AN ABSTRACT OF THE THESIS OF

Judith S. Bilyea for the Master of Science in Biology

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Title: Early detection, dispersal, and maturation of zebra mussels (*Dreissena polymorpha*) in the Marais des Cygnes River and Melvern Lake, Kansas

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Abstract

The zebra mussel (*Dreissena polymorpha*) is an aquatic invasive mollusk brought to North America via cargo ships from the Black and Caspian seas in 1986. A biofouling species, zebra mussels not only cause damage to submerged manmade materials but also outcompete native mussels, many of which are already critically endangered. Zebra mussels also destroy ecosystems by disrupting native food webs. The objectives of my study were to determine: 1) the best method for early detection of a zebra mussel infestation; 2) the distribution pattern of zebra mussels in the Marais des Cygnes River; and 3) stages of gametogenesis achieved by zebra mussels in the Marais des Cygnes River and Melvern Lake, Kansas. From June–November 2013 and May–November 2014, I investigated the invasion of zebra mussels in the Marais des Cygnes River from a source population in Melvern Lake, downstream 196 river-km. I used 30-minute inspection of shoreline, settlement structures, cross-polarized light microscopy (CPLM)

of plankton, and detection of environmental DNA (eDNA) to determine which of these methods would provide the earliest indication of a zebra mussel infestation. I chose 12 sites of three site types (lake, free-flowing river, and lowhead dam) to determine whether lowhead dams influenced the dispersal and distribution of zebra mussels downstream from the source lake. I also performed histological analysis to determine whether zebra mussels entered a mature stage of gametogenesis in the river and sought to determine whether increased veliger densities were due to instream recruitment or simply accumulation from the upstream source population. CPLM of water samples taken via plankton net tows gave the earliest indication of infestation at previously non-documented sites. The density of zebra mussels (both settled recruits and veligers) decreased with distance from the source lake, with peaks of densities at lowhead dam sites. Zebra mussels achieved sexual maturity, with correlative likelihood of reproduction, at both free-flowing and inundated (dammed) sites, consistent with the downstream–march model of dispersal.

Keywords: Aquatic Nuisance Species, Aquatic Invasive Species, Mollusk, Management, Biofouling, Infestation, Lowhead Dam, Lake, Gametogenesis, Reproduction, Spawning

EARLY DETECTION, DISPERSAL, AND MATURATION OF ZEBRA MUSSELS (*DREISSENA POLYMORPHA*) IN THE MARAIS DES CYGNES RIVER AND MELVERN LAKE, KANSAS

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PREFACE

My thesis consists of two chapters, each of which is formatted for submission to a specific peer-reviewed journal: Chapter 1—Early Detection and Dispersal of Zebra Mussels (*Dreissena polymorpha*) in a Midwestern USA Lake–River System—formatted for Management of Biological Invasions; and Chapter 2—Histological Assessment of Zebra Mussel Reproduction in a Midwestern USA River—formatted for Hydrobiologia.

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Chapter One–Early Detection and Dispersal of Zebra Mussels (*Dreissena polymorpha*) in a Midwestern USA Lake–River System

I. INTRODUCTION

The zebra mussel (Mollusca, Bivalvia, *Dreissena polymorpha,* Pallas, 1771) is an aquatic invasive species which was introduced from the Black and Caspian seas into Laurentian Great Lakes watersheds in 1986 (Herbert et al. 1989). It is easily spread between water bodies in its larval (veliger) stage by human vectors, including boats, trailers, and bait buckets (Johnson et al. 2001). This species multiplies rapidly, grows quickly (Mackie 1991; Delmott and Edds 2014), and damages manmade structures such as piping, causing impaired water use and distribution (MacIsaac 1996). Zebra mussels congregate in groups and can clog intake pipes of water treatment plants, farming irrigation, and power plants, which causes decreased efficiency and increased prices for consumers (Stice 1997; Mann et al. 2010). Damages and control costs for aquatic invasive species are almost $6.5 billion per year for the United States (ANSTF 2015).

Zebra mussels are primary consumers, and introduction outside their native environment can negatively impact the food web of an ecosystem (Dunne et al. 2002). Zebra mussels are also capable of outcompeting native mussels for food and habitat (Gillis and Mackie 1994; Ricciardi et al. 1998). They filter phytoplankton from the water, consuming valuable food resources that otherwise would be used by native filter feeders, including aquatic invertebrate larvae (Horvath et al. 1999), fishes (Padilla 1997) and unionid mussels (Williams et al. 1993). Decreased levels of phytoplankton can impact the size and number of aquatic invertebrate larvae (e.g. mayflies, dragonflies, stoneflies), which, in turn, can affect fish populations that prey on these larvae (Carpenter

et al. 1985). In addition to competing for food, zebra mussels attach to unionids, hindering movement, feeding, and absorption of oxygen by these native mussels (Marsden and Lansky 2000), which can lead to declines in their populations (Williams et al. 1993). Multiple studies have documented the decline of native mussel populations in the northern United States since the arrival of zebra mussels (Mackie 1991; Nalepa et al. 1996; Ricciardi et al. 1998; Schloesser et al. 2006).

Zebra mussels are native to lakes and reservoirs, but can disperse into rivers in their larval life stage (Mellina and Rasmussen 1994; Horvath et al. 1996). They produce large numbers of veligers that remain suspended in the water column until they metamorphose and settle on a substrate (Mackie 1991). Large lowhead dams on rivers slow water current and affect flow patterns, and lowhead dams can have similar effects (Baxter 1977; Blalock and Sickel 1996; Dean et al. 2002; Tiemann et al. 2004; Smith et al. 2015). When flowing water is impounded, it forfeits its suspended materials (Tiemann et al. 2004), including silt, leaves, and larval mussels. Kolar and Lodge (2000) suggested that dams could support aquatic invasions by creating opportunities for non-native species to colonize areas that otherwise would not have been available to them. Smith et al. (2015) showed that water behind lowhead dams in the Neosho and Cottonwood river system of eastern Kansas had a higher density of zebra mussel veligers than free-flowing sites nearby, resulting in stepping-stone environments that facilitated downstream dispersal of zebra mussels.

Zebra mussels disperse downstream into rivers from source lakes and reservoirs, forming colonies in slow-moving areas of rivers (e.g., pools, lowhead dams, other reservoirs), “seeding” downstream populations (Mackie, 1993; Horvath et al. 1996;

Bobeldyk et al. 2005; Smith et al. 2015) and have been modeled by three scenarios. The large river model (Strayer, 1991) suggests dispersal occurs in rivers >30m wide but rarely into small streams; the source–sink model (Horvath et al. 1996) hypothesizes dispersal can occur only a short distance downstream from a source; and the downstream–march model (Horvath et al. 1996), predicts progressive dispersal and successive establishment downstream due to instream recruitment.

Given the adverse impacts of zebra mussels on human infrastructure and native ecosystems, it is important to be able to detect their presence at the earliest stages of an infestation and understand their dispersal abilities so procedures can be implemented to minimize their impacts on aquatic environments. I surveyed Melvern Lake and the Marais des Cygnes River, Kansas USA, for infestation by zebra mussels and tested various techniques to determine which would give the earliest indication of an infestation: 30-minute inspection, settlement structures, cross-polarized light microscopy (CPLM), or environmental DNA (eDNA) analysis using the polymerase chain reaction (PCR).

I tracked the invasion of zebra mussels from a source population in Melvern Lake, Kansas, downstream into the Marais des Cygnes River to determine extent and pattern of dispersal. The stretch of the river surveyed has four lowhead dams (Figure 1) along its 196-km length. I included sites at lowhead dams to increase the probability of locating zebra mussels in the river and to assess effects of lowhead dams on riverine zebra mussel densities (Smith et al. 2015). Specifically, my research addressed two objectives: (1) sample a source reservoir and downstream river for the presence of zebra mussel recruits (settled individuals) and veligers (free floating larvae) utilizing various

techniques to determine optimum approach for early detection; and (2) characterize zebra mussel densities in the lake and river to determine dispersal and colonization dynamics, including at lowhead dams.

II. METHODS AND MATERIALS

Study Area

Melvern Lake (2,805 ha, 18 m max depth), an impoundment on the Marais des Cygnes River, has been infested with zebra mussels since at least June 2011 (KDWPT 2018) and was the source population for the study area. The Marais des Cygnes Basin comprises 11,147 km^2, and study sites on the river mainstream spanned Miami, Franklin, Linn, and Osage counties, Kansas, encompassing 196-km of the 349-km length of the river. Land use around sites chosen in the Marais des Cygnes is primarily agricultural, and the predominant substrates at sites were mud, rock, and gravel.

Sampling

In 2013 and 2014, I sampled Melvern Lake and the Marais des Cygnes River at 12 sites (Figure 1, Appendix A), in habitat suitable for zebra mussel veligers and recruits (e.g., flowing water and rocky areas with lesser current speed, respectively) (Ackerman and Claudi 1991; Jonsson et al. 1991; Churchill 2013). To compare densities of recruits and veligers in the slow-moving water at lowhead dams with those in free-flowing water (out of the zone of direct influence from dams, 0.44–17.0 km upstream and downstream), I distributed sites as follows: Site 1 in Melvern Lake to evaluate source population density; one site in the lake's outlet channel (Site 2) to determine whether zebra mussels were being released from the lake; six free-flowing riverine sites (sites 3, 5, 7, 9, 11, 12);

and four sites in the inundated water at lowhead dams (sites 4, 6, 8, 10). Three of the free-flowing river sites (5, 7, 9) were located between two lowhead dams and, in each case, were used to represent both a downstream and an upstream site treatment for those dams (sites 4, 6, 8); for example, free-flowing Site 7 was the downstream treatment for Site 6 and the upstream treatment for Site 8. Beginning in July 2013, access to Site 7 was restricted due to localized flooding and subsequent planting of crops.

I sampled from June to November 2013 and May to November 2014, focusing my efforts on dates with optimal spawning temperatures (>12^{o}C) (Mackie 1991; Leach 1993; Mackie 1993), for a total of 15 sample dates (Table 1). Warmer months were sampled multiple times during year 1, while cooler months were sampled only once (Table 1). In year 2, sites were sampled once per month (Table 1). I measured water temperature at approximately 1-meter depth with an alcohol thermometer during each visit and visually noted predominant substrate of the area at each site.

To compare effectiveness of each sampling method, I employed 30-minute inspection of shoreline, inspection of settlement structures, and plankton tow collection (analyzed using both CPLM and eDNA via PCR) at each site, following sample collection protocols for dreissenid mussel monitoring by the Oregon Department of Fish and Wildlife (Wells and Sytsma 2010). For recruits, 30-minute inspection and examination of settlement structures were used, while for veligers, CPLM and eDNA were employed.

Recruits

30-Minute Visual Inspection

At each site visit, I performed a 30-minute inspection for zebra mussels settled on any substrate; n=15 for each site, with the exception of Site 7 with n=5. I examined predominant substrate and any submerged items in the area, including rocks, submerged tree limbs, and other items (e.g., scrap metal, glass bottles, trash, concrete structures, riprap, unionid shells), upon which juvenile and adult zebra mussels might have settled. Mussels were placed into polyethylene containers, stored on ice, and transported to the lab, where they were preserved and enumerated.

Settlement Structures

All sites were sampled for zebra mussels with a modified "Portland Sampler" settlement structure (Wells and Sytsma 2010) (Figure 2). The sampler consisted of a variety of materials: (1) white PVC pipe; (2) black ABS pipe; (3) black plastic construction mesh; (4) 12 glass microscope slides in a (5) blue polypropylene slide box; (6) two green kitchen-scouring pads (Marsden 1992); (7) a concrete anchor; (8) cable wire; (9) eight black zip ties; and (10) a Hester-Dendy sampler with (11) a stainless steel eyelet hook (Figure 2). Each sampler had a total surface area of 1,467 cm^2 and was composed of 17.8-cm (7-inch) sections, 5.1-cm (2-inch) diameter, of both PVC and ABS piping suspended horizontally in the water column (Figure 2). Five holes, 0.9-cm (3/8-inch) diameter, were drilled through each section of pipe to allow movement of water for mussel colonization. Stainless steel cable wire was used to weave 0.5 meters (1.6 feet) of construction mesh, with 1-cm (0.39-inch) pore size, above and below the pipes, following specifications of the Oregon Department of Fish and Wildlife standard protocols (Wells

and Sytsma 2010). Two 15.24-cm (6-inch) green Scotch-Brite® (3M, St. Paul, MN) heavy-duty scouring pads were attached with black zip ties to cable wire at ends of each section of construction mesh (Figure 2). Microscope slide boxes (14.1 x 9.2 cm; 5.5 x 3.6 inches) were attached to the top of the concrete anchor with zip ties. In each slide box, two standard-size (75 x 25 mm; 3 x 1 inches) microscope slides were placed upright, side-by-side, with three slide slots between them to allow settlement space, with a total of 12 slides per box.

On the first day of sampling, one settlement structure was deployed at each site (12 total) from docks, trees, stakes, dams, or bridges, and remained continuously submerged 1–2 meters deep. Optimal depth for zebra mussels ranges 2–7 meters (Wells et al. 2012), which was an available depth at all sites, with the exception of Site 3 which averaged ~1 meter. Swift current is not conducive to zebra mussel colonization (MacIsaac 1996; Drake and Bossenbroek 2004); therefore, samplers were placed in the most suitable area found at each site. Structures were examined visually during each sampling date. On 17 of 146 occasions throughout my study, I arrived at a sample site to find the deployed settlement structure missing or out of the water (beached); I replaced or redeployed structures when this occurred. This, coupled with the eventual loss of Site 7, gave me a total of 129 settlement structure observations.

Veligers

Plankton Collection

I used a 63-μm mesh Wisconsin Sampler plankton net with a 133-mm (5-inch) diameter opening (Wildco; Yulee, Florida) to collect veligers suspended in the water column, employing oblique tow techniques (Claudi and Mackie 1994; Wells and Sytsma

2010; Holoubek et al. 2014) from the shore, a dam, or a dock at each site. A flow meter (model 2030R, General Oceanics; Miami, Florida) was attached inside the net to measure distance towed to allow calculation of volume of water sampled. As recommended for initial detection of zebra mussel veligers (Marsden 1992; Wells and Sytsma 2010), I took a large number of tows at each site (20) to increase the volume of water sampled and thus increase likelihood of veliger capture. The net was cast out 5 to 6 meters, allowed to sink to just above the substrate (in river sites) or 4–5 meters (for lake site), and retrieved at an oblique angle.

Plankton was washed from the net codpiece into an autoclaved polyethylene bottle using pure, non-denatured ethanol (Carolina Biological Supply; Burlington, North Carolina), which was added to the bottle until the sample reached 70–85% dilution (Wells and Sytsma 2010). Preserved samples were mixed by repeatedly inverting the bottle and split into two autoclaved polyethylene containers for CPLM and for eDNA analysis. Samples for PCR were kept at 4^{o} C in an insulated cooler with ice to prevent DNA degradation in the field and stored in a 4^{o}C refrigerator after transport to the lab; samples for CPLM were kept at ambient temperature during sampling and stored at room temperature in the lab.

I used separate buckets for each step of sampling to reduce the possibility of a net and rope becoming contaminated with zebra mussel DNA: a clean "transport bucket" (for transporting the net to the field); a "field bucket" (to hold the net at each site); and a "decontamination bucket" (to soak sampling gear in acetic acid to destroy viable DNA or nucleotides). To further protect from false eDNA positives, I sampled from downstream (least dense populations) to upstream (most dense populations), and decontaminated

between sites. To prevent possible transfer of veligers between sites, the plankton net and towing rope were sprayed with 10% bleach solution, soaked in 95% isopropyl alcohol for at least 10 minutes, then rinsed with pure ethanol. After completion of sampling on each date, the net and rope were decontaminated by spraying them with 10% bleach solution and immersing them in 5% acetic acid for a minimum of 2 hours (L. Dalton, Utah Division of Wildlife Resources retired, pers. comm.). Before each sampling date, the net was thoroughly rinsed with tap water and pure ethanol was passed through it as a "blank" that was examined under a cross-polarized light microscope as well as tested for eDNA to verify that no veligers remained.

Cross-Polarized Light Microscopy (CPLM)

Plankton samples were examined under a cross-polarized light microscope (Zeiss, Germany) at 25, 100, and 400X magnification, and veligers were enumerated without subsampling. Laboratory methodology followed the standard operating procedure of the U.S. Bureau of Reclamation (BOR) (BOR 2011). Viewed under a cross-polarized light microscope, zebra mussel veligers appear to glow with a Maltese cross in the center (birefringence), which aids in their detection and distinguishes them from other objects often found in samples (e.g., sand, diatoms, algae) (Johnson 1995). Although CPLM does not differentiate among bivalve species, knowledge of the size and morphology of each species allows differentiation (Johnson 1995). Veligers were identified using guides by Nichols and Black (1994), Johnson (1995), and Wells and Sytsma (2013).

I calculated veliger density via the standard rotor equation provided by the flow meter manufacturer (General Oceanics; Miami, Florida):

$$\text{Distance (meters)} = \frac{\text{difference in counts* x rotor constant**}}{999{,}999}$$

$$\text{Volume } (m^3) = \frac{3.14 \times (\text{net diameter})^2}{4} \times \text{distance}$$

*End count minus start count. (10 counts = 1 rotor revolution)
** Standard speed rotor constant = 26,873

Environmental DNA (eDNA)

Plankton samples from each site on each date were used to isolate eDNA and perform PCR to amplify zebra mussel DNA. Laboratory methodology followed the standard operating procedure of the Bureau of Reclamation (SOP No. PCR-3, Keele et al. 2012).

I isolated total DNA from pure zebra mussel DNA (positive control) (sample provided by D. Hosler, Bureau of Reclamation), a zebra mussel water sample (from a confirmed positive source at Melvern Lake, Kansas), and a non-zebra mussel-infested lake sample (confirmed negative by multiple detection methods from Clinton Lake, Kansas, by Kansas Department of Wildlife, Parks, and Tourism in 2012) using an UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California). DNA samples were incubated with GeneReleaser (BioVentures, Murfressboro, Tennessee) to sequester any potential inhibitors of PCR. I also used nuclease-free water as an additional negative control.

