Effectiveness of a nonlethal method to quantify gamete production in freshwater mussels

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Abstract: Studies on the reproductive biology of freshwater mussels have contributed to conservation of this group, but methods to study early reproductive stages are either lethal (e.g., histological technique) or useful for only qualitative assessments (e.g., nonlethal syringe technique). Using 2 common mussels (*Quadrula apiculata* and *Quadrula verrucosa*) and 2 rare mussels (*Quadrula petrina* and *Quadrula houstonensis*) distributed across 3 sites in the Navasota River and San Saba River, Texas, we validated the effectiveness of the syringe technique to quantify gamete production by examining: 1) if estimates of gamete traits (sperm concentration, egg size, and egg concentration) obtained with the syringe technique were correlated to estimates of gamete traits (sperm density, egg size, and egg density) obtained with a histological technique; and 2) if survival, growth, and body condition of individual mussels sampled with the syringe technique were negatively affected in a 2-y mark–recapture field experiment. Pearson's correlation analysis of gamete production measured over the 1st year of the study indicated sperm concentration and density and egg sizes were correlated between the 2 techniques; however, egg concentration and density were correlated in only some cases. Joint analysis of live and dead encounters from the mark–recapture experiment indicated the syringe technique had little to no effect on survival probability of mussels, and mixed models of shell growth and Fulton's K body condition index failed to detect sublethal effects of the syringe technique on mussels. The syringe technique is relatively accurate and noninvasive and can be used to study the reproductive biology of threatened and endangered mussels quantitatively. In addition, it can provide the large sample sizes often needed to study the reproductive ecology of mussels.

Key words: Unionidae, reproduction, gamete production, histology, conservation

The global decline of nonmarine mollusks, and freshwater mussels (Bivalvia:Unionidae) in particular, has led to rapid expansion of efforts to conserve them in recent decades (Lydeard et al. 2004, Lopes-Lima et al. 2014). The study of the reproductive biology of freshwater mussels has contributed greatly to conservation efforts, especially for analyzing population structure and understanding life histories (Downing et al. 1989, McIvor and Aldridge 2007, Haag 2013). However, the study of mussel reproduction has relied primarily on lethal methods. For example, Taskinen and Valtonen (1995) examined parasitism by trematodes and gamete density through dissection and optical microscopy of mussel gonads, and other investigators have analyzed sex ratios (Morton 1991), gametogenic periodicity (Haggerty and Garner 2000), and hermaphroditism (Downing et al. 1989) by creating histological thin sections of gonadal tissue. Histological methods commonly are used and preferred because they illuminate reproductive development at the cellular level. Although lethal, histology has contributed importantly to our understanding of mussel reproduction for over a century (e.g., Lefevre and Curtis 1910). However, the level of detail gained from histological analysis of gonadal tissues is not necessarily needed (e.g., quantifying nongamete germline cells) to elucidate important aspects of mussel reproduction (Henley 2002), and sacrificing live mussels is not always a viable option, especially for threatened and endangered species.

Nonlethal methods that involve the use of a hypodermic syringe needle (hereafter, syringe technique) are being used increasingly to study freshwater mussels. The syringe technique has been used to extract hemolymph from the foot of mussels for the collection of genetic material (Geist and Kuehn 2005) and from adductor muscles to assess physiological condition (Gustafson et al. 2005, Fritts et al. 2015). More commonly, the syringe technique has been used to examine reproductive traits of mussels by extract-

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ing fluid from gonads. For example, Bauer (1987) was among the first to use the syringe technique to identify sex in nonsexually dimorphic species based on the presence of male (spermatozoa) or female (oocytes) gametes in gonadal fluid. Others have used the syringe technique to examine age of maturation (postmortem; Christian et al. 2000), reproductive timing (qualitatively; Shiver 2002), and prevalence of hermaphroditism and parasitism of mussels in disturbed habitats (e.g., Moles and Layzer 2008, Galbraith and Vaughn 2011). Henley (2002) developed a protocol that uses this technique to assess sex, hermaphroditism, and gametogenic stage. In a laboratory experiment, Saha and Layzer (2008) validated the lethality and accuracy of the syringe technique but only for assessing sex in nonsexually dimorphic species and gametogenic stage qualitatively. Galbraith and Vaughn (2009) used this method to assess factors influencing timing and rate of gamete production quantitatively. Their work was the first and only attempt to use the syringe technique in a quantitative fashion.

The syringe technique is appealing to conservationists for the study of reproduction because it can be used to assess sex or reproductive condition rapidly (Henley 2002), allows large sample sizes (Galbraith and Vaughn 2009), and limits adverse effects on mussels (Saha and Layzer 2008) while providing the information necessary for conservation and management. Investigators have concluded this technique can provide accurate representation of sex and gametogenic stage without increasing mortality under laboratory conditions (Henley 2002, Saha and Layzer 2008), but the technique has not been validated properly for its use to assess gamete production in mussels quantitatively (Galbraith and Vaughn 2009), and its lethal and sublethal effects (e.g., on growth) have not been tested under natural conditions (but see Geist and Kuehn 2005). We evaluated the effectiveness of the nonlethal syringe technique to assess gamete production in freshwater mussels quantitatively. We specifically investigated whether gamete production measured with the syringe technique is correlated positively with gamete production measured with the traditional histological technique and if the syringe technique affects survival, growth, and body condition of mussels in natural populations.

METHODS

Study sites

We established 3 study sites in 2 rivers located on the western Gulf Coast slope, USA. We selected 2 sites in the lower San Saba River, a tributary of the Colorado River, Texas, and 1 site in the Navasota River, a tributary of the Brazos River, Texas (Fig. 1). The San Saba River is situated on the Edwards Plateau, which is characterized by uplands of limestone bedrock, relatively little soil cover, and semiarid to subtropical–subhumid climate (Blum et al. 1994). This river is relatively high gradient and experiences long periods of low flow and short, high-magnitude flows during heavy rainfall (Blum et al. 1994). In contrast, the Navasota River is characterized by alluvial sediments from sandy loams to clay and subtropical–subhumid climate (Clark 1973). During periods of heavy rainfall, the Navasota River experiences high flows and extended flooding.

Study species

We targeted 4 mussels of the genus *Quadrula* for this study: *Quadrula apiculata* (Say, 1829) (Southern Mapleleaf), *Quadrula houstonensis* (Lea, 1859) (Smooth Pimpleback), *Quadrula petrina* (Gould, 1855) (Texas Pimpleback), and *Quadrula verrucosa* (Rafinesque, 1820) (Pistolgrip). *Quadrula apiculata* occurs widely among Gulf Coast drainages, ranging from the Rio Grande to Mississippi River (Williams et al. 2008), and *Q. verrucosa* is distributed throughout most of the eastern USA, including Gulf Coastal and Atlantic Slope drainages (Williams et al. 2008). In contrast, *Q. petrina* and *Q. houstonensis* are both Texas endemic species, restricted to rivers of central Texas (Howells et al. 1996), are currently considered threatened in Texas (Texas Secretary of State 2010), and are federal candidates for listing under the Endangered Species Act (US Fish and Wildlife Service 2011). Gametogenic periodicity is known from only *Q. verrucosa* (Jirka and Neves 1992), which produces gametes throughout the year, typically peaking between early spring and summer. We presumed a priori that *Q. apiculata*, *Q. houstonensis*, and *Q. petrina* have a relatively similar gametogenic cycle to *Q. verrucosa* or other species in *Quadrula* (e.g., Galbraith and Vaughn 2009). Because of differences in distribution and abundance of our focal species between study sites, we studied *Q. petrina* and *Q. verrucosa* from only the San Saba River (sites 1 and 2), *Q. apiculata* from only the Navasota River (site 3), and *Q. houstonensis* in both rivers (sites 2 and 3; Table 1, Fig. 1).

