AN ABSTRACT OF THE THESIS OF

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Title: ZEBRA MUSSEL MATURATION AND SEASONAL GAMETOGENESIS IN MARION RESERVOIR, KANSAS

Abstract approved: _____

1. Based on its native distribution and temperature constraints, the invasive zebra mussel (*Dreissena polymorpha*) was not expected to colonize southern portions of the U.S., but it has now spread from the Great Lakes to the Gulf of Mexico. Temperature is critical in zebra mussel reproduction, yet no studies have compared gametogenesis in the cool north vs. the warm south.

2. I studied zebra mussel seasonal gametogenesis in Marion Reservoir, Kansas, histologically in 2011-12, examining monthly gonad development and categorizing mussels into one of five stages: resting, early development, late development, spawning, or reabsorbing. I also histologically examined multiple size classes to determine size at maturity, and measured colonizers on artificial substrates in the reservoir to determine time to maturity.

3. Adults spawned March–August (at 7.8–34.6°C), compared to mid-June–September (at 18–24°C) in the Great Lakes. Sixty percent of zebra mussels were mature at 5 mm; 100% were mature at 7 mm, compared to 7.5–10 mm in the northern U.S. and 5–12 mm in Europe. Zebra mussels reached maturity within 4 weeks compared to 5 weeks in the Great Lakes and Europe.

4. I conclude that zebra mussels in Kansas mature faster, at a smaller size, and that spawning season is longer than farther north, possibly contributing to greater annual fecundity.

Keywords: Zebra mussel, *Dreissena polymorpha*, gametogenesis, histology, spawning, growth, maturity, season.

ZEBRA MUSSEL MATURATION AND SEASONAL GAMETOGENESIS IN MARION RESERVOIR, KANSAS

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PREFACE

My thesis contains a single chapter according to guidelines of the journal Freshwater Biology where I intend to submit my manuscript for possible publication.

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Introduction

The zebra mussel (Mollusca, Bivalvia, *Dreissena polymorpha*) is an aquatic nuisance species introduced from Eurasia into the Great Lakes where it was discovered in 1988 and from which it has rapidly spread (Herbert, Muncaster & Mackie, 1989; Griffiths *et al.* 1991). It was once thought that the species would not colonize the lower Mississippi River Basin region of the United States due to warm water temperatures (Strayer, 1990), but zebra mussels have now spread past New Orleans (Allen, Thompson & Ramcharan, 1999) to river mile 10 near Venice, Louisiana (U.S. Geological Survey, 2013). Zebra mussels have detrimental impacts on industries, recreation, and native species, including unionids (Williams *et al.*, 1993) and fishes (Ludyanskiy, McDonald & MacNeill, 1993; MacIsaac, 1996). There is need to understand the life history and ecology of these biofouling and aquatic nuisance species so that more effective management methods can be developed and further spread can be controlled. To gain a greater understanding of zebra mussels and their potential distributive limits, scientists must develop extensive knowledge of their ecology and biology, including reproduction (Wacker & von Elert, 2003).

Several investigators have studied reproduction and seasonal variation of gametogenesis in zebra mussels in Europe and North America (e.g. Borcherding, 1991, Wang and Denson, 1995; Claxton and Mackie, 1998; Vailati, Bacchetta & Mantecca, 2001), and Nichols (1996) noted that minor environmental changes such as calcium, pH, and suspended nutrients can lead to substantial differences in timing of gamete production. Many studies (e.g., Borcherding, 1991; Nichols, 1996; Ram, Fong & Garton,

1996) have shown that temperature is a major factor controlling gametogenesis and spawning in zebra mussels.

In Europe, zebra mussel spawning begins in May, ends in August or September, and is followed by a resting stage in September that continues until gametogenesis is resumed between late November and February, when gametes develop, until water temperatures rise to >12˚C and spawning begins (Borcherding, 1991; Bacchetta, Mantecca & Vailati, 2001; Juhel *et al.*, 2003; Mantecca *et al.*, 2003). In Lake Erie, zebra mussels begin to spawn in mid-June (Claxton & Mackie, 1998) and cease in September (Gist, Miller & Brence, 1997). In the Erie Canal, New York, zebra mussels begin spawning in June, are spent by August or September, and resume gamete development in November (Wang & Denson, 1995). North American populations spawn at higher maximum temperatures (22–23˚C vs. 17–18˚C) and are more tolerant of high temperature (31˚C lethal limit) than those in Europe (27–28˚C lethal limit) (McMahon, 1996). In both Europe and North America, limited spawning can begin at 12˚C (McMahon, 1996). Zebra mussels most commonly begin spawning in spring and end in fall, with no spawning in winter, except in cooling reservoirs and under laboratory conditions with suitable water temperatures (12–24˚C), in which spawning has been documented year round (Stanczykowska, 1977; Nichols, 1993; Nichols, 1996). In Polish cooling reservoirs, reproduction begins earlier in spring and lasts longer into fall than in unheated lakes (Lewandowski & Ejsmont-Karabin, 1983). Higher temperatures that lead to zebra mussels reproducing earlier in spring or later in fall might allow this nuisance species to have greater fecundity during a year and thus increased rate of population growth.

Temperature is a strong negative correlate of latitude. The 40th parallel north was historically the southern distribution limit of zebra mussels (Nichols, 1996); however, recently zebra mussels spread past the 40th parallel in Europe and Asia (Spain, Turkey, and Syria) (Senckenberg collection unpub. data). The 40th parallel north, which forms the border between Kansas and Nebraska, U.S., is a point of interest in the United States given the historical European range and temperature constraints on populations farther south (Nichols, 1996). The spread of zebra mussels past the 40th parallel to the lower Mississippi River by 1991, three years after their discovery in the Great Lakes (Allen *et al.*, 1999), demonstrated their capability of colonizing southern areas despite temperatures that are lethal to European populations (McMahon & Tsou, 1990). Zebra mussels are still rapidly spreading throughout the United States, despite national attempts to stop them (U.S. Geological Survey, 2013).

As with spawning, zebra mussel growth rates are positively correlated with water temperature (Nichols, 1996), unless temperatures exceed 30˚C (McMahon & Tsou, 1990; McMahon, 1996). In Europe and the Great Lakes, zebra mussels may or may not be reproductive within their first year, depending on settling time, temperature, and calcium constraints that limit growth (Mackie, 1991; Sprung, 1992). Time to maturity determines how fast young cohorts can become reproductive and contribute to the breeding population (Caswell, 1982), and zebra mussels can mature faster in warmer climates than they do in Europe and the Great Lakes (Mackie & Schloesser, 1996; Nichols, 1996). In Kansas, temperature and calcium levels are favorable for zebra mussel shell growth (Whittier *et al.*, 2008); however, it is unknown how soon Kansas individuals mature. Growth rates are important to management because the size of zebra mussels is more

detrimental to industries than their sheer numbers —– larger mussels clog pipes faster than numerous small mussels, reducing the effectiveness of dewatering (a common management practice) (Stice, 1997).

