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Evaluating the Effectiveness of Freshwater Mussel Mitigation Strategies: Final Report

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16. Abstract
Relocation of mussels is applied as a mitigation strategy for potential impacts on threatened mussel species resulting from bridge construction or repairs. Demonstrably efficient surveys are required to document the distribution and abundance of mussels within the potentially impacted area to enable relocation. There is increased interest in propagation of threatened mussels in Texas as a potential conservation method or potential alternative mitigation strategy, but this requires more life history and genetic information. This project examined different aspects of mussel mitigation strategies by examining (1) and comparing the effectiveness of survey methods at six sites in rivers of Central Texas; (2) the seasonality of vertical migration; (3) the reproductive ecology of Lampsilis bracteata, its seasonality in brooding and gamete production and suitability of different host fish species from different sources; (4) the seasonality of gamete production of Cyclonaias species; and (5) the genetic structure of wild populations and providing crucial information for augmentation efforts and genetic management.

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Executive Summary

Relocation of mussels is applied as a mitigation strategy for potential impacts on threatened mussel species resulting from bridge construction or repairs. Demonstrably efficient surveys are required to document the distribution and abundance of mussels within the potentially impacted area to enable relocation. There is increased interest in propagation of threatened mussels in Texas as a potential conservation method or potential alternative mitigation strategy, but this requires more life history and genetic information. This project examined different aspects of mussel mitigation strategies by examining (1) and comparing the effectiveness of survey methods at six sites in rivers of Central Texas; (2) the seasonality of vertical migration; (3) the reproductive ecology of *Lampsilis bracteata,* its seasonality in brooding and gamete production and suitability of different host fish species from different sources; (4) the seasonality of gamete production of *Cyclonaias* species; and (5) the genetic structure of wild populations and providing crucial information for augmentation efforts and genetic management.

The comparison of survey methods showed that the transect method provided density estimates at a considerably lower effort compared to the adaptive cluster method, but it was most likely to miss species. The timed searches generally were most effective in detecting species and mussels per unit search effort, but they were biased towards larger and more sculptured mussels and those species that were found to burrow less deeply. Field observations showed that burrowing behavior differed not only between species, but also between substrates and the biggest differences were found between seasons. The performance of survey methods also varied with local habitat conditions, and the overall results suggested that to design effective surveys variation in detectability of mussels must be considered which depends on local habitat conditions, behavior, size and morphology of mussels, as well as experience of surveyors.

Monitoring of the reproductive ecology of threatened mussels in the San Saba and Llano River showed that the proportion of female *L. bracteata* brooding tended to be lower in the summer and the fall, and higher during winter and spring months before peak water temperatures were reached. Similarly, lower gamete densities of *C. petrina* occurred during summer, coinciding with higher temperatures. Fecundity and glochidia viability of *L. bracteata* were higher in the Llano River population compared to the San Saba population and the reproductive output of *Cyclonaias* species appeared to be more limited in the San Saba population due to

several potential stressors, including higher temperature and trematodes. Further research will need to investigate the potential impacts that trematodes and other parasites might have on the long-term persistence of these mussel populations and how they are interacting with other stressors in the system.

Highest metamorphosis success of glochidia to juvenile *L. bracteata* occurred on wild Green Sunfish and Largemouth Bass, and hatchery Largemouth and Guadalupe Bass. Average metamorphosis success was higher for some mussel-fish pairings originating from the same tributary, suggesting that mussels may be locally adapted to host fish, which should be considered in conservation and propagation efforts.

We genetically tested samples collected during surveys across five river drainages to confirm morphological identifications, measure genetic diversity, and detect genetic structuring. We also use these data to estimate the number of females needed for captive propagation efforts. The genetic analysis identified distinct groups and clades within *Cyclonaias* and also showed variation being partitioned by river basin, which should be considered in captive propagation efforts. Genetic analyses can increase the efficiency of conservation and management analyses, but the lack of reference sequences for the taxa encountered in Texas need to be addressed. In addition, managers should use all relevant data to designate Evolutionary Significant Units.

Recommendations:

1. Mussel surveys should be done by well-trained surveyors with timed searches always used as a first step to assess species presence and overall distribution of mussels at a site, followed by a quantitative method if density estimates or other population parameters need to be assessed.
2. Surveys should be accompanied by taking non-intrusive genetic samples especially of species for which the taxonomy is currently in flux or for which morphology is not a reliable tool for identification.
3. Further research into ecological and genetic differences of populations are necessary to inform conservation and mitigation strategies.

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1. Detectability affects the performance of survey methods – A comparison of sampling methods of freshwater mussels in Central Texas

by
Brittney Sanchez and Astrid Schwalb

Abstract

Designing effective surveys for freshwater mussels (Unionidae) is a challenge, because they are spatially clustered and often found in low densities. The objective of this study was to examine how the effectiveness of three different survey methods (timed searches, transect method, and adaptive cluster method) varied between different habitats at six sites in the San Saba, Guadalupe, and San Antonio Rivers in Central Texas. Species richness, the total number of mussels per search effort, species composition and size distribution obtained with different survey methods were compared between sites. Timed searches were generally the most effective method in detecting species especially when densities were low ($\leq$ 0.2 individuals per m^2) or mussels were highly clustered. The adaptive cluster method, however, was as effective as timed searches in detecting species when densities were moderate or higher (>2 ind. per m^2) and detected more species than timed searches at a site at which habitat conditions hindered searches. The performance of adaptive cluster in respect to number of mussels found per unit search effort seemed to be enhanced by sandy substrate facilitating the detection of mussels, and timed searches were less effective at sites at which habitat conditions hindered the detectability of mussels. Differences in detectability of mussels was not only associated with habitat conditions, but also with the size of mussels, their behavior and morphology. Timed searches detected a larger proportion of larger mussels that tended to be less burrowed and that had shells with more sculpturing compared to quantitative methods. In addition, surveyors with more search experience detected a larger number of mussels. Our results suggest that to design effective surveys variation in detectability of mussels must be considered which depends on local habitat conditions, experience of surveyor, behavior, size and morphology of mussels.

Introduction

Freshwater mussels can play an important role in the functioning of freshwater ecosystems affecting water clarity and chemistry by filtering water, providing physical habitat for other organisms, and enhancing benthic algae and macroinvertebrates (Vaugh et al. 2008). However,

populations of freshwater mussels have globally declined (Lopes-Lima et al. 2014). Globally, the highest diversity of Unionida exists in North America, where they are one of the most imperiled group of organisms (Haag 2012); Texas alone is home to approximately 50 native unionid species (Burlakova et al. 2011). Currently, Texas Parks and Wildlife Department (TPWD) has listed 15 of these species as threatened and one species (*Popenaias popei*) has been listed as endangered under the Endangered Species Act (Texas Register 35 2010). Declines of freshwater mussels in Texas and elsewhere in North America have been attributed to habitat loss and degradation, pollution, dewatering (groundwater pumping), and to impact of dams (Burlakova et al. 2011, Haag 2012, Inoue et al. 2014, Randklev et al. 2015).

A critical part of the successful conservation of mussel communities is a reliable account of their distribution and abundance. Mussels have a highly patchy distributions and rare species often occur at low densities (Strayer 1999, Pooler and Smith 2005, Strayer and Smith 2003, Dickson 2000). Designing an efficient sampling scheme for rare and clustered populations is challenging in general (Salehi and Smith 2005), and the patchy nature of mussel populations in particular presents substantial challenges to field sampling for population enumeration and species detection, and the costs associated with these efforts in terms of person-time and effort can be expensive. Currently, there is no standardized and accepted protocol for sampling mussels in Texas. There are, however, a number of studies which have described and compared different sampling methods for uninoids (e.g., Hornbach and Deneka 1996, Vaughn et al. 1997, Obermeyer 1998, Mecalfe-Smith et al. 2000, Smith et al. 2001, Villella and Smith 2005). Timed searches are semi-quantitative, less expensive and less time consuming than quadrat sampling and provide quick exploration of larger areas and a variety of habitats (e.g., Metcalfe-Smith 2000). Several studies have found that timed searches tend to have higher rates of detection of rare species compared to quantitative searches (Hornbach and Deneka 1996, Vaughn et al. 1997, Strayer et al. 1997, Obermeyer 1998, Smith et al. 2001), but also larger individuals and species (Hornbach and Deneka 1996, Vaughn et al. 1997, Obermeyer 1998) and mussels with sculptured shells (Miller and Payne 1993, Vaughn et al. 1997, Obermeyer 1998). It is crucial to set an adequate search time to obtain reliable estimates of mussel community composition (e.g., Metcalfe-Smith 2000), which can differ substantially between different habitat types and conditions (Smith et al. 2000).

1. Comparison of survey methods

To obtain density or demographic data quantitative methods need to be used (Vaughn et al. 1997, Dickson 2000), but these methods may underestimate unionid species richness with the exception of very small mussel beds (Vaughn et al. 1997, Hornbach and Deneka 1996). Because the patchy distribution of mussels, a potentially large number of sampling units (e.g., quadrats) are needed to obtain relatively acceptable levels of precision e.g., for mussel density (Dickson 2000). Thus, random quadrat searches are often considered inferior to transect methods for estimation of some population-level parameters (Dickson 2000), which allow for quicker and more efficient searches than quadrats (Strayer and Smith 2003). In addition, excavation of materials in each quadrat is often necessary to obtain precise density estimates (Strayer and Smith 2003) because a considerable proportion of mussels may be completely burrowed (Schwalb and Pusch 2007). Adaptive cluster sampling is a different quantitative method, which allows investigators to concentrate their efforts where mussels occur, which is useful for populations that are rare and clustered (Salehi and Smith 2005, Smith et al. 2009). For instance, if one or more mussels is found in a quadrat, the four adjacent units quadrat areas of that quadrat are then searched. If mussels are found in any of those adjacent quadrats then their adjacent quadrats are search allowing the direction of searches to focus where mussels are.

Not all methods will work under different habitat conditions and surveys may not accurately measure mussel richness or miss rare endangered species (Strayer 2008). Thus, different habitat types may require different survey methods (Burlakova et al. 2011). The objective of this overall thesis was to evaluate the relative effort and effectiveness of three different unionid mussel survey methods (timed searches, transect method, and adaptive cluster method) and to examine how their effectiveness vary in different habitats in Texas rivers. Based on a review of the literature, we developed the following predictions: (1) Timed searches will be more effective compared to quantitative methods in detecting species presence (particularly rare species) and in finding a larger number of mussels (per unit search effort) especially when density is low and/or distribution is highly clustered. (2) Adaptive cluster will be more effective than transect method when patchiness is high, and density is low. (3) Adaptive cluster and Transect methods will detect smaller individuals and smaller species than timed searches. (4) Precision in density estimates will increase as the number of quadrats increases.

1. Comparison of survey methods

Methods

Sites

Field studies were conducted at six riverine sites in the central Texas region: Guadalupe River (two sites), San Antonio River, Llano River, and the San Saba (two sites) River between Fall of 2016 and Summer 2017 (Fig. 1.1; Table 1.1). These sites covered three different ecoregions: Edwards Plateau (Llano and San Saba Rivers,), South Texas Plains (San Antonio River), and the Western Gulf Coast Plains (Guadalupe River).

Study sites varied in size so and the same area of stream (50 m stream length and 10 m width) was used to perform the following sampling methods on the six sites:

A. Timed search Method

B. Transect Method

C. Adaptive Cluster Method

Sites were visited weekly or biweekly to allow the mussels to settle back into their habitat after surveys, and to ensure similar seasonal conditions. There were two exceptions to this sampling schedule caused by high water levels preventing access to the field sites. At the Llano River the third sampling was only possible two months after the second sampling, and in the San Antonio River the second sampling was performed five months after the first sampling (Table 1.1). A different order of methods was applied to each site to avoid potential bias (Table 1.1). All unionids contained within the area were collected, identified, enumerated and then returned to the riverbed to the approximate spot in which they were found.

Timed Search Sampling Method

Three surveyors initiated sampling of the downstream boundary of each study reach and moved together upstream covering as much habitat as possible within the entire site. Sites were searched using waders, wet suits, snorkels, underwater viewers, diving and weight belts were used in deeper waters to snorkel at the bottom, and mussels were collected in mesh bags. After each person-hour (p-H, number of people multiplied by time) it was determined whether new species were found or not. Timed searches were continued until no new species were found for three consecutive 1-p-H. In addition to these data, on one date (May 3, 2017) in the San Antonio site I recorded how many mussels were found by each surveyor (n = 3 surveyors) with varying experience to examine if the number of mussels found by a surveyor varied with the amount of

previous experience searching for mussels (previous experience ranged from 3 months to 2 years).

Transect Method

To conduct a transect search method, nine 10m transects were set-up perpendicular to the flow at 5m intervals along 50 m stream length at each study site. At each transect five quadrat samples were taken. A 50cm x 50 cm quadrat was used for all sites except for the Llano and Guadalupe 2 site, for which 1m x 1m and 25cm x 25cm quadrats was used respectively (due to lower density at the former and higher densities at the latter site). During searches, substrate in each quadrat was excavated to a depth of up to 10 cm.

Adaptive Cluster Method

To conduct this search methodology, the entire search area of each site was divided into equal non-overlapping quadrat locations. The quadrat size used for each site was the same as the transect method (see above). Initially, three quadrat locations were chosen randomly and searched for mussels. If one or more mussels was found in a quadrat, the four adjacent quadrat areas were then searched and this was repeated until a total of n = 45 quadrats transect method). On two occasions, no mussels were detected in the initial 3 quadrats (Guadalupe 1 and the Llano site), and 22 random initial quadrats were added. Again, substrate in each searched quadrat was excavated to a depth of up to 10 cm. The dominant substrate type at each site was observed and average velocity at 60% of stream depth in the middle of the stream was measured.

Data Analysis

Densities (mean number of mussels per m^2) were determined for each site based on the results of the transect method and was calculated for the adaptive cluster method for comparison. The Clumping Index (Cressie 1993) was used to examine differences in patchiness between sites, which is calculated as the variance/mean ratio -1 (of density estimates). To examine whether adaptive cluster method would result in significantly higher density estimates, a paired t-test was used. To determine the effect of using 4, 3, 2, and 1 quadrat per transect instead of 5, we used a bootstrapping approach, in which the dataset was re-sampled while restricting the number of sampled quadrats and repeated 1000 times. The coefficient of variation was calculated for each scenario.

1. Comparison of survey methods

Results

Habitat Conditions

Substrate, depth and flow conditions varied considerably between sites (Table 1.2). Site depth ranged from 0.45-1.7m, with the deepest sites being Guadalupe 1 and San Saba 1. Among sites, substrate conditions ranged from predominantly bedrock (Llano) with gravel filled divots scattered throughout the site, to substrates composed of sand and cobble mixtures (San Saba 2 and San Antonio). At the San Saba 1 site, a large woody debris from previous flooding in the deeper middle (~1m depth) section of the river hindered searches, but quadrat searches were still possible because flow was relatively slow. Water velocity was fastest at Guadalupe 1, and substrate was predominately cobble. Guadalupe 2 had slow flow and substrate was predominately sand (Table 1.2).

The Llano and Guadalupe 1 sites had the lowest density (i.e. average density ≤ 0.2 individuals per m^2 as determined by transect method). Moderate densities were found at the San Saba sites and the San Antonio River (1.3 to 2.1 ind. per m^2, transect method), whereas the Guadalupe 2 site had the highest mussel density (7.1 ind. per m^2, Table 1.2). In terms of the patchiness of the mussel populations within a site, the San Antonio had the highest patchiness (clumping index = 1.1), and all other sites had clumping indices <0.5. Density estimates obtained with adaptive cluster were significantly higher compared to the transect method ($T_5 = 2.2$, $p = 0.04$). The biggest difference was found at the Llano site (20 times higher; Table 1.3), but is should be noted that considerably more than 3 random starts were used a the two sites with the lowest density (Llano and Guadalupe 1). As no mussels were found at Guadalupe 1 after 25 random starts, search with the adaptive cluster was discontinued. Densities estimated with the adaptive cluster method were about 4 times higher than the transect method at the San Antonio and San Saba 2 sites, about 3 times higher at Guadalupe 2, and 2 times higher at San Saba 1 (Table 1.3).

Species Richness

As predicted, timed searches detected a higher number of species than the transect method; however, timed searches did not necessarily detect more species than the adaptive cluster method (Fig. 1.2). The greatest number of species detected with timed searches compared to the transect method was most pronounced at the two sites with the lowest density (<0.2

mussels/m^2). For example, 4 species were found at the Guadalupe 1 site with the timed search method, whereas only 1 species were found with the transect method (no mussels were found with the adaptive cluster method, Fig. 1.2A). Timed searches were equally effective compared to transect method in detecting species at the site San Saba 2, but only two species were found at this site. Similarly, timed searches detected more species than the adaptive cluster method at the two sites with lowest density, and also at the site with the highest patchiness (Fig. 1.2B). In contrast, more species were found with the adaptive cluster method than the transect method at San Saba 1, where wooden logs and branches occurred in the middle of the river.

Species Composition

Differences between search methods were not only found for the number of species, but also their relative abundance. This was most obvious at sites where all methods found a similar number of species (i.e., Guadalupe 2, San Antonio, and San Saba sites). In general, timed searches found a higher proportion of larger-sized species. For example, a considerably higher proportion of *Tritogonia verrucosa*, (usually larger sized, average length=89 mm) was found with timed searches (50%, n = 134) at the San Antonio site compared to adaptive cluster (37%, n = 97) and transect method (29%, n = 24). This was also the case at San Saba 2, where 74% of the mussels detected with the transect methods were *T. verrucosa*, whereas 55% of the mussels detected with the other two methods were *T. verrucosa*. Similarly, 15% of all mussels detected with the transect method at the Guadalupe 2 site were *Crytonaias tampicoensis* (also, larger sized ~82 mm), while the other two methods detected a lower proportion of that species (~5%). In contrast, a higher proportion of smaller species, e.g., *Cyclonaias aurea* (average length: 53 mm), were found with the adaptive cluster method (29%) compared to timed searches (5%) at the San Antonio site.

Number of Mussels per Unit Search Effort

In general, the adaptive cluster method tended to have a similar search effort compared to timed searches (mean 3.8 p-H; range =2.8-4.2 p-H) with the exception of the Guadalupe 2 site, where the adaptive cluster method took considerably less time ~2.8 p-H. In contrast, the transect method had the lowest search effort (mean of ~2.5 p-H; range = 1.5-3.7 p-H). In accordance with prediction 2, the adaptive cluster method was more effective at detecting a higher number of

mussels per unit search effort than the transect method, especially at sites where patchiness was high (i.e., the San Antonio site) or density was high (i.e., the Guadalupe 2 site (Fig. 3 A), but also at the site with moderate density (San Saba 2, Fig. 1.3A) These three sites were also the only sites with sandy substrate. Timed searches were more effective than quantitative methods at most sites in respect to number of mussels per unit search effort (Fig. 1.3 B, C), but not at the sites with the gravel and cobble substrate (San Saba 1 and Guadalupe 1).
A comparison of the number of mussels found by surveyors with different levels of experience (ranging from >3 months to 2 years) showed that the number of mussels found increased with experience level (Fig. 1.4).

The bootstrapping analysis showed that the coefficient of variation (CV of density estimates) increased as the number of quadrats per transect was decreased from 5 to 1 (Fig.1.5). This was most pronounced for the site with the lowest density, the Llano site (CV up to ~250% with 9 compared to 45 quadrats, Fig. 1.5), followed by the site with second lowest density (Guadalupe 1, CV up to ~120%, Fig. 1.5). For the other four sites with moderate to high densities, CV ranged between 20-30% when the total number of quadrats were decreased from 45 to 27 and between 40-60% with 9 quadrats.

Mussel Size

As predicted, there were indications that timed searches tended to be biased towards finding larger individuals and those that were less burrowed. Timed searches found a higher proportion (94%) of larger mussels (i.e., those with >60mm shell length) compared to the other two methods at the San Antonio (72%), and both San Saba sites. Only the transect and adaptive cluster method found smaller individuals (<48 mm) of the following species, *A. plicata, T. verrucosa*, and *C. houstonensis* at the San Saba sites. At the San Antonio site only the transect method found smaller species of *A. plicata* (<55mm), but all methods found smaller individuals of *T. verrucosa.* In contrast, at 2 of the 6 sites there was no difference in the size frequency of mussels detected with different methods (Llano and Guadalupe 2), and timed searches also detected smaller sized mussels at these sites.

Discussion

This is the first study in Texas that examined differences in the effectiveness of three different unionid survey methods across multiple sites in four different rivers. Our findings were generally in accordance with our predictions and the results from previous studies which compared qualitative and quantitative surveys (e.g., Hornbah and Deneka 1996, Vaughn et al. 1997, Obermeyer 1998). However, there were some notable exceptions that were associated with special local habitat conditions. Firstly, while timed searches clearly outperformed quantitative methods at most sites in respect to number of mussels per unit search effort, this was not the case at sites where searching for mussels was considered more difficult (i.e., rough gravel or cobbles hindering tactile searches) and therefore the detectability of mussels. Secondly, the adaptive cluster method was only more effective compared to the transect methods at sites where local habitat conditions facilitated the detectability of mussels (i.e., sandy substrate). This is in accordance with model simulations by Smith et al. (2010), which found that performance of adaptive cluster degraded as detectability declined. Thirdly, the adaptive cluster method only detected a larger number of species compared to timed searches at the site, where surveyors avoided an area with wooden logs in the middle of the river (which was difficult to search), but in which the additional species (*Cyclonaias apiculata* and *Amblema plicata)* were found with the adaptive cluster method. Thus, habitat conditions affect the performance of survey methods by facilitating or hindering the detectability of mussels.

It has been shown previously that the detection of mussels can vary with habitat conditions such as depth, water velocity/turbulent flow, and substrate (Meador 2008, Smith and Mayer 2010, Shea et al. 2013, Wisniewski et al. 2013), but this is the first study that shows how it can affect the relative performance of different survey methods. Differences in detectability of mussels cannot only be associated with habitat conditions, but also with the size of mussels, their behavior and morphology. This study found that timed searches tended to detect a higher proportion of larger species, such as *T. verrucosa* and a smaller proportion of smaller species such as *C. aurea.* These species do not only differ in their size, but also in their burrowing behavior and morphology. *C. aurea* burrows more deeply, whereas *T. verrucosa* tends to be less burrowed and can be sometimes found laying at the surface, e.g., a survey in April 2017 at the San Antonio site found that 47% of *T. verrucosa* were completely at the surface, 30% of *A. plicata,* but only 15% of *C. aurea* (Hernandez 2016, Zachary Mitchell, Texas State University,

unpublished data). This study also found that timed searches found a larger proportion of larger species with sculptured shells such as *A. plicata*, which makes it easier to find the mussel within cobble and gravel with tactile searches. In contrast, quantitative methods found more burrowed, small, and species with smooth shells, which is consistent with findings by other studies (Hornbach and Deneka 1996, Vaughn et al. 1997).

Detection also depended on surveyor experience in this study, which has been previously shown by other studies (e.g., Wisniewski et al. 2013, Reid 2016). A study in the Flint River in Georgia, found that searchers with more experience tended to better recognize mussels from substrate, to be less affected by sampling fatigue, and better able to negotiate challenging sampling conditions (Reid 2016). Thus, in order to avoid incomplete detection and potential biases, training of field staff for the rigors of searcher fatigue and their ability to discriminate mussels in same size or larger sized substrate will be necessary.

When designing mussel surveys, it should be considered that the relative effectiveness of different survey methods varies between sites and rivers with different habitat conditions. The ideal search method for a given study and at specific sites will depend on the search goal (i.e., is the purpose to find as many number of mussels, find as many species, or find a certain species, or get an idea of the species composition of an area). Timed searches are especially useful when densities are low (e.g., Llano and Guadalupe 1) and when distribution is extremely, but predictably clustered. For example, timed searches allowed to focus on the pockets of gravel on bedrock at the Llano site and to avoid the deeper and faster areas where not mussels were found with the quantitative methods at the Guadalupe 1 site. It should be noted that our assumption was that densities would not change between sampling dates, but this may not have been the case at Guadalupe 1, where anglers were observed to use mussels as bait, and pile of shells of dead mussels were present at the shore.

Transect methods can be effective in obtaining density estimates, but the accuracy and precision will depend on the search effort, including the number of quadrats. The increase in the coefficient of variation for density estimates when the number of quadrats were reduced in the bootstrapping analyses indicated that there is a high likelihood that surveys in the same area would over-or underestimate mussel densities. This was especially pronounced at sites with lower densities. Although it was possible to calculate how the CV may change with a lower number of quadrats, a modelling approach would be necessary to predict how many quadrats

may be needed to obtain reliable density estimates of mussels. For example, a modelling study of a mussel bed in the Upper Mississippi River showed that a low CV< 0.25 was achieved with samples sizes of >500 quadrats for populations with density ≥ mussels $0.2m^{-2}$ (Smith et al. 2009). For even lower density populations, sample size would have to increase even further to achieve similar precision, which may not be feasible. Thus, density estimates of mussel species that occur in very low densities should be considered with caution.

Findings from the adaptive cluster method cannot be easily translated into a density estimate for the search area, but it has the advantage to guide searches towards areas where mussels occur, including those avoided by searchers during timed searches (e.g., when obstacles hinder searches). As previously suggested (Villella and Smith 2005, Smith et al. 2011) a combination of timed searches and quantitative methods should suffice for most survey needs. To improve the performance of timed searches, searches should also be done in areas usually avoided by searchers, where searching is hindered by habitat conditions. In summary, our results suggest that to design effective surveys variation in detectability of mussels must be considered which depends on local habitat conditions, experience of surveyor, behavior and morphology of mussels, as well as size of mussels. Future studies should examine how the effectiveness of survey methods vary in rivers with a higher species richness and mussel densities and further examine the role of detectability for the performance of survey methods.

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1. Comparison of survey methods

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Tables and Figures

Table 1.1 List of survey sampling days and methods applied.

Site	Visit 1	Date	Visit 2	Date	Visit 3	Date
Llano	Timed search	06/30/2017	Adaptive cluster	07/14/2016	Transect	09/12/2016
San Saba 1	Timed search	03/06/2017	Transect	03/15/2017	Adaptive cluster	03/22/2017
San Saba 2	Transect	03/06/2017	Adaptive cluster	03/15/2017	Timed search	03/22/2017
San Antonio	Adaptive cluster	12/01/2016	Timed search	05/03/2017	Transect	05/11/2017
Guadalupe 1	Timed search	07/07/2017	Adaptive cluster	07/14/2017	Transect	07/28/2017
Guadalupe 2	Transect	07/07/2017	Timed search	07/14/2017	Adaptive cluster	07/28/2017

1. Comparison of survey methods

Table 1.2 List of sites and their habitat characteristics (substrate, depth, and average velocity), and mussel density determined by the transect method.

