Effects of hematopoietic neoplasia on physiological processes in the soft-shell clam, Mya arenaria (Linne)

Mary-Susan Potts

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Abstract
Mya arenaria (L.) is susceptible to the disease, hematopoietic neoplasia (Hn), in which atypical cells invade the blood and connective tissue of the clam's organs. In this study impacts of Hn on the reproductive and excretory systems were examined in clams collected from New Bedford Harbor and Little Buttermilk Bay, Buzzards Bay, MA.

The relationships between Hn seasonality and reproduction were evaluated from fresh blood samples and histological analysis of gonads. Both clam populations had biannual reproductive cycles with spawnings in the spring and fall. Hn prevalence exhibited some seasonality with maxima in the fall and late winter-early spring. Reproductive activity may enhance Hn prevalence, which in turn diminishes reproductive output.

Clams between 40-70 millimeters in length showed the highest prevalence of disease. Prevalence of Hn in a single cohort increased as these clams grew into the disease-susceptible size ranges. Hn may alter the population size distribution by eliminating particular size classes.

The radiopharmaceuticals, Tc-99m-disofenin and Tc-99m-sulfur colloid, were injected into the sinus around the anterior adductor muscle, and their accumulation was determined through scintigraphy, gamma well counting and autoradiography. By 7 hrs, 60% of the Tc-99m-disofenin was localized within the kidney. Whole body levels indicate that 15% of the disofenin was lost over 7 hrs. While Hn cells increasingly invade the clam's connective tissue, it appears that the kidney may not be as heavily impacted as other organs. A reduction in phagocytosis was indicated by a decrease in hemocyte Tc-99m-sulfur colloid activity with increasing stages of Hn. This loss of phagocytic capability impairs the clam's ability to remove foreign material from circulation, transport nutrients and repair wounds. Overall, Hn affects physiological processes including reproduction and excretion and alters the population size distribution of this commercially important bivalve.

Keywords
Biology, Animal Physiology, Biology, Zoology, Agriculture, Fisheries and Aquaculture

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EFFECTS OF HEMATOPOIETIC NEOPLASIA ON PHYSIOLOGICAL PROCESSES IN THE SOFT-SHELL CLAM, *Mya arenaria* (LINNE.)

BY

MARY-SUSAN POTTS

B. A. Suffolk University, 1987

DISSERTATION

Submitted to the University of New Hampshire in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in

Zoology

May, 1993
This dissertation has been examined and approved.

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April 22, 1993

Date
DEDICATION

This is dedicated to my parents, my first and very best teachers; without their love and support this would never have been possible. Thank you for always encouraging me.
ACKNOWLEDGMENTS

This work would not have been possible without the generous gifts of time, talent and support from a number of individuals. First and foremost, I extend my deepest appreciation and gratitude to Dr. John Sasner for the care and dedication with which he guided my graduate career, and for being a friend as well as a mentor. In addition, I am indebted to the other members of my committee: Dr. Judy McDowell, Dr. Tom Foxall, Dr. Chuck Walker, and Dr. Win Watson for providing valuable expertise and thoughtful comments, suggestions and encouragement at each step of this process.

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ABSTRACT

EFFECTS OF HEMATOPOIETIC NEOPLASIA ON PHYSIOLOGICAL PROCESSES IN THE SOFT-SHELL CLAM, *Mya arenaria* (Linne.)

by

Mary-Susan Potts
University of New Hampshire, May, 1993

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GENERAL INTRODUCTION

The soft-shell clam, *Mya arenaria* (Linne.), is a well-known component of coastal marine and estuarine ecosystems. The clam is found intertidally to subtidally in sheltered habitats on both coasts of the United States, Europe and in the Western Pacific. On the East coast of the United States, *M. arenaria*'s range extends from Labrador, Canada to Cape Hatteras, North Carolina and locally south to Florida. On the West coast it occurs from Alaska to Monterey, California. The warm temperatures (approximately 12 - 15 °C) necessary for successful reproduction limit the species' northern distribution, whereas high summer temperatures (above 28 °C) limit it in the south (Lawson, 1966; Pfitzenmeyer, 1972). In addition to "soft-shell clam," *Mya arenaria*, is referred to by a number of other common names including: the "common clam," "soft clam," "steamer clam," "long-necked clam," "nannynose clam," "gaper clam," or in northern New England, simply "the clam."

*Mya arenaria*, is in the order Eulamellibranchia, Suborder Heterodonta, and Family Myidae. Its valves are oval in shape, generally average less than 13 cm in length, and are usually white or gray in color with a periostracum of yellow or brown. The siphons are dark. *M. arenaria* lives buried in a variety of sediments from rocky gravel to soft mud, but prefers firm mixtures of sand and mud or soils compacted and cemented by algal growth (Belding, 1930; Turner, 1948). Shell color and thickness are dependent upon the character of the sediments in which the animal lives (Swan, 1952;
Newell and Hidu, 1982). Clams on sandy flats have shells that are thin, brittle and white in color, while those on rocky flats have shells that are dark gray to black, and thick with rounded and blunt edges. The clam rarely leaves its burrow after it attains a size of approximately 5 cm (Belding, 1930) and is commonly found in the tidal flats of bays, inlets, rivers, and sheltered beaches between low and high water. The clam burrows to various depths, depending upon its size and the type of sediment in which it lives. Its siphon may extend up to 4 times the length of the shell (Hanks, 1960). According to Dow and Wallace (1961), clams generally burrow approximately 2.5 times their longest diameter by digging a hole with their expandable, spade-like foot. During burrowing, the small muscular foot is distended with blood and extruded from the shell through a slit in the mantle. This organ is then used in alternating cycles to probe the surrounding sediments and anchor the clam while water is jetted from around the foot opening to loosen the sediments.

Selected Aspects of Soft-Shell Clam Anatomy

The anatomy of *M. arenaria* is that of a typical bivalve mollusc (Fig. 1). General reviews of soft-shell clam anatomy can be found in the works of Belding (1930), Hanks (1960, 1969), and Dow and Wallace (1961). The body of the clam is encased within two shells hinged by a spoon-shaped projection and a ligament. The opening and closing of the valves is regulated by the activity of the anterior and posterior adductor muscles. The mantle, which secretes the shell, overhangs the body organs forming a layer of tissue between the valves and enclosing the gills and mantle cavity. The posterior edges of the mantle are drawn out into a large retractable siphon which consists of two muscular tubes. The siphon is extended during feeding and is responsible for bringing food and oxygenated water into the animal and
Figure 1. Anatomy of the soft-shell clam, *Mya arenaria*. Major organs are identified.
removing waste products. The soft-shell clam feeds on organic material, as well as microscopic plants and animals, and may filter as much as 40-50 liters of water per day (Belding, 1930; Hanks, 1960). Water containing both food and oxygen is drawn into the animal through the incumbent siphon. From there it enters the branchial chambers and passes over the large, paired gills which envelope each side of the body. Over the surface of the gills, food particles are separated from water and other materials and passed on to the labial palps for further sorting. Blood is carried within the gills in vertical vessels that course between water tubes. Oxygenation takes place as the water moves dorsally in the water tubes opposite to the direction of blood flow. Water and waste products are collected and expelled through the excurrent siphon or the opening surrounding the foot.

The visceral mass of the clam, situated between the gills, houses the digestive and reproductive organs. The digestive tract consists of the mouth, which receives food particles from the labial palps, the short, tubular, esophagus, the stomach, the digestive gland, which surrounds the stomach and secretes digestive juices, the intestine, and the crystalline style. The crystalline style is located in a sac-like structure which curves around the visceral mass. Dissolution of the style during digestion results in the release of carbohydrate-splitting enzymes and lipases. The gonads consist of highly ramified tubules bearing numerous terminal and lateral alveoli which fill the fill much of the visceral mass in ripe individuals (Stickney, 1963).

Activity and behavior in *M. arenaria* is mediated through the clam’s nervous system. This system consists of three pairs of ganglia which branch to innervate all organs of the body. The very small white ganglia, located near the mouth, in the visceral mass just below the posterior adductor muscle, and in the foot, are designated as cerebropleural, visceral and pedal,
respectively, and communicate with each other through the long delicate nerve fibers.

The soft-shell clam has an open circulatory system with a three chambered heart (1 ventricle and 2 auricles) which pumps hemolymph through sinuses. The course of circulation is through the two aortae, anterior and posterior, to the various parts of the body. Blood from the body organs collects in the sinus venosus, passes through the kidney and on to the gills. After aeration, the blood passes into the auricles, which open into the ventricle. The major excretory organs are the pericardial glands and the kidneys, which function to filter, transport and excrete nonessential molecules and ions.

The two major types of hemocytes in the hemolymph of *M. arenaria* are granulocytes and agranulocytes (Cheng, 1981; Huffman and Tripp, 1982). Both types are involved in wound and shell repair, phagocytosis, neutralization of foreign particles, nutrient transport and excretion. The origin and ontogeny of the bivalve blood cells are unknown; however, it is generally accepted that both types of hemocytes arise from differentiation of connective tissue cells (Cheng, 1981; Mix, 1976).

**Life History**

The lifespan of *Mya arenaria* is estimated at 10 to 12 years in Massachusetts (Belding, 1930), but may be longer in northern localities where growth is slower (Turner, 1948). In the Chesapeake Bay, it is uncommon to have clams older than 3 years (Pfitzenmeyer, 1972). According to Belding (1930) and Turner (1948), the growth rate of young clams is influenced by a variety of conditions including temperature, time of submergence,
availability of food, current, degree of crowding, water quality, and sediment type.

Sexes in the soft-shell clam are separate, and there is no evidence for protandry. In a given population, there is an approximately equal number of males and females. Most young *M. arenaria* are able to spawn when they are about 1 year of age. Sexual maturation is a function of size and in northern waters is attained at a size between 25 and 40 mm (Hanks, 1960). Peak reproductive output is not reached until well after the animal's first reproductive cycle (Brousseau, 1978a). Gametogenesis occurs either once or twice a year depending on the location of a population and the environmental conditions (i.e., food abundance and temperature). According to Brousseau (1978a), a 60 mm female clam produces approximately 120,000 oocytes during a single breeding season and on the order of $1.5 \times 10^6$ oocytes during its lifetime, while a male of the same size produces $10^9$ sperm over the course of its life.

Gametes of the soft-shell clam are shed into the water column, and fertilization occurs externally. Development is indirect, and the larval stages include a trochophore and veliger (Fig. 2). Until a shell is formed, the larvae of the soft-shell clam are indistinguishable from that of other bivalve larvae. The size range at which metamorphosis of the larvae in culture occurs extends from about 170 μm - 230 μm (Loosanoff et al., 1966). Attachment takes place at the end of the veliger stage, a period of approximately 10-14 days, through the formation of a byssal thread (Belding, 1930). The larvae first attach to eelgrass, filamentous algae, sand grains, shells, or other projections in the subtidal zone (Kellogg, 1900; Belding, 1930). During the first year, juvenile clams migrate over the surface of the flat, repositioning.
Figure 2. Diagrammatic representation of the life cycle of the soft-shell clam. Stages are not drawn to scale.
themselves often. The migratory behavior of juvenile clams has been described by several authors, including Belding (1930), Mattiessen (1960) and Hidu and Newell (1989). After the first year the clam establishes a permanent burrow, digging deeper into the sediments with increasing size. The high fecundity of *M. arenaria* is offset by high mortality during pelagic life, metamorphosis and early settlement with only a few out of several million eggs released by the adult female clam ever reaching maturity (Belding, 1930). Predation, disease, bottom character and water conditions are important factors in determining recruitment (Belding, 1930; Brousseau, 1978a). A life history table for *M. arenaria* in New England has been constructed by Brousseau (1978b), and the species has a type D (or type III) survivorship curve (high mortality in early life followed by period of lower and constant losses). Because the soft-shell clam is such an important commercial resource, numerous studies on growth and population dynamics can be found (Belding, 1930; Newcomb, 1935; Turner, 1948; Ayers, 1956; Brousseau, 1978b; Appeldoor, 1980).

Commercial Significance and Factors Affecting Clam Populations

The soft-shell clam is an important element in coastal food chains because it is preyed upon by many other organisms including green crabs, moon snails, horseshoe crabs, ducks, bottom-feeding fishes and people. Belding (1930) cites the blue crab, *Callinectes sapidus*, the lady crab, *Ovalipes ocellatus*, and the horseshoe crab, *Limulus polyphemus*, as important predators on Massachusetts clam populations. The green crab, *Carcinus maenus*, is most destructive in New England and has been reported to control the size of clam populations on Maine's clam flats (Glude, 1954; Hanks, 1960).
In the New England area, particularly Maine and Massachusetts, *Mya arenaria* has been harvested commercially since colonial times (Hanks, 1960; Ritchie, 1977; Hidu and Newell, 1989). A second important commercial fishery was developed in the Chesapeake Bay during the 1950's with the utilization of the hydraulic soft clam escalator (Manning and Pfitzenmeyer, 1957). Together the clam stocks from the Chesapeake Bay and along the New England coast support a multimillion dollar industry. In 1989 alone, soft-shell clams yielded 6.8 million pounds of meats valued at $19.9 million dollars (U.S. Dept. of Commerce, 1990). A clam of approximately 6.5 cm in length is marketable, and growth to marketable size is generally attained in 1.5 to 2 years in Northern waters.

Because the clam resource occurs primarily in intertidal areas very close to shore, domestic and industrial pollution has seriously affected the soft-shell clam producing areas of the East coast (Turner, 1948; Ritchie, 1977; White and Campbell, 1989). Deteriorating water quality due to industrial pollutants, such as heavy metals, petroleum oils, and pesticides, and domestic pollutants, resulting in bacterial contamination via wastewater discharges, failing septic systems, stormwater runoff, discharge from marine craft, and waste products from waterfowl and wildlife, has resulted in an increased closure of large shellfish-producing areas. According to NOAA (1991), approved estuarine shellfish growing waters in the North Atlantic region, which extends from the U.S.-Canadian border in Maine to the tip of Cape Cod in Massachusetts, declined from 88 percent of those classified as approved in 1985 to 69 percent in 1990. In Massachusetts alone, approved shellfish-harvesting areas dropped from 70 percent in 1985 to only 36 percent in 1990. Some Massachusetts areas, such as the harbors of Boston, Salem, Lynn, New Bedford, Plymouth and Provincetown have been prohibited for shellfish
harvest since the early 1900's (Belding, 1930). In 1988 the estimated annual loss in unharvested shellfish from Massachusetts was about $16 million in landed value with a total economic loss of approximately $71 million (Hickey, 1989). Other factors influencing soft-shell clam harvesting areas are toxic dinoflagellate blooms and weather extremes, such as tropical storms, hurricanes and blizzards. Red tides resulting from blooms of *Alexandrium tamarense* cause paralytic shellfish poisoning (PSP) and result in the closure of widespread areas to shellfish harvest. Hurricanes, like Agnes in 1972 in the Chesapeake Bay, have been known to devastate entire clam producing regions.

**Responses to Stress**

According to Prosser (1986), all organisms maintain some internal parameters in a constant state. Maintaining this constancy of internal state, known as homeostasis, in an ever-changing external environment, involves the continuous balancing and rebalancing of physiological processes and the coordinated activities of numerous body systems. Disturbances to the physiological steady-state condition are defined as stress (Bayne, 1975), and when an organism is challenged beyond the limits of its regulatory processes, its systems fail. Stressors often encountered by animals include temperature fluctuations, food limitations, parasitic infection, pollution, and disease.

Because of its benthic lifestyle, *M. arenaria* is susceptible to perturbations and pathological conditions resulting from environmental disturbances of both the water column and the sediment (Appeldoorn et al., 1984). Predicting the impact of these factors requires an understanding of the responses of both the individual organisms and the whole population before and after the perturbation (Capuzzo, 1981).
Capuzzo (1981) suggests that responses to a stressor can be assessed at five levels of biological organization: biochemical; cellular/tissue; organismal, including physiological and behavioral; population; and community through changes in structure and dynamics. The most important physiological changes to consider when evaluating the impact of a stressor on an individual are those that may adversely affect the organism's growth, survival, and ability to contribute to the population gene pool. Within the animal, the systems most directly impacted are those involved with maintaining homeostasis, and especially those which serve as sites of exchange with the external environment. Stressors have various impacts on organisms, and may result in such things as tissue inflammation and degeneration, repair and regeneration of damaged tissue, chromosomal damage, and the formation of neoplasms within the animal.

Neoplasia is defined as "a pathologic condition involving a disturbance in growth of tissue or growth of any new and abnormal tissue. It is characterized by an excessive proliferation of cells without apparent relation to the physiological demands of the organ involved" (Appeldoorn et al., 1984; Sindermann, 1990). The occurrence of neoplasms in commercially-valuable bivalve molluscs has been well-documented (Peters, 1988). Molluscan neoplasms exhibit most of the traits associated with malignancy by vertebrate standards including: atypical structure, often with pleomorphic, undifferentiated cells; invasive, rapid, and unencapsulated growth; abundant and abnormal mitotic figures; progressive growth terminating in death; and frequent metastasis (Sindermann, 1990). In bivalves, invasion may appear diffuse rather than metastatic as a result of the lack of dense tissues, the open circulatory system and the highly anaplastic nature of the neoplastic cells (Farley, 1976).
Hematopoietic Neoplasia

A blood disorder, characterized by increasing numbers of leukemia-like cells in the connective tissue, blood vessels and sinuses of the visceral mass, muscle, and mantle tissue, has been reported with increasing frequency over the past two decades from at least 15 species of marine and estuarine bivalves from various parts of the world (Peters, 1988). This disease was first described by Farley (1969a, b) in the oysters, Crassostrea virginica and Crassostrea gigas and the blue mussel, Mytilus edulis, and has been referred to by a variety of names, some of which are: disseminated sarcomas of possible hemic origin, hematopoietic neoplasms, diffuse neoplasm of hyaline hemocyte origin, diffuse sarcoma of possible amoebocyte stem cell origin, hemocytoblastomic sarcoma, hemocytosarcoma, or disseminated hemic sarcoma. Regardless of the name, this condition has been most intensively studied in the soft-shell clam, Mya arenaria. In the soft-shell clam, cell location, morphology and monoclonal antibody work (Cooper, 1979; Smolowitz et al., 1989; Elston et al., 1990) suggest that the neoplastic cells are derived from a type of normal circulating hemocyte. On this basis, the terms, hematopoietic neoplasia (Hn) or leukemia, will be used when referring to this condition.

