Saha
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2008

J. N. Am. Benthol. Soc., 2008, 27(1):84–89

DOI: 10.1899/07-004R.1
Published online: 11 December 2007

Evaluation of a nonlethal technique for determining sex of freshwater mussels

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Abstract. The shells of most North American freshwater mussel species are not sexually dimorphic. During the brooding period, gravid females can be identified by inspection of marsupial gills; however, it is difficult to separate nongravid females from males in species lacking sexual dimorphism. The ability to differentiate males from females throughout the year would assist mussel conservation and research. Our objective was to test the accuracy and safety of a method to determine the sex of live mussels. We used a syringe to extract ~0.2 mL of gonadal fluid from 67 *Elliptio dilatata* and 65 *Actinonaias ligamentina*. The fluid was stained and examined microscopically for developing gametes. This method was safe and effective for determining the sex of mussels. After 1 y, survival was indistinguishable between test and control groups for both species. We sacrificed 4 to 7 *E. dilatata* and *A. ligamentina* at 3-mo intervals and examined histological sections of gonads. Sex assigned from examination of gonadal fluid and histological sections agreed in most cases (*E. dilatata*: 100%, *A. ligamentina*: 89%).

Key words: Unionidae, gonadal fluid, histology, gametes, gametogenesis.

Freshwater mussels are among the most imperiled animals (Strayer et al. 2004). The lack of recruitment in many populations is well documented, but only rarely (e.g., Heinricher and Layzer 1999) is the cause known because disruption of any stage in the complex life history of mussels could result in recruitment failure. Studies of mussel reproductive biology can provide important information about population structure, such as sex ratio, and indicate disruptions in reproductive development. In particular, the ability to differentiate the sex of individuals and obtain information about gonadal activity throughout the year without sacrificing individuals would assist mussel management and research, especially for endangered species.

Some mussel species have sexually dimorphic shells, but most do not. During the brooding period, gravid females can be identified by the presence of charged marsupial gills; however, brooding seasons vary among mussel species. In bradytictic species, females brood glochidia from late summer until the following spring. In contrast, females of tachytictic species brood only for a short time during late spring to early summer. Thus, identifying females by examining them for gravidity is possible for only a brief period in tachytictic species. Moreover, not all mature females become gravid every year (Bauer 1987). Therefore, determination of sex solely by gravidity would be inaccurate because nongravid females cannot be differentiated from males in species lacking sexually dimorphic shells.

Sex of nongravid individuals can be determined by examining histological sections of gonads (van der Schalie 1970), but this procedure involves sacrificing mussels. Bauer (1987) extracted a small amount of gonadal fluid with a hypodermic syringe and determined sex of *Margaritifera margaritifera* based on the gametes present. Christian et al. (2000) also used this technique to assess sexual maturity of *Fusconaia ebena* and *Amblema plicata*. Extraction of gonadal fluid does not require sacrificing mussels, but its long-term effects on survival are unknown. Moreover, the accuracy of determining sex from gonadal fluid samples during different times of the year, especially at times when mature gametes are less likely to be found in the

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gonadal fluid, is unknown. The objectives of our study were to test the effects of gonadal fluid extraction on long-term survival of mussels and to determine the accuracy of identifying sex from gonadal fluid samples.

Methods

We selected 2 common species of mussels that are not sexually dimorphic and that represent both brooding strategies. We collected *Elliptio dilatata* (short-term brooder, mean length ±1 SE = 85.9 ± 0.7 mm) from the Duck River, Tennessee, on 25 September 2004. We collected *Actinonaias ligamentina* (long-term brooder, mean length = 100.6 ± 1.3 mm) on 27 October 2004 from the Clinch River, Tennessee. We transported mussels to a hatchery in a cooler containing river water. At the hatchery, we double-tagged the mussels with plastic shellfish tags (Lemarié et al. 2000), measured them, pried them open with a speculum, and inspected them to determine whether they were gravid. We randomly assigned individuals to 1 of 3 groups: experimental, control, or validation. The experimental and control groups each contained 40 individuals of each species, and the validation group contained 25 *A. ligamentina* and 27 *E. dilatata*. Mean lengths did not differ between the experimental and control groups for either species (*t*-test, *E. dilatata*: $p = 0.13$, *A. ligamentina*: $p = 0.34$).

