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Physiological Aspects of Zebra Mussel Reproduction: Maturation, Spawning, and Fertilization[1]

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SYNOPSIS. The prolific reproductive capabilities of the zebra mussel, *Dreissena polymorpha,* have facilitated the rapid spread and high densities of this biofouling organism since its accidental introduction into North America less than 10 years ago. Research on its reproductive mechanisms and capabilities may be valuable not only in predicting its further spread, but also in investigating basic mechanisms of reproduction and development and in developing new strategies to mitigate its impact. Since zebra mussels are dioecious and fertilization occurs externally, coordinated maturation, spawning, and other mechanisms have evolved to increase the probability of successful fertilization. The zebra mussel undergoes an annual cycle of gonadal growth and gamete maturation, culminating in one or more spawning events in late spring or early summer. Temperature, rates of temperature change, food availability, and effects of neighboring mussels seem to be critical variables that determine reproductive responses. Serotonin is a biogenic amine which is implicated in spawning behavior and can reliably trigger spawning. Serotonin is present in the gonad in neural varicosities that encircle groups of gametes, and specific serotonergic ligands can mimic or block spawning caused by serotonin. In females, serotonin reinitiates meiosis causing maturation from prophase I to metaphase I prior to spawning. Spawned oocytes contain substances that are species specific sperm chemoattractants. The sequence of binding, entry, and subsequent nuclear movements have been observed with fluorescence and scanning microscopy. Despite their negative ecological and economic impacts, zebra mussels have also provided a new and easily obtainable resource for studies of reproductive mechanisms.

INTRODUCTION

The zebra mussel (*Dreissena polymorpha*) has spread rapidly since its accidental introduction into the Great Lakes less than 10 years ago (Hebert *et al.,* 1989), and reached densities as high as 800,000/m^2 in North America (Kovalak *et al.,* 1993) and 1,700,000/m^2 in Europe (Stanczykowka and Lewandowski, 1993). High densities and rapid spread have been facilitated by high fecundity, with mature females capable of producing more than a million oocytes per year (Borcherding, 1991). In addition to

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Dreissena polymorpha, a second dreissenid species, *Dreissena bugensis,* popularly known as the quagga mussel, has also been found in the Great Lakes, and it, too, is beginning to be found in high densities in various locations in Lakes Ontario and Erie (May and Marsden, 1992; Dermott, 1993; Rosenberg and Ludyanskiy, 1994). Increased knowledge of the reproductive mechanisms of these biofouling dreissenid mussels may be useful in predicting their further spread and in developing new approaches to mitigating their impacts. Furthermore, zebra mussels are an excellent model organism for the study of reproductive mechanisms.

Since zebra mussels are dioecious and fertilization occurs externally, coordinated maturation and spawning processes are important for successful reproduction. The maturation process and changes that occur in gametes during the annual cycle and upon spawning will be delineated. Spawning is influenced by multiple factors, both environmental and internal. As will be described, the best established chemical regulator of spawning behavior is the neurohormone/transmitter serotonin; however, spawning may also be regulated by pheromones and other external influences. Finally, mechanisms that affect fertilization success will be discussed. These topics include investigations into sperm chemoattractants, binding and entry of sperm into oocytes, and mechanisms that mediate final maturation and early development of the zygote.

BIOLOGY AND ANATOMY OF ZEBRA MUSSEL REPRODUCTION

Adult zebra mussels are sessile, but highly gregarious animals. Zebra mussels are generally found in clumps or layers on hard substrates. Male and female zebra mussels are usually present in about equal numbers, although some low percentages (4–8%) of hermaphrodites have been reported (Nichols, 1993; Antheunisse, 1963). The close proximity of individuals of opposite sex within such groupings facilitates successful external fertilization. The zebra mussel is among the few freshwater bivalves having both external fertilization and a free-swimming planktotrophic larval stage (Sprung, 1987).

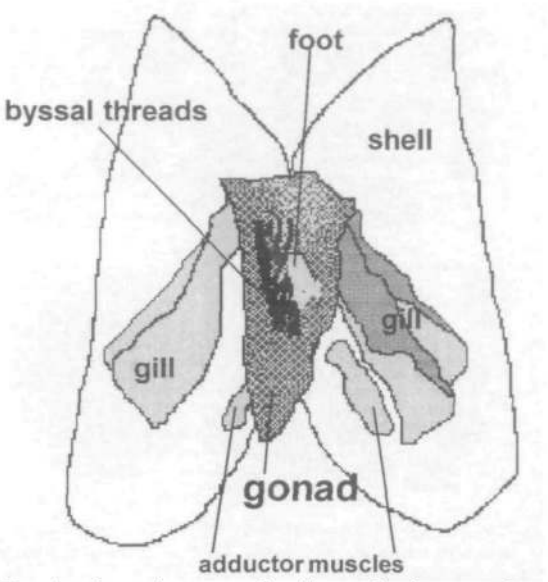

FIG. 1. Internal organs of a dissected zebra mussel, showing the location of the gonad in relation to other organs.

