**1. Training & Career Development Plan**

My primary career goal is to focus on cutting edge research that will enhance shellfish aquaculture while simultaneously allowing me the opportunity to engage with students and the general public. As a scientist, I will continue to examine molecular mechanisms of gene regulation in bivalves to better understand important physiological processes such as reproduction, growth, and immune response. As an educator, I will use my leadership role in research to train future generations of students in the concepts and approaches of applying molecular and cellular techniques to address important questions in shellfish physiology and sustainable aquaculture.

The following training objectives will result in a successful fellowship and increase my career opportunities. My first training objective is to expand my research base to include a greater understanding of bivalve reproduction and how to improve aquaculture with this knowledge. My graduate training has focused on genomic and epigenomic responses to environmental stress in bivalves (Gavery & Roberts 2010, Roberts & Gavery 2011, Gavery & Roberts 2012). I am interested in expanding on this research, specifically focusing on signal integration and gene regulation during shellfish reproductive development under the primary mentorship of Dr. Adam Luckenbach. My second training objective is to increase my teaching effectiveness through a faculty-in-training experience under the mentorship of Dr. Graham Young.

In summary, training as a USDA post-doctoral fellow would a) provide me with valuable preparation to become an independent, broadly trained researcher focused on fundamental questions important to the advancement of sustainable shellfish aquaculture, b) facilitate opportunities to create new relationships and develop collaborations with leading scientists in the field, and c) enable the development of my own teaching philosophy under the mentorship of an experienced professor. In short, training as a USDA fellow will enhance my skills as an academic researcher through the intellectual independence, broad training, and the invaluable teaching experiences I will receive.

**2. Mentoring Plan**

Scientific training will be performed under the primary mentorship of Dr. Adam Luckenbach, at NOAA’s Northwest Fisheries Science Center (NWFSC), Seattle WA. Dr. Graham Young, professor in the School of Aquatic and Fishery Sciences at the University of Washington (UW) and Executive Director of USDA-NIFA Western Region Aquaculture Center (WRAC), will serve as a collaborating mentor.

Dr. Luckenbach has an extensive background in reproductive biology and using cutting edge research approaches that directly benefit the aquaculture industry, making his involvement an ideal fit for the project. His expertise will not only assist with interpreting results from this project, but it will also expose the PD to a different taxonomic perspective via his background in sex determination, reproduction and finfish aquaculture. In addition, the facilities made available through his mentorship, namely the new shellfish hatchery at NWFSC’s Manchester Research Station, are ideally suited for this research. Dr. Luckenbach has trained numerous mentees in the last 5 years including: José M. Guzmán (NRC postdoc), Elizabeth K. Smith (post-bachelor), Douglas Immerman (UW graduate student), Louisa Harding (UW graduate student), Yoji Yamamoto (WA Sea Grant postdoc), Korrin Keyser (UW undergraduate), David C. Metzger (post-bachelor), and Tristan J. Stringer (UW undergraduate).

The collaborating mentor, Dr. Graham Young, will provide expertise and training for the education portion of this project. Dr. Young has extensive experience as a lecturer and has been a faculty mentor for the OACIS GK20 and NSF GK12 program. He has broad experience with outreach as the executive director of the Western Region Aquaculture Center. Dr. Young will provide guidance in instruction technique and advise on the development of a *Reproduction in Aquaculture* seminar series.

**3. Project Plan**

***3.1 Introduction***

Pacific oysters, *Crassostrea gigas*, are one of the most important aquaculture species in the United States. Despite their economic and ecological value, there are many aspects of their reproductive biology, including control of sex determination and reproductive maturation, which remain unclear. A greater understanding of these processes will facilitate improved efficiency in bivalve aquaculture as reproductive effort is correlated with many important traits such as growth and stress tolerance. Likewise, improved knowledge of sex determination and differentiation in *C. gigas* is easily transferable to aquaculture activities related to ploidy manipulation, sterility and other efforts to minimize trade-offs between reproduction and growth. The proposed research aims to increase our understanding of reproduction in bivalves by using controlled experimental conditions to identify factors contributing to sex determination and characterizing regulatory events controlling reproductive maturation.

