

## Gonadal Analysis

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### ABSTRACT

This document describes the procedures for determining the reproductive stage of oysters, mytilid mussels, and dreissenid mussels collected for NOAA's National Status and Trends Mussel Watch Program. Analyses are conducted on paraffin-embedded tissues sectioned at a 5- $\mu$ m thickness and stained using a pentachrome staining procedure. Each slide is examined microscopically to determine the animal's sex and stage of gonadal development. A semi-quantitative ranking is assigned.

### 1. INTRODUCTION

Assessment of the physiological state of bivalve populations requires an analysis of the state of gonadal development. Determination of reproductive stage is included as part of the Mussel Watch Project to give an indication of the amount of gametic material in bivalve tissues at the time of chemical analysis. Certain contaminants are preferentially concentrated in gonadal tissue (Ellis *et al.*, 1993; Lee, 1993; Abbe *et al.*, 1994). Others are concentrated in non-gonadal tissue (Cunningham and Tripp 1975; Mo and Neilson, 1993). Because gametic material can account for 20% to 50% of body weight in target species of oysters and mussels (Sprung, 1991; Choi *et al.*, 1993), the relative proportion of gonadal to somatic tissue and the timing of spawns (an important depuration route for some contaminants) can significantly impact the body burden of contaminants.

This description updates the methods presented in Ellis *et al.* (1998) and Powell *et al.* (1993). The original intent of the determination of reproductive stage was to assure that sampling was conducted at the same stage of the reproductive cycle so that analyses of the lipophilic organic contaminants and the trace element contaminants were not influenced by reproductive state. Unfortunately, the time required for sampling and the wide latitudinal range encompassed by the sites did not permit consistent recovery of individuals in similar stages of reproductive development at all sites. For example, typically oysters are undifferentiated in the winter. Gonads begin to develop in early spring and spawning occurs late spring through early fall. Most Gulf Coast oysters spawn at least twice during this time period. Single spawns tend to occur in the shorter summers of the mid-Atlantic region (e.g., Dittman *et al.*, 2001). The timing of the last spawn varies with latitude and with yearly variations in climate (e.g., Wilson *et al.*, 1990, 1992). Southeast Atlantic and southern Gulf sites, for example, routinely yield oysters in reproductive development or that are ready to spawn in mid-winter during the period when Mussel Watch sampling occurs. Mid-Atlantic sites are typically characterized by individuals in an undifferentiated state and thus contain significantly less lipid-rich gametic tissue than the southern animals. Mytilid mussels and dreissenid mussels have the same assortment of problems relating to latitude and interannual changes in climate (Newell, 1989; Seed and Suchanek, 1992; Borcherding, 1991). In addition, dreissenid mussels are typically collected during late August-September whereas the remaining Mussel Watch species are collected during winter. Thus, analysis of reproductive stage has proved important in identifying differences in tissue composition that might affect comparisons of contaminant data among sites and among years. Wilson *et al.* (1990, 1992) and Kim *et al.* (1999, 2001) discuss the influence of climate on reproductive stage and contaminant body burden in the Mussel Watch Program in more detail.

Oyster gonadal tissue is distributed around the body mass (Morales-Alamo and Mann, 1989). Gonads of dreissenid mussels also develop within the body, around the periphery of the viscera. In contrast to oysters and dreissenid mussels, gonadal follicles develop primarily within the mantle of mytilid mussels. Nevertheless, in none of these cases can the gonad be easily excised and weighed. Consequently, virtually all assays of reproductive stage use histological methods to recognize the changes in the germinal epithelium and germinal products that identify stages in gonadal development. The histological approach uses a semiquantitative numerical assignment to rank reproductive stage. Quantitative measures, such as egg protein content (Choi and Powell, 1993, Choi *et al.*, 1993, 1994), remain expensive and time consuming, and do not permit a concomitant histopathological analysis. Therefore, a histological examination is still the single method of choice when only one method can be used.

