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FINAL REPORT

FEDERAL AID GRANT NO. T-27-P-1

REPRODUCTIVE TIMING AND SUCCESS OF FRESHWATER MUSSELS IN THE LITTLE RIVER, OKLAHOMA

OKLAHOMA DEPARTMENT OF WILDLIFE CONSERVATION

JULY 1, 2005 through DECEMBER 30, 2008

FINAL REPORT

Reproductive timing and success of freshwater mussels in the Little River, Oklahoma

Submitted to:

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FINAL REPORT

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Abstract:
Freshwater mussels are one of the most highly threatened faunas globally. Little research has been conducted to determine what factors lead to successful reproduction (gamete development and fertilization success) in freshwater mussels. We conducted both field and laboratory studies to determine the factors that influence successful reproduction in three closely related species of freshwater mussels, *Quadrula cylindrica*, *Quadrula pustulosa*, and *Quadrula quadrula* in the Little River in southeastern Oklahoma. We also conducted an experiment to determine how sperm motility is affected by water temperature, and built the results into a model predicting the downstream movement of viable sperm under natural temperature and flow conditions. We discovered that successful gamete development in these three mussel species is closely linked to temperature, specifically the number of degree days in the field. We found in our laboratory studies, however, that temperature, photoperiod, and food availability all interact to regulate gamete development in these species. Our field data also suggested that successful reproduction in the Little River could be severely inhibited by unnatural flow and temperature regimes, unequal mussel sex ratios, and parasitism. We found that *Q. pustulosa* sperm is most viable between 15 and 25°C and that sperm has the potential to travel long distances downstream before settling out of the water column or becoming nonviable. We recommend that natural temperature and flow patterns be maintained in southeastern Oklahoma rivers to facilitate successful mussel reproduction.

Objectives:
(1) Collect data on mussel reproductive cycles and determine how reproduction is related to environmental cues including temperature, photoperiod, and food availability.
(2) Determine how fertilization success may be influenced by water temperature and flow.

Need:
(1) Timing of mussel reproduction
Freshwater mussels are one of the most critically imperiled faunas in North America. Their numbers are dwindling due to habitat destruction, population fragmentation, and introduction of invasive species. Mussel populations in southeastern Oklahoma are no exception; both the Little and Kiamichi watersheds are currently threatened by a plan to sell water to the North Texas Alliance, and both rivers are already impounded. Both of these factors influence natural water

temperature, flow, and light regimes within the river, all factors that may be important to mussel survival and reproduction. Given the current and projected demands for water in this area, it is important to determine what factors are important in regulating freshwater mussel reproduction. If we can determine the environmental cues that trigger reproduction, we should be better able to manage lakes and rivers to maintain freshwater mussel reproduction.

(2) Fertilization success of freshwater mussels

Environmental variables are not only important in triggering gamete development and release in mussels; they also may have a profound effect on how successful mussel sperm is at reaching a female mussel's eggs. High temperatures may decrease sperm viability thus decreasing reproductive success. Additionally, flow may be important for both overall reproductive success and population genetics. High flow events may wash mussel sperm downstream, facilitating gene flow among populations; however, low flow (which is usually coupled with high temperatures) may limit fertilization to nearby individuals within the mussel bed. Therefore it is important to know how the environment influences mussel sperm once it has been released.

Background:

Freshwater mussels are one of the most globally-threatened faunas due to habitat destruction, population fragmentation, and introduction of non-native species. To understand how to prevent further mussel declines, it is important that we understand the reproductive biology of these organisms and what conditions are favorable for successful reproduction. Reproduction occurs when males broadcast their sperm into the water column and females passively filter in the sperm. Female eggs are housed and fertilized in specialized brood chambers located within her gills. After reaching a particular developmental stage, mussel larvae (called glochidia) are transferred to one or several specific host fish species to survive as ectoparasites until reaching further maturity. Juvenile mussels then drop from their host fish and mature to adulthood in the epibenthos (McMahon and Bogan 2001).

Little is known about the timing and success of reproduction in most species of mussels (particularly those that are threatened or endangered), and even less is known about the environmental cues that signal reproduction. It is hypothesized that reproduction is triggered by a combination of temperature, photoperiod, and food availability (Borcherding 1995). If this is the case, mussels experiencing unnatural environmental conditions (like those in regulated rivers) may survive but fail to reproduce, contributing to a slow decline of the community. For example, hypolimnetic coldwater releases from some reservoirs have been shown to halt mussel reproduction (Layzer et al. 1993). Reservoirs are also known to severely alter temperature, light and food availability, as well as fragment mussel populations. Additionally, most southeastern streams are intermittent (i.e. dry in areas) during hot summer months, turning long stretches of river into a series of isolated pools. River regulation further exacerbates these harsh conditions, particularly in the pools which experience extremely high temperatures, high ammonia, and low dissolved oxygen. Here mussel communities encounter unnatural thermal regimes for cuing reproduction, and experience high stress levels (Spooner 2007), further decreasing potential reproductive success. Therefore, to preserve mussel community success, it is critical to understand the intricacies of mussel reproductive timing and how it relates to environmental conditions.

Although environmental signals may be important for cuing gamete development and release, successful reproduction ultimately relies on the ability of sperm to "find" the eggs of the correct species. Research on zebra mussels has shown that both sperm viability and motility are affected by water temperature (Ciereszko et al. 2001). Low motility and viability could have profound influences on how successful sperm are at reaching eggs. In addition, flow could be important to both reproductive success and overall mussel population dynamics. Sperm release during high flow events may wash sperm downstream and prevent female mussels from becoming fertilized; however, low flow may limit fertilization to nearby mussels which could affect population genetics and relatedness of individuals within a mussel bed. Since both flow and temperature can be altered by river regulation, it is pertinent to understand how fertilization success may be impacted in order to make informed management decisions and conserve mussel populations.

Southeastern Oklahoma mussel populations are threatened by a plan to sell water from the Little and Kiamichi watersheds to the North Texas Alliance. If these plans are carried out, water may be diverted from the Little River, its tributary the Mountain Fork River, and from the nearby Kiamichi River. These actions may impact the mussel populations in these rivers, particularly their reproductive success. Additionally, both of these rivers are already impounded; current patterns of water release also may be influencing the reproductive cycles of mussels. Therefore, the goal of this project was to collect data on mussel reproduction in the Little River watershed to help inform state and federal management agencies and to identify and address mussel conservation needs.

METHODS:

I. Field Study—

In August of 2005 we surveyed three mussel beds in the Little River to establish density estimates for three closely related species of mussels: the Pimpleback (*Quadrula pustulosa*), the Rabbitsfoot (*Quadrula cylindrica*) and the Mapleleaf mussel (*Quadrula quadrula*). We selected the genus *Quadrula* because it represents four Species of Greatest Conservation Need (two Tier I species, one Tier II and one Tier III) and they exist in varying abundances throughout the southeastern part of the state. In addition, we have been monitoring the populations of these three species in the Little River and know they are abundant enough for us to obtain required samples. The results of our study should be applicable to other mussel species in the region.

We sampled mussel beds using both quadrat sampling and timed searches to obtain an estimate of relative abundance of each of the three *Quadrula* species (Vaughn et al. 1997). Quadrat sampling consisted of excavating thirty, $0.25m^2$ quadrats to a depth of approximately 15 cm. Timed searches consisted of at least two hours of searching for mussels by hand, snorkel, or SCUBA in deeper areas (>0.75m). All three of the sites are located below Pine Creek reservoir, and Site 3 is located below the confluence of the Mountain Fork River which is impounded by a cold-water, hypolimnetic release dam.

Between September 2005 and August 2006, we sampled these three species on a monthly basis for one year, except during December, January, and March due to inclement weather and high water. During each monthly sampling trip, we collected, marked, weighed and measured as many individuals of each species that we could find during an approximately two-hour timed search. We collected three, small (~50 μl), gonad samples from their visceral mass using a

syringe and preserved the samples in formalin. This non-lethal technique for quantifying gamete development (gametogenesis) allows large sample sizes without sacrificing individuals, particularly threatened and endangered species. The biopsy-needle method has been used very successfully by several mussel biologists (Shiver 2002, Saha and Layzer 2008). In the laboratory, we examined gonadal samples under a microscope to quantify sperm and egg development in each of the sampled individuals. Sperm samples were quantified using a hemocytometer to estimate the approximate sperm concentration (number of sperm per milliliter) sampled from the gonad tissue (Photograph 1). All eggs and their vitellin membranes were measured (2 estimates of both length and width) to quantify change in ovum size over time (Photograph 2). We also quantified the presence or absence of sterilizing trematodes in the gonads (Photograph 3).

We estimated mussel body condition using the Fulton's K metric (K) in which body condition = $l^3w(10^6)$, where l is mussel length and w is mussel wet weight. This measurement of condition has been traditionally used in the fish literature, but has been applied with success to freshwater mussels (Spooner 2007).

We placed HOBO™ temperature and light loggers in each mussel bed. These loggers recorded measurements every 30 minutes so that we could estimate seasonal and diurnal temperature and photoperiod variation during the year of our sampling. We also measured stream flow and water column productivity (a surrogate measure of food availability) during each time period. We estimated the number of accumulated degree days as a single sine method using the University of California Statewide Integrated Pest Management Program online degree day calculator (http://www.ipm.ucdavis.edu/WEATHER/ddretrieve.html).

We also collected benthic core samples at each site on a monthly basis. These samples were taken back to the laboratory where they were homogenized with a sediment processor. Three 150 ml subsamples were collected. We then filtered each subsample and dried and weighed it to obtain dry weight estimates. We then ashed the samples in a muffle furnace to obtain estimates of ash free dry mass as a surrogate measure of benthic production. We correlated these environmental parameters (productivity, temperature, degree days, and light) to the timing of peak reproduction in the three species of freshwater mussels to make predictions about the environmental cues important for stimulating gamete development and release.

Data analysis for field study:

We analyzed mean density on a species-by-site basis using a two-way ANOVA with a Tukey's post hoc multiple comparison procedure. We used chi-square analysis to determine differences in parasite loads among sites. To do so, we used two different methods for calculating the expected parasite loads within each site: for the first method, we simply assumed that parasite loads should be equal across all three sites; in the second method, we assumed that rates of parasitism should be a function of mussel density and weighted our expected proportions by mussel density at each site.

We used ANOVA and Tukey's post hoc comparisons to test for differences in body condition among sites. Chi-square analysis was used to determine if the proportion of males of each species was equal both across all three sites and within individual sites. We also used chi-square

analysis to test for differences among sites in the incidence of hermaphroditism. We tested the null hypothesis that hermaphrodites are equally distributed among all three sites. We used ANOVA and Tukey's post hoc multiple comparison procedures to determine differences in seasonal water temperature and productivity among sites. We also tested for differences among sites in the mean hours of light (> 0 lux) reaching the benthos using ANOVA. We graphically analyzed timing of peak reproduction in each species at each site and we used regression analysis to determine which environmental variables are important in regulating gametogenesis in the field.

II. Laboratory Experiment—

Temperature, light, and food availability are often correlated in the field. During the summer of 2007, we used our field data to design a laboratory experiment testing the influences temperature and photoperiod on reproductive timing. Mussels were housed in recirculating stream mesocosms (1.5 m x 0.5 m x 0.5 m) on the University of Oklahoma campus which consisted of large fiberglass tanks lined with gravel-filled Rubbermaid containers. Each stream housed 14 *Q. pustulosa* and five *Q. cylindrica* individuals for a total mesocosm density of approximately 25 individuals per m^2. Each of 12 streams was exposed to one of four temperature and light treatments: cold/dark, cold/light, warm/dark, and warm/light. Cold treatments were kept at 5°C for the entire three month period, while warm treatments were kept at 5°C for the first month and 15°C for the second and third months. Dark treatments were exposed to 16 hours of darkness and 8 hours of light, while light treatments were exposed to 16 hours of light and 8 hours of darkness.

Mussels were fed every other day with a 2:1 mixture of commercial marine shellfish diet and *Nannochloropsis* (Instant Algae, Reed Mariculture, Campbell, California, USA). We wanted to ensure that food was not a limiting factor in this experiment. Data from (Spooner 2007) show that *Q. pustulosa* clearance rates triple from 5°C to 15°C. Therefore, mussels in cool treatments were fed approximately 3.0 mls of shellfish diet and 1.5 mls of *Nannochloropsis* while warm treatments were fed 9.2 mls of shellfish diet and 4.6 mls of *Nannochloropsis*. Partial water changes were completed every two weeks to minimize the accumulation of ammonia in the water. We collected gonad samples from a subsample of individuals in each treatment on a monthly basis to quantify reproductive development. After sampling, mussels were placed back into their respective streams to maintain mussel density throughout the experiment.

Data analysis for laboratory experiment:

We used a two way ANOVA to determine the effects of temperature and light on gamete development in the laboratory experiment. Mean mussel wet weight for the stream and time were used as covariates in this analysis.

III. Model—

To determine how temperature influences sperm viability we used similar methods to those of (Ciereszko et al. 2001). We originally intended to collect sperm samples "voluntarily" released by males after injecting them with a solution of serotonin, a neurotransmitter that is known to stimulate gamete release in certain mussel species; however, we were unable to trigger spawning in this species using this method. Instead, we collected sperm from *Q. pustulosa* individual males, taken from the Kiamichi River, using the syringe biopsy technique and diluted the sperm

in filtered pond water taken from the University of Oklahoma's Aquatic Research Facility. We exposed the sperm to temperatures of 5, 15, 25, and 35°C (all temperatures experienced by mussels with mature sperm present in their gonads in the Little River) and estimated the percentage of motile sperm after 0, 2, 4, 8, 24, and 48 hours (n=7 for each temperature).

We also analyzed mussel sperm morphology using scanning electron microscopy to calculate settling rates of sperm for our model. We collected sperm samples from five individuals of *Q. pustulosa* (taken from the Kiamichi River) using a syringe. The sperm were fixed for 30 minutes in a 3:1 solution of saturated mercuric chloride and 2% osmium tetroxide and then washed three times with distilled water. The sperm were mounted on sputter coated 22mm Thermanox® cover slips and were dehydrated in an ethanol series. Coverslips were critical point dried and sputter coated with a gold/palladium mix and viewed under the scanning electron microscope. We used Image J (Abramhoff et al. 2004) digital image analysis software to measure average head length and width, and average tail length.

To estimate settling velocity (V_s) of sperm we used Stoke's Law: $V_s = 2gr^2 (\rho_p - \rho_f)/9\mu$ where g = acceleration of gravity (m/s), r = the radius of the sperm (m), ρ_p and ρ_f are the densities of the sperm and water respectively (kg/m^3), and μ is the dynamic viscosity if water (Pa s). We used measurements of length (l), width (w), and height (h) of sperm to calculate the equivalent spherical diameter (ESD= $(lwh)^{1/3}$) which was used in place of the radius in the Stoke's Law equation. ESD of an irregularly shaped object provides an estimate of the diameter of a sphere with an equivalent volume to the object. In estimating the ESD, we considered only the dimensions of the sperm head and did not take into account the length of the tail. We obtained estimates of cell density from (Kamykowski et al. 1992) who calculated cell density for six species of marine dinoflagellates (average= 1.079 g/cm^3).

We modeled the effects of temperature and stream flow to estimate the downstream movement of sperm under varying flow regimes. To do so, we used USGS real-time discharge data (http://waterdata.usgs.gov/nwis/uv?07338500) from July (the approximate time gamete release in these three species). In particular, our models were based on four different July discharge estimates: 0.51 cubic meters per second (cms), the July 2006 average; 8.34 cms, the average July discharge from 2000-2007; 18.89 cms, the average July discharge from 1990-1999; and 58.26 cms, the maximum July discharge from 1990-2006. Estimates were based on sperm motility at 25°C, the approximate mean water temperature during the month of July. To build the model, we assumed laminar flow and a constant river width of approximately 25.5 meters (based on field data at our three sampling sites) throughout the Little River and across varying discharge. We built a regression between stream depth and discharge so that we could approximate the cross sectional area of the Little River under different discharges ($F_{(1,11)}$= 228.885, $p < 0.0001$, $R^2 = 0.95$).

Data analysis for model:

To determine the effect of temperature on sperm motility we used repeated measures ANOVA with time as the block factor and mussel wet weight and initial percent motility as covariates. To determine the dimensions of mussel sperm, we averaged the head length and width and tail length measurements from 36 subsamples (i.e. different sperm) collected from four individuals.

Results:

I. Field Study—

We found significant differences in mussel density among sites ($F_{(2,261)}= 29.249$, $p< 0.001$) and species ($F_{(2,261)}= 165.786$, $p< 0.001$) and a significant site-by-species interaction ($F_{(4,261)}= 19.918$, $p< 0.001$). All three species were significantly different from each other with *Q. pustulosa* having the highest density followed by *Q. cylindrica* and *Q. quadrula* respectively. In general, Site 2 had the highest densities of all three species, followed by Site 1 and then Site 3, with the exception of *Q. cylindrica* density, in which case Site 1 had higher densities than Site 2 (Fig. 2).

Over the course of our year-long study, we sampled a total of 460 individual mussels across species and sampling sites (Fig. 3). Of these, sterilizing trematodes were present in 17 individuals and were only found in one species, *Q. pustulosa* (Fig. 4). We used chi-square analysis and failed to reject the null that rates of parasitism should be equal among sampling sites ($\chi^2_{(2)}=2.235$, $p= 0.327$); however, we did find a significant difference among sites after weighting the expected probabilities by *Q. pustulosa* density at each site ($\chi^2_{(2)}= 12.852$, $p= 0.0016$), with higher rates of parasitism than expected at Site 3 (Fig. 5).

We found significant differences in mussel body condition as calculated by Fulton's K across sites (*Q. cylindrica*: $F_{(2,111)}= 18.507$, $p< 0.001$; *Q. pustulosa*: $F_{(2,272)}= 16.489$, $p< 0.001$; *Q. quadrula*: $F_{(2,34)}= 5.890$, $p= 0.006$). Body condition in all three species was consistently lower at Site 3 than either one or both of the other sites (Figs. 6-8). We found no effect of sex or parasitism on *Q. pustulosa* body condition except females at Site 3 had significantly lower body condition than males (Fig. 9).

We also found differences from equality in the relative proportion of males and females of each species (Fig. 10). Averaged across all sites, *Q. cylindrica* had a significantly female biased population ($\chi^2_{(1)}= 4.033$, $p= 0.0446$); however, within individual sites there were no differences from equal sex ratios. Alternatively, *Q. pustulosa* had significantly more males than females ($\chi^2_{(1)}= 8.4$, $p= 0.0037$) across all sites. Although this same pattern was seen at each of the individual sites, there was only a significant male bias at Site 1 ($\chi^2_{(1)}= 6$, $p= 0.0143$). *Quadrula quadrula* was found to have an approximately equal number of males and females across all sites and within each individual site.

We found a significant difference in the proportion of hermaphroditic individuals among sites ($\chi^2_{(2)}= 6$, $p= 0.0498$; Fig. 11). There was no difference among sites in the frequency of *Q. cylindrica* hermaphrodites ($\chi^2_{(2)}= 0.5$, $p= 0.7788$; Fig. 12). However, Site 3 had significantly more hermaphrodites of *Q. pustulosa* ($\chi^2_{(2)}= 6$, $p= 0.0498$; Fig. 13) and marginally more *Q. quadrula* hermaphrodites ($\chi^2_{(2)}= 5.2$, $p= 0.0743$; Fig. 14) than expected. The incidence of hermaphroditism ranged between 0 and 7% for Sites 1 and 2, but for Site 3 was as high as 14% in *Q. cylindrica* and 33% in *Q. quadrula*.

We found significant differences among sites in mean annual temperature ($F_{(2,906)}=10.488$, $p< 0.001$) with Site 2 having the highest mean annual temperature and Site 3 having the lowest (Fig. 15). We also found seasonal differences among sites (winter, spring, summer and fall), particularly in the winter and summer (winter: $F_{(2,267)}= 7.131$, $p= 0.001$; summer: $F_{(2,218)}=$

214.358, $p < 0.001$). Specifically, Site 3 was significantly warmer than Site 1 in the winter and significantly colder than the other sites in the summer (Fig. 16). We found peak summer temperatures occurred in August at Sites 1 and 2 and in June at Site 3. The lowest temperatures occurred during December at all sites (Fig. 17).

The average amount of light reaching the benthos varied throughout the year and is likely a function of both time of year, stream flow, and turbidity (Fig. 18). We found a significant difference in hours of light reaching the benthos among sites ($F_{(2.796)} = 9.111$, $p < 0.001$) with Site 1 receiving more light to the stream bottom than Sites 2 or 3.

We found a significant difference in mean benthic production based on site ($F_{(2,118)} = 15.160$, $p < 0.001$) and a significant season by site interaction ($F_{(6,118)} = 2.874$, $p = 0.012$). In particular, Site 1 had significantly lower benthic production than Sites 2 and 3, which were not significantly different from each other (Fig. 19). These differences varied by season except during the winter when there were differences in benthic production among sites (Fig. 20). Site 1 consistently had the lowest benthic production of all the sites during the fall, spring and summer. However, Site 3 also had lower benthic production than Site 2 during the fall.

We identified reproductively mature individuals within a range of size classes for each species (Figs. 21-23). The smallest individuals collected of each species were 58, 38, and 47 mm for *Q. cylindrica*, *Q. pustulosa*, and *Q. quadrula* respectively, and all were found to have mature gametes in their gonads. Averaged across all sites, peak sperm concentration in the gonads occurred in the summer, with *Q. cylindrica* reaching its peak slightly earlier (late May) than *Q. pustulosa* or *Q. quadrula* (mid June; Fig. 24). Declines in gonadal concentrations of sperm after the peak are attributed to gamete release and occur throughout June, July and August. There appeared to be variability in timing of reproduction among species depending on site (Figs. 25-27). For example, at Site 1, *Q. quadrula* and *Q. cylindrica* both appeared to reach their reproductive peak earlier than *Q. pustulosa* (Fig. 25); however, Site 2 mussels matched the pattern of reproductive timing seen river wide (Fig. 26). Patterns of gametogenesis are difficult to distinguish at Site 3 given the lower densities of individuals and extremely erratic sperm concentrations (Fig. 27).

Mean ovum diameter in the gonads (averaged across all three sites) followed similar seasonal patterns to sperm concentration; however, peaks in ovum diameter occurred slightly earlier than peaks in sperm concentration (Fig. 28). Patterns in ovum diameter appeared to be similar among sites, although patterns were again difficult to discern at Site 3 (Figs. 29-31).

We found a significant cubic relationship between mussel sperm concentration and the number of accumulated degree days since the start of our field sampling in both *Q. cylindrica* and *Q. pustulosa* (*Q. cylindrica*: $F_{(3,45)} = 28.6911$, $p < 0.0001$, $R^2 = 0.6338$; *Q. pustulosa*: $F_{(3,149)} = 11.5073$, $p < 0.0001$, $R^2 = 0.1718$; *Q. quadrula*: $F_{(3,14)} = 2.435$, $p = 0.1080$, $R^2 = 0.2021$; Figs. 32-34). We also found a significant relationship between ovum diameter and the number of accumulated degree days since the start of our field sampling in all three species (*Q. cylindrica*: $F_{(3,61)} = 62.5359$, $p < 0.0001$, $R^2 = 0.7426$; *Q. pustulosa*: $F_{(3,103)} = 41.2518$, $p < 0.0001$, $R^2 = 0.5325$; *Q. quadrula*: $F_{(3,15)} = 4.1011$, $p = 0.026$, $R^2 = 0.3407$; Figs. 35-37). We did not observe any correlation between reproductive maturity and mean monthly temperature or the number of hours

of light reaching the benthos. Additionally, we found no relationship between mussel length, wet weight or body condition and stage of reproduction.

II. Laboratory Experiment—

Overall, we found a marginally significant effect of light and a marginally significant temperature-by-light interaction in explaining sperm concentration in male *Q. pustulosa*, and time was a significant covariate (light: $F_{(1,25)}= 2.955$, $p= 0.098$; light x temperature: $F_{(1,25)}= 3.163$, $p= 0.087$; time: $F_{(1,25)}= 8.306$, $p= 0.008$). Treatment differences were only significant during time period 2 in which case there was a significant effect of temperature, light, and a light by temperature interaction. Specifically, males in warm and dark treatments were found to have significantly higher sperm concentrations in their gonads than males in other treatments (Fig. 38), but only during the second time period ($F_{(1,7)}= 8.532$, $p= 0.022$; temperature: $F_{(1,7)}= 3.824$, $p= 0.091$; light x temperature: $F_{(1,7)}= 5.787$, $p= 0.047$). There was no difference between treatments during time periods 1 and 3.

We found no effect of light or temperature and no light-by-temperature interaction in female *Q. pustulosa*; however, we did observe similar trends to those of the males with higher ovum diameters in warm and dark treatments (Fig. 39). We also found no significant difference among treatments within time periods.

We found no overall effect of light or temperature and no light-by-temperature interaction in male *Q. cylindrica*; however, during time period 3, we found a significant light-by- temperature interaction (light x temperature: $F_{(1,3)}= 14.273$, $p= 0.032$) in which males in cool and dark treatments had higher sperm concentrations than males in other treatments (Fig. 40). We found no significant difference among treatments within time periods 1 or 2.

Overall, we found a significant effect of temperature explaining ovum diameter in *Q. cylindrica* (temperature: $F_{(1,24)}= 6.497$, $p= 0.02$). Specifically, females in cool treatments were found to have significantly larger eggs in their gonads than females in other treatments (Fig. 41). There was no significant treatment effect within time periods in female *Q. cylindrica*.

In general, gamete production was higher in the laboratory experiment than in the field. The highest average *Q. cylindrica* sperm concentrations were between 1.4 and 8.4 (average= 3.8) times higher in the lab than the average peak concentrations in the field. Likewise, *Q. pustulosa* laboratory concentrations ranged from 1.3 to 7.4 (average= 4.5) times higher in the lab than the field. Patterns in female egg size between the lab and field were more difficult to discern. The largest mean *Q. cylindrica* egg size in the lab ranged from 0.8 to 1.7 (average= 1.2) times the highest average egg size in the field. *Q. pustulosa* egg size appeared to be smaller on average in the lab than in the field with the peak average size in the lab ranging from 0.82 to 0.96 (average= 0.88) times the average peak size in the field.

III. Model—

We found a significant effect of temperature on mussel sperm motility with initial sperm motility as a significant covariate (temperature: $F_{(3,22)}= 9.077$, $p< 0.001$; initial motility: $F_{(1,22)}= 8.629$, $p= 0.008$; Fig. 42). In particular, motility was highest at 25°C (although not significantly

different from 15°C) and much lower at 5 and 35°C (Fig. 43). Averaged across all temperatures there was no significant difference in motility after 8 hours (Fig. 44).

Q. pustulosa sperm averaged approximately 38.23 μm (±3.22 μm) in total length (head plus tail). Sperm head length averaged 3.61 μm (±0.26 μm) while head width averaged 2.13 μm (±0.26 μm) (Photograph 4). Sperm tails averaged 34.62 μm (±3.04 μm) long, approximately 9.6 times longer than sperm head length (Photograph 5). Based on head size, we calculated the ESD of *Q. pustulosa* sperm to be 2.54 μm. Using this metric, we estimated the approximate settling velocity of sperm to be 1.137 μm per second.

