2009 Project Abstract
For the Period Ending June 30, 2013

PROJECT TITLE: Vulnerability of Lakes to Endocrine Disruption
PROJECT MANAGER: Richard L. Kiesling
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: ML 2009, Chap.142, Sec. 2, Subd. 5b

APPROPRIATION AMOUNT: $297,000

Overall Project Outcome and Results

Effects of endocrine active compound (EAC) exposure to fish have been assessed predominantly at the molecular to organismal level, leaving questions regarding implications for population sustainability. One EAC, 17β-estradiol (E2), is frequently detected in aquatic environments because it is a hormone produced by vertebrates. This study assessed developmental and reproductive effects of E2 exposure on fathead minnows (FHM) and bluegill sunfish (BG). Continuous, six week exposures were conducted in outdoor tanks to simulate natural lake environments. First generation (F0) FHM and BG were exposed during sexual maturity. Second generation (F1) FHM were exposed during early development, sexual maturity, or both stages. Multiple biomarkers were measured to assess the effects of E2 exposure on fecundity, fish health, and development. Differences in the timing of egg production for both species indicate differences in lifetime fecundity between unexposed and exposed females. Exposure to E2 resulted in lower relative health and reduced expression masculine secondary sexual characteristic expression in F0 FHM. Similar results were not observed in F1 FHM. First generation BG males exposed to E2 had significantly smaller testes compared to controls. Supplemental, laboratory exposures were conducted on a separate FHM cohort to assess reproduction and larval ability to escape a predator threat. Predation tests suggest E2 exposure of the current generation has the greatest effect on larval survival. Larval FHM exposure to E2 in the F2 generation had longer escape responses and lower survival rates when compared to controls. Females exposed to E2 tended to lag behind controls in terms of larvae production after an initial period of similar activity. Results from this study suggest that exposure to E2 (in the absence of other estrogenic compounds) at environmentally relevant concentrations has subtle reproductive and developmental effects on FHM and BG and implications for long-term survival in a predator-rich environment.

Project Results Use and Dissemination

Results from this study feed into an ongoing study assessing septic system discharge to lakes and effects on bluegill fitness (Assessing Septic System Discharge to Lakes, funded by Environment and Natural Resources Trust Fund in 2010).

A manuscript was submitted to the Journal of the American Water Resources Association for inclusion in a special issue on contaminants of emerging concern (originally submitted in February 2013, revised copy submitted in July 2013). A copy of the revised manuscript is included as an attachment to this final report.

Results from portions of this study have been included in two graduate student theses at St. Cloud State University under the supervision of Co-PI, Heiko Schoenfuss.

Results have been presented at the following scientific conferences:
March 2012 – Midwest Society of Environmental Toxicology and Chemistry (Minneapolis, MN)
June 2012 – American Water Resources Association specialty conference on contaminants of emerging concern (Denver, CO)
October 2012 – Minnesota Water Resources Conference (Minneapolis, MN)

This study was discussed in conjunction with similar work in a MPR story that aired on February 20, 2013. (http://minnesota.publicradio.org/display/web/2013/02/20/environment/new-study-shows-chemicals-can-reduce-fish-survival)

Environment and Natural Resources Trust Fund 2009 Work Program Final Report

Date of Report: 9/20/2013
Final Report
Date of Work Program Approval: 5/4/2010 (date of funding agreement with custodial agency)
Project Completion Date: 6/30/2013

I. PROJECT TITLE: Vulnerability of Lakes to Endocrine Disruption

Project Manager: Richard L. Kiesling
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Web Site Address: http://mn.water.usgs.gov/projects/CED/index.html

Location: Ramsey County, MN; Winona County, MN

Total Trust Fund Project Budget:	**Trust Fund Appropriation**	**$297,000**
	Minus Amount Spent:	**$297,000**
	Equal Balance:	**$ 0**

Legal Citation: ML 2009, Chap.142, Sec. 2, Subd. 5b
Appropriation Language:
$297,000 is from the trust fund to the commissioner of natural resources for an agreement with the United States Geologic Survey and St. Cloud State University to develop quantitative data on juvenile and adult fish vulnerability to endocrine-active emerging contaminants found in Minnesota lakes. This appropriation is available until June 30, 2012, at which time the project must be completed and final products delivered, unless an earlier date is specified in the work program.

II. FINAL PROJECT SUMMARY:

Effects of endocrine active compound (EAC) exposure to fish have been assessed predominantly at the molecular to organismal level, leaving questions regarding implications for population sustainability. One EAC, 17β-estradiol (E2), is frequently detected in aquatic environments because it is a hormone produced by vertebrates. This study assessed developmental and reproductive effects of E2 exposure on fathead minnows (FHM) and bluegill sunfish (BG). Continuous, six week exposures were conducted in outdoor tanks to simulate natural lake environments. First generation (F0) FHM and BG were exposed during sexual maturity. Second generation (F1) FHM were exposed during early development, sexual maturity, or both stages. Multiple biomarkers were measured to assess the effects of E2 exposure on fecundity, fish health, and development. Differences in the timing of egg production for both species indicate differences in lifetime fecundity between unexposed and exposed females. Exposure to E2 resulted in lower relative health and reduced expression masculine secondary sexual characteristic expression in F0 FHM. Similar results were not observed in F1 FHM. First generation BG males exposed to E2 had significantly smaller testes compared to controls. Supplemental, laboratory exposures were conducted on a separate FHM cohort to assess reproduction and larval ability to escape a predator threat. Predation tests suggest E2 exposure of the current generation has the greatest effect on larval survival. Larval FHM exposure to E2 in the F2 generation had longer escape responses and lower survival rates when compared to controls. Females exposed to E2 tended to lag behind controls in terms of larvae production after an initial period of similar activity. Results from this study suggest that exposure to E2 (in the absence of other estrogenic compounds) at environmentally relevant concentrations has subtle reproductive and developmental effects on FHM and BG and implications for long-term survival in a predator-rich environment.

Amendment Request: August 15, 2013

1. Rationale and Proposal

The project was designed to develop quantitative data on juvenile and adult fish vulnerability to endocrine-active emerging contaminants (EACs) found in Minnesota lakes using mesocosms enclosures. The primary experiment used four-cubic meter mesocosms located at the USGS Upper Mississippi Ecosystem Science Center (UMESC) in La Crosse, WI. This facility is uniquely capable of housing and maintaining such an experiment in the most-cost efficient manner. However, the mesocosm design required the consecutive testing of each species as not all of them could be accommodated in the mesocosm setup at once.

In order to gain a benchmark data set to directly compare exposure effects across species, we were able to collaborate with the USGS Columbia Environmental Research Center in Yankton, SD, using their serial-diluter system to perform concurrent exposures of multiple fish. Equipped with this dilution system, the Yankton facility can reliably supply estrogen-spiked water to multiple aquaria containing sunfish and fathead minnows.

The location of this facility required several day and multi-day trips from St. Cloud, MN to Yankton, SD to assist with the setup, sample collection and takedown of the experiment. The Research Addendum ha been amended to reflect the addition of this experiment to the approved scope of work. The design criteria of this experiment are outlined in detail below in the Research Addendum.

Amendment approved by the LCCMR September 16, 2013

II. PROGRESS SUMMARY:

PREVIOUS PROGRESS SUMMARY AS OF 12/31/12:

Larval fathead minnow (FHM) predator avoidance behavior was assessed in F1 and F2 FHM generations. Fish were exposed to 17 beta estradiol (E2) in controlled indoor mesocosms at the St. Cloud State University Aquatic Toxicology Center. The fathead larvae were evaluated for the predator avoidance behavior using the C-start testing procedure. Results indicate that the relative inability of E2 exposed larvae to avoid predation may be a root cause of reduced population sustainability. This interpretation is consistent with the response seen in both exposed fathead minnows and bluegill sunfish larvae in the large outdoor mesocosm experiments already completed under this project. These recent results would also explain the fathead minnow population structure observed by Kidd and others in whole lake exposure experiments.

We are proceeding with two journal manuscripts, one of which will be submitted in February to a special issue of the Journal of the American Water Resources Association.

PREVIOUS PROGRESS SUMMARY AS OF 6/30/12:

The F1 bluegill and F2 fathead fry that were exposed to E2 during the summer of 2011 experienced high levels of over-wintering mortality, and surviving numbers were insufficient for the planned set of experiments. As a consequence, the continued assessment of larval fathead minnow predator avoidance behavior could only be performed on a second group of fathead minnows. This group is being exposed during the F1 and F2 generations in controlled indoor mesocosms at the St. Cloud State University Aquatic Toxicology Center. The fathead larvae will be evaluated for the predator avoidance behavior using the C-start testing procedure. Previous work in Dr. Schoenfuss' lab suggests that the inability of E2 exposed larvae to avoid predation may be a root cause of reduced population sustainability. This interpretation is consistent with the muted response seen in both exposed fathead minnows and bluegill sunfish larvae in the large outdoor mesocosm experiments already completed under this project.

We have proceeded with exposures of an additional cohort of fathead minnows over two generations in laboratory mesocosms. These fish will be used to assess predator avoidance performance across two generations of fathead minnows.

