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REGULAR ARTICLE

FRESHWATER MUSSEL ASSEMBLAGES IN THE BLACK RIVER, MISSOURI AND ARKANSAS

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ABSTRACT

Freshwater mussel assemblages show predictable variation according to large-scale biogeographic factors and stream-size gradients, but smaller-scale assemblage patterns are less well known. The goal of this study was to classify and delineate mussel assemblages of the Black River, Missouri and Arkansas, USA, along an upstream–downstream gradient and with regard to physiography and biogeographical regions. We analyzed mussel assemblages using nonmetric multidimensional scaling and indicator-species analysis. Our results yielded three assemblage groupings distributed along the upstream–downstream gradient and thereby considered aquatic ecological systems (100–1,000 km²) in a hierarchical spatial classification scheme. These groupings also support previously proposed biogeographical differences for mussels and fishes between the Ozark Highlands and Mississippi Alluvial Plain physiographic regions. Each group was characterized by 2–13 indicator species. Our demonstration of small-scale patterns of mussel assemblage change will be useful for conservation planning and for a better understanding of mussel assemblage dynamics.

KEY WORDS: Black River, Arkansas, Missouri, biogeography, faunal groups, hierarchical spatial classification, conservation planning

INTRODUCTION

Riverine freshwater mussel assemblages in much of North America show predictable variation according to macrohabitat and biogeographical factors (Haag 2012). Stream size is one of the most important macrohabitat factors, with species richness and assemblage composition changing predictably along stream-size gradients (Haag 2012). The North American mussel fauna is presently categorized into four major faunal regions (Mississippian, Eastern Gulf, Atlantic, and Pacific), which are further divided into 17 faunal provinces, and these biogeographical affinities also play a major role in determining assemblage structure in a given stream (Haag 2010, 2012). Although these patterns have been recognized, additional case studies are needed to evaluate their generality, particularly at smaller scales. Furthermore, hierarchical spatial classification

can be an effective approach for conservation planning (Higgins et al. 2005). Thus, identifying statistically defined faunal and assemblage groups at a variety of scales will aid in conservation planning and management.

The Black River of Missouri and Arkansas, USA, crosses physiographic and faunal boundaries and supports an important mussel resource (Harris 1999; Neves 1999). The Black River mussel fauna is part of the Mississippian faunal region (Haag 2010). The upstream portion of the watershed lies in the uplands of the Ozark Highlands, and the mussel fauna is categorized within the Interior Highlands faunal province. The downstream portion lies in the lowlands of the Mississippi Alluvial Plain, and the fauna is within the Mississippi Embayment province. A total of 53 mussel species are reported from the Black River, including 47 species from the Missouri portion and 42 species from the Arkansas portion (Hutson and Barnhart 2004; S. E. McMurray, unpublished

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Table 1. Mussel species present in our dataset for the Black River, Missouri and Arkansas. Stream-size associations depict habitats in which a particular species frequently dominates mussel assemblages in the Mississippian faunal region or habitats with which a species is otherwise strongly associated as reported by Haag (2012); species not categorized by Haag (2012) are indicated with a dash (—). Nomenclature follows Williams et al. (2017).

Taxa	Stream Size
<i>Actinonaias ligamentina</i>	Midsized
<i>Alasmidonta marginata</i>	Small
<i>Amblema plicata</i>	Generalist
<i>Arcidens confragosus</i>	Large
<i>Cyclonaias nodulata</i>	Large
<i>Cyclonaias pustulosa</i>	Midsized/large
<i>Cyclonaias tuberculata</i>	Midsized
<i>Cyprogenia aberti</i>	Midsized
<i>Ellipsaria lineolata</i>	Large
<i>Epioblasma triquetra</i>	Midsized
<i>Eurynia dilatata</i>	Small/midsized
<i>Fusconaia flava</i>	Midsized
<i>Lampsilis abrupta</i>	Large
<i>Lampsilis cardium</i>	Midsized
<i>Lampsilis reeveiana</i>	Small/midsized
<i>Lampsilis siliquioidea</i>	Small/midsized
<i>Lampsilis teres</i>	Large
<i>Lasmigona complanata</i>	Midsized
<i>Lasmigona costata</i>	Small/midsized
<i>Leptodea fragilis</i>	Midsized
<i>Ligumia recta</i>	—
<i>Ligumia subrostrata</i>	—
<i>Megalonaias nervosa</i>	Midsized/large
<i>Obliquaria reflexa</i>	Midsized/large
<i>Obovaria olivaria</i>	Large
<i>Plectomerus dombeyanus</i>	Large
<i>Pleurobema sintoxia</i>	Midsized
<i>Potamilus ohioensis</i>	Large
<i>Potamilus purpuratus</i>	Large
<i>Ptychobranhus occidentalis</i>	Small/midsized
<i>Pyganodon grandis</i>	Small
<i>Quadrula quadrula</i>	Midsized/large
<i>Reginaia ebenus</i>	Large
<i>Strophitus undulatus</i>	Small
<i>Theliderma cylindrica</i>	—
<i>Theliderma metanevra</i>	Large
<i>Toxolasma parvum</i>	Small
<i>Tritogonia verrucosa</i>	Midsized
<i>Truncilla donaciformis</i>	—
<i>Truncilla truncata</i>	—
<i>Utterbackia imbecillis</i>	—
<i>Utterbackiana suborbiculata</i>	—
<i>Villosa iris</i>	Small
<i>Villosa lienosa</i>	Small

data; J. L. Harris, unpublished data) and 44 species were present in our dataset (Table 1). The watershed's geographic and faunal heterogeneity and high species richness make the Black River a useful system in which to examine patterns of mussel assemblage composition.

The goal of this study was to classify and delineate mussel assemblages of the Black River along the upstream–downstream gradient and with regard to physiographical and biogeographical regions. We analyzed Black River mussel assemblages using nonmetric multidimensional scaling (NMDS) and indicator species.

METHODS

Study Area

The Black River watershed occupies 22,165 km² in southeastern Missouri and northeastern Arkansas, USA (Fig. 1). The Black River originates at the confluence of the East Fork and Middle Fork near Lesterville, Missouri, and flows 480 km through the Ozark Highlands and Mississippi Alluvial Plain ecoregions to its confluence with the White River near Newport, Arkansas. The upstream portion of the Black River in the Ozark Highlands, at about river kilometer 341 and upstream, is characterized by clear water, higher gradient, and shallow stream conditions with substrates dominated by gravel and sand (Chapman et al. 2002). The middle and downstream portions of the Black River on the Mississippi Alluvial Plain have lower water clarity, lower gradient, and deeper stream conditions with substrates dominated by sand and clay (Woods et al. 2004).

Mussel Assemblage Data

We compiled existing mussel sampling data from 63 Black River (BLR) sites from Black River kilometer (BRKM) 81.4 (Site BLR50.6) in Arkansas to BRKM 412.80 (Site BLR256.5) in Missouri (Rust 1993; Hutson and Barnhart 2004). Site numbers (BLR; see Fig. 1) correspond to river mile to allow easier cross-referencing with state agency collection records and U.S. Army Corps of Engineers navigation maps (USACE 1985), both of which are in English units. All sites used in this study were sampled between 1990 and 2003. Sixteen sites were located in Missouri and 47 were in Arkansas. Sampling methods included timed-search sampling in Missouri (mean = 2.2 person-hr/site; Hutson and Barnhart 2004) and 1-m² quadrat-based sampling in Arkansas. Quadrat sampling consisted of five haphazardly placed 1-m² quadrats in small mussel beds and 10–25 1-m² quadrats in large mussel beds (Rust 1993; see also Christian and Harris 2005). There were no small or large mussel beds reported between the mouth of the Black River and BRKM 81.4 (Rust 1993).

Data Analysis

We assessed patterns in mussel assemblage data with nonmetric multidimensional scaling (NMDS) based on Bray–

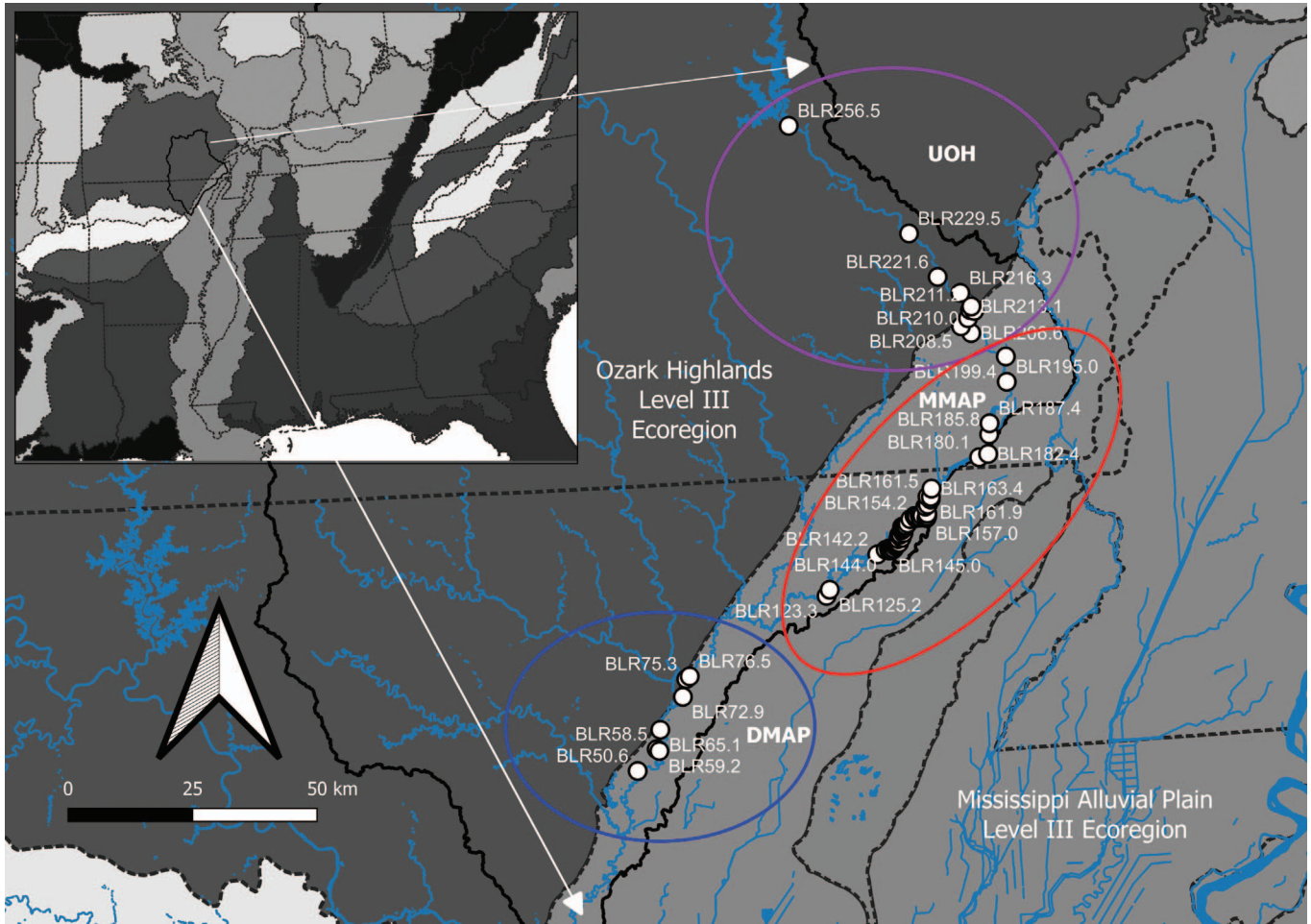


Figure 1. Map of the Black River watershed, Missouri and Arkansas, USA, and sampling sites overlain on the Level III Ozark Highlands (dark gray) and Mississippi Alluvial Plain (lighter gray) ecoregions. The solid black outline shows the watershed boundary and white circles are the Black River mussel sampling sites according to river mile (BLR). Colored ellipses indicate the geographic range of the upstream Ozark Highlands (UOH), midstream Mississippi Alluvial Plain (MMAP), and downstream Mississippi Alluvial Plain (DMAP) mussel assemblage groupings identified by nonmetric multidimensional scaling (NMDS). The inset map shows the location of the study area in the south-central USA.

Curtis dissimilarity using the vegan package (Oksanen et al. 2018) in R (R Core Team 2018). To correct for differences in sampling methods and effort among sites, we transformed assemblage data to relative abundance at each site by dividing the number of individuals of each species collected at a site by the total number of individuals (all species) collected at each site. Based on visual inspection of initial NMDS analysis, geographic groups were assigned as an *a posteriori* hypothesis of geographic clusters defining assemblage composition. Significance was evaluated by determining if between-group variation, measured as the distance between geographic group centroids, was significantly greater than within-group variation, based on a simulated distribution drawn from resampled data (analysis of similarity, ANOSIM).

We used indicator-species analysis to identify species that were uniquely characteristic of the identified geographic groups (Dufrêne and Legendre 1997). We conducted this analysis using the Indicspecies package in R (Cáceres and Legendre 2009; R Core Team 2018). This analysis assigns an

indicator value to each species in each group from 0 to 1, where 0 indicates that a species was not observed at any site in the group, and 1 indicates a species was observed at every site in the group and never outside of the group. Indicator species were identified as those having indicator values that were significant at $P \leq 0.05$ based on a permutation test.

RESULTS

The NMDS analysis revealed a geographic pattern of three clusters representing an upstream Ozark Highland (UOH) assemblage from sites BLR206.6–BLR256.5 (BRKM 332.5–412.8), a midstream Mississippi Alluvial Plain (MMAP) assemblage from sites BLR123.3–BLR195.0 (BRKM 198.4–320.9), and a downstream Mississippi Alluvial Plain (DMAP) assemblage from sites BLR50.6–BLR76.5 (BLKM 81.4–123.1) (Fig. 2). The three assemblages were significantly different based on the standard deviation of sites to their geographic-group centroids (ANOSIM, $R = 0.6809$, $P =$

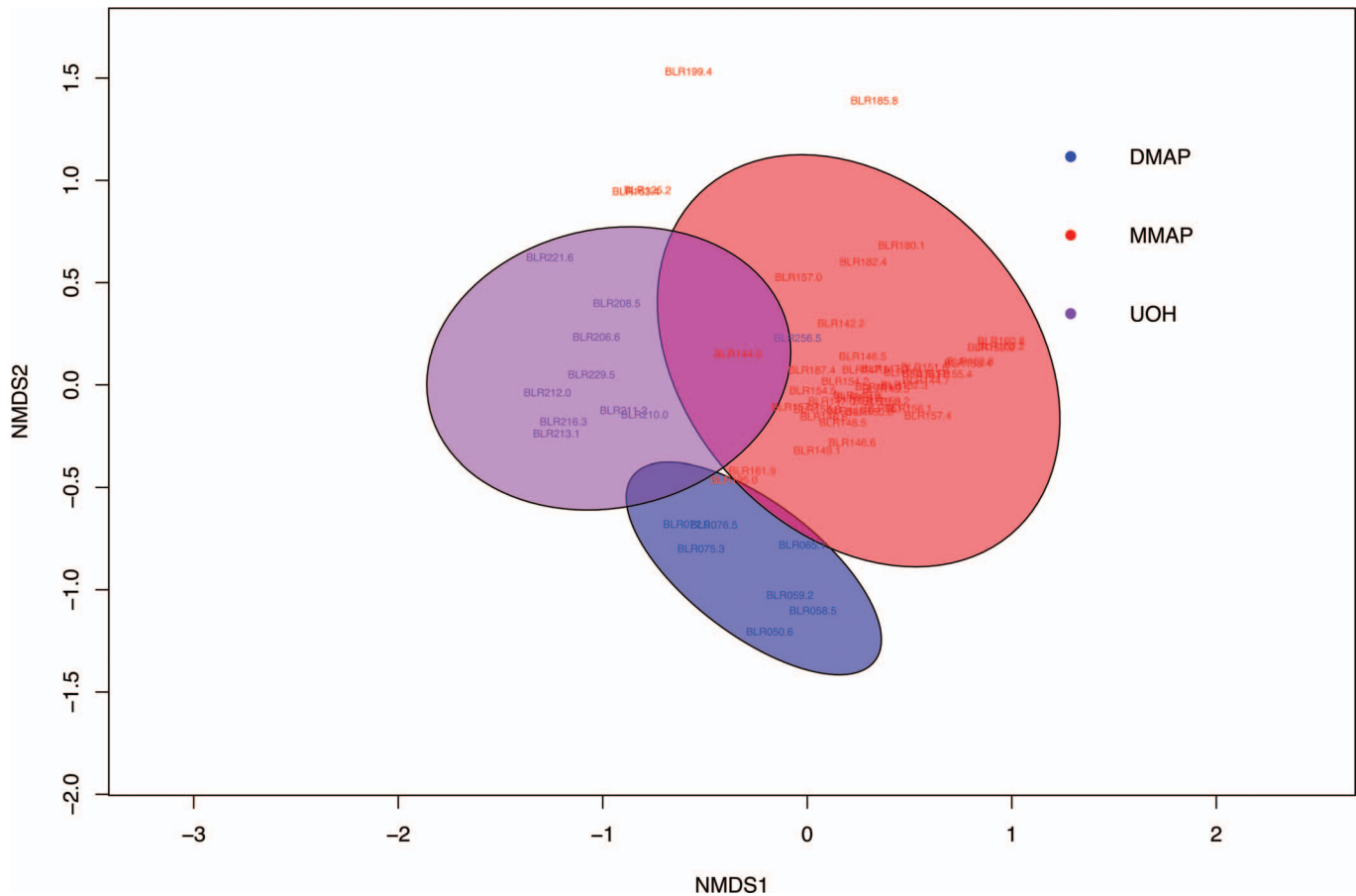


Figure 2. Nonmetric multidimensional scaling (NMDS) plot of mussel assemblages at 63 Black River sampling sites. Ellipses represent 95% confidence intervals around three geographic groupings: upstream Ozark Highlands (UOH), midstream Mississippi Alluvial Plain (MMAP), and downstream Mississippi Alluvial Plain (DMAP). Four sites on the upper part of the graph were not contained in any of the three groupings.

0.001). Four sites did not fall within the 95% confidence interval for any resulting assemblage cluster: BLR 199.4, BLR 185.8, BLR163.4, and BLR125.2, but all four sites were spatially distributed within the range of MMAP sites (Fig. 1).

Indicator-species analysis identified characteristic species for each geographic group (Fig. 3). The upstream UOH group had 13 species that were significant indicators, with the strongest being *Pleurobema sintoxia* (0.987), *Actinonaias ligamentina* (0.936), *Plectomerus dombeyanus* (0.933), *Strophitus undulatus* (0.903), *Lasmigona costata* (0.894), *Cyprogenia aberti* (0.885), and *Fusconaia flava* (0.830). The midstream MMAP group had two significant indicators, *Amblema plicata* (0.937) and *Lasmigona complanata* (0.836). The downstream DMAP group had seven significant indicators, with the strongest being *Obovaria olivaria* (1.000), *Reginaia ebenus* (0.948), *Quadrula quadrula* (0.891), and *Ellipsaria lineolata* (0.844).

DISCUSSION

Our finding of three distinct assemblages dispersed along an upstream–downstream gradient was expected. Mussel assemblages show predictable structure in which dominance

is shared by a small group of codominant species and dominance shifts along stream-size gradients (Haag 2012). The UOH assemblage was associated mostly with midsize stream species (*P. sintoxia*, *A. ligamentina*, *L. costata*, *C. aberti*, and *F. flava*), but it also included one small-stream species (*S. undulatus*). The MMAP assemblage was associated with *L. complanata*, a midsize stream species, and *A. plicata*, a stream-size generalist. Finally, the DMAP assemblage was associated with four species, *O. olivaria*, *R. ebenus*, *E. lineolata*, and *Q. quadrula*, all of which are large-stream species.

Three (BLR185.5, BLR163.4, and BLR125.2) of the four sites that did not cluster with the UOH, MMAP, or DMAP groups had *A. plicata* and *L. complanata*, indicators for MMAP; however, *A. plicata* and *L. complanata* were absent at the fourth site, BLR199.4. Overall, these four sites mostly had low overall abundances and a mixture of indicator species from a variety of groups. BLR125.2 had representation of UOH indicator species *A. ligamentina* and *F. flava* and the DMAP indicator *Q. quadrula* in addition to MMAP indicators *A. plicata* and *L. complanata*. BLR163.4 had low numbers of the DMAP indicator *Q. quadrula* in addition to low numbers of MMAP indicator species *A. plicata* and *L. complanata*.

