M.L. 2014 Project Abstract
For the Period Ending June 30, 2018

PROJECT TITLE: Evaluation of Wastewater Nitrogen and Estrogen Treatment Options
PROJECT MANAGER: Paige Novak
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2014, Chp. 226, Sec. 2, Subd. 03d as extended M.L. 2017, Chapter 96, Section 2, Subdivision 18

APPROPRIATION AMOUNT: $ 500,000
AMOUNT SPENT: $454,288
AMOUNT REMAINING: $45,712

Overall Project Outcome and Results

Wastewater treatment plants (WWTPs) discharge effluent that contains contaminants of emerging concern (CECs), including estrogens. These estrogens have caused ecological damage, such as fish feminization, with unknown long-term consequences. The most important estrogen exiting WWTPs is a chemical called estrone. In this project we studied how different treatment systems performed with respect to estrone degradation and how temperature effected degradation. We also studied how fish vulnerability changed seasonally. Finally, we combined these laboratory efforts with models of fish population dynamics to extrapolate the results.

We determined that the technology used by a treatment plant is likely to have an impact on the estrogenicity of WWTP effluent, with some technologies performing very well and others failing to remove estrone. In addition, natural seasonal fluctuations in temperature and expected fluctuations in estrone concentration can cause negative changes in exposed fish. Mathematical models were used to expand this research to whole river systems and showed that the impacts of estrone on fish populations varied depending on the characteristics of the environment. Impacts were expected to be low in systems in which fish were limited by food and high in systems in which fish were limited by predators. Therefore, fish populations in Minnesota rivers are likely to vary in their response to wastewater estrone. The cost of various wastewater improvements were calculated, which could be compared to the value associated with recreational fishing.

Overall, this research showed that low energy treatment systems do exist that are capable of excellent estrone removal, which should be considered so that multiple ecological benefits can be reaped as treatment plants upgrade. Nevertheless, modeling results suggest that the impacts of estrone vary at the population scale based on river characteristics. Therefore, the impact of estrogens at the fishery scale should be evaluated for a given river of interest.

Project Results Use and Dissemination

Information from this project has been shared broadly and multiple peer-reviewed manuscripts have been published from this work and submitted to the LCCMR.

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2014 Work Plan

Date of Status Update Report: September 4, 2018

Final Report

Date of Work Plan Approval: June 4, 2014

Project Completion Date: June 30, 2018

PROJECT TITLE: Evaluation of Wastewater Nitrogen and Estrogen Treatment Options

Project Manager: Paige J. Novak

Organization: University of Minnesota

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Location: Statewide

Total ENRTF Project Budget:	
ENRTF Appropriation:	**$500,000**
Amount Spent:	**$454,288**
Balance:	**$45,712**

Legal Citation: M.L. 2014, Chp. 226, Sec. 2, Subd. 03d
M.L. 2017, Chapter 96, Section 2, Subdivision 18

Appropriation Language:
$500,000 the second year is from the trust fund to the Board of Regents of the University of Minnesota to examine the performance of new wastewater contaminant treatment options under Minnesota weather conditions in order to understand how to improve wastewater treatment of nitrogen and estrogenic compounds, decrease costs and energy use, and safeguard aquatic species. This appropriation is available until June 30, 2017, by which time the project must be completed and final products delivered.

Carryforward (a) The availability of the appropriations for the following projects are extended to June 30, 2018: (1) Laws 2014, chapter 226, section 2, subdivision 3, paragraph (d), Evaluation of Wastewater Nitrogen and Estrogen Treatment Options.

I. PROJECT TITLE: Wastewater estrogen: removal options, fish abundance, and cost

II. PROJECT STATEMENT:
Wastewater treatment plants discharge effluent that contains contaminants of emerging concern (CECs), including estrogens. These estrogens have caused dramatic ecological effects such as fish feminization and fish population collapses, with unknown long-term consequences. The most important estrogen exiting wastewater treatment plants, in terms of contributing to the feminization potential of effluent, is a chemical called estrone, which is an estrogen that is released naturally from women via the waste stream. Although this and other estrogens are present in Minnesota lakes and rivers and can be ecologically harmful, their treatment and discharge are not regulated.

Interestingly, the discharge of estrone and other estrogens is a function of how (and how well) a treatment plant removes nitrogen. Nitrogen discharge is regulated to some extent in Minnesota and will be more heavily regulated in the future, requiring additional wastewater treatment plant upgrades. With this research we will determine how different nitrogen removal processes perform over the range of temperatures experienced in Minnesota with respect to both CEC (and in particular, estrone) and nitrogen removal so that the very best processes for the protection of Minnesota's natural resources can be put into place. In addition, we will determine how fish vulnerability changes seasonally so that treatment to extremely low levels of CECs is only required during critical periods (e.g., during egg maturation or spawning) to save energy and costs from excessive (and unnecessarily rigorous) treatment. Finally, we will combine laboratory efforts with predictive mathematical models so that we can extrapolate to cost and whole population behavior.

III. PROJECT STATUS UPDATES:

Project Status as of January 31, 2015:
We have completed design and construction for the five reactor systems to be used in this project. Initial "base case" experiments with synthetic wastewater and an influent estrone concentration of 10 µg/L have been completed and show 90% removal of estrone in the effluent. A control experiment to quantify estrone sorption within the reactor has also been completed. Three additional experiments are currently in operation: (1) conventional treatment using actual wastewater from the Metropolitan Wastewater Treatment Plant in St. Paul; (2) conventional treatment using a lower influent concentration of estrone (100 ng/L); and (3) granular aerobic sludge. These experiments will allow us to determine whether experiments performed with synthetic wastewater and slightly higher estrone feed concentrations (for ease of analysis) behave similarly to those performed with real wastewater and environmentally relevant estrone concentrations. Experiments with granular aerobic sludge represent experiments with a cutting edge lower-energy nutrient removal technology. Results for this experiment are expected by July, 2015.

We have also nearly completed the fathead minnow larval exposures (Activity 2). We collected eggs from minnow breeding groups and exposed each clutch to one of 20 treatments for 21 days (5 concentrations of estrone at 4 temperatures). We have collected data on hatching success, survival, predator avoidance behavior, and feeding efficiency sufficient for assessing the effects of temperature and exposure concentration on larvae. In addition, exposed fathead minnow larvae have been preserved appropriately for future vitellogenin analysis and assessment of growth and developmental abnormalities.

Amendment Request (07/16/2015):
The addendum is to formally request a re-budgeting of funds for this project.

As part of the project, we would like to establish one additional personnel category: undergraduate researcher. The undergraduate will assist the graduate student researchers with routine activities (running reactors) to enable the graduate student to spend more time on higher-level functions such as data analysis and estrone

analysis. All of the required rebudgeting will remain within the "**Personnel**" category and will simply move from sub-category to sub-category.

The movement of money between sub-categories will not affect project objectives or timelines.

Amendment Approved: July 20, 2015

Project Status as of July 31, 2015:
We have completed the following experiments regarding estrone degradation during biological nitrogen treatment: (1) conventional nitrification with synthetic and actual wastewater at 10 µg/L influent estrone, (2) conventional treatment using a lower influent concentration of estrone (100 ng/L) and synthetic wastewater, (3) Modified Ludzack-Ettinger (MLE) process fed actual wastewater and (4) sequencing batch reactor systems fed high (1000 mg/L) and standard (200 mg/L) wastewater carbon to study estrone removal in a granular aerobic sludge system. We found excellent removal of estrone in the conventional, MLE, and low carbon sequencing batch reactor systems (>90% removal), but no estrone removal for the high carbon sequencing batch system (the granular aerobic sludge system). Results to date suggest that aeration benefits estrone degradation and that the repeated exposure to high carbon concentrations and/or granulation is detrimental to estrone removal. We are currently operating conventional reactors at low temperatures (15°C), as well as several additional novel reactor systems for total nitrogen removal. These additional experiments will show whether low oxygen systems can be effective for estrone removal and will also demonstrate the effect of cold temperatures on estrone degradation. Results for all these experiments are expected by January 2016.

All fathead minnow experiments have been completed. Both adult flow-through exposures and larval batch exposures were conducted at estrone concentrations of 0, 25, 125 or 625 ng/L and at temperatures of 15, 18, 21, or 24°C. A total of 2,292 fish were studied. Data is currently being analyzed to determine the statistical significance of the results and to verify the estrone concentrations used during the exposures.

Population modeling and economic modeling studies will be starting shortly.

Project Status as of January 31, 2016:
We have completed all of the wastewater experiments in which estrone degradation during biological nitrogen treatment was monitored. Final analysis of the estrone concentrations in the SHARON and anammox reactors is being completed. Anaerobic estrone controls have been performed to assess sorption, but an additional abiotic control experiment may be completed in the next several months to clarify sorption of estrone to the membrane used to filter the bacteria from the effluent. This is not expected to alter our conclusions, but will provide the necessary quality assurance for a strong publication. New observations indicated that during low temperature (15°C) operation nitrification slows, but after a very short lag period, estrone degradation is unaffected. Estrone appears to be effectively degraded at the low oxygen addition required for only partial ammonia oxidation in the SHARON system, but this is currently being verified through additional estrone analysis. Effluent estrone concentrations have not yet been analyzed in the anammox experiment; nevertheless, estrone degradation is not anticipated because anammox operates under complete anaerobic conditions. Two manuscripts should be completed in the next six months: one that incorporates the temperature data from the wastewater experiments with the minnow data, and a second that focuses on estrone degradation in novel nitrogen treatment systems.

Larval and adult behavioral data have now been quantified from video tapes and the statistical data analyses assessing the effects of E1 on hatching success, early survival, fecundity, fertility, behavioral impairment (4 assays), and biochemical endpoints are largely completed. Additional analysis of the adult fathead minnow tissues is ongoing. Our initial data provide some evidence that temperature modulates the effects of estrogen exposure in both adult and larval fathead minnows, but that its influence is limited and unpredictable. A preliminary draft of a manuscript reporting the results of the behavioral assays has been written.

The mathematical model for predicting minnow and bass abundance is in development. We are focusing on the most important pathway in this model, which links environmental estrogen concentration to population-level effects via the estrone concentration in individual fish and the effect that this concentration has on the feeding, maintenance, growth, and/or reproduction of individual fish. We are using a well-tested ecotoxicological model to convert environmental estrogen concentrations to internal estrogen concentrations, and a bioenergetics approach to convert these internal concentrations to individual-level effects. We have developed and parameterized this part of the model for fathead minnows, and are now testing it to make sure that model output is consistent with results from Activity 2.

Economic modeling studies will be starting shortly.

Amendment Request (01/22/2016):
Our findings from Activity 2 (Outcome 1) indicate that temperature does not strongly or consistently modulate the effects of E1 on reproduction, development, and egg survival. However, our results suggest that temperature may interact with E1 to alter larval predator avoidance performance. In light of these findings, we propose to amend the work plan for Activity 2. Specifically, we propose to conduct a new, large-scale (40 treatment), factorial predation experiment in lieu of collecting basic data on the reproduction and survival of smallmouth bass exposed to wastewater contaminants. The proposed changes to the work plan will allow a more thorough understanding of the effects of estrogen exposure on trophic interactions under different thermal regimes. This will not alter the budget.

A second amendment request involves changing the budget such that Jay Coggins, Professor, Department of Applied Economics, can be paid from the project as well. Dr. Frances Homans has accepted a position as Department Head of the Department of Applied Economics at the University of Minnesota and has therefore brought Dr. Coggins into the project to assist with the economic/costing tasks (Activities 1 and 3). This will not result in budget changes, only the addition of Jay Coggins' name to the project so that he and Dr. Homans can allocate the currently-budgeted salary as they see appropriate.

Amendments Approved: January 27, 2016

Retroactive Amendment Request (02/5/2016):
After submitting our project status report and amendment request 1/22/16 we realized that our travel budget was overspent by $774. In addition, travel funds have been needed to pick up wastewater from the Metropolitan Wastewater Treatment (Metro) Plant in St. Paul and transport it to the laboratory for use in experiments, rather than only for project meetings. When we began the research we thought that we would be able to use synthetic wastewater created in the laboratory. We verified this assumption by performing replicate estrone and nitrogen degradation experiments with both synthetic wastewater and real wastewater from the Metro Plant. Although estrone behaved identically in the two experiments, nitrogen did not; therefore, we felt that it was important to perform experiments with real wastewater rather than synthetic so that we could be sure that our results were transferrable to actual wastewater systems. This change necessitates the collection of 100 L of wastewater per week and therefore requires the student performing the experiments to collect the wastewater using a vehicle from fleet services at the University of Minnesota. We therefore request that $1000 be moved from the "Personnel" line item to the "Travel expenses in MN" line item. We also request that the following language be added to the budget descriptor within the "Travel expenses in MN" line item: "*Travel funds are ~~extremely~~ minimal ($1,400) and are included for travel to meetings at either St. Cloud State University or the University of Minnesota for project coordination or to the Metropolitan Wastewater Treatment Plant for sample collection for use in experiments."* The proposed changes to the work plan will allow a more accurate and realistic assessment of estrone and nitrogen removal during wastewater treatment. This will not alter the overall budget or timing of the project.

Amendment Approved: February 9, 2016

Project Status as of July 31, 2016:
The experiments for Activity 1 on the degradation of estrone in different nitrogen removal systems have been completed. Estrone removal was excellent when fed to the nitrification (room temperature and cool temperature), MLE, and sequencing semi-batch systems, at 96%, 96%, and 97% mean estrone loss, respectively. The aerobic granular sludge system failed to remove estrone (14% mean estrone loss), which was perhaps not unexpected given the very high COD loading that is required for granulation to occur. Surprisingly, the anammox system also resulted in excellent estrone removal (99.8% mean estrone loss), despite the anaerobic nature of anammox. The anammox results are particularly exciting, as this is a low-energy alternative for total nitrogen removal. The estrone removal results from the SHARON reactor system were variable and unreliable, despite repeating this experiment three times. Two manuscripts (one that includes data from the minnow experiments) are in preparation and should be submitted by the next project update.

We have completed our analysis of larval and adult fathead minnow exposures at four temperatures (15, 18, 21, 24°C) and four concentrations of estrone (0 ng/L, 25 ng/L, 125 ng/L, 625 ng/L nominal concentrations). Results from the behavioral assessment of these exposed fish have been summarized in a manuscript draft that is currently being circulated among the co-authors for comment. Results from physiological and anatomical assessments of adult fathead minnows exposed in the above experiments are currently being prepared as a draft manuscript that will be circulated among co-authors shortly. Larval fathead minnows exposed in these experiments are currently processed for gene expression. RNA has been extracted from all 140 larvae and gene expression assays will be conducted shortly.

We have made some progress on the mathematical model for predicting minnow and bass abundance. The post-doc who was a part of this activity left at the end of February 2016, but has continued to work on the model in his spare time and taken steps to ensure that his replacement, who starts December 1 2016, can pick up where he left off.

The cost information on biological nutrient removal processes has been collected and summarized from recent literature. The capital cost includes the impact of design capacity of wastewater treatment facilities, but the positive relationship between the cost and corresponding treatment capacity does not seem significant for all treatment technologies. A remaining challenge is to isolate cost performance and efficiency as they depend upon treatment capacity, suitable treatment technology and spatial characteristics. Work is underway to develop and refine a set of mathematical models of the costs of alternative treatment technologies.

Retroactive Amendment Request (07/25/2016):
The addendum is to formally request a re-budgeting of funds for this project.

We would like to move funds ($4,393) from the personnel category to the laboratory supplies category to cover an over-expenditure of $4,393, which was required to complete the wastewater experiments. The cost of estrone analysis was higher than anticipated as a result of these analyses being performed in a core facility (charge-per-analysis) and resulted in overruns in the laboratory supplies category. It is difficult to estimate in advance (at the proposal stage) exactly how many samples will need to be analyzed to generate high quality data. In addition, sometimes samples need to be re-analyzed as a result of the need for dilution for better quantification of analytes. We have now completed the experimental work in Activity 1 and should not have further laboratory supply expenditures. The graduate student working on the wastewater experiments in Activity 1 graduated at the beginning of June, 2016, rather than August, 2016 as planned, and the funds that would have paid her over the summer are therefore available for this transfer.

The movement of funds between these categories will not affect project objectives or timelines.

Approved by LCCMR 7-28-2016

Project Status as of January 31, 2017:
The experiments for Activity 1 on the degradation of estrone in different nitrogen removal systems have been completed. One additional experiment was performed that showed that estradiol was not a product of estrone degradation under anammox conditions. One manuscript has been resubmitted and a second (that includes data from the minnow experiments) is in preparation.

The large-scale, factorial predation experiment with the predator, bluegill sunfish (*Lepomis macrochirus*), and prey, larvae of the fathead minnow (*Pimephales promelas*), has been completed and the data have undergone a preliminary analysis. The results of this analysis indicate the following main results: (i) sunfish exposed to 625 ng/L estrone exhibited pronounced physiological responses to estrone exposure at all four temperatures, (ii) sunfish exposed at 125 ng/L show a less pronounced and consistent response, (iii) sunfish exposed at the lowest temperature (15°C) fed less than sunfish at the three higher temperatures, (iv) independent of temperature, estrone exposed larval fathead minnows were eaten at a greater frequency then ethanol control larvae as indicated by the approximately 12% greater survival of control fish across trials, (v) estrone concentration (125 ng/L and 625 ng/L) did not affect the greater rate at which exposed larvae were eaten suggesting that a detrimental threshold of exposure exists below 125ng/L. The results of these experiments are being incorporated into the population models under development.

We are making up lost ground on the mathematical model for predicting fish abundance and the impacts of estrone. The new post-doc for this activity started December 1 2016. He brought himself up to speed by reviewing existing material and the literature, and working with the former post-doc and project leaders. We are now coding the heart of the model, which are two sub-routines that govern how individual fish allocate food energy to metabolism, growth, reproduction, etc. (and how this is impacted by estrone concentrations). We will present this work at the Minnesota Chapter of the American Fisheries Society annual meeting in February.

We are currently developing tables to be used for a cost-benefit analysis of different wastewater treatment processes, including the MLE process, nitritation-denitritation and anammox, which show both the treatment costs and the additional benefits of different nitrogen removal processes. The treatment cost is categorized into two parts: the capital cost and the operation and management cost. The additional benefit category considers the benefits of a change in fish population (in monetary units based on recreational and economic value) as a result of changes in estrone removal. The efficiency of nitrogen removal can be easily computed from the table, to assist with a comparison among different wastewater treatment processes.

Amendment Request (01/31/2017):
The addendum is to formally request a 6-month extension for this project. The project is scheduled to end 30 June 2017, but we are requesting that this be changed to 31 December 2017. The extension is necessary because of an unexpected change in personnel. The 2-year post-doc who we hired for Activity 3 left the project after 8 months (28 February 2016). We found a highly-qualified replacement in just three months, but he could not begin work until 1 December 2016. Because we lost 9 months of productivity, a 6-month extension shortens Activity 3 from 24 to 21 months; this is, however, a necessary compromise given the length of the vacancy and remaining funds. These funds are sufficient to pay this post-doc until 31 December 2017. Outcomes will be met by the new deadline.

Approved by LCCMR 5-30-2017

Project Status as of July 31, 2017:
The experiments for Activity 1 on the degradation of estrone in different nitrogen removal systems have been completed. One manuscript has been accepted for publication; a second (containing data from both Activity 1 and Activity 2) has been submitted for publication.

The experiments for Activity 2 on the effects of temperature, life stage, and estrone concentration on fathead minnows are also completed. One manuscript has been published, one submitted (including Activity 1 data, see above), and a third is in preparation.

The fish population model is now coded. The sub-routines that govern how individual fish allocate food energy to metabolism, growth, reproduction, and activity are parametrized, and we are calibrating the model to observed fish densities in Minnesota rivers. We are making good progress on incorporating estrone effects into this model. We will present our results at the Society of Environmental Toxicology and Chemistry North America 38th Annual Meeting at Minneapolis in mid-November.

Project Status as of January 31, 2018:
The experiments for Activity 1 and Activity 2 on the degradation of estrone in different nitrogen removal systems and their effects on fish at different temperatures have been completed. Three manuscripts have been published or accepted for publication on this work; 2 additional manuscripts (containing data from Activity 2) are being prepared for publication.

We calibrated the population model to reproduce realistic fish densities in different river systems. We ran simulations based on the results of Activity 2 and are also running additional simulation scenarios. A first manuscript is in preparation, one conference presentation was made in mid-November at a National meeting, and an additional conference presentation will be made in late January, 2018.

The economic analysis of alternative nutrient removal processes is well along and will be completed in the current semester. The goal of this work is to compare the cost of nutrient removal, in terms of plant upgrade and operations cost, to the value of estrone removal, in terms of fish population value.

The cost information on biological nutrient removal processes has been collected and summarized from recent literature. Depending on available technologies for wastewater treatment, total capital cost on upgrading facilities has been generalized, with a focus on comparing the cost of anammox and granular aerobic sludge facilities. Remaining steps are first, to estimate to the nitrogen removal achieved by the treatment facilities under study and the benefits of such removal and second, to link costs/benefits of nitrogen removal to the value of estrone removal. Because the valuation of fish populations is highly variable, we will compute the dollar value per fish that would be minimally necessary to justify the expense associated with an improved treatment technology.

Overall Project Outcomes and Results:
Wastewater treatment plants (WWTPs) discharge effluent that contains contaminants of emerging concern (CECs), including estrogens. These estrogens have caused ecological damage, such as fish feminization, with unknown long-term consequences. The most important estrogen exiting WWTPs is a chemical called estrone. In this project we studied how different treatment systems performed with respect to estrone degradation and how temperature effected degradation. We also studied how fish vulnerability changed seasonally. Finally, we combined these laboratory efforts with models of fish population dynamics to extrapolate the results.

We determined that the technology used by a treatment plant is likely to have an impact on the estrogenicity of WWTP effluent, with some technologies performing very well and others failing to remove estrone. In addition, natural seasonal fluctuations in temperature and expected fluctuations in estrone concentration can cause negative changes in exposed fish. Mathematical models were used to expand this research to whole river systems and showed that the impacts of estrone on fish populations varied depending on the characteristics of the environment. Impacts were expected to be low in systems in which fish were limited by food and high in systems in which fish were limited by predators. Therefore, fish populations in Minnesota rivers are likely to vary in their response to wastewater estrone. The cost of various wastewater improvements were calculated, which could be compared to the value associated with recreational fishing.

Overall, this research showed that low energy treatment systems do exist that are capable of excellent estrone removal, which should be considered so that multiple ecological benefits can be reaped as treatment plants upgrade. Nevertheless, modeling results suggest that the impacts of estrone vary at the population scale based on river characteristics. Therefore, the impact of estrogens at the fishery scale should be evaluated for a given river of interest.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Determine the performance of different wastewater treatment processes with respect to nitrogen removal, CEC and estrone removal, energy use, and cost

Description: Five laboratory-scale reactor systems will be set-up to mimic different wastewater treatment systems, including conventional (NH_3 removal-only) treatment (*CONV*) and four treatment systems designed for total nitrogen removal. Reactors will be constructed from glass and will be designed to mimic the most basic total nitrogen removal process (the Modified Ludzack-Ettinger (MLE) process), a nitritation-denitritation system (*N-D*), an *ANAMMOX* system, and a low-oxygen granular sludge process (*GRAN*). All of the reactors, except for the *GRAN* reactor, will be fed continuously and operated with solids residence times of 10-25 days depending on the reactor. A membrane separation system will be used with each reactor set-up to retain the biomass for recirculation. Experiments will initially be performed at approximately 72°F followed by a second set of experiments performed at approximately 59°F. Two exceptions are the experiments performed with the *N-D* and *ANAMMOX* reactors, which require heating of the aerobic (both, 90°F) and second anaerobic (*ANAMMOX* only, 97°F) reactors to function optimally. In these cases the feed to the reactors will be heated and the energy used for heating will be incorporated into the cost calculations. When appropriate, reactors will be aerated. The flow rate of oxygen required to meet the dissolved oxygen set-point will be monitored daily and will be incorporated into energy utilization and cost calculations. Reactors will be fed synthetic wastewater amended with estrone. Reactor effluents will be monitored for soluble COD, estrone, NH_3, NO_3^-, NO_2^-, and dissolved oxygen. Two of the experiments will be repeated in triplicate to verify reproducibility. Finally, two to three experiments will be repeated with influent secondary wastewater from the Metropolitan Plant in St. Paul, MN to which estrone has been amended. This will allow verification of the trends observed with synthetic wastewater amended with estrone with a more complex feed. The effluent will also be collected, and the CECs present will be extracted and amended to aquaria in fish exposure experiments (described under Activity 2). We have experience operating similar systems.

Summary Budget Information for Activity 1:

ENRTF Budget:	**$ 140,450**
Amount Spent:	**$ 140,311**
Balance:	**$ 139**

Activity Completion Date: February 28, 2017

Outcome	Completion Date	Budget
1. Nitrogen removal efficiency in five different wastewater treatment plant configurations	10/31/2016	$62,125
2. Estrone/CEC removal efficiency in five different wastewater treatment plant configurations	10/31/2016	$62,125
3. An estimate of the energy use for the various treatment options	02/28/2017	$5,000
4. An estimate for the cost of the various treatment options	02/28/2017	$10,000

Activity Status as of January 31, 2015:
Reactor design, configuration, and construction for the five reactor systems (conventional, Modified Ludzack-Ettinger, nitrification-denitrification, anammox, and granular aerobic sludge) have been completed. Analytical methods for quantification of estrone, dissolved organic carbon, ammonia, nitrate and nitrite have been

developed and tested. A method for total nitrogen analysis is still undergoing development due to digestion issues. Sludge samples for seeding reactors have been obtained from the Metropolitan Wastewater Treatment Plant in St. Paul and preserved for use in all experiments.

The base case study, conventional treatment with synthetic wastewater with an influent concentration of 10 µg/L of estrone, has been completed. Results show 90% removal of estrone in the effluent. An anaerobic control to account for estrone removal through sorption to sludge or membrane module has also been completed, with results pending sample analysis.

Three additional experiments are currently in operation: (1) conventional treatment using actual wastewater from the Metropolitan Wastewater Treatment Plant in St. Paul; (2) conventional treatment using lower influent concentrations of estrone (100 ng/L); and (3) granular aerobic sludge. The first two configurations will run until 2/2 and 2/9 respectively. Once (1) and (2) are complete, verifying that the base case results are applicable to real wastewater and environmentally relevant estrone concentrations, a second base case configuration and a Modified Ludzack-Ettinger configuration will be operated. The granular aerobic sludge reactor has been operating for a month and a half, and granule formation has begun, though full granulation is still not complete. We expect to operate this reactor for at least another month, and possibly longer, depending on the granulation timeline and changes in reactor performance.

Activity Status as of July 31, 2015:
Several quality assurance experiments were performed with the conventional reactor configuration to determine whether (1) synthetic wastewater could be reliably used in experiments, as opposed to real wastewater, and (2) degradation of estrone differed between reactors fed 10 µg/L versus 0.1 µg/L estrone. Results showed that although estrone degradation was identical in experiments performed with real wastewater and synthetic wastewater, the nitrogen removal differed. Therefore, all experiments are being conducted with real wastewater so that clear linkages between the different technologies designed for nitrogen removal can be made with estrone degradation performance. Results also showed that the degradation of estrone was very similar (approximately 90% degraded) in experiments fed 10 µg/L versus 0.1 µg/L estrone. Therefore, experiments are being performed with a 10 µg/L estrone feed to simplify the analytical chemistry required.

The following experiments have been completed with real wastewater feed at an influent of 10 µg/L estrone: (1) conventional nitrification, (2) Modified Ludzack-Ettinger (MLE) (3) sequencing batch reactor operation fed high concentrations of wastewater carbon (1000 mg/L) to induce sludge granulation, and (4) sequencing batch reactor operation fed low concentrations of wastewater carbon (200 mg/L) with no sludge granulation. Excellent estrone degradation was observed in the conventional, MLE, and low carbon sequencing batch systems (>90% removal), but no estrone removal occurred in the high carbon sequencing batch system containing granular sludge. These results suggest that repeated exposure to high carbon concentrations and/or granulation is detrimental to estrone removal. This is as expected based on our previous published research. We are currently operating conventional reactors at low temperatures (15°C), as well as a reactor operating with partial nitrification, called nitritation (SHARON), which is fed only very low concentrations of oxygen and is operated at a high temperature to control the microbial populations present. Preliminary results for SHARON show good estrone removal (>90%), though we have not yet been able to achieve consistent partial nitritation at this time. Two additional sets of reactors will be evaluated for estrone removal in the coming months: high-rate anaerobic treatment followed by partial nitritation by SHARON, and anammox. Results for all these experiments are expected by January 2016.

Activity Status as of January 31, 2016:
The following additional experiments have been completed since July 31, 2015: (1) cool temperature (15°C) conventional nitrification (2) cool temperature (15°C) conventional nitrification duplicate, (3) SHARON and (4) anammox. Experiments 1, 2, and 3 were conducted with 10 ug/L estrone added to real wastewater from the local Metropolitan Wastewater Treatment Plant. Experiment 4 used a synthetic wastewater feed that delivered

the required nitrite to ammonia ratio for successful anammox activity; 10 ug/L estrone was also added in this experiment.

Estrone degradation (>90% removal) was observed in both 15°C conventional nitrification experiments at the end of the 50 to 60 day experiments. The cool temperature (15°C) experiments exhibited a slight delay in the initiation of estrone degradation after experiment start up as compared to the room temperature conventional nitrification experiments performed in fall 2014. Preliminary data indicated that the room temperature experiments exhibited >90% removal prior to Day 3 of the experiment, whereas the 15°C experiments did not exhibit >90% estrone removal until after Day 3.

The SHARON and anammox experiments were successfully completed. The method of estrone analysis is currently being assessed for quality assurance and quality control prior to the analysis of the SHARON and anammox estrone samples. Preliminary results suggest that estrone removal did occur in the SHARON reactor. Final results from estrone analysis for all experiments are expected by May 2016, completing Activity 1.

Activity Status as of July 31, 2016:
All experiments for Activity 1 have been completed. Unexpectedly, estrone was effectively degraded in the anammox reactor, with 99.8% mean estrone removal in this reactor. These results are very exciting and were not anticipated, as anammox operates under anaerobic conditions, which are not expected to be conducive to estrone degradation.

Upon further analysis of the SHARON results, the estrone removal was determined to be quite variable. Because SHARON and anammox are operated in series at full-scale installations, the degradation of estrone in the anammox reactor will enable the use of this coupled system for effective estrone treatment, despite the variable performance of the SHARON system.

We are collaborating on a manuscript on the influence of temperature on estrone degradation and on the impact of estrone on fish. This manuscript is in preparation and should be completed by late summer/early fall 2016. We have also drafted a second manuscript focusing on the degradation of estrone in different nitrogen removal treatment systems. This manuscript is being reviewed by one of the coauthors and should be submitted by the end of summer.

Activity Status as of January 31, 2017:
All experiments for Activity 1 have been completed. One additional experiment was performed, as requested by reviewers of our manuscript, to determine whether estrone transformation to estradiol occurs during anammox treatment. This experiment showed that estrone was again degraded under anammox conditions but without the concomitant formation of estradiol. This is important because it shows that estrone degradation follows an unknown pathway to unidentified products.

We have resubmitted the manuscript describing this research to the top disciplinary journal in Environmental Engineering and expect a decision within two months. In addition, Kira Peterson, the Master's student who completed the research on Activity 1 and wrote her Master's thesis on this work, was awarded the Cale Anger Master's Thesis Award and the University of Minnesota Distinguished Master's Thesis Award in Mathematics, Physical Sciences and Engineering for her research. The collaborative manuscript on the influence of temperature on estrone degradation and on the impact of estrone on fish is in progress.

Activity Status as of July 31, 2017:
All experiments for Activity 1 have been completed and results accepted or submitted for publication. In this research estrone removal was compared in a laboratory-scale system that modeled current conventional wastewater treatment with removal in laboratory-scale systems designed to remove total nitrogen from wastewater: the Modified Ludzack-Ettinger (MLE) system (a two-stage anaerobic-aerobic system with recycle), a

granular activated sludge system (cycled anaerobic-aerobic), a sequencing batch reactor (cycled anaerobic-aerobic), and an anaerobic ammonia oxidation (anammox) system. Estrone removal was excellent when fed to the nitrification, MLE, and sequencing batch reactors, at >96% mean estrone loss. Excellent estrone removal occurred in a nitrification system operated at 15°C as well, though there was a slight lag in degradation. The granular activated sludge system operated in our laboratory failed to remove estrone, which was perhaps not unexpected given the high carbon loading under which our system was operated. Despite the anaerobic nature of anammox, it also resulted in excellent estrone removal (95% mean estrone loss) without concomitant 17β-estradiol production. This work demonstrates that the choice of nitrogen removal technology used by a treatment plant could have an impact on the estrogenicity of WWTP effluent, but low energy total nitrogen removal systems do exist that are capable of excellent estrone removal.

Activity Status as of January 31, 2018:
All experiments have been completed and there are no additional updates.

Final Report Summary:
In this study, the degradation of a common and persistent human estrogen, estrone (E1) was studied in several different laboratory-scale reactor configurations and in one case, at two different temperatures. E1 removal in a laboratory-scale system that modeled current conventional wastewater treatment (designed only to transform, but not completely remove total nitrogen) was compared with that in laboratory-scale systems designed to remove total nitrogen from wastewater: the Modified Ludzack-Ettinger (MLE) system (a two-stage anaerobic-aerobic system with recycle), a granular activated sludge system (cycled anaerobic-aerobic), a sequencing batch reactor (cycled anaerobic-aerobic), and an anaerobic ammonia oxidation (anammox) system. E1 removal was excellent when fed to the conventional (nitrification) system, as well as the MLE system and the sequencing batch reactor, with >96% mean E1 loss in all cases. Excellent E1 removal occurred in a nitrification system operated at 15°C as well, though there was a slight lag before degradation began, demonstrating that E1 degradation slows when the temperature drops. Although this was not a problem at 15°C, with eventual excellent E1 degradation, it is possible that at lower temperatures (5-10°C), E1 could persist. E1 did not degrade in the granular activated sludge system operated in our laboratory, which was perhaps not unexpected given the high carbon loading under which our system was operated. Interestingly, despite the anaerobic nature of anammox, E1 degraded well in this reactor system (95% mean E1 loss) without concomitant 17β-estradiol production. This work demonstrates that the choice of nitrogen removal technology used by a treatment plant could have an impact on the estrogenicity of WWTP effluent, but low energy total nitrogen removal systems do exist that are capable of excellent E1 removal. As treatment plants upgrade to implement total nitrogen removal, anammox would be a system worth considering for this reason.

ACTIVITY 2: Determine how temperature and life stage alter the reproduction and survival of fathead minnows after exposure to a common wastewater contaminant

Description: First, we will employ a staggered blocked design using a model fish species (fathead minnow, *Pimephales promelas*), at two life stages, two temperatures and five exposure treatments to determine windows of vulnerability for fish to effluent exposure. The life history of non-migrating North American fishes usually contains two life stages during which the fish are assumed to be particularly vulnerable to the effects of environmental estrogens: (i) the embryonic/early larval stage during which organogenesis occurs and (ii) the period during which adult fish produce gametes and reproduce. We will expose fathead minnow to estrone-amended water during both stages. This species was chosen because it is native to North America, widespread and abundant in many aquatic environments, readily available from controlled culture facilities, and has been used as model species for laboratory and field studies of CECs in the past. Both life stages will be exposed at four temperatures (15°C; 18°C; 21°C; 24°C) to mimic conditions across the life history stages of these fishes. Following exposure, larvae will be assessed in their ability to perform innate predator avoidance behaviors. Adult fishes (males and females) will be assessed for changes in their reproductive behavior. Fish will also be analyzed for vitellogenin concentrations (a precursor protein involved in egg production and a sign of

feminization of male fish) and their livers and reproductive organs will be evaluated for changes. Second, we will conduct a large-scale predation experiment to determine how variation in the thermal regime modulates the effects of contaminants present in wastewater effluent on natural predator-prey relationships. Juvenile (45 dph) fathead minnows (prey) and adult piscivorous bluegill sunfish (*Lepomis macrochirus*) (predators) will be exposed to E1 (i.e., 25, 125, or 625 ng L^{-1}) or maintained under control conditions (0 ng L^{-1}) at four temperatures (15°C; 18°C; 21°C; 24°C) for 45 days. Trials will be conducted in a factorial manner (i.e., exposed predator/non-exposed prey; non-exposed predator/exposed prey; exposed predator/exposed prey; non-exposed predator/non-exposed prey) (40 total treatments). The resultant data will be used to develop the fish biomass model described in Activity 3.

Summary Budget Information for Activity 2: **ENRTF Budget: $ 186,800**
Amount Spent: $ 185,130
Balance: $ 1,670

Activity Completion Date: March 31, 2017

Outcome	Completion Date	Budget
1. Reproduction and survival data for larval and adult fathead minnows exposed to estrone (E1) at seasonally-appropriate temperatures	03/31/2016	$93,500
2. Predation experiment assessing the effect of exposure to wastewater contaminants on predator-prey relationships (fathead minnows and bluegill sunfish)	03/31/2017	$93,500

Activity Status as of January 31, 2015:

Exposures, hatching success and early survival

Thus far, we have collected fathead minnow eggs from 45 different breeding groups. We randomly exposed these eggs to one of 20 different treatments (i.e., a concentration of 0, 5, 25, 125 or 625 ng L^{-1} estrone at a temperature of 15, 18, 21, or 24°C). To date, a total of 229 clutches have successfully hatched under exposed conditions, or are currently undergoing exposure. Current sample sizes for each treatment range from 10-12, or 83-100% of our minimum goal sample size of 12 replicates in each treatment. For each clutch hatched to date, we recorded the proportion of fertilized eggs that hatched (hatching success) and the proportion of hatched eggs that survived to Day 21 (larval survival). We also recorded the latency to hatching and the duration of the hatching period (in days). Preliminary analyses indicate that the data generated to date are sufficient for assessing hatching success and early survival.

Behavioral endpoints – predator avoidance and feeding efficiency

To date, we have conducted a total of 372 behavioral trials (i.e., between one and three trials per treatment replicate, per behavioral assay). Current treatment sample sizes for the predator avoidance assay (C-start assay) range from 7-12 (total 183 trials). Replicate sample sizes for the larval feeding assay range from 6-12 (total 189 trials). The treatment replicates tested to date are between 60% and 120% of our minimum goal sample size of 10 replicates in each behavioral assay. For the predator avoidance trials conducted thus far, we used a high-speed camera to generate a video of the response of a single larva to a simulated predator. These videos were stored off-line and are currently awaiting quantification by eye for several measures of escape behavior, including response latency, velocity of escape, and turning angle. To quantify feeding efficiency, we recorded the number of brine shrimp consumed by two larvae over a 1 min period. Each trial conducted to date was filmed using a digital video camera. Preliminary analyses indicate that the data that we have generated are sufficient for assessing the selected behavioral endpoints of exposure.

