Environment and Natural Resources Trust Fund

Research Addendum for Peer Review

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Project Title: Estrogen exposure analyses in Minnesota's shallow lake wildlife

Project number: 048-B

1. **Abstract** - Minnesota's smaller lakes play an important role by providing wildlife habitat, by providing opportunities for recreation, and by providing economic value for various commercial ventures. Endocrine-disrupting contaminants, including environmental estrogens (EEs) are present in Minnesota's larger lakes and streams at concentrations which have adverse impacts on wildlife. In contrast, very little is known about the sources and effects of EEs in small, shallow lakes, or about how surrounding land and associated lake management practices may exacerbate the effects of contaminants in these systems. The goals of this project are to determine whether EE exposure is common for aquatic wildlife in shallow lakes, determine land-use practices that correlate with EE exposure, and identify the effects of EE exposure on the nervous system of aquatic species. These analyses will allow us to identify which land-use and shallow lake management practices are most beneficial to minimizing EE exposure, and associate EE exposure with impacts on wildlife.

2. **Background** – Minnesota's shallow lakes are an important cultural, recreational and commercial resource. But over the last 150 years, these lakes have been affected by changes in land use and water quality. Industrial and agricultural practices, urban development, and increased recreational traffic have impacted the composition of these ecologically important lakes by changing runoff patterns and adding anthropogenic compounds to the waters that feed them. However, little is known about how land use and lake management practices impact EE levels in shallow lakes, or about how exposure to EEs impacts Minnesota's shallow-lake wildlife.

   The presence of EEs and other endocrine active compounds in aquatic ecosystems is well-known (e.g., Barber et al. 2000; Kolpin et al. 2002). Moreover, the consequences of EE exposures on wildlife have been described for many species of fish (e.g., Schultz et al., 2013), turtles (e.g., Irwin et al., 2001) and for many lake and river environments in Minnesota (e.g., Schoenfuss et al., 2008; Ferrey, 2013). The Minnesota Pollution Control Agency conducted a study in 2008 which found EEs in numerous lakes and also identified biological effects in fish consistent with EAC exposures (e.g., increased vitellogenin (VTG) levels in male fish; histomorphological changes to reproductive organs).

   In this proposal, we aim to examine EE exposure in painted turtles (*Chrysemys picta*) collected from several lakes in several different ecological regions across Minnesota to explore three questions about the impact of EEs in shallow lakes: 1) How common is EE exposure among aquatic wildlife in shallow lakes? 2) What land-use practices correlate with EE exposure (e.g., urban, agriculture- and forest-dominated ecosystems)? 3) What are the effects of EE exposure on the nervous system of aquatic species? We are using painted turtles because of a few advantages they

possess for this study. First, turtles are long-lived, so we can assess the effects of EE exposure across a fairly long time-span. Because we can estimate the age of the turtles from morphological characteristics (e.g., carapace size), this may provide a window into comparing cumulative effects of chronic EE exposure in older turtles with more acute effects in younger turtles. Second, VTG levels have previously been shown to increase in response to environmental exposure to estrogens in painted turtles, so our study has a high likelihood of success. Third, turtles for this study have already been obtained as a by-product of an earlier study examining shallow lakes from several distinct ecological regions across Minnesota; therefore, the results we obtain can be merged with geospatial data on land use practices and overall ecological health. Finally, painted turtles are a commercially important species in Minnesota, with tens of thousands harvested each year for sale to biological supply companies, as pets and as food (Gamble and Simons, 2003). Since increased VTG has been suggested to impact reproductive fitness (Irwin et al, 2001), our results may have an economic benefit by suggesting practices that will enhance turtle population health and the sustainability of Minnesota's turtle population.

We will use a multifaceted approach to answer the questions proposed in this study. To determine the distribution of EE exposure levels across Minnesota, we will develop a VTG assay and determine VTG levels in blood that has been collected from over 50 turtles in lakes from forested, agricultural, mixed-use and urban regions. To explore the effect of EE exposure on neural structures, we will examine the brains from these same turtles, looking specifically at the sexually dimorphic structures in the brain. Finally, to determine the relationship between land use, EE exposure and neural structures, we will merge this data with current and historical geospatial data on land cover, land use, and lake management practices.

