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PRIMARY RESEARCH PAPER

Temperature and food interact to influence gamete development in freshwater mussels

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Abstract Freshwater mussels are one of the most threatened faunas in North America and worldwide, but little research has examined factors leading to successful reproduction (gamete development and fertilization success) in these species. We combined field and laboratory studies to determine the environmental factors influencing successful reproduction in three closely related species of freshwater mussels in a south central U.S. river. 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. Our data indicate that successful reproduction may be inhibited by altered temperature regimes found below impoundments.

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Introduction

Freshwater ecosystems and the species that inhabit them are being decimated globally (Allan & 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 & 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 & Vaughn, 2006; Vaughn et al., 2007; Vaughn et al., 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 & Hakenkamp, 2001; Vaughn et al., 2004; Howard & Cuffey, 2006; Vaughn et al., 2007). Additionally, live mussels and their spent shells provide habitat for other benthic organisms (Spooner & Vaughn, 2006; Vaughn & 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 & Bogan, 2001). Fertilization occurs on the interior of specialized brood chambers located in the females' gills where larvae (glochidia) begin their maturation (Richard et al., 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 & 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 & 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 et al., 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 & 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 & 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

This study was conducted in three mussel beds in the Little River in southeastern Oklahoma, USA (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 & 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 et al., 2008).

Since we were interested in effects of temperature on mussel reproduction, we chose sites that were 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 Lake Broken Bow 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 Lake Broken Bow 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 & 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 & 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*,

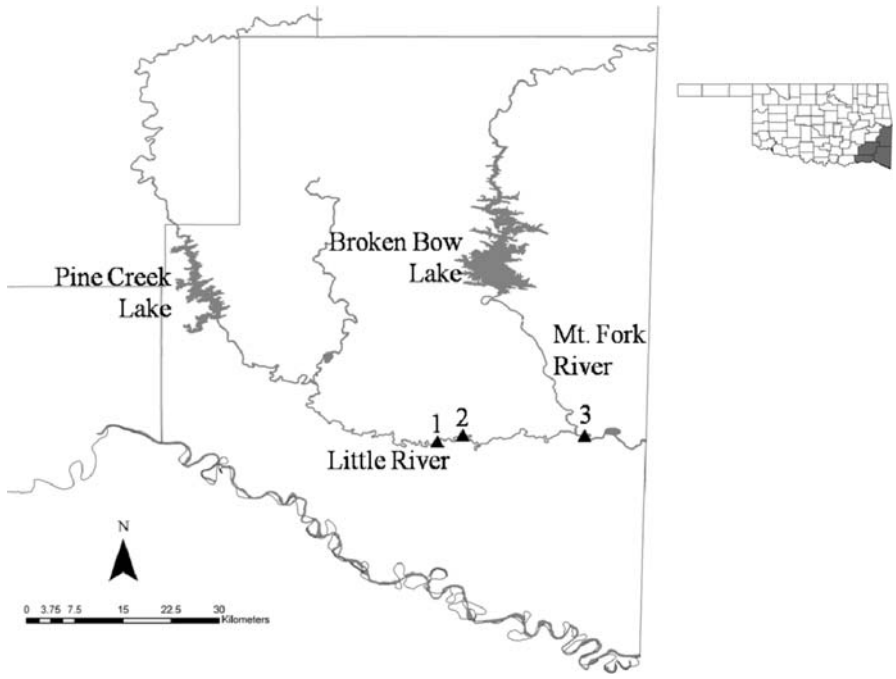

Fig. 1 Location of sampling sites (*filled triangle*) in the Little River in southeastern Oklahoma