The gonad of both males and females consists of a single Y-shaped organ occupying nearly the whole length of the body in a ripe animal (Fig. 1). The external morphology of the gonad is similar in males and females; however, the presence of oocytes or sperm can be identified by microscopic examination. The volume of the gonad in females (adjusted by an allometric equation to a "standard" 24 mm long animal) ranges from a minimum, in early winter, of approximately 20 mm^3, to a maximum, in spring and early-summer ripe animals, of approximately 100–160 mm^3 (Borcherding, 1991). Over the same period, the gonad index (volume of gonad divided by total volume of the visceral sack, consisting of most internal organs) increases from 25% to as high as 70% (Borcherding, 1991). In females, the gonad is composed of vesicles (referred to as acini (Bielefeld, 1991) or ovarian follicles (Garton and Haag, 1993)) in which the oocytes develop. In males, the vesicles in which spermatozoa develop have been referred to as lobes (Garton and Haag, 1993). Ducts from the vesicles lead to a ciliated gonoduct, which

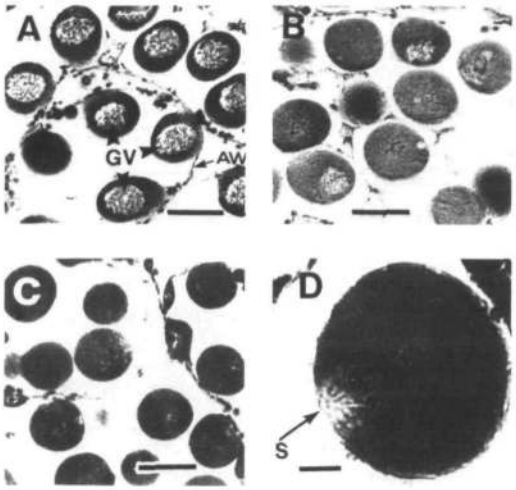

FIG. 2. Oocyte maturation in ripe ovaries prior to (A) and following (B, 30 min; C, D, 40–50 min) exposure of animals to 10^{-3} M serotonin. Abbreviations: AW, acinar wall; GV, germinal vesicle; S, spindle apparatus. Calibration bars: A, B, C, 60 μm; D, 10 μm.

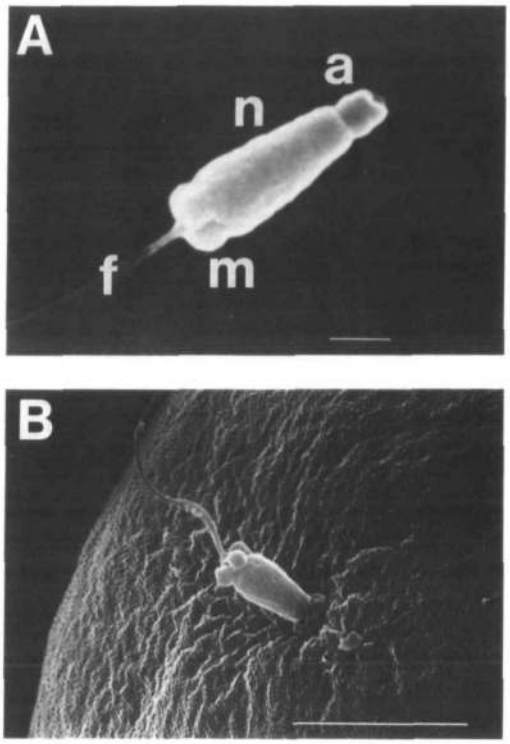

FIG. 3. Scanning electron micrographs of zebra mussel sperm. (A) Isolated sperm. (B) During fertilization, two min after addition to oocytes. Abbreviations: a, acrosomal region; n, nuclear region; m, midpiece (mitochondrial) region; f, flagellum. Calibration bars: A, 1 μm; B, 5 μm.

opens into the inner supra-branchial chamber. Muscles near the opening of the gonoduct may assist or regulate the release of gametes from the gonad (Antheunisse, 1963). Branches of the cerebrovisceral connective nerve innervate the gonad (Antheunisse, 1963) and the nerve forms plexuses of varicose fibers containing the neurotransmitter serotonin surrounding each ovarian follicle (Ram *et al.*, 1992).

SEASONAL CHANGES AND GAMETE MATURATION

Gametes undergo a sequence of histologically identifiable developmental stages annually (Tourari *et al.*, 1988; Haag and Garton, 1992; Garton and Haag, 1993; Nichols, 1993). In the most immature stage (stage 1), usually seen mid-Winter until early spring, gonads contain simple round cells of indeterminate sex <10 μm in diameter. With further development, oocytes increase in size (Borcherding, 1991) and differentiate according to their stage of meiosis. Prior to spawning, a mature (stage 4) oocyte is approximately 40–85 μm in diameter (Borcherding, 1991; Nichols, 1993), has a distinct nucleus (germinal vesicle) with a diameter greater than half of the oocyte itself (Fig. 2A), and is arrested at prophase I of meiosis. Intermediate stages of ovary development have been classified according to the percentage of stage 4 oocytes (Haag and Garton, 1992) or the diameters of the oocytes (Borcherding, 1991). In males, the small undifferentiated round cells develop an appearance that looks triangular in the light microscope and a flagellar tail appears. In scanning electron micrographs of ripe spawned sperm (Fig. 3A), the sperm head has a cylindrical acrosomal region approximately 1 μm in length; a frustrum-shaped nuclear region 2.5 μm in length, 0.85 μm in diameter at the top and 1.3 μm in diameter at its base; and a smaller midpiece region consisting of 4 spherical mitochondria-containing bulges each approximately 0.6 μm in diameter (Mojares *et al.*, 1995). Total length of the sperm head in these scanning electron micrographs (and in