Using sperm viability data from the 25°C trials (approximately the mean July temperature), we estimated how far downstream sperm could travel under four different July flow regimes (Fig. 45). However, these estimates merely consider distance moved downstream due to flow alone and do not incorporate estimates of settling velocity. When considering sperm settling rate and viability, we built three models predicting the relative importance of settling rate and viability (Figs. 46-48).

Conclusions and Recommendations:

I. Field Study—

Densities of freshwater mussel species vary across North America and likely depend on biogeography, habitat suitability for survival and reproduction, and rates of extirpation, both natural and human caused. We found differences in density in three species of *Quadrula* among three sites in the Little River with *Q. pustulosa* being the most common, followed by *Q. cylindrica* and the much rarer *Q. quadrula* (Fig. 2). Mussel densities of all three species were lowest at Site 3 than at Sites 1 or 2. Site 3 has unusual thermal and hydrologic regimes compared to the other two sites, which may be a factor affecting the mussel density at this site.

Parasite loads vary among freshwater mussels but in some areas have been found to be as high as 100% in a single species (Henley et al. 2007). While we found less than 10% of our individuals to be parasitized, trematodes were found in only one species, *Q. pustulosa*, and were found in higher abundance than predicted from mussel densities at Site 3 (Fig. 4). There was no significant difference in body condition between parasitized mussels and non-parasitized males and females (Fig. 9); however, we only sampled a relatively small number of parasitized individuals. Further analysis is needed to confirm if parasitism does indeed influence body condition or whether initial body condition makes particular individuals more or less susceptible to infection. We did not find mature gametes in any of our parasitized individuals, suggesting that these parasites completely sterilize their hosts. This has consequences for *Q. pustulosa* reproduction in that a substantial portion of the population is not reproducing, thus lowering the effective population size.

We found significantly female-biased sex ratios in *Q. cylindrica* (Fig. 10), a pattern that has frequently been observed in other mollusk species (Bauer 1987, Byrne 1998, Garner et al. 1999, McIvor and Aldridge 2007). Several of these authors suggest that the female bias could be a phase in the development of hermaphroditism within the population (McIvor and Aldridge 2007, Yusa 2007). We did found some evidence of hermaphroditism in *Q. cylindrica*; however only four hermaphroditic individuals of this species were recorded. (Henley 2002) has shown that in

hermaphrodites, male and female gametes are found in different regions of the gonad. Perhaps our biopsy technique underestimates the proportion of hermaphroditic individuals in the population. If this is the case, it makes hermaphroditism a more plausible cause for the observed sex ratio. We found a male biased sex ratio in *Q. pustulosa*, which has also been commonly observed in freshwater mussels (Downing et al. 1989, Haag and Staton 2003).

These deviations from a 1:1 sex ratio may be due to a sampling bias because our sampling methods are biased towards larger individuals (smallest individual collected was 38 mm, Figs. 21-23) (Vaughn et al. 1997). Alternatively, sex ratios could be equal at conception, with later sex-specific mortality skewing the adult sex ratio. Another possibility is that *Q. pustulosa* females have higher rates of parasite infections than males, or, as mentioned earlier, that hermaphroditism could be affecting the ratio (Yusa 2007). We were unable to confirm or refute the parasitism hypothesis since there were no mature gametes found in any of our parasitized individuals. Nonetheless, the male-biased sex ratio indicates that there are fewer female individuals available for reproduction, and thus *Q. pustulosa* abundance is likely on the decline. This was evidenced in (Galbraith et al. 2005) where *Q. pustulosa* in the nearby Kiamichi River, Oklahoma was found to have dropped in density an average of 85% across 10 established monitoring sites in a period of less than 15 years.

Hermaphrodites generally constitute a small proportion (usually less than 10%) of the population in most studies of reproduction in freshwater mussels (Haggerty et al. 1995, Garner et al. 1999, Haag and Staton 2003). We found similar proportions of hermaphrodites at Sites 1 and 2 and in *Q. pustulosa* at Site 3. However, hermaphroditism was much greater in *Q. cylindrica* and *Q. quadrula* at Site 3. In the past it has been suggested that hermaphrodites are often more common in small, isolated populations or in environments that are particularly stressful (Heard 1975). It could be that the conditions at Site 3 are particularly stressful for this species which is why the incidence of hermaphroditism is so high there relative to the other two sites. *Q. quadrula* is rare throughout southeastern Oklahoma, perhaps because it is on the edge of its range. However, data from the Kiamichi River suggest that this species is also rapidly declining as seen by an almost 14% decline in density since the early 1990's (Galbraith et al. 2005).

We found that all three species of *Quadrula* reproduce during summer months with peak timing of reproduction occurring in June and July. Peaks in female egg size were seen slightly earlier than peaks in sperm concentration (Fig. 24 & Fig. 28), suggesting that female mussels are reproductively mature earlier in the year than males. Females, however, have to transfer their mature eggs from their gonads to their gills where fertilization takes place, so it makes sense that there is a slight difference in peaks of gonad development.

Time of reproduction varied slightly among sites, but in all species it correlated with the number of accumulated degree days in a year (Figs. 32-37). This suggests that natural thermal regimes are important cues for timing of gamete development (and potentially gamete release). Site 3 appeared to have an unusual thermal regime compared to the other two sites, with warmer winter and cooler summer temperatures (Fig. 16). This site also had notably lower mussel densities (Fig. 2), mussels with poorer body condition (Figs. 6-8), higher parasite loads (Fig. 5), and higher proportions of *Q. pustulosa* and *Q. quadrula* hermaphrodites (Figs. 11-14). Additionally,

patterns of reproductive timing were variable among all species, and could be a function of the unusual thermal patterns at the site (Fig. 27 & Fig. 31).

There is evidence that reproductive success can be disrupted by cold water temperatures (Layzer et al. 1993, Heinricher and Layzer 1999) and that unusual thermal regimes can cause declines in species diversity (Heinricher and Layzer 1999, Vaughn and Taylor 1999), and is often attributed to cold water release from dams. Declines in reproduction do not appear to be a function of host fish elimination in these studies, but instead, are due to lack of appropriate reproductive cues. The thermal conditions at Site 3 are not as extreme as in many other disrupted rivers; however, it is clear from this study that this population of mussels is in poor condition. These results provide evidence that natural thermal regimes should be maintained in the Little River, particularly during spring and summer months, to facilitate mussel reproduction.

II. Laboratory Study—

The results from our laboratory study suggest that temperature and photoperiod may both play a complex role in determining the time of gamete maturation. We found that male *Q. pustulosa* respond by increasing their gonadal sperm concentrations when kept in warm, dark conditions; females, on the other hand experienced no change in egg size under any of the conditions. Alternatively, *Q. cylindrica* males responded to cool and dark environments by increasing their sperm concentrations while females responded by increasing their egg size in cool environments.

We also noted, however, that sperm concentrations in both *Q. pustulosa* and in *Q. cylindrica* were much higher in the laboratory experiments than ever observed in the field. Laboratory-kept animals were fed an extremely nutritious diet and were fed in excess such that food would not be a limiting factor in our experiment. It is likely that the effects of high quality food were able to overpower any meaningful effects of temperature or light in our experiment. Our field data offer some support of this conclusion in that Site 1 was observed to have the lowest benthic productivity of all three sites. Corresponding to this were slightly lower sperm concentrations in all three species than the other two sites. Further experimentation where food is a limiting factor is needed to make conclusions about the environmental variables that influence gametogenesis.

III. Model—

We found that *Q. pustulosa* sperm is most viable at 25°C (Figs. 42-43), the approximate mean temperature during months of reproduction. This, however, is based on sperm that were taken from mussels using the syringe biopsy technique and was not collected from mussels that voluntarily released their sperm. It is likely that males that release their sperm naturally release a higher proportion of motile sperm. We suggest further studies are needed of sperm motility in individuals that have been induced to release their sperm.

Under natural July flow regimes, sperm has the potential to be carried significant distances downstream while still remaining viable (Fig. 45), even after taking sperm settling rates into account (Figs. 46–48). This has important consequences for understanding gene flow between mussel beds. (Lefevre and Curtis 1910, Downing et al. 1993) suggested that mussel sperm in lake environments may only diffuse a maximum of 0.5 meters while remaining viable. While this may be the case in lentic systems, it is plausible in lotic systems to see sperm traveling between 0.8 and 14.9 km (Fig. 45) within the four hours during which sperm is the most viable.

When considering sperm viability and distance sperm travel downstream, it is important to remember that sperm also naturally settle out of the water column. The relative importance of sperm settling and sperm viability varies depending on how far above the substrate mussels release their gametes. If, for example, mussels release their sperm only a centimeter above the substrate (and, of course, assuming laminar flow), under all four flow regimes mussel sperm will settle out of the water column before a drop in viability ever occurs (Fig. 46). Again, even after considering settling rates, mussel sperm has the potential to travel anywhere from 1.0 to 9.1 km downstream while still motile.

However, if mussels release their sperm five centimeters above the substrate, motility plays a larger role in how far downstream fertilization can successfully take place (Fig. 47). Ideally, sperm could travel between 4.8 and 45.6 km before settling out of the water column; however, the drop in motility between four and eight hours after release limits fertilization success to distances much closer to where sperm were released. For example, at six hours after release, sperm viability is approximately 10% (almost 1/3 of original motility), and sperm have only moved between 2.4 and 22.4 km, depending on flow regime: sperm effectively travel half the distance that they could have based on settling rates alone.

A similar pattern can be observed if sperm is released 10 cm above the substrate (Fig. 48), with the maximum potential for downstream transport, based on settling alone, between 9.6 and 91.2 kilometers. However, sperm motility caps the movement of motile sperm to the same 2.4 to 22.4 km range that was observed when mussels release their sperm 5 cm above the substrate. This is between 7.2 and 68.8 km (75%) less than it could have traveled based on settling rates alone.

This shows that, although sperm cannot necessarily travel as great a distance downstream when it is released only 1 cm above the water column, its role in fertilization is never limited by a drop in motility. At release heights of 5 cm and greater, sperm has the potential to move extremely large distances downstream; however, its ability to be useful in fertilization is essentially capped to a much shorter distance downstream due to the drop in viability between four and eight hours. There has been no research investigating the height at which freshwater mussels release their gametes; research into this phenomenon would make building useful models of sperm movement more realistic. These data also suggest that, even in mussel species which do not use highly mobile host fish (e.g. darters), there is still a possibility for large amounts of gene flow among mussel beds due to sperm movement. The next step in this research would be to investigate the relative contributions of host fish movement and sperm movement to genetic diversity in mussels.

This is the first model to be developed to predict the movement of mussel sperm and should serve as a null model against which more complex models can be tested. Clearly these models are an oversimplification of reality and do not take into consideration complex flow patterns (eg. turbulence), the diffusion of sperm across the width of the stream, or the loss of sperm due to filtration by mussel beds. Nonetheless, the distance sperm travel ultimately depends on discharge. Thus, successful mussel reproduction requires adequate flow to distribute sperm to receptive females of the same species; this can best be accomplished by maintaining natural flow regimes. Drastically manipulating flow could not only alter the genetic structure of mussel

populations, but lead to failed reproduction of mussels in general should flow be so high that sperm are completely washed downstream.

Since the Little River has some of the last remaining viable populations of *Q. cylindrica* and the recently discovered endangered mussel species *Quadrula fragosa* (Galbraith et al. 2008), particular efforts should be made to assure these species are protected. Our data also indicated that even the more common mussel, *Q. pustulosa*, could be threatened by unequal sex ratios, high parasite loads, and altered thermal regimes (particularly at Site 3). Freshwater mussels are known to provide important services to the aquatic community (Vaughn and Hakenkamp 2001, Vaughn et al. 2004, Spooner and Vaughn 2006, Vaughn et al. 2007, Vaughn et al. 2008). Southeastern Oklahoma provides habitat to over forty-one of North America's 300 mussel species.

Based on our studies, we recommend the natural thermal and discharge regimes in the Little River be maintained, particularly during summer months when mussels are reproducing. Any manmade alterations to the natural flow regimes, such as proposed water sales, must be conducted in such a manner that they do not impact mussel survival or reproductive success. Finally, we recommend long-term studies of mussel reproductive success in the Little River as part of a comprehensive monitoring program. Doing so should help facilitate successful reproduction in both rare and common mussel species and maintain healthy, productive, mussel beds.

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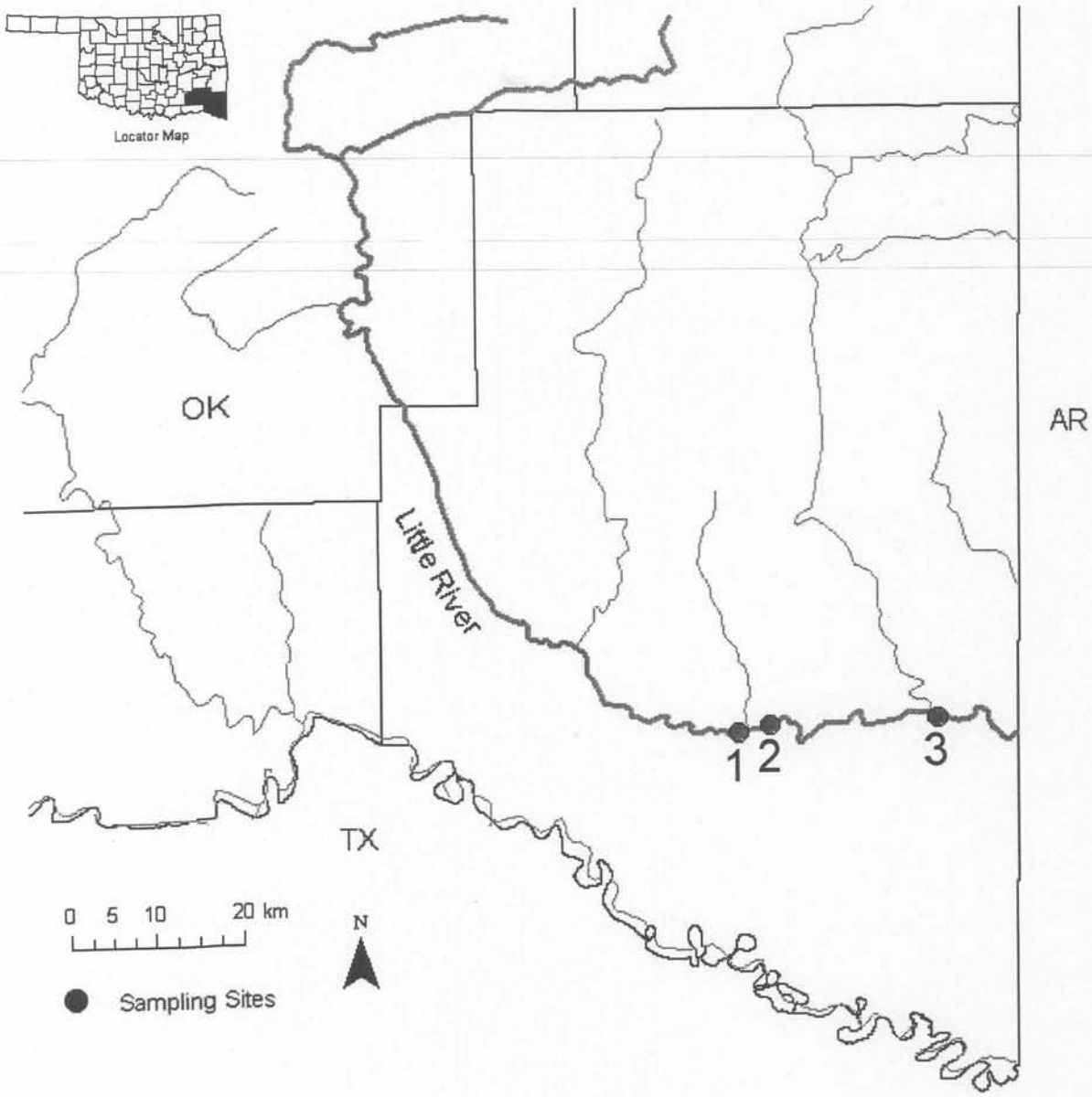


Figure 1. Location of sampling sites in the Little River in southeastern Oklahoma.

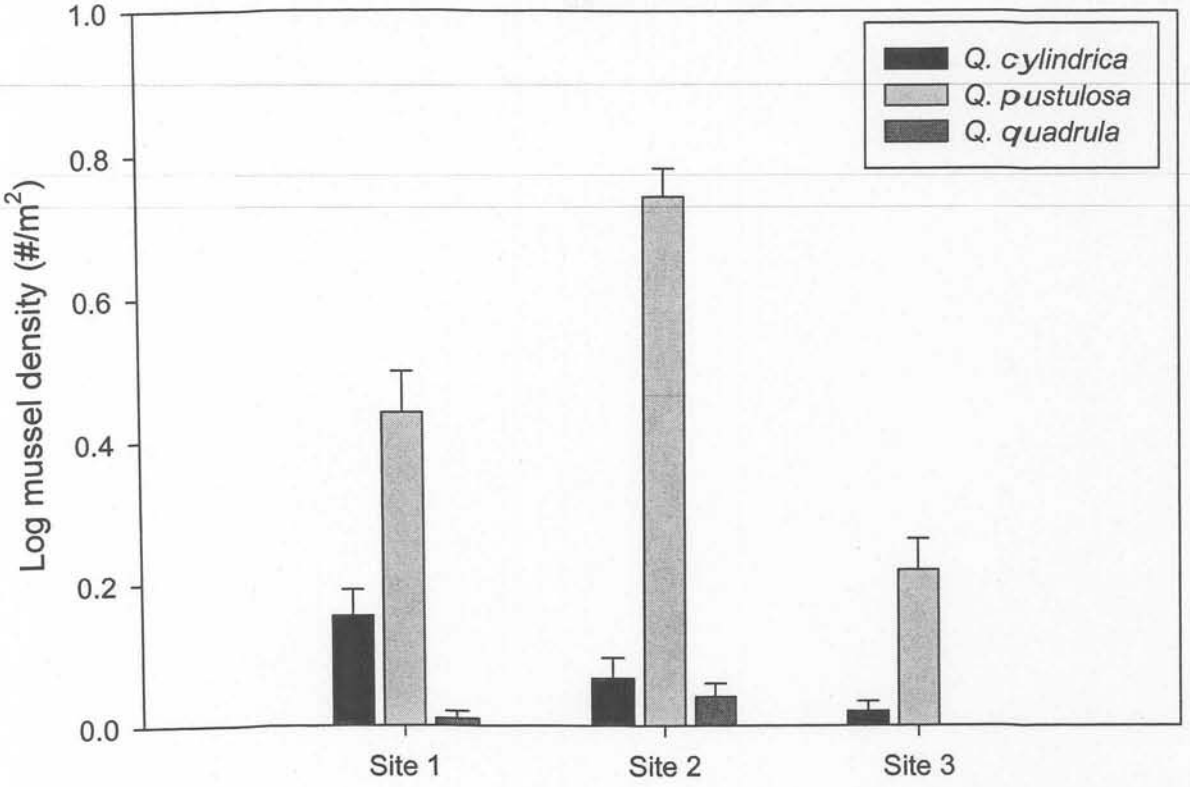


Figure 2. Mean (±SE) mussel density at each sampling site.

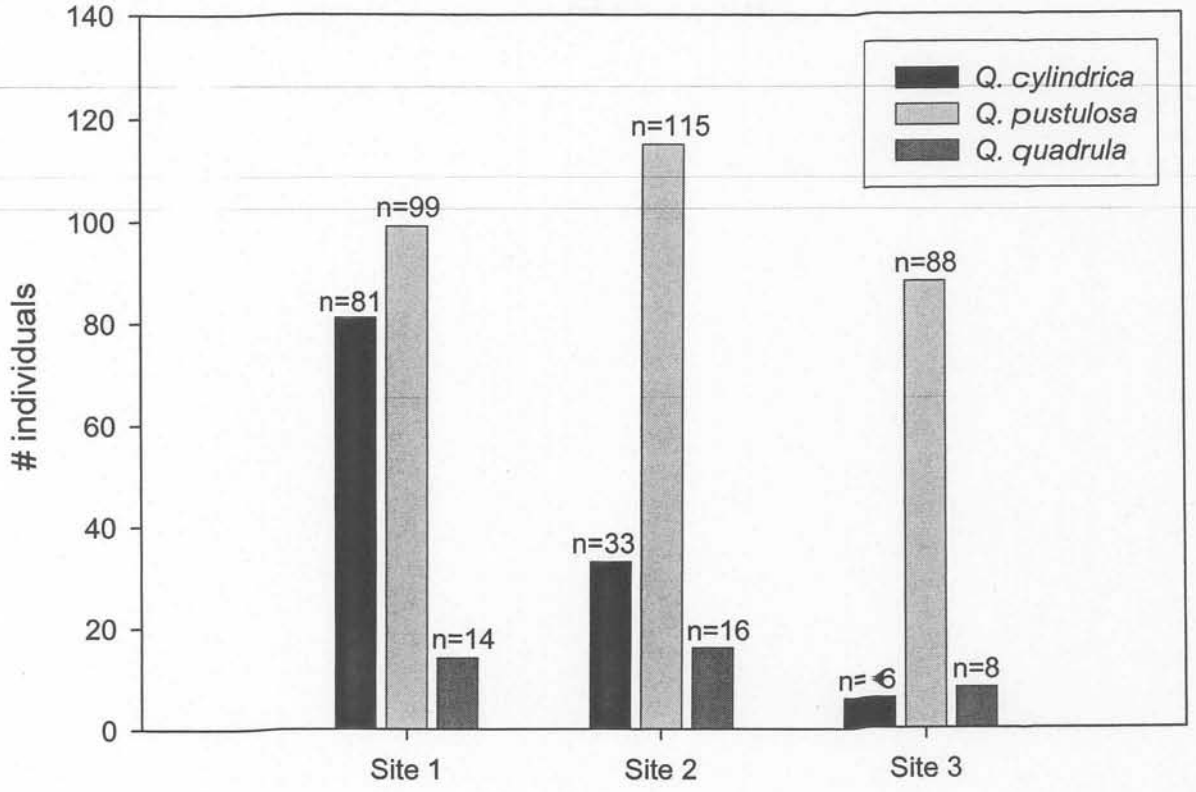


Figure 3. Number of individuals of each species collected for gonad sampling at three sampling sites in the Little River, Oklahoma.

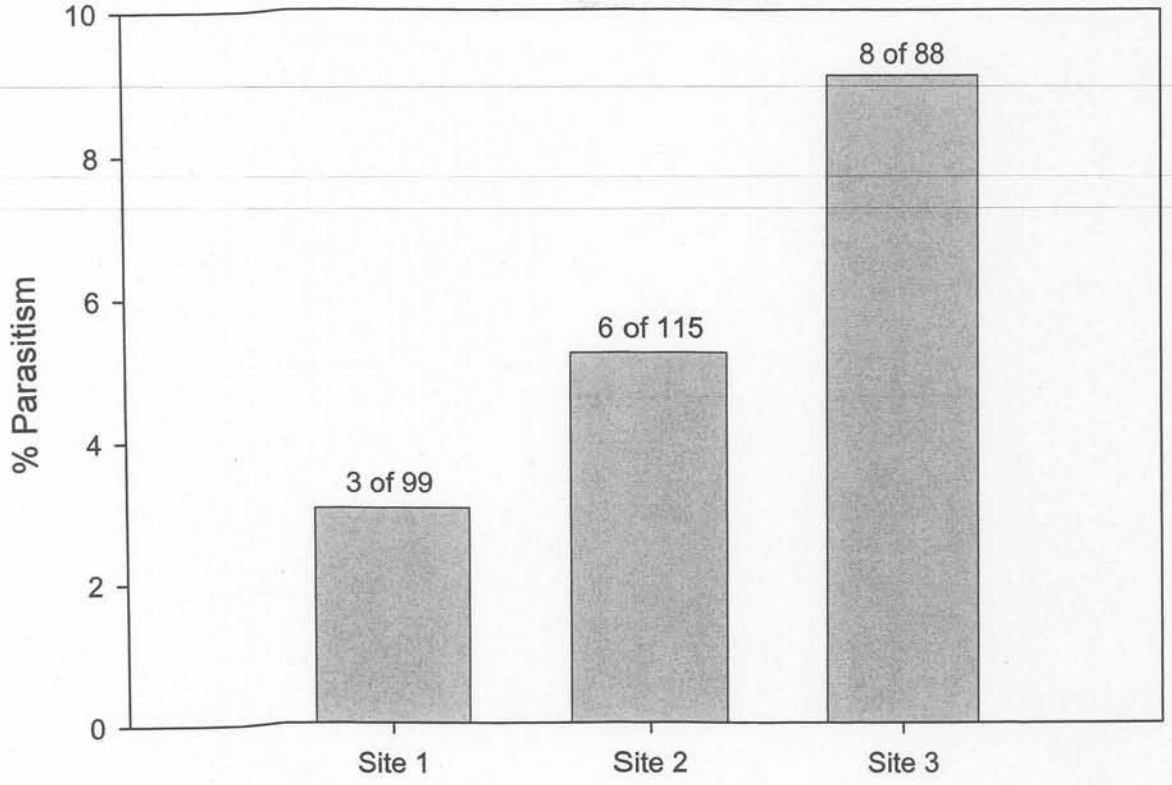


Figure 4. Percent of parasitized *Q. pustulosa* individuals at each sampling site.

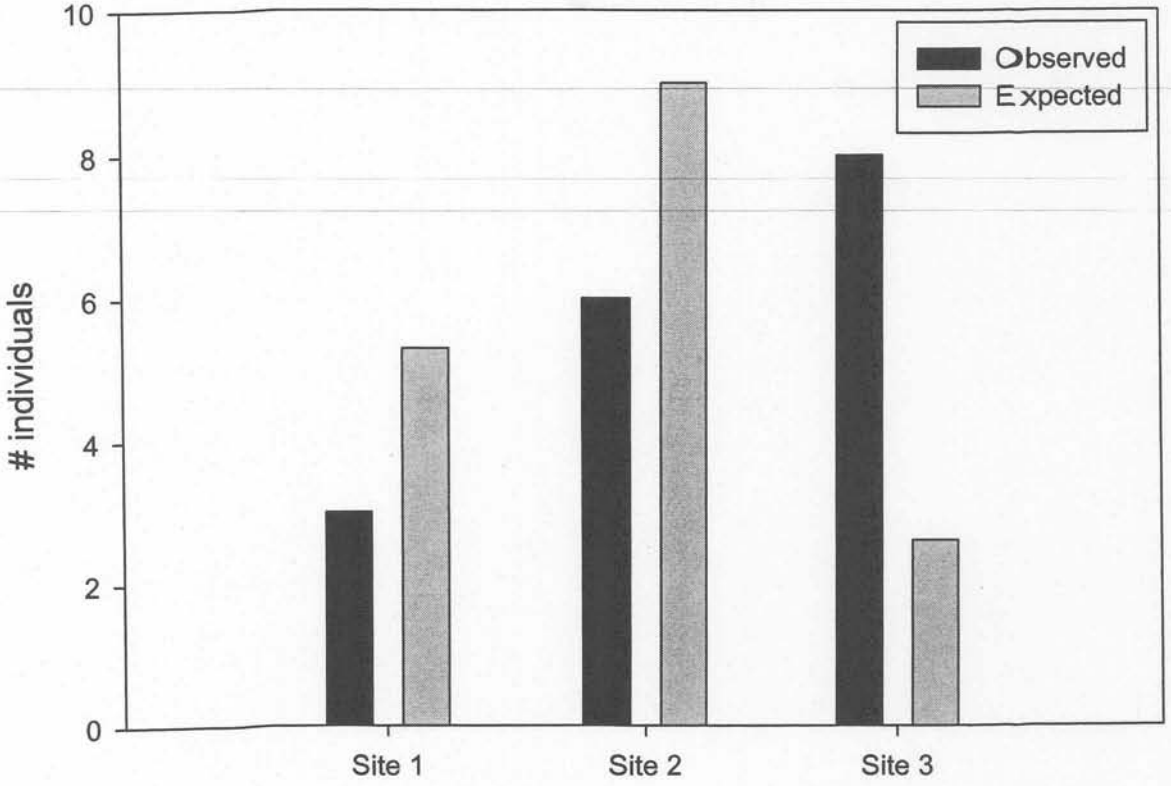


Figure 5. Results of chi-square analysis comparing observed and expected parasite loads in *Q. pustulosa* at each site. Expected rates of infection were weighted based on *Q. pustulosa* density at each site.

