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

By

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ABSTRACT

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

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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. 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. Feeding regimes, as well as the effects of different weekly prophylactic chemotherapeutants were quantified during the larval period. 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.

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THESIS 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. As a result, aquaculture methods (i.e., chemical treatments, de-adhesion procedures, feeding regimes) that address stage-specific life-history characteristics (e.g., egg adhesion) that hinder hatchery success have been employed to increase growth and survival during early life periods. However, the direct and indirect effects of these aquaculture practices on growth and survival of developing embryos to hatch and on through subsequent life periods is understudied and thus needs further investigation, especially for species of conservation concern such as Sturgeons (Acipenseridae).

According to the International Union for Conservation of Nature (IUCN), 85% of the world's Sturgeons (Acipenseridae) are in danger of extinction (IUCN, 2010). The decline and persistent low abundance of sturgeon populations worldwide have been attributed to several anthropogenic factors including over-fishing, poaching, habitat degradation, and discontinuity of migration routes utilized for reproduction (Auer and Dempsey 2013). In North America, there are nine species of sturgeon, all of which are protected and have either continued to decline in number or have remained constant at low levels of abundance for the past 25 years (FishBase 2013).

As a result of sturgeons' imperiled status, agencies have begun assessing and enumerating localized populations to evaluate viable recovery options. However, life-history traits (e.g., spawning periodicity, late maturity, spawning site fidelity) of sturgeons make recovery goals expensive and difficult to attain in time periods that are consistent with

management goals. Also, there is concern regarding stocking and the negative effects associated with out-breeding depression when non-natal brood sources are used. For Lake Sturgeon (*Acipenser fulvescens*), Welsh et al. (2010) provided genetically-based guidelines for Great Lakes recovery programs involving stocking. These guidelines provide genetic decision-trees for managers focused on recovery of remnant populations or repatriation of extirpated Great Lakes sturgeon populations. Choosing brood-source populations within a specified genetic management region increases the likelihood that biological attributes (e.g., river-imprinting, temperature regimes) related to fitness are maintained and deleterious effects avoided (Welsh et al. 2010).

Since 2004, the use of streamside rearing facilities (SRFs) has been widely advocated in the Great Lakes basin as the preferred method for culturing lake sturgeon in situations where restoration goals to enhance or repatriate populations can be met by stocking (Holtgren et al. 2007). SRFs utilize a flow-through natal water source, believed to improve fitness of progeny and in turn maximize the probability of imprinting, compared to traditional hatcheries which use non-natal well-water for rearing (Pepper 1992, Travis et al. 1998, Flagg and Nash 1999, Holtgren et al. 2007, Crossman 2011). The use of SRFs pose challenges which include increased exposure to fish pathogens and extreme temperature fluctuations during early development when mortality is already especially high. SRFs have been used for nearly a decade, however, targeted stocking goals have been difficult to achieve when SRFs are used, due in large part to high mortality rates during early life periods. Information pertaining to optimal rearing strategies during early life periods in SRFs do not exist and if evaluated may improve production results for lake sturgeon in the Great Lakes.

The need for quantitative evaluation of currently SRFs rearing methodologies is the focus of my thesis which encompasses multiple life periods during the rearing period. For example, during embryogensis there are properties (i.e, egg adhesion) and behavioral traits (i.e., phototaxis and crowding) exhibited, while advantages in riverine environments can reduce success of hatchery propagation. The effects of these different embryonic rearing conditions (i.e., mechanical and chemical egg de-adhesion, egg incubation) on potentially symbiotic or pathogenic microbial communities is also a focus of this study which may provide insight regarding potential mechanisms effecting growth and survival to hatch. Also, feeding regimes (i.e., feeding frequency, food type) employed during the larval period that reduce the occurrence of low growth and high mortality events are quantified. Chemotherapeutant prophylactics utilized during the egg and larval periods are common practice in SRFs, however the effects of these treatments on the growth and survival during rearing are not well understood and is a focus in two chapters of my thesis. Growth (body size) and survival are used as response variables for the direct and indirect effects of hatchery practices given that under stressful rearing conditions anaerobic metabolic processes associated with stress indirectly affect somatic tissue growth (body size) and may directly affect survival (Kamler 2008, Conte 1998).