*Reproductive biology*

Oysters in the genus *Crassostrea* are sequential protandrous hermaphrodites with rare instances of simultaneous hermaphroditism (Coe et al., 1936). Reproduction in oysters is associated with seasonal changes including initiation of gametogenesis in winter followed by an active phase of gametogenesis in spring, maturation and spawning in summer, and finally a reabsorption period. The gonad is a diffuse and transient tissue surrounding the digestive gland composed of both somatic and germ cells. Germ cells in oysters are derived from germinal stem cells produced during embryogenesis (Fabioux et al., 2004). During gametogenesis germ cells divide by mitosis and gonial proliferation leads to maturation.

*Sex Determination*

Sex determination is the process by which the sexual characteristics of an organism are established. There are two main types of sex-determining mechanisms, genetic sex determination (GSD) where sex is established at fertilization, and environmental sex determination (ESD) where sex is determined after fertilization in response to an environmental cue. Sex determination is poorly understood in oysters, but there is evidence to suggest that there are elements of both GSD and ESD, similar to certain fishes such as tilapia (Baroiller & D’Cotta, 2000), European sea bass (Pifferer et al., 2005), and some flatfishes (Luckenbach et al., 2009).

 The first evidence for GSD in oysters came from a study with controlled crosses in *C. virginica,* where significant differences in sex ratios were observed between families sired by the same male (Haley 1979). Subsequently, Guo et al. (1998) extended these findings by evaluating sex ratios in 86 pair-mated *C. gigas* families and also reported significant family and parental effects on sex ratio. Additional studies on GSD in oysters will be beneficial for understanding heritability of sex and possibly manipulating sex in controlled crosses. However, *C. gigas* does not show strict GSD, as environmental factors are also important in predicting sex ratios in this species.

Evidence for ESD in oysters has been previously described. Several studies have reported that temperature and food availability are associated with sexual differentiation in *Crassostrea* species (Coe 1936, Galtsoff 1964, Quayle 1988). When *C. gigas* from the same origin were grown in two sites with different temperature and food availability, they exhibited differing rates and timing of gametogenesis (Enriquez-Diaz et al., 2009). Very recently, a controlled laboratory study investigating the effects of temperature on gonadogenesis in spat (*i.e.* recently metamorphosed juveniles) provided direct evidence that sex determination is influenced by temperature in *C. gigas* (Santarre et al., 2013). While this study provided novel insights into the control of sex determination in *C. gigas*, our understanding would be greatly improved by investigating the effects of additional variables thought to be involved in sex determination, including sex steroids.

Sex steroids regulate a variety of reproductive activities in vertebrates including sex differentiation, maturation, and estrous cycles. Studies on the function of sex steroids in bivalves are incomplete. However, there is evidence to suggest that steroids, such as estrogens and androgens, are important regulators of bivalve reproduction and are involved in gametogenesis, vitellogenesis, and spawning (Croll & Wang 2007). To date, no investigation has addressed possible effects of estrogens on *C. gigas* during initial gametogenesis.

Elucidating the time period when organisms are sensitive to exogenous signals is critical for understanding and controlling reproduction. For example, it has been demonstrated in fish that sex determination and differentiation is a labile process that can be altered by treatment of sex steroids to manipulate sexual development (reviews; Pandian & Sheela 1995, Devlin and Nagahama 2002), but the timing of the treatment is pivotal to the ultimate status of the gonad (Devlin & Nagahama 2002). Similarly, exposure to estrogens skews the sex ratio of adult bivalves toward more females, but the timing of exposure is critical for the effect (Mori et al., 1969, Ciocan et al., 2010). The period when oysters are most vulnerable to exogenous signals is unknown, though recent studies investigating the expression of conserved sex differentiation-related genes (*e.g.* FoxL2, DMRT-like, vasa) suggest that this process occurs approximately 40 - 60 days post fertilization (dpf) in *C. gigas* (Naimi et al., 2009a, b, Fabioux 2004). Identification of the sensitive time-frame, along with the characterization of the triggering factors themselves, will be important to understanding reproduction and efforts to control sex in aquaculture settings.

