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SHORT COMMUNICATION

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Milt volume influences the probability of egg fertilization in lake sturgeon (*Acipenser fulvescens*, Rafinesque, 1817)

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1 | INTRODUCTION

Many freshwater fishes (e.g., walleye *Sander vitreus*, lake whitefish *Coregonus clupeaformis*) exhibit broadcast spawning where males and females form breeding congregations and synchronize gamete release. The number of spawning adults, sex ratios, and the size of individuals within spawning congregation can affect egg fertilization success that influences year class strength and population dynamics (Dammerman, Webb, & Scribner, 2018; Rowe, Hutchings, Bekkevold, & Rakitin, 2004). Egg fertilization is also influenced by aquatic conditions (e.g. water velocity, depth) at the time of spawning where encounter rates of eggs and sperm may vary (Petersen, 1991). While fertilization success plays a role in inter-annual recruitment, specific mechanisms influencing egg fertilization rates are uncertain outside of controlled laboratory settings in broadcast spawning fish species.

Lake sturgeon (*Acipenser fulvescens*) are a long-lived and highly fecund species native to the North American Great Lakes. The species exhibits delayed sexual maturity with variable spawning periodicity between sexes (Forsythe, Crossman, Bello, Baker, & Scribner, 2011) resulting in slow population growth (Peterson, Vecsei, & Jennings, 2007). Variation in spawning periodicity between sexes and considerable variation in sex by age could influence sex ratios at the time of spawning. Lake sturgeon are broadcast spawners, where eggs are released, externally fertilized in the water column and deposited downstream from spawning congregation. Eggs are distributed non-randomly on river substrate after deposition (Finley, Forsythe, Crossman, Baker, & Scribner, 2018). Despite a relatively short incubation period (7–10 days), eggs can experience high mortality due to predation and developmental arrest (Caroffino, Sutton, Elliott, & Donofrio, 2010; Forsythe, Scribner, Crossman, Ragavendran, & Baker, 2013). Lake sturgeon populations have declined over the past century with overharvest, spawning habitat degradation, and barriers to migration identified as important factors causing regional reductions (Bruch, Haxton, Koenigs, Welsh, & Kerr, 2016; Peterson et al., 2007).

Changes in lake sturgeon adult abundance and altered sex ratios at the time of spawning have been discussed in the context of rehabilitation. For example, Holey, Baker, Thuemler, and Elliott (2000) suggested that a “rehabilitated” lake sturgeon population has a sex ratio of 5:1 (males: females), at the time of spawning, to promote genetic diversity (Hay-Chmielewski & Whelen, 1997) and to ensure egg fertilizations. However, egg fertilization rates have not been estimated for lake sturgeon or placed in the context of reproductive scenarios (e.g., operational sex ratios) or physical stream processes (e.g., water velocity) that are known to vary widely within and across years in wild populations (Dammerman et al., 2018; Forsythe et al., 2012). Further knowledge of egg fertilization rates is a critical research need (Peterson et al., 2007), especially in light of depensatory effects that may occur in situations of spawning sizes which are increasingly common throughout the species range (Rowe et al., 2004). It seems reasonable to hypothesize that low probabilities of contact between eggs and sperm may be common for lake sturgeon. The objective of this study was to estimate fertilization success of lake sturgeon eggs under different milt volumes and flow conditions.

2 | MATERIALS AND METHODS

This experiment was conducted at a streamside research facility (SRF) located on the Upper Black River System (UBR; Cheboygan

County, Michigan, USA) in May 2007. The facility was a flow through design where water was pumped from the UBR through sand filters to remove particulates. Gametes were collected from adult lake sturgeon (2 unique females and 4 unique males) captured during the act of spawning on the UBR and transferred to the SRF. Gametes were stored for up to several hours before being used in this experiment. Egg fertilization trials were carried out in experimental flumes to simulate different flow conditions and to best approximate the broadcasted nature of gametes during release and downstream drift. The flumes were constructed from 10.16 cm diameter PVC pipe cut lengthwise in half for visibility, with a water depth of 2 cm, and a total length of 183 cm.

