EVALUATION OF DIET, GAMETOGENESIS, AND HERMAPHRODITISM IN FRESHWATER MUSSELS (BIVALVIA: UNIONIDAE)

by

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ABSTRACT

To determine the effects of different algal diets on freshwater mussels, tissues of *Elliptio complanata* were sampled for physiological, somatic, and gametogenic condition from August 1999 to May 2000. Treatments included mussels fed *Scenedesmus quadricauda* (S), *Neochloris oleoabundans* (N), a no feed treatment (NF), and a reference group of mussels from the Nottoway River (NR), Virginia. The levels of protein and glucose differed among treatments ($p<0.0001$), but glycogen and percentage tissue moisture did not ($p>0.17$). Production of ripe and developing gametes differed significantly among treatments ($p=0.001$), but stage of gamete development did not ($p=0.70$). Lipid levels and muscle fiber areas of treatment groups differed significantly ($p<0.0001$). Results of the feeding trial indicate that *S. quadricauda* is a suitable feed for *E. complanata*, but future experiments should identify algal species higher in carbohydrates for a mixed algal diet.

To determine sex and stage of gametogenesis, tissue histological sections from gonads of *Villosa iris* and *Utterbackia imbecillis* were evaluated. Occurrences of oogenic, spermatogenic, and hermaphroditic tissues were summarized in frequency tables. Visceral sites from which similar tissues were collected from conspecific specimens were evaluated for gametogenic stage. Sex was accurately determined in the central, visceral portion *V. iris* and female regions of *U. imbecillis*; and spermatogenic tissue was consistent in the dorsal-anterior areas of *U. imbecillis*. These areas also provided accurate determination of gamete stage in specimens. Reproductive asynchrony was observed among males and females ($p<0.02$). Male regions of *U. imbecillis* showed gamete stage

characterized by mature and developing spermatogenic tissue, while 2 groups of mussels were showed oogenic development characterized by mature oocytes and resorption of gametes. Male *V. iris* showed early gamete development without mature spermatozoa, and 2 groups of female *V. iris* showed mature and developing gametes and resorption of gametes. Protocols for biopsy tissue collection from selected visceral areas were developed for *U. imbecillis* and *V. iris* for sex determination and staging of gametogenesis. The application of this biopsy protocol should be considered population specific, and protocols appropriate for other populations and species should be developed with methods of this study.

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CHAPTER I

DIET EVALUATION FOR THE FRESHWATER MUSSEL *ELLIPTIO COMPLANATA* (LIGHTFOOT, 1786) (BIVALVIA: UNIONIDAE)

ABSTRACT

To determine the effects of different algal diets on freshwater mussels, tissues of adult (56-106 mm) eastern elliptios, *Elliptio complanata* (Lightfoot, 1786) were sampled 8 times for physiological, somatic, and gametogenic condition from August 1999 to May 2000. The treatments included a reference group collected from the source population, the Nottoway River (NR), Virginia, and 3 treatment groups held in air-driven 350 L recirculating units. Captive treatments included feeding mussels algal diets of *Scenedesmus quadricauda* (S), *Neochloris oleoabundans* (N), and a no feed treatment (NF). Production of ripe and developing gametes differed significantly among treatments ($p=0.001$), and the NF mussels ceased gametogenesis in spring. The gamete development index (GDI) evaluated maturation of gametes, and showed that mussels from the treatments and reference site did not differ significantly ($p=0.70$). Glycogen levels of the N, NF, NR and S mussels declined during the experiment and were not significantly different ($p=0.17$), but glucose levels of these groups did differ ($p<0.0001$). The patterns of protein levels of the treatments differed over time ($p<0.0001$): levels in N and S mussels remained relatively high throughout the experiment; those of NF mussels declined; and those of NR mussels increased in summer, declined in winter, and rose again in spring. The percent moisture content in tissues of all sample sources did not differ significantly ($p=0.21$), and all treatment mussels showed increases in winter and decreases in spring. Mean levels of total lipids in the mantle tissues of treatment groups differed significantly ($p<0.001$), with increases in fall and winter, a decrease in early spring, and subsequent increase by May in the NR mussels. The muscle fiber areas in the visceral masses were significantly different among the treatment mussels ($p<0.0001$); the N and S mussels showed stable fiber areas during the experiment, those of the NF treatment decreased, and those of the NR mussels declined in spring and increased by the end of the experiment. The alga *S. quadricauda* can be used as a feed for captive *E.*

complanata, but further experiments are needed to identify other algal species higher in carbohydrate content for use in a mixed algal diet.

INTRODUCTION

Approximately 72% of the 297 species and subspecies of North American freshwater mussels (Unionidae and Margaritiferidae) have been classified as extinct, endangered, threatened, or of special concern (Williams *et al*., 1993). Declines in species richness and abundance of this faunal group during this century have been attributed to habitat alteration, commercial harvest, competition from the exotic zebra mussel, *Dreissena polymorpha* (Pallas, 1771), and pollution (Bogan, 1993; Ricciardi, 1998; Neves, 1999). As part of the National Strategy for the Conservation of Native Freshwater Mussels (1998), the National Native Mussel Conservation Committee identified development of technologies and protocols for captive holding and breeding of threatened and endangered mussels as a priority for mussel conservation. Neves (1997) also identified captive propagation as important to the future of conservation and recovery of this faunal group.

The quality and quantity of suspended food are important to the physiological condition of marine and freshwater bivalves. Davis and Guillard (1957) and Walne (1970) determined that the nutritional importance of algal species varied, and that growth of larval and juvenile marine bivalves varied with algal species used as food. Whyte *et al*. (1990) found that dry tissue weight and energy reserves, such as proteins, lipids, and glycogen, decreased over time in starved and partially starved adult Pacific oysters, *Crassostrea gigas* (Thunberg, 1793). During the same experiments, these authors also reported an increase in percentage of moisture in the tissue of *C. gigas* under nutritive stress (Whyte *et al*., 1990). Gabbott and Walker (1971) attributed declines in dry flesh weight, glycogen, and proteins in adult oysters (*Ostrea edulis* Linnaeus, 1758) relocated to a hatchery to nutritive stress, as compared to the same measures in field oysters. Naimo *et al*. (2000) reported that growth, survival, and glycogen levels in tissue significantly varied with diet type in long fingernail clams, *Musculium transversum* (Say, 1829). Lomte and Jadhav (1982) showed that glycogen, proteins, and lipids decreased, and tissue water content increased, during a starvation experiment with the freshwater bivalve *Lamellidens corrianus* (Prasad, 1922). Patterson *et al*. (1999) reported significant declines in glycogen levels of mantle tissue of adult *Amblema plicata* (Say, 1817) and *Quadrula pustulosa* (I. Lea, 1831) starved for 14 d, versus glycogen levels of mussels fed

with the algal species *Neochloris oleoabundans* Chantanachat and Bold, 1962, *Scenedesmus* sp., and *Ankistrodesmus* sp.

Although energy reserves in bivalves are responsive to nutritional input, they are also dependent on seasonal energetic demands (Sastry, 1968; Bayne, 1973; Newell *et al*., 1982; Lowe *et al*., 1982; Fisher *et al*., 1996; Blanchard and Feder, 1997; Almeida *et al*., 1999). In particular, it is well established that the time-series profiles of stored glycogen, protein, and lipid levels in bivalve tissues are related to energetic demands during gametogenesis (Bayne and Thompson, 1970; Gabbott and Bayne, 1973). Mann (1979a and 1979b) reported that the levels of carbohydrates in *C. gigas* and the Manila clam, *Tapes philippinarum* (Adams and Reeve, 1850), increased prior to initiation of gametogenesis, and decrease after its onset. Bayne *et al*. (1982) also described a decline in the level of glycogen in blue mussels, *Mytilus edulis* Linnaeus, 1758, that was related to gametogenic development. In fact, temporal variations in stored glycogen, protein, and lipid were related to the energetic demands of gametogenesis in *M. edulis* (Zandee *et al*., 1980). The results of Zandee *et al*. (1980) revealed increases in glycogen, proteins, and lipids in *M. edulis* of the Dutch Wadden Sea from spring to autumn that coincided with food availability, followed by declines in glycogen and proteins from November through April. These declines were associated with low food availability and progression of gametogenesis (Zandee *et al*., 1980). Zandee *et* al. (1980) also reported an association between the sharp decline in stored carbohydrates in October with metabolic demands of oogenesis and vitellogenesis, and attributed this decline to conversion of stored glycogen to lipids for maturing eggs. During the period of November to April (gametogenesis and spawning), lipids remained relatively constant in *M. edulis* until the time of spawning in March and April, presumably for energy production during the period of carbohydrate and protein re-storage (spring through autumn).

The objectives of this study were to compare changes in the physiological and reproductive condition of eastern elliptios, *Elliptio complanata* (Lightfoot, 1786), exposed to 2 different diets and a no feed laboratory treatment; and to compare the condition of these test mussels to those in the source population. Glycogen, glucose, proteins, lipids, and percentage moisture were measured over time in the tissues of adult *E. complanata* fed algal diets of *Scenedesmus quadricauda* Turpin Brébisson sensu

Chodat, 1913 and *N. oleoabundans*. During the experiment, I also examined visceral histological sections for evaluation of cross-sectional areas of muscle fibers and reproductive condition of the mussels. The goal was to evaluate the sufficiency of *S. quadricauda* and *N. oleoabundans* as diets for *E. complanata*.

MATERIALS AND METHODS

Mussel Collection

In August 1997, approximately 500 adult *E. complanata* (mean length = 73.5 mm, SD = 7.6; mean dry weight = 2.54 g, SE = ± 0.02) were collected at the Highway 1 bridge crossing of the Nottoway River (NR), Dinwiddie County, Virginia. None of these mussels were observed as gravid at the time of collection. Eighteen of these mussels were measured and sacrificed for the excision of mantle tissue samples and visceral masses. The mantle tissues were transported on ice in vials to the Virginia Tech Aquaculture Center (VTAC), Blacksburg, Virginia, and immediately frozen at -60º C. The visceral masses were placed in embedding cassettes, and preserved in 95% ethyl alcohol at the collection site. Periodically throughout the experiment, specimens of *E. complanata* were collected from this NR reference site for excision of mantle tissues and visceral masses.

The remainder of the mussels was transported live to the VTAC in aerated river water. At the VTAC, 45 mussels were randomly assigned using a random number table to each of 9 replicate treatment units, consisting of water recirculating systems, at a density of 36 mussels/m^2 (mean DW=0.26 g/L). The shell lengths (mm) of mussels were tested for differences between treatments using ANOVA in Minitab 13 (Minitab Inc., State College, Pennsylvania). Three each of these 9 treatment units were randomly assigned and designated as *Scenedesmus* diet (S), *Neochloris* diet (N), or no feed (NF) treatments. Thus, each of these treatments was comprised of 3 replicates with 45 mussels assigned to each replicate.

Aquacultural Systems

Mussels assigned to the S, N, and NF treatments were held in nine 350-L air-driven closed recirculating systems without substratum. For a full description of the aquacultural recirculating system used in this study, see Henley *et al*. (2001). Each recirculating system consisted of an inter-connected 3.12 X 0.60 X 0.25 m polyethylene trough

(Southern States Cooperative, Richmond, Virginia)[3], and a 113 L polyethylene drum (Lab Safety Supply Inc., Janesville, Wisconsin). Water was delivered to the trough via 3.8 cm PVC tubing by airlifts from the drum, and returned to the drum from the trough by 5.1 cm PVC tubing. The usable bottom area in a trough was 1.25 m^2. To promote the suspension of algae cells in the water column, twelve 22.9 cm air diffusers were used in each treatment trough. Mean flow of these recirculating systems was 25.2 L/min, with no measurable variance as determined by a doppler flowmeter (Cole-Parmer, Vernon Hills, Illinois). The water used in the recirculating units was a mixture of 50% de-chlorinated city water and 50% well water that was sterilized with a ultra-violet filter (Lifegard, #QL-40). This mixture achieved an approximate hardness level of 250 mg/L $CaCO_3$. Air was delivered to the airlifts and subsequently to air diffusers by a Sweetwater 1.86 kW regenerative blower (Aquatic Eco-Systems, Inc., Apopka, Florida). During the experiment, water temperatures (˚ C) were measured approximately every 14 d. The temperatures of the NR samples were acquired from the Virginia Department of Environmental Quality, Richmond, Virginia for the Route 1 Nottoway River water quality-monitoring site, Dinwiddie County, Virginia (monitoring site number 5ANTW109.02).

Feed and Feeding Evaluation

During the experiment, mussels of the S and N treatments were fed either *S. quadricauda* or *N. oleoabundans* at a ration of 30,000 cells/mL/d (0.641 mg DW/mL for *S. quadricauda* and 0.26 mg DW/mL/d for *N. oleoabundans*). The cultures of *S. quadricauda* were inoculated from the juvenile fish rearing tanks at the VTAC. All other algae cultures of algae described herein, including those of *N. oleoabundans*, were inoculated from seed cultures ordered from the UTEX Culture Collection of Algae, University of Texas, at Austin. Based on a previous experiment, I determined that approximately 40% of introduced algae settled in the trough systems (Henley *et al.*, 2001). Therefore, to maintain a 30,000 cells/mL/d ration concentration in the troughs, I added a cell concentration of 50,000 cells/mL/d (0.661 mg DW/mL for *S. quadricauda* and 0.520 mg DW/mL/d for *N. oleoabundans*) in the systems once per day. Algae species

[3] Use of this and all subsequent products, brands, and suppliers does not imply endorsement by the U. S. government

were cultured in 300 L Kalwall tubes (Kalwalls Aquacenter, Leland, Mississippi), fertilized with Kent Pro-culture F2 Algae Culture formula (parts A and B) (Kent Marine Inc., Marietta, Georgia), aerated, and provided natural and artificial light (wide-spectrum florescent grow-lights). The water used for the culture of algae was a mixture of 50% de-chlorinated city water and 50% well water, as described previously. All algae cultures were semi-continuous; therefore, the algae used as feed were usually in the growth phase. The rationale for selection of these species of algae as experimental diets was pragmatic. At the beginning of the experiment, I attempted to culture and use, as a treatment feed, a tri-algal mixture of *N. oleoabundans, Bracteacoccus grandis* Bischoff and Bold, 1963*,* and *Phaeodactylum tricornutum* Bohlin 1897. Gatenby *et* al. (1997) showed this tri-algal mixture to support growth and survival in juvenile freshwater mussels, but I found that *B. grandis* and *P. tricornutum* were difficult to culture under the mixed and open culture conditions that I employed. Therefore, I chose to use *N. oleoabundans* as a treatment feed because it has also been found to support growth and survival in juvenile mussels (O'Beirn *et al*., 1998), and was relatively easy to culture under conditions at the VTAC. By using *N. oleoabundans* as an experimental feed, I sought to determine whether a diet commonly used to feed juvenile mussels could also be used to feed adult mussels. *Scenedesmus quadricauda* is an algal species naturally abundant in water tanks used for juvenile fish grow-out at the VTAC. By testing *S. quadricauda* as a feed, I sought to determine whether a naturally occurring and abundant algal species could be used as feed to support the physiological well-being of *E. complanata*.

In August, water samples were collected from the N, S, and NR sources for determination of carbohydrate, protein, and lipid levels. These samples were filtered using a 105 μm Nytex mesh, and frozen at -60º C. Algae in the NR water sample were preserved with Lugol's iodine, and later identified to genus. Relative abundances of algal species and total algal density (cells/mL) of the NR sample were determined using the means of 6 hemocytometer counts. At the VTAC, *N. oleoabundans* and *S. quadricauda* were cultured as previously described, and samples were collected during the growth phases of culture. For reference of nutrient contents, *Nannochloropsis oculata*, Droop, 1957, was also cultured using the previously described method. The frozen samples (N, S, and *N. oculata* species of algae and NR reference site samples) were sent to the Patrick

Center for Environmental Research, The Academy of Natural Sciences, Philadelphia, Pennsylvania for analyses. The N, S, and NR samples were analyzed for percentages of total carbohydrates, proteins, and lipids in dry samples using the methods of Kreeger (1993).

The algal ration (cells/ml) provided mussels in the fed treatments (N and S) at the VTAC was selected by determining the pseudofeces cell-free density (PCFD) for adult *E. complanata* (Winter, 1978; Bayne *et al*., 1989). The PCFD is the algae concentration that is just below the concentration at which pseudofeces begin to be produced by bivalves (Winter, 1977). The PCFD is theoretically close or equal to the optimal food concentration (ration); at this concentration, maximum ingestion rates and low energy consuming filtration rates occur, and all algae cells are filtered from the water column (Winter, 1977; Widdows *et al*., 1979; Bayne *et al*., 1989). To determine the PCFD, I collected 75 adult *E. complanata* (mean length = 65.7 mm, SD = 5.1; mean DW=2.27 g ± 0.023) from the study location on the Nottoway River in July of 1997. From these mussels, 60 were randomly assigned to 5 different feed rations, with 12 replicates per group. The algae concentrations of *S. quadricauda* provided in each treatment were 25,000 (0.636 mg DW/mL), 50,000 (0.661 mg DW/mL), 100,000 (0.771 mg DW/mL), 200,000 (0.811 mg DW/mL), and 300,000 (0.911 mg DW/mL) cells/mL, respectively. This PCFD experiment was conducted over 10 d, with each cell concentration test beginning every other morning. Individual mussels from each group were placed in a round 1.0 L polyethylene container with 0.5 L of water (mean DW=5.08 g/L) from a mussel-colonized tank, and aerated with a 5 cm diffuser. Prior to initiation of the experiment, mussels were not provided feed for 24 h to allow gut clearance. For the experiment, 12 mussels of each treatment were placed in their treatment units, and allowed to acclimate for 1 h prior to the introduction of algae. One hour after introduction of algae, all pseudofeces from the individual treatment containers were collected with pipettes. The pseudofeces replicate samples then were individually ashed at 500º C for 24 h for determination of ash-free dry weight (AFDW). Water temperatures during the experiment ranged from 20 to 22º C. This experiment provided an estimate of AFDW of pseudofeces per hour (mg/h) of exposure to each algal treatment concentration. For statistical purposes, the ratios (multiplied by 100) of the AFDW of pseudofeces (mg/h) to

the DW (g) of the experimental mussels were used. This allowed statistical examination of pseudofeces production on a per gram (DW) of mussel tissue. These data were transformed with the degree-arcsine-square root transformation (Sokal and Rohlf, 1995), and were analyzed using ANOVA and Tukey's multiple comparisons procedure in Minitab. Residuals of the data were normally distributed based on the Kolmogorov-Smirnov test for normality ($p>0.15$).

In July of 1998, 15 *E. complanata* were collected from the Roanoke River at the Highway 639 bridge, Roanoke County, Virginia, and randomly selected 8 of these mussels (mean length = 78.5 mm, SD = 12.6; mean DW=2.71 g ± 0.040) for inclusion in a study to determine whether rejection of *S. quadricauda* and *N. oleoabundans* was observed in *E. complanata*. The mussels were starved for 24 h prior to the experiment, and acclimated in the treatment containers for 1 h as previously. For this experiment, 1.0 L polyethylene containers were used with 0.5 L of aerated water from the tank that previously held the mussels. A mixture of *S. quadricauda* and *N. oleoabundans* of known relative abundance was introduced to the containers at a cell concentration of 100,000/mL/d (1.18mg DW/mL). At 1, 3, 6, 12, and 24 h, all pseudofeces samples were collected with pipettes from the treatment containers, and individually preserved in 1% Lugol's iodine. The relative abundances of these algae species cells in the pseudofeces samples were determined by dissolving the mucus in the pseudofeces with a papain solution (Adolph's Tenderizer, Englewood Cliffs, New Jersey), agitating the mixture, and then counting the cells in a defined area of a hemocytometer using 4 replicate counts. I examined selective rejection of *S. quadricauda* and *N. oleoabundans* by *E. complanata* by statistically comparing relative abundances of these algae species in pseudofeces to that in the feed, using t-tests for paired comparisons (Sokal and Rohlf, 1995).

Biochemical Condition

The main diet experiment was conducted between August 1997 and June 1998; during this period, the mantle tissues and visceral masses of mussels were collected from the N, NF, NR, and S treatments. For the N, NF, and S treatments, samples were obtained in September and November of 1997; and January, February, and April of 1998. From the NR reference site, tissue samples were collected in August of 1997 and February, March, and May of 1998. Data acquired from the first sampling (August 1997) from the

NR reference site served as a baseline (termed sample event zero) for evaluation of data acquired during subsequent sample events for all treatments. Six mussels were collected and sacrificed for mantle tissues and visceral masses from the sample sources on a given sample event. For sample events of the N, NF, and S treatments, tissues of 6 mussels were collected from each of the 3 treatment replicates. All samples were preserved and processed as previously described.

To determine levels of protein, glycogen, and glucose (all in mg/g dry weight of mantle tissue), collected tissue samples were thawed. From the thawed sample, 2 mantle tissue snippets were cut, blotted, and weighed. The weight of the tissue snippets ranged from 0.03 to 0.08 g. One of these snippets was used for determination of percentage moisture in mantle tissue by drying at 60º C for 48 h. The other mantle tissue snippet was processed for measurement of proteins, glycogen, and glucose. The tissue snippets destined for these tests were combined with a 0.05M Na_3PO_4 (4.8 pH) buffer, at a 1:30 ratio of tissue weight to buffer volume. This mixture was then homogenized using a variable speed Tissue Tearor (Biospec Products, Bartlesville, Oklahoma), and centrifuged at 800 g for 1 h. The supernatant was then removed for protein testing. Protein was measured that day using a portion of the supernatant; the unused portion of the supernatants were frozen at -60º C for later analyses of glucose and glycogen.

The protein concentrations (mg/g DW) of mantle tissue samples were measured using Microprotein-PR™ test kits (procedure #611; Sigma Diagnostics, St. Louis, Missouri). This procedure is colorimetric in nature, and is based on the shift in absorbance associated with the binding of pyrogallol red-molybdate complex to the basic amino acid groups of protein molecules. For this assay, 20 µL of the homogenate supernatants, standards of known concentrations, and blanks were added to 1 mL of the Microprotein-PR™ color reagent in 5 mL test tubes to create 2 replicates per tissue sample and standard concentration. These replicates were then incubated at 37º C for 3 min in a water bath, and replicate absorbances were read at 600 nm using a Spectronic 301 spectrophotometer (Milton Roy, Rochester, New York). The protein concentrations were calculated using the mean absorbances of the supernatant replicates. To compare my protein assay levels to those measured by another method, I sent 20 randomly selected tissue samples to the Patrick Center for Environmental Research, The Academy of

Natural Sciences, Philadelphia, Pennsylvania, for determination of protein (mg/g DW) using the method of Kreeger (1993). The statistical relationship between levels of protein measured by the 2 techniques was analyzed by linear regression and the two sample t-test using Minitab (Sokal and Rohlf, 1995).

Glucose and glycogen concentrations (mg/g DW) in mantle tissue samples were determined using glucose test kits (procedure #510; Sigma Diagnostics). This procedure is enzymatic, and employs glucose oxidase and peroxidase in combination with o-dianisidine dihydrochloride to produce colorimetric changes measured by absorbance. A 100 µL aliquot of each mantle tissue supernatant sample was added to each of four-15 mL test tubes, with 2 of these tubes receiving 7 µL (3.5 I.U.) of amyloglucosidase (#A-3514; Sigma-Aldrich, St. Louis, Missouri). Thus, each tissue sample was represented by 2 replicates of supernatant aliquots with and without amyloglucosidase. Also, 2 replicates of the glucose standard concentrations were created with and without the addition of amyloglucosidase. Four hundred µL of 0.05M Na_3PO_4 (4.8 pH) buffer was added to the samples without amyloglucosidase; and 393 µL of this buffer was added to the samples with amyloglucosidase. These supernatant and standard replicates (with and without amyloglucosidase) were incubated in a water bath at 37º C for 2 h to allow conversion of glycogen to glucose by the amyloglucosidase. After this incubation period, 5.0 mL of the enzyme color reagent solution from the glucose kit was added to each of the test tubes, which were then placed in a 37º C water bath for 30 min. The absorbances of these samples then were read at 450 nm with a spectrophotometer. To determine glucose and glycogen levels (mg/g DW) of mantle tissues samples, mean glucose absorbances of the samples with and without addition of amyloglucosidase were calculated from the replicates. The mean absorbances of samples without amyloglucosidase were used to determine glucose levels, and these means were subtracted from the mean absorbances of the samples with amyloglucosidase to determine levels of glycogen.

Statistical comparisons of proteins, glucose, and glycogen levels (mg/g DW), and percentage moisture of the mantle tissue samples from the N, NF, NR, and S groups were conducted using the General Linear Model (GLM) in SAS 8.01 (SAS Institute, Inc., Cary, North Carolina), with treatment groups and sample dates as factors (Sokal and Rohlf, 1995). Prior to statistical evaluations, residuals of data sets were tested for

normality with the Kolmogorov-Smirnov test (Sokal and Rohlf, 1995). The data from the sample events from all treatments and reference site were examined for differences in means with ANOVA and Tukey's multiple comparisons technique in Minitab (Sokal and Rohlf, 1995). Also, statistical relationships among levels of proteins, glycogen, glucose, as well as transformed percentage moisture, were investigated using correlation a procedure (r) in Minitab. Prior to the evaluation, the percentage moisture data were transformed with an arcsine-square root transformation (Sokal and Rohlf, 1995), and resulting values were converted to degrees (Sokal and Rohlf, 1995). The differences in mortality between the N, NF, and S treatments were statistically compared with ANOVA and Tukey's multiple comparisons technique in Minitab, using percent mortality converted by the degree-arcsine-square root transformation.

Determination of lipid levels (mg/g DW) in mantle tissue, evaluation of cross-sectional muscle fiber area (μm^2), and examination of reproductive status were conducted on a sub-sample of the treatment and NR mussels. From the overall number of mussels whose tissues were used to determine levels of protein, carbohydrates, and percentage moisture, 3 mussels were randomly selected from 1 randomly selected trough of the N, NF, and S treatments per sample date. Also, 3 mussels for each sample date were selected from the NR tissue pool per sample date. Residuals of these data also were tested for normality with the Kolmogorov-Smirnov test. The values were tested with the GLM using treatment and sample dates as factors, and data from the last sample events were compared using ANOVA and Tukey's multiple comparisons technique. Visceral masses were not collected on the August 1997 sample date. Lipid assays were conducted for this sample date, because mantle tissues were collected on the August 1997 sample date.

Lipids in mantle tissue samples were measured from dry mantle tissue by extraction with chloroform. Samples were oven dried at 102º C for 24 h, and weighed. Dry samples were pre-ground in a 15 mL Pyrex Tenbroeck hand tissue grinder (Fisher # 08-414-10C, Fisher Scientific, Pittsburgh, Pennsylvania). After initial grinding, 1.0 mL of a 2:1 chloroform to methanol solution was added to the dry material to begin the homogenizing process. After the tissue was partially homogenized, 3.5 mL of chloroform was added, and homogenization was continued until the tissue was in solution. This solution then was vacuum filtered through a 56 mm Coors porcelain Büchner filter funnel (CoorsTek,

Golden Colorado, Fisher # 10-365B) with a pre-dried and weighed 4.25 cm Whatman 40 ashless filter circle (>8μm) (Whatman, Ann Arbor, Michigan, Fisher # 09-845H). The filter circle was wetted with chloroform to ensure the adhesion to the porcelain funnel prior to filtering. The grinder glass container and pestle were rinsed twice with 1 mL of chloroform, and this rinse was filtered. Prior to initial drying by flushing with N_2 gas, the filter also was rinsed twice with 1mL of chloroform, oven dried at 102º C for 2 h, and weighed. The after-extraction, filtered dry tissue weights were subtracted from the before-extraction dry tissue weights to determine proximate total lipid levels (mg/g DW). The lipid data were statistically analyzed as previously described for protein and carbohydrate levels.

Cross-sectional Areas of Muscle Fibers

Measurements of cross-sectional areas of muscle fibers were obtained from the circum-transverse visceral muscles that were distal to the germinal tissue and just inside the body wall (muscles unnamed) (Appendix A, Photograph 1.1). These areas were evaluated using the subset of mussels previously used for lipids. Visceral masses were fixed in 95% ethyl alcohol, processed through an alcohol and xylene series, and embedded in Paraplast® embedding medium. Three 5 μm thin-sections were obtained per visceral mass, which were stained with hematoxylin and eosin. Because of the thickness of the sections, they will be termed histological sections in this study. To ensure comparative consistency of the evaluation between visceral masses, these histological sections were obtained from similar positions within all visceral masses (Morales-Alamo and Mann, 1989). Sagittal histological sections were obtained at 25, 50, and 75% of the transverse depth measurement (mm) of each visceral mass width. Cross-sectional areas (μm^2) of 50 individual muscle fibers were digitally measured using Sigma Scan Pro 4.01 (SPSS, Inc., Chicago, Illinois) from digital images captured through a Vanox T-AH2 microscope (Olympus America, Inc., Melville, New York) at 1540X magnification, using FlashBus Grabber 32 capture application (Integral Technologies, Inc., Indianapolis, Indiana). Prior to the measurement of muscle fibers, images were sharpened using Adobe Photo Shop (Adobe Systems, Inc., San Jose, California). These values were statistically analyzed as described for the protein and carbohydrate data.

Reproductive Condition

To evaluate reproductive condition of mussels, the mantle gamete area (MGA) method of Kreeger (1993) was adapted for use with freshwater mussels. This adaptation was necessary because the gonads within the visceral masses were inter-digitated with other types of tissue, such as the digestive tract and muscle. Data generated by this adapted method were termed the gamete area fraction (GAF), and represented the proportion of ripe or developing gametes within the gonad areas of examined histological sections. The stages of gametogenesis in these histological sections also were evaluated using the gonad activity method of Barber (1996), and which was termed the gamete development index (GDI) in this study. The GAF and GDI of each histological section were determined by the random point-count volumetry method of Chalkley (1943). Twenty-four randomly selected point evaluations per histological section were obtained using the corners of an ocular grid on a light microscope at 200X. The stage of the microscope was randomly moved in 2 dimensions to obtain a new field of view from which a set of four point evaluations were conducted. Only points within the gonad tissue were evaluated. If all 4 corners of the ocular grid were not within the gonad area, then the microscope stage was randomly repositioned. For determination of the GAF score per histological section, if a corner of the ocular grid overlaid a point within the gonad containing ripe or developing gametes, then a 1 was assigned. If a corner of the grid did not contact gametes in a ripe or developing condition, then a 0 was assigned. The GAF for a histological section was calculated as percentage of ones to zeros, which denoted percentage of the gonad area containing ripe or developing gametes. At the same time, the GDI score of the gonad tissue under the corners of the ocular grid was determined by assigning a 0, 1, 2, 3, 4, or 5 to represent stage of gamete development of the underlying tissue. Classification stages of gamete development used in the GDI, as set forth by Barber (1996), range from inactive (stage 0) to resorbing (stage 5) (Table 1.1 and Appendix A, Photographs 1.2 through 1.12). The mean GAF and GDI values from the 3 histological sections of each visceral mass were calculated for each individual. Degrees of the arcsine-square root transformed GAF means were used for statistical comparisons. The GAF and GDI data were analyzed using the GLM, with treatment groups and sample dates as factors. Frequently, GDI mean scores of histological sections were heavily

influenced by a preponderance of scores of 5. To investigate this influence, mean GDI values, excluding scores of 5, were statistically tested using ANOVA and Tukey's multiple comparisons technique. Differences in stages of gamete development within sample dates and treatments were tested with the GLM procedure. The GAF and GDI data from the last sample events were analyzed with two-way ANOVAs and Tukey's multiple comparison technique. Statistical relationships between the transformed GAF and GDI data and levels of protein, glycogen, glucose, as well as the transformed percentage moisture data, were investigated using correlation analysis.

RESULTS

The residuals of the protein, lipid, transformed percentages of tissue moisture, GAF, GDI, and transformed relative AFDW pseudofeces production values were normally distributed ($p>0.13$). The square root glycogen, *ln* glucose, and *ln* cross-sectional muscle fiber area transformations achieved normality of their data residuals ($p>0.09$).

Feed and Feeding Evaluation

The mean cell density of algae in the NR water sample was low, and was estimated at approximately 2,800 cells/mL, with predominantly green algae and diatoms. The genera (% relative abundance) observed in the NR sample were *Chlorella* (43.2%), *Chlamydomonas* (39.2%), *Cyclotella* (4.1%), *Navicula* (2.7%), *Pinnularia* (<1.0%), *Selenastrum* (4.1%), and *Synedra* (6.8%). Comparisons of the percentages of total carbohydrate, protein, and lipid levels of the N, NR, and S samples showed wide variation (Table 1.2). Percentages of protein in the dry N and S algae samples (37.6 and 26.4%, respectively) were greater than those of the NR (7.1%). The total lipid content of the S algae sample (23.7%) was greater than those of the other samples (8.5 and 4.2% for the N, and NR samples), and the percentage of total carbohydrates in the N sample (12.5%) was greater than those of the others (1.8 and 9.1% for the NR and S samples). For reference, the carbohydrate content of *N. oculata* was 28.%. Also, there was a clear difference between percentages of organic content among samples; algae samples showed greater percentages of organic content (93.2 and 83.2 for the N and S samples) than in the NR sample (10.1%).

The pseudofeces production ratios ([AFDW in mg/h/g DW of tissue] X 100) were significantly different among the algae treatments ($p<0.001$) (Fig. 1.1). The pseudofeces

production ratio of mussels from the 25,000 cells/ml concentration ration (mean = 0.001 mg/h ± 0.0002) was significantly different from those of mussels in the 50,000 (0.004 ± 0.0006), 100,000 (0.007 mg/h ± 0.002), 200,000 (0.007 ± 0.001), and 300,000 (0.008 ± 0.002) cells/ml ration levels ($p<0.05$). The production ratios of the 50,000, 100,000, 200,000, and 300,000 cells/ml treatments were not significantly different ($p>0.05$). During the PCFD experiment, all mussels tested in the 25,000 cells/ml treatment produced pseudofeces (Fig. 1.1). Therefore, the PCFD was seemingly below this feed concentration.

Relative abundances of *S. quadricauda* and *N. oleoabundans* in pseudofeces were not significantly different from their relative abundances in the feed provided to experimental mussels ($p>0.38$ and $p>0.34$, respectively). Over the course of this experiment, relative abundances of *S. quadricauda* and *N. oleoabundans* in feed and pseudofeces were not significantly different in the 1, 3, 6, and 24 h sample ($p>0.45$ and $p>0.42$ respectively, for *S. quadricauda* and *N. oleoabundans*). In the 12 h sample, the relative abundance of *S. quadricauda* in pseudofeces was significantly greater than that of the feed ($p<0.01$), and significantly less for *N. oleoabundans* versus the feed ($p<0.01$). In other words, the test mussels seemingly rejected *S. quadricauda* at this time in favor of *N. oleoabundans*, but no such rejection was detected throughout the rest of the experiment.

Biochemical Condition

The lengths of mussels (mm) assigned to the treatments were not significantly different ($p=0.62$). During the experiment (10 mo), mortalities of mussels in the N, NF, and S treatments were 4.4% (6 mussels), 3.0% (4 mussels), and 2.2% (3 mussels), respectively. These mortality percentages were not significantly different among the N, NF, and S treatments ($p=0.41$). Mean temperatures of the N, NF, S, and NR sample sources did not differ significantly ($p=0.54$). The overall mean for the N, NF, and S treatments was 18.5˚ C (SD=2.0), with a range of treatment means of 15.8˚ C in January to 23.7˚ C in August. Although temperatures of the NR were not significantly different from those of the other sample sources, temperatures of the NR ranged from 8.8˚ C in February to 23.5˚ C in August, with an overall mean temperature of 17.0˚ C. Temperature means for the N, NF, and S treatments on the sample dates of the experiment were not correlated to mean levels of protein and glycogen ($r=0.52$, $p=0.50$ and $r=0.20$, $p=0.47$,

respectively), positively correlated to mean levels of glucose ($r=0.52$, $p=0.05$), and inversely correlated to means of transformed percentages of moisture ($r=-0.82$, $p<0.001$). Also, mean temperatures were not statistically correlated with mean lipid content, transformed muscle fiber areas, transformed GAFs, and GDIs ($p>0.32$). The mean temperatures of the NR were not statistically related to means of any of the dependent variables measured in this study ($p>0.13$).

The protein levels (mg/g DW) of mantle tissues from the diet treatments and reference site were significantly different ($p<0.001$) (Table 1.3 and Fig. 1.2), with a significant interaction between treatments and sample dates ($p<0.001$). There were 3 different patterns seen in the protein profiles during the experiment. The first pattern, that of the S mussels, showed initial increases, elevated levels through winter, and a slight decline in spring. The second pattern was that of the NF mussels, showing an initial increase in protein levels, and a steady decline throughout the winter and spring. Thirdly, the N and NR mussels showed increases in mean protein levels in late summer, followed by declines during winter, and increases in spring; the winter decrease in the N mussels was still above the baseline level. Mean protein levels of the S mussels increased to 201% of the baseline during fall, and 162% in spring. Protein levels in tissue samples from the N and NR mussels initially increased to 200% and 229% in fall, decreased to 123% and 30% of original mean levels in winter, and then increased three fold in spring. The levels of the NF mussels initially increased to 187%, then declined to 62% by the end of the experiment. The N, NR, and S mean protein levels from mussels of the last sample events were not significantly different ($p<0.05$), and these were all significantly higher than those of the NF treatment ($p<0.05$).

