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ARTICLE

Effects of Water Filtration and Temperature on Microbial Colonization and Survival of Lake Sturgeon Eggs

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Abstract

Physical and biotic factors that affect development and survival during the embryonic period of fish are major concerns in aquaculture, particularly when individuals are produced using natural surface water sources. We quantified the effects of bacterial community taxonomic composition and abundance, water filtration treatment, and incubation temperature on the development and survival of embryos from Lake Sturgeon *Acipenser fulvescens*, a threatened Great Lakes fish species. Eggs were fertilized and incubated using a two-by-two treatment design by manipulating water treatments (treated [filtered and UV treated] and untreated stream water) and temperature (12°C and 18°C) during incubation. Egg samples from five full-sibling families were exposed to each rearing treatment. At different stages during development, eggs were analyzed for egg surface microbial community composition and abundance using terminal restriction fragment length polymorphism methods based on the 16S rRNA gene and quantitative PCR, respectively. Sanger sequencing identified egg-associated microbial taxa. Microbial community composition derived from terminal restriction fragment length polymorphism was visualized using principal component analysis. Permutational multivariate analysis of variance revealed that the composition of microbial communities that developed on egg surfaces varied as a function of water filtration and temperature. Quantitative PCR analysis revealed that significantly higher microbial abundance was present on surfaces of eggs reared in untreated stream water than on those reared in treated stream water. Temperature did not affect egg surface microbial abundance. Significantly higher egg mortality was documented for eggs reared in unfiltered stream water (mean ± SD = 77.4 ± 17.6%) than for eggs reared in treated stream water (50.3 ± 14.7%). Temperature during incubation did not affect levels of egg mortality. Sequence analysis revealed that certain microbial taxa were associated with eggs characterized by higher survival, including *Acidovorax* spp. and *Massilia* spp., suggesting that they may be candidates for natural probiotics. Filtration and UV treatment of surface water was an effective method for modification of egg microbial community composition and quantity and significantly reduced egg mortality.

Microbial communities immediately develop on fish egg surfaces upon deposition into water during reproduction (Fujimoto et al. 2013, 2018; Llewellyn et al. 2014). Microbial community composition and abundance on fish egg surfaces change over time across the incubation period (Fujimoto et al. 2018) and are also influenced by abiotic

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factors, such as streamflow rates (Fujimoto et al. 2013). While the interactions between microbes and host organisms play a significant role in a host's life history (Llewellyn et al. 2014), the interaction between microbes and a host's embryos and larvae are particularly important to the developmental sequence of the host (Vadstein et al. 2013). Population rates of recruitment are tied to rates of mortality that are especially high in fish during the early ontogenetic stages (Demetrius 2012; Forsythe et al. 2013; Crossman et al. 2014).

Egg mortality is a major concern in hatchery settings, where maximizing production is the primary goal (DeSchryver and Vadstein 2014; Liu et al. 2014). Unlike eggs reared in natural streams, rearing environments in hatcheries can be controlled to alter microbe–embryo interactions. Aquaculture operations rely heavily on the use of antibiotics (Cabello 2006; Vincent et al. 2020), formalin (Barnes et al. 2005), hydrogen peroxide (Small et al. 2003), iodine (Tendencia 2001), and UV irradiation (Komar et al. 2004), all of which impede microbial colonization and proliferation on egg surfaces. Although in the aforementioned studies antimicrobial treatments have been shown to reduce egg mortality, the mechanisms underlying these reductions are not fully understood, particularly with regard to the quantitative assessment of treatment effects on microbial abundance and alteration of microbial community taxonomic composition on egg surfaces.

While the microbes that colonize egg surfaces directly affect levels of egg mortality, indirect effects of embryo exposure to microbial communities can also be important in their early life history. The interaction between aquatic microbes and eggs can also affect embryonic development. For example, the embryos of frogs (order Anura) associated with microbial pathogens had a lower size at hatch (Uller et al. 2009), which could be attributed to conflicts in resource allocation between immune response and growth. The effects of egg surface microbial abundance and taxonomic composition on fish embryo development has not been extensively studied.

Rates of mortality and development during embryogenesis can also be affected by abiotic processes, such as temperature (Atkinson 1995). Different fish species have different developmental optimums with respect to incubation conditions, and deviation from optimum rearing temperatures can increase rates of egg mortality for sturgeon (family Acipenseridae) (Wang et al. 1985; Van Eenennaam et al. 2005; Kappenman et al. 2013). Temperature also affects the rate of development and the incubation duration (Morbey and Ydenberg 2003). Size at hatch is also affected by temperature in that embryos reared under lower temperature tend to hatch with larger size (Atkinson 1995). Interactions of microbes with the host environment are long term in nature, and perturbing the environmental variables, such as temperature, can disrupt interrelationships (Deutsch et al. 2008; Paaikmans et al. 2013). For example, one previous study found that changes in temperature affected microbial communities associated with frog hosts, which subsequently affected host mortality rates (Harris et al. 2009).

Lake Sturgeon *Acipenser fulvescens* are an iteroparous, lithophilic-spawning primitive teleost fish native to the northcentral region of North America (Peterson et al. 2007), including the Laurentian Great Lakes. Spawning is multimodal (Forsythe et al. 2012), where early- and late-reproducing adults spawn during late April through early June during periods of comparatively colder (~12°C earlier) and warmer (~18°C later) water temperatures (Forsythe et al. 2012). Egg incubation length varies as a function of temperature (Detlaff et al. 1993), whereby colder incubation temperatures prolong incubation time. In the wild, the species experiences extremely high rates of mortality during the egg stage (Forsythe et al. 2013), including developmental arrest attributed to envelopment of the egg surface (Llewellyn et al. 2014) by rapidly proliferating microbial populations.

