PROJECT COMPLETION REPORT
Genetic Marker-Assisted Selection of Northeastern Hard Clams for QPX-Resistance
Subaward # Z555103
Grant # 2012-38500-19656

PROJECT CODE: **SUBCONTRACT/ACCOUNT:**

PROJECT TITLE: Genetic Marker-Assisted Selection of Northeastern Hard Clams for QPX-Resistance

DATES OF WORK: 02/01/2013-01/31/2017 (including 1 year no-cost extension)

FUNDING LEVEL: $199,998

PARTICIPANTS:

Principal Investigators:

Bassem Allam / Professor / School of Marine and Atmospheric Sciences / Stony Brook University / Stony Brook, NY 11794-5000 / Phone: 1 631 632 8745 / Fax: 1 631 632 8915 / E-mail: bassem.allam@stonybrook.edu - Funded

Ximing Guo / Professor / Haskin Shellfish Research Laboratory / Rutgers University / 6959 Miller Avenue / Port Norris, NJ 08349 / Phone: (856) 785-0074 ext. 4324 / Fax: (856) 785-1544 / E-mail: xguo@hsrl.rutgers.edu - Funded

Roxanna Smolowitz / Associate Professor / Roger Williams University / One Old Ferry Road, Bristol, RI 02809 / Phone: (401) 254-3299 / Fax: (401) 254-3310/ Email: rsmolowitz@rwu.edu – Funded

Emmanuelle Pales Espinosa / Research Scientist / School of Marine and Atmospheric Sciences / Stony Brook University / Stony Brook, NY 11794-5000 / Phone: 1 631 632 8694 / Fax: 1 631 632 8915 / E-mail: epalesespino@notes.cc.sunysb.edu - Funded

Gregg Rivara / Aquaculture Specialist / Cornell University Cooperative Extension of Suffolk County / 3690 Cedar Beach Road / Southold, NY 11971 / Phone: (631) 852 8660 ext. 35 / Fax: (631) 852 8662 / Email: gjr3@cornell.edu - Funded

Cooperating Participants:

George (Gef) Flimlin / Marine Extension Agent / Rutgers Cooperative Extension / 1623 Whitesville Road / Toms River, NJ 08755 / Phone: (732) 349-1152 / Fax: (732) 505-8941 / Email: flimlin@aesop.rutgers.edu – Unfunded

Diane Murphy / Fisheries & Aquaculture Specialist / Cape Cod Cooperative Extension & Woods Hole Sea Grant / Box 367 / Barnstable, MA 02630 / Phone: (508) 375 6953 / Fax: (508) 362-4923/ Email: dmurphy@whoi.edu - Funded

Arnaud Tanguy / Associate Professor / University of Paris 6 / Station Biologique de Roscoff / Place Georges Teissier BP74/ 29682 Roscoff, France / Phone: +33 298 292 527 / Fax: +33 298 292 324 / Email: atanguy@sb-roscoff.fr - Unfunded

Antoinette Clemetson / Fisheries Specialist / NY Sea Grant / 3059 Sound Avenue / Riverhead, NY 11901 / Phone: (631) 727-3910 / Fax: (631) 369-5944 / Email: aoc5@cornell.edu - Unfunded

REASON FOR TERMINATION: Objectives completed and funds terminated

PROJECT OBJECTIVES:

- **Objective 1:** Select candidate genes based on sequence information generated from our prior investigations and validate single nucleotide polymorphism loci for clam genotyping

- **Objective 2:** Proof-test the link between the polymorphism of the candidate genes and QPX resistance on samples preserved from prior field work preceding and following QPX-related clam mortalities

- **Objective 3:** Validate the markers identified in Objectives 1 and 2 for the assessment of the resistance of different seed strains used for aquaculture along the east coast during QPX exposure studies

- **Objective 4:** Provide the aquaculture industry with superior germlines derived from selected clams surviving QPX-related mortalities

ANTICIPATED BENEFITS:

The genetic markers identified in this project are expected to represent a useful method for forecasting clam resistance to QPX infection. A direct outcome of this research is the identification of resistant clam stocks that will help the aquaculture industry face QPX disease outbreaks. The project will also generate important genomic information that will be made public, fostering research on this economically and ecologically important species.

PRINCIPAL ACCOMPLISHMENTS:

Objective 1: Transcriptomic sequence data from previous research (707 Million Illumina 100 bp reads) were assembled and screened for single nucleotide polymorphism (SNP). Assembled transcripts included a total of 66,378 contigs displaying SNP variations, in addition to 568 and 426 contigs displaying deletion/insertion variants or multiple nucleotide variants, respectively. An additional 19,037 contigs simultaneously displayed 2 or more types of variations. All contigs were functionally annotated and a total of 384 immune-related genes that have SNPs transcripts were used to genotype clams using genotyping by sequencing approaches (Ion AmpliSeq method in conjunction with next generation sequencing). Primer pairs were successfully designed for 373 transcripts. They were synthesized and pooled for multiplex amplification and genotyping of clams sampled before and after QPX disease outbreaks.

