Using Shotgun Proteomics to Obtain Hemolymph Markers for Determining Sex and Stage of Gonad Maturation in Oysters

PI: Frederick Goetz, Northwest Fisheries Science Center, REUT Division, Manchester Research Station, 7305 E. Beach Dr., Port Orchard, WA 98366, Tel. #360-871-8322, rick.goetz@noaa.gov
Co-PIs: **Joth Davis**, Taylor Shellfish Farms, Inc. 130 SE Lynch Road, Shelton, WA; **Dr. Steven Roberts**, University of Washington, School of Aquatic and Fishery Sciences.
Industry Collaborator: Taylor Shellfish Farms, Inc., 130 SE Lynch Road, Shelton, WA 98584

Initiative Addressed: Development and transfer federal research and development - The development of technologies and resources that can be directly and rapidly transferred to the aquaculture industry to provide blue-green jobs.

Introduction: NOAA has launched a National Shellfish Initiative, the goal of which is to increase populations of bivalve shellfish in our nation's coastal waters through commercial production and conservation activities. In the Pacific Northwest this initiative is specifically coordinated with the Washington State Shellfish Initiative; an agreement among federal and state governments, tribes and the shellfish industry to restore and expand Washington's shellfish resources to promote clean-water industries and create family-wage jobs.

An impediment to fulfilling the goals of the shellfish initiatives is the difficulty in producing sufficient shellfish seed. The need to increase seed production for commercial and restoration activities relies on increasing the efficiency of seed production. While many aspects of shellfish culture have been successfully addressed, a problem that still exists is the nonlethal identification of sex and the maturational stage of the gonads of oysters. This is extremely important since some oyster strains or populations have highly skewed sex ratios and the maturational state of the oyster is critical for knowing when to condition and how close an individual is to spawning. Various attempts have been made in the past to use nonlethal methods for sex determination and maturational stage including MRI and NMR imaging, muscle relaxants to induce gaping and, more recently, Proteinchip SELDI-TOF-MS and discriminant analysis to determine patterns of peptides that change consistently with sex and stage of maturation (Li et al., 2010, Aquaculture 309:258-264). While these approaches all have some merit, they have significant drawbacks including cost, access to instrumentation and the inability of some to differentiate sex outside of the breeding season. None of these methods have led to the development of low-cost and widely available screens for gender and maturational stage. The sequence and release of the oyster genome (Zhang et al., 2012, Nature, 490, 49-54) and the development of global proteomic approaches has provided a new avenue to study this problem. In particular, "shotgun proteomics" can now be used to identify entire proteomes of various tissues. Shotgun proteomics is the sequencing of a complex mixture of peptides using liquid chromatography (LC) and tandem mass spectrometry (MS) without prior separation such as two-dimensional gel electrophoresis. In addition, the advent of new software and bioinformatic approaches has led to the ability to quantitatively determine differences in the proteomes of different stages, treatments, and individuals (see Approach).

We propose to use shotgun proteomics in conjunction with the quantitative analysis of protein frequencies to characterize proteins that change in the hemolymph of the oyster, particularly during sexual maturation. We will also compare hemolymph proteins between males and females quantitatively at various stages of maturation to determine if gender specific proteins can be identified that could be used for sex determination. Past investigations with proteomic approaches have used the comparison of peptide fragment profiles (without protein identification) as a means to differentiate sex and maturation state. We are not suggesting that shotgun proteomics be used for the routine discrimination of sex and stage of maturation. Rather, our intent is to use this approach to characterize and identify protein markers for which specific assays could be developed that can be used by industry or researchers for determining sex and stage of maturation. These assays would not require complex and expensive instrumentation and would be more widely available to the industry. Further, they will be based on hemolymph that can be nonlethally sampled from oysters in hatchery operations and in the wild.

Approach:

Oysters and gonadal maturation: Broodstock oysters between 1 and 2 years will be obtained from Taylor Shellfish and transferred to the NOAA Manchester Research Station (Port Orchard, WA). As part of the NOAA and Washington shellfish initiatives, a new shellfish hatchery is being built at the Manchester Research Station that will produce seed of various shellfish species for restoration as well as provide an experimental platform to study various aspects of the physiology and genetics of shellfish. This hatchery will be finished in April/May and has a complete temperature controlled, filtered and UV sterilized saltwater supply. Oysters will be conditioned by increasing temperature and supplementing the diet with *Tetraselmis chuii* and *Chaetoceros neogracile.* At weekly intervals, 30 oysters will be removed, sacrificed and the hemolymph drawn directly from the heart with a syringe and the gonads taken and fixed in Bouins for histological analysis of sex and maturational stage.

Proteomic sample preparation and analysis: Following centrifugation to remove hemocytes, the hemolymph will be processed for shotgun proteomics as recently described (Timmins-Schiffman et al. 2013 in review). Briefly, proteins in the hemolymph will be alkylated with iodoacetamide and digested with trypsin. Following clean-up, samples will be separated by liquid chromatography on a nanoACUITY liquid chromatograph and then analyzed by tandem mass spectrometry on a LTQ Orbitrap XL mass spectrometer (MS) at the University of Washington Proteomics Resource (Seattle, WA, USA). High resolution full precursor ion scans will be acquired by the MS and peptide sequence and corresponding protein identification for all mass spectra will be carried out using SEQUEST (Eng et al. 1994. J. Am. Soc. Mass Spectrom., 5, 976-989) and the *Crassostrea gigas* proteome version 9 (Zhang et al. 2012. Nature, 490, 49-54). SEQUEST results will be analyzed using PeptideProphet and ProteinProphet in order to statistically evaluate peptide matches and assign protein probabilities (Nesvizhskii et al. 2003. Anal. Chem., 75, 4646-4658).

