Stage-specific responses of freshwater mussels to temperature and suspended solids

by

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Abstract

Elevated levels of suspended solids (TSS) are one of the most widespread water quality problems in streams, rivers, and lakes throughout North America, yet little is known about their effect on populations of freshwater mussels. Freshwater mussels are the most imperiled fauna in the United States and understanding the influences of TSS on their life history will be critical for implementing appropriate conservation action. The overarching objective of my dissertation is to experimentally assess the effect of either temperature or TSS or both on multiple stages of freshwater mussels' life cycle, including filtration, fertilization, brooding of glochidia (larvae), and mantle lure display. For these experiments, I manipulated pond TSS concentrations at South Auburn Fisheries Research Station, Auburn, AL through the addition of aqueous fertilizer (organic solids) or bioturbating carp (inorganic solids). In-pond filtration trials at warm temperatures, with two lentic species, two lotic species, and one habitat generalist, demonstrated a linear increase for the lentic and generalist species in particle removal with increasing suspended solids up to 50 mg/L, but no relationship for lotic species. However, lotic species particle removal was constrained at high temperatures. Trials during the spawning season showed that <30% of females per pond developed glochidia when suspended solids levels were >20 mg/L. This pattern was consistent across two species, a short- and a long-term brooder, in ponds dominated by inorganic solids and for the long-term brooder in ponds dominated by both organic and inorganic solids. In-marsupial glochidia, showed no difference in viability or metamorphosis rates between low and high TSS ponds. Finally, laboratory experiments with

flow-through pond water demonstrated the role of temperatures in regulating mantle lure display and a switch to conglutinate release as a secondary strategy for *Ligumia subrostrata*. Overall, my results show that TSS appears to have the greatest negative effect on early reproduction, which includes both fertilization and glochidia development. Determining a critical suspended solids threshold (~20 mg/L) where successful reproduction is limited aids in understanding mechanisms responsible for the decline of many mussel species in disturbed watersheds and can guide future restoration and conservation activities.

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Introduction

High levels of suspended solids are a pervasive problem in stream, rivers, and lakes throughout the world (Wood and Armitage 1997). Elevated suspended solids negatively influence ecosystem processes and aquatic biota (Waters 1995). Decreasing light penetration, caused by increased suspended solids, can shift algae and aquatic plant assemblages and also lead to changes in species interactions (Sweka and Hartman 2001, Utne-Palm 2002). Additionally, suspended sediments have been shown to disrupt fish spawning by reducing contact between sperm and egg (Galbraith et al. 2006) or through sedimentation which leads to changes in substrate conditions (Walters et al. 2003). Anoxic conditions created by sedimentation, alters biogeochemical processes, and can foster increases in interstitial un-ionized ammonia (Strayer and Malcom 2012), which can be toxic to benthic dwelling organisms (Augspurger et al. 2003).

Suspended solids can be partitioned into two main components, organic and inorganic solids. High levels of organic solids, or eutrophication, are often the result of excessive nutrient introduction into the ecosystem (Carpenter et al. 1998) and can affect both lotic (Dodds et al. 1998) and lentic ecosystems (Carpenter et al. 2001). Typically, increased nutrient levels lead to changes in algae communities, often favoring cyanobacteria which can have many toxic (Codd 1995) and unpalatable forms (Demott and Moxter 1991). These changes in algae communities often alter food web dynamics (Paerl and Tucker 1995). Furthermore, increases in nutrients, and

the resultant increase in primary production, promote hypoxic or anoxic conditions when primary producers die and decay (Paerl et al. 1998).

High levels of inorganic suspended solids in streams and rivers are typically the result of poor land use practices including agriculture, forestry, and urbanization (Wood and Armitage 1997). Input of inorganic suspended solids usually occurs during precipitation events with highest loads transported from disturbed watersheds (Gurtz et al. 1980, Houser et al. 2006). The effect of suspended sediments on aquatic ecosystems has been well studied; but nonetheless there are many gaps in our knowledge of how suspended solids influence freshwater mussel life history (Brim Box and Mossa 1999).

Freshwater mussels are experiencing unprecedented declines in species number and population abundance (Lydeard et al. 2004, Strayer et al. 2004). The southeastern United States is the global epicenter for freshwater mussel biodiversity (Graf and Cummings 2007), but this region is also plagued with high levels of extinction and endangerment (Williams et al. 1993). For example, in the state of Alabama of the approximately 175 species, 16% are extinct, 29% are endangered, 11% are threatened, 21% are of special concern, and only 22% of the species are considered to have stable populations (Benz and Collins 2004). There are many commonly cited causes of decline for freshwater mussel populations including, impoundments (Layzer et al. 1993, Vaughn and Taylor 1999), loss of host fish (Kelner and Sietman 2000), introduction of exotic species (Haag et al. 1993), exploitation (Claassen 1994), and changing climate (Haag and Warren 2008, Gough et al. 2012), but the most commonly cited problem is water quality (Downing et al. 2010). However, specific mechanisms of how water quality and related suspended solids impact mussel life history are not well known.

One reason for the decline of freshwater mussels is likely because of their complex life cycle, which has many opportunities for disruption. Adult mussels live in the benthos of streams and rivers and are relatively sedentary (McMahon and Bogan 2001). Mussels use a network of gill cilia and labial palps to filter food particles from the water column (Nichols et al. 2005). Gills are also responsible for respiration and brooding glochidia (larval mussels). Freshwater mussels reproduce by sperm casting (Mosley 2012); males release sperm into the water column and eggs are fertilized after females passively capture sperm while filtering water. Males release spermatozuegmata, which are packets containing between 1500 and 7000 individual sperm (Waller and Lasee 1997, Ishibashi et al. 2000). This adaptation is thought to assist in long distance transport of sperm, especially for mussels in low density populations (Ferguson et al. 2013). After fertilization occurs embryos develop and glochidia are brooded in the marsupial gills (Richard et al. 1991). Two brooding strategies exist for freshwater mussels, short-term and long-term (Watters and O'Dee 2000). Short-term brooding species typically spawn in the spring and early summer and hold mature glochidia several days or several weeks depending on the species (Haag and Warren 1997). In contrast, long-term brooding species typically brood in the fall and hold their glochidia overwinter until initiating transfer to hosts the following spring (Williams et al. 2008).

One of the most charismatic life history stages for freshwater mussels is their parasitic relationship with a vertebrate host, which are almost exclusively fish (Barnhart et al. 2008). Mussels have evolved many unique strategies for transferring glochidia to host fish, including (but not limited to) mantle lure display, conglutinate release (both pelagic and demersal), or free glochidia release (Haag 2012). Female mussels perform lure display using the edge of their mantle, which has been modified and elaborated to mimic prey items (Haag et al. 1995). When

fish attracted to the mantle lure strike the mussel and rupture the marsupial gill, glochidia are released on their head and gills. Conglutinates are packets of glochidia which often take the shape of fishes prey items, such as fish eggs, insects pupae, or worms (Haag and Warren 2003), which rupture in the fishes mouth and spread to the gills after consumption by the fish. Finally, species of mussels releasing free glochidia into the water column rely on random encounters with host fish, and thus these species are typically host generalist. Across all strategies, the primary advantage to mussels of this parasitic relationship is dispersal provided by host fish (Barnhart et al. 2008).

After two to four weeks attached to the fish, often depending on the water temperature (Roberts and Barnhart 1999), glochidia metamorphose into juveniles. The juvenile stage of freshwater mussel life history is the most enigmatic. During this stage mussels are thought to burrow and remain in the substrates for several months or years. This is a difficult stage to study and may present a critical bottleneck in the life history of mussels. I did not examine the juvenile stage for my dissertation, but focused on the role of suspended solids on mussel particle removal, growth, reproduction, and glochidial brooding and the affect of temperature on particle removal and mantle lure display.

Filter feeder organisms typically remove an increasing number of particles as concentration of particles increases, up to a threshold at which particle removal asymptotes (Winter 1978). Particle removal likely ceases because ingestion rates are maximized (Hornbach et al. 1984) or gills become clogged due to excessive particles (Kryger and Riisgard 1988). If gills become clogged it is expected that filtration rate will decline at relatively high particle concentrations. *Anadonta anatina* linearly increased ingestion (mg C/g/h) of particles up to 10 mg C/L (Bontes et al. 2007) and *Elliptio complanata* increased filtration rate (mg C/kg/h) up to

32 mg C/L (Eversole et al. 2008). These relatively low particle concentrations did not show a decline in removal and show a similar trend across species and food types. At extremely high inorganic solids loads (450-600 mg/L), clearance rate of three lotic species sharply declines, and may limit the overall food consumption of these animals (Aldridge et al. 1987). However, the change in total number of particles removed in this study is unclear and would have important implications for energetic balance and ecosystem services provided.

Temperature also plays a critical role in regulating filtration ability of freshwater mussel, with different species of mussel having different thermal optima A recent study showed that for three of eight species of freshwater mussels, clearance rate declined or stayed the same when temperatures increased to 35˚C, but the remaining five species continued to have increases in clearance rate up to 35˚C (Spooner and Vaughn 2008). Additionally, two lotic species demonstrated differences in clearance rate due to seasonal temperature patterns (Baker and Hornbach 2001). Both studies were completed with relative constant levels particle concentration; however, species thermal tolerance will likely interact with their ability to remove particles at high suspended solids loads.

Particle selectivity is important for many filter feeding organisms and important differences may exist for species specialized to specific habitat types. Lotic species of freshwater mussels have higher clearance rates on monocultures of bacteria than lentic species (Silverman et al. 1997). This difference in particle size selective was related to density and spacing of gill cilia on the cirril plate. In contrast, three species of freshwater from diversity of habitat preferences all preferred a single-celled Microcystis sp. (Baker and Levinton 2003). Both of these studies were conducted the laboratory and it is not known if these differences translate to particle size selection in the field with a diverse seston assemblage.

Since female freshwater mussels capture sperm from the water column using their gills it seems likely that the reproductive process would be sensitive to increases in suspended solids. Freshwater mussel fertilization has rarely been studied, even outside the context of suspended sediments. A different stage of the reproductive process that has been negatively affected by suspended solids is the attachment and metamorphosis of glochidia on host fish gills (Beussink 2007). At TSS levels of 1250 – 5000 mg/L, glochidia showed reduced attachment and metamorphosis largely due to irritation of the fish gills. Furthermore, decreased female mussel condition has been linked to decreased reproductive success (Bauer 1998). Increased suspended solid levels could positively affect female mussel condition if additional particles are food items, but may be detrimental because increased inorganic particles may drive a negative energetic balance due to the cost increased sorting requirement (Madon et al. 1998). However, no studies have assessed the potential interruption of fertilization and glochidia development due to high suspended solids levels.

The effect of elevated suspended solids levels on brooding glochidia have not been explored for freshwater mussels. A recent study demonstrated that glochidia brooded for 32 days in a solution with a common pharmaceutical (fluoxetine) showed no different in glochidia viability after immediate removal from the gill (Hazelton et al. 2013). These results indicate that in-marsupial glochidia may be buffered against suspended solid levels; however, elevated concentrations of suspended solids may limit maternal growth and respiration (Madon et al. 1998) and subsequently reduce glochidial health.

Little is known about the factors controlling mantle lure display in freshwater mussel. High levels of a common pharmaceutical, fluoxetine, has been shown to increase mantle lure display (Bringolf et al. 2010). Temperature is an important factor controlling reproductive

activity in a wide range of organisms, and thus we expect that temperature plays an important role in regulating the timing and duration of mantle lure display.

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Chapter 2

Filtration redundancy in a diverse assemblage of freshwater mussels

Freshwater mussels are a highly diverse group of primary consumers that have generally been regarded as functionally redundant, but it is increasingly recognized that some species are functionally unique or dominant. Species loss in the current freshwater mussel extinction crisis may have important implications for ecosystem services. Our aim was to explore biodiversity effects on ecosystem services by examining water filtration abilities of five species of mussels, three adapted to lotic and two adapted to lentic habitats, and mixed species assemblages, under varying contexts of total suspended solids (TSS) load, particle size selectivity, and temperature. Experimental ponds (0.1 ha) were fertilized at different rates to create a gradient in TSS and trials were conducted across seasons to capture a range of temperatures (12-32˚C). In-pond filtration trials were conducted at 5 temperatures (11.9, 16.3, 19.8, 30, 31.6˚C) and across a TSS gradient (2.4 – 48 TSS, N = 9). Trials were assessed using particle removal rates (mg/g dry mass/h). *Quadrula asperata*, *Pyganodon grandis*, *Ligumia subrostrata*, and the mixed assemblage all demonstrated a linear increase in filtration rate with increasing particle concentration at warm temperatures (>29˚C), with *Q. asperata* having significantly higher filtration rates than all other species, except *L. subrostrata*. Particle removal rates for lotic species, *Plectomerus dombeyanus* and *Reginaia ebenus* had no significant relationship and were also the only two species that had a significant unimodal temperature, indicating physiological

constraint at relatively high temperatures. *Quadrula asperata* is considered a large river habitat generalist, which may explain its unique functional trait. A high degree of redundancy was observed for particle size preference and all species demonstrated flexibility by preferring different sized particles in different filtration trials. Seston assemblages in many aquatic ecosystems are temporally variable and the redundancy observed for all species may be a response to changing environmental conditions. Overall, we found that species identity was important with regards to response to temperature and increasing TSS concentrations, but species were surprisingly redundant with regards to particle size-selectivity. Thus loss of individual species may have a greater impact on ecosystem services in our changing environment than declining biodiversity.

Introduction

The relationship between biodiversity and ecosystem services has been frequently studied, and it is generally accepted that there is a positive relationship between biodiversity and ecosystem service (Loreau and Hector 2001, McIntyre et al. 2007; but see Creed et al. 2009). Proposed mechanisms behind this relationship include complementarity, such that species interactions are positive (Cardinale et al. 2002) and sampling/selection effects wherein increasing biodiversity increases the chance of inclusion of functionally dominant species, such as keystone species and ecosystem engineers (Cardinale et al. 2006). The strength of the relationship between biodiversity and ecosystem services depends in large part on species identity. If a functional guild is comprised of functionally redundant species, loss of species within that guild will have less impact on ecosystem services than in guilds with less redundancy (Rosenfeld 2002).

Freshwater mussels in the family Unionidae exhibit their highest, worldwide, biodiversity in the southeastern U.S. with approximately 278 species (Graf and Cummings 2007). They commonly occur in dense, multiple-species groups (beds) with as many as 40 species observed within a single riffle (Haag and Warren 1997). As primary consumers, freshwater mussel species share the same trophic position (Vaughn et al. 2008) and are considered an ecologically significant filter-feeding guild. However, mussel species are declining in the southeastern U.S., and face increasing stresses due to warming temperatures and increasing frequency of drought (Karl et al. 2009). This raises important questions as to how decreases in diversity are likely to affect ecosystem services provided by mussels. The strength of these effects will depend in large part on the degree of functional redundancy exhibited by freshwater mussel communities.

Water filtration is a central ecosystem service provided by mussels. Factors likely influencing a mussel species' ability to remove particles include: total suspended solids loads (TSS), particle size, and temperature (Vaughn and Hakenkamp 2001). The quantity and quality of particles removed may subsequently affect other ecosystem processes, such as nutrient retention/excretion and biodeposition. However, the degree to which water filtration varies among species has received little attention. It is also unclear as to whether differences in filtration ability differ according to predictable patterns related to taxonomy or general habitat preferences (lotic versus lentic).

Seston density in aquatic ecosystems is temporally variable and filter feeding bivalves respond by adjusting particle removal rates. Typically, particle removal increases with increasing TSS levels, until a greater number of particles cannot be processed (Winter 1978, Bontes et al. 2007). The asymptote in particle removal may be reached because ingestion rates are maximized (Hornbach et al. 1984) and / or the gills are clogged due to excess particles

(Kryger and Riisgard 1988). Determining whether lotic and lentic species respond differently to increasing particle concentrations is important for predicting ecosystem services provided. Redundancy may occur within a habitat group if species have all adapted to similar environmental conditions (Loreau and Hector 2001); conversely, resource partition may occur to allow for the coexisting of mussels within a habitat type (Bronmark and Malmqvist 1982). Overall, it is expected that redundancy will be greater for species within habitat type, than across habitat type (Poff et al. 2003).

It has been suggested that lotic species are more efficient at clearing small particles because they have been shown to clear bacteria (2.3 μm ± 0.1) at significantly higher rates than lentic species in laboratory trials (Silverman et al. 1997). The proposed mechanism is that lotic species have a higher density of cilia per cirral plate, which allows for easier removal of bacteria from the water column. However, in a separate study, scanning electron microscopy revealed differences in gill size and cirri density for four co-occurring species of mussels (Galbraith et al. 2009), implying that mussel species within a given habitat type might differ in their ability to remove particles of different sizes. It is therefore unclear whether redundancy in size selection differs more strongly between habitat types than within habitat types. Although lotic and lentic species are generally thought to occupy discrete habitat units, species generally regarded lentic can often co-occur with lotic species in rivers by selecting lentic microhabitat, thus providing ecosystem services to the same water body.

Temperature is also important in regulating freshwater mussel filtration (Vanderploeg et al. 1995, Baker and Hornbach 2001) and likely plays an important role in dictating the quantity of particles removed at high TSS levels. Previous studies have demonstrated reduced clearance rate

at relatively high temperatures for some species, but not for others, because of differences in thermal optima across species (Spooner and Vaughn 2008).

In this study we focus on a single ecosystem service provided by freshwater mussels, removal of particles from the water column. We utilize natural seston in a semi-natural setting – experimental ponds. Many previous studies examining filtration capacity of freshwater mussels have occurred in controlled laboratory settings utilizing algal monocultures (Silverman et al. 1997, Bontes et al. 2007, Spooner and Vaughn 2008), and the results of these experiments may be difficult to accurately translate to natural environments. The primary objective of this study was to compare the abilities of various lotic and lentic species to remove suspended solids at high temperatures (29 – 32˚C) and to determine whether a multi-species assemblage was able to remove more particles than any given single species assemblage. We also examined whether all species exhibited maximum particle removal rates at high temperatures or whether some species exhibited maximum rates at cooler temperatures. Finally we compared particle size-selectivity among species. We expected that lotic and lentic species would differ in terms of particle size selection (Silverman et al. 1997) and that this specialization in differing size fractions would lead to removal of a greater quantity of suspended solids by multi-species assemblages compared to single-species assemblages.

Methods

Experimental mussels and ponds

We utilized two lentic and three lotic mussel species in our particle removal trials (Table 2-1). Lentic species *Pyganodon grandis* and *Ligumia subrostrata* were collected from Johnson Lake, MS in October, 2009 and overwintered in ponds at South Auburn Fisheries Research Unit,

Auburn University, Auburn, AL. Lotic species *Plectomerus dombeyanus, Reginaia ebenus,* and *Quadrula asperata* were collected from the Alabama River, 8 km west of Camden, AL in April, 2010 and held in ponds until initiation of experiments. We chose these species because they 1) have large population sizes in the wild, making it easy to collect enough animals for experiments; 2) are phylogenetically diverse (Table 2-1) (Graf and Cummings 2007); and 3) are widely distributed throughout the eastern U.S., with the exception of *Q. asperata*, a Mobile Basin endemic (Williams et al. 2008).

All six ponds used in the experiment contained an in-pond raceway, which provided oxygenated water and a continuous supply of seston flowing past the mussels (mean flow = 0.03 m/s). Ponds were 0.1 ha (15 x 65 m) with depths sloping from approximately 2 m at the deep end to 0.1 m at the shallow end. Raceways were created using a 15-20 m long baffle, erected 5 m from the pond bank. Air diffusers were placed at 1.2 m water depth at one end of the baffle, and through the use of a deflecting hood, created a slowly moving current (mean flow = 0.03 m/s) towards the shallow end of the pond. Mussels were housed in the raceway for the duration of the experiments.

Ponds were managed for a gradient of TSS dominated by organic particles. Liquid fertilizer (39 P:13 N: 0 K) was applied on an as-per-need basis to maintain three different secchi depths corresponding to three TSS levels: >75 cm = Low TSS (no fertilizer added), 40-75 cm = Intermediate TSS, and <40 cm = High TSS (N = 2 ponds/level). Secchi depth was used because it provides a rapid proxy of water column TSS (AM Gascho Landis unpublished data). Fertilizer was added (1 L/week/pond) when secchi depths fell below the target range. We randomly assigned mussels to one of the three TSS levels (low, intermediate, and high, corresponding to the three secchi depth ranges). Individuals were moved between ponds assigned same TSS level

for filtration trials, if one pond had TSS levels that helped complete the gradient. Mussels were acclimated to a new pond for at least 2 days prior to an experimental run. Grass carp, *Ctenopharyngodon idella,* were stocked in all ponds (N = 10) to help control aquatic macrophoytes which compete with phytoplankton for nutrients. Water samples were collected weekly for TSS analysis (see below for lab processing).

Particle removal trials

Particle removal trials were conducted opportunistically between June 16 and Aug 25, 2010 to encompass a wide range of TSS levels (2 – 48 mg/L) at consistently warm temperatures (29-32˚C), and from October 10 – December 1, 2010 to include a narrow range of TSS levels (11-21 mg/L), but to expand the range of temperatures (12-31˚C). During a given filtration trial, all five species and a mixed assemblage were tested simultaneously in the same pond (three replicate filtration chambers/species plus three control chambers with no mussels). Filtration chambers consisted of 58 L, floating, cone bottom tanks modified into downwellers (Figure 2-1). An air stone was inserted into a stand pipe, containing holes at the base, which act to draw the water in from the bottom of the chamber and expel it at the top, creating a circular current that provided thorough mixing and limited particle settling within the chamber. After filling, water was not exchanged between the chamber and the pond. During experiments, mussels were placed on platforms approximately half way up the stand pipe. Platforms were made of coarse mesh (1 x 1 cm mesh size) so as to not impede water or particle circulation.

Within a single-species filtration chamber, five mussels of the same species were held in 25 L of pond water. We used multiple mussels per chamber to minimize bias in filtration data caused by individuals having above or below average performance (Pascoe et al. 2009). In the

mixed assemblage we matched biomass among species as closely as possible, such that smaller species had multiple individuals, while larger species had only one individual. To obtain well mixed and relatively homogeneous pond water for filling the filtration chambers, sump pumps were suspended in the water column directly in front of the pond airlift. In an additional step to ensure similar starting TSS, chambers were filled sequentially in 5 L increments until all chambers were full (Vanderploeg et al. 1995).

All trials were initiated at approximately 8:00 AM to ensure consistent diurnal feeding patterns. Before trials mussels were gently scrubbed, rinsed, and weighed. At time zero a 500 mL water sample was removed from each chamber using a battery powered siphon and mussels were immediately introduced into the appropriate filtration chambers. Mussels were allowed to filter for 24 hours with 500 ml water samples collected at 2, 4, 8, 12, and 24 hrs from the time of initial samples. After completion of filtration trials on December 1, 30 mussels from each species, except *P. grandis* (n=18), were used to determine the average ratio of total wet mess (shell and soft tissue) to dry tissue mass (soft tissue only). This allowed us to estimate the total dry tissue mass within each filtration chamber based on the total wet mass recorded just prior to the run.

Water samples were processed using standard EPA protocols for suspended sediment analysis (APHA 1995). Replicate water samples (250 mL or less) were vacuum filtered on 47 mm Pall A/E glass fiber filters (1 μm). Samples were placed in an oven at 105˚C until a consistent dry weight was reached. Filters were ashed in a muffle furnace for one hour at 550˚C, volatizing the organic matter, and leaving only inorganic material. The weight of the organic fraction was obtained by subtracting the inorganic fraction from the initial dry weight (or TSS).

Calculating organic and inorganic fractions allowed us to monitor organic:inorganic ratios (O:I; calculated as O ÷ I) throughout the experiment.

Particle removal rate (mg/g dry weight/h) is the total mass of material removed from the water per unit time, and was calculated by subtracting the final sample mass ($C_{t,}$ hour 2) from initial sample mass (C_0):

$$\text{Particle removal} = V * (C_0 - C_t)/ (n * t)$$

and standardizing for volume (V), mussel dry weight (n) and time (t) (Stuart et al. 2001). Particle removal was corrected for average amount of material settling out in the control chambers. For analysis of particle removal across the TSS gradient, we only used data from time 0 and 2 in an attempt to limit the percent of particles removed, thereby minimizing change in filtration behavior due to particle depletion. We analyzed particle removal rate in our experiments, instead of the more commonly used clearance rate, because during the first two hours, in many of the trials, especially at low TSS, mussels removed >30% of particles within 2 hrs. As percent particles removed increases, clearance estimates become decreasingly accurate (Pascoe et al. 2009). Also, we were interested in realized particle depletion rates within a system that could be depleted by filtering activity, rather than in a system where TSS levels remained constant. In our experiment, we did not measure pseudofeces production. Thus particle removal is not equivalent to ingestion rate; rather it is a measure of the total material removed from the water column and represents both assimilated material and settled biodeposits (pseudofeces and feces).

We used regression analysis to assess the effects of increasing initial TSS concentrations on particle removal rate for the warm-water trials (29 – 32˚ C) for each species and the mixed assemblage. We used ANCOVA, with TSS level as a covariate, to test for differences among

species in the rate at which material was processed across the TSS gradient. Prior to running the ANCOVA we confirmed the assumption of homogeneity of slopes ($F = 1.73$, $p = 0.166$).

Conducting in-pond experiments presented two main challenges for our temperature trials: we needed consistent TSS values across different temperatures and we had a narrow window of time to assess particle removal rates at any given temperature. We conducted trials in a relatively narrow TSS range (11 – 22 mg/L) and assumed that particle removal was minimally affected by differences in TSS concentrations within this range (see results). We then fit quadratic regressions to the data to test for significant unimodal patterns in particle removal rates across declining temperatures. A significant relationship would indicate that particle removal was maximized at intermediate temperatures and inhibited above the thermal optima. A non-significant relationship would indicate that particle removal rate was not inhibited at high temperature and that particles were being removed at or near a maximum rate.

To compare particle size preference across species we collected 500 mL water samples at 0 and 24 hrs and preserved with Lugol's solution for particle analysis. Only three trials were selected for this analysis. Within those three trials, particle concentrations in the chambers of all five species leveled off within 24 hours (as indicated by significant exponential declines, see results), indicating that only unavailable or strongly "unpreferred" particles remained.

Particles in the initial (time zero) and final (24 hrs) samples were counted using a Beckman Multisizer 3 Coulter Counter®. Each sample was subsampled (200 μL), diluted with an electrolyte solution (20 mL), and particles counted for 120 s, yielding an estimate of relative particle composition. Particle measurements were divided into 300 bins between 2.64 and 60 μm, with a greater proportion of bins in the smaller size classes. To determine particles removed within each bin over the 24 hour trial we subtracted the number of particles at time 24 h in each

bin from those at time 0 h. However, particle numbers at time 24 were first corrected for the average number particles settling out in the control chambers.

To assess preference for different size particles we combined the automated bins into 4 size categories, 2.64 - 4.9 μm, 5 – 9.9 μm, 10 – 19.9 μm, and 20 – 60 μm. The division at 20 μm was chosen because it has been suggested that mussels do not remove particles >20 μm (Vaughn et al. 2008). The 20 – 60 μm category was not further subdivided because relatively few particles ever fell within this category. However particles less than 20 μm were more numerous and subdivided into three additional categories. Minimum size of detection for particles was 2.64 μm. We estimated size selectivity using Chesson's alpha:

$$\alpha = \frac{ri/pi}{\sum(ri/pi)}$$

where r_i are the particles removed for a given size class, p_i are the particles for a given size class in the filtration chamber at the start of the trial (Chesson 1978). Chesson's alpha calculates relative preference on a scale from 0 to 1, with 1 indicating the highest preference. Neutral preference is determined as 1/*m* where *m* is the number of particle classes. Values above 1/*m* are preferred and values below 1/*m* are negatively selected.

Results

Experimental ponds yielded a wide range of TSS conditions (Table 2-2). During TSS trials from June 16 through August 25, 2010 (N = 9 trials) pond temperatures were consistently high, with average temperatures during the trial ranging from 29.0 – 32.5˚C. Across all trials, TSS load was dominated by organic particles, except on one date (Table 2-2). There was no significant relationship between TSS level and O:I ratio ($R^2 = 0.02$, $p = 0.46$).