transmission electron micrographs by Franzen, 1983) was thus approximately 4 μm; however, phase optic images of heads of live sperm are approximately 5.5 μm in length, indicating about a 25% shrinkage of the electron microscopic image (Majores *et al.,* 1995). Tails averaged 50 μm in length. Quagga mussel sperm heads are curved and about 20% longer than zebra mussel sperm (Denson and Wang, 1994).

The testis is classified as stage 4 when virtually all spermatocytes have differentiated into tailed spermatozoa. Intermediate and partially spent stages have lower percentages of mature spermatozoa.

Borcherding (1991) made quantitative measurements of changes in gonad volume and gonad index as a function of water temperature and time of year in two European lakes. Haag and Garton (1992) followed seasonal changes in gonad maturation in zebra mussels in Lake Erie. In Borcherding's study, gonad volume and index fell to a minimum value in the early fall (September or October). In most years, the gonad began to increase steadily in volume throughout the winter, even while the water temperature was still falling, and it continued to increase in size as the water warmed up in the spring, until it reached maximum size in May or June. After several months of relatively constant size, gonad volume and index fell precipitously to minimum values again. At the same time, oocyte diameters changed correspondingly. In February or March, oocytes generally averaged about 25–35 μm in diameter. In May or June, a bimodal size distribution often appeared, with one oocyte cohort remaining in the range of 25–35 μm, while another cohort of ripe oocytes appeared with diameters of 40–70 μm. At the time that the precipitous decline of gonad volume occurred, all or almost all of the ripe oocytes disappeared from the gonad (interpreted as due to spawning), leaving the smaller immature cohort intact. Subsequently, with little increase in total gonad size, the second cohort ripened and then was released. In at least one case, a third cohort subsequently ripened. The initial release of oocytes, accompanying the precipitous decline in gonad volume, accounted for the largest proportion of oocytes released. Nevertheless, it was evident that several different cohorts of oocytes could ripen in mussels taken from a single location and that mussels retain the capacity to spawn even after their gonad has lost a large part of its early summer volume.

In Haag and Garton's (1992) observations, near South Bass Island in Lake Erie, gonads of both males and females matured gradually through stages 1 to 4 from December through June, 1989, stayed almost uniformly in stage 4 for over a month, and then precipitously became evacuated as all mussels spawned out in a period of less than a week. In 1990, the mid-summer decline in average maturation level was incomplete and subsequently declined more gradually over a span of three months, possibly corresponding to the ripening of several cohorts following an initial large decrease in average maturity in late July. Thus, observations of gametes extant in gonads revealed that there is frequently a coordinated and sudden decrease in the number and degree of maturation of oocytes at least once during the summer, and that additional cohorts can continue to ripen and to be released.

Gamete maturation (meiosis reinitiation) resumes just prior to spawning. In experiments by Fong *et al.* (1994*c*), animals were stimulated with serotonin, an activator of spawning, and gonads were fixed and stained at various intervals, up to an hour, after initiation of serotonin treatment. Within 30 min of serotonin treatment of females, germinal vesicles began to break down and by 40 to 50 min germinal vesicle breakdown (GVBD) was complete in most oocytes (Fig. 2B, C). At 50 min the spindle apparatus of metaphase chromosomes became evident in many oocytes (Fig. 2D). Females spawn about 60 to 100 min after serotonin treatment (Fong *et al.,* 1994*b*), and virtually all spawned oocytes are at metaphase I, with no germinal vesicle evident.

The process of meiosis reinitiation can be activated in an *in vitro* system (Fong *et al.,* 1994*c*). Similar to the time-course in intact animals, the number of oocytes having gone through GVBD in hemisected gonads in-

creased rapidly from <10% in controls to >70% 20 to 30 min after exposure of treated gonads to 10^{-4} M serotonin. In other experiments, it was shown that GVBD can be triggered *in vitro* by 10^{-4} M and 10^{-5} M serotonin, but not by 10^{-6} M serotonin.

When oocytes containing germinal vesicles are dissected from ovarian fragments, they usually undergo spontaneous GVBD. However, immersion of the gonad fragments in theophylline and forskolin, expected to increase cyclic AMP, a hypothesized inhibitor of GVBD (Huchon, 1981), enabled oocytes with intact germinal vesicles to be isolated (Kyozuka *et al.,* 1994).