Borcherding (1991) and Vailati *et al.* (2001) noted that very few studies have been conducted on the size and age at which zebra mussels reach sexual maturity; no studies have been conducted on populations south of the 40th parallel. Previous literature from northern populations (U.S. and Europe) has shown that zebra mussels become sexually mature at shell lengths of 5–12 mm (Stanczykowska, 1977; Afanasyev & Protasov, 1988 [5–5.5 mm]; Borcherding, 1991 [7–8 mm]; Garton & Haag, 1993 [8–9 mm]; Wang & Denson, 1995 [7.5–10]; Mackie & Schloesser, 1996 [8–10 mm]; Nichols, 1996 [≥ 5 mm, range 5–12]; Vailati *et al.*, 2001 [≥ 5 mm for males, ≥ 6 mm for females]). Mackie (1991) determined that zebra mussels in the Great Lakes reach maturity in approximately 5 weeks (at 8 mm), but later showed that they can grow as much as 0.5 mm per day (Mackie, 1993). In Europe, zebra mussels can grow 0.5 mm per day, but the growing season tends to be shorter than in North America (Sprung, 1992; Neumann, Borcherding & Jantz, 1993). Shell growth stops in early fall and resumes in spring in Europe (Neumann *et al.*, 1993), however in the lower Mississippi River, zebra mussels grow less in summer and more in winter (Allen *et al.*, 1999).

Most zebra mussel reproduction studies have been performed by sampling veligers (Claxton & Mackie, 1998), and many state and provincial programs, including that of Kansas, rely almost exclusively on veligers to monitor zebra mussel reproduction and population densities (J. Goeckler, Kansas Department of Wildlife, Parks and Tourism, pers. comm.). However, this may be an inconsistent method because water

currents, wind, and daily vertical migration of plankton make collection of larvae inconsistent (Nichols, 1996; Claxton & Mackie, 1998). A more reliable way to examine reproductive effort is to perform histological studies of gametogenesis in adult mussels, because reproductive output can be observed directly, as can sex ratio and possible hermaphroditism (Claxton & Mackie, 1998).

To gain knowledge of zebra mussel reproduction and maturity south of the 40th parallel, I examined zebra mussel seasonal variation in gametogenesis, size at maturity, and time to maturity in Marion Reservoir, Kansas, U.S. I predicted that, due to environmental conditions in Kansas differing from those of Europe and the Great Lakes region, timing of gametogenesis, size at maturity, and time to maturity would differ from that previously reported in the literature. Specifically, I predicted that spawning season would last longer, mussels would mature at a smaller size than those in more northern populations, and mussels would mature faster than those in Europe and the Great Lakes.

Materials and Methods

Study site

I studied zebra mussels in Marion Reservoir, Marion Co., Kansas, N38° 21' 56.50", W97° 5' 20.32", an impoundment of the Cottonwood River in the upper Neosho River basin. Zebra mussel presence was confirmed in this reservoir in 2008, and the population is well-established (B. Smith, Emporia State University, unpubl. data). Marion Reservoir has an approximate surface area of 24.9 km^2, a shoreline of 96.5 km, and a maximum depth at conservation pool of 15 m (Kansas Department of Wildlife, Parks and Tourism, 2013).

Field data collection

Zebra mussels for histological study were collected from rocks, sticks, logs, and debris to which mussels were attached. All mussels were collected near the dam of the reservoir at ~1m depth to ensure they had undergone the same approximate temperature regime (Claxton & Mackie, 1998; Mantecca *et al.*, 2003) and immediately preserved in Bouin's Fixative Solution (Thermo Fisher Scientific Inc., Fremont, CA) (Vailati *et al.*, 2001). Zebra mussels were collected between the 21st and 28th of each month from July 2011 to July 2012.

Temperature monitoring

I recorded reservoir water temperatures with iButton^{TM} (Maxim Integrated Products, Sunnyvale, CA) thermo-sensors placed in a perforated PVC pipe that allowed water to pass through. The iButtons^{TM} were programmed to record temperatures twice a

day, at 0750 h and 1950 h, from July 2011 to July 2012 to provide approximate daily maxima and minima. Data from sensors was uploaded to a personal computer after 13 months and retrieved with iButton Viewer (Embedded Data Systems, LLC, Lawrenceburg, KY).

Seasonal variation in gametogenesis

Adults ≥15 mm in length (umbo to tip of the shell) were used to analyze seasonal gametogenesis because of their relative ease of processing, and because they were certain to be mature (Sprung, 1992). I haphazardly collected at least 25 adult mussels monthly for examination.

I examined gametogenesis via standard histological procedures (Vailati *et al.*, 2001). I stored all collected specimens in Bouin's Fixative Solution for at least 3 days. The visceral sack was removed with a scalpel and forceps, and washed in an ascending isopropyl alcohol series of 50%, 70%, 80%, two washes of 95%, and two washes of 100%, for at least 30 min each, but no longer than 8 h, to dehydrate the specimens (Sheehan & Hrapchak, 1987). After dehydration, visceral sacks were cleared with Histo-Clear™ (Thermo Fisher Scientific Inc., Fremont, CA) for at least 1 h but no longer than 3 h, and placed into liquid (melted) paraffin (Paraplast Plus, Thermo Fisher Scientific Inc., Fremont, CA) in a paraffin oven (Model 4, Precision Scientific Co., Chicago, IL) and left overnight. The oven temperature was set at 60˚C, just above the melting point of paraffin (56˚C), so as not to scorch the specimens or damage the structure of the paraffin (Humason, 1962). This step was repeated with fresh paraffin to ensure no Histo-Clear™ remained in the samples. The paraffin-infiltrated visceral sacks were then placed in

containers of folded aluminum foil, filled with melted paraffin, and placed on a differential slide warmer, which allowed the paraffin to cool slowly. Once the paraffin cooled and a film developed on top, the foil containers containing the specimens embedded in paraffin were submerged into a cold-water bath at 10–15˚C. Cooled paraffin blocks were trimmed and squared for sectioning, and cut to a thickness of 10 µm (Vailati *et al.*, 2001) by a rotary microtome (Model 820, Spencer Scientific Corporation, Derry, NH). Three sections each were sliced from the anterior, central, and posterior portions of each mussel, increasing the chance that if gonads were small or underdeveloped, they would be seen in at least one section of the visceral sack (Mantecca *et al.*, 2003).

Glass microscope slides were placed on a slide warmer at 52˚C and treated with Haupt's Gelatin Fixative (Humason, 1962) for 24 h before sectioned tissue was applied, which aided adherence to the slide. Sections of tissue were floated on drops of warm water (52˚C) placed on the slides to allow compression from slicing to relax. Slides were dried on the slide warmer overnight (12 h or until dry) and then treated in a series of baths to remove the paraffin and rehydrate the tissue for staining. The bath series consisted of 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 distilled H_2O. Sections were stained with Mayer's Hematoxylin (Thermo Fisher Scientific Inc., Fremont, CA) for 3 min, washed in running tap water for 3 min, placed in Scott's Bluing Solution (Humason, 1962) for 3 min, and rinsed again in tap water for 3–5 min. Sections were counter-stained with alcoholic eosin (Thermo Fisher Scientific Inc., Fremont, CA) for approximately 8 min, rinsed in distilled H_2O for 2 sec and placed in 75% and 95% isopropyl alcohol for 1 min each before dehydrating completely in 100% isopropyl alcohol for 5–10 min. Slides

were mounted with glass coverslips and Eukitt mounting medium (Thermo Fisher Scientific Inc., Fremont, CA) (Vailati *et al.*, 2001) and examined under an Olympus BX 51 light microscope (Olympus America Inc., Center Valley, PA) equipped with a digital camera and a calibrated eyepiece. I used the gametogenic index of Juhel *et al.* (2003) to classify mussels into one of five stages of gametogenesis: resting, early development, late development, spawning, or reabsorbing (Table 1).