Objective 2: Hard clams were collected from three stocks before deployment in Massachusetts and New Jersey. The deployed clams were sampled again after field mortalities (47 – 93%) caused primarily by QPX. DNA from 56 - 64 clams were pooled in equal amounts producing 3 before and 6 after-mortality DNA pools. The pooled DNAs were used as templates for amplification of 373 candidate genes with the AmpliSeq primer panel. Amplified products were sequenced to about 1000x per gene with the Ion Torrent PGM 400 bp module. Of the 373 genes targeted, 98 genes were successfully amplified and sequenced in all 9 samples. SNPs and indels were identified and analyzed for post-mortality frequency shifts. Nine SNPs in seven genes showed consistent allele frequency shifts in all three stocks and at both sites, suggesting they may be linked to QPX-resistance or survival.

Objective 3: A total of 5 clam strains were deployed in a field site in NY in early July 2014 including 3 custom-spawned clams (SC, NJ and MA strains derived from clams that survived QPX mortalities) and 2 commercial strains (NY and a second MA strain hereby designated MA2) (5 replicates each). The 2 commercial clam strains (NY and MA2) were also deployed in MA. Deployed clams were monitored for 2 years and results showed marked difference in clam resistance to QPX disease in the MA site (disease prevalence in NY was low for all strains). Interestingly, results showed that the disease developed significantly more in the MA clam strain as compared to the NY strain (obtained from Frank M. Flowers and Sons Oyster Co. Oyster Bay, NY) with prevalence averaging 50% and 10%, respectively. DNA samples from clams collected before and after deployment are being submitted to genotyping to validate the genetic markers identified in Objective 2. The initial delay in the establishment of the award led to a delay in field deployment and final sample collection. We expect the validation step to be completed in the next 3 months.

Objective 4: The project allowed the identification of resistant clam stocks (Frank M. Flowers and Sons line). This clam line is available and will be evaluated for use in breeding programs throughout the Northeast. Upon validation, the new genetic markers will also be published and shared with stakeholders and scientists for use in marker-assisted selection programs.

IMPACTS:

- For the first time, genetic markers have been associated with survivorship following QPX outbreaks
- Identified clam stocks that are resistant to QPX disease
- Communicated study results to stakeholders for the promotion of resistant clam stocks
- Provided the industry (growers in MA, NY and NJ) with disease testing results on clam broodstock and seeds

RECOMMENDED FOLLOW-UP ACTIVITIES:

Study results served as a base for the development of a new research program recently funded by the USDA (NIFA) to validate the identified genetic markers as predictors for clam resistance to the infection (not just as being correlated to resistance). We propose to extend this research to the discovery of additional genetic markers linked to resistance and overall yield. Further, the potential benefits of integrating resistant broodstocks (Frank M. Flowers and Sons line) into the breeding programs of commercial hatcheries throughout the Northeast needs to be assessed.

SUPPORT:

Year	NRAC-USDA funding	Other support					Total support
		University	Industry (in-kind)	Other Federal	Other (in-kind)	Total	
1	68,502	14,900 [1]	Clams [2]	0	Field support [3]	14,900	83,402
2	63,123	14,900 [1]	0	0	Field support [3]	14,900	78,023
3	68,373	14,900 [1]	0	0	Field support [3]	14,900	83,273
Total	199,998	44,700 [1]	0	0	Field support [3]	44,700	244,698

[1] The university cost share is contributed by Stony Brook University as support to Allam's academic salary.
[2] Clams were provided by three commercial hatcheries located in MA and NJ (names of hatcheries are not presented here to maintain confidentiality) and NY (Frank M. Flowers and Sons, Oyster Bay, NY).
[3] Field support was provided by the New York State Department of Environmental Conservation (boat use + captain time) for accessing the field site in NY

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED:

- Peer-reviewed publications:

Wang K, Del Castillo C, Corre E, Pales Espinosa E, Allam B. (2016). Clam focal and systemic immune responses to QPX by RNA-Seq technology. BMC Genomics 17:146.

Wang K, Pales Espinosa E, Tanguy A, Allam B. (2016). Alterations of the immune transcriptome in resistant and susceptible hard clams (Mercenaria mercenaria) in response to Quahog Parasite Unknown (QPX) and temperature. Fish and Shellfish Immunology 49: 163-176.

Allam B, Pales Espinosa E. (2016). Bivalve immunity and response to infections: Are we looking at the right place? Fish and Shellfish Immunology, 53: 4-12

Dahl S, Allam B. (2016). Hard clam relocation as a potential strategy for QPX disease mitigation within an enzootic estuary. Aquaculture Research 47(11):3445-3454.

Allam B, Raftos D. (2015). Immune responses to infections. Journal of Invertebrate Pathology 131: 121-136.

In Preparation: Guo X, Wang G, Pales Espinosa E, del Castillo C, Tanguy A, Kraeuter J, Allam B. Identification of QPX-resistance markers by genome-wide candidate-gene association study in the hard clam.