Experimental design

We established 3 treatment groups of mussels: 1) syringe (experimental), mussels used to measure gamete production with the syringe technique; 2) histology (validation), mussels used to measure gamete production with the histological technique; and 3) nongamete (control), mussels in which gamete production was not measured (Table 1). Syringe treatment groups served 2 purposes in our experiment. The 1st was to validate the syringe technique by comparing gamete estimates between syringe and histology treatments, and the 2nd was to assess the effect of the syringe technique on mussels by comparing survival, growth, and body condition between syringe and control treatment groups. Mussel assemblages varied among rivers, so we used a syringe/nongamete control (sites 1 and 2) or syringe/histology (sites 2 and 3) treatment pairing to assess the accuracy of the syringe technique or examine the effects of the syringe technique on mussels, respectively (Table 1).

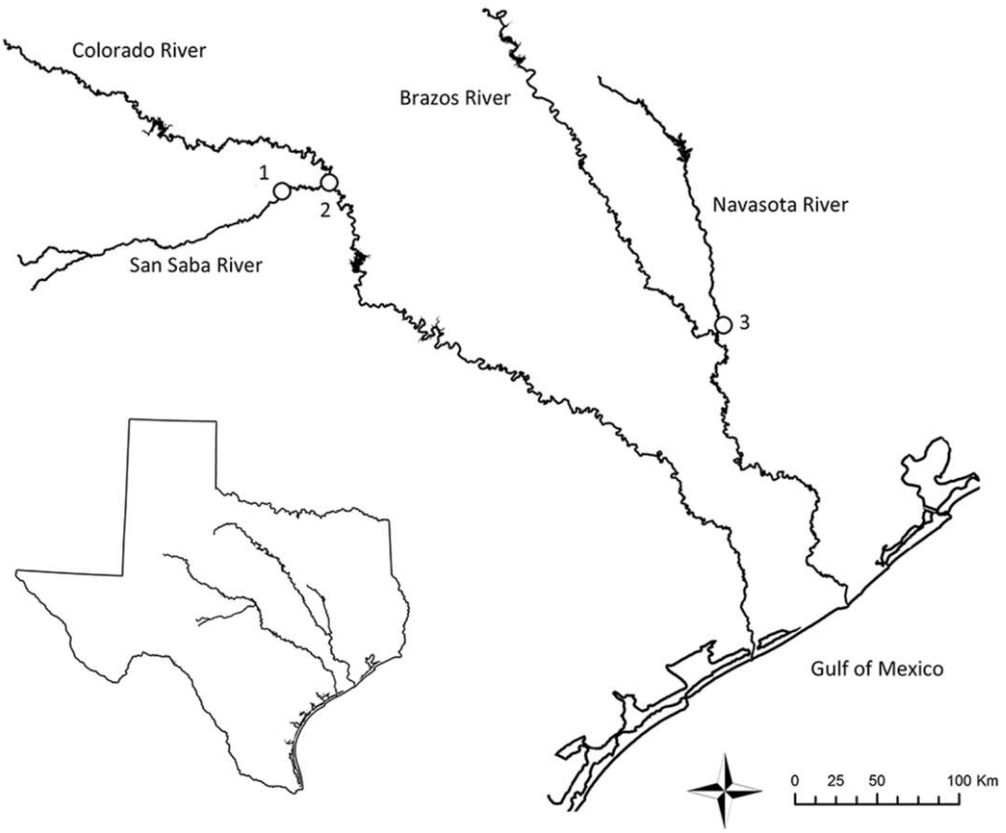

Figure 1. Experimental sites (circles) situated on the San Saba River (sites 1 and 2) and Navasota River (site 3), Texas.

Starting in July 2012, we collected adult mussels of similar size at sites 1 and 2, marked them with 12.5-mm passive integrated transponder (PIT) tags (Biomark, Boise, Idaho), and randomly assigned them to treatments (Table 1). Each PIT tag had a unique identification number and was affixed to a mussel shell with nontoxic marine epoxy putty (Kurth et al. 2007). Once we marked and assigned mussels to a treatment, we measured initial shell

Table 1. Treatment design to study the effectiveness of the syringe technique for 4 species of freshwater mussels (*Quadrula* spp.) from 3 sites. Treatments included experimental treatment for quantifying gamete production with the syringe technique (Syringe), validation treatment for quantifying gamete production with the histological technique (Histology), and nonreproduction treatment to control for the effects of the syringe technique on survival and growth (Control). Respective sample sizes (*n*) and mean (±SD, mm) initial shell length (sl) are given.

	Q. apiculata		*Q. houstonensis*		*Q. petrina*		*Q. verrucosa*	
Treatment	*n*	sl	*n*	sl	*n*	sl	*n*	sl
Site 1 (San Saba River)								
Syringe	–	–	–	–	63	55.6 ± 3.8	96	87.8 ± 6.8
Histology	–	–	–	–	–	–	–	–
Control	–	–	–	–	40	54.4 ± 4.7	40	84.7 ± 7.1
Site 2 (San Saba River)								
Syringe	–	–	105	43.9 ± 5.7	110	47.6 ± 6.5	96	79.4 ± 16.0
Histology	–	–	105	43.2 ± 7.3	100	45.5 ± 9.0	–	–
Control	–	–	40	45.2 ± 5.5	40	46.0 ± 6.1	40	77.4 ± 16.1
Site 3 (Navasota River)								
Syringe	74	54.6 ± 9.5	79	42.6 ± 5.0	–	–	–	–
Histology	78	55.4 ± 9.2	79	41.5 ± 5.7	–	–	–	–
Control	–	–	–	–	–	–	–	–

length (anterior to posterior, mm) and wetted mass (g) for estimates of growth and body condition. We placed mussels from each treatment in 5- × 5-m plots (1 treatment/plot) in which densities were kept at 8 mussels/m^2 to control for density-dependent effects on survival and growth (DiDonato 2002). At these sites (sites 1 and 2), syringe and control treatments were monitored to assess mussel survival, growth, and body condition. At site 3, we compared only gamete production between the 2 techniques (i.e., syringe and histology; Table 1) and, therefore, did not mark mussels with PIT tags or use mark–recapture methods.

We examined reproduction in syringe and histology treatments at ~4 to 6-wk intervals for 1 y. We examined survival, growth, and body condition for 2 y, which allowed us to test for sublethal effects associated with the syringe technique. During sampling trips to sites 1 and 2, we used an antenna receiver to locate 8 to 10 mussels from syringe treatments and histology treatments. For histology treatments, we preserved each individual by cutting its adductor muscles and placing it directly into 10% buffered formalin. For syringe treatments, we followed Galbraith and Vaughn (2009) and extracted gonadal fluid from each individual by inserting a 20-gauge hypodermic needle through the foot, positioned ~midline to the shell and halfway into the visceral mass. We confirmed the location of the gonads for these species a priori by examining cross and longitudinal sections of reproductive tissues (Henley 2002). We extracted 0.25 to 0.50 mL of gonadal fluid/individual, which we fixed in 10% buffered formalin and placed on ice for transport to the laboratory. We sampled gonadal fluid of mussels in syringe treatments only once and placed individuals back in their respective plots for the duration of the study. At site 3, we randomly collected mussels, sampled gonadal fluid from 8 to 10 individuals, and preserved an additional 8 to 10 individuals for histological analysis. Because mussels sampled with the syringe technique were not fitted with PIT tags at this site, we used a paint pen to mark their shells to ensure gonadal fluid was sampled only once from an individual.

