UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0039
Expires 03/31/2004

PROPOSAL COVER PAGE

1. NAME OF ORGANIZATION TO WHICH AWARD SHOULD BE MADE
Marine Biological Laboratory

3. NAME AND TITLE OF AUTHORIZED ORGANIZATIONAL REPRESENTATIVE (AOR)
Richard J. Mullen, Ph.D Manager, Research Administration

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5. ADDRESS OF AOR (If different from Item 2.)

6a. TYPE OF PERFORMING ORGANIZATION (Choose 1 only)
- 01 ☐ USDA Agency
- 02 ☐ Other Federal Agency/Department
- 03 ☐ 1862 Land-Grant University
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- ☐ Alaska Native-Serving Institution
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- ☐ State Agricultural Experiment Station
- ☐ Tribal College (other than 1994)
- ☐ Veterinary School or College

7. TITLE OF PROPOSED PROJECT (140-character maximum, including spaces)
Functional genomic analyses of production-related traits in cultured bivalves

8. PROGRAM TO WHICH YOU ARE APPLYING (Include Program Area and Number: Refer to Federal Register announcement or program solicitation where applicable)
Functional Genomics of Agriculturally Important Organisms 45.0

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14. TYPE OF REQUEST (Check only one)
■ New ☐ Renewal ☐ Supplement ☐ Resubmission ☐ Resubmitted Renewal
☐ Continuing Increment ☐ PD Transfer [PRIOR USDA Award No. ______________]

15. FEDERAL FUNDS REQUESTED (From Form CSREES-2004) $713,860

16. PROJECT DIRECTOR (PD)
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20. IF THIS IS A RESEARCH PROJECT, WILL IT INVOLVE RECOMBINANT DNA, HUMAN SUBJECTS, OR LIVING VERTEBRATE ANIMALS?
☐ No ■ Yes (If yes, complete Form CSREES-2008)

21. WILL THIS PROJECT BE SENT OR HAS IT BEEN SENT TO OTHER FUNDING AGENCIES, INCLUDING OTHER USDA AGENCIES?
■ No ☐ Yes (If yes, list Agency acronym(s) & program(s))

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SIGNATURE OF PROJECT DIRECTOR(S) (All PDs listed in blocks 16 or 19 must sign if they are to be included in award documents.)
[signatures] DATE 6/10/04

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UNITED STATES DEPARTMENT OF AGRICULTURE

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COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

Project Director(s) (PD):

PD Steven Beyer Roberts — Institution Marine Biological Laboratory

CO-PD Maureen Krause — Institution Hofstra University

CO-PD ____ Institution ____

CO-PD ____ Institution ____

Project Title: Functional genomic analyses of production-related traits in cultured bivalves

Key Words: bivalve, growth, aquaculture, SAGE, bay scallop

PROPOSAL TYPE

For National Research Initiative Competitive Grants Program Proposals Only

- [x] Standard Research Proposal
- [ ] Conference
- [ ] AREA Award
  - [ ] Postdoctoral
  - [ ] New Investigator
- Strengthening:
  - [ ] Career Enhancement
  - [ ] Equipment
  - [ ] Seed Grant
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For Higher Education Program Proposals Only:

Need Area: ____

Discipline: ____

(Approximately 250 words)

PROJECT SUMMARY

Aquaculture production of marine bivalves such as clams, oysters, scallops and mussels has grown dramatically in the recent past and will likely continue to thrive as bivalves provide an environmentally friendly agricultural commodity that can be cost-effective. In the United States, shellfish are the highest-valued farmed marine animal. However, the industry is still faced with unsatisfactory growth rates and high mortality. This has resulted in increased attention to solutions such as intensive breeding programs and marker assisted selection of brookstock. In order for such programs to be successful there needs to be a more complete understanding of the functional genetics that are involved in production traits. The research proposed here is designed to further promote the aquaculture of marine bivalves by characterizing the transcriptome of bay scallops, focusing on aspects that are associated with increased growth. The hypotheses of the PDs are that bivalves growing at a faster rate will possess a quantitatively different transcriptome, and that there is a functional relationship between growth rate and nutritional and environmental factors. In order to test these hypotheses, the goals of the research proposed here are to 1) identify transcriptome differences in fast growing bay scallops and 2) genetically characterize the relationship between growth rate and feed efficiency. To complete the first goal, two approaches will be taken. First a novel differential display technique will be used to identify genes regulated in bay scallop treated with growth promoting compounds. The second approach will be to use modified Long SAGE analysis to compare gene expression profiles in scallops that have been selected for rapid and slow growth over several generations. In order to characterize the relationship between growth rate and nutritional and environmental factors, feed conversion efficiency of scallops selected for growth will measured. Genes that correspond to traits associated with increased conversion efficiency will be identified using differential display and quantitative RT-PCR. In addition, an over-wintering trial will be conducted to examine any functional relationship between growth and survival in selected scallop lines. Upon completion of the research objectives proposed here, there will be an increased understanding of the biological role of gene sequences linked to growth in marine bivalves.