Yevich and Barszcz (1977) first reported this neoplastic condition involving high numbers of undifferentiated circulating cells in the hemolymph and connective tissue of the soft shell clam, M. arenaria, from clams collected in Maine. The disease was termed "hematopoietic neoplasia (Hn)" because of the similarity of neoplastic cells to hemocytes and because of its occurrence in vascular spaces. The Hn cells are characterized by anaplasia and hyperchromatic, enlarged nuclei, which are often lobed with large and sometimes multiple nucleoli (Farley, 1989). A high nuclear to cytoplasmic ratio is evident, and mitotic figures are commonly observed. The neoplastic
cells lack or have lost the ability to form pseudopodia, an important aspect of the cell's normal function, although they still may be able to form filipodia. Neoplastic cells fail to adhere to glass surfaces, while 90% of normal hemocytes adhere within 10 minutes (Reinisch et al., 1984).

Diseased hemocytes form a relatively homogeneous population of cells with a dense cytoplasm. Noticeably absent in these cells at the ultrastructural level are lysosomal bodies and evidence of particulate uptake by the cells (Morse, 1988). Comparative studies of phagocytosis in diseased and normal cells (Beckmann and Morse, 1988; Beckmann et al., 1992) indicate that Hn cells do not recognize or degrade foreign particles normally although they do possess some of the lysosomal enzymes necessary. The lack of phagocytic ability by Hn cells has been attributed to a disrupted cytoskeletal structure (Moore et al., 1988). Monoclonal antibody studies indicate that Hn tumor cells do not express some of the cell surface determinants found on the normal circulating hemocyte (Reinisch et al., 1983), while others are common to both normal and Hn cells (Smolowitz et al., 1989). Recently, Sunila and Dungan (1992) reported that terminal Hn clams had significantly more and several different serum proteins than normal ones. The hemolymph of terminal Hn clams has also been reported to be strongly hypoxic (Sunila, 1991). Not only do Hn-infected animals differ from normal ones on the basis of cellular morphology of the hemocytes but also in the number of circulating cells per milliliter of hemolymph. The number of circulating hemocytes/ml hemolymph in normal M. arenaria is between 1-6 x 10^6 hemocytes/ml, while in diseased clams the number can be as high as 5 x 10^8 cells/ml (Reinisch et al., 1984).
Hematopoietic neoplasia is diagnosed by examining the clam's hemolymph for circulating neoplastic cells. Clams are bled from sinuses surrounding the anterior or posterior adductor muscle. It is possible to monitor individuals over time as repeated bleeding, when carefully performed, is not deleterious to the health of the animal (Cooper, 1979; Cooper et al., 1982a). Examination of fresh hemocytes with a hemocytometer and phase-contrast microscopy (Cooper et al., 1982b) and staining with Feulgen Picromethyl Blue (Farley et al., 1986) are used for diagnosis and staging of Hn in individual hemolymph samples. The hemocytometer method is less accurate than the Feulgen Picomethyl Blue method particularly for early stages of the disease (Farley, 1989). The development of a monoclonal antibody, which is specific for *M. arenaria* neoplastic cells (Smolowitz and Reinisch, 1986), has led to a third method for disease diagnosis. This technique, referred to as indirect peroxidase immunochemical staining (IP), incorporates peroxidase staining of neoplastic cells using antibodies specific for Hn cells (Smolowitz and Reinisch, 1986).

Stages of Hn are expressed as the proportion of neoplastic cells to normal hemocytes in the hemolymph, and two severity scales have been developed (Table 1). A number of researchers (Cooper et al., 1982b; Brousseau, 1986; Farley et al., 1986; Smolowitz and Reinisch, 1986) use a severity scale of 1 to 5, while Leavitt et al. (1990) have reduced this scale to 3 stages based on observations of the survival of individuals with different levels of the disease.

According to Cooper et al. (1982a), Hn becomes a progressive and fatal disease in most cases, upon reaching a severity level of 1% Hn cells or greater. Laboratory studies (Cooper et al., 1982; Appeldoorn et al., 1984; Farley et al.,
Table 1. Classification of Hn severity. All stages are expressed as percent (%) neoplastic cells in the hemolymph.

<table>
<thead>
<tr>
<th>5 Stage System</th>
<th>3 Stage System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1: &lt;0.1%</td>
<td>Early Stage 1: &lt;15%</td>
</tr>
<tr>
<td>Stage 2: 0.1% to &lt;1%</td>
<td>Intermediate Stage 2: 16-70%</td>
</tr>
<tr>
<td>Stage 3: &gt;1% to &lt;50%</td>
<td>Advanced Stage 3: &gt;70%</td>
</tr>
<tr>
<td>Stage 4: 50 to 90%</td>
<td></td>
</tr>
<tr>
<td>Stage 5: 90 to 100%</td>
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</table>
1986) and field studies (Farley, 1989; Brousseau and Baglivo, 1991) indicate an increase in mortality with advanced stages of disease. According to Brousseau and Baglivo (1991), laboratory studies may overestimate the mortality due to neoplasia, because it may be compounded by mortality due to the added stress of the laboratory environment. Low severity levels of Hn infection may follow 1 of 3 courses: 1) progress to a higher severity level, 2) remain stable; or 3) go into remission (Cooper et al., 1982a; Leavitt et al., 1990; Brousseau and Baglivo, 1991). As the disease progresses, neoplastic cells increase in number in the hemolymph, invade and replace the soft tissue of the animal, eventually causing death.

**Causes of Hn**

The causes of hematopoietic neoplasia in the soft-shell clam are unknown. The infectious nature of the disease was established by Brown (1980), and Farley (1989) demonstrated transplantability of the condition. Oprandy et al. (1981) were able to isolate virus-like particles from Hn cells, and use them for disease transmission. Hn has also been induced in normal clams by 5-Bromodeoxyuridine, a reported activator of retrovirus activity (Oprandy and Chang, 1983). A retroviral etiology for Hn in *M. arenaria* is still open to question. Regardless of the causative factor, Hn cells are able to tolerate a broad range of environmental factors (salinity, temperature, and pH) and can survive in seawater long enough to transmit the disease to other clams either by cell transplantation or by transmission of an infectious agent (Sunila and Farley, 1989).

Earlier research of hematopoietic neoplasia in *M. arenaria* attempted to correlate the occurrence of the disease with increased levels of environmental contaminants, i.e., oil and petroleum derivatives,
polychlorinated biphenyls (PCBs), and heavy metals (Brown et al., 1977; Appeldoorn et al., 1984; Reinisch et al., 1984). In mussels, a correlation was established between neoplasm prevalence and polycyclic aromatic hydrocarbons (PAH's) in Yaquina Bay, Oregon (Mix and Schaffer, 1983). Appeldoorn et al. (1984) and Brown (1980) postulated that stress resulting from environmental conditions increases the susceptibility of clams to neoplasia. Attempts to correlate Hn prevalence with general environmental pollutants (Appeldoorn et al., 1984; Farley et al., 1986), petroleum hydrocarbons (Yevich and Barszcz, 1977; Brown et al., 1979; Appeldoorn et al., 1984), and polychlorinated biphenyls (Reinisch et al., 1984) have been inconclusive. More recently, Farley et al. (1991) have demonstrated a correlation between the pesticide, chlordane, and the prevalence of Hn in Chesapeake Bay clams, although more evidence is necessary before a cause and effect can be established. Overall, little is known as to why some locations have higher levels of Hn than others.

Hn Prevalence

Regardless of the causative factors of the disease, hematopoietic neoplasia is endemic to populations of M. arenaria along the Eastern coast of the United States. Data indicate that the disease has reached epizootic levels (prevalence of >1%) in some mid-Atlantic and New England states (Table 2). Brown et al. (1977) reported neoplasia prevalence as high as 64% in six M. arenaria populations in Rhode Island, one in Massachusetts, and three in Maine. Cooper et al. (1982a) reported Hn prevalence of 20-40% in soft-shell clam populations in Allen Harbor, RI surveyed from 1977 to 1979. It was estimated that 15-20% of the M. arenaria population in Allen Harbor, RI died each year as a result of the disease (Cooper, 1979). In 1982 and 1983, a
Table 2. Recent reports of Hn in soft-shell clam populations along the Atlantic coast of the United States (adapted from Peters, 1988). Range or peak prevalence is in parentheses.

<table>
<thead>
<tr>
<th>Location</th>
<th>Frequency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maine (oil spill)</td>
<td>11%</td>
<td>Yevitch and Barszcz (1976)</td>
</tr>
<tr>
<td>Maryland to Nova Scotia</td>
<td>0 - 40%</td>
<td>Yevitch and Barszcz (1977)</td>
</tr>
<tr>
<td>Massachusetts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annisquam River</td>
<td>12%</td>
<td>Farley (1976)</td>
</tr>
<tr>
<td>New Bedford Harbor</td>
<td>10 - 90%</td>
<td>Reinisch et al. (1984)</td>
</tr>
<tr>
<td>New Bedford Harbor</td>
<td>39%</td>
<td>Leavitt et al. (1990)</td>
</tr>
<tr>
<td>Boston Harbor</td>
<td>10%</td>
<td>Reinisch et al. (1989)</td>
</tr>
<tr>
<td>Little Buttermilk Bay</td>
<td>17%</td>
<td>Leavitt et al. (1990)</td>
</tr>
<tr>
<td>Maine and Massachusetts</td>
<td>10% (0 - 39%)</td>
<td>Brown et al. (1977)</td>
</tr>
<tr>
<td>Rhode Island (5/10 sites)</td>
<td>33%</td>
<td>Brown et al. (1976)</td>
</tr>
<tr>
<td>Allen Harbor</td>
<td>12% (0 - 64%)</td>
<td>Brown et al. (1979)</td>
</tr>
<tr>
<td>Connecticut</td>
<td>20-40%</td>
<td>Cooper et al. (1982)</td>
</tr>
<tr>
<td>Long Island Sound (3 sites)</td>
<td>6% (45%)</td>
<td>Brousseau (1987a)</td>
</tr>
<tr>
<td></td>
<td>13% (59%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13% (60%)</td>
<td></td>
</tr>
<tr>
<td>Maryland</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chesapeake Bay (5 sites)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1979 - 1983</td>
<td>0.1%</td>
<td>Farley et al. (1986)</td>
</tr>
<tr>
<td>1983 - 1984</td>
<td>42 - 65%</td>
<td></td>
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</tbody>
</table>
prevalence of 10-90% was reported for *M. arenaria* in New Bedford Harbor, MA (Reinisch et al., 1984). More recent investigations in New Bedford Harbor and Little Buttermilk Bay, both part of Buzzards Bay, MA, report overall average incidences of Hn as 39% and 17%, respectively (Leavitt et al., 1990). *M. arenaria* populations in Long Island Sound have also been reported to have epizootic levels of neoplasia (Brousseau, 1987a).

Farley et al. (1986) documented the epizootic occurrence of neoplasia in Chesapeake Bay clams, thus marking the first occurrence of neoplastic disease in a natural molluscan population that had previously been shown to be disease-free. Peak prevalences of 42-65% were noted in these populations (Farley et al., 1986). According to Farley et al. (1986), the Chesapeake Bay situation suggests that an infectious agent may have been introduced into the populations by clams transferred from New England after Hurricane Agnes (1972). Overall, the prevalence of Hn shows great geographic, annual, and seasonal variation (Brown et al., 1977; Cooper et al., 1982a) for reasons that are poorly understood. Since sampling has been irregular and restricted to the last several years and to certain geographic areas, it is difficult to determine the spatial and temporal patterns of Hn in *Mya arenaria*.

**Summary**

Malignant neoplasms were first discovered in bivalves in the late 1960's. Since that time epizootics have been identified in mussels, oysters, and several species of clams from both coasts of the United States. Features common to epizootics in bivalves include a unique cytology, cell proliferation, and a relatively high level of incidence (Farley, 1989). Hematopoietic neoplasia is a significant disease problem in the commercially important, soft-shell clam, and in areas where it is newly introduced, such as
in the Chesapeake Bay, can have a large impact on both the coastal ecosystem and the commercial fishery. While recent studies on Hn in *M. arenaria* have helped to answer some of the questions about progression of the disease and malignancy, behavior and structure of the neoplastic cells, and prevalence in various clam populations, major gaps in our understanding of Hn still remain. Questions that remain to be answered include: how the disease is transmitted between animals and from one population to another, seasonal cycling of the disease, effects of the disease on the physiological functioning of the animal, and impact of mortalities on existing clam populations.

**Areas of Research**

In light of our current knowledge about Hn and its effects on *Mya arenaria*, it is appropriate to examine the following aspects of this condition: 1) seasonal disease cycling and disease impact on clam reproductive organs; 2) disease prevalence in a single cohort of clams and effects of population size distribution; and 3) Hn effects on clam excretory system function. These areas were chosen as they are among those most important for evaluating the impact of disease on the individual organism and population consequences. Examination of seasonal disease cycling and prevalence within a cohort, as well as effects on reproduction, allows evaluation of disease at the population level. Studies of disease effects on reproduction, excretion and blood cell phagocytosis are important since alterations in these systems may adversely affect the organism's ability to survive, maintain homeostasis and contribute to the population gene pool.

This research was undertaken in conjunction with a larger project established by Dr. Judy McDowell’s group at Woods Hole Oceanographic Institution (WHOI), Woods Hole, MA, who have been studying the clam
populations of New Bedford Harbor and Little Buttermilk Bay, MA for several years. These clam populations have allowed researchers to investigate a number of aspects of the Hn problem including: 1) Hn prevalence within the clam populations (Reinisch et al., 1984; Leavitt et al., 1990); 2) effects of Hn on the physiological condition of the soft-shell clam (Leavitt et al. 1990); and 3) properties of Hn cells and development of a monoclonal antibody to Hn cell antigens (Smolowitz and Reinisch, 1986; Miosky et al., 1989; Smolowitz et al., 1989). Use of the Little Buttermilk Bay and New Bedford Harbor study sites in this research, will serve to increase the data base on these clam populations and will complement the long term studies by the WHOI group.

In this dissertation, Hn effects on reproduction and the size distribution of the population will be dealt with in Chapter I, and Hn effects on clam excretory processes will be addressed in Chapter II. Each chapter will be comprised of an introduction, materials and methods, results and discussion. In addition to the general introduction, there is a general summary.

**Description of Study Sites**

The collection sites for this study were New Bedford Harbor and Little Buttermilk Bay, both located in Buzzards Bay, MA (Fig. 3). New Bedford Harbor is located in the middle of Buzzards Bay and is part of the Acushnet River estuary. Little Buttermilk Bay is located at the northern end of Buzzards Bay and forms the upper part of Buttermilk Bay. Although both sites support large populations of *Mya arenaria*, only Little Buttermilk Bay is routinely open for commercial shellfish harvesting (Leavitt et al., 1990). New Bedford Harbor has a well-documented history of contaminant inputs which
include heavy metals, petroleum hydrocarbons, and polychlorinated biphenyls (Weaver, 1984; Farrington et al., 1986). Parts of this area have been closed for the harvesting of shellfish since the 1920's (Belding, 1930). At times, Buttermilk Bay is closed to shellfish harvesting due to bacterial contamination from failing septic systems, stormwater runoff, and waterfowl (Heufelder, 1989). According to Leavitt et al. (1990), both sites are very similar with regards to salinity, dissolved oxygen, and temperature.
Figure 3. Map of Buzzards Bay, Massachusetts showing the study sites at New Bedford Harbor (A) and Little Buttermilk Bay (B) (adapted from Leavitt et al., 1990).
CHAPTER I
EFFECTS OF HN ON REPRODUCTION AND POPULATION SIZE DISTRIBUTION IN THE SOFT-SHELL CLAM

INTRODUCTION

The prevalence of Hn in soft-shell clam populations shows great geographic, annual, and seasonal variation (Brown et al., 1977; Appeldoorn et al., 1984; Cooper et al., 1982a). Seasonal cycling of Hn has been reported in Rhode Island (Cooper et al., 1982a), the Chesapeake Bay (Farley et al., 1986), Long Island Sound (Brousseau, 1987a), and Buzzards Bay (Leavitt et al., 1990). Farley et al. (1986) and Brousseau (1987a) describe the seasonal disease cycle for clam populations in the Chesapeake Bay and Long Island Sound, respectively, as a sinusoidal curve with the maximum peak in the late fall to early winter and a minimum during the late spring and early summer. Cooper et al. (1982a) have described the Hn seasonal cycle in Rhode Island M. arenaria populations as a biphasic curve with two maxima, one in the late fall-winter and the other in the early spring, with minima during late winter and through the summer months. Clam populations at New Bedford Harbor and Little Buttermilk Bay exhibited seasonal fluctuations in Hn prevalence with a maximum in the fall and a minimum in early summer with one site (New Bedford Harbor) having a second maximum in late winter (Leavitt et al., 1990). Leavitt et al. (1990) suggest a scheme to reconcile the two apparently different observations; at sites where the Hn prevalence is low, a single yearly maximum is evident, while sites with higher levels have two maxima. The winter peak in Hn prevalence is attributed to a reduction in mortality since
low water temperatures are thought to suppress the progression of Hn (Appeldoorn et al., 1984; Cooper, 1982a; Farley, 1989; Leavitt et al., 1990). In clams from Boston Harbor, no evidence of Hn seasonality was found (Reinisch et al., 1989).