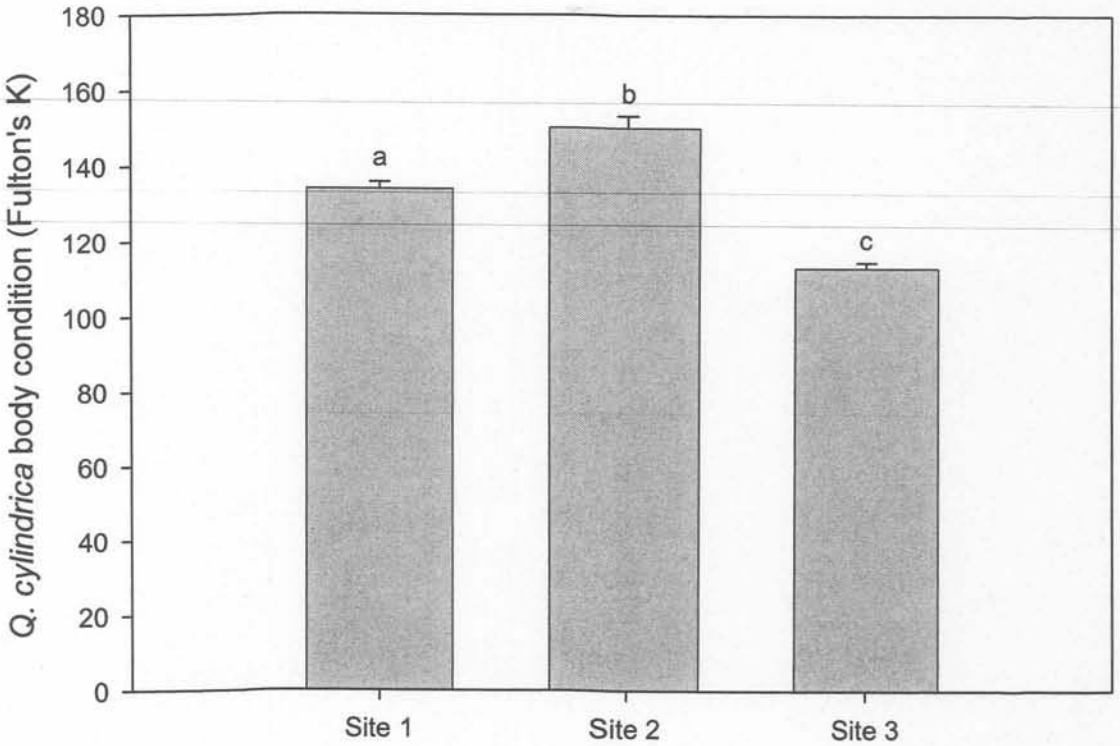


Figure 6. Mean (±SE) *Q. cylindrica* body condition at each site.

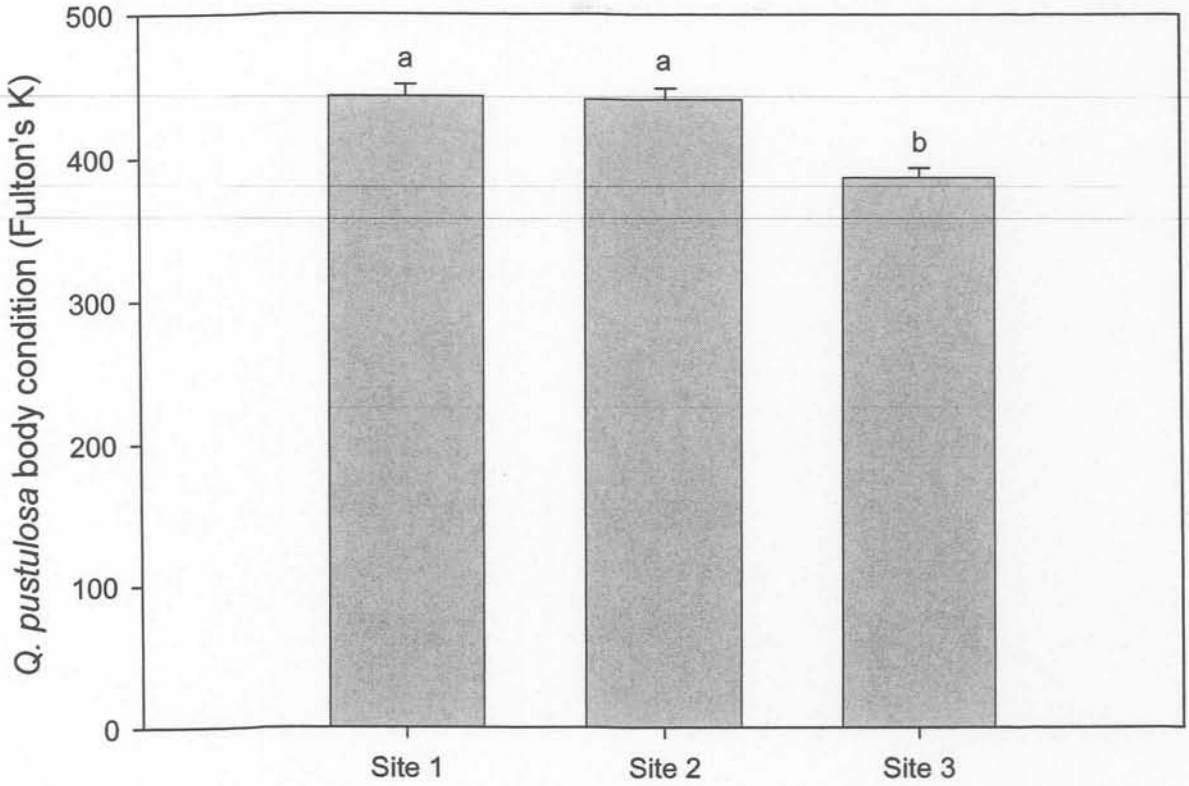


Figure 7. Mean (±SE) *Q. pustulosa* body condition at each site.

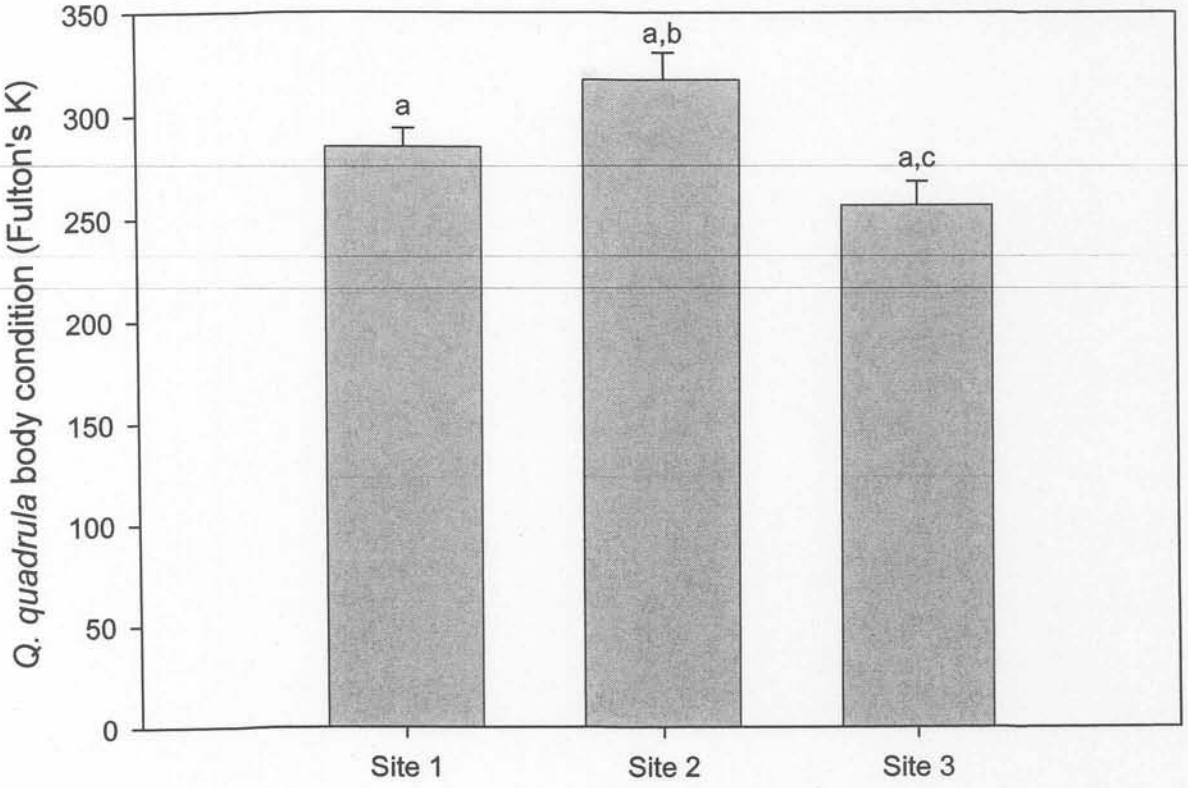


Figure 8. Mean (±SE) *Q. quadrula* body condition at each site.

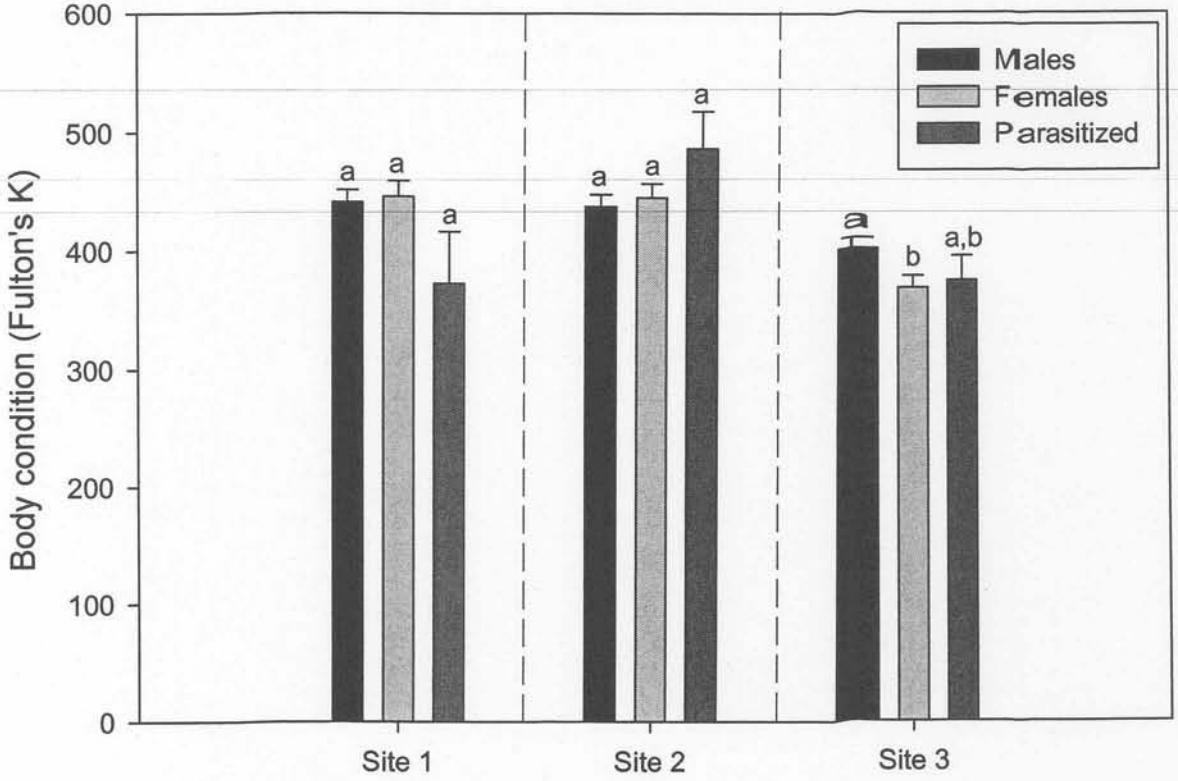


Figure 9. Mean (±SE) body condition of males, females and parasitized *Q. pustulosa* at each site.

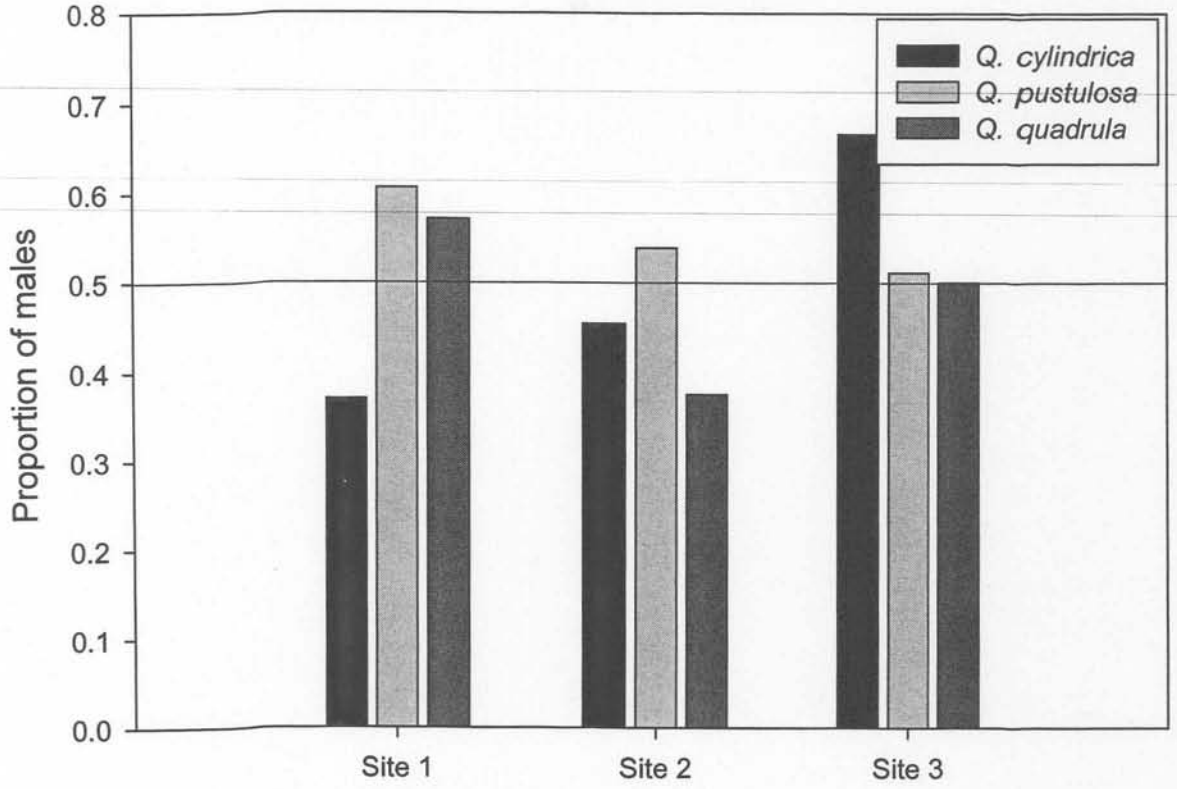


Figure 10. Proportion of males in population at each site (line = 0.5: equal proportions of males and females.)

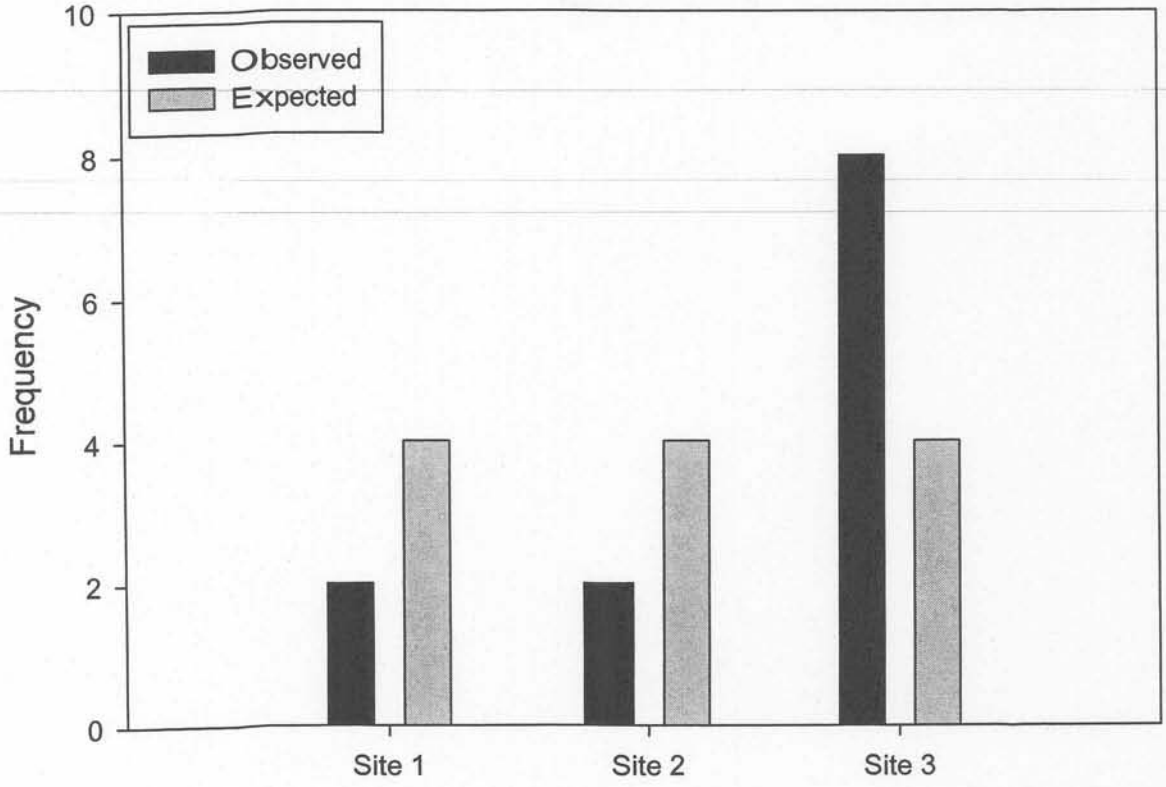


Figure 11. Chi-square results for three *Quadrula* species at each site.

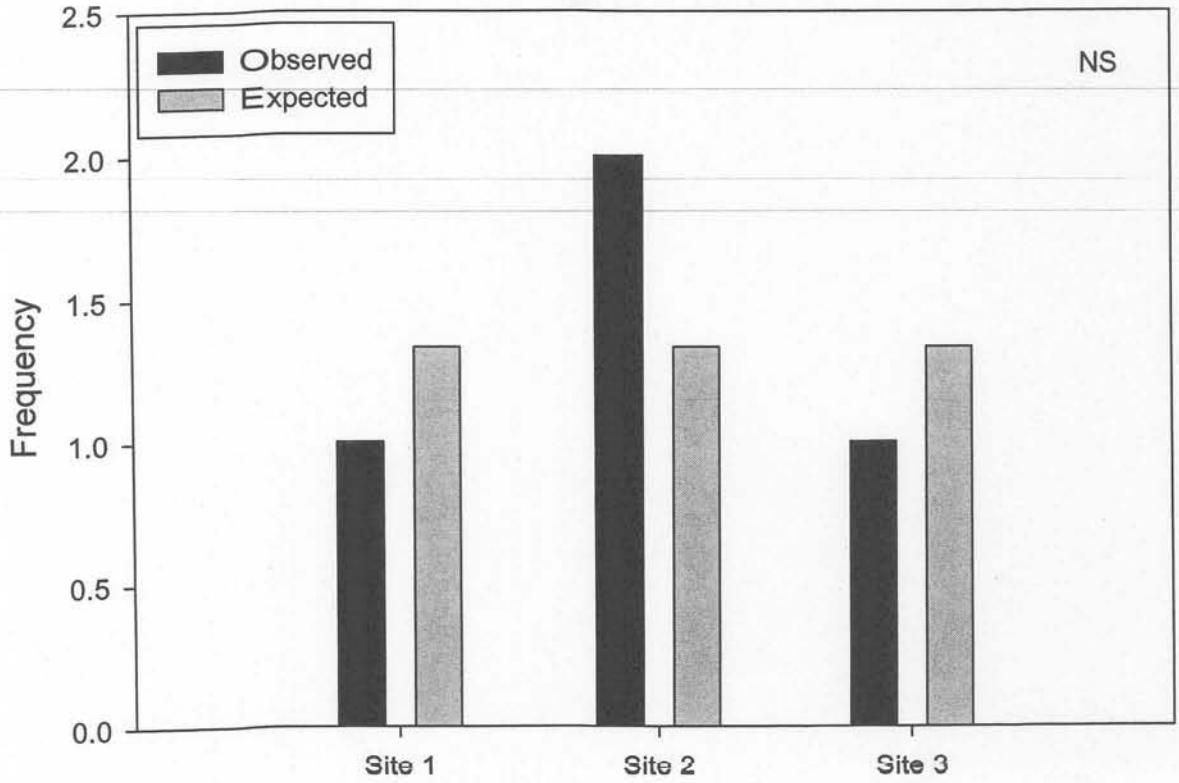


Figure 12. Chi-square results for *Q. cylindrica* at each site. Results are non-significant.

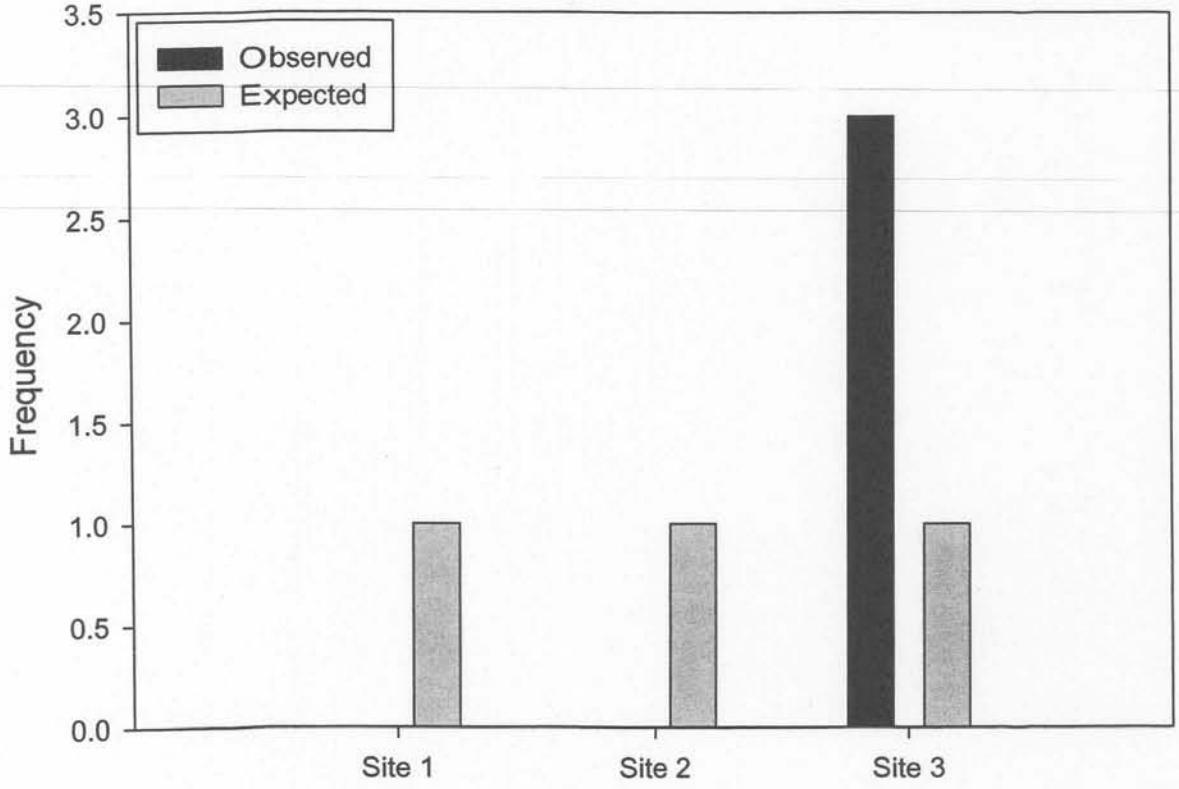


Figure 13. Chi-square results for *Q. pustulosa* at each site.

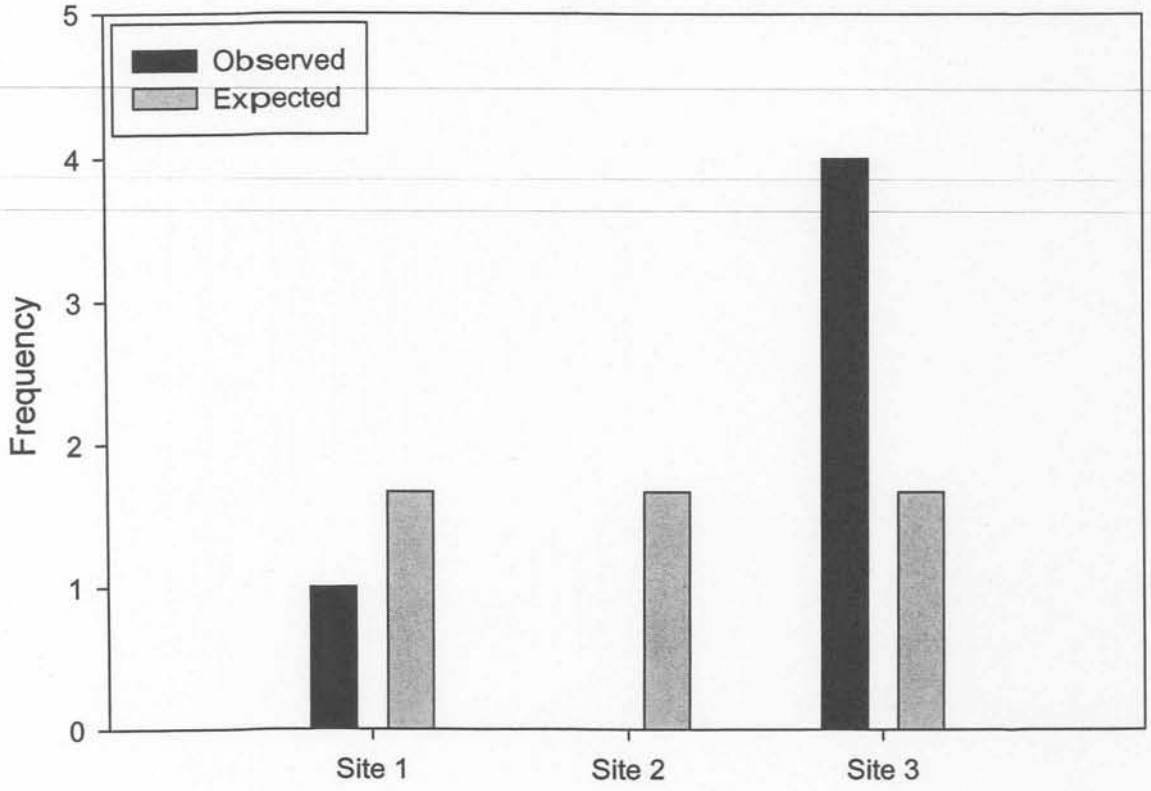


Figure 14. Chi-square results for *Q.quadrula* at each site.