Specifically, we will test the hypotheses that (i) larval E2 exposure will reduce predator avoidance performance and larval survival during active predation and (ii) subsequent F2 generation E2 exposure will further exacerbate the reduction in predator avoidance performance.

PREVIOUS PROGRESS SUMMARY AS OF 2/10/12:

The F1 bluegill fry and F2 fathead minnow fry were being reared over the winter at UMESC in preparation for another exposure to E2 during 2013 and 2012, respectively. Bluegill and fathead fry that were exposed to E2 during the summer of 2011 continued to experience over-wintering mortality in the holding tanks. In contrast, control fry of both species continue to be vigorous and exhibit high survivorship. The remaining number of exposed bluegill and fathead fry are now insufficient for the planned set of experiments.

In response to result from the original experimental design, the remaining control fry will be used for laboratory E2 exposure experiments at the SCSU facilities in April and May 2012. The design of these experiments will follow the standard E2 exposure experimental design with the addition of an external control group of naïve fish from both species. These exposure experiments will test the response of the remaining fry, now young-of-the-year, to E2 exposure under standard laboratory conditions. F2 fathead minnow fry will be allowed to spawn under exposed and control conditions, and the results F3 fry will be tested for the c-start behavior response.

PREVIOUS PROGRESS SUMMARY AS OF 12/31/11:

Five, 6-week experiments have been completed, including: F0 fathead minnow, F1 fathead minnow fry, F1 fathead minnow adult, F0 bluegill, and F1 bluegill fry. Chemical and physical properties of the mesocosms were collected at least weekly throughout each experiment. All chemical analyses and summary statistics have been completed, as of November 30, 2011. The F1 bluegill fry and F2 fathead minnow fry are being reared over the winter at UMESC in preparation for another exposure to E2 during 2013 and 2012, respectively. Bluegill fry survivorship was lower than expected, however; sufficient numbers of individuals were obtained to allow future experiments.

Initial experiments conducted in 2010 included two levels of exposure to 17β-estradiol (E2): 10ppt and 30ppt. The chemical data indicated very little difference between the observed E2 water concentrations of the two treatments and, as a result, the experimental design in 2011 was modified to include only one level of exposure at 30ppt, allowing for more replication.

Plasma vitellogenin concentration, histo-pathological, and c-start predator avoidance analyses have been completed for F0 fathead minnows and bluegills. Summary statistics have been completed for the F0 fathead minnow biological data and are in

progress for the F0 bluegill data. In-depth analysis of the biological and chemical data is in progress and expected to be complete by February 28, 2012.

PREVIOUS PROGRESS SUMMARY AS OF 12/31/10:

Following signature of a funding agreement in May 2010, mesocosm facilities were constructed in May and tested through the month of June. Adult fathead minnows were introduced into the mesocosm tanks the second of July and allowed to spawn. The first experimental exposures to 17-beta estradiol started the third week of July for adults and the first week of August for fry. Physical and chemical data were collected weekly during the experiments. Water samples from the mesocosm tanks and the feedstock tanks were collected weekly and analyzed for 17-beta estradiol using ELISA methods. Significant differences were maintained between controls and treatment doses through the 6 week experiment. Physical and chemical data were also collected weekly from the mesocosm treatment tanks. Fish survivorship was high in both adult and fry treatments, providing sufficient stocks for over-wintering of fry. Histopathology data collection continued through the end of 2010.

IV. OUTLINE OF PROJECT RESULTS*:*

Result 1: Developmental impacts of juvenile exposure to water and sediment concentrations of EACs. Budget: $182,162

Description:
Effects of EACs will be measured in a three-way, balanced design using mesocosms. Mesocosms will be installed at the USGS Upper Mississippi Ecosystem Science Center (UMESC). Three species of juvenile fish reared at the UMESC facility [fathead minnow (*Pimephales promelas)*, bluegill (*Lepomis macrochirus*), and walleye (*Stizostedion vitreum*)] will each be exposed to one of three EACs (ethynylestradiol, 4-nonylphenol (4-NP), or nonylphenol ethoxylate (NPEO)) for six weeks. Water and sediment concentrations will be monitored four times during the experiment, and fish will be evaluated using blood-chemistry biomarkers (e.g., plasma vitellogenin) and histo-pathological indices of abnormal development (e.g., intersex). Half of the fathead minnows and bluegills exposed in this experiment will be allowed to grow to sexual maturity and will be evaluated for reproductive success as part of Result 2 below.

Summary Budget Information for Result 1: **Trust Fund Budget:** $182,162
Amount Spent: $182,162
Balance: $ 0

Deliverable / Milestone	Completion Date	USGS Budget
Build and test mesocosms	September 30 2009	$45,000
Establish captive fish stock	September 30 2009	$13,000
Run F0 generation experiments	June 30 2011	$41000
Final Report for Result 1	June 30 2013	$10,000

M.L. 2009 Final Report Summary 06/30/13:

The experimental design was altered from exposure of three fish species to one of three EACs to two fish species exposed to one EAC (17-beta estradiol (E2)). A third species was not exposed to E2 due to complications with rearing and spawning a population for the experiment. The compound used for exposure was chosen over the proposed compounds due to its' ubiquitous presence in natural environments and estrogenic activity. The three proposed EACs are less estrogenic compared to E2 and often found at very low concentrations in the environment which may have been difficult to maintain in the mesocosms. In place of sediment in the mesocosms, artificial gravel nests were constructed to serve as spawning nests for bluegills (Figures 1 and 2). As a result, no sediment concentrations of E2 were obtained.

Figure 1. Example of experimental setup. Three blocks of three mesocosms were placed in outdoor enclosures. Mesocosms were equipped with biofilters and submersible pumps to aid with denitrification and circulation. Mesocosms pictured above are set up for bluegill exposures with artificial nests to promote spawning.

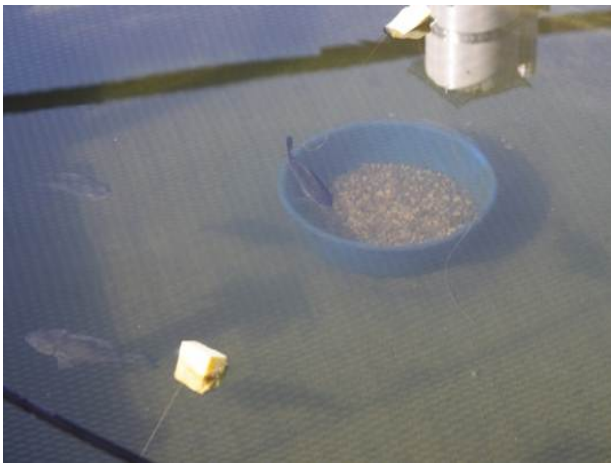

Figure 2. Adult bluegills in one of the mesocosms. Artificial nests were constructed by placing artificial gravel in bowls to promote spawning.

Second generation fathead minnow fry were obtained from control mesocosms from the initial adult experiment. Fathead minnow fry exposures were successfully conducted in Summer 2010. Significant differences between controls and treatment doses were maintained throughout the 6 week experiment (Figure 3). Survivorship in the mesocosms was high, providing sufficient stock to over-winter for a second exposure to E2 as adults the following year (Result 2). During the second exposure, fish were separated into four groups: never exposed, exposed only as juvenile, exposed only as adult, or exposed during both juvenile and adult stages. Significant developmental differences were only seen in the relative liver weight of males. Males exposed to E2 during both life stages had significantly lower liver weights (relative to body weight) compared to those only exposed as adults. Similar trends were observed in livers of male fish exposed as juveniles, regardless of their exposure treatment as an adult. Results suggest that exposure to E2 during juvenile stages when fish organs are developing may be more of a burden compared to exposure when organs have already developed.

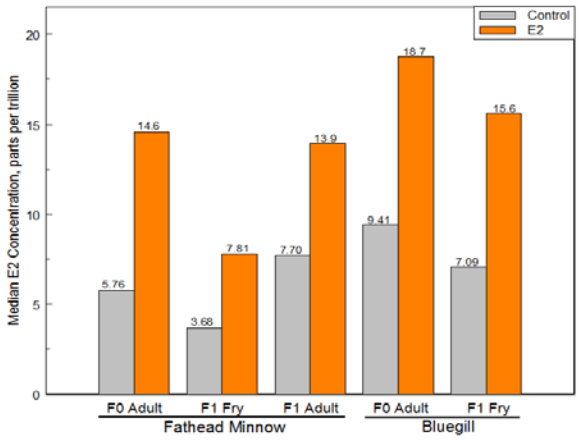

Figure 3. Median 17-beta estradiol (E2) concentrations in mesocosm tanks by treatment for each 6-week exposure. Significant differences were maintained between E2 and control treatments for all exposures ($p<0.01$). F0 = first generation, F1 = second generation.

Bluegill fry exposures were conducted in Summer 2011. Initial introduction into mesocosm tanks resulted in 100% mortality for reasons unknown (hypotheses include macroinvertebrate predation or unfavorable environmental conditions). A second attempt at introduction of bluegill fry was more successful than the first, although overall mortality was still higher than anticipated. Again, significant differences in E2 concentrations between treatments and controls were maintained throughout the 6 week exposure. Bluegill fry were transported to an indoor rearing facility for overwintering in preparation for a second exposure. However, mortality was nearly 100% resulting in cancellation of the second exposure and as a result no development effects of juvenile exposure to E2 for bluegills could be assessed.

