis of two dose-response experiments, with the number of eggs injected per dose averaging 50 and 75 in the two trials, respectively. The doses used were control (triolein), 0.02, 0.10, 0.20, 1.0, 2.0, 4.0, 10.0, and 20.0 eggEQ. The pg TEQ/g are computed from eggEQ values multiplied by 14.7 pg TEQ/g in the extract.

<table>
<thead>
<tr>
<th></th>
<th>ED50</th>
<th>LOAEL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EggEQ</td>
<td>pg TEQ/g*</td>
</tr>
<tr>
<td>Mortality</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>103</td>
</tr>
<tr>
<td>Lesions</td>
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</tr>
<tr>
<td>Hemorrhage</td>
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<td>6</td>
</tr>
<tr>
<td>Yolk-sac Edema</td>
<td>3</td>
<td>44</td>
</tr>
<tr>
<td>Cranio-deformities</td>
<td>7.4</td>
<td>109</td>
</tr>
</tbody>
</table>
Discussion

I. Egg injection bioassays with TCDD produced LD50s and toxicity thresholds similar to those from previous studies of TCDD toxicity in lake trout.

We measured a TCDD LD50 of 81 pg/g (76-101, 95% F.L.), with NOAEL and LOAEL values of 20 pg/g and 40 pg/g, respectively. As a primary component in the equation for calculating relative potencies (REPs) of individual PHH congeners (i.e. $LD50_{TCDD}/LD50_{congener} = TEF_{congener}$), the accuracy of the lake trout TCDD LD50 is essential. Several studies have looked at TCDD toxicity in lake trout early life stages, and a range of LD50 values has been reported (Table 4). Values vary from a low of 44 pg/g to a high of 85 pg/g, with differences possibly due to egg source, exposure route, exposure method, or a combination of factors (Walker et al. 1994; Zabel et al. 1995b; Guiney et al. 1996; Walker et al. 1996; Guiney et al. 1997). Nevertheless, our estimation of the TCDD LD50 in lake trout early life stages falls within the range of reported values. Our estimates of the NOAEL and LOAEL values for TCDD toxicity likewise compare to other studies. Thus we can be confident in using our value of 81 pg/g in the analysis of the effects of the complex chemical extract of Lake Michigan lake trout.

II. The REP of 2,3,7,8-TCDF in lake trout (0.032), and is similar to that determined in rainbow trout (0.028).

One objective of this study was to determine the relative potency of 2,3,7,8-TCDF in early life stage lake trout. The use of 2,3,7,8-TCDF in this study is especially relevant because it contributes the greatest mass of all dioxin-like PCDDs and PCDFs found in Lake Michigan lake trout (EPA 1991; Cook 1994; Wright and Tillitt 1999). Thus, although the relative potency (REP) for 2,3,7,8-TCDF (0.028) is the lowest of all the furans with the exception of OCDF, its contribution relative to the other furans towards total TCDD TEQs is second only to 2,3,4,7,8-PeCDF (Table 1). The LD50 for 2,3,7,8-TCDF in lake trout was 2.5 ng/g, with identical signs of toxicity (i.e. yolksac edema, hemorrhage, and craniofacial malformations) to that caused by TCDD. The LD50 of 2,3,7,8-TCDF in Erwin strain rainbow trout fry is 8.09 ng/g, and thus lake trout are significantly more sensitive to this compound (Walker and Peterson 1991). This closely
follows the pattern of relative sensitivity differences between rainbow trout and lake trout to PHH compounds. The REP of 2,3,7,8-TCDF in lake trout is 0.032. This value is similar to the REP of 0.028 determined for rainbow trout early life stage mortality (Walker and Peterson 1990). Both of these values are lower than the REP for 2,3,7,8-TCDF based on the PLHC-1 fish cell bioassay (0.09) (Tillitt unpublished). This difference may be significant in the application of REPs to ecological risk assessment, because use of the REP for 2,3,7,8-TCDF based on the PLHC-1 fish cell bioassay may overestimate the contribution of TCDF to the TCDD-equivalent concentration of a mixture.

III. Based on the use of TCDD equivalent concentrations, the Lake Michigan lake trout extract has additive effects on embryonic toxicity in lake trout.

Toxic Equivalency Factors (TEFs) relate the potency of the various PCDDs, PCDFs, and PCB congeners to the prototypic, most toxic PHH 2,3,7,8-TCDD. A TEF represents a consensus value for the relative potency of a specific PHH congener based on the results of several studies. In contrast, relative potencies (REPs) are used to describe the potencies of compounds relative to TCDD based on a single in vivo or in vitro study (Van den Berg et al. 1998). The TEF concept for PHHs assumes additivity among congeners in order to assess the risk of complex mixtures of chemicals. The REPs used here to predict the potency of the Lake Michigan extract are based on chemical potencies in early life stage rainbow trout where available (Walker et al. 1991; Zabel et al. 1995a), the PLHC-1 fish cell bioassay (Tillitt and Cantrell 1992), or from mammalian in vivo and in vitro studies (Safe 1990). TEFs for various chemicals in lake trout are similar to those values for rainbow trout. Zabel et al. (1995b) determined a REP of 0.003 for PCB 126 in causing lake trout sac fry mortality, similar to the REP of 0.005 for rainbow trout early life stages (Walker and Peterson 1990). This indicates that the relative potencies of PHH congeners are similar between lake trout and rainbow trout, although lake trout are more sensitive to PHH toxic effects. This similarly is further supported by our finding that the REP for 2,3,7,8-TCDF in lake trout (0.032) is similar to that in rainbow trout (0.028) (Walker et al. 1991). These values are both less
than the proposed 2,3,7,8-TCDF TEF of 0.05 proposed for fish for use in risk assessment (Van den Berg et al. 1998).

The total TCDD TEQs in the Lake Michigan lake trout extract, or the amount estimated to be in one Lake Michigan lake trout egg in the wild, is 14.7 pg TEQ/g egg. In comparison, values for Lake Ontario lake trout eggs collected in the same sampling year were 18.19 pg TEC/g (Cook et al. 2003), while in Lake Michigan lake trout eggs values were between 5.44 and 6.38 pg TEQ/g egg (Cook et al. 1997). The most toxic congener, 2,3,7,8-TCDD, contributed ~12% of the predicted egg TEQ, similar to past results (Cook et al. 1997). The LD50 for the Lake Michigan lake trout extract injected into lake trout was 7 eggEQ (4-11, 95% F.L.). This value is nearly identical that predicted based on injection of the extract into rainbow trout eggs (i.e., 5.5 eggEQ) (Wright and Tillitt 1999). The LD50 of the extract was predicted to be 103 pg TEQ/g of egg based on the TEQs in the original lake trout. We measured an LD50 of 81 pg/g in lake trout eggs injected with 2,3,7,8-TCDD, which is virtually identical to the LD50 value of TCDD (80 pg/g) measured by Walker et al. (1994) and in the range of LD50 values found by others (Zabel et al. 1995b; Guiney et al. 1996; Walker et al. 1996; Guiney et al. 1997). In a study involving the injection of a synthetic mixture of PHHs into lake trout eggs, Walker et al. (1996) found a similar small shift to the right of the mixture dose-response curve relative to that for 2,3,7,8-TCDD. However, the deviation from additivity was not significant when the lake trout-specific REP for PCB 126 of 0.003, rather than 0.005, was used to calculate the total TEQs for the mixture. In our case, if the lake trout-specific REP for PCB 126 is used, the LD50 for the extract is 90 pg TEQ/g egg. This example points to the need for further study of species-specific differences in relative potencies of PHH compounds in order to fine tune risk assessment of complex mixtures. Other possible explanations for such a shift include differential bioavailability of the mixture versus 2,3,7,8-TCDD in the embryos, and competitive binding of congeners with low AhR binding affinities (e.g. PCBs 77 and 126) and high affinity binders (e.g. 2,3,7,8-TCDD) (Walker et al. 1996).

The difference between the LD50 of the extract (103 pg TEQ/g) and the actual LD50 of TCDD (81 pg/g) in this study was relatively small. This indicates that the complex mixture of
PHHs in the extract taken from Great Lakes lake trout has additive effects on embryonic toxicity in lake trout. Thus, an additive model for PHHs appears to be appropriate to predict effects and conduct hazard assessments. Additivity is a major assumption of the TEF/TEQ method, most studies of fish early life stage mortality due to PHH exposure result in additive toxicity (Tillitt 1999). Zabel et al. (1995b) found additional evidence of additivity between PCB 126 and TCDD in causing lake trout early life stage mortality. A complex extract of Lake Michigan lake trout tissue appeared to act in an additive manner in producing rainbow trout early life stage mortality (Wright and Tillitt 1999). Additive interactions have also been shown between pairs of polybrominated dibenzo-p-dioxin, dibenzofuran, and biphenyl congeners in rainbow trout sac fry (Hornung et al. 1996). The additive manner of the extract in the current study also indicates that PCDDs, PCDFs, and PCBs appear to comprise almost all of the dioxin-like potency in Lake Michigan lake trout. While other organochlorine residues, including DDT, DDE, DDD, dieldrin, chlordane, toxaphene and others may be present in the mixture, they appear to be of limited importance toward lake trout embryo mortality (Schmitt et al. 1990). The possibility that these compounds contribute to the embryotoxicity observed in lake trout, or that they might produce toxic effects in later life stages, cannot be discounted.

IV. PHHs in lake trout from Lake Michigan are below a threshold for adverse effects such as hemorrhage and such sublethal lesions may contribute to the lack of recruitment in certain Great Lakes lake trout populations.

Concentrations of chlorinated hydrocarbons have declined substantially in Great Lakes lake trout over the last twenty years. Total PCB congener concentrations from whole lake trout collected in Lake Ontario from 1977 to 1993 have declined 80% (Heustis et al. 1996). Concentrations of PCBs, DDE, and 2,3,7,8-TCDD in adult lake trout from Lakes Michigan and Ontario have likewise declined (Baumann and Whittle 1988; Zacharewski et al. 1989). However, these contaminant concentrations now appear to have stabilized, albeit at substantially lower concentrations than were present at the peak of contamination 20 to 30 years ago (Baumann and Whittle 1988; Suns et al. 1993; Hebert et al. 1994). Currently, the concentrations of PHHs
present in Great Lakes lake trout are below the threshold for direct lethality (Guiney et al. 1996; Wright and Tillitt 1999; Cook et al. 2003). However, TEQs below the threshold for direct lethality may nevertheless cause sublethal effects (such as yolksac edema) which may still be contributing to the lack of lake trout recruitment in the Great Lakes (Wright and Tillitt 1999).

Using the complex extract, we found a LOAEL for mortality in lake trout of 29 pg TEQ/g egg. Even though this value is less than the TCDD LOAEL of 40 pg/g, it is still above the lake trout egg TEQ of 14.7 pg/g. Thus, the concentration of PHH compounds in Lake Michigan lake trout is below the threshold for mortality. Some studies of lake trout mortality have concluded that total TEQs of at least 30 pg/g would be necessary to induce adverse effects in lake trout (Fitzsimons 1995; Guiney et al. 1996). In contrast, based on the gross pathological lesions induced in this study, the concentrations of dioxin-like chemicals in Lake Michigan lake trout demonstrably exceed the threshold required for causing certain sublethal effects. A significant increase in sublethal effects such as hemorrhage can occur at TEQs as low as 2 pg TEQ/g egg (Table 2). Therefore, it is important to consider how these sublethal effects contribute to overall mortality in lake trout fry. It is not unreasonable to speculate that these pathological lesions, which are observed in the same patterns as seen in fish exposed to TCDD in the laboratory (Cantrell et al. 1996), may result in a decreased ability of the fry to forage, avoid predation, or compete with other species for habitat or resources in the wild.

Guiney et al. (1997) examined CYP1A induction in endothelium of lake trout embryos and fry, and its possible association with mortality due to the edema and vascular effects of TCDD. The strongest response occurred at TCDD doses greater than 88 pg/g (the approximate LD50). However, CYP1A induction was detected at doses as low as 22 pg TCDD/g egg. The LOAEL for yolksac edema in lake trout (15 pg/g) is very close to this value, suggesting that CYP1A induction in the endothelium may be linked to early lesions that result in yolksac edema (Cantrell et al. 1996; 1998; Guiney et al. 1997). These findings point to the need to examine toxic effects other than lethality in order to better determine true toxic potential of any PHH compound in early life stage lake trout. TEQs in Lake Superior, where natural lake trout reproduction has been maintained, likely never exceeded 5 pg TCDD TEQ/g egg in the past (Cook et al. 1997).
Because the LOAELs for both hemorrhage and yolksac edema (i.e. 2 pg TEQ/g and 15 pg TEQ/g, respectively) are at or below current estimated TEQs in lake trout eggs, these sublethal effects may contribute to poor lake trout recruitment in the Great Lakes (Table 2). The reduction of TEQs in lake trout eggs to below 2 pg TEQ/g may improve the survival of young lake trout.

Conclusions

The TEF/TEQ method was used to evaluate the toxicity of a complex extract from Lake Michigan lake trout to early life stage lake trout. The mixture acted in an additive manner in producing lake trout early life stage mortality. Total TEQs in the extract were estimated to be 14.7 pg TEQ/g, which is below the LOAEL for TCDD in lake trout. However, gross pathological lesions, such as the incidence of hemorrhage, occurred at a TEQ as low as 2 pg TEQ/g. The significance of the occurrence of such lesions, and the relative importance that their deleterious effects might have on the surviving fry are unknown at this point. This study confirms our earlier conclusions that PHHs in lake trout from Lake Michigan are below a threshold for adverse effects and these compounds may have implications on the lack of recruitment in certain Great Lakes lake trout populations.

Acknowledgements

The support of the University of Missouri Cooperative Unit, under Dr. Charles Rabeni, was appreciated and critical to the success of these studies. We also wish to thank those at the Columbia Environmental Research Center for their cooperation and support, especially John Meadows, Dennis Schroeder, Mark Alexander, Kathy Echols, Paul Peterman and Robert Gale for their technical assistance with the extract preparation and chemical analyses. We also thank Susannah Cantrell, Pam Alt, and Diane Nicks for their assistance in the laboratory. Thanks to John Besser, James Fairchild, and Douglas Noltie for their comments and suggestions regarding this paper.
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Chapter 2: Thiamin supplementation ameliorates 2,3,7,8-TCDD toxicity in early life stage rainbow trout and Japanese medaka

Abstract

Early Mortality Syndrome (EMS) in Great Lakes salmonids may be caused by an interaction between contaminant and nutritional factors. Adult females here accumulate dioxin-like compounds from the water, sediment, and contaminated prey. Coincidentally, they also consume diets that are either deficient in thiamin or high in thiaminase-containing prey. The degree of thiamin deficiency in adults may affect their overall health, and females, particularly those from Lake Michigan and Lake Ontario, spawn eggs that are themselves deficient in thiamin. The present study tests whether pharmacological doses of thiamin can ameliorate certain adverse effects of TCDD in rainbow trout (Oncorhynchus mykiss) and Japanese medaka (Oryzias latipes) embryos. Newly fertilized eggs of both species were experimentally exposed to 2,3,7,8-TCDD, and were then subjected to multiple thiamin supplementations. In addition, a group of rainbow trout eggs were treated to a single thiamin supplementation at water hardening, followed by injection with 2,3,7,8-TCDD. In Arlee strain rainbow trout treated with multiple thiamin supplementations, the TCDD-related mortality in the thiamin+ treatments was significantly reduced in comparison to the thiamin- treatments at TCDD doses of 100, 200, and 400 pg/g TCDD. Also, the TCDD LD50 value for thiamin-treatments (375 pg/g) was significantly lower that in thiamin+ treatments (435 pg/g). Similar responses were observed in medaka. In contrast, a single thiamin treatment at water hardening was not sufficient to ameliorate TCDD toxicity in Ervin strain rainbow trout. These data support there being an interaction between thiamin and dioxin-induced embryo toxicity. Our findings contribute to our developing understanding that these stressors (contaminants and thiamin deficiency) together play a role in producing the overt mortality and decreased egg and fry health seen in wild populations.

Introduction

Early Mortality Syndrome (EMS) is the term most commonly used to describe mortality affecting the early life stages of Great Lakes salmonids, more specifically that mortality coinciding with fry swim-up. In Great Lakes salmonids, young afflicted with Early Mortality Syndrome (EMS) exhibit lethargy, loss of equilibrium, abnormal swimming, hyper-excitability, hemorrhage, and death just prior to the onset of exogenous feeding. EMS occurs primarily in Lake Michigan and Lake Ontario salmonids, and to a lesser extent in Lake Huron and Lake Erie fish. Affected species include coho salmon (O. kisutch), chinook salmon (O. tshawytscha), steelhead/rainbow trout (O. mykiss), brown trout (Salmo trutta), and lake trout (Salvelinus namaycush).

One factor that likely plays a role in salmonid EMS is thiamin. Thiamin is already important for carbohydrate and protein metabolism in fishes (Halver, 1989), and thiamin deficiency in larval landlocked Atlantic salmon (Salmo salar) produces both gross pathological lesions (subcutaneous and pericardial edema, hemorrhage, craniofacial deformities) and
neurobehavioral effects (abnormal phototactic behaviors and ataxia) similar to EMS symptoms (Fisher et al., 1995). Furthermore, in young salmonids susceptible to early life stage mortality, both wild and laboratory-reared individuals benefit from treatment with thiamin, ameliorating the deleterious effects of thiamin deficiency (Fitzsimons, 1995; Amcoff et al., 1998; Fisher et al., 1996).

For Great Lakes lake trout, EMS and reproductive dysfunction may also be a consequence of the effects of persistent, hydrophobic contaminants (such as PCDDs, PCDFs, and PCBs, collectively termed the planar halogenated aromatic hydrocarbons or PHHs) (Wilson and Tillitt, 1996; Willford et al., 1981; Mac, 1988; Walker and Peterson, 1990). PHHs are a class of highly toxic environmental contaminants that are often found in complex mixtures and are known to be toxic to early life stage fishes (Helder, 1980, 1981; Walker and Peterson, 1990; Walker et al., 1991; Harris et al., 1994). Their adverse effects may result from bioaccumulation directly from the water and sediment, and indirectly via the consumption of contaminated prey; affected females directly deposit these lipophilic compounds into their eggs as they ready themselves for spawning (Guiney et al., 1980). Supporting this suggestion are observations that lowered egg and fry survival occurs in salmonid populations where contaminant concentrations are heightened. As examples, (1) in Lake Michigan, the survival rates of chinook salmon fry to the swim-up stage are negatively correlated with adult body burdens of PCBs with TCDD-type activity (Ankley et al., 1990; Geisy et al., 1986); (2) contaminant concentrations have been implicated as contributing to the reproductive disorders of both Atlantic salmon and coho salmon (Elson et al., 1973; Johnson and Pecor, 1969). However, interpretations of the impact of environmental contaminants (such as PHHs) on Great Lakes salmonid populations remain controversial (Fitzsimons, 1995).

Although salmonid early life stage mortality is likely linked to thiamin insufficiency in eggs and fry, other agents may have additional negative consequences. Circumstances in Lake Michigan illustrate this: although EMS only affects up to 30% of female lake trout broods, and the survival in affected broods is appreciable (from 5% to 87%), the apparent lack of successful natural reproduction here suggests that additional factors contribute to the remaining fry mortality.
(Edsall et al., 1999). Similarly, the effects of poor egg quality or physiological changes alone seem insufficient causes for the mortalities experienced by lake trout fry (Manny et al., 1995). Results such as these indicate that no single factor is the basis for Great Lakes EMS. For Great Lakes salmonids, their young may begin life "inheriting" two major impediments from their mothers: body burdens of dioxin-like compounds, and a thiamin insufficiency. In the wild, either stressor alone may not appreciably affect egg or fry health. However, in combination, their impact may be sufficient to reduce egg and fry fitness and/or yield overt mortality. It is therefore important to investigate how such PHH compounds as 2,3,7,8-TCDD and thiamin status together interact to produce salmonid early life stage mortality. As such, the objectives of this study were: (1) to investigate whether thiamin supplementation affects TCDD toxicity in early life stage rainbow trout and Japanese medaka (Oryzias latipes), and (2) to describe the effects by using the incidence of gross pathological lesions and/or mortality as indicators.

**Methods**

*2,3,7,8-TCDD Exposures with Multiple Thiamin Supplementation*

Rainbow Trout

Arlee strain rainbow trout eggs were received from the Ennis National Fish Hatchery, Ennis, Montana, and were fertilized on-site at the Columbia Environmental Research Center on December 7, 14, and 21, 1994 according to standard methods (Leitritz and Lewis, 1976). For each brood, rainbow trout eggs were pooled from two to three females, and the milt was a pooled sample from two to three males. The egg preparation and TCDD injection procedures also mirrored standard methods (Walker et al., 1996). Briefly, the fertilized and water-hardened eggs were individually inserted into the U-shaped wells in preformed agarose plates to stabilize them during injection. The injections were performed using glass micropipettes, a computer positioning device, and a regulated gas pressure injection system.

Side-by-side triplicate TCDD dose-response trials were conducted, one per week, using six injection doses of 0 (triolein alone), 50, 100, 200, 400 and 800 pg/g TCDD. The number of eggs injected ranged from 20 to 43 eggs per dose (Table 1). Following injection, the eggs were
Table 1. Experimental Design for TCDD and Thiamin Dosing.

<table>
<thead>
<tr>
<th>Trials</th>
<th>Thiamin Supplementation (Thiamin+)</th>
<th>No Thiamin Supplementation (Thiamin-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I, II, III</td>
<td><strong>Rainbow Trout: Three times weekly beginning 220 dd</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCDD Doses (pg/g):</td>
<td>TCDD Doses(pg/g):</td>
</tr>
<tr>
<td></td>
<td>0 50 100 200 400 800</td>
<td>0 50 100 200 400 800</td>
</tr>
<tr>
<td>I, II, III</td>
<td><strong>Rainbow Trout: Single supplementation at water-hardening</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCDD Doses(pg/g):</td>
<td>TCDD Doses(pg/g):</td>
</tr>
<tr>
<td></td>
<td>0 50 100 200 400 800</td>
<td>0 50 100 200 400 800</td>
</tr>
<tr>
<td>I, II, III</td>
<td><strong>Medaka</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCDD Concentrations (pg/ul):</td>
<td>TCDD Concentrations (pg/ul):</td>
</tr>
<tr>
<td></td>
<td>0, 1.25, 2.5, 5, 10</td>
<td>0, 1.25, 2.5, 5, 10</td>
</tr>
</tbody>
</table>
cultured in stainless steel wire mesh baskets at 11±1°C in a flow-through 16-tray vertical incubator supplied with aerated well water. Upon reaching the eyed stage, each egg batch for each dose was split and randomly allocated to one of twelve incubation baskets, with each half being destined for either thiamin supplementation or non-supplementation at a particular TCDD dosage level.