It has been suggested that certain environmental conditions may increase Hn susceptibility (Appeldoorn et al., 1980) or enhance disease prevalence through some undefined stress to the clams (Brown, 1980). Cooper et al. (1982a) regard spawning and water temperature as two potentially important physiological factors in stimulating the onset of Hn in the soft-shell clam since highest neoplasia prevalences have been found to occur during spawning and at water temperatures between 5 - 10 °C.

Reproduction in the Soft-Shell Clam

The reproductive system of *M. arenaria* is comprised of a pair of gonads, which encompass the intestinal loop, and simple gonoducts, which open into the mantle cavity (Belding, 1930; Stickney, 1963). On the basis of histological examination, Ropes and Stickney (1965), and Brousseau (1978a) have described the male and female reproductive cycles of *M. arenaria* as a sequence of 5 morphological phases: Inactive or Indifferent, Active or Developing, Ripe, Partially spawned, and Spent. In general, gametogenesis begins with the proliferation of gametes from between the follicle cells of the alveoli (follicles). This progressive development of gametes continues until ripe cells fill the tissues and spawning commences. According to Belding (1930), sexual cells are extruded from the reproductive organs into the upper mantle chamber, carried out the excurrent siphon, and passed into the water through rhythmic contractions. After a peak of spawning activity,
gametogenesis ceases, unspent gametes are resorbed, and follicle cells reinvade the gonadal follicles.

Seasonal development of the gonads has been described for a number of M. arenaria populations along the Atlantic Coast (Coe and Turner, 1938; Ropes and Stickney, 1965; Shaw, 1962; Pfitzenmeyer, 1972; Brousseau, 1978a, 1987b). It has been reported that clams north of Cape Cod exhibit a single spawning cycle, while clams south of Cape Cod have two spawning cycles each year (Ropes and Stickney, 1965). In those with a single annual cycle gametogenesis begins during the late winter or early spring and progresses until spawning occurs in the late spring and summer. In the biannual cycle gametogenesis begins in the winter and ends with a spring spawning in March or April. A second cycle is then initiated during the summer and concludes with spawning in the late summer or early fall. More recently it has been suggested that spawning may be a facultative event, occurring either once or twice a year depending on the location of the population and the environmental conditions (i.e., food abundance and temperature) (Bayne, 1975; Brousseau, 1978a, 1987b).

In spite of the hypothesis that Hn seasonality may be related to reproduction, data which correlate the reproductive cycle with Hn prevalence have not been provided. Previous studies have dealt exclusively with one or the other. The only clam populations on which both types of data are available occur in Long Island Sound (Brousseau, 1987a, b), and no comparisons of the reproductive cycle with disease prevalence were made.

In addition to its proposed relationship with spawning, a possible sublethal effect of Hn on the soft-shell clam may be a reduced ability to produce gametes. The primary sites of Hn cell invasion are the connective tissue of the gills, kidney, digestive tract and gonads (Yevich and Barczscz,
1976; Cooper, 1979; Brousseau, 1987b). This disease may impact the reproductive organs and capabilities by invasion and compression of these tissues by increasing numbers of neoplastic cells.

Not only may Hn have an impact on clam reproductive processes, but it may also alter the size distribution of clams within a population since Hn prevalence appears to be a function of size (age). On a site specific basis, length is an indicator of age in the soft-shell clam (Brousseau, 1978b; Newell and Hidu, 1982; Appeldoorn, 1982). Juvenile animals (<2 years old) have been reported to have a lower prevalence of Hn than adult *M. arenaria*, while clams between the ages of 3 and 4 years old have been shown to have the highest levels of Hn (Cooper et al., 1982a; Leavitt et al., 1990). A reduction in disease prevalence is noted for animals older than four years (Leavitt et al., 1990).

In light of current evidence it appears that hematopoietic neoplasia may be involved with reproductive processes in the soft-shell clam in several different ways. First, Hn may be induced by spawning or some other event in the reproductive cycle (Cooper et al., 1982a). Since each of these cycles have only been examined separately, correlations between them have not been possible. Also, as the disease progresses, increasing numbers of neoplastic cells in the hemolymph and connective tissue invade the reproductive system. Hn may impact the overall population size distribution by eliminating particular size (age) classes. When the study site at New Bedford Harbor naturally received a large set of soft-shell clams during the 1990 reproductive season, examination of Hn prevalence in a cohort became possible.

In view of past work and the questions that arise, the objectives for this study were the following: 1) to determine if the reproductive cycle and the Hn
seasonal cycle are related in two populations of *M. arenaria* on Cape Cod; 2) to examine qualitative and quantitative Hn effects on the morphology of the clam's reproductive organs and determine if diseased clams had reduced reproductive capabilities; 3) to examine Hn effects on the population size distribution by tracking disease prevalence in a cohort. This third objective included determinations of whether the cohort showed an increase in Hn as it reached the size range in which Hn prevalence was reported to be highest, and whether an increased number of clams of this size range would result in an increase in Hn prevalence for the flat as a whole. Relationships between Hn and reproduction are important to examine since effects on this system may hinder the individual's ability to survive and contribute to the population gene pool.
MATERIALS AND METHODS

Soft-shell clams were collected monthly from New Bedford Harbor (NBH) and Little Buttermilk Bay (LBB), Buzzards Bay, MA over a two year period (January 1990 to December 1991) (Fig. 3). During each collection period, 50 *M. arenaria* were taken at low tide from the exposed mid-intertidal area by hand-digging. The subtidal water temperature at time of collection was measured. Following collection, the clams were transported on ice to the UNH Coastal Marine Laboratory, Newcastle, NH where they were maintained in sand under ambient conditions in a flow-through seawater system. Clams from each study site were housed in separate tanks.

In the lab, *M. arenaria* were diagnosed for Hn using fresh blood preparations (Cooper et al., 1982b). Clams were bled by inserting a 26 g 1/2" needle into the sinus around the anterior or posterior adductor muscle. One-tenth (0.1) ml of hemolymph was withdrawn into a 1 cc syringe. A minimum of 1000 cells per sample were examined on a hemocytometer using phase contrast microscopy. If the clam was severely afflicted with leukemia (Hn stage 3), an accurate diagnosis was made after counting fewer cells. Neoplastic cells were characterized by their rounded appearance, relative lack of pseudopodia, presence of retractile granules and lack of clumping. When neoplastic cells were observed, the clam was diagnosed as positive for the disease and staged. The severity of Hn was classified on an ordinal scale of 1 to 3 according to the criteria of Leavitt et al. (1990). Slight modifications of these criteria were necessary as Hn diagnosis from fresh blood preparations is not as sensitive a method as the immunoperoxidase test (Smolowitz and Reinisch, 1986). Because of the reduced sensitivity of diagnosis from fresh blood preparation, no attempt was made to diagnose clams with less than 1%
Hn cells. The prevalence of neoplasia was reported as the percentage of neoplastic cells in the sample as follows:

- Stage 1: 1 - 15% neoplastic cells
- Stage 2: 16 - 70% neoplastic cells
- Stage 3: 71 - 100% neoplastic cells.

Permanent preparations of representative blood samples were made using the techniques of Farley et al. (1986) and staining with Toluidine Blue O (Howard and Smith, 1983). Morphometric analysis was done on both normal and Hn cells for comparative purposes according to the methods of Cooper et al. (1982b). All normal cells (granulocytes and agranulocytes) were grouped together. In this analysis, blood preparations were viewed at 1000X and scanned into a Macintosh Ilci computer using a video camera mounted on a Zeiss light microscope and Quick Capture software (Translation Inc., version 1.13). Cells (n=200) were measured for cell and nuclear diameter along their longest axis and for cell area using the image analysis software Image (NIH public domain software, version 1.22).

After Hn diagnosis, the valve length (along the longest transverse plane) of each clam was determined, and the animal was sacrificed. Prior to sacrificing, some animals were used in the radiopharmaceutical studies described in Chapter II. The visceral mass (digestive gland and gonad) was removed from each animal, and its displacement volume in water determined. Each sample was then fixed in Bouin's solution, dehydrated in alcohol and embedded in paraffin at the State of New Hampshire Veterinary Diagnostic Laboratory, and sectioned at 10 μm on a rotary microtome. The sections were stained with Harris' hematoxylin and eosin (Howard and Smith, 1983) and examined using bright field microscopy. The sex of each
animal was determined, and the clam's reproductive status assigned as one of five categories of gonadal condition (indifferent, developing, ripe, partially spawned, and spent) based on the criteria of Ropes and Stickney (1965) and Brousseau (1978a, 1987b).

In addition to qualitative observations, quantitative measurements of the impact of Hn on the size of the gonadal follicles were made. In this analysis, Hn stage 2 and 3 clams were paired with normal animals for size, sex, reproductive condition and month of collection. Several fields of view, (each 0.5 mm²) from each clam gonad were then viewed at 100X with bright field microscopy and scanned into a Macintosh IICi computer using the system described for analysis of blood preparations. From these images, the cross-sectional areas of 40-70 gonadal follicles per animal were determined using the Image software. These values were used to determine the mean gonadal follicle size for each animal. In addition, the number of gonadal follicles in each of 3 fields of view were counted and used to establish the mean number of gonadal follicles per 0.5 mm² in each clam.

Categories of Gonad Condition

The following descriptions from Ropes and Stickney (1965) and Brousseau (1978a, 1987b), as well as personal observations of male and female gonadal condition, were utilized in this study. These criteria are based solely on morphological features.

Female gonads

1. Indifferent
   Very small oocytes, each with a round nucleus and irregularly-shaped cytoplasm, appear at the periphery of alveoli. Distinctive female inclusions (Coe and Turner, 1938) are often present in the follicle cells which fill the alveoli and surround the oocytes.
2. Developing
The developing stage is marked by an increase in the size and number of oocytes and a decrease in the number of follicle cells and inclusions. In the early phase, the oocytes are sub-conical, hemispherical, or cylindrical with rounded apices and broad cytoplasmic extensions. Subsequent growth produces large, round oocytes with constricted cytoplasmic bases and a prominent amphinucleolus.

3. Ripe
A very slender stalk connects many of the largest oocytes to the basal membrane, while others appear as round cells filling the follicle lumina.

4. Partially Spawned
As the mature ova are gradually discharged, nutritive inclusions begin to reappear in the follicle cells. Small oocytes are imbedded in the follicle cells of some alveoli while others are empty.

5. Spent
Follicle cells form a thin layer covering the basal membrane of some alveoli, while in others they fill the alveoli. Unspent oocytes in the early stages of cytolysis are present as are numerous spherical lipoid droplets. The spent phase merges with the indifferent.

Male gonads

1. Indifferent
During this phase, the male gonads contain the multi-nucleated pycnotic cells and non-ypcnotic cysts produced by atypical spermatogenesis (Coe and Turner, 1938). Follicle cells fill the lumen and imbed a few primary spermatocytes or spermatogonia at the periphery of the alveoli.

2. Developing
The entire process of spermatogenesis takes place during this phase. Early development is characterized by an increase in the number of primary spermatocytes at the basal membrane and the appearance of some spermatids. The later part is marked by the migration of spermatids toward the follicle center and their arrangement into radial columns. Proliferation of the spermatids is followed by their differentiation into spermatozoa.

3. Ripe
Masses of spermatozoa arranged in radial columns with their tails oriented toward the center fill the lumina of the alveoli.

4. Partially Spawned
The follicles contain few or no spermatogonia at the basal membrane. Follicle cells occur between the basal membrane and the groups of cells undergoing spermatogenesis. A few spermatozoa remain in radiating bands in the central lumen.

5. Spent
The alveoli are almost completely filled with follicle cells, although a few spermatozoa may persist in the central alveolar area. Multinucleated, non-ypcnotic cysts and pycnotic cells reappear filling the follicle cells and surrounding small groups of spermatozoa
Immature

In immature clams, the small, and in some cases, poorly developed alveoli are filled with large numbers of follicle cells. No inclusions of any type are present.

Hn Prevalence in Cohort Clams

To examine the effects of Hn on a particular age-class of clams, the abundance of both the total clam population and cohort clams, together with their Hn prevalences, were estimated over time using a randomized block design (Zar, 1984). In this sampling scheme, an 8 m X 5 m grid, encompassing 40, 1 m² blocks was laid out around the mean low tide line at the New Bedford Harbor clam flat (Fig. 1-1). At 3 month intervals, 0.05 m² core samples were collected from 5 randomly chosen blocks. Each block was assigned a number, and the cores to be collected in each sample were chosen using a random numbers table (Ott, 1988). No block was sampled more than once. Quarterly collections were made from November 1990 through March 1992. The February 1992 sample was shifted to March 1992 because of an insufficient tidal flux on the day the cores were to be collected. All clams in the core were measured for shell length. Fifty *M. arenaria* from the cohort were then collected, transported to the lab, and diagnosed for Hn as previously described. In order to insure that the appropriate clams were collected, the size of the cohort clams was determined prior to collection, based on growth information (D. Leavitt and B. Lancaster, personal communication; Belding, 1930). From this study the mean abundance of the cohort and the population at the time of collection, as well as the prevalence of Hn in each group, were determined.
Figure 1-1. Diagram of randomized block design for estimation of cohort and population density at New Bedford Harbor clam flat (not drawn to scale). Cores collected at each quarterly sampled are listed below.

**Cores Collected**

- **Nov 90 (1)**
  - D-6, C-5, A-1, C-2, D-5

- **Feb 91 (2)**
  - C-3, B-3, E-7, E-2, A-7

- **May 91 (3)**
  - A-5, E-4, B-8, D-4, D-3

- **Aug 91 (4)**
  - E-5, A-8, C-4, D-7, E-3

- **Nov 91 (5)**
  - A-4, A-3, C-1, E-1, B-1

- **Mar 92 (6)**
  - C-6, B-5, A-6, E-8, D-1
Data Analysis

For each study site seasonal cycling of Hn was compiled by plotting the percent prevalence of neoplasia in the monthly sample over the two year time period (January 1990-92). Mean Hn prevalence by season of the year was also examined using the following designations: Winter (Jan, Feb, Mar), Spring (Apr, May, Jun), Summer (Jul, Aug, Sep), Fall (Oct, Nov, Dec) Analysis of variance (ANOVA) and t-tests were used to analyze Hn prevalence by season and by year. Differences in Hn prevalence between study sites and between years for each site were examined using Chi-square analysis.

The reproductive cycle of clams at each site was evaluated through histological examination of all gonads (n=50) from 4 months of each year (January, April, July, October) and selected gonads from other months (n ≥ 10). In addition, gonad displacement volume, shell length and month of sample were recorded. The reproductive cycle was evaluated by plotting gonad displacement volume against shell length for normal clams, 40-70 mm in length, each month. The linear slope of this relationship, which was anticipated to be highest when the clams were ripe, was then plotted against month for each site and year. The gonad displacement volume data, coupled with data from histological evaluations of reproductive condition, were then used to assess spawning in the clam populations. Correlations between seasonal disease cycle and spawning were examined. The histological sections of clam gonads were used to examine the relationship between sex (male vs. female) and Hn prevalence, as well as impact of Hn on reproductive tissues, particularly the size and number of gonadal follicles. Differences in the mean size and number of gonadal follicles between pairs of Hn Stage 3, Hn Stage 2 and normal clams were analyzed using paired t-tests.
RESULTS

Blood Cell Morphology

The two major types of hemocytes in the hemolymph of *M. arenaria* are granulocytes and agranulocytes (Cheng, 1981; Huffman and Tripp, 1982). Both types are involved in wound repair, phagocytosis, neutralization of foreign particles in the hemolymph, nutrient transport and excretion. In some cases, these hemocytes have been further categorized to include 3 cell types: a granulocyte, a large agranular cell (fibrocyte) and a small agranular cell (hyalinocyte) (Cooper, 1979). Of these, the irregularly-shaped fibrocyte is the largest and most abundant cell. This cell type with its eccentrically located nucleus and clear cytoplasm attaches readily to glass slides by extending long pseudopodia. The other two cell types form smaller populations of cells and are characterized by their small size, rounded shape, and presence or absence of cytoplasmic granules. A photomicrograph of normal hemolymph from *M. arenaria* is presented in Figure 1-2A.

In contrast to normal hemocytes, Hn cells are round, undifferentiated cells with a high nuclear to cytoplasmic ratio and a prominent nucleolus (Fig. 1-2B). Differences between the normal and abnormal cells can be appreciated from a comparison of various morphological features (Table 1-1). While normal cells range in diameter from 20 - 40 μm, Hn cells are much smaller (8-10 μm in diameter). In contrast to the normal cell's long pseudopodia, Hn cells extend short, blunt pseudopods only on poly-L-lysine coated slides. Normal hemocytes are approximately 3 times greater than Hn cells in both cell diameter and total cell area. While the nuclear dimensions of Hn and normal cells are very similar, the cytoplasm of a normal cell has an area that is approximately 7 times greater than that of a neoplastic cell. The most
dramatic differences between normal and Hn hemocytes were found in a comparison of the cells' nuclear to cytoplasmic ratios. Normal cells have a mean nuclear to cytoplasmic ratio of 1:10, while that of Hn cells 1:1. The increase in this ratio is attributed to the reduction in cytoplasmic area found in the Hn cells rather than to an increase in nuclear size.