Field data on the number of eggs released by females and sperm released by males during episodic bouts of spawning is limited in the literature. Here, a standardized volume (2.5 mL or n ~ 150) of eggs (not water hardened) from a single female were released at the leading edge of the flume while simultaneously injecting milt using a syringe (1 mL). In a hatchery setting, a standardized (and stationary) volume of sperm is typically used for egg fertilization (1 mL of milt per 200 mL water or 1:200 dilution; see Aloisi, Gordon, Starzl, Walker, & Brady, 2006). Milt is diluted to avoid negative effects of polyspermy under controlled fertilization settings. This hatchery protocol could not be replicated given milt was injected into flowing water. We estimate milt dilution in this study could be as high as 1:3,700 under the milt volumes, water velocities, and flume size evaluated. Milt and eggs were injected and released by the same two individuals over the course of the experiment to minimize variation in experimental conditions as limited gametes were available. One person released the eggs and the other individual injected milt. Several practice trials were conducted prior to collection of data to standardize the release of gametes and to positively confirm that milt and eggs where co-drifting at the point of gamete introduction into the flume. Drifting eggs were collected at the end of the flume with a mesh net and were immediately transferred to heath trays and incubated in ambient UBR water (11–15°C). After 48 hr, all eggs from each unique trial were placed in glass petri dishes and photographed (Sony Alpha DSLR camera) for developmental assessment.

Three different volumes of milt were used in this study (1ml, 2ml and 3ml). Milt was injected separately for each focal male during a trial (i.e., three syringes were used for a total of 3 mls of milt) in order to simulate three different sex ratios. We assumed sperm density and quality to be equal across males used in the experiment. Hereafter we refer to the sperm treatments as 1:1, 2:1 and 3:1 (male:female) sex ratios. All three sex ratios/volumes were replicated under high (0.55 ± 0.01 m/s) and low (0.18 ± 0.01 m/s) flow water velocities that fall within the range of flow conditions lake sturgeon experience in the UBR during spawning (Forsythe et al., 2012). Thus, the experiment included 24 trials split along 6 treatment combinations or water velocities and sex ratios (1:1 high = 7, 1:1 low = 6; 2:1 high = 2, 2:1 low = 2; 3:1 high = 3, 3:1 low = 4). We attempted to balance this experimental design among females and males but the degree of replication ultimately depended on the volume of eggs/milt collected in the field. Minimally, sperm from all 4 males were individually tested with each female (1:1 sex ratio) under high and low velocity treatments.

2.1 | Image processing and data analysis

All eggs within each photograph (trial) were individually evaluated and categorized as either fertilized, unfertilized, or undetermined by two independent reviewers (for egg classification see Figure 1; Colombo, Garvey, & Wills, 2007; Dettlaff, Ginsburg, & Schmalhausen, 2012). After 48 hr under the previously described incubation temperatures, a fertilized egg has entered the gastrulation phase of development (stages 16 &17; Detlaff et al., 2012) and thus can be visually distinguished from an unfertilized condition (Colombo et al., 2007; Dettlaff et al., 2012). For our evaluation, the presence of an advanced "yolk plug" (late gastrulation phase) was required for categorization as contrasted by eggs with a marbled appearance (unfertilized and dead). Eggs that could not be assigned to a category with a high degree of confidence due to limitations in photo quality or egg position were listed as "undetermined" and removed from analysis. Eggs that were assigned to different categories by independent reviewers were re-examined by both, to determine an agreeable classification.

A generalized linear mixed model was used to statistically evaluate the effects of sex ratio and water velocity on the percentage of eggs fertilized. Egg fertilization (dependent variable) was calculated as the proportion of total eggs categorized as fertilized. Egg fertilization was arc sign square root transformed to meet normality assumptions as evaluated using a Kolmogorov-Smirov test. Sex ratios, water velocity and the interaction between them were treated as fixed effects in the statistical model and the effect of female was included as a random effect to partially control for maternal effects or egg quality. Differences in egg fertilization success among males were also evaluated using a generalized linear mixed model procedure where male was treated as a fixed effect. In this model, egg fertilization was compared under the 1:1 sex ratio treatment where the male used in each trial could be identified. Models were fit using SPSS software and model effects were considered significant at $p < .05$ and post hoc comparisons were evaluated using a Least Significant Difference test. Model results were back transformed for computing and displaying descriptive measures (means and *SD*).

3 | RESULTS

A total of 3,192 lake sturgeon eggs were used during this experiment and subsequently evaluated for developmental condition. Unfertilized eggs accounted for the highest portion of assignment (1,549 eggs; 48.5% of the total), followed by undetermined (1,027 eggs, 32.2%) and lastly fertilized eggs (616 eggs, 19.3%). Fertilization was low on average (mean ± SE 29.6% ± 4%) but highly variable across all sex ratio and water velocity treatments (range = 7%–59%). The percent of egg fertilization was highest in the 3:1 sex ratio with