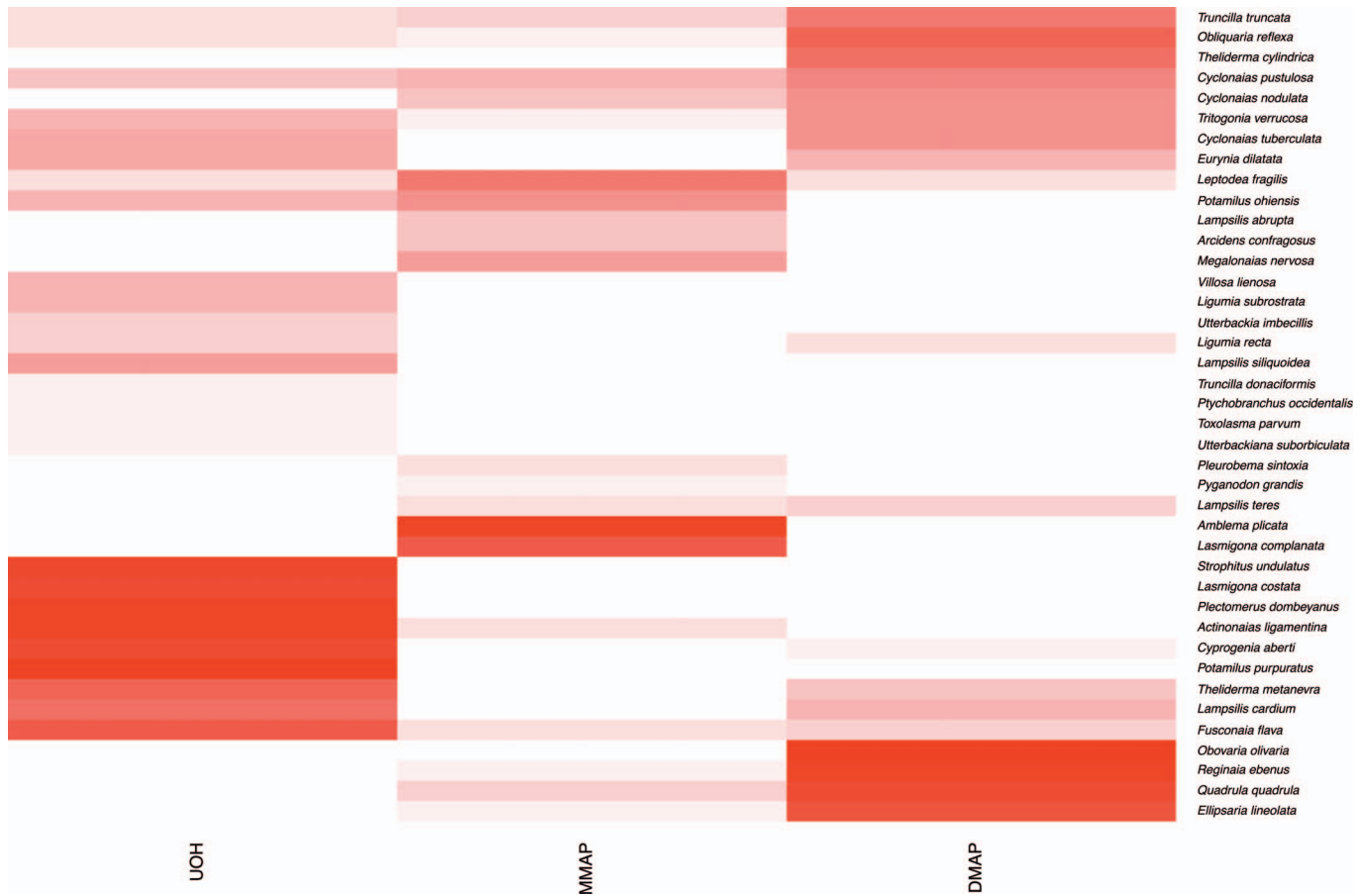


Figure 3. Heat map showing results of indicator-species analysis of mussel assemblages in the Black River within three geographic groups: upstream Ozark Highlands (UOH), midstream Mississippi Alluvial Plain (MMA), and downstream Mississippi Alluvial Plain (DMP). Deeper red coloring indicates stronger indicator relationships within each group.

BLR185.5 had low numbers of UOH indicator species *F. flava* and *P. dombeyanus* in addition to low numbers of MMA indicator species *A. plicata* and *L. complanata*. BLR 199.4 did not have any MMA indicator species but had low numbers of the UOH indicator species *A. ligamentina*. Therefore, one could argue that BLR125.2, BLR163.4, and BLR185.5 associate with the MMA group, while BLR199.4 associates with the UOH group.

Our three faunal groupings also were concordant with physiography and biogeographical affinities. The UOH assemblage showed remarkably close association with the Ozark Highlands. Furthermore, one of the indicator species for this assemblage, *Cyprogenia aberti*, is a characteristic member of the Interior Highlands faunal province (Haag 2010). The MMA assemblage may represent a transitional area between the upland UOH assemblage and the lowland DMP assemblage. Similar assemblage differences between the Ozark Highlands and the Mississippi Alluvial Plain are seen for fishes in the Black River and adjacent watersheds in southeastern Missouri (Pflieger 1970, 1997; Matthews and Robison 1988).

In our study area, stream size and physiography/biogeography are confounded because the Black River becomes larger

as it flows off the Ozark Highlands and onto the Mississippi Alluvial Plain. Consequently, we cannot assess the relative importance of these two factors in influencing mussel assemblage composition. Other unmeasured factors also likely affect these assemblages. For example, local environmental variables can be correlated with mussel assemblages (Arbuckle and Downing 2002; Poole and Downing 2004), and distribution and abundance of fish hosts also can be a strong predictor of mussel assemblage structure (Vaughn and Taylor 2000; Schwalb et al. 2013).

When our study is considered in a spatial classification framework (Higgins et al. 2005), individual mussel beds are equivalent to macrohabitats (1 to 100 km²), our three assemblage groupings (UOH, MMA, DMP) are equivalent to aquatic ecological systems (100 to 1,000 km²), the Black River represents an ecological drainage unit (1,000 to 10,000 km²), and the Mississippian faunal region (Haag 2010) represents an aquatic zoogeographic unit (10,000 to 100,000 km²). Our identification of distinct UOH, MMA, and DMP mussel assemblages provides the basis for conservation planning aimed at maximizing biodiversity within a hierarchical spatial and biogeographic context (National Native Mussel

Conservation Committee 1998; Freshwater Mollusk Conservation Society 2016).

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REGULAR ARTICLE

SEASONALITY OF GAMETE PRODUCTION OF CYCLONAIAS SPECIES IN CENTRAL TEXAS

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ABSTRACT

Reproductive traits, which can impact population health, are important life-history characteristics for freshwater mussels. Little research has been done on the reproductive ecology of mussels, and crucial information is missing for many threatened and endangered species, especially in Texas. The objective of this study was to examine gamete production, parasitic infection rates, and sex ratios of two freshwater mussel species (*Cyclonaias petrina* and *Cyclonaias pustulosa*) in the Llano and San Saba rivers in central Texas. Gamete densities and egg diameters of *C. petrina* in the Llano River varied seasonally, with peak gamete densities occurring in December and February 2017 and being significantly lower from June through September 2017, while the relative abundance of the largest size classes of eggs was highest in February 2018. Few to no differences were detected in gamete production and egg diameter sizes for *C. petrina* between rivers. *Cyclonaias pustulosa* had significantly higher sperm densities and smaller egg diameters compared to *C. petrina* but exhibited similar egg densities in the San Saba River. There were no significant differences in gamete densities between rivers and no significant correlations between shell length and gamete density. Infection rates of parasitic trematodes varied from <1% to 14%, with the highest infection rates occurring in *C. petrina* in the San Saba River. Sex ratio of *C. petrina* was slightly skewed toward females in the Llano River and toward males in the San Saba River, with *C. pustulosa* having a 1:1 sex ratio in the San Saba River. The high percentage of samples without gametes suggests that the reproductive outputs of *Cyclonaias* appear to be more limited in the San Saba River due to several potential stressors. Further research will need to investigate the relative importance of the various stressors that affect the reproductive ecology of mussels and their persistence.

KEY WORDS: Unionidae, gonadal fluid, reproduction, conservation, parasite, trematodes

INTRODUCTION

Traits such as reproductive timing and sex ratio are important life-history characteristics because they affect population dynamics and can be used to inform future conservation efforts. Yet, life-history data are still lacking for many unionid mussels (Haag 2012). While significant efforts have been put towards host-fish identification and the development of propagation techniques for freshwater mussels, less research has been done on the reproductive ecology of mussels, and crucial information is missing for many threatened and endangered species. Most information available on the reproductive ecology of freshwater mussels (family Unionidae) is from species within the tribes Amblemini and

Lampsilini of the subfamily Ambleminae, and the tribe Anodontini of the subfamily Unioninae (e.g., Heard 1975; Haag and Staton 2003; Moles and Layzer 2008; Haag 2012). Less research has been conducted on species within the tribe Quadrulini of the subfamily Ambleminae (but see, e.g., Jirka and Neves 1992; Woody and Holland-Bartels 1993; Haag and Staton 2003).

The tribe Quadrulini contains six genera: *Cyclonaias*, *Megalonaias*, *Quadrula*, *Theliderma*, *Tritogonia*, and *Unio* (Williams et al. 2017). Species in the tribe are classified as equilibrium strategists (Haag 2012) and, in general, can be characterized as being relatively long-lived (i.e., life span over 25 yr) with relatively low growth rates and late maturity. They primarily use either mucoid conglutinates or mantle magazines to attract host fish (Haag and Staton 2003; Barnhart et al. 2008; Sietman et al. 2012). *Cyclonaias*,

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Quadrula, and *Tritogonia* species are catfish specialists and *Theliderma* species are minnow specialists, whereas *Megaloniaias* is classified as a host generalist (Haag 2012). Fish hosts of *Unio* are currently unknown. Despite a general knowledge of life-history strategies and host-fish use of several species, there is still a lack of knowledge on the reproductive ecology (e.g., reproductive effort and timing) for most species within the tribe.

Reproductive effort and timing of mussels plays a critical role in determining the reproductive health and viability of populations. Since fecundity is often strongly related to body size, smaller *Cyclonaias*, *Quadrula*, and *Theliderma* species often have much lower fecundity rates (5–63-fold lower) compared to larger species such as *Megaloniaias nervosa* and *Tritogonia verruscoa* (Haag 2013). Species in *Quadrulini* are short-term brooders (tachytictic) and, in general, gamete production increases in the fall (September–November) and reaches peak concentrations in the winter or early spring before spawning occurs in May or June (although monthly variability can exist between species and populations). The lowest gamete concentrations occur during the late summer for most species within the tribe for which data are available (Yeager and Neves 1986; Jirka and Neves 1992; Garner et al. 1999; Culp et al. 2011; Hove et al. 2011; Tsakiris et al. 2016; Dudding et al. 2020). Exceptions to this reproductive pattern have been documented for *Megaloniaias nervosa* (Woody and Holland-Bartels 1993; Haggerty et al. 2005) and a northern population of *Quadrula fragosa* (Hove et al. 2012), in which brooding and spawning occurred relatively late (summer–fall) compared to other *Quadrulini* species. Information on the seasonality of gamete production is still lacking for many species of *Quadrulini*, as is the degree of variation between populations of the same species. A better understanding of the seasonality of gamete production would improve the timing of collection of brooding mussels and host fishes and ultimately make propagation methods more predictable and efficient.

In addition to reproductive timing, sex ratio and the number of reproducing individuals is crucial in determining the reproductive potential of mussel populations (Haag and Staton 2003; Berg et al. 2008; Haag 2013). Most *Quadrulini* were found to have a relatively equal sex ratio; where variation in sex ratio has been reported, most populations showed an increased proportion of males (summarized in Berg et al. 2008 and Haag 2012). However, female-dominated populations have been documented for *M. nervosa*, *Theliderma metanerva*, and *Theliderma cylindrica* (Woody and Holland-Bartels 1993; Garner et al. 1999; Galbraith and Vaughn 2009). The exact causes of skewed sex ratios are usually not well understood in freshwater mussel populations (Haag 2012), but a strongly skewed sex ratio could lead to decreased reproductive health within a mussel population. For example, parasitic trematodes have been shown to alter sex ratios and limit the reproductive output of freshwater mussels; however, the impacts of these parasites on most freshwater mussel species are not well understood (e.g., Taskinen and Valtonen 1995; Müller et al. 2015).

Texas has approximately 50 species of freshwater mussels (Howells 2014). Like other populations throughout North America, Texas mussels have experienced significant population declines (Howells et al. 1996; Burlakova et al. 2011a; Ford and Oliver 2015; Mitchell et al. 2019). In order to better understand and inform management strategies, it is imperative to examine basic life-history traits of these species. Only three studies have investigated gamete production of mussels within Texas (Tsakiris et al. 2016; Seagroves et al. 2019; Dudding et al. 2020). Hence, the objective of this study was to evaluate the reproductive timing of two congeneric species of mussels from central Texas. *Cyclonaias petrina*, endemic to this region, is a candidate for federal listing; *Cyclonaias pustulosa* is widespread throughout the Mississippi River drainage and some coastal drainages of the Gulf of Mexico. Central Texas populations of the latter were previously named *Cyclonaias houstonensis*, but that name was recently synonymized with *C. pustulosa* (Johnson et al. 2018; Lopes-Lima et al. 2019). The reproductive ecology of *C. pustulosa* has been studied relatively well compared to other species in the tribe but little research has been conducted in subtropical rivers located in semiarid regions such as central Texas (but see Tsakiris et al. 2016; Dudding et al. 2020). Almost no information on the reproductive ecology of *C. petrina* is known, although a recent study examined monthly gamete production and gamete parasitism by digenetic trematodes (Tsakiris et al. 2016). Since *C. petrina* and *C. pustulosa* were found to be genetically different in central Texas (Johnson et al. 2018), it is important to understand how they may differ in their reproductive ecology. Our objectives were to (1) describe seasonal variation in gamete production (egg and sperm densities and egg diameters) for *C. petrina* in the Llano River from February 2017 to February 2018 and for both species in the San Saba River for the periods June–November 2017 and February 2018; (2) compare gamete production between (a) populations of *C. petrina* located in different rivers and (b) two species within the San Saba River; (3) examine the relationship between shell length and gamete density; (4) quantify rates of infection with parasitic trematodes; and (5) determine sex ratios of each species.

METHODS

We used three study sites in two rivers located in central Texas: one site in the upper Llano River and two sites in the lower San Saba River (Fig. 1). Both rivers are spring-fed tributaries of the Colorado River. At the San Saba sites, the habitat consisted of riffles with a mix of sand, gravel, cobble, and boulder substrate while the Llano River site was a run with substrate consisting of mostly bedrock with patches of boulder, cobble, and silt. We chose these sites because both species occurred in high local abundances and site access was easy.

The Llano and San Saba rivers are in the Edwards Plateau ecoregion, which is generally characterized by grasslands and juniper/oak/mesquite woodlands in shallow soils underlain by

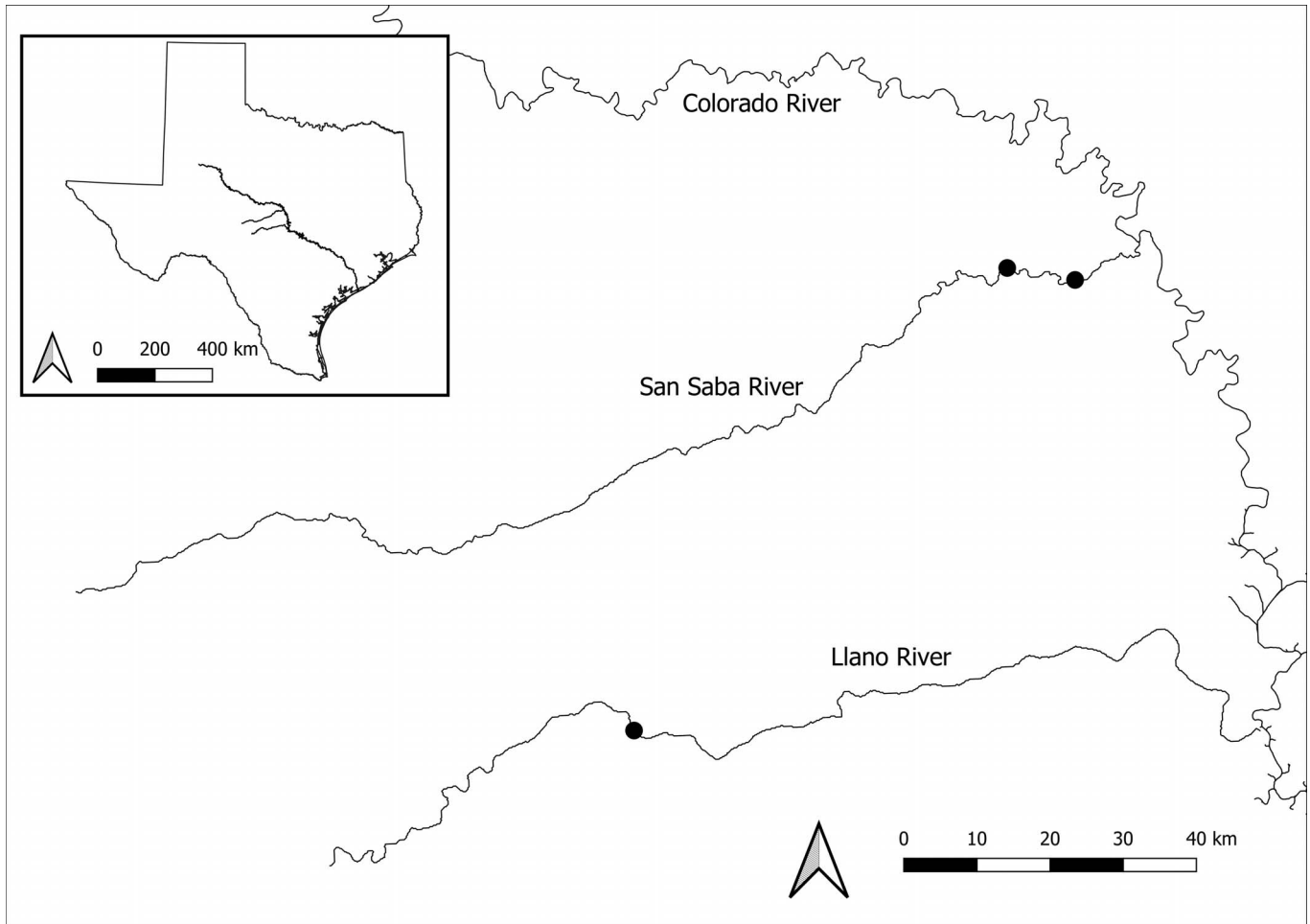


Figure 1. Site map for gamete collections in the Llano and San Saba rivers, Texas.

limestone (Griffith et al. 2007; tpwd.texas.gov). The Llano River (~169 km long) is the most urbanized tributary of the middle Colorado River but still has substantial semiarid ranchland and farmland (TPWD 1974; Heitmuller and Hudson 2009). Landcover in the San Saba River (~225 km long) is characterized by semiarid ranchland in the middle and upper stretches but is dominated by pecan orchards and row crops in the lower stretch (TPWD 1974; Griffith et al. 2007; RPS Espey 2013). Historically, the Llano River contained approximately 14 species of freshwater mussels, but it has suffered great losses in terms of species diversity over the past several decades (Strecker 1931, Howells et al 1996; Burlakova et al. 2011b; Burlakova and Karatayev 2012, Mitchell et al. 2019). It contains two candidate species for federal listing (*C. petrina* and *Lampsilis bracteata*). The loss of species richness has been less severe in the San Saba River, compared to other tributaries of the Colorado River. It still harbors around 13 of an original 16 species (Strecker 1931, Howells et al 1996; Burlakova et al. 2011b; Burlakova and Karatayev 2012; Mitchell et al. 2019), and four extant species are candidates for federal listing (*C. petrina*, *L. bracteata*, *Fusconaia mitchelli*, *Truncilla macrodon*).

Using visual and tactile methods, we collected 10 new individuals of *C. petrina* monthly in the Llano River from February 2017 to February 2018, except for October 2017 ($n=9$). The survey period was shorter in the San Saba River, where we collected individuals of *C. petrina* and *C. pustulosa* monthly from June 2017 to February 2018. We collected 10 individuals of *C. petrina* during each sampling event, but we were not able to find 10 individuals on all sampling dates for *C. pustulosa* so that sample size was six in July and August and one in September. In order to prevent unnecessary mortality as a result of mussels potentially not being able to rebury themselves in the sediment at colder temperatures (Block et al. 2013), we did not collect samples in December 2017 and January 2018 in the San Saba River because water temperatures were below 10°C at the time of sampling. All sampled mussels were uniquely marked during each survey period with shell tags to prevent resampling of gonadal fluid during future collection periods. After collecting consistently low numbers of nontagged *C. pustulosa* during the summer, we started to collect individuals at an alternate site that had similar environmental conditions and community assemblage, located approximately 9 km from our original site. A Mantel

correlogram analysis based upon data from continuous surveys completed within the lower San Saba River identified mussels from these two sites as belonging to a “single mussel patch” (Mitchell 2020). *Cyclonaias petrina* was collected from the same site in the San Saba River throughout the study.

