MICHIGAN STATE
UNIVERSITY

April 29, 2015

Jon Beard
Grant Manager
Great Lakes Fishery Trust
230 N. Washing Square, Suite 300
Lansing MI 48933

Dear Mr. Beard:

As part of the completion report to the Great Lakes Fishery Trust for project number 2011.1202.scribner, we are submitting 7 manuscripts that have either been published in, are in review with, or are being prepared for submission to peer-reviewed journals. The 7 manuscripts include the following:

Attachment #1: Jay, K., J. McGuire, and K.T. Scribner. Phenotypic and behavioral responses by lake sturgeon to ecological conditions experienced during sequential early ontogenetic stages. Submitted to Functional Ecology. In revision.

Attachment #2: Dammerman, K., J.P. Steibel, and K.T. Scribner. Increasing thermal regimes reveal cryptic genetic variation during early ontogenetic stages of lake sturgeon (*Acipenser fulvescens*). Submitted to Evolutionary Applications. In review.

Attachment #3: Bauman, J., E. Baker, T. Marsh, and K.T. Scribner. Effects of de-adhesion method and incubation condition on body size, survival, oxygen consumption rate, and microbial community composition of lake sturgeon eggs surfaces. To be submitted to Aquaculture.

Attachment #4: Bauman, J., E. Baker, T. Marsh, and K.T. Scribner. Effects of egg chemotherapeutants on body size, survival, oxygen consumption rate, and microbial diversity of lake sturgeon egg surfaces. Submitted to North American Journal of Aquaculture. In review.

Attachment # 5: Bauman, J., E. Baker, T. Marsh, and K.T. Scribner. Effects of rearing density on body size and survival of lake sturgeon (*Acipenser fulvescens*) free embryos. North American Journal of Aquaculture. In press.

Attachment #6: Bauman, J., E. Baker, T. Marsh, and K.T. Scribner. Body size and survival of hatchery and wild produced larvae as a function of feeding frequency and alternative food types. Submitted to North American Journal of Aquaculture . In review.

Attachment #7: Bauman, J., E. Baker, T. Marsh, and K.T. Scribner. Survival of lake sturgeon as a function of different chemotherapeutant prophylactics. Submitted to North American Journal of Aquaculture. In review.

College of Agriculture and Natural Resources

Department of Fisheries and Wildlife

517-353-3288
Fax: 517-432-1699

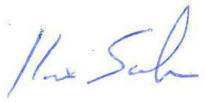
In addition to the published or submitted manuscripts, we are submitting attachments that include a summary of results for an additional project objective (Attachment 8). The final part of the report (Attachment 9) includes the standard operating procedures (SOPs) for all lake sturgeon stream-side culture activities. The final draft of SOPs and accompanying descriptions will eventually be submitted as a Michigan DNR Technical Report following further internal review and comment by Great Lakes agency biologists and fish culturalists. The Thesis by John Bauman which encompasses Attachments 3-8 and the SOPs have been distributed to lake sturgeon biologists and stream-side fish culturalists.

Attachment #8: Bauman, J., E. Baker, T. Marsh, and K.T. Scribner. Effects of different water filtration methods on the taxonomic composition of lake sturgeon egg surfaces (summary of research).

Attachment #9: Bauman, J., E. Baker, T. Marsh, and K.T. Scribner. Stream-side lake sturgeon culture standard operating procedures.

We are grateful that the Great Lakes Fishery Trust for funding this project. Thank you also for granting the no-cost extension. We are pleased with the results of the studies conducted associated with the project. We believe the data and methodological recommendations derived from the data will be widely used by fishery managers and biologists within the Great Lakes and elsewhere.

Sincerely,



Kim Scribner
Professor

Final Report

INVESTIGATIONS OF AQUACULTURE METHODOLOGIES TO ENHANCE SUCCESS OF GREAT LAKES LAKE STURGEON STREAMSIDE FACILITIES

Project number:
2011.1202.scribner

PROJECT ABSTRACT

Streamside rearing facilities (SRFs) have been widely advocated in the Great Lakes basin as the preferred method of culturing Lake Sturgeon in situations where restoration goals to enhance or repatriate populations can be met by stocking. However, over the past decade, targeted stocking goals have been difficult to achieve due in large part to low survival and low growth during early life periods. This study examined three specific early life periods encountered during streamside operation (egg, free-embryo, and larval) and aimed to quantify the effects of different methodologies on the body size and survival of lake sturgeon in a SRF. Information collected during the egg period provides insight regarding the use of different egg chemotherapeutants, de-adhesion and incubation procedures, as well as the documentation of microbial community composition on lake sturgeon egg surfaces. Among egg chemotherapeutants utilized in streamside hatcheries, hydrogen peroxide showed higher mean proportional survival compared to formalin and the control (although results were not statistically significant). Additionally, chemotherapeutants may have selected for microbial communities that have been determined in other studies to be pathogenic. The effects of de-adhesion and incubation on lake sturgeon eggs were quantified using egg survival, egg oxygen consumption rate, and body size as response variables. Results revealed that these variables, except egg survival, did not vary significantly among de-adhesion and incubation treatments. Microbial community composition data suggest de-adhesion and incubation techniques affect the bacterial community composition on the egg surface which may provide insight into mechanisms responsible for differences detected in survival. At the free-embryo period, the effects of rearing density and family on body size and survival in association with dissolved oxygen concentration were quantified to the time of emergence. A significant density and family effect on free-embryo body size at emergence was documented. Feeding regimes, as well as the effects of different weekly prophylactic chemotherapeutants were quantified during the larval period. Results from feeding regime studies revealed significant differences in body size as a function of feeding frequency in hatchery-produced larvae to 30 days post-exogenous feeding. In addition, using hatchery-produced as well as wild-caught larvae, significant differences in body size and survival as a function of alternate food types were documented. Results from the weekly prophylactic chemotherapeutant study revealed a significant treatment effect on the survival of young-of-year lake sturgeon at 49 days post-exogenous feeding. Water sources at SRFs provide 'enrichment' or stimuli associated with natural biological and physical aspects of natural stream communities. We investigated effects of temperature associated with behavioral responses and growth. We found strong evidence for genetic and environmental effects on behavior and body size that along with other study findings have significant implications for survival upon release. This investigation at multiple life periods highlights methods that improve survival and growth, as well as serves as a tool for the development of standard operating procedures for SRFs geared to enhance current production and recovery of the Great Lakes Lake Sturgeon.

Final Narrative Report

Project Title: INVESTIGATIONS OF AQUACULTURE METHODOLOGIES TO ENHANCE SUCCESS OF GREAT LAKES LAKE STURGEON STREAMSIDE FACILITIES

Grantee: Department of Fisheries and Wildlife, Michigan State University

Project Team: Kim Scribner, Edward Baker, Terence March

Contact Person: Kim Scribner, Michigan State University, scribne3@msu.edu

Grant Amount: $166,639

Start and End Dates: 12/01/2011 to 12/31/2014

Key Search Words: lake sturgeon, aquaculture, streamside rearing facility, growth, environmental effects

Background/Overview

1. *Briefly summarize the project description as outlined in the original proposal.*

We outlined a series of objectives and hypotheses directed toward overcoming factors that negatively affect growth and survival of lake sturgeon at different ontogenetic stages reared in Streamside rearing facilities (SRFs). The general objectives of the study were to evaluate different culture methods that can be tailored to accommodate site-specific variability in rearing environmental regimes and equipment and resources available to operators at each SRF.

Experimental treatments and response variables that were evaluated during different steps of streamside culture. Treatments were evaluated represent current or possible alternative methods that can be adapted in present or future SRFs.

2. *Briefly summarize any significant changes to the work performed in comparison to the plan of work originally proposed and funded. If changes were made, describe how they affected your ability to achieve the intended outcomes for the work.*

We made no changes to overall project objectives. However, based on preliminary results, we did expand project objectives to look at genetic and environmental sources of variation and their interaction. We also expanded the microbial genetic component of the project.

Outcomes

3. *To what extent and how (if at all) did this research project advance scientific knowledge of the issue?*

There is a considerable literature on sturgeon culture. However, there is a paucity of information

on the efficacy of different culture methods in systems that use natural water sources. Many different protocols exist for sturgeon culture at different life stages. However, the vast majority of protocols have not been evaluated in the context of replicated and comparative methods. The research conducted as part of this GLFT grant significantly advanced our knowledge of the effects of different culture practices on survival and growth during critical early ontogenetic stages when the majority of mortality occurs.

4. *To what extent and how (if at all) did this project contribute to the education and advancement of graduate or undergraduate students focused on Great Lakes fishery issues?*

One graduate student (John Bauman) was supported full time on the grant. Bauman's thesis (Bauman 2015) forms the basis for several of the appendices in the report.

Research by several other former or current graduate students also contributed to the Grant. Masa Fujimoto (2012) completed a PhD program in Microbiology and Molecular Genetics on microbial communtities associated with lake sturgeon egg surfaces. Current PhD student Kari Dammerman and former Masters student Katy Jay contributed papers on behavioral and phenotypic responses of lake sturgeon free embryos to thermal regimes. Current PhD students Shirah Razak and Roshan Angoshtari are working on microbial community succession associated with diet and inter-microbial interactions and biofilm production, respectively with lake sturgeon at the Black River SRF.

During each of two field seasons, the grant supported 2 undergraduate hourly technicians (total N=4) to assist with data collection and analysis. Other summer seasonal undergraduate technicians that worked at the facility made significant contributions to the project and were trained in experimental research and fish culture activities of relevance to the Great Lakes. We also have participated in K-12 education and outreach at the stream-side facilities. We regularly participate in GLFT-GLRI activities. We hosted a K-12 teacher training session during the grant along with Brandon Schroeder for the NE GLFT Hub. We participate in the Michigan DNR's Sturgeon in the Classroom program. We provide public tours of our facility and present talks at local functions in the Black Lake region.

We presented a day-long program for the Michigan State University College of Natural Science seminar series called *Frontiers in Science*, for secondary science teachers in the State of Michigan. These seminars are presented by research faculty in the Colleges of Natural Science, Engineering, and Agriculture & Natural Resources and are key elements in our Masters of Education graduate programs.

References

Bauman, J. 2015. Investigations of aquaculture methodologies to enhance success of Great Lakes lake sturgeon streamside facilities. Masters of Science Degree, Michigan State University.

Fujimoto, M. 2012. Microbial succession on the lake sturgeon egg surface: mechanisms shaping the microbial community assembly during succession and the effect of microbial successional processes on host life history traits. PhD Dissertation, Michigan State University.

5. *To what extent and how (if at all) did this work help you or others on your team build new relationships with others in the research or management communities?*

Operation of the Black Lake stream rearing facility (SRF) involves cooperation between the management, fish culture, and research sections of the Fisheries Division of the Michigan Department of Natural Resource as well as Michigan State University who is the lead at the site. The facility supports researchers and visitors from all over the country and Canada and thus provides a venue for professionals to network with other people working on sturgeon biology and management. The research expanded collaboration with colleagues in Microbiology at Michigan State University and with Michigan Sea Grant. As a result of the project we are working much more closely with tribal lake sturgeon culture biologists.

6. *To what extent and how (if at all) do the findings have action implications for fishery managers? If the research has direct management implications, do you have any knowledge of use of the finding by managers? If the research does not have direct management implications at this stage, to what extent did the research advance the process of identifying management responses to critical issues?*

This project has provide the definitive suite of analyses that have led to the development of standard operating procedures (SOPs) that have and will be adopted by the Great Lakes lake sturgeon community. We have widely engaged the Great Lakes fisheries community with emphasis on communications with lake sturgeon biologist and culturists. Our research has been communicated through conference calls and webinars as well as at scientific and lake technical meetings. Increasing, stocking has been advocated as a primary mechanism to achieve lake sturgeon restoration goals in the Great Lakes and streamside hatcheries are widely used. The research will provide guidance to all lake sturgeon culture activities in the Great Lakes basin.

7. *Considering the above or other factors not listed, what do you consider to be the most important benefits or outcomes of the project?*

Basic research has been used to direct applied lake sturgeon management. Our streamside facility was established for purposes of directing research toward lake sturgeon culture applications and ecology during early life stages.

Related Efforts

8. *Was this project a stand-alone effort, or was there a broader effort beyond the part funded by the GLFT? Have other funders been involved, either during the time of your GLFT grant or subsequently?*

We have been engaged in field and experimental research at the Black river lake sturgeon streamside research facility since 2003. Work associated with the specific objectives of the current GLFT grant were a stand-alone effort. However, the work continues an avenue of research that has focused on aspects of lake sturgeon culture. We are directing the Michigan DNR's lake sturgeon restoration program for the Cheboygan River drainage

at the site. We are also funded by the Michigan State University Center for Water Sciences associated with the microbial ecology and pathogenesis studies.

9. *Has there been any spin-off work or follow-up work related to this project? Did the work inspire subsequent, related research involving you or others?*

Work has inspired additional efforts in the microbial arena in the following areas: (a) microbe-microbe interactions and biofilm formation, (b) production of anti-microbial compounds, (c) probiotic microbes on the egg surface and in the gastrointestinal tract, (d) extension of research to aquatic fungi as sources of mortality during egg and larval stages.

Communication/Publication of Findings

10. *List publications, presentations, websites, and other forms of formal dissemination of the project deliverables, tools, or results, including those that are planned or in process.*

Seven publications were listed in the cover page of the document including:

Jay, K., J. McGuire, and K.T. Scribner. Phenotypic and behavioral responses by lake sturgeon to ecological conditions experienced during sequential early ontogenetic stages. Submitted to Functional Ecology. In revision.

Dammerman, K., J.P. Steibel, and K.T. Scribner. Increasing thermal regimes reveal cryptic genetic variation during early ontogenetic stages of lake sturgeon (Acipenser fulvescens). Submitted to Evolutionary Applications. In review.

Bauman, J., E. Baker, T. Marsh, and K.T. Scribner. Effects of de-adhesion method and incubation condition on body size, survival, oxygen consumption rate, and microbial community composition of lake sturgeon eggs surfaces. To be submitted to Aquaculture.

Bauman, J., E. Baker, T. Marsh, and K.T. Scribner. Effects of egg chemotherapeutants on body size, survival, oxygen consumption rate, and microbial diversity of lake sturgeon egg surfaces. Submitted to North American Journal of Aquaculture. In review.

Bauman, J., E. Baker, T. Marsh, and K.T. Scribner. Effects of rearing density on body size and survival of lake sturgeon (*Acipenser fulvescens*) free embryos. North American Journal of Aquaculture. In press.

Bauman, J., E. Baker, T. Marsh, and K.T. Scribner. Body size and survival of hatchery and wild produced larvae as a function of feeding frequency and alternative food types. Submitted to North American Journal of Aquaculture .

Bauman, J., E. Baker, T. Marsh, and K.T. Scribner. Survival of lake sturgeon as a function of different chemotherapeutant prophylactics. Submitted to North American Journal of Aquaculture.

We are finalizing (Appendix 9 of this report) as a technical bulletin to be published as a Michigan Department of Natural Resources Technical Report .

Presentations of grant-related subjects at professional meetings include the following:

Dammerman, K. and K. Scribner. AGA Symposium June 27-29, 2014, Seattle WA.
The effects of different thermal and discharge regimes during egg incubation on larval lake sturgeon development and behavior,

Dammerman, K. and K. Scribner. The effects of different thermal and discharge regimes during egg incubation on larval lake sturgeon development and behavior. Invited oral presentation. "Early Life History" symposium at the 144th AFS Annual Meeting, August 17 – 21, 2014, Quebec City.

Bauman, J., T. Marsh, E. Baker and K. Scribner. Enhancing the Success of Streamside Culture for Lake Sturgeon" has been accepted for a 20 minute time slot in the "Practical Application of Sturgeon Research" symposium at the 144th AFS Annual Meeting, August 17 – 21, 2014, Quebec City.

Scribner, K, T. Marsh, J. Bauman, and M. Fujimoto. Integrative Molecular and Ecological Approaches to Quantify Dynamics of Microbial Community Assembly: Applications in Aquaculture and Fish Ecology. Invited paper, Symposium on "Genomic Tools for Fisheries Management and Conservation: Promises and Challenges: at the 144th AFS Annual Meeting, August 17 – 21, 2014, Quebec City.

Scribner, K. Long-Lived Iteroparous Species, Ecological, Demographic and Genetic Complexity: Acquisition, Management and Analytical Challenges Associated with Big Data. Invited presentation at the Symposium "Big Data Science and Its Impacts on Fish Conservation and Management" at the 144th AFS Annual Meeting, August 17 – 21, 2014, Quebec City.

Fujimoto, M., B. Lovett, P. Nirenberg, J. Bauman, T. Loch, M. Faisal, K. Scribner, T. Marsh. Interactions of *Flavobacterium* With Sturgeon Eggs And Other Community Members Of The Egg Microbiome. Invited talk to Eastern Fish Health Workshop, March 2013, Raleigh, NC.

Dammerman, K, P. Steibol, and K.T. Scribner. The effects of fluctuating thermal regimes on larval lake sturgeon development and behavior. ASIH Annual meeting. Albuquerque, New Mexico from 10-15 July 2013.

Jay, K., J. McGuire, and K.T. Scribner. Phenotypic and behavioral responses by lake sturgeon to ecological conditions experienced during sequential early ontogenetic stages. American Fisheries Society Annual Meeting, September 8-10, 2012, Minneapolis, MN.

11. *Please characterize your efforts to share the findings of this research with state, federal, Tribal, and inter-jurisdictional (e.g., Great Lakes Fishery Commission) agencies charged with management responsibilities for the Great Lakes fishery, If other audiences were priority for this research, please characterize your outreach efforts to the audiences as well. (Please note: You may wish to consult midterm reports in which specific audiences for the findings, and means of outreach to these audiences, were identified.)*

The majority of aforementioned activities have been directed to Great Lake managers. In addition, we have led teleconference calls with all Great Lakes lake sturgeon streamside hatchery personnel during the spring of 2012 and 2013 to discuss project goals and objectives and

proposed methods. We solicited suggestions. We used the calls and e-mailed documents to disseminate results. During 2014 we led a webinar the included the same group of agency biologists and researchers to present results in all subject areas based on years 1 and 2 of data collection at the streamside facility.

12. *Please identify technical reports and materials attached to this report by name and indicate for each whether you are requesting that GLFT restrict access to the materials while you seek publication. (Please note that the maximum amount of time during which GLFT will restrict access to the results of funded research is six months, unless notified that more time is needed.)*

We request that none of the documents listed in the 9 Appendices (listed in page one of the cover letter associated with the report) be released until the end of the 6 month deferral period.

13. *Manuscripts: Grantees submitting one or more publications or pending publications in lieu of a stand-alone technical report must submit a cover memo that confirms that all aspects of the funded research are incorporated in the published work, and in cases of multiple publications, identifies or crosswalks the grant-funded objectives to the published article containing results.*

Please see attached cover memo.

14. *Compilation reports. Grantees working on several related sub-projects under a single grant may submit a series of sub-project reports rather than a single, integrated report. However, grantees must submit a cover sheet or introduction that outlines and crosswalks grant objectives with the location of the results in the compilation document.*

Not applicable.

Appendix 1 - Phenotypic and behavioral responses by lake sturgeon to ecological conditions experienced during sequential early ontogenetic stages

K. Jay[a], J. McGuire[b], and K.T. Scribner[a,b]

[a]Department of Fisheries and Wildlife, Michigan State University, East Lansing, MI

[b]Department of Zoology, Michigan State University, East Lansing, MI

Summary

1. Inter-individual variability in expression of phenotypic traits and behaviors can result from the combined effects of genotype and environment. For poikilothermic vertebrates, variation can be pronounced during early ontogenetic stages. Lake sturgeon (*Acipenser fulvescens*) larvae exhibit considerable plasticity in body size and timing of dispersal associated with environmental conditions that individuals experience during incubation and immediately following hatch.
2. Newly hatched yolk-sac larvae immediately burrow into stream substrate and generally remain there until endogenous yolk reserves are absorbed before emerging to disperse downstream. We quantified the effects of experimental treatments simulating different stream environmental conditions (food availability, presence of predators, conspecific density, water temperature, substrate size), individual attributes at the time of hatch (body size and levels of endogenous yolk resources) and genetic (family) effects on the duration of time spent in the substrate from hatch until emergence. Eggs from two full-sib families were incubated at 10°C or 18°C, simulating stream conditions experienced by eggs early and late in the spawning season. Once hatched, yolk-sac larvae were individually placed into 240 ml plastic containers (N=263) with rock substrate. Containers were maintained in stream water at ambient temperatures and constant (~0.03 m/sec) velocity in 4 m experimental flowing streams. Duration of time that fish remained in the substrate was recorded in days and cumulative thermal units (CTU).
3. Conditions during incubation and immediately following hatch affected emergence time. Incubation temperature, growth, utilization of endogenous yolk-sac reserves, substrate (number and mean size of rocks), and degree of temperature deviance immediately preceding emergence were all significantly associated with the timing of emergence. Larvae emerged significantly earlier in treatments of high conspecific density and in the presence of chemosensory predator cues. AIC models of best fit for emergence time included a three-way interaction among incubation temperature, treatment and degree of temperature deviance and an additional fixed effect of the percent of endogenous yolk reserves utilized.
4. Plasticity in emergence time in response to environmental conditions may allow individuals to leave areas of high risk (e.g. predators, high conspecific density) or environmental deviance that likely influence survival.

Key words *Acipenser*, lake sturgeon, yolk-sac larvae, emergence time, phenotypic variation, environmental variation, plasticity

Introduction

Organisms have the ability to behaviorally, physiologically, and phenotypically respond to new or changing environmental conditions that affect their ability to survive and reproduce. Within-generation responses to ecological factors (plasticity) result from the combined effects of genotype and environmental variables (West-Eberhard 2003) and are often differentially expressed during sequential ontogenetic stages (Wilson, Kruuk & Coltman 2005). Plasticity in expression of phenotypic traits and behavior in response to ecological variables can be advantageous, as demonstrated for numerous life history, morphological, behavioral, and physiological characteristics (Miner et al. 2005; Reylea 2001; Rudolf & Rodel 2007; Stillwell & Fox 2005; Werner & Anholt 1996; Westneat & Fox 2010). However, the role that prior conditions during early-life stages play in influencing subsequent life-stages is less well characterized (Pigliucci1998). Empirical data are needed that can evaluate the relative effects of multiple environmental variables on phenotypic and behavioral traits across ontogenetic stages.

During early ontogenetic stages, environmental conditions can affect traits that are associated with survival and life-history characteristics (Morbey & Ydenberg 2003). For example, temperatures experienced through the period of embryonic and early post-incubation development affect growth and developmental rate (Atkinson 1994) and offspring size (Fox & Czesak 2000; Ojanguren & Braña 2003; Stillwell & Fox 2005).

Development can also be influenced by resource availability and foraging risks (Dmitriew 2010; Johansson et al. 2001). Environmental conditions experienced during early development can modify developmental trajectories (West-Eberhard 2003) and can induce or constrain plastic responses (Fischer-Rousseau Pokwah &Cloutier 2010). The ages and sizes when organisms transition between life stages can also vary in response to environmental conditions (Day & Rowe 2002; Niehaus, Wilson & Franklin 2006; Orizaola, Dahl & Laurila 2010; Rudolf & Rodel 2007).

Stream environments are inherently complex (Faush et al. 2002) due to the large number of abiotic (i.e., temperature, flow, substrate) and biotic (i.e., food availability, predators, competitors) factors that can affect growth, behavior, and survival. For many broadcast-spawning fishes, eggs (and by extension larvae) are exposed to a wide suite of environmental variables. Additionally, eggs are often fertilized by multiple males (Beamesderfer & Farr 1997; Bruch & Binkowski 2002; Kempinger 1988) therefore eggs and larvae of the same or different pedigree are exposed to widely varying environmental regimes. Subsequently following hatch, early-life stages are characterized by high rates of mortality (Kempinger 1988). Thus, the degree to which individuals can respond plastically to variation in ecological factors can have significant effects on individual survival and population levels of recruitment, which are particularly important for species commercially harvested or of conservation concern.

Lake sturgeon (*Acipenser fulvescens*) are long-lived, iteroparous fish with high fecundity but low annual recruitment due to high mortality during early life stages (Billard & Leconintre 2001). Eggs are widely dispersed by stream currents, and adhere to the substrate where they remain until hatch with no parental care provided (Peterson,

Vecsei & Jennings 2007). Newly-hatched yolk-sac larvae lack many prominent structures necessary for movement, sensory perception, and resource acquisition, and as a consequence immediately burrow in stream substrate and generally remain there until endogenous yolk resources are absorbed (Auer & Baker 2002; Smith & King 2005; Kempinger 1988). Larval emergence from stream substrate and dispersal is believed to be motivated by the need to begin exogenous feeding (Smith &King 2055; Lahaye et al. 1992). However, there is considerable plasticity in the timing of emergence (Duong et al. 2011), which in part is associated with environmental conditions (e.g., temperature and discharge) that individuals experience during incubation and immediately following hatch. The timing and location of lake sturgeon spawning on the Upper Black River, Michigan, varies as a function of river temperature and discharge (Forsythe et al. 2012a). In most years, multiple spawning groups of adults reproduce "early" or "late" in the spawning season corresponding to (on average) an 8°C difference in spawning temperature, and in different spawning locations. Timing and location of spawn by 'early' and 'late' spawning groups is repeatable across years, although the composition of individuals may be different (Forsyth et al. 2012b). Low water temperatures early in the spawning season results in longer incubation times and offspring that have larger body size and greater yolk reserves at hatch compared with offspring of late spawning females (Crossman 2008), which we predict will influence the timing of larval emergence from the substrate.

In this study, we quantified associations of current and previous environmental conditions with larval morphology and behavior at three distinct ontogenetic stages and during transitions between those stages using "common garden" experiments. We tested

the primary hypothesis that inter-individual variability in time until emergence and growth was influenced by food availability, presence of predators and conspecific density. We predicted that individuals exposed to chemosensory predator cues or high conspecific densities would emerge after a shorter period in the substrate and at a smaller size compared to individuals supplied with an exogenous food resource or relative to controls (status quo ambient conditions). We also tested whether emergence time and growth were affected by 1) temperature simulating conditions during the early and late periods in the spawning season during embryo incubation 2) family effects (collectively including maternal provisioning and genetic effects), and 3) degree of deviance in temperature immediately before larval emergence.

Methods

Experimental Design and Sampling

All work was conducted at the Black Lake stream-side research facility located on the Upper Black River, Cheboygan Co., Michigan. Eggs from two early-spawning females were fertilized with sperm from two early-spawning males to produce full-sibling families. Fertilized eggs from each family were divided into two groups and incubated in trays at water temperatures typical of thermal regimes experienced by embryos in natural stream systems deposited by early-spawning (10°C) or late-spawning (18°C) adults (Forsythe et al. 2012a). Temperatures were maintained through the incubation period using a re-circulating tank and heating and chilling units. Hereafter, the 10°C and 18°C treatments are referred to as "cold" and "warm" respectively.

At hatch, all yolk-sac larvae were anesthetized in a 20 mg/L solution of tricaine methanesulfonate (MS-222) and photographed using a digital camera (E-420, Olympus America Imaging Inc., Pennsylvania, USA) for morphometric measurements using image analysis software (ImageJ 1.44; Abramoff, Magalhaes & Ram 2004). A ruler was included in all photographs to allow accurate estimation of total length, total body area and yolk sac area (a measure of endogenous energy reserves).

Immediately following photography, individuals from each family and incubation treatment (cold and warm) were placed individually into 240 ml plastic containers (N=263)(Fig. 1) and randomly assigned to one of four 4 m by 0.67 m fiberglass raceways. All containers contained gravel that simulated stream substrate, which allowed yolk-sac larvae to burrow into interstitial spaces. Substrate length and depth were measured. The containers had two, 2.5 cm by 8 cm sections removed on opposite sides and replaced with mesh screen to allow constant flow of water through the containers. Stream water at ambient temperature was pumped into the raceways at a constant velocity (0.03m/sec ± 0.003; mean±SD). Water depth was maintained in the raceways at depths approximately 1cm below the top of the containers to prevent yolk-sac larvae dispersal. Water temperature was monitored hourly using a temperature logger (550A, YSI, Ohio, USA) to estimate cumulative thermal units (CTU; Kempinger 1988). A photoperiod of 9 hrs light to 15 hrs darkness was maintained to simulate a longer night regime because larvae emerge to disperse more frequently during night-time hours (Kempinger 1988; Smith & King 2005).

Each raceway was used to examine the effect of either conspecific density, food availability or predator presence. Conspecific density was simulated by adding two yolk-

sac larvae to each container as opposed to a single individual per container in all other treatments. A substrate environment with available food sources allowing for exogenous feeding was simulated by providing a small amount of food in each container (approximately 100μL of solution containing 746± 235 brine shrimp nauplii; mean± SD) twice a day (08:00, 20:00). The effect of predators was simulated by caging predators of lake sturgeon larvae (rusty crayfish (*Orconectes rusticus*) and odonate larvae including families *Caliopterygidae*, *Caliopteryx*, *Gomphidae*, *Cordulagastridae, and Aeshnidae*; Crossman 2008) upstream from the contained yolk-sac larvae to allow predatory chemosensory cues to diffuse through the raceway. The fourth raceway served as a control.

All containers were monitored for emerged larvae at 03:00, 08:00, and 20:00. Emergence time was defined as time (days) and CTU from the time of introduction into the container until emergence from the substrate. Upon emergence from the substrate, larvae were anaesthetized and photographed to obtain measures of body size and yolk sac area. Differences in body size and yolk sac area from the time of hatch and emergence provided a measure of growth and use of endogenous reserves, respectively.

Measures of Environmental Deviance

We estimated four measures of thermal deviance to test whether the degree of deviation in conditions at or near the time of emergence relative to previous conditions during the entire period in the gravel would stimulate emergence. The first measure of deviance (ΔTE:TW; Eq. 1) was calculated as the difference in the mean daily temperature for the 24 hr period prior to emergence (TE) compared to the mean daily temperature

over the entire duration in the substrate excluding the last 24 hours (TW), where *i* is the hourly temperature for the 24 hours prior to emergence, *j* is the mean temperature for one 24 hour period and *N* is the number of days a fish was in the container excluding the last 24 hours. A positive value in the deviance in mean daily temperatures indicated that the mean temperature during the 24 hours immediately prior to emergence from the gravel was greater than the mean daily temperature over the entire period larvae spent in the gravel.

$$[\text{Eq. 1}]\ \Delta TE{:}TW = \frac{\sum_{i=1}^{24} (EmergTemp_i)}{24} - \frac{\sum_{j=1}^{N} (OverallTemp_j)}{N}$$

The second temperature deviance measure (Δ RTE:RTW; Eq. 2) was calculated as the difference between the minimum and maximum temperature in the 24 hours prior to emergence (RTE) and the average difference between the daily minimum and maximum temperature over the entire duration spent in the substrate excluding the last 24 hours (RTW), where *i* is the temperature for the 24 hours prior to emergence, *j* is the mean temperature for one 24 hour period and *N* is the number of days an individual was in the container excluding the last 24 hours.

$$[\text{Eq. 2}]\ \Delta RTE{:}RTW = (MaxTemp_i - MinTemp_i) - \frac{\sum_{j=1}^{N} (OverallMaxTemp_j - OverallMinTemp_j)}{N}$$

Deviance was also measured as the difference in incubation temperature and the mean daily temperature of the raceway at the point when the yolk-sac larvae were first placed into treatments (Temperature Deviance (day)) as well as the difference between

incubation temperature and the hourly temperature at placement (Temperature Deviance (hour)).

Statistical Methods

The effects of previous and current environmental conditions on emergence time and growth were tested using mixed model analyses. Because all individuals came from one of two full-sibling families, and are therefore non-independent, family was included as a random effect in all models [Eq. 3]. Models included one of thirteen different fixed effects: treatment (control, presence of chemosensory predator cues, conspecific density, and food), incubation temperature (cold or warm), body area growth, percent body area growth, percent yolk sac utilized, substrate depth, number of rocks characterizing container substrate, the average substrate size, container position within the raceway (edge or inside), two measurements that described the deviation from the temperature on the date of emergence compared to the mean temperature over the course of time spent in the substrate (ΔTE:TW; ΔRTE:RTW), and two measurements that described the deviation from the incubation temperature to the temperature when they were placed in the treatments (hour, day).

[Eq. 3] $y_{ij} = \mu + \alpha_i + b_j \text{ (random)} + e_{ij}$

where μ represents the intercept, α_i represents the fixed effect being tested, b_j represents the random effect, and e_{ij} represents the residual error. For example, duration (in days or CTU) was modeled as a function of treatment and the inclusion of family as a random effect.

Second, all potential combinations of models containing two fixed effects were included in a mixed model (Eq.4) and compared using Akaike Information Criterion (AIC).

[Eq. 4] $y_{ij} = \mu + \alpha_i + b_j(\text{random}) + c_k + e_{ijk}$

where μ represents the intercept, α_i represents the first fixed effect being tested, b_j represents the random effect, c_k represents the second fixed effect being tested, and e_{ij} represents the residual error. For example, duration (in days or CTU) is a function of treatment, incubation temperature, and the inclusion of family as a random effect.

Because we evaluated thirteen fixed effects, comparing all potential combinations would have resulted in 78 permutations for just two-factor models, we progressed to more complex models in a step-wise fashion using an ad-hoc analysis of models of best fit. We first evaluated the model of best fit among all two-factor models. In the event that a two-factor model was identified as the model of best fit with strong support, a third factor was added to the best two-factor model by including all potential third-factor combinations. All three-factor models were compared with each other and the best two-factor model using AIC. In the event that a highly-supported three-factor model was identified, we included a fourth factor to the best three-factor model by including all potential forth-factor combinations. The same procedures that were used for testing the models for the dependent variable of CTU were used for the dependent variables of time (in days) spent in the substrate and larval growth.

We evaluated a series of models containing interaction effects among the parameters of the model. Statistical models were first developed based on interactions among the fixed effects contained in the model of best fit, with and without additional fixed effects. Step-wise comparison of interactions among all effects in two- and three-way interactions with and without the addition of additional fixed effects was also evaluated. Finally, we evaluated models including interactions with factors that were significant in models alone, and were not represented in any of the "best" models identified. All models were then compared to identify which model best represented the data. Models with a ΔAIC of 2 or less were considered to have equal weighting and the most simplistic model among equally weighted models was selected (Burnham & Anderson 2002). All statistics were performed using the lme4 package in the statistical software "R" (R Development Core Team 2011).

Results

Larval Emergence Time

Variables associated with environmental conditions during previous ontogenetic stages (incubation temperature), and ecological and environmental conditions through the period spent in the gravel and immediately prior to emergence were significant predictors of larval emergence time. Embryos incubated at colder temperatures emerged faster in both measurements of time until emergence (Table 1, Fig. 2; GLMM; duration in days, $t = 30.10$, $P < 0.001$; CTU, $t = 3.31$, $P < 0.001$). The results of treatments varied between the different quantifications of emergence time (Table 1). Fish maintained at higher density (2 vs. 1 individuals per container) emerged sooner based on both the measures of

emergence time (GLMM; days, t = -1.93, P = 0.055; CTU, t = -3.92, P < 0.001). The presence of chemosensory cues from predators resulted in significantly sooner emergence quantified in CTU (GLMM, t = -2.77, P = 0.006), but not in days (GLMM; t = -0.86, P = 0.393). There was a negative but non-significant effect of food supplementation on larval emergence time in quantification in days (GLMM; t = 1.77, P = 0.07) and CTU (GLMM; t = 0.27, P = 0.789).

The effects of other environmental and ecological factors were similar for emergence time quantified in either days or CTU (Table 1). The number of rocks in each container was positively associated with emergence time (GLMM; duration in days, t = 2.99, P = 0.003; CTU, t = 2.46, P = 0.015), whereas the average substrate size was negatively associated with emergence time (GLMM; duration in days, t = -3.59, P < 0.001; CTU, t = -2.48, P = 0.014). Substrate depth was not associated with emergence time (GLMM; duration in days, t = -0.86, P = 0.391; CTU, t = 0.67, P = 0.504).

Temperature deviance parameters were significantly related to emergence time. Higher temperature means (ΔTE/TW) (GLMM; days, t = 21.70, P < 0.001; CTU, t = 4.40, P < 0.001) and larger ranges in temperature deviance (ΔRTE/RTW) (GLMM; days, t = 7.31, P <0.001) over the 24-hour period immediately preceding emergence resulted in a longer time to emergence. Deviance in temperature range was not significant when measured in CTU (GLMM; t = -1.04, P = 0.299). Larger deviations between the incubation temperature and the hourly or daily temperature at the time of placement into the raceway resulted in faster emergence times (Fig. 4; GLMM; duration in days, deviance in hours, t = -21.85, P <0.001, deviance in days, t = 21.74, P < 0.001; CTU, deviance in hours, t = -4.40, P <0.001, deviance in days, t = -4.01, P < 0.001). Growth

during the period in the gravel (i.e., difference in body area at hatch and time of emergence) and the amount of remaining endogenous energy reserves (as reflected in the percentage of yolk sac utilized from hatch until emergence) were also significant predictors of emergence time (Table 1; GLMM; duration in days, area growth, $t = 5.43$, $P < 0.001$; percent yolk sac utilized, $t = 4.17$, $P < 0.001$; CTU, area growth, $t = 3.29$, $P < 0.001$; percent yolk sac utilized, $t = 10.19$, $P < 0.001$). Position within the raceway was not a significant predictor of duration (GLMM; duration in days, $t = 0.93$, $P = 0.35$; CTU, $t = 0.48$, $P = 0.63$).

Individuals from family 2 emerged earlier in all treatments and incubation temperature regimes than did individuals from family 1 (Fig. 2). Yolk-sac area at hatch showed a family-specific response associated with incubation temperature. Smaller yolk sacs were observed for individuals incubated in cold temperatures in family 2, but not in family 1 (Fig. 3c). Yolk-sac area at emergence also showed a family-specific response. Individuals in family 2 exhibited greater variability in yolk sac size compared to family 1 in both warm and cold incubation temperatures (Fig. 3d). Individuals from family one showed more variability with respect to emergence time when exogenous food or chemosensory cues from predators were present. In contrast, a higher degree of variability in emergence time was observed for family 2 associated with the control and density treatments. Though only two incubation temperatures were evaluated, qualitatively, the reaction norms for larval emergence time in each treatment as a function of incubation temperature did not appear to differ in either family. However, comparatively lower variation in emergence times was observed for individuals incubated in warm compared to cold temperatures in both families.

AIC model comparison results for the best one, two, three, four, and five factors with iterations of best interaction models that reflect the stepwise process (Appendix 1), suggesting both current and prior conditions affected emergence time. Models of best fit for emergence time quantified either in days or CTU included a three-way interaction among incubation temperature, treatment, and degree of temperature deviance (ΔRTE/RTW) with an additional fixed effect of the amount of endogenous yolk reserve used.

Yolk-Sac Larval

\Food supplementation was positively associated with growth (Table 2; GLMM; food, t = 2.29, P = 0.023), whereas conspecific density and the presence of chemosensory cues from predators were not (GLMM; density, t = -1.82, P = 0.070; predators, t = -1.13, P = 0.258). Embryos incubated in warm water were smaller at hatch in both families (Fig. 3a), but all individuals, regardless of embryonic incubation temperature or family, were similar in size at emergence (Fig. 3b). Yolk-sac larval growth was not associated with incubation temperature (Table 2; GLMM, t = -0.32, P = 0.747). The number of rocks was positively associated with growth, and the average substrate size negatively influenced growth (GLMM; number of rocks, t = 3.54, P < 0.001; average substrate size, t = -3.08, P < 0.001). Substrate depth had no effect on growth (GLMM; t = 1.31, P = 0.191). Deviation in mean temperature between hatch and emergence (ΔTE:TW) was positively associated with growth (GLMM; t = 2.23, P=0.027) while ΔRTE:RTW was not (GLMM; t = 0.26, P=0.797). Time spent in the substrate until emergence did not affect growth (GLMM; duration in days, t = 0.82, P = 0.408; CTU, t = 1.01, P = 0.312).

Yolk sac utilization was not a substantial determinant of growth (GLMM; $t = 1.18$, $P = 0.241$). Location within the raceway had no impact on growth (GLMM; $t = 1.55$, $P = 0.124$). The model of best fit was a single parameter model of treatment (control, predation, food, and density) (AIC; Appendix 2).

Discussion

For aquatic poikilothermic vertebrates, little is known regarding whether the effects of previous environments constrain behavioral and phenotypic traits in subsequent life stages. Comprehensive studies are needed to quantify associations among phenotypic traits and behavior and of the dependence and fitness implications of plastic behavioral, physiological, and phenotypic responses on the timing and sequence of developmental events (ontogenic contingency; Pigliucci 1998). The paucity of information for mobile vertebrate species is due in part to difficulties of disentangling temporal and spatial dependencies of environmental variable states (e.g., as compared to plants; Banerjee et al. 2010) and the tendencies of related individuals to co-occur in similar environments during sequential ontogenetic stages. We studied a series of ontogenetic stages through early development and tested hypotheses regarding how previous and current conditions influenced morphology and behavior at subsequent life stages, as well as variation in the timing of transitions between ontogenetic stages. By considering all stages together, we can identify the important components of particular stages, as well as how the factors interact to impact a single stage and subsequent stages.

