NRAC FINAL PROGRESS REPORT

Project Title	Identification of the cause of hemic neoplasia in *Mercenaria mercenaria* and development of management methods
Reporting Period	8/31/2020 – 5/30/2021
Author (Chair)	Roxanna Smolowitz
Key Word	Hemocytic neoplasia, *Mercenaria*, tumor
Funding Level	Total funds allocated for this project to date. *NOTE: This could be reported by Year. i.e.,* *Year One: FY 2017, $ amount* 81,336 *Year Two: FY 2018, $ amount* 93,999
Participants	List participating personnel and respective institutions/agency/business; include outreach representative. Indicate funded participants with an asterisk. **Name(s)/Role(s): Roxanna Smolowitz, PI** Institution/Agency/Business: Roger Williams University Address(s): One Old Ferry Road, Bristol, RI 02809 Phone(s): 401-254-3299 Email(s): rsmolowitz@rwu.edu Funded (Yes/No): yes Rebecca Gast, Co-PI, Wood Hole Oceanographic Institution, MS 32, WHOI, Woods Hole, MA 02543; 5082893209; fax: 5084572169; rgast@whoi.edu; funded Dianne Murphy, Co-PI, Cape Cod Cooperative Extension and Woods Hole Sea Grant, Box 367, Barnstable, MA 02630; 5083756953; fax: 5082747065; dmurphy@whoi.edu; funded Joshua Reitsma (continued work started by D. Murphy who retired in 2020). Cape Cod Cooperative Extension, Box 367, Barnstable, MA 02630; 5083756953; fax: 5082747065;jreitsma@barnstablecounty.org
Project Objectives	**Objective 1**: Identify methods for transmission of the disease by conducting infection experiments to determine if cell free filtrate and/or neoplastic cells themselves can result in disease in hard clams. **Objective 2**: Examine the extent of the disease in Wellfleet in 2018 and 2019 by planting seed from 3 different hatcheries in replicates of three in 2017 at five locations within Wellfleet Harbor, MA. **Objective 3**: Identify the origin of the neoplastic cells and determine whether a retroelement is involved in the transformation. **Objective 4**: Develop a quantitative PCR test method to be used in this research on hemolymph samples, water and sediment, and ultimately for use in diagnosing the disease in hard clams. **Objective 5:** Extension will lead the field work and collaborate directly with local industry members and resource managers during the 2-year grant period and will disseminate and present results of the research.

Anticipated Benefits	State briefly how the project will benefit the aquaculture industry – directly or indirectly. *Mercenaria mercenaria* aquaculture is second only to eastern oyster aquaculture on the east coast of the U.S. Except for the disease caused by QPX, hard clams aquaculturists have enjoyed the luxury of a lack of significant diseases that might economically devastate the clam aquaculturist. However, this disease has the potential to change all that. Information from this study will provide a determination concerning HN's similarity to DN in soft shell clams and other bivalves. It will provide epidemiological data such as temperature/seasonality of the disease. If the disease is similar to disseminated neoplasia of soft shell clams then using that information, we will, as part of this study, work with regulators and aquaculturists to develop handling and movement policies resulting in important controls on how animals with this disease are treated and transferred. The quantitative diagnostic test we will develop will provide a method to make timely and accurate management decisions,
Project Progress	Summarize concisely for each objective the progress toward accomplishment to date. This has an 8,000 character limit. Note: The funding for this grant and the work resulting from it began in April of 2018. **Objective 1**: Identify methods for transmission of the disease by conducting infection experiments to determine if cell free filtrate and/or neoplastic cells themselves can result in disease in hard clams. The year one lab transmission experiment consisted of four treatment methods: injections of cell-free filtrate collected from the hemolymph of neoplastic animals, harvested neoplastic cells injected into the pericardial sac/adductor muscle sinusoid, exposure of clams to harvested neoplastic cells added to the water column in the tanks, and chronic cohabitation of naïve and infected clams in the tanks. The control consisted of naïve, untreated clams. The four treatment methods and one control were conducted in quadruplicate (20 tanks total) with duplicates hooked to one of two different heating/cooling units). Naïve clams were donated by a hatchery in NJ for the study. Neoplastic clams were donated by two aquaculturists in Wellfleet, MA. Our results were as follows (Table 1):

Table 1. Results of the first lab experiment.

