



Shaken, rattled and rolled: The effects of hatchery-rearing techniques on endogenous cortisol production, stress-related gene expression, growth and survival in larval Lake Sturgeon, *Acipenser fulvescens*

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ABSTRACT

Sturgeon, one of the most endangered families of vertebrates, are of high conservation concern with population restoration efforts worldwide. Sturgeon species are often raised in conservation hatcheries to supplement wild populations with little understanding of effects of hatchery-related stressors on early development. Lake Sturgeon hatcheries deviate from natural conditions by coating adhesive embryos in clay to prevent clumping, embryos are then added to tumbling jars, colloquially referred to as McDonald Jars; a cone or cylinder-shaped container with constant flow, causing the embryos to roll and move throughout the water column. In the wild, Lake Sturgeon embryos adhere to substrate in fast-flowing rivers, where orientation is maintained throughout embryogenesis. In this study, we examined the effects of hatchery early-rearing environment, i.e. the use of tumbling jars, on gene expression, endogenous cortisol production, growth, and survival in larval Lake Sturgeon. We compared pre- and post-hatch tumbled larvae to non-tumbled larvae that were allowed to adhere to substrate throughout embryogenesis. We hypothesized that deviating from natural rearing conditions, with the use of tumbling jars, would result in changes to cortisol production, growth and mortality in post-hatch, tumbled larvae. Endogenous cortisol production was delayed one week for fish in the tumbled treatment (two-factor ANOVA; $P < .05$) when compared to non-tumbled fish. Tumbled fish were also smaller throughout development, with lasting effects on growth 62 days after hatching ($P < .05$). The tumbled fish had 23% mortality post-hatch compared to 6% in the non-tumbled group (Cox Proportional-Hazards Model; $P < .0001$). There were differences in gene expression in early development at specific time points, between the two treatment groups, in *StAR* and *hsp70* ($P < .05$). The observed developmental effects on endogenous cortisol production and growth in the tumbled embryos could be counterproductive to the goal of producing a hatchery-reared fish that can survive in the wild. As conservation hatcheries aim to release individuals that will supplement and reproduce with wild populations, changes to developmental timing, growth and survival should be avoided. The findings of this study suggest that tumbling jars may not be appropriate in conservation hatcheries for Lake Sturgeon as they may have long-term negative consequences on individual fitness.

1. Introduction

Rearing environment during early development in fishes can have lasting effects on life-history (Jonsson and Jonsson, 2014). This is of particular concern in conservation hatcheries where common rearing practices have been shown to have unintentional effects on phenotypic development and fitness in fishes (Frankham, 2008; Fraser, 2008; Jackson and Brown, 2011). In salmonids, reduced reproductive success

in hatchery-raised fish has been shown to occur within one to two generations, compared to wild counterparts (Araki et al., 2007; Christie et al., 2014; Ford et al., 2016). While the consequences of hatchery environments have been best described in salmonids raised for conservation purposes (Frankham, 2008), conservation hatcheries as a stock enhancement strategy are also used extensively in sturgeon recovery programs. Similar to patterns observed in salmonids, effects of early rearing environment on short-term phenotypic development have

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been demonstrated in White Sturgeon, *Acipenser transmontanus* (Baker et al., 2014; Bates et al., 2014) and Lake Sturgeon, *Acipenser fulvescens* (Crossman et al., 2014; Dammerman et al., 2015; Yoon et al., 2019).

In the wild, adult Lake Sturgeon spawn over gravel, cobble or large boulders in fast-flowing rivers (LaHaye et al., 1992; Auer, 1996; McKinley et al., 1998; Chiotti et al., 2008). The embryos adhere to the substrate where they develop until hatch, the timing of which is mostly dependent on temperature (Kempinger, 1988; Johnson et al., 2006). The eggs' surface adhesiveness maintains them on the substrate, limiting egg dispersal and likely influencing survival and hatching condition (Siddique et al., 2016). Conversely, in most sturgeon conservation hatcheries, once fertilized, embryos are de-adhered by gently stirring the eggs in a fine clay or silt solution for one-hour (Sadov and Kokhanskaya, 1963; Monaco and Doroshov, 1983). Embryos are then added to tumbling jars, colloquially referred to as McDonald Jars; a cone or cylinder-shaped container with constant flow, causing the embryos to roll and move throughout the water column. This type of incubation jar (i.e., McDonald jar) was originally created by Marshall McDonald to reduce the amount of fungus on dead eggs and to decrease manual labor needed to remove dead eggs from tanks, as the tumbling motion of the jar allowed dead embryos to float to the top while viable embryos remained submerged (McDonald, 1883). However, in a study with Green Sturgeon, *Acipenser medirostris*, embryos tumbled in McDonald jars had significantly reduced survival, 66–99% lower, compared to those incubated in an upwelling system (Van Eenennaam et al., 2008). Importantly, further development post-hatch was not investigated in that study and to our knowledge, the post-hatch effects of tumbling jar incubation techniques compared to embryos allowed to naturally adhere to a surface have not been evaluated in Lake Sturgeon.

Fish reared in conservation hatcheries can be subjected to various stressors, which may negatively affect development of the hypothalamic-pituitary-interrenal (HPI) axis (Barton and Iwama, 1991; Pickering, 1993; Van Eenennaam et al., 2008; Tsalafouta et al., 2014). As a glucocorticoid, cortisol is a major regulator of energy balance and the endocrine stress response (Wendelaar-Bonga, 1997; Mommsen et al., 1999). Synthesis of cortisol is reliant on the rate limiting step of steroidogenic acute regulatory protein (*StAR*) shuttling cholesterol from the outer to inner mitochondrial membrane (Stocco, 2001). Once at the target site, cortisol binds to glucocorticoid receptors such as GR1. Glucocorticoid receptors play a role in multiple early developmental processes across vertebrates (Nesan et al., 2012; Nesan and Vijayan, 2016; Kulkarni and Buchholz, 2014). Early alterations in the development of the HPI axis has resulted in cardiovascular and skeletal deformities, behavioural changes, and mortality in zebrafish, *Danio rerio* (Nesan et al., 2012; Nesan and Vijayan, 2016; Best et al., 2017; Best and Vijayan, 2018). Additionally, common hatchery stressors during embryogenesis and early development have been linked to changes in the timing and development of the HPI axis in European Sea Bass, *Dicentrarchus labrax*, and Rainbow Trout, *Oncorhynchus mykiss* (Auperin and Geslin, 2008; Tsalafouta et al., 2014; Varsamos et al., 2006; Fokos et al., 2017). Similar stressors have also been shown to increase mRNA expression of heat shock protein *hsp70*, a gene commonly used as a biomarker in thermal stress studies for its role in helping to protect cells from protein destabilization and aggregation (Somero, 2002; Currie, 2011; Liu et al., 2017; Źwirowski et al., 2017). Furthermore, mRNA expression of *hsp70* has been shown to increase in response to non-thermal stressors such as overcrowding (Gornati et al., 2004), handling (Eissa et al., 2017), and feed deprivation (Cara et al., 2005), during early development in European Sea Bass, Yellow Perch, *Perca flavescens*, and Rainbow Trout, respectively.

