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Synchronization of Reproduction in Deertoe Mussel (*Truncilla truncata*)

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SYNCHRONIZATION OF REPRODUCTION IN DEERTOIE MUSSEL
(*TRUNCILLA TRUNCATA*)

A Master's Thesis

Presented to

the Graduate College of

Missouri State University

in Partial Fulfillment

of the Requirements for the Degree

Master of Science, Biology

By

Kendell R. Loyd

December 2017

**SYNCHRONIZATION OF REPRODUCTION IN DEERTOES MUSSEL
(*TRUNCILLA TRUNCATA*)**

Biology

Missouri State University, December 2017

Master of Science

Kendell R. Loyd

ABSTRACT

Freshwater mussels, Order Unionoida, are diverse and widespread in North America but have suffered general decline and many extinctions in recent decades. The reproductive biology of Unionoida is complex and may be vulnerable to human impacts. Male mussels release aggregates of sperm called spermatozuogmata that drift downstream. Females obtain spermatozuogmata from the water and brood the fertilized eggs internally during development. Many aspects of general reproductive biology are poorly understood, including the mechanisms that coordinate spawning and the route by which sperm meet eggs in the female. The present study focuses on reproduction in the Deertoe (*Truncilla truncata*), which spawns in the spring. Adult individuals were collected from the Minnesota River watershed in early April 2016 and held at temperature below 10°C. Gonadal sex was reliably predictable from shell and gill morphology. Effects of temperature and chemical cues on spawning were examined. Both male and female *Truncilla* spawned within 24 hours after temperature reached 13°C. At lower temperatures, spawning could be delayed for at least one year. Sperm presence had no effect on the timing of male or female spawning. Spawning females invested about 2.5× more energy in gametes than males did. Female and male investments were 97 ± 61 and 40 ± 43 calories per gram whole body mass, respectively. Sperm activated and detached from spermatozuogmata in the presence of unfertilized eggs, suggesting that a chemical signal released by the eggs triggers sperm to disassociate from spermatozuogmata. Contact with female gills, however, did not trigger dissociation.

KEYWORDS: freshwater mussel, reproduction, spermatozuogmata, temperature, fecundity, fertilization

This abstract is approved as to form and content

Chris Barnhart, PhD
Chairperson, Advisory Committee
Missouri State University

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ACKNOWLEDGEMENTS

The compilation of this thesis would not have been possible without the dedicated cooperation of so many people. For their participation, I am extremely grateful.

Thank you, first, Dr. Chris Barnhart for opening my eyes to freshwater mussels. They are too often overlooked, yet deserve lifetimes of admiration. Your friendship and mentorship have grown me as a scientist, a teacher, and a person. I will always aspire to be as passionate as you are about everything. You are an encouragement to me always.

Dr. Alicia Mathis, a special thank you for taking a chance on me by allowing me to do undergraduate research in your lab. You introduced me to the joys of behavioral science and taught me to always be inquisitive. Your extensive guidance, patience, and generosity have been the root of my success.

Dr. Day Ligon, thank you for showing me the complexities of physiological biology and making me question how our bodies interact with the environment.

To Bernard Sietman and Mike Davis, it would have been impossible for this project to happen without you. Thank you for supplying my animals and for your constant willingness to assist however you were able.

Dr. Gary Meints, thank you for opening your lab to me and a helping me with calorimetry and calculations.

My lab mates: Beth Glidewell, I appreciate your open ears and willingness to help me with everything along the way, especially the long trip to Minnesota to collect mussels. Morgan Kern, sharing an office with you has been a highlight of grad school. Thank you for all of your help and always keeping me humble to the absurdity of every “three-week deadline” I made for myself. Amy & Bryce Maynard, Kathryn Martin, Alex Herrold, Jordan Murray, Tasha Karpel: your friendships and support have been a huge help to me along the way.

To my family, especially my parents, thank you for never letting me doubt myself and for always supplying the means necessary for me to pursue my love of biology. I could not be where I am without your sacrifices.

My dearest friends, who I hope now know more about mussels than the average person: thank you for your constant encouragement, your willingness to assist me in field work whenever I asked, and, more than anything, your nearly daily visits to my office. A piece of each of you is entangled in this work. I cannot say enough about what you’ve done for me.

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INTRODUCTION

North America contains the highest diversity of freshwater mussels (Order Unionoida) in the world, with approximately 300 native species. Mussels have both historical and modern significance. Native Americans used freshwater mussels as food, and shells were used for tools and ornamentation (Parmalee and Klippel 1974; Brim-Box et al. 2006; Haag 2012). From about 1890 through 1940, North American mussels were commercially important in button manufacture, and thousands of tons of mussel shell were harvested each year, mostly in the Mississippi River Valley (Neves 1999; Anthony and Downing 2001). It was from the shell fishermen, or ‘shellers’, that many of the present-day species’ common names originated (Coker 1919). Plastics have replaced shell as raw material for buttons, but North American mussels are still commercially harvested for production of pearl nuclei used in marine pearl culture (Neves 1999; Hua and Neves 2007).

In addition to their economic value, mussels have high ecological value. Mussels occupy a unique ecological role as large-bodied freshwater suspension feeders on bacteria, phytoplankton, and detritus. Large populations can filter massive volumes of water, occasionally exceeding the stream’s daily discharge (Nalepa et al. 1991; Dillon 2000; Haag 2012). Filtering reduces microbial populations, including potentially harmful bacteria such as *E. coli*, and increases water clarity (Silverman et al. 1996). While some of the filtered material is assimilated, much of it is deposited in the benthos in the form of pseudofeces and feces, linking the pelagic and benthic food webs (Howard and Cuffey 2006; Haag 2012). These biodeposits are readily used by benthic macroinvertebrates, even increasing their populations when mussel biodeposits are high. These

macroinvertebrates are major food sources for a variety of larger species, including game fishes (Howard and Cuffey 2006).

In light of their commercial and ecological significance, conservation of freshwater mussels is particularly important (Lydeard et al. 2004). Approximately 70% of North American species are considered to be extinct, endangered, or threatened (Williams et al. 1993; Master et al. 2000; Anthony and Downing 2001). The contributory factors are complex. Habitat destruction, broadly speaking, is more important than direct exploitation (Richter et al. 1997; Anthony and Downing 2001; Lydeard et al. 2004). River damming, channelization, and unsanitary disposal of wastes such as brine and mining effluent, toxic chemicals, and raw sewage all had extensive impacts, and at least 25 mussel extinctions were documented by 1990 (Haag 2012). Impacts from impoundment, including habitat fragmentation and isolation, temperature and flow fluctuations, and exclusion of hosts, may restrict successful mussel recruitment (Bates 1962; Williams et al. 1992; Vaughn and Taylor 1999). Erosion, sedimentation, head cutting caused by channelization, and introduction of invasive species such as Zebra Mussel, *Dreissena polymorpha*, also impact mussel populations (Vannote and Minshall 1982; Richter et al. 1997; Lydeard et al. 2004; Brim-Box 2006; Newton et al. 2011).

Although many mussel declines can be linked with specific impacts, some studies report decline of mussels in areas without obvious causes. Affected populations show lack of recruitment, suggesting that reproduction or the survival of juvenile life stages are more affected than adult survival (e.g. Suloway 1981; Poole and Downing 2004; Haag 2012). The reproductive processes of mussels are complex, and many knowledge gaps

remain in basic reproductive biology. Research into these processes of freshwater mussels may be crucial to their survival and conservation.

Mussel Life History and Reproduction

Mussels inhabit the substrate of freshwater streams, wetlands, rivers, and lakes (Haag 2012). Although mussels are capable of locomotion by means of the foot, adults typically remain in a single location, usually nearly completely buried, for long periods of time (Strayer 1981). This nearly sessile life style presents challenges in regards to sexual reproduction and dispersal of offspring. Males expel gametes into the water column to drift to downstream females (spermcasting: Bishop and Pemberton 2006). Females presumably collect sperm using the same filtration and water transport system with which they feed. However, the route of the entry of sperm to the water passages and the site of fertilization remains unclear.

Fertilized eggs are brooded in the water passages of the gills of the female while the larvae, called glochidia, mature (Mackie 1984). Mature glochidia are obligate parasites on fish or, in a few cases, amphibians, likely as a means of dispersal (Howard 1951; Haag and Warren 1997; Watters and O'Dee 1998; O'Brien and Brim-Box 1999). Glochidia reach the host by a variety of mechanisms (Watters and O'Dee 1998; Barnhart et al. 2008), and attach to the host's gills or fins where they metamorphose into juvenile mussels (Arey 1932; Kat 1984). After metamorphosis, the juveniles settle to the bottom, hopefully in suitable habitat, where they assume a free-living lifestyle (Arey 1932). Mussels typically reach sexual maturity in 1–10 years, depending on species, and lifespans vary widely among species from about 5–130 years (Haag 2012).

The majority of unionoid species are gonochoristic, possessing separate male and female individuals (Lefevre and Curtis 1912; Zales and Neves 1982; Haggerty et al. 2011). However, a few species are hermaphroditic, and many gonochoristic species occasionally show gonadal hermaphroditism, though the functionality of the minority tissue is questionable (Heard 1975). Mussels possess two gonads which are fused at the midline (Cummings and Graf 2015). The gonads in both sexes are located inside the visceral mass dorsal to the foot with portions of the intestine passing through the gonad (Mackie 1984). The sex of a mature individual can be identified by histology or by examination of gonadal fluid containing gametes. External sexual dimorphism varies among species. Useful characteristics include the morphology of the demibranchs and shell (Lefevre and Curtis 1912; Utterback 1931; Heard 1975; Mackie 1984; Tankersley and Dimock 1992).

Spermatogenesis and Male Spawning. Mussel spermatozoa are uniflagellate, typically lack an acrosome, and contain 4 or 5 mitochondria (Waller and Lasee 1997). Spermatozoa develop in two different cellular pathways, termed typical and atypical. Typical spermatogenesis develops fertilizing-spermatozoa by mitotic and meiotic cellular division. Cellular stages include spermatogonial cells, primary and secondary spermatocytes, spermatids, and mature spermatozoa. Atypical spermatogenesis occurs in different gonadal locations and by different cellular processes than typical spermatogenesis, and may lead to non-fertilizing or fertilizing-spermatozoa (Yokley 1972; Shepardson et al. 2012). Atypical spermatogenesis forms multinucleated inclusions called sperm-morulae, which may be precursors to the internal globe structure of the mature sperm aggregates, called spermatozeugmata; however, very little is known

about the atypical process (Heard 1975; Lynn 1994). Gametogenic cycles are highly variable, even among populations of the same species (Heard 1975; Haggerty and Garner 2000). Some species only produce mature gametes directly prior to the spawning season (e.g. Haggerty et al. 2011). In others, gametes seem to develop more continuously (e.g. Zale and Neves 1982; Garner et al. 1999). The two spermatogenesis pathways may occur with differing intensities seasonally (Shepardson et al. 2012).

Sperm are expelled from the gonads through the genital duct by ciliary movement. They exit the genital pore into the suprabranchial chambers and out of the body via the excurrent aperture (Matteson 1948; Ram et al. 1996). Probably occurring just before or during release, the sperm of freshwater mussels aggregate into multicellular spermatozeugmata (Figure 1) (Lynn 1994; Ishibashi et al. 2000). Each spermatozeugma consists of sperm and an acellular, transparent sphere, or globe, into which sperm imbed their heads with flagella projecting radially (Lynn 1994). Though less than 100 μm in diameter, spermatozeugmata can hold a few hundred to a few thousand sperm (Coe 1931; O'Foighil 1989; Waller and Lasee 1997; Maynard 2015). The globe increases in diameter osmotically when released into water (Ishibashi et al. 2000; Cummings and Graf 2015).

Spermatozeugmata are documented in diverse species of freshwater mussel and might be a feature of the entire Order Unionoida. Similar structures have been documented in other bivalves and may have evolved convergently several times (Coe 1931; O'Foighil 1989; Falese et al. 2011). Spermatozeugmata are thought to have several functions that improve the odds of fertilization. First, spermatozeugmata probably reduce the dilution of sperm in water (O'Foighil 1989). Due to their density and lower surface:volume ratio relative to individual sperm, spermatozeugmata tend to

sink, which should concentrate them near the bottom of the water column where female mussels are located (Figure 2) (O'Foighil 1989). Second, sperm retain viability longer when contained in a spermatzeugma, suggesting that the internal environment of the globe may protect the gametes from osmotic stress in freshwater and perhaps provide energy stores for longevity (Ishibashi et al. 2000; Falese et al. 2011; Haag 2012). The longer survival time means that sperm can be transported for longer distances in flowing water, increasing the likelihood of encountering a female. Dispersal of viable spermatzeugmata has been documented as far as 16 km downstream of the male (Ferguson et al. 2013).

Oogenesis and Female Spawning. Oogonia are found within ovarian acini situated radially and symmetrically around each distal genital duct (Sereflisan et al. 2009; Lima et al. 2012). Oogonia divide into primary oocytes (Matteson 1948; Yokley 1972). Oocytes develop through a wide size range in the gonad without dividing (Matteson 1948; Lima et al. 2012; Cummings and Graf 2015). Developing oocytes are surrounded by a nutritive material that is probably assimilated as oocytes mature, being that it is most abundant when the oocytes are small and seems to disappear as they become larger (Zale and Neves 1982). Oocytes are initially attached to the gonadal wall by a small stem, which becomes the micropyle of the mature ovum (Matteson 1948; Mackie 1984). Upon detaching from the gonad wall, each oocyte develops a transparent, acellular vitelline membrane. The vitelline membrane is separated from the plasma membrane of the egg by the perivitelline space except where it connects to the ovum at the micropyle (Matteson 1948; Mackie 1984). The micropyle is an opening through which sperm may fertilize the egg. The vitelline membrane surrounding the micropyle contains

glycoprotein receptors, possibly to guide the sperm (Focarelli et al. 1990). Matteson (1948) suggested that oocytes do not advance beyond the primary stage until fertilization, but this hypothesis has not been confirmed. Unfertilized eggs may divide at least twice before development is arrested (Barnhart, unpublished observation).