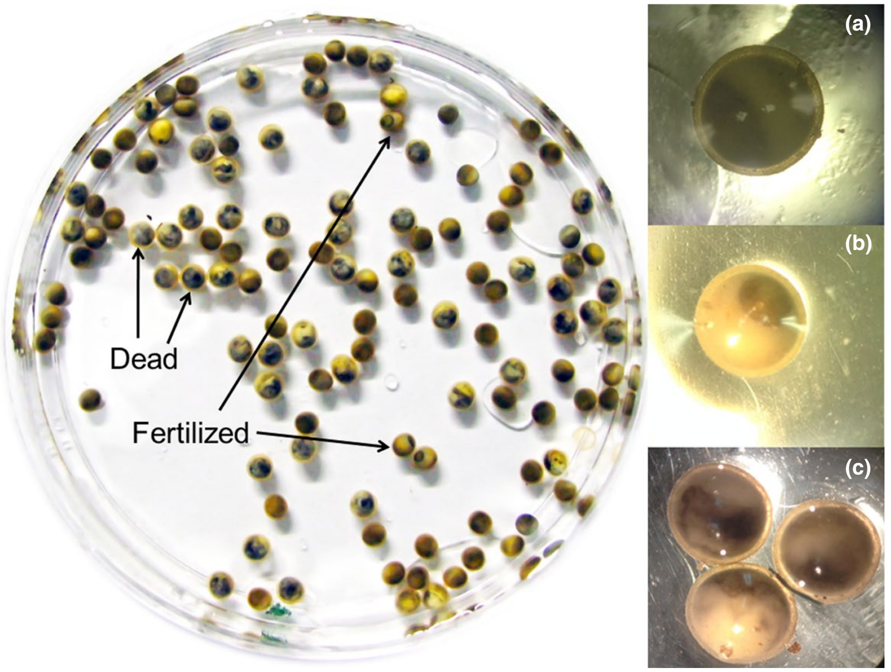

FIGURE 1 Example experimental replicate (1:1 sex ratio at high flow) ~48 hr post fertilization (left image), and reference images for in situ visual classification of egg development including (a) newly spawned egg, (b) fertilized egg with yolk plug present and (c) egg that is no longer developing (also see Dettlaff et al., 2012). Photo credit Stefan Tucker

low water velocity (mean = 51.5 ± 4% SE), while lowest fertilization occurred under a 1:1 sex ratio and high flow (mean = 21 ± 7% SE; Figure 2).

Egg fertilization success varied significantly as a function of sex ratio treatment ($F_{2, 17}$ = 7.597, p = .004). Fertilization was significantly greater (14% higher on average across flow conditions) under a 3:1 sex ratio based on least squares comparison. Water velocity was not predictive of egg fertilization ($F_{1, 17}$ = 0.773, p = .390) and there was no statistical evidence for an interaction between sex ratio and water velocity ($F_{2, 17}$ = 0.013, p = .987). Fertilization rate for a given sex ratio did not vary as a function of water velocity. The mean fertilization rate of eggs between the two females used in the experiment differed significantly ($F_{1, 22}$ = 6.173, p = .022). Fertilization was highest for Female 2 (mean + SE 36 ± 5%) compared to Female 1 (mean ± SE 24 ± 5%). Under a 1:1 sex ratio that allowed for comparison, Male 4 had the highest fertilization success on average (Male 1 mean + SE 19 ± 7%; Male 2 mean + SE 16.5 ± 5%; Male 3mean + SE 15.5 ± 5%; Male 4 mean + SE 22.0 ± 8%), but this observed variation was not statistically different.

4 | DISCUSSION

Studies seeking to estimate lake sturgeon egg fertilization success have not been attempted under any experimental situation (field or laboratory) with variable flow conditions. In this study we were