Results from my thesis research focuses on methodologies currently utilized in SRFs to quantify effects on body size and survival of lake sturgeon at multiple life periods to provide information necessary for the development of standard operating procedures that may enhance success of SRFs.

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CHAPTER I: EFFECTS OF DE-ADHESION METHOD AND INCUBATION CONDITION ON BODY SIZE, SURVIVAL, OXYGEN CONSUMPTION RATE, AND MICROBIAL COMMUNITY COMPOSITION OF LAKE STURGEON EGG SURFACE

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 mm²), and body area (BA mm²)) 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

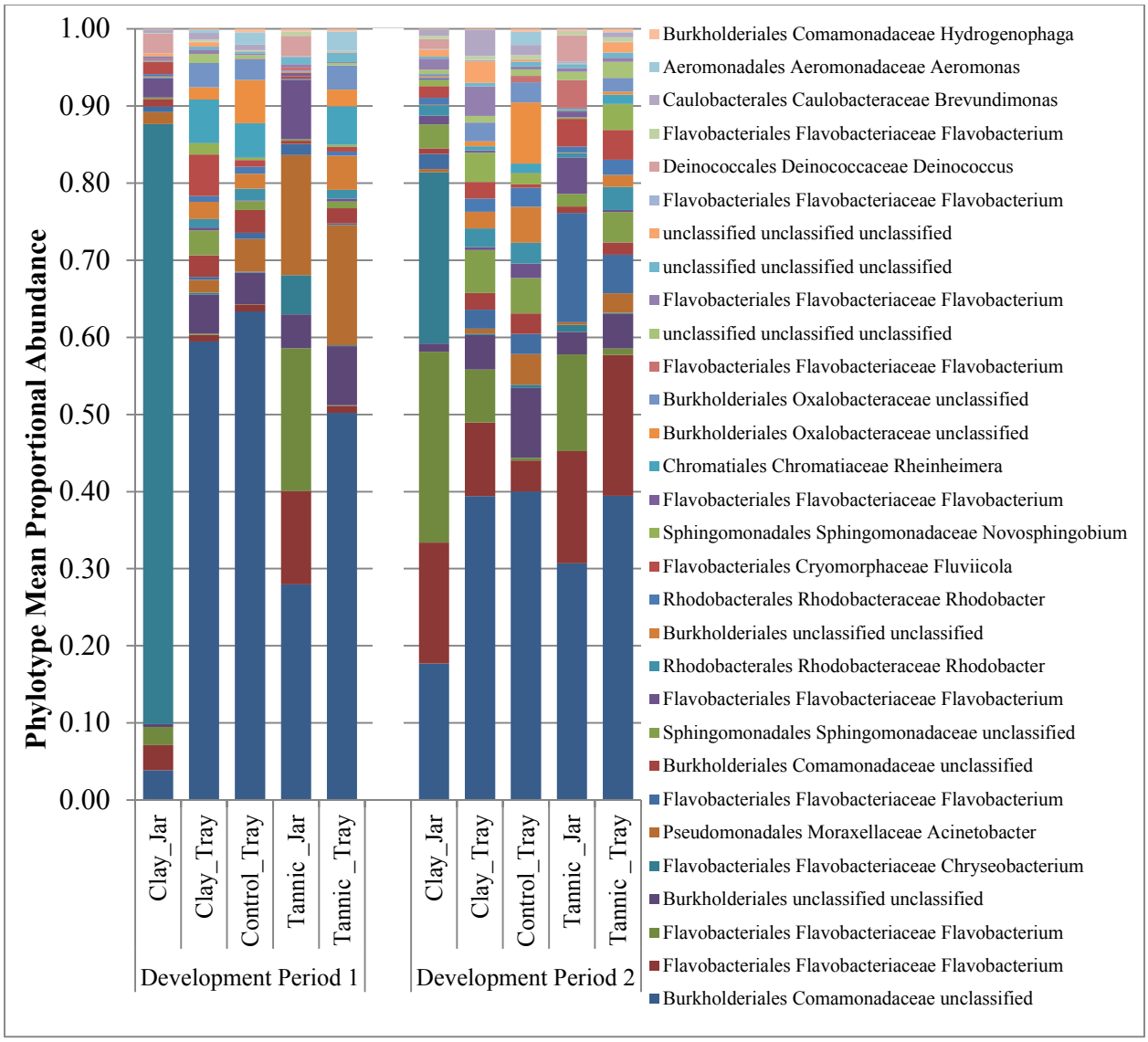

Figure 1. Microbial phylotype mean proportional abundance as a function of embryonic development time and de-adhesion and incubation treatment. Thirty major phylotypes represented in the community sample based on 5 percent contribution to total operational taxonomic units. Each unique color represents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