The gills of mussels, or ctenidia, are two pairs of structures. Each ctenidium consists of a pair of flattened, hollow demibranchs that lie in the mantle cavity on each side of the foot (Peck 1877; Cummings and Graf 2015). The demibranchs are attached to the body on their dorsal edge, but are free along their ventral edge (Lefevre and Curtis 1912). Each demibranch is hollow and internally divided by septa, also called interlamellar junctions, which divide the internal space into dorsoventral water tubes. Each water tube is sealed ventrally and open into a suprabranchial chamber dorsally (Peck 1877; Ortmann 1911; Lefevre and Curtis 1912). If an endocast of the internal water spaces can be imagined, it would be comb-like in shape, with the dorsal passage like the spine of the comb and the water tubes like the teeth.

Water is driven by cilia into the demibranchs through numerous small openings called ostia. The water flows up the water tubes dorsally to the suprabranchial chamber of each demibranch, then posteriorly to the excurrent aperture (Haag 2012; Cummings and Graf 2015). The gills use specialized cilia to collect particles and transport them ventrally to the food groove, a ciliated groove along the gill margin. Particles are transported anteriorly along the food groove to the labial palps and the mouth (Haag 2012; Cummings and Graf 2015). Particles are sorted on the labial palps. Some particles are consumed, while others are discarded in the form of pseudofeces (Sprung and Rose 1988; Cummings and Graf 2015).

The ctenidia of female mussels also play an important role in reproduction. In reproductive females, the eggs are brooded and development of the glochidia occurs within certain ‘marsupial’ water tubes (Lefevre and Curtis 1912). The location of marsupia in the demibranchs varies among taxa. It is possible that the delineation of the marsupial region may compensate for the reductions in feeding and respiration caused by the inflation of the brooding gills (Lefevre and Curtis 1912; Tankersley and Dimock 1993). In fact, the tribe Anodontini develop respiratory canals during brooding alongside the typical water tubes, which are thought to provide for continued water perfusion of the gills (Ortmann 1911; Lefevre and Curtis 1912).

During female spawning, unfertilized eggs are carried by cilia in the genital duct to the genital pore and enter the anterior end of the inner suprabranchial chambers, which normally carry the excurrent flow of water leaving the gills to the excurrent aperture (Latter 1891). What happens next is unclear, but by some mechanism the eggs are distributed down into the marsupial water tubes of the inner and/or outer demibranchs, opposite the direction of the normal flow of water. The process might involve stopping the feeding current, closing the excurrent aperture and mantle margins, and then expanding the mantle cavity to draw water into the (normally) excurrent aperture (Figure 3) (Latter 1891; Lefevre and Curtis 1912). Short ‘gulps’, perhaps aided by ciliary action, might move the eggs into the gills.

A second mystery is how sperm enter the female and contact the eggs. Most accounts assume that the eggs are fertilized in the suprabranchial chambers in transit from the oviduct into the demibranchs, but data are lacking (Matteson 1948). It is not known whether the sperm enter via the ostia or the excurrent aperture.

Spermatozeugmata are probably too big to pass directly through the gill ostia. In the freshwater bivalve genus *Corbicula* (Order: Veneroida), which also broods its embryos, ostia dimensions varied from about 10–25 μm (Medlar and Silverman 2001). Few data are available for ostia size in Unionoida, but one study reported maximum dimensions of 64 x 28 μm in *Lampsilis radiata* (Nelson and Allison 1940). Sperm introduction did not dilate the ostia or influence filtration rate (Nelson and Allison 1940). Individual sperm might pass through ostia of these dimensions, but only if not intercepted by the food capture mechanisms. The laterofrontal cilia normally capture nearly 100% of sperm-sized particles (Pletta 2013).

Sperm must be liberated from the spermatozeugmata in order to fertilize ova (O’Foighil 1989). Unionoid sperm activate and readily evacuate the globe in the presence of sodium chloride, but not in the presence of an osmotically similar sugar solution, suggesting a chemical trigger for release (Barnhart and Roberts 1997). One suggestion is that fluid in the suprabranchial chambers may cause sperm to leave the globe, but this has not been confirmed (2017 personal communication GT Watters, Ohio State University)

Reproductive Synchronization. Female unionid mussels are not known to store sperm, so the synchronization of spawning between the sexes is vital for fertilization. The timing of spawning may be important in relation to critical environmental factors, such as host abundance, water levels affecting settlement of juveniles, and food availability for juveniles. Most species spawn once per year in spring or fall, but the timing varies widely among North American unionids (Zale and Neves 1982; Haag 2012). The forces controlling this physiological behavior are not clear. Due to the

seasonality of spawning, temperature and/or photoperiod have been cited as the primary cues (Zale and Neves 1982). It is also plausible that algal concentrations, as an indicator of season, induce spawning. Nutritional status might determine whether individuals of some species forego spawning in some years, though this has received little study.

Bivalves are capable of detecting several environmental conditions. Sensory organs in the mantle edge detect tactile and photo stimulation (Cummings and Graf 2015). The osphradial sense organs of mollusks are variously hypothesized to detect sediment load, light availability, osmolality, and chemicals (Sokolov 1977; Kraemer 1981; Haszprunar 1987). However, the purpose of the osphradia in Unionids is not known. In addition to environmental cues, chemical communication among individuals could potentially be important for synchronizing spawning. Chemical cues could come in many forms. The chemical signal of an individual mussel (i.e. body odor) could provide a stimulus; however, the anatomical source of this signal would be difficult to experimentally locate, being that it may originate from many sources or be a mixture of many chemicals. Another possibility is the existence of a coordination pheromone that evolved due to its effectiveness in synchronization breeding cycles. This pheromone, however, would also be difficult to bioassay.

A coordination pheromone could, however, be associated with the sperm. For example, in the American Oyster (*Crassostrea virginica*, Order: Ostreoida), female filtration rate increases when exposed to sperm (Nelson and Allison 1940). The presence of unionid sperm in the water could similarly be a signal for unionid females to spawn. This effect would help to ensure fertilization. Hypothetically, sperm could also trigger other males to spawn as a form of competition, because males that spawn after other

nearby males might have reduced access to unfertilized eggs. Chemical cues might also facilitate congregation prior to spawning for increased fertilization success (Amyot and Downing 1998).

Deertoe Mussel

Deertoe (*Truncilla truncata* Rafinesque 1820) was selected for study because of its small body size, accessibility, and because of evidence for temperature-dependent spawning (Waller and Lasee 1997; 2015 personal communication AL Maynard and MC Barnhart, Missouri State University). Deertoe inhabits lotic environments from northern Minnesota, Michigan, and southern Ontario to the Gulf coast from eastern Texas to Alabama and east of Kansas except the Atlantic coast drainages and Florida (Oesch 1995), and is found in a variety of substrates ranging from sand-silt to large gravel (Fuller 1978; Oesch 1995). Individual lifespan is up to 18 years (Haag and Rypel 2010).

Deertoe was long assumed to be a long-term brooder, spawning in the autumn and brooding throughout winter and spring (Lefevre and Curtis 1912; Oesch 1995). This assumption was incorrect, and Deertoe are now recognized as early spring spawners and short-term brooders (Waller and Lasee 1997; present study). Captive spawning of Deertoe has been observed after exposing animals to an abrupt increase from 5°C to 10°C followed by gradual increase to room temperature, suggesting that temperature change may trigger spawning (2015 personal communication AL Maynard and MC Barnhart). Sexual dimorphism by shell morphology has not been previously described. Females exhibit a form of visual host-attraction, but only by active valve gaping. They lack a mantle flap lure that occur in females of other related species (Zanatta and Murphy 2006,

Sietman et al. in press). The only confirmed host fish for this mussel is Freshwater Drum (*Aplodinotus grunniens*) (Fuller 1978, Sietman et al. in press).

Thesis Objectives

In this study, several aspects of the reproductive biology of *Truncilla truncata* were examined. The external morphology of gonadal males and females was compared to assess external sexual dimorphism. The control and synchronization of spawning was examined in two laboratory tests: (1) the effects of temperature on spawning in males and females, and (2) the possibility of chemical communication among spawning individuals. The reproductive investment of the sexes was assessed by measuring fecundity and energy content of gametes for males and females. Finally, hypotheses regarding the path that sperm follow to meet eggs in the spawning female were tested by determining sperm activation in response to egg proximity.

METHODS AND RESULTS

Collection and Animal Care

Deertoe were collected from the lower Chippewa River, Minnesota by staff from the Minnesota Department of Natural Resources. The collection site is 1.5 km upstream of Watson, MN and the confluence of the Chippewa and Minnesota Rivers. The species is abundant and accessible at this site (2015 personal communication with BE Sietman and JM Davis, Minnesota Department of Natural Resources). Mussels were collected by hand on 5 April 2016 before water temperature had surpassed 10°C. Mussels (158 individuals) were transported to Missouri State University (MSU), Springfield, Missouri (Minnesota Collecting Permit: 20823) by van in an insulated cooler with about 75 L of aerated Chippewa River water. Water temperature was monitored repeatedly during transit using an infrared thermometer (IRT0421; Kintrex). Water temperature was kept between 2–10°C by removing or replacing bags of ice.

At MSU, the mussels were housed within a refrigerator (LV17-1-W-LED Lumavue 17.5 cu ft; Beverage Air®) or an environmental chamber (I-36VL Intellus Ultra; Percival Scientific) in unaerated shoebox containers, each containing 5 mussels and approximately 2.5 L of well water with no substrate. Temperature was recorded using temperature loggers (DS1992L iButton®; Maxim Integrated). Temperature averaged approximately 5°C, but varied between 3–10°C. Water was replaced every third day. The mussels were photographed upon arrival to record shape and unique color patterns which allowed individual identification (Figure 4). After experiments were complete, animals were engraved with an identification number using Epilog Zing 24 Laser for easier identification.

For all spawning experiments, animals that were not included in the experiment acted as controls, in that they did not spawn when not exposed to manipulated variables.

Sexual Dimorphism

Mussels were initially identified as male or female based on morphology of the shell and ctenidia. Later, sex of each mussel was verified by observing gametes from spawning and gonad biopsy. The ventral margin of females was straight or slightly convex, while that of males was concave (Figure 5). Gill morphology was also dimorphic, with the marsupia of females being visibly inflated when valves were opened slightly (Figure 6). Initial sex determination based on shell morphology was 97% accurate (i.e. the gametic sex of 153 of 158 individuals was correctly identified based on shell characters). Misidentifications were females with straighter margins mistaken for males. Sex determination from gill morphology of animals in reproductive condition was 100% accurate. All individuals were sexually mature. No hermaphroditism was observed. The sex ratio for the sample was male-biased at 99 males and 59 females (2:1), which is significantly different from 50:50 ($X^2=10.13$; $p=0.001$). Individual wet masses of the 158 specimens ranged from 1.86–106.19 g (mean 22.7 g). Females were significantly smaller than males ($t=-3.97$; $df=1$; $p<0.0001$). Mean male mass for the entire sample was 25.8 g (SD=16.4), while mean mass for females was 14.7 g (SD=7.8). Female variance in mass was less than half that of males ($p=0.005$; Levene test).

Testing Thermal Spawning Stimuli

Thermal Shock. This experiment was conducted 3 wk after collection. To confirm the experiment done by Maynard and Barnhart (2015 personal communication), three males were moved from 5°C water and placed individually into separate unaerated 1-L aquaria of 10°C well water with about 3 cm of gravel substrate. These aquaria were allowed to warm from 10°C to room temperature (about 22°C) over a period of about 3 h. Photoperiod was 24 h light. Animals were checked for signs of spawning every 6 h.

One male was observed spawning 6 h after being placed in 10°C water, but had begun spawning earlier than that. At 6 h, the water temperature was 18°C. The other two males had stopped spawning by 6 h later (12 h after experiment began). Males did not bury themselves prior to spawning and spawned while lying their sides. Spawning was easily detected because sperm sink and form a milky white layer on the bottom of the aquaria.

Gradual Temperature Increase. This experiment began 3 wk after animal collection and spanned 14 d. Five males and one female were included. The female was included to observe if female spawning would occur in the laboratory. Each mussel was placed in an unaerated 1.5-L aquarium with approximately 2 cm of gravel substrate. The aquaria were housed inside an environmental chamber (Percival Scientific: Intellus Ultra) initially set at 5°C. The environmental chamber temperature was increased by 2°C every other day until it reached 11°C, then the temperature was increased 2°C every third day. Temperature was recorded (± 0.5 C) using IButton temperature loggers. Spawning was monitored by time lapse video (Conbrov™ HD90 Mini Video Recorder). Photoperiod was 24 h light to allow for filming.

No mussels spawned at temperatures up to and including 11°C. All animals spawned within 24 h after the temperature reached above 13°C (Figure 7). Mussels, again, did not bury themselves prior to spawning and spawned lying on their sides. Sperm and eggs sank and formed a milky layer. The female was observed dumping eggs from the gills (marsupia were seen partially filled, then empty later). Gametes were identified microscopically.

Delayed Spawning of Males. This experiment was conducted nearly 1 year (50 weeks) after collection, with male mussels that had been stored unfed at <10°C to mimic winter conditions. Three males were placed individually in 1-L aquaria in an environmental chamber at 8°C and warmed to 13°C within a few hours. Temperature was recorded with temperature loggers and spawning was filmed with time lapse. Photoperiod was 24 h light to allow for filming. All 3 males spawned within 7 hours of reaching above 13°C (Figure 8).