Site	Mussel density (mean±SE) [Number of ind. /m^2]	Substrate	Depth	Average Velocity
Llano	0.02±0.02	Bedrock/gravel divots	0.5m	0.24 m/s
San Antonio	2.1±0.2	Sand/corbicula shells	0.45m	0.18 m/s
San Saba 1	1.3±0.1	Gravel/cobble/silt	1m	0.29 m/s
San Saba 2	2.0±0.5	Cobble/sand	0.45m	0.61 m/s
Guadalupe 1	0.2±0.03	Gravel/Sand	1.7m	1.1 m/s
Guadalupe 2	7.1±0.1	Sand	1.5m	0.03 m/s

1. Comparison of survey methods

Table 1.3 Species found with different methods, density estimates for adaptive cluster and transect method.

Site	Method	Species	Density [mean ± SE]
Llano	Timed search	3 (*Lampsilis bracteata, Strophitus undulatus, Cyclonaias petrina)*	
	Adaptive Cluster	2 (*Lampsilis bracteata and Cyclonaias petrina)*	0.4±0.2
	Transect	1 (*Strophitus undulatus)*	0.02±0.02
San Antonio	Timed search	5 (*Amblema plicata, Lampsilis teres, Cyclonaias aurea, Cyclonaias petrina, Tritogonia verrucosa)*	
	Adaptive Cluster	4 *(Amblema plicata, Lampsilis teres, Cyclonaias petrina, Tritogonia verrucosa)*	9.3±0.5
	Transect	4*(Amblema plicata, Lampsilis teres, Cyclonaias aurea, Tritogonia verrucosa)*	2.1±.0.2
San Saba 1	Timed search	*4(Leptodea fragilis, Cyclonaias houstenensis, Cyclonaias petrina, Tritogonia verrucosa)*	
	Adaptive Cluster	5 (*Amblema plicata, Leptodea fragilis, Cyclonaias apiculata, Cyclonaias houstenensis, Tritogonia verrucosa)*	2.9±0.1
	Transect	3 (*Cyclonaias houstenensis, Cyclonaias petrina, Tritogonia verrucosa)*	1.3±0.1
San Saba 2	Timed search	2 *(Cyclonaias petrina, Tritogonia verrucosa)*	
	Adaptive Cluster	2 *(Cyclonaias petrina, Tritogonia verrucosa)*	7.6±0.5
	Transect	2 *(Cyclonaias petrina, Tritogonia verrucosa)*	2.0±0.1

1. Comparison of survey methods

Site	**Method**	**Species**	**Density [mean ± SE]**
Guadalupe 1	Timed search	4 (*Amblema plicata, Cyrtonaias tampicoensis, Lampsilis teres, Cyclonaias aurea*)	
	Adaptive Cluster	*Unable to apply method	
	Transect	1 (*Amblema plicata*)	0.2±0.03
Guadalupe 2	Timed search	3(*Amblema plicata, Cyrtonaias tampicoensis*, and *Cyclonaias aurea*)	
	Adaptive Cluster	3(*Amblema plicata, Cyrtonaias tampicoensis*, and *Cyclonaias aurea*)	20.9±0.2
	Transect	3(*Amblema plicata, Cyrtonaias tampicoensis*, and *Cyclonaias aurea*)	7.1±0.1

1. Comparison of survey methods

Figures

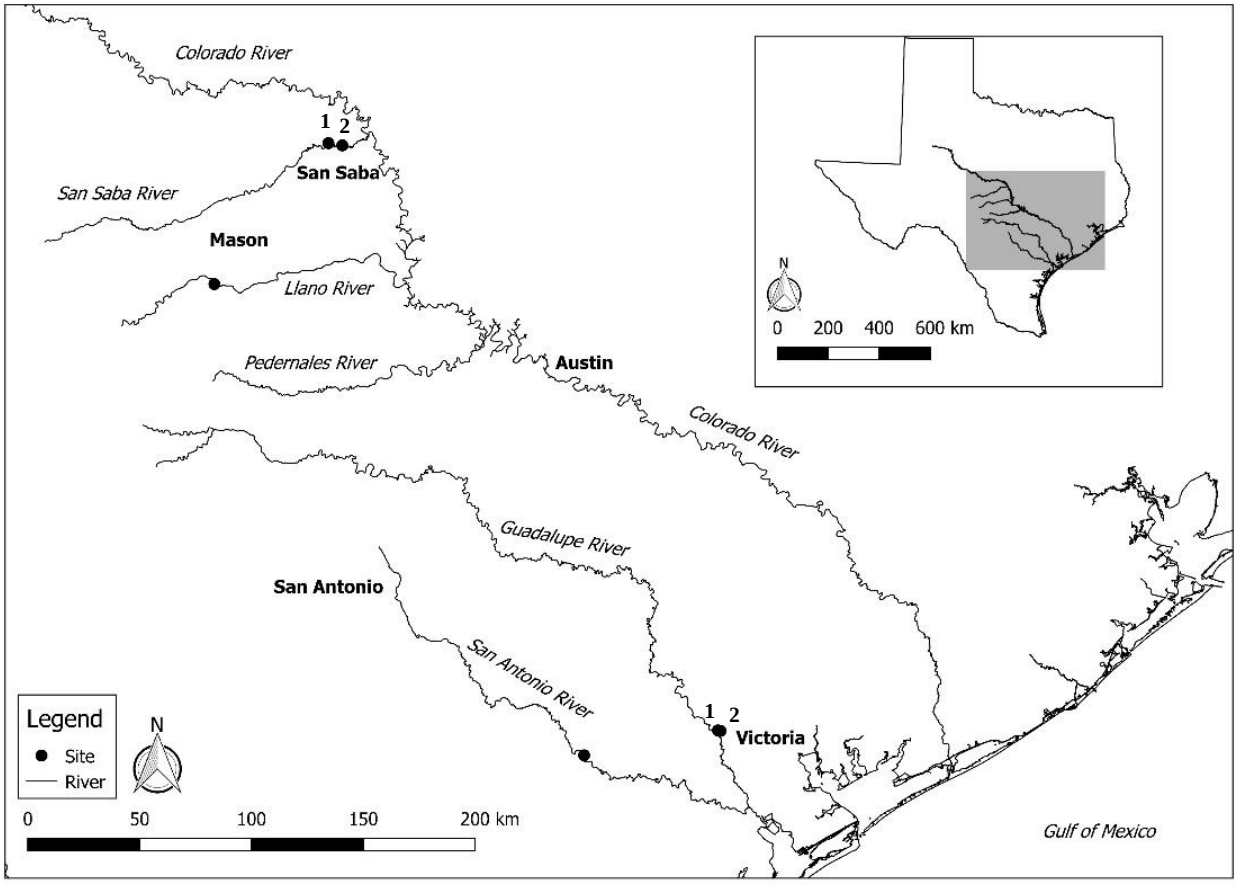
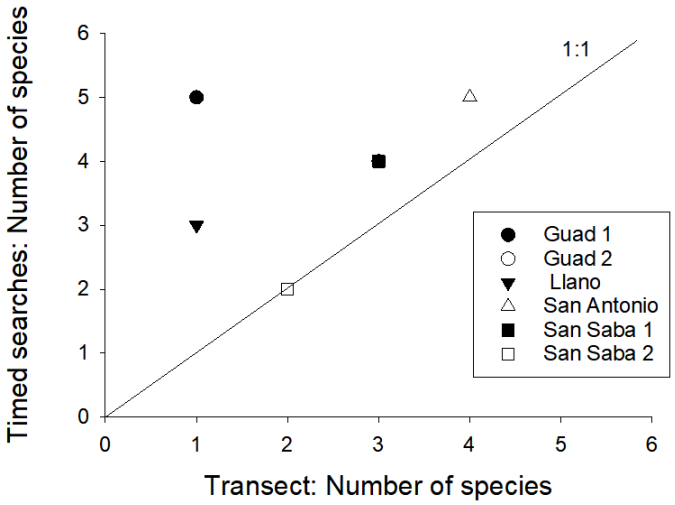


Figure 1.1 Map of study sites in the San Saba River (2 sites), Llano River, San Antonio River, and Guadalupe River (2 sites)

1. Comparison of survey methods

A



B

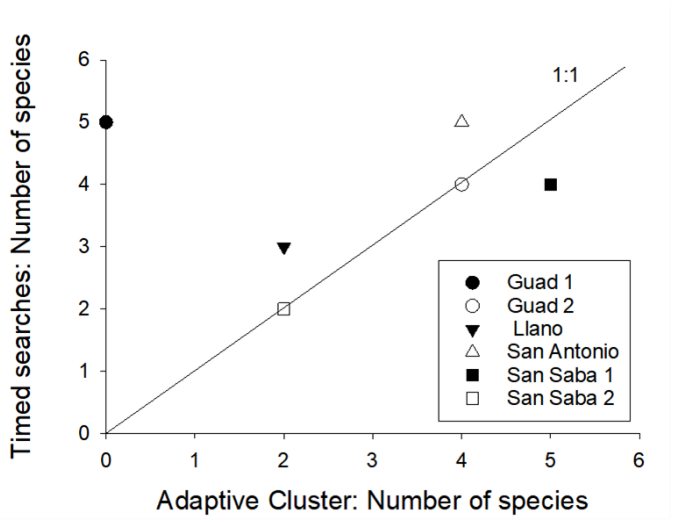


Figure 1.2 Number of species found. A) Comparison between the transect method and timed searches and B) Comparison between the adaptive cluster method and timed searches. Different symbols indicate different sites (Guadalupe 2 is "below" San Saba site 1 in panel A).

1. Comparison of survey methods

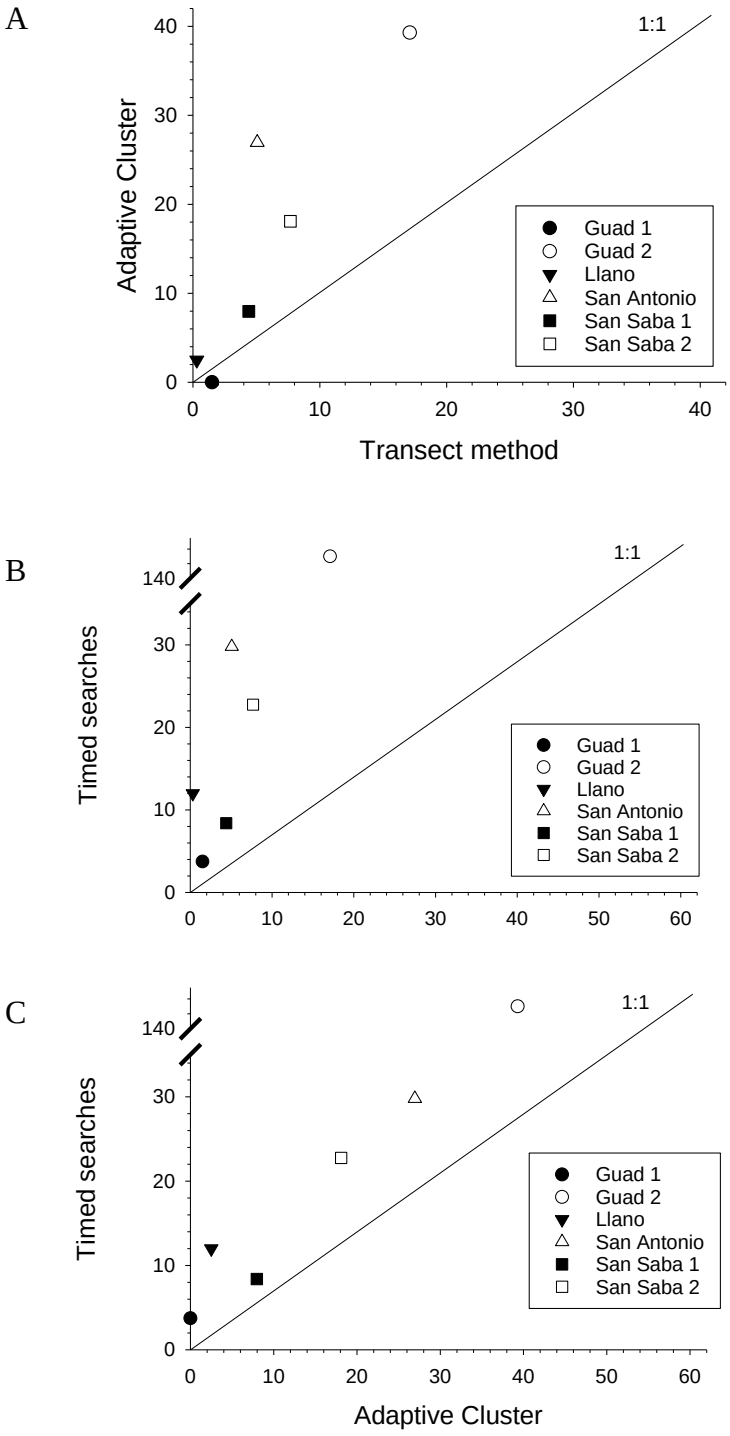


Figure 1.3 Number of mussels found per unit search effort (p-H). A) Adaptive cluster vs. transect method, B) timed searches transect method, C) timed searches vs. adaptive cluster methods.
Different symbols indicate different sites.

1. Comparison of survey methods

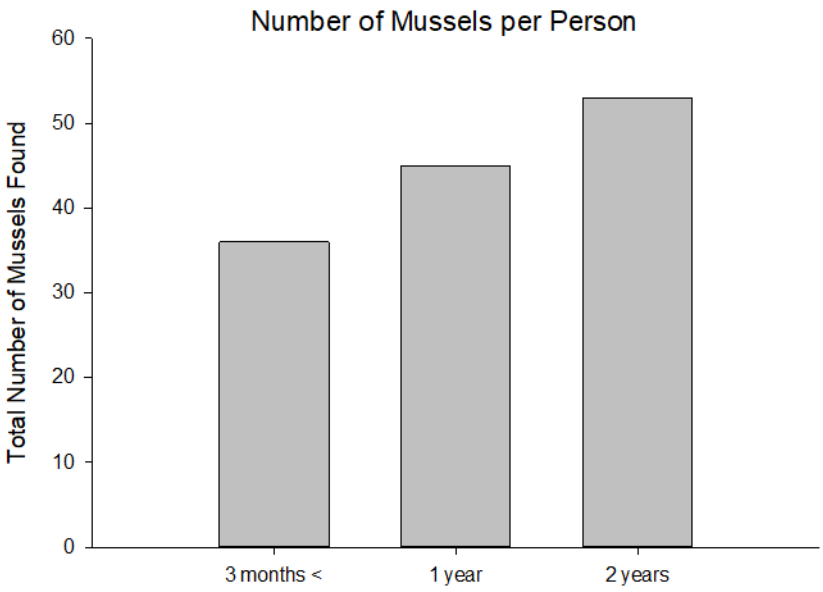


Figure 1.4 Surveyor experience: Number of mussels per person during a timed search performed at the San Antonio River on May 03, 2017.

1. Comparison of survey methods

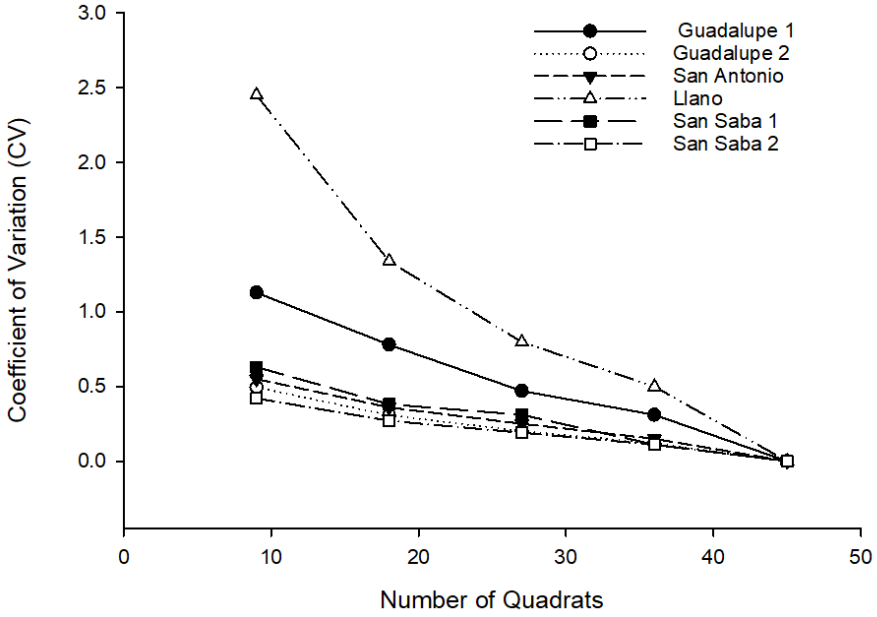


Figure 1.5 Coefficient of variation for different numbers of quadrats (i.e. 1, 2, 3, 4, and 5 quadrats for each of the 9 transects).

The data obtained with the transect method was re-sampled with a boot-strapping method.

2. Seasonality of vertical migration

by Bianca Hernandez, Zachary Mitchell and Astrid Schwalb

Abstract

Freshwater mussels can migrate vertically for several reasons including reproduction or to seek thermal refuge and these movements have been shown to be affected by various factors such as day length, flow velocity, and temperature. However, no work has focused on warmer sub-tropical rivers like those in central Texas and a better understanding of vertical movement of freshwater mussels in these rivers could better inform survey designs and facilitate conservation efforts. To gain a better understanding of seasonal variation in vertical movement of mussels in Central Texas, we examined and compared burrowing depth in the field among species and sites in the Guadalupe, and San Antonio River drainages in central Texas. Burrowing behavior was significantly affected by river, month, species, and shell length. Overall, mussels burrowed deeper in the San Antonio River (sandy) compared to the Guadalupe River (gravel). Burrowing tended to increase with decreasing temperatures, with smaller species burrowing deeper on average than larger species. Our results suggest that surveys must include excavation during colder temperatures or in softer substrates (e.g. sand) where mussels may be burrowed deeper.

Introduction

Studies on seasonal vertical migration suggest mussels burrow deeper in winter compared to summer, and burrowing has been associated with day length, water temperature, reproduction and flow velocity (Amyot and Downing 1991, 1997, 1998, Balfour and Smock 1995, Watters et al. 2001, Schwalb and Pusch 2007, Watters and Ford 2011, Gough 2012). However, seasonal vertical migration of unionid mussels has never been studied in warmer subtropical rivers like those representative of Central Texas drainages. A better understanding of vertical migration would inform survey designs and facilitate conservation efforts. For example, if surveys do not include excavations, then they may underestimate species presence/absence or actual population size when a large proportion of mussels are burrowed (Strayer and Smith 2003). However, excavations are time consuming and disturb habitat, thus should only be performed if necessary (Miller and Payne 1993, Smith et al. 2001). To gain a better understanding of seasonal variation in vertical movement of mussels in central Texas, we examined and compared burrowing depth

in the field among species and sites in the Guadalupe, and San Antonio River drainages in Central Texas.

Methods

Study Area

Field studies were conducted at two sites in the Guadalupe River (near Cuero) and San Antonio Rivers (near Kenedy) in central Texas. The Guadalupe River originates in Kerr County, Texas and flows southeasterly to the San Antonio Bay System with a drainage area of 9,769 km^2 of urban, farming, and agricultural land uses (TCEQ 2016). The San Antonio River originates in Bexar County, San Antonio, TX and flows southeastward from the San Antonio Springs to its confluence with the Guadalupe River with a drainage area of 6727 km^2 of urban and agricultural land uses (SARA 2012). Substrate at the Guadalupe site consisted of gravel, cobble and silt and were located in riffle habitats, whereas the study site in the San Antonio River was located near the bank and substrate consisted mostly of sand and dead *Corbicula* shells.

Monitoring Movement at Field Sites

Initially it was planned to survey sites monthly for one year (August 2015 to July 2016), but high water levels repeatedly made these surveys impossible (especially as wadable conditions and lower turbidity was required to observe the position of the mussel in the substrate and for the use of a PIT-tagging antenna, see below). Surveys were conducted between August 2015 and February 2016, during which temperatures decreased from 32°C to 15°C (Table 2.1 A). Sites were sampled in different months because both sites were not always accessible. In addition, initial sampling dates varied between the Guadalupe and San Antonio River, (Table 2.1), but both sites were surveyed in October 2015 and February 2016 (Table 2.1 A). Two additional surveys were done at the San Antonio site in September 2017 and April 2018, with water temperature of 28°C and 24°C respectively (Table 2.1B).

Visual and tactile searches were carried out along 10 m transects parallel to the flow, because the velocity was too strong, and/or water depths too great to orient the transects across the stream channel. Tactile searches included digging for mussels up to 5cm into the substrate. All mussels found were placed in a mesh bag and retained in the water. Each individual was measured for width, length, and height and photographed. All mussels found during a thorough

initial survey were tagged. A uniquely numbered 12mm Passive Integrated transponder (PIT) Tag (Biomark, Inc., Bowase, ID, USA) was glued on the left, posterior margin of each mussel using waterproof epoxy (LOCTITE Henkel Corporation, Rocky Hill, CT, USA). A distinctively numbered shellfish tag (Floy Tag Mfg. Inc., Seattle, WA, USA) was glued using Super Glue along the right, posterior margin of each mussel.

The location of tagged mussels was detected with a PIT-tag antenna. The use of an antenna to locate tagged mussels restricted the study area to wadeable segments of the streams. Their location on the surface or burrowed were visually examined with an underwater viewer or via snorkeling or scuba. Burrowing depth was recorded as percentage of shell burrowed on a scale of 0%, 25%, 50%, 75%, 90%, and 100% (only detected; not visible on surface). For the two additional survey in 2017/2018 burrowing depth was recorded, but mussels were not tagged.

Statistical Analysis

During this study, individual mussel burrowing percentage was repeatedly measured over time and under different conditions and because of this, mixed-effects models were used to examine the differences in burrowing depth among mussels. All statistical analyses were conducted in R version 3.5.1. Linear mixed-effect models were analyzed using the package *lme4* (Bates et al. 2015). Factors such as site, species, season and shell length were included to determine influence on burrowing behavior of freshwater mussels. Burrowing depth (% of shell burrowed) was the response variable; site (river), species, season and shell length were considered fixed effects; while mussel identity was considered a random effect. Model selection using Akaike Information Criterion (AIC_C; package *MuMIn*, Bartón 2016) was used to identify which model explained the most variation in burrowing percentage among freshwater mussels. Maximum likelihood estimation was used to calculate parameters during the model selection process. Additionally, coefficients of determination were calculated for each linear mixed-effects model by calculating the variance explained by fixed factors (marginal R^2) and by fixed and random factors (conditional R^2, Nakagawa and Schielzeth 2013). The full model was: Burrowed % ~ River + Species + Length + Month + (1|Tagged mussel).

A different statistical approach was used to assess differences in burrowing behavior of mussels that were not individually tagged during the additional surveys in combination with the original dataset for the San Antonio River (i.e., surveys from August 2015 to February 2016

combined with surveys in fall 2017 and spring 2018). Beta regression was used to examine which of the factors (season and species) significantly affected the burrowing depth of mussels within the San Antonio River. Season were classified as follows: fall (September-November), winter (December-February), spring (March-May), and summer (June-August). Post-hoc contrasts for beta regression models were tested using Tukey HSD tests.

Results

In the Guadalupe River, 98 mussels (shell lengths: 6-94 mm) were tagged in August 2015. In the October 2015 survey 67 mussels were detected with the pit-tagging antenna, whereas only 16 individuals were detected in the February 2015 survey (Table 2.1). The focus of the analyses (below) were on the 2 most abundant species: *Amblema plicata* and *Cyclonaias aurea*; with average lengths of 70 mm (range: 47 to 94 mm) and 41 mm (range: 6 to 58 mm) respectively. In the San Antonio River 145 mussels were tagged in October 2015. In December 2015, 89 individuals were detected and 79 were detected in February 2015. Mussel lengths ranged from 30 mm – 121 mm in the San Antonio River. The average length of *A. plicata* was 63 mm (range: 30-83 mm) while average length for *Q. aurea* was 45 mm (range: 30-60 mm). After the initial survey, 33% of the mussels were not detected again in the San Antonio River and 84% of the mussels in the Guadalupe River, but both sites experienced two major flood events (May and October 2015) during the monitoring period. During the additional surveys at the San Antonio River site, 195 mussels were detected in fall 2017 and 178 mussels in spring 2018 (Table 2.1. B).

The mixed effects model (Tables 2.2 and 2.3) detected significant differences in burrowing behavior of mussels between rivers ($F_{1,307}$ = 44.06, P<0.01), month ($F_{3,236}$=17.93, P<0.01), species ($F_{1,160}$ = 17.93, P<0.01), and shell length ($F_{1,161}$ = 17.93, P<0.01) which accounted for 46% of the variation in burrowing behavior (conditional R^2: 0.46). In 2015/2016 mussels at the site in the San Antonio River (a predominantly sandy site) tended to burrow deeper than in the Guadalupe River (Fig. 2.1 A, B; Table 2.3). For example, up to 71% of all mussels detected were burrowed at the 90% or greater depth interval in the San Antonio River in October, whereas only 21% were burrowed at that depth in the Guadalupe River during the same month (Fig. 2.2 A, B).

2. Seasonality of vertical migration

In the Guadalupe River both species, *C. aurea* and *A. plicata*, showed a significant increase in burrowing depth, almost doubling (1.8 times) from summer (August) to fall (October) and a slight increase (1.1 times) from fall (October) to winter (February, Fig. 2.1A, 2.2A; Table 2.3). Compared to August, burrowing depth was significantly higher in October and February. In contrast, average mussel burrowing depth in the San Antonio River did not differ much between fall and winter in 2015/2016 (Fig. 2.1B). There was no significant difference between October and December, and average burrowing depth was only slightly (2%) less in February. However, in December, the largest proportion (62 %) of mussels were completely and almost completely burrowed (≥ 90%), whereas in February only about 42% were burrowed that deep (Fig. 2.2 B). The mixed effects model indicated that burrowing depth in February was significantly different than October and December (Fig. 2.1B, D; Table 2.3). Aside from the San Antonio River in October, *A. plicata* burrowed deeper than *C. aurea* in both rivers regardless of season.

At the San Antonio site, the largest proportion of all species were at the surface in September 2017 (54%, 38%, and 28%, for *C. aurea, Tritogonia verrucosa* and *A. plicata* respectively, Fig. 2.2D) when the temperature was highest (28°C). Mussels also had the lowest average burrowing depth in September 2017, compared to all other months in which mussels were surveyed at that site (Fig. 2.1 B, C). A smaller proportion of mussels was at the surface in April 2018 compared to September 2017, but mussels were burrowed less deeply than in October and December 2015, and February 2016. There were considerable differences between species. While 61% of *C. aurea* were completely and almost completely burrowed (≥ 90%), only 20% of *A. plicata* and 5% of *T. verrucosa* were burrowed that deeply in April 2018.

