
S. Anne Böttger¹*, Emily J. Amarosa²³, Paul Geoghegan⁴, and Charles W. Walker²

Abstract - Disseminated neoplasia, a diffuse tumor of the hemic system, is characterized in many bivalve mollusks by hemolymph containing 1–100% mitotic hemocytes. Little is known about the onset and chronic distribution of neoplasia in populations of *Mya arenaria* (Soft-shell Clam), though studies have reported episodic exposure to environmental contaminants or an infectious agent as a potential cause of this disease. Here we provide the first set of continuous data on neoplasia in Soft-shell Clams, from three sites in New England where sediments have been characterized regarding their granulometry, composition, contaminants, and clam densities. When correlating sediment characteristics to terminal neoplasia (76–100% neoplastic or rounded, unattached hemocytes), New Bedford Harbor, MA, which is the most contaminated site, had the highest frequency of terminal neoplasia (maximum of 9.49% ± 0.78 SE), and the most pristine site, Ogunquit, ME, displayed the lowest frequencies (maximum of 0.47% ± 0.05 SE). Correlations of frequency of neoplasia to known environmental contaminants also suggests that fully neoplastic individuals were found only at sites of increased levels of heavy metals, PCBs, and PAHs. In addition, we documented the highest frequency of clams with terminal neoplasia from New Bedford Harbor in December (9.49% ± 0.78 SE) when seawater temperatures were low, and the lowest frequency in July (1.08 ± 0.4 SE) when seawater temperatures were highest. These results may indicate vulnerability of neoplastic clams to seasonal increases in environmental temperature and resulting oxidative stress. Based on shell measurements and a theoretical mathematical age model (which correlates susceptibility to neoplasia with age and sexual maturity), we suggest that the Soft-shell Clam is most susceptible to this disease between one and two years of age (9.5% frequency at 1 year, 22.25% incidence at 1.5 years, and 57.14% incidence at 2 years).

Introduction

*Mya arenaria* L. (Soft-shell Clam), has been a valuable commercial resource along the East coast of the USA since the 1800s and traditionally has yielded a strong annual harvest (1–5 million pounds) that provided a reliable source of income for New England fishermen. Since the 1980s, however, annual harvests have declined dramatically in Maine, Massachusetts, Rhode Island, and the Chesapeake Bay (Maryland) (Fig. 1A) resulting in significant loss of seasonal

¹Department of Biology, West Chester University, 750 S Church Street, West Chester, PA 19383. ²Department of Molecular, Cellular and Biomedical Sciences, The University of New Hampshire, 46 College Road, Durham, NH 03824. ³University of Washington Medical Center, 1959 NE Pacific Ave, Seattle WA 98195. ⁴Normandeau Associates, Inc., 25 Nashua Road, Bedford, NH 03110. *Corresponding author - sboettger@wcupa.edu.
and full-time jobs (Congleton et al. 2006). The situation has proven particularly severe in New Hampshire, where commercial clamming has been prohibited since 1951, although legal recreational harvesting still occurs. Public management of adult Soft-shell Clams, however, is limited to surveys, predator control, and seed transplantation on clam-flats (Newell 1991), and limited aquaculture efforts include hatchery culture, out-planting, and production and growth of triploid individuals (Allen et al. 1982).

Worldwide, fifteen commercially important shellfish species (e.g., *Crassostrea virginica* Gmelin [Eastern Oyster], *Mytilus* spp. [mussels], and *Ostrea edulis* L. [European Flat Oyster]) are known to be impacted by a proliferative disease of the hemocytes referred to as hemic neoplasia (Barber 2004). The common disease feature is the presence of large round cells in the hemolymph (= neoplastic

![Figure 1](image_url)