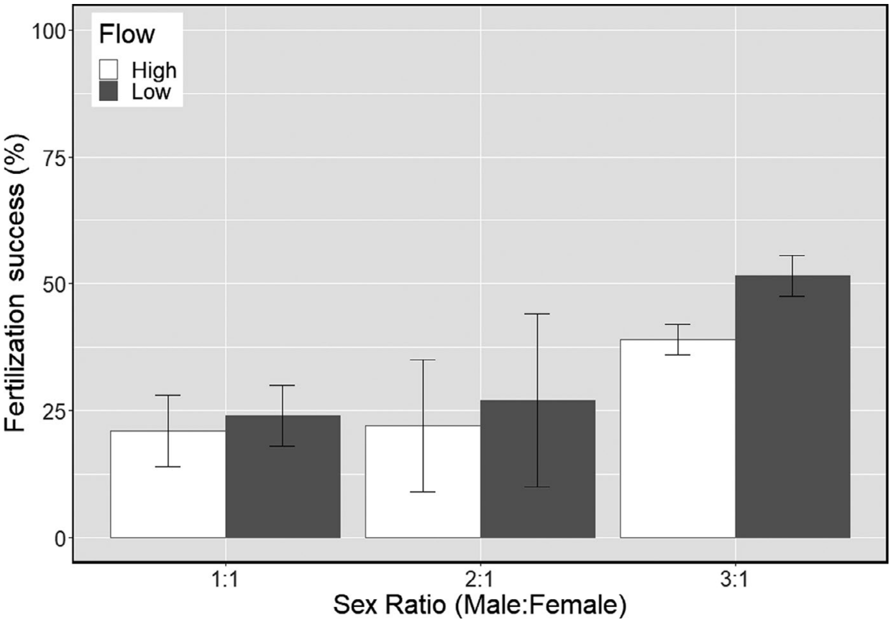

FIGURE 2 Lake sturgeon (Acipenser fulvescens) egg fertilization success (average % ± standard error) across three different sex ratios (milt volumes) and water velocity treatment combinations. Observed proportions were arc sign square root transformed for statistical analysis but displayed here in the original form

successful in estimating fertilization rates across 2 flow treatments and 3 different sex ratios. Fertilization rates were low (30%) on average and never exceeded 59% in this study. While literature on fertilization success outside of controlled purposes for aquaculture is limited, Rowe et al. (2004) report a fertilization rate ranging from 65%–97% for Atlantic cod evaluated using comparable sex ratios. As with any laboratory experiment, the challenge is to determine the degree in which the experimental conditions reflect the natural riverine conditions that lake sturgeon experience at the time of spawning. Lake sturgeon spawning behavior plays a direct role in egg fertilization, but simulating the various cues and behaviors by males and females to initiate spawning is challenging in a controlled setting (Bruch & Binkowski, 2002; Forsythe et al., 2012; Peterson et al., 2007). It is possible that our experiments were overly simplistic and the efficiency of our methods needed further evaluation. However, we believe that by simulating variation in sex ratios and water velocity, arguably two of the most important biotic and physical environmental factors associated with fertilization, we can infer that egg fertilization success may be constrained (see below) under the conditions evaluated.

Bruch and Binkowski (2002) offer that female lake sturgeon release 947–1444 eggs during episodic 2–4 s spawning bouts and eggs drift though a "cloud" of concentrated sperm at the point of release. Variable sex ratios within and among numerically depressed remnant spawning populations around the Great Lakes have been reported (1:1.06 M:F Roussow, 1957; and 9.6:1 M:F, Lyons & Kempinger, 1992). Our data supports the hypothesis that spawning events that occur under conditions of high male abundance will result in experience comparatively higher fertilization success than when fewer males are present. Across three experimental sex ratios, our results indicate that fertilization may increase by as much as 13% on average for each proportional addition to the spawning group (2:1 vs. 3:1). There was also evidence, under our controlled conditions, that increased male abundance relative to females will decrease the variability in fertilization success which can have important positive genetic implications for greater equitability of reproductive contributions and effective population size. The positive relationship between egg fertilization success and male:female relative abundance is well supported in the literature for a variety of broadcast spawning species such as sea urchins (Levitan, 2005), reef corals (Oliver & Babcock, 1992) and Atlantic cod (Hutchings, Bishop, & McGregor-Shaw, 1999).

The number of sperm released by an individual male during individual spawning bouts (2–4 s in duration) may exceed 2 billion sperm/cm^3 and is estimated to be ~50 cm^3 in volume or 100 billion sperm (Bruch & Binkowski, 2002; Dettlaff et al., 2012). Total sperm released available for fertilization depends on male relative abundance within spawning congregation (Bruch & Binkowski, 2002). Our ability to explore the upper end of male dominated sex ratios observed in nature (i.e., 9.6:1 male to female, Lyons & Kempinger, 1992) was limited in this study by access to viable gametes. Based on sperm concentrations provided above, our estimated maximum sperm contribution may be ~6 billion sperm released (1.6 mil sperm/cm^3) during the 3:1 sex ratio (assuming equal gamete quality/sperm volume per male [see below]). Like other broadcast spawning species, it is likely that the effect of increasing male number on egg fertilization success reaches an asymptote as the probability of egg/sperm contact drifting in the water column are maximized (see Rowe et al., 2004). However, many species including lake sturgeon show a decline in fertilization success due to various mechanisms such as sperm competition, egg mortality though polyspermy (multiple sperm fertilizing the same egg) and genetic incompatibilities (Dettlaff et al., 2012). In this study, we consider the methods executed may represent a lower concentration of sperm than experienced in wild scenarios and egg mortality was not attributed to polyspermy. Given that natural spawning events are often dominated by males, variable fertilization rates may be explained by polyspermy from milt-over exposure rather than milt limitation. Egg mortality due to polyspermy is complex and should be further investigated. Furthermore, the size of males participating in the spawning bout, male position in the spawning group, the synchrony of gamete release (true operational sex ratio), and the sperm allocation strategies of males within and among spawning events (Bruch & Binkowski, 2002; Forsythe et al., 2011; Smith & Baker, 2005) may also play a role in fertilization success. Future studies should explore these factors.

