



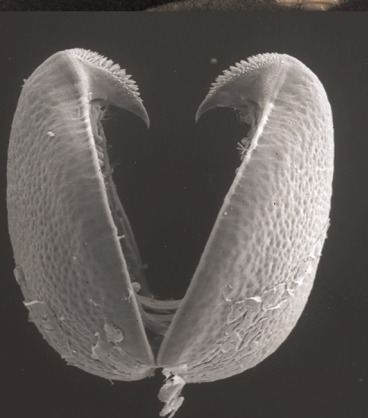

Freshwater Mollusk Biology and Conservation

The Journal of the Freshwater Mollusk Conservation Society

Volume 28 Number 1 March 2025

Freshwater Mollusk Biology and Conservation 28:1–7, 2025

DOI:10.31931/fmbc-d-23-00007

NOTE

CHROMOSOME ANALYSIS TECHNIQUES FOR THE SPINY RIVERSNAIL *IO FLUVIALIS* AND THE PLEUROCERIDAE

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ABSTRACT

A variety of shell, anatomical, and molecular characteristics continue to be used to identify pleurocerid species and aggregate them into genera and higher taxa; however, additional work is required before a comprehensive systematic revision will be possible. In a very limited study, I counted, measured, and analyzed the chromosomes in a single spread from a specimen of *Io fluvialis* to determine if those types of data could contribute to resolving pleurocerid taxonomic and evolutionary questions. The *I. fluvialis* specimen was found to have the same number of diploid chromosomes (34) as seven previously studied pleurocerids; five other species are reported to have 36 chromosomes. The karyotype and two sets of metrics derived from measuring the spread can serve as reference points for future data from this and other species. Further, recent advances in slide-based chromosome techniques and genome-mapping technology, neither of which were represented in this data set, might help resolve both taxonomic and evolutionary questions concerning pleurocerid snails and, potentially, other freshwater mollusks.

KEY WORDS: *Io fluvialis*, Pleuroceridae, chromosomes, karyotype, genome mapping

INTRODUCTION

Freshwater snails in the family Pleuroceridae, common members of many stream habitats in eastern North America, often exhibit a range of shell characteristics that complicate identifying species and sorting the species into genera (Burch 1989; Johnson et al. 2013). For many years, authors relied on external features of the shells and anatomical details to separate species and aggregate them into higher taxa (e.g., Lea 1863; Baker 1928; Burch and Tottenham 1980). Recently, authors have started using various types of RNA and other molecular data to complement and/or contradict previous groupings of populations, species, and larger groups of these snails (e.g., Minton and Lydeard 2003; Dillon and Robinson 2009; Strong and Kohler 2009). One recent paper also included information about differences in egg-laying patterns and body color as possible correlates to the clades/monophyletic groups it recognized (Whelan et al. 2022). That paper concluded that "[d]espite advances made here, additional work is required before a comprehensive systematic revision [of the Pleuroceridae] will be possible" (Whelan et al. 2022).

DNA that controls the basic form and biology of a species is arranged on the chromosomes present in the cells of that species (NHGRI 2020). While chromosome numbers have been reported for a variety of freshwater snails (Thiriot-Quievreux 2003), those numbers and other characteristics of the chromosomes have been included only rarely in taxonomic studies of these animals (Chambers 1982; Dillon 1991; Garber and Korniudhin 2003). One reason chromosomal data may not have been included in previous pleurocerid taxonomic studies is that the former typical slide preparation technique included the use of colchicine, which yields extremely contracted chromosomes that are difficult to characterize (Ronne 1989). Advances in chromosome preparation and analysis techniques (e.g., Guo et al. 2018) have led to interesting taxonomic and evolutionary results concerning other animal groups (e.g., Volleth 2013; Chueca et al. 2021). I conducted this small study to ascertain if a different slide preparation technique and other new analysis tools could provide chromosomal data that might be useful in resolving pleurocerid taxonomic and evolutionary questions.

METHODS

I collected a single live specimen of the Spiny Riversnail, *Io fluvialis,* from the Clinch River at the Route 25E bridge in Claiborne County, Tennessee (36.410, –83.500), on August 2, 1977. I made chromosome slides from this specimen on site using a slight modification of my slide preparation technique for freshwater mussels (Jenkinson 1983, 2014), summarized as follows. I broke open the shell and cut a section of mantle into ~5 × 5 mm pieces and placed them in demineralized, double-distilled water for 30 minutes. I removed the tissue samples from the water, collected any water that came off easily on paper toweling, then flooded the samples with freshly mixed 1 glacial acetic acid: 3

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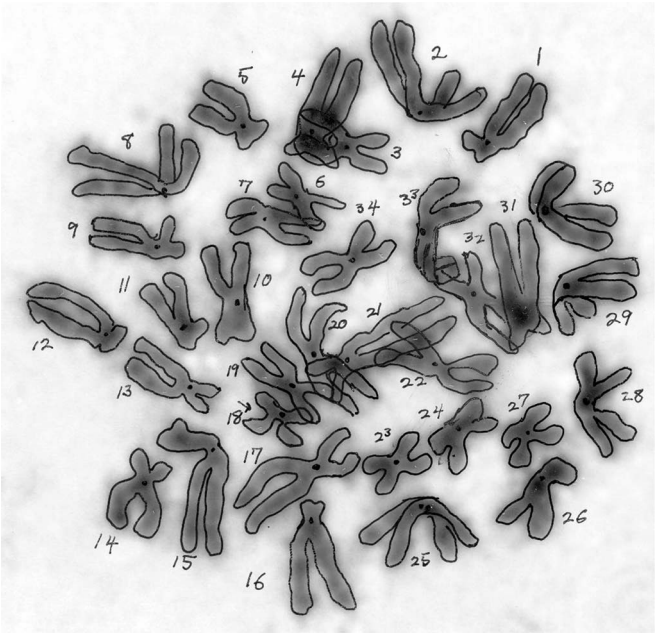

Figure 1. Photograph of the high-quality chromosome spread of *Io fluvialis* from the Clinch River in Tennessee after the individual chromosomes had been outlined while being compared with the actual spread viewed under the microscope. Dots on some of the 34 chromosomes indicate the apparent position of the centromeres when they could be identified on the spread.

absolute methanol fixative. I changed the fixative twice during 30 to 45 minutes, then rubbed each tissue sample gently on a labeled, clean, dry microscope slide. I dried the slides by waving them in the air or heating them gently over an alcohol lamp. The slides were stable at that point, and I stored them for later staining. To see unbanded, stained chromosomes, I heated the slides at 60°C for one hour, stained them in one percent Giemsa blood stain for 5 to 7 minutes, rinsed them in tap water, allowed them to dry, then covered the dry slides with an oversize coverslip using Permount mounting medium. I found chromosome spreads under 100× magnification and counted and photographed some high-quality spreads under 900× magnification.

On a print of a spread from this specimen, I outlined the individual chromosomes and, when possible, located the centromere connecting the two arms (Figure 1) while examining the spread under the microscope. I measured the arms of each chromosome on this print and used those measurements to characterize the chromosomes in two ways: Arm Ratio (r) – comparing the length of the short arms to the longer arms on each chromosome, and Percent Total Complement Length (% TCL) – dividing the overall length of each chromosome by the aggregate length of all chromosomes in the spread and converting that value to a percentage.

RESULTS AND DISCUSSION

Various types of data can be derived from chromosomes and the DNA they contain, depending on the quality of the spreads, the specific staining techniques employed, and other analytical techniques used. Only some of those possibilities were available when this project was conducted 40+ years ago.

Chromosome Number

The complete scan of a single slide from this *Io* specimen yielded two chromosome spreads, comparable to my results for freshwater mussels (Jenkinson 1983, 2014). The technique I used typically yields only a few chromosome spreads on any given slide, in part because it does not use colchicine to arrest mitosis (and, therefore, increase the number of chromosome spreads). I found that colchicine causes severe contraction in freshwater mollusk chromosomes (e.g., as illustrated in Dillon 1991), which virtually eliminates seeing many of the structural details that augment what can be learned from chromosome counts alone.

One of these two spreads included about 32 poorly separated chromosomes and the other included 34 distinct bi-armed chromosomes. The most recent compilation of gastropod chromosomal data (Thiriot-Quievreux 2003) includes chromosome counts for what are now considered (per Strong and Kohler 2009; Johnson et al. 2013) 11 North American pleurocerid species and 1 North American semisulcospirid (Table 1). This *Io* specimen has the same chromosome number (34) as seven of the previously studied species (four in the genus *Elimia* and all three in the genus *Pleurocera*). The other five species listed in Table 1 (four *Elimia* and one *Juga*) have 36 chromosomes. Differences in chromosome number may indicate separate evolutionary lineages (Blackman 1980); however, chromosome counts from additional species would be required to determine if that is the case for pleurocerid snails.

Chromosome Morphology

In the possible karyotype based on this *Io* specimen (Figure 2), the chromosomes are arranged in order of descending overall length within three classic Arm Ratio (r) categories: metacentric (m, r = 1.0–1.7), submetacentric (sm, r = 1.7–3.0), and subtelocentric (st, r = 3.0–7.0) (following Levan et at. 1964). [This spread does not include any telocentric chromosomes (t, r > 7.0).] Spaces between the groups of chromosomes in each r category on Figure 2 separate the members of each of four %TCL groupings (0–2%, 2–3%, 3–4%, and >4% of the total complement length) (following Jenkinson 2014). These two metrics are virtually unrelated to each other because r focuses solely on each individual chromosome while %TCL categorizes the relative lengths of the chromosomes in the spread. Unrecognized folding of one or more chromosome arms, however, can affect the values of both r and %TCL.

Following the shorthand used in Thiriot-Quievreux (2003), the r formula for this *Io* spread is 9m, 6sm, 2st, for a haploid (1n) total of 17. There is no present shorthand formula for the %TCL metric. The r formulas for the previously studied species listed in Table 1 include a range of values in each shape category. The five entries for *Elimia dickinsoni* and *E. floridensis* reported by Chambers are based on "large numbers of mitotic and meiotic figures" (Chambers 1982).

Table 1. Chromosome numbers and Arm Ratio data for pleurocerid and semisulcospirid snail species from previously published reports and this study. Taxa names follow Johnson et al. 2013.

		Haploid No.					
Taxa	Diploid No.	m	sm	st	t	Reference	Listed As
Pleuroceridae							
Elimia							
E. alabamensis	34	10	4	3	—	Dillon 1991	*Goniobasis alabamensis*
E. catenaria	36	10	5	2	1	Dillon 1991	*G. catenaria dislocata*
E. clavaeformis	34	8	7	2	—	Dillon 1991	*G. autocarinata*
E. dickinsoni	36	6	5	5	2	Chambers 1982	*G. dickinsoni* – Chipola River
E. floridensis	36	6	5	5	2	Chambers 1982	*G. floridensis* – Chipola River
E. dickinsoni	36	6	6	5	1	Chambers 1982	*G. dickinsoni* – Holmes Creek
E. floridensis	36	6	6	5	1	Chambers 1982	*G. floridensis* – Holmes Creek
E. floridensis	36	9	4	4	1	Chambers 1982	*G. floridensis* – Ichetucknee R.
E. floridensis	36	9	4	4	1	Dillon 1991	*G. floridensis* – Blue Springs
E. livescens	36	8	3	7	—	Dillon 1991	*G. livescens*
E. proxima	34	7	5	5	—	Dillon 1991	*G. proxima*
E. simplex	34	9	3	5	—	Dillon 1991	*G. simplex*
Io fluvialis	34	9	6	2	—	This Study	*Io fluvialis*
Pleurocera							
P. acuta	34	6	6	5	—	Dillon 1991	*Pleurocera acuta*
P. canaliculata	34	5	6	6	—	Dillon 1991	*P. canaliculata*
P. uncialis	34	8	4	5	—	Dillon 1991	*P. unciale*
Semisulcospiridae							
Juga pilicifera	36	8	3	7	—	Dillon 1991	*Juga hemphilli*