In the present study, we compared the effects of a typical hatchery rearing technique, the tumbling jar, to embryos adhered to a surface, mimicking natural conditions. Specifically, we measured whole-body cortisol production, mRNA expression of *StAR*, *GR1*, and *hsp70*, as well as growth and mortality for at least 35 days post fertilization (DPF). We tested the hypothesis that deviation from natural rearing conditions

would affect development of the HPI axis, growth and mortality by altering success in the transition from endogenous (yolk-sac) to exogenous (artemia) feeding. We predicted that embryos reared in tumbling jars would have slower growth, higher mortality post-hatch and altered gene expression patterns associated with the development of the cortisol response when compared to the embryos that could adhere to a surface. Further, we predicted that the embryo rearing treatments would have a differential effect on the timing of HPI axis development, which may be linked to changes in mortality and growth, as fish transitioned from yolk to an exogenous food source (Earhart et al. Unpublished).

2. Methods

2.1. Fish husbandry

In May 2018, gametes were collected from wild-caught female and male Lake Sturgeon captured downstream of Pointe du Bois (50°17'52 N, 95°32'51 W) on the Winnipeg River, Manitoba, Canada. Eggs and milt were transferred to the University of Manitoba, Winnipeg, Manitoba, for fertilization within 4 h of collection during which they were transported in a cooler with river water and icepacks to keep the gametes cool. Eggs from one female were mixed with sperm from one male to avoid possible family effects across treatments. About a 1:100 ratio of sperm to eggs was added for fertilization in water (Yoon et al., 2019). Fertilization was allowed to take place for 1 min, after which embryos were washed three times with dechlorinated water. The full-sibling embryos were then divided into two treatments of approximately 1000 eggs each. One treatment was immediately evenly distributed onto two mesh egg mats (25.4 cm L × 24.1 cm W; Fig. 1) and allowed to adhere to the mesh at a density of approximately 0.82 embryos cm^{-2} which remained there until hatch, hereafter referred to as the 'non-tumbled' treatment. The second treatment, hereafter referred to as the 'tumbled' treatment, was submerged into a clay solution and gently stirred by hand for one hour (i.e., Fullers Earth; Ringle et al., 1992; Dunham and Elawad, 2018) to reduce embryo adhesion after which they were rinsed thoroughly with de-chlorinated fresh water. Embryos were then added to a 7 L tumbling jar and incubated in the tumbled treatment (Monaco and Doroshov, 1983; Siddique et al., 2016) at a density of approximately 13.9 embryos cm^{-2} or 143 embryos L^{-1} , respectively. A gentle tumbling motion was achieved in the tumbling jar at a flow rate of 2 L min^{-1} , adjusted slightly throughout the incubation period to maintain this gentle rolling motion over the eggs. A linear flow of 118.9 L min^{-1} (not accounting for edge effects) across the non-tumbled eggs was achieved by positioning a small submersible fan at the upstream end of the egg mats to simulate river flow across the developing embryos. Temperature for both treatments was maintained at 12 °C until hatch (8 DPF; completed in 24 h), and oxygen maintained above 8 ppm with a 12 h light:12 h dark cycle. At hatch, larvae were transferred to four identical 9 L aquariums, two for each treatment, at equal densities of approximately 15 larvae L^{-1} , supplied with constant aeration and flow-through dechlorinated tap water. At 35 DPF, larvae were separated into three tanks each, six in total with equivalent stocking density.

Water temperature was increased to 16 °C, at a rate of 1 °C daily following transfer and all tanks had equal numbers of sinking bio-balls as substrate and refuge for the yolk-sac larvae, until exogenous feeding began, after which substrate was removed over a 7 d period. Once larvae began to emerge from substrate in their pelagic phase (Peterson et al., 2007), live artemia (Brine Shrimp Direct; Ogden, Utah) were introduced to the tanks at 15 DPF. Onset of exogenous feeding was not observed until 21 DPF. Three weeks following the onset of exogenous feeding, bloodworms (Hikari USA; Hayward, California) were slowly introduced into the diet and fish were gradually transitioned to an exclusively bloodworm diet over four weeks. Larval Lake Sturgeon were fed to satiation throughout the experiment. Tanks were checked three