The levels of protein (mg/g DW) of the re-tested tissue samples using the method of Kreeger (1993) were significantly greater than protein levels (mg/g DW) reported in this study ($p<0.001$); grand mean protein from the method of Kreeger (1993) was approximately 3 times greater, with means of 53.6 (±1.3 SE) and 162.2 (± 8.9) mg/g DW. There was no significant statistical correlation between levels of protein determined from the 2 methods ($r=0.13$, $p=0.60$).

Square root-transformed glycogen levels (mg/g DW) in mantle tissue from the sample sources did not vary significantly ($p=0.17$), and there was no significant interaction

between treatments and sample dates ($p=0.40$) (Table 1.4 and Fig. 1.3). Glycogen levels of mussels from the treatments and reference site showed significant decreases during the experiment ($p<0.05$). By the end of the experiment, mean glycogen levels of the sample sources were significantly different ($p<0.001$); glycogen levels of the N, NF, and S mussels did not significantly differ ($p>0.05$), but these were different from those of the NR ($p<0.05$).

Log-transformed glucose levels (mg/g DW) of tissue samples from all sample sources varied significantly ($p<0.0001$) (Table 1.5 and Fig. 1.4), and those of the N, NR, and S mussels declined at rates that were not significantly different ($p<0.07$). There was a significant interaction between treatments and sample dates ($p<0.0001$). Mussels of the NF treatment showed an increase in fall, a decrease in winter, and a leveling in spring. The mean glucose level of the NF mussels increased during fall to 131% of the baseline level, and then dropped to 39% in spring. Glucose in mantle tissues of the N and S treatments gradually decreased to 33% and 41% of original values in spring. The mean values of the NR mussels declined less severely than those of the N, NF, and S treatments, with ending values of 73% in spring. The mean levels of glucose of the last sample events showed 2 groupings; levels of the N, NF, and S mussels were not significantly different ($p>0.05$), but these were lower than those of the NR mussels ($p<0.05$).

The plots of glucose levels during the experiment appeared to resemble those of glycogen for all the sources (Figs. 1.3 and 1.4). The transformed levels of glycogen and glucose were correlated ($r=0.57$, $p<0.001$). Also, the levels of glycogen were correlated with the levels of protein for all treatments ($r=0.23$, $p<0.001$); this correlation was slightly stronger among the NF mussels ($r=0.47$, $p<0.001$).

The mean total lipid levels of the treatment and NR tissue samples differed significantly ($p<0.0001$), but not over time ($p=0.28$) (Table 1.6 and Fig. 1.5). Further analyses indicated that lipid levels did not vary over time within any of the treatments ($p>0.45$), except in the mussels of the NR group ($p<0.03$). Mean lipids of the N, NR, and S mussels did not vary ($p>0.17$), but the levels in these mussels were significantly different from those of the NF group ($p<0.002$). The lipid levels of specimens from the sample sources were not significantly different at the last sample events of the

experiment ($p>0.11$). Mean lipid and glycogen levels of the NF mussels were statistically correlated ($r=0.58$, $p=0.02$).

The percent moisture in the tissue samples from the treatments and reference site were not significantly different ($p=0.21$) during the experiment (Table 1.7 and Fig. 1.6), but there was an interaction between sample dates and treatments ($p=0.007$). The trends of the N and S mussels were not significantly different ($p=0.53$), and these were not different from the NR mussels ($p=0.09$). The transformed data of the N, NR, and S mussels were significantly different from those of the NF mussels ($p=0.008$). The percent moisture in mantle tissue of mussels from N, NF, and S treatments increased in winter and decreased in spring (Fig. 1.6), while those of the NR increased in late summer, maintained relatively constant levels during winter, and showed decreases in spring. At the end of the experiment, percentages of moisture in mantle tissues of the N, NF, and S mussels were not significantly different ($p>0.05$), but these were higher than those of the NR mussels ($p<0.05$) (Fig. 1.6). The transformed percentages of moisture in the mantle tissue samples were not statistically correlated to the levels of protein in mussels of the sample sources ($p>0.10$).

The changes in levels of protein, glycogen, glucose, lipids, and percentages of moisture in the mantle samples of the N and S treatment mussels did not vary significantly during the experiment ($p>0.25$). Also, levels of glycogen, glucose, and lipids of the N and S mussels did not differ significantly from those of the NF mussels ($p>07$). However, levels of protein of the N and S mussels did vary significantly over time from those of the NF mussels ($p<0.001$). Mean protein levels of the N and S treatments did not change significantly compared to those of the NR mussels ($p>0.18$).

Cross-sectional Areas of Muscle Fibers

The log-transformed cross-sectional areas of muscle fibers differed significantly during the experiment ($p<0.0001$), and there was an interaction between treatments and sample dates ($p<0.0001$) (Table 1.8 and Fig. 1.7). Mean fiber areas of the NF mussels declined during the experiment to 51% of the late summer baseline. The S mussels showed an increase in fiber areas during fall, and this increase was maintained throughout the experiment. The N mussels exhibited a decrease in winter, with an increase in spring. The NR fiber areas decreased to 74% of initial values in winter, and then increased to

184% in spring. At the end of the experiment, only the fiber areas of the NF and NR mussels were significantly different ($p<0.05$). The transformed cross-sectional areas were correlated to levels of protein in the mussels ($r=0.31$, $p=0.02$), and this relationship was stronger in the NF mussels ($r=0.54$, $p=0.04$).

Reproductive Condition

Of the 57 mussels evaluated for reproductive condition, 47.4% were identified as females, 36.8% were males, and 10.5% were indeterminate. Three hermaphrodites were observed (5.3%) during histological examinations, and these were classified as male hermaphrodites, with <20% of gonads devoted to oocyte production. None of the mussels of the N, NF, S, and NR was observed gravid during the experiment (August 1997 to May 1998).

The percentages of gonads containing ripe or developing gametes (GAF) were significantly different among treatments ($p=0.001$), and there was a significant interaction between treatments and sample dates ($P<0.0001$) (Table 1.9 and Fig. 1.8). The peak GAF was observed in January or February for the N, NF, S treatments, as well as the NR reference site (Fig. 1.8). In winter, the S and NR mussels showed the highest observed GAF values (mean GAF=84.3 and 78.2, respectively) (Table 1.9). Mean GAF values showed peaks and troughs in the time series patterns of the N, NF, S, and NR mussels, and the GAF values from these mussels showed different trends. For example, mussels from the S treatment showed a peak in GAF percentages in January, a severe decline in February, and an increase in April. Mussels from the NR site showed a peak GAF mean value in February, followed by a gradual decline to May. GAF plots for the N and NF treatment mussels were characterized by minor peaks at the beginning of the experiment, followed by increases during winter. By April, GAF values of the NF mussels had declined to zero. On the last sample dates of the experiment, there were no significant differences in mean GAF values of the N, NR, and S mussels ($p>0.05$), but these differed from those of the NF group ($p<0.05$). The transformed GAF means were inversely correlated to those of the protein levels in the NR mussels ($r=-0.77$, $p=0.003$).

The mean GDI values were not significantly different among treatments ($p=0.70$), but there was an interaction between treatments and sample dates ($p=0.01$) (Table 1.10 and Fig. 1.9). The GDI values decreased during the experiment, with all treatments showing a

preponderance of mature gametes (GDI value of 3) in winter. The mean GDI values of mussels from all treatments and the reference site for the last sample dates were not significantly different ($p<0.05$). The reason that the plots of the mean GDI values declined to lower values was due to the decreasing influence of GDI scores of 5 (resorbing; the presence of amoebocytes and phagocytes in the acini) throughout the experiment. Without the inclusion of GDI scores of 5, mature or spawning gametes maintained fairly consistent mean GDI values of approximately 3 for all treatments and reference sites, and then declined in spring. The mean GDI values and levels of protein were correlated in the NR mussels ($r=0.61$, $p=0.04$).

The transformed GAF and GDI means showed a significant negative correlation ($r=-0.29$, $p<0.03$). The statistical association between the mean GAF and GDI values of the NR was greater ($r=-0.71$, $p<0.01$). The lack of association between the GAF and GDI for the overall experiment was influenced by GDI scores of 5 (resorbing stage). When GDI scores of 5 were removed from the data, then strength of the statistical correlation between the GAF and GDI variables increased ($r=-0.69$, $p<0.001$). The preponderance of GDI scores of 5 was less pervasive in the histological sections of the NR mussels. Mean GDI values, excluding scores of 5, were significantly higher for the NR mussels (2.8 ± 0.15), than for those of the N (1.7 ± 0.19), NF (1.9 ± 0.19), and S (2.0 ± 0.17) treatments ($p<0.05$).

Use of mean GAF and GDI values ignores variation that occurred among individual mussels within sample dates and treatments. However, mussels from sample dates within treatments were not synchronous in gametic development and production. In other words, mussels from the same treatment and date often showed different percentages of ripe and developing gametes (GAF), and also were characterized by different GDI stages. This asynchrony was strongly substantiated in the statistical analyses; both the mean GAF and GDI values were significantly different among mussels of sample dates within treatments ($p<0.0001$). It was this asynchrony among mussels of sample dates within all treatments that caused the consistency of mature gamete production depicted in the GDI time series plots to occur during winter (Fig. 1.9).

A relationship between percentages of gonads containing ripe or developing gametes (GAF) and GDI stage within sample dates and treatments was evident (Figures 1.8 and

1.9). When mean GAF values increased, there was a corresponding decrease in associated mean GDI values. This is intuitive, because as the percentage of ripe or developing gametes increased in the gonad (the GAF), then GDI values would reflect a mean GDI value of 3 (mature gametes). The exception to this was the NF mussels in April. The GDI mean value for these mussels was 2.5, and this was the result of only scores of 0 (inactive) and 5 (resorbing). At the same time, these mussels had a mean GAF of 0. Thus, using both the GAF and GDI scoring systems, it was possible to have a mean GAF score of 0 (no mature or developing gametes), while recording a positive GDI. This was because of the influence of gametogenic activities (GDI stages of 0 and 5) not involved with mature gamete production.

For mussels of all sample sources, the transformed GAF means were not correlated with mean protein and lipid levels ($r=-0.04$, $p=0.76$ and $r=0.16$, $p=0.24$), but GAF means were correlated to glycogen levels ($r=0.35$, $p=0.007$). The GDI data were statistically related to glucoses levels ($r=0.29$, $p=0.03$). These relationships held true except for the mussels from the NR, which showed stronger statistical relationships between levels of protein and both GAF and GDI values ($r=-0.77$, $p=0.003$ and $r=0.61$, $p=0.04$).

DISCUSSION

Biochemical and Somatic Condition

In evaluating the suitability of *S. quadricauda* and *N. oleoabundans* as monoculture diets for captive adult *E. complanata*, it was evident that carbohydrates of the N, NF, and S treatment mussels followed similar declining patterns. By the end of the experiment, carbohydrate and moisture values of mantle tissues for N, NF, and S mussels formed a group that was set apart from those of the NR mussels. The percentages of moisture for mussels in all treatments and NR showed patterns of increase in fall and early winter, followed by decreases in spring. This pattern of moisture gain and loss occurred even in the NF mussels, possibly indicating a seasonal response. Despite this, tissue moisture levels of NR mussels were significantly lower than those of captive treatments at the end of the experiment. This may indicate that the NR mussels returned to a higher level of body condition by the end of the experimental period. In evaluating physiological condition strictly from the perspective of carbohydrates, the mussels of the N, NF, and S treatments were of lower physiological condition than those from the NR.

Several studies have noted seasonal and stress-related variations in bivalve tissue water content (Riley, 1976; Zandee *et al*., 1980; Lucas and Beninger, 1985; Whyte *et al*., 1990; Buchanan *et al*., 1998). Buchanan *et al*. (1998) observed decreases in mean dry tissue-to-wet tissue (g) ratios of eastern oysters, *Crassostrea virginica* (Gmelin, 1791) during the first week of a dietary experiment, with subsequent increases. Rheault and Rice (1996) also reported decreases in this ratio in tissue of nutritionally stressed *C. virginica*. Tissue moisture gain during starvation in *C. gigas* also has been recorded (Whyte *et al*., 1990), but Riley (1976) observed no variation in moisture content in *C. gigas* during starvation. Lucas and Beninger (1985) associated elevated tissue moisture percentages with depleted energy reserves observed in marine bivalves during starvation and in winter. These studies link water tissue content with bivalve condition, but do not provide specific explanatory hypotheses. It may be that, because of the combined effects of gametogenesis, and dietary and environmental stressors, less stored energy was available for ionic membrane transport. In this study, mussels of all sample sources showed similar patterns of increases in percent moisture in winter, with decreases in spring. Therefore, considering the small differences in tissue moisture of sample source mussels, it was difficult to distinguish seasonal responses common to all groups from treatment effects. Although I will briefly discuss results concerning the use of tissue moisture as a dependent variable in this experiment, I do not recommend its use in future dietary studies with freshwater mussels.

Although carbohydrate levels significantly declined during the experiment in N and S mussels, these mussels maintained elevated levels of protein. This is especially true for the S mussels, as the N mussels showed a decline in protein levels between January and February. After this decline, the N mussels showed an increase in protein levels in spring. Protein levels of NR mussels showed a response pattern different from those held in the N and S treatments. The NR mussels showed higher protein levels in late summer; these levels fell in winter, and then increased again in spring. The lipids of the NR mussels increased in winter, declined in early spring, followed by an increase by May. Lipids of N mussels increased in winter, and then began to decrease. Conversely, the S mussels showed relatively constant lipid levels during winter, with an increase in spring. The lipid levels of NF mussels remained relatively constant throughout the experiment, possibly at

a static standard metabolic level. Thus, if the suite of physiological measures for evaluation purposes is expanded to include proteins and lipids, then condition of the N and S mussels appears to be better than first thought. For example, carbohydrate levels of N and S mussels were lower than mussels from the NR site, but the opposite was found for levels of proteins and lipids.

Declines in proteins, carbohydrates, and the percent moisture in mantle tissue of captive bivalves have been related to diet type, ration, holding conditions, season, and gametogenic progression (Gabbott and Bayne, 1973; Bayne *et al*., 1982; Lane, 1986; Whyte *et al*., 1990; Robinson, 1992; Patterson *et al.*, 1997). Naimo *et al*. (2000) found that glycogen concentrations of *M. transversum* declined regardless of artificial diet and sediment exposure. As previously stated, Buchanan *et al*. (1998) observed declines in mean dry weight-to-wet weight ratio (g) in tissue of *C. virginica*, fed algal diets in closed recirculating systems. Buchanan *et al*. (1998) and Naimo *et al*. (2000) attributed these declines to possible poor nutrition. Bayne and Thompson (1970) observed that levels of carbohydrate and protein declined in captive *M. edulis* regardless of algal ration, and that declines were more severe at temperatures that were higher than seasonal norms of the host population's natural environment. These findings appear to support the hypothesis that there was a critical temperature maximum above which proteins and carbohydrates were more heavily utilized. Bayne and Thompson (1970) hypothesized that the effects of culture conditions increased the natural energy maintenance requirements of captive *M. edulis*, and that the declines in energy substrates were the result of increased energy demands unmet by ration input energy. A plausible explanation for the lack of significance among carbohydrate levels in N, NF, and S mussels is that the NF mussels may have decreased their metabolic rates from routine to standard rates in response to starvation (Bayne, 1973). Bayne (1973) found that during periods of starvation within the gametogenic period, *M. edulis* attains an adaptive standard metabolic rate within 25 to 30 d. Although this study was not concerned with measuring metabolic adjustment during starvation, the NF mussels did appear to achieve steady rates of decline in proteins, lipids, and cross-sectional areas of muscle fibers.

Stored lipids also make important contributions to fulfilling seasonal energetic demands in bivalves, especially in those that experience nutritional stress (Bayne, 1973;

Lane, 1986; Whyte *et al*., 1990). Zandee *et al*. (1980) observed that in *M. edulis*, lipid levels, along with proteins and glycogen, increased from spring to autumn during periods of high food availability, and remained high until spawning in spring when proteins and glycogen reached low levels. Gabbott and Bayne (1973) concluded that during starvation, *M. edulis* showed seasonal breakdown of stored carbohydrates in late summer, increased utilization of lipids in fall when carbohydrates were low, with a shift to protein catabolism in winter. Sprung and Borcherding (1991) reported that lipids were energetically important during the first 10 d of starvation in zebra mussels, *D. polymorpha*. The inter-tidal Atlantic rangia clam, *Rangia cuneata* (G. B. Sowerby I, 1831) showed a preferential decline in somatic carbohydrates during starvation, but not lipids (Lane, 1986). In my study, a simultaneous decline in carbohydrates and proteins occurred in NF mussels, while lipids showed a slight decline, if any, to a stable level. Riley (1976) also observed conservation of lipids during starvation of *C.* gigas, and postulated that preservation of lipids was important to vitellogenesis during oogenesis. While carbohydrate levels of N and S mussels declined during the experiment, proteins and lipids remained above baseline levels. This indicates that the N and S diets were sufficient in their protein and lipid contents to prevent draw-down of these stored substrates in the mantle tissue. In the NR mussels, proteins, carbohydrates, and lipids increased during spring; prior to this, there was a sharp decrease in lipid levels (Fig. A1.5). During this period, the decrease in lipid levels coincides with stabilization of the GDI in the NR mussels at about 3 (mature stage). It may be that a winter decrease in lipids in the NR mussels coincided with vitellogenesis during that period.

Carbohydrate levels in the NR water sample were lower than those of the N and S algae samples, but the glycogen levels of the N and S mussels were lower than those of the NR mussels. In addition, organic content of the NR sample was low. It is possible that a synergistic effect from factors such as effects of gametogenesis and captivity could account for the discrepancy between carbohydrate levels of water samples and associated levels of mantle tissue carbohydrates. The use of carbohydrates during winter may be important for meeting energetic demands of gametogenesis in freshwater mussels. Alternatively, the higher indoor temperature regime in captivity during winter may have heightened metabolic demands on N and S mussels, such that carbohydrate levels in their

feed were not sufficient to meet those demands. This possible diet deficiency could create an energy imbalance that may be met by depletion of carbohydrate body reserves. Zandee *et al*. (1980) found that declines in carbohydrate levels of *M. edulis* during winter and spring were associated with gametogenesis. Gabbott and Bayne (1973) observed that stored glycogen served as the main energy source during late summer and early winter in *M. edulis*, such that by the end of January glycogen levels had dropped to 12% of original levels. They attributed these declines to seasonal energetic demands of gametogenesis. It may be that rations of 30,000 cells/mL used in the N and S treatments were inadequate to meet energetic demand created by the combined effects of higher than normal winter temperatures in the laboratory and gametogenesis of these mussels. Temperatures during the ration study ranged from 20 to 22º C, while the mean temperature during the diet experiment was 18.5º C (range = 15.8 to 23.7º C). Future determination of an adequate ration for freshwater mussels should account for temperature regimes under which captive mussels should be maintained.

Although the N, S, and NR mean temperatures were not significantly different, the N and S mussels were held at higher temperatures in winter than the NR mussels, and this was likely an important factor affecting glycogen utilization by these mussels. Bayne and Thompson (1970) found that carbohydrate utilization in captive *M. edulis* was related to temperature changes relative to normal seawater temperature for source populations, and that there was a temperature threshold above which carbohydrate utilization readily occurred. In their experiments, Bayne and Thompson (1970) observed that O_2 consumption (mL O_2/g DW/h) of *M. edulis* decreased during summer and increased in winter due to gametogenesis. Mann (1979a) reported that metabolic costs associated with increased temperatures initiated substrate utilization due to food limitation. Even though the percentages of carbohydrates in the N and S diets were higher than those of the NR water sample, it is likely that the combined effects of gametogenesis and increased winter temperatures during captivity caused the N and S mussels to incur higher metabolic costs. If this was so, then carbohydrate levels in the N and S diets may have been insufficient to prevent energy reserve depletion. It is reasonable to conclude therefore that the N and S diets were insufficient in carbohydrate content for captive *E. complanata*.

Even though the N and S mussels may have incurred higher metabolic costs that decreased their carbohydrate reserves during the experiment, these mussels maintained elevated levels of protein in relation to NR mussels. This is not surprising since the percentages of proteins in the N and S diets were quite high compared to that of the NR water sample. Because of this, it may be reasonable to conclude that *S. quadricauda* provided adequate protein content as an algal feed for adult *E. complanata*, but that another algal species should be identified for feed inclusion as a source of carbohydrates.

It would be simplistic to assume that declines in the energy substrates of captive mussels are solely the result of ration or ration type, because there are also seasonal energetic demands initiated by the gametogenic cycle. Declines of stored proteins and glycogen associated with gametogenesis and low food levels have been observed from November through March in *M.* edulis (Zandee *et al.*, 1980). Deslous-Paoli and Héral (1988) also noted a decline in these substrate levels in *C. gigas* during fall and winter. Gabbott and Bayne (1973) refined the hypothesis that carbohydrates and proteins are stored during spring and summer in *M. edulis*, and utilized in fall and winter during gametogenesis. They found that glycogen served as the main energy source during summer and early winter, and that proteins became the primary energy source during winter when glycogen reserves were low. Therefore, the traditional view that carbohydrates serve as the main energy reserve for bivalves is conditional upon season and gametogenic progression. My results show that there was a simultaneous depletion of carbohydrates and proteins in the NR mussels, implying that *E. complanata* did not preferentially utilize either substrate during the experiment.

The sizes of muscle fiber diameters and cross-sectional areas have been shown to decline in fish under nutritional stress (Johnston, 1981; Moon, 1983a and 1983b; Kim and Lovell, 1995). Johnston (1981) observed declines in median muscle fiber size during starvation in plaice, *Pleuronectes platessa* (Fabricius). Kim and Lovell (1995) found that muscle fiber size did not change in fed and partially fed channel catfish, *Ictalurus punctatus* (Rafinesque), but decreased with starvation. The decrease in muscle fiber area with starvation also has been observed in American eels, *Anguilla rostrata* (Lesueur) (Moon, 1983b). Moon (1983a) concluded that the breakdown of muscle protein is an important element of the physiological fasting strategy of the eel. In my study, the fiber

areas of the N and S mussels increased to 110% and 122% of baseline values by the end of the experiment. This increase, and the elevated levels of tissue protein of the N and S mussels, indicated that their dietary protein intake was probably sufficient to avoid the breakdown of muscle protein. In the NF mussels, there were simultaneous declines in both proteins and fiber area, indicating that muscle breakdown may also be a fasting strategy in *E. complanata* to supplement declining protein levels. The NR mussels showed similarity between fiber areas and protein levels, with decreases in winter and increases in spring. It is plausible that these patterns in the NR mussels indicate that muscle protein provides a supplemental energy store that is depleted and replenished as food and energetic requirements vary. Because there was a clear treatment response, cross-sectional muscle fiber areas can be used as a dependent variable in future diet studies.

The combined patterns of carbohydrate, protein, lipid levels, and muscle fiber diameters provide a basis for discussion of differences in the physiological responses of mussels in the different feed treatments and the reference site. In the NF treatment, mussels exhibited concurrent declines in carbohydrates, proteins, and muscle fiber areas during the experiment. Although there appeared to be a patterned response of increases in percent moisture, followed by subsequent declines, in mussels of all treatments and reference site, no such similarity was evident for the patterns of carbohydrates, proteins, and lipids. Another pattern evident from the data was that all mussels, despite their physiological condition, were able to maintain some level of gamete production. All fed mussels maintained gamete production throughout the entire experiment, and even the NF mussels maintained gamete production throughout most of the experiment. Bayne and Thompson (1970) found that *M. edulis* maintained gamete production under nutritive stress and starvation conditions despite simultaneous decreases in carbohydrates and proteins. That gamete production ceased in the NF mussels after prolonged decreases in the energy substrates agrees with the findings of Bayne and Thompson (1970) and Bielefeld (1991) for *M. edulis* and *D. polymorpha*, respectively. The cessation of gametogenesis did not occur in the N and S mussels, and this indicated that these diets were of sufficient nutritive quality to enable captive mussels to maintain gamete production. It may be that declines in carbohydrates in the N and S mussels were not

critical to the gamete production of these mussels. The continued production of mature gametes in N and S treatment mussels also may have been the result of their adequate lipid levels. The inverse relationship between proteins and gamete production in the NF mussels during the experiment suggests that this substrate is important during gametogenesis for *E. complanata*. The inverse relationship in the NF mussels between muscle fiber diameters and gamete maturity (GDI stage 3) indicated that the gametes matured at the possible expense of muscle protein. That proteins were maintained during gamete production in N and S mussels also suggests that protein content in their diets was seemingly sufficient to prevent decreases in protein levels.

The hypothesis that the N and S diets were of sufficient protein content to support gamete production is upheld because no significant decreases in the levels of protein occurred, yet gamete production was maintained. Pipe (1985) reported that nutrient investment in developing eggs was maintained during a starvation experiment with *M. edulis* through simultaneous somatic decreases in carbohydrates and proteins. Pipe (1985) also noted an increase in production of ripe gametes during starvation. Based on these findings, one would expect a simultaneous depletion of carbohydrates and proteins in the N and S mussels, if these diets were of poor nutritive quality. Instead, I found that carbohydrates decreased with simultaneous maintenance of protein levels in the N and S mussels. Additionally, the data indicated that the NF mussels were able to produce at least 2 cohorts of ripe gametes during the experiment, until gamete production ceased in spring. This cessation of gamete production in the NF mussels appeared to coincide with depletion of both carbohydrates and proteins to some critical level. Even in NF mussels, priority of gametogenesis was evident; no regression or resorption of mature gametes were observed, except in acini that were classified as spawned (stage 4 in the GDI evaluation system). The regression and resorption of previously formed gametes in nutritionally stressed *M. edulis* has been reported (Bayne *et al*., 1978).

The protein levels of the tissue samples re-tested at The Academy of Natural Sciences were higher than those measured by my method. Because of the homogenization used in my protein assay method, it may be that the measured levels were a mix of total and soluble proteins. Decreases in soluble protein have been observed in response to numerous experimental variables in aquatic organisms. Reddy and Bashamohideen

(1995) observed decreases in both total and soluble proteins in organs and muscle tissue of common carp, *Cyprinus carpio* Linnaeus, 1758, with exposure to cypermethrin. Beaulieu and Guderley (1998) also reported decreases in soluble protein during starvation in Atlantic cod, *Gadus morhua* Linnaeus, 1758. Such decreases also have been observed during starvation in juvenile disk abalone, *Haliotis discus discus* (Reeve, 1846) and intermolt noble crayfish, *Astacus astacus* Linnaeus, 1758 (Takami *et al*. 1995; Huner *et al*. 1985). I detected a treatment response in proteins in this experiment. This is especially true in the NR mussels, which showed a negative correlation between levels of protein and the production of developing and ripe gametes (GAF). This relationship suggests that soluble proteins may play an important role in gamete production in *E. complanata*. The importance of protein investment in developing eggs has been demonstrated for *C. virginica*, northern quahogs, *Mercenaria mercenaria* (Linnaeus, 1758), and the foolish mussel, *M. trossulus* Gould, 1950 (Lee and Heffernan, 1991; Kreeger, 1993). Lee and Heffernan (1991) found that the major protein in eggs of *C. virginica* and *M. mercenaria* was an unidentified high-density lipoprotein, and hypothesized that it may play an important role in embryo development.

Gametogenic Activity

Variation in reproductive cycles among populations of conspecific bivalves has been noted in previous studies. For example, Lowe *et al*. (1982) studied 2 distinct populations of *M. edulis* in England and found 2 different gametogenic patterns, each with different seasonal substrate storage cycle characteristics. One population showed typical gametogenesis during winter months with associated declines in energy reserves. The other population spawned twice in one year, with gametogenesis occurring in winter and again in summer. During the winter gametogenic cycle, there was a typical drawdown of energy reserves; but during summer, gamete development was associated with storage of energy reserves because of high food availability. Newell *et al*. (1982) showed that variation among populations of *M. edulis* in their reproductive cycles along the eastern United States did not depend on latitude. In fact, the greatest temporal variation in reproductive cycles occurred between 2 populations on the same latitude near Long Island, New York. Newell *et al*. (1982) observed no relationship between water temperature and the rate of gametogenic progress, and attributed the differences in the 2

observed reproductive cycles to temporal and quantitative differences in energy content of local food resources.

Deviation from expected reproductive cycles also has been documented for freshwater mussels. Heard (1975) observed 2 populations of the Florida floater, *Utterbackia peggyae* (=*Anodonta peggyae*) (R. I. Johnson, 1965), with different reproductive patterns. Mussels from a stream population located in Holmes County, Florida exhibited one period of gametogenesis annually, while *U. peggyae* from a lake in Leon County, Florida expressed 2 distinct periods. Although developing and mature eggs were observed in the gonads of both populations during all sample events, the mussels from the stream held mature glochidia in the marsupia from July to August and again in January and February. The lake population showed gravidity only from January to February.

Lewis (1985) reported that the giant floater, *Pyganodon grandis* (= *A. grandis*) (Say, 1829) in Quebec was a short-term brooder (tachytictic breeding cycle), with gametogenesis and gravidity occurring in spring and summer, whereas *P. grandis* of Ontario, North Dakota, and Pennsylvania exhibited evidence of fertilization in late summer with glochidia over-wintering in marsupia until the following spring (Clarke 1973). Smith (1978) reported a biannual gametogenic pattern in the eastern pearlshell, *Margaritifera margaritifera* (Linnaeus, 1758), from Massachusetts that differed from previously reported tachytictic brooding. The findings of Gordon and Smith (1990) supported the hypothesis that the spectaclecase, *Cumberlandia monodonta* (Say, 1829), of Missouri is also a biannual spawner, rather than a short-term brooder. Modifications of typical breeding patterns in freshwater mussels have been attributed to genetically-based plasticity in reproductive activity, food availability, migration patterns of host-fish, and differences among local watershed flow regimes and temperature patterns (Heard, 1975; Smith, 1978; Lewis, 1985; Gordon and Smith, 1990; Woody and Holland-Bartels, 1993; Garner *et al*., 1999).

A review of the literature revealed only one publication on the period of gametogenesis for *E. complanata*. Matteson (1948) reported that the gametogenic cycle of this species in Michigan ranged from roughly November to the middle of June, with the maximum release of sperm occurring in mid-May. The start and end of the male

gametogenic period overlapped considerably that of females. Matteson (1948) recorded the presence of mature sperm in acini throughout the year, but only observed mature gametes in the sperm and ovarian ducts in spring. These findings differ somewhat from my observations. I documented a gametogenic process characterized by increases in the percentages of ripe and developing gametes in sections of the gonads, followed by the resorption (GDI stage 5) of reproductive material after final acinic discharge. This process was observed in all examined mussels from all sample sources. I also observed mature gametes in sperm and ovarian ducts throughout the experiment. This study did not measure whether mature gametes were released to the environment or held in storage, but no gravid mussels were observed during the experiment in any sample. This was unexpected, because I did not observe gametes in early stages of development, suggesting that early gametogenesis occurred prior to the first sampling of gonadal tissue in September, or that early gametogenesis occurred rapidly between sample dates. This appears to be the case for male and female N, NF, and S treatment mussels, because they showed evidence of developing 2 cohorts of ripe gametes to maturity during the experiment. This pattern differs from that exhibited by the NR mussels; these mussels only developed one cohort of mature gametes *in situ*. This difference in gametic patterns of the N, NF, and S mussels from those of the NR likely was influenced by the temperature regimes that influenced the 2 groups. Although overall mean temperatures of the captive treatments were not significantly different from that of the NR, the variation in temperature of the Nottoway River was obviously more extreme. For example, the winter minima for the captive treatments and the NR mussels were 15.5º C and 8.2º C, respectively.

Successive peaks in gametogenic activity during a single reproductive season are not uncommon in bivalves. Mann *et al*. (1994) recorded multiple major and minor peaks in the gonad area fraction (GAF) during a single reproductive season in *C. virginica* in the James River, Virginia. Such polymodal gametogenic patterns also have been recorded for species that exhibit relatively continuous spawning, such as *Mercenaria* spp. in lower latitudes and the giant reef clam, *Periglypta multicostata* (Sowerby, 1835) (Heffernan *et al*., 1989; Hesselman *et al*., 1989; García-Domínguez *et al*., 1998). Zale and Neves (1982) noted continuous presence of mature gametes throughout the year in freshwater

mussels, including the rainbow mussel, *Villosa iris* (I. Lea, 1829), mountain creekshell, *V. vanuxemensis* (I. Lea, 1838), Cumberland moccasinshell, *Medionidus conradicus* (I. Lea, 1843), and wavyrayed lampmussel, *Lampsilis fasciola* Rafinesque, 1820. Haggerty *et al*. (1995) also noted mature spermatozoa and oocytes in acini of purple wartybacks, *Cyclonaias tuberculata* (Rafinesque, 1820) over an extended period of the year.

It is noteworthy that both the percentages of developing and ripe gametes (GAF) and the reproductive stages (GDI) were significantly different in mussels of the same sample date. This asynchrony was evident within mussels of all sample sources; therefore, it may be a reproductive strategy to maintain consistent gamete production over a prolonged period. No studies have reported an analysis of reproductive asynchrony within bivalve populations or treatments. In this histological study, developing gametes were rarely observed; therefore, the GAF percentages reported herein reflect observation of mostly mature gametes. Apparently, mussels from the treatments and reference site maintained a roughly continuous spawning process that was maintained by the asynchronous gametogenic activity within sample dates.

In marine bivalve studies, the gamete area fraction (GAF) has been commonly used to evaluate reproductive condition in nutrition-related experiments (Bayne *et al*., 1978; Lowe, 1982; Mann *et al*., 1994). Kreeger (1993) modified this procedure with *M. trossulus* to estimate the mantle gamete area (MGA), based on histological section evaluation, for determination of percentage of ripe and developing gametes. Kreeger (1993) used MGA to estimate the GAF of reproductive tissue in the entire mantle. Since I could not dissect the gametic tissue from the visceral mass, I adapted the MGA for application to freshwater mussels by use of the GAF. The work of Chalkley (1943) substantiates the hypothesis that there is a relationship between the counts of target cells in histological sections using the random point-count volumetry method and their volume within the sampled tissue mass, if enough histological sections are examined. Since I did not find significant differences among the GAF percentages and GDI scores within individual mussels, my histological section sample size (n=3) was adequate to meet the assumption demands of Chalkley (1943).

The combined use of the GAF and GDI gametogenic evaluation systems was beneficial. The use of the GAF allowed detection of the cessation of production of

developing and ripe gametes in the NF mussels. This assessment showed differences between the N, NF, and S mussels that would have not been detected with only the use of the time-series energy substrate profiles. It was use of the GDI that showed no new cycle of gametogenesis in the mussels, and this denied an important endpoint to evaluate and compare the treatment mussels. The observations of mussels progressing through one gametogenic cycle to the next would have provided additional resolution to compare the treatment mussels. In future studies, such resolution can be obtained by lengthening experimental duration, and with the combined use of evaluative indices such as the GAF and GDI. Sole use of energy substrate profiles to evaluate condition of freshwater mussels, without considering gametogenic status and progression, lacks the resolution to detect biologically meaningful differences. Reproductive status and gamete development over time are of primary importance to future captive propagation efforts with threatened and endangered freshwater mussels; therefore, they should be considered important, in combination with energy substrate profiles, for evaluation of treatment effects.

Conclusions and Recommendations

Examination of time-series profiles of the levels of energy substrates, such as carbohydrates, proteins, and lipids, can provide insight into the effects of treatments and holding conditions on energetic physiology, but it is also important to consider the concurrent utilization of these substrates by mussels. The ratio of oxygen consumption to nitrogen excretion (O:N ratio), as measured in bivalves, is an established technique for determining the utilization of somatic carbohydrates and lipids, versus the utilization of stored proteins (Baker and Hornbach, 1997). Using appropriate methods for the estimation of the O:N ratio, and prior to the collection of tissues from experimental bivalves on a sample date, the oxygen consumption and ammonia excretion of selected specimens are measured (Bayne, 1973). Relative to the range of the O:N values of the experimental mussels, higher ratio estimates indicate carbohydrate- and lipid-oriented substrate utilization, and vice versa for protein-oriented utilization (Bayne, 1973). Oxygen:nitrogen ratios could provide finer resolution for the evaluation of diet treatment results in future studies. The determination of the substrate utilization disposition of mussels prior to their sacrifice could have assessed the sufficiency of their substrate reserves. For example, if the N and S mussels that exhibited low levels of carbohydrates

at the end of the experiment were utilizing more protein at the time of their collection, then their lower levels of carbohydrates may not have been of critical concern, especially since their gametic production was relatively high. To gain a finer level resolution of treatment effects, future studies similar to this one should measure levels of total carbohydrates, proteins, and lipids in mussels, as well as estimate their O:N ratios prior to the collection of tissues. The examination of utilization patterns of these energy substrates during gametogenic progression in wild and captive freshwater mussels would provide a basis for further assessment of appropriate algal diets. With such an investigation, selection of algal species for food could then be founded on energy utilization needs during gametogenesis.