Particle removal rate showed a significant, linear increase with increasing TSS for *Q. asperata*, *P. grandis*, *L. subrostrata*, and the mixed assemblage, but not for *P. dombeyanus* and *R. ebenus* (Fig 2-2). Particle removal rates were significantly different across species, while holding starting TSS constant (ANCOVA, $F = 6.2$, $p = 0.001$). *Quadrula asperata* was significantly different from all other species, except for *L. subrostrata*, showing the highest rate of increase in particle removal at high TSS. The mixed species assemblage significantly differed from *Q. asperata* but not from *L. subrostrata* or *P. grandis* and the slope of the mixed species regression line was intermediate amongst the single species regressions.

Mass-specific particle removal rates at four different temperatures (Table 2-3) were used to assess thermal constraints for all species. Significant quadratic regression was fit through the data for *P. dombeyanus*, *R. ebenus*, and the mixed assemblage, but not for *Q. asperata*, *P. grandis*, and *L. subrostrata* (Figure 2-3). This indicates that two of the lotic species (*P. dombeyanus* and *R. ebenus*) were thermally constrained, but the remaining species continued to remove particles at, or near, maximum rates even at the highest temperatures tested.

All five mussel species were able to simultaneously deplete seston during the 24 hour trial in only three of the trials (Figure 2-4 A, C, E). The starting TSS concentrations in these trials ranged from 8.8 to 20.4 mg/L. At the start of all three filtration trials, the smallest size class of particles (2.6-4.9μm) was at least 3.7 times more common than all other size classes combined (Figure 2-4 B, D, F) and the two smallest particle size classes made up between 98-99% of all particles. Although the smallest size class of particles was most abundant in starting conditions, mussel did not consistently prefer this size class.

No species selected the same particle size class across all three depletion trials (Figure 2-4 B, D, F). Furthermore, all species exhibited positive selection for the same size class(es) within

any given trial. In the trial with starting conditions of 8 mg TSS/L, all species exhibited positive selection for particles in the 20- 60 μm size class (Figure 2-4 B). In the trial with starting conditions of 15 mg TSS/L all species showed positive selection for the 5-9.9 μm size class (Figure 2-4 D). However, *P. dombeyanus* and *R. ebenus* also showed positive selection for the 2.64-4.9 μm size class, whereas the other three species only demonstrated neutral selection for this size class. In the trial with starting conditions of 20.4 mg TSS/L all species had positive selection for the two smallest particle size classes (2.5-4.9 and 5-9.9 μm) and negative selection for the two largest size classes (10-19.9 and 20-60 μm, Figure 2-4 F).

Discussion

The effect of increasing TSS concentrations on particle removal rates differed among species at the warmest temperatures tested, possibly due to temperature constraints. Whereas three mussel species removed increasing masses of particles as TSS levels increased, *P. dombeyanus* and *R. ebenus* did not. These two species were also the only ones exhibiting a significant unimodal relationship between particle removal rates and temperature, indicating particle removal was constrained at high temperatures. Pond temperatures during our trials were between 29 - 32.5˚C, but temperatures in the main channel of the Alabama River, from which they were collected, typically do not exceed 30˚C during the summer (Hoxmeier and DeVries 1997). Temperature constraint likely accounts for these species not removing increasing quantity of material, but rather removing constant quantities of particles across the TSS gradient at warm temperatures.

Neither *Q. asperata*, nor the two lentic species (*P. grandis* and *L. subrostrata*) were constrained by high temperatures and all three species exhibited a positive relationship between

particle removal and TSS. Slow moving or static waters are often warmer than running water (Lessard and Hayes 2003). Thus, it is likely that lentic species more frequently experience and are adapted to warmer temperatures. *Quadrula asperata* is considered a large river habitat generalist, and is commonly found in both lotic and lentic habitats (Haag 2012). It removed particles at a significantly higher rate than all other species, except *L. subrostrata*. Because it is a large river habitat generalist, *Q. asperata*'s high filtration rates may reflect its ability to successfully inhabit a wide range of flow conditions and corresponding solids loads.

Typically, as TSS levels increase, ingestion rate reaches a maximum level (Bontes et al. 2007), while pseudofeces production may continue to increase. However, at even higher TSS levels, ingestion and pseudofeces production combined will reach a point at which greater levels of material cannot be processed (Winter 1978). A concern for freshwater mussels is that high TSS levels, resulting from suspended sediments or algal blooms may cause gill clogging, a resultant decline in material processing, and potential cessation of feeding activity (Aldridge et al. 1987). *Crassotrea gigas*, a marine bivalve, can maintain filtration rates up to 90 mg TSS/L, because pseudofeces production allows for elimination of excess particles; but at solids levels >90 mg TSS/L there is pronounced decline in filtration rate because of physiological limitation (Barille et al. 1997). Although we did not measure pseudofeces in this study, we observed no decline in total material processing even at relatively high TSS levels (50 mg/L) in any of the five species, indicating that mussel filtration abilities were not inhibited. Thus, although warm temperatures may have reduced ecosystem services of some of the mussel species, TSS levels up to 50 mg/L did not.

We had expected that different species would differ in their efficiencies at removing differing size classes of particles based on previous studies showing differing particle size-

selectivity between lotic and lentic species (Silverman et al. 1997) and differences in cirri density of gills among mussels collected from the same habitat (Galbraith et al 2009). If so, we expected that multispecies assemblages would be more efficient at particle removal than single species assemblages, as the multispecies assemblage would remove particles more efficiently across a wider size range. However, this was not the case. The five mussel species tested exhibited a high degree of functional redundancy with regards to size-selectivity. Within any given trial, all species had positive selectivity for the same particle size classes, even though this size-selectivity was not constant across trials. All mussels appeared to be generalists, adjusting selectivity with seasonal changes in natural seston size-structure. Thus particle removal rates of the mixed species assemblage were intermediate to that of the single species assemblages, representing an average of the species present, rather than an additive or synergistic relationship.

Bacteria are increasingly recognized as a valuable food source for freshwater mussels. The studies identifying bacteria as a dominant food source, through the use of stable isotopes and fatty acid profiles, have been conducted in low order streams (Raikow and Hamilton 2001, Christian et al. 2004). In contrast, studies using stable isotopes in lentic and large river ecosystems have found phytoplankton to be the most important food items for freshwater mussels (Vander Zanden and Rasmussen 1999, Thorp and Casper 2002). We did not measure prevalence of bacteria versus phytoplankton food sources in our ponds, but likely both food sources were present and their proportion changed through time and across ponds. Consuming a wide range of particle sizes, from small bacteria to large algal cells, could be an adaptation for coping with dynamic seston assemblages, typical of both large river systems and lentic habitats. All species tested in this study are still common in the wild despite large-scale natural seston alterations resulting from eutrophication and damming of large rivers. It would be interesting to

determine whether rare, declining species are more likely to exhibit differential size-selectivity than the relatively few mussel species that still have stable population sizes.

Occurrence of cyanobacteria often increases as ecosystems productivity increases (Paerl and Tucker 1995). Cyanobacteria negatively influence aquatic ecosystems, with the specific concern of unpalatability or toxicity for filter feeding organism (Demott and Moxter 1991) and increased handling time of colonial forms (Bontes et al. 2007). However, the evidence of cyanobacteria affecting freshwater mussel feeding is mixed. *Anadonta anatina* has been shown to graze on both toxic and non-toxic forms of *Planktothrix agardhii*, a cyanobacteria species, at equally high clearance rates (Pires et al. 2007) and three species of unionids preferred *Microcystis sp*. over green algae (Baker and Levinton 2003). In contrast, *Elliptio complanata*, showed higher filtration rates on aquaculture effluent dominated by green algae (*Scenedesmus* spp. and *Ankistrodesmus* spp.) as compared to effluent dominated by cyanobacteria (*Microcystis* spp) (Stuart et al. 2001). However, in ponds dominated by cyanobacteria, mussels were able to continue feeding by selectively removing green algae (Stuart et al. 2001). Measuring cyanobacteria was outside the scope of this study, but if abundance of cyanobacteria did increase with increasing productivity, it did not appear to inhibit filtration rates.

One limitation of many previous studies is using monocultures of algae or bacteria in the laboratory to address field patterns. Using laboratory reared algae or bacterium presents a simpler, more controlled way to test physiological questions, but may not translate consumption patterns on natural seston assemblages, containing numerous species of microorganisms. Our experiments were conducted in pond raceways, which provided a more natural environment for examining filtration dynamics related to ecosystem services. Especially since many waterways in North America have increasing TSS loads, due to eutrophication and sediment runoff, and are

facing increasing temperatures due to climate change. Overall, we found that species identity was important with regards to response to temperature and increasing TSS concentrations, but species were surprisingly redundant with regards to particle size-selectivity. Thus loss of individual species may have a greater impact on ecosystem services in our changing environment than lack of biodiversity per se.

Our results also have implications for managed ecosystems. Several researchers have suggested using native freshwater mussels as "water purifiers" to maintain water quality in artificial ecosystems and to limit TSS levels in effluent. Freshwater mussels filtration capabilities have been explored in partitioned aquaculture systems (Stuart et al. 2001), caged aquaculture (Soto and Mena 1999), and waste water lagoons (Helfrich et al. 1995). In these studies, filtration capacity was assessed for only single species and did not consider the benefits of increased biodiversity on ecosystem services provisioning. Our study shows that particle removal rates were more strongly affected by species identity than by biodiversity. Thus use of appropriate single species assemblages, matched to system temperature constraints, may be just as effective as using multiple species.

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Table 2-1. Species used for in-pond filtration trials. Average and stand deviation for length, whole-body wet mass, and soft tissue dry mass calculated for N = 30, except for P. grandis, N = 18.

Species	Habitat	Tribe*	Average shell length (cm)	Average whole body wet mass (g)	Average soft tissue dry mass (g)
Plectomerus dombeyanus	Lotic	Lampsilini	112.3 ± 13.0	269.0 ± 85.0	8.0 ± 2.1
Reginaia ebenus	Lotic	Amblemini†	70.3 ± 5.9	147.0 ± 35.0	4.1 ± 0.6
Quadrula asperata	Lotic	Quadrulini	54.9 ± 6.9	81.8 ± 28.9	2.1 ± 0.4
Pyganodon grandis	Lentic	Anodontini	112.0 ± 7.2	177.5 ± 54.4	7.4 ± 2.1
Ligumia subrostrata	Lentic	Lampsilini	85.6 ± 12.6	66.3 ± 24.1	4.0 ± 0.9

*Phylogenetics based on Graf and Cummins 2007. † incertae sedis: Campbell and Lydread 2012.

Table 2-2. Temperature and total suspended solids parameters for pond conditions during in-pond filtration trials.

Date	Temperature (˚C)	Organic (mg/L)	Inorganic (mg/L)	Total (mg/L)	O:I
6/16/2010	31.5	27.2	21.2	48.4	1.3
6/24/2010	32.5	1.4	1.0	2.4	1.4
7/1/2010	30.2	5.7	3.1	8.8	1.7
7/22/2010	31.8	23.3	6.7	29.9	3.5
7/28/2010	32.0	5.5	9.7	15.2	0.6
7/30/2010	31.3	14.2	9.9	24.1	1.4
8/4/2010	32.2	4.3	2.0	6.3	2.2
8/18/2010	30.6	10.9	9.8	20.7	1.1
8/25/2010	29.0	21.1	8.8	29.7	2.5

Table 2-3. Pond water total, inorganic, organic suspended solids for the in-pond filtration trials across a temperature gradient.

Temperature (˚C)	Organic (mg/L)	Inorganic (mg/L)	Total (mg/L)	O:I
11.9	1.9	9.4	11.4	0.2
16.3	3.7	18.6	22.4	0.2
19.8	7.5	8.3	15.7	0.9
30.6	10.9	9.8	20.7	1.1
32.0	5.5	9.7	15.2	0.6

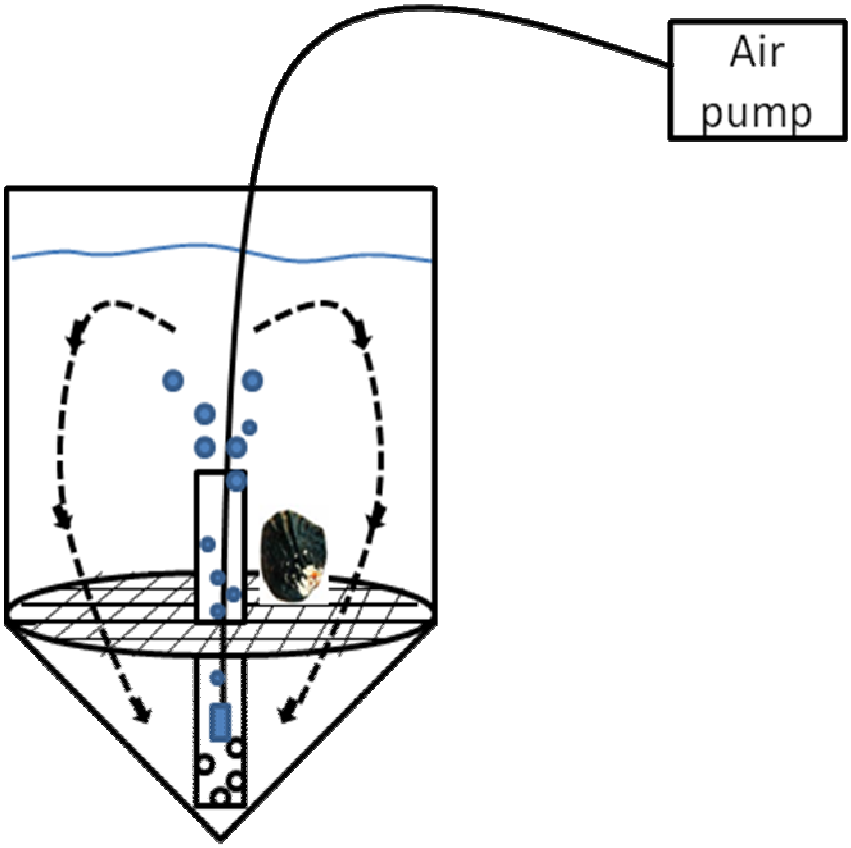

Figure 2-1. Cone bottom tanks (57 L) constructed as an aeration driven down-welling filtration chambers. Cone bottoms prevented settling of particles over 2 hour filtration trials.

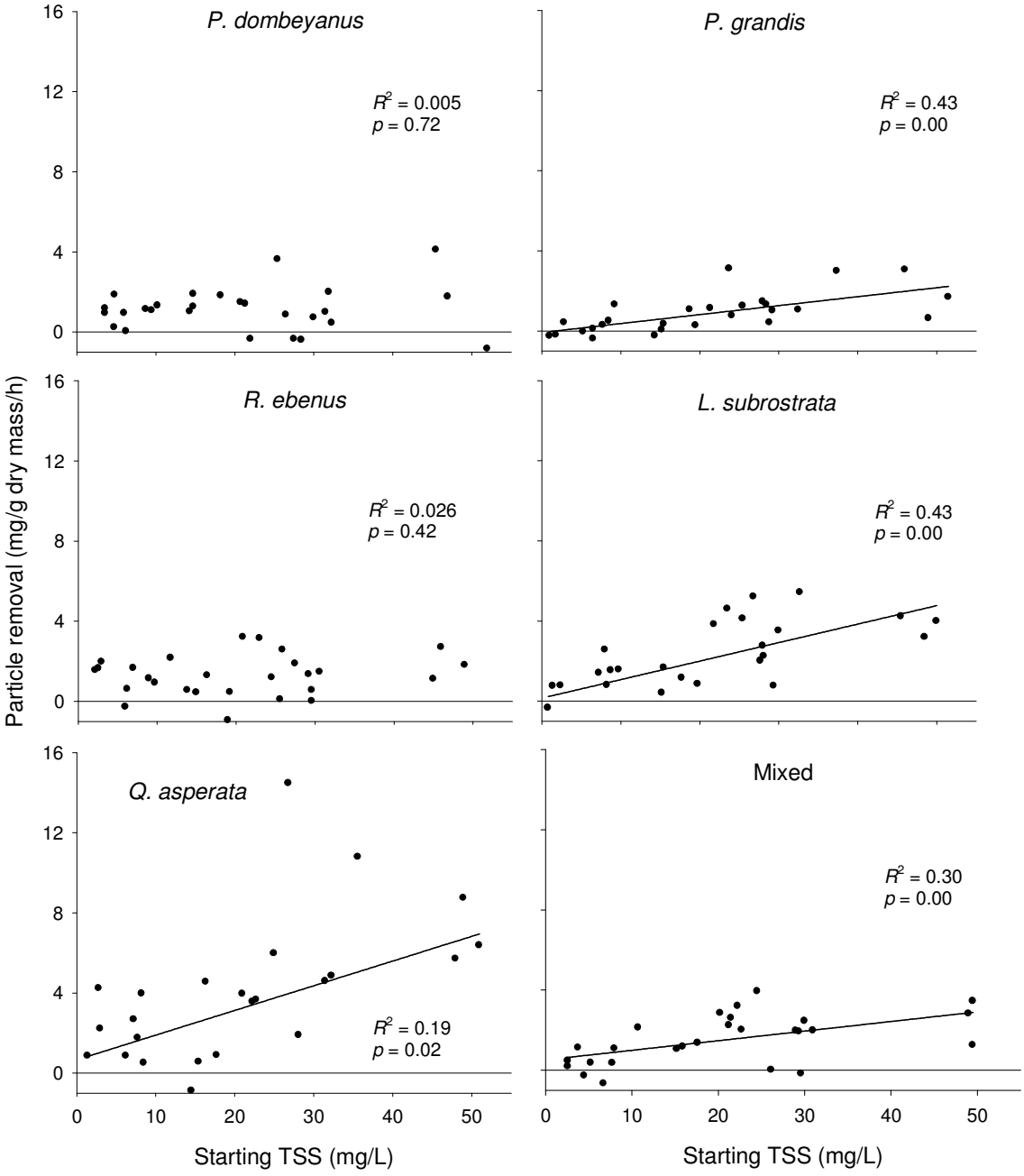

Figure 2-2. Particle removal rate for five species and a mixed assemblage across a gradient of total suspended solids at relatively constant temperature (29-32.5˚C). Significance of each trend can be found in the legend. All trials were conducted in ponds using natural seston assemblages.

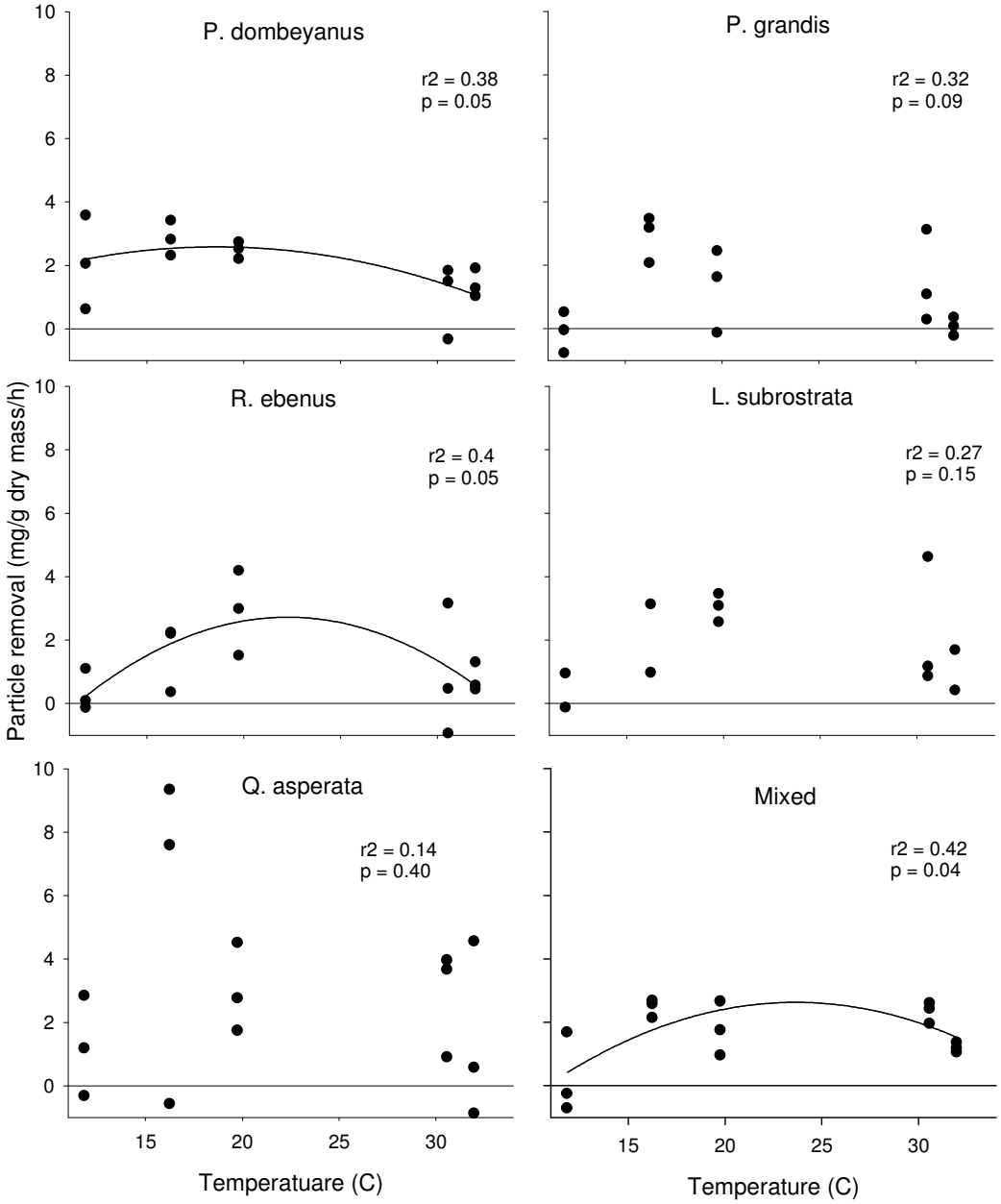

Figure 2-3. Particle removal for five species and a mixed assembles across a temperature gradient at relatively similar total suspended solids. All trials were conducted in ponds using natural seston assemblages.

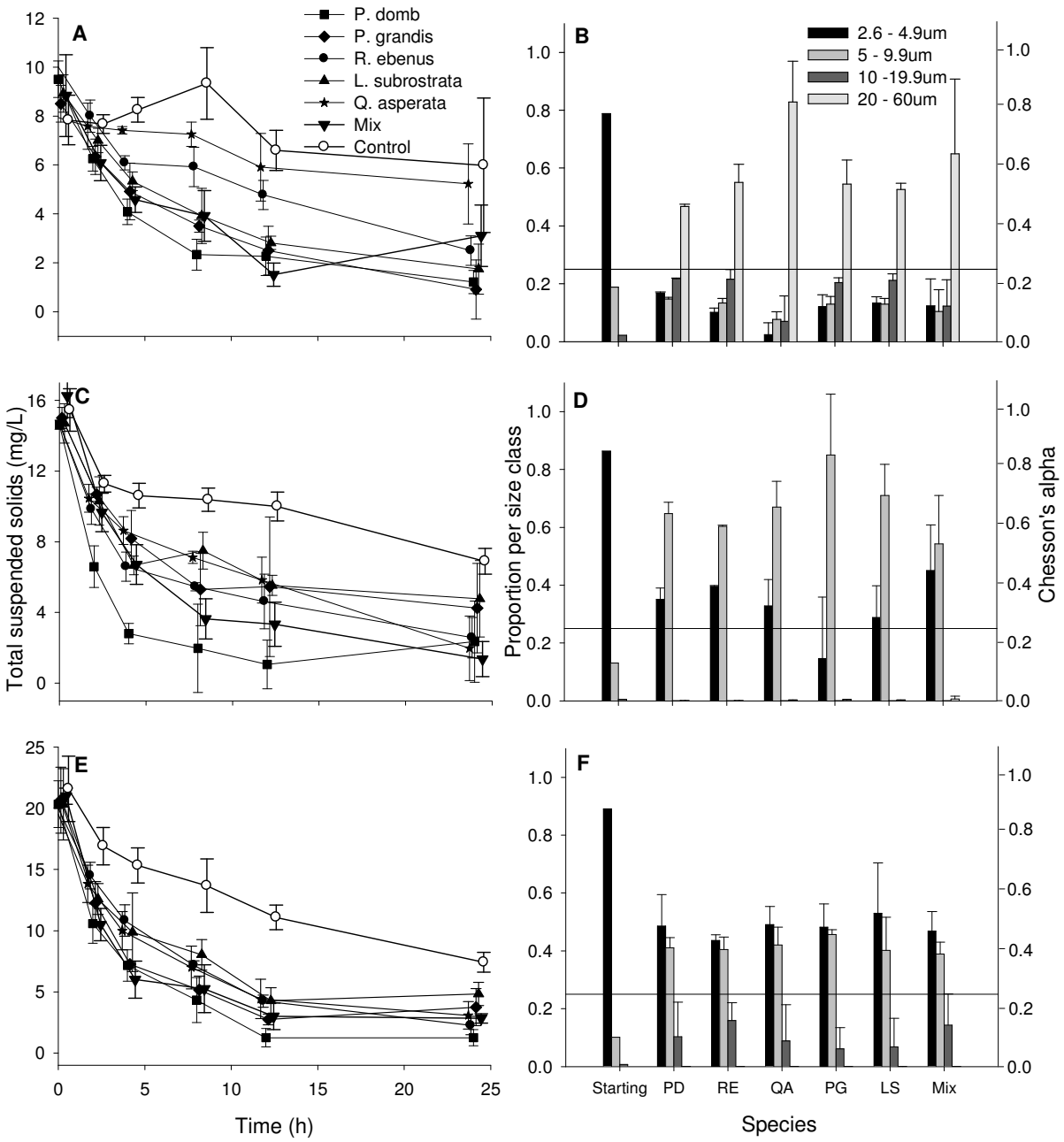

Figure 2-4. A, C, E – Depletion curves for 24 h filtration trials. All curves are significant exponential declines. Data points are offset for viewing purposes. B, D, F – The first group of bars in each graph is the starting proportion of the different size classes. The remaining 6 groups of bars are the Chesson's alpha values for each particle size class after 24 h trials. The horizontal line represents neutral selectivity.

Chapter 3

High suspended solids as a factor in reproductive failure of a freshwater mussel

Elevated suspended solids are a widespread stressor of aquatic ecosystems, but their effects on growth and reproduction in freshwater mussels are largely unknown. We fertilized experimental ponds to create a gradient in total suspended solids (TSS) and examined the effects of TSS on growth, nutritional status, reproduction, and clearance rate in *Ligumia subrostrata*. The number of females that became gravid declined sharply with increasing TSS, and no gravid females were found in the highest TSS treatments. The proportion of gravid females was not related to the TSS organic:inorganic ratio. Fertilization was an all-or-nothing phenomenon. In all females that did become gravid, 98 to 99% of eggs were fertilized regardless of TSS, and total fecundity was unrelated to TSS. Clearance rates declined sharply as TSS increased but showed a threshold relationship in which clearance was uniformly low at TSS > ~8 mg/L. Reproductive failure probably was not caused by poor body condition or nutritional status because growth (length and mass) and energetic status (measured as caloric density) were not related to TSS. We propose 2 mechanisms that implicate interference of TSS with fertilization as the cause of reproductive failure. Reduced clearance rate could decrease the chance of females encountering suspended sperm during filter feeding, or an increase in pseudofeces production could bind sperm in mucus and lead to its egestion before fertilization. Interruption of fertilization coincident with high TSS is a potential mechanism to explain the lack of mussel recruitment in many locations. Monitoring

and reduction of TSS, especially during the spawning season, may help create conditions necessary for maintenance and recovery of mussel populations. More research is needed to explore the generality of this pattern across a broad range of mussel species including those adapted to lotic environments or that use different brooding strategies.