We extracted gonadal fluid from all mussels in the experimental and validation groups by prying the mussels open with a speculum and inserting a wooden wedge between the valves. Then we inserted the needle (38 mm in length, 18-gauge) of a hypodermic syringe through the epithelial wall of the foot–visceral complex. We raised the needle tip slightly to create a small cavity within the hemocoel between the inner epithelium and the gonad and injected ~0.5 mL of deionized water to dislodge some developing gametes from the gonad. We then extracted ~0.2 mL of gonadal fluid. After fluid extraction, we removed the wooden wedge and immediately placed the mussels in plastic mesh cages filled with a sand–gravel substrate in the hatchery raceway. We retained gonadal fluid from each individual in a uniquely numbered syringe. We capped the needles and transported the syringes containing gonadal fluid on ice to the laboratory.

In the laboratory, we transferred the contents of each syringe onto a numbered glass slide. Because sperm and immature gametes were difficult to detect, we added an equal volume of 10% methylene blue solution (v/v) to the gonadal fluid and mixed the fluids by gently agitating the glass slides. We air-dried the slides for 24 h and examined them under a compound microscope at 40×, 500×, and 1000× magnification. Sex was assigned on the basis of mature or developing gametes observed in the gonadal fluid. We used a classification system adapted from Yokley (1972) and Woody and Holland-Bartels (1993) to categorize gonadal fluid containing gametes by the following stages: 0 = gametes absent or indistinguishable, sex undetermined; 1 = mostly spermatogonia or oogonia present, sex differentiated; 2 = mostly spermatids and spermatozoa or developing ovocytes and mature ova present. We differentiated cell types based on descriptions of Dinamani (1974) and Peredo and Parada (1984).

At 3-mo intervals, we removed 4 to 7 individuals from the validation group in the raceway and extracted a 2nd gonadal fluid sample before fixing the mussels in 10% formalin. We compared sex assigned from the initial and final gonadal fluid samples to determine the consistency of using this technique at different times of the year. We then prepared histological sections from the gonads from the fixed individuals. We prepared paraffin blocks from 2- to 3-mm-thick cross sections of the gonads and then cut 7-μm-thick serial sections with a rotary microtome. We counterstained tissue sections with Harris hematoxilin and eosin-Y (Humanson 1979), permanently mounted the sections on glass slides, and observed them at 40×, 500×, and 1000× magnification to determine sex and stage of gametogenesis. We classified histological sections by the same criteria used to classify gonadal fluid. We compared the sex and stage of gametogenesis determined from histological sections with determinations made from gonadal fluid samples to evaluate the accuracy of determining sex and gamete stage from gonadal fluid.

We recorded water temperature in the raceway periodically throughout the study period. We used Fisher's exact test (Zar 1999) to compare survival of individuals in the experimental and control groups after 1 y in the raceway. We used an experiment-wise type-I error of $\alpha = 0.05$ to determine significance of all statistical tests. Statistical analyses were done with Statistical Analytical Software (version 8.0; SAS Institute, Cary, North Carolina). At the end of the experiment, we measured all mussels with dial calipers to determine growth during captivity.

Results

Extraction of gonadal fluid did not affect survival. After 1 y in captivity, survival of *E. dilatata* was 72.5% in the control group and 75% in the experimental group (Fisher's exact text, $p < 0.9999$; power = 0.95). Survival of *A. ligamentina* was 85% in the control group and 75% in the experimental group (Fisher's exact text, $p = 0.2742$; power = 0.73). We could not evaluate

TABLE 1. Sex assigned to individuals in the experimental and validation groups for *Actinonaias ligamentina* and *Elliptio dilatata* from an initial gonadal fluid sample, and from a 2nd gonadal fluid sample and histological section from the validation group. Numbers in parentheses indicate individuals that died. * = includes 1 female originally classified as a male, and 1 female originally undetermined (U).

