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Spawning and gravidity of the endangered freshwater mussel *Epioblasma capsaeformis* (Bivalvia: Unionidae) in captivity for production of glochidia

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ABSTRACT

Understanding the reproductive biology of the endangered *Epioblasma capsaeformis* of the eastern United States is critical to conservation efforts at mussel hatcheries. We studied how males influenced gravidity among females held in captivity. Percent males (0%, 33%, 50%, and 67%) within a holding system was used as the predictor variable. Our response variables were percent females observed gravid, number of eggs and glochidia per gravid female, total eggs (sum of eggs and glochidia) per gravid female, and proportion of total eggs successfully fertilized and developed into glochidia. Mean percent of females gravid in the male treatments were 73%, 85%, 69%, and 60%, respectively, with no evidence that treatments differed significantly from one another. However, the treatment without males had significantly lower mean number of total eggs observed (4,533 vs. 5,868 to 7,330), with fewer viable glochidia (1,354 vs. 5,645 to 6,920). Most of the eggs in the treatment without males were unfertilized at experiment completion (3,179 vs. 206 to 410), with a much lower percentage of transformed glochidia (27% vs. 94 to 97%). Our study documents the important role that males play in fertilizing females for production of glochidia and that key reproductive processes occurred in captivity.

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Introduction

The Oyster Mussel (*Epioblasma capsaeformis*) is a rare and endangered freshwater mussel species endemic to the Tennessee and Cumberland River drainages, major tributaries of the Ohio River of the eastern United States. Listed by the U.S. Fish and Wildlife Service (USFWS) as an endangered species in 1997, the Oyster Mussel only has one remaining stronghold, a large native population of >500,000 individuals in the Clinch River in Hancock County, Tennessee (TN) (U.S. Fish and Wildlife Service 2004; Jones et al. 2014, 2018; Ahlstedt et al. 2016). A much smaller native population of perhaps hundreds up to a few thousand individuals occurs in the Nolichucky River in eastern TN, and several additional populations of hundreds to thousands of individuals are being actively restored by hatchery programmes to suitable habitat in reaches of the upper Clinch River, Virginia (VA), lower Powell River (TN), Nolichucky River (TN), Big South Fork Cumberland River (TN) and Kentucky (KY) and the Paint Rock River, Alabama (AL). While population restoration has been initiated for *E. capsaeformis* to help meet recovery needs for the species, continued success will be contingent on improving the captive rearing (*in situ*) process to increase the number and genetic diversity of progeny produced by hatcheries. Globally, freshwater mussels (order Unionida) are one of the most imperiled groups of aquatic animals in the world with many species considered threatened with future declines, listed as critically endangered or extinct (Lopes-Lima et al. 2018). Many species now are so rare that they need direct conservation action to save them, to include protecting their habitat and rearing them in captivity to replenish wild populations.

Gaining an in-depth understanding of the life history of *E. capsaeformis* to include its fish hosts, periods of spawning, gametogenesis and gravidity are considered core knowledge for captive rearing of this species (U.S. Fish and Wildlife Service 2004). Knowledge of the reproductive biology of *E. capsaeformis* and other species in need of conservation action is thus critical to biologists working at mussel hatcheries trying to propagate and release progeny to the wild in an effort to hasten species recovery (FMCS (Freshwater Mollusk Conservation Society) 2016). Key aspects of *E. capsaeformis* reproductive biology are broadly known but the specific timing of when males and females spawn, when females become gravid, when larvae are mature, and when and for how long they release theirglochidia to host fishes are life history processes that still need an improved understanding Table 1.

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Mussel larvae (i.e., glochidia) for most freshwater mussel species are obligate parasites on fish, which they use for dispersal and to metamorphose their glochidia to the juvenile stage. *Epioblasma capsaeformis* uses small (50–60 mm total length) benthic dwelling darters (Percidae) in the genus *Etheostoma* and *Percina* as its natural fish hosts (Yeager and Saylor 1995). However, the manner in which it infects glochidia on its host is quite complex and amazing. The mantle of *E. capsaeformis* is modified into a lure that is bright blue Figure 1a to attract its fish hostmm total length) benthic dwelling darters (Percidae), which it captures in a similar manner to how a 'venus flytrap' captures insects. The female mussel holds on to the fish Figure 1b for several

Table 1. Monthly occurrence of major life-history functions of the Oyster Mussel (*Epioblasma capsaeformis*) in the Clinch River, Tennessee and Virginia, U.S.A.

January	February	March	April	May	June	July	August	September	October	November	December
Gametogenesis[1]										Gametogenesis[1]	
			Release of Glochidia[2,3]								
				Glochidia Metamorphosis[2]							
				Excystment from Host Fishes[3]							
					Growth of Juvenile and Adult Mussels[3,4,5]						
								Spawning by Adults[3]			
Brooding of Glochidia by Females[3]								Brooding of Glochidia by Females[3]			

Data sources: [1]Henley et al. (2007); [2]Yeager and Saylor (1995); [3]Jones and Neves (2011); [4]Jones et al. (2014); [5]Jones et al. (2018).

