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REGULAR ARTICLE

IMPACTS OF PROPAGATION ON POPULATION GENETICS OF THE THREERIDGE MUSSEL *AMBLEMA PLICATA* (SAY, 1817)

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ABSTRACT

Freshwater mussels provide many ecosystem services, but over the past century, they have become among the most imperiled taxa in the world. Often, efforts to restore mussel populations have included the propagation and release of juveniles. We utilized microsatellites to compare the genetic diversity of propagated mussels to the source population from which the broodstock was derived. Three wild-fertilized female threeridge mussels (*Amblema plicata*) sourced from the Cedar River watershed in Minnesota were used as broodstock. We then genetically characterized a sample from the source population, a subsample of the juvenile cohort directly after transformation (Juv-0Y), and another subsample of the juvenile cohort after 1 yr of being raised in the hatchery (Juv-1Y). After correcting for sample sizes, the Juv-0Y sample set contained the greatest allelic richness, followed by the source sample set and then the Juv-1Y sample set. All three sample sets exhibited alleles that were not shared with other samples sets, henceforth referred to as "private alleles." Private alleles in Juv-0Y and Juv-1Y indicated the dams (mothers) were likely fertilized by males living upstream of the source population, outside of the sampling effort of this study. High levels of multiple paternity were observed in the juveniles from both subsamples. In total, 89 juveniles were estimated to have been sired by 58 males, increasing the amount of genetic variability in the population. Analyses indicated the Juv-1Y samples were produced nearly entirely from a single dam, indicating that differential mortality in the hatchery reduced the amount of genetic variability in the released population. The Juv-1Y sample was significantly differentiated from the source, suggesting the juvenile population did not fully represent the source population. This study highlights the importance of genetic monitoring of mussels in hatchery environments to maximize the genetic diversity of the propagules that are released.

KEY WORDS: freshwater mussel, genetic, multiple paternity, selection, microsatellite

INTRODUCTION

Freshwater mussels (Bivalvia: Unionoida; hereafter referred to as "mussels") provide important ecological services in riverine habitat building and nutrient cycling (Spooner and Vaughn 2006). As freshwater ecosystems have been altered and degraded by human activity in past centuries, mussel populations have declined steeply (Strayer and Dudgeon 2010). Historical overharvesting continues to affect mussels today, with current populations greatly reduced and highly fragmented (Lopes-Lima et al. 2014).

Environmental stressors and low population densities can negatively impact mussel reproduction before they cause mortality in adults (Haag and Rypel 2011). Successful reproduction requires the presence of suitable fish hosts for the parasitic larval stage of the mussel life cycle. This parasitic stage is likely the primary driver of mussel dispersal, with species distributions tied to host-fish movement patterns (Schwalb et al. 2013). Therefore, the parasitic stage also facilitates connectivity of species metapopulations (Modesto et al. 2018). After metamorphosis from glochidium (larva) and detachment from the host, a juvenile mussel is still nearly microscopic in size and highly vulnerable to environmental stressors. Fluctuations in water quality, chemicals, poor physical conditions, and

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predators are much more likely to kill mussels in their first few years of life than in adulthood (Wang et al. 2007; Brim-Box and Mossa 2016).

Conservation managers have turned to propagation as a means of augmenting freshwater mussel populations and dispersing mussels throughout their historic ranges (Lopes-Lima et al. 2014). Most practitioners collect gravid female mussels from the wild to begin propagation, so this system relies on an already established breeding population (Hoftyzer et al. 2008). It also requires knowledge of the fish species utilized as hosts by each mussel species, while circumventing the potential problems of host-fish absence and environmental stressors preventing larval attachment (Modesto et al. 2018). In laboratory propagation, juveniles are raised in the hatchery during the most vulnerable period of life, often for at least a year (Carey et al. 2015). After this period, the older, larger juvenile mussels are released in the wild to mature and to contribute to breeding populations (in the case of augmentation) or to establish new breeding populations (in the case of reintroduction) (Hoftyzer et al. 2008).