Total DNA was isolated from each plankton sample, as well as from the known positive and negative samples, which served as the DNA templates to potentially amplify a 381-base-pair DNA fragment unique to zebra mussels (Hosler 2011; Keele et al. 2012). Reactions were prepared in a buffer solution containing the DNA template, DNA primers

(5’ TGT CAC CAC TCA TGG GCT TGT T 3’ and 5’ TGC AGA ACA AAG GGA CCC GGT AAA 3’), dNTPs, $MgCl_2$, and Taq polymerase. Amplification was carried out in a BioRad T-100 thermocycler (Hercules, California) using an initial denaturation of 95^{o}C for 9 minutes, followed by 40 cycles of 95^{o}C/20 seconds, 59^{o}C/90 seconds, and 72^{o}C/90 seconds. A final elongation at 72^{o}C for 10 minutes was completed for each sample. All amplified samples were stored at 4^{o}C until electrophoretic analysis. Amplified DNA samples were analyzed by agarose gel electrophoresis to test for presence of the amplicon diagnostic for zebra mussels (Keele et al. 2012; D. Hosler, Bureau of Reclamation, pers. comm.) (Figure 3). Any samples containing a 381-base-pair DNA fragment displayed an amplicon the same distance from the starting wells as the positive controls, confirming zebra mussel DNA presence.

Data Analysis

Data were analyzed with PASW Statistics for Windows (SPSS version 24.0; SPSS 2016). Due to the strongly positively-skewed data resulting from the much greater zebra mussel abundance upstream and the large number of zeroes in the dataset (i.e., zebra mussel absence), the statistical assumption of a normal distribution was not met; transformation only marginally improved normality. The assumption of sphericity, critical for repeated measures ANOVA, was also violated. Thus, the data were analyzed by graphical analysis, determination of presence/absence, examination of measures of central tendency and variability, and non-parametric statistics. Zebra mussel densities were compared among site types and dates with a Friedman test, the non-parametric counterpart to a repeated measures ANOVA. To examine pairwise differences, post-hoc

multiple comparison Wilcoxon signed-rank tests were conducted with a Bonferroni-corrected overall α of 0.05 to reduce Type 1 error rate.

At sites with no previous evidence of zebra mussels, I noted which method or methods first produced a positive indication of an infestation. To compare sites that produced a positive detection on the first sample day, I noted how many sample days it took to detect zebra mussels with each remaining method. I determined which detection method had the highest percentage of positive detections, which method provided the greatest number of sites determined to be positive, and which method held any exclusivity (if there were sites that produced only positive results for one detection method). I weighed all of these factors to judge which detection method was best for early zebra mussel detection.

III. RESULTS

On each sampling date, settled zebra mussels and veligers were detected in both the lake and river, but differences existed among site types. Because the infestation had already occurred in the Marais des Cygnes, I considered sites that did not produce any positive results in the first year as "pioneer sites" (unsettled) for year 2 of sampling (sites 9, 10, 11, 12). During year 1, I found zebra mussels (settled or larval) by at least one detection method at sites 1–8; however, sites 9, 10, 11, and 12 did not produce a positive detection by any method. During year 2, I found zebra mussels farther downstream at sites 9 and 10, while sites 11 and 12 still did not produce a positive detection by any method. Sites 1, 2, 6, and 8 produced a positive detection (by at least one method) on every sample date. During 2013, the farthest downstream veliger discovery was at Site 8, 78 river-km from the source lake, however the farthest downstream discovery for both

years (by CPLM) was in July 2014 at Site 10, which is 127 river-km from the source lake. Site 10 had had no previous positive detection during the study; therefore, CPLM was the method that provided the earliest indicator for zebra mussel infestation. Thirty minute inspection had the highest number of total positive detections with 87, CPLM had the second highest with 70, settlement structures had 24, and eDNA had 21.

Of sites that produced positive detections by any method, lowhead dam sites (4, 6, 8, 10) had higher percentages of positive detections than the free-flowing sites surrounding them (3, 5, 7, 9, 11) (Table 2). This gave the zebra mussel dispersal in the river a distinct pattern of peaks of higher percentages of positive detections at lowhead dam sites vs. free-flowing sites for both settled individuals and veligers (figures 4, 5, 6). Lowhead dam sites 4, 6, 8, and 10 showed 73, 100, 100, and 0% positive detections, respectively, by 30-minute inspection, whereas free-flowing sites 3, 5, 7, 9, 11, and 12 showed 40, 60, 0, 0, 0 and 0% positive, respectively, by 30-minute inspection (Table 2).

Detection

Recruits

30-Minute Visual Inspection

I conducted 30-minute inspections for 85 hours in the Marais des Cygnes River and Melvern Lake and found 40,734 recruits at seven of the 12 sites (58%), which made this method the second best at determining positive sites for zebra mussels (following CPLM with 10 of 12 sites, 83%). For 2013 samples, the farthest downstream that recruits were found was at Site 8, which is 78 river-km from the source at Melvern Lake; recruits were found no further downstream for 2014 samples. During 2013, there were 104 sampling events with 49 positive (47.0%), and during 2014 there were 66 sampling

events with 38 positive (57.5%), an increase of 10.5% in one year. Of the total 170 sampling events (15 sample days, 12 samples per day, minus 10 missed visits to Site 7), 30-minute inspection produced 87 positive results (51.1%), the highest percentage of positive detections of all methods.

Thirty-minute inspection produced 100% positive detections (15/15) for sites 1, 2, 6, and 8, 73% positive detections (11/15) for Site 4, and 60% positive detections (9/15) for Site 5. Site 3, though closer to the source lake, produced positive detections by visual inspection only 40% of the time (6/15) (Table 2).

Settlement Structures

I examined a total surface area of 203,913 cm^2 on deployed settlement structures throughout my study. Recruits were found on deployed settlement structures at three of the 12 sites (sites 1, 2, 6) out of 13 total sample dates for this method. I found zebra mussels settled on deployed structures at Site 6 (57 river-km from the source at Melvern Lake), which was the third farthest downstream of any detection method. Of 130 sampling events (13 sample days, 12 samples per day, minus 10 missed visits to Site 7 and 17 occasions of lost or beached structures), settlement structures produced 24 positive results, an overall success rate of 18%. Individuals did not attach to settlement structures in high densities in the river, though the structure at Site 1 (Melvern Lake) was coated with juvenile recruits.

Veligers

Cross-Polarized Light Microscopy

I sampled 455,364 liters of water from the Marais des Cygnes River and 24,993 liters from Melvern Lake (Site 1). A total of 15,502 veligers were found—820 in the

river and 14,682 in the lake. I identified veligers from 10 of the 12 sites (sites 1–10), the highest number of sites positive for any method. Of 170 samples for CPLM, I found 70 positive detections for zebra mussel veligers (41%), the second highest frequency of detection for all methods except the 30-minute inspection (51%).

Though CPLM produced positives for 10 sites, it did not produce a positive at each of those sites on every sample date. At Site 1, CPLM was positive for zebra mussel veligers 100% of the time, and at Site 2 it was positive 86% of the time (13 of 15 sample dates). Site 3 had a 46% rate (seven of 15 dates); sites 4 and 6 had 53% positive detections (eight of 15 dates); Site 5 had 60% positive detections (nine of 15 dates); Site 7 had a 40% rate (two of five dates); Site 9 had 6% positive detections (one of 15 dates); and Site 10 had 13% positives (two of 15 dates). Sites 9 and 10 did not produce a positive until 2014 and were never positive by any other detection method, which demonstrates CPLM as the only method with exclusivity (Table 3). Sites 11 and 12 had no veligers (0 of 15 dates).

Environmental DNA

I detected zebra mussel DNA at two of the 12 sites in 2013 (Site 1, Melvern Lake and Site 2, Melvern Lake Outlet) and at five of 12 sites in 2014 (Site 1, Melvern Lake, Site 2, Melvern Lake Outlet, and sites 5, 6, and 8). Of 170 eDNA samples, I found 21 positive detections (12.4%), making it the least effective detection method in terms of frequency and number of sites judged to be positive. Sites that produced a positive result by CPLM but a negative result with eDNA were deemed "false negatives." Site 1 (Melvern Lake, source population) produced a positive result for every sample taken during 2013, but gave one false negative result in 2014. All samples that produced a

positive CPLM detection (70) but did not produce a positive eDNA result (49) were considered "false negatives" (70% false). The farthest downstream DNA was detected was in September 2014 at Site 8, which was 78 river-km from the source at Melvern Lake.

Dispersal

Recruits

30-Minute Inspection

Numbers of settled individuals per 30-minute inspection showed a general pattern of decrease downstream (Figure 4), with means ranging from 2,373.7 (SE=279.6) per 30-minutes in the source lake and 291.9 (SE=43.4) per 30-minutes in the lake outlet to 10.6 (SE=7.8) at Site 3, 2.5 (SE=0.7) at Site 4, 6.3 (SE=2.4) at Site 5, 14.4 (SE=4.2) at Site 6, 0.0 (SE=0.0) at Site 7, 16.3 (SE=4.5) at Site 8, and 0.0 (SE=0.0) at sites 9–12. Densities differed significantly among site types (inundated control, free-flowing control, inundated, free-flowing) (Friedman test, $\chi^2_{(3)}=41.23$, $p<0.001$). Post-hoc multiple comparisons illustrated that each site type had recruit densities significantly different from all other site types, with inundated sites having more recruits than free-flowing sites (Wilcoxon, $z=-3.033$, $p=0.002$). Densities of recruits were borderline significant for dates (Friedman test, $\chi^2_{(14)}=23.59$, $p=0.051$).

Settlement Structures

Settlement structures were colonized at only three sites: source lake (Site 1), lake outlet (Site 2), and dam 2 (Site 6). The mean number of zebra mussels colonizing settlement structures decreased with distance downstream from the lake (Figure 5). Sites

with more frequent human activity (1, 2, 4, 6, 8) had more instances of settlement structures being lost/beached (17 of 146 sampling occasions). Site 1 numbers were extrapolated from a subsample of 1 cm^2 due to the extremely high density of settled zebra mussels attached to the structure. Mean numbers were 31,351 (SE=1,868.2) in Melvern Lake, 1.16 (SE=0.6) in the lake outlet, 1.0 (SE=0.3) at Site 6, and 0.0 (SE=0.0) at all other sites. Recruit densities varied significantly among site types (inundated control, free-flowing control, inundated, free-flowing) (Friedman test, $\chi^2_{(3)}=13.62$, $p=0.003$). Recruit densities on settlement structures at Melvern Lake (inundated control) were significantly greater than at inundated river sites (4, 6, 8, 10) (Wilcoxon, $z=-2.21$, $p=0.027$); Melvern Outlet (free-flowing control) densities were not significantly different from those at free-flowing river sites (3, 5, 7, 9, 11, 12) (Wilcoxon, $z=-1.89$, $p=0.059$); and inundated river site densities were significantly greater than those at free-flowing sites (Wilcoxon, $z=-2.23$, $p=0.026$). Densities of recruits on settlement structures did not vary significantly by date (Friedman test, $\chi^2_{(12)}=10.81$, $p=0.545$).

Veligers

Cross-Polarized Light Microscopy

Mean zebra mussel veliger densities decreased with distance downstream from Melvern Lake (Figure 6), similar to the pattern seen with recruits (figures 4 & 5), though both detection methods showed peaks of higher densities at lowhead dam sites (Figure 7). Mean densities ranged from 1.05 per liter (SE=0.43) in the source lake and 0.07 per liter (SE=0.04) in the lake outlet to 0.00013 per liter (SE=0.0013) at Site 10 and 0.00 per liter (SE=0.0) at sites 11 and 12.

Veliger densities at Melvern Lake (inundated control) were significantly greater than at inundated sites in the river (sites 4, 6, 8, 10) (Friedman test, $\chi^2_{(1)}=15.00$, $p<0.001$), and Melvern Outlet (free-flowing control) veliger densities were significantly greater than at free-flowing river sites (3, 5, 7, 9, 11, 12) (Friedman test, $\chi^2_{(1)}=13.00$, $p=0.001$). Veliger densities differed significantly among individual sites (1–12) (Friedman test, $\chi^2_{(11)}=41.86$, $p<0.001$), and among site types (inundated control, free-flowing control, inundated, free-flowing) (Friedman test, $\chi^2_{(3)}=31.73$, $p<0.001$). Post-hoc comparisons illustrated significantly more veligers at Site 1 (Melvern Lake) vs. all other sites (2–12) and differences between Site 2 (Melvern Lake Outlet) and all other sites (1, lower; 3–12, greater). All site types were significantly different from other site types, with the exception of inundated sites (4, 6, 8, 10) vs. free-flowing sites (3, 5, 7, 9, 11, 12) (Wilcoxon, $z=-1.537$, $p=0.124$). Veliger densities differed significantly among sample dates (Friedman test, $\chi^2_{(14)}=47.10$, $p<0.001$) (Figure 8).

Densities and Discharge

I collected a total of 14,682 veligers and 35,605 recruits in the lentic control lake (Site 1) and 622 veligers and 4,378 recruits at the lotic control (Site 2). At the four inundated sites (4, 6, 8, 10), I collected at total of 149 veligers and 498 recruits, and at the six free-flowing sites (3, 5, 7, 9, 11, 12) I found a total of 49 veligers and 253 recruits (Table 4). Densities of veligers and recruits decreased with distance from the source lake, but both had peaks of higher numbers at the four inundated sites (Figure 4), though only recruits were significantly higher from adjacent free-flowing sites. Densities of veligers and recruits were high in the lentic control, Melvern Lake (Site 1), and the lotic control, lake outlet channel (Site 2), relative to those in the river (Figure 2), which was

expected given that the lake had a well-established population. Mean veliger density at each site type was 1.06 ± 0.43 veligers/liter at Site 1 (inundated control), 0.07 ± 0.038 veligers/liter at Site 2 (free-flowing control), 0.004 ± 0.002 veligers/liter at inundated river sites, and 0.0011 ± 0.0004 veligers/liter at free-flowing river sites. Veligers were located at all four inundated sites (4, 6, 8 10) and four of the six free-flowing sites (3, 5, 7, 9). At three of the four dams, the total number of veligers found was higher than at the adjacent free-flowing sites, with the exception of sites 9 & 10 (Figure 2); however, mean veliger density did not vary significantly between any inundated site (4, 6, 8, 10) and its adjacent free-flowing site (3, 5, 7, 9, 11).

Mean recruit density at each site type was 2,373.67 ± 279.59 recruits/30 min search at Site 1 (inundated control), 291.87 ± 43.39 recruits/30 min search at Site 2 (free-flowing control), 8.3 ± 1.77 recruits/30 min at inundated river sites, and 3.16 ± 1.57 recruits/30 min at free-flowing river sites. Recruits were located at three of the four inundated river sites (4, 6 & 8) and two of the six free-flowing river sites (3 & 5). At each lowhead dam where recruits were found, the mean number of recruits was higher than at the adjacent free-flowing sites, and recruit densities varied significantly between each inundated site and its adjacent free-flowing site.

Dam-controlled discharge of the Marais des Cygnes River from Melvern Lake remained constant (0.57 m^3/s=20 cfs) throughout 2013 given relative drought conditions (J. Franz, Natural Resource Manager, Melvern Lake, pers. comm.); mean discharge for the 30 years prior to zebra mussel detection in 2011 was 5.7 m^3/s (202.0 cfs) at the outlet (USGS 2015). The Marais des Cygnes River flowing from Melvern Lake had a median and mean discharge of 0.57 m^3/s (20 cfs) for 2013 and a median discharge of 1.3 m^3/s (47

cfs) with a mean of 1.1 m^3/s (39.1 cfs) for 2014 (USGS 2015). Due to the low and steady release from Melvern Lake in 2013, I used discharge data from the closest (0.29 km) USGS station downstream at Pomona, Kansas (station 06913000) to more accurately gauge changes in discharge of the river due to precipitation, runoff, and drought. The Marais des Cygnes River had seasonal flow variations in 2013–2014, with higher discharge in summer and fall of both years (USGS 2015) (Figure 8). Higher veliger densities did not coincide with periods of high discharge during 2013, but they did coincide in 2014 (Figure 8).

IV. DISCUSSION

My study design allowed me to compare effectiveness of detection methodologies in a lotic environment, to analyze density distributions, and to track the distance that zebra mussels had dispersed downstream. I found zebra mussels by all four detection methods employed (30-minute search, settlement structures, CPLM, and eDNA). CPLM of veligers was the first method to produce a positive detection at a study site where zebra mussels had not previously been found. Densities of settled zebra mussels and veligers decreased downstream from the source lake, with peaks of higher densities at inundated lowhead dam sites. Zebra mussels dispersed from river-km 78 in 2013 to river-km 127 in 2014. Settled densities were significantly greater at inundated sites than at free-flowing sites, and even though veliger densities were not significantly different, they showed peaks of increased densities, suggesting that inundated sites could be a refuge for zebra mussels to accumulate, settle, grow, and eventually contribute to instream recruitment.

Detection

Recruits

Thirty-minute inspection had the highest zebra mussel recruit detection rate (51%) in the Marais des Cygnes River. Few adult mollusks can visually be mistaken for zebra mussels in Kansas, due to their distinct morphological differences from native unionids. However, in areas where quagga mussels (*Dreissena rostriformis bugensis*) are present, there is a chance of misidentification due to similar morphological properties between the two species (*D. polymorpha* and *D. bugensis*) of both settled and larval mussels (Nichols and Black 1994).

Some sites had low densities of recruits, where detection was not positive on every sampling date (sites 3, 4, 5). However, many sites (1, 2, 6, 8) were positive on every sample date, giving 30-minute inspection the highest frequency of positive detections. On the other hand, 30-minute inspection never produced a positive result at a site where CPLM did not produce a positive detection at some point during the study. Throughout the study, 30-minute search never showed exclusivity (producing a positive at a site where no other detection method produced a positive result). Expenses for 30-minute inspections were relatively low, requiring only person-hours and vehicle/fuel to get to each site, which I consider the base cost for the expense calculations for all detection methods (total cost: $3,050).

Several Kansas lakes and reservoirs (e.g., Marion Reservoir, Lake Shawnee, Milford Lake, Council Grove City Lake, Winfield City Lake) have been confirmed positive for zebra mussels by KDWPT after settled individuals were initially found by a boater, angler, or swimmer (KDWPT 2018), and many other populations of zebra

mussels have been first discovered by lake users in other regions (Johnson and Carlton 1996; Havel and Stelzleni-Schwent 2000). Veligers can settle and grow to 4 mm in one-month in Kansas (Delmott and Edds 2014), which means that monthly inspections may be insufficient for very early detection and more frequent sampling may not be feasible for state/federal agencies, municipalities, industries, and researchers with limited resources.