Size at maturity

To determine the size at which Marion Reservoir zebra mussels reach sexual maturity, in July 2011, I haphazardly collected at least 20 mussels from each of 12 size classes (3–14 mm), a range that extended ±2 mm beyond previously known maturity lengths (5–12 mm) (Stanczykowska, 1977; Garton & Haag, 1993; Nichols, 1996). The same histological procedures used to examine seasonal variation were used to assess sexual maturity (Vailati *et al.*, 2001), however for mussels smaller than 6 mm I sectioned the entire mussel because they were so small that the gonads were difficult to isolate by themselves. Following Vailati *et al.* (2001), I classified mussels in spawning stage if oocytes were ≥ 40 µm or spermatozoa had a flagellum. I examined the 4–7 mm size classes first because I expected to find mature mussels within these groups, and once I discovered a size class with 100% maturity, I assumed that all larger mussels were also sexually mature (Vailati *et al.*, 2001).

Time to maturity

To determine zebra mussel time to maturity in Marion Reservoir, I conducted growth experiments in which four PVC colonization substrates were placed in the reservoir between the 22nd and 28th of each month from March to June 2012. During those months, I checked previously deployed substrates for settlement and growth (for example, in May I checked and measured mussels on all four substrates from March and all four from April). Colonization substrates were made of 5 cm-diameter white PVC piping (Cresline Plastic Pipe Co., Inc., Evansville, IN) cut to 25 cm lengths. The pipe was cut lengthwise and halves held together by hose clamps that could be opened to observe zebra mussels. The PVC substrates were placed at approximately 1 m depth (Wainman *et al.*, 1996), and attached by a 6.4 mm-diameter aircraft cable to the underside of a dock. Zebra mussels on the internal surface only were used and were measured from tip to tip along the longest axis with a ruler.

Results

Seasonal gametogenesis

I histologically examined gametogenesis in 325 adult zebra mussels (150 males, 149 females, 25 indistinguishable, and 1 hermaphrodite). Male and female zebra mussels showed similar patterns of gametogenesis, however there was slight variation in gametogenic development during March and April, when the majority of females were in late development stage but most males were in spawning stage (Fig. 1).

For males, March was the first month of spawning, with 92% (11 of 12) in spawning stage, and August was the last month, with 31% spawning, 46% reabsorbing, and 23% resting (*n*=4, 6, 3, respectively) (Figs. 1, 2). From May through July, 98% (48 of 49) of males were spawning, with only one in reabsorbing stage in July 2011. In September, 100% of mussels (*n*=25) observed were in resting stage, completely lacking gametes and making gender indistinguishable. From October to January, 98% (51 of 52) of males were in early development, with one in resting stage in October. In February, one of 13 males was in late development and the other 12 were in early development; although few males were observed in late development, presumably they did go through this stage sometime between times of observation in February–March. Males began spawning at water temperatures of 7.8–15.5° C, and peak spawning took place between April and July at 16.7–34.6° C (Fig. 1).

For females, March was also the first month of spawning, with 8% in spawning condition, 69% in late development, and 23% in early development (*n*=1, 9, 3, respectively) (Figs. 1, 3). By April, 50% (*n*=7) were spawning and 50% (*n*=7) were in late development. From May–July, 98% (50 of 51) of females were spawning, with only

one in reabsorbing stage in July 2011. August was the last month of spawning, with 8% spawning, 58% reabsorbing, and 33% resting (*n*=1, 7, 4, respectively). In September, 100% of mussels (*n*=25) were in resting stage, completely lacking gametes, making gender indistinguishable. From October to January, 100% of females (*n*=48), were in early development. In February, 18% were in late development while 82% were in early development (*n*=2, 9, respectively). Unlike males, which largely lacked an observable late development stage, females were seen in late development from February to April. As with males, spawning in females did not occur until water temperatures were 7.8–15.5° C, and peak spawning took place between 16.7–34.6° C (Fig. 1).

Water temperature

The highest water temperature recorded was 34.6˚ C in July 2011, and the lowest was 0.6˚ C in January 2012 (Fig. 1). Water temperatures gradually fell after July 2011, and were consistently below 12˚ C between November 2011 and March 2012. The minimum and maximum temperatures of each gametogenic stage in both genders occurred at approximately: 12–34.6˚ C for spawning, 15–30˚ C for resting, 0.5–12˚ C for early development, 5–22˚ C for late development, and 17–34.6˚ C for reabsorbing (Fig. 1).

Size at maturity

None of the 4-mm size class mussels I examined (*n*=20) were mature (Table 2). I was unable to determine gender or stage in nine of these mussels, however four were females in late development stage with well-developed oocytes of 20–35 µm, and two

were females in early development. Five identifiable males were much less developed, but showed beginning signs of spermatogenesis, with germinal cells present. In the 5-mm size class (*n*=20), five males and seven females were mature. Large portions of the testes were in late development in five immature males, but the five mature males possessed fully developed testes with mature gametes. In the seven mature females, there were large numbers of 20–30 µm oocytes, but each female contained numerous oocytes ≥ 40 µm, and therefore they were classified as mature. In three immature mussels, gender was unidentifiable. In the 6-mm size class (*n*=20), 11 females were mature, seven males were mature, and two mussels lacked distinguishable gonads. In the 7-mm size class (*n*=20), all 11 males and nine females were mature (Table 2).

Time to maturity

No mussels were observed on any colonization substrate placed in the reservoir in March–May until June. Seven colonizers < 4 mm were present on the April substrates checked in June, with their small size and the fact that they were not there in May indicating that they had settled recently. Because mussels discovered in June were not at mature length, I used only the colonization substrates checked in July (substrates placed in March, May, and June --- all had colonizers that month) to determine how long it took these mussels to reach a length consistent with maturity; the April substrate was not used because it had mussels growing on it from a previous month.

Mussels on the substrates checked in July (*n*=110) were 3–9 mm after one month or less of growth (Fig. 4). The majority of mussels (*n*=69, 63%) were 5–7 mm, with 6% (*n*=7) ≥ 8 mm. Thus, given that 100% of zebra mussels previously examined were mature

at 7 mm, it is clear that this species can reach maturity within one month in Marion Reservoir. The growth of some individuals (n=3, 3%) to 9 mm within one month indicates a growth rate of 0.29 mm d^{-1} over a 31-day period (9 mm in 31 days).

Sex ratio

Of the 325 adult zebra mussels examined, 150 were male, 149 were female, 1 was hermaphroditic, and 25 were in resting stage and unidentifiable as male or female. There was no significant difference in sex ratio (χ^2=0.0027, df=1, p= 0.95). The hermaphroditic mussel (Fig. 5) was taken on 23 February 2012, and was in early development stage. The mussel was predominantly male, but had developing oocytes in the same vesicles as the testis. Oocytes were observed in all three histological sections of the middle portion of the mussel, but there were no signs of oocytes in the anterior or posterior sections.