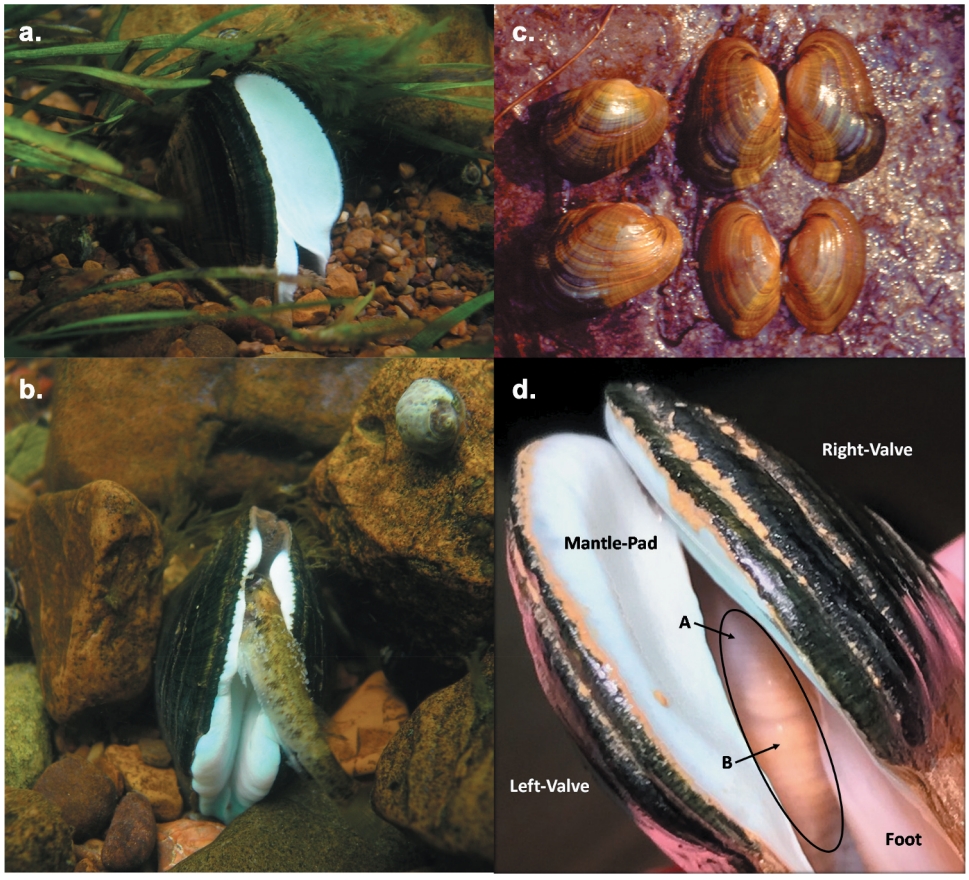

Figure 1. (**A**) Mantle-lure display of *Epioblasma capsaeformis*; (**B**) Female capturing and infesting a host fish (*Etheostoma camurum*), glochidia are attached to and visible on the body and fins of the fish; (**C**) Two live *E. capsaeformis* females (top and bottom left) and fresh-dead shells of a female (top right) and male (bottom right) showing sexual dimorphism of the shells; (**D**) A live female *E. capsaeformis* non-lethally opened 5–6 mm to show mantle-pad and gravid gill (left), colour of gill is 'clear' (arrow A) near posterior-end indicating absence of and release of eggs, and is 'yellowish' (arrow B) in center and anterior section of gill, indicating unfertilized eggs in early stage of decay and release. Photographs A and B were taken on 14 May 2007 at Frost Ford, Clinch River, Hancock County, Tennessee, U.S.A. by N. King, Freshwater Mollusk Conservation Center (FMCC), Virginia Tech. Photographs C and D were taken by J. Jones at FMCC on 11 November 2017.

minutes to infest its glochidia directly onto the host (Jones 2015; Jones et al. 2006). The male and female shells are dimorphic and easily distinguishable from each other Figure 1c, with males typically living to a maximum age of 12 years old and females living to a maximum age of 9 years old, longevity that is generally considered short-lived compared to most other freshwater mussel species (Jones and Neves 2011). Eggs of females are fertilized internally while being held in the gills by sperm released by males into the water and taken in during siphoning. Alternatively, females of some mussel species can produce both sperm and eggs in their gonadal tissue and self-fertilize, known as hermaphroditic fertilization (Van der Schalie 1970). Embryos then develop or 'brood' in the gills of the female until becoming mature glochidia. Depending on the species, mussel glochidia brood in the gills of females during either winter or summer. Females of *E. capsaeformis* are long-term winter brooders, becoming fertilized in late summer and early fall (autumn), and then they over-winter their glochidia in their outer gills for release to host fishes the following spring (Yeager and Saylor 1995). However, the exact timing of the species' spawning and gravidity have not been well documented empirically *in situ* or in a captive setting. Gametogenesis has not been documented in *E. capsaeformis*, but it is thought to occur in the winter, similar to other long-term winter brooders (Henley et al. 2007).

Many improvements to mussel propagation and culture technology have been achieved over the past 25 years, especially regarding identification of host fishes, development of algal diets, preparation and use of sediment for culturing juveniles, development of a range of aquaculture systems utilizing hatchery raceways and other systems utilizing varying degrees of water flow and renewal, and development of *in vitro* techniques that metamorphose glochidia into juveniles without use of a host fish (Zale and Neves 1982; Gatenby et al. 1996, 1997, 2003; O'Beirn et al. 1998; Beaty and Neves 2004; Jones et al. 2005; Hanlon and Neves 2006; Barnhart 2006; Chumnanpuen et al. 2011; Kunz et al. 2020). However, only a few studies in North America and Europe have examined spawning and gravidity of mussels in captivity, especially with the intent of harvesting glochidia *ex situ* for mussel propagation (Thomas et al. 2010; Mosley et al. 2014). How environmental and demographic factors such as water temperature, stream discharge, photo-period, chemical cues (e.g., natural and anthropogenic hormones), and sex ratios trigger spawning in *E. capsaeformis* and other species remain unknown, but some may be important factors for inducing reproduction in captivity.

Thus, the main goal of our study was to assess how the ratio of *E. capsaeformis* males to females influenced gravidity among females when held in captivity at the Freshwater Mollusk Conservation Center (FMCC) at Virginia Tech, Blacksburg. We measured percentages of females that were gravid and the number of eggs and glochidia that we observed as indicators of male fertilization success. Further, we compared the laboratory gravidity data to data collected from females in a wild, demographically healthy population in the Clinch River, Hancock County, TN. We wanted to know whether mussels held in captivity would spawn and become gravid at levels similar to those observed in the wild, and therefore support production of viable glochidia for propagation purposes. To accomplish this objective, we assessed monthly from August to November the percent gravidity of females at a field site in the river to those held at FMCC, and as an indicator of the timing of spawning in the wild, changes in abundance of males and females at the substratum surface.

Materials and methods

Mussel collection

A total of 240 wild, sexually-mature *Epioblasma capsaeformis* (150 non-gravid females, 90 males), measuring >35 mm long were collected from the Clinch River at Wallen Bend [River Kilometer 309.6 (River Mile 192.4): 36° 34'49.40" N and 83°00'18.28" W] in Hancock County, TN on 11 August 2017. Mussels were collected in early August based on our previous experience that females likely would not be gravid and males would not have released sperm at that time Table 1. At the site, each mussel was uniquely tagged with a shellfish tag (Hallprint, Inc., Holden Hill, New South Wales, Australia), measured for maximum shell length, sexed, and the females were examined for gravidity. Males and females are sexually dimorphic and easily identifiable by the shape of the shell. Gravidity of each mussel was determined by J.W. Jones (primary author) by slowly prying apart the valves to visually inspect the outer gills for presence of eggs or developed glochidia. Gills of gravid mussels are swollen with eggs or glochidia and white in colour. Once tagged and examined for gravidity, mussels were placed in an aerated cooler filled with river water and transported back to the FMCC at Virginia Tech for holding in recirculating aquaculture systems.

Laboratory study

Mussels were held in 32-liter circular buckets (38.1 cm diameter X 25.4 cm deep standpipe) containing ~29 L of

FMCC pond water. Water was recirculated continuously through the bucket at a rate of 2 liter per minute for 89 days (August 12 to November 8). To assess the effects of changes in water temperature on mussel spawning and gravidity, a HOBO Water Temp Pro v2 data-logger (Onset Computer Corporation, Bourne, MA) was deployed inside one of the buckets to measure water temperature every hour. After the trials, all mussels were promptly returned to Wallen Bend.