- Presentations:
  - Oral

    Allam B, Pales Espinosa E, Wang G, Smolowitz R, Murphy D, Rivara G, Guo X. (2017). Development of strategies to mitigate QPX disease in the hard clam. Northeastern Aquaculture Conference and Expo. January 11-13, 2017. Providence, Rhode Island, USA.

    Guo X, Wang G, del Castillo C, Pales Espinosa E, Tanguy A, Kraeuter J, Allam B (2016). Identification of QPX-resistance markers by genome-wide candidate-gene association study in the hard clam. World Aquaculture Society Triennial Meeting, Las Vegas, NV. February 22-26, 2016.

    Allam B, Pales Espinosa E. (2016). Bivalve immunity and response to infections: Are we looking at the right place? International Society for Fish and Shellfish Immunology. Portland, Maine, USA. June 26-July 1, 2016.

    Dahl S, Allam B. (2015). Will Climate Change Help New York Hard Clams Fight Disease? Northeastern Aquaculture Conference and Exposition. January 14-16, 2015. Portland, Maine, USA.

    Allam B. (2015). Bridging basic and applied biological science in support of shellfish aquaculture. Long Island Shellfish Managers meeting. January 30, 2015.

    Dahl S, Allam B. (2014). Will climate change help New York hard clams fight disease? Meeting of the New York Marine Science Consortium. October 18, 2014.

    Dahl S, Barnes D, Allam B. (2014). QPX disease relationships with environmental parameters monitored over a decade in a Raritan Bay (NY) hard clam fishery. 106th Meeting of the National Shellfisheries Association. March 25-29, 2012. Jacksonville, Florida, USA.

    Allam B. (2013). QPX in hard clams: current disease status and mitigation strategies. New York State's Shellfisheries Advisory Committee. March 27, 2013. NY.

    Allam B. (2013). QPX disease in the hard clam: from fundamental research to disease management strategies. University of Rhode Island. November 2013.

  - Posters

    Wang K, Del Castillo C, Pales Espinosa E, Allam B. (2014). Clam focal and systemic immune responses to QPX revealed by RNA-Seq technology. 106th Meeting of the National Shellfisheries Association. March 25-29, 2012. Jacksonville, Florida, USA.

    Wang K, Pales Espinosa E, Allam B. (2014). Effect of “heat shock” treatments on QPX disease in the hard clam, Mercenaria mercenaria. 106th Meeting of the National Shellfisheries Association. March 25-29, 2012. Jacksonville, Florida, USA.

- Non-Peer-reviewed: Published abstracts:

  Wang K, Del Castillo C, Pales Espinosa E, Allam B. (2014). Clam focal and systemic immune responses to QPX revealed by RNA-Seq technology. Journal of Shellfish Research 33(2): 661.

  Wang K, Pales Espinosa E, Allam B. (2014). Effect of "heat shock" treatments on QPX disease in the hard clam, Mercenaria mercenaria. Journal of Shellfish Research 33(2): 661.

- Students dissertations:

  - Kailai Wang (2016). Molecular characterization of clam (Mercenaria mercenaria) immune responses against Quahog Parasite Unknown (QPX): Effect of host and environmental factors. http://gradworks.umi.com/10/13/10139857.html

  - Soren Dahl (2015). Ecology of QPX disease in the hard clam Mercenaria mercenaria. http://gradworks.umi.com/10/00/10000662.html

This website describes the project: http://you.stonybrook.edu/madl/research/mas_clam/

PROJECT COMPLETION REPORT
Genetic Marker-Assisted Selection of Northeastern Hard Clams for QPX-Resistance
Subaward # Z555103
Grant # 2012-38500-19656

Project Code: Subcontract/Account #

PART II

The hard clam or northern quahog, *Mercenaria mercenaria*, is one of the most valuable seafood products in the Northeast representing the first marine resource in several states. In addition to their economic value, hard clams, like other suspension feeding bivalves, play an important ecological role in benthic-pelagic coupling by transferring energy to the benthos and cycling large amounts of particulate matter. In recent years, the focus of the clam fishery has shifted from wild harvest to aquaculture production. Since the 1990's, several Northeastern states have suffered severe losses in aquacultured hard clam stocks due to a fatal disease caused by a protistan parasite called Quahog Parasite Unknown (QPX).

The overall aim of this project was to identify genetic markers associated with clam resistance to QPX disease. We screened a large *M. mercenaria* transcriptome dataset and identified genetic variants in key candidate immune genes. We further evaluated the association between these markers and resistance to QPX by evaluating variant frequency shifts after QPX mortality events. Results allowed the identification of 9 variants that hold promise for the development of marker-assisted breeding programs. In parallel, we evaluated disease resistance between different clam stocks and were able to show marked difference in resistance between Northeastern clam stocks.

