REPRODUCTIVE ECOLOGY OF *LAMPSILIS BRACTEATA* (BIVALVIA: UNIONIDAE)

by

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ABSTRACT

The Texas fatmucket, *Lampsilis bracteata,* is a unionid mussel endemic to the Colorado and upper Guadalupe River basins of Central Texas and is one of fifteen threatened mussel species in Texas that is also a candidate for federal listing under the Endangered Species Act. A better knowledge of its reproductive ecology is needed to develop conservation and management strategies. 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 Basin in Central Texas, and to monitor and compare seasonality of reproduction between the rivers. Monthly sampling events assessed sex ratios, gamete production, gravidity period, and viability of larvae (glochidia).

Reproduction varied with season and between rivers. Gravid mussels were detected throughout the study period (February to September 2017), with largest proportions being gravid between February and June before peak water temperatures were reached in summer. Sex ratio in the Llano was female-biased, whereas it did not significantly differ from 1:1 in the San Saba River. Gamete production, fecundity and glochidia viability were consistently higher in the Llano River than the San Saba, where trematode flatworms (*Bucephalus sp.*) were found in 21% of the egg samples, but none in samples from the Llano or any sperm samples. Host fish compatibility was tested

between mussels and fish collected in both rivers using a fully-crossed study design by monitoring juvenile metamorphic (transformation) success. In addition, host compatibility was also tested with Guadalupe bass and largemouth bass from a hatchery. Highest transformation rates occurred on green sunfish, largemouth bass, and Guadalupe bass (hatchery). Average transformation success was higher for some mussel-fish pairings originating from the same tributary, but individual variation was high and the differences between mussel-fish pairings of the same species but different tributaries were not statistically significant. The results of this study suggest that *L. bracteata* could be produced in the lab using hatchery or wild fish, but propagation efforts that are currently initiated in Texas should consider ecological differences between populations, as mussel populations may be locally adapted to host fish. Further investigations of the life-history strategies of *L. bracteata* and other mussels are warranted before augmentation and reintroduction efforts are initiated.

I. 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 across the globe 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 as endangered 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 worldwide and locally, such as within the geographic distribution of *L. bracteata* (Howells 2015, Hansen et al 2015, Lydeard et al 2004, Bogan 1993).

Unionid mussels have a complex life-cycle involving a host fish on which larvae (glochidia) develop into juvenile mussels after fertilization of eggs in the suprabranchial chambers of female mussels by male-broadcasted sperm (Jirka and Neves 1992). Like other *Lampsilis* species, *L. bracteata* are long-term brooders, termed bradytictic, which are generally known to spawn in the summer, brood over the winter, and release glochidia during the following spring (Watters and O'Dee 1998, 1999). The seasonality of reproduction of *L. bracteata* is largely unknown, but gravid 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 (Barnhart et al 2008).

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 darter (Percidae), which is known to attract predatory fish in other *Lampsilis* species (Haag 2012). 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). All of these species reside in a single family (Centrarchidae) which suggests that other centrarchid fishes could also act as host fish (Haag and Warren 1997, Haag 2012).

Lab transformation is the most common form of host fish study today and provides insight into the physiological compatibility among mussel and host fish (Haag 2012). When conducting host fish experiments, however, it has to be considered that immunological resistance to glochidia may be acquired in fish with previous exposure to mussels, and 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 transformation is 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 transformation is that it provides little to no information regarding the frequency of encounters among glochidia and the host fish in the wild. However, this information could be obtained with natural infestation or natural transformation studies, but infestation incidence and density are usually low on wild fish. Further, a large number of fish are required to obtain a sufficient number of attached glochidia or transformed juveniles to draw any conclusions about mussel-host fish relationships (Barnhart et al 2008, Bauer 1994).

Captive breeding has been widely used as a conservation measure in order to augment declining populations and to reintroduce mussels to areas where they were previously extirpated (Thomas et al 2010). Increasing the knowledgebase of life history information prior to captive breeding is imperative to success of the wild population after reintroduction (Haag 2013, McMurray and Roe 2017). Local adaptations can lead to variation in reproductive ecologies (e.g. breeding, gravidity period, host fish requirements) among populations, which must be considered when collecting brood stock (Rogers et al 2001). Disjunct populations are more susceptible to local adaptation due to limited gene flow between neighboring populations. Local adaptations to, or coadaptation with, host fish may make glochidia more compatible with fish from the same river as the mussel, as opposed to allopatric host strains (Rogers, et al 2001, Eckert 2003, Taeubert et al 2010, Zanatta and Wilson 2011). Other studies have found that compatibility may be higher with allopatric fish (Osterling and Larsen 2013) or did not find any differences between sympatric and allopatric fish and mussel pairings (Caldwell et al. 2016). There is increased interest in propagation of threatened mussels in Texas as a potential conservation method, but still little is known about their life history and potential differences in host compatibility with wild fish from different basins or tributaries or hatchery fish.

The objective of this study was firstly to collect life history data on breeding, gravidity period and viability of glochidia of *L. bracteata;* and secondly, to investigate differences in host fish compatibility between mussels and wild fish originating from two rivers within the distribution of *L. bracteata* and hatchery fish. A fully-crossed study design was used with *L. bracteata* and fishes originating from two tributaries of the

Colorado River: Llano River and San Saba River. Fish cannot freely move between these tributaries, because two major dams (Buchanan Dam and Inks Dam) are located in the main stem of the Colorado River between the Llano and San Saba rivers.

II. METHODS

Sampling Sites

Mussels and fish were collected from two major tributaries of the Colorado River, the Llano and San Saba rivers (Fig. 1). Mussels were monitored monthly in these rivers from February to September 2017, and additional preliminary monitoring occurred in the Llano River from April to November 2016 (Table 1A, 2A). Higher water clarity in the Llano River more often permitted visual search techniques than the San Saba River, which required tactile search techniques. Mussel sampling continued until ten *L. bracteata* individuals were located per sampling event per site (Llano and San Saba rivers). All mussels were sampled for gonadal fluid (see details below) and were assessed for signs of gravidity and glochidia viability (if gravid).

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 handing which could affect reproductive patterns (Peredo et al 2005). Fish for host fish experiments were collected 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). After arrival at the Texas State University wet lab, fish were thermally acclimated overnight before transferring them to 10-gallon holding tanks. Fish received pellet food and/or bloodworms daily along with weekly water changes and regular water quality testing.

Environmental Parameters

Temperature at field sites was measured continuously using temperature loggers (HOBO Pro v2 and HOBO 64K). Temperature was logged hourly from February to mid-May and every 12 hours thereafter (12 a.m. and 12 p.m.). 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 number of male and female *L. bracteata* detected during each sampling event were recorded, and sex ratios between rivers were assessed using a chi-square goodness of fit test to determine if the detected sex ratio in each river differed from 1:1. Gonadal fluid was extracted monthly using a nonlethal hypodermic needle and syringe technique (Tsakiris et al. 2016) from ten *L. bracteata* (regardless to sex). Samples of 0.1-2.0 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. Gamete samples were quantified in 10 µl subsamples using a compound microscope at 400x magnification (sperm) and 100x magnification (eggs). Counts from subsamples were extrapolated to estimate gamete concentration (number/ml).