Vitellogenin analysis and developmental abnormalities

All of the larvae used to date in the C-start and predator avoidance assays (i.e., 1620 individual fish) were placed in RNAlater immediately following the completion of each trial. These samples were subsequently frozen at -20°C and are currently awaiting vitellogenin gene expression analysis. The remaining fish from each treatment

replicate were euthanized on Day 21 of exposure via an overdose of MS-222, and stored in formalin. These fish will be used to assess the effects of exposure on larval growth and development.

Activity Status as of July 31, 2015:

Adult fathead minnow flow-through exposures

We have completed the adult fathead minnow exposures using the flow-through system. We exposed a total of 672 fish (two females and one male per tank) to each of 16 different estrone treatments (i.e., a concentration of 0, 25, 125 or 625 ng L^{-1} estrone at a temperature of 15, 18, 21, or 24°C) for 30 days. Between 8 and 14 breeding groups were established in each treatment. We monitored the fish daily throughout the exposure period and recorded the number of eggs laid and the proportion of eggs that were fertilized for each breeding group. Beginning on Exposure Day 10 and continuing through Exposure Day 20, we collected one clutch from each breeding group and placed it in a commercial fry basket in the exposure tank containing the parental fish. We monitored the clutch and recorded the proportion of fertilized eggs that hatched (hatching success) and the proportion of hatched eggs that survived to Day 21 (larval survival). We also recorded the latency to hatching and the duration of the hatching period (in days). Behavioral assays (feeding rate, male-male aggression) were conducted for all adult minnows on Day 30; the fish were then immediately sacrificed and dissected (see *behavioral endpoints* and *vitellogenin analysis and histology* below). Exposures continued to Day 21 for the larvae in the fry basket. On the morning of Day 22, the larvae were tested in the same behavioral assays described above for static-exposed larvae (i.e., predation evasion and feeding rate). These data will allow for comparisons between our flow-through and static exposure set-ups.

Adult behavioral endpoints – male-male aggression and feeding rate

At the end of the exposure period (Day 30) we examined whether estrone exposure altered the aggressive intensity with which males defended their nests from intruding males, and whether exposure affected the ability of fish to capture live prey (*Daphnia pulex*). A total of 224 resident, exposed males (10-14 males per treatment) were assessed for aggressive intensity by quantifying the number of aggressive acts, and the latency to first response, performed towards a (non-exposed) intruder male over a 5-min period. A total of 384 fish were assessed for feeding rate (192 males and 192 females, 12-28 fish per treatment). Individual fish were introduced to a feeding arena and provided with 30 mature *D. pulex.* The number of daphnia consumed over a 10-min period was recorded. These behavior data are currently undergoing statistical analysis.

Adult behavioral endpoints – predator evasion and feeding rate

All of the larvae from the static exposures have now been analyzed. Several measures of predator evasion were scored from high-speed videos and feeding rate was quantified. These data are currently undergoing statistical analysis. All of the larvae from the flow-through exposures have been tested and are currently awaiting quantification for several measures of escape behavior, including response latency, velocity of escape, and turning angle.

Vitellogenin analysis and histology

Larvae: all of the larvae used in the C-start and predator avoidance assays were placed in RNAlater immediately following the completion of each trial. These samples were subsequently frozen at -20°C and are currently awaiting vitellogenin gene expression analysis. The remaining fish from each treatment replicate were euthanized on Day 21 of exposure via an overdose of MS-222, and stored in formalin. These fish will be used to assess the effects of exposure on larval growth and development.

Adults: the minnows were dissected on exposure Day 30, following behavioral testing. Each minnow was weighed and measured for standard length. The liver and gonads were dissected from each fish and weighed. Blood was collected and the presence and quantity of vitellogenin was measured from each fish using commercial ELISA kits. These data are currently undergoing statistical analysis.

Activity Status as of January 31, 2016:

Larval and adult behavioral data have now been quantified from video tapes, and the statistical data analyses assessing the effects of E1 on hatching success, early survival, fecundity, fertility, behavioral impairment (4 assays), and biochemical endpoints are largely completed. Our initial data provide some evidence that temperature modulates the effects of estrogen exposure in both adult and larval fathead minnows, but that its influence is limited and unpredictable. A preliminary draft of a manuscript reporting the results of the behavioral assays has been written.

In addition, the preparation of adult fathead tissues for histological analysis is approximately 80% complete. Guided by the results of the behavioral analyses, we have also chosen several genes for qPCR analysis, and have begun to extract RNA from preserved larvae.

Based on our initial finding that temperature has only a weak or unpredictable modulating effect on the fecundity, fertility and development of fish exposed to E1, but that temperature significantly interacts with E1 to alter larval predator avoidance performance, we have proposed to amend Activity 2 to conduct a large, factorial predation experiment to clarify how temperature interacts with wastewater contaminants to alter natural predator-prey relationships, in lieu of collecting basic survival and reproduction data for smallmouth bass.

Activity Status as of July 31, 2016:

Adult and larval fathead minnow exposures

Exposure experiments with adult and larval fathead minnows have been completed and are fully analyzed. A manuscript detailing the behavioral impact of estrone exposure at various temperatures is currently being circulated among co-authors in preparation of journal submission in the near future. A second manuscript, detailing the effects of estrone exposures at various temperatures on the physiology and anatomy of fathead minnows is currently being prepared, will be circulated among co-authors shortly and will be readied for journal submission in the coming months.

Larvae preserved in RNAlater for subsequent analysis of gene expression patterns have been processed to extract RNA. Extracts will soon be used in the gene expression analysis.

Predation trials with adult sunfish and larval fathead minnows

We developed the experimental design and standard operating procedure to examine the effect of estrone exposure at various temperatures on the predator-prey interactions of adult sunfish and larval fathead minnows. These experiments have been completed for the 15°C and 21°C treatments at all estrone concentrations. The 18°C and 24°C treatments are currently in the exposure phase and predation trials will resume in late July.

Activity Status as of January 31, 2017:

Predation trials with adult sunfish and larval fathead minnows

We executed the complete large-scale, factorial predation experiment described in our approved amendment (1/22/2016). This experiment provides the information for a more thorough understanding of the effects of estrogen exposure on trophic interactions under different thermal regimes. Our predator species, the bluegill sunfish (*Lepomis macrochirus*), was exposed at four experimental temperatures (15, 18, 21, 24°C – matching the previous fathead minnow exposure experiments) for 30 days to one of three treatments: ethanol carrier control, estrone low (125 ng/L), and estrone high (625 ng/L). Concurrently, our prey species, larvae of the fathead minnow (*Pimephales promelas*) were exposed for 30 days at the four temperatures and three concentrations of estrone. A subset of larval minnows were assessed for their feeding and predator avoidance performance after 21 days exposure to match previously established experimental protocols. Following the 30 day exposures, individual predators were placed into 800L circular tanks acclimated to the same water temperature as the exposure temperature of the specific fish treatments. Tanks contained foliage to provide a more natural habitat and cover for the prey fish. After a 90-minute acclimation period, the prey fish (larval fathead minnows) were introduced in groups of five control and five estrone exposed (either estrone low or high treatment) fish for a

total of 10 prey fish in the tank. After a 60-minute forage period, predators were removed and processed for all anatomical, physiological and histological endpoints that had previously been collected for all other adult fish analyzed in the larger project. Surviving prey were then recovered from the tank and identified (by a previously applied stain only visible at a wave-length outside the visual spectrum of fishes) by treatment. Great care was taken to randomize treatments for predator and prey and for the identifying stain.

We have completed these exposure experiments at all temperatures, all treatments and in all randomized trials. These combinations resulted in almost 300 individual (=one tank) trials. All sunfish have been processed for all endpoints while all feeding efficiency and predator avoidance performance data for larval fathead minnows have been gathered. Data for survival of prey fish in the mixed predation experiments have been compiled by treatment and have undergone a preliminary analysis. The results of this analysis indicate the following main results: (i) sunfish exposed to 625 ng/L estrone exhibited pronounced physiological responses to estrone exposure at all four temperatures, (ii) sunfish exposed at 125 ng/L show a less pronounced and consistent response, (iii) sunfish exposed at the lowest temperature (15°C) fed less than sunfish at the three higher temperatures, (iv) independent of temperature, estrone exposed larval fathead minnows were eaten at a greater frequency then ethanol control larvae as indicated by the approximately 12% greater survival of control fish across trials, (v) estrone concentration (125 ng/L and 625 ng/L) did not affect the greater rate at which exposed larvae were eaten suggesting that a detrimental threshold of exposure exists below 125ng/L. The results of these experiments have been discussed with our collaborators on this study and are being incorporated into the population models under development (Activity 3).

Adult and larval fathead minnow exposures
Larvae preserved in RNA*later* for analysis of gene expression patterns have been processed to extract RNA. Quality control of the extract indicates excellent extraction efficiencies. Gene expression analysis has begun with the evaluation of putative primers. We expect to complete gene expression analysis in spring 2017.

Activity Status as of July 31, 2017:
Adult and larval fathead minnow exposures
Gene expression analysis for larval fathead minnows has been completed. All data have been quality checked and analyzed. Results of the analysis, which indicated changes in gene expression that are consistent with observed changes in predator avoidance performance in larval fathead minnows, have been completed. The results of these investigations are currently being prepared as manuscript for submission during the next reporting period.

Activity Status as of January 31, 2018:
All exposure experiments have been completed and all data have been analyzed. Results are described above. Data not already published are currently readied for publication.

Final Report Summary:
In this study, we exposed reproductively mature and larval fathead minnows (*Pimephales promelas*) to three environmentally relevant concentrations of a common environmental estrogen, estrone (E1), at four water temperatures reflecting natural spring and summer variations. We then conducted a series of experiments to assess the independent and interactive effects of temperature and E1 exposure on individual fish and on interactions between the prey fish (fathead minnows) and their natural predators (bluegill sunfish, *Lepomis macrochirus*). Our results document important consequences of E1 exposure to the fitness, reproduction, foraging, and predator evasion of fathead minnows. Our data demonstrated significant independent effects of temperature and/or E1 exposure on the physiology, survival, and behavior of adult fish. Some endpoints (body size, growth, organ size) were more susceptible to temperature-modulating effects in female fish while others (biosynthesis of proteins) were more strongly affected in males. Conversely, larval fathead minnows were smaller and impaired in their ability to avoid predators when exposed to E1 at all temperatures. Most notably, the concentration-dependent predation survival rates declined by almost 25% in E1-exposed larval fathead

minnows when compared to control fish. The prey catching abilities of the sunfish were also impaired, although not as severely as predator avoidance behaviors in minnows, potentially mitigating the predation effects on the minnows.

Collectively, our data demonstrate that natural seasonal fluctuations in temperature and E1 concentrations are sufficient to induce sex-dependent physiological and anatomical changes in exposed fish and alter population-level dynamics. These findings improve our understanding of the outcomes of interactions between anthropogenic stressors and natural abiotic environmental factors, and suggest that such interactions can have ecological and evolutionary implications for freshwater populations and communities.

ACTIVITY 3: Conduct a cost-benefit analysis that links the cost of different wastewater treatment options to mathematical predictions of fathead minnow and smallmouth bass abundance

Description: During Activity 3 we will conduct an empirical analysis of alternative water quality trading systems, incorporating information about costs of upgrading wastewater treatment facilities and ongoing operating costs from Activity 1 and from the literature, and solve for the cost of attaining a set of water quality levels. Mathematical modeling will link these water quality levels to the equilibrium biomass of fathead minnows and smallmouth bass in a river that receives treated wastewater. To link treatment options to fish biomass, we will develop a mathematical simulation model that uses environmental cues (e.g., seasonal wastewater effluent temperature) and fish biology to predict minnow and bass biomass under various scenarios of exposure to treated effluent. This information will allow us to express the cost of treating effluent in terms of benefits related to the biomass of different fish species

Summary Budget Information for Activity 3:

ENRTF Budget: $ 172,750
Amount Spent: $ 128,847
Balance: $ 43,903

Activity Completion Date: June 30, 2018

Outcome	Completion Date	Budget
1. A predictive mathematical model that simulates minnow and bass abundance in a pristine, Minnesota river during different seasons	12/30/2015	$52,325
2. A predictive mathematical model that simulates minnow and bass abundance under exposure to treated wastewater effluent during different seasons	12/31/2017	$52,325
3. A cost-benefit analysis of treatment options and fish abundance	5/30/2018	$69,100

Activity Status as of January 31, 2015:
No work has been completed on this activity to date because results from Activity 2 will be used for Activity 3.

Activity Status as of July 31, 2015:
No work has been completed on this activity to date because results from Activity 2 will be used for Activity 3. Work will begin this summer.

Activity Status as of January 31, 2016:
The mathematical model for predicting minnow and bass abundance is in development. We began work on this model in July 2015 with a conceptual framework that describes the different parts of the model and how they fit together. Because the details of the model depend on how we incorporate estrone effects, we have decided to develop the complete model rather than a pristine version followed by an impacted one. We are focusing on the most important pathway in this model, which links environmental estrogen concentration to population-level effects via the estrone concentration in individual fish and the effect that this concentration has on the feeding, maintenance, growth, and/or reproduction of individual fish. We are using a well-tested ecotoxicological model to convert environmental estrogen concentrations to internal estrogen concentrations, and a bioenergetics

approach to convert these internal concentrations to individual-level effects. We have developed and parameterized this part of the model for fathead minnows, and are now testing it to make sure that model output is consistent with results from Activity 2. We have focused solely on fathead minnows so far because they are well studied, and because the results from Activity 2 are from fathead minnows. Late in 2015, the post-doc who is in charge of this work informed us that he will be leaving at the end of February 2016 to start his dream job. Since that time, our focus has been on hiring a replacement, and on documenting the model and related work so far so that the transition is as smooth as possible. Once we are back up and running, we can finish testing and then duplicate this part of the model for smallmouth bass and/or bluegill sunfish. We will then scale the model up to multiple individuals: first in a homogeneous environment, then in a pristine river, and then in a river that is receiving wastewater effluent as per results from Activity 1.

Activity Status as of July 31, 2016:
We have made some progress on the mathematical model for predicting minnow and bass abundance. The post-doc who was a part of this activity left for a permanent position at the end of February 2016. He has documented the model and related work to date so that his replacement, who starts December 1 2016, can begin working on the model in an effective way immediately. The former post-doc is also continuing to work on the model in his spare time. He recently replaced the energetics part of the model with a version that the replacement post-doc can easily implement, and is currently improving how fish feeding and energetics scale with temperature and updating the documentation accordingly. Last week, we shared the most recent data from Activity 2 with the former post-doc so that he can ensure that model fish respond to estrone the same way that laboratory fish do.

The cost information on biological nutrient removal processes has been collected and summarized from recent literature. Depending on available technologies for wastewater treatment, total capital cost on upgrading facilities has been generalized for Bardenpho, A^2/O, Step Feed, Biolac, Methanol, MLE, Oxidation Ditch and Sequencing Batch Reactor. The capital cost includes the impact of design capacity of wastewater treatment facilities, but the positive relationship between the cost and corresponding treatment capacity does not seem significant for all treatment technologies. A remaining challenge is to isolate cost performance and efficiency as they depend upon treatment capacity, suitable treatment technology and spatial characteristics. Assuming a 20-year project lifetime and a 6% discount rate, the total capital cost of building a new but small wastewater treatment facility has also been summarized for MLE, Sequencing Batch Reactor, Submerged Biofilter, Rotating Biological Contactor and Activated Sludge. Work is underway to develop and refine a set of mathematical models of the costs of alternative treatment technologies.

Activity Status as of January 31, 2017:
We are once again making good progress on the mathematical model for predicting minnow and bass abundance. The new post-doc for this activity began work December 1 2016. He has quickly brought himself up to speed by reviewing the files that were left by the previous post-doc. The two post-docs (former and current) have also been in communication via phone and email. We have met with Co-PI Heiko Schoenfuss to discuss results from Activity 2. A follow-up meeting is scheduled for mid-February. We are also back to coding the model, with an emphasis on using individual-scale data (growth, reproduction, and other life history traits) from both Activity 2 and the literature to parameterize the Dynamic Energy Budget models for the two fish species. These components govern how an individual fish allocates food energy to different processes (e.g., metabolism, growth, reproduction) along a gradient of estrone contamination.

Our previous work summarized the cost of different nitrogen removal technologies both in a new facility and in an upgraded facility. Based on this work, we have developed tables for a cost-benefit analysis of different wastewater treatment processes, including the MLE process, nitritation-denitritation and anammox. This table can be adjusted based on cost assumptions from the literature or from specific information obtained from experiments. The table lists both the treatment cost and the additional benefit of different nitrogen removal processes. The treatment cost is categorized into two parts: (1) the capital cost, which includes the construction

cost and the indirect cost, and (2) the operation and management cost, which includes maintenance, labor, electricity, chemicals, taxes and insurance, and miscellaneous. All the costs are converted to an annual basis using a parameter that relies on the discount rate and the life of the equipment. The additional benefit category considers the benefits of a change in fish population (in monetary units based on recreational and economic value) as a result of changes in estrone removal. In this case the change in the fish population is linked to the mathematical model developed in this project. By combining the efficiency of nitrogen removal for different wastewater treatment processes, the average cost to remove nitrogen can be computed, which can in turn be used to assist with comparisons among different wastewater treatment processes.

Activity Status as of July 31, 2017:
The population model is in the final stages of development. We have parameterized the Dynamic Energy Budget sub-routine, which describe how food is allocated to different processes within an individual of a given species. We have also obtained bass and minnow abundance data in Minnesota Rivers from the Minnesota Pollution Control Agency. We are using these data to calibrate model fish abundance in the absence of estrone. We are also beginning to integrate results from Activity 2 (metabolism, growth, and reproduction) into the model. When this step is complete, we will be able to link wastewater treatment and individual-level responses to estrone to population-level impacts on fish.

Activity Status as of January 31, 2018:
The population model was calibrated to reproduce realistic population densities and reproduction patterns based on both literature and in situ data obtained from the Minnesota Pollution Control Agency. Temperature data from a pristine river in Minnesota (Kawishiwi river) was used for model input; this was obtained from the USGS Water Data for the Nation website. Our model takes into account three kinds of mortalities: Aging, which is computed from the internal metabolism of an individual, starvation, which happens when food availability is too low for too long to enable individuals to survive, and predation, which is based on a daily probability. Inclusion of multiple mortality processes allows us to represent different types of river ecosystems, one in which fish population density is more controlled by food availability than by predation pressure (bottom-up controlled system) and a system in which fish population density is more controlled by predation (top-down controlled system).

Predator-prey scenarios:
Results from Activity 2 showed that exposure to estrone significantly alters larval predator avoidance. Consequently, we ran simulations in which prey survival probability in response to predation decreased according to these experimental results. In these simulations, estrone is homogeneously distributed in the river. Our results suggest that the outcomes at the population level are dependent on the river system (top-down or bottom-up) and that fish population density can be significantly reduced in the presence of estrone. We are now developing simulations in which estrone concentrations within the river can vary spatially.

Nest-defense scenarios:
Results from Activity 2 showed that male fathead minnows are less aggressive when exposed to estrone, which could lead to a decreased efficiency in nest defense. Although a proper link between loss of aggressiveness and reduction of nest defense efficiency does not exist at this time, we ran simulations with reduced egg survival due to increased predation (a logical consequence of reduced efficiency in nest defense). In these simulations, estrone is homogeneously distributed in the river. Consistent with our predator-prey scenarios above, impacts on fish population density appear to depend on the river system; further simulations are needed.

Reduced sperm scenarios:
We ran simulations in which sperm quality is reduced, leading to fewer reproductive events per male. In these simulations, estrone is homogeneously distributed in the river. Estrone effects on sperm quality appear to have limited impacts on population density, but further analyses are needed.

The economic analysis of alternative nutrient removal processes is well along and will be completed in the current semester. The goal of this work is to compare the cost of nutrient removal, in terms of plant upgrade and operations cost to the value of estrone removal, in terms of fish population value.

The cost information on biological nutrient removal processes has been collected and summarized from recent literature. Depending on available technologies for wastewater treatment, total capital cost on upgrading facilities has been generalized, with a focus on comparing the cost of anammox and granular aerobic sludge facilities. The cost performance and efficiency as a function of treatment capacity, treatment technology, and spatial characteristics are currently being determined. Two additional steps remain. First, we are currently estimating the nitrogen removal achieved by the treatment facilities under study in terms of economic benefit. This is a challenge, but is currently being estimated based on literature values relating home costs to nitrogen pollution. Second, we are linking costs/benefits of nitrogen removal to the value of estrone removal. Because the valuation of fish populations is highly variable, we will compute the dollar value per fish that would be minimally necessary to justify the expense associated with an improved treatment technology. This will then be carefully analyzed to determine whether these values are realistic, and under what scenarios estrone removal makes economic sense.

Final Report Summary:
We estimated the population-level effects of wastewater estrone by first developing individual-based, mathematical models that represent the full life cycle of fathead minnows and walleye in pristine Minnesota rivers (Outcome 3.1). Individual-based models simulate populations and communities by following individuals and their properties in response to environmental cues (e.g., temperature, food availability, predation rate). Each individual has a set of attributes and behaviors. Simulated rivers, all in Minnesota and based on data obtained from the US Geological Survey, differed in terms of food availability (high vs. low) and predation pressure (high vs. low). We parametrized two population models: one for the fathead minnow and one for the walleye, both culturally and economically important fish in Minnesota. We calibrated the fathead minnow model to reproduce population densities and reproduction patterns that have been reported in the literature and observed in data obtained from the Minnesota Pollution Control Agency. We calibrated the walleye model to reproduce realistic population densities and reproduction patterns based on literature data only. This process of calibration allowed us to simulate population dynamics on four rivers; one for each combinations of food availability and predation pressure. It was important for us to model these combinations because fish population densities in some rivers can be largely controlled by food availability (bottom-up controlled system) or predation (top-down controlled system). The technical document that is attached to this report ("DEB_parameterization.pdf") describes both models, details how they were parameterized and calibrated, and compares individual processes such as growth and reproduction to observed data.

We incorporated the observed effects of estrone on individual fish (Activity 2) into our population model (Outcome 3.2). Lab results indicated that temperature does not strongly or consistently modulate the effects of estrone on reproduction, development, and egg survival. However, results do suggest that estrone alters larval predator avoidance performance. We incorporated this result into our model by reducing larval survival by 10% (fatheads only) or 25% (fatheads and walleye) when estrone was present. Experiments also showed a decline in aggressive behaviors in male fathead minnows that were exposed to estrone. Because male fathead minnows must defend their nests from predation, reduced aggressiveness in male fathead minnow likely translates into reduced egg survival. We therefore included simulations in which fathead egg survival was reduced by 25% when estrone was present.

We ran our fathead and walleye models on all four simulated rivers and with the effects of estrone exposure on egg and larval survival separately and in combination. We ran each simulation for 20 years – the last 5 years of which included exposure to estrone (if present) – and then compared population sizes between the exposed and non-exposed scenarios. The attached technical document "IBMs_Methods_and_Results.pdf" describes each model, the simulations that we considered, and results.

Model results suggest that the impact of exposure to wastewater estrone on fish abundance in Minnesota rivers is context-dependent. Individuals in food-limited systems do not have enough to eat, and are therefore smaller and less reproductive. When estrone exposure increases predator-induced egg or larval mortality in these systems, the initial decrease in population size results in a decrease in competition (i.e., an increase in per capita food availability), and therefore a corresponding increase growth, size, and reproduction. In some cases, these changes are enough to fully compensate for the increased mortality due to estrone exposure - the result being that estrone is not predicted to impact fish populations. Conversely, individuals are less likely to compete for food in systems in which population density is largely controlled by predation. Individuals in such systems are therefore more likely to grow quickly and large, and have high reproductive output. An estrone-induced increase in egg or larvae mortality in this system is predicted to cause a significant decline in fish abundance because individuals in the population are already growing and reproducing at their maximum rate. These individuals are already well fed, so they are not released from competition and therefore unable to compensate for the additional mortality that is imposed by estrone. Results are intermediate to these extremes in systems in which fish are limited by some combination of food and predation.

Our overall conclusion is two-fold. First, measuring exposure effects on individuals alone is insufficient to determine whether a stressor (or the removal of a stressor through wastewater treatment) will cause population-level impacts; food web dynamics play a large role in determining this response. Second, it is insufficient to assume that population-level responses to wastewater estrone (treatment) will be the same in all rivers. Variation in river food webs (e.g., in terms of the degree of limitation by food or predators) means that fish populations in these rivers will also vary in their respond to wastewater estrone. Taken together, these results suggest that impacts of estrone should be evaluated at the population scale, and for the conditions that are appropriate for river of interest.

Our research summarized the cost information for different nitrogen removal technologies either in a new facility or in an upgraded facility. Tables were developed to be used in a cost-benefit analysis for different wastewater treatment processes. In terms of cost, the treatment cost of different nitrogen removal processes was categorized into two parts: capital cost, which included construction and indirect costs, and the operation and management cost, which included maintenance, labor, electricity, chemicals, taxes and insurance, and miscellaneous costs. All costs were converted to an annual basis. These costs can be compared to the estimated benefit to fish from the population models, based on the literature value of estimated walleye value to recreational anglers of $13.52 to $28.38 per fish (after conversion to 2017 dollars).

V. DISSEMINATION:

Description: The target audience for results from this research will be professionals in the areas of wastewater treatment and natural resource management. Specific targets will be environmental engineers and scientists in academia, industry, state agencies such as the DNR and MPCA, and environmental consultants. Results will be disseminated through scholarly publications in peer-reviewed journals such as *Environmental Science and Technology*. Results from the research project will also be presented at regional conferences such as the *Minnesota Water* conference and if possible, at targeted seminars at the DNR and MPCA. Results will be used to determine which wastewater treatment upgrades offer the most ecological protection while incorporating the value of fisheries and energy use.

Status as of January 31, 2015:
No dissemination efforts have been made, as the project is not advanced enough at this point.

Status as of July 31, 2015:
No dissemination efforts have been made, as the project is not advanced enough at this point.

Status as of January 31, 2016:
Data from this project were presented at the SETAC meeting in Duluth in March 2015 and the Minnesota Water Resources Conference in St. Paul in October 2015. In addition, one manuscript has been drafted for submission to a peer-reviewed journal.

Status as of July 31, 2016:
Three manuscripts (one collaborative between Activity 1 and Activity 2, one focused on Activity 1, and a third focused on Activity 2) are in internal review among the collaborators and should be submitted by the end of the summer/early fall.

Status as of January 31, 2017:
A manuscript detailing the behavioral impact of estrone exposure at various temperatures is currently in peer review. A second manuscript, detailing the effects of estrone exposures at various temperatures on the physiology and anatomy of fathead minnows is being circulated among co-authors and will be readied for journal submission in the coming month. The preparation of a third manuscript draft detailing the linkage between gene expression and behavioral effects is planned for spring 2017. A fourth manuscript describing the majority of the Activity 1 results has been resubmitted for peer review. We have submitted an abstract for a poster that will be presented at the annual meeting of the Minnesota Chapter of the American Fisheries Society at St. Cloud State in February. This poster will describe the project, and focus on our simulation model as an up-scaling tool that infers the population-level effects of estrone on individual patterns of energy allocation and survival.

Kira Peterson, the Master's student who completed the research on Activity 1 and wrote her Master's thesis on this work, was awarded the Cale Anger Master's Thesis Award and the University of Minnesota Distinguished Master's Thesis Award in Mathematics, Physical Sciences and Engineering for her research.

Status as of July 31, 2017:
Results from our estrone biodegradation experiments are accepted for publication in *Environmental Science: Water Research and Technology* (Peterson KN, Tan DT, Bezares-Cruz JC, Novak PJ. Estrone Biodegradation in Laboratory-Scale Systems Designed for Total Nitrogen Removal from Wastewater. *Environmental Science: Water Research and Technology*). Results from our adult fathead minnow exposure experiments have recently been published in the journal *Hormones and Behavior* (Ward JL, Cox MK, Schoenfuss HL. 2017. Thermal modulation of anthropogenic estrogen exposure on a freshwater fish, *Pimephales promelas*, at two life stages. *Hormones and Behavior* 94:21-32.). A second manuscript, detailing the effects of estrone exposures at various temperatures on the physiology and anatomy of fathead minnows is in journal review. A third manuscript detailing the linkage between gene expression and behavioral effects in larval fathead minnows is being readied for journal submission. We presented a poster at the annual meeting of the Minnesota Chapter of the American Fisheries Society at St. Cloud State in February. We also presented a poster at the 5th International symposium on Dynamic Energy Budget Theory in Tromsø, Norway. Both posters described the project, and focused on our modelling approach as an up-scaling tool that infers the population-level impacts of estrone effects on individual energy allocation and survival. We will also present a poster at the Society of Environmental Toxicology and Chemistry North America 38th Annual Meeting at Minneapolis in mid-November.

Status as of January 31, 2018:
Three manuscripts have been published on this work (1 from Activity 1, 1 from Activity 2, and a third that combines results from both Activity 1 and 2 *(Cox MK, Peterson KN, Tan D, Novak PJ, Schoenfuss HL, Ward JL. 2017. Temperature modulates estrone degradation and biological effects of exposure in fathead minnows. Science of the Total Environment. https://doi.org/10.1016/j.scitotenv.2017.10.069)*. A fourth manuscript detailing the linkage between gene expression and behavioral effects in larval fathead minnows is being readied for journal submission. Similarly, a fifth manuscript detailing the effects of estrone at various temperatures on

the predator prey interactions is currently readied for publication. A sixth publication is being written on the fish population modeling efforts.

We presented the results of some specific scenarios (Predator-prey and Nest-defense scenarios) at the Society of Environmental Toxicology and Chemistry North America 38th Annual Meeting at Minneapolis in mid-November. We will have an oral presentation about the impacts of behavioral effects at population scale at the 78th Midwest Fish & Wildlife Conference at Milwaukee, Wisconsin in late January.

Final Report Summary:
The research was widely disseminated at conferences, including the national/international conferences (the 5th International symposium on Dynamic Energy Budget Theory in Tromsø, Norway and the Society of Environmental Toxicology and Chemistry North America 38th Annual Meeting) as well as multiple local/regional meetings. Three manuscripts have been published on this research and have been sent to the LCCMR with the final project report. X manuscripts have been submitted for publication on this research and 3 manuscripts are in preparation for submittal ("*Population level impacts of measured individual behavioral impact after exposure to estrone in fathead minnow," by Vaugeois, Forbes, and Venturelli*; *"Estrone Exposure Interacts with Temperature to Alter Predator Escape Performance and Gene Expression," by Cox, Ward, Matsuura, Aing, Schoenfuss, Kohno*; *"Does Temperature Modulate the Effects of Estrogenic Exposure in a Piscivore Freshwater Fish?" by Korn, Ward, Edmiston, Schoenfuss*). Once accepted, they will be forwarded to the LCCMR.

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget Overview:

Budget Category	$ Amount	Explanation
Personnel:	$ 277,407	Over the course of the 3-year project, two years of support for two graduate students (Activity 1 and the economic aspects of Activity 3), two years of support for a postdoctoral researcher (the population modeling aspects of Activity 3), and two years of support for a research associate (Activity 2), are budgeted. Funds for the research associate will be covered under a subcontract to St. Cloud State University (see below). Funds will also be used to pay an undergraduate researcher for approximately 13 weeks. The PI (Novak) will each receive 2 weeks of salary a year for the first 2 years of the project. The Co-PIs Venturelli and Homans/Coggins will each receive 1 week of salary a year for the first 2 years of the project. No salary is requested for Schoenfuss who will be granted one semester 100% re-assign time by St. Cloud State University to focus on the analysis of biological data. The PIs will be responsible for project oversight, guidance of the graduate students and postdoctoral researchers, data interpretation and analysis, and report preparation and submission. Two graduate

		student research assistants will each devote 100% of their research time to the project over a 2-year period. Fringe benefits for graduate students include tuition, health insurance, and summer FICA. All fringe benefit rates are set by the University of Minnesota and St. Cloud State University.
Equipment/Tools/Supplies:	$ 34,393	Funds ($34,393) are requested for materials, supplies, consumables, analytical costs and upkeep associated with the LC-MS, computers (to be used only on this project), and software. Required materials include, but are not limited to: pipette tips, glassware, solid phase extraction cartridges for extractions, chemicals for standards and experiments, pumps, analytical consumables, analytical fees, solvents, reagents, gloves, digital data storage media, and laboratory notebooks. A portion of the Materials & supplies are budgeted for support of the fish exposure experiments (fish, chemicals, pumps, aquaria maintenance, etc., $54,000/3 years) and will be part of the subcontract to St. Cloud State University (see below).
Travel Expenses in MN:	$1,400	Travel funds are minimal ($1,400) and are included for travel to meetings at either St. Cloud State University or the University of Minnesota for project coordination or to the Metropolitan Wastewater Treatment Plant for sample collection for use in experiments.
Other: Subcontract to St. Cloud State University	$186,800	The subcontract amount ($186,800) will include salary for a research associate ($48,825 salary, $17,575 fringe (36% fringe rate) per year for 2 years) and supplies for experiments (fish, chemicals, pumps, aquaria maintenance, etc., $54,000 for 3 years) to complete Activity 2.
TOTAL ENRTF BUDGET:	**$500,000**	The total proposed project amount is $500,000. No indirect costs for the University of Minnesota or St. Cloud State University are included in the budget.

Explanation of Use of Classified Staff: N/A

Explanation of Capital Expenditures Greater Than $5,000: N/A

Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation: 4.15

Number of Full-time Equivalents (FTE) Estimated to Be Funded through Contracts with this ENRTF Appropriation: 2

B. Other Funds: N/A

VII. PROJECT STRATEGY:

A. Project Partners: The project team consists of the Principal Investigator (PI) Paige Novak (University of Minnesota) and co-PIs Dr. Heiko Schoenfuss (St. Cloud State University), Paul Venturelli (UMN), and Frances Homans (UMN). Dr. Jay Coggins will also assist Homans. Novak will direct Activity 1; Schoenfuss will direct Activity 2; Venturelli will direct Activity 3; Homans and Coggins will direct the cost analysis and economic modeling efforts. MCES has agreed to provide access to wastewater.

B. Project Impact and Long-term Strategy: The proposed work fits into a larger research agenda centered at UMN and St. Cloud State focused on environmental estrogens and improved wastewater treatment. The proposed research complements current and prior research in this area. This project builds on what we have learned and takes it further, factoring in cost, how CEC removal is impacted by changes in treatment (focused on nitrogen removal), and how temperature impacts both removal efficiency and fish vulnerability. It also expands the impact of the research by incorporating fish population modeling to scale the findings to a whole-state level. When taken together, this research will provide a more complete picture of how to improve treatment, decrease costs and energy use, and safeguard our fish populations.

C. Spending History: N/A

VIII. ACQUISITION/RESTORATION LIST: N/A

IX. VISUAL ELEMENT or MAP(S): See attached graphic.

X. ACQUISITION/RESTORATION REQUIREMENTS WORKSHEET: N/A

XI. RESEARCH ADDENDUM: See attached Research Addendum

XII. REPORTING REQUIREMENTS:
Periodic work plan status update reports will be submitted no later than January 31, 2015, July 31, 2015, January 31, 2016, July 31, 2016, January 31, 2017, July 31, 2017, and January 31, 2018. A final report and associated products will be submitted between June 30 and August 15, 2018.

Environment and Natural Resources Trust Fund
M.L. 2014 Project Budget

Project Title: Evaluation of Wastewater Nitrogen and Estrogen Treatment Options
Legal Citation: M.L. 2014, Chp. 226, Sec. 2, Subd. 03d
Project Manager: Paige J. Novak
Organization: University of Minnesota
M.L. 2014 ENRTF Appropriation: $ 500,000
Project Length and Completion Date: 4 Years, June 30, 2018
Date of Report: September 4, 2018

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Activity 1 Budget	Amount Spent	Activity 1 Balance	Activity 2 Budget	Amount Spent	Activity 2 Balance	Activity 3 Budget	Amount Spent	Activity 3 Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	***Determine the performance of different wastewater treatment processes with respect to nitrogen removal, CEC and estrone removal, energy use, and cost***			***Determine how temperature and life stage alter the reproduction and survival of fathead minnows and smallmouth bass after exposure to treated synthetic or real***			***Conduct a cost-benefit analysis that links the cost of different wastewater treatment options to mathematical predictions of fathead minnow and smallmouth bass***				
Personnel (Wages and Benefits)	$104,657		$0	$0		$0	$172,750		$43,903	$277,407	$43,903
Paige Novak, PI ($12,700 salary, $4,300 fringe, 33.6% fringe rate; total for 2 years; 3.8% effort),		$4,828			$0			$0			
Paul Venturelli, Co-PI ($4,300 salary, $850 fringe, 19.8% fringe rate; total for 2 years; 1.9% effort)		$5,667			$0			$5,749			
Frances Homans, Co-PI and Jay Coggins ($5,650 salary, $1,400 fringe, 24.7% fringe rate; total for 2 years; 1.9% effort)		$0			$0			$0			
One Postdoctoral Researcher ($82,400 salary, $17,100 fringe (includes healthcare); total for 2 years; performing the mathematical modeling of fish populations)		$24,487			$0			$67,834			
Two Graduate Research Assistants ($79,293 salary, $65,414 fringe (includes healthcare and tuition); total for 2 years for each student; one student will perform the research on the removal of nitrogen and CECs during wastewater treatment and the other will perform research on the cost and value of wastewater treatment upgrades with respect to the preservation of fish populations))		$65,693			$0			$55,264			
An Undergraduate Research Assistant ($5,000 salary plus fringe) to assist with the wastewater treatment reactor experiments.		$3,981			$0			$0			
Equipment/Tools/Supplies Laboratory supplies and analytical costs (includes, but is not limited to, chemicals for all analyses, supplies to maintain analytical equipment, supplies for reactor construction, and pumps ($34,393/3 years))	$34,393	$34,393	$0	$0	$0	$0	$0	$0	$0	$34,393	$0
Travel expenses in Minnesota Travel between St. Cloud and Minneapolis for research progress meetings (in state) and to the Metropolitan Wastewater Treatment Plant for sample collection for use in experiments	$1,400	$1,261	$139	$0	$0	$0	$0	$0	$0	$1,400	$139
Other: Subcontract Some of the work will be conducted at St. Cloud State University (Activity 2). The subcontract amount will include salary for a research technician ($55,000 salary, $11,400 fringe (20.75% fringe rate) per year for 2 years) and supplies for experiments (fish, chemicals, pumps, aquaria maintenance, etc., $54,000/3 years)	$0	$0	$0	$186,800	$185,130	$1,670	$0	$0	$0	$186,800	$1,670
COLUMN TOTAL	**$140,450**	**$140,311**	**$139**	**$186,800**	**$185,130**	**$1,670**	**$172,750**	**$128,847**	**$43,903**	**$500,000**	**$45,712**

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Temperature modulates estrone degradation and biological effects of exposure in fathead minnows

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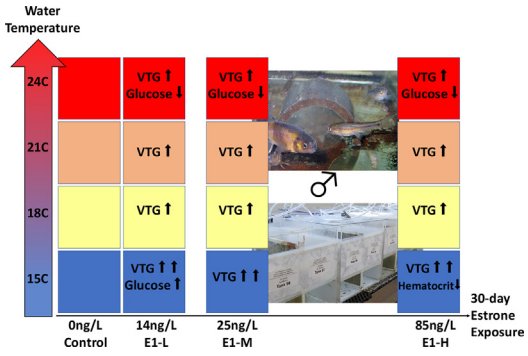
HIGHLIGHTS

- Estrone and effluent temperature interact to affect fish with unknown consequences.
- Identified effects of temperature on E1 degradation in wastewater and exposed fish.
- Ran nitrification experiments and fish estrone exposures at multiple temperatures.
- Cooler temperatures slowled E1 degradation and produced sex-specific effects in fish.
- Seasonal variation modifies E1 degradation in wastewater and E1 exposure in fish.