Since the early 2000s, millions of produced mussels of dozens of species have been released throughout the United States (Jones et al. 2006). The release of propagated mussels could have unintended consequences for wild populations (Jones et al. 2006; Hoftyzer et al. 2008; McMurray and Roe 2017). Capturing a limited number of gravid females to propagate juveniles to serve as a new population may result in a population with lowered genetic variability. Moreover, as the new population reproduces, inbreeding depression is possible (O'Grady et al. 2006). A highly variable family size being used to source the new population may lead to a lowered effective population size, thus making inbreeding more likely or increasing the rate of genetic drift (Lande and Barrowclough 1987). The geographical location from which parent mussels are collected and the location of the juvenile release is important to consider as well. If mussels are propagated and then released to interbreed with a separate extant wild population, the dilution of locally adapted alleles could result in outbreeding depression, though the likelihood of this is low based on the recency (<500 yr ago) of most mussel population fragmentation and the relative similarity of habitats that populations of the species continue to inhabit (Neves 2004; Frankham et al. 2011). It is also possible that the hatchery can alter the gene pool of a produced population. Some juvenile mortality is expected within the hatchery; however, if hatchery mortality is correlated to specific genotypes in the hatchery, it could bias the genetic diversity of the propagated juveniles and potentially introduce maladapted genotypes into wild populations (Neves 2004).

We sought to document what, if any, differences arise between the source population from which gravid female mussels are drawn and the propagated juvenile mussels produced in a hatchery. Threeridge mussels *Amblema plicata* (Say, 1817) are a common, widespread species in the American Midwest (Elderkin et al. 2007), but they are listed as a Species of Greatest Conservation Need (SGCN) in the state of Iowa (Iowa Department of Natural Resources 2015). A previous study examining the genetic structure of threeridge mussel populations found low among-population structure compared to within-population structure, especially within a single river drainage, leading to the conclusion that it was appropriate to release mussels produced from within the same watershed (Elderkin et al. 2007).

The Minnesota Department of Natural Resources propagated threeridge mussels from a population from the upper Cedar River watershed in southern Minnesota. Our first objective was to determine if the propagated juvenile population was genetically representative of the source population, thereby decreasing chances for inbreeding effects. Our second objective was to determine if there was a significant reduction in juvenile genetic diversity from the start to the end of residency in the hatchery. Our results add to the growing body of information on the genetic impacts of propagation and aid propagation practitioners who are concerned with preserving the genetic diversity of their target species while enhancing opportunities for recovery.

METHODS

Sample Collection and Data Generation

We collected nondestructive samples from 50 threeridge mussels in the upper Cedar River near Lansing, Minnesota, using buccal swabs, which were then stored in ethanol (the "source" sample set). We found three of the mussels to be gravid, and these gravid females were used for propagation at the Center for Aquatic Mollusk Programs in Lake City, Minnesota. Hatchery staff infested individual walleye (*Sander vitreus*) host fish with the glochidia from a single dam, or mother. After transformation and dropping from their hosts, 20 juveniles from each dam were collected and preserved in ethanol for a total of 60 juveniles (the "Juv-0Y" sample set). Staff combined the remaining juveniles and reared them at the hatchery for 1 yr. On July 22, 2020, staff collected 50 more juveniles and preserved them in ethanol (the "Juv-1Y" sample set). General guidelines suggest sampling 25 to 30 individuals from each population to obtain accurate allele frequencies and estimates of genetic diversity, though this may not capture all rare alleles from the population (Hale et al. 2012). The "juvenile population" refers to all the juvenile mussels that were produced, while the "released juvenile population" is the juvenile population after its release into the lower Cedar River in Iowa. As the Juv-1Y sample set was collected after 1 yr in the hatchery, immediately before juveniles were released, it alone is used to make inferences about the released juvenile population.

Genomic DNA was isolated from the buccal swabs of the source population using Qiagen's Puregene Buccal Cell Core Kit A following the "DNA Purification from a Buccal Brush" protocol (Qiagen). Genomic DNA was isolated from the whole Juv-0Y samples using Chelex 100 Resin (BioRad)

Table 1. Loci amplified in *Amblema plicata*, presented with repeating motif and number of alleles (N_a) total (all sample sets), N_a in the Source set, N_a in the Juv-0Y set, and N_a in the Juv-1Y set. — indicates no value reported because locus was eliminated from the study.

