eGC1 number: A61902

Proposal Title: *Sequencing-based approach for characterizing DNA methylation in the Pacific oyster (Crassostrea gigas)*

PI: Steven Roberts, Assistant Professor
School of Aquatic and Fishery Sciences

Abstract

DNA methylation is an epigenetic mechanism with important regulatory functions in plants and animals. While the mechanism itself is evolutionarily ancient, the distribution and function of DNA methylation is diverse both within and among phylogenetic groups. Although DNA methylation has been well studied in mammals, there are limited data on invertebrates, particularly molluscs. Recent work in our lab demonstrates a potentially important role for DNA methylation in the Pacific oyster. Using primarily *in silico* approaches, it appears that there is a significant difference in DNA methylation between functionally distinct gene families. This finding has not been experimentally corroborated at the genome level, nor is there an understanding of how DNA methylation patterns vary across tissue type and developmental stage. The specific objectives of the proposed study are to 1) characterize differences in methylation across the oyster genome, 2) evaluate cell-type variability in methylation, 3) gain information on nucleotide specific methylation patterns. This research will not only generate fundamental information on DNA methylation in molluscs but will provide an assessment of tools to study DNA methylation in non-model species. This work will set the foundation for larger research efforts on organismal responses to environmental change and broodstock improvement for aquaculture.

Description of Proposed Research

Sequencing-based approach for characterizing DNA methylation in the Pacific oyster (*Crassostrea gigas*)

PI: Steven Roberts, Assistant Professor, School of Aquatic and Fishery Sciences

A. *Introduction and Rationale*

Epigenetic mechanisms are defined as processes that alter gene activity without manipulating the underlying DNA sequence (Jablonka & Lamb, 2002). Common epigenetic mechanisms include DNA methylation, histone modifications and non-coding RNA activity. The best studied of these is DNA methylation, which refers to the addition of a methyl group to position 5 of cytosines and occurs almost exclusively in CpG dinucleotides in animals. The functional significance of DNA methylation in vertebrates includes providing genomic stability (Maloisel & Rossignol, 1998), regulation of imprinted genes (Bell & Felsenfeld, 2000) and X-chromosome inactivation (Csankovszki *et al*, 2001). In addition, DNA methylation is one mechanism that contributes to cellular differentiation across cell-types despite the fact that all cells in an organism possess the same DNA. A range of factors including diet (Wilson et al, 1984; Dolinoy *et al*, 2006), xenobiotic chemicals (Sutherland & Costa, 2003), and endocrine disruptors (Anway & Skinner, 2006) has been shown to disrupt DNA methylation patterns. DNA methylation, like many epigenetic marks, may be heritable; therefore environmentally-induced changes can be passed on for multiple generations (Anway & Skinner, 2006).

Much of the work to date on methylation has focused on mammals where approximately 70-80% of cytosines in CpG dinucleotides are methylated (Bird & Taggart, 1980), a pattern referred to as global methylation. The studies on DNA methylation in invertebrates are limited but intriguing and illustrate a wide diversity of patterns, ranging from very limited methylation in *Drosophilia melanogaster* (Gowher *et al*, 2000) and *Caenorhabditis elegans* (Simpson *et al*, 1986) to a mosaic pattern of methylation in the sea urchin (*Strongylocentrotus purpuratus*) (Bird *et al*, 1979) and *Ciona intestinalis* (Simmen & Bid, 2000; Suzuki *et al*, 2007).