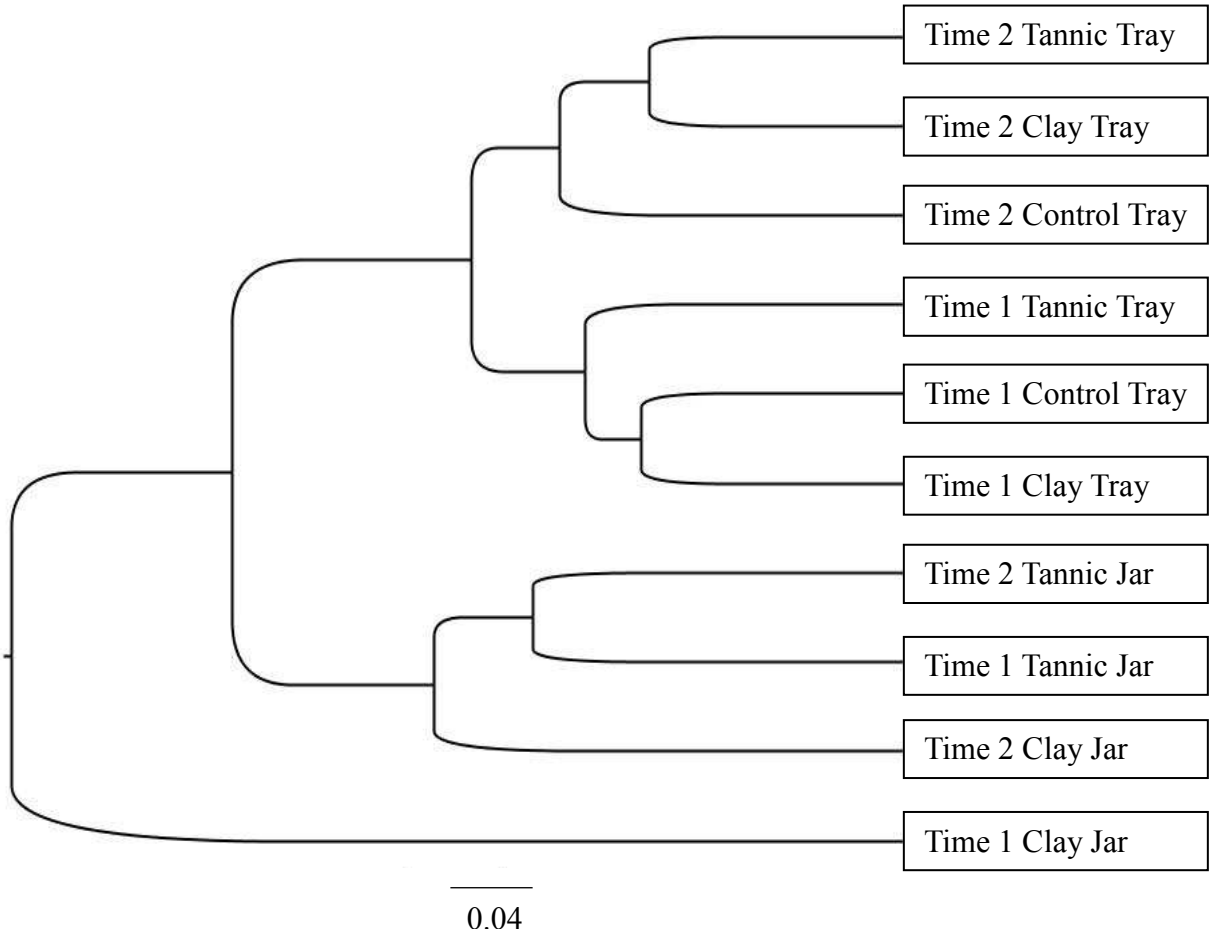

Figure 2. Neighbor-joining tree construct using Bray-Curtis dissimilarity for lake sturgeon egg microbial communities as a function of embryonic development period (Development period 1 30 to 36 CTU and development period 2 55 to 67 CTU; Kempinger 1988) and de-adhesion and incubation conditions.

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.

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CHAPTER II: EFFECTS OF EGG CHEMOTHERAPEUTANTS ON BODY SIZE, SURVIVAL, OXYGEN CONSUMPTION RATE, AND MICROBIAL DIVERSITY, OF FISH EGGS

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 mm²), and body area (BA mm²)) 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 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).