Discussion

Seasonal gametogenesis

All zebra mussels examined from September were in resting stage, and gender was not identifiable due to the lack of gametes. This same pattern of gametogenesis was observed in Germany (Borcherding, 1991) and Ireland (Juhel *et al.*, 2003). In Kansas, gametogenesis resumed in October, and zebra mussels remained in early development stage until February. In the northern U.S, in New York (Wang & Denson, 1995) and the Great Lakes (Gist *et al.*, 1997), early development was observed from November–January, but in Ireland (Juhel *et al.*, 2003) early development did not resume until December. In my study, both males and females began to enter late development by February. Northern populations typically enter late development stage as early as March in the Netherlands and New York (Antheunisse, 1963; Wang & Denson, 1995), and as late as June in the Great Lakes (Gist *et al.*, 1997), with most populations in the Great Lakes in late development in May (Haag & Garton, 1992; Claxton & Mackie, 1998). By March in Kansas, 92% of males had entered spawning stage, while 69% of females were still in late development. In April, 91% of males and 50% of females were spawning, and by May 100% of males and females were spawning. Both males and females had spawning individuals from March to August (Table 3). This is a much longer spawning season than in or near the Great Lakes, where spawning typically starts in June or July and ends in August or September (Haag & Garton, 1992; Garton & Haag, 1993; Wang & Denson, 1995; Claxton & Mackie, 1998) (Table 3). It is also longer than in Europe where zebra mussels spawn from May or June–August (Bacchetta *et al.*, 2001; Mantecca *et al*., 2003) (Table 3.). In Ireland, Juhel *et al.* (2003) observed spawning in females from

March–August; however, males did not start spawning until May (33%), hence actual spawning season was from May–August. Therefore, it appears that zebra mussels in Marion Reservoir undergo gametogenesis over a shorter period than mussels north of the 40th parallel, allowing them to begin spawning sooner and cease spawning at about the same time. This could contribute to greater annual reproduction and thereby facilitate the spread of zebra mussels.

Depending on location, the duration of zebra mussel spawning differs from a brief concentrated event to an extended one. Zebra mussels in many northern locations, including the Great Lakes, have brief spawning periods because temperatures are unsuitable for most of the year (Claxton & Mackie, 1988; Gist *et al.*, 1997). However, in power plant cooling reservoirs where temperature conditions remain > 12˚ C, spawning can persist over most or all of the year (Lewandowski & Ejsmont-Karabin, 1983; Afanasyev & Protasov, 1988). In areas where zebra mussels spawn year-round, most gametogenic stages are present at any given time (Lewandowski & Ejsmont-Karabin, 1983; Mantecca *et al*., 2003), while in areas with short spawning seasons, only one or two gametogenic stages are present (Claxton & Mackie, 1988; Gist *et al.*, 1997). In Marion Reservoir, I observed a long spawning season, and males and females were synchronous in gametogenesis 10 months of the year, with only one or two gametogenic stages present. March and April were the two months in which I observed males and females in different stages; males became ripe first and females followed. Highly coordinated spawning has also been observed in zebra mussels in Italy (Mantecca *et al.*, 2003; Binelli *et al*., 2004) and rivers in the northern U.S. (Wang & Densen, 1995); however, in these populations, gametogenesis began in females before males. In

Germany and Ireland, zebra mussel gametogenesis was non-synchronous, with most gametogenic stages being present throughout the year (Borcherding, 1991; Juhel *et al.*, 2003).

Size at maturity

Zebra mussel size at maturity varies greatly among locations, ranging 5–12 mm (Nichols, 1996) (Table 3). I examined mussels 4–7 mm for maturity, and found no 4-mm mussels mature (Table 2). In the 5-mm size class, 60% of the mussels examined were mature, and in the 7-mm size class 100% were mature (Table 2). In Italy, male zebra mussels mature at 5 mm but females do not mature until 6 mm (Vailati *et al.*, 2001), and in a Ukrainian thermal reservoir, 77% of zebra mussels were mature at 5–5.5 mm (Afanasyev & Protasov, 1998). In Ireland, zebra mussels mature at 6 mm (Juhel *et al.*, 2003). In Germany, zebra mussels mature at 7–9 mm (Walz, 1973; Borcherding, 1991; Sprung, 1992; Jantz & Neumann, 1998), and in the Great Lakes region of the U.S. they mature at 7.5–10 mm (Wang & Denson, 1995; Mackie & Schlosser, 1996). Zebra mussels may mature at slightly different sizes due to physical condition and health of the mussel, that is, how it is able to develop sufficient energy reserves for reproduction.

Vailati *et al.* (2001) found that male zebra mussels mature before females, which could be expected because zebra mussels with a smaller body size are less able to store energy for reproduction, and sperm production requires less energy than oocyte production. However, I observed that females appeared to mature and had more developed sex cells at a smaller size than males (Table 2). Females developing before males were also observed in New York (Wang & Denson, 1995) and Ireland (Juhel *et al.*,

2003). Determining why one gender sometimes develops at a smaller size than the other is an area of potential future research.

Time to maturity

The time it takes for zebra mussels to grow to a mature size is based on local factors, such as temperature, food availability, and calcium concentration (Nichols, 1996). In the lower Mississippi River basin in the southern U.S., zebra mussel growth is retarded by extreme water temperatures in summer but is comparable to that of northern populations over an entire year (Allen *et al.,* 1999). In cold climates, zebra mussel growth is much slower than in more temperate climates (Nichols, 1996). In northeastern Poland, zebra mussels may not mature until 2 years after settlement (Stanczykowska, 1977) and in parts of Germany they do not mature until one year after settlement (Sprung, 1992), but in most places they mature within their first year. In other locations in Germany, zebra mussels mature in 6–7 weeks (Borcherding, 1991) to 1 year (Walz, 1973) (Table 3). In the northern U.S., zebra mussels mature in 5 weeks (Mackie, 1991) to 1 year (Mackie & Schloesser, 1996).

In my study, zebra mussels matured in less than 1 month, with some reaching 9 mm within 4 weeks. Such rapid growth is probably due to favorable conditions in Kansas. Thus, zebra mussels are likely reproducing within 4 weeks of settlement in Kansas, and young of the year are likely contributing to the reproductive population. With a spawning season of 5 months, a 4-week time to maturity, and an additional 1-5 weeks for veliger settlement (Neumann *et al.*, 1993), there is the possibility of at least

three generations of zebra mussels per year in Kansas. Such rapid turnover could result in faster spread across Kansas and the southern U.S.

Sex ratio and hermaphroditism

The sex ratio of zebra mussels in Marion Reservoir was 1:1. In Italy, Mantecca *et al.,* (2003) also found a balanced sex ratio. Studies in the Great Lakes (Nichols, 1993) and New York (Wang & Denson, 1995) showed that females were predominant. In Ireland (Juhel *et al.,* 2003), females appeared to have a much harder time recovering from the spawning season than males, and many died throughout the winter, causing the sex ratio to change drastically. In Kansas, it seems that males and females had an equal probability of surviving summer and winter, and that spawning effort did not negatively impact one gender disproportionately.

Hermaphrodite zebra mussels are rare, with many field studies finding none (Wang & Denson, 1995; Juhel *et al.,* 2003; Mantecca *et al.,* 2003) or one (Binelli *et al.,* 2004), although in laboratory settings as many as 8% of mussels are hermaphroditic (Nichols, 1993). The single hermaphroditic zebra mussel found during my study was predominantly male, with a few oocytes intermixed with spermatocytes. Antheunisse (1963) noted that the oocytes and spermatocytes in a hermaphrodite are usually found in separate vesicles, but on occasion can be found in the same vesicle. The individual in my study contained oocytes and spermatocytes in the same vesicles. Only the middle section of the gonad contained oocytes; none was located in the anterior or posterior. This is not consistent with Antheunisse's (1963) suggestion that hermaphroditism begins in the anterior portion of female zebra mussels since my mussel was predominantly male and I

only observed female oocytes in the middle section. I collected the hermaphrodite in February when it was in early development, and I am unable to say whether the oocytes or spermatocytes would have continued to develop or been fertile, or whether this mussel could fertilize itself.