In order to better understand the role of DNA methylation in invertebrates, we recently characterized DNA methylation patterns in the Pacific oyster, *Crassostrea gigas* (Gavery & Roberts, 2010). The *in silico* analysis and experimental work suggest that intragenic methylation is important in regulating gene expression in oysters. Specifically, the predicted methylation status of *C. gigas* genes was characterized based on known hyper-mutability of methylated cytosines, which readily deaminate to thymine residues (Coulondre *et al*, 1978). This CpG mutation is not easily corrected by DNA repair machinery, and as a result consistently methylated regions of DNA are depleted of CpG dinucleotides over evolutionary time (Schorderet & Gartler, 1992). Consequently, regions of DNA with a low CpG oberserved:expected ratio (o/e) are predicted to be methylated, whereas regions with a high CpGo/e (approaching 1.0) are predicted to be unmethylated. The results of this analysis indicate that *C. gigas* genes predicted to be hyper-methylated are generally associated with housekeeping functions and those predicted to be hypo-methylated are associated with immune related processes (Figure 1). The hypothesis is that genes predicted to be hypo-methylated have greater epigenetic flexibility, which allows for higher regulatory control of these inducible classes of genes. Oysters have been shown to have high phenotypic plasticity in response to environmental changes and stress (Hamdoun *et al*, 2003; Honkoop *et al*, 2003) and it is possible that an epigenetic mark, such as DNA methylation, could provide this level of control. This finding is similar to research on *A. mellifera* where genes associated with general metabolic or housekeeping functions were predicted to be hyper-methylated, whereas caste-specific genes were preferentially hypo-methylated (Elango *et al*, 2009; Fortet *et al*, 2009). Intragenic DNA methylation has been reported in other invertebrates (Suzuki *et al*, 2007; Elango & Yi, 2008), and stands in contrast to what is reported in vertebrates where regulation of transcription by DNA methylation is accomplished primarily at the gene promoters (Boyes & Bird, 1992; Kass *et al*, 1997; Hsieh, 1994).

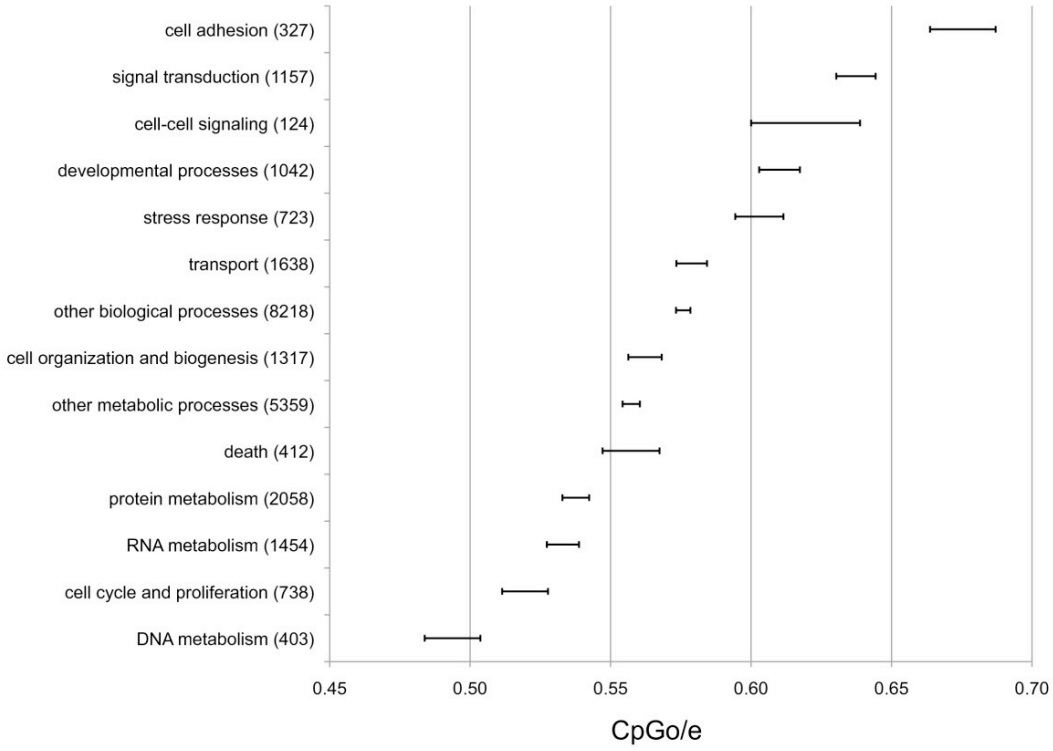

Figure 1. Differential methylation between categories of genes involved in discrete biological processes as measured by CpGo/e. Mean CpGo/e for 10,699 *C. gigas* genes (*i.e.* contigs from ESTs) categorized by GO Slim term. Bars represent mean ± 1 standard error. The number of contigs in each category is listed in parenthesis. Modified from Gavery and Roberts 2010.