The regression analysis for all years combined in the San Antonio River showed that 19% of the variation in burrowing behavior could be explained by differences in season, species, and their interaction (R^2: 0.19) (species, $X^2_{(2)} = 55.6$, $p < 0.001$; season $X^2_{(2)} = 35.4$, $p < 0.001$; interaction, $X^2_{(2)} = 26.4$, $p < 0.01$). *A. plicata* burrowed deeper (all seasons combined) compared to *C. aurea*, and *T. verrucosa* showed the lowest burrowing depth (Tukey HSD: $p < 0.05$). Mussels within the San Antonio river burrowed more with decreasing water temperatures, with a higher percentage of individuals burrowed during the winter followed by spring and fall seasons, respectively (Tukey HSD: $p < 0.05$).

2. Seasonality of vertical migration

Discussion

Our results show that mussels show seasonal variation in their burrowing behavior, but random variation in individual movement behavior was high, usually explaining a large portion of variation in movement behavior. Previous studies have shown seasonal vertical migration, where mussels burrow deeper during colder months and re-emerging as water temperatures increase (Balfour and Smock 1995, Amyot and Downing 1997, Watters et al. 2001, Schwalb and Pusch 2007, Allen and Vaughn 2009). However, most of these studies were conducted in colder climates in the Northern hemisphere with seasonal water temperatures ranging from 2°C-26°C whereas long-term average water temperatures in rivers of central Texas is 20°C, ranging between 9°C and 32°C (TCEQ 2016) for the lower Guadalupe River and 10°C and 29°C (TCEQ 2016) in the San Antonio River. Interestingly, despite climatic differences and warmer temperatures in Texas, we found the same seasonal trend in the San Marcos (data not shown here), Guadalupe, and San Antonio Rivers. where burrowing depth was deeper when water temperature cooled down. For example, burrowing depth increased between August and October 2015 in the Guadalupe River, and mussels were burrowed deeper in the San Antonio River in October 2015 compared to September 2015. This suggests that either seasonal cues such as day length rather than temperature may trigger increased burrowing behavior in the fall; or, if temperature is a cue, mussels in Texas have a different thermal tolerance and start increased burrowing at warmer temperatures.

Only the San Antonio River was sampled during spring (2018). Interestingly, there were some indications that mussels started to emerge in February when temperatures were slightly higher (18°C compared to 16°C in December) with a larger proportion of mussels being burrowed less deeply in the San Antonio River. Consistent with this trend, the average burrowing depth was deeper in April 2018 (except for *C. aurea*), but the highest proportion of mussels at the surface was recorded in September 2017. A trend of mussels to start re-emerging in February was not evident from the Guadalupe River, where temperature was slightly colder and sample size considerably lower (as many mussels were not detectable in February, likely due to losses caused by flooding). As reproduction is often triggered by changes in temperature, the re-emergence with warmer temperatures may be ultimately triggered by mussels coming to the surface for reproduction (Vaughn and Hakenkamp 2001).

2. Seasonality of vertical migration

Apart from season as the strongest driver in burrowing behavior, we also detected significant differences between species and shell length with smaller mussels being generally burrowed deeper consistent with findings by other studies (Schwalb and Pusch 2007; Allen and Vaughn 2009). Although it has been suggested that smooth-shelled species may have greater burrowing capabilities than sculptured species (Watters 1994), our results are not as clear. The smooth-shelled *C. aurea* was found to be burrowed deeper than *T. verrucosa* (with sculptured shell) regardless of season, but only burrowed deeper than *A. plicata* (also sculptured) on two sampling events. Interestingly, *A. plicata* burrowed deeper than the smooth-shelled *Fusconaia flava* in another experimental study (Allen and Vaughn 2009) and also burrowed deeper in response to dewatering than other smooth-shelled species in a recent experimental study (Mitchell et al. in press).

Our results show that surveys will be less efficient and may fail to detect larger proportions of populations during colder temperatures, especially in winter. A larger proportion of mussels burrowed can also be expected at sites with smaller substrate such as sand, in which mussels can more easily burrow. Visual searches will not suffice under these conditions.

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2. Seasonality of vertical migration

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2. Seasonality of vertical migration

Tables and Figures

Table 2.1 Survey Dates and Number of Mussels Detected

A) Dates of start and regular surveys for field studies and water temperature at sampling dates in parenthesis, and numbers of mussel marked and detection of marked mussels. In parenthesis number of species: Ap: Amblema plicata, Ct: Cyrtonaias tampicoensis, Lt: Lampsilis teres, Mn: Megalonaias nervosa, Qa: Quadrula aurea, Tv: Tritogonia verrucosa.

Sites	**Survey start**	**Re-survey**	**Marked**	**Detected**
Guadalupe River	8/28/2015 (32°C)	10/17/2015 (25°C) 2/12/2016 (15°C)	98 (Ap: 52, Qa: 27, Ct: 11, Qp: 7, Mn: 1)	67 (Ap: 46, Qa: 13, Ct: 7, Mn:1) 16 (Ap: 9, Qa: 3, Ct: 3)
San Antonio River	10/21/2015 (24°C)	12/22/2015 (16°C) 2/13/2016 (18°C)	145 (Ap: 58, Qa: 53, Tv: 30, Mn: 2, Lt: 2)	89 (Ap: 37, Qa: 33, Tv: 15, Mn: 2, Lt: 2) 79 (Ap: 30, Qa: 27, Tv:18, Mn: 2, Lt: 2)

B) Number of mussels detected during two additional surveys in fall 2017 and spring 2018 (water temperatures in parenthesis). See above for abbreviations of species names.

Sites	**Survey**	**Number of mussels**
San Antonio River	September 2017 (28°C) April 2018 (24°C)	195 (Ap: 118, Qa: 13, Tv: 64) 178 (Ap: 89, Qa: 24, Tv: 65)

Table 2.2 Summary of Akaike Information Criterion (AIC) model selection analysis examining burrowing behavior of freshwater mussels in central Texas.

Models	Predictors	K	AIC_c	ΔAIC_c	weight
4	River+Species+Length+Season+(1|PITTag)	9	3201.7	0	1
3	River+Species+Length+(1|PITTag)	6	3258.0	56.24	0
2	River+Species+(1+PITTag)	5	3258.9	57.16	0
1	River+(1|PITTag)	4	3262.4	60.63	0

Table 2.3 Summary of fixed factor coefficients and significance from the linear mixed-effects model examining burrowing behavior of freshwater mussels in central Texas.

Coefficient	Estimate	SE	df	t-value	P
(Intercept)	55.9317	9.3633	175.36	5.973	< 0.001
River: San Antonio	25.0115	3.768	307.23	6.638	< 0.001
Species: C. aurea	-8.049	3.937	160.64	-2.044	< 0.05
Shell Length	-0.2661	0.1333	161.21	-1.996	< 0.05
Month: December	27.6677	5.1924	228.83	5.329	< 0.001
Month: February	23.4749	4.8477	247.54	4.842	< 0.001
Month: October	28.409	3.8823	214.73	7.318	< 0.001

2. Seasonality of vertical migration

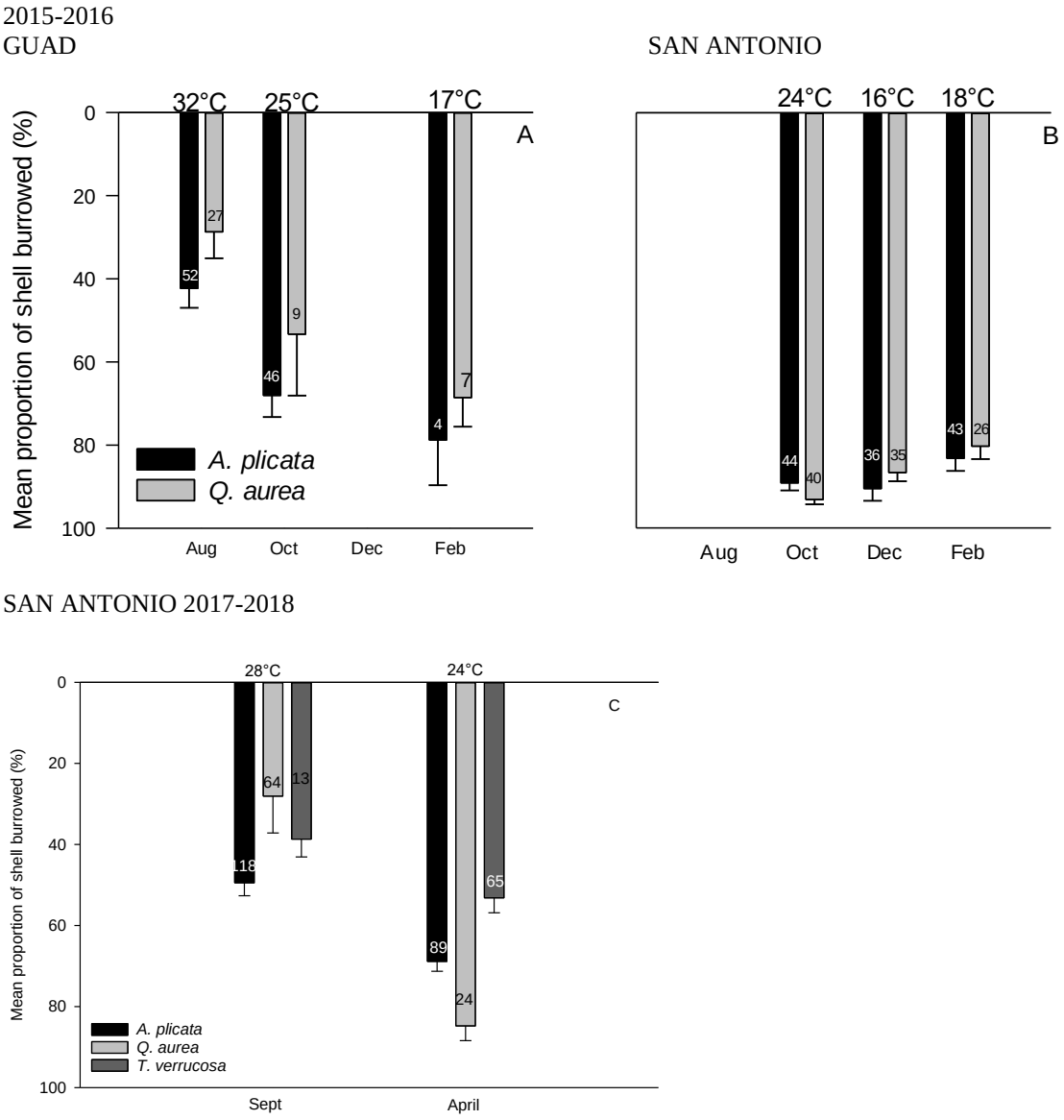
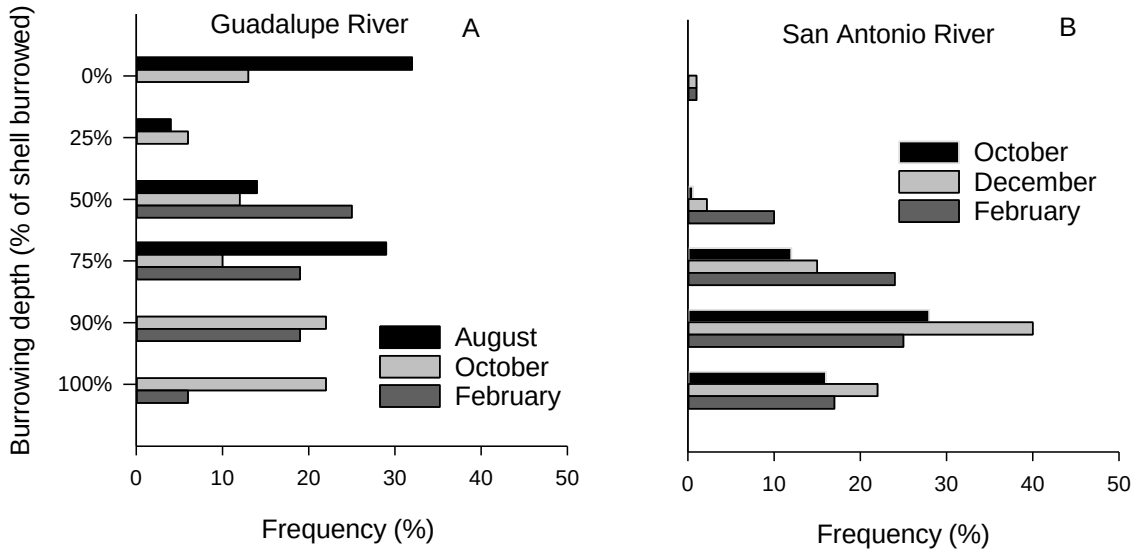


Figure 2.1 Mean proportion ±SE of shell burrowed in the Guadalupe River (A) and San Antonio River (B, C) in 2015/2016 (A, B) and 2017/2018 (C).
Different colors represent different species. *Amblema plicata* (black bars) and *Quadrula aurea* (light grey bars), *Tritogenia verrucosa* (dark grey bars) Numbers in bars represent sample size.

2. Seasonality of vertical migration

2015-2016



2017-2018, San Antonio River

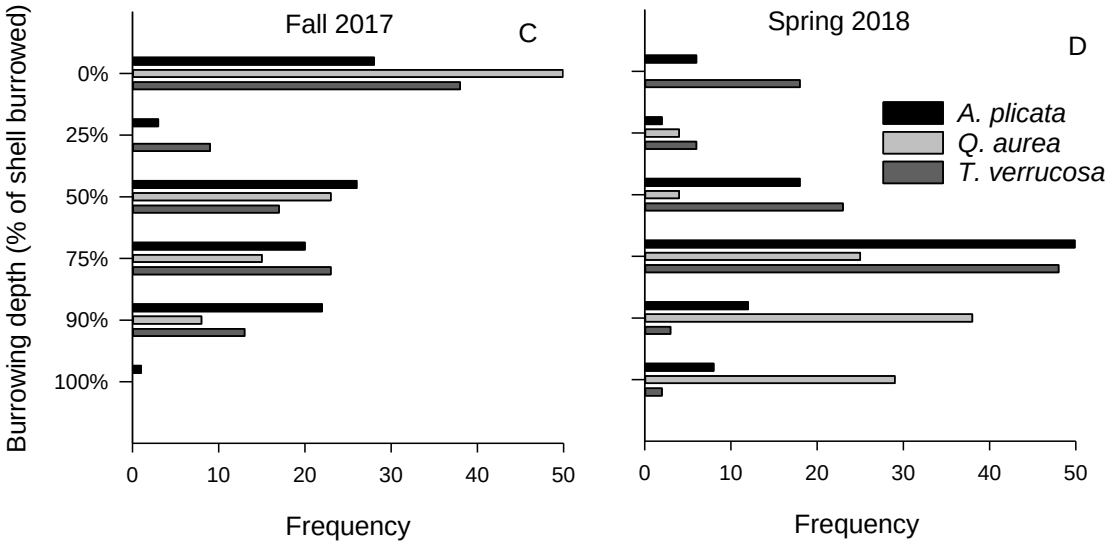


Figure 2.2 Proportion of mussels burrowed at different burrowing depth categories in the Guadalupe River (A), and the San Antonio River (B-D).
Different colors indicate (A, B) different months; (C, D) different species.

3. Reproductive ecology of the endemic Texas Fatmucket (*Lampsilis bracteata*)

Ashley Seagroves, Chris Barnhart, Thom Hardy, and Astrid Schwalb

Abstract

The Texas fatmucket, *Lampsilis bracteata,* is a unionid mussel endemic to the Colorado and upper Guadalupe River basins of Central Texas and a candidate for federal listing under the Endangered Species Act. The purpose of this study was to investigate differences in mussel host fish relationships between populations of *L. bracteata* and fish originating from the San Saba and Llano rivers of the Colorado River Basin in Central Texas, and to monitor and compare seasonality of reproduction between two populations from the San Saba and Llano Rivers. Monthly sampling events between February 2017 and February 2018 assessed gamete concentration, brooding period, viability of larvae (glochidia), and sex ratios. Reproduction varied with season and between populations. The proportion of females brooding tended to be lower in the summer and the fall, and higher during winter and spring months before peak water temperatures were reached. Sex ratio in both populations did not significantly differ from 1:1. Fecundity and glochidia viability were higher in the Llano River population compared to the San Saba population. Trematode flatworms were found in several female gonad samples from the San Saba population and in few samples of the Llano population, but no trematodes were found in any sperm samples. Host fish compatibility measured as metamorphosis success was tested between mussels and fish collected in both rivers using a fully-crossed study design. In addition, host compatibility was tested with hatchery-produced Guadalupe Bass and Largemouth Bass. Highest metamorphosis success occurred on wild Green Sunfish and Largemouth Bass, and hatchery Largemouth and Guadalupe Bass. Average metamorphosis success was higher for some mussel-fish pairings originating from the same tributary, suggesting that mussels may be locally adapted to host fish, which should be considered in conservation efforts.

Introduction

The Texas fatmucket, *Lampsilis bracteata,* is a unionid mussel endemic to the Colorado and upper Guadalupe River basins of Central Texas. Widespread imperilment of unionid bivalves has drawn great interest in the conservation of these ecologically important organisms (Williams et al. 1993, Strayer 2008). *L. bracteata* is one of fifteen threatened mussel species in Texas that is also a candidate for federal listing under the Endangered Species Act. Human impacts are largely to blame for the massive decline in freshwater mussel populations and have imposed threats upon watersheds both globally and locally, i.e., within the geographic distribution of *L. bracteata* (Bogan 1993, Lydeard et al 2004, Howells 2015, Hansen et al 2015).

Female unionid mussels are fertilized by spermcasting males and brood the developing eggs within their marsupial gills (Jirka and Neves 1992). Like most other *Lampsilis* species, *L. bracteata* are thought to be long-term brooders which spawn in the summer and brood until the following spring or summer (Howells 2000). Brooding mussels have previously been found between July and October in the San Saba River (Johnson 2012). Glochidia of unionid mussels remain on host fish for weeks to months, depending on water temperature and species, before detaching from the host as juvenile mussels.

Unionid mussels have developed a fascinating variety of strategies to attract and infest host fish (Barnhart et al. 2008). Female mussels of *L. bracteata* display a mantle lure, mimicking the appearance and movement of a small fish. Predatory host fish become infected with glochidia by attacking the lure and rupturing the mussel marsupial gill. Known host fish of *L. bracteata* include four fish of the Centrarchidae family: *Lepomis cyanellus* (Green Sunfish), *Lepomis macrochirus* (Bluegill Sunfish), *Micropterus salmoides* (Largemouth Bass) and *Micropterus treculii* (Guadalupe Bass; Johnson 2012).

Host fish species are defined by physiological compatibility and also by ecological association. Lab experiments which examine the metamorphosis to juvenile mussels are the most common form of host fish study today and provides insight into the physiological compatibility among mussel and host fish. It should be considered that immunological resistance to glochidia may be acquired in fish with previous exposure to mussels, and that smaller or hatchery fish may show a weaker immune response compared to larger or wild fish (Bauer and Vogel 1987, Dodd et al. 2005, Rogers and Dimock 2003). Lab host fish studies are an ideal precursor to captive breeding because it shows which mussels can be propagated in a lab setting

(Johnson et al. 2012, Levine et al. 2012, Hove et al. 2011). One drawback of lab host fish studies is that they provide no information regarding the frequency of encounters among glochidia and the host fish in the wild. This information could be obtained with observations of natural infestations, but such studies require collection of large numbers of fish and are usually impractical because natural infestations are infrequent (Barnhart et al. 2008, Bauer 1994).

Captive breeding has been widely used as a conservation measure to augment declining populations and to reintroduce mussels to areas where they were previously extirpated (Thomas et al. 2010). There is increased interest in propagation of threatened mussels in Texas as a potential conservation method, but still little is known about their life histories. Increasing the knowledge base of life history information is obviously important in planning for captive propagation and reintroduction (Haag 2013, McMurray and Roe 2017). One concern is the possibility that populations may exhibit local adaptations in timing of spawning and brooding, host fish requirements, or other aspects of reproduction. For example, local adaptations to genetically distinct populations of host fish may sometimes make glochidia more compatible with sympatric than allopatric host populations (Rogers, et al. 2001, Eckert 2003, Taeubert et al. 2010, Zanatta and Wilson 2011).

The objective of this study was to compare life history data between two isolated populations of *L. bracteata*, including seasonal variation in gamete development, brooding period and viability of glochidia, and compatibility among mussel and host populations. A fully-crossed study design was used with *L. bracteata* and host fish originating from two tributaries of the Colorado River in Texas: The Llano River and San Saba River (Fig. 3.1) These tributaries are separated by a stretch of the mainstem Colorado River that includes two major dams, Buchanan and Inks Dam (both constructed in 1938), thus fish and mussels cannot move freely between these tributaries.

Methods

Sampling Sites

Mussels were studied at two sites, one in each of two major tributaries of the Colorado River, the Llano and San Saba rivers (Fig. 3.1). Mussels were monitored monthly from February 2017 to February 2018. Additional preliminary monitoring occurred in the Llano River from April to November 2016 (Table 3A1). Higher water clarity in the Llano River more often

permitted visual search techniques compared to San Saba River, which required tactile search techniques. On each date, sampling continued until ten unmarked *L. bracteata* individuals were located (unmarked mussels had not been previously sampled, see below). All mussels were sampled for gonadal fluid (see details below) and were assessed for brooding and glochidia viability. All sampled mussels were uniquely marked with a Floy® Shellfish Tag (shell tag) to avoid accidental re-sampling for gonadal fluid, as mussels may experience stress from handling which could affect reproductive success (Peredo et al. 2005).

Fish for host fish experiments were collected from the two mussel field sites using backpack electroshocking and seine netting methods. Fish were transported in aerated coolers filled with site water in a 0.18% NaCl solution to reduce stress of handling and transport (Carneiro and Urbinati 2001). Fish were thermally acclimated in the laboratory overnight before being transferred to 10-gallon holding tanks. Fish received pellet food and/or bloodworms (maximum of 2-3 mL per fish) daily along with weekly water changes and regular water quality testing.

Field Environmental Parameters

Temperature at each field site was recorded with a temperature logger (HOBO Pro v2 and HOBO 64K). Temperature was logged hourly from February to mid-May 2017 and from mid-November 2017 to early February 2018, and every 12 hours (12 a.m. and 12 p.m.) from mid-May to mid-November 2017. Daily maximum and minimum temperatures at the two sites were derived from the hourly readings. For the days for which temperatures were only measured twice, the maximum and minimum temperatures were estimated based on the hourly data with linear regressions of the temperature measured at noon vs. maximum temperatures (R^2-values: 0.94 and 0.99 for the Llano and San Saba River respectively) and vs. minimum temperature (R^2 = 0.98 for both rivers). For each sampling date mean values were calculated for maximum and minimum temperatures that mussels had experienced since the last sampling.

Specific conductivity (µS/cm), dissolved oxygen (mg/L), and pH were measured in the thalweg at each mussel sampling site during monthly trips using a YSI 556 MPS. Water samples were collected for analysis of chlorophyll-*a* and total suspended solids (TSS).

Sex Ratio and Gamete Analysis

The numbers of male and female *L. bracteata* detected during each sampling event were recorded, and sex ratios were assessed using a chi-square goodness of fit test to determine if the sex ratio differed from 1:1 or differed between sites. Gonadal fluid was sampled (Tsakiris et al. 2016) from ten *L. bracteata* (regardless to sex). Samples of 0.1-3.2 ml were extracted using a 20 gage hypodermic needle (BD 5ml syringe Luer-Lok™ with BD PrecisionGlide ™ Needle) inserted into the foot (mid-length and mid-width of the shell). Gamete samples were fixed with 10% formalin, dyed with 0.01% methylene blue and transported to the laboratory for analysis. Sperm were quantified in 10 µl subsamples (transferred with micropipette, Fisherbrand Elite) with a compound microscope (400X) and Improved Neubauer hemocytometer (INCYTO DHC-N01-5). Sperm concentration (n/ml of gonadal fluid) was extrapolated from subsamples using equations 1 and 2:

Equation 1: Number/ml = # sperm in 5 small center squares * 5 * dilution factor * 104

Equation 2: Dilution Factor = Total volume (containing ethanol and methyl blue)/ Initial sample volume (gonadal fluid)

Egg concentration was estimated by counting the number of eggs in a 10 µl subsample at 100x magnification on a glass slide and extrapolating the number of eggs to 1 ml of gonadal fluid similarly to sperm concentration calculations, accounting for sample dilution. When egg quantities permitted, the diameter of 50 eggs were measured.

Brooding, Glochidia Viability and Fecundity

Female mussels were considered brooding when gills were swollen and opaque with eggs (Hove and Neves 1994). A sample of glochidia was obtained from each brooding female by flushing 1-2 water tubes (of the marsupium) with a 20-gage hypodermic needle. Glochidia viability was determined in the field by observing the closing response of ~100 glochidia to a saturated salt solution:

$$Glochidia\ Viability = \frac{(open\ glochidia - open\ glochidia\ after\ NaCl\ addition)}{(total\ glochidia)}$$

Glochidia that remained within egg membranes were considered to be immature and were not included in our measures of viability. Fecundity was estimated from the females used in the host

fish experiments (see below). The entire contents of the marsupial gills of each mussel were flushed into separate beakers, diluted with water, and then all glochidia were counted in sub-samples from the stirred suspension and the total number estimated volumetrically.

Host Fish Experiment

Mussels with glochidia that exceeded 90% viability were collected from the field, although some were collected as low as 75% when few gravid mussels were detected (see below). Mussels for host fish experiments were collected from the Llano River in March (testing wild host fish) and April 2017 (testing hatchery host fish). Mussels from the San Saba River were collected in July 2017 for host fish experiments with wild and hatchery fish. Collected mussels were transported in aerated coolers filled with a small layer of substrate and water from the collection site and transferred to flow-through tanks (Living Streams) containing natural gravel substrate and artesian well water from the Edward's Aquifer. Mussels were kept in the lab for 5-7 days before the host fish experiments and fed daily with manually-administered Rotifer Shellfish Diet 1800 (RSD: Pentair Aquatic Eco-Systems) at about 5 microliters RSD per liter of water in mussel tank. Following host fish inoculation, mussels were returned to the sampling site.

The following centrarchid fish species were inoculated with *L. bracteata* glochidia: *Lepomis auritus* (Redbreast Sunfish, only Llano mussels), Green Sunfish, *Lepomis gulosus* (warmouth), Bluegill Sunfish, *Lepomis megalotis* (Longear Sunfish), and Largemouth Bass (sample sizes shown in Table A3). Hatchery-reared Largemouth Bass and Guadalupe Bass were also inoculated with glochidia from mussels from both Llano and San Saba River.

Glochidia used for host tests were extracted from females and viability was tested. For host fish experiments with San Saba mussels, viability was >90% (95 ± 1%, mean ± SE, n = 4); for Llano mussel viability was >79% (88 ± 4%, n = 3) and >76% (82 ± 4%, n = 3) for experiments with wild and hatchery fish respectively. The combined glochidia sample was distributed between inoculation chambers, so that the concentration was ~ 4,000 viable glochidia/L. Glochidia were kept in suspension via continuous turbulent mixing by several air-stones. Fish were exposed to glochidia for 25 minutes before being transferred to randomly-selected individual tanks (1.5 L, 3 L, 10 L) in the flow-through system (Douda et al. 2016). Water temperature ranged between 19 and 23.9 °C.