Testing Chemoreceptive Spawning Stimuli

Sperm Donors. Several experiments involved exposing mussels to newly released spermatozeugmata from donor males. Males used as sperm donors were removed from storage in the 5–8°C environmental chamber, placed individually in 1-L aquaria, and allowed to warm to room temperature. A temperature logger was present with each male. Aquarium temperatures increased from 5–8°C to 20°C in fewer than 2 h. Males spawned 3–12 h after removal from the environmental chamber. Spermatozeugmata collected for experiments were less than 24 h old when used.

Effects of Sperm on Male Spawning. This experiment began about a month after collection and tested possible stimulatory effects of conspecific sperm on male spawning. Three males were placed in an environmental chamber in separate 1-L aquaria without substrate. Temperature was increased 2°C every day until reaching 10°C, then the temperature was increased 2°C every third day. Each male received sperm from a donor daily after environmental chamber temperature surpassed 10°C, so that each male was exposed to sperm of four different males for a day each, in hopes of stimulating males to spawn below 13°C. Donor sperm suspension was stirred and then split three ways between recipient males. The recipient's water was replaced completely before each new treatment. Recipients were filmed on time lapse from above. Photoperiod was 24 h light to allow for filming. Temperature was recorded using temperature loggers.

No effect of sperm exposure on spawning was detected. All males spawned within 12 h after reaching above 13°C (Figure 9).

Effects of Sperm on Male and Female Spawning. A flow-through, gravity-fed system was developed to expose male and female mussels to water with or without sperm while controlling temperature (Figure 10). Temperature was controlled by using water baths cooled by chillers (ViaAqua: Polar Bear™ CC-25). Animals were held individually in 400 mL beakers. Water levels of water baths remained below the rim of beakers and mixing wells to prevent exchange of water. The reservoir was filled with well water once daily. Solenoids (Bürkert: type 0290) delivered 1.7 L of water to each mixing well every 45 min, meaning each beaker's water would be entirely replaced every 3 h. Water levels in each mixing well would then top a stand pipe, flow to manifolds and

deliver a roughly equal amount of water to each beaker/animal. Beakers overflowed into the lower water bath, and excess water drained via a stand pipe in the lower bath.

The experiment using the flow-through system began about a month after collection. Seventeen males and fifteen females were randomly selected using a random number generator and placed in individual beakers. Both chillers were set to 5°C for the beginning of the experiment, and both chillers were kept at identical temperatures for the remainder of the experiment. A temperature logger was placed in the upper water bath and another was placed among the beakers to confirm the temperature fluctuations experienced by the animals. Temperature was increased 2°C every day. Sperm was added to one mixing pail to test if the presence of sperm could trigger spawning. The complete 1 L of milt solution (see Sperm Donors) was added to one mixing pail as temperature increased to 11°C, while at 11°C, and after increase to 13°C. Sperm additions were separated by at least 12 h. Photoperiod was 24 h.

All but one male (16 of 17) and female (14 of 15) spawned within an 11-h timeframe at a temperature of about 13°C (Figure 11). Both treatment (sperm-exposed) and control (sperm-naïve) groups spawned. Water samples were taken from each beaker and gametes were identified microscopically. Females all released eggs into the water rather than brooding them in the gills.

Fecundity

Fecundity was measured for both male and female Deertoe by collecting the total reproductive output of individuals. Males (n=15) were induced to spawn individually in unaerated 300 mL beakers until no further release of milt was observed (usually after

about 8 h), and the number of spermatozeugmata released was calculated from the volume of the suspension and from concentration measured in subsamples using a hemocytometer. Beakers were mixed continuously using a pipette and samples were drawn from the center of the water volume, to avoid possible concentration gradients in the beaker. Each male's output was measured in triplicate and the results were averaged. Total spermatozeugmata was estimated from concentration in the subsample.

Females (n=16) were likewise induced to spawn in containers. When aeration was not provided females would abort their brood (i.e. expel the eggs directly into the water or from the marsupium into the water). Technique for collecting egg samples was the same as with collection of spermatozeugmata samples. Eggs were counted in three 0.1-mL subsamples and averaged. Total number of eggs was also estimated from concentration in the subsample.

Males released an average of 295.0×10^5 spermatozeugmata, while females released an average of 5.4×10^5 eggs (Tables 1 and 2). The body mass of individual females was a significant predictor of number of eggs per spawn (Figure 12: $R^2=0.3422$; $p=0.0172$) but body mass of male mussels was not a significant predictor of the number spermatozeugmata (Figure 13: $R^2=0.0024$; $p=0.8707$). The measured average for female fecundity fell within the range found for this species by Haag (2013).

Energy Investment in Gametes

Gamete samples were prepared for calorimetry from each of four males and four females. Samples were stirred and subsamples of 40 mL were removed from each 300 mL beaker of gametes. The subsamples were centrifuged at 2500 rpm for 10 min.

The supernatant was aspirated and the pellet was resuspended in 1.5 mL of DI water, and then freeze dried at -80°C and $<100\text{ mT}$ (13.3 MPa) air pressure. Energy content of the dry gametes was measured using bomb calorimetry (Parr 1451 Solution Calorimeter). The calorimeter was calibrated with benzoic acid samples. One male sample was removed from the analysis because the thermal change within the calorimeter was not properly recorded.

The mass-specific energy content of the egg samples was $6.3 \pm 0.31\text{ kcal/gram}$ ($n=4$), and was significantly higher than the energy content of spermatozeugmata, which was $4.6 \pm 0.36\text{ kcal/g}$ ($n=3$) ($t=-6.72$; $p=0.007$). The energy contents of individual eggs and spermatozeugmata were also determined, and were multiplied by fecundity to calculate total energy investment in gametes for all individuals for which fecundity had been determined. Total energy investment per individual was divided by individual whole wet mass to determine mass-specific individual investment in gametes. Spawning females invested about $2.5\times$ more energy in gametes than males did. Female and male investments were 96.9 ± 62.6 and 39.6 ± 43.1 calories per gram whole body mass, respectively (Tables 1 and 2). These values were significantly different (Mann-Whitney U-test, $p<0.01$)

Spermatozeugmata Disassociation

Spermatozeugmata response to solutes was tested by adding small amounts of substances (Table 3) to a drop of fresh spermatozeugmata suspension under a compound microscope at $100\times$ magnification. Solid substances were placed beside the drop containing the spermatozeugmata, and then pushed into the drop using a pointed

laboratory spatula while observing the result. Liquid substances were dropped into the center of the suspension. New sperm samples were used for each attempt, and reactions were repeated at least twice to verify results. All equipment was thoroughly rinsed with DI water and dried between samples to avoid possible contamination. All substances were reagent grade.

Spermatozeugmata dissociated when the individual sperm were activated and began beating their flagella rapidly (Figure 14). The abandoned globe could usually be seen after dissociation. Sodium chloride, in pure form or in rock salt, and calcium chloride were the only chemicals tested that elicited activation (Table 3).

Spermatozeugmata dissociated in response to freshly spawned, unfertilized eggs and to water that had been in contact with freshly spawned, unfertilized eggs. However, water containing a non-brooding, unspawned female did not cause a reaction. When several eggs were washed by letting them settle for one hour in a 5-mL test tube of DI water both the eggs and the wash water still provoked a reaction. Sperm that were observed coming into contact with the egg membrane did not appear to leave the egg. Sometimes so many sperm were in contact that they were capable of pushing the egg through the water. Sperm not in contact with the eggs swam in apparently random directions.

Females that spawned in unaerated water generally released the eggs into the water either directly or soon after they had entered the marsupial gills. Females that spawned in aerated water retained the eggs in the marsupia. Eggs that were collected within 6 h of release from females, or eggs that were collected directly from the brooding gill using a 1-ml syringe and 20G needle, both triggered spermatozeugmata dissociation.

Eggs that were aged by holding in 100 mL of DI water for 3 days or collected from the brooding gill 3 days after spawning, and eggs that were collected directly from the gonad, did not lead to a reaction (Figure 15).

Suprabranchial water was collected by inserting a syringe, without puncturing tissue, through the excurrent aperture into the suprabranchial chamber of the outer gill (the outer gill is the marsupium in this species). The valves were held open using a pair of nasal spreader pliers with the tips filed down to create a thinner wedge, easing their insertion into the shell margin. All four suprabranchial chambers were easily accessible when the valves were opened until the cloaca, the point just inside the excurrent aperture where all four suprabranchial chambers converge, was visible. When spermatozeugmata were added to water from the suprabranchial chamber of non-brooding females there was no response. Although water from the suprabranchial chambers of brooding females was not tested, due to lack of females in reproductive condition, it is logical that this would elicit a response in the sperm since the water had been in contact with the eggs, and thus any chemical emitting from them.

Spermatozeugmata response to tissue from gills of females was also tested. Non-brooding marsupial gills and non-marsupial gills were dissected out by cutting near the dorsal attachment. Most of the water tubes were collected. The gills were placed in DI water on a slide. Spermatozeugmata were introduced onto the gill and observed under the compound microscope at 100× magnification.

There was no response by spermatozeugmata to either empty marsupial gill tissue or non-marsupial gill (Figure 16). Dissected gills retained ciliary activity, and

spermatozeugmata that came into contact with the surface of the gill were quickly moved to the ventral edge where they typically lost contact with the gill.

DISCUSSION

Deertoe Mussel (*Truncilla truncata*) proved to be ideal for laboratory studies of mussel reproduction. Males and females were easily distinguishable based on morphology, and their small size was practical for holding in compact space. Individuals were also easily identified due to their color, pattern, and size polymorphisms.

Spawning Stimuli

Spring spawning and short-term brooding in Deertoe is unusual in the taxonomic tribe Lampsilini, most of which spawn in the summer or fall and brood until the following spring (long-term brooding) (Sietman et al., in press). Other examples of short-term brooding Lampsilini include Fawnsfoot (*Truncilla donaciformis*) and Neosho Mucket (*Lampsilis rafinesqueana*) (Utterback 1916; Shiver 2001). Early spring spawning is also characteristic of a number of other freshwater mussel taxa, including Margaritiferidae and some species of Amblemini, Pleurobemini, and Quadrulini. It appears possible that rising temperatures in the early spring may be an important spawning cue for many of these species (Haag 2012).

Spawning of Deertoe in response to temperature rise was first reported by Waller and Lasee (1997), who collected Deertoe from the upper Mississippi River (Navigation Pool 7; La Crosse County, WI) in November 1991 and held the mussels at 12°C for 6 weeks. The male mussels spawned when they were warmed to 17°C. Temperature-dependent spawning of Deertoe from the Sac River in mid-Missouri was also observed in 2015 by AL Maynard and MC Barnhart (Missouri State University, personal communication) after mussels were exposed to a temperature increase from 5°C to room

temperature (about 22°C). In the present study, male and female Deertoe both spawned at 13°C when exposed to a gradual temperature increase. This thermal trigger could be delayed for at least a year, as long as mussels were kept below at most 11°C.

Males and females synchronously spawned above 13°C, even when exposed to sperm at a lower temperature. This suggests that there is no utilization of a spermatozoon chemical to provoke male or female spawning, or that if such a chemical exists it does not provoke male or female spawning below the critical temperature. This also suggests that there is no male spawning competition in Deertoe, as males waited to spawn at or above 13°C even when other males' sperm was present in the water column. Males did not spawn prematurely in order to maximize fertilization chances. This was unexpected, as a coordinating chemical should promote higher fertilization probabilities, especially in situations when the time of temperature increase is variable or even premature to the season. Both sperm and eggs spawned by laboratory stimulation were viable. When males and females spawned within an aerated aquarium, viable glochidia developed, and juveniles metamorphosed when infected onto Freshwater Drum in the laboratory. These juveniles survived in laboratory culture.

Embryo abortion was very common in experimentation. Reduced dissolved oxygen was likely to blame. As temperatures increase, mussels respire more and increased respiration coupled with reduced respiratory ability when gravid likely led to the expulsion of the brood. This inference is supported by observations that no abortions occurred when aquaria were aerated. Reduced DO resulting from high bacterial breakdown under high sperm concentrations, probably also played a role.

Deertoe are host-specific with only one host: Freshwater Drum. It has been suggested that Pink Heelsplitter (*Potamilus alatus*), another Drum specialist, discharge their glochidia at a time that coincides with the onset of Drum spawning (Haggerty and Garner 2000). Freshwater Drum spawn between May and July after water temperatures have reached 18°C (Swedberg and Walburg 2011). Spawning is preceded by an upstream movement out of larger streams into smaller tributaries. The eggs drift downstream for one to two days before hatching (Pflieger 1997). Deertoe spawning, in 2016, based on the laboratory-determined critical temperature of 13°C, occurred approximately 5 weeks (16 April) before the Drum spawning should have begun at 18°C (23 May) (Figure 17). In short-term brooders, egg maturation into a glochidium takes between 2–6 weeks depending on species and temperature (e.g. Yokley 1975; Weaver et al. 1991, Garner et al. 1999). In the laboratory, Deertoe glochidia take approximately 2 weeks to mature and an additional 2–3 weeks to metamorphose (2017 personal communication MC Barnhart, Missouri State University; personal observation); additionally, females are able to brood their glochidia for several weeks before infecting a host (observations in the wild: 2017 personal communication BE Sietman, Minnesota Dept. of Natural Resources). This time frame places Deertoe with mature glochidia around the time that Freshwater Drum should begin migrating upstream to spawn. The coordination between individual mussels should produce higher fertilization; however, mussels typically experience very high fertilization rates (Zales and Neves 1982). It may be that a thermal trigger provides enough spawning synchronization that selection for intraspecific reproductive coordination is low or absent. The apparent critical spawning temperature of Deertoe seems to have evolved to match the onset of upstream migrations

of their host. Annual fluctuations of the time between mussel/Drum spawning temperatures may account for some of the differences between levels of successful recruitment between years.