Dillon (1991) does not indicate the number of spreads represented by the r formulas given for any of the species reported there. Chromosome measurements are not included in either of these reports; however, Dillon does categorize the chromosomes as large, medium, and small (Dillon 1991). Both Chambers and Dillon proposed relationships among the populations or species based on suggested exchanges or other rearrangements in the lengths of the chromosomes or

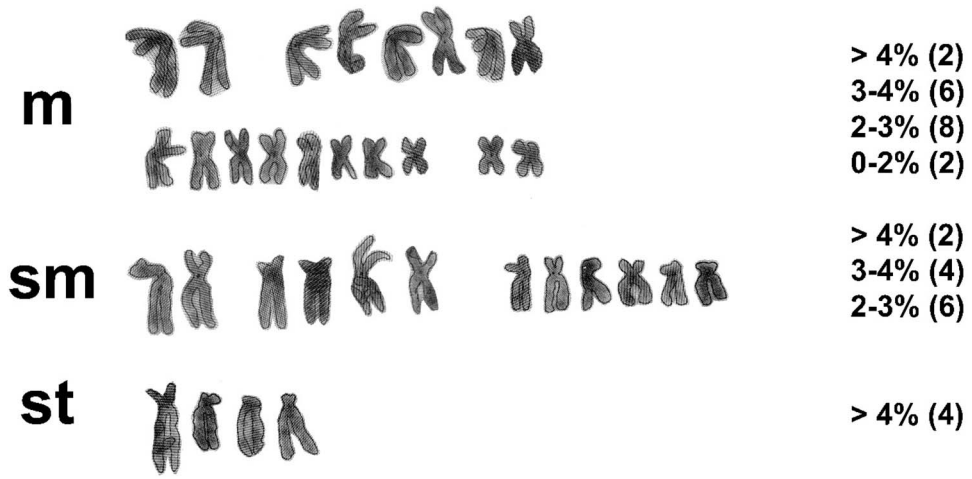

Figure 2. Suggested karyotype for *Io fluvialis* based on the measurements of the single chromosome spread. The 18 Arm Ratio metacentric (m) chromosomes fall into four Percent Total Complement Length (%TCL) categories, the 12 submetacentric (sm) chromosomes fall into three %TCL categories, and all 4 subtelocentric (st) chromosomes fall into a single %TCL category.

positions of the centromeres even though Dillon mentions that "colchicine had the undesirable side-effect of yielding rather condensed chromosomes" (Dillon 1991).

Having two analysis metrics for this *Io* chromosome spread (data presented in Table 2) makes it possible to visualize the size and shape relationships present. Plotting the r values against the %TCL values for each chromosome (Figure 3) demonstrates that both the lengths and shapes of the chromosomes vary across both metrics and do not sort easily within either set of categories. Likely pairs of chromosomes often plot close to each other, and those that do not plot together suggest possible errors in my recognizing folds or the location of the centromeres when the photograph was annotated. For individual spreads, a plot such as this could lead to a careful reexamination of the spread under a microscope and a corrected set of metrics. Plots of multiple spreads from the same animal, population, or species could lead to more precise karyotypes and a solid foundation for recognizing likely chromosomal rearrangements differentiating the taxa.

Table 2. Measurements and analysis ratios of the chromosomes in the *Io fluvialis* spread. Arm Ratio (r) is the length of each long arm (l) divided by the length of the associated short arm (s); %TCL is 100 times the combined length (c) divided by the total of all combined lengths. Reference numbers (Ref. No.) link these measurements to the numbered chromosomes on Figure 1.

Ref. No.	l	s	c	r	r cat.	%TCL	%TCL cat.
27	6.05	6.05	12.10	1.00	m	1.82	0–2
23	6.75	6.45	13.20	1.05	m	1.99	0–2
34	9.05	8.15	17.20	1.11	m	2.59	2–3
28	10.30	9.30	19.60	1.11	m	2.95	2–3
22	11.85	10.70	22.55	1.11	m	3.39	3–4
33	12.55	10.65	23.20	1.18	m	3.49	3–4
25	12.85	10.70	23.55	1.20	m	3.54	3–4
30	12.50	10.30	22.80	1.21	m	3.43	3–4
7	9.25	7.50	16.75	1.23	m	2.52	2–3
24	7.80	6.15	13.95	1.27	m	2.10	2–3
3	9.95	7.55	17.50	1.32	m	2.63	2–3
6	8.00	5.95	13.95	1.34	m	2.10	2–3
29	12.60	9.30	21.90	1.35	m	3.29	3–4
19	10.05	7.20	17.25	1.40	m	2.59	2–3
2	17.30	11.75	29.05	1.47	m	4.37	>4
10	10.50	6.85	17.35	1.53	m	2.61	2–3
18	7.95	5.05	13.00	1.57	m	1.96	0–2
8	17.65	11.00	28.65	1.60	m	4.31	>4
20	13.55	7.85	21.40	1.73	sm	3.22	3–4
14	10.35	5.75	16.10	1.80	sm	2.42	2–3
32	13.20	7.10	20.30	1.86	sm	3.05	3–4
26	10.85	5.70	16.55	1.90	sm	2.49	2–3
15	18.15	9.45	27.60	1.92	sm	4.15	>4
17	17.10	8.40	25.50	2.04	sm	3.84	3–4
31	15.65	7.00	22.65	2.24	sm	3.41	3–4
9	12.55	5.45	18.00	2.30	sm	2.71	2–3
4	14.55	6.10	20.65	2.39	sm	3.11	3–4
11	10.20	4.25	14.45	2.40	sm	2.17	2–3
13	12.65	5.05	17.70	2.50	sm	2.66	2–3
21	20.00	7.10	27.10	2.82	sm	4.08	>4
5	10.40	3.65	14.05	2.85	sm	2.11	2–3
1	14.20	3.70	17.90	3.84	st	2.69	2–3
12	17.50	3.60	21.10	4.86	st	3.17	3–4
16	16.90	3.40	20.30	4.97	st	3.05	3–4
Totals	420.75	244.15	664.90			100.00	

Chromosomal Banding

Spreads of chromosomes that are not severely contracted can be stained in ways to reveal different types of bands along their length (Ronne 1989). Those bands can be used to identify specific chromosome segments involved in relocations on or between chromosomes, supporting or correcting suggested relationships among taxa based only on shape and size data (e.g., Carvalho et al. 2005; Schmid and Steinlein 2015). I did not find an appropriate staining technique to produce banded chromosomes for freshwater mussels (Jenkinson 1983) and did not attempt it for this *Io* specimen.

Genome Mapping

Comparing populations, species, or higher taxa using chromosome number, chromosome morphology, and chromosome banding all involve making and examining microscope slides of dividing cells. Genome mapping, however, uses analytical techniques on cells from any tissue to break the chromosomes into segments, identify the DNA sequences along those segments, then recombine the segments into a virtual map of the chromosome complement (Knobloch et al. 2023). Techniques developed as part of the Human Genome Project (Hood and Rowen 2013) have advanced to the point that genome mapping has documented that early Neanderthals-Denisovans interbred with an earlier hominid before breeding with modern Eurasians (Rogers et al. 2020) and that some young boys sacrificed by the Maya more than a century ago were close relatives, including some sets of twins (Barquera et al. 2024). Similar work has explored the evolution of a wide variety of species, including domestic dogs (Ostrander et al. 2017), rice (Wang and Han 2022), and some marine bivalves (Li et al. 2024). Recently, draft genome maps have been generated for some freshwater clams and snails (e.g., Schell et al. 2017; Zhang et al. 2021; Fuchs et al. 2023); however, I am not aware of genome maps for any pleurocerid snails or any taxonomic or evolutionary studies using genome mapping data for other freshwater snails.

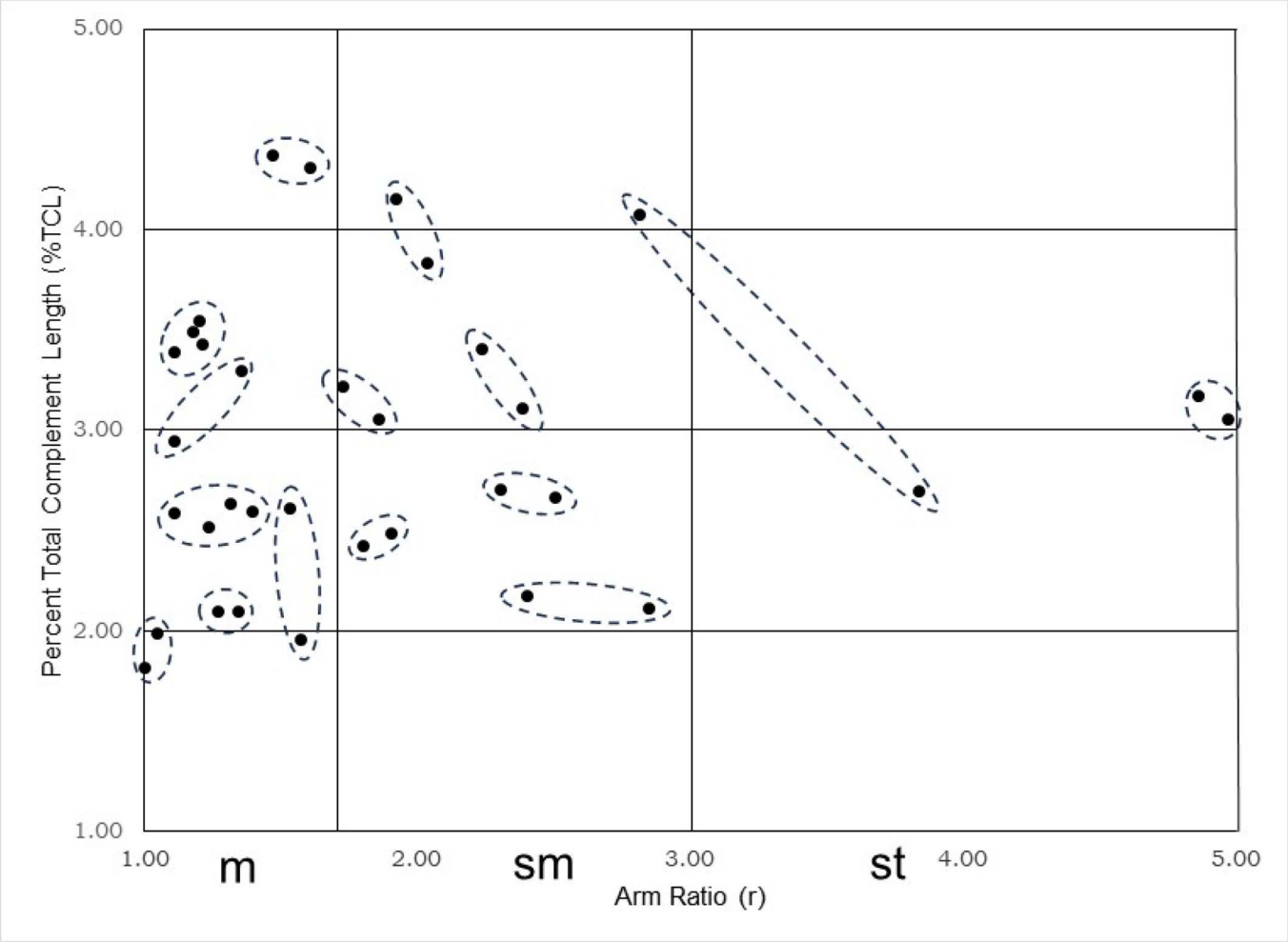

Figure 3. Arm Ratio (r) metrics for the 34 *Io fluvialis* chromosomes plotted against their Percent Total Complement Length (%TCL) metrics. Most of the chromosomes are metacentrics or submetacentrics, each representing between 2 and 4% of the total complement length. The dotted lines suggest one possible sorting of the chromosomes into pairs, with the larger ovals likely indicating some of the errors included in this single set of measurements.