Unattached glochidia were collected from the flow-through tanks by flushing them for a 10-minute interval at 12 hours and 24 hours post inoculation. Glochidia and juvenile mussels were subsequently collected every second day. Survivorship of juveniles was determined by observing foot and valve movement, and length and height (µm) were measured of a subset of juveniles (total n = 557 from Llano River, n = 256 from San Saba River). Any fish that died during the experiment were dissected, and the gills were checked for presence of encapsulated glochidia (Osterling and Larsen 2013). None of the dissected fish contained encapsulated glochidia, and fish that died during the experiment were excluded from all further analyses with the exception of Warmouth that died after juvenile detachment had ceased for that species. Total length (mm) and weight (g) were measured for each fish upon conclusion of the experiment. Remaining fish were stocked into a private pond for neighborhood fishing.

The metamorphosis success (%) was computed by dividing the number of live juveniles detached from each individual fish by the total number of glochidia and dead juveniles captured from a tank. As data were not normally distributed even after transformation of the data, a two-way ANOVA with permutation test (Anderson 2001) was used to determine whether metamorphosis success differed significantly between fish species and between fish from different rivers (origin).

For comparisons between two groups, (e.g., to examine whether metamorphosis rates differed significantly between fish from different rivers (but same fish species). Welch's t-tests instead of Student's test were used when sample sizes differed between groups. For the pairwise comparisons data were root-transformed when necessary to meet criteria of normality and homogeneity of variances.

Gonad fluid sample volumes varied between about 0.1 and 3.2 ml. Gamete concentration in the samples declined with sample volume, and the relationship was roughly linear between the logarithm of concentration and the sample volume, as would be expected from a dilution curve. We concluded that gonad samples were increasingly diluted with hemolymph as more fluid was drawn. We therefore multiplied sample volume by concentration, yielding a total number of gametes per sample. The number of gametes per sample was defined as gamete abundance. This measure was not significantly correlated with sample volume and was used for comparisons between sites and among sampling dates.

Results

Environmental Parameters

Conductivity and chlorophyll-*a* concentrations were significantly higher at the San Saba River site, and pH was significantly higher at the Llano River site (Table 3.1, Fig. 3.2G, H). There was no significant difference in dissolved oxygen (DO) or total suspended solids (TSS, Table 3.1).

Mean monthly temperatures ranged between 8 and 33°C at the Llano River site and between 9 and 31°C at the San Saba River site. Temperatures peaked in early August at both sites, and reached minima in February. Mean daily minimum temperatures were consistently lower, and mean maximum temperatures higher, at the Llano River site, than at the San Saba site (Fig. 3.2G, H).

Reproductive Monitoring

Sex ratios of males:females for *L. bracteata* collected between February 2017 and February 2018 were not statistically different from 1:1 with 0.9 males per female (n = 110) in the Llano River (X^2 (1) = 0.58, p = 0.45), and 1.2 males per female (n = 120) in the San Saba River (X^2 (1) = 1.2, p = 0.27).

Gamete abundance in gonad fluid samples varied seasonally (Fig. 3.2A-D). Sperm and egg abundance were generally high in October-February and declined to a minimum in midsummer, before increasing again before spawning in late fall-early winter (Fig. 3.2A, B). At the Llano site, the minimum of egg abundance occurred about a month earlier than the minimum sperm abundance. That seasonal pattern was less clear at the San Saba site.

Egg diameters in gonad fluid samples varied between 32 and 331 µm and a wide range of diameters was present throughout the year (Fig. A1, A2). The largest size classes of eggs were relatively least abundant in July-September at the Llano site and in July at the San Saba site. The largest size classes were most abundant in November at the Llano site and October at the San Saba site.

Brooding females were observed throughout the year except in the September-October samples at the Llano River site, and October-December samples at the San Saba site. After this barren period in the fall, brooding resumed. Brooding resumed at least a month earlier at the Llano River site than the San Saba site (Fig. 3.2 E, F). A few glochidia samples (1 out of 6

females in March 2017, and 2 out of 8 mussels in August 2017) contained a large proportion of undeveloped eggs along with a few glochidia. Preliminary monitoring in the Llano River between April and November 2016 found no brooding mussels in July 2016 (n=21), whereas brooding females were found in April (n=2) and June (n=8). Also in contrast to the sampling in 2017, no brooding mussels were found in November 2016 (n = 10).

Most of the brooding mussels (20 of 23 mussels) found in the Llano River had a high glochidia viability (>80%), with the exception of March 2017 (viability <40% in 2 of the 6 brooding mussels) and August 2017 (67% in the only brooding female found). In contrast, in the San Saba River about half of the brooding mussels (13 of 24 mussels) had dead glochidia or <1% viability). Mussels with high glochidia viability (>80%), were found in in April, May, and July 2017, and February 2018. Mean glochidia length was 217.9 ± 1.3 µm, and height was 272.2 ± 1.6µm (n = 343).

Aside from temperature, there was no obvious correlation between gamete abundance, brooding, or glochidia viability and other environmental parameters, such as Chlorophyll-*a* or TSS (data not shown).

Trematode larvae, *Bucephalus sp.* (Bucephalidae), were found in all samples from female mussels collected in February and March 2017 in the San Saba River (n = 5), and in two out of eight egg samples from the Llano River. During the remaining sampling period other unidentified parasites were found in one out of 50 egg samples from the Llano River (in August) and in four out of 40 egg samples from the San Saba River (3 from June to August, and 1 in November 2017). Overall there was no indication of lower gamete abundance in infected samples, but two out of five infected samples found between June and November 2017 contained no gametes. No trematodes were found in samples from male mussels in either system (n = 55 in Llano River, n = 67 in San Saba River).

Fecundity was higher at the Llano compared to the San Saba site and was generally higher for larger mussels (Fig. 3.3). The number of glochidia per female mussel ranged from 36,900 ± 1,100 to 49,600 ± 3,500 (rounded to the nearest hundred) in the Llano River with an average of 43,700 ± 3,700 (mean ± SE, n = 3, collected in March, length of mussels ranged from 42-28mm). In contrast, fecundity of mussels from the San Saba River were lower with 5,800 ± 500 of the smallest female (30mm length) compared to 25,200 ± 1,100 glochidia per female of the largest female (70mm) with an average of 17,500 ± 4,700 (n = 4, collected in July, Fig. 3.3).

Host Fish Experiment: Wild fish

Metamorphosis success differed significantly between host fish species for glochidia from both the Llano and San Saba rivers (Fig. 3.4). There was also considerable variation between individual host fish. For example, glochidia from Llano River mussels had the highest average metamorphosis success on Green Sunfish collected from the Llano River (45% average), which ranged between 27 and 76% (or 72 vs. 167 juveniles produced). Overall metamorphosis success tended to be highest on Green Sunfish, followed by Largemouth Bass, but was significantly lower for Bluegill Sunfish and Longear Sunfish (< 12 and < 1 % mean metamorphosis success respectively, Fig. 3.4), and 0 or <1% for Redbreast Sunfish and warmouth respectively.

The permutation ANOVA detected a significant effect of fish origin and fish species on metamorphosis success for the Llano mussels, whereas only fish species was a significant factor for the Saba mussels (Fig. 3.4). Metamorphosis success (mean values) was higher for sympatric than allopatric mussel-fish pairs in 4 of 7 comparisons, i.e. for the Llano River Green Sunfish (26% higher), Largemouth Bass (21% higher), and Bluegill Sunfish (4% higher), and the San Saba River Largemouth Bass (20% higher). However, individual variation was high, and differences were not statistically significant for the Llano River mussels: Green Sunfish (Student's t-test, $T_8 = 1.8$, $p = 0.11$), Bluegill Sunfish ($T_8 = 1.7$, $p = 0.13$), and marginally significant for the metamorphosis success of San Saba River mussels on Largemouth Bass from different origins (Welch's t-test $T_{5.6} = 2.4$, $p = 0.06$). Note that n = 2 for Largemouth Bass from Llano River (fish died), which hindered a statistical comparison.

The sloughing of undeveloped or dead glochidia was highest on day 2 (>3,800 glochidia) (Fig. A3, A4). Juvenile detachment peaked between day 18 (San Saba mussels, 4009 juveniles) and day 23 (Llano mussels, 766 juveniles). Green Sunfish and Guadalupe Bass had similar temporal patterns of detachment with the vast majority of juveniles detaching around ~15 days post inoculation. Recovery of both glochidia and juveniles from Green Sunfish and Guadalupe Bass was complete after 40 days. In contrast, juveniles detached from Largemouth Bass over a much longer period up to 48 (Llano mussels) and 62 (San Saba mussels) days post inoculation.

Host Fish Experiment: Hatchery Fish

Both Llano and San Saba mussels had a high metamorphosis success on Guadalupe and Largemouth Bass from the hatchery (Fig. 3.4). For Llano mussels, average metamorphosis success on hatchery fish were similar to wild Green Sunfish from the same river (44 and 60% in hatchery vs. 46% in wild fish, Fig. 3.4). For San Saba mussels, the metamorphosis success on hatchery fish was similar to the success rate on wild Largemouth Bass from the same river and Green Sunfish from both rivers (59 and 68% in hatchery vs. 73, 69, and 70% in wild fish, Fig. 3.4). Metamorphosis success on hatchery Guadalupe Bass and hatchery Largemouth Bass was similar in mussels from both rivers (San Saba: Student's t-test, $T_8 = 1.6$, $p = 0.15$, Llano: $T_8 = 2.0$, $p = 0.09$, Fig. 3.4). There were no significant differences in metamorphosis success between Largemouth Bass from wild (Llano and San Saba rivers) versus Largemouth Bass from hatchery origin for Llano mussels (Welch's t-test, $T_{9.2} = 1.1$, $p = 0.28$); or San Saba mussels (Welch's t-test, $T_{7.2} = 0.3$, $p = 0.74$).

Discussion

This study provides much needed information on the reproductive ecology of *L. bracteata* and is the first study to investigate host fish specificity among populations of *L. bracteata* using a fully-crossed study design. Metamorphosis success of glochidia was higher on several mussel-fish pairings from the same river. Numerous previous studies have examined host fish suitability for mussels with artificial infestation in the laboratory, but only few studies have investigated differences in host fish compatibility of mussels and fish of sympatric and allopatric river origin (e.g., Schneider et al. 2016, Bingham 2002, Caldwell et al. 2016). Only one other study examined mussel-fish pairings of different populations within the same drainage basin, but it looked at variation of infection success rather than metamorphosis success (Douda et al. 2014). Hence, to the best of our knowledge this is the first study to look at differences in metamorphosis success of mussels from different tributaries of a single river basin.

One may expect different adaptations to host fish between mussel populations that exhibit genetic differences, but a recent study on snuffbox, *Epioblasma triquetra*, in tributaries of the Laurentian Great Lakes did not find differences in metamorphosis success between sympatric and allopatric fish despite genetic differences between mussel populations (Caldwell et al. 2016). In contrast, our results suggest that different local adaptations at the sub-drainage level to host

fish may exist even though not geographically separated, which would parallel genetic differences recently found between mussel populations of the San Saba and Llano River (K. Inoue, Texas A&M, personal communication).

There is no consensus on host fish compatibility being higher with sympatric or allopatric fish. A study on freshwater pearl mussel, *Margaritifera margaritifera,* in southern Norway found allopatric fish strains to have higher number of encapsulated glochidia, a measure of host fish compatibility, in comparison to sympatric fish strains (Osterling and Larsen 2013), whereas highest infection rates and growth rates during the parasitic stage occurred on fish from within the natural distributional range of *M. margaritifera* in southern Germany (Taeubert et al. 2010). No differences in host suitability between sympatric and allopatric mussel-fish pairings were found for *E. triquetra* in the Great Lakes basin (Caldwell et al. 2016). A study on thick shelled river mussel, *Unio crassus,* in two geographically separated rivers of southern Sweden suggested that not all populations of a species may show the same adaptive tendencies in respect to host fish compatibility (Schneider et al. 2016). Populations in the Llano River may be more closely adapted to Green Sunfish from the same river and mussels in the San Saba River more closely adapted to Largemouth Bass from the same river. However, further research is needed to explore this hypothesis. We did not find mussel populations from different rivers to have adaptations to different host fish species that have been previously reported (Douda et al. 2014, Eckert 2003), but dispersal between these rivers has been restricted by the construction of major dams in the mainstem Colorado River in the 1930s, which is considered recent over evolutionary time scales.

Higher metamorphosis success should be expected from fish without previous exposure to mussels (i.e. higher in hatchery fish compared to wild fish), as laboratory experiments found that fish may acquire an immune resistance to glochidia upon exposure (Dodd et al. 2005). However, we only found minor differences in metamorphosis success on hatchery versus wild Largemouth Bass, which could be due to acquired resistance not being as common in the wild (Dodd et al. 2006). It is interesting to note that parent fish of hatchery Guadalupe Bass originated from the South Llano River, and metamorphosis success was higher on Guadalupe compared to Largemouth Bass where parents originated from a different basin (Red River basin). Unfortunately, we were not able to catch a sufficient number of Guadalupe Bass from the wild for experimental comparison between wild Guadalupe Bass and other host fish, thus future

experiments will be necessary to determine whether the differences between hatchery Guadalupe and Largemouth Bass were due to differences in species or origin of the parents.

Based on metamorphosis success alone, both wild and hatchery fish could be used for captive propagation of *L. bracteata.* However, using hatchery fish for captive propagation and reintroduction may have ecological risks, as domestication of juvenile mussels via (accidental) artificial selection may occur (Jones et al. 2006; Hoftyzer 2008). Such effects should be considered, as glochidia which metamorphose well on hatchery fish may not necessarily metamorphose well on wild fish and local adaptations may be lost. Although beneficial for retaining local adaptations in juvenile mussels for reintroduction, wild fish may be already infested with glochidia when collected and should therefore be collected well in advance of experiments to allow for detachment of wild juveniles.

With only a few host fish species from a single family, *L. bracteata* appears to have more specialized host requirements than mussels with more general host use such as Central Texas native and non-threatened Yellow Sandshell, *L. teres,* which can utilize host fish from many (5+) fish families (Ford and Oliver 2015). Our study found both Largemouth Bass and Green Sunfish (and hatchery Guadalupe Bass) to be the best host fish, while juveniles also metamorphosed on Bluegill Sunfish, but in smaller numbers. Like piscivorous Green Sunfish and Basses, Bluegill Sunfish will opportunistically consume a variety of prey, but are more limited by gape size. Thus Green Sunfish and Basses are more likely to attack a lure that resembles a darter (such as the lure of *L. bracteata*) than Bluegill Sunfish which likely feeds on smaller prey items (Mittlebach 1981, Carlander 1977). This may have facilitated a stronger adaptation of *L. bracteata* to Green Sunfish and the Basses tested in this study. In a previous study Green Sunfish produced the greatest number of juvenile mussels, followed by Bluegill Sunfish, and were considered good hosts for *L. bracteata,* whereas Largemouth and Guadalupe Bass—which produced 50% fewer juveniles than Green Sunfish in the study—appeared as less suitable hosts (Johnson et al. 2012). The longer observational timeframe (70 vs. 26 days post inoculation) used in our study compared to Johnson et al., (2012) may have contributed to the different findings. Largemouth Bass in our study produced fewer juveniles compared to Green Sunfish during the peak detachment period, but live juveniles continued to detach over a longer period of time (i.e., 45 vs. 26 days, Fig A3, A4).

Most Lampsilis species breed between late summer/fall and early spring (Barnhart et al. 2008) with the exception of *Lampsilis rafinesqueana*, which were found to be brooding between May and July (Shiver 2002). The observed brooding period of *L. bracteata* in this study appears to be much longer than previously known (July-October, Johnson et al. 2012) as brooding mussels were found throughout the study period (February 2017 to February 2018) except for October 2017. Other *Lampsilis* species such as *Lampsilis cardium* and *L. fasciola* have been reported to be brooding throughout the year (Lefevre and Curtis 1912, Stagliano 2001) or were observed brooding during most months of the year, like *Lampsilis hydiana* (Howells 2000).

L. bracteata is a long-term brooder and brooding females were observed throughout the year except in September-October at the Llano River site, and October-December samples at the San Saba site. Resumption of brooding was observed at least a month earlier at the Llano River site. The decline in the proportion of brooding females in summer co-incided with rising temperatures, but further research is needed to better understand the driving factors of the seasonal variation.

Seasonal variation in gamete concentration reflects variation in gamete production only if one assumes that the gamete fluid volume does not vary seasonally. Nevertheless, we observed some interesting patterns that could be related to seasonal variation in gamete production. Similar to brooding, variation in gamete concentration appeared to be at least partly related to temperature, as declines in egg concentration (both rivers) and sperm concentration in the Llano coincided with increasing temperatures, consistent with previous research (Galbraith and Vaughn 2009, Jirka and Neves 1992). Our results also indicated that sampling volume should be restricted to small amounts (<100 microliters), as larger amounts may dilute the gamete samples by pulling hemolymph from the hemocoel in addition to the gonads. Such dilution could explain the logarithmic decline we found between gamete concentration and sampling volume.

It is possible that lower gamete densities, brooding, fecundity, and glochidia viability in San Saba mussels, could at least in part be associated with the gonadal parasites detected a higher prevalence in female mussels from the San Saba compared to the Llano River. These gonadal parasites can castrate mussels (Haag and Staton 2003). Trematodes (Family Bucephalidae) were also detected in a congener, *Lampsilis rafinesqueana* in Missouri, USA (Shiver et al. 2002). The present study is the first to document *Bucephalus sp.* in *L. bracteata.*

Environmental differences may also play a role or interact with the presence of the trematodes, such as temperature and flow (Young and Williams 1984, Watters and O'Dee 1998). *L. bracteata* is a long-term brooder, which tend to brood during colder months, and elevated temperatures likely decrease brooding duration and glochidia viability (Zimmerman and Neves 2002). The Llano River had much higher discharges (range: 1-48 m^3s^{-1}) than the San Saba River (range: 0.4-1.3 m^3s^{-1}) during the survey period which may have contributed to the lower thermal minima seen in the Llano and allowed mussels to remain brooding and maintain glochidia viability for a longer period (Zimmerman and Neves 2002). The lower flows in the San Saba may have also contributed to the infection rate of *L bracteata* with larvae of *Bucephalus sp.*, because lower flows may allow parasites to accumulate in higher densities, (D.G. Huffman, personal communication). Tsakiris et al. (2016) reported a high incidence (> 20%) of digenetic trematodes in *C. petrina* and *C. houstonensis* in the San Saba River between July 2012 and July 2013 during an exceptional drought in Texas, whereas a more recent study in 2017 found only ~5% of *Cyclonaias* to be infected (ANS, unpublished data). In addition, mussels may also have been more stressed by higher minimum temperatures in the San Saba River.

This study has implications for captive breeding of freshwater mussels and suggests that further exploration of host fish and mussel stock origin should be evaluated. Future studies should consider longer-term survival of juvenile mussels in relation to host fish origin, as mussel propagation may require host fish from a particular location based on where the mussels originated. Monitoring juveniles through the most sensitive portion of the mussel life-cycle (the early post-parasitic stage) could better-explain the relationships between fish and mussel stock origin (Buddensiek et al. 1993). Future studies should consider the effects of mixing glochidia of parent mussels from different locations, as this could reduce local adaptations (via outbreeding depression) and make them more susceptible to changes in the environment (Denic et al. 2015; Hoftyzer et al. 2008). To avoid this problem, parent mussels should be collected locally and offspring reintroduced to the same area (Hoftyzer et al. 2008).

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Tables and Figures

Table 3.1 Summary of water quality data collected at both sites in the Llano and San Saba River as mean values of all sampling events and the range is given in parenthesis. Parameters with an asterisk indicate that there was a significant difference ($p<0.05$ after Bonferroni correction) between sites.

	Llano River	San Saba River
pH*	8.3 (8.2-8.5)	8.1 (7.9-8.2)
Specific conductivity * (µS/cm)	375 (338-410)	505 (453-547)
Chlorophyll-*a** (µg/L)	0.6 (0.2-2.8)	1.3 (0.5-2.9)
Total suspended solids (mg/L),	0.05 (0.02-0.06)	0.05 (0.04-0.07)
Dissolved oxygen (mg/L)	9.5 (5.6-14.8)	8.3(6.6-12.6)

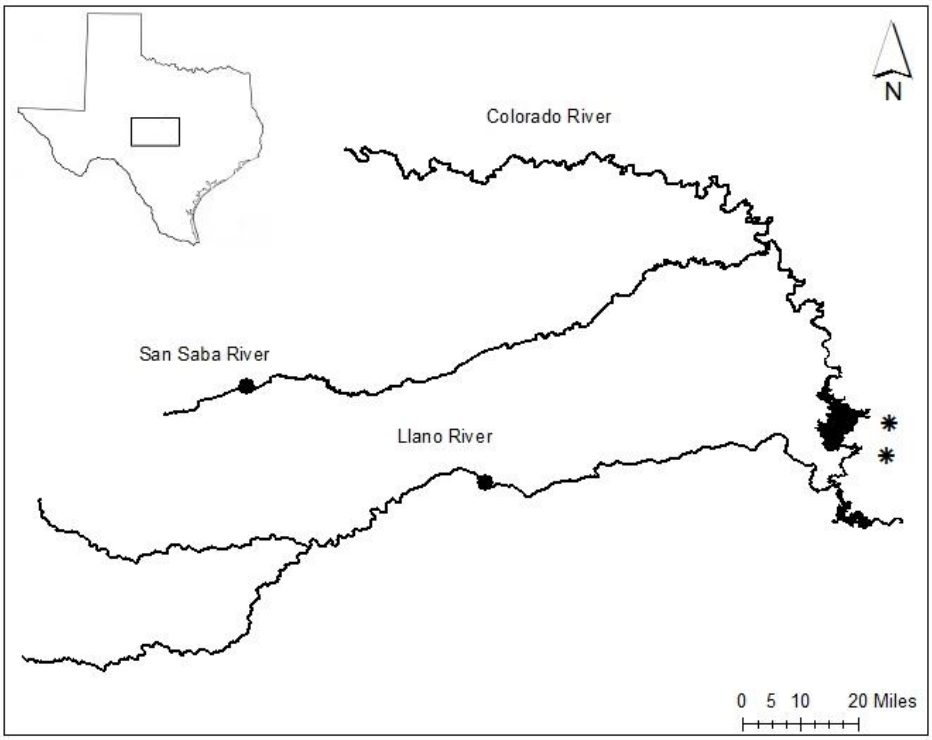

Figure 3.1 Map of sampling sites in two tributaries of the Colorado River Basin.
Sampling sites (black circles) in the San Saba River near Menard, TX and Llano River near Mason, TX. Rivers are separated by two major dams (asterisks): Buchanan Dam (upstream) and Inks Dam (downstream).

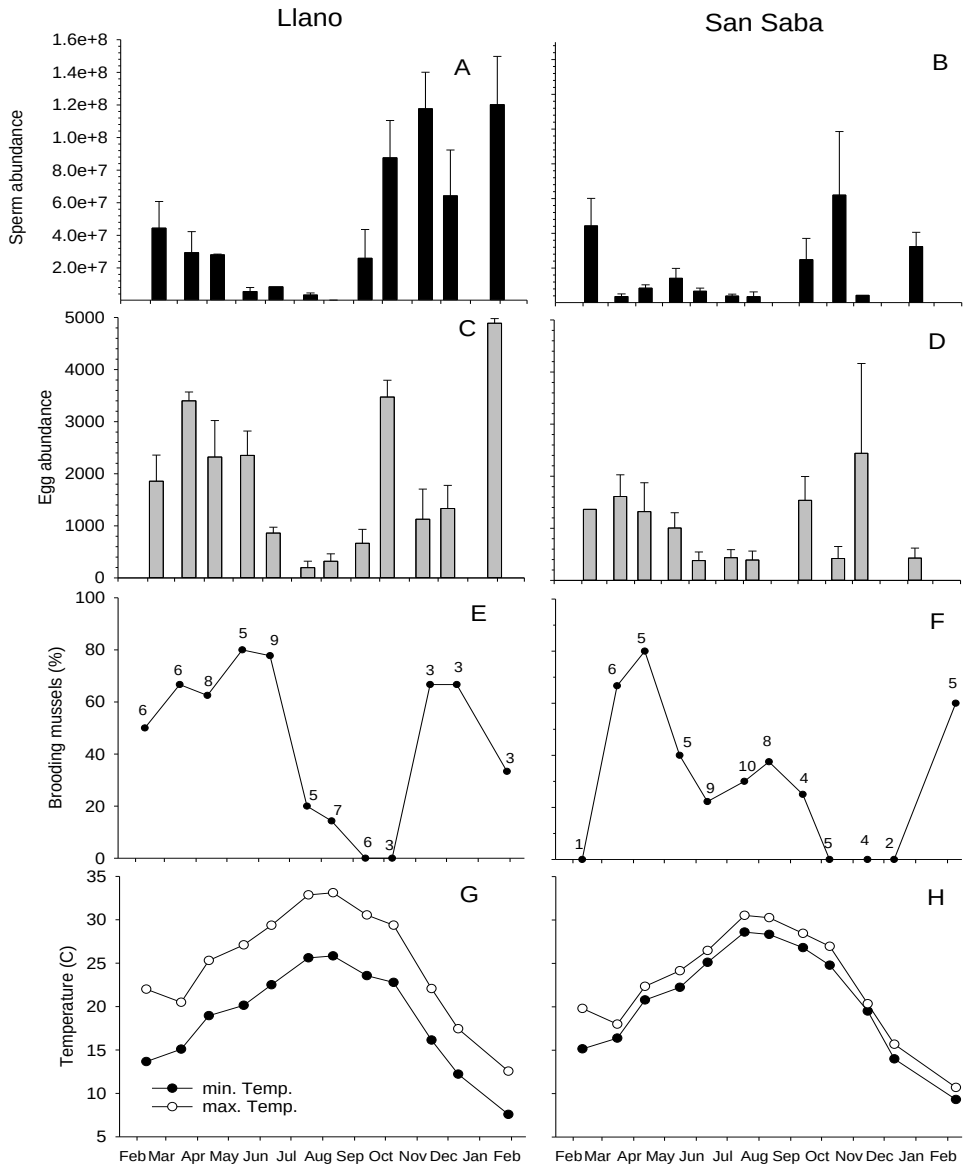

Figure 3.2 Seasonal variation of gamete abundance (mean number of gametes ± SE) for sperm (A, B) and eggs (C, D), the proportion of brooding mussels (E, F), and average minimum and maximum water temperatures (G, H) at the Llano (A, C, E, G) and San Saba site (B, D, F, H).

Numbers above data points indicate sample size. Note that the sperm abundance in August 2017 was low, but not 0.