Gravidity and glochidia Viability

Female mussels were deemed gravid when gills were swollen (Hove and Neves 1994). Mussels with flat milk-colored gills, or with partially swollen gills were assumed to have released their glochidia (Sietman et al 2012). Glochidia viability was assessed for gravid mussels by obtaining a small sample of glochidia by flushing 1-2 water tubes (of the marsupium) with a 20-gage hypodermic needle. The viability of a subsample of ~100 glochidia was determined by observing valve-closing capability upon addition of a saturated salt solution (NaCl 240 g L−1) using:

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

Host Fish Experiment

Mussels with glochidia which exceeded 90% viability were collected from the field. 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 fed daily with manually-administered Rotifer Shellfish Diet 1800 (Pentair Aquatic Eco-Systems: 5.28×10^{-6} % solution). 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 were extracted from females and viability was tested to ensure >90% of glochidia of each female were viable. The combined glochidia sample was distributed between inoculation chambers, so that the concentration was ~ 4,000 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). Unattached glochidia were removed from the 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. Viability 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). Fish mortality was monitored during the experiment and if fish died during the experiment (Appendix Table A4, A5), they were dissected, and gills were checked for presence of encysted glochidia (Osterling and Larsen 2013). None of the dissected fish contained encysted 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 (Appendix Table A4, A5).

The transformation success (%) was computed by dividing the number of live juveniles detached from each individual fish with the total number of glochidia and dead juveniles captured from a tank. Transformation success data were fourth-root transformed to meet assumptions of normality and homogeneity, and data were analyzed using a two-way ANOVA and Tukey's HSD post hoc test to determine differences within and among treatments (i.e., different fish species and different origins). Total length (mm) and weight (g) were measured for each fish upon conclusion of the experiment, and transformation success was assessed for covariance with fish weight using an ANCOVA. Remaining fish were stocked into a private pond for neighborhood fishing.

III. RESULTS

Environmental Parameters

There were small differences in physico-chemical conditions between rivers. Specific conductivity was lower in the Llano River (mean ± SE: 371±10, range: 338-410 µS/cm^2) than the San Saba River (505 ± 11 µS/cm^2, range: 453-547 µS/cm^2), whereas pH was similar (Llano: 8.3 ± 0.03, range: 8.2-8.3, San Saba River: 8.1 ± 0.03, range: 7.9-8.1). DO tended to be higher in the Llano River (9.3 ± 1.2 mg/L, range: 5.6-14.8 mg/L, measured between 12-2 p.m., Fig. A1) compared to the San Saba River (8.2 ± 2.1 mg/L, range: 6.6-12.6 mg/L, measured between 8-10 a.m.) except for June, August and September (Fig. A1).

Chlorophyll-*a* in the San Saba River (0.49-2.9 µg/L) was about 1.5-14x higher compared to the Llano River (0.21-2.76 µg/L) except in August when chlorophyll in the Llano (2.8 µg/L) was basically the same as the San Saba River (2.6 µg/L). Total suspended solids were similar in the Llano (range 0.03-0.06 mg/L, and San Saba Rivers (TSS avg. range 0.04-0.05 mg/L).

The greatest differences in temperature between rivers were in thermal minima, where the San Saba mean was three degrees higher (23 ± 2°C; range: 15-26°C) than the Llano (20 ± 2°C; range: 11-24°C). Thermal averages and maxima were similar among rivers with averages in the Llano River (range 11.2-31.1°C, average 24 ± 2°C) slightly lower than the San Saba River (14.9-31.1°C, average 25 ± 2°C) and thermal maxima slightly higher in the Llano (28 ± 1°C) than the San Saba River (27 ± 1°C, Figure 3).

Reproductive Monitoring

Sex ratios for *L. bracteata* collected between February and September 2017 were 0.5 males per female (n=72) in the Llano River, which was female-biased and significantly differed from a 1:1 sex ratio (X^2 (1) = 7.02, p<0.05), and 1.3 males per female (n=87) in the San Saba River, which did not significantly differ from a 1:1 sex ratio (X^2 (1) = 0.59, p=0.44). Gamete production was generally higher in the Llano River than the San Saba River, although large differences were observed with sperm concentrations up to 15 times higher in February and egg concentrations up to 5 times higher in March, Fig. 2.

Gamete production, gravidity, and viability varied seasonally in both rivers. Egg concentrations in the Llano were highest from February through May with peak egg production occurring in March, and up to 2 orders of magnitude lower concentrations found in June through September. Similarly, egg concentrations in the San Saba mussels were highest between February through April and up to an order of magnitude lower during May through August (Fig. 2). No eggs were found in September in the San Saba River. Decline in egg production in both rivers coincided with an increase in thermal minima in both rivers; thermal minima increased by 5 degrees in the Llano River in June (24°C) and by 6 degrees in the San Saba River in April (21°C, Fig. 2, 3). Sperm concentrations in the Llano peaked in February and were also high in March, but an order of magnitude lower April to July, and 3 orders of magnitude lower in August (Fig. 2) Sperm concentrations increased again in September to levels similar in March (Fig. 2). In the San Saba River, sperm concentrations had a similar peak as observed in the Llano

River in February, but sperm concentrations were 1-1.5 orders of magnitude lower from March through August (Fig. 2).

The peak in egg and sperm production in the Llano River in February and March was followed by an increase in the proportion of gravid females in the Llano River from 50% in February to around 80% in May and June and declined to less than 20% in July to August (Fig. 3). More than half of the monitored mussels in the Llano River showed signs of recent glochidia release in July and August. None of the females found in September were gravid. The decline in the proportion of gravid mussels in the Llano River in July coincided with temperature reaching a summer maxima (31°C). In contrast, in the San Saba River a higher proportion of gravid mussels (>60%) was only found in March and April, and remained between 20 and 40% between May and September. In addition, in March and April, the majority of San Saba mussels showed signs of recent glochidia release. Eggs (instead of glochidia) were detected in a few gravid mussels in March (one mussel containing eggs, n=6 females total) and August (two mussels, n=8), and were surrounded by membranes resembling loose conglutinates, known to be released by some Lampsilini (Haag 2012). Preliminary monitoring between April and November 2016 detected a somewhat similar seasonal variation in gravidity with all of the females that were monitored being gravid in April (n=2) and June (n=8). However, by July none of the mussels were found to be gravid (n=21), and no gravid mussels were found in November (n = 10).

Average glochidia viability in gravid mussels from the Llano River was consistently high from February through July with >80% viability, except for March, when glochidia viability was low (<40%) in 2 of the 6 tested mussels. Only 2 gravid

females were found in July and August, which also had a relatively high viability (≥ 67%, Fig. 3). San Saba River glochidia viability was high (range 70-91%, average 81%) in April, May and July and extremely low (range 0-1.5%, average <1%) in all other months sampled, Fig. 3). Aside from temperature, there was no obvious correlation in gamete production, gravidity, or glochidia viability with other environmental parameters, such as Chlorophyll-*a* (Fig. A2) or TSS (Fig. A3).

In addition, parasites were observed to occur in some of the gamete samples. Specifically, trematodes, *Bucephalus sp.*, (Bucephalidae) were found in 21% of gamete samples of female mussels (n = 33 total) in the San Saba River, but none were detected from the Llano River (*n*=46) or any samples from male mussels in either system (*n*=30 in Llano River, *n*=50 in San Saba River, Fig. A4).

Fecundity differed considerably between rivers and was generally higher for larger mussels. 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. A5).