Water-borne thiamin dosing began on Day 20 post-fertilization, at approximately 220 degree days (Table 1). Thiamin hydrochloride was obtained in powder form from Sigma Chemical Company, St. Louis, Missouri. Water from the incubator was used to prepare a new thiamin solution of 400 mg/l for each dosing period. This concentration was based on prior thiamin studies (M. Hornung, personal communication). Fifty milliliters of this solution were poured into each of six plastic trays in a separate incubator tray, after which the wire baskets containing the 0, 50, 100, 200, 400, and 800 pg/g TCDD treatments were immersed in the solution for 30 minutes (these groups were designated thiamin+). Thiamin dosing was carried out in this manner three times per week until the swim-up stage. The TCDD-dosed groups that were not supplemented with thiamin (designated thiamin-) were treated in a similar manner using incubator water only. Mortalities of the eggs or/and fry in every incubator basket were recorded every other day, and occurrences of gross pathological lesions in one replicate (Trial III) were recorded once at 450 degree days.

Medaka

Fertilized medaka eggs were obtained by manually stripping breeding females on the morning of each experiment. Eggs from 4 to 5 females were pooled, and held in glass beakers at 25°C until needed. To determine the appropriate thiamin supplementation level for the medaka young, two preliminary thiamin dose-response trials were conducted. Both involved exposures to six thiamin concentrations: 0, 0.001, 0.005, 0.01, 0.05, and 0.1 mg/ml dissolved in aquarium water. For the first trial, between 12 and 15 embryos per dosage level were tested, whereas between 28 and 32 embryos per dosage level were tested in the second trial. All medaka embryos were held and dosed in six-well microtiter. During dosing, each embryo was placed in 1 ml of thiamin solution for one hour, after which the dosing solution was replaced with fresh
aquarium water. The dosing was repeated daily, beginning on the third day post-fertilization, and continued until two days post-hatch. Embryo and fry mortalities were recorded daily.

Thiamin exposure produced dose-related mortality in medaka (Figure 1). Thiamin toxicity resulted in high mortality in the egg and newly hatched fry (data not shown); moribund embryos exhibited a slowing of blood flows and severe congestion in the yolk region. The lowest thiamin concentration that did not produce mortality rates different from the control was 0.005 mg/ml (SAS, Dunnett's Test for Difference Between Means, p=0.05, 6 d.f.). At this level, there was 20% mortality in the medaka up through 2 days post hatch versus 0% mortality in the control group and 7% mortality in the 0.001 mg/ml Consequently, a thiamin concentration of 0.005 mg/ml was used for subsequent medaka exposures.

Triplicate TCDD dose-response trials using waterbath exposures were conducted on medaka using newly fertilized embryos pooled from 4-5 females. Newly fertilized eggs were manually stripped from the females immediately before TCDD dosing was to begin. In preparation for dosing, individual eggs were placed with 10 ul of aquarium water into the wells of 12-well culture slides. To each well was added 1 ul of the TCDD dosing solution in isooctane (0, 1.25 pg/ul, 2.5 pg/ul, 5 pg/ul, or 10 pg/ul), after which the eggs were incubated in the wells for two hours at room temperature. Following dosing, each egg was transferred to the well of a 96-well microtiter plate containing 1 ml of incubator water and incubated at 27°C.

At this point, two separate dose-response groups were randomly designated: those receiving thiamin supplementation (thiamin+) and not receiving thiamin supplementation (thiamin-). Water-borne thiamin dosing of the thiamin treatments began on the third day post-fertilization and was repeated daily until two days post-hatch. Thiamin dosing involved removing the 1 ml of incubator water from each well and replacing it with 1 ml of thiamin solution for one hour, after which fresh incubator water replaced the thiamin solution. Embryos not receiving thiamin supplementation were subjected to a similar procedure using incubator water for each of the changes. Embryo and fry mortalities were recorded daily, and the incidence of hemorrhage was recorded for all three replicates at 2 days post-hatch.
Figure 1. Thiamin dose-response in Japanese medaka. Two thiamin dose-response trials were conducted with between 12 and 32 embryos per dosage level tested. From left to right, the six thiamin concentrations were 0, 0.001, 0.005, 0.01, 0.05, and 0.1 mg/ml thiamin hydrochloride, respectively. Shown are means and accompanying standard deviations where detectable. "*" represents a mortality rate that differs significantly from the control based on Dunnett's test for differences in means (p<0.05).
TCDD Exposure with Single Thiamin Supplementation at Water-hardening

Erwin strain rainbow trout green, unfertilized eggs were received from Ennis National Fish Hatchery, Ennis, Montana on January 4, 1995. Eggs arrived in a single batch, pooled from 3-4 two year old females along with a pooled batch of milt from 3-4 males. Immediately upon receipt, the eggs were split into two equal groups. Eggs in the first group (designated thiamin-) were fertilized using standard procedures (Leitritz and Lewis, 1976), after which they were placed directly into the vertical flow incubator (11°C ±1) for water hardening. Eggs in the second group (designated thiamin+) were likewise fertilized, but were then immediately immersed for one hour in a chilled 750 mg/l thiamin solution, or until water hardening was completed, after which they were also placed into the incubator.

Triplicate TCDD dose-response trials were again conducted using the same injection procedure described above. Side-by-side triplicate TCDD dose-response trials were conducted using six injection doses of 0 (triolein alone), 50, 100, 200, 400 and 800 pg/g TCDD. Forty eggs were injected per dose. Following injection, the eggs were cultured in stainless steel wire mesh baskets at 11±1°C in a flow-through 16-tray vertical incubator supplied with aerated well water. The eggs or/and fry in every incubator basket were examined on alternate days, and all occurrences of gross pathological lesions or mortalities were recorded.

Results

2,3,7,8-TCDD Exposure with Multiple Thiamin Supplementation

Rainbow Trout

For both rainbow trout and medaka, thiamin was able to ameliorate TCDD toxicity in an appreciable number of the TCDD dosage groups (Figure 2). For rainbow trout, the TCDD-related mortality in the thiamin+ treatments was significantly reduced in comparisons to the thiamin- treatments at the intermediate TCDD doses of 100, 200, and 400 pg/g TCDD (Figure 2A). No ameliorative effect was detected at the lowest doses (0 and 50 pg/g) or at the highest dose, 800 pg/g TCDD. Rainbow trout receiving thiamin supplementation (thiamin+) exhibited a
Figure 2. TCDD dose-response in (A) Arlee strain rainbow trout and (B) Medaka embryos in (†) thiamin+ and (0) thiamin- treatments. In the rainbow trout, side-by-side triplicate (thiamin+/thiamin-) were conducted, with 20-43 newly fertilized eggs injected per TCDD dose (0, 50, 100, 200, 400, and 800 pg/g TCDD). In medaka, triplicate side-by-side (thiamin+/thiamin) TCDD dose-response trials using waterbath exposures (0, 1.25, 2.5, 5, and 10 pg/ul TCDD) were conducted. "*" represents a significant difference in mortality between the thiamin+ and thiamin- groups, based on Student's t-test (p<0.05). Note the nonlinear scale for 2A. Standard deviations are shown.
significantly higher TCDD LD50 value as compared to the thiamin- fish (435 pg/g vs. 375 pg/g; Table 2).

Gross pathological lesion occurrences declined appreciably with exposure to thiamin (Table 3). Mortality in the highest TCDD dose (800 pg/g) was 100% in Trial III, and so no data on gross lesions was taken. The incidence of most gross pathological lesions was lower in the Thiamin+ treatments versus the Thiamin- treatments. This reduction of gross lesions in thiamin+ treatments was statistically significant for cardiac edema and craniofacial deformities. However, the incidence of poor blood flow to the tail actually increased in thiamin+ treated fry at all doses, and this increase was statistically significant (Table 3).

Medaka

In medaka, TCDD-related mortality was also significantly reduced by thiamin treatment at the intermediate TCDD doses of 2.25 and 5 pg/ul (Figure 2B). Thiamin was again unable to moderate the mortality caused by the highest TCDD dose used and thiamin treatment alone in the control groups (0 pg/ul) produced a detectable increase in mortality. Like in rainbow trout, the occurrence of hemorrhaging was significantly reduced by thiamin treatment at the uppermost TCDD doses of 1.25 and 2.50 pg/ul (Table 4).

TCDD Exposure with Single Thiamin Supplementation at Water-hardening

A single thiamin treatment at water hardening was not sufficient to ameliorate TCDD toxicity in Erwin strain rainbow trout (Figure 3). The thiamin+ and thiamin- treatments yielded near-identical TCDD dose-response relationships, and their respective TCDD LD50 values did not differ significantly (Table 2).

Discussion

Timing of Thiamin Supplementation

Researchers studying the effects of thiamin on fish early life stage mortality have employed a variety of thiamin supplementation schedules. For example, in a study of Baltic salmon, Amcoff et al. (1998) used thiamin immersion (varying concentrations) at both water
Table 2. TCDD LD50 (rainbow trout) and LC50 (medaka) values with 95% C.I. from Probit analysis of thiamin supplemented (thiamin+) and non-supplemented (thiamin-) treatments, either multiple or single. In the Arlee strain rainbow trout, side-by-side triplicate (thiamin+/thiamin-) were conducted, with 20-43 newly fertilized eggs injected per TCDD dose (0, 50, 100, 200, 400, and 800 pg/g TCDD). In Erwin strain rainbow trout, eggs were immersed at water hardening with a single thiamin supplementation of 750 mg/ml, followed by triplicate side-by-side (thiamin+/thiamin-) TCDD dose-response trials with 40 eggs injected per TCDD dose (0, 50, 100, 200, 400, and 800 pg/g TCDD). In medaka, triplicate side-by-side (thiamin+/thiamin) TCDD dose-response trials using waterbath exposures (0, 1.25, 2.5, 5, and 10 pg/ul TCDD) were conducted. “*” represents significant difference between values for Thiamin+ vs. Thiamin- in a given species based on Student’s t-test (p < 0.05).

<table>
<thead>
<tr>
<th>Thiamin Treatment Regime</th>
<th>Species</th>
<th>TCDD LC50^1/LC50^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Thiamin+</td>
</tr>
<tr>
<td>Multiple</td>
<td>Rainbow Trout^1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Arlee Strain)</td>
<td>435 pg/g*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(409-469)</td>
</tr>
<tr>
<td></td>
<td>Medaka^2</td>
<td>5.1 pg/µl*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.5-5.4)</td>
</tr>
<tr>
<td>Single</td>
<td>Rainbow Trout^1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Erwin Strain)</td>
<td>190 pg/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(114-237)</td>
</tr>
</tbody>
</table>
Table 3. Incidence (%) of gross pathological lesions in Arlee strain rainbow trout fry injected as eggs with 2,3,7,8-TCDD and receiving multiple thiamin supplementations, (Thiamin+) or not (Thiamin-), beginning on Day 20 post-fertilization and continuing three times per week until swim-up. Counts from a single replicate were taken at 450 degree days of development. ** indicates significant difference between Thiamin+ and Thiamin- treatments, based on two-tail paired sample t-tests.

<table>
<thead>
<tr>
<th>TCDD (pg/g)</th>
<th>Hemorrhage</th>
<th>Yolk-sac Edema</th>
<th>Cardiac Edema*</th>
<th>Craniofacial Deformities*</th>
<th>Vascular loss to tail*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thiamin+</td>
<td>Thiamin-</td>
<td>Thiamin+</td>
<td>Thiamin-</td>
<td>Thiamin+</td>
</tr>
<tr>
<td>0</td>
<td>0.0</td>
<td>3.6</td>
<td>12.1</td>
<td>4.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>17.4</td>
<td>15.8</td>
<td>65.0</td>
<td>58.1</td>
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</tr>
<tr>
<td>100</td>
<td>48.1</td>
<td>74.1</td>
<td>81.3</td>
<td>100.0</td>
<td>48.1</td>
</tr>
<tr>
<td>200</td>
<td>80.0</td>
<td>87.5</td>
<td>87.5</td>
<td>100.0</td>
<td>67.1</td>
</tr>
<tr>
<td>400</td>
<td>90.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>85.0</td>
</tr>
<tr>
<td></td>
<td>d.f. = 4, t = -1.95, p = 0.12</td>
<td>d.f. = 4, t = -0.62, p = 0.56</td>
<td>d.f. = 4, t = -2.71, p = 0.05</td>
<td>d.f. = 4, t = -3.56, p = 0.02</td>
<td>d.f. = 4, t = 3.05, p = 0.03</td>
</tr>
</tbody>
</table>

* Hemorrhage: d.f. = 4, t = -1.95, p = 0.12
* Yolk-sac Edema: d.f. = 4, t = -0.62, p = 0.56
* Cardiac Edema: d.f. = 4, t = -2.71, p = 0.05
* Craniofacial Deformities: d.f. = 4, t = -3.56, p = 0.02
* Vascular loss to tail: d.f. = 4, t = 3.05, p = 0.03
Table 4. Incidence (%) of a single gross pathological lesions, hemorrhage, in medaka fry exposed as eggs to 2,3,7,8-TCDD. Triplicate side-by-side (thiamin+/thiamin) TCDD dose-response trials using waterbath exposures (0, 1.25, 2.5, 5, and 10 pg/ul TCDD) were conducted. Water-borne thiamin dosing began on the third day post-fertilization and was repeated daily until two days post-hatch. Data shown are means (standard deviation). “*” represents significant difference between Thiamin+ vs. Thiamin- treatments at a given TCDD dose (Students t-test, p<0.05).

<table>
<thead>
<tr>
<th>TCDD (pg/ul)</th>
<th>Thiamin+</th>
<th>Thiamin-</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.4 (1.29)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1.25</td>
<td>24.85 * (3.7)</td>
<td>49.9 * (10.14)</td>
</tr>
<tr>
<td>2.5</td>
<td>35.36 * (4.77)</td>
<td>62.5 * (3.53)</td>
</tr>
<tr>
<td>5</td>
<td>100 (0)</td>
<td>100 (0)</td>
</tr>
</tbody>
</table>
Figure 3. TCDD dose-response in Erwin strain rainbow trout after a single thiamin supplementation at water hardening. Newly fertilized eggs were immersed at water hardening with a single thiamin supplementation of 750 mg/ml, followed by triplicate side-by-side (thiamin+/thiamin-) TCDD dose-response trials with 40 eggs injected per TCDD dose (0, 50, 100, 200, 400, and 800 pg/g TCDD). Mean values and standard deviations are shown for Thiamin+ (■) and Thiamin- (○) treatments.
hardening and at the sac fry stage. This study suggested that, although therapeutic thiamin treatment could limit fish early life stage mortality (=M74), its effectiveness depended on the developmental stage at which the treatment occurred. Recent work on Atlantic salmon sac fry suffering from early mortality syndrome (=Cayuga Syndrome) indicates that the long-term effects of thiamin deficiency are better reduced when thiamin immersions are administered early, i.e., at egg water hardening (Wooster and Bowser, 2000). In comparison, our results regarding TCDD-induced mortality similarly show that the ameliorative effects of thiamin supplementation depend on the timing of administration. Our results are also consistent with salmonid EMS being the consequence of a contaminant exposure/thiamin status interaction, and may assist in optimizing the concentration and scheduling of ameliorative thiamin supplementation procedures. When contaminant exposure is suspected, it may be necessary to conduct multiple thiamin treatments at several developmental time points. However, the TCDD doses used in the current study were much higher than are the levels that occur in feral fish.

*Interactions of PHHs with Vitamins, Including Thiamin*

Several studies have shown that PHHs and related compounds interfere with the metabolism of a number of vitamins. For instance, exposure to the organochlorine insecticide toxaphene resulted in a decrease in the levels of vitamin C in the bones of channel catfish, with the toxaphene exposure in vitamin C-deficient fish having an even greater adverse effect (Mayer et al., 1978; Hamilton et al., 1981). Brook trout exposed as eggs to Aroclor 1254 were vitamin C-deficient as sac fry (Mauck et al., 1978). Exposure to certain PHHs adversely affects vitamin A metabolism in a number of species (Villeneuve et al., 1971; Brouwer and Van den Berg, 1984; Spear et al., 1990; Van Birgelen et al., 1992; Pelessier et al., 1992). Boyer et al. (2000) found that exposing fish, birds, and mammals to the coplanar PCB 3,3',4,4'-tetrachlorobiphenyl resulted in retinoid (vitamin A) imbalances, with associated edema, growth inhibition, reproductive impairment, immunosuppression, teratogenesis, and susceptibility to cancer. Based on these indications, it is reasonable to question the possible effects of dioxin-like compounds such as 2,3,7,8-TCDD on thiamin metabolism and function.
As a vitamin itself, thiamin can produce interactive effects with dioxin-like compounds under laboratory conditions. For rats (*Rattus norvegicus*), Berdanier et al. (1975) described a synergistic relationship between thiamin deficiency and PCB exposure; here, the PCB-related effects (enlarged livers and increased liver lipid) were more significant in thiamin deficient/PCB treated individuals as compared to their thiamin sufficient/PCB treated counterparts. Also in rats, PCB exposure has been shown to decrease thiamin levels in the blood, brain, and sciatic nerve (Yagi et al., 1979). The question remains whether similar effects might be produced in fish such as rainbow trout and Japanese medaka.

The results above are significant because they mirror those derived from studies of salmonid early life stage mortality, namely that affected individuals benefit from treatment with thiamin. In fishes, thiamin treatment has been used successfully to treat feral Lake Ontario EMS-affected salmonid fry (Fitzsimons, 1995), naturally-occurring thiamin-deficient Atlantic salmon fry affected with EMS-like symptoms (Fisher et al., 1996, 1998), and M74-affected Atlantic salmon from the Baltic (Amcoff et al., 1998). Together, these findings strongly suggest that thiamin deficiency is contributing to the occurrence of EMS in these populations.

Thiamin supplementation may also have benefits over and above the simple reduction of thiamin deficiency. While not an antioxidant itself, thiamin may reduce the effects of the oxidative stress which can result from TCDD toxicity (Cantrell et al., 1996). In rats, Chen et al. (1999) examined the long-term effects of thiamin deficiency on the endogenous antioxidant defense systems of the liver, and found that the occurrence of such enzymatic antioxidants as glutathione and glutathione peroxidase decreased significantly in thiamin-deficient versus control animals. This outcome suggests that long-term thiamin deficiency weakens the antioxidant defense capability of the liver, and increases its cellular sensitivity to oxidative stress. If the same holds true for fish, then the high mortality experienced by Great Lakes salmonid young may result from a heightened susceptibility to PHH-induced toxicity stemming from their diet-related thiamin-deficient status. This is especially relevant, given that decreased levels of other antioxidants have been found in populations of salmonids exposed to contaminants and suspected of being thiamin-deficient (Palance et al., 1998; Pettersson and Lignell, 1998).
Since certain PHH compounds may interact with the metabolism and storage of thiamin in rats (Yagi et al. 1979; Pelessier et al. 1992), the involvement of such compounds must be considered in relation to thiamin deficiency and possible contaminant exposure of salmonid populations in the Great Lakes. Current levels of PHH compounds such as 2,3,7,8-TCDD appear to be below the no observable adverse effect level (NOAEL) for sac fry mortality (Guiney et al. 1996; Cook et al. 2003). However, contaminant levels in eggs from species such as lake trout may be above the threshold for the sublethal effects of yolksac edema and hemorrhaging (Wright and Tillitt 1999; Cook et al. 2003). Although to date no direct correlation has been found between the concentrations of PHHs and the mortality rates associated with EMS in Great Lakes lake trout, the role contaminants may have in lake trout reproductive dysfunction cannot be discounted, and further research into specific interactions between thiamin deficiency and sublethal TCDD exposure should be considered.

Acknowledgements

The support of the Missouri Cooperative Fish and Wildlife Research Unit, under Dr. Charles F. Rabeni, was appreciated and critical to the success of these studies. We also wish to thank those at the Columbia Environmental Research Center for their cooperation and support, especially Eugene Greer, Pam Alt, Diana Papoulias, and Susannah Cantrell. We greatly appreciate Wes Orr at the Ennis National Fish Hatchery for supplying the rainbow trout eggs. The reviewers of this paper are thanked for their comments and suggestions, including Dr. Edward Little and Dr. Paulo Carvalho at the Columbia Environmental Research Center and Dr. Kevin Fritsche, Dr. Robert Hayward at the University of Missouri-Columbia. This publication is a contribution from both the Missouri Cooperative Fish and Wildlife Research Unit (the U.S. Geological Survey/Biological Resources Division, Missouri Department of Conservation, The School of Natural Resources of the University of Missouri-Columbia, and the Wildlife Management Institute cooperating), and from the Missouri Agricultural Experiment Station.
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Chapter 3: Comparison of Pre-Hatch C-start Responses in Rainbow Trout and Lake Trout Embryos Using a Tactile Stimulus Test

Abstract

The C-start in teleost fishes, a type of startle response, mediates the ability to respond to abrupt, unexpected stimuli, and is characterized by a short-latency, C-type fast start acceleration. In pre-hatch fish embryos the C-start appears necessary for mechanical breakdown of the egg chorion and successful hatch, via increased embryo movement and distribution of the hatching enzymes. In later stages, the C-start plays an important role in predator avoidance. Using tactile stimulation, the C-start response was evaluated in rainbow trout Oncorhynchus mykiss at 170 degree days, when 6.6% of embryos exhibited C-starts, and lake trout Salvelinus namaycush embryos at 320 degree days, when 23% of embryos exhibited C-starts. Triplicate groups of embryos were later tested at three developmental stages; early (220 or 360 degree days for rainbow trout and lake trout respectively), middle (260 or 480 dd, respectively) and late (320 or 560 dd, respectively). The proportion of trout embryos exhibiting C-start increased through time with 100% responding at the late stage, just prior to hatch. C-starts could be obtained by repeated stimulation, and the relative activity of the embryos, based on the number of flexures per stimulus, also increased over time. Rainbow trout and lake trout showed very similar C-start responses at parallel developmental stages, and these patterns of response were similar to that reported in other fish species.

Introduction

When startled, a subcarangiform fish such as rainbow trout Oncorhynchus mykiss will laterally flex its body into the shape of the letter 'C' and then rapidly and forcefully straighten the body, propelling the individual away from the perceived threat. This "C-start" response is highly coordinated, with the initiation of the response mediated by the Mauthner cells (Diamond 1971; Eaton and Farley 1975; Zottoli 1977; Eaton et al. 1981), although the completion of the C-start response is thought to involve the participation of many other reticulospinal neurons (Zottoli et al. 1999; Eaton et al. 2001). Specifically, two other reticulospinal neurons, homologs of the Mauthner cells, are also involved in the escape behavior and appear to provide directional control of the response (Metcalfe et al. 1986; Foreman and Eaton 1993; O'Malley et al. 1996; Lui and Fetcho 1999). Although the C-start response may be elicited by a number of different types of stimuli, tactile stimulation is especially effective in early embryos and larvae (Eaton and DiDomenico 1986; Easter and Nicola 1996; Hale 1996; Saint-Amant and Drapeau 1998).