Locus name	Motif	N_a total	N_a source	N_a Juv-0Y	N_a Juv-1Y
Anec101	CATC	10	7	No data	7
Anec103	CATC	—	—	—	—
Anec114	CATC	22	17	9	15
Anec117	CATC	—	—	—	—
Anec122	CATC	—	—	—	—
Anec126	TAGA	38	27	14	16
Anec130	CATC	13	7	9	8
Anec144	TAGA	—	—	—	—
Anec103	TAGA	—	—	—	—
Aned104	TAGA	13	9	10	10
Anec106	TAGA	14	12	9	9
Aned108	TAGA	10	8	9	6
Aned126	TAGA	25	22	10	14
Aned132	TAGA	6	4	3	4
Aned134	TAGA	—	—	—	—
Aned140	TAGA	16	14	7	6

following a modified version of the protocol (Singh et al. 2018). Tissue samples were taken from the Juv-1Y samples, and genomic DNA was isolated using the QIAamp DNA mini kit according to the "Tissue" protocol (Qiagen). All extracted DNA was quantified using a Nanodrop ND1000 spectrophotometer and stored at 4°C. We used 16 microsatellite markers developed for the fat threeridge (*Amblema neislerii*; Díaz-Ferguson et al. 2011) to genotype the mussel samples collected (Table 1).

We conducted polymerase chain reaction (PCR) amplification using the BIOLASE PCR kit (Bioline, Boston, MA). Each 10 μL reaction contained 6.6 μL of sterile deionized water, 1 μL of Biolase NH_4 reaction buffer (10×), 0.6 μL of $MgCl_2$ (50 mM), 0.8 μL of dNTP's (2.5 mM each), 0.1 μL of M13 labeled forward primer (20 mM), 0.1 μL of reverse primer (20 mM), 0.05 μL of M13 labeled oligo (20 mM), 0.05 μL of Biolase DNA Taq polymerase (5 U/μL), and 1 μL of template DNA (approximately 2 ng/μL). Reactions were completed in Eppendorf Master Cycler thermocyclers under the following conditions: 95°C/5 min; (94°C/30 sec, touchdown beginning at 56°C and dropping by 0.6°C per cycle/1 min, 72°C/30 sec) × 11; (94°C/30 sec, 55°C/1 min, 72°C/30 sec) × 25; 72°C/20 min. A negative control without mussel DNA was performed with each reaction. PCR products were visualized on 1.5% agarose gels against a 100 bp DNA ladder to confirm the success of the reactions and to ensure the negative control showed no contamination. We sent products to the Iowa State University DNA Facility to determine allele sizes with capillary electrophoresis on an Applied Biosystems 3500 Genetic Analyzer.

Data Analysis

We scored raw data with the software Gene Marker (Version 3.0.1). We checked all loci for null alleles with MICRO-CHECKER (Van Oosterhout et al. 2004). Loci with possible null alleles were excluded from the data set because their presence can bias genetic analyses (Selkoe and Toonen 2006). We used GenePop version 4.7.5 (Rousset 2008) to perform Hardy-Weinberg exact tests. Exact *P* values were estimated with the Markov chain method according to the following parameters: dememorization number was 1,000, batches set to 100, and 1,000 iterations per batch. GenePop was also used to check for linkage disequilibrium within and among sample sets.

We used GenAlEx 6.5 (Peakall and Smouse 2006) to calculate statistics of genetic diversity including sample size, which was adjusted for missing data by subtracting proportionally for each missing locus (i.e., subtracting 0.10 for one missing locus as data were collected for 10 loci per individual), number of alleles, effective number of alleles, Information Index (also known as Shannon's index), observed heterozygosity, expected heterozygosity, unbiased heterozygosity, and fixation index. HP-Rare 1.0 (Kalinowski 2005) was used to calculate allelic richness and private allelic richness using rarefaction to account for unequal sample sizes between sample sets. We used GenePop 4.7.5 (Rousset 2008) to calculate pairwise F_{ST} values between the sample sets and to calculate genetic differentiation for each pair with an exact G-test. We used GenAlEx to conduct an analysis of molecular variance (AMOVA) and to visualize genetic distances between the three sample sets by generating a principal coordinates analysis (PCoA) based on a covariance matrix with data standardization. In this instance, the covariances were standardized by subtracting the mean and dividing by the standard deviation, resulting in each element representing the correlation between two variables instead of their raw covariance. We used Colony 2.0.6.6 (Wang and Jones 2010) to analyze both parentage and sibship using a full-pedigree likelihood method. We set parameters to full likelihood (FL) with male and female polygamy and ran analysis for a medium length of time. We knew and preassigned the dams of the Juv-0Y samples. The data set used for this study is available on Dryad (https://doi.org/10.5061/dryad.08kprr5cp).

RESULTS

Sample Collection and Data Generation

We successfully genotyped 45 out of 50 threeridge mussels collected from the source population, including the three dams. For the Juv-0Y subsample (immediately after transformation), we successfully genotyped 41 out of 60 collected juveniles; for the Juv-1Y subsample (after 1 yr of being raised in the hatchery), we successfully genotyped 48 out of 50 collected juveniles.