According to the Paperwork Reduction Act of 1995, an agency may not conduct or sponsor, and a person is not required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0524-0039. The time required to complete this information collection is estimated to average .50 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information.

Project Description: Introduction

In the U.S., mariculture will provide the most likely avenue for expanding the aquaculture industry as inland resources are in short supply. In particular, the culture of bivalve molluscs such oysters, clams, mussels, and scallops has increased and has the potential to make significant economic and environmental impacts. In the U.S., revenue from combined bivalve production now bypasses that from the salmon industry and bivalves are the highest valued farmed marine product (FAO 2001). As filtering feeders and first-order consumers shellfish are an important component in improving water quality in marine and estuarine environments. Recently, increased consumer demand, declining natural fisheries, and advances in culture technology have peaked interest in the bay scallop (*Argopecten irradians*) (Coastal Zone Management MA 1995; Oesterling 1998; Wikfors *et al.* 1998). These factors have resulted in an increased research focus on the molecular and biochemical factors involved in bay scallop developmental physiology (see *Preliminary Data*; pg 3). The research proposed here is designed to ***further promote the aquaculture of marine bivalves by characterizing the transcriptome of bay scallops, focusing on aspects that are associated with increased growth and survival.*** As is the case for the production of all agriculturally important organisms, getting a quality product to market in an efficient, cost-effective manner is highly desirable. Additional confounding issues that are specific to the shellfish industry and are relevant to the current proposal include the relatively high cost of feed production (microalgae must be cultured simultaneously) and high seasonal mortality rates. In the remainder of this section bivalve aquaculture and biology will be outlined, followed by a discussion of preliminary data regarding the genes involved in growth and development in the bay scallop.

Bivalve Aquaculture

Unlike other animals that are commonly produced for consumption (cows, pigs, and fish) bivalves undergo a complete metamorphosis during production. In bivalves, free-swimming larvae metamorphose into juvenile shellfish that resemble what most consumers are familiar with (two shells encapsulating soft tissue). Figure 1 illustrates the life history of a scallop. While the appearance and anatomy of adult bivalves can be different, development from unfertilized egg to spat is the same across bivalves.

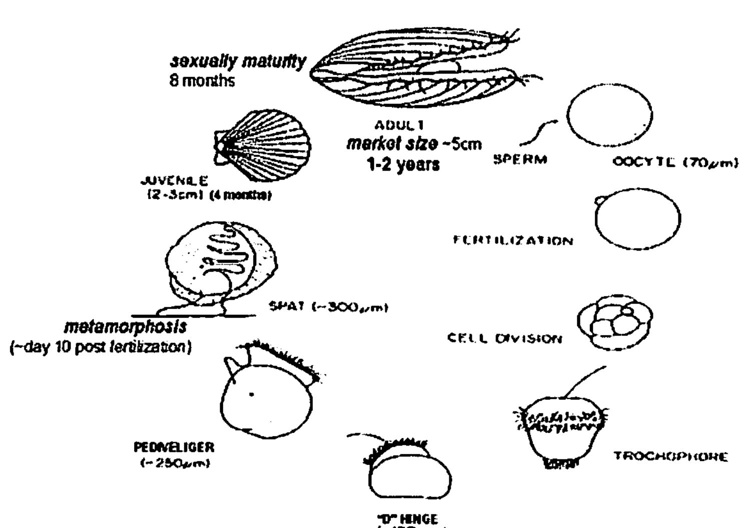

Figure 1. Schematic diagram of the scallop's basic life history. Modified from Sorgeloos *et al.* (1999)