Overwinter survival of third generation fathead minnow fry and second generation bluegill fry was insufficient to proceed with planned experiments. All remaining fish

were sacrificed for histo-pathology evaluation. As a result of the mortalities, the experimental design was modified to conduct C-start predator avoidance tests on a new cohort of fathead minnow larvae exposed to E2 in a controlled setting at St. Cloud State University (see amendment to project addendum in section IX).

Observed trends in C-start predator avoidance tests suggest that exposure of the generation being tested has greater influence compared to previous generation exposures. For example, longer latency, reduced velocity, and slower total escape response were observed in treatments containing an E2 exposure for the F2 generation. Larval fathead minnows exposed to E2 for 21 days post-hatch did not exhibit altered escape response within the generations (Figure 4). Longer latency period, reduced mean velocity and slower total escape response in E2 exposed F1 and F2 generation larvae was observed, but these differences were not significant.

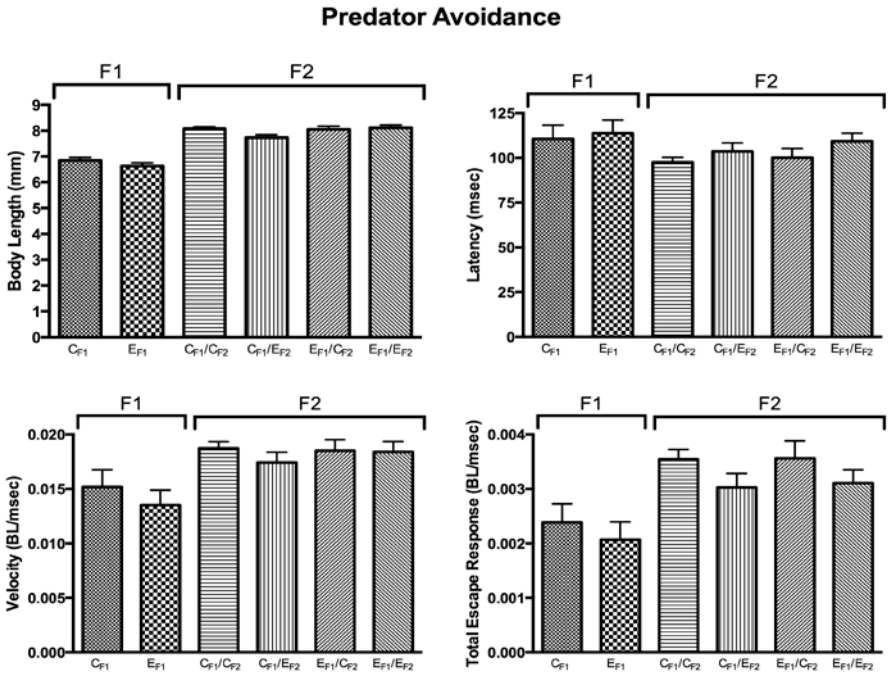

Figure 4. Mean body length, latency period, velocity, and total escape response for F1 and F2 generation treatments. C = control; E = E2 exposed; F1 = second generation; F2 = third generation

Despite the lack of difference in escape response within generations, comparisons across generations revealed some statistically significant differences. Body lengths were significantly different ($p<0.0001$). The follow up Dunn's test showed that both F1 generation treatment body length means were lower than any F2 generation measurements ($p<0.0001$ for each pairing). Higher latency periods were observed in the F1 generation, compared to F2 ($p=0.00314$ Kruskal-Wallis, not resolved using Dunn's post-test). All F2 generation velocity means were higher than the F1 generation during the first 40ms after initiation of the escape response ($p=0.0207$).

The greatest differences were observed between the E2 exposed F1 larvae and the C_{F1}/C_{F2} and E_{F1}/E_{F2} treatments in the F2 generation ($p<0.05$ for both). Similarly, the F1 generation total escape response means were lower than any of the F2 generation means. Differences were greater between the E2 exposed F1 generation and all F2 generation treatments (C_{F1}/C_{F2} , $p<0.001$; C_{F1}/E_{F2}, $p<0.05$; E_{F1}/C_{F2}, $p<0.01$; E_{F1}/E_{F2}, $p<0.05$) compared to similar comparisons between the control F1 generation and F2 generation treatments.

Survival of 21 day post-hatch F2 generation larvae during active predation was affected by exposure to E2 ($p=0.0275$) (Figure 5). Similar to F2 generation predator avoidance response, the current generation exposure had a larger effect than the exposure of any previous generation. Larvae from the F2 generation exposed to E2 had lower survival rates compared to controls.

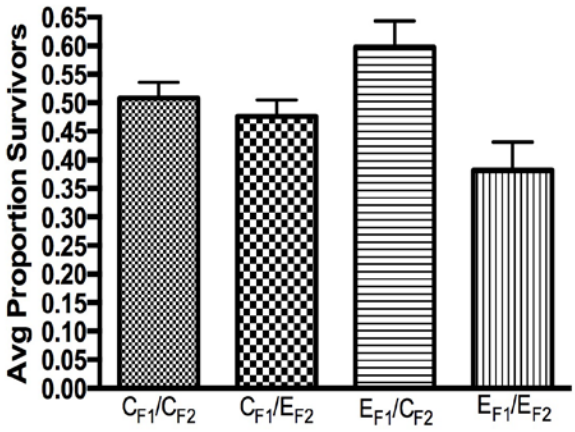

Figure 5. Mean survivorship of F2 generation larvae from active predation by bluegill sunfish in mesocosms. C = control; E = E2 exposed; F1 = second generation; F2 = third generation

Anatomical endpoints such as gonadosomatic index, hepatosomatic index, body condition factor, and presence of male secondary sex characteristics were generally not affected by E2 exposure in either sex. One exception was that females exposed to E2 had statistically significantly higher median body condition factor ($p=0.026$) compared to unexposed counterparts. Histological analysis of F1 generation adult gonads and livers revealed only sporadic pathologies (one control male exhibiting intersex).

Result 2: Reproductive impacts of juvenile and adult exposure to water and sediment concentrations of EACs. Budget: $114,838

Effects of EACs on reproductive success (i.e, mating behavior and spawning success) will be measured in a two-way, balanced design using mesocosms. Juvenile fathead minnows and bluegills reared under Result 1 above will be allowed

to spawn under controlled conditions and evaluated for reproductive output. Sexually mature adults of walleye at UMESC (and bluegill if necessary) will each be exposed to ethynylestradiol through water or sediment exposure for six weeks prior to spawning. Water and sediment concentrations will be monitored four times during the experiment, and fish will be evaluated using blood-chemistry biomarkers (e.g., plasma vitellogenin) and histo-pathological indices of abnormal reproductive physiology before and after spawning as appropriate.

Summary Budget Information for Result 2: **Trust Fund Budget:** $ 114,838
Amount Spent: $ 114,838
Balance: $ 0

Deliverable	Completion Date	USGS Budget
Rear F1 generation	December 31 2010	$30,000
Run F1 generation experiments	June 30 2011	$36,000
Complete chemical analysis	December 31 2011	$6,000
Final Report for Result 2	June 30 2013	$8,100

M.L. 2009 Final Report Summary 06/30/13:

As mentioned under Result 1, E2 was used for the exposures in place of ethynylestradiol because E2 is more ubiquitous in the environment and more estrogenic compared to ethynylestradiol. Artificial gravel nests were used in place of sediment for bluegill nests and hence, no E2 sediment concentrations are available. First and second generation (F0 and F1, respectively) adult fathead minnows and F0 bluegills were exposed to E2 for 6 weeks. Reproductive output of females was measured throughout the exposure by quantifying the number of eggs produced daily. At the conclusion of the 6 weeks, physical and histopathological biomarkers were assessed to provide indications of reproductive developmental effects in exposed fish compared to controls.

Female F0 fathead minnows were exposed to E2 during Summer 2010. Concentrations of E2 varied considerably throughout the exposure, but differences between controls and E2 treatments were maintained throughout (Figure 3). Female F0 fathead minnows exposed to E2 showed a slight delay in egg production compared to controls, although no overall statistically significant differences were observed between the two treatments. Contrary to what may be expected, both males and females exposed to E2 had significantly lower concentrations of plasma vitellogenin compared to controls. Vitellogenin concentrations were highly variable for all experiments and emphasize the need to assess multiple biomarkers in studies investigating effects of EACs on fish. Gonad development stages were not significantly different between treatments indicating development of reproductive organs was not greatly affected by adult exposure to E2. The presence of secondary sexual characteristics in F0 fathead males exposed to E2 was decreased compared to controls (Figure 6), indicating males exposed to E2 were at a reproductive disadvantage compared to those not exposed.