*Cellular and Molecular Regulators of Gametogenesis*

Exogenous factors related to reproductive maturation in *C. gigas* have been identified and include temperature, food availability, salinity, and photoperiod (Muranaka & Lannan 1984, Enriquez-Diaz et al., 2009, Fabioux et al., 2005).Because of the high reproductive investment in many marine bivalves, the period of gametogenesis is associated with intensive physiological change as a majority of the energy acquired is used for gamete production (Soletchnik et al., 1997). Because reproductive maturation is energetically costly, and significantly affects traits such as growth and stress tolerance, increasing our understanding of gene regulation during these processes is critical. A key component of understanding gametogenesis and maturation in *C. gigas* is knowledge of the cellular and molecular players involved in signaling pathways. For example, it has recently been reported that 2,484 genes are differentially expressed over the course of maturation in *C. gigas* (Dheilly et al., 2012). Studies such as these underscore the importance of understanding the factors regulating gene expression during physiologically taxing processes. One promising avenue of investigation is gene regulation through epigenetics.

Epigenetics refers to heritable processes that alter gene activity without changes to the underlying DNA sequence. Common epigenetic marks include DNA methylation, histone modifications and non-coding RNAs. All of these marks have been shown to have important roles in mediating processes involved in gonadogenesis (Pifferer 2013), however a vast majority of these studies have been performed in mammalian systems. Recently, characterization of DNA methylation patterns in *C. gigas* indicate that this epigenetic mark has an important role in regulating gene expression (Gavery & Roberts 2010, Roberts & Gavery 2012). Another important class of epigenetic marks that remain wholly uncharacterized in bivalve molluscs are regulatory microRNAs (miRNAs).

 It has been shown that miRNAs exhibit sex-biased expression in the reproductive axis of vertebrates such as chicken (Bannister et al., 2009) and Atlantic halibut (Bizayehu et al., 2012). Furthermore, miRNAs have been shown to play an important role during gonadogenesis in insects (Jin & Xie 2007, Bryan et al., 2010). Given the increasing evidence for the essential role of miRNAs in reproduction, it is likely that characterization of miRNA expression patterns during differentiation and gametogenesis will provide valuable insight into factors regulating reproduction in *C. gigas*.

***3.2 Rationale and Significance***

The proposed research aims to increase our understanding of critical reproductive processes in bivalve molluscs. This is in direct alignment with the AFRI Foundational Program Area: *Animal health and production and animal products*. ***Specifically, in the long-term, this project will enhance shellfish aquaculture production through improved growth rates***. For instance, a relationship between sex and growth has been documented in *C. gigas* with females being 10-12% heavier than males (Hedgecock et al., 1993). Being able to control gender in a hatchery or nursery setting to produce a higher number of females would improve the economics of producing oysters and is thus highly desirable to the shellfish aquaculture industry.

In addition to manipulating sex ratios, it is of great interest to control the amount reproductive effort in mature bivalves. In general, an ability to decrease reproductive investment will contribute to increased growth. Another advantage in limiting reproductive investment is that this would increase metabolic resources available to tolerate stress. Large scale oyster die-offs attributed to “summer mortality” are associated with reproductive maturation, spawning, and gamete re-absorption (Goulletquer et al., 1998, Huvet et al., 2010). ***Having the ability to manipulate exogenous factors that result in diversion of energy from reproduction would lead to more resources dedicated to maintaining homeostasis under stress associated with climate change***. The proposed research thus also aligns with the AFRI Challenge Area: *Agricultural and natural resource science for climate variability and change.* The ability to *increase* reproductive investment under controlled conditions will also have a benefit to the shellfish industry, particularly with respect to increasing production efficiency.

Induction of reproductive sterility has also been a desired trait. Sterility can preserve nutrient reserves normally utilized in gonad development, resulting in increased size and flesh quality of bivalves while also eliminating potential genetic contamination of native bivalve populations. Completion of the proposed project will yield data that may significantly improve our ability to promote shellfish aquaculture across all of these fronts.