	# with HN infections	Total # clams examined histologically from all replicates	% HN
Control	**0**	**25**	**0**
Exposed to sick clams	**7**	**29**	**24.1**
Neoplastic cells from hemolymph added to water	**2**	**26**	**7.7**
Injected with neoplastic cells	**0**	**28**	**0**
Injected with cell free filtrate	**3**	**31**	**9.7**

This work demonstrated that the disease is directly infective. Moreover, it strongly suggested that a cause, other than an infective cell may be important in the conversion of a clam from normal to neoplastic. It was surprising that injection with neoplastic cells did not cause the disease, but the current hypothesis is that the injection also caused bacterial infection (high non-HN mortality in those tanks).

Year two transmission experiments:

Left over naïve clams from year one were examined histologically as an added caution for verifying the experimental test results. Unfortunately we found one HN positive animal in the reservoir of the naïve clam sample. It was thought this animal may have been placed back in the wrong reserve tank after removing a hemolymph sample. But, in year two, as a precaution, we tested 60 naïve animals from the group of naïve clams to be used in the experiment. They were from the same hatchery from which naïve animals were obtained in year one. The experiment was started in the meantime with the same testing parameters as describe in year one. Unfortunately, the positive rate in sample of 60 animals was 8%. Thus four weeks into the experiment, we discontinued the experiment for groups that had shown low positives in year one (neoplastic cells in the water, clams injected with neoplastic cells and clams injected with cell free

filtrate), since we would not be able to tell the difference between or treatment method and the accidental inclusion of a positive "naïve" animal in the tanks. We continued the controls and the tanks with naïve animals exposed to sick animals since results from the previous year had demonstrated a significant different between the two treatments. These last two groups continued in the experiment for a total of 8 weeks before we ended the entire experiment. All animals were histologically examined from the second experiment. Results are below (Table 2).

Table 2. Results of the second lab experiment.

Treatment Method	# with HN infections	Total # of clams examined histologically from all replicates	%HN
Control	0	24	0
Exposed to sick clams	17	53	32.1
Neoplastic cells from hemolymph added to water	1	25	4
Injected with neoplastic cells	0	25	0
injected with cell free filtrate	0	25	0

While we could not trust our results from the category in which neoplastic cells were added to the water (it was not significantly different from the 8% noted in the survey of the naïve clams). The results from the naïve clams exposed to sick clams again indicated direct infection was occurring (32.1% in our experimental animals vs. 8% in the sample of 60 naïve clams and 0 % in our control animals).

We repeated the transmission experiment in the fall of 2020. Due to the pandemic and restriction on personnel, the pandemic seriously affected our ability to run the experiments. We shifted to another source of animals for the 2020 experiments. Unfortunately, that source was positive for neoplasia. So, we tried a third location early in the summer found a source of "naïve" clams (histology was conducted on 60 animals from both of these potential sources). The entire experiment was run a third time in our lab. Interestingly, none of the

naïve animals turned positive in this lab experiment. We have several potential hypotheses for this finding. First, it is possible that the animals used were more resistant to being productively infected. Second, because the innate immune system was fully active when the animals were acquired and placed into the lab set up (late summer/early fall), the lack of infection might indicate an immune component to infection resistance. Third, the salinity in the tanks unfortunately reached levels of 38 to 40 ppt during most of the experiment and it is possible that the neoplastic cells were destroyed in the high salinity water column between animals.

We are conducting an additional test this summer (at no cost to NRAC) to follow up on the last experiment.

Objective 2: Examine the extent of the disease in Wellfleet in 2018 and 2019 by planting seed from 3 different hatcheries in replicates of three in 2017 at five locations within Wellfleet Harbor, MA.

Seed from 3 hatcheries was deployed late in the year in 2018 (September instead of 2017). In addition to the replicates deployed in 4 locations as described in the grant, "left over" animals were planted in larger plots in an additional location (Town Bed). These are also being tested for HN.

Samples of clams were taken from all plots in July of 2019. All animals were negative for HN on that sampling date.

Animals were sample from the plots again in July of 2020. At that time several animals from some plots and some locations were positive for HN. Animals were sampled again in Oct. 2020. Results showed positive clams in all but one strain at one location (Figure 1). These results show the disease has spread to all strains of animals planted in the infected aquaculture location.