Seasonal gametogenesis vs. veliger abundance

I conducted a post-hoc comparison in an effort to determine whether my histological data was congruent with veliger data from Marion Reservoir collected by the Kansas Department of Wildlife Parks, and Tourism (KDWPT). The KDWPT veliger data were collected monthly April–October 2011 and April–October 2012. In each sampling period, three veliger samples were taken from the down-lake portion of the reservoir with a 63-µm Wisconsin plankton net (Wildlife Supply Company, Yulee, FL) via a vertical tow from a boat, using distance towed to calculate water volume sampled.

Veligers were first detected in April 2012 (0.01 L^{-1}) and May 2011 (1.14 L^{-1}) (Fig. 6), which was consistent with my histological observation of spawning activity in some mussels as early as March. There was an upward trend in veliger abundance following initiation of spawning, with peaks occurring in July 2011 and August 2012 (Fig. 6). Some inconsistency is reflected between the histological and veliger samples with early spawning but the peak in veliger abundance not occuring until much later in the season. Possible reasons for a later peak could include: spawning triggered by conspecifics with presence of gametes prompting further release of gametes; juvenile recruitment, with young-of-the-year beginning spawning with adults; and more than one

reproductive population within Marion Reservoir, with zebra mussels at greater depths spawning at a later time.

Introduction of zebra mussel sperm into holding tanks containing zebra mussels has been shown to trigger release of gametes in mussels that were not currently spawning (Walz, 1973; Nichols, 1993). The peaks in veliger abundance in Marion Reservoir could be explained by small initial releases of male gametes (males were in spawning stage in greater numbers first) triggering other mussels to release their gametes. However, this model does not seem to fit the pattern I observed. By May, all zebra mussels had loose gametes in the lumen, suggesting they were already spawning, and therefore the peak should be seen earlier in the year.

Zebra mussels reached a reproductive size of ≥ 5 mm within 4 weeks, so another possible scenario for the large spike of veligers 3 months after the initial spawn could be that early veligers settled, reached maturity, and then spawned along with the older adults. This model seems likely, with newly settled veligers spawning and contributing to a larger peak.

Multiple populations of zebra mussels have been documented reproducing at varying times within the same large water bodies, such as the Great Lakes (Garton & Haag, 1993) and Lake Como, Italy (Mantecca *et al.*, 2003). Populations at different depths (Mantecca *et al.*, 2003) or horizontal location (Garton & Haag, 1993) can also spawn at different times. Multiple spawning populations could account for large increases of veliger densities if zebra mussels near shore spawn at different times than those deeper in a reservoir. Marion Reservoir is a relatively small reservoir, however, and multiple populations within the same water body have only been documented in large lakes

(Garton & Haag, 1993; Mantecca *et al.*, 2003). However, I only sampled mussels at ~1m, and water this shallow would likely increase in temperature before deeper water in the reservoir. Thus, it is possible that mussels at greater depths in Marion Reservoir (max depth 15 m) could spawn later than mussels at shallower depths.

Because KDWPT trawling data showed veligers to be present during the same times that I observed spawning in adult zebra mussels histologically, it seems that the veliger sampling technique was effective in determining the presence of spawning zebra mussels in this well-established population. The veliger data validate my histological data, given that veligers were, in fact, present during the time period I histologically observed spawning zebra mussels.

Summary

I determined that Kansas zebra mussels spawned from March–August, and this conclusion was supported by KDWPT veliger surveys. Kansas zebra mussels matured as early as 5 mm and reached that length in 4 weeks or less. Because of the long spawning season, small length at maturity, and rapid growth to maturity, I demonstrated the possibility of multiple generations of zebra mussels in one year. This could lead to a prolific spread of zebra mussels in Kansas and other warm regions, increasing the negative effects on aquatic ecosystems and industries that depend on water resources.

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Table 1. Gametogenic stages in zebra mussels (modified from Juhel *et al.* 2003).

Stage	Female	Male
Resting	Ovaries slack and empty. May contain sporadic remaining ova. Many blood cells observed within the ovaries' interstitial tissue.	Connective tissue present in gonad. Follicles empty. May see slight redevelopment of unidentified gametes.
Early Development	Ovaries small with oocytes in early maturation. Central lumina lined completely by germinal epithelium. Haemocytes observed in central lumen and ovaries' interstitial tissue.	Round tubule. Thick germinal epithelium and a few germinal cells in center of lobes.
Late Development	Ovaries swollen, containing many ova and few oocytes > 40 µm. 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.

Spawning	Stalk-like (pedunculated) oocytes present. Mature oocytes observed in connective tissue. Ovaries large but showing signs of becoming slack.	Small, mature spermatozoa found in center of follicle; 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 destruction. Oocytes staining darkly (basophilic).	Connective tissue present in gonad. Triangular-shaped follicles. Many haemocytes present in tubules, generally surrounding residual spermatozoa.

Table 2. Size at maturity of zebra mussels in Marion Reservoir, Kansas, July 2011. n=20 per size class.

Size Class	Number Mature		Number Immature			Total % Mature
	♂	♀	♂	♀	?	
4 mm	0	0	7	4	9	0
5 mm	5	7	5	0	3	60
6 mm	7	11	0	0	2	90
7 mm	11	9	0	0	0	100

Table 3: Zebra mussel spawning, size at maturity, and time to maturity in Europe, northern U.S., and Kansas.*

Location (Latitude range)	Spawning Season (Months)	Months of Spawning Activity	Size at Maturity (mm)	Time to Maturity (Weeks)
Europe N52° 48.83’ – N45° 42'	3	May–Aug/Sept.	5–12	6-7
Northern U.S. N42° 56' – N41° 28'	3	June–Sept.	7.5–10	5
Kansas N38° 21'	5	Mar.–Aug.	5–7	4

*Europe: data from Borcherding, 1991 (~N50° 49' and N 50° 49'); Bacchetta *et al.*, 2001 (~N45° 53' and N45° 43'); Juhel *et al*., 2003 (N52° 48.83'); Mantecca *et al.*, 2003 (~N45° 42'); northern U.S.: data from Hagg & Garton, 1992 (~N41° 39'); Garton & Hagg, 1993 (~N41° 39'); Wang & Denson, 1995 (~N42° 56'); Gist *et al.*, 1997 (~N41° 28'); Claxton & Mackie, 1998 (N42° 43.00’, N42° 44.80’, N42° 45.80’, N42° 38.80’, N42° 40.20’, N42° 41.60’, N42° 37.50’, N42° 39.10’, and N42° 40.20’); Kansas: this study.