One hypothesis regarding how intragenic DNA methylation affects gene expression is that it simply prevents inappropriate initiation of transcription outside of promoter regions (Bird, 1995). Additionally, there are a number of new studies that indicate an active role for DNA methylation. For example, exonic DNA methylation has been shown to regulate transcription of the *phytochrome A* gene in *Arabidopsis thaliana* (Chawla *et al*, 2007). Investigation of intragenic CpG islands (≥200 bp regions with G+C content of at least 50% and CpGo/e close to expected) in humans has revealed that CpG islands in terminal exons may regulate transcription of non-coding RNAs (Medvedeva *et al*, 2010). More recently a study has shown a conserved DNA methylation-associated regulation of alternative promoters within gene bodies in mice and humans (Maunakea et al, 2010).

The application of gene-targeted methods, such as bisulfite sequencing PCR, aimed at identifying DNA methylation in the oyster is limited (Gavery & Roberts, 2010). Five genes predicted to be hyper-methylated and five predicted to be hypo-methylated (based on CpGo/e) were randomly selected for analysis. Valid PCR products were produced for only two of the genes. This is a typical result as the conversion of unmethylated cytosines results in challenges for primer specificity. Four individual clones were sequenced for each of the two products. In fragment 1, one of seven CpGs sites displayed methylation in 25% of the clones sequenced (Figure 2(a)) In a second fragment, one of two CpGs sites was determined to be methylated in 50% of the clones (Figure 2(b)).

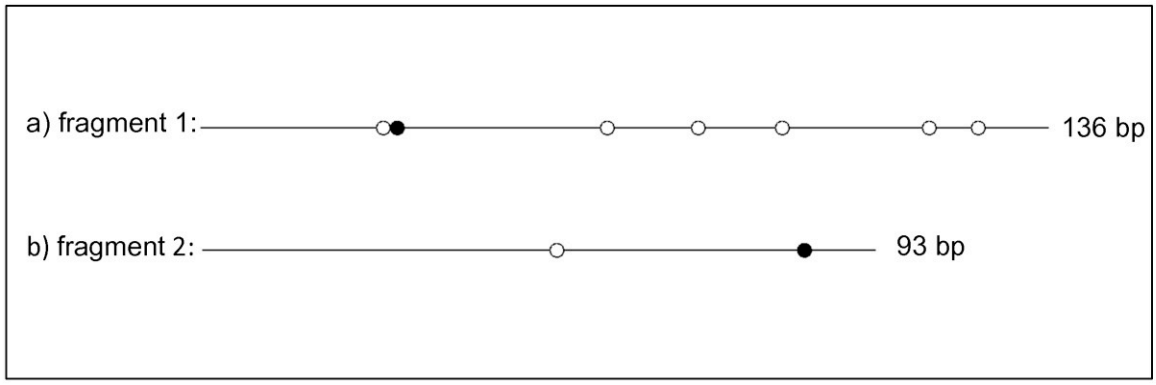

Figure 2. Methylation status of a 136 bp (a) and 93 bp (b) fragment of *C. gigas* DNA as determined by bisulfite sequencing. Solid and open circles represent methylated and non-methylated CpG dinucleotides, respectively. One of four clones was determined to be methylated at the CpG indicated by the solid circle in (a) and 2 of 4 clones were determined to be methylated at the CpG dinucleotide indicated in (b). Figure modified from Gavery and Roberts, 2010.

Several technologies have been developed for characterizing global DNA methylation patterns that alleviate issues associated with gene-targeted approaches. For instance, direct sequencing based methods provide the opportunity to investigate global DNA methylation at a high-resolution. Bisulfite treatment and methylation enrichment (*e.g.* MeDIP or MBD2) followed by high-throughput sequencing are the most common technologies being employed. Bisulfite sequencing (BS-seq) approaches utilize sodium bisulfite to deaminate cytosine, but not methylcytosine, to uracil followed by direct sequencing. This approach provides methylation interrogation at single-base resolution. However, BS-seq data can be challenging to interpret due to the reduced complexity of the sequence (A/T richness), which makes assembly against a reference genome difficult. This issue is further exacerbated when working with a non-model species where a reference genome is not available.