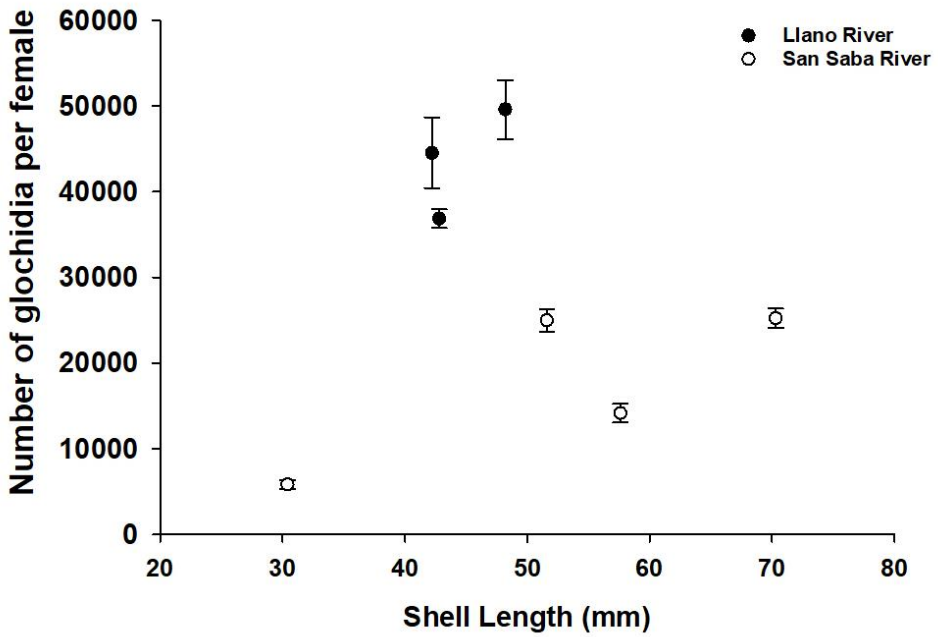

Figure 3.3 Fecundity (number of glochidia per female) in relation to shell length of mussels in the Llano (black circles) and San Saba (white circles).

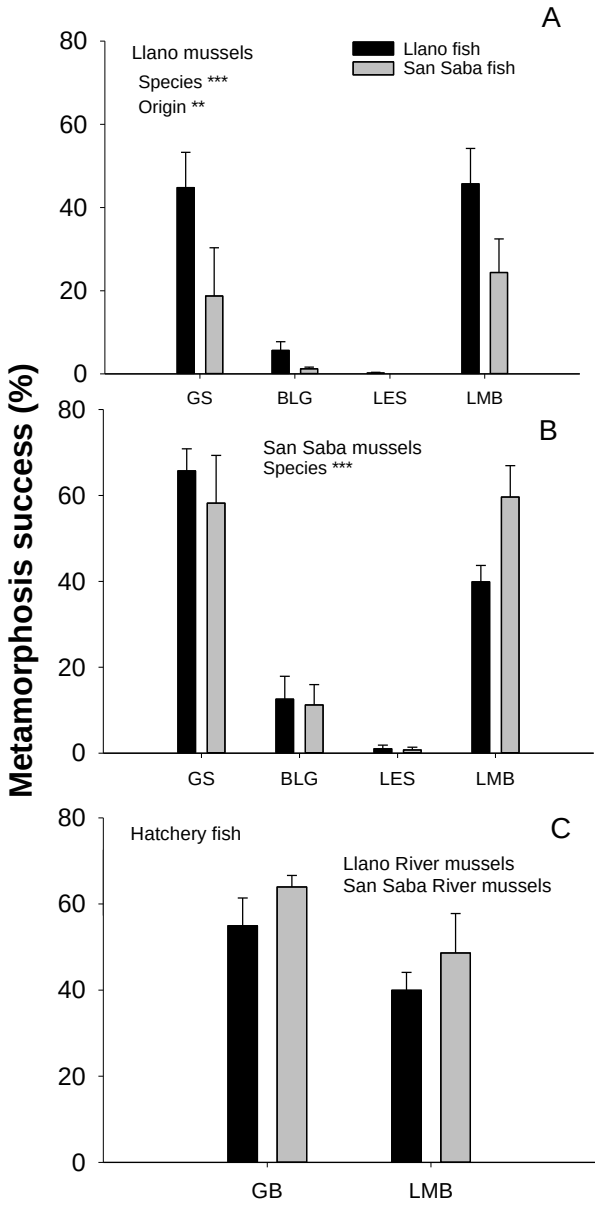

Figure 3.4 Metamorphosis success (mean ± SE) as percentage of glochidia which successfully metamorphosed into juvenile mussels on different fish species and fish from different origin.

A) Llano River glochidia on wild fish, B) San Saba River glochidia on wild fish, and C) Llano and San Saba River glochidia on hatchery fish. Species codes are as follows: RBS= Redbreast Sunfish, GS= Green Sunfish, WM= warmouth, BLG= Bluegill Sunfish, LES= Longear Sunfish, LMB= Largemouth Bass, GB= Guadalupe Bass. Significant effects detected by the ANOVA are indicated with asterisks: $p \leq 0.01$ (**), $p \leq 0.001$ (***). Sample sizes were n=5 fish, except in A) LES from San Saba (n=3) and LMB from the Llano (n=2), in B) n=4 for WM and GS from San Saba, LES and LMB from Llano, and

n=3 for WM from Llano and LMB from San Saba, and in C) Guadalupe Bass from the hatchery (n=4).

Appendix

Table 3A.1 Sampling dates and methods in the Llano River.
Sampling methods (marked with X) during each sampling event at the Llano River between April 2016 and September 2017.

Sample Date	Sex Ratio	Gravidity	Glochidia Viability	Gamete
Apr. 30th 2016	L	L	L	NA
May 24th 2016	L	L	L	NA
Jun. 24th 2016	L	L	L	NA
Jul. 14th 2016	L	L	L	NA
Nov. 10th 2016	L	L	NA	NA
Feb. 10th 2017	L, S	L, S	L, S	L, S
Mar. 16th 2017	L, S	L, S	L, S	L, S
Apr. 12th 2017	L, S	L, S	L, S	L, S
May 16th2017	NA	L, S	L, S	L, S
Jun. 12th 2017	L, S	L, S	L, S	L, S
Jul. 18th 2017	L, S	L, S	L, S	L, S
Aug. 11th2017	L, S	L, S	L, S	L, S
Sep. 13th 2017	L, S	L, S	L, S	L, S
Oct. 11th 2017	L, S	L, S	L, S	L, S
Nov. 15th 2017	L, S	L, S	L, S	L, S
Dec. 13th 2017	L, S	L, S	L, S	L, S
Jan. 29th, 2018 (L) and Feb. 9th, 2018 (S)	L, S	L, S	L, S	L, S

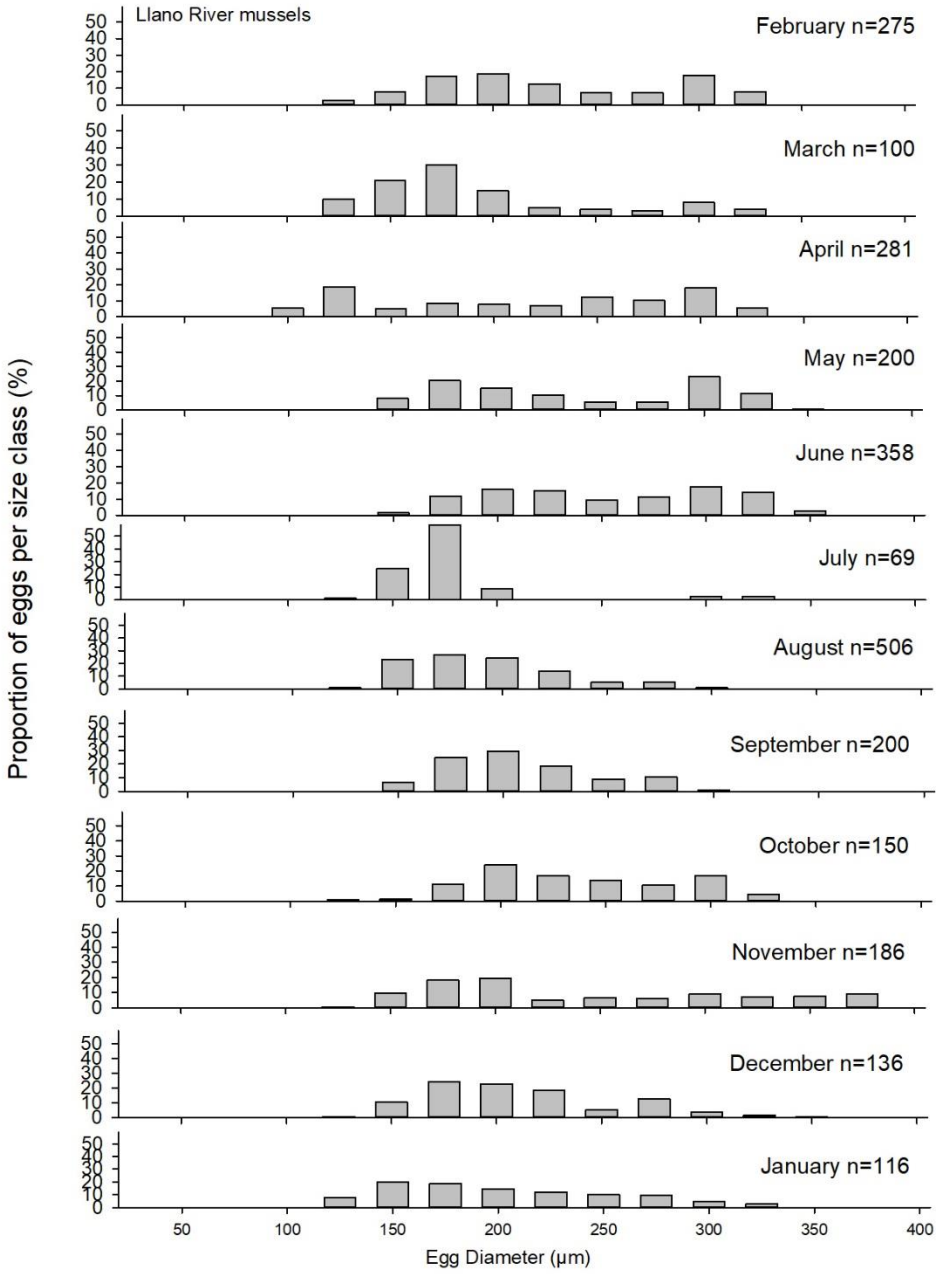

Figure 3.A1 Egg size distribution in mussels from the Llano River during monthly sampling events between February 2017 and January 2018.
Number of eggs measured per month shown in each panel. Number of individuals from which eggs were extracted are as follows: February 2017—6 individuals, March—2, April—7, May—4, June—8, July—2, August—11, September—4, October—3, November—4, December—3, January 2018—2.

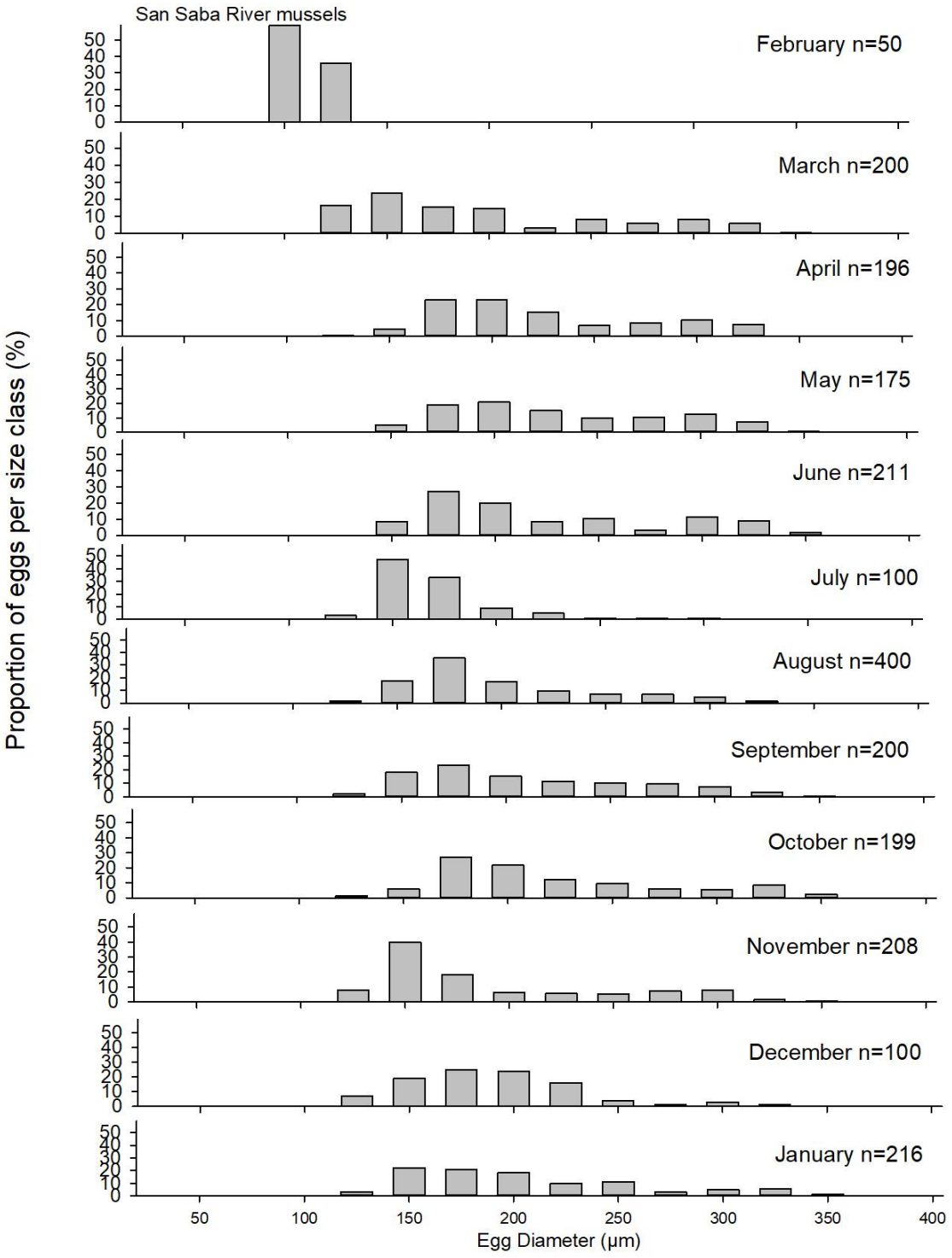

Figure 3.A2 Egg size distribution in mussels from the San Saba River during monthly sampling events between February 2017 and January 2018.

Number of eggs measured per month shown in each panel. Number of individuals from which eggs were extracted are as follows: February 2017—1 individuals, March—4,

April—4, May—4, June—7, July—2, August—8, September—4, October—4, November—4, December—2, February 2018—5.

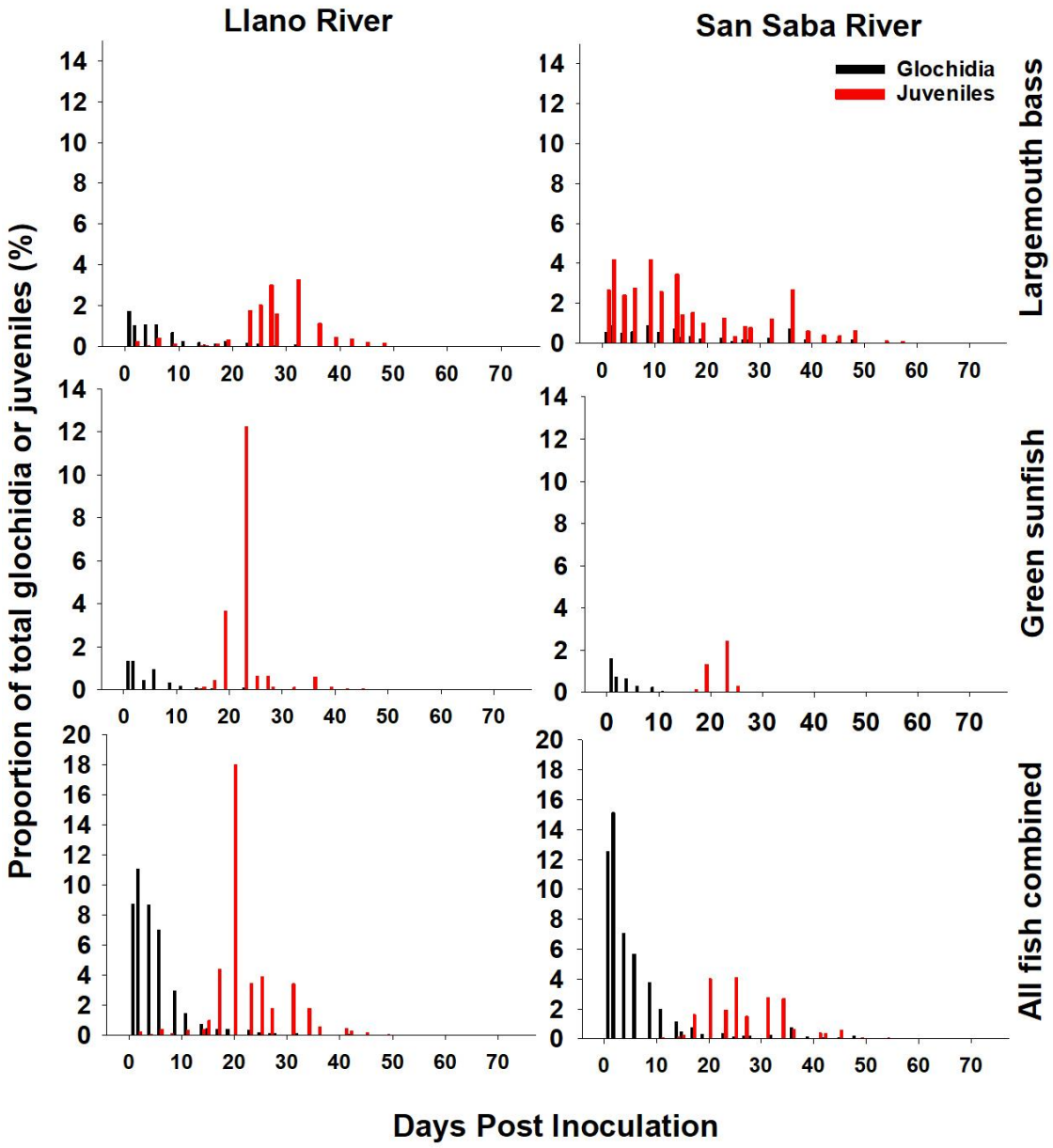

Figure 3.A3 Developmental dynamics of Llano River L. bracteata glochidia on host fish. Bars indicate the proportion of glochidia (black bars) or juveniles (red bars) recovered from Llano host fish (left panel) and San Saba host fish (right panel) the respective day after inoculation with Llano mussel glochidia.

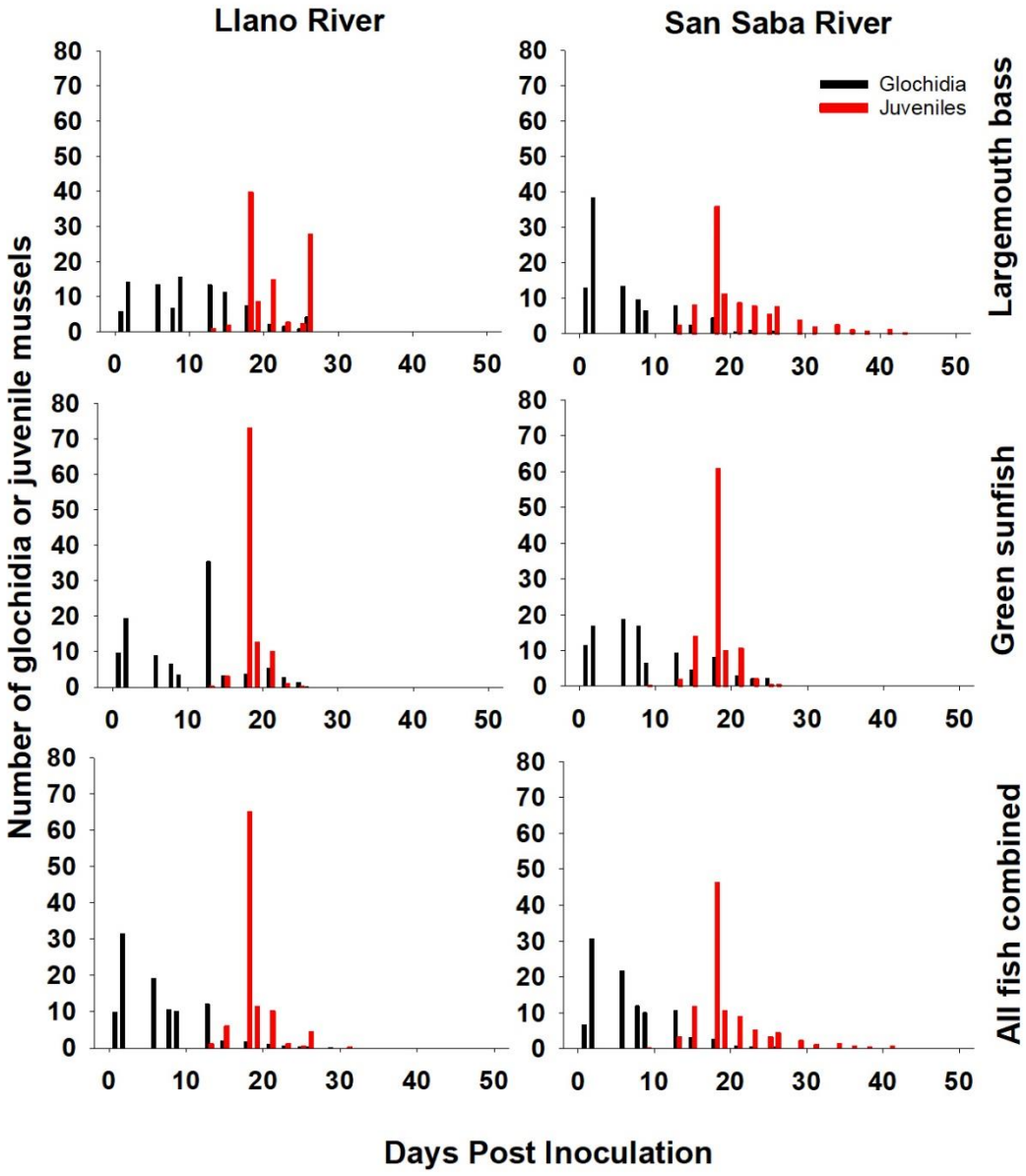

Figure 3.A4 Developmental dynamics of San Saba River glochidia on host fish.

Developmental dynamics of San Saba River glochidia on host fish. Bars indicate the number of glochidia (black bars) or juveniles (red bars) recovered from Llano host fish (left panel) and San Saba host fish (right panel) the respective day after inoculation with San Saba mussel glochidia.

4. Seasonality of gamete production of *Cyclonaias (Quadrula)* species

by Zachary Mitchell and Astrid Schwalb

Abstract

Reproductive traits are important life history characteristics for freshwater mussels, which can impact population health. Unfortunately, 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. Hence, the objective of this study was to evaluate the reproductive timing of two freshwater mussel species in central Texas, which are currently candidates for federal listing. Sex ratios varied between species and rivers. Additionally, gamete densities for both species varied seasonally and with temperature in both the Llano and San Saba Rivers. Our results show that seasonal variation in gamete density are associated with changes in temperature with gamete density being significantly lower when temperature was higher in the Llano River and a similar trend in the San Saba River. In addition, the reproductive output of *Cyclonaias* appear to be more limited in the San Saba River due to several potential stressors (e.g. parasites). Further research will need to investigate the potential impacts that these parasites might have on the long-term persistence of these mussel populations and how they are interacting with other stressors in the system.

Introduction

Reproductive traits such as reproductive timing and sex ratio are important life history characteristics for freshwater mussels, which affect population dynamics. Yet, life history data is 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, especially in Texas. For example, strongly skewed sex ratios could potentially be indicators of decreasing reproductive health within mussel populations. Similarly, long-term seasonal gamete production data can give insight into environmental and anthropogenic stressors that may be limiting the reproductive output of mussel populations during certain times of the year. For example, digenetic trematodes are known to parasitize the gonads of freshwater mussels and can affect reproduction (Laruelle et al. 2002; Tsakiris et al. 2016). Additionally, a better understanding of the seasonality of gamete

production would improve the timing of collection of brooding mussel and host fish collections to ultimately make propagation methods more predictable and efficient.

To the best of our knowledge only one study has investigated gamete production of mussels within Texas (see Tsakiris et al. 2016). Hence, the objective of this study was to evaluate the reproductive timing of two freshwater mussel species (*Cyclonaias petrina* and *C. houstonensis*; formerly *Quadrula*) in central Texas, which are currently candidates for federal listing. Our objectives were to 1) determine the sex ratios of our two species of interest, 2) quantify monthly gamete production of both males and females, and 3) quantify infection rates of parasitic digenetic trematodes within our study species in two tributaries of the Colorado River, TX.

Methods

Study Sites

We established three study sites in two rivers located in central Texas, USA. We selected one site in the upper Llano River and two sites in the lower San Saba River, both of which are tributaries of the Colorado River, TX (Fig. 4.1). The Llano River site can be characterized as a run habitat consisting of mostly bedrock with patches of rock, cobble, and silt, whereas, the San Saba sites can be characterized as riffles.

Field Sampling and Lab Processing

In the Llano River, *C. petrina* were collected monthly between February 2017 and February 2018. In the San Saba River, *C. petrina* and *C. houstonensis* were collected between June 2017 and February 2018. Since cold water temperatures can negatively impact the burrowing ability of freshwater mussels (Block et al. 2013), no samples were collected during December 2017 or January 2018 in the San Saba river as water temperatures were below 10° C. Temperature was collected with a temperature logger in the Llano River (see chapter 3), whereas temperature was only measured at each sampling event in the San Saba River (measurements taken between 0800-1100 hrs.).

Mussels were collected using visual and tactile methods. All individuals were identified, measured, uniquely tagged and sampled for gonadal fluid using a nonlethal syringe technique (see Tsakiris et al. 2016). No mussel was sampled twice during the study period to reduce handling bias. All samples were stored in 10% formalin and transported back to Texas State

University for processing. See Chapter 3 for detailed descriptions of field and lab processing techniques used for gamete samples.

Data Analysis

We used chi-square tests to assess whether sex ratios were significantly different from a male to female ratio of 1:1. The relationship between gamete densities and water temperature were evaluated with simple linear regression. Assumptions of normality and homogeneity of variance were tested using the Shapiro-Wilk and Levene's test, respectively. Densities were log10(x) transformed to better meet the assumptions of regression. We used student's t-tests to compare gamete densities between the Llano and San Saba species.