Several conclusions can be drawn from this study. Gametogenesis was maintained even in starved mussels, despite declines in carbohydrates and proteins. Although gamete production of the NF mussels eventually ceased, it appeared that priority of gamete production over resorption for *E. complanata* was maintained even during starvation. Declines in carbohydrates in the N, NF, S and NR mussels during winter indicated that metabolism of carbohydrates during gametogenesis was important. The pattern of carbohydrate declines in N and S mussels supported the hypothesis that the diets lacked sufficient carbohydrates. The N and S mussels maintained relatively high levels of protein, and possibly lipid during the experiment, compared to those of the NR reference site. There was a decrease in protein levels of the NR mussels, and this was associated with their gamete production in winter. That this decline did not occur in the N and S mussels indicated that the protein content of their diets was sufficient to prevent winter depletion, even while gamete production was maintained.

Although gamete production and maturation were maintained in the N and S mussels during the experiment, depletions of their carbohydrate levels indicate that use of *N. oleoabundans* and *S. quadricauda* as monoculture algal diets was inadequate. Also, the ration concentrations of these diets may have been insufficient to meet carbohydrate demands of mussels during the experiment. Mixes of algal species for adult mussels should provide a nutritionally complete food source. Because of high protein and lipid content, *S. quadricauda* should be included in such a mix. Further research is warranted to identify species of algae high in carbohydrate content for dietary inclusion. As reported

herein, evaluation of the carbohydrate content of *N. oculata* showed that this species is high in carbohydrates; therefore, this species is a strong candidate for feed inclusion. Because of negligible mortality and continuous gamete production, the re-circulating systems used during the captive phase of this experiment are adequate to hold adult *E. complanata*. These systems could be modified by adding a temperature control unit to simulate natural thermal regimes. Although temperature was not treated as a dependent variable in this experiment, it likely affected carbohydrate declines in N and S mussels. Dietary research with adult freshwater mussels should include measurement of total carbohydrates, proteins, and lipids in tissue, as well as evaluation of gametic production and maturity. The estimation of energy substrate utilization patterns through the O:N ratio would facilitate understanding of changes in the dietary requirements of freshwater mussels during different seasons and gametogenic stages, and help to provide information to formulate appropriate diets.

TABLES AND FIGURES

Table 1.1. Classification stages of the gamete development index (GDI) for evaluation of gametogenic activity of *Elliptio complanata*. Adapted from Barber (1996).

Gametogenic Stage	Stage Classification	Description
0	Inactive	Acini are nonexistent or elongated, with walls consisting of undifferentiated germinal epithelium.
1	Early active	Acini contain oogonia and primary oocytes, or spermatogonia, spermatocytes and spermatids, but no free oocytes or spermatozoa.
2	Late active	Free oocytes or spermatocytes and spermatids predominate the acini; there are some spermatozoa.
3	Mature	Mature gametes (oocytes and spermatozoa) fill the acini.
4	Spawned	Acini contain spaces mostly devoid of gametes; acini walls may be broken. Hemocytes and phagocytes may be sparsely present.
5	Resorbing	Acini have a shrunken appearance and contain phagocytes and products of resorbtion. Gametes are refractory, and development is not evident.

Table 1.2. Percentages dry weight (g) of total carbohydrates, proteins, lipids, and organic contents in *Neochloris oleoabundans*, *Scenedesmus quadricauda*, and Nottoway River samples (n=1). Results for *Nannochloropsis oculata* and *Selenastrum capricornutum* are included for reference. The algae samples were from semi-continuous cultures in the laboratory.

Sample	Carbohydrates	Proteins	Lipids	Organic Content
N. oculata	28.2	23.0	10.7	85.9
N. oleoabundans	12.5	37.6	8.5	93.2
S. capricornutum	11.6	18.1	12.9	91.9
S. quadricauda	9.1	26.4	23.7	83.2
Nottoway River	1.8	7.1	4.2	10.1

Table 1.3. Mean protein levels (mg/g DW) and standard errors (±SE) in mantle tissue samples of *Elliptio complanata* from the *Neochloris oleoabundans*, *Scenedesmus quadricauda* and no feed diet treatments, and the Nottoway River reference site (n = 18). Means within columns with the same letters are not significantly different ($p<0.05$). Mean level of Nottoway River mussels from sample number 1 is considered the baseline for the diet treatments.

Sample		**Diet Treatments**			
Number	**Date**	***Neochloris oleoabundans***	***Scenedesmus quadricauda***	**No Feed**	**Nottoway River**
0	08/97	34.9 ± 3.8a	34.9 ± 3.8	34.9 ± 3.8a	34.9 ± 3.8a
1	09/97	69.8 ± 9.1b	63.8 ± 2.4ab	65.1 ± 2.9b	79.8 ± 4.6
2	11/97	60.6 ± 3.2bc	71.9 ± 3.2ab	39.1 ± 5.4bc	-
3	01/98	62.1 ± 3.5b	69.0 ± 3.0ab	37.4 ± 3.8ac	-
4	02/98	42.9 ± 4.7ac	70.9 ± 2.9ab	34.1 ± 4.1a	10.4 ± 2.1
5	03/98	-	-	-	41.3 ± 4.8ab
6	04/98	57.7 ± 3.2bc	56.6 ± 2.3a	21.6 ± 2.8a	-
7	05/98	-	-	-	58.1 ± 3.0b

Table 1.4. Mean glycogen levels (mg/g DW) and standard errors (±SE) in mantle tissue samples of *Elliptio complanata* from the *Neochloris oleoabundans*, *Scenedesmus quadricauda*, and no feed diet treatments, and the Nottoway River reference site (n = 18). Means within columns with the same letters are not significantly different ($p<0.05$). Mean level of Nottoway River mussels from sample number 1 is considered the baseline for the diet treatments.

Sample		**Diet Treatments**			
Number	**Date**	***Neochloris oleoabundans***	***Scenedesmus quadricauda***	**No Feed**	**Nottoway River**
0	08/97	148.7 ± 22.1a	148.7 ± 22.1ab	148.7 ± 22.1a	148.7 ± 22.1a
1	09/97	171.7 ± 19.7a	206.8 ± 18.7a	167.9 ± 16.4ab	126.7 ± 25.6a
2	11/97	67.9 ± 13.2b	98.7 ± 14.9ab	106.2 ± 17.0abc	-
3	01/98	71.2 ± 12.7b	83.5 ± 14.7ab	90.0 ± 16.8ac	-
4	02/98	41.6 ± 8.7b	55.2 ± 12.0b	55.1 ± 8.7cd	85.6 ± 25.0a
5	03/98	-	-	-	78.1 ± 16.4a
6	04/98	31.2 ± 4.0b	46.3 ± 8.5b	26.5 ± 4.1d	-
7	05/98	-	-	-	91.3 ± 24.0a

Table 1.5. Mean glucose levels (mg/g DW) and standard errors (±SE) in mantle tissue samples of *Elliptio complanata* from the *Neochloris oleoabundans*, *Scenedesmus quadricauda*, and no feed diet treatments, and the Nottoway River reference site (n = 18). Means within columns with the same letters are not significantly different ($p<0.05$). Mean level of Nottoway River mussels from sample number 1 is considered the baseline for the diet treatments.

Sample		**Diet Treatments**			
Number	**Date**	***Neochloris oleoabundans***	***Scenedesmus quadricauda***	**No Feed**	**Nottoway River**
0	08/97	21.8 ± 1.6a	21.8 ± 1.6a	21.8 ± 1.6a	21.8 ± 1.6a
1	09/97	20.6 ± 2.2a	24.8 ± 2.2a	28.4 ± 3.5a	24.0 ± 4.4ab
2	11/97	13.4 ± 2.2b	10.4 ± 1.0b	33.9 ± 3.3a	-
3	01/98	10.2 ± 1.4bc	11.9 ± 1.3b	12.6 ± 1.1b	-
4	02/98	7.8 ± 1.0c	8.9 ± 1.0b	11.3 ± 1.5b	12.8 ± 2.8b
5	03/98	-	-	-	16.9 ± 3.4ab
6	04/98	7.2 ± 0.8c	9.0 ± 1.2b	8.5 ± 0.7b	-
7	05/98	-	-	-	15.9 ± 1.5ab

Table 1.6. Mean lipid levels (mg/g DW) and standard errors (±SE) in mantle tissue samples of *Elliptio complanata* from the *Neochloris oleoabundans*, *Scenedesmus quadricauda*, and no feed diet treatments, and the Nottoway River reference site (n = 3). Means within columns with the same letters are not significantly different ($p<0.05$). Mean level of Nottoway River mussels from sample number 1 is considered the baseline for the diet treatments.

Sample		**Diet Treatments**			
Number	**Date**	***Neochloris oleoabundans***	***Scenedesmus quadricauda***	**No Feed**	**Nottoway River**
0	08/97	69.6 ± 11.3a	69.6 ± 11.3a	69.6 ± 11.3a	69.6 ± 11.3a
1	09/97	87.9 ± 19.5a	87.9 ± 19.5a	57.9 ± 7.5a	99.5 ± 13.1ab
2	11/97	76.2 ± 8.2a	76.2 ± 8.2a	65.8 ± 14.1a	-
3	01/98	78.9 ± 10.3a	78.9 ± 10.3a	49.3 ± 4.9a	-
4	02/98	70.9 ± 9.4a	70.9 ± 9.4a	52.1 ± 9.9a	115.3 ± 12.1abc
5	03/98	-	-	-	56.5 ± 18.2ab
6	04/98	102.7 ± 15.6a	102.7 ± 15.6a	51.4 ± 10.3a	-
7	05/98	-	-	-	85.1 ± 7.2abc

Table 1.7. Mean percentage moisture levels and standard errors (±SE) in mantle tissue samples of *E. complanata* from the *Neochloris oleoabundans*, *Scenedesmus quadricauda*, and no feed diet treatments, and the Nottoway River reference site (n = 18). Means within columns with the same letters are not significantly different ($p<0.05$). Mean level of Nottoway River mussels from sample number 1 is considered the baseline for the diet treatments.

Sample		**Diet Treatments**			
Number	**Date**	***Neochloris oleoabundans***	***Scenedesmus quadricauda***	**No Feed**	**Nottoway River**
0	08/97	84.6 ± 0.6a	84.6 ± 0.6ab	84.6 ± 0.6a	84.6 ± 0.6a
1	09/97	84.0 ± 0.8a	84.9 ± 0.7ab	84.0 ± 0.6a	89.0 ± 0.5b
2	11/97	87.4 ± 0.5b	86.5 ± 0.5ab	85.8 ± 0.8a	-
3	01/98	87.7 ± 0.9b	87.8 ± 0.7a	86.5 ± 1.1a	-
4	02/98	87.7 ± 0.8b	87.6 ± 1.1a	90.7 ± 0.4	87.9 ± 1.4ab
5	03/98	-	-	-	81.4 ± 1.4c
6	04/98	84.1 ± 0.8a	83.2 ± 1.0ab	85.9 ± 0.7a	-
7	05/98	-	-	-	79.1 ± 0.9c

Table 1.8. Mean cross-sectional areas (μm^2) and standard errors (±SE) of muscle fibers of the visceral mass of *E. complanata* from the *Neochloris oleoabundans*, *Scenedesmus quadricauda*, and no feed diet treatments, and the Nottoway River reference site (n = 3). Means within columns with the same letters are not significantly different ($p<0.05$).

Sample		**Diet Treatments**			
Number	**Date**	***Neochloris oleoabundans***	***Scenedesmus quadricauda***	**No Feed**	**Nottoway River**
0	08/97	-	-	-	-
1	09/97	33.6 ± 2.5abcd	30.3 ± 1.9	38.5 ± 6.6	37.6 ± 1.7a
2	11/97	29.3 ± 2.3ab	37.7 ± 2.3a	26.7 ± 6.1a	-
3	01/98	30.3 ± 1.7abc	36.3 ± 7.0a	27.2 ± 4.1a	-
4	02/98	40.0 ± 6.7	38.1 ± 4.6a	27.9 ± 6.2a	27.7 ± 3.9
5	03/98	-	-	-	33.7 ± 3.6a
6	04/98	37.1 ± 2.5ad	36.8 ± 1.5a	19.7 ± 5.7	-
7	05/98	-	-	-	61.1 ± 8.1

Table 1.9. Mean gamete area fraction (GAF) and standard errors (±SE) in gonadal tissue samples of *E. complanata* from the *Neochloris oleoabundans*, *Scenedesmus quadricauda*, and no feed diet treatments, and the Nottoway River reference site (n = 3). Means within columns with the same letters are not significantly different ($p<0.05$).

Sample		**Diet Treatments**			
Number	**Date**	***Neochloris oleoabundans***	***Scenedesmus quadricauda***	**No Feed**	**Nottoway River**
0	08/97	-	-	-	-
1	09/97	32.9 ± 9.1a	48.1 ± 9.2a	25.0 ± 6.1a	31.5 ± 5.0
2	11/97	15.3 ± 5.0a	56.0 ± 8.4ab	45.4 ± 8.2ab	-
3	01/98	45.4 ± 11.6a	84.3 ± 2.3ab	30.1 ± 5.7ab	-
4	02/98	48.1 ± 9.4a	23.6 ± 12.2abc	69.4 ± 2.6b	78.2 ± 3.1
5	03/98	-	-	-	56.0 ± 3.8a
6	04/98	27.3 ± 7.5a	38.9 ± 10.9abc	0.0 ± 0.0	-
7	05/98	-	-	-	48.1 ± 3.3a

Table 1.10. Mean gamete development index (GDI) and standard errors (±SE) in gonadal tissue samples of *E. complanata* from the *Neochloris oleoabundans*, *Scenedesmus quadricauda*, and no feed diet treatments, and the Nottoway River reference site (n = 3). Means within columns with the same letters are not significantly different ($p<0.05$).

Sample		**Diet Treatments**			
Number	**Date**	***Neochloris oleoabundans***	***Scenedesmus quadricauda***	**No Feed**	**Nottoway River**
0	08/97	-	-	-	-
1	09/97	4.1 ± 0.29ab	3.7 ± 0.28a	4.5 ± 0.11a	4.7 ± 0.05
2	11/97	4.4 ± 0.17a	3.3 ± 0.24a	3.2 ± 0.40ab	-
3	01/98	3.0 ± 0.31abc	3.0 ± 0.08a	3.9 ± 0.37abc	-
4	02/98	2.7 ± 0.33c	3.0 ± 0.11a	2.8 ± 0.14bcd	2.7 ± 0.08a
5	03/98	-	-	-	3.0 ± 0.12a
6	04/98	2.1 ± 0.22c	2.3 ± 0.19	2.5 ± 0.29bd	-
7	05/98	-	-	-	2.9 ± 0.33a

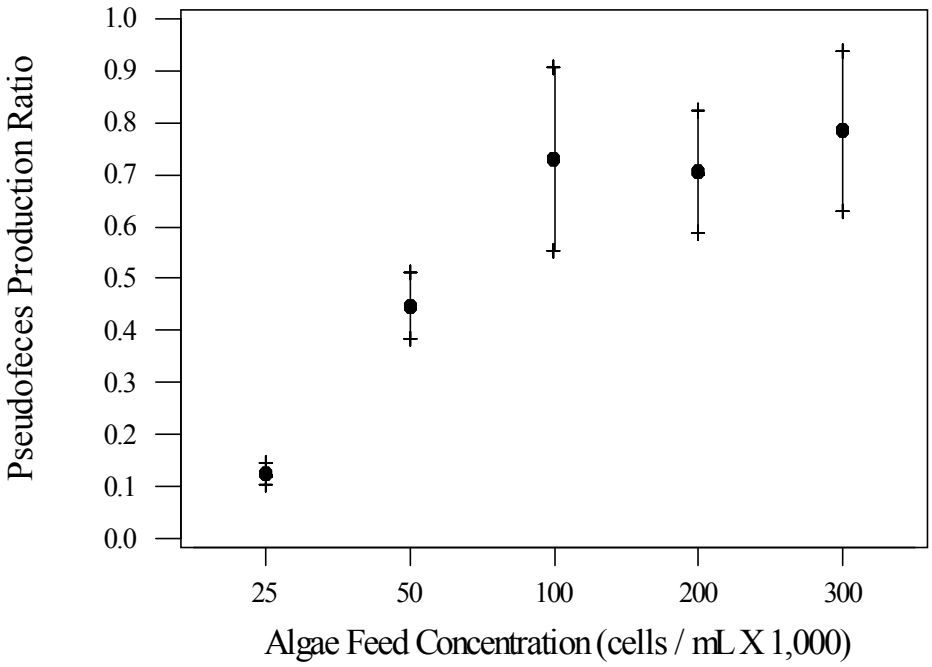

Fig. 1.1. Mean pseudofeces production ratio (AFDW in mg/h ± SE) for *Elliptio complanata* under various feed concentrations (cells/ml X 1,000) of *Scenedesmus quadricauda* (n=12). Pseudofeces production ratio equals AFDW mg/h divided by tissue dry weight (mg) multiplied by 100. Ratios of 50, 100, 200, and 300 (X 1,000) algae concentrations are not significantly different ($p<0.05$), but those of the 25 and 50 (X 1000) concentrations are not ($p>0.05$).

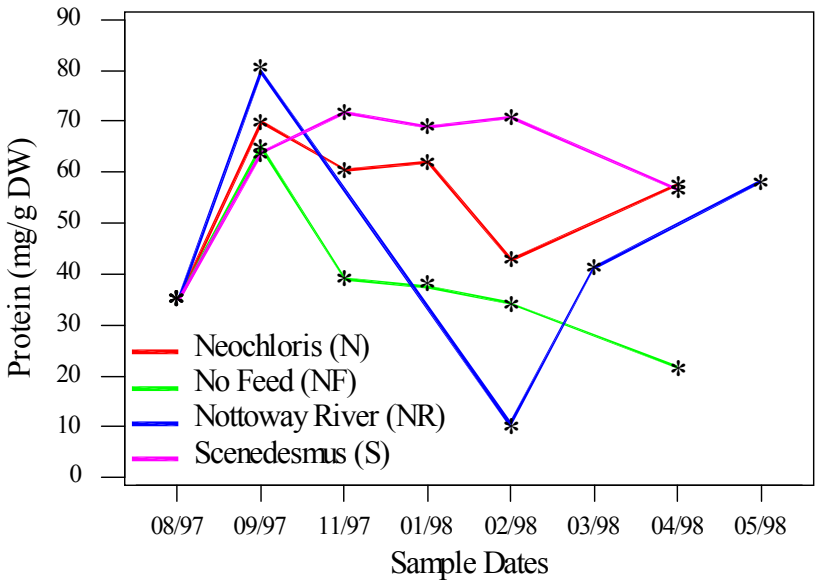

Fig. 1.2. Mean protein levels (mg/g DW) in mantle tissue of *Elliptio complanata* from the *Neochloris oleoabundans* (N), no feed (NF), and *Scenedesmus quadricauda* (S) diet treatments (n=18), and the Nottoway River (NR) reference site (n=6).

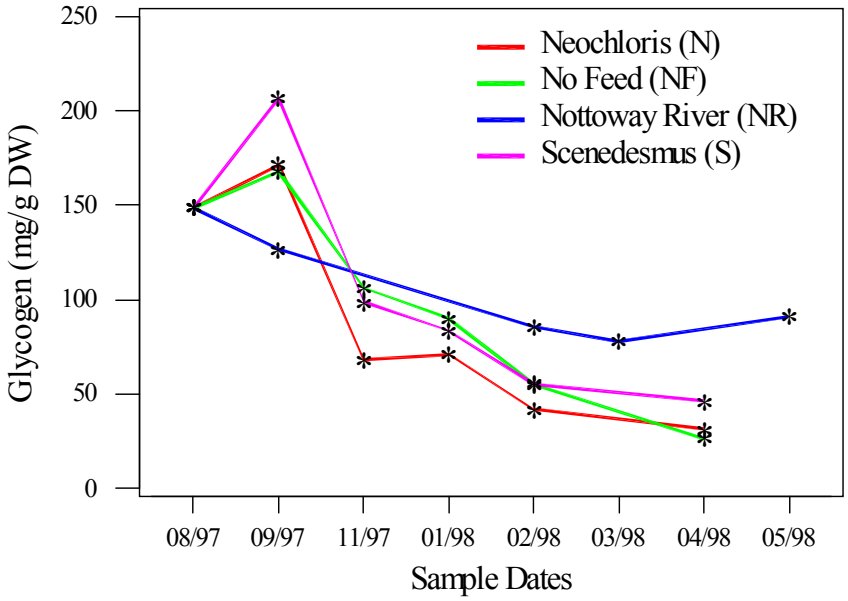

Fig. 1.3. Mean glycogen levels (mg/g DW) in mantle tissue of *Elliptio complanata* from the *Neochloris oleoabundans* (N), no feed (NF), and *Scenedesmus quadricauda* (S) diet treatments (n=18), and the Nottoway River (NR) reference site (n=6).

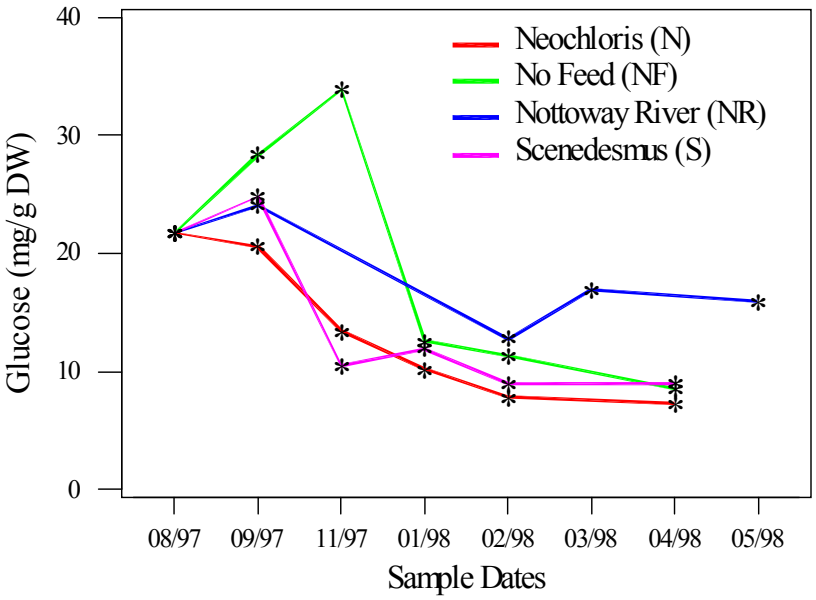

Fig. 1.4. Mean glucose levels (mg/g DW) in mantle tissue of *Elliptio complanata* from the *Neochloris oleoabundans* (N), no feed (NF), and *Scenedesmus quadricauda* (S) diet treatments (n=18), and the Nottoway River (NR) reference site (n=6).

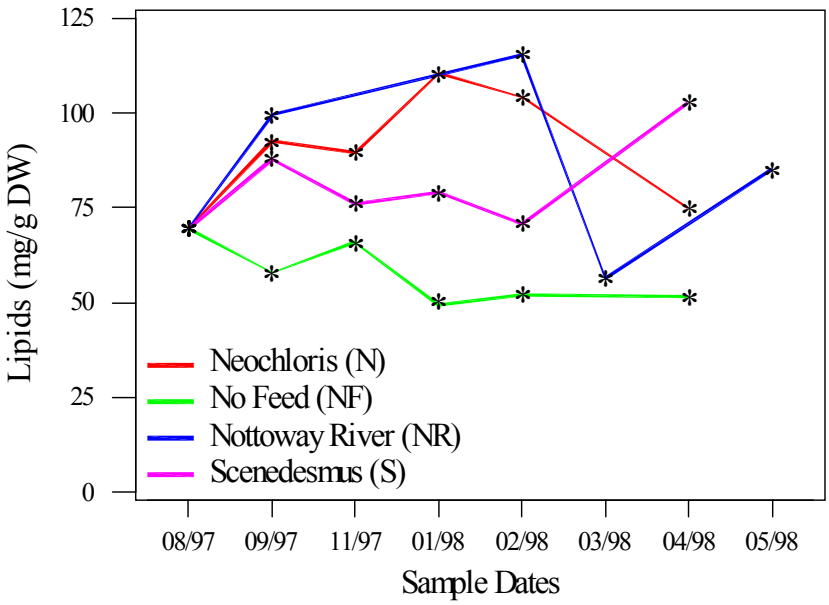

Fig. 1.5. Mean lipid levels (mg/g DW) in mantle tissue of *Elliptio complanata* from the *Neochloris oleoabundans* (N), no feed (NF), and *Scenedesmus quadricauda* (S) diet treatments (n=3), and the Nottoway River (NR) reference site (n=3).

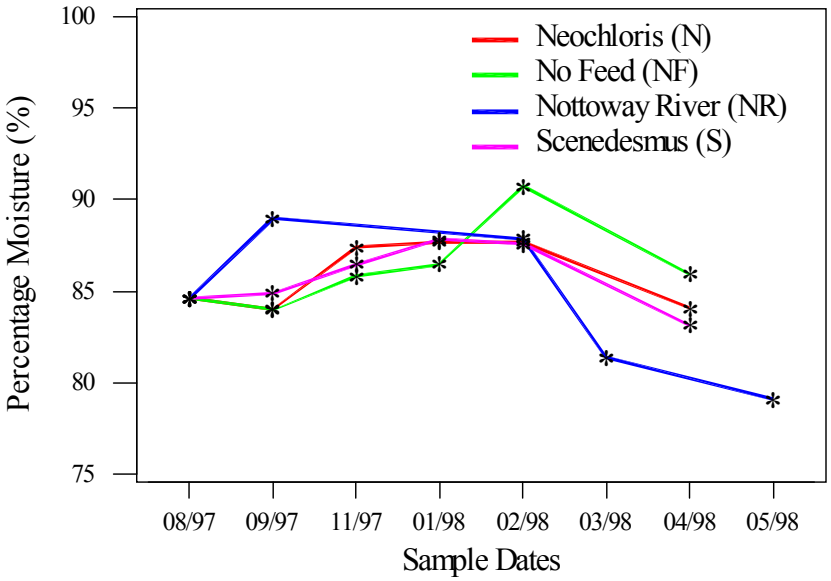

Fig. 1.6. Mean percentage moisture in mantle tissue of *Elliptio complanata* from the *Neochloris oleoabundans* (N), no feed (NF), and *Scenedesmus quadricauda* (S) diet treatments (n=18), and the Nottoway River (NR) reference site (n=6).

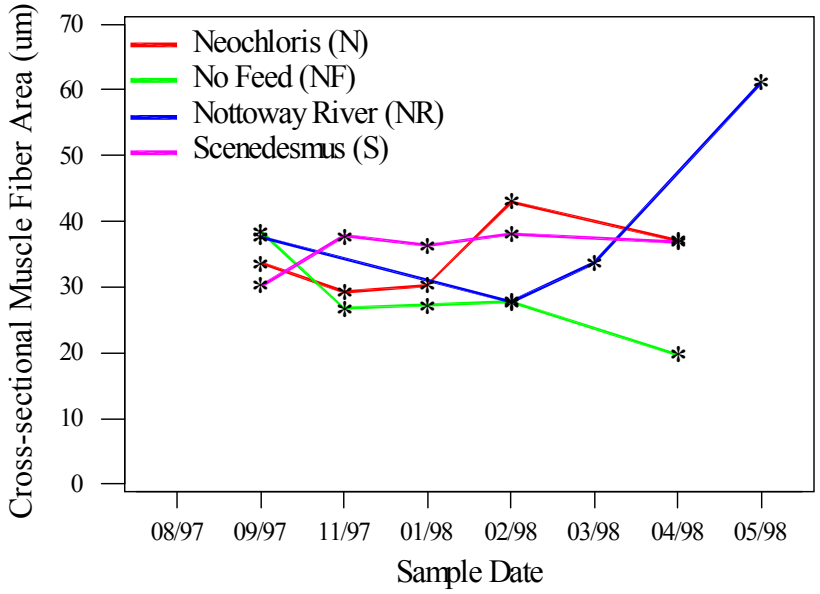

Fig. 1.7. Mean cross-sectional areas (μm^2) of muscle fibers of the visceral mass tissue of *Elliptio complanata* from the *Neochloris oleoabundans* (N), no feed (NF), and *Scenedesmus quadricauda* (S) diet treatments (n=3), and the Nottoway River (NR) reference site (n=3).

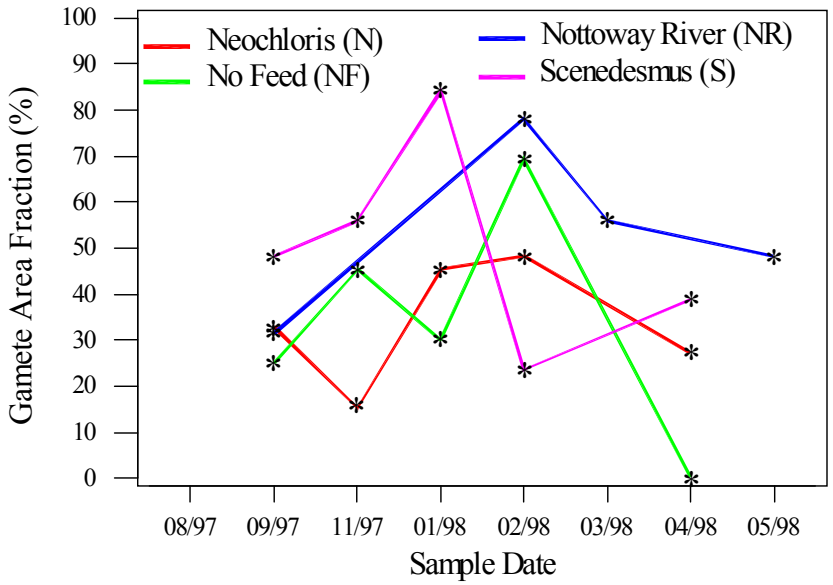

Fig. 1.8. Mean gamete area fraction (GAF) in gonadal tissue samples of *Elliptio complanata* from the *Neochloris oleoabundans* (N), no feed (NF), and *Scenedesmus quadricauda* (S) diet treatments (n=3), and the Nottoway River (NR) reference site (n=3).

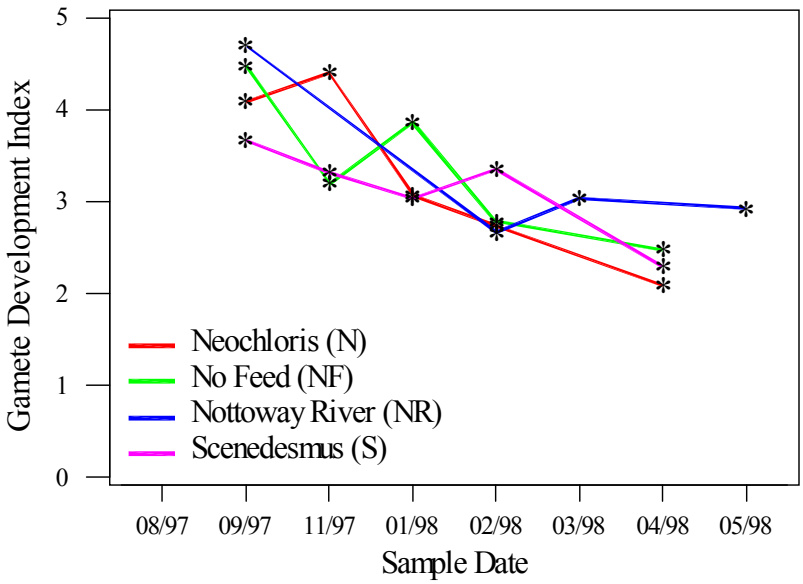

Fig. 1.9. Mean gamete development index (GDI) in gonadal tissue samples of *Elliptio complanata* from the *Neochloris oleoabundans* (N), no feed (NF), and *Scenedesmus quadricauda* (S) diet treatments (n=3), and the Nottoway River (NR) reference site (n=3).

CHAPTER II

DETECTION OF HERMAPHRODITISM AND EVALUATION OF GAMETOGENESIS IN GONADS OF *UTTERBACKIA IMBECILLIS* (SAY, 1829) AND *VILLOSA IRIS* (I. LEA, 1829) (BIVALVIA: UNIONIDAE)

ABSTRACT

To develop protocols for determination of gender and stage of gametogenesis in mussels, labeled quadrats within germinal tissue histological sections of rainbow mussels, *Villosa iris* (I. Lea, 1829), and paper pondshells, *Utterbackia imbecillis* (Say, 1829), were evaluated. *Villosa iris* and *U. imbecillis* were collected in May 1999 and February 2000, respectively. Occurrence frequencies of oogenic, spermatogenic, and hermaphroditic tissues were summarized in frequency contingency tables, and visceral sites from which similar tissues were collected from every conspecific specimen were evaluated for gametogenic stage. Sex was consistently and accurately determined in the central portion of the viscera of male and female *V. iris* and female regions of *U. imbecillis*, and spermatogenic tissue was consistently observed in the dorso-anterior areas of *U. imbecillis*. The tissues of these visceral positions also consistently provided accurate determination of gamete stage of development observed in individual specimens. Reproductive asynchrony was observed among males and females of these species ($p<0.02$).

All examined male regions of *U. imbecillis* showed gamete development characterized by mature and developing spermatogenic tissue, while 2 groups of *U. imbecillis* showed oogenic development characterized by mature oocytes and resorption of gametes, respectively. Male *V. iris* showed early gamete development without mature spermatozoa, and 2 groups of female *V. iris* also showed different stages of oocyte development. One group was characterized by mature and developing gametes, and the other group of females showed resorption of gametes. Protocols for collection of non-lethal biopsy tissues from selected visceral areas are provided for specimens of *U. imbecillis* and *V. iris* for sex determination and staging of gametogenesis. The application

of this biopsy protocol may be species specific, and the suitability of these protocols for other species should be tested.

INTRODUCTION

A landmark study by Morales-Alamo and Mann (1989) established that locations from which histological thin-sections were obtained from gonads of eastern oysters, *Crassostrea virginica* (Gmelin, 1791) affected results of reproductive evaluations of gonads. These findings showed that areas of tissue containing gametes could vary among histological sections, and the authors concluded that results of reproductive evaluations could be compared if histological sections were obtained from similar positions within viscera. The hypothesis that gamete development in dioecious species of freshwater mussels varies among gonadal regions has been untested. However, segregation of spermatogenic and oogenic tissues in gonads has been documented in hermaphrodites of some species by histological examination of tissue histological sections. Avelar and Mendonça (1998) found that spermatic tissues in *Diplodon gratus* (Wagner, 1827) were concentrated in dorsal portions of gonads, with oocyte production in ventral areas. Kat (1983) recorded that spermatogenic acini in hermaphrodites of paper pondshells, *Utterbackia imbecillis* (Say, 1829) were observed in the dorso-posterior portions of gonads. Heard (1975) also documented regional separation of spermatogenic and oogenic tissues in gonads of *U. imbecillis*, but did not comment on areas of centralization.

Regional partitioning of male and female acini has not been observed in all hermaphroditic specimens of freshwater mussels. Heard (1975) observed that most specimens of *Anodonta* showed intermingled male and female acini without clear gonadal organization. Within the genus *Elliptio*, male and female tissues were regionally organized in viscera of some hermaphroditic specimens, and not in others of the same species (Heard, 1979). Grande *et al*. (2001) observed no regional organization of male and female tissues in gonads of hermaphroditic *Margaritifera auricularia* (Spengler, 1793), with male and female acini dispersed throughout the viscera. To date, 8 species of freshwater mussels have been described as commonly hermaphroditic, and 41 species as occasionally hermaphroditic (Table 2.1). However, occurrence of hermaphroditism can vary among conspecific populations. Heard (1975) and Kat (1983) examined specimens of *U. imbecillis* that were not hermaphroditic, in disagreement with Sterki (1898a and 1898b) and van der Schalie (1966) who concluded that *U. imbecillis* was normally hermaphroditic. Incidence of hermaphroditism also has varied among different

populations of eastern elliptios, *Elliptio complanata* (Lightfoot, 1786), which are predominately hermaphroditic in some populations, and not at all in others (Kat, 1983; Downing *et al*., 1989).

Histological examinations of freshwater mussel gonads have provided information for description and evaluation of germinal development using various methods. Methods of tissue histological section evaluation have included measurement of mean oocyte size, numbers of oocytes per acini, and numbers and types of germinal cells per transect (Haggerty *et al*., 1995; Garner *et al*., 1999); determination of gamete development stage (Woody and Holland-Bartels, 1993; Heinricher and Layzer, 1999); estimation of percentage of gonad area in histological sections devoted to mature and developing gametes (Peredo and Parada, 1986); and general descriptions of germinal cells observed (Peredo and Parada, 1984; Jirka and Neves, 1992; Avelar and Mendonça, 1998). Without highly detailed descriptions of histological methods, it is not possible to determine whether previous studies have employed systematic sampling for selection of histological section positions from viscera. However, application of a sampling method that systematically defines histological section positions within viscera could yield data on regional consistency or variance in stages of spermatogenesis and oogenesis within the gonads of freshwater mussels.