Fecundity and Energy Investment in Gametes

The observed differences in reproductive investment by males and females may provide clues to reproductive strategy of Deertoe. Females invested 2.5 times more energy in gametes. Differences in the energy allocation between sexes suggests a divergence in life histories. If females experience greater mortality than males, perhaps as a result of attracting a predatory host, then there should be selective pressure for greater female reproductive success at younger ages and an increased energy allocation. Females were smaller than males (Tables 1 and 2) which could be the result of slower growth and/or higher rate of mortality. Higher rate of mortality is supported by the skewed sex ratio (smaller number of females) in the field sample. Higher rate of mortality is also supported by the lower variance in female size, which would result from a smaller number of old females. Further research into sex ratios, fecundity, and energy allocation of freshwater mussels is needed.

Spermatozeugmata Disassociation

In the laboratory, spermatozeugmata retained flagellar movement and spherical integrity for over 4 days in unaerated water at 22°C. Spermatozeugmata degraded more slowly when kept at lower temperatures. Free sperm, as well as spermatozeugmata were always present in milt. After one day, samples typically contained many dead free sperm

and many live spermatozeugmata, suggesting free sperm do not live as long as aggregated sperm. Spermatozeugmata, over time, did begin losing sperm and occasionally began losing their spherical shape, taking on a more asymmetrical appearance.

Sperm activation and spermatozeugmata dissociation can be triggered by the addition of sodium chloride (NaCl) or calcium chloride (CaCl₂). Sperm were also activated by the presence of fresh mussel eggs, which suggests that activation and dissociation are triggered by a chemical emitted from the eggs. This chemical may diffuse out of the egg only for a short time, explaining the lack of a reaction to older eggs. The interactions between mussel sperm and eggs are poorly understood. It has been suggested, however, that spermatozeugmata may be capable of chemotaxis, since spheres that are not completely covered with sperm usually only have sperm on one side and are capable of directional movement (Barnhart and Roberts 1997).

In the hydrozoan *Orthopyxis caliculata* (Order: Leptomedusae), the eggs secrete a chemotactic factor, which is only present in mature ova (Miller 1978). In sea urchins and some other echinoderms (Order: Echinoida), eggs emit chemotactic sperm-activating peptides (SAPs). These peptides are species-specific and bind to receptors in the sperm head, which activates the receptors' guanylyl cyclase (RGC) activity, activating latent intracellular cyclic guanosine monophosphate (cGMP), which then opens calcium channels in the plasma membrane (Ward et al. 1985). The influx of Ca²⁺ into the sperm activates motility by increasing mitochondrial ATP generation and activating dynein ATPase, thus stimulating flagellar movement (Hardy et al. 1994). The influx of Ca²⁺ through the plasma membrane of the tail allows the sperm to swim up the gradient

formed by the emitting SAPs, sensing the gradient to the egg with their tails (Ward et al. 1985). SAPs may function as both sperm-attracting and sperm-activating compounds in sea urchins.

Deertoe sperm increase flagellar beat and disassociate from the spermatzeugma when near freshly laid eggs. The presence of SAPs in freshwater mussels is unknown. However, manual activation by NaCl and CaCl₂, but not other chloride salts, suggest that the trigger mechanism involves Na⁺ and/or Ca²⁺ ions. If the eggs are secreting SAPs or similar substances, the response may involve cation channels. Somehow, exposure to these cations imitates the presence of eggs and elicits a response in the sperm. A possible environmental concern is that both NaCl and CaCl₂ are used as de-icer for roadways and could elicit a premature disassociation of spermatzeugmata (Table 3) (Helmenstine 2017; Prosser et al. 2017). Since Deertoe spawn just after winter melt, pollution by these chemicals in the form of runoff could pose a serious threat to mussel fertilization success.

How the sperm encounter the eggs is still a mystery. Sperm must be disassociated from the spermatzeugma to fertilize eggs, but it is not known when or where this dissociation occurs (O'Foighil 1989). Spermatzeugmata are apparently too large to pass through the gill ostia (Nelson and Allison 1940). Individual sperm might pass through the ostia if disassociation of the spermatzeugmata occurred in the mantle cavity, and if the ciliary filtration feeding mechanisms were stopped. The feeding/respiratory water current into the gills presents another issue. If the feeding/respiratory current is operating, any chemical emitting from the eggs would be carried out of the water tubes and the excurrent aperture, away from the mantle cavity where the spermatzeugmata would accumulate. The chemical signals would not reach the spermatzeugmata, and in

present observations the spermatozeugmata do not dissociate by simply coming into contact with the gills (Figure 16).

If female mussels somehow fully reversed the flow of water in the ctenidia, spermatozeugmata would be brought in through the excurrent aperture, thus avoiding the filtration feeding mechanisms all together. This reversal would also aid the deposition of eggs into the marsupia, which would otherwise have to proceed against the flow of water. The genital pores are in the anterior end of the suprabranchial chambers. Latter (1891) and Lefevre and Curtis (1912) state that the eggs are apparently transported by cilia from the genital pore to the cloaca. Then their movement is reversed and they backtrack anteriorly into the marsupia by short gulps created by first closing all the ventral edges of the mantle and leaving only the siphons open, then relaxing the adductor muscles, allowing the ligament to gape the valves apart. The hydrostatic pressure outside the shell would be greater than that inside and this vacuum effect could cause water to rush inside through the siphons and could move the eggs forward and into the water tubes. This process has received little further study, however, and seems inefficient for collection of sperm. When Deertoe open their valves, the separation where the apertures protrude does not spread as drastically as the rest of the margin. It would seem that a technique of 'gulps' could provide brief intakes of water through the incurrent aperture if the excurrent aperture and mantle edge were closed.

Another possibility is that female spawning might involve a complete reversal of ciliary flow. By bringing water in through the traditionally excurrent aperture would readily expose the spermatozeugmata to eggs and deliver fertilized ova to the marsupia.

During regular filtering, it is also conceivable that since spermatozeugmata seem to bounce off the gill due to ciliary movement that they would accumulate within the mantle cavity of a filtering female (a possible form of short-term sperm storage). The female could accumulate spermatozeugmata in the mantle cavity during spawning, then periodically pause the feeding current. During these pauses, hypothetically the chemical cue emitting from the eggs would diffuse out of the gill and activate the sperm. Sperm, could enter the ostia and fertilize eggs. The actual mechanism remains elusive, but is apparently efficient, because mussels typically have very high fertilization success (Zales and Neves 1982). More studies need to be done to fully understand the process of fertilization in freshwater mussels.

REFERENCES

- Amyot JP, Downing JA. 1998. Locomotion in *Elliptio complanata* (Mollusca: Unionidae): a reproductive function? *Freshwater Biology* 39:351-358.
- Anthony JL, Downing JA. 2001. Exploitation trajectory of a declining fauna: a century of freshwater mussel fisheries in North America. *Canadian Journal of Fisheries and Aquatic Sciences* 58:2071-2090.
- Arey LB. 1932. The formation and structure of the glochidial cyst. *Biological Bulletin* 62:212-221.
- Barnhart MC, Haag WR, Rosten WN. 2008. Adaptations to host infection and larval parasitism in Unionoida. *Journal of North American Benthological Society* 27:370-394.
- Barnhart MC, Roberts A. 1997. Reproduction and fish hosts of Unionids from the Ozark Uplifts. Pages 14-20 in KS Cummings et al. editors. *Conservation and management of freshwater mussels II: Initiatives for the future*. Upper Mississippi Conservation Committee, Rock Island, Illinois, USA.
- Bates JM. 1962. The impact of impoundment on mussel fauna of Kentucky Reservoir, Tennessee River. *The American Midland Naturalist* 68:232-236.
- Bishop JDD, Pemberton AJ. 2006. The third way: spermcast mating in sessile marine invertebrates. *Integrative and Comparative Biology* 46:398-406.
- Brim-Box J, Howard J, Wolf D, O'Brien C, Nez D, Close D. 2006. Freshwater mussels (Bivalvia: Unionidae) of the Umatilla and Middle Fork John Day rivers in eastern Oregon. *Northwest Science* 80:95-107.
- Coe WR. 1931. Spermatogenesis in the California Oyster (*Ostrea lurida*). *Biological Bulletin* 6:309-315.
- Coker RE. 1919. Fresh-water mussels and mussel industries of the United States. *Bulletin of the Bureau of Fisheries* 36:15-17.
- Cummings KS, Grad DL. 2015. Class Bivalvia. Pages 423-506 in JH Thorp and DC Rogers editors. *Ecology and General Biology: Thorp and Covich's Freshwater Invertebrates*. Academic Press. London, UK.

- Dillon RT Jr. 2000. *The Ecology of Freshwater Molluscs*. Cambridge University Press. New York, NY.
- Falese LE, Russell MP, Dollahon NR. 2011. Spermcasting of spermatozeugmata by the bivalves *Nutricula confuse* and *N. tantilla*. *Invertebrate Biology* 130:334-343.
- Focarelli R, Rosa D, Rosati F. 1990. Differentiation of the vitelline coat and the polarized site of sperm entrance in the egg of *Unio elongatulus* (Mollusca, Bivalvia). *Reproductive Biology* 254:88-96.
- Fuller SLH. 1978. Fresh-water mussels (Mollusca; Bivalvia: Unionidae) of the Upper Mississippi River: observations at selected sites within the 9-foot channel navigation project on behalf of the U.S. Army. Philadelphia, PA: The Academy of Natural Sciences of Philadelphia Division of Limnology and Ecology. Biosurvey Monitored by Army Engineer District, St. Paul and Rock Island.
- Garner JT, Haggerty TM, Modlin RF. 1999. Reproductive cycle of *Quadrula metanevra* (Bivalvia: Unionidae) in the Pickwick Dam Tailwater of the Tennessee River. *The American Midland Naturalist* 141:277-283.
- Haag WR. 2012. *North American Freshwater Mussels*. New York, NY: Cambridge University Press.
- Haag WR. 2013. The role of fecundity and reproductive effort in defining life-history strategies of North American freshwater mussels. *Biological Reviews* 88:745-766.
- Haag WR, Rypel AL. 2010. Growth and longevity in freshwater mussels: evolutionary and conservation implications. *Biological Reviews* 86:225-247.
- Haag WR, Warren ML. 1997. Host fishes and reproductive biology of 6 freshwater mussel species from the Mobile Basin, USA. *Journal of North American Benthological Society* 16:576-585.
- Haggerty TM, Garner JT. 2000. Seasonal timing of gametogenesis, spawning, brooding, and glochidial discharge in *Potamilus alatus* (Bivalvia: Unionidae) in Wheeler Reservoir, Tennessee River, Alabama, USA. *Invertebrate Reproduction & Development* 38:35-41.

- Haggerty TM, Garner JT, Crews AE, and Kawamura R. 2011. Reproductive seasonality and annual fecundity in *Arcidens confragosus* (Unionidae: Unioninae: Anodontini) from Tennessee River, Alabama, USA. *Invertebrate Reproduction & Development* 55:230-235.
- Hardy DM, Harumi T, Garbers DL. 1994. Sea Urchin sperm receptors for egg peptides. *Seminars in Developmental Biology* 5:217-224.
- Haszprunar G. 1987. The fine morphology of the osphradial sense organs of the Mollusca III. Placophora and Bivalvia. *Philosophical Transactions of the Royal Society of London* 315(1169):37-61.
- Helmenstine AM. 2017. What is road salt? Chemical composition of road salt and how it works. *ThoughtCo*. 13 April 2017. <<https://www.thoughtco.com/chemical-composition-of-road-salt-609168>>.
- Howard AD. 1951. A river mussel parasitic on a salamander. *Natural History Miscellaneous* 77:1-6.
- Howard JK, Cuffey KM. 2006. The functional role of native freshwater mussels in the fluvial benthic environment. *Freshwater Biology* 51:460-474.
- Hua D, Neves RJ. 2007. Captive survival and pearl culture potential of the pink heelsplitter (*Potamilus alatus* Say, 1817) (Bivalvia: Unionidae). *North American Journal of Aquaculture* 69:147-158.
- Ishibashi R, Komaru A, Kondo T. 2000. Sperm sphere in Unionid mussels (Bivalvia: Unionidae). *Zoological Science* 17:947-950.
- Kat PW. 1984. Parasitism in the Unionacea (Bivalvia). *Biological Reviews* 59:189-207.
- Kraemer LR. 1981. The osphradial complex of two freshwater bivalves: histological evaluation and functional context. *Malacologia* 20:205-216.
- Latter OH. 1891. Notes on Anodon and Unio. *Proceedings of the Zoological Society of London*. 1891:52-59.
- Lefevre G, Curtis WC. 1912. Studies on the reproduction and artificial propagation of fresh-water mussels. *Bulletin of the Bureau of Fisheries* 30:105-201.

- Lima P, Monteiro SM, Sousa M, MacHado J. 2012. A histological study of oogenesis in the freshwater mussel *Anodonta cygnea* (Linnaeus, 1758) in Mira Lagoon, Portugal. *Malacologia* 55:251-261.
- Lydeard C, Cowie RH, Ponder WF, Bogan AE, Bouchet P, Clark SA, Cummings KS, Frest TJ, Gargominy O, Herbert DG, Hershler R, Perez KE, Roth B, Seddon M, Strong EE, Thompson FG. 2004. The global decline of nonmarine mollusks. *BioScience* 54:321-330.
- Lynn JW. 1994. The ultrastructure of the sperm and motile spermatozeugmata released from the freshwater mussel *Anodonta grandis* (Mollusca, Bivalvia, Unionidae). *Canadian Journal of Zoology* 72:1452-1461.
- Mackie GL. 1984. Bivalves. Pages 351-418 in Tompa AS, Verdonk NH, van den Biggelaar. Editors. *The Mollusca*. Academic Press, Orlando, FL, USA.
- Master LL, Stein BA, Kutnere LS, Hammerson GA. 2000. Vanishing assets: Conservation status of U.S. species. Pages 93-118 in Stein BA, Kutnere LS, Adams JS (editors). *Precious heritage: the status and biodiversity in the United States*. Oxford University Press, New York.
- Matteson MR. 1948. Life history of *Elliptio complanatus* (Dillwyn, 1817). *The American Midland Naturalist* 40(3):690-723.
- Maynard AL. 2015. Sex-associated mitochondrial genes: tools for understanding unionid reproduction and development. Master's thesis: Missouri State University, Springfield, MO.
- Medler S, Silverman H. 2001. Muscular alteration of gill geometry *in vitro*: implications for bivalve pumping processes. *The Biological Bulletin* 200:77-86.
- Miller RL. 1978. Site-specific agglutination and the timed release of a sperm chemo-attractant by the egg of the leptomedusan, *Orthopyxis caliculata*. *Journal of Experimental Zoology* 205:385-391.
- Nalepa TF, Gardner WS, Malczyk JM. 1991. Phosphorus cycling by mussels (Unionidae: Bivalvia) in Lake St. Clair. *Hydrobiologia* 219:239-250.