We assessed survival, growth, and body condition in syringe and nongamete treatments ~quarterly for 2 y (7 encounter periods). During each assessment, we collected mussels by locating PIT tags with an antenna receiver combined with visual and tactile searches in the study plots. We searched for mussels until all individuals were recovered or PIT tags were no longer detected with the antenna receiver. This search effort typically took 1 to 3 d/site. We also spent time searching the entire study area (50 m up- and downstream) to recover dead mussels that became dislodged from the plots. Movement in and around the plots was minimal because of the complexity of the substrate (i.e., sand, small and large gravel and cobbles), but in rare cases live recaptures did occur downstream of plots. We placed all mussels collected in mesh bags, which we kept submerged in areas with sufficient flow. We organized data collected on recapture occasions as follows for each mussel: not encountered, live encounter, or dead recovery. We measured shell length (mm) and wetted mass (g) to estimate yearly proportional shell growth (mm/y) and Fulton's K body condition factor:

$$\text{shell growth rate} = \frac{\text{new shell length} / \text{initial shell length}}{\text{time (y) since the beginning of the study}} \quad \text{(Eq.1)}$$

and

$$\text{Fulton's K} = \frac{\text{wetted mass}}{\text{shell length}^3} \times 10^6. \quad \text{(Eq.2)}$$

Quantification of gamete production

We quantified gamete production in syringe treatments from gonadal fluid by estimating mean sperm concentration (number/mL) for males and mean egg concentration (number/mL) and diameter (μm) for females (Galbraith and Vaughn 2009). We assessed sex by identifying male or female gametes in a small drop of each sample after adding methylene blue to samples to help identify gametes (see Saha and Layzer 2008 for details and descriptions of gametes). We quantified sperm concentration with a hemocytometer and a compound microscope (400×), a technique that has been used to assess blood or reproductive cell density in humans and has been applied successfully to nonhuman subjects (e.g., Navarro et al. 1998). For females, we mixed the contents of each sample, placed 3 μL on a glass slide by means of an automatic pipettor (GeneMate; ISC BioExpress, Kaysville, Utah), and counted the number of eggs with the aid of a compound microscope (100×). We estimated egg concentration by extrapolating the number of eggs to 1 mL of gonadal fluid. We estimated mean egg diameter by measuring 50 randomly selected eggs with an ocular micrometer.

We fixed mussels sacrificed for histological examination in 10% buffered formalin for ≥2 wk and subsequently transferred them to 70% ethanol. We conducted tissue and slide preparation following Kiernan (1999). In the laboratory, we dissected mussels by excising the visceral mass and cutting a 2–4-mm section situated slightly anterior of the midline of the shell. We chose this area to mirror the location at which gonadal fluid was sampled with the syringe technique. We then dehydrated gonadal tissue in a graded ethanol series (to 100%), cleared it in toluene, and embedded it in paraffin wax. We cut transverse sections of gonadal tissues (7 μm) with a Spencer 820 rotary microtome (American Optical, Buffalo, New York). We mounted tissues sections on glass slides and stained them with hematoxylin and eosin. We quantified gamete production by

counting and measuring the number of gametes through the center of 10 randomly selected follicles (methods described by Jones et al. 1986, Haggerty et al. 1995, Haggerty and Garner 2000). Using the eyepiece pointer on a compound microscope (1000×), we counted the number of sperm/follicles along transects by moving the microscope stage along an *x*- or *y*-axis, and we measured the diameter of the first 50 eggs along transects positioned randomly through the entire tissue section. We measured only eggs that were sectioned through the nucleus, and we estimated diameter for each egg by averaging length and width measurements.

Statistical analyses

We used Pearson's product-moment correlation to test whether mean monthly estimates of gamete production measured with the syringe technique (sperm concentration, egg diameter, and egg concentration) were correlated with gamete production measured with the histological technique (sperm density, egg diameter, and egg density). This analysis allowed us to test whether the times of peak gamete production estimated from the 2 techniques were positively correlated, which would indicate the ability of the syringe technique to estimate gametogenic periodicity accurately in relation to the traditional histological technique. We analyzed each syringe–histology pairing (i.e., the same species at the same site) separately because of differences in timing of peak gamete production among species and sites. Prior to the analysis, we scaled all estimates of gamete production to a mean of 0 and standard deviation (SD) of 1. We compared the strength of linear correlation among treatments with the correlation coefficient r, and we used the t-statistic to test for a significant trend between gamete estimates. We performed these analyses in R (version 3.2; R Project for Statistical Computing, Vienna, Austria) and set $\alpha = 0.05$ for all statistical tests. We considered a correlation among gamete estimates statistically significant, if $p < 0.05$ and marginally significant when $0.05 < p < 0.10$.

We used a joint live encounter and dead recovery mark–recapture analysis in *RMark* (Laake 2013) to develop models in MARK (White and Burnham 1999) with the primary aim of modeling the effects of the syringe technique on survival probability (Lebreton et al. 1992, Burnham 1993). Four parameters can be estimated with this model: 1) survival probability (S), the probability of surviving the duration of an encounter interval; 2) recapture probability (p), the probability of being observed, conditional on being alive and in the study area; 3) recovery probability (r), the probability of being observed and reported dead; and 4) fidelity (F), the probability that an individual will remain in the sampling area. Key assumptions necessary to implement this model include: 1) all marked individuals have the same probability of surviving and being recaptured, 2) tags were not lost, 3) dead recovery rates are constant, 4) encounter intervals were relatively short in duration, and 5) dead recoveries occurred outside the sampling area (Lebreton et al. 1992, Burnham 1993). This model can differentiate between temporary and permanent emigration ($1 - F$), so survival probability is considered true survival. However, because we recovered dead individuals only in the same sampling area as live recaptures (violating an important assumption for estimating fidelity), we fixed $F = 1$ for all models, making our estimates of S analogous to apparent survival.

To estimate survival, recapture, and recovery probabilities we developed a candidate set of biologically relevant additive models based on our knowledge of freshwater mussel biology and stream ecology. We considered 4 predictor variables as potential sources of variation: sample date (time), site, species (sp), and treatment (treat). For each of the 3 parameters, we included time and site effects because variation in environmental conditions in streams over time and space can influence survival, recapture, and recovery rates. We modeled species effects with survival probability because mortality schedules inherently vary among species as a result of trade-offs among life-history traits (Stearns 1992). We also modeled treatment effects with survival probability because we wanted to test whether the syringe technique significantly affects survival. Mussels belonging to the syringe treatment groups were not all sampled with the syringe technique at the same time. We accounted for this variability by including treatment effects as a time-varying, categorical covariate. Behavioral differences can influence recapture probability among species of mussels (Villella et al. 2004), but we did not include species effects to estimate recapture and recovery probabilities because all mussels were marked with PIT tags and placed in study plots. PIT tags improve detection of mussels significantly (Kurth et al. 2007), negating differences among species and influence on recapture probability. Treatment effects were not considered for recapture and recovery probabilities because the syringe technique cannot significantly influence these parameters. Thus, our global model was $S_{\text{treat+time+sp+site}}\, p_{\text{time+site}}\, r_{\text{time+site}}\, F_1$.

We used a bootstrap goodness-of-fit test implemented in *MARK* to test for adequate fit of the global model. We assessed the level of fit by ranking and counting the number of models from 1000 simulations with deviance ≥ observed deviance. Our model lacked fit ($p = 0.001$), so we corrected for overdispersion by estimating the variance inflation factor ($\hat{c} = 1.73$) by dividing observed $\hat{c}$ by mean estimated $\hat{c}$ from the bootstrap simulations. After correcting for overdispersion, we used an information-theoretic approach to assess the candidate models (Burnham and Anderson 2002). We ranked the models based on lowest quasi-likelihood Akaike's Information Criterion corrected for small sample sizes (QAICc) to identify the most parsimonious

model (Burnham and Anderson 2002). We considered the top-ranked models within ΔQAICc < 2 to have substantial support, but models fitting this criterion with a difference of only 1 parameter and minimal difference in maximum log-likelihood typically are not considered competitive because of the inclusion of an uninformative parameter (Burnham and Anderson 2002, Arnold 2010). Thus, to make further inferences regarding the best-approximating model(s) and the importance of variables, we averaged parameter estimates from the top competitive models to account for model selection uncertainty. We also used QAICc weights (w) to assess the relative importance of each model based on the ratios among weights (i.e., evidence ratios), and we estimated relative variable importance ($w_{+(j)}$) by summing w across all candidate models that contained each predictor variable x_j (Burnham and Anderson 2002). The higher $w_{+(j)}$ value (ranging from 0 to 1) indicates higher support for a particular variable. The advantage of investigating $w_{+(j)}$ is that inferences can be drawn beyond variables occurring in the best-approximating model (Burnham and Anderson 2002, Wagenmakers and Farrell 2004) but only when the variables occur in equal numbers throughout the candidate model set, as was the case in our analyses.