Embryonic Incubation and Yolk Sac Utilization

Environments experienced by females at the time and location of spawning can significantly influence offspring phenotypic traits (Mousseau & Fox 1998), and can collectively contribute to embryonic and larval developmental time (Einum & Fleming 2000; Kamler 2002) and dispersal (Duong et al. 2011; Edwards et al. 2007). Lake sturgeon exhibit bi-modal distribution of spawning times (Forsythe et al. 2012b), thus temperatures at the time of egg release by females vary, which can influence transition time between early ontogenetic stages. Embryos from early spawning females experience colder incubation temperatures whereas embryos of late spawning events experience warmer incubation temperatures. Here, individuals exposed to colder embryonic incubation temperatures (10^{o}C vs. 18^{o}C) emerged from the substrate after shorter periods (Table 1). However, Duong et al. (2011) found that in naturally produced lake sturgeon, offspring produced by adults from early spawning periods (cold) spent longer periods (days and CTU) in the substrate. Colder incubation temperatures have also been shown to be associated with slower yolk-sac absorption in lake sturgeon (Wang, Binkowski, & Doroshov 1985) and other species including shortnose and Atlantic sturgeon (Hardy and Litvak 2004), which may also affect emergence time.

In our experiment, embryonic incubation temperature was held constant whereas rearing temperatures were allowed to fluctuate with the natural environment. As a result, there was a sharp decline in water temperature that was only experienced by yolk-sac larvae from the warm incubation temperatures (Fig. 4). Thus, we cannot disentangle whether differences observed between the two treatments resulted from incubation temperature effects or from a dramatic temperature change that was experienced post-hatch but prior to emergence.

Pre-emergence Conditions

Pre-emergence conditions experienced by the yolk-sac larvae can also influence the timing of emergence. Higher conspecific density was associated with faster emergence times. Based on CTU data, the presence of chemosensory cues from predators shortened emergence time. Substrates characterized by larger (and thus fewer) rocks were associated with faster emergence times likely due to the size of interstitial spaces. Additionally, yolk-sac larval growth was influenced by post-hatch temperatures, food supplementation, and was marginally influenced by conspecific density (Table 2). Thus, individuals appear to be gaining information from their surroundings to emerge from the substrate when there is increased conspecific competition, high-risk of predations, or areas that do not provide sufficient cover. Larvae that emerge earlier in these environments may allow them to travel to areas where risks of mortality are lower.

Temperature Deviance

Temperature deviance prior to emergence affected the timing of emergence. Larger deviations between the incubation temperature and the mean daily and hourly ambient water temperature at the point when the yolk-sac larvae were first placed into the containers resulted in faster emergence times (both CTU and duration in days). In contrast, more variability in temperature throughout the duration in the substrate resulted in longer emergence times. In both measurements of time until emergence (CTU, duration in days), larger deviations in the mean temperature of the 24 hour period immediately prior to emergence resulted in longer times until emergence. Longer times

until emergence were observed when there were large deviations between the average minimum and maximum temperatures between the 24 hours prior to emergence and the minimum and maximum of the incubation temperature when emergence was measured in days, but not when measured as CTU. Collectively, these results suggest that individuals do not emerge when conditions are highly variable (and potentially unpredictable), but emerge when conditions are strikingly different from incubation conditions (e.g. seasonal variation triggering warmer conditions). Kempinger (1988) also reported a peak in larval drift corresponding with a sharp increase in water temperature. Therefore, temperature conditions, and specifically deviations in temperatures relative to incubation temperatures, contribute to the timing of emergence.

Anthropogenic activities can influence short-term changes in stream temperature. For example, dams can influence water temperature, as well as increase diel deviations in water temperatures . Such temperature fluctuations could affect emergence timing and potentially influence survival and mortality of larvae.

Multi-factor Model Selection to Predict the Transition to Emergence

Despite differences in significance among univariate models between the different measurements of time until emergence (CTU vs. duration in days), model selection identified that the same factors contributed to emergence time. Emergence time was best explained by a model that included a three-way interaction between incubation temperature, treatment, and temperature deviance (ΔRTE/RTW) plus the inclusion of the additional fixed effect of the percent of endogenous yolk reserves used (AIC analysis, Appendix 1). Growth was best explained by treatment, specifically food supplementation

and potentially density, suggesting that pre-emergence environments across multiple ontogenetic stages influence both growth and emergence time.

The long-term effects of early-stage environmental conditions on morphology and behavior have been established for several aquatic organisms (Luning 1992; Black 1993; Dittman & Quinn 1996) and our results demonstrate the importance of environmental conditions to the early life-stages of lake sturgeon, and potentially other long-lived, iteroparous fishes.

Acknowledgements

Support for this project was provided by the Michigan Department of Natural Resources through Federal Aid Project and the Great Lakes Fisheries Trust. Additional support was provided by the Michigan Agricultural Experiment Station at Michigan State University and the Partnership for Ecosystem Research and Management (PERM) cooperative program between the Michigan Department of Natural Resources and Environment and Department of Fisheries and Wildlife at Michigan State University. We gratefully acknowledge the assistance during periods of data collection and analysis provided by Ryan Hastings, John Bauman, Jared Homola, Greg Hill, Nikki Hegna, and Tori Roznowski. We thank members of the Scribner lab for comments on an earlier draft of the paper.

References

Abramoff, M.D., Maalhaes, P.J. & Ram, S.J. (2004) Image processing with ImageJ. *Biophotonics International*, **11**, 36-42.

Atkinson, D. (1994) Temperature and organism size-a biological law for ectotherms? *Advances in Ecological Research*, **25**, 1-58.

Auer, N.A., & Baker, E.A. (2002) Duration and drift of larval lake sturgeon in the Sturgeon River, Michigan. *Journal of Applied Ichthyology*, **18**, 557-564.

Banerjee, S., Finley, A.O., P. Waldmann, and T. Ericsson. 2010. Hierarchical spatial process models for multiple traits in large genetic trials. *Journal of the American Statistical Association*, **105**, 506-521.

Beamesderfer, R.C.P. & Farr, R.A. (1997) Alternatives for the protection and restoration of sturgeons and their habitat. *Environmental Biology of Fishes*, **48**, 407-417.

Billard, R. & Lecointre, G. (2001) Biology and conservation of sturgeon and paddlefish. *Reviews in Fish Biology and Fisheries*, **10**, 355-392.

Bruch, R.M. & Binkowski, F.P. (2002) Spawning behavior of lake sturgeon (Acipenser fulvescens). *Journal of Applied Ichthyology*, **18**, 570-579.

Black, A.R. (1993) Predator-induced phenotypic plasticity in *Daphnia pulex*: Life history and morphological response to *Notonecta* and *Chaoborus*. *Limnology and Oceanography*, **38**, 986-996.

Crossman, J. (2008) *Evaluating collection, rearing and stocking methods for lake sturgeon (Acipenser fulvescens) restoration programs in the Great Lake*s. PhD Dissertation, Michigan State University, East Lansing.

Burnham, K.P. & Anderson, D.R. (2002) *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*. Springer, New York.

Day, T. & Rowe, L. (2002) Developmental thresholds and the evolution of reaction norms for age and size at life-history transitions. *The American Naturalist*, **159**, 338-350.

Dmitriew, C.M. (2010) The evolution of growth trajectories: what limits growth rate? *Biological Review*, **2010**, 000-020.

Dittman, A.H. & Quinn, T.P. (1996) Homing in pacific salmon: Mechanism and ecological basis. *The Journal of Experimental Biology*, **199**, 83-91.

Duong, Y., Scribner, K.T., Crossman, J.A., Forsythe, P. & Baker, E. (2011) Environmental and maternal effects on timing and duration of dispersal of larval lake sturgeon (*Acipenser fulvescens*). *Canadian Journal of Fisheries and Aquatic Sciences*, **68**, 643-654..

Edwards, K.P., Hare, J.A., Werner, F.E. & Seim, H. (2007) Using 2-dimensional dispersal kernels to identify the dominant influences on larval dispersal on continental shelves. *Marine Ecology Progress Series*, **352**, 77-87.

Einum, S. & Fleming, I. (2000) Selection against late emergence and small offsring in Atlantic salmon (*Salmo salar*). *Evolution*, **54**, 628-639.

Fausch, K.D., Torgersen, C.E., Baxter, C.V. & Li, H.W. (2002) Landscapes to riverscapes: bridging the gap between research and conservation. *Bioscience*, **52**, 483-498.

Fisher-Rousseau, L., Pokwah Chu, K. & Cloutier, R. (2010) Developmental plasticity in fish exposed to a water velocity gradient: a complex response. *Journal of Experimental Biology*, **314**, 67-85.

Forsyth, P.S., Scribner, K.T., Crossman, J.A., Ragavendran, A., Baker, E.A., Davis, C. & Smith, K.K. (2012) Environmental and lunar cues are predictive of the timing of river entry and spawning-site arrival in lake sturgeon *Acipenser fulvescens*. *Journal of Fish Biology*, **81**, 35-53.

Forsyth, P.S., Crossman, J.A., Bello, N.M., Baker, E.A. & Scribner, K.T. (2012) Individual-based analyses reveal high repeatability in timing and location of reproduction in lake sturgeon (*Acipenser fulvescens*). Canadian Journal of Fisheries and Aquatic Sciences, **69**, 60-72.

Fox, C.W. & Czesak, M.E. (2000) Evolutionary ecology of progeny size in arthropods. *Annual Reviews of Entomology*, **45**, 341-369.

Hardy, R.S. & Litvak, M.K. (2004) Effects of temperature on the early development, growth, and survival of shortnose sturgeon, *Acipenser brevirostrum*, and Atlantic sturgeon, *Acipenser oxyrhynchus*, yolk-sac larvae. *Environmental Biology of Fishes*, **70**, 145-154.

Johansson, F., Stoks, R., Rowe, L. & De Block, M. (2001) Life History plasticity in a damselfly: effects of combined time and biotic contraints. *Ecology*, **82**, 1857-1869.

Johnsson, J.I., Bohlin, T. (2005) Compensatory growth for free? A field experiment on brown trout, *Salmo trutta*. *Oikos*, **111**, 31-38.

Kamler, E. (2002) Ontogeny of yolk-feeding fish: an ecological perspective. *Reviews in Fish Biology and Fisheries*, **12**, 79-103.

Kempinger, J.J. (1988) Spawning and early life history of lake sturgeon in the Lake Winnebago system, Wisconsin. *American Fisheries Society Symposium Series*, **5**, 110-122.

Lahaye, M., Brandchaud, A., Gendron, M., Verdon, R. & Fortin, R. (1992) Reproduction, early life history, and characteristics of the spawning grounds of the lake sturgeon (Acipenser fulvescens) in Des Prairies and L'Assomption rivers, near Montreal, Quebec. *Canadian Journal of Zoology*, **70**, 1681-1689.

Luning, J. (1992) Phenotypic plasticity of *Daphnia pulex* in the presence of invertebrate predators: morphological and life history responses. Oecologia, **92**, 383-390.

Morbey, Y.E. & Ydenberg, R.C. (2003) Timing games in the reproductive phenology of female pacific salmon (*Oncorhynchus* spp.). *America Naturalist*, **161**, 284-298.

Miner, B.G., Sultan, S.E., Morgan, S.G., Padilla, K.D. & Relyea, R.A. (2005) Ecological consequences of phenotypic plasticity. *Trends in Ecology and Evolution*, **20**, 685-692.

Mousseau, T.A. & Fox, C.W. (1998) *Maternal Effects as Adaptations*. Oxford University Press, New York.

Niehaus, A.C., Wilson, R.S. & Franklin, C.E. (2006) Short- and long- term consequences of thermal variation in the larval environment of anurans. *Journal of Animal Ecology*, **75**, 686-692

Ojanguren, A.J. & Brana, F. (2003) Thermal dependence of embryonic growth and development in brown trout. *Journal of Fish Biology*, **62**, 580-590.

Orizaola, G., Dahl, E. & Laurila, A. (2010) Compensating for delayed hatching across consecutive life-history stages in an amphibian. *Oikios*, **119**, 980-987.

Peterson, D.L., Vecsei, P. & Jennings, C.A. (2007) Ecology and biology of the lake sturgeon: a synthesis of current knowledge of a threatened North American Acipenseridae. *Review in Fish Biology and Fisheries*, **17**, 59-76.

Pigliucci, M. (1998) Developmental phenotypic plasticity: where internal programming meets the external environment. *Current Opinion in Plant Biology*, **1**, 87-91.

R Development Core Team (2011) R: A Language and Environment for Statistical Computing. Vienna, Austria.

Relyea, R. (2001) Morphological and behavioral plasticity of larval anurans in response to different predators. *Ecology,* **82**, 523-540.

Rudolf, V.H.W. & Rodel, M.O. (2007) Phenotypic plasticity and optimal timing of metamorphosis under uncertain time constraints. Evolutionary Ecology, **21**, 121-142.

Smith, K.M. & King, D.K. (2005) Dynamics and extent of larval lake sturgeon *Acipenser fulvescens* drift in the Upper Black River, Michigan. *Journal of Applied Ichthyology,* **21**, 161-168.

Stillwell, R.C. & Fox, C.W. (2005) Complex patterns of phenotypic plasticity: interactive effects of temperature during rearing and oviposition. *Ecology,* **86,** 924-934.

Wang, Y.L., Binkowski, F.P. & Doroshov, S.I. (1985) Effect of temperature on early development of white and lake sturgeon, *Acipenser transmontanus* and *A. fulvescens. Enivronmental Biology of Fishes*, **14**, 43-50.

Werner, E.E. & Anholt, B.R. (1996) Predator-induced behavioral indirect effects: consequences to competitive interactions in anuran larvae. *Ecology,* **77**, 157-169

West-Eberhard, M.J. (2003) *Developmental plasticity and evolution*. Oxford University Press, Oxford.

Westneat, D.F. & Fox, C.W. (2010) *Evolutionary Behavioral Ecology*. Oxford University Press, Inc., New York.

Wilson, A.J., Kruuk, L.E.B. & Coltman, D.W. (2005) Ontogenetic patterns in heritable variation for body size: using random regression models in a wild ungulate population. *American Naturalist,* **166**, 177-192.

Table 1. Mixed model analysis of the effect of incubation and pre-emergence environmental conditions on the time from hatch until larval emergence in days and cumulative thermal units (CTU). All models include a random effect of "family".

Dependent Variable: Duration	Fixed Effects		Beta Estimate	t-value	p-value
Days	Treatment	Control (Intercept)	13.89	11.82	<0.001
		Density	-1.46	-1.93	0.055
		Food	1.34	1.77	0.077
		Predator	-0.65	-0.86	0.393
Days	Incubation temperature	Cold (Intercept)	9.88	9.47	<0.001
		Warm	7.80	30.19	<0.001
Days	Area Growth		0.26	5.43	<0.001
Days	Percent yolk sac utilized		0.16	4.17	<0.001
Days	Substrate Depth		-0.32	-0.86	0.391
Days	Number of Substrate Rocks		0.06	2.99	0.003
Days	Average Substrate Size		-0.20	-3.59	<0.001
Days	ΔRTE/RTW		1.51	7.31	<0.001
Days	ΔTE/TW		1.55	21.70	<0.001
Days	Temperature Deviance (Hour)		-1.15	-21.85	<0.001
Days	Temperature Deviance (Day)		-1.18	-21.74	<0.001
Days	Raceway Position	Intercept (Edge)	13.44	11.16	<0.001
		Inside	0.51	0.93	0.35
CTU	Treatment	Control (Intercept)	171.46	13.19	<0.001
		Density	-18.82	-3.92	<0.001
		Food	1.29	0.27	0.789
		Predator	-13.35	-2.77	0.006
CTU	Incubation temperature	Cold (Intercept)	158.12	12.00	<0.001
		Warm	11.51	3.31	<0.001
CTU	Area Growth		1.06	3.29	<0.001
CTU	Percent yolk sac utilized		2.16	10.19	<0.001
CTU	Substrate Depth		1.60	0.67	0.504
CTU	Number of Substrate Rocks		0.35	2.46	0.015
CTU	Average Substrate Size		-0.91	-2.48	0.014

CTU	ΔRTE/RTW		-1.54	-1.04	0.299
CTU	ΔTE/TW		3.30	4.40	<0.001
CTU	Temperature Deviance (Hour)		-2.23	-4.01	<0.001
CTU	Temperature Deviance (Day)		-2.29	-3.98	<0.001
CTU	Raceway Position	Intercept (Edge) Inside	162.89 1.72	12.16 0.48	<0.001 0.63

Table 2. Results of mixed model analysis of the effect of incubation and pre-emergence environmental conditions on growth from hatch to emergence. All models include a random effect of "family".

Dependent Variable: Duration	Fixed Effects		Beta Estimate	t-value	p-value
Growth	Treatment	Control (Intercept)	0.39	6.75	<0.001
		Density	-0.15	-1.82	0.070
		Food	0.19	2.29	0.023
		Predator	-0.09	-1.13	0.258
Growth	Incubation temperature	Cold (Intercept)	0.39	9.22	<0.001
		Warm	-0.02	-0.32	0.747
Growth	Percent egg sac utilized		0.01	1.18	0.241
Growth	Substrate Depth		0.05	1.31	0.191
Growth	Number of Rocks		0.01	3.54	<0.001
Growth	Average Substrate Size		-0.02	-3.08	<0.001
Growth	ΔRTE/RTW		0.01	0.26	0.797
Growth	ΔTE/TW		0.03	2.23	0.027
Growth	Edge		0.09	1.55	0.124
Growth	CTU		0.00	0.83	0.408
Growth	Duration in Days		0.01	1.01	0.312

Appendix 1. AIC model comparisons for environmental conditions that best predict time until larval emergence in days and cumulative thermal units (CTU). Only the best two-factor and three-factor models were presented as a comparison with more complex models. Interaction models are only shown where the inclusion of an interaction term significantly improved a model beyond the less supported non-interaction model shown based on ΔAIC.

Dependent Variable: Duration	First Parameter	Second Parameter	Third Parameter	Forth Parameter	Fifth Parameter	AIC	ΔAIC	
Days	Incubation Temp*Treatment*RTE.RTW	Yolk sac utilized				984		*
Days	Incubation Temp * Treatment * RTE.RTW	Yolk sac utilized	Substrate Depth			989	5	
Days	Incubation Temp*Treatment	Yolk sac utilized				994	10	
Days	Incubation Temp*Treatment	Yolk sac utilized	RTE.RTW			995	11	
Days	Incubation Temp*RTE.RTW	Treatment	Yolk sac utilized			1003	19	
Days	Incubation Temp*TE.TW	Treatment	Yolk sac utilized			1006	22	
Days	Incubation Temp	Treatment	Yolk sac utilized			1007	23	
Days	Incubation Temp	Treatment	Yolk sac utilized			1007	23	
Days	Incubation Temp	Treatment	Yolk sac utilized	Substrate Depth		1010	26	
Days	Incubation Temp	Treatment	Yolk sac utilized	Yolk sac utilized		1011	27	
Days	Incubation Temp	Treatment	Yolk sac utilized	Substrate Size		1012	28	
Days	Incubation Temp	Treatment	Yolk sac utilized	Number of Rocks		1015	31	
Days	Incubation Temp*Treatment*Yolk sac utilized					1031	47	
Days	Incubation Temp*Treatment*Yolk sac utilized	RTE.RTW				1031	47	

Days	Incubation Temp*Treatment*Yolk sac utilized	Substrate Depth				1035	51	
Days	Incubation Temp*Treatment*Yolk sac utilized	Area Growth				1036	52	
Days	Incubation Temp*Treatment*Yolk sac utilized	TE.TW				1036	52	
Days	Incubation Temp*Treatment*Yolk sac utilized	Average Substrate Size				1038	54	
Days	Incubation Temp*Treatment*Yolk sac utilized	Number of Rocks				1041	57	
Days	Incubation Temp	Yolk sac utilized				1054	70	
CTU	Incubation Temp*Treatment*RTE.RTW	Yolk sac utilized	Substrate Depth			2290		
CTU	Incubation Temp*Treatment*RTE.RTW	Yolk sac utilized				2292	2	*
CTU	Incubation Temp*RTE	Treatment	Yolk sac utilized			2348	58	
CTU	Incubation Temp* Treatment					2373	83	
CTU	Treatment	Yolk sac utilized	Incubation Temp	RTE.RTW	Substrate Depth	2374	84	
CTU	Treatment	Yolk sac utilized	Incubation Temp	RTE.RTW	TE.TW	2376	86	
CTU	Treatment	Yolk sac utilized	Incubation Temp	RTE.RTW	Area Growth	2378	88	
CTU	Treatment	Yolk sac utilized	Incubation Temp	RTE.RTW	Average Substrate Size	2378	88	
CTU	Treatment	Yolk sac utilized	Incubation Temp	RTE.RTW	Number of Rocks	2380	90	
CTU	Treatment	Yolk sac utilized	Incubation Temp			2383	93	
CTU	Incubation Temp* Treatment* RTE.RTW					2393	103	
CTU	Treatment	Yolk sac utilized	Incubation Temp	TE.TW		2395	105	

CTU	Treatment	Yolk sac utilized	Incubation Temp	Substrate Depth		2399	109
CTU	Treatment	Yolk sac utilized	Incubation Temp	Area Growth		2401	111
CTU	Treatment	Yolk sac utilized	Incubation Temp	RTE.RTE		2401	111
CTU	Treatment	Yolk sac utilized				2402	112
CTU	Treatment	Yolk sac utilized	Incubation Temp	Average Substrate Size		2402	112
CTU	Treatment	Yolk sac utilized	Incubation Temp	Number of Rocks		2406	116

*Model of best fit

Appendix 2. AIC model comparisons for environmental conditions that best predict growth rate, with only the best two-factor models were presented as a comparison against more complex models.

Dependent Variable	Parameter 1	Parameter 2	AIC	ΔAIC	
Growth	Incubation Temperature	TE.TW	369.3		
Growth	Treatment		370.3	1	*
Growth	Number of Rocks		373.1	3.8	
Growth	Treatment	Edge	373.2	3.9	
Growth	Average Substrate Size		374.1	4.8	
Growth	Treatment	Number of Rocks	375.7	6.4	
Growth	Treatment	Incubation Temperature	375.9	6.6	
Growth	Treatment	Substrate Depth	376.2	6.9	
Growth	Average Substrate Size	Substrate Depth	376.4	7.1	
Growth	Edge		376.5	7.2	
Growth	Treatment t	Average Substrate Size	376.6	7.3	
Growth	ΔTE.TW		377	7.7	
Growth	Number of Rocks	Edge	377	7.7	
Growth	Treatment	ΔTE.TW	377.6	8.3	
Growth	Average Substrate Size	Edge	377.7	8.4	
Growth	Treatment	ΔRTE.RTW	377.9	8.6	
Growth	Substrate Depth		378	8.7	
Growth	Number of Rocks	Incubation Temperature	378.5	9.2	
Growth	Incubation Temperature		378.8	9.5	
Growth	Number of Rocks	Substrate Depth	378.9	9.6	
Growth	Number of Rocks	ΔTE.TW	379.1	9.8	
Growth	Incubation Temperature	Average Substrate Size	379.3	10	
Growth	Duration in Days	Incubation Temperature	379.4	10.1	
Growth	Average Substrate Size	ΔTE.TW	379.9	10.6	
Growth	Treatment	Duration in Days	380.5	11.2	
Growth	ΔRTE.RTW		380.6	11.3	
Growth	Number of Rocks	ΔRTE.RTW	380.7	11.4	
Growth	Edge	ΔTE.TW	380.7	11.4	
Growth	Treatment	Yolk sac utilized	380.8	11.5	

Growth	Substrate Depth	ΔTE.TW	381.6	12.3
Growth	Average Substrate Size	ΔRTE.RTW	381.7	12.4
Growth	Substrate Depth	Edge	381.8	12.5
Growth	Number of Rocks	Average Substrate Size	382.1	12.8
Growth	Incubation Temperature	Edge	382.1	12.8
Growth	Duration in Days		382.3	13
Growth	ΔRTE.RTW	ΔTE.TW	382.7	13.4
Growth	Yolk sac utilized		382.8	13.5
Growth	Number of Rocks	Yolk sac utilized	383	13.7
Growth	Duration in Days	Number of Rocks	383.2	13.9
Growth	Average Substrate Size	Yolk sac utilized	383.5	14.2
Growth	Incubation Temperature	Substrate Depth	383.7	14.4
Growth	ΔRTE.RTW	Edge	384	14.7
Growth	Treatment	CTU	384.2	14.9
Growth	Duration in Days	Average Substrate Size	384.2	14.9
Growth	Duration in Days	ΔTE.TW	384.7	15.4
Growth	ΔRTE.RTW	Substrate Depth	385.3	16
Growth	Incubation Temperature	ΔRTE.RTW	385.8	16.5
Growth	Duration in Days	Edge	385.9	16.6
Growth	Edge	Yolk sac utilized	386.1	16.8
Growth	CTU		386.5	17.2
Growth	Duration in Days	Substrate Depth	387.1	17.8
Growth	Number of Rocks	CTU	387.1	17.8
Growth	Yolk sac utilized	ΔTE.TW	387.5	18.2
Growth	Substrate Depth	Yolk sac utilized	387.9	18.6
Growth	CTU	Average Substrate Size	388.1	18.8
Growth	Incubation Temperature	Yolk sac utilized	388.5	19.2
Growth	Duration in Days	ΔRTE.RTW	389.7	20.4
Growth	CTU	Edge	390	20.7
Growth	ΔRTE.RTW	Yolk sac utilized	390.2	20.9
Growth	CTU	ΔTE.TW	391	21.7
Growth	CTU	Substrate Depth	391.5	22.2
Growth	Incubation Temperature	CTU	392	22.7
Growth	Duration in Days	Yolk sac utilized	392.5	23.2
Growth	CTU	ΔRTE.RTW	393.9	24.6
Growth	Duration in Days	CTU	395.8	26.5
Growth	CTU	Yolk sac utilized	396.5	27.2

*Model of best fit

Fig. 1. Diagram of raceways used to quantify the treatment effects of density, food supplementation, and presence of chemosensory cues from predators (predators housed in an enclosure (A)) on time (days or cumulative thermal units) until emergence from the substrate. Each dot represents an individual container with gravel substrate.

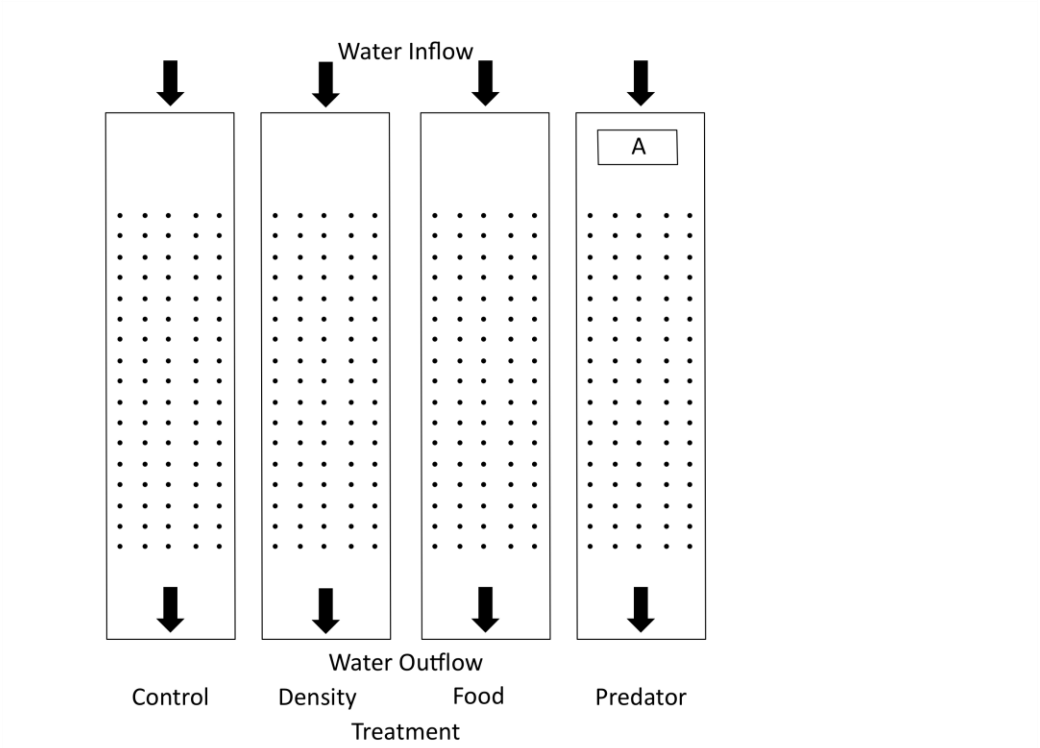
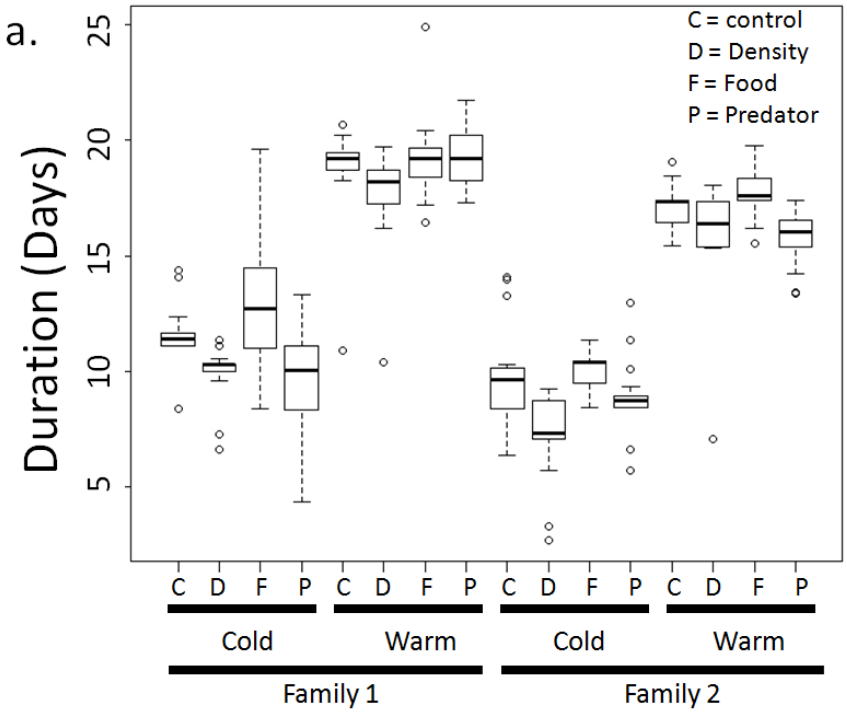


Fig. 2. The effects of family, egg incubation temperature, and treatment on the duration from hatch to larval emergence calculated in (a) days and (b) cumulative thermal units (CTU) shown as box (25-75% quartiles) and whisker plots (95% CI).



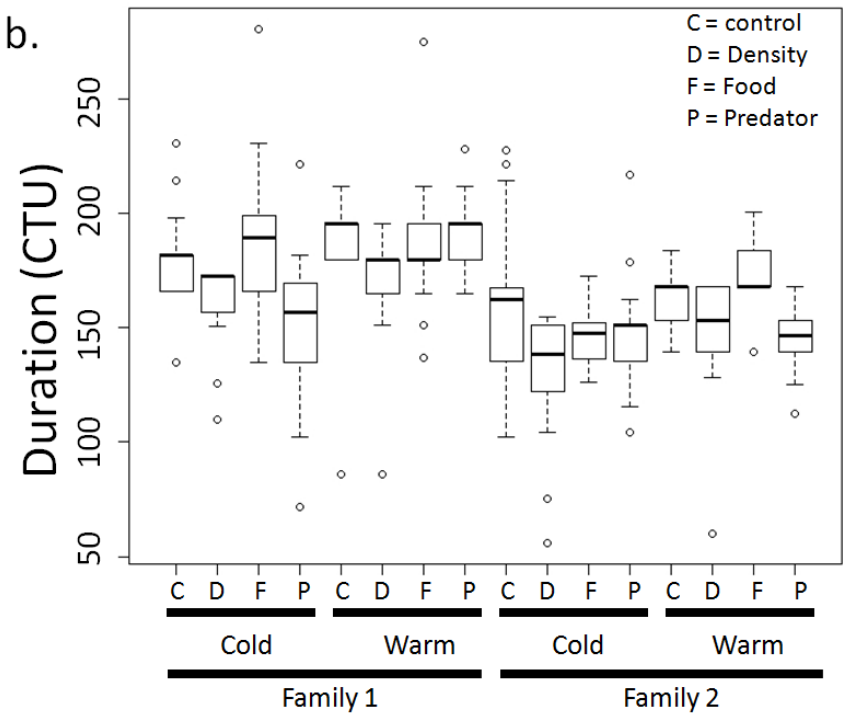

b.
C = control
D = Density
F = Food
P = Predator
Duration (CTU)
50
100
150
200
250
C D F P C D F P C D F P C D F P
Cold
Warm
Cold
Warm
Family 1
Family 2

Fig. 3. The effects of family and incubation temperature on body size and yolk sac size at the time of hatching (a, c) and emergence from the substrate (b, d) shown as box (25-75% quartiles) and whisker plots (95% CI).

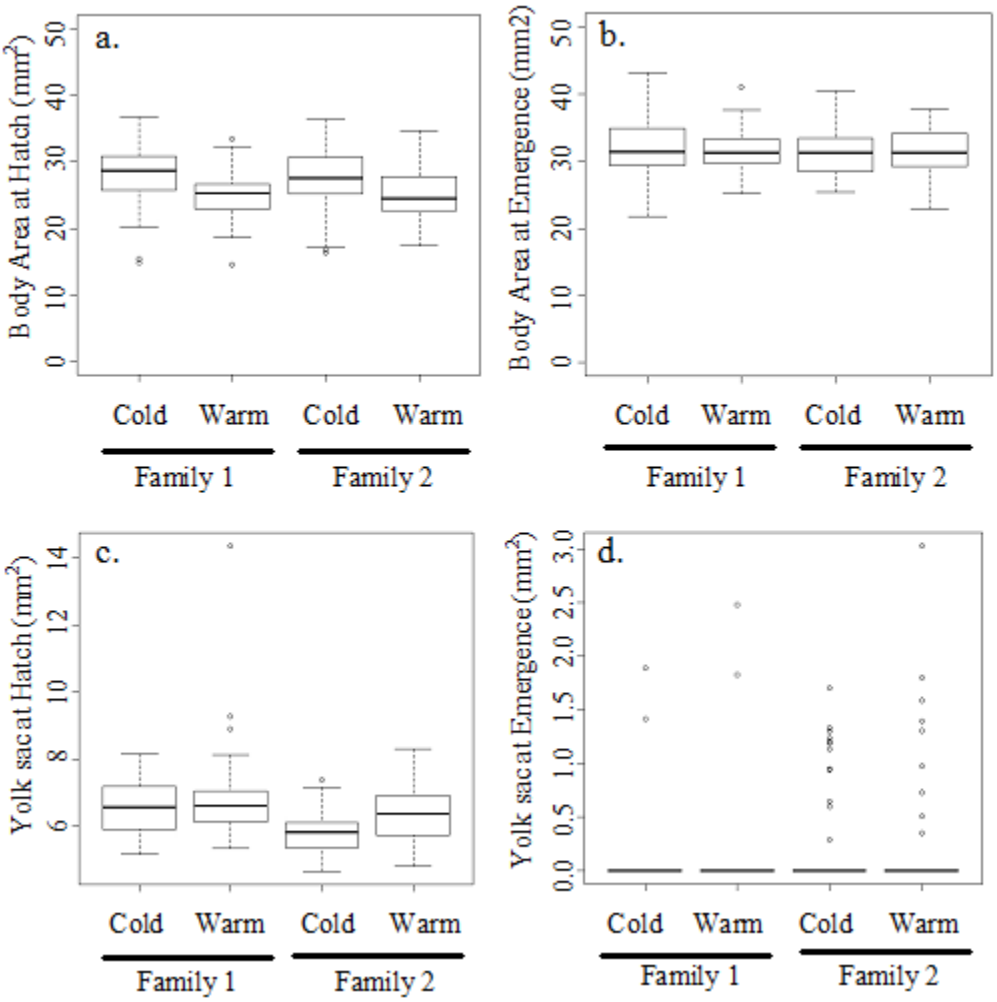
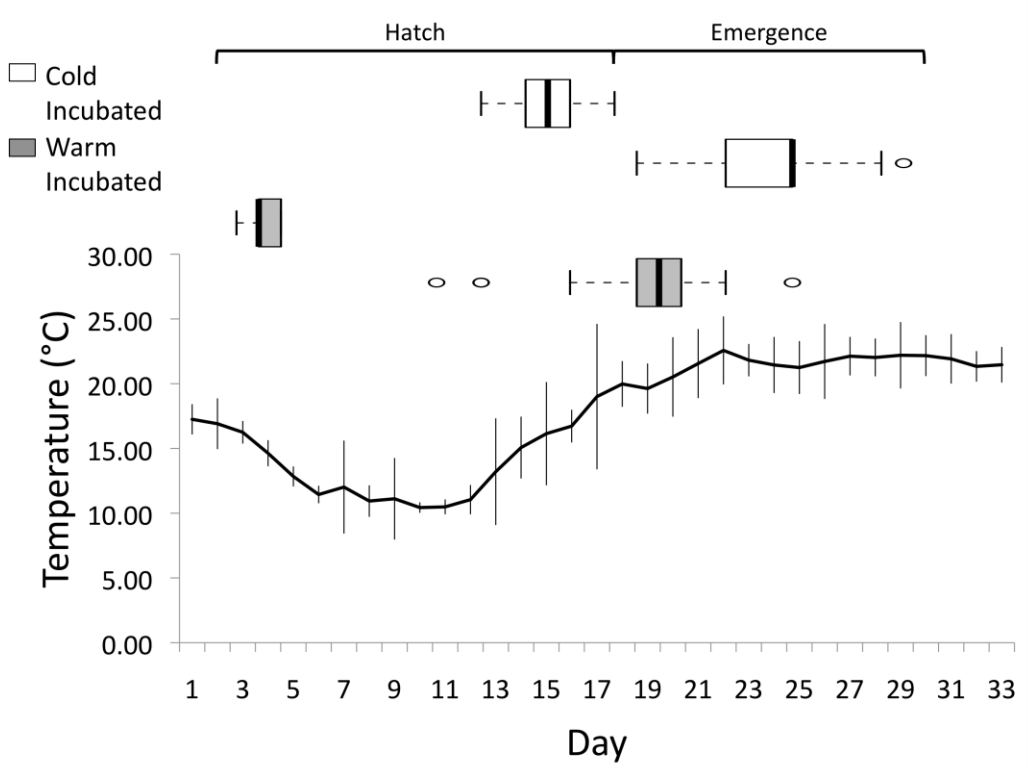


Fig. 4. Ambient water temperature (°C ±SD) over the duration of the experiment with box plots representing the day the yolk-sac larvae were placed into the containers (hatch) and emergence from the substrate (emergence). White and grey box plots represent yolk-sac larvae incubated in cold (10°C) and warm (18°C) temperature regimes of both families and across all treatments (food, predator, density, control), respectively.



Appendix #2 - Increasing thermal regimes reveal cryptic genetic variation during early ontogenetic stages of lake sturgeon (*Acipenser fulvescens*)

Authors: K.J. Dammerman[1], J.P. Steibel[2,3], K.T. Scribner[1,3]

Affiliations/Addresses of the authors:
[1]Department of Zoology, Michigan State University, 228 Farm Lane Rm 203, East Lansing, MI 48824, USA
[2]Department of Animal Science, Michigan State University, 474 S Shaw Lane, East Lansing, MI 48824, USA
[3]Department of Fisheries and Wildlife, Michigan State University, 480 Wilson Road Rm 13, East Lansing, MI 48824

Abstract
Climate change is affecting thermal conditions worldwide. Understanding organismal responses associated with predicted changes are essential for predicting future population persistence. However, few studies have examined the effects of both increased mean and variance in temperature on organismal traits, particularly during early life stages. Using lake sturgeon from Black Lake, MI, we tested whether phenotypes and behavior would vary among families incubated in different thermal regimes. Eggs were reared at two constant (10 and 18°C) and two fluctuating temperature treatments (10-19°C) representing temperatures larvae experience in the river and a simulated anthropogenic disturbance. Body length, body area, and yolk-sac area were quantified at hatch. Traits quantified at emergence (i.e. the onset of exogenous feeding) included time to emergence, body length at emergence, and growth from hatch to emergence. A significant family-by-treatment interaction was detected for traits measured at hatch. The greatest range in phenotypic variance was observed among individuals reared in the constant warm (18°C) treatment. Families also varied in growth (h^2=0.33) and the timing of emergence (h^2=0.24). Results demonstrate that increases in mean thermal regimes may reveal cryptic genetic variation potentially leading to differential survival between genotypes thereby altering the genetic architecture of the population.

Keywords: environmental change, temperature, genotype-by-environment interaction, cryptic genetic variation, selection, ectotherms, lake sturgeon

Introduction

Climate change and variability are altering thermal regimes on a global scale (IPCC 2013). Within the next century, mean temperatures are predicted to increase by approximately 2 to 5ºC due to human influences (Estay et al. 2014). Additionally, increases in the magnitude and frequency of extreme climatic events are altering the variability in thermal patterns (Pincebourde et al. 2012; Bauerfeind and Fischer 2014). Thermal changes are known to directly affect biological functions in wild populations. For instance, previous studies have documented advances in the timing of reproduction (Parmesan 2006), faster growth rates (Drinkwater 2005), and decreases in species abundance (Jonsson and Jonsson 2009) in response to increasing temperatures. Therefore, understanding the ecological and evolutionary responses of wild populations to predicted thermal changes due to climate change has become a major goal for ecologists and climate researchers (Hansen et al. 2012; Walther et al. 2002).