Host Fish Experiment: Wild fish

Host fish experiments with mussels from the Llano and San Saba rivers resulted in a total number of 7,566 live mussel juveniles. Average transformation success differed

significantly between host fish species for glochidia from both the Llano and San Saba rivers (Tables 1 and 2, and Fig. 4), although there was considerable variation between individual host fish. For example, glochidia from Llano mussels had the highest average transformation success on green sunfish from the Llano River (45% average), which ranged between 27 and 76% (or 72 vs. 167 juveniles produced). The 2-way ANOVA detected a significant effect of fish origin and fish species for the Llano mussels (Table 1) but not for the Saba mussels (Table 2). Average transformation success was considerably higher for mussel-fish pairings from the same river for the Llano River green sunfish and San Saba River largemouth bass, and was somewhat higher for San Saba River green sunfish. However, none of these differences were statistically significant (Fig. 4, Table 1, 2).

Transformation success was significantly lower for bluegill sunfish and longear sunfish (< 12 and < 1 % mean transformation success respectively), and 0 or <1% for redbreast sunfish and warmouth. Weight of the fish had no significant effect on transformation success of the Llano mussel glochidia (ANCOVA: $F_{3,27}$=2.03, p=0.13) nor San Saba glochidia (ANCOVA: $F_{4,32}$=0.56, p=0.70).

The number of detached glochidia decreased continuously with time in mussels from both rivers with detachment being highest on day 2 (>3,800 glochidia). Minimal detachment of both glochidia and juveniles occurred after 40 days (Fig. 5, Fig. A6). Juvenile detachment peaked between day 18 (San Saba mussels, 4009 juveniles) and day 23 (Llano juveniles 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. 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 has a high transformation success on Guadalupe and largemouth bass from the hatchery (Fig. 4). For Llano mussels, transformation success on hatchery fish were similar to wild green sunfish from the same river (44 and 60% in hatchery vs. 46% in wild fish) but higher than the mean transformation success on wild largemouth bass from both rivers (Llano River: 46%, San Saba River: 28%) and San Saba green sunfish (19%, Fig. 4). For San Saba mussels, the transformation 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. 4). Differences in transformation success on hatchery Guadalupe bass and largemouth bass were marginally significant for mussels from both rivers (ANOVA $F_{1,8}$=4.48, p=0.07). There were no significant differences in transformation success between largemouth bass from wild (Llano and San Saba rivers) versus largemouth bass from hatchery origin for Llano mussels (ANOVA $F_{1,10}$=1.91, p=0.20); or San Saba mussels (ANOVA $F_{1,11}$=0.09, p=0.78).

Glochidia growth on host fish

Glochidia in both rivers grew on host fish during the transformation to juveniles (on average approximately 50 µm for San Saba mussels and 100 µm for Llano mussels; Fig. 6). Llano juveniles were significantly larger (T_{588}=14, P<0.001, Fig. 6) than San

Saba juveniles despite similarly-sized initial glochidia (T_{33}=0.73, p=0.47). For Llano glochidia, mean shell length increased from 218 µm (range: 165-292 µm, n=313) to 314 µm as juveniles (range: 197-497 µm; n=557, Fig 5). For San Saba mussels mean glochidia length increased from 214 µm (range: 180-287 µm, n=29) to 269 µm (range: 153-367 µm, n=256) as juveniles. Glochidial growth rates were lower in hatchery fish (approximately 40 µm for San Saba mussels and 70 µm for Llano mussels).

IV. 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 with mussels and host fish from two tributaries of the Colorado River in Central Texas. Average transformation success of glochidia was considerably 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 looked at variation of infection success rather than transformation success (Douda et al. 2014). Hence, to the best of our knowledge this is the first study to look at differences in transformation success and growth on host fish of mussels from fish populations 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 transformation 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).

At this point, we believe that there is not clear pattern in the published literature that demonstrates host fish compatibility with sympatric and allopatric fish. A study on freshwater pearl mussel, *Margaritifera margaritifera,* in southern Norway found allopatric fish strains to have higher number of encysted 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 parings 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 later. We did not find mussel populations from different rivers to have adaptations to different host fish species, as other studies have found (Douda et al. 2014, Eckert 2003), but dispersal between these rivers has been restricted by the construction of major dams in the mainstem Colorado in the 1930s, recent for evolutionary time scales.

Higher transformation 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 transformation 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 transformation success was significantly 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 transformation 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 transform well on hatchery fish may not necessarily transform 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.

Our study found both largemouth bass and green sunfish (and hatchery Guadalupe bass) to be the best host fish, while juveniles also transformed 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).

The female-biased sex ratio in the Llano, could be due to more females occurring at the surface for reproduction, e.g., to display mantle lures and to attract host fish (Yusa, 2007). Individuals at the surface and luring females are more likely to be sampled during visual searches, and lures were visible in the Llano River (clear water conditions) but not the San Saba River (higher turbidity). This is consistent with the findings of other studies which detected female-biased sex ratios in other *Lampsilis* species, e.g. southern pocketbook, *L. ornata*, and wavy-rayed lampmussel, *L. fasciola*, and also contributed to detection of females as opposed to males without lures (Haag and Staton 2003, Zanatta et al 2007).

The observed gravidity period of *L. bracteata* in this study appears to be much longer than previously known (July-October, Johnson et al 2012) as gravid mussels were found from the beginning of the study period (February) until September. In agreement with previous studies, gamete production appeared to be related to temperature, as declines in egg production in both rivers coincided with increased thermal minima (Galbraith and Vaughn 2009, Jirka and Neves 1992). The detection of eggs in the marsupia of gravid *L. bracteata* in March in the San Saba coincided with detection of mussels with signs of recent glochidia release, suggesting asynchronous reproduction among individuals. Larger individuals tended to have a higher fecundity, which is not surprising as the relationship between size of females and number of glochidia is well established (Haag 2012).

Lower gamete production, gravidity, fecundity, and glochidia viability in San Saba mussels, could at least in part be associated with the gonadal parasites detected in the female mussels from the San Saba River. These gonadal parasites can castrate mussels (Haag and Staton 2003). Although gonadal parasites have been documented in other unionid mussels, this study is the first that we know of to identify the parasitic trematode and document the *Bucephalus sp.*, in *L. bracteata* (Shiver 2002).

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: 38-1700 cfs) than the San Saba River (range: 14-46 cfs) during the survey period

which may have contributed to the lower thermal minima seen in the Llano and allowed mussels to remain gravid 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). Finally, mussels in well-known populations, such as at the San Saba Site which has become increasingly popular for mussel research, may become stressed due to handling and relocation (Peredo et al 2005) which could potentially affect their reproduction.

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. Glochidia of *L. bracteata* grew on host fish, which has also been shown for other *Lampsilis* species such as *L. fragilis* and *L. laevissimus* (Coker and Surber 1911, Barnhart et al 2008).

This study has implications for captive breeding of freshwater mussels and opens the floor for further exploration of host fish and mussel stock origin. 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 reintroduced to same area (Hoftyzer et al 2008).

TABLES

Table 1. Two-way ANOVA of host Fish for Llano River mussels. Two-way ANOVA considering transformation success of Llano River glochidia on wild-caught fish collected from the Llano and San Saba rivers.