For oysters, a dorsal-ventral slice of tissue is taken and fixed in Davidson's fixative. Dreissenid and mytilid mussels are preserved whole, and a dorsal-ventral slice is taken after fixation. Tissue slices are embedded in paraffin,

sectioned, and stained using a pentachrome staining protocol. Stained sections are examined under a compound microscope, and sex and the state of gonadal development determined. Fixation follows the method described in Preece (1972). The staining procedure is an adaptation of Masson's (1928) trichrome procedure (Ellis *et al.*, 1998). Reproductive stage in oysters is determined using a semiquantitative scale adapted from Ford and Figueras (1988). The scale developed by Seed (1975, 1976) for determining gonad index in mussels was adopted for mytilid mussels and dreissenid mussels.

## 2. EQUIPMENT, REAGENTS, SOLUTIONS, AND SAMPLE PREPARATION

### 2.1. Equipment

Cover slips - various sizes. Fisher Scientific, Pittsburgh, PA.  
Disposable cassettes - HistoPrep tissue capsules, 15-182-218. Fisher Scientific, Pittsburgh, PA.  
Disposable microtome blades, 12-634-11. Fisher Scientific, Pittsburgh, PA.  
Drying oven, 13-254-1. Fisher Scientific, Pittsburgh, PA.  
Embedding rings - HistoPrep embedding rings, 12-652-1OB. Fisher Scientific, Pittsburgh, PA.  
Frosted microslides, 12-552. Fisher Scientific, Pittsburgh, PA.  
Microscope, compound, Zeiss, 12-070-20. Fisher Scientific, Pittsburgh, PA.  
Microtome, HM330. Microm GmbH. D-6900 Heidelberg, Germany.  
Slide staining set, Tissue-Tek II. Miles Laboratories, Inc. Westmont, IL.  
Slide staining holder, Tissue-Tek, 4466. Miles Laboratories, Inc. Westmont, IL.  
Stainless steel molds, various sizes. Fisher Scientific, Pittsburgh, PA.  
Tissue embedding system, HistoCentre 2, 6400012. Shandon, Inc., Pittsburgh, PA.  
Tissue processor, Citadel 2000, 69800006. Shandon, Inc., Pittsburgh, PA.  
Vacuum infiltrator, Tissue-Tek II, 4613. Miles Laboratories, Inc. Westmont, IL.  
Water bath - Fisher Tissue PrepModel 134 Flotation Bath, 15-464. Fisher Scientific, Pittsburgh, PA.

### 2.2. Reagents

Acetone HistoPrep ( $\text{CH}_3\text{COCH}_3$ ), [67-64-1], HC300-1GAL. Fisher Scientific, Pittsburgh, PA.  
Acid fuchsin, certified stain ( $\text{C}_{20}\text{H}_{17}\text{N}_3\text{O}_9\text{S}_3\text{Ca}$ ) [136132-76-8], A3908. Sigma Chemical Co., St. Louis, MO.  
Ammonium hydroxide ( $\text{NH}_4\text{OH}$ ) [1336-21-6], A6899. Sigma Chemical Co., St. Louis, MO.  
Aniline blue powder, certified stain [28631-66-5], A967-25. Fisher Scientific, Pittsburgh, PA.  
Chromotrope powder 2R, ( $\text{C}_{16}\text{H}_{10}\text{N}_2\text{Na}_2\text{O}_8\text{S}_2$ ) [4197-07-3], C3143. Sigma Chemical Co., St. Louis, MO.  
Ethanol ( $\text{C}_2\text{H}_5\text{OH}$ ) [64-17-5], R8382. Sigma Chemical Co., St. Louis, MO.  
Fast green FCF, certified stain ( $\text{C}_{37}\text{H}_{34}\text{N}_2\text{O}_{10}\text{S}_3\text{Na}_2$ ) [2353-45-9], F7252. Sigma Chemical Co., St. Louis, MO.  
Ferric ammonium sulfate ( $\text{Fe NH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ ) [7783-83-7], F1018. Sigma Chemical Co., St. Louis, MO.  
Formaldehyde, 37% solution ( $\text{CH}_2\text{O}$ ) [50-00-0], F1635. Sigma Chemical Co., St. Louis, MO.  
Glacial acetic acid ( $\text{C}_2\text{H}_4\text{O}_2$ ) [64-19-7], A0808. Sigma Chemical Co., St. Louis, MO.  
Glycerin ( $\text{C}_3\text{H}_8\text{O}_3$ ) [56-81-5], G7893. Sigma Chemical Co., St. Louis, MO.  
Hematoxylin powder, certified stain ( $\text{C}_{16}\text{H}_{14}\text{O}_6$ ) [517-28-2], H3136. Sigma Chemical Co., St. Louis, MO.  
Orange G powder, certified stain ( $\text{C}_{16}\text{H}_{10}\text{N}_2\text{O}_7\text{S}_2\text{Na}_2$ ) [1936-15-8], O7252. Sigma Chemical Co., St. Louis, MO.  
Paraffin - Paraplast tissue embedding media (melting pt. 56 °C), 12-646-111, Fisher Scientific, Pittsburgh, PA.  
Permout mounting media, SP15-500. Fisher Scientific, Pittsburgh, PA.  
Phosphomolybdic acid ( $20\text{MoO}_3 \cdot 2\text{H}_3\text{PO}_4 \cdot 48\text{H}_2\text{O}$ ) [51429-74-4], P7390. Sigma Chemical Co., St. Louis, MO.  
Phosphotungstic acid ( $12\text{WO}_3 \cdot \text{H}_3\text{PO}_4 \cdot \text{H}_2\text{O}$ ) [12501-23-4], P4006. Sigma Chemical Co., St. Louis, MO.  
Sodium chloride ( $\text{NaCl}$ ) [7647-14-5], S9625. Sigma Chemical Co., St. Louis, MO.  
Sulfuric acid ( $\text{H}_2\text{SO}_4$ ) [7664-93-9], S1526. Sigma Chemical Co., St. Louis, MO.  
Tissue Clear III, SH3-4. Fisher Scientific, Pittsburgh, PA.  
Tissue Dry, SH5-20. Fisher Scientific, Pittsburgh, PA.  
Xylene, histological grade ( $\text{C}_6\text{H}_4(\text{CH}_3)_2$ ), X3S-4. Fisher Scientific, Pittsburgh, PA.

