5.2.11 Disseminated Neoplasia of Bivalve Molluscs

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A. Name of Disease and Etiological Agent

Disseminated neoplasia (DN) is a general grouping of proliferative disorders in the hemolymph of several bivalve species. It is generally believed that the transformed and abnormal circulating cells arise from a circulating cell (hemocyte) line but this hypothesis remains unproved and the source of hematopoietic tissue is not known with certainty in bivalve molluscs. It is clear that the cells are of host origin (Gee et al. 1994). There may actually be a complex of diseases with morphological similarities that arise from different tissues and possibly derive from infection by different etiological agents. This disease is known also known as and referred to in the literature as hemic, hematopoietic, or hemocytic neoplasia (HCN), disseminated sarcoma, hemic proliferative disease, leukocytic neoplasia, hemocytic leukemia, sarcomatous neoplasia, sarcomatoid proliferative disorder, and atypical hemocyte condition. Limited evidence suggests a retroviral etiology, but this, as well as the hemic origin of the affected tissue, has not been demonstrated in many of the species affected.

B. Known Geographical Range and Host Species of the Disease

The disease with morphologically similar or identical characteristics occurs in a variety of bivalve molluscs worldwide. Although the diseases are identical or nearly so in various species and are transmissible within a species, a common etiology has not been established. In addition, experiments attempting to demonstrate cross-species transmissibility have failed (e.g. Kent et al. 1991). The affected molluscs include:

1. *Adule californica*: west coast of North America

2. *Arctica islandica* (mahogany quahog): Rhode Island Sound, eastern North America

3. *Cerastoderma edule* (common cockle): south coast of Ireland near Cork, coast of Galacia, northwest Spain and Brittany, France.

4. *Saccostrea commercialis* (Australia rock oyster): Australia

5. *Crassostrea gigas* (Japanese or Pacific oyster): Matsushima Bay, Japan and Brittany, France

6. *Crassostra rhizophorae*: Brazil

7. *Crassostrea virginica* (Eastern or American oyster): discontinuously on the Atlantic and Gulf coasts of North America

8. *Macoma balthica* (duck clam): Chesapeake Bay, eastern North America, Finland, Gulf of Gdansk, Poland

9. *Macoma calcarea*: Baffin Island, Canada

10. *Macoma nasuta*: Yaquina Bay, Oregon

11. *Macoma irus*: Yaquina Bay, Oregon

12. *Mya arenaria* (soft shell clam): Atlantic coast of North America, discontinuously from Chesapeake Bay to Nova Scotia

13. *Mya truncata*: Baffin Island, Canada

14. *Mytilus edulis* (bay or blue mussel, also includes what may be *Mytilus trossulus* on the Pacific coast of North America): Pacific coast of North America discontinuously from Yaquina Bay, Oregon to sites in British Columbia, Canada; Atlantic coast of North America in the state of Maine; European sites in England, Denmark and Finland

15. *Mytilus galloprovincialis*: coast of Galacia, northwest Spain

16. *Ostrea chilensis* (Chilean oyster): Chiloe, Chile

17. *Ostrea lurida* (*Ostreola conchaphila*), (Olympic oyster): Yaquina Bay, Oregon

18. *Ostrea edulis* (European flat oyster): Mali-Ston area of Yugoslavia near Dubrovnic; Ria de Noya, Galacia, Spain; Mediterranean and Brittany region of France

19. *Tagelus plebeius* (razor clam): Chesapeake Bay, USA

C. Epizootiology

Some cultured populations appear to be 100% infected if individuals are monitored over several months. According to a 1992 review (Elston et al. 1992) prevalence of the disease is given in a variety of papers ranging from fractional percentages to 65% and is listed as unknown in many cases. These are generally point-in-time prevalence estimates. It is also clear that due to the transmissible nature of the disease, prevalences tend to be higher in densely cultured populations than in less dense wild populations. Annual mortality rates are reported to reach nearly 100% in some populations of affected species. Such high mortality is

generally found only in cultured populations. In other cases, particularly in wild populations, the occurrence is very low and no mortality has been established. Mortality is clearly density dependant but other factors are undoubtedly important as well, but specific environmental factors that induce or enhance the disease are not well understood. Although much research has been conducted to determine if various types of pollution contribute to the disease, no single factor has been identified.