The culture of shellfish has to accommodate for these early developmental changes and is commonly broken into three major phases; 1) spawning of broodstock and rearing of planktonic larvae; 2) nursery production (usually post-metamorphosis to spat size; ~2-8 mm) and 3) growing out in open water to bring the bivalve to market size (Castagna and Duggan 1971; Castagna 1975; Castagna and Kraeuter 1981; Widman *et al.* 2001). During the first two stages a significant amount of time and resources must be dedicated to micro-algae production to

supplement the diet of larval and post-metamorphosed shellfish. Compared to production of land based animals, commercial bivalve production is relatively new. It has only been in the past decade that many of the physical constraints faced in all phases of bivalve culture have been overcome, including optimization of environmental parameters in hatcheries and engineering of structures to hold adult bivalves (Gosling 2003). While some species of bivalves can grow out on natural bottoms, other species such as scallops are grown in mesh cages on racks, suspended mesh cages, lantern nets, or individually suspended on long lines. Farmed shellfish commonly will spend a majority of their life in open waters and financial expenditures decrease dramatically when animals are transferred from the hatchery to field-based culture, and consume only ambient food. However, the longer time a shellfish is cultured in open water the more likely they are to be exposed to severe environmental conditions, predators, and disease, factors that contribute to high rates of mortality. For this reason, reducing the time it takes for scallops and other bivalves to reach a harvestable size is a critical goal for the aquaculture industry.

Scallop Biology

Bivalves belong in the phylum Mollusca and share certain common morphological characteristics. For example, bivalves have a calcareous shell with two valves that are hinged dorsally. The two valves are attached by an elastic hinge ligament that allows the two valves to open and close via the action of adductor muscles. The shell encloses and protects the internal organs including the intestines, gonads, and gills as seen in Figure 2. While various bivalve species are morphologically similar, bay scallops have characteristics that set them apart. For example, bay scallops have a higher growth rate, can jet propel themselves through the water as adults, are hermaphroditic, and have a single large adductor muscle (Figure 2). The muscle is composed of two different types of muscle fibers. The cross-striated muscle, also known as the phasic adductor, is the most obvious structure when examining the insides of a scallop. The major function of this muscle is the quick action necessary for opening and closing the shell, the major means of locomotion. In contrast, the smooth, tonic muscle provides sustained contractions for long-term closure.

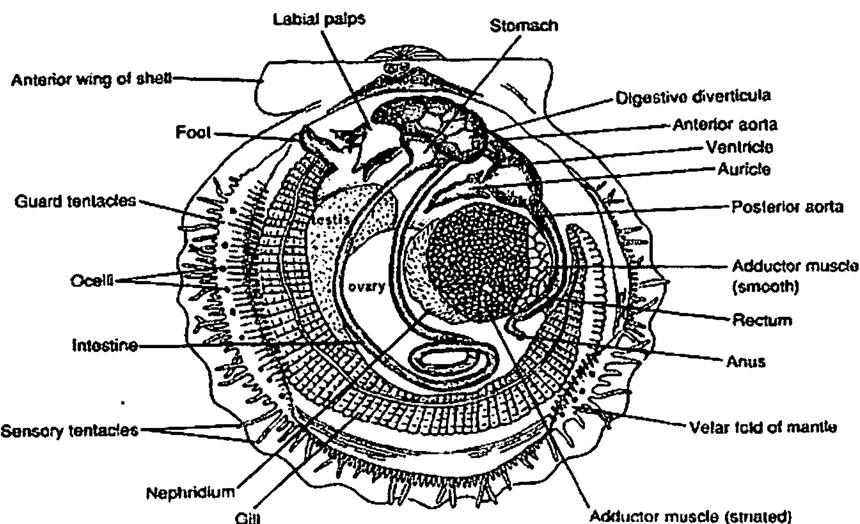

Figure 2. Diagram of the internal organs of a scallop. Modifed from Ruppert and Barnes (1994) (Invertebrate Zoology 6th Ed.)