### 2.3. Solutions

Ferric alum mordant: 25 g ferric ammonium sulfate dissolved in 500 mL distilled water.

Basic ethanol: 26 mL ammonium hydroxide in a solution of 3370 mL 95% ethanol and 630 mL distilled water.

Phosphomolybdic acid solution: 5 g phosphomolybdic acid crystals dissolved in 495 mL distilled water.

1% acetic acid: 20 mL glacial acetic acid in 1980 mL distilled water.

1% acid acetone: 20 mL glacial acetic acid in 1980 mL acetone

Groat/Weigert hematoxylin working solution: 245 mL distilled water, 5 mL sulfuric acid, 5 g ferric ammonium sulfate, 245 mL 95% ethanol, and 2.5 g hematoxylin powder.

Acid fuchsin working solution: 1.5 g acid fuchsin powder dissolved in 495 mL distilled water, to which is added 5 mL glacial acetic acid.

Phosphotungstic acid solution: 10 g phosphotungstic acid crystals dissolved in 490 mL distilled water.

Orange G/Chromotrope: 4 g orange G powder and 1 g chromotrope powder dissolved in 495 mL distilled water to which was added 5 mL glacial acetic acid.

Fast green/Aniline blue working solution: 5 g fast green FCF, 4 g aniline blue powder dissolved in 495 mL distilled water to which was added 5 mL glacial acetic acid.

Davidson's fixative solution: 1 part glycerin, 1 part glacial acetic acid, 2 parts 37% formaldehyde, 3 parts 95% ethanol, and 3 parts isotonic sodium chloride (usually 20 - 30‰).

### 3. SAMPLE COLLECTION AND FIXATION

#### 3.1. Sampling

From 1986 to 1994, the same oysters were used for organic contaminant analysis and gonadal analysis at all Gulf coast sites (e.g., Powell *et al.*, 1993). The use of the same animals for gonadal analysis and for analysis of contaminant body burden potentially biases the latter analyses because digestive gland tissue and gonadal tissue, that contribute disproportionately to the tissue taken for histological analysis, may contain a higher than average body burden of certain contaminants. Sericano *et al.* (1993) showed that this source of error resulted in an underestimation of true body burden no greater than 10% when a 5-mm slice was removed from the large oysters normally sampled in the Mussel Watch Program. This potential error would be much larger for the smaller mytilids and dreissenids. To avoid this error, separate samples have always been obtained for gonadal analysis of mussels and East Coast oysters. Beginning in 1995, the same protocol was adopted for Gulf Coast oysters. The number of animals analyzed per site has also changed. Through 1994, 15 animals were analyzed per site. Thereafter, five were analyzed per site. Thus, the present sampling method requires the assumption that individuals collected from a common collection area will have experienced similar chemical loading and that the five animals analyzed for gonadal analysis are representative of the animals pooled for contaminant analysis.