Synthesis

This chromosome number, possible karyotype, and set of measurement data are presented and used here in spite of the fact that they are based on just one chromosome spread. Typically, chromosome counts, karyotypes, and chromosome metrics are developed after measuring a number of high-quality spreads of the population or species being studied (e.g., Leitão et al. 1999). The chromosomes in this single *Io fluvialis* spread appeared to be sufficiently separated and detailed to warrant constructing a possible karyotype and associated metrics; however, additional high-quality spreads should be examined to confirm the chromosome count and, probably, adjust the karyotype for *Io fluvialis*. Regardless of its limitations, this spread has met the purpose of this study by providing a focus for exploring the types of data and analyses that chromosomes can provide.

Modern researchers interested in looking at freshwater snail (and/or freshwater mussel) chromosomes should be able to take advantage of numerous technical improvements that were unavailable or insufficiently perfected 40+ years ago. Techniques to culture cells from test animals (e.g., Quinn et al. 2009) would render large numbers of chromosome spreads available for study. Digital photography and computer-assisted measuring devices (e.g., as mentioned in Thiriot-Quievreux 2002 and Leitão et al. 1999) would make the recovery and examination of chromosome data much easier and, probably, much more accurate. Techniques to identify banding patterns on chromosomes (e.g., Ronne 1989; Bayani and Squire 2004) would enable the investigator to locate and follow rearrangements of linkage groups across populations, species, and related taxa (e.g., Martínez-Lage et al. 1996; De Jong et al. 1999; Schmid and Steinlein 2015). All of these advances would enhance the use of slide-based chromosomal data.

Genome mapping appears ideally suited to addressing a wide variety of biological and evolutionary topics (e.g., Shastry 2007; Saavedra and Bachère 2006; Cheng et al. 2024), including the potential for untangling complex

taxonomic and evolutionary issues (Parey et al. 2023; Steenwyk and King 2024). While not yet tested, genome mapping also appears capable of making similar contributions to addressing taxonomic and evolutionary questions concerning North American pleurocerids (as well as freshwater mussels).

ACKNOWLEDGMENTS

I have encouraged others to look at freshwater mollusk chromosomes for years, without much success. Eventually, I was motivated to publish this Note by the obvious frustration of pleurocerid biologists continuing to try to determine the boundaries of species and higher taxa even as researchers investigating other groups were using chromosomal data and genome mapping to identify specific traits and describe long, complex evolutionary lineages. Two anonymous reviewers and two journal editors all felt this topic warranted publication but agreed that my initial submission required further development. Addressing those positive comments and suggestions resulted in this more complete and focused Note; I hope it will encourage snail and mussel biologists to add chromosome-based data sets to their analyses.

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Freshwater Mollusk Biology and Conservation 28:8–21, 2025

DOI:10.31931/fmbc-D-22-00006

REGULAR ARTICLE

PROPAGATION AND RELEASE OF FRESHWATER MUSSELS (BIVALVIA: UNIONIDAE) FOR TWO NATURAL RESOURCE DAMAGE ASSESSMENT AND RESTORATION CASES IN THE CLINCH AND POWELL RIVERS IN VIRGINIA AND TENNESSEE

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ABSTRACT

Two Natural Resource Damage Assessment and Restoration cases in the upper Tennessee River basin are among the first and largest cases in the United States involving injury to freshwater mussels due to the release of hazardous substances. The Certus, Inc. spill of a rubber accelerant occurred in 1998 in the upper Clinch River in Virginia, killing an estimated 18,621 mussels, including individuals of three endangered species. The Lone Mountain Processing, Inc. spill of coal slurry occurred in 1996 in the Powell River in Virginia, affecting mussels over a 105-miles river section. Settlement money was used to propagate and release mussels at multiple sites in both rivers. We compiled the number of mussels produced from 2003 to 2018 on host fishes and the number of mussels released from 2004 to 2019 at population restoration sites by the Virginia Department of Wildlife Resources' Aquatic Wildlife Conservation Center (AWCC) and Virginia Tech's Freshwater Mollusk Conservation Center (FMCC). A total of 8,456,191 juvenile mussels of 34 species was produced by AWCC and FMCC, with 861,845 mussels of 26 species released at sites in Virginia and Tennessee. Of the released mussels, 150,680 were 20–40 mm long and 1–3 yr old. The remaining mussels (711,165) were typically a few weeks old and <1 mm long and were released from 2004 to 2008. By 2010, both facilities were growing mussels to larger sizes before release, which allows mussels to settle into substrate more quickly and thus improves their chances of survival. Of the mussels produced from 2010 to 2019, mean survival to a stockable size (20–40 mm) was 4.8% for AWCC and 6.1% for FMCC, with no species experiencing survival >22%. Our data show that production must be much higher than the target number of mussels to be released, and they allow researchers to better estimate the amount of production necessary to reach restoration goals.

KEY WORDS: freshwater mussels, restoration, propagation, culture, Clinch and Powell rivers

INTRODUCTION

Two of the first and largest Natural Resource and Damage Assessment and Restoration (NRDAR) cases in the United States involving injury to freshwater mussels were the Certus, Inc. and Lone Mountain Processing, Inc. (LMPI) spills in the upper Tennessee River basin in Virginia. As part of the settlements between Certus, Inc., LMPI, and the Department of Interior (DOI), on behalf of the United States of America, approximately $4,000,000 in natural resource damages was used to compensate for the injuries from these two spills by propagating and releasing mussels in both rivers (Commonwealth of Virginia and U.S. Department of the Interior 2003; Jones 2003). Because of the large amount of mussel propagation required, both the Virginia Department of Wildlife Resources' Aquatic Wildlife Conservation Center (AWCC) and the Freshwater Mollusk Conservation Center (FMCC) at Virginia Polytechnic Institute and State University (Virginia Tech) were needed to achieve restoration goals, and hence were funded and staffed using settlement money from both cases. These facilities were responsible for producing and releasing

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(a) Spill of Octocure-554 Revised turned the Clinch River milky-white for 7 river miles from Cedar Bluff, VA, downstream to Richlands, VA.

(b) U.S. Fish and Wildlife Biologist Leroy Koch examining mussels killed during the Certus, Inc. chemical spill.

Figure 1. Photographs of the Certus, Inc. chemical spill that occurred in the Clinch River at Cedar Bluff, Virginia, on August 27, 1998.

freshwater mussels at restoration sites in the Clinch and Powell rivers in Virginia and Tennessee over a 16-yr period.

NRDAR Case Backgrounds

The Comprehensive Environmental Response, Compensation, and Liability Act of 1980 (CERCLA) and the NRDAR program (43 CFR Part 11) provide the regulations and processes for assessments of injury to natural resources (such as mussels), the recovery of damages from responsible parties, and the design and implementation of restoration activities. CERCLA gives authority to federal and state trustees to "[a]ssess damages for injury to, destruction of, or loss of natural resources." In this context, an *injury* is "a measurable adverse change in the chemical or physical quality or viability of a natural resource," while *damages* are the "amount of money sought by the natural resource trustee as compensation for injury ... of natural resources." The NRDAR program provides guidelines and a framework for evaluating natural resources and their services injured by the release of a hazardous substance and, if appropriate, for restoring them back to baseline conditions or the equivalent. The U.S. Fish and Wildlife Service, acting on behalf of the DOI, evaluated injuries to natural resources due to the Certus, Inc. and LPMI spills.

The Certus, Inc. chemical spill released 1,350 gallons of Octocure-554 revised, a rubber accelerant, into a tributary of the Clinch River when a tanker truck overturned on U.S. Route 460 in Tazewell County, Virginia, on August 27, 1998. The river turned a snowy white color downstream of the spill (Fig. 1a) and took at least 12 h to clear. The spill occurred during summer low-flow conditions and affected all organisms in the Clinch River within an approximately 11-miles impact zone from Cedar Bluff, Virginia, downstream to Richlands, Virginia (Fig. 2) but dissipated over several days. The spill killed an extensive proportion of the fish population, as well as most aquatic macroinvertebrates, including an estimated 18,621 mussels of 13 species. Among those 13 were three mussel species listed as federally endangered, the Golden Riffleshell (*Epioblasma aureola*), Purple Bean (*Venustaconcha trabalis)*, and Rough Rabbitsfoot (*Theliderma strigillata*) (Table 1; Fig. 1b) (U.S. Fish and Wildlife Service 2004). The spill eliminated a large portion of the last known reproducing population of the *E. aureola*, making it one of the worst kills of an endangered species since passage of the Endangered Species Act in 1973 (U.S. Fish and Wildlife Service 2004).

A total of 6,207 dead mussels were collected from the surface of the substrate immediately following the spill, including 250 individuals of the three federally listed endangered species (Table 1). At any given time, only a fraction of mussels is

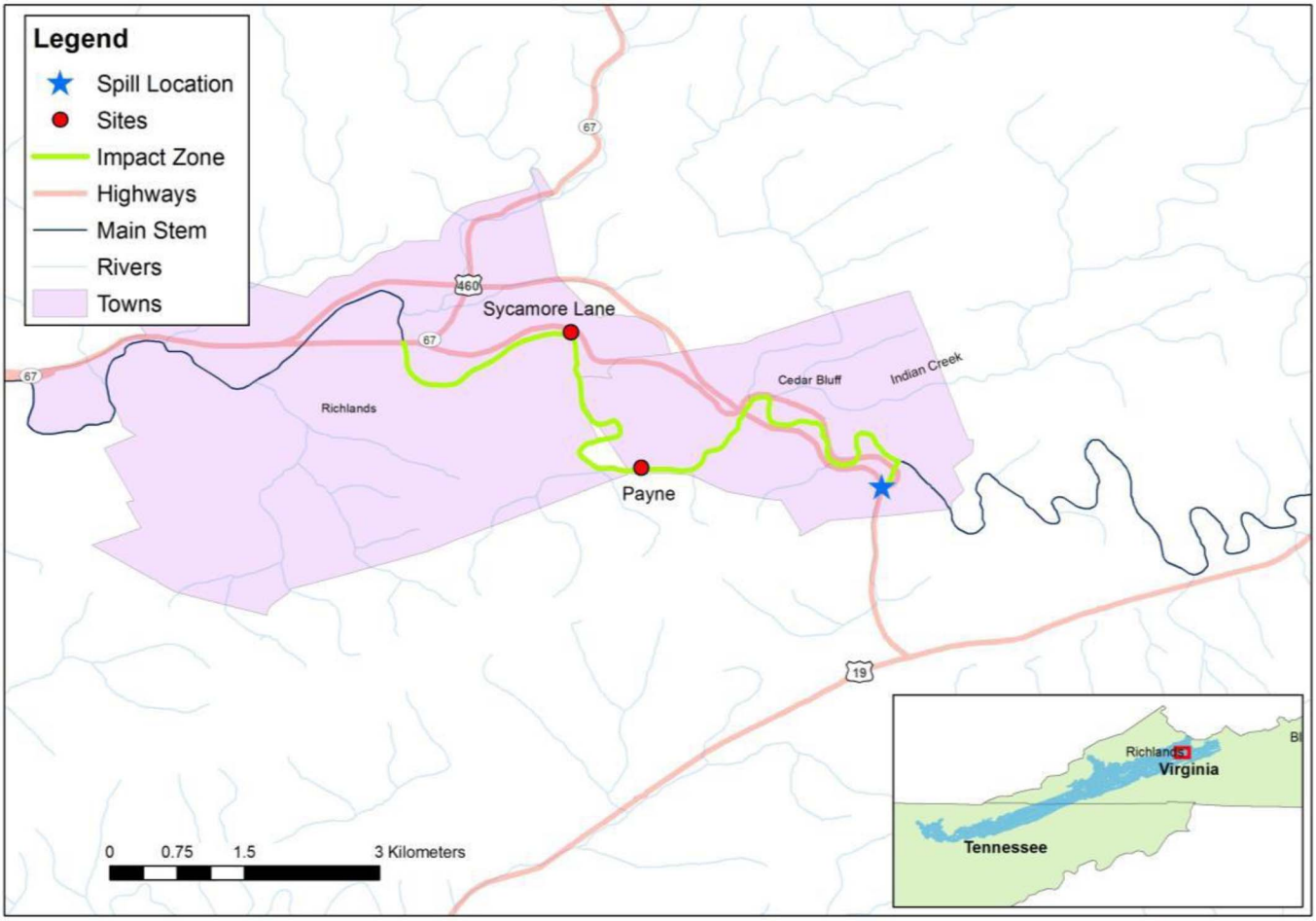


Figure 2. Impact zone of the Certus, Inc. chemical spill in the Clinch River, Tazewell County, Virginia on August 27, 1998.

expected to be on the substrate surface and available for capture or collection (Schwalb and Pusch 2007). To include buried mussels in the injury quantification, the total number of dead mussels was multiplied by three, although no quantitative sampling was conducted in the spill zone to validate this multiplier. This extrapolation resulted in an estimated injury of 18,621 mussels, including 750 individuals of the three endangered species, which was used as the baseline condition and ultimate restoration target for the Certus, Inc. NRDAR case (Table 1). At the time of this spill, more sophisticated methods of injury quantification had not been developed for NRDAR incidents involving mussel species.