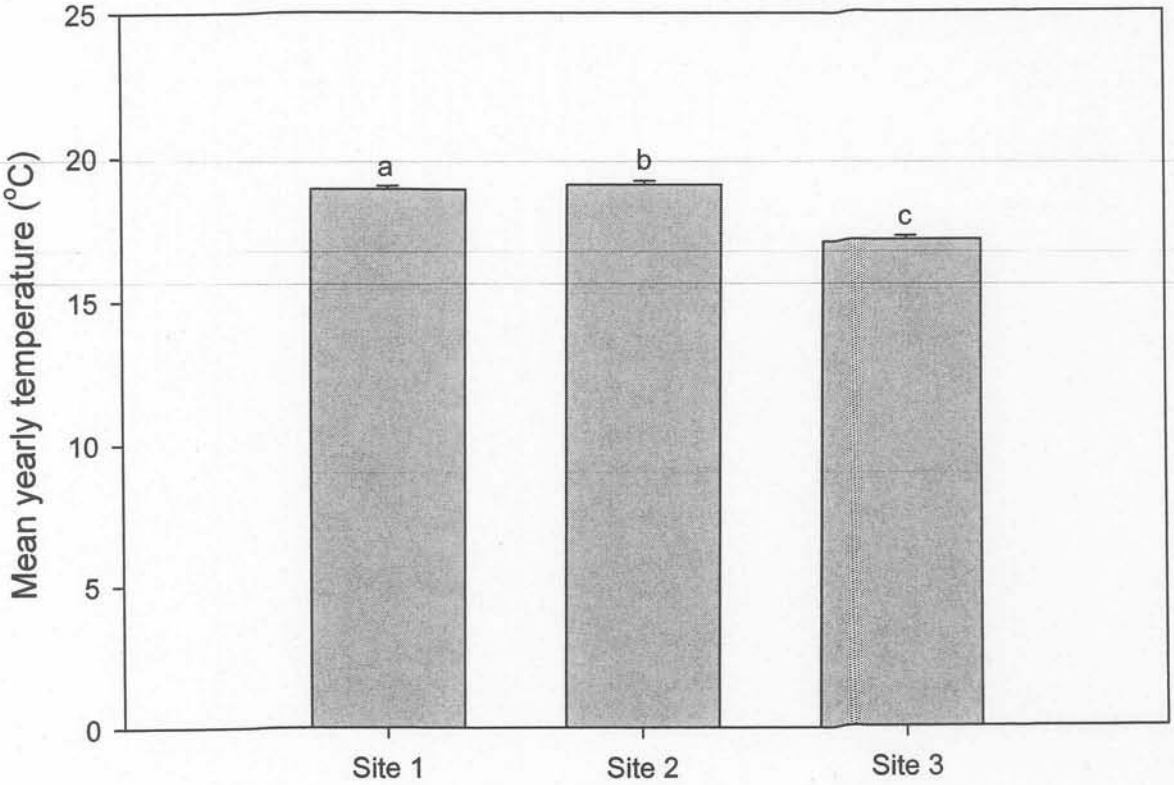


Figure 15: Mean monthly temperature (±SE) at each site.

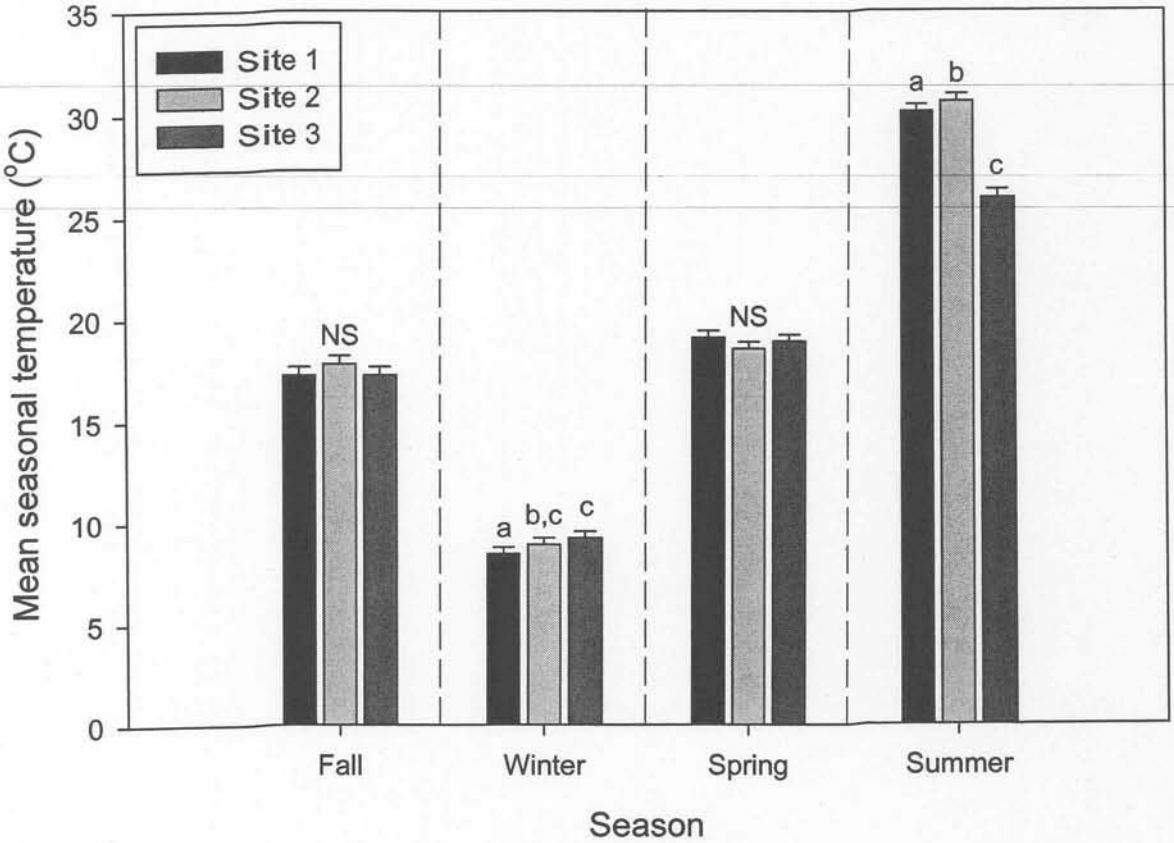


Figure 16. Differences in mean seasonal temperature among sites.

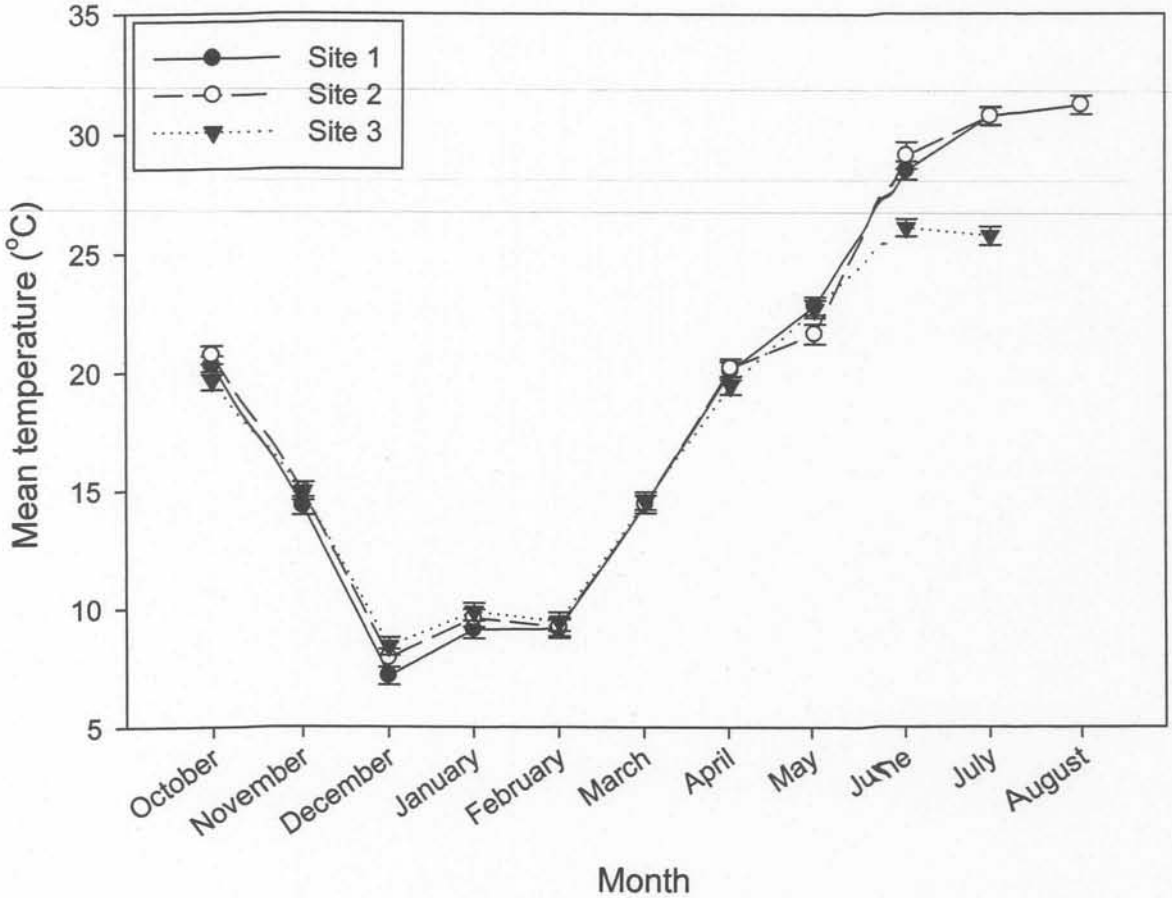


Figure 17. Mean monthly temperature (±SE) at each site.

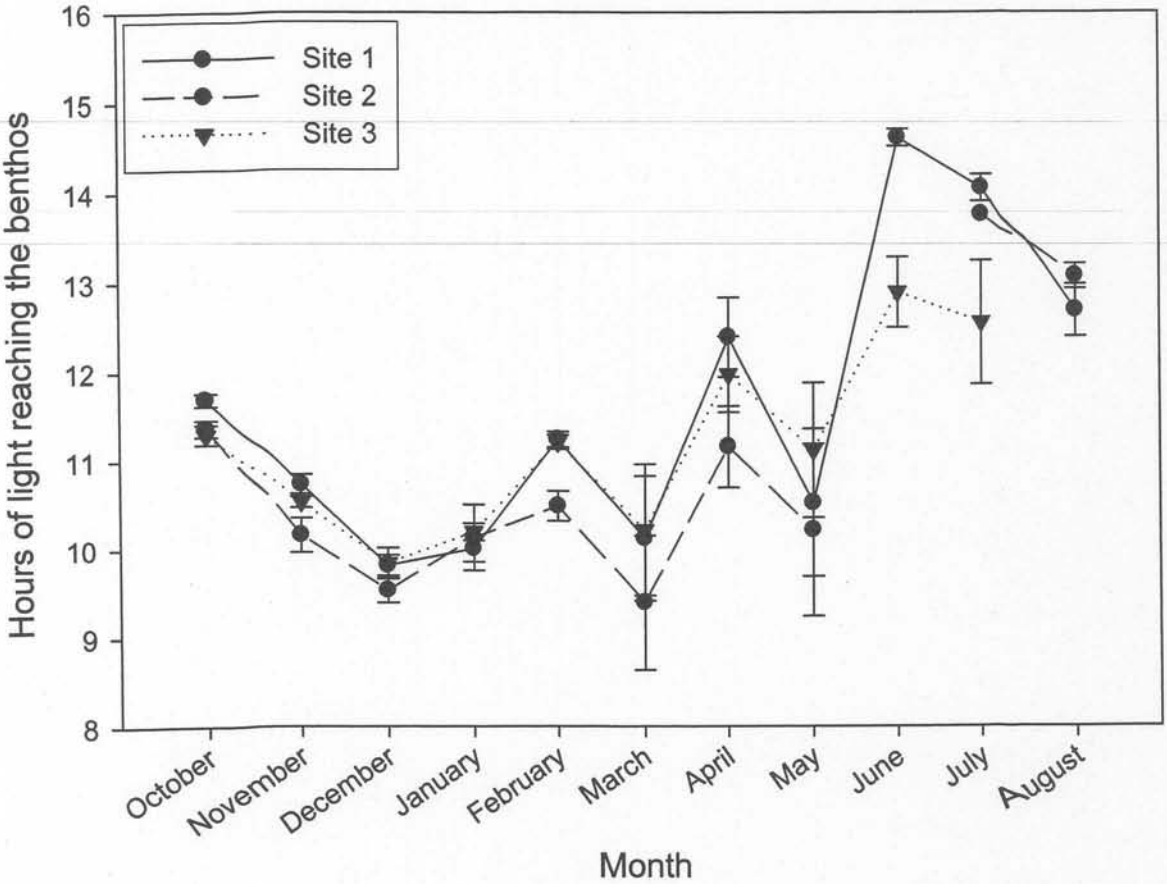


Figure 18. Mean (±SE) hours of light reaching stream bottom at each site.

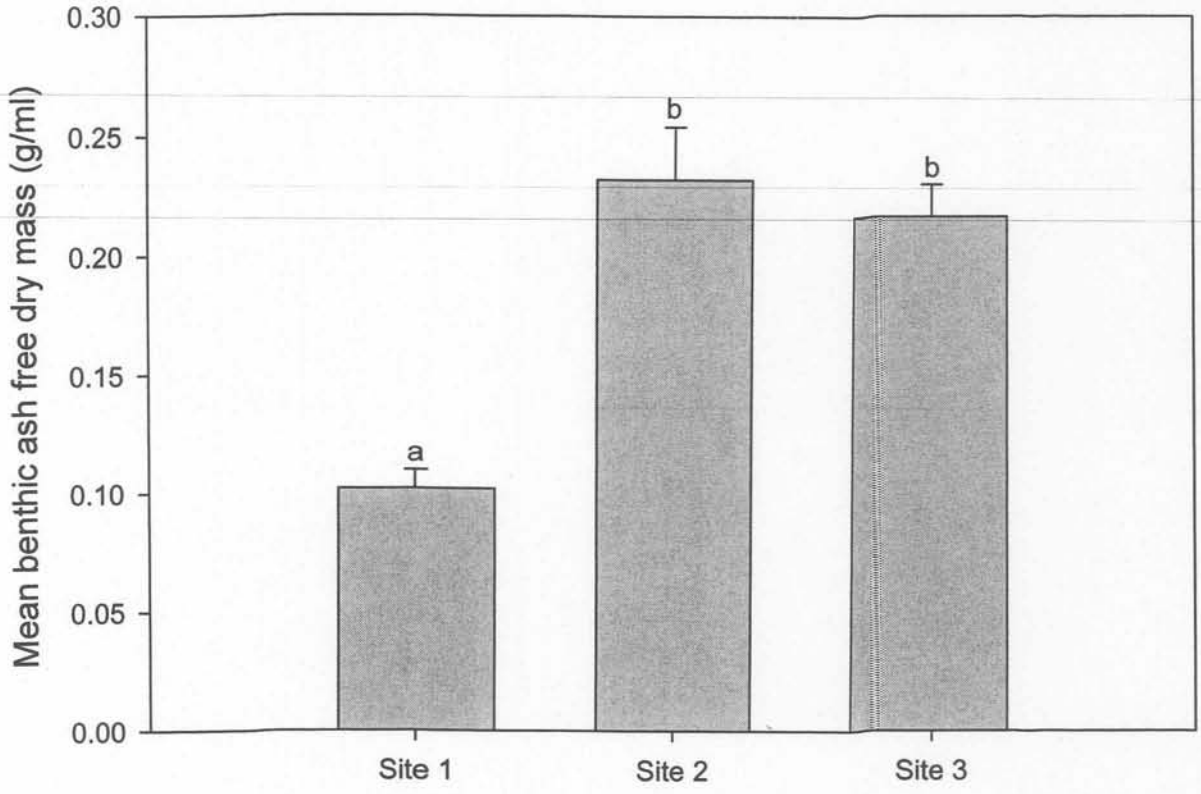


Figure 19. Ash free dry mass means (±SE) from benthic core samples.

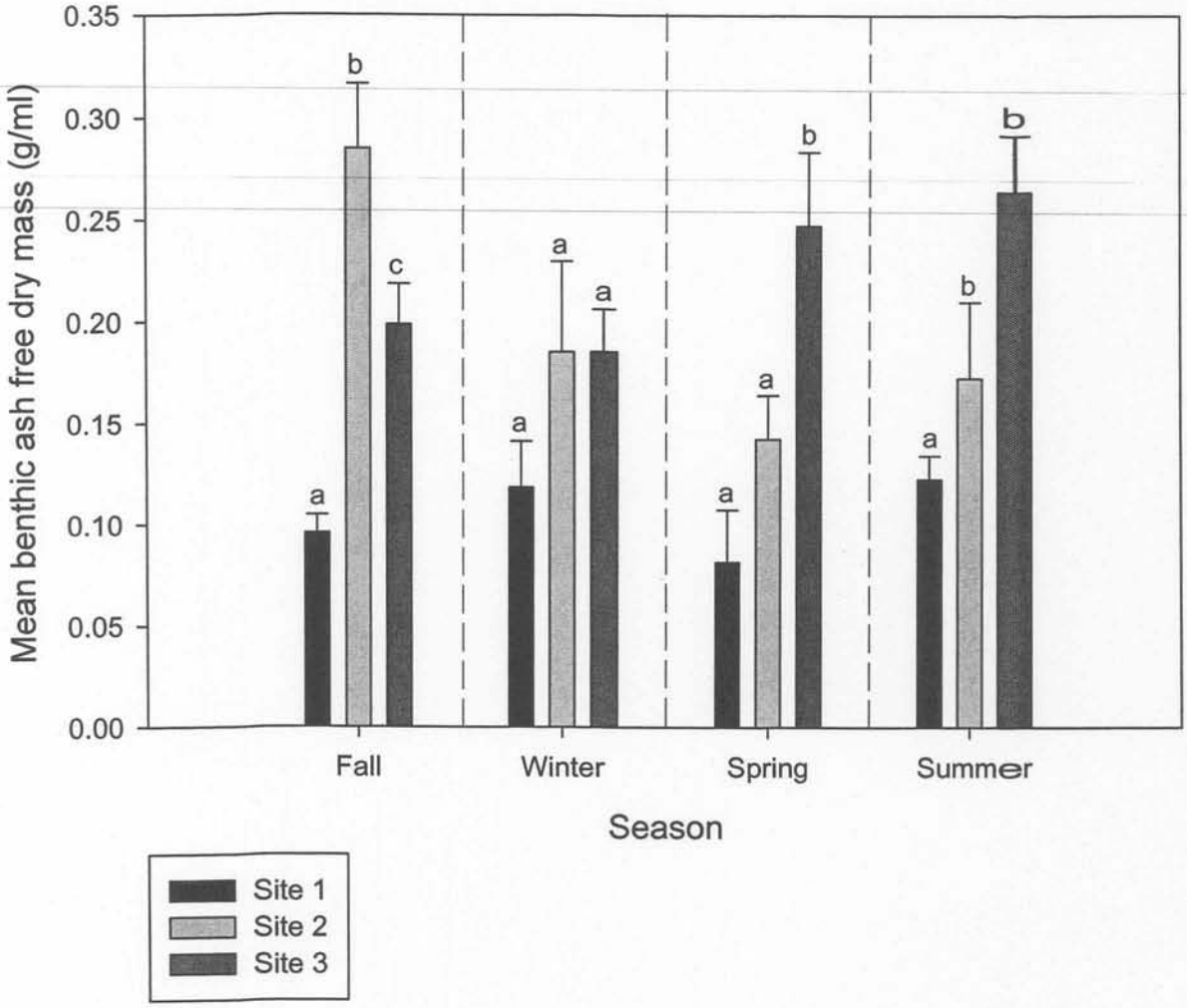


Figure 20. Ash free dry mass means (±SE) across season from benthic core samples.

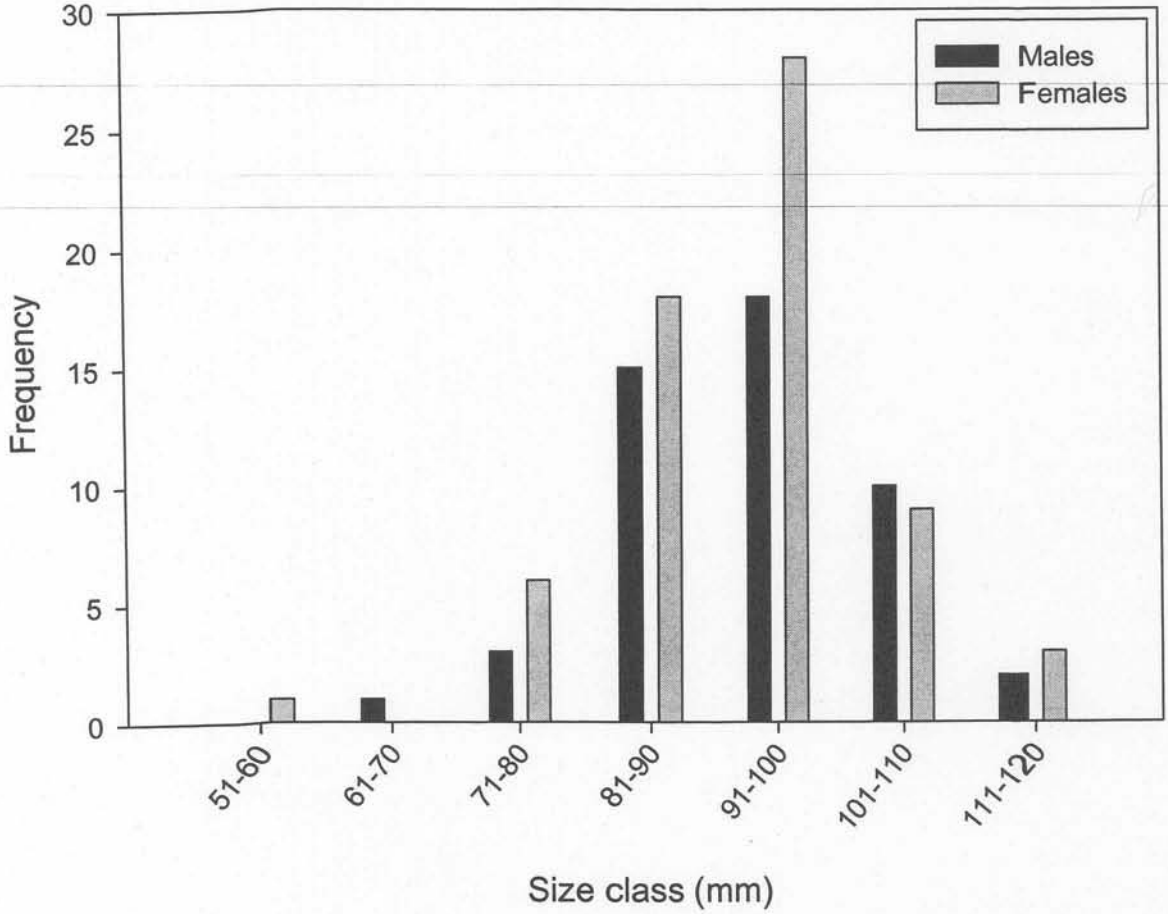


Figure 21. *Q. cylindrica* size class frequencies across all three sampling sites.

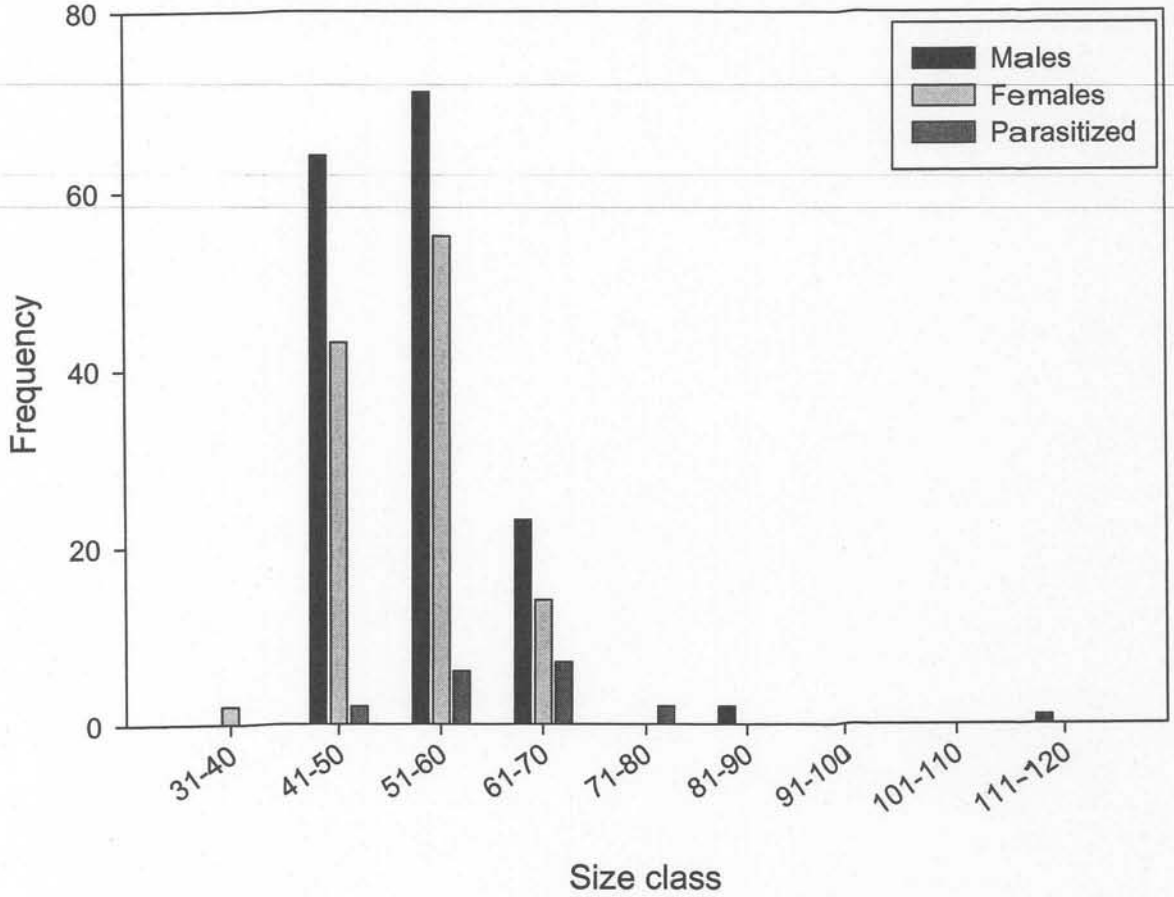


Figure 22. *Q. pustulosa* size class frequencies across all three sampling sites.