Results

We collected gamete samples from 254 mussels, which included 201 samples from *C. petrina* (129 from Llano River, 72 from San Saba River), and 53 samples of *C. houstonensis* from the San Saba River. Gametes were found in 76 % (n = 193) of samples from both species. The majority of samples without gametes (44 of 61 samples) were collected from the San Saba River. Trematodes were found in 5% (n = 13) of all collected gametes samples, which were almost exclusively (12 of 13 samples) from the San Saba River.

The sex ratio for *C. petrina* in the Llano River were dominated by females (0.6 males per female, $X^2_1 = 7.13$, $p < 0.01$, n=110), whereas in the San Saba River *C. petrina* was dominated by males (2.2 males per female; $X^2_1 = 5.16$, $p < 0.05$, n = 38). The sex ratio for *C. houstonensis* in the San Saba River did not significantly differ significantly from a 1:1 sex ratio ($X^2_1 = 0.02$, $p > 0.05$, n = 41).

Gamete density varied seasonally and with temperature in the Llano River (Fig. 4.2 A-D), where gamete density of *C. petrina* declined with increasing temperatures (Sperm density: $F_{(1,11)} = 12$, $p < 0.01$, adj. $R^2 = 0.48$; Egg density: $F_{(1,11)} = 4.55$, $p = 0.05$, adj. $R^2 = 0.23$; Fig. 4.3). Highest sperm densities for *C. petrina* in the Llano River occurred between December and February of 2017 and 2018, whereas the lowest concentrations (3 orders of magnitude lower) were found in July and September 2017 (Fig. 4.2A). Similarly, egg density of *C. petrina* in the Llano River were highest during February 2017 and then tended to decline during warmer months (1 order of magnitude) (Fig. 4.2C).

There was no significant relationship between gamete density and temperature in the San Saba River, in which less sampling events occurred (n = 7 instead of 13, Fig. 4.2 B,D,F). In the San Saba River, sperm density was highest during July 2017 and February 2018 for both species. Sperm density in *C. petrina* decreased substantially (3 orders of magnitude lower) into November 2017 (Fig. 4.2B). Such a pattern was not observed for *C. houstonensis* in the San Saba River, with individuals containing similar sperm densities over time. Egg densities were lowest for both species in the San Saba River in June 2017 and tended to increase (1 order of magnitude higher) to October 2017 (Fig. 4.2D). In most cases, *C. petrina* and *C. houstonensis* from the San Saba River exhibited a similar pattern for sperm and egg density when compared to their Llano River counterparts (student's t-test: $p > 0.05$; Fig. 4.2 C,D).

Egg diameters varied between 30 and 435 µm and differently sized eggs were present throughout the year (Fig. 4.4 to 4.6). The largest size classes of eggs for *C. petrina* and *C. houstonensis* were relatively least abundant in August 2017. The largest size classes were most abundant in February 2018 for *C. petrina* at the Llano site and San Saba site and in September for *C. houstonensis* in the San Saba River.

We noted a logarithmic decline of *C. petrina* gamete densities with sample volumes, but not in *C. houstonensis* samples. The relationship for *C. petrina* was more pronounced for eggs ($R^2 = 0.22$, $P < 0.001$, n = 78) compared to sperm ($R^2 = 0.12$, $P < 0.01$, n = 67, samples from both rivers combined). Thus, gamete densities may have been estimated in samples with higher sample volume. However, samples with considerably higher volume (>1mL, sperm: n = 31, egg: n = 10) were spread out relatively evenly between months. Thus, the seasonal pattern and magnitude of change in gamete density between months would likely be similar to our results, as month was still a significant factor when the variables month, sampling volume, and their interaction were included in the linear model. All factors together explained 50% of the variation on egg densities ($F_{(22,55)} = 4.5$, $p < 0.001$) and 38% of the variation in sperm densities ($F_{(24,42)} = 2.7$, $p < 0.01$).

Discussion

Our results show that seasonal variation in gamete density are associated with changes in temperature with gamete density being significantly lower when temperature was higher in the Llano River and a similar trend in the San Saba River. In addition, the reproductive output of

Cyclonaias appear to be more limited in the San Saba River due to several potential stressors (see below). Our results also indicated that sampling volume should be restricted to small amounts (100µl), as larger amounts may dilute the gamete samples by pulling hemolymph from the hemocoel in addition to the gonads (Chris Barnhart, pers. comm.), thereby potentially explaining the logarithmic decline we found between gamete density and sampling volume for *C. petrina*.

Sex ratios for *C. petrina* were significantly different from 1:1 in both rivers, but such biased sex ratio is often thought to be caused by sampling bias and low sample sizes and likely has limited ecological value, especially if the deviation is less than 2:1 (Haag 2012). The largest deviation was observed in the San Saba River, but the lower sample size (n=38) in that river may have contributed to this result.

Although seasonal variation in gamete density reflects variation in gamete production only if one assumes that the gamete fluid volume does not vary seasonally (see also Chapter 3), we observed some interesting patterns that could be related to seasonal variation in gamete production. Our results indicate that gamete density in *Cyclonaias* species vary seasonally with peak gamete production occurring in the fall and winter months, which is similar to the findings of another study within central Texas that examined the reproduction and survival of four species (*C. petrina, C. houstonensis, Quadrula apiculata, Tritogonia verrucosa*) for one year (Tsakiris et al. 2016). Similarly, to our study, Tsakiris et al. (2016) saw the lowest gamete concentration during summer and fall months, however they recorded peak gamete production 1-3 months later than in our study. Additionally, Jirka and Neves (1992) reported low levels of active gametogenesis throughout the year in *Cyclonaias tuberculata* with a pulse of mature gametes being produced and held during late fall and winter months, as seen in our *Cyclonaias* species. *Cyclonaias petrina* showed similar seasonal patterns of gamete production in both the Llano and San Saba Rivers, however this pattern was clearer for the site in the Llano River due to a larger number of samples. 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).

Most tachytictic brooders spawn primarily during the spring and summer months (Haag 2012), but no brooding *Cyclonaias* were found during our sampling events. Our gamete data suggest that the collection of host fish and mussels for captive propagation of *Cyclonaias* spp.

should be started around the end of April/May when egg and sperm densities have largely decreased, and water temperatures begin to increase.

Similar to findings for *L. bracteata* (see Chapter 3), reproduction appeared to be more limited at sites in the San Saba River, where a larger number of samples without gametes were found. It should be noted that many of these samples without gametes were collected during warmer months in the San Saba River and those individuals might not have had any gametes present at that time as peak gamete production largely occurred during the winter months. Furthermore, some mussels may experience reproductive senescence due to various stressors (e.g. higher water temperatures), resulting in a lack of gametes, however this theory has not been well studied in freshwater mussels (Haag 2012). Alternatively, the higher incidence of trematodes in the San Saba River may also have played a role. Digenetic trematodes have been known to feed on gonadal tissue in some mussel species and substantially lower or eliminate the reproductive output of individuals (Fuller 1974; Taskinen and Valtonen 1995; Gangloff et al. 2008). Parasitic infestation rates in mussels are usually low (< 6%; Haag and Stanton 2003; Haag 2012), but some studies have shown relatively high (20-30%) infestation rates in multiple species (Zale and Neves 1982; Tsakiris et al. 2016). Tsakiris et al. (2016) reported high infestation levels (> 20%) of digenetic trematodes in *C. petrina* and *C. houstonensis* 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. Interestingly, the mean monthly discharge during our study period was significantly higher (mean discharge: 64 cfs) at our San Saba sites compared to flow conditions during the Tsakiris et al. (2016) study (mean discharge: 41 cfs). The decreased discharge within the San Saba River could have concentrated parasitic trematodes within the channel, leading to higher infection rates. In the San Saba River, trematodes were found in gamete samples of *Lampsilis bracteata* during cooler months (February-April, chapter 3) whereas parasites were mostly found during the summer and fall months (June-November) for both *Cyclonaias* species. Further research will need to investigate the potential impacts that these parasites might have on the long-term persistence of these mussel populations and how they are interacting with other stressors in the system.

Acknowledgments

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Figures

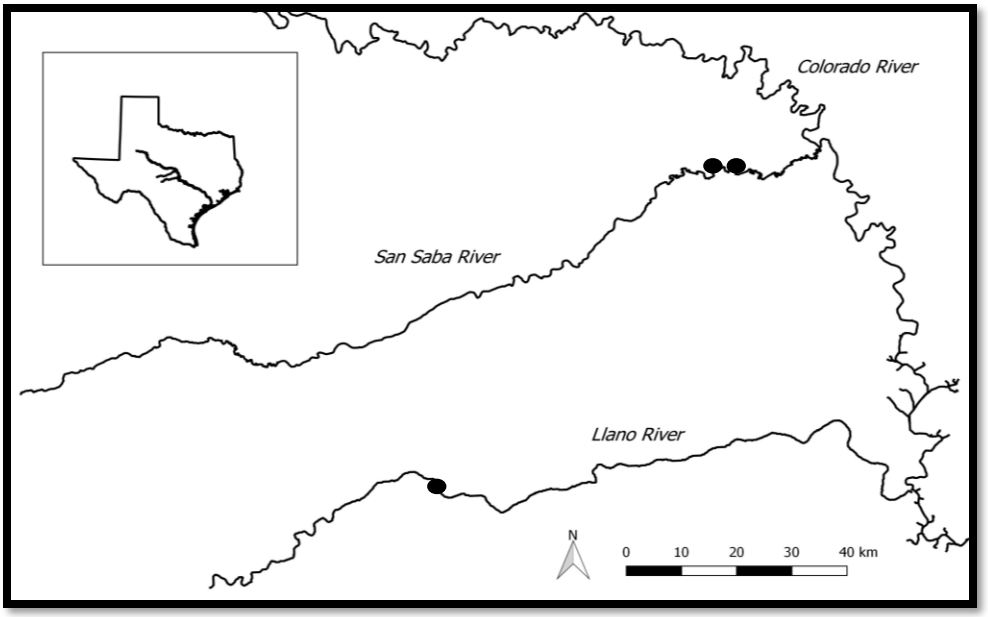

Figure 4.1 Site map for gamete collections in the Llano and San Saba Rivers, TX.

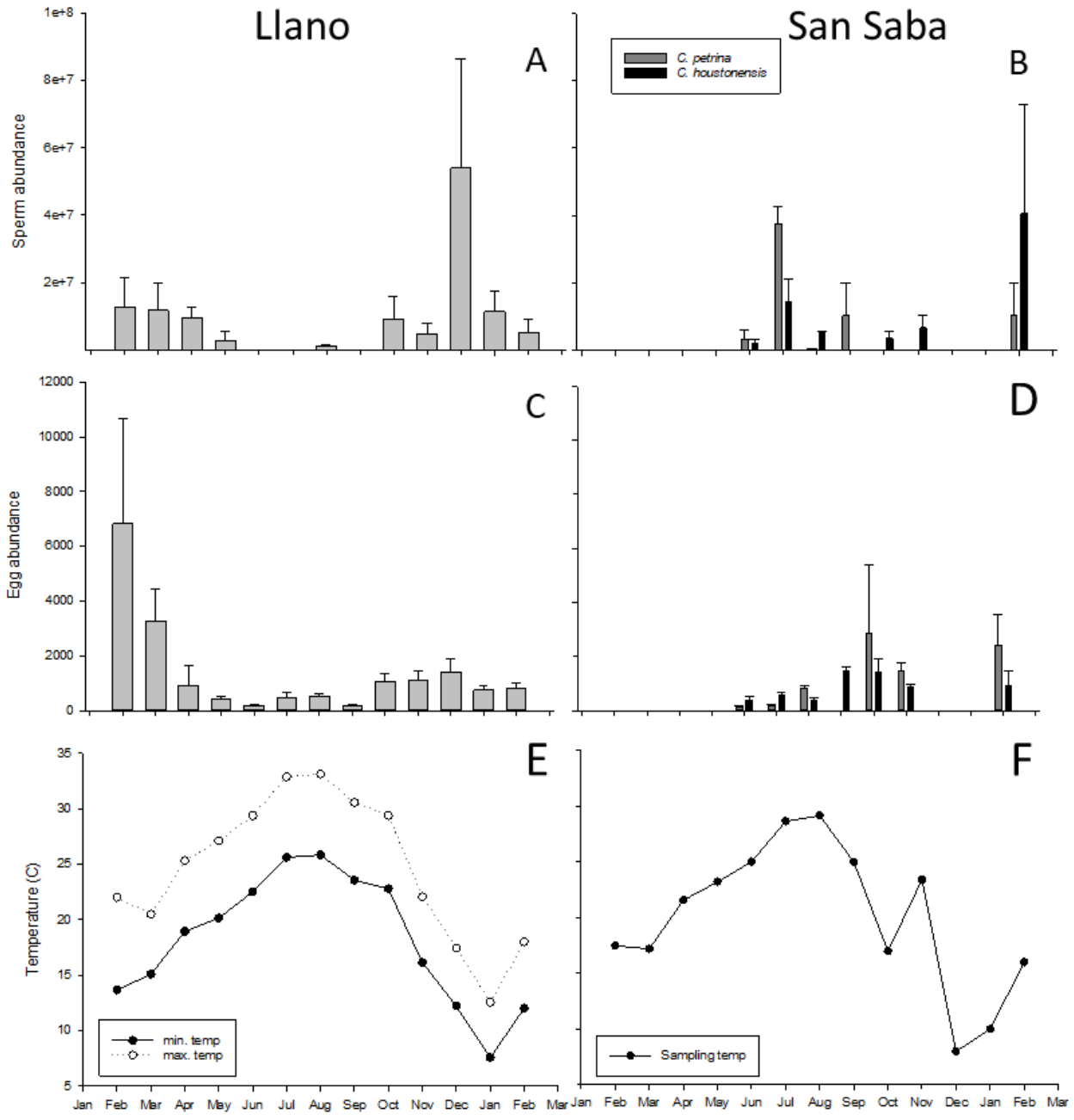

Figure 4.2 Monthly gamete production (mean ± SE) for two candidate species of mussels in the Llano and San Saba Rivers, TX.

Gray bars denote *C. petrina* and black bars denote *C. houstonensis*. Temperatures for the Llano River (E) represent mean monthly maximum and minimum recorded on data loggers, whereas temperature data for the San Saba River (F) represent measurements taken during sampling events (taken between 0800-1100 each month).

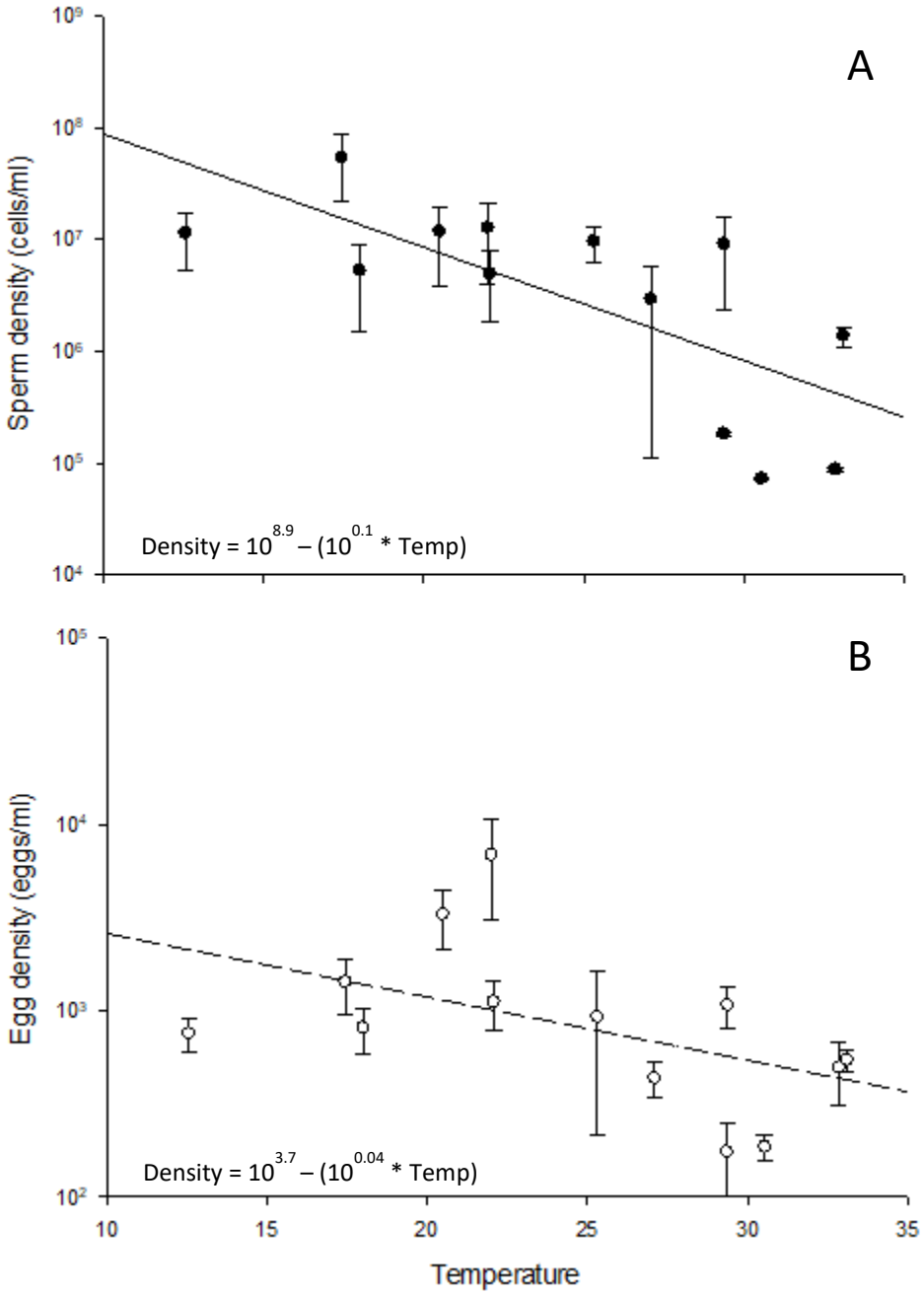

Figure 4.3 Relationship between *C. petrina* sperm (A) and egg (B) density and water temperature within the Llano River, TX.

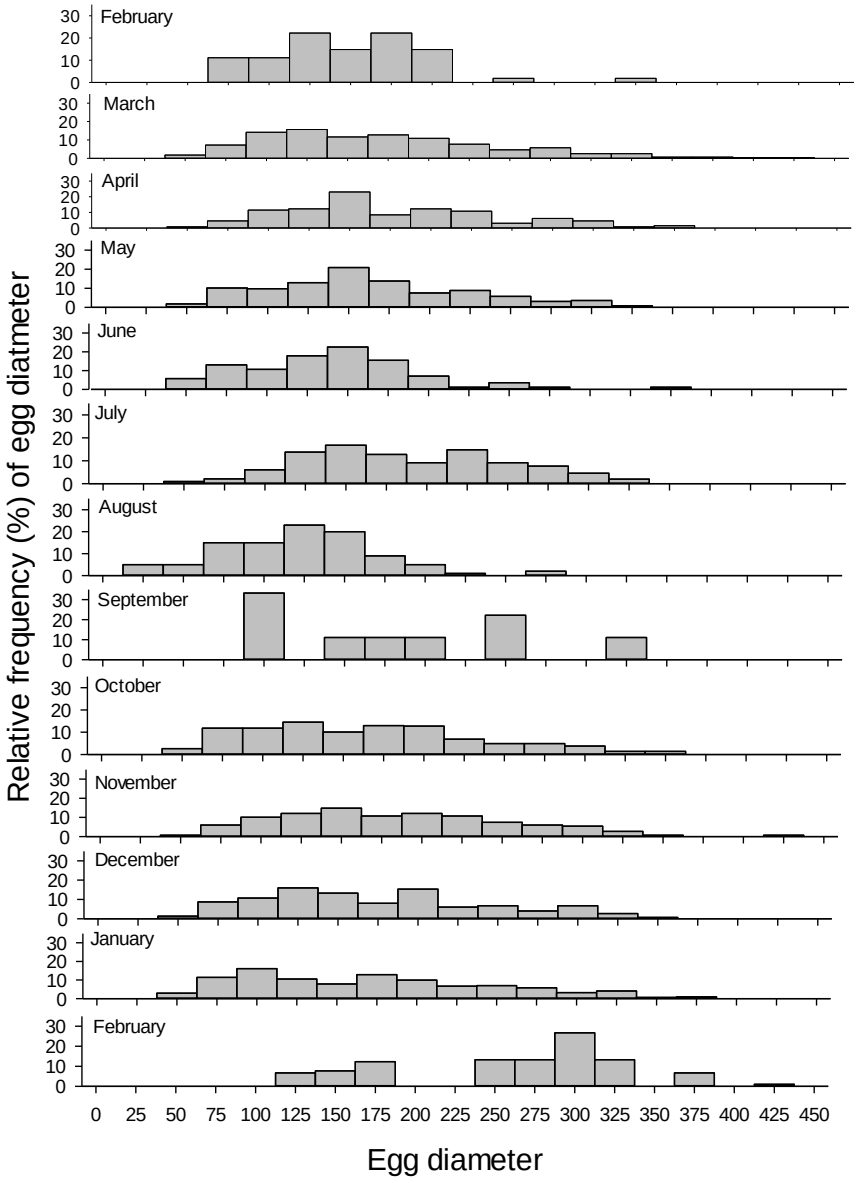

Figure 4.4 Egg size distribution of *C. petrina* from the Llano River during monthly sampling events between February 2017 and February 2018.

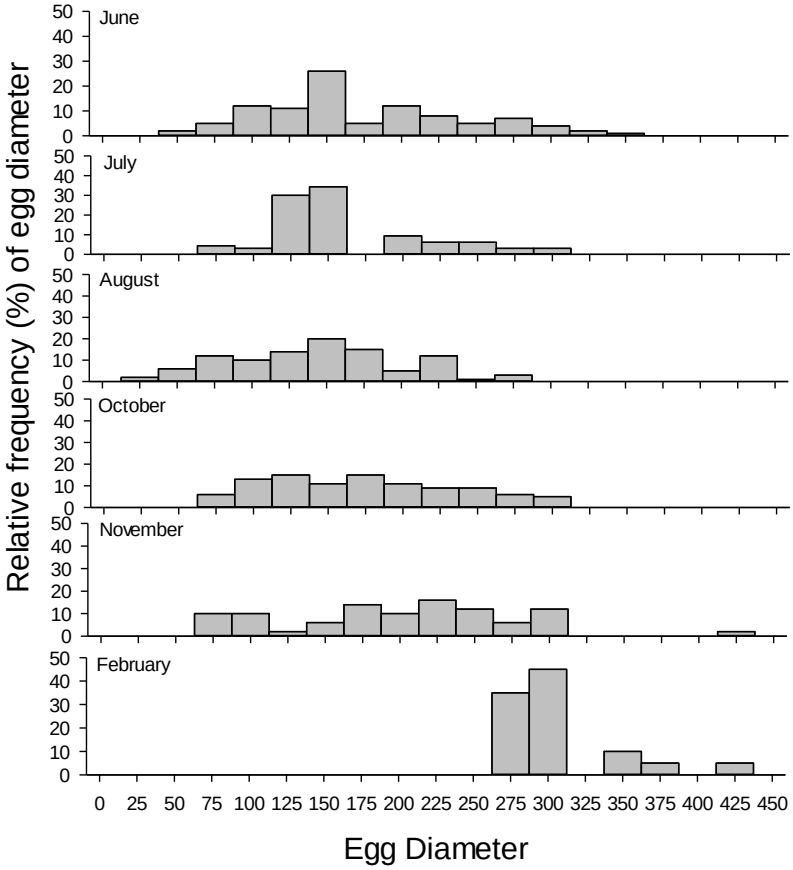

Figure 4.5 Egg size distribution of C. petrina from the San Saba River during monthly sampling events between June 2017 and February 2018.

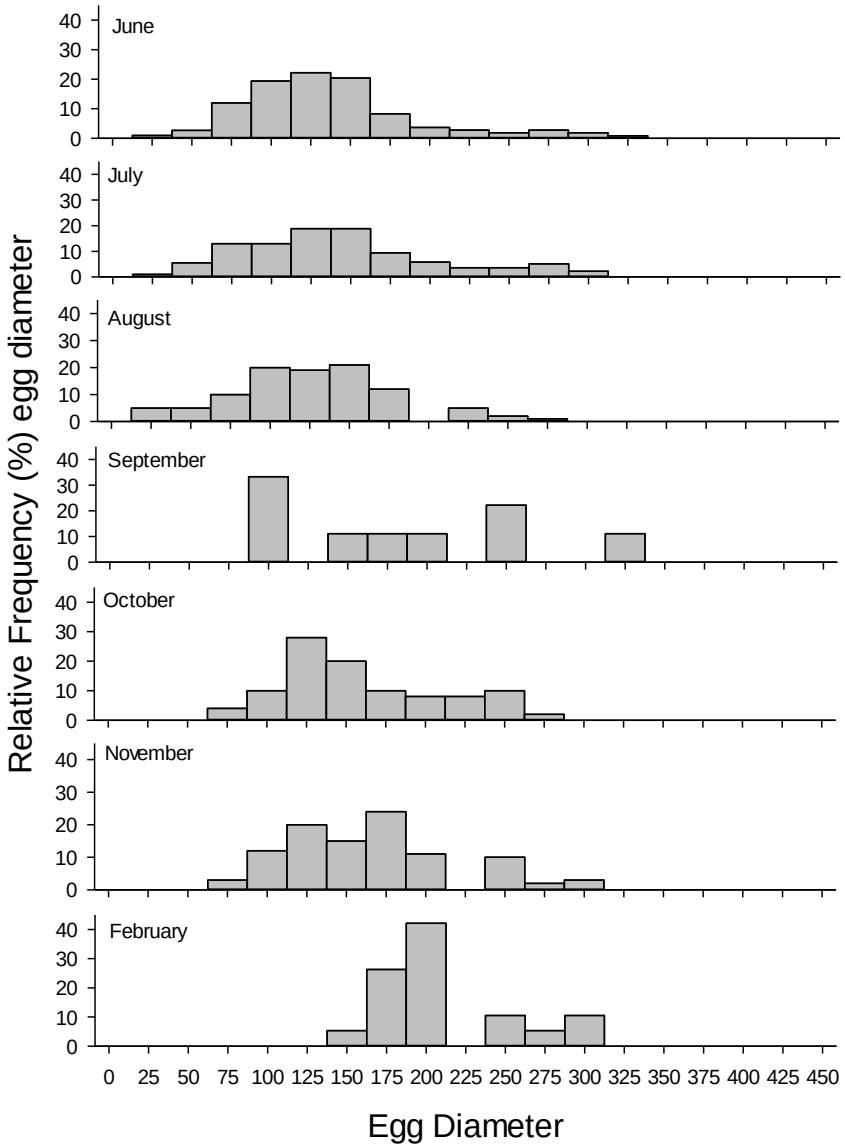

Figure 4.6 Egg size distribution of *C. houstonensis* from the San Saba River during monthly sampling events between June 2017 and February 2018.