**Seasonal Cycling of Hn**

The monthly prevalence of Hn at each study site is presented in Figure 1-3. Data from New Bedford Harbor suggest a seasonal cycling of Hn prevalence with maxima in the late summer-fall (August-October) and winter (January-February) and a minimum during March to July. The fall maximum during 1991 begins earlier, lasts longer, and is of greater amplitude than in 1990 (July-December). It appears as though the two maxima (fall and winter) are run together during 1991. At Little Buttermilk Bay, Hn was highest during the late fall and winter (October-January) of 1990, and late summer and fall (July-November) of 1991. Some indication of a secondary maximum is present during the early spring (March) of both years of the study. Overall Hn prevalence at both sites is consistently high in the fall and into the early winter with suggestions of secondary maxima in the early spring.

Hn prevalence varied significantly both annually and with the source of the clams. Chi-square analysis indicated that there was a significantly higher prevalence of Hn in clams from NBH than from LBB (Table 1-2A). At both sites Hn prevalence was higher during 1991 than in 1990 but significantly different only at NBH (Table 1-2B, C). Figure 1-4 represents the mean Hn prevalence by season of the year for NBH and LBB. ANOVA indicates that Hn prevalence varies significantly with season at both sites during 1991, but
only at LBB in 1990. In this analysis, the highest Hn prevalences were found to occur during the summer and fall of 1991 at NBH and during the fall of 1990 and summer and fall of 1991 at LBB. In addition, Hn prevalences at NBH were significantly higher during the summer and fall of 1991 than in 1990, while at LBB only the summer of 1991 differed significantly from that of 1990. The way in which months are grouped into each season (Fall, Winter, Spring and Summer) does not necessarily reflect the seasonal cycle of Hn prevalence discussed previously. Overall, grouping the data into four seasons of the year provides further evidence of the annual and seasonal variation in Hn prevalence at and between each site, and highlights the large increase in Hn prevalence at NBH during the summer and fall of 1991.

Hn and Reproduction

Representative gonadal sections of each phase of the reproductive cycle are presented for male and female clams in Figure 1-5(A and B, respectively). The criteria for each of these phases were as previously described. Reproductively active individuals (those with developing, ripe or partially spawned gonads) were found in all months examined at both New Bedford Harbor and Little Buttermilk Bay (Fig. 1-6). The lowest levels of gametogenic activity were observed in October of 1990 and November of 1991 at NBH and in October of both years at LBB.

At New Bedford Harbor, development of gametes began in the winter (February) of 1990 with some clams ripe by March and a larger proportion ripe (60%) during April. A second ripening of gonads is suggested by the fact that over 50% of the clams had developing gametes in July with a few ripe, but by the October sample, most individuals were partially spawned or spent. This spawning pattern is repeated in the 1991 samples with development of the
gametes being initiated in the winter (100% developing in December) and concludes with spawning in April (70% ripe). A second cycle was initiated in July. By August most clams (70%) had developing gametes with some individuals ripe, however, most (90%) were ripe or partially spawned by September.

Spawning patterns similar to those at New Bedford Harbor are suggested in the data from Little Buttermilk Bay. In 1990, development of gametes at LBB was thought to have begun in the winter, since by March 60% of the clams had developing gametes with some individuals ripe or partially spawned. By April, 75% of the clams were either in the ripe or partially spawned condition. A second reproductive cycle may have been initiated in the early summer since some individuals were again developing in July. A few, however were ripe. In the October sample, most clams were again indifferent with a few partially spawned or spent. During 1991, development of gametes was initiated in the winter with over 60% of the individuals with developing gametes by March. This cycle concludes with spawning in early spring (April-May). A second ripening of gonads is speculated to have occurred in August - September, as developing gametes were encountered in clams in July. By October, 60% of the individuals were indifferent, while 40% were partially spawned or spent. Further histological analysis of gonads from the summer and fall is necessary to document the occurrence of a second spawning at LBB during each year of the study.

Analysis of gonad displacement volume by shell length and month of sample was also performed in order to help describe the reproductive cycle of clams at Little Buttermilk Bay and New Bedford Harbor. A summary of the relationship between gonad displacement volume, shell length and month for normal and Hn 1 clams (40-70 mm) is presented in Table 1-3. Based on
these data, it appears that the relationship between gonad volume and clam length does vary with month. Based on $R^2$ values, there is close agreement with linearity in the gonad vol/shell length data for some months of the year, but not for others. The slope of gonad volume/clam length was plotted along with the histological analysis of spawning against month in order to examine spawning in the clam populations (Fig. 1-7). Peaks in the data are present during those months in which spawning was thought to be occurring based on histological evidence. Both of these factors together support the presence of a biannual spawning cycle in these clam populations, with one spawning taking place in early spring (March-May) followed by a second one in the late summer-early fall (August-October) during each year of the study. The presence of ripe clams in the middle of summer (July) suggests that some clams may be either spawning once a year or are on a slightly different cycle than the majority of the population (spawning in mid-summer and late fall). According to Brousseau (1987), larger (older) clams are more likely to undergo multiple spawnings than smaller (younger) ones.

Comparisons of spawning and temperature data (Fig. 1-8) for the two sites reveal that spawning takes place at temperatures above 12 °C. The reproductive cycle of Hn clams (stage 2 and 3) appears to mirror that of the population as a whole (Fig. 1-9). A comparison of the seasonal data of Hn prevalence with the clam populations' reproductive cycle reveals that Hn prevalence is highest in the population around the times of spawning (early spring and fall) (Fig. 1-10). It appears that spawning follows high Hn prevalences in the late winter - early spring and is concurrent with the Hn fall peak at each site.

Based on the analysis of gonads, the proportions of male and female
clams at New Bedford Harbor and Little Buttermilk Bay did not differ significantly from 1:1 (Table 1-4). Male and female gonads were indistinguishable in immature individuals. One individual from Little Buttermilk Bay was a bilateral hermaphrodite. In clams diagnosed with Hn and whose gonads were examined histologically, the male to female sex ratio also did not differ significantly from 1:1. Based on these findings, it can be concluded that Hn prevalence is not related to the sex of the clam.

Hematopoietic neoplasia has both qualitative and quantitative effects on the reproductive system of M. arenaria. One of the primary sites of Hn cell invasion in the soft-shell clam is the connective tissue of the gonads (Cooper, 1979; Brousseau, 1987). Based on histological observations, this invasion is not apparent, however, until the clam reaches an Hn stage of 2. In Hn stage 1 clams, neoplastic cells were not obvious anywhere in the gonadal tissue (Fig. 1-11A). These clams were indistinguishable from non-diseased individuals. Variations in the extent of gonadal invasion were evident in Hn stage 2 individuals (Fig. 1-11B, C). These variations reflect the wide range of Hn cells present in the hemolymph of stage 2 clams (16-70%). The gonads of Hn stage 3 clams appeared pale and shrunken with a watery consistency. Internally, the gonads of virtually all Hn stage 3 clams were heavily infiltrated with neoplastic cells (Fig. 1-11D, E). The gonadal follicles appear reduced in size as the connective tissue around them becomes increasingly packed with neoplastic cells. In some cases, the gonadal follicles appear as islands surrounded by a "sea" of neoplastic cells. In Hn stage 2 and 3 clams, formation of gametes within the follicles appears normal, if somewhat reduced, except in very extreme cases of infiltration. In some severely infiltrated animals, it was impossible to determine the sex of the individual (Fig. 1-11F). In addition, one clam showed evidence of a gonadal neoplasm.
In this animal, the center of the follicle was filled with neoplastic cells rather than the surrounding connective tissue as described by Yevich and Barszcz (1977).

The effects of Hn on reproductive capabilities in the soft-shell clam were examined quantitatively through comparisons of mean gonadal follicle size and number in normal and Hn stage 2 and 3 clams (Table 1-5). This analysis revealed that the gonadal follicles were significantly smaller in Hn stage 2 and stage 3 clams (23% and 32%, respectively) than in their non-diseased counterparts. Hn stage 1 clams were excluded from comparisons because their gonads, upon inspection, were indistinguishable from those of normal clams. In order to examine the point at which the difference in follicle size becomes significant, Hn stage 2 clams were further analyzed as 2 groups: those in which less than 50% of the hemolymph was comprised of Hn cells, and those with greater than 50% Hn cells. The mean gonadal follicle size in both of these categories of Hn stage 2 clams differed significantly from that of non-diseased individuals. These data suggest that Hn begins to have an impact on the gonadal follicles at a stage in which 15% or more of the clams circulating cells are neoplastic and continues as the disease progresses. Although actual gametes were not counted, the data suggest that Hn clams have reduced reproductive capabilities since Hn clams have smaller gonadal follicles and thus less space in which gametes may develop. No differences in the mean number of gonadal follicles per 0.5mm² were evident between any of the groups of animals examined. Since Hn stage 2 and 3 clams appeared to be spawning at the same time as the general population and no reductions in the number of follicles were present, resorption of the gonadal follicles themselves was not apparent. While no conclusions can be drawn about
arrestment of gametogenesis or resorption of the gametes, some evidence of these processes exists in the fact that some of the most heavily burdened Hn animals were excluded from comparisons as they could not be paired with a corresponding non-diseased individual or their sex was indeterminable.

Hn Prevalence and Clam Size

The size frequency distribution of clams in each monthly sample is presented in Figure 1-12. Clam lengths are grouped in 10 mm increments over the length range of the clams collected. In general, the Little Buttermilk Bay flat was populated by tremendous numbers of small clams with some larger ones, while the flat at New Bedford Harbor had a more random distribution of size classes. At NBH most of the 1990 samples were dominated by larger clams (>60 mm). Smaller clams began to appear with greater frequency beginning in August - September of 1990. Prior to this, the NBH clam flat had received a very large set of soft-shell clams for the first time in a few years (Leavitt, personal communication). This cohort formed a large portion of the clams collected throughout the remainder of the sampling period (Aug. 1990 - Dec. 1991). Their growth into larger size classes is evident in the shifting peaks in the size frequency data. At LBB most monthly samples during 1990 were dominated by clams 50-70 mm in length. Beginning in December of 1990 and continuing through June 1991, the samples from LBB took on a bimodal distribution as a result of a new cohort reaching collectable size during the late winter. This group then dominated the monthly samples from July 1991 until December 1991.

Hn prevalence with respect to clam length(size) is presented in Figure 1-13. Chi-square analysis revealed that Hn prevalence was independent of clam length at LBB but dependent upon length at NBH (p<0.05). At NBH, the
greatest prevalences of Hn were found in clams ranging from 40-70 mm in length. Although not significant, some suggestion of increased Hn prevalence is evident in 40-60 mm clams from Little Buttermilk Bay.

**Hn Prevalence within a Cohort**

As previously stated, the study site at New Bedford Harbor received a large set of clams during 1989-90. This cohort was identifiable in the monthly sample size frequency data beginning in August of 1990. Data from field grid samples over the next 18 months allowed the determination of the mean abundance of both the total and cohort soft-shell clams populations per 0.05 m² (Fig. 1-14). Inspection of the data reveals that this particular cohort does indeed make up a large proportion of the total clam population during the time frame of this study. The prevalence of Hn in this cohort at each sample is presented in Figure 1-15. These data grouped by 10 mm increments in shell length and month are presented in Figure 1-16. Hn prevalence in the cohort was stable during the first 3 quarters sampled (Nov. 1990 - May 1991). Beginning in August of 1991 and continuing to the end of sampling, a large increase in Hn prevalence, and especially Hn stage 2 and 3, was evident in this group. This increase in Hn prevalence began when the cohort reached the 50-70 mm size range. The cohort data supports the conclusions from the population data and from Leavitt et al., (1990) that clams in the size range of 40 - 70 mm have the highest prevalence of Hn. Seasonal changes in Hn prevalence in the cohort clams mirrored those seen in the total clam population (Figure 1-17). Hn prevalence in the cohort, as in the total population, was highest during the late summer - fall.
Figure 1-2. Soft-shell clam hemocytes fixed and stained with Toluidine Blue O. Scale bar = 10 μm. A) Normal clam hemocytes. Cell diameters measured at longest axis = 20-40μm. B) Hn Cells. Cell diameters measured at longest axis = 7-10μm.
Table 1-1. Morphometrics of normal and Hn soft-shell clam hemocytes. Values shown are mean ± S.E.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>n</th>
<th>Diameter</th>
<th>Area</th>
<th>Diameter</th>
<th>Area</th>
<th>Area</th>
<th>Ratio</th>
<th>Ratio</th>
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<tr>
<td></td>
<td></td>
<td>μm</td>
<td>S.E.</td>
<td>μm²</td>
<td>S.E.</td>
<td>μm</td>
<td>S.E.</td>
<td>μm²</td>
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<td>81.1</td>
<td>1.6</td>
<td>6.5</td>
<td>0.1</td>
<td>30.3</td>
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</table>

*a No distinction was made between cell types (i.e., granulocytes and agranulocytes).

*b This value is the longest axis of the cell.

*c This value is determined by subtracting the area of the nucleus from the area of the cell.

*d This value is determined by dividing the area of the nucleus by the area of the cytoplasm.
Figure 1-3. Hn prevalence in soft-shell clams from New Bedford Harbor (A) and Little Buttermilk Bay (B) (n=50). Note that the prevalence of Hn was usually greater at New Bedford Harbor than Little Buttermilk Bay.
Table 1-2. Hn prevalence at New Bedford Harbor and Little Buttermilk Bay during 1990-91 (A) and during each year at New Bedford Harbor (B) and Little Buttermilk Bay (C). Chi-square analysis was used in comparisons of Hn prevalence between sites and between years at each site.

A) New Bedford Harbor and Little Buttermilk Bay

<table>
<thead>
<tr>
<th>Site</th>
<th>Sample size</th>
<th>Number of Hn clams</th>
<th>% Hn</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Bedford Harbor</td>
<td>1169</td>
<td>288</td>
<td>24.6% **</td>
</tr>
<tr>
<td>Little Buttermilk Bay</td>
<td>1190</td>
<td>224</td>
<td>18.8%</td>
</tr>
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</table>

B) New Bedford Harbor

<table>
<thead>
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<th>Year</th>
<th>Sample size</th>
<th>Number of Hn clams</th>
<th>% Hn</th>
</tr>
</thead>
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<tr>
<td>1990</td>
<td>569</td>
<td>112</td>
<td>19.7%</td>
</tr>
<tr>
<td>1991</td>
<td>600</td>
<td>176</td>
<td>29.3% **</td>
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</table>

C) Little Buttermilk Bay

<table>
<thead>
<tr>
<th>Year</th>
<th>Sample size</th>
<th>Number of Hn clams</th>
<th>% Hn</th>
</tr>
</thead>
<tbody>
<tr>
<td>1990</td>
<td>590</td>
<td>97</td>
<td>16.4%</td>
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<tr>
<td>1991</td>
<td>600</td>
<td>127</td>
<td>21.2% ns</td>
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</table>

** Values significantly different at p<0.001.
ns Values not significantly different.
Figure 1-4. Seasonal prevalence of Hn at New Bedford Harbor (A) and Little Buttermilk Bay (B). Values shown are mean ± S.E. with the following designations: Winter = Dec, Jan, Feb; Spring = Mar, Apr, May; Summer = Jun, Jul, Aug; Fall = Sep, Oct, Nov.

A) New Bedford Harbor

B) Little Buttermilk Bay

*Indicates significant difference at p<0.05 level in t-tests on arcsin transformed data.
Figure 1-5A. Sections of gonadal tissue from female clams at each stage of the reproductive cycle: a) Indifferent, b) Developing, c) Ripe, d) Partially spawned, e) Spent. Scale bar = 50 μm.
Figure 1-5B. Sections of gonadal tissue from male clams at each stage of the reproductive cycle: a) Indifferent, b) Indifferent with atypical spermatogenesis occurring, c) Developing, d) Ripe, e) Partially spawned, f) Spent. Scale bar = 50 μm.
Figure 1-6. Proportion of clams from New Bedford Harbor (A) and Little Buttermilk Bay (B) at each developmental phase of the reproductive cycle. Males and females are combined (n ≥10).

A) New Bedford Harbor

B) Little Buttermilk Bay
Table 1-3. Monthly gonad displacement volume/shell length data.