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 might influence gamete development in *Q. pustulosa*, *Q. cylindrica*, and *Q. quadrula*. We sampled these three species on a monthly basis from September 2005 to August 2006. We did not collect samples during December, January, and March because high discharge precluded safely snorkeling or diving at our sites. During each monthly sampling trip, we collected, marked, weighed, and measured as many individuals of each species as we could find during an approximately 2-h timed snorkel search (Vaughn et al., 1997). We gently inserted a 20 gauge hypodermic needle into the junction of the foot and visceral mass slightly anterior to the midline of the mussel and approximately half-way through the thickness of the viscera. By drawing back on the syringe and carefully moving the tip of the needle in and out of the viscera, we were able to collect small ($\sim$50 µl) gonad samples from each mussel's visceral mass. It was evident when gonadal tissue was collected because of its milky white (or orange in *Q. cylindrica*) color; the few samples in which we accidentally collected gastrointestinal contents (evident by the bright green color) were not analyzed. We preserved the samples in 10% 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 compound microscope (400× magnification) and 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 using a compound microscope at 40× magnification (two estimates of both length and width) to quantify change in ovum size. To our knowledge, 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 & Layzer, 2008). Although further studies are necessary to compare this sampling technique with traditional histological sampling (particularly the variability in the two techniques), the consistency of

our data with the literature (see 'Discussion') 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 min with HOBO (Onset, Pocasset, MA, USA) loggers for use in our estimation of seasonal and diurnal temperature and photoperiod variation. 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 (Baskerville & Emin, 1969; UC IPM, 2008). To calculate degree days, we defined the limits of growth for all species to occur between 10 and 30°C based on metabolic rate data for *Q. pustulosa* (Spooner & Vaughn, 2008). We determined the maximum and minimum daily temperatures from the temperature logger data and used a single sine method to calculate the number of accumulated degree days since the start of our sampling.

There is evidence that some mussels species can feed on both suspended and non-suspended material (Nichols et al., 2005), although the extent to which mussels do both in the wild is still unknown. Therefore, we quantified both benthic and water column food availability at each site. 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°C for 72 h) and weighed to obtain dry weight estimates. We ashed samples in a muffle furnace at 550°C for 1 h 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 expected to see an increase in sperm concentration and mean ovum diameter in the gonads as reproduction progressed, followed by a decrease in both after either sperm were released or eggs had been shunted to the gills for fertilization. We used ANOVA with 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 analysis 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 gamete 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 pattern of wave-like periodicity (and thus 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 to test the influences of temperature regime and photoperiod on reproductive timing. During the spring of 2007, we collected mussels from the Little River and acclimated them to 5°C for 2 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 & Vaughn, 2009). Each mesocosm housed 14 *Q. pustulosa* and five *Q. cylindrica* individuals for a total mesocosm density of 44 individuals m^{-2}. Total mussel densities at our three field sites were 35, 98, and 16 individuals m^{-2} for sites 1, 2, and 3, respectively; densities in our experiment fell within this natural range. *Q. 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). To determine how temperature influences gamete release, we allowed mussels to first experience the same thermal regime, and then changed that thermal regime for a subset of treatments. We did this by maintaining all treatments at 5°C for 1 month, and then warming some treatments to 15°C for the second and third months. Treatments that were kept at 5°C for all 3 months are referred to as "cold" and those that started at 5°C and were warmed to 15°C are called "warm." Light treatments were 16 h light/8 h dark ("light") versus 8 h light/16 h dark ("dark"). The mean number of hours of daylight in the field ranged from a minimum of 9.8 (±0.18) hours in December at site 3 to a maximum of 14.6 (±0.09) hours in June at site 1. Therefore, we are confident that the light regimes in the experiment approximated light conditions in the field.

Since 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, and successfully used for feeding freshwater mussels (Randy Reed, Reed Mariculture, personal communication). Data from Spooner and Vaughn (2008) show that *Q. pustulosa* clearance rates triple from 5 to 15°C. Therefore, mussels in warm treatments were fed approximately thrice 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 2 weeks to minimize ammonia accumulation.

Table 1 Experimental design for stream mesocosm experiment

Treatment	*N*	Temp (°C)		Light (hours of light:dark)
		Month 1	Months 2–3	
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

Cold treatments were maintained at 5°C for 3 months while warm treatments were kept at 5°C for 1 month and increased to 15°C for the second and third months

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 (Table 2; Fig. 2). Site 1 received more hours of daylight to the benthos than the other sites, but had lower benthic organic matter in 3 out of 4 seasons than sites 2 and 3 (Table 2). Site 3 had lower water column chlorophyll *a* than site 2 but did not differ significantly from site 1 (Table 2).

