Royalty Research Fund Proposal

Proposal: ***Maturation processes in the marine mollusc, Panopea generosa***

eCG1 Number: A88598

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Abstract:
Maturation processes in marine molluscs are not well-characterized and large gaps in our understanding remain. Many studies have concluded that steroids are involved in mollusc reproduction, but the majority of these studies were targeted at vertebrate steroids that may not play a role given the apparent lack of functional receptors for androgens and estrogens. The top-down approaches used in the past focused on reproduction molecules from divergent phyla may be missing the mark for the phylum mollusca. An entirely different mechanism for reproductive maturation processing may be at play. While the existing evidence does not definitively rule out a role for steroids, the validity of a different, bottom-up approach is evident. Combining high-throughput proteomic data acquisition with comprehensive bioinformatic data analysis as proposed here, will lead to the discovery of factors controlling maturation processes in this diverse phylum.

Description of Research

A. Introduction and Rationale

Marine Mollusc Reproductive Biology

Maturation processes in marine molluscs are not well-characterized and large gaps in our understanding remain. Many studies have presumed that steroids are involved in mollusc reproduction, but the majority of these studies were targeted at vertebrate steroids (reviewed in Scott, 2012) that may not play a role in reproduction, given the apparent lack of functional receptors for androgens and estrogens (Fernandes et al. 2011). Despite characterizations of gonadal differences in lipids (e.g. Palacios et al., 2007), targeted proteins (e.g. Arcos et al. 2009), specific genes (e.g. Anantharaman & Craft, 2012; Ciocan et al. 2011), and RNA/DNA ratios (e.g. Li et al. 2000), these top-down approaches may be missing the mark. As suggested by Scott (2013), an entirely different mechanism for maturation processing may be at play. For example, Li et al. (2010) found a series of unidentified proteins using a combination of chromatography and mass spectrometry (SELDI-TOF-MS) that appear to be involved in maturation in the Eastern oyster, *Crassostrea virginica*. Using a 'shotgun' metabolomics approach, Cubero-Leon et al. (2012) were unable to find any evidence of differential steroid metabolism in developing or mature gonads of the mussel, *Mytilus edulis*. Cubero-Leon et al. (2012) did find highly significant differences in phospholipid metabolites possibly related to vitellogenesis. While the existing evidence does not definitively rule out a role for steroids, we do have ample evidence to suggest the validity of a taking a non-biased, comprehensive, bottom-up approach as proposed here.

Given the current fragmented understanding of maturation in marine molluscs, coupled with recently developed proteomic and transcriptomic approaches (reviewed in Slattery et al., 2012), we have a clear opportunity to approach the maturation processes in marine mollusks in a new way. Specifically, a shotgun proteomics approach will be used to examine sex-specific protein expression profiles throughout maturation. This approach obviates any preconceptions stemming from previous work on vertebrates or model invertebrates.

Commercial and Environmental Applications

The goal of the proposed research is to initiate a basic research line of inquiry that also has the potential for commercial and environmental application. This will be accomplished by shedding new light on the question of maturation dynamics in molluscs, with the primary objective of characterizing sex determination and maturation status in geoduck clams (*Panopea generosa*).

Geoduck are large, burrowing clams that have been commercially fished since 1970. Commercial farming of geoduck was initiated 15 years ago; farmed geoduck now generate over 20 million dollars in annual sales and are the region's most valuable farmed shellfish on a per

acre basis. Despite their value, relatively little is known about this species; and aside from a few DNA microsatellite loci (Kaukinen et al. 2004; Vadopalas & Bentzen, 2000; Vadopalas et al. 2004), ***no genomic or proteomic resources exist for geoduck clams***. The rapid development of geoduck aquaculture close to wild populations of conspecifics has raised concerns over farmed-wild interbreeding and the loss of genetic diversity. To address these concerns, geoduck hatcheries are currently encouraged to use practices to manage genetic risk. To this end, the production of commercial geoduck larvae (referred to as *seed*) uses large numbers of wild broodstock to maximize genetic diversity in outplanted animals (Recommended Broodstock Practices, Washington Department of Fish and Wildlife).

