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HEATHER SUSAN GALBRAITH
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REPRODUCTION IN A CHANGING ENVIRONMENT: MUSSELS, IMPOUNDMENTS, AND CONSERVATION

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF ZOOLOGY

BY

Dr. Caryn C. Vaughn, Chair

Dr. Jeff Kelly

Dr. Rosemary Knapp

Dr. Scott Russell

Dr. Gary Wellborn

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Abstract

As humans alter the environmental landscape, there is an increasing need to understand the relationship between species and the environment, how changes to the environment translate to populations and communities, and how to develop management practices that reduce or reverse our negative impacts. Different animals respond differently to environmental change as do different developmental life stages within the same species. Reproduction, for example, is often the most sensitive time period of an organism's life, yet has been largely ignored in conservation biology, partly due to the difficulties in studying reproduction (e.g. complex life cycles, migration, delayed reproduction). Nonetheless, it is vitally important that we have some basic understanding of the reproductive process in order to facilitate sound management of critically imperiled fauna. Freshwater mussels are one such globally imperiled group of invertebrates with over 70% of species considered threatened. However, very little is understood about their complex life cycle, particularly the portion of reproduction up to and including fertilization. The research detailed in my dissertation broadens our understanding of this portion of the mussel reproductive cycle and how it is being impacted by humans.

My first chapter explores the environmental variables that are important in regulating timing of gametogenesis in mussels. Using a year-long field sampling regime I found that water temperature, and in particular the number of degree days during which growth occurs, is an important correlate for the number of mature gametes present in adult mussel's gonadal tissue. Using a 3-month long laboratory study I confirmed these findings; however, I also discovered the potential for a food quality by temperature

interaction in this study as mussels in my experiment were fed high quality food and had substantially more gametes present in their gonads than were ever observed in the field.

My second chapter explores how environmental variables affect the process of fertilization in freshwater mussels. I conducted a sperm viability experiment in which I manipulated water temperature (5, 15, 25, and 35^{o}C) and measured the percentage of viable mussel sperm that were motile over time. I found that mussel sperm are viable for extensive periods of time, but that the highest motility was observed in the 15 and 25^{o}C temperature range. I combined these data with a modeling approach to determine how mussel population dynamics and gene flow could be impacted by different thermal and flow regimes. I discovered that mussel sperm has the potential to move extremely long distances downstream, but that ultimately sperm transport is a function of stream velocity and height above the sediment at which sperm are released. Reproductive success, however, is a function of the proportion of sperm that have remained viable over time.

The research detailed in my third chapter examines the role of impoundments on the reproductive success and population attributes of freshwater mussels. Using data collected in my year-long field study, I found that mussels below a cold-water release impoundment had lower overall mussel densities, higher proportions of hermaphroditic individuals, higher prevalence of sterilizing trematodes, and lower body condition relative to mussels found above the impoundment. I also found that patterns in timing of gamete development were also unusual below the cold-water release dam. I outline a conceptual model by which alterations in temperature, stream flow, light, and food availability caused by impoundments could lead to overall negative density-dependence in mussel populations. These first three chapters illustrate the importance of natural

temperature and flow regimes in maintaining healthy reproduction in freshwater mussel communities, information that is critical for managing rivers that provide habitat to mussels.

As humans continue to alter riverine landscapes, we are also likely to impact the evolutionary trajectories of species residing there. Unfortunately, another aspect of mussel biology that is also understudied is the evolution of the great diversity of freshwater mussels, particularly in North America. Several evolutionary hypotheses have been proposed for the evolution of these organisms, yet none have been tested. The goal of my fourth chapter was to address freshwater mussel evolution from the perspective of mechanisms of reproductive isolation, since barriers must exist between species to maintain distinct species identities. I examined the role that habitat use and timing of reproduction may play in isolating co-occurring, closely related mussel species of the genus *Quadrula*. I found that habitat overlap among closely related species varies (although is often high), but could be one isolating mechanism. Timing of reproduction, however, overlaps almost entirely among these species and is likely not a factor maintaining species identity in this genus. Further research into other isolating mechanisms is required to increase our understanding of reproductive barriers and evolution of freshwater mussels.

Chapter 1. Temperature and food interact to influence gamete development in freshwater mussels

SUMMARY

1. Freshwater mussels are one of the most threatened faunas in North America and globally, but little research has examined factors leading to successful reproduction (gamete development and fertilization success) in these species.

2. We combined field and laboratory studies to determine environmental factors influencing successful reproduction in three closely related species of freshwater mussels in a south central U.S. river.

3. Successful gamete development in the field was linked to temperature, specifically the number of accumulated degree days. Laboratory studies confirmed this finding, but also suggested that temperature and food availability interact to regulate gamete development.

4. Our data indicate that successful reproduction may be inhibited by altered temperature regimes found below impoundments.

INTRODUCTION

Freshwater ecosystems and the species that inhabit them are being decimated globally (Allan and Flecker, 1993). One of the most threatened freshwater groups in North America is freshwater mussels (Bivalvia: Unionoida) (Williams *et al.*, 1993; Strayer *et al.*, 2004). Freshwater mussels are a guild of benthic, filter-feeding bivalves that provide important ecosystem services to the aquatic community (Vaughn and Hakenkamp, 2001). Mussels can be found in dense, multi-species aggregations known as mussel beds; here they can dominate the benthic biomass and their influences on ecosystem function can be significant, particularly during periods of low flow and high temperature (Spooner and Vaughn, 2006; Vaughn, Spooner and Galbraith, 2007; Vaughn,

Nichols and Spooner, 2008). They are important in nutrient cycling and couple benthic and pelagic compartments by filtering suspended material and depositing feces and pseudofeces (undigested food particles) to the benthos (Vaughn and Hakenkamp, 2001; Vaughn, Gido and Spooner, 2004; Howard and Cuffey, 2006; Vaughn *et al.*, 2007). Additionally, live mussels and their spent shells provide habitat for other benthic organisms (Spooner and Vaughn, 2006; Vaughn and Spooner, 2006). Therefore, understanding and maintaining mussel species diversity and abundance has implications for entire stream ecosystems.

Reproduction in freshwater mussels occurs when male mussels cast their gametes into the water column. Females, filtering phytoplankton and other seston from the water, passively collect the ejected sperm (McMahon and Bogan, 2001). Fertilization occurs on the interior of specialized brood chambers located in the females' gills where larvae (glochidia) begin their maturation (Richard, Dietz and Silverman, 1991). Glochidia are released to complete their development as obligate ectoparasites on fish hosts after which they detach from their hosts and become free-living in the epibenthos (McMahon and Bogan, 2001).

Few studies have examined the timing and success of reproduction in unionid mussels and little is known about the factors that signal reproduction. Studies on other freshwater bivalves such as zebra mussels suggest that reproduction is ultimately cued by a combination of temperature, photoperiod, and food availability (Borcherding, 1995; Wacker and von Elert, 2003) and research in both zebra mussels and marine mollusks suggests that signaling molecules such as serotonin and available energy reserves serve as physiological cues for reproduction (Zandee, Kluytmans and Zurburg, 1980; Ram *et al.*,

1993; Urrutia *et al.*, 1999; Masseau *et al.*, 2002). How the combined effect of these factors may influence the reproductive success of freshwater mussels depends on which factors are most important for cuing gametogenesis in different species. Therefore, one goal of this study was to use a combined laboratory and field approach to investigate the factors that are important signals for reproduction in freshwater mussels.

Mussel populations have been steadily declining in recent history due to habitat destruction, population fragmentation, and introduction of non-native species (Strayer, 1999; Vaughn and Taylor, 1999; Watters, 2000). One factor linked to mussel decline is the widespread impoundment of rivers. Impoundments have been shown to negatively impact mussels at all stages of life (Vaughn and Taylor, 1999); however, because reproduction is often the most sensitive time during development and impoundments are known to drastically alter the physical properties of rivers (Baxter, 1977; Allan, 1995; Poff *et al.*, 1997), it is likely that dams could influence the timing of reproduction in downstream mussel populations. Therefore, a second goal of this study was to document effects of impoundments on the timing of reproduction in freshwater mussels.

MATERIALS AND METHODS

Study Sites

The present study was conducted in three mussel beds in the Little River in southeastern Oklahoma, U.S. (Fig. 1). The Little River is located in the Ouachita Mountains region of the Interior Highlands and is a tributary of the Red River. This region is a center of speciation for both aquatic and terrestrial organisms including fish, crayfish, mussels, salamanders, and caddisflies (Mayden, 1985; Moulton and Stewart,

1996). The Little River is approximately 350 km long and drains two major tributaries in Oklahoma, the Glover and Mountain Fork rivers. This river contains a healthy and diverse mussel fauna with over 37 species of unionids (Galbraith, Spooner and Vaughn, 2008).

Because we were interested in effects of temperature on mussel reproduction, we chose sites known to have different thermal regimes. The Little River is influenced by two impoundments. The mainstem of the river is impounded by Pine Creek Reservoir (Fig. 1). The Mountain Fork River is impounded by Broken Bow Lake which is formed from a hypolimnetic (cold water release) dam. It is used to generate hydropower and maintain a non-native trout hatchery downstream. Cold water released below Broken Bow Lake enters the Little River at its confluence with the Mountain Fork River, approximately 64 km downstream from Pine Creek Reservoir, and substantially changes the thermal regime of the river (Vaughn and Taylor, 1999). Sites 1 and 2 were located above the confluence of the Little River with the Mountain Fork River and site 3 was directly below this confluence.

Study species

Quadrula is among the most widespread and speciose genera of freshwater mussels in North America (Parmalee and Bogan, 1998) and includes dominant species as well as several species that are either federally endangered (e.g. *Q. fragosa*) or listed as species of special concern (*Q. cylindrica*). We studied three species of *Quadrula*, the Pimpleback (*Q. pustulosa*, Lea 1931), the Rabbitsfoot (*Q. cylindrica*, Say 1817) and the Mapleleaf mussel (*Q. quadrula*, Rafinesque 1820). These three species vary in their relative abundance within southeastern Oklahoma and across North America.

Field Study

We conducted a year-long field study to estimate factors (water temperature, light reaching the benthos, and food availability) that influence gamete development in *Q. pustulosa*, *Q. cylindrica* and *Q. quadrula*. Between September 2005 and August 2006, we sampled these three species on a monthly basis, except during December, January, and March due to inclement weather and high water. During each monthly sampling trip, we collected, marked, weighed and measured as many individuals of each species as we could find during an approximately two-hour timed snorkel search (Vaughn, Taylor and Eberhard, 1997). We collected small (~50 µl) gonad samples from each mussel's visceral mass with a syringe and preserved the samples in buffered formalin. In the laboratory, we examined gonadal samples under a microscope to quantify gamete status in each individual. Sperm samples were quantified using a hemocytometer to estimate the sperm concentration or sperm standing crop present in the gonads (number of developed sperm per milliliter). All eggs and their vitellin membranes were measured (2 estimates of both length and width) to quantify change in ovum size. This is the first study to use syringe gonad sampling in a quantitative fashion. This non-lethal sampling technique allows for large sample sizes without sacrificing individuals, particularly of threatened and endangered species (Shiver, 2002; Saha and Layzer, 2008). Although further studies are necessary to compare this sampling technique with traditional histological sampling, the consistency of our data with the literature suggests that that this method is an unbiased method for quantifying the number of mature gametes in a non-lethal manner.

For each site, we recorded temperature (°C) and light (lux) every 30 minutes with HOBO (Onset, Pocasset, MA, U.S.A) loggers for use in our estimation of seasonal and diurnal temperature and photoperiod variation during the year of our sampling. We estimated the number of accumulated degree days from the start of our study using the University of California Statewide Integrated Pest Management Program online degree day calculator (UC IPM, 2008; Baskerville and Emin, 1969). To calculate degree days, we defined the limits of growth for all species to occur between 10 and 31^{o}C based on metabolic rate data for *Q. pustulosa* (Spooner and Vaughn, 2008). We determined the maximum and minimum daily temperatures from the temperature logger data and a single sine method to calculate the number of accumulated degree days since the start of our sampling.

To quantify food availability we collected benthic core samples at each site seasonally (four times over the course of the year) and monthly water column chlorophyll *a* samples. Core samples were homogenized with a sediment processor in the lab. We then filtered three 150-ml subsamples which were dried (100^{o}C for 72 hours) and weighed to obtain dry weight estimates. We ashed samples in a muffle furnace at 550^{o}C for 1 hour to obtain estimates of ash free dry mass as a measure of benthic organic matter. For water column chlorophyll, we filtered three 1-l samples of river water onto glass fiber filters, and extracted and quantified chlorophyll *a* spectrophotometrically using the acetone method (APHA, 1996).

We determined the timing of peak reproduction for each species at each site by plotting sample date against either log concentration of sperm observed in the gonads or proportion of female eggs in the 80th size percentile based on our measurements of

diameter (a standardized estimate of reproductive state to account for differences in egg size across species). We used ANOVA and a Tukey post hoc multiple comparison procedure to determine seasonal differences in daily water temperature, benthic organic matter, and water column chlorophyll *a* among sites. ANOVA was used to test for differences among sites in mean hours of light per day (> 0 lux as measured by HOBO loggers) reaching the benthos. We used multiple regression with forward selection to determine which environmental parameters (benthic organic matter, water column chlorophyll *a*, temperature, degree days, and light) explained the most variation in timing of peak maturation in the three species of mussels. Egg data were arcsine transformed and sperm data were log transformed to meet assumptions of ANOVA. Because we used a sine function to calculate our degree day data, we *a priori* decided that a cubic function should be used to relate timing of reproduction to degree days. We expected that reproductive output would follow a third degree polynomial function over time with a period of low reproductive output, gradually increasing over time, and again dropping off after spawning. Therefore, we included three degree day terms in our regression analysis: degree days, degree days^2, and degree days^3.

Laboratory Experiment

Based on the results of our field study, we designed a laboratory experiment testing the influences of temperature and photoperiod on reproductive timing. During the summer of 2007, we collected mussels from the Little River and acclimated them to 5^{o}C for two weeks prior to the start of the experiment. Mussels were housed in re-circulating stream mesocosms that consisted of large fiberglass tanks lined with gravel-filled plastic containers (Allen and Vaughn, 2009). Each mesocosm housed 14 *Q. pustulosa* and five

Q. cylindrica individuals for a total mesocosm density of 44 individuals m^{-2}. Mussel densities at our three field sites were 35, 53, and 16 individuals m^{-2} for sites 1, 2 and 3, respectively; densities in our experiment fell within this natural range. *Quadrula quadrula* was not abundant enough in the field for use in this experiment. Each of 12 mesocosms was exposed to one of four temperature and light treatments: cold/dark, cold/light, warm/dark, and warm/light (Table 1).

Because we wanted to ensure that food was not limiting in this experiment, mussels were fed every other day with a 2:1 mixture of commercial marine shellfish diet and *Nannochloropsis* (Instant Algae, Reed Mariculture, Campbell, California, USA) used for brood stock conditioning in marine mussels. Data from Spooner and Vaughn (2008) show that *Q. pustulosa* clearance rates triple from 5°C to 15°C. Therefore, mussels in warm treatments were fed approximately 3 times the amount of food of cool-treatment mussels to account for the added metabolic demands due to increased temperature (Borcherding, 1995). Partial water changes were completed every two weeks to minimize ammonia accumulation. We collected gonad samples from a subsample of individuals in each treatment at the start of the experiment and monthly thereafter to quantify reproductive development. Individual mussels were never sampled more than once and after sampling were placed back into their respective mesocosms to maintain mussel density throughout the experiment. We used a two-way ANCOVA to evaluate effects of temperature and light on gamete development in the laboratory experiment; time was used as a covariate in this analysis.

RESULTS

Field Study

We found significant differences among sites in mean annual temperature, mean hours of light reaching the benthos, benthic organic matter, and water column chlorophyll *a* and significant site by season interactions in benthic organic matter and mean water temperature (Table 2). Specifically, site 3 was significantly colder than the other sites in the summer and warmer than the other sites in the winter (Fig. 2). Site 1 received more hours of daylight to the benthos than the other sites, but had lower benthic organic matter than sites 2 and 3. Site 3 had lower water column chlorophyll *a* than site 2 but did not differ significantly from site 1.

We sampled a total of 460 individual mussels across species and sampling sites over the course of our year-long study. We identified reproductively mature individuals across a range of size classes for each species. The smallest individuals collected of each species were 58, 38, and 47 mm for *Q. cylindrica*, *Q. pustulosa*, and *Q. quadrula*, respectively, and all were found to have mature gametes in their gonads. Averaged across all sites, peak sperm concentration in the gonads occurred in the summer, with *Q. cylindrica* reaching its peak slightly earlier (late May) than *Q. pustulosa* or *Q. quadrula* (mid June; Table 3). Declines in gonadal concentrations of sperm after the peak are attributed to gamete release and occur throughout June, July and August. There was variability in timing of reproduction among species across sites. At site 1, *Q. quadrula* and *Q. cylindrica* both appeared to reach their reproductive peak earlier than *Q. pustulosa* (Table 3); however, site 2 mussels matched the pattern of reproductive timing seen river-wide (Table 3). While peaks in reproduction at site 3 were generally similar to those at

sites 1 and 2 (Table 3), overall patterns of gametogenesis are difficult to describe given that this site had much lower mussel densities and variable sperm concentrations.

Mean proportion of ova with a diameter in the 80th percentile (averaged across all three sites) had seasonal patterns similar to those for sperm concentration; however, peaks in ovum diameter occurred slightly earlier than peaks in sperm concentration (Table 3). Riverwide, *Q. quadrula* appeared to reach peaks in ovum size approximately one to two months later than the other two species. However, patterns in ovum diameter varied within and among sites. At site 1, all three species reached peak reproductive status at approximately the same time of year (Table 3), while at site 2 *Q. cylindrica* peaked slightly earlier than the other two species (Table 3). Peaks in egg size at site 3 were similar to those observed at sites 1 and 2 (Table 3), but it was again difficult to discern any seasonal patterns at site 3 (Table 3).

Multiple regression revealed significant relationships between environmental variables and reproductive status for each species except for *Q. quadrula* males (Table 4). Reproductive status in all three species was related to at least one degree day term (Table 4; Fig. 3). Reproduction in *Q. pustulosa* males and *Q. cylindrica* females also was dependent on mean monthly water temperature. However, we found that all of the environmental parameters were significantly correlated with one another except for seasonal benthic organic matter, which was not significantly correlated with any of the degree day terms or mean temperature. There was no relationship between mussel length or wet weight and stage of reproduction.

Laboratory Experiment

Temperature and light had no effect on sperm concentration in *Q. cylindrica*, there was no light by temperature interaction, and time was not a significant covariate (temperature: $F_{(1,16)}= 0.04$, p= 0.84; light: $F_{(1,16)}= 1.35$, p= 0.26; light x temperature: $F_{(1,16)}= 1.47$, p= 0.24; time: $F_{(1,16)}= 0.03$, p= 0.86). There was, however, a significant effect of temperature in female *Q. cylindrica*, no effect of light, and again no light by temperature interaction (temperature: $F_{(1,19)}= 6.85$, p= 0.02; light: $F_{(1,19)}= 0.58$, p= 0.46; light x temperature: $F_{(1,19)}= 1.27$, p= 0.27). However, time was a significant covariate ($F_{(1,19)}= 6.14$, p= 0.02). In particular, females in the cool treatments had a larger proportion of developed eggs in their gonads than females in the warm treatments (Fig. 4).

We found no main effect of temperature or light on sperm concentration in *Q. pustulosa* (temperature: $F_{(1,26)}= 0.06$, p= 0.81; light: $F_{(1,26)}= 2.23$, p= 0.15) but did find a marginally significant light by temperature interaction, with time as a significant covariate (light x temperature: $F_{(1,26)}= 4.17$, p= 0.05; time: $F_{(1,26)}= 10.54$, p= 0.003). In particular, males in warm/dark treatments had the highest sperm concentrations compared to other treatments. There was an effect of temperature in female *Q. pustulosa*, but there was no effect of light, no light by temperature interaction and time was not a significant covariate (temperature: $F_{(1,28)}= 5.20$, p= 0.03; light: $F_{(1,28)}= 0.07$, p= 0.79; light x temperature: $F_{(1,28)}= 1.43$, p= 0.24; time: $F_{(1,28)}= 0.37$, p= 0.55). Specifically, females in warm treatments had a larger proportion of developed eggs than females in cool treatments (Fig. 4).

In males, gamete concentration in the gonads was higher in the laboratory experiment than in the field. *Quadrula cylindrica* sperm concentrations were between 1.6 and 4.6 times higher (mean = 3.0) in the lab than the mean peak concentrations measured in the field. Likewise, *Q. pustulosa* laboratory sperm concentrations ranged from 1.7 to 6.8 times higher (mean = 3.8) in the lab than the field. The proportion of developed eggs in the laboratory study, however, was lower or equal in size to that observed in the field. *Quadrula cylindrica* egg size in this study ranged from 0.3 to 1.0 times (mean = 0.5) the average egg size during peak reproduction in the field. Similarly, *Q. pustulosa* egg size in the lab ranged from 0.3 to 1.0 times (mean = 0.5) the average peak size found in the field.

DISCUSSION

Densities of freshwater mussel species vary across North America and likely depend on biogeography, habitat suitability for survival and reproduction, and rates of extirpation, both natural and human caused (Strayer, 2008). As mussel densities are rapidly declining globally, it is urgent that we understand the factors contributing to these declines in greater detail. Determining the environmental cues that are important for successful reproduction and how disrupting these cues can influence mussel reproduction is necessary for management and conservation of mussel communities. Although later stages of mussel reproduction (glochidial development and encystment on host fish) have received considerable attention in the literature, there have been few quantitative studies of factors that regulate gamete production in mussels. To our knowledge, ours is the first

study to use a combined field and laboratory approach to determine factors that trigger gametogenesis in unionid mussels.

Both the field and laboratory studies described here suggest that thermal regimes are important cues for timing of gamete development (and potentially gamete release). Time of reproduction varied slightly among sites, but in all species was correlated with number of accumulated degree days, a measure of the total amount of heat to which an organism has been subjected. Degree days are an important developmental cue for many aquatic invertebrate species (Ward and Stanford, 1982) including freshwater mussels. Hruska (1992) suggested that time for glochidial metamorphosis in the pearl mussel, *Margaritifera margaritifera*, was related to degree days. Our results indicate that number of annually accumulated degree days also may be important in governing earlier stages of the reproductive cycle. This makes sense given the natural variability in temperature from year to year and that mussel growth and development, like that of most aquatic ectotherms, is constrained between a minimum and maximum temperature range (Burky, 1983; Willmer, Stone and Johnson, 2005).