We eliminated 3 of the 16 microsatellite loci due to poor amplification (Anec117, Anec144, Aned103) and 3 others

Table 2. Summary statistics by population. Mean and standard error over all loci for each population. *N* is sample size (with missing data subtracted from original sample size), N_a is number of alleles (averaged across all loci), N_e is number of effective alleles (averaged across all loci), *I* is Information Index, H_o is observed heterozygosity, H_e is expected heterozygosity, uH_e is unbiased expected heterozygosity, F_{IS} is fixation index.

Pop	*N*	N_a	N_e	*I*	H_o	H_e	uH_e	F_{IS}
Source								
Mean	42.1	12.7	5.955	1.933	0.754	0.784	0.793	0.045
SE	0.862	2.329	0.975	0.18	0.067	0.042	0.042	0.061
Juv-0Y								
Mean	21.2	8	3.464	1.421	0.667	0.64	0.654	−0.06
SE	2.529	1.238	0.531	0.186	0.078	0.078	0.079	0.054
Juv-1Y								
Mean	48	9.5	3.31	1.462	0.769	0.651	0.657	−0.183
SE	0	1.319	0.437	0.13	0.055	0.047	0.047	0.022

(Anec103, Anec122, Aned134) for returning larger-than-expected numbers of homozygotes for most allele size classes, indicating the possible presence of null alleles. We retained the 10 remaining microsatellite loci for analysis: Anec101, Anec114, Anec126, Anec130, Aned104, Aned106, Aned108, Aned126, Aned132, and Aned140. Across all 10 loci, we identified a total of 173 alleles, with the number of alleles per locus ranging from 7 to 38. No genotypes from Anec 101 were successfully called for the Juv-0Y set (Table 1).

Data Analysis

Hardy-Weinberg equilibrium.—The source population sample evinced a deficit of heterozygotes at locus Anec130 ($P = 0.0000$, S.E. $= 0.0000$) and locus Aned132 ($P = 0.0222$, S.E. $= 0.002$) and an excess of heterozygotes at locus Aned104 ($P = 0.0474$, S.E. $= 0.0074$). Overall, the source population sample was heterozygote deficient ($P = 0.0119$, S.E. $= 0.0038$). Overall, the Juv-0Y sample was found to be within Hardy-Weinberg equilibrium, and the Juv-1Y sample was found to exhibit an excess of heterozygotes ($P = 0.0000$, S.E. $= 0.0000$). Across all samples, linkage disequilibrium was detected at 4 out of 45 locus pairs (8% of pairs): Anec101 and Anec114 ($P = 0.029798$), Anec126 and Aned106 ($P < 1.54\text{e-}06$), Anec126 and Aned126 ($P < 1.59\text{e-}06$), and Aned106 and Aned126 ($P < 4.82\text{e-}07$). Because the purpose of this study was to compare samples from a population and a group of offspring from that population, and not to investigate population substructure, we retained all loci for the study.

Summary statistics, heterozygosity, and private alleles.—The sizes of the different sample data sets were adjusted for each locus with missing data (Table 2 and Fig. 1). The source set contained the greatest number of alleles averaged across all loci, followed by the Juv-1Y set. Across all loci, the Juv-0Y set averaged only 1.5 fewer alleles than the Juv-1Y set, despite having half of the adjusted sample size. After rarefaction was conducted to make comparisons of populations with