Gills in scallops (and other bivalves) are large and in most species function to exchange gas and collect food. The internal (mantle) cavity is divided by the gills into inhalant and exhalent chambers. Water enters through the inhalant opening (siphon) and is moved through by cilia on the gills and mantle surface. The labial palp functions to sort food materials from the gills to either the mouth for digestion or along rejectory tracts as pseudofeces. Ciliary action moves food materials into the stomach which is imbedded in the digestive gland (diverticula). At the posterior end of the stomach is the style sac, from which the crystalline style (not shown in figure) originates. The crystalline style, produced by the style sac, is an unusual structure that continually dissolves, releasing digestive enzymes and added to at the style sac. There are tubules connecting the stomach to the digestive gland which is the major site of intracellular digestion. The digestive gland as well as the stomach, style sac, and intestines produce an array of enzymes that are responsible for the breakdown and absorption of carbohydrates, fat and proteins (Reid 1968; Mathers 1973; Langdon and Newell 1996; Le Pennec *et al.* 2001; Le Pennec and Le Pennec 2002; Le Pennec and Le Pennec 2003). Digestive gland lipids and carbohydrates and proteins from the muscle are primary repositories for energy storage during winter months when the feeding slows or ceases altogether (Barber and Blake 1985, Epp *et al.* 1988, Bricelj and Krause 1992)

While bivalves do not have a central nervous system similar to higher vertebrates, they do have 3 major ganglia that are essentially a small mass of neuronal tissue containing neurosecretory cells. These ganglia are referred to as the cerebral, visceral and pedal ganglia. The majority of neurosecretory cells are located in the cerebral ganglia. Researchers have demonstrated compounds (hormones) released from these cells have can regulate physiological processes such a reproduction and growth. For example, researchers have shown that the activity of neurosecretory cells in the mussel increases with the developing gonad (De Zwann and Mathieu 1992). Researchers have also identified the growth promoting factors including insulin-like peptides in the gangia of mussels (Kellnercousin *et al.* 1994; Kellnercousin *et al.* 1994; Danton *et al.* 1996).

Preliminary Data: Genes Involved in Bay Scallop Growth and Development

While oysters and clams are two of the commonly produced shellfish products, recently there has been significant interest in the bay scallop, *Argopecten irradians*. This is primarily a result of increased consumer demand, declines in natural populations, advances in culture technology (Oesterling 1998; Wikfors *et al.* 1998; Gosling 2003), and the coinciding availability of functional genetic research focused on bay scallop growth. Some of this genomics work is being done in the lab of the PD (S. Roberts) and is focused on identifying factors regulated during larval competence and metamorphosis in the bay scallop (USDA grant # 2002-03633). By understanding what specific factors are involved in the control of early development, scallop larvae could be stimulated to set and begin to grow faster, potentially decreasing mortality rates and decreasing the time needed to get a bay scallop to market size.

One approach that is being used characterize genetic control of development is Expressed Sequence Tag (EST) analysis. Bay scallop larvae were taken at 3 different developmental stages corresponding to taken prior to ("D"-hinge), during (pediveliger) and following-metamorphosis (spat) (Figure 1). This approach was chosen because single-pass DNA sequences of approximately 800 bps not only provides rudimentary data regarding differential expression of specific gene products, but also significantly contributes to the lack of gene sequence information known about the bay scallop. To date over 2000 sequences generated from this research are available to the public as part of the National Center for Biotechnology

Information's EST database (dbEST) (http://www.ncbi.nlm.nih.gov/dbEST/index.html). Several of these genes have been putatively identified based on sequence homology. One example of a novel gene product has been putatively identified as iodothyronine deiodinase (thyroid hormone deiodinating enzyme). In vertebrates such as frogs, thyroid hormones has and important role in controlling metamorphosis. To our knowledge, there have been no reports of the presence of thyroid hormone in bivalves. Preliminary PCR results show that this gene is differentially expressed through development. Interestingly, in adults this gene appears to be expressed in relative low amounts in muscle and digestive tissue (i.e. crystalline style).