Methylation enrichment approaches such as methyl DNA immunoprecipitation sequencing (MeDIP-seq) and methylated DNA binding domain sequencing (MBD2-seq), isolate a portion of the genome based on degree of methylation. These data are more straightforward to analyze as DNA is not converted prior to sequencing and coverage is increased as representation in the genome is reduced. A relative disadvantage is that nucleotide specific information is lacking. For methylation enrichment approaches, genomic DNA is randomly sheared and methylated fragments are enriched by separation of highly methylated fragments using either immunoprecipitation with a monoclonal antibody that specifically binds 5-methylcytidine (MeDIP), or by preferential binding of methylated fragments to methyl-CpG binding domain of human MBD2 protein (MBD2). The resulting bound fractions, enriched in methylated DNA, can then be directly sequenced. MBD2 approaches have a couple of advantages compared to the MeDIP procedures with respect to downstream sequencing. First, the end product in MeDIP is single stranded DNA, which increases manipulation to make doublestranded DNA necessary for library preparation. Second, MBD2-seq allows for fractionation based on methylated density to generate pools of densely methylated, moderately methylated and unmethylated fractions. This can be beneficial both in reducing the genome to increase coverage. Moderately methylated and unmethylated regions are of particular interest in the oyster.

B. Objectives

For the current proposal, MBD2-seq will be used to characterize global DNA methylation patterns in the Pacific oyster. In addition, an integrative method to combine MBD2 selection with bisulfite sequencing will be developed. To our knowledge this has not been done in a non-model organism.

The specific objectives of the current study are to:

1) Characterize differences in methylation across the Pacific oyster genome
2) Evaluate cell-type variability in methylation
3) Gain information on nucleotide specific methylation patterns

C. Procedure

To carry out the research objectives of this proposal, next-generation sequencing technology (SOLiD 4) will be used to characterize DNA methylation in oysters. Methylation enrichment will be used to identify portions of the genome that are densely, moderately, and un-methylated. As DNA methylation can vary between tissue type and developmental stage, multiple adult tissues will be examined as well as larval oyster samples. In order to examine DNA methylation at the nucleotide specific level, bisulfite sequencing will be performed. The schematic below illustrates the proposed methodology. Specific technical aspects are provided below.