Figure 1. (A) Landings (lbs*10^6) and value ($/lb) for *Mya arenaria* (Soft-shell Clams) in New England (ME, MA, and RI), the Mid-Atlantic Region (NY and NJ), and the Chesapeake Bay (MD). Market values have been combined for these areas (Maine Department of Marine Resources, Augusta, ME; Massachusetts Division of Marine Fisheries, Boston, MA; and Maryland Department of Natural Resources, Annapolis, MD; pers. comm.). (B) Nomarski image of living, freshly collected hemocytes. Normal clam hemocytes (inset) and neoplastic clam hemocytes in vivo. (C) Prevalence of clams with neoplastic hemocytes under different pollution conditions (Barber 2004). (D) Frequencies of clams with neoplastic hemocytes were compiled for locations including Alaska (AK), Maryland (MD), New York (NY), Connecticut (CT), Rhode Island (RI), Massachusetts (MA), New Hampshire (NH), Maine (ME), and New Brunswick (NB). Data was collected between 2002 and 2009. Clam populations in Alaska were added as a West Coast comparison, since Soft-shell Clams did not naturally occur on the West Coast but were imported from East Coast flats.
clam hemocytes, also called cancerous clam hemocytes [Fig. 1B]; Walker et al. 2009), although differences exist in hemocyte structural characteristics and pathology between bivalve species. Neoplastic hemocytes are mitotic, have a nuclear to cytoplasmic volume ratio of 1:1, and contain one or more prominent nucleoli and one to several vesicles containing neutral triglycerides and lipids (Walker et al. 2009).

While considerable information exists on the molecular basis for disseminated neoplasia, and disease onset has been linked to environmental contaminants and infectious agents, including retroviral particles (Oprandy and Chang 1983), the cause(s) for this disease remain largely unknown (Walker et al. 2009). Based on limited, site-specific data, several environmental contaminants (hydrocarbons and polychlorinated biphenyls [PCBs]) have been correlated to disease incidence (Fig. 1C; information compiled from Barber 2004). However, no single pollutant or group of contaminants has been definitively associated with this or other bivalve-disseminated neoplasias, and the disease has in fact been reported from unpolluted sites (Pekkarinen et al. 1993).

Disseminated neoplasia in Soft-shell Clams has been recorded in populations from Prince Edward Island to the Chesapeake Bay (review in Barber 2004), with incidence of the disease varying from 1–100% hemocyte involvement. Once established, this disease results in 40–100% mortalities in resident populations. Recently, we have also documented this disease for sites between Maryland and Nova Scotia (Fig. 1D). These data were compiled from 2002–2009, providing survey information during stable environmental conditions, not following catastrophic events like previous studies. However, no data were available tracking the disease over a long period of time in specific chronically affected populations.

In this study, we have followed the prevalence of clam neoplasia at three different sites in New England over multiple years. This study examines neoplasia frequencies at the different locations and correlates them to environmental contaminant loading, sediment composition, environmental temperatures, and ages of the animals developing the disease.

**Materials and Methods**

**Field site descriptions**

Collection sites for *Mya arenaria* were located on Marsh Island in New Bedford Harbor (NBH), MA (41°38.0’N, 70°55.0’W); Hampton Harbor (HH), NH (42°54.0’N, 70°49.0’W); and Ogunquit (OQ), ME (43°25.3’N, 70°59.4’W). All three collection sites displayed similar sediment granulometry, with sediments composed mainly of sand (determined through sieve analysis by Geotesting Services, MA; above 89% for all three sites). Amounts of silt and clay were as low as 3.35% (NBH), and the proportion of gravel was low as 0.05% (OQ). All sediments were low in organic content (determined through analyzing wet weights, dry weights, and ashing) and generally associated with sandy areas, with a minimum of 3.78% organic contents (NBH) (Fig. 2).
NBH is an EPA Superfund Site, where shellfish show a high degree of contamination with PCBs (average of 14,725 mg/kg dry weight in clams and mussels), heavy metals, and dichlorodiphenyltrichloroethane (DDT), and elevated levels of polycyclic aromatic hydrocarbons (PAHs; average of 6015 mg/kg dry weight [DW] in clams and mussels). Sediment samples (analyzed by Columbia Analytical Services now ALS, Rochester NY using gas chromatography/mass spectroscopy for PCBs and PAHs, gas chromatography/electron capture detector for pesticides, and optical emission spectrometry for heavy metal analyses) from NBH also contain high levels of PCBs (4500 mg total PCBs/kg DW), PAHs (7134 mg total PAHs/kg DW), and heavy metals (total of 13,000 mg/kg DW, including aluminum, cadmium, chromium, copper, iron, lead, mercury, nickel, silver, and zinc) which exceed NOAA ER-M levels (Jones et al. 1998). HH sediments contained 80 mg/kg DW total PCBs, 1600 mg/kg PAHs, and 7800 mg/kg total heavy metals. At the relatively pristine site in OQ, organic contaminants are considered undetectable in sediments or shellfish tissues (Fig. 2), and heavy metal contaminants are present at low detectable levels totalling 2400 mg/kg DW.