Correspondingly, we found temperature, and for male *Q. pustulosa* a photoperiod by temperature interaction, to be significant to gamete development in our laboratory experiment. The results of this experiment are somewhat difficult to interpret, however, particularly for females. For both *Q. pustulosa* and *Q. cylindrica*, our data show that proportion of eggs in the 80th percentile declined from the beginning of the experiment. This decline could be due to the fact that females were collected from the field during the peak of their reproductive cycle and that mature eggs were then being transferred to the brood pouch (leaving smaller eggs to be sampled from the gonads). Alternatively,

experimental conditions may have caused females to resorb their mature eggs, a phenomenon that is known to occur in mussels (Henley, 2002). It was also unexpected that *Q. cylindrica* males and females had higher numbers of mature gametes under cold temperatures, especially because water temperatures rarely drop as low as 5^{o}C in southeastern Oklahoma. *Quadrula cylindrica* does reach its reproductive maturity slightly earlier than *Q. pustulosa* so it is plausible that gametogenesis is triggered by cooler temperatures. An alternative explanation could be that gamete development is compromised by cold temperature. All of these questions require further study.

Both *Q. pustulosa* and *Q. cylindrica* had higher sperm concentrations in laboratory experiments than we observed in the field. Laboratory-kept animals were fed a highly nutritious diet (protein concentration of food fed to laboratory mussels was three to nine times higher than Little River seston (Galbraith, unpublished data)) and were fed in excess, so that food would not be a limiting factor in our experiment. Thus, it is possible that effects of high quality food overpowered any effects of temperature or light in our experiment and indicates that food and food quality may be limiting resources in the field. Borcherding (1995) showed that zebra mussel gametogenesis was dependent on temperature, but was also reliant on food availability. Similarly, Wacker and von Elert (2003) stressed the importance of temperature and food quality, specifically polyunsaturated fatty acids (PUFAs) in zebra mussel reproduction. Our field data offer some support to the importance of food availability to reproduction: site 1 had the lowest benthic organic matter of all three sites and correspondingly lower sperm concentrations in all three species (Table 3). Further studies examining effects of both food quality and

quantity are needed to make conclusions about environmental variables that influence gametogenesis.

We found that all three species of *Quadrula* reproduce during summer months with peak gamete concentrations present during May and June, thus placing gamete release during June, July and August. These patterns in timing of reproduction are consistent with that reported for other members of the genus *Quadrula* and other mussel species in general (Yeager and Neves, 1986; Haggerty *et al.*, 1995; Garner, Haggerty and Modlin, 1999). Peak female egg size was seen slightly earlier than peak sperm concentration, suggesting that female mussels are reproductively mature earlier in the year than males. Females need to be ready for males to release their sperm; however, females have to transfer their mature eggs from their gonads to their gills where fertilization takes place (McMahon and Bogan, 2001). Both are potential factors that need to be further examined in the context of understanding female mussel receptivity.

The thermal regime at site 3 was different than at the other two sites, with warmer winter and cooler summer temperatures (Fig. 2). This site receives substantially colder water from the upstream tributary during summer months, released to generate electricity and to maintain a trout hatchery downstream of the dam. It also has lower water column chlorophyll *a* than the other two sites. While peaks in reproduction were similar among all sites (Table 3), general patterns of reproduction varied among all species at site 3, and could be a function of the altered physical and potentially chemical properties we observed here. However, because mussel densities were so low at this site, it is difficult to determine whether patterns in reproductive timing truly differ from the other sites or is simply due to the fact that not enough individuals could be sampled to observe a clear

pattern. The trends do suggest that *Q. quadrula* males peak later in the year at site 3 than they do at sites 1 or 2, which may be a function of the unusual physical properties of this site (cold water, low food, etc.).

Reproductive success in mussels can be disrupted by cold temperatures and unnatural thermal regimes (Layzer, Gordon and Anderson, 1993; Heinricher and Layzer, 1999; Watters, 2000). Heinricher and Layzer (1999) showed that *Megalonaias nervosa* individuals that had stopped reproducing below a hypolimnetic release dam were capable of reproduction following translocation to a river with suitable reproductive cues. In that study inappropriate thermal cues below the dam were considered the most likely explanation, although food availability was not ruled out as a causative factor. Layzer *et al.* (1993) suggested that mussel extirpations in the Caney Fork River, Tennessee were due to direct effects of altered temperature on mussel reproduction. Temperature and food availability may be responsible for the unusual patterns in gamete development observed at site 3, but further research must examine other differences that may exist between these sites (e.g. toxins, water chemistry) that could be causing the unusual patterns in gametogenesis.

Impoundments also indirectly affect mussel reproduction by altering host fish species' migration patterns and presence and by impacting juveniles. Dams can limit the movement of migratory host fish species, which can potentially influence gene flow between mussel populations (Layzer *et al.*, 1993; Watters, 1996, 2000). Decreases in thermally sensitive-host fish populations also have been documented in rivers with altered thermal regimes (Davenport and Warmuth, 1965). Since most mussel species are obligate ectoparasites on fish and many are host fish specialists, declines in host fish

abundance can have severe implications for mussel reproductive success. Juvenile mussel recruitment can also be negatively impacted by high water velocities, siltation and lack of food below impoundments (Layzer and Madison, 1995; Vaughn and Taylor, 1999; Watters, 2000).

Impoundments and other anthropogenic factors are known to have detrimental impacts on aquatic organisms at all stages of life, not just the reproductive stage. However, reproduction is often one of the most vulnerable time periods for organisms, making them particularly sensitive to changes in environmental conditions. Our results emphasize the importance of maintaining natural thermal regimes and potentially food availability in regulated rivers to facilitate successful mussel reproduction.

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LITERATURE CITED

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Table 1. Experimental design for stream mesocosm experiment.

Treatment	N	Temp (^{o}C) Month 1	Temp (^{o}C) Months 2-3	Light (hrs of light:dark)
Cold/Dark	3	5	5	8:16
Cold/Light	3	5	5	16:8
Warm/Dark	3	5	15	8:16
Warm/Light	3	5	15	16:8

Table 2. Results of two-way ANOVAs comparing temperature, light, benthic organic matter, and water column chlorophyll *a* among sites and seasons.

Factor	**F (df)**	***p***
Water temperature		
Site	11.26 (2,926)	<0.001
Season	1721.68 (3,926)	<0.001
Site x Season	13.66 (6,926)	<0.001
Light reaching benthos		
Site	2.92 (2,787)	0.06
Season	43.86 (3,787)	<0.001
Site x Season	0.97 (6,787)	0.45
Benthic production		
Site	15.16 (2,118)	<0.001
Season	1.11 (3, 118)	0.35
Site x Season	2.87 (6,118)	0.01
Water column chlorophyll		
Site	5.82 (2,51)	<0.001
Season	12.52 (3,51)	<0.001
Site x Season	1.08 (6,51)	0.39

Table 3. Mean (± SE) sperm concentration, mean (± SE) proportion of eggs in the 80th size percentile, and month of peak reproduction for each sex at each study site and averaged across all three study sites.

Site	Species	Summer peak in male maturity	Mean sperm concentration (#/ml x 10^8)	Summer peak in female maturity	Mean proportion of eggs in 80th size percentile
All sites	*Q. cyindrica*	May	4.85 (0.63)	April	0.53 (0.11)
	Q. pustulosa	June	2.97 (0.32)	April	0.50 (0.10)
	Q. quadrula	June	2.76 (2.1)	June	0.44 (0.28)
Site 1	*Q. cyindrica*	May	4.11 (0.44)	April	0.60 (0.10)
	Q. pustulosa	June	2.76 (0.47)	April	0.52 (0.27)
	Q. quadrula	May	1.62 (0.91)	April	0.38 (0)
Site 2	*Q. cyindrica*	May	7.05 (0)	May	0.34 (0.04)
	Q. pustulosa	June	4.48 (0.62)	April	0.49 (0.11)
	Q. quadrula	June	1.05 (0.90)	May	0.29 (0.02)
Site 3	*Q. cyindrica*	May	5.61 (1.84)	None	0.00
	Q. pustulosa	May	3.11 (0.58)	May	0.30 (0.04)
	Q. quadrula	July	8.93 (0)	June	1.00 (0)

Table 4. Results of multiple regression and significant factors explaining the importance of environmental variables on patterns in gamete development over time.

	Adj. R^2	**F (df)**	**Explanatory variables**
Males			
Q. cylindrica	0.61	23.14 (3,39)	degree days, degree days^2, degree days^3
Q. pustulosa	0.15	12.53 (2,125)	degree days3, mean temperature
Q. quadrula			no significant model
Females			
Q. cylindrica	0.19	13.99 (1,54)	mean temperature
Q. pustulosa	0.19	22.85 (1,92)	degree days^3
Q. quadrula	0.24	5.66 (1,14)	degree days

FIGURE LEGENDS

Figure 1. Location of sampling sites (▲) in the Little River in southeastern Oklahoma. Latitude and longitude coordinates for the sites are as follows: Site 1: 33.93992, -94.76927; Site 2: 33.949203, -94.73382; Site 3: 33.94822, -94.56965.

Figure 2. Mean (± SE) monthly temperature during the 2005-2006 study period at three sampling sites in the Little River, Oklahoma. Data collected at 30 minutes intervals using continuous data loggers.

Figure 3. Relationship between the number of accumulated degree days since the start of our field season and sperm concentration for *Q. cylindrica* (a), *Q. pustulosa* (b), and *Q. quadrula* (c) and the proportion of eggs in the 80^{th} percentile based on size for *Q. cylindrica* (d), *Q. pustulosa* (e), and *Q. quadrula* (f).

Figure 4. Treatment means (± SE) for males (a, b) and females (c, d) of *Q. cylindrica* and *Q. pustulosa* averaged across time periods for the laboratory experiment.

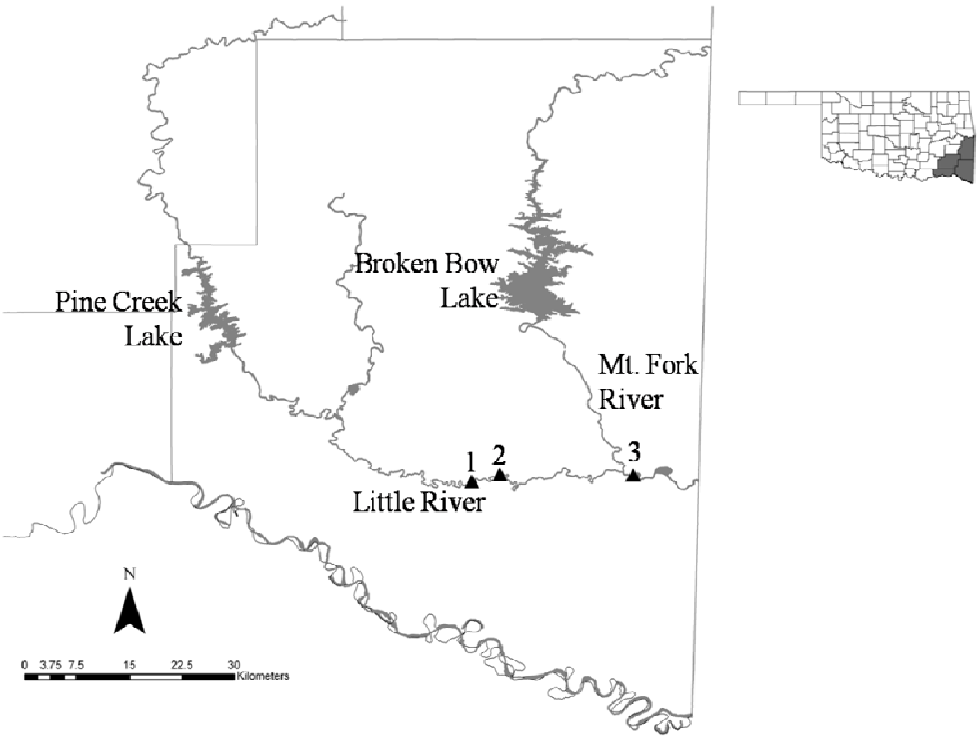
Broken Bow
Lake
Pine Creek
Lake
Mt. Fork
River
1
2
3
Little River
N
0 3.75 7.5 15 22.5 30
Kilometers

Figure 1.

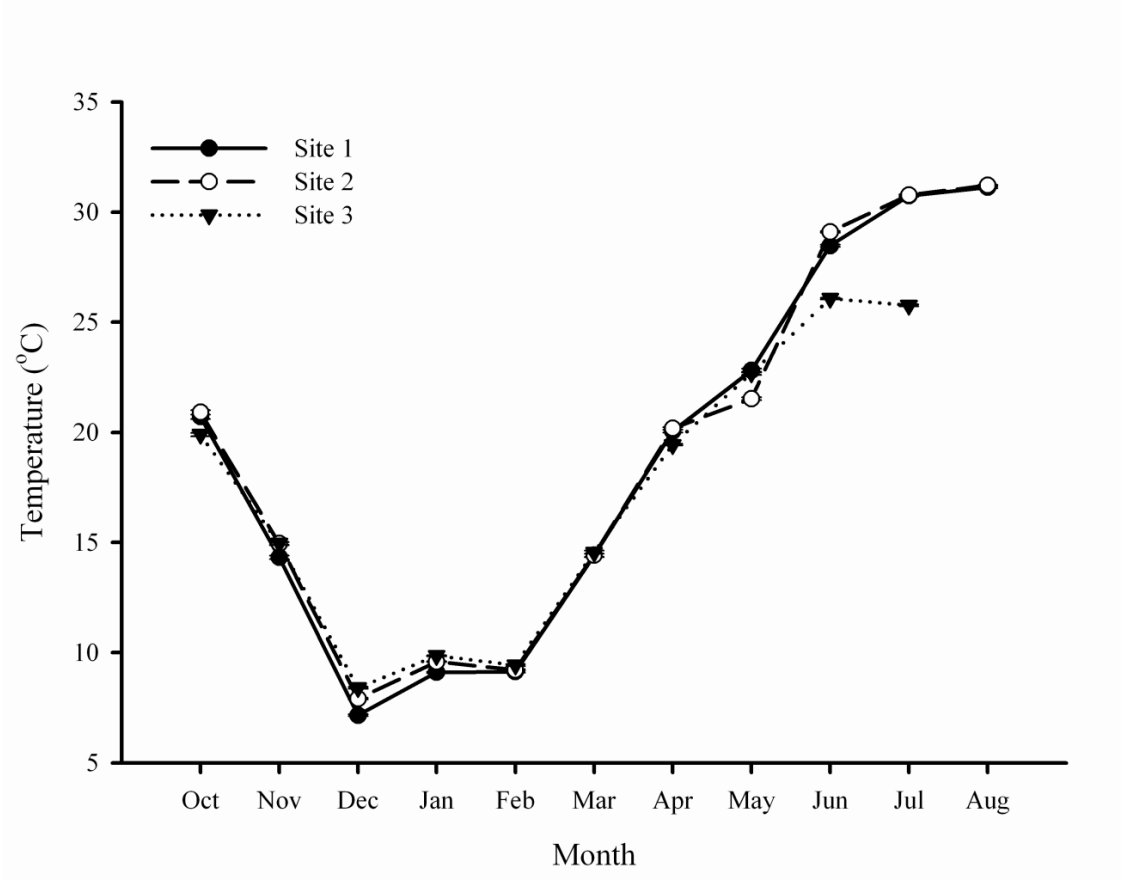
Site 1
Site 2
Site 3
Temperature (°C)
35
30
25
20
15
10
5
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Month

Figure 2.

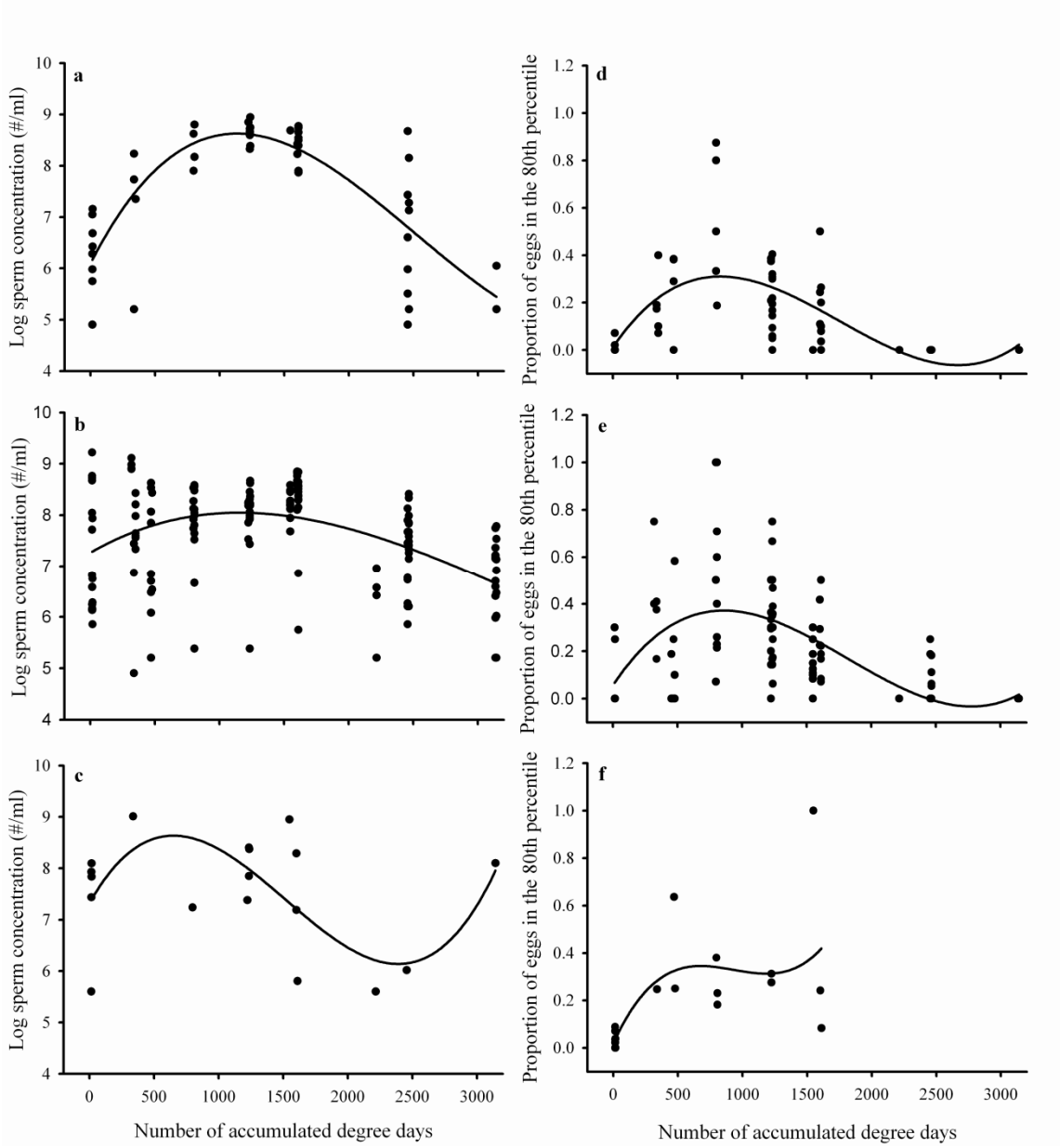
a
b
c
d
e
f
Log sperm concentration (#/ml)
Proportion of eggs in the 80th percentile
Number of accumulated degree days
0
500
1000
1500
2000
2500
3000
4
5
6
7
8
9
10
0.0
0.2
0.4
0.6
0.8
1.0
1.2

Figure 3.

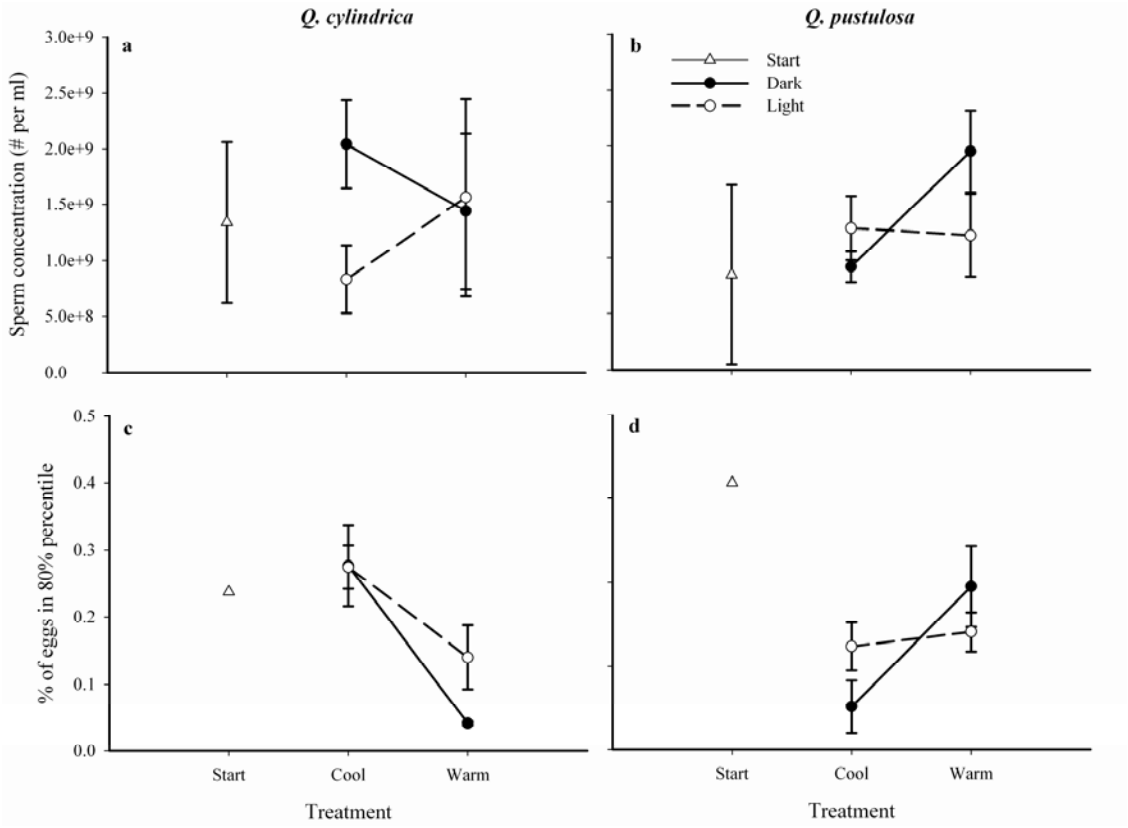
Q. cylindrica
Q. pustulosa
a
b
c
d
Start
Dark
Light
Sperm concentration (# per ml)
3.0e+9
2.5e+9
2.0e+9
1.5e+9
1.0e+9
5.0e+8
0.0
% of eggs in 80% percentile
0.5
0.4
0.3
0.2
0.1
0.0
Start
Cool
Warm
Treatment

Figure 4.