Introduction

Elevated levels of total suspended solids (TSS) are a ubiquitous water-quality problem in the US (Wood and Armitage 1997, Carpenter et al. 1998, Biggs 2000) and are a threat to many aquatic organisms (Richter et al. 1997). Suspended solids can be composed of organic or inorganic particles. Increased organic solids (phytoplankton, bacteria, fungi, etc.) and eutrophication often result from excess nutrient inputs and can negatively affect aquatic organisms via decomposition and resultant hypoxic/anoxic conditions (Paerl 1988, Vitousek et al. 1997, Camargo and Alonso 2006). Elevated organic solids also can increase food resources, resulting in increased individual growth rates and biomass production (Deegan and Peterson 1992). High concentrations of inorganic solids (sand, silt, clay, etc.) originate from erosion related to agriculture, forestry, and urbanization, and can alter feeding patterns, substrate composition, and food web dynamics (Waters 1995). Understanding the net effects of TSS on the health of aquatic organisms is essential for establishing and implementing effective water-quality guidelines and regulations.

Freshwater mussels (Order Unionoida) have declined dramatically in much of North America, but in many cases, the causes of these declines are unknown. Anthropogenic increases in deposited and suspended solids have been widely invoked as causes of mussel declines (e.g., Brim Box and Mossa 1999), but direct evidence for these effects is scant (Haag 2012).

Furthermore, the effects of TSS on freshwater mussels may vary widely according to context. Translocation of *Hyridella depressa* (family Hyriidae) from an oligotrophic lake to a nutrient-enriched stream below a sewage treatment plant resulted in increased growth rates, potentially because of higher food availability (Walker et al. 2001). Mean annual shell growth of *Diplodon chilensis* (Hyriidae) in Chilean lakes was strongly positively correlated with TSS concentration measured indirectly as Secchi depth (Valdovinos and Pedreros 2007). However, mussels were largely extirpated from lakes with the shallowest SDs (hypereutrophic lakes), possibly indicating a threshold above which increased nutrients and resultant organic solids have a negative effect. TSS loads dominated by inorganic particles can decrease growth rates of zebra mussels (Order Veneroida) (Osterling et al. 2007). Intermittent exposure to extremely high levels of suspended sediment led to decreased clearance rates (the volume of water cleared of particles per unit time) for 3 unionid species and was proposed as a cause of decreased growth or starvation (Aldridge et al. 1987). However, 2-mo exposure to high levels of inorganic suspended sediments (>80 nepholometric turbidity units [NTU]) did not decrease energy density of *Amblema plicata* (Howard 1999).

One of the hallmarks of many mussel declines is absence of recent recruitment, a pattern suggesting that some factor limits reproduction. Sedimentation might negatively affect mussel recruitment (Brim Box and Mossa 1999), but these effects are poorly understood and appear to vary widely. Male mussels (Unionidae) fed excessive food in the laboratory had significantly higher sperm production than individuals in the wild (Galbraith and Vaughn 2009). Fecundity of *Hyridella depressa* below a sewage effluent was ~2× that of mussels upstream of the effluent (Walker et al. 2001), and food availability was positively related to offspring production in fingernail clams (*Sphaerium striatinum)* (Beekey and Karlson 2003). In contrast, recruitment

strength of *Margaritifera margaritifera* was negatively related to turbidity (suspended sediment) and deposited sediment, but the mechanism for this relationship was unclear (Osterling et al. 2010). The only direct evidence of negative effects of suspended sediment on mussel reproduction is the observation that very high concentrations of suspended clay (1250–5000 mg/L) caused reduced attachment and metamorphosis success of parasitic mussel larvae (glochidia) on host fishes in the laboratory (Beussink 2007).

The effects of TSS on fertilization of mussel eggs have not been examined directly. Male mussels release sperm into the water, and sperm are captured by the female gills during filter feeding and moved by gill cilia to the suprabranchial chamber where eggs are fertilized (McMahon and Bogan 2001). Consequently, sperm capture and egg fertilization probably are sensitive to changes in TSS that influence feeding dynamics and gill function (Tankersley 1996). High TSS may decrease clearance rate, decreasing the likelihood of capturing sperm, because energetic demands can be met from a smaller volume of water (Winter 1978). Increased TSS may lead to an increase in pseudofeces production and decrease in particle selectivity (Schneider et al. 1998), increasing the chance that captured sperm will be rejected in mucus-bound pseudofeces. Thus, high concentrations of suspended organic particles may present a trade-off between increased growth, caloric density, and fecundity and decreased fertilization success.

We assessed the effects of TSS on clearance rate, growth, caloric density, and reproductive success of mussels in pond experiments. We added liquid fertilizer in experimental ponds to create a gradient of TSS concentrations and explored the balance of potential positive and negative effects. On the basis of available information, we hypothesized that increasing TSS dominated by organic suspended solids would lead to initial increases in growth, caloric density, and reproductive success because of higher food availability, but ultimately would lead to a

decline in growth, caloric density, and egg fertilization as TSS exceeded a limiting threshold.

Methods

Experimental design

Our study species was *Ligumia subrostrata*, a sexually dimorphic species that is a common constituent of lentic mussel assemblages. This species typically releases all glochidia by late summer, and the subsequent egg clutch is deposited in the gills and fertilized by October (Gascho Landis et al. 2012). We collected mussels used in the experiment in November 2009 from ponds at the South Auburn Fisheries Research Station, Department of Fisheries and Allied Aquaculture, Auburn University. These individuals recruited to the ponds in early spring 2009 as part of a separate experiment, and despite their young age (<9 mo), 90% of females were reproductively mature and gravid by November (JAS and WRH, unpublished data). Mussels were overwintered in the laboratory in tanks with flow-through pond water until initiation of the experiment in April 2010, at which time all individuals were ~1 y old. We expected all mussels to be reproductively active and to exhibit high growth rates because of their young age. We tagged all individuals with uniquely numbered Hallprint® tags (Hallprint Pty Ltd, Hindmarsh Valley, South Australia) and recorded shell length (longest anterior–posterior dimension) and total wet mass (shell plus soft tissue) of each mussel at the start of the experiment.

We conducted experiments in six 0.1-ha ponds (20 × 60 m, 2 m maximum depth) at South Auburn Fisheries Research Station. We increased the organic content of TSS by adding aqueous pond fertilizer (N:P:K = 10:31:0) to ponds to stimulate primary production. We assigned 2 ponds to each of 3 treatments (low, intermediate, and high TSS). We did not fertilize low-TSS ponds. We added fertilizer as needed (1 L fertilizer/pond) to maintain Secchi depth

measurements between 40 and 75 cm in intermediate-TSS ponds and <40 cm in high-TSS ponds. We used air lifts and baffles to provide oxygenation and even mixing of water and food in each pond. We stocked 10 grass carp, *Ctenopharyngodon idella*, in each pond to control submerged aquatic vegetation and to reduce competition for nutrients between phytoplankton and rooted aquatic plants. We monitored inorganic and organic solids because bioturbation by the fish potentially could have added inorganic particles to the water column.

We calculated the trophic state index (TSI) based on SD (Carlson 1977) weekly for each pond as an index of productivity. The index is scaled from 0 (highly oligotrophic) to 100 (highly hypereutrophic). The threshold between eutrophic and hypereutrophic is 70. Trophic state was calculated using the equation $TSI = 10(6 - \log_2 SD)$.

We placed 38 mussels in each pond on 24 April 2010 and removed them on 11 November 2010. We suspended mussels 45 cm from the surface in pocket nets (20 × 30 cm) made from 1.25-cm^2 plastic mesh (2 pocket nets/pond with 19 mussels/net; Fig. 3-1A) because mussels can obtain resources from the sediments by pedal feeding (Raikow and Hamilton 2001, Nichols et al. 2005). Suspension prevented contact with the sediment and allowed us to link feeding and growth rates to TSS loads more directly. Sex ratios in each pocket net were 1 male: 2.4 female. The number of individuals in each pocket net decreased from 13 female and 6 males at the start to an average of 12 females and 5 males at the end of the experiment because some mussels escaped. The sex ratio of 1:2.4 (male:female) was relatively constant throughout the experiment. We placed males and females side by side in pocket nets to minimize sperm limitation. We did not handle mussels after we placed them in ponds, but we cleaned pocket nets periodically to maximize water circulation.

We did not track the timing of spawning during the experiment to avoid disrupting

fertilization of experimental animals. However, we tracked spawning of *L. subrostrata* suspended in pocket nets (pond TSS = 7–11 mg/L) during autumn 2011 to confirm reproductive timing. We held *L. subrostrata* in ponds between the 2010 and 2011 trials. In 2011, we used a 22-gauge needle to extract fluid from the gills of 5 females weekly for 6 wk from 22 August to 26 September (n = 30 females) and examined the extract for eggs or glochidia. We first observed fertilized eggs in the gills on 26 September, indicating spawning probably was initiated during the preceding 6 d (20–25 September). On 31 October, we examined the gills of all females, and 62% were brooding glochidia. We set the spawning period in 2010 from 7 September to 21 October (from 2 wk before and 4 wk after the earliest spawning in 2011).

Laboratory analysis

We collected water samples once weekly from each pond throughout the 7-mo experiment. We measured TSS directly by vacuum filtration of 50 to 200 mL of sample water (depending on particle concentration) through precombusted, preweighed 47-mm Pall A/E glass-fiber filters (1 μm) (APHA 1995). We dried filters at 105°C for a minimum of 6 h, cooled them in a desiccator for 15 min, weighed then combusted them at 550°C for 1 h, cooled them in a desiccator for 15 min, and reweighed them to determine total dry mass and organic solids content, respectively. We recorded SD weekly coincident with water-sample collection and measured dissolved O_2 (DO) on 5 occasions throughout the experiment.

At the completion of the experiment, we recorded shell length and total wet mass of each mussel to assess growth, which was expressed as % change in length or mass. We used bomb calorimetry (Parr 1417 microcalorimeter; Parr Instrument Company, Moline, IL) to measure whole-body (minus the gills) caloric density of 3 fertilized and 3 unfertilized females from each

pond when available. We removed gills of unfertilized females before analysis because gills of fertilized females used for calorimetry were removed for quantification of fertilization (see below). We dried tissues at 60°C until a stable dry mass was reached, then pulverized and homogenized them with a mortar and pestle. We analyzed 2 pellets of homogenized tissue (0.02–0.03 g each) from each mussel. If results for the 2 samples differed by >5%, we analyzed a 3rd pellet.

At the end of the experiment, we quantified reproductive effects as: 1) the proportion of females that became gravid in each pond, 2) the proportion of fertilized:unfertilized eggs/female, and 3) individual fecundity (total number of glochidia or fertilized eggs/female). We assessed gravidity of females by gently prying apart the shell valves and examining the gills (Tankersley and Dimock 1993b). Gravid females had swollen, distended gills, and we considered individuals with flaccid gills not gravid. We examined the flaccid gills of a subset of females (n = 3, 70× magnification) and found neither unfertilized eggs nor glochidia in any flaccid gills. To estimate the proportion of fertilized eggs in gravid females, we sacrificed females and examined the gills of up to 6 gravid individuals from each pond. In some ponds, we examined <6 mussels because we found few or no gravid individuals. We flushed gills with water, diluted the contents with 1000 to 2000 mL, and counted glochidia and unfertilized eggs in three 1-mL subsamples. We estimated total fecundity by multiplying the mean number of glochidia or fertilized eggs in the 3 subsamples by the dilution volume. We chose 6 males from each pond for gamete extraction (Saha and Layzer 2008) and quantified mature sperm as % of total cells.

Clearance rates

We conducted 8 in-pond clearance-rate trials across a TSS gradient representative of

conditions in our experiment (3–48 mg TSS/L; see results). We had a complete TSS gradient across 5 ponds, and TSS conditions in the 6th pond did not expand the range of TSS. Therefore, we used 5 of the 6 experimental ponds for clearance trials and ran multiple trials in 2 of the ponds. We conducted trials periodically from June to August 2010 on an opportunistic basis that coincided with attainment of desired TSS values across the gradient. Pond temperatures were similar across this time period with daily maxima ranging from 30 to 35°C. These temperatures were slightly higher than those during the estimated spawning period (7 September–21 October) when mean daily temperatures were >28°C until 26 September 2010, but fell to 22° by the end of the spawning period.

We used different mussels in clearance trials than in the fertilization component of the experiment to avoid negative effects of handling on their growth and reproduction. However, all mussels came from the same source stock and were housed in the same experimental ponds. We moved mussel between ponds with similar TSS levels for clearance trials because the number of mussels was limited. Before each trial, we acclimated individuals for ≥48 h in the ponds in which clearance trials were to take place. We ran each trial with 3 replicates conducted in individual static filtration chambers. Chambers were 57 L cone-bottom tanks set up as down-welling systems using an airlift (Fig. 3-1B). Each chamber contained 25 L of water and 5 mussels (males and nongravid females), and the total mussel biomass was similar across chambers (720–886 g). We set mussels on a platform of 1.25-cm^2 mesh to allow continual flow of water past the mussels and to allow feces and pseudofeces to settle to the bottom of the tank and limit their resuspension in the water column (Fig. 3-1B). We filled and ran 3 control chambers (no mussels) simultaneously to correct for settling of solids during the trial. We floated the chambers in the pond so water temperatures would remain similar to pond conditions. We filled chambers in 5-L

increments with a sump pump placed near the pond airlift to ensure that the water was well mixed and all chambers had similar starting conditions (Vanderploeg et al. 1995). We collected 500 mL of water from each chamber at time 0 before mussels were added to the chambers. We then added mussels, ran trials for 2 h, and collected 500-mL water samples at the end of the trial. We processed water samples for TSS using methods described above.

We calculated clearance rates with the following equation to standardize for water volume and mussel biomass in the trial:

$$F = (V/nt)\ln(C_0/C_t)$$

where F is clearance rate (L g^{-1} mussel wet mass h^{-1}), V is the volume of water in the filtration chamber, t is the duration of the trial, n is the biomass of mussels in the chamber (modified from Riisgard 2001 in which n is number of mussels), and C_0 and C_t are the initial and final TSS concentrations, respectively (Riisgard 2001). Before we calculated F, we corrected C_t by subtracting the mean mass of solids that settled in control chambers to yield solids removed by the mussels.

Data analysis

We characterized TSS in each pond by calculating the proportion of days with TSS > 20 mg/L and organic:inorganic ratios (O:I) > 1 over the entire experiment and for the putative spawning season only. We used a TSS threshold of 20 mg/L because previous investigators have shown that filter feeding is disrupted above this level (Hornbach et al. 1984, Way et al. 1990). O:I > 1 indicates dominance by organic particles.

We used a regression approach to examine the effect of TSS (and the related TSI and O:I) on mussel growth, caloric density, and reproductive output. Independent variables were normally

distributed (Shapiro-Wilks' test: TSS, $p = 0.09$; TSI, $p = 0.44$; O:I, $p = 0.55$). We arcsin$\sqrt{(x)}$-transformed proportional data (growth, proportion of females gravid, and proportion of fertilized eggs) to achieve normal distributions. We used mean TSS in each pond during the entire experiment for analysis of mussel growth (mass and length) and caloric density because growth and energy storage would have occurred throughout this period. We used regression to analyze change in size (growth), caloric density, proportion of eggs fertilized, and fecundity for each individual. We used mean TSS in each pond during the putative spawning season (7 September–21 October) for analyses of fecundity and proportion of fertilized eggs because TSS during this period was most likely to affect reproduction.

We used analysis of variance (ANOVA) to test for differences among clearance rates across 8 TSS treatments (1.9, 7.6, 8.9, 15.3, 20.3, 28.5, 30.1, 48.0 mg/L). We used Tukey's Honestly Significant Difference to identify treatment means that differed (SYSTAT 12; Systat Software, Chicago, Illinois).

Results

We succeeded in creating a gradient of TSS by fertilizing ponds (cf. Fig. 3-2A, C, E; Table 1), but ponds with high TSS levels were highly variable. Trophic state of all ponds was eutrophic or hypereutrophic, but only fertilized ponds became hypereutrophic. O:I was usually >1 except in 1 pond that was dominated by inorganic suspended solids (O:I < 1) for 60% of the spawning season (Fig. 3-2B, D, F, Table 3-1). Overall, O:I was variable within and among treatment levels, and mean TSS and mean O:I were not related during the experiment ($R^2 = 0.39$, $p = 0.184$). DO levels were >4.5 mg/L in the water surrounding the pocket nets throughout the experiment.

All mussels except 1 individual survived the 7-mo experiment, and all mussels grew and showed positive change in length and wet mass during the experiment (Table 3-2). However, neither measure of growth was related to TSS (Fig. 3-3A, B). Nutritional status, as indicated by caloric density, also was unrelated to TSS (Fig. 3-3C).

The proportion of females that became gravid during the experiment was strongly related to TSS, and this relationship was best characterized by an exponential decline (Fig. 3-4A). At the lowest mean TSS, 88% of females were gravid, but this percentage declined rapidly with increasing mean TSS, and no gravid females were found at TSS >20 mg/L. Ponds with no gravid females also had a higher proportion of days with TSS >20 mg/L for the duration of the experiment and during the spawning season (Table 3-1). The proportion of gravid females was negatively related to TSI, and no gravid females were found in hypereutrophic conditions (Fig. 3-4B). The proportion of gravid females was not related to mean O:I ($R^2 = 0.063$, $p = 0.631$).

Neither the proportion of fertilized eggs nor total fecundity of gravid females was related to TSS (Fig. 3-4C, D). Mean proportion of fertilized eggs ranged from 0.98 to 0.99 even in ponds with relatively high TSS that exhibited a low percentage of gravid females. Sperm production was not related to TSS. At the end of the experiment, mature sperm cells made up >90% of all cells in each gamete extract for 97% of males ($n = 36$) in all ponds, regardless of TSS, TSI, or O:I.

Clearance rate was negatively related to TSS (Fig. 3-5). Clearance rate appeared to show a threshold relationship in which clearance dropped abruptly at TSS > ~8 mg/L but remained similar at successively higher levels.

Discussion

Contrary to our expectations, growth of mussels was not related to TSS. We saw neither an increase in growth with moderate increase in TSS nor a decrease in growth or survival at the highest levels of TSS as reported by previous investigators (Walker et al. 2001, Valdovinos and Pedreros 2007). All of our ponds were eutrophic, so food probably was never limiting, even at the lowest TSS. The lack of negative effects of high TSS on growth might have been a result of the prevalence of organic particles, which are potential food items, and lower TSS (maximum ≈ 100 mg/L) than in other studies, which showed decreases in growth or increased catabolism of stored energy reserves at very high concentrations of primarily inorganic suspended sediment (e.g., >600 mg/L; Aldridge et al. 1987, Osterling et al. 2007). In addition, *L. subrostrata* typically occurs in eutrophic environments and probably is well adapted to these conditions.

In contrast, TSS had profound effects on reproduction. The percentage of brooding females decreased sharply with increasing TSS and TSI, and complete reproductive failure occurred in hypereutrophic ponds with TSS >20 mg/L. However, fertilization of eggs appeared to be an all-or-nothing phenomenon. Fecundity and the percentage of fertilized eggs did not differ between the few females that became gravid at TSS >~15 mg/L and the many females that became gravid in low-TSS ponds. We did not find females that were brooding unfertilized eggs in any treatment. In the high-TSS ponds, females that were not gravid at the end of the experiment might not have produced eggs at all, perhaps in response to high TSS, or they could have produced eggs that were resorbed or released after failing to become fertilized. The percentage of fertilized eggs in gravid females typically is high in most mussel species except those in which unfertilized eggs impart structure to conglutinates (e.g., *Cyprogenia, Dromas*, *Fusconaia, Pleurobema*) (Haag and Staton 2003, Barnhart et al. 2008, Moles and Layzer 2008), and we have consistently observed a high percentage of fertilized eggs in wild

populations of *L. subrostrata* (Haag in press). Brooding glochidia in the gills can reduce respiratory and feeding efficiency (Richard et al. 1991, Tankersley and Dimock 1993a). Thus, the rarity of unfertilized eggs in many species suggests that females do not retain unfertilized or partially fertilized broods to avoid reduced gill function.

Our results appear most consistent with direct physical interference with fertilization by high TSS. We see at least 2 potential mechanisms for physical interference. First, clearance rates were ~75% lower at intermediate to high TSS than at low TSS, and the likelihood that females encountered sperm during filter feeding may have been similarly reduced. The TSS threshold above which clearance rate was substantially reduced (~8–15 mg/L) was broadly similar to the TSS threshold above which fertilization success decreased sharply (~15–25 mg/L). The apparent discrepancy between these thresholds may be a result of the temporally variable TSS concentrations in the ponds, which could have fluctuated enough to provide brief windows of conditions more favorable for fertilization. Second, high TSS probably increased production of pseudofeces, thereby increasing the probability that sperm were bound in mucus during attempts to clear the gills of heavy accumulations of particulate matter. Asian clams (*Corbicula* spp.) and fingernail clams (*Sphaerium*) initiated pseudofeces production at 17 to 20 mg TSS/L (Fuji 1979, Hornbach et al. 1984, Way et al. 1990). Zebra mussels (*Dreissena polymorpha*) initiated production at 27 mg/L, but production continued to increase as TSS increased (Lei et al. 1996, Schneider et al. 1998). These thresholds are remarkably concordant with the TSS threshold above which we saw reproductive failure. However, thresholds for pseudofeces production in unionid mussels are not well known.

Reduced clearance rates and increased pseudofeces production are not mutually exclusive and would be expected to occur synergistically to reduce sperm acquisition and egg fertilization.

Furthermore, the mode of sperm transfer in mussels helps to explain the all-or-none basis of egg fertilization. Male freshwater mussels do not release sperm singly, but rather in hollow, spherical aggregates called spermatozeugmata, each of which contains ~3600 to 9000 sperm (Barnhart and Roberts 1997, Waller and Lasee 1997). Spermatozeugmata have not been reported in *L. subrostrata*, but they occur in at least 18 other species, including all 5 North American unionid tribes, a pattern suggesting that they are a general feature of freshwater mussels (Haag unpublished data). A brood of a 61-mm *L. subrostrata* (the approximate mean size of females at the end of the experiment) contains ~75,000 eggs (Haag in review), and could be fertilized completely by only 15 spermatozeugmata (assuming 5000 sperm in each spermatozeugmata). However, reduced clearance rate and increased production of pseudofeces could incrementally reduce acquisition of spermatozeugmata and egg fertilization below some critical level necessary for retention and brooding by female mussels.

Our results are concordant with physical interference by TSS with egg fertilization, but we find this mechanism puzzling as an explanation for reproductive failure in mussel populations. The extent to which high TSS may interfere with reproduction in the wild depends on several factors. Levels of organic suspended solids sufficient to cause reproductive failure in our study are probably frequent in eutrophic lentic habitats, and it is interesting that mussel species like *L. subrostrata* that are characteristic of these habitats should be so sensitive to high TSS. However, we have occasionally observed a very low incidence of gravid females of *L. subrostrata*, *Pyganodon grandis*, and *Utterbackia imbecillis* in lentic habitats in Mississippi (see Haag In Press and references therein). The typical late summer and autumn fertilization and subsequent overwintering of the brood in the female gills of these and other species of the tribes Anodontini and Lampsilini, which dominate lentic habitats, may have evolved, in part, to

coincide with decreases in day length and primary productivity and resultant lower TSS.

Previous explanations for reproductive failure or variation in female reproductive output do not account for the patterns in our study. Several authors proposed food limitation as a factor in determining female mussel reproductive output. In *M. margaritifera*, egg production occurred only when females exceeded a minimum body-mass threshold, and increasing energetic surpluses above this threshold were associated with increased fecundity (Bauer 1998). However, food limitation or other energetic constraints do not seem likely explanations for complete reproductive failure in our study because neither growth nor caloric content were related to TSS. Sperm limitation also has been proposed as an explanation for reproductive failure (Downing et al. 1993, Galbraith and Vaughn 2009). This explanation is unlikely for our results because males and females were in close proximity in experimental pocket nets. Moreover, no evidence indicated that sperm production was affected by TSS. Mature sperm cells dominated gamete extracts of all males regardless of treatment.

Hypoxia is often associated with hypereutrophic environments because of high microbial O_2 demand and could negatively affect female metabolic processes or egg survival in the gills. For example, O_2 stress has been proposed as a trigger for females to abort their broods to alleviate reductions in gill efficiency associated with brooding (Aldridge and McIvor 2003). All of our DO measurements were >4.5 mg/L, but these measurements were made during the day, and nighttime DO levels dropped sharply during suspension of phytoplankton photosynthesis. Nighttime drops in DO are a characteristic feature of lentic environments and could have caused females to abort broods, particularly in high TSS ponds, but all of our ponds were constantly aerated to minimize O_2 stress. Moreover, reproductive failure was observed in a high TSS pond dominated by inorganic solids during the spawning season (Pond E, Fig. 2F) and in a high TSS

pond dominated by organic solids (Pond F, Fig. 2F). Diurnal variation in DO should have been less severe in ponds dominated by inorganic solids because of lower biological O_2 demand. Enrichment of water bodies by agricultural fertilizers and resultant increases in primary productivity and decomposition rates can lead to increases in NH_3. Mussels, especially in the glochidia and juvenile stage, are inordinately sensitive to NH_3, and NH_3 toxicity is proposed as a cause of mussel declines in many areas (Augspurger et al. 2003, Wang et al. 2008, Strayer and Malcom in review). Consequently, high NH_3 levels in hypereutrophic ponds could have caused death and abortion of the brood but allowed survival of adult females. We did not measure NH_3 levels in ponds, but several observations are inconsistent with this factor as a cause of reproductive failure. First, adult mussels in all ponds experienced negligible mortality. If adult mussels were stressed by NH_3, we would have expected to see at least some mortality or depressed growth in hypereutrophic ponds. Second, NH_3 levels are typically higher in sediments than in the water column. In the sediments, NH_3 can adsorb to sediment particles and lower DO can reduce the ability of bacteria to detoxify NH_3 (Frazier et al. 1996, Strayer 2008). In our study, mussels were suspended in the water column where they presumably would have been exposed to low NH_3 concentrations.

Primary productivity is typically lower in streams than in lentic habitats, and suspended sediment generally is of less direct importance to aquatic organisms than deposited sediments because, even in agricultural watersheds, very high levels of suspended sediments usually occur for only short periods after storm-flow events (Waters 1995, Borah et al. 2003, Schwartz et al. 2011). Our data show that negative effects on mussel reproduction could occur even with the modest increases in TSS routinely associated with an array of human landscape impacts. For example, 2 y after clear cutting, average monthly TSS concentrations in an Appalachian stream

at base flow ranged from 22 to 57 mg/L for much of the year (including in October, the main period of egg fertilization for many mussel species) and were ~10× as high as in an undisturbed watershed (Gurtz et al. 1980). In the Kaskaskia River basin in Illinois (USA), mean TSS at base flow was 17 mg/L in areas of intensive agricultural, and 14 mg/L in urbanized areas (Miller et al. 2011). These levels approach or exceed thresholds above which complete reproductive failure occurred in our study. Inputs of suspended sediment in streams may be composed primarily of inorganic particles from landscape erosion, and these effects may further limit reproduction by reducing food acquisition and nutritional status of female mussels below critical levels needed for egg production.

Our study provides some of the first data demonstrating a negative effect of suspended sediment on mussel reproduction, and they suggest that elevated TSS could be an important factor in mussel declines in some situations. Additional research is needed to clarify the mechanisms responsible for reproductive failure at high TSS and to examine other factors affecting early reproductive stages. The generality of our results should be evaluated across a range of species' life histories and habitat conditions. Measurement of TSS, especially during periods of egg fertilization, should be part of efforts to identify causes of mussel declines and of evaluations of sites for mussel reintroduction.

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Table 3-1. Characteristics of total suspended solids (TSS) and trophic state index (TSI) in experimental ponds over a 7-mo experiment (April–November) and during the presumed spawning season (7 September–21 October) exposing *Ligumia subrostrata* to varying TSS levels. TSS = 20 mg/L is the threshold above which previous studies have shown disruption of filter feeding. An ratio of organic:inorganic particles (O:I) >1 indicates dominance of organic particles. TSI was calculated for the spawning season only.