	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio	P-value
Species	3	22.2	7.4	15.3	<0.001
Origin	1	3.8	3.8	7.9	0.01
Species:Origin	3	2.5	0.8	1.7	0.19
Residuals	27	13.1	0.5		

Table 2. Two-way ANOVA for host Fish of San Saba River mussels. Two-way ANOVA considering transformation success of San Saba River glochidia on wild-caught fish collected from the Llano and San Saba rivers.

	Degrees of Freedom	Sum of Squares	Mean Square	F-ratio	P-value
Species	4	42.7	10.7	38.3	<0.001
Origin	1	0.3	0.3	0.9	0.34
Species:Origin	4	0.2	0.04	0.1	0.97
Residuals	33	9.2	0.3		

FIGURES

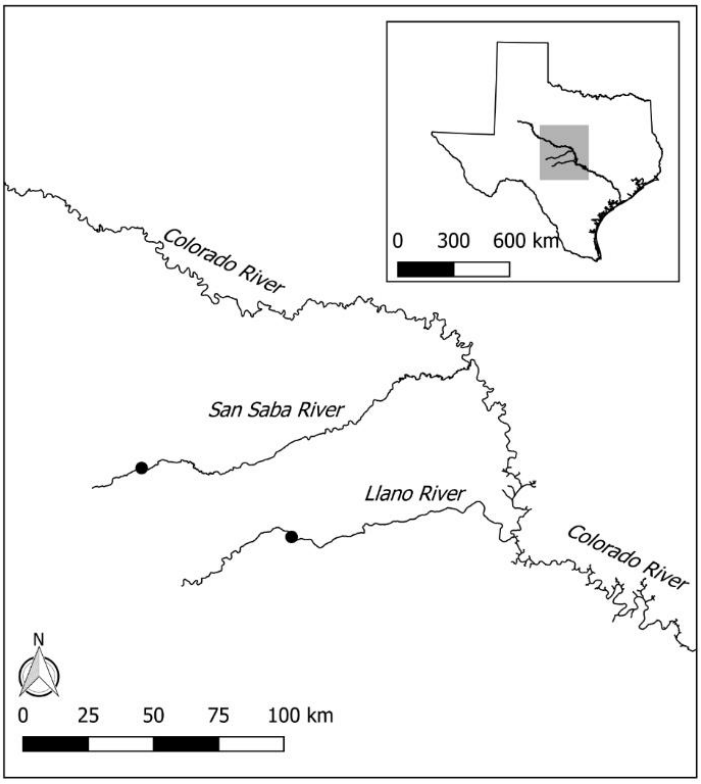

Figure 1. Map of sampling sites in two tributaries of the Colorado River Basin. Sampling sites in the Llano River near Mason, TX and San Saba River near Menard, TX. Map by Zachary A. Mitchell.

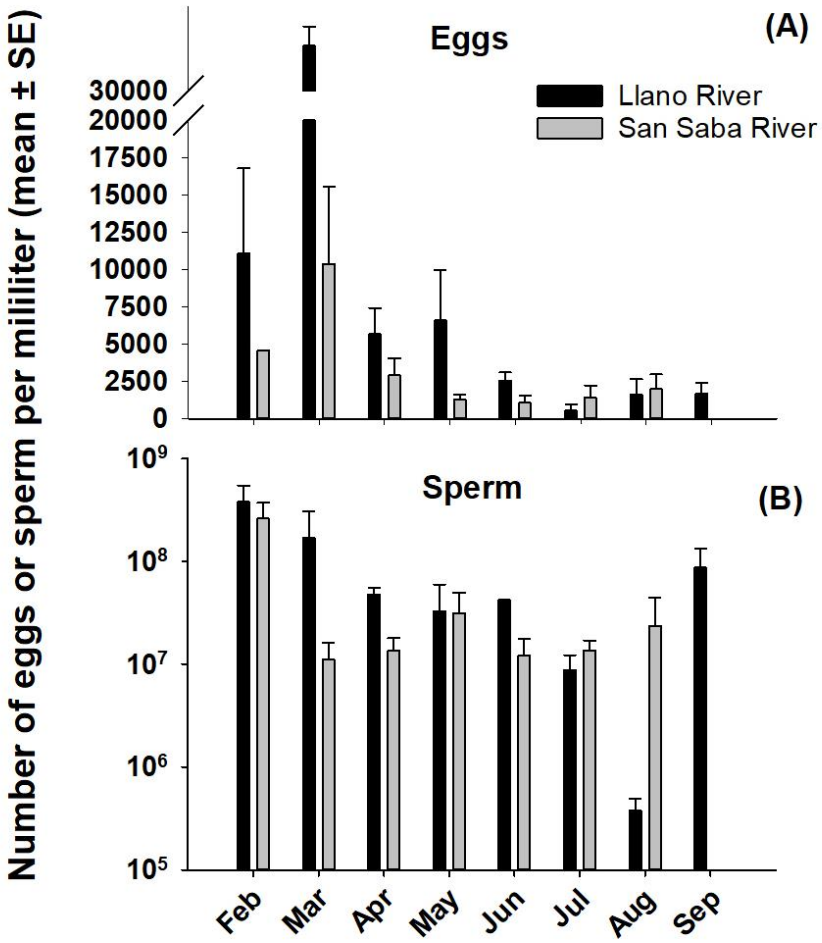

Figure 2. Variation in egg and sperm concentrations between February and September 2017 in the Llano and San Saba River. Egg (panel A) and sperm (panel B) concentrations of L. bracteata in the Llano (black) and San Saba (gray) rivers from February-September 2017. Sperm is shown on a logarithmic scale.