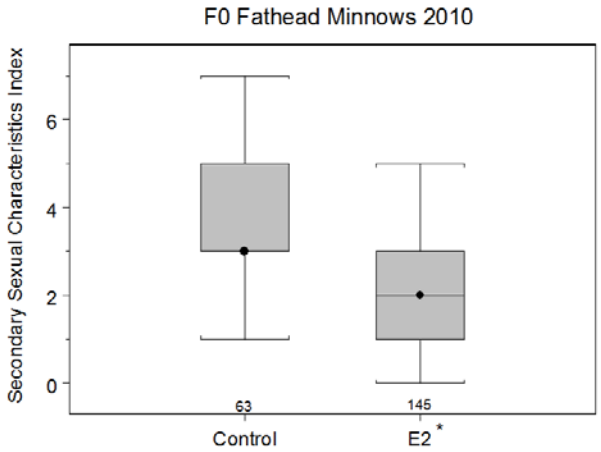

Figure 6. Boxplot summary of secondary sexual characteristic scores for male F0 adult fathead minnows. The Secondary Sexual Characteristics Index (SSCI) is computed by summing individual scores for the presence of tubercles, thickness of dorsal fat pad, and color of banding. * = significantly different from controls ($p<0.01$)

F1 fathead minnows were used for two exposure periods, one during juvenile (Summer 2010) and one during the adult life stage (Summer 2011). This resulted in four treatment groups: never exposed, exposed as juvenile only, exposed as adult only, and exposed during both juvenile and adult stages. Although overall egg production was not significantly different among treatments females only exposed as juveniles produced more eggs, faster compared to other treatments for several weeks throughout the exposure. This observation corresponds well with the differences seen in relative liver weight of F0 fathead minnows. Again, exposure to E2 during the juvenile phase while organs and processes are establishing themselves may have a greater impact compared to fish exposed after development is complete. No other statistical differences were observed for plasma vitellogenin concentration or biological indices among treatments for this group of fish.

Reproductive effects on bluegills are only available for the F0 adults because of complications with rearing bluegill fry (see Result 1). Males exposed to E2 had relatively smaller testes in relation to fish size compared to controls. Similarly, a slight shift in testes development stage was observed between treatments. Plasma vitellogenin was not significantly different between treatments in either sex, but concentrations were generally higher in fish exposed to E2 compared to controls. Similar to observations from the F0 fathead exposure, female bluegills exposed to E2 began spawning a few days after controls. Females exposed to E2 also generally produced fewer eggs compared to controls.

Since fecundity was measured in terms of total numbers of eggs produced in the outdoor mesocosm exposures, reproduction in the laboratory exposures was assessed by analyzing the number of viable offspring hatched. Exposure to E2 affected reproductive output of female fathead minnows, as evidenced by the total larvae produced during the breeding assay (Figure 7). Over the first 9 days the numbers of hatched larvae for E2 exposed fathead minnows mirrored closely the control minnows. This trend continued over the next five days while the reproductive

output plateaued for both treatments. After this lag however, the control minnows then resumed reproduction at a much higher rate than those exposed to E2. Due to the chaotic nature of fathead minnow reproduction, most of the larvae produced for control minnows were from two spawning tanks so the data was standardized to the number of larvae produced per female and the prior trend was saved through this standardization.

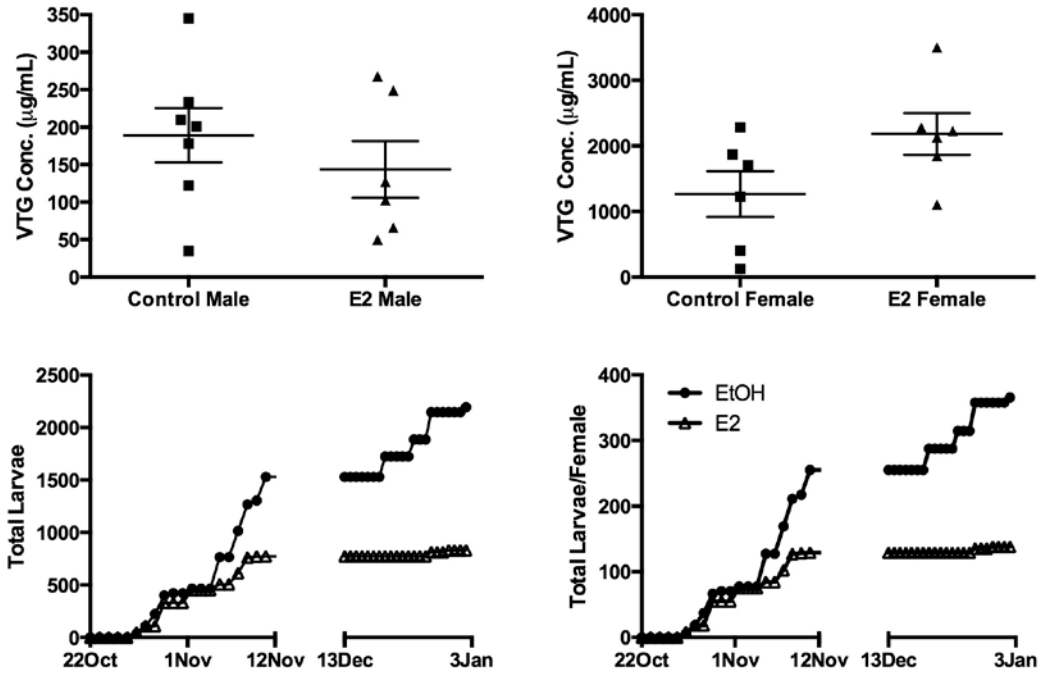

Figure 7. Top graphs depict plasma vitellogenin concentrations in males and females. Bottom graphs depict total larvae produced and total larvae per female.

V. TOTAL TRUST FUND PROJECT BUDGET:

Personnel: $229,235

Equipment/Tools/Supplies: $60,566

[Turbo-evaporator for processing chemical extractions from mesocosm sediment samples = $2307]

[Tanks, plumbing, air-lifts, and effluent treatment filters for mesocosms = $20,155]

[Supplies for Enzyme-linked immunosorbent (ELISA) chemical assays of EDC compounds in water and fish from mesocosms = $31,500 (SCSU) budget]

[Laboratory supplies and analysis kits for chemical determinations of EACs in sediment extracts = $6604

Travel: $7086

Other (Printing and Shipping)**:** $ 113

Personnel	229,235
Equipment/Tools/Supplies	60,566
Travel	7,086
Other	113
Total	297,000

TOTAL TRUST FUND PROJECT BUDGET: $297,000

VI. PROJECT STRATEGY:

A. Project Partners: This project is a continuing partnership between the United States Geological Survey (USGS) and Dr. Heiko L. Schoenfuss, Professor and Director of the Aquatic Toxicology Laboratory, Department of Biological Sciences, St. Cloud State University. Team members from the USGS include Dr. Richard Kiesling (project Leader) and Mark Gaikowski (USGS-UMESC Mesocosm Facility Director). Team members from the USGS will manage mesocosm installation and experimental protocols, exposure experiments, chemical analysis, and spawning experiments. Team members from St. Cloud State University will manage histopathology, behavioral experiments and spawning experiments. All team members will participate in writing the final report and communicating results to state user groups.

Funding by Partner	**Budget**
1. USGS Cooperative Water Program	$189,100
2. St. Cloud State University	$107,900
TOTAL	$297,000

B. Project Impact and Long-term Strategy:

1. Project provides direct estimate of how juvenile fish of common lake species respond to environmentally meaningful EAC exposures in a lake setting. Most EACs are found at very low concentrations in water (Kolpin and others; 2002; Lee and others; 2004) but reach higher concentrations in sediment (e.g., Mayer and others, 2006; Pojana and others 2007; Kim and Carlson, 2007). Despite these low concentrations, research has identified developmental and reproductive effects on fish species at environmentally relevant concentrations. The proposed work will extend this work to include important lake species under chronic exposure through either water or sediment exposure pathways.

2. Project provides an estimate of the importance of longevity and other reproductive characteristics in the magnitude of fish response to EAC exposure during different growth stages/ages.

In Minnesota, endocrine disruption has been observed in short- and long-lived fish species including vitellogenin induction in male fathead minnows, male carp, and walleye (Folmar and others, 1996, 2001; Lee and others, 2000). Vitellogenin in male carp was also observed at numerous sites downstream of WWTP discharges throughout central Minnesota (Lee and others, 2000). Two ongoing studies in Minnesota have recently identified additional fish species affected by EACs in tributaries of the Mississippi and the St. Croix Rivers (Jahns and others in prep; Lee and others in review) as well as urban lakes (Schoenfuss and others – unpublished data). Taken as a whole, these results indicate that Minnesota fish communities are vulnerable to reproductive impacts. This study helps answer how vulnerable adult fish are to EAC exposure and whether life-history characteristics mitigate EAC exposure.

C. Other Funds Proposed to be Spent during the Project Period:

Budget Source	Budget
1. USGS Cooperative Water Program Funding	$133,000
2. USGS In-kind Contribution	$28,000

D. Spending History:

Funds have been spent to complete work outlined above. Details of expenditures are available on Attachment A.

VII. DISSEMINATION: Results were communicated to local groups, state agencies and national peer groups through presentations at regional and national meetings including the following:

- Results have been presented at the following scientific conferences:
- March 2012 – Midwest Society of Environmental Toxicology and Chemistry (Minneapolis, MN)
- June 2012 – American Water Resources Association specialty conference on contaminants of emerging concern (Denver, CO)
- October 2012 – Minnesota Water Resources Conference (Minneapolis, MN)

Results were also communicated through scientific reports including a manuscript accepted for publication in the Journal of the American Water Resources Association and parts of two Masters theses at St. Cloud State University. The JAWRA article will be included in a special issue on contaminants of emerging concern. A copy of the revised manuscript is included as an attachment to this final report. Results from portions of this study have also been included in two graduate student theses at St. Cloud State University under the supervision of Co-PI, Heiko Schoenfuss. Copies of these documents are also attached to this final report. One additional scientific journal article is in preparation as of 9/20/2013.