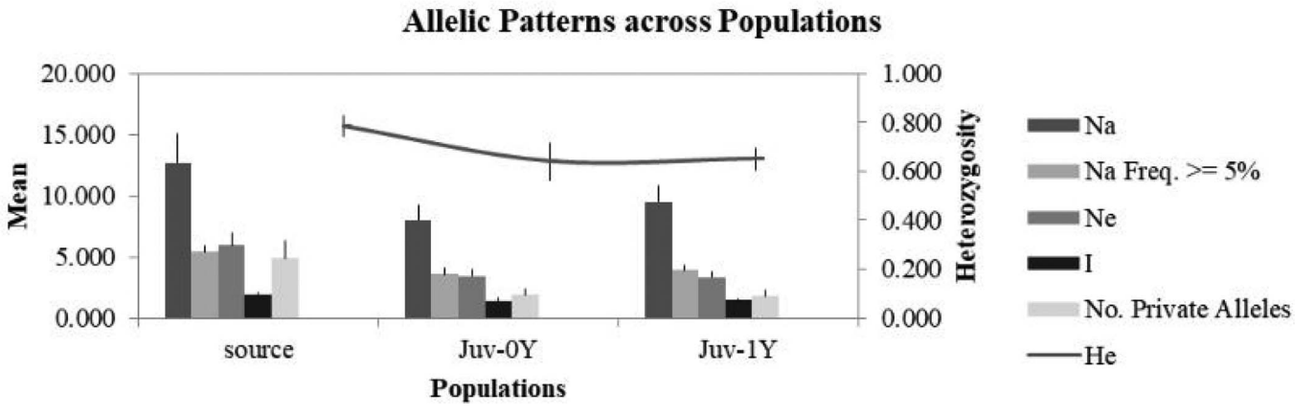

Figure 1. Allelic patterns by population. The labels on the left *x* axis correspond to the bar graph representing the mean values for each population of each variable. N_a is number of alleles, N_a Freq. $\geq 5\%$ is the number of different alleles with a frequency greater than or equal to 5%, N_e is the number of effective alleles, No. Private Alleles is the number of private alleles in the population. The labels along the right *x* axis correspond to the line chart representing expected heterozygosity (H_e) for each population.

Table 3. Rarefied allelic richness per locus, by population.

	Source	Juv-0Y	Juv-1Y
Average across all loci	5.33	6.01	4.07
Anec101	4.44	3.62	3.69
Anec114	6.64	5.15	6.01
Anec126	7.57	5.16	4.80
Anec130	4.65	4.43	3.54
Aned104	5.21	5.36	4.12
Aned106	5.27	3.89	3.91
Aned108	4.80	4.93	4.07
Aned126	6.74	4.46	4.43
Aned132	2.59	2.66	2.11
Aned140	5.35	4.11	4.01

different sample sizes more meaningful, the Juv-0Y set had the greatest allelic richness, followed by the source set, and then the Juv-1Y set (Table 3).

The Information Index (I) indicated that the source set ($I = 1.933$) was the most genetically diverse, while the Juv-0Y ($I = 1.421$) and Juv-1Y ($I = 1.462$) sets exhibited similar diversity. Observed heterozygosity (H_o) was 0.754 in the source set, decreased to 0.667 in the Juv-0Y set, and increased to 0.769 in the Juv-1Y set. H_o was slightly lower than H_e (expected heterozygosity) in the source set, resulting in an F_{IS} value of 0.045. In the Juv-0Y set, H_o was slightly higher than H_e, resulting in an F_{IS} value of -0.06. In the Juv-1Y set, H_o was higher than H_e, resulting in an F_{IS} value of -0.183.

All sets of samples exhibited private alleles. In the source set, 49 private alleles were exhibited in 33 out of 45 individuals (73%). In the Juv-0Y set, 19 private alleles were exhibited by 20 out of 41 individuals (49%). In the Juv-1Y set, 18 private alleles were exhibited by 15 out of 48 individuals (31%). An additional three alleles were found in both the Juv-0Y and Juv-1Y sets that were not found in the source set, resulting in a total of 40 alleles found in the juvenile sets that were not found in the source set. After rarefaction, the source set had the greatest number of private alleles, followed by the Juv-0Y set; the Juv-1Y set had the fewest private alleles (Table 4).

Table 4. Rarefied private allelic richness per locus, by population.

	Source	Juv-0Y	Juv-1Y
Average across all loci	1.89	1.07	0.87
Anec101	0.00	No data	0.00
Anec114	2.27	0.78	1.83
Anec126	4.76	2.04	1.85
Anec130	1.52	1.44	0.51
Aned104	1.18	1.56	0.84
Aned106	1.95	1.27	1.14
Aned108	0.99	1.13	0.37
Aned126	3.28	1.23	1.35
Aned132	0.64	0.69	0.27
Aned140	2.30	0.61	0.56

Table 5. Pairwise population F_{ST} values. All values are significantly different from $F_{ST} = 0$.

	Source	Juv-0Y
Juv-0Y	0.0254	
Juv-1Y	0.0625	0.0615

Population comparison.—All sample sets were significantly differentiated (Table 5). The source and Juv-0Y sets were the most similar, followed by the two juvenile sets. The source and Juv-1Y sets were the most genetically distinct from each other. AMOVA results indicated a global F_{ST} of 0.149 ($P = 0.001$) with 15% of molecular variation detected among populations, 10% among individuals, and 75% within individuals.