#### 3.2. Tissue preparation

##### 3.2.1. Oyster tissue preparation

Five of the 12 oysters processed for Dermo analysis (Ashton-Alcox *et al.*, this volume) are chosen randomly for gonadal analysis. A 5-mm thick cross-section of tissue is removed from the oyster using a scalpel or scissors. The determination of reproductive stage is based on a histological evaluation of the maturation stage of oyster gonads located within/around the visceral mass. The tissue section is obtained such that the dorsal-ventral aspect passes through the digestive gland and gill tissue just posterior to the palps (Figure 1 in Ellis *et al.*, 1998). This aspect provides example sections of most oyster tissues for histopathological analysis (Kim *et al.*, this volume), while also providing a representative cross-section of oyster gonad. Each section is immediately placed in a tissue cassette and the cassette placed in a jar filled with Davidson's fixative for 48 hr. After 48 hr, the fixative is decanted, 70% ethanol added and the tissues are allowed to stand until processing.

##### 3.2.2. Mytilid mussel tissue preparation

Determination of reproductive stage for mussels is based on a histological evaluation of the maturation stages of mussel gonads, most of which are located in the mantle (Newell, 1989). Five animals are analyzed but a few more are preserved for reasons discussed subsequently. The tip of a sharp knife is carefully inserted between the shells at

the ventral lip and run dorsally between the shells until the posterior adductor muscle is cut so that the shells remain in an open position. Care is taken to cut no further than the adductor muscle to avoid cutting into the digestive gland immediately below the adductor muscle. Shucking of fresh mussels usually results in severe damage to the mantle tissue lying next to the shell. Therefore the mussels are placed whole in a wide-mouth jar filled with Davidson's fixative after the adductor muscle has been cut. Because the entire animal is being preserved, the specimens are left in fixative for at least a week to ensure preservation of all tissues. After this time, the fixative is decanted and 70% ethanol is added for storage until processing.

Once preserved, the tissue hardens and becomes easier to detach from the shell. To excise the preserved mussel meat from its shell, a knife is carefully run between the mantle and the lip of each valve, detaching the mantle from the shell. At this time, byssal threads are completely removed from the byssal gland to avoid later difficulties in tissue sectioning. Five specimens are chosen from each site and their anterior-posterior lengths are measured using a ruler. A 5-mm thick cross-section is then removed using a scalpel. The cross-section is obtained such that the dorsal-ventral aspect passes through the digestive gland and gills at an angle across the body and such that ventral edge of the cross-section is slightly towards the posterior-ventral margin. Each cross-section is placed in a tissue cassette and processed immediately after dissection. If the mantle tissue is damaged during the shucking procedure, the specimen is replaced by one of the additional specimens preserved from the same site because the wound could result in the loss of gametic material and lead to an erroneous evaluation.

### 3.2.3. Dreissenid mussel tissue preparation

Most of the gonad of a dreissenid mussel is concentrated within the visceral mass (Borcherding, 1990). Due to their small size however, dissection of living tissue without destroying the gonads is difficult. Therefore, dreissenid mussels collected from each site are preserved whole in Davidson's fixative, without cutting the adductor muscle. They are left in fixative for one week to allow adequate time for tissue fixation. After this time, 20 to 30 mL of acetic acid is added to enhance decalcification of the shell. The shell is properly decalcified when it is no longer hard.

After decalcification, the Davidson's fixative is replaced with 70% ethanol according to the procedure followed for mytilid mussels and stored for later embedding. Prior to embedding, byssal threads are cut away from the byssal gland to minimize difficulty in sectioning the tissue. A 5-mm thick cross-section is taken from five individuals as described for mytilid mussels. Each section is placed in a tissue cassette and processed for embedding immediately thereafter.