Finally, results from this study were discussed in conjunction with similar work in a MPR story that aired on February 20, 2013. The story is archived at (http://minnesota.publicradio.org/display/web/2013/02/20/environment/new-study-shows-chemicals-can-reduce-fish-survival)

VIII. REPORTING REQUIREMENTS: Periodic work program progress reports will be submitted every six months not later than 12/31/09, 06/30/10, 12/31/10, 06/30/11, 12/31/11, 6/30/12, and 12/31/12. A final work program report and associated products will be submitted between June 30 and August 1, 2013 as requested by the LCCMR.

IX. RESEARCH PROJECT ADDENDUM:

2009-2012 LCCMR Proposal – Assessing the Vulnerability of Lake Fish Communities to Endocrine Disruption from Water and Sediment Using Pond Mesocosms

2. Overview of the Study

Fathead minnows and bluegill sunfish will be exposed for six weeks to two environmentally realistic concentration of the endocrine active compounds 17b Estradiol (E2) during two sensitive ontogenetic stages: during larval development and during reproduction. F0 adults will be exposed and assessed for the reproductive output and physiological alterations consistent with EAC exposure. F1 generation offspring will be exposed from prior of hatching to six weeks post-hatch. A subset of exposed F1 generation fish will be assessed for the induction of vitellogenin or their ability to perform C-start predator avoidance behaviors. The remaining larvae will be reared to adulthood (one year for fatheads, two years for bluegill). Adult F1 generation fish will be placed into reproductive scenarios for six weeks and reproductive output will be assessed. During this reproductive period, a subset of fish will be exposed for a second time to establish three F1 generation adult treatments: (1) control-never exposed; (2) control exposed as larvae only; (3) exposed as larvae and as adults. Following exposure, fish will be processed for vitellogenin and histopathology.
In addition, walleye or other top predator will be exposed for six weeks in the same exposure scenario and assessed for reproductive, physiological and behavioral endpoints during year 2 of the study.

3. Experimental Design

3.1. Sub-system Design

Each of the six replicate exposure sub-systems will consist of four 200 gallon treatment / exposure tanks connected to a central 200 gallon balancing reservoir. The over all design is for a recycling static-renewal exposure with daily replacement of 5% of total tank volume. Daily water renewal will be necessary to prevent accumulation of waste products from fish metabolism. In addition to the daily water exchange, the balancing reservoir will be equipped with a bio-filter to help process accumulated nitrogenous wastes. The balancing reservoir will receive a constant dose of 17-B estradiol (E2) dissolved in ethanol and delivered by peristaltic pump at a constant flow rate.

Concentrations of E2 will be maintained at 10 or 30 ng/L for six weeks. Culture water will be recycled between all four treatment tanks in a sub-system and the central balancing reservoir. The four exposure tanks will be equipped with center-mounted outflow connections positioned approximately 20 cm below the water surface. Outlets will be double screened to prevent exchange of fish between treatment tanks and the balancing reservoir. Each treatment tank will be equipped with an airlift standpipe that delivers recycled water from the balancing reservoir to each tank. A

single air compressor will run the four airlift standpipes that deliver recycling water from the balancing reservoir to the individual treatment tanks. Water will return to the balancing reservoir by gravity equalization of water level through the center-mounted outflow connections.

Effluent generated from the 5% daily renewal rate of each sub-system will be processes using a flow-through UV exposure unit rated at 20 gallons per minute. The combined effluent flow rate for all six sub-systems is estimated to be less than 15 gallons per hour. Following UV exposure, effluent will be passed through an ozonation system prior to discharge to the UMESC holding pond. Treatment efficiency will be tested in an off-site facility prior to field deployment.

The sub-systems will be assembled in one of two 0.01 acre concrete ponds enclosed in wire fencing to prevent predation on the fish. Three sub-systems will be placed in each of two concrete ponds, and replicate sub-systems will be evenly split between the two concrete ponds. Each sub-system will be rinsed three times with well water from the UMESC supply wells. Once they have been drained, all sub-system tanks will be filled with ambient water from one of the 0.10 acre fish ponds at the UMESC facility. These source ponds are filled with well water and allowed to develop a natural planktonic community. Once the tanks are filled, the surrounding concrete pond will be filled with well water to provide a thermal buffer. The surrounding pond water will be continuously mixed with four air-lift standpipes and temperature will be regulated with additions of well water.

Treatment systems will be allowed to equilibrate with the continuous E2 addition and to develop stable physical and biological conditions prior to addition of fish. Efforts will made to establish acceptable food levels in the treatment tanks. Plankton dynamics will be monitored in the sub-systems

3.2. Fathead Minnow Exposures

F0 generation adult fathead minnows and F1 generation eggs are being placed randomly into two of the four exposure tanks of each of the six identical systems. Two systems have been exposed prior to fish placement with the carrier control ethanol, two have been exposed to 10 ng/L E2, and two have been exposed to 30 ng/L E2. Fish are maintained in the exposure scenarios for six weeks. F1 generation larvae (which will hatch within the first three days of exposure) will receive supplemental feed of newly hatched brine shrimp. Adult fathead minnows (10 males, 10 females per tank) receive supplemental frozen brine shrimp daily. Adult fathead minnow tanks will contain 10 nest sites which will be exchanged daily (eggs are counted daily and the discarded). After six weeks exposure, all adult fathead minnows are sacrificed in MS-222, a blood sample is taken, fish are measured for weight and length, and livers and gonads are excised, weight and preserved in 10% buffered formalin for later histopathological analysis. A subset of ten larvae per tank (n=20 per exposure scenario, n=40 per treatment) are being assessed for their ability to perform C-start predator avoidance behaviors. Following the behavioral observation, these larvae and ten additional larvae (n=2- per tank,

n=80 per treatment) are sacrificed in MS-222 and preserved for later vitellogenin analysis of the homogenate.

The remaining larval fish are transferred to rearing facilities at UMESC and reared to adulthood. Upon reaching maturity (Year 2), F1 generation adults are split into two groups; one group of ten males, ten females per tank will be sacrificed and assessed for vitellogenin and histopathology. A second set of ten males and ten females per tank are reintroduced to the exposure scenarios and allowed to spawn (similar to F0 generation fish in Year1). Three treatments are prepared based on the results of the prior analysis of Year 1 effects:

(1) Control – F1 generation fish that were never exposed;
(2) F1 generation fish exposed as larvae only; and
(3) F1 generation fish exposed as larvae and now again as adults.

The lowest effects concentration determined in Year 1 of the study (10 or 30 ng/L) will be used for the Year 2 exposures. Reproductive output will be quantified through daily counting of eggs on all spawning tiles similar to Year 1. Following the six week exposure all fish will be sacrificed and assessed for vitellogenin and histopathology.

3.3. Bluegill Sunfish Exposures

In Year 1 of the study, bluegill sunfish eggs and adult F0 generation bluegill sunfish will be exposed for six weeks to 10 or 30 ng/L E2 for six weeks. Exposures will utilize the same setup previously used for fathead minnows (see above). Two pairs of mature male and female bluegill will be placed into two of every four tanks in each exposure scenario (n=2 pairs per tank, 4 pairs per scenario, 8 pairs per treatment). These pairs will be separated by a stainless steel mesh barrier to avoid territorial interactions among the male fish. Bluegill reproductive output will be monitored daily for the six week exposure duration. F1 generation larvae (hatched during the first few days of the exposure) in the other two tanks of each exposure scenario will be exposed for six weeks as well. Following the six week exposure, all F0 generation adults will be sacrificed and assessed for plasma vitellogenin concentrations and histopathology. A subset of 10 F1 generation larvae per tank (n=40 per treatment) will be assessed for their ability to perform a predator avoidance C-starts. These larvae and ten more (n=20 per tank, n=80 per treatment) will be preserved after observation for later determination of vitellogenin concentration in whole larvae homogenate. The remaining larvae are reared to adult hood (two year rearing time to Year 3). Upon reaching maturity (Year 3), F1 generation adults are split into two groups; one group of ten males, ten females per tank will be sacrificed and assessed for vitellogenin and histopathology. A second set of two pairs of fish per tank are reintroduced to the exposure scenarios and allowed to spawn (similar to F0 generation fish in Year1). Three treatments (n=16 spawning pairs per treatment) are prepared based on the results of the prior analysis of Year 1 effects: (

1) Control – F1 generation fish that were never exposed;
(2) F1 generation fish exposed as larvae only; and (

3) F1 generation fish exposed as larvae and now again as adults.

The lowest effects concentration determined in Year 1 of the study (10 or 30 ng/L) will be used for the Year 3 exposures. Reproductive output will be quantified through daily counting of eggs in the nest of each spawning pair (similar to Year 1). Following the six week exposure all fish will be sacrificed and assessed for vitellogenin and histopathology.