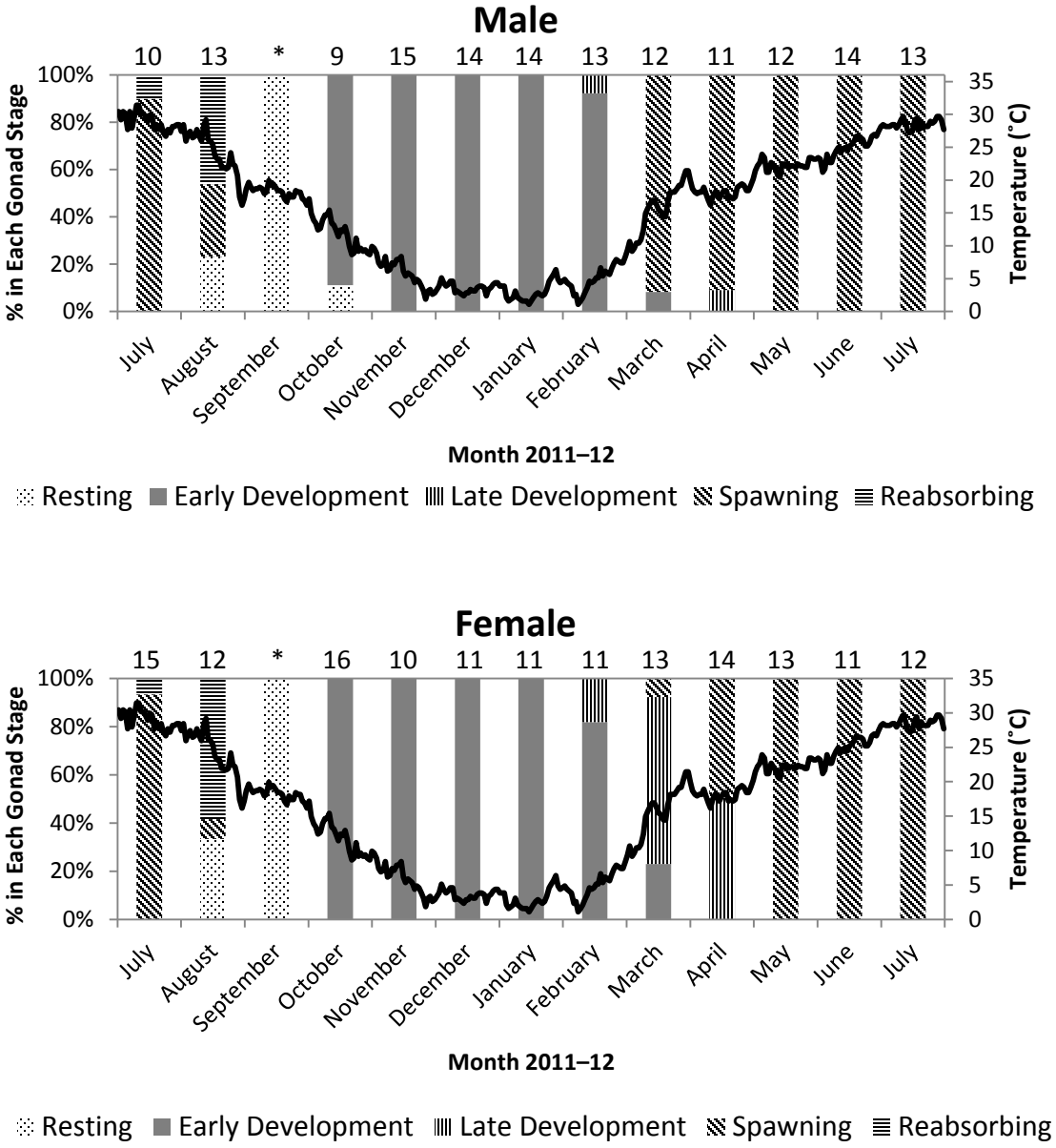

Figure 1. Frequency of gametogenic stages in male and female adult (≥ 15 mm) zebra mussels in Marion Reservoir, Kansas, July 2011–July 2012. Black line represents mean daily temperature. Numbers above bars represent sample size each month (*n*=25 total male and female; February hermaphrodite not included). Asterisk indicates gender indeterminable; resting mussels lacked gametes, and all mussels were in resting stage in September.

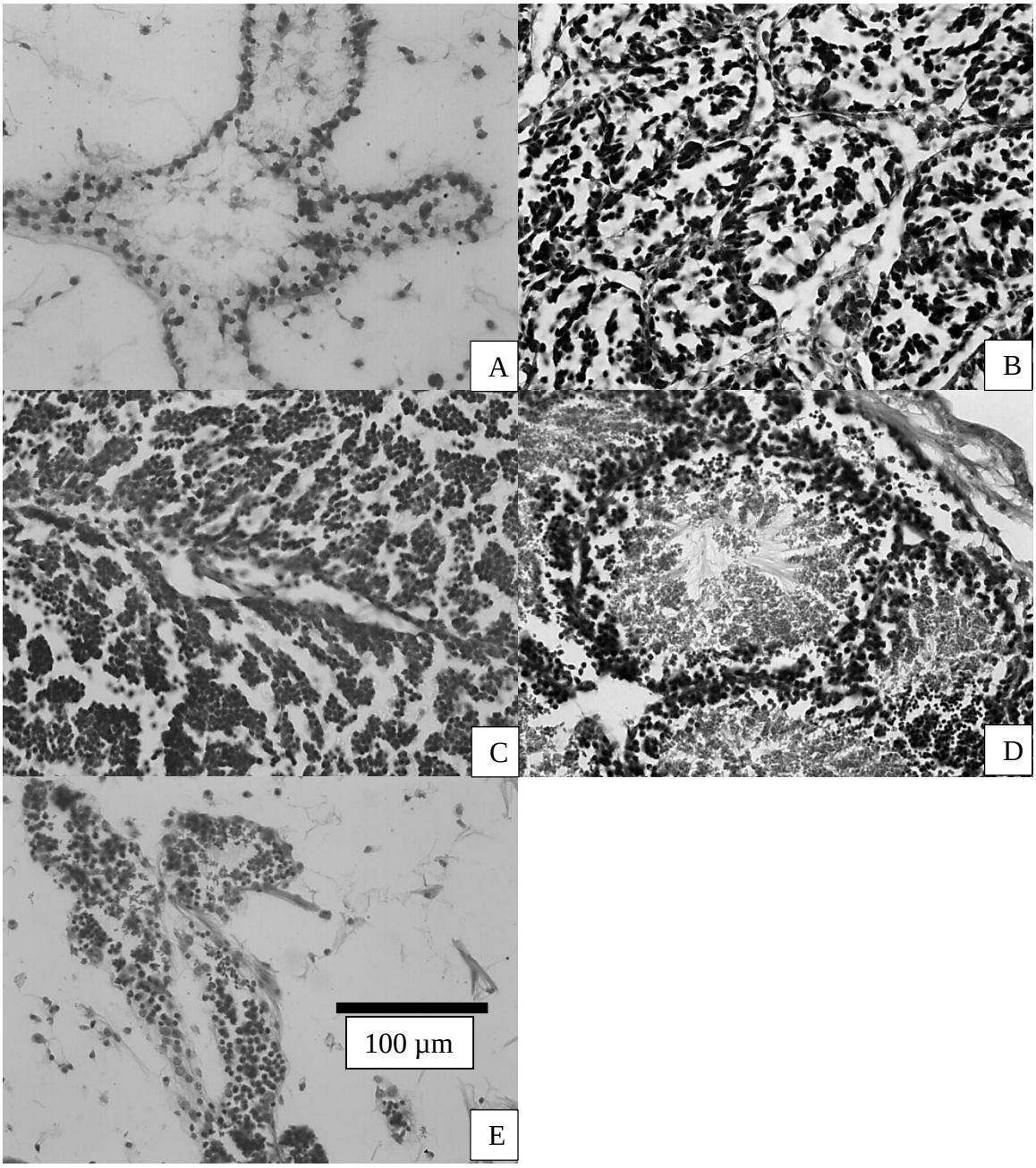

Figure 2. Stages of zebra mussel spermatogenesis in Marion Reservoir, Kansas, July 2011–July 2012, at 400x. A: resting; B: early development; C: late development; D: spawning; E: reabsorbing.