3. Hypothesis

Our approach has been crafted to determine whether EE exposure in shallow lakes varies across the state, whether EE exposure levels correlate with land use practices, and whether such exposure impacts wildlife. Although there is not a true hypothesis being tested *per se* (i.e., there is not a control group with an experimental group receiving measured amounts of estrogen active compounds), our premise is that differences in land use within the shallow lake watersheds will lead to measurable differences in EE exposure (as measured by VTG levels). We want to explore this question further by determining whether such differences in EE exposure can be manifest in neural structures, most notably in sexually dimorphic structures that may impact reproductive fitness. The proposed work will represent an important contribution to our understanding of the effects of EEs and EACs in Minnesota's shallow lakes, and may have far-reaching impact in terms of wildlife management, land-use practices, and watershed use.

4. Methodology

i) Assessing the distribution of EE exposure levels in shallow lake wildlife.

We will develop an assay to examine vitellogenin (VTG) levels in painted turtles (*Chrysemys picta*), which have been shown to vary with EE exposure in this species (Irwin et al, 2001). The VTG assay will be developed using methods similar to previously published reports (e.g., Bartell and Schoenfuss, 2012; Irwin et al., 2001). Plasma vitellogenin will be measured by antibody-capture competitive ELISA incorporating a species-validated anti-vitellogenin antibody and purified vitellogenin as standard. Polyclonal antisera will be produced by ProSci Inc. (San Diego, CA)

from purified painted turtle plasma vitellogenin. Microtiter plate wells will be coated with 600 ng species-validated vitellogenin in carbonate coating buffer (pH 9.6). A pre-competition step will be performed with the antibody (1:20,000 final dilution) and either standard vitellogenin, sample plasma or control plasma in 1% BSA/PBS (pH 7.5). After incubation this mixture will be loaded into the wells and incubated at room temperature for 1 h, followed by secondary antibody (anti-rabbit IgG-HRP, Sigma-Aldrich, St. Louis, MO) at a concentration of 1:10,000. The substrate tetramethylbenzidine (TMB) will be added and incubated for 20 min at room temperature and color development measured at 620 nm on a Thermo Multiscan plate reader (Waltham, MA). Each plate will contain a set of standards for standard curve generation, and will be read precisely at 20 min post-TMB addition. Average VTG levels for males and females will be calculated for each lake, ecological region, and statewide. Comparisons will be made using analysis of variance, and significant main effects (sex, lake, ecological region) and interactions will be explored using appropriate post-hoc analyses (e.g., Tukey's HSD).

ii) Determining the effects of EE exposure on nervous system structures.

We will analyze brain regions associated with foraging and reproductive behavior in turtles for which blood vitellogenin levels are available. This will include examining the nucleus paraventricularis (NPV), a structure that is sexually dimorphic and may be related to reproductive behaviors in the turtle. Brains were fixed in the skull with 4% paraformaldehyde for >92 hours, then removed and placed in a cryoprotectant solution (25% sucrose) for 48 hours. Serial sections were cut at 40 microns using a cryostat (Leica Instruments) and transferred to well plates containing phosphate buffered saline (PBS) and 0.1% sodium azide until further processing. Sections were stained with Cresyl violet, dehydrated, mounted on gelatin-coated slides and coverslipped. Preliminary data on the size of the NPV was collected in the same manner as the proposed work, as follows. Sections of turtle brain that contain the NPV will be marked and prepared for counting. All procedures from this point on are carried out with the experimenter blind to the identity and sex of the specimen. Using a microscope connected to a computer running Neurolucida software (MicroBrightField), experimenters will circumscribe the NPV for every section from each animal. Each section will be subject to circumscription by at least two experimenters, and the average area for each section will be computed. These areas will be combined into a 3-D volumetric measurement by the software. Preliminary results have shown that the difference in size of the NPV between males and females varies widely throughout the state. Therefore, average volumes for males and females will be computed for each lake and ecological region where turtles were collected. Comparisons will be made using analysis of variance, and significant main effects (sex, lake, ecological region) and interactions will be explored using appropriate post-hoc analyses (e.g., Tukey's HSD). These results, taken together with EE exposure data, will suggest whether exposure to EEs might affect behavior related to survival and reproduction in this species.

iii) Identifying land use and lake management practices that reduce EE exposure.