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; Fig. 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

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*	Post hoc comparisons
Water temperature			
Site	11.261 (2,926)	<0.001	
Season	1721.676 (3,926)	<0.001	
Interaction	13.660 (6,926)	<0.001	F: Site 1 = Site 2 = Site 3
			W: Site 1 < Site 2[a], Site 2 = Site 3, Site 1 < Site 3
			SP: Site 1 = Site 2 = Site 3
			SU: Site 1 < Site 2[a], Site 2 > Site 3, Site 1 > Site 3
Light reaching benthos			
Site	2.918 (2,787)	0.055	Site 1 > Site 2, Site 2 = Site 3, Site 1 > Site 3
Season	43.86 (3,787)	<0.001	F > W, F = SP, F < SU, W = SP, W < SU, SP < SU
Interaction	0.965 (6,787)	0.448	N/A
Benthic production			
Site	15.160 (2,118)	<0.001	
Season	1.114 (3,118)	0.346	
Interaction	2.874 (6,118)	0.012	F: Site 1 < Site 2, Site 2 > Site 3, Site 1 < Site 3
			W: Site 1 = Site 2 = Site 3
			SP: Site 1 = Site 2, Site 2 < Site 3, Site 1 < Site 3
			SU: Site 1 = Site 2, Site 2 = Site 3, Site 1 < Site 3
Water column chlorophyll			
Site	5.818 (2,51)	<0.001	Site 1 = Site 2, Site 2 > Site 3, Site 1 = Site 3
Season	12.517 (3,51)	<0.001	F < W[a], F = SP, F > SU[a], W = SP, W > SU, SP > SU
Interaction	1.084 (6,51)	0.385	N/A

All post hoc comparisons are significant at $P \leq 0.05$ unless otherwise noted. *F* fall, *W* winter, *SP* spring, and *SU* summer

[a] Designates marginal significance of post hoc comparison ($P \leq 0.10$)

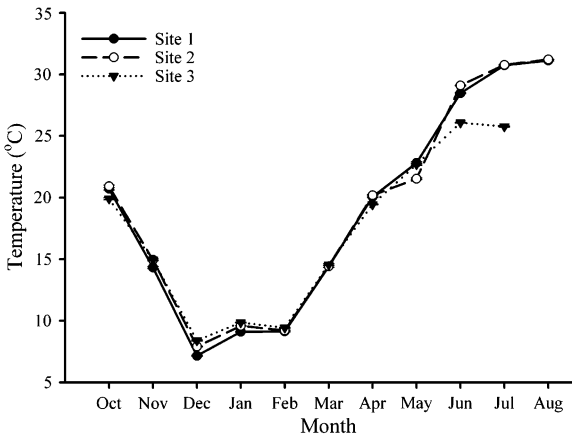

Fig. 2 Mean (±SE) monthly temperature during the 2005–2006 study period at three sampling sites in the Little River, Oklahoma. Data collected at 30 min intervals using continuous data loggers

Q. pustulosa (Fig. 3); however, site 2 mussels matched the pattern of reproductive timing seen river-wide (Fig. 3). While peaks in reproduction at site 3 were generally similar to those at sites 1 and 2 (Fig. 3), overall patterns of gametogenesis are difficult to describe given that this site had much lower mussel densities and variable sperm concentrations and because we were unable to sample this site in April due to high reservoir releases from Lake Broken Bow.