5. Examining population augmentation as an alternative to relocation with genetic tools, and using the genetic structure of wild populations to determine the minimum number of gravid females required to mimic wild genetic diversity under captive propagation

Abstract

We genetically identified 462 mussels captured via surveys and opportunistically across five river drainages in Texas. While our field sampling focused on threatened species within the genus Cyclonaias (Quadrula), we also collected 12 other mussel genera to validate our genetic assays. Haplotype network and phylogenetic analyses show distinct groups and clades within Quadrula, for the most part. Three species (Q. aurea, Q. houstonensis, and Q. pustolosa) show lower levels of genetic divergence compared to other species (Q. petrina, Q. nodulata, C. necki sp. nov., Q. mortoni, Q. apiculata, and Q. nobilis). Clades of Q. aurea and Q. houstonensis are polyphyletic but associate with drainage but not by river. Microsatellite data show Q. houstonensis and Q. aurea form distinct genetic demes and the variation is partitioned by drainage. Species groups also show contrasting patterns of demographic histories, but generally Q. aurea and Q. mortoni show higher haplotype richness, while Q. houstonensis, Q. petrina, and Q. nobilis show lower richness based on our sample. Numbers of females required for captive propagation can be scaled by these patterns of genetic diversity.

Introduction

North America has the richest freshwater bivalve fauna in the world, yet mussels are one of the most imperiled groups of organisms on the continent. The diverse mussel fauna in Texas includes several regional and Texas endemics with the largest number of endemics found in Central Texas. Across North America, dramatic declines have occurred owing to changes in land use, habitat destruction (e.g., channel modification, dams), pollution, introduced species, and commercial exploitation (Lydeard et al. 2004). In 2009, 15 of the 52 known native mussel species (Unionidae) in Texas were listed as threatened in the state (Texas Register 2010). Specifically, *Quadrula aurea*, *Q. houstonensis*, and *Q. petrina* were listed as candidates for federal protection (Federal Register, Vol. 81, No. 232, 2016). We acknowledge the recent

reclassification of *Quadrula* species into the genus *Cyclonaias* as supported by strong genetic evidence (Texas A&M Institute of Renewable Natural Resources IAC# 314-5283-2RR); although, we retain the current taxonomy in this report. The protection and conservation of freshwater mussels are only possible through a better understanding of their ecology; more so, ecological patterns should be assessed and measured for distinct evolutionarily significant units, because variances among ecological correlates may overlap and obscure true distributions if identifications are incorrect. Our goal is to use genetic markers to catalog and measure genetic variation among Texas mussels and to determine the minimum number of gravid females required to mimic wild genetic diversity under captive propagation. The focus of this report is to highlight our results on the genetic diversity in wild populations of *Quadrula* and sympatric mussel species from different basins of Central Texas.

Methods

Fine and Coarse-scale Makers

Tissue samples were obtained by brush-swabbing the mantle and foot of live mussels (Henley et al. 2006), which is a non-lethal, non-consumptive method. We extracted DNA from those swabs using the GeneJet DNA extraction kit (ThermoFisher, Inc.). Previously published primers were used to amplify mitochondrial 12S (course scale) and ND1 (fine-scale) sequences (Serb et al. 2003). Amplicons were cycle sequenced using BigDye v3.1 chemistry and sequenced on an ABI 3500xl (Applied Biosystems, Inc.) and the resulting chromatograms were edited and trimmed in Geneious 9.1.4. We also investigated the population genetics of *Quadrula* and *Lampsilis* species using previously published microsatellite markers (fine-scale).

Mitochondrial Sequence Data

To date, we have sequenced 462 mussels collected in Texas at the ND1 locus. Using MUSCLE as implemented in Geneious 9.1.4, we created an alignment of all newly generated sequences and trimmed the alignment to 526 bp that were congruent across all individuals. A translation alignment was used to ensure spurious indels were absent. The alignment was used to build an initial neighbor-joining tree to visualize broad scale sequence similarity across individuals. All the sequences were passed through a *de novo* assembly set at 95% minimum

overlap identity to create contigs of closely related taxa. The consensus sequences of these contigs were used to perform a BLAST search to assign taxonomy. Using custom R scripts, we visualized the NJ tree and mapped the river drainage of origin on the branch tips to assess clustering of genetic diversity according to river and broad taxonomic group (Fig. 5.1).

Quadrula *Haplogroups*

Based on raw sequence similarity, we partitioned the dataset (n = 235) to include only individuals most likely within the *Quadrula* species group, regardless of origin or morphological call, and *Tritogonia verrucosa* (formerly known as *Quadrula verrucosa*). We used POPART to generate a median-joining network scaled by sample size and representing river of origin for each sample (Fig. 5.2). Five major haplogroups were resolved by 20 to 48 mutational changes with a generally strong association to river (Fig. 5.3). The major haplogroups correspond to named species *Tritogonia verrucosa, Q. nodulata, Q. nobilis, Q. apiculata,* and *Q. petrina.* One shallow haplogroup corresponds to *Q. aurea, Q. pustulosa, Q. mortoni, and Q. houstonensis.* Another haplogroup sister to the *Q. petrina* group is most likely a newly described species *Cyclonaias necki* (Burlakova et al. 2018).

Phylogenetic References

We compared the haplotype variation within Texas rivers to closely related sequences accessioned into GenBank (see Fig. 5.4 for accession numbers and reported species names) and an unpublished dataset of *Q. pustulosa* from Canada (Harding et al. unpublished) owing to the close phylogenetic affinity to *Q. aurea, Q. houstonensis*, and *Q. petrina*. We used MUSCLE, implemented through Geneious 9.1.4 (Biomatters Ltd.), to align reference sequence data with Texas *Quadrula.* The de-replicated alignment (430 bp) was used to generate a Bayesian phylogeny to infer phylogenetic placement of Texas individuals. We inferred five clades within the *Quadrula* species group. Clade 1 was broadly distributed and corresponds to *Q.* aff. *pustulosa*, while Clades 2, 3, and 4 showed phylogenetic affinity to known references for *Q.* aff. *aurea*, *Q.* aff. *mortoni*, and *Q.* aff. *houstonensis*, respectively (Fig. 5.4). Clade 6 was well-supported and sister to Clade 5 (*Q. petrina*), this evolutionarily significant unit is most likely *Cyclonaias necki* recently described by Burlakova et al. 2018.; although, they used COI sequence

data to diagnose this taxon while we used ND1. Thus, we will have to confirm the assignment using their marker system. Clade 1 is particularly problematic as it shows very little resolution among named groups, which are polyphyletic with respect to *Q. aurea*, *Q. pustulosa*, and *Q. houstonensis*.

Microsatellite Data

Using the mitochondrial assignments, we tested a panel of 19 loci for *Quadrula*, which were initially designed for *Q. fragrosa* (Hemmingsen *et al*., 2009; Roe & Boyer, 2015), and 15 loci for *Lampsilis*, which were initially designed for *L. abrupta* (Eackles & King, 2002). We found that most loci amplified inconsistently, failed to amplify, or were monomorphic in our sample. Only five loci were polymorphic for *Quadrula* (QfD103, QfC109, QfC114, QfR9, and QfD5) and four loci were polymorphic for *Lampsilis* (LabD92, LabD213, LabC23, and LabD71).

Quadrula *Population Genetics*

Using a threshold of 25% missing data, we were able to genotype 131 individuals within the *Quadrula* group including 45 reference samples from Canadian *Q. pustulosa* (Harding *et al*. unpublished), which we analyzed using Bayesian clustering via STRUCTURE. Our model assumed admixture, correlated allele frequencies across populations, and no linkage between loci. We ran 20,000 iterations as a burn-in period and then 30,000 iterations for values of *K* ranging from 1 to 8 with five runs per value of *K*. The resulting data were summarized with Structure Harvester Web v0.6.94 (Earl and vonHoldt 2012) that implements the Evanno method for inferring the most likely number of populations (*K*) given the data (Evanno *et al*. 2005). Individual assignment proportions were summarized using CLUMPP (Jakobsson & Rosenberg, 2007) and visualized using custom R scrips. The L(*K*) plateaued at $K = 4$ and ΔK peaked at $K = 3$ (62.3) with ΔK for $K = 4$ was the second highest value (44.9). Given the values of L(*K*) and ΔK (Fig. 5.5), we used $K = 4$ as the number of genetic demes that best represents the data. For the most part, there was a strong association between genetic deme and haplogroup (Fig. 5.6) with many individuals showing high (> 0.90) assignment probabilities. We were able to confidently place individuals unidentifiable in the field into an ESU and found instances of misidentifications. More so, the nuclear data show support for morphological assignments with

regards to *Q. aurea* and *Q. houstonensis*. In the mitochondrial data, *Q. mortoni* shows strong association to locality and morphology, although the nuclear data show admixture or uncertainty in deme assignment. Only two representatives from the Clade 6 were genotyped, and they showed similar levels of admixture between the deme representing *Q. petrina* and *Q. aurea*; although, this may indicate homoplasy owing to marker bias. Several other individuals also exhibited admixture or incongruent assignments to their respective haplogroups. Using *Q. pustulosa* as an outgroup, several individuals showed shared alleles, which is peculiar given their geographic distance.

Glochidia Genetics

The ability to genetically type individual glochidia is required before assessing diversity among glochidia sampled directly from females or from water column collections. We attempted to measure the limit of detection by extracting 1, 10, 50, and 100 glochidia in 50 ul of PrepMan® Ultra Sample Preparation Reagent (ThermoFisher, Inc.). This helped to prevent any loss of DNA owing to inefficient binding of nucleic acids to the silica column in the GeneJet protocol. We then attempted a semi-nested PCR reaction using ND1 primers. Among the glochidia PCR tests, the first reaction in the semi-nested protocol resulted in non-detectable bands on an agarose gel, although the second PCR using a newly designed internal primer resulted in positive PCR bands of the expected size for all glochidia numbers tested. This showed that we can generate sufficient amplicons to identify a single glochidium using this semi-nested PCR protocol.

We compared diversity between captive *L. bracteata* females and their glochidia to wild populations. While we were able to successfully amplify and sequence ND1 from glochidia, the amplification of microsatellites was less effective, likely because of lower copy number or the lack of species specific primers. Despite these limitations we assessed the allelic diversity of *L. bracheata* in the wild (w), captive gravid females (f), and individual glochidia (g) collected from captive females. Only assessing female allelic diversity will underestimate the number of females needed to mimic natural populations, because the allelic contribution of males is unaccounted for. Allelic diversity of glochidia should include the alleles present in both parents and potentially multiple parents in the case of multiple paternity. We found that allele diversity was relatively low in two of the polymorphic loci, potentially owing to the loci not being optimized for this taxon. Although, a third locus showed a large amount of allelic diversity. As

expected the diversity in the wild population is typically higher than in captivity, and most alleles are shared with mother. We detected several alleles not found in the female population indicating that these alleles have originated from males (Fig. 5.7). A sample size of eight captive females captures on average 45% of the allelic diversity present in the wild population. Their progeny, on the other hand, represent 60% of the measured diversity (Table 5.1). These values will likely change based on increased sampling, although an additional eight females, sixteen total, from across the range may produce progeny that are closer to the wild population diversity.

Demographic Histories

Using the partitions based on morphology, nuclear DNA, and mitochondrial DNA, we assessed each clade for signatures of population expansions or contractions via haplotype networks and mismatch distributions. In a general sense, unimodal mismatch distributions skewed to the right indicate a demographic expansion, while those skewed to the left might indicate a more recent population expansion after a contraction. Distributions that are multimodal might suggest a stable population given appropriate sample sizes. In comparison, *Q. apiculata* shows signatures of population stability (or alternatively multiple ESUs), while *Q.* aff. *aurea, Q. mortoni,* and *Q. nobilis* show signatures of a demographic expansion after a contraction (Fig. 5.8). Clade 4 (*Q.* aff. *houstonensis*) and *Q. petrina* show evidence of a recent population contraction, or decline; although, if grouped according to genetic deme, which combines Clade 4 and Clade 1.1, the population shows a pattern of stability. We lacked sufficient sampling for *Q. nodulata* and *C. necki sp. nov.,* although populations of *T. verruscosa* show evidence of a significant bottleneck. We also assessed haplotype richness within each phylogenetic clade via rarefaction using the *rarecurve* function of the *vegan* package in R (Fig. 5.9). The results show that *Q. aurea* and *Q. mortoni*, are relatively haplotype rich (15 – 25) based on our sample (n > 20), thus many females (~25) would be needed in captive breeding efforts to mimic the genetic diversity (mtDNA) in the wild. Other species for which we have sufficient sampling, *Q. nobilis, Q. petrina*, and *Q. houstonensis*, show lower haplotype richness (5 – 10), and thus we estimate at least 10 females would be needed for captive propagation. These estimates are assuming that each female would carry of each of the unique mtDNA haplotypes we have detected. In these rarefaction analyses, we only included individuals from Clade 2 (*Q.* aff. *aurea*) and Clade 4 (*Q.* aff. *houstonensis*) as Clade 1.1 and Clade 1.2 form unique

haplogroups and thus may represent different ESUs. Additional regional sampling may concomitantly reveal additional haplotypes and will affect these estimates.

General Recommendations

The field identification of freshwater mussels is complicated by variation in expertise and cryptic diversity. Misidentifications can potentially obscure which abiotic and biotic factors are relevant to the maintenance of mussel populations, and thus affect management strategies. To increase the efficiency of conservation and management actions, mussels should be genetically tested whenever possible, although we acknowledge that smaller mussels can be difficult to sample. In association with morphological and environmental accessory data, the genetic data can be used to assign individuals to an ESU, which may or may not correspond to a formally described taxon.

In our investigation, we encountered instances of misidentifications and a dearth of genetic resources for Texas mussels including the lack of reference data and optimized genetic markers. In future population assessments, we recommend developing clade specific nuclear markers (microsatellites) or using a reduced genomic representation approach to assess fine-scale patterns of diversity (GBS, ddRAD, or ezRAD). Using both mitochondrial and nuclear markers is important, because there may be incongruence in the partitioning of genetic variation (e.g. Clade 1.1 and Clade 4) used to define ESUs. One glaring deficiency is the lack of reference sequences for the taxa encountered in Texas, more specifically whole mitochondrial genomes, which will serve to increase the number of comparable base pairs across taxa, populations, and sub-populations, and may help to resolve short branch lengths. Caution should also be exercised in captive propagation efforts, because admixture may be occurring between recently diverged taxa, as evidenced by our microsatellite data. This study along with others currently in progress will provide genetic reference data that will be helpful in future population surveys and captive propagation efforts.

Other practical avenues could possibly include field-based methods for genetic identification using these well-characterized mitochondrial genomes and known spatial distribution of genetic diversity. With regard to conservation strategies, we suggest that managers use all relevant data to designate ESUs as opposed to relying solely on taxonomy, which is in flux and has been based on morphological assessments that can be subjective based

on collector experience or on characters showing wide, overlapping variance among populations and between species.

Tables and Figures

Table 5.1 Number of alleles present in each group per locus.
In parentheses, the number of shared alleles with the wild populations for captive females f(w), and the number of shared alleles with the wild population and females for glochidia g(w,f).

Loci	Sample size	LabC23	LabD92	LabD71
Wild	19	3	23	4
Captive Females	8	1(1)	6(3)	3(3)
Glochidia	23	2(2,1)	5(3,2)	4(4,3)

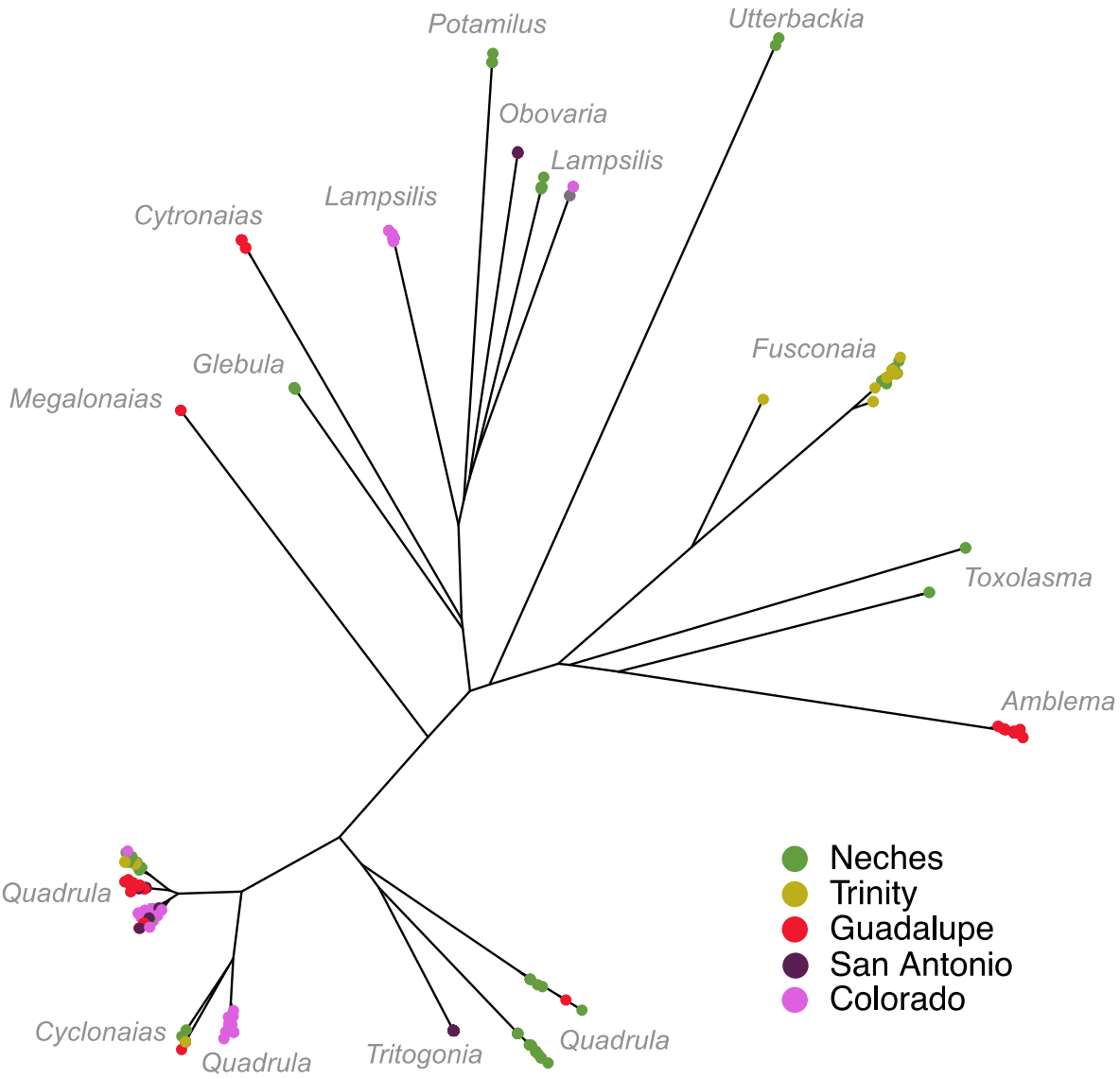

Figure 5.1 Unrooted neighbor-joining tree based on a pairwise distance matrix using a Jukes-Cantor model.
Closest Genbank reference taxon (genus-level) is overlaid in grey.

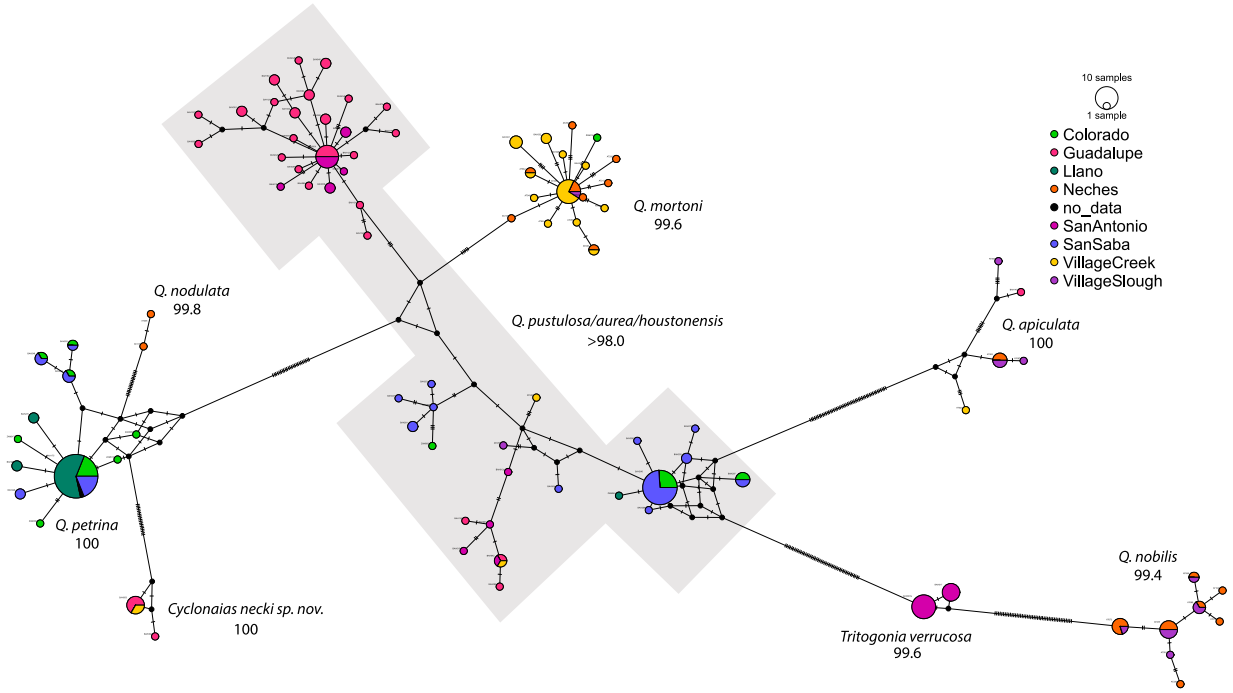

Figure 5.2 Median-joining network of ND1 haplotypes likely within *Quadrula*. *Tritogonia verruscosa* was included as a comparative outgroup.
Colors indicate water body of origin.

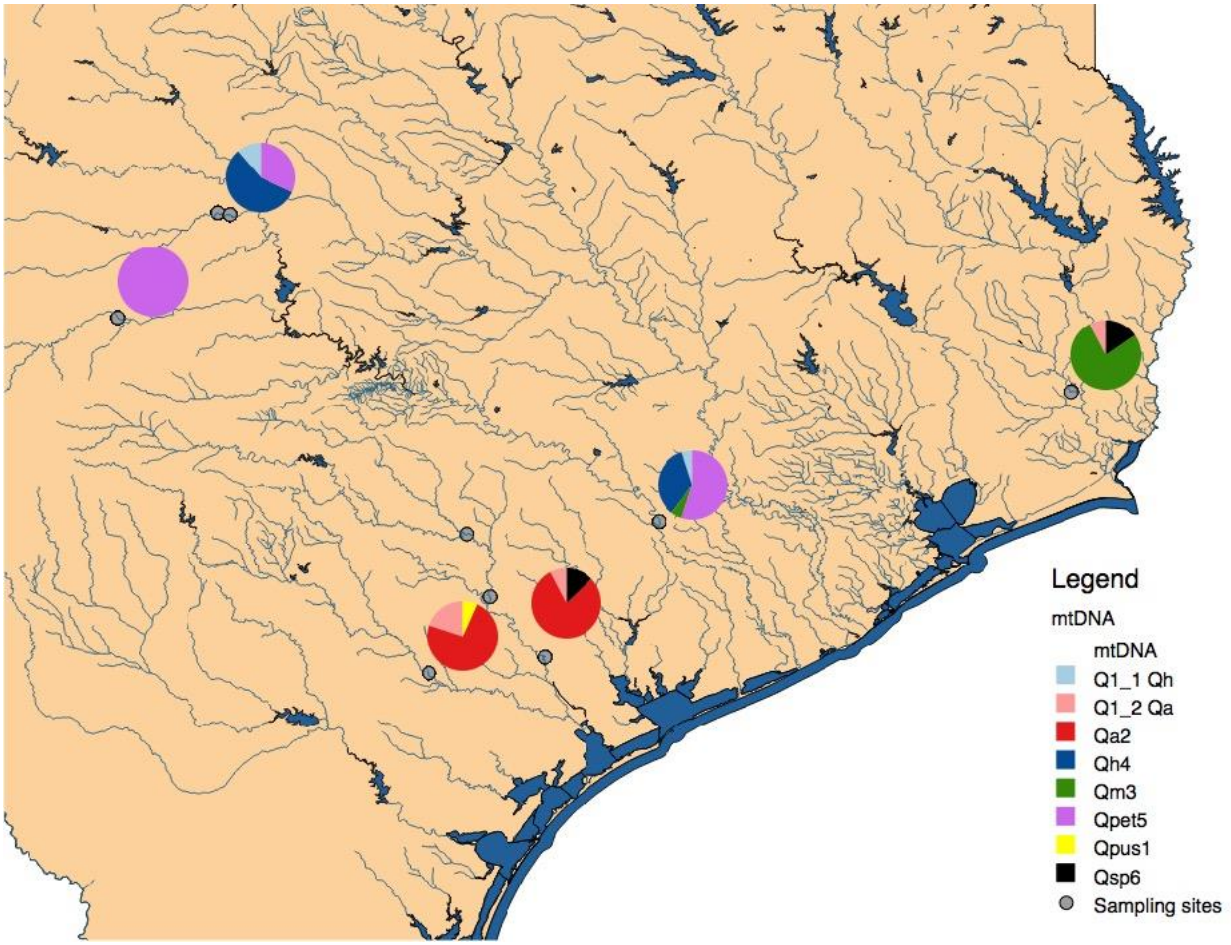

Figure 5.3 Sampling sites and proportion of haplogroups per site.