In areas around lowhead dams, settled zebra mussels were found on the concrete face of the dam, at the side of the dam, and on rocks just upstream. I also found many zebra mussels settled primarily on the underside and sides of rocks, except at Melvern Lake, where they also settled on top of rocks, possibly due to high population density and less availability of surfaces of suitable substrates. My observations were made 2 and 3 years after initial zebra mussel detection in the lake and may not be generalizable to water bodies with a different infestation chronology. Once a population has increased in density, it is possible that their settling behaviors could change, such as choosing any exposed hard surface to attach to (tops of rocks), not just more "protected" areas (undersides and sides of rocks).

Settlement structures were the least effective (positive detections at only three sites), and third most expensive method in terms of time invested and materials purchased (with eDNA and CPLM being the most expensive methods, respectively). Settlement structures require materials, person-hours to assemble and deploy, person-hours to examine once deployed, and materials/person-hours to replace lost structures (total cost: $3,770). However, for agencies that wish to monitor known zebra mussel populations in a water body, settlement structures afford the opportunity to have a large determined

surface area that can be checked periodically and easily to facilitate abundance assessments. Many different types of settlement structures are used by state and federal agencies, and they all have value because zebra mussels do attach to them, however, certain types of materials may be more effective. Anecdotally, I found zebra mussels attached to the ABS piping in greater densities than the PVC piping, which could be due to zebra mussels being negatively phototaxic (Toomey et al. 2002). I also observed that the density of zebra mussels on the scouring pad was very high, and density became much higher when the pad folded over (due to the weight of attached zebra mussels), causing zebra mussels to accumulate in mass in the concave area of the fold.

Sites with mud or clay substrate often had silt accumulation in many areas of the settlement structures, which may have lowered the overall effectiveness of detection. Perhaps selecting sites with lower sedimentation could increase the effectiveness of settlement structures at a site.

Sites with more frequent human activity (1, 2, 4, 6, 8) had greater instances of settlement structures being lost or beached. I recommend camouflaging structures and cables, especially at sites with frequent human visitors. If camouflaging is not an option, durable signs should be posted stating the purpose of the structures; this, however, can draw the attention of curious people. When choosing a location to deploy settlement structures, it is sometimes necessary to forfeit more favorable habitat for areas where the settlement structures will remain intact and undisturbed.

Veligers

When settled zebra mussels have not been documented at a site previously, CPLM of water samples taken via plankton net tows offers a longer period of advanced

warning (compared to 30-minute inspection) before a zebra mussel population establishes. Though eDNA could potentially give a more advanced warning in the future, I found CPLM to be a more reliable detection method at this time. Hosler (2011) found that, in the absence of settled mussels, CPLM gave the earliest indication of a zebra mussel infestation in Colorado. Although CPLM produced positive detections farther downstream than any other detection method in my study (127 river-km), the frequency of positive detections (41%) was less than for 30-minute inspections (51%); however, veliger densities change seasonally due to spawning, which could account for the lower CPLM detection rate. CPLM detected zebra mussels at three sites (7, 9, 10) where no previous positive detection had occurred. However, CPLM was the second most expensive (after eDNA) (requiring a plankton net and a cross-polarized microscope), and it was the most time-consuming of the detection methods, entailing several days in the lab to process samples for each day of field sampling (total cost: $7,270).

Without proper training and practice identifying larval mussels under a cross-polarized microscope, larval zebra mussels (in "D" phase of metamorphosis, Wells and Sytsma 2013) can be easily mistaken for larval Asian Clams (*Corbicula fluminea*). Guides to larval zebra mussel identification include those by Nichols and Black (1994), Johnson (1995), and Wells and Sytsma (2013), with the latter containing a detailed and helpful dichotomous key, which can effectively eliminate misidentification.

The presence of veligers did not guarantee that DNA would be detected via PCR. This result is similar to findings by Hosler (2011) who had CPLM and eDNA samples from the same location that contradicted one another (false positive (positive eDNA but negative CPLM) or false negatives (negative eDNA but positive CPLM)). This was

demonstrated well at my Site 2, which had high settled and larval densities (100% positive detections via 30-minute search and 85% positive detections via CPLM), but only two positive DNA detections out of 15 samples. In each case, false negatives contained very low densities of veligers compared to Site 1, which produced positive eDNA detections on every sample date except one. A low density of free-floating DNA (from naturally occurring cell lysis, decay of dead bodies, excrement, sloughing) can also lead to false negatives (Jerde et al. 2011).

No false positive results occurred, with any detection method, throughout my study. Every positive eDNA detection was corroborated by another detection method (Appendix B). False positive results are a potential hazard when performing eDNA analysis (Jerde et al. 2011; Frischer et al. 2012; Biggs et al. 2014), but good decontamination and quarantine practices with plankton nets and samples can avoid this. All equipment must be decontaminated after use (Wells and Sytsma 2010; Wells et al. 2012) and kept in areas where DNA contamination cannot occur, including during transport between sites, storage, and end of sampling day (BOR 2014). All bottles must be autoclaved or sterilized before use (Jerde et al. 2011). I used separate buckets for each step of sampling to reduce the possibility of a net and rope becoming contaminated with zebra mussel DNA. To further protect from false DNA positives, I sampled from downstream (least dense populations) to upstream (most dense populations), decontaminated between sites, and poured "blank" samples through the net that were tested for DNA as well. No blank sample produced a positive result throughout the study.

False negatives (a positive CPLM result with a negative eDNA result) were common in my eDNA samples in the Marais des Cygnes River. I found recruits at eight sites and veligers at 10 sites along the river, yet only five sites (1, 2, 5, 6, 8) produced positive results for eDNA, and infrequently at that (Site 1, 10 of 15 samples; Site 2, 2 of 15; Site 5, 1 of 15; Site 6, 3 of 15; and Site 8, 1 of 15). Throughout my study, I collected 48 samples that gave false negative results from the Marais des Cygnes River and one sample that gave a false negative from Melvern Lake. Several possible scenarios could have led to false negatives. Some samples could have had low densities of veligers, though Maruyama et al. (2014), who studied bluegill sunfish (*Lepomis macrochirus*), found that adult fish released DNA 3–4 times faster than juveniles, so a positive eDNA relationship may be more dependent on biomass rather than density. Hosler (2011) found that DNA testing is more accurate when zebra mussel veligers are at higher densities. Though eDNA was not a reliable method in the Marais des Cygnes River due to the many false negative results, Melvern Lake, even with its high density of veligers, produced false negatives 7% of the time (1 of 15 samples).

It is possible that the primers chosen for this study may not be the best for low densities of veligers or DNA, which could lead to false negatives as well. Flowing water could also contribute to false negatives by distributing veligers and free-flowing DNA throughout the water column. The rate at which zebra mussels release DNA or the rate of DNA decay in flowing systems is unknown. Other factors that could contribute to false negatives include the method of handling samples before analysis, type and quality of preservative, PCR inhibitors, and human error. The lab setup and materials needed to perform environmental DNA analysis on a large scale are much more expensive than any

of the other methods I employed (Frischer et al. 2012) (total cost: $9,431). With the rapid advances in molecular genetics, in the near future, improved eDNA technologies may give promise; however, for my study, it was unreliable and expensive.

Various methods of detection were employed in an attempt to find different life stages of zebra mussels, though detecting veligers first would be preferred, as it would give an earlier indication of infestation than would finding settled individuals. Because the Marais des Cygnes River is a lotic environment, results I obtained there may not be applicable to lakes (lentic environments), due to potentially confounding factors that remain untested, such as veliger dilution by flow, differential settling and survival in varying velocities, greater variation in temperature. For example, flow could distribute veligers differently in the water column of a river than in a lake. The distribution of settled zebra mussels may be based more on the availability of slower-moving water (< 2m/s) (Bobat et al. 2004) than on substrate type or site depth (Wells and Sytsma 2010), as can be the case in a lake or reservoir (Smith et al. 2016). Furthermore, temperature variation may affect detection in rivers by shifting settlement toward deeper portions that maintain a more constant daily temperature profile than shallow portions, though this may be true only for younger mussels with their temporary byssal threads (O'Neill and MacNeill 1991).

Dispersal

Zebra mussels were first detected in Melvern Lake in 2011 and were discharged downstream into the Marais des Cygnes River through reservoir releases. I detected zebra mussels at the first 10 sites along the Marais des Cygnes River by employing 30-minute inspection (positive at sites 1–6 & 8), settlement structures (positive at sites 1, 2,

& 6), CPLM (positive at sites 1–10), and eDNA (positive at sites 1, 2, 5, 6, & 8) methodologies. Zebra mussel veligers were found 78 river-km downstream from the source in 2013 and 127 river-km downstream in 2014, showing that zebra mussels continued to disperse downstream in densities high enough to be detected. The rate of dispersal seemed to be moving slowly but consistently. However, the dispersal rate could change once the zebra mussels move beyond the portions of river with many lowhead dams, which have been known to facilitate dispersal of aquatic invasive species (Havel et al. 2005; Smith et al. 2015). Zebra mussels in the Marais des Cygnes River fit the downstream–march model of dispersal; however, they also display aspects of the source–sink model, due to the continuous feed of veligers from the upstream population source of Melvern Lake.

Although Site 3 was the closest free-flowing site to the source population in Melvern Lake and the Melvern Outlet, it had the lowest mean density of the eight sites where recruits were found and the fourth lowest veliger density of the 10 sites where veligers were found. One possible explanation for the low density of recruits at Site 3 is that any veligers discharged from Melvern could have been too small (60–150 μm) (Wells and Sytsma 2013) to settle-out of the water column by the time they reached Site 3 (20 river-km from the source), due to the time needed for them to settle. Veligers in North America remain suspended in the water column for 1 week to 2 months before growing large enough to settle out and attach to a hard surface (Fraleigh et al. 1993; Martel et al. 1994; Horvath et al. 1996; Horvath et al. 1999). If a newly metamorphosed veliger traveled at the speed of discharge from the source lake (0.57 m^3/s) (USGS 2015), it would reach Site 3 in 9.8 hours and Site 12 (196 river-km downstream) in 96 hours,

which could be too short a timeframe for the veliger to settle out of the water column. Veligers large enough to settle (~158 μm) (Nichols and Kollar 1991) soon after they had left the source might not have survived due to the characteristics of the site (e.g. shallow, swift current with sand or mud substrate). Optimal depth for zebra mussels ranges 2–7 meters (Wells et al. 2012), but average depth of Site 3 was less than 1 meter, while other sites were ~1–2 meters deep. Site 3 was a riffle with no areas of pooling and had a swifter current than the other free-flowing sites (5, 7, 9, 11, 12) (personal observation); swift current is not conducive to zebra mussel colonization (MacIsaac 1996; Drake and Bossenbroek 2004).

Three years after initial zebra mussel sightings in Melvern Lake, recruits were dense in the lower portion of the lake ($\bar{x}$=2,374 (SE=279.6) per 30-minute inspection). The highest veliger density observed at any site was 4.25/liter in July 2014 at the source lake. Mean densities of both recruits and veligers were lower in the Marais des Cygnes River than in the source lake and they decreased with distance downstream. Smith et al. (2015) found that zebra mussel densities (settled and larval) in the Neosho and Cottonwood rivers, Kansas, decreased with distance downstream from the source, with mean number of recruits in the Cottonwood River dwindling from 309 per 30-minute search (2.8 river-km downstream) to 0 per 30-minute search (24 to 199 river-km downstream), and mean veliger density decreased from 1.45 per liter (2.8 river-km downstream) to 0.03 per liter (199 river-km downstream). I found that the mean number of recruits in the Marais des Cygnes River declined sharply from 292 (SE=43.39) per 30-minute search (0.3 river-km downstream) and 11 (SE=7.85) per 30-minute search (19.8 river-km downstream) to 0 per 30-minute search (110 to 196 river-km downstream).

Mean veliger density also decreased from 0.06 (SE=0.04) per liter (0.3 river-km downstream) to 0 (SE=0.0) per liter (143 to 196 river-km downstream).

Drought years may have led to low densities of veligers in the Marais des Cygnes River ($\bar{x}$=0.002, SE=0.0012/liter) compared to other lake-river coupled systems that have had zebra mussels for the similar amounts of time, such as Smith et al's (2015) findings in the Neosho River, Kansas (0.08/liter, less than a year after introduction) and the Cottonwood River, Kansas (0.20/liter, 3 years after introduction). Following increased discharge from Melvern Lake in 2014 (~1.1 m^3/s=39.1 cfs), I found larval zebra mussels at sites 9 and 10, downstream from 2013's furthest extent, Site 8; increased discharge in 2014 may have dispersed veligers farther downstream. Zebra mussels will likely move farther downstream and densities will likely continue to rise with increases in discharge and over time.

The extent of zebra mussel dispersal downstream likely depends both on discharge and time since colonization. Zebra mussels are capable of more dense populations in lentic environments than can be found in rivers (Mellina and Rasmussen 1994), giving rise to several potential limiting factors for zebra mussels in lotic systems. Nutrient availability is very important for veligers to be able to grow and metamorphose (Ackerman et al. 1994), and if they are unable to filter sufficient nutrients to survive, it could lead to lower densities. Settled zebra mussels are negatively phototaxic, seeking dark areas that provide protection (Korgina 1982; Toomey et al. 2002); however, many portions of rivers might not provide areas where zebra mussels can attach, especially sites with sand or clay substrate, potentially limiting recruitment in a river. I generally found zebra mussels settled toward the underside or on the sides of rocks where they could be

protected from damage or predation in the flowing river, but in Melvern Lake they settled on the underside as well as on top of rocks and under a marina dock, possibly due to high population density in the lake forcing recruits to settle in less desirable areas. Zebra mussels could also be more inclined to settle in protected areas of the river due to the flow of the lotic environment compared to their native lentic environment.

Effects of Lowhead Dams

Impoundments can support invasion of non-native species by creating opportunities for colonization in habitats that otherwise would not have been available (Kolar and Lodge 2000; Muirhead and MacIsaac 2005; Smith et al. 2015). Humans are altering waterways with dams and reservoirs, building suitable habitat that facilitates dispersal of non-native species (Havel et al. 2005). Lowhead dams can aid dispersal of zebra mussels by providing areas of slow-moving water, similar to their preferred lentic habitat (Strayer 1991; Neary and Leach 1992; Johnson et al. 2008). Smith et al. (2015) found that densities of recruits and veligers were greater at lowhead dams than in other portions of the Neosho and Cottonwood rivers, Kansas, and that these dams were acting as stepping-stones for zebra mussel dispersal downstream.

In the Marais des Cygnes River, Kansas, settled and larval zebra mussel densities generally declined with increasing distance from Melvern Lake, however peaks of higher densities occurred in the inundated waters behind lowhead dams (sites 4, 6, 8, 10; Figure 8), similar to Smith et al.'s (2015) findings in the Cottonwood and Neosho rivers, Kansas. These higher densities were likely related to the flow at each site, which typically is slower at sites inundated by lowhead dams (Tiemann et al. 2004). As shown by Smith et al. (2015) in the Neosho and Cottonwood rivers, Kansas, the lowhead dam "peak" and

free-flowing “trough” patterns of zebra mussel (recruits and veligers) distribution I found (figures 6 and 8) suggest that these dams could be providing zebra mussels with areas of more suitable flow than free-flowing areas of the river. These habitats facilitate dispersal downstream (Schiemer et al. 2001) and give zebra mussels a better chance to settle, accumulate, and reproduce in the Marais des Cygnes River.

Future Detection Methods

Scanning electron microscopy (SEM), quantitative PCR (qPCR), and flow cytometry are future possible detection methods for zebra mussels (Hosler 2011). SEM is much more expensive initially than other detection methods, given the upfront cost of the microscope, and requires more training to complete than other methods, although it is more accurate than CPLM and eDNA because it provides a more detailed image for identification of the suspect organism (Hosler 2011). The Bureau of Reclamation uses SEM in circumstances where CPLM and eDNA testing contradict one another (Hosler 2011).

Quantitative PCR (qPCR) has been celebrated as an early detection method for zebra mussels and is said to be more accurate than conventional PCR because conventional PCR has low sensitivity, poor precision, and requires post-PCR processing (Singh et al. 2014). Quantitative PCR uses target-specific primers to amplify portions of DNA, like conventional PCR, but qPCR also uses a fluorescent probe that provides extra specificity for the base pair region being amplified (Pilliod et al. 2016), which allows the DNA to be counted by a computer detecting the fluorescence. Because no steps are required after the PCR run, qPCR is also much faster than PCR followed by gel electrophoresis. Image-based flow cytometry is a detection method in which water

samples, usually taken by plankton net tow, are run through a cytometer that counts objects that pass over its lens (Frischer et al. 2012); however, image flow cytometers take a picture of each particle, which leaves the researchers to sift through thousands of pictures to identify which particles are zebra mussel veligers (Frischer et al. 2012).

Conclusions

Zebra mussels can be devastating to the aquatic environments they invade, and they are spreading to new water systems, including the Marais des Cygnes River basin in Kansas. There is no simple answer regarding which method is "best" for early detection of zebra mussels. When interpreting results of my study, it is important to remember that it was conducted during the very early stages of a zebra mussel invasion and during a drought. However, I found that, in waters that contain low-density populations (such as the Marais des Cygnes River), performing a large number of plankton net tows and spending large amounts of time looking for settled zebra mussels greatly increased detection. To maximize the likelihood of detecting veligers, particularly early in an invasion when there are low densities, plankton tows should sample a large volume of water, at suitable temperatures, and in multiple locations where veligers are most likely to occur, such as marinas, downwind areas, coves, eddies, and inundated waters (reservoirs, dams, and lowhead dams) (Marsden 1992).

Thirty-minute inspection, settlement structures, CPLM, and eDNA all had their positive and negative aspects. Thirty-minute inspection produced positive detections more frequently than the other methods (51% success rate), but detected zebra mussels only 78 km downstream and at only seven of 12 sites. Visual and tactile inspections are relatively cheap to perform, with person-hours being the single cost, but a limited area

can be examined. CPLM produced positive detections at more sites (10 of 12) and farther downstream (127 km) than the other methods and had a 41% success rate. CPLM was the only method that produced a positive detection at sites (7, 9, 10) that had not previously produced a positive detection by any other method, making it the only method to be exclusively positive at a site. Despite its relative effectiveness in early detection, CPLM is very time consuming and relatively expensive in terms of time required for collection of samples (person-hours), examination of samples (person-hours), and materials used (cross-polarized microscope, plankton net, preservative), and requires microscopy training for veliger identification. Deploying settlement structures in the river was less effective (18%) than 30-minute inspections and CPLM, and only produced positives at three of 12 sites. Settlement structure construction and deployment are relatively simple, though structures need a few weeks to accumulate biofilm before zebra mussels will attach (Wainman et al. 1996). Detecting eDNA via PCR was the least effective method in the Marais des Cygnes River. Though eDNA produced positives at more sites (five of 12) than settlement structures (three of 12), it had only 14% total positives and 49 confirmed false negatives (veligers present in sample, but negative eDNA results); however, it was 93% effective with samples from Melvern Lake, which had a dense population of zebra mussels and veligers.