For males, the major reported change between ripe gonadal sperm and spawned sperm is an increase in motility (Miller *et al.,* 1994). As analyzed by a computerized sperm analysis system (CASA), spawned sperm were >95% motile and exhibited curvilinear velocities (VCL) greater than 100 μm/sec, whereas sperm stripped from ripe gonads were less motile (28 ± 6%) and slower (VCL = 58 ± 8 μm/sec). Stripped sperm were nevertheless capable of fertilizing oocytes, at least at high titre (10^8 sperm/ml; Mojares *et al.,* 1996; criterion for fertilization: cleavage of oocytes within two hours of exposure to sperm). Although sperm spawned in response to serotonin are more motile than sperm stripped directly from male gonads, we could demonstrate no significant effect of serotonin directly on sperm kinematics of either released sperm or stripped sperm. The apparent lack of a direct effect of serotonin on sperm motility may mean either that treatment conditions were not appropriate for demonstrating an effect of serotonin on sperm motility, or that serotonin may ordinarily activate sperm motility indirectly by stimulating the release of other substances within the gonad. Further research on the mechanisms of sperm activation is needed.

ACTIVATION OF SPAWNING

Aquatic organisms that rely on external fertilization must maximize the likelihood that sperm will encounter recently released eggs. The length of time that gametes are viable upon release to the environment is limited, and likelihood of gamete interaction is further reduced by dilution in the surrounding water. One of the mechanisms that has evolved to increase fertilization success is synchronization of spawning. Environmental influences, including chemicals, temperature, and photoperiod, may regulate synchronous development and possibly provide specific trigger stimuli for activating simultaneous spawning of male and female *Dreissena polymorpha.* Spawning itself appears to be triggered by a neurally controlled system involving the neurotransmitter serotonin.

Serotonin—the internal mediator

Much of the evidence for the role of serotonin in initiating spawning in zebra mussels has been alluded to above. This evidence includes (1) Efficacy at triggering spawning: Serotonin stimulates spawning in both male and female zebra mussels by injection or by external application in surrounding aquarium water (Ram *et al.,* 1993*a*). External application stimulates spawning at concentrations of 10^{-4} M and above; whereas, injection is effective at approximately a 10-fold lower concentration (Ram and Nichols, 1993*b*). As little as 5 min application of serotonin is enough to trigger a complete spawning episode (Fong *et al.,* 1994*c*). At room temperature male zebra mussels usually spawn within 30 min, whereas, female mussels take an hour or more (Ram *et al.,* 1993*a,* 1994; Fong *et al.,* 1994*b*). (2) Presence in gonads: Serotonin is found in nerves that innervate the gonad and in varicosities in the walls of the ovarian follicles. Its presence has been demonstrated immunohistochemically and by HPLC of gonads from both males and females (Ram *et al.,* 1992). (3) Effect on isolated gonad fragments: Serotonin can activate oocyte maturation in isolated gonad fragments (Fong *et al.,* 1994*c*). Several critical pieces of evidence for the role of serotonin have yet to be demonstrated, however, including: (1) release of serotonin into the gonad in response to natural activators of spawning and (2) capability of serotonin receptor antagonists at blocking spawning activated by natural activators. An obstacle to demonstrating these last two lines of evidence is that reliable natural activators of

spawning for zebra mussels, other than serotonin, have yet to be demonstrated in laboratory experiments (see below on some possible candidates, however).

The receptor mediating spawning has been characterized pharmacologically by determining effects of specific serotonergic ligands. 8-OH-DPAT and serotonin were about equally effective at stimulating spawning (Ram *et al.,* 1992; Fong *et al.,* 1993); TFMPP, 2-methylserotonin, and alpha-methylserotonin were all less effective than 8-OH-DPAT and serotonin at inducing spawning (Fong *et al.,* 1993). Like serotonin, 8-OH-DPAT has also been shown to reinitiate meiosis in gonad fragments (Fong *et al.,* 1994*c*). In studies of possible antagonists, ketanserin and propranolol had no effect; mianserin, NAN-190, and cyproheptadine had partial inhibitory effects (Fong *et al.,* 1993), and methiothepin was a very effective antagonist (Fong *et al.,* 1994*a, b*). Methiothepin was at least two orders of magnitude more effective than other antagonists, and significant long-lasting effects on spawning latency of females were obtained at methiothepin concentrations as low as 10^{-8} M (Fong *et al.,* 1994*b*). Metergoline had mixed agonist/antagonist properties, stimulating males to spawn at concentrations as low as 10^{-8} M, while blocking spawning at 10^{-4} M (Fong *et al.,* 1994*a, b*). Ergotamine was the most effective activator of spawning in females, stimulating spawning at concentrations as low as 10^{-5} M, and inducing spawning in males down to 10^{-6} M (Duncan and Ram, unpublished observations). The pharmacological profile for inducing/blocking spawning does not fit readily into vertebrate serotonin receptor categories, but it does resemble competitive binding properties of 5HTlym, a molluscan receptor recently cloned from the freshwater snail *Lymnaea.* 5HTlym binds methiothepin, ergotamine, and metergoline with 2 to 4 orders magnitude higher affinity than it does serotonin (Sugamori *et al.,* 1993), matching to some extent the relative efficacy of these compounds at activating or inhibiting spawning in zebra mussels. The differences in efficacy of some serotonergic agents between males and females may indicate the presence of different receptors in the two sexes or may reflect differences in accessibility of the receptors.