Research Aim I. Identification of single nucleotide variants

Transcriptomic sequence data generated by our group from previous research (Wang *et al.*, 2016a) were compiled and screened for SNP detection. A total of 707 Million raw Illumina (100 bp in length) reads were filtered and trimmed according to length and quality score (min length 60 nt, end trimming quality 25, min quality filtering: 20 on 75 % of the read length) using the FASTX-Toolkit software v 0.0.13. rRNA cleaning was performed using the riboPicker software v 4.0.3 against SILVA database v111. High quality filtered sequence reads were subsequently used for de novo assembly using the de Bruijn graph assembler Trinity using the default parameters. Annotation of this de novo assembled transcriptome was performed using Blastx search against National Center for Biotechnology Information (NCBI) nonredundant sequences (nr) database with the E-value threshold setting at 1E-06. Putative gene functions were predicted by sequence similarity search against Gene Ontology (GO) database and assigning GO annotation terms to each mapped transcript. Protein domain search and enzyme annotation were also performed using InterPro scan and the Kyoto Encyclopedia of Genes and Genomes (KEGG). The annotated assembly was then exported to CLC Genomics Workbench bioinformatics software where variant searches were performed by mapping individual reads to the assembly using default parameters. A quality score of 30 was used to filter variants and only maintain high quality calls.

Assembled transcripts included a total of 66,378 contigs displaying single nucleotide variants, in addition to 568 and 426 contigs displaying deletion/insertion variants or multiple nucleotide variants, respectively (Figure 1). An additional 19,037 contigs simultaneously displayed 2 or more types of variations.

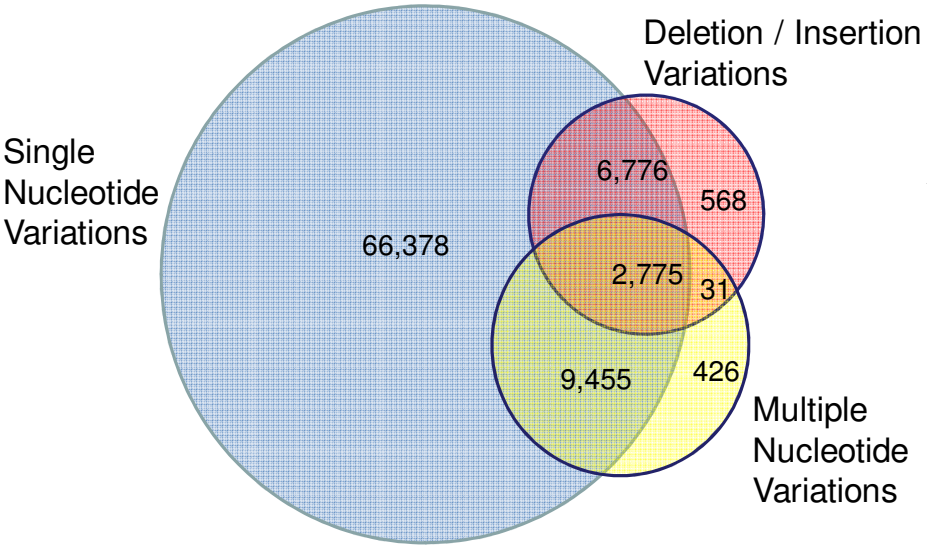


Figure 1. Number of transcriptomic sequences showing different types of genetic variants.

Research Aim II. Identification of genetic variants associated with QPX disease resistance

A total of 903 transcripts were identified to play a role in immune defenses and showed at least 1 type of genetic variation. These were further screened to identify those displaying single nucleotide polymorphism (SNP) variations that are appropriate for genotyping by sequencing. Therefore, 384 immune-related genes that have SNPs and conserved flanking sequences that are suitable for primer design were chosen for genotyping using the AmpliSeq method in conjunction with high throughput sequencing technologies. This technique allows the use of next generation sequencing technology for cost-effective genotyping of a large number of SNPs. Primer pairs were designed using the Ion AmpliSeq™ Designer to amplify the candidate 384 genes. Primers were synthesized and pooled in a single-tube for multiplex amplification of each experimental sample. Among the 384 primer pairs, 373 pairs were shown to amplify target clam DNA. These were used for probing SNP frequency shifts associated with clam survivorship following QPX epizootics.

In this framework, the study contrasted SNP frequencies shifts in clams before and after exposure to QPX mortalities. Samples used in this work were generated from a previous field study described earlier by Kraeuter *et al.* (2011). Briefly, gill samples were obtained from three genetically-distinct clam stocks before deployment in Massachusetts and New Jersey. These included a clam strain from South Carolina, a strain from New Jersey, and a strain from Massachusetts. The deployed clams were sampled again after field mortalities (47 – 93%), caused

primarily by QPX. DNA was extracted from 56 - 64 clams from each group before pooled in equal amounts producing 3 before and 6 after-mortality DNA pools. The pooled DNAs were used as templates for amplification of the candidate genes with the AmpliSeqTM primer panel. Amplified products were purified before sequenced with the Ion Torrent PGM 400 bp module. Frequencies of each SNP were compared in samples collected before and after QPX-related mortality in a total of 6 paired comparisons, to identify significant or consistent changes.