<table>
<thead>
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<th>Month</th>
<th>Slope (gonad vol/ shell length)</th>
<th>R²</th>
<th>n</th>
<th>Slope (gonad vol/ shell length)</th>
<th>R²</th>
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<tr>
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<td>0.39</td>
<td>34</td>
<td>0.180</td>
<td>0.58</td>
</tr>
<tr>
<td>Mar</td>
<td>0.302</td>
<td>0.44</td>
<td>47</td>
<td>0.233</td>
<td>0.63</td>
</tr>
<tr>
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<td>0.64</td>
<td>44</td>
<td>0.102</td>
<td>0.44</td>
</tr>
<tr>
<td>May</td>
<td>0.090</td>
<td>0.37</td>
<td>44</td>
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<td>0.71</td>
</tr>
<tr>
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<td>0.141</td>
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<td>0.192</td>
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<td>0.52</td>
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<td>0.56</td>
</tr>
<tr>
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<td>0.56</td>
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<td>0.094</td>
<td>0.69</td>
</tr>
<tr>
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<td>0.094</td>
<td>0.69</td>
</tr>
<tr>
<td>Aug</td>
<td>0.113</td>
<td>0.80</td>
<td>32</td>
<td>0.104</td>
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<tr>
<td>Sep</td>
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<td>0.39</td>
<td>34</td>
<td>0.180</td>
<td>0.58</td>
</tr>
<tr>
<td>Oct</td>
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<td>0.44</td>
<td>47</td>
<td>0.233</td>
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</tr>
<tr>
<td>Nov</td>
<td>0.215</td>
<td>0.64</td>
<td>44</td>
<td>0.102</td>
<td>0.44</td>
</tr>
<tr>
<td>Dec</td>
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<td>0.37</td>
<td>44</td>
<td>0.135</td>
<td>0.71</td>
</tr>
<tr>
<td>Jan</td>
<td>0.141</td>
<td>0.69</td>
<td>45</td>
<td>0.192</td>
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</tr>
<tr>
<td>Feb</td>
<td>0.151</td>
<td>0.52</td>
<td>43</td>
<td>0.115</td>
<td>0.56</td>
</tr>
<tr>
<td>Mar</td>
<td>0.141</td>
<td>0.56</td>
<td>43</td>
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<td>0.69</td>
</tr>
<tr>
<td>Apr</td>
<td>0.113</td>
<td>0.80</td>
<td>36</td>
<td>0.084</td>
<td>0.80</td>
</tr>
<tr>
<td>May</td>
<td>0.135</td>
<td>0.73</td>
<td>38</td>
<td>0.097</td>
<td>0.72</td>
</tr>
<tr>
<td>Jun</td>
<td>0.149</td>
<td>0.88</td>
<td>33</td>
<td>0.089</td>
<td>0.82</td>
</tr>
<tr>
<td>Jul</td>
<td>0.141</td>
<td>0.56</td>
<td>43</td>
<td>0.094</td>
<td>0.69</td>
</tr>
<tr>
<td>Aug</td>
<td>0.113</td>
<td>0.80</td>
<td>32</td>
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<td>0.67</td>
</tr>
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<td>0.267</td>
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<td>0.180</td>
<td>0.58</td>
</tr>
<tr>
<td>Oct</td>
<td>0.302</td>
<td>0.44</td>
<td>47</td>
<td>0.233</td>
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</tr>
<tr>
<td>Nov</td>
<td>0.215</td>
<td>0.64</td>
<td>44</td>
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<td>0.44</td>
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<tr>
<td>Dec</td>
<td>0.090</td>
<td>0.37</td>
<td>44</td>
<td>0.135</td>
<td>0.71</td>
</tr>
</tbody>
</table>

56
Figure 1-7. Relationship (slope) of gonad volume to shell length for 40-70 mm clams for each month of the study at New Bedford Harbor (A) and Little Buttermilk Bay (B). Bars indicate spawning in the population based on histological data.

A) New Bedford Harbor

B) Little Buttermilk Bay
Figure 1-8. Comparison of combined mean monthly water temperatures for New Bedford Harbor and Little Buttermilk Bay and spawning. Bars indicate spawning in the populations.
Figure 1-9. Proportion of Hn stage 2 and 3 soft-shell clams from New Bedford Harbor and Little Buttermilk Bay combined at each developmental phase of the reproductive cycle. Males and females are combined (n ≥ 5).
Figure 1-10. Comparison of Hn prevalence and spawning in soft-shell clams from New Bedford Harbor (A) and Little Buttermilk Bay (B) (n=50). Bars represent spawning based on histological and monthly gonad vol/shell length data.

A) New Bedford Harbor

B) Little Buttermilk Bay
Table 1-4. Hn prevalence in male and female soft-shell clams from New Bedford Harbor (A) and Little Buttermilk Bay (B).

### A) New Bedford Harbor

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
<th>Immature</th>
<th>Unknown</th>
<th>Ratio Males : Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Clams</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obs.</td>
<td>498</td>
<td>235</td>
<td>255</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>% of Total</td>
<td>47.2</td>
<td>51.2</td>
<td>1.2</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Hn Clams</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obs.</td>
<td>177</td>
<td>80</td>
<td>94</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>% of Total</td>
<td>45.2</td>
<td>53.1</td>
<td>0.6</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>

### B) Little Buttermilk Bay

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
<th>Hermaphrodites</th>
<th>Immature</th>
<th>Unknown</th>
<th>Ratio Males : Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Clams</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obs.</td>
<td>461</td>
<td>207</td>
<td>231</td>
<td>1</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>% of Total</td>
<td>44.9</td>
<td>50.1</td>
<td>0.2</td>
<td>4.6</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Hn Clams</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obs.</td>
<td>129</td>
<td>58</td>
<td>65</td>
<td>0</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>% of Total</td>
<td>45.0</td>
<td>50.4</td>
<td>3.9</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1-11. Gonadal tissues affected by increasing stages of Hn:
A) Hn stage 1, B) Hn stage 2 (low level: 16-50%), C) Hn stage 2 (high level: 51-70%), D) Hn stage 3: male, E) Hn stage 3: female, F) Hn stage 3: sex could not be determined. Scale bar = 50 μm.
Table 1-5. Comparison of mean gonadal follicle size and number of gonadal follicles per 0.5 mm$^2$ between pairs of normal and Hn stage 2 and 3 soft-shell clams. Values shown are means (+ S.E.).

<table>
<thead>
<tr>
<th></th>
<th>Normal (16-70%)</th>
<th>Stage 2 (&lt;50%)</th>
<th>Normal Stage 2 (&gt;50%)</th>
<th>Normal Stage 3 (&gt;70%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>23</td>
<td>7</td>
<td>60</td>
</tr>
<tr>
<td>Length (mm), $\bar{x}$</td>
<td>56.7 (2.0)</td>
<td>57.0 (2.3)</td>
<td>55.6 (4.9)</td>
<td>57.0 (1.3)</td>
</tr>
<tr>
<td>S.E.</td>
<td></td>
<td>(2.0)</td>
<td>(2.2)</td>
<td>(1.3)</td>
</tr>
<tr>
<td>Comparison</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gonadal Follicle Size ($\mu m^2$), $\bar{x}$</td>
<td>21.1 (1.3)</td>
<td>21.3 (1.5)</td>
<td>20.7 (2.9)</td>
<td>18.9 (0.8)</td>
</tr>
<tr>
<td>S.E.</td>
<td></td>
<td>(1.2)</td>
<td>(1.4)</td>
<td>(0.7)</td>
</tr>
<tr>
<td>No. of follicles/0.5mm$^2$, $\bar{x}$</td>
<td>18.9 (1.1)</td>
<td>20.2 ns</td>
<td>19.3 (0.7)</td>
<td>20.1 ns</td>
</tr>
<tr>
<td>S.E.</td>
<td></td>
<td>(1.1)</td>
<td>(0.6)</td>
<td></td>
</tr>
</tbody>
</table>

* Paired observations significantly different at p<0.001.

**ns** Paired observations not significantly different.
Figure 1-12. Monthly size frequency distribution of clams sampled from New Bedford Harbor (A) and Little Buttermilk Bay (B) during 1990 (1) and 1991 (2).

January

February

March

April

May

June

July

August

September

October

November

December

Frequency

40
30
20
10
0
40
30
20
10
0
40
30
20
10
0
40
30
20
10
0
40
30
20
10
0
40
30
20
10
0
40
30
20
10
0

Shell Length (mm)

66

January

February

March

April

May

June

July

August

September

October

November

December

Shell Length (mm)
B-1) Little Buttermilk Bay, 1990

January

February

March

April

May

June

July

August

September

October

November

December

Shell Length (mm)
Figure 1-13. Hn prevalence in different sizes of soft-shell clams from New Bedford Harbor (A) and Little Buttermilk Bay (B).

A) New Bedford Harbor

B) Little Buttermilk Bay
Figure 1-14. Total and cohort soft-shell clam abundance at New Bedford Harbor based on field grid samples of 5 cores (mean ± S.E.)
Figure 1-15. Hn prevalence in cohort soft-shell clams from New Bedford Harbor at each quarterly sample (n = 50).
Figure 1-16. Hn prevalence in the New Bedford Harbor cohort clams at each quarterly sample expressed as a function of size.
Figure 1-17. Comparison of Hn prevalence in the total and cohort soft-shell clam populations at New Bedford Harbor at each quarterly sample (n=50).
DISCUSSION

Hematopoietic neoplasia, a progressive and potentially fatal condition, is a significant disease problem in the soft-shell clam, *Mya arenaria*. This condition has been reported from clam populations all along the Atlantic coast of the United States, and like epizootics in other bivalves, is characterized by a unique cytology, extensive cell proliferation, a high level of prevalence (Farley, 1989) and a seasonal disease cycle (Leavitt et al., 1990). The Hn cells form a relatively homogenous population of cells within the hemolymph of the clam. Since the discovery of this condition, several methods of diagnosis have been developed including: 1) examination of fresh hemocytes with a hemocytometer and phase-contrast microscopy (Cooper et al., 1982b), 2) fixation and staining of hemocytes with Giemsa, Wright's stain (Cooper et al., 1982b) or Feulgen Picromethyl Blue (Farley et al., 1986) followed by examination with bright-field microscopy, and 3) indirect peroxidase immunochromical staining (IP) which incorporates peroxidase staining of neoplastic cells using antibodies specific for Hn cells (Smolowitz and Reinisch, 1986). According to Peters (1988), the monoclonal antibodies, while extremely sensitive, may be of limited value due to their restricted availability. The hemocytometer method, is more convenient to use but less accurate than either the Feulgen Picromethyl Blue method or the IP staining for early stages of the disease (<1% Hn cells) (Smolowitz and Reinisch, 1986). Because of its accessibility and relative ease of use for evaluating large numbers of clams, the hemocytometer method was chosen for use in this study.

Regardless of the preparation or staining methods used, the Hn cells are anaplastic in appearance with lobed nuclei, prominent nucleoli and a
high nuclear to cytoplasmic ratio. Mitotic figures are commonly observed. A comparison of the morphometrics for normal and neoplastic cells in fixed blood smears stained with Toluidine Blue O provided further indications that these cell types were distinctly different. The cell measurements from this method of preparation fell within the ranges of those reported by Cooper (1979) and suggest that this may be another possible staining procedure for the examination of Hn cells. In all cases, the methods of preparation utilized should accompany the measurements, since these values are influenced by such things as settling time of the cells, fixation, and staining. While morphometric analysis of the cells in histological preparations is useful, it permits only a two-dimensional description of the cells. Future studies that include cell comparisons based on volume and three-dimensional analysis are necessary to explore further differences between Hn and normal hemocytes.

The limitations of the method of diagnosis must also be considered in analyses of Hn prevalence. While the fresh bleed method is accurate for >1% Hn cells in the hemolymph, it is less so for lower levels (Smolowitz and Reinisch, 1986). Since this study involved bleeding large numbers of clams and because the IP staining method was not available, the simpler hemocytometer method was utilized even though it meant sacrificing accuracy. Because of this limitation, no attempts were made to detect Hn in clams in which less than 1% of the circulating cells were neoplastic. This bias may have resulted in an underestimate of Hn levels in the clam populations and must be kept in mind in all analyses of disease prevalence.

The average prevalence of Hn was 18.8% for Little Buttermilk Bay and 24.6% for New Bedford Harbor. These values are slightly higher and somewhat lower, respectively, than those reported by Leavitt et al. (1990) at
the same sites (average Hn prevalence at LBB = 17.0% and at NBH = 39.3%).
These data, together with the annual and seasonal (Winter, Spring, Summer, Fall) Hn prevalences, further highlight the variations in Hn levels within the populations over time. In addition, Hn prevalence varies significantly with the source of the clams. In agreement with the results reported by Leavitt et al. (1990), New Bedford Harbor, the more polluted site, had consistently higher levels of Hn in the clam population than Little Buttermilk Bay. In spite of numerous attempts to correlate Hn prevalences with general environmental pollutants (Appeldoorn et al., 1984; Farley et al., 1986), petroleum hydrocarbons (Yevich and Barszcz, 1977; Brown et al., 1979; Appeldoorn et al., 1984), polychlorinated biphenyls (Reinisch et al., 1984), and chlordane (Farley et al., 1991) it is still unknown why some areas support higher levels of Hn prevalence than others.

The overall seasonal patterns of Hn prevalence at both sites of the present study are in agreement with those reported by Leavitt et al. (1990) for the same two sites and with the results of Cooper et al. (1982a) from Allen Harbor, Rhode Island. At both sites there were indications of a biphasic seasonal disease cycle with the first peak occurring in the fall followed by a second peak during the late winter-early spring. Minima were observed during the winter and summer months. Differences between this study and that of Leavitt et al. (1990) are apparent both in the overall prevalence of Hn, particularly with regards to the numbers of Hn stage 1 clams, and the precise Hn seasonal pattern which appeared to vary slightly from year to year. These differences may, in part, be attributable to the methods used in Hn diagnosis, and to normal annual and seasonal variations.

Based on suggestions by Cooper et al. (1982b) that Hn seasonality may be related to spawning, comparisons of the clam's reproductive cycle and Hn
seasonal cycle were made. In general, the reproductive system of the soft-shell clam is similar in both males and females and is comprised of a pair of gonads and simple gonoducts. The gonads themselves consist of highly ramified tubules bearing numerous terminal and lateral alveoli (Stickney, 1963). In a well-nourished, ripe individual, the alveoli (follicles) fill much of the visceral mass. The sex of the soft-shell clam can only be determined through histological examination of the gonads or examination of the sexual products after the ovaries or testes have been cut open.

*Mya arenaria* is a dioecious bivalve with reports of hermaphrodites occurring at very low frequencies (Coe and Turner, 1938; Otto, 1972, 1973; Porter, 1974; Brousseau, 1978a, 1987b; this study). In all studies containing information on the proportion of male to female clams, sex ratios of 1:1 were reported (Coe and Turner, 1938; Porter, 1974; Brousseau, 1978a, 1987b; this study). Based on the 1:1 male to female sex ratio of neoplastic clams in this study, it can be concluded that Hn prevalence is not related to the sex of the soft-shell clam. While similar findings have been reported for hematopoietic neoplasms in *Mytilus edulis* (Peters, 1988), and *Cerastoderma edule* (Twomey and Mulcahy, 1988) and for hematopoietic and gonadal neoplasms in *Mya arenaria* (Yevich and Barszcz, 1977; Appeldoorn et al., 1984), this study is the first to present data to establish the relationship between sex (male vs. female) and Hn prevalence.

Seasonal development of the gonads has been well-described for a number of *M. arenaria* populations. In common with many others along the U.S. Atlantic coast, especially those from south of Cape Cod (Pfitzenmeyer, 1972; Brousseau, 1987b) and at Cape Ann, MA (Brousseau, 1978a), the clam populations of New Bedford Harbor and Little Buttermilk Bay exhibited a biannual spawning cycle during each year of this study. In this cycle gamete
development commenced in the winter with spawning in April - May, followed by a second cycle of gametogenesis that concluded with spawning in August - October. While further histological evaluation of clam gonads from more sampling dates is necessary to determine the precise period of spawning in these populations, comparisons of spawning and the Hn seasonal cycle indicate that Hn prevalence is highest around the times of spawning. At both NBH and LBB, spawning followed the late winter - early spring maximum and occurred in conjunction with or slightly preceded the fall maximum. Comparisons of the current spawning information with Hn prevalence data from Leavitt et al. (1990) suggest similar conclusions. No conclusions of this sort, however, can be drawn from the data of Brousseau (1987a, b) on clam populations in Long Island Sound. The clams in this area were spawning in either the spring and summer on an annual cycle or in the summer and fall on a biannual cycle with neoplasia prevalences highest during the late fall - winter (Brousseau, 1987a, b). The only suggestion that peak Hn prevalence was related to spawning was in the observation that the spring spawnings were in some cases preceded by high Hn prevalence in the population. The other factor that needs to be considered in evaluating these correlations is that the secondary (winter) peak in Hn prevalence that occurs at some sites, is thought to result from reduced mortality because of low ambient water temperatures, rather than an increase in disease incidence (Brown et al., 1977; Appeldoorn et al., 1980; Farley, 1987; Leavitt et al., 1990). In this study and others (Cooper, 1979; Leavitt et al., 1990), the fall - winter peak in Hn prevalence was comprised of large numbers of Hn stage 2 and 3 individuals which may live longer in colder water temperatures.

In view of the conflicting evidence, no firm conclusions about correlations between Hn seasonality and spawning are possible. In general, it
appears that spawning is not consistently preceded, followed or concurrent with high Hn prevalence in the clams. While spawning and increased Hn prevalence may not have a cause-and-effect relationship, some other more loosely defined relationship is suggested by the observations that 1) high Hn prevalence and spawning do occur closely in time in some cases, and 2) Hn prevalence appears to be highest in those clams approaching or in the midst of their peak reproductive outputs.

The gametogenic process represents a very significant energy expenditure by bivalve molluscs, and spawning leaves the individual with depleted nutrient reserves (Bayne, 1975). In healthy Mytilus edulis, reproduction is associated with decreased growth and increased mortality (Bayne et al., 1978). One possible explanation for the occurrence of peak Hn prevalence around the time of spawning is that the reproductive process itself is acting in conjunction with other factors (e.g., temperature extremes or environmental contaminants) as a stressor on the clam. Appeldoorn et al. (1984) hypothesized that the neoplastic disease of the soft-shell clam was a result of a synergistic interaction between a virus and chemical carcinogen. Results of bioassay studies by Brown (1980) indicate that nonspecific stress in the clam enhances its susceptibility to neoplasia. In M. arenaria, it is possible that gametogenesis or spawning enhances the development or progression of Hn in the clam and thus contributes to high Hn prevalence in the population.