Pond	% days during experiment with TSS > 20 mg/L	% days during spawning with TSS > 20 mg/L	% days during experiment with O:I > 1	% days during spawning with O:I > 1	TSI
A	**15**	**16**	**84**	**67**	**64**
B	**4**	**0**	**83**	**84**	**66**
C	**46**	**16**	**86**	**84**	**73**
D	**46**	**55**	**93**	**100**	**60**
E	**90**	**100**	**56**	**40**	**70**
F	**57**	**84**	**100**	**100**	**67**

Table 3-2. Growth of female *Ligumia subrostrata* in experimental ponds from April to November. L_0 and M_0 are mean initial length and mass, respectively; L_1 and M_1 are mean final length and mass, respectively. Mean, min (minimum), and max (maximum) change refer to % changes (e.g., $[L_1 - L_0]/L_0 \times 100$) across all individuals in each pond. *n* = number of mussels.

	Length (mm)					Wet mass (g)				
Pond (*n*)	L_0	L_1	Mean % change	Min. % change	Max. % change	M_0	M_1	Mean % change	Min. % change	Max. % change
A (24)	48.4	58.9	21.5	6.7	33.4	10.8	18.3	73.8	17.4	131.1
B (23)	48.2	63.8	30.6	7.9	59.0	10.7	24.4	133.3	24.5	331.1
C (27)	48.4	62.4	28.7	16.0	38.4	10.6	22.2	106.3	40.0	193.3
D (18)	48.4	60.1	22.5	5.9	37.3	10.8	20.1	87.2	22.6	155.0
E (17)	49	61.8	31.9	13.7	53.7	11.1	21.5	125.2	54.4	225.9
F (26)	47.9	59.3	24.8	1.7	41.2	10.1	18.6	96.3	17.5	180.9

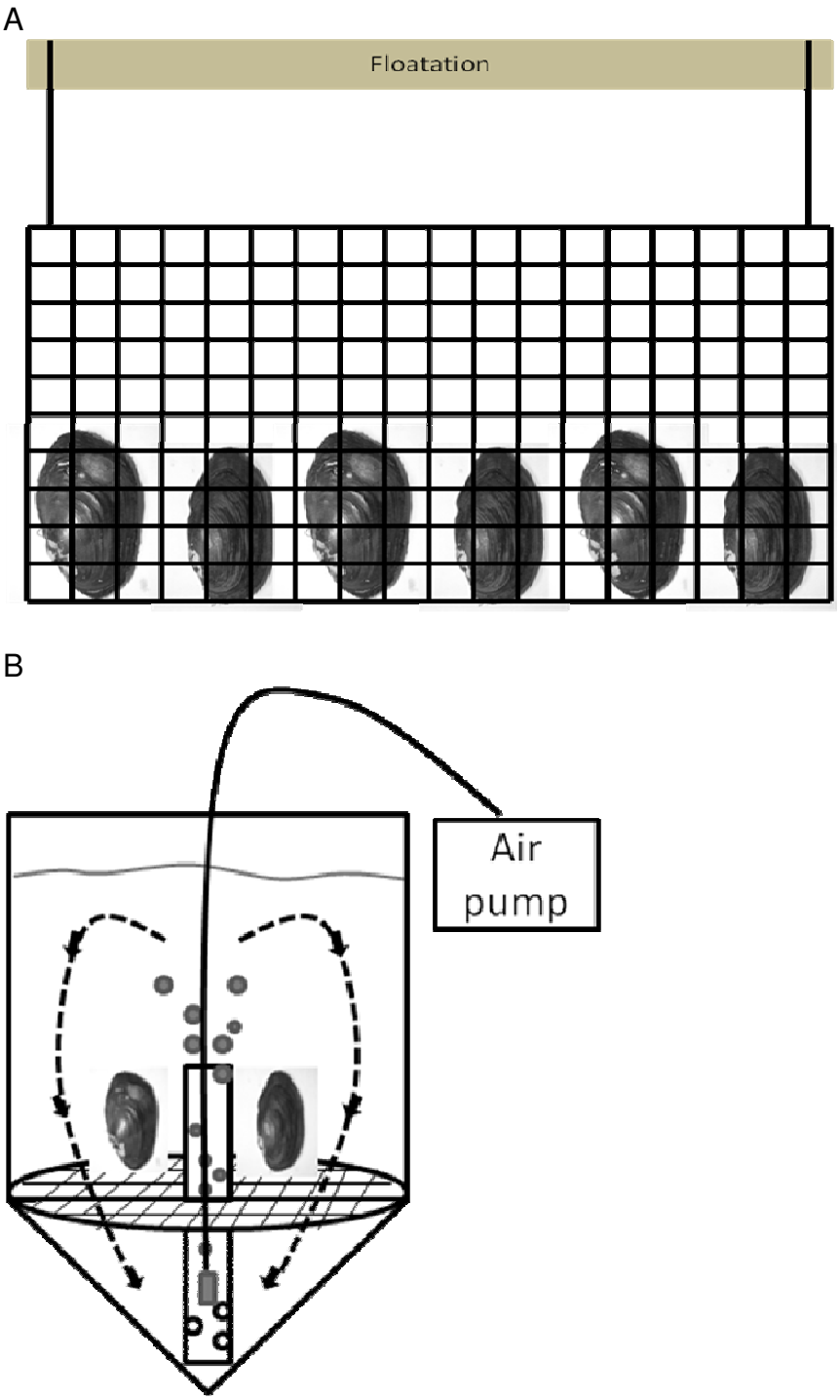

Fig 3-1. A.—Floating pocket nets (20 × 30 cm), constructed of 1.25-cm^2 plastic mesh, used to house mussels during the reproduction experiments. Mussels are not drawn to scale. B.—Aerator-driven, upwelling, cone-bottom clearance rate chambers (57 L) with a 1-cm^2 platform for holding the mussel while allowing the water to circulate throughout the tank. Arrows represent the direction of the flow.

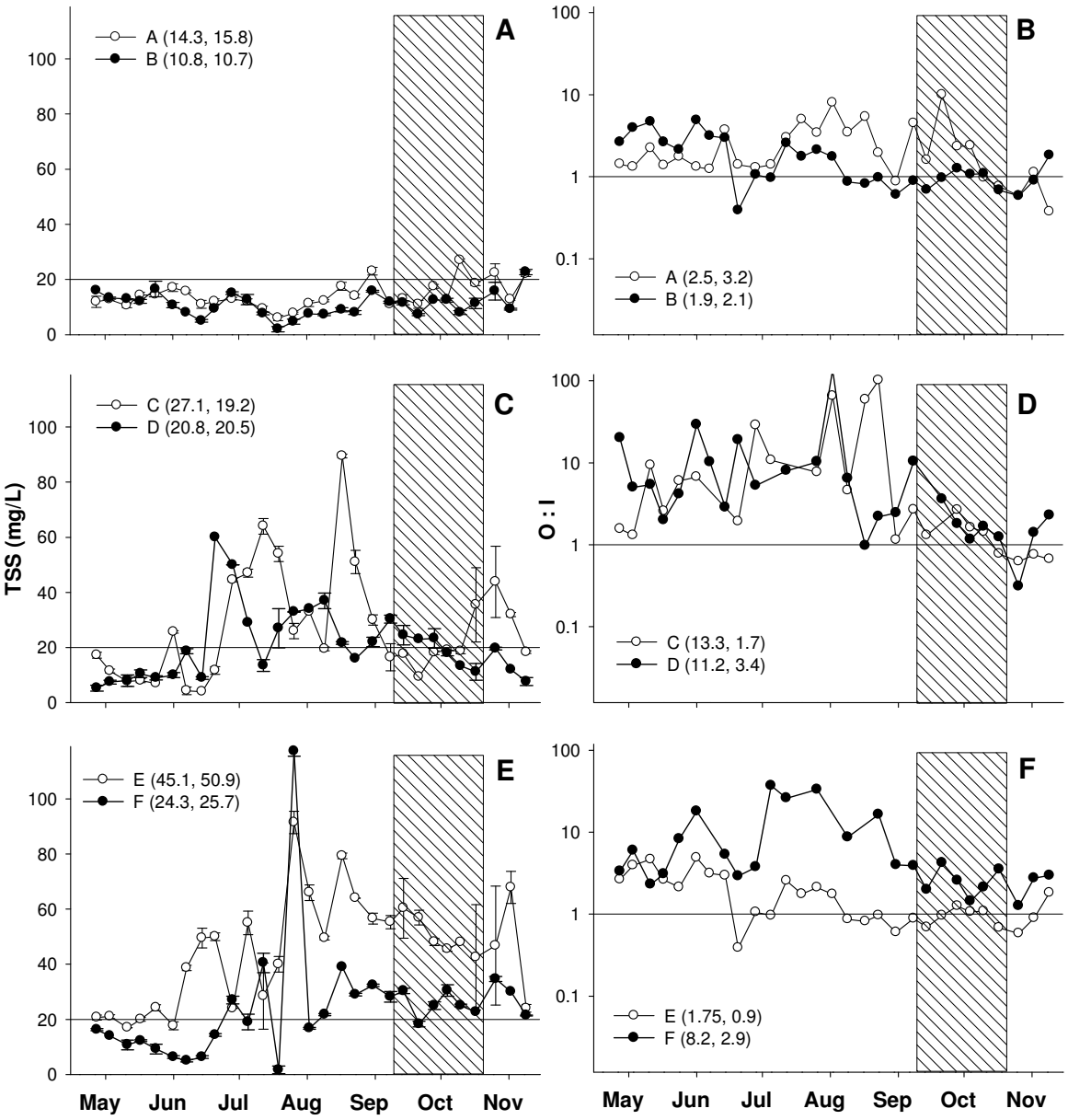

Figure 3-2. Weekly total suspended solids (TSS) (A, C, E) and organic:inorganic ratios (O:I) (B, D, F) in experimental ponds in low (A, B), intermediate (C, D), and high (E, F) TSS treatments over a 7-mo growth and fertilization experiment with freshwater mussels. Letters in figure legends correspond with pond letter codes in Tables 1, 2. Shaded areas show the assumed spawning period (7 September–21 October). The horizontal line in A, C, and E indicates the 20-mg TSS/L threshold, above which previous studies have shown disruption of filter feeding (see text). The horizontal lines in B, D, and F indicate O:I = 1, above which TSS are dominated by organic particles. Numbers in parentheses show mean TSS and O:I for the entire experiment and the spawning season, respectively.

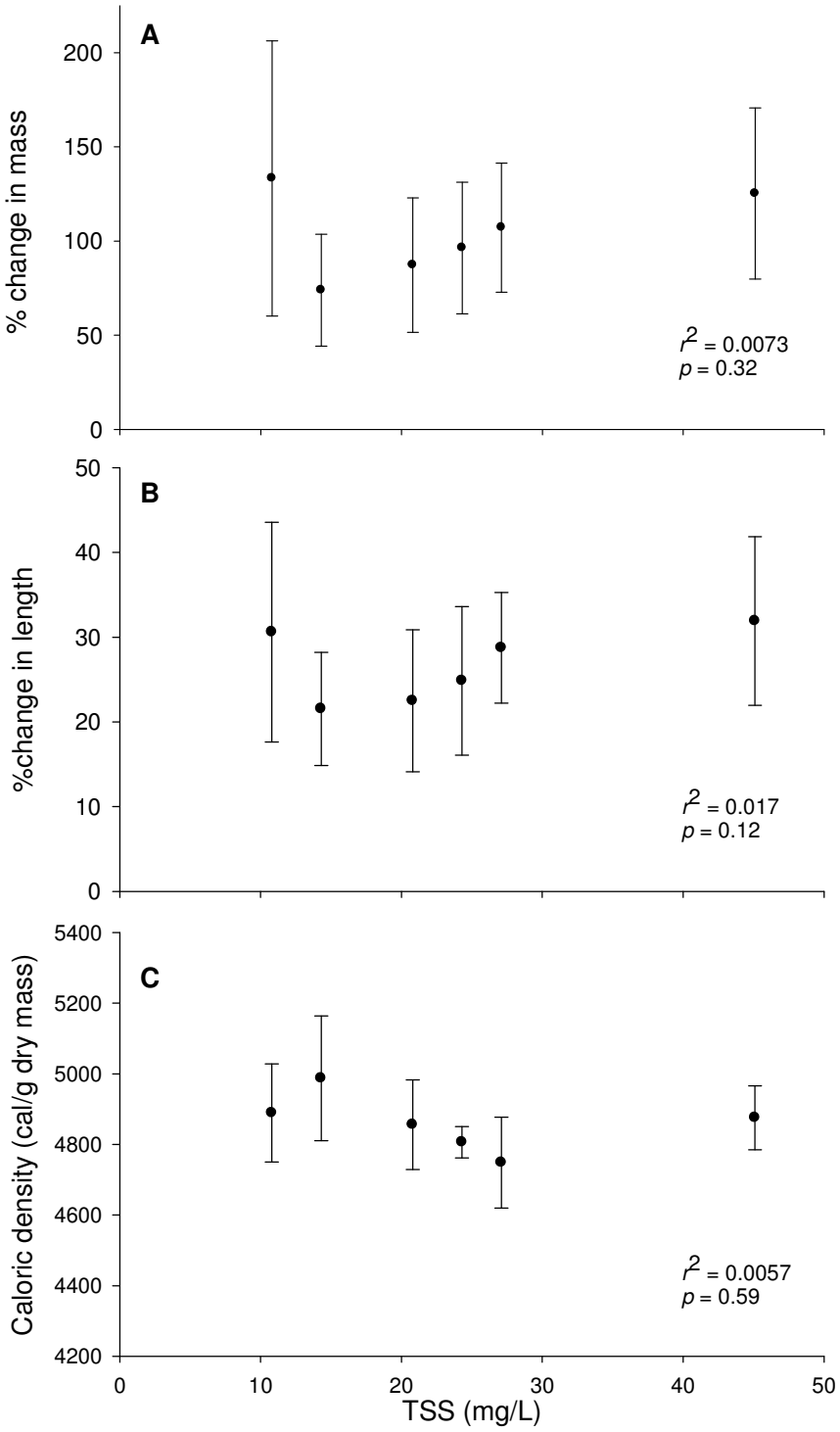

Fig. 3-3 Mean (±1 SD) % change in mass (A) and length (B), and whole-body caloric density (C) of freshwater mussels in individual ponds along a gradient of total suspended solids (TSS) during a 7-mo experiment.

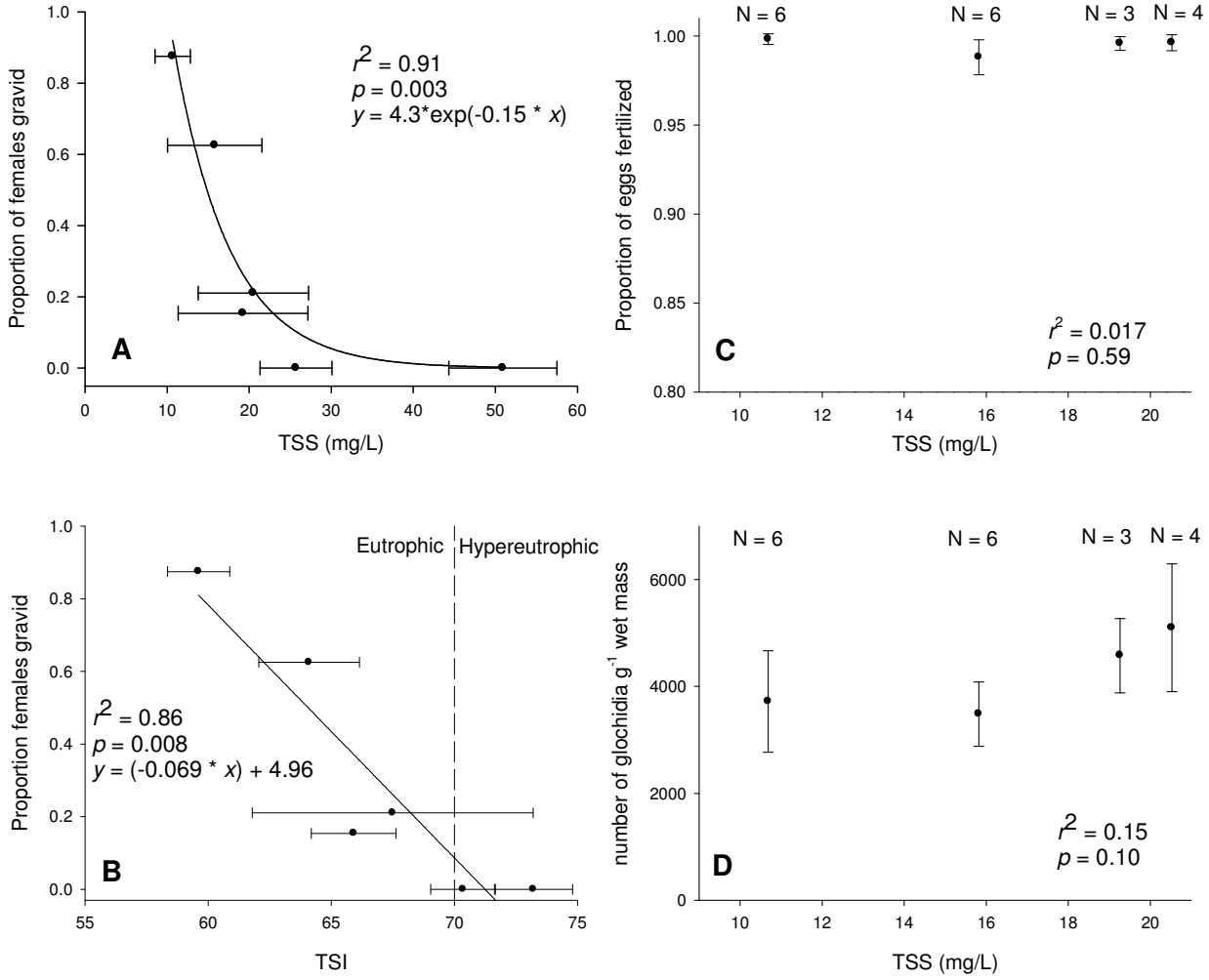

Fig 3-4. Relationships between mean proportion of mussel females gravid and mean (±1 SD) total suspended solids (TSS) (A) and trophic state index (TSI) (B), between proportion of eggs fertilized and mean (±1 SD) total suspended solids (TSS) during the presumed spawning period (C), and between mean (±1 SD) number of glochidia/female and mean TSS (D) in each pond during a 7-mo experiment. In all panels, data points indicate mean values for individual ponds. On panel B, the dashed line shows the threshold between eutrophic and hypereutrophic conditions. On panels C and D, sample sizes are the number of female mussels examined. No females were examined from ponds lacking reproduction (n = 2).

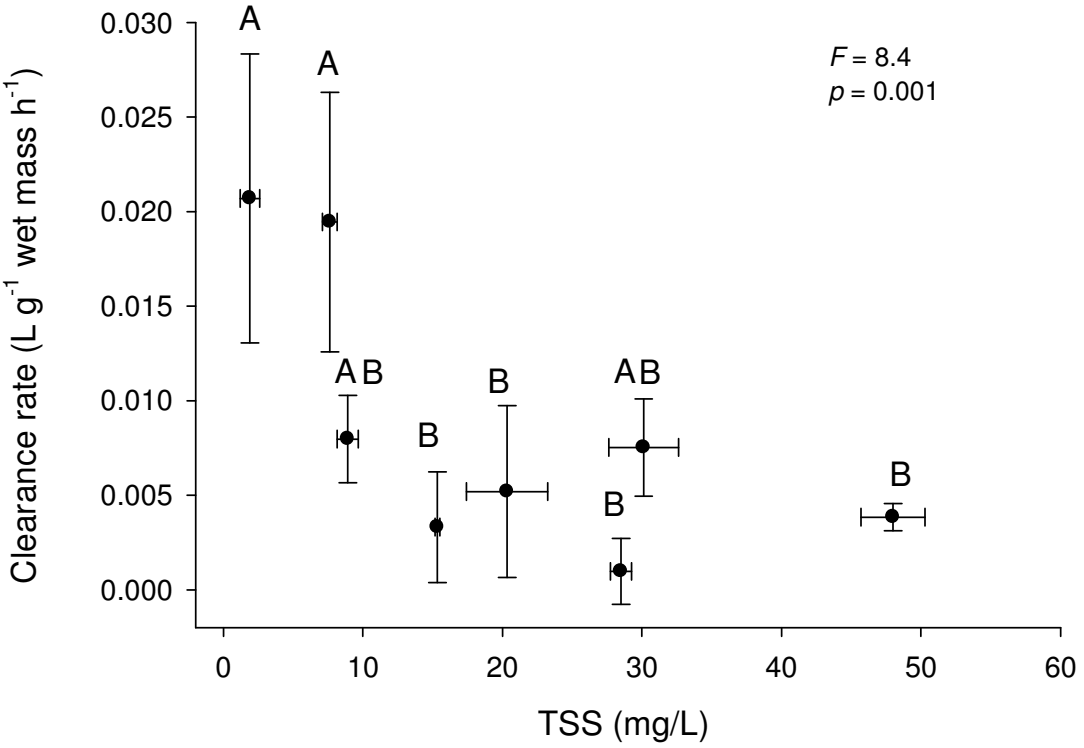

Fig. 3-5 Mean (±1 SD) clearance rate of mussels and mean (±1 SD) total suspended solids (TSS) during the period of clearance trials in a 7-mo pond experiment. Mean clearance rates with the same letter were not significantly different.

Chapter 4

Stage-specific disruption of freshwater mussel reproduction by high suspended solids in short- and long-term brooders

Inorganic suspended solids are one of the most widespread water quality problems in streams and rivers throughout North America. Freshwater mussels are among the most imperiled taxa and yet little is known about how their life history is influenced by suspended solids. In a previous study, we showed that increasing concentrations of total suspended solids (TSS) resulted in a decreased proportion of gravid female mussels; however, the generality of this pattern and the specific stage(s) at which reproduction was inhibited remained unknown. In this study, we examine stage-specific disruption of reproduction in a short-term brooding species (*Reginaia ebenus*) and a long-term brooder (*Ligumia subrostrata*). For the long-term brooder, we also examined effects of chronically high TSS concentrations on glochidial viability over a ~ 5 month brooding period and on metamorphosis success at the end of that period. A high proportion of female *Reginaia ebena* became gravid across the entire TSS gradient (11 to 92 mg/L), but few glochidia developed at TSS >20 mg/L. In contrast, only a low proportion of female *Ligumia subrostrata* became gravid at high TSS concentrations, but all gravid females eventually produced fully developed glochidia. For *L. subrostrata*, neither glochidia viability nor metamorphosis success was reduced by chronic exposure to high TSS concentrations. High TSS concentrations can disrupt reproduction in both short- and long-term brooding species, but the specific mechanism appears to differ between brooding strategies. High TSS does not appear

to negatively affect egg fertilization in short-term brooders, but it appears to inhibit embryo development. In long-term brooders, high TSS appears to interfere with sperm acquisition or egg fertilization, but it does not appear to adversely affect subsequent development of eggs that do become fertilized.

Introduction

Suspended sediments are one of the most widespread water quality problems in the United States. Increases in inorganic suspended sediments are typically correlated with poor land use practices related to forestry, agriculture, urbanization, and exotic bioturbating fish such as common carp (*Cyprinus carpio*) and grass carp (*Ctenopharyngodon idella*) (Wood and Armitage 1997, Lougheed et al. 1998). In general, increases in inorganic suspended sediments negatively affect aquatic ecosystems by decreasing light penetration, disrupting foraging dynamics, and causing sedimentation of substrates (Waters 1995). Although, the effect of suspended sediments on aquatic ecosystems and biota has received considerable attention from aquatic ecologists, little of this research has focused directly on freshwater mussels (Brim Box and Mossa 1999).

Freshwater mussels are the most imperiled group of organisms in the United States (Williams et al. 1993, Strayer et al. 2004). Many factors have been suggested as responsible for the decline of freshwater mussels, but water pollution and water quality problems are among the most commonly cited (Downing et al. 2010). Even though poor water quality, including suspended sediments, has been implicated by many studies, specific life stages affected, and mechanisms behind these effects, remain elusive (Lydeard et al. 2004).

Previous studies have observed limited or no recruitment of freshwater mussels in streams in disturbed watersheds with high concentrations of suspended solids (Houp 1993, Wolcott and Neves 1994) The mechanisms are unclear, but several potential explanations exist. At very high concentrations of suspended clay (1250-5000 mg/L), attachment and metamorphosis success of parasitic mussel larvae (glochidia) was reduced on host fishes in the laboratory (Beussink 2007). Experiments simulating high inorganic suspended sediment caused by barge traffic, demonstrated a decline in clearance rate for three riverine mussel species (Aldridge et al. 1987). The authors suggested this might lead to poor condition and eventual starvation under chronic exposure. In an observational study, *Margaratifera margaratifera* had lower recruitment levels and adult growth in streams with relatively high concentrations of suspended and settled sediments (Osterling et al. 2010), suggesting that poor condition caused by high suspended solids may be the mechanism behind reduced reproductive output (Bauer 1998, Osterling et al. 2010). However, in a previous study we showed that moderately high organic suspended loads (>20 mg TSS/L) reduced reproductive success of *Ligumia subrostrata* even though condition, as assessed by caloric content and growth of females, was unaffected (Gascho Landis et al. 2013).

Two primary brooding strategies are recognized among mussel species, short- and long-term brooders (Haag 2012). Short-term brooding species typically spawn in the spring or early summer and only brood glochidia for a period of days to weeks before initiating transfer of glochidia to host fish. Long-term brooding species typically spawn in the fall and brood glochidia for several months over the winter and begin releasing glochidia in the spring. Brooding glochidia appears to incur a cost to female mussels by reducing respiratory efficiency in long-term brooders (Tankersley and Dimock 1993, Aldridge and McIvor 2003). High levels

of suspended solids decrease respiration rates in zebra mussels (Madon et al. 1998) and marine clams (Grant and Thorpe 1991); thus it is likely that brooding freshwater mussels would experience additional respiratory stress in the presence of elevated suspended solids. This may limit the development of glochidia and lead to the expulsion of puerile conglutinates (Lefevre and Curtis 1912, Aldridge and McIvor 2003). However, difference in gill structure and function between short- and long-term brooders may affect successful reproduction in conditions with high TSS concentrations.

Effects of stressors on glochidia viability is typically examined utilizing acute assays on glochidia that are exposed after being removed from the gills. In these experiments the glochidial stage is repeatedly shown to be a sensitive life stage to toxicants (Bringolf et al. 2007, Wang et al. 2007), but in natural settings glochidia typically spend little time outside the marsupium. In studies assessing chronic exposure of brooded glochidia to toxicants demonstrate little effect on glochidia viability (Jacobson et al. 1997, Hazelton et al. 2012). Long-term effects of high TSS on brooding glochidia viability are unknown and may differ from the long-term effects of toxicants, because chronic high concentrations of inorganic dominated TSS may impair maternal growth and respiration (Aldridge et al. 1987, Madon et al. 1998) and consequently compromise glochidia health.

In the current study, we assessed whether the previously documented negative relationship between reproductive success and suspended solids in long-term brooding species also held true for short-term brooding species. By examining gill contents multiple times throughout the spawning period, we also determined the stage at which the reproductive process was inhibited. If the fertilization process was interrupted by high suspended solids, we expected to see no, or greatly reduced numbers of, fertilized eggs. However, if fertilization occurred but

embryo development was stymied by high solids loads, we expected to see fertilized eggs during initial examinations, but no mature glochidia on subsequent examinations. Finally, we assessed effects of high suspended solids on overwinter brooding success of a long-term brooder as measured by changes in glochidia viability through time, and eventual juvenile transformation success.