The principal goal for the Certus, Inc. NRDAR case was to restore the 18,621 mussels of 13 species, including the three endangered species, to approximate baseline conditions (U.S. Fish and Wildlife Service 2004). The bulk of settlement funds went toward supporting propagation of all impacted mussel species at sites within the spill area; to reduce the risk of stocking mussels only at a single, relatively short, rural-suburban stream reach, several sites in the Upper Clinch River in Russell County, Virginia, from Nash Ford downstream to Cleveland Islands, also were stocked with mussels (Fig. 3). Sites outside the impact zone were chosen because *Epioblasma capsaeformis* and *Epioblasma brevidens* were used as surrogate species for the critically endangered *E. aureola*, as this species was difficult to successfully propagate and monitor in 2004 and even years later. However, historically, *E. capsaeformis* and *E. brevidens* did not occur in the impact zone and had to be stocked downstream, necessitating the use of additional sites where they had occurred historically or currently.

The LMPI spill occurred when a coal slurry impoundment failed at a coal-processing plant in Lee County, Virginia, on October 24, 1996. Coal slurry entered a system of unused underground mineworks and ultimately exited to the surface at Gin Creek (U.S. Fish and Wildlife Service 2003). As a result, 6,000,000 gallons of coal slurry were released into a series of tributaries of the Powell River. The resulting "blackwater," a mixture of water, coal fines, and clay, impacted a large section of the Powell River, and coal particle sediment ultimately was deposited as far downstream as Norris Reservoir, Tennessee, 65 miles downstream from the release site.

Although both the Certus and LMPI cases involved injuries to mussels, there were key differences between them. The Certus spill killed almost every mussel within a relatively short, 6.8 river-miles length of stream (i.e., acute impact).

Table 1. Mussel age and kill estimates from the Certus, Inc. chemical spill that occurred in the Clinch River at Cedar Bluff, Tazewell County, Virginia, on August 27, 1998 (U.S. Fish and Wildlife Service 2004). Kill estimate was determined by multiplying the number collected by three to account for dead mussels buried in the substrate and thus not observed at the substratum surface.

Species	Min. Age	Max. Age	Mean Age	No. Collected	USFWS† Kill Estimate
Actinonaias pectorosa	6	32	15.5	135	405
Epioblasma aureola	2	11	4.9	178	534
Lampsilis fasciola	8	33	18.5	962	2,886
Lampsilis ovata	5	38	14.2	62	186
Lasmigona costata	4	33	16.5	84	252
Medionidus conradicus	2	14	6.2	219	657
Pleuronaia barnesiana/ Pleurobema oviforme[*]	4	51	18.8	610	1,830
Ptychobranchus fasciolaris	7	85	31.0	579	1,737
Ptychobranchus subtentus	9	55	21.9	35	105
Theliderma strigillata	11	63	44.5	20	60
Venustaconcha trabalis	4	29	11.3	52	156
Cambarunio iris	2	20	7.2	3,247	9,741
Leaunio vanuxemensis	6	22	11.4	24	72
Total				6,207	18,621

*Species are very similar in morphological appearance and were enumerated together.
†U.S. Fish and Wildlife Service.

Although the LMPI spill was a discrete event, coal slurry remained in the river for months afterward and was periodically resuspended during high-discharge events. Thus, the LMPI spill exposed mussels to chronic levels of contaminants (e.g., polycyclic aromatic hydrocarbons and trace metals), potentially causing sublethal effects to mussels over 105 river-miles of the main stem of the Powell River as well as several smaller tributaries. It impacted 15 species of federally listed endangered mussels (three were listed after the spill), 15–20 nonlisted mussel species, and critical habitat of two fish species listed as federally threatened. The Virginia Department of Environmental Quality also estimated that at least 11,240 fish of various species were directly killed (U.S. Fish and Wildlife Service 2003). These fishes included species that serve as hosts to endangered mussels.

At the time of these two NRDAR cases, propagation technology was refined enough that most of the affected species could be propagated (U.S. Fish and Wildlife Service 2004). However, no full-time, professionally staffed hatcheries were dedicated to mussel propagation, and the state of propagation technology was underdeveloped compared to today, especially for rare species. Thus, the propagation and restoration activities that resulted from these cases represented the first large-scale applications of captive propagation as a restoration strategy for freshwater mussels in an NRDAR context.

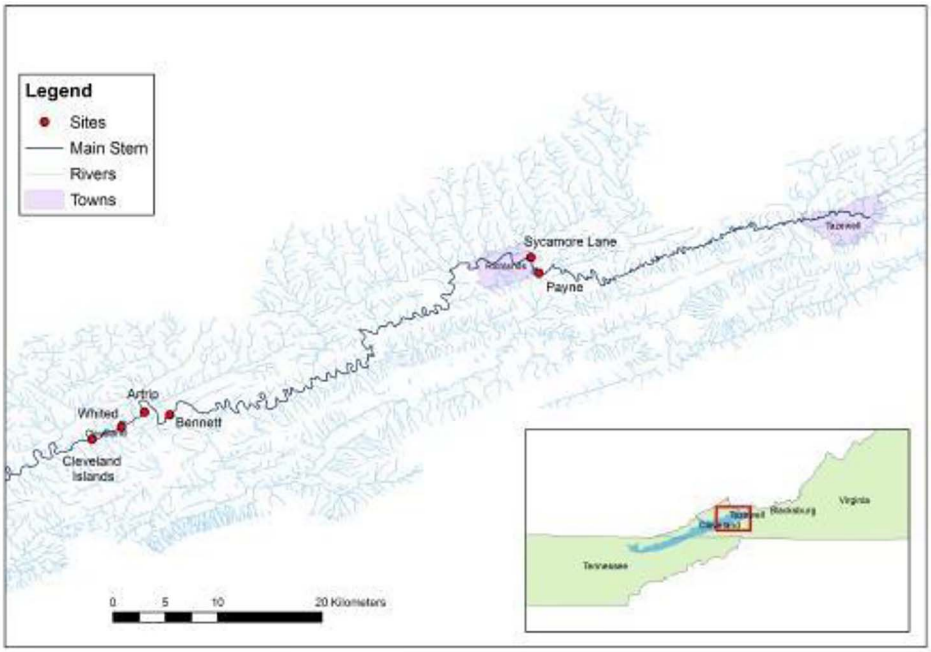
Objectives

Although propagation is now a commonly used restoration strategy for NRDAR cases involving injury to mussels and there are many mussel hatcheries in the United States, Canada, and abroad dedicated to this purpose, a comprehensive examination of restoration activities by mussel propagation facilities has never been conducted for an NRDAR case or a similar large-scale mussel restoration project. The purpose of this study was to examine the production and release data for AWCC and FMCC for restoration in the Certus, Inc. and LMPI NRDAR cases. We refer to *production* as the number of juvenile mussels produced from host fishes artificially infested with mussel glochidia (i.e., number of metamorphosed and excysted juveniles from host fish) and *releases* as the number of hatchery-reared or translocated mussels released at restoration sites. These data are important for several reasons. First, production data can give managers an idea of the general capacity of a propagation facility, which is useful for determining the number of facilities needed for future restoration efforts. Second, the total number of mussels released at restoration sites can be used to track overall success compared to restoration goals. Third, comparing the difference between production and releases at restoration sites can provide information on how much production is needed to reach stocking goals. For example, if the goal is to release 20,000 mussels and only 10% of produced mussels survive to be released, then 200,000 mussels should be produced at the hatchery. This information can be used to determine the level of effort needed for restoration in the future. Finally, these data can be used in further analyses to determine whether restoration goals for the Certus, Inc. and LMPI NRDAR cases were successful (compared to a simple count of releases), as well as to develop tools and resources for injury and damage assessment of mussels in future NRDAR cases. For example, these data can be combined with operating costs of each facility to determine the cost to produce and release a single mussel. Thus, the goals of this project were to (1) summarize the number of mussels produced by AWCC and FMCC associated with the Certus, Inc. and LMPI NRDAR cases, (2) summarize the number of mussels released at restoration sites in the Clinch and Powell rivers as part of the restoration efforts for these two NRDAR cases, and (3) broadly assess survival of propagated mussels at AWCC and FMCC during the grow-out period at each hatchery to determine the percentage survival from production to their eventual release at 1–3 yr old.

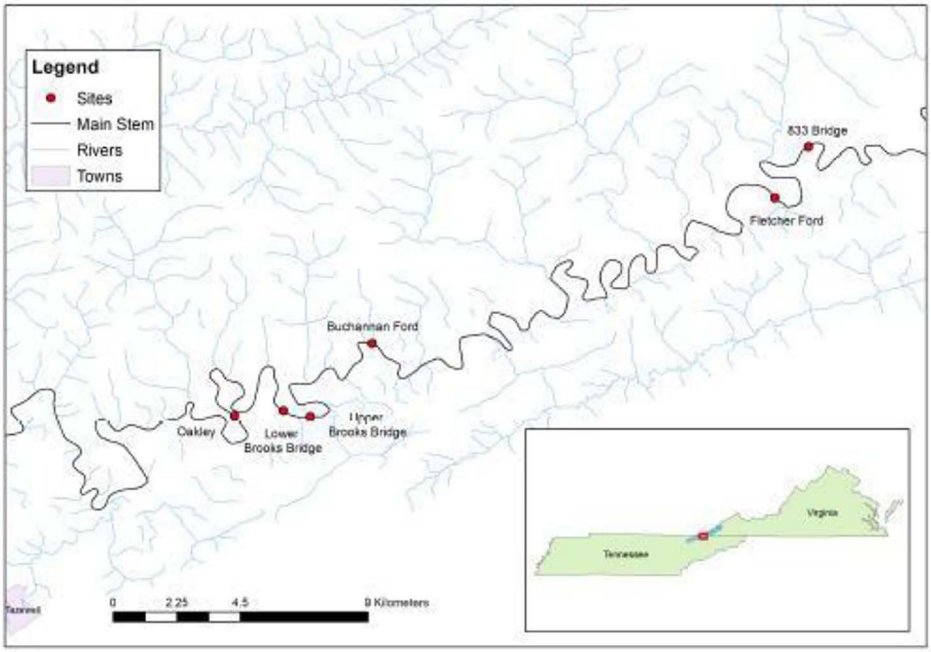
METHODS

Mussel Production and Release Data

We summarized, checked for accuracy, and collated data records for total numbers of newly transformed juvenile mussels produced and total number of mussels released in the



(a) Clinch River sites in Virginia.



(b) Powell River sites in Tennessee and Virginia.

Figure 3. Locations of restoration and monitoring sites in the Clinch (a) and Powell (b) rivers for the Certus, Inc. and LMPI NRDAR cases. Sites represent restoration and monitoring sites for the LMPI, Inc. and Certus, Inc. NRDAR cases.