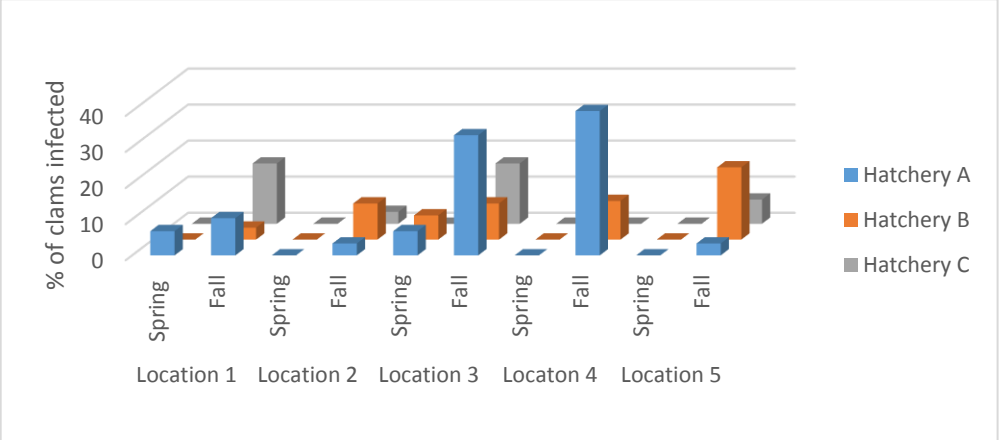


Fig. 1. Results of the spring and fall 2020 sampling of experimental plots in the field showing the % of each clam strain (from 3 different hatcheries) infected with HN.

Water and sediment from all locations has also been sampled and extracted for PCR/qPCR evaluation once specific primers are developed.

Objective 3: Identify the origin of the neoplastic cells and determine whether a retroelement is involved in the transformation.

To date hemolymph from uninfected and infected clams and tissue from both types have been explored for ITS sequences followed by cloning and sequencing of plasmid clones (8/sample).
Consensus sequences from both normal and neoplastic animals and tissues from both match hard clam sequences in the NCBI Database. No sequence was associated only with infected clams. While this information did not provide a neoplastic specific sequence, it did confirm that the neoplastic cells originated from the hard clam and not from another species of clam/bivalve.

Metzger (2018) tested one hard clam (without neoplasia) with primers in order to identify steamer-like elements. Three hits were described in that paper. Our work showed positive hits with three sequences. SLE4M (a Mya version of Steamer) and SLEP a (a degenerative primer set) both recovered sequences from both normal and neoplastic hard clam samples, so provided no neoplastic specific primers. However SLEPa1 primers (used by Metzger on Politipates aureus) did results in a specific hard clam HN sequence which does not match anything in the database or any sequences previously published by Metzger. However it did not recognize all of the neoplastic animals tested.

We investigated the COI gene of our HN vs. normal hemocyte cells. Previous, Baker et al. (2008) showed that the COI gene has many haplotypes in hard clams along the entire east coast of the U.S. and they produced a relatedness genetic tree showing the haplotype relationships. According to Metzger et al. (2015), the neoplastic cells should most probably have one origin cell and so would all have the same COI haploptype and that the haplotype of the HN cell would be different from the tissue haplotype in the infected clam. We found that as expected the COI haplotypes varied in the normal hemocytes from uninfected clams. We found as predicted that the haplotypes of the HN cells were different than the tissue haplotypes in infected clams. But we also found that the COI haplotypes varied in the HN cells indicating that more than one probable transformation to a HN cell had occurred! This was not expected and raised the question again of what is causing the transformation of hard clams cells to become HN cells (Tables 3 and 4).

Table 3.

COI Haplotypes of Normal Hemocytes and HN Cells

Normal Hemocytes	HN Cells
1,7,8,12,13,41	**1,3,9,13,51,56**

Table 4. A comparison of haplotypes of HN cells with the tissue haplotypes of the tissue from the infected individual clam.

Infected clams haplotypes		
Animal #	HN cells	Clam tissues
1	51	1
2	51	13
3	1	13
4	1	53

Because we could not identify a gene associated only with the HN cells with which we could develop a specific qPCR diagnostic test method, we began to look at RNA expression in the HN Cells (vs normal hemocytes).
A distinct difference in the RNA expression data was found between normal clam hemocytes and HN infected clam hemocytes (Figure 2).