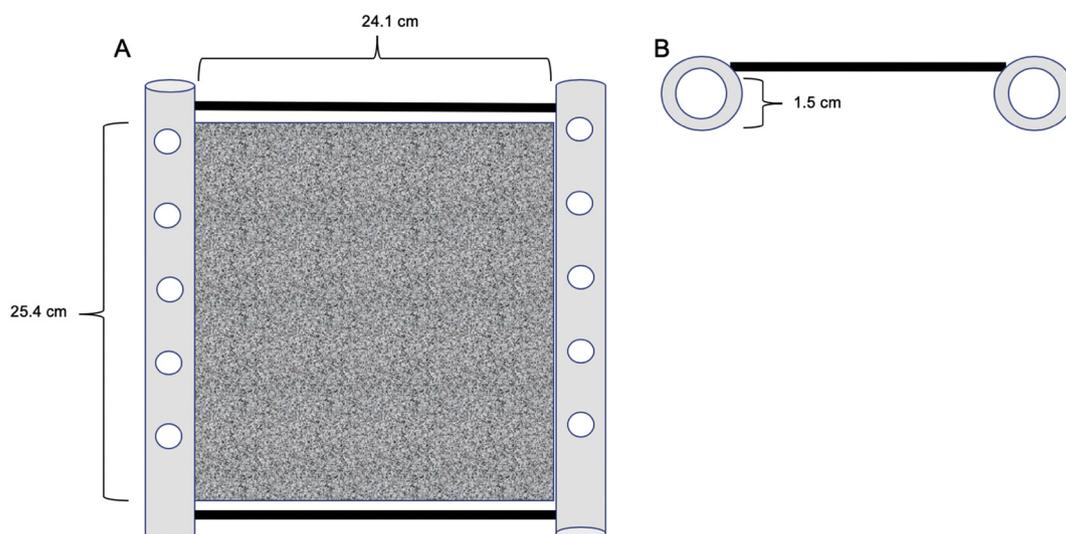


Fig. 1. Diagram of egg mat used to incubate non-tumbled embryos. A) Top view B) Front view. Grey cylinders represent 1.3 cm (1/2 in) PVC pipe with drilled holes to ensure submersion. Black bars represent stabilizing poles used to prevent mat from moving. The mat was made from plastic-coated mesh screen. Dimensions on diagram are of the mesh mat, not including PVC. The mat was suspended on top of PVC pipes, with silicone, to lift the mat off the tank bottom.

times daily to remove debris and mortalities, which were recorded throughout the experiment. All animals used in this study were reared and sampled according to animal use and care guidelines established by the Canadian Council for Animal Care and approved by the Animal Care Committee at the University of Manitoba (#F15-007).

2.2. Growth

Sampling for growth was done haphazardly from replicate tanks throughout the experiment and each tank was treated the same, with equal number of embryos or larvae removed at each sampling point, until 35 DPF. Embryos were sampled daily ($n = 8$) to assess any changes in the diameter of the embryo, including the chorion, which was measured to the nearest 0.01 mm using digital calipers. Following hatch, larvae were measured weekly for whole-body length using calipers to the nearest 0.01 mm, wet mass to the nearest 0.001 g. Yolk-sac volume, in mm^3 , was calculated as the product of the length, width, and height of the yolk-sac as measured by calipers at the widest point for each dimension, at 10 and 14 DPF. Specifically, the larvae were positioned on their side for measuring the height and length of the yolk-sac and positioned on their back to measure the width. Following the onset of exogenous feeding, larvae were measured weekly for length (mm; $n = 8$) and wet mass (g; $n = 8$) until 35 DPF, after which they were sampled at 49, 60, and 70 DPF ($n = 8$ for length and $n = 16$ for mass). All length and body mass measurements were completed following euthanasia by immersion in an overdose of anesthetic (MS-222 0.5 g L^{-1}) buffered with equal volumes of sodium bicarbonate. Larvae were sampled haphazardly, with 5, 5, and 6 larvae sampled from one of the three tank replicates, respectively. Energy density was estimated based on dry to wet mass ratio using the technique and equation recently described in Yoon et al. (2019). Fulton's condition factor (Fulton, 1911) was calculated as $K = ((\text{mass}/\text{length}^3) \times 100)$.

2.3. Baseline cortisol levels

Individual embryos were haphazardly sampled from tumbled and non-tumbled treatments every second day until hatch (which started at 7 DPF), and then larvae were sampled weekly until 35 DPF. An equal number of embryos and larvae (4 from each tank) were removed at each sampling time point. To obtain baseline cortisol levels, individuals were euthanized as described above. The number of individuals needed at each time-point was dictated by the minimum detectable limit of the

cortisol assay (see below). An $n = 8$ was used for each time point, but at the embryonic stage, five embryos were pooled for each sample ($n = 40$), and then immediately following hatch, 2–3 individuals were pooled for each sample ($n = 16$ –24). Total body length and wet mass were recorded for all individuals and samples were then frozen at -80°C until analysis. To avoid introducing bias due to diurnal changes in cortisol (Lankford et al., 2003), sampling was completed between 15:00–16:00 h each day.

Whole-body samples were homogenized with phosphate-buffered saline (PBS; 0.9% NaCl in PB and pH 7.4) and cortisol was extracted using solid-phase extraction with C18-Sep-Pak cartridges (Waters Corporation; Milford, MA, USA) as previously described (Zubair et al., 2012). Samples were then frozen at -80°C until further use. On the day of the assay, each sample was individually reconstituted in 250 μL of radioimmunoassay (RIA) buffer (90 mL Mili-Q water, 10 mL PBS, 0.9 g NaCl, and 0.5 bovine serum albumin). Following reconstitution, each sample was split into duplicates with 100 μL sample $^{-1}$. One hundred μL of titrated cortisol ~ 5000 disintegrations min^{-1} (Perkin Elmer, Waltham, MA, USA) and 100 μL of cortisol antibody (1:100,000; rabbit anti-cortisol polyclonal antibody; Fitzgerald Industries, Acton, MA, USA) were then added to each duplicate. After vortexing and 1 h incubation at room temperature, samples were incubated overnight at 4°C . The following day the assay was terminated by the addition of 100 μL charcoal separation buffer (1.25 g charcoal and 0.125 g dextran in 25 mL RIA buffer) to each sample. Following vortexing, samples were left on ice for 15 min and then centrifuged at 4°C for 30 min at 2500g (Hereaus Multifuge X3R, Thermo Fisher Scientific). The supernatant was decanted into a 6 mL scintillation vial and 4 mL of scintillation fluid was then added (Ultima Gold, Perkin Elmer). Samples were then counted, for 5 min, on a liquid scintillation counter (Tri-Carb 3110 TR; Perkin Elmer). The minimum detectable limit for the RIA was 0.013 ng g^{-1} and serial dilutions of plasma showed good parallelism with the standard curve (data not shown). Unknown cortisol values were determined by interpolating against a standard curve, generated with each assay. Intra-assay variation ($n = 10$) and inter-assay variation ($n = 10$) were 9% and 7% respectively and extraction efficiency was $98.9\% \pm 1.15\%$ ($n = 10$).