Although serotonin has also been demonstrated to trigger spawning in marine bivalves (reviewed in Ram *et al.,* 1992), the studies described above in zebra mussels are the first to characterize the serotonin receptors involved in the spawning response. The accompanying process of GVBD has, however, been studied extensively in isolated clam (*Spisula* and *Ruditapes*) oocytes, including pharmacological analysis (Kadam and Koide, 1989; Krantic *et al.,* 1991; Gobet *et al.,* 1994). The binding profile of serotonergic ligands to *Spisula* oocyte membranes matches their efficacy at triggering (agonists; *e.g.,* serotonin, 8-OH-DPAT) or inhibiting (antagonists; *e.g.,* ritanserin) GVBD and, as in zebra mussel spawning studies, does not agree with vertebrate serotonin receptor categories (Krantic *et al.,* 1993*a, b*; Bandivdekar *et al.,* 1991). Investigators of *Spisula* and *Ruditapes* did not test methiothepin, the most effective ligand inhibiting spawning in zebra mussels.

External factors regulating spawning

Earlier European studies have shown that the occurrence of *Dreissena* veligers in the plankton is distinctly seasonal, displays annual and geographic variability, and thus indicates that adult spawning is synchronized by specific environmental cues (reviewed by Sprung 1989, 1993). Seasonal changes in water temperature are critical in regulating gonadal maturation and spawning. However, seasonal changes in photoperiod and phytoplankton abundance may also influence spawning, and some authors (Walz, 1978; Starr *et al.,* 1990; Ram *et al.,* 1992) have suggested that chemical cues from phytoplankton and conspecifics may be the most important trigger for spawning. Numerous observations and experiments have been made on the influence of temperature and chemicals on spawning responses, but there are few observations on reproductive effects of photoperiod and light intensity.

Temperature. Annual and geographic variation in temperature have been reported as the primary factor explaining timing of reproduction of *Dreissena* (Galperina, 1978; Sprung, 1993; many others). Veligers

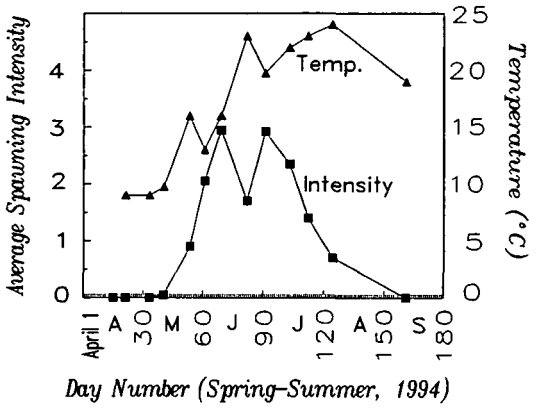

FIG. 4. Spawning of freshly collected zebra mussels in response to 10^{-3} M serotonin. On each indicated date, 40 mussels collected by scraping from steel bulkheads at Belle Isle, Detroit River, Detroit, MI were tested. Water temperature at the collecting sites is indicated; spawning tests were done at approximately 22°C. Spawning intensity was rated 0 (no spawning) to 4 (most intense spawning), according to Ram *et al.* (1993*a*).

typically appear in plankton when water temperature exceeds 12°C, the minimum temperature allowing gonad maturation (Borcherding 1991; Sprung 1993). Temperatures approaching 30°C inhibit gametogenesis (Tourari *et al.* 1988). In tests of responsiveness to serotonin, animals that had been maintained at 12°C were tested at 4°C, 12°C, 20°C and 27°C. Peak responsiveness was obtained at 20°C, with intermediate levels of response being obtained at 12 and 27°C and no spawning obtained at 4°C (Ram *et al.*, 1993*a*). A possible inhibitory effect of high temperatures may also account for the observation, illustrated in Figure 4, that for animals collected from the field in summer 1994 and tested immediately with serotonin, a decrease in responsiveness in late June coincided with a period during which water temperature rose rapidly. High temperature may also account for the decrease in veliger densities seen in mid-summer in western Lake Erie in 1989 (Fig. 5). Although high temperature initially may be inhibitory, mussels may also acclimate and regain responsiveness. Responsiveness to serotonin, which was lower than maximal levels when mussels were first placed at 27°C, rose again to maximal levels within one to two weeks of acclimation at the higher temperature (Fong *et al.*, 1995).

Another factor may be the pattern and rate of warming. In Polish lakes heated by power station cooling water, spawning begins earlier and persists later than in nearby nonheated lakes (Lewandowski and Ejsmont-Karabin, 1983; Stanczykowska *et al.*, 1988). In North America, veliger larvae are present earlier in the shallower, warmer portion of Lake Erie (Fraleigh *et al.*, 1993;