A second, more quantitative molecular approach being taken to understand the internal factors controlling metamorphosis is the isolation of differentially expressed genes (DEGs). This refers to all the genes that are expressed differentially in mRNA level of different samples. To accomplish this objective, developing bay scallops samples were taken as described above ("D"-hinge, pediveliger, spat). After RNA isolation, a Genefishing™ DEG Kit (Seegene), based on Annealing Control Primers (ACP) technology, was used to PCR amplify differentially expressed genes. An example of a gel with the differentially expressed genes present is seen in Figure 3. The premise behind this approach is, that the up or downregulation of a specific gene at a given developmental stage is linked to physiological factors controlling somatic changes. A hypothesis is that genes isolated using this approach can function in the format of structural proteins or catalytic enzymes. In addition, the genes identified could be metabolites, which are a consequence of the action of catalytic enzymes and are by products of organogenesis. The PCR products representing regulated genes are indicated with lower case letters and have recently been sequenced. The proteins with the highest degree of similarity with the deduced amino (BlastX ref) of each band are; "a" - heat shock protein 70 [AAO38780], "b" - Chymotrypsin-like serine proteinase precursor [P35003], and "c" - pheromone receptor Rcb3 B47 [AAQ96349].

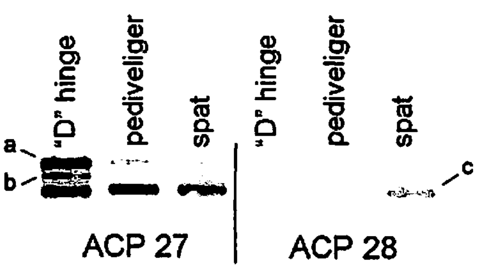

Figure 3. PCR products run out on agarose gel, showing differentially expressed genes (lower case letters) in developing bay scallops (N=~100k).

A directed, targeted candidate gene approach has also been taken by the PD of the current proposal (Roberts) to identify genes involved in development and growth in the bay scallop. The basic principle behind this technique is that conserved regions among candidate genes families are used for designing degenerative primers to be used for PCR. One specific candidate gene that has significant implications of for animal production it myostatin. Myostatin is a member of the transforming growth factor-β (TGF-β) superfamily, and has been established as a regulator of development and growth in mammals (McPherron *et al.* 1997; Lee and McPherron 1999; Lee and McPherron 2001). Myostatin was first characterized in mice (developing somites), where disruption of this gene resulted in a significant increase in muscle mass (McPherron *et al.* 1997).

Figure 4. Belgian Blue that has been selected for "double muscling". Researchers have recently determined that a natural mutation myostatin resulted in this phenotype (McPherron and Lee 1997)

Naturally occurring mutations in MSTN were soon attributed to the 'double muscle' phenotype observed in some breeds of cattle (i.e. Belgian Blue; (McPherron and Lee 1997)) (Figure 4). A reduction in myostatin expression results in increased cell proliferation, with both hyperplastic muscle growth (Thomas *et al.* 2000). Roberts was one of the first researchers to characterize myostatin expression in salmonids (Roberts and Goetz 2001) and has since examined protein expression (Roberts and Goetz 2003; Biga *et al.* 2004; Roberts *et al.* 2004), transcriptional regulators (Roberts and Goetz 2003), and myostatin's relationship with transgenic overexpression of growth hormone in coho salmon (Roberts *et al.* 2004).

More recently, Roberts, along with colleagues at Colorado State University (Hyun-Woo Kim, Donald L. Mykles) and the Great Lakes WATER Institute (Frederick Goetz), identified and characterized a myostatin-like cDNA from the bay scallop. (see *Appendix pg 73* for accepted pre-print of the corresponding manuscript – ***Characterization of a myostatin-like gene from the bay scallop, Argopecten irradians.*** **Biochemica et Biophysica Acta**) This gene codes for a 382 amino acid myostatin-like protein (sMSTN). The sMSTN sequence is most similar to mammalian myostatin, containing a conserved proteolytic cleavage site (RXXR) and conserved cysteine residues in the C-terminus. Based on quantitative RT-PCR, the sMSTN gene is predominantly expressed in the adductor muscle, with limited expression in other tissues. Using the sMSTN sequence, a *Ciona* myostatin-like gene was also identified from the *Ciona intestinalis* genome. These findings indicate that the myostatin gene has been conserved throughout evolution and suggests that myostatin likely plays a role in muscle growth and development in invertebrates, as it does in mammals.