Laboratory trials were conducted to assess the effects of four different percentages of males on spawning success, with five replicate buckets per treatment, for a total of 20 experimental units. Treatment 1 (12 females and 0 males per replicate, i.e., 0% males) assessed the outcomes of hermaphroditism and subsequent self-fertilization of females. Treatment 2 (8 females and 4 males per replicate, i.e., 33% males) and Treatment 4 (4 females and 8 males per replicate, i.e., 67% males) tested the impact of a female-skewed and male-skewed sex ratio, respectively. Treatment 3 (6 females and 6 males per replicate, i.e., 50% males) represented the approximate sex ratio observed in the Clinch River (TN) estimated in previous quantitative surveys (Jones and Neves 2011; Jones et al. 2014, 2018). Females were examined for gravidity at the time of their collection at Wallen Bend on August 11, and then again in the laboratory on September 25 (Day 45) to check for the onset of gravidity and at the end of the study on November 8 (Day 89) to determine the overall percent gravidity of females (Table 1). Total number of females that became gravid by the end of the trial was determined within each experimental unit. On Day 89 at the end of the study, eggs and glochidia were extracted non-lethally by J.W. Jones from only the left gill using a water-filled syringe from a subset of three gravid females per replicate from each treatment. The needle of the water-filled syringe was inserted near the posterior-end of the gill (see Figure 1, photograph D, arrow A) and was used to carefully flush the gill with water from posterior to anterior end of the gill until all of the eggs and glochidia were removed. Throughout this study, our usage of the term 'eggs' assumes they were not fertilized by Day 89 and hence we assume they are not fertilized embryos. Contents were flushed into a petri-dish and when finished the needle was removed, and the inside of each female mussel then was rinsed with a squirt-bottle to flush-out any remaining eggs and glochidia. The eggs and glochidia extracted from each female were counted completely using a dissecting microscope and a plastic petri-dish with a bottom marked into 1 cm squares to facilitate counting.

Proportion of females gravid was compared among percent-male treatments using generalized linear models (GLMs) with binomial error distribution and logit link using function glm() from the base R package *stats* (R Core Team 2020). We used generalized linear mixed models (GLMMs) to test for sex-ratio treatment effects on production of eggs and glochidia (numbers of glochidia and unfertilized eggs in the marsupial outer gills of gravid females). Replicate bucket was treated as a random effect in GLMMs to account for the non-independence of observations from the subsample of females from a common bucket. We used Poisson GLMMs with log link for modeling egg and glochidia count data and we used a binomial GLMM with logit link for modeling proportion data. Function glmer() of the *lme4* package (v.1.1–23; Bates et al. 2015) was used for GLMMs, and the glht() and mcp() functions of the *multcomp* package (v.1.4–13; Hothorn et al. 2008) were used to make multiple pairwise comparisons among treatments using Tukey contrasts.

We fit binomial GLMs for proportion of females gravid as a function of treatment, with weighted proportions (via the 'weights' option in function glm()) to account for different numbers of females in each treatment and death of some females before experiment termination. We fit three models for gravidity: one each for gravidity as observed in September and November separately, and a third model for cumulative gravidity – that is, percent of females that became gravid at any time during the experiment. The September and November models serve to evaluate potential treatment effects on gravidity at different times in the mating season, whereas the cumulative gravidity metric represents overall gravidity success that might be expected in the wild after completion of a full mating season.

We used three Poisson GLMMs to model mean counts of eggs, mean counts of glochidia, and mean sum of egg and glochidia counts as a function of treatment. To explore the rate of successful fertilization, the proportion of total eggs observed that were fertilized and became glochidia was calculated as mean count of glochidia divided by sum of egg and glochidia counts. We then fit a binomial GLMM using that proportion as a function of treatment. All analyses were conducted with test level alpha = 0.05 using R statistical software v.4.0.2 (R Core Team 2020).

We used model results to assess how sex ratios may influence gravidity rates, overall egg production, and fertilization success. Where significant treatment effects were present, we identified the sex ratio that produced the greatest number of viable glochidia. In this study, potential sources of reproductive variability included: sex ratio, gametic stage of individuals, transportation stress, acclimation to captivity, and flow dynamics in the holding tanks.

Field study

To determine how spawning and gravidity of mussels held at FMCC compared to those in the wild, we conducted a field study at Frost Ford in the Clinch River, Hancock Co., TN. Quantitative (0.25-m^2) quadrat sampling was conducted four times at approximately monthly intervals in August, September, October and November of 2016 and 2017 at Frost Ford [River Kilometer 291.8 (River Mile 181.3): 36°31′49.73″ N and 83°09′02.96″ W]. Sampling was conducted within a predetermined 25 by 25-meter survey plot (625 m^2) within the approximate mid-point of the mussel shoal. Approximately 50 quadrats were sampled in total from three passes (15–17 quadrats sampled per pass) with a spacing of 6 meters between quadrats, with a random start for each pass determined using a random number generator (Strayer and Smith 2003). Substratum in quadrats was not excavated; rather, the substratum surface was observed for presence of *E. capsaeformis* males and females, and their reproductive behaviour was observed as follows: males at or protruding above substratum level, typically with excurrent siphon dilated indicating possible release of sperm, and females at or protruding above substratum level, lying on side, slightly agape indicating possible capture of sperm. Individuals of *E. capsaeformis* observed during quadrat sampling were removed from the substratum, sexed, measured using calipers, and carefully replaced to the same point of collection within the quadrat. Data from quadrat sampling was used to infer the timing of spawning, and to measure factors possibly acting as cues to spawning, such as water temperature and discharge.

Each quadrat was treated as a replicate observation for purposes of statistical analysis. On most survey dates, 50 quadrats were sampled, with exceptions occurring in 2016 in September (n = 48), October (n = 49), and November (n = 46). Analysis of raw mussel counts per quadrat ensured that differences in sample size were accounted for, and enabled use of Poisson error distribution in the generalized linear models to accommodate the skewed data distribution. All analyses were conducted with test level alpha = 0.05 using R statistical software v.4.0.2 (R Core Team 2020).

For each of the eight surveys, we calculated mean total abundance of mussels in each quadrat, which we scaled to estimate the total abundance of mussels in the 625-m^2 survey plot by multiplying the mean mussel abundance per 0.25-m^2 quadrat by 2,500. Abundance of females and abundance of males within the survey plot were estimated in the same manner. We estimated 95% confidence limits for mean total abundance per quadrat by first fitting an intercept-only generalized linear model to total abundance (with Poisson error distribution and log link) using function glm() from the base R package *stats* (R Core Team 2020). Then, we used the confint.glm() function in the *MASS* package (v.7.3–-51.6; Venables and Ripley 2002) to compute the confidence limits of the Poisson model 'fitted' values (simply the mean total abundance), which we exponentiated to convert from log-scale to count-scale. Mean density and associated confidence limits were derived by dividing the respective statistics for total abundance by 625 m^2.