Common to studies that have investigated hermaphroditism and gametogenesis in freshwater mussels is lethal collection of viscera for gonadal histological sections. To develop protocols for non-lethal determination of gender, hermaphroditism, and stage of gamete development, spatial consistencies in oogenic and spermatogenic tissues of gonads of conspecific mussels must be identified for determination of suitable biopsy sites. A way to accomplish this is to evaluate predefined regions within histological sections that have been obtained from similar locations in viscera. Evaluations of male and female tissues could be based on occurrence frequencies of each of spermatogenic and oogenic tissues within regions of histological sections of mussels examined. A summary contingency table of male, female, and hermaphroditic occurrence frequencies for regions within all histological sections would provide a summary of the spatial occurrence of male and female tissues. Consistently high frequencies of male and female tissue within the cells of the contingency table would indicate sites suitable for biopsy

sampling, for determination of sex and evaluation of consistency of spermatogenic and oogenic development within visceral masses.

One objective of this study was to develop histological protocols for gender determination, including hermaphroditism, and staging of gametogenesis in populations of rainbow mussels, *Villosa iris* (I. Lea, 1829) and *U. imbecillis* (Say, 1829). *Villosa iris* is an occasional hermaphrodite (van der Schalie, 1966), whereas *U. imbecillis* is typically hermaphroditic among populations (Heard, 1975). This objective sought to determine whether there were regions within the visceral mass that could be reliably sampled by biopsy for determination of sex and gametogenic stage. To accomplish this, quadrats were formed within tissues of regularly spaced histological sections, and the occurrence of male and female gametes within the quadrats was recorded. A second objective was to determine whether the sample of mussels exhibited the same stages of gamete development, or whether they exhibited reproductive asynchrony among individuals.

MATERIALS AND METHODS

During May 1999, 12 male and 12 female adult *V. iris* were collected from the North Fork Holston River, Smyth County, Virginia. In February 2000, 16 adult *U. imbecillis* were collected from Haleyville City Lake, an impoundment of Clear Creek, Winston County, Alabama, and mailed to Virginia Tech, Blacksburg, Virginia. Mean lengths of these mussels were 43.7 mm (SD = 7.9) for male *V.* iris, 38.8 mm (SD = 4.5) for female *V. iris*, and 70.6 mm (SD = 3.2) for specimens of *U. imbecillis*. All mussels were sacrificed for excision of visceral masses (Appendix B, Photograph 2.1), which were placed in histology cassettes, fixed in Bouin's fixative for 1 wk, repeatedly washed in water, and transferred to 95% ethyl alcohol. Viscera were trimmed to fit cassettes by cutting through projected transverse planes perpendicular to sagittal planes of bodies, at posterior junctions of the labial palps and the viscera (Appendix B, Photograph 2.2). Therefore, anterior portions of viscera above these transverse cut planes were not included in histological sampling. Tissue masses were processed with alcohol and embedded in Paraplast wax. After tissue processing, and prior to paraffin embedding, each visceral mass was measured for its maximum sagittal width (mm). Specimens of *V. iris* were sexed initially using shell morphology. Female identity was verified by

gravidity, and in the case of non-gravid mussels, by the presence of a dark coloration along the distal edges of gill demibranchs.

Of the original number of visceral masses collected, 3 males and 3 females of *V. iris* and 3 *U. imbecillis* were selected for initial histological sectioning to determine an appropriate histological method of sampling. Thin-sections (5μm) were obtained from each tissue mass at 10, 20, 30, 40, 50, 60, 70, 80, and 90% of its maximum sagittal width measurement (Fig. 2.1). Because of the width of the sections, they will be termed histological sections in this study. These will be referred to as histological sections 1 through 9, respectively. Widths were measured from sides of the visceral masses proximate to the left valve. Each visceral mass was sampled from the same positions relative to its sagittal width measurement. Histological sections used for determination of sex, hermaphroditism, and gametogenic stage were stained with hematoxylin and eosin (H and E). All histological photographs in Appendix B were stained with H and E, unless otherwise noted.

Areas of viscera were evaluated for sex by overlaying a hypothetical grid over histological sections. Tissues of each histological section were roughly triangular; thus, the triangular grid was composed of 9 quadrats (Fig. 2.1). The tissues of quadrats were nominally scored with 0, 1, 2, and 3 for occurrence of no gametes, male gametes, male and female (hermaphrodite) gametes, and female gametes. This evaluation system was termed the sexual status index (SSI).

Because only 1 *V. iris* was identified as a hermaphrodite in the first round of histological section observations, 9 additional male and female *V. iris* were histologically sectioned using the systematic sampling method previously described. Also, to increase the sample size of *U. imbecillis*, 3 additional mussels were histologically sectioned. Thus, sample sizes used in this study were 12 male and 12 female *V. iris*, and 6 *U. imbecillis*. In the course of evaluating the histological sections of *V. iris*, 5 male mussels showed heavy infestations of an unidentified parasite in their gonads. These mussels were not counted in the observational sample size; therefore, sample size of *V. iris* in this study was 19 (7 males and 12 females). The smaller sample size of *U. imbecillis* was judged sufficient because all examined mussels were hermaphrodites, and preliminary examination of

histological sections showed a consistent organization of male and female gamete regions within the visceral masses.

To determine whether a pattern of organization of male, female, and hermaphroditic tissues (quadrats) existed in gonads of *V. iris and U. imbecillis*, observed frequencies of the SSI in mussels were compared among individuals, histological section positions in viscera, and quadrats within histological sections using contingency tables. Frequencies of the SSI in contingency tables were used to determine quadrats in histological sections, specimens, and species that provided the highest probability of sex determination using the FREQ procedure in SAS 8.02 for Windows (SAS Institute, Inc., Cary, North Carolina). Protocols for determination of sex and hermaphroditism in the 2 species were developed using quadrats, within histological section positions, that showed the highest relative frequencies of SSI scores.

To determine whether a predictable organization of gamete development stage occurred in histological sections and quadrats within histological sections, histological sections at 3, 5, and 7 of sagittal visceral mass widths were evaluated from 6 male and 6 female *V. iris* and 6 *U. imbecillis* using an adapted version of the Gamete Development Index (GDI) (Barber, 1996). The GDI scores of the gonad tissues in the quadrats were determined by assigning a 0, 1, 2, 3, 4, or 5 to represent stage of gamete development. Classification stages of gamete development used in the GDI, as set forth by Barber (1996), range from inactive (stage 0) to resorption (stage 5). Each of the 9 quadrats in histological sections was assigned a GDI score that ranged from 0 (inactive) to 5 (resorption) based on characteristics of gamete development (Table 2.2). Appendix B, Photographs 2.3 through 2.8 provide visual examples of GDI stages observed in this study. These log-transformed GDI values were statistically compared among individual mussels, histological sections, and quadrats using ANOVA in Minitab. Results were used to develop gonad-sampling protocols for evaluation of gametogenic stage within *V. iris* and *U. imbecillis*, and to determine whether reproductive asynchrony occurred among the individuals examined.

In the course of the histological investigations of this study, the presence of what appeared to be a large gonosinus was serendipitously observed in most specimens of *V. iris* and *U. imbecillis*. The consistence of occurrence of this large gamete-collecting sinus

in the gonads was determined in specimens using the spatial quadrat and histological section method previously described. Six *U. imbecillis* and 20 *V. iris* were evaluated using the frequencies of occurrence in tissue areas. For further investigation of the cell lining of the gonosinus, one thin-section per mussel was stained with periodic acid/Schiff (PAS) for elaboration of glycoproteins.

RESULTS

UTTERBACKIA IMBECILLIS

All specimens of *U. imbecillis* were found to be hermaphrodites. For an example of male and female acini in a histological section of *U. imbecillis*, see Appendix B, Photograph 2.9. Histological sections were primarily composed of oogenic tissues, and spermatogenic tissues were always centralized in the dorso-anterior portion of all viscera. Mussels in this study showed spermatogenic tissues in quadrats 7 through 9, but occurrence within these quadrats varied among histological sections (Table 2.3). There were areas in all viscera of *U. imbecillis* that showed spermatogenic and oogenic tissues in 100% of the specimens (Fig. 2.2). In most mussels, male tissue occupied quadrat 7 in histological section 7, and the probability of occurrence of spermatogenic tissue at this position in viscera was 0.87 (Table 2.3). Even though most mussels held male tissue at quadrat 7 at 70% of the visceral mass depth, results of biopsy sampling at this position in the viscera were associated with a probability of error. Examination of the data showed that 1 mussel had hermaphroditic tissues in quadrat 7 of histological section 7, and because both tissue types were observed, a hermaphroditic SSI score (2) was assigned to the quadrat. However, it is important to emphasize that spermatogenic tissue was observed at this position in all specimens. Based on this data, biopsy sampling at this visceral position would have correctly detected the presence of spermatogenic tissues in 83% of the biopsy samples, but would have failed in 17%. If biopsy tissues were obtained from the dorsal and anterior portion of the quadrat, then spermatogenic tissue would have been collected from every specimen. Several visceral areas consistently contained oogenic tissues in all mussels, especially in the central region of the gonads. Quadrats 5 and 6 at 30 to 60% of the visceral mass depth (histological sections 3 through 6) contained female tissue in 100% of the mussels (Table 2.3). Biopsy sampling from the

central area of visceral masses at quadrat 5 in histological section 5 would have consistently detected female tissue in all instances.

Since all *U. imbecillis* were hermaphrodites, it was appropriate to determine gamete stage of male and female regions in the viscera. GDI means of the male and female regions of mussels differed significantly ($p<0.001$), indicating that the regions were characterized by different stages of gamete development. All male quadrats were characterized by GDI stage 2 (late active), and GDI values were identical among mussels, histological sections, and quadrats ($p=1.0$, SE $=0.0$). The GDI provided reliable data for accurate determination of spermatogenic stage in all mussels only when tissue of the dorso-anterior area of quadrat 7 of histological section 7 was examined.

Female regions of mussels were characterized into 2 groups. Three females consistently showed GDI values of 3 (mature) within histological sections and quadrats of their gonads ($p=1.0$, SE $=0.0$), and 3 mussels showed values of 4 (spawned) ($p=1.0$, SE $=0.0$). As described, the *U. imbecillis* were collected in February. Evaluation of oogenic tissue in quadrat 5 of histological section 5 consistently and reliably provided the GDI stage that was characteristic of the specimens of both female groups. The GDI evaluation also showed the asynchrony between male and female regions of the gonads, as well as between the 2 groups of female gonadal regions. Thus, biopsy sampling at the 2 visceral positions yielded both accurate male and female sex determination and evaluation of oogenic stage for all mussels. The consistency of results indicates that they are appropriate sites for tissue biopsy sampling.

VILLOSA IRIS

Examination of SSI frequencies indicated that detection of hermaphroditism in *V. iris* was difficult (Table 2.4). Only 2 out of the 19 mussels were indisputably hermaphroditic (10.5%); with 1 male hermaphrodite and 1 female hermaphrodite (Appendix B, Photographs 2.10 and 2.11). Within these 2 mussels, hermaphroditic quadrats were extremely rare. Hermaphroditism was evident in all histological sections except histological section 9, toward the right valve of the mussels. The most common occurrence of hermaphroditic tissue was in quadrat 5 in the center of viscera. The majority of quadrats contained hermaphroditic tissues in only 5% of the observations. It is important to note, within viscera of *V. iris*, that the probability of error associated with

detection of hermaphroditism ranged from 0.89 to 1.0 (Table 2.4). With such a low incidence of hermaphroditism in specimens of *V. iris*, examination of histological sections appears to be the only reliable method of detection. Because of the small sample size of observed *V. iris* hermaphrodites (n = 2), it is not possible to recommend an appropriate method for detection of hermaphroditism in the sampled population.

Without regard to sexual identification based on external characters, frequencies of spermatogenic tissues in quadrats were low, with highest observed frequency of 0.32 in quadrats 4, 5, 6, 8, and 9 (Table 2.4); male tissue was only evident consistently in histological sections. For all mussels, the highest observed frequency of female tissue was 0.86 in quadrat 7 of histological section 3; but female tissue was evident throughout the viscera in most quadrats and histological sections.

When data from viscera were partitioned by sex based on shell characters, then frequencies associated with the occurrences of male and female tissues in quadrats greatly increased (Tables 2.5 and 2.6). Many quadrats within multiple histological sections showed spermatogenic tissues in 100% of the mussels, especially in quadrats 4 and 5 of all histological sections except 9 (Table 2.5 and Figs. 2.3 and 2.4). Within quadrats and histological sections, consistency of sex determination also was observed in females (Table 2.6). Here again, multiple quadrat and histological section locations were associated with observed oogenic tissue in 100% of the specimens, especially in quadrat 5 of histological sections 3 through 6. Because oogenic tissue was observed in quadrats 5, 7, 8, and 9 of histological section 3, and quadrats 5, 8, and 9 of histological section 5, in 100% of the examinations, these positions were selected for evaluation of oogenic stage among the mussels. For males, spermatogenic tissue was observed in quadrats 4 through 9 in histological sections 3, 5, and 7 in 100% of the examinations. Therefore, these positions were evaluated for stages of gamete development.

Transformed GDI means differed significantly between tissues of male and female *V. iris* ($p<0.001$). GDI values of females differed significantly among mussels ($p<0.02$), but those of males did not ($p=1.0$). All quadrats in males received GDI scores of 1 (early active). Asynchrony of gamete development in females was evident. As with the female regions of *U. imbecillis*, there were 2 groups of female *V. iris*; 1 group of 3 mussels was characterized by GDI values of 2 (late active) in all quadrats within histological sections,

and the other group of 3 mussels showed consistent GDI scores equal to 5. Tissues of quadrat 5 in histological section 5 of both males and females showed consistency for determination of sex and stage of gametogenesis among specimens. At this location, which was at the center of viscera at 50% of the sagittal width, reliable staging of oogenesis also was obtained from females of the 2 reproductively asynchronous groups.

Gonosinus and Gonopore

Important to the success of non-lethal sampling of freshwater mussel viscera for determination of oocyte diameter and counts of mature gametes is the position of the gonosinus. Mature gametes exit an acinus through a ciliated gonoduct (Appendix B, Photograph 2.12). It appears that groups or clusters of acini supply mature gametes to such gonoducts (Appendix B, Photographs 2.13 and 2.14), which in turn merge into larger gonoducts (Appendix B, Photograph 2.15). In this way, mature gametes from regional aggregations of acini are transported via the ciliated gonoducts to what appears to be a collecting gonosinus (Appendix B, Photograph 2.16). The sinus was evident in 5 of the 6 *U. imbecillis* in quadrat 7 in histological sections 5 and 7 (50 and 70% of the visceral depth), occupying a large area of the dorso-anterior portion of the viscera. Nineteen of the 20 mussels showed the sinus in histological section 7, and 1 specimen in histological section 5. Variance in size of this sinus was apparent in *V. iris*, from widening of the gonoduct to what appeared to be a very large sinus (Appendix B, Photographs 2.16 and 2.17). In *U. imbecillis*, this sinus holds both sperm and oocytes (Appendix B, Photograph 2.18), thus the gonoducts from both male and female acini eventually empty to the sinus. It may be that the dorso-anterior portion of the viscera is a collection site for mature gametes, and that the large gonoduct merely expands with numbers of gametes contained.

The gonosinus is in communication with a gonopore (Appendix B, Photograph 2.19). The gonopore appears to be a muscular sphincter that possesses a ciliated closure flap of muscle (Appendix B, Photograph 2.19). The gonoducts are lined with ciliated simple cuboidal or columnar epithelial cells (Appendix B, Photograph 2.12). However in *U. imbecillis*, the ciliated epithelial lining of the gonosinus changes from that of the gonoduct to simple columnar cells with prominent nuclei (Appendix B, Photograph 2.20) that are secretory in function (Appendix B, Photograph 2.21). It must be stressed that

future histological plastic embedding technique may demonstrate that the lining of the gonosinus is actually composed of separate, but paired, ciliated and secretory cells. The secretory granules stain positive for glycoproteins using PAS stain (Appendix B, Photograph 2.22). After secretion, the secretory granules enter the lumen of the gonosinus (Appendix B, Photograph 2.23), where they dissolve (Appendix B, Photograph 2.24). The existence of these granules is undocumented in the literature; possibly, they are involved in nourishment or maturation of gametes in the gonosinus in preparation for fertilization. Not all of the secretory cells produce the granules simultaneously; it appears that cohorts of these cells secrete the granules at the same time. Importantly, the nucleated columnar cells (Appendix B, Photograph 2.20), and the secretion of the granules (Appendix B, Photograph 2.21), were only observed in *U. imbecillis*, and not in *V. iris*.

DISCUSSION

Hermaphroditism

All of the *U. imbecillis* were hermaphrodites that showed spatial separation of spermatogenic and oogenic tissues. Although the incidence of hermaphroditism in *U. imbecillis* may vary among populations (van der Schalie, 1970; Heard, 1975), zonal segregation of male and female tissues has been observed in all hermaphroditic specimens (Heard, 1975; Kat, 1983). However, locations of visceral areas that contain testicular acini may vary among populations. All examined specimens contained spermatogenic material in the dorso-anterior portions of the viscera. This finding is in disagreement with findings of Kat (1983), who observed that male acini were localized in the dorso-posterior area. Spermatogenic tissue also has been observed in the anterior portion of viscera in yet another population of *U. imbecillis*, but specific somatic locations were not provided (Johnston *et al*., 1998). Avelar and Mendonça (1998) observed male tissue in the dorsal areas of hermaphrodites of *Diplodon gratus* (Wagner, 1827). Heard (1992) described one gonopore in Unionidae, located in the dorso-anterior visceral area. Centralization of testicular tissue in this area may result in decreasing exposure of sperm and egg, since oocyte production is localized ventrally. This may decrease the likelihood of self-fertilization (selfing). However, a gonoduct system that drains male and female acini, and empties to a common gonosinus for sperm and oocytes,

could facilitate selfing. Interestingly, I observed no evidence of internal self-fertilization, indicated the presence of polar bodies of oocytes or the presence of zygotes, in the gonoducts and gonosinuses. This does not exclude the possibility of self-fertilization. In fact, some populations of *U. imbecillis* show evidence of high degrees of selfing (Hoeh *et al.*, 1998). Whether sperm are activated in the acini, gonosinus, or after exiting the gonosinus, has not been determined. Hoeh *et al.* (1998) hypothesized that fertilization is affected by release of sperm and oocytes from separate and adjacent nephridial and genital openings in giant floaters, *Pyganodon grandis* (Say, 1829). However, this hypothesis was not supported by *U. imbecillis*, as it would be unlikely that gametes are sorted by an anatomical mechanism after exiting the gonosinus for presentation to 2 separate visceral exit paths. The presence of male and female gametes in the sinus provides indirect evidence that there is only 1 common gonopore in *U. imbecillis*.

The presence of mature sperm in the gonosinus was surprising since male regions of *U. imbecillis* were characterized by an early active stage of gametogenesis (GDI stage 2). Testicular acini contained developing gametes with some mature gametes. Possibly, mature spermatozoa were being transported from acini to the gonosinus soon after maturity. This hypothesis is proposed because no areas of the male regions showed evidence of resorption of gametes, indicating that no recent cohorts of sperm had been produced in other areas of the viscera. These results imply that release of sperm from acini is a process mediated by presence, rather than numbers, of gametes. Apparently, sperm are not held in acini of *U. imbecillis* for a massive release to the gonosinus.

Eight species of freshwater mussels have been classified as simultaneous hermaphrodites (SH) (Table 2.1). By strict definition, a functionally simultaneous hermaphrodite exhibits mature male and female gametes in gonads at the same time (Coe, 1943; Hoeh *et al*., 1995). Co-occurrence of mature sperm and oocytes was observed in *U. imbecillis*; therefore, they could be technically considered SH. This definition ignores the possibility that mature male and female gametes may not be present in the gonad, and that spermatogenic and oogenic gametes may mature at different rates. What is critical is the simultaneous availability of mature gametes after spawning for fertilization. A less constraining term for description of freshwater bivalve species that regularly exhibit hermaphroditism is normal hermaphrodite (NH). This description also accommodates

variance in hermaphroditic expression among populations, as with *U. imbecillis* (van der Schalie, 1970; Heard, 1975), but acknowledges hermaphroditism as the normal reproductive mode of the species. The NH classification recognizes that mussels may be functional hermaphrodites by the descriptive system of Coe (1943).

Of the North American species that are NH, 1 is of the family Margaritiferidae, and 7 of the Unionidae (Table 2.1). Occurrence within the Unionidae crosses subfamily boundaries, with 4 Anodontinae, 2 Lampsilinae, and 1 Unioninae. Common to all Unionidae that are NH is preferred habitat of standing, quiet, or slow moving water (van der Schalie, 1970; Heard, 1975). However, the margaritiferid western pearlshell, *Margaritifera falcata* (Gould, 1850), also is a NH, but prefers swift moving water (Sterki, 1898a). Although no study has been conducted on whether the actual occurrence of hermaphroditism is facultative in NH species, allocation of testicular tissue in *U. imbecillis* has been related to degrees of self-fertilization among populations (Johnston *et al*., 1998). Kat (1983) set forth several inter-related hypotheses to explain variance in male gonadal allocation in species that are NH, including hormonal systems that respond to environmental influences, reliance on self-fertilization and decreased somatic investment in testicular tissue, and habitation of lentic conditions and subsequent decreased dispersion of sperm. Johnston *et al*. (1998) showed that higher incidence of selfing was correlated to lower proportions of male tissue among populations of *U. imbecillis*. Hoeh *et al*. (1995) speculated that regular occurrence of hermaphroditism in *U. imbecillis* is the result of historical hybridization with the Florida floater, *U. peggyae* (R. I. Johnson, 1965), that disrupted the sex determination system of *U. imbecillis*.

One male and one female hermaphrodite were discovered among the 19 *V. iris*. The male and female acini of these hermaphroditic specimens were sporadically dispersed throughout the viscera, with no apparent spatial distinction. Spermatogenic and oogenic tissues of these hermaphrodites constituted <5% of the gonads. The finding that hermaphroditism in *V. iris* is rare agrees with the conclusion of van der Schalie (1970), who classified this species as an occasional hermaphrodite (OH). Freshwater bivalve species that show OH have been observed in several families (Table 2.1). Of the 41 North American unionoid species that show OH, 17 are Unioninae, 14 are Anodontinae, 10 are Lampsilinae; and 3 are Margaritiferidae (Table 2.1). These species occur in a wide range

of habitats, and it is difficult to determine whether the occurrence of OH is facultative in nature. However, the incidence of OH can vary among populations, as observed with eastern pearlshells, *M. margaritifera* (Linnaeus, 1758) (van der Schalie, 1966; Smith, 1979; Bauer, 1987; Hanstén *et al*., 1997; Grande *et al*., 2001). It appears from these findings that hermaphroditism in *M. margaritifera* is more common among Spanish populations, where population densities may be lower than in those of North America (Grande *et al*., 2001).

Of the 12 female *V. iris*, 93% were gravid, including the female hermaphrodite. The male *V. iris* was not gravid. Conversely, only 33% of the *U. imbecillis* were gravid. Hermaphroditic activity in organisms of low-density populations theoretically increases the probability of fertilization (Ghiselin, 1969; Charnov *et al*., 1976; Heath, 1977). This may be especially true for mussels of lentic systems, such as *U. imbecillis*, where successful current dispersal of sperm to females is improbable (Kat, 1983). However, the low percentage of gravidity of *U. imbecillis* possibly shows low utility of hermaphroditism in the source population. One would expect that if self-fertilization were prevalent, and if simultaneous production of mature spermatozoa and oocytes promotes self-fertilization, then the incidence of gravidity among *U. imbecillis* specimens would have been higher than observed in this study.

There was no consistent pattern in the developmental oocyte stages that characterized the gravid and non-gravid specimens of *U. imbecillis*. Both gravid and non-gravid mussels were characterized by GDI stages 3 (late active) or 4 (mature). Apparently, mature oocytes were either not being fertilized, or they were being retained in the gonosinuses for later fertilization. Although difficult to substantiate, some mussels may have been retaining mature oocytes in the sinuses, while others delivered their oocytes for fertilization. That 93% of the *V. iris* were gravid implies that facultative hermaphroditism in the population did not occur in the examined mussels, and that OH in this population may be accidental in nature (Coe, 1943). This high occurrence of gravidity implies that the dioecious mating system of the source population is effective for their lotic environment, and that cross-fertilization is probable.

The low occurrence of hermaphroditism among populations of OH species of freshwater mussels (Table 2.1) suggests that the dioecious mating system is the norm for

most species. Kat (1983) observed that the occurrence of OH in triangle floaters, *Alasmidonta undulata* (Say, 1817), and eastern lampshells, *Lampsilis radiata* (Gmelin, 1791) was associated with trematodal infections. Although 5 male *V. iris* were observed to be heavily infested with parasites during this study, none of the *V. iris* hermaphrodites were infested. Therefore, the cause of OH in *V. iris* probably was not related to parasitic infestation. However, variance in OH expression has been noted among populations. Kat (1983) observed no evidence of OH in 28 northern populations of *E. complanata* collected from a variety of habitats and population densities. Downing *et al*. (1989) found 6% hermaphroditism in specimens of *E. complanata* from a lake in Québec, and Heard (1979) noted that OH was rare in specimens from a Florida creek. Hermaphroditic occurrence varies also among populations of *M. margaritifera*. Van der Schalie (1970) found hermaphroditism in 1 of 12 specimens of *M. margaritifera* from the Yellowstone River. Smith (1979) and Hanstén *et al*. (1997) did not find hermaphrodites in populations from New England and Finland, although 50% of specimens from a Spanish population were monecious (Grande *et al*., 2001).

Since OH has been reported in specimens from such wide habitats and regions, it is difficult to draw conclusions as to the causation of OH in species of freshwater mussels. Van der Schalie (1970) posited that an interaction between environmental influences and genetic sex-determining mechanisms might be responsible for OH expression. Kat (1983) hypothesized that juvenile mussels exhibit a primary male phase, and remain male if a presumptive "single or complex of sex-determining hormones" stay low. According to this hypothesis, hermaphroditic and female expression would occur with relatively higher levels of such a hormone. Kat (1983) believed that parasitic influence was a plausible explanation for possible hormonal disruption. The problem with *post priori* testing of this hypothesis with results of published reports is that data on parasitic infestation of specimens are not noted. Again, no evidence of infestation was observed in the *V. iris* used in this study.

Determination of Gender and Hermaphroditism

An objective of this study was to develop histological protocols for determination of sex and hermaphroditism in *V. iris* and *U. imbecillis*. All *U. imbecillis* showed similar visceral organization of spermatogenic and oogenic tissue, with male tissues occurring in

the dorso-anterior area of the viscera. Thus, there were areas within the viscera that showed remarkable consistency of occurrence of male and female tissue among specimens. Because of this consistency, selection of visceral sites for non-lethal collection of tissue for determination of hermaphroditism in *U. imbecillis* was possible. Although sampling of spermatogenic tissues in quadrat 7 of histological section 7 would have possibly failed to collect male tissue in 17% of biopsies, results indicated that if the dorso-anterior area of this quadrat was sampled, spermatogenic tissue would have been collected in 100% of the mussels. Oogenic tissue always was observed in the tissue area of quadrat 5 in the middle of the tissue masses (histological section 5) of specimens. Thus, the feasibility of confirming hermaphroditism in 100% of specimens by non-lethal biopsy sampling was substantiated.

Confirmation of hermaphroditism in species believed to be NH would prove valuable in studies concerned with the relationship between degree of self-fertilization and visceral investment in testicular tissue (Kat, 1983; Hoeh *et al*., 1998; Johnston *et al*., 1998). The development and application of similar sampling protocols for other species of freshwater mussels would allow verification of hermaphroditism prior to sacrifice for measurement of testicular and oogenic tissue areas. Application of the biopsy protocol presented herein for other populations of *U. imbecillis* would provide a means for resolving the apparent disagreement on whether *U. imbecillis* is universally hermaphroditic (Heard, 1975; Hoeh *et al*., 1995).

Without prior knowledge of the spatial organization of male and female tissues in viscera of NH, collection of biopsy samples from randomly selected visceral sites for determination of hermaphroditism would likely lead to incorrect conclusions. For example, biopsy samples obtained from the middle of viscera of *U. imbecillis* (quadrat 5 of histological section 5) would have failed to detect spermatogenic tissue in 100% of the specimens, and this would have led to the false conclusion that some of the specimens were not hermaphrodites. By examining tissues of the 2 separate visceral locations of the mussels (quadrat 7 of histological section 7 for spermatogenic tissues, and quadrat 5 of histological section 5 for oogenic tissues), the correct conclusion that all of the specimens were hermaphrodites would have been drawn. Thus, prior knowledge of the visceral organization of spermatogenic and oogenic tissues was important. Since studies of NH in

species of freshwater mussels normally obtain data from examination of histological sections, databases of spatial organizations of male and female tissues in viscera could be obtained. If predictable organizational occurrences of spermatogenic and oogenic tissue are observed in other species that are NH, then non-lethal protocols for determination of hermaphroditism could be developed to prevent needless sacrifice.

Hermaphroditism, and the occurrence of hermaphroditic tissue in individuals, was rare in *V. iris*; therefore, the selection of specific visceral locations for non-lethal tissue biopsy sampling was not possible. Too few hermaphroditic *V. iris* were examined such that no conclusions can be drawn as to the visceral organization of spermatogenic and oogenic tissues. Only examinations of histological sections were suitable for hermaphroditic detection in this species. However, occurrence of spermatogenic and oogenic tissues wase evident in every histological section of both *V. iris* hermaphrodites, except histological section 9. If male and female tissues are prevalent throughout the viscera of other OH species of freshwater mussels, then examination of sagittal histological sections from the middle of viscera should detect hermaphroditism. Further research with other OH species should be conducted to test this hypothesis. The rarity of hermaphroditism, as well as the lack of organizational structure within hermaphroditic viscera, implies that OH in the source population of *V. iris* may be incidental in nature (Coe, 1943; van der Schalie, 1970), and may be related to disruption of the sex determining hormonal system hypothesized by Kat (1983).

There were locations in the viscera of male and female *V. iris* that showed reliable consistency of spermatogenic and oogenic tissue occurrences among specimens. These consistencies allowed for selection of sites to collect biopsy samples for determination of sex. Conveniently, gender would have been accurately determined in all specimens with inspection of tissue from the middle of the viscera (quadrat 5 of histological section 5). It is logical that fluid or biopsy samples would be obtained from the middle of the viscera by most researchers. However, only the application of the quadrat and histological section evaluation system of this study substantiated that this visceral position was suitable for reliable sampling. The assumption that the mid-visceral location is suitable for fluid or biopsy sampling in other populations of *V. iris* or other species of freshwater

mussels should be verified through evaluation of spatial consistencies of testicular and oogenic tissue occurrences within viscera.

In commenting on the reliability of evaluations of gametogenic fluids from randomly selected visceral sites, Grande *et al*. (2001) stated "Knowing the chaotic distribution of the gonadal tissues inside the mussel foot, it is easy to imagine that previous results obtained by this method were unreliable, since only a few parts of the gonad can be studied." My results show that spermatogenic and oogenic tissues of *V. iris* and *U. imbecillis* were not chaotically distributed within the viscera. For example, there were specific sites within all conspecific mussels that yielded consistent results on sexual identity and gametogenic stage. However, the comments of Grande *et al*. (2001) indicate the importance of precise visceral site selection for biopsy and gametogenic fluid collection, and that it must be based on prior knowledge of the spatial organization of sexual tissues in the gonad. Examination of all cells in Tables 2.3, 2.5, and 2.6 that have observed frequencies of <1.0 shows that biopsy sampling from randomly selected visceral sites for sex determination may produce data that lead to false conclusions. Occurrence frequencies of <1.0 show that at many visceral sites there is a probability of error associated with determination of gender. Whether associated error probabilities are >0.0 can only be determined through either research that establishes probable detection errors, as in this study, or *post priori* validation of results by examination of histological sections, as conducted by Burton *et al*. (1996) with blue mussels, *Mytilus edulis* Linnaeus, 1831. Burton *et al*. (1996) used squash preparations for microscopic determination of sex in *M. edulis*, and then substantiated the accuracy of their results by examination of histological sections.

Evaluation of Gametogenic Stage

The second objective of this study was to develop protocols for determination of gametogenic stage of the specimens. Because there were gonadal locations that showed consistency of spermatogenic and oogenic stage in the conspecific mussels, the development of these protocols was feasible. The same visceral locations that provided reliable data for sex determination in *V. iris* and *U. imbecillis* were suitable for evaluation of germinal tissue as well. The gametogenic stages of tissues in these locations were representative of the germinal development within the entire gonad. This was because

spermatogenic stage did not vary within germinal tissues of individual viscera. Biopsy samples at the selected visceral areas also were suitable for identification of asynchronous gametogenesis within species. Because sampling of this study occurred at single points in time (May for *V. iris* and February for *U. imbecillis*), the methods developed in this study need to be validated for determination of sex and gametogenic stage over repeated samples.

Application of the quadrat and histological section system described here can provide useful data for determination of gonadal locations appropriate for biopsy tissue collection in other species of freshwater mussels. Studies concerned with evaluation of annual gametogenic cycles could determine gonadal locations for reliable collection of tissue biopsies during initial pilot studies through evaluation of histological sections. Thereafter, spermatogenic and oogenic stage of development could be determined non-lethally with biopsies. Reproductive assessments, such as those conducted by Lewis (1985), Gordon and Smith (1990), Haggerty *et al*. (1995), and Garner *et al*. (1999), could be accomplished in the future by non-lethal means, if data on the spatial organization of sexual tissue are collected for development of species-specific survey protocols.

The gonosinuses of *V. iris* and *U. imbecillis* are anatomical features from which mature gametes could be non-lethally sampled by fluid collection. This sinus appears to be a collection site for sperm and oocytes prior to release from the gonad for fertilization. Counts of mature gametes, as well as measurement of oocyte diameters, have been used to determine gamete maturity and reproductive stage (Bauer, 1987; Haggerty *et al*., 1995; Garner *et al*., 1999). Extraction of gamete fluids from the gonosinus with syringe and needle could be an easy and non-lethal method for such evaluations, especially since mature gametes may be concentrated in this sinus prior to and during spawning.

Gametogenic Asynchrony

The males and females of *V. iris*, as well as the male and female regions of *U. imbecillis*, were characterized by different stages of gametogenesis. Male gametes of both species showed a less mature state of development than female gametes. Asynchronous gamete development among mussels was documented for *Diplodon chilensis* (Gray, 1828) (Peredo and Parada, 1986). However, gametogenic synchrony was observed among males and females of *D. gratus* (Avelar and Mendonça, 1998). Haggerty *et al*.,

(1995) recorded greater numbers of spermatozoa per gonadal transect in early spring than numbers of oocytes per acini in purple wartybacks, *Cyclonaias tuberculata* (Rafinesque, 1820). Haggerty *et al*. (1995) noted that spawning between males and females of *C. tuberculata* was synchronous. Possibly, the rates of spermatogenic and oogenic development may be different, with male gametes maturing faster than female gametes. Heard (1975) and Kat (1983) observed gametes in different stages of development, and inferred that differential maturity rates between spermatogenic and oogenic tissues existed.

Females of *V. iris* and female gonadal regions of *U. imbecillis* also showed different stages of oocyte development. Mussels of the 2 groups of *U. imbecillis* had consistent GDI scores of 3 (mature) and 4 (spawned), respectively; and members of the 2 groups of *V. iris* showed values of 2 (late active) and 5 (resorbing). As described, the *U. imbecillis* were collected in Alabama in February 2000, and the *V. iris* were collected in Virginia in May 1999. Clearly, the 2 group of female *V. iris* were characterized by different stages of gametogenic activity, and were therefore asynchronous. However, a larger sample size of the *U. imbecillis* could have revealed that the specimens were members of one reproductively synchronous group that was in the process of releasing gametes. The GDI evaluation system necessitates that gametogenic tissue be categorized with distinct scores that represent stages of gametogenic activity within acini. A more biologically meaningful nominal evaluation system should be developed that is based a combination of acinic activity (GDI) and gamete development milestones, including stages of meiosis. The problem with inclusion of meiotic characters in such a scheme is that gamete development within an individual acinus often includes gamete in different stages of gamete development, as in GDI stages 1 and 2. Resolution of this issue is important in future research. For now, and in my opinion, a biologically meaningful evaluation system would necessarily include an inactive stage (GDI stage 1), a mature stage (GDI stage 3), a spawning stage (no GDI stage equivalent), a spawned stage (GDI stage 4), and a resorbing-discharge stage (GDI stage 5 with modification). The GDI stage 5 should be modified because not all unspawned gametes and residual reproductive by-products are resorbed, but are also discharged from the acini via the gonoducts and gonosinus, presumably for external expulsion.

Asynchrony of oocyte development among females has not been directly documented, but evidence suggests that mussels within local populations may exhibit different gametic stages. Woody and Holland-Bartels (1993) found that not all males and females of washboard mussels, *Megalonaias nervosa* (Rafinesque, 1820), were characterized by the same gametogenic development stages. Smith (1978) observed most males of *M. margaritifera* with developing spermatogenic tissue, while some males held prodigious numbers of mature sperm. Asynchronous spawning among mussels of a population of *D. chilensis* may indicate asynchronous gamete development that could explain extended spawning periods (Peredo and Parada, 1986). The continuous presence of mature gametes in populations of *U. imbecillis* and *U. peggyae* reported by Heard (1975) may have been the result of individuals, or spermatogenic and oogenic regions within individuals, under various stages of gametogenesis. This gametogenic asynchrony would result in production of mature gametes over an extended period.