Methods

Pond environments

We evaluated spawning and brooding success of freshwater mussels in seven, 0.1 ha ponds at the South Auburn Fisheries Research Unit of Auburn University. An airlift and baffle system was used to create a 15 m long raceway within each pond and maintain similar experimental conditions between this and the previous study with *L. subrostrata* (Gascho Landis et al. 2013). Each raceway provided aeration and an area of slowly flowing water (0.03 m/s) to reduce localized depletion of food resources due to filtering by experimental mussels. We also felt that moving water would reduce stress on the riverine species (*Reginaia ebenus*, formerly *Fusconaia ebena*, (Campbell and Lydeard 2012). To create a gradient of total suspended solids (TSS), dominated by inorganic particles at high TSS concentrations, we manipulated abundances of strongly (common carp) and weakly (grass carp) bioturbating species among experimental ponds to reduce growth of aquatic vascular plants and promote suspension of bottom sediments. For high TSS concentrations we added 6 common carp and 20 grass carp to each of two ponds, for intermediate TSS concentrations we added 20 grass carp to each of two ponds, and for low TSS concentrations we added 2 - 5 grass carp per pond to three ponds.

We collected weekly water samples (1 L) from the raceway at approximately 45 cm from the surface to track TSS concentrations during the fertilization and brooding portions of the reproductive cycle of each mussel species. Total suspended solids were measured by filtering 80 – 200 ml of sample water (depending on particle concentration) through pre-ashed and pre-weighed 47 mm Pall A/E glass fiber filters (1 μm) (APHA 1995). Filters were dried at 105˚C for a minimum of 6 hours, cooled in a desiccator for 15 min, weighed, then ashed at 550° C for 1 h, cooled in a desiccator for 15 min, and reweighed to determine total dry mass and organic solids content, respectively.

We suspended mussels 45 cm from the surface in pocket nets (20 × 30 cm) made from 1.25-cm^2 plastic mesh (27 mussels/net) to prevent mussels from obtaining food resources from the sediments via pedal feeding (Raikow and Hamilton 2001, Nichols et al. 2005). Suspension of mussels allowed us to directly link growth and reproduction to TSS loads. Sex ratios in each pocket net were 1 female: 0.8 male. We placed males and females side by side in pocket nets to minimize sperm limitation, and cleaned pocket nets periodically to maximize water circulation. Temperature loggers were attached to several pocket nets to monitor temperatures throughout the spawning and brooding season. We monitored dissolved oxygen concentrations weekly during the *L. subrostrata* fertilization and brooding trials.

Reginaia ebenus fertilization trials

Reginaia ebenus were collected from Alabama River 8 km west of the town of Camden on February 15, 2011. *Reginaia ebenus* is primarily a large river species (Williams et al. 2008), but previous trials demonstrated that it exhibits high survival in pond raceways (AMGL and JAS

unpublished data). It is a short-term brooder, gravid from May to August (Coker et al. 1920), and is one of the most abundant species in Alabama (Williams et al. 2008).

Reginaia ebenus is not sexually dimorphic and requires gamete extraction for sex determination (Saha and Layzer 2008). Upon returning to the laboratory all mussels were examined for gametes so equivalent sex ratios could be placed in each pond (15 females and 12 males; 1 : 0.8). Sex ratio of the animals returned from the field was 1:1, but we used a slightly greater proportion of females in each pond to increase our sample size. .

To determine the influence of TSS on fertilization success and subsequent embryo development, we examined female mussels four times during the spawning period, each sampling event separated by 5-7 days (5/20, 5/26, 5/31, 6/7/2011). It was difficult to determine the exact start date of the spawning season for *R. ebenus*; however, a concurrent study, in a similar pond environment, documented evidence of spawning on May 13, 2011 (Mosley 2012). Our first gill extractions were performed a week after the first confirmation of spawning, to ensure that females had the opportunity to become fertilized. On each sampling occasion we extracted a small amount of fluid from the gill of each female using a 22 gauge needle and examined the contents under a microscope at 40 X magnification. Extract from the gill was classified as empty, unfertilized eggs, fertilized eggs, or glochidia. We distinguished between fertilized and unfertilized eggs by the presence/absence of cell division. Glochidia included all embryos that had a detectable shell. If gills contained glochidia, the female mussel was sacrificed to assess fecundity (# glochidia per gram of gravid female) and fertilization efficiency (glochidia or fertilized eggs / (glochidia or fertilized eggs + unfertilized eggs)).

In contrast to long-term brooders, short-term brooders do not retain glochidia for extended periods of time and may abort broods when disturbed via handling (Haag and Warren

2003, Barnhart et al. 2008). To determine the relationship between fertilization success, glochidial development, and TSS concentrations we first regressed the proportion of females observed with fertilized eggs or mature glochidia on the first sampling event against average TSS concentrations. This eliminated the effects of handling, but may have missed late spawning females. Thus, we also regressed both the proportion of females observed with either fertilized eggs or glochidia at least once during the four checks against average TSS. This reduced the problem of late spawning, but increased the potential influence of handling. To determine whether females ceased reproductive activity after handling, we calculated the proportion of females handled on the first day that possessed either fertilized eggs or glochidia on at least one of the subsequent three sampling events. To determine whether post-handling reproductive activity differed with TSS concentration, we first separated ponds into two groups based on previously identified favorable (<20 mg / L, n = 4 ponds) and unfavorable (> 20 mg / L, n = 3 ponds) TSS concentrations (Gascho Landis et al. 2013). We then calculated the proportion of handled females that possessed fertilized eggs and the proportion that possessed glochidia on at least one of the final three sampling dates in favorable and in unfavorable TSS ponds, separately. We used a G test of independence to examine for differences between TSS groupings for the proportions of females with fertilized eggs and for proportion of females with glochidia.

Ligumia subrostrata fertilization trials

Ligumia subrostrata is a lentic, long-term brooding species commonly found throughout the eastern United States (Corey et al. 2006). This species typically spawns in late summer or early fall and broods their glochidia until the following spring (Gascho Landis et al. 2012). *Ligumia subrostrata* used in the fertilization trials were a mix of animals collected from wild

populations (Jackson Lake, MS) and their progeny, produced in ponds as the result of prior experiments at South Auburn Fisheries Research Unit (WRH and JAS unpublished data). *Ligumia subrostrata* is a sexually dimorphic species, and thus no gamete extracts were performed to identify sexes. Males and females from both sources were mixed together and 15 females and 12 males were randomly assigned to each pond, to maintain the same sex ratio as the *R. ebenus* fertilization trial.

All mussels were uniquely tagged using Hallprint tags attached with cyanoacrylate glue (Lemarie et al. 2000). Total wet mass of each individual was recorded at the start and completion of the fertilization trial. Growth was quantified in terms of the proportional increase in wet mass ((final – initial) / initial). We used ANOVA with a Tukey HSD post-hoc analysis to test for differences in growth between ponds over the 3 month trial. All data was arc-square root transformed before analysis, but non-transformed data are shown in our figures. For this analysis, we used data only from female mussels produced at South Auburn Fisheries Research Unit, because they were all of the same age (2 yr.), were expected to still be experiencing relatively rapid growth rates, and comprised the majority of experimental mussels.

In a separate 0.1 ha pond (tracking pond) we used periodic gill extracts to track the timing of *L. subrostrata* spawning to 1) determine when to examine experimental mussels for early stage fertilization, and 2) to determine the approximate duration of the spawning season during which to calculate average TSS concentrations. We placed 30 female and 24 male mussels in pocket nets (8 males and 10 females per net) and monitored spawning in the tracking pond for 11 weeks. Beginning on August 22, 2011, we examined 5, randomly selected female mussels per week for six weeks. Because of the limited number of mussels, during weeks 7 to 11 we re-examined each group of 5 mussels in the same order they were initially examined from

week 1 to 6. On week 8 we combined two groups of mussels (originally examined on week 2 and 3, N = 8, 2 suffered mortality) for a larger sample size to confirm spawning activity. On each sampling occasion we performed a gill extract using a 22 gauge needle and syringe and examined the contents under a microscope (40X). We monitored secchi depth in this pond throughout the spawning period and it was always between 1.1 and 1.4 m, corresponding to TSS concentrations of 7-11 mg/L (AMGL and JAS unpublished data)

We first examined and classified gill contents of experimental females on October 11 (following methods detailed above), two weeks after the first observation of fertilization in the tracking pond. Females were returned to their respective ponds until several weeks after the close of the spawning season (November 11), at which time all females were again removed and brought to the lab for a second examination of gill contents. After gills of all females had been examined, we sacrificed 6 brooding female mussels per pond, when available, to assess fecundity and fertilization efficiency. We excised both gills from each female and repeatedly flushed gills with artificial fresh water (AFW: 50 mg $CaCO_3$, 25 mg CaCl, 50 mg Na_2CO_3, and 5 mL 30‰ salt water/L deionized water) until all glochidia had been removed. Glochidia from individual females were diluted and mixed in 1000 – 2000 mL AFW, depending on the number of glochidia, and 3, 1 mL subsamples were counted. We estimated total fecundity by multiplying the mean number of glochidia in the three subsamples by the dilution volume. During fecundity estimates we also counted the number of unfertilized eggs to estimate fertilization efficiency (see above). We used a salt test to determine glochidia viability of three gravid mussels per pond. Ten, 100-200 µL subsamples were withdrawn from the glochidial sample of each female and the numbers of open and closed glochidia in each subsample were enumerated. Several drops of 35‰ salt water were added to each subsample, which was then recounted for the number of open

and closed glochidia. To calculate glochidia viability we used the following formula (R. Brigolf personal communication):

$$\text{Viability} = \frac{(\#\text{ of open glochidia initially} - \#\text{ of glochidia open after adding salt})}{\text{total number of glochidia}}$$

Our initial intent was to use regression analysis to assess reproductive success (proportion gravid females, fertilization efficiency, and fecundity) across a TSS gradient; however, in this experiment our pond gradient during spawning was limited to low and high TSS concentrations with no intermediate values (see results). We therefore grouped ponds with similar TSS concentrations to compare reproduction between nominally high (average TSS = 79.1 ± 0.35, n = 2) and low (average low TSS = 9.4 ± 2.6, n = 4) ponds. We used Fishers exact test for proportions to compare proportion of gravid females between the two groupings, and ANOVA to compare fecundity, fertilization efficiency, and glochidia viability across all ponds. The pond with the lowest TSS concentrations was not included in the analysis because mussels in this pond grew significantly less than those in every other pond (see results), indicating confounding effects of poor condition.

Ligumia subrostrata glochidia health during brooding

To assess the influence of TSS concentration on *L. subrostrata* glochidia viability and transformation throughout the brooding period we used a subset of ponds from the fertilization trials, but only made comparisons between low (n = 2 ponds) and high (n = 2 ponds) TSS environments. All mussels (N = 66) used in this trial were brooding age-0 female produced from a single, low TSS pond (hereafter referred to as the source pond) as part of a concurrent study at

South Auburn Fisheries Research Unit (WRH and JAS unpublished data). Total suspended solids were not recorded throughout the growing and spawning season from the source pond; however, secchi depth from May – October was always between 1.1 and 1.6 m, corresponding to TSS concentrations of ~8-10 mg/L (AMGL unpublished data). In November 2011 brooding females were removed from the source pond, placed in pocket nets, and randomly assigned to each of four experimental ponds (15 brooding females per pond). To determine the average glochidia viability at the start of the trial, a salt test (see above) was conducted on glochida from six female mussels from the source pond. These females were not used in the subsequent experiment. Once a month in December, January, February, and March three unique females were collected from each experimental pond and a 22 gauge needle containing 5 ml of AFW was used to flush glochidia from the gills of each female. We then assessed glochidia viability using a salt test. Following gill flushing, mussels were returned to the same experimental pond from which they had been collected. To test for monthly differences in glochidia viability between ponds, we used ANOVA with a Tukeys HSD post-hoc test.

To determine whether TSS affected female mussel growth during the brooding season, we recorded length and mass of each individual on November 15, 2011, and then again for the subset of mussels (N = 3) collected monthly from each pond for glochidia viability. We used ANOVA to test for differences in proportion increase in mass (arc-sine square root transformed) between ponds for each month of the brooding period.

We allowed three mussels to brood glochidia in each experimental ponds through March without gill extractions, at which time we infested a known host - bluegill *Lepomis macrochirus* (Stern and Felder 1978) with glochidia of females from each experimental pond. However, in two ponds, 1 low and 1 high, 1 female had already released glochidia and thus only 2 females

were used. Females were sacrificed and gills removed and flushed with AFW to collect glochidia. Glochidia from the 2-3 females of a given pond were pooled and placed in one of four 10 L inoculation baths at a density of 1100 individuals/L (one inoculation bath per pond). Twenty bluegill (mean length = 91.9 mm ± 5.5 S.D., mean mass = 8.2 g ± 1.8 S.D., n = 20), collected from a pond without unionid mussels, were placed simultaneously in each inoculation bath for 15 minutes (n = 80 total bluegill). Fish were then transferred to AHAB tanks (Aquatic Habitats) at a density of 5 fish per three liter tank, with all fish within a given tank infested with glochidia from the same pond. All sixteen AHAB tanks (4 tanks per experimental pond) were held within a single recirculating system with 150 μm mesh cups at the outflow of each tank to collect sloughed glochidia and juveniles. A 25 μm inline filter was mounted on the water supply line going from the common sump to the AHAB tanks to intercept any sloughed glochidia or juveniles that unexpectedly bypassed the 150 μm mesh cups. Water temperature was maintained at 22˚C ± 1. Fish placement was randomly stratified between the two shelves of the recirculating system with two tanks per treatment on each shelf.

Outflow filter cups were examined every other day for sloughed glochidia and metamorphosed juveniles. Because some fish died during the experiment, we quantified daily production as the observed number of glochidia or juveniles divided by the number of live fish in the tank. We calculated the metamorphosis (%) for each tank as the cumulative number of juveniles produced per live fish per day, divided by the cumulative number of glochidia initially attaching per fish (sloughed glochidia plus juveniles; (McNichols et al. 2011). We used ANOVA to test for differences in metamorphosis rates among glochidia brooded in the four different ponds.

Results

Reginaia ebenus fertilization trials

The ponds exhibited a wide range of TSS concentrations (10.8-92.3 mg/L) during the spawning season (Table 4-1). There was a significant negative exponential relationship between O:I ratios and TSS ($R^2 = 0.65$, $p = 0.02$), such that ponds with highest TSS concentrations were dominated by inorganic suspended solids and ponds with lower TSS concentrations were dominated by organic suspended solids. Differences in pond TSS concentrations did not translate into differences in average daily and maximum temperatures among ponds during the spawning season ($F = 1.3$, $p = 0.28$; $F = 1.1$, $p = 0.37$, respectively).

Fertilization of female *R. ebenus*, indicated by the presence of fertilized eggs or glochidia in the gills, occurred in all ponds across the TSS gradient. On the initial sampling date we found no significant relationship between TSS and proportion of females with fertilized eggs or glochidia, although no individuals from ponds with TSS >20 mg/L contained glochidia (Figure 4-1A, B). It appeared that timing of spawning differed between females within the same pond such that some had only fertilized eggs in their broods while others had only glochidia (Figure 4-1B). No mixed broods (fertilized eggs plus glochidia) were observed in any female. Across all sampling occasions, a high proportion of females in each pond became fertilized (>0.67), with the exception of one pond (0.3 females fertilized). We found no significant relationship between the proportion of females fertilized and pond TSS concentration when all sampling events were considered (Figure 4-1 C). In contrast, across all sampling occasions, proportion of females with mature glochidia declined exponentially with increasing TSS such that 20% or less of females produced glochidia at TSS concentrations >20 mg/L (Figure 4-1 D). Due to a low sample size of females with glochidia at high TSS, and because some females were removed for a concurrent

host identification trial, we were not able to examine fecundity and fertilization efficiency across the TSS gradient.

Across all TSS concentrations, 44 % of females handled and returned to their pond on May 20, 2011 exhibited either fertilized eggs or glochidia on subsequent sampling events (Table 4-2). Females in ponds with TSS >20 mg/L were just as likely to exhibit fertilized eggs after the initial handling event as females from ponds with TSS <20 mg/L (23% vs. 27%, G = 0.155, d.f. = 0.69, p = 0.69). However, in ponds with TSS >20 mg/L females were significantly less likely to develop glochidia as compared to females from low TSS ponds (9% vs 45%, G = 13.0, d.f. = 1, p = 0.00; Fig. 4-2).

Ligumia subrostrata fertilization trials

During the *L. subrostrata* spawning season, the two ponds with common carp exhibited high TSS concentrations while one pond containing only 2 grass carp exhibited extremely low TSS concentrations (Table 4-1). However, we were not able to create a gradient in pond TSS concentrations between these two extremes: no ponds exhibited average TSS concentrations between 13 and ~70 mg TSS/L. Similar to the *R. ebenus* experiment, ponds with high TSS concentrations were dominated by inorganic particles, whereas ponds with low TSS concentrations were dominated by organic particles. Daily average and maximum temperatures were similar across ponds throughout the spawning season (F = 1.1, p = 0.34; F = 0.95, p = 0.42, respectively) and dissolved oxygen concentrations were also similar across ponds (F = 1.5, p = 0.21) and were never below 7 mg/L.

Female *L. subrostrata* with either fertilized eggs or glochidia were first observed in the tracking pond on September 26, 2011, indicating that spawning was initiated sometime during

the previous week (September 19-26; Figure 4-3). The proportion of females fertilized continued to rise each week and peaked on October 17, when 80% (4 out of 5) of females had glochidia. Thus, we considered the main spawning season to be September 19 – October 17.

Female mussels in the pond with lowest TSS (1.8 mg/L ± 1.6) grew significantly less than mussels in all other ponds (ANOVA; $F = 7.6$, $df = 6$, $p < 0.00$, Tukeys HSD test; all $p <= 0.01$), but no differences were observed among all other ponds (Figure 4-4).

Gill examinations of female *L. subrostrata* in the experimental ponds two weeks after the first observation of spawning (October 11, Figure 4-5 A, B), showed broods of either fertilized eggs or glochidia, but never a mix of eggs and glochidia together in the same brood. Similar to the *R. ebenus* experiment, a mix of brood types occurred among females within the same pond (Fig. 4-5 B) suggesting that fertilization was not synchronous. However, on the subsequent gill extraction (November 11, 2011), all individuals previously observed with fertilized eggs now contained glochidia, even in the high TSS ponds (Figure 4-5 C, D). No females that were unfertilized in October became fertilized by November. A significantly lower proportion of females in the two highest TSS ponds produced fertilized eggs or glochidia than in the next four lower TSS ponds in both October and November ($Z = -4.1$ and $p = 0.00$; $Z = -4.3$, $p = 0.00$, respectively).

No significant differences in fertilization efficiency (fertilized eggs / (glochidia + fertilized eggs): $F = 0.3$, d.f. = 5, $p = 0.91$) or fecundity (# glochidia / g dry weight: $F = 1.3$, d.f. = 5, $p = 0.29$) were found among ponds (Figure 4-6 A, B). Additionally, there was no difference in glochidia viability (salt test) among TSS concentrations ($F = 1.6$, d.f. = 5, $p = 0.24$), with average viability always >85% (Figure 4-6 D).

Ligumia subrostrata: glochidia health during brooding

The two ponds with common carp maintained high TSS concentrations (>50 mg/L) over the winter brooding period, whereas the two ponds without common carp had consistently low concentrations of TSS (<20 mg/L, Table 4-1). All ponds exhibited O:I ratios < 1, likely due to senescence of the algae community. Pond temperatures were highly variable over the winter brooding period, but average daily temperatures remained relatively warm (>10˚C, Table 4-1, Figure 4-7). Dissolved oxygen concentrations were not significantly different ($F = 0.4$, $p = 0.75$) and was never below 8 mg/L)

Average glochidia viability (salt test) at the start of the brooding period on November 11, 2011 was 84% ± 9 S.D. Glochidia viability was significantly lower in December and January at the highest TSS concentration (Figure 4-7 A, $F = 3.9$, d.f. = 3, $p = 0.05$; $F = 20.2$, d.f. = 3, $p = 0.00$, respectively). However, during February and March there was no significant difference in glochidia viability among TSS concentrations ($F = 2.3$, df = 3, $p = 0.15$; $F = 0.71$, df = 3, $p = 0.59$, respectively). Brooding mussels continued to grow over the winter and proportion increase mass was not significantly different among TSS concentrations during any month (Figure 4-7B, December: $F = 3.5$, df = 3, $p = 0.6$; January: $F = 1.0$, df = 3, $p = 0.44$; February: $F = 2.6$, df = 3, $p = 0.12$, March: $F = 2.7$, df = 3, $p = 0.11$). Metamorphosis success was not significantly different for glochidia brooded in ponds with contrasting TSS concentrations (Figure 4-8, $F = 0.83$, d.f. = 3, $p = 0.50$).

Discussion

High concentrations of suspended solids affected reproductive success of both short- and long-term brooding mussel species. Ponds dominated by high inorganic TSS produced similar

declines in reproductive success as observed in previous studies with ponds dominated by organic TSS (Gascho Landis et al. 2013). In the previous study, reproduction was eliminated for *L. subrostrata* at TSS >20 mg/L. In the current study, *R. ebenus* showed a similar, significant decline in proportion of females becoming fertilized at TSS >20 mg/L. While we lacked a complete gradient for the *L. subrostrata* trials in the current study, mussels in ponds with average TSS >20 mg/L had low reproductive success, but mussel in ponds <20 mg/L had relatively high reproduction success. An interesting exception to this pattern was the low proportion of females fertilized occurring in the pond with an average TSS of ~1mg/L. Low growth rates in this pond suggest females were most likely food limited and not able to meet the energetic demands required for reproduction. Similarly, populations of *M. margaritifera* had a low proportion of brooding females in low productivity environments (Bauer 1998, Osterling et al. 2010). .

Although we observed a similar pattern of low fertilization for both short- and long-term brooders at TSS >20mg/L, it appears that different stages of the reproductive process are interrupted between species. Fertilization success of *R. ebenus* was not negatively affected, but subsequent development of fertilized eggs to the glochidia stage was limited. In contrast, fertilization success of *L. subrostrata* was limited, but subsequent development of fertilized eggs to the glochidia stage was not affected. This difference between short- and long-term brooding species is intriguing and may be related to differences in habitat use; *R. ebenus* is a large river habitat specialist, while *L. subrostrata* prefers lentic habitats (Williams et al. 2008). Gill anatomy is different between some lotic and lentic species, with lotic species having a greater cilia density on the cirral plate than lentic species (Silverman et al. 1997). This difference was hypothesized to be responsible for higher clearance rates of small particles by lotic species, although these results were based on a relatively small sample size of species. While *R. ebenus* and *L.*

subrostrata gill anatomy and structure has not directly been examined, potential differences in gill structure may play an important role in sperm capture. *Reginaia ebenus* may be more efficient at sorting sperm particles, especially in face of interference from high inorganic particle concentrations. Furthermore, large river, filter-feeding specialists may also be adapted to sorting inorganic particles which occur frequently in these ecosystems (Soeken-Gittinger et al. 2009) and could explain why *R. ebenus* had relatively high proportion of fertilized females even at high TSS concentrations.

Additionally, differences in gill use between species may explain the stage at which reproduction was interrupted. Most Lampsiline mussel species (including *L. subrostrata*) brood glochidia in the posterior portion of the two outer gills (Haag 2012), likely because they are long-term brooding species and cannot compromise the function of all four gills for the duration of a 6-8 month brooding period. In contrast, all Amblemine (inclusive of *Reginaia ebenus*) brood glochidia using the entirety of all four gills (Williams et al. 2008), but are short-term brooders, holding developing eggs and glochidia for only 2-6 weeks before releasing. While *R. ebenus* became fertilized, embryos may not have developed because of the additional respiratory stress due to brooding at high TSS concentrations. These embryos may have been parturitioned so as not to compromise the health of the mother (Aldridge and McIvor 2003). An alternate explanation is that the females at high TSS concentrations released their glochidia between gill extractions as a result of handling. This seems unlikely because *R. ebenus* across the TSS gradient were handled similarly, and mussels from low TSS ponds continued to develop glochidia after handling. If mussels in high TSS ponds did produce or continued to produce glochidia we should have observed them at a similar frequency. In contrast, the embryos of the

few *L. subrostrata* females (n = 4) that became fertilized in high TSS ponds developed into mature glochidia, likely because females retain two gills with the primary function of respiration.

Many other factors influence freshwater mussel reproduction and could provide alternate explanations for low reproductive success at high TSS. However, we feel that physical interference with particle capture, and compromised gill function, remain the most likely explanations for the observed reductions in fertilization success and early embryological development at high TSS concentrations. Temperature is an important factor in freshwater mussel reproduction (Galbraith and Vaughn 2009) and varying water reflectance associated with TSS concentration may affect warming rates (Butler 1962, Kara et al. 2004). If high TSS ponds warmed more quickly it could have caused earlier spawning, such that we missed the spawning season in those ponds but not the others. However, we did not observe differences in temperature between ponds. Similarly, high TSS concentrations could have led to lower DO concentrations due to high biological oxygen demand associated with decomposition of organic material or due to nighttime algal respiration. However in this study, high TSS ponds were dominated by inorganic rather than organic particles. Also, all ponds were aerated and dissolved oxygen concentrations remained above 7 mg/L. Freshwater mussel condition, measured as individuals with above average weight, has also been linked to reproduction (Bauer 1998). The cost of sorting large quantities of inorganic particles might limit energy acquisition (Schneider et al. 1998) and limit excess energy available for growth and reproduction. Although we observed depressed growth rates at the lowest TSS concentrations, we saw no differences in percent growth increases for *L. subrostrata* across the range of TSS (for TSS >6 mg/L) indicating that mussel condition was not affected at high TSS concentrations.

In contrast to fertilization success, glochidia health during the overwinter brooding period of *L. subrostrata* appeared minimally affected by high concentrations of suspended solids. Neither glochidial viability nor transformation success differed between low and high TSS ponds at the end of the brooding period. Marsupial gills appear to buffer brooding glochidia from environmental contaminants in the water column. Previous studies examining viability and metamorphosis success of in-marsupial glochidia found little effect of long-term exposure to contaminants as long as glochidia remained in the marsupium, but did find significant effects of contaminants if glochidia were exposed after removal from the marsupium (Jacobson et al. 1997, Hazelton et al. 2013). Additionally, all brooding females in our study grew at similar rates throughout the winter (> 20% increase in mass). Thus, we found no evidence that high TSS levels stressed either adult mussels or their brooding glochidia over the winter months when temperatures were cool (mean daily temperature < 15 C) and dissolved oxygen concentrations were relatively high (> 8 mg/L). However, we only tested TSS loads up to 80 mg/L and it is possible that negative responses occur at higher concentrations.

Although glochidia viability did not differ among ponds by the end of the brooding period, viability was significantly lower in high TSS ponds at the beginning of the brooding period. The mechanism behind this pattern is unclear. It is possible that dead or unhealthy glochidia were either resorbed or expelled from the marsupium by the third month, thereby improving the observed viability of the remaining brood. This would have resulted in a decrease in glochidia per brood by the third month. Alternately, it may be that high TSS levels resulted in initial poor health of glochidia, but these glochidia survived and eventually regained their viability. In this case, fecundity (glochidia per brood) would not have decreased over the winter months. Unfortunately, we did not quantify fecundity at the beginning and end of the brooding

experiment, and it is unknown whether individual glochidia can be sorted, selectively resorbed, or expelled during the brooding process. If glochidia were absorbed or expelled during the overwintering period and fecundity did in fact decrease, we may have missed an important negative effect of high TSS concentrations during the brooding phase.

Both eutrophication (organic particles) and erosion (inorganic particles) are likely to have negative influences on freshwater mussel reproduction at TSS >20 mg/L. This study suggests the seasonal timing and duration of high TSS concentrations is important for predicting the response of a freshwater mussel population. High TSS events occurring primarily during the spawning, fertilization, and early embryo developmental stages are likely to have the greatest detrimental effect. The periods of vulnerability differ between short-term and long-term brooding species due to seasonal differences in spawning periods (spring/summer vs fall).