GRAPHICAL ABSTRACT



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ABSTRACT

Environmental pollutants, including estrogens, are widespread in aquatic environments frequently as a result of treated wastewater effluent discharged. Exposure to estrogens has been correlated with disruption of the normal physiological and reproductive function in aquatic organisms, which could impair the sustainability of exposed populations. However, assessing the effects of estrogen exposure on individuals is complicated by the fact that rates of chemical uptake and environmental degradation are temperature dependent. Because annual temperature regimes often coincide with critical periods of biological activity, temperature-dependent changes in estrogen degradation efficacy during wastewater treatment could modulate biological effects. We examined the interactions between ambient water temperature and degradation of estrone (E1) during wastewater treatment. In addition, we exposed mature fathead minnows (*Pimephales promelas*) to three environmentally relevant concentrations of E1 at four different water temperatures (15 °C, 18 °C, 21 °C, and 24 °C) to reflect natural seasonal variation. E1 degradation occurred with and without the support of robust nitrification at all temperatures; however, the onset of E1 degradation was delayed at cooler water temperatures. In addition, we observed significant interactive effects between temperature and E1 exposure. Female morphometric endpoints were more susceptible to temperature-modulating effects while physiological endpoints were more strongly affected in males. Collectively, the data demonstrate that natural seasonal fluctuations in temperature are sufficient to affect E1 degradation during wastewater treatment and induce sex-dependent physiological and anatomical changes in exposed fish.

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0048-9697/

1. Introduction

All vertebrates naturally excrete estrogens in their urine and feces. Among the estrogens secreted, 17β-estradiol (E2) has received the greatest attention as a result of its presence in wastewater effluent (Kolpin et al., 2002) and estrogenic potency (Van den Belt et al., 2004). However, E2 quickly biodegrades into the more stable E1 under aerobic conditions (Vajda et al., 2008; Writer et al., 2012), which tends to be more persistent in the environment (Johnson and Williams, 2004; de Mes et al., 2005) and present at environmental concentrations frequently in the tens of ng/L and may can exceed 100 ng/L (Alvarez et al., 2013; Ankley et al., 2017; Chen et al., 2010; Matthiessen et al., 2006). Fortunately, E1 is also degraded biologically in wastewater treatment systems, with conditions that support robust nitrification also supporting E1 degradation (Shi et al., 2004; Tan et al., 2013). In addition, during nitrification at temperatures between 16 and 26 °C, researchers (Suarez et al., 2010) have observed that 99% of the E1 and E2 present in the influent degraded. Nevertheless, nitrification is sensitive to temperature and researchers have also shown that both ammonia and estrogen degradation can slow dramatically with decreasing temperature (Raman et al., 2001; Wild et al., 1971). As a result, decreased water temperatures could feasibly increase estrogenic concentrations in aquatic ecosystems.

The presence of estrogens in aquatic ecosystems is correlated with disruption of the normal physiological and reproductive function of aquatic organisms. Estrogens present in municipal and industrial effluents have been causally linked to widespread sexual perturbation in exposed wild fish populations (Jobling et al., 1998; Routledge et al., 1998). Most dramatically, exposure of an experimental lake to a synthetic estrogen (17α-ethynylestradiol) caused the collapse of the resident fathead minnow population (Kidd et al., 2007; Palace et al., 2009). In laboratory studies, plasma vitellogenin (VTG), an egg yolk precursor protein produced by female fish for reproduction, has been shown to be elevated in males exposed to estrogens (e.g., Hemmer et al., 2002; Shappell et al., 2010). Estrogen exposure also decreases reproductive success through reduced fecundity and fertilization success (Dammann et al., 2011; Panter et al., 1998; Parrott and Blunt, 2005; Thorpe et al., 2003).

Organisms live in dynamic environments, and abiotic environmental conditions can interact with chemical challenges to produce complex organismal responses (Brian et al., 2008). Ambient temperature exerts an environmental influence on ectothermic aquatic organisms, including teleost fish (Jin et al., 2009). However, assessing the effects of temperature and estrogenic exposure on individual aquatic organisms is complicated by the temperature dependent rates of E1 degradation in the environment and rates of E1 uptake by aquatic organisms (Gordon, 2003; Heugens et al., 2001; Starner et al., 1999). Established experimental protocols for toxicological experiments often fail to take abiotic environmental factors such as temperature into account (Jin et al., 2009). Most fishes, including fathead minnows, are ectothermic; therefore, water temperature has the potential to exert effects at multiple levels of organization, from gene expression and biosynthesis (e.g., the rate of VTG expression; Brian et al., 2008; Körner et al., 2008) to ecological responses (i.e., the timing of reproduction; Gillet and Quétin, 2006). Whereas temperature and environmental estrogens have been individually shown to influence the physiology and reproductive functioning of fish, the biological effects resulting from the interaction between these two factors are understudied (Jin et al., 2009; Körner et al., 2008) and are potentially complex. Because annual temperature regimes often coincide with critical periods of biological activity (i.e., reproduction during spring warming of aquatic environments), temperature-dependent changes in E1 degradation efficacy during wastewater treatment could have a dramatic impact on fish present in receiving waters.

The goals of the current study were to (i) examine the interactions between ambient water temperature and E1 degradation during wastewater treatment, and (ii) to assess the biological response in fish exposed to E1 under several temperature regimes. Specifically, we tested the hypotheses that (i) cooler water temperatures adversely affects E1 degraders and therefore E1 degradation in model wastewater treatment systems, and (ii) cooler water temperatures exacerbate the physiological and reproductive effects of E1 exposure on adult fathead minnows. Alternatively, the biological effects of E1 exposure on adult fathead minnows could be exacerbated at higher water temperatures, possibly as a result of the increased organismal metabolism, or there could be no effect of water temperature on exposed organisms. Previous studies have shown that chemical toxicants can interact with the thermal conditions in complex ways to influence physiological impairment and survival (Heugens et al., 2001; Gordon, 2003); therefore, these alternative hypotheses would account for these potential outcomes.

2. Materials and methods

2.1. Experimental design

2.1.1. Estrone removal experiments

Duplicate laboratory-scale nitrification experiments were performed at room temperature (21 ± 2 °C) (mean standard ± deviation) for 31 days (Peterson et al., 2017) and duplicate nitrification experiments were performed at 15 °C for 48 and 55 days. A clean water control reactor was also monitored and those results are presented elsewhere (Peterson et al., 2017). Each reactor was seeded with 10 mL of preserved concentrated activated sludge from the Metropolitan Wastewater Treatment Plant (Metro WWTP) in St. Paul, MN, as described elsewhere (Peterson et al., 2017). The reactors were fed with primary effluent collected from the Metro WWTP and amended with 10 μg/L E1. The high E1 concentration was chosen to allow for detailed tracking of E1 degradation without the need for large volume extraction. The influent, as described elsewhere (Peterson et al., 2017), contained the following: 299 ± 80 mg/L (n = 8) mean chemical oxygen demand, 47 ± 7.8 mg/L (n = 16) total nitrogen and 6.2 ± 3.1 μg/L (n = 16) E1. Reactors were operated as continuous flow systems and had a reactor volume, HRT, and SRT of 0.8 L, 5 h, and 10 days, respectively. A cross flow filtration membrane (Spectrum Labs Minikros® 750 kDa mPES) was used as a clarifier for the laboratory-scale reactors, allowing the uncoupling of the solids residence time (SRT) from the hydraulic residence time (HRT). The membrane was backwashed as described elsewhere (Peterson et al., 2017), to avoid excessive pressure drop across the membrane. In the 15 °C experiments the reactors were maintained in a cold room (10 °C) while continuously mixed on a heated stir plate. Dissolved oxygen (DO) was maintained at >5 mg/L in the reactors throughout the experiments using a diffuser continuously feeding air. Effluent samples were analyzed for ammonia, nitrate, nitrite and E1 concentrations twice weekly.

2.1.2. Flow-through exposure experiments

Four successive month-long flow-through exposure experiments were conducted in the Aquatic Toxicology Laboratory at St. Cloud State University (MN) between March and July 2015. Previously published flow-through exposure protocols (Hyndman et al., 2010; Schoenfuss et al., 2008; Shappell et al., 2010) were modified to accommodate a 4 × 4 factorial experimental design consisting of graded concentrations of E1: low (E1-L), medium (E1-M), and high (E1-H), at 12.5, 25, and 65 ng/L E1 respectively, including an ethanol control (0 ng/L E1; 0.0002% ethanol v/v) at four temperatures (15 °C, 18 °C, 21 °C, and 24 °C). Concentrations of E1, especially in the low and medium treatment, were chosen to be of immediate environmental relevance (Alvarez et al., 2013; Ankley et al., 2017; Chen et al., 2010; Matthiessen et al., 2006). Estrone (Sigma-Aldrich, St. Louis, MO) was dissolved in 100% ethanol to generate treatment-specific concentrated aliquots used to make fresh E1 stock solutions on every third day of the exposure period. Estrone concentrations in these concentrated aliquots were confirmed

analytically prior to the commencement of exposures. Treatment-specific concentrated aliquots were diluted in 10 L of water and pumped (Cole-Palmer Masterflex 7523-40 peristaltic pump) at a consistent rate through stainless steel tubing into a continuous flow of groundwater (100 mL/min/aquarium) to achieve the desired exposure concentrations. A mixing tank with two internal chambers was utilized to ensure complete mixing of ground water and E1 stock solution prior to the mixture being distributed equally to all aquaria within a treatment. The final treatment specific flow rate provided approximately 12 volume exchanges per 24 h to assure a near constant E1 concentration in the exposure system to avoid substantial temperature-specific differences in E1 degradation across fish exposure treatments.

Mature (6 months old) fathead minnows (*P. promelas*) were obtained from a dedicated laboratory fish supplier (Environmental Consulting and Testing, Superior, WI). Exposures were conducted all four concentrations of E1 for 30 days at one temperature at a time due to facility size restrictions. A total of 28 aquaria (volume: 12 L; dimensions: 30.5 × 30.5 × 30.5 cm) were divided in half by the addition of a stainless-steel mesh partition to accommodate two breeding groups, each consisting of one male and two females (56 breeding groups total; 10–14 aquaria per treatment). All fish were assessed for morphological and histopathological endpoints at the end of the 30-day exposure in accordance with approved St. Cloud State University IACUC protocols (# 8–73). Fish were maintained in accordance to US EPA guidelines (Denny, 1987), with the exception of water temperature which was adjusted to meet the experimental objectives. Fish were fed a diet of frozen blood worms (*Glycera* spp.) and brine shrimp (*Artemia* spp.) ad libitum twice daily (Brine Shrimp Direct, Ogden, UT) and maintained on a 16:8 h light/dark photoperiod.

2.2. Chemical analyses

2.2.1. Flow-through exposure water quality and chemistry

Water quality parameters were recorded daily and included temperature, pH, dissolved oxygen, conductivity, salinity, and oxidation reduction potential using a YSI (model 556 MPS; Yellow Springs, OH). Every third day total hardness, free chlorine, total chlorine, pH and alkalinity were screened for using AquaChek 5 in 1 test strips (Hach Company, Loveland, CO). The concentration of the E1 stock solution was verified using LC/MS/MS. Water sample collection occurred the day after an E1 chemical solution renewal; water samples were collected from the outflow of the mixing tanks in 1-L high density polyethylene bottles and stored at −20 °C until further analysis.

2.2.2. Water chemistry analysis

Methods for monitoring chemical oxygen demand (COD), pH, dissolved oxygen, ammonia, total nitrogen, nitrate, and nitrite in the laboratory-scale wastewater reactors are described in detail elsewhere (Peterson et al., 2017). Briefly, COD, ammonia, and total nitrogen were measured colorimetrically. Dissolved oxygen and pH were measured with a Vernier optical dissolved oxygen probe and a Vernier pH sensor. Nitrate and nitrite were measured in filtered samples (0.2-μm) by ion chromatography with detection limits of approximately 0.2 mg/L as nitrogen.

2.3. E1 analysis

Methods for sample extraction and E1 analysis are described in detail elsewhere (Peterson et al., 2017). Briefly, samples of 10–100 mL were extracted via solid phase extraction (except for concentrated treatment-specific aliquots in ethanol) after labeled surrogate (13,14,15,16,17,18-^{13}C6-estrone) amendment. Extracts were cleaned using a silica gel column. Extracts were blown down to dryness and resuspended in methanol:water (60:40, v/v) and an internal standard (2,4,16,16-D_4-estrone) was added before analysis. Analysis was performed on an Agilent 1100 series Liquid Chromatograph (LC) with a 4000 QTRAP triple quadrupole mass spectrometer as described elsewhere (Peterson et al., 2017). Recovery averaged 64 ± 17%. Typical limits of quantification were 11.5 ng/L E1 sample (Peterson et al., 2017).

2.4. Biological endpoints

2.4.1. Plasma VTG analysis

Heparinized capillary tubes were used for blood collection from the severed caudal vasculature of anesthetized minnows (0.01% neutral buffered MS-222 solution; Argent Laboratories, Redmond, WA). Male fish yielded on average 50–100 μL whole blood while female fish often yielded less than half as much. Whole blood was centrifuged at 3600 × *g* for 5 min at 4 °C, before plasma was separated and stored at −80 °C for subsequent analysis. Plasma volume varied substantially between fish (males: ~20–50 μL; females <5–30 μL) and at times prevented vitellogenin analysis especially in female fish. However, to maintain the integrity of the experimental design, plasma samples were not pooled. Quantification of plasma VTG was achieved through a competitive antibody-capture ELISA (Parks et al., 1999) requiring a small volume of blood plasma (5 μL) for analysis. A two-fold serial dilution was used to prepare an eight-point standard curve ranging from 4.8 μg/mL to 0.00375 μg/mL. Further elaboration of this method can be found in Parks et al. (1999) and Shappell et al. (2010).

2.4.2. Blood glucose and hematocrit

Blood glucose was measured using a digital glucose monitor (TRUEbalance Blood Glucose Monitor, Moore Medical, Farmington, CT), which utilizes a 1 μL sample of whole blood obtained from the caudal vasculature of each fish. To measure hematocrit (i.e., percent packed red blood cells), heparinized capillary tubes were used to collect whole blood. Hematocrit tubes were sealed with a clay plug and centrifuged at 3600 × *g* for 5 min (HERMLE Z200A, Labnet International Inc., Woodbridge, NJ) before hematocrit was determined using a Spiracrit Micro-Hematocrit Tube Reader (Clay Adams Inc., New York, NY).

2.4.3. Organosomatic indices

Fish were euthanized using a 0.01% neutral buffered MS-222 solution (Argent Laboratories, Redmond, WA) prior to dissection. Each fish was measured for total length (TL, in mm) and standard length (SL, in mm) using a metric ruler and for total body weight (in grams, using a digital scale precise to 0.01 g; Acculab Vicon, Edgewood, NY). Body condition factor (BCF), an overall measurement of fish health, was calculated using the formula (BCF = [body weight / total length^3] × 100,000; Fulton, 1904). Liver and reproductive organs (i.e., ovaries and testes) were collected and the mass of each organ was recorded using a digital scale precise to 0.001 g (Mettler Toledo AG245, Columbus, OH). A hepatosomatic index value (HSI; liver weight / whole body weight × 100) and a gonadosomatic index value (GSI; gonad weight/whole body weight × 100) were calculated for each fish, respectively.

2.4.4. Secondary sex characteristics

Secondary sexual characteristics (SSC) were measured using a scoring system based on prominence of characters using a scale from 1 (least prominent) to 3 (very prominent) to score the fatty dorsal pad, tubercles, and banded coloration intensity, following methods described in Smith (1978).

2.4.5. Histopathology

Excised livers and gonads were placed in histological cassettes, and fixed in 10% neutral buffered formalin. Dehydration protocols described in Carson (1997) were used to process tissue samples in a Leica ASP 300 Automated Tissue Processor. Paraffin embedded tissues were sectioned on a Reichert-Jung 2030 cassette microtome (5 μm sections). Tissues were stained with hematoxylin and eosin counter-stain procedures described in Carson (1997) using a Leica ST5010 Autostainer XL. Liver hepatocyte vacuolization and developmental stage were assessed using

bright-field microscopy. Liver vacuolization was based on the prominence of vacuoles in hepatocytes within the field of view, 1 (few vacuoles visible), 2, (small vacuoles visible throughout the tissue section), 3, (vacuoles prominent and widespread), and 4, vacuoles large and the dominating feature of the tissue sections) (Fig. S1). Reproductive maturity was based on the percentage of four main germ cell types during gametogenesis (i.e., spermatogenesis and oogenesis) within the field of view (Fig. S2). The presence of pathologies such as eosinic fluid or intersex were noted when observed during evaluation. The assessment of four to six histological sections was blinded with the observer unaware of the treatment of each observed section.

2.5. Data analysis

Males and females were analyzed separately using multivariate analysis of variance (MANOVA) with concentration, temperature, and concentration × temperature specified as fixed factors and SL, body weight, gonad weight, liver weight, hematocrit, blood glucose level, plasma VTG concentration, BCF, GSI, and HSI specified as dependent variables. Dependent variables were log transformed where needed to improve normality. The model for males also included an overall SSC score for each male, based on the degree of development of the tubercles and dorsal pad, and the intensity of banding color. Preliminary analysis revealed that these characters were highly correlated; therefore, each male was assigned a SSC score based on principal components analysis (PCA). The PCA extracted a single component that explained 68% of the variation. Where appropriate, pair-wise post hoc tests (Least Significant Difference; LSD) were used to compare dependent variables across levels for significant effects. All analyses were conducted using SPSS (v21), apart from the multiple comparisons analysis for the histological data, which utilized a two-way ANOVA using Prism 6.1A Graph-Pad Software, followed by Tukey's multiple comparisons post hoc test. Calibration curves for E1, ^{13}C-labeled E1, nitrite, nitrate and COD were generated from a simple linear regression of the samples in either Excel or R. Reported P-values were generated with R software utilizing a two sample, two-sided, un-pooled Student's *t*-test.

3. Results

3.1. E1 removal experiments

Results from the nitrification experiments performed at room temperature are shown in Fig. 1, with complete ammonia oxidation to nitrate occurring by Day 20 in the duplicate experiments (also see Peterson et al., 2017). Delayed onset of nitrification was observed in the 15 °C experiment, as expected, with complete ammonia oxidation to nitrate occurring by Days 36 and 40 in the duplicate experiments (Fig. 1). E1 removal was excellent in all experiments, with effluent E1 concentrations of 257 ± 112 ng/L (0.26 ± 0.11 μg/L) after Day 3 in the room temperature experiments and 58 ± 38 ng/L (0.06 ± 0.04 μg/L) after Day 10 in the 15 °C experiments. These concentrations are slightly higher, but within the same order of magnitude as the concentrations used in the minnow exposure experiments. The onset of E1 degradation was delayed approximately 3 days, however, though only slightly compared to the onset of nitrification, which was delayed approximately 25–30 days in the 15 °C experiments (Fig. 1).

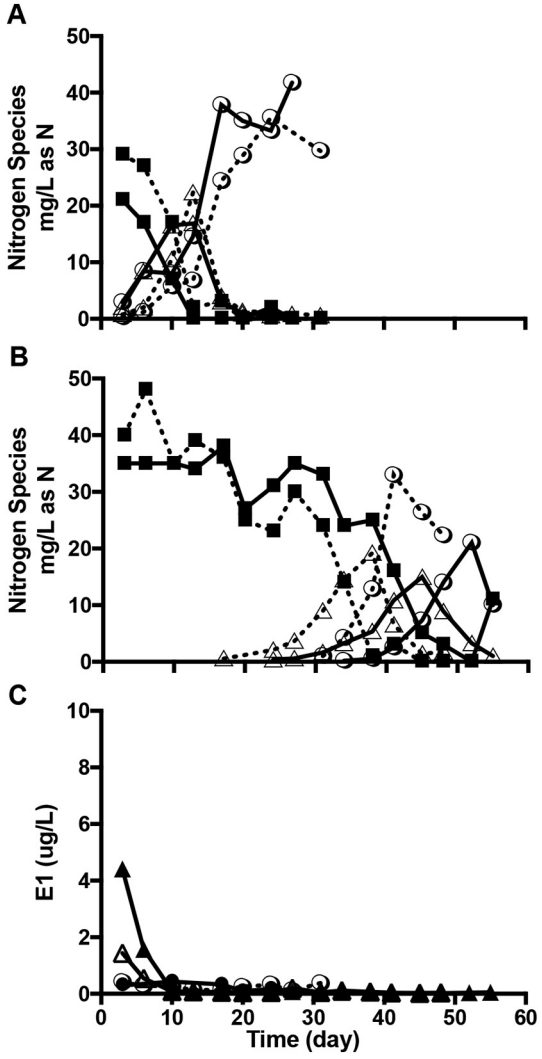


Fig. 1. Comparison of ammonium, nitrite, nitrate, and E1 concentrations over time in the laboratory-scale wastewater reactors. Panel A shows reactor performance in terms of nitrogen speciation at room temperature, Panel B shows reactor performance at 15 °C. Here dotted lines and solid lines show data from replicate experiments. In Panels A and B, closed squares (■) are ammonium, open circles (○) are nitrate, and open triangles (△) are nitrite. Panel C shows E1 transformation in the two reactors, with the open (△) and closed triangles (▲) representing E1 concentration in the duplicate 15 °C reactors and the open (○) and closed circles (●) representing E1 concentration in the duplicate room temperature reactors.

3.2. Flow-through exposure experiments water quality and E1 concentrations

Environmental conditions were stable throughout the duration of all four temperatures (Table 1). Verification of concentrated treatment-specific E1 aliquots in ethanol confirmed concentrations near the target values for the concentrated stock solutions (E1-L: nominal E1 concentrated aliquot concentration 67.5 μg/mL, measured 62.9 μg/mL; E1-M: 337.5 μg/mL, measured 290 μg/mL; E1-H: 1687.5 μg/mL, measured 1435 μg/mL). Collapsing over all concentrations, environmental conditions are as follows: 15 °C experiment: pH = 7.9 ± 0.3; conductivity = 0.90 ± 0.04; salinity = 0.45 ± 0.02; ORP = 225.3 ± 64.5; 18 °C experiment: pH = 7.7 ± 0.2; conductivity = 0.90 ± 0.07; salinity = 0.45 ± 0.01; ORP = 266.9 ± 22.5; 21 °C experiment: pH = 8.14 ± 0.2; conductivity = 0.63 ± 0.42; salinity = 0.31 ± 0.21; ORP = 167.5 ± 38.0; and 24 °C experiment: pH = 7.72 ± 0.3; conductivity = 0.92 ± 0.07; salinity = 0.46 ± 0.01; ORP = 125.3 ± 0.28. E1 measured concentrations were 14.26 ± 3.1, 25.43 ± 9.3 and 84.83 ± 40.4 ng/L for the E1-L (n = 7), E1-M (n = 8) and E1-H (n = 11) treatments respectively (Table 1).

3.2.1. Fish survival

In total, 642 fish (428 females and 214 males) were analyzed for the experiment. Fish survival across all thermal regimes was excellent (range: 92–99%) and similar when compared across all 16 treatments using a chi-square test of independence (χ^2 = 0.99, df = 14, P = 0.95). Collapsing across all concentrations, percent survival was observed to be the lowest in the 24 °C temperature exposure at 92%.

Table 1
Descriptive water chemistry of measured E1 concentrations collapsed across all temperatures and the environmental conditions, temperature and dissolved oxygen, in four subsequent 30-day exposure experiments with mature fathead minnows (mean ± SD shown for all measured parameters with number of samples in parenthesis).

Treatment (abbreviation)	Measured E1 concentration (ng/L)	Nominal temperature (°C)	Measured temperature (°C)	Measured dissolved oxygen (mg/L)
Ethanol control (EtOH)	No E1 detected	15	16.8 ± 0.572 (31)	9.73 ± 1.35 (31)
		18	18.9 ± 0.722 (33)	7.97 ± 1.53 (33)
		21	21.2 ± 0.561 (20)	7.19 ± 0.02 (2)
		24	24.5 ± 0.524 (31)	6.95 ± 0.24 (31)
E1 low (E1-L)	14.26 ± 3.01 (7)	15	17.2 ± 0.761 (31)	9.19 ± 1.29 (31)
		18	18.5 ± 0.432 (34)	8.77 ± 1.14 (34)
		21	21.2 ± 0.492 (20)	6.99 ± 0.00 (1)
		24	24.2 ± 0.590 (31)	7.71 ± 0.21 (31)
E1 medium (E1-M)	25.43 ± 9.31 (8)	15	16.8 ± 0.745 (31)	9.62 ± 0.95 (31)
		18	18.7 ± 0.532 (34)	7.97 ± 1.31 (34)
		21	21.0 ± 0.561 (20)	6.29 ± 0.00 (1)
		24	24.3 ± 0.591 (31)	7.71 ± 0.21 (31)
E1 high (E1-H)	84.83 ± 40.54 (11)	15	16.5 ± 0.459 (30)	10.12 ± 1.23 (30)
		18	18.3 ± 0.318 (34)	8.78 ± 1.24 (34)
		21	21.1 ± 0.594 (20)	6.66 ± 0.00 (1)
		24	24.3 ± 0.597 (31)	7.71 ± 0.28 (31)

Collapsing across all temperatures, the E1-M concentration had the lowest percent survival at 95%.

3.2.2. Physiological and anatomical endpoints for males and females

For both male and female subjects, the MANOVA models revealed significant overall effects of E1 exposure, temperature, and concentration × temperature on organismal endpoints (Table 2).

3.2.2.1. Effects of E1 exposure on male organismal endpoints. There were significant effects of E1 exposure on plasma VTG concentrations in male fathead minnows ($F_{3,169} = 15.85$; $P < 0.001$; Fig. 2A) with lower concentration in control males (E1-L, E1-M, and E1-H; all Ps < 0.001). Significant effects of E1 concentration on blood glucose ($F_{3,169} = 5.10$; $P = 0.002$; Fig. 2B) and hematocrit ($F_{3,169} = 4.88$; $P = 0.003$) were also observed. Blood glucose levels were higher in males exposed to E1-L than in males exposed to either E1-M or E1-H ($P < 0.001$). Hematocrit was significantly lower in E1-H exposed males compared with control ($P = 0.037$) and E1-L ($P < 0.001$) exposed males, and lower in males exposed to E1-M than E1-L ($P = 0.013$).

3.2.2.2. Effects of temperature on male organismal endpoints. Temperature had a significant effect on plasma VTG concentration in males ($F_{3,169} = 5.17$, $P = 0.002$; Fig. 2A) with VTG concentrations higher at 15 °C than at 18 °C ($P = 0.018$) or 21 °C ($P < 0.001$; Fig. 2B). Temperature also had a significant effect on blood glucose levels ($F_{3,169} = 5.10$, $P = 0.002$), subjects maintained at 24 °C had significantly lower blood glucose levels compared to subjects maintained at cooler temperatures (vs 15 °C: $P = 0.046$; 18 °C: $P = 0.017$; 21 °C: $P = 0.001$).

Temperature also had significant independent effects on morphological indices with greater values at lower temperatures. Significant effects of temperature were observed for male gonad weight ($F_{3,170} = 5.12$, $P = 0.002$) and liver weight ($F_{3,169} = 13.96$, $P < 0.001$). Gonad weight at 15 °C was significantly greater than at 21 °C ($P = 0.001$) or 24 °C ($P = 0.008$). Liver weights were higher at the two lowest temperatures (15 °C and 18 °C) than they were at either of the two higher temperatures (21 °C and 24 °C) (all Ps < 0.003). Significant effects of temperature on BCF ($P = 0.002$) and GSI ($P < 0.001$) were also observed. Post hoc analysis showed that the body condition of males maintained at the highest temperature (24 °C) was significantly reduced compared to that of males maintained at lower temperatures (15 °C: $P = 0.011$; 18 °C: $P = 0.027$; 21 °C: $P = 0.009$). The average GSI was greater at 15 °C than at any of the higher temperatures ($0.032 \le Ps < 0.001$). There was a significant effect of temperature on the degree of development of male SSC ($F_{3,169} = 4.59$, $P = 0.004$). Post hoc tests indicated that male SSC scores were higher at 24 °C than at 15 °C ($P = 0.005$). An extremely significant effect of temperature was observed for male vacuolization severity for liver histology ($F_{3,250} = 10.02$, $P < 0.0001$). Males maintained at cooler temperatures (15 °C and 18 °C) displayed higher levels of liver vacuolization severity than males maintained at 21 °C and 24 °C.

3.2.2.3. Interactions between E1 exposure and temperature on male organismal endpoints. A significant interaction between water temperature and E1 exposure concentration was observed for plasma VTG concentration ($F_{9,170} = 2.99$, $P = 0.002$); in general, the differences in plasma VTG concentration between control and exposed males was more pronounced at cooler temperatures. Post hoc analysis showed that plasma VTG levels were significantly reduced in control males compared to exposed males at temperatures 15 °C, 18 °C, and 21 °C ($0.034 \le Ps < 0.001$; Fig. 2A). A significant temperature × concentration interaction was also observed for blood glucose ($F_{9,169} = 3.74$, $P < 0.001$; Fig. 2B). Post hoc tests revealed that control and E1-L males maintained at 18 °C had significantly elevated blood glucose compared to E1-M and E1-H exposed males, in addition to males at 24 °C (control vs E1-L; Fig. 2B).

3.2.2.4. Effects of E1 exposure on female organismal endpoints. Female endpoints were not nearly as impacted by exposure as those of males; however, a statistically significant effect of E1 exposure on hematocrit ($F_{3,215} = 2.95$, $P = 0.034$) was observed, and post hoc tests revealed

Table 2
Summary of significant results for the independent and interactive biological effects of temperature and E1 exposure on exposed male and female fathead minnows relative to the ethanol carrier control. The direction of arrows indicates worsening effects as temperature or concentration, respectively, increases. For example, the induction of plasma vtg in male fathead minnows is greater at colder temperatures (second column) and at higher concentrations (3rd column).

	MALES			FEMALES		
	Temp.	Conc.	Temp × Conc	Temp.	Conc.	Temp × Conc.
Physiological endpoint						
Plasma vtg	↓	↑	↓			
Blood glucose	↓	↓	↓			↓
Hematocrit		↓			↓	
Morphological endpoint						
TL						
SL				↓		↓
Total weight				↓	↓	↓
Liver weight	↓			↓	↓	↓
Gonad weight	↓			↓		
BCF	↓			↓	↓	
HSI				↓		↓
GSI	↓			↓		
Vacuolization severity	↓			↓		
Mature sex cells		↓		↓		
SSC	↑			–		–

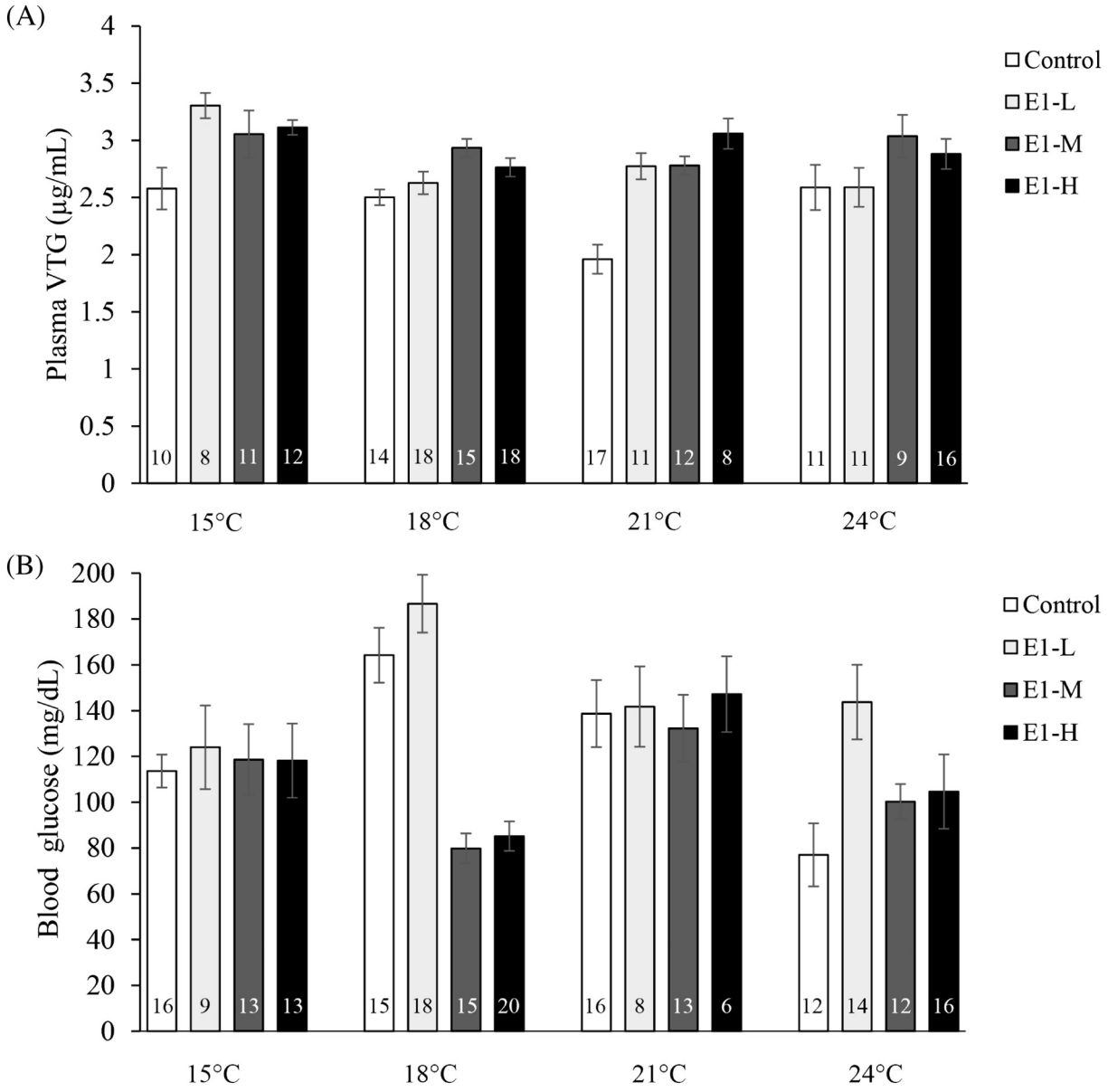


Fig. 2. Significant interactions of E1 concentration and temperature effects on male physiological endpoints (A) log VTG concentrations (µg/mL); and (B) blood glucose levels (mg/dL). White bars represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black bars represent the E1-L, E1-M, and E1-H exposure treatments; bars and error bars depict means ± SEM.

the hematocrit of control females was greater than E1-H exposed females ($P = 0.015$). In contrast to the results for males, however, female morphology was affected by E1 exposure (Fig. 4). In particular, significant effects of E1 concentration were observed on female fish total weight ($F_{3,215} = 4.64$, $P = 0.004$) and liver weight ($F_{3,215} = 5.71$, $P = 0.001$). Post hoc tests indicated that the weights of females exposed to E1-M had a significantly reduced total weight compared to females exposed to E1-L ($P = 0.024$). In addition, the liver weights of fish exposed to E1-M were less than the liver weights of control fish ($P = 0.030$) and fish exposed to E1-L ($P = 0.007$). Significant effects of E1 concentration were observed for BCF ($F_{3,215} = 3.66$, $P = 0.013$) and HSI ($F_{3,215} = 3.42$, $P = 0.018$). The BCF of subjects exposed to E1-M was reduced compared with that of fish exposed to E1-L ($P = 0.01$).

3.2.2.5. Effects of temperature on female organismal endpoints. A significant effect of temperature was observed in female fish for SL ($F_{3,215} = 15.24$, $P < 0.001$; Fig. 4A), total weight ($F_{3,215} = 28.28$, $P < 0.001$; Fig. 4B), gonad weight ($F_{3,215} = 6.99$, $P < 0.001$), liver weight ($F_{3,215} = 25.64$, $P < 0.001$; Fig. 4C), BCF ($F_{3,215} = 6.88$, $P < 0.001$), GSI ($F_{3,215} = 9.09$, $P < 0.001$), and HSI ($F_{3,215} = 12.95$, $P < 0.001$; Fig. 4D). Female fish maintained at 15 °C and 18 °C had bigger gonads (by weight) compared with those maintained at 21 °C ($P = 0.03$ and $P = 0.005$, respectively), and at 18 °C compared to 24 °C ($P = 0.008$). Females maintained at the coldest temperature (15 °C) had a greater BCF and a higher GSI than those maintained at the highest temperature (24 °C; all Ps < 0.001). Females maintained in cooler water also had greater HSI values; HSI was higher at 15 °C and 18 °C compared to at 21 °C (Ps < 0.001) and greater at 18 °C than 24 °C ($P = 0.023$). A significant effect of temperature was evident for liver vacuolization severity in females ($F_{3,277} = 8.28$, $P < 0.0001$) with vacuolization most severe in females maintained at 15 °C compared to females maintained at 24 °C ($P = 0.025$). Female sexual maturity was decreased at higher temperatures (24 °C) compared to lower temperatures (15 °C). Significant independent effects of temperature were evident throughout all the anatomical endpoints tested, with TL being the only exception to this pattern.

3.2.2.6. Interactions between E1 exposure and temperature on female organismal endpoints. A significant temperature × concentration interaction was detected for female blood glucose ($F_{9,215} = 2.43$, $P = 0.012$; Table 2). At 18 °C, subjects exposed to E1-L had higher blood glucose levels than those exposed to E1-M ($P < 0.001$) or E1-H ($P < 0.001$) (Fig. 3).