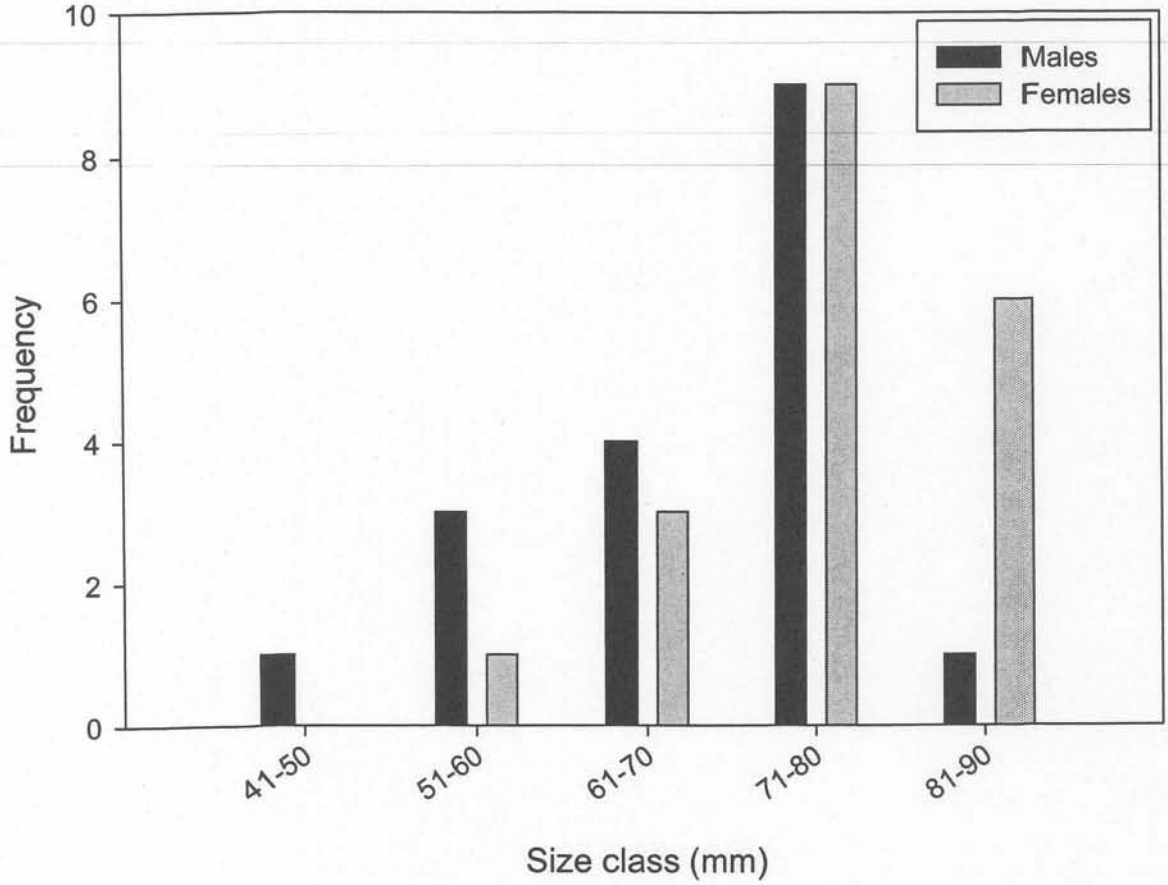


Figure 23. *Q. quadrula* size class frequencies across all three sampling sites.

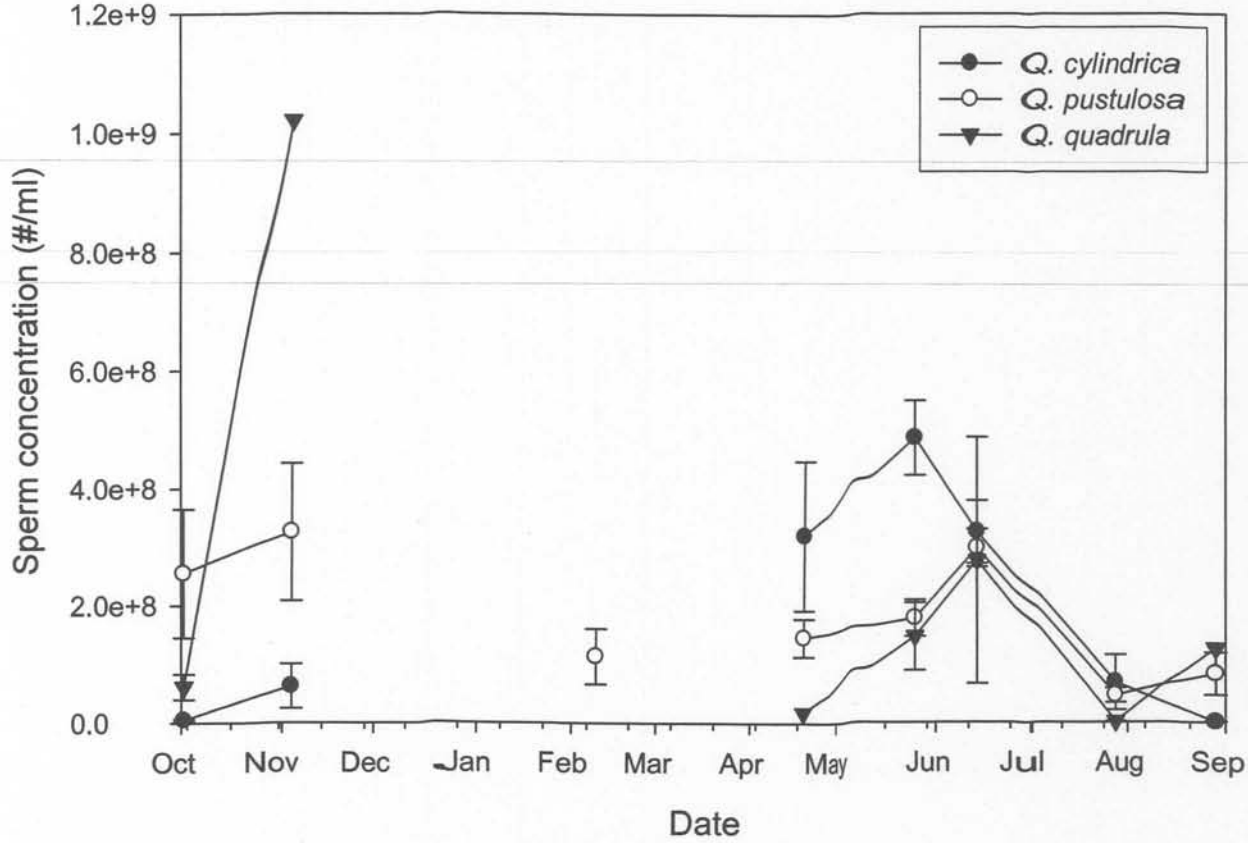


Figure 24. Mean monthly sperm concentration (±SE) for each species averaged across all three sites.

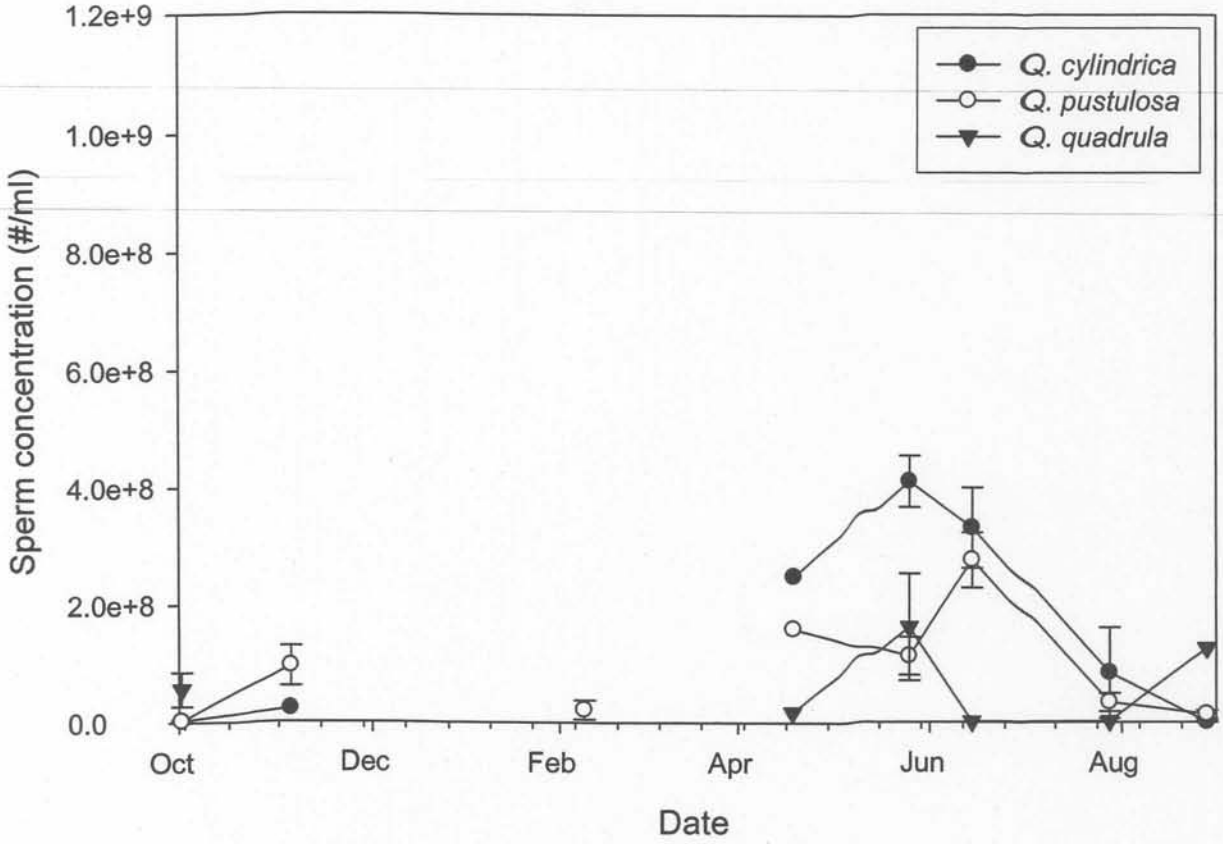


Figure 25. Mean monthly sperm concentration (±SE) for each species at Site 1.

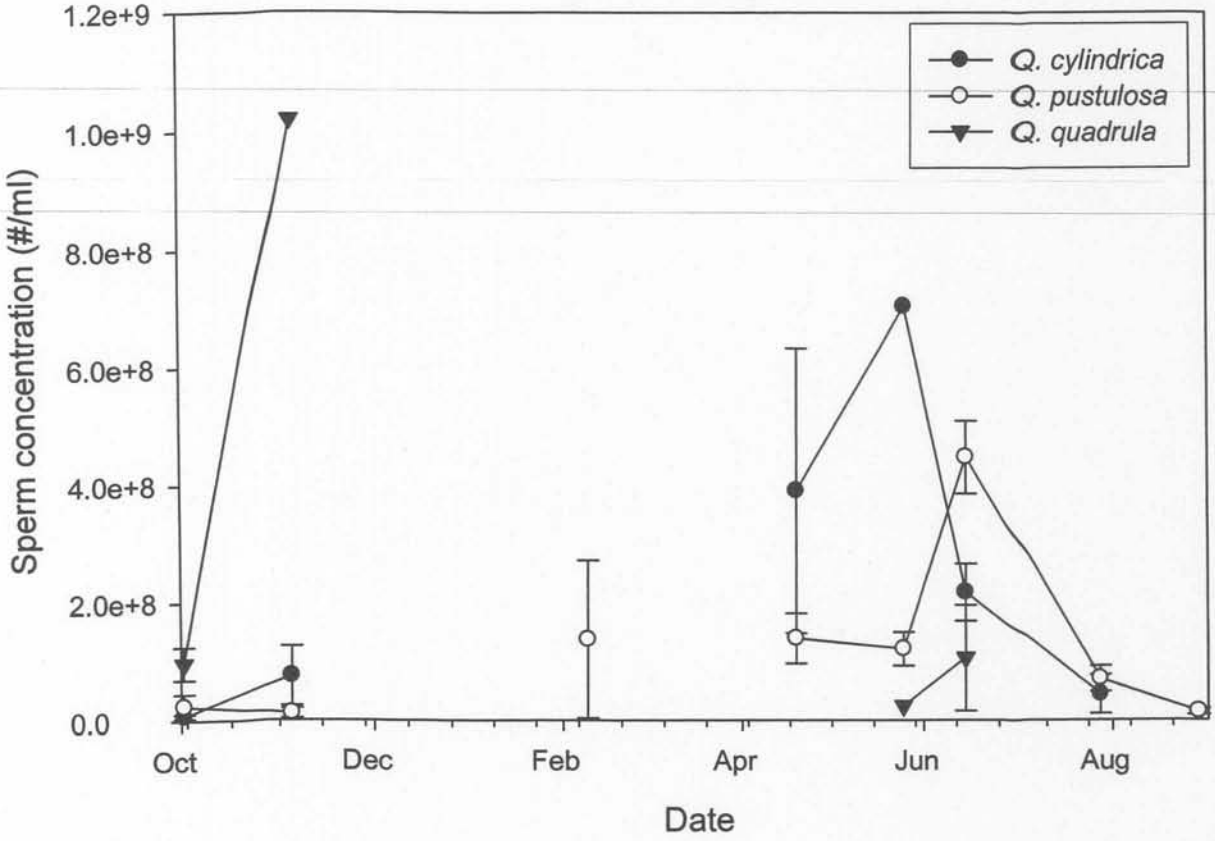


Figure 26. Mean monthly sperm concentration (±SE) for each species at Site 2.

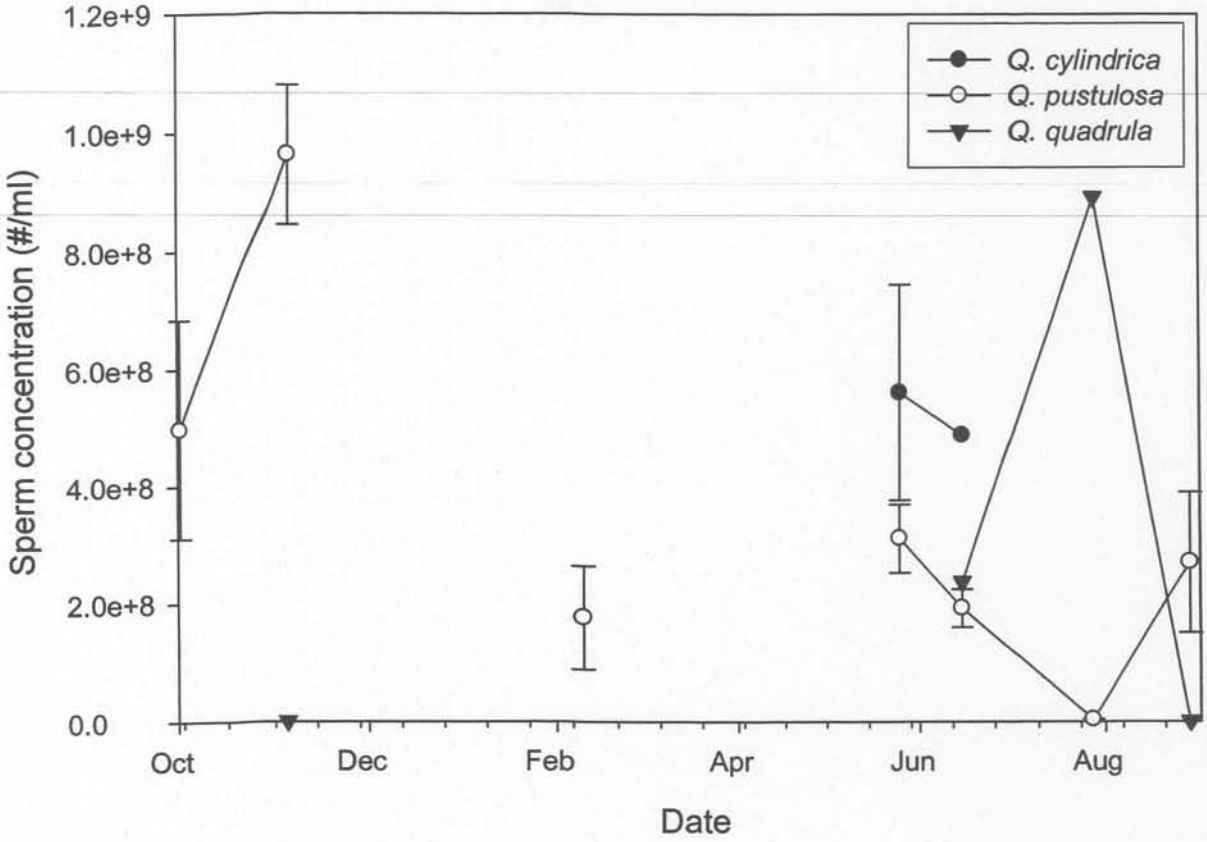


Figure 27. Mean monthly sperm concentration (±SE) for each species at Site 3.

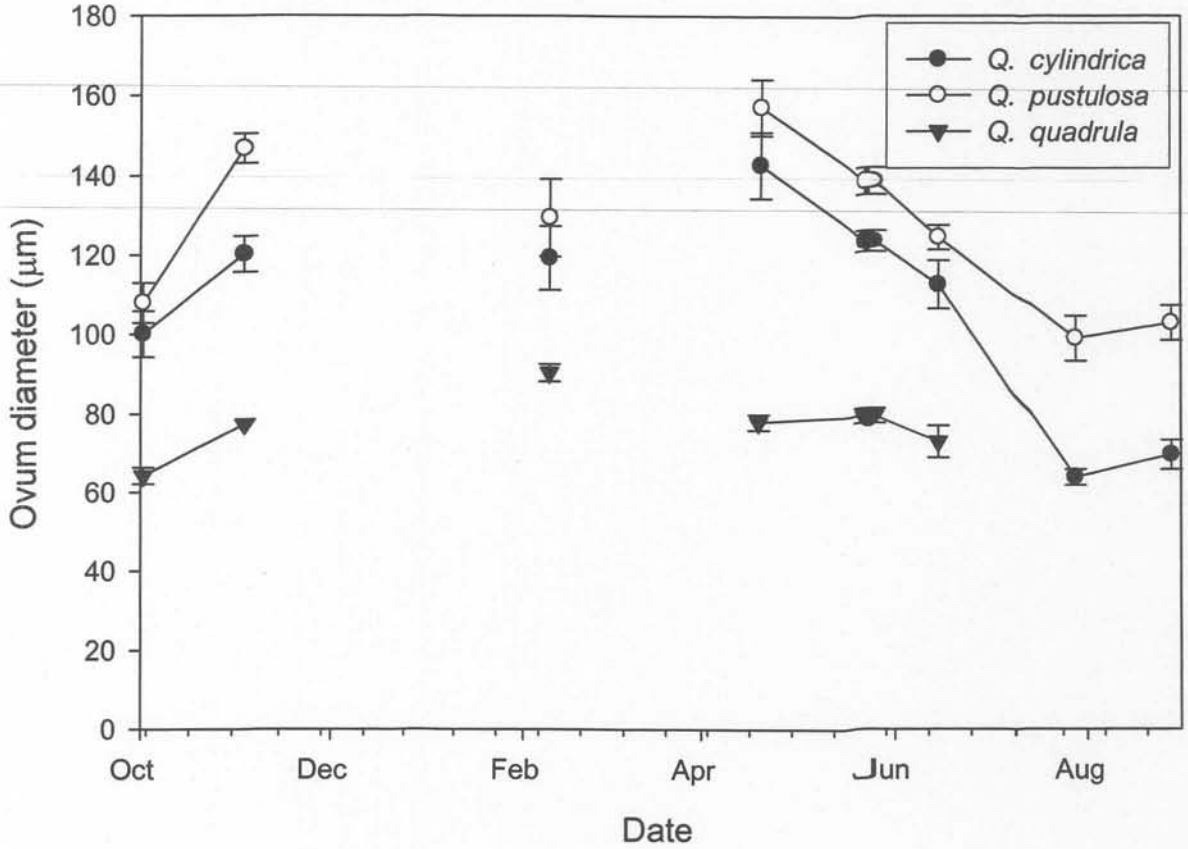


Figure 28. Mean monthly ovum diameter (±SE) for each species averaged across all 3 sites.

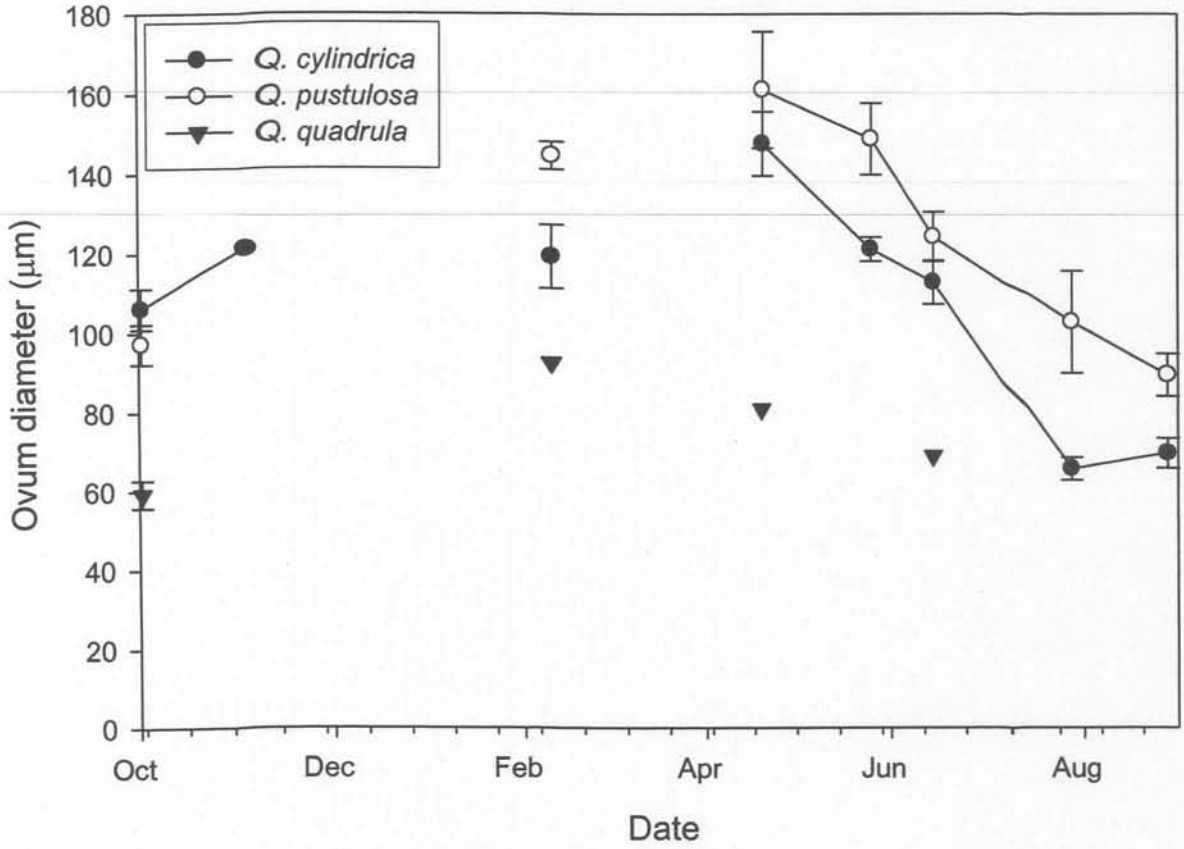


Figure 29. Mean monthly ovum diameter (±SE) for each species at Site 1.

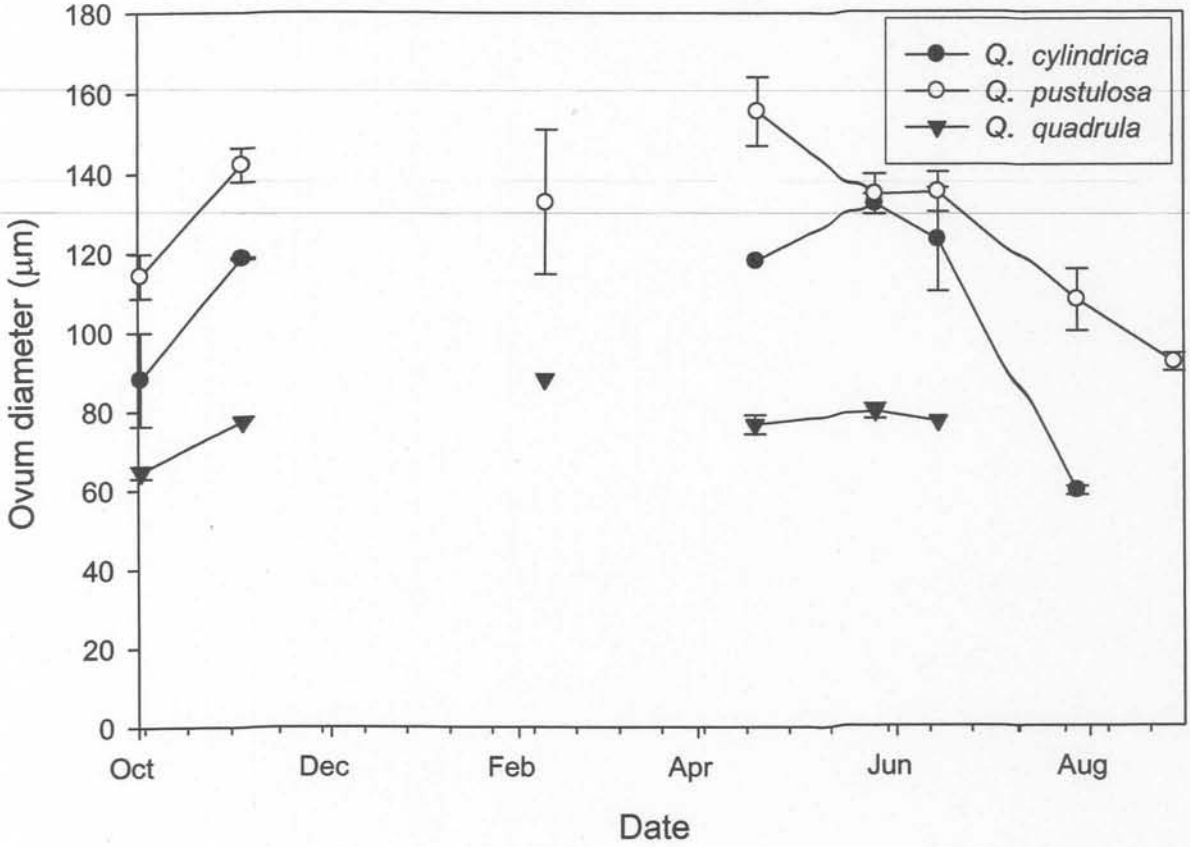


Figure 30. Mean monthly ovum diameter (±SE) for each species at Site 2.