We used principal coordinate analysis to visualize genetic similarity within and between groups (Figs. 2, 3). The Juv-0Y sample set was the least tightly clustered sample set, while the Juv-1Y set was the most tightly clustered. The source set was located between the Juv-0Y and Juv-1Y sets and overlapped somewhat with each. There was little overlap between Juv-0Y and Juv-1Y. Axis 1 explained 23.06% of variation, Axis 2 explained 6.06% of variation, and Axis 3 explained 4.82% of variation.

Multiple paternity.—Parentage analysis revealed high levels of multiple paternity in all three broods. In the combined Juv-0Y and Juv-1Y sets (89 juveniles total), 56 juveniles were assigned to dam D191 and were sired by at least 33 males, 17 juveniles were assigned to dam D231 and sired by at least 11 males, and 13 juveniles were assigned to dam D185 and sired by at least seven males. No male sired more than three juveniles, and no male sired juveniles from more than 1 dam.

Differential mortality by dam.—The juveniles experienced high levels of mortality in the hatchery, with less than half of all juveniles surviving between the sampling events immediately after transformation and 1 yr later. Juv-0Y samples were

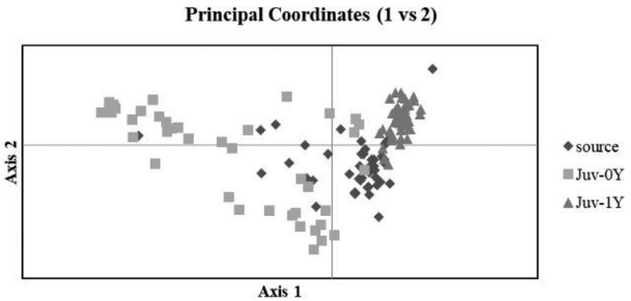

Figure 2. Principal coordinates analysis (PCoA) via covariance matrix with data standardization.

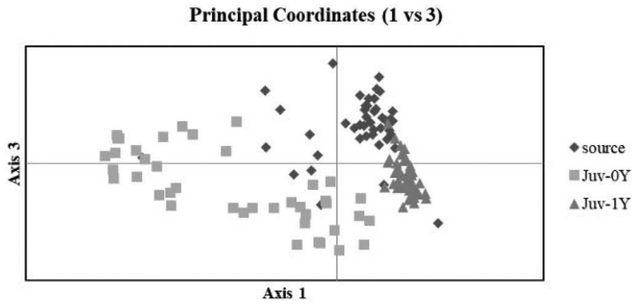

Figure 3. Principal coordinates analysis (PCoA) via covariance matrix with data standardization.

purposely taken proportionately from each of the three dams. Of the 41 samples successfully genotyped, 11 (27% of Juv-0Y) were from dam D191, 17 (41% of Juv-0Y) were from dam D231, and 13 (32% of Juv-0Y) were from dam D185 (Fig. 4). After obtaining the Juv-0Y subsample, the remaining juveniles were pooled and raised together. Analysis of the Juv-1Y set found that 45 out of 48 (94% of Juv-1Y) juveniles in the subsample were the offspring of one dam, D191 (Fig. 5). Two juveniles (4% of Juv-1Y) were from dam D231, and one juvenile (2% of Juv-1Y) was from dam D185.

DISCUSSION

Captive propagation can be an important tool for the conservation of rare species, and in some instances, it may have prevented the extirpation of populations (Hebdon et al. 2004). However, it can significantly impact the genetic structure and evolutionary trajectory of target populations (Waples and Drake 2004; McMurray and Roe 2017). Thus, while the propagation of freshwater mussels has the potential to greatly aid the conservation of the growing number of species that are imperiled, care must be taken to ensure that release of

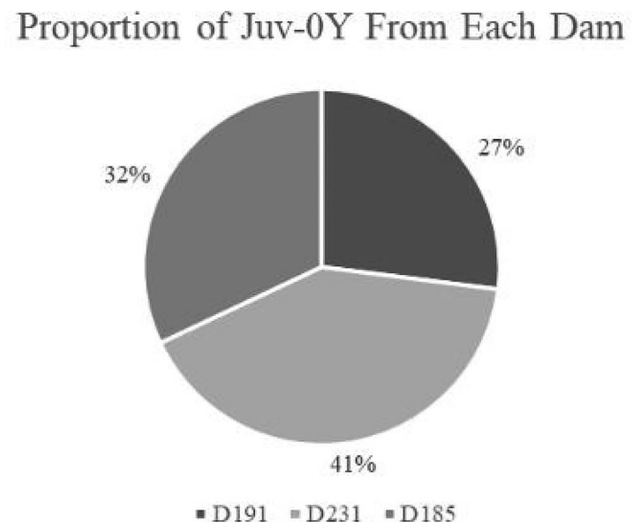

Figure 4. Proportion of Juv-0Y individuals from dams D185, D191, and D231.