Gonadal fluid was sampled from all individuals using a nonlethal syringe (BD 5-mL syringe Luer-Lok™ with BD PrecisionGlide™ needle; BD, Franklin Lakes, New Jersey, USA) technique (see Tsakiris et al. 2016). Gamete samples were fixed with 10% formalin, dyed with 0.01% methylene blue, and transported to the laboratory for analysis. Since our study species are not sexually dimorphic, mussels were sexed from collected gamete samples (Saha and Layzer 2008). Sperm were quantified in 10-μL subsamples (transferred with micropipette; Fisherbrand Elite Fisher Scientific Co., Pittsburgh, Pennsylvania, USA) with a compound microscope (400×) and improved Neubauer hemocytometer (INCYTO DHC- N01-5; INCYTO, Covington, Georgia, USA). Sperm concentration (n/mL of gonadal fluid) was extrapolated from subsamples using Equations 1 and 2:

$$\text{Number/mL} = \# \text{ sperm in 5 small center squares} * 5 \\ * \text{dilution factor} * 10^4 \quad [1]$$

Dilution factor =

$$\frac{\text{Total volume (containing formalin and methyl blue)}}{\text{Initial sample volume (gonadal fluid)}} \quad [2]$$

We estimated egg concentration and diameter by counting and measuring the number of eggs in a 10-μL subsample at 100× magnification on a glass slide and extrapolating the number of eggs to 1 mL of gonadal fluid after accounting for dilution, similar to sperm estimates. The presence or absence of trematodes within each gamete sample was recorded.

We used one-way analysis of variance (ANOVA) to examine differences in mean gamete densities and egg diameter sizes sampled in different months. We did a separate analysis for each species, at both sites and for each sex. Two-way ANOVA was used to determine whether gamete densities (i.e., egg or sperm densities) and egg diameters differed significantly between (1) rivers (Llano vs. San Saba) and sampling period (month) for *C. petrina*, and (2) between species (*C. petrina* and *C. pustulosa*) and sampling period within the San Saba River. Two-way ANOVA tests only compared months in which data were available for both rivers, both species, or both sexes. Multiple comparisons for ANOVA tests were examined using a Tukey honestly significant difference (HSD) post hoc analysis. Pearson product-moment correlations were used to estimate the association between gamete density and mussel-body size (shell length measured anterior to posterior) for each sampling period for both species (males and females separately) and rivers. A two-way ANOVA was also used to determine whether length of mussels differed significantly between mussels with and without gametes and between sampling periods. We employed Student's *t*-tests to compare the body sizes of mussels that

were infected with trematodes to those that were not. We used chi-square goodness-of-fit tests to assess whether sex ratios were significantly different from a male to female ratio of 1:1. Assumptions of normality and homogeneity of variance were tested using the Shapiro-Wilk and Levene's tests, respectively. Densities were log₁₀(x) transformed to better meet the assumptions for all analyses.

RESULTS

We collected gamete samples from 252 mussels, which included 199 samples from *C. petrina* (129 from Llano River, 70 from San Saba River), and 53 samples from *C. pustulosa* in the San Saba River. Length (mean ± SD) of collected *C. petrina* was 49.1 ± 7.7 mm and 59.8 ± 10.7 mm for the Llano and San Saba rivers, respectively. Length of *C. pustulosa* was 65.7 ± 6.1 mm in the San Saba River. Gametes were found in 76% (*n* = 191) of samples from both species. Most samples without gametes (42 of 61 samples) were collected from the San Saba River, where 43% of *C. petrina* and 23% of *C. pustulosa* were found without gametes. Samples in which no gametes were found were collected from mussels with a wide range of body sizes (37–72 mm). Size did not differ significantly between mussels with and without gametes for all sampling periods ($F_{[1,232]} = 2.48$, $P = 0.12$). In both rivers, no significant correlations between shell length and gamete density were found for either species of each sex during each sampling period ($r = 0.16$ – 0.78 ; $P > 0.05$ in all cases).

In the Llano River, differences in gamete densities between months were statistically significant for both sperm ($F_{[12,27]} = 7.39$, $P < 0.001$) and eggs ($F_{[12,57]} = 19.3$, $P < 0.001$). Sperm densities of *C. petrina* were highest in December 2017 and lowest (three orders of magnitude lower) in the summer and early fall (June–September 2017; Fig. 2A). Egg densities of *C. petrina* were highest in February and March 2017 and tended to decline during warmer months (one order of magnitude lower) before slightly increasing the following fall (October–December 2017; Fig. 2B). In the San Saba River, gamete densities were not different between months for *C. petrina* (sperm: $F_{[6,33]} = 1.07$, $P = 0.4$; eggs: $F_{[5,8]} = 3.32$, $P = 0.06$) or *C. pustulosa* (sperm: $F_{[6,34]} = 0.33$, $P = 0.91$; eggs: $F_{[5,13]} = 1.45$, $P = 0.27$; Fig. 2C, D). There was no statistically significant difference in sperm and egg densities (Tukey HSD: $P > 0.05$) of *C. petrina* between rivers when months in which data available for both rivers were compared, except for higher sperm densities in July 2017 in the San Saba River. For all months combined, *C. pustulosa* had significantly higher sperm densities (1.5-fold higher) compared to *C. petrina* ($F_{[1,34]} = 8.57$, $P < 0.01$) in the San Saba River (Fig. 2C), but exhibited similar egg densities ($F_{[1,21]} = 0.04$, $P = 0.85$; Fig. 2D).

Egg diameters varied between 30 and 435 μm and differently sized eggs were present throughout the year (Figs. 3–5). Mean egg diameter size varied significantly between months for both *C. petrina* (Llano River: $F_{[12,1861]} = 5.58$, $P < 0.001$; San Saba River: $F_{[4,183]} = 7.01$, $P < 0.001$) and *C.*

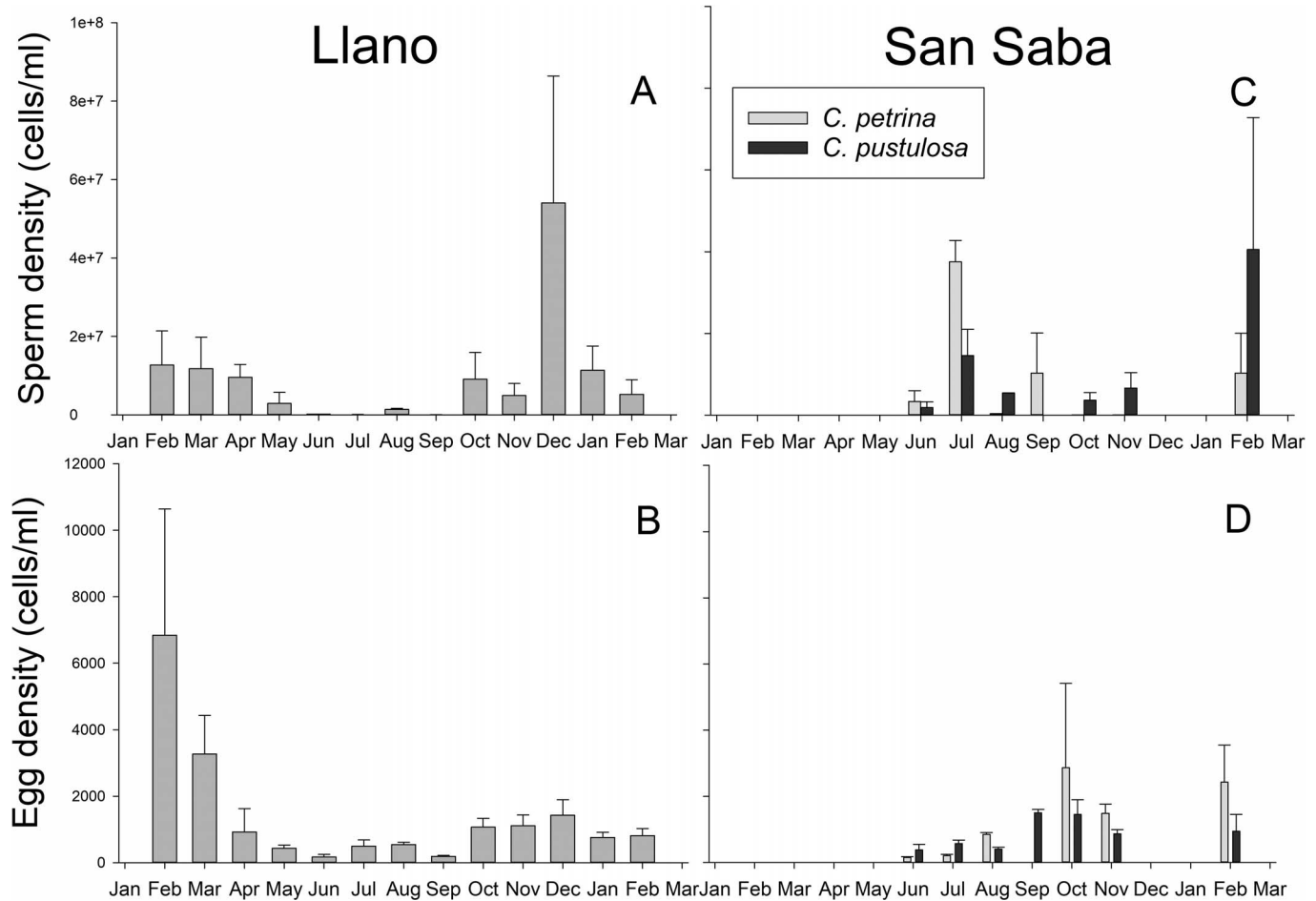


Figure 2. Monthly gamete production (mean \pm SD) for two candidate species of mussels in the Llano and San Saba rivers, Texas. Gray bars denote *Cyclonaias petrina* and black bars denote *Cyclonaias pustulosa*.

pustulosa (San Saba River: $F_{[6,360]} = 8.69$, $P < 0.001$). The relative frequency of the largest size classes of eggs for both species was highest in February 2018 in both rivers and lowest in August 2017 in the San Saba River (Figs. 4, 5). In the Llano River it was lowest for *C. petrina* in February, June, and August 2017 (Fig. 3). There were no significant differences in mean egg diameters of *C. petrina* between the Llano River (182.9 μm) and the San Saba River (195.4 μm) when comparing months in which data was available for both sites (Tukey HSD: $P > 0.05$). Overall, *C. pustulosa* had a significantly smaller mean egg diameter (mean: 158.2 μm) compared to *C. petrina* in the San Saba River (all months combined; Tukey HSD: $P < 0.001$), but both species showed similar monthly trends (Figs. 4, 5).

Trematodes were found in 5% ($n = 13$) of all collected gamete samples, primarily from June to November, and almost exclusively (12 of 13 samples) from the San Saba River. Parasite incidence for *C. petrina* and *C. pustulosa* in the San Saba River was 14% ($n = 10$) and 4% ($n = 2$), respectively, and no differences in body size were found between mussels that were infected with trematodes and those that were not ($t =$

-0.53 , $df = 250$, $P > 0.05$). No gametes were found in samples that contained trematodes.

The sex ratio for *C. petrina* in the Llano River was skewed toward females (0.6 males per female, $\chi^2_1 = 7.13$, $P < 0.01$, $n = 110$), whereas in the San Saba River *C. petrina* was skewed toward males (2.2 males per female; $\chi^2_1 = 5.16$, $P < 0.05$, $n = 40$). The sex ratio for *C. pustulosa* in the San Saba River did not significantly differ from 1:1 ($\chi^2_1 = 0.02$, $P > 0.05$, $n = 41$).

DISCUSSION

We found that gamete density of *C. petrina* was lower during the summer and early fall months (June–September) and highest during the winter and early spring months (December–March, depending upon sex), which is in accordance with previous studies on *Cyclonaias* species (Jirka and Neves 1992; Haag and Staton 2003; Galbraith and Vaughn 2009; Tsakiris et al. 2016; Dudding et al. 2020). In addition, the higher percentage of samples without gametes in the San Saba River compared to the Llano River suggests that the reproductive potential of *C. petrina* may be more limited in

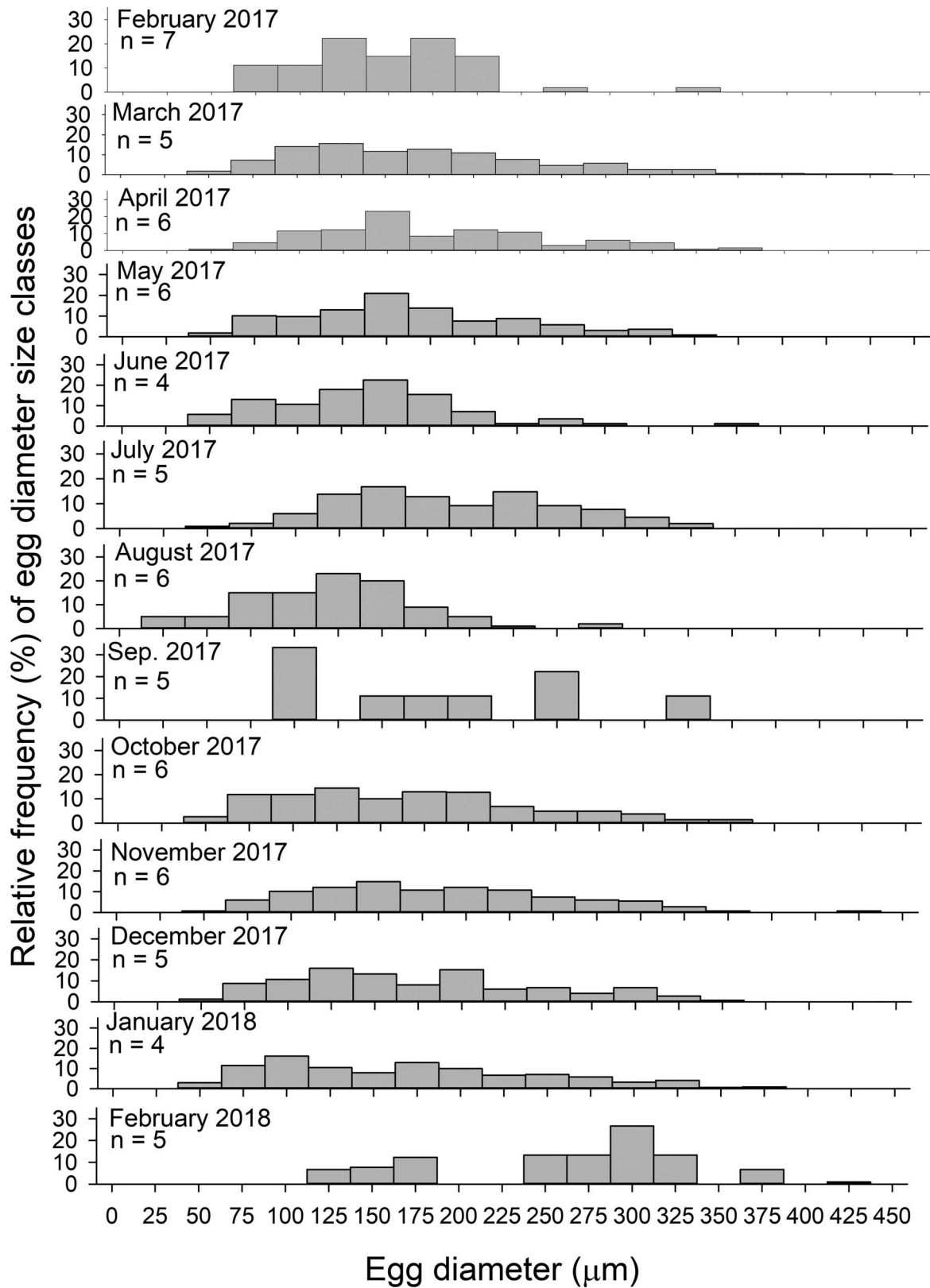


Figure 3. Egg size distribution of *Cyclonaias petrina* from the Llano River during monthly sampling events from February 2017 through February 2018.

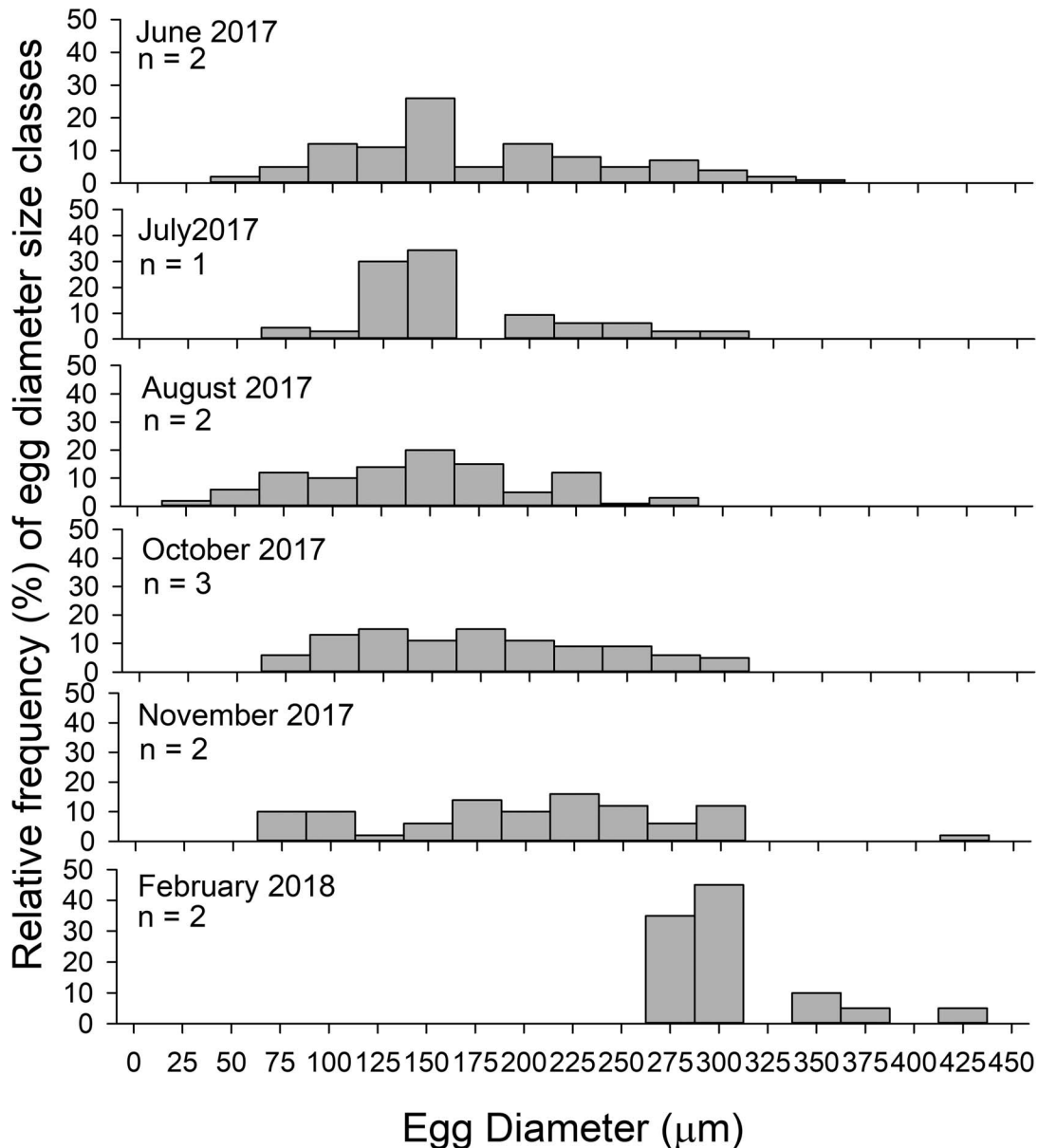


Figure 4. Egg size distribution of *Cyclonaias petrina* from the San Saba River during monthly sampling events from June 2017 through February 2018.

the former, which could be due to several potential stressors (see below).

We observed gamete densities increasing in the fall (October–November) and peaking in winter and early spring (December–March). However, seasonal variation in gamete densities represents variation in gamete production of mussels only if it is assumed that gamete fluid volume does not vary seasonally (Seagroves et al. 2019). Garner et al. (1999) suggested that mussels may benefit from starting gamete production in the fall to utilize increased nutrients within the river. Similar to our study, Tsakiris et al. (2016) reported the lowest gamete concentration of *Cyclonaias* during summer and early fall (June–September); however, they recorded peak gamete production 1–3 mo later than in our study. Low levels of active gametogenesis were found throughout the year in

Cyclonaias tuberculata with a pulse of mature gametes being produced and held during fall and early spring months (Jirka and Neves 1992), similar to our results. There seems to be little difference in gamete production between the two *Cyclonaias* species in the San Saba River, which is consistent with previous findings (Tsakiris et al. 2016).