From a neurobiological perspective, the C-start response has been extensively studied in the zebrafish Brachydanio rerio (Kimmel et al. 1980; Eaton and Hackett 1984; Lui and Fetcho
1999) and the goldfish *Carassius auratus* (Zottoli 1977; Eaton et al. 1981; Eaton et al. 1982; Zottoli et al. 1999). In these and in other species, the avoidance maneuvers of the C-start response are likely initiated by neural impulses conducted from the brain to the caudal musculature via the Mauthner cells, a single pair of large myelinated neurons extending caudally from the brainstem and down the length of the spinal cord. The fact that escape responses can be elicited in fish after deletion of the Mauthner cells indicates that other neurons are also involved (Eaton et al. 1982; O’Malley et al. 1996). Important for our investigation, the Mauthner cells develop very early in fish embryogenesis, and the C-start response is exhibited prior to hatch (Kimmel et al. 1974; Eaton et al. 1977). Zebrafish exhibit the C-start in response to a touch stimulus as early as 21 hours after fertilization, at which time primary organogenesis has occurred but the animal is still in the early stages of physiological differentiation (Saint-Amant and Drapeau 1998). Similarly, Atlantic salmon *Salmo salar* exhibit active, spontaneous C-type trunk movements early in development (Peterson and Martin-Robichaud 1983).

Although the main functions ascribed to the C-start response of free-swimming larval and adult fishes are predator escape (Eaton et al. 1977; Webb 1981; Webb 1982; Blaxter and Batty 1985) and possibly the terminal phase of prey capture (Canfield and Rose 1993), its role in egg-bound embryonic fishes likely differs. Here, embryo movements, including spontaneous C-starts, are believed to be important in distributing the enzymes responsible for rupture of the egg capsule (Willemse and Denuce 1973; Yamagami 1981; Shoots et al. 1982). Eaton and Nissanov (1985) also suggest that early development of the Mauthner cells may aid the embryo in bursting free from the egg capsule during predator attack.

Although research on the Mauthner cell system and the C-start response in young fish is extensive (e.g., Kimmel 1972; Eaton and Farley 1973; Zottoli 1977; Eaton et al. 1981; Eaton and Nissanov 1985; Eaton and DiDomenico 1986), relatively few species have been considered, even though the system occurs and develops similarly in a wide variety of teleost fishes (Zottoli 1978; Eaton and Hackett 1984; Goehner and Pfeiffer 1996). We contend that it is important to consider the ecological and taxonomic diversity of the C-start response in furthering our understanding of this behavior; furthermore, understanding the reason for the precocious development of this
system requires additional description, characterization, and tests of the function of the C-start response through development in different fish species. We chose two species, rainbow trout and lake trout, which are both commercially and ecologically important species. The comparative study of these closely related fish species is particularly important because the more readily available rainbow trout is often used as a surrogate for lake trout in certain types of laboratory testing. It was our aim to develop a simple, inexpensive, and repeatable assay for testing C-start response that could be implemented under a variety of testing and laboratory conditions. As such, the present study was conducted to:

(1) develop a simple in ovo tactile stimulus test for eliciting the C-start response in rainbow trout and lake trout embryos, and

(2) use this assay in describing the ontogeny of the behavior at several pre-hatch developmental time points, paying particular attention to how the behavior relates to embryo hatching success.

**Methods**

_Egg Culture_

A standard shipment of unfertilized rainbow trout eggs (pooled from 2 or 3 females, with milt pooled from 2 or 3 males) was obtained from the Ennis National Fish Hatchery, Ennis, MT, on June 3, June 10, and June 17, 1998, and eggs were fertilized on arrival at Columbia, MO according to standard methods (Leitritz and Lewis 1976; Walker et al. 1996). Lake trout eggs were obtained newly fertilized from National Research and Development Lab, Leetown Science Center, Wellsboro, PA on October 16, 1998. Milt pooled from 2 or 3 males was used to fertilize two batches of lake trout eggs from two separate females.

On receipt, the rainbow trout eggs were warmed slowly to 10°C and the lake trout eggs to 8°C; they were then placed in monolayers into stainless steel rearing baskets in separate 16-tray vertical flow Heath Stacks® for incubation at these temperatures. Daily inspections provided data concerning the frequency and timing of egg mortalities; dead eggs were picked and discarded immediately on detection.
C-start Test

The three-stage C-start test we devised began by selecting rainbow trout eggs (total of 90 eggs per test per stage) or lake trout eggs (total of 60 eggs per test per stage), which were then separately pipetted into individual pre-formed wells in agarose plates. These shallow wells (U-shaped in cross-section) allowed each egg to be held firmly in place during subsequent tactile stimulation. Each loaded plate was then returned to the vertical flow incubator for 24 hours, during which the embryos all oriented themselves uniformly, head and back uppermost against the egg capsule. No mortality was observed during this part of the procedure.

Our test involved positioning one of the three egg-bearing agarose plates for each species under a Nikon stereoscopic microscope (15x magnification). Each egg was then stimulated manually; this involved using a 10 mm blunt glass probe to briefly touch each chorion surface at a standardized location (dorso-lateral position of thorax anterior to the dorsal fin) and with a consistent force. The force applied was just enough to dimple the egg capsule. Three stimuli were applied in sequence to each egg at 10-second intervals (0 sec = ‘1st stimulus’; 10 sec = ‘2nd stimulus’; 20 sec = ‘3rd stimulus’), after which the next egg was brought into view.

For each stimulus applied to each egg, we recorded the absence or presence of a C-start response; specifically, the presence of a response involved a trunk flexure, always first in the opposite direction of the stimulus, into a "C" shape with the nose and tail being brought into close proximity. In addition to the absence or presence of a C-start response, the number of consecutive flexures that occurred in the following 10-second period were recorded. Slight movement by the embryo, although not considered a C-start response, was noted where it occurred.

We conducted complete C-start tests three times during the embryonic development of each species (Table 1). Because rainbow trout develop more rapidly than do lake trout (Leitritz and Lewis 1976), we tested the two species at matched developmental stages (percentage of time to hatch), even though the number of degree-days (i.e., mean daily temperature multiplied by day post-fertilization) each species had experienced increasingly diverged. At each stage, we
Table 1. C-start testing time points, corresponding percent of full term values, and percent response in rainbow trout and lake trout. Full term corresponds to hatch, with values based on average time to hatch in each species given the average daily water temperature. Percent response represents the percent of embryos exhibiting C-starts at a given time point. ** indicates a significant difference between means values for rainbow trout and lake trout based on student’s t-test, p<0.05. (a. First preliminary C-start test in rainbow trout, lake trout not tested; and b. Second preliminary C-start test, three sets of ten eggs each tested in both species).

<table>
<thead>
<tr>
<th>Time point</th>
<th>Rainbow Trout</th>
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<th>Lake Trout</th>
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<td></td>
<td>Degree Day</td>
<td>% of Full Term</td>
<td>% Response</td>
<td>Degree Day</td>
<td>% of Full Term</td>
<td>% Response</td>
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<td>b</td>
<td>170</td>
<td>52</td>
<td>6.6*</td>
<td>320</td>
<td>56</td>
<td>23*</td>
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<td>360</td>
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<td>97</td>
<td>100</td>
<td>560</td>
<td>98</td>
<td>100</td>
</tr>
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</table>
measured the diameters of all eggs that were tested, after which the entire set of eggs was
discarded so as to avoid testing any single egg more than once.

To determine when in the developmental sequence in ovo C-start responses might first be elicited, we conducted two preliminary trials on rainbow trout at 140 and 170 degree days, and one preliminary trial on lake trout at 320 degree days (Table 1). These preliminary trials considered a limited number of embryos: for rainbow trout, at 140 degree days the trial consisted of a single set of ten rainbow trout eggs and at 170 degree days the trial consisted of three sets of ten eggs each; for lake trout the preliminary trial at 320 degree days consisted of three sets of ten eggs each (Table 1).

Results

Ontogenetic Comparison of C-Start Occurrences

Rainbow Trout: No rainbow trout embryos responded at 140 degree days of incubation in our preliminary scoping trial (Table 1). In contrast, embryo responses to tactile stimulation were elicited in these embryos at 170 degree days of incubation, although only 6.6% did so, and the responses occurred only to the first tactile stimulation. At 220 degree days of development, 63% of rainbow trout embryos exhibited C-start responses to at least one of the tactile stimulations; this proportion increased to 92% at 260 degree days, and to 100% at 320 degree days (Table 1). There were no differences in egg diameter between embryos that did or did not exhibit C-start responses at any point in development (Students T-test p<0.05).

To determine the relationship between % of full term and percent of embryos exhibiting C-starts, the arcsine√X-transformed percentage data were fit to a least squares curve (Figure 1). Arc-sin transformation is useful in stabilizing the variance of proportion data (Snedecor and Cochran 1989). For rainbow trout, the regression equation was: arcsine√% Exhibiting C-start = 1.8 (% Full Term) - 73.7 (r² = 0.80, n=310, p<0.01). Using this formula, rainbow trout would not be expected to exhibit C-start responses prior to 42% of full term (hatch), or 140 degree days of development.
Figure 1. The proportion of embryos exhibiting C-start responses as a function of the percentage of full term (hatch) for rainbow trout and lake trout embryos. Points represent arcsine-square-root transformations and have been fitted by least-squares regression. The designations “a”, “b”, “early”, “middle”, and “late” correspond to the time points tested (see Table 1).
Lake Trout: Two sets of 10 lake trout eggs each were first tested at 320 degree days of incubation, at which time 23% exhibited C-start responses to at least one of the tactile stimulations (Table 1). At 360 degree days, 40% of lake trout embryos exhibited C-starts. Although these 360 degree-day lake trout were developmentally equivalent to the 220 degree-day rainbow trout (Table 1), significantly fewer responded ($\chi^2=5.609$, 1 d.f., P=0.018). Similarly, although the 480 degree-day lake trout were developmentally equivalent to the 260 degree-day rainbow trout, significantly fewer (77%) again responded ($\chi^2=10.664$, 1 d.f., P=0.001) (Table 1). However, because all the lake trout embryos responded at 560 degree days of incubation, no statistical difference in response frequency was found in comparison to the developmentally equivalent 320 degree day rainbow trout (also 100% response).

In lake trout, linear regression was performed on the arcsine $\sqrt{X}$-transformed percentage data to determine the relationship between C-start response occurrence and developmental stage (Figure 1). The linear regression model for lake was: arcsine $\sqrt{\%}$ Exhibiting C-start = 1.6 (% Full Term) - 70.8 ($r^2 = 0.84$, n=210, p<0.001). Using this formula, it is estimated that C-start responses in lake trout could first be elicited via tactile stimulation at 45% of full term, or at 257 degree days of development.

Comparisons of C-Start Response

To index and compare the embryo responses to tactile stimulation, we tallied the number of flexures with which each fish responded with to each successive tactile stimulus. For both species, the number of flexures per 1st stimulus increased as development advanced (Figure 2), with the proportion of embryos failing to exhibit C-start decreasing from 34 and 60% at the early stage to 3 and 5% at the late stage for rainbow trout and lake trout, respectively. The proportion of embryos exhibiting three or more flexures in response to the 1st stimulus increased from 4 and 5% to 23 and 25% for rainbow trout and lake trout, respectively. Very similar responses occurred after the 2nd and 3rd stimuli (Figure 2). Comparing between species, rainbow trout and lake trout exhibited statistically similar average numbers of flexures per stimulus at each consecutive tactile stimulus and developmental stage, except for the response to the 2nd stimulus at the middle and late stages (Table 2) when rainbow trout exhibited significantly greater activity. In both species,
Table 2. Flexures per stimulus after 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> stimulations in rainbow trout and lake trout at early, middle, and late time points. Values are mean (standard deviation). ** Indicates significant differences between values for rainbow trout and lake trout at a given time point based on students t-test (p < 0.05). Removed outliers based on significance level (5%) of the Maximum Normal Residual (MNR) (Snedecor and Cochran 1989).  

- Mean and standard deviation after removing data for two embryos exhibiting 13 flexures each, and
- Mean and standard deviation after removing data for one embryo exhibiting 17 flexures.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Early</th>
<th>Middle</th>
<th>Late</th>
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<td></td>
<td>Rainbow Trout</td>
<td>Lake Trout</td>
<td>Rainbow Trout</td>
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<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; (0 sec.)</td>
<td>0.84 (0.81)</td>
<td>0.80 (1.58)</td>
<td>1.64 (2.07)</td>
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<td>2&lt;sup&gt;nd&lt;/sup&gt; (10 sec.)</td>
<td>0.54 (0.72)</td>
<td>0.55 (1.36)</td>
<td>1.89** (2.59)</td>
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<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; (20 sec.)</td>
<td>0.41 (0.60)</td>
<td>2.05 (4.20)</td>
<td>1.59 (2.71)</td>
</tr>
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</table>
Figure 2. The proportion of rainbow trout and lake trout embryos exhibiting 0, 1, 2, and 3 or more flexures after the first, second, and third tactile stimulus. Data from the early, middle, and late developmental stages are shown.
the average number of flexures per 1st stimulus (a measure of relative embryo activity) increased over time and peaked late in development and shortly before hatch.

The pattern identified above for the average number of flexures per first stimulus was not evident in the 2nd stimulus data even when outlier values, based on calculation of the significance levels (5% and 1%) of the Maximum Normal Residual (MNR) for a normal sample, from the few embryos that exhibited unusually numerous flexures were excluded from the analysis (Table 2, in bold; Snedecor and Cochran 1989). These few embryos exhibited responses that were highly atypical, flexing over twenty times after tactile stimulation. Using this modified data set, the only significant differences between species occurred in response to the 2nd stimulus at the both the middle (1.67 vs. 0.90 flexures per stimulus) and late (1.59 vs. 1.05 flexures per stimulus) stages. Again, in the 3rd stimulus data, there were no significant differences in embryo responses between species. Thus rainbow trout and lake trout embryos mostly behaved in a very similar manner in terms of C-start expression in response to tactile stimulation.

**Discussion**

*Mauthner Neuron Involvement and Ontogeny of the C-start in Rainbow and Lake Trout*

The Mauthner neuron-initiated C-start system is one of only a few examples of an established causal connection between the activity of a particular cell in the vertebrate nervous system and a defined behavioral response (Zottoli 1977; Eaton et al. 1981; Eaton and Hackett 1984; Nissanov and Eaton 1989). In intact teleost fish, the Mauthner cell serves as a command neuron that initiates a C-start response each time it fires an action potential (Zottoli 1977; Eaton et al. 1981; Nissanov and Eaton 1989). However, when the Mauthner cells are absent, non-Mauthner cells are capable of initiating C-starts (Eaton et al. 1982; O’Malley et al. 1996; Liu and Fetcho 1999; Zottoli et al. 1999).

The initial exhibition of the C-start response in fishes follows development of the Mauthner neurons (Eaton and Farley 1973; Kimmel et al. 1978). For example, the Mauthner neurons in zebrafish develop very early in embryogenesis and are involved in producing the early C-start responses (Eaton and Nissanov 1985; Eaton and Didomenico 1986). For rainbow trout,
Ballard (1973) detected Mauthner neurons only once the embryos reached stage 23 of development (hatching). In contrast, in the present investigation we determined that rainbow trout embryos began responding with C-starts much earlier (at approximately 52% of full term degree days, just prior to Ballard's stage 17) and likewise in lake trout (at approximately 56% of full term degree days, just prior to Ballard's stage 17). This finding suggests that the Mauthner neurons in these salmonids may possibly be present and functional long before hatch and thus much earlier than previously thought, although they were not specifically identified in each test organism.

Developmentally, the initial exhibition of the C-start response in other fishes has previously been shown to occur before egg hatch (Eaton and Farley 1973; Kimmel et al. 1974; Taylor and McPhail 1985b; Eaton and DiDomenico 1986). For example, C-start responses in zebrafish have been observed as early as 28 hours post-fertilization, corresponding to 30% of full-term embryo development (Grunwald et al. 1988), and can be consistently elicited via tactile stimulation in 100% of embryos as of 48 hours post-fertilization, two days before normal hatching begins or at approximately 50% of full-term embryo development (Easter and Nicola 1996). In the present study, C-start responses could not be elicited in rainbow trout at 140 degree days (42% of full-term). Some rainbow and lake trout embryos did show a response at 170 and 320 degree days of incubation (52% and 56% of full-term degree days), respectively. We do not know whether C-start responses could have been elicited at earlier time points in these salmonids, although our regression models indicate this to be likely. In addition, closely related Atlantic salmon Salmo salar exhibit spontaneous C-starts at approximately 45% of full term degree days (Peterson and Martin-Robichaud 1983). It appears that C-start activity is somewhat delayed in the trout species studied in comparison to the pattern observed in zebrafish, when hatching occurs at 96 hours post-fertilization.

C-start Development and Its Role in Hatch

Hatching in fishes involves the softening of the chorion by hatching enzymes (Hoar and Randall 1969). For Japanese medaka Oryzias latipes, the onset of respiratory movements in the embryo is a prerequisite for the secretion of the hatching enzyme (Yamagami 1981; Yamagami
1988); this may also be true for other species. In salmonids, hatching enzymes appear to generally disrupt the chorion, leading to widespread weakening (Jobling 1995). Less active fish embryos (those exhibiting fewer in ovo C-starts) may be less efficient at distributing the hatching enzyme throughout the perivitteline fluid, leading to slower digestion of the chorion and thus delayed or disrupted hatch (Hayes 1942; Peterson et al. 1980). In such embryos, the concentration of hatching enzyme in one location often leads to partial hatch or half-hatch, which is characterized by the partial emergence of the embryo from a small opening in the chorion followed by death. Hatch is delayed or absent in anesthetized zebrafish embryos (Saint-Amant and Drapeau 1998). External factors such as pH and temperature are also known to influence chorion weakening (Hayes 1942; Haya and Waiwood 1981; Yamagami 1981).

Following the breakdown of the proteinaceous layer of the chorion, hatching is assisted by the rapid flexing movements of the fish embryo (Willemse and Denuce 1973). Successful hatching requires that the outer layer of the egg zona radiata be physically ruptured, presumably by the movements of the embryo inside. Delayed hatch in Atlantic salmon embryos was partially attributable to reductions in embryo movement and less rapid rupture of the outer layer of the zona radiata (Eaton et al. 1977); consequently, embryo movements appear critical to the hatching process (Hayes 1942; Peterson et al. 1980; Peterson and Martin-Robichaud 1983). The pattern of increasing embryo activity up until hatch in both rainbow trout and lake trout, as measured by the number of flexures per stimulus, is consistent with this theory.

Eaton and Nissanov (1985) suggest that spontaneous Mauthner cell firings, resulting in spontaneous C-start movements, play an important role in the hatching of undisturbed embryos; in zebrafish, these firings occurred up until the normal time of hatching, but only rarely thereafter. Saint-Amant and Drapeau (1998) found that such spontaneous behavior begins at 17 hours post-fertilization (i.e., 35% of full term), peaks just prior to the onset of tactile-stimulated responses at 21 hours post-fertilization (44% of full term), and gradually declines in occurrence in zebrafish embryos. Similarly in the present study, spontaneous embryo C-start behavior in rainbow trout was present as early as 140 degree days (42% of full term), before responses to tactile stimulation could be elicited, and continued up until the onset of hatching. Tactile-stimulated
responses in rainbow and lake trout increased significantly in both occurrence and intensity until just prior to the time of normal hatch. Because this pattern of increase matches that seen in zebrafish, we surmise that the C-start responses that we artificially elicited in our salmonids may play a similar role in hatching.

*Embryonic C-start and Predator Avoidance*

One of the main roles of C-start movements in post-hatch larval fishes is thought to be predator avoidance (Webb 1982; Blaxter and Batty 1985), and considerable experimental evidence substantiates this view. Larval responsiveness corresponds to escape ability, and improves during ontogeny in many species (Taylor and McPhail 1985a; Higgs and Fuiman 1996; Hale 1996). In northern anchovy *Engraulis mordax* larvae, a C-start response to predator attack by a biting planktivore increases the probability of escape (Webb 1981). When together with carnivorous protozoa, larval zebrafish exhibit significantly more C-starts than when such predators are absent (Eaton and Nissanov 1985). For young coho salmon *Oncorhynchus kisutch*, Taylor and McPhail (1985a) correlated differences in susceptibility to predation with differences in the degree to which C-start responses featured within the overall suite of escape movements. Thus in post-hatch larval fishes, the function of C-start movements is fairly well defined.

For pre-hatch fishes, the function of the in ovo C-start response remains unclear. Their occurrences are spontaneous, but can also be elicited in response to external stimuli. Some embryo C-start responses are strong enough to break the egg envelope (Eaton et al. 1977); consequently, Eaton and Nissanov (1985) proposed that pre-hatch C-start movements might allow embryos to emerge prematurely from the egg capsule and move to a more protected area in order to escape a predator. Rainbow trout and lake trout may be similarly advantaged, in that what is purportedly an antipredatory behavior in hatched larval fishes may also be so in pre-hatch individuals. The fact that C-starts in these fish can be repeatedly activated in ovo at relatively high frequency may support this protective feature of the early responsiveness, given that habituation to tactile stimulation can occur shortly after hatch in some species.

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References


Chapter 4: 2,3,7,8-Tcdd-exposure decreases embryo C-start response in rainbow trout and lake trout

Abstract

Rainbow trout and lake trout prehatch C-start responses were examined in embryos injected as eggs with 2,3,7,8-Tcdd. The C-start in teleost fishes is a type of startle response, and mediates the ability to respond to abrupt, unexpected stimuli. The C-start has several functions in pre-hatch fish embryos, including assisting in the mechanical breakdown of the egg chorion, successful hatch, and predator avoidance in later stages. Using tactile stimulation, the C-start response was evaluated in rainbow trout (Oncorhynchus mykiss) and lake trout (Salvelinus namaycush) pre-hatch embryos at three developmental stages (early, middle, late). The proportion of embryos exhibiting C-starts in response to tactile stimulation decreased in Tcdd-injected rainbow trout and lake trout embryos, and the number of C-starts exhibited per tactile stimulus also declined with increasing dose of Tcdd at most of the time points for both species. C-start responses per stimulus were decreased in rainbow trout even at 75 pg/g Tcdd, a relatively low dose. In addition, there was a highly negative correlation between half-hatch mortality and the proportion of embryos exhibiting C-starts at the late time point in both rainbow trout (0.9595) and lake trout (0.9359). These results suggest that early C-start responses may be a useful endpoint in assessing early life stage toxicity of 2,3,7,8-Tcdd and related compounds in fishes.

Introduction

Planar halogenated hydrocarbons (PHHs) are a class of toxic lipophilic, bioaccumulative contaminants that have the potential to cause adverse effects on survival, growth, and reproduction in certain fish species. The early life stages of fish are known to be particularly sensitive to the toxic effects of PHHs such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (Tcdd), with salmonid species such as rainbow trout (Oncorhynchus mykiss) and lake trout (Salvelinus namaycush) tending to be the most sensitive fish species to Tcdd-induced early life stage toxicity. As evidence of the latter, rainbow trout LD50 values of between 230 pg/g and 488 pg/g have been reported for various strains of the species (Walker and Peterson 1991). Lake trout respond to even lower exposure levels, with LD50s ranging from 44 pg/g to 85 pg/g (Guiney et al. 1996; Guiney et al. 1997; Walker et al. 1994; Walker et al. 1996; Zabel et al. 1995; Wright and Tillitt, unpubl.)

Although the acute toxicity of Tcdd has been well documented in a variety of fish species, the possible sublethal behavioral effects of the low doses more likely to be present in aquatic environments are not well known. This is especially true as regards the early life stages of fish, which are generally the most sensitive to chemical exposure (Spitsbergen et al. 1991;
Walker and Peterson 1990; Walker et al. 1991). Sublethal affects such as hemorrhaging, yolk sac edema, and craniofacial deformities have been produced in both rainbow trout and lake trout fry following exposure to a complex chemical extract of Lake Michigan lake trout (Wright and Tillitt 1999; Wright and Tillitt unpubl.) and these effects were seen at very low exposure levels. As yet unknown is what, if any, behavioral effects may be produced from similarly low levels of TCDD.