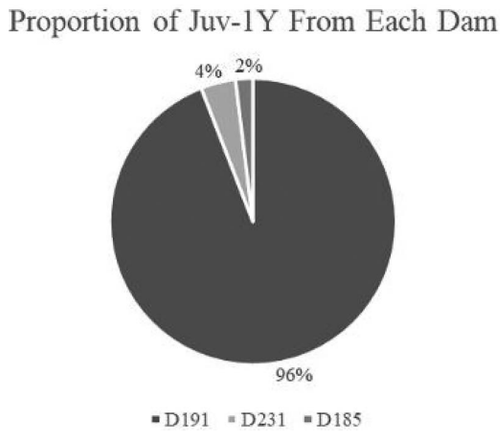

Figure 5. Proportion of Juv-1Y individuals from dams D185, D191, and D231.

propagated juvenile mussels maximizes the benefits and minimizes the risks to the target species/populations. Our examination of the genetic characteristics of propagated juvenile threeridge mussels and their source population provides insight into how propagation of mussels can be improved.

The ratio of H_o to H_e in the source population was comparable to nine other threeridge mussel populations analyzed using the same set of microsatellite markers (Olson and Vaughn 2020) and may be typical for this species. The source population exhibited a deficit of heterozygotes, although heterozygosity was well within the range observed for a variety of other freshwater mussel species (Inoue et al. 2015; Paterson et al. 2015; Chong et al. 2016; Schwarz and Roe 2022). Low levels of heterozygosity might indicate high relatedness of individuals in the source population, with potential occurrences of inbreeding (Harmon and Braude 2010).

The Juv-1Y sample exhibited excess heterozygosity, as would be consistent with a recent reduction in population size (Barker et al. 2009). Mortality in the hatchery, as in any bottleneck event, could have reduced the number of alleles more than it reduced the overall measured heterozygosity (in the sense of Nei's [1987] gene diversity), thus leaving the signature of excess heterozygosity (Piry et al. 1999). Populations naturally recover from genetic bottlenecks through immigration, connectivity with other populations, and mutation giving rise to new alleles (McEachern et al. 2011; Jangjoo et al. 2016). Our results indicate that augmented populations might require multiple infusions of genetic diversity either naturally via gene flow or through additional introductions.

All sample sets displayed a substantial number of private alleles, and the combined juvenile populations exhibited 40 alleles not detected in the source population (19 in Juv-0Y, 18 in Juv-1Y, 3 in both Juv-0Y and Juv-1Y). This observation could be evidence for fertilization of female mussels by sires located upstream of the source population. Male freshwater mussels broadcast sperm into the water column to be filtered by females (Haag 2012). In a study of the population structure of the plain pocketbook mussel *Lampsilis cardium* Rafinesque,

1820, a male mussel was found over 16 km upstream of the dam it fertilized (Ferguson et al. 2013). Dispersal of sperm over long distances allows for gene flow between spatially distinct mussel beds within a watershed. We found 49 alleles in the source population that were not represented in either juvenile set, while the juvenile sets combined had 40 alleles that were not represented in the source population. The Juv-0Y sample set was significantly different from the source population based on alleles present, but the two sets were similar in terms of genetic variability, with the Juv-0Y exceeding the source population in rarefied allelic richness. The three dams successfully produced a cohort of juveniles that were representative of the genetic variability of the source population based on allelic richness. However, the sample sets were significantly different based on the identity of the alleles exhibited. Private alleles were present in 20 out of 41 Juv-0Y juveniles, indicating nearly half of the juveniles were sired by males outside of the source sample. The sum of private alleles within those 20 juveniles, across all loci, was 19.