Surprisingly, few studies on the reproductive ecology of Quadrulini mussels reported monthly changes in egg size (but see Yeager and Neves 1986; Garner et al. 1999; Haggerty et al. 2005; Tsakiris et al. 2016). Such changes tend to closely follow changes in gamete densities, with egg size being positively correlated with egg density (Yeager and Neves 1986; Garner et al. 1999; Haggerty et al. 2005; Tsakiris et al. 2016). Similar to our results, most short-term brooders that spawn in the spring and early summer (i.e., not including *M.*

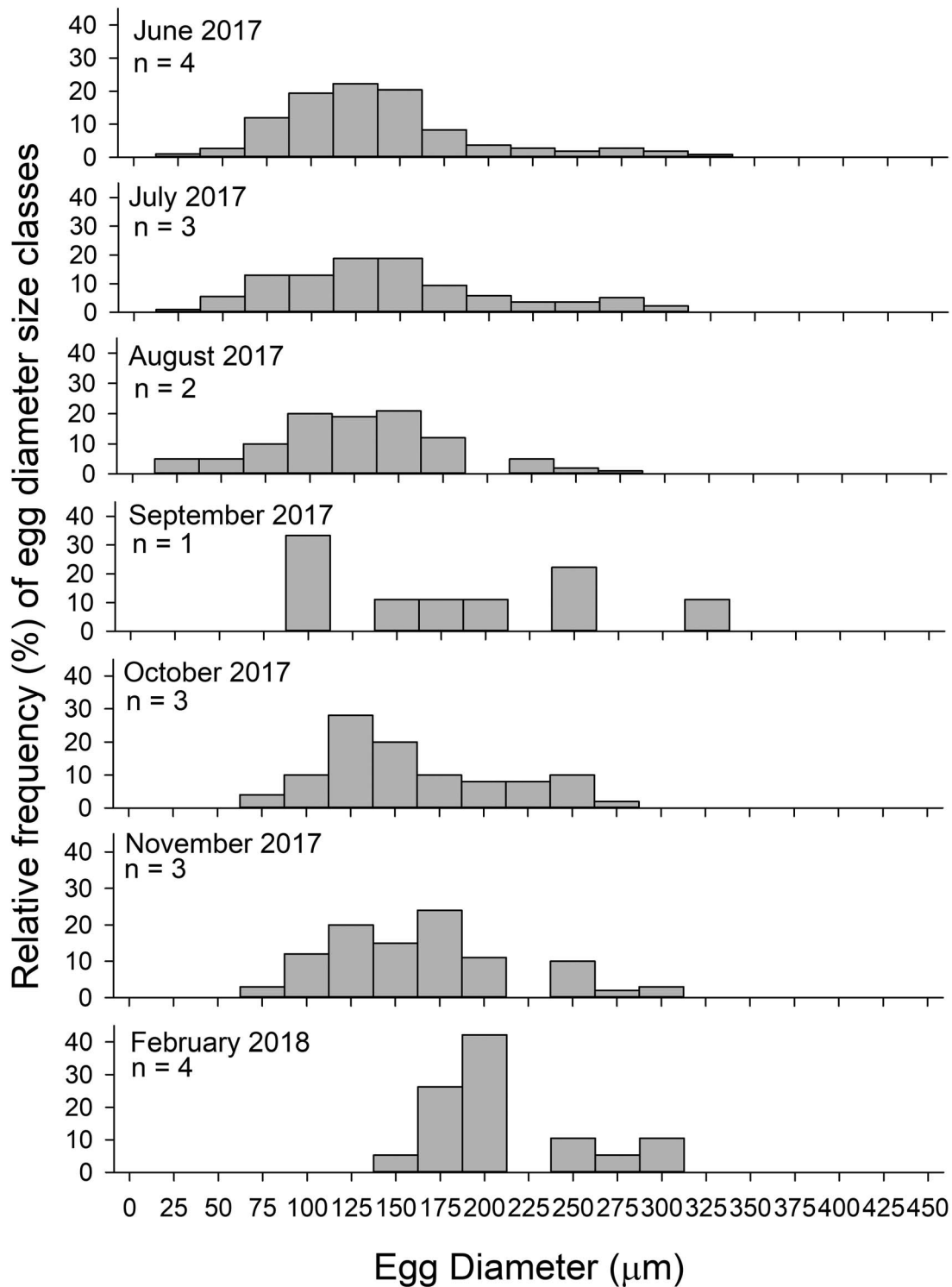


Figure 5. Egg size distribution of *Cyclonaias pustulosa* from the San Saba River during monthly sampling events from June 2017 through February 2018.

nervosa) have increases in egg diameters throughout the fall and reach maximum size from late winter to spring (February–April), before decreasing in size during the late summer (August–September; Yeager and Neves 1986; Garner et al. 1999; Tsakiris et al. 2016). Decreases in egg size within the gonads has been interpreted as an indicator of spawning, with

mature eggs presumably moving into the suprabranchial chamber to be fertilized (Garner et al. 1999). Substantial decreases in egg density for Llano River females in April 2017 (a pattern not seen in the San Saba River) also suggest that eggs had been released by the females from the gonads. Even though no brooding females were found in our study,

decreased egg densities and the estimated brooding time for tachytictic species (2–6 wk; Yokley 1972; Weaver et al. 1991; Garner et al. 1999) suggests that glochidia release occurred between April and June, similar to other species of *Quadrulini* (Yeager and Neves 1986; Jirka and Neves 1992; Haggerty et al. 1995; Garner et al. 1999; Haag and Staton 2003; Dudding et al. 2019).

Researchers have shown that gametogenesis can occur year round in many tachytictic species (Holland-Bartels and Kammer 1989; Jirka and Neves 1992; Garner et al. 1999; Tsakiris et al. 2016), which was observed in *C. petrina* in the Llano River, but not in the San Saba River. Similar to findings from another study investigating *Lampsilis bracteata* in central Texas (Seagroves et al. 2019), reproduction appeared to be more limited at sites in the San Saba River compared to the Llano River as suggested by a larger number of samples without gametes found in the San Saba River compared to the Llano River (43% vs. 11% when comparing months in which both rivers were sampled). Detrimental environmental conditions in the San Saba River may play a role, either by causing reproductive senescence in mussels as exhibited in populations of other taxa (Nussey et al. 2013) or by making mussels more susceptible to diseases and parasites (see below). Lower water quality in the San Saba River has been documented by the Texas Commission on Environmental Quality (TCEQ), with the lower San Saba River classified as impaired since 2008 due to elevated levels of *Escherichia coli*, likely resulting from nonpoint sources of pollution such as agriculture runoff and improper or lack of sewage treatment (TCEQ 2019).

As suggested by other studies, some mussels may experience decreased reproductive output due to factors other than water quality. Reproductive senescence caused by old age has been suggested (Bauer 1987; Downing et al. 1993; Haag and Staton 2003) but not supported for the vast majority of freshwater mussel species (Haag 2012). Based on age and growth data of *Cyclonaias* spp. in our study area, reproductive senescence due to old age likely did not play a role in explaining the lack of gametes within the majority of our samples because most of our mussels were less than 15 yr of age (Hayes 2020).

The higher incidence of trematodes in the San Saba River versus the Llano River may influence reproductive output in the former, potentially explaining the lack of gametes within our samples. Digenetic trematodes have been known to feed on gonadal tissue, lower reproductive output, negatively impact physiological condition, and limit growth of freshwater mussels (e.g., Taskinen 1998; Gangloff et al. 2008; Müller et al. 2015). Prevalence of parasitic trematodes in freshwater mussels has been shown to be related to season, mussel body size, age, and sex, with higher infection rates being found in larger and older females during warmer months (Huehner 1984; Taskinen et al. 1994; Taskinen and Valtonen 1995; Müller et al. 2015; Seagroves et al. 2019). Our study found increased prevalence of trematodes during the summer and fall months (June–November), but no relationship with mussel body size. Our data did not allow us to test for relationships

between parasite prevalence and mussel sex or age. Parasitic infection rates in mussels are usually low (<6%; Haag and Staton 2003), but some studies have shown relatively high (20–36%) infection rates in multiple species (Zale and Neves 1982; Tsakiris et al. 2016; Dudding et al. 2020). In the San Saba River, during congruent sampling trips, bucephalid trematodes (Bucephalidae) and unidentified parasites were found in gamete samples of *Lampsilis bracteata* (6% infection rate for all individuals) consistently and in low abundance from February 2017 to February 2018 but were not found in April and September 2017 (Seagroves et al. 2019). Conversely, parasites in our study were found mostly during the summer and fall months (June–November) in both *Cyclonaias* species. The differences in trematode infection patterns between our study and Seagroves et al. (2019) might be explained by differences in mussel or parasite life-history strategies or differential habitat use between mussels of different genera. Tsakiris et al. (2016) reported high infection levels (>20%) of digenetic trematodes in *C. petrina* and *C. pustulosa* within the San Saba River between July 2012 and July 2013 during an exceptional drought in Texas. Our lower infection rates within the San Saba River could be a product of different sampling sites, lower sample sizes, or differences in environmental conditions at the time of sampling.

Sex ratios for *C. petrina* were statistically different from 1:1 in both rivers, but *C. pustulosa* showed no difference from a 1:1 ratio. Reported sex ratios for other *Quadrulini* populations are close to equal or slightly male-biased (Yeager and Neves 1986; Jirka and Neves 1992; Haggerty et al. 1995; Garner et al. 1999; Haag and Staton 2003). Sex ratios less than 2:1 likely do not have any ecological significance (Haag 2012); however, in our study the ratio for *C. petrina* in the San Saba River slightly exceeded this. This deviation could be caused by differences in sex-specific stressors (e.g., increased female parasitism in which all gonads are removed; Kuris 1974; Müller et al. 2015) or low sample size (e.g., Ricklefs and Miller 2000; Haag 2012). In our study, the number of individuals without gametes present and those infected with trematodes was highest for *C. petrina* in the San Saba River. Possibly, the male-skewed sex ratio in the San Saba River could be the result of increased parasitism of female mussels. We cannot draw definitive conclusions because we were unable to determine the sex of *C. petrina* in the absence of gametes and because our small sample size ($n=40$) resulted in lower statistical power ($\beta=0.60$) for detecting differences in sex ratio. A sample size of about 100 individuals is needed to obtain accurate estimates of sex ratio within a mussel population (Haag 2012). Thus, more data would need to be collected in order to verify our results of a skewed sex ratio.

The results of this study have increased our insight into the reproductive ecology of *Cyclonaias* populations, which is important for their management and conservation, to predict and understand the impact of climate change and other human activities, and to improve the timing of collection of brooding mussels and host fish to facilitate propagation methods. Furthermore, our study highlights that the reproductive

potential of a species of freshwater mussel can vary substantially between tributaries within a single river basin. Further research is needed to investigate the relative importance of various stressors affecting the reproductive ecology of these mussels and the effects of reproductive variation on population persistence.

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REGULAR ARTICLE

HOST FISHES AND LIFE HISTORY OF THE ROUND HICKORYNUT (*OBOVARIA SUBROTUNDA*)

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ABSTRACT

The Round Hickorynut, *Obovaria subrotunda*, is declining throughout its range, but little life history information exists for the species. We examined host use and glochidial size in Buck Creek (Cumberland River drainage) and the Licking River (Ohio River drainage), Kentucky, and we examined age and growth in Buck Creek. Glochidia of *O. subrotunda* from Buck Creek metamorphosed on five darter species (Percidae)—*Etheostoma baileyi*, *Etheostoma blennioides*, *Etheostoma gore*, *Etheostoma variatum*, and *Percina stictogaster*—but not on 43 other fish species from nine families; host use was broadly similar to previous studies in the Duck River, Tennessee, and Lake St. Clair, Ontario. Glochidia from the Licking River metamorphosed only on Eastern Sand Darters (*Ammocrypta pellucida*) and not on 11 other darter species, including two that produced juveniles in the Buck Creek trial. Glochidial metamorphosis was higher on *A. pellucida* than on any other species, suggesting that sand darters are primary hosts for *O. subrotunda*, but sand darters do not occur in several streams occupied by *O. subrotunda*. Such major differences in host use may indicate phylogenetic divergence between these populations. Glochidial size differed significantly between the Buck Creek and Licking River populations, suggesting that it may have taxonomic value also. *Obovaria subrotunda* was relatively short-lived (13 yr) and fast-growing ($K = 0.22$), and age-at-maturity was estimated at 2–3 yr. Males and females had similar growth rates, but males were substantially larger (L_{∞} : males = 53.7 mm; females = 39.2 mm). Life-history data support categorizing *O. subrotunda* as a periodic life-history strategist, and this view provides benchmarks for assessing population health and responses to watershed conditions.

KEY WORDS: Unionidae, conservation, age and growth, age-at-maturity, *Ammocrypta*

INTRODUCTION

The Round Hickorynut, *Obovaria subrotunda*, is distributed throughout the Ohio River basin and in the Lake St. Clair and Lake Erie drainages (Williams et al. 2008). Although the species has a G4 global ranking of “apparently secure” (NatureServe 2019), it is declining throughout much of its range and is currently under review for listing under the U.S. Endangered Species Act (USFWS 2011).

Life-history information for *O. subrotunda* is scant and exists primarily for populations in the Duck River, Tennessee (Tennessee River drainage; Ehlo and Layzer 2014), and Lake

St. Clair, Ontario (McNichols 2007). In the Duck River population, males grew faster than females, female sexual maturity was reported at one year of age, and glochidia transformed on three darter species (*Etheostoma blennioides*, *Etheostoma obama*, and *Etheostoma flabellare*) and the Banded Sculpin (*Cottus caroliniae*). In Lake St. Clair, glochidia transformed on three darter species (*Etheostoma exile*, *E. flabellare*, and *Percina maculata*). Glochidia of a related species, the Alabama Hickorynut, *Obovaria unicolor*, transformed primarily on sand darters (*Ammocrypta* spp.) and the Gulf Darter (*Etheostoma artesiae*, Haag and Warren 2003). Glochidia of *O. subrotunda* were described by Ortmann (1911, 1919), Surber (1915), and Hoggarth (1988, 1999), but no one

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Table 1. Results of host identification trial for *Obovaria subrotunda* from Buck Creek, Pulaski Co., Kentucky. The number of fish inoculated in parentheses is the number that survived to the end of the trial. Metamorphosis success is the proportion of inoculated glochidia that metamorphosed into juvenile mussels.

Fish Species ¹	No. of Fish Inoculated	No. of Glochidia Inoculated/Fish	No. of Juveniles Recovered	Days to Metamorphosis	Metamorphosis Success	No. of Juveniles/Fish
<i>Etheostoma baileyi</i>	17 (6)	15	8	10	0.089	1.3
<i>Etheostoma blennioides</i>	10 (7)	25	3	13	0.017	0.4
<i>Etheostoma gore</i>	2 (1)	15	1	18	0.067	1.0
<i>Etheostoma variatum</i>	10 (3)	20	12	13	0.200	4.0
<i>Percina stictogaster</i>	7 (6)	20	19	10	0.158	3.2

¹Fish families and species that did not produce juvenile mussels (number of fish inoculated): Atherinidae, *Labidesthes sicculus* (1); Catostomidae, *Hypentelium nigricans* (10), *Moxostoma erythrurum* (10), *M. macrolepidotum* (1), *Minytrema melanops* (1); Centrarchidae, *Ambloplites rupestris* (2), *Lepomis cyanellus* (2), *L. gulosus* (2), *L. macrochirus* (2), *L. megalotis* (2), *L. microlophus* (1), *Micropterus dolomieu* (1), *M. salmoides* (2), *Pomoxis annularis* (1); Cottidae, *Cottus caroliniae* (4); Cyprinidae, *Camptostoma anomalum* (1), *Carassius auratus* (1), *Cyprinella spiloptera* (4), *C. whipplei* (3), *Luxilus chrysocephalus* (5), *Lythrurus fasciolaris* (10), *Nocomis micropogon* (4), *Notropis rubellus* (11), *Pimephales notatus* (1), *P. promelas* (2), *Semotilus atromaculatus* (3); Fundulidae, *Fundulus catenatus* (1); Ictaluridae, *Ameiurus melas* (2), *A. natalis* (2), *Noturus elegans* (2); Percidae, *Etheostoma caeruleum* (10), *E. flabellare* (3), *E. jimmycarter* (4), *E. maydeni* (1), *E. nigrum* (11), *E. spectabile* (4), *E. virgatum* (10), *E. zonale* (12), *Nothonotus bellus* (2), *N. camurus* (9), *Percina caprodes* (4), *P. maculata* (3), Sciaenidae, *Aplodinotus grunniens* (1).

has assessed variation in glochidial size among populations. Additional life-history information for *O. subrotunda* is needed to better understand its ecology and to inform conservation strategies for the species.

We examined life-history aspects of *O. subrotunda* in two populations in Kentucky: Buck Creek and the Licking River. We examined host use and glochidial size in both populations, as well as age and growth and age-at-maturity in the Buck Creek population. We discuss our results in the context of existing information for *O. subrotunda* and other *Obovaria*.

METHODS

Mussel and Fish Collection for Host Identification

We collected one female and five male *O. subrotunda* from Buck Creek, Pulaski County (hereafter Co.), Kentucky (Cumberland River drainage), between March and November 2003; the female was not gravid at the time of collection (September). We transported mussels to the Center for Mollusk Conservation (Kentucky Department of Fish and Wildlife Resources, Frankfort, Kentucky) and placed them together in sand and gravel substrate in a flow-through raceway fed by water from South Elkhorn Creek. The female became gravid in captivity by January 2004.

We collected 3 female and 13 male *O. subrotunda* from the Licking River, Bath Co., Kentucky, in September 2015. We transported mussels to Minor Clark Hatchery (Kentucky Department of Fish and Wildlife Resources, Farmers,

Kentucky) and placed them together in sand and gravel substrate in a flow-through raceway fed by water from the Licking River. Two females were gravid by March 2016.

For the Buck Creek host trials, we collected 48 potential host-fish species (22 genera, 9 families; Table 1) with a backpack electrofisher or seine in September 2003. We collected fishes from the Red River, Menifee Co., Kentucky; Elkhorn Creek, Franklin Co., Kentucky (both Kentucky River drainage); Green River, Casey Co., Kentucky; Horse Lick Creek, Rockcastle Co., Kentucky (Cumberland River drainage); and Taylorsville Lake (Salt River), Spencer Co., Kentucky. The Buck Creek host trials showed that *O. subrotunda* was a specialist on darters (Percidae; see Results). Therefore, for the Licking River host trials, we collected 11 species of darters (Table 2) with a backpack electrofisher or seine from September to October 2015, from the Licking River, Bath Co., Kentucky. For both trials, we maintained fishes over the winter in aquaria in the laboratory until initiation of host trials. We fed fishes blackworms, crayfish, minnows, or pelletized fish food, depending on the dietary habits of each species.

Host Identification

We extracted glochidia from female *O. subrotunda* on May 1, 2004, and May 17, 2016, for the Buck Creek and Licking River host trials, respectively. For the Licking River trial, we combined glochidia from two females. We extracted glochidia by flushing the gills with water through a syringe. We tested

Table 2. Results of host identification trial for *Obovaria subrotunda* from the Licking River, Bath Co., Kentucky. The number of fish inoculated in parentheses is the number that survived to the end of the trial. Metamorphosis success is the proportion of inoculated glochidia that metamorphosed into juvenile mussels.

Fish Species ¹	No. of Fish Inoculated	No. of Glochidia Inoculated/Fish	No. of Juveniles Recovered	Days to Metamorphosis	Metamorphosis Success	No. of Juveniles/Fish
<i>Ammocrypta pellucida</i>	9 (8)	125	366	20–23	0.366	45.8

¹Fish species (all Percidae) that did not produce juvenile mussels (number of fish inoculated, days to rejection): *Etheostoma blennioides* (10, 2), *E. caeruleum* (10, 2), *E. flabellare* (3, 2–4), *E. nigrum* (4, 2–4), *E. variatum* (12, 2–4), *E. zonale* (10, 2), *Nothonotus camurus* (1, 2), *Percina copelandi* (3, 2–4), *P. oxyrhynchus* (2, 2), *P. sciera* (1, 2).