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 (Figs. 3, 4). River-wide, *Q. quadrula* appeared to reach peaks in ovum size approximately from 1 to 2

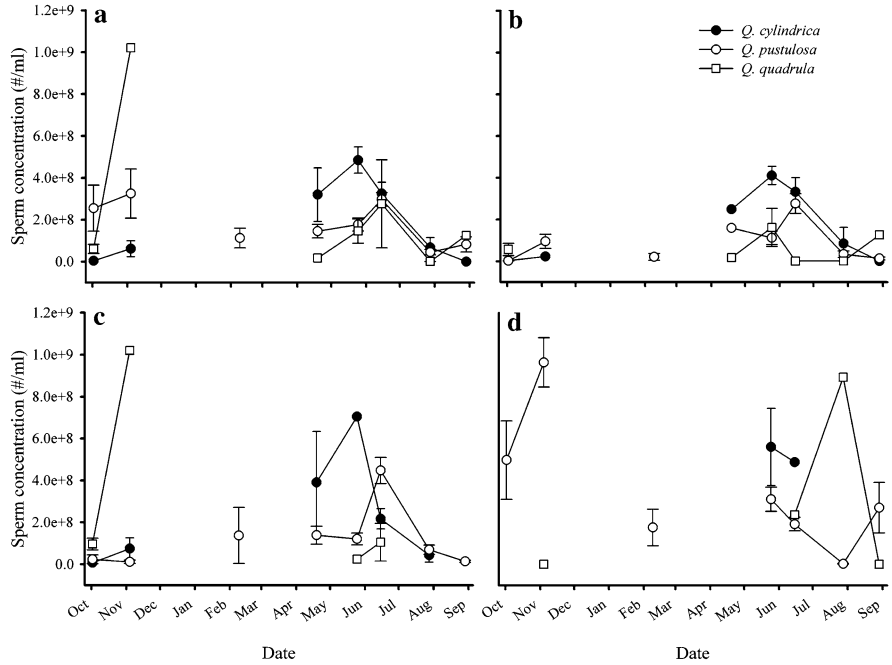

Fig. 3 Mean (± SE) sperm concentration over time for *Q. cylindrica*, *Q. pustulosa*, and *Q. quadrula* averaged across all three sites (**a**) and at site 1 (**b**), site 2 (**c**) and site 3 (**d**)

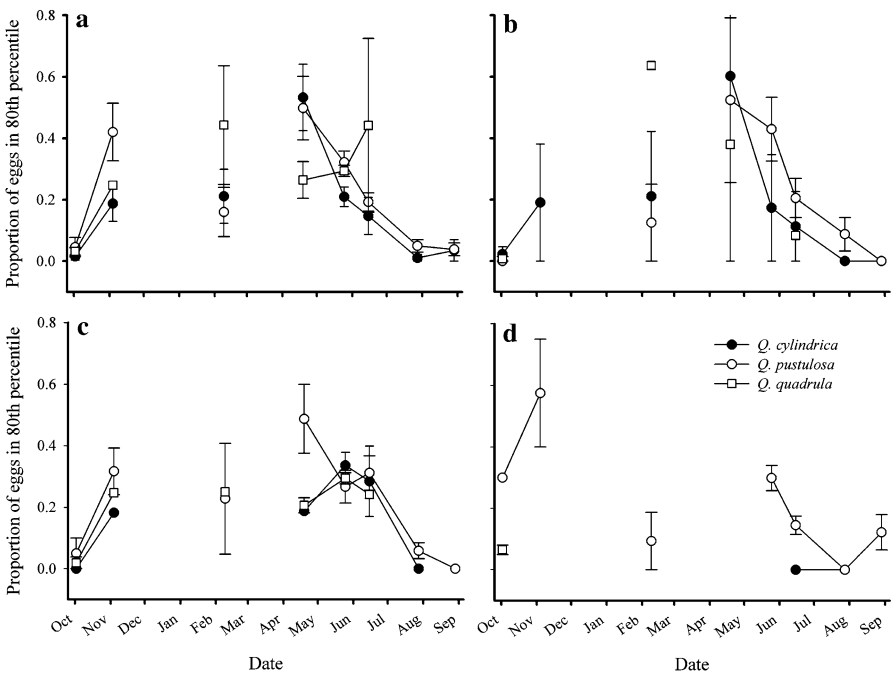

Fig. 4 Mean (±SE) proportion of eggs in the 80th percentile based on size over time for *Q. cylindrica*, *Q. pustulosa*, and *Q. quadrula* averaged across all three sites (**a**) and at site 1 (**b**), site 2 (**c**) and site 3 (**d**)

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 (Fig. 4), while at site 2 *Q. cylindrica* peaked slightly earlier than the other two species (Fig. 4). Peaks in egg size at site 3 were similar to those observed at sites 1 and 2 (Fig. 4), but it was again difficult to discern any seasonal patterns at site 3 (Fig. 4).