Clinch and Powell rivers by AWCC and FMCC. Mussels were produced from 2003 to 2018 and released from 2004 to 2019, which was considered the restoration period. Production data (i.e., 1-day-old mussels counted after dropping off fish hosts into the tanks of fish-holding systems) included all juvenile mussels produced at these facilities over this period. By necessity, the juvenile production data had to include mussels produced for all projects conducted during this period because it was impossible to allocate the number of juveniles produced on a project-by-project basis. Only when mussels were grown to a larger stockable size could they then be allocated to a specific project. Although

Table 2. Location information for 14 restoration and monitoring sites for the Certus, Inc. and Lone Mountain Processing, Inc. Natural Resource and Damage Assessment and Restoration mussel restoration cases in the Clinch and Powell rivers, Tennessee and Virginia. River-miles are used to correspond to the United States Geological Survey's topographic maps. RDC = right descending channel and LDC = left descending channel. Sites listed as "no" may have been monitored as part of an effort outside of this project; see text for details.

Site	River	River-Mile	Monitoring*	Latitude, Longitude
Indian Creek, Cedar Bluff, Virginia	Clinch	324	No	37.088809°, −81.765915°
Payne Property, Virginia	Clinch	322.1	Yes	37.081642°, −81.778816°
Sycamore Lane, Virginia	Clinch	320	Yes	37.095162°, −81.785898°
Bennett Property, Virginia	Clinch	277.5	Yes	36.959511°, −82.097550°
Artrip, Virginia	Clinch	274.5	Yes	36.961647°, −82.119429°
Whited Property, Virginia	Clinch	272.7	Yes	36.948771°, −82.139325°
Cleveland Islands—RDC, Virginia	Clinch	270	Yes	36.938084°, −82.164613°
Cleveland Islands—LDC, Virginia	Clinch	270	No	36.937047°, −82.166494°
State Route 833 Bridge, Virginia	Powell	120.2	No	36.620940°, −83.284570°
Fletcher Ford, Virginia	Powell	117.3	No	36.604622°, −83.295228°
Buchannan Ford, Tennessee	Powell	99.2	No	36.558269°, −83.423269°
Upper Brooks Bridge, Tennessee	Powell	95.3	Yes	36.534982°, −83.442999°
Lower Brooks Bridge, Tennessee	Powell	94.7	Yes	36.536824°, −83.451406°
Oakley Property, Tennessee	Powell	89.7	Yes	36.535212°, −83.467035°

*Site was quantitatively monitored from 2015 to 2017 with data available in Hyde and Jones (2021).

concurrent restoration projects were not the focus of our assessment of the Certus, Inc. and LMPI NRDAR cases, they constituted part of the total production capacity of both facilities and helped with the operational costs of each. Therefore, we compiled and reported the total number of mussels reared to stockable size for all projects and used this number to determine the overall survival rates per facility (see below).

Until 2009, mussels generally were released within days or weeks of excysting from host fishes. By 2010, all propagated mussels were allowed to grow to older ages and larger sizes in both facilities to ensure higher survival when released at restoration sites. Therefore, we designated all mussels released at population restoration sites into two categories: those released at <6 mo old (typically 2–4 wk old and <1 mm long) and those released at >6 months old (typically 1–2 yr old and 20–40 mm long). The state of propagation technology, knowledge of source populations (both mussels and host fishes), species life histories, and appropriate mussel habitat and associated restoration sites all contributed to wide variation in the production per species each year, timing of subsequent releases, and restoration for specific projects. Mussels released by AWCC in the Powell River from 2004 to 2014 were designated to replace mussels injured by the LMPI, Inc. spill, while mussels released from the headwaters of the Clinch River near Tazewell, Virginia, river-mile (RM) 350.5, downstream to St. Paul, Virginia (RM 255.7), were designated to replace mussels lost from the Certus, Inc. spill. Mussels released by FMCC in the Powell River from 2004 to 2014 were designated to the LMPI NRDAR case, while mussels released in the Powell River after 2014 were designated to other projects due to funding for LMPI ending in 2014. Mussels released by FMCC from the headwaters of the Clinch River near Tazewell, Virginia (RM 350.5), to Cleveland Islands near Cleveland in Russell County, Virginia (RM 270), were designated to the Certus, Inc. NRDAR case. All scientific names of mussels follow Williams et al. (2017).

These data were summarized by facility (AWCC or FMCC), project (Certus, Inc. or LMPI), and each individual population restoration and monitoring site in the Clinch and Powell rivers where mussels were released. As part of additional studies, these data were used to estimate the expected number of surviving mussels at monitoring sites and to determine costs of producing mussels per facility (Hyde and Jones 2021; Hyde 2022). Estimates of expected numbers of surviving mussels at release sites also were compared to quantitative monitoring data collected from 2015 to 2017 to estimate the actual number of mussels surviving at each site.

Study Areas

For the Certus, Inc. NRDAR case, two release sites, the Payne Property (RM 322.1) and Sycamore Lane (RM 320), were in the Clinch River, Tazewell County, Virginia, within the immediate impact zone of the spill (Table 2). These sites were chosen because they generally had the best mussel habitat in the 11-miles impact zone. Mussels also were released in the lower 400 m of Indian Creek, to include individuals of the critically endangered *E. aureola*; the creek confluences with

Table 3. Total juvenile mussels produced by Aquatic Wildlife Conservation Center (AWCC) and Freshwater Mollusk Conservation Center (FMCC) from 2003 to 2018 for the Certus, Inc. and Lone Mountain Processing, Inc. Natural Resource and Damage Assessment and Restoration cases in the Clinch and Powell rivers in Virginia and Tennessee.

Species	AWCC	FMCC	Total
Actinonaias ligamentina	119,440	1,371	120,811
Actinonaias pectorosa	744,601	5,183	749,784
Alasmidonta viridis	5,623	0	5,623
Cambarunio iris	400,940	448,498	849,438
Cyprogenia stegaria	13,091	8,994	22,085
Dromus dromas	91,395	35,760	127,155
Epioblasma aureola	4,591	18,079	22,670
Epioblasma brevidens	602,545	460,569	1,063,114
Epioblasma capsaeformis	445,260	701,865	1,147,125
Epioblasma triquetra	42,356	21,082	63,438
Eurynia dilatate	42,873	0	42,873
Fusconaia cor	2,552	0	2,552
Fusconaia cuneolus	698	0	698
Hemistena lata	168	56	224
Lampsilis abrupta	427,172	0	427,172
Lampsilis fasciola	1,573,212	225,510	1,798,722
Lampsilis ovata	738,943	84,175	823,118
Lasmigona costata	80,664	0	80,664
Lasmigona holstonia	190,965	0	190,965
Leaunio vanuxemensis	203,024	42,947	245,971
Lemiox rimosus	76,746	8,643	85,389
Ligumia recta	168,343	1,079	169,422
Medionidus conradicus	25,508	28,443	53,951
Plethobasus cyphyus	523	0	523
Pleurobema oviforme	46	0	46
Pleuronaia barnesiana	1,171	0	1,171
Pleuronaia dolabelloides	457	0	457
Potamilus alatus	0	7,849	7,849
Ptychobranchus fasciolaris	8,988	60,431	69,419
Ptychobranchus subtentus	61,277	63,838	125,115
Strophitus undulatus	5,617	0	5,617
Theliderma cylindrica	588	187	775
Theliderma intermedia	0	1	1
Venustaconcha trabalis	131,825	20,429	152,254
Total	6,211,202	2,244,989	8,456,191

the Clinch River at RM 324 in Cedar Bluff, Virginia. Mussels were released at four additional sites in the Clinch River, the Bennett Property (RM 277.5), Artrip (RM 274.5), the Whited Property (RM 272.7), and the left and right descending channels at Cleveland Islands (RM 270), approximately 40 miles downstream of the immediate impact zone in Russell County, Virginia (Fig. 3a). These sites were selected to decrease the risk of released mussels being impacted by a single future event at the release sites in the impact zone. Canoes were used to examine potential sites in Russell County, and the criteria used for selection were presence of good physical habitat, presence of native mussel fauna, observed recruitment of juveniles (mussels <20–30 mm), and the presence of fish hosts.

For the LMPI NRDAR case, six sites in the Powell River were selected as release sites because of the presence of diverse existing mussel assemblages and suitable habitat, including the 833 Bridge (RM 120.2) and Fletcher Ford (RM 117.3) in Lee County, Virginia, and Buchannan Ford (RM 99.2), Upper Brooks Bridge (RM 95.3), Lower Brooks Bridge (RM 94.7), and the Oakley Property (RM 89.7) in Claiborne County, Tennessee (Fig. 3b). All sites where mussels were released for this project were not monitored, such as Indian Creek and the left descending channel at Cleveland Islands. However, six sites in the Clinch River were monitored from 2015 to 2017, the two in Tazewell County and the four in Russell County, and similarly, the latter three sites in the Powell River over the same period, with monitoring data and additional project information available at https://www.doi.gov/sites/doi.gov/files/orda-mussel-restoration-monitoring-final-report-508-checked_0.pdf.

Together, these sites represent the principal sites used for population restoration for these two NRDAR cases. We use the term "population restoration" to refer to the translocation of propagated mussels from the laboratory to locations within the indigenous range of the mussel species. Population restoration sites included both reinforcement sites (release of mussel species into an existing population of conspecifics) and reintroduction sites (release of a mussel species in areas from which it has been extirpated) (IUCN/SSC 2013). All population restoration sites were typically 100–300 m long and were considered high-quality mussel habitat. In the Clinch River, all sites other than the Payne Property and Sycamore Lane (i.e., in the impact zone of the Certus, Inc. spill) were considered reinforcement sites. All sites in the Powell River were considered restoration sites, as they were in the area affected by the spill.

Survival of Propagated Mussels at AWCC and FMCC

From 2010 to 2019, we estimated survival of hatchery-reared mussels to stocking size (e.g., 20–40 mm long) by assessing the number of mussels surviving from production as age-0 excysted juveniles to their eventual release at typically 1–2 yr, by dividing the number of mussels >6 mo old released in each year by the number of mussels produced in the previous year. Because we could not separate out the juvenile production data on a project-by-project basis, the survival analysis included releases for the Certus, Inc. and LMPI NRDAR cases and from all the other projects conducted over the years at both facilities. We chose 2010 to begin the analysis because all releases from this year onwards were >6 mo

Table 4. Total mussels >6 mo old released by the Aquatic Wildlife Conservation Center (AWCC) and Freshwater Mollusk Conservation Center (FMCC) from 2004 to 2019 for the Certus, Inc. and Lone Mountain Processing, Inc. (LMPI) Natural Resource and Damage Assessment and Restoration cases in the Clinch and Powell rivers in Virginia and Tennessee.

	AWCC			FMCC			Totals		
Species	Certus	LMPI	Total	Certus	LMPI	Total	Certus	LMPI	Grand
Actinonaias pectorosa	730	3	733	0	0	0	730	3	733
Cambarunio iris	263	2,977	3,240	9,861	1,077	10,938	10,124	4,054	14,178
Cyprogenia stegaria	38	0	38	0	0	0	38	0	38
Dromus dromas	8	0	8	0	27	27	8	27	35
Epioblasma aureola	710	0	710	0	0	0	710	0	710
Epioblasma brevidens	20,765	2,476	23,241	15,853	2,350	18,203	36,618	4,826	41,444
Epioblasma capsaeformis	9,533	1,930	11,463	15,767	9,468	25,235	25,300	11,398	36,698
Epioblasma triquetra	389	231	620	1,768	33	1,801	2,157	264	2,421
Eurynia dilatata	909	0	909	0	0	0	909	0	909
Fusconaia cor	135	0	135	0	0	0	135	0	135
Lampsilis fasciola	9,325	1,729	11,054	5,061	0	5,061	14,386	1,729	16,115
Lampsilis ovata	1,996	1,294	3,290	4,462	500	4,962	6,458	1,794	8,252
Lasmigona costata	82	0	82	0	0	0	82	0	82
Lasmigona holstonia	1,053	0	1,053	0	0	0	1,053	0	1,053
Leaunio vanuxemensis	9,987	0	9,987	4,228	0	4,228	14,215	0	14,215
Lemiox rimosus	261	63	324	33	0	33	294	63	357
Ligumia recta	1,239	250	1,489	0	0	0	1,239	250	1,489
Medionidus conradicus	1,925	0	1,925	2,800	0	2,800	4,725	0	4,725
Plethobasus cyphyus	3	0	3	0	0	0	3	0	3
Pleuronaia barnesiana	99	0	99	0	0	0	99	0	99
Pleuronaia dolabelloides	100	0	100	0	0	0	100	0	100
Ptychobranchus fasciolaris	460	0	460	2,236	0	2,236	2,696	0	2,696
Ptychobranchus subtentus	472	0	472	1,526	200	1,726	1,998	200	2,198
Venustaconcha trabalis	1,990	0	1,990	5	0	5	1,995	0	1,995
Total	62,472	10,953	73,425	63,600	13,655	77,255	126,072	24,608	150,680

old and allowed us to estimate survival separately for each facility.