Species	Group	Method of determination	No.		
			Male	Female	U
E. dilatata	Experimental	Gonadal fluid	22 (5)	18 (5)	0
	Validation	Gonadal fluid (initial sample)	15 (2)	12 (0)	0
	Validation	Gonadal fluid (2nd sample)	13	12	0
	Validation	Histological section	13	12	0
A. ligamentina	Experimental	Gonadal fluid	20 (5)	16 (4)	4 (2)
	Validation	Gonadal fluid (initial sample)	12 (3)	10 (2)	3 (2)
	Validation	Gonadal fluid (2nd sample)	8	10*	0
	Validation	Histological section	8	10*	0

possible effects on growth because mussels grew little during their 1 y in captivity (0.5 mm for *E. dilatata*, no measurable growth for *A. ligamentina*).

We observed gametes in the initial gonadal fluid samples from all *E. dilatata* ($n = 67$) and in 58 of 65 samples from *A. ligamentina* (Table 1). At 1000×, the heads and occasionally the tails of sperm were observable (Fig. 1A). In both species, the sperm head

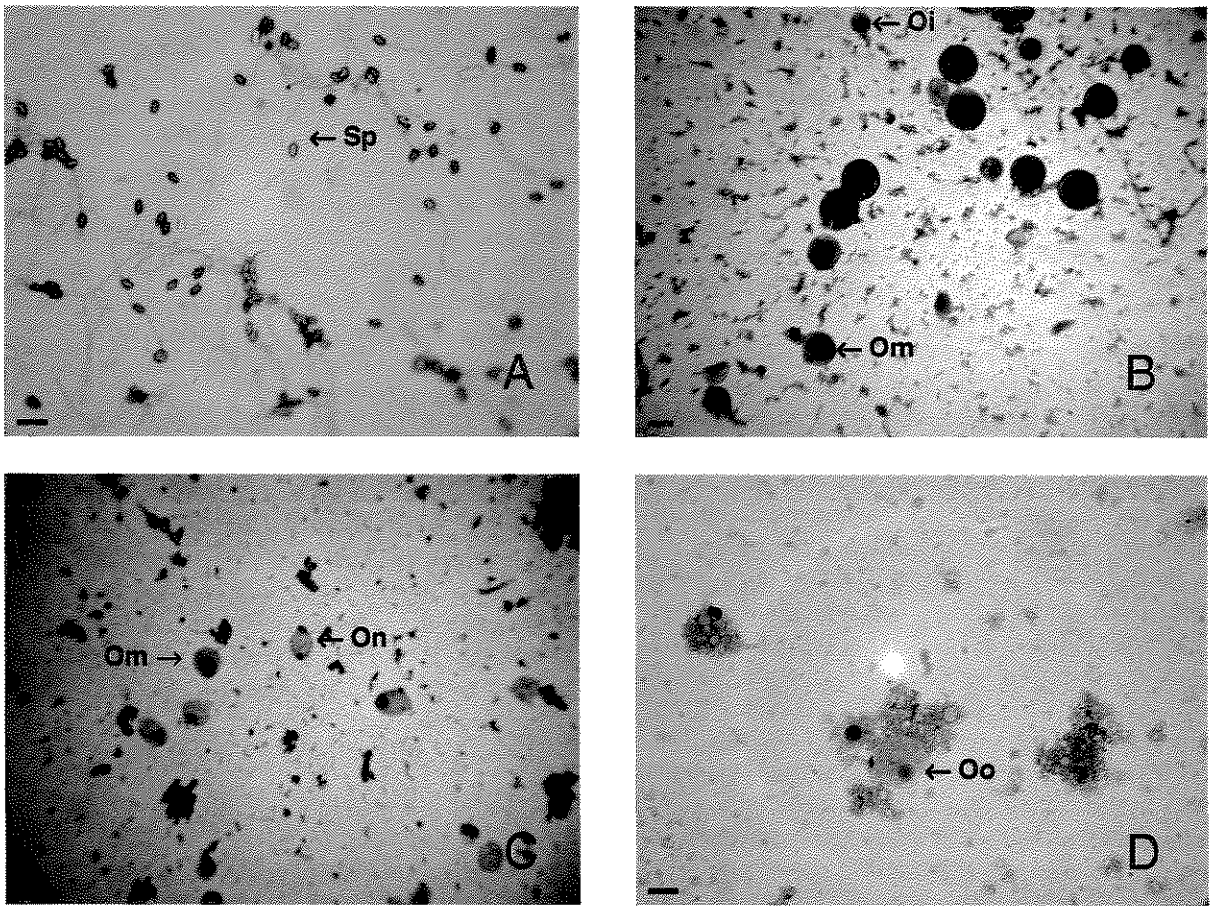

FIG. 1. A.—Gonadal fluid of male *Elliptio dilatata* showing mature sperm (Sp) with a brilliant light-blue head and an undulating tail (1000×). Scale equals 10 μm. B.—Female gonadal fluid of *E. dilatata* containing spherical mature ovocyte (Om) with a peripheral light-blue albuminous ring, which is lacking in an immature ovocyte (Oi) (40×). Scale equals 200 μm. C.—Gonadal fluid of female *Actinonaias ligamentina* showing ovoid Om and a nonviable ovum (On) (40×). Scale equals 200 μm. D.—Gonadal fluid of female *A. ligamentina* showing immature oogonium (Oo) (1000×). Scale equals 10 μm.

TABLE 2. Gamete stage predominating in the initial gonadal fluid extracted from the experimental and validation groups of *Elliptio dilatata* (25 September 2004) and *Actinonaias ligamentina* (30 October 2004). Numbers of gravid individuals are in parentheses.

	E. dilatata		*A. ligamentina*		
Stage	Male	Female	Male	Female	Undetermined
0	–	–	–	–	7
1	4	6 (0)	15	16 (13)	–
2	33	24 (0)	17	10 (5)	–

was oblong, biconcave, and stained bright blue. Mature ova of *E. dilatata* were suspended in an albuminous fluid and individually encased within a spherical vitelline membrane. The darkly stained ova were positioned centrally with the peripheral albuminous ring stained bright blue; in immature ovocytes, the albuminous ring was absent (Fig. 1B). In *A. ligamentina*, mature ova were enclosed within ovoid shaped vitelline membranes, and the ovum was located at the narrower end (Fig. 1C). Occasionally in *A. ligamentina*, the vitelline casings lacked ova, a characteristic suggesting that they became nonviable (Fig. 1C). Immature oogonia were mostly amoeboid and observable only at >500× magnification (Fig. 1D).