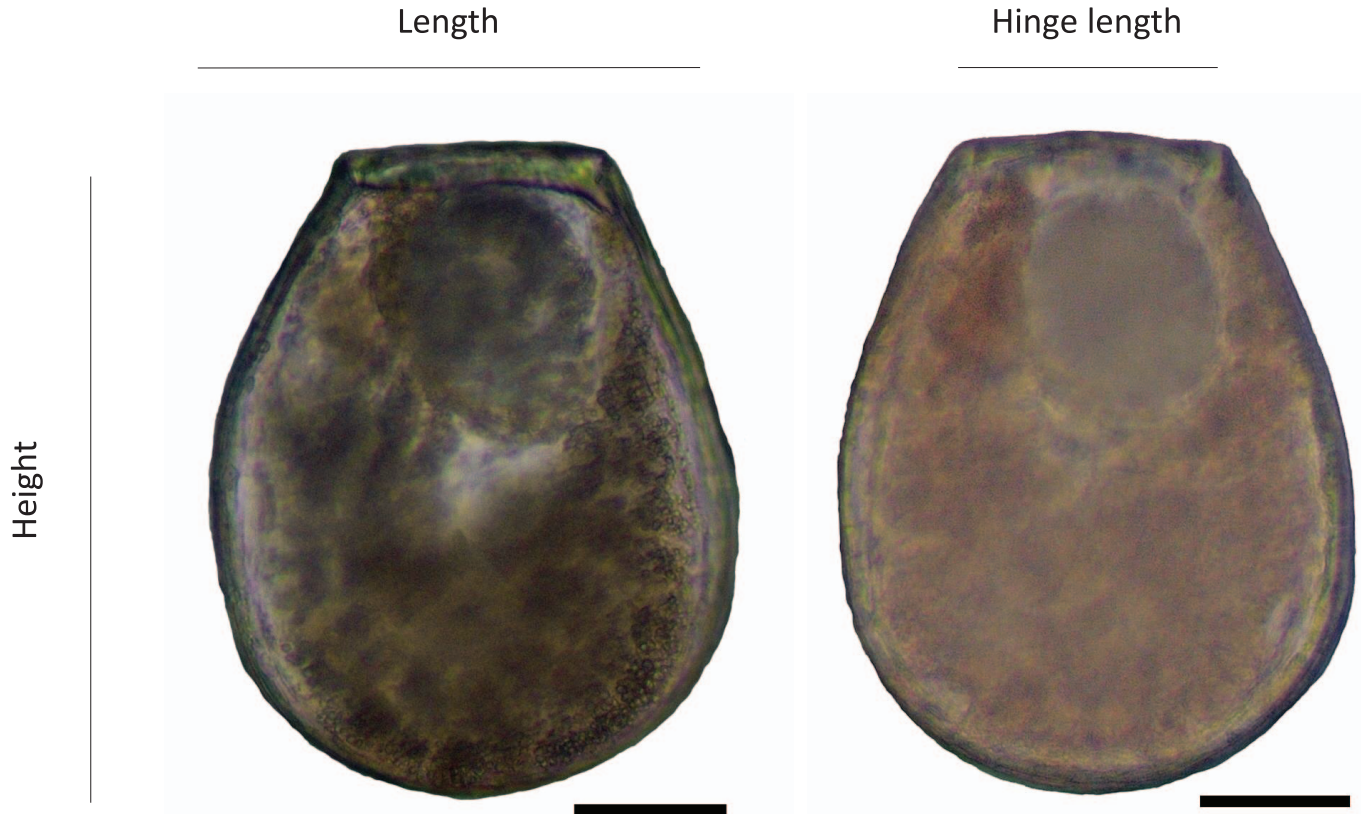


Figure 1. Glochidia of *Obovaria subrotunda* from Buck Creek, Kentucky (left) and the Licking River, Kentucky (right). Scale bar = 50 μm . Darker coloration of the Buck Creek specimen is due to alcohol storage.

viability of glochidia by exposing a subset to a NaCl solution. Glochidia for the Licking River trial exhibited 100% viability. Viability was not quantified for the Buck Creek trial, but most glochidia exhibited rapid valve closure in response to salt.

We conducted host trials for both populations generally following Zale and Neves (1982) and Rogers et al. (2001). We anesthetized fishes in MS-222 (0.15 g/L) and inoculated them by pipetting glochidia directly onto the gill filaments of each fish. For the Buck Creek trial, we inoculated each fish with 15–200 glochidia, depending on fish size (Table 1), and we counted glochidia in a Petri dish before drawing them into a pipette. For the Licking River trial, we inoculated each fish with about 125 glochidia, determined volumetrically based on prior estimates of the number of glochidia in a 0.125 ml aliquot. After inoculation, we placed fishes in a 5-L recovery chamber for about 10 min and then transferred them to 1- to 9-L chambers in a recirculating AHAB system (Pentair Aquatic Habitats, Apopka, FL). We placed 1 to 12 individual fish in each chamber, depending on the size of fishes, maintained them in the AHAB systems at 23–25°C, and fed them as described previously.

For the Licking River trial, we anesthetized all fishes on days 2 and 4 postinoculation and examined their gills for encysted glochidia. All individuals of all species except *A. pellucida* had rejected all glochidia by day 4 and were removed from the trial; we did not examine *A. pellucida* after

day 4 because all individuals were heavily infested on that day. We did not examine fishes after inoculation in the Buck Creek trial. In both trials, we examined material on the bottom of each chamber daily for transformed juveniles, beginning 10 days after inoculation and continuing until 7 days after the last juvenile mussels were observed. We collected material from the bottom of each AHAB chamber on a 100- μm filter screen and examined the material under a binocular microscope. We considered suitable host-fish species as those that produced active juveniles with a well-developed foot. We evaluated the robustness of juvenile mussel production from each suitable host species by calculating a measure of metamorphosis success as total number of juveniles produced/number of inoculated fish that remained alive at the end of the experiment/number of glochidia inoculated on each fish. We also calculated the number of juveniles produced/fish to allow comparison of our results with those of McNichols (2007) and Ehlo and Layzer (2014).

Glochidial Size

We collected a haphazardly selected sample of 25 glochidia each from Buck Creek and the Licking River; Licking River glochidia were from the composited sample from two females. We measured glochidial shell length, height, and hinge length (Fig. 1) of each individual to the

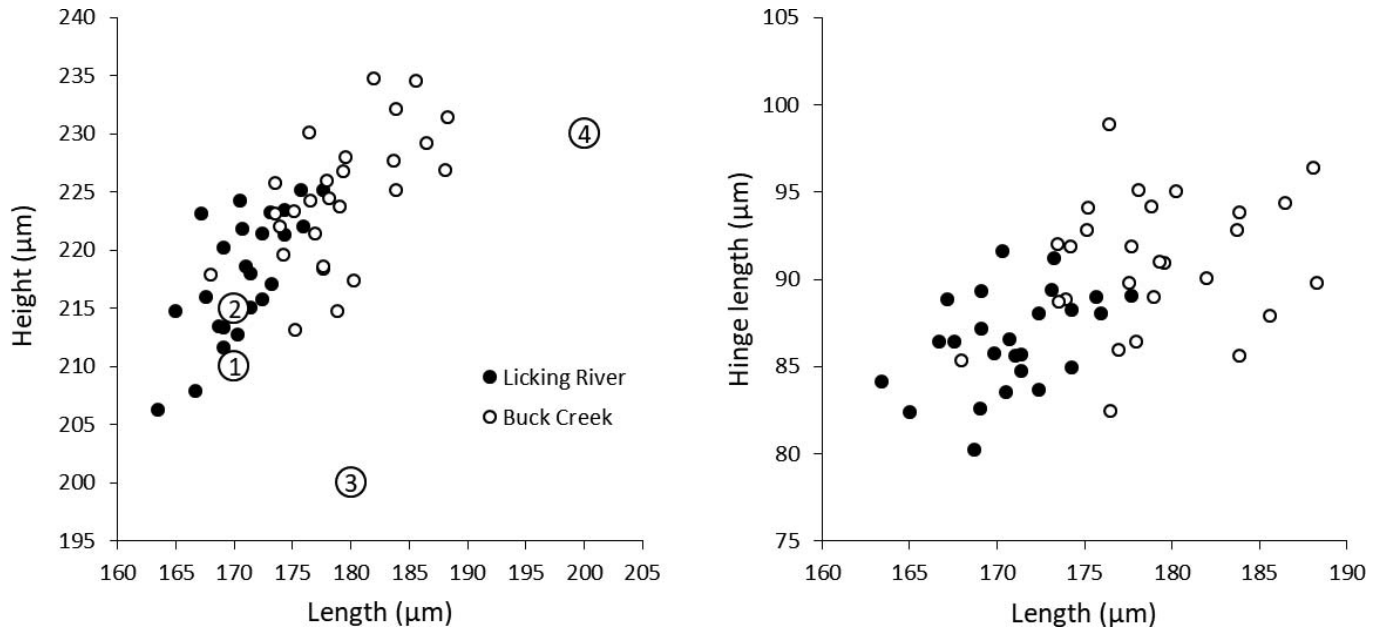


Figure 2. Glochidial size of *Obovaria subrotunda* from Buck Creek and the Licking River, Kentucky. Numbered points refer to single measurements for (1) Fish Creek, Ohio; (2) Wabash River, Indiana; (3) Big Darby Creek, Ohio; and (4) Crooked Creek, Indiana. Hinge length was not available for all of the last four populations. See text for data sources.

nearest 0.1 μm at 20× magnification using a compound microscope and imaging software. We examined bivariate plots between size measurements and tested for differences in size between populations using MANOVA and ANCOVA. We also compared glochidial size in our study populations with published glochidial measurements for *O. subrotunda* from Fish Creek and Big Darby Creek, Ohio (Hoggarth 1988), the Wabash River, Indiana (Surber 1915), and Crooked Creek, Indiana (Ortmann 1911, 1919).

Age and Growth

We obtained shells of 21 male and 17 female *O. subrotunda* collected from Buck Creek in 1983 and 1984 from the Branley A. Branson Museum of Zoology, Eastern Kentucky University (EKU collection numbers 118, 127, and 130). We measured shell length (greatest anterior-posterior dimension, nearest 0.1 mm) of each individual and determined its sex based on shell sexual dimorphism (Ehlo and Layzer 2014). We estimated the age of each individual by counting external growth rings (annuli), assuming one annulus/year (Haag and Commens-Carson 2008). To obtain additional information about length-at-age, particularly for younger ages, we back-measured shell length at all interpretable annuli for all individuals (Michaelson and Neves 1995; Haag and Rypel 2011). We constructed von Bertalanffy growth models separately for each sex. We tested for differences in length-at-age between sexes using ANCOVA with length as the dependent variable, sex as the independent variable, and $\ln(\text{age})$ as the covariate. We estimated age-at-maturity by noting the earliest external annulus at which shell sexual dimorphism became evident.

RESULTS

Host Identification

Glochidia of *O. subrotunda* from Buck Creek metamorphosed only on five darter species (Percidae): *E. baileyi*, *E. blennioides*, *E. gore*, *E. variatum*, and *Percina stictogaster* (Table 1). Metamorphosis success was highest on *E. variatum* (0.200) and *P. stictogaster* (0.158) and was about 50% less or lower on the other three species. No juvenile mussels were produced on any other fish species, including 12 other darter species and *Cottus caroliniae*. Glochidia from the Licking River metamorphosed only on *A. pellucida* (Percidae) and not on 11 other darter species, including two species that produced juveniles in the Buck Creek trial (*E. blennioides* and *E. variatum*; Table 2). Metamorphosis success was substantially higher on *A. pellucida* (0.366) than on any species in the Buck Creek trial.

Glochidial Size

Glochidial size differed significantly between populations (MANOVA: Wilks' $\lambda = 0.480$, $F_{3,46} = 16.64$, $P < 0.0001$; Fig. 2). All three measurements of glochidial size differed significantly between populations, and mean values were 3–5% smaller in the Licking River than in Buck Creek (univariate ANOVA, $F_{1,48} = 18.09$ – 45.37 , $P < 0.0001$ for all tests; Table 3). Despite overall size differences, glochidia were proportionally similar in both populations (Fig. 2). Glochidial height was strongly related to length (two-factor ANOVA: $F_{1,46} = 31.01$, $P < 0.0001$), but stream and the length \times stream interaction were not significant (stream, $F_{1,46} = 1.82$, $P = 0.182$; length \times stream, $F_{1,46} = 1.83$, $P = 0.183$).

Table 3. Glochidial size of *Obovaria subrotunda* from six populations. Values for Buck Creek and the Licking River are means \pm SD. Means with different superscripted letters within a column are significantly different (all $P < 0.0001$). Values for the other four streams are single reported values; a dash (—) indicates that the value was not reported. See text for data sources.

Stream	Height (μm)	Length (μm)	Hinge Length (μm)
Buck Creek, Kentucky	224.5 \pm 5.7 ¹	179.0 \pm 4.9 ¹	90.9 \pm 3.8 ¹
Licking River, Kentucky	217.9 \pm 5.3 ²	170.8 \pm 3.4 ²	86.5 \pm 2.8 ²
Fish Creek, Ohio	210.0	170.0	80
Wabash River, Indiana	215.0	170.0	—
Big Darby Creek, Ohio	180.0	200.0	90
Crooked Creek, Indiana	230.0	200.0	—

An ANCOVA model omitting the interaction term similarly showed a strong effect of length on height ($F_{1,47} = 28.85$, $P < 0.0001$), but no effect of stream when the effect of length was accounted for ($F_{1,46} = 0.01$, $P = 0.929$; least square mean heights: Licking River = 221.2 μm , Buck Creek = 221.1 μm). Glochidia from Fish Creek, Ohio, and the Wabash River, Indiana, were similar in size to glochidia from the Licking River (Table 2). Glochidia from Big Darby Creek, Ohio, and Crooked Creek, Indiana, differed substantially in size from each other and from other populations.

Age and Growth

Males ranged from 4 to 13 yr old (median age = 5.0 yr), and females ranged from 4 to 12 yr old (median = 6.5). The von Bertalanffy growth constant K was lower for males (0.239 ± 0.026 SE) than females (0.272 ± 0.055), but asymptotic length (L_∞) was substantially higher for males (52.8 mm \pm 1.7 SE) than females (36.9 \pm 1.8; Fig. 3). The slopes of the regressions of length on $\ln(\text{age})$ differed between males and females (ANCOVA, age \times sex interaction: $F_{1,207} = 62.14$, $P <$

0.0001), indicating that male and female size did not differ by a constant factor across ages; rather, length-at-age relationships showed that males became increasingly larger than females with increasing age (Fig. 3 and Table 4). Female shells first showed discernible evidence of sexual dimorphism between the ages of 3 and 5.

DISCUSSION

Our host trials, and those from the Duck River and Lake St. Clair (McNichols 2007; Ehlo and Layzer 2014), support *O. subrotunda* as a host specialist largely on darters (Percidae). The only nondarter host that facilitated glochidial metamorphosis was *Cottus carolinae* (Cottidae), but that species produced juvenile mussels only in the Ehlo and Layzer (2014) study and not in our Buck Creek trial. We did not test sculpins in the Licking River trial, but *C. carolinae* does not occur in the Licking River system, and *Cottus bairdi* occurs only sporadically in small streams in the system and is not reported from the mainstem Licking River in association with *O. subrotunda* (Burr and Warren 1986). *Cottus* spp. were not tested by McNichols (2007).

In addition to the lack of metamorphosis on *C. carolinae*, results of our host trial from Buck Creek differed in several ways from those from the Duck River and Lake St. Clair (McNichols 2007; Ehlo and Layzer 2014). Together, all three studies show that *O. subrotunda* is able to successfully parasitize relatively few darter species, and host use only loosely follows phylogenetic relatedness in darters. No species within the genus *Nothonotus* (*Nothonotus aquali*, *Nothonotus bellus*, *Nothonotus camurus*, *Nothonotus rufilineatus*) or the subgenera *Allohistium* (*Etheostoma maydeni*) or *Boleosoma* (*Etheostoma nigrum*) served as hosts in any study. No species in the subgenus *Oligocephalus* (*Etheostoma caeruleum*, *Etheostoma spectabile*) served as hosts, with the exception of *Etheostoma exile*, which produced the highest number of juveniles in Lake St. Clair (4.8/fish). The species that produced the highest numbers of juveniles in Buck Creek and the Duck River were in the subgenus *Etheostoma* (*Etheostoma blennioides*, Duck River, 1.3 juveniles/fish; *Etheostoma variatum*, Buck Creek, 4.0 juveniles/fish). However, *E. blennioides* produced the lowest number of juveniles of any suitable host species in Buck Creek (0.4 /fish) and did not serve as a host in

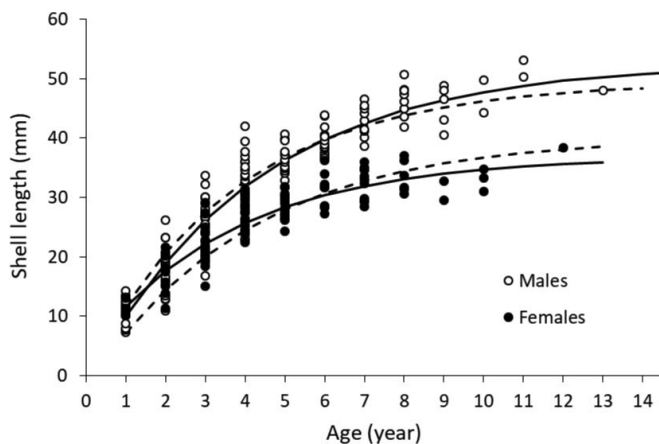


Figure 3. Length-at-age for *Obovaria subrotunda* from Buck Creek, Pulaski Co., Kentucky. Data points are direct estimates of length-at-age inferred from external shell rings. Solid lines are length-at-age predicted by von Bertalanffy growth models for each sex [$L_t = L_\infty(1 - e^{-K(t-t_0)})$, where L_t = length at time t , and t = age (year); males: $L_\infty = 52.8$, $K = 0.239$, $t_0 = 0.118$; females: $L_\infty = 36.9$, $K = 0.272$, $t_0 = -0.391$]. Dashed lines are von Bertalanffy growth curves for *O. subrotunda* from the Duck River, Tennessee (Ehlo and Layzer 2014).

Table 4. Length-at-age data for *Obovaria subrotunda* from Buck Creek, Pulaski Co., Kentucky. Ages without direct observations are indicated by a dash (—). Sample size (*N*) refers to the number of observations for each age. Predicted mean length is from a von Bertalanffy growth model (see Fig. 3). Growth increment refers to the predicted increase in shell length relative to the previous year.

Age (Year)	<i>N</i>	Mean Observed Length (Range, mm)	Predicted Mean Length (mm)	Growth Increment (mm)
Males				
1	15	10.8 (7.3–14.2)	10.0	10.0
2	17	18.2 (10.9–26.2)	19.1	9.1
3	21	25.8 (16.8–33.7)	26.2	7.1
4	19	31.7 (22.9–42.0)	31.9	5.6
5	11	36.5 (32.9–40.7)	36.3	4.4
6	10	39.4 (36.7–43.9)	39.8	3.5
7	8	42.8 (38.6–46.6)	42.5	2.8
8	6	46.3 (41.8–50.7)	44.7	2.2
9	3	47.4 (40.5–48.8)	46.4	1.7
10	2	47.1 (44.4–49.8)	47.8	1.3
11	2	51.8 (50.3–53.2)	48.8	1.1
12	0	—	49.7	0.8
13	1	48.0	50.3	0.7
Females				
1	5	11.8 (10.1–13.2)	11.6	11.6
2	11	17.0 (11.3–21.7)	17.6	6.0
3	16	22.0 (15.0–29.1)	22.2	4.6
4	17	26.9 (22.4–31.4)	25.7	3.5
5	16	28.3 (24.3–31.9)	28.4	2.7
6	10	31.0 (27.2–36.2)	30.4	2.0
7	9	32.4 (28.5–35.9)	31.9	1.5
8	6	33.4 (30.6–37.0)	33.1	1.2
9	2	31.2 (29.6–32.8)	34.0	0.9
10	3	33.0 (31.0–34.7)	34.7	0.7
11	0	—	35.2	0.5
12	1	38.3	35.6	0.4

Lake St. Clair; two other species in the subgenus were not suitable hosts (*Etheostoma blennioides*, *Etheostoma zonale*). Similarly, *Etheostoma flabellare* (subgenus *Catonotus*) produced low numbers of juveniles in the Duck River (0.3/fish) and Lake St. Clair (0.1/fish), but it did not produce juveniles in Buck Creek, and two other members of the subgenus were unsuitable (*Etheostoma crossopterygion*, *E. virgatum*). Two members of the *E. stigmaeum* complex (subgenus *Doration*) produced moderate numbers of juveniles (*Etheostoma obama*, Duck River, 0.6/fish; *Etheostoma gore*, Buck Creek, 1.0/fish), but a third species in the complex was not a suitable host (*Etheostoma jimmycarteri*). *Percina stictogaster* produced the second-highest number of juveniles in the Buck Creek trial (3.2 juveniles/fish), but no other *Percina* were suitable hosts, including *Percina maculata*, which produced the highest number of juveniles in Lake St. Clair (4.8/fish); *Percina* spp. were not tested by Ehlo and Layzer (2014). It is difficult to directly compare juvenile production in our study with that

reported by McNichols (2007) or Ehlo and Layzer (2014) because those studies did not quantify the number of glochidia inoculated on each fish. Nevertheless, inconsistencies between these studies suggest subtle differences in host use between the Buck Creek, Duck River, and Lake St. Clair populations.

Results of our host trial from the Licking River were radically different from all other studies. *Obovaria subrotunda* from the Licking River metamorphosed only on Eastern Sand Darters, *A. pellucida*, and the number of juveniles produced (45.8/fish) was substantially higher than on any fish species from other populations. Higher production is due in part to the greater number of glochidia inoculated on each fish in the Licking River trial, but the higher metamorphosis success (0.366) suggests that *A. pellucida* is a more robust host for *O. subrotunda*. Furthermore, no metamorphosis occurred on fish species that were suitable hosts for the other populations (*E. blennioides*, *E. flabellare*, and *E. variatum*). Host use in the Licking River was similar to *Obovaria unicolor*, which metamorphosed consistently only on two *Ammocrypta* species and *E. artesia*, and 10 other darter species were either marginal hosts or unsuitable (Haag and Warren 2003). *Ammocrypta* spp. were not tested in the Buck Creek, Duck River, or Lake St. Clair trials.