- Nelson TC, Allison JB. 1940. On the nature and action of diantlin: a new hormone-like substance carried by the spermatozoa of the oyster. *Journal of Experimental Zoology* 85:299-338.
- Neves RJ. 1999. Conservation and commerce: management of freshwater mussel (Bivalvia: Unionoidea) resources in the United States. *Malacologia* 41:461-474.
- Newton TJ, Zigler SJ, Rogala JT, Gray BR, Davis M. 2011. Population assessment and potential functional roles of native mussels in the Upper Mississippi River. *Aquatic Conservation: Marine and Freshwater Ecosystems* 21:122-131.
- O'Brien CA, Brim-Box J. 1999. Reproductive biology and juvenile recruitment of the shinyrayed pocketbook, *Lampsilis subangulata* (Bivalvia: Unionidae) in the Gulf Coast Plain. *The American Midland Naturalist* 142:129-140.
- O'Foighil DÓ. 1989. Role of spermatozeugmata in the spawning ecology of the brooding oyster *Ostrea edulis*. *Gamete Research* 24:219-228.
- Oesch RD. 1995. Missouri Naiades: a Guide to the Mussels of Missouri. Missouri Department of Conservation. Jefferson City, MO.
- Ortmann AE. 1911. The anatomical structure of certain exotic naiades compared with that of the North American forms. *The Nautilus* 24:103-108.
- Parmalee PW, Klippel WE. 1974. Freshwater mussels as a prehistoric food resource. *American Antiquity* 39:421-434.
- Peck RH. 1877. The minute structure of the gills of lamellibranch Mollusca. *Quarterly Journal of Microscopical Science* 17:43.
- Pflieger, W.L. 1997. The Fishes of Missouri. Missouri Department of Conservation. Jefferson City, MO.
- Pletta M. 2013. Particle capture by freshwater bivalves: implications for feeding ecology and biopesticide delivery. Master's thesis: Missouri State University, Springfield, MO.
- Poole KE, Downing JA. 2004. Relationship of declining mussel biodiversity to stream-reach and watershed characteristics in an agricultural landscape. *Journal of North American Benthological Society* 33:114-125.

- Prosser RS, Rochfort Q, McInnis R, Exall K, Gillis PL. 2017. Assessing the toxicity and risk of salt-impacted winter road runoff to the early life stages of freshwater mussels in the Canadian province of Ontario. *Environmental Pollution* 230:589-597.
- Ram JL, Fong PP, Garton DW. 1996. Physiological aspects of zebra mussel reproduction: maturation, spawning, and fertilization. *American Zoologist* 36:326-338.
- Richter BD, Braun DP, Mendelson MA, Master LL. 1997. Threats to imperiled freshwater fauna. *Conservation Biology* 11:1081-1093.
- Sereflisan H, Cek S, Sereflisan M. 2009. Histological studies on gametogenesis, hermaphroditism, and the gametogenic cycle of *Anodonta gabillotia pseudodopsis* (Locard, 1883) in the Lake Golbasi, Turkey (Bivalvia: Unionidae). *Journal of Shellfish Research* 28:337-344.
- Shepardson SP, Heard WH, Breton S, Hoeh WR. 2012. Light and transmission electron microscopy of two spermatogenic pathways and unimorphic spermatozoa in *Venustaconcha ellipsiformis* (Conrad 1836) (Bivalvia: Unionoida). *Malacologia* 55:263-284.
- Shiver, Melissa. 2001. Reproductive biology of the Neosho mucket, *Lampsilis rafinesqueana*. Master's thesis: Missouri State University, Springfield, MO.
- Sietman BE, Hove MC, Davis JM. Host attraction, brooding phenology, and host specialization on freshwater drum by 4 freshwater mussel species. *Freshwater Science* (in press).
- Silverman H, Lynn JW, Achberger EC, Dietz TH. 1996. Gill structure in zebra mussels: bacterial-sized filtration. *American Zoology* 36:373-384.
- Sokolov VA, Kamardin NN. 1977. Osphradial chemoreception in lamellibranch mollusks, *Unio pectorum* and *Anodonta cygnea*. *Journal of Evolutionary Biochemistry and Physiology* 18:56-61.
- Sprung M, Rose U. 1988. Influence of food size and food quality on the feeding of the mussel *Dreissena polymorpha*. *Oecologia* 77:526-532.
- Strayer DL. 1981. Notes on the microhabitats of unionid mussels in some Michigan streams. *The American Midland Naturalist* 106:411-415.

- Suloway L. 1981. The unionid (Mollusca: Bivalvia) fauna of the Kankakee River in Illinois. *The American Midland Naturalist* 105:233-239.
- Swedberg DV, Walburg CH. 2011. Spawning and early life history of the freshwater drum in Lewis and Clark Lake, Missouri River. *Transactions of the American Fisheries Society* 99:560-570.
- Tankersley RA, Dimock RV Jr. 1992. Quantitative analysis of the structure and function of the marsupial gills of the freshwater mussel *Anodonta cataracta*. *Biological Bulletin* 182:145-154.
- Utterback, W. I. 1916. The Naiades of Missouri. VI. *The American Midland Naturalist* 4(8): 339-354.
- Utterback WI. 1931. Sex behavior among Naiades. *Proceedings of the West Virginia Academy of Science* 5:43-45.
- Vannote RL, Minshall GW. 1982. Fluvial processes and local lithology controlling abundance, structure, and composition of mussel beds. *Proceedings of the National Academic Sciences* 79:4103-4107.
- Vaughn CC, Taylor CM. 1999. Impoundments and the decline of freshwater mussels: a case study of an extinction gradient. *Conservation Biology* 13(4):912-920.
- Waller DL, Lasee BA. 1997. External morphology of spermatozoa and spermatozeugmata of the freshwater mussel *Truncilla truncata* (Mollusca: Bivalvia: Unionidae). *The American Midland Naturalist* 138:220-223.
- Ward GE, Brokaw CJ, Garbers DL, Vacquier VD. 1985. Chemotaxis of *Arbacia punctulata* spermatozoa to resact, a peptide from the egg jelly layer. *The Journal of Cell Biology* 101:2324-2329.
- Watters GT, O'Dee SH. 1998. Metamorphosis of freshwater mussels glochidia (Bivalvia: Unionidae) on amphibians and exotic fishes. *The American Midland Naturalist* 139:49-57.
- Weaver LR, Pardue GB, Neves RJ. 1991. Reproductive biology and fish hosts of Tennessee Clubshell *Pleurobema oviforme* (Mollusca: Unionidae) in Virginia. *The American Midland Naturalist* 126:82-89.

- Williams JD, Fuller SLH, Grace R. 1992. Effects of impoundments on freshwater mussels (Mollusca: Bivalvia: Unionidae) in the main channel of the Black Warrior and Tombigbee Rivers in western Alabama. *Bulletin Alabama Museum of Natural History* 13:1-10.
- Williams JD, Warren ML Jr, Cummings KS, Harris JL, Neves RJ. 1993. Conservation status of freshwater mussels of the United States and Canada. *Fisheries* 18:6-22.
- Zales AV, Neves RJ. 1982. Reproductive biology of four freshwater mussel species (Mollusca: Unionidae) in Virginia. *Freshwater Invertebrate Biology* 1:17-28.
- Zanatta DT, Murphy RW. 2006. Evolution of active host-attraction strategies in the freshwater mussel tribe Lampsilini (Bivalvia: Unionidae). *Molecular and Phylogenetic and Evolution* 41:195-208.

Table 1. Fecundity and energy investment of males. Rows represent individual mussels. Whole mass (WM) refers to the whole wet mass of individual mussels including shell and tissue. SZ represents spermatozoeugmata. Number of sperm was approximated using an estimate of 8500 sperm per spermatozoeugmata (Waller and Lasee 1997). Energy content was estimated by multiplying the number of spermatozoeugmata by caloric content per spermatozoeugma. Data in last row are mean \pm standard deviation (N).

Whole Mass (g)	Number of SZ	SZ per gram WM	cal per gram WM
1.86	666 667	358 423	52.15
2.12	666 667	314 466	53.85
5.54	45 333 633	8 182 966	179.54
12.66	19 669 667	1 553 686	15.44
13.42	31 333 933	2 334 868	14.20
15.29	42 666 667	2 790 495	23.82
16.29	27 333 633	1 677 939	36.81
20.27	22 000 900	1 085 392	51.23
22.86	54 333 633	2 376 799	34.09
24.40	22 666 667	928 962	0.67
26.29	43 666 667	1 660 961	20.38
28.93	71 002 100	2 454 272	7.86
31.27	22 003 900	703 674	6.90
57.24	37 033 900	646 993	61.22
66.18	2 007 800	30 338	35.60
22.97 \pm 18.23 (15)	29 492 429 \pm 20 183 107 (15)	1 806 682 \pm 1 965 448 (15)	39.58 \pm 43.13 (15)

Table 2. Fecundity and energy investment of females. Rows represent individual mussels. Whole mass (WM) refers to the whole wet mass of individual mussels including shell and tissue. Number of eggs represents the estimated number of eggs released from each spawning female. Energy content was estimated by multiplying the number of eggs by the caloric content per egg. Data in last row are mean \pm standard deviation (N).

Whole Mass (g)	Number of Eggs	Eggs per gram WM	cal per gram WM
7.75	165 000	21 290	51.97
8.12	252 000	31 034	68.47
8.54	306 000	35 831	60.35
10.31	492 000	47 721	65.78
10.12	207 000	20 455	1.44
10.32	699 000	67 733	99.80
11.19	210 000	18 767	115.23
12.81	207 000	16 159	67.82
13.36	6 000	449	44.72
14.51	306 000	21 089	153.47
16.23	603 000	37 153	54.94
20.36	993 000	48 772	119.48
25.89	360 000	13 905	57.92
30.03	513 000	17 083	217.82
32.02	2 127 000	66 427	156.85
36.48	657 000	18 010	213.63
16.75 \pm 9.34 (16)	540 000 \pm 497 417 (16)	32 095 \pm 18 069 (16)	96.86 \pm 61.64 (16)

Table 3. Triggers of disassociation of spermatozeugmata to common laboratory compounds, solutions, and female reproductive substances. Each trial consisted of adding the stimulus compound to a drop of spermatozeugmata suspension and observing the result under a compound microscope. Several males were used. Reaction and number of attempts is included.

Stimulus	Response	Number of trials
<i>Compounds and Solutions</i>		
Sodium Chloride (NaCl)	Dissociated	>10
Confectioner Sugar	None	5
Magnesium Chloride (MgCl)	None	5
Potassium Chloride (KCl)	None	5
Calcium Chloride (CaCl ₂)	Dissociated	5
Ammonium Chloride (NH ₄ Cl)	None	3
Borax (Na ₂ B ₄ O ₇)	None	3
Calcium Sulfate (CaSO ₄)	None	3
Calcium Hydroxide (Ca(OH) ₂)	None	3
Calcium Carbonate (CaCO ₃)	None	3
Sodium Bicarbonate (NaHCO ₃)	None	3
Sodium Fluoride (NaF)	None	3
Citric Acid (C ₆ H ₈ O ₇)	None	3
Phosphate Buffer pH 4	None	3
Phosphate Buffer pH 7	None	3
Phosphate Buffer pH 10	None	3
DI water	None	>10
<i>Female Reproductive Substances</i>		
Aborted unfertilized eggs	Dissociated	5
Water with female	None	5
Washed unfertilized eggs	Dissociated	2
Water from egg wash	Dissociated	2
Non-marsupial gill	None	3
Empty marsupial gill	None	3
Fresh unfertilized eggs from gills	Dissociated	5
3-day old unfertilized brooded eggs	None	3
3-day old unfertilized aborted eggs	None	4
Eggs from gonad	None	5
Suprabranchial fluid	None	3

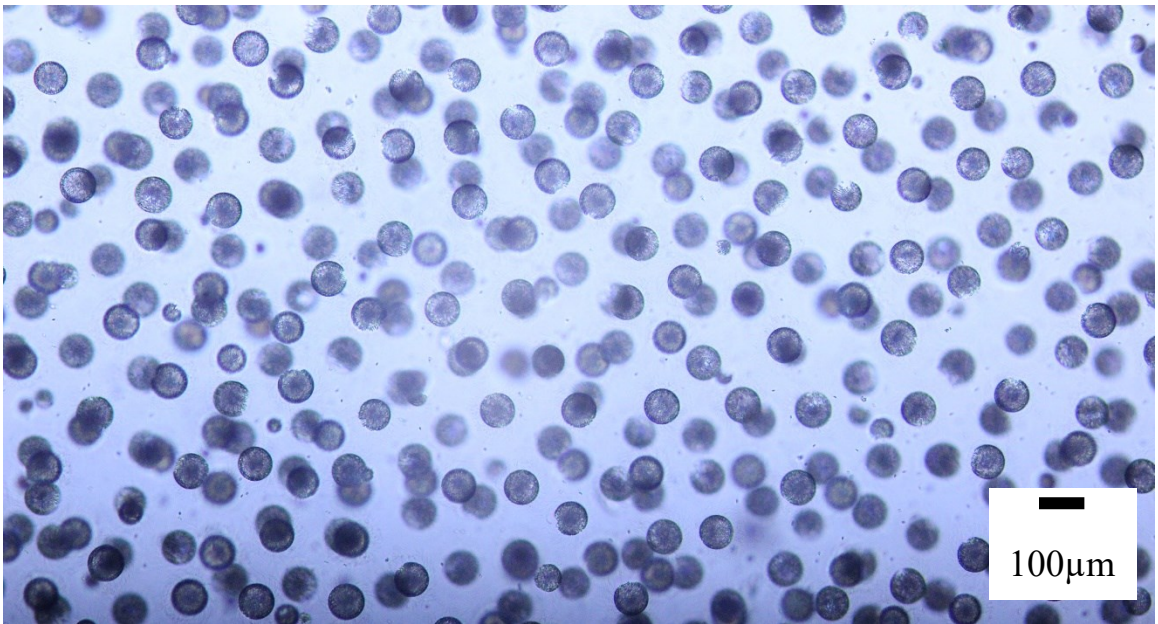


Figure 1. Freshly spawned spermatozeugmata of *Truncilla truncata*



Figure 2. Male *Truncilla truncata* spawning. Note that spermatozeugmata are denser than water.