We used linear mixed models (LMMs) and generalized additive mixed models (GAMMs) to examine variation in Fulton's K condition factor and yearly proportional growth rate, respectively. LMMs are useful regression analyses for grouped data (e.g., repeated measures on experimental units) because of their flexibility in handling covariance structures and unbalanced designs (Pinheiro and Bates 2006). We modeled both response variables with time as a continuous fixed variable and allowed all possible combinations of species, site, and treatment effects as categorical grouping variables. We modeled the lowest experimental unit (mussel) as a random effect and allowed intercepts to vary to account for heterogeneity and nonindependence of (repeated) measurements over time (Pinheiro and Bates 2006). We used *mgcv* package in R (Wood 2001) to model growth with GAMMs because of the nonlinear rate at which mussels grow over time (Zuur et al. 2009). We implemented GAMMs with a Gaussian identity link function and cubic smoothing splines to characterize the nonlinear relationship between time and growth (Zuur et al. 2009, 2014). Exploratory analysis of normalized residuals indicated heterogeneity, so we $\sqrt{(x)}$-transformed growth to meet model assumptions. We evaluated the fit of the smoothing term by the effective degrees of freedom (edf), where edf > 1 is defined by the degree of nonlinearity. We tested significance with an F-ratio test (Zuur et al. 2014). We used LMMs to model Fulton's K with *lme4* package in R (Bates 2010) because these data displayed a linear trend over time. Because length–wet mass ratios vary widely among species (i.e., shell morphology varies in size and mass relative to tissue mass), we also included species as a random effect to account for this variation (Bates et al. 2015). For both GAMM and LMM, we implemented model selection by means of the lowest-ranked AIC value to identify the most parsimonious model, and we used evidence ratios and $w_{+(j)}$ based on AIC w to measure relative support of the models and individual variables.

RESULTS

We studied 1185 mussels from 4 species across 2 rivers, which included 875 mussels marked with PIT tags. We extracted 0.37 ± 0.1 mL (mean ± SE) of gonadal fluid from 528 individual mussels with the syringe technique, and we successfully quantified gametes (sperm or eggs) from ~77.8% (n = 411) of these samples. Of the other gonadal fluid samples, 21.8% (n = 115) contained no gametes, largely because digenetic trematodes had parasitized mussel gonads, and the remaining 0.4% (n = 2) contained neither trematodes nor gametes. Of the 339 mussels sacrificed for histological analysis, which excludes 23 mussels initially marked and not sampled, we were able to assess gamete production from 290 individual mussels (85.5%), and the other 49 (14.5%) lacked gametes because they were parasitized by trematodes.

Accuracy of the syringe technique

Mean scaled estimates of gamete production measured with the syringe technique (egg concentration, egg size, and sperm concentration) and the histological technique (egg density, egg size, and sperm density) were comparable among the 12 syringe–histology treatment comparisons (4 species–site groups × 3 gamete estimates) when plotted over time (Fig. 2A–L). In most cases, the timing of highest and lowest points of gamete production aligned exactly across treatments, especially for egg size (Fig. 2B, E, H, K). However, the timing of some peak estimates between sperm concentration and sperm density (*Q. houstonensis* at site 3; Fig. 2J) and egg concentration and egg density (*Q. apiculata* at site 3; Fig. 2I) were off by 1 sampling date. Gamete estimates from syringe and histology treatments were positively correlated based on Pearson's correlations (Fig. 3A–L). Egg size was correlated among treatments for all 4 groups (r = 0.88–0.92, p < 0.05; Fig. 3B, E, H, K). Sperm concentration and sperm density also were correlated: 1 group (*Q. petrina*, site 2) was significantly correlated (r = 0.94; Fig. 3A), and 3 groups were marginally significantly correlated (r = 0.61–0.64; Fig. 3D, G, J). In contrast, egg concentration and egg density were correlated in only some groups. They were correlated for *Q. apiculata* at site 3 (r = 0.78, p = 0.02; Fig. 3I) and marginally so for *Q. houstonensis* at site 2 (r = 0.64, p = 0.06; Fig. 3F). They were not correlated (p > 0.10) for the other 2 groups (*Q. petrina* at site 2 and *Q. houstonensis* at site 3; Fig. 3C, L).