3.4. Walleye Exposures

During year 2 of the study (2011), we will expose adult walleye (F0 generation) and eggs/newly hatched larvae (F1 generation) to two concentrations of E2 for six weeks following the same exposure protocol outlined in 2.2 and 2.3 and assessing the same endpoints as outlined in 2.5. This exposure will be conducted early in the season (March) and no fish will be reared to adulthood from this experiment. All fish will be assessed at the end of the six week exposure period for plasma or homogenate vitellogenin concentrations, histopathological endpoints and, in the case of the larvae, C-start predator avoidance performance.

3.5. Endpoints & Total Fish Sample Numbers

I. **Vitellogenin (vtg) analysis**. VTG analysis for fathead minnows will utilize purified fathead minnow vitellogenin and an in-house developed monoclonal vtg antibody. This ELISA has been used on several thousand fathead minnows and has been peer reviewed extensively. For bluegill sunfish vtg analysis, we will first purify vtg form a gravid female bluegill sunfish, purify the vtg and then develop a polyclonal vtg antibody specific for the bluegill sunfish. To assess vtg concentrations in larval fish, we will produce homogenates of 2 larval fish each, centrifuge the homogenate and the conduct the ELISA analysis on the supanate. For adult fish, we will measure plasma vtg concentrations separately for each fish.

   i. YEAR 1.

      a. Fathead minnow larvae: 80 larvae per treatment x 3 treatments = 240 larvae/2 (two larvae per homogenate) = 120 vtg analyses

      b. Fathead minnow adults: 10 males/10 females per tank, 40 males/40 females per treatment x 3 treatments = 120 males; 120 females → 240 vtg analyses of adult fathead minnows

      c. Bluegill sunfish larvae: 80 larvae per treatment x 3 treatments = 240 larvae/2 (two larvae per homogenate) = 120 vtg analyses

      d. Bluegill sunfish adults: 2 males/2 females per tank, 8 males/8 females per treatment x 3 treatments = 24 males; 24 females → 48 vtg analyses of adult bluegill sunfish

   ii. YEAR 2

a. F0 generation adult walleye exposed for 6 weeks: 48 vtg analyses
b. F1 generation juvenile walleye exposed for six week: 120 vtg analyses
c. F1 generation adult fathead minnows (no further exposure): 240 vtg analyses
d. F1 generation adult fathead minnows (secondary exposure experiment): 240 vtg analyses

iii. YEAR 3

a. F1 generation adult bluegill sunfish (no further exposure): 48 vtg analyses
b. F1 generation adult bluegill sunfish (secondary exposure experiment): 96 vtg analyses

iv. TOTAL:

a. Larval fathead minnow vtg: 120
b. Larval bluegill sunfish vtg: 120
c. Laval walleye vtg: 120
d. Adult fathead minnow vtg: 720
e. Adult Bluegill sunfish vtg: 240
f. Adult walleye vtg: 48

II. Histopathology

Histopathological analysis will follow well established protocols developed at the US EPA (2008 *Histology and histopathological guidelines for phase 1b of the OECD fish screening assay for EDCs*. EPL Project No. 481-017.) and applied extensively at the St. Cloud State University Aquatic Toxicology Laboratory. Briefly, tissues of interest (liver, testis, ovaries) are excised from the animals immediately following their sacrifice with MS-222. Tissues from each animal are placed together in a individually labeled histo-cassette and immersed in 10% buffered formalin for later processing. Upon return to the laboratory, tissues are dehydrated and paraffin embedded using a Leica automated tissue processor. After embedding, tissues are sectioned at 5µm using a Leica motorized microtome. Tissue sections are stained following established staining protocols for Haematoxylin & Eosin stains or for other stains (i.e., reticular stain) as appropriate. After coverslipping and drying, tissues parameters are ranked on a 0-4 scale based on the presence and abundance of cell types in livers, ovaries, and testis following standardized procedures outlined by the US EPA (2008). The analysis of tissues will be conducted by a trained histologist with years of experience analyzing fish tissue sections.

i. YEAR 1
   a. Fathead minnows: 240
   b. Bluegill sunfish: 48
ii. YEAR 2
   a. Walleye: 48
   b. Fathead minnows:480
iii. YEAR 3
   a. Bluegill sunfish:144

III. C-START PREDATOR AVOIDANCE PERFORMANCE

The C-start predator avoidance performance of exposed larvae will be measured using a trigger-activated system with a small light-emitting diode (LED) and a vibrating electronic chip attached to the base of the filming arena to provide a stimulus. When activated, the system causes a short vibrational stimulus (< 1 s) marked in the field of view by the appearance of the LED light used to determine time zero for data analyses. The filming arena consists of a 5-cm diameter glass Petri dish positioned on top of a 1-mm grid. The larval escape behavior in the filming arena will be recorded using a high-speed digital video cameras (Redlake MotionScope M1, Tucson, AZ) at 1,000 frames per second. Larvae will be fed 30 min prior to testing and a resultant time limit of 6 h will be set for data collection to avoid an observed drift in response due to time since last feeding. Individuals will be placed singularly and in random order into the filming arena and allowed to acclimate. Once a larval fish swims into the center portion of the grid (marked with a square), the trigger is depressed and the vibrational stimulus provokes a predator avoidance response (Fig. 3). Each larval fish is used for only one performance recording. High-speed video sequences of predator avoidance behaviors (Fig. 3.) are used to calculate the time to induction of behavior (latency period), escape velocity (velocity during the first 40 ms after the initiation of an evasive maneuver; body length/ms to exclude any size differences as confounding factors among individual fish), and total escape response [body length/ (latency in ms+40 ms)]. Videos will not be considered if the latency response is <5 ms (false start).

i. YEAR 1
   a. Fathead minnows: 120
   b. Bluegill sunfish: 120
ii. YEAR 2
   a. Walleye: 120
   b. Fathead minnows: 120
iii. YEAR 3
   a. Bluegill sunfish: 120

Experimental Methods

Addendum to Methods section of the Original Study Plan

Fish. Juvenile fathead minnows were obtained from the USGS Columbia Environmental Research Center, Columbia, MO. Juvenile bluegill sunfish were obtained from 10,000 Lakes Aquaculture, Inc., Osakis, MN. Upon arrival all fish were gradually acclimated to 25 ºC. The fish were fed live *Artemia* nauplii 2-3 times/day *ad libitum*. St. Cloud State University Institutional Animal Care and Use Permits were obtained for these experiments (permit # 0609).

Experimental design. The 21-day study was conducted in a flow-through proportional diluter system that delivered the test waters to 67-L glass aquaria. The aquaria were divided into three chambers; two replicate exposure chambers that contained about 20 L of water and one small chamber with a drain that received the overflow from the exposure chambers. There was no water connection between the two exposure chambers, so they were judged to be true replicates. The system was calibrated to deliver 0.5 L of test water to each exposure chamber every 15 minutes. The two species were exposed simultaneously to two concentrations of 17β-estradiol at 10 and 30 ng/L and a water control for 21 days. There were two replicates per treatment for each species, for a total of 12 experimental units, and the replicates were located in the same aquaria. The treatments were randomly assigned to the exposure aquaria. The test concentrations of E2 (17ß-estradiol, Sigma-Aldrich Co., St. Louis, MO) were created from stock solutions prepared in 100% reagent-grade ethyl alcohol (EMD Chemicals, Philadelphia, PA) and delivered to the diluter by automated pipettes (Micromedic model 25000 automatic pipette with 200-μL glass pumps and stainless steel pistons; ICN Micromedic Systems, Horsham, PA) through Teflon® tubing. The chemical deliveries were initiated by the dilutor system.

The study was initiated by impartially stocking 40 mixed-sex juveniles into each exposure chamber. Each exposure chamber included 10 more fish than what was planned to be sampled to allow for possible mortalities. The size of the fish stocked varied between species; the mean (± SD) weights were 0.151 (± 0.043) g for fathead minnows and 1.562 (± 0.573) g for bluegill sunfish. While the size of fish varied between species, both species were exposed in their juvenile stage soon after sexual differentiation. The fish were fed a concentrated solution of live brine shrimp nauplii two times per day at a rate of 0.5 mL concentrated solution per gram biomass (1.0 mL concentrate/gram/day) and the ration was normalized for mortality and sampling. The exposure chambers were brushed and siphoned as needed. A subsample of 10 fish was randomly collected every seven days from each exposure chamber for analysis of whole-body vitellogenin concentrations, mortality, a body condition index, and the histological sex. Mortality was monitored daily in all tanks prior to the feeding and all dead fish were removed daily by netting. Survival percent was calculated while taking into account the loss of fish through sampling.

Water analysis. Frozen water samples were shipped to Wooster College (Wooster, OH) and analyzed by direct injection liquid chromatography tandem mass spectrometry (1200 LC and 6410 mass spectrometer; Agilent, Santa Clara, CA) with negative electrospray ionization. Multiple-reaction monitoring was employed to monitor two precursor-to-product ion transitions for E2. The quantitative and qualitative precursor-to-product ion transitions were *m/z* 271 → *m/z* 145 and *m/z* 271

→ *m/z* 183, respectively. Concentrations were determined from a standard curve constructed from an authentic standard of E2 (98%, Sigma Aldrich).