The higher metamorphosis success on *Ammocrypta* in the Licking River trial and the variable results from other darter species in other trials could indicate that *Ammocrypta* is a primary host for *O. subrotunda*, and other darter species are only marginally suitable hosts. This explanation is plausible for the Lake St. Clair population, where *O. subrotunda* co-occurs with *A. pellucida* (Derosier 2004). However, no species of *Ammocrypta* are reported from Buck Creek or the Duck River, and there are few (mostly historical) records of that genus anywhere in the Cumberland or Tennessee river systems (Burr and Warren 1986; Etnier and Starnes 1993). The current or former presence of large populations of *O. subrotunda* throughout those river systems (Parmalee and Bogan 1998; Ehlo and Layzer 2014; Haag and Cicerello 2016) suggests that other darter species are capable of supporting those populations and host use may differ substantially from populations elsewhere in the Ohio River basin, such as the Licking River, where sand darters may be a primary host. The distributions of *O. subrotunda* and *A. pellucida* in the middle and upper Ohio River basin are remarkably concordant (compare distributional maps in Burr and Warren 1986 and Haag and Cicerello 2016, and Trautman 1981 and Watters et al. 2009), and Clark (1977) proposed *A. pellucida* as a host for *O. subrotunda* based on the frequent co-occurrence of the two species in the Maumee River system (Lake Erie drainage). Declines in sand darter populations may partially explain declines in *O. subrotunda* populations in this region (see Trautman 1981). Additional studies of *O. subrotunda* host use in the middle and upper Ohio River basins and Great Lakes basin are needed to better evaluate this potential relationship.

Glochidial size differed significantly between the Buck Creek and Licking River populations. Glochidial size and proportions reported from two other populations (Fish Creek,

Ohio; Wabash River, Indiana) were similar to the smaller glochidia from the Licking River. Glochidial size and proportions from two other populations differed substantially from either of our populations: glochidia from Big Darby Creek had much lower height but were similar in length to Buck Creek glochidia; glochidia from Crooked Creek, Indiana, were similar in height to Buck Creek but had much greater length. Data from these four other populations appear to be based on observations of single glochidia and may suffer from several sources of error. Although our data represent measurements of multiple glochidia, they are from a small number of females and may reflect differences among individual females rather than differences among populations. Nevertheless, these findings are the first to our knowledge to suggest differences in glochidial size among populations.

The Cumberland River system supports high endemism of aquatic species, and recently, several cryptic species have been described from the region (e.g., Powers et al. 2004; Lane et al. 2016). The differences in host use and glochidial size between Buck Creek and Licking River *O. subrotunda* raise the possibility of some degree of phylogenetic divergence between those populations. Host use can vary among populations of the same mussel species from different drainage basins in some cases but not in others (Riusech and Barnhart 2000; Caldwell et al. 2016; St. John White et al. 2017). However, differences are usually manifested as differences in glochidial metamorphosis success on shared host species, rather than radically different patterns of host use, such as those we observed. More data from other populations are needed to examine patterns of variation in glochidial size in more detail, but glochidial size should be considered as a potentially informative taxonomic character (see O'Brien et al. 2019).

Our age and growth data depict *O. subrotunda* as a relatively short-lived, fast-growing species. Our maximum observed age (13 yr) was similar to the median life span for the tribe Lampsilini (15 yr) but is substantially lower than median life span for any other North American unionid tribe except the Anodontini (Haag and Rypel 2011). Similarly, the value of the von Bertalanffy growth constant, K (0.25, mean of males and females), is near the median for the Lampsilini (0.27), and higher than other tribes except the Anodontini. It is possible that our age and growth data are biased due to our reliance on external shell annuli. However, *O. subrotunda* have distinctive external annuli because of their light shell color and relatively rapid growth, and external annuli can provide accurate length-at-age measurements in those situations (Haag and Commens-Carson 2008). Furthermore, our estimates of life span, K , maximum size (L_{∞}), and length-at-age were very similar to those of Ehlo and Layzer (2014) from the Duck River based on internal annuli (maximum age = 14 yr; K : males = 0.272; females = 0.247; L_{∞} : males = 49.5 mm; females = 40.3; see Fig. 3).

We did not see shell sexual dimorphism until three to five years of age, but it is possible that maturity occurs before shell characters become evident. Ehlo and Layzer (2014) reported

female maturity at age one based on the minimum observed size of mature individuals (20 mm) and von Bertalanffy predictions of length-at-age. However, their von Bertalanffy model predicts that a 20-mm female would be slightly less than 3 yr old (predicted mean size of a 3-yr-old individual = 20.1 mm). The smallest mature male they observed (22 mm) is predicted by their von Bertalanffy model to be 2+ yr old. Our estimates of K for males and females predict sexual maturity at age 3.0 and 2.6, respectively, based on a relationship generated for several unionid species [age-at-maturity = $0.69(K^{-1.031}) - 1$; Haag 2012). Direct observations are necessary to determine age-at-maturity more precisely, but our data and those of Ehlo and Layzer (2014) support a relatively early age-at-maturity of about 2–3 yr.

Our data and those of Ehlo and Layzer (2014) support categorizing *O. subrotunda* as a periodic life-history strategist, similar to *O. unicolor* (Haag 2012). In this view, *O. subrotunda* is expected to have periodic, but large, bouts of recruitment and has the potential for rapid population growth under favorable conditions. For example, up to 40% of individuals in populations in the Duck River were recruits <1 yr old, and recent population increases in that river are attributed to watershed and landscape improvement (Ehlo and Layzer 2014). The population of *O. subrotunda* in Buck Creek has declined rapidly in the last 30 yr (Haag and Cicerello 2016; M. McGregor, unpublished data), but the causes of this decline are unknown.

Our host-use findings provide the impetus for assessing host availability as a potential causal factor in widespread declines of *O. subrotunda*. This information also improves our ability to propagate *O. subrotunda* for conservation, but a better understanding of population-specific host use across the range of the species is needed. Phylogenetic divergence between populations of *O. subrotunda* would have important conservation implications, but no firm conclusions about this can be made at this time; assessing this possibility requires a molecular genetics approach. The life-history information we provide for *O. subrotunda* can help derive more explicit benchmarks for assessing population health and responses to watershed conditions.

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REGULAR ARTICLE

GENETIC DIVERSITY IN THE THREATENED FRESHWATER MUSSEL *LAMPSILIS POWELLII*

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ABSTRACT

North America is home to the greatest share of the world's freshwater mussel diversity; however, more than 70% of its ~300 species are endangered or threatened. *Lampsilis powellii*, the Arkansas Fatmucket, is endemic to Arkansas and is now restricted to upstream reaches of the Ouachita and Saline rivers, but the species is declining within this small range. Conservation actions such as augmenting or reintroducing populations may be necessary, but they require knowledge of the distribution of genetic variation within and among extant populations. We analyzed population structure between the South Fork Ouachita River and Saline River using a 607-base-pair region of the mitochondrial *COI* gene and 14 microsatellites designed for *Lampsilis abrupta*. *COI* sequences showed little variation, and the most common haplotype was present in both rivers. Our mtDNA sequences were indistinguishable from those of *L. siliquioidea* deposited on GenBank, but we were unable to make conclusions about the taxonomic distinctiveness of *L. powellii*. Microsatellites showed heterozygote deficiencies for most loci and revealed little evidence of population structure between the two rivers. Overall, our results show low genetic diversity in *L. powellii*, which may reflect its small population size due to its long history of geographic isolation compounded by anthropogenic habitat destruction and fragmentation. Further genetic analyses of lampsiline taxa are needed to establish species limits for *Lampsilis* in the Interior Highlands.

KEY WORDS: unionid, lampsiline, mtDNA, microsatellites, population genetics

INTRODUCTION

Small, isolated populations lose rare alleles through genetic drift, and such reductions in genetic diversity can make species more vulnerable to extinction because greater diversity increases adaptability and long-term population persistence (Reed and Frankham 2003; Hoffman et al. 2017). As a result, imperiled species face increased probabilities of extinction because small populations leave them vulnerable to the interacting effects of genetic drift, demographic change, and environmental stochasticity. Such species are at risk of entering a so-called extinction vortex (Gilpin and Soulé 1986). More than 70% of North American freshwater mussel species (families Unionidae and Margaritiferidae) are imper-

iled, and many survive only in small, isolated populations (Williams et al. 1993).

Lampsilis powellii (Lea, 1852), the Arkansas Fatmucket, is a federally threatened unionid mussel with a narrow historical distribution restricted to Interior Highlands portions of the Ouachita, Saline, and Caddo rivers in south-central Arkansas, USA (U.S. Fish and Wildlife Service 1990). Populations of upland fishes and mussels in many Interior Highlands streams are proposed to have been isolated from each other historically by long stretches of lowland habitat in the Mississippi Embayment, created when the lower reaches of these streams were buried under sediment deposited by high sea levels during interglacial periods (Mayden 1988; Haag 2010). The range of *L. powellii* has been fragmented further by dams, which destroyed suitable habitat for this species. Dams also restrict fish movement, which, in turn, restricts mussel dispersal and gene flow because mussel larvae are obligate parasites on fishes. *Lampsilis powellii* currently survives in

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only two isolated populations separated by impoundments, in the upper Ouachita and upper Saline river systems.

We examined population genetic structure and genetic variation of *L. powellii* from remaining populations using a 607-base-pair (bp) region of the mitochondrial *COI* gene and 14 nuclear microsatellite loci. Given historical and recent barriers to dispersal, we expected to observe reduced genetic diversity and limited connectivity among populations compared to more widespread mussel species. We identified patterns of genetic variation and population structure in *L. powellii*, which will be useful for assessing its conservation status and implementing population restoration efforts.

METHODS

We obtained swab samples from the mantle or foot of 42 individuals: 15 individuals from one site on the South Fork of the Ouachita River, and 27 individuals from four sites on the Saline River (Fig. 1). Genomic DNA was extracted using ArchivePure DNA Cell/Tissue kits (5 Prime, Gaithersburg, MD, USA). The mitochondrial *cytochrome c oxidase subunit I* (*COI*) gene was amplified with primers LCO22me2 and HCO700dy2 using conditions developed by Walker et al. (2006) following the specifications included with GoTaq Master Mix (Promega Corporation, Madison, WI, USA) in 20 μ L reactions. Clean-up of polymerase chain reactions (PCRs) was performed with a QIAquick gel extraction (Qiagen, Inc., Germantown, MD, USA) with 35 μ L of the PCR product. We used the above primers for cycle sequencing and carried out reactions using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Waltham, MA, USA) with the default protocol. Cycle sequencing products were purified with the EDTA/sodium-acetate/ethanol protocol included with the BigDye kit and analyzed on an ABI Genetic Analyzer (Applied Biosystems).

We assembled, edited, and aligned sequences with Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA) and verified an open reading frame and absence of primer sequences. DNASP v5.10 (Librado and Rozas 2009) was used to estimate population genetic indices from mtDNA sequences, including number of haplotypes (H), mean number of bp differences (K), and mean nucleotide diversity (π) over the pooled data set and within each river. A 95% confidence parsimony network was created in PopArt v1.7 (Leigh and Bryant 2015), and multiple connections between haplotypes were simplified using methods described by Fetzner and Crandall (2003).

Fourteen of 15 microsatellite primers designed for *Lampisilis abrupta* (Eackles and King 2002) were successfully optimized in *L. powellii*. Forward primers for each PCR were labeled with a 5' fluorescent tag (6-FAM, NED, PET, or VIC) for visualization. We amplified microsatellite loci in 10 μ L reactions using GoTaq Master Mix (Promega), 0.5 μ M of fluorescently labeled forward and reverse primer, and 10 ng of DNA template carried out under the following conditions: initial denaturing at 94°C for 2 min; 35 cycles of 94°C for 40 s,

variable annealing temperature for 40 s (Appendix A1), 72°C for 1 min; and a final extension at 72°C for 5 min. We performed fragment analysis on an ABI 3730 Genetic Analyzer with LIZ600 size standard (Applied Biosystems). PEAKSCANNER v1.0 (Applied Biosystems) was used to score alleles, and TANDEM v1.07 (Matschiner and Salzburger 2009) was used to assign integer numbers to DNA fragment sizes.

We estimated null allele frequencies using the Brookfield I (1996) method as calculated in MICROCHECKER (van Oosterhout et al. 2004). We used GenAlEx v6.3 (Peakall and Smouse 2006) to estimate mean number of observed alleles per locus (N_A) and number of private alleles (N_P) and to calculate observed and expected heterozygosities (H_O and H_E) for each population. Allelic richness (A_R) was computed via rarefaction using FSTAT v2.9.3 (Goudet 1995) to estimate the total number of alleles in the population given the sample size. GENEPOP v4.0.10 (Rousset 2008) was used to conduct exact tests of pairwise linkage disequilibrium and to test whether genotype frequencies met Hardy–Weinberg expectation.

Population genetic structure was visualized by conducting a principal coordinate analysis (PCoA) in GenAlEx using a covariance matrix created from all polymorphic loci. A permutational multivariate analysis of variance (PERMANOVA; $n = 9999$ permutations) was used to determine the significance of the PCoA clusters utilizing the adonis function within the vegan package (Oksanen et al. 2017) in R (R Core Team 2016). FreeNA was used to assess the degree of genetic differentiation among rivers by calculating pairwise F_{ST} values employing the ENA (excluding null alleles) correction, which has been shown to effectively correct for the positive bias of F_{ST} that may result from the presence of null alleles (Chapius and Estoup 2007).

RESULTS

We successfully sequenced mitochondrial DNA for 29 individuals and recovered nine *COI* haplotypes with a length of 607 bp. Sequences are available on GenBank (accession nos. MT762680–MT762708).

Three clusters of haplotypes were present, separated by 34–46 mutational steps (>5% of total sequence length; Fig. 2). The first cluster consisted of a common haplotype found in 13 individuals from the Saline River and seven individuals from the Ouachita River, two individuals of a second haplotype limited to the Saline River that differed from the common haplotype by two bp, and a singleton haplotype from the Ouachita River that differed from the common haplotype by four bp. Our most common haplotype was identical to a GenBank *L. siliquioidea* haplotype from the upper Mississippi River (accession no. MK672781.1; see Inoue et al. 2019).

A second cluster contained three singleton haplotypes from two sites in the Saline River that differed from each other by four or five bp. These haplotypes differed from those in the first cluster by 34–39 bp and corresponded to *Lampisilis hydiana* sequences on GenBank (accession no. MK672683.1).

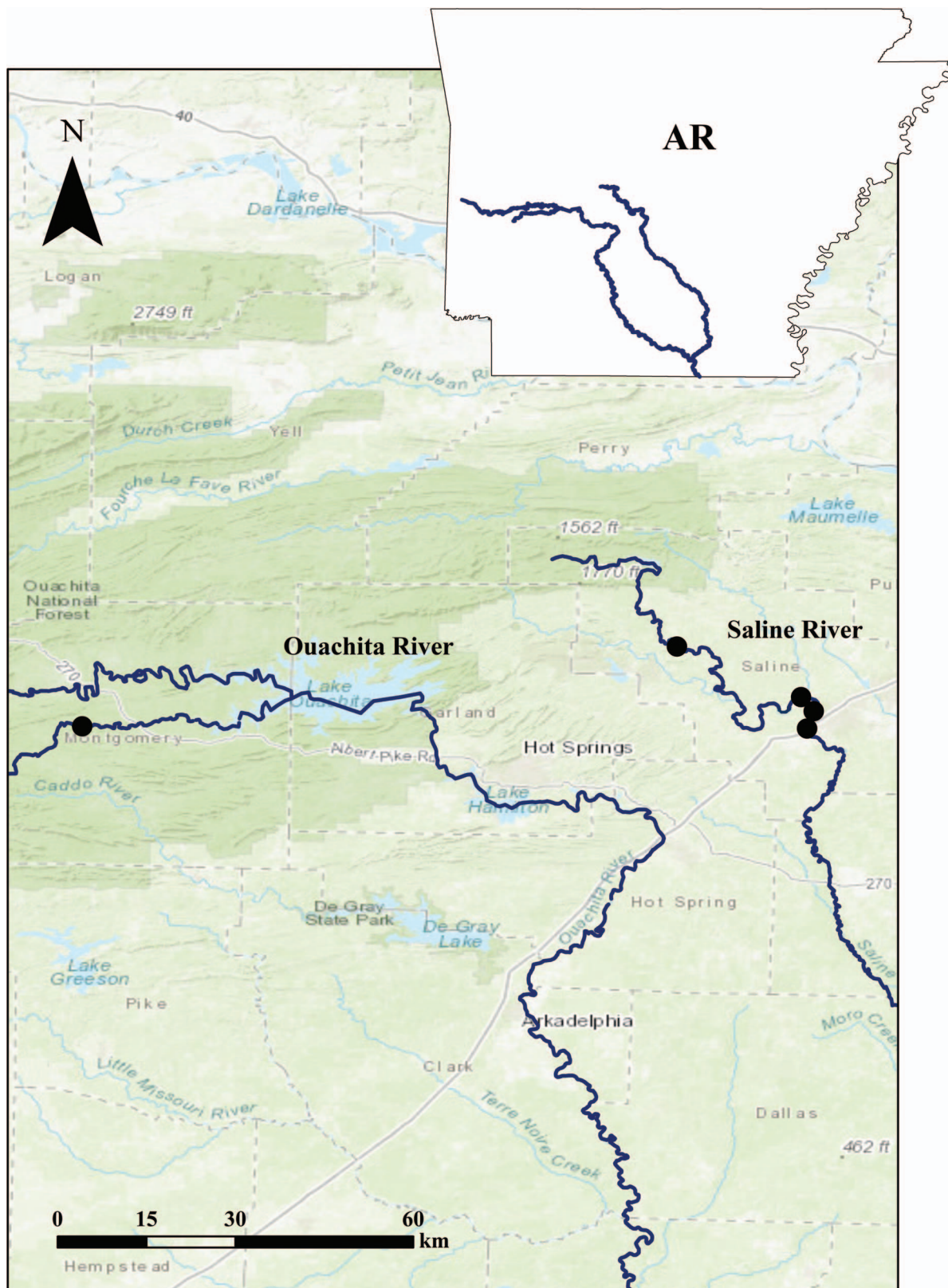


Figure 1. Sampling localities for *Lampsilis powellii* in Arkansas, USA.

Table 1. Summary statistics for *COI* sequences and 14 microsatellite loci for *Lampsilis powellii* in the Ouachita and Saline rivers, Arkansas, USA. Individuals identified as *Lampsilis hydiana* and *Actinonaias ligamentina* based on *COI* sequences are not included (see text). N = number of individuals sampled. Genetic diversity metrics are number of haplotypes (H), mean number of bp differences between all pairs of individuals (K), nucleotide diversity (π), mean number of observed alleles (N_A), rarefied allelic richness (A_R), mean number of private alleles (N_P), mean observed heterozygosity (H_O), and mean expected heterozygosity (H_E).

River	<i>COI</i>				Microsatellites				
	n	H	K	Π	N_A	A_R	N_P	H_O	H_E
Ouachita	8	2	0.75	0.001	5.77	3.82	3.21	0.42	0.68
Saline	15	2	0.25	0.000	9.07	4.97	6.50	0.44	0.75
Overall	23	3	3.00	0.002	7.43	4.30	NA	0.43	0.72

A third cluster contained three singleton haplotypes from the Saline River that differed from each other by six to nine bp. These haplotypes differed from those in the first cluster by 43–46 bp and were most similar to GenBank sequences for *Actinonaias ligamentina* (accession no. MK672757.1). We omitted from further analysis the six individuals in clusters two and three representing *L. hydiana* and *A. ligamentina*.

There was little genetic diversity among the 23 remaining *COI* sequences representing *L. powellii*, and patterns were similar for both rivers (Table 1). There were two haplotypes in each river, the mean number of base-pair differences between all individuals was three, and nucleotide diversity was low.

Microsatellite analyses also showed low genetic variation. We scored a total of 172 alleles over the 14 loci examined, ranging from four to 19 per locus (Tables 1 and 2). Microsatellite genotypes in GENEPOP format are deposited

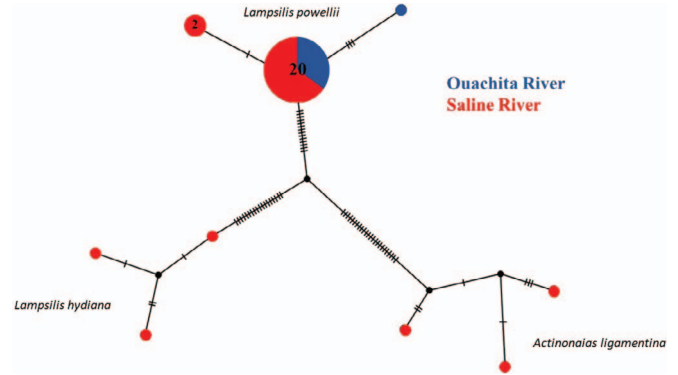


Figure 2. Haplotype network for samples identified as *Lampsilis powellii* derived from 607 bp region of the mitochondrial cytochrome *c oxidase subunit I* (*COI*) gene. Each circle represents a unique haplotype. The number within each circle represents the number of individuals having that haplotype; circles without numbers represent a single individual. Black dots and tick marks represent missing haplotypes.

in Dryad (<https://doi.org/10.5061/dryad.3r2280gd6>). Mean rarefied allelic richness and heterozygosity across the 14 loci were similar between rivers (Table 1). The mean number of private alleles was twice as high in the Saline than in the Ouachita. We found considerable evidence of null alleles (present at 82.1% of all river-by-locus pairs) and no evidence of linkage disequilibrium. Eighteen of 28 locus-by-river combinations deviated from Hardy–Weinberg expectations after Bonferroni correction, with all showing a heterozygote deficiency consistent with the presence of null alleles.