Further support for the idea that reproduction is a stressor that may increase Hn is present in the relationship between Hn prevalence and size (age) of the clams. Sexual maturation in the soft-shell clam is a function of size and is generally attained between 20 and 40 mm or at about 1 year of age (Belding, 1930; Hanks, 1960; Pfitzenmeyer, 1972; Porter, 1974). Peak
reproductive outputs are not reached until well after the animal's first reproductive cycle (Brousseau, 1978a). The results of this study, as well as those of Cooper et al. (1982a) and Leavitt et al., (1990), indicate that Hn prevalence is highest in clams 3 and 4 years old (40-70 mm) and somewhat lower in clams both older and younger. Hn prevalence appears to be highest in those clams approaching or in the midst of their peak reproductive outputs. It is in these animals that the stress of reproduction would be at its greatest level and would be most likely to enhance Hn.

The fact that the natural reproductive process may be acting as one of the stressors on the soft-shell clam's system may also help to explain some of the geographic trends in Hn prevalence. In general the trend in Hn prevalence is for more contaminated areas like Searsport, ME (Yevich and Barszcz, 1977), Allen Harbor, RI (Cooper et al., 1982a), and New Bedford Harbor, MA (Reinisch et al., 1984; Leavitt et al., 1990; present study), and for areas, like the Chesapeake Bay, where the disease is newly introduced (Farley et al., 1986) to have higher prevalences than less contaminated areas where Hn levels are thought to be at background levels (Brown, 1980). It is possible that while in polluted areas, reproduction and a host of contaminants are acting synergistically to enhance Hn prevalence in the clam population, reproduction alone may act with the disease to enhance prevalence at more pristine sites.

The physiological effects of Hn on the soft-shell clam are dependent upon the stage of the disease. While Cooper et al. (1982b) reported a correlation between the level of Hn and the amount of tissue degradation, Leavitt et al. (1990) found that neoplastic clams show a reduction in both total carbon composition and percent carbon in their tissues and suggested that this reduction is a reflection of the disruption of normal organ function by Hn.
cells in heavily diseased clams. The qualitative and quantitative effects of Hn on reproductive tissues are readily apparent from histological sections of gonadal material. The gonads of early Hn clams (Hn stage 1) were indistinguishable from those of normal individuals, and normal gametogenesis appeared to be occurring. As Hn infiltration of the tissues progressed, the disorder caused a significant reduction in the size and appearance of the germinal follicles. In some cases, extensive invasion lead to the loss of sexual characteristics and the arrest of gametogenic development. The significant reduction in the size of the gonadal follicles was present beginning at a point when more than 15% of clam's circulating cells were neoplastic (Hn stages 2 and 3). This reduction in follicle size suggests that increasing disease leads to a reduction in reproductive capabilities in the clams since there is less space available for gametes. Further work involving comparisons of actual gamete production in normal and Hn clams is necessary in order to firmly establish this conclusion. While the germinal follicles were certainly disrupted by Hn cells, in most cases there was no apparent loss of follicles from the gonad. Only in the most heavily diseased individuals were there any indications of follicle resorption. In these clams, the gonadal tissue was almost entirely taken over by neoplastic cells.

Based on examinations of blood and gonadal tissues, it appears that Hn, as it progresses, is an increasingly stressful condition for the diseased clam. In spite of this, gametogenesis appears to be occurring normally, if at somewhat reduced levels, in most Hn stage 2 and some Hn stage 3 clams. This continuation of gametogenesis may depend not only on the physiological state of the clam, but also on environmental conditions (temperature and nutrients) and extent of Hn invasion when development of the gametes is
initiated. According to Bayne (1975), stressed mussels devote a large proportion of their resources to the growth and maturation of the gametes in spite of the significant energy drain placed on other body systems. While *Mytilus* can continue gametogenesis during temperature and nutritive stress, as long as the gametes are not ripe, stress that occurs after complete gamete maturation results in resorption of the sperm and eggs, and recession of the gonad (Bayne, 1975). This continuation of gametogenesis may depend upon a supply of nutrients, either from the external environment or internally stored reserves (Bayne, 1975). In the case of the soft-shell clam, nutrient reserves are concentrated in the follicle cells of the gonad (Coe and Turner, 1938). Any factors, such as environmental contaminants, temperature and salinity extremes, and/or disease, that interfere with the organism’s nutrient supply or ability to lay down reserves for future cycles of gametogenesis has a potential impact on the growth, maturation, or vitality of the gametes or on the resulting larvae. While further work is necessary to examine the effects of Hn on the development of the gametes and the resulting larvae, the data in general suggest that Hn reduces the reproductive capabilities of diseased individuals and impedes their ability to contribute to the population gene pool.

In addition to its impacts on reproduction, Hn may directly alter the size distribution of soft-shell clam populations by removing particular size classes through mortality. As previously stated, Hn prevalence is greatest in clams 40-70 mm in length and lower in clams both older and younger (Cooper et al., 1982b, Leavitt et al., 1990; this study). The tracking of Hn prevalence through a cohort supports the view that juvenile clams show an increase in Hn prevalence upon reaching their second summer (Cooper et al., 1982b). While further work is necessary to monitor growth and insure that
only a single cohort was included in these analyses, a large increase in Hn prevalence was evident in cohort clams upon reaching the most disease-susceptible size range during the fall and winter of 1991-92. Disease progression is suggested by the fact that increased numbers of Hn stage 1 clams present in the August 1991 sample appear to give way to large numbers of Hn stage 2 and 3 clams in the November 1991 and February 1992 samples. Mortality of these clams is anticipated based on the results of previous field and laboratory studies (Cooper et al., 1982b; Farley et al., 1986; Farley, 1989; Brousseau and Baglivo, 1991; Farley et al., 1991).

Overall, the cohort data suggest that size distribution of clams in the population should be considered in examining trends in Hn seasonality and may help to explain some of the annual variation that is characteristic of this condition. Because this cohort was such a large part of the total population, changes in this group were in part responsible for the results seen in the NBH clam population as a whole. In response to the objectives to be addressed by this study the following statements can be made: 1) the cohort did experience an increase in Hn as it grew into the sexually mature size range in which Hn prevalence was highest; and 2) the increased number of clams of this size range appeared to result in an increase in Hn prevalence for the flat as a whole. While further sampling is still necessary, it is suspected that a large portion of the clams in this disease-susceptible size range will be removed by mortality. The presence of a large cohort in the size range in which Hn prevalence is highest at a time of year when disease is routinely high in the population served to amplify the results seen for the population as a whole.

Overall, the results of this study provide further evidence that the blood disorder, hematopoietic neoplasia, has a significant impact on populations of the soft-shell clam, *Mya arenaria*. In particular this condition
has been shown to affect soft-shell clam reproductive tissues and size distribution within a population. Because Hn adversely affects the organism's ability to survive and contribute to the population gene pool, this condition must be considered in the evaluation of soft-shell clam population dynamics as well as in the development of appropriate management strategies for this commercially valuable species.
CHAPTER II
EFFECTS OF HN ON THE EXCRETORY SYSTEM
OF THE SOFT-SHELL CLAM

INTRODUCTION

The excretory system of the soft-shell clam, as in most bivalves, functions, in conjunction with the circulatory system, to filter, transport, accumulate, and eliminate wastes and foreign material. The major excretory organs are the kidneys and the pericardial gland, also referred to as Keber's gland, which is associated with the pericardial cavity (Fig. 1). Normal hemocytes play an important role in excretion since they are the cells that phagocytize foreign matter and transport it to the kidney for storage and/or elimination. The presence of hydrolytic enzymes (Huffman and Tripp, 1982), and an increase in lipase activity in actively phagocytizing hemocytes of M. arenaria (Cheng and Yoshino, 1976) indicate that hemocytes have a lysosomal function.

The excretory system of soft-shell clams with hematopoietic neoplasia may be compromised by: 1) invasion and compression of the kidney and pericardial gland tissue by increasing numbers of neoplastic cells; and 2) an inability to phagocytize and transport nutrients and foreign particles since Hn cells have an altered morphology compared to normal hemocytes. While in vitro studies examining phagocytosis in normal and Hn hemocytes indicate that Hn cells may recognize and ingest some foreign particles, but not others (Beckmann et al., 1992), little is known about the ability of Hn animals
to clear foreign materials from their body fluids. Effects of Hn on clam kidney and hemocyte function are important to examine since disruption of normal function in these areas may lead to a decreased ability to remove waste products and foreign matter from its body and an inability maintain normal homeostasis.

One way of examining and imaging both normal and pathological conditions in vertebrates and especially humans has been through the practice of nuclear medicine and the use of radiopharmaceuticals (Bernier et al., 1989). Recently, the applicability of radiopharmaceuticals has been extended to marine organisms (Burn et al., 1990a, b; Potts et al., 1992; Burn et al., in press). Prior to these studies, the distribution of these agents in non-mammalian vertebrates or invertebrates had not been investigated. The goal of this study was to examine effects of Hn on the soft-shell clam excretory system, in particular clam kidney function and hemocyte phagocytosis, through the use of radiopharmaceuticals. In order to apply the techniques of nuclear medicine to the study of the Hn condition in the clam, it was first necessary to determine if these agents could reliably be used in this animal. Once this was established, the radiopharmaceutical technology could then be used to explore Hn effects on the soft-shell clam excretory system.

Radiopharmaceuticals are defined as tracer compounds composed of a physiologically active molecule coupled to a gamma ray-emitting nuclide (Chilton and Witkowski, 1986). The primary advantage of gamma ray-emitting nuclides over the more traditional beta particle-emitting radiotracers, $^{14}C$ and $^3H$, is their ability to be detected externally, imaged, and quantified in vivo using a scintillation (gamma) camera. Distribution and localization of the radiopharmaceutical reflects both the properties of the
compound itself and specific physiological functions, such as blood flow, bone mineralization, hepatocyte activity, antigen-antibody specificity and phagocytosis.

Based on preliminary data, two radiopharmaceuticals, Tc-99m-disofenin and Tc-99m-sulfur colloid, were chosen for use in Hn and normal soft-shell clams. This work represents the first reported attempts at using radiopharmaceuticals to study disease-related changes in organ function in non-mammalian vertebrates or invertebrates.

**Tc-99-m-Disofenin**

The use of iminodiacetic acid (IDA) to chelate technetium(Tc)-99m to various lidocaine analogs has played a prominent role in nuclear medicine studies of hepatobiliary function since its introduction in the mid-1970's (Krishnamurthy and Turner, 1990). The IDA-type radiopharmaceuticals are dimers comprised of a centrally located Tc-99m atom bound to two IDA molecules (Fig. 2-1A) (Burns et al., 1977; Loberg and Fields, 1978; Costello et al., 1983). Hepatocyte uptake of these compounds is mediated through the terminal lidocaine analogue, and both uptake and excretion are influenced by substitutions on the lidocaine phenyl ring (Chilton and Witkowski, 1986; Krishnamurthy and Turner, 1990). One of the most commonly used Tc-99m-IDA agents is diisopropylacetanilide iminodiacetic acid (referred to as DISIDA or disofenin), a small lipid-soluble molecule (mw = 348 daltons), in which the methyl groups are replaced with isopropyl groups at positions 2 and 6 on the phenyl rings (Fig. 2-1B). In mammals, this agent is useful for liver studies because it combines high hepatic specificity with rapid clearance. In other organisms, Tc-99m-disofenin, localizes in organs which handle lipophilic compounds, including the teleost liver, lobster hepatopancreas and antennal
Figure 2-1. General structure of an Tc-99m-IDA-type radiopharmaceutical (A) and specific structure of Tc-99m-disofenin (= Tc-99m-disopropylacetanilide iminodiacetic acid) (B) - (adapted from Chilton and Witcowski, 1986).
A) Lidocaine Analog

B) Diisopropylacetanilide iminodiacetic acid (Disofenin)
gland and bivalve excretory organs (Burn et al., in press). Because Tc-99m-disofenin localizes within the clam kidney and because Hn is known to impact the clam kidney, this agent was used to examine Hn effects on soft-shell clam excretory organ function.

**Tc-99m-Sulfur Colloid**

In healthy humans, intravenously injected Tc-99m-sulfur colloid is rapidly cleared from the blood as the colloid particles are phagocytized by cells of the reticuloendothelial (RE) system. In normal humans, 80-90% of the injected dose localizes in the Kupffer cells of the liver, 5-10% in the phagocytic cells of the spleen and the remainder in the reticulo-endothelial system of the bone marrow (Chilton and Witkowski, 1986; Bernier et al., 1989). Metabolism of the colloid is limited, and approximately 85% of the radiopharmaceutical is permanently localized within the liver (Bernier et al., 1989). The primary clinical application of this radiopharmaceutical is liver imaging in the evaluation of functional liver diseases, such as cirrhosis, hepatitis, or metabolic disorders, and the detection of hepatic lesions and abnormalities. According to Bernier et al. (1989), Tc-99m-sulfur colloid probably exists as Tc-99m-heptasulfide co-precipitated with colloidal sulfur particles. The colloidal particles are generated from acid (phosphorous or hydrochloric) decomposition of sodium thiosulfate. The precise structure of this agent is unknown. Since normal clam blood cells are phagocytic cells, it was thought that these cells would take up this radiocolloid, and that this agent could be used to examine differences in blood cell function for normal and Hn clams.

Overall, the aims of this study fell into two categories: 1) to establish if radiopharmaceuticals could be reliably injected, retained, distributed and then concentrated within the body and especially the excretory systems of normal
and Hn soft-shell clams; and 2) to assess effects of Hn on soft-shell clam excretory functions and the animal's ability to clear foreign material from the circulatory system.
MATERIALS AND METHODS

**Tc-99m-Disofenin and Tc-99m-Sulfur Colloid**

Normal and Hn clams from New Bedford Harbor and Little Buttermilk Bay were paired according to location and shell length in order to obtain the same size range of animals in each sample. The animals were then transported to Massachusetts General Hospital (MGH)-East, Charlestown, MA, where they were maintained in aerated, ambient (15-18 °C) seawater. This temperature range was chosen to reflect field conditions at the time of collection and to minimize further stress on the clams. Injections of either Tc-99m-disofenin (n=109) or Tc-99m-sulfur colloid (n=64) were made into the sinus surrounding animal's anterior adductor muscle using a 1/2", 25 g needle.

Both Tc-99m-disofenin and -sulfur colloid were prepared from commercial kits (Hepatolite, E.I. duPont de Nemours & Co., Billerica, MA, and Tesuloid, Squibb Diagnostics, New Brunswick, NJ, respectively) using sterile seawater in place of saline. Average injected dose per animal was 0.5 millicuries (mCi) (in 0.05 ml) for disofenin and 0.1 mCi (in 0.05 ml) for sulfur colloid. Distribution of the radiopharmaceutical was determined by scintigraphy (imaging on a gamma camera) using an Ohio Nuclear Sigma 410 Radioisotope Camera and Multichannel Analyzer combined with a Technicare 560 Computer. Imaging times of 0.08 hr (initial), 0.5 hr, 1 hr, 2 hrs, 3 hrs, 5 hrs, and 7 hrs post-injection were used for Tc-99m-disofenin, while images of clams injected with Tc-99m-sulfur colloid were collected at 0.08 hr (initial), 1 hr, 2 hrs, and 3.5 hrs. Scintigrams were obtained using a parallel collimator which yields contact print-like images with a maximum
resolution of approximately 6 mm. The images were 256 x 256 pixels in size and were acquired for 30 or 60 seconds each. Clams were imaged out of water and maintained in aerated, ambient seawater (15-18 °C) between imaging sessions.

Representative animals injected with Tc-99m-disofenin were also imaged at various times using a 1 mm pinhole collimator. The 1 mm pinhole collimator provides images with a resolution approaching 1 mm within a local region. In order to compensate for the pinhole collimator’s reduced sensitivity, imaging times were increased to 30 minutes.

Upon completion of imaging, the biodistribution of each radiopharmaceutical within the clam tissues was determined by removing various organs or pieces of organ (gill, kidney, Keber's gland, digestive gland) from individuals (n=8 and 17 for Tc-99m-disofenin and -sulfur colloid, respectively) and counting the activity in each tissue in a gamma well counter. Prior to counting, the weight (in grams) of each tissue piece was determined. The gonadal tissue from each animal was then prepared for histological examination as described in Chapter I.

**Tc-99m-Disofenin**

In order to further evaluate the localization of Tc-99m-disofenin within the kidney of the soft-shell clam, autoradiography was performed on selected kidneys of clams from New Bedford Harbor. Normal and Hn clams (n=35) collected in March 1992 from New Bedford Harbor were maintained at MGH-East, Charlestown, MA, injected with Tc-99m-disofenin, and imaged at 0.08 hr, 2 hrs, and 4 hrs, as described above. After 4 hrs, the kidneys were removed from clams (n=12) selected on the basis of initial injection and
kidney uptake of Tc-99m-disofenin. Following removal, the kidney tissues were fixed in 10% neutral buffered formalin, frozen in Tissue Tek® O.C.T. medium at -20 °C and sectioned at 10 μm on a freezing microtome. Sections were placed on alcohol-cleaned glass slides, and used for macro- or microautoradiography.

In macroautoradiography, the slides with kidney tissue were placed against the emulsion surface of x-ray film (Dupont Chronex 429 single emulsion x-ray film) in a film cassette. The cassette was stored in a -80 °C freezer for approximately 18 hrs during which the radiation from the sections directly exposed the film. After 18 hrs, the films were removed from the cassette and developed on a Kodak X-omat automatic film processor. The slides were stained with Harris’ hematoxylin. Views of the kidney tissue and corresponding image were then scanned into an IBM 286 personal computer through a television camera (Cohu solid state, model 5100) using a Datatranslation 2853 8-bit monochrome framegrabber. The images were viewed on the computer monitor through the use of ImagePro software (Media Cybernetics).