TABLES AND FIGURES

Table 2.1. Documentation of hermaphroditism and percentages of occasional hermaphrodites in species of unionoid freshwater mussels. Species denoted by * are normal hermaphrodites, and all others are occasional hermaphrodites.

Species Name	Percentage Hermaphrodites of Specimens	Citation
Margaritiferidae		
Cumberlandia monodonta (Say, 1829)		van der Schalie, 1966
Margaritifera auricularia (Spengler, 1793)		Grande *et al*., 2001
M. falcata (Gould, 1850) *		Heard, 1970
M. margaritifera (Linnaeus, 1758)	8.3	van der Schalie, 1970
Unionidae		
Anodontinae		
Alasmidonta marginata Say, 1818		van der Schalie, 1970
A. undulata (Say, 1817)		Kat, 1983
Anodonta anatina (Linnaeus, 1758)		Pekkarinen, 1993
A. californiensis I. Lea, 1852		Heard, 1975
A. couperiana I. Lea, 1840		Heard, 1975
A. cygnea (Linnaeus, 1758) *		Bloomer, 1930
A. woodiana (Martens, 1874)		Dudgeon and Morton, 1983
Lasmigona complanata (Barnes, 1823)		van der Schalie, 1970
L. compressa (I. Lea, 1829) *		van der Schalie, 1970
L. subviridis (Conrad, 1835) *		van der Schalie, 1970
Pseudanodonta complanata (Rossmässler, 1835)	2.0	Pekkarinen, 1993
Pyganodon grandis (Say, 1829)	14.3	van der Schalie, 1970
Strophitus undulatus (Say, 1817)	1.6	van der Schalie, 1970
Unio crassus (Küster, 1836)		Pekkarinen, 1993
U. pictorum (Linnaeus, 1758)	4.0	Pekkarinen, 1993
U. tumidus (Philipsson, 1788)		Pekkarinen, 1993
Utterbackia imbecillis (Say, 1829) *		Sterki, 1898a
U. peggyae (R. I. Johnson, 1965)		Heard, 1975
Lampsilinae		
Lampsilis cariosa (Say, 1817)		van der Schalie, 1970
L. radiata (Gmelin, 1791)		Kat, 1983
Potamilus alata (Say, 1817)		van der Schalie, 1970
P. obiensis (Rafinesque, 1820)		van der Schalie, 1970
Ptychobranchus fasciolaris (Rafinesque, 1820)		van der Schalie, 1970
P. subtentum (Say, 1825)		van der Schalie, 1970
Toxolasma parvus (Barnes, 1823) *		van der Schalie, 1970
T. pullus (Conrad, 1838) *		Kat, 1983
Vanustaconcha ellipsiformis (Conrad, 1836)	4.0	van der Schalie, 1970
Villosa iris (I. Lea, 1829)		van der Schalie, 1969
Unioninae		
Cyclonaias tuberculata (Rafinesque, 1820)	0.3	Haggerty *et al*., 1995
Elliptio arctata (Conrad, 1834)		Heard, 1979
E. buckleyi (I. Lea, 1843)		Heard, 1979
E. complanata (Lightfoot, 1786)	6.0	Downing *et al*., 1989

Table 2.1 (cont.). Documentation of hermaphroditism and percentages of occasional hermaphrodites in species of unionoid freshwater mussels.

Species Name	Percentage Hermaphrodites of Specimens	Citation
E. dilatata (Rafinesque, 1820)		van der Schalie, 1970
E. icterina (Conrad, 1834)		Heard, 1979
E. producta (Conrad, 1836)		van der Schalie, 1970
Fusconaia ebena (I. Lea, 1831)		van der Schalie, 1970
F. flava (Rafinesque, 1820)		van der Schalie, 1970
Gonidea angulata (I. Lea, 1838)		van der Schalie, 1970
Megalonaias nervosa (Rafinesque, 1820)		Heinricher and Layzer, 1999
Pleurobema cordatum (Rafinesque, 1820)		van der Schalie, 1970
P. sintoxia (Rafinesque, 1820)		van der Schalie, 1970
Quadrula metanevra (Rafinesque, 1820)	2.0	Garner *et al*.,1999
Q. quadrula (Rafinesque, 1820)		van der Schalie, 1970
Tritogonia verrucosa (Rafinesque, 1820)		van der Schalie, 1970
Uniomerus tetralasmus (Say, 1831) *		Sterki, 1898b
Hyriidae		
Diplodon r. gratus (Wagner, 1815)		Avelar and Mendonça, 1998

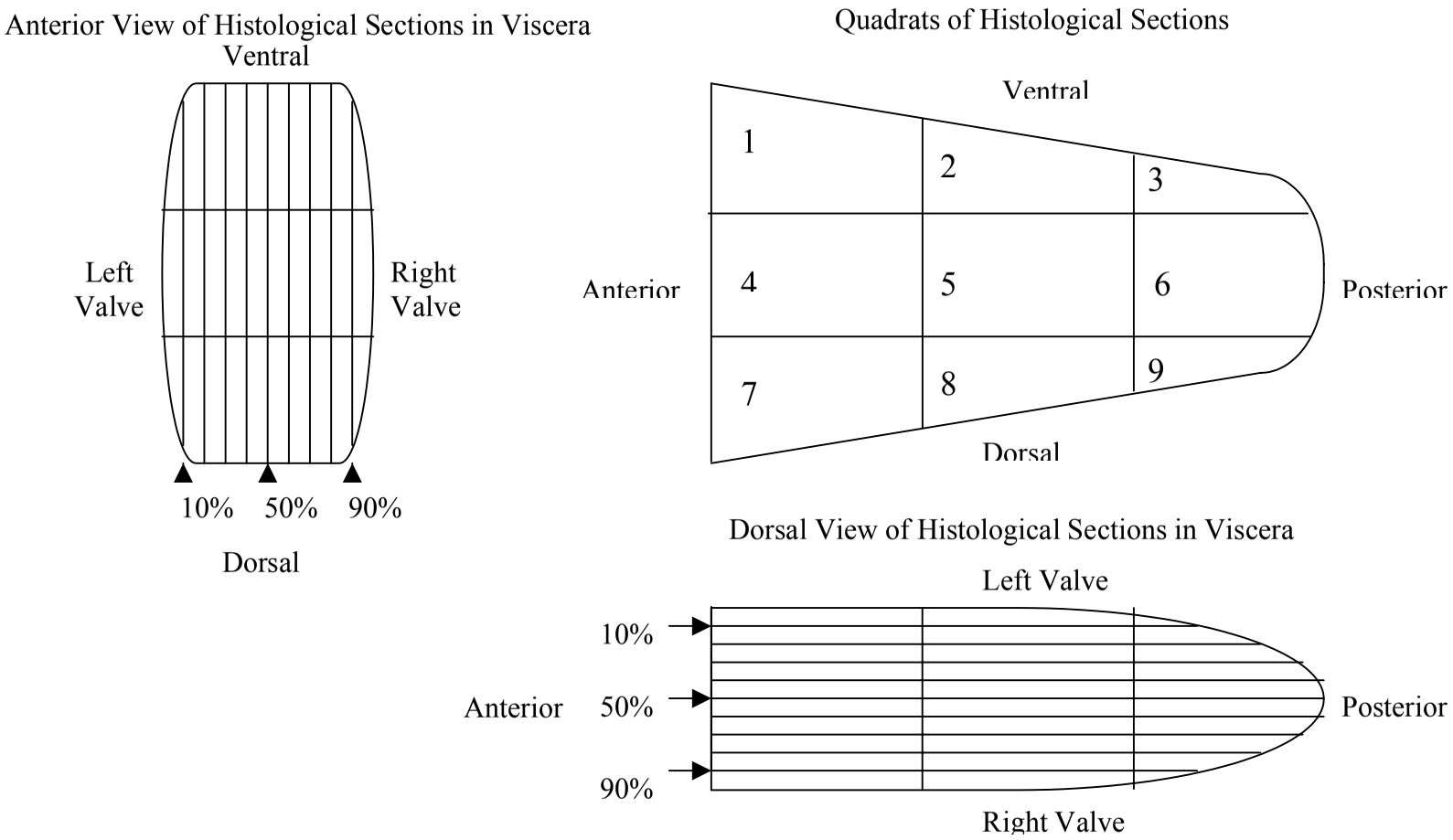

Figure 2.1. Schematic views of sampling grid over tissue samples, and orientation of histological sections and quadrats within viscera.

Table 2.2. Classification stages of the gamete development index (GDI) for evaluation of gametogenic activity of *Villosa iris and Utterbackia imbecillis*. Adapted from Barber (1996).

Gametogenic Stage	Stage Classification	Description
0	Inactive	Acini are nonexistent or elongated, with walls consisting of undifferentiated germinal epithelium.
1	Early active	Acini contain oogonia and primary oocytes, or spermatogonia, spermatocytes and spermatids, but no free oocytes or spermatozoa.
2	Late active	Free oocytes or spermatocytes and spermatids predominate the acini; there are some spermatozoa.
3	Mature	Mature gametes (oocytes and spermatozoa) fill the acini.
4	Spawned	Acini contain spaces mostly devoid of gametes; acini walls may be broken. Hemocytes and phagocytes may be sparsely present.
5	Resorption	Acini have a shrunken appearance and contain phagocytes and products of resorbtion. Gametes are refractory, and development is not evident.

Table 2.3. Observed frequencies of spermatogenic, oogenic, and hermaphroditic tissues in quadrats of histological sections from *Utterbackia imbecillis* (n = 6). All mussels were hermaphrodites. Male, female, and hermaphroditic quadrats with the highest frequencies are highlighted. Histological section number equals percentage of sagittal depth in viscera.

		Quadrat Number								
Histological section (X 10%)	**Sexual Status**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	No sex	0.33	0.33	0.67	0.0	0.0	0.17	0.0	0.0	0.17
1	Hermaphrodite	0.00	0.00	0.00	0.67	0.17	0.17	0.83	**1.00**	0.17
	Female	0.67	0.67	0.33	0.33	0.83	0.67	0.00	0.00	0.50
	Male	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.17
	No sex	0.67	0.67	0.83	0.0	0.0	0.0	0.0	0.0	0.00
2	Hermaphrodite	0.00	0.00	0.00	0.67	0.17	0.00	0.83	0.67	0.00
	Female	0.33	0.33	0.17	0.33	0.83	**1.00**	0.00	0.17	**1.00**
	Male	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.17	0.00
	No sex	0.67	0.67	0.67	0.00	0.00	0.00	0.00	0.00	0.00
3	Hermaphrodite	0.00	0.00	0.00	0.33	0.00	0.00	0.67	0.83	0.17
	Female	0.33	0.33	0.33	0.67	**1.00**	**1.00**	0.33	0.00	0.83
	Male	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.17
	No sex	0.83	0.83	0.83	0.00	0.00	0.00	0.00	0.00	0.17
4	Hermaphrodite	0.00	0.00	0.00	0.17	0.00	0.00	0.67	0.83	0.00
	Female	0.17	0.17	0.17	0.83	**1.00**	**1.00**	0.00	0.00	0.67
	Male	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.17	0.17
	No sex	0.67	0.67	0.67	0.00	0.00	0.00	0.00	0.00	0.00
5	Hermaphrodite	0.00	0.00	0.00	0.17	0.00	0.00	0.50	0.67	0.17
	Female	0.33	0.33	0.33	0.83	**1.00**	**1.00**	0.00	0.17	0.83
	Male	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.17	0.00

Table 2.3 (cont.). Observed frequencies of spermatogenic, oogenic, and hermaphroditic tissues in quadrats of histological sections from *Utterbackia imbecillis*.

		Quadrat Number								
Histological section (X10%)	**Sexual Status**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	No sex	0.67	0.50	0.50	0.00	0.00	0.00	0.00	0.00	0.00
6	Hermaphrodite	0.00	0.00	0.00	0.17	0.00	0.00	0.50	0.67	0.00
	Female	0.33	0.50	0.50	0.83	**1.00**	**1.00**	0.00	0.33	**1.00**
	Male	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00
	No sex	0.83	0.50	0.50	0.17	0.17	0.17	0.00	0.00	0.17
7	Hermaphrodite	0.00	0.00	0.00	0.33	0.17	0.00	0.17	0.50	0.00
	Female	0.17	0.50	0.50	0.50	0.67	0.83	0.00	0.33	0.83
	Male	0.00	0.00	0.00	0.00	0.00	0.00	**0.83**	0.17	0.00
	No sex	0.80	0.80	0.80	0.00	0.00	0.00	0.20	0.00	0.00
8	Hermaphrodite	0.00	0.00	0.00	0.20	0.20	0.00	0.60	0.80	0.00
	Female	0.20	0.20	0.20	0.80	0.80	**1.00**	0.00	0.20	**1.00**
	Male	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.00
	No sex	0.80	0.80	0.80	0.80	0.60	0.40	0.40	0.40	0.40
9	Hermaphrodite	0.00	0.00	0.00	0.20	0.00	0.00	0.20	0.20	0.00
	Female	0.00	0.20	0.20	0.00	0.40	0.00	0.00	0.40	0.60
	Male	0.20	0.00	0.00	0.00	0.00	0.60	0.40	0.00	0.00

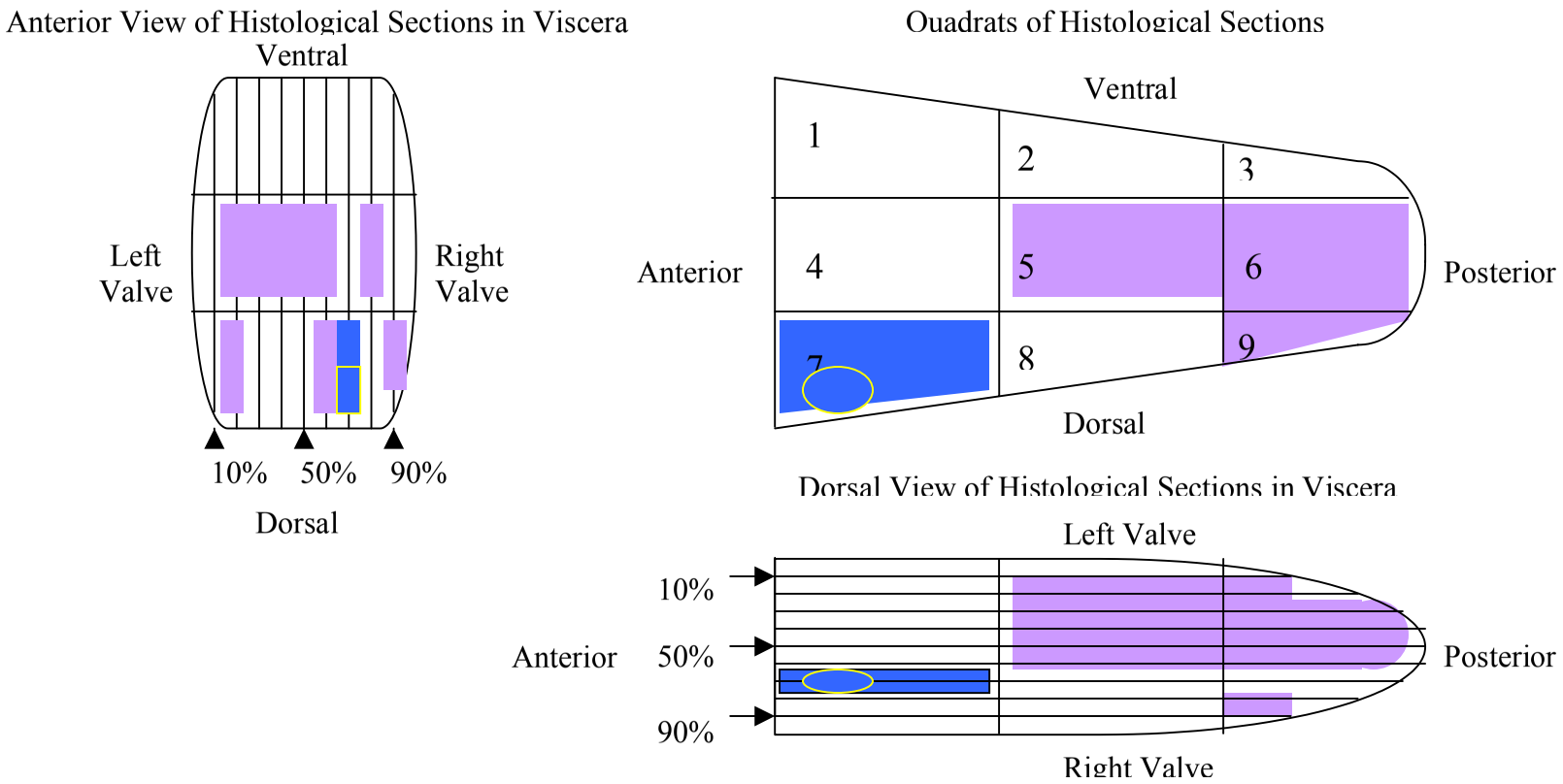

Figure 2.2. Quadrats of *Utterbackia imbecillis* gonads containing oogenic (purple) and spermatogenic (blue) tissues in 100% of specimens. Only the dorso-anterior area of quadrat 7 in thin-section 7 (yellow outline) contained spermatogenic tissue in all mussels.

Table 2.4. Observed frequencies of spermatogenic, oogenic, and hermaphroditic tissues in quadrats of histological sections from *Villosa iris* (n = 19). Two hermaphrodites were observed. Male, female, and hermaphroditic quadrats with the highest frequencies are highlighted. Histological section number equals percentage of sagittal depth in viscera.

		Quadrat Number								
Histological section (X 10%)	**Sexual Status**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	No sex	0.89	0.95	0.89	0.05	0.11	0.21	0.05	0.05	0.11
1	Hermaphrodite	0.00	0.00	0.00	0.00	0.05	0.00	0.05	0.05	0.0
	Female	0.00	0.0	0.05	0.63	0.53	0.47	0.63	0.58	0.58
	Male	0.11	0.05	0.05	**0.32**	**0.32**	**0.32**	0.26	**0.32**	**0.32**
	No sex	0.89	1.00	0.95	0.05	0.00	0.00	0.00	0.00	0.00
2	Hermaphrodite	0.00	0.00	0.00	0.05	0.05	0.00	0.05	0.00	0.00
	Female	0.05	0.00	0.05	0.58	0.63	0.68	0.68	0.68	0.68
	Male	0.05	0.00	0.00	**0.32**	**0.32**	**0.32**	0.26	**0.32**	**0.32**
	No sex	0.84	1.00	1.00	0.05	0.0	0.05	0.00	0.00	0.05
3	Hermaphrodite	0.00	0.00	0.00	0.00	0.05	0.00	0.05	0.00	0.00
	Female	0.11	0.00	0.00	0.63	0.63	0.63	**0.86**	0.68	0.68
	Male	0.05	0.00	0.00	**0.32**	**0.32**	**0.32**	0.26	**0.32**	0.26
	No sex	0.84	0.79	0.89	0.11	0.00	0.05	0.05	0.00	0.05
4	Hermaphrodite	0.00	0.00	0.00	0.00	0.05	0.05	0.00	0.05	0.05
	Female	0.05	0.11	0.05	0.58	0.63	0.58	0.68	0.68	0.63
	Male	0.11	0.11	0.05	**0.32**	**0.32**	**0.32**	0.26	0.26	0.26
	No sex	0.79	0.74	0.95	0.05	0.00	0.05	0.05	0.00	0.00
5	Hermaphrodite	0.00	0.00	0.00	**0.11**	**0.11**	0.00	0.05	0.05	0.00
	Female	0.11	0.11	0.00	0.58	0.63	0.63	0.63	0.68	0.68
	Male	0.11	0.16	0.05	0.26	0.26	**0.32**	0.26	0.26	**0.32**

Table 2.4 (cont.). Observed frequencies of spermatogenic, oogenic, and hermaphroditic tissues in quadrats of histological sections from *Villosa iris*.

		Quadrat Number								
Histological section (X 10%)	**Sexual Status**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	No sex	0.89	0.89	0.95	0.05	0.00	0.00	0.05	0.00	0.00
6	Hermaphrodite	0.00	0.00	0.00	0.05	0.05	0.05	0.05	0.05	0.00
	Female	0.00	0.00	0.00	0.58	0.63	0.63	0.63	0.63	0.68
	Male	0.11	0.11	0.05	**0.32**	**0.32**	**0.32**	0.26	**0.32**	**0.32**
	No sex	0.84	0.95	0.89	0.11	0.05	0.05	0.05	0.05	0.11
7	Hermaphrodite	0.00	0.00	0.05	0.05	0.05	0.00	0.05	0.05	0.05
	Female	0.05	0.00	0.00	0.53	0.58	0.63	0.63	0.58	0.53
	Male	0.11	0.05	0.05	**0.32**	**0.32**	**0.32**	0.26	**0.32**	**0.32**
	No sex	0.95	0.95	0.95	0.11	0.05	0.16	0.11	0.05	0.16
8	Hermaphrodite	0.00	0.00	0.00	0.00	0.05	0.05	0.05	0.00	0.00
	Female	0.00	0.05	0.00	0.58	0.58	0.53	0.63	0.63	0.53
	Male	0.05	0.00	0.05	**0.32**	**0.32**	0.26	0.21	**0.32**	**0.32**
	No sex	0.95	1.00	1.00	0.63	0.74	0.74	0.63	0.74	0.74
9	Hermaphrodite	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Female	0.00	0.00	0.00	0.11	0.16	0.11	0.16	0.16	0.11
	Male	0.05	0.00	0.00	0.26	0.11	0.16	0.21	0.11	0.16

Table 2.5. Observed frequencies of spermatogenic tissues in quadrats of histological sections from male *Villosa iris* (n = 7). Frequencies of 1.0 for male quadrats are highlighted. Histological section number equals percentage of sagittal depth in viscera.

		Quadrat Number								
Histological section (X10%)	**Sexual Status**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	No sex	0.60	0.80	0.80	0.00	0.00	0.00	0.00	0.00	0.00
	Male	0.40	0.20	0.20	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**
2	No sex	0.80	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00
	Male	0.20	0.00	0.00	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**
3	No sex	0.80	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00
	Male	0.20	0.00	0.00	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**
4	No sex	0.60	0.60	1.00	0.00	0.00	0.00	0.00	0.00	0.00
	Male	0.40	0.40	0.00	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**
5	No sex	0.60	0.60	0.80	0.00	0.00	0.00	0.00	0.00	0.00
	Male	0.40	0.40	0.20	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**
6	No sex	0.60	0.60	0.80	0.00	0.00	0.00	0.00	0.00	0.00
	Male	0.40	0.40	0.20	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**
7	No sex	0.60	0.80	0.80	0.00	0.00	0.00	0.00	0.00	0.00
	Male	0.40	0.20	0.20	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**
8	No sex	0.80	1.00	0.80	0.00	0.00	0.20	0.20	0.00	0.00
	Male	0.20	0.00	0.20	**1.00**	**1.00**	0.80	0.80	1.00	1.00
9	No sex	0.80	1.00	1.00	0.20	0.60	0.40	0.20	0.60	0.40
	Male	0.20	0.00	0.00	0.80	0.40	0.60	0.80	0.40	0.60

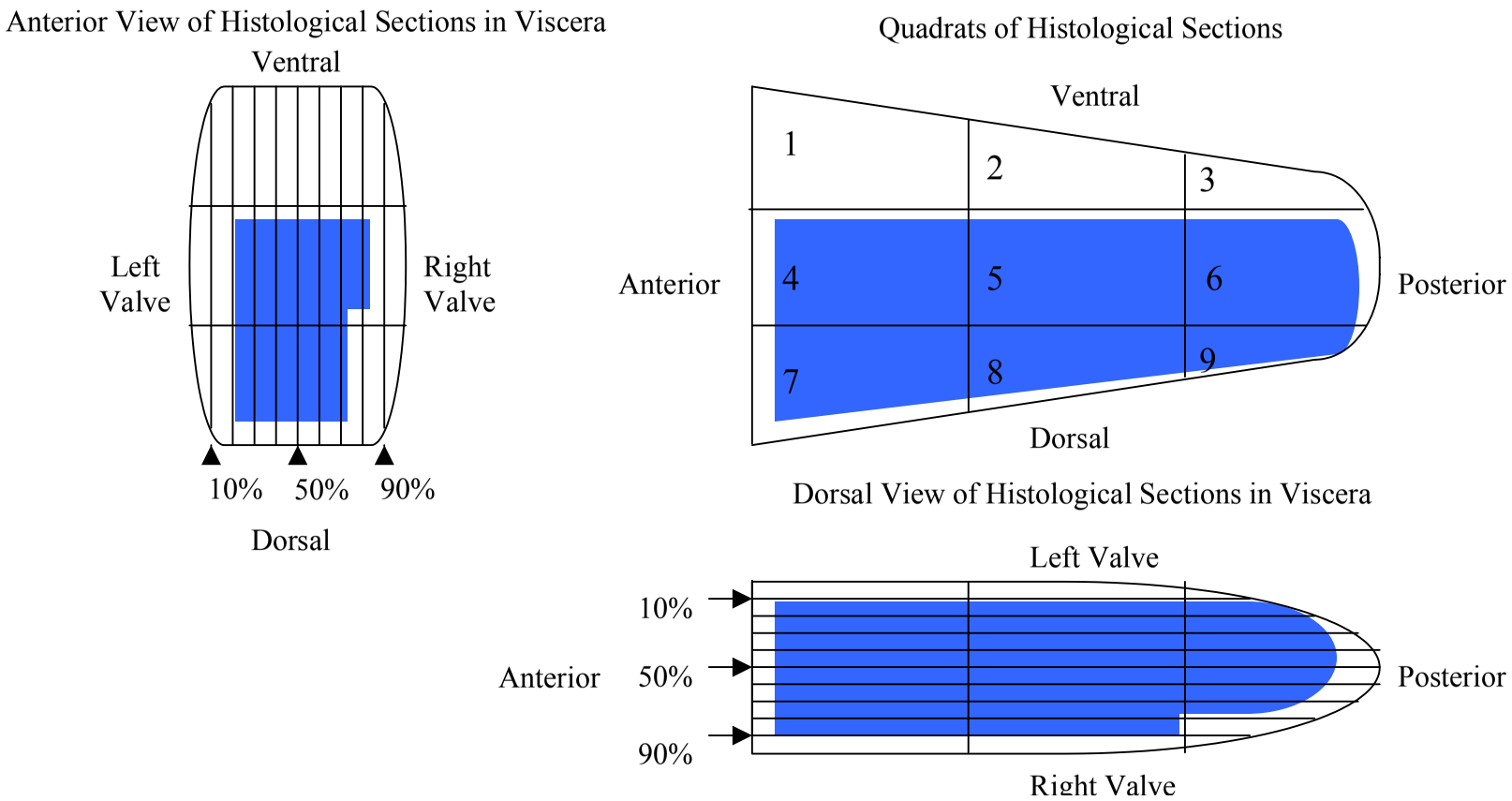

Figure 2.3. Quadrats of *Villosa iris* gonads containing spermatogenic (blue) tissues in 100% of male specimens.

Table 2.6. Observed frequencies of spermatogenic tissues in quadrats of histological sections from female *Villosa iris* (n = 12). Frequencies of 1.0 for female are highlighted. Histological section number equals percentage of sagittal depth in viscera.

		Quadrat Number								
Histological section (X 10%)	**Sexual Status**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	No sex	1.00	1.00	1.00	0.08	0.17	0.33	0.08	0.08	0.17
	Female	0.00	0.00	0.00	0.92	0.83	0.67	0.92	0.92	0.83
2	No sex	0.92	1.00	1.00	0.83	0.00	0.00	0.00	0.00	0.00
	Female	0.08	0.00	0.00	0.92	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**
3	No sex	0.92	1.00	1.00	0.08	0.00	0.08	0.00	0.00	0.00
	Female	0.08	0.00	0.00	0.92	**1.00**	0.92	**1.00**	**1.00**	**1.00**
4	No sex	0.92	0.92	1.00	0.08	0.00	0.08	0.00	0.00	0.08
	Female	0.08	0.08	0.00	0.92	**1.00**	0.92	**1.00**	**1.00**	0.92
5	No sex	0.83	0.92	1.00	0.08	0.00	0.08	0.08	0.00	0.00
	Female	0.17	0.08	0.00	0.92	**1.00**	0.92	0.92	**1.00**	**1.00**
6	No sex	1.00	1.00	1.00	0.08	0.00	0.00	0.08	0.00	0.00
	Female	0.00	0.00	0.00	0.92	**1.00**	**1.00**	0.92	**1.00**	**1.00**
7	No sex	0.92	1.00	1.00	0.16	0.08	0.08	0.08	0.08	0.17
	Female	0.08	0.00	0.00	0.83	0.92	0.92	0.92	0.92	0.92
8	No sex	1.00	0.92	1.00	0.17	0.08	0.17	0.08	0.08	0.25
	Female	0.00	0.08	0.00	0.83	0.92	0.83	0.92	0.92	0.75
9	No sex	1.00	1.00	1.00	0.83	0.75	0.83	0.75	0.75	0.83
	Female	0.00	0.00	0.00	0.17	0.25	0.17	0.25	0.25	0.17

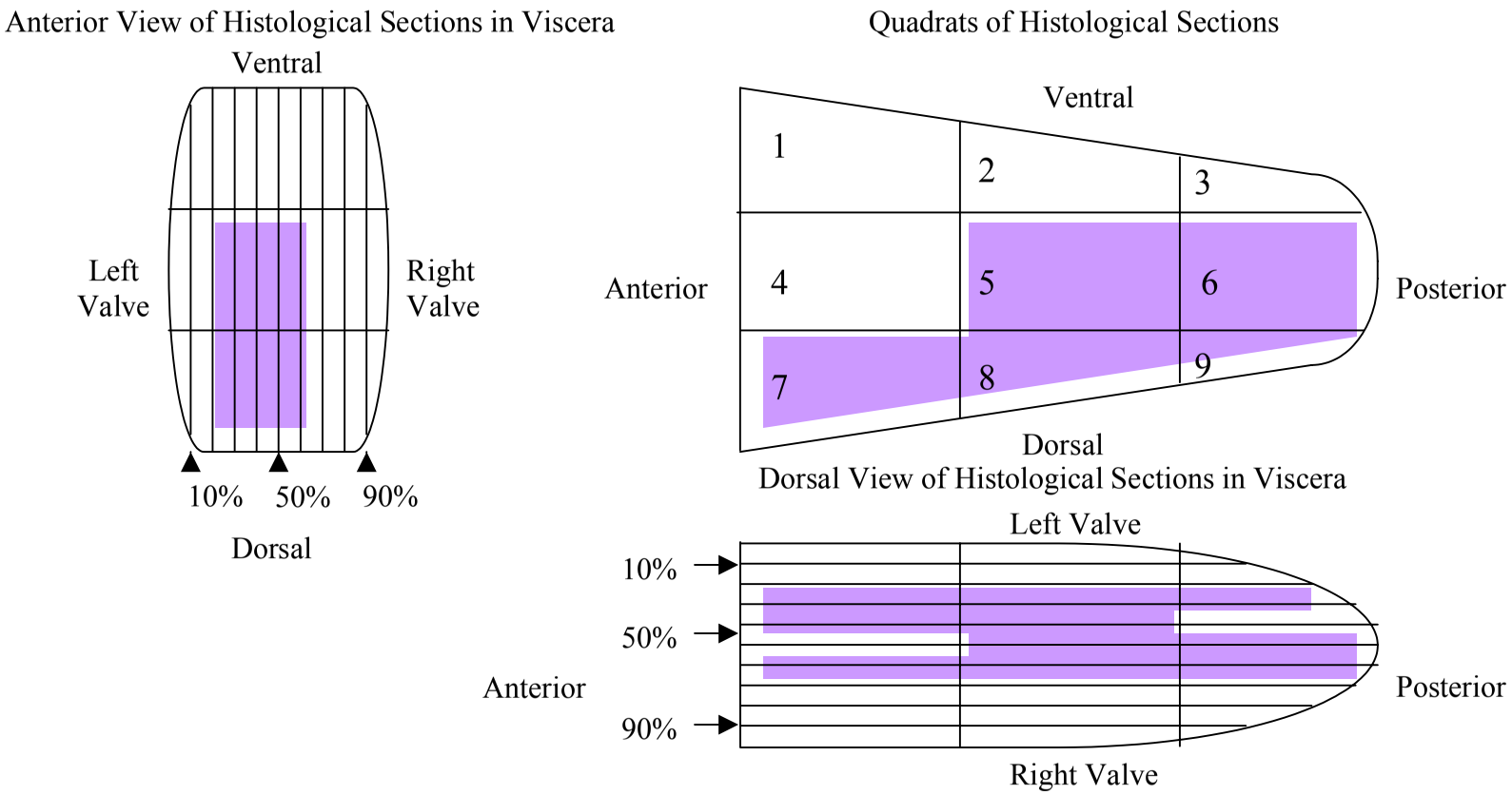

Figure 2.4. Quadrats of *Villosa iris* gonads containing oogenic (purple) tissues in 100% of female specimens.