Figure 2. Sediment characteristics including granulometry (%), components (% water and inorganic and organic components) and loads of contaminants including heavy metals (compiled results for aluminum, cadmium, chromium, copper, iron, lead, mercury, nickel, silver and zinc [mg/kg]), polychlorinated biphenyls (PCBs [mg/kg]) and polyaromatic hydrocarbons (PAHs [mg/kg]).
Natural clam densities, clam distributions, and other characteristics were evaluated using ten 1-m² quadrats/site, and sites also differed significantly for all these factors. NBH displays average clam densities of 4.26 ± 0.57 individuals/m², with adult animals found suspended in the sediment between 10–30 cm depth. The upper sediments down to 10 cm depth also showed average juvenile clams (spat) densities of 6.71 ± 1.71 individuals/m², with the largest amounts of spat occurring in the summer months (maxima of 12 individuals/m² detected in July/August of each year). HH sediments contained lower densities of adult clams (1 ± 0.35 individuals/m²). Clams there were found at depths between 15–25 cm, though no spat were recovered during neoplasia surveys. Overall the clam flats at HH were the most dynamic, with sediments showing significant erosion and loss or redistribution of animals following prolonged rain storms, possibly associated with stronger bay and river currents. At OQ, densities of adult clams were highest (7.33 ± 1.01 individuals/m²). Adults were generally found there between 20–30 cm depth, and no spat were recovered from sediments down to 35 cm depth during our surveys.

**Clam collections**

Between 2002–2007, Soft-shell Clams (n = 100–200) were collected by hand at the lowest tide of each month from sand flats in NBH and OQ. Soft-shell Clams were collected once annually in October from HH by Normandeau Associates.

**Classification of disease in clams**

To assess the degree of neoplasia for each specimen, a small aliquot of hemolymph (50 µl) was removed from the pericardial sinus, added to a 96-well flat-bottom plate, and incubated for 2 hours at 8 °C. We examined hemolymph samples at 160X on a Zeiss IM inverted microscope. Clams were classified as: normal (0–25% round, non-motile neoplastic hemocytes); early incipient neoplastic hemocytes (26–50%); late incipient neoplastic hemocytes (51–75%); or fully neoplastic and therefore terminally ill clams (76–100% neoplastic hemocytes) (Taraska and Boettger 2013). For this study, we restricted our analysis to clams that were terminally ill and would likely die within 3–10 weeks.

**Age estimation**

To estimate the age of fully neoplastic and therefore terminally ill Soft-shell Clams, shell lengths were overlaid with a growth curve developed by Brousseau (1978, 1979). The von Bertalanffy equation allows mathematical modeling of growth over time (Brousseau 1978, 1979). The nonlinear von Bertalanffy equation can be used to describe mollusk growth rate and convert animal shell length into age, assuming a relationship between size and age. Brousseau (1978) applied the von Bertalanffy equation using constants (a = asymptotic length value, b = constant related to initial conditions, k = growth coefficient) for Gloucester, MA. In our study, shell lengths of terminally neoplastic clams were measured to the nearest 0.1 mm using digital vernier calipers from all collections and sites, and then separated into age classes based on the von Bertalanffy equation and percentages of fully neoplastic clams calculated for each age class.
Statistical analyses

All data were calculated as means with standard errors (SE). Terminal neoplasia levels were compared between sites using descriptive statistics followed by a one-way ANOVA on ranks followed by a Tukey pairwise comparison (SigmaStat, Systat Software Inc., Point Richmond, CA). All statistical analyses were preceded by assessments of the assumption of normality (Kolmogorov-Smirnov test) and homoscedacity (Spearman Rank correlation). Principle component analyses (PCA) followed by linear regressions was used to characterize the effect of environmental factors (i.e., temperatures and contaminants) in relationship to prevalence of clam neoplasia.

Results

Prevalence of disseminated neoplasia

Highest overall frequencies of terminal neoplasia were recorded from NBH, which also displayed the highest quantities of contaminants, including metals, PCBs, and PAHs. The frequency of terminal neoplasia in clams collected from NBH July–September was low ($P < 0.001$) (July: 1.08% ± 0.4, August: 1.24% ± 0.2, and September: 1.77% ± 0.7), when seawater temperatures were highest at that site (20.08 ± 0.5, 21.44 ± 0.6, and 19.73 ± 0.7 °C, respectively) (Fig. 3; seawater temperatures were downloaded from NOAA buoys [BUZM3 = Buzzards Bay, MA; IOSN3 = Isle of Shoals, NH; and WELM1 = Wells Harbor, ME]). Highest average frequencies of terminal neoplasia were recorded for NBH during December and January (9.49% ± 0.78 and 7.50% ± 1.22, respectively) when seawater temperatures were below 5 °C.