To infer timing of spawning in the wild, we used generalized linear models to assess whether mean mussel abundance and density varied by month for each survey year. Mussel count per quadrat was modeled as a function of survey month (with Poisson error distribution and log link) using function glm(). For each survey year, we fit models for total abundance, as well as for female and male abundance separately. We used the glht() and mcp() functions of the *multcomp* package (v.1.4–13; Hothorn et al. 2008) to make multiple pairwise comparisons of abundance among months using Tukey contrasts. We conducted no analysis of monthly differences using density data directly, instead simply concluding that statistical differences in abundance among survey months equated to identical temporal differences in density, as the latter is derived from abundance (count) data.

To assess the influence of discharge and temperature on mussel spawning and gravidity, two Onset HOBO Water Temp Pro v2 data-loggers were deployed just outside of the sampling area at Frost Ford to record water temperature data, and temperature and discharge data were downloaded from the USGS gauge (03527220) near Looney's Gap, Hancock County, TN. Following quadrat sampling, 20–30 female *E. capsaeformis* were collected from outside the 625-m^2 survey plot at Frost Ford. These additional female mussels were measured, checked for gravidity, and placed back in the substratum at their collection location outside of the main survey plot. Environmental data were examined to assess how gravidity changed over time, and what percentage of sexually mature female *Epioblasma* mussels were gravid per time period each year.

Results

Laboratory study

We detected no effects of sex ratio treatment on percent of females gravid as observed in September (df = 3, χ^2 = 4.4731, p = 0.2147), or November (df = 3, χ^2 = 4.5809, p = 0.2052), nor for cumulative gravidity (df = 3, χ^2 = 4.3585, p = 0.2253). Mean (± SE) cumulative

percent gravidity in the 0, 33, 50, and 67% male treatments were 73% (± 6%), 85% (± 7%), 69% (± 10), and 60% (± 8%). Gravidity in the 33% male treatment was nominally higher than in other treatments, whereas gravidity in the 67% male treatment was nominally lower than in other treatments, with comparable gravidity observed within a treatment at each time point Figure 2.

Sex ratio treatment had a significant effect on number of eggs (df = 3, χ^2 = 58.498, $p < 0.0001$), number of glochidia (df = 3, χ^2 = 17.640, $p = 0.0005$), sum of eggs and glochidia (df = 3, χ^2 = 18.443, $p = 0.0004$), and proportion of total eggs observed that were glochidia (df = 3, χ^2 = 42.469, $p < 0.0001$). Mean (± SE) number of (unfertilized) eggs observed in the 0% (3,179 ± 644) male treatment was significantly greater than mean egg counts in other treatments (Tukey contrasts, all pairwise $p < 0.0001$; Figure 3A), with mean (± SE) egg counts in the 67% (200 ± 42), 33% (223 ± 117), and 50% (430 ± 258) male treatments being no different from one another (Tukey contrasts, all pairwise $p \geq 0.2$). Number of glochidia observed followed a similar but inverse pattern, with mean (± SE) glochidia count significantly lower in the 0% male treatment (1,354 ± 591; Tukey contrasts, all pairwise $p \leq 0.0054$). Glochidia counts were not significantly different among the 33% (5,645 ± 629), 67% (6,555 ± 1043), and 50% (6,834 ± 493) male treatments (Tukey contrasts, all pairwise $p \geq 0.9909$; Figure 3B). The sum of eggs and glochidia was not different among 33% (5,868 ± 548), 67% (6,755 ± 1032), and 50% (7,264 ± 448) male treatments (Tukey contrasts, all pairwise $p \geq 0.1728$). The 0% male treatment had significantly lower sum of eggs and glochidia (4,533 ± 613) than in the 50% and 67% male treatments (Tukey contrasts, all pairwise $p \leq 0.0116$), but was not significantly different from the 33% male treatment (Tukey contrast, $p = 0.1761$; Figure 3C). Proportion of total eggs observed that were fertilized was not significantly different among treatments containing males (Tukey contrasts, all pairwise $p \geq 0.92$). The 0% male treatment exhibited significantly lower mean (± SE) fertilization success rate (28 ± 10%; Tukey contrasts, all pairwise $p < 0.0001$), whereas = 88% of observed eggs in the 33% (92 ± 5%), 50% (94 ± 3%), and 67% (88 ± 9%) male treatments were fertilized Figure 3D.

A total of six mussels died during the study (all females), which represents 2.5% mortality over all collected mussels, but is less than half the natural mortality rate in the wild for the species over a similar 3-month period as observed by Jones and Neves (2011). Two mussels died in Treatment 2, one on September 13 and another on October 6; one mussel died in Treatment 3

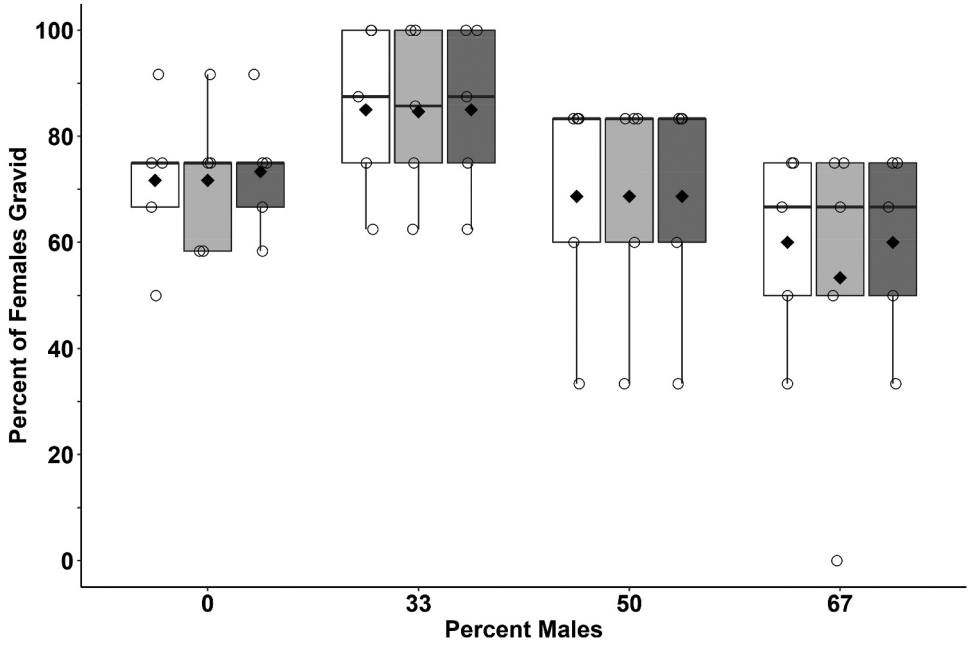

Figure 2. Boxplots of percent of *Epioblasma capsaeformis* females observed gravid as held in 0, 33, 50, and 67% male treatments. Mussels were held in recirculating aquaculture systems at the Freshwater Mollusk Conservation Center, Blacksburg from August to November, 2017 (See Methods). Percent gravidity as observed for each female in September (white boxplots), November (light gray boxplots), and cumulatively (i.e., females observed gravid at any time during the experiment; dark gray boxplots). With replicate observations (open circles) and treatment-wise mean values (black diamonds). There were no significant differences in cumulative percent gravidity among treatments (binomial generalized linear model on proportions; model df = 3, χ^2 = 4.36, $p = 0.2253$).