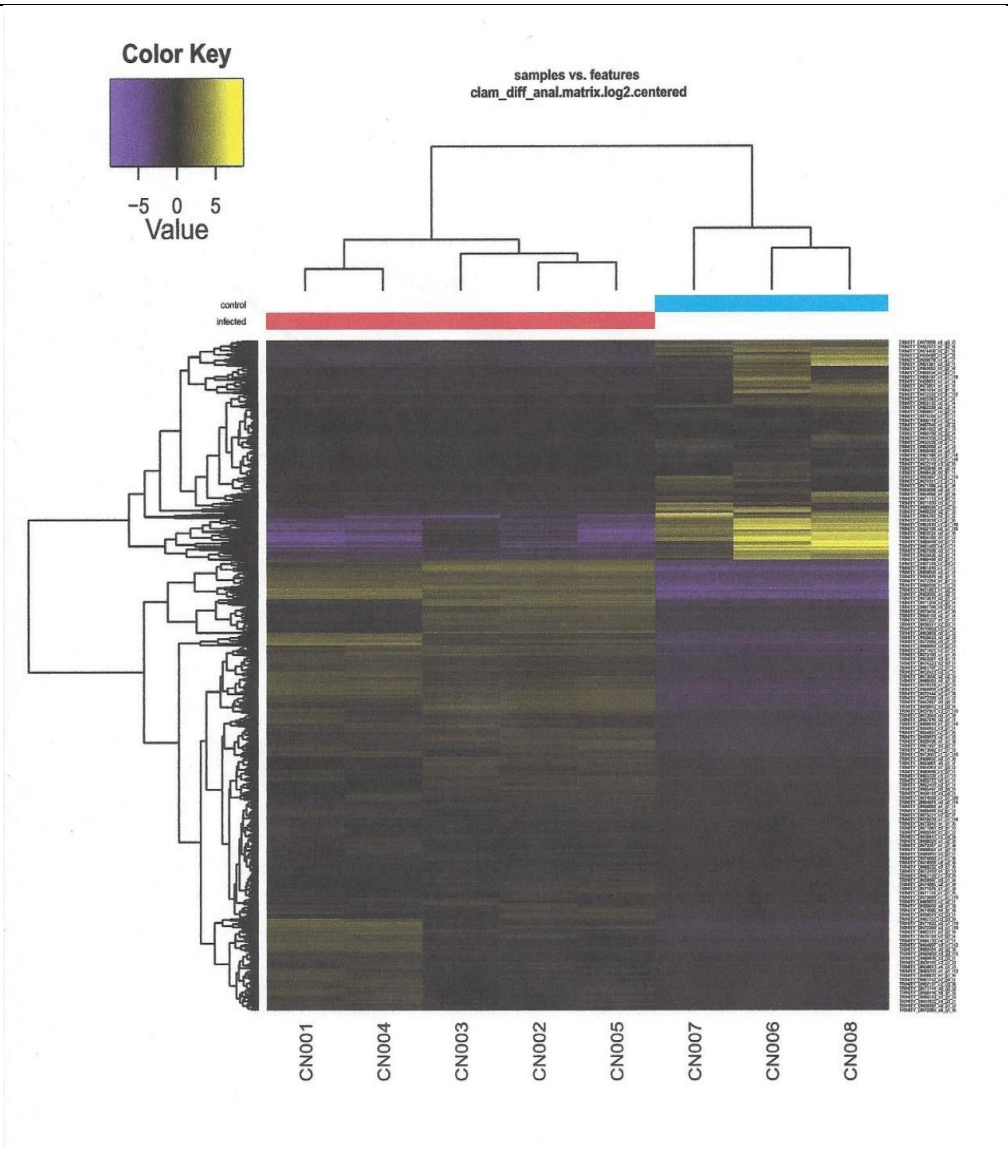


Fig. 2. RNASeq data. CN001-005 are infected clams. CN006-008 are normal clams

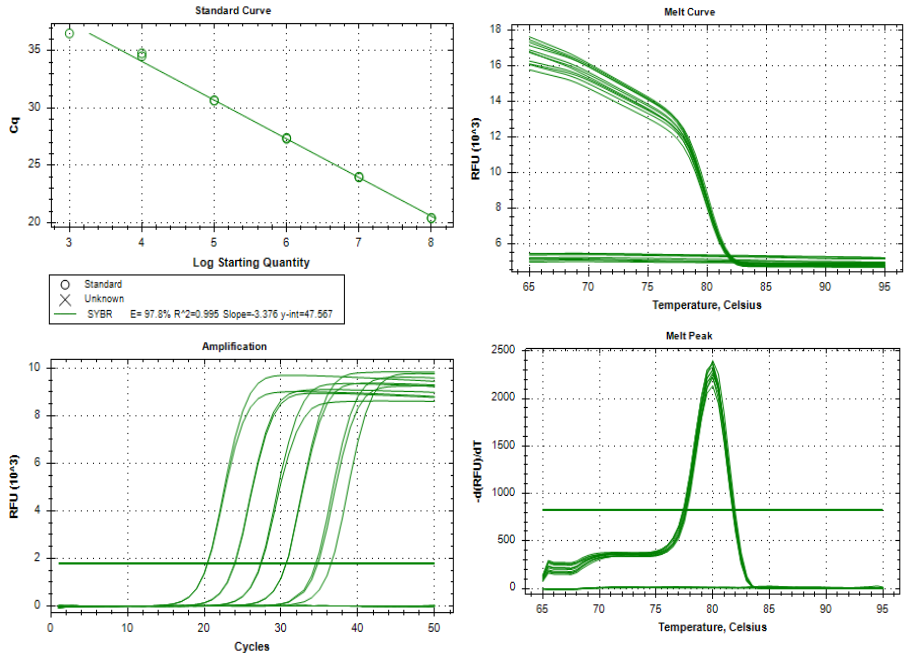
Using the RNASeq data, Gast has recently identified HN specific upregulated transcripts (not show here). These can now be used to develop a new HN diagnostic test in a new study.

Objective 4: Develop a quantitative PCR test method to be used in this research on hemolymph samples, water and sediment, and ultimately for use in diagnosing the disease in hard clams.

We evaluated our samples for the presence of Steamer-like retroelements present in other bivalve neoplasia (Mya) as potential targets for qPCR. Primers available in the literature were used on our samples and we identified two retroelements; these were similar to SLE4 and SLEPa1 elements previously found in *Mercenaria*. We designed primer sets for each of these (SLEM4 and

SLEMPa) and developed qPCR tests using these as standards (Figure 3).