Examination of histological sections indicated that the sex of the surviving 25 *E. dilatata* and 16 of 18 *A. ligamentina* was correctly identified by the initial gonadal fluid extraction (Table 1). We could not determine the sex of 1 *A. ligamentina* in the validation group from the initial gonadal fluid sample, but we determined it was a female from the 2nd sample collected in July. We misclassified 1 *A. ligamentina* from the initial sample as a male but correctly classified this female from the 2nd gonadal fluid sample collected in July. Examination of histological sections of both individuals confirmed that they were female. Therefore, based on our initial gonadal fluid extraction, we determined sex accurately for all *E. dilatata* and 89% of the *A. ligamentina*. However, examination of gonadal fluid extracted immediately before sacrificing mussels (2nd sample) enabled us to identify the sex of all individuals of both species correctly.

Gonadal activity varied between species and among seasons. Examination of initial gonadal fluid samples indicated that gametes of most *E. dilatata* were in stage 2 in September, whereas gametes of *A. ligamentina* were predominantly in stage 1 in October (Table 2). Moreover, proportionately more stage 2 gametes were observed in gonadal fluid collected from *A. ligamentina* in April and July than during October and January (Table 3). In contrast, gonadal fluid of most *E. dilatata* contained stage 2 gametes throughout the year. Overall, classification of predominant gamete stages based on examination of histological sections was similar to that determined from gonadal fluid samples; 3 of 4 misclassifications were females (Table 3). Hermaphroditism was not observed in either gonadal fluid or from histological sections of gonads of either species. In the control and experimental groups combined, 25 female *A. ligamentina* survived for 1 y. Each year, 19 of these individuals were gravid in October; however, only 13 individuals were gravid in both years.