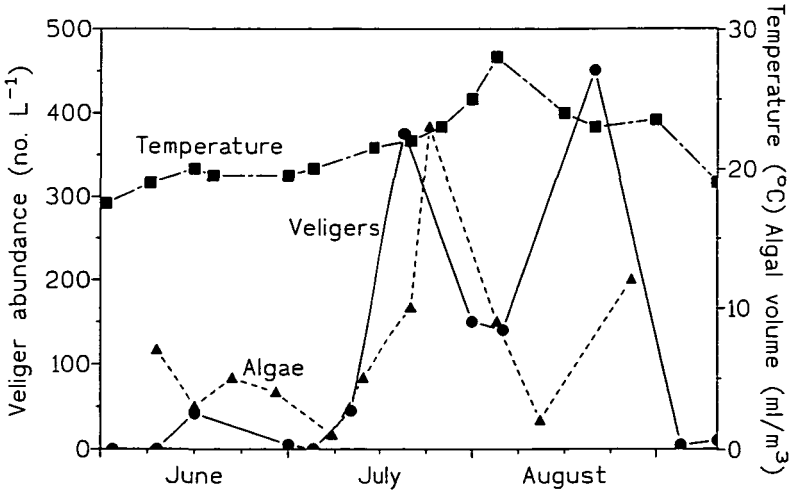

FIG. 5. Changes in algal density (measured as algal volume; D. Culver, unpublished data), zebra mussel veliger density, and temperature (Garton and Haag, 1993) in western Lake Erie in 1989.

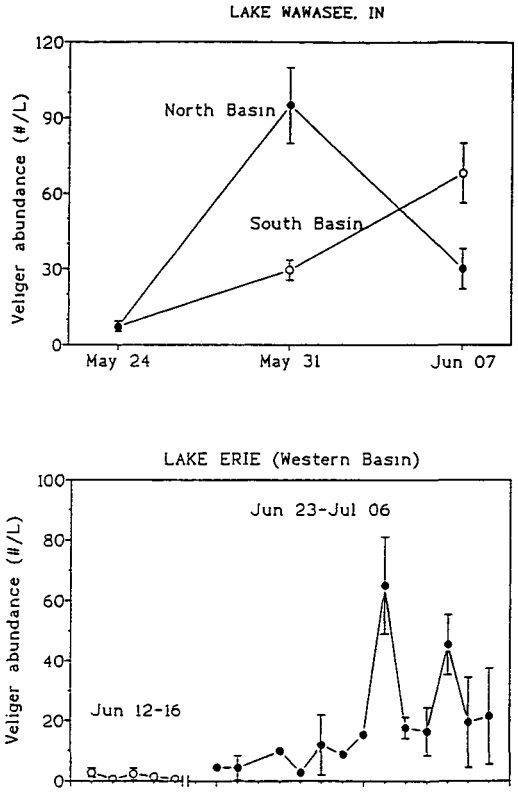

FIG. 6. Veligers appeared earlier in Lake Wawasee, IN than in western Lake Erie in 1994. The temperature in Lake Wawasee (both basins) rose from 14°C to 21°C between May 17 and May 24 and reached 24°C by June 7. In Lake Erie the temperature was 21°C on June 12 and did not reach 24°C until late June.

Garton and Haag, 1993; Garton, unpublished data). Likewise, spawning began in early May in Lake Wawasee, an inland lake in northern Indiana that warms faster and earlier than the Great Lakes, in contrast to mid-June (and early July for peak spawning) in western Lake Erie (Fig. 6; Glover *et al.*, 1994).

The minimum temperature requirement for gamete maturation has been reported as 12°C (Walz, 1978; Nichols 1993; Stoeckel and Garton, 1993); however, higher temperatures and considerable variation occurs in the temperatures at which spawning actually begins (Sprung, 1989; Garton and Haag, 1992; Haag and Garton, 1992). In Lake Erie, *Dreissena* spawning has typically occurred at water temperatures above 18°C, and in some years little spawning occurred until temperatures reached 22–23°C (Garton and Haag, 1992), considerably higher than reported in several European lakes (Sprung, 1989; Borcherding, 1991). This difference is likely the result of dissimilarities in seasonal temperature patterns and rates of warming in the spring. The lakes studied in northern Europe have lower average temperatures and narrower temperature ranges than lakes in the mid-central region of the United States. Thus, the rate at which water temperature increases in the spring determines the temperatures at which spawning actually occurs once the minimum temperature for gamete maturation has been exceeded.

Temperature shock has been used to trigger spawning in bivalves. Tourari (1988) elicited spawning about half the time with temperature shock, and others have reported its use for obtaining zebra mussel gametes for larval viability experiments (*e.g.*, Wright, personal communication). In contrast, animals exposed to a slow rise in temperature from 12°C to ambient (20°C–25°C), as untreated controls in spawning experiments in the Ram laboratory, have never spawned within the duration of the experiment (usually 4–6 hr, >1,000 control animals recorded). Drastic temperature change is unlikely to occur under natural conditions and therefore probably does not act as a physiological spawning activator; however, temperature shock may be useful to elicit spawning in some laboratory studies.

Phytoplankton and chemical cues. Coordinating spawning with phytoplankton blooms may increase the survival of planktotrophic larvae to settling stage by increasing larval growth rate and reducing the length of time the planktonic phase is exposed to predation. In lakes, phytoplankton abundance varies seasonally, and seasonal patterns of abundance varies among years. Although data are limited, the temporal pattern of *Dreissena* larval density in Lake Erie in 1989, which may have been influenced by temperature as noted above, also appears to be correlated with phytoplankton abundance (Fig. 5).