A suite of behaviors occur in developing early life stage salmonids. These may include behaviors related to hatching, emergence, phototaxis, rheotaxis, swimming, feeding, and predator avoidance (Dill 1977; Hoar and Randall 1969). The C-start escape behavior, a type of startle response, is one of the earliest behaviors manifested in fish embryos and is visible even in prehatch embryos (Eaton et al. 1977; Kimmel et al. 1974; Wright et al. 2003). This response is initiated by two Mauthner cells, located in the hindbrain (Zottoli 1977; Eaton and Farley 1973; Eaton and Farley 1975; Eaton et al. 1981; Diamond 1971). The Mauthner cells appear early in embryogenesis, differentiate rapidly, and reach mature morphological form at about hatch in teleost fish. The embryo possesses the full, functional neural circuitry for the initiation of coordinated C-starts while in the egg capsule; consequently, embryonic responsiveness to tactile stimuli is one of the earliest behavioral tests that can be conducted on a fish (Saint-Amant and Drapeau 1998; Eaton and DiDomenico 1986; Easter and Nicola 1996; Hale 1996). In free-swimming fish larvae and adults, the C-start functions in predator escape (Eaton et al. 1977; Webb 1981; Webb 1982; Blaxter and Batty 1985) whereas in prehatch embryos it likely functions to distribute the hatching enzyme (Willemse and Denuce 1973; Yamagami 1981; Shoots et al. 1982). The neurobiology of the C-start response has been studied extensively in the zebrafish (Brachydanio rerio) (Eaton and Hackett 1984; Kimmel et al. 1980; Lui and Fetcho 1999) and the goldfish (Carassius auratus) (Zottoli 1977; Eaton et al. 1981; Eaton et al. 1982; Zottoli et al. 1999). Recent work has described the ontogeny of the behavior in rainbow trout and lake trout embryos (Wright et al. 2002).

The C-start response has been used as a behavioral endpoint in the study of chemical exposure in juvenile medaka (Rice et al. 1997). These fish also exhibit altered startle reactions and escape behavior in response to several organic chemicals (Carlson et al. 1998). We have
previously developed a simple *in ovo* tactile stimulus test for eliciting the C-start response in rainbow and lake trout embryos, and have used this assay in describing the ontogeny of the behavior at several prehatch developmental time points (Wright et al. 2003). The objective of this study was to test whether and how tactile-stimulated C-start response is affected in rainbow trout and lake trout embryos injected as newly fertilized eggs with TCDD. In addition, the relationship between C-start activity and hatching success was examined.

**Methods**

*TCDD Injection of Eggs (LD0, LD50, LD100)*

Rainbow Trout: Green unfertilized Erwin strain rainbow trout eggs were obtained from Ennis National Fish Hatchery, Ennis, MT, on June 3, June 10, and June 17, 1998. Rainbow trout eggs were pooled from 2-3 three-year-old females, and the milt was a pooled sample from 2-3 males. Upon arrival, the eggs and milt were slowly warmed separately to 10°C. Eggs were fertilized on-site at the Columbia Environmental Research Center, Columbia, MO, according to standard methods (Leitritz and Lewis 1976; Walker et al. 1996). Briefly, sperm motility was tested by placing a drop of milt and a drop of 1% saline solution on a glass microscope slide and examining the slide under a microscope for sperm movement and number. Eggs were placed in a cool, dry mixing bowl, and 30-40 ml of 1% saline solution was added to the eggs along with the milt. The mixture was gently mixed by hand for approximately one minute, and then the eggs were strained to dispose of wastewater and were placed directly into plastic screened rearing baskets in separate 16-tray vertical flow Heath Stacks® at 10°C (± 1°C).

The egg preparation and TCDD injection procedures followed standard methods (Walker et al., 1996). Briefly, prior to injection with TCDD, the fertilized and water-hardened eggs were individually inserted into U-shaped wells in preformed agarose plates. Each plate's 36 eggs were then injected using pre-filled glass micropipettes (15° bevel, 8-12µm diameter) and a regulated gas pressure injection system to deliver picoliter volumes of the injection solution. Triplicate TCDD dose-response trials were conducted, one per week, using three injection doses of 0 (triolein alone), 200 and 800 pg/g TCDD. These TCDD doses roughly correspond to the early life
stage LD0, LD50, and LD100 values for this strain of rainbow trout (Walker et al. 1991). Our sample sizes were 100 injected eggs per dose per replicate. Following injection, the eggs were cultured in plastic mesh baskets at 10±1°C in the flow-through 16-tray vertical incubator supplied with aerated well water. Nonviable and dead eggs were culled daily. Mortality was monitored carefully during hatch, and the number of half-hatch mortalities (embryos that died partially emerged from the egg capsule) were recorded for each TCDD treatment groups.

Lake Trout: Green, unfertilized lake trout eggs and milt were received on October 15, 1998 from Hiawatha National Fish Hatchery, Brimley, Michigan. Eggs and milt were allowed to slowly warm to within one degree of incubator temperature (8°C). Lake trout eggs were pooled from 2-3 three-year-old females, and the milt was a pooled sample from 2-3 males. Eggs were fertilized on-site using the same method as was used for the rainbow trout.

Triplicate groups of 100 eggs per dose were injected with 0, 50, and 100 pg/g TCDD. The egg preparation and injection techniques were identical to those used for rainbow trout egg injections. Following injection, the lake trout egg replicates were placed in plastic mesh baskets at 8±1°C in the flow-through 16-tray vertical incubator supplied with aerated well water. Nonviable and dead eggs were culled daily, and half-hatch mortalities were also recorded.

C-start Assay

Tests of embryo C-starts were conducted at three developmental time points measured as Temperature Units, T.U. (i.e., degree days, daily water temperature in °C multiplied by day post-fertilization). The three-stage C-start test began by randomly selecting embryos from the respective treatment groups, and placing them by group into individual pre-formed wells in agarose plates. These shallow wells (U-shaped in cross-section) allowed each egg to be held firmly in place during subsequent tactile stimulation. Each loaded plate was then returned to the vertical flow incubator for 24 hours, during which the embryos all oriented themselves uniformly, head and back uppermost against the egg capsule. No mortality was observed during this part of the procedure.

The test involved positioning one of the embryos-bearing agarose plates under a stereoscopic microscope (15x magnification). Each embryo was then stimulated manually; this
involved using a 10 mm diameter blunt glass probe to briefly touch each chorion surface at a standardized location (dorso-lateral position of thorax anterior to the dorsal fin) and with a consistent force. The force applied lasted approximately 1 sec, and was just enough to dimple the egg capsule. Three stimuli were applied in sequence to each egg at 10-second intervals (0 sec = '1st stimulus'; 10 sec = '2nd stimulus'; 20 sec = '3rd stimulus'), after which the next embryo was brought into view.

For each stimulus applied to each embryo, the absence or presence of a C-start response was recorded; specifically, the presence of a response involved a trunk flexure, always first in the opposite direction of the stimulus, into a "C" shape with the nose and tail being brought into close proximity. In addition to the absence or presence of a C-start response, the number of consecutive flexures that occurred in the following 10-second period were recorded. Slight movement by the embryo, although not considered a C-start response, was noted where it occurred.

Rainbow trout embryos from the 0, 200, and 800 pg/g TCDD triplicates were tested for C-start activity at three developmental time points: Early (220 T.U.), Middle (260 T.U.), and Late (320 T.U.). In terms of developmental stage, these time points correspond to 67%, 79%, and 97% of full term (i.e., hatch), respectively.

The C-start responses of lake trout embryos were tested using an identical assay to that described above. The triplicate groups of embryos from the three TCDD doses (0, 50, and 100 pg/g) were each tested at 360 T.U. (Early), 480 T.U. (Middle), and 560 T.U. (Late). Again, in terms of developmental stage, these time points correspond to 63%, 84%, and 98% of full term (hatch), respectively.

Sublethal TCDD Injections

The purpose of this portion of the study was to monitor individual embryos through time, and to quantify their C-start responses, mortality rates (egg and hatching), and the occurrence of gross pathological lesions such as hemorrhaging and edema for these individuals. Eagle Lake strain green unfertilized rainbow trout eggs were received on February 7, 1999 from Ennis National Fish Hatchery, Ennis, Montana. The egg batch was a pooled sample from 2-3 three-
year-old females, and the milt was a pooled sample from 2-3 males. Upon arrival, the eggs and milt were slowly warmed separately to 10°C, after which eggs were fertilized on-site according to the methods described above.

Triplicate groups of 16 eggs each were injected with 0 and 75 pg/g TCDD (total of 48 eggs per dose), following the methods described above. The LD50 for Eagle Lake strain rainbow trout is 488 pg/g (338-580) (Walker and Peterson 1991). Each replicate was then placed into separate, random plastic mesh baskets at 10±1°C in the flow-through 16-tray vertical incubator supplied with aerated well water. Nonviable and dead eggs were culled daily.

C-start Test: Twenty-four hours prior to the first C-start test, triplicate sets of 10 eggs each were randomly chosen from the original 16-egg batches, and were placed into labeled U-shaped wells in preformed agarose plates. These individuals remained in these labeled plated for the remainder of the study. The C-start response of embryos was tested at 300 T.U., and 410 T.U., corresponding to 68% and 93% of full term embryo development. Testing was conducted in the same manner as described above. In addition the C-start response, the presence of gross pathological lesions was recorded for the individual embryos. Unlike in the standard C-start tests, the same embryos were left in the agarose plates after the first testing at 300 dd and were replaced in the incubator for later testing at 410 dd. After the second C-start test, the plates were again placed into the incubator and were carefully monitored until hatch was complete.

Hatch began at 440 T.U. and was complete by 500 T.U. Status of hatch was recorded for individual embryos daily; embryos were designated as follows: 1) ‘Egg’, meaning that hatching had not occurred, 2) ‘Hatch’, meaning that hatching was complete and the individual was free from egg capsule and was resting on the agarose surface, 3) ‘Half-hatch’, meaning that the embryo died partially emerged from the egg capsule, and 4) ‘Abnormal Hatch’, meaning that the embryo was alive and resting on the surface of the agarose plate, but was only partially emerged from the egg capsule.
Results

The Effect of TCDD on the Percentage of Embryos Exhibiting C-starts

The proportion of embryos exhibiting C-starts in response to tactile stimulation decreased in TCDD-injected rainbow trout and lake trout embryos (Figure 1). In rainbow trout, the proportion of embryos exhibiting C-starts was significantly different among TCDD treatments at both the middle ($F = 101.33$, 2 d.f., $p < 0.001$) and late ($F = 5.89$, 2 d.f., $p < 0.001$) developmental time points (Figure 1A). At both of these time points, both the 200 pg/g and 800 pg/g treatment means were significantly lower than control means (Dunnett's Multiple Comparisons Test, $p < 0.05$). At the early time point, the proportion of embryos exhibiting C-starts was reduced from 63.3% in the 0 pg/g treatment to 53.3% and 45.7% in the 200 pg/g and 800 pg/g treatments, respectively; however, this reduction was not statistically significant. At the middle and lake time points, this decline was even greater. At the middle time point, the percentage of embryos exhibiting C-starts fell from 93.3% in the 0 pg/g groups to 66.7% and 26.7% in the 200 pg/g and 800 pg/g groups, respectively, and at the late time points, the percentage declined from 100% in the 0 pg/g to 50% and 6.7% in the 200 pg/g and 800 pg/g groups, respectively (Figure 1A).

The pattern was very similar in lake trout embryos. At both the early and late time points there was a significant difference among treatment means (One-way ANOVA, $F = 10.33$, 2 d.f., $p = 0.01$ and $F = 256.5$, 2 d.f., $p < 0.001$, respectively). However, the difference in means was not significant at the middle time point ($F = 4.76$, 2 d.f., $p = 0.06$) (Figure 1B). At the earliest testing time point, the percentage of embryos exhibiting C-starts decreased from 40% in the 0 pg/g treatment to 35% in the 50 pg/g embryos and significantly declined to 10% in the 100 pg/g group. At the middle time point, the proportion of lake trout embryos exhibiting C-starts declined from 80% to 65% to 50% in the 0 pg/g TCDD, 50 pg/g TCDD and 100 pg/g TCDD groups, respectively. At the late time point, the percentage significantly declined from 100% to 55% to 25% in the 0 pg/g TCDD, 50 pg/g TCDD and 100 pg/g TCDD, respectively (Dunnett's Multiple Comparison Test, $p < 0.05$; Figure 1B).
C-starts Per Tactile Stimulus in TCDD-injected Embryos

In addition to a decrease in the proportions of embryos exhibiting C-starts, the numbers of C-starts exhibited per tactile stimulus also declined with increasing TCDD dosage at most of the time points for both species (Figure 2). In rainbow trout at the middle and late time points, there was a significant difference between C-start intensity among TCDD treatment groups ($F = 12.14$, 2 d.f., $p = 0.008$, and $F = 15.05$, 2 d.f., $p = 0.005$, respectively based on One-way ANOVA). At these time point, the numbers of C-starts per stimulus declined across the three TCDD dosage groups, with means for the 200 pg/g and 800 pg/g groups significantly reduced (Dunnett’s Multiple Comparison Test, $p < 0.05$). For the middle time point, C-starts per stimulus declined from 1.67 at 0 pg/g to 0.76 at 200 pg/g to 0.36 at 800 pg/g. Similarly, at the late time point, C-starts per stimulus declined from 1.27 to 0.74 to 0.33 at 0 pg/g, 200 pg/g, and 800 pg/g, respectively.

In lake trout, the mean C-start intensity was significantly different among treatment groups at all three time points (One-way ANOVA, $p < 0.05$; Figure 2B). At the earliest time point, the numbers declined from 0.77 in the 0 pg/g group to 0.50 in the 50 pg/g group, and significantly declined to 0.05 in the 100 pg/g group (Dunnett’s Multiple Comparison Test, $p < 0.05$). At the middle and late tests, C-starts per stimulus declined from 1.1 and 1.4 in the 0 pg/g groups respectively, to 0.61 and 0.43 in the 50 pg/g groups, respectively (Dunnett’s Multiple Comparison Test, $p < 0.05$). The number of C-starts per stimulus significantly reduced to 0.36 at the middle time point and was 0.17 at the late time point in the 100 pg/g treatments (Dunnett’s Multiple Comparison Test, $p < 0.05$, Figure 2B).

Half-Hatch Mortality

In rainbow trout, embryos exhibiting lower C-start responses due to high levels of TCDD exposure were more likely to die partially emerged from the egg (i.e., half-hatch mortality). There was an highly negative correlation between half-hatch mortality and the proportion of embryos exhibiting C-starts at the late time point, 97% and 98% of full term development in rainbow trout (-0.9595) and lake trout (-0.9359), respectively (full term corresponds to complete hatch) (Figure
Figure 1. Percentage of embryos exhibiting C-starts in (A) rainbow trout and (B) lake trout injected as eggs with 2,3,7,8-TCDD. For rainbow trout, triplicate groups of 100 newly fertilized eggs were injected with 0 (triolein), 200 or 800 pg/g TCDD, respectively. For lake trout, triplicate groups of 100 eggs per dose were injected with 0, 50 and 100 pg/g TCDD. Mean percentages with standard deviations are shown at three developmental time points (Early, Middle, Late). In rainbow trout, these time points correspond to 220 temperature units (T.U.), 260 T.U., and 320 T.U., respectively while in lake trout they correspond to 360 T.U., 480 T.U., and 560 T.U., respectively. ** indicates significant differences compared to 0 pg/g TCDD dose based on One-way ANOVA followed by Dunnett’s Multiple Comparison Test, p < 0.05.
Figure 2. C-starts per stimulus versus 2,3,7,8-TCDD doses in (A) rainbow trout embryos and (B) lake trout embryos at three developmental time points (Early, Middle, Late). Data points represent mean values with standard deviations. For rainbow trout, triplicate groups of 100 newly fertilized eggs were injected with 0 (triolein), 200 or 800 pg/g TCDD, respectively. For lake trout, triplicate groups of 100 eggs per dose were injected with 0, 50 and 100 pg/g TCDD. Mean percentages with standard deviations are shown at three developmental time points (Early, Middle, Late). In rainbow trout, these time points correspond to 220 temperature units (T.U.), 260 T.U., and 320 T.U., respectively while in lake trout they correspond to 360 T.U., 480 T.U., and 560 T.U., respectively. "*" indicates significant differences compared to 0 pg/g TCDD dose based on One-way ANOVA followed by Dunnett’s Multiple Comparison Test (p < 0.05).
Rainbow trout embryos injected with 0 pg/g TCDD as eggs exhibited a 100% embryo C-start response rate and only 2% embryo half-hatch mortality, while in the 200 pg/g TCDD treatment group only 50% of embryos exhibited C-starts and 30.3% died while partially emerged from the egg capsule (Figure 3A). In the 800 pg/g TCDD group, a half-hatch mortality rate of 76.4% corresponded to only 6.7% of embryos exhibiting C-start at the late testing time point, shortly before the onset of hatch (Figure 3A).

A similar inverse relationship between half-hatch mortality and the proportion of embryos exhibiting C-starts existed in lake trout. In the 0 pg/g TCDD treatment group, in which 100% of the embryos exhibited C-starts at the late time point, 6.3% died while partially emerged from the egg capsule (Figure 3B). In the 50 pg/g TCDD group, 55% of the embryos exhibited C-starts and 26.1% experienced half-hatch mortality. In embryos injected as eggs with 100 pg/g TCDD, only 25% exhibited C-starts in response to tactile stimulation while half-hatch mortality was 38.5% (Figure 3B).

Sublethal TCDD Injections

In this portion of the study, individual rainbow trout embryos injected with either 0 pg/g TCDD or 75 pg/g TCDD were monitored through two C-start testing time points (corresponding to 68% and 93% of full term embryo development) as well as through hatch. There was no significant difference in the proportion of embryos from the 0 pg/g group exhibiting C-starts vs. the number of embryos from the 75 pg/g group exhibiting C-starts at either the first time point ($t = 3.35, 2$ d.f., $p = 0.19$) or the second time point ($t = 1.11, 2$ d.f., $p = 0.57$). In terms of C-starts per tactile stimulation, the numbers did not differ significantly for both treatment groups at the first testing time point (0.82 at 0 pg/g TCDD, 0.71 at 75 pg/g TCDD, $t = .341$, $p = 0.65$, 59 d.f.). However, there was a significant decrease in the number of C-starts per stimulus exhibited by those embryos injected as eggs with 75 pg/g TCDD group, with 0.98 C-starts per stimulus, versus 1.5 C-starts per stimulus exhibited by those embryos from the 0 pg/g TCDD ($t = 6.59$, $p = 0.02$, 47 d.f.). In addition, significantly more of the embryos from the 75 pg/g TCDD treatment groups exhibited gross pathological lesions, 16.6% versus 3.3% in the 0 pg/g TCDD groups ($t = 7.36$, 2
Figure 3. Mean percentage (with standard deviations) of embryos exhibiting C-starts at increasing doses of 2,3,7,8-TCDD, compared to mean percentage (with standard deviations) of half-hatch mortalities in (A) rainbow trout and (B) lake trout. For rainbow trout, triplicate groups of 100 newly fertilized eggs were injected with 0 (triolein), 200 or 800 pg/g TCDD, respectively. For lake trout, triplicate groups of 100 eggs per dose were injected with 0, 50 and 100 pg/g TCDD.
d.f., \( p = 0.03 \) and exhibited abnormal hatch or half-hatch mortality (13.3% vs. 0% in the 0 pg/g TCDD groups, \( t = 6.15, 2 \text{ d.f.}, p = 0.05 \)).

To compare the number of C-starts per stimulus in embryos that exhibited normal versus abnormal hatch, mean values for abnormally hatching embryos, and for the normally hatching embryos, normally hatching embryos from the 0 pg/g TCDD treatments, and normally hatching embryos from the 75 pg/g TCDD treatments were calculated separately for the 1\textsuperscript{st}, 2\textsuperscript{nd}, and 3\textsuperscript{rd} stimulus (Table 1). Abnormally hatching embryos included both those that died partially emerged from the chorion, and those that partially emerged but lived. When the respective means were compared, embryos that hatched normally exhibited significantly more C-starts per 1\textsuperscript{st} stimulus than those that hatched abnormally (0.50 vs. 1.27, respectively \( t = -2.07, 58 \text{ d.f.}, p = 0.02 \)) (Table 1). When the normally hatching embryo results were separated into those from the 0 pg/g TCDD treatment and those from the 75 pg/g TCDD treatment and compared separately to the abnormally hatching embryos, the same pattern held \( t = -1.95, 32 \text{ d.f.}, p = 0.02 \), and \( t = -2.05, 28 \text{ d.f.}, p = 0.02 \) for Abnormal vs. Normal/0 pg/g and Abnormal vs. Normal/75 pg/g, respectively) (Table 1). However, no significant difference existed in the C-start response count per 1\textsuperscript{st} stimulus between the normally hatching embryos in the 0 pg/g TCDD treatment and the normal fish from the 75 pg/g TCDD treatment (Table 1).

There were no significant differences in C-start counts of abnormally and normally hatching embryos for the 2\textsuperscript{nd} stimulus; C-start counts were 1.75 and 1.46 in the abnormal and normal hatch groups, respectively. At the 3\textsuperscript{rd} stimulus, there was no (0) c-starts in the abnormally hatching embryos (Table 1), and this was significantly different from both the 0.91 average counts observed in the normally hatching embryos \( t = -1.72, 58 \text{ d.f.}, p = 0.04 \) and the 1.4 average C-starts exhibited in just the normally hatching embryos from the 0 pg/g TCDD treatment group \( t = -2.43, 32 \text{ d.f.}, p = 0.01 \). Also for the 3\textsuperscript{rd} stimulus, there was a significant difference \( t = -4.3, 54 \text{ d.f.}, p = 0.0003 \) between the number of C-starts in normally hatching embryos from the 0 pg/g TCDD group and the number of C-starts exhibited in normally hatching embryos from the 75 pg/g TCDD group (1.4 and 0.35, respectively) (Table 1).
Table 1. C-start magnitude in rainbow trout embryos based on successful hatch and TCDD treatment. Hatch began at 440 T.U. and was complete by 500 T.U. Status of hatch was recorded for individual embryos daily; embryos were designated as follows: 1) ‘Egg’, meaning that hatching had not occurred, 2) ‘Hatch’, meaning that hatching was complete and the individual was free from egg capsule and was resting on the agarose surface, 3) ‘Half-hatch’, meaning that the embryo died partially emerged from the egg capsule, and 4) ‘Abnormal Hatch’, meaning that the embryo was alive and resting on the surface of the agarose plate, but was only partially emerged from the egg capsule. I. Mean C-start magnitude values (thrashes per stimulus) with standard deviations for 1st, 2nd, and 3rd stimuli. II. P-values (and degrees of freedom) based on t-test comparisons of treatments. **P-values** represent a significant difference between groups.