Interactive effects of exposure concentration and temperature were observed for female morphological indices such as SL ($F_{9,215} = 3.07$, $P = 0.002$; Fig. 4A), total weight ($F_{9,215} = 2.35$, $P = 0.015$; Fig. 4B), liver weight ($F_{9,215} = 3.23$, $P = 0.001$; Fig. 4C), and HSI ($F_{9,215} = 2.78$, $P = 0.004$; Fig. 4D) (Table 2). Post hoc tests indicated that at 15 °C females exposed to E1-L were shorter than those in the E1-M ($P < 0.001$) or E1-H ($P = 0.03$) treatments (Fig. 4A). At 21 °C and 24 °C fish exposed to varying concentrations of E1 were similar in size. Differences among the exposure groups in body weight were more pronounced at cooler temperatures. At 15 °C, females exposed to E1-L were heavier (by weight) than fish in the control ($P = 0.015$), E1-M ($P < 0.001$), or E1-H ($P = 0.014$) groups. At 18 °C, control fish also weighed more than fish exposed to E1-M ($P < 0.001$) or E1-H ($P = 0.023$) (Fig. 4B). Liver weight was negatively and approximately linearly correlated to exposure concentration at 18 °C; control females had livers that were significantly heavier than females exposed to E1-M ($P < 0.001$) and E1-H ($P < 0.005$), females exposed to E1-L had heavier livers than those exposed to E1-M ($P = 0.001$), and females exposed to E1-M had heavier livers than those exposed to E1-H ($P = 0.025$) (Fig. 4C). Similarly, control and E1-L exposed fish at 18 °C also had higher HSI values than fish exposed to E1-M or E1-H ($0.037 < P < 0.001$) (Fig. 4D). Plasma VTG concentrations for females exposed to alternative concentrations of E1 were not dependent on temperature, suggesting that temperature does not modulate plasma VTG synthesis in exposed females, when compared to unexposed females. Collectively, the results for females suggest that temperature interacts with E1 exposure more intimately concerning morphological endpoints rather than physiological endpoints.

4. Discussion

This study examined the extent to which ambient water temperature modulates the physiological and reproductive effects of E1 exposure on adult fathead minnows. In addition, we investigated how cooler water temperature affects E1 degradation rates during wastewater treatment. The results revealed three key findings. First, E1 exposure concentration and water temperature exerted significant independent effects on fish physiology and morphology in both sexes. Cumulatively, the data suggest that temperature is the main driving force affecting morphological indices, whereas E1 exposure concentration primarily affects physiological endpoints. Second, we found evidence supporting the hypothesis that water temperature modulates the effects of estrogen exposure on both males and females, with the effects of exposure more pronounced at cooler water temperatures. Third, extensive E1 degradation was found to occur both with and without the support of efficient nitrification at all temperatures corroborating results from prior studies.

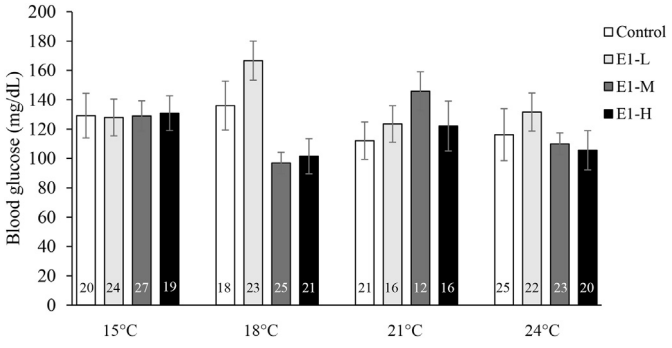
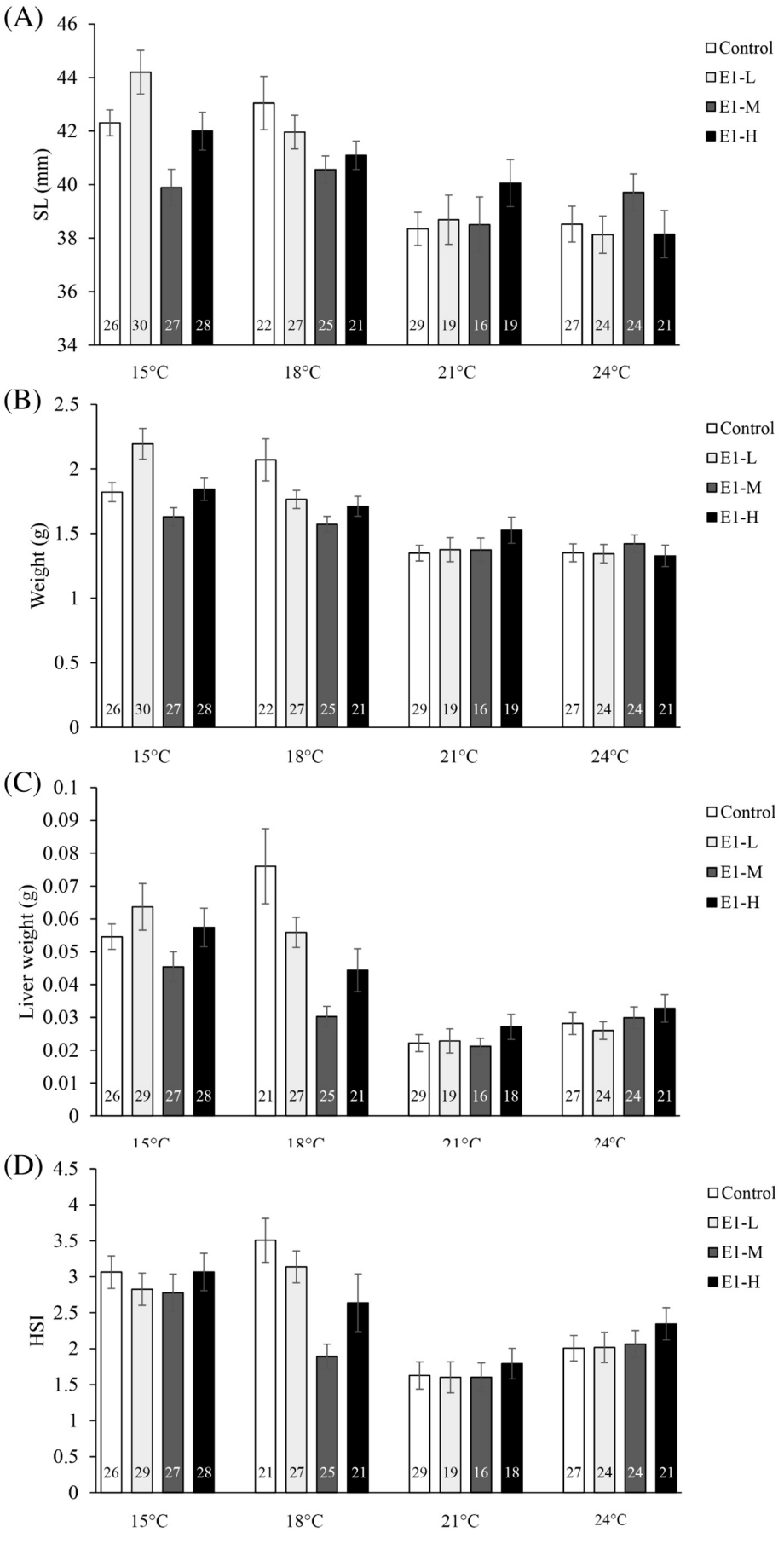


Fig. 3. Significant interaction of E1 concentration and temperature effect on female blood glucose levels (mg/dL). White bars represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black bars represent the E1-L, E1-M, and E1-H exposure treatments; bars and error bars depict means ± SEM.

Temperature increases the metabolic rate of ectotherms (Johnston and Dunn, 1987), subsequently increasing the rate of enzymatic activity (Voet et al., 2013). Enzymes are required for hepatic synthesis of the VTG protein (Hadley and Levine, 2006). In males, the presence of VTG in the blood, and related transcription in the liver, are considered to be specific biomarkers for estrogen-mimetic contaminants in the environment (Hoyt et al., 2003). Research on salmonid fishes injected with a natural steroid estrogen has suggested that estrogenic potency increases with temperature (Korsgaard et al., 1986). However, the results of this study demonstrated an opposite phenomenon. Unexposed males did exhibit lower plasma VTG concentrations than exposed males (Fig. 2A) but male fish maintained at 15 °C had higher concentrations of plasma VTG induction than males maintained at 18 °C or 21 °C. Further, VTG levels were significantly elevated in exposed males compared with control males only at the cooler water temperatures. The high volume turnover rates in the flow-through exposure system make it unlikely that high temperatures dramatically reduced E1 bioavailability (Raman et al., 2001) to exposed fathead minnows. Instead, males experiencing higher water temperatures may forgo vitellogenesis in order to maintain homeostasis at a higher metabolic demand (Luquet and Watanabe, 1986). This hypothesis is supported by the reduced blood glucose concentrations in E1 exposed male fish at higher temperatures (Fig. 2B) suggesting limited energetic reserves needed for VTG biosynthesis.

By contrast, we did not detect significant independent effects of exposure or interactive effects of exposure and temperature on plasma VTG induction in females (Fig. 3). Watanabe et al. (2007) pooled six large exposure studies and systematically evaluated several experimental endpoints (i.e. E2 concentrations, plasma VTG concentrations) to determine baseline natural variability in fathead minnows. They determined that the mean concentration of E2 in unexposed females was 6.07 ± 4.0 ng/mL, whereas the mean concentration of E2 in unexposed males was 0.43 ± 0.30 ng/mL. Because females naturally produce estrogens, the concentrations used for this experiment may have been insufficient to elicit a biological exposure response.

Hematological endpoints can shed insight on fish tolerance to a stressor, such as thermal stress, and the overall physiological state of the organism (Del Río Zaragoza et al., 2008). Temperature increases induce hepatic glycogenesis and glucose concentration in the blood (Radhakrishnaiah and Parvatheswararao, 1984). Temperature-induced insulin fluctuations may be correlated to changes in insulin receptor internalization and turnover rate in hepatic tissue (Larsen et al., 2001); in addition, insulin receptor binding affinity is thought to be positively correlated with increased temperatures (Freychet et al., 1971). Ekman et al. (2008) examined the biochemical effects of 17α-ethynylestradiol exposure on male fathead minnow metabolites and found that glycogen, the stored form of glucose, was decreased in both exposure groups. The results of that study suggested that glycogen-derived glucose is an important component of meeting the energetic demands of estrogen exposed individuals, but may become depleted under intense physical demands. A similar phenomenon was observed in the current study in which unexposed males and females, or males and females exposed to E1-L, displayed higher blood glucose levels when maintained at lower temperatures than E1-M or E1-H exposed individuals, with few exceptions. This may indicate that individuals displaying higher levels of glucose can combat physiological stress, maintaining metabolic homeostasis more readily than their heavily exposed counterparts. However, the ability to buffer the effects of metabolic stress induces by VTG synthesis in male fish may not be limitless. The increased blood glucose concentrations in E1-L exposed males at lower temperatures co-occurred with a significant increase in hepatocyte vacuolization suggesting an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E1-H
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Weight (g)
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27 24 24 21
15°C
18°C
21°C
24°C
Control
E1-L
E1-M
E1-H
(C)
Liver weight (g)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
26 29 27 28
21 27 25 21
29 19 16 18
27 24 24 21
Control
E1-L
E1-M
E1-H
(D)
HSI
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
26 29 27 28
21 27 25 21
29 19 16 18
27 24 24 21
15°C
18°C
21°C
24°C
Control
E1-L
E1-M
E1-H

intense recruitment of glycogen reserve from liver hepatocytes that may not be sustainable during prolonged exposures. This effect may be more apparent in the current study at low temperature as basic metabolic needs increase in a temperature-dependent manner in ectotherms. Thus, fish maintained at the higher temperatures may have depleted their glycogen storage prior to the termination of the exposure experiments.

The anatomical endpoints in this study were substantially influenced by temperature in both males and females. In females, all but one anatomical endpoint (TL) yielded a statistically significant effect of temperature. In addition, the effects of exposure on SL, total weight, liver weight, and HSI were significantly affected by temperature (Fig. 4). Male anatomy was also influenced by temperature to a lesser degree, but not by exposure. Overall, unexposed and E1-L exposed females maintained at lower temperatures were longer, heavier, and had bigger livers than E1-M or E1-H exposed females. Metabolic response to temperature changes is vital to organismal homeostasis under seasonal temperature fluctuations (Bruneaux et al., 2014). Fish maintained at lower temperatures experience a reduced metabolism, which may lead to a decreasing capacity for detoxifying processes, resulting in the internalization of toxins for longer periods of time. Exposure to toxins can result in the accumulation of glycogen or fatty vacuoles in fish livers, hypothesized to occur due to decreased breakdown of macromolecules stemming from the physiological stress of hepatocellular toxicity (Wolf and Wolfe, 2005). In this experiment, male and female liver histology both displayed significantly greater vacuole severity at cooler temperatures (15 °C and 18 °C) compared to warmer temperatures (21 °C and 24 °C), providing support for the hypothesis that cooler water temperatures decrease metabolism, leading to reduced or impaired detoxifying pathways.

These findings are particularly important to fish populations in northern effluent dominated aquatic ecosystems as fish are more likely to reproduce at cooler temperatures than in more southern latitudes (Shappell et al., 2018). E1-L and E1-M exposure concentrations in the current study represent “worst case scenarios” for environmental E1 concentrations as found in some effluent dominated systems (Schultz et al., 2013) but are lower than reported total estrogenicity in other effluent dominated systems (Alvarez et al., 2013; Kolpin et al., 2002; Schultz et al., 2013). Fish populations in northern latitudes are also frequently drawn to effluent outfalls as temperature refugia during cold winter months when ambient water temperature may approach freezing. Offspring produced during cooler spring water temperatures may, therefore, experience greater exposure consequences than summer broods as has been determined in a separate but related study (Ward et al., 2017). Therefore, the current study highlights differences in the effects of E1 exposure based on regional climatic conditions.

As expected (Suarez et al., 2010; Wild et al., 1971), the onset of nitrification was delayed in the reactors operated at 15 °C when compared to those operated at room temperature (Fig. 1). While there was also an apparent delay in E1 degradation (Fig. 1), comparable and low (<0.4 μg/L) effluent E1 concentrations were observed in the reactors operated at 15 °C and room temperature within the first week of reactor operation. This demonstrated that efficient nitrification was not required for E1 removal, only the conditions that could support nitrification (long SRT, low bulk organic carbon concentrations, aeration). Although lower temperatures (<15 °C) could result in a further decline, or even eventual cessation, of E1 degradation, the maintenance of conditions that support nitrification, even in the absence of robust nitrification itself should also support biological E1 degradation. Therefore, given that minnows do not generally reproduce at temperatures <15 °C, the absence of robust nitrification should still protect those species during sensitive life stages (i.e. reproduction).

4.1. Conclusion

Collectively, the results of this experiment demonstrate that fluctuations in temperature over a natural spring-summer range of variation modulate the effects of estrogenic exposure on adult fathead minnow physiology, morphology, and affect E1 degradation. The significant interactions between temperature and E1 exposure observed in this experiment suggest that female fish may be more susceptible to the temperature-modulating effects of estrogenic exposure on morphometric endpoints, but males may be more susceptible to the interactive effects of temperature and E1 exposure on physiological endpoints. The results also indicate that E1 removal under treatment conditions that support nitrification is robust, even at cooler temperatures, though a delay in E1 degradation was observed. Collectively, the data demonstrate that natural seasonal fluctuations in temperature are sufficient to induce significant physiological and anatomical changes in fish upon exposure, and highlight that the responses of males and females can vary markedly under identical exposure regimes. The effects observed in the current study warrant further investigations into the reproductive effects of chemical and non-chemical stressor interactions in laboratory and resident fish populations.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.10.069.

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Fig. 4. Significant interaction of E1 concentration and temperature effect on female morphological endpoints (A) standard length - SL (mm); (B) total weight (g); (C) liver weight (g); and (D) hepatosomatic index - HSI. White bars represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black bars represent the E1-L, E1-M, and E1-H exposure treatments, respectively; bars and error bars depict means ± SEM.

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Add-my-pet procedure for fathead minnow *Pimephales promelas* and walleye *Sander vitreus*

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1 Introduction

The present document presents a full life cycle model at the individual scale for the growth, reproduction and maintenance in *Pimephales promelas* and *Sander vitreus* based on the Dynamic Energy Budget (DEB) theory [Kooijman, 2010]. Both models are DEB model with type M acceleration, which means that during part of the life cycle metabolism accelerates. This model is a one-parameter extension of standard DEB model. This document is organized as followed. Firstly, we briefly describe the standard DEB model and the DEB model with type M acceleration. Then, we briefly introduce the parameter estimation method that was used in this work. Finally, we present the data used in this parameter estimation and provide a comparison of the models outputs and data used.

2 Methods

2.1 DEB model

The standard DEB model describes the entire life cycle of an organism through three life stages. We here introduce state variables and fluxes (table 2.1) from a life cycle point of view. In DEB theory, life cycle is described in 3 stages and the transition between one stage to the other depends on a state variable, called maturity E_H.

During the first life stage, named the embryonic stage, the organism does not feed, so the flux for assimilation $\dot{p}_A$ is null. The organism uses the available energy in reserve compartment (E), with a fixed allocation rate (κ), to grow in structure (V). So a proportion κ of the mobilization flux $\dot{p}_C$ goes to the structure (V) for its maintenance $\dot{p}_S$ and its growth which thus equals $\kappa\dot{p}_C - \dot{p}_S$. What remains of the mobilization flux (*i.e.* $(1-\kappa)\dot{p}_C$) is allocated to maturity E_H for maintenance ($\dot{p}_J$) and maturity increase. So the increase in maturity is $(1-\kappa)\dot{p}_C - \dot{p}_J$. The maintenance of the structure ($\dot{p}_S$) depends on temperature trough the Arrhenius relationship ($c(T)$), and also on its surface and/or volume. The maintenance of maturity depends on temperature, with the same Arrhenius relationship ($c(T)$), and on the amount of maturity.

The second stage is the juvenile stage. It starts when the organism is able to feed on the environment, *i.e.* when maturity has reached a fixed threshold (E_H^b). Therefore,

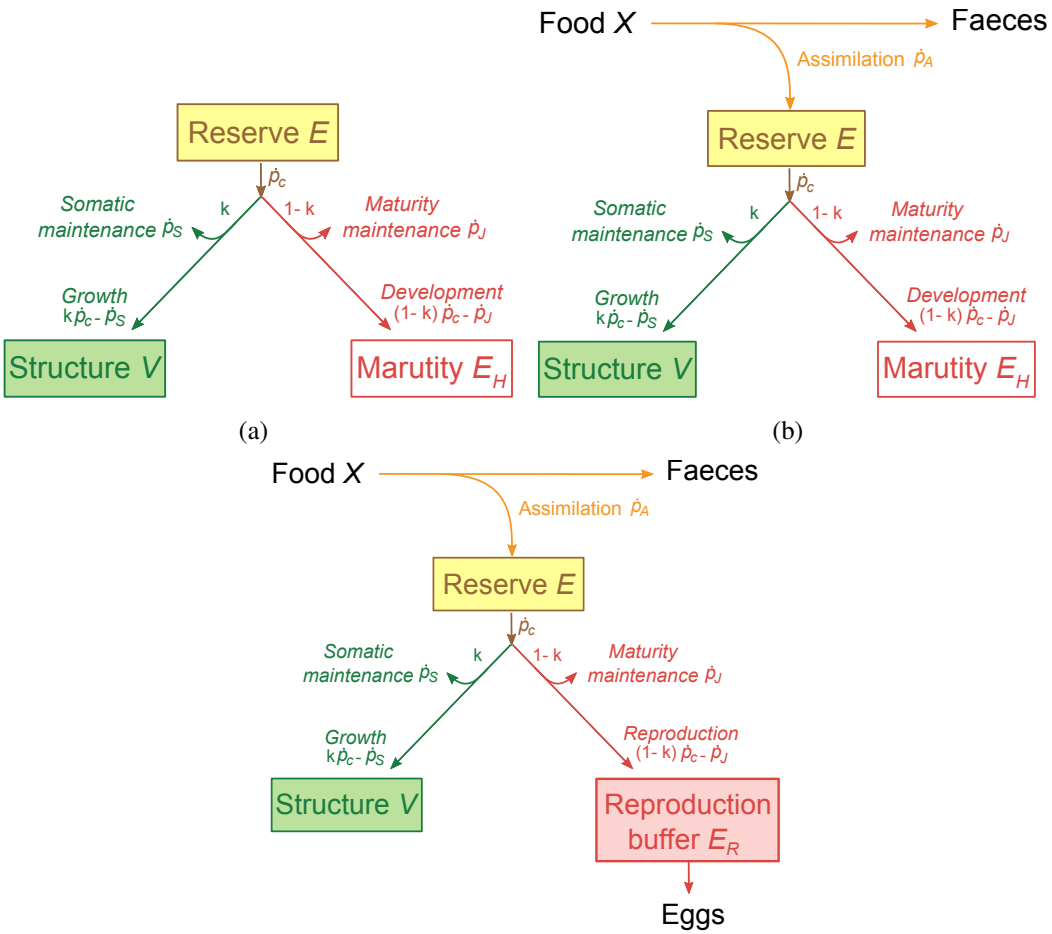

Figure 1: **Schematic representation of the three life stages of the standard DEB model.** (a) An embryo uses reserve to grow and develop. (b) At birth, a juvenile starts feeding, and (c) at puberty, an adult starts allocating energy to reproduction.

the assimilation flux $\dot{p}_A$ is no longer null. The assimilation depends on temperature, environmental food condition ($f(X)$) and the structural surface of the organism. The assimilated energy supplies the reserve compartment from which energy is allocated to growth or maturation, still with the same κ allocation rule.
When maturity has reached a fixed amount of energy (E_H^p), the organism enters the adult stage. From this moment, named puberty, energy that was previously allocated to maturity is now allocated to reproduction. Nevertheless, the maturity maintenance does not cease, and the allocation rate to growth or maturity/reproduction is still the same.

The metabolic acceleration occurs between birth and a moment defined as the metamorphosis (when maturity reaches a threshold value (E_H^j), before puberty) which might or might not correspond with changes in morphology.
For more details on DEB theory, see Kooijman [2010].

Table 1: Equations of the standard DEB model for ectotherm.

Differential equations
$\frac{\mathrm{d}}{\mathrm{d}t}E = \dot{p}_A - \dot{p}_C$
$\frac{\mathrm{d}}{\mathrm{d}t}V = \frac{1}{[E_G]}\dot{p}_G = \frac{1}{[E_G]}(\kappa\dot{p}_C - \dot{p}_S)$
$\frac{\mathrm{d}}{\mathrm{d}t}E_H = (1-\kappa)\dot{p}_C - \dot{p}_J$ if $E_H < E_H^p$, else $\frac{\mathrm{d}}{\mathrm{d}t}E_H = 0$
$\frac{\mathrm{d}}{\mathrm{d}t}E_R = 0$ if $E_H < E_H^p$, else $\frac{\mathrm{d}}{\mathrm{d}t}E_R = (1-\kappa)\dot{p}_C - \dot{p}_J$
Fluxes equations
$\dot{p}_A = c(T)f(X)\{\dot{p}_{Am}\}L^2$ if $E_H \geq E_H^b$, else $\dot{p}_A = 0$
$\dot{p}_C = c(T)\{\dot{p}_{Am}\}L^2\frac{ge}{g+e}\left(1 + \frac{L}{gL_m}\right)$; with $e = \frac{E}{E_m} = \frac{E}{V}\frac{\dot{v}}{\{\dot{p}_{Am}\}}$ and $L = V^{\frac{1}{3}}$
$\dot{p}_S = c(T)[\dot{p}_M]L^3$
$\dot{p}_J = c(T)\dot{k}_J E_H$
Scaled food and temperature functions
$f(X) = \frac{X}{X+K}$
$c(T) = exp\left(\frac{T_A}{T_{ref}} - \frac{T_A}{T}\right)$

2.2 Parameter estimation

The parameters of the standard DEB model were estimated using the co-variation method [Lika et al., 2011]. This method uses the simplex method to simultaneously minimize the weighted sum of squared deviations between model predictions and observations for a considerable number of data sets. Two types of data are used: the uni-variate data and the zero-variate data. Uni-variate data consist of sets of time-series observations of an organism, like growth versus time. The zero-variate data are composed of pseudo-data and real-data. Pseudo data are composed of parameter values which are supposed to be highly conserved among all the taxa, so they serve as a kind of prior knowledge on the organism. Real data are observations such as maximum length and weight at birth and/or puberty and/or death, lifetime and number of egg produced. A weight coefficient can be assigned to each uni-variate data set, and both kinds of zero-variate data set. In the present study we chose to assign the same weight to every data set.

Table 2: Parameters, state variables and forcing variables of the standard DEB model.

Symbol	Value	Units	Definition
		State and forcing variables	
E		J	Reserve density
V		cm^3	Structural volume
E_H		J	Cumulated energy invested into development
E_R		J	Reproduction buffer energy
X		$J \cdot l^{-1}$	Food density
T		K	Temperature
$f(X)$			Scaled functional response
$c(T)$			Temperature correction factor
		Primary parameters for *Pimephales promelas*	
$[\dot{p}_M]$	85.598	$J \cdot cm^{-3} \cdot d^{-1}$	Volume-specific somatic maintenance rate
$\{\dot{p}_T\}$	0	$J \cdot cm^{-2} \cdot d^{-1}$	Surface-area-specific somatic maintenance rate
$[E_G]$	5220.5	$J \cdot cm^{-3}$	Volume-specific cost for structure
$\dot{v}$	0.0201	$cm \cdot d^{-1}$	Energy conductance
κ	0.9105		Fraction reserve used for growth + maintenance
$\dot{k}_J$	0.0020	d^{-1}	Maturity maintenance rate coefficient
E_H^h	0.0768	J	Maturity threshold at hatching
E_H^b	0.1391	J	Maturity threshold at birth
E_H^j	9.3675	J	Maturity threshold at metamorphosis
E_H^p	546.82	J	Maturity threshold at puberty
κ_R	0.95		Fraction of the reproduction buffer fixed into eggs
		Auxiliary and compound parameters for *Pimephales promelas*	
T_A	12000	K	Arrhenius temperature
T_{ref}	293.15	K	Temperature
δ_M	0.1609		shape coefficient
		Primary parameters for *Sander vitreus*	
$[\dot{p}_M]$	108.990	$J \cdot cm^{-3} \cdot d^{-1}$	Volume-specific somatic maintenance rate
$\{\dot{p}_T\}$	0	$J \cdot cm^{-2} \cdot d^{-1}$	Surface-area-specific somatic maintenance rate
$[E_G]$	5224.5	$J \cdot cm^{-3}$	Volume-specific cost for structure
$\dot{v}$	0.0485	$cm \cdot d^{-1}$	Energy conductance
κ	0.9743		Fraction reserve used for growth + maintenance
$\dot{k}_J$	0.002	d^{-1}	Maturity maintenance rate coefficient
E_H^h	0.1948	J	Maturity threshold at hatching
E_H^b	2.4333	J	Maturity threshold at birth
E_H^j	503.22	J	Maturity threshold at metamorphosis for female
E_H^j	335.73	J	Maturity threshold at metamorphosis for male
E_H^p	1313.2	J	Maturity threshold at puberty for female
E_H^p	81032	J	Maturity threshold at puberty for male
κ_R	0.95		Fraction of the reproduction buffer fixed into eggs
		Auxiliary and compound parameters for *Sander vitreus*	
T_A	8000	K	Arrhenius temperature
T_{ref}	293.15	K	Temperature
δ_M	0.1677		shape coefficient

3 Results

3.1 Data versus model outputs for *Pimephales promelas*

Table 3: Real versus estimated data values used for parameter estimation of DEB model for *Pimephales promelas*.

Data (dimension)	Value	Source	Modeled
Age at hatching (d)	4.5	Braunbeck et al. [1998], Jeffries et al. [2015]	4.5
Age at birth for female (d)	6	Sommer [2011]	6.087
Age at birth for male (d)	6	Sommer [2011]	6.678
Age at puberty for female (d)	135	Sommer [2011]	133.8
Age at puberty for male (d)	135	Sommer [2011]	112.7
Age at death (d)	1460	Sommer [2011]	1460
Weight at birth (10^{-4} g)	3.393	Braunbeck et al. [1998]	3.548
Weight at puberty (g)	0.7857	Braunbeck et al. [1998]	0.835
Weight at death for female (g)	3	Sommer [2011]	3.358
Weight at death for male (g)	5	Sommer [2011]	5.185
Length at birth (cm)	0.5	Wang [1986]	0.3552
Length at puberty (cm)	4	Braunbeck et al. [1998]	4.515
Length at death for female (cm)	8.09	Collected from Lab data from SCSU	7.424
Length at death for male (cm)	10.1	Etnier and Starnes [1993]	9.61
Number of egg per day ($\#/d$)	30	Watanabe et al. [2007]	33.35

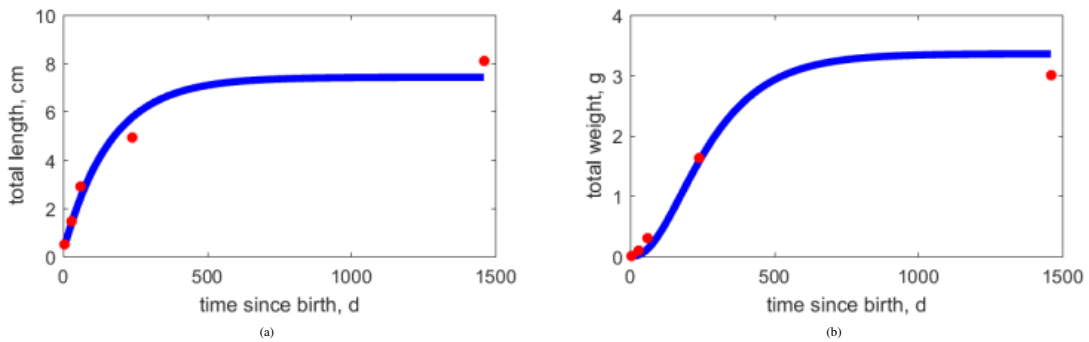

Figure 2: Model outputs versus length data (a) and weight data (b) for female *Pimephales promelas*. Model outputs are the blue lines and data are the red dots.

We used a previously developed version of DEB model for *Pimephales promelas*[1]. We modified some zero-variate data and added some new ones in order to model both female and male fathead minnows (table 3).
We also added uni-variate data on length and weight both for female (figure 2) and male (figure 3). These data were extracted from Saint-Cloud State University (SCSU) laboratory experiments.

[1] `https://www.bio.vu.nl/thb/deb/deblab/add_my_pet/entries_web/Pimephales_promelas/Pimephales_promelas_res.html` - version of 2011/03/17

Zero-variate data (table 3) are accurately reproduced by the model. Life-cycle events (*i.e.* ages at hatching, birth, puberty and death) at different temperatures are particularly well estimated, which guarantees that the DEB model estimations are in accordance with reality. Lengths and weights observed at each of these life-cycle events are also well reproduced by the model as well as the reproduction rate.
Pimephales promelas exhibits a sexual dimorphism that is taken into account by the DEB model. The female (figure 2) and male (figure 3) growth patterns are accurately simulated by the model both for length and weight growth.

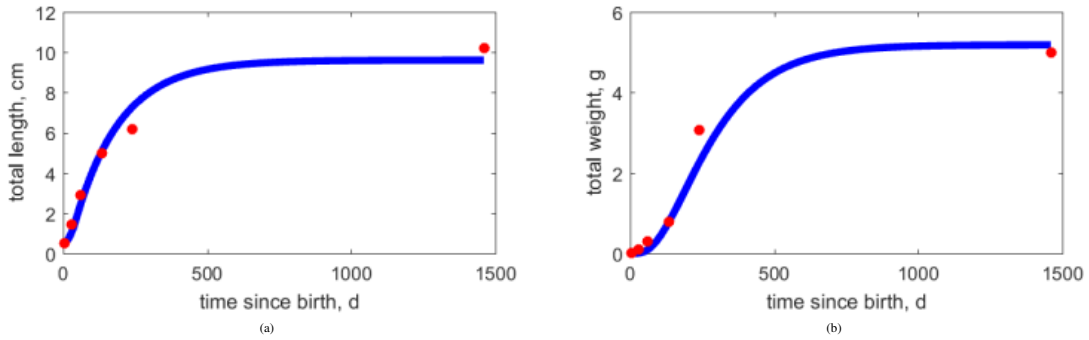

Figure 3: Model outputs versus length data (a) and weight data (b) for male *Pimephales promelas*. Model outputs are the blue lines and data are the red dots.

3.2 Data versus model outputs for *Sander vitreus*

Data used for the parameter estimation were all extracted from literature. Zero-variate data are shown in table 4. Uni-variate data include data on length, growth (figures 4 and 5), weight versus length data (figure 6), egg versus length data (figure 7) and incubation time data versus temperature data (figure 8).

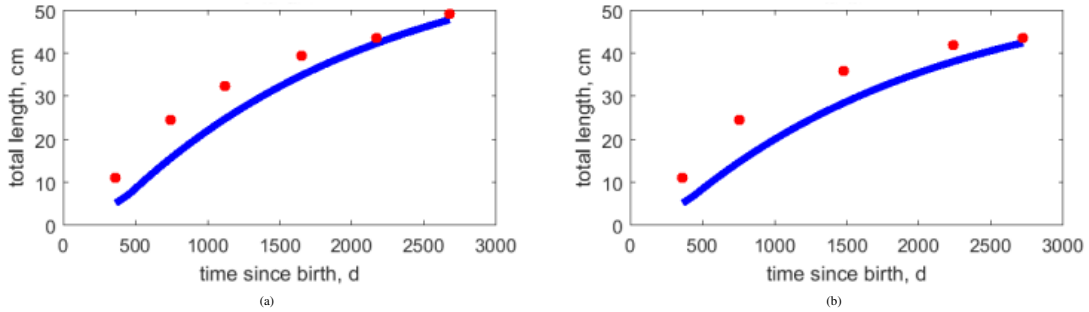

Figure 4: Model outputs versus length data for female (a) and male (b) *Sander vitreus*. Model outputs are the blue lines and data are the red dots. Data are extracted from Colby et al. [1979]

Table 4: Real versus estimated data values used for parameter estimation of DEB model for *Sander vitreus*.

Data (dimension)	Value	Source	Modeled
Age at hatching (d)	14	Nelson [1968]	13.83
Age at puberty for female (d)	1095	Bozek et al. [2011]	827.2
Age at puberty for male (d)	730	Bozek et al. [2011]	792.2
Age at death (d)	6023	Bozek et al. [2011]	6023
Weight at birth (g)	0.031	Bozek et al. [2011]	0.0308
Weight at puberty for female (g)	587.9	Honsey et al. [2017]	660.4
Weight at puberty for male (g)	436.9	Honsey et al. [2017]	417.9
Weight at death for female (g)	2763	Honsey et al. [2017]	2585
Weight at death for male (g)	1605	Honsey et al. [2017]	1727
Length at hatching (cm)	0.65	Nelson [1968]	0.6622
Length at birth (cm)	1.5	Nelson [1968]	1.492
Length at puberty for female (cm)	40	Bozek et al. [2011]	41.45
Length at puberty for female (cm)	35	Bozek et al. [2011]	35.59
Length at death for female (cm)	67	Bozek et al. [2011]	65.33
Length at death for male (cm)	54	Bozek et al. [2011]	57.11
Number of egg per day (#/d)	2055	Bozek et al. [2011]	1034

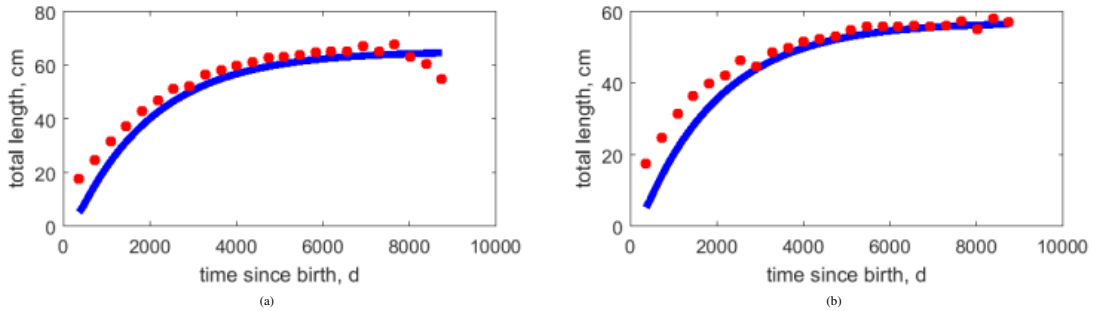

Figure 5: Model outputs versus length data for female (a) and male (b) *Sander vitreus*. Model outputs are the blue lines and data are the red dots. Data are extracted from Honsey et al. [2017]

The DEB model well reproduce all the zero-variate data (tabel 4). Ages at hatching, birth, puberty and death at different temperatures are all well estimated both for the male and the female. Age at puberty for female is slightly underestimated by the model. Nevertheless, both length and weight at puberty for the female walleye are accurately reproduced. Lengths and weights observed at each of the other life-cycle events are also well reproduced by the model. Walleye exhibits a sexual dimorphism. The female grows larger in length and weight compared to the male walleye. This is also accounted in the model and accurately reproduced both for length (figures 4 and 5) and weight (figure 6) for male and female.

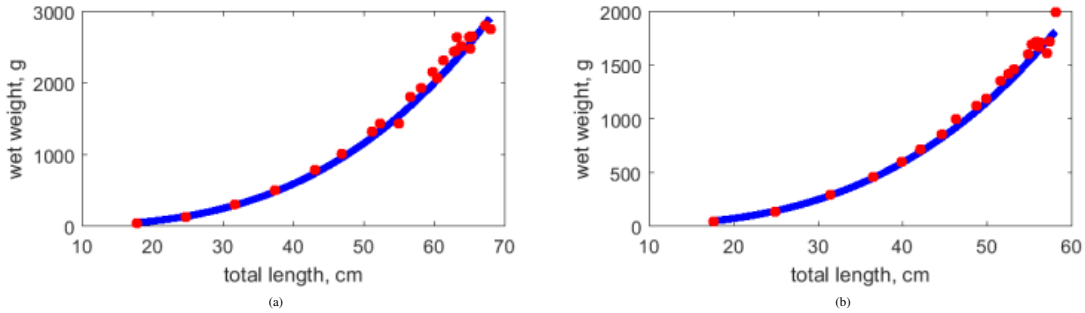

Figure 6: Comparison of model outputs and length versus weight data for female (a) and male (b) *Sander vitreus*. Model outputs are the blue lines and data are the red dots. Data are extracted from Honsey et al. [2017]

Female reproduction (eggs number) as function of length is also well reproduced by the model. Figure 7 shows data on reproduction at two different food and temperature conditions in lake Erie that are well reproduced as well. Incubation time (time to hatch) as function of temperature (figure 8) is accurately reproduced. The accurate reproduction of these data at different food and temperature conditions validate the realistic behavior of the model in different modeling contexts.