Multiple regression analysis revealed significant relationships between environmental variables and reproductive status for each species except for *Q. quadrula* males (Table 3). Reproductive status in

Table 3 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)	P	Explanatory variables
Males				
Q. cylindrica	0.613	23.14 (3,39)	$P < 0.001$	Degree days, degree days^2, degree days^3
Q. pustulosa	0.154	12.53 (2,125)	$P < 0.001$	Degree days^3, mean temperature
Q. quadrula			$P > 0.050$	No significant model
Females				
Q. cylindrica	0.191	13.99 (1,54)	$P < 0.001$	Mean temperature
Q. pustulosa	0.190	22.85 (1,92)	$P < 0.001$	Degree days^3
Q. quadrula	0.237	5.66 (1,14)	$P = 0.032$	Degree days

all three species was related to at least one degree day term (Table 3; Fig. 5). Reproduction in *Q. pustulosa* males and *Q. cylindrica* females also was dependent on mean monthly water temperature. 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 × 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 × temperature: $F_{(1,19)} = 1.27$, $P = 0.27$). 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. 6).

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 × 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 × 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. 6).

In males, gamete concentration in the gonads was higher in the laboratory experiment than during summer peaks observed in the field. *Q. 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. *Q. 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

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

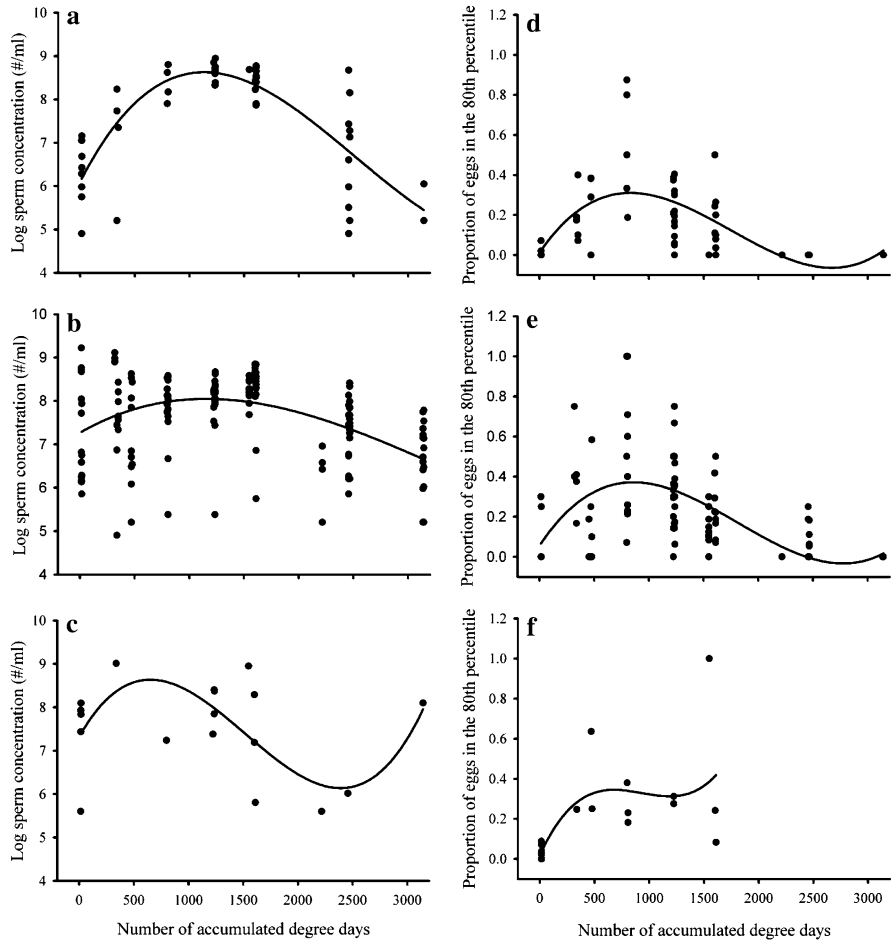

Fig. 5 Relationship between the number of accumulated degree days since the start of our field season and sperm concentration for *Q. cylindrica* (a), *Q. pustulosa* (b), and *Q. quadrula* (c) and the proportion of eggs in the 80th percentile based on size for *Q. cylindrica* (d), *Q. pustulosa* (e), and *Q. quadrula* (f)