Lake Sturgeon are a species of conservation concern in many regions of the species' range. Hatchery production has been widely embraced as an important component of species recovery planning. Streamside facilities are widely used across the Great Lakes (Holtgren et al. 2007), whereby surface waters from resident streams are used during incubation and rearing of early larval stages. Stream waters typically contain highly abundant and taxonomically diverse microbial communities (Fujimoto et al. 2013, 2018). Additional research is warranted that investigates the effects of environmental conditions, such as temperature, that are commonly encountered in sturgeon conservation aquaculture and the effects of treatments that are targeting microbial communities during early life stages.

The objective of this study was to characterize changes in egg-associated microbial community composition and abundance during the Lake Sturgeon egg incubation period under different water treatments and incubation temperatures. We further quantified the effects of the water treatments and water temperature on embryonic mortality rates and embryo size at hatch. We hypothesized that (1) water treatments and incubation temperature would affect microbial community composition and abundance on egg surfaces and (2) microbial communities that developed on egg surfaces and rearing temperature would affect embryonic development and mortality. This study has implications for understanding a complex interaction among microbes, early life stages of fish hosts, and the environments in surface water hatcheries. We specifically focus on methods available to change egg surface microbial community abundance and taxonomic composition and concomitantly lower egg mortality.

METHODS

Study site.— This experiment was conducted in a Lake Sturgeon streamside hatchery located on the upper Black River in Cheboygan County, Michigan. The experiment was conducted in May 2009 during the annual spawning season. The hatchery (45°23'35.13"N, 84°20'0.06"W) is a flow-through system that uses Black River water. Stream water from the retaining pond above the Kleber Dam on the Black River was pumped to the hatchery and passed through sock filters (100 and 50 μm) to remove large particulate matter. Flow rates into the hatchery were ~740 L/min.

Experimental design.— The experiment included two factors, each with two levels during egg incubation: water treatment (no treatment or UV treament and filtration) and temperature (12°C and 18°C corresponding to average stream temperature during the early and late portions of the spring spawning period). Water was used in a recirculation system and was either hatchery water (no treatment following general 100- and 50-μm coarse filtration) or UV treated (25 W; Emperor Aquatics) and filtered with a 1-μm filter cartridge (SpectraPure) at two temperatures, cold (12 ± 1°C) and warm (18 ± 1°C). These temperatures represent the natural variation encountered for spawning Lake Sturgeon in the Black River system (Forsythe et al. 2012).

The study was replicated with five different female–male full sibling crosses (family codes: BC, CE, DG, EI, and FK). For each treatment, approximately 200 eggs from each female were fertilized with 1 mL of sperm from a single male on a sterile polyethylene mesh screen (0.040-in × 0.053-in mesh and 0.019 inch thickness; Industrial Netting, Maple Grove, Minnesota) in a heath tray filled with 1 L of either untreated or treated (filtered and UV-treated) water. Fertilized eggs were left for 30 to 40 min, allowing adhesion to the polyethylene mesh screens, which served as substrate for eggs throughout incubation. Heath trays were 30 cm long by 23 cm wide by 4 cm deep with a volume of 2.76 L. Water flow rate through the heath trays was ~5.5 L/min. Eggs were reared in their respective water and temperature treatments until hatch.

Egg samples from each female and treatment were collected for DNA extraction and microbial community analysis at six time points. At warm temperatures (e.g., 18 ± 1°C) the incubation period was approximately half the duration of that of eggs exposed to the coldwater treatment (12 ± 1°C) (Wang et al. 1985), though the number of cumulative thermal units (Kempinger 1988) was comparable at each sampling time and at the hatch (~72 cumulative thermal units). For warm temperature rearing conditions (warm–treated, warm–untreated), egg samples were collected at day 0 (1 h postfertilization) and days 1, 2, 3, 4, and 5 postfertilization. Eggs from warm rearing conditions hatched at day 6 in this experiment. For cold treatments (cold–treated, cold–untreated), egg samples were collected at day 0 (1 h postfertilization) and days 2, 4, 6, 8, and 10 postfertilization. All eggs from the cold treatments hatched at day 11, 12, or 13. With this sampling scheme, the developmental stages of the embryos were roughly synchronized between the two water treatments. The developmental stages of the embryos were confirmed by visual observations of stage-specific embryonic developmental features (Detlaff et al. 1993). For each egg-sampling event, 10 live eggs were collected and placed in a sterile 2-mL Eppendorf tube that was filled with 80% ethanol and stored at 4°C.

Extraction of DNA and analysis.— Egg samples from three families (CE, DG, EI) and four treatment groups (water–temperature treatment combinations) were collected at six time points ($n = 72$ total samples). The DNA was extracted from the surfaces of eight eggs per sample using the PowerSoil Kit (Mo Bio Laboratories, California) according to the manufacturer's protocol. Terminal restriction fragment polymorphism (TRFLP) based on the 16S rRNA gene was performed to characterize microbial community structure (Liu et al. 1997; Marsh 1999). The detailed PCR amplification procedures for TRFLP were as described by Fujimoto et al. (2013). Briefly, purified 16S rRNA PCR products were digested with restriction endonuclease MspI (Gibco BRL). Two replicates of each digested DNA sample were sent to Michigan State University's sequencing facility, and the DNA fragments were separated on an ABI 3100 Genetic Analyzer automated sequencer (Applied Biosystems Instruments, Foster City, California) in GeneScan mode. The size and abundance (peak height) of the terminal restriction fragments were calculated using GeneScan 3.7 software. Each terminal fragment corresponds to a phylotype, and peak height indicates relative phylotype abundance. The TRFLP profiles were processed with T-Align software (http://inismor.ucd.ie/~talign/index.html) to align terminal restriction fragments across egg samples from different treatments. The output of T-Align was used for microbial community analysis. Principle component analysis (PCA) was performed on TRFLP data (specifically microbial phylotype relative abundance) from the egg samples exposed to different treatments in order to elucidate underlying patterns across samples. The PCA was conducted using the "prcomp" function of the R software version 2.10.0 (R Development Core Team 2009). The statistical significance of the treatment effects were assessed using permutational multivariate analysis of variance (PERMANOVA) in R with vegan package "adonis" function.