Figure 3. qPCR Standard curve development results for SLEM4



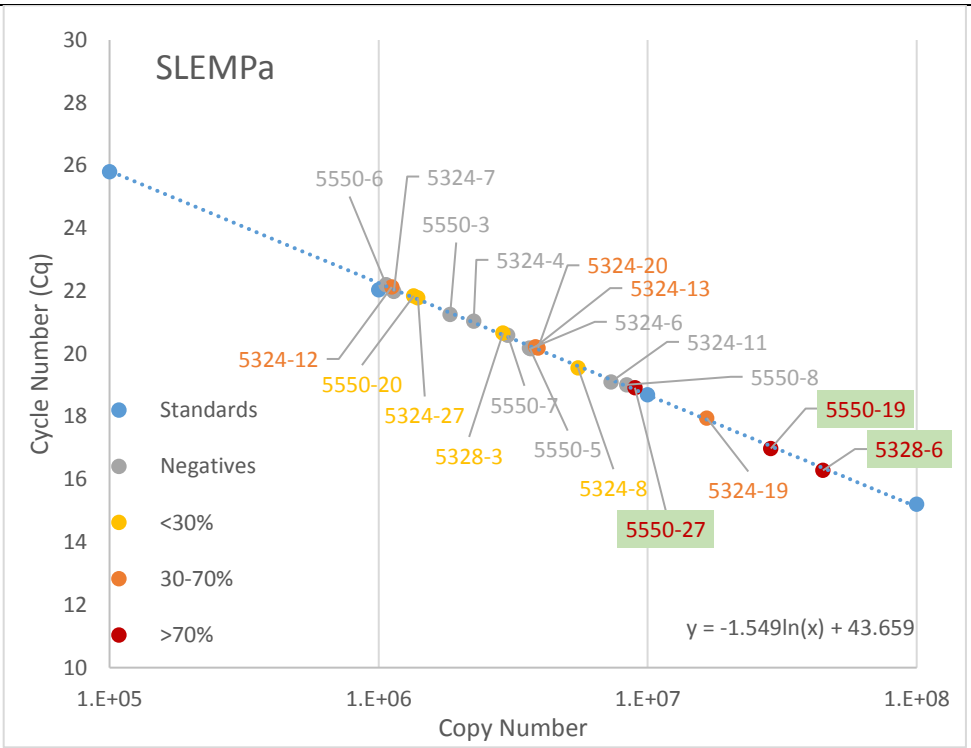


Figure 4. qPCR evaluation of positive and negative hemolymph (clam blood) samples for SLEMPa as identified by case number (ADL identification system) and level of neoplasia (determined histologically as negative, <30% neoplastic cells, 30-70% neoplastic cells and >70% neoplastic cells in the vascular system). There is no clear distinction between positive and negative animals using these primers.

They were then tested with uninfected, low infection and high infection samples from our clams (as determined histologically). While highly infected clams gave clear thresholds of detection, distinction between low infection and uninfected samples was not possible (Figure 4). This makes the assay unsuitable for use in examining clams or environmental samples. Work continues to find primers that will be specific for HN.

Further work (haplotypes) did not provide a specific gene sequence that could be used to develop a diagnostic test. However this spring, we were able to identify specific upregulated RNA that could be used to develop a diagnostic test. Additionally, the genome of *Mercenaria mercenaria* was recently published, opening up a new ability to find HN associated DNA genes that could be used. Continuation of the work, to develop a diagnostic test and further understanding of the cause of the disease, will need new funding.

Objective 5: Extension will lead the field work and collaborate directly with local industry members and resource managers during the 2-year grant period and will disseminate and present results of the research.
The yearly monitoring has been conducted and will be used to communicate our

	progress to the aquaculturists in Wellfleet. Smolowitz and Reitsma have presented data to date to in shellfish training courses and at meetings of the shellfish officers as well as a Cape Cod aquaculturist training course held annually. Smolowitz has also presented findings to date to the RI Biosafety committee (which included USDA-APHIS and MA Marine Fisheries representatives).
Accomplishments:	
Outreach Overview	Describe in general how your results have been extended to the intended users. OR, if they haven't yet, explain when & how this will occur. *Objective 5a:* Extension worked with Wellfleet hard clam growers and resource managers to deploy and maintain the naïve clams in test plots over the course of the study. *Objective 5b:* R. Smolowitz was invited by Extension to a meeting of all shellfish constables on Cape Cod to explain finding to date and answer questions. *Objective 5c:* Extension will present research results in year 3 in the fall of 2021 to the public in Wellfleet. We have presented results at several NSA conferences also. In addition, a fact sheet will be produced for the industry which will explain the nature of the disease and include management recommendations at in the fall of 2021. *Objective 5d:* Extension will organize two regional workshops to share results of this study and importance of recognizing emerging diseases and their potential economic impact on industry. Workshops will also include education on biosecurity and basic disease management recommendations. This will occur later in 2021. **Note:** The Pandemic outbreak has curtailed engagement with aquaculturists. However we will try to develop Zoom meeting in the coming months.
Targeted Audiences	Provide information on the **target audience** for efforts designed to **cause a change in knowledge, actions, or conditions**. The target audience for results of this research are the aquaculturists, regulators and disease specialists.
Outputs:	Outputs are tangible, measurable products (website, events, workshops, products [AV, curricula, models, software, technology, methods, websites, patents, etc.], trainees, etc.). Do NOT include publications as they're listed separately. Outputs are new markers for HN and normal clams as well as development of in laboratory methods for transmission. Potential management methods are being discussed with regulators, extension agents and nursery managers.
Outcomes/Impacts:	Describe how findings, results, techniques, or other products that were developed or extended from the project generated or contributed to an