RESULTS

Juvenile Mussel Production

Total numbers of juvenile mussels produced by both AWCC and FMCC from 2003 to 2018 for the LMPI and Certus, Inc. NRDAR cases varied from 134,130 to 1,077,786 juveniles per year with a total of 8,456,191 juveniles of 34 species (Table 3). *Lampsilis fasciola* was the species with the largest number produced with 1,798,722 individuals, while *Theliderma intermedia* had the fewest individuals produced, just one mussel. Of the 8,456,191 mussels produced, 6,211,202 were produced at AWCC and 2,244,989 were produced at FMCC (Table 3). Overall, a total of 32 species were produced at AWCC, with *L. fasciola* as the species with the most individuals produced (Table 3). A total of 22 species were produced at FMCC, with *E. capsaeformis* as the species with the most individuals produced (Table 3). See Hyde and Jones (2021) for a detailed breakdown of production by year.

Total Mussels Released

From 2004 to 2019, AWCC and FMCC released 861,845 mussels representing 26 species—ranging from three *Plethobasus cyphus* to 181,995 *L. fasciola*—to replace mussels lost from the Certus, Inc. and LMPI NRDAR cases. Beginning in 2010, almost all mussels were released at larger sizes by each facility to ensure higher survival and retention at monitoring sites. Of the 861,845 total mussels released, 150,680 were 20–40 mm long and generally 1–3 yr old (Table 4; Fig. 4). Of these 150,680 older mussels, 126,072 individuals representing 24 species were released in the Clinch River, Virginia, for the Certus, Inc. NRDAR case (Table 4). *Epioblasma brevidens* and *E. capsaeformis* were

(a) *Epioblasma capsaeformis* released in Powell River, September 24, 2012.

(b) *Epioblasma triquetra* released at the Bennett Property in the Clinch River, VA.

(c) *Lemiox rimosus* released at the Bennett Property in the Clinch River, VA.

(d) Release of *Ptychobranchus subtentus*, *Ptychobranchus fasciolaris*, *Villosa vanuxemensis* and *Lampsilis fasciola* in the Clinch River, VA, at Sycamore Lane, on September 26, 2014.

Figure 4. Photographs of juvenile mussels that were released for the Certus, Inc. and LMPI NRDAR cases in the Clinch and Powell rivers in Virginia and Tennessee.

the species with the greatest number of mussels released (Table 4). For the LMPI NRDAR case, 24,608 older mussels representing 11 species were released, and *E. capsaeformis* was the species with the most released individuals (Table 4).

Mussels Released by AWCC

Of the total mussels released of all ages, 632,002 individuals representing 25 species were released by AWCC to replace mussels lost from the Certus, Inc. and LMPI NRDAR cases. Releases ranged from three *P. cyphyus* to 179,832 *Actinonaias pectorosa*. Of the total number released, 73,425 individuals representing 24 species were >6 mo old (Table 4). Of these older mussels, 62,472 individuals representing 24 species were released for the Certus, Inc. NRDAR restoration project (Table 4). *Epioblasma brevidens* and *E. capsaeformis* were the species with the greatest numbers of mussels released (Table 4). For the

Table 5. Total mussels released >6 months old by Aquatic Wildlife Conservation Center and Freshwater Mollusk Conservation Center for the Certus, Inc. and Lone Mountain Processing, Inc. Natural Resource and Damage Assessment and Restoration cases at each population restoration and monitoring site in the Clinch and Powell rivers, Virginia and Tennessee, from 2004 to 2019. Sites listed represent those monitored annually from 2015 to 2017, except for Indian Creek, which was not monitored. All other restoration sites are included in "Other Sites" for each river.

	Clinch River								Powell River			
Species	Payne Property	Sycamore Lane	Bennett Property	Artrip	Whited Property	Cleveland Islands, Right Descending	Indian Creek	Other Sites*	Upper Brooks Bridge	Lower Brooks Bridge	Oakley Property	Other Sites*
Actinonaias pectorosa	22	0	10	0	0	0	598	100	0	0	0	3
Cambarunio iris	3,699	5,963	0	0	0	0	208	254	0	0	0	4,054
Cyprogenia stegaria	0	0	38	0	0	0	0	0	0	0	0	0
Dromus dromas	0	0	4	0	0	0	0	4	1	26	0	0
Epioblasma aureola	0	300	0	0	0	0	300	110	0	0	0	0
Epioblasma brevidens	0	0	11,979	5,131	0	3,587	0	15,921	1,194	1,120	18	2,494
Epioblasma capsaeformis	0	0	10,013	1,801	1,028	3,757	0	8,701	2,883	3,337	1,187	3,991
Epioblasma triquetra	0	0	1,764	0	0	0	0	393	33	0	0	231
Eurynia dilatata	61	0	356	119	0	0	259	114	0	0	0	0
Fusconaia cor	0	0	68	67	0	0	0	0	0	0	0	0
Lampsilis fasciola	3,135	3,317	1,686	1,243	200	0	1,541	3,264	0	0	0	1,729
Lampsilis ovata	1,355	3,175	265	300	0	0	511	852	0	0	0	1,794
Lasmigona costata	69	0	10	0	0	0	3	0	0	0	0	0
Lasmigona holstonia	0	21	0	0	0	0	1,032	0	0	0	0	0
Leaunio vanuxemensis	4,369	1,829	970	1,694	69	0	2,395	2,889	0	0	0	0
Lemiox rimosus	0	0	133	50	0	0	0	111	0	0	0	63
Ligumia recta	0	0	467	311	0	0	0	461	0	0	0	250
Medionidus conradicus	177	3,250	142	0	0	0	564	592	0	0	0	0
Plethobasus cyphyus	0	0	3	0	0	0	0	0	0	0	0	0
Pleuronaia barnesiana	0	99	0	0	0	0	0	0	0	0	0	0
Pleuronaia dolabelloides	0	0	50	50	0	0	0	0	0	0	0	0
Ptychobranchus fasciolaris	741	1,584	196	0	0	0	175	0	0	0	0	0
Ptychobranchus subtentus	164	1,686	89	0	0	0	59	0	100	100	0	0
Venustaconcha trabalis	20	193	295	300	0	0	224	963	0	0	0	0
Total	13,812	21,417	28,538	11,066	1,297	7,344	7,869	34,729	4,211	4,583	1,205	14,609

*Site localities are given in Table 2.

LMPI NRDAR restoration project, 10,953 older mussels representing nine species were released (Table 4). *Cambarunio iris* was the species with the greatest number of mussels released (Table 4).

Mussels Released by FMCC

Of the total mussels released of all ages, 212,041 individuals representing 15 species were released by FMCC to replace mussels lost from the LMPI and Certus, Inc. NRDAR cases. Total mussels released per species ranged from 58 *Pleuronaia barnesiana* to 75,495 *E. capsaeformis*. Of the total number of mussels released, 77,255 individuals representing 13 species were >6 mo old (Table 4). Of these, 63,600 individuals representing 12 species were released to replace mussels lost from the Certus, Inc. NRDAR restoration project (Table 4). *Epioblasma brevidens* was the species with the most mussels released (Table 4). For the LMPI NRDAR restoration project, 13,655 mussels representing seven species were released, and *E. capsaeformis* was the species with the greatest number of mussels released (Table 4).

Number of Mussels Released at Restoration and Monitoring Sites

Six population restoration sites for the Certus, Inc. NRDAR case were monitored from 2015 to 2017 in the Clinch River (Hyde and Jones 2021). At these sites, 84,976 individuals >6 mo old were released (Table 5). Within the Clinch River impact zone of the Certus, Inc. spill, 15,314 mussels representing 11 species were released at the Payne Property. Most of these mussels were *L. fasciola*, *C. iris*, and *Leaunio vanuxemensis* (Table 5). At Sycamore Lane, the second site in the impact zone, 21,417 mussels representing 11 species were released. The greatest number of mussels released was of *C. iris*, followed by *L. fasciola*, *Lampsilis ovata*, *Medionidus conradicus*, *Ptychobranchus fasciolaris*, and *Ptychobranchus subtentus* (Table 5). In Indian Creek, one of the unmonitored sites, 7,869 mussels representing 13 species were released, including 300 individuals of *E. aureola* (Table 5).

Downstream of the impact zone in the Clinch River, 28,538 mussels representing 20 species were released at the Bennett Property, the majority of which were *E. capsaeformis* and *E. brevidens*. At Artrip, 11,066 mussels representing 11 species were released, with the majority being *E. capsaeformis*

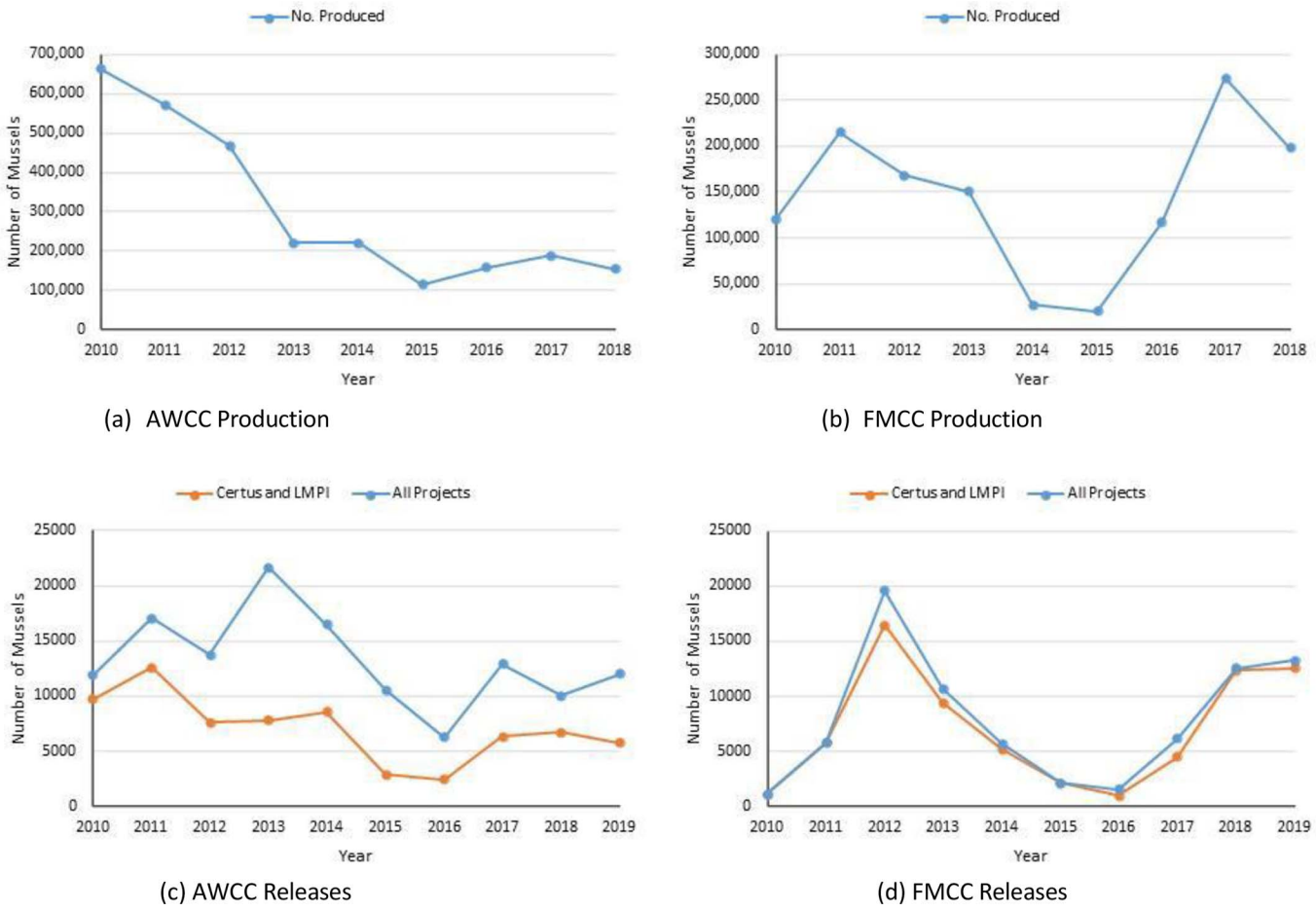


Figure 5. Numbers of mussels produced and numbers of >6-mo-old mussels released by AWCC (a and c) and FMCC (b and d) from 2010 to 2019.

and *E. brevidens*. Only 1,297 mussels representing three species were released at the Whited Property, most of which were *E. capsaeformis*. At Cleveland Islands in the right descending channel (RDC), 7,344 mussels were released, most of which were *E. capsaeformis* (Table 5). In addition, 12,241 mussels were released in the left descending channel (LDC), most of which were *E. capsaeformis* and *E. brevidens* and are included in the "Other Sites" column of Table 5; detailed information about these sites is available in Hyde and Jones (2021). Further, the LDC at Cleveland Islands was not monitored as part of this project but was monitored in 2011 and 2012 by Carey et al. (2015).