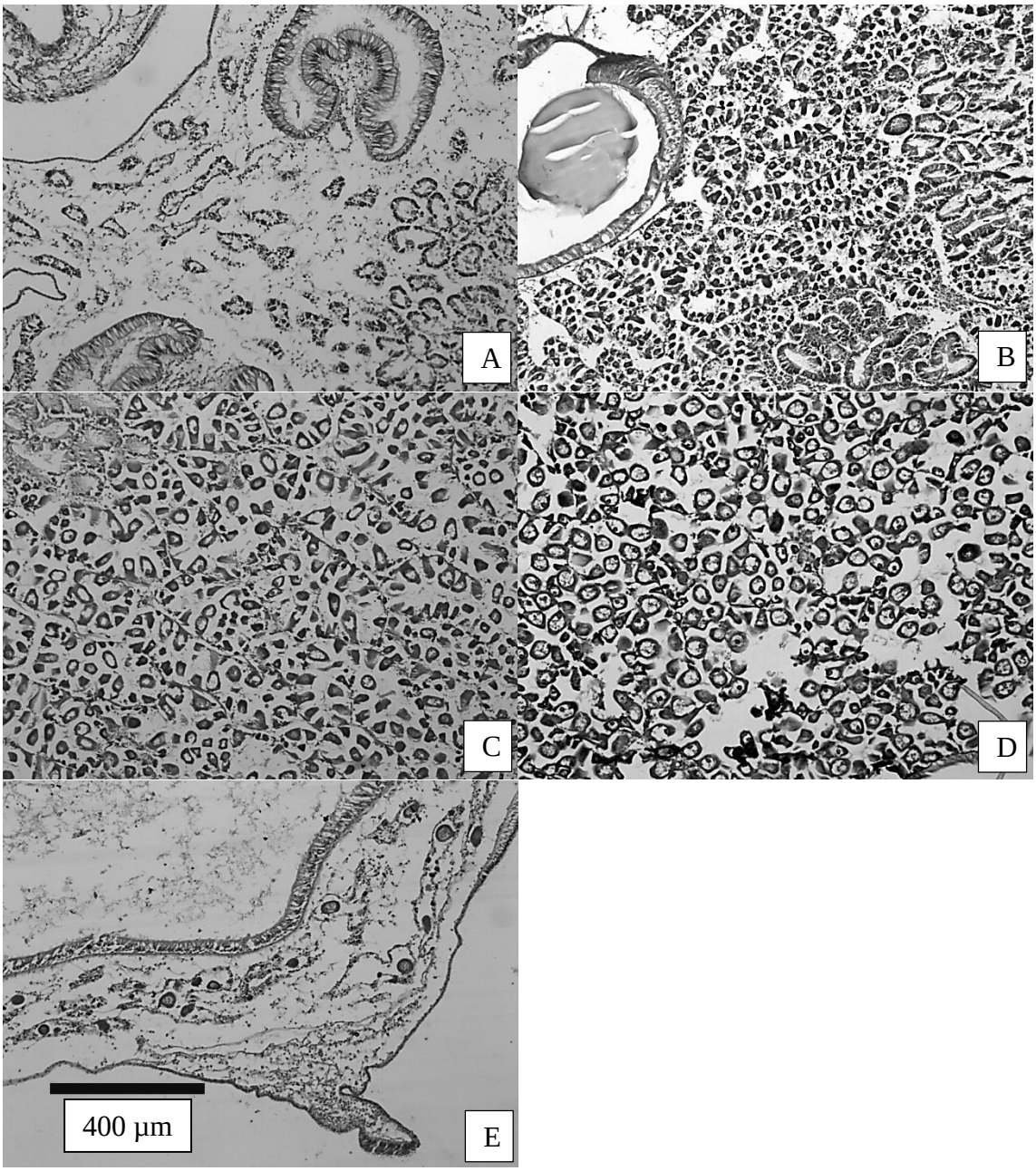

Figure 3. Stages of zebra mussel oogenesis in Marion Reservoir, Kansas, July 2011–July 2012, at 100x. A: resting; B: early development; C: late development; D: spawning; E: reabsorbing.

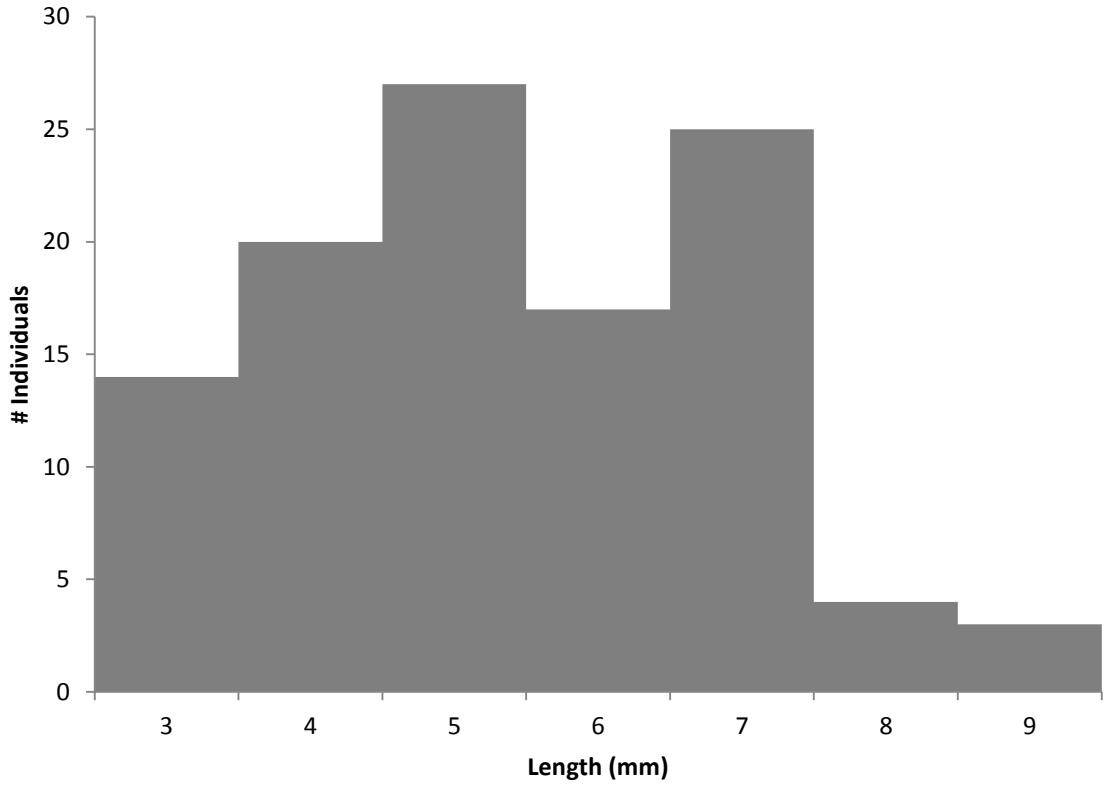

Figure 4. Number of settled zebra mussels (*n*=110) by size class on PVC substrates in Marion Reservoir, Kansas, in July 2012, from substrates placed in June 2012.