It appears that the disease is highly infectious and dense populations of farmed shellfish maintain a high prevalence and severity of infection because close proximity facilitates the known capability for direct transmission from one animal to another. In all species in which seasonality has been investigated, the disease is reported to have highest prevalence (percentage of infected individual shellfish) during fall and winter months, typically from October through March. The prevalence drops in the spring and summer, due to the deaths of heavily diseased individuals over the winter. In farmed populations, it is clear that the disease is maintained from year to year, in spite of high mortality rates. This suggests that some individuals are either resistant to infection or do not develop advanced disease which causes death. In fact, remission has been observed in some individuals (e.g. Elston et al. 1988).

D. Disease Signs

Common clinical signs for all affected species have not been established. The following are known to apply in some cases:

1. Failure to produce mature reproductive follicles.

2. Epizootic mortalities that spread geographically in such a way as to suggest an infectious etiology.

3. Presence of high concentrations of large transformed cell types in mantle cavity fluid.

4. Swollen tissues in advanced stages of DN caused by massive systemic proliferation of transformed cells.

E. Disease Diagnostic Procedures (see Table 1)

1. Presumptive Diagnosis

Wet mount preparations of hemolymph can be prepared by bleeding the bivalve from the posterior adductor muscle with a hypodermic needle, then placing 0.1- 0.5 ml hemolymph onto a slide coated with 0.1% poly-L-lysine. The sample can be observed directly with phase contrast microscopy.

Presence of large (typically 6 to 8 μm, but often up to 10 μm diameter) hemocytes which fail to spread on a glass slide (after 15 minutes) as observed live in wet mount preparations (Figure 1). Normal hemocytes will rapidly attach and spread out on a glass substrate (Figure 2).

Also, after 15 minutes (to allow the normal cells to spread out), these samples can be fixed in 1% glutaraldehyde/ 4 % formaldehyde, and stained with Geimsa. However,

a Fuelgen stain with a picro-methy blue counterstain provides very good differentiation of normal and diseased nuclei from cytoplasmic components (Moore 1993) for a permanent record.

Slide Preparation
Dip slides 10 times in a solution of 1% HCl in 70% ethanol.
Dip in 3 changes of dH_2O.
Dip in 0.1% poly-L-lysine (MW 90,000; Sigma) in dH_2O. This solution is stored frozen and re-used indefinitely.
Air dry vertically, store indefinitely at 20°C.

Schiff's Reagent
Boil 200 ml dH_2O. Remove from heat, add 1.0 g basic fuchsin. Cool to 50°C, add 20 ml 1.0 N HCl, cool to 25°C. Transfer to a clean bottle, add 1.0 g sodium metabisulfite ($Na_2S_2O_5$), shake vigorously. Store in the dark for two days until straw colored. Add 0.5 g charcoal, shake and filter; reagent should be colorless. Store at 4°C. Discard when the reagent turns purple.

Picro-methyl Blue, 0.03%
Methyl blue, 0.15 g.
Picric acid, saturated aqueous solution, 500 ml.

Staining Procedure (Adapted from Farley 1969b).
Methanol, 3 min.
10% neutral buffered formalin, 3 min.
dH_2O, 1 min x 2.
5 N HCl, 30 min.
dH_2O, 1 min x 2.
Schiff's reagent, 20min.
dH_2O, 1 min x 2.
Running tap water, 2min.
dH_2O, 1 min.
0.03% picromethyl blue, 3 min.
70% ethanol, 1 min x 2.
100% ethanol, 1 min.
Hemo-de, 2 min x 2.
Mount.