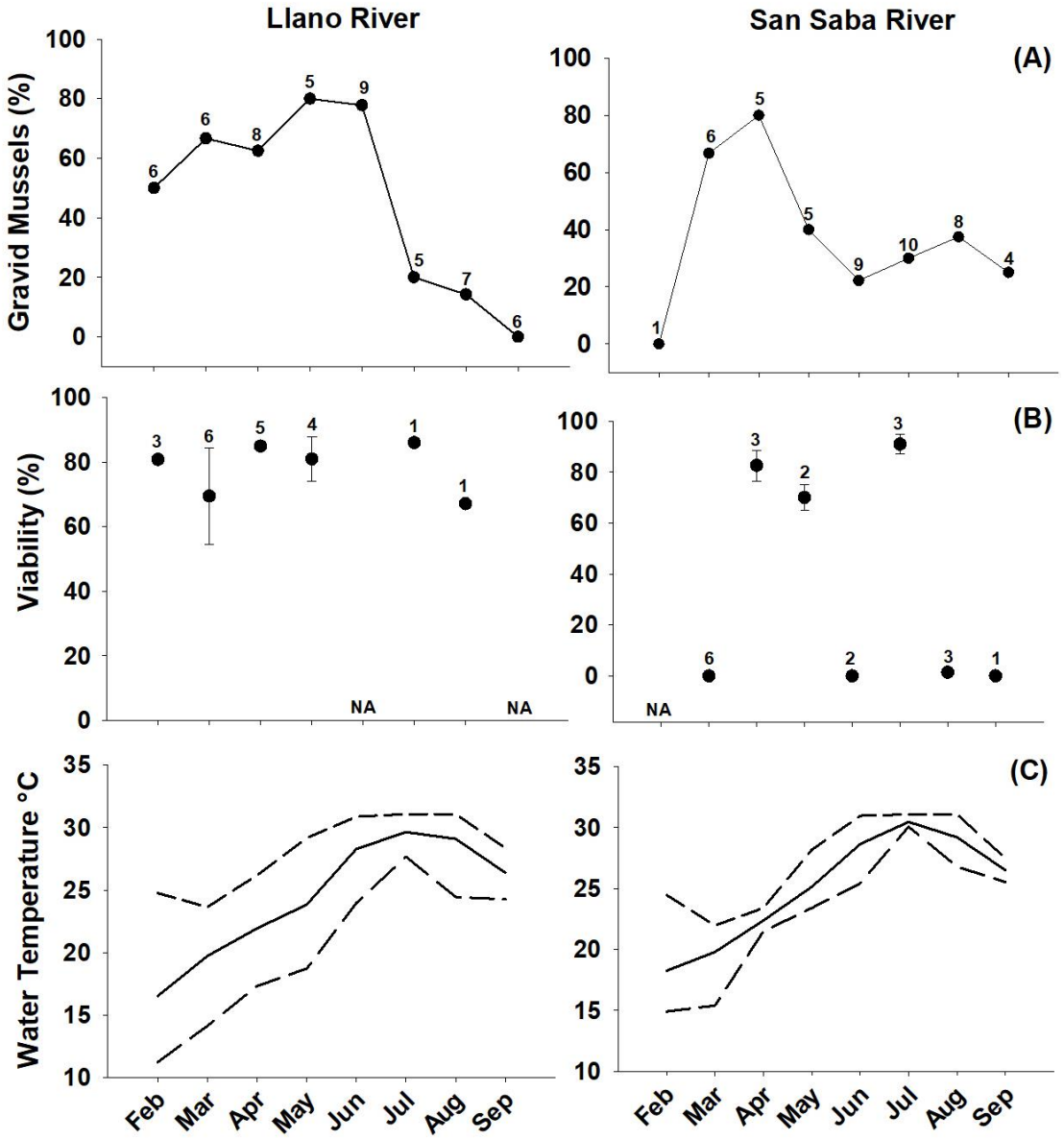

Figure 3. Seasonal variation of the proportion of gravid mussels, glochidia viability and temperature. Seasonal variation of the proportion of female mussels that were gravid (panel A), glochidia viability (mean % ± SE; panel B), and mean monthly water temperatures (°C; panel C) in the Llano (left) and San Saba (right) rivers expressed as monthly thermal averages (solid line), monthly thermal maxima (dashed line above) and monthly thermal minima (dashed line below). "NA" is noted for months in which no viability measurements were collected or when no gravid mussels were detected. Numbers above data points indicate sample size.

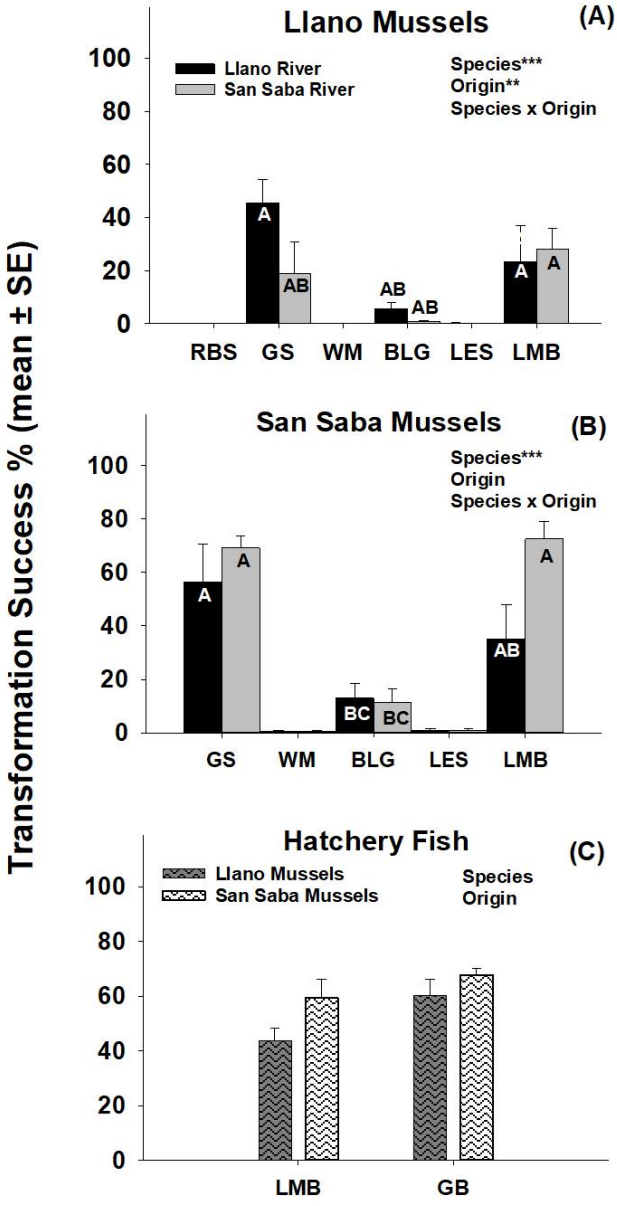

Figure 4. Transformation success of juvenile mussels on different fish species and fish from different origin. Proportion (%, mean ± standard error) of glochidia that successfully transformed into juvenile mussels using A) Llano River glochidia on wild fish, B) San Saba River glochidia on wild fish, and C) transformation success of glochidia of the Llano River (dark gray chevrons) and San Saba River (white chevrons) on hatchery fish (panel C). In panels A+B: grey bars represent fish from San Saba River, and black bars represent fish from Llano River. 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: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