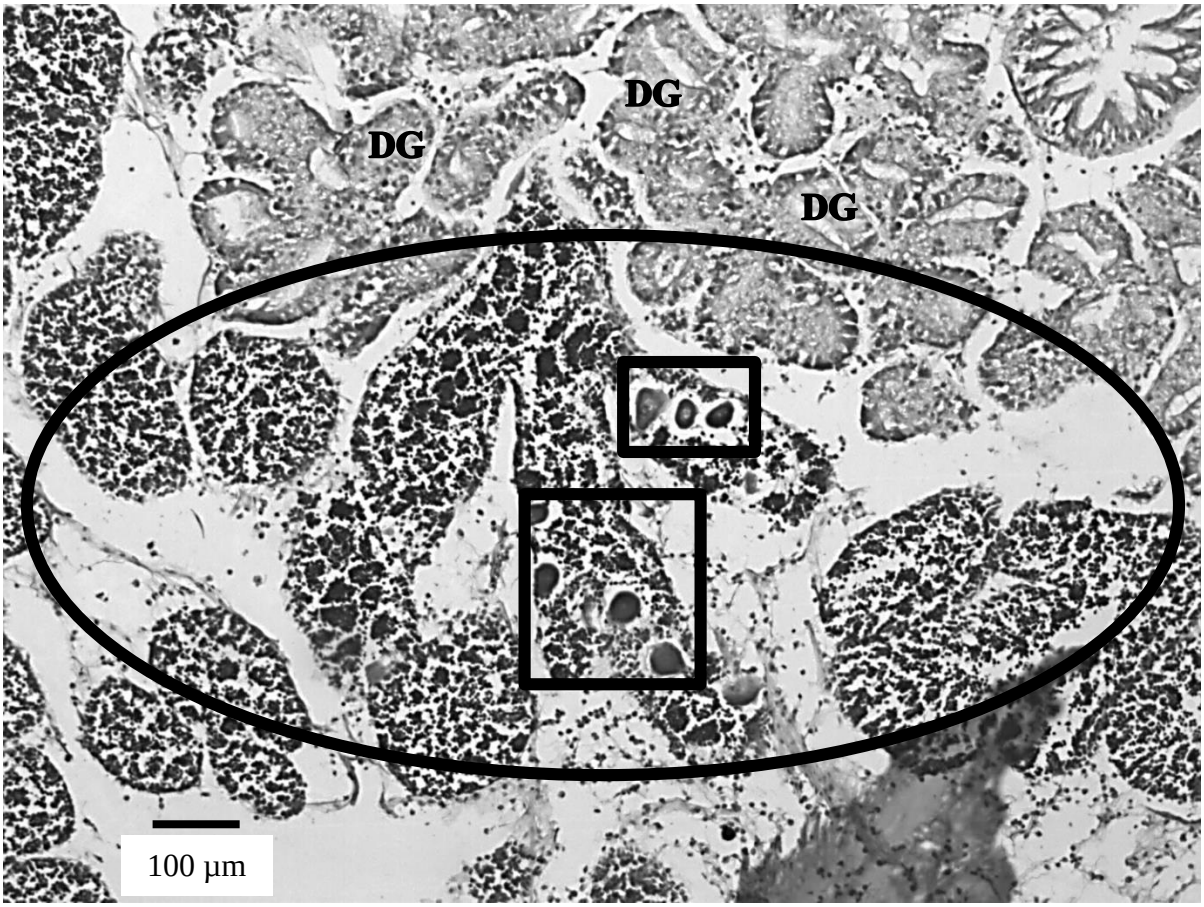

Figure 5. Hermaphroditic zebra mussel collected 23 February 2012 in Marion Reservoir, Kansas. Oval shows testis lobes; oocytes are identified by black boxes within testis. DG=digestive glands. 400x.

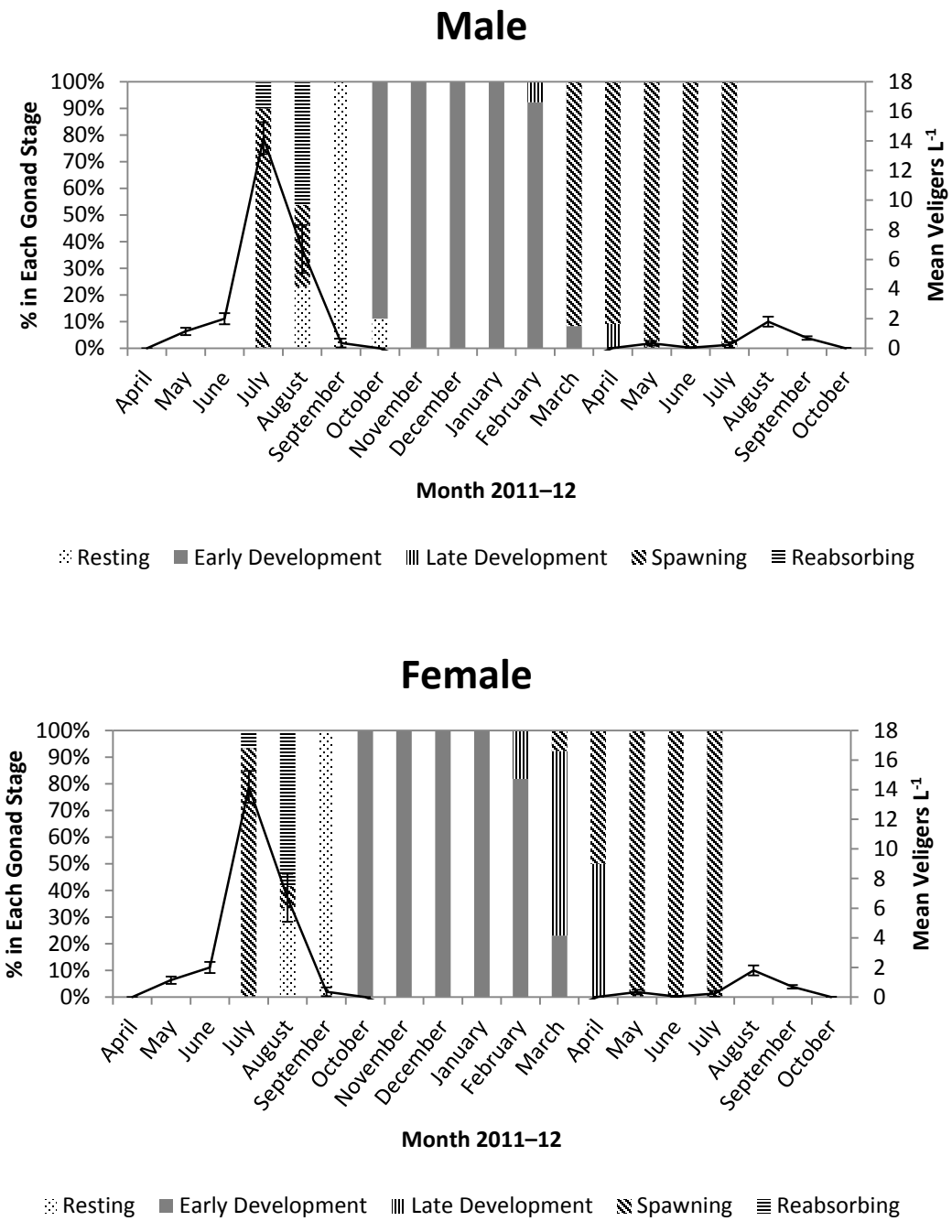

Figure 6. Mean veliger densities (L^{-1}) from three plankton tows, compared to stages of male and female zebra mussel gametogenesis in Marion Reservoir, Kansas 2011-12. Black line represents veliger density; error bars = ±1 standard deviation. Veliger densities include samples from months in which histological analysis was not conducted.

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