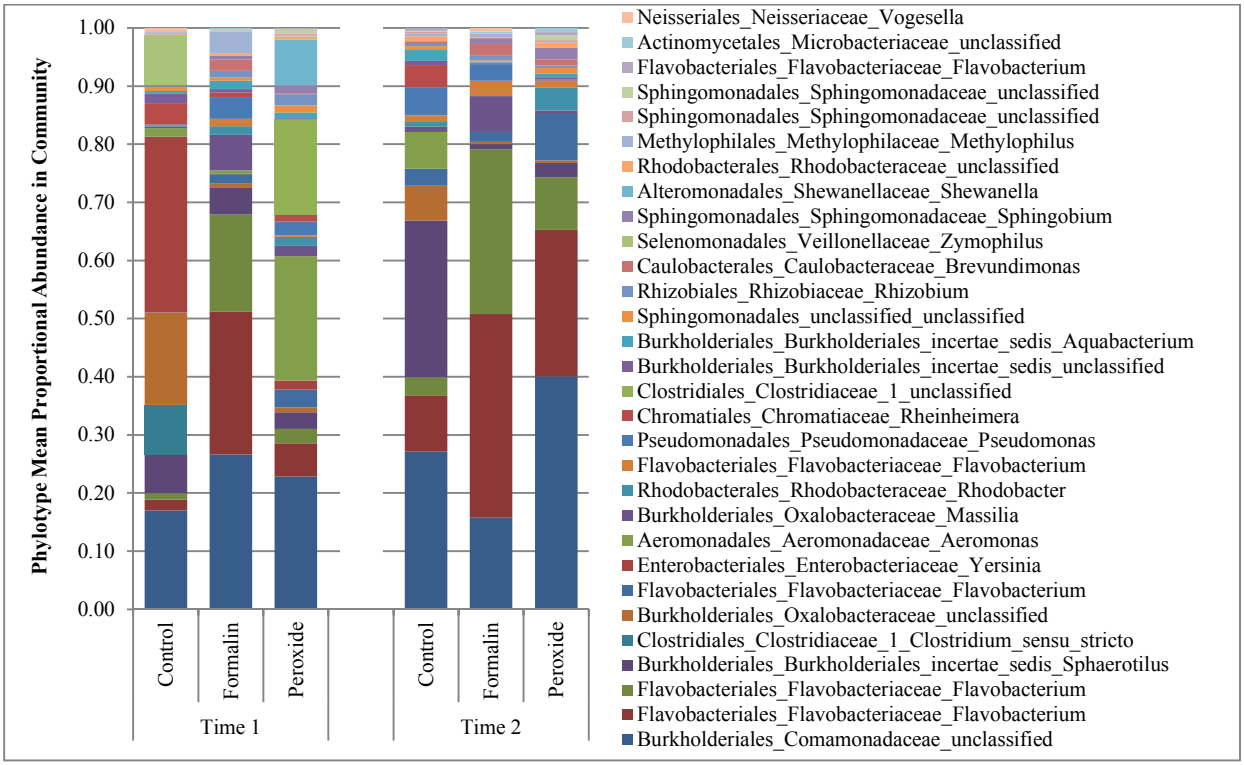

Figure 3. Microbial phylotype mean proportional abundance as a function of embryonic development time and chemotherapeutant treatment. Thirty major phylotypes represented in the community sample based on 5 percent contribution to total operational taxonomic units. Each unique color represents a unique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

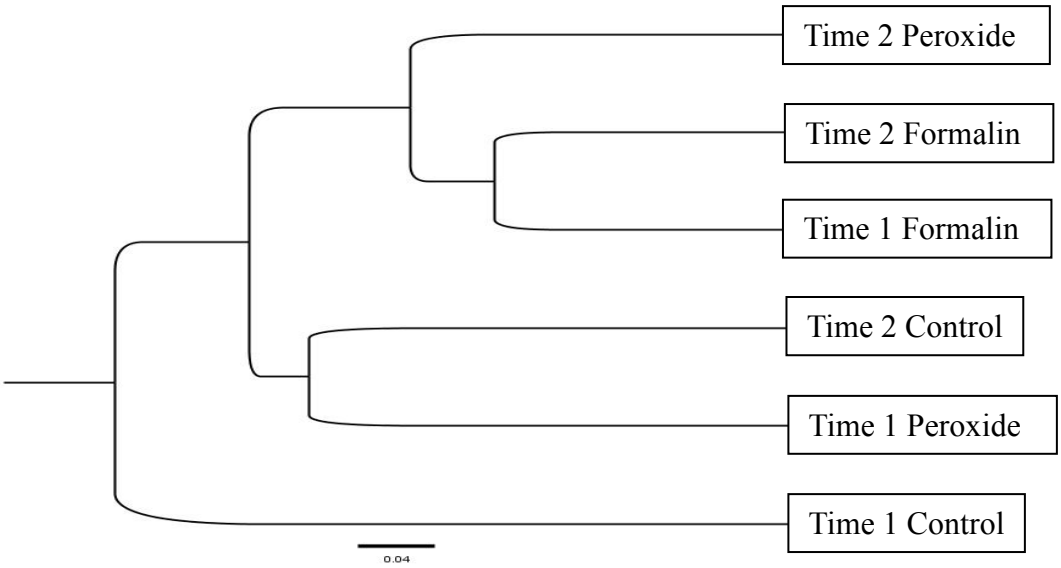

Figure 4. Neighbor-joining tree construct using Bray-Curtis dissimilarity for lake sturgeon egg microbial communities as a function of embryonic development period (Development period 1 30 to 36 CTU and development period 2 55 to 67 CTU; Kempinger 1988) and chemotherapeutant treatment.

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.

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LITERATURE CITED

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CHAPTER III: EFFECTS OF REARING DENSITY ON BODY SIZE AND SURVIVAL OF LAKE STURGEON (ACIPENSER FULVESCENS) FREE-EMBRYOS

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 m², 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.

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LITERATURE CITED

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CHAPTER IV: BODY SIZE AND SURVIVAL OF HATCHERY- AND WILD-PRODUCED LARVAE AS A FUNCTION OF FEEDING FREQUENCY AND ALTERNATE FOOD TYPE

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 ($F_{1, 29} = 5.54$, $P = 0.0255$) on weight per fish at 30 days post-exogenous feeding was detected however, no effect of family was detected ($F_{1, 29} = 3.09$, $P = 0.0894$). Mean 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) ($t_{29} = -2.35$, $P = 0.0255$) (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 ($F_{1, 29} = 0.29$, $P = 0.5939$ and $F_{1, 29} = 0.09$, $P = 0.7661$, 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 ($F_{5, 39} = 113.36$**,** $P < 0.0001$). Mean 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.