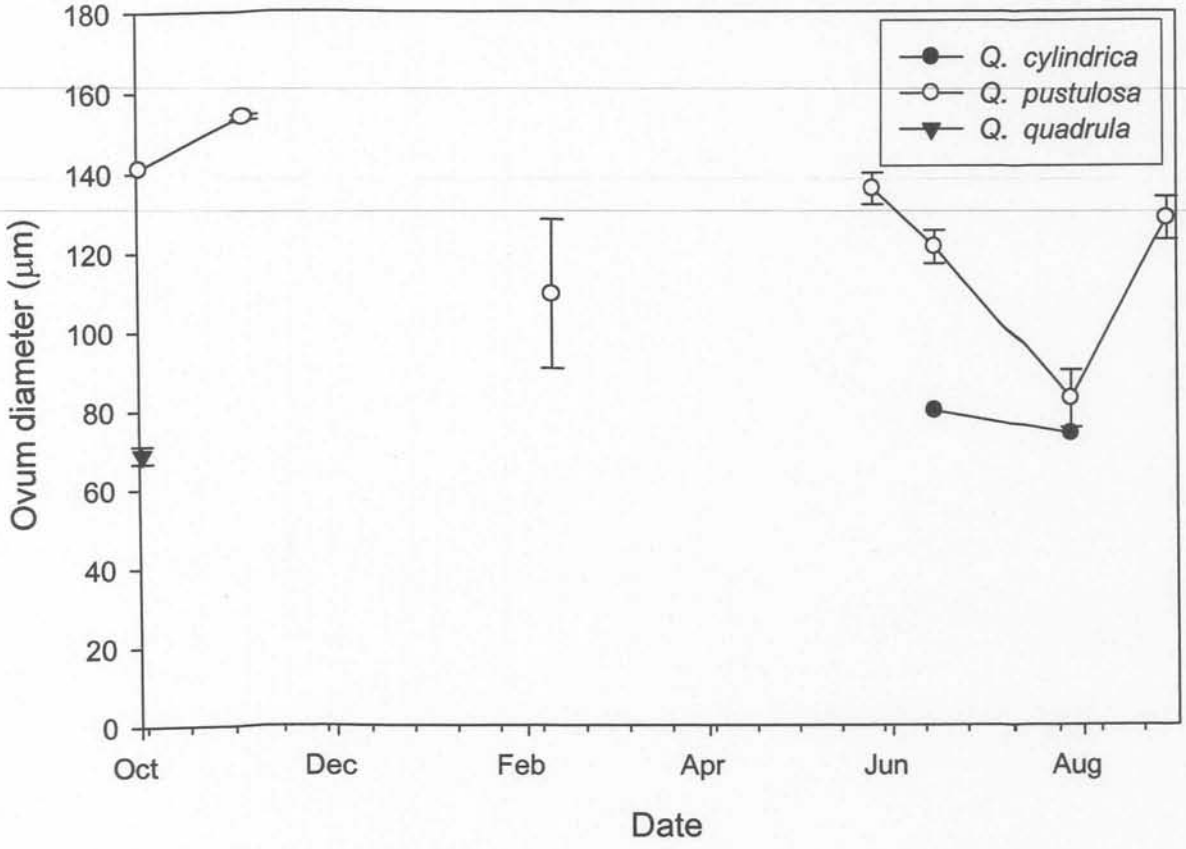


Figure 31. Mean monthly ovum diameter (±SE) for each species at Site 3.

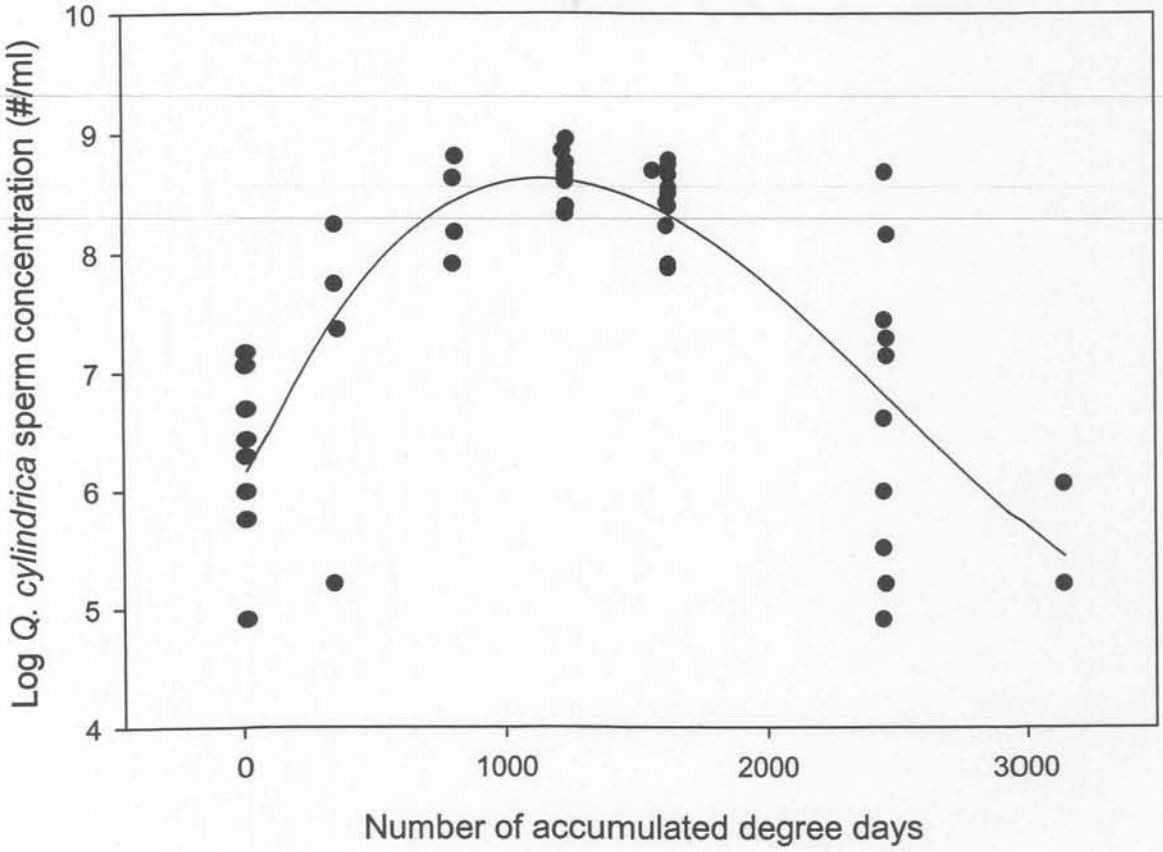


Figure 32. Relationship between number of accumulated degree days since the beginning of sampling and *Q. cylindrica* sperm concentration.

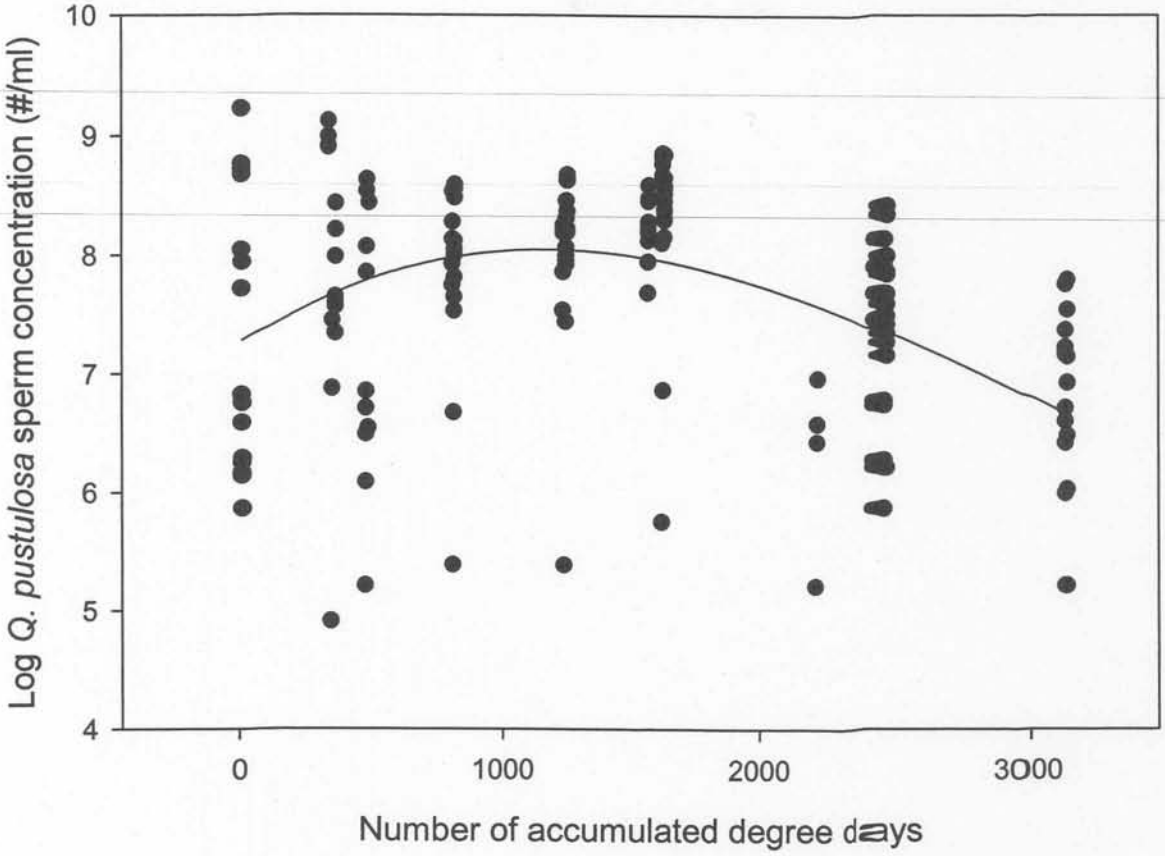


Figure 33. Relationship between number of accumulated degree days since the beginning of sampling and *Q. pustulosa* sperm concentration.

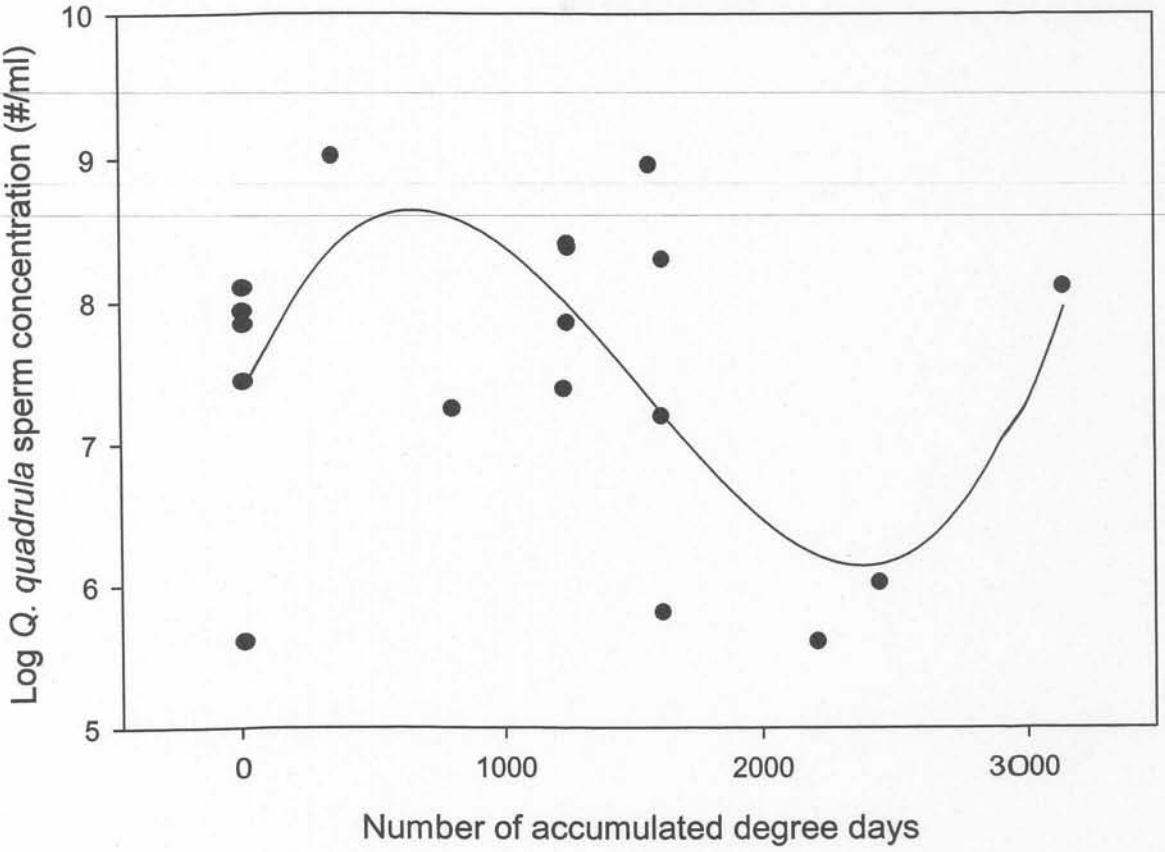


Figure 34. Relationship between number of accumulated degree days since the beginning of sampling and *Q. quadrula* sperm concentration.

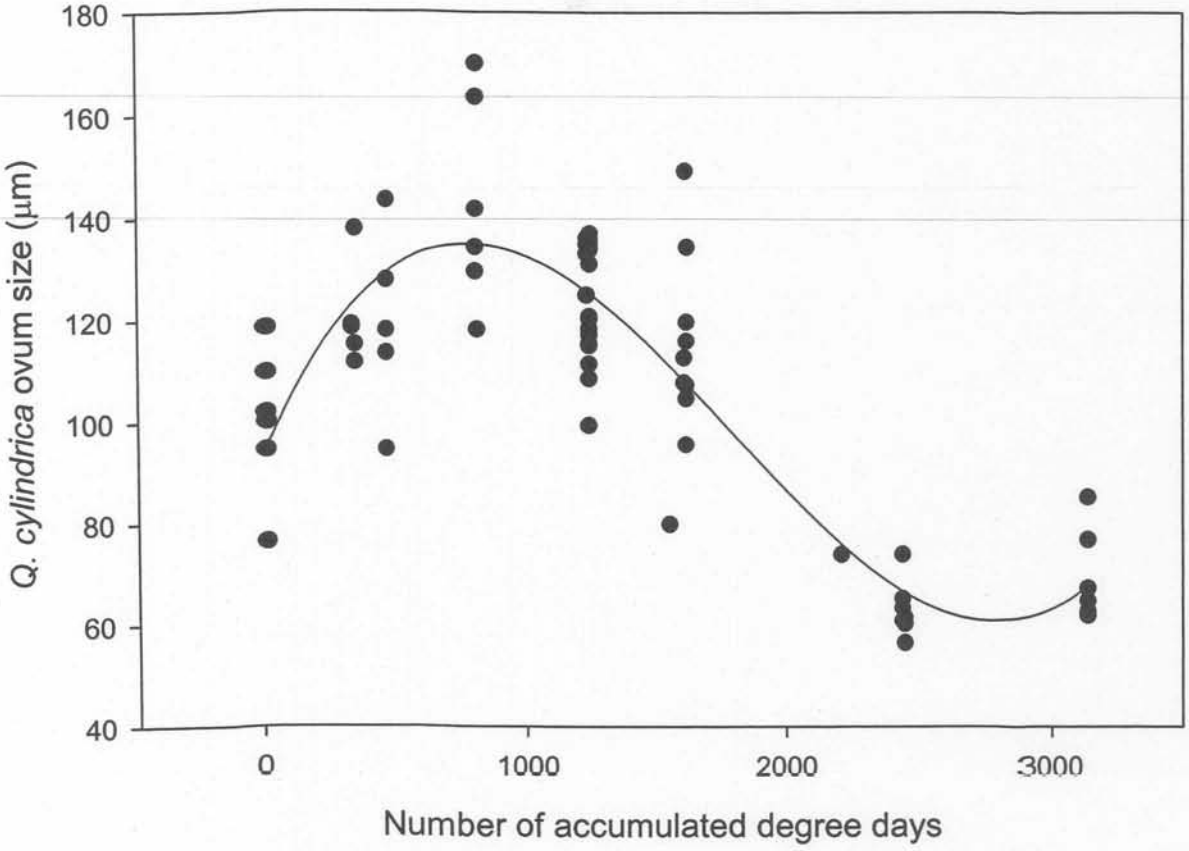


Figure 35. Relationship between number of accumulated degree days since the beginning of sampling and *Q. cylindrica* ovum diameter.

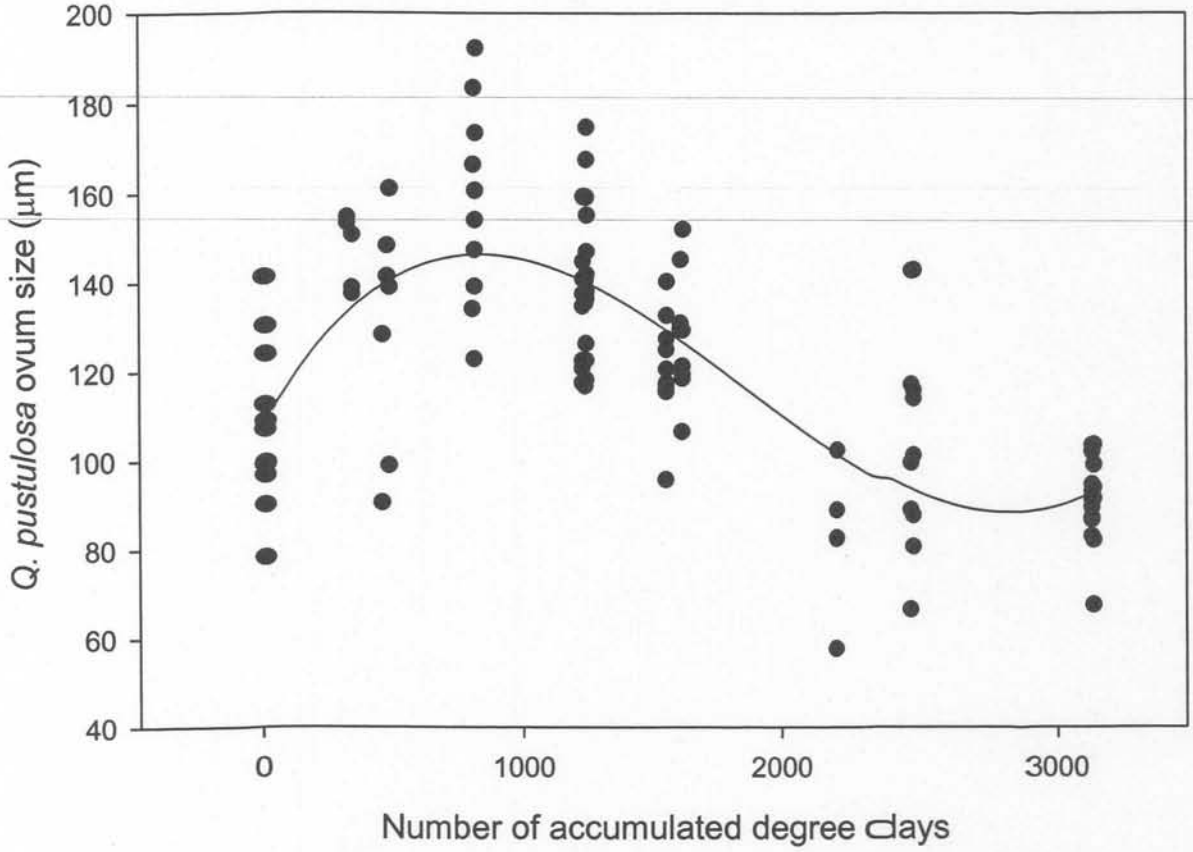


Figure 36. Relationship between number of accumulated degree days since the beginning of sampling and *Q. pustulosa* ovum diameter.

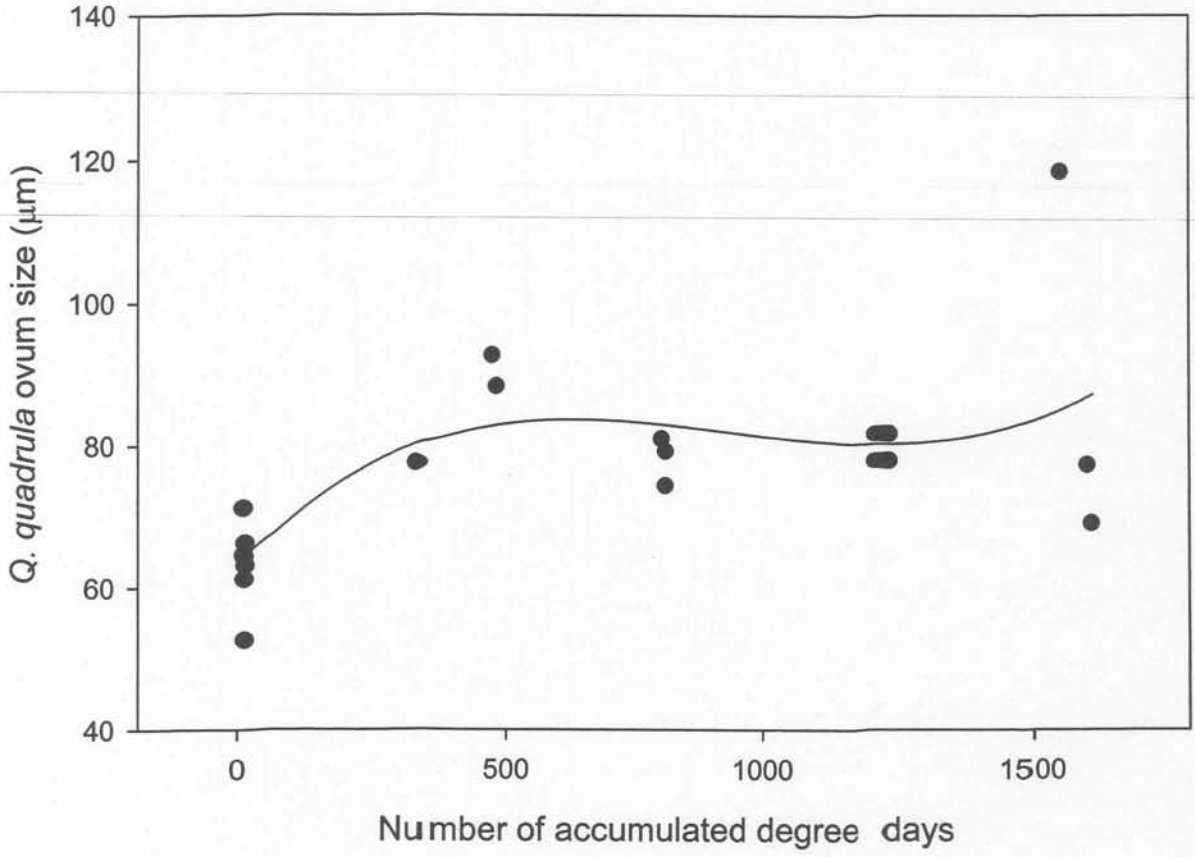


Figure 37. Relationship between number of accumulated degree days since the beginning of sampling and *Q. quadrula* ovum diameter.

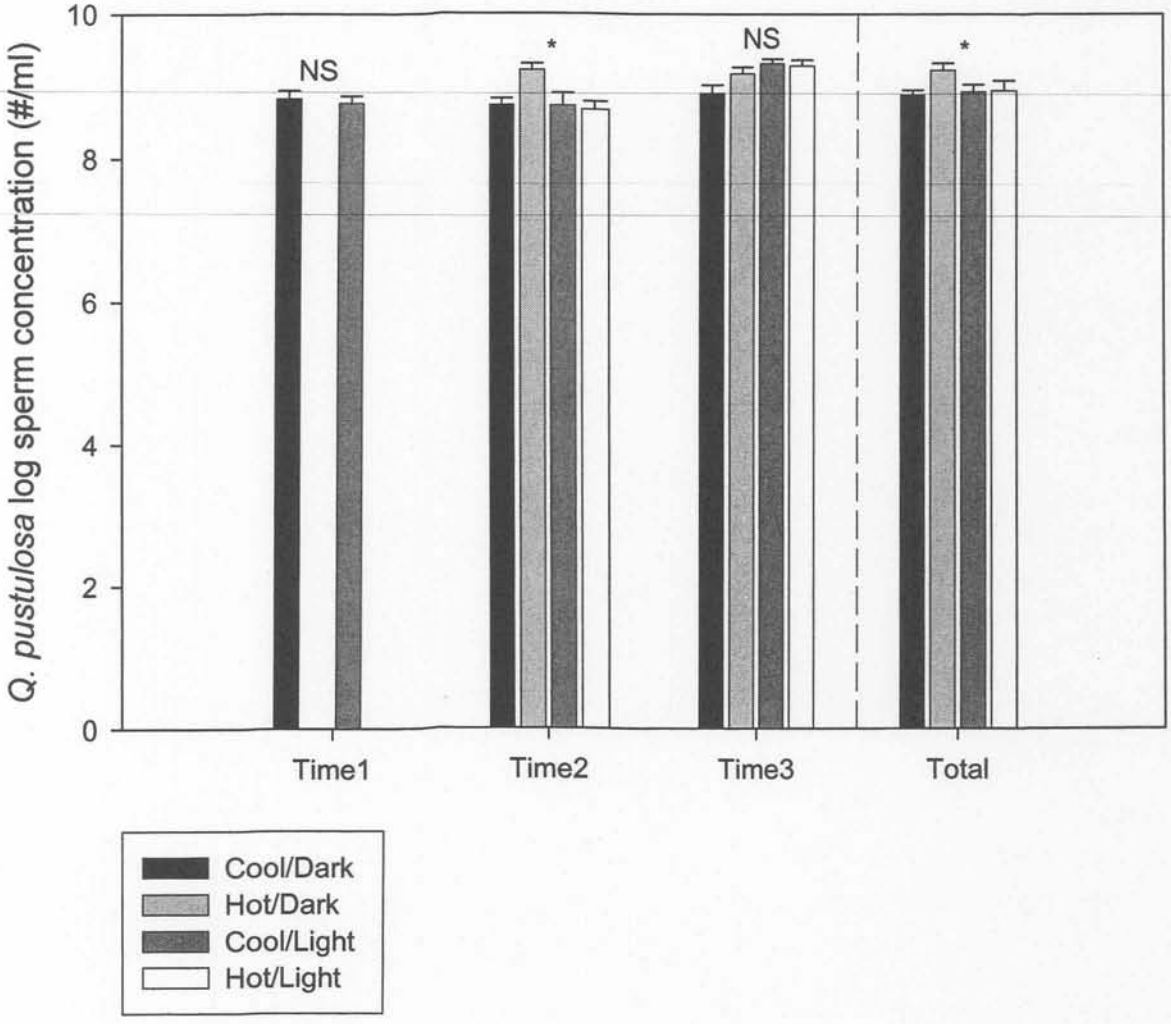


Figure 38. Mean (±SE) *Q. pustulosa* sperm concentration for each treatment and time period.