In order to better understand myostatin's function in scallop's members of the PD's lab are currently examining myostatin gene expression in different populations and experimentally treating scallops with compounds presumed to effect mysotatin expression and in turn growth physiology. One approach being taken by undergraduate students (Phoenix Becker, University of Maine; Adam Bissonnette, Saint Anselm College, NH) working with Dr. Roberts, is treating scallops with a dietary supplement advertised as a "Myostatin Neutralizing Growth Factor Complex". The main ingredient is a MyoZap™ CSP3, based on sulfated polysaccharides (SP) from *Cystoseira canariensis* (C) a brown seaweed. Researchers have demonstrated that natural sulfated polysaccharides isolated from this macroalgae bind to the myostatin protein in serum (Ramazanov *et al.* 2003). It is possible since the scallop's diet is primarily algae, this compound will affect growth. At the time this proposal was being prepared preliminary feeding trials were being

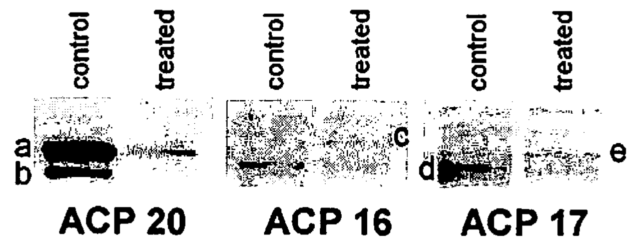

Figure 5. PCR products from RNA from adult scallops (N=2) treated with MyoZap and not treated (contols). Differentially expressed genes are indicated with lower case letters.

perfomed at the MBL in order to empirically determine dose, based on factors such as clearance rate. As this experiment has just been initiated (June 2004) there are no results in terms of phenotypic effects. However, after five days of treatment (600 mg MyoZap™ CSP3 / day via 4 hour immersion feeding) scallops from that were treated and controls were sacrificed, and total RNA was extracted from adductor muscle tissue to identify differentially expressed genes. The GeneFishing DEG system was used and representative gels can be seen in Figure 4. To date only bands "b" and "d" have been putatively identified based on DNA sequencing and are arginine kinase and cyclin T, respectively. This data is very preliminary as differential

expression has not been verified (i.e. quantitative RT-PCR) and the sample size was small (N=2). Neverthelesss, the possibility that myostatin decreased in the treated samples and a downregulation of cyclin T is observed is consistent with what has been shown in mammalian systems. One suggested mechanism of action of myostatin through regulation of p21 (a cyclin dependent kinase (cdk) regulator (Thomas *et al.* 2000). Thus, the downregulation of cyclin T (a cdk regulator shown to me important in muscle growth (Sano and Schneider 2003) suggest that myostatin function in scallops could be similar to vertebrates.

Project Description: Rationale and Significance

Why bivalves?

The culture of marine shellfish and finfish make up a large percentage of seafood production and is the fastest growing segment within the aquaculture industry (FAO 2001). Unfortunately, the U.S. is relying on seafood imports to meet their seafood demand. According to the FAO, U.S. consumers spent an estimated $52.3 billion for fisheries products, importing $9.0 billion of edible seafood in 1999. In contrast, the U.S. only exported $2.8 billion in fisheries products for a deficit of $6.2 billion. The U.S. trade deficit in seafood is the largest for any agricultural commodity and the second largest, after petroleum, for any natural resource product (Tlusty *et al.* 2001). This, in part, has caused the U.S. Department of Commerce to call for a 5-fold increase in aquaculture production by 2025.

It makes sense to focus much of this effort into mariculture of bivalves such as scallops. Two of the main reasons for this are that bivalves provide an environmentally friendly agricultural commodity that can be cost-effective. In marine and estuarine aquatic systems, a major cause of poor water quality is eutrophication. Excess organic waste from municipalities and commercial activities such as finfish aquaculture can contribute significantly to eutrophication. Sedimentation of such waste negatively affects benthic communities. In addition, nitrogenous waste (mainly in the form of ammonia) in high quantities can be toxic to fish and shrimp. Bivalves are filter feeders and can remove excess organics, nutrients, and particulates (Newell *et al.* 1999; Rice 1999; Rice *et al.* 1999). Simply increasing the number of bivalves in open waters can have a substantial effect by removing harmful nutrients. Bivalves could also be raised in conjunction with finfish to improve water quality.