## 4. SLIDE PREPARATION

### 4.1. Tissue embedding

Individual tissue samples are prepared for embedding in paraffin using an established dehydration protocol (Table 2). The solutions used for dehydration, clearing, and infiltration are changed frequently to maintain solution purity. The tissue embedding sequence uses an automated tissue processor that processes tissue in plastic cassettes through the dehydration-clearing series and into paraffin. Embedding can also be done manually by moving the tissues through the sequence. The paraffin is melted in an embedding center with temperature set at 60°C. Newly melted paraffin should always be used in the final infiltration and embedding steps.

After the tissues are infiltrated with paraffin (Table 1), they are transferred to a vacuum infiltrator set at 60°C and placed under a vacuum for 30 min. Tissues are transferred to a holding tank of melted paraffin and removed singly to stainless steel molds. The tissues are oriented with the cross-sectional face down for sectioning, and a plastic mold embedding ring is placed on top. The ring is filled with paraffin and the mold moved to a cold plate of the embedding system. As the tissue/paraffin cools and hardens, the paraffin shrinks. Care must be taken to use sufficient paraffin to cover the tissue after this shrinkage. The mold is left on the cold plate until the tissue-paraffin block is removed. The block is then placed in a freezer until sectioning.

### 4.2. Tissue sectioning

The paraffin blocks are first cut at 20  $\mu\text{m}$  to expose an entire tissue cross-section and then sliced at 5  $\mu\text{m}$  using a microtome. Tissue sections may be cut singly or into contiguous sections. The sections are placed on the surface of a water bath maintained at 45-50°C and allowed to expand. Once the sections expand to full size, a microscope slide is held at an angle and slid under one or more of the tissue sections. The sections are then lifted out of the water and onto the slide. The sections are positioned on the slide in the orientation in which they will be stained and read. The slide is allowed to air dry until it can be placed in a slide rack. The slide rack is placed in a drying oven at 40°C. After drying overnight or longer, the slides are ready to stain.

Table 1. Tissue embedding sequence\*.

Dehydration		Clearing	
Tissue Dry	60 min	Tissue Clear	60 min
Tissue Dry	120 min	Tissue Clear	120 min
Tissue Dry	120 min	Tissue Clear	120 min
Tissue Dry	120 min		
Tissue Dry	120 min	Infiltration	
Tissue Dry	120 min	paraffin	120 min
Tissue Dry	60 min	paraffin	120 min
		paraffin in vacuum infiltrator	30 min

\* In cases where the sequential solutions are the same, each transfer is a transfer to a fresh solution.

#### 4.3. Tissue staining

Sections are deparaffinized and hydrated using a xylene-ethanol series (Table 2). Following hydration, slides are stained in a pentachrome series, dehydrated in a series of acetic acid dips followed by acetone, cleared in xylene and mounted in Permount (Table 2). The pentachrome staining procedure is an adaptation of the trichrome stain of Masson (1928) as modified by Gurr (1956) (Ellis *et al.*, 1998). The modifications include the addition of aniline blue to the fast green working solution, substitution of chromotrope 2R/orange G for Ponceau de Xylidene, and the addition of phosphotungstic acid prior to the orange G/chromotrope stain; the procedure is now a pentachrome technique. The addition of these stains yields better differentiation of tissue types and mucins. Times required for each step are flexible in both the staining procedures discussed here and in the previous embedding protocol. Different tissue types may require different times. All solutions, especially the xylene and ethanol ones, should be changed frequently. Slides should not be allowed to dry during transfers. Solutions to common embedding, sectioning, and staining problems are discussed in Preece (1972) and most other manuals of histological technique.

#### 5. ANALYSIS

Each slide is examined microscopically to determine sex and stage of gonadal development. A histopathological examination can also be made at this time (Kim *et al.*, this volume). Careful examination of early developmental stages is needed to positively distinguish males or females in early stages of development from individuals as yet undifferentiated. Occasional hermaphrodites will also be found (all target species normally have separate sexes). The stage in the gametogenic cycle is assigned based on the maturity of the follicles and gametes and a numerical value is assigned as described in Tables 3 and 4.

Cases of renewed gonadal development following spawning are common in oysters (stage 7), particularly along the Gulf of Mexico coast (Supan and Wilson, 2001). These animals typically have a few remaining large, mature ova and many developing ova that would normally be found in stages 3 or 4. Accordingly, for oysters, further data reduction can better be achieved by comparing the number of individuals with substantial gonadal development with those having little gonadal volume using an egg/eggless ratio, calculated as

Table 2. Tissue staining sequence.