For early detection of zebra mussels, I recommend that after initial visual and tactile inspection of suitable habitat, if no settled zebra mussels are located, the water body be tested via CPLM of plankton net tows. To maximize the likelihood of detecting veligers, a large volume of water should be sampled, at suitable temperatures (during spawning seasons) and in multiple locations where veligers are most likely to occur.

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VI. TABLES

Table 1. Sample date and mean temperature (SD) at sites in the Marais des Cygnes River and Melvern Lake, Kansas (n=12 except where * denotes n=11).

Sample Date	Mean Temp ^{o}C (SD)
14 Jun 2013	25.5 (4.5)
27 Jun 2013	27.2 (3.9)
10 Jul 2013	26.5 (3.5)
24 Jul 2013	26.9 (3.8)
31 Jul 2013*	23.7 (2.9)
31 Aug 2013	27.7 (3.3)
22 Sep 2013*	19.8 (1.8)
4 Oct 2013*	20.6 (0.8)
9 Nov 2013*	10.3 (1.1)
19 May 2014*	18.9 (1.9)
7 Jul 2014*	26.2 (2.5)
23 Aug 2014*	28.1 (1.9)
28 Sep 2014*	21.1 (1.5)
26 Oct 2014*	18.3 (1.4)
22 Nov 2014*	10.5 (1.1)

Table 2. Percent positive detections for zebra mussel presence in Marais des Cygnes River and Melvern Lake, Kansas, June–November 2013 and May–November 2014, for each of four detection methods: 30-minute inspection, settlement structures, cross-polarized light microscopy (CPLM), and environmental DNA (eDNA). (n=15 for each site shown in parentheses). Lowhead dam sites in bold.

Site Number	30 Minute Inspection	Settlement Structures	CPLM	eDNA
1	100	100 (12)	100	80
2	100	31 (6)	86	13
3	40	0 (12)	46	0
4	**73**	**0 (6)**	**53**	**0**
5	60	0 (13)	60	6
6	**100**	**54 (13)**	**53**	**0**
7	0	0 (3)	40	0
8	**100**	**0 (13)**	**33**	**15**
9	0	0 (13)	6	0
10	**0**	**0 (13)**	**13**	**0**
11	0	0 (13)	0	0
12	0	0 (13)	0	0
Overall %	51	18	41	14

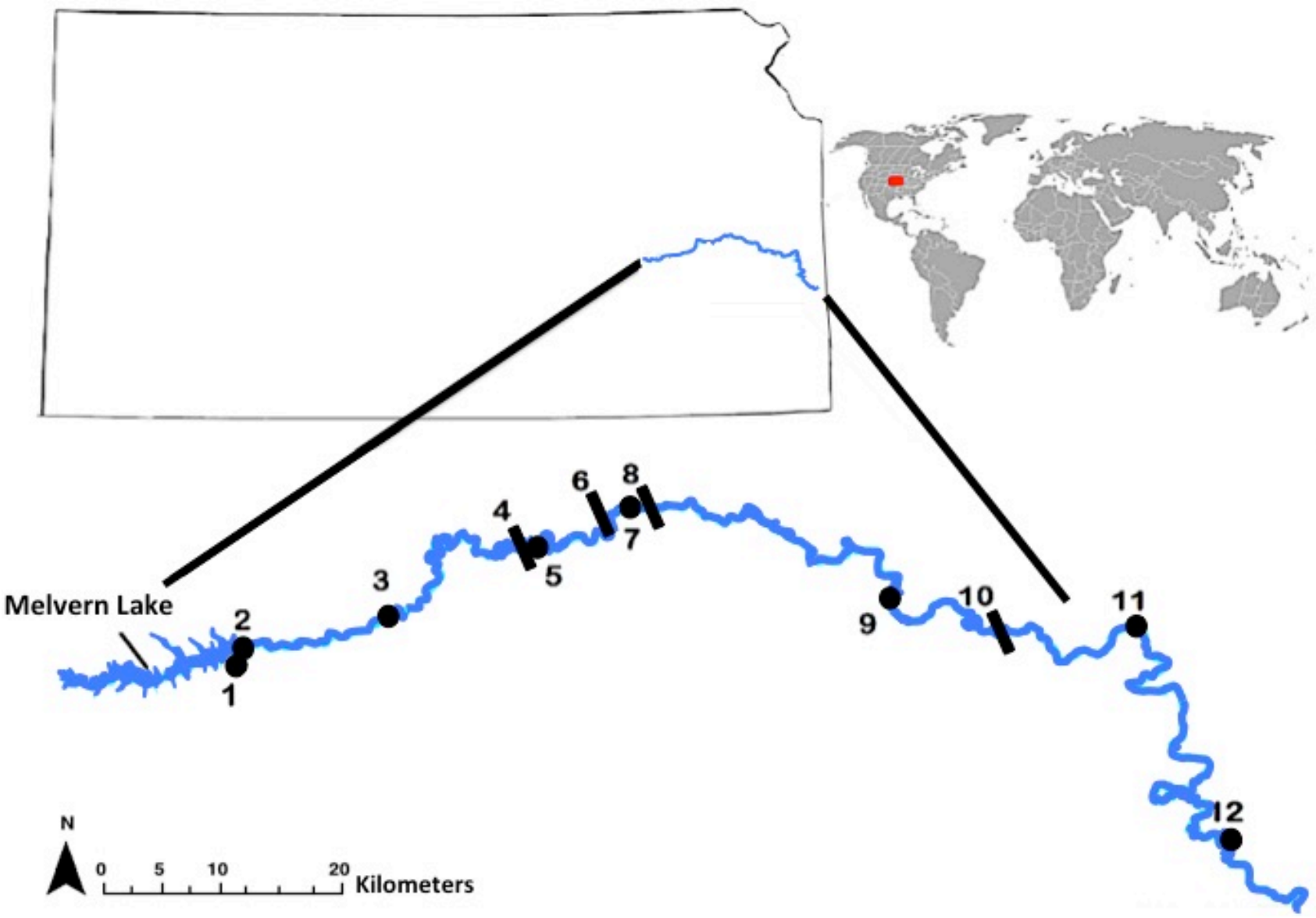
Table 3. Sites positive for zebra mussel presence in the Marais des Cygnes River and Melvern Lake, Kansas, June–November 2013 and May–November 2014, for each of four detection methods: 30-minute inspection, settlement structures, cross-polarized light microscopy (CPLM), and environmental DNA (eDNA). Lowhead dam sites in bold. Sites 7, 9, and 10 were exclusively positive using CPLM.

Method	Site #											
	1	2	3	**4**	5	**6**	7	**8**	9	**10**	11	12
30 Minute Inspection	+	+	+	**+**	+	**+**		**+**				
Settlement Structures	+	+				**+**						
CPLM	+	+	+	**+**	+	**+**	+	**+**	+	**+**		
eDNA	+	+			+	**+**		**+**				

Table 4. Total and mean (SE) number of zebra mussel veligers and recruits at each site at Melvern Lake and in the Marais des Cygnes River basin, Kansas, 2013–2014. Sites inundated lowhead dams denoted in bold (4, 6, 8, 10).

Site #	Veligers			Recruits		
	Total	Mean per Liter	SE	Total	Mean per 30 min	SE
1	14682	1.06	0.43	35605	2373.67	279.59
2	622	0.067	0.038	4378	291.87	43.39
3	19	0.002	0.0015	159	10.6	7.85
4	**35**	**0.013**	**0.009**	**37**	**2.47**	**0.68**
5	22	0.003	0.002	94	6.27	2.44
6	**62**	**0.0023**	**0.0012**	**216**	**14.4**	**4.16**
7	2	0.0016	0.0011	0	0	0
8	**48**	**0.0013**	**0.0008**	**245**	**16.33**	**4.54**
9	6	0.0001	0.0001	0	0	0
10	**4**	**0.0001**	**0.0001**	**0**	**0**	**0**
11	0	0	0	0	0	0
12	0	0	0	0	0	0

VII. FIGURES


Melvern Lake
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Kilometers

Figure 1. Marais des Cygnes River along a 196-km stretch from Melvern Lake (source population, Site 1) in eastern Kansas, USA, showing the 12 sites (source population, Site 1; free-flowing sites 3, 5, 7, 9, 11, 12; and four lowhead dams (differentiated with black bars) sites 4, 6, 8, and 10) sampled for zebra mussels in 2013–2014. ArcMap 10.2.1.

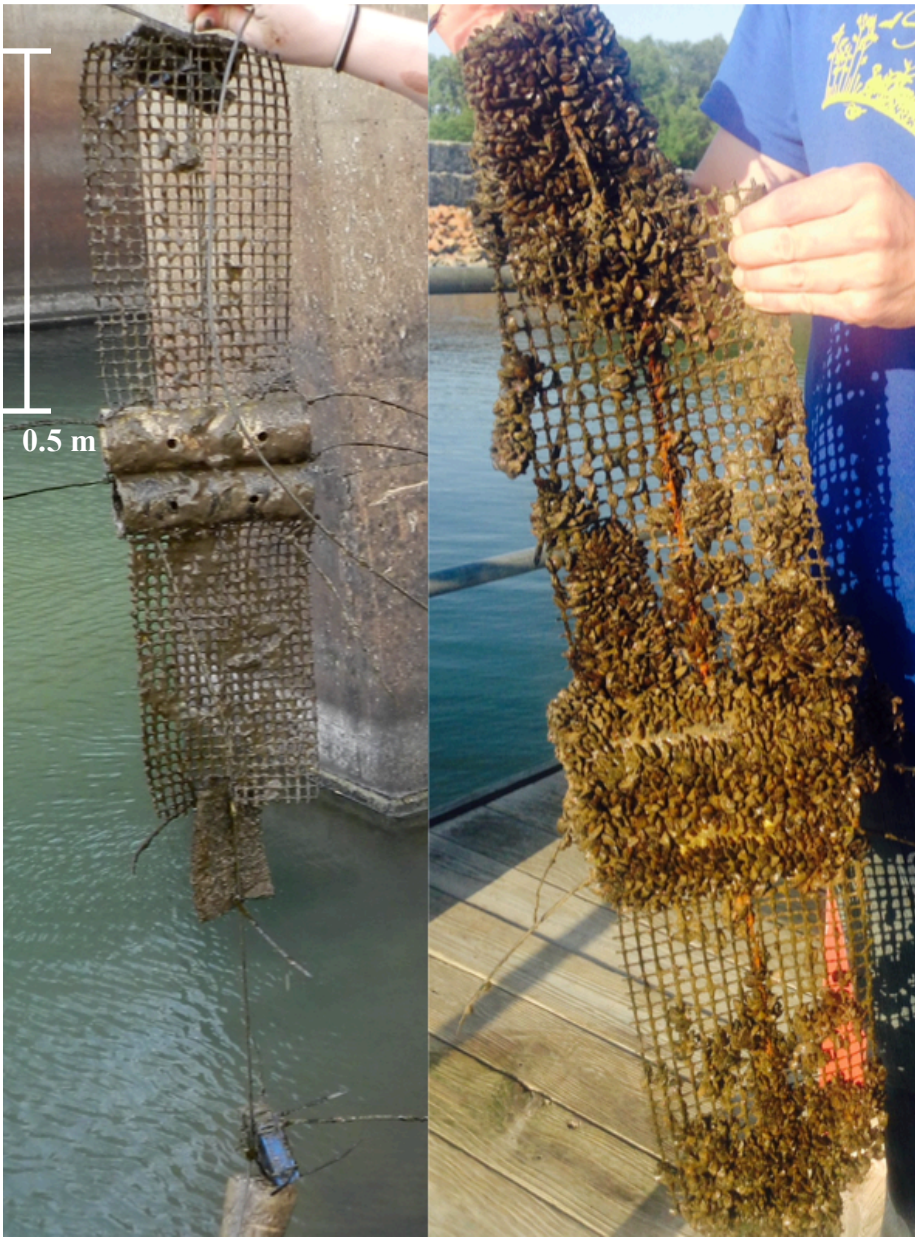

Figure 2. Modified "Portland Sampler" before (left) and after (right) colonization by zebra mussels, deployed at 12 sites in the Marais des Cygnes River and Melvern Lake during 2013–2014.

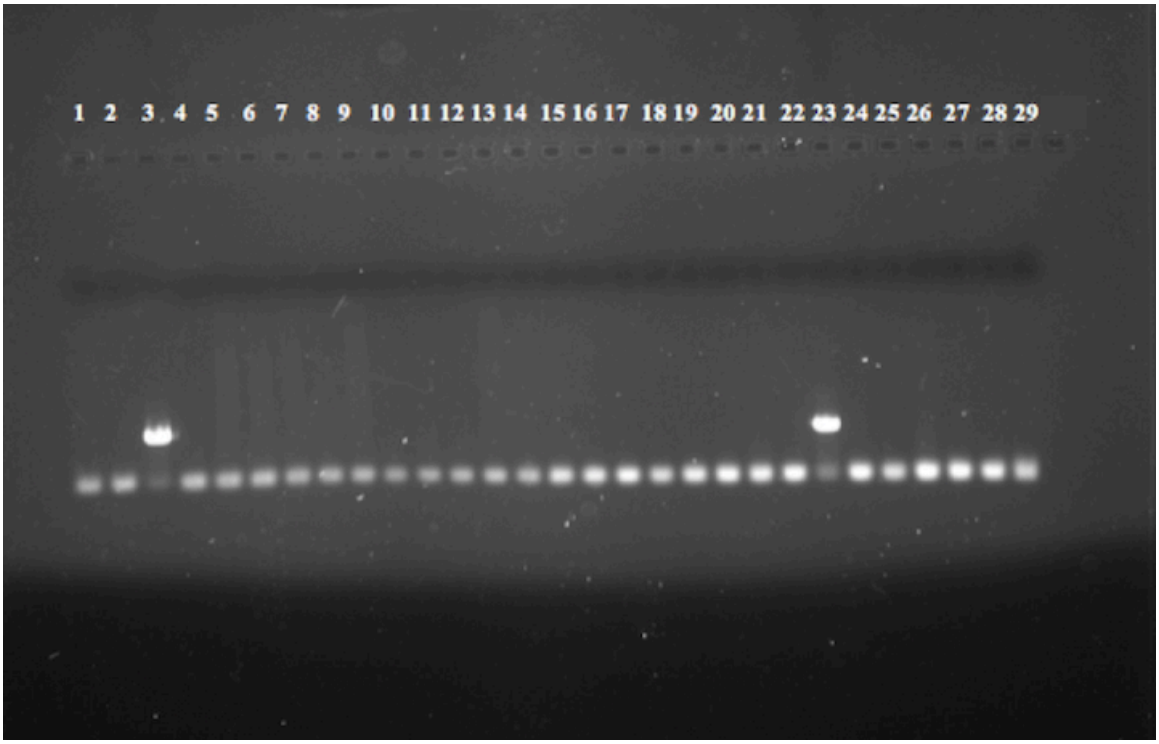

Figure 3. Agarose gel from electrophoresis for zebra mussel detection. Lane 1=nuclease-free water (negative control); 2=Clinton Lake (negative control); 3=pure zebra mussel sample, positive (isolated and donated by Bureau of Reclamation); 5–22, 24–29=negative samples from Marais des Cygnes River (sites 2–12, 7 July 2014 and 23 Aug 2014); and (23) positive sample from Melvern Lake (Site 1, 7 July 2014).

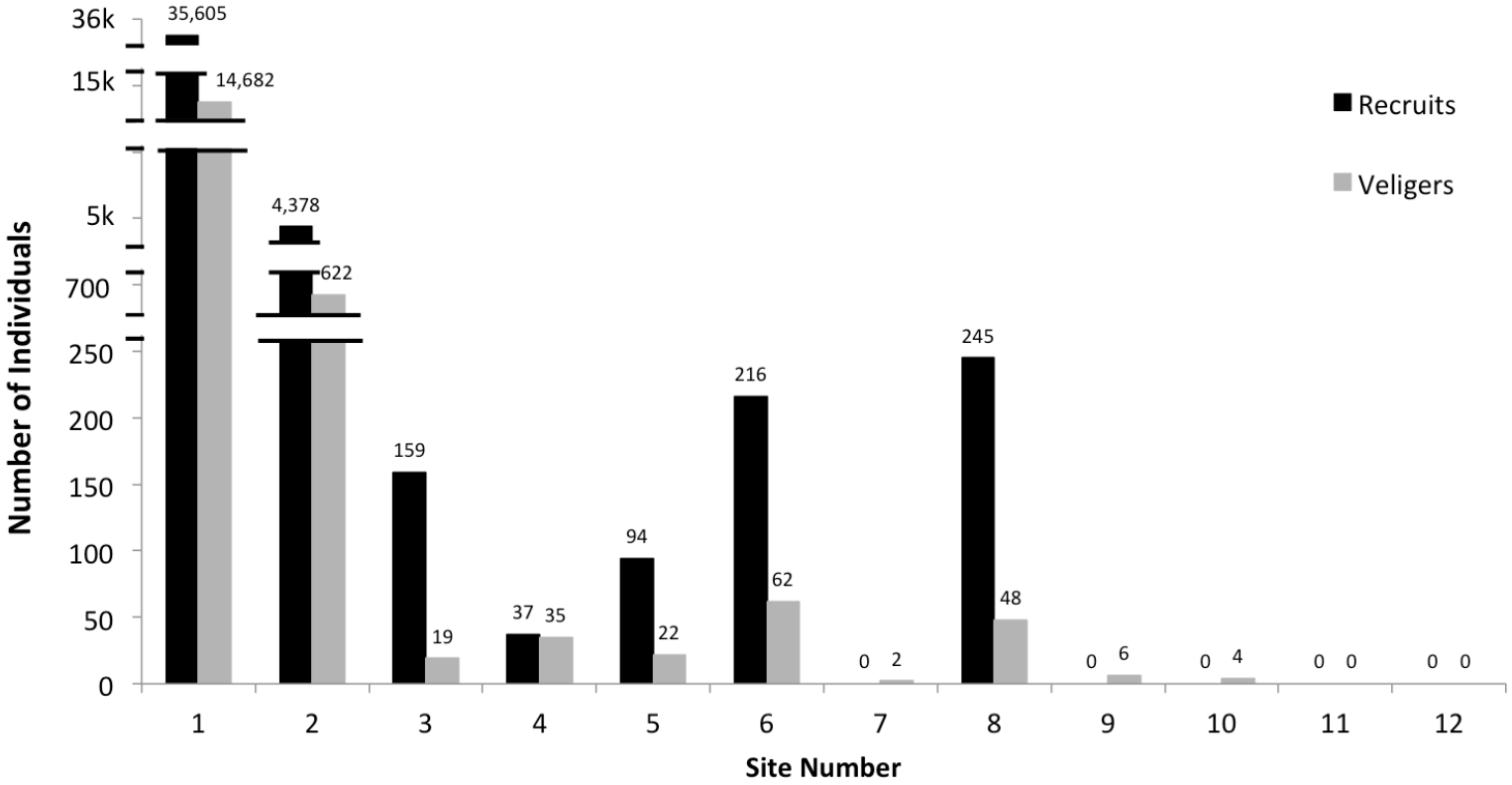

Figure 4. Total number of zebra mussels (recruits/30 min search) and veligers (CPLM) from the Marais des Cygnes River and Melvern Lake, Kansas, June–November 2013 and May–November 2014. Due to the high numbers at Site 1 (Melvern Lake) and Site 2 (Lake Outlet), recruit and veliger numbers are set above a broken axis. n=15 for each site, except Site 7, where n=5. Sites 4, 6, 8, and 10 were inundated by lowhead dams.