Figure 3. Apertures of a female *Truncilla truncata*. The incurrent aperture is on the right and the excurrent aperture is on the left.



Figure 4. *Truncilla truncata* used in this study showed high color polymorphism, as well as an array of sizes and ages.

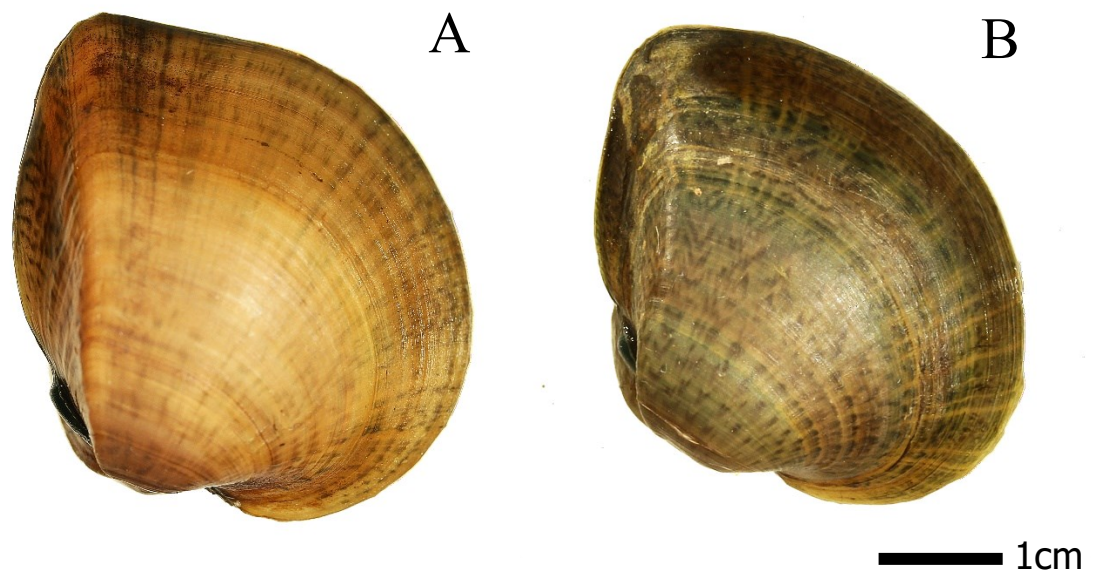


Figure 5. Shell dimorphism in *Truncilla truncata*. Male shells (A) typically possess a more defined posterior ridge, a slightly concave margin, and a sulcus. Female shells (B) typically possess a less defined posterior ridge, a neutral or slightly convex margin, and no sulcus.

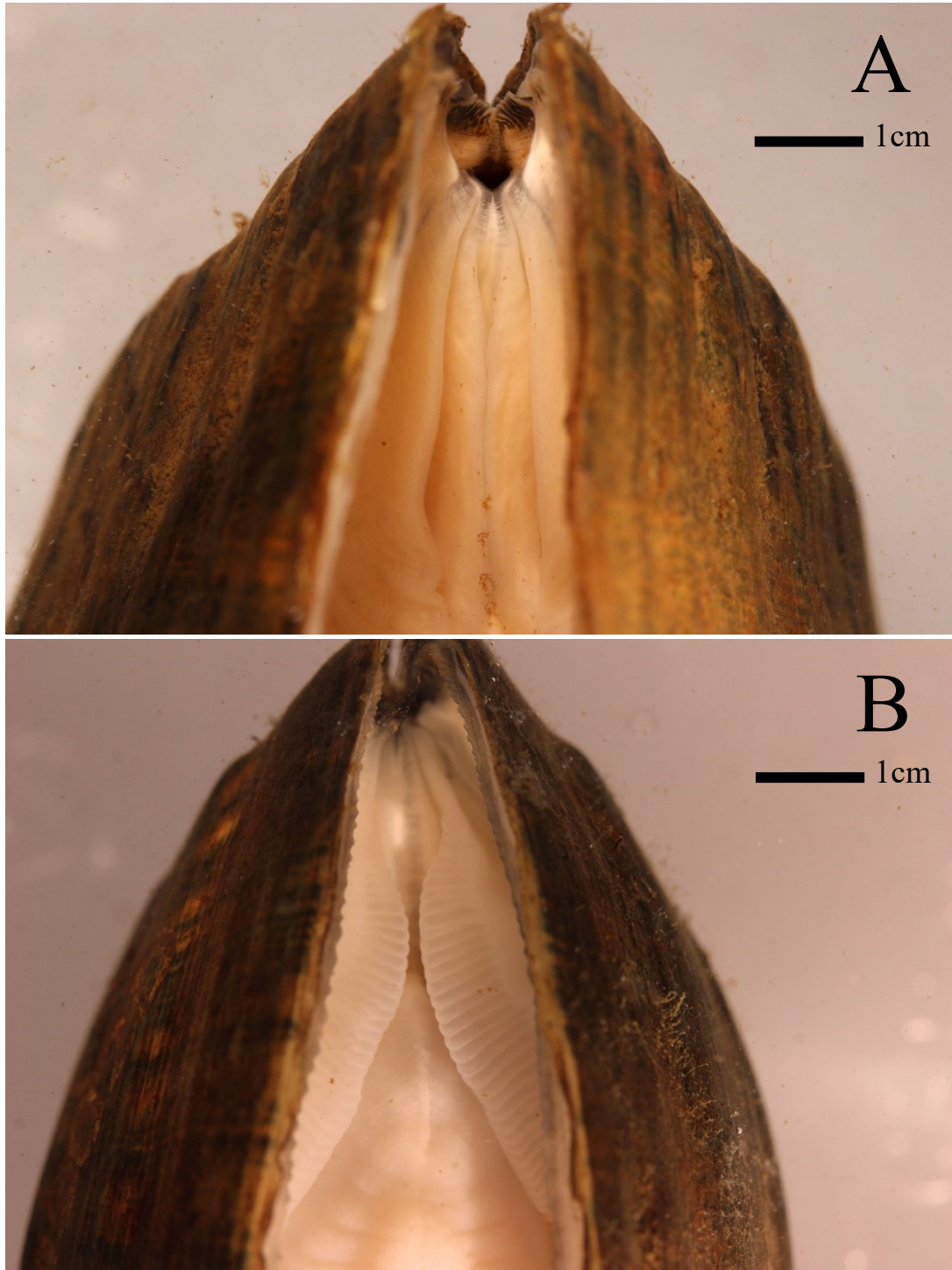


Figure 6. Gill dimorphism in *Truncilla truncata*. The outer demibranchs of males (A) are morphologically identical to the inner demibranchs, while the outer demibranchs of females (B) are highly inflated and extended in order to serve as marsupia. This female is not gravid.

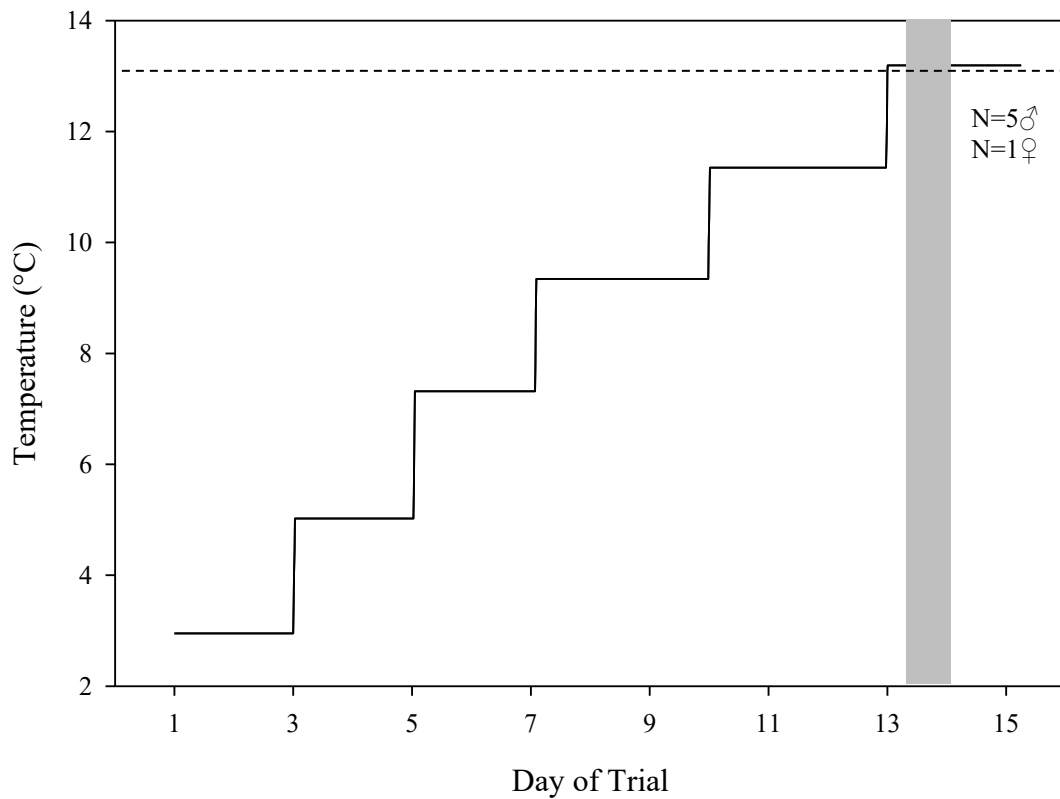


Figure 7. Identification of critical spawning temperature by gradual temperature increase. The range of time for observed spawning is indicated by the shaded band. The dotted line shows 13°C. The number and sex of individuals in the experiment are indicated. Day 1 was 21 April 2016 (2 weeks after collection)

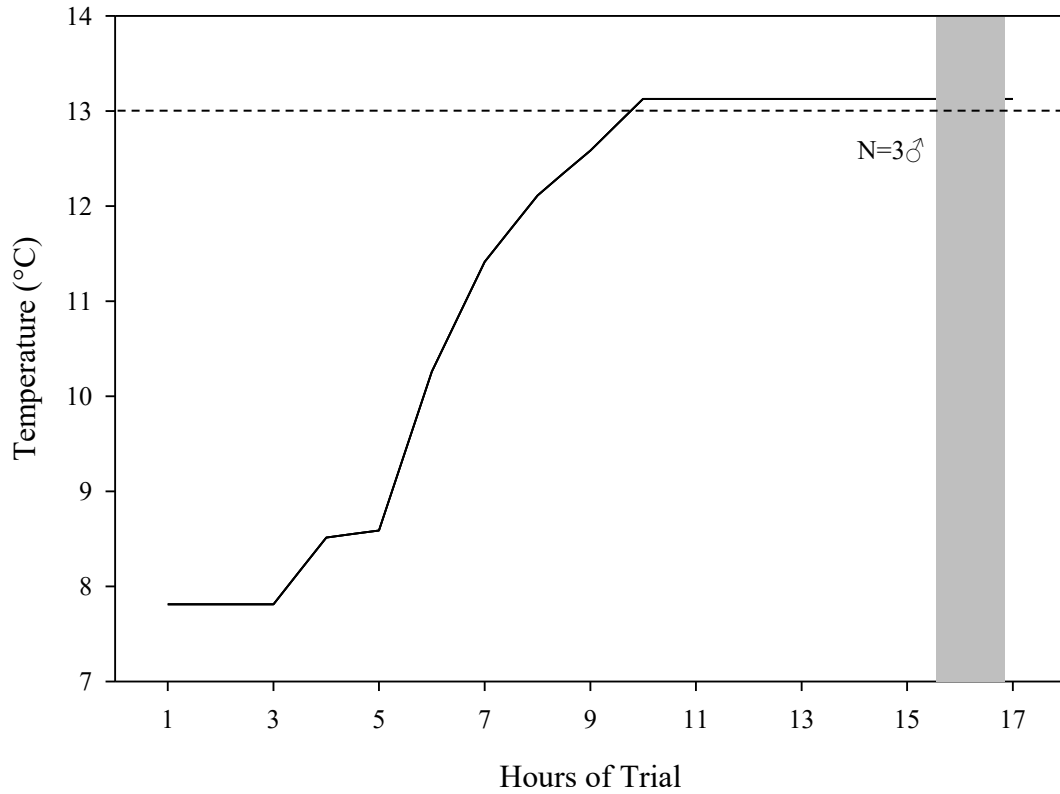


Figure 8. Testing of critical spawning temperature after extended winter. The range of time for observed spawning is indicated by the shaded band. The dotted line shows 13°C. The number and sex of individuals in the experiment are indicated. Experiment was conducted on 23 May 2017 (50 weeks after collection)