Parentage analyses in this study found high levels of multiple paternity, with broods of 56, 19, and 14 juveniles being fertilized by 33, 15, and 10 sires, respectively. Multiple paternity has been observed before in other species of freshwater mussels. Ten broods of the triangle sail mussel *Sinohyriopsis cumingii* (Lea, 1852) composed of 23 to 29 offspring each were fertilized by 2 to 4 males (Bai et al. 2012), while nearly every juvenile was found to be fertilized by a different male in 15 broods of *M. margaritifera* (Wacker et al. 2019). In the largest brood, 43 juveniles were sired by at least 32 different males. Multiple paternity increases the genetic variability of juveniles from a single dam. Reproductive methods differ between unionid species; multiple paternity, although phylogenetically widespread, has not been documented in every species, nor always to the same degree when observed (Bai et al. 2012; Ferguson et al. 2013; Hewitt et al. 2018; Wacker et al. 2019; Garrison et al. 2021). Therefore, the evidence available for a particular species must be considered when determining the minimum number of females needed to produce a genetically representative juvenile population. Our multiple paternity results highlight the importance of collecting female mussels that were fertilized in the wild, rather than fertilizing females in a hatchery setting. Wild fertilization allows the chance for many male mussels upstream of the female to sire juveniles, maximizing genetic variability in each brood.

The unexpected finding that nearly all the mussels sampled from the released cohort were from a single dam and the high mortality in the hatchery indicates the possibility that mortality in the hatchery biased the produced juveniles in favor of a single dam. The highly variable rate of survival reduced the genetic variability initially captured in the produced juvenile population and contributed to significant genetic differentiation between the source population and Juv-1Y. Frequently, propagation has been found to alter population genetic variability and structure in both bivalve and fish species (Heath et al. 2003; Osborne et al. 2006; Carlson et al. 2007; Hornick and Plough 2019; Geist et al. 2021). The alteration of the selective pressures faced by juveniles raised in the hatchery, either by removing selection present in the wild and/or by inducing artificial selection, can promote maladaptive traits and reduce fitness in subsequent generations (Heath et al. 2003). In some bivalve species, propagation has been shown to successfully produce representative populations when following practices such as controlling for even contributions from brood stock and contributing to new populations via broods from multiple years (Hornick and Plough 2019; Geist et al. 2021). When guidance from the literature is unavailable, the resources to conduct genetic studies should be included in the cost of propagation to ensure that the latter is providing more benefits than harm to populations in the long term.

Freshwater mussel populations commonly exhibit low effective population sizes (N_e) compared to total population sizes (N). Analysis of nine beds of threeridge mussels in Oklahoma found ratios of effective population size to total population size (N_e/N) ranging from 0.002 to 0.219, with a mean of 0.071 (Olson and Vaughn 2020). Frankham (1995) reviewed data for 102 species and found mean estimates of N_e/N ranging from 0.10 to 0.11. A small N_e makes inbreeding more likely and means the population would be likely to lose genetic diversity more quickly through genetic drift (Lande and Barrowclough 1987). The source population of threeridge mussels in the Cedar River was not thoroughly surveyed, so N_e/N data were unavailable for this study, but we recommend that estimates of N_e should be conducted in the future to develop a baseline for freshwater mussel species. Uneven family size, as was observed in the produced juveniles in this study, can reduce N_e, so the population may be at risk of the adverse effects that come with low N_e (McMurray and Roe 2017). It is possible to mitigate problems of low N_e/N by equalizing family size—i.e., by releasing equal numbers of juveniles from different broods (Harmon and Braude 2010; McMurray and Roe 2017).

Threeridge mussel populations from 10 river drainages in the central United States exhibited little genetic structure between beds within the same river drainage (Elderkin et al. 2007). Many studies of mussel species have reported similar findings, assuaging concerns of outbreeding depression resulting from translocating propagated mussels within a river (Ferguson et al. 2013; Galbraith et al. 2015; Jones et al. 2015; Inoue and Berg 2017). However, some genetic structure between upstream and downstream beds of threeridge mussels in the Little River in Oklahoma was found (Olson and Vaughn 2020). A study examining the Texas hornshell mussel *Popenaias popeii* (Lea, 1857) in the Black River of New Mexico also detected genetic structuring within the river (Inoue et al. 2015). Our project used the breeding population of threeridge mussels found closest to the release site within the same watershed to produce juveniles, and it is recommended that future propagation efforts do the same.

Our study revealed changes in the genetic diversity and variation between the source population and the population of juveniles produced for introduction. Reductions in genetic diversity during the propagation process offset the addition of genetic variation due to multiple paternity. Moving forward, the propagation of freshwater mussels will undoubtedly play a role in their conservation. We encourage hatchery managers to embrace a perspective that includes the preservation of genetic diversity as well as the production of juvenile mussels. Maintaining genetic variation in mussel populations will help maintain the adaptive potential of these species in a changing environment.

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