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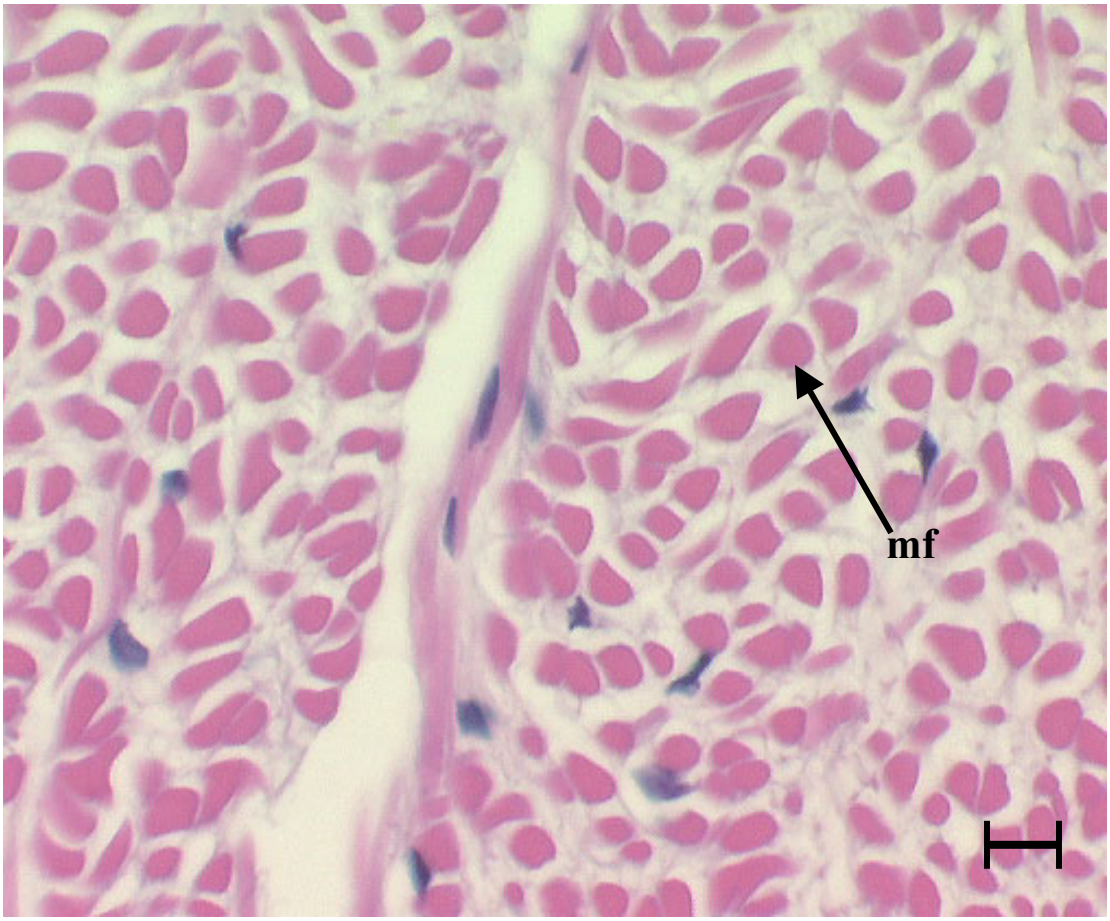
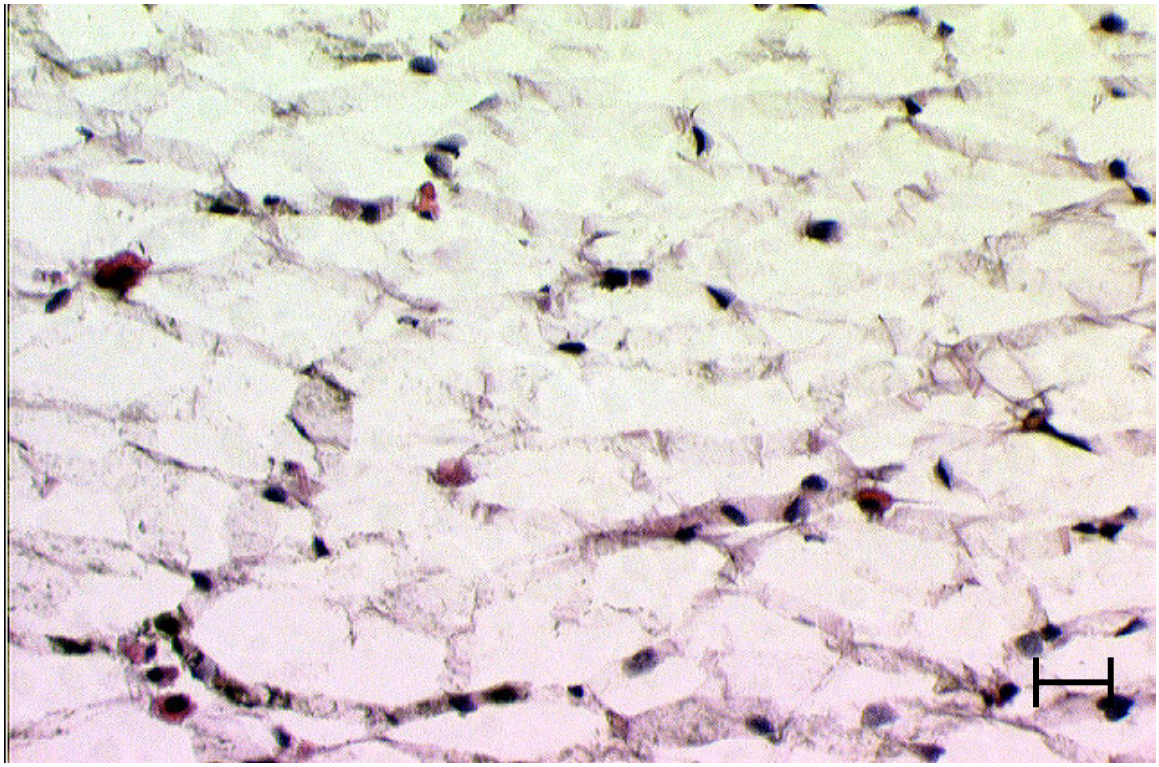
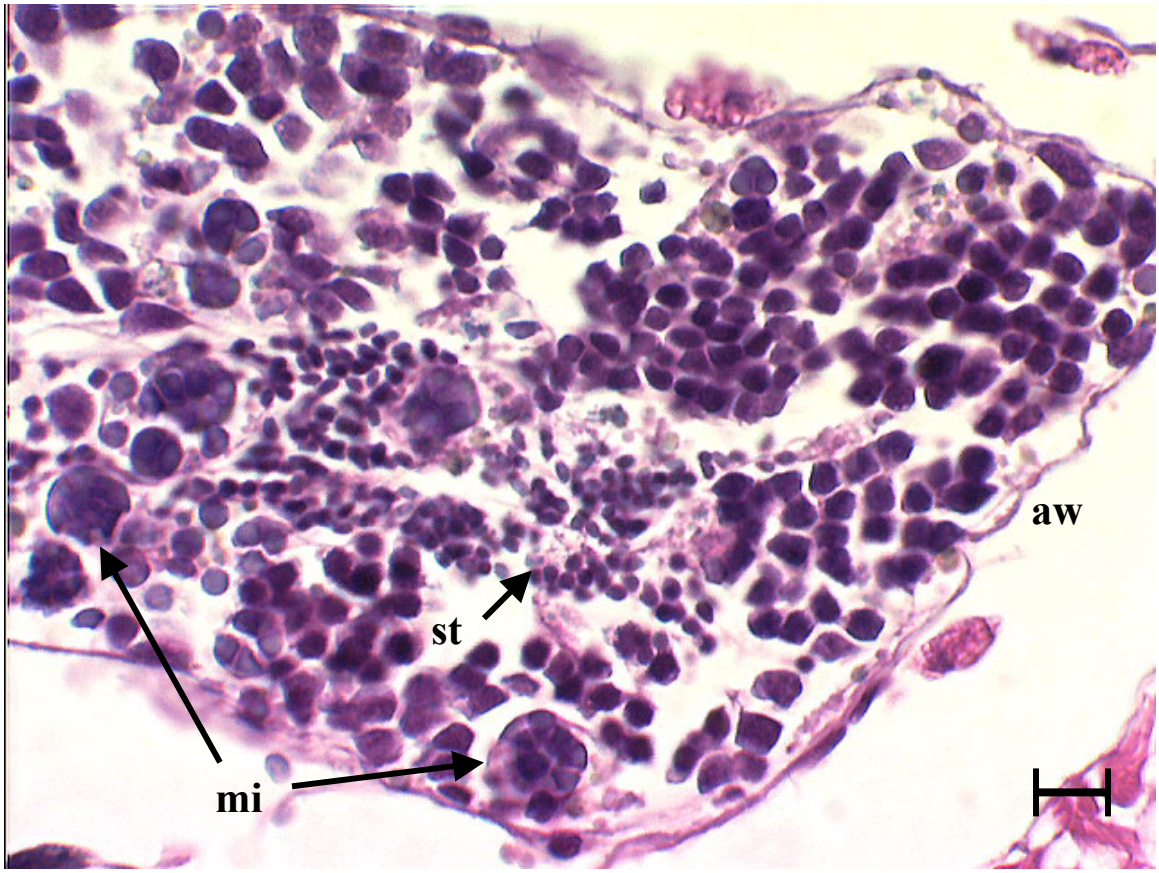
APPENDIX A



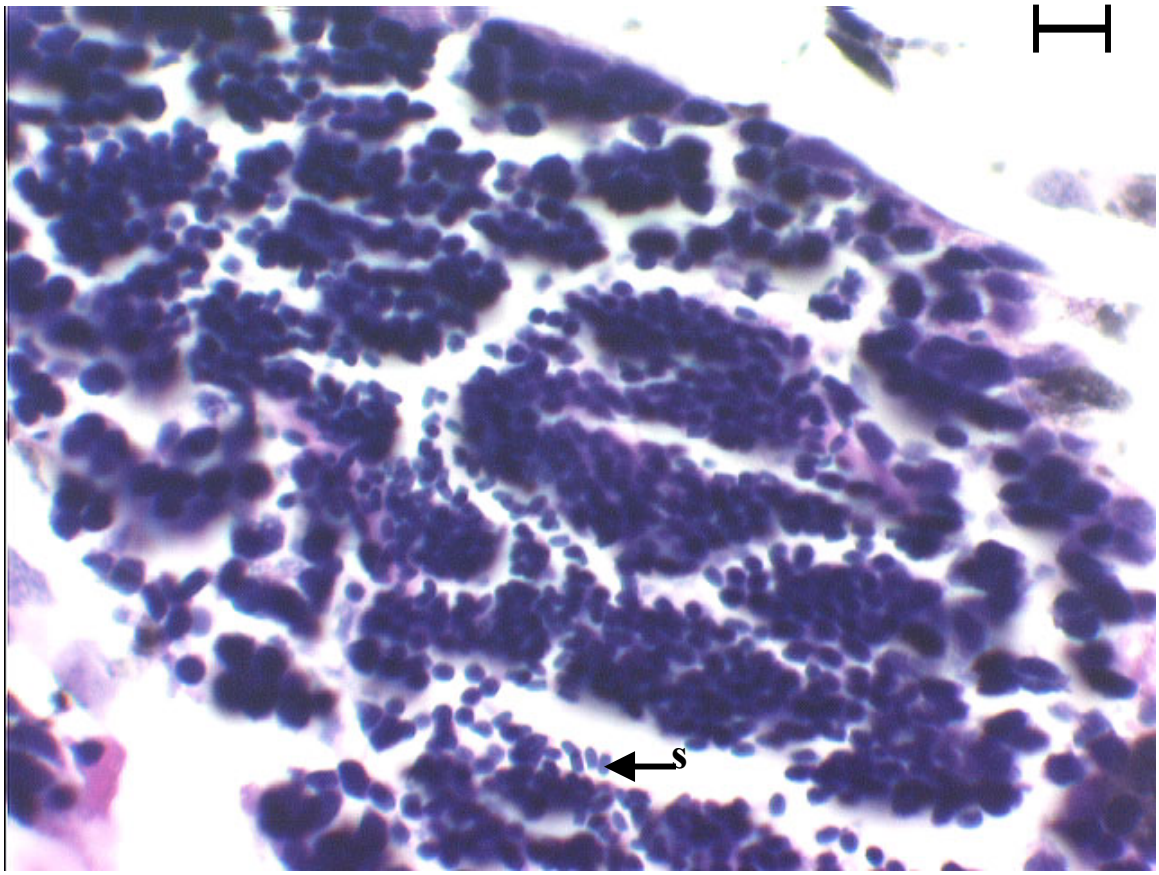
Photograph 1.1. Example of digital image of muscle fibers (mf) used to measure cross-sectional muscle fiber areas of *Elliptio complanata*. Image captured at 1540X. Bar equals 5 µm.



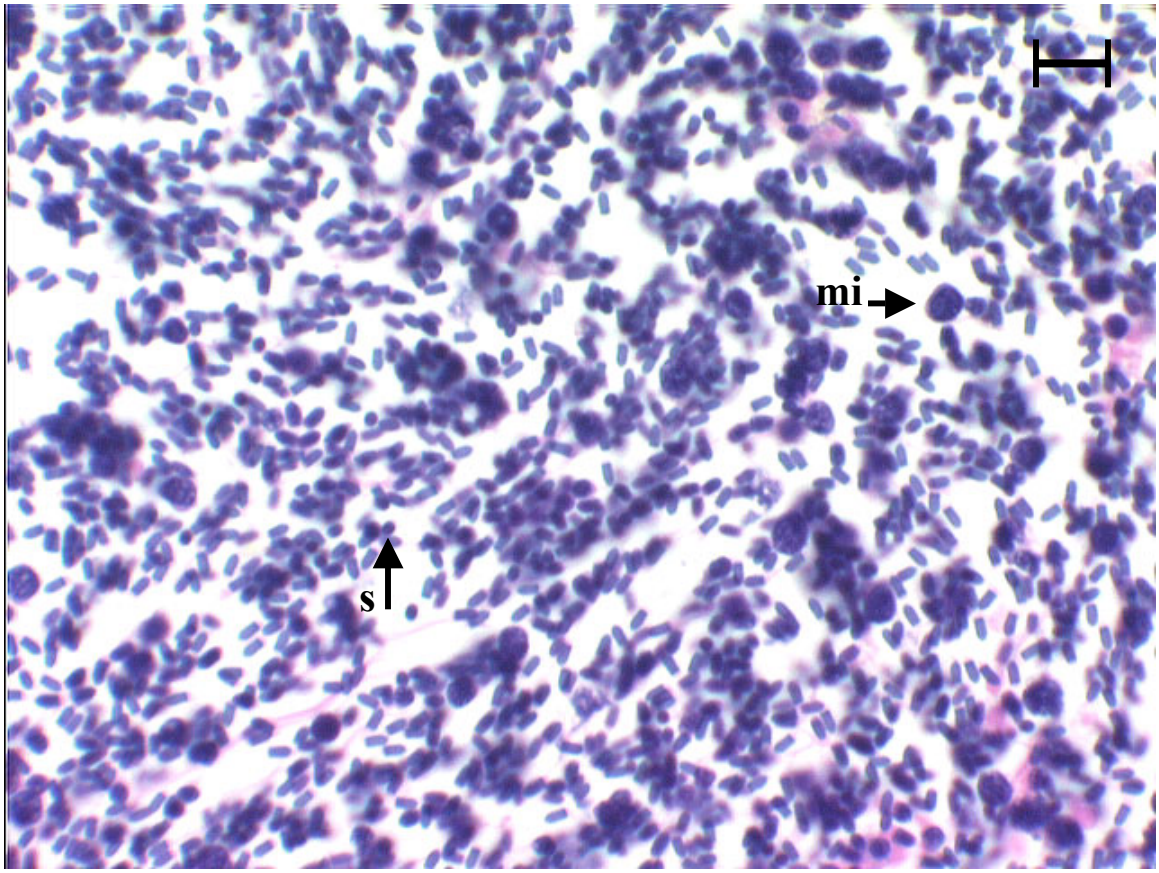
Photograph 1.2. Example of GDI Stage 0 (inactive) in visceral mass of *Elliptio complanata*. Note lack of acini within connective tissue. Image captured at 390X. Bar equals 30 µm.



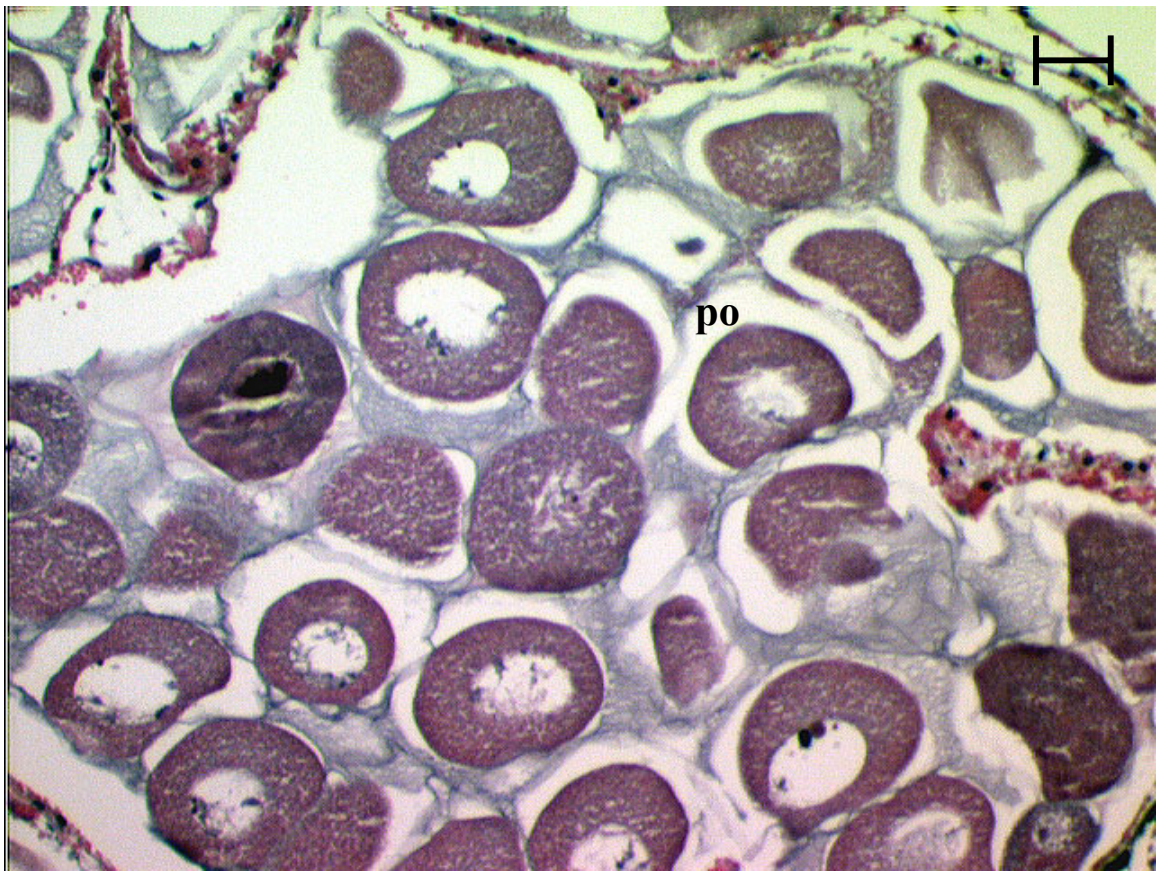
Photograph 1.3. Example of GDI Stage 1 (early active) stage in visceral mass of male *Elliptio complanata*. Note spermatids (st), multi-nucleated inclusions (mi), lack of free spermatozoa, and acinar wall (aw). Image captured at 1540X. Bar equals 5 µm. GDI stage 1 was not observed in females of this study, therefore no example is presented.



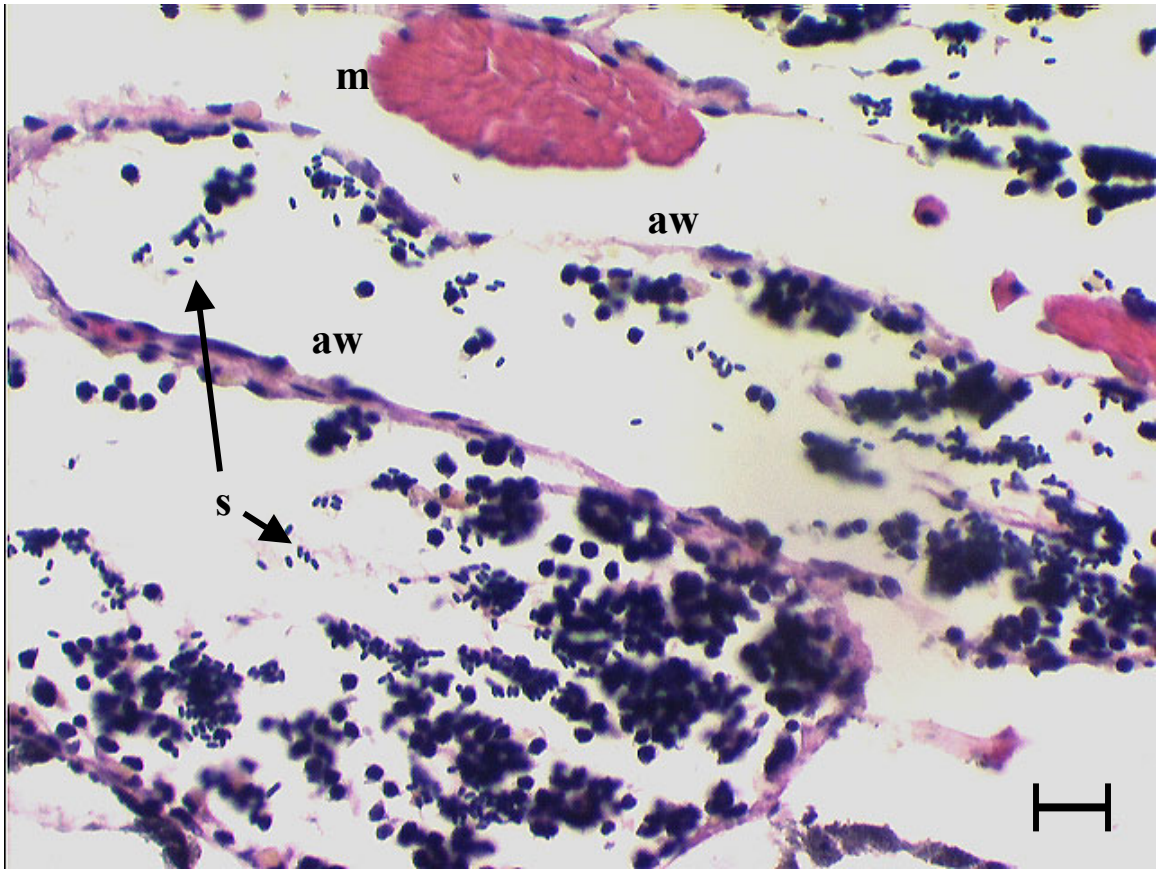
Photograph 1.4. Example of GDI Stage 2 (late active) in male *Elliptio complanata*. Note acinus is filled with developing gametes and some mature spermatozoa (s). Image captured at 1540X. Bar equals 5 µm. GDI stage 2 was not observed in females of this study, therefore no example is presented.



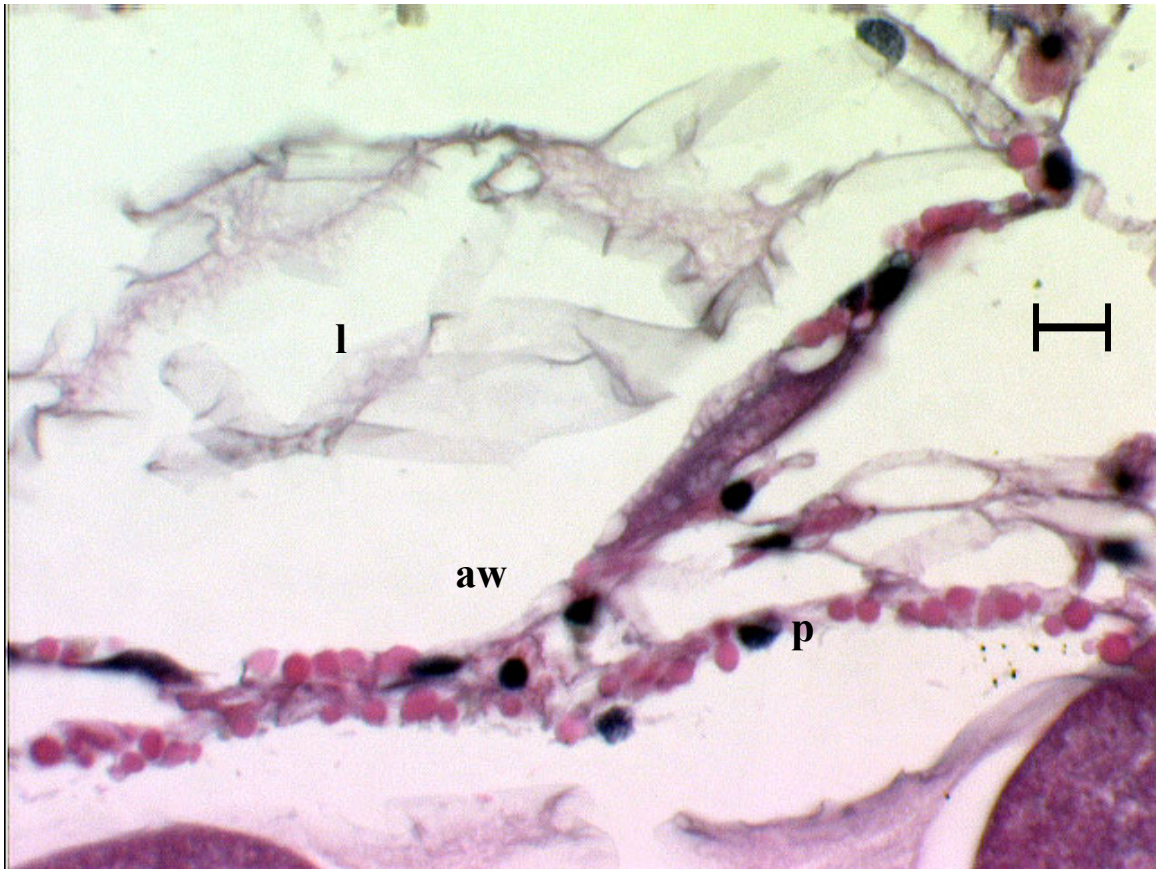
Photograph 1.5. Example of GDI Stage 3 (mature) in male *Elliptio complanata*. Note lumen of acinus is filled with mature spermatozoa (s) and some multi-nucleated inclusions (mi). Image captured at 1540X. Bar equals 5 µm.



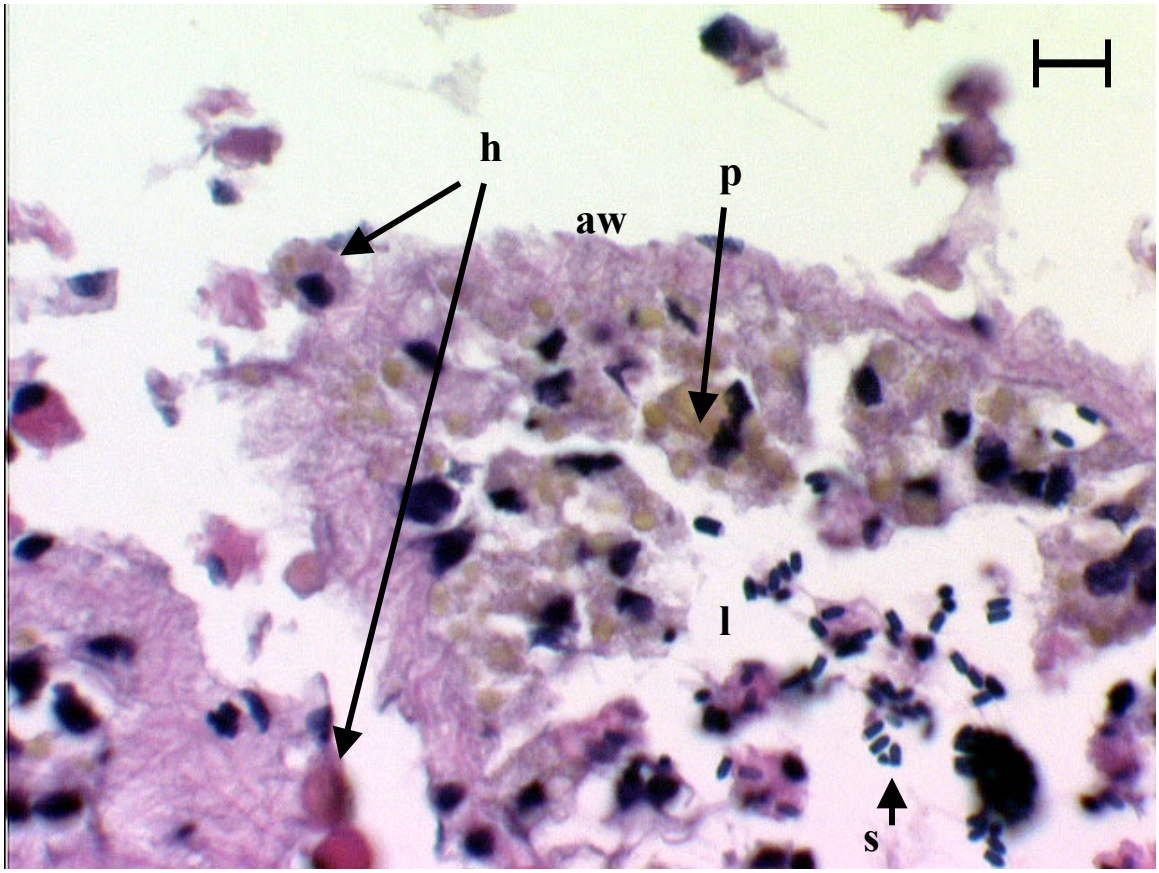
Photograph 1.6. Example of GDI Stage 3 (mature) in female *Elliptio complanata*. Note lumen of acinus is filled with mature primary oocytes (po). Image captured at 390X. Bar equals 30 µm.



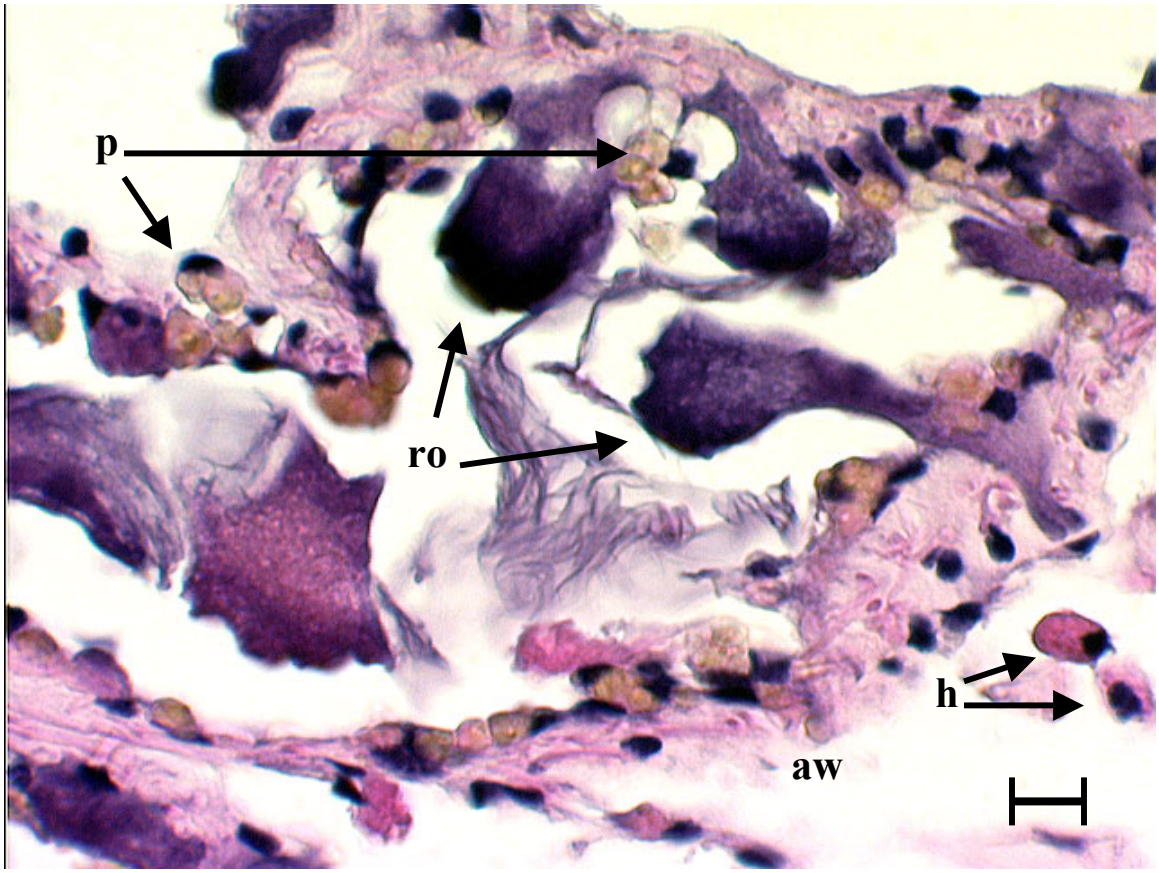
Photograph 1.7. Example of GDI Stage 4 (spawned) in male *Elliptio complanata*. Note acinus is nearly empty of spermatozoa (s), acini walls (aw) have significantly thinned and weakened, and presence of muscle tissue (m). Image captured at 1540X. Bar equals 5 μm.



Photograph 1.8. Example of GDI Stage 4 (spawned) in female *Elliptio complanata*. Note lumen (l) of acinus is empty of free oocytes, acinar wall (aw) has significantly weakened, and presence of phagocytes (p) along acini walls. Image captured at 1540X. Bar equals 5 μm.

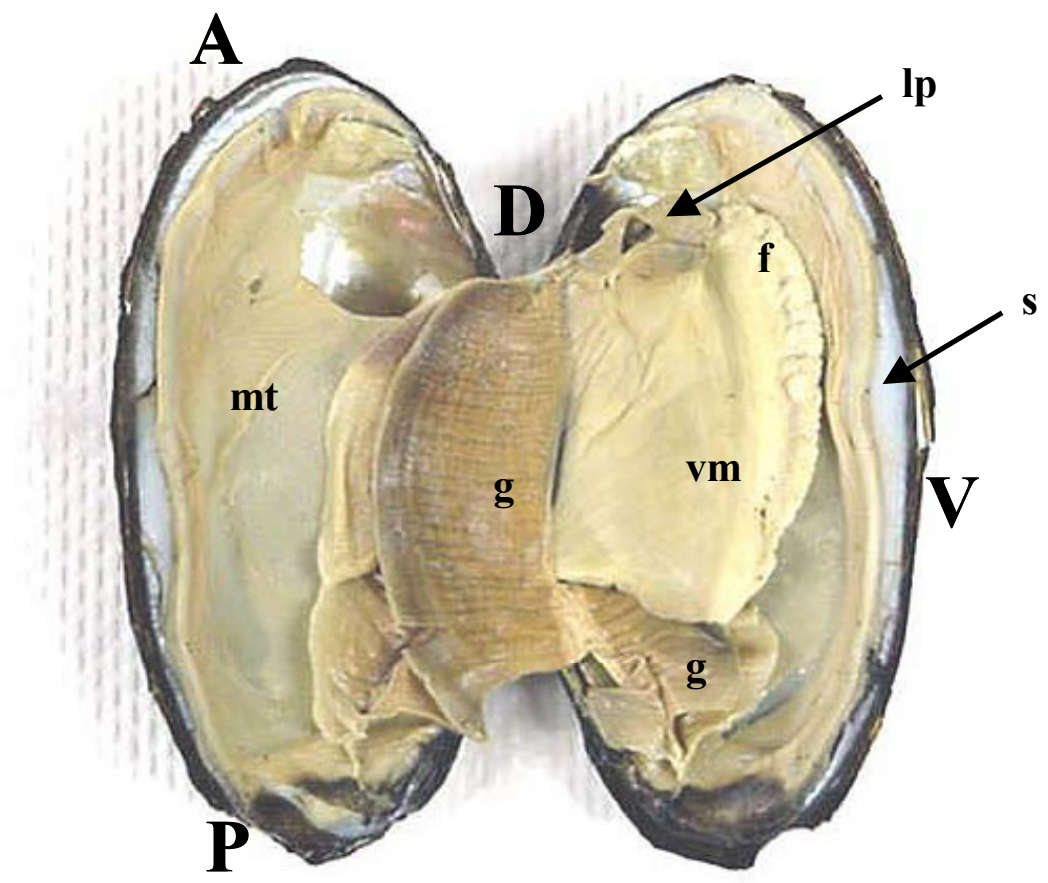


Photograph 1.9. Example of GDI Stage 5 (resorbing) in male *Elliptio complanata*. Note presence of phagocytes (p) along acinar wall (aw), presence of hemocytes (h), and some spermatozoa (s) in lumen (l) of acinus. Image captured at 1540X. Bar equals 5 µm.

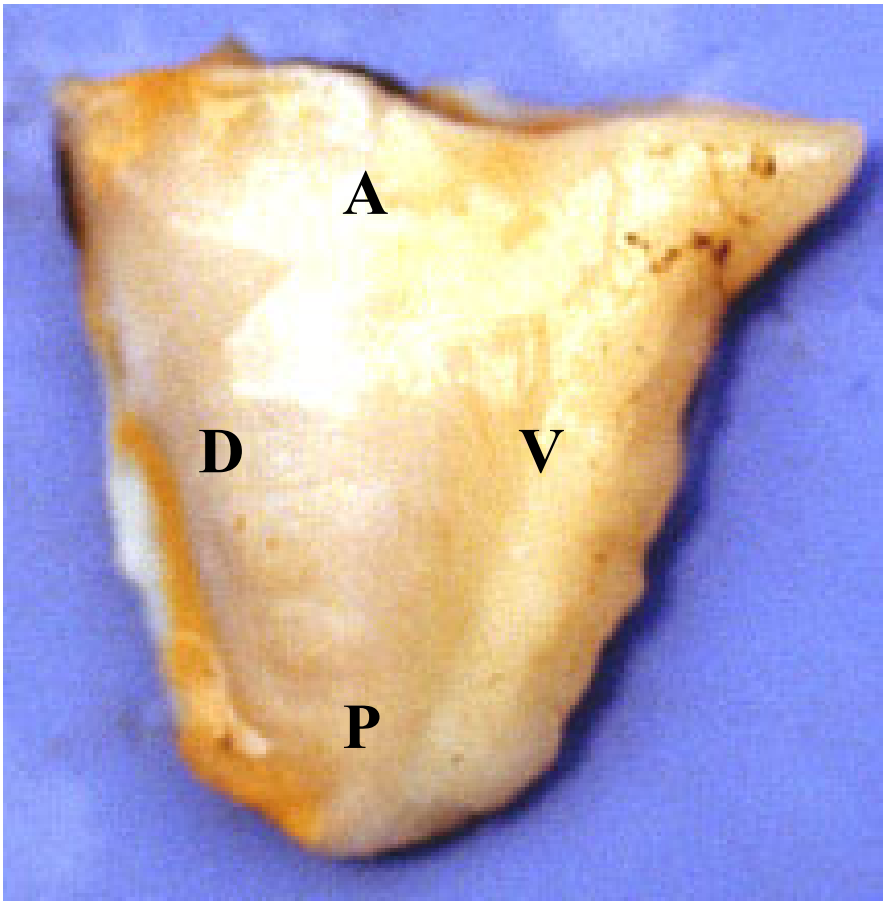


Photograph 1.10. Example of GDI Stage 5 (resorbing) in female *Elliptio complanata*. Note phagocytes (p) along acinar wall (aw), presence of hemocytes (h), resorbing oocytes (ro). Image captured at 1540X. Bar equals 5 µm.