An ongoing impediment to fulfilling the conservation goals of state and federal shellfish initiatives is the difficulty in producing sufficient shellfish seed for commercial and restoration activities; remedying this bottleneck relies on increasing the efficiency of seed production. While many aspects of shellfish culture have been successfully addressed, ***one important roadblock to efficient production is the nonlethal identification of sex and maturational stage***. Highly skewed sex ratios in some populations and the inability to determine when to induce spawning based on maturation state presents an economic challenge for shellfish hatcheries. For the burgeoning geoduck aquaculture industry, broodstock remain costly to acquire, condition, and maintain in the hatchery setting. Geoduck are repeat broadcast spawners that must be spawned volitionally merely to determine gender.

Hatchery personnel must increase the temperature of seawater and introduce large rations of microalgae to essentially coax the broodstock to spawn. Typically, few of the available broodstock are fully mature and spawn during a given attempt by hatchery personnel; their maturation status and gender are unknown until after their first spawn. In terms of personnel and the number of broodstock required, plus the amount of cultured microalgae that is essentially wasted by unknowingly trying to induce unripe individuals to spawn, these spawn attempts are costly to the industry. In addition, broodstock management and breeding guidelines designed to increase genetic diversity in hatchery seed by involving large numbers of broodstock are frequently thwarted by spawn attempts that succeed for only a small fraction of available broodstock.

In other marine bivalve species such as oysters, various attempts have been made in the past to use nonlethal methods for determination of sex and maturational stage including MRI and NMR imaging (Davenel et al. 2006), and muscle relaxants to induce gaping (Suquet et al., 2010). Proteinchip SELDI-TOF-MS and discriminant analysis have recently been used to determine patterns of peptides that change consistently with sex and stage of maturation (Li et al., 2010). While these approaches have 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 that can be applied in a hatchery setting, and none have been applied to geoduck clams. The recent advances in proteome characterization including sequencing of a complex mixture of peptides using liquid chromatography (LC) and tandem mass spectrometry (MS) without prior separation offers an ideal approach that has the potential for the development of a simple protein assay that could be deployed in a hatchery setting.

B. Objectives

The overall goal of this project is to develop a fundamental understanding of processes controlling marine mollusc reproductive maturation. In order accomplish this goal the specific research objectives of this proposal are to

1) Identify proteins that play a role in geoduck reproductive maturation
2) Characterize tissue specific transcriptomic resources for the geoduck

The first objective will provide a discovery-driven framework for understanding reproductive process in marine molluscs, something that has suffered from presumptions rooted in mammalian biology (see introduction; Scott 2012, 2013). Completion of this objective will immediately provide resources to be used for more comprehensive, research efforts. The second research objective is primarily designed to support accurate identification of proteins that play a role in geoduck reproductive maturation. A major additional benefit of completing this objective is the genomic resources necessary to pursue other funding opportunities associated with conservation genetics, gene regulation, and environmental physiology.

C. Procedure

Experimental design and sampling:

Geoduck will be obtained through cooperation with the Washington Department of Fish and Wildlife (Robert Sizemore, WDFW research scientist, pers. comm. 9-18-2013), and will be maintained at the new shellfish facility at NOAA's Manchester Field Station (Frederick R. Goetz, Supervisory Research Scientist, NOAA, pers. comm. 9-10-2013) in cooperation with the Puget Sound Restoration Fund (Betsy Peabody, Executive Director, pers. comm. 9-5-2013). The maturation period will be controlled using standard aquaculture practices, which include gradually increasing water temperature and microalgal ration.

Reproductive maturation (going from undifferentiated gonad to spawning) takes approximately 2.5 months in geoducks. Geoducks will be maintained at the NOAA Manchester Field Station and at weekly intervals, 10 geoducks will be removed, sacrificed and the hemolymph drawn with a syringe and the gonads taken and fixed in Bouins for histological analysis of sex and maturational stage.

Gonad histological analysis:
Gonad samples will be processed for histology and slides will be examined microscopically and classified according to stage of gonadal maturity. To gauge maturation in histological sections, following Goodwin (1976) and Ropes (1968), each gonad will be scored as inactive (0), early active (1), late active (2), ripe (3), partially spawned (4), or spent/resorbed (5).

Proteomic analysis:
After gonad histological analysis is completed, 10 reproductively immature males, 10 reproductively immature females, 10 reproductively mature males, and 10 reproductively mature females will be selected for proteomic analysis. 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 identify proteins specific to gender and/or maturational stage. Proteomic analysis will be carried out on both hemolymph and gonad tissue, thus a total of 80 individual samples will be characterized. Protein 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) and the geoduck proteome database as developed as part of research objective two of the current proposal. SEQUEST results will be analyzed using PeptideProphet and ProteinProphet in order to statistically evaluate peptide matches and assign protein probabilities (Nesvizhskii et al. 2003).