Organisms that are of particular conservation concern under predicted climatic changes are ectotherms given that temperature directly affects their physiology including growth, reproduction, and locomotion (Jonsson and Jonsson 2009; Deutsch et al. 2008). For example, one well-documented trend among ectotherms is the temperature-size rule in which rearing temperature is inversely related to body size (Diamond and Kingsolver 2010; Kingsolver and Huey 2008). Individuals that are reared in warm temperatures grow faster, but are typically smaller than individuals reared in cold temperatures (Atkinson 1994; Forster et al. 2012). Numerous studies have examined phenotypic trait changes in response to rearing temperatures in ectotherms given that changes in traits associated with fitness (i.e. body size) typically occur during early life stages which are characterized by high mortality, and are considered to be the most susceptible to thermal fluctuations (Jonsson and Jonsson 2009). Additionally,

environmental conditions experienced during early ontogeny have long-term consequences on individual fitness by affecting developmental rates, phenotypes, physiology, and behavior at later ontogenetic stages (i.e. ontogenetic contingency, Diggle 1994; Orizaola et al. 2010; Huey et al. 2012; Crespi and Warne 2013; Pittman et al. 2013; Dammerman et al. 2015).

Changes in phenotypic and behavioral trait variation are commonly visualized as thermal reaction norms that are constructed by rearing individuals of known genotype over a range of temperatures that remain constant and quantifying the variation in phenotypes that are expressed (Angilletta 2009). These plots have been useful for modeling the effects of climate change by exposing individuals to ecologically relevant changes in temperature and comparing their phenotypes to individuals reared under current thermal conditions in a laboratory setting. However, few studies have reared individuals under fluctuating incubation temperatures (i.e. diurnal temperature changes) that mimic conditions encountered in the wild (Bauerfeind and Fischer 2014). Recent research has highlighted that rearing individuals under fluctuating temperatures more accurately represents responses to climate change given that organisms encounter daily fluctuations in temperature (Niehaus et al. 2012; Bauerfeind and Fischer 2014), and temperature variability is predicted to affect traits associated with fitness to a greater extent than mean temperature alone (Paaijmans et al. 2013).

In this study, we reared different families of lake sturgeon (*Acipenser fulvescens*; Rafinesque 1817) under constant and fluctuating thermal egg incubation conditions and quantified phenotypic and behavioral variation at hatch and at the timing of emergence. Lake sturgeon are long-lived ectotherms and a useful species for examining the effects of thermal changes because spawning events, embryogenesis, and larval development occur across a range of temperatures. In the spring, adults migrate to riverine areas for spawning (Auer 1996). The

timing of spawning is multi-modal, and has been observed when water temperature is between 8.8 – 21.1˚C (Bruch and Binkowski 2002; Forsythe et al. 2012). During spawning events, females release demersal, adhesive eggs which are fertilized by multiple males (Thiem et al. 2013). Fertilized eggs from full and half-sibling families attach to the substrate and incubate without parental care under site-specific conditions until hatch (Duong et al. 2011). Larvae typically hatch within 5-14 days dependent on water temperature (Smith and King 2005; Kempinger 1988) then burrow into the substrate to endogenously feed on yolk-sac reserves (Hastings et al. 2013). Yolk-sac utilization and the timing of emergence when larvae disperse downstream to begin exogenously feeding are dependent on several abiotic conditions within larval rearing sites including temperature (Duong et al. 2011).

Our first objective was to determine whether larval phenotypes and the timing of emergence would vary among families reared in constant and fluctuating thermal incubation environments. We predicted that phenotypic variation within the fluctuating treatments would be greater than observed under constant treatments (Niehaus et al. 2012). Additionally, we predicted that phenotypes would vary among families given the large variance attributed to genetic (family) effects quantified in a recent study on lake sturgeon responses to river flow regimes (Dammerman et al. 2015). Our second objective was to assess whether thermal environments experienced during egg incubation would affect larval growth and behavior during subsequent ontogenetic stages. Quantifying trait changes conditional on environments experienced during previous ontogenetic stages increases understanding of how fluctuations in local thermal regimes will affect phenotypic trait variation and survival of different genotypes during critical developmental stages of ectotherms.

Materials and Methods

Study site

During the 2012 spawning season, adult lake sturgeon were sampled daily using long-handled dip nets on the Upper Black River (UBR). The UBR is the largest tributary of Black Lake located in Cheboygan County, Michigan (Fig. 1; Smith and King 2005). Adults migrate into the UBR from late April to early June to spawn among shallow (~1-3 meters) rocky areas (Baker and Borgeson 1999; Forsythe et al. 2012). Spawning activities observed early in the season occur when water temperature is approximately 10°C. By the later part of the season, spawning adults typically encounter water temperatures closer to 18°C. The wadable conditions of the stream and presence of a streamside rearing facility provide the opportunity to collect gametes from spawning adults and conduct experimental temperature manipulations and monitoring of larval traits.

Fertilizations

Gametes were collected from five females and ten males on May 3rd, 4th, and 6th of 2012. Eggs were collected from spawning females, placed in sealed plastic bags, and stored with river water to maintain eggs at ambient river temperature. Milt was collected using 20-mL syringes and placed on ice. Gametes were transported to the streamside rearing facility and fertilizations were conducted within twelve hours of collection. Approximately 200 eggs from each female were placed on 1-mm mesh screens within polymerized vinyl chloride couplings (31.90cm^2). Couplings were used to keep families separate. Each female's eggs were fertilized with 0.5mL of sperm from two males to create half-sibling families. Fertilized eggs were widely distributed within the couplings and left undisturbed for 30 minutes to allow adhesion to the mesh screens.

Thermal treatments

Fertilized eggs were incubated in heathtrays where temperature was controlled to produce two constant and two fluctuating thermal treatments (Fig. 2). Constant treatments, cold (10°C) and warm (~18°C), represented mean temperatures that characterized the early and late season spawning periods, respectively (Forsythe et al. 2012). Fluctuating treatments consisted of ambient river temperature (natural fluctuations of 1-3 degrees daily in river temperature) and a variable treatment simulating an anthropogenic disturbance with a range of 1-9 degrees of change per day. Water temperature within each treatment was measured daily using Onset HOBO pressure loggers (Cape Cod, Massachusetts, USA). Fertilized eggs were slowly acclimated to treatment temperatures (two-degree change per hour) prior to placement in heathtrays. Families were replicated among treatments and dead eggs were removed daily to prevent fungal infections.

Larval traits

Thirty larvae from each family within each treatment (N=1200 total) were anesthetized with tricaine methanesulfonate (MS-222; 25 mg/mL) and photographed at hatch using a digital camera. Photographs were analyzed using Image J analysis software (Version 1.34, free-ware) to quantify three phenotypic traits: body length (mm), body area (mm^2), and yolk-sac area (mm^2; Dammerman et al. 2015). Eighteen individuals were subsampled from five of the half-sibling families within each treatment (N=360 total) and randomly assigned to individual plastic incubation chambers (12.7cm by 6.35cm) to monitor until the timing of emergence. Incubation chambers contained gravel substrate to provide cover during the endogenous feeding period, ambient river water, and mesh siding to allow for continual water passage (~0.1m/sec). Chambers were randomly placed across three fiberglass raceways (3.7m by 0.67m) with respect to family and thermal incubation treatment to prevent any influence of chamber location on the

timing of emergence. Incubation chambers were monitored daily until larvae emerged from the substrate with depleted yolk-sac reserves to disperse downstream and begin exogenously feeding. Larvae were anesthetized and photographed again to quantify body length at emergence (mm), growth from hatch to emergence (mm), and the time from hatch to emergence (days).

Individuals within the thermal treatments were predicted to hatch at different times given that stream temperatures experienced during the endogenous feeding stage directly affect yolk-sac utilization and timing of emergence in fishes including sturgeon (Duong et al. 2011; Hardy and Litvak 2004). Therefore, we tested the influence of the ambient river temperature experienced while in the incubation chambers on the three traits measured at the time of emergence. Temperature data was collected hourly within the streamside facility using a YSI 5200 Recirculating System Monitor (Xylem, Inc.). For each individual, we calculated the mean temperature (MeanT) and range in temperature (daily maximum minus minimum, RangeT) experienced during the endogenous feeding period in the incubation chamber.

Statistical analysis

Statistical analyses were performed using the program, R (R Development Core Team) and the Bayesian inference package, MCMCglmm (Hadfield 2010). General linear mixed-effect models were used to test the contribution of Family, Treatment, and a Family-by-Treatment interaction on the larval traits measured at hatch. Traits were analyzed separately by fitting the full model:

Eq. (1) $Trait(y_{ijk}) = \mu + Treatment_i + Family_j + (Family\ x\ Treatment)_{ij} + \varepsilon_{ijk}$

where μ is the population mean, (x) represents an interaction between variables, and ε_{ijk} was the random residual error. Treatment was fit as a fixed effect. Family and the Family-by-Treatment interaction terms were fit as random effects where Family ~ $N(0, \sigma_F^2)$, Family x Treatment ~

$N(0, \sigma_{FS}^2)$, and $\varepsilon \sim N(0, \sigma_{\varepsilon}^2)$. Models were fit using a Family term as opposed to fitting the breeding values as a random effect (i.e. animal model, Wilson et al. 2010) given the possible presence of a genotype-by-environment interaction and the difficulty in convergence of the animal model. Traits quantified at the time of emergence were analyzed using the full model with the addition of MeanT and RangeT experienced while in the incubation chamber as fixed effects. Low correlation between MeanT and RangeT (Pearson's correlation = 0.31) allowed us to test the variables in the same model.

All models included a burn-in of 10,000, thinning interval of 100, and a total of 200,000 iterations which were chosen after examining diagnostic plots when using default starting values in the MCMCglmm package. Random effects were fit using a non-informative prior that follows an inverse-gamma distribution with a scale and shape of 0.001. A sensitivity analysis was conducted to determine there was no effect of the prior on the posterior distribution. Model selection was based on Deviance Information Criterion (DIC) which incorporates model fit and complexity based on an expected deviance parameter and the effective number of parameters estimated in the model (Spiegelhalter et al. 2002). We compared DIC estimates for the full model against all possible simpler models. The model with the lowest DIC estimate was selected as the model of best fit except when models were within two DIC values of each other. In that case, the simplest and less complex model was chosen as the model of best fit (Spiegelhalter et al. 2002). Parameter estimates were calculated as the modes of the posterior distributions estimated from the model of best fit. The 95% highest posterior density (HPD) was also estimated for each parameter. For traits where the model of best fit included a Family effect but no Family-by-Treatment interaction, the mode of the posterior distribution of the narrow-sense

heritability (h^2) and 95% HPD intervals were computed. Heritability estimates reported were obtained using the heritability derivation from Dammerman et al. 2015 (see Appendix 1).

Results

Mean body length at hatch ranged from 10.19 (±0.09) to 13.84 (±0.09) mm among families across the four treatments. A large range in phenotypic variation among families was observed within the fluctuating treatment groups; however, the greatest range was observed in the warm thermal treatment with Families D and H differing approximately 3-mm in size (Fig. 3). The cold treatment group showed the least amount of variation with families varying up to 1.2-mm in size. A significant Family-by-Treatment interaction was detected for body length (Table 1), and explained approximately 39% of the phenotypic variance observed (Table 2). Mean body area and mean yolk-sac area at hatch also varied significantly among families across treatments ranging from 16.82 (±0.17) to 28.46 (±0.32) mm^2 and 6.91 (±0.11) to 8.11 (±0.14) mm^2, respectively. Family-by-Treatment interactions were significant for both traits (Table 1), explaining approximately 43% of the variation observed in body area and 13% of the variation observed in yolk-sac area (Table 2).

Approximately 87% of the larvae from the five half-sibling families emerged from the substrate to begin exogenously feeding. Individuals began to emerge approximately 10 days after being placed in the incubation chambers. Families showed significant variation in the mean time spent in the incubation chambers ranging from 12.67 (±0.34) to 14.21 (±0.26) days; however, no Family-by-Treatment interaction or effect of the temperatures experienced while in the incubation chambers (MeanT and RangeT) were detected for the timing of emergence (Table 1). The mode of the heritability (h^2) distribution explaining the similarity in the timing of emergence

between larvae from the same family and 95% HPD were estimated as 0.24 (0.002, 0.706; Table 2).

Families also showed significant variation in growth from hatch to emergence ranging from 10.97 (±0.35) to 15.39 (±0.47) mm among families. Individuals that were incubated as eggs in the two fluctuating treatments differed approximately 1.3 to 1.7-mm in growth (Fig. 4). Similar to the traits measured at hatch, the largest range in mean growth among families was observed for individuals that were reared as eggs in the warm treatment with families differing approximately 2.7-mm in size (Fig. 4). No Family-by-Treatment interaction or effect of temperatures experienced while in the incubation chambers (MeanT and RangeT) were detected (Table 1). The mode of the h^2 distribution for growth and 95% HPD were estimated as 0.33 (0.021, 0.904; Table 2). Variation in mean body size at the timing of emergence was low among families ranging from 24.57 (±0.20) to 25.45 (±0.16) mm, and no significant effects were detected (Table 1).

Discussion

The early life stages of many ectothermic species are characterized by high levels of mortality (Vitt and Caldwell 2014; Fuiman and Werner 2002). Accordingly, it is during these early ontogenetic stages that selection may have a considerable effect. Increased temperatures encountered during early ontogeny are known to affect phenotypic and behavioral traits in ectothermic species (Atkinson 1996; Angilletta et al. 2004). However, there is a limited understanding of how thermal variability during egg incubation will affect trait variation at hatch and during sequential ontogenetic stages. Empirical work quantifying the effects of both increased mean and variability in temperature are vital to predict the ecological and evolutionary responses of populations to future climatic regimes. In this study, we experimentally manipulated

egg incubation temperatures in a common garden experiment and demonstrated large effects of thermal regimes, genetic (family) effects, and their interaction on phenotypic trait variation during consecutive early life stages.

Rearing temperatures influence phenotypic variation observed at hatch

Rearing temperature had a significant effect on the range of phenotypic variation observed in the traits measured at hatch. The largest range in body length at hatch was observed among families reared in the warm (~18˚C) treatment (Fig. 3). Although lake sturgeon have been observed spawning in temperatures up to 21.1˚C (Bruch and Binkowski 2002), the gametes used in our experiment were taken from adults that typically spawn during the early part of the season when water temperatures are closer to 10˚C. Therefore, the large range in phenotypic variation observed in the warm thermal treatment may have been due to the presence of cryptic genetic variation revealed under stressful thermal conditions. Cryptic genetic variation is not commonly observed under typically encountered conditions, and is often exposed in stressful environments (Badyaev et al. 2005; Ledón-Rettig et al. 2014). Results indicate that increases in mean thermal regimes (not just thermal variability) may reveal cryptic genetic variation expressed as differences among families thereby increasing phenotypic variation and the likelihood of exposing variant phenotypes to selection.

Cryptic variation can also be exposed when a genotype-by-environment interaction is present (Schlichting 2008; Paaby and Rockman 2014). In our common garden experiments, Family-by-Treatment interactions detected for body length, body area, and yolk-sac area measured at hatch indicate that families were responding differently to the same thermal incubation environments. A large proportion of the observed phenotypic variation in the traits (~13-43%) was explained by the Family-by-Treatment interaction. Other studies (Beacham 1988

and 1990), found that genotype-by-temperature interactions explained a substantial portion of observed variation in morphometric traits in *Oncorhynchus gorbuscha* and *Oncorhynchus keta* after rearing individuals under different thermal regimes indicating that water temperature has a significant effect on the genotypic expression of traits during early development. Additionally, the cryptic genetic variation observed in phenotypic traits at hatch may lead to differential survival among genotypes if selection favors larger individuals. Therefore, consideration of parentage, interaction effects, and cryptic genetic variation is important to understand how predicted thermal changes will affect phenotypic variation within wild populations.

Fluctuating and constant rearing temperatures influence the range of phenotypic variation observed in larval traits

In the variable and ambient treatments where temperatures fluctuated throughout the day, variation in body length at hatch among families was over 1.5 times as large as variation observed in the constant cold treatment (Fig. 3). Although diurnal temperature fluctuations were much larger in the variable than the ambient treatment, families showed similar responses in the ranges of phenotypic variation observed in the two fluctuating treatments. Results indicate that rearing individuals at a constant temperature reflecting the mean thermal regime observed in the wild will not accurately represent the extent of phenotypic variation that would be observed and on which selection would act on in wild-reared larvae when even modest daily temperature fluctuations occur. Similarly, Niehaus et al. (2012) found that growth and developmental rates of striped marsh frogs (*Limnodynastes peronii*) reared in fluctuating thermal treatments were continually under-predicted based on reaction norms constructed from individuals reared at constant rearing temperatures. Therefore, rearing individuals under fluctuating treatments

provides a more accurate representation of phenotypic trait variance and any potential genotype-by-environment interactions expected in the wild in response to climatic changes.

Rearing temperatures affect trait variation at a subsequent ontogenetic stage

Thermal conditions experienced during embryogenesis are known to affect phenotypic traits associated with survival such as shape, color, behavior, and size at developmental stages beyond hatch in several taxa of ectotherms (e.g. reptiles, Goodman 2008; fishes, Martell et al. 2005; amphibians, Orizaola et al. 2010). In our study, larvae reared in the fluctuating thermal treatments showed lower overall growth than those reared in the constant treatments indicating that egg incubation temperature affects trait variation at a subsequent ontogenetic stage (Fig. 4). Additionally, families within the warm treatment (Fig. 4) show the greatest range in growth indicating that differences observed among families in body size at hatch are persisting to a later ontogenetic stage. The persistence of a large range of variation in growth in the warm treatment and presence of non-zero heritability ($h^2 = 0.33$) for growth indicates that thermally-induced, cryptic variation could potentially be maintained across ontogenetic stages.

Given that body size during early life stages is associated with survival with larger individuals typically having lower levels of mortality (Perez and Munch 2011; Brown and Shine 2004; Fischer et al. 2011), differential growth among families may lead to changes in the genetic architecture of the population if selection favors genotypes that produce larger offspring during critical development periods. In our study, we're limited in our ability to predict the accommodation of thermally-induced phenotypic variation and survival in the population given that we only measured individuals to emergence and that the long generation time of lake sturgeon requires long-term monitoring. However, the early life stages of fishes are where

mortality is highest (>99%; Chamber and Trippel 1997) indicating that our study is beneficial for predicting future survival.

Several abiotic and biotic factors affect the timing of transition between early ontogenetic stages (Day and Rowe 2002). In larval fishes, the timing of emergence is typically under stabilizing selection (Crozier et al. 2008) and can be dependent on heritable variation, maternal effects, and/or environmental conditions such as temperature (Skoglund et al. 2011; Curry et al. 1995; Einum and Fleming 2000). In our experiment, there were no effects of thermal regimes experienced while in the incubation chambers. Alternatively, approximately 24% of the variation observed in the timing of emergence was attributed to differences among families. Heritability estimates for behavioral traits are rare in wild populations of fishes even though several behaviors are believed to have a heritable component (Mittelbach et al. 2014). Our estimate of h^2=0.24 for emergence time is comparable to other reported h^2 estimates of 0.15-0.20 for behavioral traits in fishes (Chervet et al. 2011; Carlson and Seamons 2008). However, our estimate represented the upper-limit of the narrow-sense heritability (resembling heritability in the broad-sense) given that we were unable to separate out additive genetic variance from other genetic effects. Despite the limitation, our h^2 estimate provides insight on the potential change in emergence time and ability of the population to respond genetically (i.e. changes in gene frequency) to changes in thermal regimes.

Families differ in growth at a subsequent ontogenetic stage

We observed no differences in body size at the time of emergence although families differed in the timing of emergence and growth from hatch to emergence. One explanation is that families that had smaller body sizes at hatch may have remained in the substrate longer to allow more time to grow thereby emerging from the substrate at the same size as individuals that spent

less time in the substrate and were larger at hatch. Alternatively, differences in growth and time spent in the substrate may be attributed to differences among families in endogenous yolk reserves. Differences between maternal provisioning of endogenous yolk-sac reserves have been well-documented in ectotherms where offspring with larger yolk reserves typically have higher survival and grow to a larger body size (Kamler 2005; Dziminski and Roberts 2006; Gagliano and McCormick 2007). In our experiment, we observed differences in yolk-sac area at hatch due to a Family-by-Treatment interaction. Therefore, differences in yolk sac area among families at hatch may have led to differential growth and time spent in the substrate feeding on endogenous yolk-sac reserves until the timing of emergence.

Conclusions and future directions

Our findings have important implications regarding the impact of predicted thermal changes due to climate change on phenotypic variation in ectothermic species. Understanding how cryptic genetic variation is maintained across sequential ontogenetic stages in wild populations is vital to understanding how populations can respond to environmental change (Ledón-Rettig et al. 2014). Additionally, reaction norms constructed from rearing individuals at constant temperatures are not reliable indicators under variable conditions. Therefore, researchers must construct "realized" thermal reaction norms by adding naturally occurring (e.g. diel) varying temperature into experimental designs for treatments (Paaijmans et al. 2013). Given our findings on behavioral and phenotypic traits that are tied to survival, further empirical work addressing how incubation conditions and differences among families affect trait variation and survival at later ontogenetic stages would be beneficial to predict changes in population levels of genetic diversity due to environmental perturbations. Knowledge within these research areas is

essential to understand the ecological responses and evolutionary potential of a population to the impacts of climate change.

Acknowledgements

Funding for this study was provided by the Michigan Department of Natural Resources, The International Association for Great Lakes Research, The Great Lakes Fishery Trust, and U.S. Fish and Wildlife Service. Research was conducted under animal use and care procedure number (03/14-042-00) from the Michigan State University Institutional Animal Care and Use Committee. We thank John Bauman, Nathan Barton, Jim Holser, Annie VanSickle, and James Garavaglia for assistance in construction of the experiment and collection of data.

Data Arching Statement

Data for this study is part of a graduate student's thesis and will be made available at the Dryad Digital Repository (datadryad.org) after the manuscript is accepted for publication.

Literature Cited

WORK IN PROGRESS: Discussion references not included yet

Angilletta, M.J. 2009. Thermal adaptation: a theoretical and empirical synthesis. New York, NY: Oxford University Press

Atkinson 1994

Atkinson, D. 1996. Ectotherm life history responses to developmental temperature. In I.A. Johnston and A.F.Bennett (eds.), Animals and temperature: Phenotypic and evolutionary adaptation, pp. 183–204. Cambridge University Press, Cambridge.

Auer 1996

Baker and Borgeson 1999

Bauerfeind and Fischer 2014

Bruch and Binkowski 2002

Dammerman et al. 2015

Diamond and Kingsolver 2010

Diggle 1994

Deutsch et al. 2008

Duong et al. 2011

Estay et al. 2014

Field, C. B. (Ed.). (2012).

Forster et al. 2012

Forsythe, P.S., J.A. Crossman, N.M. Bello, E.A. Baker, K.T. Scribner. 2012. Individual-based analyses reveal high repeatability in timing and location of reproduction in lake sturgeon (*Acipenser fulvescens*). Canadian Journal of Fisheries and Aquatic Science 68:1-13

Fuiman and Werner 2002. Fishery science: the unique contributions of early life stages.

Hadfield, J.D. 2010. MCMC methods for multi–response generalised linear mixed models: the MCMCglmm R package. Journal of Statistical Software 33(2):1-22

Hansen et al. 2012

Hardy and Litvak 2004

Hastings et al. 2014

Hoffman et al. 2003

Huey et al. 2012

Intergovernmental Panel on Climate Change. 2012. Summary for policymakers. In C.B. Field, V. Barros, T.F. Stocker, D. Qin, D.J. Dokken, K.L. Ebi, M.D. Mastrandrea, K.J. Mach, G.K. Plattner, S.K. Allen, M. Tignor, and P.M. Midgley (eds.) Managing the risks of extreme events and disasters to advance climate change adaptation: special report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge.

Intergovernmental Panel on Climate Change. 2013. Climate change 2013: the physical science basis. Contribution of working group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York.

Jonsson and Jonsson 2009

Kempinger 1988

Kingsolver and Huey 2008

Ligon, D.B. and M.B. Lovern. 2009. Temperature effects during early life stages of the Alligator snapping turtle (*Macrochelys temminckii*). Chelonian Conservation and Biology 8(1):74-83.

Mearns et al. 1997

Niehaus et al. 2012

Norberg et al. 2012

Ojanguren, A.F., F.G. Reyes-Gavilán and R.R Muñox. 1999. Effects of temperature on growth and efficiency of yolk utilisation in eggs and pre-feeding larval stages of Atlantic salmon. Aquaculture International 7:81-87

Orizaola et al. 2010

Paaijmans et al. 2013

Parmesan 2006

Pincebourde et al. 2012

Schulte et al. 2011

Skelly et al. 2007

Smith and King 2005

Spiegelhalter, D.J., N.G. Best, B.P. Carlin, A. Van Der Linde. 2002. Bayesian measures of model complexity and fit. Journal of the Royal Statistical Society: Series B 64(4):583-639

Thiem et al. 2013

Vitt and Caldwell 2014. Herpetology: An introductory biology of amphibians and reptiles

Walther et al. 2002

Wilson et al. 2010

Tables

Table 1. Step-wise model comparisons and DIC-values used in model selection for the six larval traits quantified at hatch and emergence in the experiment.

Ontogenetic Stage	Larval Trait	Model	DIC
Hatch	Body Length	μ + Treatment + *Family* + *Family*Treatment* + ε	2739.86*
		μ + Treatment + *Family* + ε	3135.40
		μ + Treatment + ε	3334.08
		μ + ε	3637.86
	Body Area	μ + Treatment + *Family* + *Family*Treatment* + ε	5687.97*
		μ + Treatment + *Family* + ε	6123.70
		μ + Treatment + ε	6290.38
		μ + ε	6821.93
	Yolk-sac Area	μ + Treatment + *Family* + *Family*Treatment* + ε	2668.96*
		μ + Treatment + *Family* + ε	2779.48
		μ + Treatment + ε	2824.39
		μ + ε	3045.09
	Time to Emergence	μ + Treatment + MeanT + RangeT + *Family* + *Family*Treatment* + ε	1219.22
		μ + Treatment + MeanT + RangeT + *Family* + ε	1218.40
		μ + Treatment + MeanT + *Family* + ε	1218.43
		μ + Treatment + *Family* + ε	1217.83*
		μ + Treatment + ε	1233.12
		μ + ε	1372.54
Emergence	Emergence Body Length	μ + Treatment + MeanT + RangeT + *Family* + *Family*Treatment* + ε	1125.42
		μ + Treatment + MeanT + RangeT + *Family* + ε	1125.11
		μ + Treatment + MeanT + *Family* + ε	1126.24
		μ + Treatment + *Family* + ε	1124.31
		μ + Treatment + ε	1128.53
		μ + ε	1124.33*
	Total Growth	μ + Treatment + MeanT + RangeT + *Family* + *Family*Treatment* + ε	1146.03
		μ + Treatment + MeanT + RangeT + *Family* + ε	1145.74
		μ + Treatment + MeanT + *Family* + ε	1143.58
		μ + Treatment + *Family* + ε	1141.91*
		μ + Treatment + ε	1166.68
		μ + ε	1207.01

*Indicates the model of best fit.

Table 2. Variance components and 95% highest posterior density (HPD) estimated from the models of best fit. Heritability (h^2) estimates are provided for traits where the model of best fit included a significant family effect, but no family-by-treatment interaction.

Phenotypic Traits	Component	Var	±95% HPD
Body Length	Family*Treatment	0.35	(0.00, 0.26)
	Family	<0.01	(0.20, 0.62)
	Residual	0.56	(0.51, 0.60)
Body Area	Family*Treatment	4.85	(0.00, 2.06)
	Family	0.02	(2.85, 8.10)
	Residual	6.56	(6.01, 7.04)
Yolk-sac Area	Family*Treatment	0.08	(0.00, 0.05)
	Family	<0.01	(0.05, 0.15)
	Residual	0.53	(0.49, 0.57)
Time to Emergence	Family	0.12	(0.00, 1.60)
	Residual	3.21	(2.69, 3.69)
	h^2	0.24	(0.00, 0.71)
Total Growth	Family	0.19	(0.02, 1.81)
	Residual	2.37	(2.08, 2.86)
	h^2	0.33	(0.02, 0.90)

*Indicates an interaction between the components

Figure Legends

Fig. 1 The study location on the Upper Black River, the largest tributary of Black Lake, Michigan showing the spawning locations where adult lake sturgeon were sampled during the 2012 spawning season

Fig. 2 The four egg incubation temperatures (warm, cold, variable, and ambient) in which individuals were reared in during the 2012 spawning season

Fig. 3 Thermal reaction norm for body length at hatch for lake sturgeon larvae subjected to the four thermal treatments. A significant genotype-by-environment (G-by-E) interaction was

detected, and most of the variation among families was observed for larvae that hatched from eggs incubated in the warm (18°C) treatment

Fig. 4 Thermal reaction norm for larval lake sturgeon growth measured from hatch to the timing of emergence. A significant family effect was detected (h^2=0.33) indicating that differential size among families measured at hatch was persisting to a later ontogenetic stage

Figures

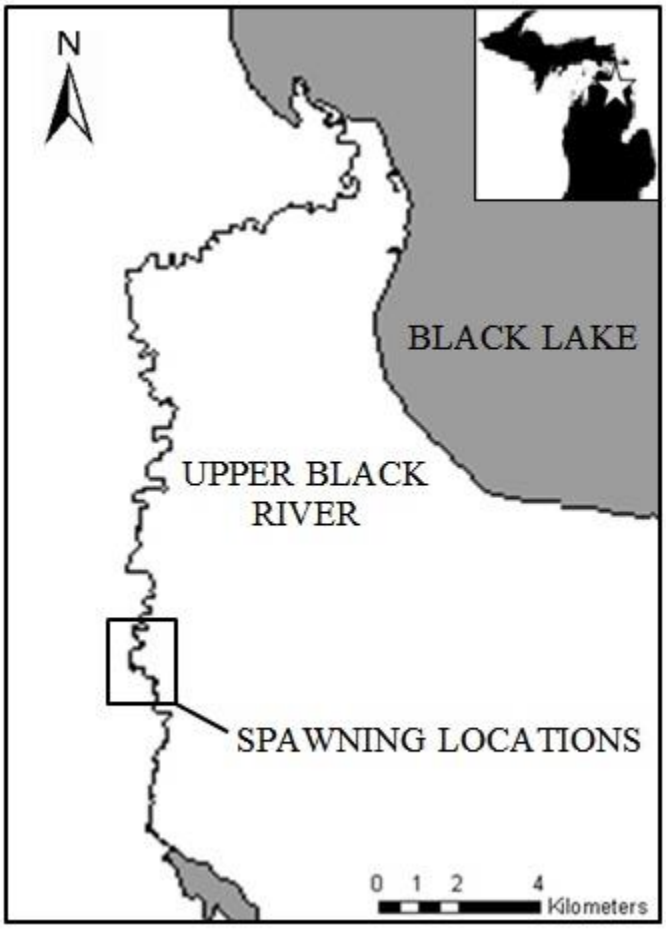


Fig. 1

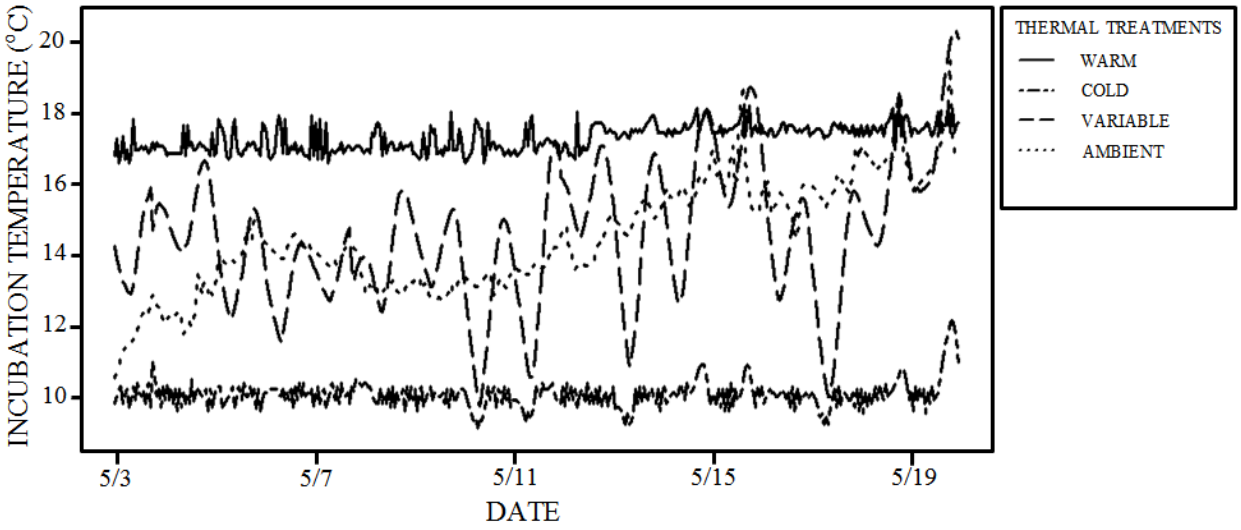

INCUBATION TEMPERATURE (°C)
20
18
16
14
12
10
5/3
5/7
5/11
5/15
5/19
DATE
THERMAL TREATMENTS
WARM
COLD
VARIABLE
AMBIENT

Fig. 2

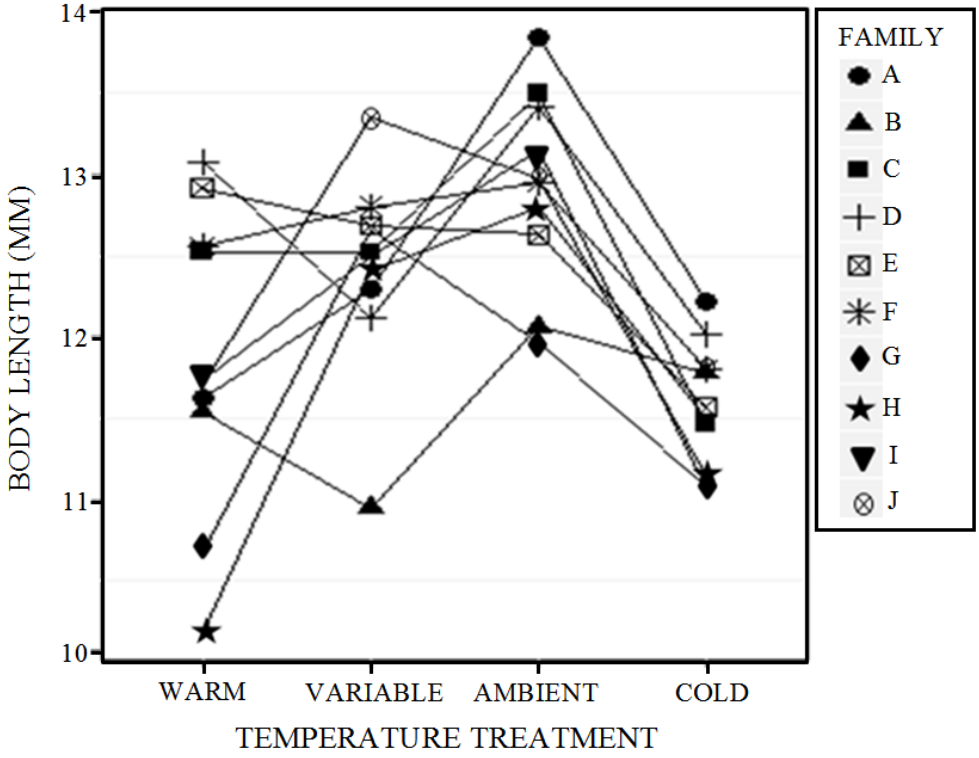

BODY LENGTH (MM)
14
13
12
11
10
WARM
VARIABLE
AMBIENT
COLD
TEMPERATURE TREATMENT
FAMILY
A
B
C
D
E
F
G
H
I
J

Fig. 3

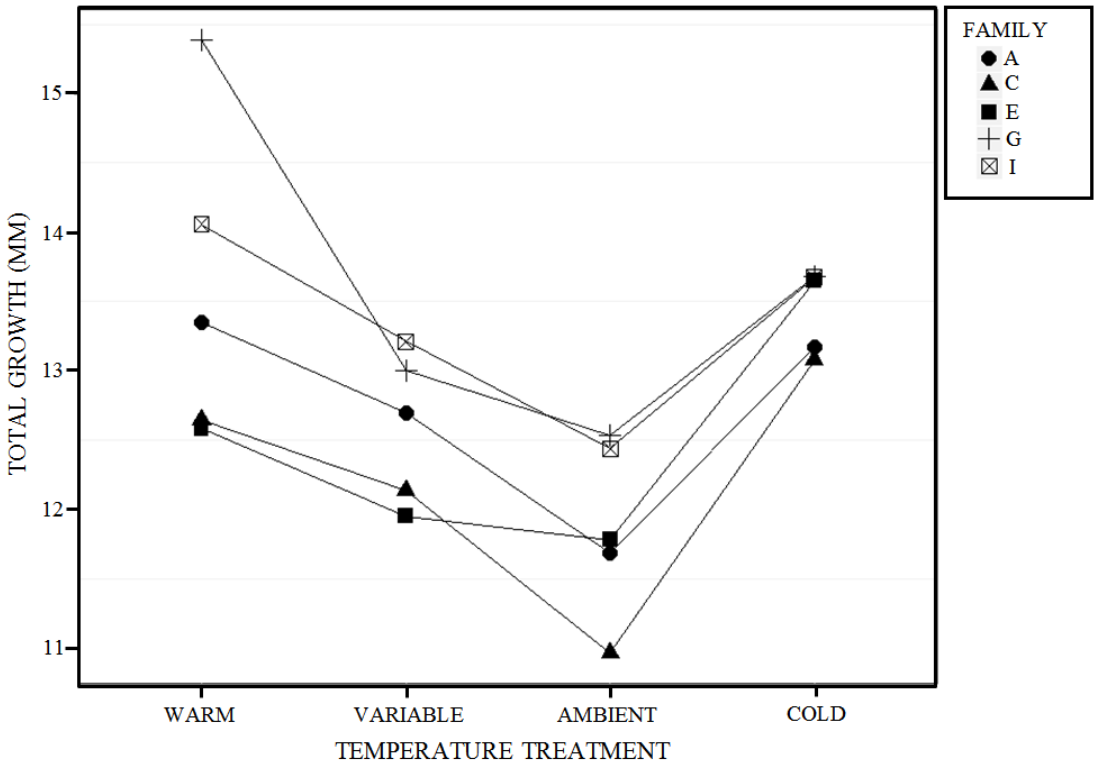

FAMILY
A
C
E
G
I
TOTAL GROWTH (MM)
15
14
13
12
11
WARM
VARIABLE
AMBIENT
COLD
TEMPERATURE TREATMENT

Fig. 4

Appendix # 3 – Effects of de-adhesion method and incubation condition on body size, survival, oxygen consumption rate and microbial community composition of lake sturgeon egg surfaces

John M. Bauman[a], Edward A. Baker[b], Terry L. Marsh[c] and Kim T. Scribner[a,d]

[a]Department of Fisheries and Wildlife, Michigan State University, 13 Natural Resources Building, East Lansing, MI 48824, USA.

[b]Michigan Department of Natural Resources Fisheries Division, 484 Cherry Creek Road, Marquette, MI 49855, USA.

[c]Department of Microbial and Molecular Genetics, Michigan State University 6169 Biomedical Physical Sciences, East Lansing, MI 48824, USA.

[d]Department of Zoology, Michigan State University 288 Farm Lane, East Lansing, MI 48824, USA.

ABSTRACT

In the wild, many fish have developed ecological, physiological, and behavioral reproductive traits that place fertilized gametes in locations within aquatic habitats that are conducive to survival during egg incubation. However, under artificial or semi-natural rearing conditions these unique ecological adaptations confront hatchery personnel with logistical difficulties that compromise production efforts. This study was conducted using Lake Sturgeon eggs to quantify the effects of different egg de-adhesion and incubation methods on the body size and survival of fish eggs. Furthermore, this study aimed to quantify egg oxygen consumption rates in association with the relative abundance and community composition of microbes on the

egg surface at different egg developmental periods. Results revealed that body size parameters did not vary significantly as a function of different de-adhesion and incubation methods. However, significantly higher survival was documented among individuals incubated in trays (except the control) compared to jars. Oxygen consumption rate varied between two developmental periods but did not vary as a function of different de-adhesion and incubation methods. Using 16S rRNA sequence data, this study documented significant differences in microbial community composition at two developmental periods which may be related to unique binding properties of microbial taxa and egg surface substrates. Evaluation of de-adhesion and incubation procedures in association with body size, survival, and the effects of microbial communities provides hatchery managers with best-practices information that can be used to develop standard operating procedures for Lake Sturgeon during the egg period.

INTRODUCTION

High mortality during early life periods can reduce rates of recruitment for many fish species, compromising recovery efforts using aquaculture and stocking as primary means of recovery. Species reared in artificial conditions experience barriers (i.e., egg quality, egg adhesion) to successful fish production due to many species-specific life history traits, particularly during the egg period. As a result, aquaculture methods, including de-adhesion and incubation conditions, have been employed to increase survival during egg incubation and early larval periods. Direct and indirect effects of these aquacultural practices on growth and survival of developing embryos to hatch is understudied and thus needs further investigation.