Clone library analysis of the 16S rRNA gene.— A subset of the egg samples used for TRFLP analyses including four samples from the CE family collected on day 1 and day 4 from both filtered and unfiltered treatment groups in the warm temperature regime were used to create clone

libraries to identify microbial species present on the egg surface in the two different water treatments at two developmental time points. The 16S rRNA gene of the extracted community DNA was amplified using primer 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and primer 1389R (5′-ACG GGC GGT GTG TAC AAG-3′), using the same PCR conditions as used for TRFLP. The PCR amplicons were purified and ligated into a vector plasmid pCR2.1 with kanamycin resistance using a TOPO cloning kit (Invitrogen, Carlsbad, California), and the vector plasmid was transformed into competent *Escherichia coli* cells. Vector-bearing *E. coli* clones were isolated on LB kanamycin plates with X-gal. A total of 96 white colonies per sample were picked and inoculated in LB broth with kanamycin and grown overnight at 37°C. The insertion of the amplicon into the vector plasmid was confirmed using M13 primers. Broth cultures were sent to Michigan State University's sequencing facility, and the cloned 16S rRNA gene was sequenced using a 27F primer. The sequences of the clone library were identified using the Ribosomal Database Project pipeline (Cole et al. 2009), and the microbial community structures of different samples were compared at the genus level.

Quantitative PCR analysis.— Estimates of microbial abundance for the 72 egg samples were determined using quantitative PCR (qPCR) with SYBR green. The qPCR was performed using universal bacterial primers 331F (5′-TCCTACGGGAGGCAGCAGT-3′) and 519R (5′-CG TATTACCGCGGCTGCT-3′) targeting the conserved sequences within the 16S rRNA gene, as previously described (Smith-Vaughan et al. 2006). The qPCR reaction was conducted in a 25-μL reaction volume, containing 3 μL of template DNA, 12.5 μL of master mix (2×) (mixture of DNA polymerase, buffers, and SYBR green; SABIoscience, Maryland), and 0.16 μM of each primer in the final concentration. The PCR was performed using the iQ5 (Bio-Rad) thermal cycler according to the following protocols: an initial denaturation at 94°C for 5 min followed by 43 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and an extension at 72°C for 30 s. Fluorescence signals were detected at the end of the extension for each cycle.

A standard curve for the relationship between 16S rRNA gene copy number and cycle threshold (Ct) values was constructed using a series of dilutions of the bacterial genomic DNA *Flavobacterium johnsoniae* ATCC 17061 that is known to have six 16S rRNA gene copies in its genome (Leach 2006). Standard curves were constructed with each group sampled, and the PCR efficiency of the standard curves was found to range from 87% to 100%. For determination of microbial abundance, serial dilutions were made for each sample in triplicate for each dilution. Replicate dilutions were PCR amplified along with the standard. The Ct values for both the standard and samples were determined at 600 relative fluorescence units, where the relationship between relative fluorescence units and Ct was linear in the log-transformed view. The PCR efficiency for each sample was comparable to that of the standard (data not shown). The 16S rRNA gene copy number for each sample was determined by substituting the Ct value of one of the sample dilutions into the equation of the standard curve and multiplying it by the dilution factor.

Mortality assessment during incubation.— The number of dead eggs in each treatment group was recorded daily for warm treatments and every other day for coldwater treatments throughout the incubation period. The arrest of embryonic development was determined by visual observation of developmental stages of embryos (Dettlaff et al. 1993; Colombo et al. 2007). All dead eggs were removed from the incubation tray when detected. The number of hatching larvae for each treatment was recorded, and the percentage of eggs that died during incubation was calculated as the total number dead eggs divided by the total number of live and dead eggs. The effect of water treatment and temperature on egg mortality was assessed using a general linear model using the "lm" function in the R software version 2.10.0 (R Development Core Team 2009).

Larval body size at hatch.— Immediately after hatching, a subset of larvae (up to $N = 30$ per treatment, family, and replicate, depending on levels of mortality during incubation) were anaesthetized using MS-222 (tricaine methanesulfonate) according to approved Michigan State University Animal Use and Care protocols. The anesthetized larvae were photographed using a digital single reflex lens Olympus camera with a millimeter ruler as a size standard. Total body length, an indicator of development, was quantified from digital images using ImageJ software. The effects of water treatment and temperature and their interaction on larvae size were assessed using a general linear model with fixed variables and a linear mixed-effect model with family effect as an additional random variable. The statistical significance of the treatment effect on larvae size was confirmed after accounting for family effect as a random variable using the mixed-effect model. The linear mixed-effect model was performed using "lme" function in R version 2.10.0.