Chapter 2. Effects of temperature and stream flow on sperm motility and dispersal in freshwater mussels

Abstract

Freshwater mussels use a spermcasting reproductive strategy that is unique among freshwater invertebrates, although more common in marine invertebrates. We determined how sperm motility (estimated as percent motile sperm) is affected by temperature in freshwater mussels and then modeled the combined effects of temperature and stream flow on downstream movement of motile mussel sperm. We found that sperm of *Quadrula pustulosa* are most motile between 15 and 25^{o}C and that sperm can travel long distances downstream under certain flow regimes. The distance sperm can travel, however, depends in part on their release height above the substrate, and the percent motile sperm available for fertilization influences how far downstream successful reproduction can occur. Our findings highlight the importance of maintaining natural thermal and flow regimes to insure successful reproduction in freshwater mussels.

Key words: Unionid, fertilization, spermcast, discharge, climate change, impoundment

Introduction

Organisms living in stream ecosystems have evolved a variety of morphological, behavioral, and physiological adaptations to cope with a flowing environment (Vogel 1994). Many of these adaptations revolve around extreme events like floods and droughts and involve either avoiding these events or developing life history strategies that are synchronized with them (Vogel 1994, Lytle and Poff 2004). Many aquatic organisms, for example, time their reproduction around particular temperature and flow regimes to either take advantage of newly available resources or reduce natal mortality

due to harsh conditions (Lytle and Poff 2004). Although environmental variability is not predictable on a yearly basis, long-term averages in flow and temperature have shaped organisms' responses to these parameters (Poff et al. 1997, Lytle and Poff 2004). Historical stream flow and temperature regimes, however, are being altered on a large scale by human activity. In particular, impoundments (Poff et al. 1997, Vaughn and Taylor 1999), clearing of riparian habitat (Castelle et al. 1994), and increased climatic variability due to climate change (Mulholland et al. 1997, Parmesan 2006) are shifting natural variability in stream ecosystems. Studies suggest that alterations in flow and temperature are causing declines in species diversity and abundance in both fish and aquatic invertebrates (Allan and Flecker 1993, Mulholland et al. 1997, Poff et al. 1997, Burgmer et al. 2007).

Freshwater mussels (Bivalvia: Unionoida) are one of the most globally-threatened faunas (Williams et al. 1993, Strayer 1999, Vaughn and Taylor 1999, Lydeard et al. 2004, Strayer et al. 2004). Mussels have a reproductive strategy that results in generally low recruitment (McMahon and Bogan 2001) which, in combination with their long lifespan, makes them particularly susceptible to anthropogenic influences (Watters 2000). Reproduction occurs when males release their gametes into the water column and females passively filter in the sperm (McMahon and Bogan 2001); we refer to this process as spermcasting (Bishop and Pemberton 2006) as opposed to broadcasting where both sexes release their gametes. Eggs develop and are fertilized in specialized brood chambers located within the females' gills. Mussel larvae (called glochidia) are transferred to one or several specific host fish species to survive as ectoparasites until reaching further maturity (Barnhart et al. 2008). Juvenile mussels then drop from their

host fish and mature to adulthood in the epibenthos (McMahon and Bogan 2001). Although spermcasting is a common reproductive strategy in many sessile marine invertebrates (Bishop and Pemberton 2006), freshwater mussels are the only known freshwater organisms to use this strategy.

Reproduction in freshwater mussels is thought to be triggered by a combination of temperature, photoperiod, and food availability (Mackie 1984, Hruska 1992, Borcherding 1995). If this is the case, mussels experiencing altered environmental conditions may survive but fail to reproduce, contributing to a slow decline of the community. Reservoirs may alter temperature, light and food availability, as well as fragment host fish populations and thus mussel populations (Baxter 1977, Vaughn and Taylor 1999, Watters 2000). In the southern U.S., streams often dry into a series of isolated pools in hot summer months. River regulation may exacerbate these harsh conditions by holding water in upstream reservoirs and prolonging drought conditions downstream. This practice can lead to extremely high temperatures (as high as 40^{o}C), elevated ammonia, and low dissolved oxygen (Spooner and Vaughn 2000, Gagnon et al. 2004, Cooper et al. 2005). Under such conditions, mussel communities encounter unnatural thermal regimes for cuing reproduction, and mussels experience high stress levels (Spooner and Vaughn 2008), further decreasing potential reproductive success. Alternatively, hypolimnetic release impoundments can have the opposite effect on stream temperature and flow by releasing unnaturally cold water into rivers; this has also been shown to decrease mussel reproductive success (Layzer et al. 1993, Heinricher and Layzer 1999, Watters 2000).

Although environmental signals may be important for cuing gamete development and release, successful reproduction ultimately depends on the ability of viable sperm to

travel to the eggs of the correct species. In zebra mussels, both sperm viability and motility are affected by water temperature (Ciereszko et al. 2001). Low motility and viability should decrease the fertilization success of sperm (Mojares et al. 1995, Ciereszko et al. 2001). In addition, stream flow may be important to both reproductive success and overall mussel population dynamics. Sperm release during high flow events may result in sperm being washed quickly downstream and prevent females' eggs from becoming fertilized; however, low flow may restrict fertilization to only nearby mussels, which could affect population genetics and relatedness of individuals within a mussel bed. Because both flow and temperature can be altered by river regulation, it is important to understand how these factors may impact fertilization success. Here, we first asked how mussel sperm motility is affected by temperature. We then developed a model to predict how temperature and stream discharge interact to govern the downstream movement of viable freshwater mussel sperm.

Methods

Study organisms

Mussels used in this study were collected from the Kiamichi River and hydrological data used for modeling were from the Little River. These adjacent rivers are located in the Ouachita Highlands of southeastern Oklahoma, U.S., and are known for their high mussel and fish biodiversity (Moulton and Stewart 1996, Master et al. 1998, Matthews et al. 2005, Galbraith et al. 2008). Our study focused on *Quadrula pustulosa*. We chose this species because it occurs throughout the Mississippi drainage (Parmalee and Bogan 1998) and is common in the Kiamichi and Little Rivers. It is a medium-sized

(mean shell height at our sites is 54 mm) habitat generalist and thus likely serves as a good surrogate for other mussel species.

Quantifying sperm motility

To determine how temperature influences sperm motility and, by extension, viability, we used methods similar to those of Ciereszko et al. (2001). We collected sperm from individual *Q. pustulosa* males using a syringe biopsy technique (Saha and Layzer 2008) and diluted the sperm in filtered pond water. Pilot work showed that mussels survived and reproduced in the filtered pond water (Vaughn et al. 2004, pers. obs.). We exposed the sperm to temperatures of 5, 15, 25, and 35^{o}C (all temperatures that are experienced by mussels with mature sperm present in their gonads in southeastern Oklahoma; pers. obs.) and counted the percentage of motile sperm after 0, 2, 4, 8, 24, and 48 hours of exposure (n=7 for each temperature). To do this we quantified the number of moving sperm in one grid cell of a hemacytometer (4 nl) for each temperature and time period and divided this by the total number of sperm in the cell to obtain the percent motile sperm in a sample. We counted a mean (± SE) of 150 ± 8 sperm for each mussel per temperature for each of the 6 sampling time periods. To determine the effect of temperature on percent motile sperm, we used repeated measures ANCOVA on arcsine transformed percent motile sperm values with time as the block factor and arcsine transformed initial percent motile sperm as a covariate. We acknowledge that sperm motility does not directly equate to viability and that, in many spermcasting species, sperm are not activated until they are exposed to some chemical cue, undergo a morphological shift, or come into contact with females (Burighel and Martinucci 1994b, Johnson and Yund 2004, Temkin and Bortolomi 2004). Also, males

that release their sperm naturally likely release a higher proportion of motile sperm than we observed by collections made using the syringe biopsy technique.

Model development and parameter estimates

Our model incorporated the movement of sperm due to stream discharge and gravitational settling and combined this movement with our estimates of percent motile sperm. To estimate settling velocity (V_s) of sperm, we used Stoke's Law: $V_s = 2gr^2 (\rho_p - \rho_f)/9\mu$ where g = acceleration of gravity (m/s), r = the radius of the sperm (m), ρ_p and ρ_f are the densities (mass/volume) of the sperm cells and water, respectively (kg/m^3), and μ = dynamic viscosity of water (Pa s). We used the mean sperm head length (l), width (w), and height (h) (see below) to calculate the equivalent spherical diameter ($ESD = (lwh)^{1/3}$) of sperm and the ESD/2 was used in place of the radius in the Stoke's Law equation. ESD of an irregularly shaped object provides an estimate of the diameter (ESD/2 estimates the radius) of a sphere with an equivalent volume to the object (Jennings and Parslow 1988, Kamykowski et al. 1992). In estimating the ESD, we considered only the sperm head dimensions. Because flagella are likely to increase sperm resistance to settling, our estimates using only sperm head size should bias our model towards faster settling rates than if we had included the flagellum dimensions in our model.

We used scanning electron microscopy to examine *Q. pustulosa* sperm morphology for use in settling rate calculations for our model. We collected sperm samples from 5 males using a syringe (Saha and Layzer 2008). The sperm were fixed for 30 min in a 3:1 solution of saturated mercuric chloride and 2% osmium tetroxide (Parducz 1967, Small and Marszalek 1969) and then washed 3 times with distilled water. The sperm were mounted on sputter coated 22-mm Thermanox® cover slips and were

dehydrated in an ethanol series. Coverslips were critical point dried and sputter coated using 60% gold/40% palladium, mounted, and viewed using a JEOL JSM-880 high-resolution scanning electron microscope. We used ImageJ digital image analysis software to measure the sperm dimensions along three perpendicular axes (head length, width and height), and tail length (Abramhoff et al. 2004). We then averaged the head length and width and tail length measurements from 36 sperm cells collected from each of 4 individuals for use in our model.

We were unable to find information on the density (kg/m^3) of freshwater mussel sperm in the literature. Therefore, we substituted the mean density of the single-celled freshwater green alga, *Chlorella vulgaris* in our model (average= 1079 kg/m^3 (Oliver et al. 1981). This algal species is similar in shape (prolate spheroid) and size (slightly larger than mussel sperm: mean diameter = 5.71 μm) to freshwater mussel sperm (Oliver et al. 1981). We recognize that there are probably subtle differences in cell density between plant and animal cells; however, due to lack of a suitable alternative, we feel that using *Chlorella* density is appropriate for this model.

Buried mussels come to the streambed surface to release gametes that can then be transported by the current (Watters et al. 2001). We estimated the potential downstream movement of sperm by building a model that incorporated varying stream discharge regimes and gamete release locations. Sperm release behavior and the height above the substrate of gamete release have not been confirmed in bivalves (Mackie 1984, G. T. Watters, Ohio State University, personal communication); thus, we built our model using heights that represent what we considered a realistic range based on the size of this species (mean shell height is 5.4 cm): 1, 5, and 10 cm above the substrate. We consider

it reasonable to assume that mussels are capable of forcibly ejecting their sperm up to 10 cm above the substrate because we have observed mussels using their exhalant siphon to "pump" feces and pseudofeces (undigested food particles) this distance into the water column (pers. obs.).

We used United States Geological Survey (USGS) real-time stream discharge data for the Little River (http://waterdata.usgs.gov/nwis/uv?07338500) for midsummer, the approximate time of gamete release in this species (pers. obs.). In particular, our model was based on 4 different July stream discharge estimates: 0.51 m^3/s, the mean July 2006 discharge; 8.34 m^3/s, the mean July discharge for 2000-2006; 18.89 m^3/s, the mean July discharge for 1990-1999; and 58.26 m^3/s, the maximum July discharge for 1990-2006. We assumed laminar flow and a constant river width of 25.5 m (based on field data from three mussel beds reported in Galbraith et al. 2008) throughout the Little River and across varying stream discharge rates. We used linear regression between stream depth (measured in the field) and stream discharge (from USGS) to estimate the cross sectional area of the Little River under different discharges ($F_{(1,11)}= 228.90$, $p< 0.0001$, $R^2= 0.95$) so that we could use these data to obtain a linear flow estimate in m/s.

We used simple vector addition to sum the flow rate and the sperm settling rate to obtain estimates of downstream movement of sperm (settling distance downstream = flow rate + sperm settling rate). To account for variations in our model outcome due to variability in sperm size, we estimated settling rates and downstream movement of sperm based on mean sperm size (± SD). We incorporated our data on percent motile sperm at 25°C into our model to estimate the relative importance of stream discharge and percent motile sperm on fertilization success.

Results

Sperm motility and morphology

Temperature significantly affected the percentage of motile sperm, and percent sperm initially motile was a significant covariate (temperature: $F_{(3,22)} = 11.02$, $p < 0.001$; initial percent motile sperm: $F_{(1,22)} = 9.16$, $p = 0.006$; Fig. 2). The percentage of motile sperm was highest at 25°C, although not significantly different from 15°C and marginally different from 35°C. Despite the appearance of an increase in percent motile sperm between 8 and 48 hours at some temperatures, there was no significant difference in percent motile sperm between 8, 24, and 48 hours at any temperature; this artifact was due to high variability in percent motile sperm during these later time periods.

Q. pustulosa sperm (Fig. 1) had a mean (± SE) total length (head plus tail) of 38.23 ± 3.22 μm. Mean sperm head length was 3.61 μm (±0.26 μm) and head width averaged 2.13 μm (±0.26 μm). Mean sperm tail length was 34.62 μm (±3.04 μm), approximately 9.6 times longer than sperm head length. Based on mean head dimensions, we calculated the ESD of *Q. pustulosa* sperm to be 2.54 μm.

The model

Using the percent motile sperm at 25°C, we first estimated how far downstream viable sperm could travel under 4 different July flow regimes due to flow alone without considering settling velocity (Fig. 3). We then predicted downstream transport distances and observed the relative importance of settling rate and percent motile sperm under 3 different release heights (Figs 4). Based on our estimates of sperm size, we calculated the mean settling velocity of sperm to be 0.28 μm/s; however, using the mean sperm radius ± 1 SD, we calculated that settling rate could vary between 0.23 and 0.35 μm/s.

We found that under natural midsummer flow regimes, sperm has the potential to be carried considerable distances downstream and remain viable (Fig. 3), even after taking sperm settling rates into account (Fig. 4, Table 1). Due to variation in sperm size, we calculated that sperm settling rates can vary around the mean by between 18-25% (Table 1).

Our model predicts that the relative importance of sperm settling and percent motile sperm varies with gamete release height. For example, if mussels release their sperm 1 cm above the substrate, mussel sperm has the potential to travel from 3.8 to 36.5 km downstream; however, the drop in percent motile sperm between 4 and 8 h after release limits fertilization success to distances much closer to where the sperm were released. For example, 6 h after release, only approximately 10% of sperm are still motile (almost 1/3 of the original value), but by then sperm have only moved between 2.4 and 22.4 km, depending on flow regime: sperm effectively travel half the distance that they could have based on settling rates alone (Fig. 4).

A similar pattern is observed if sperm are released at 5 or 10 cm above the substrate (Fig. 4), with sperm having a maximum potential for downstream transport of between 19.2 and 182.5 km if released at 5 cm and between 38.4 and 364.9 km if released at 10 cm. However, sperm motility effectively restricts the movement of viable sperm to the same 2.4 to 22.4 km range that was observed when mussels release their sperm 1 cm above the substrate. This distance is between 90 and 95% shorter than sperm could have traveled based on settling rates alone.

Discussion

Our study suggests that *Q. pustulosa* sperm are most viable between 15 and 25^{o}C (Fig. 2), and that mussel sperm have the ability to travel large distances downstream depending on stream discharge and height at which sperm are released into the water column. We found that although sperm cannot necessarily travel as great a distance downstream when released only 1 cm above the sediment, a decline in percent motile sperm only limits their role in fertilization to half the distance they could have traveled before settling. At release heights of 5 cm or higher, sperm have the potential to move hundreds of kilometers downstream; however, their ability to be useful in fertilization is restricted to a distance within only 5-10% of the potential distance they could have travelled based on settling rates alone.

Lefevre and Curtis (1910) and Downing et al. (1993) suggested that mussel sperm in lake environments may only diffuse a maximum of 0.5 m while remaining viable. Whereas this may be the case in lentic systems, it is plausible in lotic systems for sperm to travel many more kilometers over the 4 h during which sperm are the most viable, depending on release height and stream discharge. There are no current data on release height of mussel sperm, and in fact, some mussels may release their sperm into the interstitial pore water in the sediment to fertilize nearby females. Nichols et al. (2004) showed that mussels are capable of filtering benthic (i.e. non-suspended) algal particles so this strategy may be used by females to filter sperm released to the benthos by adjacent males. Further research into this phenomenon is necessary, especially given its important consequences for understanding gene flow between mussel beds. Our data suggest that, even in mussel species that do not use highly mobile host fish species (e.g. minnows or

darters), high rates of gene flow among mussel beds are still possible due to sperm movement. Mussel bed monitoring sites established by C. Vaughn in the Little River range between 1.4 and 127 km apart (Reagan 2008). Based on our model, these are reasonable distances for sperm to travel between beds provided there is adequate flow. The relative contributions of host fish movement and sperm movement to genetic diversity in mussel populations has not been investigated and is an obvious follow-up to this study.

While our model includes estimates of the movement of individual sperm cells, several species of freshwater mussel have been discovered to release sperm in concentrated spermatozeugmata, or sperm balls (Lynn 1994, Waller and Lasee 1997, Ishibashi et al. 2000, G. T. Watters, Ohio State University, personal communication). These sperm packages can contain several thousand sperm, can range up to 80 μm in diameter, and are suspected to be an efficient sperm delivery system in many invertebrate taxa (Lynn 1994, Waller and Lasee 1997, Ishibashi et al. 2000). We did not observe any spermatozeugmata in *Q. pustulosa* using our sampling method; however, it is possible that prior to natural gamete release sperm are packaged in similar structures. If true, this would increase the settling velocity and potentially increase the longevity of the encapsulated sperm. We used Stoke's Law to estimate the settling rate of hypothetical spermatozeugmata assuming a diameter of 80 μm and a density similar to that of an individual sperm cell (i.e. the density of *Chlorella vulgaris*). Using the same model we used for our sperm, we incorporated settling rates with July flow regimes and sperm release heights to predict how far downstream spermatozeugmata could travel (Fig. 5). Sperm packaging drastically decreases the distance that sperm travel downstream before

settling from the water column. Under the highest July flow regimes and the highest release height, spermatozeugmata could travel a maximum of 0.36 km downstream, a considerable decrease from the range observed in individual, free-swimming sperm cells. Ishibashi et al. (2000) observed spermatozeugmata in five species of freshwater bivalves and found that sperm encapsulated in spermatozeugmata were active for at least 48 h after release. If this is the case, sperm viability should never be a limiting factor in freshwater mussel fertilization downstream of release. Further investigation into the effects of sperm packaging on viability and travel downstream is needed as this has implications for population and conservation genetics of these organisms.

Once sperm have been released into the water column and begin travelling downstream (assuming they are not packaged into spermatozeugmata), sperm concentrations within the water column could be extremely dilute, particularly during high flow, decreasing the chances of successful fertilization (Haggerty et al. 1995). However, mussels in southeastern Oklahoma reproduce during summer months when water levels are generally low and temperatures warm. Vaughn et al. (2004) and Spooner and Vaughn (2008) have shown that mussels under these conditions have high clearance rates, which would facilitate sperm filtration by females. Additionally, recent work suggests that marine animals adopting the spermcasting reproductive strategy can still achieve high levels of successful reproduction with sperm concentrations two to three orders of magnitude more dilute than required by typical broadcast spawners (Pemberton et al. 2003, Bishop and Pemberton 2006). This conclusion is supported by several studies in which high levels of fecundity (>85% of females) have been observed in naturally reproducing freshwater mussel populations even when mussel densities were low

(Haggerty et al. 1995, Haag and Staton 2003), although other studies report fecundities slightly lower than this (65-75%) (Yeager and Neves 1986, Haggerty et al. 1995, Garner et al. 1999).

Spermcasting marine species can also achieve successful reproduction at population densities much lower than broadcast spawners (Phillippi et al. 2004), partly because females have the ability to store sperm (Foighil 1985, Bishop and Ryland 1991, Burighel and Martinucci 1994a). Foighil (1985) found that sperm storage can last between 1 to 4 months in the marine bivalve, *Mysella tumida*, and Bishop and Ryland (1991) found sperm storage up to a month in the ascidian, *Diplosoma listerianum*. Although no work has yet documented sperm storage capabilities in freshwater mussels, there is recent evidence indicating that multiple paternity does occur (Christian et al. 2007). Although sperm storage may not need to be invoked to explain multiple paternity, it is one possible explanation for the observation that female mussels brood only a single clutch at a time even when there is high variability in timing of gamete release within a species (Haag and Staton 2003, Galbraith and Vaughn 2008). Research into the mechanism of multiple paternity and the potential for sperm storage in mussels is critical for our understanding the dynamics of freshwater mussel reproduction.

We have presented here the first model for predicting the movement of freshwater mussel sperm. It should serve as a basic model against which more complex models can be tested. This model is clearly very simple and does not take into consideration complex stream flow patterns, diffusion of sperm across the width of the stream, or loss of sperm from the water column due to filtration by mussel beds and by other filter-feeding animals. Additionally, our "high end" estimates of the distance sperm can travel is based

on extremely high July water flow for this region (the highest July flow in almost 20 years). Under high flow regimes where water residence times are low, mussels would have a more difficult time clearing sperm from the water column (Vaughn et al. 2004). The actual distance sperm can travel and be successfully used in reproduction is probably much closer to those found under our more moderate flow estimates.

Despite the limitations of our model, the model demonstrates that the distance that motile sperm travel ultimately depends on stream discharge and temperature. Successful mussel reproduction requires adequate flow to distribute sperm to receptive females of the same species and appropriate thermal conditions such that sperm are viable upon reaching a female. Thus, as temperature and flow regimes continue to be altered by climate change and river regulation, the distance motile sperm are carried downstream, and ultimately mussel reproductive success, is likely to change. Climate change models have predicted an increase in seasonal air temperature by as much as 4^{o}C over the coming years (Mulholland et al. 1997). In addition, river regulation in the southern U.S. often results in rivers becoming a series of isolated pools in the summer with temperatures sometimes exceeding 40^{o}C (Spooner and Vaughn 2000). Although we did not measure percent motile sperm at temperatures between 25 and 35^{o}C, we do know that motility drops off somewhere between these two temperatures (Fig. 2). This means that as water temperatures rise, the distance motile sperm can travel downstream will be restricted closer to the site of gamete release. Freshwater mussels have evolved in rivers where there is flow and temperature variability across years, which likely leads to some years of good and other years of poor recruitment. However, our model indicates that drastically

manipulating flow and temperature could alter not only the genetic structure of mussel populations, but could lead to overall reproductive failure of mussels.