2. Confirmatory Diagnosis

Observation of transformed circulating cells (Figure 3) in hemocytological preparations with large (6 μm and larger compared with normal fixed nuclear diameters of 3 to 5 μm), often irregular nuclei with prominent single or multiple nucleoli and relatively little cytoplasm. These cells will fail to spread on glass microscope slides in advanced stages. The relative proportion of abnormal cells required to confirm a diagnosis has not been definitively determined since apparently certain normal hemocyte stem cells are morphologically similar to the transformed

cells. If the disease is present, it will be progressive in many individuals and the abnormal cells will eventually comprise nearly 100% of the blood cell population in the advanced disease.

Histological examination also confirms the disease. The abnormal hemocytes are systemically distributed and are well differentiated from normal cells (Figure 4) by the above criteria even though the normal cells will not be spread out as they are on a glass substrate. In early stages of the disease, abnormal hemocytes are located in discrete foci in the connective tissue and vascular spaces (Figures 5 and 6) of connective tissue around the digestive gland. As the disease progresses, enlarged focal areas may be observed around the major digestive organs including the stomach, intestine and style sac. In advanced disease, the vascular spaces of the entire animal are filled and tightly packed with the abnormal cells. Histological preparation of a transverse section of the body, including visceral connective tissue and gills, or other organs typcially rich in circulating cells, is a good choice for observation of abnormal hemocytes. The cells are easily recognized in conventional 6 µm thick paraffin sections of tissues that have been previously fixed in either Davidson's fixative or 10% formalin in seawater.

Note: The characteristics of DN cells in *Tagelus plebeius* differ from those detected in other bivalves. The hypertrophied cells and nuclei are only about twice as large as those of normal hemocytes, marginal cytoplasm is acidophilic, and they show filopodia. Many circulating *T. plebeius* DN cells show blebbing of hypertrophied nuclei or are binucleate, but mitotic figures are rare. (Figure 7)

F. Procedures for Detecting Subclinical Infections

Use of hemocytological or histological examination as above.

G. Procedures for Transportation and Storage of Samples

Hemolymph samples and tissues for histology can be prepared and fixed as described above. If live animals can be shipped within 48 hours to the diagnostic laboratory, this is preferable because wet mounts and hemocytological preparations can be made for a preliminary diagnosis. To ship live animals, obtain required permits and ship fresh bivalves by courier service. Pack the animals in a moist insulated container with a frozen gel-pack. Insulate the animals from the gel-pack to prevent freezing of the tissues. Add sufficient packing material to protect them from breakage. Alternatively, shucked (shellfish removed from their shells) animals or sections placed in histological cassettes can be fixed in Davidson's shellfish fixative or 10% formalin in seawater. After a minimum of 24 hours fixation, drain the excess fixative and place the tissues or tissue sections in a sealable plastic bag with paper towels moistened with fixative. Seal this bag in a vacuum packed plastic bag and ship to the diagnostic laboratory by courier service.

The methods currently available for surveillance, detection, and diagnosis of DN are listed in Table 1. The designations used in the Table indicate: – = the method is presently unavailable or unsuitable; + = the method has application in some situations, but cost, accuracy, or other factors severely limits its application ; ++ = the method is a standard method with good diagnostic sensitivity and specificity; and +++ = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity.

Table 1. *DN surveillance, detection and diagnostic methods*

Method	**Screening**			**Presumptive**	**Confirmatory**
	Larvae	Juveniles	Adults		
Gross signs	–	–	–	–	–
Direct BF/LM		+++	+++	+++	+++
Histopathology		+++	+++	+++	+++
Bioassay	–	–	–	–	–
Transmission EM[1]	–	–	–	–	–
Antibody-based methods[2]	–	+	+	+	+
DNA probes – *in situ*[3]	–	–	–	–	–
RT-PCR[4]	–	–	–	–	–

BF = bright field; LM = light microscopy; EM = electron microscopy; RT-PCR = reverse-transcription polymerase chain reaction.

[1] Electron microscopy could be used for diagnosis but histology is less time consuming and expensive and equally reliable, as well as more extensive in terms of the tissues that can be examined. Electron microscopy is therefore not an appropriate diagnostic tool.