Of the 373 genes targeted, 194 genes (52%) were successfully amplified and sequenced. Single-nucleotide polymorphisms (SNPs) and indels were identified and analyzed for post-mortality frequency shifts. About 0.55 Million reads were generated for each pool, averaging 850x coverage per gene. A total of 777 SNPs were identified in 140 genes, at a density of 1.4% or 1 SNP/70 bp. Among these, a total of 70 SNPs showed significant allele frequency shifts before and after field deployment, with 9 SNPs in 7 genes showing consistently allele-frequency shifts across all clam strains in both field sites (Table 1). The genes include interferon-induced guanylate-binding protein 2 (GBP2), cytochrome b-245 light chain-like (CYB245), a metalloproteinase domain-containing protein 10 (MP10), inhibitor of apoptosis 1 (IAP1), sparc-related modular calcium-binding protein 1-like (SMOC1), hemagglutinin/amebocyte aggregation factor (HAAF), and programmed cell death protein 7-like (PCDP7).

Table 1. SNPs with allele-shifts in the same direction (9 SNPs in 7 genes). Stock-State combinations are shown in separate columns (e.g. M-MA and M-NJ designate the Massachusetts clam strain deployed in Massachusetts or New Jersey, respectively).

				After/before frequency ratio, stock-state						
Gene	**Position**	**Ref**	**Variant**	**M-MA**	**M-NJ**	**N-MA**	**N-NJ**	**S-MA**	**S-NJ**	**P-value**
GBP2	28	C	T	0.67	0.84	0.88	0.86	0.85	0.74	0.0029
CYB245	117	G	A	0.80	0.78	0.90	0.67	0.88	0.84	0.0023
MP10	83	C	T	0.68	0.78	0.83	0.68	0.82	0.74	0.0004
	127	G	A	0.72	0.90	0.74	0.65	0.85	0.92	0.0073
	244	A	G	0.77	0.84	0.75	0.76	0.79	0.85	0.0002
IAP1	116	G	A	0.77	0.85	0.94	0.65	0.63	0.97	0.0074
SMOC1	171	-	A	0.96	0.88	0.96	0.91	0.94	0.77	0.0094
HAAF	72	C	T	1.07	1.12	1.03	1.01	1.03	1.08	0.0084
PCDP7	486	A	T	1.48	1.99	1.25	1.04	1.29	1.27	0.0088

In particular, a novel mutation in a gene involved in the homeostasis of reactive oxygen species (CYB245) was shown to be associated with increased clam survival (Figure 2). This mutation represents a prime candidate for the validation of genetic markers associated with QPX resistance.

Figure 2. Enhanced clam survival was associated with a novel non-synonymous mutation in CYB245 gene.

Most SNPs that shifted in opposite directions may represent sampling or genotyping artifacts. However, some SNPs may be linked to the resistant allele in opposite phases in different populations and therefore shift in opposite directions. Some of the genes that display opposite shifts in different clam strains and/or field sites are given in Table 2. They include tumor necrosis factor ligand superfamily member 6 (TNF), TNF receptor-associated factor 7 (TRAF7), and transforming growth factor-beta receptor-associated protein 1 (TGFBR1). If these opposite shifts are indeed caused by opposite linkage phase, they can still be used for marker-assisted selection. Further confirmation is needed.

Table 2. SNPs with allele-shifts in different directions. See legend of Table 1 for details.

				After/before frequency ratio, stock-state					
Gene	**Position**	**Ref**	**Variant**	**M-MA**	**M-NJ**	**N-MA**	**N-NJ**	**S-MA**	**S-NJ**
TNF	126	T	C	**1.62**	**1.26**	**1.29**	**1.11**	**0.54**	**0.74**
	170	C	T	1.48	1.17	1.26	1.10	0.61	0.76
	226	T	C	1.28	1.10	1.13	1.02	0.71	0.78
TRAF7	306	A	T	1.02	1.24	0.79	0.79	1.40	1.35
	337	A	G	1.01	1.23	0.79	0.78	1.38	1.28
	423	T	C	1.30	1.60	0.60	0.61	2.21	1.96
	430	C	T	**1.32**	**1.61**	**0.61**	**0.61**	**2.29**	**2.02**
TGFBR1	109	A	T	0.77	0.97	0.81	0.98	1.01	1.22
	226	C	T	0.74	0.92	0.80	0.99	1.06	1.24
	256	T	C	0.64	0.90	0.77	0.96	1.07	1.27
	314	AA	-	**0.55**	**0.89**	**0.74**	**0.94**	**1.16**	**1.33**