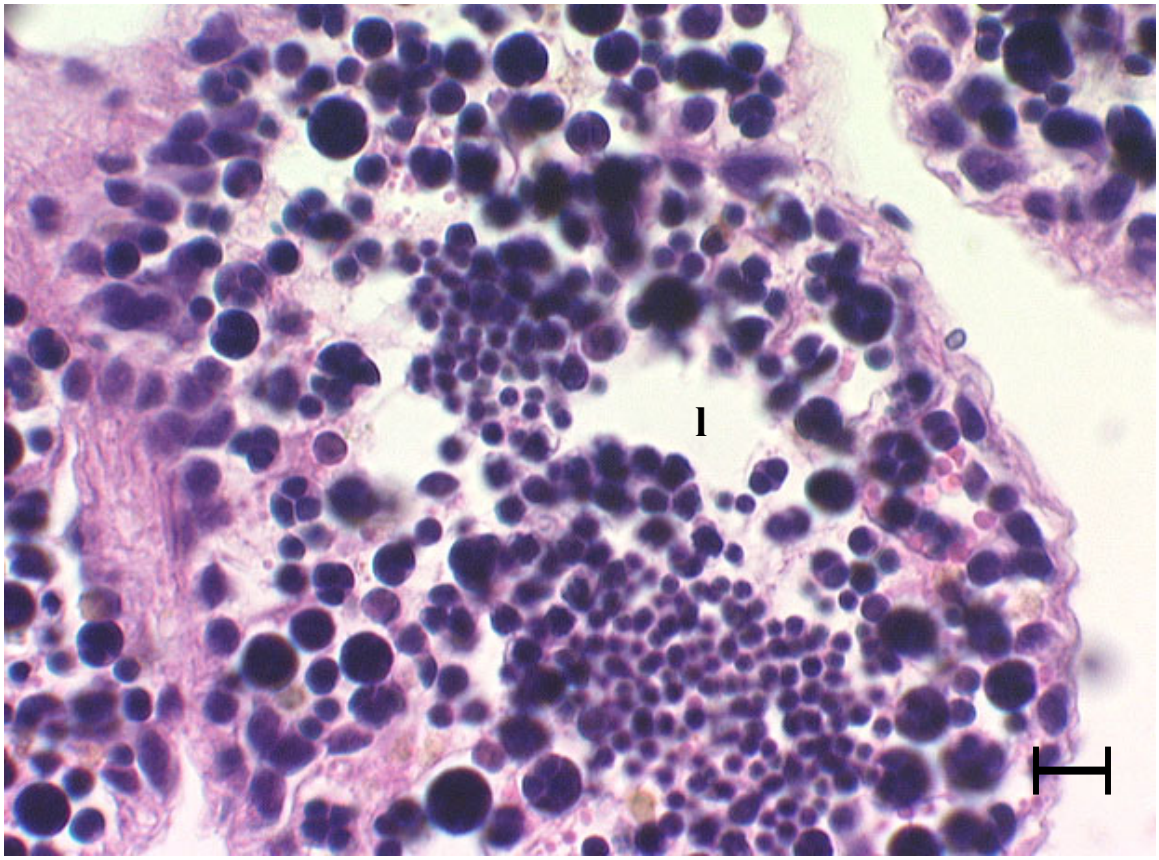
APPENDIX B



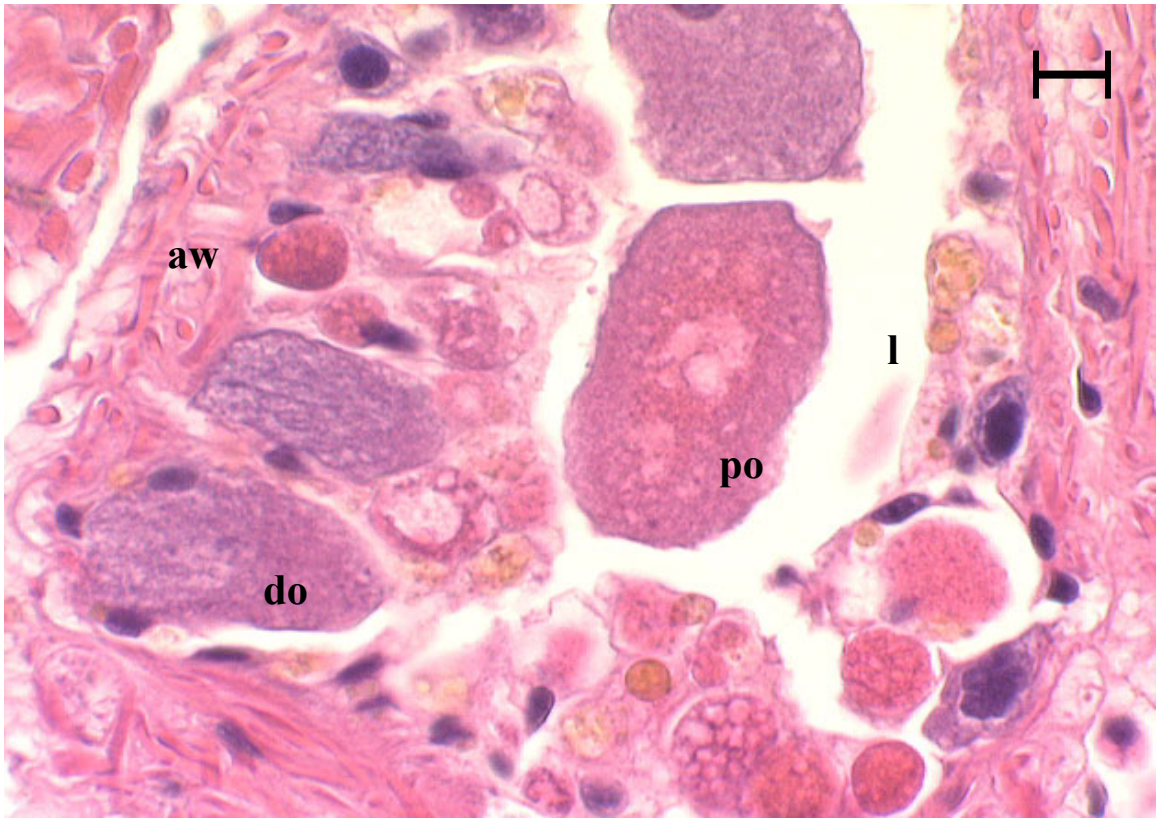
Photograph 2.1 Gross exterior anatomical features of a preserved freshwater mussel, including visceral mass (vm), gills (g), mantle tissue (mt), labial palp (lp), and shell (s). Anatomical orientation descriptors are anterior (A), posterior (P), dorsal (D), and ventral (V).



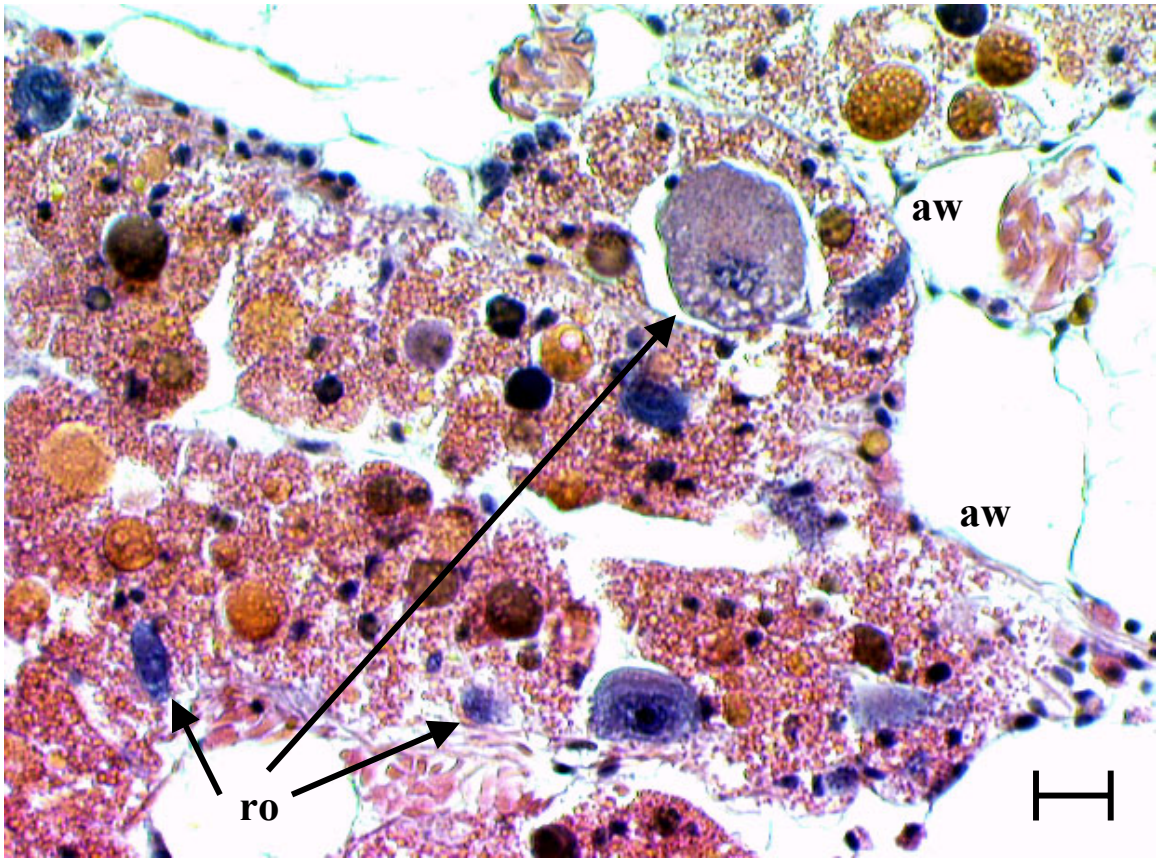
Photograph 2.2 Excised visceral mass of *Villosa iris* used for fixation, embedment, and thin-sectioning. Anatomical orientation descriptors are anterior (A), posterior (P), dorsal (D), and ventral (V).



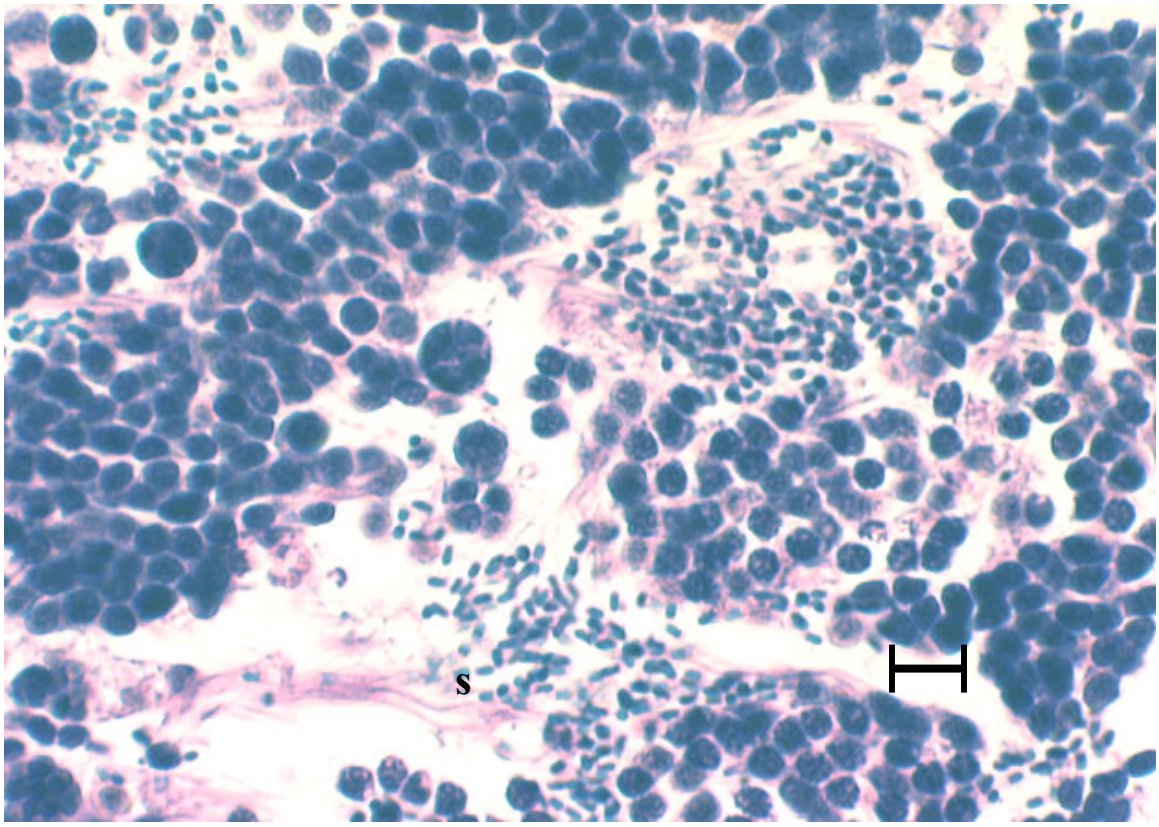
Photograph 2.3. Example of GDI Stage 1 (early active) in visceral mass of male *Villosa iris*. Note absence of free spermatozoa in lumen (l) of acinus. Image captured at 1540X. Bar equals 5 µm.



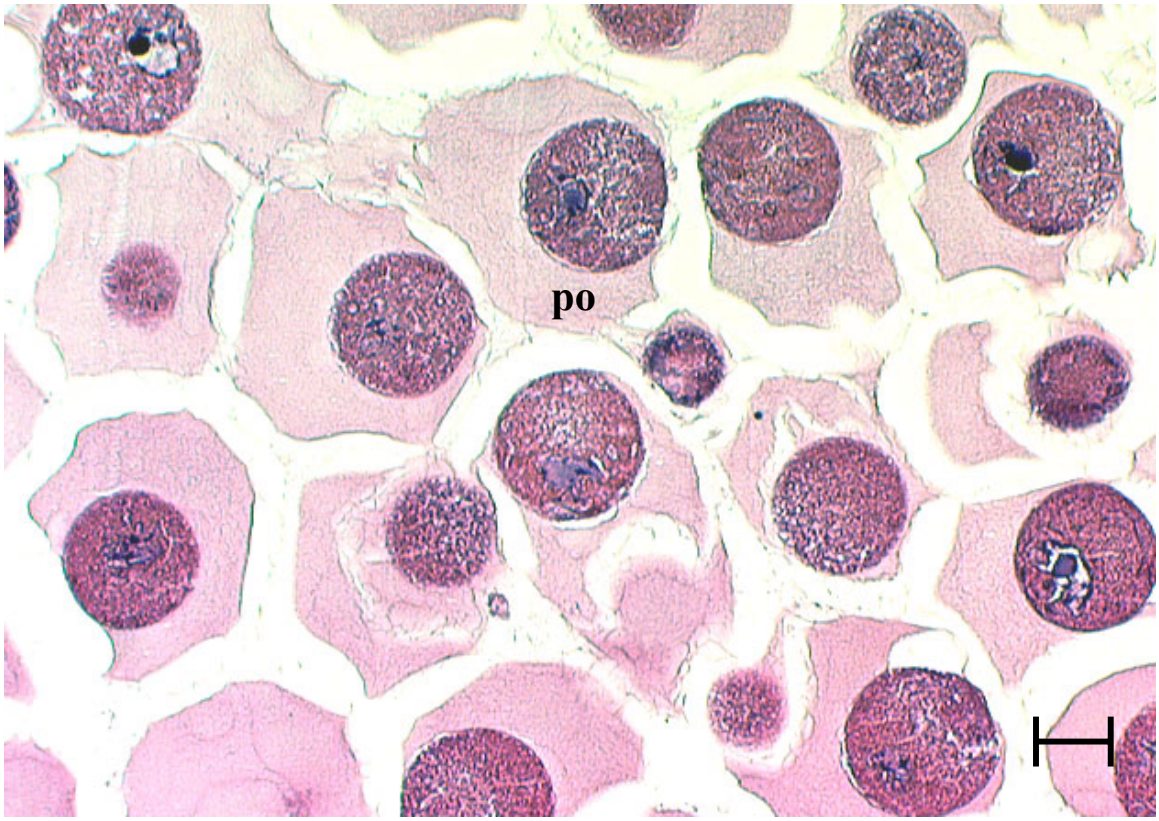
Photograph 2.4. Example of GDI Stage 2 (late active) in female *Villosa iris*. Note acinus lumen (l) contains free primary oocytes (po) and acinar wall (aw) contains developing oocytes (do). Image captured at 1540X. Bar equals 5 µm.



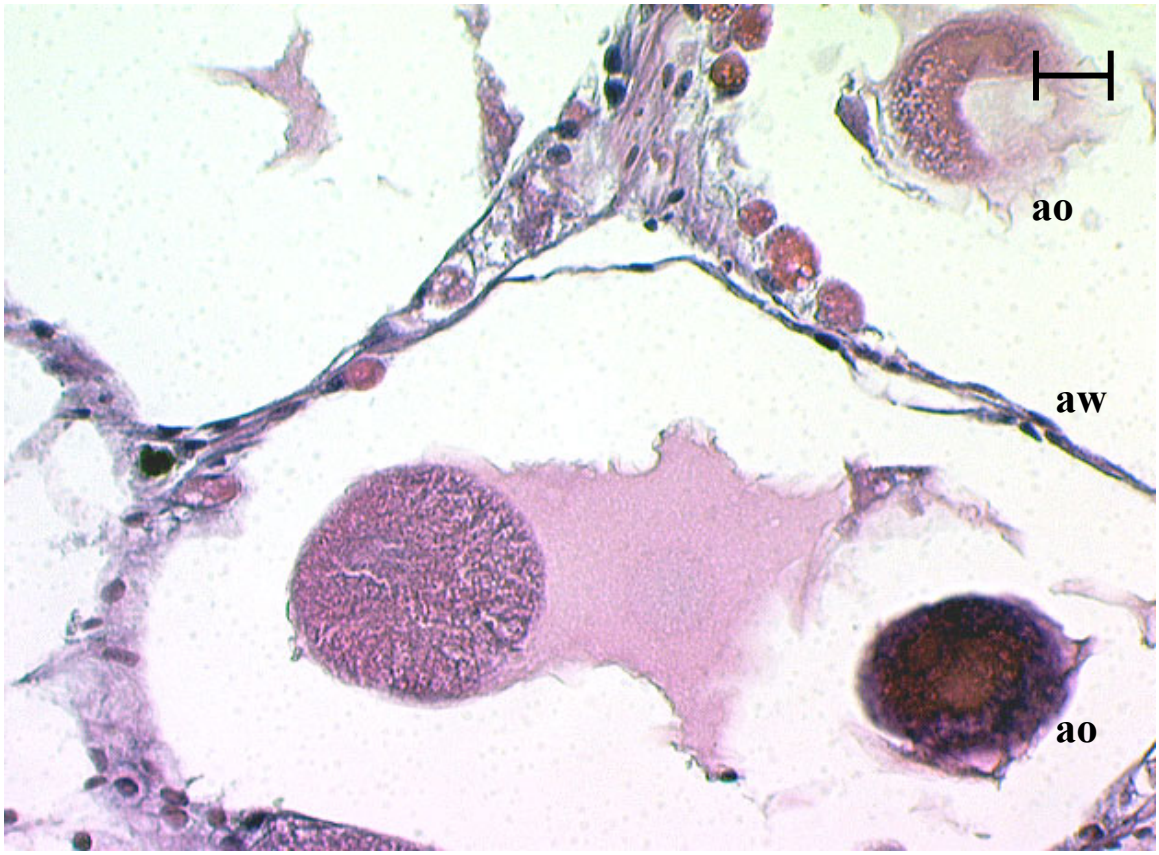
Photograph 2.5. Example of GDI Stage 5 (resorption) in female *Villosa iris*. Note resorbing oocytes (ro) and acinar walls (aw) that have significantly weakened. Image captured at 390X. Bar equals 30 µm.



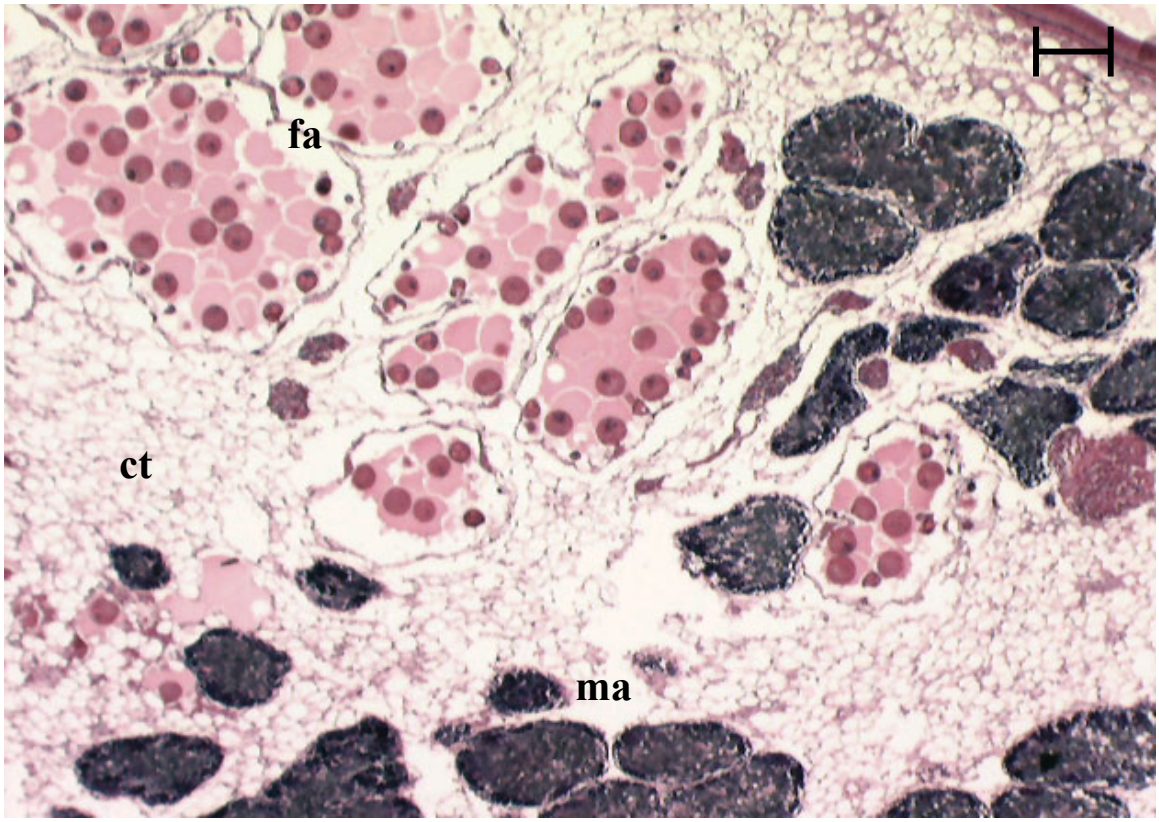
Photograph 2.6. Example of GDI Stage 2 (late active) in male *Utterbackia imbecillis*. Note acinus is filled with developing gametes and some mature spermatozoa (s). Image captured at 1540X. Bar equals 5 µm.



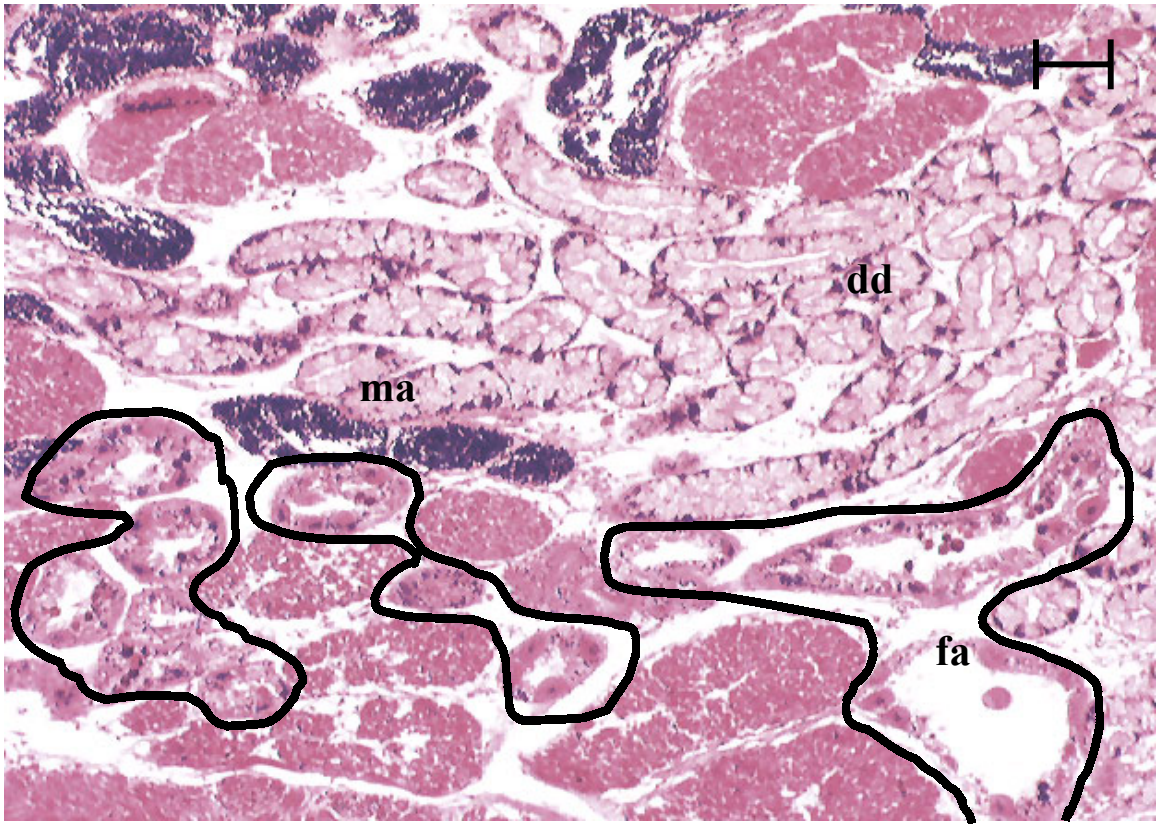
Photograph 2.7. Example of GDI Stage 3 (mature) in female *Utterbackia imbecillis*. Note lumen of acinus is filled with mature primary oocytes (po). Image captured at 390X. Bar equals 30 µm.



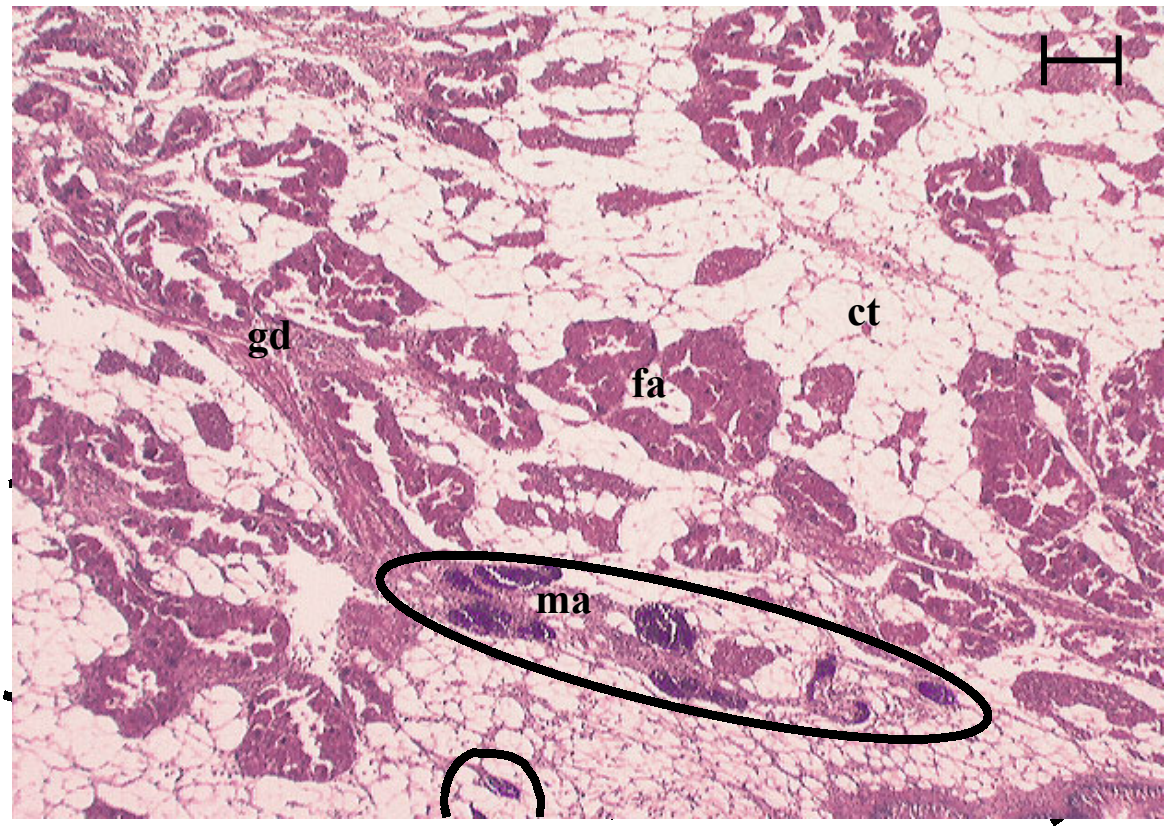
Photograph 2.8. Example of GDI Stage 4 (spawned) in female *Utterbackia imbecillis*. Note acinar wall (aw) has significantly thinned and weakened, and presence of atretic oocytes (ao). Image captured at 870X. Bar equals 15 µm.



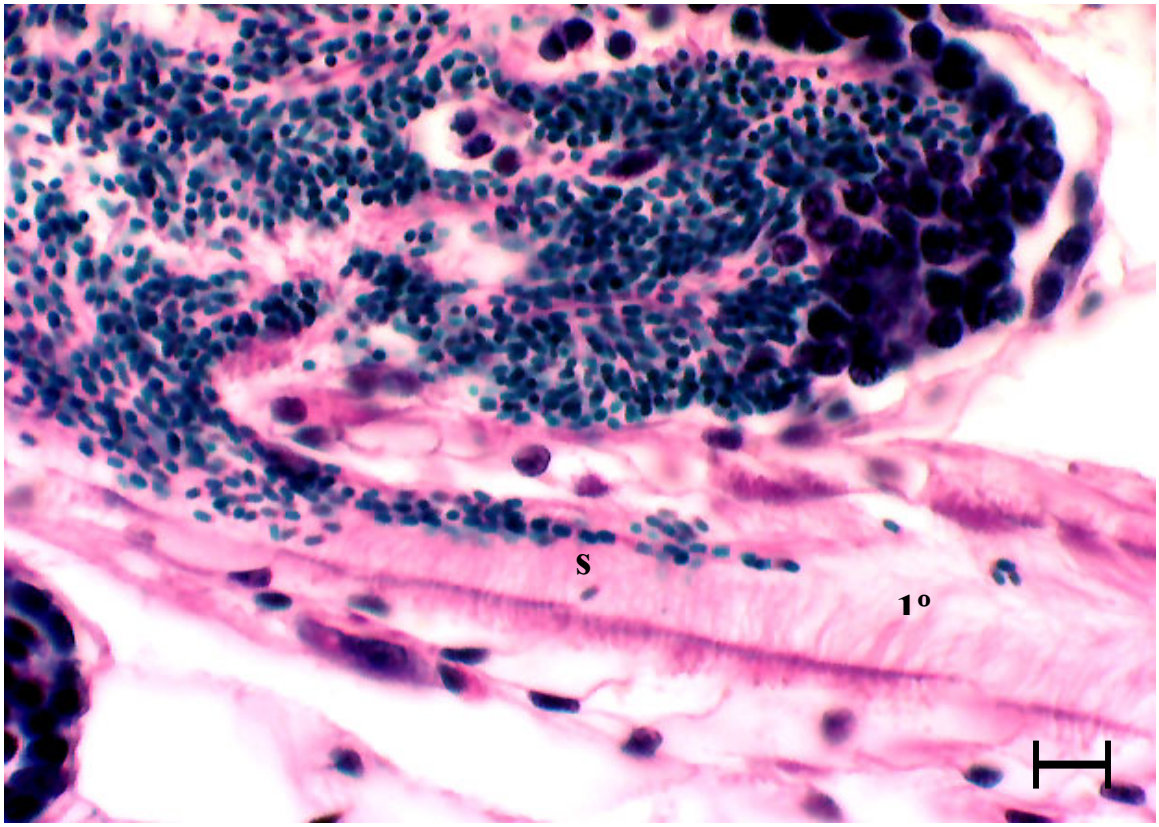
Photograph 2.9. Occurrence of hermaphroditic tissues in *Utterbackia imbecillis*. Note male acini (ma) in GDI stage 2 (late active), female acini (fa) in stage 3 (mature) and connective tissue (ct). Image captured at 154X. Bar equals 60 μm.



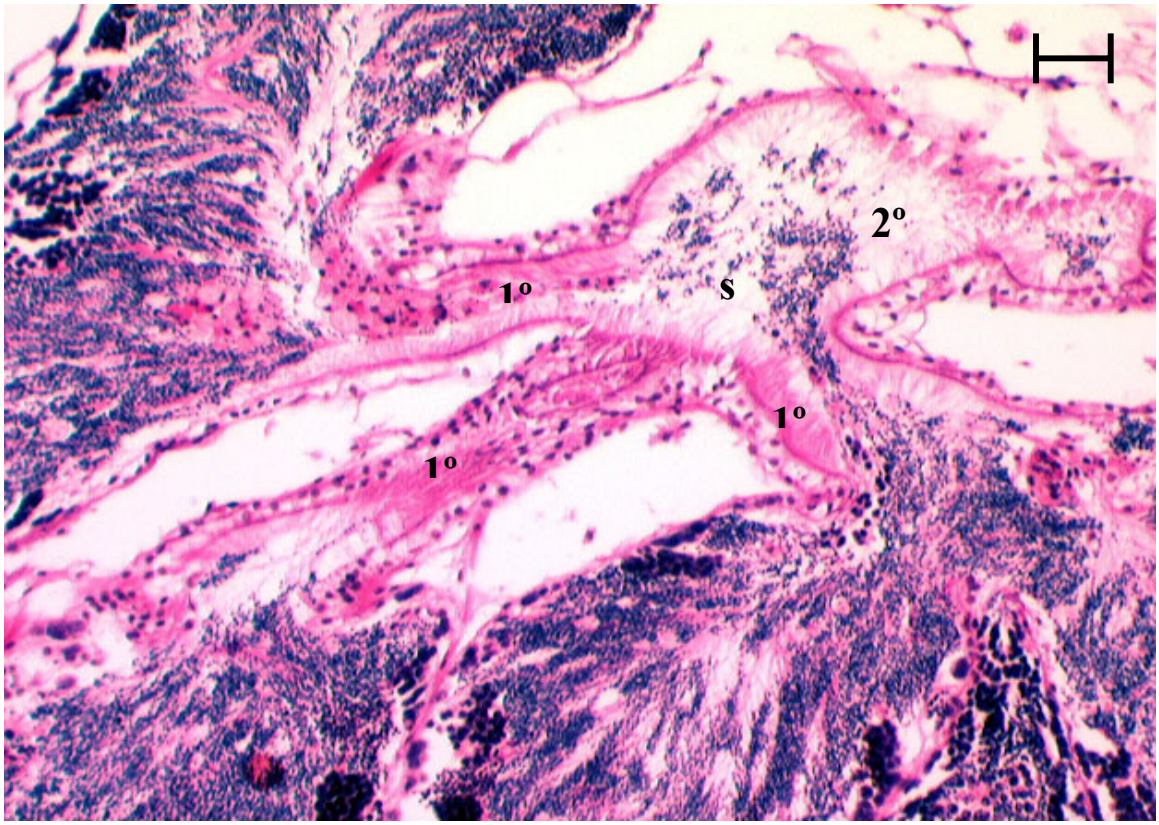
Photograph 2.10. Occurrence of oogenesis in male hermaphrodite of *Villosa iris*. Female acini (fa) outlined. Male acini (ma) are dark blue. Note digestive diverticula (dd). Image captured at 154X. Bar equals 60 μm.



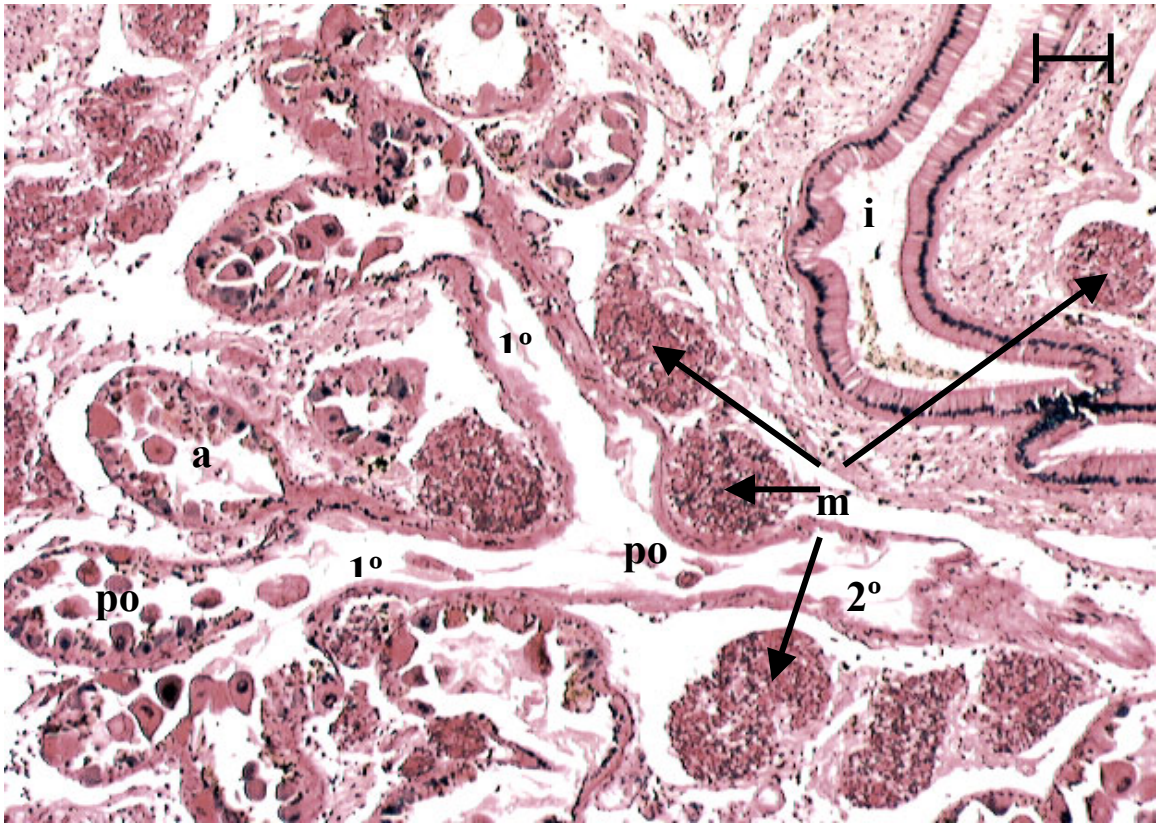
Photograph 2.11. Occurrence of spermatogenesis in female hermaphrodite of *Villosa iris*. Male acini (ma) outlined. Note female acini (fa), connective tissue (ct), and gonoduct (gd). Image captured at 72X. Bar equals 120 µm.