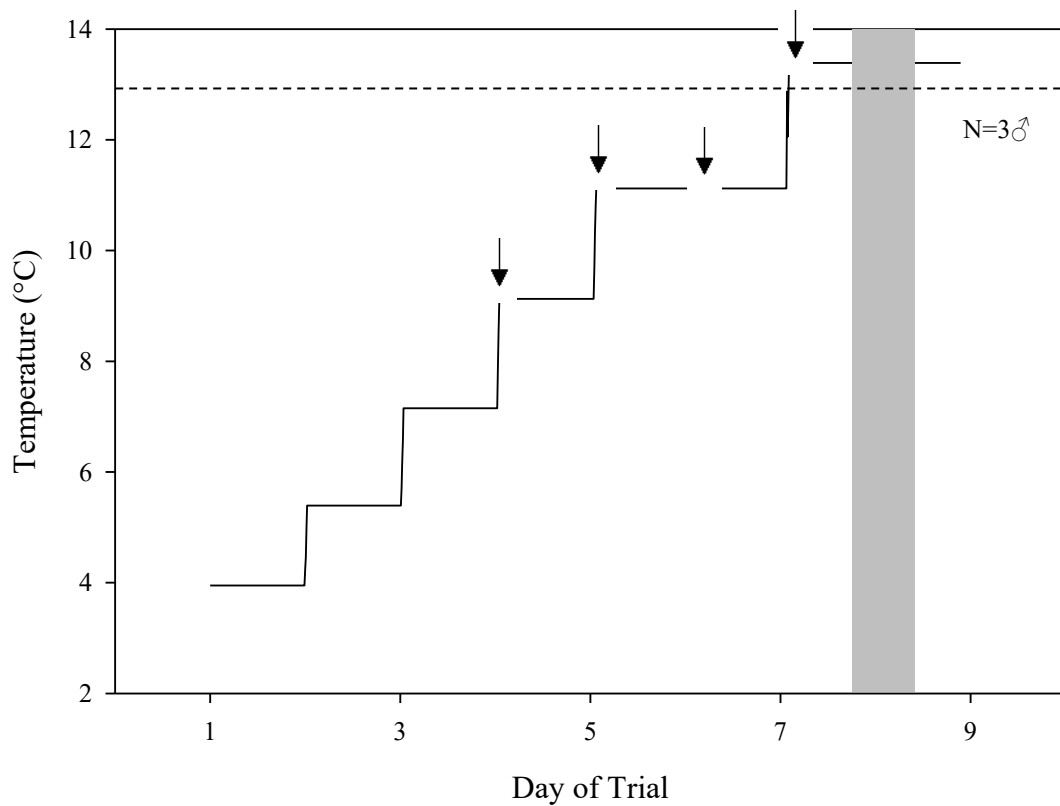


Figure 9. Testing of male critical spawning temperature by gradual temperature increase in the presence of spermatozoa. Sperm exposures are indicated with black arrows. The range of time for observed spawning is indicated by the shaded area. The dotted line shows 13°C. The number and sex of individuals in the experiment are indicated. Day 1 was 11 May 2016 (5 weeks after collection)

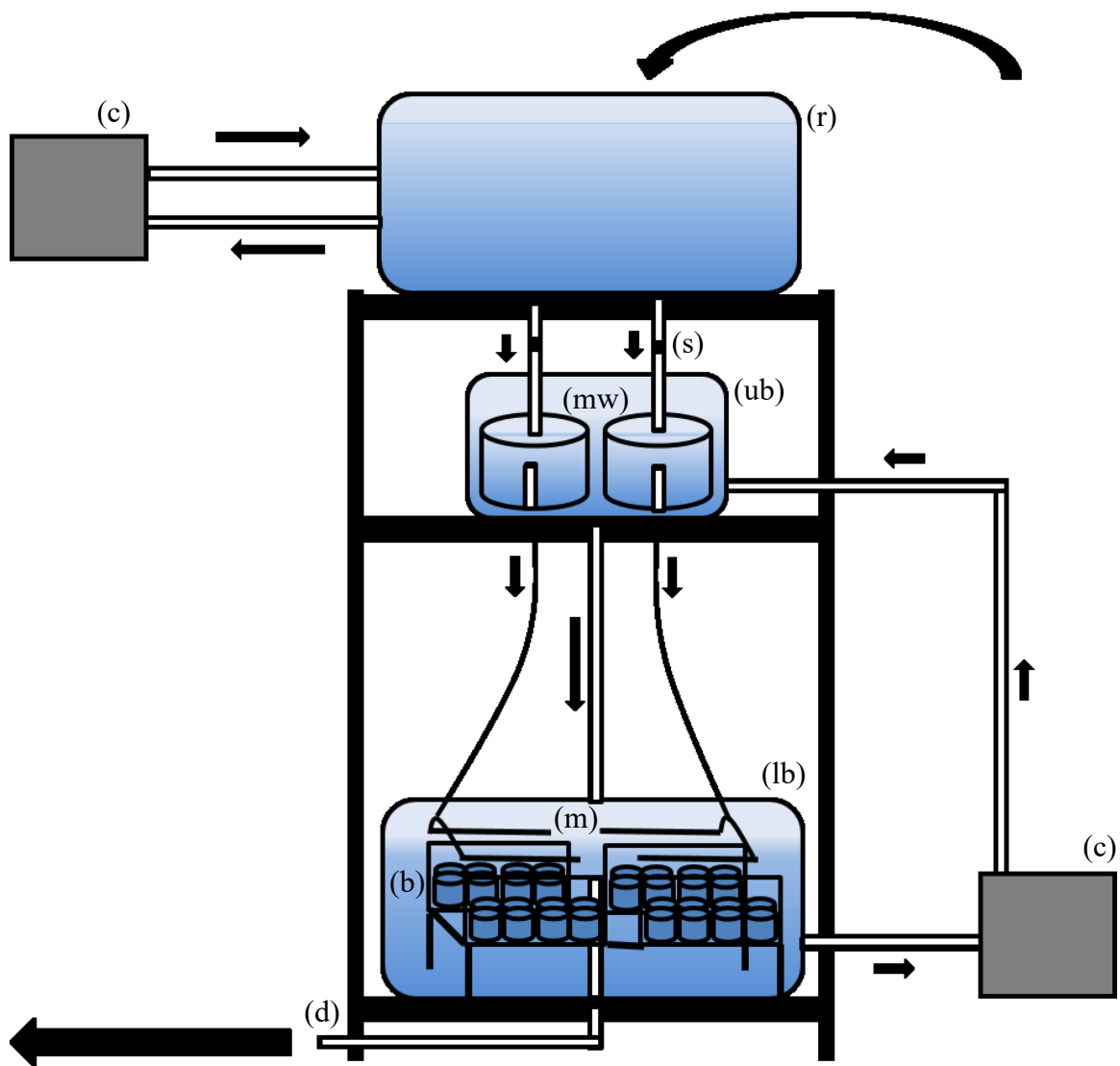


Figure 10. Diagram of gravity-fed system for sperm delivery. Arrows indicate direction of water flow. The entire system is temperature controlled by chillers (c). Water being added to the reservoir (r) was deposited via solenoids (s) into the mixing wells (mw), held inside the upper water bath (ub). Water in the mixing wells and water in the upper water bath could not mix. Water exited the mixing wells through stand-pipes and was fed by rubber tubing to manifolds (m) and into beakers (b), each holding an individual animal. Beakers would overflow into the lower water bath (lb), which was rid of excess water by a stand-pipe leading to the drain (d). Water level in the water baths remained below the rim of mixing wells and beakers to prevent contamination. Sperm could be added to one mixing well and not the other, allowing for exposure of some animals without exposing others.

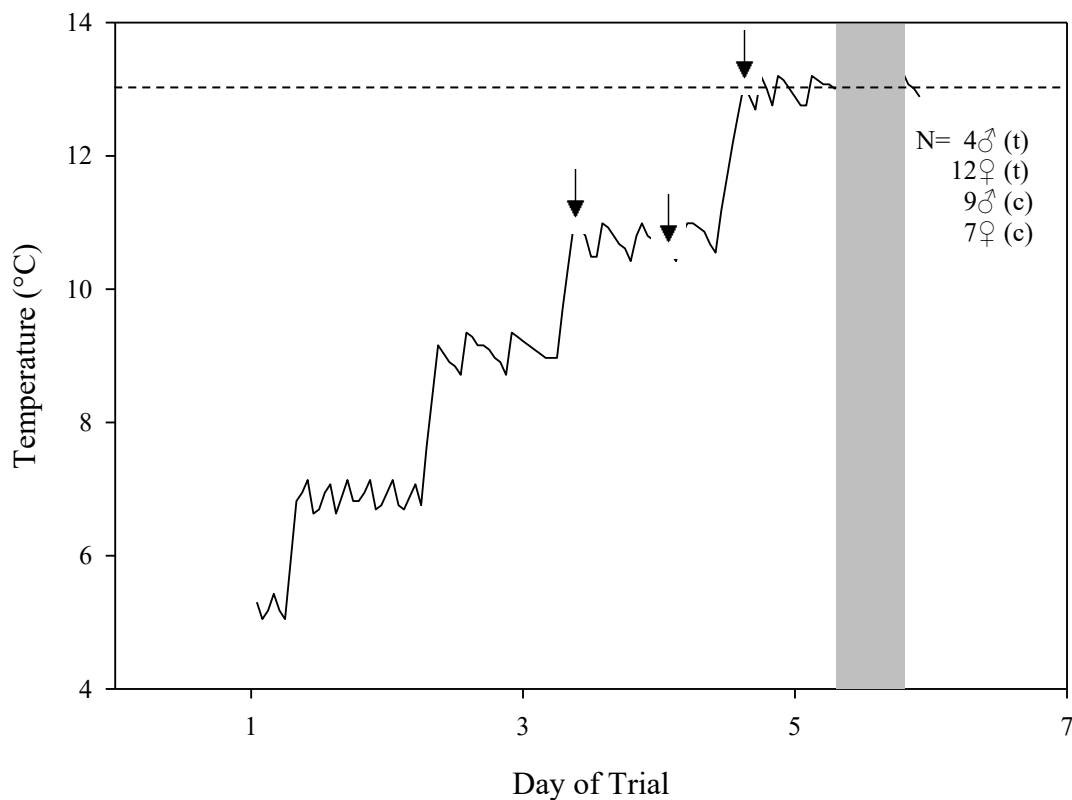


Figure 11. Testing of male and female critical spawning temperatures by gradual temperature increase in the presence of spermatozoa. Figure represents treatment (t) group and control (c) group. Sperm exposures to the treatment group are indicated with arrows. Control group was not exposed to sperm at any time during the experiment. The range of time for observed spawning for both groups is indicated by the shaded band. The dotted line shows 13°C. The number and sex of individuals in the experiment are indicated. Day 1 was 18 May 2016 (6 weeks after collection)

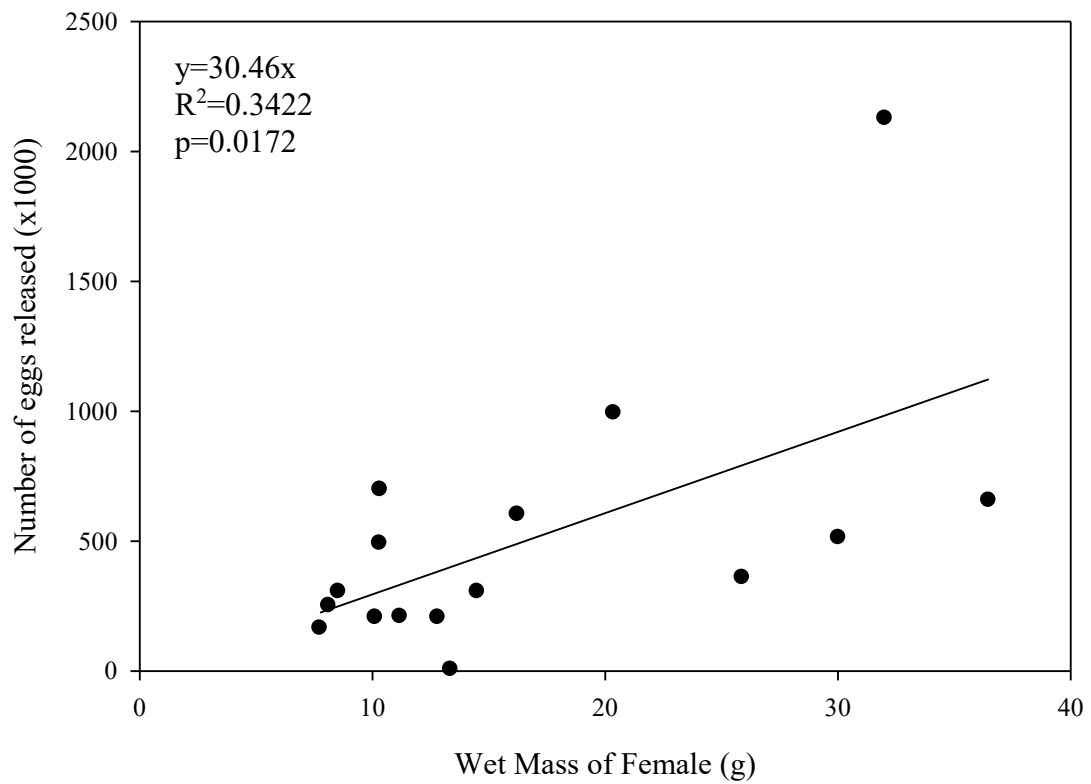


Figure 12. Mass of females as a predictor of number of eggs released. Line of regression is shown.