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Discussion

*Behavioral toxicology and the use of C-starts*

Endpoints of behavioral toxicity tend to be more sensitive to chemical exposure effects than are endpoints such as growth, pathology and survival (Little 1985). In addition, environmentally relevant concentrations of contaminants may be below a threshold for overt mortality, but may be above the threshold for sublethal effects. Demonstrating this, extracts of Lake Michigan lake trout caused sublethal gross pathological lesions such as yolksac edema in both lake trout and rainbow trout fry, and at concentrations below the lowest observable adverse effect level (LOAEL) for mortality (Wright and Tillitt 1999; Wright and Tillitt unpubl). Doses well below the LD50 for TCDD caused gross and histopathological lesions in rainbow trout, and growth was retarded even at a very low TCDD dose (0.1 ppb) (Helder 1981). Thus, studies that address sublethal effects of potential contaminants in fish are essential.

Behavioral toxicology studies in fish early life stages are especially important due to the increased sensitivity of this developmental period in most fish species (Kleeman et al. 1988; Spitsbergen et al. 1991). Behavioral toxicology studies of fish early life stages have been less extensive compared to those of adults, yet the ontogeny of many behaviors necessary for survival occurs early in development (Dill 1977; Noakes 1978). Of those conducted, reduced and altered emergence success, and decreased foraging efficiency was caused by benzo[a]pyrene egg exposure of coho salmon fry (Ostrander et al. 1988), and the emergence behaviors in rainbow trout were adversely affected by egg benzo[a]pyrene exposure (Ostrander 1990). However, the behavioral effects of exposure to PHHs in salmonid early life or juvenile stages have not been extensively studied. Exposure of young rainbow trout to TCDD and TCDF did induce behavioral impairments that became progressively worse over time and with increasing concentration (Mehrle et al. 1988). In Atlantic salmon, phototactic behavior and predator avoidance were altered in fry exposed as eggs to PCBs (Fisher et al. 1994), and fry exposed as eggs with a sublethal dose of PCBs were significantly more vulnerable to predation that control fry. However, broader study of the effects of PHHs and related compounds on fish early life stages is needed.
In particular, the study of sublethal behavioral effects of dioxin-like compounds on the early life stages of a variety of fish species would benefit the overall base of knowledge related to PHHs.

Sublethal behavioral testing of contaminants in fish involves several challenges, including developing procedures for the use of fish early life stages, as well as choosing clearly defined endpoints that have direct ecological significance (Little et al. 1993). The use of C-start responses in fish embryos addresses both of these issues. Because its ontogeny (both physiological and behavioral) is well defined in pre-hatch and larval fishes of several species, the C-start assay is clearly applicable to early life stage studies (Eaton and Farley 1973; Kimmel et al. 1974; Taylor and McPhail 1985b; Saint-Amant and Drapeau 1998). Also, the ecological effects of altered C-starts and startle response behaviors on predator avoidance are well established (Eaton and Hackett 1984; Taylor and McPhail 1985a; Eaton and DiDomenico 1986).

In juvenile and adult fish, C-starts and startle response can be altered by exposure to certain environmental contaminants. Carbaryl and phenol exposure in juvenile medaka affected Mauthner cell to motorneuron transmission, whereas chlorpyrifos, carbaryl, phenol, and 2,4-dinitrophenol resulted in neuromuscular effects (Carlson et al. 1998). Five compounds with different modes of action altered the startle responses (tactile stimulated) in medaka (Rice et al. 1997). Exposure of rainbow trout to TCDD and TCDF caused the loss of their startle response to an overhead stimulus, consisting of waving a hand above the aquaria (Mehrle et al. 1988). Although the C-start response has been characterized in embryo and larval fish, few have been conducted at the earliest life stages, and therefore additional toxicological studies of this behavior in the early life stages of fish are needed.

*Relationship Between C-starts and Hatch*

The physiological and biochemical dynamics of hatch among teleosts involves both the secretion of chorionase, a hatching enzyme, and the mechanical circulation of this enzyme via embryo movement to ensure uniform distribution over the internal surface of the egg chorion prior to hatch (Yamagami 1981). The time between the secretion of the hatching enzyme and hatch is affected by the degree of embryo movement (Hayes 1942). Successful hatch can be affected by a variety of factors. In Atlantic salmon eggs, low pH caused both delayed onset of hatch, and an
increase in the incidence of half-hatch mortality (Peterson et al. 1980; Haya and Waiwood 1981), possibly due to decreased chorionase activity. However, declining embryo activity leading to less efficient distribution of the hatching enzyme throughout the perivitteline fluid may also have been involved, thus supporting our finding that TCDD-induced decreases in C-start activity can lead to declining embryo activity.

Adverse effects on hatch due to chemical exposure have been previously reported. It is clear from the present study that the incidence of half-hatch mortality is TCDD dose-dependent, and is highly negatively correlated with C-start response intensity in rainbow trout and lake trout embryos. For other studies, delayed hatching was caused by benzo[a]pyrene egg exposure of coho salmon fry (Ostrander et al. 1988). In a variety of fish species elevated half-hatch mortality appears characteristic of PHH toxicity. In pike, (Esox lucius), TCDD exposure of newly fertilized eggs caused delayed hatch and up to 90% half-hatching and abnormal hatch. This was thought to be a consequence of fry underdevelopment (Helder 1980). TCDD-related hatching mortality is also seen in Japanese medaka (Wisk and Cooper 1990) and brook trout (Salvelinus fontinalis) (Walker and Peterson 1994). In both lake trout and rainbow trout, TCDD exposure of eggs produced dose-related increased in the incidence of mortality during hatch (Spitsbergen et al. 1991; Walker et al. 1991; Walker et al. 1992). For lake trout, Spitsbergen et al. (1991) described half-hatch as involving the emergence of 25-30% of the yolk sac from the egg capsules. Profound neuromuscular weakness of the embryos was noted, indicating the possibility that reduced C-starts may not represent direct effects on M-cell activity, but may be due to lack of sufficient muscle strength for the response. For Spitsbergen et al. (1991), the criteria for abnormal hatch (the embryo was free-swimming but not completely emerged from the eggshell) were similar to that used in the present study. In addition, fry that failed to completely shed their eggshells on their tails were more susceptible to subsequent fungal infections (Spitsbergen et al. 1991). Thus, even in embryos that are free swimming after abnormal hatch, there exists the possibility of adverse effects on survival.
Ecological Significance of Altered C-start

The ecological significance of altered C-start response due to embryonic exposure to TCDD and related compounds is likely to be complex. Although the performance of C-start responses improves through development in species such as chinook salmon, coho salmon, rainbow trout, and lake trout (Taylor and McPhail 1985b; Hale 1996; Wright et al. 2002), hatchability due to TCDD exposure may decrease thus reducing sac fry numbers. In rainbow trout, half-hatch mortality was significantly elevated at a TCDD dose of 200 pg/g (Wright 1995). Fisher et al. (1994) found no difference in hatching success between Atlantic salmon controls and those exposed as eyed embryos to a PCB mixture, however the nominal concentrations of the mixture were below the lowest observable adverse effect concentration (LOAEC) for mortality. More subtle effects of TCDD exposure on salmonid egg hatchability as it relates to C-start responses require additional study.

Sublethal alterations in C-start responses can directly affect embryo and fry survival. C-starts are an essential part of the startle response to external stimuli such as the presence of a potential predator (Eaton and DiDomenico 1986; Taylor and McPhail 1985a). Because predation is thought to be the main source of larval mortality in many fish species (Bailey 1984; Bailey and Batty 1984), any physiological or behavioral changes that increase the risk of predation would be significant. Even in prehatch embryos, in which some C-start activity may be strong enough to break the egg envelope, such movements might function in premature emergence from the egg capsule in response to the presence of a predator (Eaton et al. 1977; Eaton and Nissanov 1985). In the wild, lake trout egg predation by crayfish may contribute to early mortality (Savino and Miller 1991), although Savino and Henry (1991) found no difference in the predation susceptibility of early life stages of lake trout between normal and contaminated offspring of Lake Michigan females.

In addition to roles in hatch and predator avoidance, the C-start response may play a role in the terminal phase of prey capture (Canfield and Rose 1993). Because the most critical stage in the survival of young fish is likely to be the transition to active feeding, disruptions in the timing of initial feeding, feeding behavior or success could negatively impact fish survival (Twongo and
MacCrimmon 1976). Although, the participation of Mauthner-initiated C-starts in prey capture remains unclear (Budick and O’Malley 2000), studies like ours provide insight into the likely effects of contaminants on early life stage fish in the wild.

Summary

The use of embryo C-start response to tactile stimulation appears to be a useful endpoint in behavioral studies of 2,3,7,8-TCDD toxicity. This response is common to all teleosts and has been well characterized in the embryo and larval stages of a number of fish species. Because C-starts can be elicited in fish embryos very early in development, this endpoint may be especially useful in early life stage studies of chemical exposure. In the present study, 2,3,7,8-TCDD exposures of rainbow trout and lake trout eggs produced dose-related decreases in both the proportion of embryos exhibiting C-start responses, and in the relative activity of each response in terms of C-starts per stimulus. Decreased C-start activity was correlated with increased half-hatch mortality, supporting the role of C-starts in successful hatch. Even at a relatively low 2,3,7,8-TCDD dose of 75 pg/g, C-start activity was significantly decreased and the incidence of half-hatch mortality was elevated. In the wild, alterations in C-start activity of fish embryos exposed to contaminants such as PHHs could potentially affect hatching success, predator avoidance, and other behaviors in which C-starts may play a role.

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Chapter 5: Feeding Efficiency in Young Lake Trout and Rainbow Trout Injected as Eggs with 2,3,7,8-TcDD

Abstract

External feeding in salmonids begins a short time after the absorption of the yolk sac following emergence, and this period of change from endogenous to exogenous feeding is often marked by high mortality from both predation and starvation. Impaired feeding behavior in fish early life stages may result in reduced growth, and therefore feeding behavior is an important endpoint in early life stage behavioral toxicity studies. The feeding efficiency and total number of prey eaten were studied in rainbow trout and lake trout fry injected as eggs with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In addition, these endpoints as well as strike latency were considered at two different prey densities in rainbow trout injected as eggs with TCDD. Although the capture rate generally was not significantly affected by TCDD dose, the number of prey ingested was significantly lower at 225 pg/g and 450 pg/g in rainbow trout and at 100 pg/g in lake trout at three different time points. Similarly, rainbow trout feeding at two different prey densities consumed fewer prey at 225 pg/g and 450 pg/g, and strike latency was significantly delayed. In general, age was a significant covariable for both feeding efficiency and number of prey eaten. Results suggest that both feeding efficiency and total number of prey are important endpoints in early life stage feeding studies; however other measurements (i.e., strike latency) maybe more subtle indicators of lower, environmentally relevant contaminant levels.

Introduction

Young salmonids such as rainbow trout (Oncorhynchus mykiss) and lake trout (Salvelinus namaycush) undergo a behavioral, morphological and physiological shift as they develop from embryonic endogenous feeders to free-swimming larval and juvenile exogenous feeders (Dill 1977; Balon 1981). The feeding behavior of late-larval and juvenile salmonids requires both motor coordination and sensory interpretation (vision, lateral line) and a young fish must accomplish searching, detection, capture, and ingestion of food successfully in order to acquire food. External feeding in salmonids begins a short time after the absorption of the yolk sac following emergence. At this time, fry begin to actively search out prey items, and may begin competing with other fry for food. This period of change, accompanied by dispersal of the young away from spawning areas, is often marked by high mortality from both predation (Hones et al. 1995; Krueger et al. 1995; Kiggs and Fuiman 1996) and starvation (Savino and Henry 1991).

Impaired feeding behavior in fish early life stages may result in reduced growth, and rapid growth is important in larval and juvenile fishes because predation risk decreases with increasing
prey size (Pedersen 1997). Consequently, feeding behavior is an important endpoint in early life stage behavioral toxicity studies (Little et al. 1993). Any additional stresses on the young, including contaminant-induced changes in behaviors or physiological processes related to feeding, could potentially reduce the animal’s chances of survival.

Many studies in fish have documented contaminant-induced alterations of key early behaviors such as predator avoidance capabilities, prey capture rates and startle responses (Beitinger 1990; Carlson et al. 1998). In addition, morphological alterations caused by contaminant exposure can affect related behaviors. For example, contaminant-induced changes in eye structure could greatly diminish prey capture rates (Savino et al. 1993). Sublethal morphological changes in craniofacial features such as jaw and maxilla structure are known to occur in contaminant-exposed fish larvae (Wright et al. 1999). The effects of such chemicals on behaviors in fish early life stages are not as studied.

Studies of early life stage salmonids have shown numerous contaminant-induced changes caused by exposure to planar halogenated aromatic hydrocarbons (PHHs), a class of persistent, lipophilic compounds in that includes 2,3,7,8-tetrachlorodibenzo-p-dioxin (Walker et al. 1991; Spitsbergen et al. 1991; Walker et al. 1992; Hornung et al. 1999). These compounds are known to be present in aquatic systems and are especially toxic to early life stage fishes. As evidence of the latter, both rainbow trout and lake trout are sensitive to the effects of TCDD at concentrations below 500 parts per trillion, and sublethal concentrations cause certain sublethal morphological effects that include craniofacial malformations (Wright and Tillitt 1997, Wright and Tillitt unpublished).

It has been well established that acute exposure to dioxin causes mortality in both rainbow trout and lake trout young. However, little work has focused on whether chronic exposure to dioxin at sub-lethal concentrations affects the critical initial feeding behavior of trout larvae. Consequently, the goal of the present study was to determine whether feeding efficiency in young rainbow trout and lake trout is altered by sub-lethal 2,3,7,8-TCDD exposure.
Methods

TCDD Egg Injection, and Rearing of Eggs and Fry

Rainbow trout: Unfertilized Eagle Lake strain rainbow trout eggs and milt, pooled from 2-3 two-year-old females and 2-3 males, respectively, were obtained from Ennis National Fish Hatchery, Ennis, Montana in March 1998. Upon receipt at the Columbia Environmental Research Center (CERC), the eggs were warmed slowly to ambient incubator temperature (10°C ± 1) and then fertilized on site. The fertilized eggs were immediately placed into incubator trays to water-harden for one hour. Within 48 hours of fertilization, triplicate groups of 150 eggs each were injected with 0, 75, 225, or 450 pg/g 2,3,7,8-TCDD according to methods previously described (Walker et al. 1996; Wright et al. 1997). Consequently, 1800 fertilized eggs were used in total: each of the four dioxin dosages was applied to 450 eggs, and these were subdivided into three treatment groups each of 150. The twelve treatment groups were then placed into plastic rearing baskets and randomly assigned by group to locations in a 16-tray Heath Stacks® vertical flow incubator. Within-group mortalities were recorded twice weekly until hatch, and then daily for the remainder of the study. At twenty days post-swim-up, all remaining fry were moved from the incubator to a flow-through aquaria system. The system consisted of 10 40L glass aquaria arranged 2x5 inside of a large, chilled water bath (14°C ± 1). Each aquarium was fitted with two to three plastic mesh cages, so that treatment groups sharing an aquarium were kept separated. Fresh water was supplied to each individual aquarium at a rate of 5 liters every 30 minutes. Fry were fed on a standardized trout flake diet once daily to satiation, and switched to a trout chow diet starting at approximately 40 days post-hatch.

Lake trout: Unfertilized Seneca Lake strain lake trout eggs and milt, pooled from 2-3 three year old females and 2-3 males, respectively, were received on October 15, 1998 from Hiawatha National Fish Hatchery, Brimly, Michigan. Upon receipt at CERC, the eggs and milt were allowed to slowly warm to within one degree of incubator temperature (8°C), after which the eggs were fertilized and water-hardened on-site using the same method as was used for the rainbow trout. Three groups of 100 eggs each were injected with 0 (control) and 100 pg/g TCDD, respectively. Consequently, 600 fertilized eggs were used in total: each of the two dioxin
dosages was applied to 300 eggs, and these were subdivided into three treatment groups each of 100 eggs. The egg preparation and injection techniques were identical to those used for rainbow trout injection. Following injections, the six treatment groups were placed in plastic mesh baskets and randomly assigned by group to locations in a Heath Stacks® flow-through 16-tray vertical incubator supplied with aerated well water at $8 \pm 1^\circ$C. Nonviable and dead eggs were culled twice weekly up until hatch, and then daily for the remainder of the study. At 30 days post-swim-up, the remaining lake trout fry were moved from the incubator to the flow through aquaria system described above. The fry were initially fed on a standardized trout flake diet, followed by a trout chow diet starting at approximately 45 days post-hatch.

Feeding Assay

One week prior to the first feeding test (described below) the fry of both species were switched to a combination diet of trout chow and *Daphnia magna*. On the fifth day, a prey size suitability test was conducted to determine the size of *D. magna* to be used for the subsequent tests. *Daphnia* were first sorted into three size classes by measuring total length (tip of head to tail end) under a Wolfe Stereomicroscope. Three fish from each treatment (i.e., the four TCDD dosage groups) were placed separately into one of 12 test chambers; each consisting of a 2.5 liter glass jar placed inside one of the 40 liter glass aquaria. Next, 20 prey of each size class (Small, 0.90-1.3 mm, Medium 1.4-1.8 mm, Large 1.9-2.3 mm) were added to each test chamber, and the number of prey eaten by each fry was determined. This test was repeated using three lake trout fry from both the 0 TCDD dosage group and the 100 pg/g TCDD dosage group. Based on this test, small *D. magna* were used for the remainder of the trout feeding studies, as this test confirmed that small prey could be consumed by all of the dosage group fish.

For rainbow trout, the feeding assay consisted of three trials per TCDD dosage done at each of three time points: 60-65 days post hatch, 75-80 days post hatch, and 100-105 days post-hatch. Measured as degree days (the number of days post-fertilization multiplied by the average daily temperature, these time points corresponded to 950-1000, 1100-1150, and 1350-1400 degree days. Lake trout were tested at two time points, 60-63 days post-hatch and 75-78 days post hatch, corresponding to 960-984 degree days and 1080-1104 degree days, respectively.
Each trial used 10 fry per TCDD dose. Twenty-four hours prior to each feeding test, 10 individual fry from each TCDD treatment were randomly selected and placed one each into 10 test chambers (2.5 L glass jars) maintained in a 14°C ± 1 water bath. The test chambers were visually isolated from one another by an opaque covering surrounding each jar. Test fry were denied food for a total of 24 h during this acclimation period.

To begin each feeding test, 20 prey of appropriate size were pipetted into a test chamber. Each fry was observed for 30 minutes, and both the number of prey eaten and the total number of strikes was recorded. After thirty minutes, each fry was carefully removed from the chambers using a small net, taking care not to remove any remaining prey. The water from each test chamber was then strained through a 125 μm mesh net and the number of prey remaining was counted. For each fry tested, total length (using a mm ruler and a dissecting microscope) and wet weight (using a digital scale) measurements were taken, after which the fry were euthanized using MS-222.

In addition to the above test, rainbow trout fry from the four TCDD treatment groups (0, 75, 225, or 450 pg/g 2,3,7,8-TCDD) were tested using two different prey densities at 130-135 days post-hatch only (1650-1700 degree days). Before the start of this test, 20 fry per treatment were randomly chosen and 10 were assigned to each of two feeding regimes: low density (4 small prey/L or 10 prey per 2.5 L chamber) or high density (8 small prey/L or 20 prey per 2.5 L chamber). These fry were placed individually into test chambers for a 24 h acclimation period. To begin each feeding test, the appropriate number of prey were pipetted into a test chamber. Each fry was observed for 30 minutes, and two endpoints were monitored: feeding behavior (both the number of prey eaten and the total number of strikes per capture) and strike latency (seconds to first strike). After thirty minutes, each fry was carefully removed from it's test chamber and the number of prey remaining was counted. For each fry tested, total length and wet weight measurements were taken, after which the fry were euthanized.

Statistical Analysis

For feeding behavior, treatment differences in capture rate and the number of prey ingested were analyzed using One-way ANOVA followed by Dunnett’s Multiple Comparison Test
(p = 0.05) to determine treatment effects. An ANCOVA was performed to test for group differences based on age (degree day) and 2,3,7,8-TCDD dose. The ANCOVA used to analyze feeding at the different time points included TCDD dosage and degree day as the covariates and mean capture rate or number of prey ingested as response variables.

Results

TCDD Effects on Feeding Efficiency

Rainbow Trout: For capture rate, the treatment means were significantly different among groups at 950-1000 dd (F = 85.59, 3 d.f., p < 0.001), at 1100-1150 dd (F = 8.55, 3 d.f., p < 0.001) and at 1350-1400 dd (F = 90.64, 3 d.f., p < 0.001). The mean capture rate (strikes per capture) was significantly affected by TCDD dose at the highest dose (450 pg/g) at all time points (Dunnett’s Multiple Comparison Test p < 0.05, Figure 1A). At the earliest time point, the capture rate decreased from 42% in the 0 pg/g TCDD group to 25% in the 450 pg/g TCDD group, with decreases from 53.4% to 48.2% and 57.8% to 50.7% at the middle and late time points, respectively.

Likewise, the mean number of prey ingested was significantly different at the earliest (F = 39.1, 3 d.f., p < 0.001), middle (F = 48.82, 3 d.f., p < 0.001), and the latest time point (F = 46.61, 3 d.f., p < 0.001) based on One-way ANOVA, p = 0.05. Rainbow trout fry exposed as eggs to 225 pg/g and 450 pg/g TCDD ingested significantly fewer prey as compared to control fish at all of the time points tested based on Dunnett’s Multiple Comparison Test, p < 0.05 (Figure 1B). Age was significantly correlated with both mean capture rate (F = 109.12, 1 d.f., p < 0.001, $r^2 = 0.81$) and number of prey ingested (F = 1503.2, 1 d.f., p < .001, $r^2 = 0.85$). Overall, mean capture rate increased from the first time point to the second and third time points, and the mean number of prey ingested increased by 2-3 time.

Lake Trout: The mean capture rate did not significantly differ among TCDD treatments (Figure 2A), although age was a significant covariate (F = 250.98, 1 d.f., p < 0.001, $r^2 = .95$). Similar to the results for rainbow trout, the mean number of prey ingested by lake trout exposed as eggs to 100 pg/g TCDD was significantly lower than in the controls at both the early time point
Figure 1. Feeding behavior at three time points in rainbow trout fry injected as eggs with 2,3,7,8-TCDD. Triplicate groups of 150 newly fertilized eggs each were injected with 0 (triolein), 75, 225, or 450 pg/g TCDD. Feeding trials were conducted at three time points: 950-1000 dd, 1100-1150 dd, and 1350-1400 dd. A) Mean capture rate, or the total number of strikes per capture, and B) the number of prey ingested in a thirty minute trial. “*” indicates significant difference from 0 pg/g TCDD (control) based on One-way ANOVA followed by Dunnett’s Multiple Comparison Test (p<0.05).
Figure 2. Feeding behavior at two time points in lake trout fry injected as eggs with 2,3,7,8-TCDD. Three groups of 100 newly fertilized eggs each were injected with 0 (triolein), and 100 pg/g TCDD. Feeding trials were conducted at two time points (960-984 dd, 1080-1104 dd). A) Mean capture rate, or the total number of strikes per capture, and B) the number of prey ingested in a thirty minute trial. ** indicates significant difference from 0 pg/g TCDD (control) based on Student’s t-test (p < 0.05).