The activity and maturity of lake sturgeon gametes can play a significant role in fertilization success. Sperm must be able to reach/penetrate the egg and the egg must be able to accept the sperm (Dettlaff et al., 2012; Rurangwa, Kime, Ollevier, & Nash, 2004) or success declines. Aspects of gamete condition (i.e., swimming directionality of sperm and degree of egg ripeness/ polarization) were not directly measured in this study but was a concern and the motivation for recording individual identity. There was no statistical difference in fertilization success among the 4 males used in this study (1:1 sex ratio) and thus we conclude that variation in sperm quality, which is observed in lake sturgeon (see Toth, Ciereszko, Christ, & Dabrowski, 1997), did not drive variation in fertilization success. In contrast, the 12% difference in fertilization rates between females could be related to a number of maternal effects (egg size; Duong, Scribner, Crossman, Forsythe, & Baker, 2011; Trippel, 1998), the maturation of eggs at the time of collection (Dettlaff et al., 2012) or due to the decline of egg condition leading up to the experiment. We suggest that studies focused on gamete health, gamete development, and maternal effects at the time fertilization would improve our understanding of fertilization probabilities.

Water velocity is a key physical stream factor that has been important in spawning duration and spawning site selection (Auer 1996a, Forsythe et al., 2011; Forsythe et al., 2012; Smith, Smokorowski, & Power, 2017). However, the effects of water velocity on egg fertilization was minimal in this study. Several factors may explain this result. For instance, Finely et al., (2018) recently reported that lake sturgeon eggs drift and deposit up to 50m downstream of spawning groups. While the bulk of fertilization likely happens at the point of gamete release male-female proximity, our experimental flumes allowed co-occupancy in water (< 2m) over a fraction of the distance that eggs and sperm may interact under natural conditions. Thus fertilization rates reported here might be underestimated across all treatments. Furthermore, the estimated interaction time of eggs and sperm in our simulations was approximately 3.32 s under high flow and 10.16 s under low flow within a laminar environment. Lake sturgeon actively spawn under considerably higher and turbulent river flow conditions than we were able to simulate experimentally (e.g., 1.90 m^3/s; Smith et al., 2017). Thus, the hypothesized negative effect of water velocity on egg fertilization may not be realized until higher water velocities are experienced.

The confirmation of egg fertilization was challenging due to egg position in photos. During early development, lake sturgeon eggs are capable of advancing through several phases despite being unfertilized and can continue abnormal development under polyspermy fertilization (Dettlaff et al., 2012). The result is variation in appearance (i.e., marbling, irregular yolk plug shape, large and small yolk plug size, etc.,) that can be difficult to confidently classify the condition of egg via photos taken in bulk as in this study. Eggs that were 'mixed' in appearance during this study were classified as "undetermined" and therefore our estimates of fertilization should be viewed as conservative. Despite this approach, we note that the fertilization success in this study would have averaged at best 51% (range 19%–78%) if every unknown egg was treated as fertilized. The ability to handle eggs, manipulate position and observe them under a dissecting microscope "in situ" would be an improvement to the methods used in this study.

Rehabilitation practices for lake sturgeon are diverse (Bruch et al., 2016), and while all seek to increase adult abundance, few strategies take into consideration scenarios that reduce depensatory effects of sperm limitation and attempt to increase egg fertilization success. Estimates of low and variable fertilization success suggest that mortality during the egg stage should not be viewed as the starting point for population modelling and restoration activities (Caroffino et al., 2010). At a minimum, we believe that operational sex ratios should be monitored and considered in modeling of recruitment in the context of egg fertilization rates, especially for small populations. Altering flow regimes that create mixed physical cues may disperse adults during optimal times of spawning that can potentially reduce reproductive success (Smith et al., 2017). Protecting habitats that congregate spawning activity, while maximizing adult abundance, should continue to be a focus of restoration effort and may indirectly elevate fertilization probabilities and thus overall population trajectories.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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