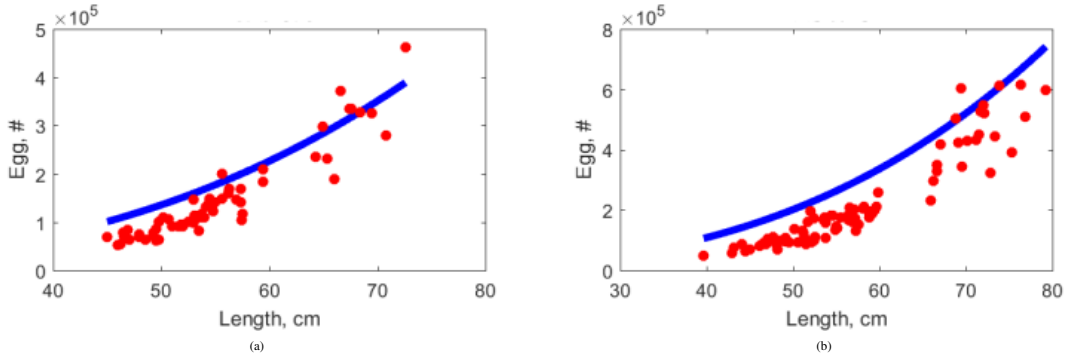

Figure 7: Comparison of model outputs and egg versus length data for East Erie Lake (a) and West Erie Lake (b) for *Sander vitreus*. Model outputs are the blue lines and data are the red dots. Data are extracted from Wolfert [1969]

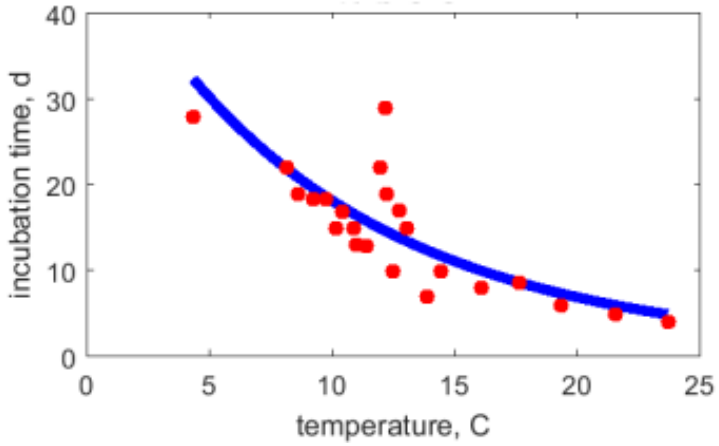

Figure 8: Comparison of model outputs and incubation time versus temperature data for*Sander vitreus*. Model outputs are the blue lines and data are the red dots. Data are extracted from Wolfert [1969]

4 General conclusion

The DEB models calibrated for *Pimephales promelas* and *Sander vitreus* provide an accurate representation of the full life-cycle in different simulation contexts both for male and female individuals.

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Individual Based Models for the fathead minnow *Pimephales promelas* and the walleye *Sander vitreus*

M.Vaugeois, P.A. Venturelli, V.E. Forbes

1 Introduction

This document first provides a description of the DEB-IBM models developed for the fathead minnow *Pimephales promelas* and the walleye *Sander vitreus* following the ODD protocol (Overview, Design concepts, Details) for describing individual- and agent-based models [Grimm et al., 2006, 2010]. Then, it presents the simulations results and conclusions about this study.

2 Methods

The two models are developed in Java using the "SimAquaLife" framework [Dumoulin, 2007], which is an individual-based process-oriented framework for aquatic life simulation. Both models are based on same methodologies and share the same objective which is to predict population-level effects of exposure to estrone in different river systems. Therefore, the IBMs are very similar and only differ by the value of their parameters, the initialization and the reproduction sub-models.
The following description is written as the description of one model. We will clearly specify any difference between the two models.

2.1 Purpose

The purpose of this model is to predict effects of exposure to estrone at the population level for different river system types and for different species.

2.2 Entities, state variables, and scales

There are two types of entities in the model, the individuals and spatial units. We used the scaled version of the Dynamic Energy Budget (DEB) model with metabolic acceleration to represent the energy budget of individuals. Individuals are characterized by four state variables (see table 1), namely: the structure (noted L, with the dimension of cm); the

scaled reserve (U_E, d.cm); the scaled maturity (U_H, d.cm); and the scaled reproduction (U_R, d.cm). For more details or introduction on Dynamic Energy Budget theory [Kooijman, 2010], see [van der Meer, 2006, Nisbet et al., 2000, Sousa et al., 2010, Jusup et al., 2016]. The ageing of individuals is set the same way as in [Martin et al., 2012]. Briefly, in DEB theory ageing is described by two state variables, namely the damage inducing compounds ($\ddot{q}$), and damage ($\dot{h}$). This is based on the idea that free radicals cause irreversible damage on DNA (damage inducing compounds) that creates damage, and probability to die by ageing is proportional to the amount of damage. Regarding the inter-individual variability, we set it by introducing a normally distributed number ($\mu = 1$ and $\sigma = 0.05$), the scatter multiplier, which impacts four of the eight standard DEB parameters (table 2).

Table 1: State variables.

Name	Notation	Dimension
Volumetric structural length	L	cm
Scaled reserve	U_E	$d.cm^2$
Scaled maturity	U_H	$d.cm^2$
Scaled reproduction	U_R	$d.cm^2$
Ageing acceleration	$\ddot{q}$	$d.cm^2$
Hazard rate	$\dot{h}$	$d.cm^2$
Prey density	X	$J.cm^{-2}$

The model has ten spatial units, each representing one hectare zone of a river. Individuals decide to move upstream or downstream or to stay in their current zone with an equal probability (see 2.3).
Each zone is characterized by 3 quantitative state variables: the prey density (X, $J.cm^{-2}$), the temperature (T, in C) and the surface area (in m^2). The prey density changes daily (*i.e.* at each time step) and independently in each zone according to a logistic growth minus what is eaten by fishes in the zone. The logistic growth function has two parameters, the intrinsic growth rate (here noted a_E, with the dimension of d^{-1}) and the carrying capacity (here noted k_E, with dimension of $J.m^{-2}$). Temperature is the same in all zones and changes daily according to temperature data recorded in Kawishiwi River, MN. Data were downloaded from USGS website (https://waterdata.usgs.gov/nwis).

The model integration is done following the classical Runge-Kutta method thanks to Apache Commons Math library (release 3). One time step represents one day and simulations are run for 20 years. When considered, exposure to estrone starts at 15 years.

2.3 Process overview and scheduling

At each time step, the following action are executed. First, individuals are listed zone by zone. Then, in each zone, individuals first update their DEB state variables. Consequently to this update, organisms can die for 3 reasons: or they can no longer pay their maintenance cost (starvation), or their death is due to ageing, or they die by predation (stage-dependent

Table 2: Parameters.

Name	Notation	Dimension
Standard parameters		
Fraction of mobilized energy to soma	κ	wd
Fraction of reproduction energy fixed in eggs	κ_R	wd
Somatic maintenance rate coefficient	$\dot{k}_M$	d^{-1}
Maturity maintenance rate coefficient	$\dot{k}_J$	d^{-1}
Scaled maturity at hatching	U_H^h	$d.cm^2$
Scaled maturity at metamorphosis	U_H^J	$d.cm^2$
Scaled maturity at birth	U_H^b	$d.cm^2$
Scaled maturity at puberty	U_H^p	$d.cm^2$
Energy conductance	$\dot{v}$	$cm.d^{-1}$
Energy investment ratio	g	wd
Ageing parameters		
Weibull ageing acceleration	$\ddot{h}_a$	d^{-2}
Gompertz stress coefficient	S_G	wd

survival rate to predation). Starvation death is deterministic (depends on state variable) and the other two (*i.e.* ageing and predation) are stochastic (*i.e.* depends on a random selection, see 2.3). The next process is the reproduction. Only adult individuals with a certain amount of energy allocated to reproduction (U_R, d.cm) are able to reproduce. Females can only reproduce if a ready-to-reproduce male is present in the zone. The last process executed is the movement. This process is also a stochastic process. Individuals have 50 percent chance to move or to stay in their current zone. If they choose to move, they have 50 percent chance to move upstream or downstream.

2.4 Design concepts

2.4.1 Basic principles

The model is based on Dynamic Energy Budget theory for describing the energetic of individuals. The DEB model describes how individuals feed, allocate energy for growth and reproduction, and die. Food is limited in our model and its availability depends both on fish density and the logistic growth parameter of food defined in the spatial units. DEB model allow to represent two kinds of mortalities, ageing or starvation. We introduced a third one that represent the mortality due to predation.

The goal of this model is to estimate the population scale effects of exposure to Estrone in different river system types. Consequently we will model different system types, each one characterized by different sets of parameters.

2.4.2 Emergence

All the following measurements emerge from the behavior of individuals, their metabolism, the indirect interaction of individuals through competition for food and the predation: fish density, average individual size (length and weigh), average number of reproducing events per female, average number of eggs per reproducing events, population level mortality rates (starvation and predation), Fulton's condition index [Shin et al., 2005] (*i.e.* ratio of weight of cubic length).

2.4.3 Adaptation

While DEB parameters are different among individuals, they stay constant over the simulation for each individuals. So, there is no adaptive behavior. At any time, individuals can chose to stay or to move from one zone to another.

2.4.4 Objectives

The objective of this work is to compare effects of estrone at the population scale level for different types of river systems. In order to do that, we modeled a river environment with two controlling precesses, the food availability and the stage-dependent predation. Then, several simulations were ran with different sets of parameters for food logistic growth and stage-dependent survival rates to predation. Finally, we analyze the outputs to see in which conditions (*i.e.* set of parameters) a realistic population pattern is observed.
The pattern we want to reproduce is based on literature data and is as follow for the fathead minnow:

1. The average fish population (individuals between 1.5 and 9.8 cm) density must be between 1 individuals per m^{-2} and 16 individuals per m^{-2} based on the Minnesota Pollution Control Agency (MPCA) (see figure 1);

2. The average number of reproduction events per female per year must be between 14 and 26 [Gale and Buynak, 1982];

3. The average spawning interval (average time between two reproduction events) for female must be between 2.5 and 5.5 days [Watanabe et al., 2007, Jensen et al., 2001];

4. The average number of eggs per clutch must be between 80 and 140 [Watanabe et al., 2007, Ankley et al., 2001].

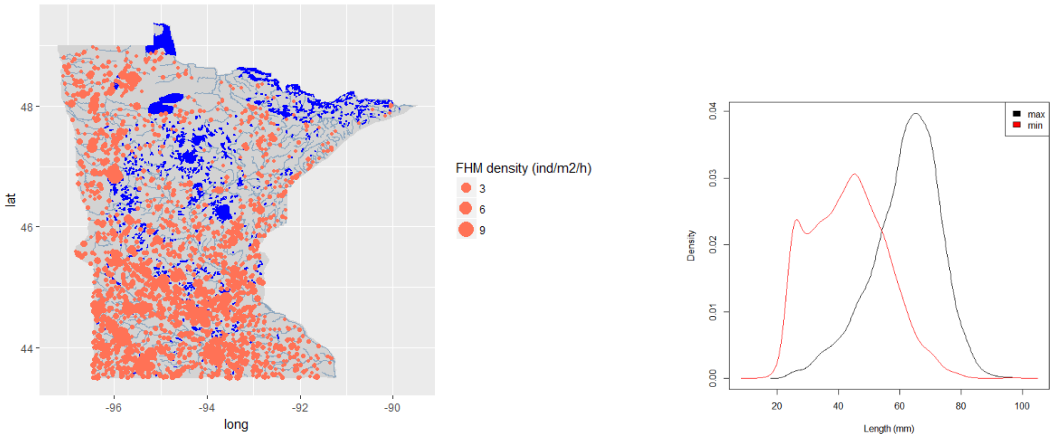
Regarding the walleye, the pattern we want to reproduce is based on literature data and is as follow:

1. Proportional stock density (PSD) must be comprised between 10 and 60 [Cichosz, 2009, Nate et al., 2011]. PSD is a population structure index that is calculated as follow [Anderson, 1996]: $PSD = 100 * \frac{Numberoffishwithlength>=38cm}{Numberoffishwithlength>=25cm}$

2. Relative stock density (RSD) must be comprised between 1 and 40 [Cichosz, 2009]. RSD is another population structure index that is calculated as follow [Anderson, 1996]: $RSD = 100 * \frac{Numberoffishwithlength>=45cm}{Numberoffishwithlength>=25cm}$

3. Reproduction rate (number of eggs per reproduction) must be comprised between 50000 and 700000 per female individuals Bozek et al. [2011].

4. Number of adults walleye per hectare must be comprised between 1 and 60 [Nate et al., 2011].



(a) (b)

Figure 1: Fathead minnow density in Minnesota (a). Distribution functions of maximum (black) and minimum (red) size measured when fish were collected by electro-fishing (b). Minimum size limit is 1.5 cm and maximum size limit is 9.8cm. Data obtained from the Minnesota Pollution Control Agency.

2.4.5 Learning

There is no learning process in the present model.

2.4.6 Prediction

There is no prediction process in the model.

2.4.7 Sensing

There is no Sensing process in the present model.

2.4.8 Interactions

Individuals interact via reproduction. Individuals interact indirectly via competition for food.

2.4.9 Stochasticity

Some DEB parameters change among individuals. We followed the same methodology than [Martin et al., 2012], based on [Kooijman et al., 1989], to set variability among individuals. Briefly, the surface area specific maximum ingestion rate $\{\dot{J}_{XAm}\}$ is multiplied by a scatter multiplier to introduce differences between individuals. It impacts 4 DEB parameters: the half-saturation coefficient K (see equation **??**) that is multiplied by the value of the scatter multiplier; the energy investment ratio g, that is divided by the value of the scatter multiplier as it equals $\frac{[E_G]\dot{v}}{\kappa\{\dot{P}_{Am}\}}$, with $\{\dot{P}_{Am}\} = \{\dot{J}_{XAm}\}\kappa_X\mu_X$; and the scaled maturity threshold for birth U_H^b and puberty U_H^b, that are divided by the value of the scatter multiplier because we use the scaled DEB model (which is the standard DEB model scaled by $\{\dot{P}_{Am}\}$, see [Kooijman et al., 2008] for more details on scaling standard DEB model).
Another source of stochasticity comes from the order with which organisms are updated. In this work, individuals are randomly selected at each time step, zone by zone.
Another source of stochasticity comes from destination decision (stay or upstream or downstream).
The last sources of stochasticity comes from the probability to die by ageing and the probability to to die by predation.

2.4.10 Collectives

There is no aggregation behavior in the model.

2.4.11 Observations

We collected observations on fish density, number of eggs per reproductive event per female, number of reproducing event per female, average time between two reproductive events (spawning interval) for female, average size of individuals (length and weigh), average population Fulton's condition index (ratio of weight over cubic length).

2.5 Initialization

At the beginning of each simulation food density is set to the value of the carrying capacity in all zones.
For the fathead minnow, all simulations starts at the 151^{th} day of the year in accordance with the reproduction period. For the first 3 years of simulation, 40 individuals are introduced (at the egg stage, with a sex ratio of 0.5) each day with a temperature higher than 15 degree Celsius.
For the walleye, all simulations starts at the 91^{th} day of the year. At this day, 1000 larvae are introduced (with a sex ratio of 0.5). This is repeated for the first 4 years of simulation at this day of the year.

We introduced variability among individuals as described in part 2.4.9. The initial set of DEB parameters (*i.e.* the one on which we apply a scatter multiplier) is the one from the add-my-pet procedure (see the document entitled "Add-my-pet procedure"). The initial value of the DEB state variables are: $L = 0.001$; $U_R = 0$; $U_H = 0$. The initial

amount of scaled reserve U_E is calculated for each individuals from the bisection method, as in [Martin et al., 2012].

2.6 Input data

One temperature data set (recorded in Kawishiwi River, MN), provided by the USGS, is inputed in the model. The temperature is the same in every zone of the river and is set identical from one year to the other.

2.7 Sub-models

There are 4 sub-models. 2 sub-models concern individual life cycle: the *Deb sub-model*, which includes DEB state variables update, death due to starvation, ageing and prey dynamics ; and the *mortality sub-model*, which includes the death due to ageing and predation. The other 2 sub-models are for reproduction and movement.

2.7.1 DEB sub model

- Calculate delta reserve considering feeding and mobilized energy
- If maturity is inferior to maturity threshold for puberty, then calculate delta maturity. Otherwise calculate delta reproduction buffer
- Calculate delta length ; If delta length is inferior to 0, then recalculate structure, reserves, maturity, and reproduction buffer based on starvation rules
- If mobilized energy is inferior to energy needed for maintenance, then die
- Calculate delta ageing acceleration
- Calculate delta hazard based on ageing (*i.e.* probability of dying related to ageing)
- Calculate delta prey density based on feeding
- Update prey density
- Update DEB state variables

2.7.2 Mortality sub-model

- If random number is inferior to probability of dying related to ageing, then die
- If random number is inferior to probability of dying related to predation, then die

2.7.3 Reproduction sub-model

For the fathead minnow:

- If the environmental temperature is superior or equal to 15 degree Celcius:
  - If female reproduction buffer is superior to threshold for reproduction and if a ready to reproduce male is in the zone, then reproduce:
    - calculate egg initial energy amount
    - calculate number fertilized eggs spawned
    - update reproduction buffer accordingly
  - If male reproduction buffer is superior to threshold for reproduction and if a ready to reproduce female is in the zone, then reproduce:
    - calculate energy amount needed for reproduction event
    - update reproduction buffer accordingly

For the walleye:

- If the environmental temperature is comprised between 4 and 11 degree Celcius and the time in the year is before the 151^{th} day of the year:
  - If female reproduction buffer is superior to threshold for reproduction and if a ready to reproduce male is in the zone, then reproduce:
    - calculate egg initial energy amount
    - calculate number fertilized eggs spawned
    - update reproduction buffer accordingly
  - If male reproduction buffer is superior to threshold for reproduction and if a ready to reproduce female is in the zone, then reproduce:
    - calculate energy amount needed for reproduction event
    - update reproduction buffer accordingly

2.7.4 Movement

- If not egg, then:
  - If random number is inferior to probability of moving, then move
  - If random number is inferior to probability of moving upstream, then move upstream

2.8 Simulations

2.8.1 Preliminary simulations for the fathead minnow

We ran 20 replicates of each simulation. A simulation has a specific combination of 4 parameters:

- The intrinsic growth rate::
  $a_E \in [0.4, 0.8, 1.5]$;
- The carrying capacity:
  $k_E \in [500.00, 2000.0, 4000.0]$;
- The juvenile annual survival rate:
  $asr_J \in [0.005, 0.01, 0.025, 0.05, 0.1]$;
- The adult annual survival rate:
  $asr_A \in [0.4, 0.8]$.

The total number of simulations is thus 1800. From these simulations, we selected the ones that reproduced the pattern described in the part 2.4.4. The number of selected sets of parameters was 50 out of 90 possible. Each one of this 50 selected sets of parameters represent a different river system.

2.8.2 Preliminary simulations for the walleye

We ran 20 replicates of each simulation. A simulation has a specific combination of 4 parameters:

- The intrinsic growth rate::
  $a_E \in [0.4, 0.8, 1.5]$;
- The carrying capacity:
  $k_E \in [5000.00, 10000.0, 20000.0]$;
- The egg annual survival rate:
  $asr_E \in [0.02, 0.05, 0.1]$;
- The larvae annual survival rate:
  $asr_L \in [4.73e^{-8}1.44e^{-5}]$;
- The juvenile annual survival rate:
  $asr_J \in [0.09, 0.15]$;
- The adult annual survival rate:
  $asr_A \in [0.6, 0.8]$.

The total number of simulations is 4320. From these simulations, we selected the ones that reproduced the pattern described in the part 2.4.4. The number of selected sets of parameters was 31 out of 216 possible. Each one of this 31 selected sets of parameters represent a different river system.

2.8.3 Simulations with exposure to estrone

Effect on larval survival rate to predation for fathead minnow:

The observed effects of exposure to estrone indicated that temperature does not strongly or consistently modulate the effects of estrone on reproduction, development, and egg survival. However, results suggested that estrone alter larval predator avoidance performance [Ward et al., 2017] which motivated the set up of a predation experiment. Based on the results of this experiment [Korn, 2018], we set up simulations in which the larval survival probability was affected by 10 and 25 percent, the latter corresponding to the observed effect during the experiments.
For each previously selected set of parameters, we ran 20 replicates of simulations in which fish are exposed to estrone starting at the year 15. We then measured effects at the population scale after 5 years exposure.

Effect on egg survival rate to predation for fathead minnow:

Experiments showed that the number of aggressive acts by male fathead minnow decreased when they were exposed to estrone [Ward et al., 2017]. A reduced aggressiveness in male fathead minnow could result in a reduced efficiency to defend the nest from predator. Consequently, the egg survival rate to predation could be affected. In order to investigate how a potential reduction of male nest defense ability could translate at the population scale, we set up simulations in which the egg survival probability was affected by 25 percent.
For each previously selected set of parameters, we ran 20 replicates of simulations with exposure to estrone starting at the year 15. We then measured effects at the population scale after 5 years exposure.

Simulations with combined effects of estrone exposure on larval and egg survival rates to predation for fathead minnow:

For each previously selected set of parameters, we ran 20 replicates of simulations with exposure to estrone starting at the year 15. The considered effects were a 25 percent decrease of both larval and egg survival rate to predation. We then measured effects at the population scale after 5 years exposure.

Simulations for the walleye:

For the walleye, we considered two effects. First, we considered that walleye larvae could be affected in the same way as the fathead minnow when exposed to estrone. We thus ran simulations with a 25 percent decrease of the larval survival rate to predation.
Secondly, taking into account the results of the fathead minnow IBM that showed a population decrease due to exposure to estrone when food was limiting, we also ran simulations with the walleye IBM in which food availability was reduced.
We did not considered effects on egg survival rates to predation because walleye are broadcast spawners.
For each previously selected set of parameters, we ran 20 replicates of simulations with exposure to estrone starting at the year 15. We then measured effects at the population scale after 5 years exposure. We ran 3 sets of simulations:

- The considered effect was a 25 percent decrease of larval survival rate to predation.

- The considered effect was a 25 percent decrease of the environmental food carrying capacity.
- The considered effect was a combination of the two previously considered effects (25 percent decrease for both).

3 Results

3.1 IBMs simulations for fathead minnow:

3.1.1 Effect on larval survival rate to predation:

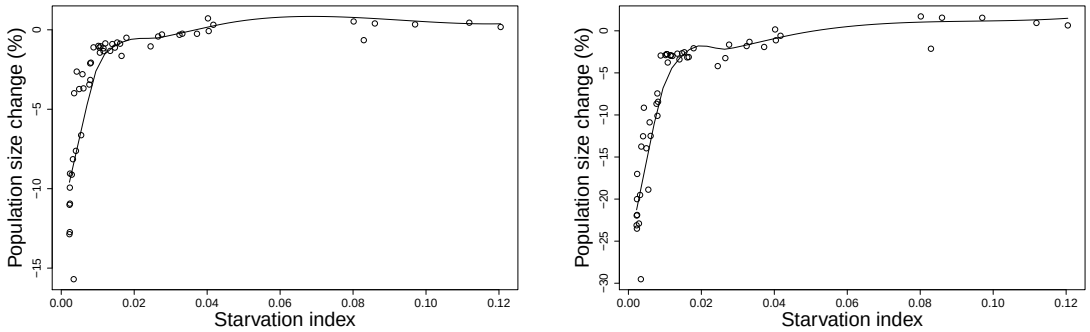


Figure 2: Relative population decline (in percent) after 5 year exposure to estrone as function of the starvation index. Considered effect is a 10 percent (a) and a 25 percent (b) decrease of the larval survival rate to predation. Each point represents the average value of 20 simulations of one particular system. The line is a non-parametric regression of the points (LOESS).

The figure 2 presents how population density changes when fish are exposed to estrone. We can see that all river system types do not show the same answer. For a 10 percent decrease in larval survival rate to predation, the consequence on the fish population density can either be null or decrease by more than 15 percent. Similarly, the population density can either decrease by about 30 percent or do not decrease at all when a 25 percent decrease in larval survival rate is considered. Actually, the population-level effect depends on whether the fish population is more controlled by predation or by food availability. Both control types occur in any system but one of the two has more hold on the population size than the other.
Depending on the relative strength of these two controlling processes, individuals will be more likely to die by starvation or by predation. Therefore, we can characterize the different types of system by comparing the different ratios of individuals who died by starvation over all dead individuals in the systems. This index, that we will named starvation index, will inform us on which is the major process controlling the population size. If it is low,

we can say that the system is more controlled by predation than food availability and vice versa. On the figure 2, we can see that the more a system is controlled by predation, the more it is likely to be importantly impacted by an exposure to estrone.

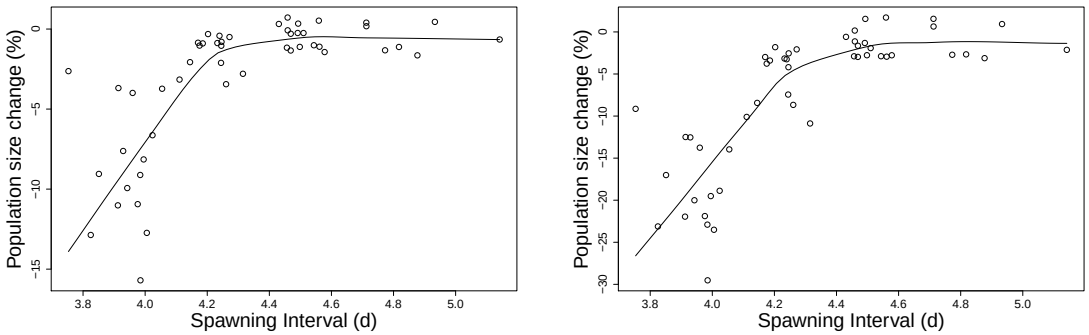


Figure 3: Relative population decline (in percent) after 5 year exposure to estrone as function of the Fulton's condition index (noted K index) before exposure to estrone. Considered effect is a 10 percent (a) and a 25 percent (b) decrease of the larval survival rate to predation. Each point represents the average value of 20 simulations of one particular system. The line is a non-parametric regression of the points (LOESS).

The figure 3 shows how a relative decrease in population size is related to the value of the Fulton's condition index (ratio of weight over cubic length, noted K index) before the exposure to estrone. We can see that the K index value before exposure to estrone is high (around 0.75 $10^2.cm^{-3}$) in the case of a system which population density will be strongly impacted by the exposure. On the opposite, this value is small (around 0.55) in systems that will not be impacted by exposure to estrone. This means that individual are bigger before exposure in systems that will be strongly impacted by an exposure to estrone.
We can see the same pattern on the figure 4. This figure shows the average size of individuals in different system before being exposed to estrone as function of the relative population decrease after exposure. Here again, we can note that systems with smaller average individual size will be the less impacted by exposure to estrone, whereas the systems with higher average individual size will be more likely be strongly impacted.
Figure 5 presents population changes after exposure as function of the average female spawning interval before any exposure to estrone. It shows that the systems that will be the more impacted are the ones that show a short spawning interval before exposure (*i.e.* higher reproduction rate). On the contrary, systems with longer average spawning intervals before exposure show almost no population decline after exposure.

3.1.2 Effect on egg survival rate to predation:

In these simulations with a decreased egg survival rate to predation, we observed the same pattern as we did with the simulations in which we considered a decrease in larval survival rate to predation. The systems that are more controlled by predation (those with a low

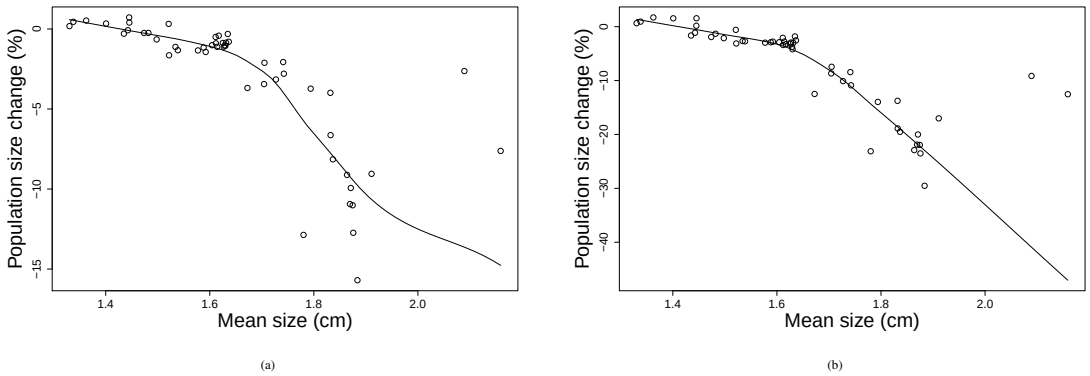


Figure 4: Relative population decline (in percent) after 5 year exposure to estrone as function of the mean individual size before exposure to estrone. Considered effect is a 10 percent (a) and a 25 percent (b) decrease of the larval survival rate to predation. Each point represents the average value of 20 simulations of one particular system. The line is a non-parametric regression of the points (LOESS).

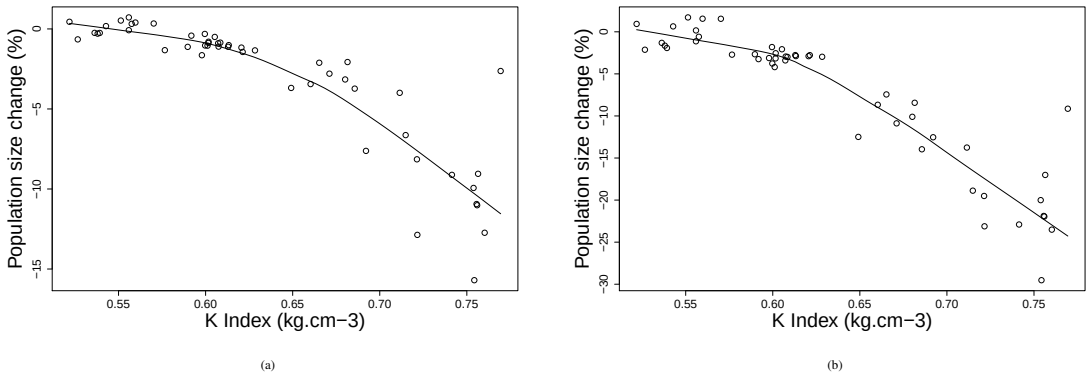
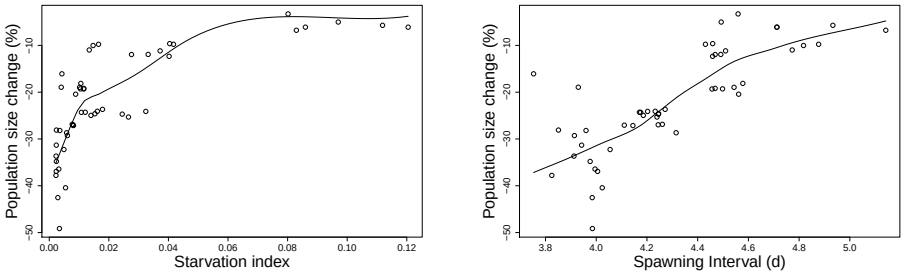


Figure 5: Relative population decline (in percent) after 5 year exposure to estrone as function of the spawning interval before exposure to estrone. Considered effect is a 10 percent (a) and a 25 percent (b) decrease of the larval survival rate to predation. Each point represents the average value of 20 simulations of one particular system. The line is a non-parametric regression of the points (LOESS).

starvation index) are the ones that show the biggest impacts on fish population density after exposure to estrone (figure 6(a)). Nevertheless, the strength of the population decline is more important than it was with simulations considering effects on larval survival rate. Firstly, a decrease in 25 percent of the egg survival rate to predation can causes a population decline as high as 50 percent. Moreover, systems that were not strongly impacted before (with starvation index between 0.01 and 0.04) are now significantly more impacted.

The relationship between relative population decline after exposure and Fulton's condition index before exposure (figure 6(b)) shows a slightly different pattern compared to the previous case. It is now convex whereas it was concave before.



(a) (b)

(c) (d)

Figure 6: Relative population decline (in percent) after 5 year exposure to estrone as function of the starvation index (a), the Fulton's condition index (noted K index) before exposure to estrone (b), the mean individual size before exposure to estrone (c) and the spawning interval before exposure to estrone (d). Considered effects are a 25 percent decrease of egg survival rate to predation. Each point represents the average population decline for 20 simulations of one particular system. The line is a non-parametric regression of the points (LOESS).

The pattern of fish population decline after exposure as function of the average individual size before exposure (figure 6(c)) has also changed compared to the previous case. It is now more linear at the beginning instead of being concave.
The shape of the relationship between relative population decline after 5 year exposure to estrone as function of spawning interval before exposure (figure 6(d)) is more linear compared to the simulations in which a decrease in the larval survival rate was considered. All these different patterns indicate that the population-level effects of impact on egg survival rate are stronger than those on larval survival rate, both in intensity and in the spectrum wideness of potentially impacted systems.

3.1.3 Combined effects on egg and larval survival rates to predation:

Figure 7 shows how population is affected when a 25 percent decrease of both egg and larval predation rate is considered. We can notice that all types of system are impacted. The minimum impact is a 10 percent population decrease and the maximum impact is a 50 percent decrease. Moreover, a large proportion of systems shows a population decrease

of 20 percent or more. This proportion as well as the intensity of the impacted river systems are higher to what was observed when we considered the two effects separately. The patterns of both the Fulton's condition index, the average size and the reproduction rate (spawning interval) are identical to what was observed when we considered the two effects separately.

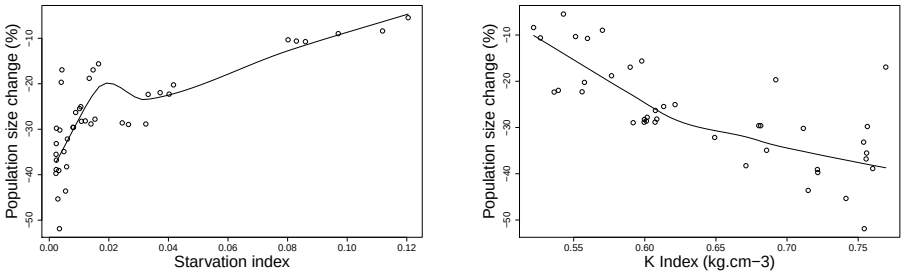


Figure 7: Relative population decline (in percent) after 5 year exposure to estrone as function of the starvation index (a), the Fulton's condition index (noted K index) before exposure to estrone (b), the mean individual size before exposure to estrone (c) and the spawning interval before exposure to estrone (d). Considered effects are a 25 percent decrease of both the egg and the larval survival rates to predation. Each point represents the average population decline for 20 simulations of one particular system. The line is a non-parametric regression of the points (LOESS).

3.2 IBMs simulations for walleye:

3.2.1 Effect on larval survival rate to predation:

the population-level effects of a decrease in larval survival rate to predation for the walleye (figure 7) are very similar to those observed for the fathead minnow. Similarly to what we observed for the fathead minnow, the figure 7(a) shows that the population size change is dependent on the river system type. Nevertheless, the intensity of the observed effect on population size is lower than what was observed for the fathead minnow. The maximum population decrease is between 15 and 20 percent whereas it was between 25 and 30

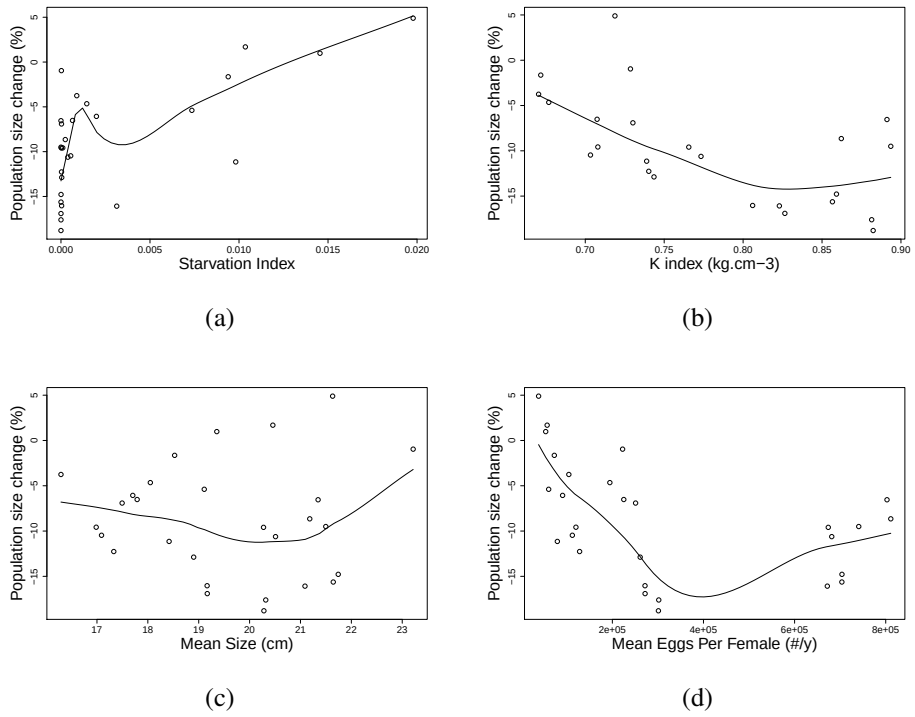


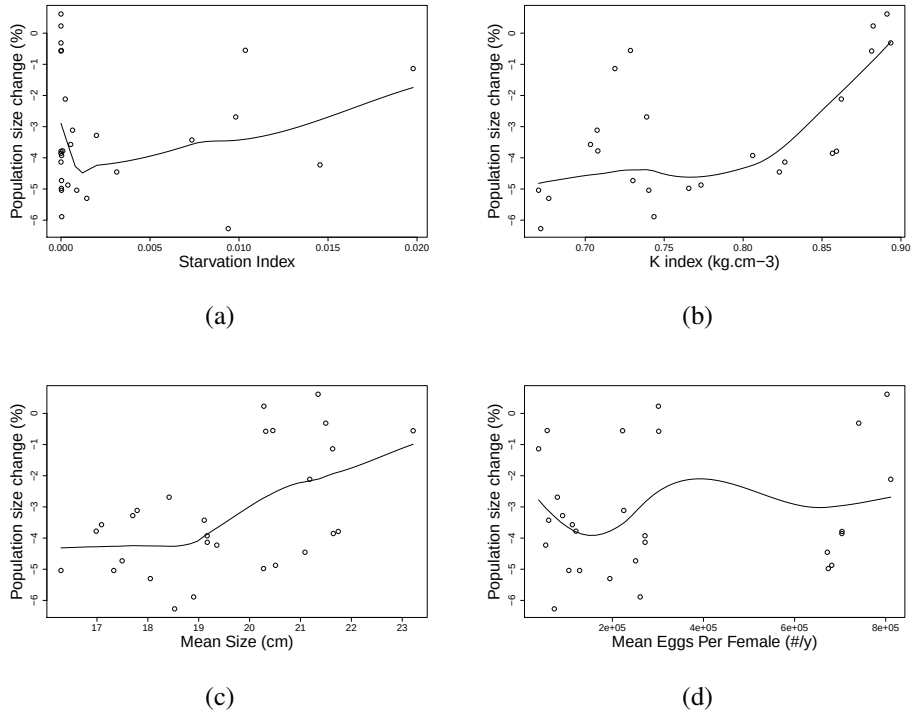
Figure 8: Relative population decline (in percent) after 5 year exposure to estrone as function of the starvation index (a), the Fulton's condition index (noted K index) before exposure to estrone (b), the mean individual size before exposure to estrone (c) and the number of eggs per female before exposure to estrone (d). Considered effect is a 25 percent decrease of the larval survival rate to predation. Each point represents the average population decline for 20 simulations of one particular system. The line is a non-parametric regression of the points (LOESS).

percent for the fathead minnow.
The pattern of Fulton's condition index versus population size change is also similar to what was previously observed with the fathead minnow. It is lower in less affected systems (figure 7(b)) and higher in more impacted systems.
The average size (figure 7(c)) shows a different pattern than what was observed with the fathead minnow. In the present case, the average size is not very different between the two types of system.
Regarding the number of eggs per female versus population size change (figure 7(d)), it shows that females in less impacted system produce less eggs compared to systems that will be impacted. This is similar to what was observed with the fathead minnow.