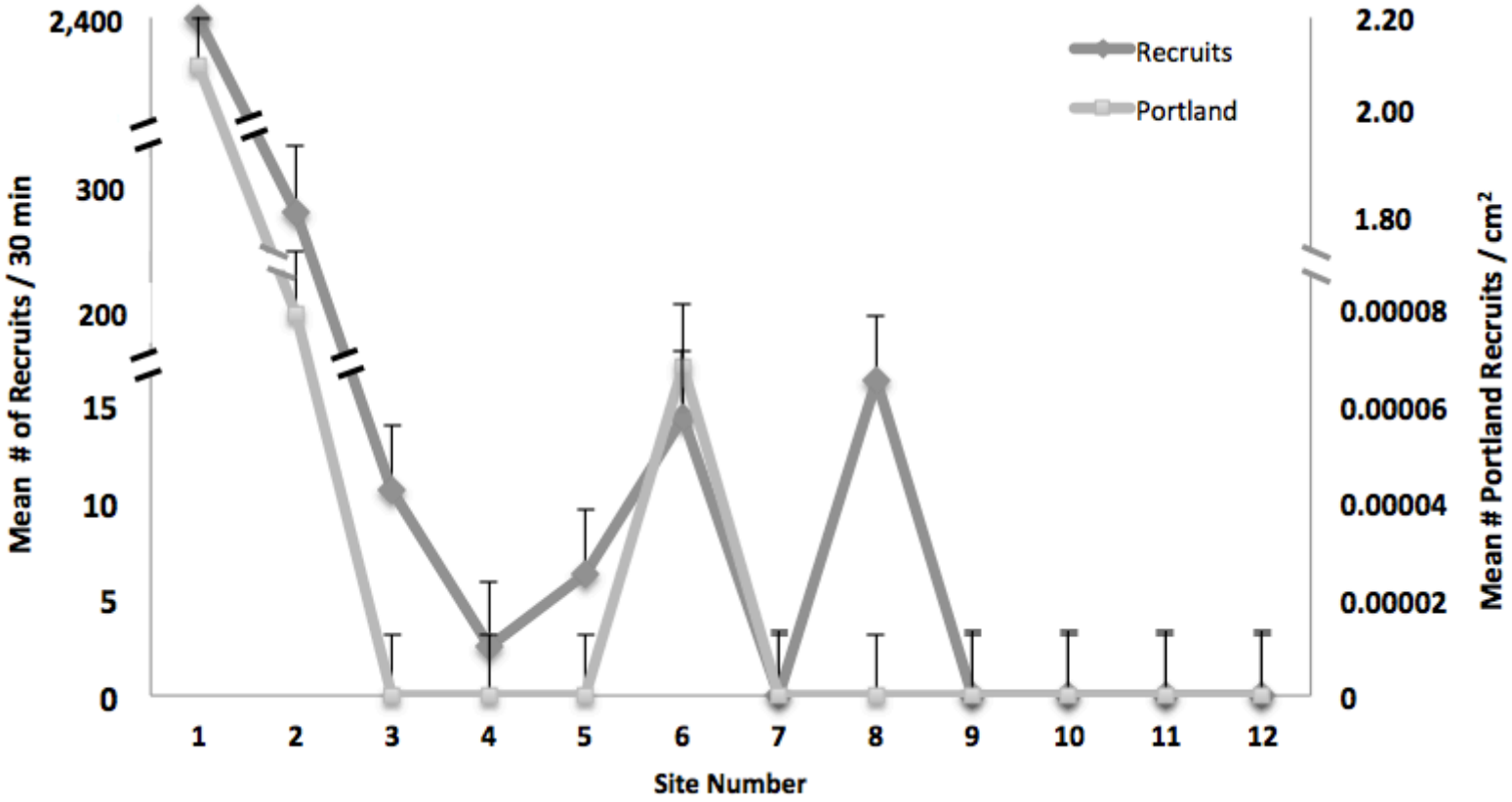

Figure 5. Mean (SE) number of recruits per 30-minute search and per cm^2 on Portland Samplers from the Marais des Cygnes River and Melvern Lake, Kansas, June–November 2013 and May–November 2014. Due to high numbers at Site 1 (Melvern Lake) and Site 2 (Lake Outlet), their recruits and Portland recruits are set above a broken axis. n=15 for each recruit site; n=13 for each Portland recruit site, except Site 7, where n=5.

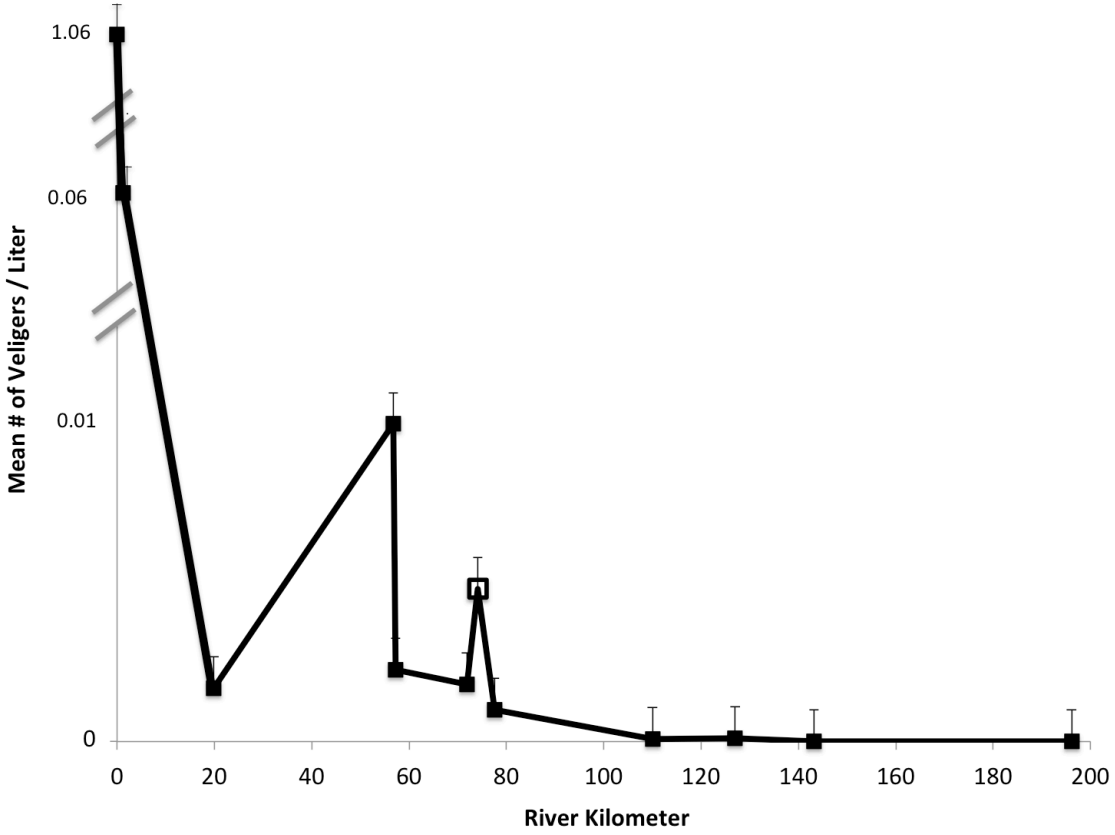

Figure 6. Mean (SE) zebra mussel veliger density at 12 sites along 196 river-kilometers of the Marais des Cygnes River, Kansas, sampled June–November 2013 and May–November 2014. Melvern Lake (Site 1) is set on a broken axis due to its high veliger density compared to other sites. n=15 for each site, except Site 7 (open square), where n=5.

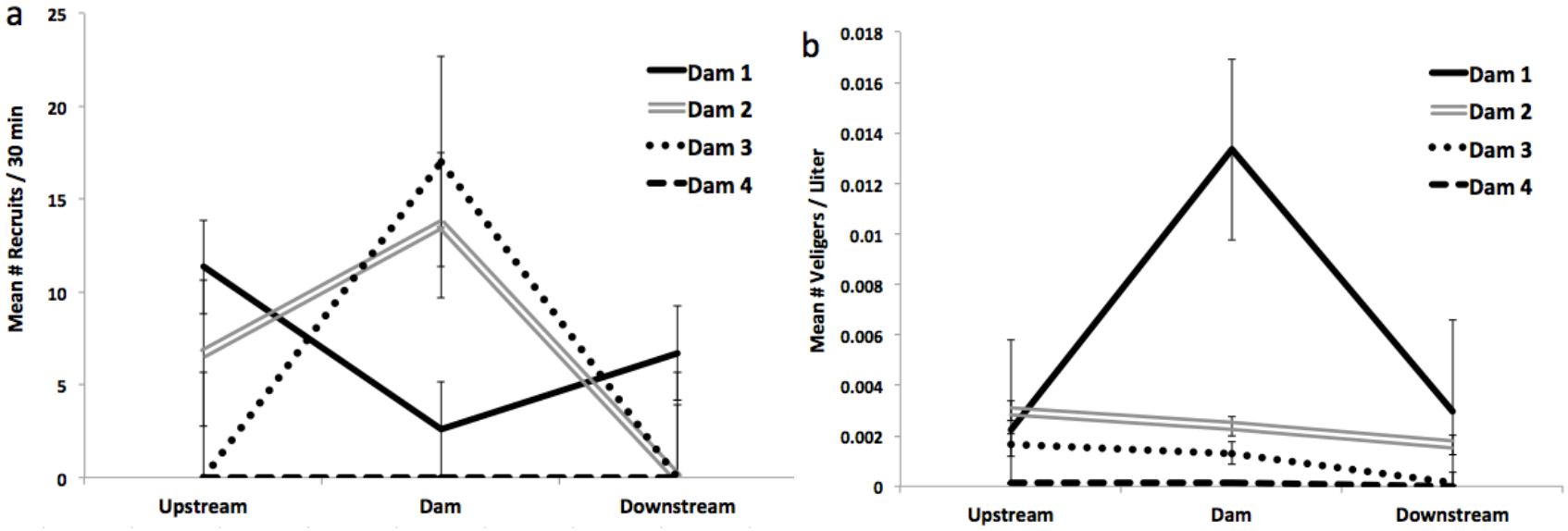

Figure 7. Mean (±SE) (a) recruited zebra mussels per 30-minute inspection and (b) veligers per liter upstream from, at, and downstream from lowhead dams along the Marais des Cygnes River, Kansas, June–November 2013 and May–November 2014. No recruits were found at sites 9, 10, 11 or 12, which included Dam 4 (thick dashed line (b)).

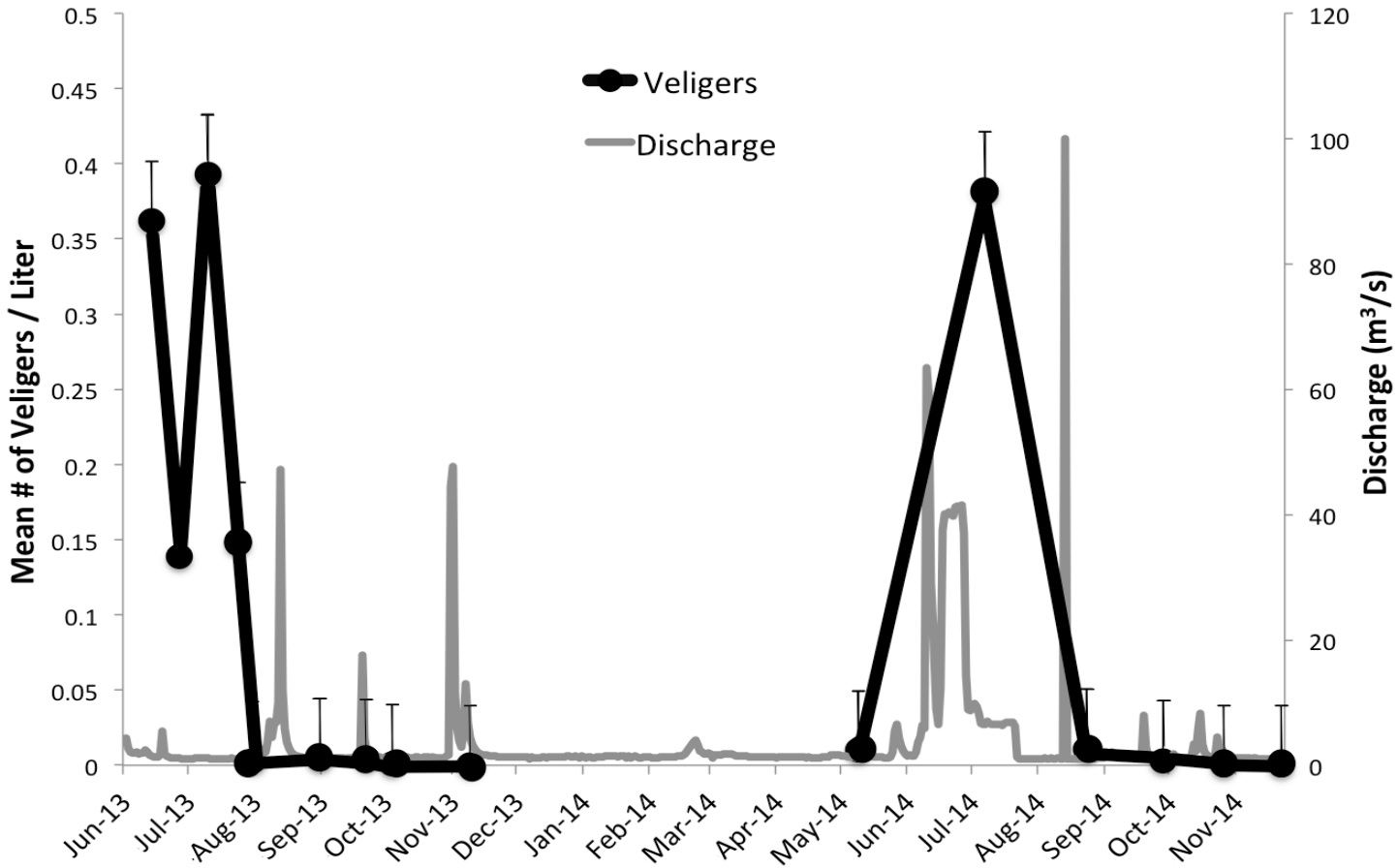

Figure 8. Mean (SE) zebra mussel veliger density (bold line) for each month, June 2013 to November 2014, with mean discharge (m^3/s) per day of the Marais des Cygnes River (grey line) from the Pomona, Kansas, USGS station 06913000, located between Site 3 and 4, June 2013–December 2014.

VIII. APPENDICES

Appendix A. Site number, distance from source reservoir, county, and GPS location for sites sampled in Melvern Lake (source) and Marais des Cygnes River, Kansas, USA, in 2013–2014. Lowhead dam/inundated sites listed in bold.

Site Number	Distance from Source (km)	County	GPS	
1	0	Osage	38.50031274	-95.71436147
2	0.3	Osage	38.51139204	-95.70710562
3	19.9	Osage	38.5367886	-95.56408809
4	**56.8**	**Franklin**	**38.58724489**	**-95.41950589**
5	57.2	Franklin	38.58980781	-95.41591442
6	**71.8**	**Franklin**	**38.61293008**	**-95.34092643**
7	74.1	Franklin	38.62088313	-95.32200373
8	**77.5**	**Franklin**	**38.61829858**	**-95.29325489**
9	110	Franklin	38.53611326	-95.07265621
10	**127**	**Franklin**	**38.50301031**	**-94.95594253**
11	143.2	Miami	38.50465714	-94.833733
12	196.1	Linn	38.3207837	-94.7510341

Appendix B. Positive zebra mussel detections in Melvern Lake (source) and the Marais des Cygnes River, Kansas, USA for each detection method for each sample date in 2013–2014.

	Visual Inspection												Settlement Structures												CPLM												eDNA											
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
14-Jun-13	+	+		+		+		+																	+	+	+			+		+					+											
27-Jun-13	+	+		+		+		+																	+	+	+	+	+	+	+						+	+										
10-Jul-13	+	+		+	+	+		+					+	+											+	+	+	+	+		+	+					+											
24-Jul-13	+	+		+	+	+		+					+												+	+		+	+	+							+											
31-Jul-13	+	+	+	+	+	+		+					+	+											+	+				+		+					+											
31-Aug-13	+	+		+		+		+					+	+											+	+	+	+	+								+											
22-Sep-13	+	+		+		+		+					+					+							+												+											
4-Oct-13	+	+	+		+	+		+					+												+	+			+								+											
9-Nov-13	+	+				+		+					+					+							+	+											+											
19-May-14	+	+		+	+	+		+					+					+							+	+		+	+	+		+					+											
7-Jul-14	+	+				+		+					+					+							+	+	+	+	+	+		+	+	+			+											
23-Aug-14	+	+	+	+	+	+		+					+	+				+							+	+	+	+	+	+							+											
28-Sep-14	+	+	+	+	+	+		+					+					+							+	+	+	+	+	+				+			+	+				+		+				
26-Oct-14	+	+	+	+	+	+		+					+					+							+																+	+						
22-Nov-14	+	+	+		+	+		+					+												+	+											+					+						

CHAPTER TWO–HISTOLOGICAL ASSESSMENT OF ZEBRA MUSSEL REPRODUCTION IN A MIDWESTERN USA RIVER

I. INTRODUCTION

Background

The zebra mussel (Mollusca, Bivalvia, *Dreissena polymorpha* (Pallas, 1771)) is an aquatic invasive species that wreaks havoc on North American waterways and freshwater ecosystems by negatively affecting water quality, damaging submerged materials (clogging piping, fouling boat machinery), and outcompeting native fauna for food and habitat (Herbert et al., 1989; Griffiths et al., 1991). Zebra mussels are native to the lentic habitats of the Black and Caspian seas, and they were transported to the Laurentian Great Lakes watersheds in 1986 as a result of international shipping (Herbert et al., 1989). Since their introduction, zebra mussels have spread rapidly, owing to a combination of their planktonic larvae (veligers) and dispersal by human vectors (e.g., boats, trailers, bait buckets) (Johnson et al., 2001), enabling them to colonize many lakes and rivers in the United States.