In the wild, many fish have developed ecological, physiological, and behavioral traits that place fertilized gametes in locations within aquatic habitats that are conducive to survival through incubation (Balon 1975). Egg adhesion (or 'stickiness'), which is characteristic of litho-philic and litho-pelagophilic fish species (Balon 1975), serves as an example of how ecological adaptation can, under artificial conditions, make production of such species difficult in aquaculture settings. For example, in aquatic environments eggs are extruded into the water column, initiating enzymatic reactions that hydrolyze glycoproteins (Cherr and Clark 1984, Hansen and Olafsen 1999) causing eggs to adhere to substrates in habitats critical for embryogenesis (i.e., high oxygen availability, limited predation) (Balon 1975, Kamler 2008). In addition, as documented more recently using meta-genomic techniques (Cole et al. 2009, Nelson et al. 2014), adhesive egg surfaces are quickly colonized by symbiotic as well as pathogenic members of the aquatic microbial community. After initial colonization, egg surfaces experience a rapid succession in microbial community composition which can affect offspring phenotype and survival (Fujimoto et al. 2013).

In contrast, in aquacultural settings eggs are incubated in high numbers and adhesive properties of the eggs can become problematic. For example, mass clumping in incubation devices can cause egg asphyxiation and can increase levels of microbial infection by bacterial (Barnes et al. 2009) and eukaryotic oomycetes (Van Den Berg et al. 2013), negatively affecting growth and survival during embryogenesis (Barnes et al. 2009). As a result, physical and chemical de-adhesion techniques have been used for egg de-adhesion for several fish species (*Cyprinus carpio, Silurus glanis, Tinca tinca*; Linhart et al. 2003: *Acipenser fulvescens*; Bouchard and Aloisi 2000: *Acipenser baerii*; Feledi et al. 2011) in aquaculture programs.

Physical egg de-adhesion includes the use of milk, talc, or more commonly Fuller's Earth solution (hereinafter referred to as 'clay') whereby charged particles bind to adhesive glycoproteins released after initial contact of the egg with water (Doroshov 1983). Chemical compounds such as alcase, or Woynarovich solution and tannic acid (hereinafter collectively referred to 'tannic acid') removes the glycoprotein from the outer surface of the egg (Kowtal et. al 1986). Despite wide use of de-adhesion compounds, limited information pertaining to the effects of egg de-adhesion on the growth, respiration and survival of embryo to hatch exists. Given advances in 16S meta-genomic sequencing techniques and bioinformatics tools (Cole et al. 2009, Nelson et al. 2014, Schloss et al. 2014), studies aimed to quantify the effects of these de-adhesion compounds and incubation devices on growth, respiration and survival at hatch in association with microbial communities present during embryogenesis are warranted.

The purpose of this study was to quantify the effects of different de-adhesion methods and incubation conditions on the body size and survival to hatch of Lake Sturgeon. Furthermore, quantification of egg respiration rates in association with the relative abundance and taxonomic composition of microbial communities during different egg development periods may provide

insight regarding the potential physiological mechanisms responsible for observed results. Lake Sturgeon were used in this study due to egg properties (Cherr and Clark 1982, Cherr and Clark 1984), similar to those of other aquaculture species, as well as their conservation status which has prompted a need for aquaculture standard operating procedures. The hypotheses of this study are 1) body size and survival at hatch will differ as a function different de-adhesion and incubation techniques, 2) physical and chemical de-adhesion treatment of egg surfaces will result in different bacterial taxa colonizing the egg.

METHODS

Study site

This study was conducted at the Black River Streamside Rearing Facility (BR-SRF), which is supplied with ambient river water (~680 L/min) from the Kleber Reservoir, located near primary spawning areas for lake sturgeon in the upper Black River in Cheboygan County, Michigan. The study took place in May 2013 when BR-SRF water temperature ranged from 9.5 to 17.6^{0}C with a mean of 14.3^{0}C. Water used for rearing in the BR-SRF is passed once through a high output UV sterilizer (Pentair #E50S).

Gamete collection, fertilization, and incubation

Gametes were collected from adult lake sturgeon spawning in the upper Black River following procedures described by Crossman et al. (2011). Gametes were transported to the BR-SRF for fertilization of four full-sibling families, which took place within twelve hours of collection. Eggs were fertilized using a 1:200 milt dilution of ambient river water immediately poured over the eggs and allowing 90 seconds for fertilization. Excess milt was then removed and eggs were rinsed once with ambient river water. On 9 May (one male and one female: Family 1), 11 May (two males and two females Family 2 and Family 3), and 3 June (one male and one female: Family 4) 2013, de-adhesion procedures for each family were administered immediately after fertilization. Fertilizations for each family were performed in duplicate to provide replicates for each experimental treatment.

Experimental treatments

The purpose of this experiment was to quantify the effects of different de-adhesion and incubation procedures on lake sturgeon body size and survival at hatch. In addition, this study was designed to document microbial community taxonomic composition and relative abundance

on the egg surface as a function of different de-adhesion and incubation conditions in association with oxygen consumption rate during embryogenesis and body size at hatch. Microbial and oxygen experiments focused on two development periods, determined by calculating cumulative daily water temperature units (CTU) (Kempinger 1988), representing two time periods (30 to 36 CTU and 55 to 67 CTU) during embryogenesis when significant mortality occurs in Lake Sturgeon culture (Scribner and Marsh, unpublished data).

De-adhesion treatment. – Immediately following fertilization, eggs were divided into three de-adhesion treatment groups; 1) clay de-adhesion, 2) tannic acid, and 3) control de-adhesion. De-adhesion procedures were performed simultaneously.

Clay (Fuller's Earth) de-adhesion. – Clay de-adhesion procedures began by applying a Fuller's Earth solution (Sigma Aldrich) and gently mixing for 50 min. After 50 min, Fuller's Earth was rinsed from the eggs and a 15 min, 50 ppm Iodophor disinfection treatment was administered. Following a 10 min rinse to remove residual Iodophor using ambient river water, eggs were split into two groups of 100 to 150 randomly selected eggs to be incubated using two different rearing apparatuses.

Tannic acid de-adhesion. – The tannic acid de-adhesion procedure (Kowtal et al. 1986) began by treating eggs with a 0.4% urea (Sigma Aldrich) and 0.3% sodium chloride (Sigma Aldrich) solution for 5 min, followed by a 1.0 min rinse with 0.1% tannic acid (Kowtal et al. 1986). Tannic acid was partially decanted three times over the period of three min. After three min, eggs were rinsed and a 15 min, 50 ppm Iodophor disinfection treatment was administered as described for the clay treatment. Following a 10 min rinse to remove residual Iodophor using ambient river water, eggs were split into two groups of 100 to 150 randomly selected eggs to be incubated using two different rearing apparatuses.

***Control de-adhesion.* –** The control for de-adhesion was not provided any de-adhesion treatment after fertilization. After fertilization eggs were rinsed and a 15 min, 50 ppm Iodophor disinfection treatment was administered as described for the clay treatment. Eggs for control de-adhesion were not split into two groups following iodiphor treatment.

Incubation treatment. – After de-adhesion procedures were performed, three groups of 100 to 150 eggs (one from clay, tannic acid, and the control for de-adhesion) from each family were placed at random into each of two common incubation units used for hatchery production, with one exception.

***Mini egg-hatching jar.* –** Following the rinse to remove residual Iodophor using ambient river water, one groups of 100 to 150 eggs from each de-adhesion treatment was added to a mini egg-hatching jar (Pentair J32, Apopka, FL) hereinafter referred to as "jar". Due to logistical difficulty and the potential for mass mortality, eggs from control de-adhesion groups were not incubated in a jar. Jar flow rate was set to provide eggs a gentle roll and was checked twice daily throughout incubation.

***Heath tray.* –** Following the rinse to remove residual Iodophor using ambient river water, one group of 100 to 150 eggs from each de-adhesion treatment (including the control) was added to a 7.6 cm diameter PVC coupling with 1.0 by 1.0 mm mesh on top (removable) and bottom, hereinafter referred to as 'tray', to represent Heath tray incubation conditions. Couplings were placed into a Heath tray for incubation and the flow rate was set to approximately 19 L per min and checked twice daily.

Data collection

Body size. – At hatch, we used a digital camera to photograph and measure a random subsample (n=25) of fish from each treatment, each family (except Family 4), and each replicate for mean

body size (total length (TL mm), yolk-sac area (YSA mm^2), and body area (BA mm^2)) using Image J software (v.1.43u).

Survival. – Incubation units from each treatment, each family (except family 4) and each replicate were checked daily to enumerate and remove dead eggs and to quantify proportional survival at hatch.

Oxygen consumption rate. – At each of two development periods, eight eggs from one family (only family 4), each treatment and each replicate were sub-sampled to quantify oxygen consumption rate (mg/L / 5 min / eight eggs) using two, 24-well SensorDish reader plates (PreSens Precision Sensing, GmbH, Regensburg, Germany) (Naciri et al. 2008). Each well was filled with 1.5 mL of ambient river water, and eight eggs from each treatment were placed circumpolar to the electro-florescence sensor, and then topped with 0.5 mL of autoclaved mineral oil. Oxygen concentration (mg/L) was recorded every 30 sec for up to one hr. After 1 hr, eggs were removed from the SensorDish reader and placed into separate 2.0 mL posi-click tubes, filled with 1.5 mL of 95% ethanol, for downstream microbial 16S rRNA extraction. Oxygen concentration data were plotted using Excel (time on x-axis, oxygen concentration y-axis) to determine the oxygen consumption rate. Oxygen consumption was quantified by identifying the initial decline (time 1), and selecting the tenth (5 min) measurement (time 2) following the initial decline point. Oxygen consumption was calculated for each treatment group and each family by subtracting time 2 from time 1 (mg/L over the 5 min period for eight eggs). Oxygen consumption due to microbial respiration was assumed to be negligible (Boucher 2012).

Daily egg chemotherapeutant

Beginning two days post-fertilization; eggs in jars and trays were exposed to daily chemotherapeutant treatment of 500 ppm hydrogen peroxide treatment for the duration of 15

min. Daily chemical treatments were performed until 24 hours prior to hatch as determined by observing embryo development stage 32 to 34 (Detlaff et al. 1993).

Microbial DNA extraction

Microbial genomic DNA was extracted from 8 eggs per treatment per development period (from those used in oxygen consumption rate experiment), using a modified DNeasy Blood & Tissue QIAGEN Kit (QIAGEN Group, 2006). Modified steps include the initial incubation of samples in an enzymatic lysis buffer at 37^0C for 30 min followed by bead-beating (Fujimoto et al. 2013) for 10 min. After bead-beating, steps were followed according to manufacturer's protocols. To ensure sufficient DNA was extracted for analysis, eight eggs from each sample were pooled during the extraction process (Fujimoto et al. 2013). Polymerase chain reaction (PCR) amplification and sequencing of 16S rRNA gene was used to estimate the microbial community composition as a function of chemotherapeutants and development time period during incubation. PCR was conducted in a 50 uL reaction volume, containing 10 uL template DNA (6 to 110 ng/uL), 0.4 uL of AquPrime HiFi Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA), 5.0 uL 10X PCR Buffer II, 1.0 uL 27 Forward Primer, 1.0 uL 1389 Reverse Primer, and 32.6 uL sterile water. Reactions were performed using the following thermocycle conditions; initial denaturation step at 95ºC for 2 min, then 30 cycles of denaturation at 95ºC for 20 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 7 min (Fujimoto 2013).

Processing 16S microbial sequencing data

Sequencing analyses for the 16S rRNA gene were generated using the Illumina MiSeq platform using paired-end reads (Kozich et al. 2013). Sequences were categorized using operational taxonomic units (OTUs) at 97% similarity threshold. To statistically and graphically

describe similarity and dissimilarity among treatment groups, program Mothur (Schloss 2014) was used to compute alpha (Simpson 1949, Shannon 1948) and beta (Bray Curtis 1957) diversity indices. Dendrograms (using program FigTree) and lower-triangle dissimilarity matrices were created based on Bray-Curtis values. Furthermore, to visualize relationships in diversity among samples, a comparison of dominant phylotypes as function of de-adhesion, incubation, and developmental period were graphed as a stacked column chart using Microsoft Excel.

Statistical analysis

Differences tested among treatments included mean (±SE) body size (TL, YSA, and BA) at hatch, and proportional survival at hatch from families 1, 2, and 3. At two developmental periods, oxygen consumption rate and alpha diversity (using Simpsons and Shannon values) were tested using family 4. Due to unbalanced design (missing jar control) de-adhesion and incubation groups were merged as a single treatment (total of 5 treatments). All analyses and summary statistics were performed using SAS (SAS Institute version 9.3 Cary, NC). Egg incubation tray (coupling) and jar, which included 100 to 150 eggs were the experimental units for all response variables used in the analysis. A p value < 0.05 was considered statistically significant for all analyses. A general linear model using analysis of variance was used to analyze all response variables. We used Tukey-Kramer multiple pair-wise comparison tests for all response variables.

RESULTS

Effects of de-adhesion method and incubation conditions on body size and survival

Total length at hatch. – The effect of de-adhesion and incubation on mean (±SE) TL at hatch was not significant ($F_{4,23} = 1.04$, $P = 0.41$). At hatch, mean TL was greatest in clay jar (TL: 13.51 ±0.045) and smallest among tannic jar treatment groups (TL: 13.33 ±0.072), although differences were not statistically significant (Table 1). The effect of family on the mean TL at hatch was not significant ($F_{2,23} = 1.17$, $P = 0.33$) (Table 1).

Yolk-sac area at hatch. – The effect of de-adhesion and incubation treatment on mean YSA at hatch was not statistically significant ($F_{4,23} = 2.62$, $P = 0.06$) (Table 1). At hatch, mean (±SE) YSA was greatest in control tray (YSA: 7.80 ±0.12) and smallest among tannic jar (YSA: 7.57 ±0.16) treatment groups, although differences were not statistically significant (Table 1). The effect of family on YSA at hatch was significant ($F_{2,23} = 40.86$, $P < 0.0001$). Multiple pair-wise comparison tests revealed a significant difference in mean YSA between families 1 (YSA: 7.35 ±0.08) versus family 2 (YSA: 8.01 ±0.05) ($t_{23} = -8.85$, $P < 0.0001$), family 1 versus family 3 (YSA: 7.79 ±0.04) ($t_{23} = -6.02$, $P < 0.0001$), and family 2 versus family 3 ($t_{23} = 2.83$, $P = 0.02$) (Table 2).

Body area at hatch. – The effect of de-adhesion and incubation treatments on mean (±SE) BA at hatch was not statistically significant ($F_{4,23} = 1.51$, $P = 0.23$). At hatch, mean BA was greatest in clay tray (BA: 29.03 ±0.16), and smallest among tannic jar (BA: 27.93 ±0.34) treatment groups, although differences were not statistically significant (Table 1). The effect of family on the mean BA at hatch was not significant ($F_{2,23} = 2.31$, $P = 0.12$) (Table 2).

Survival at hatch. – The effect of de-adhesion and incubation treatment on mean (±SE) survival at hatch was significant ($F_{4,23} = 5.77$, $P = 0.002$) (Table 1). At hatch, survival was greatest in

clay tray (0.45 ±0.08), and lowest in control tray (0.19 ±0.07) treatment groups (Table 1). Multiple pair-wise comparison tests revealed a significant difference in mean survival between clay tray (0.45 ±0.08) and control tray (0.19 ±0.07) treatment groups ($t_{23} = 4.09$, $P = 0.004$). Additionally, pair-wise comparison tests revealed a significant difference in mean survival between control tray (0.19 ±0.07) and tannic tray (0.43 ±0.09) treatment groups ($t_{23} = -3.75$, $P = 0.008$). The effect of family on survival at hatch was statistically significant ($F_{2,23} = 25.42$, $P < 0.0001$) (Table 2). Multiple pair-wise comparison tests revealed a significant difference in mean proportional survival at hatch between family 1 (0.16 ±0.05) versus family 2 (0.32 ±0.05) ($t_{23} = -3.09$, $P = 0.01$), family 1 versus family 3 (0.52 ±0.04) ($t_{23} = -7.11$, $P < 0.0001$), and family 2 versus family 3 ($t_{23} = -4.02$, $P = 0.002$) (Table 2).

Table 1. Lake sturgeon mean (±SE) body size (total length (TL), yolk-sac area (YSA), and body area (BA)), and proportional survival to hatch as a function of de-adhesion and incubation treatment. Identical lowercase letters within columns represent treatments that are not significantly different (Tukey-Kramer, $P < 0.05$).

Treatment	TL	YSA	BA	Survival
Clay Jar	13.51 ±0.04	7.62 ±0.15	28.89 ±0.29	0.31 ±0.07 yz
Clay Tray	13.50 ±0.03	7.78 ±0.14	29.03 ±0.16	0.45 ±0.08 y
Control Tray	13.46 ±0.11	7.80 ±0.12	28.77 ±0.57	0.19 ±0.07 z
Tannic Jar	13.33 ±0.07	7.57 ±0.16	27.93 ±0.34	0.28 ±0.08 yz
Tannic Tray	13.49 ±0.09	7.80 ±0.12	28.48 ±0.40	0.43 ±0.09 y

Table 2. Lake sturgeon mean (±SE) body size (total length (TL), yolk-sac area (YSA), and body area (BA)), and proportional survival to hatch as a function of family. Identical lowercase letters within columns represent treatments that are not significantly different (Tukey-Kramer, $P < 0.05$).

Family	TL	YSA	BA	Survival
1	13.53 ±0.08	7.35 ±0.08a	28.82 ±0.38	0.16 ±0.05 x
2	13.45 ±0.04	8.01 ±0.05b	28.13 ±0.22	0.32 ±0.05 y
3	13.40 ±0.05	7.79 ±0.04c	28.90 ±0.23	0.52 ±0.04 z

Effects of de-adhesion method and incubation conditions on oxygen consumption rate

Developmental period 1. – The effect of de-adhesion and incubation on mean (±SE) oxygen consumption rate at developmental period 1 was not statistically significant ($F_{4,5} = 0.77$, $P = 0.59$) (Table 3). At developmental period 1, the mean oxygen consumption rate was highest in tannic jar (0.35 ±0.03) and lowest in control tray (0.28 ±0.01) (Table 3), although differences were not statistically significant.

Developmental period 2. – The effect of de-adhesion and incubation treatment on mean oxygen consumption rate at developmental period 2 was not statistically significant ($F_{4,5} = 0.88$, $P = 0.53$) (Table 3). At developmental period 2, the mean oxygen consumption rate was highest in tannic tray (1.04 ±0.04) and lowest in control tray (0.49 ±0.14) treatment groups (Table 3), although differences were not statistically significant.

Table 3. Lake Sturgeon mean (±SE) egg oxygen consumption rate at two developmental periods (30 to 36 CTU and 55 to 67 CTU; Kempinger 1988) as a function of different de-adhesion methods and incubation conditions.

Treatment	Development Period 1	Development Period 2
Clay Jar	0.30 ±0.06	0.85 ±0.48
Clay Tray	0.33 ±0.01	0.92 ±0.27
Control Tray	0.28 ±0.01	0.49 ±0.14
Tannic Jar	0.35 ±0.03	0.55 ±0.03
Tannic Tray	0.30 ±0.03	1.04 ±0.04

Effects of de-adhesion method and incubation conditions on microbial diversity

Alpha-diversity. –

***Developmental period 1.* –** The effect of de-adhesion and incubation treatments on mean Shannon diversity at developmental period 1 was statistically significant ($F_{4,5}$ = 17.12, P = 0.004) (Table 4). Multiple pair-wise comparison tests revealed that the mean Shannon diversity for clay jar (1.06 ±0.03) was statistically lower than the tannic jar (2.09 ±0.02: t_{23} = -7.73, P = 0.003), tannic tray (1.84 ±0.02: t_{23} = -6.05, P = 0.01), clay tray (1.79 ±0.04: t_{23} = -5.72, P = 0.01), and control tray (1.60 ±0.21: t_{23} = -4.31, P = 0.04) treatment groups (Table 4). The effect of de-adhesion and incubation on mean Simpson's diversity at developmental period 1 was statistically significant ($F_{4,5}$ = 28.38, P = 0.001). Multiple mean comparison tests revealed Simpson's diversity was significantly higher in clay jar (0.61 ±0.02) compared to clay tray (0.37 ±0.02) (t_5 = 4.05, P = 0.05) (Table 4). Additionally, the mean Simpson's diversity in tannic tray (0.29 ±0.01) was significantly higher compared to tannic jar (0.17 ±0.01) (t_5 = -4.29, P = 0.04) (Table 4). Multiple mean comparison tests revealed that the mean Simpson's diversity in clay jar (0.61 ±0.02) was significantly higher than tannic jar (0.17 ±0.01) (t_5 = 10.18, P = 0.0009) (Table 4).

However, mean Simpson's diversity in clay tray (0.37 ±0.02) was not significantly different from tannic tray (0.29 ±0.01) (t_5 = 1.85, P = 0.44).

Microbial phylotypes that contributed 5 percent or higher of the mean proportional abundance in clay jar include: Flavobacteriaceae (0.78) (Figure 2). In addition, in the clay jar treatment, phylotypes identified as Flavobacteriaceae included only one species (Flavobacteriaceae Chryseobacterium). Phylotypes that contributed 5 percent or higher of the mean proportion abundance in clay tray included: Comamonadaceae (0.59), Chromatiaceae (0.06), Cryomorphaceae (0.05), and Burkholdariales unclassified (0.05) (Figure 2). Phylotypes that contributed 5 percent or higher of the mean proportional abundance in control tray included: Comamonadaceae (0.63) and Oxalobacteraceae (0.06) (Figure 2). Phylotypes that contributed 5 percent or higher of the mean proportional abundance in tannic jar included: Comamonadaceae (0.28), Flavobacteriaceae (0.43), and Moraxellaceae (0.16) (Figure 2). In addition, in the tannic jar treatment, phylotypes identified as Flavobacteriaceae included 4 different species (Flavobacterium n=3 (0.38), Chryseobacterium n=1 (0.05)). Phylotypes that contributed 5 percent or higher of the mean proportional abundance in tannic tray included: Comamonadaceae (0.50), Moraxellaceae (0.16), Burkholdariales unclassified (0.08), Chromatiaceae (0.05) (Figure 2).

Developmental period 2. – The effect of de-adhesion and incubation on Shannon diversity at developmental period 2 was statistically significant ($F_{4,5}$ =7.31, P = 0.03) (Table 4). Multiple comparison tests revealed that mean Shannon diversity in clay jar (2.14 ±0.04) was significantly lower than that in clay tray (2.38 ±0.01) and control tray (2.37 ±0.05) (t_5 = -4.61, P = 0.03 and t_5 = -4.48, P = 0.03, respectively) (Table 4). The effect of de-adhesion and incubation on mean Simpson's diversity at developmental period 2 was not statistically significant

($F_{4,5}$=4.37, p=0.0687). Mean Simpson's diversity at development period 2 was highest in tannic tray (0.202 ±0.008) and lowest in tannic jar (0.160 ±0.005) (Table 4), however differences were not statistically significant.

Microbial phylotypes that contributed to 5 percent or higher of the man proportional abundance in clay jar included: Flavobacteriaceae (0.65) and Comamonadaceae (0.16) (Figure 2). In addition, in the clay jar treatment, phylotypes identified as Flavobacteriaceae included 3 different species (Flavobacterium n=2 (0.47), Chryseobacterium n=1 (0.18)). Phylotypes that contributed 5 percent or higher of the mean proportional abundance in clay tray included: Comamonadaceae (0.39), Chromatiaceae (0.10), Cryomorphaceae (0.07), and Moraxellaceae (0.06) (Figure 2). Phylotypes that contributed 5 percent or higher of the mean proportional abundance in control tray included: Comamonadaceae (0.48), Moraxellaceae (0.09), Rhodobacteraceae (0.05), and Aeromonadaceae (0.05) (Figure 2). Phylotypes that contributed 5 percent or higher of the mean proportional abundance in tannic jar included: Comamonadaceae (0.31), Flavobacteriaceae (0.15), Burkholdariales unclassified (0.14), Moraxellaceae (0.13), and unclassified (0.05) (Figure 2). In addition, in the tannic jar treatment, phylotypes identified as Flavobacteriaceae included one species (Flavobacterium n=1 (0.15)). Phylotypes that contributed 5 percent or higher of the mean proportional abundance in tannic tray included: Comamonadaceae (0.39), Moraxellaceae (0.18), and Aeromonadaceae (0.05) (Figure 2).

Table 4. Mean (±SE) alpha diversity (Simpson's and Shannon diversity) of lake sturgeon egg microbial communities at two developmental periods (Development period 1 30 to 36 CTU and Development period 2 55 to 67 CTU; Kempinger 1988) and de-adhesion and incubation methods. Identical lowercase letters within columns represent treatments that are not significantly different (Tukey-Kramer, $P < 0.05$).

Development Period	Treatment	Simpson	Shannon
1	Clay Jar	0.39 ±0.02 a	1.06 ±0.03 y
1	Clay Tray	0.63 ±0.02 b	1.79 ±0.04 z
1	Control Tray	0.58 ±0.07 ab	1.60 ±0.21 z
1	Tannic Jar	0.83 ±0.01 c	2.09 ±0.02 z
1	Tannic Tray	0.71 ±0.01 b	1.84 ±0.02 z
2	Clay Jar	0.83 ±0.01	2.14 ±0.04 z
2	Clay Tray	0.82 ±0.01	2.38 ±0.01 y
2	Control Tray	0.81 ±0.01	2.37 ±0.05 y
2	Tannic Jar	0.84 ±0.01	2.27 ±0.04 yz
2	Tannic Tray	0.80 ±0.01	2.24 ±0.04 yz

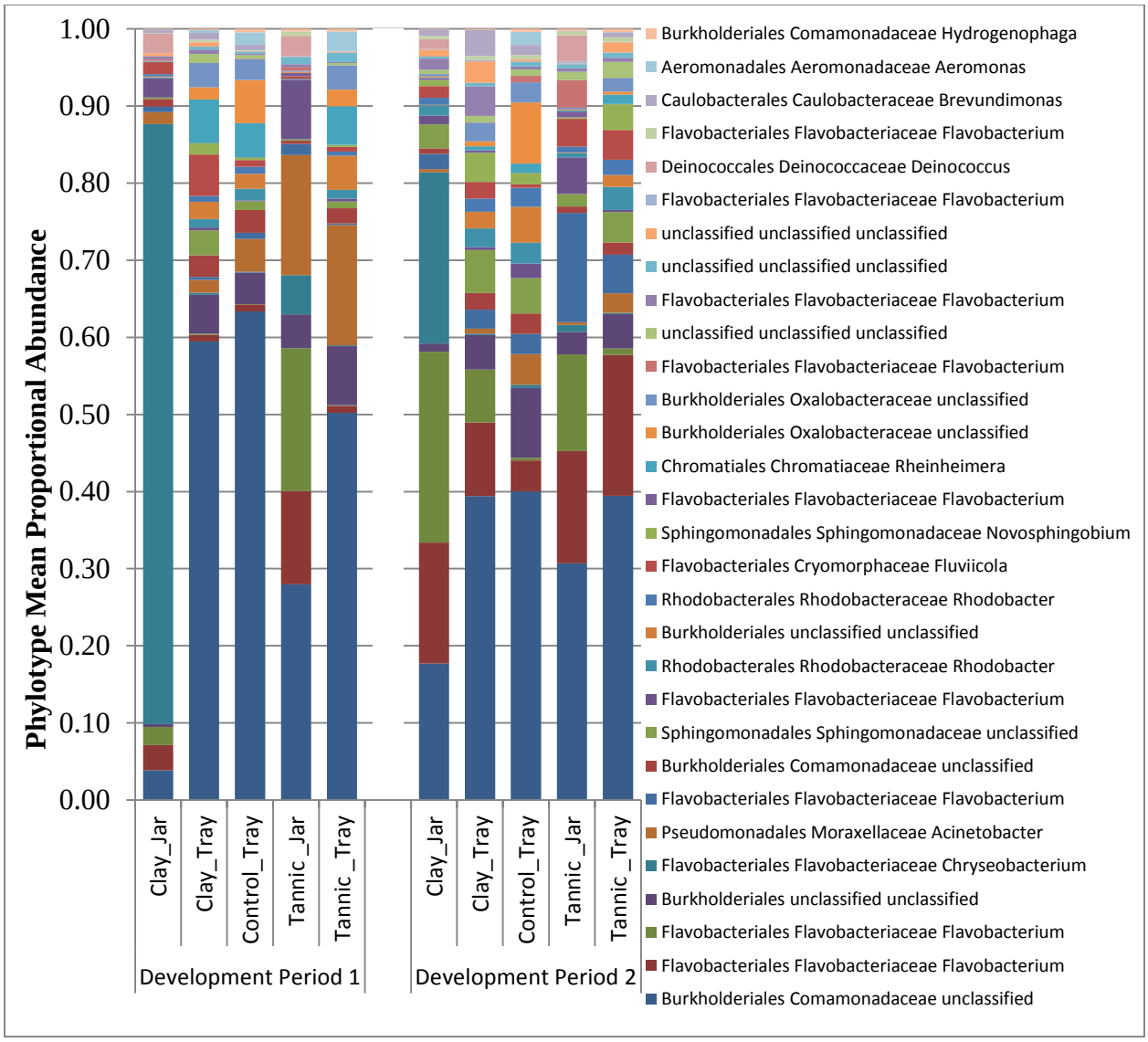


Figure 1. Microbial phylotype mean proportional abundance as a function of embryonic development time and chemotherapeutant treatment. Thirty major phylotypes represented in the community sample based on 5 percent contribution to total operational taxonomic units. Each unique color represents a unique species phylotype based on 97% similarity of operational taxonomic units.

Beta-diversity. –

***Developmental period 1.* –** Among de-adhesion and incubation treatments greater dissimilarity was observed among those incubated in jars compared to those incubated in trays (Figure 2: Table 5). Control tray at developmental period 1 was more dissimilar to those incubated in jars (Figure 2: Table 5).

***Developmental period 2.* –** Among de-adhesion and incubation treatments greater dissimilarity was observed among those incubated in jars compared to trays (Figure 2: Table 5). Control tray at developmental period 2 was more dissimilar to those incubated in jars (Figure 2: Table 5).

Table 5. Mean (±SE) Bray-Curtis dissimilarity index of lake sturgeon egg microbial community as a function of development period (Development period 1 30 to 36 CTU and development period 2 55 to 67 CTU; Kempinger 1988), de-adhesion, and incubation.

	Time	**1**	**1**	**1**	**1**	**1**	**2**	**2**	**2**	**2**	**2**
Time	**Treatment**	**Clay Jar**	**Clay Tray**	**Control Jar**	**Tannic Jar**	**Tannic Tray**	**Clay Jar**	**Clay Tray**	**Control Tray**	**Tannic Jar**	**Tannic Tray**
1	**Clay Jar**	**0.000**									
1	**Clay Tray**	**0.853**	**0.000**								
1	**Control Tray**	**0.855**	**0.229**	**0.000**							
1	**Tannic Jar**	**0.771**	**0.620**	**0.620**	**0.000**						
1	**Tannic Tray**	**0.889**	**0.284**	**0.271**	**0.509**	**0.000**					
2	**Clay Jar**	**0.614**	**0.666**	**0.715**	**0.393**	**0.737**	**0.000**				
2	**Clay Tray**	**0.834**	**0.355**	**0.441**	**0.477**	**0.455**	**0.488**	**0.000**			
2	**Control Tray**	**0.834**	**0.345**	**0.327**	**0.540**	**0.329**	**0.621**	**0.302**	**0.000**		
2	**Tannic Jar**	**0.803**	**0.568**	**0.616**	**0.323**	**0.624**	**0.425**	**0.414**	**0.523**	**0.000**	
2	**Tannic Tray**	**0.839**	**0.352**	**0.431**	**0.483**	**0.440**	**0.494**	**0.221**	**0.297**	**0.369**	**0.000**

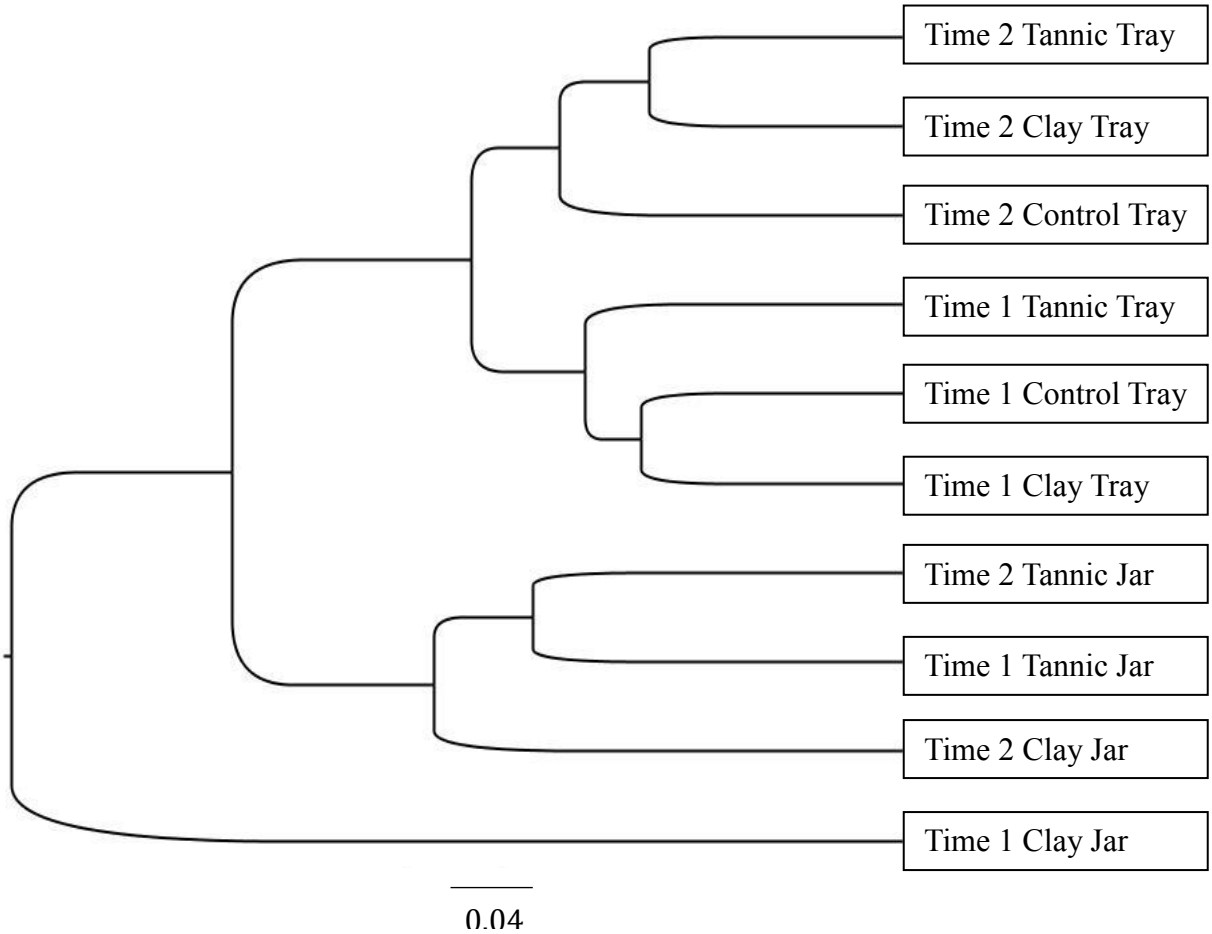


Figure 2. Neighbor-joining tree construct using Bray-Curtis dissimilarity for lake sturgeon egg microbial communities as a function of embryonic development period (Development period 1 30 to 36 CTU and development period 2 55 to 67 CTU; Kempinger 1988) and de-adhesion and incubation conditions.

DISCUSSION

The effects of de-adhesion and incubation on lake sturgeon eggs were quantified using egg survival, egg oxygen consumption rate, and body size as response variables. Results revealed that these variables, except egg survival, did not vary significantly among de-adhesion and incubation treatments. Also, this study incorporated a novel approach by using 16S genomic data to quantify community taxonomic composition of bacteria present on the egg surface in response to different de-adhesion methods and incubation conditions utilized in aquaculture. Results suggests de-adhesion and incubation techniques affect the bacterial community composition on the egg surface which may provide insight into the mechanisms responsible for differences detected in survival.

Effects of de-adhesion method and incubation conditions on body size and survival

Body size at hatch. – No significant effects of de-adhesion or incubation were identified using body size parameters (total length, yolk-sac area, and body area) measured. However, the larger mean total length at hatch in clay jar groups compared to those from other de-adhesion and incubation treatments is encouraging given that these de-adhesion and incubation methods are among those most often utilized in aquaculture facilities. Body size, more specifically total length, is important given results from other studies that suggest body size at hatch is positively correlated with timing and likelihood of exogenous feeding during the larval period and subsequent survival to later life periods (Gisbert et al. 2000, Blaxter and Hempel 1963).

Egg Survival. – The effect of de-adhesion and incubation treatments on the mean proportion of survival to hatch was significant among treatments. Currently, most rearing facilities utilize jars during incubation to limit space utilized for egg production. However in this study, individuals incubated in trays exhibited greater survival (except de-adhesion control) compared to those

incubated in jars. Differences observed between incubation devices (i.e., jar versus tray) may be explained by the rolling action that jar-incubated eggs experience compared to those in trays that remain still. In addition to benefits of space, anecdotal evidence has suggested that eggs in jars 'knock' into one another while rolling in the jar which might inhibit microbial growth at the egg surface and improve survival at hatch. However, results from this study show that survival is higher among egg groups incubated in trays. Therefore, rolling within the jars may 1) remove or inhibit symbiotic microbial taxa that potentially aid in egg development and subsequent survival or 2) removes most bacteria except those that are pathogenic and may have greater binding affinity and also act as a potential egg pathogen (i.e., Flavobacterium). Sensitivity to friction associated with the rolling action encountered in the jar incubators during early embryogenesis may also explain high mortality in jars compared to trays. Green sturgeon are reported to be more sensitive to similar jar apparatuses which may explained by differences in egg chorion thickness (Van Eenennaam et al. 2008), however it is unknown if this is the case for lake sturgeon which warrants further attention.

Differences in survival observed between clay tray and tannic tray compared to control tray might be explained by the reduced adhesive surface area available to be colonized by microbes. Clay particles adhere to the glycoprotein that is released through the egg surface (Doroshov et al. 1983); while tannic acid removes this adhesive glycoprotein layer (Kowtal et al. 1986) processes may both provide less suitable substrates available for microbes to adhere. Control tray groups did not receive any de-adhesion compound and as a result greater adhesive surface area may have been available for colonization by potentially pathogenic microbes.

Effects of de-adhesion method and incubation conditions on oxygen consumption rate

During egg incubation, oxygen consumption rate was measured to identify physiological stress associated with different de-adhesion and incubation treatments. Results revealed that oxygen consumption rate did not vary significantly among de-adhesion and incubation treatments. Higher oxygen consumption rates at the second development period relative to the first are consistent with embryonic stage of development for all de-adhesion and incubation groups. To date, no known study has documented oxygen consumption rates for lake sturgeon eggs. Oxygen consumption rate data provided herein are beneficial to future studies investigating oxygen consumption rates of developing embryos at two critical developmental periods.

Effects of de-adhesion method and incubation conditions on microbial diversity

The effects of de-adhesion and incubation on community alpha diversity (species richness) were significant. Relative to all treatment groups, trends were observed when comparing beta-diversity among jars and trays. These data suggest that de-adhesion and incubation techniques employed prior to egg incubation alter the microbial community on the egg surface.

When comparing the body size and survival at hatch from family 1 through 3 with that of the microbial diversity data from family 4, it is interesting to note that only those incubated in jars were documented to be colonized with Flavobacteriaceae at both developmental periods. Flavobacteriaceae is a known fish pathogen and is ubiquitous in hatchery environments (Loch et al. 2013). This might explain the lower mean survival (albeit not statistically significant) observed among the clay jar and tannic jar, compared to that of the clay tray and tannic tray. Among those incubated in trays, Comamonadaceae (unclassified) dominated the major

phylotypes at both developmental periods, however, it is unknown if the species within the groups are pathogenic. Based on body size and survival of groups incubated in trays from family 1 through 3, it would suggest that this phylotypes may be symbiotic. However, additional studies are needed with growth and microbe data collected from similar families to further support this.

Conclusions

Relative to parameters measured in this study (egg survival, egg oxygen consumption rate, microbial community composition, and body size at hatch), benefits that may result from employing different de-adhesion and incubation methods during embryogenesis were apparent in terms of mean survival at hatch. Although mean differences were not significant, incubating eggs in trays resulted in higher mean survival to hatch. In addition, genomic interrogation of egg microbe communities demonstrated that microbial community composition on the egg surface is changed as a function of different de-adhesion and incubation methods. Furthermore, phylotypes documented on egg surfaces may act as potential egg pathogens or symbionts. Therefore additional studies are needed to further demonstrate costs or benefits with respect to growth and survival in relationship with different microbial communities on egg surfaces. Additional information gathered about the relative abundance of phylotypes, using quantitative PCR would also provide information to interpret oxygen consumption data. Lastly, it is unclear if microbial communities on the fish egg surface effect growth and survival from hatch through subsequent ontogenetic stages. Therefore additional studies are needed to further support this hypothesis. Additional information gathered about the specific abundances of certain phylotypes, using quantitative PCR may also provide researchers additional information related to parameters used in this study highlighting potential mechanisms for the observed results. Lastly, it is unclear if

microbial communities on the fish egg surface, as a function of different de-adhesion and incubation conditions, effects growth and survival from hatch through ontogenetic contingency which warrants further investigation as well.