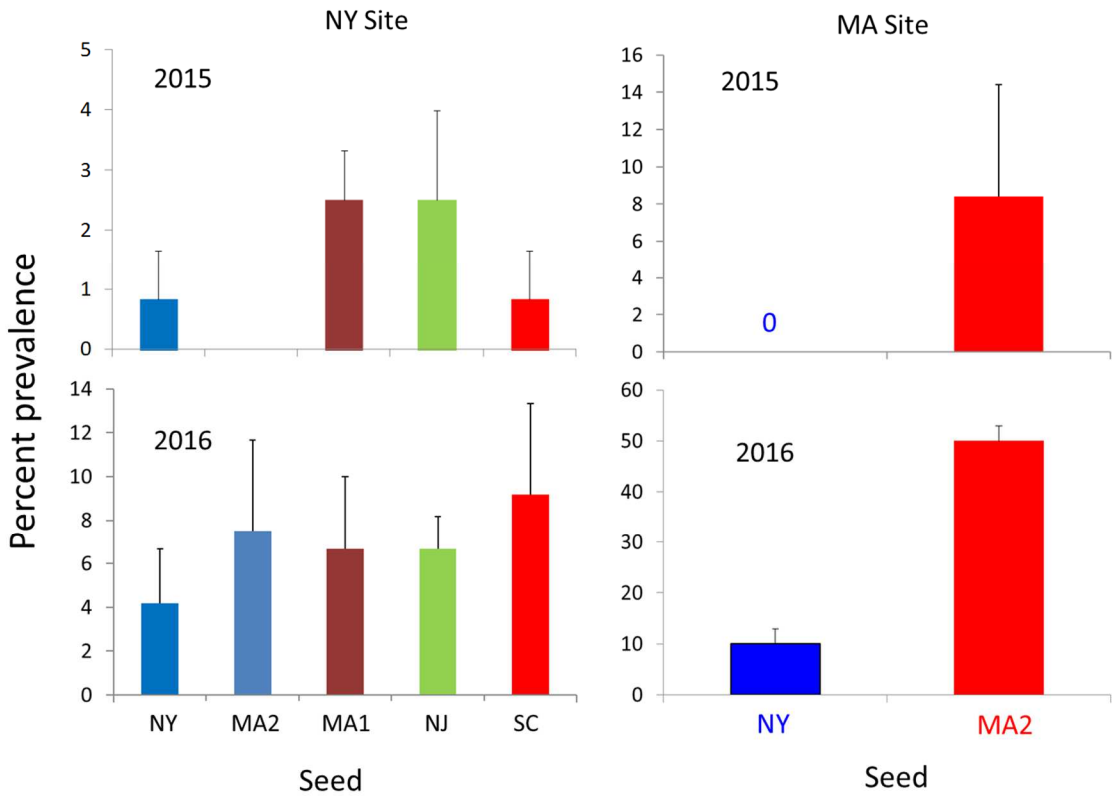
<u>Research Aim III. Identification of resistant Northeastern clam stocks</u>

Three strains of QPX survivor clams resulting from a prior NRAC-funded project (to Dr John Kraeuter *et al.*) were transported to the Suffolk County Marine Environmental Learning Center (SCMELC) in Southold, NY where they were conditioned for spawning as per industry standard. Twenty clams from each group were individually spawned, with different strains kept in separate tanks. Larvae were cultured using industry-standard techniques and post sets were grown in land-based upwellers before being placed in a floating upweller system. We also secured seed from 3

commercial sources (1 from each of the following states: MA, NY and NJ) to be included in the study. All seed was tested for pathology before transfer to field sites and 1 of the commercial seed (NJ strain) was tested positive for QPX and hence could not be imported for field deployment in NY and MA. Therefore, a total of 5 clam strains were deployed in a field site in NY in early July 2014 including the 3 custom-spawned clams (SC, NJ and MA) and 2 commercial strains (NY and a second MA strain hereby designated MA2). Clam deployment in the MA site was limited, however, to the 2 commercial strains (NY and MA2) since there were no sufficient custom-spawned clams left (because of higher than expected mortality during the harsh winter of 2014) to ensure a statistically-robust field deployment in MA (3 replicate plots each). Deployed clams were sampled in June (NY) and October (MA) 2015 and October 2016 (both sites) for the assessment of QPX disease prevalence.

Results showed the presence of QPX in the deployed clams in both field sites but higher prevalence was noted in the MA site (Figure 3). Significant differences between strains were noted in the MA site only with disease prevalence being markedly higher in the MA2 strain as compared to the NY strain. In parallel to disease prevalence, mortality rates were also higher among the MA2 clams deployed in MA as compared to their NY counterparts (Figure 4). These findings are counter

Figure 3. QPX disease prevalence in different clam stocks deployed in NY (left panels) and MA (right panels) and surveyed in 2015 (top row) and 2016 (bottom row). *Significantly higher disease prevalence was measured in the MA2 clam strain deployed in MA as compared to the NY strain deployed in the same site (note that different panels have different Y-scales).*



intuitive as the expectation was that local strains would perform best in local environments, highlighting the need for a better understanding of genetic x environment interactions in aquaculture operations.