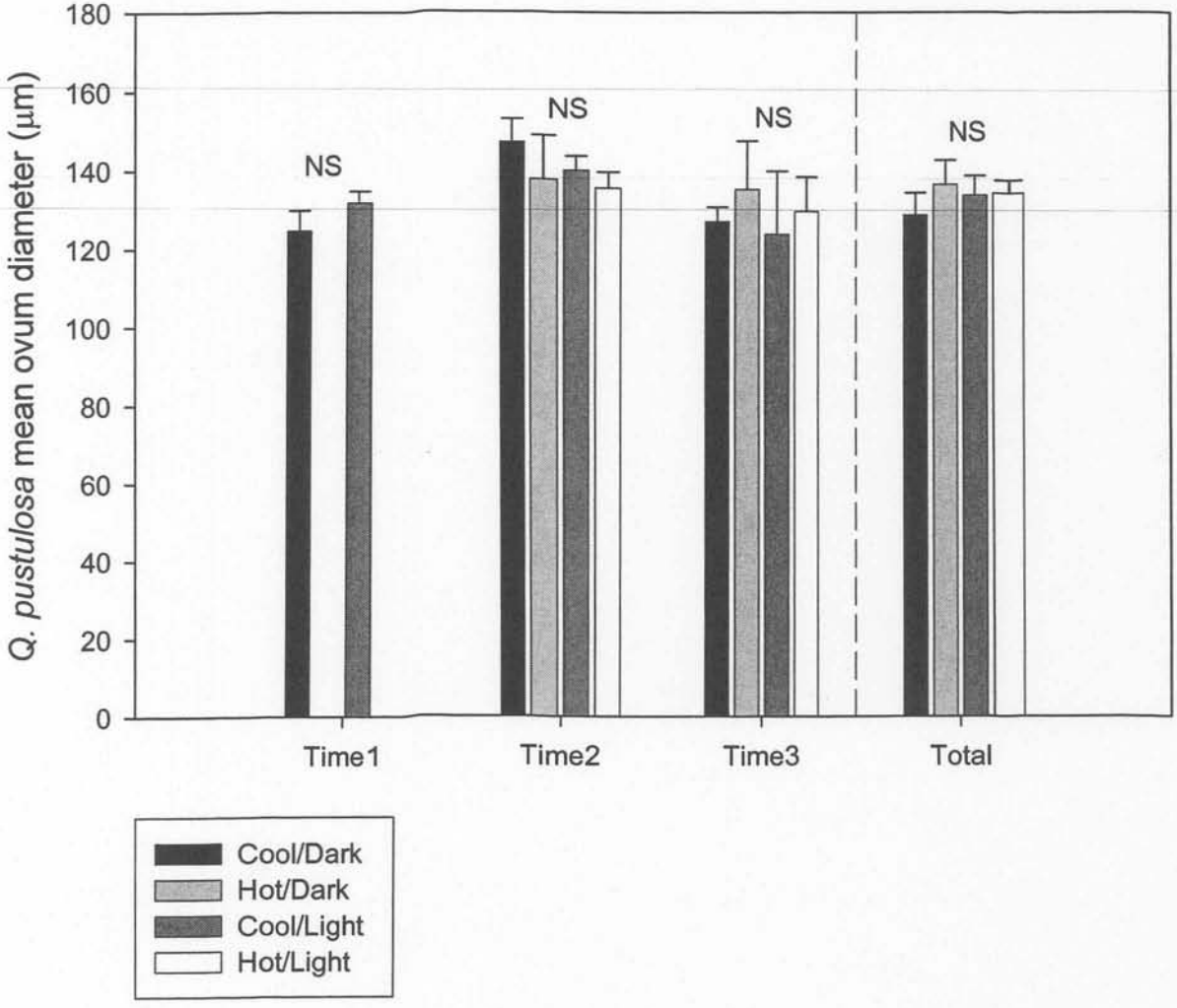


Figure 39. Mean (±SE) *Q. pustulosa* ovum diameter for each treatment and time period.

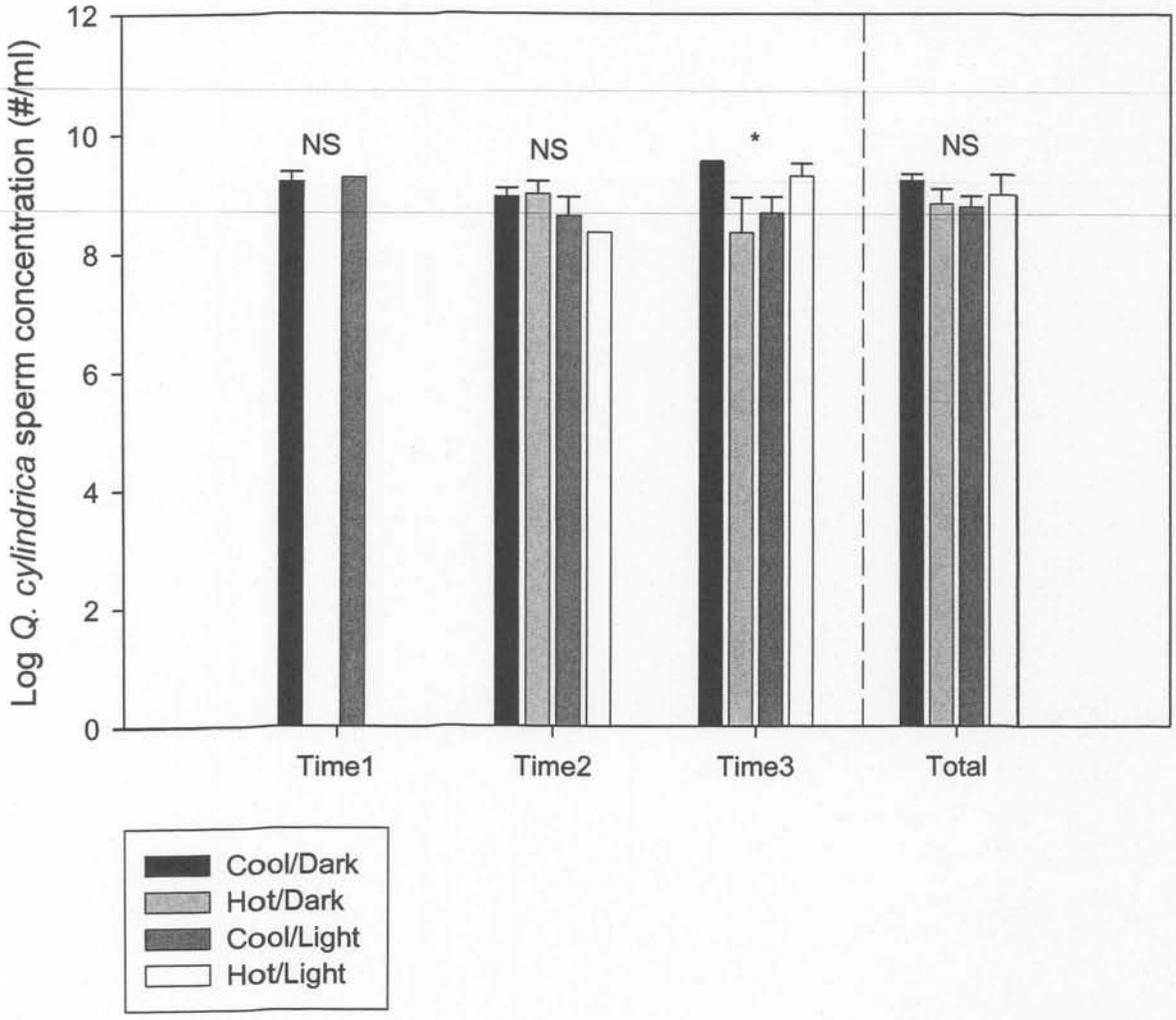


Figure 40. Mean (±SE) *Q. cylindr−ica* sperm concentration for each treatment and time period.

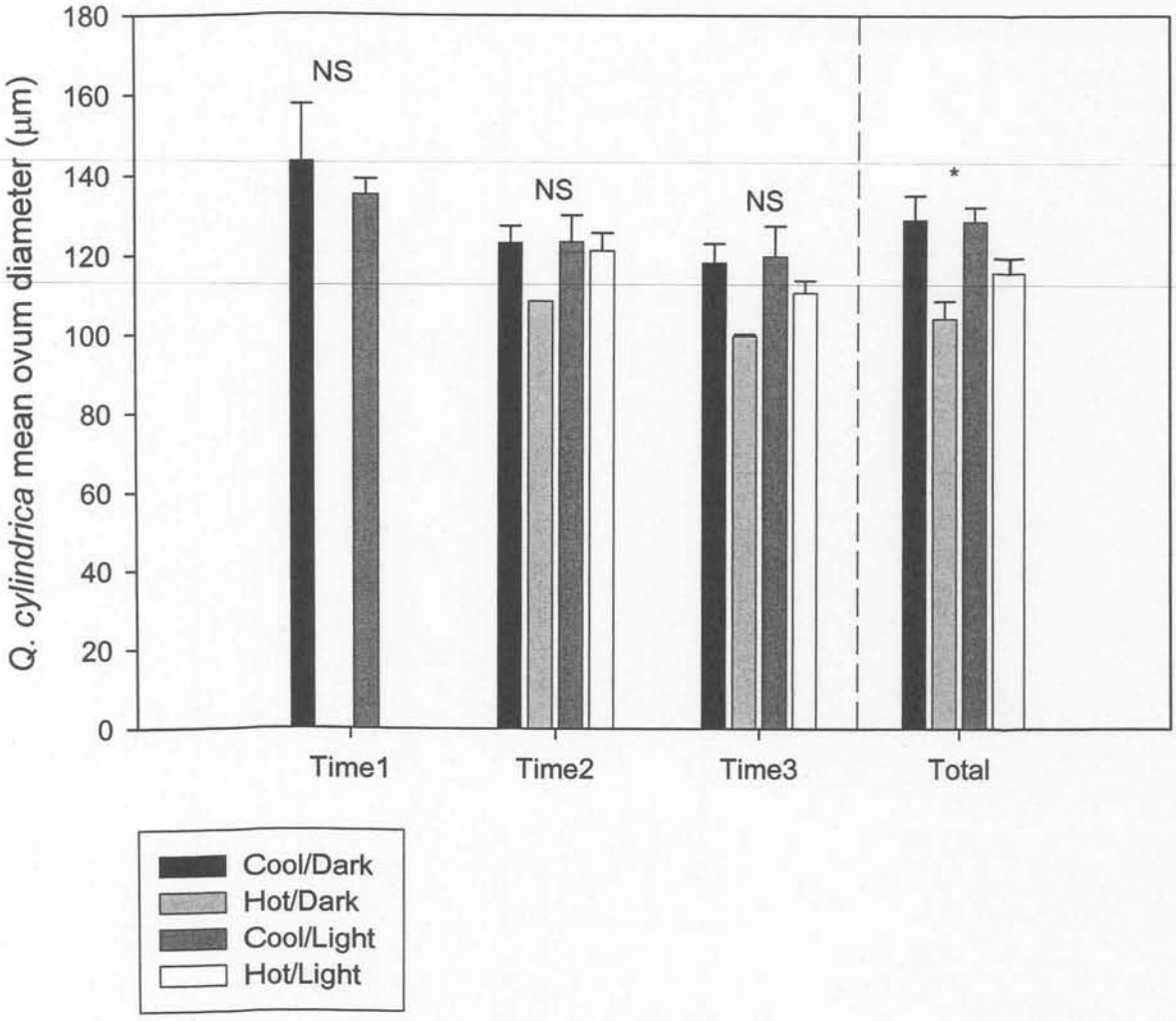


Figure 41. Mean (±SE) *Q. cylindrica* ovum diameter for each treatment and time period.

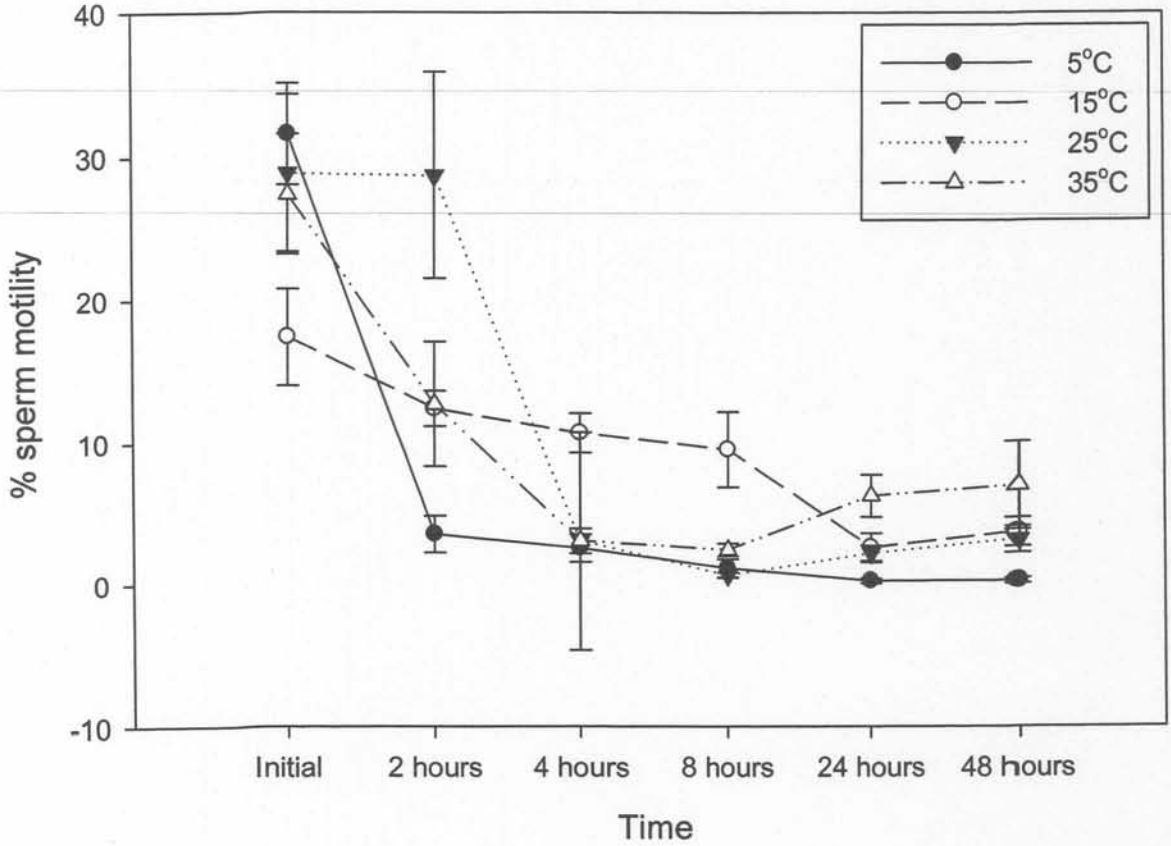


Figure 42. Mean (±SE) sperm motility over time at four different temperatures.

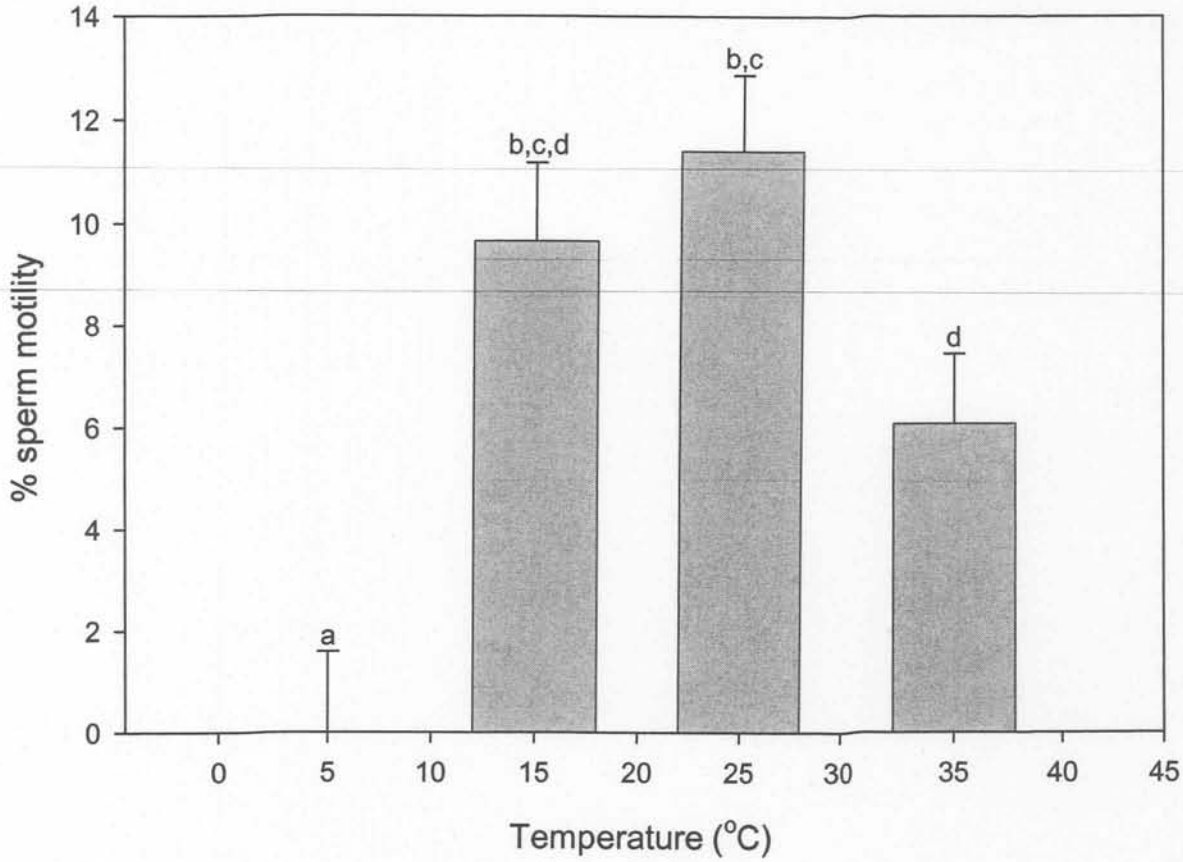


Figure 43. Mean (±SE) sperm motility at four temperatures.

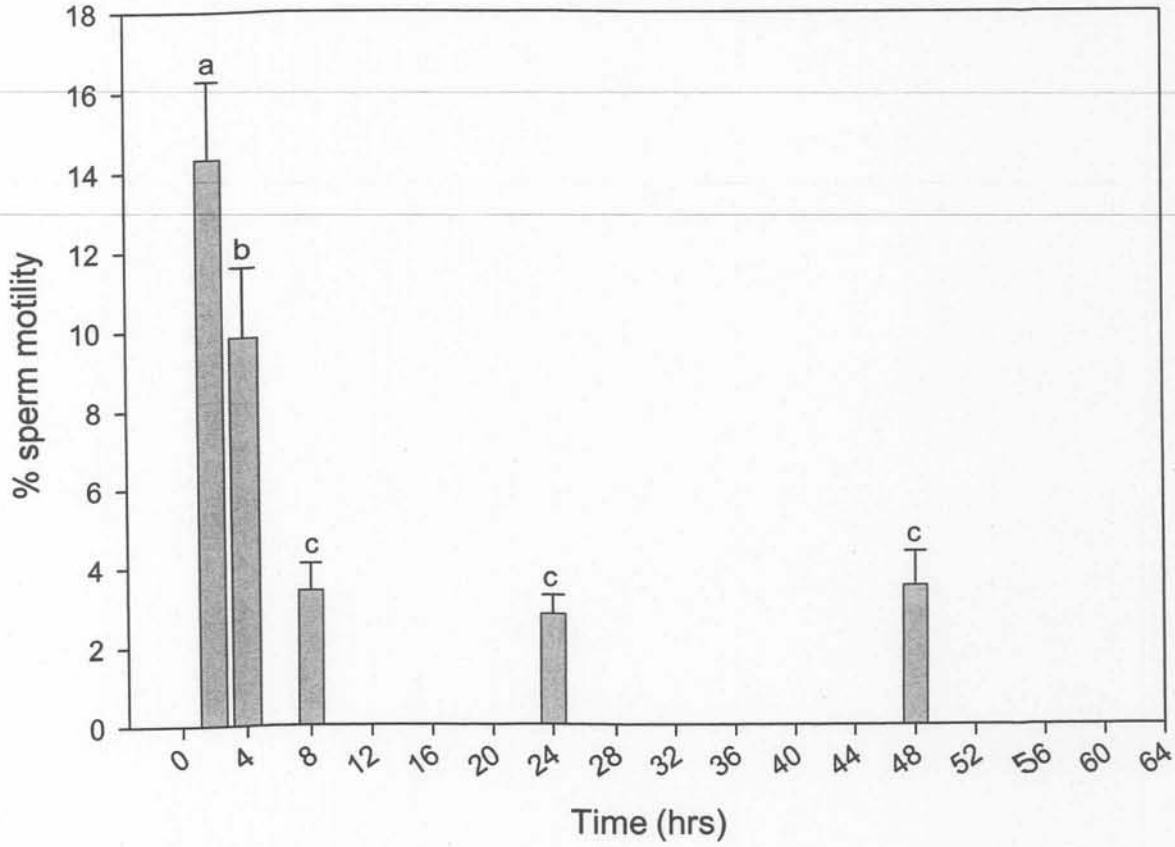


Figure 44. Mean (±SE) sperm motility averaged across time.

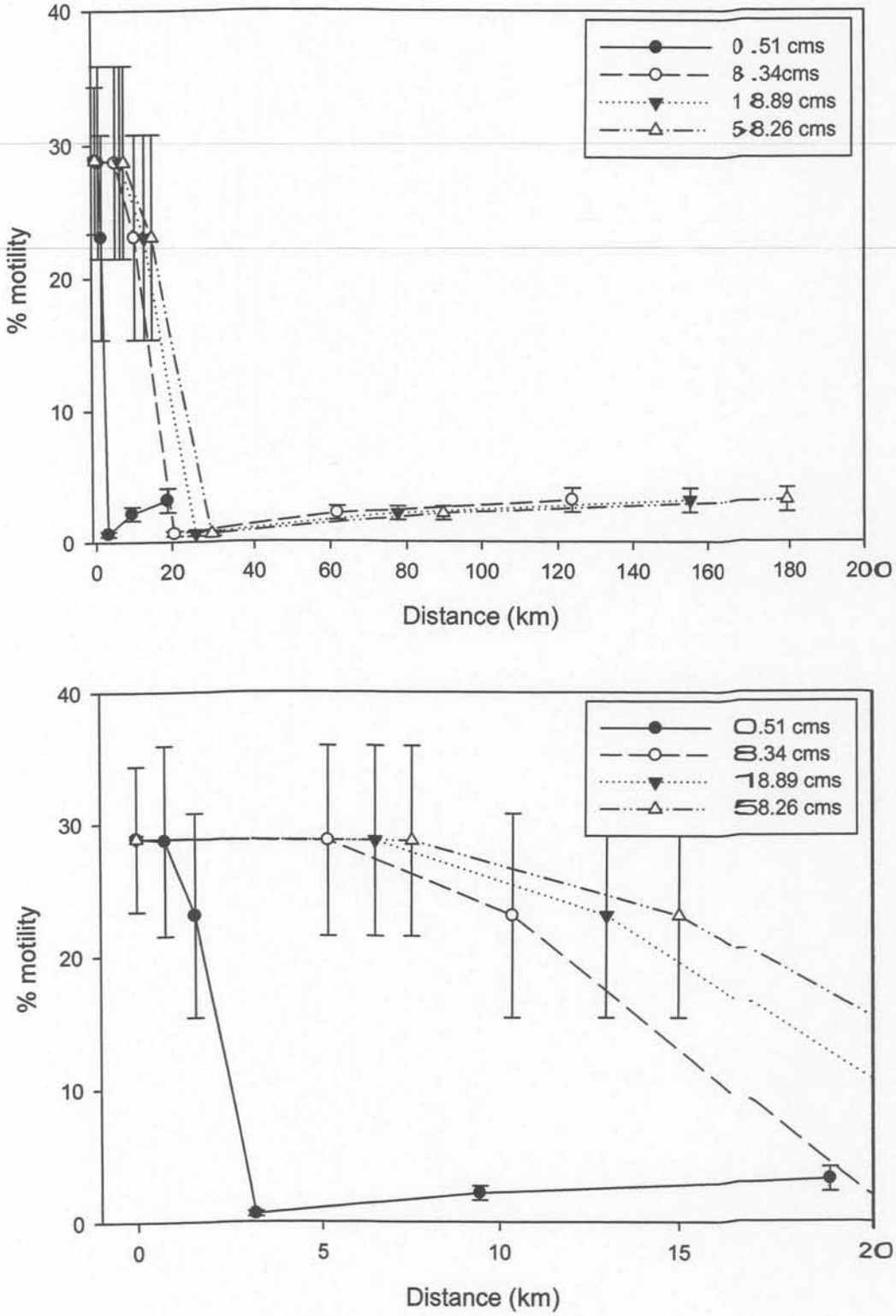


Figure 45. (a.) Distance downstream sperm can move (without considering sperm settling) under 4 different July flow regimes versus sperm motility at 25°C. (b.) Expanded view of (a.).

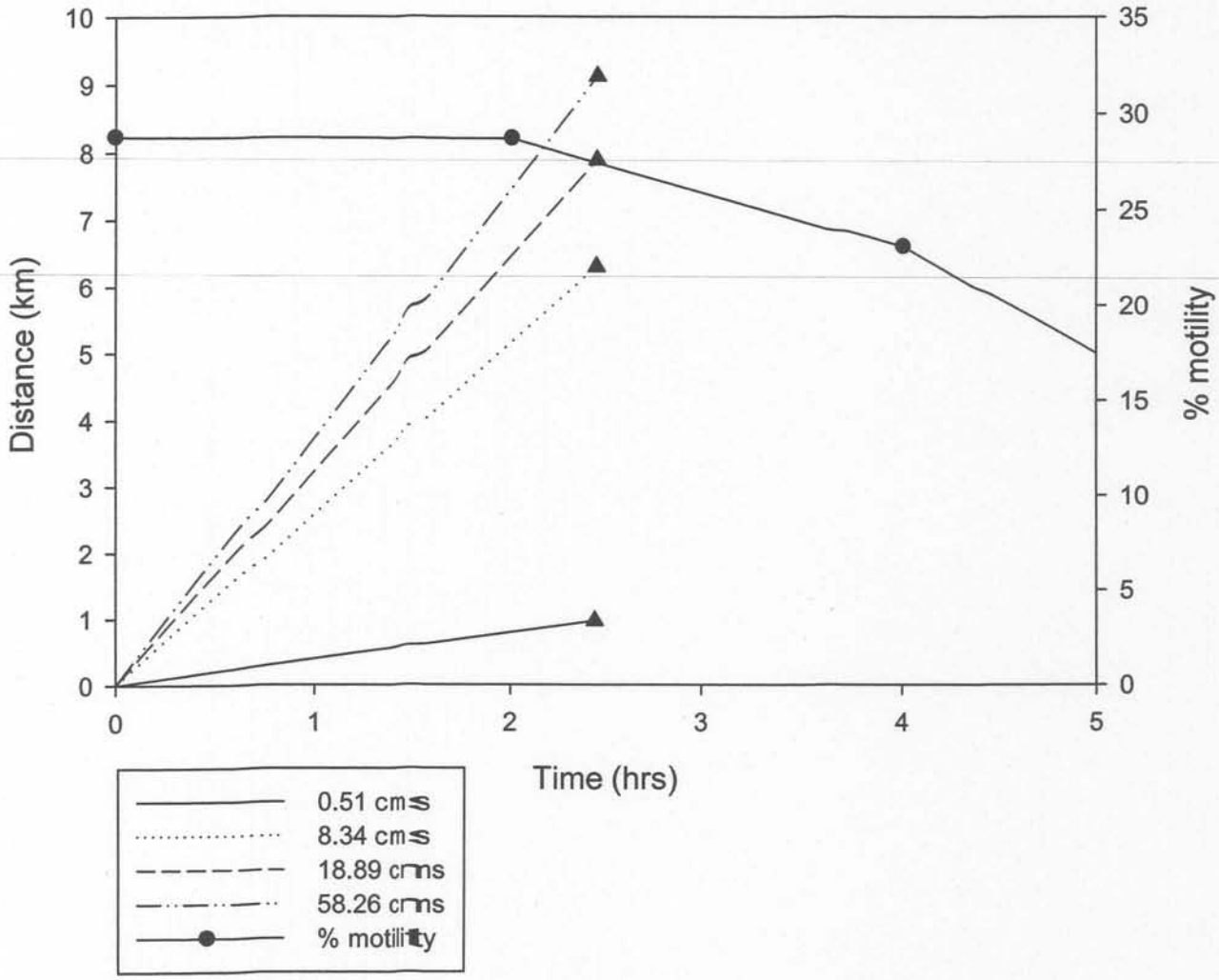


Figure 46. Results of model predicting the downstream distance sperm can move until settling out of the water column (marked by triangles) versus sperm motility at 25°C. Model assumes mussels release sperm 1 cm above sediment.