RESULTS

The effects of water treatment and rearing temperature on the egg-associated microbial communities were examined using TRFLP. A total of 146 microbial phylotypes were detected from egg surfaces using TRFLP across the 72 samples. On average, 28.0 ± 8.2 phylotypes were detected per sample. The PCA plots using TRFLP phylotype relative abundance data revealed that the effects of developmental stage and water treatment on microbial

community compositions were dominant over the effect of rearing temperature (Figure 1). Principal component 1 (PC1) revealed that microbial communities associated with eggs collected at 1 h postfertilization differed compositionally from the communities on eggs collected during the remainder of the incubation period (Figure 1). The

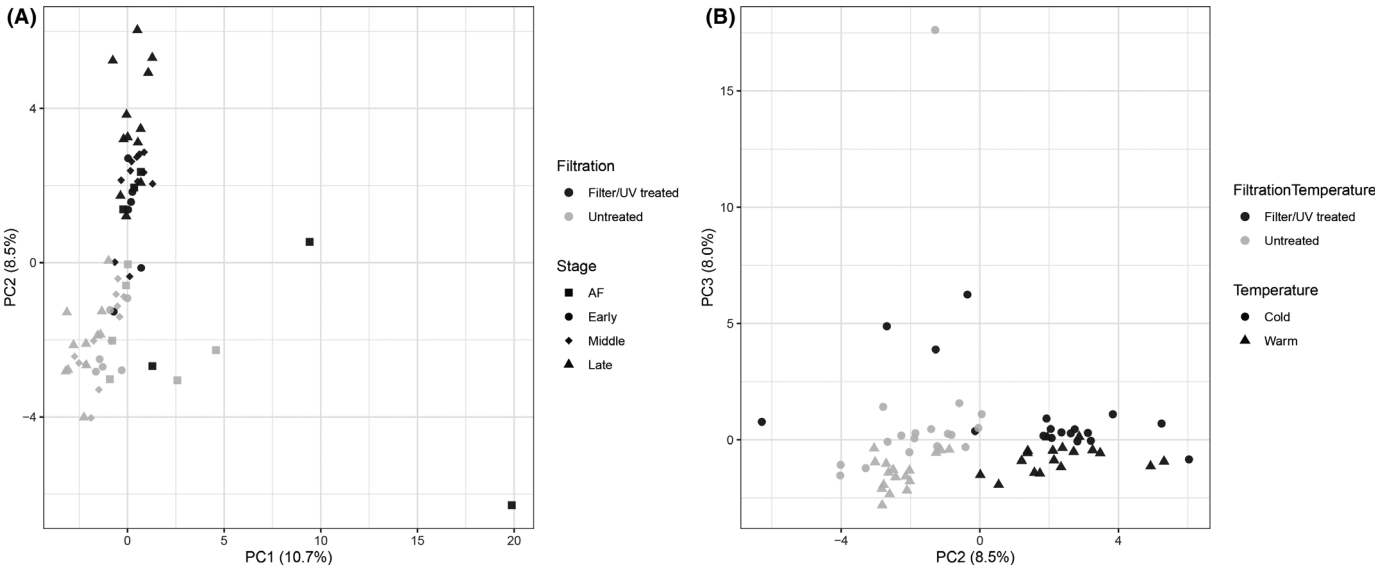

FIGURE 1. Principal component analysis plot of TRFLP data from 72 egg samples exposed to different water treatments and temperature regimes. Panel **(A)** displays clustering associated with temporal variation (PC1) and water treatment (PC2). The stage label "AF" stands for after fertilization. Early, middle, and late indicate egg developmental stages: "early" corresponds to day 1 for warm and day 2 for cold, "middle" corresponds to days 2 and 3 for warm and days 4 and 6 for cold, and "late" corresponds to days 4 and 5 for warm and days 8 and 10 for cold. Panel **(B)** displays clustering due to water treatment (PC2) and temperature (PC3).

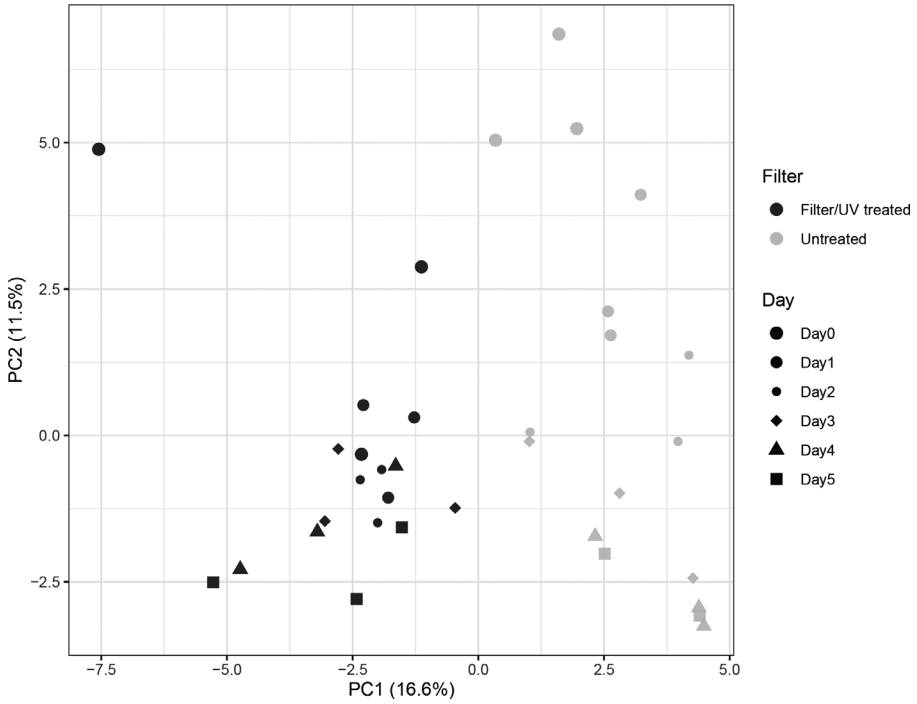

FIGURE 2. Principal component analysis plot of microbial phylotype variation based on TRFLP data. The plot shows associations between water treatment and microbial community composition on eggs incubated at warm temperatures. In the plot, PC1 and PC2 account for 16.6% and 11.5% of all TRFLP microbial phylotype variation, respectively.

variation in egg microbial communities associated with water treatment was captured by principal component 2 (PC2), which separated the communities reared in treated stream water from those reared in untreated stream water. The microbial communities on eggs treated with UV treatment and filtration were significantly different in taxonomic composition from those in untreated water (PERMANOVA: $P = 0.001$, $R^2 = 0.193$). Principal component 3 (PC3) captured variation in the egg microbial community composition associated with incubation temperature (Figure 1). The taxonomic composition of microbial communities reared in warm temperatures differed significantly from those reared in cold water (PERMANOVA: $P = 0.001$, $R^2 = 0.082$).