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 & 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 (i.e., gametogenesis). 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 et al., 2005).

Similarly, we found temperature, and for male *Q. pustulosa* a marginally significant photoperiod by temperature interaction, to influence 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 because females were collected from the field during the peak of their reproductive cycle when mature eggs were 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 has been observed in mussels (Henley, 2002). Concurrent gonad and brood pouch sampling in future studies should help to tease apart these two possibilities. It was also unexpected that *Q. cylindrica* females had higher numbers of mature gametes under cold temperatures, especially because water temperatures rarely drop as

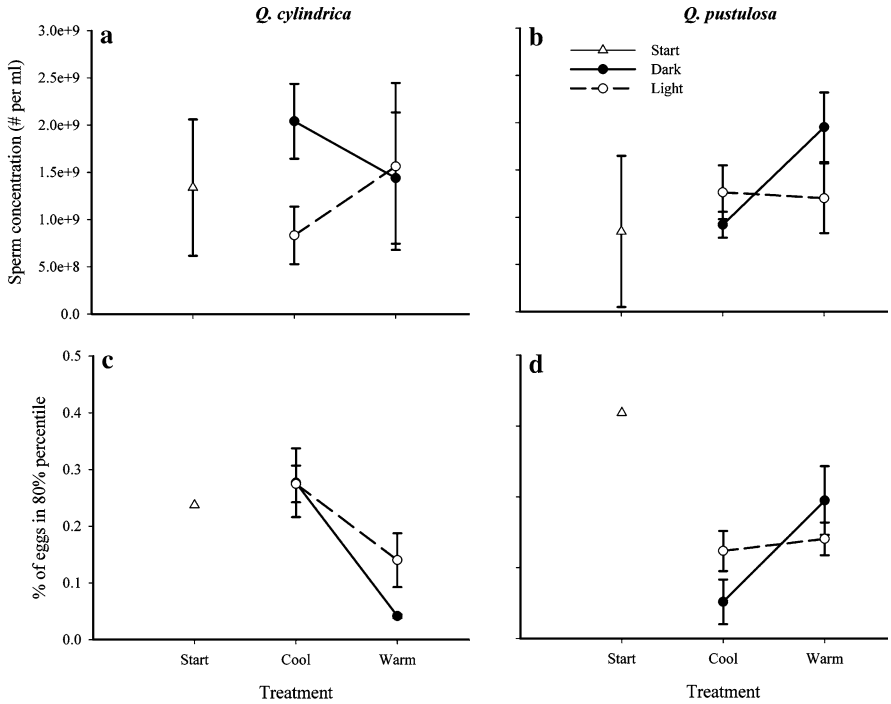

Fig. 6 Treatment means (± SE) for males (**a**, **b**) and females (**c**, **d**) of *Q. cylindrica* and *Q. pustulosa* averaged across time periods for the laboratory experiment

low as 5°C in southeastern Oklahoma. *Quadrula cylindrica* does reach its reproductive maturity slightly earlier than *Q. pustulosa,* thus it is possible that gametogenesis is triggered by cooler temperatures. However, a more likely explanation is that development of *Q. cylindrica* eggs was halted in a state of suspended maturation due to the decrease in metabolic rate associated with cold temperature. All of these questions require further study.

Both *Q. pustulosa* and *Q. cylindrica* had higher sperm concentrations in our laboratory experiments than we observed in the field. Because we did not want food to be a limiting factor in the experiment, mussels in the experiment were fed in excess and 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)). Thus, it is possible that effects of abundant, high quality food overpowered any effects of temperature or light in our experiment.