Discussion

Sex can be ascertained with a high level of accuracy from gonad fluid samples collected from individuals containing mature gametes or during periods of active

TABLE 3. Predominant gamete stage for *Elliptio dilatata* and *Actinonaias ligamentina* observed in gonadal fluid extracted immediately before sacrificing individuals in the validation group. Numbers in parentheses indicates stage determined from examination of histological sections.

		E. dilatata		*A. ligamentina*	
Season	Stage	Male	Female	Male	Female
Autumn (September)	1	0 (0)	0 (1)	1 (1)	2 (2)
	2	3 (3)	3 (2)	2 (2)	1 (1)
Winter (January)	1	0 (0)	1 (1)	1 (1)	2 (2)
	2	3 (3)	2 (2)	1 (1)	0 (0)
Spring (April)	1	0 (0)	1 (0)	1 (2)	1 (1)
	2	3 (3)	2 (3)	1 (0)	1 (1)
Summer (July)	1	1 (1)	2 (2)	0 (0)	1 (0)
	2	3 (3)	1 (1)	1 (1)	2 (3)

gametogenesis. We correctly identified the sex of all *E. dilatata* in the validation group and found no evidence of pausing females (sexually mature females not producing gametes in a given year). We misidentified the sex of 1 pausing female *A. ligamentina* and could not determine the sex of a 2nd pausing female. However, we correctly identified other pausing female *A. ligamentina* from the initial gonadal fluid samples.

Gonadal fluid can be safely extracted from mussels. The safety of this technique means that it is a feasible means of accurately determining the sex of live individuals of endangered freshwater mussel species. Sublethal effects were not evaluated directly in our study. However, individuals in the control and experimental groups burrowed soon after being placed in the raceway and remained partially buried throughout the study. Furthermore, gametogenesis seemed normal in the validation groups, and individuals in the experimental groups became gravid during captivity. Thus, we conclude that any sublethal effects were minimal and short term.

Observation of mature and developing gametes in gonadal fluid is crucial for positive identification of sex. Mature eggs were easily seen in gonadal fluid samples at 40×, but sperm and immature gametes could not be detected. Spermatogenesis typically occurs throughout the year, but oogenesis is restricted to a much shorter period (Ghosh and Ghose 1972, Weaver et al. 1991, Woody and Holland-Bartels 1993). Depending upon species and brooding strategy, mature ova might be absent from gonadal fluid samples for much of the year. Therefore, stained gonadal fluid samples should be examined at a magnification ≥500× to identify sperm or immature gametes.

We did not attempt to quantify gametes, but mature ova seemed to be more numerous in the last sample collected before the expected period of spawning for each species. Nonetheless, mature ova were not present in all female *A. ligamentina*. Bauer (1987) found that only 64% of female *M. margaritifera* spawned in a given year and 36% of the females were pausing. Pausing is also characteristic of the life history of *A. ligamentina* (JBL, unpublished data), and some females might pause for ≥2 y. Our finding of 24% pausing females each year is probably a minimum because any female in the control group that was pausing for ≥2 y would not have been identifiable.

Permits for conducting research on endangered species typically require use of techniques demonstrated to be nonlethal. Nonlethal techniques have been developed for obtaining tissue biopsies for glycogen analysis (Berg et al. 1995, Naimo et al. 1998) and extracting hemolymph samples for assessing health of mussels (Gustafson et al. 2005). The use of gonadal fluid samples provides a nonlethal alternative to sacrificing individuals to determine sex or to monitor gametogenesis.

Acknowledgements

We thank Dan Allen, Erica Dyer, Jason Hunt, Malissa Mengel, Kendall Moles, Chris Morton, Nevin Welte, and Malabika Laha for field and laboratory assistance. We also thank Robert Hall and Gail Takes of Cookeville Pathology for use of equipment and guidance on histological techniques, and John Gunderson for help with the digital photomicrographs. We thank S. Brad Cook, Wendell Haag, Kendall Moles, and David Strayer for helpful comments on the manuscript. Funding for this research was provided by the US Fish and Wildlife Service, and the Center for Management, Utilization, and Protection of Water Resources at Tennessee Tech University.

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Received: 11 January 2007
Accepted: 8 September 2007