Improving aquaculture production of the most prolific shellfish species in the United States will have significant economic and ecological returns. Pacific oysters are a key cultured shellfish species in the U.S. with production estimated at over 40,000 tons/year (FAO 2006). In Washington State alone, shellfish companies bring $270 million to the economy and provide 3,200 jobs to those living in rural coastal communities. As, benthic filter-feeders, oysters also play an important role in estuarine food webs and contribute to the removal of excess organics, nutrients, and particulates (Newell & Ott 1999, Officer et al., 1982). Like other shellfish, oysters are also carbon fixers, incorporating carbon into their shell thereby helping reduce atmospheric carbon dioxide levels (Newell 2004). This important ecological role of oysters also aligns the project with the AFRI Challenge Area: *Agricultural and natural resource science for climate variability and change.*

***3.3 Approach***

The proposed project sets out to gain a better understanding of fundamental aspects of shellfish reproductive biology using the Pacific oyster (*Crassostrea gigas*) as the basis for investigation. The research will focus on two critical periods in reproductive development, sex determination and gonadal maturation. The specific research objectives of this proposal are to:

*1) Identify how exogenous factors contribute to sex determination.*

*2) Characterize regulatory events controlling reproductive maturation.*

*3) Develop an integrative education schema focused on reproductive biology of shellfish.*

In the remainder of this section, each objective will be described in detail including the specific methods, expected outcomes, and any potential pitfalls.

**Objective 1: Identify how exogenous factors contribute to sex determination**

Although there are observations linking exogenous signals to sex determination, deterministic relationships between environmental factors and reproductive processes are poorly understood. In order to increase our understanding of these processes, the proposed research associated with Objective 1 will investigate the effects of two exogenous signals (temperature and estradiol-17β) on initial sex determination in *C. gigas*.

 Recently it has been shown that *C. gigas* spat raised at different temperatures exhibit different sex ratios(Santarre et al., 2013). Here, the authors reported that sex ratios were significantly different between spat raised at 18°C and 28°C, with a skew toward more males at higher temperature. On the other hand, the effects of sex steroids on sex determination and gametogenesis in molluscs remain controversial (Scott 2013). Some studies have indicated that treatment with estradiol-17β can affect sex ratios in adult *C. gigas*. However, the effect is only observed when animals are treated early in gametogenesis (*e.g.* Mori et al., 1969). To my knowledge, there have been no studies looking at the effects of estrogens during the window of initial sex determination in juvenile oysters. This is an interesting developmental stage for investigation as we would expect a preponderance of males (protandry), and deviations from this expectation would provide strong evidence in support of exogenous control of sex determination.

 For the current proposal a factorial experimental design will be implemented where the effect of three temperatures (18°C, 23°C and 28°C) and/or estradiol-17β (E2) treatment on sex determination will be assessed. Temperature regimes are based on Santarre et al. (2013) and E2 will be delivered via a 50 ng/L bath. This dosage is based on previous bath exposures performed in bivalve species (Andrew et al., 2010; Ciocan et al., 2010). *A novel and transformative component of the experiment to be carried out here is that multiple (5) distinct genetic lines will be incorporated into the replication of the experiment in order address the variability of the temperature response among oysters with different genetic backgrounds*.

 Incorporating genetic variability into the experiment will significantly contribute to our understanding of the combination of factors influencing sex determination in bivalves. It has been previously shown that temperature responsiveness has a genetic component in certain fish species (Conover and Kynard, 1981). For the proposed research, five families generated by controlled single-pair matings will be produced from unrelated individuals and larvae will be raised in a hatchery setting.

It is hypothesized that temperature will significantly alter sex ratios, with a greater proportion of males at 28°C relative to cooler rearing temperatures. Secondly, we anticipate that the E2 treatment will skew ratios toward females. Finally, it is hypothesized that the effect of either treatment will vary between genetically distinct families.

*Specific methods*

This work will take place at NWFSC, Manchester Research Station’s shellfish hatchery (see letter of collaboration). Standard protocols for spawning, larval rearing and nursery practices will be followed (Helm & Bourne 2004). After metamorphosis, 300 spat from each family will be placed into each of the six experimental conditions (*i.e.* 18°C, 23°C or 28°C with or without E2 treatment). Treatments will take place in upwelling systems with controlled temperatures. Sampling frequency will be temperature dependent, following Santarre et al. (2013). For each time point, 10 individuals will be measured, weighed and sampled for histology (whole body). Standard histology and H&E staining of the gonads will be performed per the methods of Luckenbach et al. (2003). Five additional animals will be sampled and tissue (whole body) will be stored at -80°C to allow for potential molecular characterization. Once sex can be determined via histological methods, 50 oysters will be sampled to assess sex ratio. The remaining oysters will be placed in grow-out bags in the field until 1 year of age when they will be measured and weighed to assess the effects of each treatment on growth and will also be monitored through gametogenesis for Objective 2 (see below).