We will integrate EE exposure data and brain morphology data with previously-collected GIS data sets containing information about ecological and land-management to test whether watershed land use is related to EE levels in turtles, leveraging the wide collection range to assess whether these relationships vary across the state. In particular, we will combine the geospatial information collected during the collection of the turtle blood and brains with land use data available from the Minnesota Geospatial Information Office (for example, data on land use practices

is available http://www.mngeo.state.mn.us/chouse/land_use.html). This process will involve merging land-use data with the vitellogenin level data and brain morphology data. Additionally, because some of the turtles in our study are relatively long-lived, we will explore the degree to which vitellogenin levels may correlate with historical land use, to the extent that this data is available. To facilitate this, we will explore correlations between vitellogenin levels, brain structure and data available on historical land cover and land use from the data clearinghouse of the Minnesota Geospatial Information Office. For both recent and historical data sets, these correlations will help us identify land use patterns that are most favorable for reducing EE exposure in shallow lake wildlife..

5. Results and Deliverables

We expect that the VTG assay will result in measurable differences in EE exposure for turtles from different ecological regions of Minnesota. Previously published data (e.g., Irwin, 2001) suggests that for female turtles, EE exposure will result in higher circulating VTG levels. We will compare these levels across different ages of turtles, and we will compare them with levels reported in the literature, to estimate the possible long-term cumulative effects of such exposure. Although VTG levels do not increase in male painted turtles after acute exposure to estrogens (Irwin, 2001), our results for blood collected from male turtles may help identify any effects of longer-term exposure on VTG levels. In addition, the overall results of this assay will suggest whether there are statewide differences in EE exposure in shallow lakes.

We expect that there will be differences in brain structure for turtles found in lakes with different levels of EE exposure. In particular, our preliminary results suggest that the difference in the size of the NPV between male and female animals will vary depending on the ecological region where the turtles were collected. These results will give us a better idea of how land use and EE exposure might impact shallow lake wildlife.

Finally, by correlating the findings from these two aims with geospatial data on historical and current land use, we hope to identify specific conditions in which EE exposure is limited or more favorable. This will allow us to suggest provisional guidelines for best practices (e.g., land use and watershed management practices) to ensure the health and sustainability of Minnesota's shallow lake wildlife.

6. Timetable

Development of VTG assay will start in June, 2014 and will take approximately eight to 12 months to complete. Running the collected blood on the assay will take a further six to eight months (including data analysis), with a proposed completion by January or February of 2016. Final analyses and writing papers for submission to scientific journals will be completed by June, 2017.

Examining the effects of EE exposure on nervous system structures will begin in June 2014 and will take 18-20 months to complete (including data analysis), with a proposed completion by January 2016. Final analyses and writing papers for submission to scientific journals will be completed by June, 2017.

Correlating data from the VTG assay and brain morphology studies will begin as data analysis is completed for each study. Comprehensive merging of datasets will begin around February 2016 and will take approximately 8 months to complete, with a proposed completion by December 2016. Final analyses and writing papers for submission to scientific journals will be completed by June, 2017.

7. **Budget** – The budget is unchanged from the original proposal, and is reproduced below. Note that the proposed project leverages over $100,000 of funding and in-kind services, in addition to work already performed by faculty and students at the University of St. Thomas (including sample collection, preliminary specimen preparation and analyses). Thus, the proposal provides a high impact for relatively low cost, to deliver an important investigation of how land- and lake-management practices correlate with exposure and effects of EEs.

TOTAL ENRTF REQUEST BUDGET 3 years

BUDGET ITEM	AMOUNT
Personnel: Kurt Illig, overall project director; brain morphology work; personnel training and management; data analysis, writing and dissemination; four months work over three years.	$ 32,000
Personnel: 8 undergraduate researchers providing 24 person-months work over three years.	$ 36,000
Contracts: Stephen Bartell, Normandale Community College, VTG assay development.	$ 14,000
Equipment/Tools/Supplies: *VTG development supplies and consumbles*	$ 36,000
Equipment/Tools/Supplies: *Brain structure analysis supplies and consumables*	$ 18,000
TOTAL ENVIRONMENT AND NATURAL RESOURCES TRUST FUND $ REQUEST =	$ 136,000

OTHER FUNDS

SOURCE OF FUNDS	AMOUNT	Status
Other Non-State $ Being Applied to Project During Project Period: University of St. Thomas funding for student reserachers	$ 18,000	Secured
Other State $ Being Applied to Project During Project Period: *N/A*	$ -	
In-kind Services During Project Period: extensive equipment list	$ 24,000	Secured
Remaining $ from Current ENRTF Appropriation (if applicable): *N/A*	$ -	
Funding History: *UST supplied funding for faculty research time*	$ 2,000	Secured
Funding History: *UST supplied funding for undergraduate researchers*	$ 4,000	Secured
Funding History: *UST supplied funding for equipment, consumables*	$ 16,000	Secured