[2] An immunocytochemical assay using a monoclonal antibody effective for detecting DN cells in *Mya arenaria* has been developed by Smolowitz and Reinisch (1986). This monoclonal antibody has not been effective for detecting DN cells in any other bivalve species, but has been a helpful tool in diagnosis of DN in *M. arenaria*.

[3] DNA probes are not available for most species and not widely available for any species.

[4] RT-PCR is more useful as a research tool and is not an appropriate application because there are less expensive definitive diagnostic tools available.

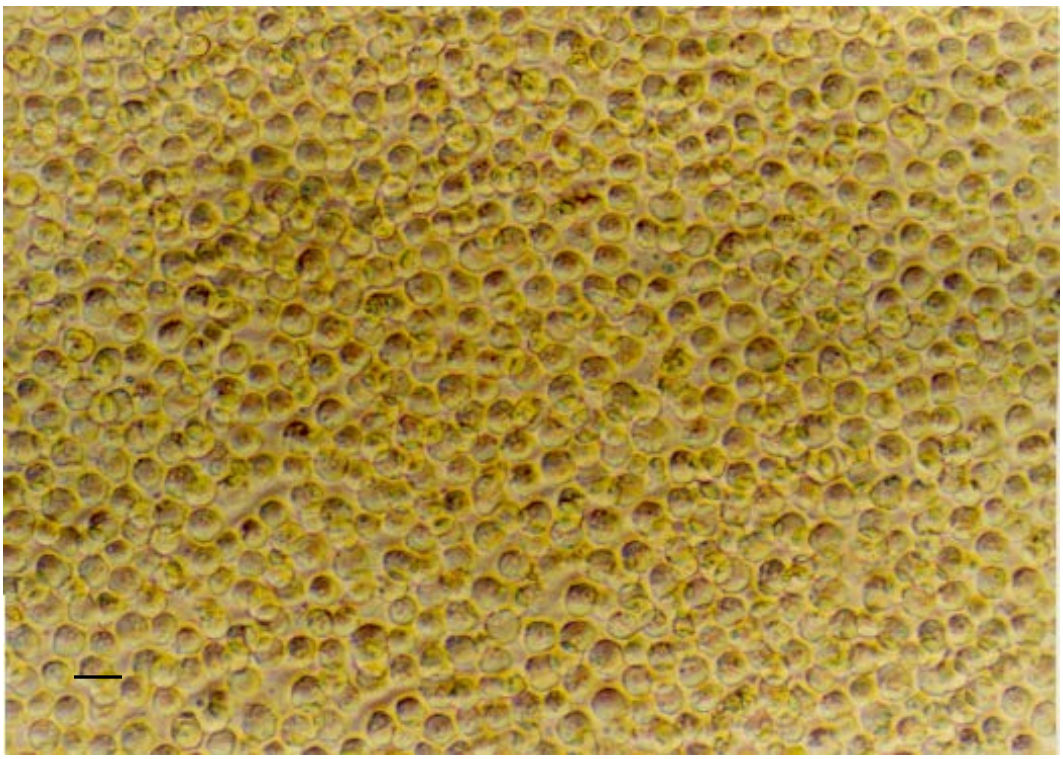

Figure 1. Neoplastic cells from *Mya arenaria*, the soft shell clam. This animal was in the final stages of the disease Scale bar = 10 um, phase contrast.