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Table 1. Variation in sperm settling distance due to variability in sperm size. Values represent settling distance based on mean sperm radius (settling distance), settling distance based on mean – 1 SD in sperm radius (longest settling distance), and settling distance based on mean + 1 SD in sperm radius (shortest settling distance).

Discharge (m^3/s)	Sperm release height (cm)	Settling distance (km)	Longest settling distance (km)	Shortest settling distance (km)
0.51	1	3.8	4.8	3.1
8.34	1	25.2	31.5	20.6
18.89	1	31.6	39.5	25.8
58.26	1	36.5	45.6	29.9
0.51	5	19.2	24.0	15.7
8.34	5	125.9	157.4	103.1
18.89	5	157.8	197.3	129.2
58.26	5	182.5	228.1	149.4
0.51	10	38.4	48.1	31.5
8.34	10	251.9	314.9	206.2
18.89	10	315.6	394.6	258.4
58.26	10	364.9	456.3	298.8

Figure legends

Figure 1. Scanning electron micrographs of *Q. pustulosa* sperm illustrating an individual spermatozoon (A), a sperm head (B), and the protrusion caused by mitochondria (arrow) and insertion point of the flagellum with the sperm head (C).

Figure 2. (A) Estimated marginal means (±1 SE) of percent motile *Q. pustulosa* sperm over time at four different temperatures. Mean for initial percent motile sperm is the grand mean averaged across all temperature treatments (the covariate in our ANCOVA). (B) Mean (±1 SE) percent motile sperm at four temperatures averaged over 48 hours.

Figure 3. (A) Distance sperm can move downstream (without considering sperm settling) under four different July flow regimes versus percent motile sperm at 25^{o}C. (B) Expanded view of (A).

Figure 4. Results of model predicting the distance sperm can travel downstream until settling out of the water column (marked by triangles) under four July flow regimes versus percent motile sperm at 25^{o}C (gray line). Model assumes mussels release sperm 1 cm (A), 5 cm (B) and 10 cm (C) above sediment.

Figure 5. Results of model predicting the distance hypothetical spermatozeugmata (sperm packages) can move downstream until settling out of the water column (marked by triangles) under four July flow regimes. Model assumes mussels release spermatozeugmata 1 cm (A), 5 cm (B) and 10 cm (C) above sediment.

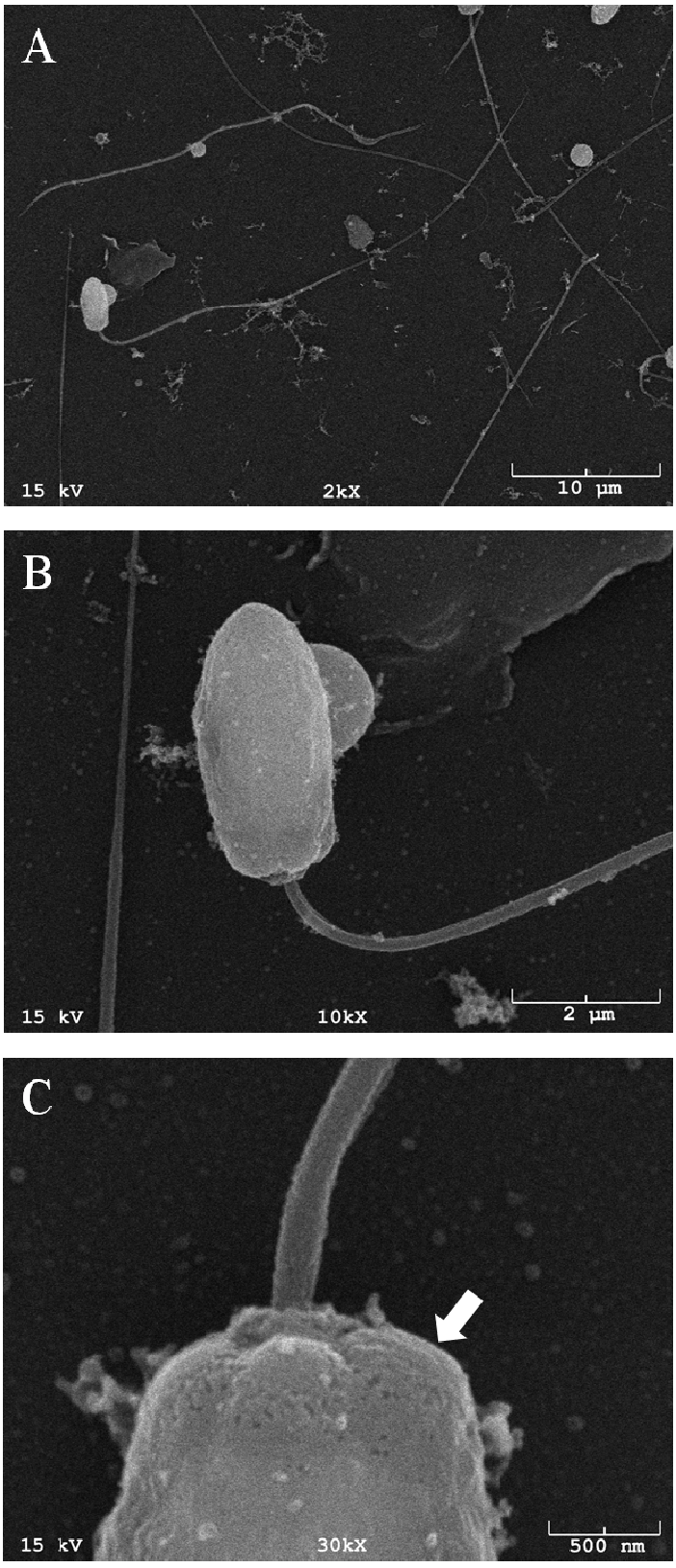

Figure 1.

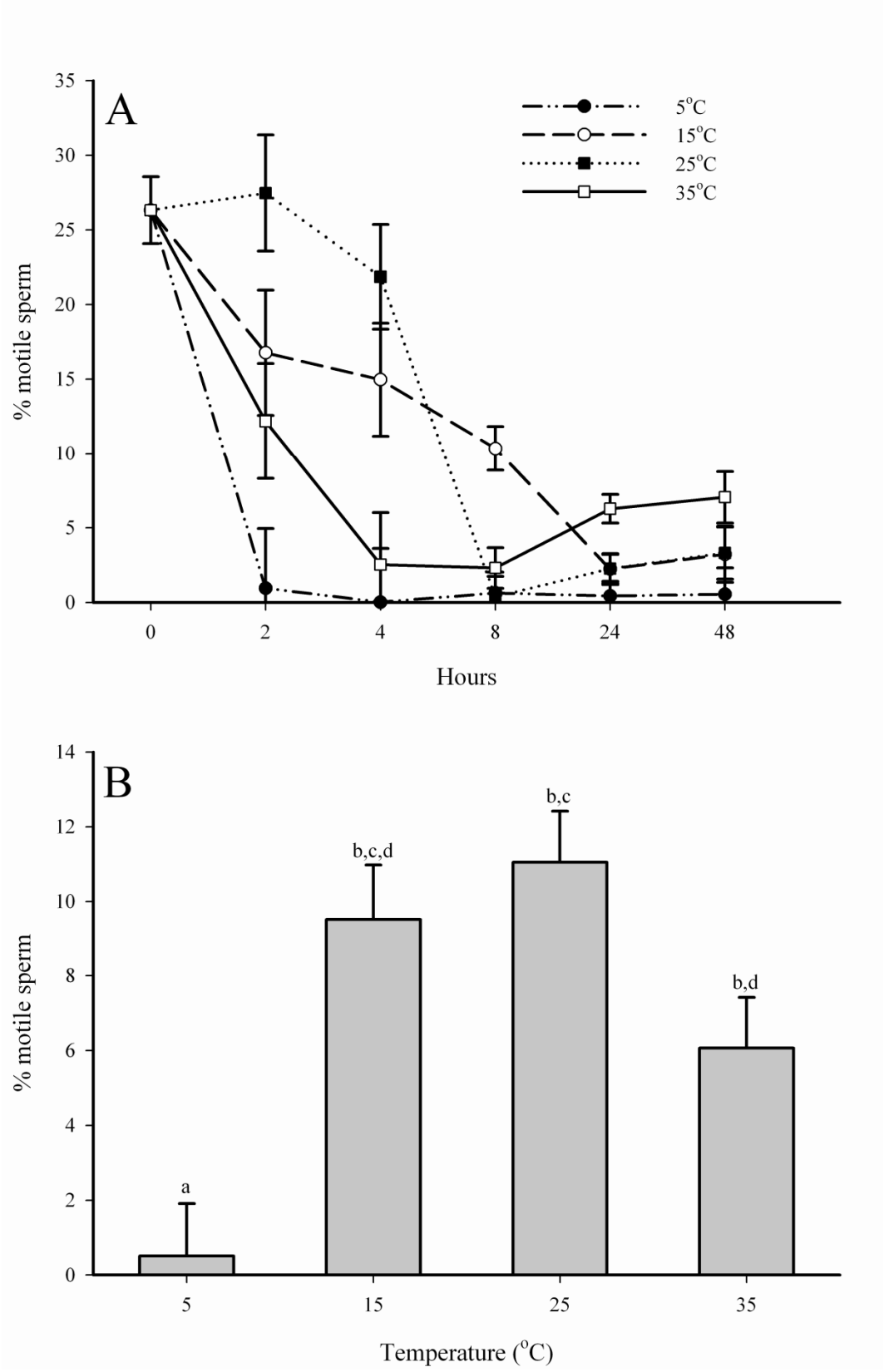
A
5°C
15°C
25°C
35°C
% motile sperm
Hours
0
2
4
8
24
48
B
% motile sperm
a
b,c,d
b,c
b,d
5
15
25
35
Temperature (°C)

Figure 2.

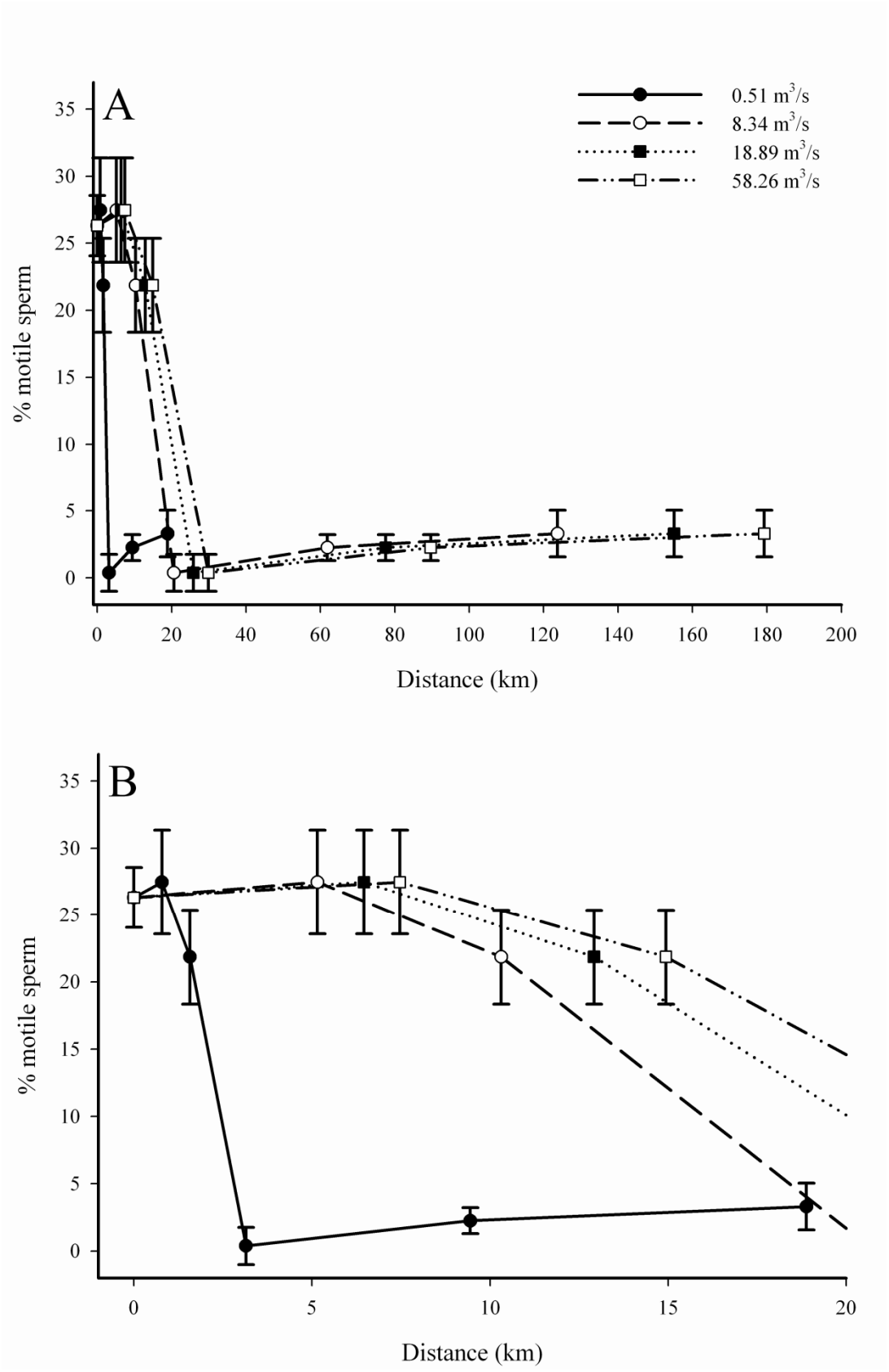
A
0.51 m3/s
8.34 m3/s
18.89 m3/s
58.26 m3/s
% motile sperm
Distance (km)
B
% motile sperm
Distance (km)

Figure 3.

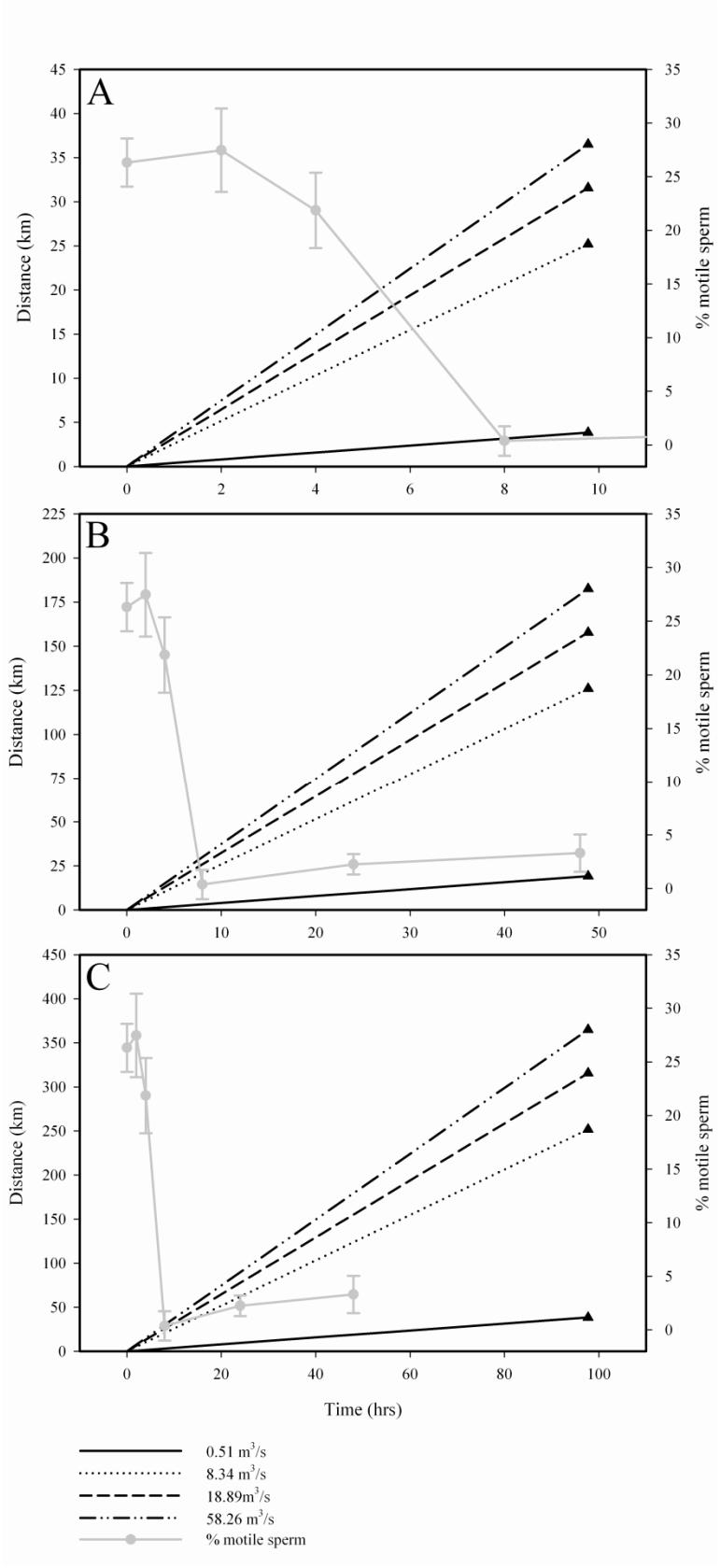
A
B
C
Distance (km)
% motile sperm
Time (hrs)
0.51 m3/s
8.34 m3/s
18.89m3/s
58.26 m3/s
% motile sperm

Figure 4.

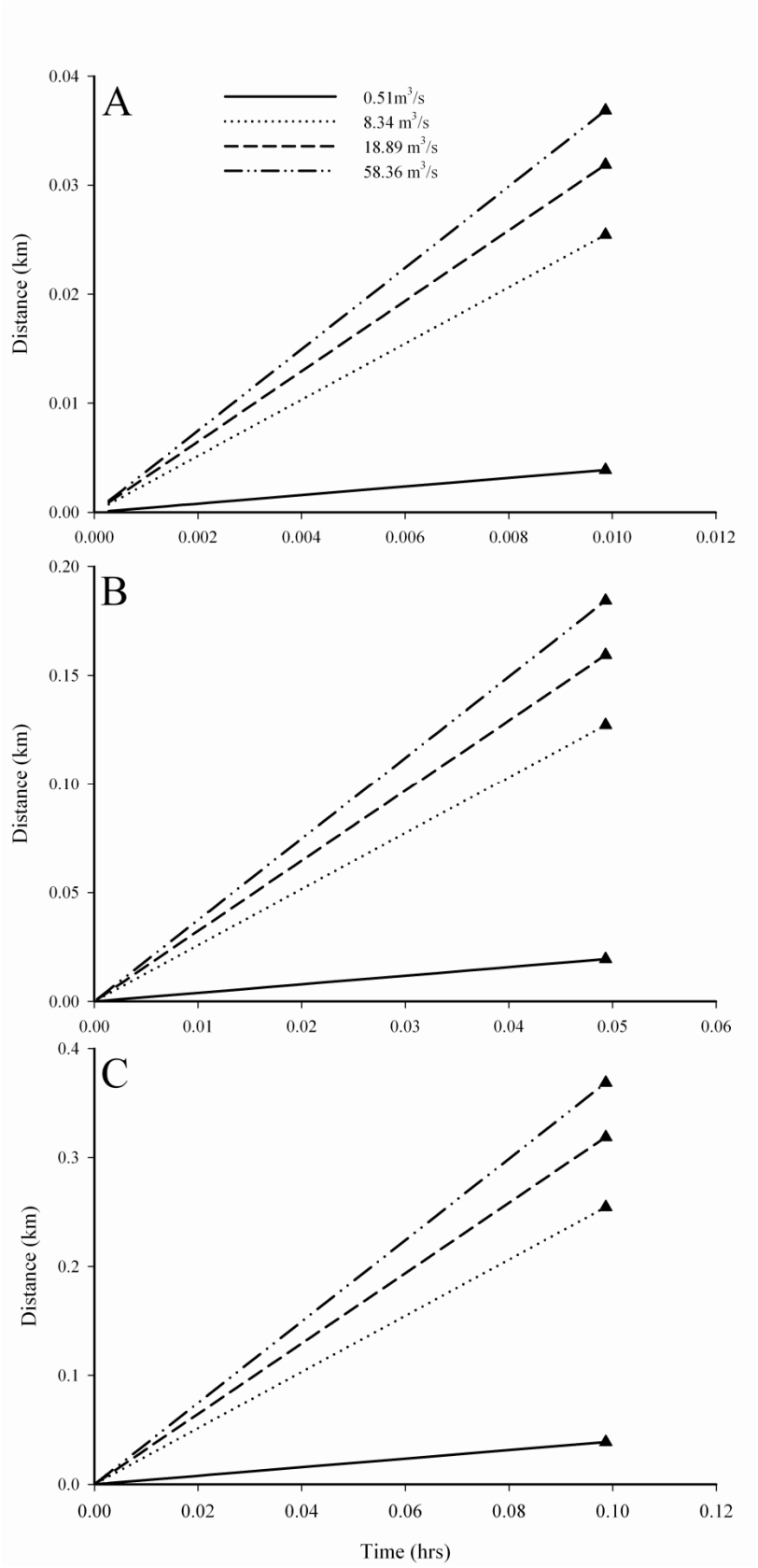
A
0.51m3/s
8.34 m3/s
18.89 m3/s
58.36 m3/s
Distance (km)
0.04
0.03
0.02
0.01
0.00
0.000
0.002
0.004
0.006
0.008
0.010
0.012
B
Distance (km)
0.20
0.15
0.10
0.05
0.00
0.00
0.01
0.02
0.03
0.04
0.05
0.06
C
Distance (km)
0.4
0.3
0.2
0.1
0.0
0.00
0.02
0.04
0.06
0.08
0.10
0.12
Time (hrs)

Figure 5.

Chapter 3. Potential effects of reservoir management on the condition and reproductive traits of downstream mussels

Abstract

Impoundments are known to have dramatic influences on aquatic ecosystems; however, these effects can vary depending on management, including water release patterns. We examined the effects of two dams on freshwater mussel reproductive traits: one dam that mimicked natural flow patterns where the amount of water released (outflow) approximately equaled the amount of water coming into the reservoir (inflow) and one dam where outflow did not mimic natural flow patterns. We found that mussel reproductive traits, including density, percent hermaphrodites, percent of the population infected by sterilizing trematodes, body condition and sex ratio, were all disrupted below the dam with unnatural flow regimes. Although there were still some population disturbances below the more naturally-regulated impoundment, these were not as severe. Potential mechanisms by which impoundments can influence reproductive characteristics of freshwater mussels and ultimately lead to slow population demise include effects on gamete viability, reproductive cues, and stress responses, all of which ultimately lead to negative density-dependence (allee effects).