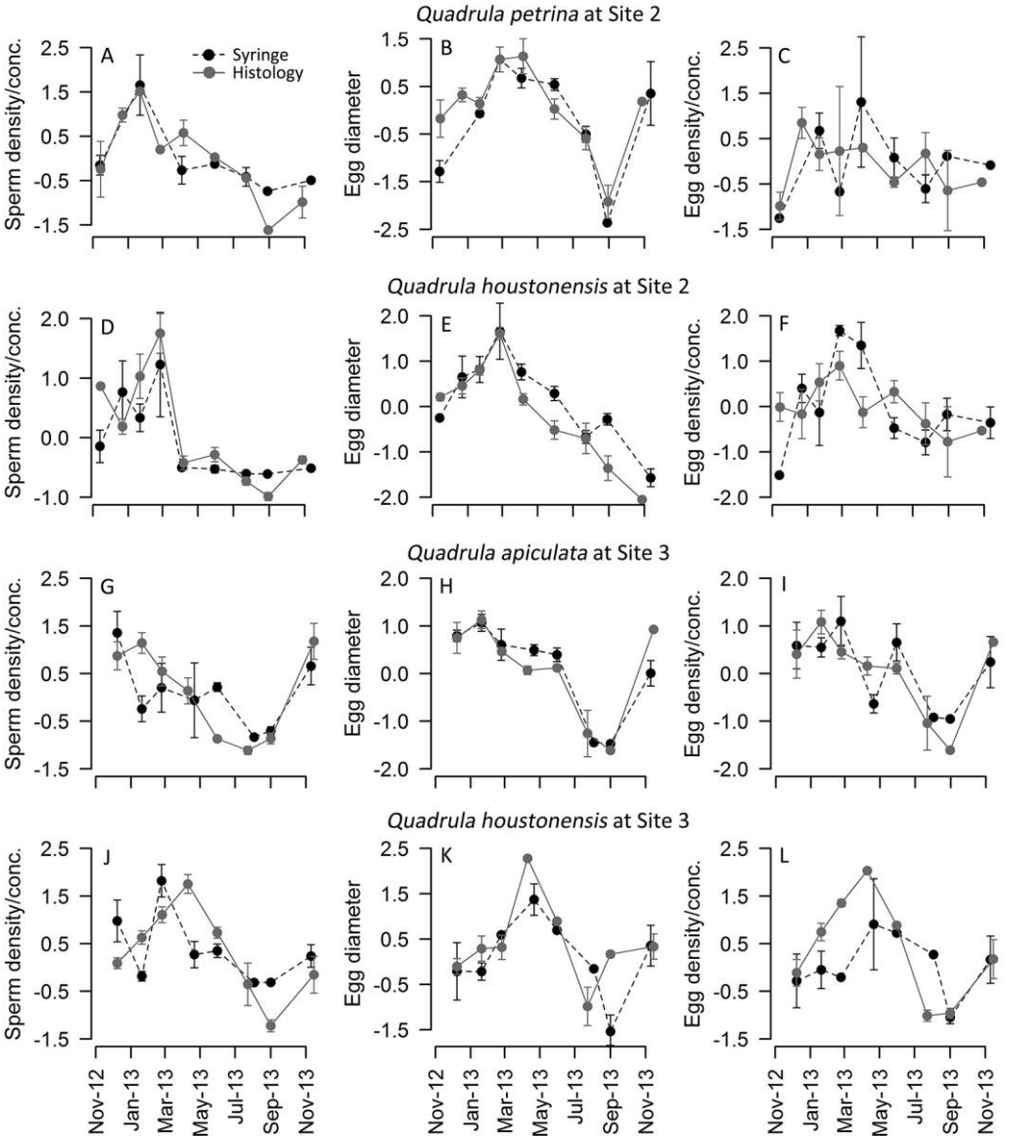

Figure 2. Mean (±1 SE) scaled estimates of gamete production comparing syringe and histology techniques for *Quadrula petrina*, site 2 (A–C), *Quadrula houstonensis*, site 2 (D–F), *Quadrula apiculata*, site 3 (G–I), and *Quadrula houstonensis*, site 3 (J–L). Estimates of gamete production include sperm concentration (syringe treatment) or density (histology treatment) (A, D, G, J), egg size (B, E, H, K), and egg concentration (syringe treatment groups) or density (histology treatment groups) (C, F, I, L).

Survival, recapture, and recovery probabilities

Our candidate model set consisted of 128 models, for which we estimated 3 parameters (survival [S], recapture [p], and recovery [r] probabilities) represented by 4 variables (time, species [sp], site, and treatment [treat]). The best-approximating model ($S_{(\text{time + sp + site})}\, p_{(\text{time + site})}\, r_{\text{time}}$, QAICc = 1291.55) indicated that survival probability varied with time, species, and site; recapture probability varied with time and site; and recovery probability varied with time (Table 2). All 3 parameters were consistently time dependent (Table 2). Based on a criterion of ΔQAICc < 2, the top 3 QAICc ranked models were supported. The 2nd-best-approximating model ($S_{(\text{treat + time + sp + site})}\, p_{(\text{time + site})}\, r_{\text{time}}$, QAICc = 1291.94) was similar to the 1st but included treatment as an additional factor explaining survival probability, whereas the 3rd-best-fit model ($S_{(\text{time + sp + site})}\, p_{(\text{time + site})}\, r_{(\text{time + site})}$, QAICc = 1293.42) was similar to the 1st except site was an additional factor explaining recovery probability (Table 2). However, only the 1st and 2nd top-ranked AIC models were supported because the 3rd model had a similar maximum log-likelihood value as the 1st model and differed by only 1 parameter, which indicates that it is less parsimonious than the 1st and 2nd top-ranked models (Table 2). In addition, QAICc w of the 1st model indi-

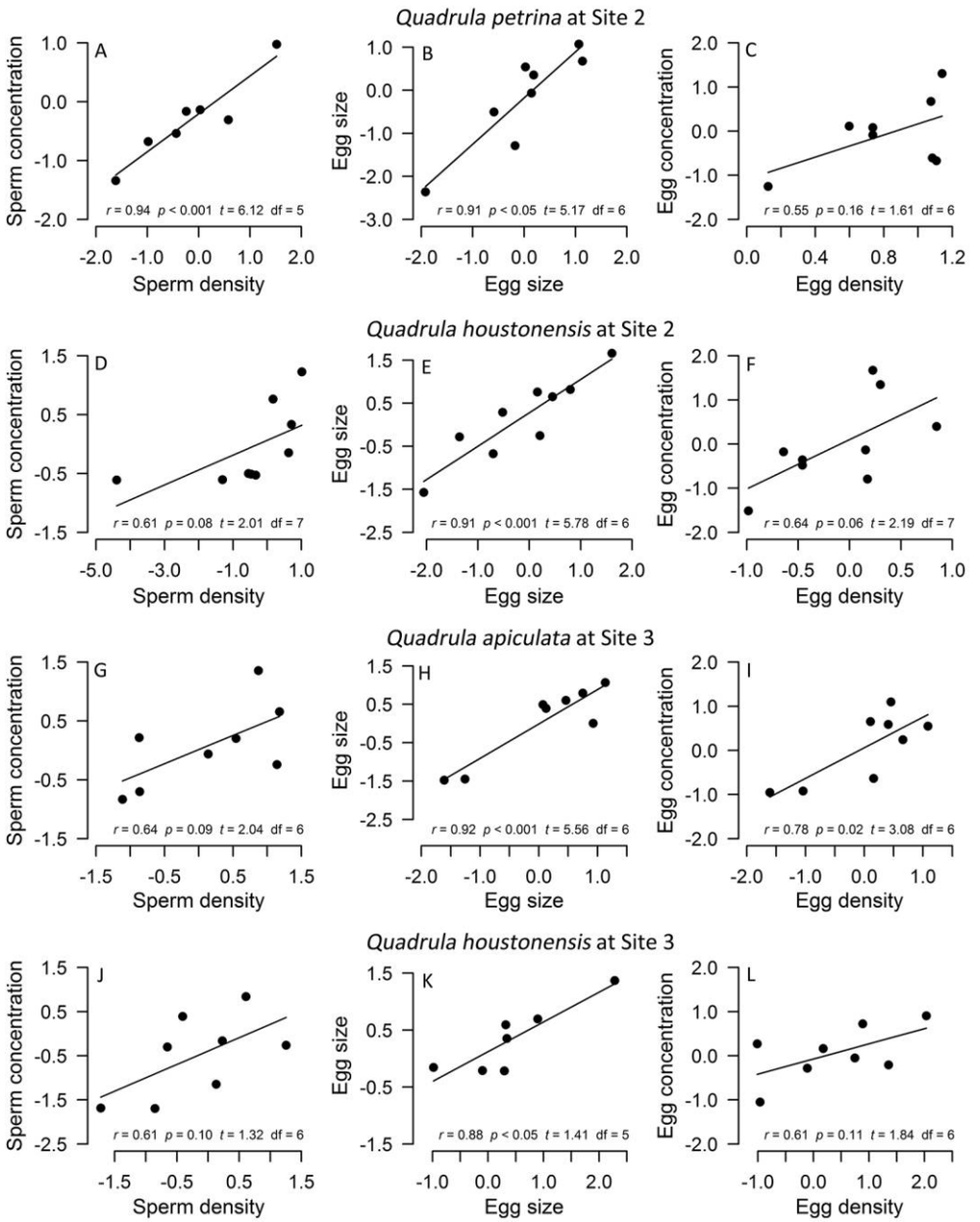

Figure 3. Pearson's correlations comparing gamete production between syringe and histology techniques for *Quadrula petrina*, site 2 (A–C), *Quadrula houstonensis*, site 2 (D–F), *Quadrula apiculata*, site 3 (G–I), and *Quadrula houstonensis*, site 3 (J–L). Estimates of gamete production include sperm concentration with sperm density (A, D, G, H), egg size (B, E, H, K), and egg concentration with egg density (C, F, I, L).

cated 2.5× greater support than for the 3rd model, whereas w of the 1st model was only 1.2× times that of the 2nd model. When considering the importance of variables individually, species, site, and time all significantly explained variability in survival probability ($w_{+(j)}$ = 0.999 for all 3 variables; Table 3), whereas treatment was 2.2× less important than the other variables considered ($w_{+(\text{Treat})}$ = 0.452; Table 3). Time and site were both relatively important variables in explaining recapture probability ($w_{+(\text{time})}$ = 1.0000, $w_{+(\text{site})}$ = 0.9167; Table 3). Time was also important in explaining recovery probability ($w_{+(\text{time})}$ = 0.9940), whereas site had considerably less support (3.5×) explaining recovery probability ($w_{+(\text{site})}$ = 0.2817; Table 3).