2.4. Gene expression

Ten larvae were haphazardly selected at 10, 14, 21, 28 and 35 DPF from each treatment and transferred to an overdose of MS-222 as

described above. Larvae were sampled equally from all tank replicates at each time point, i.e. 5 from each tank. The individuals were then added to a vial containing 500 μL of RNAlater (Invitrogen; Thermo Fisher Scientific). Samples were stored at 4 °C for 24 h and then transferred to –80 °C until subsequent analyses. Total RNA was extracted from whole-body homogenates using a Purelink RNA mini kit following the manufacturer's instructions (Invitrogen; Ambion Life Technologies). Total RNA purity and concentration was assessed for all samples using a Nanodrop One (Thermo Fisher Scientific) and integrity was tested by gel electrophoresis. Samples were then stored in –80 °C until further use.

The cDNA was synthesized from 1 μg of DNase treated RNA using a qScript cDNA synthesis kit following the manufacturer's instructions (Quantbio; Beverly, Massachusetts). The cDNA reactions were run on a SimpliAmp Thermal Cycler (ThermoFisher; Waltham, Massachusetts) with the following conditions: 1 cycle of 22 °C for 5 min, 1 cycle of 42 °C for 30 min and 1 cycle of 85 °C for 5 min, and then held at 4 °C. The cDNA was then stored in a –20 °C freezer until further use. Post cDNA synthesis, qPCR was conducted using a 10-fold dilution of cDNA template for all target genes with the exception of *hsp70* for which cDNA template was diluted by 100-fold.

Real-time quantitative polymerase chain reaction (RT-qPCR) for each gene was completed with a master mix of Bio-Rad SsoAdvanced Universal SYBR Green Supermix (5 μL per sample) and nuclease-free water (2 μL per sample) (Bio-Rad). Genes of interest included *StAR*, *GRI*, and *hsp70* (Table 1). *StAR* primers were based on published primers designed from White Sturgeon and share 60% homology with other vertebrates (Kusakabe et al., 2009). All other primers were created using sequences from a Lake Sturgeon ovary transcriptome produced through pyrosequencing (Hale et al. 2009). Primer efficiency was validated by using a 1:10 dilution standard curve for all primers (Table 1). All reactions were run using a QuantStudio 5 (Applied Biosystems; Thermo Fisher Scientific) on a 384-well plate and under the same conditions: 2 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Melt curves were determined by denaturation for 15 s at 95 °C, a decrease for 1 min down to 60 °C and followed by a gradual increase of 0.075 °C s⁻¹ to 95 °C. Amplification data was analyzed using the delta delta Ct method described by Livak and Schmittgen (2001) normalizing genes of interest to the relative expression of two reference genes, *RPL7* and *EFA1- α* .

2.5. Mortality

Mortalities were checked for and recorded 3 times daily in all tanks. Fertilization success was observed in both treatments (< 80% of embryos) and unfertilized eggs or dead embryos were removed 3 times daily. Volume of embryos removed was recorded, but the exact count of dead individuals was not recorded until hatch. Following hatch (8 DPF), all individuals were counted and were divided evenly among replicate tanks. Any obvious deformed larvae, such as kinked tails or edemas, were culled and not included in analysis. These were rarely observed in either treatment and none were observed at the time of hatch. Mortalities were counted and recorded at all 3 checks and these 3 daily checks were then combined into one number for that day. Cumulative mortality was determined by the summation of daily mortalities over

the first 35 days.

2.6. Statistical analysis

Whole-body cortisol, body measurements and mRNA abundance of target genes were analyzed using two-factor ANOVAs. Embryo treatment, developmental day and their interaction were included in the model as fixed effects (embryo treatment x developmental day). Tank effects were included in original models as random effects, but were not significant for embryo diameter, larval length, weight, or any gene expression. Therefore, tank effects were not included in the final model. Statistical analyses were performed using R v3.5.2 (Core Team, 2013) with a significance level (α) of 0.05. Tukey's honestly significant difference post-hoc tests, from the “multcomp” package (Hothorn et al., 2008) in R were used to assess significance. Normality of data and homogeneity of variance were assessed using Shapiro-Wilks and Levene's tests, respectively, as well as visual inspection of normal probability plots and fitted residuals. If normality or homogeneity of variance assumptions were violated, either a rank or log transformation was applied to the data.

A Cox proportional hazards model, conducted in R using the “survival” package (Therneau, 2015), was used to evaluate differences in mortality between treatments in the first 35 DPF. This model is used for assessing time to event survival data (Cox and Oakes, 1984; Jeffrey et al., 2018), which is therefore appropriate for our dataset. Assumptions of the model were assessed and there was no significant relationship between residuals and time. Embryo treatment was included in the model as a fixed effect. Tank effects were assessed as above and found to be not statistically significant and therefore were not included in the final model.

3. Results

3.1. Growth

Embryo diameter, throughout pre-hatch development, was larger in non-tumbled eggs compared to the tumbled treatment (Treatment $P < .0001$, Day $P < .0001$ and Interaction $P < .0001$) and significantly different at 1–5 DPF ($P < .0005$; Table 2). Twenty-four h after fertilization the tumbled treatment embryos had a diameter of 3.26 mm and the non-tumbled treatment had a diameter of 3.51 mm. Hatch began at 8 DPF and was completed by 10 DPF, with the majority of larvae hatching on day 9. Yolk-sac volume of larvae was measured at both 10 and 14 DPF. At 10 DPF, non-tumbled larvae had yolk-sacs that were 19.8% larger than their tumbled counterparts ($P < .0001$; Table 2); however, by 14 DPF there were no significant differences in yolk-sac volume between treatments.