LITERATURE CITED

Balon, E. K. 1975. Reproductive guild of fishes: A proposal and definition. Journal of Fisheries Research Board of Canada 32(6):821-864.

Barnes, M. E., D. Bergmann, J. Jacobs, and M. Gabel. 2009. Effect of *Flavobacterium columnare* inoculation, antibiotic treatments and resident bacteria on rainbow trout *Oncorynchus mykiss* eyed egg survival and external membrane structure. Journal of Fish Biology 74:576-590.

Blaxter, J. H. S., and G. Hempel. 1963. The influence of egg size on Herring larvae (*Clupea harengus* L.). J. Const. Int. Explor. Mer. 28:211-240.

Bouchard, J. H. and D. B. Aloisi. 2002. Investigations in concurrent disinfection and de-adhesion of lake sturgeon eggs. North American Journal of Aquaculture 64:212-216.

Boucher, M. A. 2012. The effect of substrate rearing on the growth, development, and survival of larval white sturgeon (*Acipenser transmontanus*) during early ontogeny. The University of Northern British Columbia Master's Thesis 1-97.

Bray, J. R., and J. T. Curtis. 1957. An ordination of the upland forest communities southern Wisconsin. Ecological Monographs 27:325:349.

Cherr, G. N., and W. H. Clark. 1982. Fine structures of the envelope and micropyles in the eggs of the White Sturgeon, *Acipenser transmontanus* Richardson. Developmental Growth and Differentiation 24(4):341-352.

Cherr, G. N., and W. H. Clark. 1984. Jelly release in the eggs of the White Sturgeon, Acipenser transmontanus: An enzymatically mediated event. The Journal of Experimental Zoology 230:145-149.

Crossman, J. A., P. S. Forsythe, K. T. Scribner, and E. A. Baker. 2011. Hatchery rearing environment and age affect survival and movements of stocked juvenile lake sturgeon. Fisheries Management Ecology 18:132-144.

Doroshov, S. I., W. H. Clark, P. B. Lutes, R. L. Swallow, K. E. Beer, A. B. McGuire, and M. D. Cochran. Artificial propagation of the White Sturgeon (*Acipenser transmontanus*) Richardson. Aquaculture 32:93-104.

Feledi, T., B. Kucska, and A. Ronyai. 2011. Effect of different fertilization and egg-de-adhesion methods on the artificial propagation of Siberian sturgeon. Archives of Polish Fisheries 19:119-122.

Gisbert, E., P. Williot, and F. Castello-Orvay. Influence of egg size on growth and survival of early stages of Siberian sturgeon (Acipenser baeri) under small scale hatchery conditions. Aquaculture 183:83-94.

Fujimoto, M., J. A. Crossman, K. T. Scribner, and T. A. Marsh. 2013. Microbial community assembly and succession on Lake Sturgeon egg surfaces as a function of simulated spawning stream flow rate. Microbial Ecology 66:500-511.

Hansen, G. H., and J. A. Olafsen. 1999. Bacterial interactions in early life stages of marine cold water fish. Microbial Ecology 38:1-26.

Jeon, Y., S. Park, J. Lim, J. Chun, and B. Kim. 2015. Improved pipeline techniques for reducing erroneous identification by 16S rRNA sequences using the Illumina MiSeq platform. Journal of Microbiology 53(1):60-69.

Kamler, E. 2008. Resource allocation in yolk-feeding fish. Reviews in Fish Biology and Fisheries 18:143-200.

Kempinger, J. J. 1988. Spawning ad early life history of lake sturgeon in the Lake Winnebago system, Wisconsin. American Fisheries Society Symposium 5:110-122.

Kowtal, G. V., W. H. Clark, and G. B. Cherr. 1986. Elimination of adhesiveness in eggs from the White sturgeon, *Acipenser transmontanus*: Chemical treatment of fertilized eggs. Aquaculture 56:139-143.

Kozich, J. J., S. L. Wescott, N. T. Baxter, S. K. Highlander, and P. D. Schloss. 2015. Improved pipeline for reducing erroneous identification of 16S rRNA sequences using the Illumina MiSeq platform.

Linhart, O., M. Rodina, D. Gela, M. Flajshans, and M. Kocour. 2003. Enzyme treatment for elimination of egg stickiness in tench (Tinca tinca L.), European catfish (Silurus glanis L.) and common carp (Cyprinus carpio L.). Fish Physiology and Biochemistry 28:507-508.

Naciri, M., D. Kuystermans, and M. Al-Rubeai. 2008. Monitoring pH and dissolved oxygen in mammalian cell culture using optical sensors. Cytotechnology 57:245-250.

Nelson, M. C., H. G. Morrison, J. Benjamino, S. L. Grim, ad J. Graf. 2014. Analysis, optimization and verification of illumina-generated 16S rRNA gene amplicon surveys. PLOS ONE 9(4):1-14.

Schloss P. D., D. Gevers, and S. L. Westcott. 2014. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PloS ONE. 6:e27310.

Shannon, C. E. 1948. A mathematical theory of communication. Reprinted for the Bell System Technical Journal with corrections 5(1):3-55.

Simpson, E. H. 1949. Measurement of diversity. Nature 163:688-689.

Van Den Berg, A. H., D. McLaggan, J. Dieguez-Uribeondo, and P. Van West. 2013. The impact of the water moulds *Saprolignia diclina* and *Saprolegnia parasitica* on natural ecosystems and the aquaculture industry. Fungal Biology Reviews 27:33-42.

Appendix #4 – Effects of egg chemotherapeutants on body size, survival, oxygen consumption rate, and microbial diversity of lake sturgeon eggs.

John M. Bauman[a], Edward A. Baker[b], Terry L. Marsh[c] and Kim T. Scribner[a,d]

[a]Department of Fisheries and Wildlife, Michigan State University, 13 Natural Resources Building, East Lansing, MI 48824, USA.

[b]Michigan Department of Natural Resources Fisheries Division, 484 Cherry Creek Road, Marquette, MI 49855, USA.

[c]Department of Microbial and Molecular Genetics, Michigan State University 6169 Biomedical Physical Sciences, East Lansing, MI 48824, USA.

[d]Department of Zoology, Michigan State University 288 Farm Lane, East Lansing, MI 48824, USA.

ABSTRACT

Microbial communities including pathogens in water-sources used for aquaculture represent a significant source of mortality during the egg period for many fish species. As a result, preventative measures are integrated in aquaculture protocols to ensure that infection outbreaks are prevented or reduced. In this study, Lake Sturgeon eggs were used to quantify the effect of different chemotherapeutants on the survival and body size at hatch. Oxygen consumption rates were also quantified in association with the relative abundance and community composition of microbes on the egg surface at different developmental periods. Of the parameters measured in this study (egg survival, egg oxygen consumption rate, microbial

community composition, and body size at hatch), benefits of treating lake eggs using peroxide or formalin were not evident. 16S rRNA gene sequencing was used to quantify relative abundance and community composition of microbes as a function of fish egg chemotherapeutants. Microbial analysis data showed that diversity of microbial communities on the egg surfaces were altered as a function of chemotherapeutant treatment, indicating a shift towards pathogenic taxa (i.e., Flavobacteriaceae) particularly during early embryogenesis. Downstream effects of these treatments and altered microbial community structures on the growth and survival at subsequent life periods are unknown, warranting additional studies.

INTRODUCTION

Microbial communities including pathogens in water-sources used for aquaculture represent a significant source of mortality during the egg period for many fish species (Barnes et al. 2005[Bacterial], Wagner et al. 2012[Bacterial], Van Den Berg et al. 2013[oomycete], Meyer 1991[oomycete]). Egg surfaces provide suitable substrates for microbial taxa to attach and proliferate during incubation (Hansen and Olafsen 1989, Fujimoto et al. 2013). Importantly, pathogenic microbes, (e.g., *Flavobacterium*) can negatively affect production of developing eggs by attaching to the adhesive glycoprotein matrices that envelope the outer surface of the egg (Cherr and Clark 1984, Hansen and Olafsen 1999) and degrading the chorion (Hansen et al. 1992, Barnes et al. 2009). Additional research suggests that bacterial communities colonize the egg surface in great abundance causing developmental arrest (Forsythe et al. 2014) due possibly to oxygen depletion (Barker 1989).

In addition to pathogenic bacteria, a common eukaryotic oomycete known as Saprolegnia, which causes *Saprolegniosis*, is a disease commonly found on fish egg surfaces, and is believed to inhabit surfaces of dead or unfertilized eggs (Van Den Berg et al. 2013). It is

therefore only through subsequent 'hyphal infection' that viable eggs raised in nearby incubation devices become infected (Van Den Berg et al. 2013) likely resulting in suffocation and mortality (Meyer 1991). In addition to egg surface properties, hatchery environments (e.g., water sources, incubation devices) expose developing eggs to sub-optimal incubation conditions and provide favorable environments for pathogenic taxa to colonize the egg surface causing direct (mortality) and indirect effects (growth) (Hansen et al. 1992[Bacterial], Van Den Berg et al. 2013[oomycete]). As a result, preventative measures are integrated in aquaculture protocols to ensure that infection outbreaks are prevented or reduced.

Preventative measures include the use of ultraviolet irradiation which is believed to disrupt replication of and reduce microbial abundance (Sharrer et al. 2005) in aquaculture water-sources. Additionally, chemical treatments (hereinafter referred to as "chemotherapeutants") administered during egg incubation have reduced microbial abundance and increased hatch success in some species (Barnes 2009[Bacterial], Stephenson et al. 2003[Bacterial] Van Der Berg et al. 2013[oomycete]), although results vary. Two chemotherapeutants commonly used to reduce microbial abundance and improve hatch success are formalin and hydrogen peroxide, both of which are currently listed by the U. S. Food and Drug Administration as approved drugs for treatment of freshwater finfish eggs (Bowker 2011). Formalin (37%) is typically used to treat eggs for 15 min at 1,500 uL/L concentration using a constant flow water supply (Bowker 2011). Hydrogen Peroxide (35%) is typically used to treat eggs for 15 minutes at 500 ppm concentration using a constant flow water supply (Bowker 2011). Despite the wide use of these chemotherapeutants, little information exists pertaining to the effects on microbial community abundance and diversity following treatment, or the potential effects of colonization on egg respiration, growth and hatch success. Recent advances in meta-genomic 16S rRNA techniques

and software involving high throughput data management tools (Cole et al. 2009, Nelson et al. 2014, Schloss et al. 2014) allows documentation of changes in community abundance in response to chemotherapeutants in aquaculture settings. Also, since approved chemotherapeutants were initially and most commonly assessed using salmonids, further research is needed to evaluate the applicability of common chemotherapeutants for other fish species, including those of conservation concern, such as Sturgeons (IUCN, 2010).

Sturgeons are highly fecund, migratory litho-pelagophiles (Balon 1975) with unique egg properties that differ from most teleosts (Cherr and Clark 1982, Cherr and Clark 1984, Detlaff et al. 1993). The economic value of roe and flesh gathered from mature adults, as well as the conservation status of many sturgeon species has prompted recovery efforts through the use of traditional as well as conservation hatchery programs (Memis 2009, Bronzi et al. 1999). Conservation hatchery programs, such as those which utilize streamside rearing facilities (SRFs) for Lake Sturgeon (*Acipenser fulvescens*) (Holtgren et al. 2007), have been in place for nearly a decade in an attempt to recover or repatriate populations in the North American Great Lakes. However, desired annual production levels have been difficult to achieve due in large part to high mortality during early development, including the egg period.

The objectives of this study were to: 1) quantify the effects of different egg chemotherapeutants on body size and survival at hatch, and 2) quantify the respiration rates of eggs and the relative abundance of microbial communities on egg surfaces as a function of different chemotherapeutants used in aquaculture. The research hypotheses of this study were: 1) egg survival will differ significantly as a function of different chemotherapeutants used during incubation, 2) body size at hatch will differ significantly based on the use of different chemotherapeutants, 3) egg chemotherapeutants reduce the relative abundance of microbial and

oomycete communities on the egg surface, and 4) egg chemotherapeutants alter the microbial and oomycete community composition and in particular, reduce the relative abundance of pathogenic microbes.

METHODS

Study site

The Black River Streamside Rearing Facility (BR-SRF) is supplied with ambient river water (~680 L/min) from the Kleber Reservoir, located near primary spawning areas for Lake Sturgeon in the upper Black River in Cheboygan County, Michigan. Water used for rearing in the BR-SRF is passed once through a high output UV sterilizer (Pentair #E50S). This study was conducted in May 2012 and water temperatures ranged from 13.1 to 17.0^{0}C (mean 14.9^{0}C).

Gamete collection, fertilization, and incubation

Gametes were collected from one male and one female lake sturgeon (5 May and 11 May, 2012,) during spawning in the upper Black River following procedures described by Crossman et al. (2011). Gametes were transported to the BR-SRF for the fertilization of two full-sibling families, which took place within twelve hours of collection. Eggs were fertilized using a 1:200 milt dilution of 0.22 um filtered, re-circulated UV treated ambient river water and immediately poured over the eggs allowing 90 seconds for fertilization. Excess milt was then removed and eggs were rinsed once with 0.22 um filtered, re-circulated UV-treated river water. Egg de-adhesion procedures began by applying a Fuller's Earth solution (Sigma Aldrich) and gently mixing for 50 min. After 50 min, Fuller's Earth was rinsed from the eggs and a 15 min, 50 ppm Iodophor disinfection treatment was administered. Following a 10 min rinse to remove residual Iodophor using ambient river water, three batches of 150 to 200 randomly selected eggs from each family were placed into three separate 7.6 cm diameter PVC couplings with 1.0 by 1.0 mm mesh on the top (removable) and bottom. Each coupling (experimental unit) was randomly assigned to one of two disinfectant treatment groups and a control and transferred to Heath trays for incubation with a flow rate of 19.0 L/min.

Experimental chemotherapeutant treatments

The purpose of this experiment was to quantify the effects of chemotherapeutants on lake sturgeon body size and survival at hatch. In addition, the microbial community composition and relative abundance on the egg surface was quantified as a function of different chemotherapeutants used during incubation in association with oxygen consumption rate. Microbial and oxygen experiments focused on two development periods, determined by calculating cumulative daily water temperature units (30 to 36 CTU and 55 to 67 CTU; Kempinger 1988), during embryogenesis when significant mortality occurs (Scribner and Marsh, unpublished data). Beginning two days post-fertilization, eggs in couplings were exposed to daily chemotherapeutants treatment: 1) 15 min, 500 ppm constant-flow hydrogen peroxide treatment, 2) 15 min, 1667 uL/L constant-flow formalin treatment, or 3) a control (no chemical treatment). Daily chemical treatments were performed until 24 hours prior to hatch.

Data Collection

Body size. –At hatch, we used a digital camera to photograph and measure body size (total length (TL mm), yolk-sac area (YSA mm^2), and body area (BA mm^2)) for a random subsample ($3 \leq n \leq 25$) of fish from each treatment, and each family using Image J software (v.1.43u).

Survival. – Beginning at 3 days post-fertilization, dead eggs were removed and recorded at the start of each day to quantify cumulative proportional survival at hatch.

Oxygen measurement. – At each of two development periods, eight eggs from each treatment and each family (replicate) were sub-sampled to quantify mean oxygen consumption rate (mg/L / 5 min / eight eggs) using a 24-well SensorDish reader plate (PreSens Precision Sensing, GmbH, Regensburg, Germany) (Naciri et al. 2008). Each well was filled with 1.5 mL of ambient 0.22 um, UV treated river water, and eight eggs from each treatment (including a negative control –

no eggs) were placed circumpolar to the electro-florescence sensor, and then topped with 0.5 mL of sterile mineral oil. Oxygen consumption rate was recorded every 30 sec for up to one hr. After 1 hr, eggs were removed from the SensorDish reader and placed into separate 2.0 mL posi-click tubes, filled with 1.5 mL of 95% ethanol, for microbial 16S rRNA extraction. Oxygen consumption rate data were plotted using Excel (time on x-axis, oxygen concentration y-axis) and visually inspected to determine the initial point of decreasing oxygen concentration (representing oxygen consumption) which has been shown to vary (up to 10 min from initial measurement) (Warkentin et al. 2007, Strecker et al. 2011). Oxygen consumption was quantified by identifying the initial decline point (time 1), and selecting a second time point (5 min later) (time 2). Oxygen consumption was calculated for replicates of each treatment group and each family by subtracting oxygen levels at time 2 from time 1 (mg/L). Selection criteria for time 1 and time 2 was necessary to capture the initial respiration rate prior to the point that embryo may begin to 'adapt' to potentially hypoxic conditions (Strecker et al. 2011). Microbes were not considered a significant source of oxygen loss during this experiment (Boucher 2012).

Microbial and Oomycete DNA extraction

Microbial genomic DNA was extracted from 8 eggs per treatment per time point using a modified DNeasy Blood & Tissue QIAGEN Kit (QIAGEN Group, 2006). Modified steps include the initial incubation of samples in an enzymatic lysis buffer at 37^0C for 30 min followed by bead-beating (Fujimoto et al. 2013) for 10 min. After bead-beating, steps were followed manufacturer's protocols. To ensure sufficient DNA was extracted for analysis, eight eggs from each sample were pooled during the extraction process (Fujimoto et al. 2013). Amplification of 16S rRNA gene was used to estimate the microbial community composition as a function of chemotherapeutants and development time period during incubation. Polymerase chain reaction

(PCR) was conducted in a 50 uL reaction volume, containing 10 uL template DNA (6 to 110 ng/uL), 0.4 uL of AquPrime HiFi Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA), 5.0 uL 10X PCR Buffer II, 1.0 uL 454 HMP Forward Primer, 1.0 uL Barcode Reverse Primer, and 32.6 uL sterile water. Reactions were performed using the following thermocycle conditions; initial denaturation step at 95ºC for 2 min, then 30 cycles of denaturation at 95ºC for 20 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 7 min (Fujimoto et al. 2013).

Processing16S Sequencing Data

16S sequencing analysis was performed using default settings in program Mothur (Schloss 2014). Sequences were assigned to operational taxonomic units (OTUs) using a 97% similarity threshold. To statistically and graphically describe similarity and dissimilarity among samples, program Mothur was used to compute alpha (Simpson 1949, Shannon 1948) and beta (Bray and Curtis 1957) diversity indices and construct a dendrogram and lower-triangle dissimilarity matrix based on Bray-Curtis values. Furthermore, to visualize relationships in diversity among samples, a comparison of dominant phylotypes as function of chemotherapeutant and developmental period were graphed as a stacked column chart using Microsoft Excel.

Statistical Analysis

Trait differences among chemotherapeutant treatments tested included mean (±SE) body size (TL, YSA, and BA) at hatch and mean proportional survival at hatch. Furthermore, oxygen consumption rate and alpha diversity of egg microbial communities were tested at two development periods as a function of different chemotherapeutants. Parameters were tested using SAS (SAS Institute version 9.3 Cary, NC). Egg incubation coupling, which contained 150 to 200 eggs, was the experimental unit for all response variables used in the analysis. A p value < 0.05 was considered statistically significant for all analyses. A general linear model using

analysis of variance was used to analyze all response variables except proportional survival. Data for total length and yolk-sac area were log-transformed to validate the normality assumption. Data for oxygen consumption was square-root transformed to validate the normality assumption. A generalized linear mixed model using analysis of variance was used to analyze survival data. Survival to hatch was modeled assuming a beta-distribution and reported as proportional survival. We used Tukey-Kramer multiple pair-wise comparison tests for all response variables.

RESULTS

Effects of chemotherapeutants on body size at hatch

The effect of chemotherapeutant treatments on body size at hatch was not statistically significant (TL: $F_{2,3} = 3.20$, $P = 0.1801$; YSA: $F_{2,3} = 0.63$, $P = 0.5908$; BA: $F_{2,3} = 0.1.87$, $P = 0.2975$). Mean (±SE) body size at hatch was smallest for individuals hatching from eggs treated with formalin (TL = 12.30 ±0.54, YSA = 8.14 ±0.16, BA = 24.76 ±3.64) and largest for individuals from control groups (TL = 13.81 ±0.35, YSA = 8.39 ±0.29, BA = 30.51 ±0.91) with the exception of body area which was largest in individuals that hatched from eggs treated with peroxide (TL = 13.41 ±0.41, YSA = 8.54 ±0.30, BA = 30.39 ±1.83) (Table 6).

Effects of chemotherapeutants on survival at hatch

The effect of chemotherapeutant treatments on mean proportion of eggs surviving to hatch was not significant ($F_{2,3} = 1.78$, $P = 0.3097$). Mean (±SE) proportional survival in the control (0.43 ±0.18) was lower than that observed in hydrogen peroxide (0.62 ±0.03), and higher than that observed in with formalin (0.32 ±0.21) (Table 6).

Effects of chemotherapeutants on embryo oxygen consumption

The effects of chemotherapeutant treatments on mean (±SE) oxygen consumption rate (mg/L /5 min for eight eggs) at development periods 1 and 2 were not statistically significant (time 1: $F_{2,3} = 0.07$, $P = 0.9305$, time 2: $F_{2,3} = 1.00$, $P = 0.4642$). Compared to a control (0.32 ±0.01 mg/L) at development period 1, mean oxygen consumption rate was higher in groups treated with peroxide (0.34 ±0.01 mg/L), and lower in groups treated with formalin (0.31 ±0.10 mg/L); however, differences were not statistically significant (Table 6). Compared to the control (0.50 ±0.11 mg/L) at development period 2, mean oxygen consumption rate was higher in groups treated with formalin (0.97 ±0.15 mg/L), and in groups treated with peroxide (0.67 ±0.37 mg/L); however, differences were not statistically significant (Table 6). During the course of all oxygen trials, oxygen sensor wells filled with 1.5 mL of ambient 0.22 um UV treated river water and did not contain eggs (negative control) served as a control, and gradually increased in oxygen concentration (0.06 ±0.04 mg/L /5 min).

Table 6. Lake Sturgeon mean (±SE) body size (total length (TL), yolk-sac area (YSA), and body area (BA)), and proportional survival at hatch as well as oxygen consumption rate at two developmental periods (Development period 1 30 to 36 CTU and development period 2 55 to 67 CTU; Kempinger 1988).

Treatment	TL	YSA	BA	Survival	Oxygen Period 1	Oxygen Period 2
Control	13.81 ±0.35	8.39 ±0.29	30.51 ±0.91	0.43 ±0.18	0.32 ±0.01	0.50 ±0.11
Formalin	12.30 ±0.54	8.14 ±0.16	24.76 ±3.64	0.32 ±0.21	0.31 ±0.10	0.97 ±0.15
Peroxide	13.41 ±0.41	8.54 ±0.30	30.39 ±1.83	0.62 ±0.03	0.34 ±0.01	0.67 ±0.37
Overall	13.17 ±0.35	8.35 ±0.14	28.55 ±1.61	0.46 ±0.09	0.32 ±0.03	0.71 ±0.14

Effects of chemotherapeutants on microbial alpha-diversity

The effect of treatment on the mean (±SE) Simpson index at development periods 1 and 2 was not statistically significant (development period 1: $F_{2,3} = 0.75$, $P = 0.5456$, development

period 2: $F_{2,3} = 0.61$, $P = 0.6007$). Compared to the control at development period 1 (0.67 ±0.09), mean Simpson index was higher in groups treated with peroxide (0.73 ±0.04), and highest in groups treated with formalin (0.78 ±0.03) (Table 7). Compared to the control at development period 2 (0.82 ±0.02), mean Simpson index was lower in groups treated with peroxide (0.75 ±0.06) and lowest in groups treated with formalin (0.73 ±0.08) (Table 7).

The effect of treatment on the mean Shannon index at development periods 1 and 2 were not statistically significant (development period 1: $F_{2,3} = 2.06$, $P = 0.2732$, development period 2: $F_{2,3} = 0.75$, $P = 0.5430$). Compared to the control at development period 1 (1.28 ±0.14), mean Simpson index was higher in groups treated with peroxide (1.79 ±0.40), and highest in groups treated with formalin (2.03 ±0.17) (Table 7). Compared to the control at development period 2 (2.08 ±0.09), mean Shannon index was lower in groups treated with peroxide (1.84 ±0.16) and lowest in groups treated with formalin (1.70 ±0.33) (Table 7).

Table 7. Microbial alpha diversity estimated for lake sturgeon eggs exposed to different chemotherapeutants at two developmental periods (Development period 1 30 to 36 CTU and development period 2 55 to 67 CTU; Kempinger 1988). Alpha diversity indices include Simpson, Shannon diversity indices (Simpson's and Shannon diversity indices; mean (±SE)).

Treatment	Simpson Period 1	Simpson Period 2	Shannon Period 1	Shannon Period 2
Control	0.67 ±0.09	0.82 ±0.02	1.28 ±0.14	2.08 ±0.09
Formalin	0.78 ±0.03	0.73 ±0.08	2.03 ±0.17	1.70 ±0.33
Peroxide	0.73 ±0.04	0.75 ±0.06	1.79 ±0.40	1.84 ±0.16
Overall	0.73 ±0.03	0.77 ±0.03	1.70 ±0.18	1.87 ±0.12

Microbial phylotypes which contributed 5 percent or higher of the mean proportional abundance in the control group at development period 1 included: Enterobacteriaceae (0.30), Comamonadaceae (0.17), Oxalobacteraceae (0.16), Veillonellaceae (0.09), Clostridiaceae (0.09),

and Burkholdariales (0.07) (Figure 3). In addition, for the control at development period 1, phylotypes identified as Flavobacteriaceae included 3 species and comprised approximately 0.03 of the mean proportional contribution to the microbial community (Figure 3). Phylotypes which contributed to 5 percent or higher of the mean proportional abundance in the formalin group at developmental period 1 included: Comamonadaceae (0.27), Flavobacteriaceae (0.25), Flavobacteriaceae (0.17), Oxalobacteraceae (0.06), and Burkholdariales (0.05) (Figure 3). In addition, for formalin at developmental period 1, Flavobacteriaceae included 5 species and comprised approximately 0.44 of the mean proportional contribution to the microbial community (Figure 3). Phylotypes which contributed to 5 percent or higher of the mean proportional abundance in the peroxide group at developmental period 1 included: Comamonadaceae (0.23), Aeromonadaceae (0.21), Clostridiaceae (0.16), Shewanellaceae (0.08), and Flavobacteriaceae (0.06) (Figure 3). In addition, for peroxide at developmental period 1, Flavobacteriaceae included 5 species and comprised approximately 0.12 of the mean proportional contribution to the microbial community (Figure 3).

Phylotypes which contributed 5 percent or higher of the mean proportional abundance in the control group at development period 2 included: Enterobacteriaceae (0.27), Veillonellaceae (0.27), Comamonadaceae (0.10), Burkholdariales (0.06), Burkholdariales (0.06), and Neisseriaceae (0.05) (Figure 3). In addition, for the control at development period 2, phylotypes identified as Flavobacteriaceae included 3 species and comprised approximately 0.01 of the mean proportional contribution to the microbial community (Figure 3). Phylotypes which contributed to 5 percent or more of the mean proportional abundance in the formalin group at developmental period 1 included: Comamonadaceae (0.35), Oxalobacteraceae (0.28), Enterobacteriaceae (0.16), and Aeromonadaceae (0.06), (Figure 3). In addition, for formalin at

developmental period 2, Flavobacteriaceae included 3 species and comprised approximately 0.01 of the mean proportional contribution to the microbial community (Figure 3). Phylotypes which contributed to 5 percent or higher of the mean proportional abundance in the peroxide group at developmental period 2 included: Enterobacteriaceae (0.40), Comamonadaceae (0.25), Oxalobacteraceae (0.09), and Chromatiaceae (0.08) (Figure 3). In addition, for peroxide at developmental period 2, Flavobacteriaceae included 3 species and comprised approximately 0.05 of the mean proportional contribution to the microbial community (Figure 3).

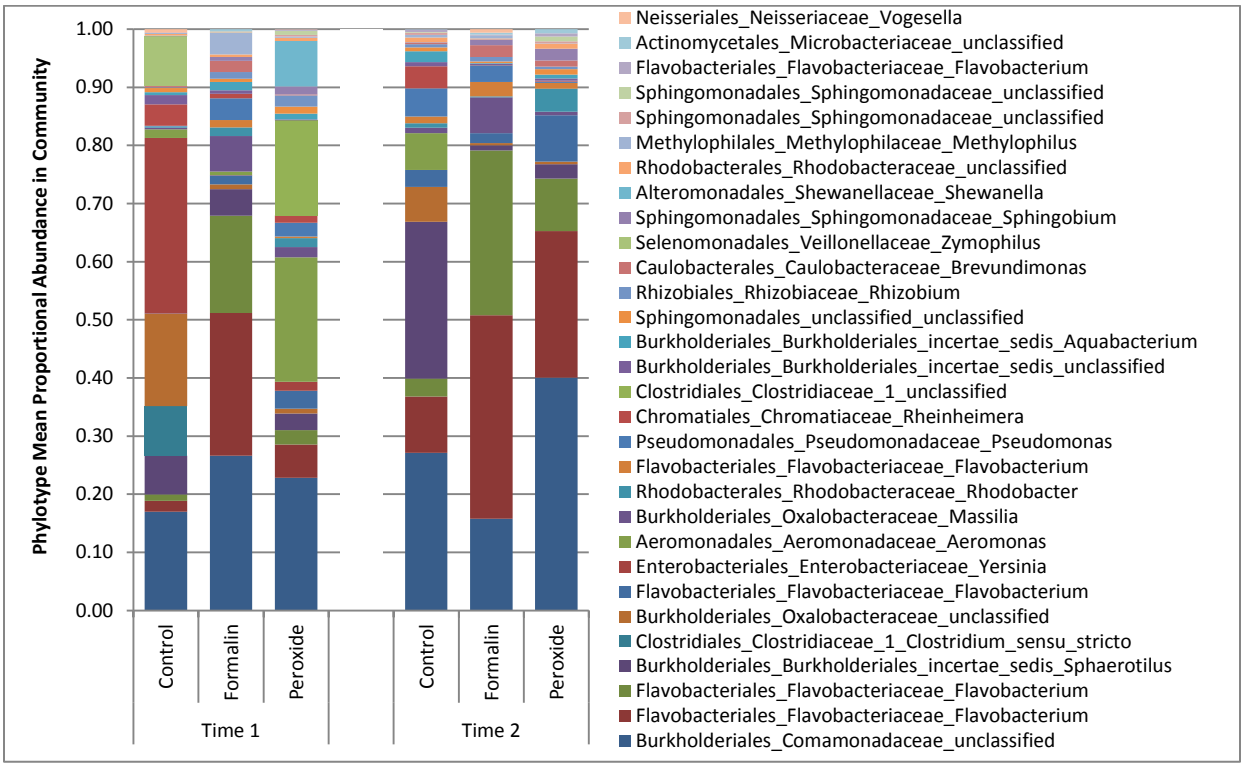


Figure 3. Microbial phylotype mean proportional abundance as a function of embryonic development time and chemotherapeutant treatment. Thirty major phylotypes represented in the community sample based on 5 percent contribution to total operational taxonomic units. Each unique color represents a unique species phylotype based on 97% similarity of operational taxonomic units.

Effects of chemotherapeutants on microbial beta-diversity

Among treatments comparisons at developmental period 1 between peroxide and formalin (0.73 ±0.30) suggests moderate dissimilarity as a function of chemotherapeutant treatment (Table 8: Figure 3). Mean Bray-Curtis dissimilarity index between egg microbial communities at developmental period 2 for the control suggests moderate dissimilarity compared to peroxide (0.51 ±0.06) and formalin (0.64 ±0.16) (Table 8: Figure 3). An additional comparison at developmental period 2 between peroxide and formalin (0.46 ±0.11) communities suggest lower dissimilarity with respect to the control (0.57 ±0.11) at development period 2 (Table 8: Figure 3).

Table 8. Mean (±SE) Bray-Curtis dissimilarity index of lake sturgeon egg microbial community as a function of development period (Development period 1 30 to 36 CTU and development period 2 55 to 67 CTU; Kempinger 1988) and chemotherapeutant.

	Time	1	1	1	2	2	2
Time	Treatment	Control	Formalin	Peroxide	Control	Formalin	Peroxide
1	Control	0.00 ±0.00					
1	Formalin	0.84 ±0.20	0.00 ±0.00				
1	Peroxide	0.70 ±0.33	0.73 ±0.30	0.00 ±0.00			
2	Control	0.66 ±0.42	0.52 ±0.06	0.63 ±0.29	0.00 ±0.00		
2	Formalin	0.93 ±0.07	0.30 ±0.06	0.84±0.16	0.64 ±0.16	0.00 ±0.00	
2	Peroxide	0.78 ±0.30	0.43 ±0.05	0.65 ±0.46	0.51 ±0.06	0.46 ±0.11	0.00 ±0.00

Time 2 Peroxide

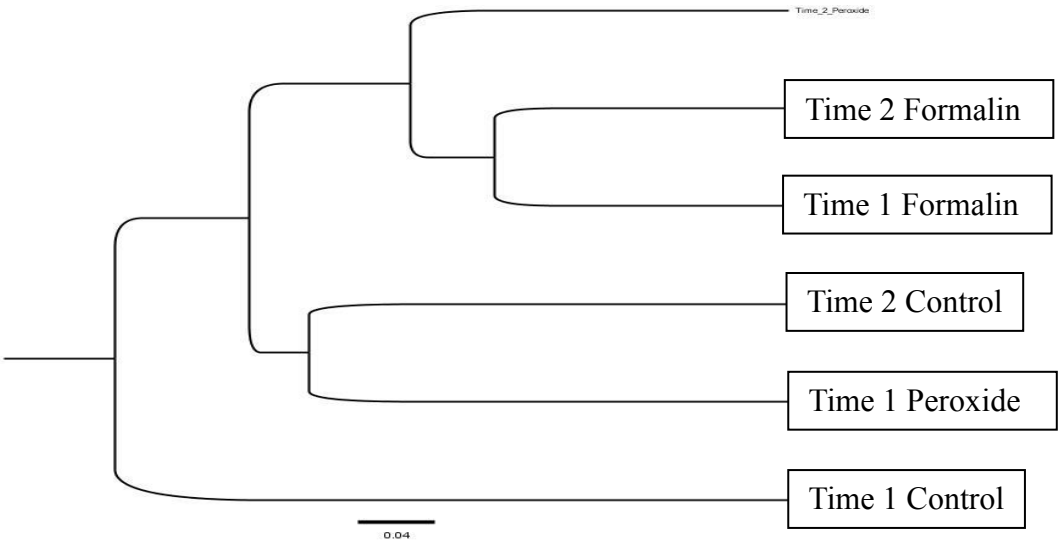


Figure 3. Neighbor-joining tree construct using Bray-Curtis dissimilarity for lake sturgeon egg microbial communities as a function of embryonic development period (Development period 1 30 to 36 CTU and development period 2 55 to 67 CTU; Kempinger 1988) and chemotherapeutant treatment.

DISCUSSION

In this study, the effects of chemotherapeutants on lake sturgeon eggs and post-hatch free-embryos were quantified using egg survival, egg oxygen consumption rate, and body size data. Results revealed that these variables did not vary significantly among treatments. This study incorporated a novel approach by using 16S genomic data to quantify the community composition of bacteria present on the egg surface in response to different chemotherapeutants utilized in aquaculture. In this study peroxide, among chemotherapeutants commonly utilized in streamside hatcheries, showed a higher mean proportional survival compared to formalin and the control (although results were not statistically significant). Additionally, chemotherapeutants may have selected for microbial communities that have been determined in other studies to be

pathogenic, which warrants additional studies and possible refinement of hatchery egg-treatment protocols.

Impact of chemotherapeutants on body size, survival, and oxygen consumption

Body size at hatch. – No significant effect of chemotherapeutant was identified using body size parameters (total length, yolk-sac area, and body area) measured. However, the larger mean total length at hatch from eggs in the control groups compared to those from the same families treated with formalin or peroxide can be of biological significance for larvae during subsequent life periods. For example, studies have reported that larger size at hatch is positively correlated with the timing and likelihood of first-feeding, which is associated with larval survival (Gisbert et al. 2000, Blaxter and Hempel 1963). In this study, survival was not monitored beyond hatch, therefore, additional studies are needed to quantify the effects of chemotherapeutants on survival during subsequent life periods.

Egg Survival. – The effect of chemotherapeutants on the mean proportion of survival to hatch was not significant. However, though variability was high across replicates we did observe 20 to 30% differences in survival as a function of chemotherapeutant which is of practical significance to hatchery managers. For example, if survival at the egg period limits hatchery production, use of hydrogen peroxide as a chemotherapeutant may be beneficial, without reducing body size as was demonstrated when formalin was applied. We failed to detect significant differences among treatments likely due to high variability among replicates. For the purposes of these experiments, we utilized family as a replicate which may have contributed to observed variability.

Survival, measured as proportional hatch success, was similar across treatments, compared to other studies. For example, Rach et al. (1998) showed that hatch success between

peroxide groups compared to controls were 57% and 40%, respectively and in an additional study using formalin (Rach et al. 1997), treated eggs exhibited a 54% hatch rate versus 42% in the control. However, in this study we failed to detect significant differences between treatments likely due to the variability in replicates. For the purposes of these experiments, we utilized family as a replicate which may have contributed to observed variability. Additional studies are needed to account for variability that may be associated with family (genetic effects).

Oxygen consumption rate. – In this study, oxygen consumption rate was measured in attempt to identify physiological stress associated with chemotherapeutants use. Results from this study revealed that oxygen consumption rate did not vary significantly among treatment. Chemotherapeutants are commonly administered during egg development to treat or prevent microbial colonization of the egg surface which may contribute to egg asphyxiation (Barker 1989). However, we did not observe differences in oxygen consumption rate of controls compared to groups treated with either formalin or peroxide.

Higher oxygen consumption rates at the second development period relative to the first are consistent with embryonic stage of development. In this study, no effect of treatment was observed for oxygen consumption rates at either developmental period. Results suggest that 1) eggs are not affected (or stressed) by chemotherapeutant treatment and therefore do not alter respiration rates, 2) the species composition of microbial communities and the abundance of microbes present did not negatively affect egg oxygen consumption rates, or 3) experimental manipulations of eggs were equally stressful and contributed to undetected differences due to chemotherapeutant across all treatments. Furthermore it could be that the timing of oxygen consumption trials, relative to when chemotherapeutants were administered provided ample time for eggs to normalize post-treatment. In this study, oxygen consumption rates were administered

several hours after application of chemotherapeutants. To date, no known study has documented oxygen consumption rates for lake sturgeon eggs. Oxygen consumption data summaries provided herein are beneficial to future studies investigating oxygen consumption of developing embryos and two critical development periods.

Impacts of chemotherapeutants on egg microbial diversity

There was no effect of chemotherapeutants on the alpha diversity (species richness) within treatment groups at two developmental periods sampled during embryogenesis. However, there are notable differences at developmental period 1 with regard to microbial beta-diversity that deserves attention. At development period 1 for example, dissimilarity indices suggest that peroxide and formalin treated groups select for dissimilar microbial communities. Phylotypes present in formalin and peroxide groups were dissimilar compared the controls, especially at developmental period 1, and the increase in abundance of pathogens (i.e., Flavobacteriaceae; Barnes et al. 2009, Loch et al. 2013) among these two chemotherapeutants compared to the controls is a concern.

Conclusions

Relative to parameters measured in this study (egg survival, egg oxygen consumption rate, microbial community composition, and body size at hatch), benefits of treating lake sturgeon eggs using peroxide or formalin were not apparent. However, using the novel genomic interrogation of egg microbe communities it was demonstrated that beta diversity on the egg surfaces are altered as a function of chemotherapeutant treatment which is a concern. The downstream effects of these treatments and those altered microbial community structures on growth and survival at subsequent life periods is important, warranting additional studies.

LITERATURE CITED

Balon, E. K. 1975. Reproductive guild of fishes: A proposal and definition. Journal of Fisheries Research Board of Canada 32(6):821-864.

Barker, G. A., S. N. Smith, and N. R. Bromage. 1989. The bacterial flora of Rainbow Trout, *Salmo gairdneri* Richardson, and Brown Trout, *Salmo trutta* L., eggs and its relationship to developmental success.

Barnes, M. E., D. Bergmann, H. Stephenson, M. Gabel, and R. J. Cordes. 2005. Bacterial numbers from landlocked fall Chinook Salmon eyed eggs subject to various formalin treatments as determined by scanning electron microscopy and bacteriological culture methods. North American Journal of Aquaculture 67(1):23-33.

Barnes, M. E., D. Bergmann, J. Jacobs, and M. Gabel. 2009. Effect of *Flavobacterium columnare* inoculation, antibiotic treatments and resident bacteria on rainbow trout *Oncorynchus mykiss* eyed egg survival and external membrane structure. Journal of Fish Biology 74:576-590.

Blaxter, J. H. S., and G. Hempel. 1963. The influence of egg size on Herring larvae (*Clupea harengus* L.). J. Const. Int. Explor. Mer. 28:211-240.

Boucher, M. A. 2012. The effect of substrate on the growth, development, and survival of larval white sturgeon (Acipenser transmontanus) during early ontogeny. University of Northern British Columbia Master's Thesis.

Bowker, J., M. Gaikowski, A. Goodman, L. Sprague, and M. Bowman. 2011. Guide to using drugs, biologics, and other chemicals in aquaculture. U. S. Fish and Wildlife Service Aquatic Animal Drug Approval Partnership Program http://www.fws.gov/fisheries/aadap/home.htm 1-62.