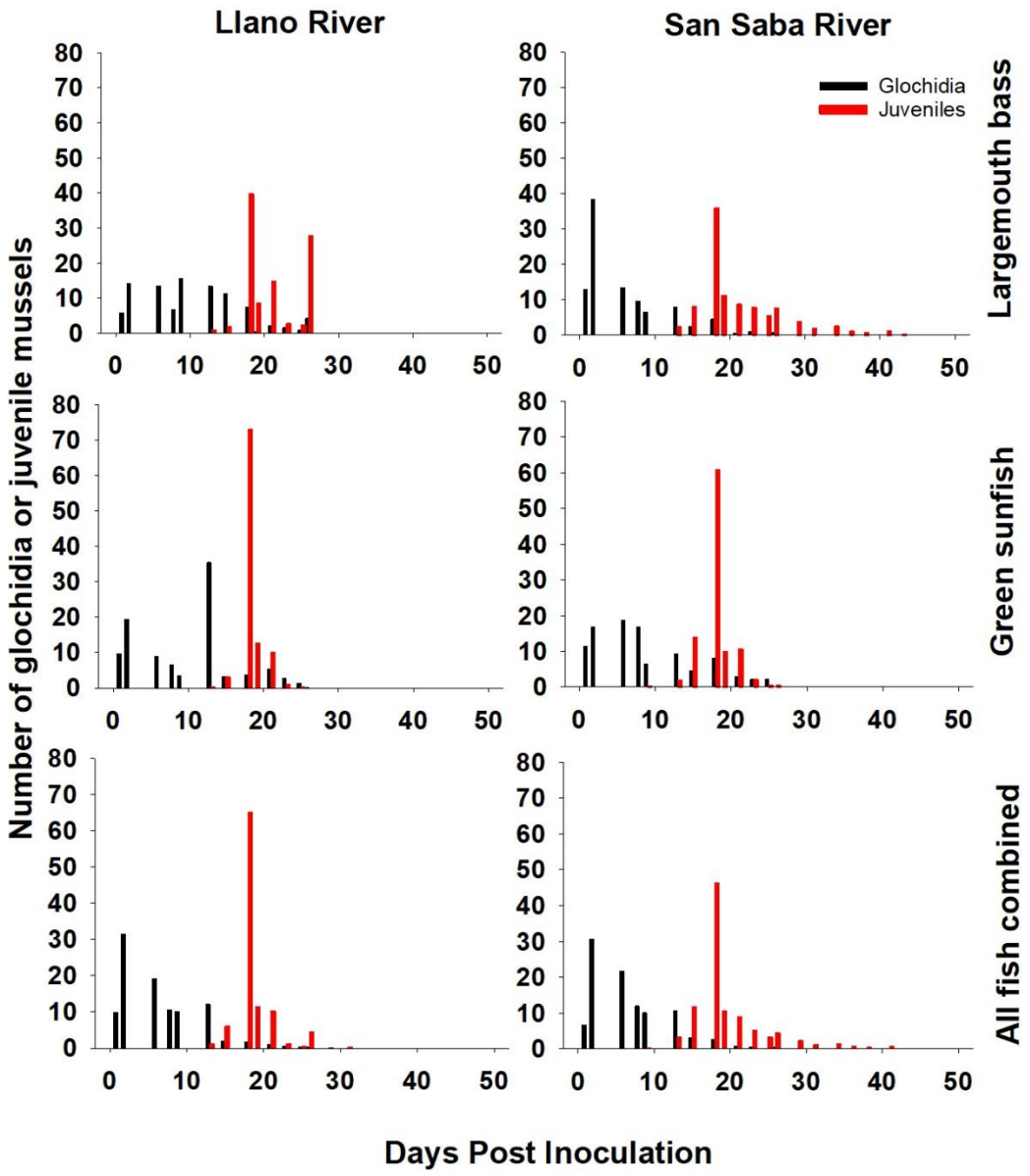

Figure 5. Developmental dynamics of San Saba River glochidia on host fish. Developmental dynamics of San Saba River glochidia on host fish. Bars indicate the number of glochidia (black bars) or juveniles (red bars) recovered from Llano host fish (left panel) and San Saba host fish (right panel) the respective day after inoculation with San Saba mussel glochidia.

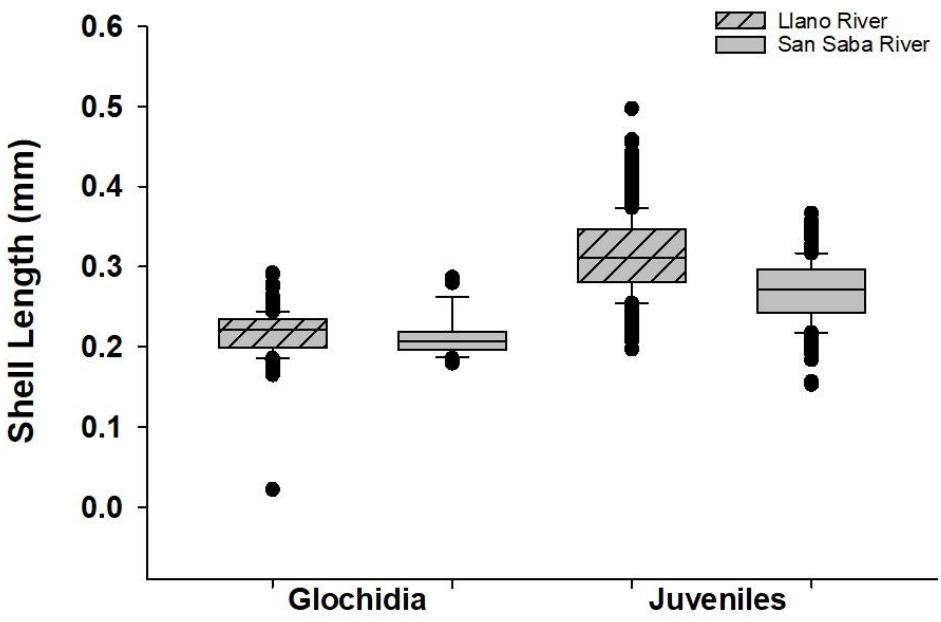

Figure 6. Glochidia and juvenile mussel sizes. Shell length (mm) of glochidia and juvenile mussels from parent mussels collected in the Llano (stripes) and San Saba (solid gray) rivers. Boxes represent 25th, median, and 75th percentiles. Whiskers represent the 10^{th} and 90^{th} percentiles. Points represent outliers. Sample sizes were 313 (Llano glochidia), 29 (San Saba glochidia), 557 (Llano juveniles), and 256 (San Saba juveniles) respectively.

APPENDIX

Table A1. 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.

	Sex Ratio	**Gravidity**	**Glochidia Viability**	**Gamete**
Apr. 30th 2016	X	X	X	
May 24th 2016	X	X	X	
Jun. 24th 2016	X	X	X	
Jul. 14th 2016	X	X	X	
Nov. 10th 2016	X	X		
Feb. 10th 2017	X	X	X	X
Mar. 16th 2017	X	X	X	X
Apr. 12th 2017	X	X	X	X
May 16th2017		X	X	X
Jun. 12th 2017	X	X	X	X
Jul. 18th 2017	X	X	X	X
Aug. 11th2017	X	X	X	X
Sep. 13th 2017	X	X	X	X

Table A2. Sampling dates and methods for the San Saba River. Sampling methods (marked with X) during each sampling event at the San Saba River between April 2016 and September 2017.

	Sex Ratio	**Gravidity**	**Glochidia Viability**	**Gamete**
Feb. 10th 2017	X	X	X	X
Mar. 16th 2017	X	X	X	X
Apr. 12th 2017	X	X	X	X
May 16th 2017		X	X	X
Jun. 12th 2017	X	X	X	X
Jul. 18th 2017	X	X	X	X
Aug. 11th 2017	X	X	X	X
Sep. 13th 2017	X	X	X	X

Table A3. Sample Sizes for Host Fish Inoculations. Sample sizes of host fish used in analyses.

Mussel Origin:	Llano mussels			San Saba mussels		
Fish Origin:	Llano	San Saba	Hatchery	Llano	San Saba	Hatchery
redbreast sunfish	*n*=5	*n*=5	NA	NA	NA	NA
green sunfish	*n*=5	*n*=5	NA	*n*=5	*n*=4	NA
warmouth	*n*=5	*n*=5	NA	*n*=3	*n*=4	NA
bluegill sunfish	*n*=5	*n*=5	NA	*n*=5	*n*=5	NA
longear sunfish	*n*=5	*n*=3	NA	*n*=4	*n*=5	NA
largemouth bass	*n*=2	*n*=5	*n*=5	*n*=4	*n*=3	*n*=5
Guadalupe bass	NA	NA	*n*=5	NA	NA	*n*=4

Table A4. Fish mortalities during the host fish experiment using mussels from the Llano River. DPI = days post inoculation. Num. encysted is the number of glochidia encysted on the gills of dissected fish. Analyses (Y/N) refers to whether or not fish were used in further host fish analyses.