Biological measurements. At the end of the experiment, whole body weights were measured to the nearest 0.001 g, and total lengths (TL) were measured to the nearest 0.5 mm. Fultons' body condition factor, a measure of the overall metabolic status of the fish, was calculated by dividing the body weight by the total length cubed and multiplying by a constant ((weight, g/(TL, mm^3))*100,000) for each fish.

The posterior section of each fish was then separated from the anterior for histological sex determination. A cut was made anterior of the anal pore, allowing for gonadal tissue to be processed for histological evaluation of fish sex. This approach ensured that the more anteriorly located liver (site of vitellogenin biosynthesis) would remain with the carcass allowing for a more accurate analysis of whole-body vitellogenin concentrations. The posterior section was preserved in a 10% neutral buffered formalin solution until the tissues could be histologically processed at St. Cloud State University (SCSU - St. Cloud, MN). Tissues were embedded in paraffin after dehydration in a series of ethanol and xylene baths. Embedded tissues were sectioned at 4 µm using a Reichert-Jung cassette microtome. Sectioned tissues were stained using a haematoxylin and staining protocol modified after Gabe (1976). Because the fish were exposed after sexual differentiation, sex was preferably determined through the actual presence of ovarian or testicular tissue. As ovarian differentiation precedes testis differentiation, an absence of ovarian tissue but presence of undifferentiated reproductive tissue was identified as male.

The anterior section of the fish was weighed to the nearest 0.001 g, placed in a cryovial, packed on wet ice in a cooler, and transferred to SCSU, where the samples were stored at -80 ºC until analysis. At SCSU, fathead minnow and bluegill sunfish were thawed on ice and homogenized using a 1X phosphate buffered saline (PBS) solution (0.075M, pH 7.5) at a 1:1 body section weight to PBS solution volume ratio. Following homogenization, all samples were centrifuged to isolate supernatant (13,000 rpm for 15 min) and supernatant was transferred to a -80 ºC freezer until analysis using a competition antibody-capture ELISA. Microtiter plates were coated (except for one microtiter well per assay plate) using purified fathead minnow vitellogenin prepared previously for fathead minnow and purified bluegill sunfish vitellogenin for bluegill sunfish assays. Standard curve dilutions for fathead minnows were prepared as seven-step twofold serial dilutions with a range of 4.8 ug/mL to 0.0375ug/mL by diluting purified fathead minnow vitellogenin in PBS+BSA buffer. Standard curve dilutions for bluegill sunfish were prepared as seven-step twofold serial dilutions with a range of 8 ug/mL to 0.0625 ug/mL by diluting purified bluegill sunfish vitellogenin in PBS+BSA buffer. Internal standards were also included at 1:50 and 1:100 dilutions of control fathead minnow vitellogenin for fathead minnows and control bluegill sunfish vitellogenin for bluegill sunfish. A maximum binding control, a true-blank control and a BSA-coated well control were also included on each plate. Samples were analyzed at three concentrations each. Plates were read at 620 nm on a Multiskan EX (Thermo Electron, Waltham, MA) plate reader.

All data sets were analyzed for assumption of normality using the Kolmogorov-Smirnov test for normality (PASW Statistics 18, IBM Corporation, Somers, NY; Prism 4.01 statistical package, GraphPad Software Inc., Oxnard, CA). One-way ANOVA followed by Bonferroni post-hoc test was used to analyze data that met assumptions of normality. Kruskal-Wallis analysis followed by Dunn's post-hoc test was used to analyze data that did not meet normality assumptions. Aquarium effects were tested for (Mann-Whitney U test) and not found, consequently data from both exposure chambers from the same treatment were combined for subsequent statistical analysis. Sex ratios were tested using Chi-Square analysis. An assumed sex ratio of 50% males and 50% females was used for analysis. Survival was compared using a Chi-Square analysis with Yates correction for low sample sizes. A significance level of 95% ($p < 0.05$) was used for all tests.

End Amendment to Methods: 9/16/2013

Amendment Addendum to the Original Study Plan

Rationale and Proposal

Over the past two years, we have exposed multiple generations of fathead minnows and bluegill sunfish in mesocosms to environmentally relevant concentrations of the ubiquitous endocrine active steroid hormone 17ß-estradiol (E2). These exposures yielded interesting and at times unexpected results that are outlined in our current progress report. As is often the case in multi-generational experiments, maintaining constant exposure conditions and adequate survival proved challenging. As a consequence, the proposed assessment of larval fathead minnow predator avoidance behavior could only be performed on the F1 generation of fathead minnows. The lack of larvae for an F2 generation predator avoidance test is especially disappointing as recent results suggest that the inability of E2 exposed larvae to avoid predation may be a root cause of reduced population sustainability in this species.

Given the remaining timeline for this study, we have an opportunity to amend the already obtained results by exposing an additional cohort of fathead minnows over two generations in laboratory mesocosms and test their predator avoidance performance across two generations of fathead minnows. Specifically, we propose to test the hypotheses that (i) larval E2 exposure will reduce predator avoidance performance and larval survival during active predation and (ii) subsequent F2 generation E2 exposure will further exacerbate the reduction in predator avoidance performance.

Experimental Design

To reduce some of the challenges of our previous experiments, the proposed experiment will be conducted at the *St. Cloud State University Aquatic Toxicology*

Laboratory (Co-PI Schoenfuss and the graduate students Daniel Rearick and William Heikkila).

Twenty newly hatched larval fathead minnows from F0 generation unexposed adult fathead minnows will be placed randomly into twenty four (to account for some larval mortality) one liter glass beakers for 21 day controlled static renewal exposures to one of two treatments:

- ethanol carrier control ($F1_C$) (n=200)
- 20ng/L 17ß-estradiol (E2) ($F1_{E2}$) (n=200)

The rationale for these exposures is two fold: (i) static renewal exposures in beakers provide more controlled exposure conditions with little anticipated mortality (McGee et al., 2009) and (ii) approximate the measured exposure concentrations of our previous mesocosm exposure experiment (median exposure: 19ng/L E2).

Following the 21-day exposure, a subset of 40 random larvae from each treatment will be assessed for their predator avoidance performance in our standardized assay (McGee et al., 2009; Painter et al., 2009; Schultz et al., 2012). Two 500 L mesocosms will be stocked with 100 larvae from each treatment. Half of those larvae will be stained before stocking to allow for later identification under fluorescent light. Each mesocosm will also contain standardized structures (3/4" pipe elbows and artificial floating substrate) to provide cover for the larval fish. Finally, one 3" bluegill sunfish (a native predator of larval fathead minnows) will be added to one, but not the other mesocosm. Over the course of the summer, four pairings of two mesocosms will be established in this fashion.

This experimental design accomplishes three objectives: (i) it allows for the assessment of larval predator avoidance performance in E2 exposed larval fathead minnows under controlled conditions; (ii) it provides an opportunity to assess the actual survival of exposed and control larvae in the same mesocosm in the presence of a native predator; and (iii) it allows for four complete replications of the experiment. Each mesocosm pairing will be maintained for five weeks before the survival of the juvenile fathead minnows will be evaluated using the stain that was applied to mark one treatment (chosen at random for each mesocosm). The mesocosm containing the predator will allow us to assess the effect of reduced predator avoidance on survival in the presence of unexposed conspecifics and a native predator, while the mesocosm without a predator will provide F1 generation fish that will be reared to adulthood for the F2 generation exposure.

F1 generation fish removed from the mesocosm without predator will be reared in flow-through aquaria to adulthood (maturity is reached at 6 months) and then split into single-treatment spawning pairs to assess reproductive output of previously exposed vs. unexposed fathead minnows (F1 generation adults will be sacrificed

after the spawning assessment is completed and assessed for plasma vitellogenin concentration and histopathological changes to reproductive organs). Eggs from these pairings will provide the F2 generation larvae to generate four treatments:

- $F1_C$ x $F2_C$: unexposed F2 larvae from unexposed F1 fish
- $F1_C$ x $F2_{E2}$: exposed F2 larvae from unexposed F1 fish
- $F1_{E2}$ x $F2_C$: unexposed F2 larvae from exposed F1 fish
- $F1_{E2}$ x $F2_{E2}$: exposed F2 larvae from exposed F1 fish

Replicating the experimental design from the F1 generation fish, we will test 40 larvae from each F2 generation treatment 21 days post-hatch in our standardized predator avoidance assay. All larvae (100/treatment) will then be randomly placed into 500L mesocosms containing a bluegill sunfish in each mesocosm (there is no need to generate mesocosm without predators as these fish will not need to be reared to adulthood). The following three pairings will be established in triplicate:

- $F1_C$ x $F2_C$ & $F1_C$ x $F2_{E2}$
- $F1_C$ x $F2_C$ & $F1_{E2}$ x $F2_C$
- $F1_C$ x $F2_C$ & $F1_{E2}$ x $F2_{E2}$

Following the five-week mesocosm predation period, all remaining larvae will be removed to assess juvenile survival in the presence of unexposed conspecifics and a native predator.