Key words: Unionid, impoundment, temperature, flow, sex ratio, parasitism, hermaphrodite

Introduction

All animals must acquire and assimilate energy, grow and reproduce. Success in doing so ultimately depends on an animal's physiological and ecological requirements. Different species vary in these requirements and different life stages within a single species can also have diverse physiological and ecological optima. In particular, reproduction is one of the most critical time periods in an organism's life and is therefore crucial to conservation efforts. For example, there are often narrow windows during which conditions are optimal for reproduction, and reproductive adults, juveniles, or gametes can be extremely sensitive to stressors during this time (Snell, 1986; Gosselin and Chia, 1995; Eriksson and Baden, 1997; Willmer et al., 2005). Additionally, reproduction is energetically costly: both males and females can invest heavily in reproduction through a variety of mechanisms including nuptial gifts, calling behavior, mate guarding, brooding and matrotrophy (Trexler, 1997; Chaparro and Thompson, 1998; Vahed, 1998; Watson et al., 1998; Sullivan and Kwiatkowski, 2007).

Even though reproduction is particularly sensitive to environmental stressors and change, understanding how changing environmental conditions impact reproduction can be difficult. For example, many long-lived organisms delay reproduction until late in life, making it difficult to detect changes in their reproductive success (Rowe, 2008). Some animals migrate during their reproductive period, while others have complex life cycles that are not well understood, further complicating the study of their reproduction (Holmes, 2007; Petranka, 2007; Fraser and Bernatchez, 2008). Unfortunately, most conservation research has focused primarily on adult organisms, neglecting the larval, embryonic, and gametic stages that make reproduction a "bottleneck" to species

conservation: failure to understand this most sensitive time period could lead to failure in conservation efforts.

A widespread and significant environmental change that has impacted many organisms globally is the large-scale impoundment of rivers. In the 20th century alone, more than 75,000 dams over 2 meters high have been constructed in the United States (Poff et al., 2007). Impoundments alter physical characteristics of rivers including flow, temperature, light, material cycling and availability, and sediment loads, not only in impounded sections of rivers but upstream and downstream as well (Benke, 1990; Allan and Flecker, 1993; Poff et al., 2007). These altered conditions result in a wide variety of direct and indirect effects on aquatic organisms including mortality, disruption of reproductive cues, blocked fish migration and disturbance of entire food webs (Poff et al., 1997; Poff and Hart, 2001; Lytle and Poff, 2004). Although we have a generally good understanding of acute impacts of impoundment, more information and synthesis is needed on chronic, cumulative effects of dam-induced disturbances on affected organisms (Strayer et al., 2004).

Impoundments have been constructed for a variety of human needs including irrigation, hydroelectricity, drinking water, flood control, and recreation (Allan and Flecker, 1993; Poff and Hart, 2001). Impoundments differ in their ecological impacts depending on how drastically the river is altered and on subsequent management of reservoir releases (Poff et al., 1997). These ecological effects vary with dam location, dam height, degree of water regulation, and how closely water management mimics natural stream conditions (Poff and Hart, 2001; Poff et al., 2007). For example, water released below impoundments can result in both abnormally high and low flows,

sometimes on a daily basis, and often at the "wrong" time of year (Poff et al., 1997; Richter and Richter, 2000).

Freshwater mussels (Bivalvia: Unionoida) are a group of benthic, filter-feeding bivalves that provide important ecosystem services in lakes and rivers (Spooner and Vaughn, 2006; Vaughn et al., 2007; Vaughn et al., 2008). Freshwater mollusks in general are one of the most globally imperiled faunas, with over 700 species listed on the IUCN (the World Conservation Union) Red List as threatened or endangered (Lydeard et al., 2004; Strayer et al., 2004). Mussels possess a suite of traits that make them highly susceptible to habitat alteration (Watters, 2000). Adults are sedentary with limited dispersal capabilities and restricted refugia from disturbance (McMahon and Bogan, 2001; Spooner, 2007). Mussels are long-lived (< 6 to 100 years), have delayed reproduction (age at maturity 6 - 12 years), and juvenile survivorship is low, in turn making overall recruitment low (McMahon and Bogan, 2001). Mussels also have a complicated life history that depends on their larvae developing as ectoparasites on host fish species; therefore, successful dispersal of mussels is also fish-dependent (McMahon and Bogan, 2001; Barnhart et al., 2008). Many mussel species are host fish specialists and require encystment on particular host fish species to successfully develop (Haag and Warren, 1998; Barnhart et al., 2008). Because of these unique life history characteristics, addressing conservation needs for reproducing mussel populations has been largely neglected.

Mussels evolved in rivers that typically experienced seasonal periods of low and high flow, and recent studies indicate that natural, temporal variability in flows is important for successful recruitment (Vaughn and Taylor, 1999; Gore et al., 2001;

Hardison and Layzer, 2001). Because impoundments are known to severely alter natural flow regimes (Baxter, 1977; Poff et al., 1997; Poff and Hart, 2001), mussel communities have been strongly, negatively impacted by widespread dam construction (Bogan, 1993; Watters, 2000). Numerous studies have documented mussel declines below impoundments (Suloway et al., 1981; Miller et al., 1984; Williams et al., 1992; Layzer et al., 1993; Vaughn and Taylor, 1999; Garner and McGregor, 2001). Layzer et al. (1993) and Heinricher and Layzer (1999) showed that cold water released below dams decreases mussel reproductive success downstream. Impoundments also impede fish host movement between mussel patches, thus limiting dispersal and potentially gene flow among mussel populations (Watters, 1996, 2001; Barnhart et al., 2008). In most cases, multiple factors likely interact to lead to declines in mussel populations below impoundments; however, little work has examined the mechanisms underlying these declines.

Here, we examine a suite of reproductive traits in naturally occurring freshwater mussel populations in a southern U.S. river and relate these to the effects of two impoundments with different management practices. We compare mussel reproductive traits among three sites that are variable in their physical characteristics and are located at different distances downstream from these two impoundments. We have evidence suggesting that timing of gamete development may be impacted below a cold-water release impoundment (Galbraith, 2009, Chapter 1). Here, we examine other reproductive and population level characteristics of mussels, including sex ratio, percent hermaphroditism, body condition, and parasite load, to evaluate the reproductive success

and future stability of three mussel populations. We then use these data to generate testable hypotheses as to the mechanisms of mussel decline below impoundments.

Materials and Methods

Study Area and Species

Our study was conducted in the Little River in southeastern Oklahoma, U.S. (Fig. 1), in the Ouachita Mountains region of the Interior Highlands. This region is a center of speciation for both aquatic and terrestrial organisms including mussels, and the Little River itself harbors 37 species of unionid mussels (Mayden, 1985; Moulton and Stewart, 1996; Galbraith et al., 2008). The Little River is impacted by two impoundments. The mainstem river is impounded by Pine Creek Lake, which is used for flood control, municipal water supply, and recreation (OWRB, 2007). The river also receives inflow from a tributary, the Mountain Fork River, which is impounded by Broken Bow Lake. This second reservoir is primarily used to generate hydroelectric power and to provide pulses of cold water for a non-native, stocked trout fishery.

We chose sites known to have abundant, diverse mussel assemblages (Vaughn and Taylor, 1999; Galbraith et al., 2008) but which we thought would be differentially impacted by impoundments. Site 1 was located approximately 60 km below Pine Creek Lake, Site 2 was located approximately 5 km below Site 1 (65 km below Pine Creek Lake). Both Sites 1 and 2 were located above the inflow from Broken Bow Lake (Fig. 1). Site 3 was located approximately 85 km below Pine Creek Lake and 40 km below Broken Bow Lake (Fig. 1). Site 3 has significantly colder summer temperatures and warmer winter temperatures than the other two sites (Table 1) because of its location

below Broken Bow Lake (Galbraith, 2009, Chapter 1). Site 3 also has lower water column chlorophyll *a* than the other 2 sites (Galbraith, 2009, Chapter 1). However, all three sites vary to some extent in their flow regimes, food availability (both water column chlorophyll *a* and benthic ash free dry mass), and light availability (Galbraith, 2009, Chapter 1). All sites were on the relatively pristine USFWS-operated Little River National Wildlife Refuge and experienced similar watershed land use (OWRB, 2007).

We focused our study on three related species in the genus *Quadrula*: the pimpleback (*Quadrula pustulosa*), the rabbitsfoot (*Q. cylindrica*) and the mapleleaf mussel (*Q. quadrula*). The species we chose co-occur in many rivers across North America including the Little River (Parmalee and Bogan, 1998). Several members of this genus are either federally endangered (e.g. *Q. fragosa*) or listed as species of special concern (*Q. cylindrica*). Therefore we were particularly interested in the reproductive biology of this genus. Because of their widespread distribution throughout North America, however, we feel that data collected on these species will also pertain to other mussel species.

Sampling

In August 2005 we quantitatively sampled mussels at the three sites to estimate population densities. For each site we evacuated 30, randomly-placed 0.25 m^2 quadrats to a depth of approximately 15 cm, removed, identified and measured mussels, and returned mussels to their original location (Vaughn et al., 1997; Strayer and Smith, 2003).

From September 2005 through August 2006, we semi-quantitatively sampled (timed searches) the three *Quadrula* species at each site on a monthly basis (except during December, January, and March due to inclement weather and high water). Timed

searches consisted of at least two hours of searching for mussels by hand, snorkel, or SCUBA in deeper areas (> 0.75 m). We collected, marked, weighed and measured as many individuals of each species as we could find. We also collected a small (~50 µl) gonad sample from the visceral mass with a syringe and preserved the samples in buffered formalin. This non-lethal technique for determining sex in non-sexually dimorphic species allows large sample sizes without killing individuals, particularly threatened and endangered species (Shiver, 2002; Saha and Layzer, 2008). In the laboratory, we examined gonad samples under a microscope to identify male and female mussels and to quantify presence or absence of sterilizing trematodes (Jokela et al., 1993).

We gathered online reservoir intake and release data from the U.S. Army Corps of Engineers for both Pine Creek Lake (http://www.swt-wc.usace.army.mil/PINE.lakepage.html) and Broken Bow Lake (http://www.swt-wc.usace.army.mil/BROK.lakepage.html). The reservoir data that we examined were collected between January 1995 and December 2006.

Data analyses

For all of our analyses, individual mussels were only represented once in each analysis (i.e. recaptured mussels were not included). We analyzed log of mean mussel density (from quadrat data collected in August, 2005) on a species-by-site basis using a two-way ANOVA followed by a Tukey post hoc comparison. We used chi-square analysis to determine differences in parasite presence or absence among sites. To do this, we pooled all parasitized individuals collected during the year-long field study within each site. It is a general assumption in models of parasite infection that parasite

transmission is a function of host density (May and Anderson, 1979; Toft et al., 1991; Loot et al., 2005). Therefore, we assumed that rates of parasitism should be a function of mussel density and weighted our expected proportions in our chi-square by species density at each site.

We estimated mussel body condition for each individual collected over the course of the year using the Fulton's K metric in which body condition (K) = $l^3/w(10^6)$, where l is mussel length and w is mussel wet weight (including the shell). This measure of condition has been traditionally used in the aquaculture literature (Mgaya and Mercer, 1995), but has been applied with success to freshwater mussels (Spooner and Vaughn, 2008). Low body condition would refer to individuals with lower wet weight relative to body length whereas high body condition would refer to individuals that were heavier than predicted based on length. We used ANOVA and a Tukey post hoc comparison to test for differences in body condition among sites. Because these three species differ in their body size and shape (and potentially growth constraints) we did not compare differences in body condition among species using this metric.

We used chi-square to determine if proportion of males of each species (i.e. sex ratio) was equal both across sites and within individual sites using sex data collected over the course of the year-long study. We also used chi-square to test for differences among sites in incidence of hermaphroditism (both female and male gametes present in the same gonad), again using the mussels collected during the year-long field study. Hermaphroditism is often common in freshwater mussel populations, particularly small, isolated populations (Heard, 1975). We tested the null hypothesis that hermaphrodites are equally distributed among all three sites.

Results

We found significant differences in mussel density (Table 2) among sites ($F_{(2,261)}$= 29.25, p< 0.001) and species ($F_{(2,261)}$= 165.79, p< 0.001) and a significant site-by-species interaction ($F_{(4,261)}$= 19.92, p< 0.001). All three species were significantly different from each other with *Q. pustulosa* having the highest density followed by *Q. cylindrica* and *Q. quadrula* respectively. In general, Site 2 had the highest densities of all three species, followed by Site 1 and then Site 3, with the exception of *Q. cylindrica* density, in which case Site 1 had higher densities than Site 2.

Sterilizing trematodes were present in only 17 of 460 individuals and only in *Q. pustulosa*. We found a significant difference in parasite load among sites ($\chi^2_{(2)}$= 12.85, p= 0.002), with higher rates of parasitism than expected at site 3 (Fig. 2).

There were significant differences in mussel body condition across sites (*Q. cylindrica*: $F_{(2,111)}$= 18.51, p< 0.001; *Q. pustulosa*: $F_{(2,272)}$= 16.49, p< 0.001; *Q. quadrula*: $F_{(2,34)}$= 5.89, p= 0.006) (Fig. 3). Body condition in all three species was consistently lowest at site 3. We found no effect of sex or parasitism on *Q. pustulosa* body condition except females at site 3 had significantly lower body condition than males (Fig. 3), meaning that females had lower wet weight relative to their body length than males.

There were also differences from equality in the relative proportion of males and females of each species (Fig. 4). Averaged across all sites, *Q. cylindrica* had a significantly female-biased population ($\chi^2_{(1)}$= 4.03, p= 0.045); however, within individual sites there were no differences from equal sex ratios for this species. There were significantly more *Q. pustulosa* males than females ($\chi^2_{(1)}$= 8.4, p= 0.004) across all sites.

Although this same pattern was seen at each of the individual sites, there was only a statistically significant male bias at site 1 ($\chi^2_{(1)}$= 6, p= 0.01). *Quadrula quadrula* had an approximately equal sex ratio across all sites and within each individual site.

We found a significant difference in the proportion of hermaphroditic individuals among sites ($\chi^2_{(2)}$= 6, p= 0.05; Fig. 5). There was no difference among sites in the frequency of *Q. cylindrica* hermaphrodites ($\chi^2_{(2)}$= 0.5, p= 0.78). However, site 3 had significantly more hermaphroditic *Q. pustulosa* ($\chi^2_{(2)}$= 6, p= 0.05) and marginally more *Q. quadrula* hermaphrodites ($\chi^2_{(2)}$= 5.2, p= 0.07) than expected. Incidence of hermaphroditism ranged between 0 and 7% for sites 1 and 2, but for site 3 was as high as 14% in *Q. cylindrica* and 33% in *Q. quadrula*.

There were differences in reservoir release patterns between Pine Creek and Broken Bow reservoirs (Fig. 6). Pine Creek reservoir releases almost identically mimicked reservoir inflow during the entire year, with slight deviations during February and March. On the other hand, Broken Bow releases varied considerably from inflow; reservoir release was substantially higher than inflow during summer months when natural stream flow is generally low. These differences in reservoir release patterns translated into differences in water temperature at the mussel beds (Table 1).

Discussion

River regions both above and below a dam often experience unseasonal temperature and flow regimes, anoxic conditions, altered patterns of sediment deposition and erosion, and lower particulate organic matter concentrations (Allan, 1995). However, the way an impoundment is managed can influence how a dam impacts

downstream populations. Pine Creek Lake is an example of an impoundment where releases mimic natural variability in rainfall and tributary inflow received by the reservoir. Broken Bow Lake, on the other hand, is an example of a reservoir where summer release patterns exceed the amount of water entering the reservoir from tributary inflow and rainfall. As a consequence, the mussel populations below Broken Bow Lake experience colder than normal summer temperatures (Table 1), higher flow, and potentially limited food availability in the form of phytoplankton (Galbraith, 2009, Chapter 1) compared to populations below Pine Creek Lake (sites 1 and 2). These patterns translate into long-term trends of declining reproductive success in mussels below Broken Bow Lake.

Densities of all three mussel species were lower at site 3 than at sites 1 and 2. In addition, we found that mussels at site 3 exhibited more signs of stress than mussels at sites 1 and 2, with higher rates of parasitism in *Q. pustulosa* (Fig. 2) and lower body condition for all three mussel species (Fig. 3). Further, we found a higher frequency of hermaphroditism in both *Q. pustulosa* and *Q. quadrula* at site 3 (Fig. 5). We do not know the mechanisms by which altered temperature and flow regimes are impacting mussel reproduction below Broken Bow Lake and similar reservoirs, but they likely include multiple pathways, all of which ultimately may result in the long-term demise of mussel populations (Fig. 7). Disruptions in appropriate reproductive cues below impoundments have resulted in a complete failure of some mussel species to reproduce (Layzer et al., 1993; Heinricher and Layzer, 1999), and we have some evidence that inappropriate reproductive cues could be influencing gamete development in the Little

River, leading to a negative density-dependent feedback loop (Fig. 7ii.) (Galbraith, 2009, Chapter 1).

Freshwater mussel sperm are only motile within a narrow thermal range (Galbraith, 2009, Chapter 2); non-motile sperm cannot be used for reproduction (Ciereszko et al., 2001). This could lead to poor recruitment in areas below impoundments with inappropriate thermal regimes, thus lowering overall mussel densities over time (Fig. 7i). It is also plausible that unusual flow and temperature patterns could directly cause stress or could alter the nutrient dynamics and thus food availability to downstream mussel beds (Fig. 7iii) (Elser and Kimmel, 1985). Marine mussels exposed to extreme temperatures and low food availability experience sub-lethal, physiological stress effects as well as direct mortality (Incze et al., 1980; Dahlhoff et al., 2002). Therefore we can expect that these factors would also influence freshwater mussel body condition thereby decreasing survivorship and eventually overall mussel density.

Hermaphrodites often are more common in small, genetically isolated populations or in environments that are particularly stressful as an adaptation to counteract Allee effects (i.e. as a means of reproducing even when mates are rare) (Ghiselin, 1969; Heard, 1975). Mussels at site 3 may have reached a low density threshold, causing individuals to resort to hermaphroditism to increase the chances of successful fertilization. If this is the case, negative density dependence will likely cause these populations to continue to decline (Strayer et al., 2004). Hermaphrodites generally constitute a small proportion (usually less than 10%) of freshwater mussel populations (Haggerty et al., 1995; Garner et al., 1999; Haag and Staton, 2003). However, we found 14% of *Q. cylindrica* and 33%

of *Q. quadrula* were hermaphroditic at site 3, suggesting that these species are declining at this site. Little is known about the factors (temperature, genetics) that govern sex determination in freshwater mussels. Whether altered thermal regimes below impoundments could directly cause hermaphroditism by interfering with "normal" sex-determination needs to be investigated.

Lower body condition below impoundments also may increase susceptibility to parasite infection (Gangloff et al., 2008). Sterilizing trematode loads vary among freshwater mussels, but in some areas have been found to be as high as 100% in a single species (Henley et al., 2007). We found less than 10% of the individuals in our study to be parasitized, trematodes were found in only one species, *Q. pustulosa*, and were highest at site 3. Although there was no significant difference in body condition between parasitized mussels and non-parasitized males and females, we only sampled a relatively small number of parasitized individuals. Further analysis is needed to confirm if low body condition makes particular individuals more susceptible to infection or if the parasites themselves lower body condition of mussels post-infection (Gangloff et al., 2008). We did not find mature gametes in any of our parasitized individuals, suggesting that these trematodes completely sterilize their hosts. This has severe consequences for *Q. pustulosa* reproduction; a substantial portion of individuals are not reproducing, thus lowering the effective population size.

Our sex ratio data point to a decrease in effective population size throughout the Little River, regardless of distance below an impoundment. We found significant female-biased sex ratios in *Q. cylindrica* but found a male-biased sex ratio in *Q. pustulosa*. Deviations from a 1:1 sex ratio appear to be common in freshwater mussels (Bauer, 1987;

Downing et al., 1989; Byrne, 1998; Garner et al., 1999; Haag and Staton, 2003; McIvor and Aldridge, 2007). In our system, sex ratios could be equal at conception, with later sex-specific mortality skewing the adult sex ratio. Other possibilities are that one sex (females in *Q. pustulosa* and males in *Q. cylindrica*) has higher rates of parasite infections or that the skewed sex ratio is simply a phase in the development of hermaphroditism within the population (McIvor and Aldridge, 2007; Yusa, 2007). We were unable to confirm or refute the parasitism hypothesis since there were no mature gametes found in any of our parasitized individuals. Nonetheless, the male-biased sex ratio in *Q. pustulosa* indicates that there are fewer female individuals available for reproduction, and thus *Q. pustulosa* abundance is likely on the decline. This was demonstrated by (Galbraith et al., 2005) where *Q. pustulosa* in the nearby Kiamichi River, Oklahoma was found to have dropped in density an average of 85% across 10 established monitoring sites in a period of less than 15 years. A final explanation for unequal sex ratios could be that our sampling methods under sampled one sex or the other; however, we have no reason to believe that this is the case.

We acknowledge that there are limitations to this study particularly that we only examine 3 sites and 2 impoundments located in a narrow geographic range. Other studies, however, have supported the finding that unnatural temperature regimes can have profound, chronic influences on reproduction and population dynamics of species located below impoundments, including not only mussels but fish and other invertebrate species (Munn and Brusven, 1991; Layzer et al., 1993; Voelz et al., 1994; Heinricher and Layzer, 1999; Clarkson and Childs, 2000; Haxton and Findlay, 2008). This study, in combination with these others, suggests that improper water management and disruption of thermal

cues is a serious issue that warrants further investigation, particularly on a larger, continental scale.

Our data support a conceptual model in which mussel reproduction below impoundments is influenced by multiple pathways and feedback loops (Fig. 7). The proposed mechanisms are not comprehensive and other aspects of mussel populations could be impacted by impoundments (energy assimilation, toxin loads, etc.). Additionally, reproductive trends that we observed may not necessarily be caused by impoundments alone. The Little River and most other rivers across North America have been heavily impacted by other forms of human disturbance (clearing of riparian vegetation, agricultural run-off, channelization, etc.) which could further feed into the conceptual model of mussel decline. Nonetheless, our data are indicative of the complex, indirect effects of impoundments on mussel reproductive traits.