Phytoplankton may act as a chemical cue for spawning to occur, as has been sug-

gested for the marine bivalve *Mytilus,* which can be stimulated to spawn by algal cultures or extracts (Smith and Strehlow, 1983; Starr *et al.,* 1990). Recently, Ram *et al.* (1995) have demonstrated that zebra mussels can be stimulated to spawn by extracts of the marine algae *Rhodomonas, Fucus,* and *Phaeodactylum* but not by extracts of green algae and cyanobacteria. These results support the hypothesis that algae contain chemicals that stimulate zebra mussel spawning; however, since these extracts were from marine algae, it is important to determine whether freshwater algae that are present when zebra mussels begin to spawn can also induce spawning.

Phytoplankton quality and/or abundance can also influence spawning patterns indirectly. In *Dreissena,* energy is shifted from growth to reproduction as a response to stress. Reproductive effort, the proportion of total energy devoted to reproduction, increases when mussels are provided with a low quality diet (*i.e.,* low in lipids) (Stoeckmann and Garton, 1994). Denson *et al.* (1994) reported that zebra mussels overwintering in "poor condition" (low biochemical indices) spawned earlier in the spring than mussels overwintering in "good condition." Thus, timing of reproduction reflects the availability of food for larvae and the energy status of the adult.

In a laboratory study of the effect of starvation (30 days) on zebra mussels, Bielefeld (1991) observed that for animals that came from a population of ripe animals that had not yet spawned, starvation for 30 days caused total gonadal resorption in only 6% of the specimens studied. In most animals, starvation caused little or no gonadal degeneration. Correspondingly, in experiments in the Ram laboratory (Fong *et al.,* 1995), animals which had been starved for up to 21 days still spawned at high rates (>90%) in response to serotonin.

Aside from the possible chemical or nutritive stimulus provided by phytoplankton, spawning may also be regulated by pheromones released by neighboring mussels. A common observation for zebra mussels (Walz, 1978; Sprung, 1987, 1989; Nichols, 1993) and other bivalves (reviewed by Ram *et al.,* 1992; Ram and Nichols, 1993*b*) is that gonad extracts trigger spawning. Ram *et al.* (1995) have recently shown that serotonin-free water in which sperm or oocytes had been released and gametes removed by centrifugation could trigger spawning in other zebra mussels. The hypothesis is that in the field once a few zebra mussels have begun to spawn in response to phytoplankton stimuli, chemicals associated with released gametes may provide positive feedback inducing synchronous spawning in a large number of neighboring mussels (Ram *et al.,* 1992). Evidence for this phytoplankton/pheromone/positive feedback model awaits identification of phytoplankton and gamete-associated spawning activator chemicals and demonstration that these occur in the environment in association with observed spawning peaks.

Salinity

As zebra mussels move downstream in the Mississippi, Hudson, and other river basins, they will encounter brackish estuarine environments. In order to assess effects of brackish water on reproduction of zebra mussels, Fong *et al.* (1995) tested the effect of acclimation to freshwater (ordinary aquarium water) and salinities (dilutions of Instant Ocean in aquarium water) of 1.75, 3.5 and 7.0 parts per thousand (ppt) at 12°C, 20°C, and 27°C on serotonin-elicited spawning. In non-acclimated mussels, 7.0 ppt inhibited spawning at 12°C, 20°C, and 27°C, but after several days acclimation at 3.5 ppt, serotonin could elicit spawning in salinities of 5.0, 6.0 and 7.0 ppt at all acclimation temperatures. Thus, acclimation of reproductive responses to higher salinities can occur in zebra mussels.

FERTILIZATION

After spawning has occurred, mechanisms that attract and bind sperm and oocytes to one another increase the probability of successful fertilization. In many species, sperm exhibit chemotactic responses towards oocytes (Miller, 1985). Recently, the presence of sperm chemotaxis in zebra mussels and quagga mussels has been demonstrated (Miller *et al.,* 1994). Ethanolic extracts of freshly spawned oocytes attracted recently spawned sperm, as observed with

video-micrography. Test solutions at the tip of a micropipet rapidly caused sperm density to increase near the tip, usually within 20 sec. Attractant responses were species specific: Strong sperm attractant responses were observed to conspecific extracts from both zebra mussels and quagga mussels; whereas, tests of zebra mussel extracts on quagga mussel sperm and vice-versa demonstrated a greater than 100-fold difference in responsiveness to extracts from the other species. These studies by Miller *et al.* (1994) were the first to demonstrate sperm attraction to oocyte extracts in a bivalve.

These chemoattractant responses may function to increase the likelihood that sperm will encounter oocytes of the same species and hence lead to successful fertilization. Although zebra-quagga mussel hybrids have been demonstrated in the laboratory (Nichols and Black, 1994), genetic analysis of species specific isozymes of mussels in Lake Ontario, where mixed populations of both species occur, indicates that hybrids do not occur in nature (May and Marsden, 1992). Species specificity of the sperm attractant chemicals may be one mechanism that underlies such reproductive isolation.

Mechanisms of sperm binding to oocytes have also been investigated in zebra mussels. The surface of the oocyte has a uniform array of cylindrical microvilli covered by an egg envelope, also called chorion, vitelline layer, or glycocalyx (Conn *et al.*, 1991; Kyozuka, *et al.*, unpublished observations). The oocyte surface is relatively uniform and does not have a specialized sperm entry site (micropyle), as is found on some vertebrate oocytes.