From an economic standpoint, it is clear that the culture of bivalves can generate significant revenues for the aquaculture industry. In 2001, U.S. aquaculture of clams and oysters alone was a 70 million dollar industry (FAO 2001). In the U.S., revenue from combined bivalve production has bypassed that of the salmon industry and is the highest valued farmed marine animal (FAO 2001).

Why Scallops?

In addition to the benefits described above for bivalves in general, there are several unique qualities that set bay scallops apart from other bivalves. Scallops have a very high growth rate and can reach sexual maturity in six months. Bay scallops reach market size in 1-2 years in the wild, or nine months under laboratory conditions (Widman *et al.* 2001). In comparison, oysters and clams may take over four years to reach market size in the wild.

Bay scallops are one of the few bivalves capable of escape swimming. Because bay scallops are mobile, grow-out usually occurs in stacked cages and suspended nets as opposed to bottom culture like oysters and clams. Therefore, the use of valuable submerged land is not necessary, grow-out is not limited to inland waters, and culture methods can easily be developed

so they do not interfere with the remaining commercial shellfish industry.

A single, large adductor muscle has evolved in the bay scallop that acts to open and close the shell with great force. This muscle is the most prominent feature observed when the shell is removed, and is also the primary portion of the scallop that is desired by seafood consumers. As with any animal muscle, the scallop adductor muscle is rich in protein and glycogen. The great taste and high nutritional value has put the muscle of scallops in high demand for many years. When bay scallops are available, their market value is extremely high. For example, in the New England area, local bay scallops can sell for $16-22 per pound in the retail market.

Why focus on growth?

Rapid growth of post larval scallops is one the primary goals of selective aquaculture breeding programs because 1) decreasing time to harvest, particularly of the juvenile stage that require cultured algae, could dramatically decrease the cost of culture, 2) once juvenile scallops are moved from the hatchery to cages or, more often, to the field, size is a threshold from predation (Tettlelbach 1985, Tettelbach and Feng 1986, Tettelbach 1988, Pohl *et al.* 1991), and 3) **the mass of the final commercial product, the adductor muscle, is highly correlated with overall size** (i.e. Bricelj and Krause 1992, Thomson and MacDonald 1992, Krause and Bricelj 1995).

Multiple external factors contribute to juvenile and adult growth, including temperature, food availability, quality, and particle size, water flow, suspended sediments, reproductive condition and oxygen availability (Reviewed in (Bricelj and Shumway 1991, Thompson and MacDonald 1991, Gosling 2003), but variability for growth within a single population and observations from full-sib and mass spawning in the hatchery indicates a strong genetic component, as well. Several groups have successfully used selective breeding through truncation selection of juveniles or adults to increase the rate of bay scallop growth (really time to achieve a particular size threshold) (Crenshaw et al. 1991, Stiles et al. 1997, 1998), as well as growth in other bivalves (Wada 1984, 1986, Haskin and Ford 1987, Newkirk and Haley 1982, 1983, Mallet 1986, Hadley 1988, Hadley et al. 1991, Sheridan 1997, Rawson and Hilbish 1990) Realized heritability estimates for growth for the bay scallop *A. irradians* range from 0.21 to 0.50, and for other bivalves from .1 to .9 (Mallet *et al.* 1986, Rawson and Hilbish 1990, Hadley 1988, Toro and Newkirk 1990, Ibarra 1999, Ibarra *et al.* 1999), provide evidence of exploitable genetic variation for bivalve aquaculture. ***Still, we have little understanding of the specific genetic factors underlying variability for growth, which will be necessary if we are to advance the relatively primitive state of bivalve, especially scallop, aquaculture to a more productive and profitable level.*** Therefore, we propose to take a functional genomic approach to increase the understanding of the biological role of gene sequences in bivalve growth.
Our specific hypotheses include:

1) Bivalves growing at a faster rate will possess a quantitatively different transcriptome, as a result of activation of genes controlling growth and corresponding metabolites.

2) There is a functional relationship between growth rate and nutritional and environmental factors.