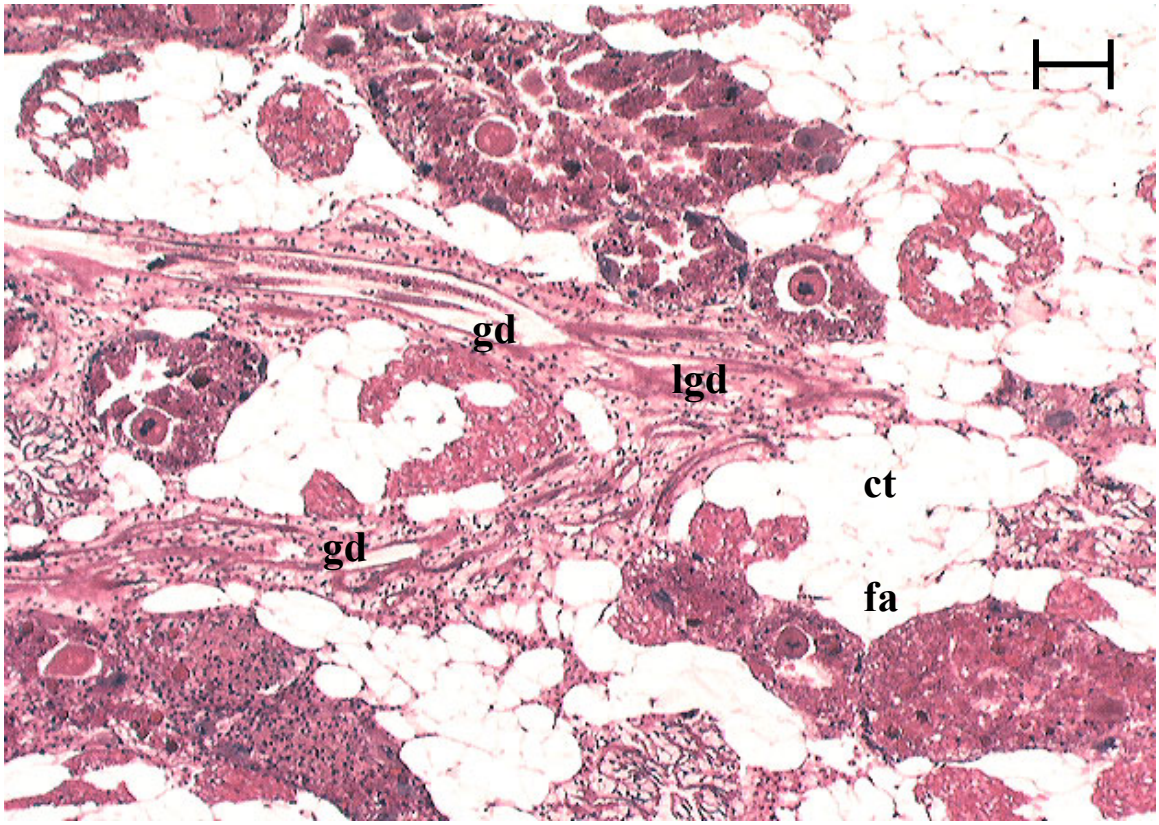
Photograph 2.12. Connection of primary gonoduct to acinus in male specimen of *Utterbackia imbecillis*. Note sperm in ciliated primary gonoduct (1º) containing spermatozoa (s). Image captured at 1540X. Bar equals 5 µm .



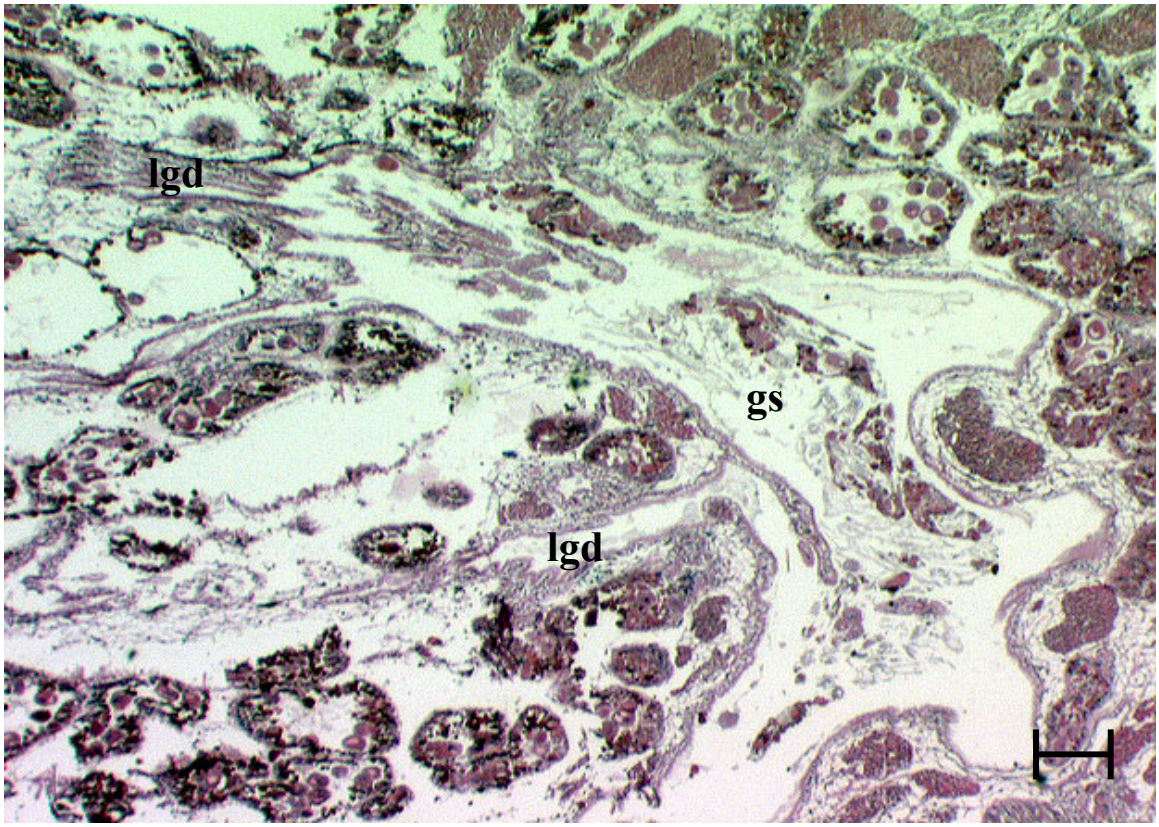
Photograph 2.13. Connection of primary gonoducts (1º) from acini to secondary gonoduct (2º) in specimen of *Utterbackia imbecillis*. Note spermatozoa (s) in ciliated gonoducts. Image captured at 870X. Bar equals 15 µm.



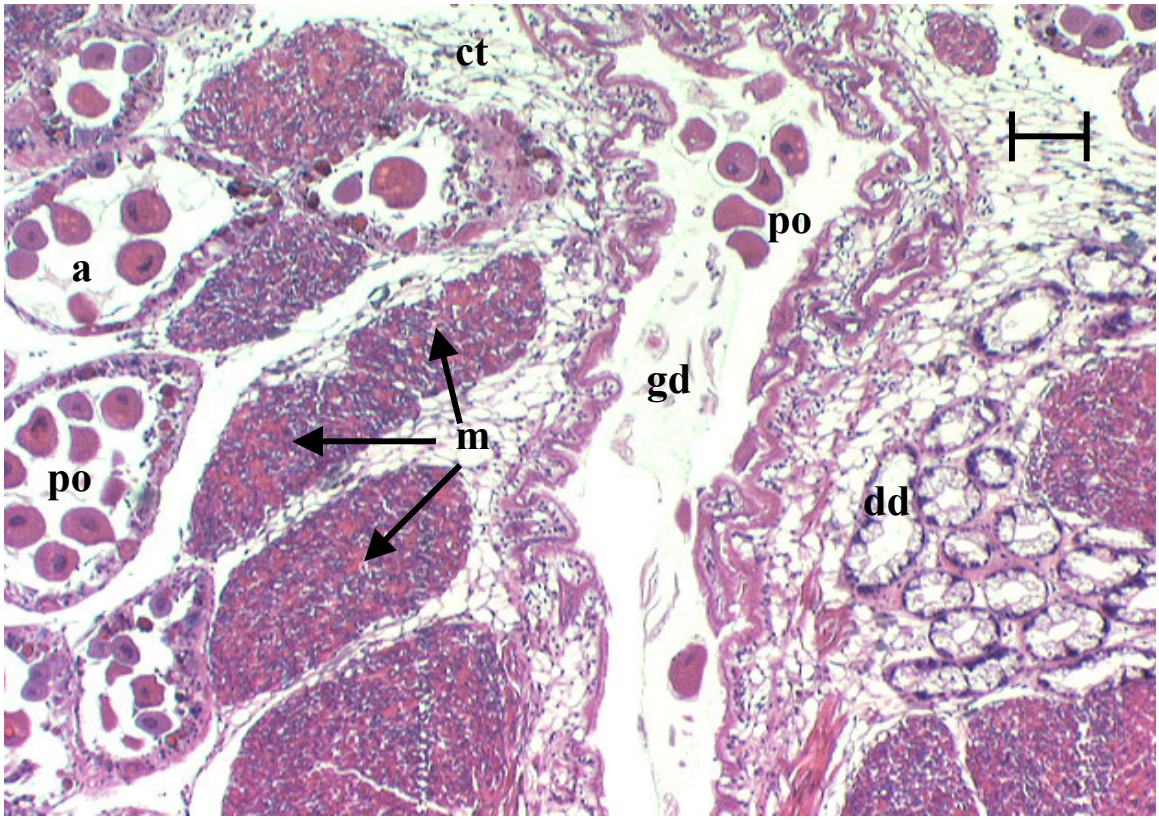
Photograph 2.14. Confluence of primary gonoducts (1º) from acini to secondary gonoduct (2º) in female specimen of *Villosa iris*. Note primary oocytes (po) in acini (a), muscle (m), and intestine (i). Image captured at 154X. Bar equals 60 µm.



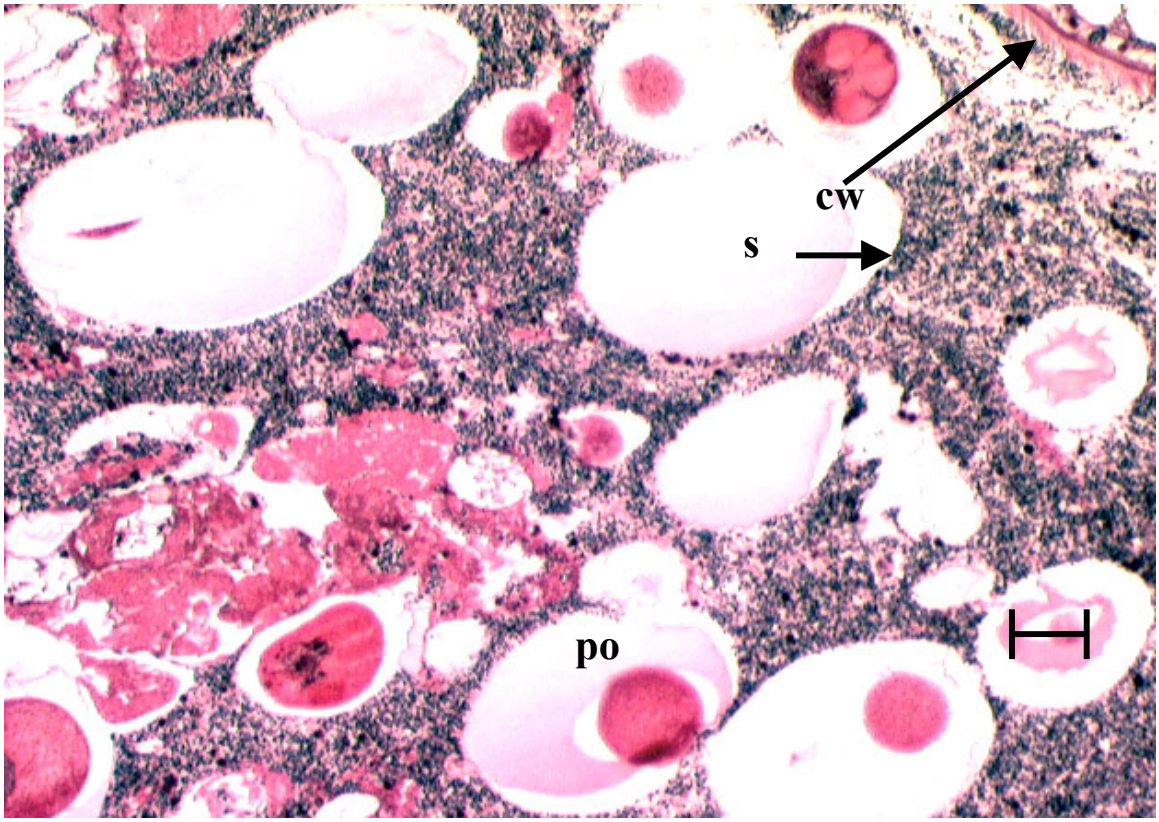
Photograph 2.15. Confluence of gonoducts (gd) to larger gonoduct (lgd) in female specimen of *Villosa iris*. Note female acini (fa) and connective tissue (ct). Image captured at 154X. Bar equals 60 μm.



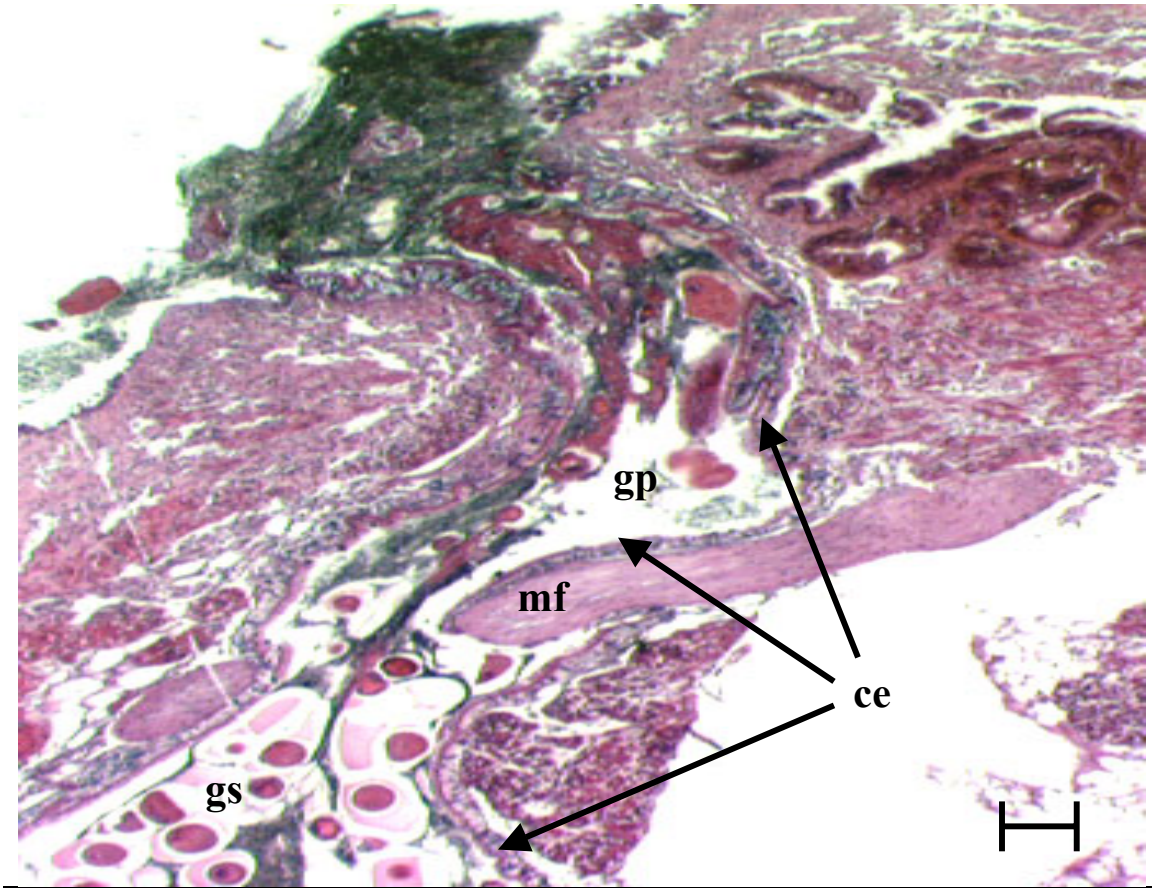
Photograph 2.16. Termination of large gonoducts (lgd) into the gonosinus (gs) in female specimen of *Villosa iris*. Image captured at 72X. Bar equals 120 µm.



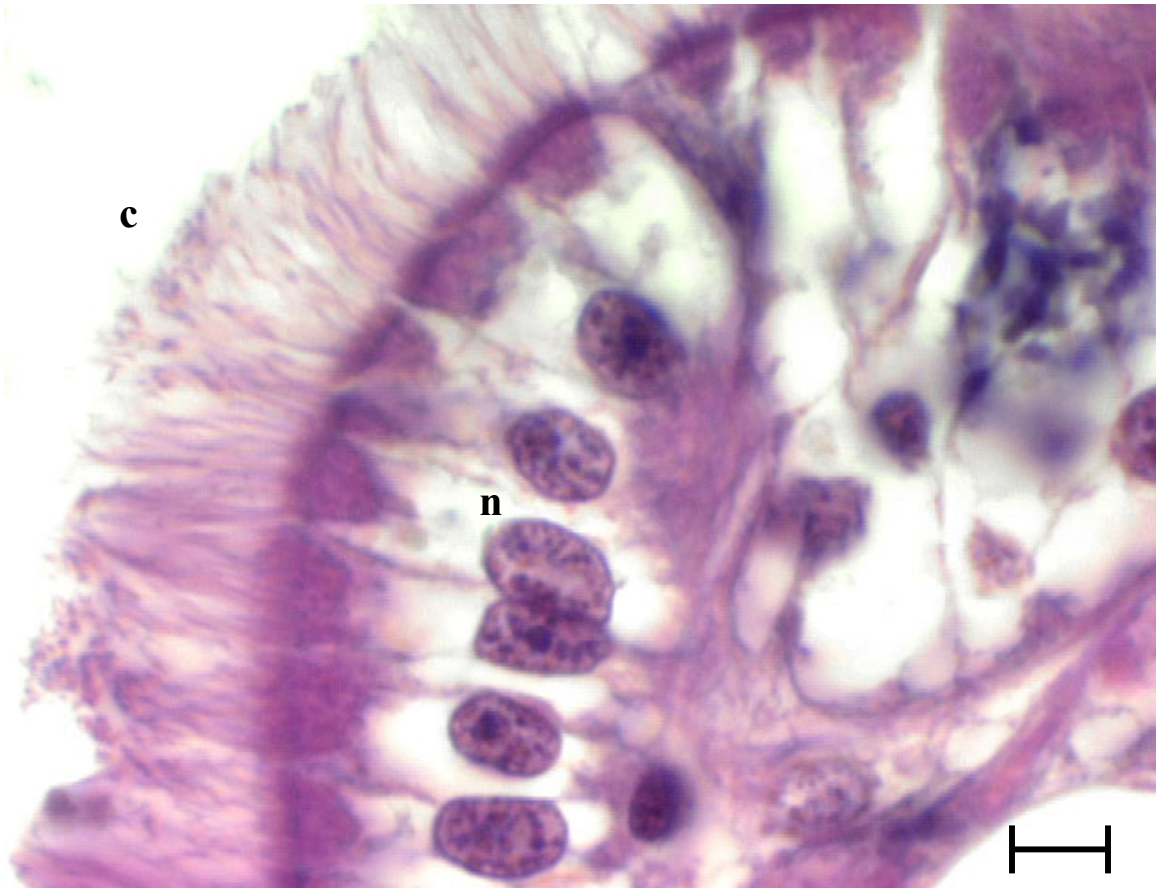
Photograph 2.17. Enlargement of gonoduct (gd) in female specimen of *Villosa iris*. Note primary oocytes (po) in gonoduct and acini (a), connective tissue (ct), muscle (m), and digestive diverticula (dd). Image captured at 154X. Bar equals 60 μm.



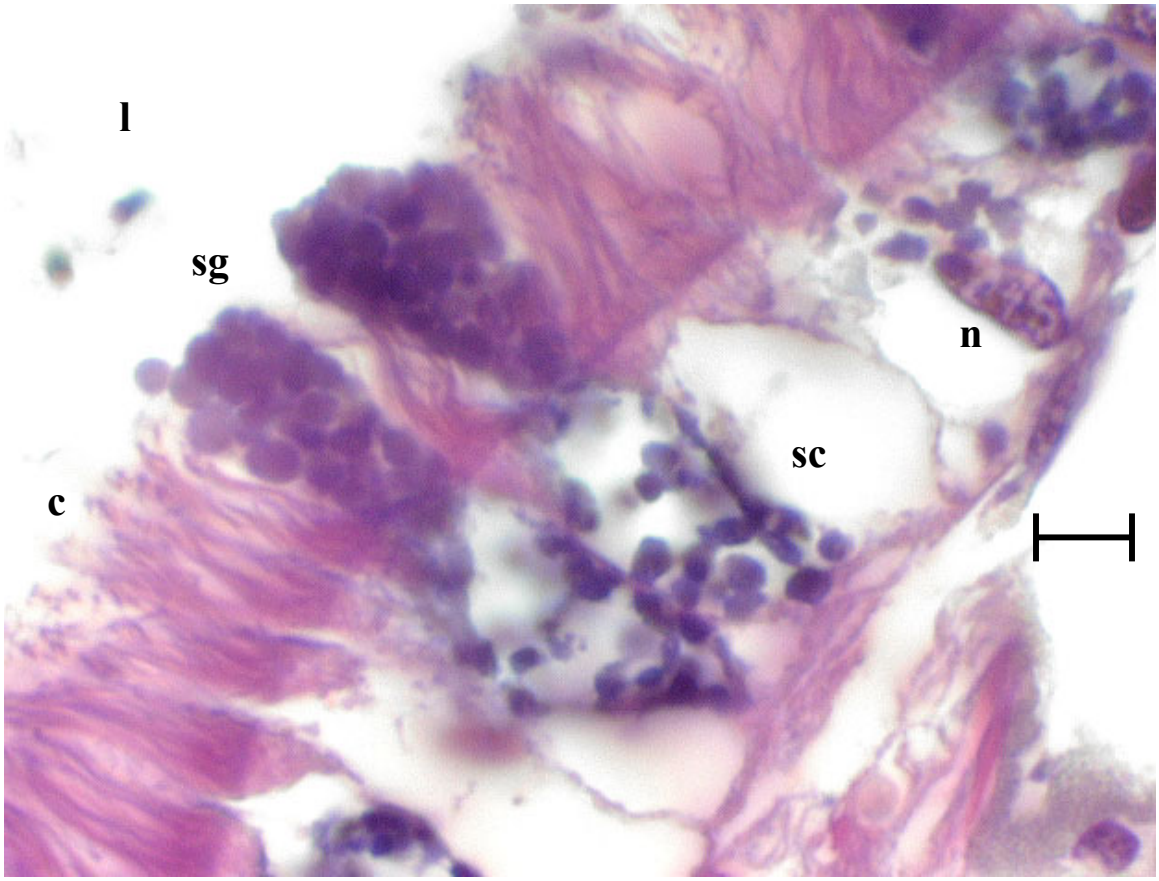
Photograph 2.18. Presence of spermatozoa (s) and mature primary oocytes (po) in gonosinus of *Utterbackia imbecillis*. Note ciliated wall (cw) of the gonosinus in top right corner of photograph. Image captured at 390X. Bar equals 30 μm.



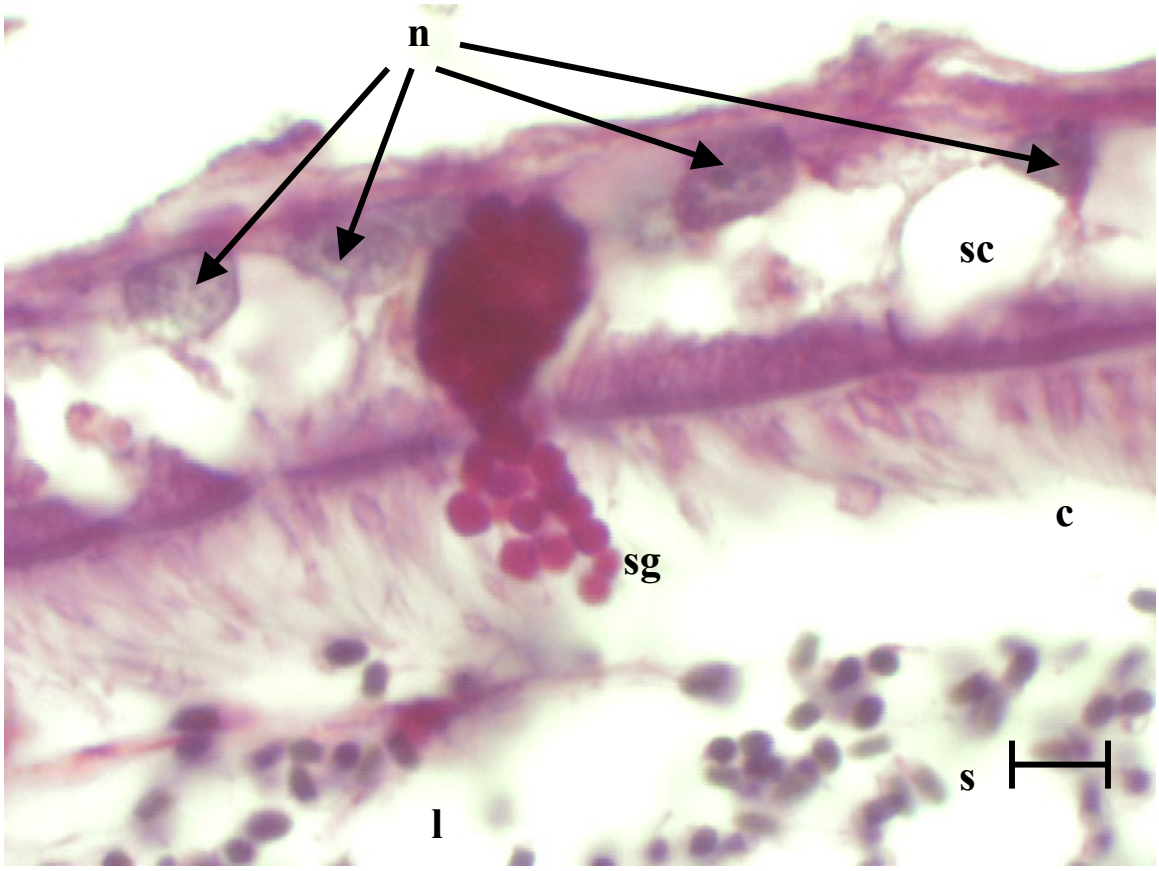
Photograph 2.19. Anatomical communication of gonosinus (gs) with gonopore (gp) in *Utterbackia imbecillis*. Note ciliated epithelium (ce) of gonosinus, gonopore, and muscular flap (mf) of gonopore. Image captured at 154X. Bar equals 60 μm.



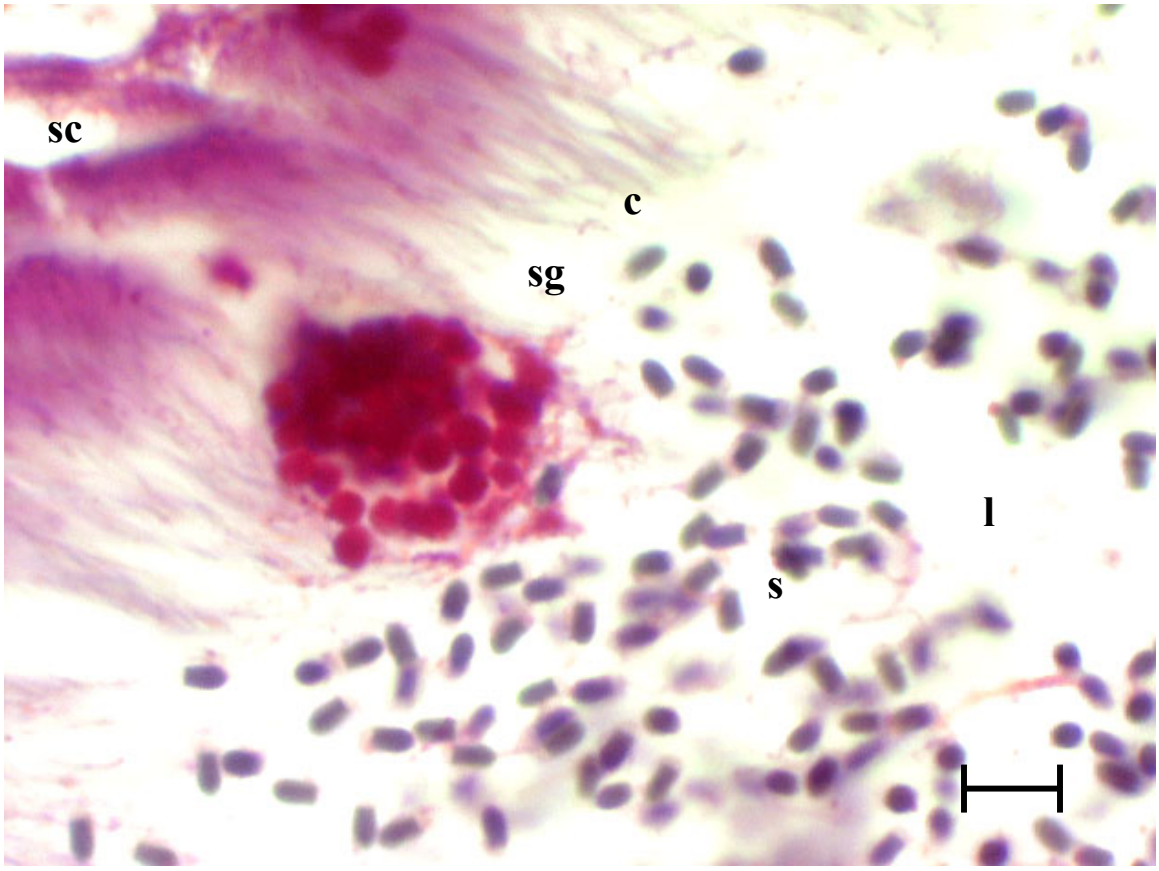
Photograph 2.20. Simple columnar ciliated epithelial cells of the gonosinus lining in *Utterbackia imbecillis*. Note cilia (n) and nuclei (n). Image captured at 3800X. Bar equals 3 μm.



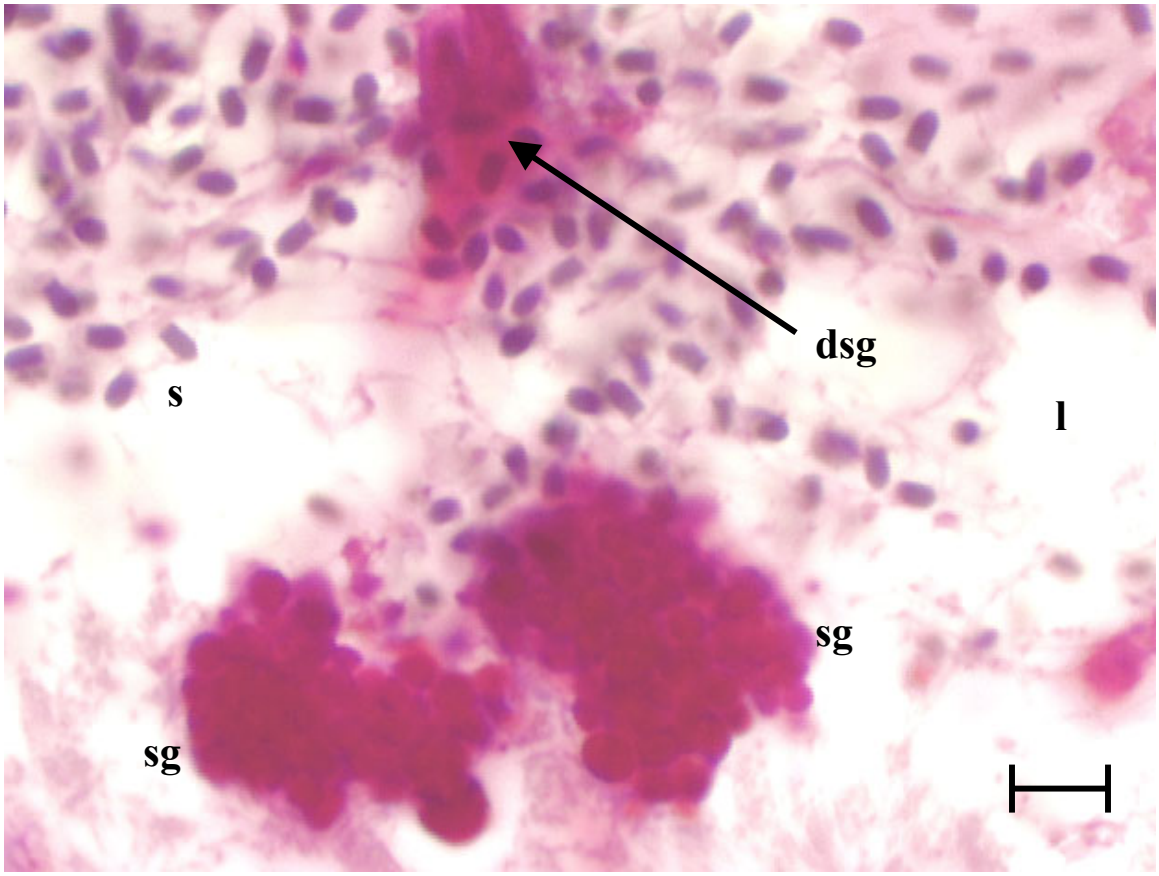
Photograph 2.21. Secretory cells (sc) of ciliated gonopore epithelium in *Utterbackia imbecillis*. Secretory granules (sg) discharged to lumen (l) of gonosinus. Note cilia (c) and nucleus (n). Image captured at 3800X. Bar equals 3 μm.



Photograph 2.22. Secretion of secretory granules (sg) from secretory cells (sc) to gonosinus lumen (l) of *Utterbackia imbecillis*. Note cilia (c), nucleus (n), and spermatozoa (s). Tissue stained with periodic acid/Schiff (PAS), and secretory granules stained positive for glycoprotein. Image captured at 3800X. Bar equals 3 µm.



Photograph 2.23. Secretory granules (sg) in lumen (l) of gonosinus of *Utterbackia imbecillis*. Note cilia (c), secretory cell (sc), and spermatozoa (s). Tissue stained with periodic acid/Schiff (PAS), and secretory granules stained positive for glycoprotein. Image captured at 3800X. Bar equals 3 µm.



Photograph 2.24. Secretory granules (sg) and dissolving granules (dsg) in gonosinus lumen (l) of *Utterbackia imbecillis*. Note spermatozoa (s). Tissue stained with periodic acid/Schiff (PAS), and secretory granules stained positive for glycoprotein. Image captured at 3800X. Bar equals 3 μm.

VITA

William F. Henley was born in Cherry Point, NC on July 13, 1952, and thereafter lived in many places. He completed high school in 1971 at Brookfield East High School, Brookfield, Wisconsin. He earned an undergraduate degree in Psychology in 1976 from Stephen F. Austin State University, Nacogdoches, Texas, and M. A. in Communication from the same university in 1978. After many years of work in home construction and remodeling, the realization that he could waste his life scared him greatly; therefore, he started graduate work in fisheries. He is married to Kathy Henley (Nurse Practitioner), and has one son, McKeever (11 years old).

In August of 1993, he was accepted as a graduate student at Virginia Polytechnic Institute and State University. In July of 1996, he completed the requirements for the Master of Science in Fisheries. In April 2002, he completed his Ph.D. with the same department.

His research expertise is the evaluation of survey technique used with aquatic invertebrates, and captive breeding of endangered fish and aquatic invertebrates. He hopes to teach and conduct research within a university setting.