*Expected outcomes*

First, exogenous factors contributing to sex determination in *C. gigas* will be identified and characterized. Second, the research will provide insight into the relationship between GSD and ESD in oysters. Finally, this research will ultimately be used to derive best management practices for bivalve hatcheries and nurseries to increase the efficiency of bivalve production. For example, hatcheries may use temperature or steroid manipulation to increase the number of faster growing female oysters. The ability to manipulate sex will also be a valuable tool in broodstock management programs.

*Potential pitfalls*

A primary challenge with this type of research activity includes having sufficient hatchery and nursery facilities to perform the experiment and maintain larvae. This challenge is addressed by using a new state-of the-art shellfish facility at the Manchester Research Station (see letter of collaboration). In addition, one question that is not addressed in this proposal is whether adult oysters (>1 year old) respond similarly or are as sensitive to exogenous signals (*e.g.* temperature) as spat. Although this research is outside the scope of the two-year proposal, these results would provide the foundation towards answering this question in future studies.

**Objective 2: Characterize regulatory events controlling reproductive maturation**

Reproductive maturation is an energetically costly process that can have detrimental impacts on immune function and growth in shellfish. A complete understanding of the molecular regulatory processes that control reproductive maturation in shellfish is currently lacking. The general approach for this objective is to follow cohorts used as part of Objective 1 and characterize how factors manipulated in the experiment (temperature, E2) influence later growth and sexual maturation. Furthermore, the underlying epigenetic mechanisms contributing to the phenotype will be characterized by evaluating miRNA expression patterns across maturation stages.

 Specifically, in the second year of this project, remaining oysters from each treatment from Objective 1 (n=100/family/treatment) will be measured and weighed to determine if temperature or E2 exposures early in development influenced growth after one year. All individuals will then be placed in a controlled conditioning system where temperature and food availability can be manipulated to stimulate gametogenesis. Oysters from each treatment group will be sampled at the beginning of the conditioning process, and at weekly intervals thereafter to monitor gametogenesis via histological methods. In addition, gonad tissue samples will be taken and frozen at -80°C for miRNA expression analysis. Micro RNA expression will be analyzed in gonad tissue throughout the differentiation processes using miRNA enrichment and next-generation sequencing techniques. A subset of samples from two of the treatment groups will be selected based on histological results. Total RNA from oyster gonad tissue for each gametogenic stage starting from the undifferentiated gonad (stage 0) to the fully mature gonad for both males and females (stage 3) will be isolated. Samples will be enriched for small RNAs prior to library preparation and high-throughput sequencing. Characterization of miRNA species, expression levels between sexes and reproductive stages, and identification of their gene targets will be performed. In addition, relationships between miRNA and gene expression levels (characterized by Dheilly et al., 2012) will be investigated.

*Specific Methods*

Total RNA isolation, enrichment of small RNAs, and high-throughput library preparation and 36-bp, single end sequencing will be performed using standard techniques (*e.g.* Gavery & Roberts 2012; Sunkar et al., 2008). Filtering and alignment of reads, classification of small RNA candidates into known categories, prediction of novel miRNAs, and identification of differentially expressed miRNAs between tissues will be performed using the web server mirTools (Zhu et al., 2010).

*Expected outcomes*

Overall, the activities of Objective 2 will enhance our understanding of gene regulation during gametogenesis in a commercially important aquaculture species, and produce new genomic resources in the form of annotated and characterized miRNA species.

*Potential pitfalls*

Here I propose to characterize and annotate miRNAs during oyster gametogenesis. It is likely that there are also other epigenetic features (*e.g.* DNA methylation histone modifications, long non-coding RNA) that contribute to gene regulation during these processes. Although a completely comprehensive dataset is not within the scope of this two year project, the data generated through this research will provide specific baseline gene regulation information in oysters and valuable information on invertebrate gene regulation. This dataset will also serve as a model for the integration of epigenetic data sources in agricultural genomics applications.