![Graph A: Mean Capture Rate](image1.png)

![Graph B: Number of Prey Ingested](image2.png)
Both TCDD dose and age (degree days) were strongly correlated with the number of prey ingested (F = 19.02, 1 d.f., p < 0.001, \( r^2 = 0.94 \)).

**Density Dependent Feeding Behavior in Rainbow Trout**

At 1650-1700 degree days of development, feeding behavior was tested in rainbow trout for two different prey densities: low density (4 prey/L) or high density (8 prey/L, the prey densities used in the three earlier tests). There was no significant difference in the mean capture rates among the TCDD treatments (Figure 3A). However, there were significant differences between capture rates at 4 prey/liter versus 8 prey/liter for the 0 pg/g (t = -3.33, 18 d.f., p = 0.001), the 75 pg/g (t = -4.48, 18 d.f., p < 0.001) and the 225 pg/g treatments (t = -3.58, 18 d.f., p = 0.002). Capture rates in the 450 pg/g TCDD group did not differ between the two prey densities (t = -0.55, 18 d.f., p = 0.6). These capture rates were similar to those seen in rainbow trout at 1350-1400 degree days. There were significant differences in the mean numbers of prey ingested among the TCDD levels at both prey densities (Figure 3B). At 4 prey per liter, the number of prey ingested was significantly lower at the two highest TCDD dose (F = 62.14, 3 d.f., p < 0.001, Dunnett’s Multiple Comparison Test p < 0.05). There were 14.1 prey ingested by fry from the 225 pg/g TCDD group compared to 20.4 in the 0 pg/g TCDD group and there were 4.5 prey ingested by fry from the 450 pg/g TCDD group (Figure 3B). The same pattern was seen at 8 prey per liter (F = 42.14, 3 d.f., p < 0.001, Dunnett’s Multiple Comparison Test p < 0.05). There were 21.1 prey ingested by fry from the 225 pg/g TCDD group compared to 31.2 in the 0 pg/g TCDD group and there were 18.1 prey ingested by fry from the 450 pg/g TCDD group. Rainbow trout fry ingested significantly more prey at the higher prey density than at the lower prey density at all TCDD doses (0 pg/g: t = -8.54, 18 d.f., p < 0.001; 75 pg/g: t = -12.98, 18 d.f., p < 0.001; 225 pg/g: t = -6.46, 18 d.f., p < 0.001; 450 pg/g: t = -11.34, 18 d.f., p < 0.001).

Strike latency was also affected by TCDD exposure in rainbow trout at both the lower prey density (F = 62.14, 3 d.f., p < 0.001) and at the higher prey density (F = 116.59, 3 d.f., p < 0.001) (Figure 4). At the lower prey density, fish from the two highest TCDD treatments (225 and 450 pg/g) exhibited a significantly delayed first strike in comparison to the controls (Dunnett’s
Figure 3. Feeding behavior in TCDD-exposed rainbow trout at two feeding densities at 1650-1700 degree days. Rainbow trout fry are from triplicate groups of 150 newly fertilized eggs each were injected with 0 (trolein), 75, 225, or 450 pg/g TCDD. A) Mean capture rate (total number of strikes per capture) at low (4 prey per liter) and high (8 prey per liter), and B) number of prey ingested at low (4 prey per liter) and high (8 prey per liter). ** indicates significant difference from 0 pg/g TCDD (control) based on One-way ANOVA followed by Dunnett’s Multiple Comparison Test (p < 0.05). † indicates significant difference between 4 prey/liter and 8 prey/liter means at a given TCDD dose (Student’s t-test, p < 0.05).
Figure 4. Strike latency (time in seconds to first strike) in TCDD-exposed rainbow trout at two feeding densities at 1650-1700 dd. Prey densities are 4 prey per liter and 8 prey per liter. Rainbow trout fry are from triplicate groups of 150 newly fertilized eggs each were injected with 0 (triolein), 75, 225, or 450 pg/g TCDD. “∗∗∗” indicates significant difference from 0 pg/g TCDD (control) based on One-way ANOVA followed by Dunnett’s Multiple Comparison Test (p<0.05). “†” represents a significant difference between strike latency at low and high prey densities at a given TCDD dose (Student’s t-test, p < 0.05).
Multiple Comparison Test \( p < 0.05 \). Likewise, at the higher prey density, this significant delay in first strike was seen at both the 225 pg/g dose and at the 450 pg/g dose (Dunnett’s Multiple Comparison Test \( p < 0.05 \)). In addition, a comparison of like TCDD doses, i.e. 0pg/g vs. 0 pg/g, revealed that strike latency was significantly delayed at the lower prey density. While this delay in the lower prey density compared with the higher prey density was seen at all TCDD doses including 0 pg/g (9.3 sec vs. 5 sec, \( t = 3.39, 18 \) d.f., \( p = 0.003 \)), it was increasingly more pronounced with increasing TCDD dose. Strike latency in the lower and higher prey densities was 11.5 sec and 8 sec (\( t = 3.45, 18 \) d.f., \( p = 0.003 \)), 20.3 sec and 14.8 sec (\( t = 3.54, 18 \) d.f., \( p = 0.002 \)) and 59.2 sec and 35.8 sec (\( t = 35.8, 18 \) d.f., \( p < 0.001 \)) in the 75 pg/g, 225 pg/g and 450 pg/g TCDD groups, respectively.

**Discussion**

*Importance of early feeding behaviors*

Smaller fish are more susceptible to predation, thus any decrease in fish growth due to reductions in feeding rates could also result in decreased survival. Foraging behavior involves evaluating multiple parameters (food availability, hunger, competition, risk of predation), and the time of first feeding is often accompanied by high mortality (Latta 1962, Elliot 1984). Salmonids such as rainbow trout and lake trout first feed a few weeks post-hatch, when the yolk is nearly exhausted (Twongo and MacCrimmon 1976; Swedberg and Peck 1984). In the development of early feeding behaviors, flexibility is a distinct advantage. Learning, via changes of behavior with experience, helps the individual contend with environmental variability (Dill 1983). Young trout are opportunistic feeders, and forage in a variety of habitats (Swedberg and Peck 1984). Fish whose behavior is altered by contaminants may be less likely to cope effectively with such variability. Rapid growth is important in larval and juvenile fishes because predation-related mortality decreases with increasing size (Pedersen 1997; Pepin 1993). Also, larger fish can consume larger prey and have greater feeding efficiency (Mayer and Wahl 1997).

Exposure of fish eggs and larvae to environmental contaminants is known to produce a variety of early life stage alterations in behaviors. Benzo[a]Pyrene (BaP) caused a decrease in
upstream orientation performance of rainbow trout (Ostrander et al. 1990). Hatch, emergence, upstream orientation, and swimming performance were all negatively affected by BaP exposure in coho salmon (Ostrander et al. 1988). Increased predation and decreased capture rates have been shown in many species of fish exposed to environmental contaminants (Little et al. 1985). For this reason, careful study of adverse behavioral effects of a potential environmental pollutant on fish early life stages is especially important, as the period of change from endogenous to exogenous feeding is a critical time in fish development (Wiggins et al. 1985).

Mild starvation due to reduced feeding may lead to further adverse effects even if feeding should resume. In lake trout, although fish can withstand short periods of food deprivation (Gunn and Keller 1981), reduced feeding and short term starvation produce indirect behavioral effects such as reduced capture efficiency and increased handling time with the fry more vulnerable to predation (Jonas and Wahl 1998). Consequently, the long-term effects of initial feeding problems may not be immediately apparent.

When a fish comes into contact with a patch of prey, the initial burst of feeding is greatest within the first minute and then gradually declines (Confer and O’Bryan 1989). Thus, a delay in the initial burst of feeding would likely reduce overall prey consumed. In the current study, rainbow trout exposed to TCDD exhibited significantly delayed strike latency, or time to initial snapping at prey by fish, compared to controls and regardless of prey densities. Latency time decreases with increased experience with prey in salmonids, and contributes to the development of successful feeding behaviors and growth rates (Godin 1978). Therefore, alterations in the strike latency in fish exposed to contaminants may be subtle but significantly adverse.

Foraging involves many complex factors; for example, in salmonid species in which large numbers of fry emerge simultaneously, food may become a limiting resource as the fry compete (Wankowski and Thorpe 1979; Welker et al. 1994). For lake trout in Lake Superior young of the year must compete with other species such as slimy sculpin (Cottus cognatus) for such items as mysid, calanoid, chironomid pupae, and planktonic cladocerans (Hudson et al. 1995), and later in development juvenile fish of various salmonid species compete for alewife (Alosa
pseudoharengus). Because of such potential competition, any delays in the initiation of feeding in fish in the wild could interfere with feeding success, especially if competition with non-impacted fish is occurring.

**Effect of TCDD on Behaviors Related to Feeding**

Indirectly, the physical changes that result from contaminant exposure may also result in behavioral alterations. Swimming behavior, through its effect on prey encounter and feeding rates, can affect the survival of larval fish (Blaxter 1969; Webb 1976; Taylor and McPhail 1985; Letcher and Rice 1997). Consequently, any effects on fin or muscle structure, or other physiological systems involved in swimming performance, could potentially affect feeding success. PHHs cause sublethal pathologic lesions, such as hemorrhaging, although such lesions were not recorded in the current study (Wright et al. 1999).

Because salmonids rely on vision for foraging and predator avoidance, changes in the structure or function of the eye could impact feeding success and survival. Vision is the primary sensory modality employed in prey detection in teleost larvae, and salmonids such as lake trout and rainbow trout are visual predators (Confer et al. 1978). In swim-up rainbow trout fry, TCDD exposure can result in visual deficits and prey capture rate decreases (Carvalho and Tillitt 2004). Other physical changes to the eye (i.e., cataracts) caused by contaminant exposure can interfere with feeding behavior of rainbow trout fry (Little et al. 1985; Savino et al. 1993; Hose et al. 1984) or even affect photobehavior and emergence (Carey and Noakes 1981). Delayed emergence will have consequences for growth and survival in that fry may begin feeding later than competitors and lag behind in growth.

In addition to alterations in the structure or function of the eye, contaminants may produce skeletal malformations of the cranium and jaw structure that could also impede feeding. Benzo[a]pyrene is known to cause such deformities in rainbow trout (Hose et al. 1984). Craniofacial malformation (domed skulls, foreshortened maxillas, and deformed jaw structures) are a characteristic symptom of TCDD-exposure (Hornung et al. 1999; Wright and Tillitt 1996; Spitsbergen et al. 1991). Johnson et al. (1998) found that TCDD exposure to brook trout young via parental dietary exposure caused pathologies such as edema and an increased occurrence of
exophthalmia. Such deformities can occur at TCDD concentrations well below the LD50 values (29 pg/g lowest observable adverse effect level (LOAEL) in lake trout, vs. 44-85 pg/g LD50) (Wright and Tillitt unpublished). Although the presence of ocular or skeletal defects was not specifically considered in the present study, this is one possible indirect link to changes in feeding behaviors that should be studied further.

**Contaminant Effects on Feeding**

Past studies have indicated that chemical exposure causes generalized adverse effects on feeding behaviors and growth in salmonids. For example, both TCDD and tetrachlorodibenzofuran (TCDF) caused a decrease in feeding of juvenile rainbow trout, involving both less feeding overall and a slower response time to food in comparison with control (Mehrle et al. 1988). And embryonic exposure to PCBs caused decreased weight and length in 13 week-post-hatch Atlantic salmon (*Salmo salar*) fry, showing that the effects on growth can be long lasting, although feeding behavior was not quantified (Savino and Henry 1991). Other chemical exposures in salmonids have caused a range of adverse effects, including a decrease in feeding efficiency, impairment of prey capture and visual capabilities, inhibition of the motivation to feed, and increased vulnerability to predation (Gunn and Noakes 1987; Ostrander et al. 1988; Little et al. 1990; Johnson et al. 1998). However, few studies have looked at such effects in fry just beginning to feed. Adverse effects on behaviors like feeding efficiency at such a critical developmental period would likely reduce growth and survival of the fry.

In the current study, exposure as eggs to 2,3,7,8-TCDD caused decreased feeding in both rainbow trout and lake trout. Feeding behavior can be a sensitive indicator of toxicity (Little et al. 1985); however, relatively high doses of TCDD were needed to produce adverse effects in the current study. Decreases in feeding were seen in rainbow trout at 50% of the LD50 (TCDD dose causing 50% mortality) or 225 pg/g (Walker and Peterson 1990). Similarly, feeding behavior in lake trout was significantly altered at the approximate LD50, 100 pg/g. Adverse effects in rainbow trout on strike latency were seen at the same TCDD dose level. No significant difference in size was found between control fish and those TCDD-exposed ones, although
differences in feeding success were found. It is possible, however, that differences in size would have become apparent had the study continued for a longer period of time.

Our results suggest that not only are feeding efficiency and total number of prey important endpoints in early life stage feeding studies, but in addition more subtle measurements such as strike latency and other related behaviors should be considered due to their possible occurrence at lower TCDD doses. Such sublethal effects that can occur in response to lower levels of contaminant exposure are important considerations, as contaminants such as TCDD and other PHHs are present at very low concentrations in the environment. While there is the potential for sublethal behavioral effects to be exhibited in fish exposed to low environmental levels of pollutants, the endpoints used in the current study were unable to detect such subtle effects.

**Acknowledgements**

The support of the Missouri Cooperative Fish and Wildlife Research Unit, under Dr. Charles F. Rabeni, was appreciated and critical to the success of these studies. We also wish to thank those at the Columbia Environmental Research Center for their cooperation and support, especially Alan Allert. We greatly appreciate Wes Orr at the Ennis National Fish Hatchery and personnel at the Hiawatha Forest National Fish Hatchery for supplying rainbow trout and lake trout eggs, respectively. Thanks to the reviewers of this paper, including Dr. Ron Hayward and Dr. Paulo Caravalho, for their comments and suggestions. This publication is a contribution from both the Missouri Cooperative Fish and Wildlife Research Unit (the U.S.G.S./Biological Resources Division, Missouri Department of Conservation, The School of Natural Resources of the University of Missouri-Columbia, and the Wildlife Management Institute cooperating) and from the Missouri Agricultural Experiment Station.

**References**


Chapter 6: Thiamin and 2,3,7,8-TCDD interact to cause sublethal adverse behavioral effects in rainbow trout and lake trout embryos and fry

Abstract

Recent research has considered whether a thiamin deficiency, caused by a change in the diet of Great Lakes lake trout, could have triggered the continuing reproductive disorder known as Early Mortality Syndrome, or EMS. It remains unclear if the low levels of PHHs still present in certain areas of the Great Lakes still have the potential to negatively impact lake trout early life stage mortality, perhaps even interacting in some way with effects related to thiamin deficiency. We obtained lake trout eggs from different sources including hatchery reared females, laboratory reared females fed a thiamin deficient diet, and Lake Michigan females (one producing eggs with very low thiamin levels and one producing eggs with thiamin levels sufficient for normal development). This study looked at three specific endpoints: (1) the presence of EMS, (2) the embryo C-start response, and (3) feeding behavior in post-swim-up fry. Low initial egg thiamin was correlated with low C-start response and high EMS mortality, and thiamin supplementation ameliorated both effects. In terms of C-start response, there was a hierarchy of response depending on egg source, with the highest response occurring in embryos with sufficient initial thiamin and the lowest response occurring in embryos with very low initial egg thiamin. Overall, feeding efficiency did not differ significantly among fry from different egg sources. More study into the subtle interactions of thiamin and contaminant status on sublethal effects such as C-start magnitude could provide valuable information on the nature of EMS in Great Lakes lake trout.

Introduction

The plight of the Great Lakes lake trout (Salvelinus namaycush) and their diminished reproductive success has received much attention from researchers since the early 1970s. Ever since severe health and reproductive disorders were first noted in the lake trout from the lower Great Lakes, questions regarding the initial cause(s) and contributing factors have been debated by fisheries biologists, hatchery managers, toxicologists, nutritionists, and others interested in returning these populations to a self-sustaining status.

Once a healthy and productive natural fishery, today’s lake trout populations in the lower Great Lakes are of hatchery origin, their reproductive success being sufficiently low that continued stocking is required. The decline of this fishery dates to the early twentieth century when commercial harvesting increased and the exotic sea lamprey (Petromyzon marinus) was introduced: by the late 1950s lake trout were considered extinct in Lake Michigan (Holey 1995; Willford et al., 1981; Eshenroder and Amatangelo 2002). Soon after, an intensive rehabilitation and stocking program was implemented, and by the mid 1970s lake trout were once again
abundant in Lake Michigan. Although these fish attain sexual maturity and are capable of reproducing, their young do not survive. Consequently, the populations are not self-sustaining.

It is believed that the reasons for this reproductive failure have changed over time. Initially, after stocked lake trout became abundant in the 1970s, excessively high egg and fry mortality was caused by industrial contaminants, planar halogenated aromatic hydrocarbons (PHHs). This class of compounds includes the polychlorinated dibenzofurans (PCDFs), coplanar polychlorinated biphenyls (PCBs), and certain polychlorinated dibenzo-p-dioxins (PCDDs) including the most toxic of the PHHs, 2,3,7,8-TCDD. PHHs are highly toxic to fish, especially their early life stages (Walker et al. 1994; Zabel et al. 1995; Hornung et al. 1999). The sensitivity of lake trout to the effects of TCDD is very high. Studies comparing effect levels of 2,3,7,8-TCDD with toxicity thresholds showed that the levels present in Lake Michigan could account for 100% of the lake trout early life stage mortality (Mac et al. 1985). A retrospective analysis of lake trout in Lake Ontario came to the same conclusion (Cook et al. 2003).

More recently, research has focused on whether a nutritional deficiency caused by changes in what lake trout are foraging on, could have triggered the continuing reproductive disorder known as Early Mortality Syndrome or EMS (Fitzsimons 1995; Fisher et al. 1996; Marcquenski and Brown 1997). This syndrome is characterized by the death of post-swim-up fry, preceded by a suite of behavioral and physical alterations that include abnormal swimming, skeletal malformations and hemorrhage (Honeyfield et al. 1998). The symptomology of EMS differs substantially from that of TCDD exposure, the latter of which includes reduced egg hatching, yolk-sac fry mortality, and an array of skeletal and physiologic abnormalities that include cranial and jaw deformities and circulatory problems that lead to severe edema (Walker et al. 1990; Walker et al. 1991). In addition, the timing of the fry mortality differs substantially between the two syndromes: TCDD-related mortality occurs mainly at hatch and in pre-swim-up fry whereas EMS occurs in fry shortly after swim-up. Researchers now believe that a nutritional deficiency (i.e., thiamin deficiency caused by adult female lake trout consumption of thiaminase-containing and thus thiamin-depleting alewife (Alosa pseudoharengus), is responsible for the low
yield of naturally reproduced lake trout young (Brown et al. 2005). Evidence supporting this hypothesis continues to accumulate.

At the present time, it appears that Great Lakes contaminant levels are no longer sufficient to directly account for the continuing mortality of lake trout fry (Cook et al. 2003; Wright and Tillitt 1999). It is also apparent that thiamin deficiency in the eggs of affected females causes mortality in lake trout fry manifesting as EMS (Fitzsimons et al. 2001). What is not clear at this point is whether the low residual levels of PHHs present in certain Great Lakes locations still have the potential to cause increased mortality in early life stage lake trout, and the degree to which thiamin deficiency may exacerbate these effects. Our understanding of the causes of Great Lakes lake trout reproductive failure is incomplete: at this point no single cause can fully account for the lack of natural reproduction in these fish.

Previous studies have demonstrated (i) that very low PHH concentrations (i.e. 2,3,7,8-TCDD) can produce sublethal physical and behavioral alterations in lake trout eggs and fry, and (ii) that eggs and fry from feral Lake Michigan females are prone to thiamin deficiency in addition to their exposure to these TCDD levels. Additionally, it is known that both TCDD and thiamin deficiency cause neurodegenerative effects with subsequent behavioral alterations in developing fish (Mehrle et al. 1989; Lindstrom et al. 1998). Thus, the objective of the present investigation was to evaluate behavioral effects of differing dioxin exposure in conjunction with differing degrees of thiamin deficiency (i.e. a dioxin/thiamin interaction). To accomplish this goal required using lake trout fry whose thiamin and contaminant exposures both varied. In addition, to maximize the applicability of our conclusions to existing lake trout populations, we deemed it important to obtain eggs from feral fish for this set of studies.

**Methods and Materials**

We assessed three specific endpoints in this study: (1) the presence of EMS, (2) the embryo C-start response, and (3) feeding behavior in post-swim-up fry. How 2,3,7,8-TCDD exposure affects embryo startle response and feeding behavior has been previously
characterized, forming a basis for comparison. A summary of the experimental design is shown in Table 1.

Egg Sources

We obtained lake trout eggs from three different sources (Table 1):

A. Hatchery-reared females free of PHH contamination and whose thiamin levels were normal, serving as controls;

B. Laboratory reared females free of PHH contamination and whose thiamin levels were rendered minimal;

C. Lake Michigan females, both of which exhibited low-level PHH accumulations, one of which produced eggs with very low thiamin levels and the other of which produced eggs with thiamin levels considered sufficient for normal development.

A. Hatchery

Green, unfertilized lake trout eggs and milt were received on October 15, 1998 from Hiawatha Forest National Fish Hatchery, Brimly MI. Upon receipt at the Columbia Environmental Research Center (CERC), eggs and milt were allowed to slowly warm to within one degree of incubator temperature ($8^\circ$C). The lake trout eggs were pooled from 2-3 three-year old females, and were fertilized with milt pooled from 2-3 males. The fertilized eggs were immediately placed into incubator trays to water-harden for one hour. Following water-hardening, lake trout egg replicates designated for the various study endpoints (C-start, EMS, and Feeding) were placed in separate plastic mesh baskets at $8\pm1^\circ$C in a flow-through 16-tray vertical incubator supplied with aerated well water. Nonviable and dead eggs were culled twice weekly up until hatch, and then daily for the remainder of the study. No thiamin analysis was conducted on the Control eggs, and there was no thiamin supplementation provided at water hardening.

B. Laboratory

On October 23rd, 1998 the green unfertilized eggs of 2-3 three year old females and the milt from 2-3 males were received from the Research and Development Laboratory, B.R.D, U.S.G.S., Wellsboro, PA. The eggs, as well as pooled sperm from hatchery reared male lake trout, had been stripped on site and samples of unfertilized eggs were taken for rapid thiamin
Table 1. Experimental design for the study of the interaction of thiamin status and contaminants in lake trout young.