We averaged parameter estimates for the 2 top QAICc-ranking models because of model-selection uncertainly (Table 2). Despite some evidence indicating that treatment was

Table 2. Fifteen best-approximating models ranked by lowest quasi-likelihood Akaike Information Criterion corrected for small sample sizes (QAICc) from joint live encounter and dead recovery analysis of 3 species of freshwater mussels (*Quadrula petrina, Quadrula houstonensis,* and *Quadrula verrucosa*) at 2 sites in the San Saba River, Texas. Model parameters included survival probability (S), recapture probability (p), dead recovery probability (r), and fidelity (F) and were tested for variation among time (time), treatment (treat), site (site), and species (sp). Only S was tested for treatment and species effects, and F was fixed at 1 for all models. ΔQAICc, Akaike weights (w_i), −2log-likelihood (−2ln[L]), and number of parameters (k) are given for each candidate model. * indicates estimates were averaged because of model selection uncertainty.

Model	k	QAICc	ΔQAICc	w_i	−2ln(L)
$S_{(time + sp + site)}\ p_{(time + site)}\ r_{(time)}\ F_{(1)}$*	21	1291.55	0.000	0.358	2161.33
$S_{(treat + time + sp + site)}\ p_{(time + site)}\ r_{(time)}\ F_{(1)}$*	22	1291.94	0.384	0.296	2158.49
$S_{(time + sp + site)}\ p_{(time + site)}\ r_{(time + site)}\ F_{(1)}$	22	1293.42	1.871	0.141	2161.07
$S_{(treat + time + sp + site)}\ p_{(time + site)}\ r_{(time + site)}\ F_{(1)}$	23	1293.81	2.256	0.116	2158.23
$S_{(time + sp + site)}\ p_{(time)}\ r_{(time)}\ F_{(1)}$	20	1296.35	4.798	0.033	2173.12
$S_{(treat + time + sp + site)}\ p_{(time)}\ r_{(time)}F_{(1)}$	21	1296.74	5.187	0.027	2170.30
$S_{(time + sp + site)}\ p_{(time)}\ r_{(time + site)}\ F_{(1)}$	21	1298.21	6.656	0.013	2172.84
$S_{(treat + time + sp + site)}\ p_{(time)}\ r_{(time + site)}\ F_{(1)}$	22	1298.60	7.046	0.011	2170.02
$S_{(time + sp + site)}\ p_{(time + site)}\ r_{(1)}\ F_{(1)}$	16	1301.73	10.180	0.002	2196.40
$S_{(treat + time + sp + site)}\ p_{(time + site)}\ r_{(1)}\ F_{(1)}$	17	1302.11	10.560	0.002	2193.57
$S_{(time + sp + site)}\ p_{(time + site)}\ r_{(site)}\ F_{(1)}$	17	1303.73	12.181	0.001	2196.37
$S_{(treat + time + sp + site)}\ p_{(time + site)}\ r_{(site)}\ F_{(1)}$	18	1304.11	12.562	0.001	2193.54
$S_{(time + sp + site)}\ p_{(time)}\ r_{(1)}\ F_{(1)}$	15	1306.42	14.865	0.000	2208.00
$S_{(treat + time + sp + site)}\ p_{(time)}\ r_{(1)}\ F_{(1)}$	16	1306.80	15.230	0.000	2205.17
$S_{(time + site)}\ p_{(time + site)}\ r_{(time)}\ F_{(1)}$	19	1308.03	16.477	0.000	2196.82

an important predictor of variability in survival probability, the small differences in mean model estimates between syringe and control treatments suggested that treatment effects have little biological significance (Fig. 4A–E). Most differences in mean survival estimates between treatments were within 0.01–0.03 probability, and the largest difference was for *Q. petrina* at site 1, which varied in probability as little as 0.03–0.05 (Fig. 4A). These small differences support earlier conclusions that treatment had little influence in explaining survival. Regardless of treatment, survival probability was generally high for most species and sites but declined slightly over time (Fig. 4A–E). Over the 2-y period of our study, *Q. petrina* had the lowest survival, which ranged from 0.53 to 0.84 (site 1) and 0.75 to 0.93 (site 2). *Quadrula verrucosa* survival ranged from 0.80 to 0.95 (site 1) and 0.91 to 0.98 (site 2), and *Q. houstonensis* ranged from 0.83 to 0.96 (site 2) (Fig. 4A–E). Recapture probabilities averaged over the 2 best-approximating models were also high and varied little over time. They ranged from 0.77 to 1.00 and 0.87 to 1.00 across sites 1 and 2, respectively (Table 4). For most recapture periods, recapture probability was >0.97, except for March 2014, when estimates dropped significantly at both sites and represented the lowest recapture rates. In contrast, mean model estimates for recovery probability varied widely over time and ranged from 0.34 to 0.93 (Table 4). Recovery probabilities were lower earlier in the study, increased by >100% by April 2013, and then steadily declined (Table 4).

Growth and body condition

Of the 8 GAMMs in the candidate set, the best-approximating model explaining variability in growth included time (s[time] = smoothing term), site, and species ($Growth_{s(time) + site + Sp}$, AIC = −7003.40; Table 5). The 2nd top-ranked AIC model also was supported and included treatment as an additional predictor variable ($Growth_{s(time) + treat + site + sp}$, AIC = −7001.46; Table 5). AIC w indicated 2.6× more support for the 1st model than the 2nd, and $w_{+(j)}$ (i.e., summed AIC w) indicated that site ($w_{+(site)}$ = 0.8485) and species ($w_{+(sp)}$ = 0.9662) were highly supported, whereas treatment ($w_{+(treat)}$ = 0.2741) was weakly supported (Table 5). Overall, the coefficients derived from the best-approximating model indicated that growth decreased over time (Table 6) and that this decreasing trend was significantly nonlinear (edf = 4.937, F = 910.8, p < 0.001; Table 6).

Of the 8 LMMs explaining variability in Fulton's K condition index, the best-approximating model varied with time and species (Fulton's $K_{time + sp}$, AIC = 26523.18; Table 5). The 2nd (Fulton's $K_{time + treat + sp}$, AIC = 26523.98; Table 5) and 3rd (Fulton's $K_{time + sp + site}$, AIC = 26524.66; Table 5) best-fit models also were well supported. Based on AIC w, the 1st best-approximating model was 1.5× better supported than the 2nd and 2.1× better supported than the 3rd. Relative variable importance indicated that treatment ($w_{+(treat)}$ = 0.3997) and site ($w_{+(site)}$ = 0.3208) were weakly supported in the candidate model set, which indi-

Table 3. Relative variable importance ($w_{+(j)}$) of grouping variables, species (sp), site (site), time (time), and treatment (treat), for the parameters survival probability (S), recapture probability (p), dead recovery probability (r), growth, and Fulton's K body condition index. Number (No.) of models is the number of times a variable occurred in the candidate model set, and $w_{+(j)}$ was estimated by summing Akaike weights (w_i) across all candidate models that contained each predictor variable.

Parameter and grouping variable	No. of models	Importance $w_{+(j)}$
S		
Sp	64	0.9999
Site	64	0.9999
Time	64	0.9999
Treat	64	0.4521
p		
Site	64	0.9167
Time	64	1.0000
r		
Site	64	0.2817
Time	64	0.9940
Growth		
Sp	4	0.9662
Site	4	0.8485
Treat	4	0.2741
Fulton's K		
Sp	4	1.0000
Site	4	0.3208
Treat	4	0.3997

cated the added variables in the 2nd (treatment) and 3rd (site) best-fit models were not important in explaining heterogeneity in Fulton's K index. Coefficients from the 1st top-ranked model indicated a linear increase in Fulton's K over time, and Fulton's K was highly dependent on species from both fixed (time and species) and random effects (species and mussel; Table 6).