Overall body mass was significantly higher in the non-tumbled treatment (Treatment $P < .0001$, Day $P < .00001$, Interaction $P < .05$). Specifically, body mass was significantly different at 28, 35, 49 and 70 DPF ($P < .05$; Table 2) and fish in the non-tumbled treatment were 26.1% heavier than their tumbled counterparts by the end of the experiment (70 DPF). Overall, length was significantly greater in the non-tumbled treatment (Treatment $P < .0001$ and Day $P < .0001$). Length showed a similar trend to mass, with significant

Table 1

List of forward and reverse primer sequences used in this study for larval Lake Sturgeon (*Acipenser fulvescens*) *StAR*, *GRI*, *hsp70*, *RPL7* and *EFA1- α* . Efficiencies are listed as %.

Gene	Forward	Reverse	Efficiency %
<i>StAR</i>	CCGAGCAAAAAGGCTTCA	TTGGGCCGAAGAACAATACAG	99.7
<i>GRI</i>	GCCGGGATCAACAGCTACTT	CCAGGTGTGGACAACCTCGAT	104.9
<i>hsp70</i>	CGTTCACCTCGGACTTTAACTTTAATTT	CGTTCACCTCGGACTTTAACTTTAATTT	99.5
<i>RPL7</i>	TGCTTAGGATTGCTGAGCCG	GATCTTCCGTGACCCCGTT	98.0
<i>EFA1-α</i>	GGTGTGCTTCCAGCTTCTTACCG	ACTGCTCACATGCGCTGCA	98.8

Table 2

Body measurements in non-tumbled and tumbled treatments throughout development in larval Lake Sturgeon (*Acipenser fulvescens*). Significance determined via two-factor ANOVA ($P < .05$) followed by Tukey's Honestly significant difference post-hoc analysis. Data are represented as a mean \pm SEM ($n = 8-16$ per treatment) asterisks represent significant differences between treatments at each sampling time point. Sample number (n) is in parentheses after each SEM.

Days post fertilization	Non-tumbled	Tumbled
Embryo diameter (mm)		
1	3.51 \pm 0.04 (8)	3.26 \pm 0.03* (8)
2	3.63 \pm 0.04 (8)	3.37 \pm 0.02* (8)
3	4.11 \pm 0.02 (8)	3.46 \pm 0.05* (8)
4	4.25 \pm 0.02 (8)	4.12 \pm 0.01*(8)
5	4.55 \pm 0.04 (8)	4.29 \pm 0.04* (8)
6	4.57 \pm 0.06 (8)	4.57 \pm 0.03 (8)
7	4.58 \pm 0.04 (8)	4.52 \pm 0.04 (8)
8	5.13 \pm 0.06 (8)	4.87 \pm 0.09* (8)
Yolk-Sac volume (mm³)		
10	18.62 \pm 0.42 (8)	15.54 \pm 0.47* (8)
14	11.34 \pm 1.44 (8)	10.25 \pm 0.8 (8)
Wet mass (mg)		
14	20.61 \pm 1.91 (8)	19.24 \pm 2.48 (8)
21	32.3 \pm 2.28 (8)	30.16 \pm 1.75 (8)
28	50.83 \pm 5.54 (8)	38.49 \pm 2.09* (8)
35	100.34 \pm 6.28 (16)	85.69 \pm 12.05* (16)
49	347.23 \pm 52.22 (15)	309.61 \pm 44.04 (16)
60	732.32 \pm 121.28 (16)	678.39 \pm 57.56 (15)
70	1107.14 \pm 131.85 (16)	883.89 \pm 119.32* (15)
Length (mm)		
14	15.07 \pm 0.17 (8)	14.56 \pm 0.31 (8)
21	20.99 \pm 0.19 (8)	19.99 \pm 0.19* (8)
28	25.04 \pm 0.19 (8)	23.06 \pm 0.22* (8)
35	31.9 \pm 0.35 (8)	30.29 \pm 0.57* (8)
49	46.74 \pm 0.99 (8)	45.3 \pm 0.75 (8)
60	58.80 \pm 1.18 (8)	59.27 \pm 1.42 (8)
70	71.67 \pm 1.25 (8)	65.97 \pm 0.9* (8)
Fulton's condition factor (mg mm³ (100))		
14	0.6 \pm 0.04 (8)	0.63 \pm 0.05 (8)
21	0.35 \pm 0.01 (8)	0.38 \pm 0.01 (8)
28	0.32 \pm 0.01 (8)	0.32 \pm 0.01 (8)
35	0.32 \pm 0.01 (8)	0.33 \pm 0.01 (8)
49	0.32 \pm 0.02 (8)	0.31 \pm 0.01 (8)
60	0.32 \pm 0.01 (8)	0.33 \pm 0.02 (8)
70	0.3 \pm 0.01 (8)	0.3 \pm 0.01 (8)
Energy density (kJ.g⁻¹)		
28	3012.9 \pm 68.2 (8)	2950.4 \pm 56.8 (8)
35	2274.4 \pm 86.5 (8)	2145.7 \pm 24.3 (8)
49	2364 \pm 97.5 (8)	2425.3 \pm 23.8 (8)
60	2542.23 \pm 93.1 (8)	2599.2 \pm 65.2 (8)

differences apparent at 21, 28, 35 and 70 DPF ($P < .05$; Table 2) and fish in the non-tumbled treatment were 8.6% longer than their tumbled counterparts at 70 DPF. Fulton's condition factor and energy density estimates (Table 2) showed no significant differences between non-tumbled and tumbled treatments at any time-point throughout development.

3.2. Baseline whole-body cortisol

Baseline whole-body cortisol levels were significantly higher in non-tumbled larvae at 8 and 10 DPF ($P < .05$; Fig. 2). The non-tumbled treatment showed significant increases in cortisol production beginning at 8 DPF, whereas this was delayed until at least 14 DPF in the tumbled treatment. While the magnitude of increase in whole-body baseline cortisol was similar between treatments over time, the time at which significant increases occurred throughout development differed between treatments.