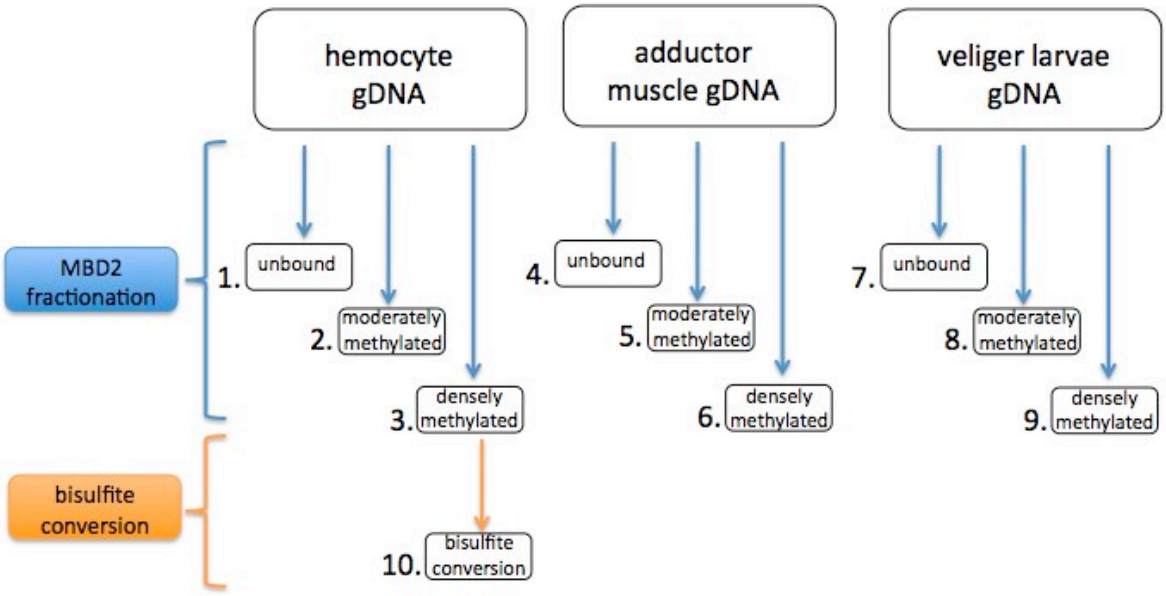

Figure 3. Schematic illustrating libraries for DNA sequencing. Three libraries fractionated based on degree of methylation will be generated from three oyster cell-types. One bisulfite treated library will be made from densely methylated DNA from hemocytes. Hemocytes are selected based on their role in immune function.

Animal Collection & DNA Isolation

Adult and larval oysters used in this study will be provided from a local commercial shellfish hatchery (Taylor Shellfish, Shelton, Washington). Muscle tissue and hemocytes will be isolated from a single adult oyster and preserved for DNA isolation. These two cell types are selected based on their diverse function and the role of hemocytes in immune function. For construction of larval oyster libraries, pooled samples will be used. Hemocytes and larvae will be centrifuged and the supernatant discarded. DNAzol (MRC) will be used to isolate DNA from all samples according to the manufacturer's protocol.

Methylation Enrichment

Methylation enrichment will be performed using the Methyl-Miner Kit (Invitrogen) which binds fragmented double-stranded genomic DNA using biotin labeled Methyl Binding Domain 2 protein (MBD2), and can be eluted using a salt gradient. DNA will first be fragmented to ~150 bp, the appropriate size to be used in the down-stream library preparation, using sonication (Covaris). The fragmented DNA will be incubated with methyl-CpG binding domain of human MBD2 protein, coupled to paramagnetic bead via a biotin linker. The bound fraction (methylated fraction) will be eluted with a high salt concentration buffer. The moderately methylated and unbound fraction will also be retained (Figure 3).

Library Construction

DNA libraries will be prepared using the SOLiD DNA Fragment Library Kit (Life Technologies). Briefly, this involves end-repairing the DNA fragments, ligating adaptors (P1 and P2), size selecting the DNA, then performing nick-translation and amplification via PCR with primers specific to the adaptor sequencing. DNA will be analyzed using a Bioanalyzer (Agilent) prior to emulsion PCR. Initially, an octect (1/8 of a slide) will be sequenced from each library. Sequencing will be carried out at the UW high-throughput sequencing facility (UW HTGU).

Library Construction – Bisulfite Sequencing

Library construction for the sodium bisulfite treated library will be similar to the above procedure with the following exceptions: the top strand of the P1 adaptor will be synthesized with 5-methyl cytosine instead of cytosine to prevent modification during bisulfite conversion and during nick-translation 2′-deoxycytidine-5′-triphosphate (dCTP) will be replaced with 5-methyl-2′-deoxycytidine-5′-triphosphate (5mC-dNTP) in the original dNTP mixture (standard dNTPs for A, G, and T will be used). Bisulfite conversion will be performed in solution according to Renade *et al*, 2009. After bisulfite conversion, PCR amplification will be performed as described above. Note that only one strand will amplify during PCR (the bottom strand is not protected during bisulfite conversion), which will simplify downstream analysis. While the genome reduction (MBD2) will increase coverage and assist in analysis, the bisulfite sequence aspect will also serve as a general proof of concept for the workflow. For this proposal only one library will be bisulfite treated.