	outcome/impact. **Outcomes/impacts are defined as changes in Knowledge, Action, or Condition.** Data from laboratory experiments show that the neoplasia is contagious. This has implications for movement of animals from nursery areas to previously uninfected areas. Molecular work shows the HN cell is of *Mercenaria mercenaria* origin and does not originate from other clam species such as *Mya arenaria*. This is important since many aquaculturists are worried about cross-species infections.
Impacts Summary	Provide short statements (2-3 sentences) about each of the following: (pre-established fields for Researchers to complete short statement answers) 1. **Relevance:** Issue – what was the problem? A new potentially contagious neoplastic disease has been identified in cultured hard clams in MA. The tumorous infection is similar, but not the same as the disease described in soft shell clams on the east coast of the U.S. 2. **Response:** What was done? The Barnstable Cooperative Extension (Diane Murphy/J. Reitsma) and the Aquatic Diagnostic Laboratory at Roger Williams University have been monitoring the disease in the last 11 years. More recently, the USDA-APHIS has provided support for regional as well as local monitoring for the disease. The disease has spread throughout Wellfleet Bay, MA and has been found in other locations in MA within the past two years and most recently in RI. Recently HN has been identified in clams from 2 other states. This study is the first to try to understand the disease and its cause. 3. **Results:** How did your work make a difference **(change in knowledge, actions, or conditions)** to the target audiences? Precautions for movement of nursery and adult clams needs to be addressed by regulators and aquaculturists. 4. **Recap:** One- sentence summary Our work has shown this is a transmissible disease of nursery to adult hard clams, is an important new disease and has resulted in new management methods in the affected states.
Publications	Follow the format to list publications in the following categories: • Presentations: o Oral – Smolowitz, 2018 NSA meeting. Title of presentation: An update on hemocytic neoplasia in hard clams cultured in MA o Oral – Smolowitz, 2019 NSA meeting. Title of presentation: An update on hemocytic neoplasia in hard clams cultured in MA. o Oral – Smolowitz, Shellfish Disease training session for Massachusetts Shellfish Officers Association Course, Feb. 2019 o Oral – Smolowitz, Shellfish Disease training session for Barnstable County Aquaculture course, April, 2019

	○ Oral – Smolowitz, Shellfish Disease training session for Massachusetts Shellfish Officers Association Course, Feb. 2020 (Zoom) ○ Oral – Smolowitz, 2021 NSA meeting. Title of presentation: An update concerning the ongoing research and occurrence of Neoplasia and QPX in the northern quahog (=hard clam) in Massachusetts, USA (Zoom) ○ Oral- Smolowitz, May, 2021, RI Biosafety Committee, Title of presentation: An update concerning the ongoing research and occurrence of Neoplasia and QPX in the northern quahog (=hard clam) in Massachusetts, USA (Zoom) ○ Posters • Peer-reviewed: None ○ Print (journal, etc.) ○ Digital (websites, videos, etc.) • Non-Peer-reviewed: None ○ Extension factsheets ○ Popular articles
	Provide the following information for **every** student that worked with you during the reporting period: • Name: Zachary Forbes • Whether Degree was completed during the reporting period (name, yes/no): yes • New or Continuing Student: continuing • Capstone/Thesis Title (actual or anticipated): no • Date of Graduation: BS in May 2021 • Provide link to thesis/dissertation document: • Name: Jemma Dickson • Whether Degree was completed during the reporting period (name, yes/no): no • New or Continuing Student: Continuing • Capstone/Thesis Title (actual or anticipated): no • Date of Graduation: Dec. 2021 • Provide link to thesis/dissertation document: • Name: Sandra Remson • Whether Degree was completed during the reporting period (name, yes/no): no • New or Continuing Student: continuing • Capstone/Thesis Title (actual or anticipated): no • Date of Graduation: May 2023 • Provide link to thesis/dissertation document:

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<tr><td></td><td colspan="4">

- Name: Hannah Cameron
- Whether Degree was completed during the reporting period (name, yes/no): no
- New or Continuing Student: continuing
- Capstone/Thesis Title (actual or anticipated): no
- Date of Graduation: May 2022
- Provide link to thesis/dissertation document:

- Name: Casey Dunbar
- Whether Degree was completed during the reporting period (name, yes/no): no
- New or Continuing Student: continuing
- Capstone/Thesis Title (actual or anticipated): URI Surf summer award/presentation
- Date of Graduation: May 2023
- Provide link to thesis/dissertation document:

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<tr><td rowspan="2">Partnerships</td><td colspan="4">List any partners that you worked with on your project. Provide the following information for each Partner:</td></tr>
<tr><td>Partner

USDA-APHIS</td><td>Specific Type
Monitoring of clams for HN in additional locations in MA and other states.</td><td>Level
Smolowitz/ADL Contract work for USDA</td><td>Nature of Partnership

Contract (2019 and 2020)</td></tr>
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