The PCA analysis on eggs reared at 18°C revealed that PC1 accounted for the effect of water treatment on the egg microbial community, separating the microbial community reared in filtered and UV-treated water from that reared in untreated stream water (Figure 2). Variability in the egg-associated microbial community due to egg developmental stage was captured by PC2 (Figure 2). Eggs collected at 1 h postfertilization (day 0) were also separated from the rest. The temporal trend was not random. The taxonomic composition of microbial communities diverged over time from the initial community composition as seen based on multivariate ordination based on Bray-Curtis distance that summarized taxonomic compositional heterogeneity (Figure 2). The incubation periods had a significant effect in explaining microbial communities in warm stream water (PERMANOVA: $P = 0.002$, $R^2 = 0.53$), while the effect of incubation period on microbial communities was not significant in warm UV-treated and filtered water (PERMANOVA: $P = 0.418$, $R^2 = 0.30$).

TABLE 1. Estimates of the relative abundance of microbial taxa on Lake Sturgeon egg surfaces using clone libraries based on the 16S rRNA gene for the two water treatments (U = untreated, F = filtered and UV treated; at the warm [W] rearing temperature) at two developmental stages (day 1 and day 4) for one family. The numbers represent the relative abundance in each community. Genera that showed marked differences in relative abundance between treated and untreated water are highlighted. Singletons are not included in the table.

Phyla/class	Further classification	WU1	WF1	WU4	WF4
Cyanobacteria	Unclassified Chloroplast	0.00	2.08	0.00	0.00
Alphaproteobacteria	*Caulobacter*	2.20	1.04	0.00	0.00
Alphaproteobacteria	*Rhodobacter*	2.20	0.00	4.40	0.00
Alphaproteobacteria	*Rhizobium*	4.40	4.17	2.20	3.26
Alphaproteobacteria	Unclassified Rhizobiales	0.00	1.04	5.49	5.43
Alphaproteobacteria	*Sphingomonas*	0.00	0.00	3.30	2.17
Alphaproteobacteria	*Novosphingobium*	6.59	5.21	10.9	3.26
Alphaproteobacteria	*Sphingobium*	0.00	1.04	1.10	7.61
Alphaproteobacteria	Unclassified Sphingomonadaceae	2.20	2.08	5.49	6.52
Betaproteobacteria	*Methylophilus*	2.20	0.00	0.00	0.00
Betaproteobacteria	*Methylotenera*	4.40	0.00	1.10	0.00
Betaproteobacteria	Unclassified Methylophilaceae	4.40	0.00	9.89	0.00
Betaproteobacteria	*Polynucleobacter*	1.10	0.00	2.20	0.00
Betaproteobacteria	*Massilia*	1.10	11.4	0.00	3.26
Betaproteobacteria	*Hydrogenophaga*	1.10	0.00	1.10	1.09
Betaproteobacteria	*Acidovorax*	1.10	10.4	5.49	8.70
Betaproteobacteria	*Curvibacter*	4.40	1.04	4.40	4.35
Betaproteobacteria	*Rhodoferax*	0.00	0.00	5.49	0.00
Betaproteobacteria	*Pseudorhodoferax*	0.00	0.00	0.00	2.17
Betaproteobacteria	*Pelomonas*	2.20	7.29	0.00	4.35
Betaproteobacteria	Unclassified Comamonadaceae	3.30	2.08	3.30	4.35
Betaproteobacteria	*Aquabacterium*	2.20	7.29	1.10	3.26
Betaproteobacteria	*Leptothrix*	2.20	0.00	2.20	0.00
Betaproteobacteria	*Paucibacter*	1.10	5.21	1.10	0.00
Betaproteobacteria	Unclassified Burkholderiales incertae sedis	1.10	9.38	10.9	16.3
Betaproteobacteria	Unclassified Burkholderiales	15.38	25.0	3.30	15.2
Gammaproteobacteria	*Rheinheimera*	5.49	0.00	1.10	0.00
Gammaproteobacteria	*Pseudomonas*	1.10	2.08	1.10	5.43
Sphingobacteria	*Flectobacillus*	8.79	0.00	0.00	0.00
Flavobacteriia	*Flavobacterium*	9.89	0.00	3.30	0.00

Since the water treatment was the dominant factor in explaining the microbial communities that developed on fertilized eggs, we identified the dominant microbial taxa that were associated with each water treatment group using clone libraries. A total of 47 genera were identified based on 16S rRNA gene clone libraries (Table 1). There was an effect of developmental stage on the microbial taxa that were found on egg surfaces. At day 1, genus *Acidovorax* and genus *Massilia* were 10 times more abundant on eggs reared in treated water, while genera *Flavovacterium* (9.89%) and *Rheinheimera* (5.89%) were more dominant in untreated stream water relative to filtered and UV-treated water, in which the presence of neither of the genera was detected (less than the detection level).