DISCUSSION

We successfully used the syringe technique to extract gonadal fluid from freshwater mussels. Gametes were observed in most samples (77.8%) but were completely absent from samples that contained digenetic trematode parasites, which castrate mussels (Taskinen and Valtonen 1995, Laruelle et al. 2002). Histological investigation of mussels parasitized with trematodes showed that gonads were in fact devoid of gametogenic tissues, which suggests that the absence of gametes in fluid extractions was not because we had failed to locate the gonads. Moreover, we attribute our high extraction rate success to an a priori histological examination of the viscera of individuals belonging to our target species, as recommended by Henley (2002), which enabled us to select and target a suitable location from which to sample gonadal fluid with a syringe needle accurately and consistently. Saha and Layzer (2008) had high success when using the syringe technique to extract gametes from *Elliptio dilatata* (Spike) for sex determination but had slightly lower success with *Actinonaias ligamentina* (Mucket), which is a species known to pause gametogenesis (Jirka and Neves 1992). The success rate of extracting gametes may vary over the course of a year for species reported to have reduced or inactive periods of gametogenesis (e.g., *Quadrula cylindrica*, Yeager and Neves 1986; *Cyclonaias tuberculata*, Haggerty et al. 1995). In contrast, use of species known to produce gametes year-round, including the species examined in our study, might result in higher extraction-rate success (e.g., *Villosa nebulosa*, Zale and Neves 1982; *Elliptio dilatata*, Jirka and Neves 1992; *Anodonta anatina*, Hinzmann et al. 2013).

Gamete production was estimated in mussels by means of the syringe technique with relatively high accuracy. Mean egg diameter had the highest correlated estimates among treatment groups, which was not unexpected because direct measurements of egg diameter were made with both techniques. The only difference observed was that mean egg diameter tended to be smaller when measured with the histology technique (mean ± SE, 130.4 ± 19.3 μm) than the syringe technique (157.3 ± 19.3 μm), an artifact probably attributable to tissue shrinkage from embedding and thin-sectioning during slide preparation (Kiernan 1999). Measurements obtained from eggs collected via the syringe technique may be closer to the actual size of the eggs, which is not relevant to quantifying gametogenic periodicity. Sperm concentration estimated from the syringe technique was generally correlated with sperm density estimated from the histological technique but with some variability among treatments. This variability was small in most cases but high enough that peak estimates did not align on the same sample period for a few treatment comparisons, which could be attributed to limited sample sizes in some treatments, particularly in treatments where parasitism by trematodes was high (e.g., *Q. petrina* and *Q. houstonensis* at site 2). Trematode parasites occurring at low levels, but potentially not detected in either syringe or histological techniques, could have increased variability in our estimates. Taskinen and Valtonen (1995) noted that reproduction in mussels infected with trematodes could be affected significantly even at low parasite loads. The life cycle of trematodes also may affect gamete estimates disproportionately across seasons (Taskinen et al. 1994). Increasing sample size and the frequency of sampling in future research may reduce this variability. In contrast, correlations among egg

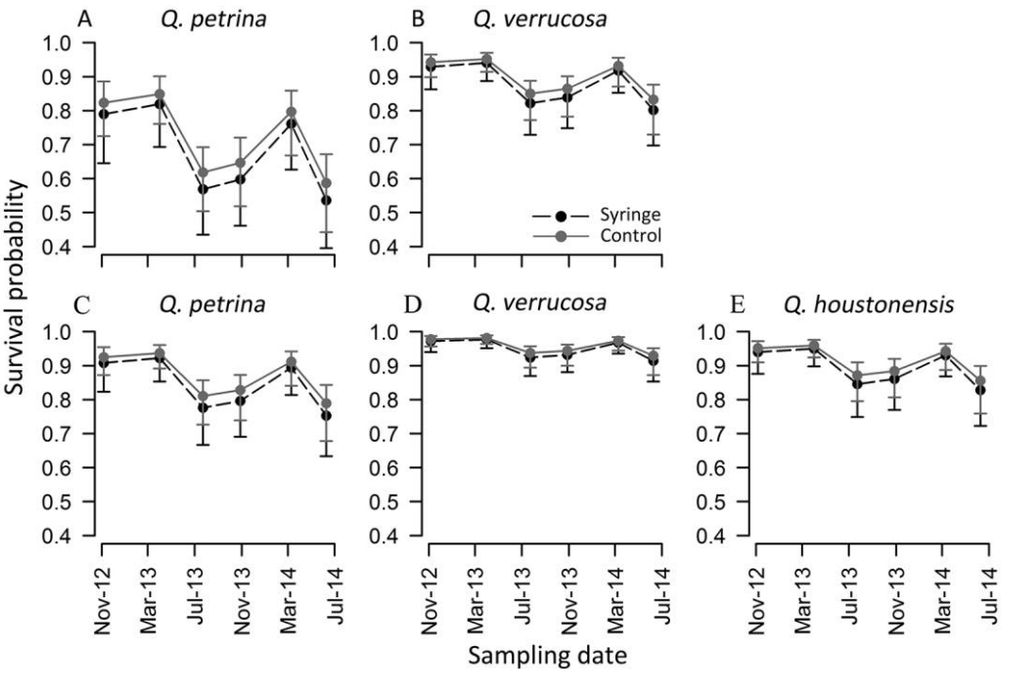

Figure 4. Mean (±1 SE) survival probability of *Quadrula petrina* (A) and *Quadrula verrucosa* (B) at site 1, *Quadrula petrina* (C), *Quadrula verrucosa* (D), and *Q. houstonensis* (E) at site 2 in San Saba River, Texas. Estimates from the 2 best-approximating quasi-likelihood Akaike Information Criterion with small sample size models were averaged to account for model-selection uncertainty (Table 2).

concentration and egg density were significant in only some cases (e.g., *Q. houstonensis* at site 2 and *Q. apiculata* at site 3) and not in others (e.g., *Q. petrina* at site 2 and *Q. houstonensis* at site 3). This result suggests that the syringe technique is less accurate when quantifying egg concentration than the other gamete estimates. We conclude that quantification of gamete production can be accomplished with reasonable accuracy with the syringe technique, particularly when estimating sperm concentration and egg diameter.

Our mark–recapture analyses indicated high recapture probability, which varied by time and site, whereas (dead) recovery probability varied by time. Seasonal environmental conditions (e.g., temperature, turbidity, and flow) that could affect mussel behavior and our ability to find mussels (e.g., Villella et al. 2004, Wisniewski et al. 2013) probably had minimal influence on recapture probability because mussels were marked with PIT tags. Most estimates were high except for a slight drop in March 2014, which we attribute to a technical malfunction of the antenna receiver. Despite the presumed invasiveness of inserting a syringe needle into the viscera of mussels, we failed to find negative effects of the syringe technique on survival. Support for treatment effects was apparent in some models, but differences in survival probability among control and sy-

Table 4. Parameter estimates and 95% confidence intervals (CI) for recapture probability (p) and dead recovery (r) for *Quadrula* spp. on each sampling date in the San Saba River, Texas, averaged over the 2 best-approximating models (Table 2).

	Site 1		Site 2		Sites 1 and 2	
Date	p	CI	p	CI	r	CI
15 July 2012	–	–	–	–	0.44	0.20–0.69
6 November 2012	0.98	0.97–1.00	0.99	0.99–1.00	0.44	0.19–0.68
1 April 2013	0.98	0.97–0.99	0.99	0.98–1.00	0.93	0.84–1.01
23 July 2013	0.97	0.96–0.99	0.99	0.98–1.00	0.69	0.52–0.86
28 October 2013	0.97	0.96–0.99	0.99	0.98–1.00	0.45	0.19–0.70
10 March 2014	0.77	0.71–0.83	0.87	0.84–0.90	0.34	0.16–0.52
9 June 2014	1.00	1.00–1.00	1.000	1.00–1.00	–	–

Table 5. Candidate model set for generalized additive mixed models (GAMMs) used to analyze growth over time and linear mixed models (LMMs) used to analyze Fulton's K condition index over time. Models are ranked according to their lowest Akaike Information Criterion (AIC). ΔAIC, AIC weight (w_i), and log-likelihood (ln[L]) are given for each model. Variables are time, site (site), species (sp), and treatment (treat). For GAMM models, s(time) indicates smoothing term was applied for time.