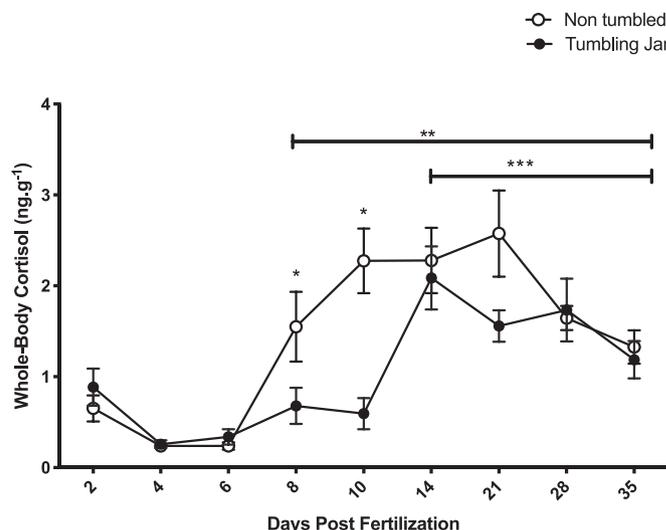


Fig. 2. Whole-body cortisol (ng g^{-1}) throughout development in tumbled (closed circles) and non-tumbled (open circles) treatments in larval Lake Sturgeon (*Acipenser fulvescens*). * indicates significant difference between treatments $P < .05$ (two-factor ANOVA). Statistical differences in developmental period for whole-body cortisol are indicated by ** and *** in the non-tumbled and tumbled treatments, respectively. The non-tumbled treatment had significantly higher cortisol 8–35 DPF when compared to 4–6 DPF ($P < .0001$, two-factor ANOVA) and the tumbled treatment had significantly higher cortisol 14–35 DPF when compared to 4–10 DPF ($P < .05$, two-factor ANOVA). Data are expressed as a mean \pm SEM; $n = 7-8$.

3.3. mRNA transcript expression

StAR mRNA expression was 1.7 fold higher ($P < .05$; Fig. 3A) in non-tumbled larvae relative to their tumbled counterparts at 14 DPF. In the non-tumbled treatment, at 14 DPF, there was significantly higher expression when compared to 28 and 35 DPF ($P < .05$) where expression was downregulated. In the tumbled treatment at 21 DPF, there was significantly higher expression when compared to 28 DPF ($P < .05$).

No significant increases in *GR1* mRNA expression were apparent between treatments throughout development (Fig. 3B). When compared to 10 DPF, both non-tumbled and tumbled larvae at 21 DPF showed significant 3.66 and 3.68-fold increases in *GR1* expression, respectively ($P < .05$).

hsp70 mRNA expression was 2.3-fold higher ($P < .05$; Fig. 3C) in tumbled larvae at 21 DPF compared to non-tumbled larvae. There were no differences in expression throughout development for non-tumbled larvae, however in the tumbled treatment, expression was significantly elevated at 21 DPF compared to 28 DPF ($P < .05$).

3.4. Mortality

In the first 30 days, mortality was 6% in the non-tumbled treatment and 23% in tumbled treatment ($P < 1.07\text{e-}10$; Fig. 4) with the vast majority occurring before the onset of exogenous feeding at 21 DPF. During hatch, from days 8–10 there was 1.9% mortality in the non-tumbled treatment vs. 0.17% in the tumbled treatment ($P < .05$), most likely due to the increased fungal growth on the egg mat, although this was not quantified in the experiment. Starting at 11 DPF treatments had similar mortality percentages, 2.6% and 2.8% in the non-tumbled and tumbled respectively. The majority of mortalities occurred between 12 DPF and 21 DPF, during the yolk-sac absorption phase. The tumbling jar increased from 3% to 21% over this period, where the non-tumbled increased from 3% to 6%. Following the onset of exogenous feeding mortality dropped to negligible values in both treatments.