8. **Credentials**

The project will be directed by Kurt R. Illig, PhD, who has over 20 years of experience conducting research on the nervous systems of model and non-model organisms. He obtained his PhD from the University of Wisconsin-Madison, completed a postdoctoral fellowship at the University of Wisconsin Medical School, and was a faculty member at the University of Virginia for 7 years before taking his current position at the University of St. Thomas. He has received several research grants, and published peer-reviewed publications and authored book chapters on comparative neuroscience, and has presented papers and invited talks at dozens of national and international conferences. He also has nearly five years of administrative experience managing deadlines and budgets as the Director of the Neuroscience Program at the University of St. Thomas.

Dr. Illig's research involves comparative neuroanatomy and neurophysiology, especially in the context of survival mechanisms for the individual and the species. This research has involved various collaborators including over 20 undergraduate researchers. Undergraduate students, who will be performing some of the work in this proposal, are a regular and skilled part of Dr. Illig's research team, and their inclusion in this proposal extends the value of the proposal to include an educational component. Dr. Illig has worked closely with this team for two years as they gathered initial samples and preliminary data, and their extensive experience together ensures not only an educational benefit to the students but also the clear confidence that the outcomes of this proposal will be realized.

Dr. Illig's laboratory at the University of St. Thomas is ready and well-equipped to perform the analyses in this proposal. In fact, the laboratory has already been working with many of the samples collected, and have made substantial initial progress on the proposed work (including preliminary data included in the proposal). Development of the vitellogenin assay will be contracted to a recognized and established expert off-site (Dr. Stephen Bartell) who has developed vitellogenin assays for other non-model organisms in Minnesota (e.g., Bartell and Schoenfuss, 2012). Within the Biology Department at the University of St. Thomas, Dr. Illig has a team of collaborators with the skills and experience to help frame the results in the context of greater ecological questions, including Dr. Kyle Zimmer, who has spent years gathering GIS data on shallow lakes in Minnesota, Dr. Dalma Martinovic-Weigelt, an environmental toxicologist whose work includes the impacts of EE exposure on aquatic organisms, and Dr. Timothy Lewis, a wildlife ecologist whose work includes the painted turtles in Minnesota's shallow lakes.

9. Dissemination and Use

The results of all proposed studies will be disseminated by presentations at national and local conferences, and publication in scientific journals as appropriate (e.g., *The Journal of Comparative Neurology*). Further, our results will be made available to interested parties at the Minnesota Pollution Control Agency, the Minnesota Department of Natural Resources, interested local city and county government officials and other interested parties, with the dual goal of dissemination our results and to determine appropriate responses to those results.

Literature Cited

Barber LB, Brown GK, Zaugg SD (2000) Potential endocrine disrupting organic chemicals in treated municipal wastewater and river water. *ACS Symposium Series,* 747: 97-123.

Bartell SE, Schoenfuss HL (2012) Affinity and matrix effects in measuring fish plasma vitellogenin using immunosorbent assays: Considerations for aquatic toxicologists. *ISRN Toxicology* 2012: Article ID 942804 doi:10.5402/2012/942804

Ferry M. (2013) *Pharmaceuticals and endocrine active chemicals in Minnesota Lakes*. Saint Paul: Minnesota Pollution Control Agency.

Gamble T, Simons AM (2003) *The commercial harvest of painted turtles in Minnesota.* Saint Paul: Minnesota Department of Natural Resources.

Irwin LK, Gray S., Oberdorster E. (2001) Vitellogenin induction in painted turtle, *Chrysemys picta*, as a biomarker of exposure to environmental levels of estradiol. *Aquatic Toxicology* 55: 49-60.

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

Schultz, MM, Minarik TA, Martinovic-Weigelt D, Curran EM, Bartell SE, Schoenfuss HL. Environmental estrogens in an urban aquatic ecosystem: II. Biological effects. *Environment International*, 61: 138-149.

Schoenfuss, H.L., S.E. Bartell, T.B. Bistodeau, R.A. Cediel, K.J. Grove, L. Zintek, K.E. Lee and L.B. Barber. 2008. Impairment of the reproductive potential of male fathead minnows by environmentally relevant exposures to 4-nonylphenol. *Aquatic Toxicology* 86: 91-98.