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Table 1. Mean seasonal temperature (± SE) for each of our three sampling sites. Temperature was recorded every 30 minutes with HOBO™ loggers. Data taken from Galbraith (2009), Chapter 1.

Site	Season	Mean temperature (^{o}C)
Site 1	Fall	17.26 (0.57)
	Winter	8.44 (0.16)
	Spring	19.08 (0.47)
	Summer	30.13 (0.18)
Site 2	Fall	17.80 (0.54)
	Winter	8.89 (0.14)
	Spring	18.52 (0.44)
	Summer	30.60 (0.14)
Site 3	Fall	17.25 (0.47)
	Winter	9.22 (0.14)
	Spring	18.89 (0.43)
	Summer	25.91 (0.14)

Table 2. Mean (±SE) mussel density at each site for each species. Densities were estimated from quadrat data collected in August, 2005.

Site	Species	Mussel density (# individuals/m^2)
Site 1	*Q. cylindrica*	2.4 (0.62)
	Q. pustulosa	10.27 (2.04)
	Q. quadrula	0.13 (0.13)
Site 2	*Q. cylindrica*	1.07 (0.50)
	Q. pustulosa	20.67 (2.16)
	Q. quadrula	0.53 (0.25)
Site 3	*Q. cylindrica*	0.27 (0.19)
	Q. pustulosa	3.73 (0.83)
	Q. quadrula	0 (0)

Figure Legends

Figure 1. Location of sampling sites in the Little River in southeastern Oklahoma.

Figure 2. Results of chi-square analysis comparing observed and expected parasite loads in *Q. pustulosa* at each study site. Mussels were collected across the year-long sampling period and numbers in parentheses represent the total number of *Q. pustulosa* collected over the course of the year at each site. Expected levels of infection were weighted based on *Q. pustulosa* density at each site.

Figure 3. Mean (± SE) body condition of unparasitized males and females and parasitized (sex cannot be determined in parasitized individuals) *Q. pustulosa* at each site. Bars with different letters are significantly different from one another within a site based on ANOVA and Tukey post hoc comparisons. Significant differences between sites are reported in the results section. Numbers in parentheses represent the total number of individuals of each sex collected over the year-long field study.

Figure 4. Proportion of males in the population at each study site collected throughout the course of our year-long field study (line = 0.5: equal proportions of males and females). Total number of individuals of each species collected at each site over the course of the year is represented in parentheses.

Figure 5. Chi-square results for hermaphroditism in the three *Quadrula* species at each study site. Frequency refers to the number of observed (or expected) hermaphroditic individuals collected over the course of the year-long field study.

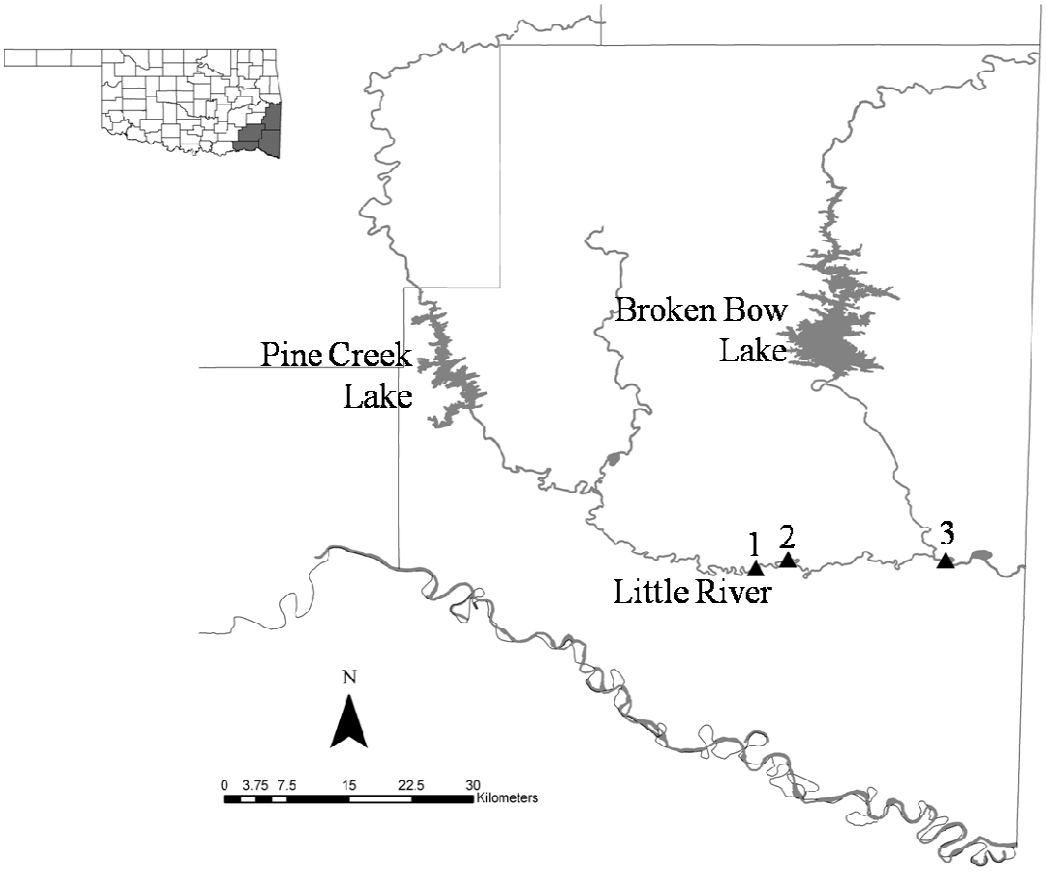
Broken Bow
Lake
Pine Creek
Lake
1
2
3
Little River
N
0 3.75 7.5 15 22.5 30
Kilometers

Figure 1.

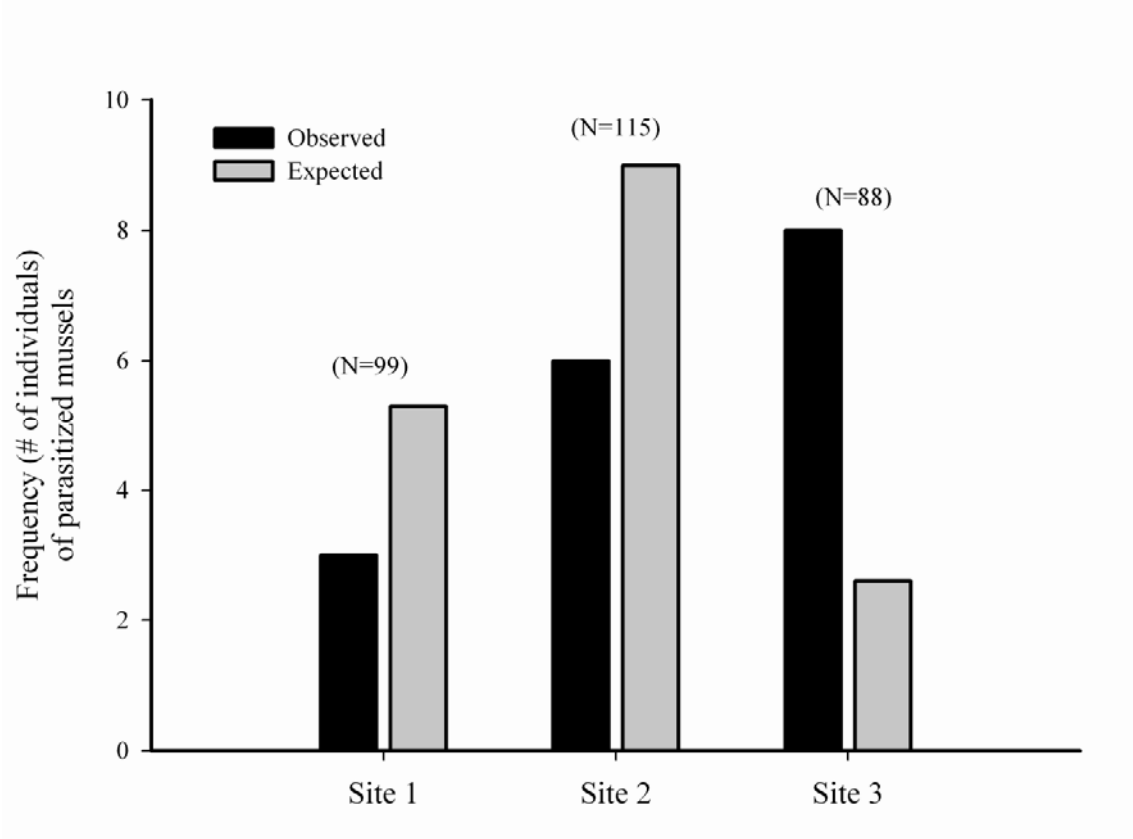
Observed
Expected
Frequency (# of individuals) of parasitized mussels
10
8
6
4
2
0
(N=99)
(N=115)
(N=88)
Site 1
Site 2
Site 3

Figure 2.

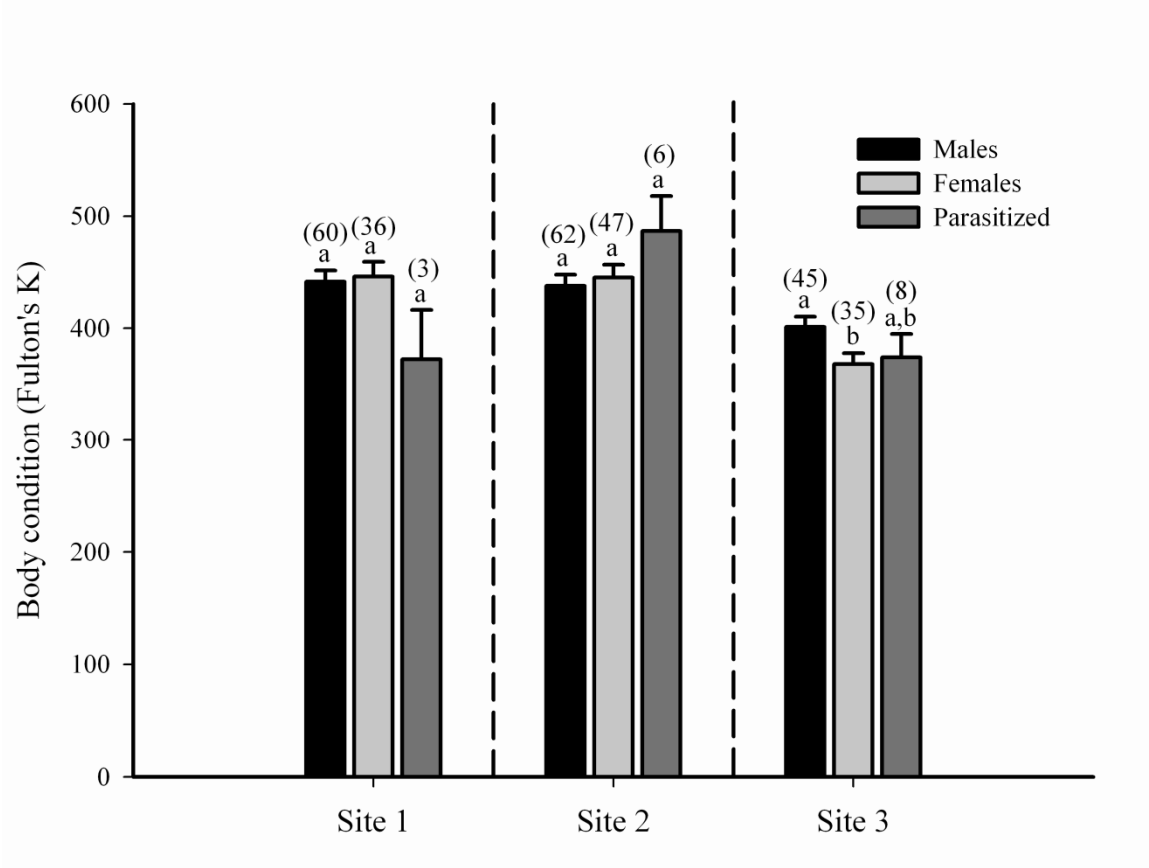
Body condition (Fulton's K)
600
500
400
300
200
100
0
Males
Females
Parasitized
(60) a
(36) a
(3) a
(62) a
(47) a
(6) a
(45) a
(35) b
(8) a,b
Site 1
Site 2
Site 3

Figure 3.

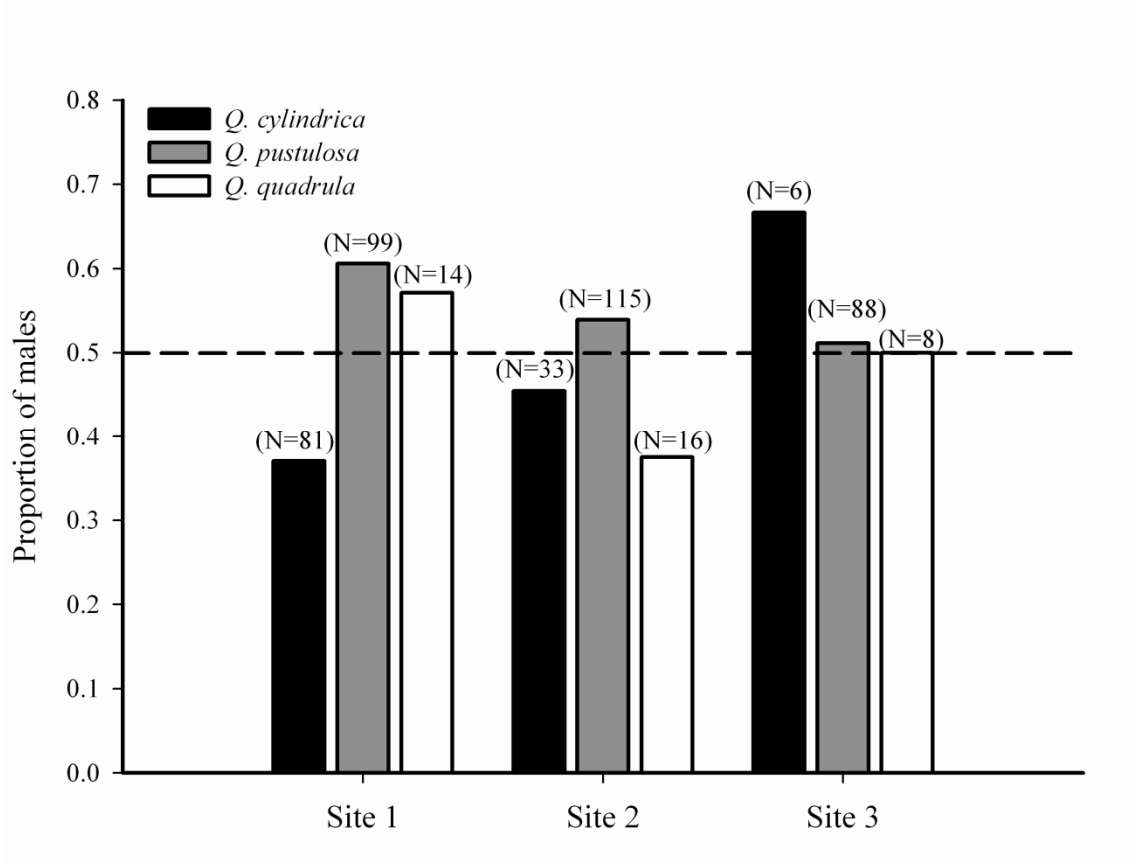
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Proportion of males
Q. cylindrica
Q. pustulosa
Q. quadrula
(N=81)
(N=99)
(N=14)
(N=33)
(N=115)
(N=16)
(N=6)
(N=88)
(N=8)
Site 1
Site 2
Site 3

Figure 4.

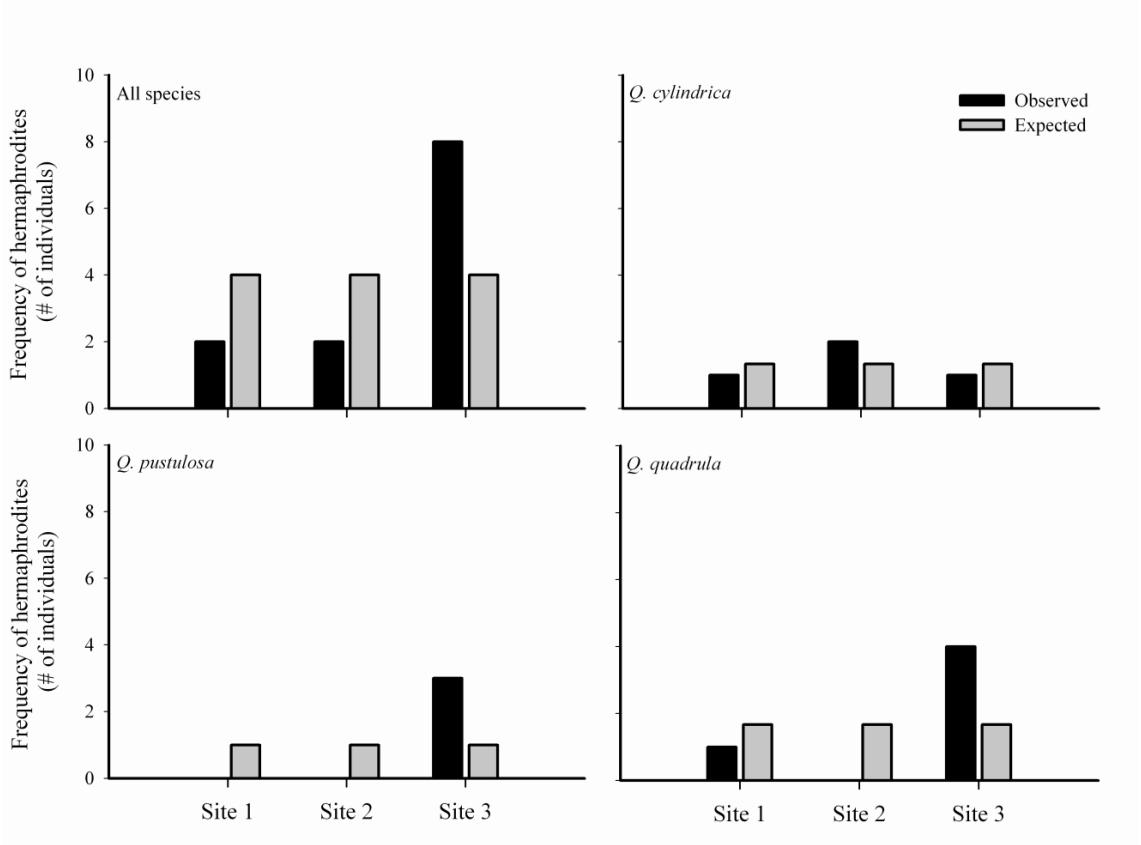
All species
Q. cylindrica
Observed
Expected
Q. pustulosa
Q. quadrula
Frequency of hermaphrodites (# of individuals)
Frequency of hermaphrodites (# of individuals)
0
2
4
6
8
10
Site 1
Site 2
Site 3
Site 1
Site 2
Site 3

Figure 5.

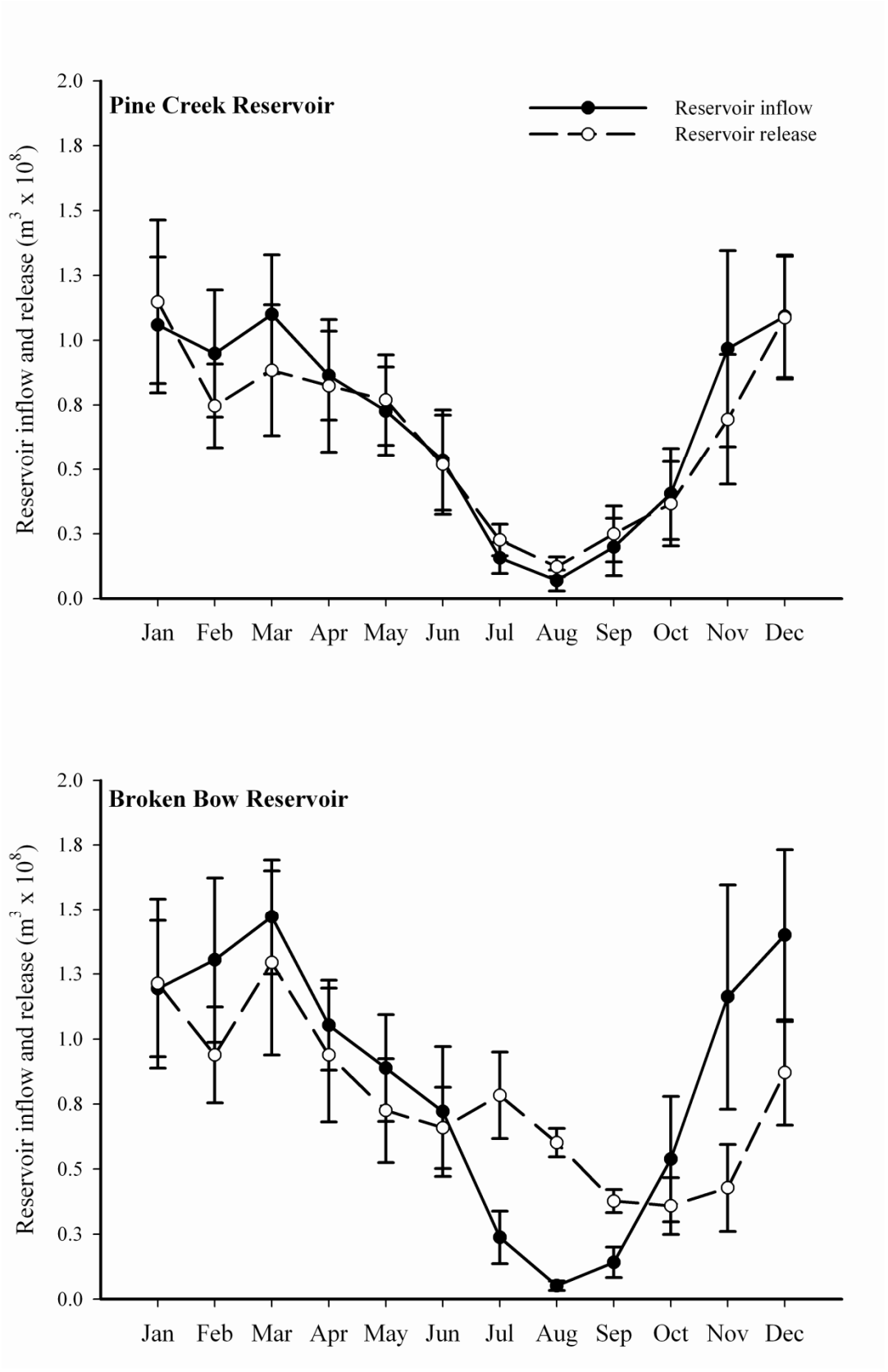
Pine Creek Reservoir
Reservoir inflow
Reservoir release
Reservoir inflow and release (m3 x 108)
2.0
1.8
1.5
1.3
1.0
0.8
0.5
0.3
0.0
Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec
Broken Bow Reservoir
Reservoir inflow and release (m3 x 108)
2.0
1.8
1.5
1.3
1.0
0.8
0.5
0.3
0.0
Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec

Figure 6.