Bray, J. R., and J. T. Curtis. 1957. An ordination of the upland forest communities southern Wisconsin. Ecological Monographs 27:325:349.

Bronzi, P., H. Rosenthal, G. Arlati, and P. Williot. 1999. A brief overview on the status and prospects of sturgeon farming in Western and Central Europe. Journal of Applied Ichthyology 15:224-227.

Cherr, G. N., and W. H. Clark. 1982. Fine structures of the envelope and micropyles in the eggs of the White Sturgeon, *Acipenser transmontanus* Richardson. Developmental Growth and Differentiation 24(4):341-352.

Cherr, G. N., and W. H. Clark. 1984. Jelly release in the eggs of the White Sturgeon, Acipenser transmontanus: An enzymatically mediated event. The Journal of Experimental Zoology 230:145-149.

Crossman, J. A., P. S. Forsythe, K. T. Scribner, and E. A. Baker. 2011. Hatchery rearing environment and age affect survival and movements of stocked juvenile lake sturgeon. Fisheries Management Ecology 18:132-144.

Detlaff, T. A., A. S. Ginsburg, O. I. Schmalhausen, G. G., Gause, and S. G. Vassetzky. 1993. Sturgeon fishes: Developmental biology and aquaculture. Springer-Verlag 1st Edition.

Forsythe, R. S., K. T. Scribner, J. A. Crossman, A. Ragavendran, and E. A. Baker. 2013. Experimental assessment of the magnitude and source of Lake Sturgeon egg mortality. Transactions of the American Fisheries Society 142:1005-1011.

Fujimoto, M., J. A. Crossman, K. T. Scribner, and T. A. Marsh. 2013. Microbial community assembly and succession on Lake Sturgeon egg surfaces as a function of simulated spawning stream flow rate. Microbial Ecology 66:500-511.

Gisbert, E., P. Williot, and F. Castello-Orvay. Influence of egg size on growth and survival of early stages of Siberian sturgeon (Acipenser baeri) under small scale hatchery conditions. Aquaculture 183:83-94.

Hansen, G. H., and J. A. Olafsen. 1989. Bacterial colonization of Cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus*) eggs in marine aquaculture. Applied and Environmental Microbiology 55(6):1435-1446.

Hansen, G. H., O. Bergh, J. Michaelsen, and D. Knappskog. 1992. *Flexibacter ovolyticus* sp. Nov., a pathogen of eggs and larvae of Atlantic Halibut, *Hippoglossus hippoglossus* L. International Journal of Systematic Bacteriology 42(3):451-458.

Hansen, G. H., and J. A. Olafsen. 1999. Bacterial interactions in early life stages of marine cold water fish. Microbial Ecology 38:1-26.

Holtgren, J. M., S. A. Ogren, A. J. Paquet, and S. Fajfer. 2007. Design of a portable streamside rearing facility for Lake Sturgeon. North American Journal of Aquaculture 69:317-323.

Kempinger, J. J. 1988. Spawning ad early life history of lake sturgeon in the Lake Winnebago system, Wisconsin. American Fisheries Society Symposium 5:110-122.

Memis, D., E. Ercan, M. S. Celikkale, M. Timur, and Z. Zarkua. 2009. Growth and survival rate of Russian Sturgeon (*Acipenser gueldenstaedtii*) larvae from fertilized eggs to artificial feeding. Turkish Journal of Fisheries and Aquatic Sciences 9:47-52.

Meyer, F. P. 1991. Aquaculture disease and health management. Journal of Animal Science 69:4201-4208.

Naciri, M., D. Kuystermans, and M. Al-Rubeai. 2008. Monitoring pH and dissolved oxygen in mammalian cell culture using optical sensors. Cytotechnology 57:245-250.

Nelson, M. C., H. G. Morrison, J. Benjamino, S. L. Grim, ad J. Graf. 2014. Analysis, optimization and verification of illumina-generated 16S rRNA gene amplicon surveys. PLOS ONE 9(4):1-14.

Rach, J. J., M. P. Gaikowski, G. E. Howe, and T. M. Schreier. 1998. Evaluation of the toxicity and efficacy of hydrogen peroxide treatments on eggs of warm- and coolwater fishes. Aquaculture 165:11-25.

Rach, J. J., G. E. Howe, and T. M. Schreier. 1997. Safety of formalin treatments on warm- and coolwater fish eggs. Aquaculture 149:183-191.

Schloss P. D., D. Gevers, and S. L. Westcott. 2014. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PloS ONE. 6:e27310.

Shannon, C. E. 1948. A mathematical theory of communication. Reprinted for the Bell System Technical Journal with corrections 5(1):3-55.

Sharrer, M. J., S. T. Summerfelt, G. L. Bullock, L. E. Gleason, and J. Taeuber. 2005. Inactivation of bacteria using ultraviolet irradiation in a recirculating salmonid culture system. Aquacultural Engineering 33:135-149.

Simpson, E. H. 1949. Measurement of diversity. Nature 163:688-689.

Stephenson, H., M. Gabel, and M. E. Barnes. 2003. Microbial inhibition in response to treatments of hydrogen peroxide and formalin on landlocked fall Chinook Salmon eyed eggs, as determined by scanning electron microscopy. North American Journal of Aquaculture 65(4):324-329.

Strecher, R., T. B. Seiler, H. Hollert, T. Braunbeck. 2011. Oxygen requirements of zebrafish (*Danio rerio*) embryos in toxicity tests with environmental samples. Comparative Biochemistry and Physiology 318-327.

Van Den Berg, A. H., D. McLaggan, J. Dieguez-Uribeondo, and P. Van West. 2013. The impact of the water moulds *Saprolignia diclina* and *Saprolegnia parasitica* on natural ecosystems and the aquaculture industry. Fungal Biology Reviews 27:33-42.

Wagner, E. J., R. W. Oplinger, and M. Bartley. 2012. Laboratory production scale disinfection of salmonid eggs with hydrogen peroxide. North American Journal of Aquaculture 74(1):92-99.

Warkentin, M., H. M. Freese, U. Karsten, and R. Schumann. 2007. New and fast method to quantify respiration rates of bacterial and plankton communities in freshwater ecosystems by using optical oxygen sensor spots. Applied Environmental Microbiology 73(21):6722-6729.

IUCN, 2010. http://www.iucnredlist.org/details/223/0 (January 2015).

Appendix #5 - Effects of rearing density on body size and survival of lake sturgeon free-embryos

John M. Bauman[a], Edward A. Baker[b], Terry L. Marsh[c] and Kim T. Scribner[a,d]

[a]Department of Fisheries and Wildlife, Michigan State University, 13 Natural Resources Building, East Lansing, MI 48824, USA.

[b]Michigan Department of Natural Resources Fisheries Division, 484 Cherry Creek Road, Marquette, MI 49855, USA.

[c]Department of Microbial and Molecular Genetics, Michigan State University 6169 Biomedical Physical Sciences, East Lansing, MI 48824, USA.

[d]Department of Zoology, Michigan State University 288 Farm Lane, East Lansing, MI 48824, USA.

ABSTRACT

Experiments were conducted to identify aquaculture conditions that improve growth and survival. We quantified the effects of rearing density, family, and dissolved oxygen concentration on free-embryo Lake Sturgeon (*Acipenser fulvescens*) body size and survival to the time of emergence. Experiments were conducted using free-embryos from two full-sibling families that were reared in four densities in 3.0 L aquaria. A significant density and family effect on free-embryo body size at emergence was documented. Mean (±SE) body size (mm) at emergence for rearing density of 9,688 (22.9 ±0.16) individuals per m^2 was significantly greater than mean body size of rearing densities of 19,375 (22.4 ±0.14) and 32,292 (21.8 ±0.17) individuals per m^2. Mean body size at emergence differed significantly between families (22.8

±0.13 mm versus 22.1 ±0.11 mm). Mean (±SE) dissolved oxygen concentration (mg/L) decreased significantly as a function of increasing fish density. Mean dissolved oxygen concentration from 3,229 individuals per m^2 treatment was significantly greater than those from the 19,375 (7.77 ±0.018) and 32,292 individuals per m^2 (7.71 ±0.035) treatments. Mean survival to emergence decreased as rearing density increased (0.976 ±0.008 versus 0.928 ±0.023; P = 0.97) from the 9,688 individuals per m^2 treatment to 32,292 individuals per m^2 treatment. There was no significant difference in survival between families. Results reveal that rearing density of free-embryos should be considered because of the effects on body size at emergence. These results are useful for development of standard operating procedures in traditional and conservation aquaculture facilities where free-embryos are raised.

INTRODUCTION

Survival and growth of free-embryo, and through subsequent ontogenetic life periods is important to commercial as well as conservation aquaculture production programs. Rearing conditions experienced at the free-embryo stage affects body size of larvae and is a significant predictor of the likelihood of first-feeding and of mortality linked to starvation (Cushing 1972, Heming and Buddington 1988, Hardy and Litvak 2002). However, there are few studies that identify aquaculture conditions that improve free-embryo body size at emergence and initiation of exogenous feeding, and results vary among taxa (Heming and Buddington 1988, Kamler 2008).

During the free-embryo stage, fish rely entirely on endogenous yolk-sac reserves for energy and growth development (Heming and Buddington 1988, Kamler 2008). The rate or efficiency at which yolk-reserves are utilized for growth is dependent upon abiotic (i.e., dissolved oxygen, light, temperature) and biotic factors (i.e., maternal provisioning) (Heming and Buddington 1988, Kamler 2008). These factors dictate, how yolk reserves are allocated with respect to either body tissue development or for competing anaerobic processes such as those experienced during respiration in response to stress (Kamler 2008). If free-embryos are reared under stressful conditions, there can be profound direct (mortality) and indirect (lower growth) effects (Bates 2014, Boucher 2014). For example, stressful rearing conditions, such as those experienced by negatively phototactic free-embryo in the absence of refugia, increases the rate at which yolk-reserves are devoted to respiration (for locomotion) rather than to somatic development (Hansen and Moller 1985, Finn et al. 1995, Bates 2014, Boucher 2014). Additionally, biotic factors such as maternal provisioning (i.e., egg size) are documented to have significant direct and indirect effects on free-embryos (Gisbert et al. 2000, Kamler 2008, Regnier

et al. 2012). In general, environmental conditions experienced during early life stages can also affect traits during later ontogenetic stages (ontogenetic contingency; Diggle 1994), and thus performance in aquaculture settings. Understanding the direct and indirect effects of environmental conditions on yolk-reserve allocation, and thus body size, helps define aquaculture practices that reduce stressful conditions and improve growth (e.g., as estimated by body size at time of emergence).

Sturgeons, given their high market value and worldwide conservation status, are an important species group (IUCN 2010, Bronzi et al. 1999, Holtgren et al. 2007) in aquaculture. Recent research conducted with White (*Acipenser transmontanus*) and Lake (*Acipenser fulvescens*) sturgeons has improved our understanding of species-specific behaviors (Hastings et al. 2013, Boucher et al. 2014) which in turn has informed research needs for best aquaculture practices. For example, Boucher et al. (2014) showed that providing cover to free-embryo rearing tanks improved body size and survival at emergence, as well as survival to subsequent life periods. However, other attributes of the rearing environment such as rearing density and dissolved oxygen concentration may also be important when rearing free-embryo sturgeons (Ceskleba 1985), yet data are lacking.

Our primary objective was to evaluate the effects of rearing conditions on free-embryo body size and survival at emergence. Specifically, we quantified the effects of rearing density and family, as well as dissolved oxygen concentration, on free-embryo lake sturgeon body size and survival at emergence. Our hypothesis was that body size and survival would decrease as a function of increasing rearing density from the time of hatch to emergence.

METHODS

Study site

Use of conservation streamside rearing facilities, such as the Black River Streamside Rearing Facility (BR-SRF) have been widely advocated in the Great Lakes basin as the preferred method for culturing lake sturgeon in situations, where restoration goals to repatriate or enhance populations can be met by stocking (Holtgren et al. 2007). The BR-SRF is supplied with ambient river water (~680 L/min) from the Kleber Reservoir, located near primary spawning areas for lake sturgeon in the upper Black River in Cheboygan County, Michigan. This study was conducted in June 2013 and the BR-SRF water temperature ranged from 17.7 to 20.5°C with a mean of 19.06°C.

Fertilization and incubation

Gametes were collected from two male and two female lake sturgeon spawning in the upper Black River following procedures described by Crossman et al. (2011). Gametes were transported to the BR- SRF for fertilization, which took place within four hours of collection. Prior to fertilization, we subsampled 20 eggs from each female and preserved them separately in 90% ethanol to measure egg size at a later date. Approximately 200 mL of eggs per female were placed into separate dry bowls. Milt samples from a separate male per female were activated using a 1:200 dilution of ambient river water and immediately poured over the eggs allowing 90 seconds for fertilization. Excess milt was then removed and eggs were rinsed once with ambient river water. Egg de-adhesion procedures began by applying a Fuller's Earth solution (Sigma Aldrich) and gently mixing for 50 min. After 50 min, Fuller's Earth was rinsed from the eggs and a 15 min, 50 ppm Iodophor disinfection treatment was administered. Following a 10 min rinse to remove residual Iodophor using ambient river water, eggs were transferred to Aquatic

Eco-Systems (Pentair) J32 Mini Egg-hatching jars for incubation. Beginning two days post fertilization; eggs were treated daily using a 500 ppm, 15 min bath treatment of hydrogen peroxide until 24 hours prior to hatch. Preserved eggs (n=20 per female) were photographed with a ruler for scale and egg diameter was measured at the greatest linear distance (mm ±SE) using Image J software (v.1.43u).

Experimental treatments

Density experiments were conducted using free-embryos from two full-sibling families in four different rearing densities (n=50, n=150, n=300, n=500 offspring per 3.0 L tank; 3,229, 9,688, 19,375, and 32,292 individuals per m^2). Densities are representative of currently utilized protocols in sturgeon production facilities (DiLauro et al. 1998, Deng et al. 2003, Boucher et al. 2014, and Wisconsin DNR unpublished data). Rearing density was based on total rearing area of the tank bottom. We kept families separate for the duration of this experiment to account for differences in body size or survival associated with family. At hatch, free-embryos were randomly placed into tanks by family until four replicates of each tank density level were filled (32 tanks in total). During the free-embryo period, lake sturgeon seek refuge in substrate (Hastings et al. 2013). Therefore, we utilized 3.0 L polycarbonate tanks (Aquatic Habitats) filled with a single layer (n=24) of 2.54 cm^3 sinking Bio-Balls (Pentair #CBB1-S) covering the tank bottom. Tank flow was set at a rate of 57 L/hr (~20 changes per hr) and checked daily. We used a 12 hr light and 12 hr dark environment. Mortality was recorded at the start of each rearing day to quantify survival to emergence. Dissolved oxygen (mg/L) was recorded (YSI ProODO Optical DO/Temp Meter) multiple times daily in each tank and reported as mean dissolved oxygen concentration. At emergence, we used a digital camera to photograph and

measure a random subsample (n=30) of fish from each treatment, each family and each replicate for body size (total length (TL) mm) using Image J software (v.1.43u).

Statistical analysis

We tested for differences in mean (±SE) egg size, mean body size at emergence, mean dissolved oxygen concentration, and mean proportional survival as a function of fixed effects including rearing density and family, using SAS (SAS Institute version 9.3 Cary, NC). Tank was the experimental unit for all response variables used in the analysis. Normality assumptions were assessed using the Shapiro-Wilk Test. Homogeneity of variance assumptions were assessed using the Levene's Test. A p value < 0.05 was considered statistically significant for all analyses.

Comparison of mean egg size data between families were conducted using a two-sample t-test. A general linear model using analysis of variance was used to analyze body size at emergence and mean dissolved oxygen concentration. A generalized linear model using a logit link function and analysis of variance was used to analyze survival data. Survival to emergence data were modeled as a binomially distributed variable and reported as the proportion of survival. We used Tukey-Kramer multiple pair-wise comparison tests for all response variables.

RESULTS

Effects of rearing density and family on body size at emergence

Body size at emergence decreased as a function of increasing rearing density. We documented a significant density ($F_{3, 25} = 31.61$, $P < 0.0001$) and family ($F_{1, 25} = 82.36$, $P < 0.0001$) effect on free-embryo body size at emergence. Multiple pair-wise comparison tests revealed no significant difference in mean body size at emergence for rearing densities of 50 (TL = 22.7 ±0.21) versus 150 (TL = 22.9 ±0.16) fish per tank ($t_{25} = 0.91$, $P = 0.7988$) (Table 9). However, multiple pair-wise comparison tests revealed that mean body size at emergence for rearing density of 50 fish per tank was significantly greater than mean body size of rearing densities 300 (TL = 22.4 ±0.14) and 500 (TL = 21.8 ±0.17) fish per tank ($t_{25} = -3.10$, $P = 0.0228$ and $t_{25} = 7.77$, $P < 0.0001$, respectively) (Table 9). In addition, mean body size at emergence for rearing density of 150 (TL = 22.9 ±0.16) fish per tank was significantly greater than mean body size of rearing densities of 300 (TL = 22.4 ±0.14) and 500 (TL = 21.8 ±0.17) fish per tank ($t_{25} = 4.16$, $P = 0.0017$ and $t_{25} = 8.96$, $P < 0.0001$, respectively) (Table 9). Multiple pair-wise comparison tests also revealed that mean body size at emergence for rearing density of 300 (TL = 22.4 ±0.14) fish per tank was significantly greater than rearing density of 500 (TL = 21.8 ±0.17) fish per tank ($t_{25} = 4.95$, $P = 0.0002$) (Table 9). Multiple pair-wise comparison tests revealed that mean body size at emergence for Family 2 (TL = 22.8 ±0.13) was significantly greater than Family 1 (TL = 22.1 ±0.11) ($t_{25} = -9.08$, $P < 0.0001$). Mean egg diameter measured from Female 2 (3.06 ±0.025) was significantly larger compared to mean egg diameter from Female 1(2.78 ±0.028) ($t_{19} = -7.57$, $P < 0.0001$).

Table 9. Lake sturgeon mean (±SE) body size (mm) differs at emergence as a function of rearing density (individuals per tank) and between families. Fixed effects with identical lowercase letters are not significantly different (Tukey-Kramer, $P < 0.05$).

Rearing Density	Family 1	Family 2	Overall
50	22.3 ±0.07	23.3 ±0.04	22.7 ±0.21 v
150	22.5 ±0.14	23.2 ±0.05	22.9 ±0.16 v
300	22.0 ±0.11	22.7 ±0.06	22.4 ±0.14 w
500	21.5 ±0.21	22.1 ±0.16	21.8 ±0.17 x
Overall	22.1 ±0.11 y	22.8 ±0.13 z	22.4 ±0.11

Effects of rearing density and family on dissolved oxygen concentration

Mean dissolved oxygen concentration decreased as a function of increasing fish density. We documented a significant density effect on dissolved oxygen concentration ($F_{3, 10.34} = 22.62$, $P < 0.0001$). However, the effects of family were not significant ($F_{1, 7.67} = 2.03$, $P = 0.1934$). Multiple comparison tests revealed no significant difference in mean dissolved oxygen concentration between 50 fish per tank (7.89 ±0.004) and 150 fish per tank (7.86 ±0.009) density treatment levels ($t_{8.39} = -2.75$, $P = 0.0802$) (Table 10). However, mean dissolved oxygen concentration from 50 fish per tank treatment levels were statistically greater than those from the 300 fish per tank (7.77 ±0.018) and 500 fish per tank (7.71 ±0.035) treatment levels ($t_{7.32} = -6.91$, $P = 0.0002$; $t_{6.09} = 5.20$, $P = 0.0019$, respectively) (Table 10). Additional mean comparison tests determined mean dissolved oxygen concentrations from 150 fish per tank treatment levels were statistically greater than those from the 300 fish per tank and 500 fish per tank treatment levels ($t_{11.2} = 4.55$, $P = 0.0045$; $t_{6.96} = 4.20$, $P = 0.0078$, respectively) (Table 10). Mean dissolved oxygen concentration in the 300 fish per tank density treatment level was not significantly different from the 500 fish per tank density treatment level ($t_{8.47} = 1.62$, $P = 0.4101$) (Table 10).

Table 10. Mean (±SE) dissolved oxygen concentration (mg/L) as a function of rearing density (individuals per tank) and family. Fixed effects (density and family) with identical lowercase letters are not significantly different (Tukey-Kramer, $P < 0.05$).

Rearing Density	Family 1	Family 2	Overall
50	7.89 ±0.005	7.88 ±0.007	7.89 ±0.004 y
150	7.85 ±0.003	7.87 ±0.018	7.86 ±0.009 y
300	7.81 ±0.011	7.73 ±0.018	7.77 ±0.018 z
500	7.79 ±0.031	7.65 ±0.034	7.71 ±0.035 z
Overall	7.84 ±0.012	7.78 ±0.027	7.81 ±0.016

Effects of rearing density and family on survival to emergence

Mean survival to emergence decreased as rearing density increased from n=150 to n=500 fish per tank (0.976 ±0.008 versus 0.928 ±0.023), respectively, however differences were not statistically significant ($F_{3, 25} = 0.08$, $P = 0.9721$) (Table 11). There was also no significant difference in survival between families ($F_{1, 25} = 0.01$, $P = 0.9274$) (Table 11).

Table 11. Mean (±SE) proportional survival to emergence as a function of rearing density (individuals per tank) and family. Fixed effects with identical lowercase letters are not significantly different (Tukey-Kramer, $P < 0.05$).

Rearing Density	Family 1	Family 2	Overall
50	0.995 ±0.005	0.940 ±0.020	0.971±0.014 z
150	0.972 ±0.010	0.980 ±0.013	0.976±0.008 z
300	0.946 ±0.025	0.961 ±0.003	0.954±0.012 z
500	0.929 ±0.055	0.928 ±0.019	0.928±0.023 z
Overall	0.963±0.013	0.953±0.009	0.958±0.008

DISCUSSION

Investigations focused on lake sturgeon during the ontogenetic period immediately after hatch, extends previous research for a life period for which there is little information pertaining to body size and survival. We documented significant effects of rearing density and also family on the body size of free-embryos at emergence, and demonstrated a corresponding, potentially related effect associated with dissolved oxygen concentration. We have also extended previous literature (Gisbert et al. 2000, Regnier et al. 2012, and others) that suggested maternal provisioning (i.e., egg size) has indirect effects on body size.

Effects of density on body size, dissolved oxygen concentration, and survival

Body size. – Mean body size decreased significantly as a function of increasing rearing density. Body size of fish raised in densities of 150 fish per tank (or 9,688 individuals per m^2) or less was significantly larger (+0.5 to 1.1 mm) at emergence compared to other groups with greater densities, indicating that density should be considered when rearing free-embryo. Some facilities raise free-embryo sturgeon at densities which exceed 21,000 per m^2 , which based on our data would result in a significant decrease in body size at emergence. Similar studies for demersal larval-species such as *Clarius batrachus* and *Clarius gariepinus* have also shown that increasing density results in a decrease in body size (Sahoo et al. 2004, Hossain et al. 1998). These studies attributed decreased growth to increased competition for or reduced availability of food (Irwin et al. 1999, Sahoo et al. 2004). However, during the free-embryo period fish are not feeding exogenously which directs attention to other potential stress mechanisms such as the availability of dissolved oxygen.

Dissolved oxygen. – Dissolved oxygen concentrations were significantly lower in higher rearing density treatments, which may explain difference in mean body size at emergence. Lower levels

of dissolved oxygen can reduce the rate at which yolk-reserves are utilized for tissue development instead of competing anaerobic metabolic processes (Kamler 2008). In our study, free-embryo reared at higher densities may have devoted more yolk-reserves to respiration in lower oxygen conditions rather than to developing tissue for body size. Similar studies have shown that lower dissolved oxygen concentrations experienced during the embryo period indirectly affect body size (Pichavant et al. 2001, Beuntello et al. 2000, Kamler 2008). At the end of our study, the lowest recorded mean dissolved oxygen concentration was 7.60 mg/L which is not characteristic of hypoxic rearing conditions. However, the lowest oxygen concentrations recorded in this study may have been stressful, potentially causing negative indirect effects on body size at emergence.

Survival. – Mean survival decreased as rearing density increased from 150 fish per tank to 500 fish per tank; however treatment differences were not significant. Survival to emergence of free-embryo was high in all tanks (mean 96%) and was comparable to other sturgeons studies (Gisbert et al. 2000, Boucher 2014). Our results suggest that although indirect effects on body size were evident, no direct effects on survival to emergence were associated with rearing free-embryos in high densities. Similar to results have been reported for other fish species (Hossain et al. 1998). However, reduced body size at emergence, as well as other associated stresses resulting from high density conditions, may lead to lower survival during subsequent ontogenetic life periods (Bates 2014, Boucher 2014). We did not continue the study beyond the onset of exogenous feeding to validate this.

Effects of family on body size, dissolved oxygen, and survival.

Body size, dissolved oxygen, and survival. – Mean egg size and body size of free-embryo from family 2 were significantly larger than those from family 1. The difference in body size is likely due to initial egg size, which would suggest a maternal effect that has been documented before for sturgeons (Gisbert et al. 2000), as well as other fish species (Regnier et al. 2012). Our analysis was limited by the numbers of families represented (n=2). However, our results suggest that additional studies with a greater number of families are needed to assess the potential impacts of family on free-embryo body size. Aquaculture facilities that mix offspring from several families during early rearing may provide free-embryos born from females with larger eggs an advantage as larvae at emergence or initiation of exogenous feeding. Given that these larvae are larger, they may out-compete others in the hatchery environment during early life periods which could result in unequal family sizes at the end of the production cycle or stock-out.

Rearing density of free-embryos in traditional as well as conservation aquaculture facilities should be considered due to the indirect effects on body size. Larger body size at emergence or at initiation of exogenous feeding is documented to increase the likelihood of first-feeding at this critical period, reducing mortality linked to starvation (Cushing 1972, Yufera and Darias 2007). Based on our study which utilized lake sturgeon free-embryo and a total rearing area of 0.015 m^2, if free-embryo rearing density exceeds 9,688 individuals per m^2, body size at emergence will likely be significantly negatively affected. These results are useful for development of standard operating procedures in traditional and conservation aquaculture facilities where free-embryos are raised.

LITERATURE CITED

Bates, L. C., M. A. Boucher, and J. M. Shrimpton. 2014. Effect of temperature and substrate on whole body cortisol and size of larval white sturgeon (*Acipenser transmontanus* Richardson, 1836). Journal of Applied Ichthyology 1-5.

Beuntello, J. A., D. M. Gatlin III, W. H. Neill. 2000. Effects of water temperature and dissolved oxygen on daily feed consumption, feed utilization and growth of channel catfish (*Ictalurus punctatus*). Aquaculture 182:339-352.

Boucher, M. A., S. O. McAdam, and J. M. Shrimpton. 2014. The effect of temperature and substrate on the growth, development and survival of larval white sturgeon. Aquaculture 430:139-148.

Bronzi, P., H. Rosenthal, G. Arlati, and P. Williot. 1999. A brief overview on the status and prospects of sturgeon farming in Western and Central Europe. Journal of Applied Ichthyology 15:224-227.

Ceskleba, D. G., S. AveLallemant, and T. F. Thuemler. 1985. Artificial spawning and rearing of lake sturgeon, *Acipenser fulvescens*, in Wild Rose State Fish Hatchery, Wisconsin, 1982-1983. Environmental Biology of Fishes 14(1):79-85.

Crossman, J. A., P. S. Forsythe, K. T. Scribner, and E. A. Baker. 2011. Hatchery rearing environment and age affect survival and movements of stocked juvenile lake sturgeon. Fisheries Management and Ecology 18:132-144.

Cushing, D. H. 1972. The production cycle and numbers of marine fish. Symposium of the Zoological Society of London 29:213-232.

Deng, D., S. Koshio, S. Yokoyama, S. C. Bai, Q. Shao, Y. Cui, and S. S.O Hung. 2003. Effects of feeding rate on growth performance of white sturgeon (*Acipenser transmontanus*) larvae. Aquaculture 217(1-4):589-598.

Diggle, P. K. 1994. The expression of andromonoecy in *Solanum hirtum* (Solanaceae): phenotypic plasticity and ontogenetic contingency. American Journal of Botany 81(10):1354-1365.

DiLauro, M. N., W. F. Krise and K. Flynn-Aikins. 1998. Growth and survival of lake sturgeon larvae fed formulated diets. The Progressive Fish Culturist 60(4):293-296.

Finn, R. N., I. Ronnestad, and H. J. Fyhn. 1995. Respiration, nitrogen and energy metabolism of developing yolk-sac larvae of Atlantic halibut (*Hippoglossus hippoglossus* L.). Comparative Biochemistry and Physiology 111A(4):647:671.

Gisbert, E., P. Williot, and F. Castello-Orvay. 2000. Influence of egg size on growth and survival of early stages of Siberian sturgeon (*Acipenser baeri*) under small scale hatchery conditions. Aquaculture 183:83-94.

Hansen, T. J., and D. Moller. 1985. Yolk absorption, yolk sac constrictions, mortality, and growth during first feeding of Atlantic Salmon (*Salmo salar*) incubated on astro-turf. Canadian Journal of Fisheries and Aquatic Sciences 42:1073-1078.

Hardy, R. S., and M. K. Litvak. 2004. Effects of temperature on the early development, growth, and survival of shortnose sturgeon, *Acipenser brevirostrum*, and Atlantic sturgeon, *Acipenser oxyrhynchus*, yolk-sac larvae. Environmental Biology of Fishes 70:145-154.

Hastings, R. P., J. M. Bauman, E. A. Baker, K. T. Scribner. 2013. Post-hatch dispersal of lake sturgeon (*Acipenser fulvescens*, Rafinesque, 1817) yolk-sac larvae in relation to substrate in an artificial stream. Journal of Applied Ichthyology 29:1208-1213.

Heming, T. A., and R. K. Buddington. 1988. Yolk absorption in embryonic and larval fishes. Fish Physiology XIA:407-446.

Holtgren, J. M., S. A. Ogren, A. J. Paquet, and S. Fajfer. 2007. Design of a portable streamside rearing facility for lake sturgeon. North American Journal of Aquaculture 69(4):317-323.

Hossain, M. A. R., M. C. M. Beveridge, and S. Haylor. 1998. The effects of density, light and shelter on the growth and survival of African catfish (*Clarias gariepinus* Burchell, 1822) fingerlings. Aquaculture 160(3-4):251-258.

Irwin, S., J. O. Halloran, and R. D. FitzGerald. 1999. Stocking density, growth, and growth variation in juvenile turbot, *Scopthalmus maximus* (Rafinesque). Aquaculture 178:77-88.

Kamler, E. 2008. Resource allocation in yolk-feeding fish. Reviews in Fish Biology and Fisheries 18:143-200.

Pichavant, K., J. Person-Le-Ruyet, N. Le Bayon, A. Severe, A. Le Roux, and G. Boeuf. 2001. Comparative effects of long-term hypoxia on growth, feeding and oxygen consumption in juvenile turbot and European sea bass. Journal of Fish Biology 59:875-883.

Regnier, T., V. Bolliet, P. Gaudin, and J. Labonne. 2012. Female effects in offspring energetic status and consequences on early development in yolk feeding Brown Trout (*Salmo trutta*). Journal of Experiment Zoology 317A:347-358.

Sahoo, S. K., S. S. Giri, and A. K. Sahu. 2004. Effect of stocking density on growth and survival of *Clarius batrachus* (Linn.) larvae and fry during hatchery rearing. Journal of Applied Ichthyology 20:302-305.

Yufera, M., and M. J. Darias. 2007. The onset of exogenous feeding in marine fish larvae. Aquaculture 268:53-63.

IUCN, 2010. http://www.iucnredlist.org/details/223/0 (November 2014).

Appendix # 6 – Body size and survival of hatchery and wild-produced larvae as a function of feeding frequency and alternative food type

John M. Bauman[a], Edward A. Baker[b], Terry L. Marsh[c] and Kim T. Scribner[a,d]

[a]Department of Fisheries and Wildlife, Michigan State University, 13 Natural Resources Building, East Lansing, MI 48824, USA.

[b]Michigan Department of Natural Resources Fisheries Division, 484 Cherry Creek Road, Marquette, MI 49855, USA.

[c]Department of Microbial and Molecular Genetics, Michigan State University 6169 Biomedical Physical Sciences, East Lansing, MI 48824, USA.

[d]Department of Zoology, Michigan State University 288 Farm Lane, East Lansing, MI 48824, USA.

ABSTRACT

During the transition from endogenous to exogenous feeding and for several weeks post-exogenous feeding larval fishes typically exhibit periods of low growth and high mortality in aquaculture. Therefore, studies that evaluate the effects of different feeding strategies (e.g., feeding frequency, food type) for larval fish growth and survival are important to aquaculture productivity. In this study, the effects of commonly utilized feeding strategies on the body size and survival of lake sturgeon larvae during this critical early life period were quantified. Results from this study revealed significant differences in body size as a function of feeding frequency in hatchery-produced larvae to 30 days post-exogenous feeding. In addition, using hatchery-

produced as well as wild-caught larvae, significant differences in body size and survival as a function of alternate food types were documented. When hatchery-produced larvae were utilized, families were kept separate to quantify family effects in response to hatchery feeding strategies, which is lacking in the literature. Results from this study provide feeding strategy information that can be used to develop standard operating procedures for lake sturgeon, a species of conservation concern.

INTRODUCTION

Larval fishes commonly experience periods of reduced growth and high mortality in aquaculture settings. Ontogenetic periods during which growth and survival can be negatively affected include the period of transition from endogenous to exogenous feeding as well as several weeks following initiation of exogenous feeding (Kamler 1992). During these critical periods, nutrient deprivation or starvation can contribute to low growth and high mortality (Heming et al. 1982, Li and Mathias 1982, Theilacker 1986, Alves et al. 1999, Kamali et al. 2006). In aquaculture settings, if proper feeding strategies are not employed at the onset of exogenous feeding, larvae reach a "point of no return", whereby larvae either fail to feed exogenously or discontinue feeding, resulting in significant mortality (Blaxter and Hempel 1963, Kamler 1992). Therefore, studies that evaluate the effects of different feeding strategies (e.g., feeding frequency, food type) for larval fish growth and survival are important to aquaculture productivity.

Feeding frequency, the number of times fish are fed daily, has been found to significantly affect growth and survival of larval fish (Mollah and Tan 1982, Wang et al. 1998, Hwoan Cho et al. 2003). For example, Li et al. (2014) measured physiological stress-factors of Blunt Snout

Bream (*Megalobrama amblycephala*) and found those that fed at greater frequency exhibited greater immunity to disease and exhibited greater growth and survival. Similar results were observed by Tung and Shiau (1991) whereby higher feeding frequency resulted in greater growth and survival due to more efficient carbohydrate metabolism. Investigations of alternate food in aquaculture settings have also found food type to significantly affect growth and survival of larval fish as well as reduce annual production costs (Hamre et al. 2013). For example, Naess et al. (1995) determined that feeding live *Artemia* to Atlantic Halibut (*Hippoglossus hippoglossus*) during early periods of development resulted in greater growth and survival compared to those fed live-wild zooplankton. Improved growth and survival were attributed to greater assimilation efficiency or greater palatability. More notably, formulated commercial diets as alternative food types have been studied extensively in efforts to identify low-cost, nutrient-rich alternatives to live food (Hamre 2013). However, despite extensive research of alternate feeding strategies (e.g., feeding frequency and alternate food type) results are highly variable and warrant evaluation of species-specific feeding strategies, particularly for species of economic and conservation concern such as sturgeons (Rochard 1990, Auer 1996).

Sturgeons are highly marketable species, sought for their roe and flesh worldwide, yet according to the International Union for Conservation of Nature (IUCN), 85% of the world's sturgeons are in danger of extinction (IUCN, 2010). Several anthropogenic factors have contributed to low levels of abundance including over-fishing, poaching, habitat degradation, and interruption of migration routes utilized for reproduction (Rochard 1990, Auer 1996). As a result, aquaculture programs are in place to rebuild natural populations and recovery programs aim to repatriate or improve recruitment of remnant populations (Memis et al. 2009). However, low growth and high mortality during the larval period, attributed to inadequate feeding

strategies (Buddington and Christofferson 1988), serve as a bottleneck to successful aquaculture production efforts for sturgeons. Research on sturgeons in aquaculture settings has demonstrated that species-specific preferences for live food, the palatability or attractiveness of certain food types, and the use of live food in combination with artificial or formulated food, all offer benefits in the form of improved growth and survival during early life periods (Kappenman et al. 2011). However, species-specific data are lacking regarding optimal feeding strategies (Giberson and Litvak 2003), such as feeding frequency or beneficial alternate food types for larval lake sturgeon (*Acipenser fulvescens*).

Lake sturgeon have been cultured for over 100 years (Post 1890, Leach 1920), yet documentation of the optimal feeding frequency and food type, on growth and survival of larvae are limited. Alternate food types have been investigated in some capacity for larval lake sturgeon and live food outperformed other formulated food types (Anderson 1984, Ceskleba 1985, DiLauro et al. 1998). However, these studies were confounded because larvae were fed *Artemia* prior to the onset of experiments that introduced alternate food. Research has been conducted to improve juvenile growth and survival in aquaculture (Moreau and Dabrowski 1996); however, the larval period is when mortality is especially high (Harkness and Dymond 1961, Ceskleba 1985) and thus this ontogenetic period is deserving of further attention. High mortality of larval lake sturgeon negatively affects the cost-effectiveness of current aquaculture restoration programs, such as streamside rearing facilities (SRFs). SRFs, as opposed to traditional hatcheries, have been widely advocated in the Great Lakes basin as opposed to traditional hatcheries as the preferred method for culturing lake sturgeon in situations where restoration goals to repatriate or enhance populations can be met by stocking (Holtgren et al. 2007). However, recent experience has shown that targeted stocking goals (~1,000 fish per year,

per facility) are difficult to achieve when SRFs are used, due in large part to high mortality rates during the larval period.

Experiments have not been conducted to quantify and compare the effects of feeding frequency and alternate food types on growth and survival of larval lake sturgeon, neither have the effects of different sources of progeny for rearing been experimentally evaluated (Crossman et al 2014). Therefore, the objectives of this study were to: 1) quantify and compare the effects of feeding frequency on the body size and survival of hatchery-produced larval lake sturgeon and, 2) quantify and compare the effects of alternate food types on body size and survival of hatchery produced and wild captured larval lake sturgeon. Results from this study can be utilized in the development of standard operating procedures for larval lake sturgeon which currently experience high mortality in aquaculture settings.

METHODS

Study Site

This study was conducted in spring of 2013 and 2014, at the Black River SRF (BR-SRF) on the upper Black River, located in Cheboygan County, Michigan USA. The BR-SRF (316 m^2) provides conditions suitable for the evaluation of culture techniques utilized for sturgeon at multiple life periods because of the large facility size and accessibility to gametes and larvae. The BR-SRF is provided with 681 L/minute of ambient Black River water from Kleber Reservoir.

Feeding rate

To ensure larval lake sturgeon were fed consistently in all experiments, we utilized previously established dry-weight feeding rates for sturgeon (Deng et al. 2003), whereby larvae

in all tanks were fed 26% body weight daily (BWD), 26% BWD, 13% BWD, and 11% BWD during the first, second, third, and fourth weeks post-exogenous feeding, respectively. For all treatments that included *Artemia*, fish were fed live *Artemia* nauplii (Brine Shrimp Direct, Premium Grade 90% Hatch Rate: Great Salt Lake strain). *Artemia* were hatched overnight and harvested in the morning prior to the first feeding of the day. In addition, prior to the first feeding each day, reservoir detritus retained by serial filtration through 100 then 50 micron Bag Filter Vessels (Pentair Aquatic Eco-Systems) was used in three experimental food types (hereinafter referred to as "filtrate"). Similar to Agh et al. (2012), a wet- to dry-weight conversion was used to calculate feeding rates of *Artemia* and 50 micron filtrate material.

Experimental treatments

Collection, fertilization, and incubation of hatchery-produced larvae. –

The purpose of using hatchery-produced larvae for this study was to quantify and compare the effects of feeding strategies on body size and survival of a progeny source commonly utilized in traditional and streamside aquaculture. Gametes were collected from four male and four female lake sturgeon spawning in the upper Black River following procedures described by Crossman et al. (2011). Gametes were transported to the BR-SRF for fertilization, which took place within four hours of collection. Approximately 50 mL of eggs per female were placed into separate dry bowls. Milt from one male per female (full-sibling family) was activated using a 1:200 dilution of ambient river water and immediately poured over the eggs allowing 1.5 minutes (min) for fertilization. Excess milt was removed and eggs were rinsed once with ambient river water. Egg de-adhesion was completed by applying a Fuller's Earth solution to the fertilized eggs (Sigma Aldrich) and gently mixing for 50 min. After 50 min, Fuller's Earth was rinsed from the eggs and a 15 min, 50 ppm Iodophor disinfection was administered. Eggs

were rinsed for 10 min to remove residual Iodophor using ambient river water. Eggs were kept separate by family and incubated in Aquatic Eco-Systems (Pentair) J32 Mini Egg-hatching jars. Beginning two days post-fertilization; eggs were treated daily using a 15 min, 500 ppm bath treatment of hydrogen peroxide until 24 hours prior to hatch. At hatch, free-embryo lake sturgeon were raised until endogenous resources were absorbed and fish began a 'swim-up' behavior (approx. 7-10 days post-hatch). During the free-embryo period, lake sturgeon seek refuge in available substrate (Hastings et al. 2013). Therefore free-embryos were raised in 10 L polycarbonate tanks (Aquatic Habitats) with a single layer of 2.54 cm^3 sinking Bio-Balls (Pentair #CBB1-S) covering the tank bottom.