Microbial quantities on the egg surfaces revealed by qPCR increased in a log-linear fashion for eggs that were fertilized and reared at warm temperature in untreated stream water during the incubation period (Figure 3; slope $= 0.218$, $P < 0.001$). These communities increased from approximately $10^{5.6}$ 16S rRNA gene copies per egg on average at 1 h postfertilization to approximately $10^{7.2}$ 16S rRNA gene copies per egg at 5 d after fertilization (Figure 3). Eggs fertilized and reared in untreated stream water had higher microbial abundance than eggs reared in filtered and UV-treated water for all time points tested at the warm temperature (Figure 3). Microbial quantities on eggs reared under the cold temperature regime also increased log-linearly, though more slowly than in the warm water treatment (slope $= 0.128$, $P < 0.001$) during the incubation (Figure S1 in the supplement provided in the online version of the article). Similar to warm temperature incubations, the microbial quantities associated with eggs reared in cold untreated stream water were significantly higher than with those reared in filtered and UV-treated water ($F_{1,\ 210} = 34.21$, $P < 0.001$; Figure 4). There was no significant effect of temperature on microbial quantity on the egg surfaces (Figure S2 in the supplement provided in the online version of the article; $F_{1,\ 210} = 0.235$, $P = 0.63$).

Manipulation of aquatic microbial communities using the filter and UV treatment influenced rates of egg mortality. Mortality of eggs reared in treated stream water (50.3%) was significantly lower than that of those reared in untreated stream water (77.4%) (Figure 5; $F_{1,\ 17} = 13.42$,

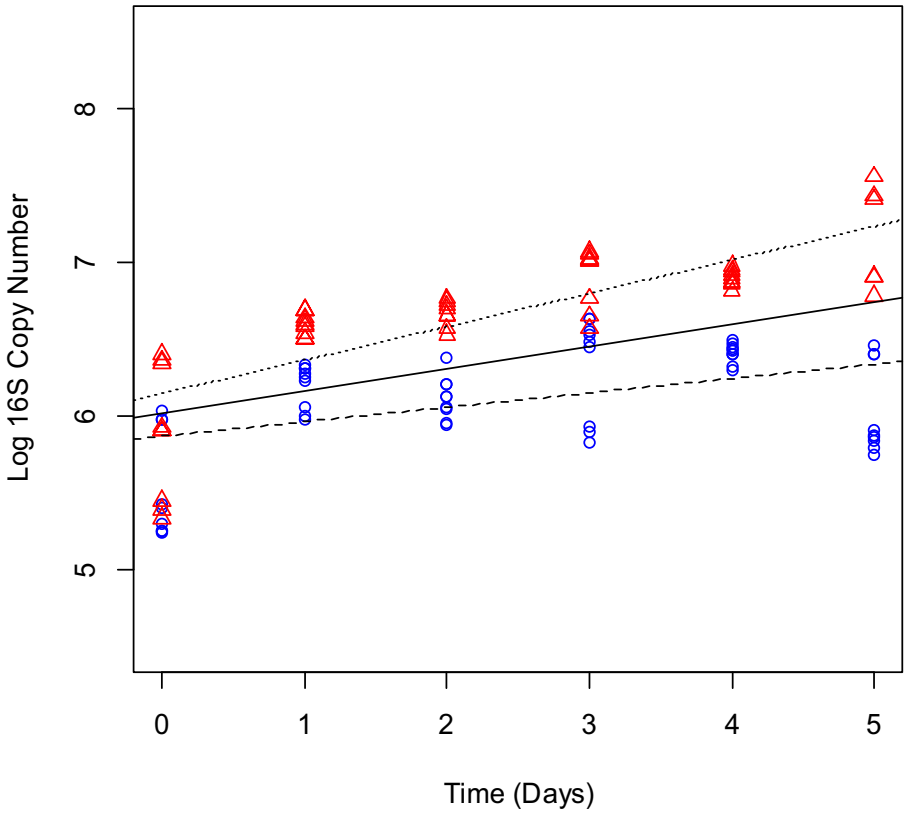

FIGURE 3. Relationships between time (days postfertilization) and microbial quantity present on egg surfaces in the warmwater regime. Microbial abundance as 16S rRNA gene copy number was measured using quantitative PCR. Eggs reared in untreated water (triangles) had higher microbial quantity than those reared in treated water (circles) at all time points. The dashed lines represent linear regression lines for warm (upper) and cold (lower) water treatments, while the solid line is the linear regression line for all data combined. [Color figure can be viewed at afsjournals.org]