The quantification of protein differences between samples will be performed by Protein Quantification and Peptide Quality Control (PQPQ) software (Forshed et al. 2011). 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).

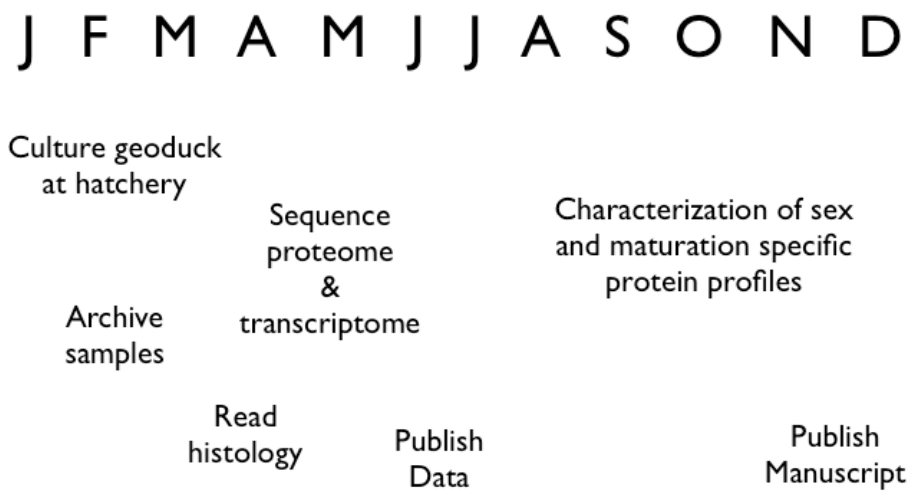
Transcriptomic Analysis:
Transcriptomic analysis will be carried on the same 40 samples selected for proteomic analysis (10 reproductively immature males, 10 reproductively immature females, 10 reproductively mature males, and 10 reproductively mature females). The primary reason a transcriptome is being produced is to provide the necessary database for analysis of the proteomic data. Four gonad tissue RNA-Seq libraries will be constructed (immature male, immature female, mature male, mature female) for transcriptomic analysis. Total RNA will be extracted from gonad tissue

using Tri Reagent. RNA-seq libraries will be constructed from pooled mRNA and sequenced at the University of Washington High Throughput Genomics Unit on the Illumina Hi-Seq 2000 platform.

Sequence analysis will be performed with CLC Genomics Workbench. De novo assembly will be performed on quality trimmed reads, and sequences will be annotated by comparing contiguous sequences (contigs) to the UniProtKB/Swiss-Prot database (http://uniprot.org) using the BLASTx algorithm (Altschul et al. 1997) with a 1.0E-5 e-value threshold. A database of deduced proteins will be generated and used from proteomic data analysis (above). Gene attributes will be fully characterized using the gene ontology (GO) database as well as comparing sequences to proteomes of other taxa. While a direct objective of the current proposal, we will also screen the RNA-Seq libraries for putative SNPs that could be used for future projects associated with conservation genetics (See *Need for RFF support*).

D. Time Schedule

The proposed research can be accomplished during the desired support period. Below is schematic representation of majority research activities and when they will occur over the course of the project.



E. Need for RRF Support

This project builds on technical approaches being used in my lab, but represents a shift in research direction from environmental influences on physiology to developing a fundamental understanding of maturation processes in marine molluscs. The work proposed here is focused on the geoduck, an economically and environmentally important species that I have never studied.

This bottom up approach to biological discovery, while an important first step, can be difficult to fund through traditional grant channels. This is particularly the case in this scenario where we are suggesting the presumption of a traditional estrogen and testosterone system might not provide the entire story regarding reproductive control. Nevertheless, discovery of factors involved in maturation is a prerequisite to the design of specific experiments in the pursuit of research questions.

The proposed research represents a valuable opportunity to not only expand my research scope and direction, but also to initiate an international collaboration with investigators at the Center for Scientific Research and Higher Education at Ensenada (CICESE) Mexico, who have secured funding to generate whole genome sequence data for geoduck and have expressed interest in collaborating with me via complementary lines of inquiry (Axa Rocha-Olivares, CISESE, personal communication Aug 14, 2013).