Feeding frequency with hatchery-produced larvae 2013. – The purpose of this experiment was to quantify the effects of feeding frequency and family on body size and survival of hatchery-produced larvae at 30 days post-exogenous feeding. At the onset of exogenous feeding, 50 larvae **w**ere placed into each of 32 (2 feeding frequencies and 2 families with 8 replicates) 3.0 L polycarbonate tanks (Aquatic Habitats). Each tank was then randomly assigned to one of two feeding frequency treatments with eight replicates. Feeing frequency treatments included: 1) fish fed 12 times daily and 2) fish fed 3 times daily. Feeding frequency treatments were designed to mimic current SRF feeding protocols of twelve or three times per day. Fish were acclimated in tanks for 24 hours prior to the start of the experiment. At 30 days post-exogenous feeding the body size (total length (mm)), and batch weight (g), were determined for all surviving fish. Mortality was recorded at the start of each rearing day to quantify survival.

Alternate food types with hatchery-produced larvae 2013. –The purpose of this experiment was to quantify the effects of alternate food type and family on body size and survival of hatchery-produced larvae at 30 days post-exogenous feeding. At the onset of

exogenous feeding, 25 larvae **w**ere placed into each of 24 (3 alternate food types and 2 families with 4) 3.0 L polycarbonate tanks (Aquatic Habitats). Each tank was then randomly assigned to one of three alternate food types, chosen to evaluate and compare alternate food types currently utilized in SRFs. Alternate food types utilized for this experiment included; 1) 100% recommended BWD of *Artemia*, 2) 50% recommend BWD of *Artemia* & 50% recommended BWD of Otohime B2 Larval Diet (particle size 0.36 to 0.60 mm), and 3) 100% recommended BWD of Otohime B2 Larval Diet. Otohime B2 Larval Diet hereinafter referred to as "Otohime". Surviving fish from each tank were batch weighed every other day to adjust food rates according to Deng et al. (2003). Mortality was recorded at the start of each rearing day to quantify survival. Significant mortality occurred in some treatments so this experiment was ended prematurely at 14 days post-exogenous feeding.

Collection of wild-produced larvae. –

Dispersing larvae, which offer greater genetic diversity than hatchery-produced larvae, are utilized in some SRFs for stocking (Holtgren et al. 2007; Duong et al. 2010; and Crossman et al. 2014). However, higher mortality among these wild-produced larvae compared to hatchery-produced larvae has been observed and may be associated with wild larvae imprinting to natural food in the river prior to capture for rearing (DiLauro et al. 1998, Crossman et al 2014). The purpose of using wild-caught larvae for this study was to quantify the effects of alternate food type on body size and survival of an additional progeny source utilized in sturgeon aquaculture. Therefore, wild dispersing-larvae were captured downstream of spawning areas using D-frame drift nets and transported to the BR-SRF for rearing. Detailed information regarding methodology and timing of drift capture can be found in Auer and Baker (2002) as well as Smith and King (2005).

Alternate food types with wild-caught larvae 2014. – The purpose of this experiment was to quantify the effect of alternate food types on body size and survival of wild-caught larvae at 30 days post-exogenous feeding. Twenty-four hours post-capture, 20 larvae were placed into each of 48 (6 alternate food types with 8 replicates) 3.0 L polycarbonate tanks (Aquatic Habitats). Each tank was randomly assigned to one of six food types designed to mimic those currently utilized in SRFs including a 50 micron filtrate material gathered from a nearby reservoir (meant to represent natural forage types in the river). Alternate food type for this experiment included; 1) 100% of recommended BWD of *Artemia*, 2) 50% of recommend BWD of *Artemia* & 50% of recommended BWD of Otohime B2 Larval Diet, 3) 100% of recommended BWD of Otohime B2 Larval Diet, 4) 90% of recommended BWD of Otohime B2 Larval Diet and 10% of recommended BWD of 50 micron reservoir filtrate, 5) 90% of recommended BWD of *Artemia* and 10% of recommended BWD of 50 micron reservoir filtrate, and 6) 45% of recommended BWD of *Artemia* 45% of recommended BWD of Otohime B2 Larval Diet and 10% of recommended BWD of 50 micron reservoir filtrate. Fish were acclimated to the tanks for 24-48 hours prior to the start of the experiment. Surviving fish from each tank were batch weighed every other day to adjust food rations according to Deng et al. (2003). Mortality was recorded at the start of each rearing day to quantify survival. Significant mortality occurred in some treatments so this experiment was ended at 14 days post-exogenous feeding.

Statistical analysis

All statistical analyses to quantify response variables were performed using SAS (SAS Institute version 9.3 Cary, NC). Summary statistics (mean and standard error) for all response variables were calculated and reported in the Results section. Tank was the experimental unit for all response variables used in the analysis. The assumption of normality was evaluated using

the Shapiro-Wilk Test. Analysis of variance (ANOVA) homogeneity of variance assumption was analyzed by Levene's Test. Body size measurements were analyzed using a general linear model. A generalized linear model was used for proportional survival data which were modeled using a beta-distribution and reported as proportional survival. A p value < 0.05 was considered statistically significant for all results.

RESULTS

Feeding frequency with hatchery produced larvae in 2013

The effects of feeding frequency and family including their interaction term were quantified at 30 days post-exogenous feeding using the following response variables; mean (±SE) total length, mean (±SE) weight per fish, and mean (±SE) proportional survival. Interaction terms for response variables, total length, weight per fish, and survival as a function of feeding frequency by family were insignificant ($F_{1, 28} = 0.11$, $P = 0.7371$; $F_{1, 28} = 0.00$ $P = 0.9829$; and $F_{1, 28} = 0.03$, $P = 0.8587$, respectively) and were removed from each model.

Total length at 30 days post-exogenous feeding. – A significant feeding frequency treatment effect ($F_{1, 29} = 4.87$, $P = 0.0354$) on total length of larvae at 30 days post-exogenous feeding was detected. However, there was no significant effect of family ($F_{1, 29} = 3.79$, $P = 0.0614$)**.** Mean total length was significantly greater in groups fed 3 times per day (46.83 ±0.33 mm) versus 12 times per day (45.73 ±0.40 mm) ($t_{29} = -2.21$, $P = 0.0354$) (Table 1). Larvae fed three times per day were 2.4% greater in total length at 30 days post-exogenous feeding than those fed twelve times per day.

Weight per fish at 30 days post-exogenous feeding. – A significant feeding frequency treatment effect ($\boldsymbol{F_{1, 29}} = 5.54$, $P = 0.0255$) on weight per fish at 30 days post-exogenous feeding

was detected however, no effect of family was detected ($\boldsymbol{F_{1, 29}} = 3.09$, $P = 0.0894$). **M**ean weight per fish was significantly greater in groups fed 3 times per day (0.41 ±0.01) versus 12 times per day (0.38 ±0.01) ($\boldsymbol{t_{29}} = -2.35$, $P = 0.0255$) (Table 12). Larvae fed three times per day were 6.5% greater in weight per fish at 30 days post-exogenous feeding than those fed twelve times per day.

Survival at 30 days post-exogenous feeding. – The effects of feeding frequency treatment and family on mean proportional survival at 30 days-post exogenous feeding were not significant ($\boldsymbol{F_{1, 29}} = 0.29$, $P = 0.5939$ and $\boldsymbol{F_{1, 29}} = 0.09$, $P = 0.7661$, respectively). Mean proportional survival was higher in groups fed 3 times per day (0.98 ±0.54) compared to those fed 12 times per day (0.95 ±2.80), however, differences were not statistically significant (Table 12). Mean proportional survival was higher in family 2 (0.97 ±0.83) compared to family 1 (0.95 ±2.79), however, differences were not statistically significant (Table 12).

Table 12. Hatchery produced larvae: mean total length (mm ±SE), weight per fish (g ±SE), and proportional survival (±SE) at 30 days post-exogenous feeding as a function of feeding frequency and family. Response variables within the same column followed by similar lowercase letters are not significantly different ($P < 0.05$).

Feeding Frequency	Total Length (mm)		Weight per Fish (g)		Proportional Survival	
	Family 1	Family 2	Family 1	Family 2	Family 1	Family 2
12 per day	45.16 ±0.61 a	46.30 ±0.46 a	0.37 ±0.02 a	0.40 ±0.01 a	0.93 ±0.06 a	0.98 ±0.01 a
3 per day	46.43 ±0.26 b	47.22 ±0.60 b	0.40 ±0.01 b	0.42 ±0.01 b	0.97 ±0.01 a	0.98 ±0.01 a

Alternate food types with hatchery-produced larvae 2013

The effects of alternate food type and family including their interaction term were quantified at 14 days post-exogenous feeding (due to high mortality in some treatments) using the following response variables; mean (±SE) weight per fish (g), and mean (±SE) proportional survival. Interaction terms for the models which included response variable weight per fish, as

well as the model for proportional survival were insignificant ($F_{2, 15}$ = 1.82 P = 0.1964 and $F_{2, 4}$ = 0.31 P = 0.7520, respectively) and were removed from each model.

Weight per fish at 14 days post-exogenous feeding. – The effect of alternate food type on weight per fish was significant among alternate food types ($F_{2, 17}$ = 489.68, P < 0.0001) and between different families ($F_{1, 17}$ = 15.97, P < 0.0009). Mean weight per fish was significantly greater in groups fed 100% *Artemia* (0.33 ±0.01), versus 50% *Artemia* & 50% Otohime (0.24 ±0.01) and 100% Otohime (0.06 ±0.01) (t_{17} = -12.30, P < 0.0001 and t_{17} = 31.26, P < 0.0001, respectively) (Table 13). Additionally, mean weight per fish in groups fed 50% *Artemia* & 50% Otohime was significantly greater than those fed 100% Otohime (t_{17} = 20.50, P < 0.0001). Mean weight per fish was significantly greater in family 2 (0.24 ±0.04) versus family 1 (0.22 ±0.03) (t_{17} = -4.00, P < 0.0001).

Survival at 14 days post-exogenous feeding. – The effect of alternate food types on mean proportional survival were significant at 14 days post-exogenous feeding ($F_{2, 6}$**,** P = 0.0002), however, not between different families ($F_{1, 6}$**,** P = 0.1261). Mean proportional survival between groups fed 100% *Artemia* (0.97 ±0.02) and groups fed 50% *Artemia* & 50% Otohime (0.99 ±.0.1) was not statistically significant **(**t_6 = 0.33, P = 0.9411) (Table 13). However, mean proportional survival was significantly greater in groups fed 100% *Artemia* and those fed 50% *Artemia* & 50% Otohime, when compared to those fed 100% Otohime (0.35 ±0.06) **(**t_6 = 7.90, P = 0.0005 and t_6 = 6.74, P = 0.0013, respectively) (Table 13).

Table 13. Hatchery produced larvae: mean weight per fish ((g) ±SE) and proportional survival (±SE) at 30 days post-exogenous feeding as a function of food type and family. Response variables within the same column followed by similar lowercase letters are not significantly different ($P < 0.05$).

	Weight per Fish (g)		Proportional Survival	
Food Type	Family 1	Family 2	Family 1	Family 2
100% *Artemia*	0.31 ±0.004 a	0.35 ±0.006 x	0.98 ±0.02 a	0.95 ±0.04 a
50% *Artemia* & 50% Otohime	0.22 ±0.011 b	0.25 ±0.005 y	0.99 ±0.01 a	0.98 ±0.02 a
100% Otohime	0.06 ±0.006 c	0.06 ±0.010 z	0.43 ±0.05 b	0.30 ±0.08 b

Alternate food types with wild-caught larvae 2014

The effects of alternate food type were quantified at 14 days post-exogenous feeding (due to high mortality in some treatments) using the following response variables; mean (±SE) weight per fish, and mean (±SE) proportional survival.

Weight per fish at 14 days post-exogenous feeding. – The effect of alternate food type on mean weight per fish was significant at 14 days post-exogenous feeding ($F_{5, 39} = 113.36$**, $P <$ 0.0001). M**ean weight per fish in groups fed 100% *Artemia* (0.41 ±0.02) and those fed 90% *Artemia* & 10% filtrate (0.37 ±0.01) were significantly greater than those fed 50% *Artemia* & 50% Otohime (0.29 ±0.02), 100% Otohime (0.03 ±0.01), 90% Otohime & 10% filtrate (0.02 ±0.002), and 45% *Artemia* & 45% Otohime & 10% filtrate (0.26 ±0.01), at 14 days post-exogenous feeding (Table 14).

Survival at 14 days post-exogenous feeding. – Survival varied significantly among alternate food types ($F_{5, 28} = 55.86$, $P < 0.0001$). Mean proportional survival of fish 100% *Artemia* (0.94 ±0.018), 50% *Artemia* & 50% Otohime (0.90 ±0.023), 90% *Artemia* & 10% filtrate (0.96 ±0.020), and 45% *Artemia* & 45% Otohime & 10% filtrate (0.94 ±0.031) was significantly greater than those fed 100% Otohome (0.13 ±0.052) and 90% Otohime & 10% filtrate (0.11

±0.032) at 14 days post-exogenous feeding (Table 14). Mean proportional survival was highest among alternate food types that included *Artemia* (0.94 ±0.018) compared to those that did not include (0.12 ±0.042) *Artemia*.

Table 14. Wild-produced larvae: mean weight per fish ((g) ±SE) and proportional (±SE) survival at 14 days post-exogenous feeding as a function of food type portioned by recommend %BWD. Response variables within the same column followed by similar lowercase letters are not significantly different ($P < 0.05$).

Food Type	Weight per Fish (g)	Proportional Survival
100% *Artemia*	0.41 ±0.024 a	0.94 ±0.018 a
50% *Artemia* & 50% Otohime	0.29 ±0.020 b	0.90 ±0.023 a
100% Otohime	0.03 ±0.006 c	0.13 ±0.052 b
90% Otohime & 10% filtrate	0.02 ±0.002 c	0.11 ±0.032 b
90% *Artemia* & 10% filtrate	0.37 ±0.014 a	0.96 ±0.020 a
45% *Artemia* & 45% Otohime & 10% filtrate	0.26 ±0.010 b	0.94 ±0.031 a

DISCUSSION

During the transition from endogenous to exogenous feeding and for several weeks post-exogenous feeding, larval fishes typically exhibit periods of low growth and high mortality in aquaculture. In this study, we quantified the effects of commonly utilized feeding strategies on the body size and survival of lake sturgeon larvae during this critical early life period. We identified significant differences in body size as a function of feeding frequency in hatchery-produced larvae to 30 days post-exogenous feeding. In addition, using hatchery-produced as well as wild-caught larvae, we identified significant differences in body size and survival as a function of alternate food types. When hatchery-produced larvae were utilized, families were kept separate to quantify family effects in response to hatchery feeding strategies, which is lacking in the literature. Results from this study provide feeding strategy information that can be

used to develop standard operating procedures for lake sturgeon, a species of conservation concern.

Feeding frequency with hatchery-produced larvae 2013

At the end of a thirty-day experiment, we quantified the effects of two commonly utilized feeding frequencies and family on the body size and survival of lake sturgeon larvae. Increasing feeding frequency has been documented to improve growth and survival of larvae for many fish species (Tung and Shiau 1991, Cho et al. 2003, Li et al. 2013 and others). However, in this study, mean body size (total length) of lake sturgeon larvae fed three times per day was significantly greater than those fed twelve times per day. Our results are similar to those reported for demersal species which found that fewer feedings either improved, or did not reduce growth (Petkam and Moodie 2001, Giberson and Litvak 2003).

Observed differences in body size (total length) in this study could be due to differences in size or energy content of *Artemia* as a function of feeding time (Sorgeloos et al. 2001). For example, fish fed three times per day received a third of the total daily ration soon after *Artemia* had been harvested, whereas fish fed 12 times per day did not receive a third of daily rations in total until four hours later. Sorgeloos et al. (2001) determined that *Artemia* nauplii kept alive may develop, after several hours, into a second larval stage which contains lower amounts of amino acids and is not as digestible compared to those sampled soon after *Artemia* hatching (Leger et al. 1987). In addition, the size of more developed nauplii, despite a reduced energy content, may be larger and exhibit greater swimming ability and evasion from predators (Sorgeloos et al. 2001), which may make feeding more difficult for larval sturgeon fed 12 times per day. We did not measure energy content or size of *Artemia* in this study. Therefore, additional studies using food types which do not lose energy content or change in size as a

function of time are needed to further support our results. Survival of larvae to 30 days post-exogenous feeding was not significantly different as a function of feeding frequency, however, it was higher than expected and higher compared to previous studies for sturgeon and will be discussed later.

After thirty days post-exogenous feeding we did not detect differences in larval body size or survival between families in this experiment. While acknowledging a small sample size, these results are surprising given the amount of research which associates body size of larvae during early and later ontogenetic life periods to genotypic origin (Nunez et al. 2011). It could be however, that genetic effects diminish as a function of age similar to early life periods of Chinook salmon (*Oncorhynchus tshawytscha*) (Heath et al. 1999). Similar aquaculture studies are needed with larger sample sizes of different families to quantify the effects of family on body size.

Alternate food types with hatchery-produced larvae 2013

At two weeks post-exogenous feeding we observed significant differences in body size (weight per fish) as a function of three alternate food types. Larvae fed live *Artemia* alone or in combination with formulated food types exhibited greater body size (weight per fish). These data are similar to other studies (Petkam and Moodie 2001, Agh et al. 2012, Poitroswska et al. 2013). Previous research suggested that sturgeons imprint to food provided at the onset of exogenous feeding (Buddington and Christofferson 1988, DiLauro et al. 1998), which warranted additional studies using alternate food types fed to hatchery-produced larvae not pre-exposed to other food types, namely *Artemia*. Our results add to previous research for hatchery-produced larvae that may have been confounded by having fed *Artemia* prior to initiating experimental food type studies (Anderson 1984, DiLauro et al. 1998). However, similar to DiLauro et al.

(1998), we did not observe hatchery-produced larvae feeding on formulated food types (Otohime B2 Larval Diet) which suggests that larval lake sturgeon may not imprint to or may not prefer formulated feed up to 14 days post-exogenous feeding. These findings are further supported by our results that show significantly smaller body size (weight per fish) of hatchery-produced larvae fed Otohime B2 Larval Diet. Conversely, Kappenman et al. (2010) reported promising results for Otohime during a study of hatchery-produced larval pallid sturgeon (*Scaphirhynchus. albus*) stating that larvae fed Otohime exhibited mean growth rates of 7.9% per day and survival rates from 54 to 72%. This could be due to the fact that Kappenman et al. (2010) mixed Otohime B2 Larval Diet in equal proportions with a smaller particle sized, more easily ingestible, Otohime B1 Larval Diet providing a range of food particle size from 0.20 to 0.62 mm. However, *Artemia* nauplii typically range in size from 0.43 to 0.52 mm (Leger et al. 1987) so a gape-limitation due to particle size of formulated foods such as Otohime B2 Larval Diet (0.36 to 0.62 mm) used in this study is unlikely.

Mean proportional survival of hatchery-produced larvae was higher when fed food types which contained *Artemia* versus those which contained only Otohime B2 Larval Diet, although differences were not statistically significant. Survival of hatchery-produced larvae fed food types containing *Artemia* were higher than expected and higher compared to previous studies.

Using hatchery-produced larvae, we documented significant differences in body size (weight per fish) at two weeks post-exogenous feeding between two different families reared separately during this experiment. Previous research has documented maternal and paternal effects on body size during early and subsequent ontogenetic life periods for fishes (Nunez et al. 2011). Differences in body size by family of sturgeon could be due to differences in maternal provisioning (i.e., egg size and hatched free-embryo size), as observed in Siberian sturgeon

(*Acipenser baeri*) (Gisbert et al. 2000). In this study, we did not measure egg size or size at hatch, and we only used two families for comparing potential family effects. Therefore, additional studies are needed to quantify and compare the effects of family on body size and survival of larvae.

Alternate food types with wild-produced larvae 2014

At two weeks post-exogenous feeding we observed significant differences in body size, measured as weight per fish, as a function of six alternate food types for wild-produced larvae. Larvae fed live *Artemia* alone or in combination with formulated food types and filtrate exhibited greater body size which is comparable to similar aquaculture studies (Petkam and Moodie 2001, Agh et al. 2012, Poitroswska et al. 2013). However, our research using wild-caught larvae extends upon previous research by providing results relative to a progeny source for which no alternate food type information existed previously.

Early rearing of wild-caught larvae has been difficult in aquaculture due to the inability of larvae to transition to *Artemia* near the onset of exogenous feeding (Holtgren et al. 2007, Crossman et al. 2014). It was suggested that wild larvae may have already begun to feed in the river and thus imprinted to natural food types (DiLauro 1998, Crossman et al. 2014). However, comparisons of body size suggest, and visual observations confirmed, that wild-produced larvae only fed on *Artemia* in this study. These results are similar for hatchery-produced larvae and similar to DiLauro et al. (1998), given that we did not observe wild-produced larvae feeding on formulated (Otohime B2 Larval Diet) food. In addition, we did not observe wild-produced larvae feeding on food that was derived from filtrate. Our findings that suggest lake sturgeon only fed on *Artemia*, are further supported by the results which show significantly smaller body

size as a function of decreasing proportions of *Artemia*. These data suggest that feeding rates, or the amount of food provided may be causing high mortality rates during larval periods.

Mean proportional survival of wild-produced larvae was significantly higher when fed food types which contained *Artemia* versus those which contained either only Otohime or a mixture of Otohime and filtrate. Survival of wild-produced larvae fed *Artemia* was higher than expected and higher compared to previous studies.

Survival of larvae in feeding frequency and alternate food type experiments

Survival of hatchery- and wild- produced lake sturgeon larvae fed *Artemia* in all experiments was high compared to that experienced in traditional (Anderson 1984, Ceskleba 1985, DiLauro et al. 1998, Crossman 2014) or in SRF (Holtgren et al. 2007, Crossman et al. 2014) aquaculture settings. When fed Artemia, larvae in all experiments in this study exhibited mean proportional survival rates near 0.96 (or 96%). We attribute high survival to the use of an *Artemia* wet- to dry-weight conversion and the use of feeding rates pre-determined for white sturgeon (Deng et al. 2003). Recommended feeding rates for lake sturgeon are not readily available in the literature warranting investigation of optimal feeding rates for lake sturgeon during the larval period.

As larvae begin exogenous feeding, several factors affect survival in hatchery environments including tank complexity (i.e., water volume, water flow), as well as aquaculture management practices (i.e., disease treatment, tank cleaning) (Conte et al. 1988, Crossman et al. 2014). In our study, we used 3.0 L polycarbonate aquaria that allowed us to utilize hatchery space efficiently while populating experimental treatment groups with multiple families and many replicates. At no time during any experiment were fish given any disease treatment or any

preventative prophylactic. Furthermore, tanks were cleaned once weekly which may have reduced handling stress compared to facilities which clean tanks daily.

Conclusions

In this study, we quantified the effects of commonly utilized feeding strategies on the body size and survival of larvae during the critical early life period. We identified significant differences in body size as a function of feeding frequency in hatchery produced larvae to 30 days post-exogenous feeding. Based on results from these experiments we recommend feeding lake sturgeon larvae *Artemia* three times per day to improve growth. Continued investigations seeking more economical food types which either maintain or improve growth without compromising survival are still needed. Furthermore, survival in traditional and SRF aquaculture settings is likely to improve if optimal feeding rates are established for larval lake sturgeon.

LITERATURE CITED

Agh, N., F. Noori, A. Irani, N. M. Makhdom. 2012. First feeding strategy for hatchery produced Beluga sturgeon, Huso huso larvae. Iranian Journal of Fisheries Sciences. 11(4):713-723.

Alves, D., J. L. Specker, and D. A. Bengston. 1999. Investigations into the causes of early larval mortality in cultured summer flounder (*Paralichthys dentatus* L.). Aquaculture. 176:155-172.

Anderson, E. R. 1984. Formulated propagation of lake sturgeon Acipenser fulvescens (Rafinesque), under hatchery conditions in Michigan, Michigan Department of Natural Resources, Fisheries Division, Fisheries Report 1898, Ann Arbor.

Auer, N. A. (1996). Importance of habitat and migration to sturgeons with emphasis on lake sturgeon. Canadian Journal of Fisheries and Aquatic Sciences, 53(S1), 152-160.

Auer, N. A., & Baker, E. A. (2002). Duration and drift of larval lake sturgeon in the Sturgeon River, Michigan. Journal of Applied Ichthyology, 18(4-6), 557-564.

Blaxter, J. H. S., and G. Hempel. 1963. The influence of egg size on Herring larvae (*Clupea harengus* L.). J. Const. Int. Explor. Mer. 28:211-240.

Buddington, R. K., & Christofferson, J. P. (1985). Digestive and feeding characteristics of the chondrosteans. Environmental Biology of Fishes, 14(1), 31-41.

Ceskleba, D. G., S. AveLallemant, and T. F. Thuemler. 1985. Artificial spawning and rearing of lake sturgeon, *Acipenser fulvescens*, in Wild Rose State Fish Hatchery, Wisconsin, 1982-1983.

Cho, S. H., Lim, Y. S., Lee, J. H., Lee, J. K., Park, S., & Lee, S. M. (2003). Effects of Feeding Rate and Feeding Frequency on Survival, Growth, and Body Composition of Ayu Post-Larvae *Plecoglossus altivelis*. Journal of the world aquaculture society, 34(1), 85-91.

Crossman, J. A., Forsythe, P. S., Scribner, K. T., & Baker, E. A. (2011). Hatchery rearing environment and age affect survival and movements of stocked juvenile lake sturgeon. Fisheries Management and Ecology, 18(2), 132-144.

Crossman, J. A., K. T. Scribner, C. A. Davis, P. S. Forsythe, and E. A. Baker. 2014. Survival and growth of lake sturgeon during early life stages as a function of rearing environment. Transaction of the American Fisheries Society. 143:104-116.

Conte, F. S. (Ed.). (1988). Hatchery Manual for the White Sturgeon (Acipenser Transmontanus Richardson): With Application to Other North American Acipenseridae (Vol. 3322). UCANR Publications.

DiLauro, M. N, W. F. Krise, and K. Fynn-Aikins. 1998. Growth and survival of lake sturgeon larvae fed formulated diets. The Progressive Fish Culturist. 60:293-296.

Deng, D., S. Koshio, S. Yokoyama, S. C. Bai, Q. Shao, Y. Cui, and S. S. O. Hung. 2003. Effects of feeding rate on growth performance of white sturgeon (*Acipenser transmontanus*) larvae. Aquaculture. 217:589-598.

Giberson, A. V., and M. K. Litvak. 2003. Effect of feeding on growth, food conversion efficiency, and meal size of juvenile Atlantic sturgeon and Shortnose sturgeon. North American Journal of Aquacutlure. 65:99-105.

Gisbert, E., P. Williot, and F. Castello-Orvay. 2000. Influence of egg size on growth and survival of early stages of Siberian sturgeon (Acipenser baeri) under small scale hatchery conditions. Aquaculture 183:83-94.

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L. E., & Izquierdo, M. (2013). Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. Reviews in Aquaculture, 5(s1), S26-S58.

Harkness, W. J. K., & Dymond, J. R. (1961). The lake sturgeon: the history of its fishery and problems of conservation. Fish & Wildlife Branch, Ontario Department of Lands and Forests.

Hastings, R. P., Bauman, J. M., Baker, E. A., & Scribner, K. T. (2013). Post-hatch dispersal of lake sturgeon (*Acipenser fulvescens*, Rafinesque, 1817) yolk-sac larvae in relation to substrate in an artificial stream. Journal of Applied Ichthyology, 29(6), 1208-1213.

Heath D. D., C. W. Fox, and J. W. Heath. 1999. Maternal effects on offspring size: variation through early development of Chinook salmon. Evolution. 53(5):1605-1611.

Heming, T. A., J. E. McInerney, and D. F. Alderice. 1982. Effect of temperature on initial feeding in alevins of chinook salmon (*Oncorhynchus tshawytscha*). Canadian Journal of Fisheries and Aquatic Sciences. 39:1554-1562.

Holtgren, J. M., Ogren, S. A., Paquet, A. J., & Fajfer, S. (2007). Design of a portable streamside rearing facility for lake sturgeon. North American Journal of Aquaculture, 69(4), 317-323.

Kamali, A., Z. Kordjazi, and R. Nazary. 2006. The effect of the timing of initial feeding on growth and survival of ship sturgeon (*Acipenser nudiventris*) larvae: a small-scale hatchery study. Journal of Applied Ichthyology. 22(Suppl. 1):294-297.

Kamler, E. (1992). Early life history of fish: an energetics approach (p. 267). London: Chapman & Hall.

Kappenman, K. M., M. Toner, J. Illgen, and F. T. Barrows. 2011. The effect of diet on growth and survival of first feeding pallid sturgeon *Scaphirhynchus albus*. Journal of Applied Ichthyology. 27:755-760.

Leach, G. C. 1920. Artificial propagation of sturgeon. Part I. Review of sturgeon culture in the United States. Report of the U.S. Fishery Commission. 1919:3-5.

Leger, P., D. A. Bengtson, P. Sorgeloos, K. L. Simpson, and A. D. Beck. 1987. The nutritional value of Artemia: a review. In: Sorgeloos, P., Bengtson, D. A., Decleir, W., Jaspers, E. (Eds.), Artemia research and its applications. Ecology, Culturing, Use in Aquaculture, vol. 3. University Press, Wetteren, pp. 357-372.

Li, S., and J. A. Mathias. 1982. Causes of high mortality among culture larval walleye. Transaction of the American Fisheries Society. 111(6):710-721.

Li, X. F., Tian, H. Y., Zhang, D. D., Jiang, G. Z., & Liu, W. B. 2014. Feeding frequency affects stress, innate immunity and disease resistance of juvenile blunt snout bream *Megalobrama amblycephala*. Fish & shellfish immunology. 38(1):80-87.

Memiş, D., Ercan, E., Çelikkale, M. S., Timur, M., & Zarkua, Z. (2009). Growth and survival rate of Russian sturgeon (Acipenser gueldenstaedtii), larvae from fertilized eggs to artificial feeding. Turkish Journal of Fisheries and Aquatic Sciences, 9(1), 47-52.

Mollah, M. F. A., & Tan, E. S. P. 1982. Effects of feeding frequency on the growth and survival of catfish (*Clarias macrocephalus* Gunther) larvae. Indian Journal of Fisheries. 29(1&2):1-7.

Moreau, R., & Dabrowski, K. (1996). Feeding stimulants in semipurified diets for juvenile lake sturgeon, Acipenser fulvescens Rafinesque*. Aquaculture Research, 27(12), 953-957.

Næss, T., Germain-Henry, M., & Naas, K. E. (1995). First feeding of Atlantic halibut (< i> Hippoglossus hippoglossus</i>) using different combinations of< i> Artemia</i> and wild zooplankton. Aquaculture, 130(2), 235-250.

Nunez, J., D. Castro, C. Fernandez, R. Dugue, F. Chu-Koo, F. Duponchelle, C. Garcia, and J. Renno. 2011. Hatching rate and larval growth variation in Pseudoplatystoma punctifer: maternal and paternal effects. Aquaculture Research. 42:764-775.

Petkam, R. and G. E. Moodie. 2001. Food particle size, feeding frequency, and the use of prepared food to culture larval walking catfish (Clarias microcephalus). Aquaculture. 194:349-362.

Piotrowska, I., B. Szczepkowska, M. Kozlowski, K. Wunderlich, and M. Szczepkowski. 2013. Results of the larviculture of Atlantic sturgeon (Acipenser oxyrinchus) fed different types of diets. Archives of Polish Fisheries. 21:53-61.

Post, H. 1890. Sturgeon: experiments in hatching. Transactions of the American Fisheries Society. 19:36-40.

Smith, K. M., & King, D. K. (2005). Dynamics and extent of larval lake sturgeon Acipenser fulvescens drift in the Upper Black River, Michigan. Journal of Applied Ichthyology, 21(3), 161-168.

Sorgeloos, P., P. Dhert, and P. Candreva. 2001. Use of the brine shrimp, Artemia spp., in marine fish larviculture. Aquaculture. 200:147-159.

Theilacker, G. H. 1986. Starvation-induced mortality of young sea-caught Jack Mackerel, *Trachurus symmetricus*, determined with histological and morphological methods. Fisheries Bulletin. 84(1):1-17.

Tung, P. H., & Shiau, S. Y. (1991). Effects of meal frequency on growth performance of hybrid tilapia,< i> Oreochromis niloticus× O. aureus</i>, fed different carbohydrate diets. Aquaculture, 92, 343-350.

Wang, N., Hayward, R. S., & Noltie, D. B. 1998. Effect of feeding frequency on food consumption, growth, size variation, and feeding pattern of age-0 hybrid sunfish. Aquaculture. 165(3):261-267.

Rochard, E., Castelnaud, G., & Lepage, M. (1990). Sturgeons (Pisces: Acipenseridae); threats and prospects. Journal of Fish Biology, 37(sA), 123-132.

IUCN 2010. http://www.iucn.org/?4928/Sturgeon-more-critically-endangered-than-any-other-group-of-species.

Appendix # 7 – Survival of larval lake sturgeon as a function of different chemotherapeutant prophylactics

John M. Bauman[a], Edward A. Baker[b], Terry L. Marsh[c] and Kim T. Scribner[a,d]

[a]Department of Fisheries and Wildlife, Michigan State University, 13 Natural Resources Building, East Lansing, MI 48824, USA.

[b]Michigan Department of Natural Resources Fisheries Division, 484 Cherry Creek Road, Marquette, MI 49855, USA.

[c]Department of Microbial and Molecular Genetics, Michigan State University 6169 Biomedical Physical Sciences, East Lansing, MI 48824, USA.

[d]Department of Zoology, Michigan State University 288 Farm Lane, East Lansing, MI 48824, USA.

ABSTRACT

In aquaculture settings, stress in fish increases as a result of unfavorable rearing conditions (e.g., water quality, water source) or common production practices (e.g., handling, disease treatment) and interferes with physiological processes that aid in the defense against pathogens. Therefore, the development of therapeutic treatment protocols that limit stress-induced infection or reduce the occurrence of high mortality events in aquaculture is essential to successful fish production. In this study the effects of different chemotherapeutants (including a control) on the survival of larval Lake Sturgeon were quantified at the end of a 5 week study which began at two weeks post-exogenous feeding. The use of sodium chloride followed 24 hr

later by a hydrogen peroxide treatment resulted in higher mean proportional survival at the end of this study. Results provide information that can be used to develop standard operating procedures for lake sturgeon, a species of conservation concern.

INTRODUCTION

Stress-, as well as pathogen-induced mortality is common in aquaculture settings, especially during early life periods, and often resulting in significant losses (Post 1987, Subasinghe et al. 2001, Conte 2004). Therefore, developing therapeutic treatment protocols that limit stress-induced infection or reduce the occurrence of high mortality events in aquaculture is essential to successful fish production. In aquaculture settings, stress in fish increases as a result of unfavorable rearing conditions (e.g., water quality, water source) or common production practices (e.g., handling, disease treatment) and interferes with physiological processes that aid in the defense against pathogens (Conte 2004, Davis 2006). For example, mucosal layers that aid in ionic regulation as well as act as a primary defense mechanism against pathogens are compromised in situations of stress (Esteban 2012). Furthermore, if defenses (i.e., mucosal layers) are compromised, external pathogenic bacteria, such as *flavobacterium columnare*, colonize external surfaces (e.g., skin and gills) and cause infection often resulting in significant mortality (Esteban 2012, Tripathi et al. 2005). In response to high mortality and a growing need for approved therapeutic regimes, culture managers have experimented with a variety of external disinfectant treatment strategies (hereafter referred to as "chemotherapeutants").

Common treatment strategies include the use of chemotherapeutants 1) to treat infected fish as a consequence of visual detection of disease or in response to high mortality events, or 2) administer weekly chemotherapeutant prophylactics to reduced stress and prevent incidences of

high mortality associated with pathogen infection (Bowker et al. 2011). Chemotherapeutant prophylactics used to reduce stress and prevent most prevalent disease-causing bacteria include chloramine-t (CT), hydrogen peroxide (H_2O_2), and sodium chloride ($NaCl^-$) (Bowker et al. 2011). CT is an external disinfectant found to effectively treat fish with, or by prophylaxis prevent, external bacterial infections (Thorburn and Moccia 1993, Gaikowski et al. 2008), particularly those associated with flavobacteriosis (Bowker 2011). Similarly, hydrogen peroxide is an oxidative external disinfectant that has been used in aquaculture since the 1930s (Tort 2000), and has been shown to reduce or eliminate infections improving survival across multiple species and multiple life periods (Speare and Arsenault 1997, Rach et al. 2000, Rach et al. 2003). For example, H_2O_2 has been used to control mortality associated with finfish egg saprolegniasis, as well as mortality of larval and juvenile fish infected with external pathogens such as *flavobacterium* (Bowker et al. 2011). $NaCl^-$ is one of the most commonly used chemotherapeutants for the control and treatment of external pathogens (Schelkle et al. 2011, Noga 2000) as well as for osmoregulatory aid (Swarm and Fitzgerald 1992, Francis-Floyd 1995, Bowker 2011). In addition, $NaCl^-$ use is believed to be associated with the 'shedding' of the mucosal layers, which exposes potential pathogens to treatment (Piper et al. 1982). The toxicity and effectiveness of chemotherapeutants utilized in aquaculture differs by fish species, treatment regime, treatment concentration, as well as the life period during which treatments are administered (Sanchez et al. 1996, Gaikowski et al. 1999, Magondu et al. 2011, Schelkle et al. 2011). Given that approved chemotherapeutants were initially and most commonly assessed using salmonids, further research is needed to evaluate the applicability of common chemotherapeutants for other fish species, including those of conservation concern, such as Lake Sturgeon (*Acipenser fulvescens*).

Lake sturgeon are a species of conservation concern throughout most of their historic range. Where restoration goals to enhance lake sturgeon populations can be met by stocking, streamside rearing facilities (SRFs) are used (Holtgren et al. 2007). SRFs utilize a natal water source and are believed to improve the probability of imprinting, compared to traditional hatcheries which use non-natal well-water for rearing (Flagg and Nash 1999, Holtgren et al. 2007). However, the use of SRFs pose challenges which include increased exposure to extreme temperature fluctuations and fish pathogens during early development when mortality is especially high. In the last decade, targeted stocking goals in SRFs have been difficult to achieve due in large part to high mortality during early life periods. Although unconfirmed, the mortality is attributed to common bacteria, such as *flavobacterium columnare* (Holtgren et al. 2007), which are ubiquitous in nearby water-sources. These challenges and the lack of data pertaining to the efficacy of chemotherapeutants utilized in lake sturgeon culture warrant refinement and evaluation of current disease prevention strategies in aquaculture facilities.

Currently, quantitative data pertaining to the use of chemotherapeutant prophylactics are lacking for lake sturgeon beyond the egg period. Therefore, the objective of this study was to quantify and compare survival of young-of-year lake sturgeon raised in a SRF exposed to different chemotherapeutant prophylactics. Our hypothesis was that survival of young-of-year lake sturgeon will differ as a function of different chemotherapeutant prophylaxis treatments.

METHODS

Study Site

This study was conducted from 26 June to 30 July 2013 at the BR-SRF which was supplied with ambient river water (~680 L/min) from the Kleber Reservoir, located near primary spawning areas for lake sturgeon in the upper Black River in Cheboygan County, Michigan. Use of SRFs, such as the Black River Streamside Rearing Facility (BR-SRF) has been widely advocated in the Great Lakes basin as the preferred method for culturing lake sturgeon. The mean water temperature recorded during this study was 22.7ºC, and ranged from 20.0 to 26.3ºC.

Study Fish

Hatchery-produced larvae gamete collection, fertilization and incubation. –

Gametes were collected from two male and two female lake sturgeon spawning in the upper Black River. Gametes were transported to the BR-SRF for fertilization, which took place within four hours of collection. Approximately 200 mL of eggs from each female were placed into a separate dry bowl. Milt samples from a separate male per female were activated using a 1:200 dilution of ambient river water and immediately poured over eggs allowing 90 seconds for fertilization. Excess milt was removed and eggs were rinsed once with ambient river water. Egg de-adhesion procedures began by applying a Fuller's Earth solution (Sigma Aldrich) and gently mixing for 50 min. After 50 min, Fuller's Earth was rinsed from the eggs and a 15 min, 50 ppm Iodophor disinfection treatment was administered. Following a 10 min rinse to remove residual Iodophor using ambient river water, eggs were transferred to Aquatic Eco-Systems (Pentair) J32 Mini Egg-hatching jars for incubation.

Wild naturally-produced larvae field collection and incubation. –

Naturally produced, fertilized eggs were collected from the Upper Black River at two spawning locations approximately three days post-fertilization. Eggs were transported to the

BR-SRF and incubated, separated by capture location (Site B, and Site C), in Aquatic Eco-Systems (Pentair) J32 Mini Egg-hatching jars.