For microautoradiographic analysis, slides with kidney tissue were dried in cold acetone, and coated with Kodak autoradiography emulsion. The emulsion was allowed to air dry on the slides in the dark for 1 hr. After drying, the slides were stored in a -80 °C freezer for approximately 18 hrs during which time the radiation from the sections exposed the emulsion on the slides. After 18 hrs, the slides were developed, fixed, stained with Harris’ hematoxylin, and photographed using bright field microscopy.

For additional comparative purposes, kidneys were removed from Hn and normal clams (n=14), fixed in Bouin’s solution, dehydrated in alcohol
and embedded in paraffin at the State of New Hampshire Veterinary Diagnostic Laboratory. They were then sectioned at 10 \( \mu \text{m} \) on a rotary microtome, stained in Harris' hematoxylin and eosin, and examined using bright field microscopy.

**Tc-99m-Sulfur Colloid**

Following imaging of dams injected with Tc-99m-sulfur colloid, each animal was bled from the sinus around the posterior adductor muscle using a 1 cc syringe with a 26 g, 1/2" needle. Approximately 0.1 ml hemolymph was withdrawn from each dam for evaluation of activity in whole blood. As much hemolymph as possible (2-4 mls) was then withdrawn from each clam and centrifuged for 20 minutes at 1,000 G in a refrigerated centrifuge in order to separate the sample into serum and cells. The serum was decanted from the sample and placed in a separate tube. Each sample was weighed. Activity of the whole blood, cells and serum was assessed through gamma well counting.

**Data Analysis**

For both Tc-99m-disofenin and -sulfur colloid, initial (0.08 hr) clam scintigrams were used to evaluate the accuracy of radiopharmaceutical injection. An injection was considered to be effective and suitable for further study when it was initially localized to the region around the clam’s adductor muscle with little spread through other tissues. Efficiency of injection was determined for both radiopharmaceuticals used, as well as for both normal and Hn clams. Only those animals regarded as having localized injections were used in further analyses.

Whole body activity as well as that within specific organs was
determined through analysis of parallel-hole scintigrams at each time interval for each radiopharmaceutical. These counts were used to establish the rate of radiopharmaceutical accumulation within specific tissues, as well as the degree to which each agent was excreted in both normal and Hn clams. For Tc-99m-disofenin, the scintigrams at each time interval were analyzed for both whole body and kidney activity, while clams in the Tc-99m-sulfur colloid study were analyzed only for whole body activity. All data were expressed as activity (counts) per region and corrected for dose (0.5 mCi and 0.1 mCi for Tc-99m-disofenin and -sulfur colloid, respectively), image acquisition time and amount of decay. The half-life for Tc-99m is 6.02 hours, and the formula for the decay correction is:

\[
\text{decay corrected counts} = \text{counts} \left( e^{(0.693/6)(t)} \right); \ t = \text{time(hours) post-injection.}
\]

Whole body values were then converted to percent(%) activity remaining with 100% at 0.5 hr and 1 hr for Tc-99m-disofenin and -sulfur colloid, respectively, and plotted over time. The 0.5 hr and 1 hr values were used as 100% to insure that only activity actually within the animal’s circulatory system was used in further calculations. For clams in the disofenin study, percent activity in the kidney over time was evaluated by dividing kidney counts by whole body counts for each image time. The mean percent activity remaining and percent kidney activity at each time interval for Tc-99m-disofenin were then calculated and compared for each disease stage using 2-way ANOVA and multiple range tests (Zar, 1984). Only mean percent activity remaining over time was analyzed for Tc-99m-sulfur colloid.

Biodistribution data were corrected for dose and decay as described above and expressed as corrected counts per minute (CCPM)/gram(G) tissue.
The biodistribution of each radiopharmaceutical within the body of Hn and normal clams was analyzed and compared using t-tests.
RESULTS

Injection Efficiency

In order to establish the utility of radiopharmaceuticals in normal and Hn clams, it was first necessary to reliably get the agent into the animal and localized within the area around the anterior adductor muscle. Similar degrees of accuracy of radiopharmaceutical injection were achieved in both normal and Hn clams (Table 2-1). These similarities in injection efficiency indicate that diseased and normal clams were able to handle radiopharmaceutical injections equally well and that the integrity of the area around the adductor muscle was not drastically compromised based on this measure.

Initial distribution

Scintigrams at 0.08 hr and 0.5 hr indicate that both radiopharmaceuticals were distributed to all parts of the clam within the first 0.5 hr (Fig. 2-2 A, B and Fig. 2-10A, B). A more precise time interval for distribution is difficult to establish due to the indistinct and variable shape of the animal within the shell. While some activity distributed from the point of injection to all parts of the clam within two minutes, residual radiopharmaceutical dispersed slowly from the muscle for up to 0.5 hr.

Tc-99m-Disofenin

Based on scintigram and biodistribution data, the organs in *M. arenaria* which show the greatest uptake of Tc-99m-disofenin were the excretory organs (kidney and Keber’s gland). Representative scintigrams taken with a parallel-hole collimator at each image time are presented in
Figure 2-2. In this sequence of images, the activity is seen to spread from the initial injection site (sinus surrounding the anterior adductor muscle) throughout the animal (0.5 hr-1 hr), and accumulates within the kidney beginning at approximately 1 hr post-injection in some animals and by 2 hrs in all. The kidney appears as an increasingly glowing white spot of activity over time. By 5 hrs, most of the activity appeared to have localized within this organ. A closer view of the clam kidney taken through the pinhole collimator is presented in Figure 2-3.

The results of scintigram analysis for kidney accumulation of Tc-99m-disofenin are presented in Figure 2-4. The mean ratio of kidney to whole body disofenin activity increased over time in each group of animals. By 7 hrs, 60% of the whole body disofenin activity had accumulated within the kidney. The kidneys of Hn and normal clams showed consistent patterns of disofenin accumulation. No significant differences in the extent or rate of kidney disofenin accumulation were noted between normal and Hn Stage 2 or 3 clams. Because of the limited numbers of Hn clams from Little Buttermilk Bay, it was not possible to separate clams by study site in order to analyze for site differences in kidney function.

The change in percent whole body disofenin activity over time for Hn and normal clams is presented in Figure 2-5. On average, approximately 15% of the initial activity present at 0.5 hr was eliminated by 7 hrs. Patterns of disofenin excretion or loss were consistent in Hn Stage 2, Hn Stage 3 and normal clams throughout the experiment. Over 7 hrs, however, Hn Stage 3 clams removed or lost significantly greater quantities of disofenin from their systems than normal ones or Hn Stage 2 clams.

Biodistribution data confirmed that Tc-99m-disofenin was concentrated primarily in the kidney and to a lesser extent in Keber's gland after 7 hrs.
Analyses of the biodistribution data by t-tests indicate that there were no significant differences (p<0.05) in mean Tc-99m-disofenin activity per gram of tissue for any organs between normal and Hn stage 2 clams.

Analysis of clam kidneys processed through macro- and microautoradiography further supports the fact that Tc-99m-disofenin accumulated very specifically within the animal's kidney tissue. In this procedure mirror-like images of the kidney tissue in section were obtained on film and compared with the original histological section stained with hematoxylin (Figure 2-7A). X-ray images generated by gamma radiation were formed only in areas that were directly over kidney tissue. No corresponding images were generated on the films in areas that were in contact with other tissues such as muscle. The film images were blurred in appearance due to the scattering inherent in gamma radiation and the thickness of the emulsion used. The results obtained from microautoradiography were similar to those of macroautoradiography. On these slides, emulsion was exposed and appeared as dark silver grains only in regions directly over kidney tissue (Figure 2-7B). Specific localization of Tc-99m-disofenin in particular cell types within the kidney was not possible due to difficulties in preserving the orientation of the tissues in section and the methods necessary for autoradiography using gamma radiation.

When examined histologically, the major portion of the kidney, the distal region, appears as an extensively folded epithelium arranged in tubules. A comparison of kidney tissue from normal and Hn clams (Fig. 2-8) reveals that while Hn cells can be found in the connective tissue and spaces around the kidney epithelium, the overall structure of the tissue appears uncompromised and is similar to that from normal clams. Additional
comparisons with various other tissues (muscle, digestive gland (Fig. 2-9), and gonad (see Fig. 1-11)) throughout the body of the soft-shell clam suggest that the kidney may not be as heavily impacted by the disease as other organs.

**Tc-99m-Sulfur Colloid**

Representative parallel hole scintigrams showing Tc-99m-sulfur colloid activity within *M. arenaria* for each image time are presented in Figure 2-10. Both the scintigrams themselves and an analysis of mean percent whole body sulfur colloid activity over time (Fig. 2-11) indicate that there was little excretion of this agent from the clams over 3.5 hrs. There were no differences in excretion of Tc-99m-sulfur colloid between normal and Hn Stage 2 and 3 clams. A closer inspection of the scintigrams from clams injected with Tc-99m-sulfur colloid revealed that some activity failed to spread beyond the initial site of injection throughout the experiments. The experiments with sulfur colloid were of shorter duration than those with disofenin due to the nature of the colloid particles and the unchanging appearance of clam scintigrams after 3 hours.

The radiocolloid, Tc-99m-sulfur colloid, appeared to be taken up by normal hemocytes (phagocytic cells) within the soft-shell clam (Fig. 2-12). Within the hemolymph, the blood cells contained significantly greater quantities of Tc-99m-sulfur colloid than the serum of either normal or Hn clams. While there were no significant differences, the cell fraction showed a clear trend of decreasing Tc-99m-sulfur colloid activity associated with increasing levels of Hn (Fig. 2-13). Serum sulfur colloid levels however, remained constant (Fig. 2-14).

The only other tissue, besides the hemocytes, which accumulated Tc-99m-sulfur colloid within the soft-shell clam was Keber’s (pericardial) gland.
(Fig. 2-15). No significant differences in accumulation of Tc-99m-sulfur colloid were seen for any organ of normal and Hn clams.
Table 2-1. Efficiency of Radiopharmaceutical Injection

<table>
<thead>
<tr>
<th>Agent</th>
<th>Hn Localized Injections</th>
<th>Hn Total</th>
<th>Normal Localized Injections</th>
<th>Normal Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disofenin Injection</td>
<td>42</td>
<td>54</td>
<td>42</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>78%</td>
<td></td>
<td>76%</td>
<td></td>
</tr>
<tr>
<td>Sulfur Colloid Injection</td>
<td>29</td>
<td>32</td>
<td>25</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>91%</td>
<td></td>
<td>78%</td>
<td></td>
</tr>
<tr>
<td>Grand Total</td>
<td>71</td>
<td>86</td>
<td>67</td>
<td>87</td>
</tr>
<tr>
<td>Overall Injection Efficiency</td>
<td>83%</td>
<td></td>
<td></td>
<td>77%</td>
</tr>
</tbody>
</table>
Figure 2-2. Distribution and kidney accumulation of Tc-99m-disofenin in 8 soft-shell clams following injection into the sinus surrounding the anterior adductor muscle as shown on the scintillation camera with a parallel-hole collimator at (A) 0.08 hr-initial image, (B) 0.5 hr, (C) 1 hr, (D) 2 hrs, (E) 3 hrs, (F) 5 hrs, (G) 7 hrs. Siphon end of the clam is pointing upwards.
Figure 2-3. Distribution of Tc-99m-Disofenin in a clam 7 hrs after injection as shown on a scintillation camera with 1 mm pinhole collimator. Increased uptake is visible in the kidney (K), as compared to the siphon (S), or the visceral mass (VS).
Figure 2-4. Uptake of Tc-99m-disofenin in the kidney of Hn and normal soft-shell clams. Values shown are mean ± S.E.
Figure 2-5. Change in whole body concentration of Tc-99m-disofenin in Hn and normal Mya arenaria over time. Values are expressed as percentage (mean ± S.E.) of initial injected dose. * Indicates significant differences (p<0.05) between Hn stage 3 and both Hn stage 2 and normal clams.
Figure 2-6. Tissue levels of Tc-99m-disofenin in Hn and normal *M. arenaria* at 7 hrs. Values shown are dose and decay corrected counts min$^{-1}$ g$^{-1}$ (mean ± S.E.).
Figure 2-7A. Comparison of hematoxylin stained kidney tissue (a) with its corresponding image on x-ray film generated through macroautoradiography (b) and viewed on the computer. Kidney (K) and muscle (M) tissue are identified.

Figure 2-7B. Kidney tissue processed through microautoradiography from soft-shell clams injected with Tc-99m-disofenin. Scale bar = 100 μm.
Figure 2-8. Kidney tissue from normal (A) and Hn stage 3 (B) clams. Hn cells (HN) can be seen in the connective tissue. Scale bar = 50 μm.
Figure 2-9. Comparison of muscle tissue (A) and digestive gland tissue (B) from normal (a) and Hn stage 3 (b) clams. Scale bar = 50 μm.
Figure 2-10. Distribution of Tc-99m-sulfur colloid in 8 soft-shell clams following injection into the sinus around the anterior adductor muscle as shown on the scintillation camera with a parallel hole collimator at (A) 0.08 hr, (B) 1 hr, (C) 2 hrs, (D) 3.5 hrs. Siphon end of the clam is pointing upwards.
Figure 2-11. Change in whole body concentration of Tc-99m-sulfur colloid in Hn and normal Mya arenaria over time. Values shown are percent of the initial dose (mean ± S.E.).
Figure 2-12. Biodistribution of Tc-99m-sulfur colloid in Hn and normal soft-shell clam blood after 4 hrs. Values shown are dose and decay corrected counts $\text{min}^{-1}\text{g}^{-1}$ (mean ± S.E.).
Figure 2-13. Levels of Tc-99m-sulfur colloid in the cell fraction of normal and Hn soft-shell clam blood after 4 hrs. Values shown are dose and decay corrected counts min$^{-1} g^{-1}$ (mean ± S.E.).

Figure 2-14. Levels of Tc-99m-sulfur colloid in the serum of Hn and normal soft-shell clam blood after 4 hrs. Values shown are dose and decay corrected counts min$^{-1} g^{-1}$ (mean ± S.E.).
Figure 2-15. Tissue levels of Tc-99m-sulfur colloid in Hn and normal *M. arenaria* after 4 hrs. Values shown are dose and decay corrected counts min$^{-1}$g$^{-1}$ (mean ± S.E.).
DISCUSSION

In Mya arenaria, the uptake of Tc-99m-disofenin is mediated by the excretory organs (kidneys and pericardial gland). Fed by the circulatory system, the excretory organs function to transport, accumulate, and eliminate wastes and foreign matter from the clam’s body. Reviews of bivalve excretion can be found in the works of Martin and Harrison (1966), Potts (1967, 1968), Martin (1983), and Morse (1987). In M. arenaria, the kidneys consist of a single pair of fused, sac-like ducts lying beneath the pericardial chamber, one on each side of the body. At one end these ducts open into the pericardial cavity, while the other empties into the mantle cavity. The pericardial glands are located along the margin of the atria and excrete directly into the pericardial space.

According to Morse (1987), urine production begins by ultrafiltration of the hemolymph past the filtration barrier at the base of the pericardial gland cells into the pericardial gland lumen, which is confluent with the pericardium. The ultrafiltration barrier is thought to be formed by the pedicels (cytoplasmic extensions) from the pericardial gland cells (podocytes) and the underlying basal lamina (Meyhöfer et al., 1985). Meyhöfer et al. (1985) provided evidence that particles of a molecular weight of 40,000 daltons are passed through the barrier and that large particles (400,000 daltons) are retained at the basal lamina. The basal lamina is therefore thought to be the principal filter in the ultrafiltration process (Meyhöfer and Morse, 1986; Morse, 1987). In the urinary spaces, primary urine is modified through reabsorption and secretion, and based on ultrastructural evidence, Meyhöfer et al. (1985) and Morse (1987) have proposed that the surface of the podocytes is the site of absorption. Since the disofenin molecule is too small to have
been retained (mw = 348 daltons), its presence in the pericardial gland provides physiological evidence for such absorption.

From the pericardial gland lumen, the filtrate passes into the pericardium and on to the kidneys through the ciliated renopericardial ducts. In the kidney lumina, the urine is modified by secretion and reabsorption prior to being discharged into the mantle cavity. The epithelium of the kidney tubules also has an extensive blood supply. Disofenin absorption by the epithelial cells is indicated by the high levels and very specific concentration in the kidney tissue, as seen in the scintigrams, biodistribution data and autoradiography results. It is unknown whether the urine or the blood is acting as the source.

While the proximal region of the soft-shell clam kidney is characterized by columnar epithelial cells with an apical border of microvilli, the distal region, which has an extensively folded epithelium arranged in tubules, is lined by columnar cells with expanded apices and scattered microvilli (Morse, 1987; Seiler and Morse, 1988). Based on structural evidence, the proximal and distal regions of the kidney of *M. arenaria* have been implicated as the sites of reabsorption and secretion, respectively, in the process of urine formation (Seiler and Morse, 1988). Because the major portion of the kidney is the distal region, the extensive localization of disofenin in the kidney tissue suggests that this region may be the one responsible for the accumulation. Although the autoradiography results clearly show that disofenin is localized within the cells of the kidney tissue, it was not possible to ascertain in precisely which cell types because of difficulties in preserving the spatial orientation of the tissue.