Analysis

Sequencing data will be analyzed using CLC Genomics WorkBench (CLC Bio) along with publicly available databases (NCBI, SWISS-PROT, GigasBase) and our own unpublished *C. gigas* RNA-Seq libraries. The PI has significant experience with this data format, characterizing RNA-Seq libraries from trout (Goetz *et al.* 2010), chum salmon (Seeb *et al.* in final revision), and several shellfish species (unpublished). Analysis will include quality trimming, d*e novo* assembly, and BLAST. Comparisons among libraries will be made within CLC Genomics Workbench (ChIP-Seq, RNA-Seq), Microsoft Access, and Galaxy tools (http://main.g2.bx.psu.edu/). One of the most challenging aspects of this project is going to be the analysis of the bisulfite sequencing with the absence of a sequenced genome. To increase coverage,

library representation will be reduced for bisulfite sequencing (Figure 3, library 10) and comparable non treated library (Figure 3, library 3) (see above). Bisulfite data will be analyzed using CLC Genomics WorkBench and custom scripts.

There is clearly an advantage when sequenced-based approaches are used with model organism systems. A completed genome greatly improves the ability to assemble and analyze data. For this study we are confident the genome reduction approach of isolating DNA with methyl binding domain will allow us to obtain enough sequence coverage. The oyster genome is 824 megabases (Hedgecock *et al*, 2005) and based on some preliminary MeDIP procedures in the lab it is estimated that approximately 20% of the genome is highly methylated. We plan to sequence an octet on the SOLiD 4 system (UW HTGU) that is expected to generate close to 12 gigabases (ABI Specification Sheet). The estimated coverage would therefore be over 50x. This an exaggerated estimation as it does not take into consideration low quality data, sequencing errors, and sub-optimal recoveries. However, based on handling similar types of data in RNA-Seq analysis we are confident in the ability to analyze this data. Furthermore, it is likely that the oyster genome will be released in the near future. Recently the genomics institute BGI in Shenzhen, China stated in a press release that they had sequenced the Pacific oyster genome (Nature News, 2010). This data would assist in the analysis of the data in Fall of 2011, however if it is not necessary for completion of this project.

D. Time Schedule

Sample preparation will begin in Winter 2011 to include, DNA isolation, methylated DNA enrichment and bisulfite treatment. Library construction will occur in late Spring through Summer months. Autumn quarter will primarily involve data analysis and manuscript preparation.

E. Need for RRF Support

In silico research indicates DNA methylation is present in oysters and plays an important role in controlling gene expression. There are significant implications for this work particularly in regard to environmental science and aquaculture. As described above, environmental conditions can impact DNA methylation and have the potential for population as well as ecosystem level effects. With respect to the aquaculture industry, a better understanding of DNA methylation patterns could benefit oyster broodstock selection programs as patterns associated with desired traits are elucidated. In order to address these issues and apply for external funding through agencies such as the EPA, NOAA, and USDA, it is important to 1) demonstrate comprehensive experimental corroboration of the *in silico* work as well as 2) develop an effective workflow for characterizing global DNA methylation in non-model species. Completion of this research would therefore directly address the mission of the Royalty Research Fund by providing an opportunity to increase the applicants' competitiveness for subsequent funding. Furthermore, this project aligns with the mission of the Royalty Research Fund by advancing a new direction of research for junior faculty. While the PI has a background in molecular biology and gene expression, epigenetics will be a new area of research focus.

Budget

01 Salary	
Steven Roberts, Assistant Professor (1 month summer salary)	8,040
Research Assistant, 50%, $1781 per month, 6 months, Winter 2011 and Fall 2011	10,686

04 Travel	
Travel	400

05 Supplies and Materials	
Reagents for methylation enrichment and bisulfite treatment	1,000
SOLiD Library Construction and Sequencing	8,500
general consumables (e.g., plastics, tips, gloves, sample tubes)	500

07 Retirement and Benefits	
01-10 Assistant Professor (25.8%)	2,074
01-40 Research Assistant (14.7%)	1,571

08 Operating Fee/Tuition	
08-05 Research Assistant, Two quarters, 2010 rate	7,112

TOTAL BUDGET 39,883

Budget Justification

Salary

Funds are requested for one month of summer salary for Assistant Professor Steven Roberts. In addition to developing the experimental approaches, he will spend time on data processing and analysis. Funds are requested for 2 quarters of graduate student support. Responsibilities will include sample preparation, library construction, and analysis.