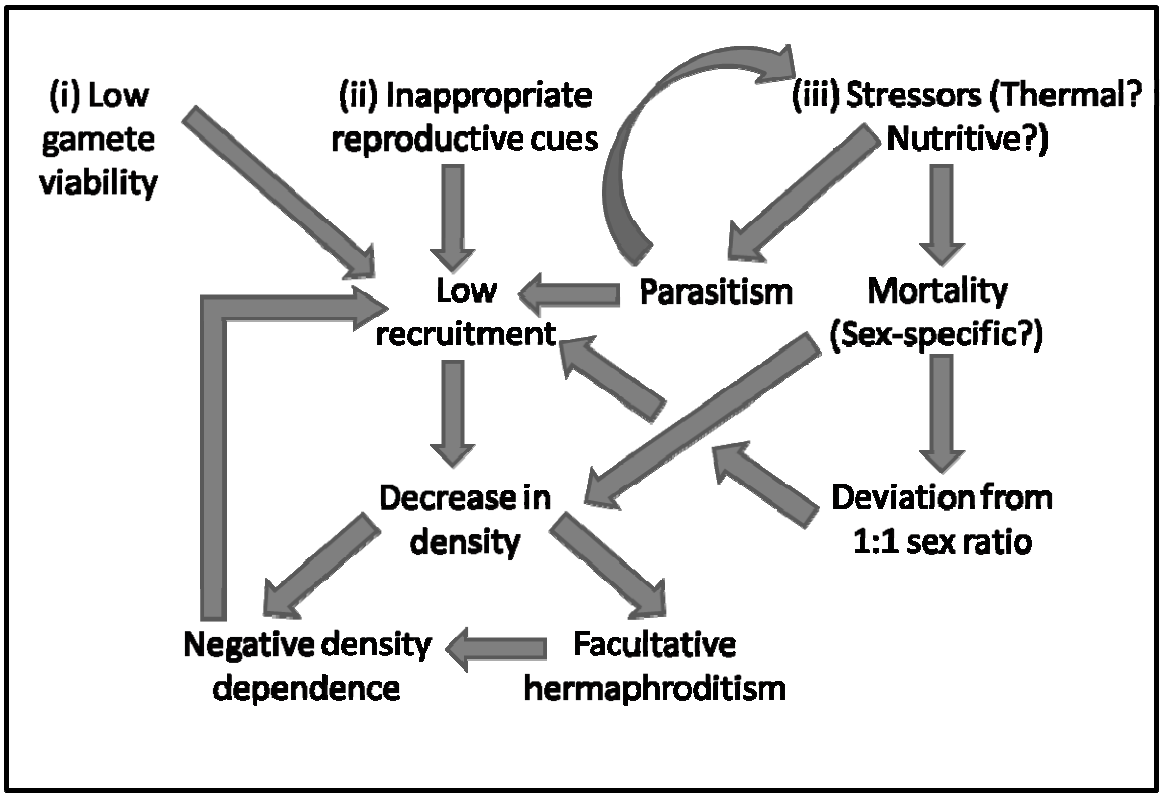
(i) Low gamete viability
(ii) Inappropriate reproductive cues
(iii) Stressors (Thermal? Nutritive?)
Low recruitment
Parasitism
Mortality (Sex-specific?)
Decrease in density
Deviation from 1:1 sex ratio
Negative density dependence
Facultative hermaphroditism

Figure 7.

Chapter 4. Temporal and spatial differences in gamete release in a spermcast spawning species: potential mechanisms of reproductive isolation in closely-related freshwater mussels

ABSTRACT

High species richness in broadcast spawning invertebrates is paradoxical in that speciation should be rare in organisms with high dispersal and gene flow. Freshwater mussels coexist in dense aggregations that can contain multiple, closely-related species. In addition, most species spawn at the same time of year, providing the opportunity for heterospecific fertilization. We examined the role of habitat separation and reproductive timing as potential mechanisms of reproductive isolation among several members of a single freshwater mussel genus, *Quadrula*. We found little overall overlap in habitat use among these closely-related species; however, this overlap varied depending on whether or not a mussel species was rare or abundant in the mussel bed. We also found high overlap in reproductive timing, suggesting that other barriers to reproduction could be at work to ensure conspecific fertilization. Further knowledge of isolating mechanisms should lead to a better understanding of the speciation process in spermcast spawning organisms.

Key words: Unionid, speciation, pre-zygotic, habitat, geographic information system (GIS)

INTRODUCTION

The great species richness of many broadcast spawning aquatic invertebrates remains paradoxical with respect to the speciation process. Most species whose life histories include releasing their gametes into the water column have high dispersal and thus high gene flow, which should slow genetic differentiation and mitigate speciation.

However, these populations often contain numerous, closely-related species, indicating that speciation might not be as rare as once thought (Palumbi, 1994; Rawson et al., 2003). Many of these species (e.g. corals) spawn simultaneously, further increasing the chances for heterospecific fertilization (Harrison et al., 1984; Levitan et al., 2004).

The process of speciation depends on barriers to reproduction developing among species. These barriers can include one or a combination of pre- and post-zygotic factors (Mayr, 1964; Futuyma, 1998). Several of the most common pre-zygotic mechanisms of reproductive isolation among broadcasters include spatial and temporal differences in gamete release, gamete incompatibility, and sperm chemotaxis (Palumbi, 1994) while post-zygotic mechanisms include decreased hybrid fitness (death, sterility)(Futuyma, 1998; Coyne and Orr, 2004). Understanding the order in which isolating mechanisms have evolved can shed light on the process of speciation and whether it occurred in allopatry or sympatry.

North American freshwater mussels present an evolutionary paradox similar to that found in broadcast spawning marine invertebrates. These mussels typically occur as dense, speciose aggregations and use a spermcasting reproductive strategy which is similar to that of broadcasting (Bishop and Pemberton, 2006). Reproduction occurs when male mussels release their sperm into the water column. Females passively collect the ejected sperm as they filter phytoplankton from the water. Fertilization occurs on the interior of the females' gills where larvae (glochidia) then begin to mature. Larvae are eventually released to complete their development as obligate ectoparasites on fish hosts (McMahon and Bogan, 2001; Barnhart et al., 2008). Most mussel species reproduce during a narrow time window over the summer (Yeager and Neves, 1986; Haggerty et al.,

1995; Garner et al., 1999; Haag and Staton, 2003). Mechanisms for ensuring fertilization of a conspecific (i.e. the sperm of one species correctly "finding" and fertilizing the egg of the same species) are virtually unstudied in freshwater mussels. As females passively filter phytoplankton and sperm suspended in the water column, the potential to filter the sperm of a closely-related species seems high. Indeed there is some evidence that hybridization can occur among closely-related mussel species (Kat, 1986; Cyr et al., 2007).

The process of speciation in general is poorly understood in freshwater mussels, partly because their life cycle is complicated by a parasitic stage. Thus, an understanding of freshwater mussel speciation relies on a speciation model for the host fish as well. To date, no studies have analyzed the overlapping phylogenies between mussels and their host fish. Unfortunately, traditional notions of speciation by geographic isolation do not satisfactorily explain mussel diversity for many species (Graf, 1997). Alternatively, Graf (1997) has presented a model of sympatric speciation via the formation of host races. While this model was not designed to universally explain speciation in all mussel species, it too has some problems. First, it assumes that speciation occurred via mussel species switching host fish that are present at different times of year; however, many closely-related mussel species share host fish species (Cummings and Watters; Parmalee and Bogan, 1998). Second, the model assumes that viable sperm cannot diffuse across large distances between populations. Galbraith (2009, Chapter 2) and Berg et al. (2008) have suggested, however, that sperm can travel large distances, at least in stream habitats. Similarly, the gametes of other spermcasting species have been shown to diffuse long distances while still achieving high fertilization success (Bishop and Pemberton, 2006).

How do we begin to understand speciation in freshwater mussels and other speciose groups that broadcast their gametes? A logical starting place is to examine mechanisms of reproductive isolation among closely-related species. This may reveal information about the order in which reproductive barriers arose and, in turn, how the speciation process proceeded. Here we examined spatial and temporal partitioning as potential mechanisms of reproductive isolation in several closely-related freshwater mussel species.

MATERIALS AND METHODS

Study Area and Species

Our study was conducted in the Little River in southeastern Oklahoma, U.S., in the Ouachita Mountains region of the Interior Highlands. This region is a center of speciation for both aquatic and terrestrial organisms including fish, crayfish, macroinvertebrates and mussels, and the Little River itself harbors 37 species of unionid mussels (Mayden, 1985; Moulton and Stewart, 1996; Galbraith et al., 2008). In this region, mussels occur in aggregations called mussel beds that can vary in size from several meters square to several thousand meters square (Vaughn and Spooner, 2006). For example, in the Little River, some beds contain as many as 30 species in a single bed and mussel biomass can reach as high as 25 kg/m^2 (Spooner and Vaughn, 2008). Our study focused on 3 mussel beds in a protected area (the U.S. Fish and Wildlife Service Little River National Wildlife Refuge) that we knew contained diverse, healthy mussel assemblages (Fig. 1) (Vaughn and Taylor, 1999; Spooner and Vaughn, 2009).

Quadrula is one of the most widespread and speciose genera of freshwater mussels in North America (Parmalee and Bogan, 1998). It contains over 20 recognized species in 3 species groups - *quadrula*, *metanevra*, and *pustulosa* (Serb et al., 2003). The Little River currently has or has historically contained multiple species of *Quadrula* including *Q. pustulosa*, *Q. cylindrica*, *Q. quadrula*, *Q. fragosa*, *Q. metanevra*, *Q. nodulata*, and *Q. apiculata*, along with species recently re-classified into this genus, *Tritogonia verrucosa* and *Fusconaia flava* (Serb et al., 2003). Our study focused on one species from each species group: *Q. pustulosa*, *Q. cylindrica* (*metanevra* group), and *Q. quadrula*. In addition, we included a fourth species, *Tritogonia verrucosa*, which was recently reclassified to the genus *Quadrula* (Serb et al. 2003) in the habitat overlap portion of our study. These 4 species co-occur in many rivers across North America, including the Little River (Parmalee and Bogan, 1998).

Habitat Overlap

We created continuous distribution maps for each species at the 3 sites, and used these to estimate habitat overlap. We quantitatively sampled mussels at each of our 3 sites in August, 2005. At each site, we evacuated 30, randomly placed, 0.25-m^2 quadrats to a depth of approximately 15 cm, removed, identified and measured mussels, and returned mussels to their original location (Vaughn et al., 1997; Strayer and Smith, 2003). We made a grid with 2 measuring tapes, one stretching along the length of the river and the other running perpendicular to flow, and assigned each quadrat a set of x and y coordinates. We plotted these coordinates in a geographic information system (GIS) and calculated the area of the mussel bed encompassed by our quadrats (referred to hereafter as mussel bed area). We also used GIS to create a continuous distribution map for each

species based on presence or absence of that species in a quadrat (Fig. 2). To do this, we used an inverse distance weighted function, which is a multivariate interpolation technique used to assign values to unknown points based on the values of nearby points (Walker et al., 2008). After creating the continuous distribution maps for each species alone, we then repeated the process for all 2-species combinations to produce maps predicting where both species would be found together. We calculated the area of the mussel bed over which there was an 80% or higher probability of the two species co-occurring and divided this by the area of the mussel bed. This allowed us to estimate the percentage of the mussel bed over which each species pair co-occurred.

Abundance of the 4 species varied among sites (Fig. 3). *Quadrula pustulosa* was abundant across sites whereas *Q. cylindrica* and *Q. quadrula* were relatively rare. These differences in abundance could result in differences in "perceived overlap" depending on whether a species is common or rare. For example, a rare species might always co-occur in quadrats with a common species, so from a rare species perspective overlap with the common species would be high. However, the common species would infrequently co-occur with the rare species, so from its perspective overlap would be low. To estimate this perceived overlap, we calculated the percent overlap of each 2-species combination based on (1) the distribution of the rarer species of the pair and (2) the distribution of the more abundant species of the pair. We did this by dividing the area of overlap of both species by the total area of the rarest species to quantify what we refer to as the "rare species percent overlap" and by dividing the area of overlap of both species by the total area of the most abundant species to quantify the "dominant species percent overlap."

The theory of limiting similarity predicts that similar species (in this case, closely related species) cannot coexist (i.e. should have low habitat overlap) (Abrams, 1983). Therefore, we might expect to see a constrained relationship between genetic divergence among species and habitat overlap. Species with low genetic divergence should have low habitat overlap and species with higher genetic divergence could have higher habitat overlap or alternatively, could have no overlap at all. This would result in a positive, triangularly-shaped constraint envelope between genetic distance and habitat overlap. To test this, we used GenBank mitochondrial ND1 sequences for the 4 species and calculated percent divergence (p-distances) with MEGA 4 (Tamura et al., 2007). We used correlation to examine directional associations between percent genetic divergence and percent habitat overlap for each site.

Timing of Reproduction

We determined timing of reproduction by quantifying sperm and egg development over time. We used timed searches to semi-quantitatively sample *Q. pustulosa, Q. cylindrica* and *Q. quadrula* at the 3 sites on a monthly basis from September 2005 through August 2006 (except during December, January, and March due to inclement weather and high water). For further details on our exact sampling techniques, refer to Galbraith (2009, Chapter 1). We graphically analyzed timing of peak reproduction for each species at each site by plotting sample date against either the log concentration of sperm or the proportion of eggs in the 80^{th} percentile based on size (a standardized estimate of reproductive state to account for differences in egg size among species). We assumed that declines following the peak in sperm concentration were due to spawning.

RESULTS

Habitat overlap between 2-species pairs was generally low, in most cases less than 10% (Table 1). An exception was *Q. pustulosa* and *T. verrucosa*, whose distribution overlapped 70% at Site 2 (Table 1). However, rare species perceived overlap with more abundant species was quite high, being 100% in many cases (Table 1). Dominant species perceived overlap was variable and depended upon particular site and species combination (Table 1). We found no significant correlations between genetic divergence and habitat overlap at any of our sampling sites (Fig. 4).

We found variable patterns of overlap in the spawning periods of all 3 species both within sites and riverwide (Figs. 5 and 6). In general, the concentration of mature sperm in the gonads peaked between mid-May and mid-June (Fig. 5) for all species. Female egg size peaked slightly earlier, with maximum size reached between mid-April and late-May (Fig. 6).

DISCUSSION

Our results suggest that differences in habitat use among closely related mussel species could serve as an isolating mechanism preventing heterospecific fertilization. The effectiveness of this mechanism likely depends on the overall abundance and thus the "perceived overlap" of the species in question. The common species *Q. pustulosa* and *T. verrucosa* overlapped very little with the rare species *Q. quadrula* and *Q. cylindrica*. In this scenario, this low overlap should prevent heterospecific fertilization between *Q. pustulosa* males and *Q. quadrula* or *Q. cylindrica* females. In contrast, from the perspective of the rare *Q. quadrula* and *Q. cylindrica*, their overlap with the more

dominant species is high. Thus, in this case there would be a high potential for heterospecific fertilization with the dominant species (*Q. pustulosa* and *T. verrucosa*) in the absence of other barriers or factors preventing fertilization.

Although there is the potential for habitat overlap to act as an isolating mechanism, other factors indicate that habitat overlap is not the predominant isolating mechanism. First, if habitat overlap is an isolating mechanism, we should see a positive constraint envelope between genetic divergence among species and habitat overlap. However, we found no significant relationships and no observable trends between habitat overlap and species' phylogenetic relationships (Fig. 4), suggesting that habitat differences are not (or at least are no longer) an isolating mechanism among closely-related species.

Several studies have demonstrated that reproductive isolation and genetic distance are correlated in nature (Coyne and Orr, 1989; Knowlton et al., 1993; Sasa et al., 1998). In other words, hybridization is unlikely to occur among species that are genetically divergent. This may explain why we observed no correlation between genetic distance and habitat overlap in our study: genetic divergence among these species is relatively high such that hybridization is no longer a concern and species can therefore coexist. Second, near-bed flow patterns in mussel beds are turbulent and complex (Vogel, 1994; O'Riordan et al., 1995; Commito and Rusignuolo, 2000) and are likely to carry mussel sperm in unpredictable patterns depending on ever-changing flow conditions. Unless habitat separation is great or perpendicular to flow, there might still be a high potential for currents to carry sperm between microhabitats and thus for heterospecific mating to occur (Galbraith, 2009, Chapter 2).

In a previous study (Galbraith, 2009, Chapter 1) we observed variable overlap in the timing of reproduction among species at our study sites, making it unclear if temporal isolation is acting as a barrier to reproduction among *Quadrula* species. We did find some differences in reproductive timing across sites that are likely due to differences in spawning cues among sites (Galbraith, 2009, Chapter 1). More importantly, however, we observed differences in peak reproduction among species within sites. If sperm is staying within a given mussel bed, then these differences in reproductive timing might be evidence of niche partitioning. However, data collected to date (Galbraith, 2009, Chapter 2) suggest that sperm can travel long distances downstream and may not be staying within a bed. Therefore, if male spawning in one species starts in May at an upstream bed, then reproduction has technically begun riverwide for that species (provided that that downstream females are receptive) despite slight differences in the timing of sperm release at downstream beds. Our data suggest then, that temporal reproductive niche partitioning is probably unlikely.

Given that the vast majority of mussel species reproduce in summer (Yeager and Neves, 1986; Haggerty et al., 1995; Garner et al., 1999; Haag and Staton, 2003) and that many mussel beds contain numerous species, the number of temporal "spawning niches" that could be carved out seems to be relatively small. Thus it is not completely surprising that reproductive timing is an unlikely barrier to reproduction among closely-related species. However, temporal isolation has been found between species of corals whose spawning periods are only separated by 2 hours, which provides just enough time for the gametes of one species to move out of reach of the gametes of the second species (Levitan et al., 2004). While this might be a unique situation, further investigation into

the timing of broadcast spawning on a much narrower time scale is warranted in freshwater mussels.

Closely related species that are near each other often spawn simultaneously and sometimes interbreed. This phenomenon has been documented in a variety of broadcast spawning marine invertebrate species including corals, sea cucumbers, and sea urchins (McEuen, 1988; Levitan, 2002; Levitan et al., 2004). For example, in mass spawning events in the Indowest Pacific, over 100 species of closely-related corals (genus *Acropora*) simultaneously release their gametes (Harrison et al., 1984). In many of these simultaneously reproducing species, it has been difficult to define clear genetic differences among species: hybridization is common in the laboratory and often in nature in areas of sympatry. Since closely related freshwater mussel species reproduce simultaneously, they also should have a high potential for hybridization. Unfortunately, little research has examined the role of hybridization in freshwater mussel communities and the extent to which closely-related species can hybridize is unknown (Cyr et al., 2007). What appear to be morphological "intermediates" are relatively common in mussels, but could well be a function of physical processes differentially acting on shell formation and not a function of hybridization (Watters, 1994). Further research into the prevalence of hybrid freshwater mussels is necessary to begin to fully understand the barriers to reproduction among closely related species.

Although there is some evidence that some *Quadrula* species may be able to hybridize (Serb et al., 2003), there is no genetic evidence for hybridization in the species we studied. This suggests that some form of reproductive barrier is preventing interbreeding. There are multiple potential pre-zygotic reproductive barriers that we did

not examine, including sperm chemotaxis, gamete incompatibility, and behavioral or mechanical incompatibility (Palumbi, 1994; Futuyma, 1998). Mechanical incompatibility is probably more common among terrestrial organisms and those with internal fertilization (Bierne et al., 2003); however, the roles of chemotaxis and gamete incompatibility are much more likely in freshwater mussels and other broadcast spawners. Laboratory experiments have revealed some degree of gamete incompatibility between closely-related species of corals and in the marine mussels *Mytlius edulis* and *M. trossulus*, suggesting strong barriers to interspecific fertilization (Rawson et al., 2003; Levitan et al., 2004). Additionally, Miller et al. (1994) showed that zebra and quagga mussel (*Dreissena polymorpha* and *D. bugensis* respectively), sperm exhibit species-specific attraction to oocyte extracts of the same species and less (although some) interspecific sperm attraction was always present between the two. Recent research has also focused on the evolution and selection of sperm and egg surface proteins in the gamete recognition process and as a pre-zygotic isolating mechanism in broadcasting organisms (Swanson and Vacquier, 1998; Vacquier, 1998; Riginos and McDonald, 2003). Further investigation into the roles of these prezygotic isolating mechanisms would be a logical next step for understanding reproductive barriers in *Quadrula* species, freshwater mussels, and broadcast spawners in general.

Postzyogtic isolating mechanisms may also play a role in limiting reproduction among heterospecifics. Decreases in hybrid fitness including fetal death, hybrid sterility and increased susceptibility of hybrids to pest, parasites and disease have been shown to act as selective agents against hybridization (Bert et al., 1993; Bert and Arnold, 1995; Futuyma, 1998). For example, Bert et al. (1993) showed that hybrids of species in the

broadcasting marine clam genus *Mercenaria* were significantly more prone to gonadal tumors than were purebred individuals. In addition, Bierne et al. (2002) demonstrated that *M. edulis*/*M. galloprovincialis* hybrids had decreased survivorship during the larval stage of development (although, interestingly, they exhibited higher growth rates than purebred larvae). Once we have a better understanding of the role of hybridization in freshwater mussel communities, further research into hybrid fitness could yield some interesting patterns and lend further insight into the speciation process.

This is some of the first work examining mechanisms of reproductive isolation in freshwater mussel species. As little is known about mussel speciation, further investigation into the barriers to reproduction among closely-related species could shed some light on the process of evolution in this unique and highly threatened taxon. Most work on reproductive isolation has been conducted in broadcast spawning organisms, not spermcasters (such as freshwater mussels, some marine bivalves, ascidians, sponges, and some corals). While many of the same isolating mechanisms likely apply to spermcasting species, the opportunity for female choice and selection on the part of females is much greater in spermcasting organisms than in broadcast spawners. Sperm storage, which is common in spermcasters (Bishop and Pemberton, 2006), may provide opportunity for female sperm choice which could either facilitate or hinder barriers to reproduction among closely related species and warrants further investigation.