Deparaffinization	
xylene	5 min
xylene	5 min
xylene	5 min
100% ethanol	3 min
100% ethanol	2 min
Hydration	
95% ethanol	2 min
10% ethanol	2 min

distilled water	2 min
Staining series	
Ferric Alum Mordant	10 min
Running tap water	quick dip
Groat/Weigert Hematoxylin*	30-45 min
Running tap water	5 min
Acid Fuchsin stain	1.5 min
Running tap water**	5 min
Phosphotungstic acid	2 min
Orange G/Chromotrope stain	1.5 min
Running tap water**	5 min
Phosphomolybdic acid	2 min
Fast Green/Aniline Blue stain	3 min
Dehydration	
1% acetic acid	20-25 dips
1% acetic acid	20-25 dips
1% acetic acid	20-25 dips
1% acid acetone	20-25 dips
1% acid acetone	20-25 dips
1% acid acetone	20-25 dips
Clearing	
xylene	5 min
xylene	5 min
Mounting	
Mounting in Permout	24 hr to dry

\* A basic ethanol dip can be used to blue the hematoxylin, if necessary.

\*\* At these steps, no stain should remain between the slides and holding grooves in the slide rack.

$$= \frac{\text{the number of individuals at stages 3, 4, 5, 6 and 7}}{\text{the number of individuals at stages 1, 2 and 8}}$$

For mytilids and dreissenids, the analogous ratio is calculated as

$$= \frac{\text{the number of individuals at stages 3, 4 and 5}}{\text{the number of individuals at stages 0, 1 and 2}}$$

Table 3. Oyster development stages adapted from Ford and Figueras (1988) by Powell *et al.* (1993).

Developmental Stage	Value	Description
Sexually undifferentiated	1	Little or no gonadal tissue visible
Early development	2	Follicles beginning to expand
Mid development	3	Follicles expanded and beginning to coalesce; no mature gametes present
Late development	4	Follicles greatly expanded, and coalesced, but considerable connective tissue remaining; some mature gametes present
Fully developed	5	Most gametes mature; little connective tissue remaining
Spawning	6	Gametes visible in gonoducts
Spawned	7	Reduced number of gametes; some mature gametes still remaining; evidence of renewed reproductive activity
Spawned	8	Few or no gametes visible; gonadal tissue atrophying

Table 4. Mytilid and dreissenid development stages adapted from Seed (1975, 1976) by Hillman (1993).

Reproductive stage	Description
Resting/spent gonad	
Stage 0	Inactive or undifferentiated
Developing gonad	
Stage 1	Gametogenesis has begun; no ripe gametes visible
Stage 2	Ripe gametes present; gonad developed to about one-third of its final size
Stage 3	Gonad increased in mass to about half the fully ripe condition; each follicle contains, in area, about equal proportions of ripe and developing gametes
Stage 4	Gametogenesis still progressing, follicles contain mainly ripe gametes
Ripe gonad	
Stage 5	Gonad fully ripe, early stages of gametogenesis rare; follicles distended with ripe gametes; ova compacted into polygonal configurations; sperm with visible tails
Spawning gonad	
Stage 4	Active emission has begun; sperm density reduced; ova rounded off as pressure within follicles is reduced
Stage 3	Gonad about half empty
Stage 2	Gonadal area reduced; follicles about one-third full of ripe gametes
Stage 1	Only residual gametes remain; some may be undergoing cytolysis

Abnormal gonadal development is commonly observed in mytilid mussels. This is often characterized by unusual development of gametes at the base of the follicles. The cells resemble those of a germinoma (Peters et al. 1994) and are differentiated from normal cells by being either enlarged or by appearing to have an enlarged nucleus. In other cases, underdeveloped, small gonadal follicles are observed. These occupy a smaller portion of the mantle tissue. Follicles may be filled with cellular debris (Figure 1; see also Figure 10 in Ellis *et al.*, 1998). Sometimes cells adhere to each other, forming accumulations and empty spaces among developing cells. Occasionally, fibrosis occurs, with proliferation of fibroblasts inside the follicles and in the interfollicular connective tissue. Abnormal gonadal development is often associated with degeneration of Leydig tissue around the follicles and hemocytic infiltration into the surrounding tissues (Figure 11 in Ellis *et al.*, 1998). The approach used to score instances of abnormal gonadal development uses a scale that rates the spatial coverage of the condition (e.g. fraction of follicles affected), but not the degree of effect in each follicle (Table 5). Normally, the entire follicle is completely affected or unaffected.