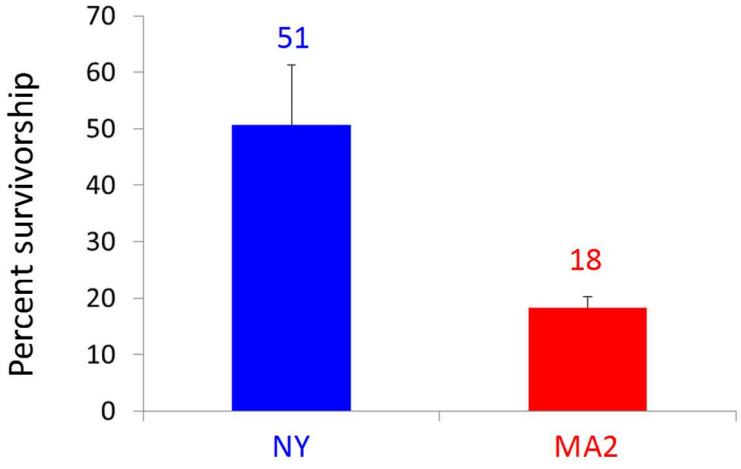


Figure 4. Percent survivorship evaluated at the end of the field deployment in MA. *Significantly higher mortality was measured in the MA2 clam strain as compared to the NY strain.*

Genetic material from survivors and pre-deployment stocks are currently being used in the framework of a new project funded by the USDA to validate genetic markers identified under Research Aim II.

Discussion/Comments

A genetic basis for clam resistance to QPX disease has been previously reported during field (Ford *et al.,* 2002; Ragone-Calvo *et al.*, 2007; Dahl *et al.*, 2010) and laboratory (Dahl *et al.* 2008) trials. The finding of genetic bases for QPX resistance is not surprising as host-defense against pathogens is controlled by many genes, and variation at these genes leads to differences in resistance and survival. Results from the present study support this scenario and allowed, for the first time, the identification of nine genetic variants potentially associated with QPX resistance.

Some of the identified genes carrying significant mutations play a central role in immunity. For example, guanylate-binding protein 2 (GBP2) is an essential part of the interferon-induced defense in vertebrates and is a primary player in antiviral immunity. Its role in immunity has been recently expanded as it was shown to include confer resistance against infection by bacterial pathogens (*Listeria monocytogenes* and *Mycobacterium bovis*; Kim *et al.*, 2011) as well as the protozoan parasite Toxoplasma gondii (Degrandi *et al.*, 2013). Similarly, different members of the metalloprotease family have been shown to play important roles in fundamental physiological processes, such as cell proliferation, differentiation, adhesion, migration, apoptosis, and inflammation (Le *et al.*, 2007; Vanlaere and Libert, 2009). In the hard clam, Wang *et al.* (2016a and b) showed a significant upregulation of several metalloproteases in response to QPX infection and suggested these to play a primary role in clam immunity and resistance to the infection. In this

context, mutation in MP10 gene may regulate clam resistance to the infection. Finally, cytochrome b-245 is a primary component of the microbicidal NADPH oxidase system of phagocytes and mutation in this gene can lead to alteration in the ability of blood cells to produce cytotoxic reactive oxygen species, leading to increased susceptibility to microbial infections (Panday *et al.*, 2015; Bast *et al.*, 2017).

Interestingly, our results showed that some markers displayed divergent selection at our two experimental sites, indicating allele-specific local adaptation. These findings are not surprising in light of a growing body of evidence supporting genotype-environment interactions in aquacultured stocks. This complexity has been highlighted by recent studies in oysters by Frank-Lawale *et al.* (2014) who evaluated a large program for the development of oyster lines in Virginia. Similar findings were also reported by Proestou *et al.* (2016) who deployed selected oyster lines from five geographic areas (ME, RI, CT, NJ, and VA) across the Northeast and mid-Atlantic coasts. In this context, additional studies are needed to understand the basis for the divergent selective pressure on the various loci.

Overall, the genetic variants identified in this study hold promise for marker-assisted selection (MAS) of QPX-resistant clam stocks. MAS provides several advantages as compared to traditional selective breeding of survivor clams. For example, exposure to diseases in the field is highly variable, and some clams may survive by chance rather because they are genetically resistant. Similarly, disease pressure may be absent in some years when breeding decisions have to be made. Finally, typical commercial hatchery practices have been shown to result in a small number of individuals contributing to the gene pools of cultured populations, reducing genetic variability and leading to inbreeding depression. With genetic markers for resistance, selection could continue when disease pressure is low. For these reasons, MAS has been a popular choice for producing resistant varieties of aquacultured species such as the Japanese flounder (Fuji *et al.*, 2007; Ozaki *et al.*, 2012) and the Atlantic salmon (Moen *et al.*, 2009) and is a very appealing approach for shellfish selection.

In this study, we report a better resistance to QPX disease and resulting mortalities in the MA field site among a NY clam strain as compared to a strain that originated from MA (MA2). These findings are intriguing since MA has been heavily hit by QPX epizootics since the 1990's while QPX disease has not been a major hamper to clam aquaculture operations in NY (wild clams are more severely hit by QPX than aquacultured clams in NY despite episodic development of the disease in aquacultured stocks; Allam, unpublished). These findings highlight the need for a better understanding of genotype x environment interactions among aquacultured stocks and warrant a more thorough evaluation of the benefits of integrating the NY line in breeding programs throughout the Northeast.

In summary, our study allowed the identification of genetic variants associated with clam survivorship following QPX epizootics. These variants need to be validated as markers for resistance before being proposed for marker-assisted selection. Such outcome will represent a major progress in mitigating the devastating effects of QPX disease on hard clam aquaculture in the Northeast.