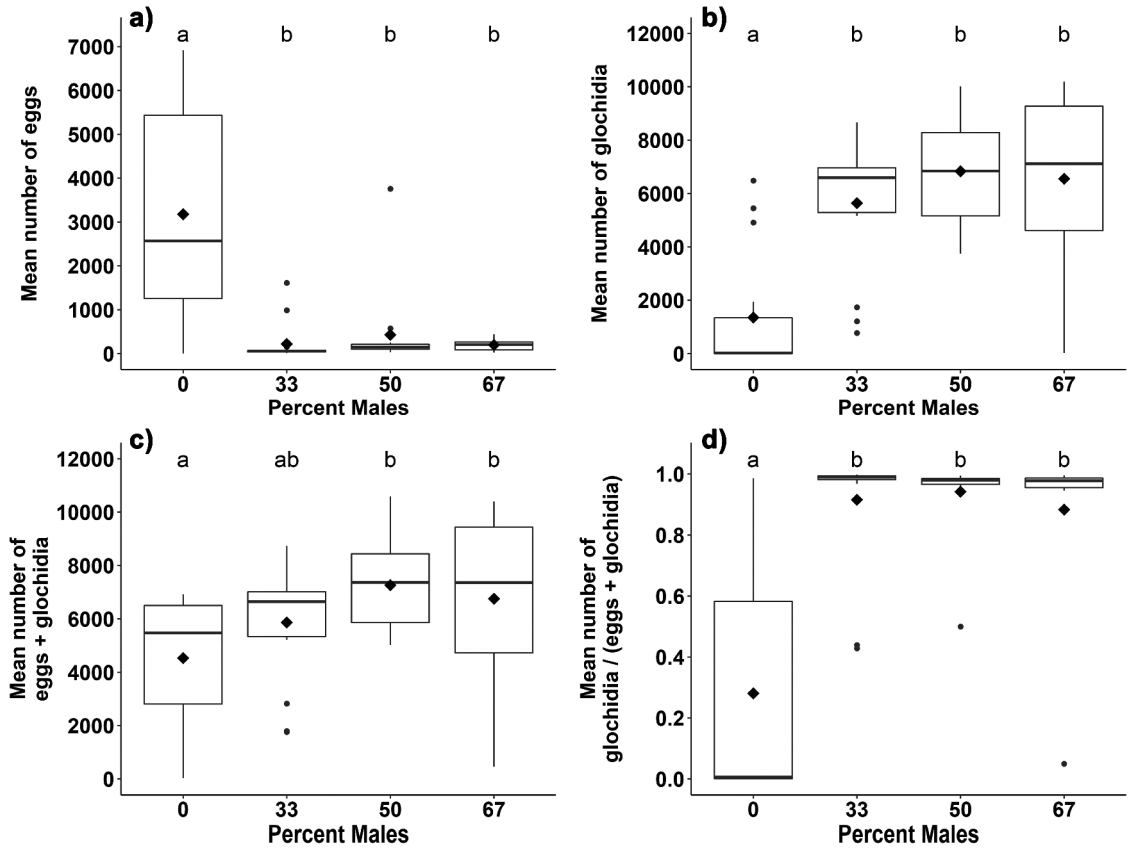

Figure 3. Boxplots of four endpoints related to eggs and glochidia observed in *Epioblasma capsaeformis* females held in 0, 33, 50, and 67% male treatments as measured in a subset of three females in each of five replicates per treatment at experiment termination. A) mean number of eggs observed per female, B) mean number of glochidia observed per female, C) mean sum of number of eggs plus glochidia observed per female, D) proportion representing mean number of glochidia divided by sum of eggs plus glochidia observed per female. With treatment-wise means (black diamonds). For each endpoint, treatments with different letters above boxplots are significantly different from one another – Panels A-C: Poisson generalized linear mixed models on counts with Tukey contrasts ($\alpha = 0.05$; all sig. diff. p as follows – A: $p < 0.0001$, B: $p \leq 0.0054$, C: $p \leq 0.0116$); Panel D: binomial generalized linear mixed model on proportions with Tukey contrasts (all sig. diff. $p < 0.0001$).

on August 21; three mussels died in Treatment 4, one each on August 21, September 13, and October 21. All observations of percent gravid females for September and November were made based on total mussels living at time of observation. Cumulative gravidity included counts of successful gravidity observed in September even if that female was observed dead subsequently.

In 2017, maximum mean daily water temperature at FMCC occurred on August 22 at 29°C, remained at or above 25°C throughout most of August, and then ranged between 24°C throughout most of September and October, and began to cool in late October Figure 5A.

Field study

In 2016, estimated density and abundance of male plus female *E. capsaeformis* observed at the substratum surface in our 625-m^2 survey plot at Frost Ford were significantly different among months (df = 3, $\chi^2 = 31.044$, $p < 0.0001$), ranging from a low of 1 m^{-2} and 600 individuals in August to a high of 4.4 m^{-2} and 2,760 individuals in September Table 3, which was a significant (Tukey $p < 0.0001$) four-fold increase over this one-month time period Figure 3. Density and abundance in October and November were significantly less (Tukey $p = 0.0467$ and 0.0078, respectively) than their respective values observed in September, having declined to about half their values from that month; August values were significantly less than in October (Tukey $p = 0.0298$), but were not significantly different from November values (Tukey p = 0.1611; Figure 4). Densities and abundances of males were nominally greater than for females in September and October, but no obvious difference or pattern in the sex ratio was observed over the four sampling intervals (Figure 4).

In 2017, estimated density and abundance of males plus females within the survey-plot were not

significantly different among months (df = 3, $\chi^2 = 5.1953$, $p = 0.1580$), ranging from a high of approximately 2 m^{-2} and 1200–1300 individuals in August and September, to a low of 1 m^{-2} and 650 individuals in October (Table 3; Figure 4). Density and abundance of males were nominally greater than for females during all four sampling events (Table 3).

Of the female mussels (n = 200) collected outside of the survey plot at Frost Ford and checked for gravidity in 2016 and 2017 and at Wallen Bend in 2016, none were gravid during the August sampling events (Table 2). In contrast, some percentage of females always was gravid during the September, October and November sampling events conducted at Frost Ford, ranging from 68% gravid in 2016 and 46–55% gravid in 2017. Of the female mussels that were collected at Wallen Bend in August 2016, checked for gravidity on site, and then transported to and held at FMCC for the laboratory part of this study, gravidity of females was 73% for both the September and November sampling events (Table 2).

In 2016, maximum mean daily water temperature in the Clinch River occurred on July 27 at 30°C, remained at or above 25°C throughout July and most of August, and then began to cool in late September and October Figure 5B. In 2017, maximum mean daily water temperature in the Clinch River peaked on July 23 at 29°C, remained below 25°C throughout most of August, and then began to cool in early September and October Figure 5C. In 2016 and 2017, mean daily discharge in the Clinch River remained low at < 14.2 cubic meters per second (<500 cubic feet per second (cfs)) from mid-August through October Figure 6.