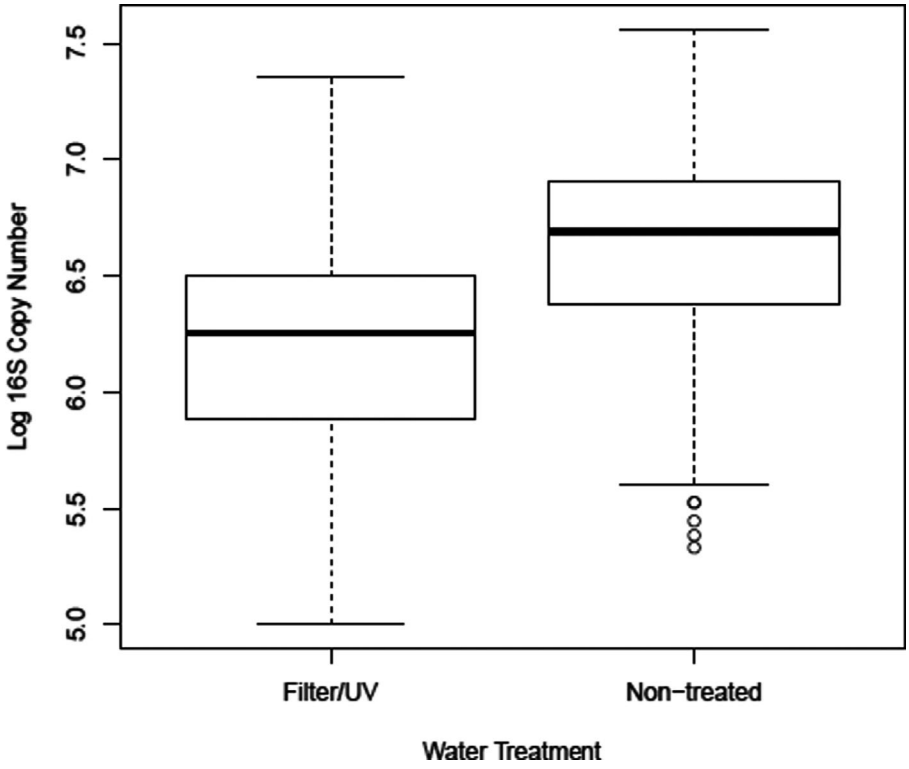

FIGURE 4. Box plot of microbial abundance (measured using quantitative PCR) on the .egg's surface as a function of different water treatments. Eggs reared in untreated water had a significantly higher microbial load than those reared in filtered and UV-treated water ($F_{1,\ 210} = 34.21$, $P < 0.001$). Egg samples from both coldwater and warmwater treatments at all time points were included for this analysis. The horizontal line in each box represents the median, the box dimensions represent the 25th to 75th percentile ranges, the whiskers show the 10th to 90th percentile ranges, and the circles show outliers.

$P = 0.002$). Microbial quantities on egg surfaces at the first sampling period (day 1 and day 2 for warm and cold incubated eggs, respectively) was a significant predictor of egg mortality ($F_{1,\ 10} = 6.89$, $P = 0.025$). However, incubation temperature did not significantly affect egg mortality ($F_{1,\ 17} = 0.308$, $P = 0.59$). There was also an effect of family on egg mortality as evidenced by a moderately lower egg mortality of family CE compared with other families ($P =$ 0.15, 0.19, 0.09, and 0.20 for CE–DG, CE–EI, CE–FK, and CE–BC, respectively). There was no significant difference in egg mortality among families BC, DG, EI, and FK.

Rearing water temperature had a significant effect on the development of embryos. Embryos reared in cold temperature had significantly longer body length at hatch than those reared in warm temperature (cold = 14.52 ± 0.81 mm [mean ± SD], warm = 13.65 ± 0.82 mm; $F_{1,\ 332} =$ 95.8, $P < 0.001$).

DISCUSSION

Our study focused on the effect of water treatments and temperature on Lake Sturgeon egg mortality and development within a surface water aquaculture setting. We demonstrated that water treatments in a hatchery that used surface water can affect the quantity and phylogenetic composition of the microbial community that colonizes the egg surface. We also demonstrated that microbial taxonomic composition and abundance, in turn, were associated with Lake Sturgeon embryo mortality rates. Water temperature during incubation significantly affected the development of embryos. Collectively, results revealed the complexity of host–microbe–environment interactions during critical early ontogenetic stages.

The composition of different microbial communities on egg surfaces varied when waters were treated with filtration and UV treatment. Results suggest that the aquatic community that eggs were exposed to during the fertilization and incubation periods was important in determining the taxonomic structure, assembly, and successional change on the egg surfaces. Data are consistent with our previous research with Lake Sturgeon and Channel Catfish *Ictalurus punctatus* that also revealed that colonization by egg-associated microbial communities is not random but is influenced by water microbial community composition (Fujimoto et al. 2013; Abdul-Razak et al. 2019).

We detected considerable temporal variation in the egg-associated microbial community membership within each water treatment (filtered and UV treated or untreated).