In contrast to our laboratory results, we did not find food to be a statistically significant factor in regulating timing of reproduction in the field. This could be because: (1) food has no effect on reproduction (2) we did not adequately measure food availability with the metrics used in this study, or (3) food does affect reproduction, but the variability among our sites was not strong enough to detect any food effects in our analyses. We cannot adequately tease these alternatives apart with our field data, but because we found that mussels fed to excess in the laboratory increased their sperm output, we think that alternative 3 is the most plausible, and that food is likely limiting in the field. Our field data hint at the importance of food availability to reproduction despite the non-significance of food in our analysis: site 1 had the lowest benthic organic matter of all three sites and correspondingly lower sperm concentrations in all three species (Fig. 3). 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. Further studies across a broader food availability gradient would be necessary to determine the true role that food availability plays (if any) in regulating reproductive output.

We found that all three species of *Quadrula* reproduce during summer months with peak gamete concentrations in May and June, indicating that

gametes are released during June, July, and August. Given the cold water temperatures in December and January, the low sperm concentrations and egg size observed in February, and the extremely low metabolic rate of mussels at these cold water temperatures (Spooner & Vaughn, 2008), we find it highly unlikely that mussels were reproductively active in the winter months when we were unable to collect gonad samples. These patterns in reproductive timing are consistent with that reported for other members of the genus *Quadrula* and other mussel species in general (Yeager & Neves, 1986; Haggerty et al., 1995; Garner et al., 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 & Bogan, 2001). Both are potential factors that need to be further examined in the context of understanding female mussel receptivity.

Reproductive success in mussels can be disrupted by cold temperatures and unnatural thermal regimes (Layzer et al., 1993; Heinricher & 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. In our study, the thermal regime at site 3 was different than at the other two sites, with warmer winter and cooler summer temperatures (Fig. 2; Table 2). This site receives substantially colder water during summer months because of hypolimnetic releases from Lake Broken Bow to generate electricity and to maintain a trout hatchery downstream of the dam. It also has lower water column chlorophyll *a,* thus presumably lower food availability, than the other two sites. While peaks in reproduction were similar among all sites (Figs. 3 and 4), general patterns of reproduction were highly variable among all species at site 3, and could be a function of the different temperature and food patterns we observed here. Since 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 are simply due to the fact that not enough individuals could be sampled to observe a clear pattern. Thus, these data should still be considered preliminary.

The large variation in our data is most likely a function of our sampling technique. To our knowledge, syringe biopsies have only been used qualitatively (i.e., to sex non-dimorphic species), not quantitatively (Shiver, 2002; Saha & Layzer, 2008). Histological thin sectioning is the most common means of quantitatively addressing questions of reproductive timing in mussels (Downing et al., 1993; Haggerty et al., 1995; Garner et al., 1999; Heinricher & Layzer, 1999) and is probably the most accurate method. Unfortunately, histological sampling is not a workable option for many rare mussel species where sacrificing even a few individuals can impact populations. Histological sampling is also quite time consuming. An important take home message from this study is that we can use non-lethal gonad pulls to estimate reproductive patterns, even though the data may be slightly more variable than histology. This has important implications for understanding the reproductive biology of threatened and endangered species.

Densities of freshwater mussel species vary across North America and likely depend on biogeography, habitat suitability for survival and reproduction, and both natural and human caused rates of extirpation (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. While this study did not examine all potential environmental factors that could regulate timing of reproduction (including stream flow, food quality, chemical cues, etc.), we found that temperature is an important environmental cue. 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, this is the first study to use a combined field and laboratory approach to determine factors that trigger gametogenesis in unionid mussels.

Conclusions

We observed peaks in reproductive timing in three species of *Quadrula* between May and June. Our combined field and laboratory data suggest that reproductive timing in these three species of mussels is predominantly regulated by water temperature. Although preliminary, and in need of further study, our data also suggest that altered thermal regimes below a hypolimnetic release impoundment could be responsible for high variability in reproductive timing. 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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