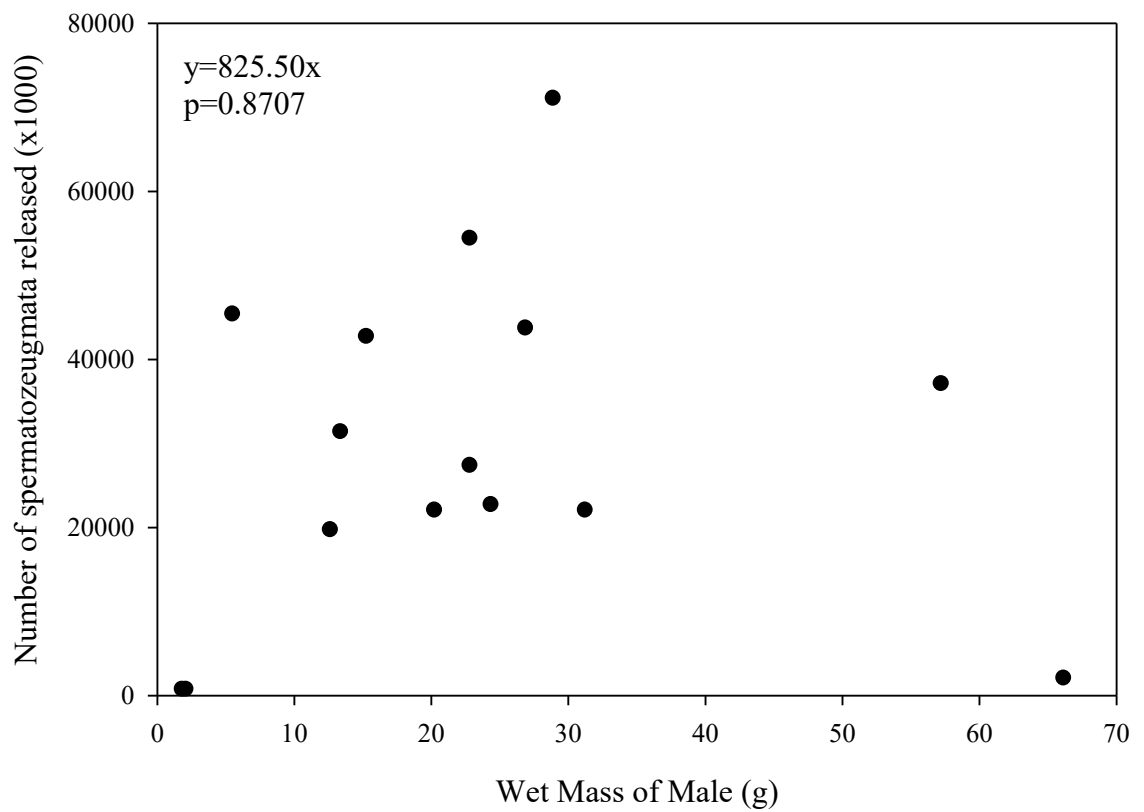


Figure 13. Mass of males as a predictor of the number of spermatozeugmata released. Regression was attempted and not significant.

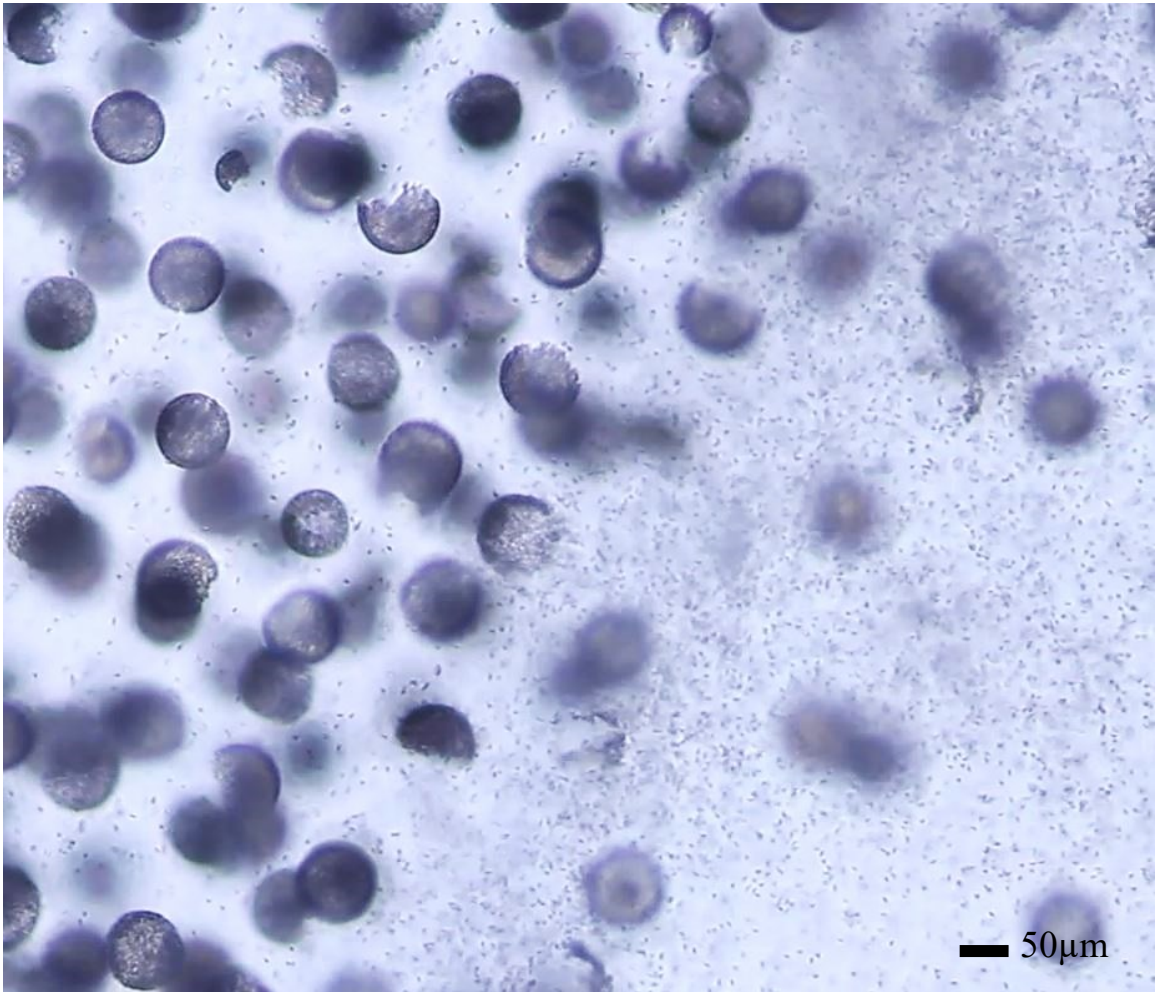


Figure 14. Spermatozeugmata disassociating from exposure to sodium chloride. The sodium chloride solution is diffusing into the frame from the lower right.



Figure 15. Freshly released spermatozeugma (center) not reacting to unfertilized eggs that had been extracted from the gonad

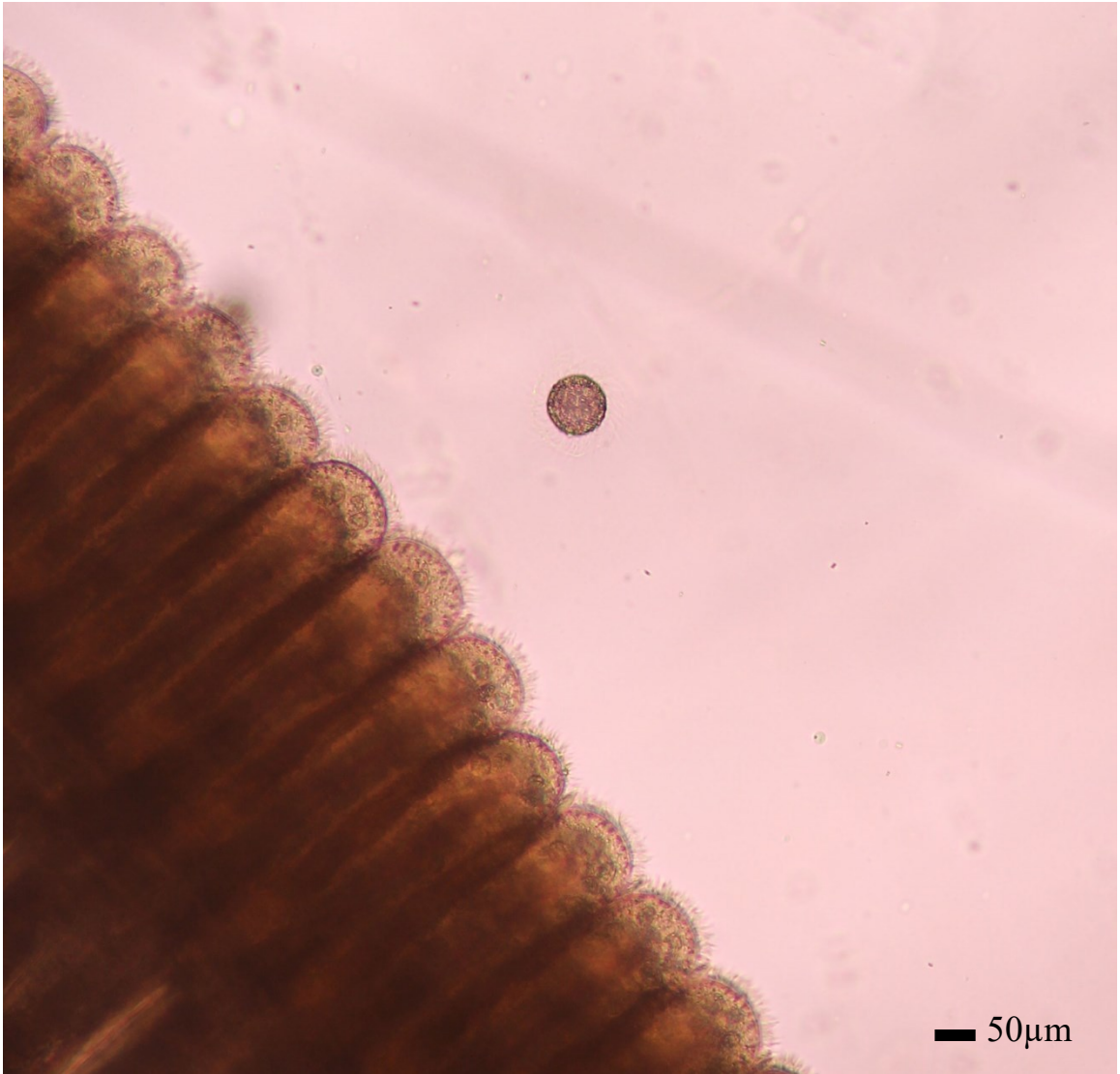


Figure 16. Freshly spawned spermatozeugma not reacting to a female gill

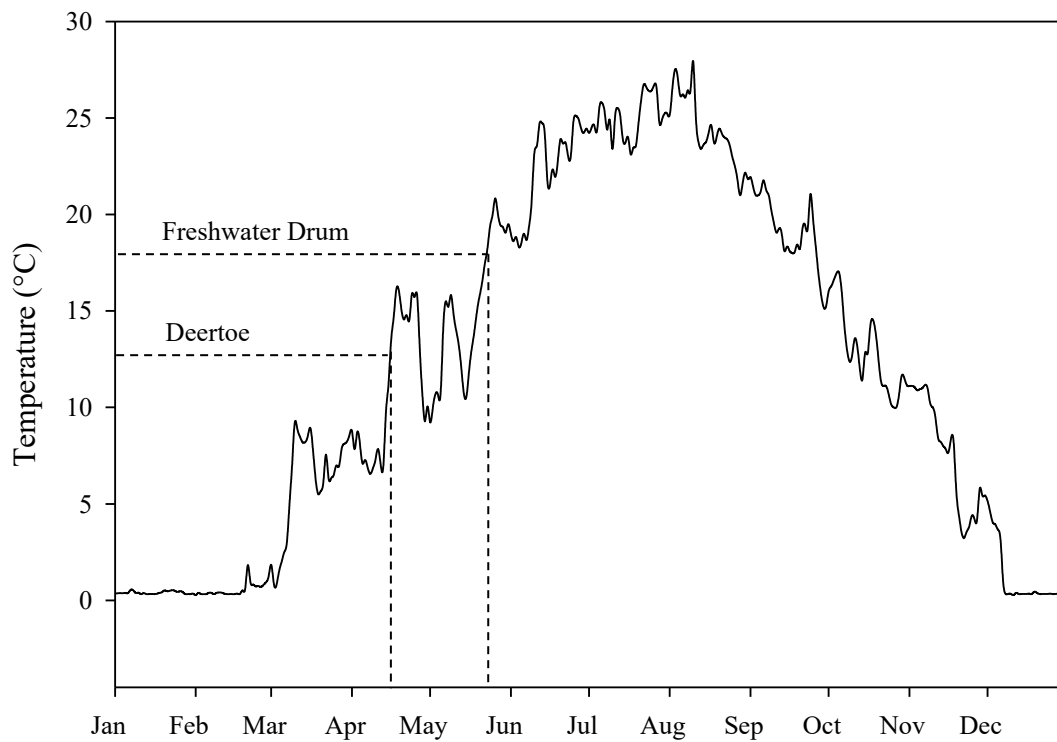


Figure 17. Daily water temperature for the Minnesota River at Mankato, MN for 2016 (USGS data). Dotted lines represent predicted wild spawning times for both Deertoe Mussel (13°C) and their host, Freshwater Drum (beginning at 18°), within the Minnesota River watershed.