<table>
<thead>
<tr>
<th>Lake Trout Egg Source</th>
<th>Condition</th>
<th>Egg Treatment</th>
<th>Endpoint Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T-1</td>
<td>T+2</td>
</tr>
<tr>
<td>A) Hatchery</td>
<td>Experimental Control: No PHH contamination Normal thiamin levels</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>B) Laboratory</td>
<td>Thiamin Deficient: No PHH contamination Low thiamin levels</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>C) Lake Michigan</td>
<td>Contaminant Exposed: Low contaminants Normal thiamin levels (&gt;2.5 nmol/g)</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Lake Michigan</td>
<td>Contaminant Exposed/Thiamin Deficient: Low contaminants Low thiamin levels (&lt;0.5 nmol/g)</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

A) Hiawatha Forest National Fish
B) Research and Development Laboratory, BRD, USGS, Wellsboro, PA
1. T- indicates no thiamin supplementation during egg water hardening
2. T+ indicates thiamin supplementation of 750 mg/l during egg water hardening
analysis. On receipt at CERC, the remaining eggs from each female were then divided in half: half were fertilized with pooled sperm and received ambient water for water hardening, whereas the other half were fertilized with pooled sperm and received thiamin-supplemented water (750 mg/L) during water hardening. Following water-hardening, the entire lot was subdivided into groups destined for examination using the various study endpoints (C-start, EMS, and Feeding; see below). Each group was placed in separate plastic mesh baskets in a flow-through 16-tray vertical incubator supplied with aerated 8±1°C well water. Nonviable and dead eggs were culled twice weekly up until hatch, and then daily for the remainder of the study.

C) Lake Michigan (LM)

Reproductively mature adult lake trout females and males were collected by U.S. Fish and Wildlife personnel from Lake Michigan, near Sturgeon Bay, using gill nets on 10/28/98. Eggs were stripped on site, and samples were taken from each egg batch for separate thiamin analysis. The eggs were then divided in half: half were fertilized with pooled sperm and received ambient water for water hardening, whereas the other half were fertilized with pooled sperm and received a thiamin-supplemented water (750 mg/L) during water hardening for approximately one hour. Several batches of fertilized, water-hardened eggs were then express-shipped to the Columbia Environmental Research Center, where they were slowly warmed to within 1 degree of incubator water temperature.

Based on the initial thiamin content determinations, the eggs from two females were chosen for further study: Lake Michigan/Normal and Lake Michigan/Low. The egg batch from each was subdivided into groups destined for examination using the various study endpoints (C-start, EMS, and Feeding; see below) were then placed in separate plastic mesh baskets at 8±1°C in a flow-through 16-tray vertical incubator supplied with aerated well water. Nonviable and dead eggs were culled twice weekly up until hatch, and then daily for the remainder of the study.

Thiamin Analysis

Unfertilized eggs taken for thiamin analysis were sent to Dale Honeyfield at the Research and Development Laboratory, B.R.D, U.S.G.S., Wellsboro, PA for rapid thiamin analysis, following standard procedures (Brown et al. 1998). Briefly, egg tissue from each egg/female
batch was homogenized and the thiamin extracted over several steps using trichloroacetic acid (TCA). The thiamin compounds (thiamin pyrophosphate TPP, thiamin monophosphate TMP and free thiamine (TH)) in the extracted supernatant were then measured by reversed phase high-performance liquid chromatography. Egg thiamin levels were reported as total thiamin (nmol thiamin per gram egg).

Endpoint Tests

Three indicators of effect (endpoints) were tested, each of which was behavior-based. Tables 1 and 2 summarize how many individuals from which egg sources were tested using each endpoint.

C-start Response

The C-start response test was conducted according to methods described by Wright et al. (2003). The three-stage (Early Stage: 360 degree days. Middle Stage: 480 degree days, and Late Stage: 560 degree days) C-start test began by randomly selecting lake trout eggs from the separate replicates, and then carefully pipetting them into individual pre-formed wells in agarose plates. These shallow wells (U-shaped in cross-section) allowed each egg to be held firmly in place during subsequent tactile stimulation. Each loaded plate was then returned to the vertical flow incubator for 24 hours, during which the embryos all oriented themselves uniformly, head and back uppermost against the egg capsule. No mortality was observed during this part of the procedure.

The C-start test involved positioning one egg-bearing agarose plate under a Nikon stereoscopic microscope (15x magnification). Each egg was then stimulated manually; this involved using a 10 mm blunt glass probe to briefly (~1 sec duration) touch each chorion surface at a standardized location (dorso-lateral position of thorax anterior to the dorsal fin) and with a consistent force. The force applied was just enough to dimple the egg capsule. Three stimuli were applied in sequence to each egg at 10-second intervals (0 sec = '1st stimulus'; 10 sec = '2nd stimulus'; 20 sec = '3rd stimulus'), after which the next egg was brought into view.

For each stimulus applied to each egg, the absence or presence of a C-start response was recorded; specifically, the presence of a response involved a trunk flexure, always first in the
Table 2. Numbers of lake trout embryos or fry from different egg sources tested for each of three endpoints: presence of Early Mortality Syndrome (EMS), embryo C-start response, and feeding behavior.

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>EMS</th>
<th>Embryo C-start response</th>
<th>Feeding behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Hatchery</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>3 groups of 10 (Early, Middle, and Late)</td>
<td>3 groups of 10</td>
</tr>
<tr>
<td><strong>B) Laboratory</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-</td>
<td>108</td>
<td>10 (Early and Middle) 3 groups of 10 (Late)</td>
<td>6</td>
</tr>
<tr>
<td>T+</td>
<td>122</td>
<td>10 (Early and Middle) 3 groups of 10 (Late)</td>
<td>3</td>
</tr>
<tr>
<td>Lake Michigan (Normal T)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-</td>
<td>66</td>
<td>10 (Early and Middle) 3 groups of 10 (Late)</td>
<td>10</td>
</tr>
<tr>
<td>T+</td>
<td>79</td>
<td>10 (Early and Middle) 3 groups of 10 (Late)</td>
<td>10</td>
</tr>
<tr>
<td>Lake Michigan (Low T)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-</td>
<td>54</td>
<td>10 (Early and Middle) 3 groups of 10 (Late)</td>
<td>10</td>
</tr>
<tr>
<td>T+</td>
<td>71</td>
<td>3 groups of 10 (Late)</td>
<td>10</td>
</tr>
</tbody>
</table>
opposite direction of the stimulus, into a "C" shape with the nose and tail being brought into close proximity. In addition to the absence or presence of a C-start response, the number of consecutive flexures that occurred in the following 10-second period was recorded. Slight movement by the embryo, although not considered a C-start response, was noted where it occurred.

*Early Mortality Syndrome (EMS)*

Subsamples of all treatment groups were set aside shortly following water-hardening, and were grown out through the swim-up fry stage in order to check for the presence of EMS mortality. The behavioral symptoms of EMS in fry are well characterized, and include abnormal swimming behavior (such as swimming on the side or swimming in circles) and the occurrence of lethargy shortly before the onset of exogenous feeding. Fry exhibiting these symptoms of EMS were carefully monitored, and the deaths of such fry were recorded daily. Specifically, abnormal swimming behavior and lethargy were used as identifiers of EMS in fry. All fry that exhibited such symptoms died within a few days of the onset of symptoms.

*Feeding Efficiency*

At thirty days post-swim-up, all remaining lake trout fry set aside for feeding studies were moved from the vertical flow incubator to a flow through aquaria system. The system consisted of 10 40 L glass aquaria arranged 2x5 inside of a large, chilled water bath (14°C ±1). Each aquarium was fitted with two to three plastic mesh cages, so that treatment groups sharing an aquarium were kept separated. Fresh water was supplied to each individual aquarium at a rate of 5 liters every 30 minutes. Replicate groups of fry were kept in separate mesh baskets within each glass aquaria. Fry were fed on a standardized trout flake diet for the first two weeks, followed by a trout chow diet starting at approximately 45 days post-swim-up. One week prior to the first feeding test, fry were started on a combination diet of trout chow and small (0.9-1.3 m) *Daphnia magna*.

The feeding assay consisted of trials conducted 75 days post swim-up. Twenty-four hours prior to each feeding test, individual fry for each treatment were randomly placed one each into a test chamber consisting of a 2.5 L glass jar placed inside one of the glass aquaria. Test fry
were denied food during this 24 h acclimation period. At the start of each feeding test, 20 prey of appropriate size were pipetted into each of the test chambers. Prey size had been previously established based on prey size that all the fry group members could ingest. Tests lasted for one hour, at which point each fry was removed from its tank, water from the test jar was strained through a 125 μm mesh net, and the number of prey remaining was counted. For each fry tested, wet weight measurements were taken, after which the fry was euthanized using MS-222 in accordance with IACUC guidelines.

Results

1) C-start Response

Both the proportion of embryos responding to a tactile stimulus, and the intensity of the C-start response to stimulus varied similarly across the treatments (Figure 1A and 1B). The results indicate a hierarchy of responses depending on egg source and initial thiamin content (see Table 1). The highest proportions of embryos exhibiting C-starts occurred in the Hatchery group and groups with high initial egg thiamin content (Lake Michigan/Normal T+ and Lake Michigan/Normal T-). Interestingly, a higher proportion of embryos from the Laboratory T+ treatments exhibited C-starts at the Early and Middle time points than both the Hatchery embryos and embryos from the Lake Michigan/Normal T- group. The lowest responses occurred in Lake Michigan/Low T+, Lake Michigan/Low T-, and Laboratory T- respectively. This ordered decline was generally for each of the three time points (Early, Middle, and Late), but was most pronounced at the Late stage, when the proportion responding ranged from a high of 100% in the Hatchery embryos and Lake Michigan/Normal T+ embryos to a low of 34% in Laboratory T- embryos (Figure 1A). However, the mean numbers of embryos responding in all other egg groups were not significantly different than in Hatchery embryos (F = 2.57, 4 d.f., p = 0.07, One-way ANOVA).

C-start intensity likewise declined following the same pattern. C-start intensity was highest in the Hatchery, LM/Normal T+ and LM/Normal T- embryos and lowest in the embryos with low initial egg thiamin content (i.e., LM/Low T+, LM/Low T-, Laboratory T+, and Laboratory T-)
Figure 1. (A) Overall C-start occurrence and (B) C-start intensity in lake trout embryos from different eggs sources and thiamin treatments, in order of declining initial egg thiamin content. Values for three time points (early, middle, and late) are shown. “LM” indicates embryos from the Lake Michigan groups. No significant differences between means from egg sources based on One-way ANOVA.
T-). As with the proportion of embryos exhibiting C-start responses, late stage C-starts intensity showed the greatest decline, from 1.95 in the Hatchery group to a low of 0.28 in Laboratory T- (Figure 1B). However, this reduction in C-start intensity was not significantly different from Hatchery embryo responses (F = 2.22, 6 d.f., p = 0.10).

Thiamin supplementation had a significant effect on the C-start intensity, based on Student’s t-test (p < 0.05, Table 3). In embryos from the Laboratory group, C-start intensity increased from 0.28 in the T- group to 0.76 in the T+ group. Likewise, in Lake Michigan/Normal embryos, intensity increased from 1.08 to 1.61 in the T- and T+ groups, respectively. Lake Michigan/Low embryos exhibited a similar increase with thiamin supplementation, from 0.40 to 0.82 C-starts per stimulus.

2) EMS

Fry from the Hatchery and Lake Michigan/Normal (regardless of thiamin supplementation) groups did not exhibit any signs of EMS (Table 4). Fry from these groups possessed initial egg thiamin levels of >5.0 and 2.57 nmol/g, respectively. However, fry from both the Laboratory and Lake Michigan/Low groups experienced high mortality attributed to EMS. The initial egg thiamin levels in the T- groups were 0.022 and 0.25 nmol/g, respectively, for the Laboratory and Lake Michigan/Low groups. Thiamin supplementation lowered EMS mortality in both of these groups, from 94.4% in Laboratory/T- to 74.6% in Laboratory/T+, and from 94.4% in Lake Michigan/Low T- to 47.9% in Lake Michigan/Low T+ fry.

Across the treatment groups, the proportions of fish exhibiting EMS mortality and C-start responses were negatively correlated at the late time point (560 degree days) (Figure 2A). Groups with high C-start response proportions (i.e., >80%) exhibited no mortality attributed to EMS, whereas groups with C-start response proportions below 60% exhibited greater than 90% EMS mortality. Likewise, across treatment groups, the proportion of fish exhibiting EMS mortality and the intensity of the C-start responses were negatively correlated at the late time point. Treatment groups with mean C-start intensity less than one thread per stimulus exhibited very
Table 3. Effect of thiamin supplementation on C-start intensity in lake trout embryos tested at the late time point (560 degree days). “T-” no thiamin supplementation, “T+” thiamin supplementation. Significance based on one-tailed Student’s t-test.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Mean C-start Intensity (with standard deviations)</th>
<th>t-statistic, d.f., p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatchery</td>
<td>T- 1.97 (1.57)</td>
<td>--</td>
</tr>
<tr>
<td>Hatchery</td>
<td>T+ --</td>
<td>--</td>
</tr>
<tr>
<td>Laboratory</td>
<td>T- 0.28 (0.67)</td>
<td>t = -2.34, 58 d.f., p = 0.01</td>
</tr>
<tr>
<td>Laboratory</td>
<td>T+ 0.75 (0.88)</td>
<td></td>
</tr>
<tr>
<td>Lake Michigan/Normal</td>
<td>T- 1.08 (0.93)</td>
<td>t = -1.68, 58 d.f., p = 0.04</td>
</tr>
<tr>
<td>Lake Michigan/Normal</td>
<td>T+ 1.61 (1.46)</td>
<td></td>
</tr>
<tr>
<td>Lake Michigan/Low</td>
<td>T- 0.40 (0.76)</td>
<td>t = -2.82, 58 d.f., p = 0.003</td>
</tr>
<tr>
<td>Lake Michigan/Low</td>
<td>T+ 0.82 (0.29)</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Initial egg thiamin (nmol/g) content and percent occurrence of Early Mortality Syndrome (EMS) in lake trout fry, with (T+) and without (T-) thiamin supplementation at water hardening.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Initial Total Egg Thiamin (nmol/g)</th>
<th>% EMS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T-</td>
</tr>
<tr>
<td>Hatchery</td>
<td>&gt;5.0*</td>
<td>0</td>
</tr>
<tr>
<td>Laboratory</td>
<td>0.022</td>
<td>94.4</td>
</tr>
<tr>
<td>Lake Michigan/Normal</td>
<td>2.57</td>
<td>0</td>
</tr>
<tr>
<td>Lake Michigan/Low</td>
<td>0.25</td>
<td>94.4</td>
</tr>
</tbody>
</table>

* Based on average egg thiamin levels of lake trout eggs from hatchery-reared females (Dale Honeyfield, personal communication).
Figure 2. EMS mortality versus occurrence of C-start response (A) and C-start magnitude (B) in lake trout embryos. % EMS Mortality is based on EMS occurrence in single replicates (see Table 2).
high EMS mortality (48%-94%) whereas groups with C-start intensity above one thrash per stimulus experienced no EMS mortality (Figure 2B).

3) Feeding Efficiency

Feeding efficiency in the Lake Michigan/Normal group, measured as mean capture rate and mean numbers of prey ingested, did not differ significantly from the Hatchery group, nor did the fry weights for these groups (Table 5). Fry originating from Lake Michigan eggs with low thiamin (Lake Michigan/Low) exhibiting a lower mean capture rate than did control fry and the mean number of prey they ingested was lower, but neither of these differences was statistically significant (Table 4). The mean capture rate and mean number of prey ingested in the Laboratory groups, both non-thiamin supplemented (T-) and thiamin supplemented (T+), were significantly higher than in the Hatchery groups, as were the mean fry weights (Table 4). Rearing densities for the Laboratory/T- and Laboratory/T+ were much lower than in all the other groups, due to low initial egg numbers and high subsequent EMS mortality.

Discussion

Experimental Issues

Thiamin levels in lake trout eggs vary widely, depending on the source of the eggs. Total thiamin in unfertilized eggs from hatchery reared females is generally very high; for example, unfertilized hatchery-reared lake trout eggs contain from 8.2 to 12 nmol/g total thiamin (Dale Honeyfield, personal communication). Thiamin content analysis was not conducted on the Hatchery eggs for this study; however we have no reason to believe that their thiamin levels were in any way dissimilar to those reported for eggs from other hatchery reared females.

In unfertilized eggs from twelve Lake Michigan females, the mean total egg thiamin levels ranged from 0.4 to 13.5 nmol/g across females, averaging 3.4 nmol/g (Dale Honeyfield, personal communication). This variation likely reflects fish-to-fish differences in the diet of adult females prior to and during oogenesis (Brown et al. 2005c). For the purposes of this study, we deliberately chose eggs from two Lake Michigan females that had very different egg thiamin levels. Those from the first female contained 2.57 nmol/g thiamin, which is well above the
Table 5. Feeding behavior (capture rate and prey ingested) and wet weight (g) of lake trout fry 75-days post swim-up. Values shown are group means and standard deviations in (). Capture rate = strikes per capture. ** indicates significant difference (Student’s t-test, p < 0.05) from control.

<table>
<thead>
<tr>
<th></th>
<th>Mean Capture Rate (%)</th>
<th>Mean # Prey Ingested</th>
<th>Fry Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatchery</td>
<td>0.55 (0.15)</td>
<td>24.1 (5.23)</td>
<td>0.74 (0.22)</td>
</tr>
<tr>
<td>Laboratory/T-</td>
<td>0.83** (0.04)</td>
<td>30.3 (6.25)</td>
<td>1.08** (0.15)</td>
</tr>
<tr>
<td>Laboratory/T+</td>
<td>0.85** (0.05)</td>
<td>32.1 (7.0)</td>
<td>1.07** (0.13)</td>
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<tr>
<td>Lake Michigan/Normal T-</td>
<td>0.55 (0.06)</td>
<td>25.2 (3.88)</td>
<td>0.80 (0.20)</td>
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<td>Lake Michigan/Normal T+</td>
<td>0.58 (0.09)</td>
<td>26.2 (2.10)</td>
<td>0.73 (0.22)</td>
</tr>
<tr>
<td>Lake Michigan/Low T-</td>
<td>0.24 (0.25)</td>
<td>12.5 (9.98)</td>
<td>0.81 (0.21)</td>
</tr>
<tr>
<td>Lake Michigan/Low T+</td>
<td>0.30 (0.14)</td>
<td>13.8 (7.29)</td>
<td>0.60 (0.11)</td>
</tr>
</tbody>
</table>
threshold below which EMS increases significantly. EMS typically occurs when total egg thiamin levels drop below 1.0 nmol/g (Brown et al. 1998). The eggs from the second female contained much lower egg thiamin levels, 0.25 nmol/g; this level would be considered very deficient and low enough to produce very high EMS mortality in lake trout (Brown et al. 1998). Out of all the females collected for this study, only these two were available for use; many of the eggs from other females were non-viable.

Recent work has focused on the use of thiamin antagonists, both in adult females and in fry, to investigate the etiology of early mortality syndrome (Honeyfield et al. 2005; Amcoff et al. 2002; Fitzsimons et al. 2001). The Laboratory eggs in this study, from females fed a diet designed to lower egg thiamin, were the product of such an attempt. The very low egg thiamin levels (0.022 nmol/g) achieved are approximately 2% of the amount considered sufficient for fry survival in lake trout. In fact, a challenge of this study was rearing enough surviving eggs and fry from this particular batch for later feeding studies. Consequently, the fry numbers we actually tested for feeding behavior were very low in comparison to the Hatchery group.

Treating eggs with thiamin during water hardening ameliorates the symptoms of EMS (Fitzsimons 1995; Fisher et al. 1996; Hornung et al. 1998). Immersing coho salmon (Oncorhynchus kisutch) and steelhead (Oncorhynchus mykiss) eggs in a thiamin solution at water hardening both significantly increases total thiamin levels and decreases EMS (Hornung et al. 1998). Lake trout eggs supplemented with thiamin during water hardening generally increase their thiamin content, with the final egg thiamin levels being dependent on their starting levels (Brown et al. 2005a). In the present study, we did not analyze the total egg thiamin in our thiamin supplemented eggs due to logistical constraints. Regardless, the large decrease in EMS mortality and increase in C-start behavior in our thiamin supplemented embryos and fry relative to the non-supplemented individuals indicates that thiamin levels increased after treatment.

**Effects of thiamin supplementation**

As anticipated, groups with very low egg thiamin levels exhibited high EMS mortality rates. Specifically, the Laboratory and Lake Michigan/Low eggs suffered from substantial EMS deaths, whereas the Hatchery and Lake Michigan/Normal eggs, with total egg thiamin levels that
exceeded 2 nmol/g, experienced 0% EMS mortality. Seen from another perspective, however, the single thiamin supplementation of 750 mg/L at water hardening in the Laboratory and Lake Michigan/Low groups proved insufficient to totally reduce EMS mortality, even though fry survival increased from ~5% to ~25% in the Laboratory groups and from ~5% to ~50% in the Lake Michigan/Low groups. This outcome indicates that a single thiamin supplementation at water hardening is insufficient to produce high fry survival in egg batches with very low initial thiamin levels. Multiple, more concentrated or longer-lasting supplementations may be necessary in such situations.

Notable was the 100% survival of eggs from the Lake Michigan/Normal group. These eggs, with sufficient initial egg thiamin levels, showed no signs of EMS and experienced minimal mortality from fertilization through swim-up. Based on analyses of egg thiamin levels from Lake Michigan females, it is known that thiamin content varies enough in lake trout from Lake Michigan as to be low enough in some to cause thiamin deficiency that results in EMS, or high enough in other individuals to yield no EMS-related mortality in their offspring. Even in fish with thiamin levels below the threshold for EMS, the variation in EMS mortality can be high (Brown et al. 1998). This being the case, it seems likely that some percentage of the eggs spawned by feral lake trout in the lower Great Lakes survive through to swim-up and beyond. Nonetheless, negligible numbers of these fry appear to survive. Consequently, it seems reasonable to conclude that other factors are contributing to the recruitment failure being experienced by many Great Lakes lake trout populations.

*C-start Effects*

It is generally accepted that contaminant levels in the eggs of feral Great Lakes lake trout are currently below the threshold for early life stage mortality. Although historical contaminant levels were once much higher, contaminants are no longer considered a sole impediment to natural reproduction in lake trout populations in the lower Great Lakes (Cook et al. 2003). Nonetheless, it remains unclear whether contaminant levels, especially the levels of the most toxic PHH congeners which are very persistent in the environment, may still be having subtle behavioral impacts of the early life stages of lake trout.
One such sublethal effect for lake trout young is alteration of their C-start response (occurrence and intensity); sublethal PHH levels have recently been shown to significantly reduce C-start intensity in lake trout embryos (Wright et al. unpublished). To briefly review, C-starts in subcarangiform fish such as lake trout are characterized by a lateral flexing of the body into the shape of the letter 'C' when startled, followed by rapid and forceful straightening of the body, propelling the individual away from the perceived threat. In addition, the C-start response may influence egg hatchability, as well as survival of the fry. In egg-bound embryonic fishes, embryo movements, including spontaneous C-starts, are believed to be important in distributing the enzymes responsible for rupture of the egg capsule (Willemse and Denuce 1973; Yamagami 1981; Shoots et al. 1982) and may possibly aid the embryo in bursting free from the egg capsule during predator attack (Eaton and Nissanov 1985).

The C-start response functions to facilitate predator escape in free-swimming larval and adult fishes (Eaton et al. 1977; Webb 1981; Webb 1982; Blaxter and Batty 1985) and may be involved in the terminal phase of prey capture (Canfield and Rose 1993). The onset of exogenous feeding and predator avoidance are critical components in the survival of lake trout fry. Therefore, impaired C-start performance, whether due to contaminants or thiamin deficiency, could significantly impact the growth and survival of young lake trout.