Our study determined that suspended solids have complex, stage-specific effects on freshwater mussels' reproductive cycle. While high suspended solids did not have a negative influence on brooding glochidia, the consistent negative impacts of TSS concentrations > 20 mg / L on early reproductive processes across species (short- and long-term brooders) and solids type (inorganic or organic) is concerning. This bottleneck in the reproductive process may be responsible for observed declines in some mussel populations. The seasonal timing and duration of high TSS spates are likely to be important predictors regarding the reproductive success of freshwater mussel populations. High TSS events occurring during the spawning and early embryo developmental stages are likely to have the greatest detrimental effect on both long- and short-term brooding species, but temporal periods of vulnerability may differ between these groups due to seasonal differences in timing of spawning (spring/summer vs fall). Elevated TSS concentrations are now ubiquitous throughout North America (Waters 1995) due to erosion and

eutrophication. Efforts need to be undertaken to curb quantities of sediments and nutrients entering streams and rivers. Without substantial improvements in watershed management the outlook for many freshwater mussel assemblages is grim (Poole and Downing 2004).

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Table 4-1. Temperatures, total suspended solids (TSS), and organic:inorganic ratios (O:I) for ponds in all three trials (mean ± 1 SD).

Pond treatment	Average Temp (C)	Max Temp(C)	Spawning period TSS	Spawning period O:I	Entire Trial TSS	Entire Trial O:I
***R. ebenus* spawning**						
2 grass carp	29.6 ± 2.8	31.3 ± 2.9	14.1 ± 3.3	4.8 ± 39	23.4 ± 19	2.3 ± 3.1
2 grass carp	29.6 ± 2.5	31.4 ± 2.6	15.5 ± 2 .4	10.7 ± 10.5	16.4 ± 8.7	5.8 ± 8.4
5 grass carp	29.8 ± 2.3	31.3 ± 2.4	10.8 ± 3.4	7.2 ± 8.1	6.3 ± 4.9	8.4 ± 7.5
20 grass carp			22.5 ± 2.6	7.2 ± 11.0	20.4 ± 5	3.3 ± 7.2
20 grass carp	29.6 ± 2.7	31.3 ± 2.8	17.4 ± 2.1	5.7 ± 6.2	19.4 ± 6.6	2.6 ± 4.4
6 c. carp/20 grass carp	28.5 ± 3	30.2 ± 3	92.3 ± 19	0.3 ± 0.09	72.0 ± 24.4	0.3 ± 0.07
6 c. carp/20 grass carp	29.3 ± 2.5	31.1 ± 2.5	41.4 ± 10.2	1.2 ± 1.1	24.2 ± 16.2	1.0 ± 0.7
***L. subrostrata* spawning**						
2 grass carp			1.0 ± 0.5	12.5 ± 10.6	1.8 ± 1.6	5.7 ± 7.5
2 grass carp	23.9 ± 2.7	25.5 ± 2.8	9.8 ± 1.6	2.3 ± 1.1	12.2 ± 6.5	1.2 ± 0.9
5 grass carp	22.8 ± 2.3	24.3 ± 2.4	5.3 ± 1.2	11.2 ± 8.7	5.9 ± 2.7	4.5 ± 6.1
20 grass carp	23.9 ± 2.6	25.1 ± 2.7	11.9 ± 2.3	3.6 ± 5.9	12.9 ± 4.1	1.5 ± 3.2
20 grass carp			10.4 ± 3.0	1.6 ± 1.6	12.1 ± 5.5	1.0 ± 1.0
6 c. carp/20 grass carp	23.3 ± 2.7	24.6 ± 2.8	79.4 ± 11.4	0.4 ± 0.08	79.1 ± 17.6	0.3 ± 0.08
6 c. carp/20 grass carp			78.9 ± 5.0	0.3 ± 0.08	68.1 ± 13.6	0.3 ± 0.08
***L. subrostrata* brooding**						
15 grass carp	14.3 ± 3	15.0 ± 3.1			6.9 ± 2.4	0.4 ± 0.17
15 grass carp	14.6 ± 2.9	15.4 ± 3.1			11.5 ± 4.5	0.3 ± 0.08
6 c. carp/20 grass carp	13.8 ± 3.1	14.8 ± 3.3			82.8 ± 27.7	0.2 ± 0.07
6 c. carp/20 grass carp	14.1 ± 2.9	14.9 ± 2.9			59.4 ± 8.2	0.2 ± 0.09

Table 4-2. Number of *Reginaia ebenus* with continued reproductive activity after initial sampling event on 5/20/2012. Initial sample column shows the reproductive state on the first sampling. The number in parenthesis is the sample size on the initial date. Final four columns show reproductive status on subsequent sampling events. All females (n= 25) that exhibited glochidia on the initial sampling date were immediately sacrificed for estimates of fecundity and fertilization efficiency.

		Subsequent samples combined (5/26, 5/31, 6/7)			
Initial sample (5/20)		Empty gills	Unfertilized eggs	Fertilized eggs	Glochidia
Empty Gills	(28)	17	0	3	8
Unfertilized eggs	(11)	5	0	1	5
Fertilized eggs	(40)	19	1	14	6

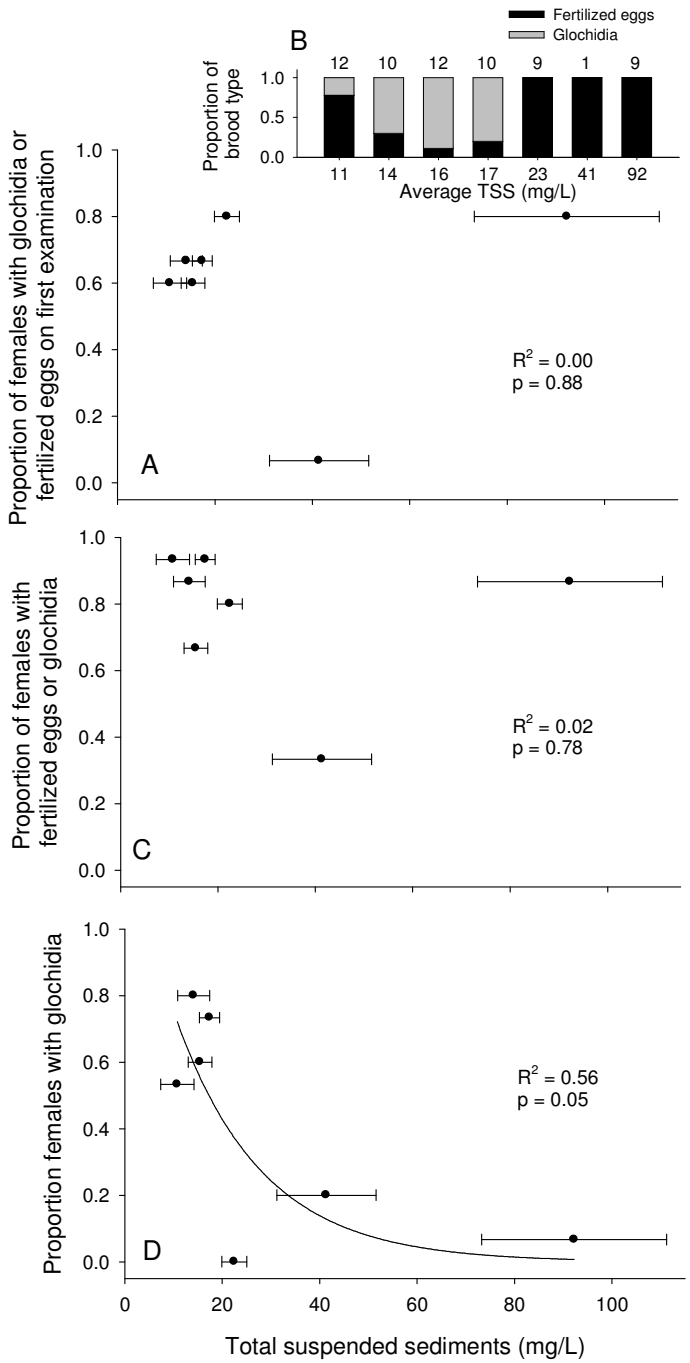

Figure 4-1. A) Proportion of female *F. ebenus* with fertilized eggs or mature glochidia on the first handling occasion (5/20/2011). B) Proportion of broods at each TSS level consisting of fertilized eggs or glochidia. Number above each bar gives the total number of broods. C) Proportion of females producing either fertilized eggs or glochidia at least once across all four sampling occasions. D) Proportion of females producing glochidia at least one date across all four sampling occasions. Horizontal error bars represent ±1 standard deviation in total suspended solids over the spawning period.

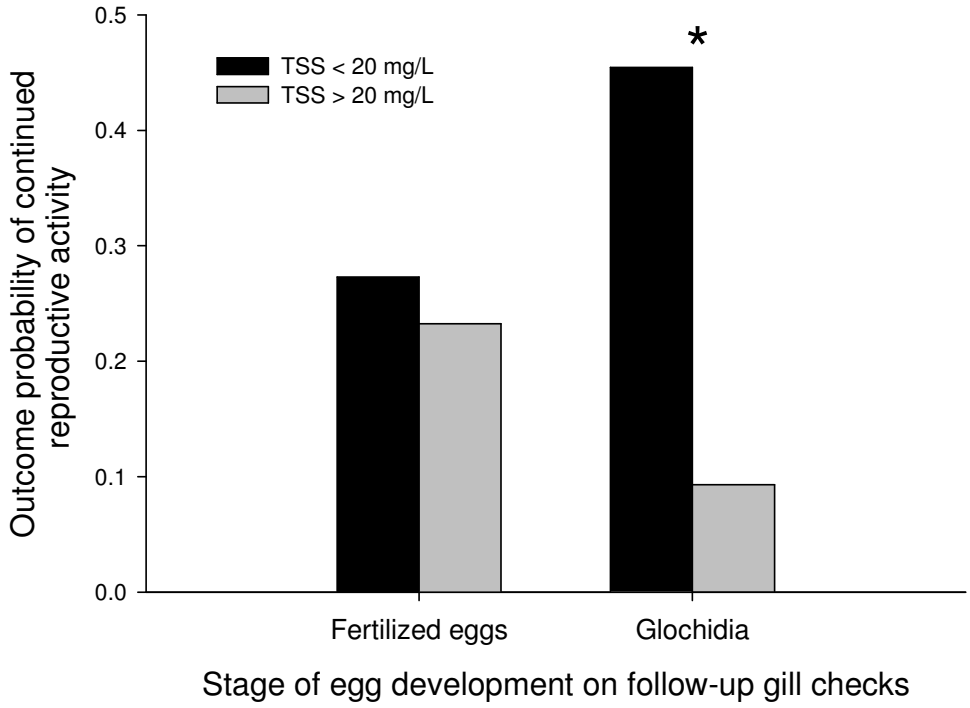

Figure 4-2. Probability of continued reproductive activity (fertilized eggs or glochidia) by *R. ebenus* after the initial gill extraction on 5/20/2011 in low (< 20 mg/L) and high (>20 mg/L) TSS ponds. Asterisk denotes significant difference.

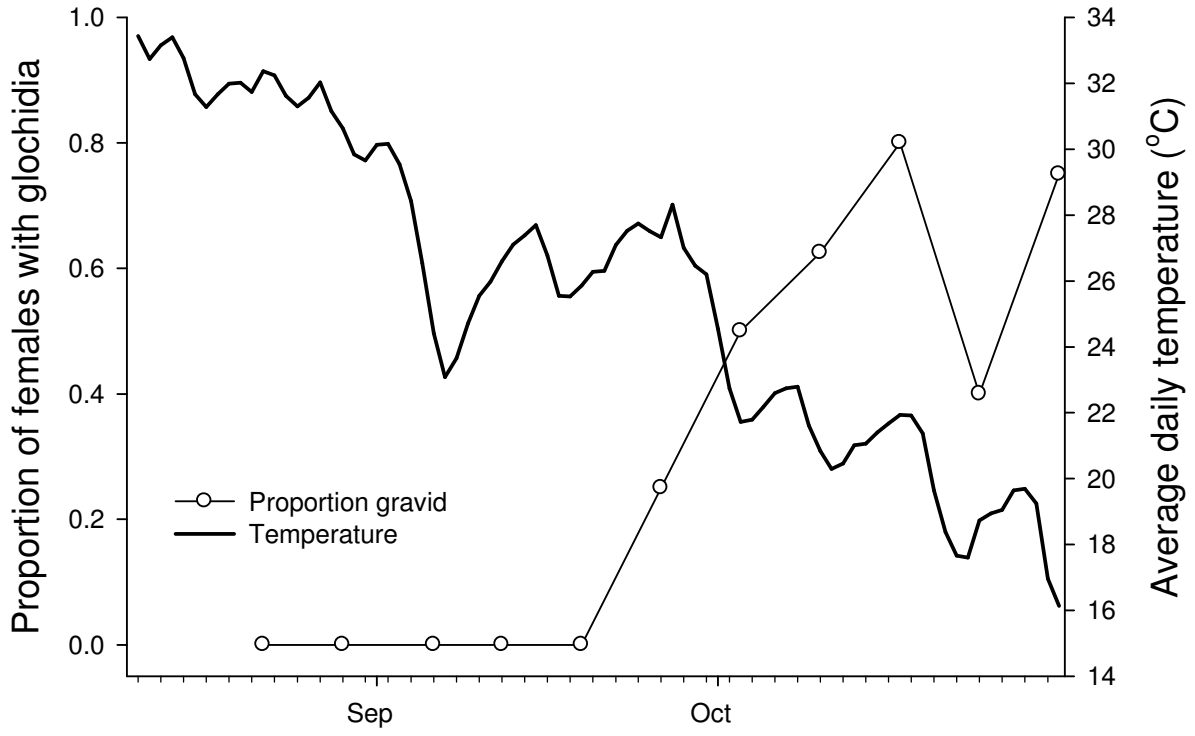

Figure 4-3. Observed spawning period for *L. subrostrata*, as indicated by the presence of fertilized eggs or glochidia in the gills. Spawning initiated the week of September 19th and was finished by October 15th.

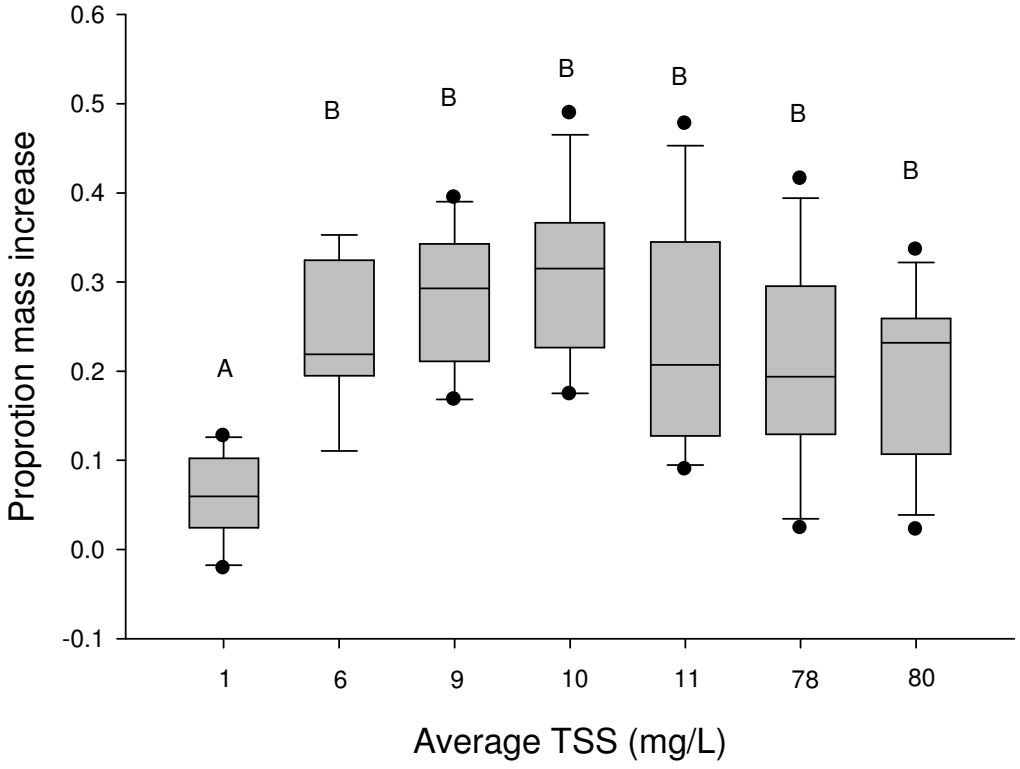

Figure 4-4. Proportion mass increase of 2 year old *L. subrostrata* during the 3 month trial period at each TSS concentration. Note the x-axis is not a continuous scale.

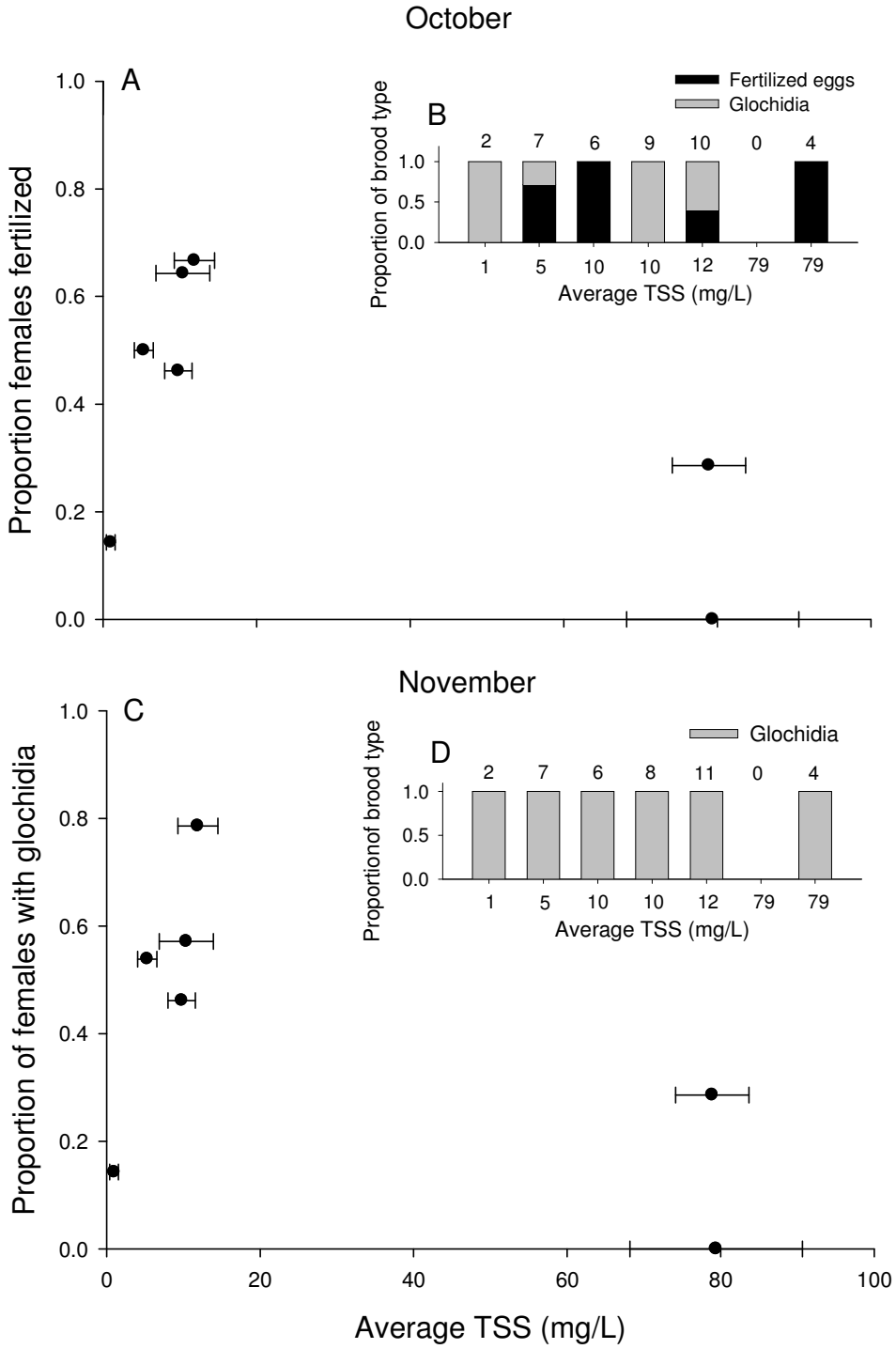

Figure 4-5. A) Proportion of *L. subrostrata* females with evidence of fertilization (either fertilized eggs or glochidia), in October. B) Proportion of brood type (fertilized egg only or glochidia only) among females within each pond in October. Numbers at the top of each bar are the total number of broods observed. C) Proportion of females with glochidia in November. Error bars represent ±1 standard deviation in total suspended solids over the spawning period. D) Proportion of brood type among females within each pond in November.

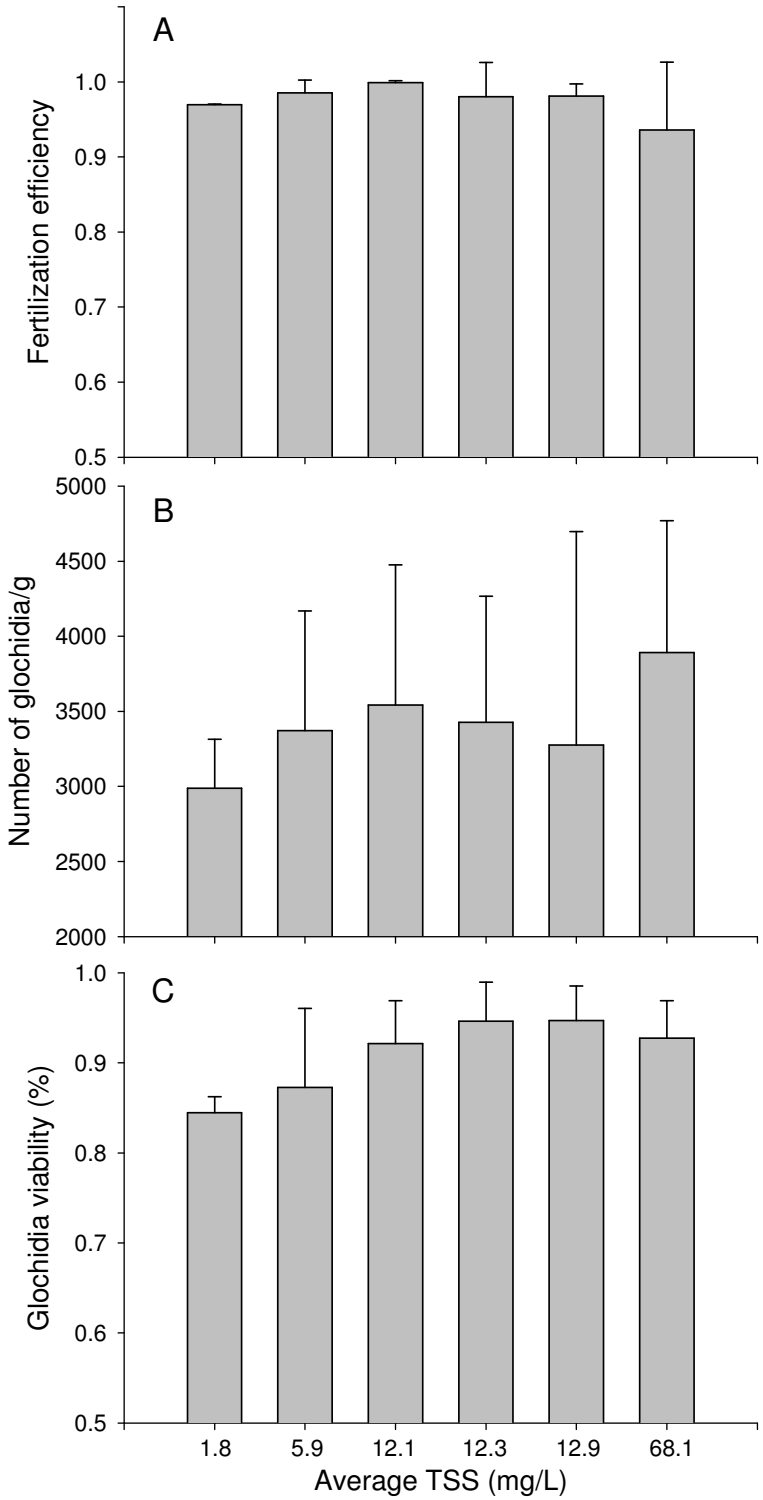

Figure 4-6. A) Fertilization efficiency, B) fecundity, and C) glochidia viability of *L. subrostrata* across a range of suspended solids during the spawning season. Note the x-axis is not a continuous scale.

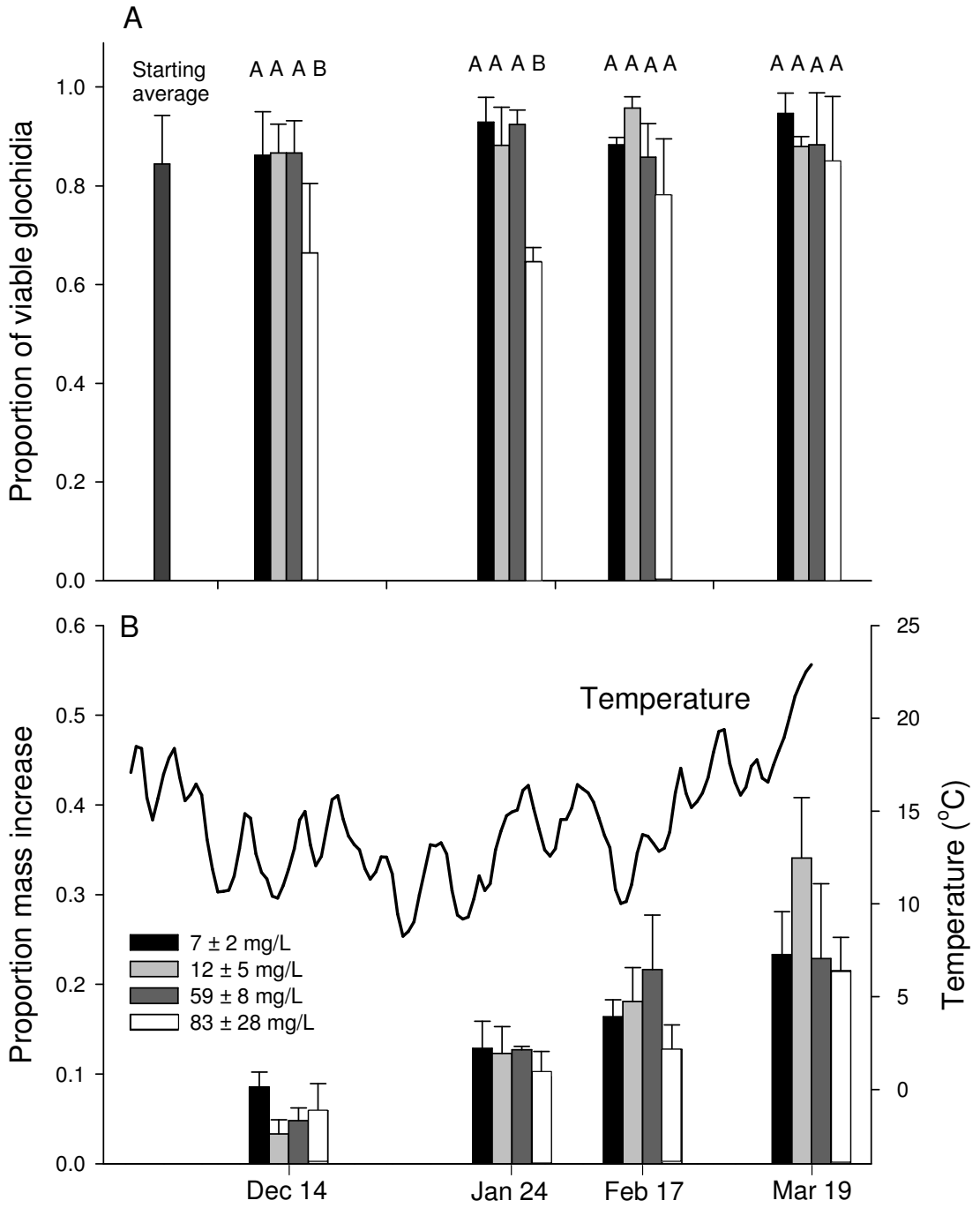

Figure 4-7. Monthly estimates of A) glochidia viability and B) growth of female mussels across TSS concentrations during the overwinter brooding period for *L. subrostrata*. Average daily temperature is shown in the bottom panel. Each bar represents data from one pond. Error bars represent ±1 SD. Letters above bars indicate significant differences between ponds on a given sampling date.