The majority of prior efforts to understand maturation processes in molluscs have been applied to either environmental toxicology (endocrine disruption) or aquaculture (biochemical manipulation to increase production and / or decrease environmental impacts). As a consequence, little work has been focused on basic understandings of maturation processes. Completion of the research described here will facilitate the pursuit of funding for these more fundamental lines of inquiry including examining cellular regulatory processes and protein interactions.

In addition to seeding grants for basic research, the new information gained should provide a strong foundation for future grant applications for applied work, designed to decrease production costs and increase sustainable production of farmed shellfish. Assuming that this research identifies proteins that are correlated to gonadal maturation and/or gender, the next logical step in the applied arena would be to develop a “dipstick” assay that can detect their presence and relative quantities in nonlethally sampled hemolymph. This assay would be highly desirable from shellfish producers at both the commercial and restoration level.

Budget:

01 Salaries and Wages	
PI Steven Roberts, Associate Professor ($8554 x 1 month salary; 8.3%)	8,554
Brent Vadopalas, Research Scientist ($7129 x 1.4 month salary; 11.6%)	9,981
Subtotal	18,535
03 Other Contractual Services	
UW Proteomics Resource LC/MS time (60hours 122/hour)	7,320
UW HTGS (4 libraries at $300 each x 1 lane PE 72bp at 2500)	3,700
Subtotal	11,020
04 Travel	
Travel to Manchester Shellfish Hatchery	630
39.8 miles RT @ $0.565/mile = $22.49 + RT ferry $22.40 total = $44.89 RT * 14 trips	
Subtotal	630
05 Supplies and Materials	
Histology: 12 sampling dates, 10 specimens, $25/specimen	3000
Molecular Sample Preparation	1500
Subtotal	4500
07 Retirement and Benefits	
PI (25%)	2,139
Research Scientist (30.9%)	3,084
Subtotal	5,025
TOTAL BUDGET	39,710

Budget Justification:

Funds are requested for the PI as well as Research Scientist. The Research Scientist will primarily be responsible for sampling as well as assisting in data analysis. A total of $11,020 is requested for services from UW cores facilities. This includes the proteomic analysis of 80 individual samples (40 gonad and 40 hemolymph) as well the library construction and sequencing of four RNA-Seq libraries. Funds are needed to support travel to the Manchester Shellfish Hatchery with mileage breakdowns provided.

Supplies and Materials include those needed for histological analysis of 120 gonad tissues samples taken ($25/specimen). $1500 is requested to cover supplies and materials needed for nucleic acid and protein extraction and sample preparation. Retirement and benefit rates are calculated at 25% for the PI and 30.9% for the Research Scientist.

Curriculum Vitae: Steven Beyer Roberts

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Appointments

2013-Present Associate Professor - University of Washington
2007-2013 Assistant Professor - University of Washington
2003-2006 Assistant Research Scientist - Marine Biological Laboratory, MA

Recent Publications

Storer CS*, Quinn TP and Roberts SB. (2013) Quantitative PCR analysis used to characterize physiological changes in brain tissue of senescent sockeye salmon. Biogerontology. 10.1007/s10522-013-9448-1

Burge CA, Mouchka ME, Harvell CD and Roberts SB. (2013) Immune response of the Caribbean sea fan, *Gorgonia ventalina*, exposed to an Aplanochytrium parasite as revealed by transcriptome sequencing.. discussed Frontiers in Physiology. 4:180. 10.3389/fphys.2013.00180

Timmins-Schiffman EB* Nunn BL, Goodlett DR and Roberts SB. *S*hotgun proteomics as a viable approach for biological discovery in the Pacific oyster. Conservation Physiology. doi: 10.1093/conphys/cot009

Timmins-Schiffman EB* Friedman CS, Metzger DC, White SJ and Roberts SB. (2012) Genomic resource development for shellfish of conservation concern. Molecular Ecology Resources. doi:10.1111/1755-0998.12052

Timmins-Schiffman EB* and Roberts SB. (2012) Characterization of genes involved in ceramide metabolism in the Pacific oyster (*Crassostrea gigas*). BMC Research Notes 5:502 doi:10.1186/1756-0500-5-502