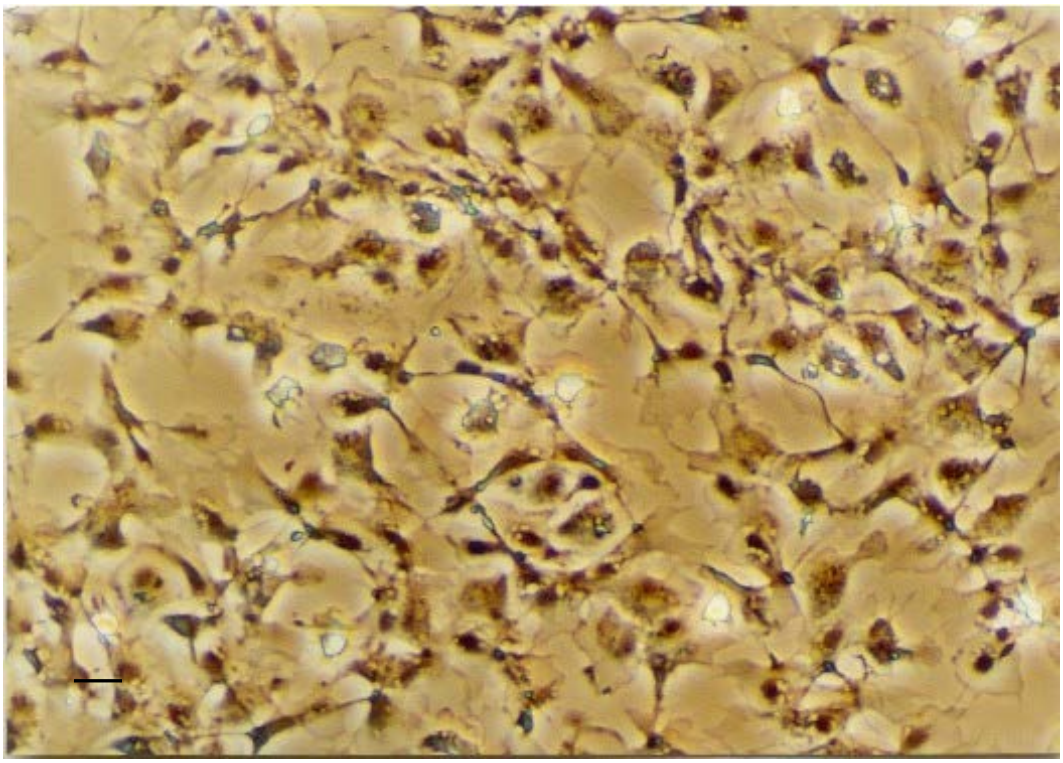

Figure 2. Normal hemocytes from *Mya arenaria* (Scale bar = 10 um, phase contrast).