In HH, clams were collected only once a year, and as a result, correlation with seawater temperatures was not possible. However, frequencies of terminally neoplastic clams in HH in October of 2002–2007 (3.665% ± 0.553) were significantly lower ($P = 0.021$) than from those collected from NBH during October (5.938% ± 0.733). Levels of contaminants were significantly ($P < 0.001$) lower in HH than NBH, but there were no significant differences ($P = 0.437$) in sediment granulometry and organic contents.

During all collections from OQ (2002–2007; $n = 100$) fully neoplastic clams were found at the lowest frequencies compared to NBH and HH. Terminally affected clams were found in OQ between December and February only (December: 0.13% ± 0.01, January: 0.32% ± 0.02, and February: 0.47% ± 0.05), during times when seawater temperatures were below 5 °C. The OQ clam bed was significantly ($P < 0.001$) lower in environmental contaminants compared to either NBH and HH.

There was no relationship at the different sites between clam densities and terminal neoplasia ($P = 0.46$) development, sediment granulometry and contaminant load ($P = 0.48$), or sediment granulometry and terminal neoplasia ($P = 0.4$).

Estimated ages of neoplastic clams

Overlaying shell sizes of terminally neoplastic clams from all locations with clam ages estimated using the von Bertalanffy equation with growth constants
for Gloucester, MA (Brousseau, 1978, 1979) suggests that the highest frequency ($P < 0.001$) of fully neoplastic clams occurred at average shell sizes of 35–50 mm, which corresponds to estimated ages of 1 to 2 years (9.5 to 57.25%, respectively; Fig. 4). Neoplasia was not observed in clams estimated to be older than 4 years.

**Discussion**

Previous studies correlated disseminated neoplasia in Soft-shelled Clams with episodic environmental contamination events or with presence of retrovirus within tissues (Barber 2004). Based on limited, highly site-specific data, several environmental contaminants have been circumstantially linked to hemocyte cancer in Soft-shelled Clams and *Mytilus edulis* L. (Blue Mussel). Reports show significant correlation with oil, PAHs, PCBs, industrial and municipal waste, pesticides, and heavy metal pollution (review in Barber 2004). Our longer-term study of clam populations sampled over a five-year period at three New England sites of differing contaminant levels showed that terminal neoplasia in clams was directly correlated to elevated levels of PAHs, PCBs, and heavy metals ($P < 0.001$), adding further support to the findings of those earlier studies.

![Figure 3. Prevalence of terminal neoplasia in New Bedford Harbor, Buzzard’s Bay, MA (dark grey bars), Hampton Harbor, NH (light grey bars) and Ogunquit, ME (white bars). Frequencies of Soft-shell Clams with fully neoplastic hemocytes (76–100% round, unattached hemocytes) were determined in monthly collections at New Bedford Harbor and Ogunquit and one annual fall collection in Hampton Harbor (sample years 2002–2007); Seawater temperatures (line graph; means ± SE) were downloaded from NOAA buoys BUZM3 (Buzzards Bay, MA), IOSN3 (Isle of Shoals, NH) and WELM1 (Wells Harbor, ME) and were averaged for all three location because seawater temperatures did not differ significantly between stations.](image-url)
Contaminants may act directly on tissues (Gardner 1993) and cells, and relationships between environmental contaminants and proliferative disease have previously been reported, particularly for molluscan hemocyte disorders. Indirect responses to environmental contaminants are also likely to be involved but have received less attention (Pipe and Coles 1995). Such indirect responses might result in environmental stressors reducing the effectiveness of the bivalve innate immune system (Drynda et al. 1998). Innate immunity in bivalves depends mainly on phagocytosis by hemocytes, and elevated levels of PCBs, PAHs, and copper are known immunosuppressants that decrease phagocytosis by hemocytes (Anderson 1993). Increased levels of phenol can cause hemocyte lysis and a dose-dependent decrease in granulocytes, but humoral factors have not been documented to be affected by pollution (Anderson 1993). Immunosuppression resulting from pollution could compromise clam innate immune defenses against parasites and other potential pathogens such as viruses. Earlier studies pointing out enviromental and/or viral challenges coupled with the data from the current study suggest that: (a) higher concentrations of animals with neoplastic hemocytes are found at contaminated sites (current study) while (b) disease transmission may be accomplished by using hemolymph from neoplastic animals lacking cells (Walker et al. 2009: cells were removed using centrifugation and filtration of neoplastic hemolymph), indicating impact of an infectious agent. Disease etiology is therefore likely not a matter of whether environmental contaminants or virus/infectious agent are involved but how they are involved.