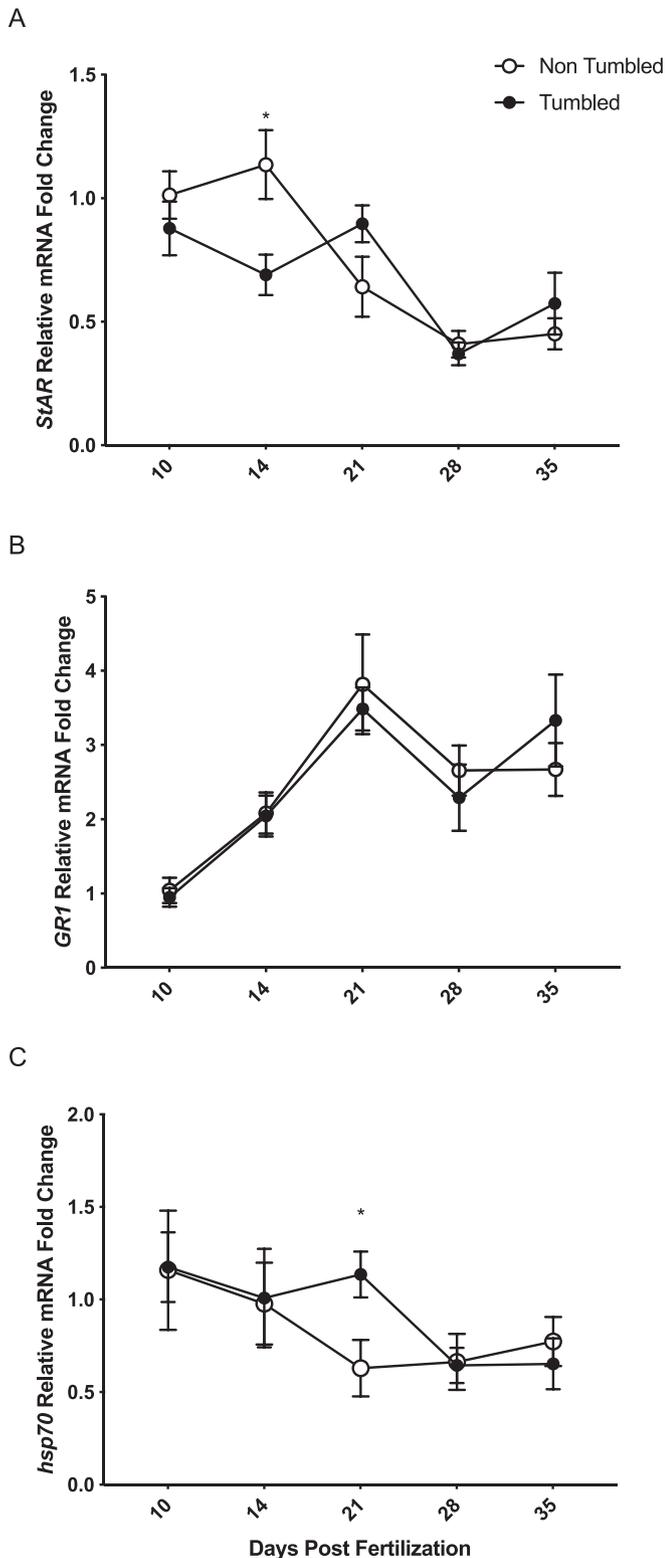


Fig. 3. Expression of A) *StAR*, B) *GRI*, C) *hsp70* in tumbled (closed circles) and non-tumbled (open circles) in larval Lake Sturgeon (*Acipenser fulvescens*). * indicates significance between tumbled and non-tumbled fish ($P < .05$; two-factor ANOVA). Data are expressed as mean \pm SEM [*StAR*: n = 4–8, Non-tumbled: 10DPF = 4, 14DPF = 7, 21DPF = 7, 28DPF = 7, 35DPF = 8; Tumbled: 10DPF = 5, 14DPF = 8, 21DPF = 7, 28DPF = 7, 35DPF = 6. *GRI*: n = 4–8, 10DPF = 4, 14DPF = 7, 21DPF = 8, 28DPF = 7, 35DPF = 8; Tumbled: 10DPF = 5, 14DPF = 8, 21DPF = 7, 28DPF = 8, 35DPF = 7. *hsp70*: n = 4–8, 10DPF = 4, 14DPF = 7, 21DPF = 6, 28DPF = 7, 35DPF = 8; Tumbled: 10DPF = 5, 14DPF = 8, 21DPF = 7, 28DPF = 8, 35DPF = 7].

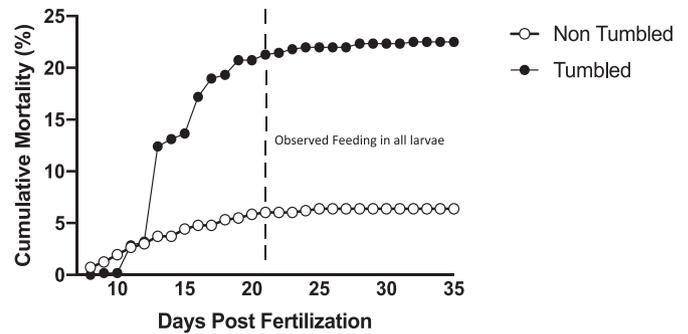


Fig. 4. Cumulative mortality (%) of larval Lake Sturgeon (*Acipenser fulvescens*) treatments throughout development of tumbled (closed circles) and non-tumbled (open circles). Exogenous feeding was first observed at 21DPF for both treatments. Data are expressed as cumulative % mortality from hatch to 35 DPF. Significance between treatments was found at $P = 1.07e-10$, $n = 580$. Statistics completed via Cox proportional hazards model.

4. Discussion

Tumbling jars are a widely accepted rearing practice and have been used in sturgeon hatcheries for decades (Monaco and Doroshov, 1983; Českleba et al., 1985; Dettlaff et al., 1993). Here we have demonstrated significant effects of embryo incubation techniques in one full-sibling family of Lake Sturgeon on all measured parameters during embryogenesis and the first two months post-hatch, with significant differences in embryo diameter as early as 1 DPF. Growth differed between the two treatments, with the tumbled treatment being smaller at many time points pre- and post-hatch. Similarly, first-feeding Sea Bass exposed to “unpredictable chronic low intensity stress”, simulating hatchery practices during development, were smaller in both total length and weight than control fish, however they reported no differences in survival between treatments (Tsalafouta et al., 2015). In Green Sturgeon, tumbling jars at different flow rates led to 68–99% higher mortality compared to upwelling incubators during embryogenesis (Van Eenennaam et al., 2008). The authors attributed this high mortality to the relative thinness of the Green Sturgeon chorion along with the high-impact tumbling compared to the less vigorous upwelling incubation system. Recently, the vigorous stirring, while covering embryos in clay prior to the tumbling jar, has been linked to spontaneous polyploidy in White Sturgeon, which was further correlated with embryo mortality (Van Eenennaam et al., 2020).

In our study, we extended the sampling period beyond hatch and determined an increase from 6%, in the non-tumbled treatment, to 23% in mortality in the tumbled treatment following hatch. The majority of mortalities occurred prior to exogenous feeding. Previous studies using tumbling jars have reported mass mortalities during transition to exogenous feeding in Acipenserids (Gisbert and Williot, 1997; Gessner et al., 2009), a period considered critical in the development of sturgeon larvae (Conte et al., 1988; Nilo et al., 1997). Specifically, Gisbert and Williot (1997) reported most mortalities 9 and 10 days after hatching, and started to decrease 12- and 13-days post hatch, post exogenous feeding. This is similar to the findings in our study, corresponding to 17 and 18 DPF, where there is high mortality, and 20 and 21 DPF, during exogenous feeding, respectively. Gessner et al. (2009) reports exogenous feeding began at day 12, coinciding with both decreased cortisol and mortality after the transition. This is also similar to the results in the current study, as there was an increase in mortality and cortisol prior to all fish transitioning to exogenous feeding. While the absolute days do not necessarily match across studies, we are comparing the specific period of diet transition where impact on mortality rate appears to be quite similar. Tumbling jars are a widely accepted sturgeon rearing technique during embryogenesis, and they may be one causal factor to these observed early mortalities. Importantly,

genetic and environmental variables were controlled for in both treatments post-hatch, implicating rearing environment during embryogenesis as the likely causal factor in the observed differences in growth and mortality. An additional study using multiple families are needed to confirm the stark differences between treatments within one family in this study.