In addition to establishing the cells within which disofenin is localized, future studies should include the determination of whether this agent is
sequestered within concretions in the kidney cells. Part of the bivalve system for removing substances from its body involves the accumulation and concentration of wastes and foreign materials into large, distinct concretions by the lysosomal-vacuolar system in kidney and pericardial gland cells (Potts, 1967; Pirie and George, 1979; Martin, 1983; Morse, 1987; Seiler and Morse, 1988; Sullivan et al., 1988). According to Reinberger et al. (1979) and Seiler and Morse (1988), pollution stress enhances concretion formation as the animal attempts to excrete the excess foreign substances. Studies have shown that pollutants, such as heavy metals, accumulate primarily within the bivalve kidney and specifically within kidney concretions (George and Pirie, 1979; Robinson et al., 1985; Sullivan et al., 1988). Because of its ability to concentrate pollutants into concretions, the bivalve kidney has potential as an indicator of environmental pollution (Reinberger et al., 1979). In addition to the possibility of concretion formation, the cyclical nature of the bivalve excretory process must also be considered as large numbers of these concretions build-up in the cells before they are shed into the kidney lumen (Morse; 1987). While this phenomenon does not have an impact on kidney disofenin accumulation in the short term, it must be considered in longer term studies involving disofenin removal from the kidney.

Temperature influences many of the physiological processes in the soft-shell clam and both the rate and extent of disofenin localization in the kidney are no exception. The rate of uptake and excretion of disofenin at 15-18 °C was much greater (with 60% localized in the kidney and 15% excreted or lost over 7 hrs), than at 6 °C where 13% was removed or lost over 27 hrs (Burn et al. in press). This finding is not surprising considering that: 1) metabolic rate in the soft-shell clam reached maximum levels at 20 °C in Chesapeake Bay
clams and was depressed at both high and low temperatures (Kennedy and Mihursky, 1972); 2) burrowing is most active at 18 °C and remains high between 9 and 21 °C (Pfitzenmeyer and Drobeck, 1967); and 3) a linear relationship exists between temperature and heartbeat (Lowe and Trueman; 1972). These factors together indicate that the temperature at which functions proceed at their maximum rate is around 18 °C for clams south of Cape Cod. The optimum temperature for clams north of Cape Cod is somewhat lower as evidenced by the reports of Gilfillan et al.; (1976) who reported food assimilation occurs, although at reduced levels, as low as 1-2 °C, and Dow (1972) who reported that the optimum temperature for the development of M. arenaria is 7.6 °C in Maine. The data from the current study indicate that temperature plays an important role in regulating clam excretory organ function as measured by its ability to accumulate disofenin. Under the conditions of these experiments the clams were probably operating within their optimum range. The data also suggest that both temperature and prior conditioning or source of the clams must be taken into consideration in studies of this type.

Based on the 7 hr disofenin uptake data, there is little or no change in physiological functioning of the clam kidney with regard to disofenin localization with advancing disease stage. While it is possible that differences in kidney function with advancing disease stage were too subtle to be adequately detected by the methods used, it is unlikely in light of histological evidence. While Hn cells can be found in the connective tissue and spaces around the kidney epithelium, the overall structure of the tissue appears uncompromised and is similar to that from normal clams. The kidney tissue
does not appear to be as heavily impacted by Hn as other organs based on both radiopharmaceutical and histological data.

While clam kidney function may not be heavily impacted by Hn, examination of disofenin levels in the whole body of the clam over time leads to very different conclusions. Even though the groups of animals handled injection of radiopharmaceutical in comparable manners, Hn stage 3 clams removed or lost significantly greater quantities of disofenin from their systems than either Hn stage 2 or normal clams. The possibilities for removal of disofenin from the clam's system are either via the excretory system or by other unknown mechanisms. The kidney is implicated as the major organ responsible for removal of disofenin from the clam's system as high levels of this agent in extra-renal tissues were evident in clams in which kidney uptake did not occur. Since the kidneys of normal and diseased clams are functioning similarly, as discussed above, the latter choice becomes the more likely mechanism in this case.

A correlation between the level of Hn and the amount of tissue degradation has been reported based on tissue sections (Cooper et al. 1982b). Visible signs of distress in Hn animals include emaciation, a pale digestive gland and a receded mantle. According to Appeldoorn et al. (1984), M. arenaria become increasingly cachexic as Hn progresses. Leavitt et al. (1990) showed that high levels of Hn results in a reduced energy composition of the clam’s soft tissues and that Hn animals are metabolically compromised. Histological comparisons of various body tissues between normal and Hn stage 3 animals indicate that tissue integrity may be drastically compromised by the massive invasion of Hn cells. It is therefore possible that disofenin may be leaking out of the clam through these compromised tissues or being lost through some other unknown mechanism. Based on the
radiopharmaceutical aspects of this study, this alteration is not evident until the clam reaches an Hn stage of 3 (>70% Hn cells). Hn stage 2 clams showed no differences in their ability to remove or lose disofenin when compared to normal ones or Hn stage 3 clams. Because of the wide range of Hn cell levels (15-70% Hn cells) in Hn stage 2 clams, there is a great deal of variability inherent in this group making the precise level at which tissues become compromised by the disease difficult to establish.

Tc-99m-sulfur colloid is concentrated within the hemocytes in the blood of the soft-shell clam. Normal hemocytes are important components of the excretory system as they phagocytize foreign matter and transport it to the kidney for storage and/or elimination. These cells initiate the excretory flow when they deliver wastes to the pericardial gland (Morse, 1987; Seiler and Morse, 1988). In their roles in phagocytosis and internal defense of tissues, hemocytes are involved in recognition, adherence, uptake, and internal degradation of foreign particles. In some cases particle-laden hemocytes pass through epithelial barriers (diapedesis) into digestive and excretory lumina for removal and are thereby directly lost from the system (Morse, 1987; Fisher, 1988). This process has been reported to be active in the removal of both foreign material, such as ink particles (Stauber, 1950), as well as soluble substances (Feng, 1965). The presence of Tc-99m-sulfur colloid in Keber’s gland, as evidenced in the biodistribution data, suggests that this agent is either transported in the serum of the hemolymph to the excretory organs and filtered from the blood, or is transported in the hemocytes for direct removal via diapedesis, or both. Based on the patterns of distribution, the unchanging nature of the scintigrams, and the very limited excretion of sulfur colloid (less than 5%) over 3 hrs, it appears that the clam handles this compound differently than it does disofenin. The fact that a proportion of
this radiopharmaceutical does not move from the initial injection site to spread throughout the body suggests that something may be happening within the body of the clam to keep sulfur colloid in this location.

Hemocytes play a crucial role in the inflammatory response and wound repair processes of marine bivalve molluscs. Once an injury has occurred, a specific sequence of events, including infiltration of the wound site by hemocytes, plug formation and replacement of damaged tissues with hemocytes, removal of necrotic tissue and restoration of normal tissue architecture, lead to healing (Feng, 1988; Sparks and Morado, 1988). According to Fisher (1988), the essential component of inflammation in bivalves is the migration of hemocytes to an infection site and the ability to form cellular aggregations. This tendency for hemocytes to form aggregations or clumps can be witnessed during the preparation of blood smears on glass slides. After standing, the cells tend to migrate apart (Auffret, 1988; Feng, 1988; personal observations). While the factors governing the formation of cell aggregations are unknown, it has been well-documented that hemocyte infiltration and formation of cell clumps can be induced in various bivalves by a variety of stimuli including: intracardial injection of tissue extracts (Bang, 1971), avian and vertebrate erythrocytes (Tripp, 1958; Feng and Feng, 1974), India ink particles, (Stauber, 1950), turpentine and talc (Pauley and Sparks, 1965, 1966) and incision wounds (Sparks and Morado, 1988). One possible explanation for the lack of spreading of Tc-99m-sulfur colloid away from the initial injection site in the present study is that the colloid particles may have induced hemocyte migration to the injection site and this infiltration then limited radiopharmaceutical flow throughout the body. Other possibilities are that some of the sulfur colloid particles were retained by cells of the anterior adductor muscle or through some other unknown mechanism. The
time frame of the experiment (3 hrs) and unchanging appearance of the
scintigrams over longer periods fall within the range during which this
phenomenon is reported to occur. Stauber (1950) reported that hemocyte
infiltration and phagocytosis in eastern oysters begins between 2 and 4 hrs
following injections of India ink particles, while Tripp (1958) reported the
occurrence of similar events within 10 min to 6 hrs in oysters injected with
vertebrate erythrocytes.

As in many other physiological activities, temperature plays an
important role in cell aggregation formation and bivalve defense-related
activities, and warmer temperatures enhance metabolic activity (Fisher, 1988).
Foley and Cheng (1975) demonstrated that binding and ingestion of bacteria by
hemocytes of the quahog, *Mercenaria mercenaria*, were greatly reduced at 4 °C
compared to 22 and 37 °C. Reduced hemocyte infiltration was also evident at
4 °C in oysters injected with avian erythrocytes (Feng and Feng, 1974). Based
on this information, the temperature conditions of the present study (15-
18 °C) can be presumed to have allowed the hemocytes to operate well within
their optimum range, if indeed hemocyte infiltration was occurring. Further
studies involving histological evaluation of the injection site and
surrounding tissues are necessary to determine if sulfur colloid induces an
inflammatory response within the soft-shell clam.

Because the clams were bled from the sinus around the posterior
adductor muscle, at the opposite end from the injection site, and because of
the limited distribution throughout the body, the blood levels of sulfur
colloid may be somewhat underestimated. Despite this fact, the blood
biodistribution data indicate a reduction in the amount of activity associated
with the hemocytes as the severity of Hn increases. There is a clear linear
trend of decreasing Tc-99m-sulfur colloid in the cell fraction of the hemolymph with advancing disease state. Although further studies are necessary to ascertain whether the sulfur colloid particles are actually ingested by the cells or merely associated with them, this reduction in activity suggests a reduction in phagocytosis by circulating neoplastic cells. This finding has previously been demonstrated in vitro in comparative studies of phagocytosis in diseased and normal cells by Beckmann and Morse, (1988) and Beckmann et al. (1992). In these studies, Hn cells did not recognize or degrade foreign particles normally even though they possessed some of the lysosomal enzymes necessary. According to Beckmann et al. (1992), Hn cells have increased acid phosphatase, nonspecific esterases and β-glucuronidase activity when compared to normal cells, while only normal cells have β-N-acetylglucoaminidase activity. It has also been demonstrated that Hn cells may recognize and ingest some foreign particles (concanavalin A), but not others (yeast) (Beckmann et al., 1992). This inability to phagocytize foreign matter has been attributed to an altered cytoskeletal framework in the Hn cell (Moore et. al., 1988).

The reduction in Tc-99m-sulfur colloid activity associated with the cells is somewhat confounded by the lack of a corresponding increase in activity associated with the serum as Hn stage increases. This lack of increase in serum activity may in part be a result of the methods used for the separation of cells and serum. After centrifugation of the whole blood, serum was decanted from the cell pellet. No attempts were made to insure that complete separation was achieved. In addition, it was very difficult in some cases, particularly in dealing with the normal blood, to separate cells and serum and obtain an accurate weight for the normal cells. Further studies are necessary to refine the techniques for the use of Tc-99m-sulfur colloid in the
investigation of phagocytosis in normal and Hn hemocytes. In spite of the limitations, the reduction in hemocyte phagocytosis observed with advancing disease state may have serious consequences for the clam (Beckmann et al., 1988; Moore et al., 1988; Morse, 1988; Beckmann et al., 1992; present study). Overall, this reduced phagocytic capability may contribute to a decreased ability to remove foreign materials from the circulation, transport nutrients and repair wounds.

The overall conclusions to be derived from this study fall into two categories; the first dealing with the utility of radiopharmaceuticals in the examination of invertebrate organ function, and the second with the physiological effects of Hn on the soft-shell clam excretory system. Not only does this work represent the first reported attempts at using radiopharmaceuticals in the study of disease-related changes in organ function in non-mammalian vertebrates or invertebrates, it also demonstrates their applicability and utility in the assessment of both morphological and physiological alterations in a non-mammalian species. The results of this study establish that the radiopharmaceuticals, Tc-99m-disofenin and -sulfur colloid can be reliably injected, retained and distributed within the body and especially the excretory systems of normal and Hn soft-shell clams. While future studies are necessary to ascertain the precise localization of Tc-99m-disofenin within the clam kidney and to more firmly establish the relationship between Tc-99m-sulfur colloid levels and normal and diseased hemocyte phagocytosis, in general, it can be concluded that Tc-99m-disofenin and -sulfur colloid are useful for examining Hn effects on soft-shell clam excretory function and blood cell phagocytosis. The soft-shell clam and perhaps other invertebrates may provide good model systems for the use of this traditionally mammalian-oriented technology.
With regards to the second area, this study indicates that despite the extensive infiltration of, and impact on, various body systems by Hn cells, there is neither radiopharmaceutical or histological evidence that kidney function or architecture is greatly disrupted. The integrity of other body tissues, such as the gonad, digestive gland, and muscle, however, has been shown to be compromised by this disease. In addition, Hn cells are postulated to have a decreased phagocytic capability as compared to normal cells. Overall, this disruption of normal function both in the hemocytes and tissues may leave diseased animals with an increased susceptibility to both internal and external stresses (e.g., bacterial invasion, starvation due to a reduction in nutrient transport, an inability to repair wounds, build-up of wastes, other diseases) and an inability to maintain homeostasis. As has been demonstrated with other physiological processes, temperature plays an important role in clam excretory system function as evidenced by its effects on the distribution and accumulation of radiopharmaceuticals in the clam’s body. Based on the results of this study, it can be concluded that hematopoietic neoplasia has an impact on excretory processes in the soft-shell clam, *M. arenaria* and these effects can be demonstrated through the techniques of nuclear medicine.
GENERAL SUMMARY

The blood disease, hematopoietic neoplasia (Hn), adversely affects the soft-shell clam's reproductive and excretory systems, although it does not appear to impact the kidney as much as other organs. Hn may be involved with reproductive processes in several ways. First, Hn prevalence may be enhanced within the individual and/or within the population by the reproductive process. In particular, fall spawnings at both New Bedford Harbor and Little Buttermilk Bay occurred simultaneously with high Hn prevalence. Hn prevalence was also highest in those animals in the midst of their peak reproductive outputs. Gametogenesis and spawning are energetically expensive processes which may reduce fitness and thereby enhance the development or progression of Hn. Gamete development may alter gonad structure to indirectly enhance Hn cell invasion of reproductive tissue.

Further histological work is necessary to examine how these spawning peaks relate to Hn prevalence. Differences between early spring and fall spawnings should be explored, since only the latter consistently occurred in conjunction with high Hn prevalence. Future studies should determine whether there are significant reductions in the clam's energy stores following spawning and how this reduction could enhance Hn in the animal. Hn appears to be enhanced equally in males and females, since Hn prevalence is not related to the sex of the individual.

Physiological effects of the disease increase with increasing disease stage as neoplastic cells invade the connective tissue of the body systems. Hn cell
invasion of the gonad leads to a reduction in gonadal follicle size providing less available space for gamete development. In some Hn animals, gametogenesis appeared to be proceeding normally although in less space, while in others, virtually the entire gonad was taken over by Hn cells, and gametogenesis was arrested. Future studies should examine the environmental conditions, including disease state, under which gametogenesis may proceed normally in the soft-shell clam. A further evaluation of reproductive capabilities, including actual quantification of gametes, in Hn and normal clams would be useful. The impact of Hn on the vitality of the gametes and the resulting larvae should also be explored.

The results of this study establish that radiopharmaceuticals can be reliably injected, retained, and distributed within the body of bivalves. In addition, these agents are potentially useful tools for examining physiological processes and disease-related changes in other invertebrates. Despite the extensive invasion of, and impact on, various body systems by Hn cells, there is neither radiopharmaceutical or histological evidence that kidney function or architecture is greatly disrupted. The integrity of other body tissues, such as the gonad, digestive gland, and muscle, however, are severely compromised by the disease. While Tc-99m-disofenin is highly specific for kidney tissue, future studies are necessary to determine how disofenin is handled and by which kidney cells. For long term studies, it will also be necessary to establish whether this agent is sequestered within kidney concretions and how, or if, it is removed from the clam’s body.

Hn cells have been shown to have reduced phagocytic capabilities \textit{in vitro} (Beckmann et al., 1992) and appear to have similar capabilities \textit{in vivo} (this study). More work is necessary to refine the techniques for using Tc-99m-sulfur colloid to study hemocyte phagocytosis in Hn and normal soft-
shell clams. The reduction in hemocyte phagocytosis and the overall disruption of tissue may leave diseased clams with an increased susceptibility to bacterial invasion, other diseases, and starvation, as well as an inability to repair wounds and remove foreign matter and wastes. Based on the results of this study, it can be concluded that Hn impacts excretory processes in the soft-shell clam, and these effects can be demonstrated through the techniques of nuclear medicine.

In addition to its effects on reproduction and excretion, Hn alters the size distribution by eliminating particular size classes from a flat. Hn should be regarded as one of the factors shaping the structure of clam populations. Conversely, this study also suggests that the population size distribution needs to be considered in examining trends in Hn seasonality, since the presence of a large disease-susceptible cohort may determine the results seen in the population as a whole. Future studies should more closely follow Hn prevalence within a single cohort to more precisely define disease impact on the size distribution of clams within the population.

Overall, the results of this study provide further evidence that Hn has a serious impact on the soft-shell clam. Based on its effects on the clam’s reproductive and excretory system and in light of the definition of stress as a disturbance to the physiological steady-state (Bayne, 1975), it appears that Hn causes soft-shell clams to be severely compromised. Because Hn adversely affects the organism’s ability to survive, reproduce, maintain homeostasis and may alter the population size distribution, this condition must be considered in the evaluation of clam population dynamics. Knowledge of the disease is important for appropriate management strategies for this ecologically and commercially important species.
LITERATURE CITED


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