Smith et al. (2015) found that zebra mussel larval and adult densities decreased with distance downstream in two Kansas source lake–river systems, but were greater in the impounded waters behind lowhead dams than at nearby free-flowing sites. They hypothesized that, rather than zebra mussels simply accumulating behind these dams, the concrete structure and inundated environment facilitated attachment, recruitment, and dispersal of populations downstream by creating habitat suitable for reproduction. Impoundments, including lowhead dams, aid downstream dispersal of many aquatic invasive species, including Eurasian watermilfoil (*Myriophyllum spicatum*), spiny water

fleas (*Bythotrephes longimanus*), rainbow smelt (*Osmerus mordax*), rusty crayfish (*Orconectes rusticus*), and zebra mussels (Schneider et al., 1998; Allen & Ramcharan, 2001; Havel et al., 2005; Johnson et al., 2008). Lowhead dams, which are typically <5 meters high, cause flowing rivers to slow (Tiemann et al., 2004) and drop suspended loads of particles, including zebra mussel larvae (Horvath et al., 1996). Such dams may support dispersal of zebra mussels by providing an area of slow-moving water, more similar to their native lentic habitat (Strayer, 1991; Neary & Leach, 1992; Johnson et al., 2008), where they can reproduce more readily than in the fast-moving water of a river.

Zebra mussels disperse downstream into rivers from source lakes and reservoirs, forming colonies in slow-moving areas of rivers (e.g., pools, lowhead dams, other reservoirs) and "seeding" downstream populations (Mackie, 1993; Horvath et al., 1996; Bobeldyk et al., 2005; Smith et al., 2015). It is unknown, however, whether these mussels actually spawn instream (downstream–march model) or are entirely products of an upstream source (lake/reservoir) population spawning (source–sink model) (Horvath et al., 1996). Evidence for instream production is lacking, but a dispersal pattern where individuals spawn instream (downstream–march model) and are also continuously seeded by upstream sources (source–sink) seems most likely. Although Smith et al. (2015) hypothesized that instream zebra mussels were contributing veligers downstream, their study was not designed to test for maturation and spawning, thus they could not demonstrate that increased veliger densities at lowhead dam impoundments were not simply accumulations from source populations in upstream reservoirs. I examined seasonal gametogenesis of zebra mussels in a source lake–river system interspersed with lowhead dams, assessing the downstream–march hypothesis that zebra mussels in this

river are capable of spawning and thus potentially contributing to the instream population.

Many investigators have studied reproduction of zebra mussels (Borcherding, 1991; Claxton & Mackie, 1998; Karatayev et al., 1998; Vailati et al., 2001; Juhel et al., 2003; Churchill, 2013; Delmott & Edds, 2014); however, only Jantz and Neumann (1998) have investigated reproduction histologically in rivers. Jantz and Neumann's (1998) examination of zebra mussel tissues revealed two spawning periods per year that correlated with water temperature in the Rhine River, Germany. Temperature plays an important role in zebra mussel reproductive cycles (Mackie, 1991; Ludyanskiy et al., 1993; Nichols, 1996; Delmott & Edds, 2014), as these mussels undergo seasonal maturation that correlates with water temperature (Jantz & Neumann, 1998; Nichols, 1996). In Europe, zebra mussel spawning begins when the water temperature reaches 15^{o}C or higher (Stanczykowska, 1977; Karatayev et al., 1998); however, spawning in North America has been reported starting at 7.8^{o}C or higher (Delmott & Edds, 2014), though this was demonstrated in lentic habitat.

Five reproductive stages have been described in zebra mussels: resting, early development, late development, spawning (mature), and reabsorbing, a change that can be seen microscopically by observing the reproductive organs (Claxton & Mackie, 1998; Juhel et al., 2003; Delmott & Edds, 2014). Several reports have documented zebra mussels achieving sexual maturity at as little as 4–5 mm (Mackie, 1991; Nichols, 1996; Vailati et al., 2001; Delmott & Edds, 2014), though these studies focused on mussels in lentic habitats. I examined zebra mussel gonads histologically to determine whether they achieved a mature stage (i.e., spawning stage) of gametogenesis in a lotic habitat,

comparing free-flowing vs. inundated sites. I also examined seasonal gametogenesis of zebra mussels in a source lake–river system interspersed with lowhead dams, assessing the downstream–march hypothesis that zebra mussels in rivers are capable of spawning and thus potentially contributing to the instream population. Knowing whether zebra mussels reproduce at lowhead dams would add to our knowledge of the factors influencing zebra mussel dispersal in rivers and could help managers establish a timeline for an aquatic invasion by knowing the distance upstream of a potential source population, which could help halt or slow their further spread in the United States (Wacker & von Elert, 2003). To combat their spread, researchers must determine whether zebra mussels can reach sexual maturity in rivers, and which areas of rivers could provide conditions conducive to zebra mussel settlement and reproduction.

Given the lentic nature of zebra mussel native habitat of the Black and Caspian seas and potential stress that river current may have on individuals settled in free-flowing areas (MacIsaac et al., 1992; Claudi & Mackie, 1994), I predicted that zebra mussels in those areas would not achieve a mature stage of gametogenesis, but that the inundated areas at lowhead dams would act as refuges, facilitating not only settlement but also spawning. I also predicted that a greater proportion of mature gametocytes would be found at the same time that greater densities of veligers were found in the water column at all site types (inundated control, free-flowing control, inundated river, free-flowing river) but focusing on those in the river. Finally, given the effect of temperature on reproduction, I predicted that if zebra mussels were capable spawning in the river, their chronology of gametogenesis would be different from that of the source lake due to differing temperatures for these bodies of water. I predict that zebra mussels at river sites

would reach maturity later in the year and would finish spawning earlier than at either of the control sites.

II. MATERIALS AND METHODS

Study Area

I sampled the Marais des Cygnes River and Melvern Lake at 12 sites spanning 196 river kilometers through Linn, Miami, Franklin, and Osage counties in eastern Kansas, USA (Fig. 1). Melvern Lake, which impounds and then feeds the Marais des Cygnes River, tested positive for zebra mussel presence in 2011 (KDWPT, 2011), and by 2013–2014 had a well-established population. I chose river sites based on access, location relative to lowhead dams, and presence of habitat suitable for settled zebra mussels (e.g., rocks, logs, dam faces, unionid shells, etc.), and avoided areas that were less suitable habitat (i.e., sandy, silty, or muddy) (Smith et al., 2015). Sites were distributed as follows (Fig. 1): Site 1 in Melvern Lake as a lentic control/established and potential source population; Site 2 at Melvern Lake outlet channel as a lotic (free-flowing) control; six free-flowing riverine sites (sites 3, 5, 7, 9, 11, 12); and four sites in the inundated water at lowhead dams (sites 4, 6, 8, 10). Lowhead dam sampling sites were located immediately upstream from each dam. As a contrast to the lowhead dam water retention zones, two free-flowing sites were located near each dam, one upstream and one downstream; each free-flowing site was located far enough (0.44–17.0 km) to be out of the direct influence of the dam's inundation (Smith et al., 2015). Free-flowing sites located between two lowhead dams (sites 5, 7, 9) were considered both a downstream comparison site for the dam immediately upstream and an upstream site for the dam downstream.

Field Collection

I collected zebra mussel veligers on 11 sample dates from June 14, 2013–July 7, 2014 (Appendix A). I used a Wildco® (Yulee, FL) Wisconsin Sampler plankton net with a 63-μm mesh, 133-mm (5-inch) diameter opening, and 6-m rope, employing 20 oblique tows from the shore, dam, or dock at each site (Claudi & Mackie, 1994; Wells & Sytsma, 2010; Holoubek et al., 2014). A flow meter (model 2030R, General Oceanics; Miami, Florida) was attached inside the net to measure distance towed to allow calculation of volume of water sampled. The net was cast 5 to 6 meters, allowed to sink, and reeled in at an angle; depth sampled varied by habitat available (river, 1–2 meters; lake, 4–5 meters). I measured surface temperature at approximately 1-meter depth with an alcohol thermometer during each visit (Appendix B).

I also collected settled zebra mussel recruits by hand on the 11 sample dates. Three samples of recruits from the control lake (Site 1, October & November) and free-flowing control (Site 2, November) were exposed to high temperatures in the field and subsequently unusable for histological analysis; therefore, the lentic control samples for recruits represent nine sample dates and the lotic control samples for recruits represent 10 sample dates.

Veliger Identification

Veliger samples were examined under a cross-polarized light microscope (Zeiss, West Germany) at 25, 100, and 400X, and were enumerated without subsampling. Laboratory methodology followed the standard operating procedure of the Bureau of Reclamation (BOR, 2011). Viewed under a cross-polarized light microscope, zebra mussel veligers appear to glow with a Maltese cross in the center (birefringence), which

aids in their detection and distinguishes them from other objects often found in samples (e.g., sand, diatoms, algae) (Johnson, 1995). Although cross-polarized light microscopy does not differentiate among bivalve species, knowledge of the size and morphology of each species allows differentiation (Johnson, 1995). Veligers were identified using guides by Nichols and Black (1994), Johnson (1995), and Wells and Sytsma (2013).

Gametogenesis

To assess zebra mussel reproductive capability in rivers, and to study whether instream zebra mussels were furthering downstream dispersal, I histologically examined variation of gametogenesis (resting, early development, late development, mature, or reabsorbing stage) by site type (inundated control, free-flowing control, inundated river, or free-flowing river). Collected zebra mussels varied in size, but to ensure they were old enough to be capable of sexual maturity (Delmott & Edds, 2014), I chose the largest individuals (at least 7 mm, though mostly >15 mm) from each sample site and sample date for gonad histology. Number of individuals found at river site types were low; however, all individuals found were 7 mm or larger, so they could be analyzed histologically (Appendix B). I stored collected specimens in containers of ambient water on ice (4^{o}C) and transported them to the lab, where individuals were immediately placed in Bouin's Fixative Solution (Thermo Fisher Scientific Inc., Fremont, CA) for at least 3 days (Delmott & Edds, 2014).

I processed zebra mussels using standard histological procedures (Vailati et al., 2001; Delmott & Edds, 2014). I removed the shell and visceral sac for optimal submersion in an ascending isopropyl alcohol dehydration series of 50%, 70%, 80%, two turns in 95%, and two turns in 100%, for at least 30 min each (Sheehan & Hrapchak,

1987). I cleared each sample using Histo-Clear™ (Thermo Fisher Scientific Inc., Fremont, CA) for 1 hour. Samples were then placed into liquid (melted) paraffin (Paraplast Plus, Thermo Fisher Scientific Inc., Fremont, CA) in a paraffin oven (Model 4, Precision Scientific Co., Chicago, IL) set at 60°C and left for 12–24 hrs. This step was repeated with fresh paraffin to ensure samples were fully permeated. Samples were then placed into a mold with liquid paraffin and allowed to cool slowly by being positioned on a differential slide warmer. Paraffin that is cooled too quickly will sink in the middle of the container, so this step was done slowly. Once the paraffin cooled to the touch and hardened, samples were placed in a 0–4°C cooler for 5 min for complete solidification. Molded blocks were trimmed to enable sectioning, and sliced to 10 µm (Vailati et al., 2001) using a rotary microtome (Model 820, Spencer Scientific Corporation, Derry, NH). Sections were sliced from the anterior, middle, and posterior portions of each mussel, which allowed me to locate the gonads even if they were immature or undersized (Mantecca et al., 2003).

I warmed glass microscope slides to ~52˚C on the slide warmer, evenly coated each slide with Haupt's Gelatin Fixative (Humason, 1962), and allowed them to dry for at least 24 hrs. I used drops of warm tap water (~52˚C) to float slices of paraffin-embedded tissue, allowing them to relax from their compressed form caused by slicing, and left slides on the slide warmer for at least 12 hrs until completely dry. Slides were then rehydrated in series (10 min in Histo-Clear, 5–10 min each in 100%, 75%, 50%, and 25% isopropyl alcohol, and then 10 min to full rehydration in dH_2O) to remove the paraffin and rehydrate the tissue, enabling it to be stained. Tissues were stained with Mayer's Hematoxylin (Thermo Fisher Scientific Inc., Fremont, CA) for 3 min, washed with tap

water for 3 min, submerged in Scott's Bluing Solution (Humason, 1962) for 3 min, and rinsed again in tap water for 3–5 min. Each slide was then counter-stained with alcoholic eosin (Thermo Fisher Scientific Inc., Fremont, CA) for 8 min, rinsed with dH_2O for ~2 sec, placed in 75% isopropyl for 1 min, and 95% isopropyl alcohol for 1 min. Slides were then dehydrated completely by submerging in 100% isopropyl alcohol for 5–10 min, and mounted with glass coverslips and Eukitt mounting medium (Thermo Fisher Scientific Inc., Fremont, CA). Samples were examined under a Zeiss light microscope (Zeiss, Germany)**.** I noted sex and classified samples based on a modified gametogenic index (Juhel et al., 2003; Delmott & Edds, 2014), labeling each as one of five stages: resting, early development, late development, mature, or reabsorbing (Table 1).

Data Analysis

Data were analyzed with PASW Statistics for Windows (SPSS version 24.0; SPSS 2016). Due to the strongly positively-skewed data resulting from the much greater zebra mussel abundance upstream, plus the large number of zeroes in the dataset (i.e., zebra mussel absence), the statistical assumption of a normal distribution was not met; transformation only marginally improved normality. The assumption of sphericity, critical for repeated measures ANOVA, was also violated. Thus, the data were analyzed by graphical analysis, determination of presence/absence, examination of measures of central tendency and variability, and non-parametric statistics. Zebra mussel densities were compared among dates (June 14, 2013–July 7, 2014) and seasons (spring, summer, fall) with a Friedman test, the non-parametric counterpart to a repeated measures ANOVA. Post-hoc multiple comparison Wilcoxon signed-rank tests were conducted to

examine pairwise differences between groups, Bonferroni-corrected at an overall α of 0.05 to reduce Type 1 error rate.

III. RESULTS

Maturation

Male and female zebra mussels reached maturity (figs. 2, 3) at all site types (inundated control, free-flowing control, inundated river, and free-flowing river), though not on each sample date, suggesting the same relationship between time/temperature and maturity that has been documented in previous studies (Fig. 5) (Stanczykowska, 1977; Mackie, 1991; Nichols, 1996; Jantz & Neumann, 1998; Karatayev et al., 1998; Delmott & Edds, 2014). In total, a majority of recruits from the control sites (Site 1, 54.4% & Site 2, 56.0%) were in a mature stage of gametogenesis; 52.2% of recruits from inundated river sites were mature, compared to 31.2% mature at free-flowing river sites. In contrast, free-flowing river sites had a greater percentage of recruits in the resting phase (37.6%) compared to the inundated control (15.5%), the free-flowing control (17.0%), and inundated river sites (10.8%) (Fig. 4).

Of the 90 recruits analyzed histologically from the source lake (inundated control, Site 1), 38 were male and 52 were female, and of the 100 recruits analyzed from the outlet channel (free-flowing control, Site 2), 54 were male and 46 were female (Table 2). At Site 1, 63.2% of males and 48.1% of females were sexually mature, while Site 2 had 64.8% mature males and 45.7% mature females (Table 2). All five stages of gametogenesis were found among individuals at both inundated and free-flowing control sites (figs. 4, 5).

I found a total 342 recruits at three of the four inundated sites (4, 6, 8), of which 67 males and 69 females were analyzed histologically. At inundated sites, 58.2% of males and 46.4% of females were sexually mature; thus, sex did not seem to be a discriminating factor for maturity in the river. All stages of gametogenesis were found among the inundated river recruits (figs. 4, 5).

Of the 16 settled zebra mussels found at free-flowing sites (3, 5, 7, 9, 11, 12), four were male and 12 were female (Table 2), five of which were mature (50% of males and 25% of females); thus, site type (inundated or free-flowing) did not prevent zebra mussels from maturing in the river. Thirteen recruits were from Site 5, which was the second closest free-flowing site to the source lake, at 57 river kilometers; the remaining three individuals were not mature and were found at Site 3, which was the closest free-flowing site to the source lake, at 20 river kilometers. All five stages of gametogenesis were found among these free-flowing individuals (figs. 4, 5).

Mature Gametes vs. Veliger Density

Peaks of veliger density coincided with times of high percentages of settled individuals that were in a mature stage of gametogenesis as well as with the peak water temperatures (June & July 2013, July 2014) (Fig. 6). The 14 June 2013 and 7 July 2014 samples had the highest veliger densities (0.36 veligers/liter and 0.39 veligers/liter, respectively) and the highest percentage of mature individuals (66.7% and 58.2%, respectively), while 4 October 2013 and 9 November 2013 had the lowest veliger densities (0.0009 veligers/liter and 0.00009 veligers/liter, respectively) and the lowest percentage of mature individuals (8.3% and 0%, respectively) (Fig. 6).

Veliger densities were statistically different among sample dates (June 2013–July 2014) (Friedman test, $\chi^2_{(10)}$=37.575, p<0.001). Dates with the highest veliger densities (14 June 2013 & 7 July 2014) were significantly greater than the dates with the lowest densities (4 October 2013 & 9 November 2013). The 14 June 2013 sample date had significantly greater veliger densities than both 4 October 2013 (Wilcoxon test, z=-1.992, p=0.046) and 9 November 2013 (Wilcoxon test, z=-2.023, p=0.043), and 7 July 2014 had significantly greater densities than both 4 October 2013 (Wilcoxon test, z=-2.549, p=0.011) and 9 November 2013 (Wilcoxon test, z=-2.668, p=0.008). Proportion of mature individuals found was significantly different by sample date (Friedman test, $\chi^2_{(10)}$=21.976, p=0.003), with July 2013 having more mature individuals than November 2013 (Wilcoxon test, z=-1.826, p=0.033).