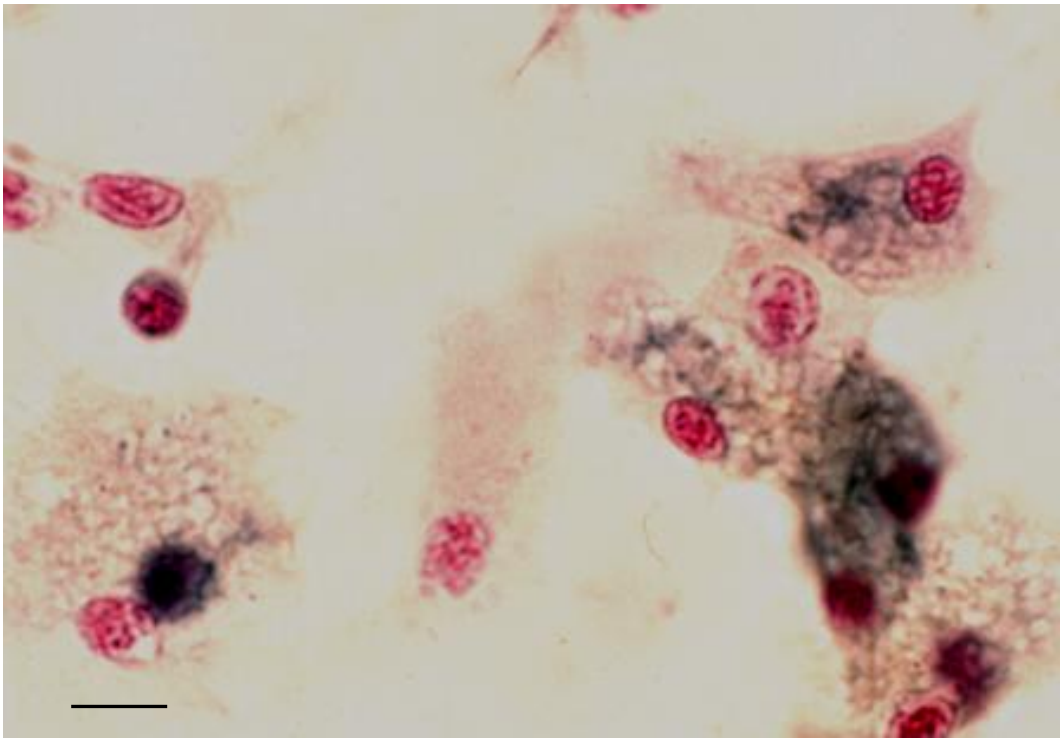

Figure 3. Hemocytological preparation of normal blood cells from *Mytilus*. Feulgen picro-methyl blue stain. Scale bar = 10 µm.

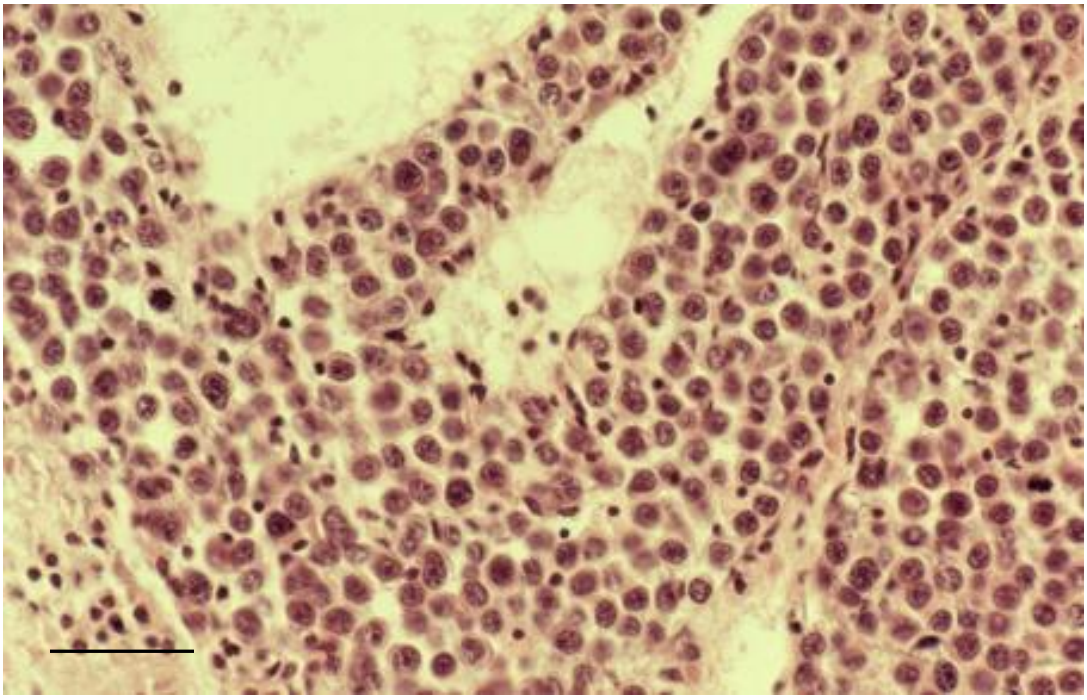

Figure 4. Hemocytological preparation of affected blood cells of *Mytilus* with hemic neoplasia in terminal stages of disease. The blood contains predominantly neoplastic blood cells that poorly attach or spread on glass substrate. Blood cells exhibit a high nucleus to cytoplasm ratio and prominent nucleoli. Scale bar = 10µm. (Feulgen-picro-methyl blue stain described in Farley, 1969b)