Discussion

The main goal of our study was to determine how the number of male *E. capsaeformis* influenced reproductive success of females when held in captivity. However, we also were interested in determining whether the species would reproduce in captivity at FMCC, and to assess how reproductive success in captivity compared to that in a demographically healthy population in the wild. We have shown that key reproductive processes such as spawning and development of glochidia occurred *ex situ*. Specifically, we documented several reproductive processes by the mussels held in captivity: (1) eggs of females were transported from the gonads to the outer gills for fertilization, (2) males released sperm and fertilized the eggs of females, and (3) fertilized eggs (embryos) of females were brooded in their gills and developed into viable glochidia.

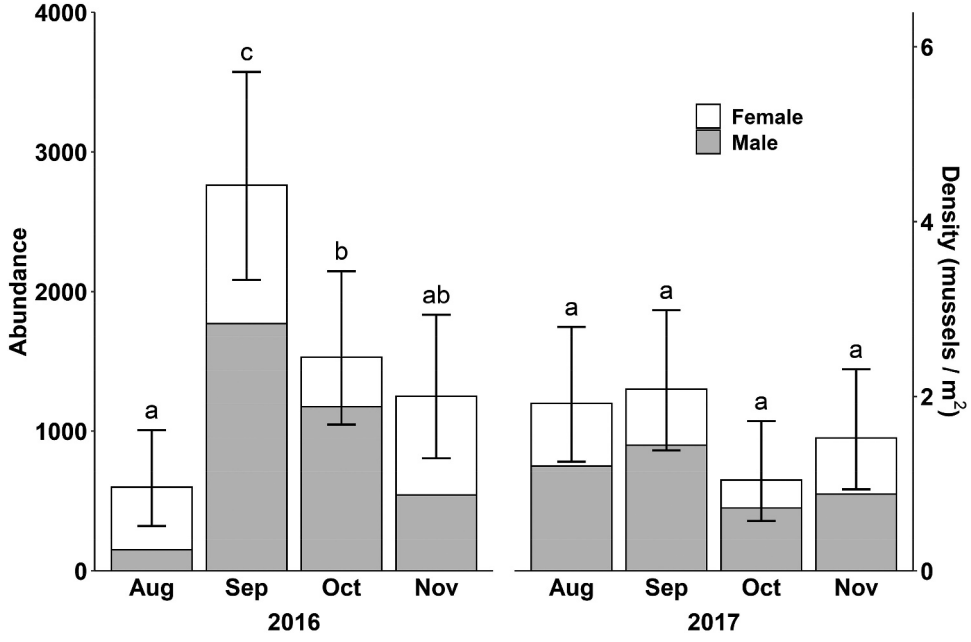

Figure 4. Mean densities (mussel/m^2) and abundances (total estimated mussels) of *Epioblasma capsaeformis* observed at the substratum surface in the Clinch River at Frost Ford (River Mile 182), Hancock County, Tennessee. Mussels were sampled by counting them in 0.25-m^{-2} quadrats, which were distributed using a systematic-random survey design in a 625-m^{-2} grid at the site. No quadrat excavation of substratum was conducted. Sampling was conducted once per month from August to November in 2016 and 2017. Error bars are 95% confidence intervals for total abundance or density values (both sexes combined). Within each year, table entries for months with the same letter above the column are not significantly different from one another (Poisson generalized linear models on counts with Tukey contrasts, α = 0.05).

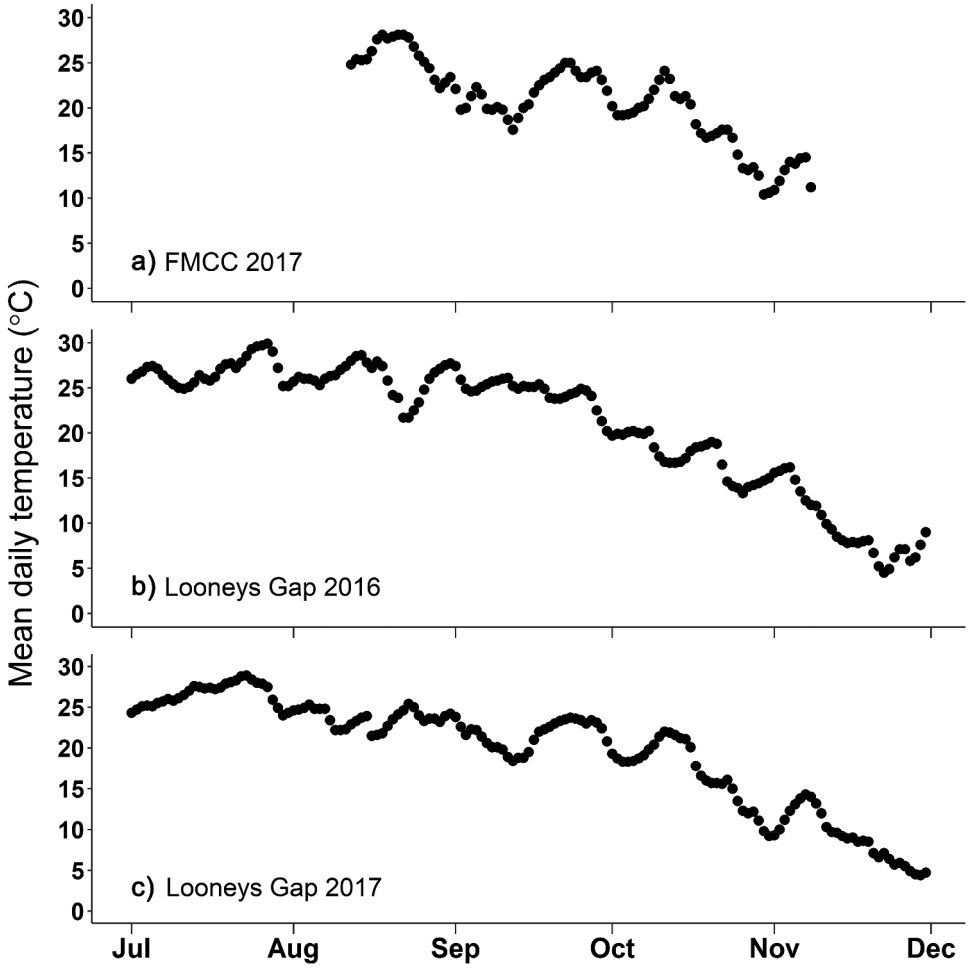

Figure 5. **A**) Mean daily water temperature (°C) recorded in recirculating water systems at the Freshwater Mollusk Conservation Center (FMCC), Blacksburg, Virginia used to hold *Epioblasma capsaeformis* in captivity from August 11 until 8 November 2017. Mean daily water temperature (°C) in the Clinch River, Hancock County, Tennessee near Looney's Gap, Virginia (River Mile 198) recorded at USGS Stream Gauge (03527220) in: **B)** 2016 and **C)** 2017.