Seasonal Variation

Veliger densities were statistically different among seasons (spring, summer, fall) (Friedman test, $\chi^2_{(2)}$=14.157, p=0.001), showing significantly greater densities in spring vs. fall (Wilcoxon test, z=-2.758, p=0.006) and summer vs. fall (Wilcoxon test, z=-3.622, p<0.001) but not spring vs. summer (Wilcoxon test, z=-1.420, p=0.156). Seasons were defined by using the lowest water temperature gathered on each date: 19 May–14 June was designated as spring (<15^{o}C), 27 June–31 August as summer (>15^{o}C), and 22 September–9 November (<15^{o}C) as fall.

Inundated river sites mirrored the seasonal variation in gametogenesis at both control sites more closely than at free-flowing river sites. Mature and late development stage mussels were present in the inundated September sample (specifically, individuals found at Site 6), and mature individuals were present at inundated sites into October,

however not at free-flowing river sites. In May samples, mature and late development mussels were found exclusively at the inundated control site (70% & 30%, respectively), free-flowing control site (70% & 30%, respectively), and inundated river sites (59% & 41%, respectively); however, May samples at free-flowing river sites contained mature (75%) and resting (25%) mussels exclusively (Fig. 5).

IV. DISCUSSION

Dispersal dynamics of zebra mussels in the Marais des Cygnes River were similar to those found in other lake–river systems (e.g., Mackie, 1995; Smith et al., 2015), with decreasing densities downstream from the source lake and peaks of higher densities (both veliger and recruits) at sites inundated by lowhead dams. Lowhead dams slow the river to the extent that veligers are capable of settling out of the water column (Mackie, 1995; Tiemann et al., 2004) and attaching in the inundated areas including on the concrete structure. Though recruits were also found at free-flowing sites, the lowhead dams formed hydraulic retentions zones where higher densities were recruited.

Both sexes achieved a mature stage of gametogenesis at all site types, showing that neither sex nor site type was a factor in the ability to achieve sexual maturity in this river. All five stages of gametogenesis were found at each site type (inundated control, free-flowing control, inundated, and free-flowing), which suggests that riverine populations could feasibly become self-sustaining.

In both sample years, veliger density peaked during June (2013) and July (2013 & 2014), which coincided with the peak number of recruits in a mature stage of gametogenesis (Fig. 6), suggesting that these mature individuals are contributing to river populations. However, veligers can remain suspended in the water column for 1 week to

2 months before growing large enough to settle out and attach to a hard surface (Fraleigh et al., 1993; Martel et al., 1994; Horvath et al., 1996; Horvath & Lamberti, 1999), and given that Tiemann et al. (2004) demonstrated that phytoplankton did not accumulate behind lowhead dams because retention time was insufficient, it is likely that any progeny created from an instream population of zebra mussels seeds downstream populations rather than contributing to the localized area where it was spawned.

Inundated sites mirrored the seasonal variation in gametogenesis at both control sites more closely than at free-flowing sites. Mature individuals were present at inundated river sites into October, however not at free-flowing river sites, suggesting that reproduction at free-flowing river sites ended earlier than at other site types. Mature and late development mussels were the only stage present at both control sites and the inundated river sites in May; however, free-flowing river sites contained mature and resting mussels, suggesting a potential delay in reproduction at free-flowing river sites at the beginning of spawning season.

Horvath et al. (1996) offered support for the source–sink model of zebra mussel dispersal in the St. Joseph River basin, Indiana–Michigan, with recruits dispersing only a short distance downstream from the source (10–12 kilometers). However, in the Marais des Cygnes River, Kansas, I found veligers 127 kilometers downstream from the source lake, and recruits 78 kilometers from the source lake, making the source–sink model improbable for this lake–river system. The high densities of mature recruits that I observed 72–78 kilometers downstream from the source lake at lowhead dams (sites 6 & 8) showed that veligers survived long-distance transport, settled, and were capable of achieving sexual maturity. Zebra mussel dispersal in this river seemed to best fit the

downstream–march model, with lowhead dams serving as stepping–stones for further downstream dispersal, while the source lake continued to contribute to the riverine population. If this conclusion is correct, veligers and recruits will likely continue to colonize further downstream, though it could take many years, due to low densities and potential veliger mortality due to river turbulence. Comparison of the genetic structure of zebra mussel metapopulations in rivers versus source lakes could give more conclusive answers regarding lake–river dispersal dynamics and instream recruitment.

Summary

In the Melvern Lake and Marais des Cygnes River system, zebra mussels achieved a mature stage of gametogenesis at all site types. During months when the highest proportions of zebra mussel recruits were mature, there were also highest densities of zebra mussel veligers, and during months when the lowest proportions of zebra mussel recruits were mature, there were lowest densities of veligers. Seasonal variation of gametogenesis varied among site types, with inundated river sites mirroring both the inundated control site and the free-flowing control site more closely than the free-flowing river sites. Thus, even though zebra mussels are capable of reproducing at each site type, differences between site types could have a profound effect on seasonal reproductive cycles. Evidence for instream production is lacking, but a dispersal pattern where individuals spawn instream (downstream–march model) and are also continuously seeded by upstream sources (source–sink) seems likely.

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VI. TABLES

Table 1. Gametogenic stages in zebra mussels (modified from Juhel et al., 2003 and Delmott & Edds, 2014).

Stage	Female	Male
Resting	Ovaries slack and empty. Contain sporadic remaining ova and blood cells within the ovarian interstitial tissue. Connective tissue also present in ovaries.	Connective tissue present in testes. Follicles empty. May see few early development stage or unidentifiable gametes.
Early Development	Ovaries small with ova and oocytes in early maturation. Central lumina lined completely by germinal epithelium. Haemocytes observed in central lumen and ovarian interstitial tissue.	Round tubule. Edge of testes lined with thick germinal epithelium, a few germinal cells in center of lobes.
Late Development	Ovaries swollen, containing many ova and few oocytes >40um. Germinal epithelia no longer active, forming discontinuous layers, often with one germ cell lining central lumina.	Tubule completely filled by reproductive cells in different maturational stages. Small, mature cells present in center of tubule, large germinal epithelium at periphery.
Mature	Stalk-like (pedunculated) oocytes present. Mature oocytes observed in connective tissue. Ovaries large but showing signs of becoming slack. Germinal epithelium begins to redevelop.	Strong definition of reproductive cells with small, mature spermatozoa found in center of follicle and large germinal cells found at periphery. Spermatozoa tails visible in center of tubule.
Reabsorbing	Many haemocytes observed in ovaries' interstitial and connective tissues. Ovaries slack, with signs of tissue degradation. Oocytes stain much more darkly (basophilic).	Connective tissue present in gonad. Triangular follicles. Many haemocytes present in tubules, generally surrounding residual spermatozoa.

Table 2. Percent of mature male and female zebra mussels at each site in Melvern Lake (Site 1) and the Marais des Cygnes River (2–12), Kansas, 2013–2014. For site locations, see Figure 1. Sites inundated by lowhead dams denoted in bold (4, 6, 8, 10).

Site #	Males (n)	Females (n)
1	63.2 (38)	48.1 (52)
2	64.8 (54)	45.7 (46)
3	0 (2)	0 (1)
4	**61.5 (13)**	**45.5 (11)**
5	100 (2)	27.3 (11)
6	**77.8 (27)**	**45.5 (33)**
7	0 (0)	0 (0)
8	**37.0 (27)**	**48.0 (25)**
9	0 (0)	0 (0)
10	**0 (0)**	**0 (0)**
11	0 (0)	0 (0)
12	0 (0)	0 (0)

VII. FIGURES

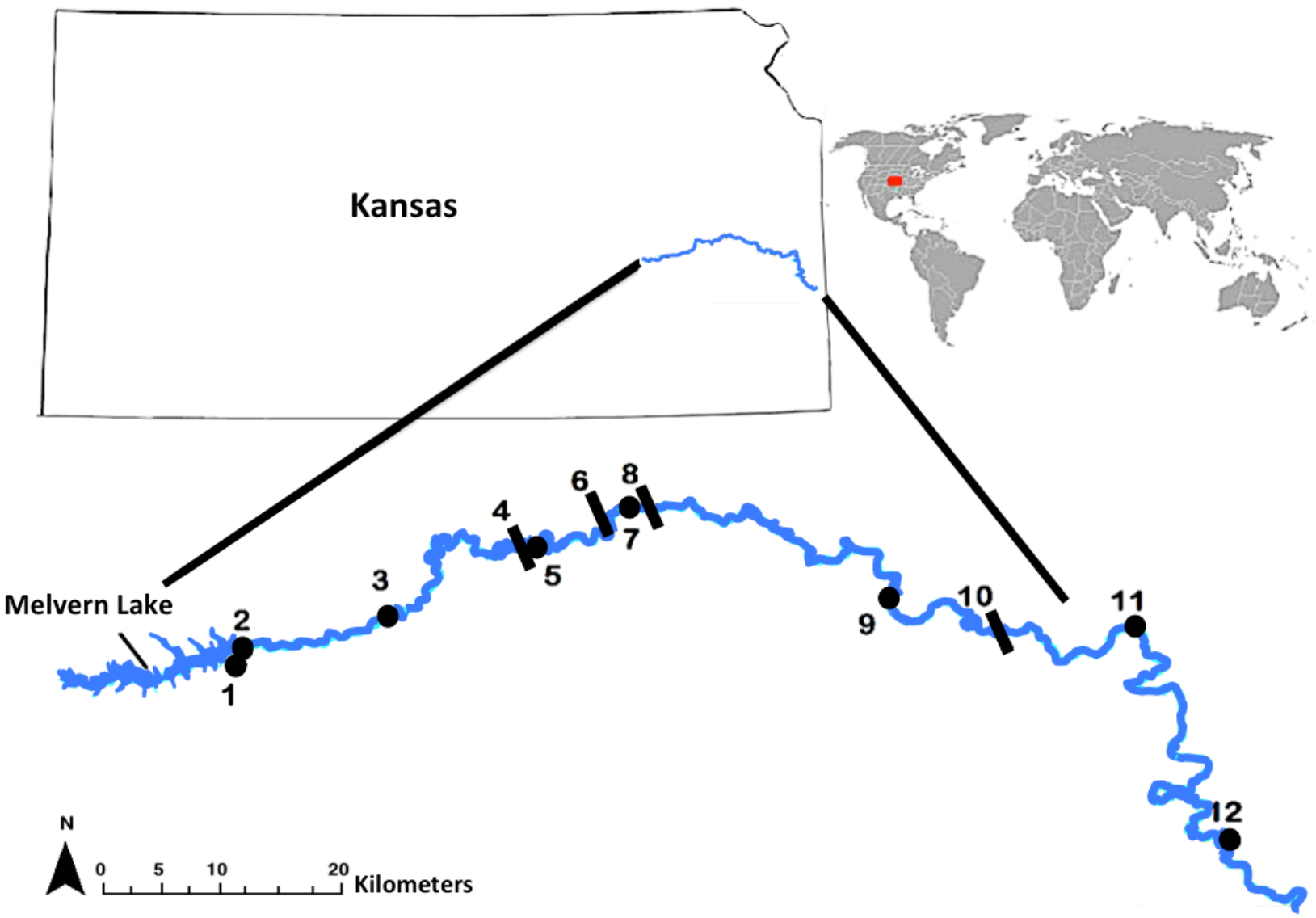

Kansas
Melvern Lake
1
2
3
4
5
6
7
8
9
10
11
12
N
0
5
10
20
Kilometers

Figure 1. Melvern Lake and the Marais des Cygnes River, which spans 196 river kilometers through Linn, Miami, Franklin, and Osage counties, Kansas, USA. Zebra mussel sampling sites in 2013–2014 consisted of three site types: control lake (inundated control, Site 1), free-flowing river control (lake outlet, Site 2), free-flowing river (sites 3, 5, 7, 9, 11, and 12), and inundated behind lowhead dams (black bars, sites 4, 6, 8, and 10).

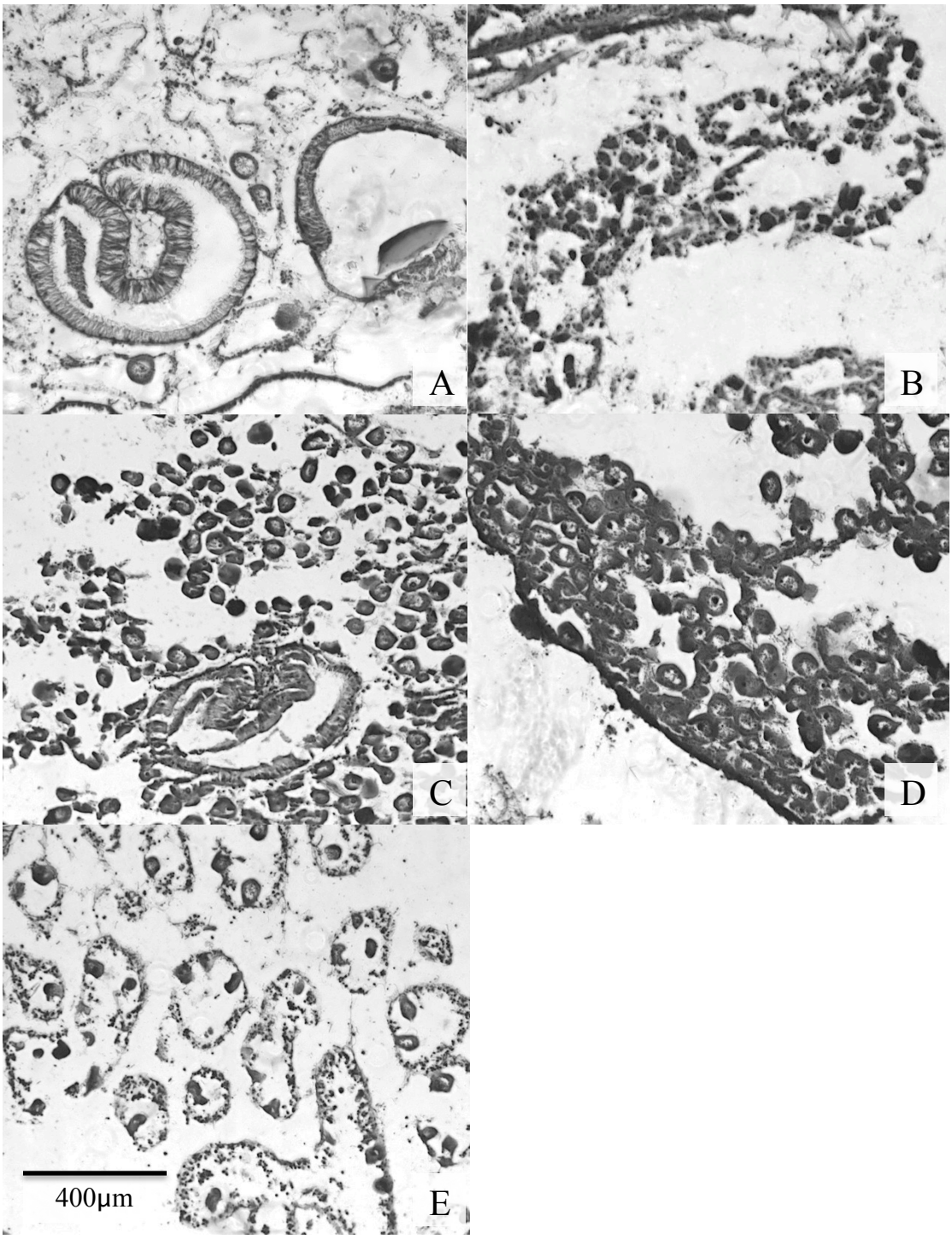


Figure 2. Stages of zebra mussel oogenesis in the Marais des Cygnes River, Kansas, June 2013–July 2014 at 400X. A: resting; B: early development; C: late development; D: mature; E: reabsorbing.

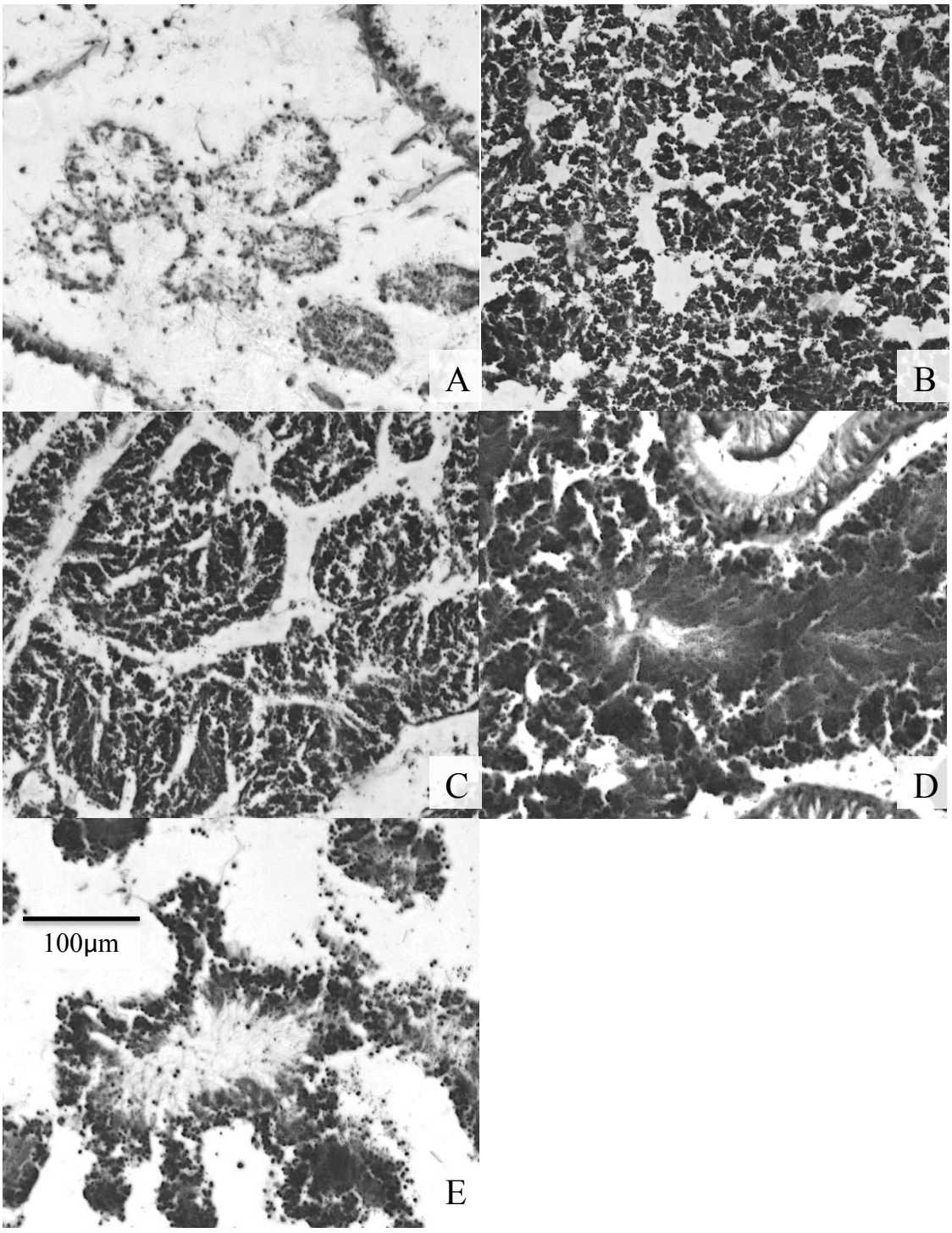


Figure 3. Stages of zebra mussel spermatogenesis in the Marais des Cygnes River, Kansas, June 2013–July 2014 at 400X. A: resting; B: early development; C: late development; D: mature; E: reabsorbing.