3.2.2 Effect on food availability:

When considering a decrease of food availability, the population-level effects are limited (figure 9(a)). The maximum decrease is about 6 percent. It appears that in this case, the effects are the same whatever the considered system. As the population size effects

are limited, we can not give any interpretation to the patterns observed for the Fulton's condition index (figure 9(b)), the average individual size (figure 9(c)) or the reproduction rate (figure 9(d)).



(a) (b)

(c) (d)

Figure 9: Relative population decline (in percent) after 5 year exposure to estrone as function of the starvation index (a), the Fulton's condition index (noted K index) before exposure to estrone (b), the mean individual size before exposure to estrone (c) and the reproduction rate before exposure to estrone (d). Considered effect is a 25 percent decrease of the environmental food carrying capacity. Each point represents the average population decline for 20 simulations of one particular system. The line is a non-parametric regression of the points (LOESS).

3.2.3 Combined effects on larval survival rate to predation and food availability:

When a decrease of the food availability and a decease of the larval survival rate to predation are simultaneously considered, population-level effects are very similar to the case when only exposure effect on larval survival rate was considered (figure 8(a)). The intensity of the population decline is the same, as well as how much a system is impacted considering his own properties.
The pattern of Fulton's condition index versus population size change is similar to what was previously observed with effects on larval survival rate (figure 8(b)).
It is also the case of the average size versus population size change (figure 8(c)) and the number of eggs per female versus population size change (figure 8(d)).

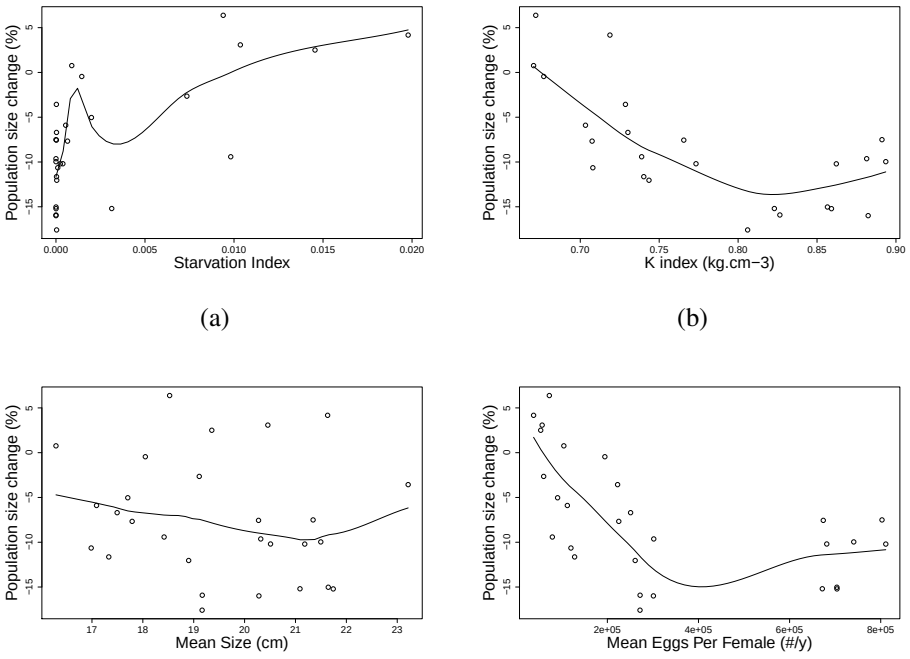


Figure 10: Relative population decline (in percent) after 5 year exposure to estrone as function of the starvation index (a), the Fulton's condition index (noted K index) before exposure to estrone (b), the mean individual size before exposure to estrone (c) and the reproduction rate before exposure to estrone (d). Considered effects are a 25 percent decrease of the environmental food carrying capacity and a 25 percent decrease of the larval survival rate to predation. Each point represents the average population decline for 20 simulations of one particular system. The line is a non-parametric regression of the points (LOESS).

4 Conclusions

River systems are not all equals when facing exposure to a stressor. Some of them are more vulnerable than the others. When a population is exposed to a stressor, all the individuals are impacted at the same level. However, the impact at the population scale can be more or less important. In the present work, we showed how different river systems can be impacted after 5 years exposure to estrone.

What makes the difference in how a fish population will be impacted is the properties of the river system in which individuals live. In the wild, the observed fish density results of the interactions between many processes. Basically, you can have two populations of the same size in two systems driven by different processes. In this work we only focused on two of them, the intra-specific competition for food and the predation by other species. We first showed that a realistic population pattern can be reproduced by different types of system. Actually, the population density can either be controlled by the food availability and the strength of intra specific competition for food or by the predation pressure exercised by other species.

The overall response of the system to a stressor will thus depends on the system properties. In systems in which population density is more controlled by predation, individuals are more likely to grow without limitation of food. Because of the high predation, the system almost never reach the environmental carrying capacity. Therefore, on average, individuals will grow and reproduce at their optimal capacity. The population will show a higher average size, a higher Fulton's condition index and a shorter spawning interval (*i.e.* higher reproduction rate). On the other side, in a system in which individuals are limited in food, individuals will struggle for feeding. Consequently, their growth and reproduction will not be optimal. The population will show a low average size, a lower Fulton's condition index and a larger spawning interval (*i.e.* shorter reproduction rate).

When facing a stressor like estrone, those two types of population will show a very different response. In the system in which population size is more controlled by food availability, individuals are not reproducing and growing at their best. Increasing mortality due to predation on egg or larval stage will result in a reduction of the number of individuals that will escape predation. Individuals surviving predation will be less numerous and the competition for food will be less important. Consequently, juveniles and adults will grow and reproduce slightly more. In some cases, this higher *per capita* reproduction rate will fully compensate the increased predation loss due to estrone exposure. In other cases, it will not be compensated at all. This corresponds to system in which population size is more controlled by predation pressure. In those types of system, increasing the predation pressure on egg or larvae will result in a population decrease, as the individuals in the population are not able to increase their reproduction and growth. In between these two extreme cases, there are other ones in which the compensation effect will only partially counterbalance the effects of exposure to estrone.

Exposing a system to a stressor will result in a modification of its equilibrium. The process that had the most control over the system before the exposure could lose some of its hold for the benefit of another process. With exposure to estrone, we can say that if it is only some systems that are negatively impacted, none of them is positively impacted.

Depending on the considered effect and its intensity at the individual scale, the population scale impacts will be more or less important. For instance, when considering effect on larval survival rate to predation, a 25 percent impact showed a significantly higher population decrease. Also, when comparing a 25 percent effect on larval survival rate with a 25 percent effect on egg survival rate, we showed that the impact on the population size is more intense when considering effect on egg survival rate to predation. Moreover, we also showed that more system types are in danger when considering the effect on egg survival rate. These results suggest that more experiments are needed regarding the impact on egg survival rate due to predation. So far, experiments showed that male aggressiveness are affected when exposed to estrone. Nevertheless, how this decrease in aggressiveness relates to egg survival rate to predation remains to be determined.

We conclude that measuring effects of stressors (*e.g.*, estrone) on individuals is not sufficient to determine whether or not such stressor causes impacts at the population scale. Intra-specific and inter-specific interactions are responsible for the emergence of the population dynamics. Individuals of the same species can respond very differently to a stressor depending on their environment. Also, populations of the same species living in two different systems can show very different impacts of exposure to a stressor. This indicates that accurately predicting impacts of chemicals on populations in natural systems requires incorporation of key ecological properties of the system. When considering a potential impact on any river system, this one should be extensively investigated.

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Estrone biodegradation in laboratory-scale systems designed for total nitrogen removal from wastewater†

Kira N. Peterson,‡ David T. Tan,§ Juan C. Bezares-Cruz and Paige J. Novak *

Changes in regional regulations are causing a shift towards the implementation of total nitrogen removal technologies. Conventional nitrification systems do not remove total nitrogen, instead only oxidizing ammonia and ammonium in the influent to nitrate. Conventional nitrification does, however, result in degradation of estrone (E1), a major contributor to the estrogenicity of wastewater treatment plant (WWTP) effluent. The objective of this research was to provide guidance on the impact that changes in wastewater treatment practices could have on E1 degradation. This was accomplished by comparing E1 removal in a laboratory-scale conventional nitrification system with that in a range of idealized laboratory-scale systems designed to remove total nitrogen from wastewater: the modified Ludzack-Ettinger (MLE) system (a two-stage anaerobic–aerobic system with recycle), a granular activated sludge system (cycled anaerobic–aerobic), a sequencing batch reactor (cycled anaerobic–aerobic), and an anaerobic ammonia oxidation (anammox) system. As anticipated, E1 removal was excellent when fed to the nitrification, MLE, and sequencing batch reactors, at >96% mean E1 loss. The granular activated sludge system operated in our laboratory failed to remove E1, which was perhaps not unexpected given the high COD loading under which our system was operated. Despite the anaerobic nature of anammox, it also resulted in excellent E1 removal (95% mean E1 loss) without concomitant 17β-estradiol production. This work demonstrates that the choice of nitrogen removal technology used by a treatment plant could have an impact on the estrogenicity of WWTP effluent, but low energy total nitrogen removal systems do exist that are capable of excellent E1 removal.

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Water impact

New rules regarding nitrogen levels in wastewater treatment plant (WWTP) effluents may result in widespread implementation of total nitrogen removal technologies. Conventional nitrification systems do not remove total nitrogen, instead only oxidizing ammonia and ammonium in the influent to nitrate. These systems do provide the additional benefit of degrading estrone (E1), a human hormone and major contributor to the estrogenicity of WWTP effluent, however. The objective of this research was to provide guidance on the impact that changes in wastewater treatment practices could have on E1 degradation. This was accomplished by comparing E1 removal in a laboratory-scale conventional nitrification system with that in a range of idealized laboratory-scale systems designed to remove total nitrogen from wastewater: the modified Ludzack-Ettinger (MLE) system (a two-stage anaerobic–aerobic system with recycle), a granular activated sludge system (cycled anaerobic–aerobic), a sequencing batch reactor (cycled anaerobic–aerobic), and an anaerobic ammonia oxidation (anammox) system. This work demonstrates that the choice of nitrogen removal technology used by a treatment plant could have an impact on the estrogenicity of WWTP effluent and that low energy total nitrogen removal systems do exist that are capable of excellent E1 removal.

Introduction

As we exceed planetary boundaries on nutrients, regional regulatory directives to remove total nitrogen from wastewater are likely to increase.[1–3] Traditionally, a combination of nitrification followed by denitrification has been used for total nitrogen removal from wastewater. This process is reliable, but is also energy- and in some cases, material-intensive. Energy costs are significant for an individual plant, typically accounting for 15–40% of a wastewater treatment plant's (WWTP's) budget.[4] Between 30 and 50% of a wastewater treatment

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† Electronic supplementary information (ESI) available: Further details on the experimental set-up, analytical procedures, and additional effluent E1 and N data for several of the experiments are presented in Supporting Information. See DOI: 10.1039/c7ew00164a

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plant's energy consumption comes from aeration during aerobic operation.[5,6] Pumping, as required in some nitrification–denitrification systems, also consumes a significant portion of energy, consuming from 10–15% of the total energy used at a plant.[5] Other denitrification systems rely on the addition of an external carbon source, which may represent a substantial operating cost. Finally, an additional resource constraint for many WWTPs is land availability; a plant without additional space for the expansion of treatment capacity must consider the footprint of any new technology to be added, including a denitrification step coupled to nitrification.

Fortunately, viable new processes that facilitate the transformation of influent ammonium (NH_4^+) to harmless dinitrogen gas (N_2) while minimizing energy use and/or chemical addition, and in some cases plant footprint, are increasing in number.[7] Anaerobic ammonia oxidation (anammox) and granular activated sludge are two promising emerging nitrogen removal technologies.[7] Anammox microorganisms anaerobically convert stoichiometric quantities of NH_4^+ and nitrite (NO_2^-) to N_2 in a single step without oxygen input.[8] Some oxygen is required to generate NO_2^-, but the aeration, and therefore energy requirements, are much lower than that of traditional nitrification.[9] Granular activated sludge systems utilize sequencing batch reactors (SBRs); this facilitates small wastewater treatment plant footprints through simultaneous COD removal, nitrification, and denitrification in one reactor, very long cell, or solids, residence times (SRT), and high biomass concentrations.[10,11] Microbial granules form with nitrifiers in contact with the bulk liquid on the outside of the granule, and denitrifiers shielded from dissolved oxygen on the inside of the granule.[12] A granular activated sludge system is operated with intermittent aeration, reducing energy costs, and a very short sedimentation period to select for large, fast-settling granules,[13] which also facilitates excellent settling. High chemical oxygen demand (COD) loading and high shear in the reactor are additional parameters that promote granule formation.[11]

Beyond predictability, one additional benefit of traditional nitrification and denitrification is the fact that estrogens, such as estrone (E1), can be effectively degraded concomitant with nitrification.[14,15] E1, a natural human estrogen excreted in urine, is one of the major estrogens present in wastewater effluent and is subject to variable removal.[16–20] E1 is biodegraded during aerobic wastewater treatment,[21–23] primarily by slow-growing heterotrophic organisms that aerobically degrade E1 while also degrading multiple low-concentration organic substrates.[14,24] As a result, E1 degradation is favored in the presence of low concentrations of microbiologically derived carbon,[24,25] long cell residence times,[14,23,26] and aeration.[22,23] Nevertheless, the range of conditions under which E1 degradation can occur is broader and has not been fully explored. Two pure cultures have been isolated that are capable of using E1 (1 mM) as their sole electron donor and nitrate as their electron acceptor,[27,28] and one study has also shown excellent E1 degradation (approximately 100%) in a one-stage nitritation/anammox process in the presence of low dissolved oxygen concentrations (0.6–1.2 mg L^{-1}).[29] Another recent study[30] demonstrated approximately 62% E1 loss as a result of both abiotic and biological degradation and sorption in an anammox batch reactor treating synthetic urine. Abiotic nitration of E1 has also been shown to occur in the presence of high concentrations of NO_2^-, but this is not a significant pathway for E1 removal under typical wastewater conditions.[31]

Given the potential energy and material savings and smaller footprints of alternative total nitrogen removal technologies, it is important to better understand how estrogens might degrade in these newer processes, so that process decisions can be made based on a more complete ecological risk analysis. It is not intuitive how the conditions under which anammox or granular activated sludge systems operate will impact E1 degradation, however.[14,24] Therefore, the objective of this research was to experimentally determine, in idealized systems at the laboratory scale, whether E1 was degraded during the steady state operation of a traditional nitrification–denitrification (modified Ludzack-Ettinger), anammox, and granular activated sludge system; a traditional nitrification system served as a positive control. This research should help plants simultaneously consider E1 removal and their chemical, energy, and physical footprint as they adapt to stricter nitrogen regulations.

Experimental section

Reactor seed

Each reactor experiment, excluding the anammox experiments, was seeded with a 10 mL aliquot of concentrated activated sludge collected once from the Metropolitan WWTP in St. Paul, MN. More information on the preparation of the aliquots is provided in the ESI† (section S1). Two anammox experiments were performed and both were seeded with 50% by volume sludge taken from a full-scale DEMON System (York River WWTP, Seaside, VA) and stored at 4 °C until use. After an upset in the first anammox experiment (days 13–20), the reactor was reseeded with an additional 10% by volume DEMON sludge on day 20 of that experiment.

Overall reactor set-up and operation

Lab-scale nitrification and nitrogen removal experiments were performed using three unique reactor systems, described in detail in the ESI† (section S2). These systems were tested in five different experimental set-ups: traditional nitrification (duplicated); modified Ludzack-Ettinger (MLE), anammox (duplicated), granular activated sludge, and sequencing batch mode.

The influent composition, reactor volume, hydraulic retention time (HRT), SRT, and temperature used for each reactor set-up and experiment are given in Table 1. Peristaltic pumps were used to control the influent flow rate in all experiments. In every experiment the reactor influent solution was amended with 10 μg L^{-1} E1. To prevent the addition of solvent to the influent solution, the required volume of E1 in

Table 1 Reactor operation

Experiment	Reactor type	Influent composition	Reactor volume (L)	HRT (hours)	SRT (days)
Nitrification	CSTR[a]	Wastewater	0.8	5	10
MLE	CSTR	Wastewater	Anaerobic: 0.2 Aerobic: 0.8	10	10
Anammox	SBR[b]	Synthetic wastewater	Two experiments conducted: 1 and 0.25	12	Not controlled[c]
Granular activated sludge	SBR	Synthetic wastewater	2	12	Not controlled
Sequencing batch reactor	SBR	Synthetic wastewater	2	12	Not controlled

[a] Continuously stirred tank reactor. [b] Sequencing batch reactor. [c] Solids were not purposefully wasted from experiment.

methanol was added to empty plastic influent containers the day before the wastewater or synthetic wastewater was added, to allow the methanol to volatilize. In the case of the second (0.25 L) anammox experiment, the E1 was added to the influent container dissolved in water. All reactors were operated at a temperature of 21 ± 2 °C (average ± standard deviation, used throughout), except for the anammox reactors, which were operated at 30 °C. The nitrification and MLE reactors were continuously stirred.

Nitrification and modified Ludzack-Ettinger experiments

The nitrification experiments, performed in duplicate, utilized the schematic shown in Fig. SI-1.† The MLE experiment was performed in the reactor shown in the schematic in Fig. SI-2.† A membrane (Minikros® 750 kDa mPES cross flow filtration membranes, Spectrum Labs) was used as a clarifier in these experiments to separate biomass from the liquid, enabling the decoupling of the SRT and HRT. During use, membranes were backwashed daily, or when the pressure in the membrane feed lines exceeded 5 PSI.

Primary effluent was collected weekly at the Metropolitan WWTP and held at 4 °C until used. As stated above, E1 was amended to the wastewater prior to feeding the reactors. After E1 amendment, the COD, total nitrogen, and E1 in the influent to the nitrification and MLE reactors were measured to be 299 ± 80 mg L^{-1} (n = 8), 47 ± 78 mg L^{-1} (n = 16), and 6.2 ± 3.1 μg L^{-1} (n = 16), respectively, where n is the number of replicate samples analyzed. The influent flow rates were 2.67 mL min^{-1} and 1.67 mL min^{-1} for the nitrification and MLE experiments, respectively. Air was introduced to the aerobic reactors (see Fig. SI-1 and SI-2†) *via* a diffuser. The aerobic reactor in the nitrification system maintained a DO of greater than 5 mg L^{-1} throughout the duplicate experiments. The aerobic reactor in the MLE experiment was adjusted to maintain a DO of >2 mg L^{-1}. The anaerobic reactor in the MLE experiment received recycled oxygenated mixed liquor at two times the influent flow rate (2Q), and maintained a DO of less than 0.2 from day 0 to 13 and less than 0.4 from day 13 to 30. On day 30 and day 33 the DO in the anaerobic reactor increased to 0.5–0.7 mg L^{-1} then returned to ≤0.4 mg L^{-1} until the end of the experiment. To control the SRT, 80 and 100 mL of mixed liquor was removed daily from the nitrification and aerobic MLE reactors, respectively. The E1 concentration in the effluent was analyzed with time.

Anammox experiment

Two anammox experiments were conducted, nearly identical to each other with the exception of the reactor size, one conducted in a 1 L SBR and one conducted in a 0.25 L SBR. Experiments were conducted according to the schematic in Fig. SI-3† and the operational parameters in Table 1. The anammox feed, adapted from van de Graaf *et al.*[32] (Table SI-1†), was amended with 10 μg L^{-1} E1. After amendment, E1 was measured in the influent at 4.5 ± 0.6 μg L^{-1} (n = 3) in the 1 L reactor and 13.0 ± 0.8 μg L^{-1} (n = 3) in the 0.25 L reactor, indicating some initial E1 loss in the feed bottle to the 1 L reactor. The SBR operation was based on that of Dapena-Mora *et al.*[33] and López *et al.*[34] During the experiment the reactors were continuously flushed with either 95% N_2/5% CO_2 (1 L reactor) or 100% N_2 (0.25 L reactor) *via* a diffuser to maintain anaerobic conditions. A control box was used to automate the SBR sequence, which was: fill to 100% volume with synthetic influent solution over the course of 4.5 hours, react 1 hour, settle for 15 minutes, draw down to 50% volume for 10 minutes, rest for 5 minutes. The 0.25 L reactor was operated without E1 feed for approximately 60 days prior to the addition of E1 to ensure that the nitrogen removal performance was as expected and was indicative of anammox activity.

Granular activated sludge and sequencing batch reactor experiments

The granular activated sludge and standard SBR experiments were operated according to the schematic in Fig. SI-4† and the operational parameters in Table 1. The two experiments were identical except that the influent to the standard SBR experiment contained a much lower COD (200 mg L^{-1}) compared to that in the granular activated sludge experiment (1000 mg L^{-1}), resulting in sludge that failed to granulate. Operation of granular activated sludge systems differs widely;[11,12] operation of this system was focused on establishing conditions in which biomass granulated and >50% total nitrogen removal occurred. The influent, freshly prepared each day and described in the ESI† (Table SI-2), was adapted from the *Syntho* medium of Boeije *et al.*[35] and was amended with 10 μg L^{-1} E1. After amendment, E1 was measured in the influent of the granular activated sludge and SBR experiment at 12.1 ± 2.3 μg L^{-1} (n = 3) and 10.5 ± 0.6 μg L^{-1} (n = 3), respectively. Total nitrogen in the influent was approximately 86 and 57 mg L^{-1} for the granular activated

sludge and SBR experiments, respectively. As with the anammox experiment, a control box was used to automate the reactor operating sequences, which were: fill to 2 L with 1 L influent solution, react anaerobically for 2 hours, aerate for 3.5 hours, settle for 5 minutes, draw down from 2 L to 1 L. During the aeration phase, air was introduced through a disc diffuser at the bottom of the reactor column (shown in Fig. SI-4†) with an upflow velocity of about 2 cm s^{-1}. Aeration was controlled with a solenoid valve coupled to the control box. Fig. SI-5† shows a photo of the granules that formed in the granular activated sludge experiment.

Abiotic control experiments

Three negative control experiments were also performed. One experiment was operated identically to the wastewater-fed nitrification experiments, except that only tap water and E1 were fed to the reactor system; this experiment was used to determine whether E1 sorption to the membrane clarifier or other reactor materials was significant. E1 was measured in the influent at 10.5 ± 1.2 μg L^{-1} (n = 2). A second batch sorption experiment was performed to determine the extent of E1 sorption to the solids (*i.e.*, killed biomass) in the system. This experiment was performed in batch to minimize the volume of sodium azide-contaminated waste generated. In this experiment, E1 dissolved in methanol (10 μg L^{-1}) was added to triplicate 500 mL glass bottles and the methanol was allowed to fully evaporate. Mixed liquor from the Metropolitan WWTP was diluted 50% by tap water to approximate the VSS in the biologically active reactors (586 mg L^{-1}, sampled in triplicate at both time = 0 and time = 4 days), amended with sodium azide (50 mM), mixed for 24 hours, and added (250 mL per bottle) to the bottles. Samples (well-mixed) for E1 analysis were taken over a four-day period. The final abiotic control experiment was performed in a manner identical to one of the anammox experiments, with the 0.25 L reactor fed only medium and E1 and operated as an SBR. E1 was measured in the influent (10.3 ± 0.2 μg L^{-1}, n = 3) and in the reactor liquid after three consecutive react cycles (hours 6, 12, and 18).

Water quality, ammonia, and total nitrogen measurements

Volatile suspended solids (VSS), dissolved organic carbon (DOC), COD, pH, and DO were monitored as described in the ESI† (section S3). Ammonia (measured as ammonium) and total nitrogen were measured colorimetrically *via* HACH Method 10031 and HACH Method 10072, respectively. Blanks were measured during each analysis and periodic standards were measured for quality assurance. Standards averaged 103 ± 6% of expected for the total nitrogen analysis and 97 ± 2% for the ammonium analysis, agreeing well with the HACH preprogrammed calibration curve.

Nitrate (NO_3^-) and NO_2^-

NO_2^- and NO_3^- were measured in filtered (0.2 μm) samples on a 761 Compact or 930 Compact Flew Metrohm ion chromatograph outfitted with an AS-18 column and 20 μL sample loop. The eluent was 3.2 mM sodium carbonate and 1 mM sodium bicarbonate. Gravimetric standards containing sodium nitrite and sodium nitrate salts in ultrapure water were prepared to generate calibration curves with at least 6 points. Typical limits of quantification were less than 0.2 mg L^{-1} as nitrogen (mg-N L^{-1}) for both NO_3^- and NO_2^-.

E1 and 17β-estradiol (E2) analysis

Samples (10–100 mL, depending on the experiment) were collected for E1 or 17β-estradiol (E2) analysis, described in the ESI† (section S4). Solid phase extraction and clean-up procedures were adapted from Tan *et al.*[14] Additional details are provided in the ESI† (section S5). Average E1 recovery for all samples, with the exception of the influent samples to the granular activated sludge and standard SBR experiments, was 60 ± 17.5%. E1 recovery was poor, 2–17%, in the influent to the granular activated sludge and SBR experiments as a result of the high COD in the influent interfering with the SPE. One sample (one time point in one triplicate reactor) in the abiotic sorption experiment had a recovery of 1% and was therefore discarded.

An Agilent 1100 series Liquid Chromatograph (LC) with a 4000 QTRAP triple quadrupole mass spectrometer was used to measure E1 and E2. The chromatography was performed on a Synergi 4u Polar-RP 80A 150 × 2.00 mm 4 μm particle size column (Phenomenex). A binary gradient was used for compound separation. The mass spectrometer was operated in negative ion, selected reaction monitoring mode. Additional details are provided in the ESI† (section S5). Blanks of 60 : 40 methanol : water, as well as periodic method blanks were analyzed. Standard curves consisted of seven to nine external standards; an internal standard was also used (see the ESI†). Limits of quantification were 2.3 μg E1 L^{-1} solvent extract and 18.2 μg E2 L^{-1} solvent extract, which corresponds to approximately 11.5 ng E1 L^{-1} sample and 91 ng E2 L^{-1} sample. E1, E2, and the ^{13}C-labeled surrogate were corrected using the internal standard. The ^{13}C-labeled surrogate contained a significant amount of unlabeled E1, up to 6 μg E1 L^{-1} solvent extract, corresponding to 12 ng E1 L^{-1} sample. Though extremely low, given that approximately 10 μg E1 L^{-1} sample was fed to the reactors, this addition of E1 was treated similarly to a standard addition and subtracted out, based on a calibration curve developed for E1 at each surrogate recovery concentration. This curve had a limit of quantification of 0.16 to 0.3 μg E1 L^{-1} solvent extract, corresponding to a concentration of 0.3 to 0.6 ng E1 L^{-1} sample.

Data analysis

The E1 and E2 sample concentrations were calculated as follows:

$$\text{Aqueous sample concentration} = \frac{[\text{In Vial Concentration}]}{(\text{Recovery}) \times (\text{Concentration Factor})}$$

Here the concentration factor was the sample volume/solvent extract volume, and recovery was the percent surrogate recovered, expressed as a fraction.

Limits of quantification for E1, E2, ^{13}C-labeled E1, NO_2^-, NO_3^-, and DOC were produced by generating a 95% confidence interval around the calibration curve using Excel or R. The confidence interval for the lowest standard was chosen as the limit of quantification for the NO_2^-, NO_3^-, DOC and invial E1 and E2 concentrations.

Reported *p*-values were generated with R or Excel software utilizing a two-sample, two-sided, un-pooled *t* test.

NO_3^- as a percent of nitrogen removal was calculated to determine whether the nitrogen species in the effluent of the anammox reactor corresponded to the expected theoretical stoichiometry of nitrogen removal during the anammox process. Expected theoretical stoichiometry of anammox is:[36]

$$1NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ \rightarrow 0.986N_2 + 0.161NO_3^- + 0.071C_{1.74}O_{0.31}N_{0.20} + 2.002H_2O$$

Nitrogen removal occurs when N_2 is generated. Therefore, from this theoretical stoichiometry, NO_3^- as a percent of nitrogen removal is:

$$\left(\frac{0.161\ \text{mol}\ NO_3^-}{0.986\ \text{mol}\ N_2}\right)\left(\frac{1\ \text{mol}\ N_2}{2\ \text{mol}\ N_2\text{–N}}\right)\times \left(\frac{1\ \text{mol}\ NO_3^-\text{–N}}{1\ \text{mol}\ NO_3^-}\right)\left(\frac{14\ \text{g mol}\ NO_3^-\text{–N}}{14\ \text{g}\ N_2\text{–N}}\right) = 8\%$$

The corresponding value in the anammox effluent samples was calculated as follows:

$$NO_3^-\ \text{as \% of nitrogen removal} = \frac{\left[NO_3^-\text{–N}\right]_E}{\left[\text{Total N}\right]_I - \left[NH_4^+\text{–N}\right]_E - \left[NO_3^-\text{–N}\right]_E - \left[NO_2^-\text{–N}\right]_E}$$

Results and discussion

The average influent E1 concentration in these experiments was 8.4 ± 3.7 μg L^{-1} (n = 27), with the exception of the 1 L anammox experiment, in which the average influent E1 concentration was lower ($P < 0.0001$), at 4.5 ± 0.6 μg L^{-1} (n = 3), suggesting that sorption of the E1 to the feed container, or perhaps some abiotic nitration, may have occurred.[31] The overall performance of all experiments, with respect to nitrogen removal and effluent E1 concentration, is summarized in Table 2.

Abiotic E1 loss

With a log K_{OW} of 3.13,[37] E1 had the potential to sorb to the plastic feed containers, tubing, reactors, membranes, and biomass in these experiments. Abiotic E1 loss was therefore assessed. Results are shown in Fig. 1 and Table 2. Sorption to the reactor materials used in the nitrification experiments resulted in some loss of E1, on average 5 μg L^{-1}, or 46% loss of the nominally fed 10 μg L^{-1} E1. Loss appeared to stabilize rapidly in the effluent, suggesting that sorption to the tubing and membranes occurred, but reached equilibrium quickly and never decreased below about 4 μg L^{-1} E1. Very little sorption to biomass or to the anammox SBR reactor was observed (Fig. 1, Table 2).

Nitrification and MLE experiments

The conventional nitrification and MLE experiments performed as expected with respect to nitrogen removal (Table 2, Fig. SI-6†). In the nitrification experiments, complete NH_4^+ removal was achieved by day 17, at which point approximately 62% of the influent NH_4^+ was converted to NO_3^-. After day 24, the MLE reactor stabilized at approximately 68% total nitrogen removal with a NO_3^--rich effluent (Table 2, Fig. SI-6†). Based on an internal recycle rate of 2Q, 67% total nitrogen removal was expected.[38]

As expected based on the literature,[14,15,21–23] E1 removal was also excellent in the nitrification and MLE experiments (Table 2, Fig. SI-7†). In the nitrification experiment, E1 was removed to <0.44 μg L^{-1} E1 throughout the experiment (Fig. SI-7†), which was significantly different than the effluent E1 concentration in the abiotic experiments ($P < 0.0001$). Similarly, in the MLE experiment E1 was present in the effluent at a concentration <0.42 μg L^{-1} throughout the experiment (Fig. SI-7†); again, significantly different from the effluent E1 concentration in the abiotic experiments ($P < 0.0001$). Assuming a nominal influent E1 concentration of 10 μg L^{-1}, both the conventional nitrification and MLE systems were capable of approximately 98% E1 removal.

These conventional nitrification and nitrification/denitrification technologies were expected to degrade E1 effectively as a result of the reactor conditions: low, consistent concentrations of dissolved organic carbon, constant aeration during nitrification, and a long solids residence time.[14,23,26] The removal performance observed in these experiments was also consistent with that observed in the literature.[15,39] Suarez *et al.*[39] utilized side-by-side nitrifying and denitrifying lab scale treatment systems to assess estrogen and personal care product removal. Excellent (99%) removal of E1 + E2 was observed in the aerobic nitrifying treatment system and good (72%) removal of the same was observed in the denitrifying treatment system. Analysis of a German full-scale plant also noted 98% removal of E1 + E2 after treatment with conventional nitrification and denitrification combined with phosphate removal.[15]

Anammox experiment

Strong evidence for anaerobic ammonia oxidation, in addition to excellent E1 removal, was observed during the

Table 2 Performance after days 3, 17, 13, 25, and 20 in the abiotic controls, nitrification, MLE, granular activated sludge, and sequencing batch reactor experiments, respectively. Anammox data is for the stable period between days 23 and 40

	Effluent DOC (mg L^{-1})	Reactor liquor VSS (mg L^{-1})	Total nitrogen removal (%)	Effluent [E1] (μg L^{-1})
Abiotic control clean water	NA[a]	NA	NA	4.9 ± 1.2 (n = 4)
Abiotic control Azide-killed	NA	586 ± 73 (n = 6)	NA	9.4 ± 1.9 (n = 11)
Abiotic SBR control	NA	NA	NA	10.6 ± 0.4 (n = 3)
Nitrification	10.5 ± 1.2 (n = 8)	710 ± 150 (n = 8)	38 ± 7 (n = 3)	0.26 ± 0.11 (n = 14)
MLE	11.9 ± 1.3 (n = 6)	300 ± 129 (n = 6)	68 ± 7 (n = 6)	0.23 ± 0.13 (n = 7)
Anammox (1 L)	14.5 ± 3.0 (n = 6)	200 ± 190 (n = 6)	77 ± 7 (n = 6)	0.01 ± 0.01 (n = 5)
Anammox (0.25 L)	n.d.[b]	1793 ± 140 (n = 3)	69 ± 16 (n = 11)	1.40 ± 0.36 (n = 3)
Granular activated sludge	31.3 ± 8.8 (n = 15)	n.d.[b]	73 ± 5 (n = 15)	10.4 ± 3.9 (n = 8)
Sequencing batch reactor	3.2 ± 0.8 (n = 12)	n.d.	27 ± 7 (n = 12)	0.44 ± 0.30 (n = 6)

[a] NA indicates that this parameter is not applicable. [b] n.d. indicates that this parameter was not determined.

laboratory-scale anammox experiment (Fig. SI-8, 2,† and Table 2). Based on stoichiometry, if a pure culture of anaerobic ammonia oxidizing organisms are present, the proportion of produced NO_3^-–N to the total nitrogen removed should be equal to 8% (see Data analysis).[36] The NO_3^- concentration as a percent of total nitrogen removed was near this value (12.4 ± 5% after day 2 in the first 1 L anammox experiment and 7.7 ± 3% after day 19 for the second 0.25 L experiment) (Fig. SI-8†). Between days 23 and 40 in the first experiment and days 19 and 67 in the second experiment total nitrogen removal ranged from 45–89% of the total influent nitrogen (Fig. 2), which agreed well with a similar laboratory-scale SBR study in which the average nitrogen removal was 78%.[33] A reactor upset, evidenced by floating biomass, NO_2^-–N accumulation to between 25 and 50 mg L^{-1}, and decreased total nitrogen removal efficiency, occurred between days 13 and 20, and on the final day of the first experiment (day 42) (Fig. 2A). Throughout the two anammox experiments, the effluent E1 concentration was 0.5 ± 0.7 μg L^{-1} (Fig. 2, Table 2), with 0.01 ± 0.01 μg L^{-1} effluent E1 in the 1 L reactor and 1.40 ± 0.36 μg L^{-1} effluent E1 in the 0.25 L reactor. These values were significantly different than the effluent E1 concentration in the abiotic experiments ($P < 0.0001$). Assuming a nominal influent E1 concentration of 10 μg L^{-1}, this corresponded to an average removal of 89–99.7% E1. Because the E1 concentrations in the influent were stable at 4.5 ± 0.6 μg L^{-1} (n = 3) and 13.0 ± 0.8 μg L^{-1} (n = 3) for the 1 L and 0.25 L experiments, respectively, abiotic E1 nitration was not responsible for the low effluent E1 concentrations.[31] In the second anammox experiment, both E1 and E2 were monitored in the effluent and E1 transformation to E2 was not observed, with influent E2 concentrations (likely as a result of impurities in the E1 feed) of 0.10 ± 0.01 (n = 3) and effluent E2 concentrations of 0.24 ± 0.14 (n = 3). These values were not significantly different from one another (P = 0.22).

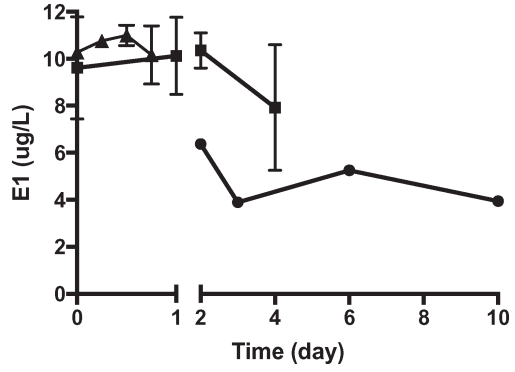

Fig. 1 E1 concentrations in the control experiments. Filled circles (●) show E1 effluent concentrations in the clean water flow-through control experiment. Closed squares (■) show E1 concentrations in the azide-killed biomass batch experiments. Closed triangles (▲) show E1 concentrations in the clean water SBR control experiment.