Populations of *L. powellii* showed little evidence of significant divergence. The first PCoA axis separated the two rivers but explained only 11.6% of total microsatellite

Table 2. Genetic diversity at 14 microsatellite loci in *Lampsilis powellii* from the Ouachita and Saline rivers, Arkansas, USA. N = number of individuals genotyped. Genetic diversity metrics are allelic richness (N_A), observed and expected heterozygosity (H_O and H_E), and null allele frequency. * indicates deviation of heterozygosity from Hardy–Weinberg expectation after sequential Bonferroni correction. Bold values indicate null allele frequencies that are significantly different from zero at $P \geq 0.05$.

Locus	Ouachita River					Saline River				
	N	N_A	H_O	H_E	Null allele	N	N_A	H_O	H_E	Null allele
LabC02	8	5	0.63	0.72	0.05	15	6	0.20*	0.49	0.19
LabC23	8	4	0.38	0.60	0.14	15	2	0.20	0.49	0.14
LabC24	8	3	0.38	0.57	0.12	15	4	0.47*	0.61	0.09
LabC67	8	3	0.88	0.62	0.00	15	11	0.40*	0.86	0.25
LabD10	8	7	0.50*	0.77	0.15	14	9	0.50*	0.83	0.18
LabD29	8	7	0.38*	0.80	0.23	14	10	0.50*	0.81	0.18
LabD31	7	6	0.14*	0.81	0.37	15	15	0.40*	0.92	0.27
LabD70	8	1	0.00*	0.00	0.00	15	6	0.27*	0.51	0.16
LabD71	8	7	0.25*	0.81	0.31	15	12	0.40*	0.89	0.26
LabD99	8	5	0.00*	0.70	0.40	13	10	0.23*	0.85	0.33
LabD111	8	11	1.00	0.87	0.00	15	15	0.93	0.92	0.00
LabD187	8	5	0.50	0.63	0.08	10	10	0.80	0.88	0.04
LabD206	8	6	0.13*	0.76	0.36	14	6	0.80	0.60	0.11
LabD213	8	11	0.75*	0.89	0.07	14	11	0.43*	0.88	0.24

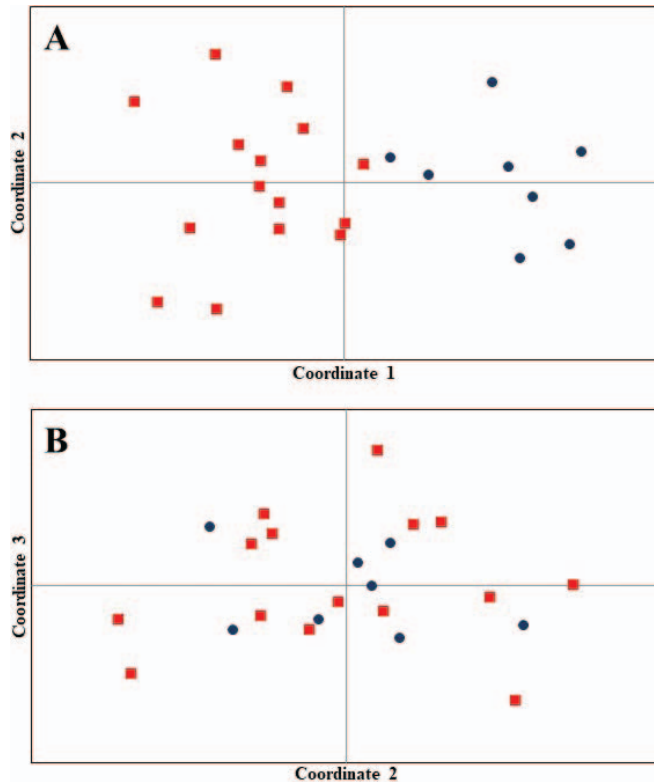


Figure 3. Results of principal coordinates analysis (PCoA) for 14 microsatellite loci of *Lampsilis powellii* from Arkansas, USA. Red squares represent individuals from the Saline River, and blue circles represent Ouachita River individuals.

variation (Fig. 3a). The second and third axes accounted for 20.5% and 27.3% of the variation, respectively; however, we could not infer any geographical patterns from these axes (Fig. 3b). The ENA-corrected estimate of pairwise F_{ST} among rivers was 0.076, while the PERMANOVA failed to reject the null hypothesis of no difference between rivers ($F = 1.72$, $P = 0.11$). The uncorrected estimate of F_{ST} (0.085) was similar to the ENA-corrected estimate; its slightly higher value was likely due to the presence of null alleles.

DISCUSSION

Lampsilis powellii seems to have maintained only a limited amount of intraspecific genetic diversity at both mtDNA and nDNA loci compared with other mussel taxa (Elderkin et al. 2007, 2008; Jones 2009). This limited amount of genetic diversity may be a result of the small sample sizes in our study, which is a common issue with rare species. However, the number of mtDNA haplotypes we found was lower than the number found in other endangered mussel species, even though sample sizes were comparable (Zanatta and Murphy 2008; Jones 2009; Menon et al. 2019). In the Ouachita River, *Cumberlandia monodonta* showed similarly low levels of mtDNA diversity with the presence of a single *COI* haplotype (Inoue et al. 2014b). Additionally, genetic diversity in *L.*

powellii was lower than in other unionid studies that employed the Eackles and King (2002) microsatellite primers (Kelly and Rhymer 2005; Menon et al. 2019). For example, genetic diversity was lower in *L. powellii* than in Great Lakes populations of *L. cardium* (N_A range: 3.3–13.7; H_O range: 0.42–0.85; Hewitt et al. 2019). Additionally, mean allelic richness was lower in *L. powellii* than for *L. siliquioidea* in the St. Clair River ($A_R = 8.7$ –11.4; Rowe and Zanatta 2015). The latter two comparisons are noteworthy because those populations are geologically young and colonized the Great Lakes region only after Pleistocene glaciation. Mussel populations in the Interior Highlands are likely much older and would be expected to contain higher genetic diversity. The low genetic diversity of *L. powellii* may reflect its small population size due to its long history of isolation compounded by anthropogenic habitat destruction and fragmentation.

The Interior Highlands of North America is a biodiversity hotspot with high endemism for a variety of aquatic organisms including mussels, fishes, crayfishes, and salamanders (Mayden 1985; Haag 2010). The region's distinctive aquatic fauna is believed to have been formed by vicariant pre-Pleistocene events, followed by repeated periods of isolation due to Pleistocene glaciations (Mayden 1988), resulting in genetically divergent populations. Not only do isolated populations with restricted gene flow lose genetic diversity, they also can exhibit an increase in population structuring (Paetkau et al. 1995). However, the limited genetic divergence between rivers that we found suggests a single panmictic population in relatively recent times. Despite the presence of the potential dispersal barrier of lowland habitats in the lower reaches of the Saline and Ouachita rivers, a mobile fish host may have allowed some dispersal across this barrier (e.g., Galbraith et al. 2015). Host fishes for *L. powellii* are currently unknown, but most *Lampsilis* are specialists on black basses (*Micropterus*), which can undertake substantial movements (Haag 2012; Schall et al. 2019). Identification of fish hosts of *L. powellii* is needed for inferring its potential dispersal ability and gene flow.

Interestingly, we were unable to differentiate *L. powellii* from *L. siliquioidea* *COI* sequences from the Mississippi River (Inoue et al. 2019). Whole mitochondrial genome sequencing identified a divergent protein-coding region, the *f-orf* gene, which distinguished between *L. siliquioidea* and *L. powellii* (Robicheau et al. 2018). However, when we examined the *COI* region of the whole mtDNA genome submitted to GenBank by Robicheau et al. (2018; accession no. NC_037720) and a partial *COI* gene sequence (Breton et al. 2011; accession no. HM849705) labeled *L. powellii*, they matched sequences identified as *L. hydana* from another study (Inoue et al. 2019). At this time, we are unable to make conclusions about the taxonomic distinctiveness of *L. powellii*. Because the available genetic data are limited to mtDNA sequences and a few microsatellite loci, a high-resolution genomic approach may be necessary to elucidate the taxonomic position of *L. powellii* among other *Lampsilis* species from this region.

Lampsilis siliquioidea and *L. powellii* are difficult to

distinguish morphologically (Harris et al. 2004; Harris et al. 2010; Krebs et al. 2013). Geometric morphometric analyses can be used to differentiate morphologically similar species (Inoue et al. 2014a; Riccardi et al. 2019), and this technique also may be useful for differentiating *L. powellii* from other co-occurring lamsilines from the Interior Highlands. The presence in our dataset of samples referable to *A. ligamentina* and *L. hydiana* from individuals originally identified in the field as *L. powellii* underscores the difficulty of identifying lamsiline species in this region of high evolutionary diversification.

Our results have important conservation implications. The lack of a clear genetic signal for differentiating *L. powellii*, *L. siliquioidea*, and *L. hydiana* shows that further research is necessary to evaluate the phylogenetic status of these species. Our results suggest that between-river genetic divergence in *L. powellii* is very low, although both populations contain private microsatellite alleles. However, our small samples sizes make this conclusion preliminary; population genomic methods are likely necessary to determine whether significant between-river divergence exists for *L. powellii*. Resolution of these issues is critical for designing conservation strategies for the small populations of *L. powellii* that remain in the Saline and upper Ouachita basins.

ACKNOWLEDGMENTS

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Appendix A1. Annealing temperature (T_A) for 14 microsatellite loci for *Lampsilis powellii*.

Locus	Primer Sequences	T_A (°C)
LabC02	F: ATGGACACCAGAAAGAAAAGG R: GAAGTCACAAGGTCAGGATCTC	52.9
LabC23	F: CAGTTGTTCCACTGTCGTAAAG R: TGGGACTAACATGGTGGTTAAG	59.1
LabC24	F: TGGACCTATTCTTGCTTGTTG R: GTTCTTTTCGCCTCCATGTATAG	59.1
LabC67	F: AGTCTCTGGCTCAACCAACTC R: CAAATCAATTACTGCCTTTTC	55.7
LabD10	F: TTGTATAAACGGTCATGGAAAAC R: CCGTGACCACTTCTTCTAAAAC	59.1
LabD29	F: TGTTCCTTAGTTTATATTTATGGTTTGC R: GCAGAAAATCTCCAGTTTATGG	55.7
LabD31	F: CTGCAGAACATCGAATGC R: AAATGACAACAAAGTGAGGTTATATG	59.1
LabD70	F: GACCGCCTCTTCTAAAACCTCTC R: AACATCGCTTTCCATTAATCAC	59.2
LabD71	F: GAAGGACATCAAGGTCAATCAG R: GGACACGGTCAAGTACAAAATAC	59.1
LabD99	F: TTGAATGAGTCACGAGTATTTAATG R: TTAAGAATCGAAAATGCTCAATC	55.7
LabD111	F: TGCATCAACTCTATTCCACAACC R: CAATGATAATGTAAATGTAAGCCTATC	55.7
LabD187	F: TCAAGTCTGCAATTTATTTTATGC R: TGATTCTTTCCCTAGAAAATACAAG	55.7
LabD206	F: AAGTGTAGAGGCAGAGAACTGAC R: TCACTGATACAGCATACATAATATAC	55.7
LabD213	F: ATACACAGGGTGCTCTAAATGC R: TTGCCAAAACAACATAGTTCC	59.1

NOTE

DISCOVERY OF THE BLUE RIDGE SPRINGSNAIL, *FONTIGENS OROLIBAS*, HUBRICHT, 1957 (GASTROPODA: EMMERICIIDAE) IN EAST TENNESSEE AND ITS CONSERVATION IMPLICATIONS

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ABSTRACT

The study of spring- and subterranean-associated microsnail species in the Appalachian karst region has focused disproportionately on the northern Appalachian Valley and Ridge (AVR), leaving many areas in the southern Appalachians unexplored. Consequently, biological inventories of subterranean habitats have been initiated in the southern AVR, particularly in the state of Tennessee. In 2013 and 2018, several previously unknown populations of a microsnail species were discovered from caves in eastern Tennessee. Through both morphological and molecular analysis, we identified these populations as the Blue Ridge Springsnail, *Fontigens orolibas*. These newly discovered populations represent a significant range extension of *F. orolibas*. As such, we reassess the conservation status of *F. orolibas* under NatureServe criteria and emphasize the need for further sampling efforts in the southern AVR for microsnails.

KEY WORDS: freshwater snails, microsnails, *Fontigens orolibas*, southern Appalachian region

INTRODUCTION

Currently, the freshwater snail genus *Fontigens* Pilsbry, 1933 is the only recognized North American group among the family Emmericiidae (subfamily Fontigentinae; Hershler

et al. 1990; Wilke et al. 2013). Compared with other hydrobioid microsnails (i.e., freshwater snails ranging from 2 to 5 mm of the family Hydrobiidae sensu lato; Davis 1979), most *Fontigens* species exhibit broad geographic distributions but are known primarily from karst landscapes of the central and eastern United States (Hershler and Holsinger 1990; Hershler et al. 1990; Culver et al. 2003). At present, there are 10 *Fontigens* species that are fully or partially restricted to springs and subterranean habitats (Hershler et al. 1990; Liu et al. in press). Six of these species are from the Appalachian karst region within the Appalachian Valley and Ridge (AVR) physiographic province (Hershler et al. 1990). Five of the six are endemic to the northern Appalachian karst in the states of Maryland, Pennsylvania, Virginia, and West Virginia (Fig. 1), where the majority of research efforts on aquatic subterranean-associated animals (i.e., stygofauna) has occurred (Holsinger et al. 1976; Holsinger and Culver 1988; Fong et al. 2007; Fong and Culver 2018). These five species are as follows: Appalachian Springsnail *Fontigens bottimeri*; Virginia Springsnail *Fontigens morrisoni*; Blue Ridge Springsnail *Fontigens orolibas*; Organ Cavesnail *Fontigens tartarea*; and Greenbrier Cavesnail *Fontigens turritella*. The other species known from the Appalachians—the Watercress Snail *Fontigens nickliniana* Lea, 1838—is the most geographically widespread of all *Fontigens*, occurring throughout the AVR, including in Tennessee, Alabama, and Georgia (TAG) karst, and into the northeastern and central USA. Comparatively, underground habitats in the southern extent of the Appalachian karst (or TAG) have been studied very

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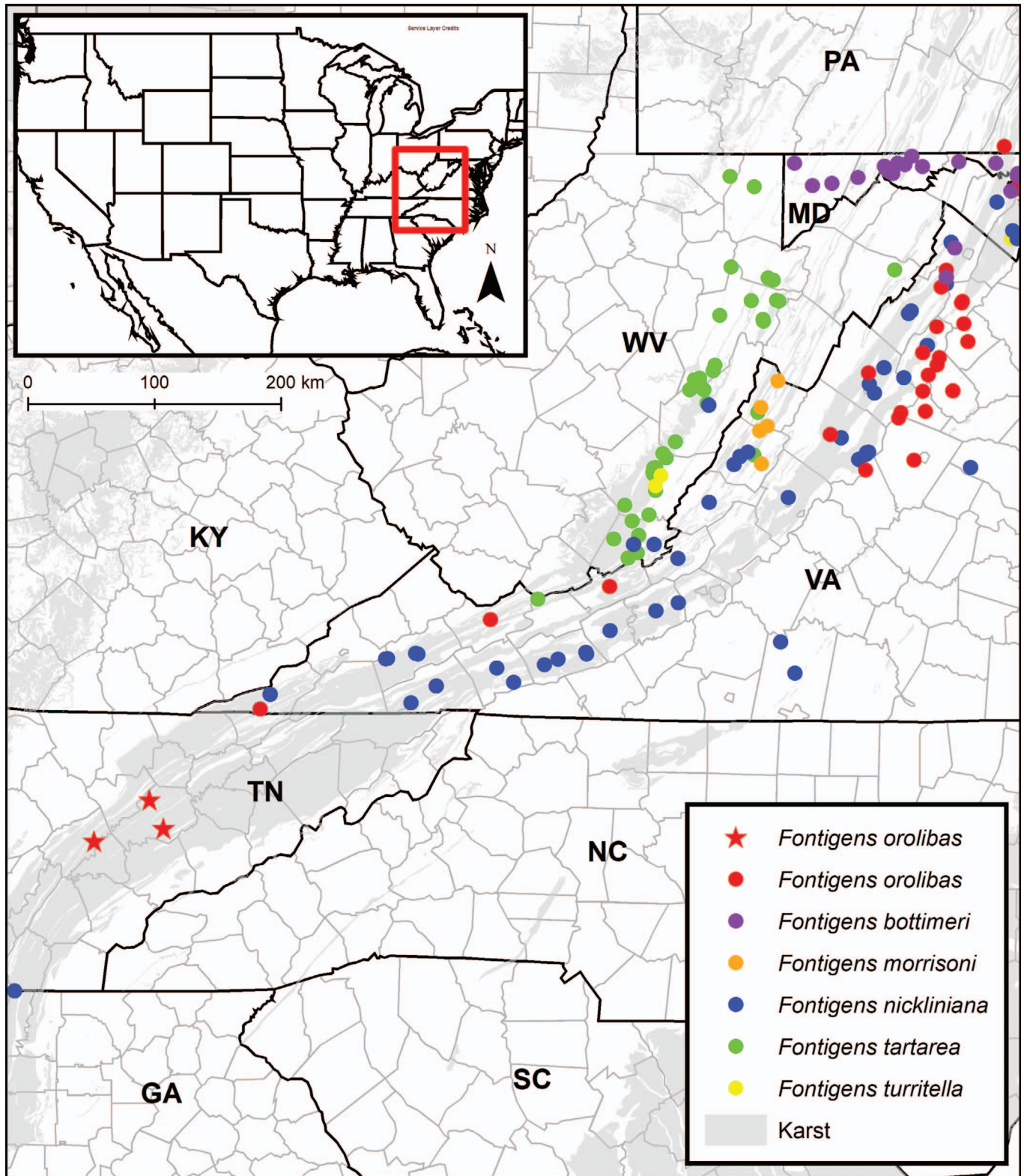


Figure 1. Geographic distributions of all *Fontigens* species occurring in the Appalachians. Newly discovered *Fontigens orolibas* populations are denoted with a star.

Table 1. Detailed site descriptions of Tennessee caves sampled in this study.

Tennessee									
Cave									
Survey no.	County	Cave name	Visitation	Personnel involved	Lithology	Cave description	Water depth	Benthic habitat	Watershed
TKN24	Knox	Cruze Cave	One trip May 2013, one monthly trip from March–October 2014, one trip June 2018, one trip February 2019	A.S. Engel, S. Engel, D. Fong, M.L. Niemiller, M.E. Slay, M.L. Porter, S. Keenan, S.J. Taylor, K.S. Zigler, A. Paterson, K. Brennan, T. Brown, A. England, N.S. Gladstone, E.B. Pieper	Ordovician Knox Dolomite	Main passage traversable for about 300 m with cave stream throughout	~0.1 to <0.25 m depth	Small to large rocks in sections of passage, small cobble and fine silt/sand in other sections	Melton Hill Lake watershed of the Clinch River, which flows into Watts Bar Lake and the Tennessee River
TKN103	Knox	Pedigo Cave	Two trips July 2018, one trip December 2018	N.S. Gladstone, E.B. Pieper, M.L. Niemiller	Cambrian Maynardville Limestone	Ca. 35 m of traversable passage, with stream flow in small room near cave terminus	2-m-deep pool	Fine silt, sand, and gravel mixed with larger cobble and smooth-faced rocks	Melton Hill Lake watershed of the Clinch River, which flows into Watts Bar Lake and the Tennessee River
TRN6	Roane	Eblen Cave	One trip May 2013, one trip December 2018	A.S. Engel, S. Engel, D. Fong, M.L. Niemiller, M.E. Slay, M.L. Porter, S. Keenan, S.J. Taylor, K.S. Zigler, A. Paterson, K. Brennan, T. Brown, A. England, N.S. Gladstone, E.B. Pieper, E.T. Carter, L.E. Hayter	Copper Ridge Dolomite	1,020 m of traversable passage, with 200 m of cave stream	<0.3 m deep	Large rocks, mixed with cobble/fine silt/sand throughout passage	Cave stream flows into Mill Creek on the surface, which is in the Clinch River watershed of the Tennessee River



Figure 2. *Fontigens orolibas* specimens. Top: Cave specimens collected from Cruze Cave (TKN24), Knox Co., Tennessee (1, 2); Pedigo Cave (TKN103), Knox Co., Tennessee (3); and Eblen Cave (TRN6), Roane Co., Tennessee (4). Photo credit: N.S. Gladstone. Bottom: Live *F. orolibas* from TKN103. Photo credit: M.L. Niemiller.

little (Niemiller and Zigler 2013; Niemiller et al. 2019). Only *F. nickliniana* has been described previously within TAG, from two spring sites.