References

Bast A, Kubis H, Holtfreter B, Ribback S, Martin H, Schreiner HC, Steinmetz I. 2017. NADPH oxidase contributes to resistance against *Aggregatibacter actinomycetemcomitans*-induced periodontitis in mice. *Infection and immunity 85*(2), e00849-16.

Dahl SF, Perrigault M, Allam B. 2008. Laboratory transmission studies of QPX disease in the hard clam: Interactions between different host strains and pathogen isolates. Aquaculture 280, 64-70.

Dahl SF, Thiel J, Allam B. 2010. Field performance and QPX disease progress in cultured and wild-type strains of hard clams in New York waters. Journal of Shellfish Research 29, 83-90.

Degrandi D, Kravets E, Konermann C, Beuter-Gunia C, Klümpers V, Lahme S, Pfeffer K. 2013. Murine guanylate binding protein 2 (mGBP2) controls *Toxoplasma gondii* replication. Proceedings of the National Academy of Sciences 110(1), 294-299.

Ford SE, Kraeuter JN, Barber RD, Mathis G. 2002. Aquaculture-associated factors in QPX disease of hard clams: density and seed source. Aquaculture 208: 23-38.

Frank-Lawale A, Allen SK, Degremont L. 2014. Breeding and domestication of Eastern oyster (*Crassostrea virginica*) lines for culture in the mid-Atlantic, USA: line development and mass selection for disease resistance. Journal of Shellfish Research 33: 153-165.

Fuji K, Hasegawa O, Honda K, Kumasaka K, Sakamoto T, Okamoto N. 2007. Marker-assisted breeding of a lymphocystis disease-resistant Japanese flounder (*Paralichthys olivaceus*). Aquaculture 272, 291-295.

Kim BH, Shenoy AR, Kumar P, Das R, Tiwari S, MacMicking JD. 2011. A family of IFN-γ–inducible 65-kD GTPases protects against bacterial infection. Science 332(6030), 717-721.

Kraeuter JN, Ford S, Bushek D, Scarpa E, Walton WC, Murphy DC, Flimlin G, Mathis G. 2011. Evaluation of three northern quahog (=Hard Clam) *Mercenaria mercenaria* (Linnaeus) strains grown in Massachusetts and New Jersey for QPX-resistance. Journal of Shellfish Research. 30, 805-812.

Le NT, Xue M, Castelnoble LA, Jackson CJ. 2007. The dual personalities of matrix metalloproteinases in inflammation. Front. Biosci. 12**:**1475-1487.

Moen T, Baranski M, Sonesson AK, Kjøglum S. 2009. Confirmation and fine-mapping of a major QTL for resistance to infectious pancreatic necrosis in Atlantic salmon (*Salmo salar*): population-level associations between markers and trait. BMC Genomics 10, 368.

Ozaki A, Araki K, Okamoto H, Okauchi M, Mushiake K, Yoshida K, Tsuzaki T, Fuji K, Sakamoto T, Okamoto N. 2012. Progress of DNA marker-assisted breeding in maricultured finfish. Bulletin of Fisheries Research Agency 35, 31-37.

Panday A, Sahoo MK, Osorio D, Batra S. 2015. NADPH oxidases: an overview from structure to innate immunity-associated pathologies. Cellular & molecular immunology 12(1), 5-23.

Proestou DA, Vinyard BT, Corbett RJ, Piesz J, Allen SK, Small JM, Li C, Liu M, DeBrosse G, Guo X (2016) Performance of selectively-bred lines of eastern oyster, *Crassostrea virginica*, across eastern US estuaries. Aquaculture 464: 17-27

Ragone-Calvo LM, Ford SE, Kraeuter JN, Leavitt DF, Smolowitz R, Burreson EM. 2007. Influence of host genetic origin and geographic location on QPX disease in hard clams, *Mercenaria mercenaria*. Journal of Shellfish Research 26: 1-11.

Vanlaere I, Libert C. 2009. Matrix metalloproteinases as drug targets in infections caused by gram-negative bacteria and in septic shock. Clinical microbiology reviews 22(2), 224-239.

Wang K, Pales Espinosa E, Del Castillo C, Corre E, Allam B. 2016b. Clam focal and systemic immune responses to QPX infection revealed by RNA-seq technology. BMC Genomics.

17(1):146.
Wang K, Pales Espinosa E, Tanguy A, Allam, B. 2016a. Alterations of the immune transcriptome in resistant and susceptible hard clams (*Mercenaria mercenaria*) in response to Quahog Parasite Unknown (QPX) and temperature. Fish and Shellfish Immunology, *49*, 163-176.

PROJECT COMPLETION REPORT

Genetic Marker-Assisted Selection of Northeastern Hard Clams for QPX-Resistance
Subaward # Z555103
Grant # 2012-38500-19656

SIGNATURE PAGE

PROJECT CODE: **SUBCONTRACT NO:**

PROJECT TITLE: **Genetic Marker-Assisted Selection of Northeastern Hard Clams for QPX-Resistance**

PREPARED BY: Bassem Allam

Project Coordinator of Subawardee Date: 06/05/2017