Our data also supports other studies that show seasonality in the incidence of neoplasia, with fewer neoplastic clams found during the summer months.

Figure 4. Average ages of Soft-shell Clams with neoplasia based on theoretical age model. Shell lengths (diameter in mm ± SE) were measured for 167 clams of stage 4 neoplasia (terminal), and ages were determined using the theoretical von Bertalanffy’s growth curve (differential equation permits mathematical modeling of the growth of a population continuously with time).
(Brousseau 1987, Leavitt et al. 1990). An increase in food availability and a decrease in dissolved oxygen availability have been recorded in marine environments (Minier et al. 2000) in the summer affecting ATP production. In addition, Soft-shelled Clams reproduce during the late spring and additionally during the fall, depending on seasonal temperatures (S.A. Böttger, pers. observ.). Gametogenesis requires increased utilization of resources and ATP production. Such oxidative and reproductive stress might underly the seasonal increase in occurrence of terminal neoplasia among clams, and could also result in increased mortality of fully neoplastic clams containing increased numbers of hemocytes and hypoxic hemolymph (Walker et al. 2009).

Previous studies suggested elevated levels of neoplastic hemocytes occur in animals <2 years and >4 years of age (Leavitt et al. 1990), while our data suggest that the highest incidence of neoplasia occurs in clams between the ages of 1–2.5 years (30–56 mm shell length), with the highest frequency at 2 years of age (57.14%). These results indicate that the disease impacts clams that are of harvestable size (between 44–75 mm shell length; Beal 2002) and suggests a potentially significant loss to the commercial clamming industry. Brousseau (1978) suggested that reproductive maturity occurs in *M. arenaria* (Gloucester, Massachusetts) at ≈1.6 years of age, which corresponds to the life-stage at which we found the highest frequencies of neoplasia. Onset of reproduction is a physiologically stressful time which may render animals even more susceptible to development of diseases such as neoplasia. In addition, sites with the high frequencies of terminal neoplasia included in a survey of native populations were also noted to have animals that were generally smaller (50–80 mm), while populations with no occurrence of terminal neoplasia could reach sizes of >100 mm (S.A. Böttger, pers. observ.). These observations indicate that populations with higher frequencies of terminal neoplasia may be skewed towards younger individuals.

Our studies show that frequencies of terminal clam neoplasia are correlated with chronic environmental contamination, which is likely involved in the disease transmission by compromising their innate immune system and making them more susceptible to infectious agents. We also determined that incidence of neoplastic hemocytes was greatest in Soft-shelled Clams during cold months and in animals between 1.5 and 2 year in age. These results indicate that disease development is not only dependent on contaminants and infectious agents but is also influenced by environmental temperatures and the age of the clams. Further efforts are needed to investigate the etiology of the disease and the involvement of contaminants and viruses.

Acknowledgments

We would like to thank former undergraduate and graduate students from the Walker lab and volunteers from Normadeau Associates for their assistance collecting clams. Financial assistance for this project was provided to C.W. Walker (NA08NMF4270416) and S.A. Böttger (NA10NMF4270215) by the NOAA Saltonstall Kennedy program. Sampling of native populations was aided by Marie-Josée Abgrall (University of New
Brunswick), Leophane Leblanc and Eric Tremblay (Kouchibouguac National Park, NB, Canada), Denis Marc-Nault (Maine DMR), Stephen Jones (University of New Hampshire), Jeff Kennedy and Glenn Casey (DMF Massachusetts), Inke Sunila (Connecticut Department of Agriculture), Marta Gomez-Chiarri (University of Rhode Island), Gregg Rivara (Cornell Cooperative Extension, NY), David Bushek (Haskin Shellfish Research Laboratory, NJ), and Chris Dungan (Maryland DNR)

Literature Cited