The excellent E1 removal observed during the anammox experiments was somewhat unexpected. E1 degradation is typically associated with aerobic systems with low organic carbon concentrations and a long SRT.[24,26,39] E1 degradation was observed in a nitritation/anammox process[29] and in a recent study assessing the degradation of E2 and E1 during the anammox treatment of synthetic urine.[30] Nevertheless, experiments assessing the potential of E1 to degrade under anaerobic conditions with anaerobic digester sludge, activated sludge, and upflow anaerobic digester sludge showed little potential for E1 to degrade.[40] It was therefore assumed that E1 degradation might not occur, or would be slow, as was recently observed in an anammox experiment.[30] Excellent and rapid E1 degradation did occur without concomitant E2 formation, however. In this experiment, the long SRT may have allowed anaerobic E1 degraders, such as E1-utilizing denitrifiers,[27,28] to grow in the initial sludge sample and begin degrading E1 soon after experiment initiation. Alternatively, the anammox microorganisms themselves may have played a part in E1 degradation.

Granular activated sludge and standard SBR experiments

In the laboratory-scale granular activated sludge experiment, stable granules formed (Fig. SI-5†), 61–87% of the influent nitrogen was removed, and nitrogen removal was stable (Table 2). The performance of this system agreed well with the total nitrogen removal reported in a continuously aerated granular activated sludge experiment (65–82%)[41] and in a full-scale granular activated sludge plant (60%).[42] Effluent DOC averaged 31.3 ± 8.8 mg L^{-1} (Table 2), showing that

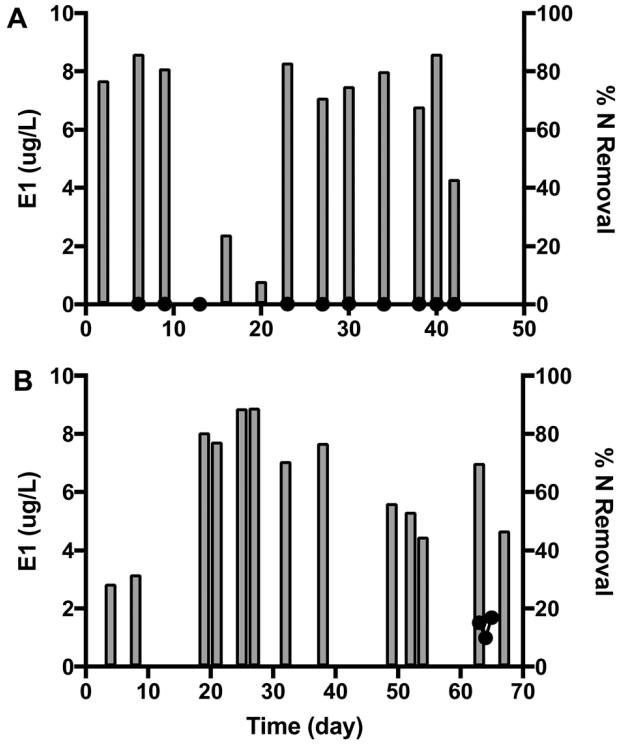

Fig. 2 E1 and total nitrogen removal in the anammox experiments, with (A) showing results from the 1 L reactor and (B) showing results from the 0.25 L reactor. Here, closed circles (●) show effluent E1 concentrations and grey bars show percent total nitrogen (%N).

stable overall carbon removal >90% occurred throughout the experiment as well. Unlike the other reactor systems investigated, the granular activated sludge system provided only very limited E1 removal, with an average effluent E1 concentration of 10.4 ± 3.9 μg L^{-1} after day 25 (Table 2, Fig. 3). This was not significantly different than the effluent concentration measured in the abiotic experiments (P = 0.26). It is possible that high influent COD concentrations select for fast-growing bacteria at and near the surface of the granules, where aerobic conditions existed. This would select against slower-growing E1 degrading microorganisms, as has been observed by others.[14,24] Alternatively, abundant electron donor could have altered the expression of metabolic pathways, switching off "scavenging" functions and as a result, hindering E1 removal.[24] A third, albeit unlikely possibility is that the reactor configuration had a negative impact on E1 removal.

To clarify the importance of high COD concentrations *versus* reactor configuration, an additional SBR experiment was performed, identical to the granular activated sludge experiment except that the influent COD was lowered from 1 g L^{-1} to 200 mg L^{-1} COD. No granule formation was observed in this experiment, and while organic carbon was degraded effectively, nitrogen removal was negatively affected (Table 2, Fig. 3), likely because of the short settling time. Nevertheless, with the lower influent COD concentration and all other operating parameters identical, E1 removal was excellent, unlike the removal observed in the granular activated sludge experiment. Indeed, the effluent E1 concentrations in this SBR experiment were ≤0.82 μg L^{-1} E1 after day 20, significantly differing from the effluent E1 concentrations in the abiotic experiments ($P < 0.0001$) (Fig. 3). If a nominal influent E1 concentration of 10 μg L^{-1} is assumed, this corresponds to an average E1 removal of 96%. This level of E1 removal was similar to that observed during the nitrification, MLE, and anammox experiments. It is therefore likely that the lower COD concentrations allowed E1 degraders to compete and grow despite the loss of nitrogen removal capacity. As stated above, granulation did not occur in this reactor without high influent COD concentration and loading. Others, however, have observed granulation at lower influent COD concentrations and loadings;[12] therefore, it is possible that under those conditions E1 degradation would occur in a granulated system. The excellent E1 removal performance observed in the standard SBR experiment agreed with results from the literature in which excellent (60–90%) E1 removal was observed in similarly operated NO_2^--accumulating SBRs.[43]

Environmental implications and extrapolation of results

The degradation of E1 is only beneficial if its degradation products no longer contribute to the estrogenicity of the

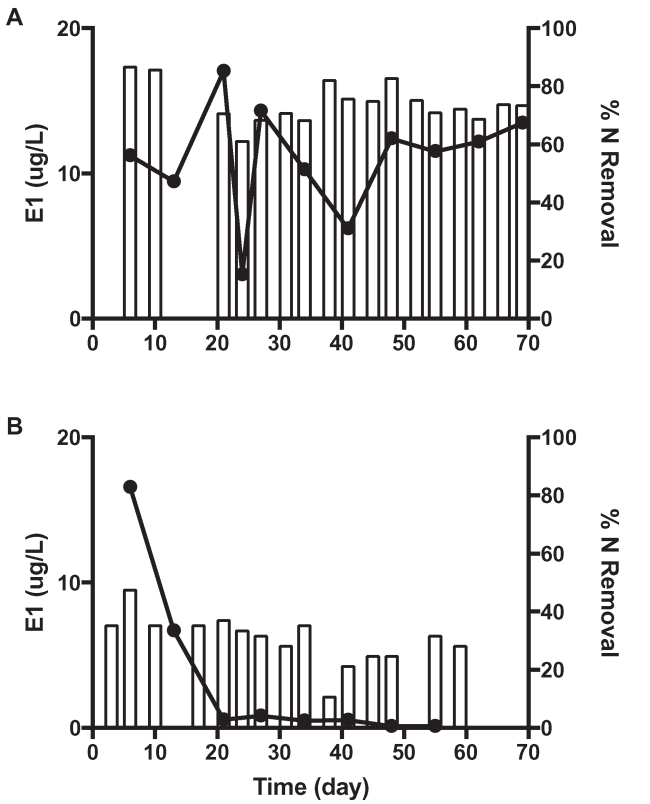

Fig. 3 Effluent E1 concentrations and total nitrogen removal in the granular activated sludge (panel A) and sequencing batch reactor (panel B) experiments. Here, solid lines with closed circles (●) show effluent E1 concentration and bars show percent nitrogen (%N) removed.

effluent. In conventional aerobic systems this is a reasonable assumption based on the current literature, where estrogen degradation in aerated activated sludge has been associated with decreased estrogenicity. In one study,[44] 95–100% removal of total estrogens in a conventional aerated full-scale WWTP operated with nutrient removal corresponded to 87–99% removal of estrogenicity. In another study,[45] the aerated stage of treatment in a survey of five WWTPs was associated with the greatest decrease in estrogenicity. These results indicate that the removal of E1 in conventional nitrification and nitrification–denitrification processes is likely to be associated with a corresponding removal of estrogenicity. This coupled removal of E1 and estrogenicity cannot be assumed, however, in the absence of oxygen, such as in an anammox process. Indeed, the products of E1 transformation, although shown not to be E2, were unidentified in our experiments. E1 has also been shown to slowly transform to unknown products during anammox treatment of synthetic urine.[30] Further study is needed to determine the products associated with E1 removal in this system and to determine whether the removal of estrogenicity occurs as well.

In this study, the nitrogen removal technologies that were capable of E1 removal shared one trait: the presence of low organic carbon concentrations. Effluent DOC was about 10 to 15 mg L^{-1} in the nitrification, MLE, and anammox reactor systems, indicating that organic carbon was present in each system, but at relatively constant, low concentrations. The presence of low concentrations of organic carbon combined with a long SRT should stimulate the growth of multiple substrate degrading heterotrophs, the microorganisms implicated in E1 degradation in aerated systems.[14] The influent to the aerobic granular sludge reactor operated in this research contained a high COD, consisting of entirely soluble synthetic wastewater constituents (Table SI-2†), which likely fostered the rapid growth of aerobic heterotrophs. Although the effluent COD was relatively low (approximately 30 mg L^{-1}), the feast-famine conditions in this reactor either selected against slower growing E1-degrading microorganisms[14,24] or altered the expression of the "scavenging" functions of E1-degrading multiple substrate utilizers.[24] It is possible that granular activated sludge systems operated differently[12] could degrade E1, or under certain conditions denitrifiers capable of growth on E1 could be active.[27,28] Nevertheless, nitrogen removal technologies that result in the presence of low and relatively constant concentrations of organic carbon are more likely to remove E1 than those technologies in which high, or highly fluctuating, concentrations of organic carbon are present.

If there is a need for total nitrogen removal and concerns about effluent estrogen, such as at plants that discharge to effluent-dominated receiving bodies, low energy treatment options do exist. Indeed, E1 removal was comparable during MLE and anammox treatment. Anammox has an advantage over conventional nitrogen removal in that the partial nitritation phase consumes much less oxygen than conventional nitrification, and therefore, much less energy.[9] This offers a distinct advantage if the anammox process can be reliably mainstreamed. This work demonstrated that anammox technology can remove an important estrogenic contaminant, E1, while also effectively removing total nitrogen. As long as the degradation products of E1 produced during anammox are not harmful and estrogenic, anammox treatment has the potential to be a practical and effective treatment method for wastewaters that are both nitrogen-rich and estrogenic. Finally, given that implementation of anammox requires aeration to accomplish partial nitritation, E1 removal would likely be further enhanced in such a process.

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Thermal modulation of anthropogenic estrogen exposure on a freshwater fish at two life stages

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ABSTRACT

Human-mediated environmental change can induce changes in the expression of complex behaviors within individuals and alter the outcomes of interactions between individuals. Although the independent effects of numerous stressors on aquatic biota are well documented (e.g., exposure to environmental contaminants), fewer studies have examined how natural variation in the ambient environment modulates these effects. In this study, we exposed reproductively mature and larval fathead minnows (*Pimephales promelas*) to three environmentally relevant concentrations (14, 22, and 65 ng/L) of a common environmental estrogen, estrone (E1), at four water temperatures (15, 18, 21, and 24 °C) reflecting natural spring and summer variation. We then conducted a series of behavioral experiments to assess the independent and interactive effects of temperature and estrogen exposure on intra- and interspecific interactions in three contexts with important fitness consequences; reproduction, foraging, and predator evasion. Our data demonstrated significant independent effects of temperature and/or estrogen exposure on the physiology, survival, and behavior of larval and adult fish. We also found evidence suggesting that thermal regime can modulate the effects of exposure on larval survival and predator-prey interactions, even within a relatively narrow range of seasonally fluctuating temperatures. These findings improve our understanding of the outcomes of interactions between anthropogenic stressors and natural abiotic environmental factors, and suggest that such interactions can have ecological and evolutionary implications for freshwater populations and communities.

1. Introduction

Human-mediated environmental changes to aquatic ecosystems are occurring at an unprecedented rate, with potentially severe repercussions for resident wildlife. Habitat alteration or loss (e.g., nutrient loading, increased sedimentation, or physical changes due to land-use), invasive species introductions, over-harvesting, and influxes of aquatic contaminants have globally recognized, clear, and adverse effects on the health and viability of aquatic biota (Global Biodiversity Outlook II, 2006; Keister et al., 2010). Such stressors are typically studied in isolation; however, interactions among multiple anthropogenic stressors, or between stressors and natural abiotic environmental factors such as dissolved oxygen, pH, salinity, UV radiation or temperature (Crain et al., 2008; Häder and Gao, 2015; Heugens et al., 2001; Holmstrup et al., 2010; Laskowski et al., 2010) have the potential to modulate or exacerbate the impacts of human-mediated environmental change at both individual and population levels. For example, interactions between inputs of inorganic nutrients and organic matter have been shown to alter the dynamics of food webs in marine intertidal ecosystems (O'Gorman et al., 2012). Changes in the toxicities of aquatic contaminants in response to variation in UV-B exposure or salinity are also well documented (Hall and Anderson, 1995; Pelletier et al., 2006). Although the outcomes of these multi-factor interactions are often cumulative or synergistic, they can also be unpredictable (Christensen et al., 2006; Muthukrishnan and Fong, 2014; O'Gorman et al., 2012; Shears and Ross, 2010; see also Crain et al., 2008; Darling and Cote, 2008), or vary across space or time (Molinos and Donohue, 2010; Newman and Clements, 2008), including life stage (Przeslawski et al., 2015; Salice et al., 2011). Thus, concerted efforts to understand the impacts of anthropogenic change under more complex, real-world scenarios are of key importance for predicting and mitigating adverse effects on aquatic ecosystems.

Freshwater fish populations are often geographically restricted, and are likely to be especially vulnerable to declines in abundance or extirpation due to anthropogenic stress (Dudgeon et al., 2006; Heino et al., 2009). Among the most pressing threats to freshwater fish is chemical pollution; urban, industrial and agricultural runoffs, and wastewater treatment plants, continually discharge contaminants into rivers and streams (Kolpin et al., 2002), many of which bind to organismal hormone receptors and disrupt the normal endocrine functioning of

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exposed individuals (Kuiper et al., 1998). Because rates of introduction typically exceed chemical half-lives (Daughton, 2002), endocrine disrupting chemicals (EDCs) are common in the environment during critical life stages, such as during early development or at reproductive maturity. Exposure to EDCs has been shown to induce a variety of adverse molecular, behavioral, and physiological effects in both juvenile and adult fish (Bhandari et al., 2015; McGee et al., 2009; Niemuth and Klaper, 2015; Saaristo et al., 2010; van Aerle et al., 2002; Ward and Blum, 2012). Furthermore, empirical work and population modeling have convincingly demonstrated that these individual-level effects can dramatically impair the viability and sustainability of aquatic populations (Brown et al., 2015; Kidd et al., 2007; Palace et al., 2009).

Efforts to assess the impacts of EDCs on natural populations, however, are complicated by the fact that rates of chemical degradation in the environment (Starner et al., 1999), and uptake and elimination by organisms (Gordon, 2003), are dependent on the ambient temperature of the environment (Cairns et al., 1975; Heugens et al., 2001). In fish and other ectothermic aquatic species, temperature governs a wide array of fundamental physiological processes, including sexual determination, rates of early development, cellular signaling, biochemical reactions, and basal metabolic activity (Crockett and Londraville, 2006; Ospina-Alvarez and Piferrer, 2008), with potential to modulate the responses of organisms to toxicants in various ways (Brown et al., 2015; Hallare et al., 2005; Heugens et al., 2001, 2003; Khan et al., 2006). For example, increases in temperature have been shown to exacerbate EDC-induced production of vitellogenin (*vtg*; an egg yolk protein precursor normally only found in females) in juvenile salmonids (Körner et al., 2008; Korsgaard et al., 1986; Mackay and Lazier, 1993), and to influence EDC-induced skewed sex ratios in zebrafish (Brown et al., 2015). At higher temperatures, EDC exposure also synergistically increases mortality and impairs embryogenesis (Osterauer and Kohler, 2008). Cumulatively, the data collected to date suggest that chemical toxicants can interact with the thermal conditions to influence mortality and physiological impairment (Gordon, 2003; Heugens et al., 2001).

By comparison, little is known regarding the interactive effects of temperature and EDC exposure on the behavior of fish and other aquatic organisms (Manciocco et al., 2014). This deficit is significant, because an individual's behavior represents integrated physiological and developmental responses to the environment (Clotfelter et al., 2004), and altered inter- and intraspecific trait-mediated behavioral interactions that impact individual fitness, such as predator-prey relationships, competition for resources, or reproduction, have potential to reduce population abundances and alter the structure and function of aquatic communities (Clotfelter et al., 2004; Kidd et al., 2014). In this study, we conducted a factorial experiment in the laboratory to determine the extent to which temperature modulates the survival, development, reproductive physiology and interspecific (foraging ability, predator evasion) and intraspecific (male-male competition) behavioral interactions of a freshwater fish, the fathead minnow (*Pimephales promelas*), exposed to a common environmental estrogen, estrone (E1), during larval development and at sexual maturity. Our aims were threefold; first, we tested the general hypothesis that temperature modulates the dose-dependent effects of estrogen exposure at both larval and adult life stages. Second, we assessed the extent to which independent and interactive effects of E1 exposure and temperature differ across fitness contexts, specifically predator evasion, foraging efficiency, and territorial defense. Third, we compared the general susceptibility of fish to behavioral impairment during early development and at sexual maturity. To date, most single studies have focused on the effects of exposure at a single life stage (but see Oliveira et al., 2009; Parrott and Blunt, 2005; Schultz et al., 2012 for examples to the contrary); but growth and survival during the early stages of life, and successful reproduction at maturity, all directly impact individual fitness. Thus, knowledge regarding the effects of contaminant exposure at multiple life stages is a prerequisite to accurately assessing and predicting impacts under complex, real-world scenarios.

2. Material and methods

2.1. Experimental design

To test the hypothesis that the biological effects of estrogen exposure are modulated by ambient temperature, we exposed breeding groups of fathead minnows (two mature females, one male) to a low, medium or high concentration of E1 (i.e., $E1_{LOW}$, $E1_{MED}$, or $E1_{HIGH}$) dissolved in EtOH, or to EtOH alone (Control), at one of four temperatures (15, 18, 21, 24 °C) for 30 days (16 total treatments; 10–14 breeding groups per treatment). These temperatures reflect natural spring and summer seasonal variation in northern temperate streams, rivers and lakes and are well within the thermal tolerance limits for *P. promelas* (Pyron and Beitinger, 1993). Throughout the exposure period we monitored the fecundity and fertility of females and males. Beginning on day 10 and lasting through day 17, we collected one clutch of eggs from each breeding pair and placed it in a breeding basket in the parental aquarium. On days 29 and 30, we tested the parental subjects in two behavioral assays designed to assess the independent and interacting effects of temperature and estrogen on the foraging ability of males and females, and the territorial aggression of resident male fish towards a conspecific male intruder. We conducted two additional assays to assess the predator escape performance and foraging ability of exposed and control 21-day-old larval fish reared at different temperatures. All subjects were sacrificed immediately following the completion of testing via a lethal concentration of $NaCO_2$-buffered MS-222 (Western Chemical, WA, USA). The subjects were dissected (adults) or stored in RNA*later*® (Thermo-Fisher Scientific, MA, USA) (larvae) for use in a separate study. All procedures, and care and maintenance protocols, were approved by the Institutional Animal Care and Use Committee (protocol number 8-73) at St. Cloud State University.

2.2. Subjects, housing and maintenance

Six-month-old, reproductively mature *P. promelas* were purchased from a laboratory culturing facility (Environmental Consulting and Testing, WI, USA) and shipped to St. Cloud State University at bi-monthly intervals between March and July 2015. We chose *P. promelas* to test the hypothesis that the biological effects of estrogen exposure are modulated by ambient temperature because this species is widespread in North America, and considered to be a model species for ecotoxicology research (Ankley and Villeneuve, 2006). Upon arrival (day 0), the fish were introduced directly into the exposure apparatus and permitted to acclimate to their surroundings for 24 h before the experiment was started (day 1). During this time holding temperatures were increased or decreased as necessary to reach the experiment-specific ambient water temperature. The fish were maintained under a 16 h light: 8 h dark photoperiod, and fed an ad libitum diet of frozen brine shrimp (*Artemia franciscana*, San Francisco Bay Brand Inc., CA, USA) and bloodworms (*Glycera* spp.) twice daily for the duration of the experiment. F1 generation larvae were fed newly hatched brine shrimp (Brine Shrimp Direct, UT, USA) twice daily, beginning two days after hatching. The aquaria were cleaned of debris and monitored daily for mortality.

2.3. Exposure chemicals

Estrone is a common natural estrogen discharged in wastewater effluent, and is representative of a broad class of steroidal hormones and other chemicals with estrogenic activity. In a U.S. national survey, Kolpin et al. (2002) reported concentrations of E1 in rivers ranging from <5 ng/L to 112 ng/L.

Powdered estrone (≥99% purity) was obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in 100% ethanol (1687.5 μg/mL). In accordance with EPA guidelines for short-term exposure studies, this solution was then serially diluted with EtOH to produce low, medium (5×) and high (25×) treatment stock solutions with nominal concentrations of

67.5, and 337.5, and 1687.5 μg/mL, verified for accuracy before the start of the experiment using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The three E1 solutions, and an EtOH solvent control, were stored in amber glass bottles at 4 °C for the duration of the experiment. For all treatments, aqueous exposure solutions were prepared every three days in darkened glass carboys via the addition of an appropriate quantity of stock solution to 10 L of conditioned, non-chlorinated well water (two carboys per treatment). Each solution was thoroughly mixed by agitating the bottles for 10 s and the neck of each bottle was covered tightly with aluminum foil.

2.4. Exposure apparatus and regime

The fish were maintained throughout the exposure period in 12 L plexi-glass aquaria (30.5 × 30.5 × 30.5 cm) divided in half by the addition of a stainless steel mesh partition to accommodate two breeding groups, each of which was composed of one male and two females (total of 56 breeding groups; 10–14 aquaria per treatment). Each aquarium was covered on the sides and back with neutral-colored contact paper, and equipped with an airstone, a semi-circular polypropylene spawning tile, and a mesh basket to prevent egg predation. An LED strip light placed ~30 cm above each aquarium provided illumination.

Water amended with E1, or an equivalent volumetric percentage of EtOH (0.0002% v/v), was continuously gravity-fed to the aquaria from eight stainless steel mixing chambers (two chambers per treatment). Each mixing chamber served eight aquaria. A Cole-Palmer Masterflex 7523-40 peristaltic pump (Vernon Hills, IL) was used to draw the exposure solutions from the carboys into the mixing chambers via stainless steel tubes. A continuous flow of ground water from a dedicated well was added to the exposure solution in each mixing tank at a flow rate of approximately 900 mL/min for estimated final E1 aquarium concentrations of 5, 25, and 125 ng/L (i.e., low, medium and high treatments). Throughout the 30-day exposure period, the incoming ground water was maintained at a constant temperature via a thermostat-controlled head tank. Due to space and equipment restrictions, separate 30-day exposures were conducted for each temperature. The temperature order, and the spatial locations of the E1 mixing tanks relative to one another were randomized at the start of the study.

Water quality parameters including dissolved oxygen, total dissolved solids, pH, salinity and temperature were measured daily using a handheld multi-parameter sampling instrument (model 556 MPS, YSI Instruments, OH, USA). The presence of chlorine was monitored twice weekly using water quality test strips (Hach, CO, USA). In addition, water samples were collected in 1 L high-density polyethylene (HDPE) containers from the outflow of the stainless steel mixing tanks at three-day intervals throughout the exposure period and frozen at −20 °C for chemical analysis of E1. The E1 concentrations of two randomly selected samples of each of the 12 treatments were measured at the conclusion of the experiment via LC-MS/MS (Axys Analytical Services, Sydney, BC, Canada; Method MLA-075, 2014) (total n = 21 samples, 6-8 samples per concentration).

2.5. Reproduction, survival and growth

During the exposure reproductive groups were assessed daily for spawning activity. Fecundity (number of eggs laid in each clutch), and fertilization success (the proportion of fertilized eggs, identified by the presence of eyespots) were recorded for each clutch. Once eyespots appeared (~3 days) we removed the spawning tile and replaced it with a fresh one. However, between exposure days 10 and 17 we collected one clutch from each breeding group and placed it into a breeding basket located in the parental aquarium. From these we recorded latency to first hatch (in days, from the time that the eggs were laid) and the number of eggs that successfully hatched to produce free-swimming larvae; these larvae were reared in the exposure tanks for 21 days, beginning from the day that the eggs were laid, and then used in the behavioral assays.

2.6. Behavioral assays

We performed four behavioral experiments designed to assess the independent and interactive effects of temperature and estrogen exposure on intra- and interspecific interactions in three contexts (reproduction, foraging, and predator evasion). All trials were performed in conditioned well water (aerated for 24 h prior to use). Larval assays were conducted on day 21 of larval exposure in a testing chamber at room temperature between 0800 h and 1300 h, under differential lighting. Depending on the experiment and the number of surviving larvae, we tested the responses of one to six subjects from each clutch. Because male territory defense tests were conducted in situ (i.e., in each focal male's home tank), all adult assays were conducted on day 29 or 30 between 0800 h and 2200 h at the ambient temperature maintained during exposure. Each larval or adult subject was only used once in a given assay.

2.7. Experiment 1: effects of E1 and temperature on larval escape performance

The 'C-start' is a broadly conserved evasive locomotor fixed-action pattern response exhibited by fishes and other aquatic organisms (Domenici and Blake, 1997; Hale et al., 2002). The response is initiated by the perception of a stimulus, and is manifested by bending the body into a C-shape followed by a bout of burst swimming away from the stimulus at a 90° angle (see Supplementary video 1). Importantly, variation in predator avoidance performance correlates with the probability of surviving a predatory attack (Walker et al., 2005).

We assessed larval escape performance using an established methodology for the quantification of fast-start locomotive mechanics (McGee et al., 2009). Briefly, at the start of each trial, a random subject was placed into a clear-bottomed, 5-cm-diameter testing arena containing 10 mL of conditioned well water. The arena was centered on a pad containing a vibrational chip used to deliver a non-point source stimulus to the subject. The pad was covered with a 1 mm × 1 mm grid to allow for quantification of the response, and illuminated via a Kessil A150 fiber optic light source (Richmond, CA) angled 20 cm above the arena. Subjects were permitted to acclimate to the arena for 1 min, after which the stimulus (~0.5 s in duration) was delivered. Subject responses were recorded using a Redlake MotionScope (Tucson, AZ) high-speed camera (1000 frames s^{-1}) positioned ~25 cm vertically above the test arena.

One observer, blinded to the treatment identity of the fish, quantified the latency to the induction of the escape response, escape velocity, turning angle, and total escape response from the videos using the software program ImageJ (National Institutes of Health, Bethesda, MD). Two anatomical landmarks, the anterior tip of the snout and the posterior tip of the tail, were digitized on each video and used to calculate standard body length (BL). Two additional landmarks were digitized on the grid at a 1 mm distance to account for scale. Latency was recorded as the length of time to induction of movement (in ms). Velocity was calculated during the first 40 ms after the initiation of movement, and adjusted to body lengths (BL) per ms per Blob et al. (2007). The total escape response of each subject was calculated as BL / (latency in ms + 40 ms); we included this value because it simultaneously takes into account changes in both velocity and latency (McGee et al., 2009). We measured the turning angle as the angle of rotational movement, relative to the initial head position at the onset of the stimulus. Trials with latency responses <6 ms were considered false starts and were discarded prior to statistical analysis.

2.8. Experiment 2: effects of E1 and temperature on larval foraging ability

We conducted a larval foraging assay to assess differences in foraging ability among the treatments. The night before each trial, we placed two random test subjects from the same clutch in a 3.8-cm-diameter

feeding arena containing 10 mL of conditioned well water. Test subjects were deprived of food for 18 h prior to testing to ensure complete evacuation of their digestive system, confirmed in preliminary tests by viewing the transparent larvae under a microscope. Trials were recorded using a Canon NTSC Optura 20 digital Hi-8 video camera positioned 40 cm above the test arena, and illuminated by a Kessil A150 fiber optic light source.

Subjects were permitted to acclimate to the arena for 1 min at the start of each trial. Following the acclimation period, we administered a prey aliquot consisting of a known quantity of freshly hatched *Artemia* nauplii (mean ± SD, 31 ± 4; range, 22–37) to the center of the arena via a glass pipette. The subjects were permitted to forage freely for 60 s, and then immediately euthanized via the lethal addition of 2 mL Na_2CO_3-buffered MS-222 administered directly to the test arena with a dedicated glass pipette. The test subjects were removed from the arena, and 2 mL of formalin was added to euthanize the surviving *Artemia*. One observer, who was blind to the treatment identity of the test subjects, counted the number of surviving *Artemia* under an Olympus dark-field microscope (Center Valley, PA). We subtracted the number of remaining prey from the initial quantity to obtain the number of prey items eaten in each trial. Similar to the procedure described for experiment 1, we used ImageJ to measure the BL of each fish in each trial from video images; we examined the relationship between size and consumption in preliminary analyses (see 'Statistics').

2.9. Experiment 3: effects of E1 and temperature on adult foraging ability

For consistency with experiment 2, we determined the ability of individual adult fish to capture and consume prey using an appropriately modified procedure. Test subjects were deprived of food for 24 h prior to testing. At the start of each trial, one male or female fish was placed in a 20-cm-diameter stainless steel arena equipped with a 240-μm mesh bottom that permitted the passage of water. The arena was located in the center of a plastic chamber (40 × 30 × 23 cm) containing water at the appropriate exposure temperature (i.e., 15, 18, 21, or 24 °C). One end of a piece of flexible PVC tubing was positioned at the center of the arena just under the water surface, to allow for food delivery. The other end of the tube was attached to a syringe mounted on a retort stand outside of the apparatus that was not visible to the test subject.

Subjects were permitted to acclimate to the arena for 10 min at the start of each trial. Following the acclimation period, we gravity-fed a prey aliquot of 30 mature *Daphnia pulex* in 10 mL conditioned well water to the center of the arena via the feeding tube. The subject was permitted to forage freely for 5 min before the test was stopped, and the subject removed from the arena via a hand net and measured for BL. We then removed the arena from the water chamber, drained the water, and counted the number of *Daphnia* that remained on the mesh. The arena was thoroughly washed between trials to remove any potential odor cues. For the same reason, we also replaced the water in the chamber between trials.

2.10. Experiment 4: effects of E1 and temperature on male territorial aggression

All tests examining the independent and interactive effects of E1 exposure and temperature on the agonistic behavior of male minnows were conducted by a single observer; daily observations made during the exposure period indicated that all males readily established territories under the provided spawning tile. In each test we presented the focal subject (i.e., the resident male) with a randomly selected, non-exposed, conspecific male intruder within a 9-cm-diameter × 12-cm-tall cylindrical glass jar capped with fine mesh. The size of the jar permitted limited movement of the stimulus male, thereby minimizing variation in intruder behavior across trials (Ward and McLennan, 2006). In total, we used 26 stimulus males. Each male was used between 3 and 16 times and males were placed in an 8 L tank and given a 1 to 3 h rest period after ~20 to 30 min of testing. After testing, we recorded the size (BL) of each stimulus male using digital calipers. The BL of the resident male was similarly obtained at the conclusion of the experiment. The resident (mean ± SD, 53.09 ± 4.98; range, 43.00–79.00) and stimulus males (52.01 ± 4.12; range, 44.81–61.61) tested were comparable in size across all treatments. However, we calculated the size ratio of the two males in each test and included this value as a covariate in preliminary statistical analyses (see 'Statistics').

To begin each trial, the jar was placed in the center of the tank, at a distance of 15 cm from the spawning tile. The behavior of each focal subject towards the intruding male was directly observed for 5 min from behind a blind, and the frequencies of two well-described aggressive behaviors, butting and strikes (McMillan and Smith, 1974; Pyron and Beitinger, 1989), were recorded in real time. A butt was defined as a slow approach towards the intruding male that culminated in closed-mouth contact between the snout of the resident male and the jar. A strike was defined as a fast approach towards the intruding male, accompanied by propulsive tail beats that culminated in either a closed-mouth bump or open-mouth snap. In addition, we recorded the latency (in s) to the first agonistic response.

2.11. Statistics

We compared the level of spawning activity among treatments using a chi-square test based on the total number of clutches produced in each treatment. We similarly used a chi-square test to compare the survival of adult fish among treatments. The effects of exposure concentration and temperature on clutch size, fertilization success, hatching latency, and larval survival were examined via ANOVAs; larval growth (BL on day 21) was tested via Generalized Estimating Equations (GEE) (Hardin and Hilbe, 2012), to account for possible genetic correlations among individuals from the same clutch. As appropriate, we arcsine or log transformed the proportions of fertilized eggs, surviving larvae, and larval BL to meet parametric assumptions. We used pairwise post-hoc tests (Least Significant Difference; LSD) to compare dependent variables across levels for significant effects.

We directly compared the among-treatment responses of larvae to the simulated predator (experiment 1), and their foraging abilities (experiment 2), using GEE models. For experiment 1 we fit marginal models to each of our three continuous response variables (latency to first response, escape velocity and total escape response) using an identity link function. For experiment 2, we fit the model to the number of prey eaten specifying a Poisson distribution with a log link function. For each model we selected and validated the appropriate correlation structure using the Quasi Likelihood Under Independence Model Criterion (QIC) (Hardin and Hilbe, 2012; Pan, 2001). All models tested the effects of E1 concentration, temperature, and the interaction between these two terms. In preliminary models for experiment 2 we also included the average BL of the two larvae in each trial as a covariate, to account for differences in prey consumption due to size. We did not find a significant main effect of size on the number of prey eaten in each trial, nor evidence of significant interactive effects with temperature or concentration. Therefore, we removed the size term prior to final analyses.

We examined whether adult fish from the 16 treatments differed in the number of prey consumed (experiment 3) using a Generalized Linear Model (GLM) with a negative binomial distribution and log link function, which corrects for the presence of zeros in the dataset (O'Hara and Kotze, 2010). We specified temperature, E1 concentration, and sex (male or female) as fixed factors in the model and included the interaction terms. In preliminary models we examined the influence of size on prey consumption by including BL as a covariate. We did not find a significant main effect of size on the number of prey eaten in each trial, nor evidence of significant interactive effects with temperature or concentration. Therefore, we removed the size term prior to final analyses.

We examined variation in male agonistic behavior towards conspecific males (experiment 4) using one-way ANOVAs with either the

frequency of agonistic displays or latency to response specified as the dependent variable, and E1 concentration, temperature, and the interaction between these terms specified as fixed factors. Preliminary analysis indicated that the numbers of butts and strikes were significantly positively correlated (Pearson correlation: $r = 0.54$, $P < 0.001$). Therefore, we additively combined these values for each male prior to statistical analysis. We also included the size ratio of the two males in each test as a covariate in preliminary statistical analyses but did not find significant main or interactive effects of the size difference between the resident and stimulus males on the number of, or delay in, agonistic responses performed by the resident male. We removed the size term prior to final analyses. Unless otherwise indicated, data were analyzed using SPSS (v 21) (IBM, New York, USA). For ANOVAs, we estimated the influence of each fixed effect on subject responses, relative to the other factors in the model, via the partial variance statistic (η^2). For all analyses, we examined the effect size for significant pairwise posthoc comparisons via Cohen's d.

3. Results

3.1. Exposure conditions

Measured E1 concentrations (mean ± SD) were 13.82 ± 7.15, 22.27 ± 9.13 and 65.39 ± 27.70 ng/L for the $E1_{LOW}$ (n = 6 samples), $E1_{MED}$ and $E1_{HIGH}$ (n = 7 and 8 samples for the $E1_{MED}$ and $E1_{HIGH}$ treatments, respectively) exposure treatments; all three values were within the environmental range reported by Kolpin et al. (2002). Water temperatures remained stable through the exposure period; over all concentrations the mean (±SD) daily temperatures recorded for the 15, 18, 21, and 24 °C treatments were 15.82 ± 0.82, 18.36 ± 0.74, 20.94 ± 0.64, and 23.45 ± 0.87 °C, respectively. Water quality measurements (dissolved oxygen = 8.30 ± 1.74 mg/L; pH = 7.76 ± 0.30; conductivity = 0.91 ± 0.06 mS/cm; salinity = 0.45 ± 0.01 g/L; and chlorine: undetectable) were also comparable throughout the experiment, and were well within tolerance limits for the study species.

3.2. Reproduction, survival and growth

A total of 642 adult *P. promelas* was used in this study (428 female, 214 male). Adult survival on day 30 was high (range: 92%–99% across treatments) and similar across the 16 treatments ($\chi^2 = 0.99$, df = 14, $P = 0.95$). The number of clutches produced during the exposure period was variable, ranging from 12 (in the $E1_{HIGH}$ treatment at 21 °C) to 54 (in the control and $E1_{MED}$ treatments, both at 24 °C) (Fig. 1a); however, the amount of spawning activity did not differ statistically among treatments ($\chi^2 = 1.120$, df = 9, $P = 0.99$).

We observed a significant effect of E1 exposure on the mean number of eggs laid in a single clutch (Table 1). Post-hoc tests indicated that females exposed to $E1_{HIGH}$ laid significantly fewer eggs than those in the $E1_{LOW}$ treatment ($P = 0.006$; Fig. 1b; Cohen's d = 0.26). We also found a significant effect of water temperature on clutch size (Table 1). In general, females maintained at lower temperatures laid more eggs than those maintained at higher temperatures (Fig. 1b). Post-hoc tests revealed that clutches laid at 21 °C had significantly fewer eggs than those laid at 15 °C ($P < 0.001$; Cohen's d = 0.60) or 18 °C ($P < 0.001$; Cohen's d = 0.81). Clutch sizes were also smaller at 24 °C compared with 15 °C ($P = 0.001$; Cohen's d = 0.32). By contrast, we did not observe a significant interaction between temperature and concentration on clutch size, or significant independent or interactive effects of concentration and temperature on fertilization success (Table 1; Fig. 1c); the proportion of fertilized eggs ranged from 0.60 ± 0.32 for fish maintained under control conditions at 15 °C to 0.81 ± 0.21 for fish exposed to $E1_{LOW}$ at 15 °C. Water temperature, but not E1 concentration or the temperature *x* concentration interaction, had a significant effect on hatching latency (Table 1). Collapsing across all concentrations, hatching latency was negatively related to temperature, with significant step-wise reductions in the duration of embryonic development observed with each temperature increase (all Ps < 0.001; Cohen's d = 1.02–1.38) (Fig. 1d).