Rearing environment also resulted in differences between treatments in the timing of endogenous cortisol production and mRNA expression of the target genes. Incremental increases in whole-body cortisol in the non-tumbled treatment were observed at time of hatch until first-feeding. This has also been described in White Sturgeon (Simontacchi et al., 2009), but it should be noted these larvae were reared in tumbling jars and there was endogenous production observed prior to hatch, which is different than in our study. However, in the tumbled treatment in our study, large increases in baseline cortisol were not observed until 14 DPF (7 days post-hatch), suggesting a delay in HPI axis development. This is similar to whole-body cortisol in Atlantic Sturgeon, *Acipenser oxyrinchus*, also raised in tumbling jars, where baseline cortisol slightly increased at the time of hatch, with a large increase at the time of exogenous feeding (Gessner et al., 2009). Species differences should also be taken into consideration when comparing across sturgeon species, as both White and Atlantic sturgeon have different life histories than Lake Sturgeon. Further, Rainbow Trout reared at different densities during embryogenesis demonstrated an impaired cortisol response when reared at high densities (Ghaedi et al., 2013) and altered induced cortisol responses following an acute stressor post-hatch (Ghaedi et al., 2015). In the current study, the non-tumbled treatment had significantly higher *StAR* mRNA expression compared to the tumbled group at 14 DPF and the potential delay in *StAR* mRNA expression in the tumbled group coincided with the delay observed in whole-body cortisol. In teleosts, expression of *StAR* mRNA, similar to cortisol, has been shown to begin increasing post-hatch until exogenous feeding, and then decreasing or plateauing after the transition to exogenous feeding (Alsop and Vijayan, 2008; Tsalafouta et al., 2014; Kiillerich et al., 2018). One week during larval development of fishes represents a significant period when substantial metabolic organization is occurring (Gisbert et al., 2001; Boucher et al., 2018). With the pivotal role cortisol plays in controlling metabolic activity (Mommensen et al., 1999), a delay in production may have significant consequences on developmental trajectory in fish incubated in tumbling jars.

The observed differences in timing of whole-body cortisol concentrations and *StAR* expression may be related to the observed differences in growth. Non-tumbled larvae in the present study used more of their yolk-sac from 10 to 14 DPF and had increased survival when transitioning to exogenous feeding compared to tumbled larvae. The majority of mortalities in the tumbled treatment occurred during yolk-sac absorption, potentially indicating a difference in yolk-sac utilization between the two treatments. The differences in mortality during yolk-sac absorption coincides with the timing differences in cortisol levels and growth in the two treatments. Similarly, Tilapia, *Oreochromis mossambicus*, treated with cortisol during yolk-sac absorption, demonstrated increased absorption and growth when compared to those not treated with cortisol (Mathiyalagan et al., 1996). Further, Pacific Threadfin larvae, *Polydactylus sexfilis*, treated with thyroid hormone (triiodothyronine - T3) and cortisol, had accelerated gut differentiation, increased nutrient uptake during first-feeding and increased survival (Brown and Kim, 1995). The same combination of hormones was shown to enhance growth and multiple metabolic enzyme activities in newly-hatched South Asian Carp, *Catla catla* (Khangembam et al., 2017). Therefore, the delay in endogenous cortisol production, observed in the tumbled treatment in this study, could be associated with decreased success before first-feeding that may be linked to cellular mechanisms in the stress response.

In the present study, a significant increase in mRNA expression of *hsp70* was observed during first-feeding in the tumbled treatment, but was absent in the non-tumbled treatment. Increases in *hsp70* mRNA

expression have been demonstrated under a variety of tissues and environmental stressors in fish (Currie, 2011). Sea Bass reared in high stocking densities demonstrated increased expression of *hsp70* in their brain and liver (Gornati et al., 2004), and in a later experiment, their skin and muscle under hatchery transport conditions (Poltronieri et al., 2007). Elevated *hsp70* expression was also observed in the livers of Yellow Perch exposed to an acute handling treatment, at a variety of temperatures and salinities (Eissa et al., 2017). Further, increased *hsp70* protein expression has been reported in food deprived, Gilthead Sea Bream, *Sparus aurata*, Rainbow Trout (Cara et al., 2005), and Rohu, *Labeo rohita* (Yengkokpam et al., 2008). Differences in trends of whole-body cortisol between treatments may explain the observed differences in *hsp70* mRNA expression patterns, as elevated cortisol levels have been shown to reduce expression levels of *hsp70* in rainbow trout, *Oncorhynchus mykiss*, and tilapia, liver and gill tissue (Basu et al., 2001). However, this response was not noted in skeletal muscle or liver in rainbow trout (Currie et al., 2010) and recently high cortisol levels were concurrent with increases in expression of *hsp70* in heat stressed juvenile Lake Sturgeon (Yusishen et al. submitted). Clearly, the interaction between cortisol and heat shock proteins in sturgeon requires further investigation. The long-term effects of changes in cortisol production and associated gene expression during development, in a long-lived species such as Lake Sturgeon, are still unknown. In short-lived species such as Zebrafish, abnormal cortisol levels during early development have been shown to affect the magnitude and timing of the endocrine stress response and boldness behaviour later in life (Nesan and Vijayan, 2016; Best et al., 2017). If similar patterns result in Lake Sturgeon, possible long-term effects could include a reduction in survival; clearly contradictory to the goals of conservation hatcheries.

Conservation hatcheries aim to promote the development of a "wild" phenotype. The present study suggests that for the development of the HPI axis, growth and survival, tumbling jars may not be appropriate for Lake Sturgeon. Tumbling jars were originally created for commercial aquaculture use, to reduce fungal growth, the amount of labour required, and overall costs, and were subsequently introduced to conservation hatcheries to facilitate mass production of fish (McDonald, 1883; Monaco and Doroshov, 1983; Dettlaff et al., 1993). The practice of rolling fertilized eggs with clay can reduce fungal infestation of embryos. However, the potentially negative effects on development and survival of those fish that do hatch, and ultimately may be reintroduced to the natural system, may outweigh those benefits. This study was limited by only one family, and should be replicated with multiple families. Further, it would be worthwhile to investigate different densities, flows and incubator types on eggs during early development. Finally, we examined only a few possible developmental and phenotypic effects of rearing environment during embryogenesis, thus further investigation is required to better understand the longer-term impacts of early development in tumbling jars, in all sturgeon species of conservation concern.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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