Proteomic bioinformatics: The quantification of protein differences between samples will be performed by Protein Quantification and Peptide Quality Control (PQPQ) software (Forshed et al. 2011. Molecular & Cellular Proteomics 10: 10.1074/mcp.M111.010264, 1–9). In addition, the proteins identified from the SEQUEST analysis will also be annotated against the

UniProtKB/Swiss-Prot database and the Associated Gene Ontology terms will be used to classify sequences based on biological process as well as to categorize genes into parent categories (GO Slim).

Gonad analysis: Gonad samples will be processed for histology and slides will be examined microscopically and classified according to stage of gonadal maturity (e.g, as in Li et al., 2010, Aquaculture 309:258-264). Differences in protein frequencies and types of proteins will then be compared between sexes at various maturational stages (i.e. gender differences) and within a sex between maturational stages (i.e., maturity differences) to determine if proteins specific to gender and/or maturational stage can be identified.

Potential for follow-up: Assuming that this research identifies proteins that are correlated to gonadal maturation and/or gender, then it will be important to try and develop a "dipstick" assay that can detect their presence and relative quantities in hemolymph sampled nonlethally. This would most likely start as an enzyme-linked immunosorbent assay (ELISA) and then be streamlined if it works. This will require protein and antibody production but there are companies that do this routinely for a fee. We envision that an assay could be developed for $60,000. The shotgun proteomics approach can also be used to detect proteins that are induced and circulating in the hemolymph during pathogenesis. Identification of shellfish that are infected would have applicability in hatchery operations as well as assessing the health and well-being of shellfish in the wild. In the current project, we will be collecting hemocytes in the processing of hemolymph and some of these samples will be analyzed by shotgun proteomics to compare them to the transcriptomics that we have already described from oyster hemocytes (Roberts et al., 2009. Marine Biotechnology 11(1): 24-44). The results of this analysis may lead to the identification of hemocyte proteins that could be used for detecting pathogenesis or the health of oysters. We envision that further proteomic work to encompass health of oysters could be accomplished for $70,000.

Target user groups and technology transfer plan: We will certainly transfer the results of our research directly to our industry collaborator, Taylor Shellfish. But these results will also be available for any other producers or researchers as well. The results will be disseminated through publications, presentations (e.g., Aquaculture America) as well as through the website of the NOAA Northwest Fisheries Science Center and the PI and CoPI.

Timeline for completion of work:

Year		Conditioning of oysters and sampling hemolymph and gonads	Processing of proteins from samples for proteomics	Proteomic analysis by LC/MS at the Proteomic Reource Center	Bioinformatic analyses of proteomic output	Histological preparation of gonad samples to produce slides	Overall data analysis and paper preparation	Present data at Aquaculture America
2013	April	■						
	May	■	■					
	June	■	■	■		■		
	July		■	■		■		
	August			■	■	■		
	September				■	■		
	October				■		■	
	November						■	
	December						■	
2014	January						■	
	February							■
	March							

Line item Budget:

Bivalve Proteomic Investigation
NOAA Fisheries

1. PERSONNEL	Pay periods	Hours	Rate	Total
Salary ZT3	3	80	$34.83	$2,786
Leave Surcharge: 0			$0.00	
Benefit Surcharge: 29.75%	0.298		$10.36	$829
Subtotal: Labor + Benefits				**$3,615**
2. TRAVEL (Attendence at scientific meeting, summer 2013)				$1,500
Travel subtotal:				**$1,500**

3. SUPPLIES & EQUIPMENT				
Supplies	**Quantity**	**Unit Type**	**Unit Price**	**Total**
supplies for oyster conditioning/rearing	1		$1,000	$1,000
histological processing and slide preparation	200	sample	$10	$2,000
proteomic analysis: LC/MS time at University of Washington Proteomics Resource	80	hours	$122	$9,797
proteomic analysis software	1	programs	$3,203	$3,203
reagents, glassware and plasticware for sample preparation for proteomic analysis	1		$2,000	$2,000
Equipment (NONE)				$0
Supplies & equipment subtotal:				**$18,000**

4. GRANTS/CONTRACTS	Quantity	Unit Type	Unit Price	Total
UW- grant to support graduate student	1	Job	$44,000	$44,000
Grants/Contracts subtotal:				**$44,000**
5. NWFSC Indirect Cost (2% of Total Direct Costs)				**$1,312**
6. NOAA PROJECT TOTAL				**$68,427**

Money to non-NOAA partners: In the budget we have a contract to fund a graduate student working on part of this project for their thesis research. We already have contracts that will enable us to direct this money to our UW collaborator, Steven Roberts, for support of this student, and have successfully done this in the past.