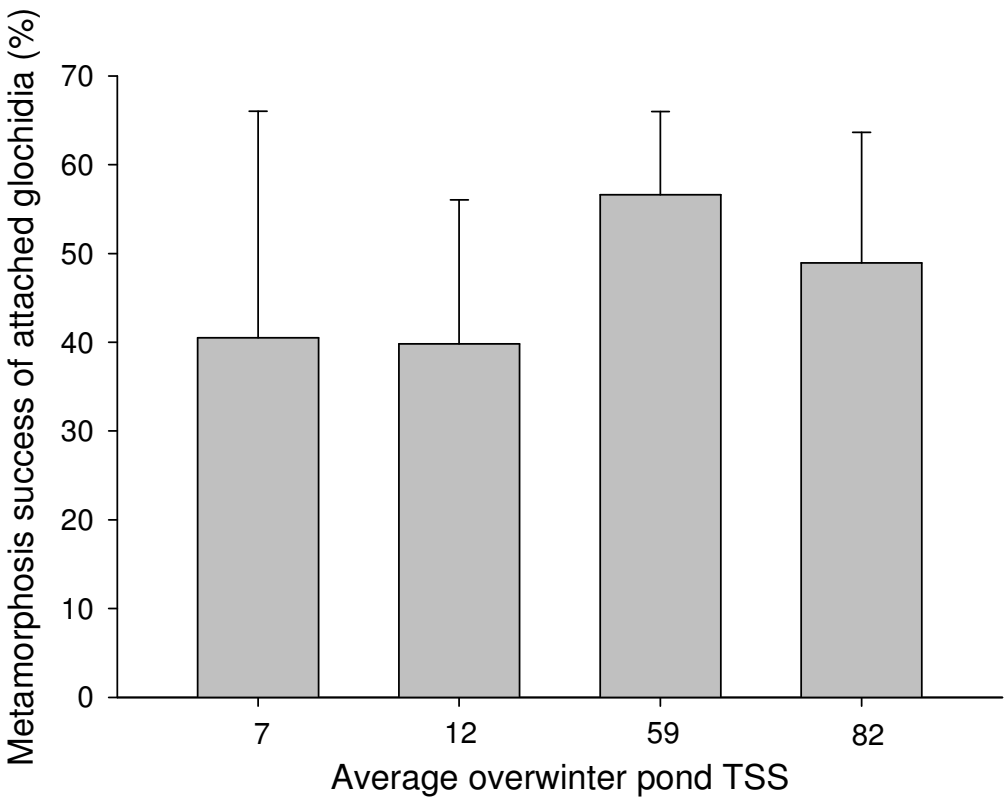

Figure 4-8. Metamorphosis success of glochidia on bluegill in March after brooding for four months in ponds with differing TSS concentrations. No significant differences were found. Note that scale on the x-axis is not continuous.

Chapter 5

Effects of temperature and photoperiod on lure display and glochidial release in a freshwater mussel

Freshwater mussels use an array of strategies to transfer their parasitic larvae (glochidia) to fish hosts. We examined the effects of temperature, photoperiod, and female gravidity on mantle lure display and conglutinate release by *Ligumia subrostrata* (Say, 1831) in 2 laboratory experiments. In the 1st experiment, we examined the use of these strategies in 4 temperature treatments (5, 15, 25, 35°C) and 3 photoperiods (10:14, 12:12, and 14:10 h light: dark). In the 2nd experiment, we observed infection strategies under ambient conditions with flow-through pond water. Water temperature appeared to be the primary cue governing use of these strategies. Lure display occurred over a protracted period but the highest display frequency occurred between ~11 and 20°C. Lure display declined rapidly above this range and ceased altogether >28°C. Release of conglutinates increased coincident with the decrease in lure display but, at ambient temperatures, occurred over a protracted period similar in duration to lure display. Females that were not gravid at the beginning of the experiment did not display lures, and gravid females whose gills were flushed of glochidia displayed for only a short period during which frequency of display was much lower than gravid individuals. *Ligumia subrostrata* exhibits a temperature-mediated switch between alternate host strategies. We present evidence that lure display is a primary strategy for host infection and conglutinate release is a secondary, bet-hedging strategy to reduce wastage of glochidia that ultimately must be cleared from the gills at the end of the reproductive

season.

Introduction

Reproductive behavior in exothermic, aquatic organisms often is cued by environmental factors. Temperature and photoperiod initiate gonadogenesis and spawning behavior in many taxa including fish, crustaceans, and mollusks (Aiken and Waddy 1989, Fong et al. 1995, Carscadden et al. 1997, Verween et al. 2009). These seasonal cues are important because the timing of spawning and other reproductive behaviors is strongly related to recruitment success (Kautsky 1982, Wieland et al. 2000). For example, in freshwater mussels, spring recruitment can maximize growth in the 1st y of life allowing greater accumulation of energetic reserves necessary for winter survival and attainment of reproductive maturity (Beaty and Neves 2004). However, if reproduction occurs too early, cold temperatures and low food availability may reduce growth and survival of recruits (Hanlon and Neves 2006). Understanding how abiotic factors control reproductive behaviors can help identify conditions necessary for successful recruitment.

Freshwater mussels have an unusual life history in which larvae (glochidia) are obligate parasites on the gills or fins of fishes. Female mussels brood fertilized eggs in their gills until they develop into mature glochidia. After maturity, glochidia are brooded for an additional period that ranges among species from short-term brooders that retain glochidia for only a few weeks to long-term brooders that retain glochidia for up to 6 to 7 mo, usually over the winter (e.g., Watters and O'Dee 2000). In both brooding strategies, glochidia infect host fishes after they are released from the female or extracted from the female gill by fish attacks on mantle lures (see below). The factors that trigger glochidial release are poorly known in most species but

are assumed to include a combination of water temperature and photoperiod (Watters and O'Dee 2000, Hastie and Young 2003). The timing of glochidial release probably is crucial for successful recruitment. Temperature and photoperiod may influence the location, abundance, and activity level of potential hosts (Martel and Lauzon-Guay 2005), and the strength of the host immune system, which determines how well glochidia can transform into juvenile mussels (Roberts and Barnhart 1999).

Mussels have a variety of strategies to increase the likelihood of that glochidia will encounter suitable host fishes (Barnhart et al. 2008). The best known of these strategies are mantle lures and conglutinates. Mantle lures are modifications of the mantle margins that are variously pigmented and elaborated to resemble small fishes, crayfish, insect larvae, or other prey items of host fishes. These lures elicit attacks from fishes during which fishes become infected with glochidia. Conglutinates are small, discrete packets containing glochidia and usually represent the contents of a single gill water tube. Similar to mantle lures, conglutinates resemble small worms, insect larvae, or other fish prey items, and attacks from host fishes result in infection by glochidia. Both release strategies involve mimicry of host prey items, but they differ in the factors that trigger glochidial release. The factors that trigger conglutinate release have not been studied directly, but release appears to occur in response to water temperature or photoperiod cues, and conglutinates are often released en masse within a few days (Neves and Widlak 1988, Hove and Neves 1994). In contrast, species that display mantle lures release glochidia primarily in response to attacks on the lures by fishes during which fishes rupture the gravid gills (Haag and Warren 2000, Barnhart et al. 2008). These species often display lures for months (Kramer 1970, Haag and Warren 2003), during which time they may infect multiple host individuals. Some species display lures at night and others by day (Haag and Warren 2000), but

the factors that influence seasonal periods of lure display have not been studied.

Many mussel species appear to rely on a single host-infection strategy, but others may use both primary and secondary strategies. For example, *Hamiota* infects hosts primarily by releasing large conglutinates (superconglutinates) that resemble small fishes, but conglutinate release is preceded by a short period of display of mantle lures with lures being reduced in most species (Haag et al. 1995, 1999). Many species in the tribe Lampsilini that infect hosts primarily by display of mantle lures also may release conglutinates. These conglutinates contain mature glochidia but are often loose and poorly formed and appear to be released mostly late in the brooding season. Consequently, whether this behavior is necessary simply to make room for the next brood or represents a secondary infection strategy in not known (Corey et al. 2006, Barnhart et al. 2008).

We examined factors that influence mantle lure display and conglutinate release in the freshwater mussel *Ligumia subrostrata* (Say, 1831). First, we ran a laboratory experiment in which we manipulated temperature and photoperiod to examine the influence of these factors on lure display and conglutinate release. Second, we examined temporal patterns of lure display and conglutinate release under ambient conditions reflective of the natural period of host infection for this species (late winter–spring) and how lure display is affected by gravidity. We discuss how these results extend our understanding of the timing of mussel reproduction and the relative benefits of alternate host infection strategies in mussels.

Methods

Study species

Ligumia subrostrata occurs in lentic conditions, including ponds and backwater habitats

of streams, throughout central North America (Cummings and Mayer 1992, Williams et al. 2008). Like most species in the tribe Lampsilini, *L. subrostrata* is a long-term brooder that broods glochidia over the winter and releases them in late winter and spring. Known or suggested fish hosts for *L. subrostrata* are sunfishes (*Lepomis* spp.) and black basses (*Micropterus* spp.) (Lefevre and Curtis 1912, Stern and Felder 1978, WRH, unpublished data). Gravid females attract hosts by displaying a mantle lure consisting of small papillae that are fluttered to reveal the gravid gills within. Displays occur primarily during the day (Corey et al. 2006). *Ligumia subrostrata* also releases conglutinates that are proposed to represent an alternate host-infection strategy (Corey et al. 2006).

We obtained gravid female *L. subrostrata* from ponds at South Auburn Fisheries Research Station, Department of Fisheries and Allied Aquaculture, Auburn University. All mussels were young-of-the-year individuals that recruited in late winter or spring 2009 as part of a separate, ongoing experiment (JAS and WRH, unpublished data). Mussels were collected from ponds in November 2009. Despite their young age, 90% of female mussels in the ponds were fully gravid at the time of collection. Length of female mussels ranged from 43.3 to 53.3 mm (mean = 48.6 mm). After collection, mussels overwintered in the laboratory in flow-through tanks supplied with pond water at ambient outdoor temperature.

Experiment 1: temperature and photoperiod manipulation

To examine the influence of temperature and photoperiod on infection strategies, we held mussels in 4 temperature-controlled water baths (~100 L) and regulated day length with a timer. We tested 4 temperature (5, 15, 25, 35°C), and 3 photoperiod (10:14, 12:12, 14:10 h light:dark [L/D]) treatments. We maintained the 5 and 15°C treatments with chillers and the 25 and 35°C

treatments with aquarium heaters. All temperatures remained within ±1°C of the treatment temperature throughout the experiment.

We randomly assigned 10 female mussels to each treatment. However, to minimize stress on the mussels, we did not examine females for gravidity prior to the start of the experiment. We examined all mussels for gravidity at the end of the experiment or immediately upon death. Mussels that were not gravid upon examination and had not released any conglutinates during the course of the experiment were considered to have been nongravid at the beginning of the experiment and were not considered in subsequent data analysis. The final number of gravid mussels in each treatment was 9, 8, 7, and 9 in the 5, 15, 25, 35°C temperature treatments, respectively.

Within each temperature treatment, we held each mussel individually in a 1-L cup filled with artificial fresh water (AFW: 50 mg $CaCO_3$, 25 mg CaCl, 50 mg Na_2CO_3, and 5 mL 30‰ salt water/L deionized water). A preliminary trial showed no difference in display activity in AFW and pond water. We used a foam platform to float cups in the water bath with ~90% of the cup suspended below the waterline. Holding mussels in individual cups isolated individuals from each other in the water bath and allowed us to record glochidia released during the experiment. We fed mussels 0.05 mL of Reed Mariculture shellfish diet (Reed Mariculture, Campbell, California) every other day as AFW was replaced in each cup. We brought replacement water to treatment temperature before adding it.

We initiated the experiment on 27 February 2010 when ambient pond water was at 10 to 12°C. We moved animals from flow-through holding tanks to the experimental containers and acclimated them to experimental temperatures by setting all water baths initially at 12°C and subsequently adjusting by 1°C/d until treatment temperatures were reached. We acclimated

mussels at their respective temperature treatments for 3 to 6 d before the start of the experiment and held them at a constant temperature throughout the remainder of the experiment. In each temperature treatment, we manipulated photoperiod to simulate increasing day length progressing from 10:14 to 12:12 to 14:10 h L:D, similar to what mussels would experience in late winter and spring. Unlike gradual changes in day length in nature, our transitions were abrupt and were designed to evaluate obvious behavioral differences among distinctly different light regimes. We observed mussels for 7 d at each photoperiod. At the end of 7 d, we increased day length and allowed mussels to acclimate for 3 d before beginning the next observation period. We made observations of lure display during acclimation periods but these observations were not used in data analysis (see Fig. 1).

We observed mussels 3 times/d during each temperature/photoperiod combination. We focused on daytime display behavior because Corey et al. (2006) found that *L. subrostrata* did not display at night. We did not make night-time observations, but our results confirmed that this species displays vigorously during the day. We made observations 1 h after lights came on (0700–0900 h, dawn), at mid-day (1200–1400 h), and 1 h before lights turned off (1900–2100 h, dusk). We scored mussel display activity following methods published by Haag and Warren (2000) where 0 = no display, 1 = partial display, and 2 = full display. A 0 score was given when a mussel was filtering normally with the valves slightly open, but the mantle lure was not extended. A 1 score was given when the valves were slightly more agape and the mantle papillae were extended beyond the shell margin, but papillae were not actively moving. A 2 score was given when the valves gaped widely, the mantle lure was fully extended, and papillae fluttered actively. In full displays, gravid gills often were visible between the shell valves. We used analysis of variance (ANOVA) to examine differences in the number of animals in full display

across the 3 daily observations (dawn, midday, and dusk) for each temperature and photoperiod (n = 12 comparisons). We grouped observations for specific time periods (dawn, midday, and dusk) across all days in each temperature treatment.

Full displays are most likely to elicit fish attacks and result in transfer of glochidia (Haag and Warren 2000), so we focused on this display category in our analysis. We calculated the proportion of animals in full display (number in full display/total number of animals per treatment) 3 times/d (morning, noon, evening) for each of the 4 temperature treatments. We averaged these proportions to obtain the daily mean proportion of mussels in full display for each temperature treatment. If a mussel died during the experiment, we adjusted the total number of animals per treatment accordingly. We used a Kruskal–Wallis test to compare average proportion in full display across temperature treatments for each photoperiod and across photoperiods separately for each temperature treatment (SYSTAT 12; Systat, Chicago, Illinois). We excluded the 35°C treatment from the analysis of the 12:12 and 14:10 photoperiods because of high mortality. We were unable to assign photoperiod randomly to individuals in each temperature treatment because the number of water baths available was limited. Therefore, we were unable to distinguish directly between the effects of time in experimental conditions and photoperiod. In addition to observing display behavior, we collected expelled conglutinates every other day coincident with water changes. From these data, we examined: 1) the number of days at experimental temperatures before each individual first released conglutinates, 2) the occurrence of glochidial dumping by individual mussels (all glochidia released in 2 d), and 3) the number of days until 100% of conglutinates were released.

Display frequency was low and conglutinate release did not occur at 5°C regardless of photoperiod, so we conducted an additional trial with these animals to examine the effect on

display behavior of manipulating water temperature while holding photoperiod constant. Upon completion of the longest daylight trial, we held photoperiod at 14:10 h L:D but raised water temperature in the 5°C treatment by 1°C/d up to 12°C, and then held temperature constant for 7 d. We scored display behavior for each mussel once daily throughout the 14-d this trial. Successive observations were not independent, so we used a nonparametric runs test to assess if the daily progression of display scores occurred in a random or nonrandom sequence (Zar 1999).

Experiment 2: ambient temperature and photoperiod

We conducted a 2nd experiment to examine: 1) how gravidity status affects lure display behavior, and 2) how the relative use of mantle display and conglutinate release strategies changes over time at ambient conditions during the natural period of host infection for this species (late winter–spring; JAS and WRH, personal observation). We obtained 20 gravid female mussels from our flow-through tanks used to overwinter mussels in the laboratory. Mussels were of similar size and age to those used in Experiment 1, but none were used in previous experiments. We allowed 10 mussels to retain glochidia and emptied 10 of glochidia by using a syringe with a 22-gauge needle to flush water through the gills (Khym and Layzer 2000).

We held all mussels in the laboratory in flow-through tanks from 27 February to 9 July 2010. We randomly assigned 1 gravid and 1 flushed mussel to each of ten 3-L Aquatic Habitat Animal Bank (AHAB) tanks that received unfiltered pond water. In the tanks, we held each mussel in a shallow cup made of polyvinyl chloride pipe (19 mm tall × 25 mm diameter) that was glued to acrylic sheeting. This shallow cup allowed mussels to be positioned upright in a natural filtering position and enabled us to observe the mussels easily without disturbing them. Water temperature closely mimicked that of ambient, outside conditions because tanks received

pond water continually. On average, tank temperatures stayed within 0.4 ± 1.2°C (SD) of ambient temperature in an adjacent 0.1-ha pond. We used fluorescent lights and timers to adjust photoperiod continually to match ambient, outside conditions. Photoperiod progressed from 11.25:12.75 to 14.25:9.75 h L:D over the duration of the experiment. We cleaned tanks weekly to remove pseudofeces and other solids that settled from the pond water. We recorded temperature once daily between 1200 and 1500.

We recorded mantle displays once daily (between 1200 and 1500) for the duration of the experiment and used the scoring system described previously. For each day, we determined the proportion of individuals in full display for both gravid and flushed mussels. We used nonlinear regression to fit the following logistic model to the relationship between proportion of individuals displaying (arcsine[x]-transformed) and day of the experiment:

$$y = a/(1 + \exp[-(x - x_0)/b])$$

where y is proportion in full display, a is the y-intercept, x is day of the experiment, x_0 is a threshold value (day of experiment), and b is the slope of the decay of display proportion from the baseline to 0. The y-intercept represents the mean maximum proportion of individuals in full display throughout the experiment, and the threshold value is the day of the experiment at which the proportion of full display decayed to ½ the mean maximum value. We estimated logistic models separately for gravid and flushed females. For both groups, we estimated water temperature on the threshold day with a linear regression describing the relationship between temperature and day of experiment ($R^2 = 0.948$, temperature = 0.171[day] – 12.6).

We also collected and counted conglutinates from the bottom of each tank every 1 to 2 d. Most glochidia were released in tightly bound conglutinates and not as free glochidia. We assumed that all conglutinates in the tanks were from the gravid individuals. We calculated

conglutinate production for a given day as the proportion of the total production over the course of the experiment. Temperatures reported for conglutinate release are those recorded between 1200 and 1500. We terminated the experiment when no conglutinates were found in any of the mussel tanks for 7 consecutive days (9 June 2010). We then examined all mussels for glochidia remaining in the gills.

We quantified the time that displaying individuals spent actively moving their lures. Lure movement occurs sporadically in displaying individuals, and this measurement provided an additional and more fine-scaled measure of how much time was devoted to attracting hosts actively. We quantified lure movement by observing each mussel once weekly and recording the proportion of 10 min spent in full display. Observations began 6 d after the start of the experiment and continued weekly for 105 d. We were interested in measuring lure movement only for individuals in full display. We measured lure movement initially on Wednesdays (1200–1400). If an individual was not in full display on that day, we attempted observation on the following 2 d (Thursday and Friday). If a mussel did not display on any of the 3 visits, we excluded it from the analysis. We made these measurements only for gravid mussels and used linear regression (arcsine[x]-transformed proportion) to examine the relationship between proportion of time spent in full display and temperature.

Results

Experiment 1: temperature and photoperiod manipulation

Thirty of 33 gravid females were in full display at some point during the experiment. The 3 individuals not in full display were in the coldest (5°C, 1 individual) and hottest (35°C, 2 individuals) treatments. None of the mussels that were considered to have been nongravid at the

beginning of the experiment (n = 7 across all treatments; see Methods) ever displayed mantle lures (partial or full displays). Mussels held at 35°C had high mortality. Eight of the 10 individuals died between days 19 and 25, and only 2 survived to the end of the experiment. No mussels died in the 5, 15, and 25°C temperature treatments. No significant differences in number of mussels in full display were found among dawn, midday, and dusk within temperature treatments at each photoperiod (ANOVA, all $p > 0.37$).

Mean and maximum observed frequency of full display was low at 5°C but increased at successively higher temperatures (Fig. 5-1A–D). However, the temporal pattern of display differed widely among temperatures. The proportion of individuals in full display differed significantly among photoperiods in the 5, 25, and 35°C treatments (p = 0.058, 0.007, and 0.001, respectively; Fig. 5-1A, C, D), but not at 15°C (p = 0.313; Fig. 1B). In the 5°C treatment, display differed only between the 10:14 and 14:10 h photoperiods (higher at 14:10 h, Kruskall–Wallis post hoc analysis, p = 0.01), and showed a gradual but modest increase over the duration of the experiment (Fig. 5-1A). At both 25 and 35°C, display differed between 10:14 h and both 12:12 and 14:10 h, but not between the latter 2 photoperiods (Fig. 5-1C, D). In contrast to the 5°C treatment, display frequency at 25 and 35°C was highest at the beginning of the experiment but declined rapidly to low levels by about day 10, before the end of the 10:14 h photoperiod. Display frequency remained essentially at 0 throughout the 12:12 and 14:10 h photoperiods (Fig. 5-1C, D).

All gravid mussels released conglutinates or free glochidia in the 25 and 35°C treatments but no individuals released conglutinates at 5 or 15°C (Fig. 5-1A–D). Conglutinates were white and tear-drop shaped, and averaged 6.9 ±1.2 mm (SD) in length (range: 4.4–9.1 mm, n = 25; Fig. 5-2). Conglutinates were composed primarily of mature glochidia (mean = 99% of total

propagules, $n = 5$ conglutinates) and broke apart easily because of the weak cohesion between degenerated egg membranes surrounding the glochidia. At 25°C, individuals initiated conglutinate release 8 to 14 d after the experimental temperature had been reached (Fig. 5-1C). At 35°C, individuals initiated conglutinate release between days 3 and 13 (Fig. 5-1D). By the end of the experiment, all surviving individuals in the 25 and 35°C treatments had released all of their glochidia and had empty marsupia. One mussel that died during the experiment (day 21) still retained a portion of its glochidia. The duration of glochidial release differed among temperatures. At 25°C, only 2 of the 7 individuals dumped all conglutinates in a single episode (i.e., within 2 d, corresponding to the interval between conglutinate collection). All but 1 of the other individuals released conglutinates in 2 episodes over 4 to 6 d, with each episode representing ~½ of total conglutinate production. One individual released conglutinates 7 times over 14 d. At 35°C, 75% of individuals dumped all conglutinates in a single episode. For all individuals at both temperatures, conglutinate release began as the proportion of individuals in full display began to decline, and for most individuals, release was complete by the end of the 10:14 h photoperiod (Fig. 5-1C, D).

Increasing the temperature of the 5°C treatment to 12°C while photoperiod remained constant (14:10) resulted in a marked increase in the proportion of mussels in full display (Fig. 5-3). The runs test indicated a significant nonrandom pattern in display activity ($p < 0.05$). The proportion of individuals in full display increased rapidly when the temperature reached about 11°C (day 7), and remained high for the remainder of the experiment. No conglutinates were released by any mussel during this component of the experiment.

Experiment 2: Ambient temperature and photoperiod

All mussels survived the 139-d study as ambient temperature increased from 10 to 32°C. Both gravid and flushed (nongravid) mussels displayed mantle lures during the experiment, but the duration and frequency of full display was much lower in nongravid mussels (Fig. 5-4A). Ninety percent of flushed mussels exhibited full display at some point. From the logistic model (overall model $R^2 = 0.58$), the maximum proportion of flushed individuals in full display was only 0.25, but daily observations ranged as high as 0.60. Maximum proportion of full displays occurred at temperatures <15°C. The threshold value indicated that the proportion declined to 50% of the mean maximum value on day 24 at 16.5°C. Full displays by flushed mussels ceased mostly by day 48 (15 April, 22°C) with the exception of 1 individual that displayed on day 111 (17 June). In contrast, all gravid mussels displayed during the experiment, and the maximum proportion of individuals in full display was 0.86 (overall logistic model $R^2 = 0.86$). The frequency of full display was consistently high throughout the first 46 d of the experiment (27 February–13 April, 11–20°C) and did not decline to 50% of the mean maximum value until day 56 (22.1°C). Display did not cease until day 116 (22 June, 32°C).

Release of conglutinates coincided with a decrease in lure display frequency (Fig. 5-4B). Conglutinate release began as early as day 25 (23 March, 14.4°C), but some individuals did not release conglutinates until as late as day 68 (5 May, 25.6°C). The mean date of onset of conglutinate release across individuals was day 40 (21.2°C), which corresponded closely to the end of the period of maximum lure display. Within individuals, the rate of conglutinate release over time was roughly linear and occurred incrementally over a protracted period in sharp contrast to the episodic dumping of conglutinates by most individuals at higher temperatures in Experiment 1. Across individuals, only 25% of conglutinates were released by day 65 at water temperatures ~23°C, at which time lure display had declined to <30%. Conglutinate release

spanned an average of 88 d across individuals (range 52–99 d) and ceased by day 139 (9 July, 31.8°C). Individual mussels released an average of 140 conglutinates (range = 69–201) during the experiment. Conglutinates were similar in appearance to those released in Experiment 1, but differed in that they did not break apart easily and more readily maintained their shape (Fig. 5-2). No free glochidia were observed. At the end of the experiment, gills of all individuals were completely empty.

The amount of time that displaying individuals spent actively moving their lures showed a temporal pattern similar to the proportion of individuals in full display. The frequency of lure movement declined exponentially with increasing temperature ($R^2 = 0.59$, $p < 0.001$) (Fig. 5-5). Frequency declined from 0.33 to 0.13 as temperatures increased to 22.1°C and declined further to 0.05 as temperatures increased to 32°C.

Discussion

Lure display and conglutinate release by *L. subrostrata* are strongly influenced by water temperature. In both the manipulative and ambient experiments, the highest lure display activity occurred within a narrow range of water temperatures from ~11 to 20°C. Lure display appeared to increase abruptly when the lower end of this temperature range was reached, but declined quickly above this range. Proportion of time spent in full display also declined steadily as temperatures increased. Similar patterns of lure display were seen for individuals in artificial freshwater and in natural pond water, a result suggesting that display is cued directly by temperature and not by algal abundance or other factors correlated with water temperature. The low display frequency of individuals in the static 15°C treatment was surprising but suggests that periods of changing temperatures are also important cues for lure display, as demonstrated by the

temperature increase experiment. In the ambient experiment, conglutinate release occurred over a broad temperature range (15–32°C) but predominantly >20°C, and its duration was similar to that of lure display. This result suggests that the switch from lure display to conglutinate release and the duration of release are strongly mediated by temperature.

We were unable to evaluate directly the influence of photoperiod on lure display or conglutinate release, but photoperiod appeared to have only a small influence on reproductive behavior. Display increased dramatically in Experiment 1 when temperature was increased from 5 to 12°C even though photoperiod was held constant. In the static 5°C treatment, lure display increased modestly with increasing photoperiod but no corresponding increase in display occurred in the 15°C treatment. Increasing photoperiod could possibly cause a hormonal or other physiological response in anticipation of increased temperatures normally associated with increasing day length, and this response may stimulate lure display to some extent. However, conglutinates were never released at cooler static temperatures regardless of photoperiod, and at high temperatures, conglutinates were released abruptly before an increase in photoperiod.

Display of mantle lures was strongly associated with gravidity. All gravid females displayed lures at some point in both experiments, but individuals that were not gravid (naturally) at the beginning of the experiment never displayed. Gravid individuals whose gills were flushed artificially at the beginning of the experiment had much lower frequency of display and displayed for only a brief period compared to gravid individuals that were not flushed. This residual display behavior suggests that either some glochidia remained in the gills after flushing, or elevated hormonal levels resulted in a continued display even after gills were emptied. Display behavior can be affected by pharmaceutical hormones in waste water discharge (Bringolf et al. 2010), but the natural role of hormonal or other physiological mediators of lure

display and glochidial release are uninvestigated in freshwater mussels.