Both C-start response and EMS are neurological in nature. The avoidance maneuvers of the C-start response are likely initiated by neural impulses conducted from the brain to the caudal musculature via the Mauthner cells, a single pair of large myelinated neurons extending caudally from the brainstem and down the length of the spinal cord (Kimmel et al. 1980; Eaton and Hackett 1984; Lui and Fetcho 1999; Zottoli 1977; Eaton et al. 1981; Eaton et al. 1982; Zottoli et al. 1999). Impairment of C-start function thus suggests impact at the neurological level. As regards EMS, it's hallmark features include loss of equilibrium, spiral swimming, lethargy, and hyperexcitability (Honeyfield et al. 1998), evidence again of impacts occurring at the neurological level. Although subtle, these neurological impacts in early life stage fishes can negatively affect survival and growth. However, no known study exists in which C-start response was correlated or quantitatively linked to predator-related mortality.
**C-start testing: Harbinger of EMS**

Both the overall percentage of embryos exhibiting C-start, and the mean C-start intensity were negatively correlated with EMS mortality in the present study. The indications from this study were that if less than 80% of late stage embryos exhibit C-start responses, then the occurrence of later EMS mortality was likely to be substantial (>70%). C-start intensity was an even better predictor than overall late-stage C-start response; embryos exhibiting less than one C-start thrash per stimulus had 50% or higher EMS mortality. Thus, we submit that our simple C-start testing of pre-hatch lake trout embryos could be used by hatchery managers to conduct early assessments of the likelihood of subsequent EMS mortality. Where cost or availability prohibit chemical thiamin analyses, assessing the proportion of embryos exhibiting C-starts and mean C-start intensity at various embryo developmental stages could provide an “early warning” of the likelihood of EMS arising, allowing culture termination decisions to be made before significant additional costs are incurred. Further study and testing of this approach is warranted.

**Feeding Efficiency Effects**

Lake trout fry exhibited similar feeding efficiencies regardless of their initial egg thiamin levels or maternal contaminant exposure. Fry from the Hatchery and Lake Michigan groups displayed mean prey capture rates and prey ingestion numbers that did not differ significantly (Table 4). However, the mean capture rates of fry from the Laboratory groups (from hatchery females that spawned eggs very low in thiamin) were significantly higher than capture rates in the Hatchery group. This difference was likely due to the significantly larger fry size in this group at the time of their feeding testing. This size differential was likely a consequence of rearing density differences: whereas the other test groups were reared at densities of 15-25 fry per rearing chamber, the low survival of the Laboratory fry reduced their rearing densities to 6-12 fry per chamber by the start of the feeding tests. Although these larger fry did not consume more prey that did the smaller fry from the remaining groups, their capture efficiencies were significantly higher (Table 4).

Because the thiamin levels in the fry tested in the feeding experiment were not assayed, we cannot quantify the degree to which fry from the Laboratory and Lake Michigan groups began
exogenous feeding with a thiamin deficiency. Once they began feeding, however, they would have ingested dietary thiamin because the standard trout flake and chow they were fed from 45 days post-swim-up contained the recommended amount of thiamin for fry growth and development (Avault 1996). Thus, once feeding begins, it thus appears that fry initially deficient in thiamin can improve their survival and feed on par with thiamin sufficient fry, as long as the forage provides adequate amounts of thiamin.

**PHHs**

Reproductive dysfunction in Great Lakes lake trout is a complex and complicated phenomenon. Possible contributors to the problem include ecosystem changes, nutrition, and contaminants (Marcquenski and Brown 1997). Although the impact of contaminants such as PHHs was once considered a significant impediment to successful natural reproduction in Great Lakes fishes, it is now widely accepted that such impacts are no longer characterized by overt mortality. Although specific contaminant data was not conducted on the two egg batches from Lake Michigan females used in the current study, lake trout egg batches collected in the same area of Lake Michigan in 1998 had low levels of PHHs: 1.5-7.35 pg/g TCDD-equivalents (based on [TCDD and TCDFs]) and 1.33-12.83 pg/g TCDD-equivalents (based on [PCBs]) (Stratus 1999). Contaminants such as PHHs persist in the Great Lakes environment and in the fishes therein, but occur at levels below those that cause overt mortality (Cook et al. 2003). The most toxic PHH, 2,3,7,8-TCDD, has an LD50 of approximately 58 pg/g based on maternal transfer (Walker et al. 1994) and close to 80 pg/g based on egg injection (Walker et al. 1994; Walker et al. 1996). In contrast, it has been estimated that a feral lake trout egg contains just under the equivalent of 15 pg/g of TCDD-equivalent (Wright and Tillitt, 1999), a level far below that required to directly elicit mortality and thus reduce recruitment. However, contaminant burdens of 15 pg/g of TCDD equivalent or less in trout eggs do cause hemorrhaging and yolk-sac edema (Wright and Tillitt, 1999). In addition, sublethal changes in C-start responses in rainbow trout and lake trout have been shown in response to TCDD doses below the lethal dose (Wright et al, unpubl). Therefore, while not causing direct lake trout embryo and fry mortality, contaminants may be reducing survival via these more subtle, sublethal means.
Current research is investigating the possibility that the documented thiamin deficiency in lake trout eggs results from adult females consuming diets in thiaminase-containing alewife, the consequence being high fry mortality just prior to the onset of exogenous feeding (i.e., EMS). There is mounting evidence that EMS is a major contributor to the reproductive dysfunction being experienced by lake trout and other Great Lakes fishes (Brown et al. 1995b; Honeyfield et al. 2005). Indeed, high EMS mortality rates occur where egg thiamin levels are low, and treating eggs with thiamin can markedly reduce EMS mortality (Fitzsimons et al. 2001).

However, the almost total lack of recruitment in certain Great Lakes lake trout populations indicates that stressors other than just thiamin deficiency-induced EMS must be involved. The existence therein of lake trout eggs with thiamin levels that exceed the threshold below which EMS occurs indicates that at least some eggs and fry should survive to first feeding. After this point, it must be stressors experienced by the fry that are causing the remaining mortality.

In early life stage fishes, salmonids especially, the switch from yolk-sac feeding to exogenous feeding is a critical life history event. Young fishes must locate and capture sufficient food, and they must avoid the increased predation that accompanies the seeking of prey. During this critical period, behavioral changes that render the young more susceptible to predation will likely result in increased mortality, and reductions in the occurrence and intensity of the C-start response is an example of just such a behavioral change.

In the present study, the C-start responses of lake trout fry decreased depending on egg source, with the highest response occurring in Hatchery embryos and Lake Michigan embryos with high initial egg thiamin content and the lowest responses occurring in both Laboratory and Lake Michigan embryos with low initial egg thiamin levels. Responses of embryos with sufficient thiamin and no level of environmental contamination (i.e. Hatchery-reared) were similar to those of embryos with sufficient thiamin and some degree of maternally-transferred contaminant level (i.e. those from Lake Michigan females). From these results, thiamin deficiency appears to be the stronger predictor of impaired C-start response. However, additional study of the interaction of contaminant exposure and thiamin deficiency on lake trout C-start intensity, fry growth, and survival is needed.
Also meriting consideration is that EMS tends to be more apparent in areas of the Great Lakes where contamination has been long-term. Similarly, the occurrence of the synonymous M74 syndrome in Baltic Sea Atlantic salmon (*Salmo salar*) appears to be contaminant-related, with an increase in M74 occurrence being linked to elevated levels of dioxin-like contaminants (Paasivirta et al. 1995). The cause(s) of thiamine deficiency in fish and other species continue to be studied in relation to contaminant exposure (Sepulveda et al. 2004). One possible explanation for this apparent association is that an interaction exists between thiamin deficiency and how thiamin is involved in the metabolism of certain contaminant compounds. As such, the presence of even small contaminant quantities may increase a fish’s requirements for thiamin; conversely, the effects of certain environmental contaminants may be more apt to be evident when thiamin is deficient. The research on this issue is meager; for rats (*Rattus norvegicus*) we know that PCBs and DDT can reduce thiamin levels (Yagi et al. 1979; Stacpoole et al. 1990), but comparable studies have not been conducted in fish. The occurrence of such subtle contaminant-related effects merit further study, especially as regards whether their impacts on early life stage lake trout survival is direct or indirect.

**Summary**

The causes of the reproductive disorder in Great Lakes lake trout, known as Early Mortality Syndrome, remain unclear. Thiamin deficiency of lake trout eggs appears to be a major impediment to embryo and fry survival. Fry from eggs with initial egg thiamin levels of less than 2 nmol/g exhibit very high EMS mortality, and this mortality can be ameliorated by immersion of eggs in a thiamin solution. In addition, thiamin deficiency significantly reduces embryo C-start response, a behavior that affects later behaviors such as predator avoidance, and reduced C-start response was correlated with high EMS mortality. However, even with the depth of research supporting the effects of thiamin deficiency on EMS, the additional potential impacts of low levels of PHHs are not clear. Very low levels of PHHs such as 2,3,7,8-TCDD can induce pathological lesions in lake trout fry, and the question remains whether such low contaminant levels can also produce subtle behavioral alterations. In the present study, thiamin-sufficient lake trout from Lake Michigan females had similar overall C-start response and feeding efficiency to that of Hatchery
embryos. C-start magnitude appeared to be lower in the Lake Michigan embryos; however this difference was not statistically significant. Further study, with larger sample sizes and expanded treatment groups, could contribute important additional information.

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Chapter 7: Can lake trout reproduction in the lower Great Lakes ever become self-sustaining again?

This question underlies all past and current research into the causes of lake trout (Salvelinus namaycush) reproductive dysfunction. The dramatic decrease in PHH concentrations in adult fish, and the failure of lake trout populations to establish natural reproduction in both Lake Ontario and Lake Michigan, has prompted much research into which and how other factors may be affecting lake trout reproduction.

There is now strong evidence that nutritional thiamin deficiency plays a role in lake trout reproductive dysfunction (Brown et al. 2005). The diets of lake trout in all of the Great Lakes except Lake Superior are now dominated by alewife (Alosa pseudoharangus) and rainbow smelt (Osmerus mordax), exotic species that contain thiaminase, an enzyme that destroys thiamin in salmonids that consume it (Ji and Adelman 1998; Tillit et al. 2005). Fitzsimons (1995) found that thiamin supplementation reversed or prevented the gross signs of, and mortality associated with, the swim-up syndrome seen in Lake Ontario lake trout fry. There is also evidence that the low thiamin levels in lake trout eggs from Lake Michigan and Lake Ontario are associated with an increased risk of early mortality syndrome (EMS) in the fry (Marcquenski and Brown 1997; Wright and Tillitt 1999). The potential of there being interactions with other factors also remains.

In Lake Michigan, where there is no evidence of successful natural reproduction, less than 30% of lake trout egg lots that were sampled between 1996 and 1998 developed EMS (Edsall et al. 1999). Those groups that did develop EMS contained low total egg thiamin levels (generally but not always less than 1 nmol/g) and their survival past swim up ranged from 5% to 87% (Edsall et al. 1999). These findings suggest that other factors may account for a significant part of the mortality in Lake Michigan lake trout fry. Factors such as poor egg quality, or changes in the physiology of hatchery lake trout once in the wild, may interact with thiamin deficiency to yield the fry mortality which occurs (Manny et al. 1995). Since certain PHH compounds may interact with the metabolism and storage of thiamin in rats (Rattus norvegicus) (Yagi et al. 1979; Pelessier et al. 1992), the involvement of such compounds in fishes must be considered.
Although a direct correlation has yet to be established between concentrations of PHHs and EMS-associated mortality in Great Lakes lake trout, the role that contaminants may have in lake trout reproductive dysfunction cannot be discounted.

**Brief History of Great Lakes Lake Trout Reproductive Dysfunction**

Because the nature of lake trout reproductive dysfunction has changed over time, the investigative research of it has too. From an historical perspective, we know that lake trout populations in the Great Lakes were once healthy and abundant. Due mainly to over-fishing and the impacts of exotic sea lamprey (*Petromyzon marinus*), populations declined by the middle of the 20th century. Rehabilitation efforts began in the 1960s and 1970s, and have been somewhat successful at increasing adult lake trout populations. However, stocked lake trout do not appear capable of natural recruitment: eggs are spawned but the young do not survive.

The initial reproductive problems experienced by lake trout in the lower Great Lakes were likely due to very high contaminants burdens, especially those of PHHs such as 2,3,7,8-TCDD (Cook et al. 2003). Early life stage lake trout are extremely sensitive to the effects of such contaminants (Walker et al. 1991). The early life stage mortality due to PHH contamination was termed “blue-sac”, and was characterized by high hatching and sac-fry mortality preceded by such gross pathological symptoms as yolk-sac edema, hemorrhaging, cardiac edema, and craniofacial abnormalities (Walker et al. 1991). However, succeeding concentrations of PHHs have steadily declined in contaminated areas of the Great Lakes, and are now at levels below the lowest observable effect level (LOAEL) for lake trout fry mortality (Wright and Tillitt 1999; Cook et al. 2003). Despite adult lake trout survival being high in all five Great Lakes, there appears to be a reproductive bottleneck (a period of high mortality in lake trout populations) which occurs between the swim-up stage of development (when fry begin to feed exogenously) and the first year age class.

With this historical perspective in mind, recent research has begun to address a number of questions regarding the continued lake trout recruitment failure problem. Research to date
indicates that:

- natural reproduction leads to a self-sustaining population of lake trout only in Lake Superior (Hansen 1995);
- in the other lakes, very low thiamin levels in eggs, caused by an adult diet rich in thiaminase-containing prey, may be contributing to the occurrence of high fry mortality (termed early mortality syndrome or EMS) in affected lake trout populations (Brown et al. 1998; Fitzsimons et al. 1999; Brown et al. 2005; Tillitt et al. 2005);
- EMS, characterized by a suite of behavioral and physical alterations (including abnormal swimming, skeletal malformations, and hemorrhaging followed by death as swim-up occurs), is exhibited in the offspring of feral lake trout females when reared under laboratory conditions (Fitzsimons et al. 1999);
- EMS typically occurs when total egg thiamin levels drop below 2.0 nmol/g, and mortality approaches 100% when thiamin levels fall below 1 nmol/g (Brown et al. 1998);
- treating eggs and fry with thiamin hydrochloride can appreciably or completely reduce EMS (Fitzsimons 1995; Fitzsimons et al. 2001; Brown et al. 2005);
- EMS mortality in Great Lakes salmonids varies widely (Wolgamood et al. 2005);
- adults can be significantly impacted by thiamin deficiency: adult Great Lakes salmonids exhibit abnormal behavior and mortality, and the offspring of affected fish are likely to exhibit high levels of EMS mortality (Brown et al. 2005);
- sublethal levels of PHHs in lake trout can cause behavioral and physical alterations that are apt to affect their ability to survive (Wright and Tillitt 1999; Wright and Tillitt, unpubl.).

**Major Impediments, Interrelationships, and Possible Solutions to Lake Trout Reproduction**

The impediments to establishing self-sustaining lake trout populations in the lower Great Lakes are many, and include such diverse issues as the impacts of sport fishing (Breider et al. 2002), low genetic diversity (Krueger and Ihssen 1995), the effects of parasitic sea lamprey (Kitchell 1990; Krueger et al. 1995; Schneider et al. 1996), and degraded spawning habitat
(Edsall and Kennedy 1995; Marsden et al. 1995). These impediments will require the combined attention of researchers and fisheries managers to overcome.

Of particular concern in this discussion are those impediments specifically related to the survival of early life stage lake trout. I see the three main barriers to lake trout egg and fry survival as being (1) the continuing body burdens in adults of persistent environmental contaminants such as PHHs, (2) the dominance of thiaminase-containing alewife, an exotic species that is both a major part of adult lake trout diets (Miller and Holey 1992; Tillitt et al. 2005), and a predator of lake trout young (Krueger 1995) and (3) heavy predation pressure on lake trout eggs and fry by both native and non-native aquatic species. Moreover, there may be complex interrelationships between these three stressors: for example, predator avoidance behaviors in fry can be altered by both contaminant exposure and thiamin deficiency. These interrelationships are depicted in Figure 1. Each stressor needs to be evaluated both separately, and in terms of how it interacts with the other major stressors.

**PHHs**

Contaminants such as PHHs can still be affecting lake trout reproduction through the sublethal physical and behavioral changes they cause in fry. Even the low PHH levels in lake trout today can cause gross sublethal effects such as yolk-sac edema and hemorrhaging (Chapter 1). Perhaps more significant are the possible effects of PHHs (i.e., TCDD) on such early behaviors as the C-start response and feeding (Chapters 4 and 5). Because C-start responses in later life stage fishes are critical for predator avoidance and serve other key functions, any adverse effects due to low contaminant levels may affect survival.

There appears to be an interaction between TCDD and thiamin, although the exact nature of the interaction remains uncertain. For example, I demonstrate that thiamin supplementation can reduce TCDD-induced mortality and related symptoms in apparently thiamin-sufficient rainbow trout (*Oncorhynchus mykiss*) and medaka (*Oryzias latipes*) fry (Chapter 2). I also show that thiamin-sufficient lake trout embryos from feral contaminant-exposed females exhibit slightly decreased C-start responsivity in comparison to hatchery-reared nominally
Figure 1. Great Lakes Lake Trout Reproductive Dysfunction: The impacts of three major stressors (PHHs, Alewife, and Predation)

1. PHHs
   - Altered behaviors, (i.e., predator avoidance, feeding efficiency)
   - Increased susceptibility to thiamin deficiency and EMS?

2. Alewife
   - Thiaminase, thiamin deficiency and EMS
   - Thiamin deficiency causes altered behaviors (i.e., predator avoidance, feeding efficiency)
   - Predation on lake trout young

3. Predation
   - Predation on lake trout e.l.s.
   + Lake trout preying on potential early life stage predators (i.e., alewife, slimy sculpin)

(+) Positive pressure in terms of lake trout restoration
(-) Negative pressure in terms of lake trout restoration
contaminant-free counterparts (Chapter 6). Such small impacts, combined with additional stresses from thiamin deficiency and predation, may likely contribute to the lack of lake trout recruitment.

There are several possible mechanisms whereby environmental toxins such as PHHs might interact with thiamin physiology or the development of thiamin deficiency. First, PHH compounds such as TCDD may directly break down thiamin. This occurs in rats (Yagi et al. 1979), but has not been shown in fish. Second, an increase in drug-metabolizing enzymes induced by dioxin-like compounds may contribute to the development of thiamin deficiency via more indirect pathways. However, the evidence of this is as yet sparse. Third, thiamin may be involved in the antioxidant system in fish, although there is little evidence that thiamin itself acts as an antioxidant. In rats, research suggests that long-term thiamin deficiency weakens the liver’s antioxidant defense capability and increases cellular sensitivity to oxidative stress (Chen 1999). If the same is true in fish, then the basis exists for there being an interaction between diet-induced thiamin deficiency and PHH-induced oxidative stress in Great Lakes salmonids. This might explain the greater than expected mortality in early life stage individuals of these species, (i.e., they may be more susceptible to PHH-induced toxicity due to their thiamin-deficient state). The main point here is that additional research may show that current applications of thiamin to eggs and fry in hatcheries are insufficient. Additional thiamin may be beneficial in the long term, and may forestall the later behavioral deficits caused by PHH exposure.

Dominance of Alewife

Alewives make up a large portion of the diet of lake trout in the Great Lakes (Miller and Holey 1992; Tillitt et al. 2005). High lake trout fry mortality due to thiamin deficiency-induced EMS has been linked to lake trout diets rich in thiaminase-containing prey such as the exotic alewife. A recent report suggested that Lake Michigan lake trout will exhibit ~25% fry EMS mortality if alewife and rainbow smelt continue to comprise the majority of their diet (Bronte et al. 2003). Alewives cause further impacts by being a predator of lake trout fry (Krueger 1995).
As regards current efforts to protect the eggs of feral females from the “EMS bottleneck”, my demonstration that adverse sublethal behavioral effects can result from stressors other than thiamin deficiency means that future models of fry survival must now incorporate the contribution of these additive stressors. Thiamin supplementation can only provide protection from thiamin deficiency-related mortality, whereas long-term behavioral deficits may superimpose additive mortality on post-swim-up fry.

The embryo C-start response can potentially be used to predict EMS occurrence rates in swim-up fry (Chapter 6). This assay could be used where thiamin analysis is unavailable, because it is simple and requires little in the way of equipment to conduct (Chapter 3). The current hatchery practice is to supplement eggs if thiamin deficiency is suspected. Although no direct mortality usually results from this treatment, it does necessitate the potentially unnecessary handling of sensitive eggs. Although assays for direct analysis of thiamin in eggs continue to be refined, most still involve sending samples off-site for processing. Using the simple, inexpensive C-start assay for evaluating early embryo health offers both time and money savings. I recognize, however, that having developed a simpler assay for evaluating EMS in lake trout embryos does not address the root problem of how to achieve higher early life stage lake trout survival.

**Predation**

The final stressor, predation, may be especially important in this discussion because it is affected by both of the other stressors. Both PHHs and thiamin deficiency cause alterations in the key early behaviors necessary for predator avoidance in salmonids, the C-start response being chief among these (Chapters 4-6). There are many potential predators of lake trout eggs and fry in the Great Lakes (Jones et al. 1995). Predation on lake trout egg and sac-fry occurs on the spawning reefs: consequently, gaining a better understanding of which predators may inhabit these reefs during spawning season could lead to construction of new lake trout reefs away from potential predators. Predation on swim-up fry and fingerlings is also a consideration. Slimy sculpin (*Cottus cognatus*) are both predators of and competitors with young lake trout (Hudson et
al. 1995); their interactions with lake trout would be bettered should the predator avoidance or feeding capabilities of lake trout be compromised. Alewives are another important predator of early life stage lake trout; ironically, the alewife’s impacts on trout predator avoidance behavior may make alewives more successful predators of lake trout fry.

Summary

What possible solutions exist to the three main impediments to the survival of early life stage lake trout? There is very little that can be done to limit contaminant exposure. Although PHH levels are declining in the Great Lakes ecosystem, these are very persistent compounds that will continue to be present for some time. It may be possible to limit the negative impacts that such compounds cause. For example, reducing the predation pressure on lake trout young would potentially counter their reduced predator avoidance capabilities. Improving the thiamin status of lake trout would likewise negate the additive effects of PHH exposure on their already thiamin-deficient young.

The abundance of alewives is likely a pivotal factor that limits the survival of lake trout young. It is estimated that the numbers of lake trout currently being stocked, especially in Lake Michigan, are below those required historically to maintain a self-sustaining population (Bronte et al. 2003). If lake trout numbers would increase in those Great Lakes that currently lack self-sustaining populations, then greater control of the alewife populations might be achieved. With lower alewife numbers, alewife predation on early life stage lake trout would diminish. In addition, if the dietary dependence of lake trout on alewife was diminished (hopefully through replacement with prey low in thiaminase), then the overall thiamin status of lake trout adults and their offspring would improve. Either might negate the impacts of contaminants. However, if alewives continue to dominate the Great Lakes, their negative impacts on lake trout populations make restoration uncertain.
References:


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Vita

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