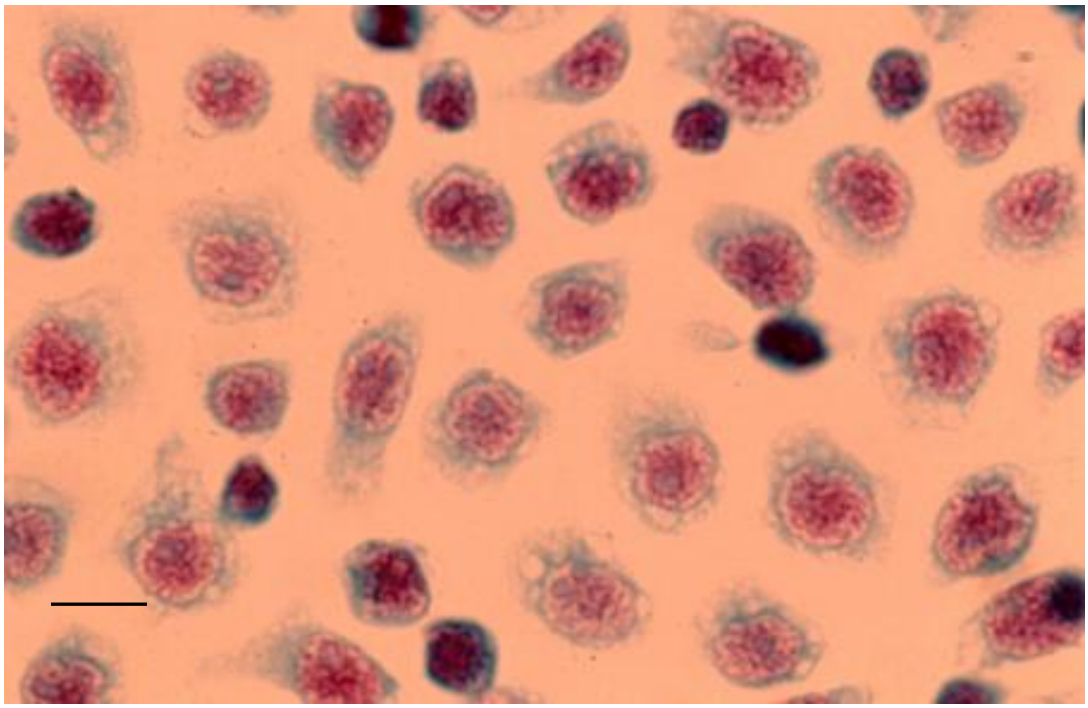

Figure 5. Histological section of *Mytilus* with DN showing the massive proliferation of neoplastic cells in the vascular spaces of the gills. Hematoxylin and eosin, Scale bar = 50 µm.