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LITERATURE CITED

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CHAPTER V: SURVIVAL OF LAKE STURGEON AS A FUNCTION OF DIFFERENT CHEMOTHERAPEUTANT PROPHYLACTICS

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.

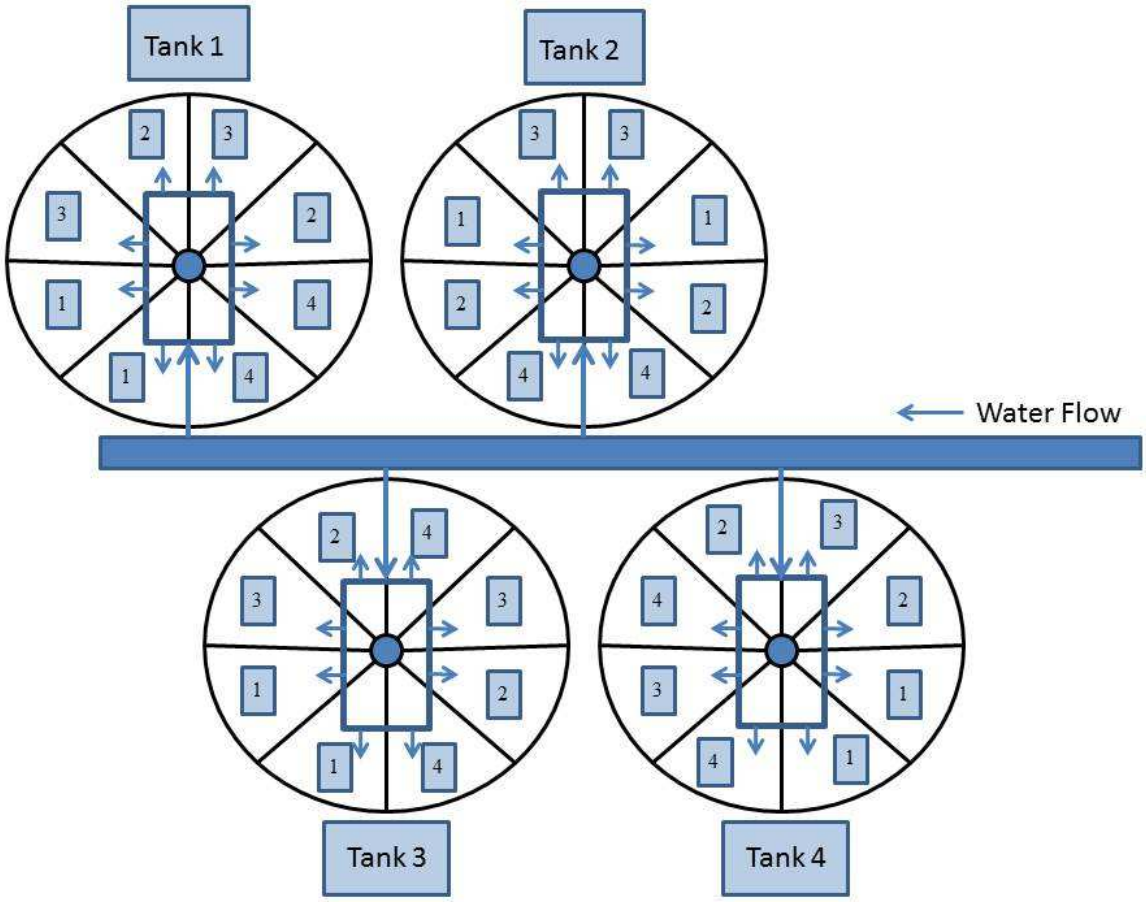

Figure 5. Experimental design of larval chemotherapeutant study. Each 1.2 m diameter tank held separate, 400 fish from hatchery- and wild naturally-produced fish which were split into four tanks and sub-divided into eight equal-sized sections (50 fish per section). Each partition was randomly assigned to one of four weekly treatment types, each with two replicates. 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.

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