A better understanding of reproductive isolation in broadcasters and spermcasters in general, however, could help to resolve the speciation paradox that exists in these organisms. Additionally, knowledge of the evolution of reproductive barriers over time could help elucidate whether speciation occurred in allopatry or sympatry in many of

these organisms. An alternative (but not necessarily mutually exclusive) explanation for this paradox is that broadcasting and spermcasting species simply have lower extinction rates rather than an unusually high rate of speciation. Further research is needed to address this question in general. Understanding the process by which such great diversity has arisen and been maintained in these organisms may be important for conserving their diversity in the future as human interference disrupts natural speciation processes in freshwater ecosystems (Hunter, 2006).

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Table 1. Estimated habitat overlap for pairwise species combinations at three sites in the Little River, Oklahoma. See text for definitions. Rare species from each pair is marked with an *.

Site	Bed area (m^2)	Species pairs	% Overlap	Corrected % Overlap (rare)	Corrected % Overlap (dominant)
Site 1	711	*Q. cylindrica** & *Q. pustulosa*	8.06	100.00	3.65
		Q. cylindrica & *Q. quadrula**	0.37	100.00	21.20
		*Q. cylindrica** & *T. verrucosa*	1.49	37.04	60.42
		Q. pustulosa & *Q. quadrula**	0.37	100.00	18.52
		Q. pustulosa & *T. verrucosa**	2.27	56.43	2.78
		*Q. quadrula** & *T. verrucosa*	0.37	100.00	3.34
Site 2	449	*Q. cylindrica** & *Q. pustulosa*	0.96	100.00	4.58
		Q. cylindrica & *Q. quadrula**	0.24	18.09	0.99
		*Q. cylindrica** & *T. verrucosa*	0.74	76.81	0.54
		Q. pustulosa & *Q. quadrula**	1.31	100.00	1.06
		Q. pustulosa & *T. verrucosa**	69.74	100.00	9.15
		*Q. quadrula** & *T. verrucosa*	0.96	73.40	71.68
Site 3	431	*Q. cylindrica** & *Q. pustulosa*	0.72	100.00	24.64
		*Q. cylindrica** & *T. verrucosa*	0.42	58.00	1.34
		Q. pustulosa & *T. verrucosa**	0.55	79.17	1.37

FIGURE LEGENDS

Figure 1. Three sampling sites in the Little River, southeastern Oklahoma, USA.

Figure 2. Example of a continuous probability distribution for a single mussel species (left) and for two overlapping species (right) generated by inverse distance weighting. Light regions represent areas of low species occurrence or overlap and dark areas represent a high probability of finding that species or species pair.

Figure 3. Mean (±SE) density (based on quadrat data) of each mussel species by site.

Figure 4. Correlation between genetic p-distance between species pairs and three measures of habitat overlap: total percent overlap, percent overlap of each 2-species combination based on the distribution of the rarest species of the pair (rare species % overlap) and percent overlap of each 2-species pair based on the distribution of the most abundant species of the pair (dominant species % overlap).

Figure 5. Mean (±SE) sperm concentration in the gonads over time for three species averaged across all three sites (a), and at Site 1 (b), Site 2 (c) and Site 3 (d).

Figure 6. Mean (± SE) proportion of eggs in the 80^{th} percentile based on size over time for three species averaged across all three sites (a) and at Site 1 (b), Site 2 (c) and Site 3 (d).

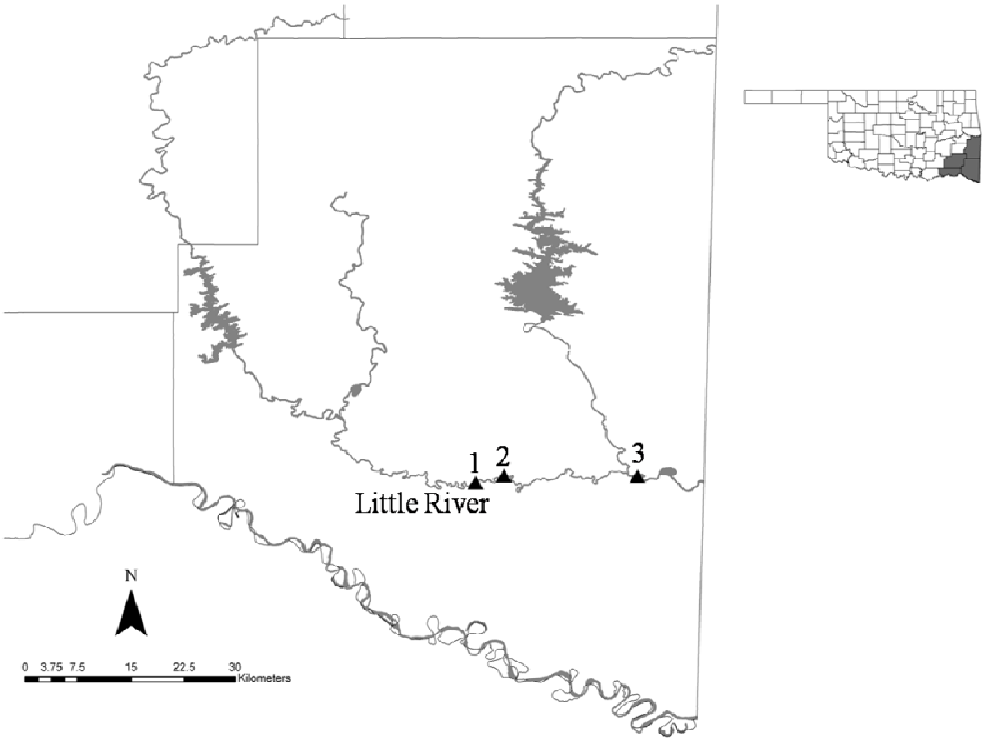

Figure 1.

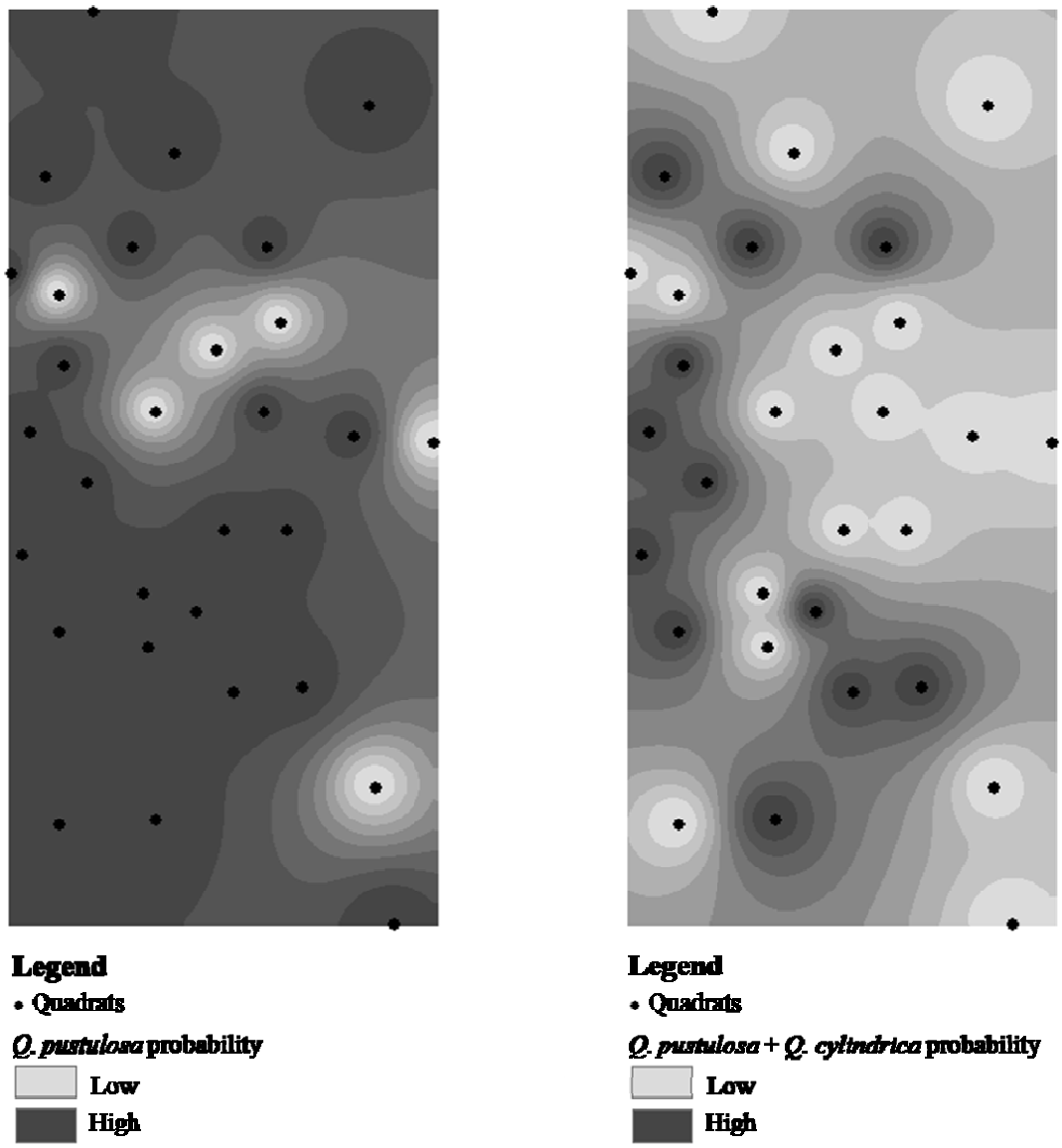
Legend
• Quadrats
Q. pustulosa probability
Low
High
Legend
• Quadrats
Q. pustulosa + Q. cylindrica probability
Low
High

Figure 2.

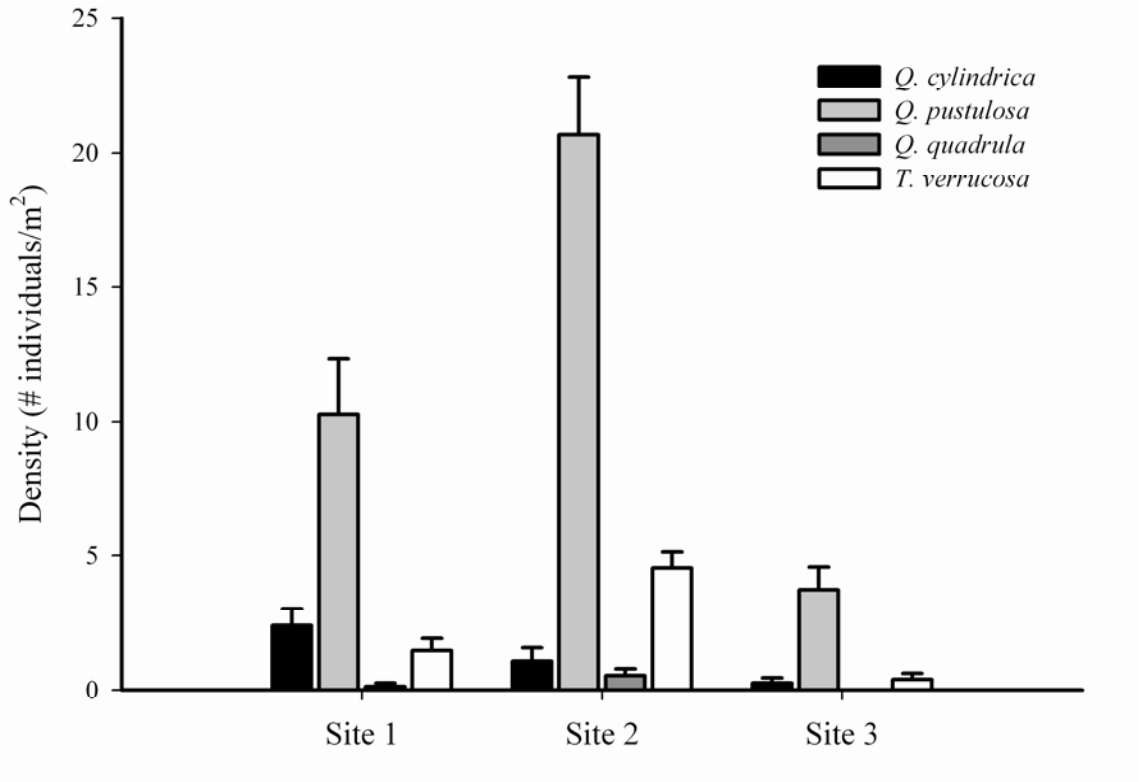
25
20
15
10
5
0
Density (# individuals/m^2)
Q. cylindrica
Q. pustulosa
Q. quadrula
T. verrucosa
Site 1
Site 2
Site 3

Figure 3.

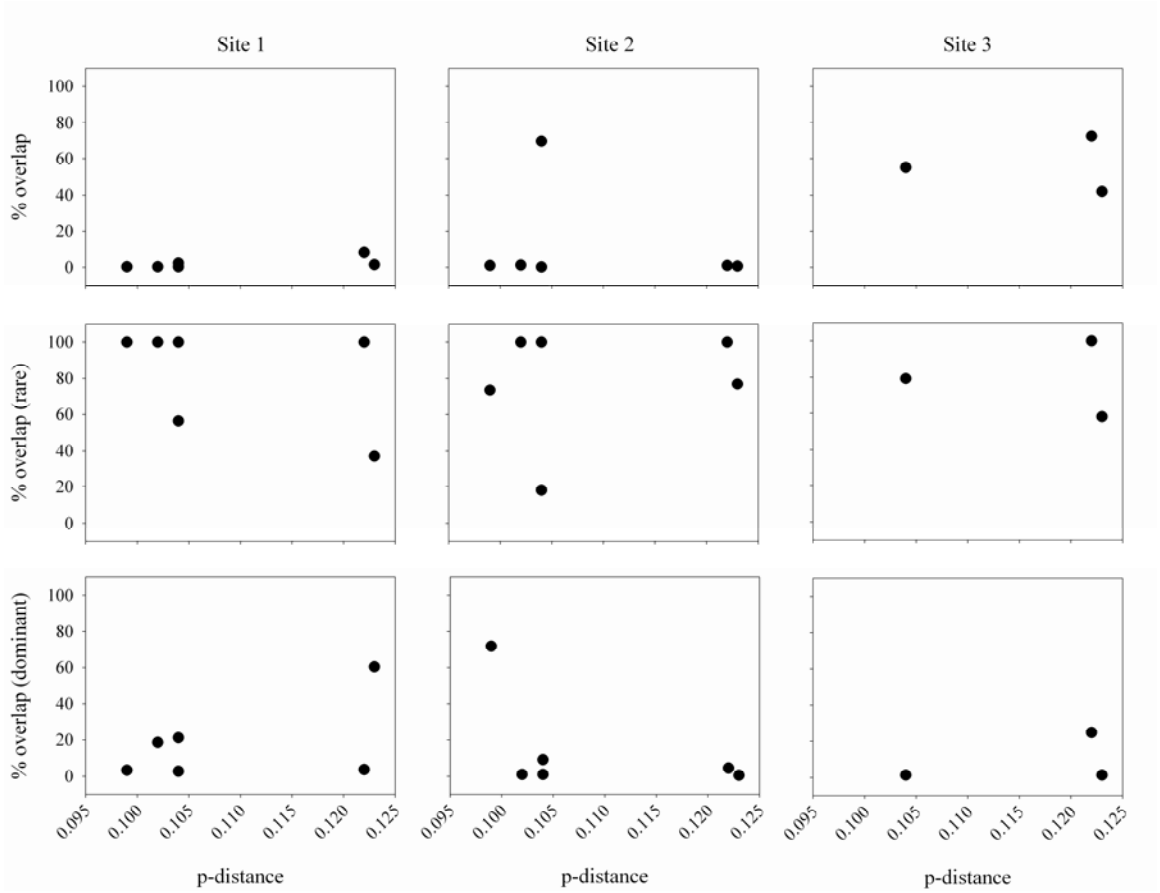
Site 1
Site 2
Site 3
% overlap
% overlap (rare)
% overlap (dominant)
100
80
60
40
20
0
0.095
0.100
0.105
0.110
0.115
0.120
0.125
p-distance

Figure 4.

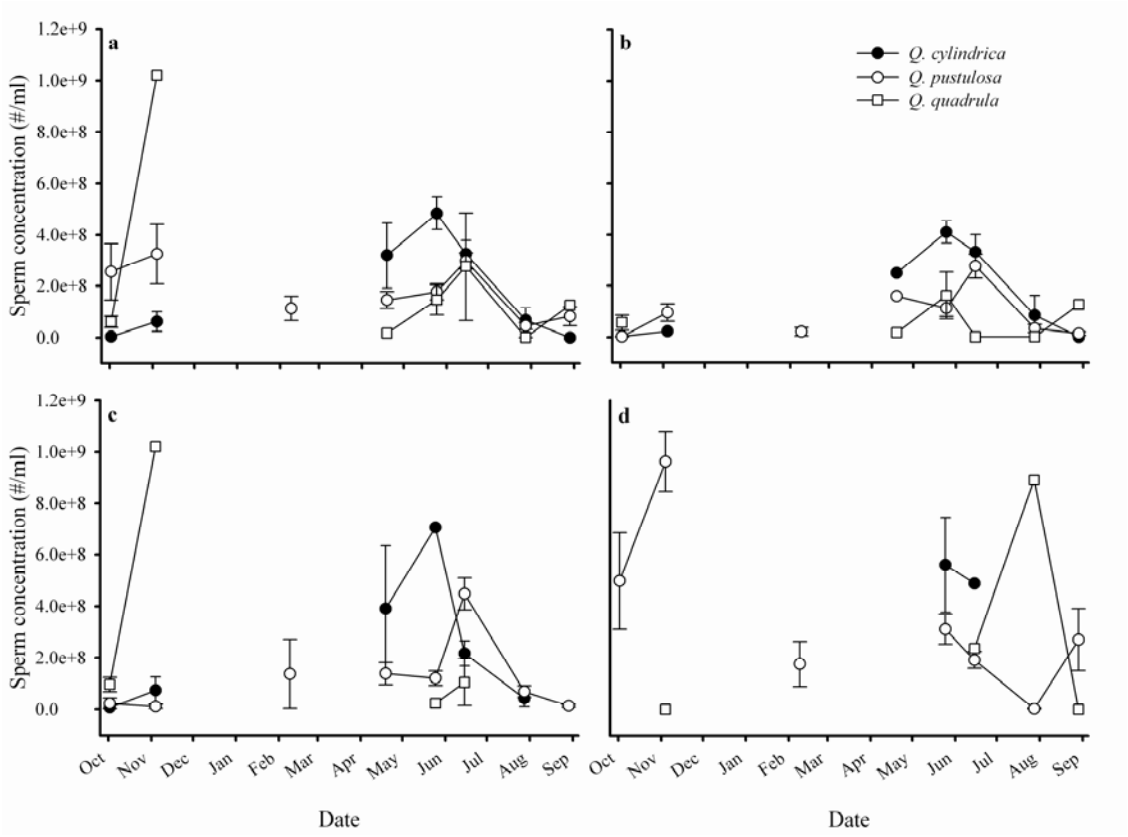
a
b
c
d
Q. cylindrica
Q. pustulosa
Q. quadrula
Sperm concentration (#/ml)
1.2e+9
1.0e+9
8.0e+8
6.0e+8
4.0e+8
2.0e+8
0.0
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Date

Figure 5.

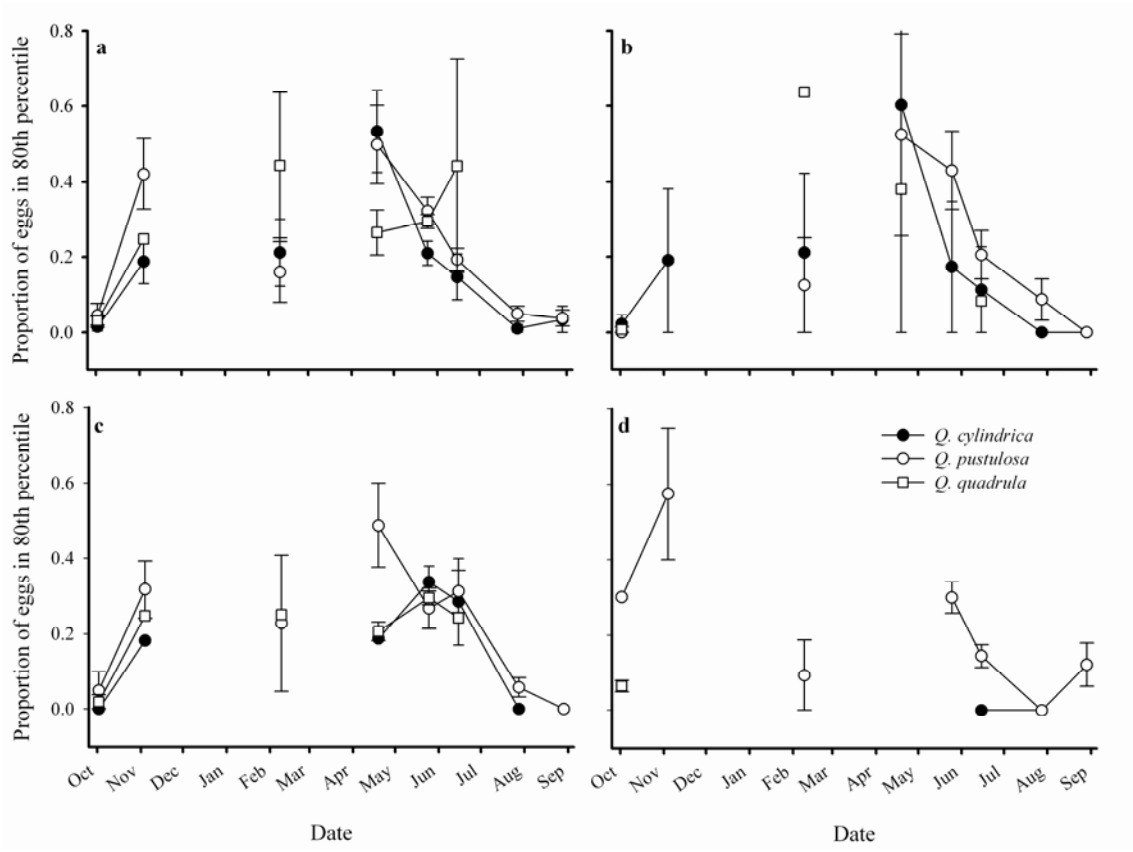
a
b
c
d
Proportion of eggs in 80th percentile
0.0
0.2
0.4
0.6
0.8
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Date
Q. cylindrica
Q. pustulosa
Q. quadrula

Figure 6.