Table 5. Semi-quantitative scale for abnormal gonadal development in mussels.

<u>Score</u>	<u>Description</u>
0	Normal gonad
1	Less than half the follicles are affected
2	About half the follicles are affected
3	More than half the follicles are affected
4	All follicles affected

## 6. CONCLUSIONS

The procedures described provide a semiquantitative ranking of reproductive stage but no quantification of the amount of gametic tissue present. The strengths of this approach are that it provides an assessment of sexual stage in the gametogenic cycle and allows for a concomitant histopathological analysis, with a single sample preparation protocol. The procedure cannot be performed on pooled samples. Thus, a direct correspondence between, for example, hydrocarbon body burden and stage in the gametogenic cycle may be difficult, because subsampling of individual animals will result in a certain degree of bias in the measurement of contaminant body burden, normally around 10% in adult oysters (Sericano *et al.*, 1993) and more for smaller individuals and species. This bias, therefore, will be size, contaminant, and time-of-year dependent.

If a quantitative gonadal/somatic index is desired, the technique of Choi and Powell (1993) should be used. The latter technique measures the concentration of egg protein present; however, it is not compatible with a concomitant histopathological analysis in that the standard histological preparation for assessing reproductive stage is not used in the quantitative analysis and tissue subsampling for histology cannot be done on the same individuals to be analyzed quantitatively for gonadal-somatic index. Choi *et al.* (1993) have further discussed the relative value of various approaches to gonadal evaluation. Overall, these authors found that the same general trends could be identified using either the semiquantitative or quantitative technique in most species, because normally an increase in gonadal volume occurs more or less simultaneously with advancement in reproductive stage and because gamete maturation occurs more or less evenly throughout the entire gonad.

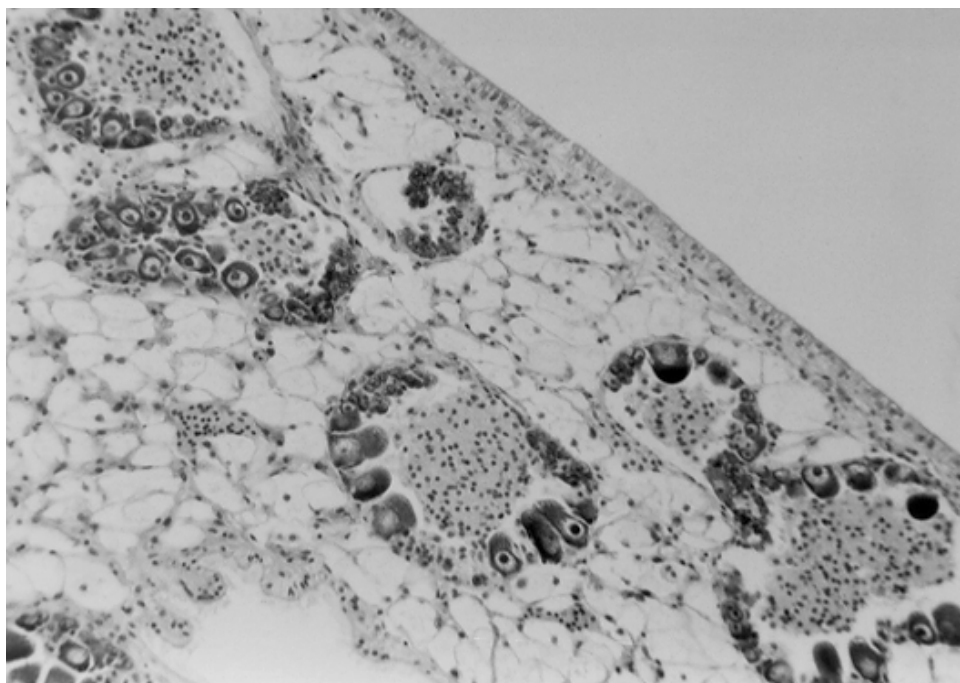


Figure 1. *Mytilus edulis* follicle with abnormal gametic tissue infiltrated with hemocytes.

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