Fish species	Fish river	Num. Fish	DPI	Num. encysted	Analyses (Y/N)
Largemouth Bass	Llano	2	4, 23	0,0	N

Table A5. Fish mortalities during host fish experiment using mussels from the San Saba River. DPI = days post inoculation. Num. encysted is the number of glochidia encysted on the gills of dissected fish. Analyses (Y/N) refers to whether or not fish were used in further host fish analyses.

Fish species	Fish river	Num. Fish	DPI	Num. encysted	Analyses (Y/N)
Largemouth Bass	Llano	1	19	0	N
Green Sunfish	Llano	1	1	0	N
Warmouth	Llano	2	36, 41	0,0	Y, Y
Warmouth	San Saba	1	31	0	Y

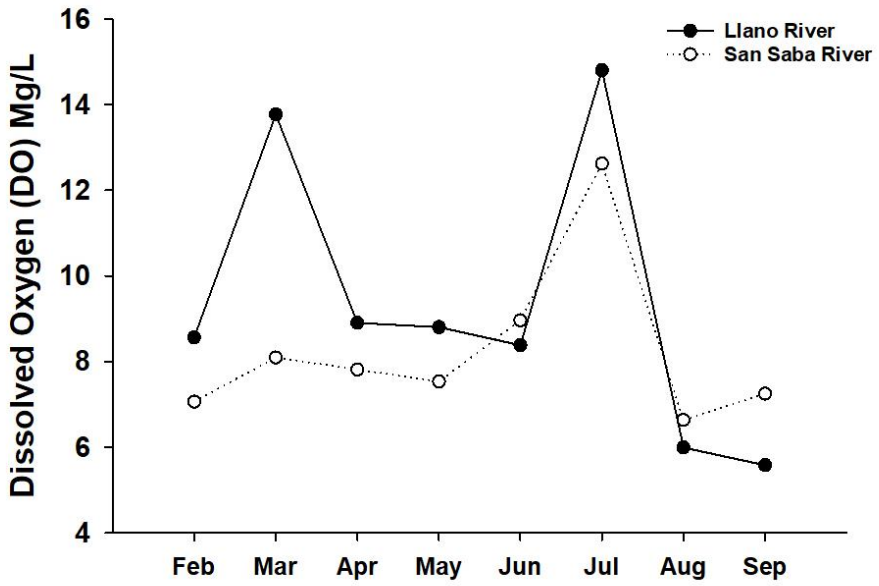

Figure A1. Dissolved Oxygen at sample sites. Dissolved oxygen (mg/L) was measured monthly at each sampling site in the Llano (black) and San Saba (hollow) rivers.

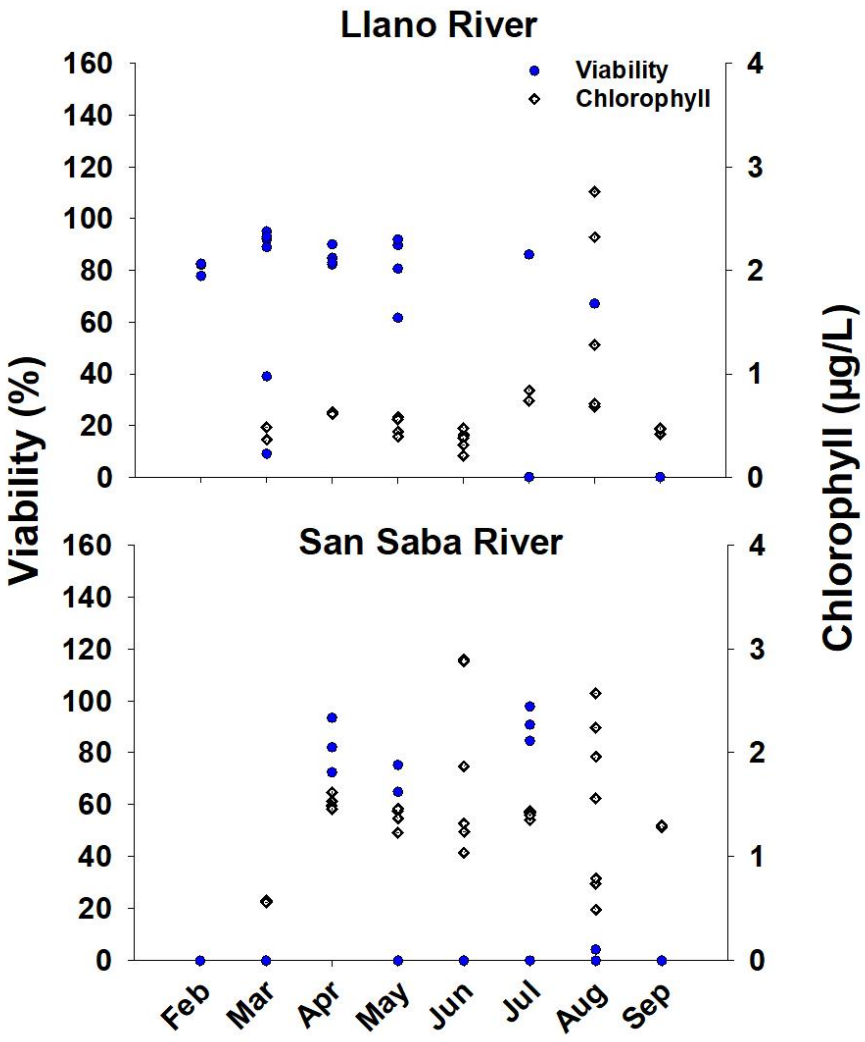

Figure A2. Chlorophyll-*a* and glochidia viability at sampling sites. Glochidia viability (%; blue circles) and chlorophyll-*a* (µg/L; hollow diamonds) in the Llano (top) and San Saba (bottom) rivers from February-September 2017.

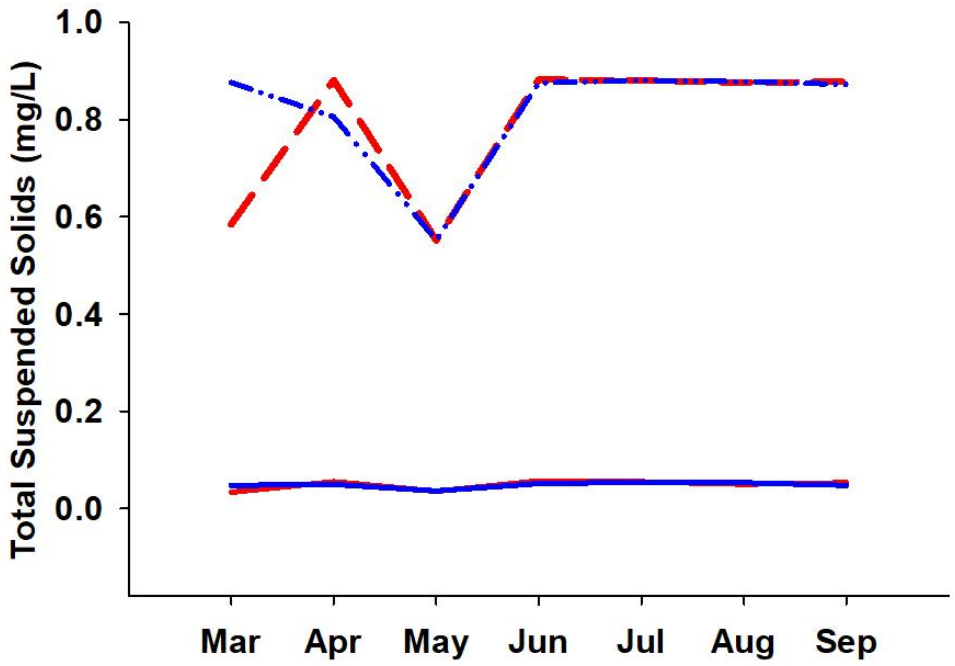

Figure A3. Total Suspended Solids at sample sites. Particulate organic matter (POM, mg/L, solid lines) and nonvolatile suspended solids (NVSS, mg/L, dashed lines) were measured in the Llano (red) and San Saba (blue) rivers from February through September 2017. Combined, these parameters can be referred to as total suspended solids.