Storer CG*, Pascal CE, Roberts SB, Templin WD, Seeb LW, Seeb JE. (2012) Rank and Order: Evaluating the Performance of SNPs for Individual Assignment in a Non-Model Organism. PLoS ONE 7(11): e49018 doi:10.1371/journal.pone.0049018

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Burge CA, Douglas N, Conti-Jerpe I, Weil E, Roberts SB, Friedman CS, CD Harvell. (2012) Friend or foe: the association of Labyrinthulomycetes with the Caribbean sea fan, *Gorgonia ventalina*. Diseases of Aquatic Organisms. 101:1-12 doi:10.3354/dao02487

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Roberts SB, Hauser L, Seeb LW, Seeb JE (2012) Development of genomic resources for Pacific herring through targeted transcriptome pyrosequencing. PLoS ONE 7(2): e30908. doi: 10.1371/journal.pone.0030908

Roberts SB and Gavery MR (2012) Is there a relationship between DNA methylation and phenotypic plasticity in invertebrates? Frontiers in Physiology. 2:116. doi:10.3389/fphys. 2011.00116

Gavery MR* and Roberts SB. (2012) Characterizing short read sequencing for gene discovery and RNA-Seq analysis in Crassostrea gigas. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 7:2 94-99 June 2012. doi:10.1016/j.cbd.2011.12.003

Morera D, Roher N, Ribas L, Balasch JC, Doñate C, Callol A, Boltaña A, Roberts SB, Goetz G, Goetz FW, Mackenzie SA. (2011) RNA-Seq reveals an integrated immune response in nucleated erythrocytes. PLoS ONE 6(10): e26998. doi:10.1371/journal.pone.0026998

Roberts SB, Sunila I, Wikfors G. (2011) Immune response and mechanical stress susceptibility in diseased oysters, *Crassostrea virginica*. Journal of Comparative Physiology B. 182:1 41-48, doi:10.1007/s00360-011-0605-z

Seeb JE, Carvalho G, Hauser L, Naish K, Roberts SB, Seeb LW. (2011) Single-nucleotide polymorphism (SNP) discovery and applications of SNP genotyping in nonmodel organisms. highly cited Molecular Ecology Resources, 11 1–8, doi:10.1111/j.1755-0998.2010.02979.x

* *indicates student author*

Synergistic Activities (selected)

-Board of Directors: Pan-American Marine Biotechnology Association (2009-present)

-Panel Member: NSF-Evolutionary Processes (2011), USDA-SBIR (2007)

-Organizer: USDA WERA099: Broodstock Management, Genetics and Breeding Programs for Molluscan Shellfish. (2010 - 2012)

-Faculty Mentor: Ocean and Coastal Interdisciplinary Science (OACIS) GK-20 Program (2011-2013)

-Advocate for open notebook science and data sharing. The public can follow our research activities on Tumblr, Twitter, Facebook, YouTube, and Flickr. All students and staff maintain open access electronic lab notebooks and share resources using a wiki-based platform.

Other Research Support

Evaluation of Putatively QPX-Resistant Strains of Northern Hard Clams Using Field and Genetic Studies
Source of Support: USDA/NRAC
Award Amount: $79,503
Award Period: 3/1/2008 – 2/28/2011
Relationship: Taxa, marine invertebrate

Threats to Bivalve Aquaculture and Fisheries: The Influence of Emerging Diseases and Environmental Change
Source of Support: NOAA
Award Amount: $243,000
Award Period: 9/1/2009 – 2/29/2012
Relationship: Taxa, marine invertebrate

Sablefish Broodstock Development and Functional Genomics
Source of Support: NOAA [Contract]
Award Amount: $349,407
Award Period: 9/15/2011 – 9/14/2013
Relationship: none

Sablefish Reproductive Life History and Genetics
Source of Support: NOAA / JISAO
Award Amount: $379,053
Award Period: 9/15/2012 – 6/30/2013
Relationship: none

DNA Methylation as a Mechanism to Increase Adaptive Potential in Invertebrates
Source of Support: National Science Foundation
Award Amount: $243,090
Award Period: 5/1/2012 – 4/30/2014
Relationship: Taxa, marine invertebrate; RNA-Seq used

Alleviating Regulatory Impediments to Native Shellfish Aquaculture
Source of Support: NOAA Aquaculture Program
Award Amount: $427,371
Award Period: 9/1/2012 – 8/30/2014
Relationship: Taxa, marine invertebrate

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