For the LMPI NRDAR case, 9,999 individuals >6 mo old were released at three sites in the Powell River monitored from 2015 to 2017. Of these, 4,211 mussels representing five species were released at Upper Brooks Bridge, and 4,583 mussels representing four species were released at Lower Brooks Bridge. Most of these were *E. capsaeformis* and *E. brevidens* (Table 5). Only 1,205 mussels were released at the Oakley Property, almost all of which were *E. capsaeformis* (Table 5). Mussels also were released at the Route 833 Bridge, Fletcher Ford, and Buchannan Ford in the Powell River (Table 5, "other sites"). These sites were not monitored as part of this study but have been monitored in the past (Eckert et al. 2007; Johnson et al. 2012). At the 833 Bridge site, 1,706 mussels were released, mostly *C. iris*; 7,964 mussels were released at Fletcher Ford, mostly *E. brevidens* and *E. capsaeformis*; and 1,997 mussels were released at Buchannan Ford, mostly *E. capsaeformis* (Table 5).

Production and Hatchery Survival of Propagated Mussels at AWCC and FMCC

Production of mussels at AWCC was highest in 2010 (662,930), and from 2013 to 2019 remained between 100,000 to just over 200,000 (Fig. 5a). Release of mussels >6 mo old to replace mussels lost from the Certus, Inc. and LMPI cases by AWCC was highest in 2011 (12,547), decreased to 2,406 in 2016, and then increased to intermediate values in 2017–19 (Fig. 5c). Release of mussels >6 mo old for all projects by AWCC was highest in 2013 (21,672), decreased to 6,256 in 2016, and then increased to intermediate values in 2017–19 (Fig. 5c). The most-produced species at AWCC was *L. fasciola*, followed by *E. brevidens* and *Lamosilis abrupta* (Table 6). *Epioblasma brevidens* was the species with the most mussels released for the Certus, Inc. and LMPI cases, followed by *E. capsaeformis* and *L. fasciola*.

Table 6. Mussels produced and released by Aquatic Wildlife Conservation Center (AWCC) and Freshwater Mollusk Conservation Center (FMCC) >6 mo old at restoration and monitoring sites from 2010 to 2019. Percent survival is number (No.) released divided by number produced. Total number of released mussels does not match Table 4 as this number does not include >6-mo-old mussels released before 2010 and does include mussels released as part of projects other than the Certus, Inc. and Lone Mountain Processing, Inc. Natural Resource and Damage Assessment and Restoration cases.

	AWCC			FMCC		
Species	No. Produced	No. Released	% Survival >6 mo	No. Produced	No. Released	% Survival >6 mo
Actinonaias pectorosa	88,958	708	0.8%			
Alasmidonta viridis	5,623	82	1.5%			
Cambarunio iris	49,519	4,475	9.0%	272,946	9,977	3.7%
Cyprogenia stegaria	6,467	129	2.0%	1,898	0	0.0%
Dromus dromas	55,069	21	0.0%	11,884	27	0.2%
Epioblasma aureola	4,574	710	15.5%			
Epioblasma brevidens	482,472	46,165	9.6%	250,176	19,006	7.6%
Epioblasma capsaeformis	233,767	23,246	9.9%	312,638	30,852	9.9%
Epioblasma triquetra	19,764	1,580	8.0%	19,150	1,901	9.9%
Eurynia dilatata	7,069	909	12.9%			
Fusconaia cor	2,282	273	12.0%			
Fusconaia cuneolus	698	29	4.2%			
Hemistena lata	148	0	0.0%			
Lampsilis abrupta	427,172	7,887	1.8%			
Lampsilis fasciola	588,147	14,649	2.5%	187,695	5,852	3.1%
Lampsilis ovata	191,698	3,323	1.7%	32,626	2,960	9.1%
Lasmigona costata	66,390	82	0.1%			
Lasmigona holstonia	137,940	3,334	2.4%			
Leaunio vanuxemensis	88,339	12,775	14.5%	29,136	1,322	4.5%
Lemiox rimosus	73,754	1,418	1.9%	8,309	33	0.4%
Ligumia recta	74,968	1,969	2.6%	295	0	0.0%
Medionidus conradicus	25,052	3,059	12.2%	27,965	2,800	10.0%
Plethobasus cyphyus	523	3	0.6%			
Pleuronaia barnesiana	1,171	99	8.5%			
Pleuronaia dolabelloides	457	100	21.9%			
Potamilus alatus				7,634	0	0.0%
Ptychobranchus fasciolaris	3,556	460	12.9%	59,605	2,236	3.8%
Ptychobranchus subtentus	16,888	1,134	6.7%	55,757	1,726	3.1%
Strophitus undulatus	5,617	39	0.7%			
Theliderma cylindrica	588	0	0.0%			
Venustaconcha trabalis	97,857	3,772	3.9%	10,869	235	2.2%
Total	2,756,527	132,430	4.8%	1,288,583	78,927	6.1%

Production of mussels at FMCC decreased from 214,585 in 2011 to 19,825 in 2015, and increased to a high of 273,966 in 2017 (Fig. 5b). Release of mussels >6 mo old for the Certus, Inc. and LMPI cases by FMCC was highest in 2012 (16,400), decreased to 981 in 2016, and then increased to 12,528 in 2019 (Fig. 5d). Release of mussels >6 mo old for all projects by FMCC was highest in 2012 (19,569) decreased to 1,533 in 2016, and then increased to 13,231 in 2019 (Fig. 5d). The most produced species at FMCC was *E. capsaeformis*, followed by *C. iris*, *L. fasciola* and *E. brevidens* (Table 6). *Epioblasma capsaeformis* had the highest number of mussels >6 mo old released (Table 6).

Mean survival of mussels produced from 2010 to 2019 to a stockable size (20–40 mm) was 4.8% for AWCC and 6.1% for FMCC, with no species experiencing survival >22% (Table 6). At AWCC, the species with the highest survival to release at >6 mo old was *Pleuronaia dolabelloides* (21.9%), followed by *E. aureola* (15.5%) (Table 6). At FMCC, *M.*

conradicus had the highest survival to release at >6 mo old (10.0%), while all other species' survival was less than 10% (Table 6).

DISCUSSION

The Certus, Inc. and Lone Mountain Processing, Inc. NRDAR cases were the first involving injuries to freshwater mussels in the United States. Consequently, these cases provided a unique opportunity to conduct mussel restoration at a larger scale than previously practiced. Before these cases, there were no full-time, professionally staffed hatcheries to propagate freshwater mussels, and mussel propagation technology was less developed in the mid-1990s.

The settlement money from these cases allowed the hiring of full-time professional-level personnel at both AWCC and FMCC. This investment of resources supported consistent improvement in culture technology of freshwater mussels. For example, numerous host fishes were identified for mussel species whose hosts were previously unknown, allowing for larger-scale production of juveniles, including various minnow, darter, and sculpin species as hosts for numerous endangered mussel species affected by the spills (Rogers et al. 2001; Jones and Neves 2002; Jones et al. 2004, 2010). During these projects, there was a transition away from the propagation and release of very young juveniles (<6 mo old). Before 2008, most mussels released for these projects were typically 2–4 wk old and <1 mm long. However, these mussels had very low survival after release, indicated by the lack of older mussels when sites were monitored from 2000 to 2004 (J. Jones, unpublished data). Early successes of growing mussels to larger sizes and older ages had occurred from 2003 through 2008, and by 2009, both AWCC and FMCC began to release mussels that had grown large enough to have higher survival rates in the wild. This shift was further supported by Carey et al. (2015), who found high survival of *E. capsaeformis* released at 1–2 yr of age compared to releases of 8-wk-old juveniles (zero observations). By 2010, both facilities were almost exclusively releasing only individuals typically 20–40 mm long and 1–3 yr old. These larger individuals were able to settle more quickly into substrate, increasing their survival rate (Jones et al. 2005). This transition to the release of older individuals necessitated the development of techniques to culture and maintain mussels in the hatchery over the course of 1–3 yr.

While production varied greatly among facilities and years, it was always much higher than the number of mussels released. Survival of mussels to larger sizes suitable for release never exceeded 20% in any year, and the total average was less than 5% (Tables 6). These numbers highlight the challenges of propagating freshwater mussels for the purposes of restoration. Given low survival to larger sizes, the target number of mussels produced must be much higher than the target number of mussels to be released for a given restoration project. Our data provide valuable estimates to establish these targets for future restoration projects.

The cooperative nature of these projects promoted collaboration among a number of stakeholders throughout southwest Virginia and northeast Tennessee. The Mussel Recovery Group (MRG) was formed in 2004 to include federal, state, and nongovernmental partners that encouraged the sharing of information for the most efficient use of the resources of AWCC and FMCC. The development of new culture techniques and technology, as well as ongoing partnerships forged during these projects, demonstrate the efficacy of using mussel propagation for restoring mussel populations impacted by chemical spills in the future (Hyde 2022).

This study is the first to compile mussel production and release data for two mussel hatcheries over a 16-yr period. Our data will help biologists determine production capacity for future restoration projects and how much production is needed to meet restoration goals.

ACKOWLEDGMENTS

Financial support for this project was received from the U.S. Department of the Interior's Office of Restoration and Damage Assessment, Washington, D.C., the U.S. Fish and Wildlife Service and the Virginia Department of Wildlife Resources, with whom we have collaborated extensively on this project, especially staff at AWCC who helped with gathering and organizing data for this project. We thank the editors and an anonymous reviewer at FMBC for their edits and comments on the manuscript. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

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DOI:10.31931/fmbc-d-24-00003

NOTE

COLLECTIONS OF THE INVASIVE NEW ZEALAND MUDSNAIL, *POTAMOPYRGUS ANTIPODARUM* (J.E. GRAY, 1843), IN THE OHIO RIVER BASIN

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ABSTRACT

New Zealand Mudsnails, *Potamopyrgus antipodarum* (J.E. Gray, 1843) (hereafter NZMSs), are small freshwater gastropods that have been introduced into multiple continents outside of their native range in New Zealand. Although NZMSs are known to be relatively common and widespread in the western United States and the Great Lakes Basin, fewer populations are known from the country's Mid-Atlantic region. Herein, we present the first records of this nonnative invasive species in the Ohio River Basin, based on recent collections in two tributaries of the Monongahela River in Allegheny County, Pennsylvania. It is likely that NZMSs were introduced into the Ohio River Basin from other invaded sites in Pennsylvania by angling gear or similar vectors.