**Objective 3: Integrative education schema focused on reproductive biology of shellfish**

An essential component of the proposed project is to develop and implement an outreach and education plan. The three parts of this objective will increase my skills in instruction and curriculum development, allow me to engage the public in the research, and increase my professional interactions in the aquaculture sciences.

The traditional education component associated with this objective includes both formal classroom instruction and one-on-one mentoring. The formal classroom setting includes serving as a co-developer and co-instructor in the UW course, *FISH310: Biology of Shellfishes*. Training will take place through real experience in the practical aspects of teaching a required undergraduate course for fisheries majors. Specific pedagogical skills will be developed through utilization of the resources made available through UW’s Center for Teaching and Learning. I will also develop a new, 2-part lab exercise that leverages research carried out as part of Objectives 1 and 2. I will develop an activity to identify sex in mature oysters, perform standard hatchery techniques to spawn oysters and observe fertilization using light microscopy. In addition to formal instruction I will also be involving an undergraduate student in research Objective 1. The School of Aquatic and Fishery Sciences at UW requires all undergraduates to complete an independent research project and I will co-mentor at least one of these students as part of a project that complements my research activities.

Disseminating my research activities to the general public is currently an important aspect of my work and this will continue to be a part of my post-doctoral research. I have kept an open electronic lab notebook throughout my time as a graduate student and have experience developing web tools for my own personal research and specific projects (see http://students.washington.edu/mgavery). I have effectively used social media to share information about science, aquaculture, and education. A dedicated web-based portal will be developed as part of this project, similar to those I developed highlighting my graduate student research. This sharing of science will provide greater transparency to the research while also contributing to the third aspect of Objective 3, professional development.

For the third part of the education objective I will organize a seminar series, *Reproduction in Aquaculture*, at the University of Washington. Through this objective I will gain networking experience and insight from other researchers, develop possible future collaborations, and engage graduate and undergraduate students in research they may not have the opportunity to learn about otherwise. The seminar series will streamed-lived on the web and will facilitate online participation. The content will also be archived and made available to the public.

*Expected outcomes*

This objective includes a critical focus on outreach and education through formal classroom instruction, undergraduate training, public outreach, and facilitation of knowledge among experts in their field. These activities will not only contribute to the education of our next generation of scientists and educators, but will also promote responsible consumers, environmental stewards, and conscientious constituents. In the long-term, public education will contribute to increased demand and improved public opinion of aquaculture development.

*Potential pitfalls*

I do not foresee substantial potential pitfalls with regard to this research objective. An important aspect of the formal classroom instruction training will be to receive evaluations and feedback from students as well as soliciting feedback from peers (*e.g.* faculty). This will be accomplished via real-time evaluations (see Evaluation plan).

**3.4 Timeline**



**Figure 1.** Timeline depicting the approximate time frame for important aspects of each objective. In addition to the tasks listed above, this project will also include travel to attend an AFRI NIFA Fellows PD’s meeting and National Shellfisheries Association Meeting in Year 2.

**4. Evaluation Plan**

*Objective 1,* *Factors contributing to sex determination*: A primary milestone associated with identifying the factors contributing to sex determination will be the completion of the experimental trial performed during year 1. Given prior research and the experimental design, I am confident we will have a greater understanding of sex determination in oysters based on this work. These results will be presented at national meetings and a publication is expected during the first part of year 2. As described above, all research activities and results will be made available online in real-time throughout the project.

*Objective 2, Epigenetic regulation of gametogenesis*: Research Objective 2 is designed to identify factors regulating reproductive maturation. For this objective there are two primary milestones. The first will be the completion of the conditioning trial and quantitative assessment of reproductive status through morphometric and histological analysis. Based on these results a suite of miRNA libraries will be constructed and sequenced which will constitute the second primary milestone. The analyses of the combined data will be made available during the second year of the fellowship as described above.

*Objective 3,* *Educational Development*: The progress of Objective 3 will be evaluated in real-time in order to improve the effectiveness of the teaching plan. Student learning will be evaluated through the use of pre- and post-assessments during lab exercises and lectures. The formal end of quarter student review will be supplemented with specifically designed evaluation forms used to assess the new labs and lectures. In addition, I will be soliciting direct feedback of classroom instruction from my collaborating mentor.