Since sugar residues have been suggested to be important in sperm-oocyte binding in other organisms, their presence and possible role in mediating fertilization has been investigated in zebra mussels (Kyozuka *et al.*, 1993; Kyozuka, Fong, and Ram, unpublished data). Using fluorescent-labeled lectins (proteins or glycoproteins that bind to specific sugars), Kyozuka *et al.* (1993) found binding of concanavalin A (Con A) and wheat germ agglutinin (WGA), and no binding of *Dolichos* lectin (DL) to egg envelopes. Fucose-binding protein (FBP) exhibited binding in some studies (Kyozuka, Fong, and Ram, unpublished data), but relatively little in others (Kyozuka *et al.*, 1993). Specificity of the binding was indicated by lack of staining with lectins prereacted with their specific sugar. Thus, the egg envelope contains sugar residues related to methyl-α-D-mannopyranoside (mannoside), the Con A ligand; N-acetyl-D-glucosamine, the WGA ligand; and possibly D-fucose, the FBP ligand. Residues related to N-acetyl-D-galactosamine, the DL ligand, apparently are not present. The acrosomal region of spawned sperm bound all four lectins, and in addition, WGA also stained the sperm surface in both the nuclear and midpiece regions (Kyozuka, Fong, and Ram, unpublished data). The possible role of mannoside-related sugar residues in mediating fertilization is indicated by the fact that fertilization could be blocked by pre-treatment of oocytes with Con A, but not by pre-treatment with WGA, FBP, or DL. Another possiblity, however, is that Con A may inhibit fertilization by binding sperm before they can reach their normal binding/entry site on the oocyte.

After sperm-oocyte binding has occurred, the acrosomal rod, contained in the sperm acrosomal region, protrudes through the egg envelope. Eventually, sperm and oocyte membranes fuse, and the sperm nucleus enters the oocyte. The entire process has been observed by scanning electron microscopy (Kyozuka, Fong, and Ram, unpublished observations) and is complete within five minutes. Two minutes after sperm are added to oocytes, the oocyte surface, which has a uniform pattern of microvilli over its entire surface prior to sperm binding, is pushed up around the entering sperm (Fig. 3B), and by five minutes all but the tail has entered the oocyte.

Chromosomal movements following fertilization have been followed using the fluorescent chromosomal stain Hoechst 33342 (Kyozuka *et al.*, 1993). Protrusion of the first polar body occurs 15–20 min after fertilization at room temperature. The second polar body is protruded 30–40 min after addition of sperm. This is followed by fusion of male and female pronuclei at 40–45 min (Fig. 7) and bipolar cleavage about 60 min

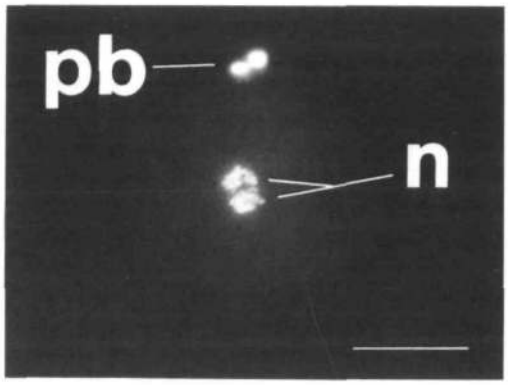

FIG. 7. Fertilized zebra mussel oocyte about 40–45 min after fertilization. Nuclei are stained with Hoechst 33342. Abbreviations: pb, polar bodies; n, male and female pronuclei. Calibration bar: 45 μm.

after sperm addition. Similar observations have been reported by Misamore *et al.* (1994). Recent studies of the mechanisms mediating polar body extrusion in zebra mussel oocytes indicate that it can be triggered in unfertilized oocytes by a calcium ionophore (A23187) and blocked by low pH (pH 6.0) (Ram *et al.*, 1996*a*).

CONCLUSIONS

Ample levels of phytoplankton and moderate mid-summer temperatures, especially in large lakes such as the Great Lakes, provide conditions that are highly suitable for zebra mussel reproduction. Furthermore, zebra mussel reproductive mechanisms are able to acclimate to more extreme conditions. Together with large areas of suitable substrate, a highly interconnected lake, river and barge canal system, and limited areas of salt water incursion, environmental conditions appear to be almost ideal for zebra mussels to spread and reproduce throughout North American freshwater systems. Zebra mussels are highly prolific, highly mobile at the larval stage, and superbly adapted to North American conditions, and thus appear to be here to stay. They have had, and will continue to have, negative ecological and economic impacts. However, zebra mussels have also provided a new and easily obtainable resource for studies of reproductive mechanisms. As described in this review, significant progress has been made in understanding mechanisms activating spawning and oocyte maturation. Zebra mussels may also be suitable for studies of pheromonal control of reproduction, interactions of energy metabolism and reproduction, and fertilization and early developmental mechanisms. It remains possible, as well, that increased understanding of reproductive mechanisms in zebra mussels may lead to novel approaches to controlling their spread and impact.

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