The timing of lure display and conglutinate release and its relationship to water temperature probably has evolved to correspond with optimal conditions for glochidia encystment and development. At low temperatures, glochidia can transform successfully on hosts, but glochidial development is slowed or suspended and requires a lengthy period of encystment (Howard and Anson 1922, Watters and O'Dee 1999, Steingraeber et al. 2007) during which chances of host mortality increase. However, glochidial survival decreases rapidly with increasing temperature (Fisher and Dimock 2000, Zimmerman and Neves 2002), and transformation success and the breadth of suitable host species can decrease at higher temperatures (Roberts and Barnhart 1999).

Release of conglutinates by *L. subrostrata* appears to represent an alternate strategy for host infection. Secondary or alternate host-infection strategies of mussels are best known in the genus *Hamiota*, in which females first display mantle lures and then release the entire remaining glochidial contents of the gills in elaborate superconglutinates (Haag et al. 1999). However, unlike *L. subrostrata*, mantle lures of most *Hamiota* are greatly reduced and displayed only briefly, suggesting that superconglutinates represent a primary strategy for host infection (Hartfield and Butler 1997, Roe and Hartfield 2005). Other species that infect fishes primarily with conglutinates produce conglutinates that may represent a considerable cost to the female, either by encasing glochidia in elaborate and highly pigmented membranes (*Ptychobranchus*), or by the presence of nondeveloping structural eggs that decrease fecundity by up to 50% (e.g., *Cyprogenia, Dromas, Fusconaia*, and *Pleurobema*; Barnhart et al. 2008). In contrast, conglutinates of *L. subrostrata* appear to be formed simply as unelaborated molds of the interlamellar gill spaces in which they are brooded and receive no additional female investment in structure or pigmentation. However, *L. subrostrata* conglutinates are similar in shape and size

to those of species that use conglutinates as a primary infection strategy (e.g., *Fusconaia, Pleurobema*; see Haag and Warren 2003), and it is plausible that *L. subrostrata* conglutinates also elicit attacks from fishes. We did not test the viability of glochidia in conglutinates, but glochidia were fully formed and largely free of egg membranes similar to mature glochidia of other lampsilines.

In other lampsiline species that display mantle lures, including *Ligumia*, release of unelaborated conglutinates has been explained primarily as a necessity to clear the gills for deposition of the subsequent brood in late summer or autumn, but also potentially as a secondary infection strategy. Our results are consistent with this idea. At ambient temperatures, mantle lures of *Ligumia* were displayed for an extended period spanning ≥2 to 3 mo, showing the importance of this behavior. Conglutinate release occurred primarily during the decline or cessation of lure display. However, individuals released cohesive conglutinates over a protracted period roughly equal to the duration of lure display, rather than dumping all glochidia in a relatively short burst. Many species release puerile conglutinates composed of unfertilized eggs or immature glochidia in response to handling or stress, but these releases occur rapidly, over a few hours or days, and do not appear to be involved in host infection (Aldridge and McIvor 2003, Haag and Warren 2003, Barnhart et al. 2008). In the static 25 and 35°C treatments, individual *L. subrostrata* released conglutinates rapidly. The release period was shortest at 35°C, and conglutinates disassociated readily, suggesting that this release may have been a stress response to clear the gills quickly and to improve respiratory efficiency at high temperatures (see Aldridge and McIvor 2003). In contrast, at ambient temperatures, conglutinate release was protracted, occurred primarily at temperatures >23°C, and conglutinates were more cohesive, suggesting that this behavior represents a secondary infection strategy and not a stress response.

Ligumia subrostrata exhibited a temperature-mediated switch in infection strategies from display of mantle lures to release of glochidia. The relative importance to population growth of lure display vs conglutinate release is unknown. However, several pieces of evidence suggest that lure display is the primary mode of host infection, and release of conglutinates represents a secondary, bet-hedging strategy. First, lures of *L. subrostrata* are elaborate structures that effectively mimic fish prey items and are displayed for extended periods, but conglutinates are simple, unadorned structures that appear to be mainly artifacts of compaction within the gills and receive no additional female investment. Second, lure display always preceded conglutinate release, even at high temperatures that may be stressful to adult mussels. Infection of hosts via mantle lures and subsequent recruitment of juvenile mussels earlier in the growing season probably increases the fitness of these individuals by maximizing growth and allowing attainment of reproductive maturity in their 1^{st} y (WRH and JAS, unpublished data). Release of glochidia remaining in the gills after lure display must occur eventually to make room for the subsequent brood and perhaps also to minimize O_2 stress associated with brooding or lure display at high summer temperatures. However, an extended period of release of cohesive conglutinates may be a bet-hedging strategy to reduce wastage of residual glochidia.

Other aspects of mussel reproductive biology including gametogenesis, fecundity, and recruitment success are intimately linked to temperature, and human-caused changes in stream temperatures can disrupt these processes (Layzer et al. 1993, Moles and Layzer 2008, Galbraith and Vaughn 2009). Changes in stream temperature may alter dynamics of host infection. In streams with depressed temperatures from hypolimnetic dam release, gravid females may not display or may display later in the season resulting in lower juvenile fitness. Increases in stream temperature associated with global climate change or other factors could shorten the display

period or shift the conglutinate release period to earlier in the year. A shorter period of lure display could cause greater reliance on conglutinate release. If conglutinate release is less efficient than mantle lures in transferring glochidia to hosts (e.g., Haag and Warren 1998), such a switch could negatively affect recruitment and population growth.

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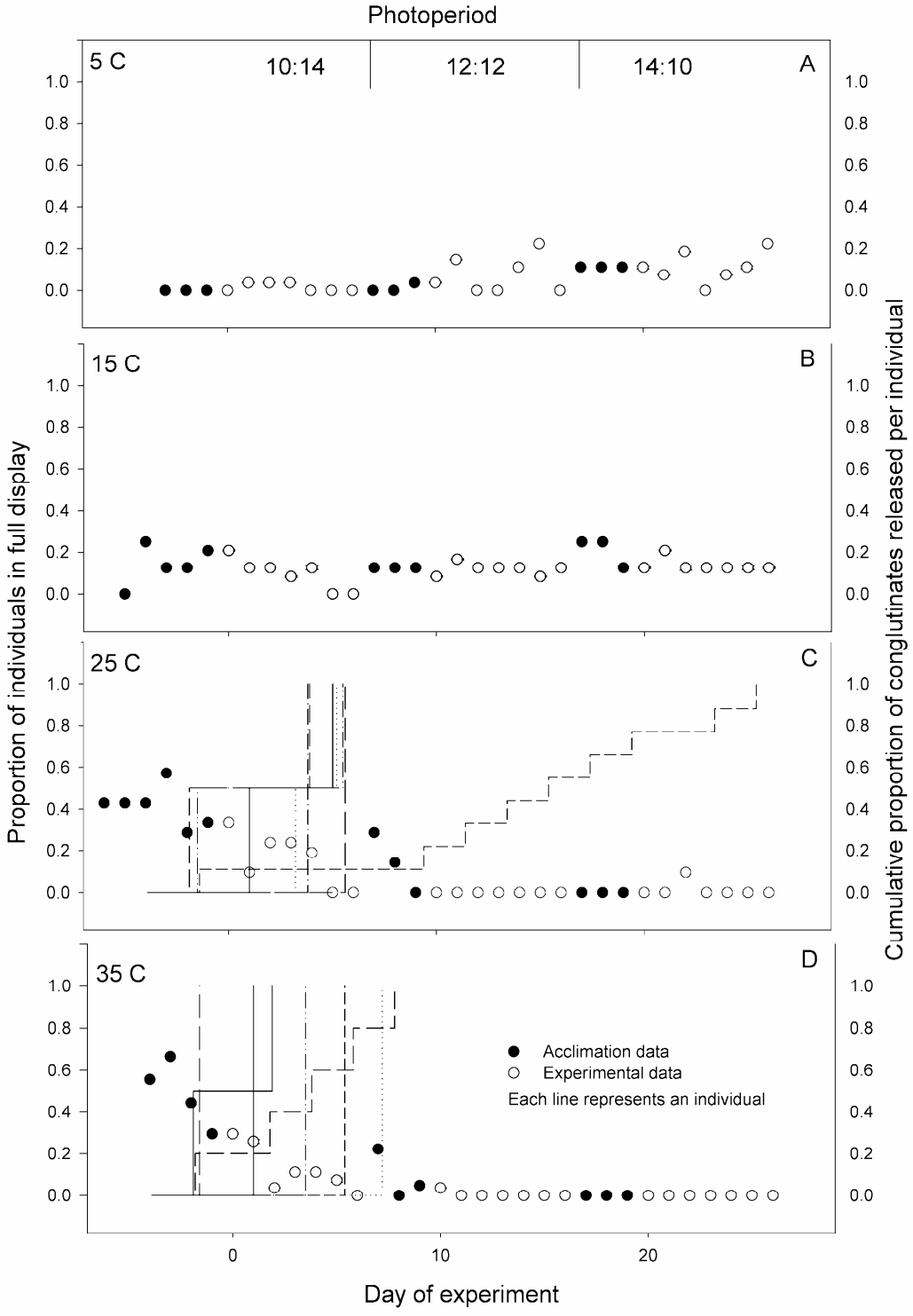

Figure 5-1. Mean (±1 SE) proportion of mussels in full display (circles) and proportion of conglutinates released (lines) with increasing photoperiod and time while maintained at 5 (A), 15 (B), 25 (C), or 35°C (D). Lines represent the cumulative proportion of conglutinates released by individual mussels.

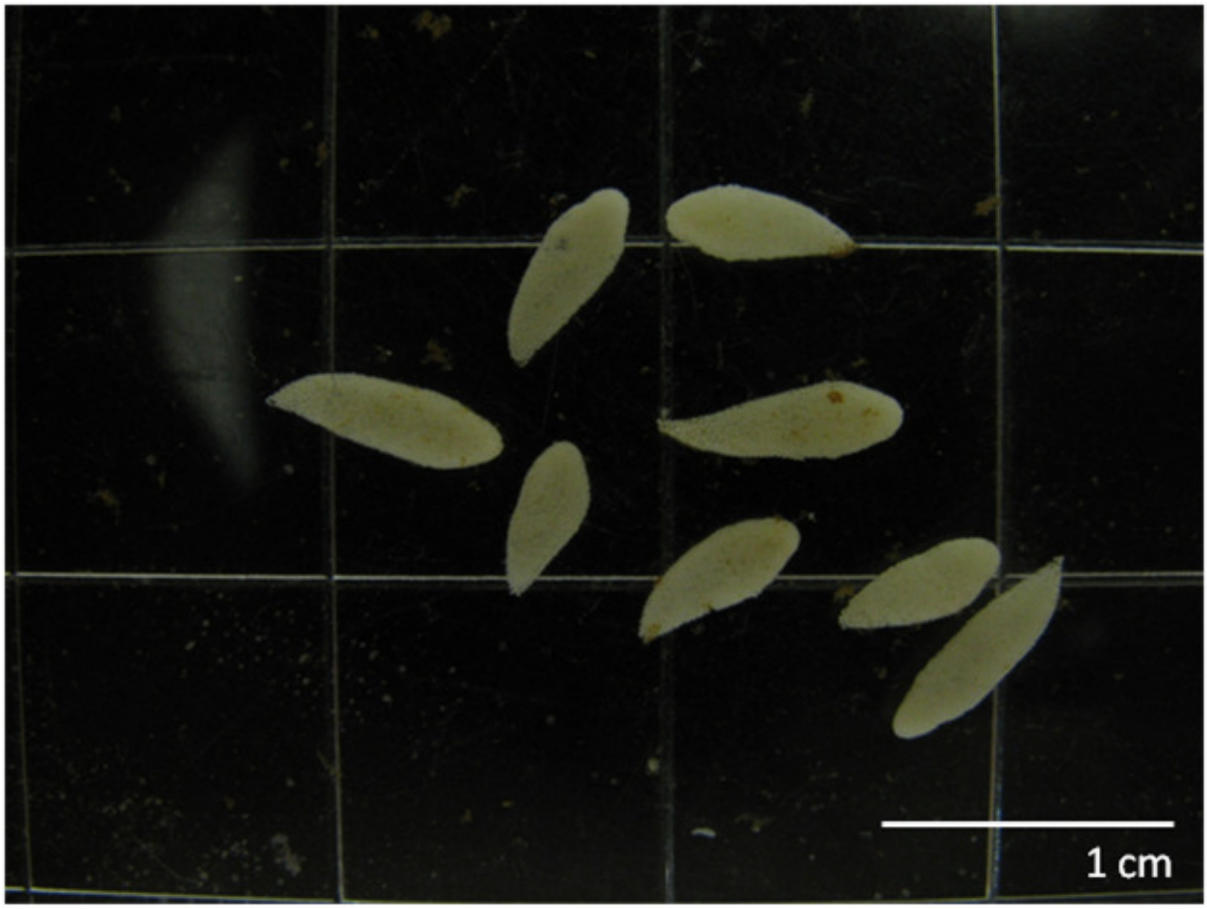

Figure 5-2. *Ligumia subrostrata* conglutinates (cohesive conglutinates from Experiment 2)

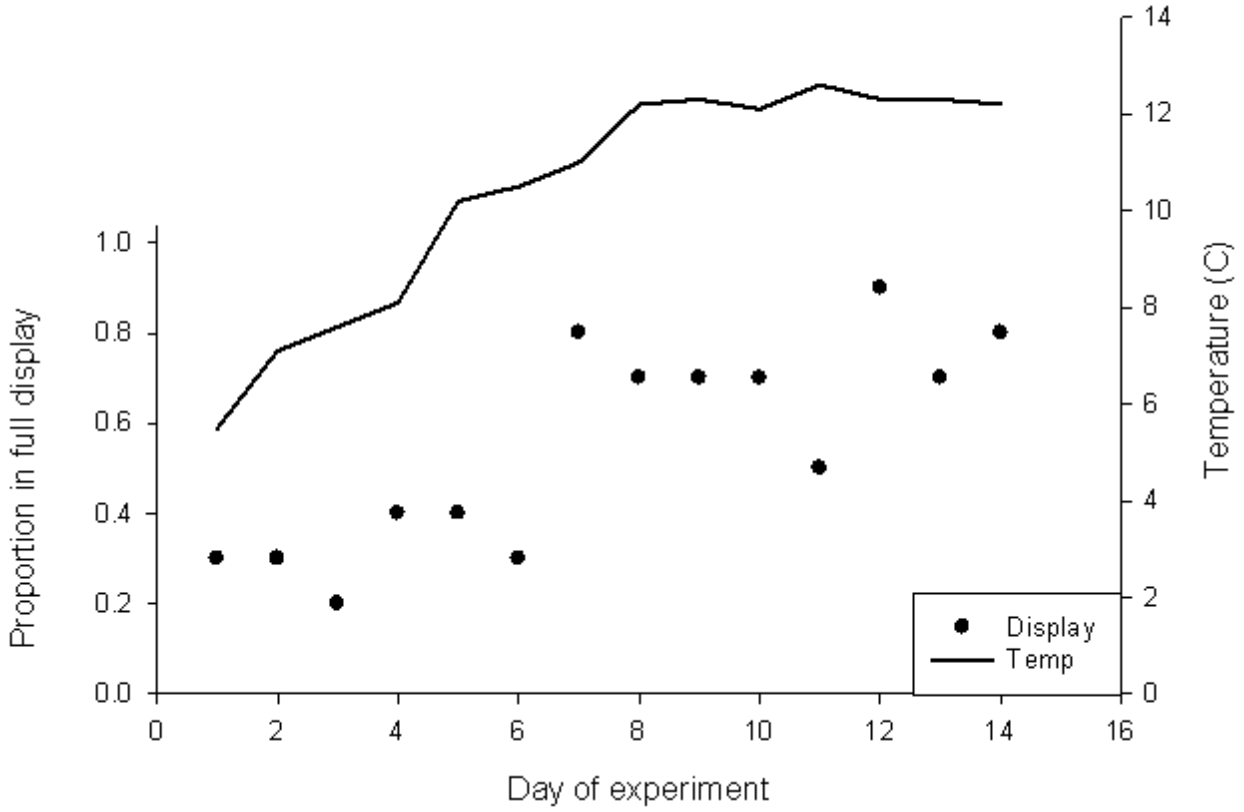

Figure 5-3. Experiment 1. Frequency of *Ligumia subrostrata* lure display during an increase in water temperature from 5 to 12°C under a constant photoperiod of 10:14 h light:dark (L:D). Data points represent the proportion of individuals in full display on each day. The solid line is daily water temperature.

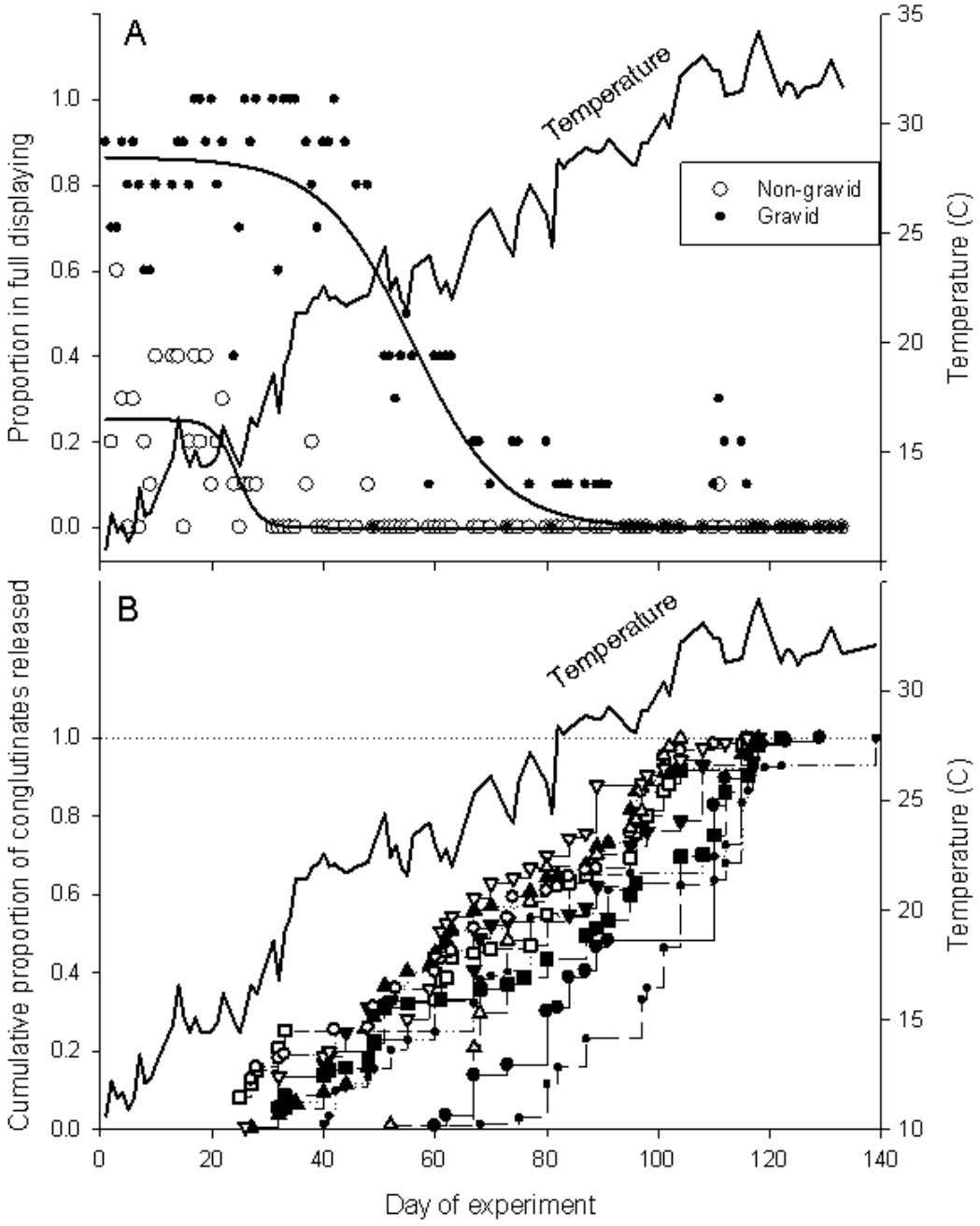

Figure 5-4. Experiment 2. A.—Proportion mussels in full display and temperature vs time under ambient increases in photoperiod and temperature. Trend lines for gravid and nongravid mussels were derived from a logistic model (see text). B.—Cumulative proportion of released conglutinates and temperature vs time. Each symbol and line combination represents an individual mussel.

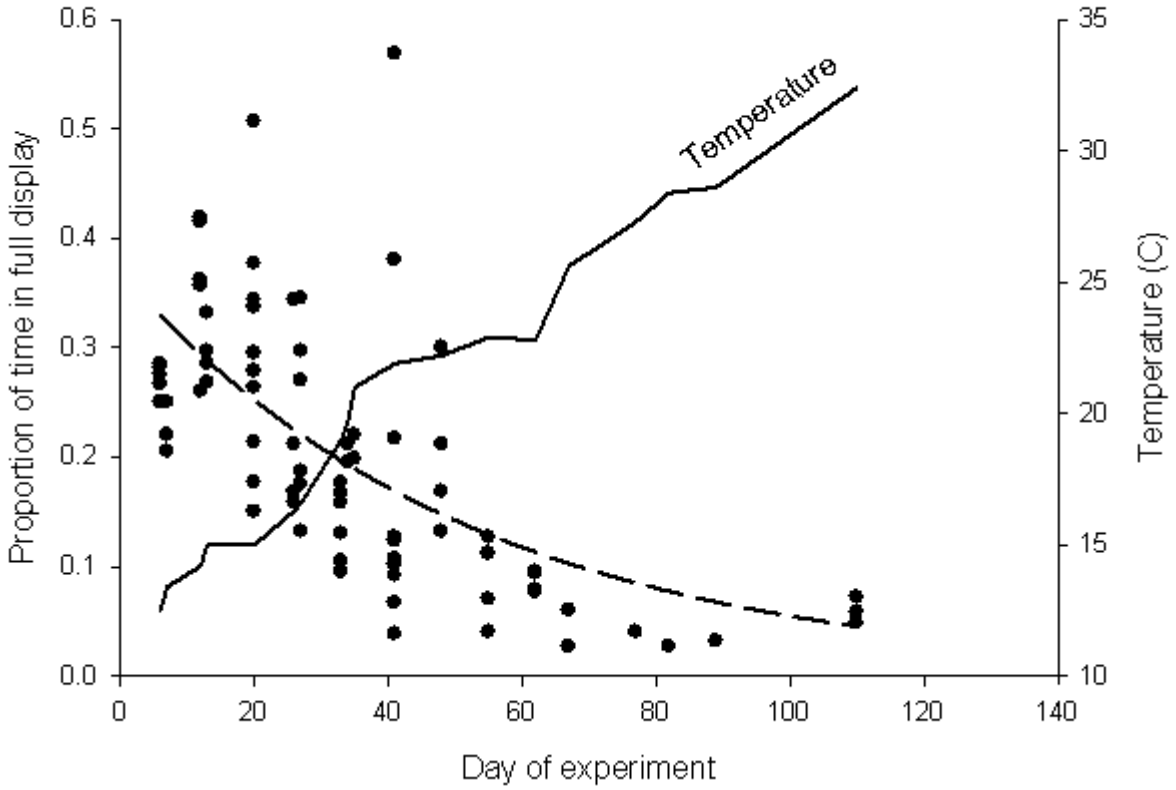

Figure 5-5. Experiment 2. Proportion of time spent in full display at ambient increases in temperature and photoperiod from 27 February to 9 July 2010.

Chapter 6

Conclusions

Freshwater mussels in the United States, especially in the Southeast, are facing an extinction crisis (Strayer et al. 2004). Many factors are linked to the decline of freshwater mussel populations, specifically impoundments, habitat destruction, and poor water quality (Downing et al. 2010); however, a strong link between suspended solids and mussel population declines has not been made. In a review of the literature Brim Box and Mossa (1999) made a strong argument for the widespread affects of suspended sediments on freshwater mussels. While many of the claims were logical, unfortunately at that time there was little scientific evidence to support the claims. My research is able to add concrete evidence of the negative impacts of suspended solids on freshwater mussels.

Overall, my results suggest that the life history stage most affected by suspended solids is early reproduction, including both fertilization and glochidia development. While it is fortunate that many life stages appear to be minimally influenced by suspended solids (particle removal, growth, glochidia viability, juvenile transformation success, and glochidia transfer (Gascho Landis and Stoeckel, unpublished data)), the disruption of the reproductive stage is perhaps the most deleterious since a lack of reproduction would necessarily eliminate subsequent developmental stages. When combining results from the three separate in-pond reproduction

trials, using different species and solids types, we observed a low proportion of females developed glochidia at TSS levels >20 mg/L; however, reproduction was not always entirely eliminated. It may be possible for populations to exist in turbid environments (as is observed), but reproduction is likely occurring at less than maximum rates and this mechanism may be responsible for the slow declines seen in many species.

Although the patterns resulting across my experiments are consistent and strong, caution must be exercised before generalizing these results. I only examined two species from the approximately 300 species found in the United States, so extrapolating the results broadly may not be prudent. Experiments examining effects of suspended solids on reproduction of additional species are needed. Since we conducted tests on species that are relatively common it would be useful to examine species that have restricted ranges because they may show even greater sensitivity to elevated suspended solids loads. Also, we did not examine any species that are habitat specialist in small streams. These species may be particular sensitive because of evolving in systems that typically have low levels of suspended solids. It is also important to conduct field surveys to assess if patterns observed in natural populations are consistent with those in experimental ponds.

Timing and duration of elevated suspended solids loads will be important when considering potential effects on freshwater mussel reproductive success. If suspended solids loads are high during spawning it will likely have the greatest impact on mussel populations, but may have little impact during the rest of the year. However, in species-rich mussel beds, which can contain species with a wide range of spawning and brooding behaviors, spawning may occur for at least one species throughout the majority of the year (January - November). Some species of short-term brooders may begin spawning in late winter, while others spawn in June and July

(Haag and Warren 1997). Long-term brooding species may spawn between August and November (Williams et al. 2008). Thus, high levels of suspended solids could have a negative effect at almost any time of the year. In our experiments we examined the influence of chronic exposure. The effects of acute exposure are unknown, but would likely have less effect. In many small streams and rivers high levels of suspended sediments typically only last for relatively short periods of time (Houser et al. 2006), on the order of hours to days, and correspond to precipitation and high discharge events. In contrast, suspended solids loads in large rivers can last for several weeks or months (Soeken-Gittinger et al. 2009). Thus, long-term, elevated levels of suspended solids may be more common in large rivers and is where they could have the greatest impact on mussel populations. It is difficult to make broad generalization about the effect of suspended solids because of the large variation in species reproductive timing and aquatic systems lack a universal pattern in timing and duration of high suspended solid loads. Assessing the effect of suspended solids in the field will require case specific investigations.

Requiring appropriate watershed management will likely lead to the greatest reductions in suspended sediment loads. Several studies have shown a correlation between the degree of watershed disturbance and persistence of mussel populations (Poole and Downing 2004, Hopkins 2009). Thus, it seems likely that improvements that reduce disturbance will lead to decreases in run-off derived solids and potentially increases in species numbers and total abundance. Strategies for ameliorating watershed degradation include revegetating riparian corridors, constructing wetlands, reforesting low quality crop lands, control of storm water runoff, and best management practices for forestry and agriculture.

One fact is clear concerning the current freshwater mussel extinction crisis – one variable is not entirely responsible for the decline of freshwater mussel species and abundance. My

research indicates that suspended solids clearly have the ability interfere with the reproductive success of freshwater mussels, but is certainly not the only cause of mussel declines. Suspended solids likely play a synergistic role, along with the putative agents of stream and river degradation (Haag 2012). The magnitude of the role of suspended solids will be difficult to parse out from other conservation threats and will likely never be completely understood (Brim Box and Mossa 1999). However, my hope is that this research lays a foundation and is a catalyst for continued research on this important topic.

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