Figure A4. Photo of *Bucephalus sp*. metacercariae larvae. Photos of *Bucephalus sp*., parasitic trematode, detected in egg sample from *L. bracteata* in the San Saba River detected in February 2017.

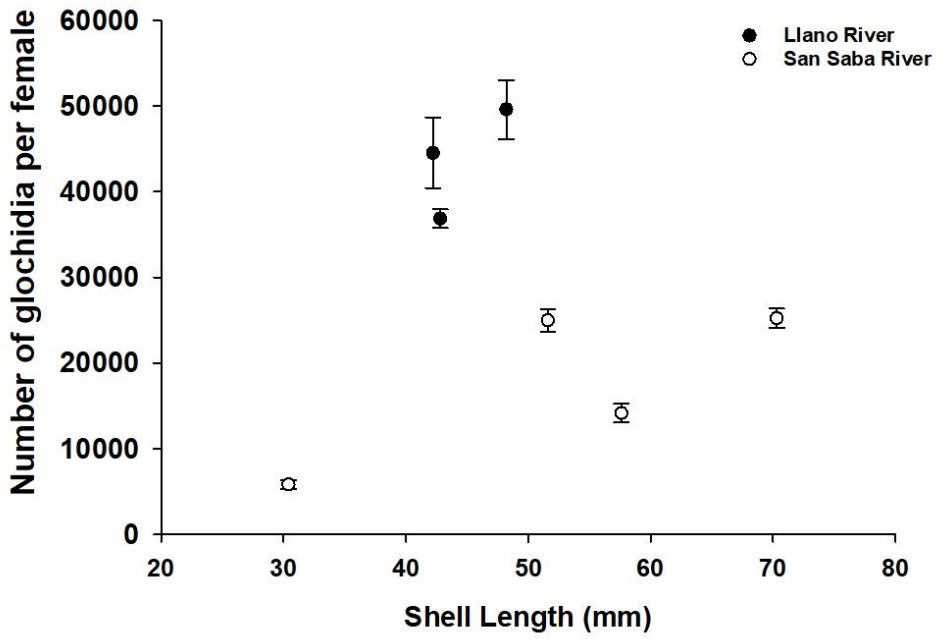

Figure A5. Fecundity in the Llano and San Saba rivers. Number of glochidia per tested *L. bracteata* female in the Llano (black circles) and San Saba (hollow circles) rivers showed with shell length (mm).

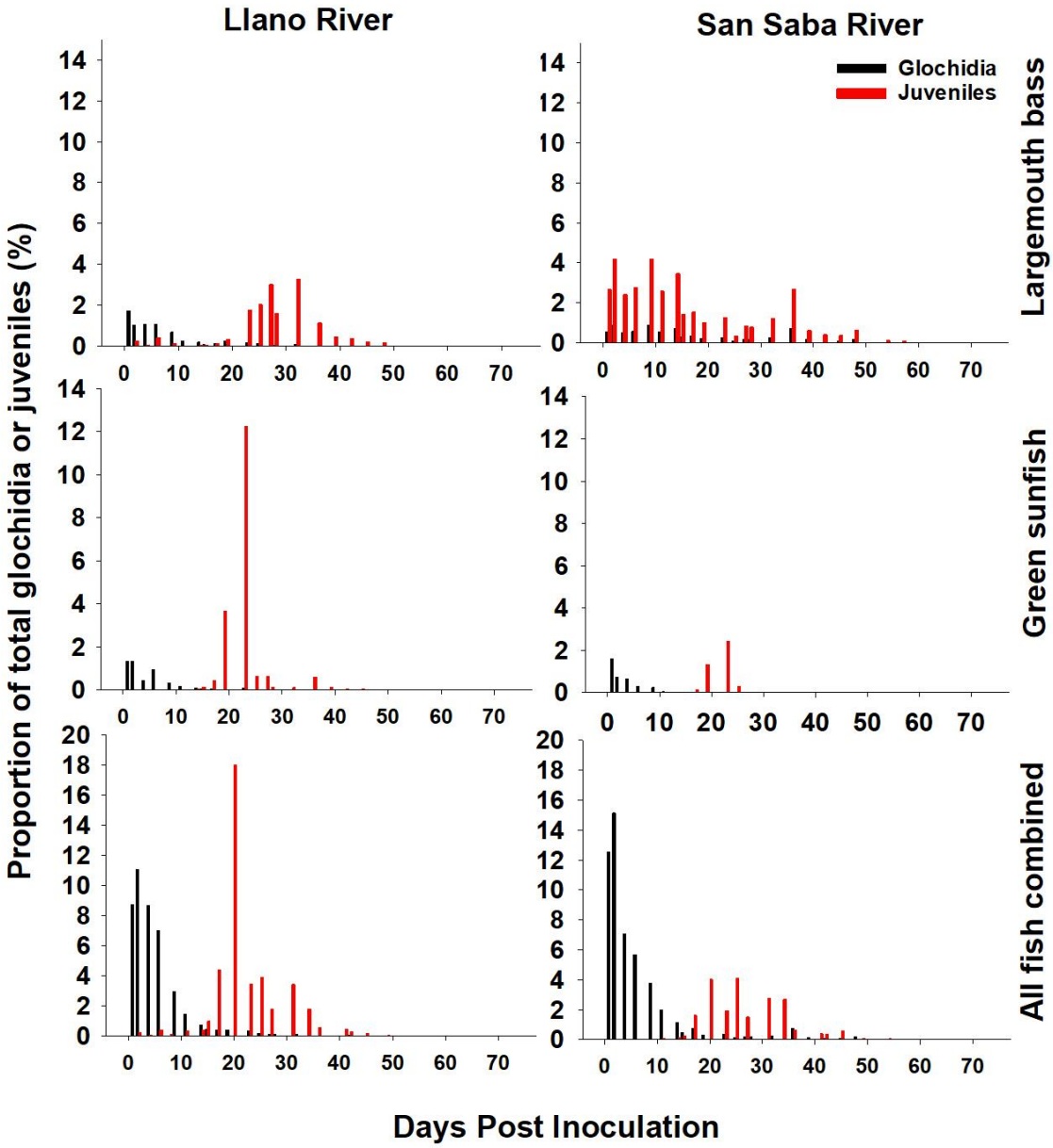

Figure A6. Developmental dynamics of Llano River *L. bracteata* glochidia on host fish. Bars indicate the proportion of glochidia (black bars) or juveniles (red bars) recovered from Llano host fish (left panel) and San Saba host fish (right panel) the respective day after inoculation with Llano mussel glochidia.

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