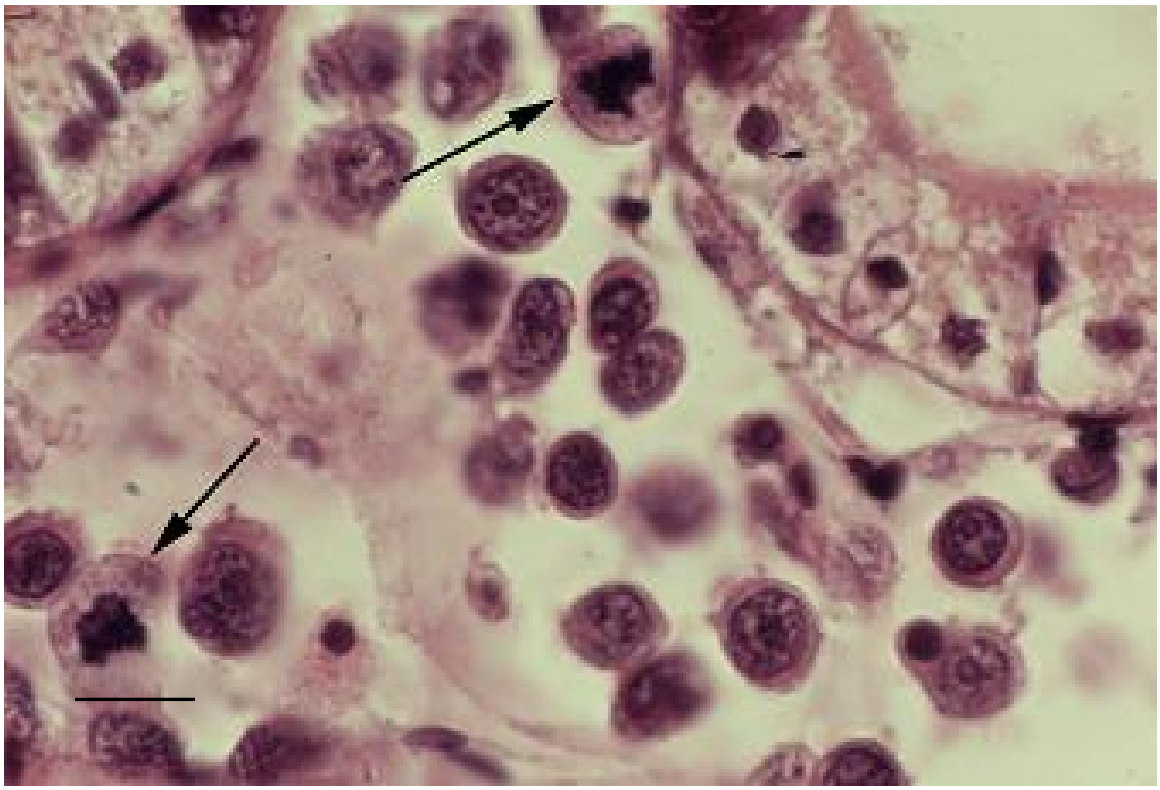

Figure 6. Histological section of the connective tissue between digestive tubules in *Mytilus* with DN showing typical enlarged hemocytes with large nuclei and scant cytoplasm. Two such cells in mitosis are shown at the arrows. Hematoxylin and eosin. Scale bar = 10 µm.

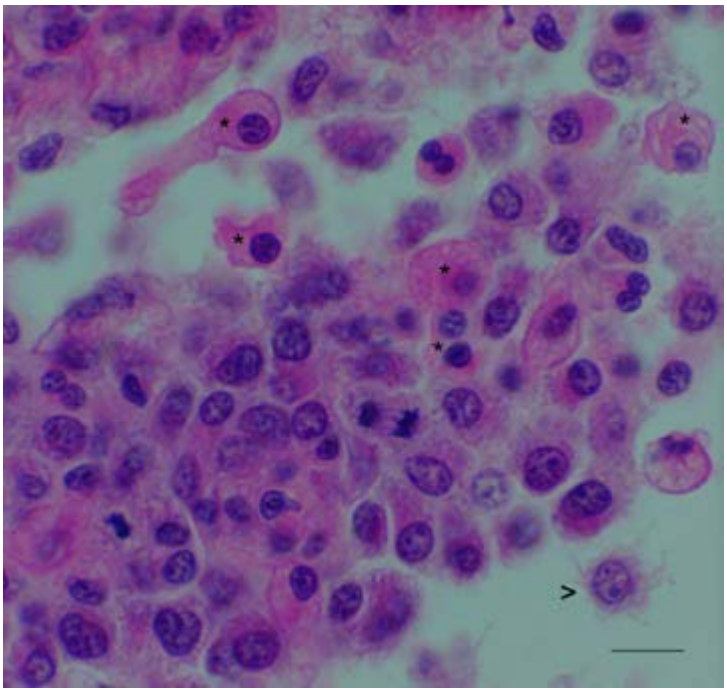

Figure 7. *Tagelus plebeius r*azor clam DN disease in H&E-stained histological section of gill vasculature. Large DN cells with hypertrophied, often bi-lobed, nuclei with prominent nucleoli predominate among circulating cells. Normal hemocytes are labeled (asterisks) for comparison. Metaphase and telophase mitotic DN cells are shown, as are several bi-nucleate DN cells, and a DN cell with prominent filopodia (arrowhead). Scale bar, 10µm.

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