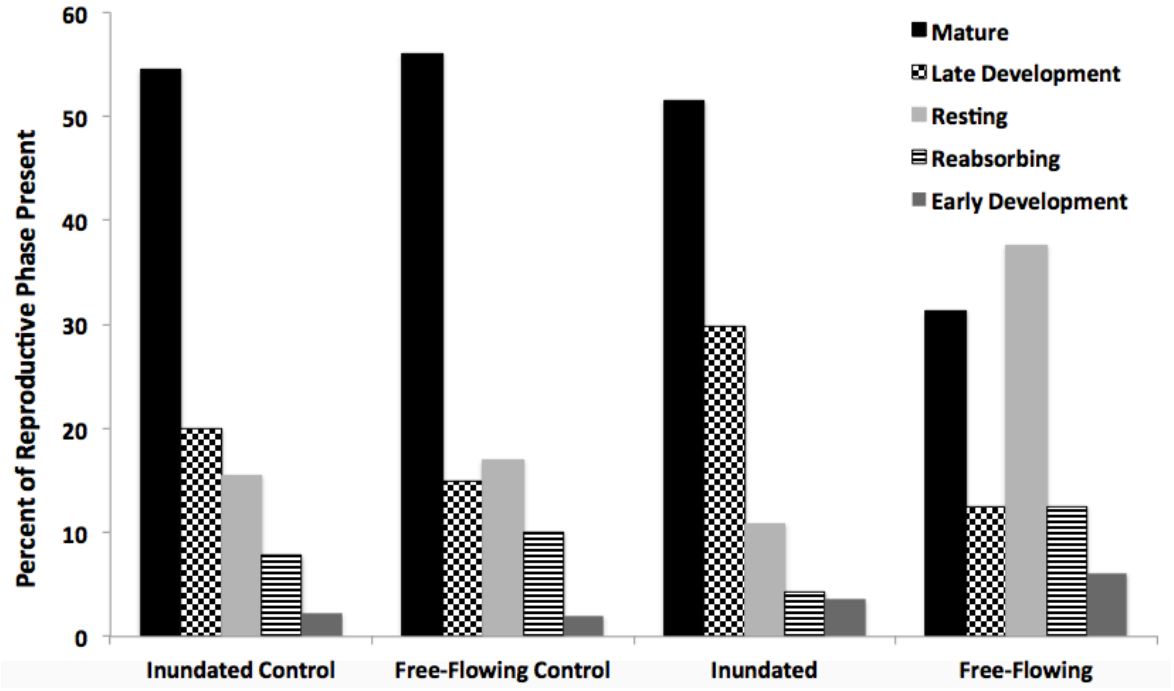
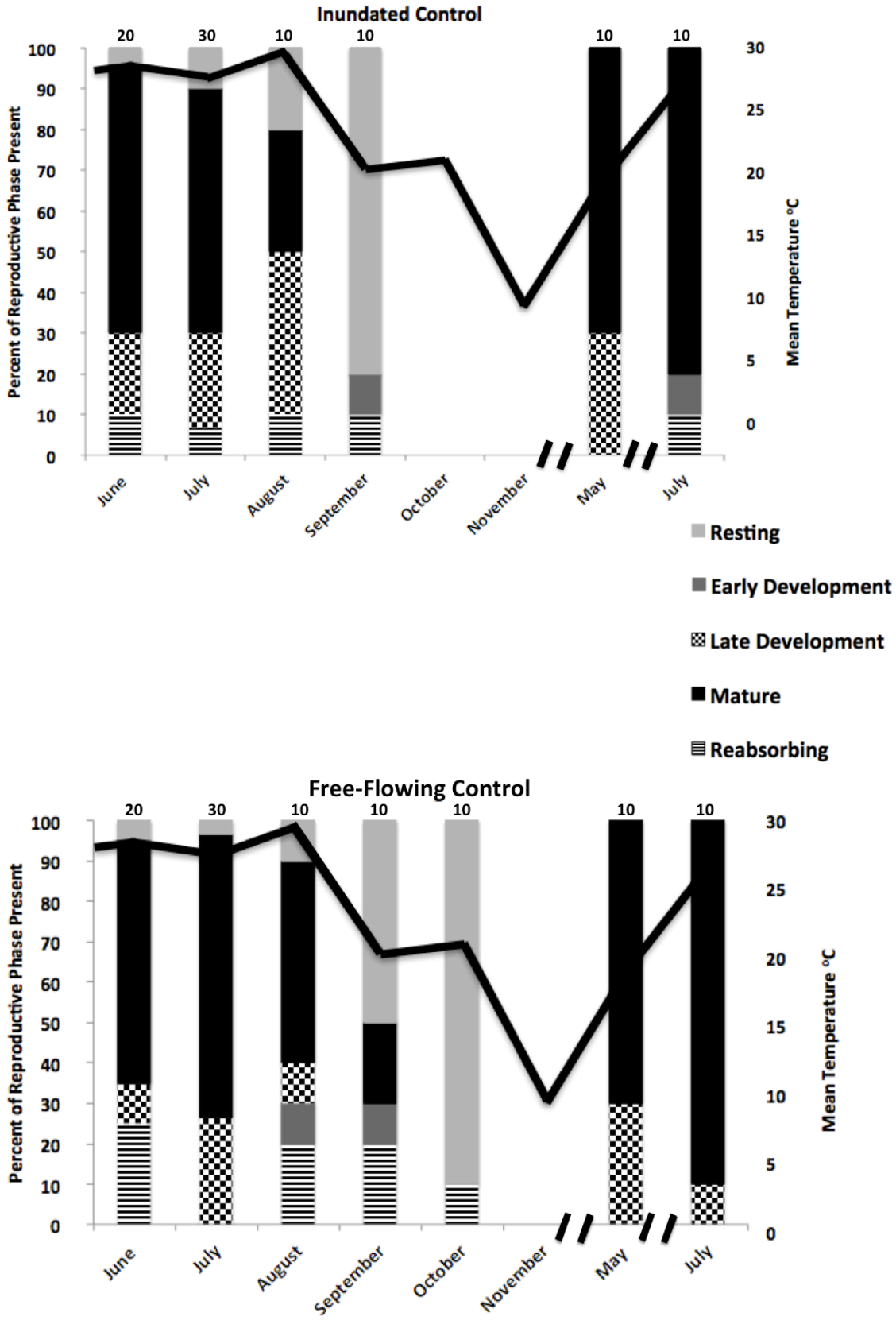


Figure 4. Percent of zebra mussel reproductive stages present across site types (inundated control (n=90), free-flowing control (n=100), inundated river (n=138), free-flowing river (n=16)) in Melvern Lake and the Marais des Cygnes River, Kansas, June 2013–July 2014.


Inundated Control
20
30
10
10
10
10
Percent of Reproductive Phase Present
Mean Temperature °C
June
July
August
September
October
November
May
July
Resting
Early Development
Late Development
Mature
Reabsorbing
Free-Flowing Control
20
30
10
10
10
10
10
Percent of Reproductive Phase Present
Mean Temperature °C
June
July
August
September
October
November
May
July

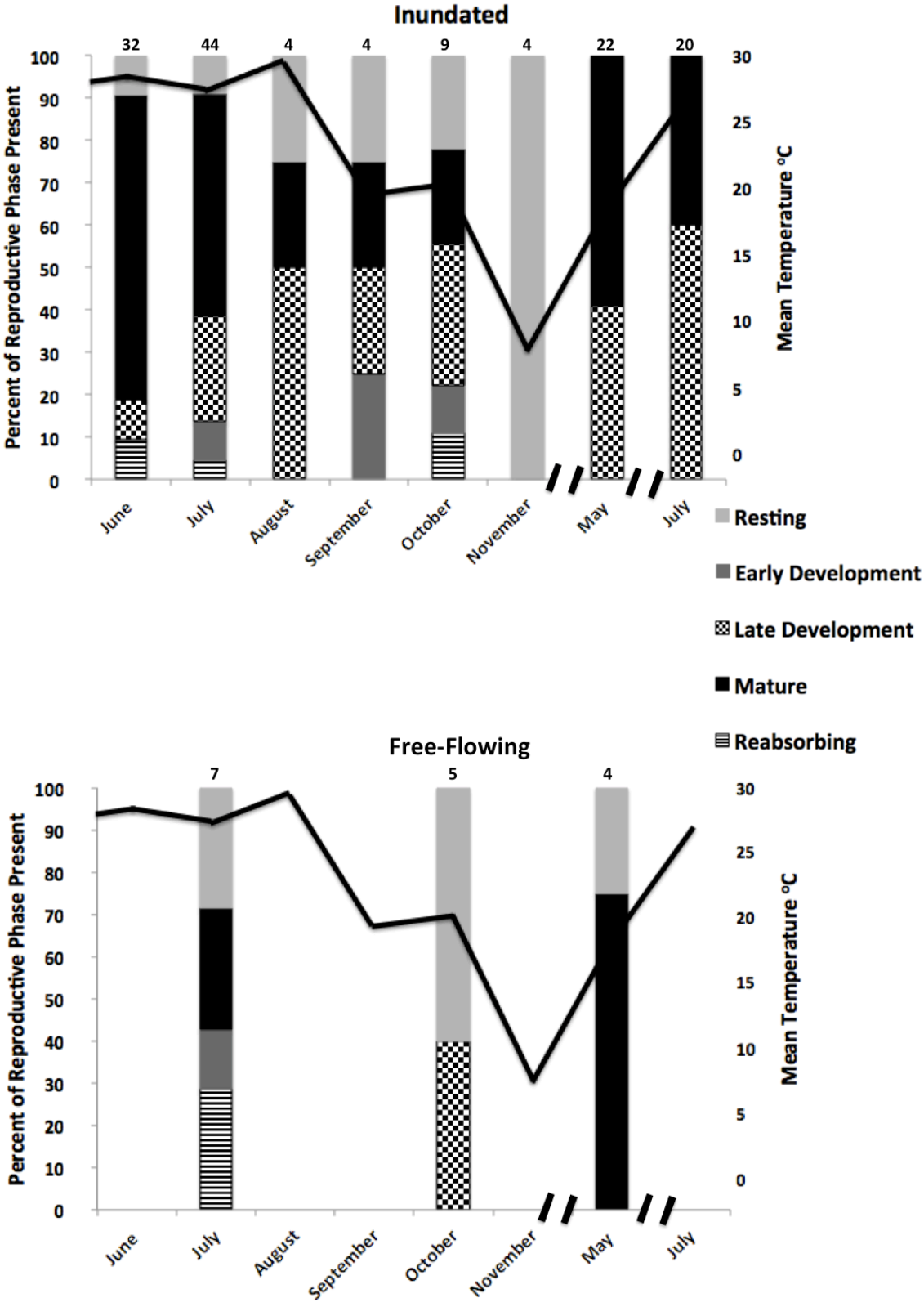

Inundated
32
44
4
4
9
4
22
20
Percent of Reproductive Phase Present
Mean Temperature °C
June
July
August
September
October
November
May
July
Resting
Early Development
Late Development
Mature
Reabsorbing
Free-Flowing
7
5
4
Percent of Reproductive Phase Present
Mean Temperature °C
June
July
August
September
October
November
May
July

Figure 5. Percent of zebra mussel reproductive stages present in recruits from each month and site type (inundated control, free-flowing control, inundated, free-flowing) in Melvern Lake and the Marais des Cygnes River and, Kansas, June 2013–July 2014, with black lines denoting mean water temperature. Numbers on top of bars denote number of individuals analyzed histologically at that site type during corresponding month.

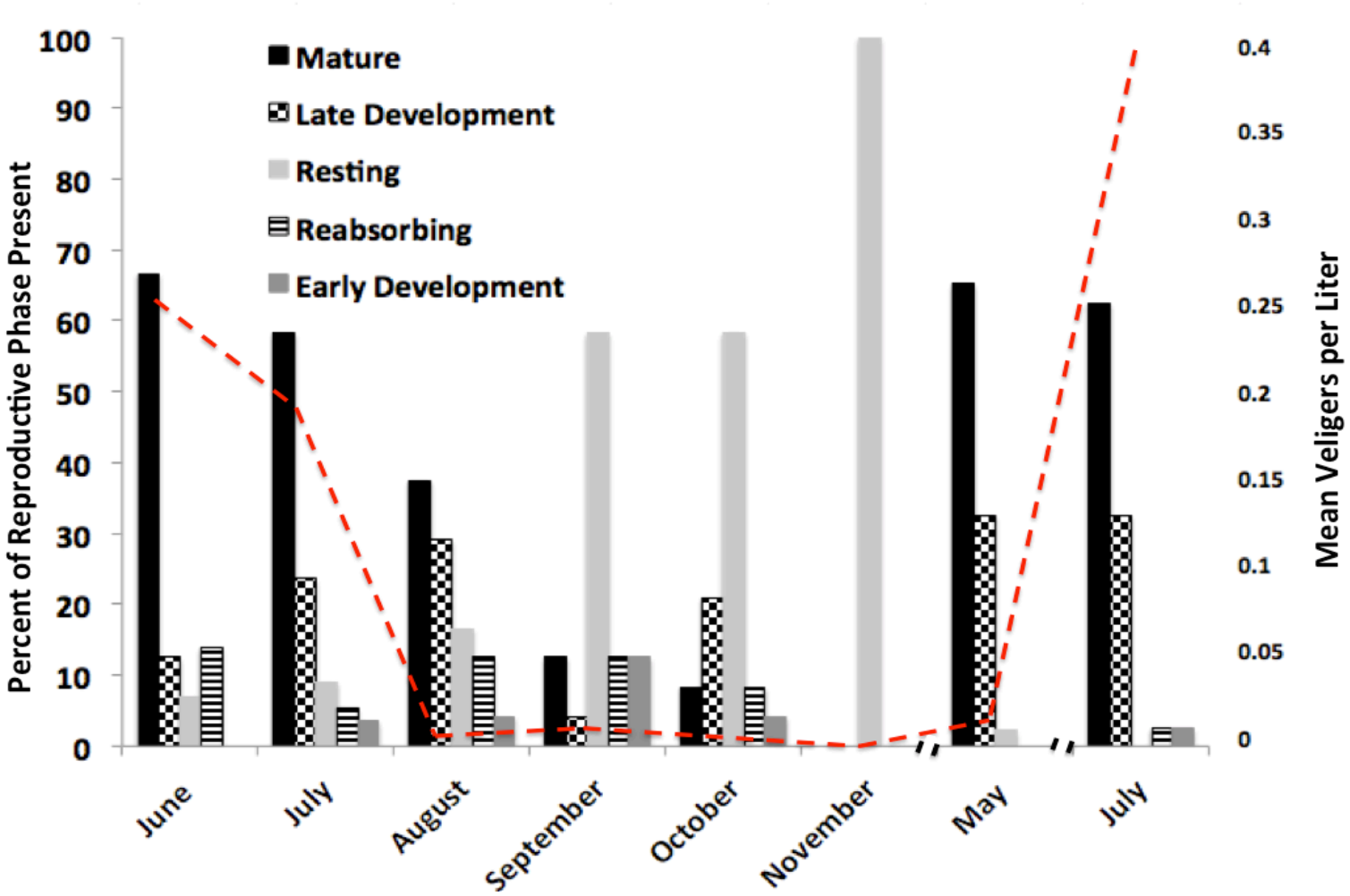


Figure 6. Percent of recruits present for each stage of sexual maturity averaged across site types, with corresponding mean veliger density (dashed line) for each month in Melvern Lake and the Marais des Cygnes River, Kansas, June 2013–July 2014.

VIII. APPENDICES

Appendix A. Sample number and date for zebra mussel sampling performed in Melvern Lake and the Marais des Cygnes River, Kansas, 2013–2014.

Sample Number	Date
1	14 Jun 13
2	27 Jun 13
3	10 Jul 13
4	24 Jul 13
5	31 Jul 13
6	31 Aug 13
7	22 Sep 13
8	4 Oct 13
9	9 Nov 13
10	19 May 14
11	7 Jul 14

Appendix B. Zebra mussel sample size for each site type, with corresponding mean (SD) water temperature (^{o}C) for each sample month in Melvern Lake and the Marais des Cygnes River, Kansas, 2013–2014.

	Inundated Control	Free-flowing Control	Inundated	Free-flowing	Mean Temp (SD)
June	20	20	32	0	26.3 (4.2)
July	30	30	43	7	25.7 (3.6)
August	10	10	4	0	27.7 (3.4)
September	10	10	4	0	19.8 (1.8)
October	0	10	9	5	20.6 (0.8)
November	0	0	4	0	10.3 (1.0)
May	10	10	22	4	18.9 (2.0)
July	10	10	20	0	26.2 (2.6)

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Early detection, dispersal, and maturation of zebra mussels (*Dreissena polymorpha*) in the Marais des Cygnes River and Melvern Lake, Kansas

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Signature of Graduate School Staff

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Date Received