Travel

$400 is requested to partially cover travel costs associated with presenting research at regional meetings.

Budget Justification continued

Supplies and Materials

A total of $10,000 is requested for materials and supplies to carry out the research objectives described as part of this project. $1000 is requested to cover costs of supplies for methylation enrichment and bisulfite treatment. $8,500 is requested to cover library preparation and sequencing to be performed at the UW HTGU. Funds ($500) are also requested to cover the common supplies and consumables used in work of this nature (i.e., gloves, pipettor tips, sample vials, plates, etc)

CURRICULUM VITAE – STEVEN BEYER ROBERTS

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B.S. – North Carolina State University (Raleigh, NC) – 1997
Natural Resources – Concentration in Marine and Coastal Resources

Professional Experience *2006-Present* · Assistant Professor
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2006-Present · Adjunct Assistant Scientist
Marine Biological Laboratory, Woods Hole, MA

2003-2006 · Assistant Research Scientist
Marine Biological Laboratory, Woods Hole, MA

Select Publications

Gavery M* and Roberts S. (2010) DNA methylation patterns provide insight into epigenetic regulation in the Pacific oyster (*Crassostrea gigas*). BMC Genomics 11:483 http://www.biomedcentral.com/1471-2164/11/483

Mathger L, Roberts S, Hanlon R. (2010) Evidence for distributed light sensing in the skin of cuttlefish, *Sepia officinalis.* Biology Letters. doi:10.1098/rsbl.2010.0223

Goetz F, Rosauer D, Sitar S, Goetz G, Simchick C, Roberts S, Johnson R, Murphy C, Bronte C, Mackenzie S. (2010) A genetic basis for the phenotypic differentiation between siscowet and lean lake trout (*Salvelinus namaycush*). Molecular Ecology, 19 176–196

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* *indicates student author*

Select Publications continued

Roberts SB, Gueguen Y, de Lorgeril J, Goetz F. (2008) Rapid accumulation of an interleukin 17 homolog transcript in *Crassostrea gigas* hemocytes following bacterial exposure. Developmental and Comparative Immunology. Volume 32, Issue 9, Pages 1099-1104

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* *indicates student author*

Recent Invited Presentations

Shellfish as indicators of environmental change. Gordon Research Conference on Oceans and Human Health. University of New England, Biddeford, ME. June 15, 2010.

Overview of Shellfish Activities at the University of Washington. USDA-WERA099: Broodstock Management, Genetics and Breeding Programs for Molluscan Shellfish. San Diego, CA. February 27, 2010.

Changes in the environment and changes in expression: insight from oysters. University of Southern California: Marine Science Seminar Series. Los Angeles, CA. February 2, 2010.

Current Research Support - Roberts
Title: Evaluation of putatively QPX-resistant strains of northern hard clams using field and genetic studies
Source of Support: USDA / Northeastern Regional Aquaculture Center
Award Amount: $263,490
Award Period: 03/01/2008 – 12/31/2010
Relationship: none

Title: 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 - 8/30/2011
Relationship: Focuses on same organism

Title: Effects of ocean acidification on declining Puget Sound molluscan calcifiers
Source of Support: Washington Sea Grant
Award Amount: $478,092
Award Period: 3/1/2010 - 2/28/2013
Relationship: Similar technical approach: SOLiD sequencing

Past Research Support - Roberts
Title: Development of genetic markers to assess disease resistance in the eastern oyster
Source of Support: USDA / Northeastern Regional Aquaculture Center
Award Amount: $154,066
Award Period: 9/1/2006 - 1/30/2008
Relationship: none

Title: Production of myostatin gene knockouts in zebrafish and the effects of specific myostatin interacting proteins
Source of Support: USDA - NRI
Award Amount: $195,862
Award Period: 1/1/2005 - 11/30/2008
Relationship: none

Title: Assessing withering syndrome resistance in California Black Abalone: Implications for conservation and restoration
Source of Support: California Sea Grant
Award Amount: $15,067
Award Period: 6/1/2007 - 5/30/2008
Relationship: none

Remaining Start-up funds: $50,732

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