Table 2. Number and percentage of gravid female *Epioblasma capsaeformis* observed in the Clinch River, Hancock County, TN at Frost Ford (FF) and Wallen Bend (WB), and for female mussels held at the Freshwater Mollusk Conservation Center (FMCC), Virginia Tech, Blacksburg, VA. Mussels held at FMCC were collected from WB, examined for gravidity on 11 August 2017, and transported to the facility the same day.

Year	Month & Day	Location	Not Gravid	Gravid	% Gravid
2016	August 16	FF	20	0	0
2016	September 15	FF	15	15	50
2016	October 13	FF	8	17	68
2016	November 10	FF	9	11	55
2017	August 24	FF	30	0	0
2017	September 18	FF	14	17	55
2017	October 12	FF	16	14	47
2017	November 6	FF	22	10	46
2017	August 11	WB	150	0	0
2017	September 25	FMCC	39	107	73
2017	November 8	FMCC	39	105	73

Since none of the female mussels collected in the Clinch River at Wallen Bend were gravid, it is clear that these mussels transported eggs to their gills and became gravid in captivity. Further, 73% of the females held at FMCC became gravid, a level higher than that observed at Frost Ford (46–60%), indicating that their reproductive condition was at least comparable to or perhaps exceeded the condition of females in the wild.

Table 3. Mean densities (mussels/m^2) and abundances (total estimated mussels) of *Epioblasma capsaeformis* observed in the Clinch River, Hancock County, TN at Frost Ford in 2016 and 2017. Sampling was conducted with 0.25-m^2 quadrats which were placed using a systematic-random sampling design (see Methods). Only mussels observed at the substratum surface were counted and sexed; thus, no excavation of substratum was conducted in the quadrats. Means values are the average of density or abundance in the entire survey plot area as estimated at the quadrat level. CI = Confidence Interval.

Year	Sample Date	Sex	Mean Density	Lower 95% CI	Upper 95% CI	Mean Abundance	Lower 95% CI	Upper 95% CI
2016	16-Aug	TOTAL	0.96	0.51	1.61	600	321	1006
		FEMALE	0.72	0.35	1.30	450	216	811
		MALE	0.24	0.06	0.62	150	37	389
	15-Sep	TOTAL	4.42	3.33	5.71	2760	2082	3572
		FEMALE	1.58	0.97	2.41	990	609	1504
		MALE	2.83	1.98	3.90	1771	1240	2435
	13-Oct	TOTAL	2.45	1.67	3.43	1531	1046	2145
		FEMALE	0.57	0.25	1.11	357	153	691
		MALE	1.88	1.21	2.75	1173	757	1720
	10-Nov	TOTAL	2.00	1.29	2.93	1250	806	1833
		FEMALE	1.13	0.62	1.86	707	389	1163
		MALE	0.87	0.44	1.52	543	272	953
2017	24-Aug	TOTAL	1.92	1.25	2.79	1200	782	1746
		FEMALE	0.72	0.35	1.30	450	216	811
		MALE	1.20	0.69	1.91	750	432	1196
	18-Sep	TOTAL	2.08	1.38	2.98	1300	862	1866
		FEMALE	0.64	0.29	1.19	400	183	744
		MALE	1.44	0.87	2.21	900	546	1382
	12-Oct	TOTAL	1.04	0.57	1.71	650	358	1070
		FEMALE	0.32	0.10	0.74	200	62	465
		MALE	0.72	0.35	1.30	450	216	811
	06-Nov	TOTAL	1.52	0.93	2.31	950	584	1443
		FEMALE	0.64	0.29	1.19	400	183	744
		MALE	0.88	0.46	1.51	550	286	942

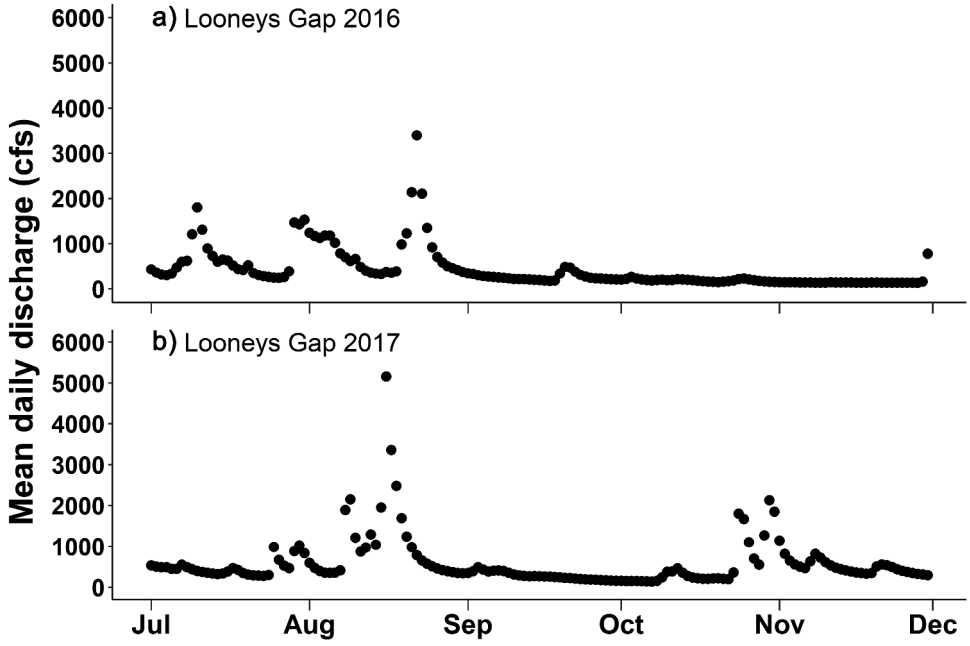

Figure 6. Mean daily discharge (cfs, cubic feet/second) in the Clinch River at Frost Ford (River Mile 182), Hancock County, Tennessee near Looney's Gap, Virginia (River Mile 198) recorded at USGS Stream Gauge (03527220) from July 1 to November 30 in: **A)** 2016 and **B**) 2017. Mussel quadrat surveys were conducted monthly from August through November each year (8 surveys total).

One of the more interesting results from our study was observed in the treatment with no males. While 73% of the females became gravid in this treatment, a frequency similar to those in the other three treatments, the mean number of glochidia observed was much lower, and conversely, the mean number of remaining unfertilized eggs much higher. Thus, several observations merit discussion here. First, while a similar number of eggs were likely transported to the gills of female mussels across all four treatments, the number

of unfertilized eggs at the end of the study was much higher in the treatment with no males, indicating that males released sperm in captivity, resulting in higher fertilization rates in the other three treatments. Second, because some female mussels in the treatment with no males not only became gravid, but their eggs developed into glochidia, some level of self-fertilization via hermaphroditism may have occurred among the female *E. capsaeformis*. Hermaphroditism in female Golden Riffleshell (*Epioblasma aureola*), a closely related congener of *E. capsaeformis*, was recently shown by Henley (2019), who documented histologically that some females contained both sperm and eggs in the acini of their gonads. Hermaphroditism is well documented among various unionid taxa in North American (Van der Schalie 1970; Heard 1975, 1979; Kat 1983; Downing et al. 1989). Thus, we suspect that some proportion of female *E. capsaeformis* utilized this reproductive strategy in our study, which may explain why some were able to produce viable glochidia without the presence of males, albeit at much lower levels.