Larval survival on day 21 varied across treatments from 4% ± 5% (in the $E1_{LOW}$ treatment, at 24 °C) to 26% ± 16% (in the control treatment, at 15 °C) (Fig. 1e). The number of larvae maintained at 21 °C that survived to day 21 was insufficient for behavioral or statistical analysis; therefore, we excluded this temperature treatment in subsequent analyses. Although we did not observe significant main effects of either temperature or E1 concentration on larval survival, we did observe a significant temperature *x* concentration interaction (Table 1); mean (±SD) survival at 15 °C (25% ± 16%) was significantly greater for control subjects than for subjects exposed to $E1_{LOW}$ (9% ± 12%, $P = 0.002$; Cohen's d = 1.09), $E1_{MED}$ (9% ± 17%, $P = 0.004$; Cohen's d = 0.93) or $E1_{HIGH}$ (5% ± 8%, $P < 0.001$; Cohen's d = 1.53). Survival did not differ among exposure levels at 18 °C or 24 °C (all Ps > 0.05).

Rearing temperature, but not concentration or the associated interaction, had a significant effect on larval growth (Fig. 1f). Larval size (body length) on day 21 was positively linearly related to the ambient temperature (Table 1). Posthoc tests indicated that larvae reared at 24 °C were significantly larger than those reared at 15 °C or 18 °C (both Ps < 0.001; Cohen's d = 1.33 and 0.94, respectively). Larvae reared at 18 °C were also larger than those raised at 15 °C ($P = 0.004$; Cohen's d = 0.30).

3.3. Experiment 1: effects of temperature and E1 concentration on predator evasion performance

We examined the innate, evasive locomotor responses of 152 larvae (n = 3–19 across treatments), depending on the number of surviving individuals available for testing. We found significant main effects of concentration and temperature on escape velocity, as well as a significant interaction between the two factors (Table 2). Overall, velocity was negatively related to temperature (Fig. 2a); average (±SD) escape velocities of larvae reared at 15, 18 and 24 °C were 0.030 ± 0.020, 0.024 ± 0.016, and 0.019 ± 0.017 BL/ms, respectively. Pairwise post-hoc tests indicated that larvae reared at 15 °C exhibited faster escape velocities than those maintained at either 18 °C ($P = 0.026$; Cohen's d = 0.33) or 24 °C ($P = 0.002$; Cohen's d = 0.59), but no other significant pairwise differences were observed (all Ps > 0.05). By contrast, escape velocities showed an inverted 'U' shaped distribution with respect to exposure concentration (Fig. 2a); average (±SD) escape velocities of larvae exposed to control, $E1_{LOW}$, $E1_{MED}$ and $E1_{HIGH}$ were 0.022 ± 0.016, 0.026 ± 0.017, 0.026 ± 0.017 and 0.022 ± 0.017 BL/ms, respectively; larvae exposed to $E1_{MED}$ were significantly faster than subjects in either the control ($P = 0.006$; Cohen's d = 0.18) or $E1_{HIGH}$ ($P = 0.003$; Cohen's d = 0.23) treatments. Pairwise comparisons indicated that escape velocities were statistically similar among all other treatments (Ps > 0.05). We also observed a significant interaction between E1 concentration and temperature that influenced escape velocity (Table 2), indicating that temperature modulates the effects of E1 exposure on escape performance; at 15 °C subjects exposed to $E1_{MED}$ exhibited a significantly enhanced escape speed compared to control subjects ($P = 0.005$; Cohen's d = 0.23). Escape velocities were similar among control and exposed subjects at higher temperatures (all pairwise Ps > 0.05 at 18 °C and 24 °C).

Temperature, but not exposure concentration, also had a significant effect on latency (Table 2); collapsing over all temperatures, the mean (±SD) latencies in the 15, 18 and 24 °C treatments were 87.97 ± 96.95, 130 ± 134.95 and 171.91 ± 195.40 ms, respectively, suggesting that latency is negatively related to thermal regime (Fig. 2b). Posthoc tests indicated that the latencies of subjects reared at 24 °C were significantly longer than those raised at 15 °C ($P = 0.013$; Cohen's d = 0.54); no other significant pairwise differences were found (Ps > 0.05).

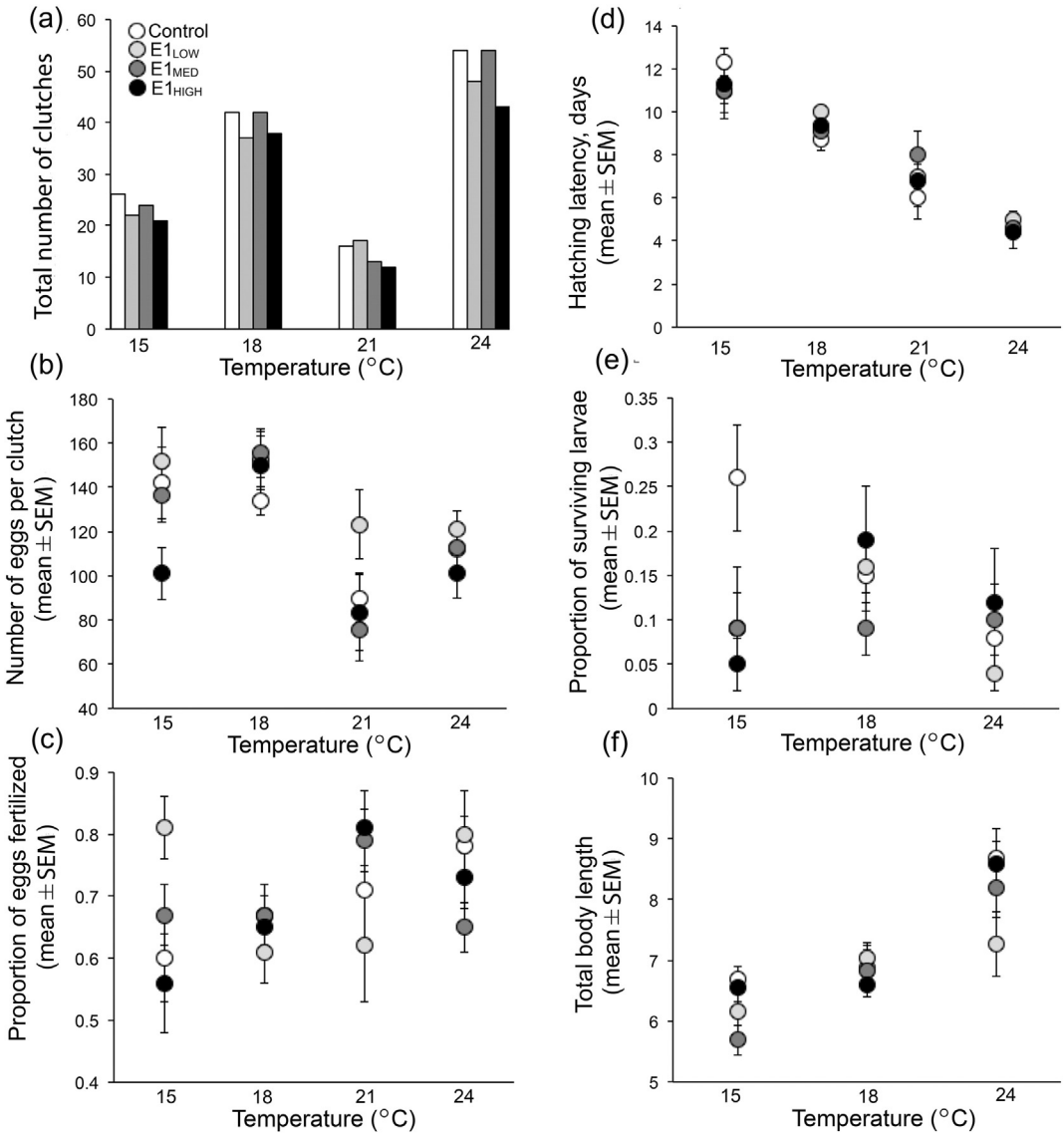

Fig. 1. Reproduction, growth, and survival of fathead minnows (*Pimephales promelas*) over a 30-day exposure period at four ambient temperatures. (a) Total level of spawning activity observed in each factorial temperature and E1 exposure concentration combination (n = 12–54); (b) female fecundity, indicated by the number of eggs (n = 12–54); (c) male fertilization success, measured as the proportion of eggs laid that showed evidence of eyespots (n = 10–51); (d) hatching latency, measured as the length of time (in days) to the first day of hatching (n = 3–12); (e) larval survival, measured as the proportion of successfully hatched eggs per clutch that survived to day 21 (n = 3–12); (f) body size (total length) of larvae on exposure day 21 (n = 3–19). For all panels, white bars or symbols represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black bars or symbols represent the $E1_{LOW}$ (14 ng/L), $E1_{MED}$ (22 ng/L), and $E1_{HIGH}$ (65 ng/L) exposure treatments. Points and error bars depict means ± SEM.

We did not detect significant effects of exposure concentration or temperature, nor a significant temperature *x* concentration interaction in GEE models examining the total escape response or the turning angle ($0.06 \le P \le 0.89$; Table 2; Fig. 2c, d). However, the total escape response exhibited a linear trend consistent with the interpretation that performance decreases with increasing temperature (Fig. 2c); over all concentrations tested, the mean (±SD) total escape responses for subjects reared at, 15, 18 and 24 °C were 0.008 ± 0.007, 0.006 ± 0.007, and 0.005 ± 0.007, respectively.

3.4. Experiment 2: effects of temperature and E1 concentration on prey capture success of larval minnows

We conducted a total of 144 foraging trials (n = 3–23 across treatments, depending on the number of surviving larvae available for testing). The average proportion of prey successfully identified, localized and captured by larvae in each of the 12 treatments is shown in Fig. 3a. The GEE revealed a significant effect of temperature on capture success (Table 3). Overall, prey capture increased linearly with rearing temperature; collapsing over all concentration levels, the mean (±SD) numbers of prey consumed at 15, 18, and 24 °C were 11.06 ± 5.64, 14.98 ± 5.85, and 17.15 ± 5.50, respectively. Post-hoc tests revealed that prey capture was significantly enhanced at each step-wise increase in temperature ($0.012 \le P < 0.001$; Cohen's d = 0.38–1.09). We did not detect a significant main effect of E1 concentration on prey capture success (Table 3). However, we did find a significant temperature *x* concentration interaction (Table 3), suggesting that temperature modulates the effects of estrogen exposure on larval foraging ability. Post-hoc comparisons indicated that at the lowest temperature tested (15 °C), subjects exposed to $E1_{MED}$ consumed significantly fewer prey than those in the Control ($P = 0.013$; Cohen's d = 0.89) or $E1_{LOW}$ treatments ($P = 0.001$; Cohen's d = 1.71). Capture success did not differ among fish reared at different exposure concentrations at higher temperatures (18 or 24 °C; all pairwise Ps > 0.05).

Table 1
Results of ANOVAs or GEE examining the effects of temperature (15, 18, 21 and 24 °C) and E1 concentration (control, $E1_{LOW}$, $E1_{MED}$, $E1_{HIGH}$) on the survival, reproduction and growth of fathead minnows, *Pimephales promelas*, at two life stages. Significant effects are given in bold ($\alpha < 0.050$).

Parameter	Effect	F	*df*	P	η^2
Fecundity	Concentration	2.67	3492	**0.047**	0.016
	Temperature	13.78	3492	<**0.001**	0.077
	Concentration × temperature	1.19	9492	0.297	0.021
Fertility	Concentration	0.59	3390	0.623	0.004
	Temperature	1.34	3390	0.260	0.009
	Concentration × temperature	1.72	9390	0.083	0.038
Hatching latency	Concentration	0.24	3103	0.871	0.007
	Temperature	59.20	3103	<**0.001**	0.633
	Concentration × temperature	0.70	9103	0.700	0.058
Larval survival	Concentration	1.90	3,85	0.136	0.051
	Temperature	2.39	2,85	0.098	0.043
	Concentration × temperature	2.49	6,85	**0.029**	0.134
Larval size	Concentration	4.56	3	0.297	
	Temperature	48.30	2	<**0.001**	
	Concentration × temperature	11.35	6	0.078	

3.5. Experiment 3: effects of temperature and E1 concentration on prey capture success of adult minnows

The average number of prey successfully identified, localized and captured by either male or female subjects in each of the 16 treatments are shown in Fig. 3b. A total of 364 subjects (183 male and 181 female) were used in foraging trials (n = 12–28 across treatments). The GLM revealed a significant main effect of temperature (15, 18, 21, or 24 °C) on the number of prey captured (Table 4). Similar to the findings for experiment 2, capture rates were greater at higher temperatures. Pairwise post-hoc tests indicated that subjects consumed more prey at 24 °C than at lower temperatures (21, 18, or 15 °C; all Ps ≤ 0.006; Cohen's d = 0.39–0.86). Subjects also consumed more prey at 21 °C than 18 °C (P = 0.011; Cohen's d = 0.49). Only one exception to this general trend was observed; subjects consumed more prey at 15 °C than at 18 °C (P = 0.005; Cohen's d = 0.50).

By contrast, we did not observe significant main effects of either sex or E1 concentration on subject responses, nor significant interaction terms involving these factors (Table 4). These results indicate that male and female subjects captured and consumed prey at similar rates, and that capture success was unaffected by E1 exposure.

3.6. Experiment 4: effects of temperature and E1 concentration on territorial aggression

We examined the responses of 207 males towards a conspecific intruder (n = 8–16 across treatments). Resident males consistently responded to the presence of an intruder by approaching the restrained male and performing aggressive displays (bumps and strikes); 127 of the 207 subjects tested (61%) exhibited at least one act of territorial aggression towards the intruder. Of the 80 subjects who failed to exhibit an aggressive response, 32 (40%) were exposed to $E1_{HIGH}$. Accordingly, there was significant main effect of E1 exposure concentration on the number of aggressive acts (i.e., the combined number of bumps and strikes) performed by resident males (Table 5). Males exposed to $E1_{HIGH}$ performed fewer aggressive acts than males exposed to $E1_{MED}$ (P = 0.005; Cohen's d = 0.66) or control subjects (P = 0.005; Cohen's d = 0.62) (Fig. 4a). No other significant pairwise differences among exposure treatments in the number of aggressive were observed (all Ps > 0.05).

Temperature also had a significant effect on male intraspecific interactions (Table 5). Males maintained at 21 °C were significantly more aggressive than those maintained at any other tested temperatures (i.e., 15, 18, or 24 °C) (0.048 ≤ Ps ≤ 0.001; Cohen's d = 0.36–0.71) (Fig. 4a). Subjects maintained at other temperatures did not differ in the number of territorial aggressive acts performed (all Ps > 0.05). However, we did not find evidence that temperature significantly modulates the effects of E1 exposure (Table 5).

By contrast, neither E1 concentration nor temperature had independent or interactive effects on the latency to first response by resident males (Table 5), although there was a trend for latency to decrease with increasing temperature (Fig. 4d); over all concentrations the average latencies (±SD) of subjects maintained at 15, 18, 21 and 24 °C were 92.70 ± 85.58, 97.03 ± 80.55, 77.62 ± 84.72, and 56.94 ± 71.24 s, respectively.

Table 2
Results of GEE models examining the effects of ambient temperature (15, 18, and 24 °C) and E1 concentration (control, $E1_{LOW}$, $E1_{MED}$, $E1_{HIGH}$) on larval predator evasion performance. Significant effects are given in bold ($\alpha < 0.050$).

Parameter	Effect	χ^2	*df*	P
Velocity	Concentration	10.71	3	**0.013**
	Temperature	10.00	2	<**0.007**
	Concentration × temperature	14.37	9	**0.026**
Latency	Concentration	1.76	3	0.625
	Temperature	6.87	2	**0.032**
	Concentration × temperature	10.32	6	0.112
Total escape response	Concentration	3.57	3	0.312
	Temperature	4.88	2	0.087
	Concentration × temperature	12.02	6	0.061
Angle of escape	Concentration	2.80	3	0.423
	Temperature	1.30	2	0.521
	Concentration × temperature	2.29	6	0.891

4. Discussion

In this study, we examined the extent to which variation in the ambient temperature modulates the behavior and physiology of fish exposed to an estrogenic contaminant at two life stages. Our results yielded several main findings; first, we confirmed that E1 and temperature independently influence aspects of the survival, reproduction and behavior of both larval and mature fish. Second, we found some evidence indicating that the effects of contaminants can vary with thermal regime. Third, taken together our data suggest that individuals in earlier life stages are more susceptible to the modulating effects of natural abiotic variation on anthropogenic stressors.

4.1. Independent and interacting effects of temperature and EDC exposure on behavior

4.1.1. Predator evasion

The C-start startle response is almost entirely regulated by a pair of large, easily identifiable Mauthner cells (M-cells) found in the hindbrain (Eaton et al., 2001). The response is initiated when one of the two M-cells is activated and the signal is propagated along the axon, causing the contralateral trunk muscles to contract into the characteristic "C" shape (Eaton et al., 2001). Both changes in temperature and exposure to contaminants have the potential to influence escape performance through their effects on the activity of neural M-cells. Temperature alterations shift the balance between excitatory and inhibitory transmission onto the M-cells with corresponding changes in behavior (Preuss and Faber, 2003; Szabo et al., 2008). M-cell to motoneuron transmission is also affected by a variety of chemicals (Carlson et al., 1998), resulting in impaired escape performance (McGee et al., 2009; Painter et al., 2009). Moreover, these effects are exacerbated at higher temperatures (Xia et al., 2015). For example, McGee et al. (2009) showed that juvenile *P. promelas* exposed as embryos to 50 ng/L of E1 exhibited a delay in the initiation of the C-start response compared to control fish, and a reduction in total escape performance. Juvenile qingbo, *Spinibarbus sinensis*, exposed to perfluorooctane sulfonate for four weeks at 28 °C also exhibited riskier behavior and impairment of the response compared with fish exposed at 18 °C (Xia et al., 2015). With the exception that larvae

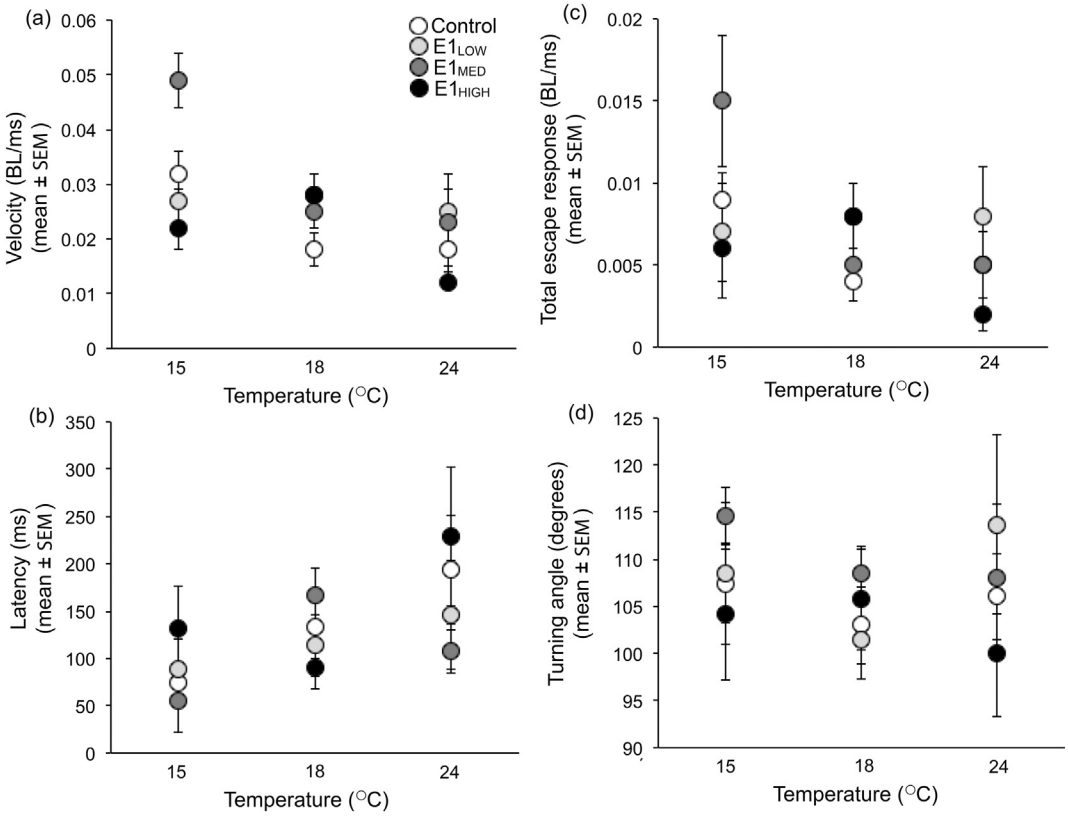

Fig. 2. Responses of larvae exposed to E1 or an EtOH solvent control (control), at three ambient temperatures. Sample sizes ranged from 3 to 19 across treatments. (a) Escape velocity (BL/ms) over the first 40 ms of the response; (b) latency (in ms) to the induction of the response; (c) total escape response (BL/ms); (d) turning angle, relative to the initial position of the head (in degrees). White circles represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black circles represent the $E1_{LOW}$ (14 ng/L), $E1_{MED}$ (22 ng/L), and $E1_{HIGH}$ (65 ng/L) exposure treatments. Points and error bars depict means ± SEM.

reared at 15 °C and exposed to an intermediate concentration of E1 (i.e., $E1_{MED}$) had a *faster* escape velocity compared with subjects exposed to higher or lower levels of estrogen, we did not find that exposure to E1 significantly impaired the fast-start startle response. However, consistent with previous work suggesting that the effects of exposure on aquatic organisms are magnified at higher temperatures (Noyes et al., 2009), compared to control subjects, fish exposed the $E1_{HIGH}$ under warmer conditions (24 °C) showed response latencies that were on average 18% longer and swimming speeds that were 50% slower; combined, these factors contributed to a 2.6-fold reduction in the total escape performance of larvae exposed to $E1_{HIGH}$ at 24 °C.

Studies investigating the influence of temperature on the startle response generally report that C-start kinematics and behavioral responsiveness are either unaffected by, or improve in parallel with, temperature over a limited thermal window (e.g., Batty and Blaxter, 1992; Webb, 1978; reviewed in Domenici and Blake, 1997). Consistent with these findings, total escape responses and turning angles exhibited by the larval *P. promelas* in our study were relatively stable across the 9 °C temperature range tested here. Interestingly however, larvae reared at 24 °C had slower swimming speeds and longer response latencies than those reared at 15 °C. One potential explanation for these results is that the trials were conducted at room temperature (~21 °C), which represented an acute increase in temperature for the fish acclimated at 15 °C or 18 °C and a decrease in temperature for fish acclimated at 24 °C. Fish acclimated to colder temperatures frequently show an improved swimming performance at warmer temperatures, and vice versa (Johnson et al., 1996; Krupczynski and Schuster, 2013; Preuss and Faber, 2003). For example, short-horned sculpins, *Myoxocephalus scorpius*, acclimated to 5 °C demonstrated higher swim velocities when tested at 15 °C compared to when tested at 5 °C (Beddow et al., 1995).

4.1.2. Male-male aggression

Estrogen-induced reductions in the nesting behavior (Brian et al., 2006) and aggression (Bell, 2001; Colman et al., 2009; Saaristo et al., 2010) of male fish are well described. Consistent with these previous studies, the resident male minnows in our study exposed to the highest concentration of E1 exhibited more than a 2.5-fold reduction (across all temperatures) in aggression towards conspecific intruders compared to control subjects. These data are consistent with evidence demonstrating that estrogenic EDCs decrease levels of androgens (Coe et al., 2008; Salierno and Kane, 2009) that regulate the expression of reproductive behavior in fish (Liley and Stacey, 1983; Mayer et al., 2004).

The reproductive males in our study were also generally more aggressive at higher temperatures (i.e., 21 °C and 24 °C, which corresponds to the optimal thermal breeding regime; Brian et al., 2011; Smith, 1978); at 21 °C and 24 °C males performed approximately 2-fold and 1.5-fold more aggressive displays than at 15 °C, respectively. At these same higher temperatures, males performed approximately 2.5-fold and 2-fold more aggressive displays than at 18 °C. Higher levels of aggression at warmer temperatures have also been reported in other species, including dwarf cichlids (*Apistogramma agassizii*) (Kochhann et al., 2015), damselfish (*Pomacentrus bankanensis*) (Biro et al., 2010) and mosquitofish (*Gambusia holbrooki*) (Carmona-Catot et al., 2013) and likely reflect the regulatory influence of exogenous proximate cues on endogenous reproductive physiology (Munro et al., 1990). In seasonally reproducing fish such as *P. promelas*, reproductive behavior (i.e., courtship, male-male aggression, and parental care) typically begins in the spring once ambient temperatures reach 18 °C (Smith, 1978), with maximal growth and survival of offspring occurring between 20 °C and 24 °C (Brian et al., 2011). Significantly, heightened aggression at the optimal breeding temperature eroded the aggressive disparity

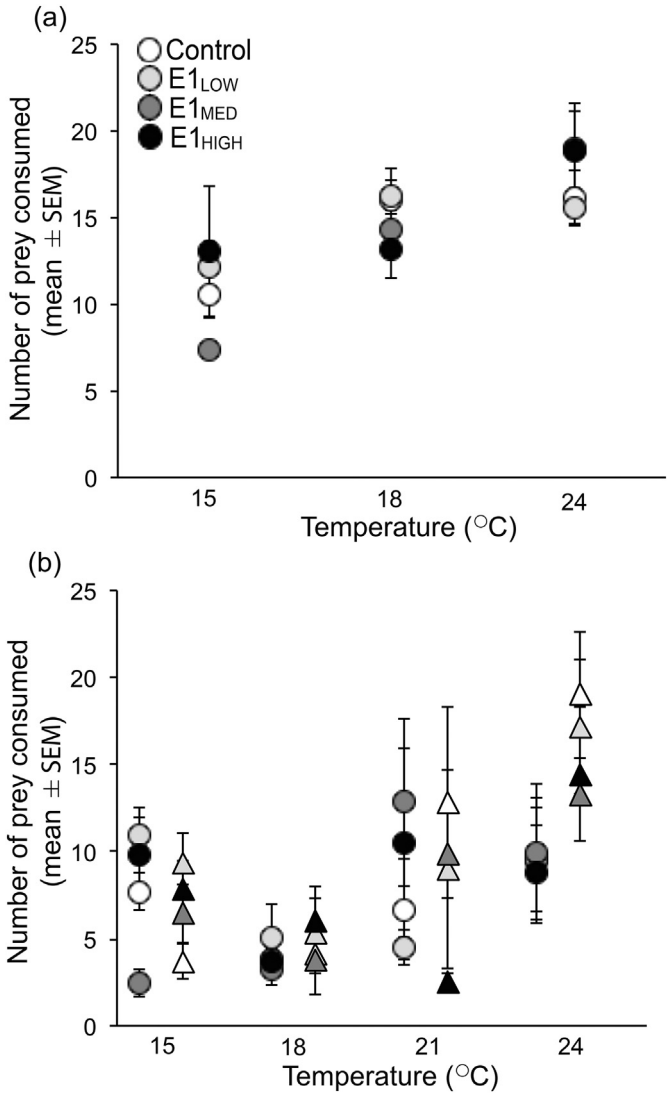

Fig. 3. Prey consumption by (a) larvae or (b) adult subjects exposed to varying concentrations of E1 or solvent (control) at different ambient temperatures. Sample sizes across treatments in (a) ranged from 3 to 23. Sample sizes in (b) ranged from 12 to 28. White symbols represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black symbols represent the $E1_{LOW}$ (14 ng/L), $E1_{MED}$ (22 ng/L), and $E1_{HIGH}$ (65 ng/L) exposure treatments, respectively. Points and error bars depict means ± SEM. Circles and triangles in (b) represent the number of prey consumed by males and females, respectively.

between males exposed to high concentrations of E1 and those in other treatments; at this temperature all males were highly aggressive. One potential explanation for these results is that higher levels of circulating androgens under peak spawning conditions compensate for the effects of estrogen exposure. In fish, 11-ketotestosterone (11-KT) is the main androgen associated with aggression and dominance (Taves et al., 2009) and endogenous levels of 11-KT and testosterone are highest during the pre-spawning and spawning periods (Borg, 1994; van Breukelen et al., 2015). Dominant males (e.g., territory-holding males such as those in the present study) also have higher levels of circulating androgens than subordinate males (e.g., non-territorial males) (Parikh et al., 2006).

Table 3
Results of GEE examining the effects of ambient temperature (15, 18, and 24 °C) and E1 concentration (control, $E1_{LOW}$, $E1_{MED}$, $E1_{HIGH}$) on the consumption of live prey by larval *Pimephales promelas*. Significant effects are given in bold ($\alpha < 0.050$).

Effect	F	*df*	P
Concentration	5.80	3	0.122
Temperature	30.66	2	<**0.001**
Concentration × temperature	22.14	6	<**0.001**

Table 4
Results of GLM examining the effects of temperature (15, 18, 21 and 24 °C), E1 concentration (control, $E1_{LOW}$, $E1_{MED}$, $E1_{HIGH}$) and sex on the consumption of live prey by adult *Pimephales promelas*. Significant effects are given in bold ($\alpha < 0.050$).

Effect	F	*df*	P
Concentration	1.32	3	0.725
Temperature	49.69	3	<**0.001**
Sex	1.23	1	0.268
Concentration × temperature	15.37	9	0.081
Concentration × sex	2.23	3	0.526
Temperature × sex	4.60	3	0.203
Concentration × temperature × sex	14.99	9	0.091

4.1.3. Prey capture success

The bioenergetic demands of prey capture, consumption and metabolism are costly to any organism, but are especially costly to organisms under environmental stress (Heugens et al., 2001; Sokolova, 2013; Sokolova and Lannig, 2008). Exposure to heavy metals, pharmaceuticals, and other contaminants has been shown to impair foraging success, and reduce the biomass and growth of adult and juvenile fish both in the lab and in the field (see reviews by Sloman and McNeil, 2012; Weis and Candelmo, 2012). By contrast, few studies have examined the effects of estrogen exposure on the foraging ability of fish; Cagle (2014) found no effect of 17β-estradiol on the feeding behavior of male *Betta splendens*. Hallgren et al. (2014) reported that juvenile roach (*Rutilus rutilus*) exposed to 50 ng/L 17α-ethinylestradiol showed reduced foraging success, measured as the number of *D. magna* captured and consumed over a given internal of time. With the exception that larvae reared at 15 °C and exposed to an intermediate concentration of E1 (i.e., $E1_{MED}$) captured fewer prey than unexposed or $E1_{LOW}$ subjects, neither larval nor adult prey capture rates were significantly affected by exposure to E1. The differences between our findings and those of Hallgren et al. (2014) could potentially reflect species-specific differences in sensitivity, or the comparatively higher estrogenicity of synthetic estrogen 17α-ethinylestradiol relative to estrone (Van den Belt et al., 2004).

However, prey capture rates were positively related to temperature for both larvae and adult *P. promelas*. Similar associations between prey capture success and elevated temperatures have been reported for a number of fish species, including Australian bass, *Macquaria novemaculeata* (Grigaltchik et al., 2012); dottyback, *Pseudochromis fuscus*, (Allan et al., 2015); perch, *Perca fluviatilis*; ruffe, *Gymnocephalus cernuus* (Bergman, 1987); and short-horned sculpin, *M. scorpius* (Beddow et al., 1995). In our study, adult and larval *P. promelas* maintained at 24 °C had capture rates that were on average 1.85-fold and 1.66-fold greater than those observed for age-matched individuals maintained at 15 °C. Further work is necessary to determine the causal mechanisms underpinning prey-capture success in *P. promelas*;

Table 5
Results of ANOVA examining the effects of temperature (15, 18, 21 and 24 °C) and E1 concentration (control, $E1_{LOW}$, $E1_{MED}$, $E1_{HIGH}$) on aggressive displays performed by a territorial male *Pimephales promelas* towards a conspecific intruder, and the latency to first aggressive response. Significant effects are given in bold ($\alpha < 0.050$).

Parameter	Effect	F	*df*	P	η^2
Aggressive displays	Concentration	3.80	3191	**0.011**	0.056
	Temperature	5.90	3191	**0.001**	0.086
	Concentration × temperature	0.98	9191	0.462	0.044
Latency	Concentration	1.50	3111	0.219	0.039
	Temperature	1.55	3111	0.205	0.040
	Concentration × temperature	0.73	9111	0.685	0.055

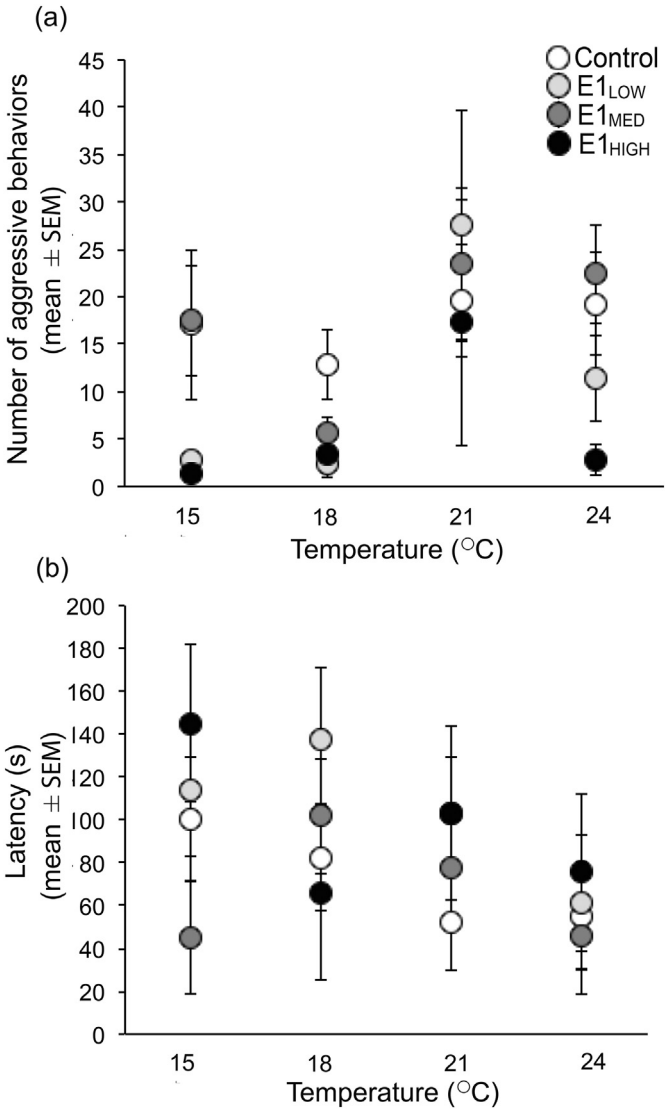

Fig. 4. Aggressive responses of resident males exposed to varying concentrations of E1 at different ambient temperatures (n = 8–16). (a) Combined number of butts and strikes; (b) latency to first aggressive act (s). White circles represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black circles represent the $E1_{LOW}$ (14 ng/L), $E1_{MED}$ (22 ng/L), and $E1_{HIGH}$ (65 ng/L) exposure treatments. Points and error bars depict means ± SEM.

however, temperature-dependent variation in the outcomes of predator-prey interactions could reflect direct differences in attack rate (Grigaltchik et al., 2012; Persson, 1986), physiological mechanisms and/or kinetics associated with a predatory strike (Allan et al., 2015), or hunger; although all of the subjects in our study were fasted for the same length of time, elevated temperatures may have induced shifts in the metabolic rate with an associated increase in energy requirements (Clarke and Johnston, 1999; Noyes et al., 2009).

4.2. Survival, reproduction and growth of P. promelas at two life stages

In natural populations, persistence is dependent not only on the reproductive success or survival of the adult cohort, but also on the survival of the F1 generation to maturity. In our study, larvae exposed to E1 at 15 °C were 2.8 times ($E1_{LOW}$) to 5 times ($E1_{HIGH}$) less likely to survive to day 21 than their unexposed counterparts, suggesting that the adverse effects of E1 exposure on early survival are more prevalent at this temperature. In addition, female *P. promelas* in the $E1_{HIGH}$ treatment laid fewer eggs than females exposed to a low concentration of E1; a finding that was also particularly pronounced at 15 °C. Reductions in reproductive output and/or survival following estrogen exposure are well-documented in *P. promelas* (e.g., Dammann et al., 2011; Parrott and Blunt, 2005; Thorpe et al., 2007). Moreover, these effects of exposure can be transgenerational (Schwindt et al., 2014), and can reduce recruitment rates below those needed for population persistence (Kidd et al., 2007).

Temperature also had a significant effect on the duration of embryonic development and growth of larval *P. promelas*. Although rates of development can vary widely among species, development within species is largely a function of temperature (Jobling, 1997). Accordingly, eggs laid at 24 °C exhibited a significantly shorter hatching latency compared to eggs laid at 15 °C. Larvae reared at 24 °C were also considerably larger than those reared at 15 °C on day 21. By contrast, neither male fertility nor adult survival was affected by exposure to E1 or temperature.

5. Conclusion

Our data show that (i) variation in behavioral responsiveness, kinematics, and the outcomes of intraspecific and interspecific interactions can occur within a relatively narrow range of seasonally fluctuating temperatures, and that (ii) changes in the thermal regime can interact with chemical stressors in ways that further influence physiology, survival and behavior. The significant interactions between E1 and temperature that we observed could all be traced to differences between exposure treatments at the coldest temperature tested (15 °C), potentially reflecting a slower rate of microbial E1 degradation under colder thermal regimes (Cox et al., in review). Support for this hypothesis comes from the finding that concentrations of *vtg* in male *P. promelas* used in this study were significantly higher at 15 °C than at warmer temperatures (Cox et al., in review). Alternatively, metabolic changes associated with elevated temperatures may have resulted in increased rates of degradation and elimination (Noyes et al., 2009). Moreover, all of the significant interactions between temperature and exposure that we observed occurred in larvae, consistent with previous studies indicating that individuals in exposed populations are particularly susceptible to perturbation by EDCs during the early ontogenetic stages of life (Guillette et al., 1996; Liney et al., 2005; Sloman and McNeil, 2012; van Aerle et al., 2002). Greater susceptibilities of fishes at early life stages can be expected, because the developmental processes of most fishes are heavily regulated by the endocrine system (Janz and Weber, 2000). Taken together, our data suggest that the effects of EDC exposure may be more pronounced on the offspring of early-spring breeding individuals than on the offspring of individuals breeding later in the season.

Changes in global air and water temperatures due to climate change (O'Reilly et al., 2015) and/or increases in aquatic contaminant loads have potential to directly or indirectly alter the structure and function of populations and communities through changes in survival, reproduction, or altered behavioral interactions (Clotfelter et al., 2004; Tuomainen and Candolin, 2011; Van Zuiden et al., 2016). While most populations are expected to be impacted by climate change or pollution to some extent, ectothermic species such as fish, amphibians and reptiles, and populations living at the edge of their physiological tolerance range, are likely be particularly vulnerable to interactions between shifting thermal regimes and contaminants (Noyes et al., 2009). Further studies are necessary to improve our understanding of the effects of interactions among multiple anthropogenic stressors, and between stressors and natural abiotic environmental factors. Such studies are likely to be of key importance to identifying vulnerable populations and predicting population dynamics under changing real-world scenarios.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2017.05.015.

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