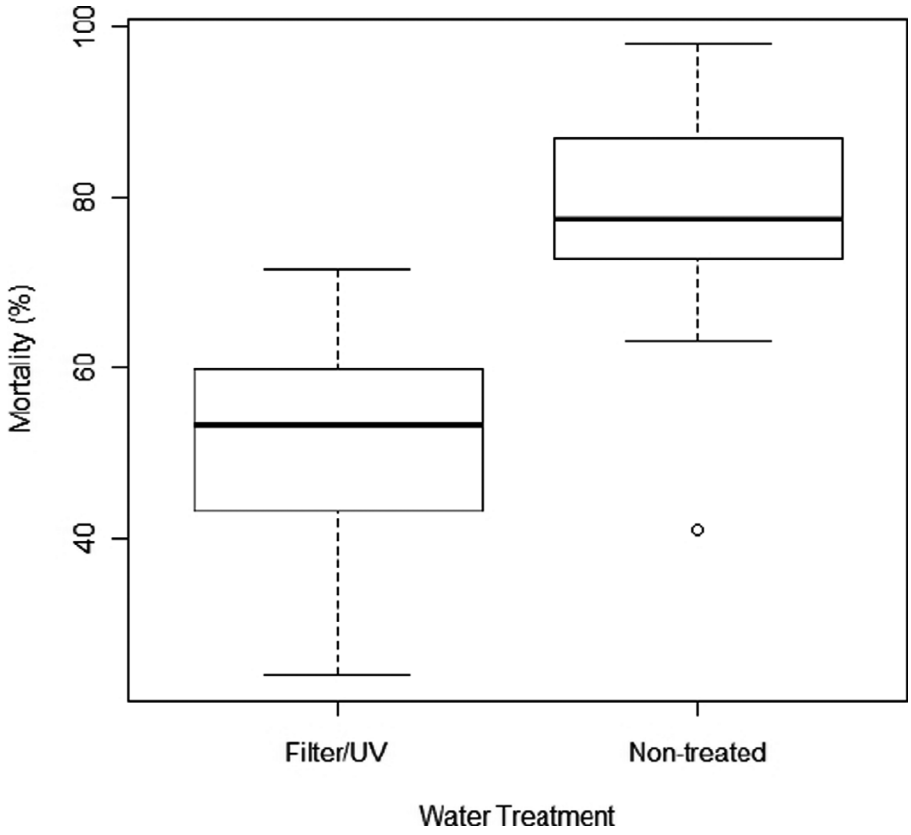

FIGURE 5. Box plot of egg mortality associated with the different water treatment groups. Lake Sturgeon eggs reared in untreated water had significantly higher egg mortality than those reared in treated water ($F_{1,\ 17} = 13.42$, $P = 0.002$). The treated group had 10 replicates and the untreated group had 9 replicates. The horizontal line in each box represents the median, the box dimensions represent the 25th to 75th percentile ranges, the whiskers show the 10th to 90th percentile ranges, and the circles show outliers.

Microbial communities on the egg surfaces changed over time, diverging from the initial community. The same trend was observed in our previous study in which we observed compositional changes in microbial communities associated with stream flow conditions (Fujimoto et al. 2013). The observed temporal variation could be derived from several factors, including changes in excreted metabolites by the egg (Steele et al. 2001; Fraune et al. 2010), changes in lysozyme from maternally provisioned to egg secreted (Fraune et al. 2011; Nematollahi et al. 2003), or microbe–microbe interactions (Fujimoto et al. 2018).

In addition to temporal trends of the egg-associated composition of the microbial community over the course of embryogenesis (Figure 2), the increase in microbial quantity over the same period was noteworthy. The qPCR analysis revealed that microbial colonization on the egg surfaces during fertilization was rapid. Over $10^{5.6}$ 16S rRNA gene copies per egg were detected immediately following fertilization (within 1 h). Microbial abundance increased in a log-linear and continuous fashion two orders of magnitude to over $10^{7.0}$ 16S rRNA gene copies per egg over a 5-d period that could be attributed to both continuous dispersal from communities in the water column or to growth of preexisting community members on egg surfaces since the time of colonization during fertilization.

Using Sanger sequencing of isolates from clone libraries, several microbial taxa were identified whose relative abundance increased or decreased on egg surfaces when subjected to different water treatments and at different time points. The finding of putative probiotic taxa is significant because lower egg mortality was observed in filtered and UV-treated water than in untreated water. Microbes that were dominant on eggs reared in untreated water, including genus *Flavobacterium,* may have negative effects on egg survival. *Flavobacterium* is a genus known to include fish pathogens (Agarwal et al. 1997; Stringer-Roth et al. 2002; Nematollahi et al. 2003). Microbes that were abundant on egg surfaces in treated water included genera *Acidovorax* and *Massilia*, which could have had a beneficial effect on egg survival. Findings of potential benefits of *Acidovorax* and *Massilia* are consistent with previous fish microbial literature. Jiang et al. (2020) describes *Acidovorax* sp. as a dominant intestinal microbial taxon of early larval developmental stages in Korean Rockfish

Sebastes schlegelii. The study compared wild versus hatchery larva, suggesting that *Acidovorax* may be transmitted from parents to offspring and may be a native probiotic. Califano et al. (2017), in a study of microbiome development during the larviculture of Gilthead Seabream *Sparus aurata*, found that *Massilia*, which is a common soil microbial taxa (Kämpfer et al. 2011), is an important host-associated taxa during larval development. Carlson et al. (2017) studied the effects of antibiotic treatment on host microbiome community structure and documented that *Massilia* was an abundant component of the skin mucosa of Western Mosquitofish *Gambusia affinis* and was particularly susceptible to broad-spectrum antibiotics.

The difference in egg mortality between the two treatments could also be attributed to differences in microbial quantity on egg surfaces between the two treatments. The high microbial abundance on the egg surface alone could prevent diffusion of oxygen to eggs, creating stressful hypoxic conditions (Komar et al. 2004), or reduce outflow of metabolic compounds from inside the egg. Based on the difference in microbial community abundance early during incubation (Figure 3), microbial abundance at the beginning of incubation was a significant predictor of the cumulative egg mortality. Data suggest that the first few days are a critical time point that dictates the trajectory of egg microbial community abundance and taxonomic composition and concomitantly egg survival. Both microbial quantity and composition appear to affect the mortality and the effects appear to be interdependent.

Although water temperature had an effect on microbial community composition, temperature did not affect egg mortality rates. Water temperature, on the other hand, significantly changed the duration of the incubation period and the size of larvae at hatch. The significantly longer body length in coldwater-reared eggs compared with warmwater-reared eggs was considerable. The association between incubation temperature and body size has also been demonstrated in other animal systems, a phenomenon termed the "temperature–size rule" (Angilletta et al. 2004; Walters and Hassall 2006). This effect of temperature on development may be ecologically important because it may affect the timing of initiation of exogenous feeding (Schiemer et al. 2003; Laurel et al. 2008; Garrido et al. 2016), which in turn could affect the probability of posthatch survival.

In conclusion, this study demonstrated the effect of water treatment to improve Lake Sturgeon egg survival that can be attributed to microbial quantity and changes in the microbial community composition on the egg surface. We also identified microbial taxa that are associated with low egg mortality (e.g., *Acidovorax* spp. and *Massilia* spp.), which will help guide further studies on identification of microbes for probiotic egg treatments as probiotic bacteria have been shown to be effective biological control agents in aquaculture (Verschuere et al. 2000).

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