The importance of males in captivity to increase production of glochidia is further corroborated by our observation of the fate of unfertilized eggs held in the gills of females. When eggs are first transported to the outer gills, the colour of the swollen and gravid gill appears uniformly white. This colour is due to the white colour of the newly transported eggs, and also, individual eggs are tightly packed together against other eggs, giving the appearance of a uniform white colour. When the eggs are fertilized and develop into glochidia, the colour of the gill changes from white to a greyish-white and the gill contents have a more granular appearance. This colour and texture change is subtle and due to the translucent greyish colour of the glochidial valves, while the granular appearance of the gill is due to space being created between the glochidia after the breakdown of the vitelline membranes of the developing embryos.

However, if the eggs remain unfertilized in the gill for too long (e.g., >2–3 months), they begin to break down, as they cannot remain in the gills indefinitely. The eggs essentially decay in the gills, first turning colour from white to yellow Figure 1d and finally to dark brown, disintegrating and ultimately are expelled from the gill. The expelling of eggs and glochidia from the marsupial gills by female mussels, especially abruptly, is known to occur in certain species when they are stressed due to handling or from an environmental stressor such as low oxygen levels, for example as shown in *Unio crassus* (Zajac and Zajac 2020). Although the expelling of unfertilized eggs because they are dead and decaying is not well documented. At the end of our laboratory study, when we extracted the gill contents from female mussels that still contained unfertilized eggs, we observed these colour changes and disintegrated eggs, especially in the treatment with no males. In this treatment, the mean number of glochidia plus eggs contained in females was much lower, suggesting that expelling of disintegrated eggs occurred during the study and contributed to this lower fecundity Figure 2. Hence, the higher number of unfertilized eggs in the treatment with no males highlights the importance of males for increasing fertilization success and productivity of glochidia in captivity.

The transport of eggs from the gonads to the gills (i.e., onset of gravidity), for female *E. capsaeformis* held in captivity at FMCC likely occurred quickly over a 2–3-week period beginning in early September. By September 25th when the mussels were first checked for gravidity, 73% of the mussels were gravid, which exceeded the proportion of female mussels observed gravid in the Clinch River in 2016 and 2017. None of the mussels checked for gravidity in the river in mid-to-late August were gravid, while 50% or more of the mussels in the river were gravid by mid-September Table 2. Thus, our laboratory and field observations in 2016 and 2017 indicate that most mussels became gravid in the first two weeks of September. Further, our laboratory study results showed that a large proportion (27%) of female mussels held in captivity were not gravid in late September, nor did these mussels become gravid by the end of the study in early November, indicating that these mussels were not going to become gravid at least in the season that we observed them. These observations were corroborated by the even higher proportion (40–50%) of non-gravid mussels sampled in the river, suggesting that many females do not become gravid in the fall, and hence will not release glochidia the following spring. However, Eads and Levine (2013) showed that *Villosa constricta*, another long-term brooding species, but native to Piedmont streams of the Atlantic Slope of North America, became gravid over an extended fall and winter time period from August to March. Perhaps monitoring of marked females of *E. capsaeformis* over a longer time period would reveal a similar pattern.

While the onset of gravidity for *E. capsaeformis* appears to be occurring in early September, the exact timing of fertilization is less certain. Our quadrat data showed that males were most abundant at the substratum surface in mid-September, suggesting that they were releasing sperm to fertilize females. For example, in 2016 there was a > 10-fold increase in male abundance at the substratum surface in the September compared to the August sample. In 2017, differences in male abundance per month were less pronounced, but still

nominally greatest in September. While we did not test for sperm release directly, the timing of sperm release by males could be tested in future studies using environmental DNA. Since we only extracted glochidia at the end of the laboratory study in November, we do not know exactly when the fertilized eggs developed into glochidia, which would have helped determine when fertilization occurred. However, we surmise that release of sperm by males typically begins in late August and early September, overlaps with the onset of female gravidity and continues into October. The timing of gravidity and spawning of *E. capsaeformis* appears to be adapted to when water temperature is high in late summer and early fall, allowing eggs and fertilized embryos to develop quickly, and when stream discharge is on average at its lowest, allowing high fertilization success. We hypothesize that lower discharge allows males and females to emerge and spawn at the substratum surface without being swept away, thereby increasing sperm density in the water and female fertilization rates. Once the eggs are fertilized, development into glochidia likely occurs quickly in 2–4 weeks. Thus, we recommend that hatchery staff trying to spawn *E. capsaeformis* in captivity hold males and females in close proximity to each other starting in mid-August through late October to ensure fertilization success.

Summary and conclusions

Our study has helped elucidate key aspects of the reproductive biology of *E. capsaeformis*, namely timing of the species' spawning, gravidity, and maturation of eggs/embryos to glochidia. Our observations that the females in the 50% male treatment had the greatest nominal mean glochidia count with the lowest variance, along with the greatest nominal fertilization success rate, suggests that maintaining a 1:1 ratio of males to females likely is a good breeding strategy in a hatchery setting to maximize fertilization success, production of glochidia, and high genetic diversity of progeny. However, hatchery managers should consider exploring a range of male proportions, e.g., from 50 to 67%, to test fertilization success of females. Male proportions in the Clinch River were generally > 50% at presumably peak spawn in September and October, and in the lab, the 67% male treatment performed nominally better than the 33% male treatment.

While some low level of self-fertilization via hermaphroditism may have occurred among females in our study, males clearly played a critical role in fertilization of eggs and thus productivity of glochidia among females. Our study has demonstrated that mussel spawning can be conducted in captivity to obtain viable glochidia for propagation of *E. capsaeformis*. Control and manipulation of spawning by chemical (e.g., exposure to serotonin), physical (e.g., increasing or decreasing water temperature), and demographic (e.g., manipulating sex ratios) methods in a hatchery is a key technical element contributing to the success of rearing oysters and many other shellfish and fish species (Gibbons and Castagna 1984; Valez et al. 1990). Development of controlled spawning techniques will be critical to increasing production efficiency and capacity at mussel hatcheries. The current practice of reliance on males to fertilize females in the wild, and then collection of those gravid mussels *in situ* for mussel propagation limits the effectiveness of hatchery programs managing critically endangered mussel species. Our findings demonstrate that fertilization can occur in captivity and at higher rates than in the wild to overcome this hurdle.

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Disclosure statement

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