Parameter and model	AIC	ΔAIC	w_i	ln(L)
Growth				
s(time) + site + sp	−7003.40	0.0000	0.6057	3509.70
s(time) + treat + site + sp	−7001.46	1.9437	0.2292	3509.73
s(time) + sp	−6999.71	3.6920	0.0956	3506.86
s(time) + treat + sp	−6997.74	5.6628	0.0357	3506.87
s(time)	−6995.97	7.4325	0.0147	3502.99
s(time) + site	−6995.18	8.2251	0.0099	3503.59
s(time) + treat	−6993.99	9.4108	0.0055	3503.00
s(time) + treat + site	−6993.21	10.1942	0.0037	3503.60
Fulton's K				
time + sp	26523.18	0.0000	0.4061	−13254.59
time + treat + sp	26523.98	0.7933	0.2731	−13253.99
time + sp + site	26524.66	1.4752	0.1942	−13254.33
time + treat + sp + site	26525.51	2.3306	0.1266	−13253.76
time	26545.20	22.0145	0.0000	−13267.60
time + treat	26546.00	22.8167	0.0000	−13267.00
time + site	26546.65	23.4710	0.0000	−13267.33
time + treat + site	26547.52	24.3362	0.0000	−13266.76

ringe treatments were not biologically meaningful. Saha and Layzer (2008), who conducted a 1-y laboratory experiment, found no evidence of increased mortality from gonadal fluid extractions with the syringe technique. Other investigators who have used the syringe technique to extract hemolymph from the foot of mussels for molecular analysis (Geist and Kuehn 2005) and from adductor muscles for physiological analysis (Gustafson et al. 2005) also noted high survival. Moreover, we failed to detect sublethal effects on mussels based on our mixed-model analyses of growth and Fulton's K index, despite the fact that growth did vary significantly with time. Mark–recapture methods could bias growth estimates because of factors associated with PIT tags or increased handling (Waller et al. 1999, Haag 2009, Wilson et al. 2011), but these biases probably were not an issue because mussels in both control and syringe treatments were marked with PIT tags. We did not test for the effects of handling associated with the syringe technique (i.e., syringe treatment mussels were handled more than control treatment mussels), but the absence of support for treatment effects in our models indicated the added handling in the syringe treatment group was not important. Overall growth of mussels could have been influenced by trematodes, but we controlled for the effects of trematodes on growth compared between treatments (syringe vs control) by including parasitized and unparasitized mussels in the analyses because parasitism of mussels in control treatments was unknown.

Our results suggest that mussels are not affected adversely by the syringe technique on lethal or sublethal levels. However, this result does not necessarily preclude the possibility of inflicting stress on mussels in other ways or of causing permanent, long-term effects. The reproductive anatomy of bivalves is relatively complex. The gonads, intestinal tract, digestive gland, and kidney are housed within the visceral mass and generally are fused throughout the anteroventral to posteroventral region, depending on the species (Cummings and Graf 2009). Thus, it would be relatively easy to damage these organs by inserting a syringe needle into the viscera of a mussel. We did notice a slight dark yellow discoloring in several samples of gonadal fluid, suggesting we had inserted the needle through the intestinal tract of the mussels. This suggestion was confirmed by the presence of undigested food particles (e.g., phytoplankton) in these samples when examined under the microscope. Unfortunately, the fates of these individuals are unknown because they were either not recaptured following collection of gonadal fluid or came from site 3, where mark–recapture analyses were not conducted. Galbraith and Vaughn (2009) also noted that inserting the needle into the visceral mass evidentially led to extraction of intestinal fluids. In addition, the effects of the syringe technique on

Table 6. Coefficients for the top generalized additive mixed model (GAMM) for growth and linear mixed model (LMM) for Fulton's K condition index, including fixed and random effects coefficient for each model. Approximated estimates for smoothing term (s[time]) consist of effective degrees of freedom (edf), *F* statistic, and significance level (*p*).

Effect or term	Estimate	SE	*t*	Variance	SD	edf	*F*	*p*
Growth: s(time) + site + sp								
Fixed								
Intercept	0.271	0.010	26.97					
s(time)	−0.114	0.002	−54.8					
Site (site 1)	−0.032	0.013	−2.38					
Species (*Q. petrina*)	0.027	0.014	1.92					
Species (*Q. verrucosa*)	0.051	0.014	3.50					
Random								
Mussel				0.115	0.041			
Smooth term								
s(time)						4.937	910.8	<0.001
Fulton's K Index: time + sp								
Fixed								
Intercept	403.7	56.0	7.21					
Time	283.4	0.0	25.80					
Species (*Q. petrina*)	−63.2	79.2	−0.80					
Species (*Q. verrucosa*)	−257.3	79.1	−3.25					
Random								
Mussel				894.9	29.9			
Species				3129.4	55.9			
Residuals				190.0	13.8			

reproduction itself are not completely understood. Saha and Layzer (2008) did examine gonadal tissues histologically in mussels after extracting gametes twice with the syringe technique and concluded that reproduction was not significantly affected. However, investigators should extract gonadal fluid from a mussel only once to avoid permanent damage to reproductive tissues. Mussels should be marked or tagged properly subsequent to using the syringe technique to avoid this issue. As such, implementation of the syringe technique should be done cautiously, and resource managers should consult the small but growing body of literature on the use of the syringe technique prior to implementation (e.g., Bauer 1987, Henley 2002, Shiver 2002, Geist and Kuehn 2005, Gustafson et al. 2005, Moles and Layzer 2008, Saha and Layzer 2008, Galbraith and Vaughn 2009, Fritts et al. 2015).

Nonlethal methods that can be used in conservation studies of freshwater mussels are needed to prevent further endangerment of this group. The syringe technique has been used previously to sample genetic material (Geist and Kuehn 2005), examine physiological condition (Gustafson et al. 2005), and qualify reproduction traits (Saha and Layzer 2008). Our results demonstrate that the syringe technique is minimally invasive to freshwater mussels and validate its use for the study of various aspects of freshwater mussel biology. Given its ability to quantify reproduction accurately, the syringe technique can now be used in place of the histological technique to study reproductive traits, such as timing and duration of spawning periods (Zale and Neves 1982, Smith et al. 2003) and gametogenic periodicity (Haggerty et al. 1995, Haggerty and Garner 2000). Caveats notwithstanding, the benefits of the syringe technique are that it could be used to study threatened and endangered species and could be used to help resolve the reproductive status of mussel populations in future conservation efforts (Saha and Layzer 2008). For example, physiochemical changes in aquatic systems, such as through hypolimnetic impoundment releases or increased levels of pollutants, can suppress gamete production and spawning in freshwater mussels (Heinricher and Layzer 1999, Bringolf et al. 2010). The syringe technique could be used to investigate the reproductive viability of populations exposed to such abnormal conditions, or it could be used in relic populations experiencing low recruitment rates from unknown causes. Furthermore, implementing this technique would be less costly, relatively easy to learn, and more time efficient than histological methods (Saha and Layzer 2008). The syringe technique opens the door to a new avenue of broader ecological research on mussels because more individuals (sampled nonlethally) can be used to explore

aspects of reproductive ecology (e.g., Galbraith and Vaughn 2009), physiological condition (Fritts et al. 2015), and genetic structure of populations (Geist and Kuehn 2005). Despite the advantages of the syringe technique in the study of mussel reproduction, a continuing need exists for histological research on mussels because it remains the only way to investigate certain aspects of gametogenesis, including germ cell differentiation. We predict that the syringe technique will be most useful in conservation studies of threatened and endangered species or ecological studies that require larger sample sizes.

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