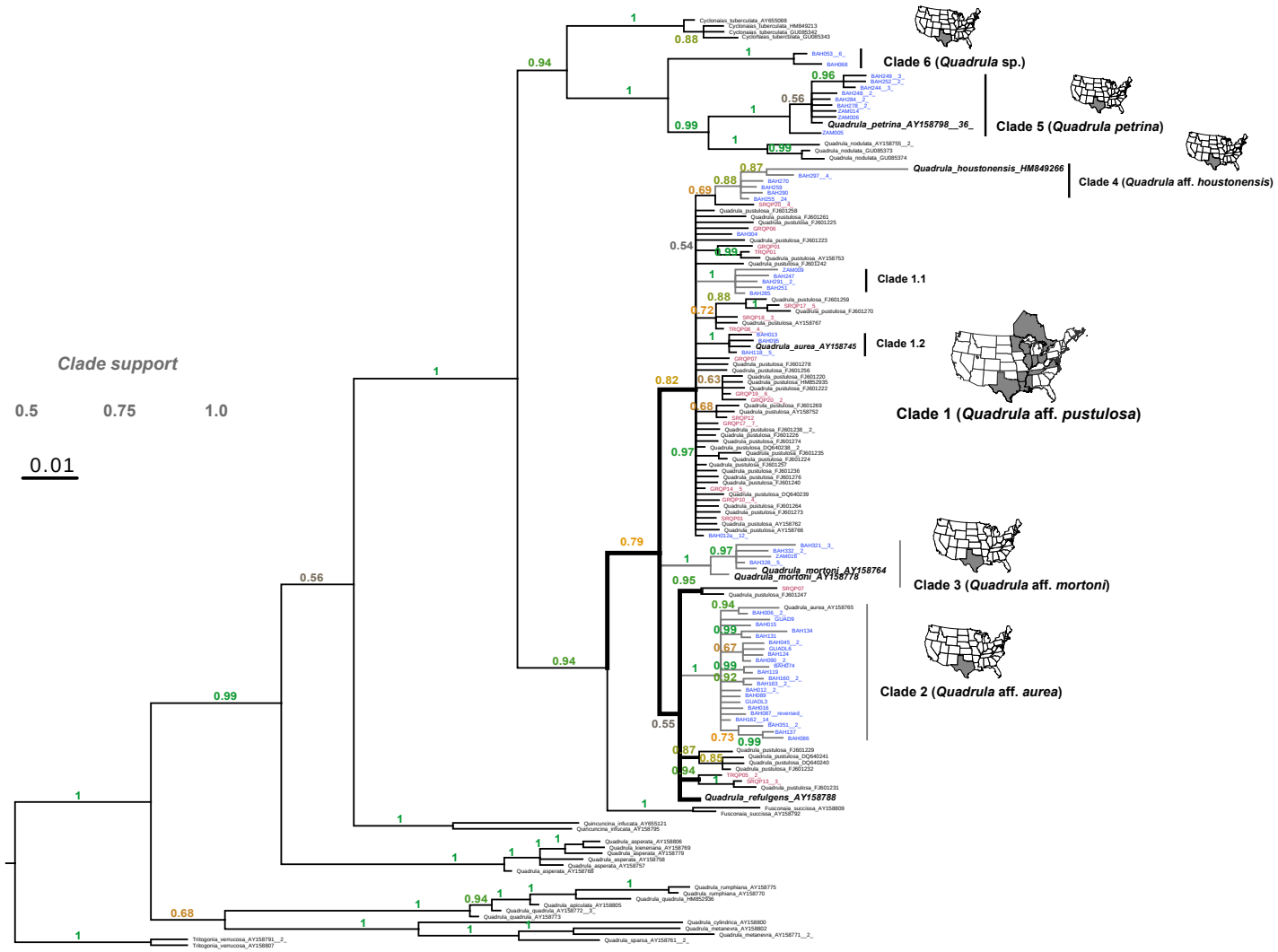

Figure 5.4 A rooted Bayesian phylogeny using ND1 sequence data collected from Texas Quadrula in comparison to data available on Genbank and unpublished sequences (Harding et al. unpublished).
Likely ESUs based on these data are given clade designations.

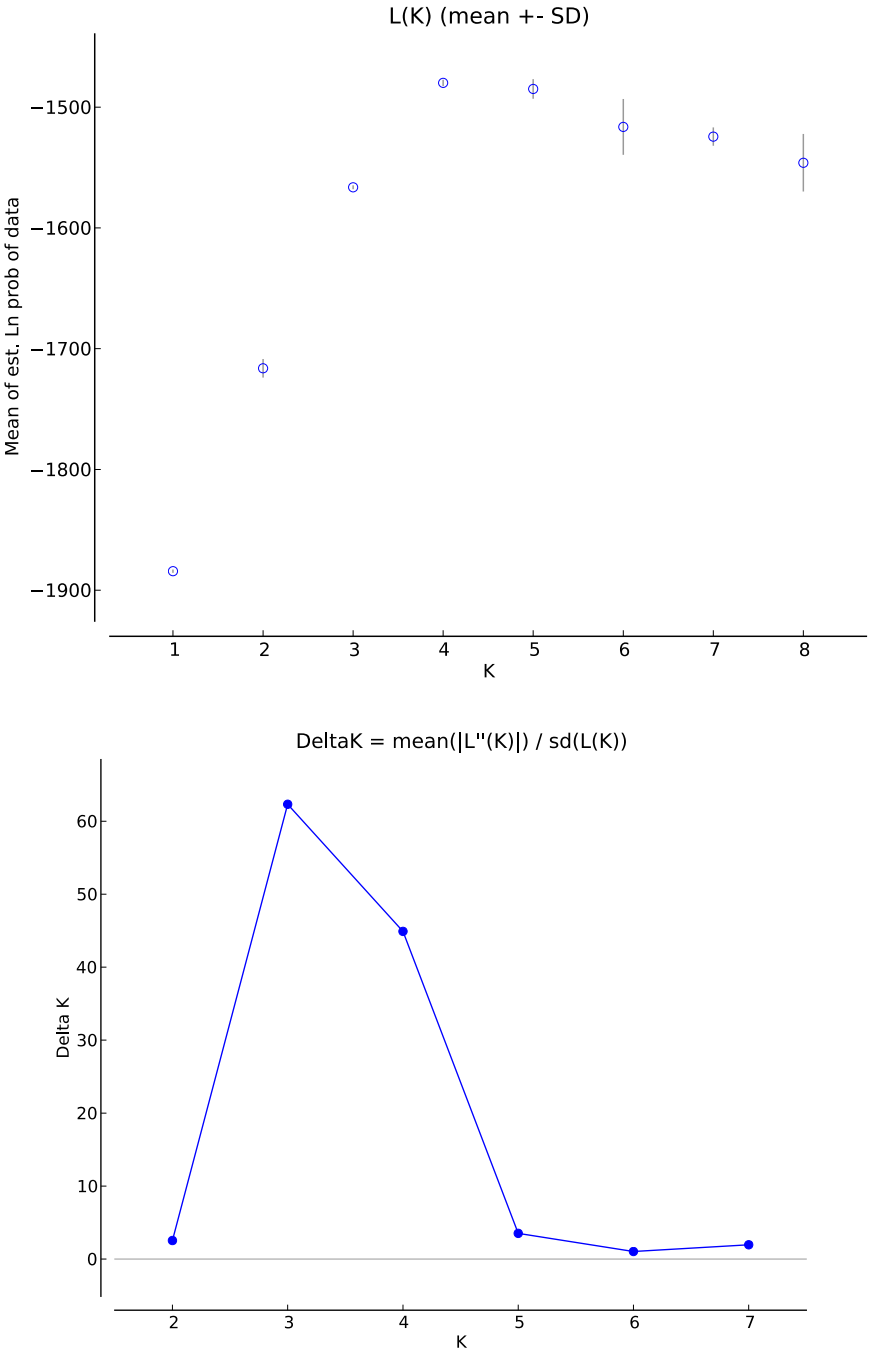

Figure 5.5 A) Log probability of data from analysis of four polymorphic microsatellite loci in STRUCUTRE suggesting four likely demes given the data. B) □*K* plot inferred suggesting either three or four likely genetic demes among the individuals sampled.

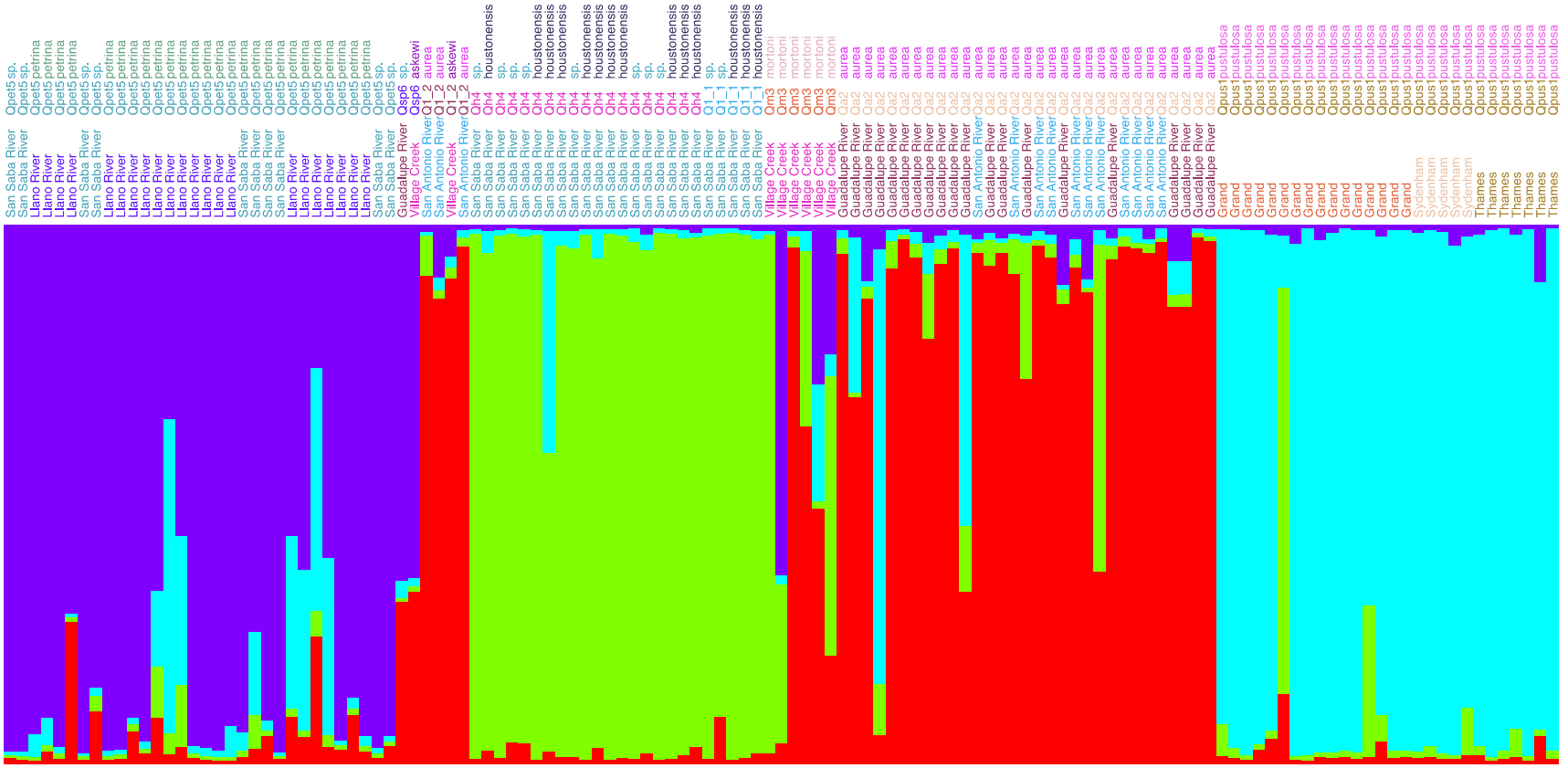

Figure 5.6 Barplot of assignment probabilities to a genetic deme per individual.
Source location, mitochondrial haplogroup, and morphological assignment are noted above.

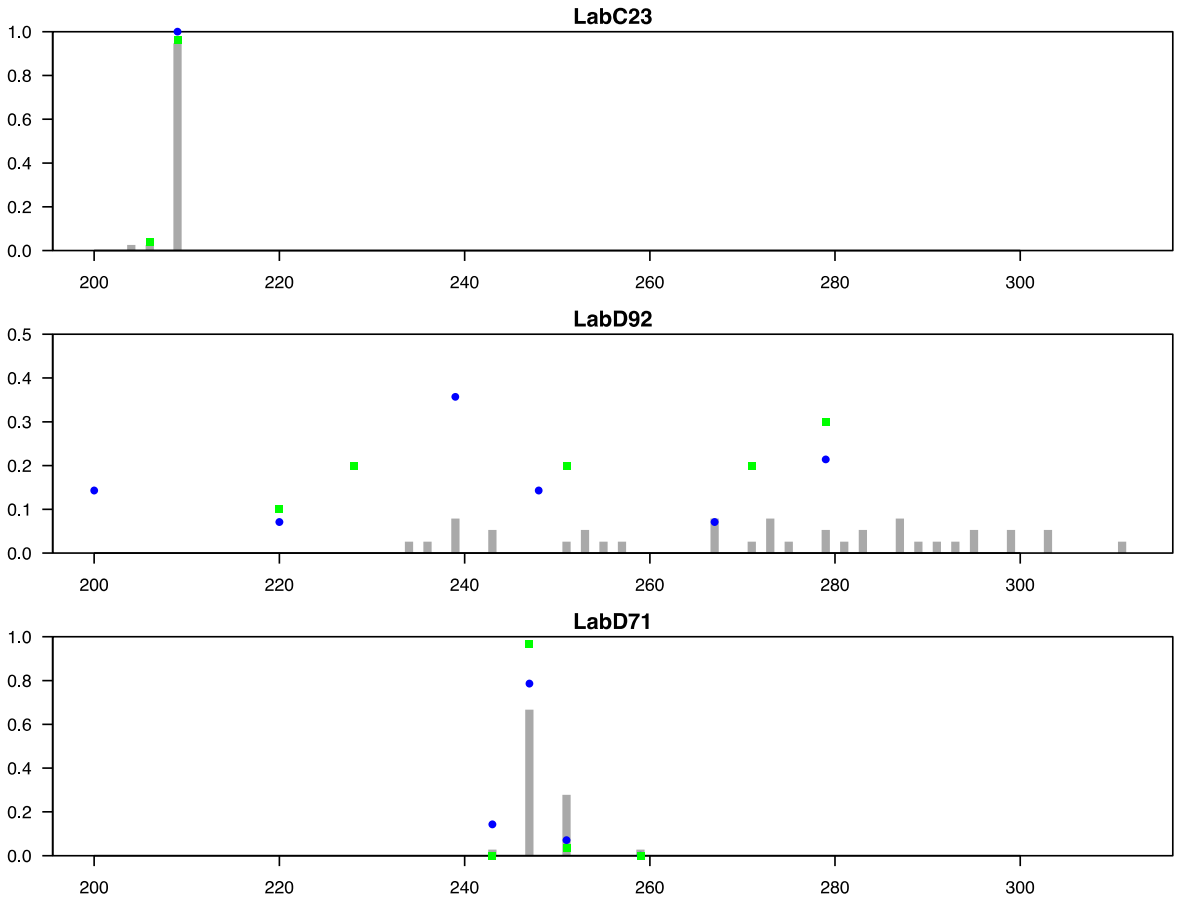

Figure 5.7 Allelic distributions for wild *Lampsilis braceata* (grey) with proportions for captive females (blue) and their glochidia (green) superimposed.

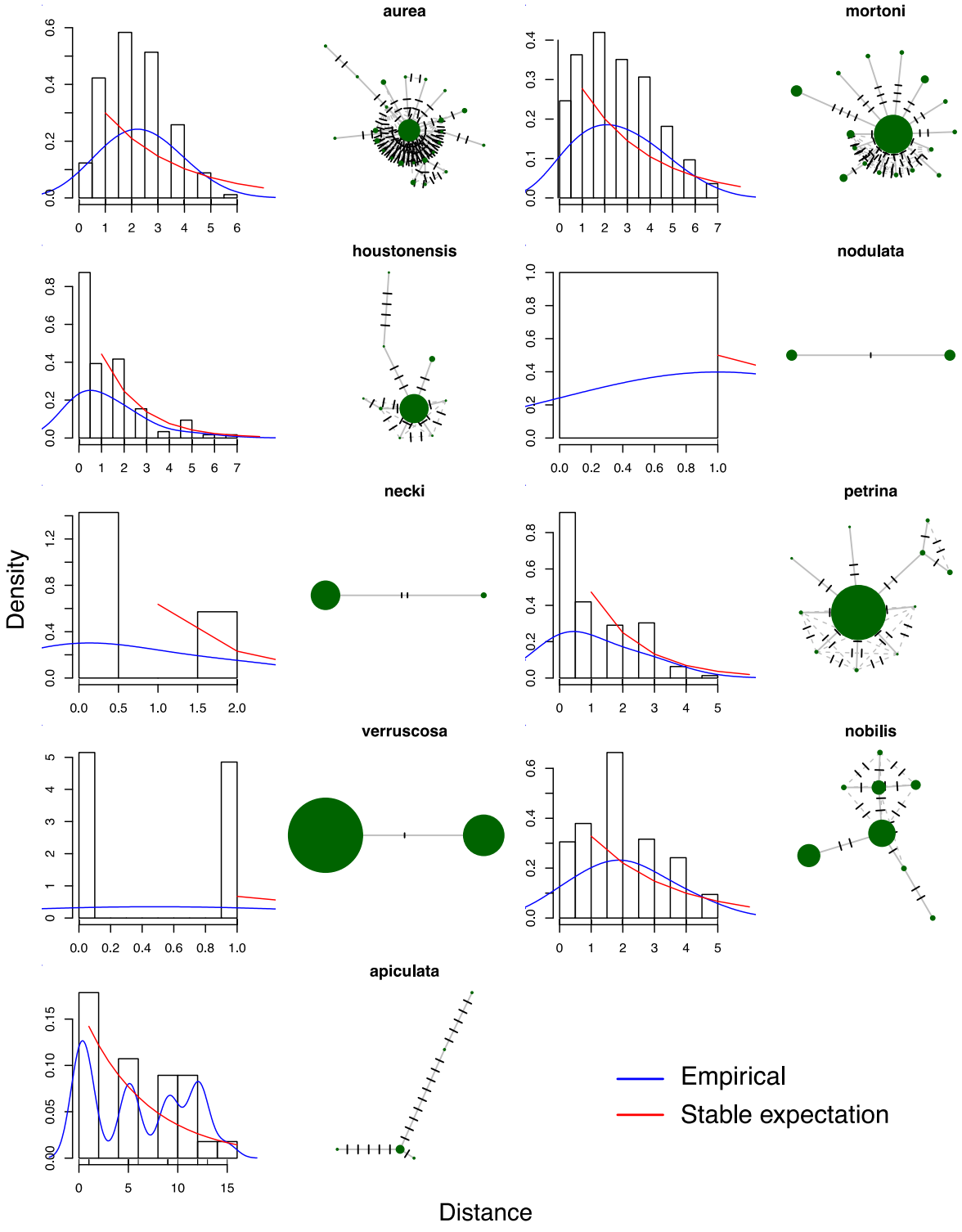

Figure 5.8 Mismatch distributions and haplotype networks per clade.

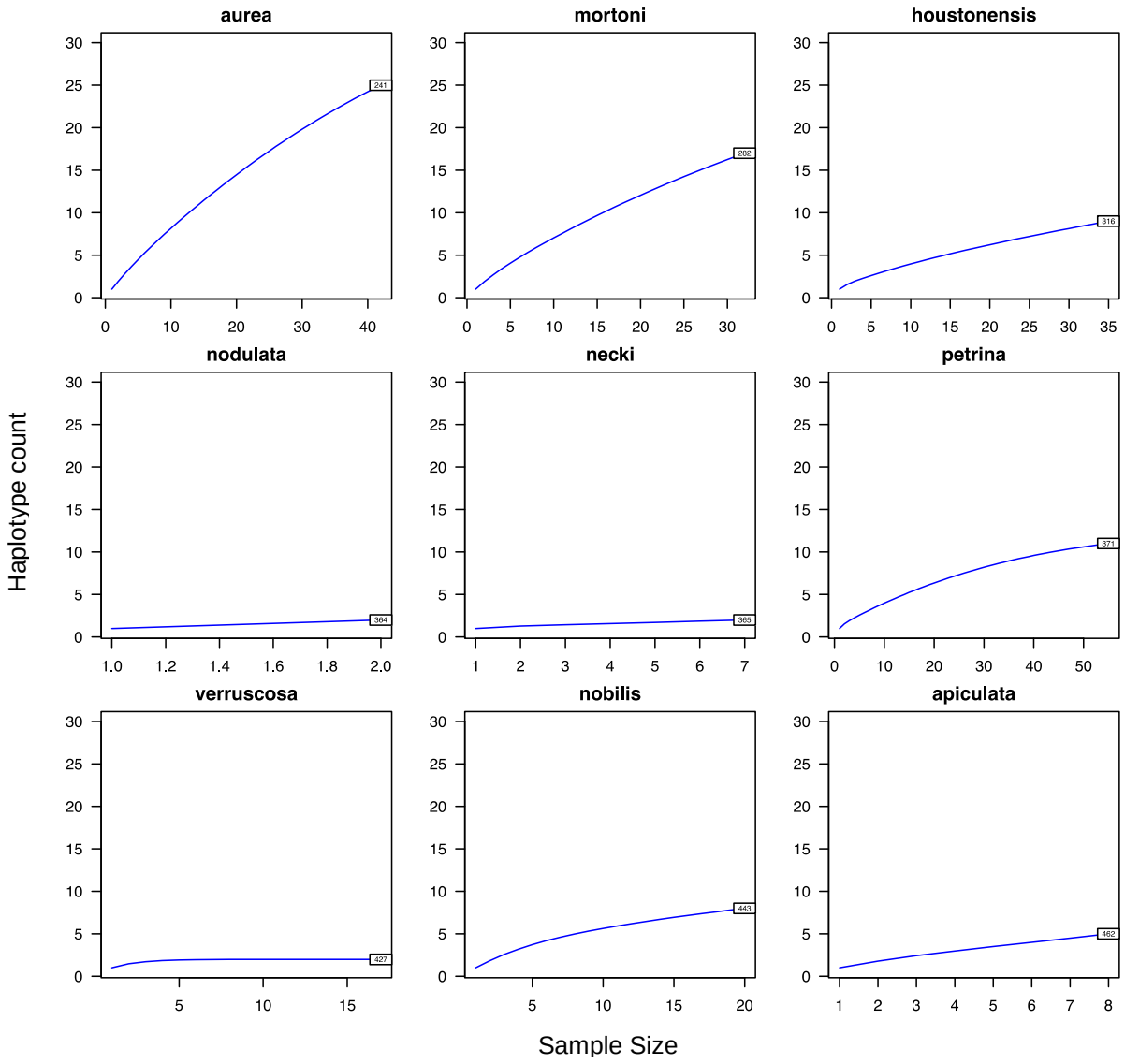

Figure 5.9 Rarefaction curves for each taxonomic group based on phylogenetic affinity (curve label indicates specific clade from a NJ tree).

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Supplementary material: Identification of known host fish and potential sources for propagation work

Based on our literature review we found that Ford & Oliver 2015 provided the best review of known or potential host fish for Texas. Few host fish were known for threatened species and host fish for related species were gathered in Table S.1.

Table S.1 List of threatened species in Texas and their known or potential host fish. Using the data compilation in Ford et al. 2015, suspected host fish of threatened Texas mussels were selected based on known hosts of mussels from the same genus.

Threatened Mussel	Known Host	Potential Host
Lampsilis bracteata	*Lepomis cyanellus* *Lepomis macrochirus* *Micropterus salmoides* *Micropterus treculii*	*Scaphirhynchus platorynchus* *Lapomis auritus* *Lepomis gulosus* *Lepomis humilis* *Lepomis marginatus* *Lepomis megalotis* *Lepomis microlophus* *Micropterus punctulatus* *Pomoxis annularis* *Pomoxis nigromaculatus* *Cyprinella venusta* *Ictalurus furcatus* *Ictalurus punctatus* *Lepisosteus oculatus* *Lepisosteus osseus* *Lepisosteus platostomus* *Lepisosteus spatula* *Etheostoma lepidum* *Etheostoma stigmaeum* *Perca flavescens* *Sander canadensis* *Sander vitreus* *Poecilia reticulata* *Xiphophorus hellerii* *Ambystoma tigrinum*
*Obovaria jacksoniana**		*Scaphirhynchus platorynchus* *Etheostoma asprigene* *Etheostoma caeruleum* *Etheostoma lepidum*

Threatened Mussel	Known Host	Potential Host
		Etheostoma stigmaeum *Perca flavescens* *Percina caprodes* *Percina maculata* *Percina phoxocephala* *Percina sciera* *Percina shumardi*
Potamilus amphichaenus		*Lepomis gulosus* *Pomoxis annularis* *Cyprinella lutrensis* *Notemigonus crysoleucas* *Fundulus notatus* *Aplodinotus grunniens*
Potamilus metnecktayi		*Lepomis gulosus* *Pomoxis annularis* *Cyprinella lutrensis* *Notemigonus crysoleucas* *Fundulus notatus* *Aplodinotus grunniens*
Truncilla cognata		*Sander canadensis* *Aplodinotus grunniens*
Truncilla macrodon		*Sander canadensis* *Aplodinotus grunniens*
Fusconaia lananensis		*Lepomis macrochirus* *Micropterus punctulatus* *Pomoxis annularis* *Pomoxis nigromaculatus* *Dorosoma cepedianum* *Cyprinella lutrensis* *Cyprinella venusta* *Hybopsis amnis* *Notemigonus crysoleucas* *Notropis texanus*

Threatened Mussel	Known Host	Potential Host
		Pimephales promelas *Pimephales vigilax* *Semotilus atromaculatus* *Esox americanus* *Fundulus notatus* *Ictalurus punctatus* *Noturus nocturnus* *Percina sciera* *Gambusia affinis*
All *Quadrula* species; *Q. aurea, Q. houstonensis, Q. (Fusconaia) mitchelli, Q. petrina*		*Scaphirhynchus platorynchus* *Lepomis cyanellus* *Lepomis megalotis* *Micropterus punctulatus* *Micropterus salmoides* *Pomoxis annularis* *Pomoxis nigromaculatus* *Dorosoma cepedianum* *Cyprinella lutrensis* *Ameiurus melas* *Ameiurus nebulosus* *Ictalurus furcatus* *Ictalurus punctatus* *Pylodictis olivaris*
Uniomerus declivis		*Notemigonus crysoleucas*

**Obovaria jacksoniana* is the only species in Texas within the genus. Suspected hosts are based on known hosts of other *Obovaria* species outside of Texas: *Obovaria unicolor, Obovaria olivaria, Obovaria subrotunda.*

Table S.2 Potential sources of fish for mussel propagation work, their sizes, and prices

Source	Species	Fish Size (in)	Price/Fish
Johnson Lake Management Service, San Marcos, (512) 396-1231	Largemouth Bass	1.5-3.0	$1.25
		3.0-6.0	$3.00
	Bluegill	1.0-3.0	$0.40
		3.0-5.0	$0.80
		>5	$3
	Redear Sunfish		$0.45
			$1.00
	Channel Catfish	4.00-6.00	$0.60
	Fathead Minnows		N/A, requires minimum order
	Golden Shiners		N/A, requires minimum order
All sizes available in spring/summer months (multiple broods per season)			
Vollmar Pond and Lake Management (830) 992-0928 Fredericksburg, TX	Largemouth Bass	3	$1.25
	Bluegill	1.00-3.00	$0.50
	Redear Sunfish	1.0-3.0	$0.60
	Channel Catfish	4.0-6.0	$0.60
Brenham Fisheries Brenham, TX	Largemouth	3	$1.15
	Bluegill	3	$0.65
	Channel	6.00-8.00	$1