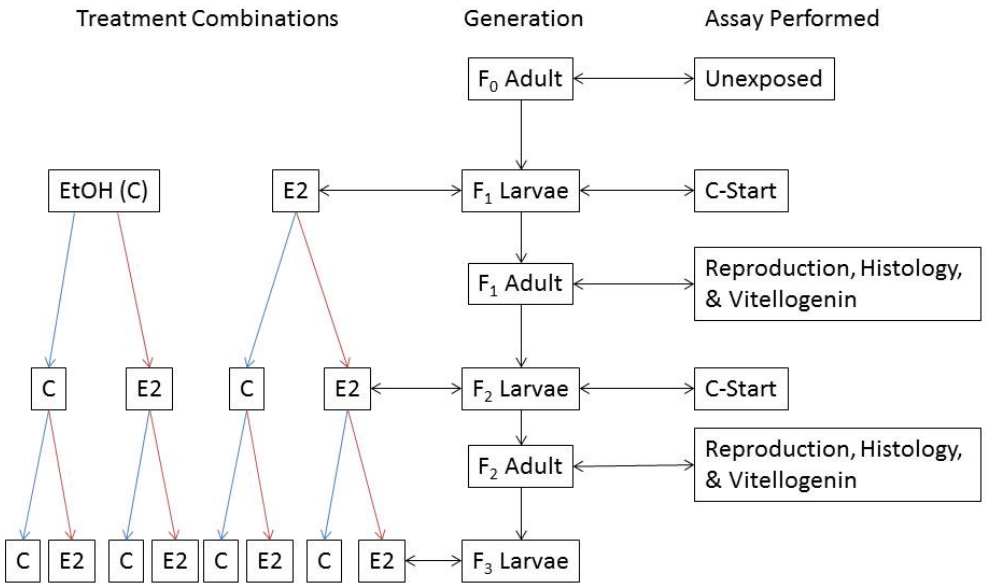

Figure 8. Graphical depiction of study design and assays performed during laboratory exposures conducted for project amendment.

Budget

The proposed amended research can be conducted within the original budget.

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Schultz, M.M., Bartell, S.E., Schoenfuss, H.L. *In press.* Effects of Triclosan and Triclocarban, Two Ubiquitous Environmental Contaminants, on Anatomy, Physiology and Behavior of the Fathead Minnow (*Pimephales promelas*). *Archives of Environmental Contamination and Toxicology.*

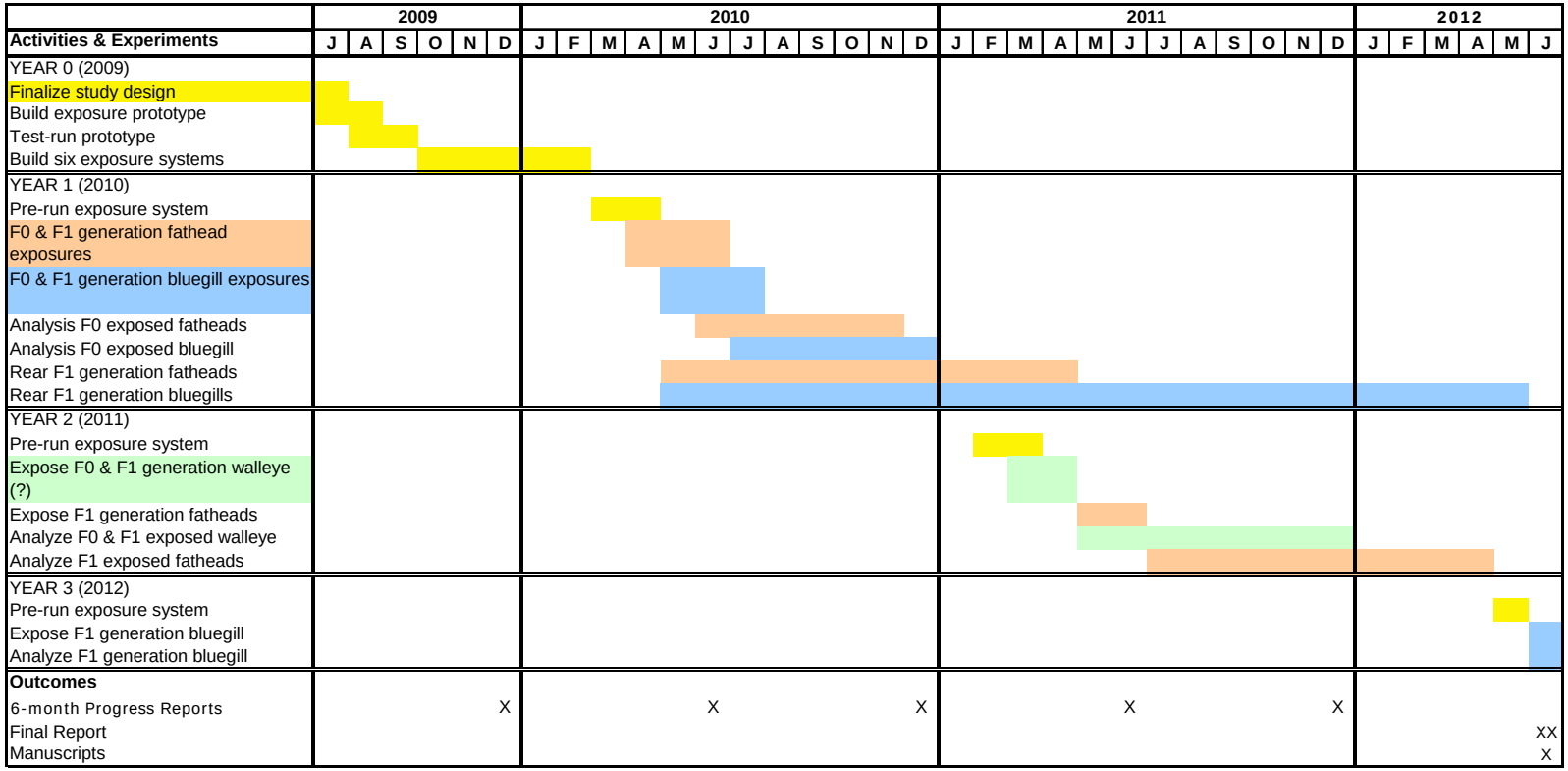
IV. ESTRADIOL ANALYSIS

Estradiol analysis utilizes a commercially available 17ß-Estradiol Kit (Cayman Chemicals, Ann Arbor, MI) based on enzyme-linked immunosorbent assay (ELISA) methodology. Assays will be conducted by a research fellow (S. Bartell) with 15+ years in ELISA development and application. Briefly, water samples will be added to a 96 well plate, exposed to a E2 specific antibody and after several washes, challenged with a second antibody with a fluorescent tag. Fluorescence of each sample well will be assessed against a standard curve of known E2 values to determine final E2 concentration in the sample water. This technique has allowed for sample analysis to a detection limit of roughly 2 ng/L - 5 times below the lower nominal values expected in this study.

i. YEAR 0: 100 analyses
ii. YEAR 1: 672
iii. YEAR 2: 336
iv. YEAR 3: 336

Sediment samples will be collected at the beginning and the end of each mesocosms deployment, extracted and concentrated using N_2 gas evaporation or solid-phase extraction (SPE), and analyzed using the ELISA as outlined above for the water samples. Other components of the mesocosm food-webs (e.g., algae and invertebrates) will also be analyzed for 17ß-Estradiol at the end of each mesocosms experiment.

Result Completion Timeline with Deliverables:



Attachment A: Budget Detail for 2009 Projects - Summary and a Budget page for USGS

Project Title: Vulnerability of Lakes to Endocrine Disruption

Project Manager Name: Richard L. Kiesling

Trust Fund Appropriation: $ *297,000*

2009 Trust Fund Budget	**Revised Result #1 Budget 6/20/13**	**Amount Spent** *(date)*	**Balance** *(date)*	**Revised Result #2 Budget 6/20/13**	**Amount Spent** *(date)*	**Balance** *(date)*	**TOTAL BUDGET**	**TOTAL BALANCE**
	Developmental impacts of juvenile exposure to water and sediment concentrations of EACs. USGS Budget: $108,861.96			*Reproductive impacts of juvenile and adult exposure to water and sediment concentrations of EACs. USGS Budget: $80,238.04*				
BUDGET ITEM								
PERSONNEL: wages and benefits (rate)	80,487	80,487	0	76,848	76,848	0	157,335	0
Richard Kiesling ~~(6%) -~~ Project Manager: USGS ~~($14,000)~~ 36% FTE								
~~Steve Redmann (8%) - Fish Culturalist: USGS ($40,000)~~								
~~Sue Schleis (12%) -~~ Technician: USGS ~~($55,000)~~ 51% FTE								
Hydrologist ~~(90%)~~: USGS ~~$60,000)~~ 54% FTE								
Other direct operating costs:								
Non-capital Equipment / Tools								
Turbo-evaporator for extractions	2,307	2,307	0			0	2,307	0
Printing	0	0	0		0	0		0
Supplies *(list specific categories)*								
Mesocosms	20,155	20,155	0			0	20,155	0
Sediment Extraction	4,200	4,200	0	2,404	2,404	0	6,604	0
Travel expenses in Minnesota								
USGS travel	1,600	1,600	0	986	986	0	2,586	0
Travel outside Minnesota *(where?, for what purpose?)*								
Shipping	113	113	0		0	0	113	0
COLUMN TOTAL	**108,862**	**108,862**	0	80,238	**80,238**	**0**	**189,100**	0