Egg incubation and chemical treatment

Hatchery- and naturally-produced eggs were treated daily using a 500 ppm, 15 min bath treatment of hydrogen peroxide until 24 hours prior to hatch determined by observing embryo development stage 32 to 34 (Detlaff et al. 1993).. After hatch and during the free-embryo period, lake sturgeon seek refuge in available substrate (Hastings et al. 2013), therefore free-embryo were raised in 10 L polycarbonate tanks (Aquatic Habitats) with a single layer of 2.54 cm^3 sinking Bio-Balls (Pentair #CBB1-S) covering the tank bottom. Wild free-embryos were raised until endogenous resources were absorbed and fish began a 'swim-up' behavior (approx. 7-10 days post-hatch). At the onset of exogenous feeding we removed Bio-Balls and began feeding brine shrimp three times daily.

Experimental treatments

At twelve days post-exogenous feeding, we transferred 400 fish from each family and each spawning location group into four 1.2 m diameter tanks which were sub-divided into eight equal-sized sections (50 fish per section). Each partition was randomly assigned to one of four weekly treatment types, each with two replicates (Figure 5). This study began at fourteen days post-exogenous feeding (two-day tank acclimation) and continued for thirty-five days to quantify and compare the effects of different prophylactic chemotherapeutants when mortality in SRFs is highest. Chemotherapeutants administered in this study include those commonly utilized in traditional hatcheries and SRFs. Weekly prophylactic treatments in this study included: 1) 60 min, 15 ppm CT bath, 2) 15 min, 60 ppm H_2O_2, 3) 3 parts per thousand (ppt) $NaCl^-$ bath for 15 min followed 24 hr later by a 15 min, 60 ppm H_2O_2 bath, and 4) a control (no chemical

treatment). Fish were fed three times daily, except on treatment days, feeding was delayed until all treatments had been performed. Each week, all fish from each treatment type (including controls) were transferred using a small aquarium dip net to 10 L polycarbonate tanks with one aerator in each tank. Fish were administered respective treatments, briefly rinsed, and placed back into their rearing tank. All treatments were administered on the same day, once per week except treatment 3, which included an additional treatment the following day with H_2O_2. Controls were handled in the same manner as all other treatment groups, however, similar to treatment 1, were held for 60 min in their 'treatment' tank before being rinsed and returned to their rearing tanks. Mortalities were removed from the tanks each day and recorded to quantify survival at the end of the study. The experiment lasted 35 days (49 days post-exogenous feeding) to encompass the period of high mortality documented in all SRFs.

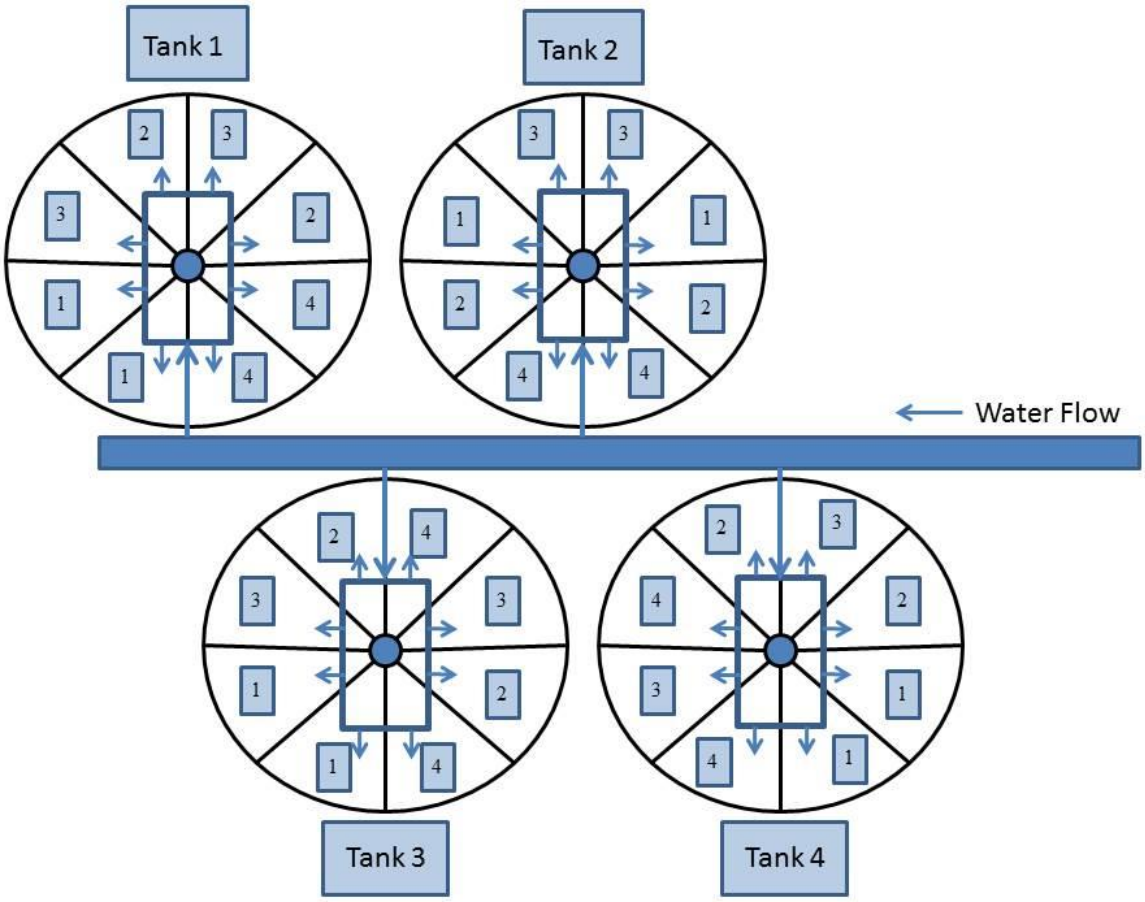


Figure 1. Experimental design of larval chemotherapeutant study. Each 1.2 m diameter tank held hatchery- and wild naturally-produced fish separate. Tanks were sub-divided into eight equal-sized sections (50 fish per section, 400 fish total per tank). Each partition was randomly assigned to one of four weekly treatment types, each with two replicates. Chemotherapeutant treatments included: 1) 60 min, 15 ppm CT bath, 2) 15 min, 60 ppm H_2O_2, 3) 3 parts per thousand (ppt) $NaCl^-$ bath for 15 min followed 24 hr later by a 15 min, 60 ppm H_2O_2 bath, and 4) a control (no chemical treatment). Arrows indicate water flow.

Statistical Analysis

Analyses to quantify mean proportional survival (±SE) as a function of treatment, was performed using SAS (SAS Institute version 9.3 Cary, NC). A p value < 0.05 was considered statistically significant for all analyses. Progeny from each family and each capture location were housed separately in four different tanks. Analyses were conducted and reported by treatment. Tank section was the experimental unit for all analyses, not individual fish. A generalized linear model using analysis of variance (ANOVA) was used to analyze proportional survival data. Survival data were modeled using a beta-distribution and reported as the proportion of survival. We used Tukey-Kramer multiple comparison tests for all response variables.

RESULTS

Survival at 35 days (49 days post-exogenous feeding)

This study documented a significant effect of treatment on mean proportional survival at the end of this study ($F_{3,28} = 9.89$, $P = 0.0001$). Mean proportional survival in groups treated weekly with NaCl followed 24 hr later by peroxide was significantly higher than those treated weekly with peroxide ($t_{28} = -3.57$, $P = 0.0067$) (Table 15), and those treated weekly with chloramine-T ($t_{28} = -4.93$, $P = 0.0002$). However, mean proportional survival between those treated weekly with $NaCl^-$ followed 24 hr later by peroxide was not significantly different from the control ($t_{28} = -1.29$, $P = 0.5775$) (Table 15). Mean proportional survival in control groups was significantly higher than those treated weekly with chloramine-T ($t_{28} = -3.84$, $P = 0.0034$) (Table 15).

Table 15. Mean number of fish surviving each week and proportional survival (±SE) at the end of this study as a function of different commonly used chemotherapeutant prophylactics. CT =

60 min, 15 ppm chloramine-T bath, H_2O_2 = 15 min, 60 ppm H_2O_2 bath, $NaCl^-$ & H_2O_2 = 3 parts per thousand (ppt) $NaCl^-$ bath for 15 min followed 24 hr later by a 15 min, 60 ppm H_2O_2 bath, and Control = no chemical treatment). Identical lower-case letters in the same column are not significantly different (Tukey-Kramer, $P < 0.05$).

Chemotherapeutant	Mean number of live fish				End (±SE)
	Week 1	Week 2	Week 3	Week 4	
CT	24.6	20.1	19.4	19.1	19.1 (0.38 ±0.09) z
H_2O_2	31.5	25.6	25.3	25.3	25.0 (0.51 ±0.06) z
$NaCl^-$ & H_2O_2	46.5	43.5	42.6	42.3	41.5 (0.83 ±0.03) y
Control	43.8	36.1	35.6	35.4	35.1 (0.70 ±0.06) yz

DISCUSSION

This study was conducted to quantify the effects of weekly prophylactic chemotherapeutants on the survival of hatchery- and naturally-produced young-of-year lake sturgeon. CT and H_2O_2 are the most commonly utilized chemotherapeutant prophylactics in SRF culture. However, these treatments showed the lowest mean proportional survival compared to the remaining treatment and the control which suggests that modifications to current chemotherapeutant prophylactic treatment regimens are necessary to improve aquaculture success in SRFs through the early rearing period.

Lower survival exhibited by tanks treated with CT or H_2O_2 was surprising given that CT and H_2O_2 have been used as a weekly chemotherapeutant prophylactic in lake sturgeon culture under similar treatment and rearing conditions (e.g., fish age, water temperature) with promising albeit not quantitative results (Ceskleba et at. 1985, Gaikowski et al. 2008, Holtgren et al. 2007). Differences in survival in our study as a function of CT or H_2O_2 treatments could have been due to stress associated with the initial treatment acting alone or in concert with rearing conditions

that may have increased the toxicity of treatments utilized in this study (i.e., pH, temperature). Several studies, using other species (i.e., salmonids) have noted significant mortality occurs soon after initial treatment with either CT or H_2O_2 which may indicate that the additional stress associated with treatment resulted in increased mortality of already sick, stressed fish (Gaikowski et al. 1999, Rach et al. 2000). Furthermore, given that we used a flow-through natal water source, temperature as well as pH was subject to change during treatment which could have increased the toxicity of CT or H_2O_2 treatments (Bills et al. 1988). However, we did not measure pH or other water quality parameters to verify such changes.

In three of the four tanks used in this study, $NaCl^-$ & H_2O_2, which included a 3 ppt $NaCl^-$ treatment followed 24 hr later by a 60 ppm H_2O_2 treatment, exhibited the highest mean proportional survival compared to other treatments. Currently, we are unaware of any literature which reports two treatments in tandem as a form of weekly chemotherapeutant prophylaxis. The purpose of using $NaCl^-$ followed 24 hr later by H_2O_2 was to first shed the mucosal layer exposing any potential pathogens (Piper et al. 1982, King and Farrell 2002), and then treat using H_2O_2 which is a known external disinfectant for pathogens such as *flavobacterium columnare* (Esteban 2012). There are two potential explanations for higher survival among those groups from $NaCl^-$ & H_2O_2 treatment groups. First, fish may not have been infected at all during the course of the study and moralities observed in other treatments were a by-product of handling and/or treatment stress. Therefore, higher survival in $NaCl^-$ & H_2O_2 treatment groups could be explained by the fact that $NaCl^-$ reduced stress-induced mortality that may have been associated with handling and/or treatment (Swarm and Fitzgerald 1992, Francis-Floyd 1995, Bowker 2011). Controls among all tanks exhibited higher mean proportional survival compared to H_2O_2 and CT

(although not significant higher than H_2O_2) and given that these groups were handled in a similar manner suggests that mortality was caused by the effect of treatments rather than handling. Therefore, the initial $NaCl^-$ bath in treatment 3, may have reduced the stress associated with chemical treatment that followed 24 hr later. Second, fish may have been infected with pathogens that were only treatable by first having the fish mucosal layer shed using the 3 ppt $NaCl^-$ treatment followed by the 60 ppm H_2O_2 treatment. CT and H_2O_2 may not have been able to disinfect pathogens which may have been embedded in the mucosal layer which could explain the lower mean proportional survival. However, the latter is not well supported based on the data from the controls. Controls showed higher mean proportional survival compared to those from treatments CT and H_2O_2 which would suggest that mortalities may have been caused by treatment rather than pathogen-induced infection.

Conclusions

The results suggest that SRFs which treat fish weekly using a 3 ppt $NaCl^-$ bath for 15 min followed 24 hr later by a 60 ppm H_2O_2 bath for 15 min treatment, can increase mean proportional survival during 30 days when mortality is high. Given that similar results were demonstrated among different tanks and among different progeny source, we recommend discontinuing the use of CT and H_2O_2 as stand-alone treatments. These data may be used to develop standard operating procedures to reduce high mortality that occurs during early rearing of lake sturgeon in SRFs.

LITERATURE CITED

Bills, T. D., L. L. Marking, V. K. Dawson, and J. J. Rach. 1988. Investigations in fish control: Effects of environmental factors on the toxicity of Chloramine-T to fish. U.S. Fish and Wildlife Service LaCrosse, WI USA.

Bowker, J., M. Gaikowski, A. Goodman, L. Sprague, and M. Bowman. 2011. Guide to using drugs, biologics, and other chemicals in aquaculture. U. S. Fish and Wildlife Service Aquatic Animal Drug Approval Partnership Program http://www.fws.gov/fisheries/aadap/home.htm 1-62.

Ceskleba, D. G., S. AveLallemant, and T. F. Thuemler. 1985. Artificial spawning and rearing of lake sturgeon, *Acipenser fulvescens*, in Wild Rose State Fish Hatchery, Wisconsin, 1982-1983. Environmental Biology of Fishes. Vol. 14(1), 79-85.

Conte, F. S. 2005. Stress and welfare of cultured fish. Applied Animal Behaviour Science 36:205-223.

Crossman, J. A., K. T. Scribner, C. A. Davis, P. S. Forsythe, and E. A. Baker. 2014. Survival and growth of lake sturgeon during early life stages as a function of rearing environment. Transaction of the American Fisheries Society. 143:104-116.

Davis, K. B. 2006. Management of physiological stress in finfish. Aquaculture 68(2):116-121.

Esteban, M. A. 2013. An overview of the immunology of fish larvae. Reviews in Aquaculture 5(1):S1-S25.

Flagg, T. A., and C. E. Nash. 1999. A Conceptual Framework for Conservation Hatchery Strategies for Pacific Salmonids. Seattle, WA: U.S. Department of Commerce, NOAA Technical Memorandum NMFS-NWFSC-38, 48.

Francis-Floyd, R. 1995. The use of salt in aquaculture. Retrieved from 307 University of Florida, Cooperative 306 Extension Service: Fact Sheet, 86, pp. 1-3.

Gaikowski, M. P., J. J. Rach, and R. T. Ramsay. 1999. Acute toxicity of hydrogen peroxide treatments to selected lifestages of cold-, cool-, and warmwater fish. Aquaculture. Vol. 178, 191-207.

Gaikoski, M. P., W. J. Larson, and W. H. Gingerich. 2008. Survival of cool and warm freshwater fish following chloramine-T exposure. Aquaculture 275:20-25.

Hastings, R. P., J. M. Bauman, E. A. Baker, and K. T. Scribner. 2013. Post-hatch dispersal of Lake Sturgeon (*Acipenser fulvescens*, Rafinesque, 1817) yolk-sac larvae in relation to substrate in an artificial stream. Journal of Applied Ichthyology 29:1208-1213.

Holtgren, J. M., S. A. Ogren, A. J. Paquet, and S. Fajfer. 2007. Design of a Portable Streamside Rearing Facility for Lake Sturgeon. North American Journal of Aquaculture. Vol. 69(4), 317-323.

King, K. and P. Farrell. 2002. Sensitivity of juvenile Atlantic Sturgeon to three therapeutic chemicals used in aquaculture. North American Journal of Aquaculture 64:60-65.

Magondu, E. W., J. Rasowo, E. Oyoo-Okoth, and H. Charo-Karisa. 2011. Evaluation of sodium chloride (NaCl) for potential prophylactic treatment and its short-term toxicity to African catfish *Clarias gariepinus* (Burchell 1822) yolk-sac and swim-up fry. Aquaculture. Vol. 319, 307-310.

Noga, E. J. 2000. Fish disease: Diagnosis and treatment. Wiley Publishing & Sons, Ames Iowa USA .

Piper, R. G., I. B. McElwain, L. E. Orme, J. P. McCraren, L. G. Fowler, and R. J. Leonard. 1982. Fish Hatchery Management. U.S. Fish and Wildlife Service, Washington D.C.

Post, G. 1987. Textbook of Fish Health. TFH Publications, Inc., Neptune City, NJ. 1-288.

Rach, J. J., M. P. Gaikowski, and R. T. Ramsay. 2000. Efficacy of hydrogen peroxide to control mortalities associated with bacterial gill disease infection on hatchery-reared salmonids. Journal of Aquatic Animal Health 12(2):119-127.

Rach, J. J., S. M. Schleis, M. Gaikowski, and A. Johnson. 2003. Efficacy of hydrogen peroxide in controlling mortality associated with external columnaris in Walleye and Channel Catfish fingerlings. North American Journal of Aquaculture 65:300-305.

Sanchez, J. G., D. J. Speare, N. McNair, and G. Johnson. 1996. Effects of a Prophylactic Chloramine-T Treatment on Growth Performance and Condition Indices of Rainbow Trout. Journal of Aquatic Animal Health. Vol. 8, 278-284.

Schelkle, B., R. Doetjes, and J. Cable. 2011. The salt myth revealed: Treatment of gyrodactlid infections on ornamental guppies, Poecilia reticulate. Aquaculture 31(1):74-79.

Spear, D. J. and G. J. Arsenault. 1997. Effects of intermittent hydrogen peroxide exposure on growth and columnaris disease prevention of juvenile rainbow trout (*Oncorhynchus mykiss*). Canadian Journal of Fisheries and Aquatic Sciences 54:2653-2658.

Subasinghe, R. P., M. G. Bondad-Reantaso, S. E. McGladdery. 2001. Aquaculture development, health and wealth. In: Subasinghe, R. P., P. Bueno, M. J. Phillips, C. Hough, S. E. McGladdery, and J. R. Arthur (Eds.). 2000. Aquaculture in the third millennium. Technical Proceedings of the Conference on Aquaculture in the third millennium, Bangkok, Thailand 20-25.

Swarm, L., and S. Fitzgerald. 1992. Use and Application of Salt in Aquaculture. North Central Regional Aquaculture Center Publication, No. 105, pp 1-2.

Thorburn, M. A., and R. D. Moccia. 1993. Use of chemotherapeutics on trout farms in Ontario. Journal of Aquatic Animal Health 5(2):85-91.

Tort, M. J. 2000. Studies of hydrogen peroxide for the treatment of bacterial gill disiease in fish. Cornell University Dissertation 1-204..

Tripathi, N. K., K. S. Latimer, C. R. Gregory, B. W. Ritchie, R. E. Wooley, and R. L. Walker. 2005. Development and evaluation of an experimental model of cutaneous disease in koi Cyprinus carpio. Journal of Veterinary Diagnostic Investigation 17:45-54.

Appendix # 8 – Assessment of microbial community composition and diversity associated with different stream water filtration methods

John M. Bauman[a], Edward A. Baker[b], Terry L. Marsh[c] and Kim T. Scribner[a,d]

[a]Department of Fisheries and Wildlife, Michigan State University, 13 Natural Resources Building, East Lansing, MI 48824, USA.

[b]Michigan Department of Natural Resources Fisheries Division, 484 Cherry Creek Road, Marquette, MI 49855, USA.

[c]Department of Microbial and Molecular Genetics, Michigan State University 6169 Biomedical Physical Sciences, East Lansing, MI 48824, USA.

[d]Department of Zoology, Michigan State University 288 Farm Lane, East Lansing, MI 48824, USA.

Background

Stream water sources are typically not suitable for fish culture activities without some means of filtration of course particulates. There are numerous filtration methods available to stream-side lake sturgeon culturalists. Many lake sturgeon culture trailers use sand filters that are commonly used for swimming pools. Other facilities use 'sock' filters. As an extension of research conducted and reported under Appendix 4, we further compared microbial communities associated with lake sturgeon eggs exposed to water filtered using sand or sock filtration devices.

Methods

Eggs from a single female were fertilized by a single male using protocols outlined in Appendices 3-7. Eggs were divided evenly into a heath tray in two separate areas of the Black River Streamside Rearing Facility. Eggs were reared in replicate PVC couplings in health trays until hatch. Daily levels of egg mortality were quantified as in Appendices 3-7. At hatch, body size was estimated. For the purpose of this summary, we only present data for eggs that were not exposed to chemotherapeutant treatments during egg incubation. Water was filtered using a sand filter and 100 and 50 micron sock filters. Statistical analyses were conducted as described in Appendix 5 for body size at hatch and percent survival to hatch.

Results

No significant differences in body size or survival was observed at hatch between offspring reared in sand or sock filters (data not shown). However, we detected substantial differences in egg microbial communities (Figure 1).

The pie diagrams (Figure 1) show the relative abundances of each of the major microbial taxa on the egg surface. The dendrogram (Figure 2) compares eight communities derived from sturgeon eggs that were incubated in water derived from either a sand or sock filter. The two closest pairs of communities were sand and sock filtrate in peroxide treated eggs from families 1 and 2, sampled at times 1 and 2. These communities were dominated (70-75%) by two phylotypes of *Burkholderia* and two of *Flavobacterium*. The next closest pair was from controls (no disinfectants) of family 2-time 2. This pair of communities are more divergent. While the community derived from the sock remains similar to the previously described communities in that *Burholderia* and *Flavobacterium* are the dominant phylotypes (74%), the community derived from the sand filters diverged with two gamma-proteobacteria (*Aeromodales* and *Pseudomonadales*) contributing approximately 25% to the community. The two most divergent communities were derived from peroxide treated sand and sock filtrates from Time 1 of Family 2. In the sand filtered water samples the community was dominated by an *Aeromonas* (42%), *Clostridia* (33%) and *Alteromonas* (16%) that in total comprise 91% of the community. The samples from sock filtered water was 92% *Clostridia*. It should be noted that the appearance of *Clostridium*, a strict anaerobe, came as a surprise. It probably indicates that *Clostridial* cells transiently present in the water were capable of binding to the eggs. However in a well-aerated incubation system these cells would not survive long. The indications from these data are that the dominant selective pressures of the formation of the community were first, the differences between eggs from different families and second, differences between the sand and sock filters, both at early time points, and finally the peroxide treatment. The early community structure in the sand and sock filtered water from peroxide treated eggs of family 2 are the most divergent, yet these communities converge and by the second time point are quite similar. The controls, which are subject to all populations within the stream, is the second most divergent pair of communities. Finally the peroxide treated sample of Family 1-Time 1 showed only minor differences between sock and sand filtrate and the same was true of the peroxide treated in family 2.

Discussion

We did not follow yolk sac fry after hatch. Thus, the effects of different microbial communities on the surfaces of developing eggs cannot be established. However, give other work on effects of microbial communities and survival and resources utilization during embryogenesis (Fujimoto 2012) we suggest that further investigation of effects of anaerobic microbial populations on subsequent life stages is warranted.

Literature Cited

Fujimoto, M. 2012. Microbial succession on the lake sturgeon egg surface: mechanisms shaping the microbial community assembly during succession and the effect of microbial successional processes on host life history traits. PhD Dissertation, Michigan State University.

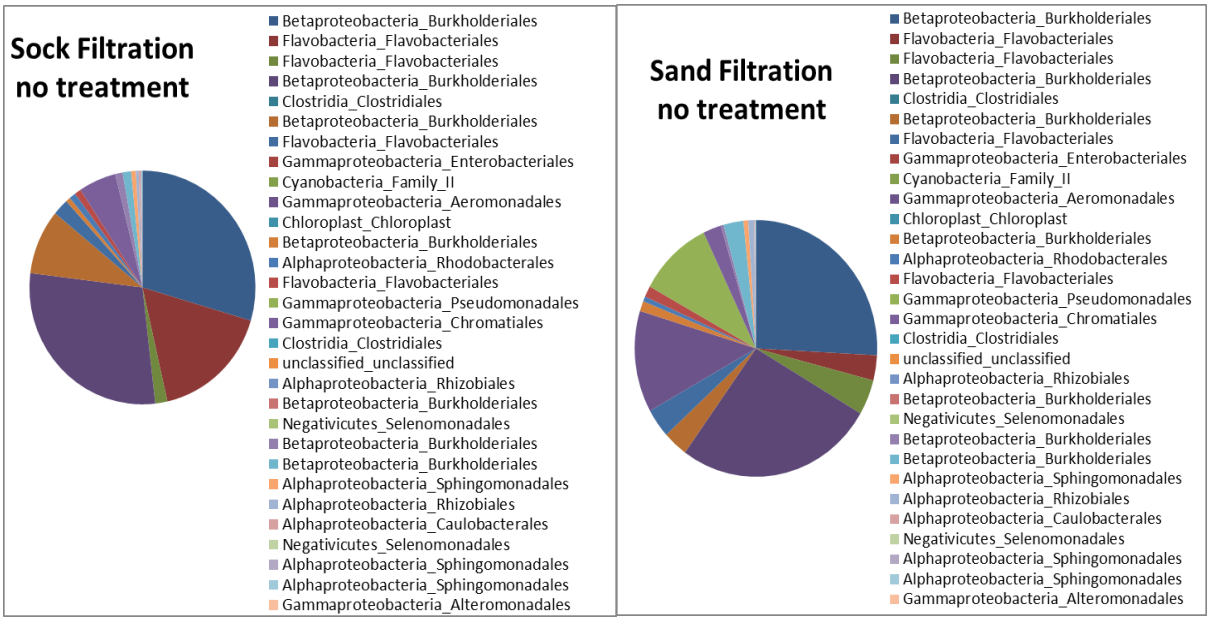
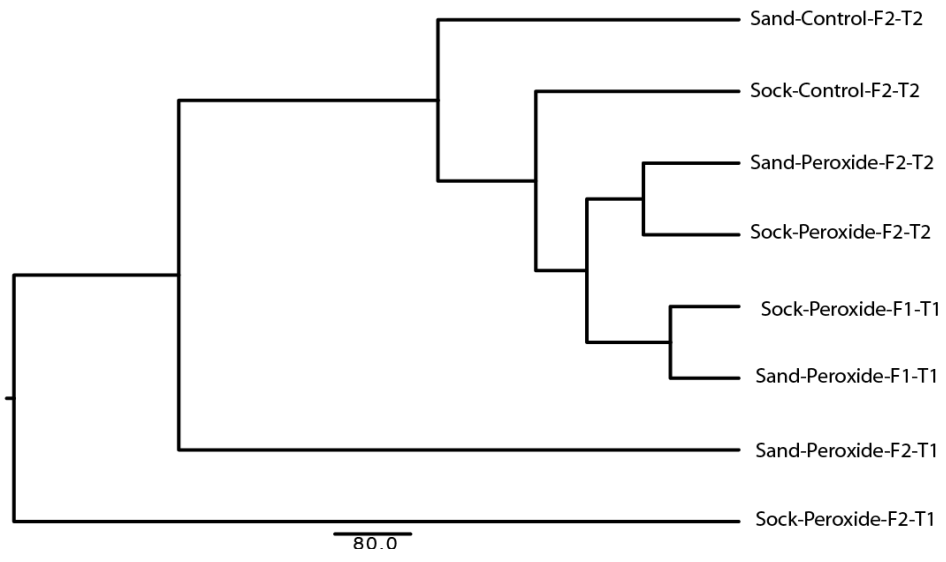


Figure 1. Pie diagrams describing the relative frequency of occurrence of microbial taxa found on the surface of sturgeon eggs during period 2 (as in Appendix 2).

Figure 2. Dendrogram characterizing similarities among microbial communities as a function of filtration (sand vs sock filters), time of sampling (day 2 and 6 during incubation) and family (eggs from 2 females, F1 and F2) were used.



Appendix # 9 - Draft of Standard Operating Procedures for Streamside lake sturgeon culture

John M. Bauman[a], Edward A. Baker[b], Terry L. Marsh[c] and Kim T. Scribner[a,d]

[a]Department of Fisheries and Wildlife, Michigan State University, 13 Natural Resources Building, East Lansing, MI 48824, USA.

[b]Michigan Department of Natural Resources Fisheries Division, 484 Cherry Creek Road, Marquette, MI 49855, USA.

[c]Department of Microbial and Molecular Genetics, Michigan State University 6169 Biomedical Physical Sciences, East Lansing, MI 48824, USA.

[d]Department of Zoology, Michigan State University 288 Farm Lane, East Lansing, MI 48824, USA.

Problem statement

Stream-side rearing facilities (SRFs) have been widely advocated in the Great Lakes basin as the preferred method of culturing lake sturgeon (*Acipenser fulvescens*) in situations where restoration goals to enhance or repatriate populations can be met by stocking. However, recent experience has shown (Lake Michigan Lake Sturgeon Task Group, unpubl. data) that targeted stocking goals are inherently difficult to achieve, due in large part to high mortality rates at egg and larval stages, and variable and low larval and juvenile growth within and among these facilities. The variable successes of current SRFs point to the need for detailed evaluation and refinement of operational methods that can be tailored to address site-specific environmental conditions and logistical concerns. Successful lake sturgeon rearing in SRFs will depend on increased vigilance and site-specific operating procedures to accommodate different equipment available for fertilization, water quality management, pathogen treatment, and rearing and to address the challenges presented by variability in environmental conditions, especially temperature and water quality. There is considerable variation in current operations of SRFs and no way for SRF managers to objectively and quantitatively evaluate the effectiveness of different methods given constraints of SRF size, man power, and budgets.

Different methods are used for providing lake sturgeon gametes and larvae to raise in SRFs. In

most cases direct gamete collections are undertaken to supply eggs for incubation directly in SRFs. In other instances lake sturgeon are allowed to spawn naturally and either fertilized eggs are collected directly from the substrate in the spawning river or larvae that have hatched from eggs in the river are collected as they drift downstream from spawning sites (*Crossman et al. 2011; Aquaculture 310: 312-324*). Direct gamete collection has proven to be the most reliable method of providing large numbers of fish to raise in SRFs, but has not produced consistent results in terms of numbers of fish stocked. The number of fish stocked has varied widely among SRFs and among years for the same SRF (Table 1). Because of the effects of, and interactions among many variables that affect egg and larval growth and survival (e.g., Table 2) during different stages of lake sturgeon SRF culture (Fig. 1), SRFs are not able to manipulate variables in a regimented manner to quantify costs and/or benefits of different culture methods without compromising production goals. The reasons for variable success of these efforts are not clear and point to the need for directed research into optimal operating procedures to improve SRF production. Further, the fact that operational protocols (e.g., fish density, feeding regimes, water quality management) at these SRFs have varied from year to year as investigators attempt to maximize production, provides a clear indication of the need for directed research that can be replicated in a controlled setting and in a short time period.

Our Black River SRF is large and provides a suitable setting for testing and refining rearing protocols for use in other SRFs. We are able to manipulated and statistically evaluate multiple methods simultaneously using large numbers of family groups and large sample sizes of eggs and larvae to evaluate specific aspects of streamside rearing operational methods.

Below we provide further detail concerning methods developed in the context of research conducted at the Black River SRF. We provide SOPs for egg, free embryo and larval stages.

Black Lake Lake Sturgeon Spawning Protocol

Protocol 1 -- Milt Extraction

1. -- Place fish on back, one person pinning the head of fish underwater and another person sitting on the bank holding the tail so that the uro-genital opening remains free of the water. Handling time varies by fish but lasts a maximum of 5 minutes for males and females.

2. -- Make sure water is flowing over gills, but that the water is not too deep to control the fish and to keep the posterior ventral side of the body dry

3. -- Wipe around the uro-genital area ensuring no water contamination

4. -- Make sure the syringe and hands are dry, water activates the sperm and is only viable for a short time once activated

5. -- Keep the tail of the fish level so that the milt can pool and be collected

6. -- Gently massage the outer sides of the abdomen (moving from anterior to posterior) and collect milt with the syringe, watch out for fecal matter, do not collect milt that comes into contact with water or if fecal material is extruded with the milt. Clean area and get fresh milt

7. -- If fish splashes around make sure to keep syringe away and dry and always clean and dry the uro-genital area before collecting

8. -- Collect approx 2-3 ml of milt and leave some air in syringe to oxygenate the milt. The milt is expressed on the dried surface of the cloacal opening and suction draws the milt into the barrel of the syringe without need for a needle.

9. -- Label tube with male information, PIT and date

10. -- Transfer to a small Ziploc bag, label, double bag to be safe or have a larger Ziploc to put all in together and place in cooler on ice or with cold pack

Protocol 2 -- Egg Collection

1. -- Place fish on back, one person pinning the head of fish and another holding the tail. Handling time varies by fish but lasts a maximum of 5 minutes for males and females.

2. -- Make sure water is flowing over gills, but that the water is not too deep to control the fish and to keep things dry

3. -- Wipe around the uro-genital area ensuring no water contamination.

4. -- Keep the tail slightly elevated and angled towards the collector

5. -- Collect eggs in large Ziploc bags, make sure everything is dry, including hands

6.-- Person at head, rub abdomen gently from anterior to posterior, going slow while the person with bag collects the eggs as they come, keep bag away if fish fights, re wipe area and try again, sometimes the fish fighting helps loosen eggs as they can tense up easily

7. -- Label bag with female information, PIT and date, double up Ziploc bags to ensure no water getting in. Float in a cooler of water that is at ambient river temperature. If a cooler is not readily available, float the eggs in another Ziploc bag with river water, not on ice. Change water in the bag as needed to avoid an increase in temperature.

Protocol 3 -- Fertilization

Temperatures must be equivalent to the current river temperature

1. -- Label mixing bowls, heath trays and make solutions needed (See Below)

2. -- Divide eggs into equal volumes dependant upon the number of males to be used

3. -- Dilute sperm at a ratio of 1:200. i.e. 5mls per 1000ml's of water

4. -- Pour immediately over eggs until the eggs are totally covered

5. -- Mix gently with hand for 1-2 minutes

6. -- Move immediately into de-adhesion and disinfection protocols

Protocol 4 -- De-adhesion and Disinfection

1. -- Prepare de-adhesion solution in a bucket (~ 90ml of Fullers Earth per 1L of water)

2. -- Prepare disinfection solution (50mg/L iodine)

3. -- Add disinfection solution to de-adhesion solution

4. -- Mix the two solutions thoroughly and add eggs

5. -- Mix with hands for 45-60 minutes till eggs are no longer clumping. No gloves are used by personnel when in the hatchery during fertilization. The clay is commercially purchased and is autoclaved by the manufacturer. There is no adverse effect. .

6. -- Pour off excess water and add clean water, repeat till eggs are well rinsed

7. -- Transfer eggs to labeled heath trays. Make sure eggs are spread out as much as possible and not on top of one another, use multiple trays if needed

Protocol 5 -- Daily Disinfection until Hatch

To be conducted once per day until hatch

1. -- Calculate volume (ml) of formalin needed using the following equation:

$$\frac{\text{Flow Rate (Gallon/}_{min}) * (3785) * 15\text{mins}}{600}$$

*Where duration will always be 15mins at a concentration of 17ppm

*Note that only 75gal of formalin waste at 17ppm will be generated during a time when effluent from the entire operation is 250gal/min which over 15 min will be 3750gal. This will dilute the formalin effluent to concentrations below the EPA effluent levels (2.1 ppm peak effluent limit)

2. -- Fill formalin container with measured amount, top off with water to fill line and place on top of heath tray stacks

3. -- Remove container when empty, allow system to flush and then re-open valve to main outflow.

Important: Use proper safety equipment when using formalin including eye protection (safety glasses or face shield), rubber gloves, rubber apron, and power2d air purifying respirator or contained face respirator.

A personal respirator (Powered air purifying respirators) will be used by anyone involved with egg disinfection using the SOPs.

Protocol 1 -- Formalin Treatment

To be conducted for eggs once per day until hatch

1. -- Calculate volume (ml) of formalin needed using the following equation:

$$\frac{\text{Flow Rate (Gallon/}_{\text{min}}\text{)} * (3785) * 15\text{mins}}{600}$$

*Where duration will always be 15mins at a concentration of 17ppm. Note that the 17ppm concentration is for a volume of 75gals over a 15 min period (5 gal. min). The flow rate of water coming into the total effluent volume out of the hatchery is 250 gal/min. Over 15 min, that equals 3750 gals into which a 17ppm formalin mix of 75 gal will be diluted. The EPA mandated limit for discharge of formalin is 2.1ppm. We are under the limit so the effluent will be returned to the stream

2. -- Fill formalin container with measured amount, top off with water to fill line and place on top of heath tray stacks

3. -- Remove container when empty, allow system to flush and then re-open valve to main outflow

Personal protection equipment - Use proper safety equipment when using formalin. Formalin treatments will be administered to eggs in heath trays inside the incubation room of the stream-side facility. There is a fan in the room and the room is well ventilated. Individuals handling

formalin will wear eye protection (safety glasses or a face shield. Individuals will also wear rubber gloves and a rubber apron. A powered air purifying respirator will be used.

Treatments of Juvenile fish

Fish in all tanks are observed multiple times per day. Visible signs of declining health that may warrant use of non-antibiotic treatments including salt or peroxide include lethargic behavior, abnormal breathing (rapid gill movements), and lack of feeding. Use of non-antibiotic treatments are widely applied to address issues associated with external parasites and microbial infection on the skin and gill surfaces. This point has been added to the SOP.

Protocol 2 -- Salt Treatment

Water source – the source of water for the facility and treatment is stream water. This water is passed through 100 micron and 50 micron filters upon entry to the facility.

Salt: NaCl, can be purchased at any feed stores (Non mineral block)

Measure: Hydrometer can be purchased from any pet store or Walmart. A 1M solution is titrated into the incoming water system at empirically determined rates to result in a 5ppt concentration. Rates of incoming water will be determined per tank as incoming water rates vary depending on tank size. Incoming water will circulate the NaCl solution. When a 5ppt concentration is reached the fish are retained at this level for 20 min. Afterwards water levels of incoming water are increased and the solution is drawn down to return the tank to ambient conditions.

Salt treatments are conducted as a preventative measure due to the abundant and diversity microbial community in the incoming water source at the streamside hatchery. Salt baths should be conducted at a concentration of 5ppt for a period of 20 minutes once per week from when the fish achieve one inch total length (~1-1.5 months). Multiple weekly salt baths may be necessary if ambient water temperature is consistently high. If fish exhibit lethargic behaviors or increased respiration prior to receiving the salt bath, bath concentrations may need to be doubled. Keep close visual contact should be maintained during this period to monitor fish for different behavior (increased activity as a sign of positive effect). Transfer fish to freshwater immediately following the treatment.

Protocol 3 -- Peroxide Treatment

If visual appearance based on daily inspection dictates that salt and/or peroxide treatment is warrented. The salt or peroxide baths are prepared in a separate container and the fish are transferred to the container for the treatment. Fish are returned to their tank following treatment.

The nets and other instruments that come into contact with fish from a specific tank are disinfected with betadyn before further use.

As a strong oxidizer, H_2O_2 should attack any Bio fouling in the tank or water lines and it will kill most bacteria or fungus or protozoa that attach to the gills of the fish. As with any chemical treatment, it is always best to do a small batch of fish first as water chemistry, temp, fish condition, etc. all play as factors in how well the fish handle the treatment. There is a sliding scale as the sicker (weaker) the fish are, the less chemical strength they can handle. Also the very first treatment in a while will work on the organic material or biofilm. Then the second treatment will be stronger because of less organics in the tanks. Also the larger the fish are the more gill surface area they have and are therefore more susceptible to higher chemical concentrations, so you would start with a lower concentration. These are all generalities, as stated earlier always try a small batch of fish before treating an entire tank.

For feeding sturgeon fingerlings, starting treatment levels should be at 50 ppm H_2O_2 for 15 minutes. Flow through is fine, or if there are very few fish, a static treatment would also work. Then every other day, do the next treatment, gradually working up to 75 ppm and then 100 ppm. 100 ppm is as high as we would want to go.

Usually 3 or 4 treatments are enough to stop mortalities. If you are doing a preventative treatment, once or twice a week should work.

To calculate treatments use the following formulas

Standing bath

$$\frac{\text{Volume of water treated X final concentration desired (ppm) X correction factor}}{\text{strength of chemical (decimal)}}$$

Example: (5 gallons of water-- times 50 ppm-- times .00378 --) divided by .10 equals 9.45 grams or Milliliters of chemical needed if you had 10 percent active H2O2 as the strength. (.00378 is the correction factor for grams or milliliter per gallon)

Flow through treatments

$$\frac{\text{flow rate X treatment time X final concentration x correction factor}}{\text{strength of chemical (decimal)}}$$

Example (5 gallons per minute-- times 15 minutes-- times 50 ppm—times .00378) divided by .20 equals 70.9 grams or milliliters of chemical if you had 20 percent active H2O2.

**Note: Based on previous observations fish exhibit increased activity during exposure to all concentrations of peroxide. Keep a close eye on the fish at all times during the treatment as a cease in activity may be an indication that the peroxide is having negative effects on the fish and should abort treatment immediately. Be sure to purge tanks twice to remove excess

peroxide and to facilitate fish recovery. If possible, add extra air stones in the tanks during treatments since the fish will be stressed out and require more oxygen.

Personal protection equipment – The peroxide treatment will be administered to fish in tanks inside the stream-side facility. Individuals handling peroxide will wear eye protection (safety glasses or a face shield. Individuals will also wear rubber gloves and a rubber apron. A powered air purifying respirator will be used. All personnel using powered air purifying respirators will be trained in their use.