KEY WORDS: invasive species, New Zealand mudsnail, Ohio River Basin, *Potamopyrgus antipodarum*, survey

INTRODUCTION

New Zealand Mudsnails, *Potamopyrgus antipodarum* (J.E. Gray, 1843) (hereafter NZMSs), are minute (typically <6 mm in total length) freshwater snails native to New Zealand and associated islands that were introduced and have become established on several continents outside of their native range (Geist et al. 2022). Once established in an aquatic ecosystem, NZMSs can attain exceptionally high benthic densities (Hall et al. 2003) and may alter aquatic food webs or compete with and cause declines in native freshwater gastropods and other benthic aquatic macroinvertebrates (Karens et al. 2010; Larson and Black 2016; Preston et al. 2024). Food web shifts caused by NZMSs also may impact the quality of trout fisheries (Vinson and Baker 2008). Vectors attributed to NZMS introductions to novel ecosystems include stowaways in ship ballast water and aquacultural products; fish stocking from infested aquaculture; inadvertent transport on boats and fishing gear, such as wading boots; and transport by fishes or waterfowl (reviewed by Geist et al. [2022]). The species' small size, its resistance to desiccation and disinfectants, and its exclusively asexual reproduction exacerbate the risk of introduction and establishment of populations outside of its native range (Geist et al. 2022).

New Zealand Mud Snails are known to be relatively common and widespread in the western United States (Geist et al. 2022; Benson et al. 2024), but fewer populations are known from the eastern United States outside of the Great Lakes Basin (Dillon et al. 2019, 2023). Three clonal strains of NZMS are known from the United States: US1, US2, and US3, with US1 widespread in the western United States and also known from the eastern United States, US2 primarily inhabiting the Great Lakes Basin, and US3 known only from one river basin in Idaho (Proctor et al. 2007; Levri et al. 2012, 2020).

In Pennsylvania, NZMSs have been known from Lake Erie for almost two decades (Levri et al. 2007) and were first discovered in a tributary within the West Branch Susquehanna River subbasin approximately one decade ago (Pearce and Morgan 2014). Recent surveys have found that NZMSs have significantly expanded their range in portions of the Susquehanna and Delaware river basins (Levri et al. 2020; Hartzell and Macelko 2022) and have led to the first record of the species within the Potomac River Basin (Hartzell and Frederick 2023). These recent collections of NZMSs within Pennsylvania's Atlantic Slope basins warrant surveys in the adjacent Ohio River Basin (Hartzell and Macelko 2022). Herein, we report results of surveys for NZMSs in waters of the Ohio River Basin in southwestern Pennsylvania. We collected NZMS within two tributaries of the Monongahela River, representing the first records of this invasive species in the Ohio River Basin.

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Table 1. Waters surveyed for New Zealand Mudsnail (*Potamopyrgus antipodarum* [NZMS]) in the Ohio River Basin, Pennsylvania, USA, during this study.

Stream or Impoundment	County	NZMS Collected
Back Creek	Fayette	No
Bens Creek	Cambria	No
Big Sewickley Creek	Beaver	No
Buffalo Creek	Armstrong	No
Bull Creek	Allegheny	No
Casselman River	Somerset	No
Cornplanter Run	Armstrong	No
Deer Creek	Allegheny	No
Elklick Creek	Somerset	No
Elton Sportsmen's Dam	Somerset	No
Flaugherty Creek	Somerset	No
Flaugherty Run	Allegheny	No
Fourmile Run	Westmoreland	No
Glade Run	Somerset	No
Hinckston Run	Cambria	No
Howells Run	Cambria	No
Indian Creek	Fayette	No
Jacobs Creek	Westmoreland	No
Laurel Hill Creek	Somerset	No
Laurel Run	Cambria	No
Linn Run	Westmoreland	No
Little Paint Creek	Somerset	No
Little Piney Creek	Somerset	No
Long Run	Allegheny	No
Loyalhanna Creek	Westmoreland	No
McClintock Run	Somerset	No
Meadow Run	Fayette	No
Mill Creek	Westmoreland	No
Miller Run	Somerset	No
Mingo Creek	Washington	No
Montour Run	Allegheny	No
Noels Creek	Cambria	No
North Branch Blacklick Creek	Cambria	No
North Branch Little Conemaugh River	Cambria	No
North Fork Big Sewickley Creek	Beaver	No
Patterson Creek	Armstrong	No
Peters Creek	Allegheny	Yes
Pine Creek	Allegheny	No
Piney Creek	Somerset	No
Piney Creek	Somerset	No
Sewickley Creek	Westmoreland	No
Stewart Run	Cambria	No
Stonycreek River	Somerset	No
Traverse Creek	Beaver	No
Tub Mill Run	Somerset	No
Turtle Creek	Allegheny	Yes
Whites Creek	Somerset	No
Youghiogheny River	Somerset	No

METHODS

From June to September 2023, we conducted physical surveys for NZMSs among 47 streams and 1 impoundment across eight counties within the lower Ohio River Basin in Pennsylvania (Table 1). Survey protocol closely followed methods published previously (Hartzell and Macelko 2022). In brief, we used visual survey techniques at each site, with at least 20 person-min of survey effort. Surveys were done by wading and examining substrates, woody debris, macrophytes, and the undersides of rocks for snails resembling the NZMS. Previously, surveyors were trained to survey for the NZMS at a known NZMS site elsewhere in Pennsylvania. We considered a site to be negative for NZMS if no specimens were found after 20 person-min, although we acknowledge that our methods may have missed very low densities of this invasive species. We identified specimens via microscopy with relevant gastropod keys (Dillon et al. 2019, 2023) and by one of the authors (S. M. Hartzell) who had previous experience identifying NZMS. Identifications were independently verified by Dr. Robert T. Dillon, Jr., of the Freshwater Gastropods of North America Project. We submitted collection records for positive NZMS sites to the U.S. Geological Survey's Nonindigenous Aquatic Species Database (Benson et al. 2024) and to the Pennsylvania iMapInvasives program (https://www.paimapinvasives.org/).

RESULTS AND DISCUSSION

We confirmed the presence of NZMS in 2 of the 48 waterbodies surveyed (Table 1). Both waterbodies with NZMS are tributaries of the Monongahela River Allegheny County: Peters Creek and Turtle Creek. We did not quantify the number of snails found per minute at invaded sites. We found NZMS in relatively high densities at two sites in Peters Creek (GPS coordinates 40.2725′ N, 79.9654′ W and 40.2835′ N, 79.9371′ W) and in relatively low densities at one site in Peters Creek and one site in Turtle Creek, respectively (40.2979′ N, 79.9048′ W and 40.3918′ N, 79.7582′ W).

To our knowledge, based on surveys of the literature (e.g., Dillon et al. 2023) and records for nonnative freshwater gastropods on the U.S. Geological Survey's Nonindigenous Aquatic Species Database (Benson et al. 2024), these appear to be the first records of NZMS collected within the Ohio River Basin. All of Pennsylvania's major drainage basins now have NZMSs present (Fig. 1). Although the exact means of introduction is

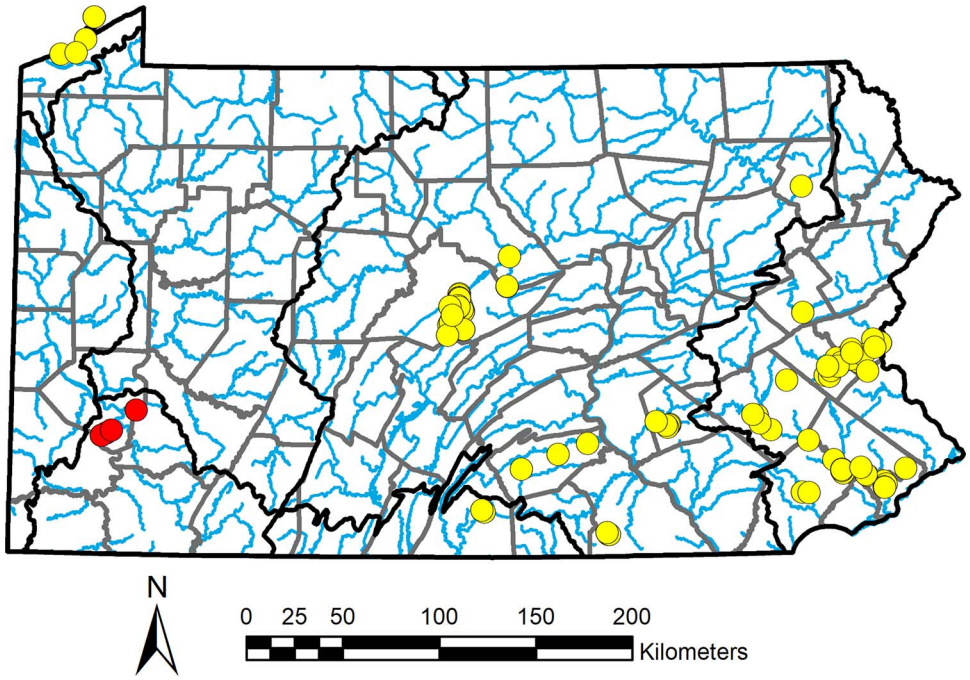

Figure 1. Known distribution of New Zealand Mudsnail (*Potamopyrgus antipodarum*) in Pennsylvania, USA. Red dots indicate recent collection sites in the Ohio River Basin. (Note that although the species was collected at four sites, only three dots are visible on the map because two sites overlap due to spatial resolution.) Yellow dots indicate collection sites in other Pennsylvania drainage basins.

unknown, it is likely that NZMSs were spread to the Ohio River Basin sites via angling gear from previously invaded sites. This vector has been implicated in the spread of NZMS elsewhere in Pennsylvania (Hartzell and Macelko 2022; Hartzell and Frederick 2023), and both Peters Creek and Turtle Creek contain sections managed as stocked trout fisheries with public fishing access. Further comparative studies focused on genetic analyses of NZMSs across various drainage basins of the Mid-Atlantic United States may help reveal pathways of spread and the clonal strain of the NZMS collected within the Ohio River Basin. Although NZMSs collected from Spring Creek in Centre County, Pennsylvania (Susquehanna River Basin), have been identified as the US1 clone, which is widespread in the United States (Levri et al. 2020), no other inland populations of NZMS in the Mid-Atlantic United States appear to have been investigated genetically to determine clonal lineage.

Given that NZMSs were collected in only 2 of 48 waters surveyed, the current range of this invasive species in the Ohio River Basin appears to be very limited. Therefore, it is imperative to prevent its further spread. Although the impacts of NZMS in the Ohio River Basin are unknown, this region supports a moderate diversity of native freshwater gastropod species (reviewed by Dillon et al. [2023]). Negative impacts on native freshwater gastropods and other invertebrates, as observed previously (e.g., Karens et al. 2010; Larson and Black 2016; Preston et al. 2024), are therefore likely. Future studies are needed to monitor native gastropod communities across the study area and evaluate potential impacts of NZMS. In addition, the Pennsylvania Fish and Boat Commission (PFBC) has prepared a statewide control plan for NZMS that recommends several educational strategies, such as posting signage, to reduce the species' spread (PFBC 2023). Last, further monitoring for NZMS in the form of physical surveys and possibly environmental DNA collections (e.g., Woodell et al. 2021) should continue within the Ohio River Basin and adjacent waters.

ACKNOWLEGMENTS

We thank Dr. Robert Dillon, Jr., for assistance in confirming specimen identifications, and we thank Heather Smiles and Gary Smith for support of this work and review of an initial draft of the manuscript. Because this work was done by PFBC staff, we note that no permits were required by the authors for the work reported herein, because the PFBC is the agency with jurisdictional authority over aquatic snails in Pennsylvania. The findings and conclusions of this work are those of the authors and may not necessarily represent the views of the PFBC.

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Freshwater Mollusk Biology and Conservation

ISSN 2472-2944

Editorial Board

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