Since 2012, ongoing biological inventories of cave systems in the AVR of eastern Tennessee have uncovered several new populations of freshwater snails from cave streams. These include recently described stygobiotic species of the genus *Antrorbis* from two caves (Gladstone et al. 2019). The others were of a *Fontigens*-like snail, with two populations being found within the Melton Hill Lake watershed of the Clinch River, which flows into Watts Bar Lake and the Tennessee River, and a third within the Clinch River watershed of the Tennessee River. Using morphological and molecular data, we diagnose these three new *Fontigens*-like snail populations as the Blue Ridge Springsnail *F. orolibas*. In light of confirming these new populations as *F. orolibas*, we also reassess the conservation ranks of this species using NatureServe criteria (Master et al. 2009).

METHODS

Field Sampling

All biological surveys involved at least two, and as many as 12, researchers. Scientific research and collection in the caves were permitted, with renewals, by the Tennessee Department of Environment and Conservation (TDEC) and the Tennessee Wildlife Resource Agency (TWRA) (TDEC number 2013-026 and TWRA number 1605). Table 1 presents the timeline of population discovery and site summaries. A single population was first discovered in 2013 from a cave system in Knox Co., Tennessee (TKN24), and other populations were found in a different cave in Knox Co. (TKN103) and in a cave in Roane Co. (TRN6), Tennessee (Fig. 1). Monthly surveys were performed at the original locality (TKN24; physicochemical measurements, abundance data, and microhabitat descriptions can be found in Keenan et al. [2017]). We report only individual Tennessee Cave Survey

Table 2. Mean shell measurements (mm) for specimens examined from each locality. Standard deviations in parentheses. * represents populations with measurements from Hershler et al. (1990).

Locality	NW	SH	SW	W	D	T	AS
Cruze Cave, Knox Co., Tennessee ($n = 24$)	4.31 (0.13)	1.97 (0.09)	1.44 (0.07)	1.25 (0.09)	0.55 (0.06)	6.03 (0.93)	1.22 (0.05)
Pedigo Cave, Knox Co., Tennessee ($n = 4$)	4.56 (0.19)	1.83 (0.11)	1.32 (0.07)	1.3 (0.07)	0.58 (0.05)	6.71 (0.76)	1.38 (0.07)
Eblen Cave, Roane Co., Tennessee ($n = 3$)	4.42 (0.24)	2.08 (0.14)	1.51 (0.11)	1.35 (0.16)	0.61 (0.02)	7.14 (1.01)	1.26 (0.05)
*Hawksbill Shelter, Madison Co., Virginia ($n = 10$)	4.58 (0.36)	2.53 (0.15)	1.46 (0.10)	1.27 (0.08)	0.56 (0.04)	5.57 (0.85)	1.24 (0.05)
*Blue Ridge Parkway, Augusta Co., Virginia ($n = 9$)	5.03 (0.08)	3.27 (0.17)	1.69 (0.08)	1.23 (0.07)	0.50 (0.08)	7.61 (1.10)	1.39 (0.13)
*Witheros Cave, Bath Co., Virginia ($n = 13$)	4.23 (0.07)	1.63 (0.10)	0.99 (0.05)	1.39 (0.08)	0.55 (0.03)	5.41 (0.78)	1.22 (0.08)
*Tawneys Cave, Giles Co., Virginia ($n = 10$)	4.40 (0.18)	2.07 (0.12)	1.20 (0.06)	1.44 (0.35)	0.57 (0.03)	6.04 (0.80)	1.21 (0.04)
*Harveys Cave, Giles Co., Virginia ($n = 9$)	4.22 (0.23)	1.98 (0.14)	1.14 (0.10)	1.40 (0.12)	0.65 (0.08)	7.44 (1.20)	1.23 (0.08)
*Indian Run Shelter, Rappahannock Co., Virginia ($n = 12$)	4.25 (0.00)	2.45 (0.10)	1.38 (0.06)	1.35 (0.09)	0.62 (0.05)	6.22 (0.74)	1.18 (0.05)
*Browntown Valley Overlook, Warren Co., Virginia ($n = 10$)	5.03 (0.08)	2.76 (0.22)	1.42 (0.09)	1.23 (0.06)	0.62 (0.08)	6.40 (0.77)	1.26 (0.10)

NW = number of whorls; SH = shell height; SW = shell width; W = whorl expansion rate; D = distance of generating curve; T = translation rate; AS = aperture shape.

(TCS) inventory numbers rather than exact geographic coordinates, but cave system descriptions and location data are maintained by the TCS (<http://www.subworks.com/tcs/>) and are available from the authors upon request.

Morphological Analysis

Specimens collected from each site were preserved in 100% ethanol and transferred to the laboratory for morpho-

logical analysis and imaging. Upon completing the re-evaluation of these materials, the specimens were deposited in the Auburn Museum of Natural History. We used a Jenoptik SUBRA full high-definition microscope camera to photograph and evaluate shells (Fig. 2). Standard shell measurements included in Hershler et al. (1990) were recorded for specimens, including number of whorls, shell height, shell width, whorl expansion rate, distance of generating curve, translation rate, and aperture shape (Table 2). We also compared the shell

Table 3. Sequence and locality information. All *Fontigens* sequences without a GenBank accession number were generated and provided by Liu et al. (in press). Numeric code next to locality information represents population identification shown in phylogeny.

Taxon	Locality	GenBank accession
<i>Fontigens antroecetes</i>	Stemler Cave, St. Clair Co., Illinois (22)	MT425002
<i>Fontigens bottimeri</i>	Wetzels Spring, Washington, District of Columbia (6)	MT425003
	Ogden's Cave, Frederick Co., Virginia (11)	MT425004
<i>Fontigens cryptica</i>	Spring in the Bernheim Cedar Grove Wildlife Corridor, Bullitt Co., Kentucky (12)	MT425005
<i>Fontigens morrisoni</i>	Spring at Mustoe, Highland Co., Virginia (13)	-
	Martin Fen, LaGrange Co., Indiana (3)	MT425007
	Blowing Springs, Bath Co., Virginia (14)	MT325008
<i>Fontigens nickliniana</i>	Spring at Lantz Mills, Shenandoah Co., Virginia (15)	MT425015
	Fleenor Spring, Washington Co., Virginia (16)	MT425020
	Kalamazoo, Michigan	JX970609
	Cruze Cave, Knox Co., Tennessee	TBD
	Hawksbill Spring, Page Co., Virginia (17)	MT425028
<i>Fontigens orolibas</i>	Spring at the Humpback Visitor Center, Augusta Co., Virginia (18)	MT425029
	Hugh Young Cave, Tazewell Co., Virginia (19)	MT425030, MT425031
<i>Fontigens tartarea</i>	Organ Cave, Greenbrier Co., West Virginia (1)	MT425032, MT425033
<i>Bithynia tentaculata</i>		MK308073
<i>Bythinella austriaca</i>		FJ028979
<i>Bythinella pannonica</i>		HQ149623
<i>Bythinella viridis</i>		FJ029102
<i>Emmericia expansilabris</i>		KC810061

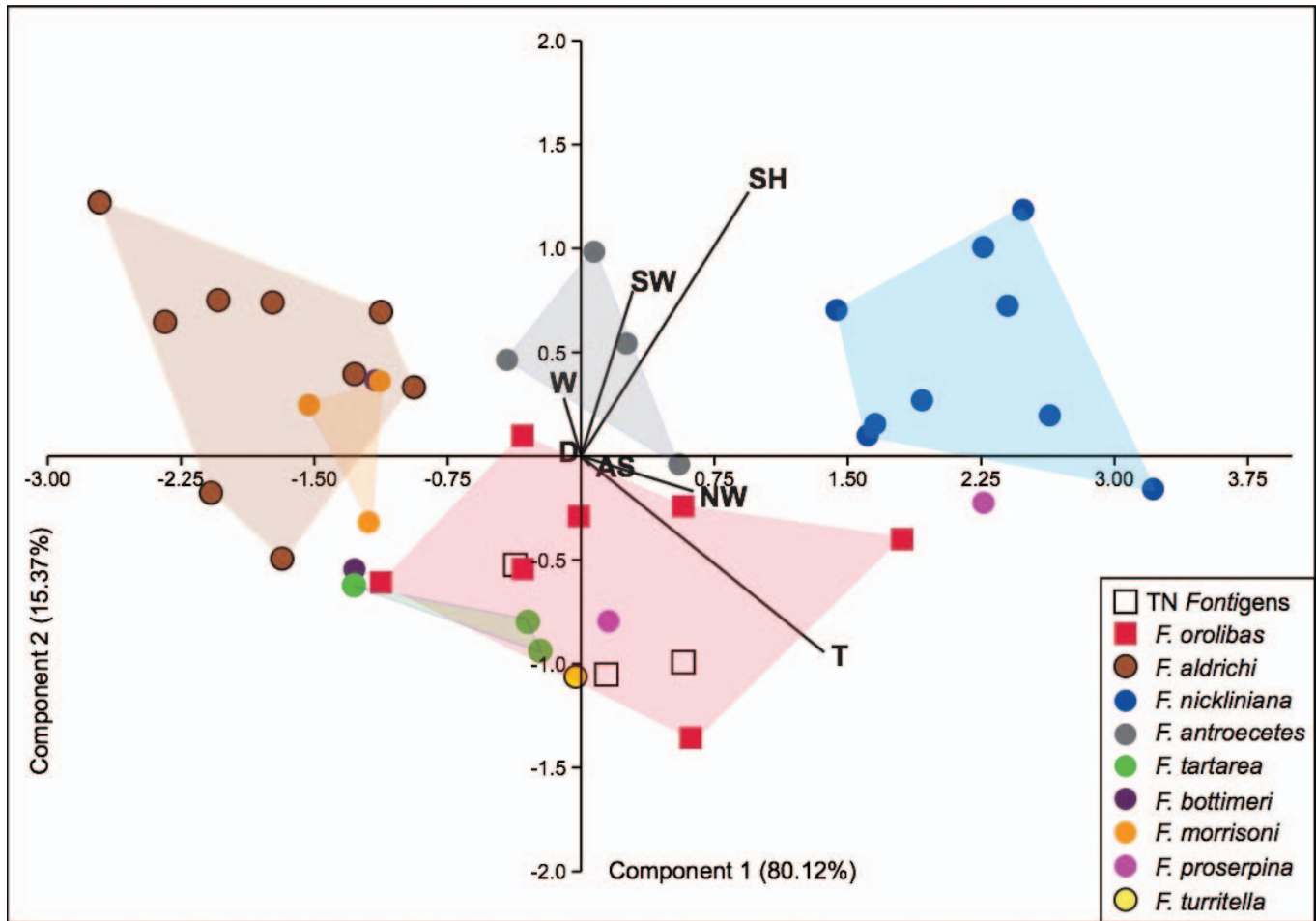


Figure 3. Principal components analysis of *Fontigens* spp. morphological data from Hershler et al. (1990) and this study. The Tennessee *F. orolibas* specimens are shown with an open square. Each point represents an individual specimen. Component 1 accounts for 80.12% of variability and component 2 accounts for 15.3% of variability. Other abbreviations defined in the text.

measurements of all individual *Fontigens* species included in Hershler et al. (1990) with three specimens from the TKN24 site via principal components analysis (PCA) with paleontological statistics software (Hammer et al. 2001). All seven shell measurements were included as components in the analysis and each individual specimen presented by Hershler et al. (1990) is included separately.

Molecular Methods and Phylogenetic Analysis

Genomic DNA was isolated from three specimens from the TKN24 population using the Qiagen DNeasy blood and tissue kit, following the manufacturer's protocol. We amplified a 638-base-pair fragment of mitochondrial cytochrome oxidase subunit 1 (CO1) locus using LC01490 and HC02198 primers (Folmer et al. 1994). PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and sequenced in both directions with BigDye chemistry at Eurofins MWG Operon (Louisville, KY, USA). Forward and reverse sequences were quality trimmed at the ends and assembled into contigs in DNA Baser v4.36 (Heracle BioSoft)

and aligned using MAFFT (Katoh and Standley 2013). These sequences were then compared with CO1 sequences from several other *Fontigens* species generated by Liu et al. (in press) (Table 3).

The CO1 phylogeny was generated using a maximum-likelihood approach in IQ-TREE 2 (Minh et al. 2020) with the model-testing function to infer the best-fit substitution model for each codon partition under the corrected Akaike's information criterion. We implemented a general time-reversible model with corrections for a discrete gamma distribution (GTR + Γ) for the first and second codon positions, and the Hasegawa, Kishino, and Yano model with a discrete gamma distribution and a proportion of invariant sites (HKY + Γ + I) for the third codon position. Branch support was assessed with 10,000 ultrafast bootstrap replicates (Hoang et al. 2017).

Conservation Status Reassessment

On the basis of the taxonomic identity of these populations as *F. orolibas*, we reassessed the conservation statuses of the

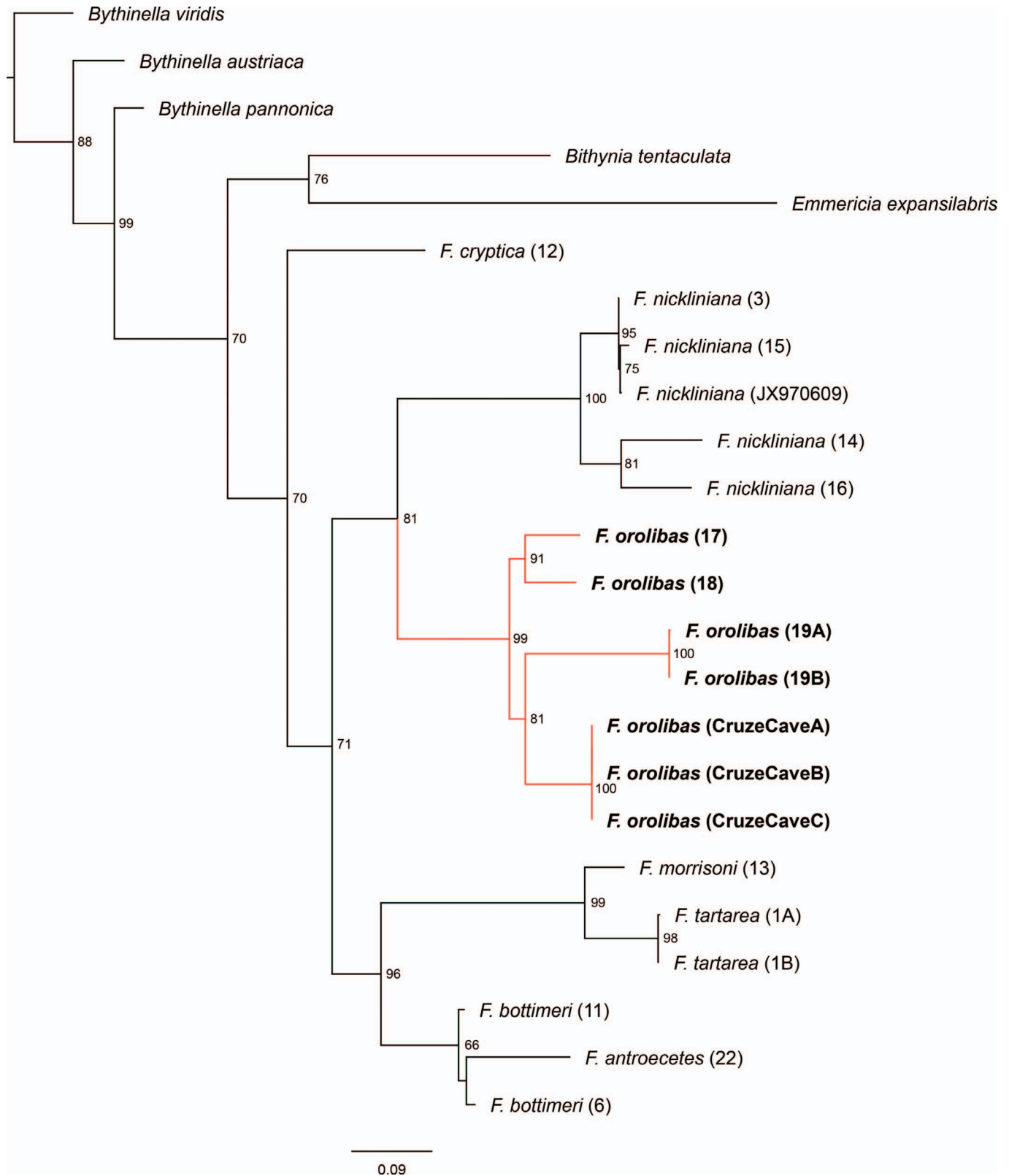


Figure 4. Mitochondrial cytochrome oxidase subunit 1 (CO1) phylogeny of *Fontigens* species with sequence data generated from this study and by Liu et al. (in press). Internal values represent bootstrap support and the scale bar represents branch length in units of sequence divergence. The species *Bythinella viridis* was rooted as the outgroup. Location identification numbers are shown to the right of the species name and listed in Table 3.

species using NatureServe criteria (Master et al. 2009). Conservation status, as designated by NatureServe, is calculated on the basis of several risk categories, including range/distribution, abundance/population condition, threat impacts, and population trends (for details on calculation procedure, see Faber-Langendoen et al. 2012). Each risk category as assessed through NatureServe was calculated with the NatureServe Rank Calculator v3.1932 (Faber-Langendoen et al. 2012). Abundance data for *F. orolibas* are virtually nonexistent, but the earlier efforts of Keenan et al. (2017) represent the first systematic survey of a single population to date. Consequently, population trends and viability information could not be completely assessed for the species. Geographic range size (as assessed by the geographic coordinates associated with each population) was calculated as extent of occurrence (EOO) and area of occupancy (AOO) using the web-based program GeoCAT (Bachman et al. 2011). To determine potential threats to *F. orolibas* throughout its range, we identified whether populations occurred on state or federally protected lands using the U.S. Geological Survey Protected Areas Database v1.3 (shapefile available at <http://gapanalysis.usgs.gov/padus/>). For each known population, we also examined history of disturbance of each site (if known), the adjacent human population density according to the 2018 U.S. Census from the U.S. Census Bureau (TIGER/Line®), and land cover associations according to the 2016 National Land Cover Database (Yang et al. 2018).

RESULTS AND DISCUSSION

Shell measurements from all new populations in Tennessee showed considerable similarity with *F. orolibas* shells measured in Hershler et al. (1990). Results of the PCA also showed that our three *Fontigens* specimens from the TKN24 populations are within the range of morphological variability of *F. orolibas*. Principal component 1 accounts for 80.12% of variability and is highly influenced by translation rate, shell height, and number of whorls. Separation of taxa along principal component 2, which accounts for 15.3% of observed variability, is influenced by changes to shell height and shell width (Fig. 3). The resulting phylogeny shows 99% bootstrap support at the internal node for all *F. orolibas* individuals including the TKN24 population (Fig. 4), further supporting the species identification. These combined results confirm that the population at TKN24 is indeed *F. orolibas*, and as such we infer that the other two newly discovered populations are also *F. orolibas* on the basis of geographic proximity and near morphologic indistinguishability (Figs. 1, 2).

The population discoveries in eastern Tennessee extend the known geographic range of *F. orolibas* to 26 localities from 19 counties in four states and increase the EOO to 55,631 km² and the AOO to 124 km². The current NatureServe rank of *F. orolibas* is Vulnerable (G3), and our reassessment does not change this status. Despite a significant increase to its known range, there persists a near complete lack of information regarding threats, baseline monitoring of population trends,

and population viability for this species. This information deficiency is likely due to the difficulty of surveying for and studying *F. orolibas* populations, making it problematic to identify both overarching and population-specific threats that could be mitigated by land conservation or management efforts. However, although no direct threat assessments have been reported for *F. orolibas*, freshwater snails restricted to such subterranean or spring habitats are broadly considered at elevated risk of local extirpation or extinction owing to habitat degradation such as groundwater pollution or extraction (Lysne et al. 2008; Hershler et al. 2014). Currently, eight of the 26 known localities of *F. orolibas* are within federally protected lands (i.e., Shenandoah National Park in Virginia), and these populations are most likely secure. Though almost all previous collection events of *F. orolibas* in its northern range are not accompanied by formal reports of abundance, the findings of Keenan et al. (2017) suggest that high population densities can occur within cave ecosystems, with the TKN24 site continuing to have hundreds of individuals throughout the cave stream since it was last visited in 2019 (N.S. Gladstone personal observation).

Some localities occur in areas of high human population density and increased urbanization, with ~27% of *F. orolibas* populations occurring within 5 km² of urban land cover, including two of the three new localities reported here. Both newly discovered cave populations in Knox Co., Tennessee are immediately adjacent to suburban neighborhoods, and both have increased levels of pollution and other forms of habitat degradation (Keenan et al. 2017). Given the lack of sampling within the AVR in eastern Tennessee, it is likely that other *F. orolibas* populations exist within the updated range extent and into the southern AVR. We hope the discovery of these previously unreported populations promotes further study of this species.

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