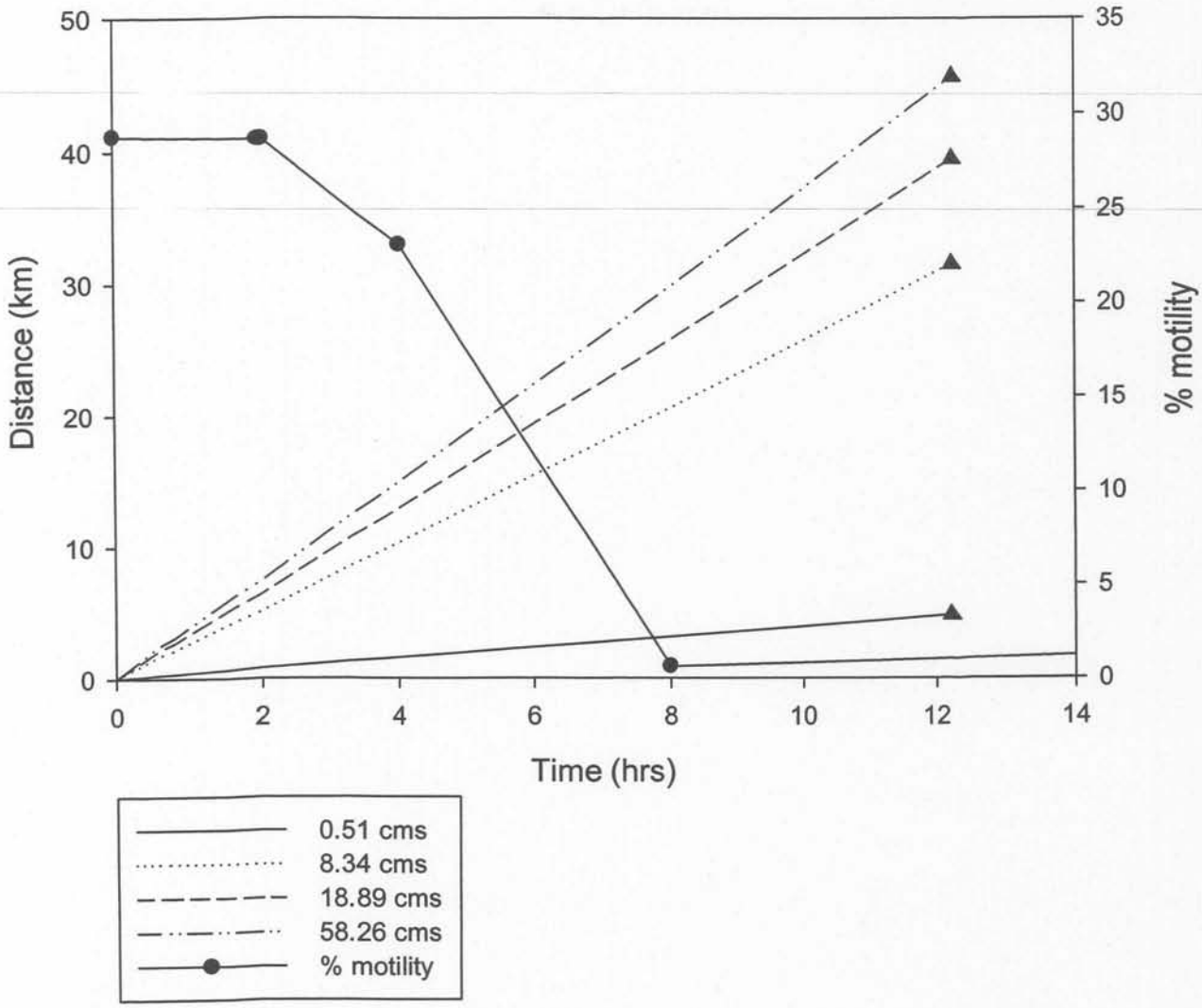


Figure 47. Results of model predicting the downstream distance sperm can move until settling out of the water column (marked by triangles) versus sperm motility at 25°C. Model assumes mussels release sperm 5 cm above sediment.

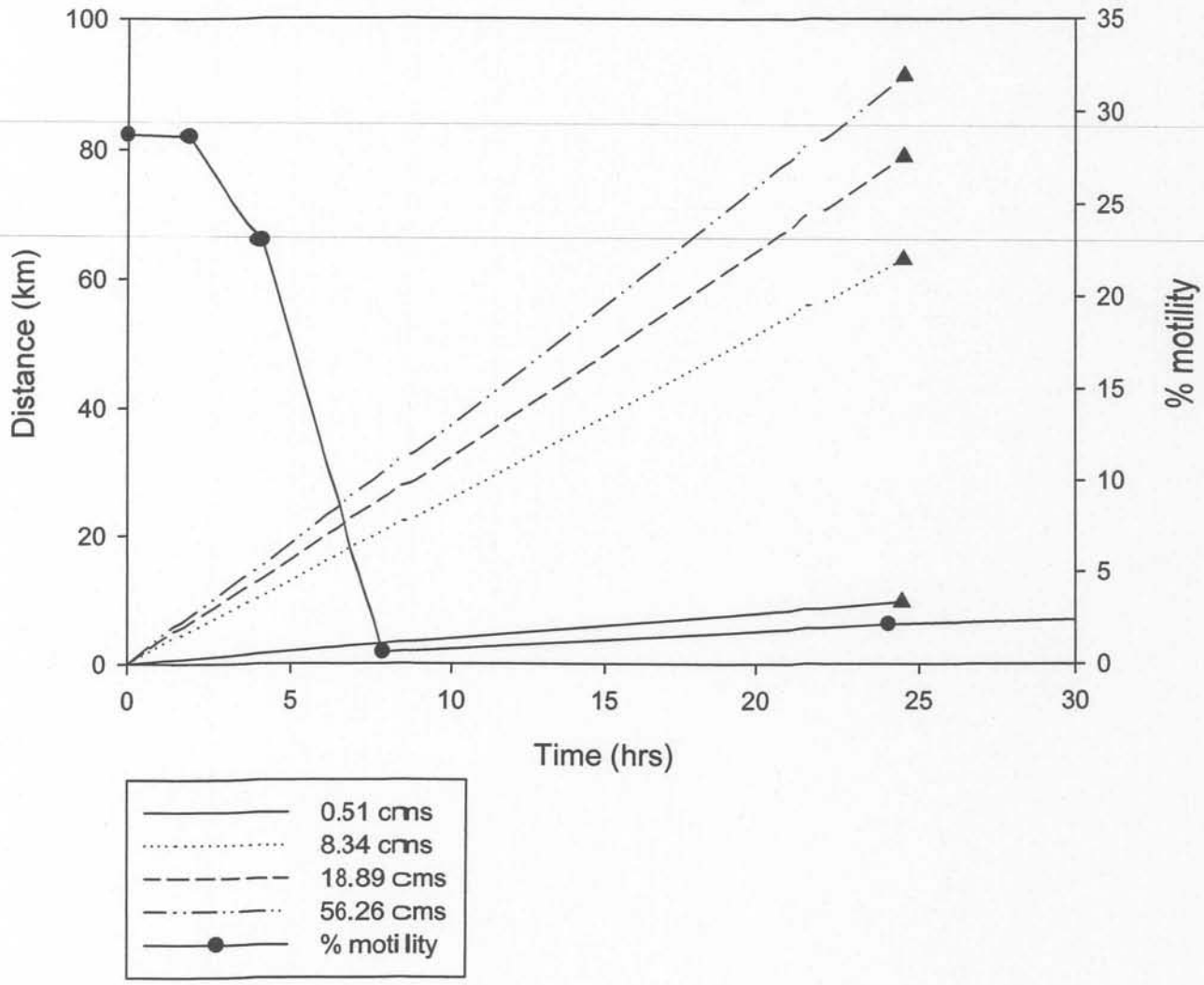
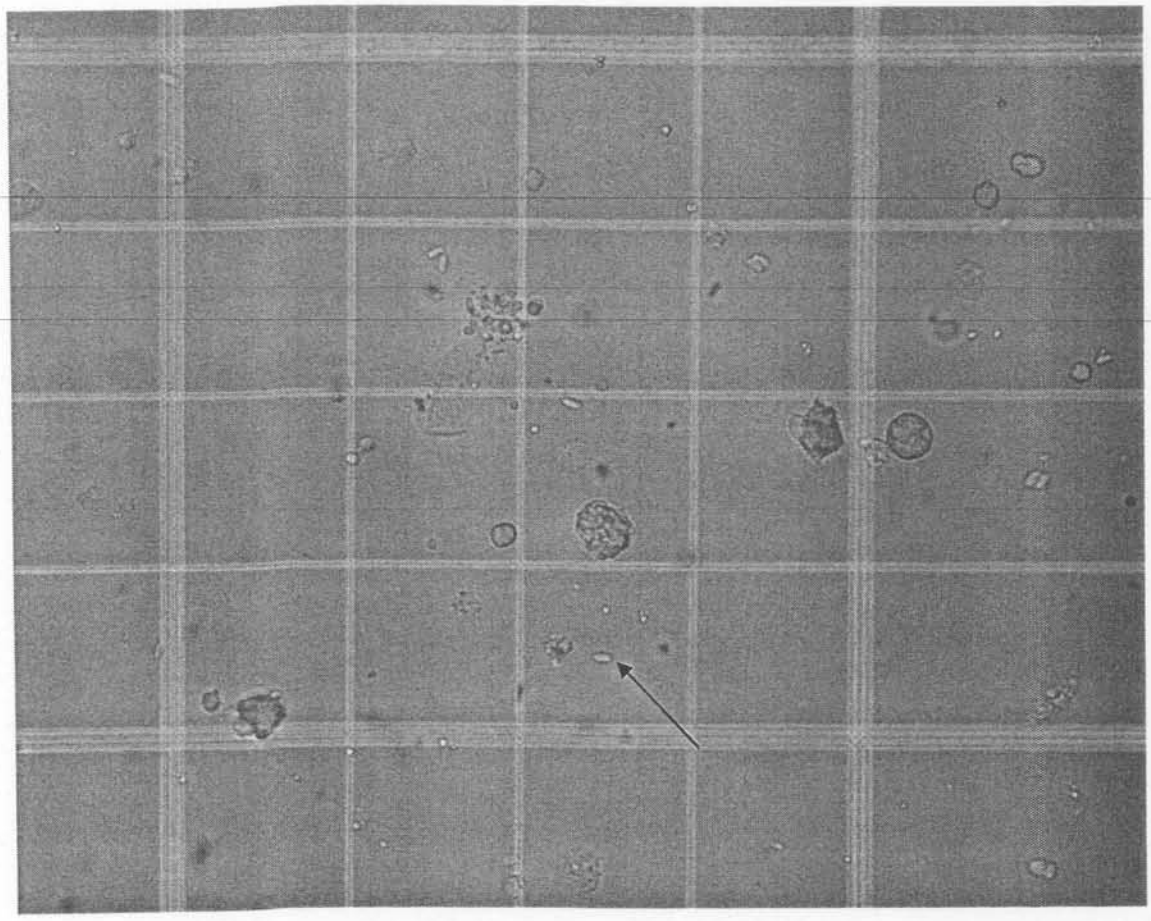
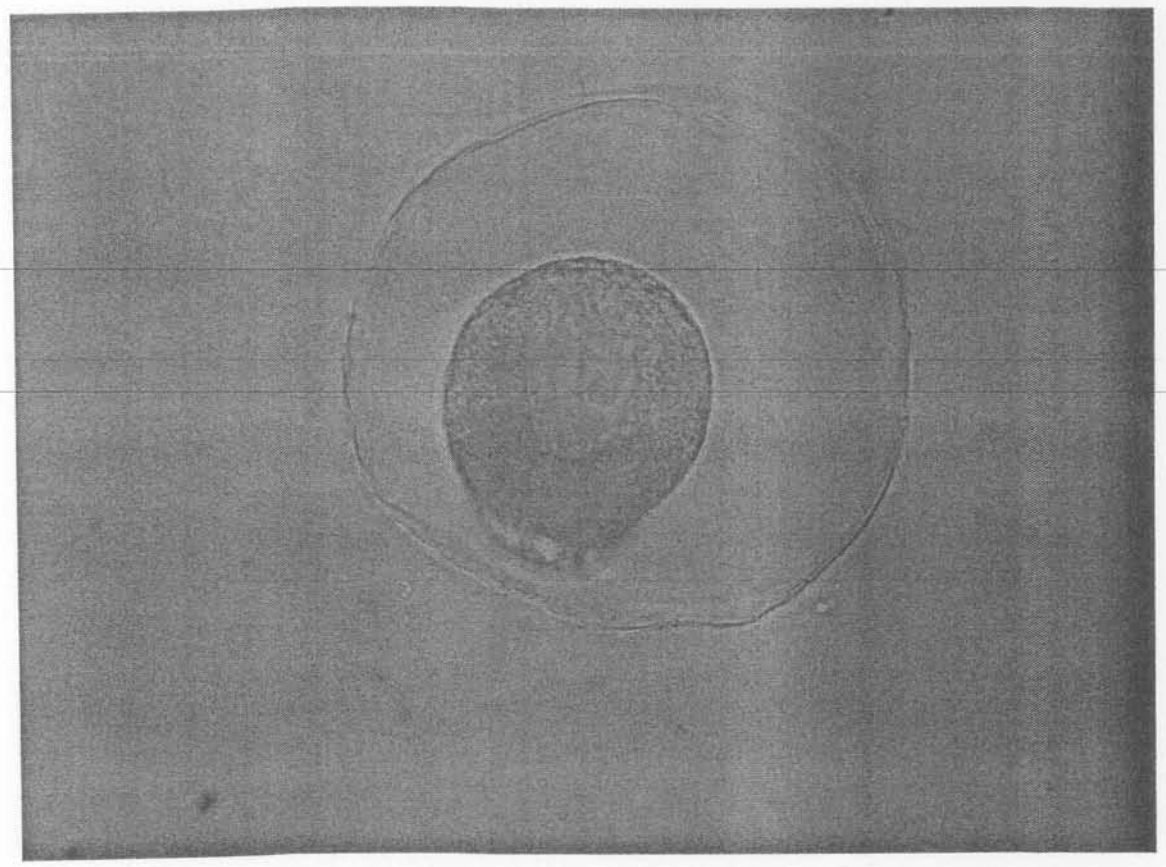


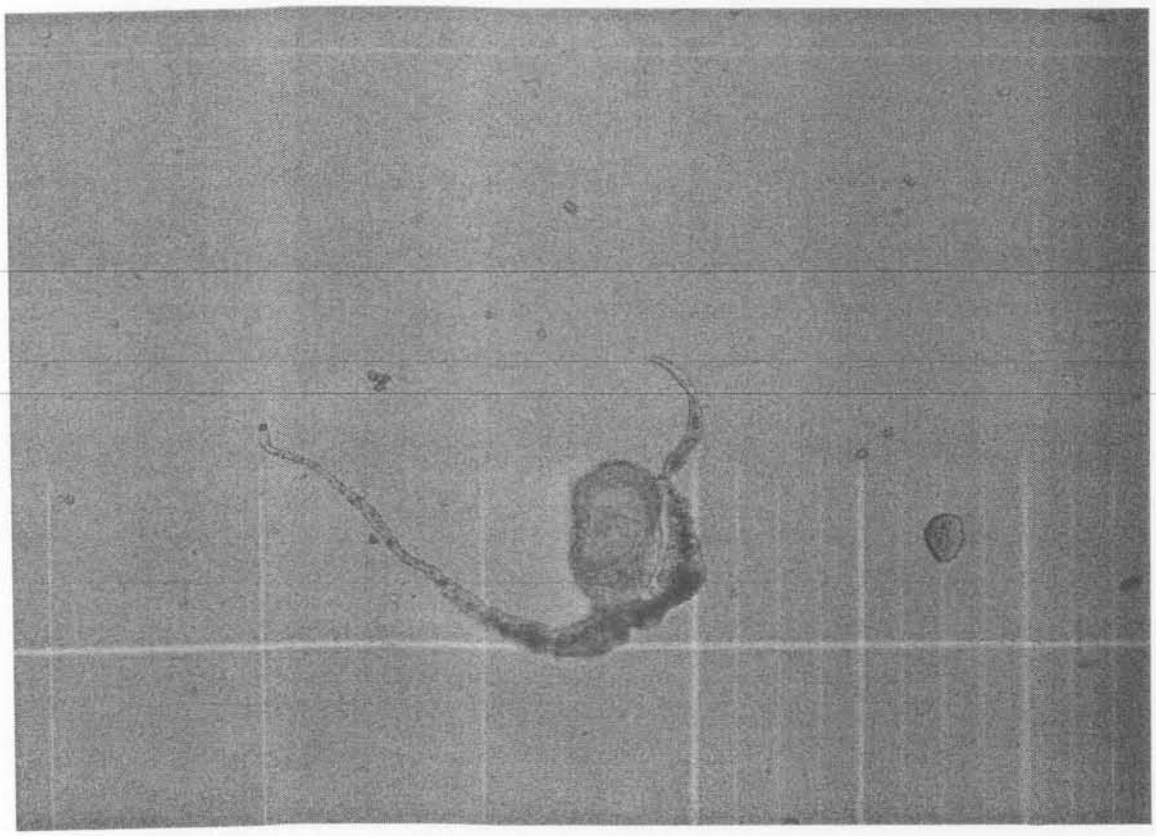
Figure 48. Results of model predicting the downstream distance sperm can move until settling out of the water column (marked by triangles) versus sperm motility at 25°C. Model assumes mussels release sperm 10 cm above sediment.



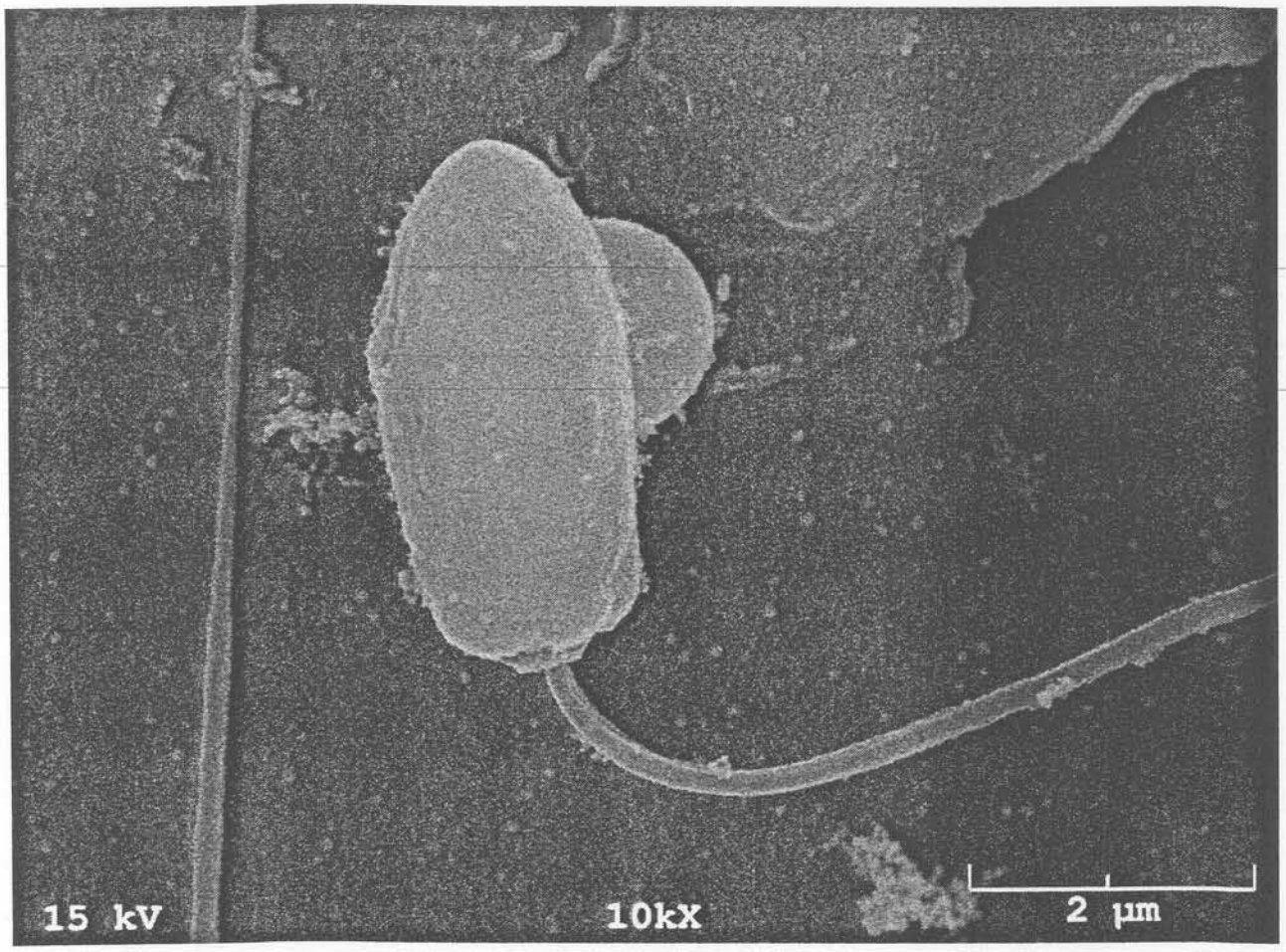
Photograph 1. Sperm cells (indicated by arrow) from *Q. pustulosa*.



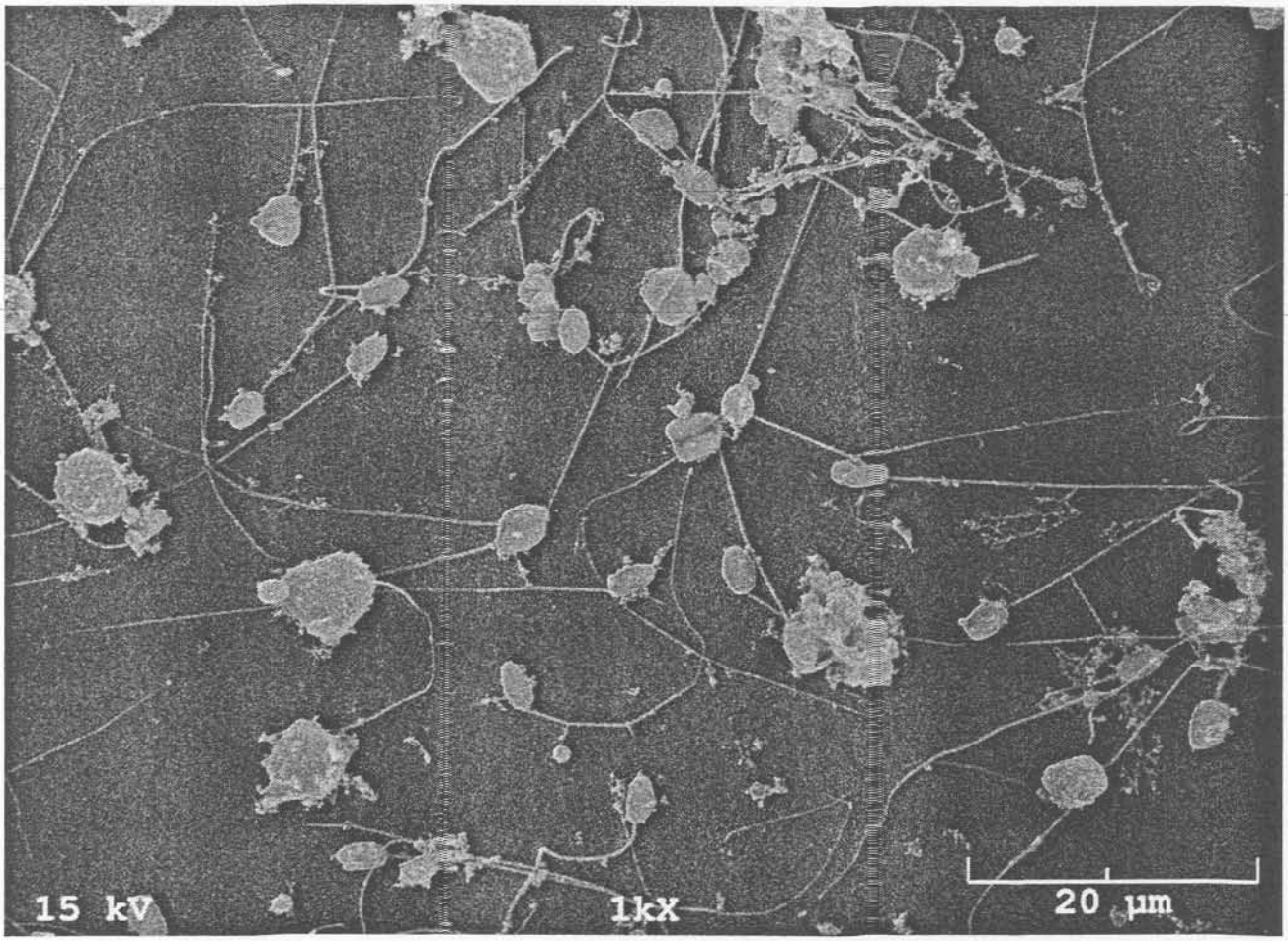
Photograph 2. Developed egg cell with vitellin membrane from *Q. pustulosa*.



Photograph 3. Sterilizing trematode found in *Q. quadrula*.



Photograph 4. Scanning electron microscope image of the head of a *Q. pustulosa* sperm.



Photograph 5. Scanning electron microscope image of many *Q. pustulosa* sperm.

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