Project Description: Approach

General: As described above, bivalve aquaculture is a excellent avenue for expanding the agriculture industry, but our lack of understanding or ability to manipulate production-related traits, and the unpredictability of the industry due to variable survival and growth rates have constrained its development. The current proposal focuses on the bay scallop as a cultured species that will greatly benefit from an understanding of the biological role of genes and their link to function as it relates to agriculturally related traits. In addition, the bay scallop is an excellent model system for examining growth and survival in marine bivalves. In order to test the hypotheses stated above, we will be collaborating with the National Marine Fisheries Service's Northeast Fisheries Center in Milford, Connecticut. The Milford laboratory was established over 70 years ago and has been the birthplace for many of the early techniques used by shellfish hatcheries in the United States (Chew 2002). Dr. Sheila Stiles and Dr. Gary Wikfors (see Collaborative Arrangements pg 28) have individually and collectively made significant contributions to bivalve aquaculture in the fields of genetics and microbiology, respectively.(Longwell and Stiles 1973; Wikfors and Patterson 1994; Wikfors *et al.* 1994; Qin-Zhao *et al.* 1995; Wikfors and Smolowitz 1995; Wikfors *et al.* 1996; Wikfors *et al.* 1996; Ghosh *et al.* 1997; Stiles *et al.* 1997; Smith and Wikfors 1998; Stiles *et al.* 1998; Wikfors *et al.* 1998; Brown *et al.* 2000; Picozza *et al.* 2000; Wikfors and Ohno 2001; Hegaret *et al.* 2004).

Specific research objectives

I. Identify transcriptome differences in fast growing bay scallops

A. Analyze genes differentially expressed from scallops treated with growth promoting compound

B. Identify gene expression profiles in scallops selected for rapid versus slow growth

II. Genetically characterize relationship between growth rate and nutritional and environmental factors

A. Measure feed conversion efficiency for individual scallops from rapid and slow growth lines

B. Identify genes differentially expressed in tissues associated with increased feed efficiency

C. Compare over-wintering mortality rates in rapid and slow growth lines

In order to complete these goals our research will include tissue-specific gene expression profiling methods such as differential display (DEG) and serial analysis of gene expression (SAGE). These molecular approaches will be used to identify and link gene sequences with artificially manipulated growth, genotypic growth, feed conversion efficiency and survival. The remainder of this section will describe the specific research objectives, methods to be carried out, and our expected results.

Specific Research Objective I.A: Analyze genes differentially expressed from scallops treated with growth promoting compound

Genes identified in controlling growth in bivalves would help the industry by providing markers that could be used for selection and this information could directly be used to scientifically evaluate current culture practices. For example, if a strong expression of a specific gene correlated positively with increased growth rates, then environmental conditions (i.e. temperature and light) could be optimized to enhance productivity. One of the means by which we will begin to analyze these genes is to expand the current research underway (June 2004) in the lab of the PD (Roberts). As described in the Introduction, bay scallops treated with MyoZap CSP3, express differentially regulated genes (Figure 5). This compound's main ingredient are sulfated polysaccharides (SP) from *Cystoseira canariensis* (C) (brown macro-algae) that binds to myostatin protein in serum (Ramazanov *et al.* 2003). In the event that the current proposal is funded, the experiments will continue until September 2004, with relevant morphometric data taken. While the preliminary data was generated from animals treated on a daily basis, other cohorts are being treated on a weekly basis and will likely be the animals (and controls) assayed for the current proposal. Data provided by other researchers (Moriyama and Kawauchi 2004) examining the use of recombinant growth hormone to accelerate growth in the cultured shellfish, abalone *(Haliotis discus hannai)* suggests this regime of treatment has the potential to enhance growth. Adductor muscle and neuronal tissue will be taken for the treated and control (N=30) and frozen at -80C for analysis of differentially expressed genes.

Methods: Isolation of Differentially Expressed Genes (DEGs)

In order to compare the differentially expressed genes in the treated and non-treated scallops the recently developed differential display approach will be used (GeneFishing DEG Kits – Seegene). This system is based on Annealing Control Primers (ACP) technology (Kim YJ *et al.* 2004) (and described below). The reason this approach is being used is that it is economical, fast, and easily managed to compare a large number of different samples. This technology is a significant improvement on conventional radioisotope based methods used in the past. The use of the ACP's specificity and a two-stage PCR results in reproducibility and the elimination of false positives, two of the major problems with other differential

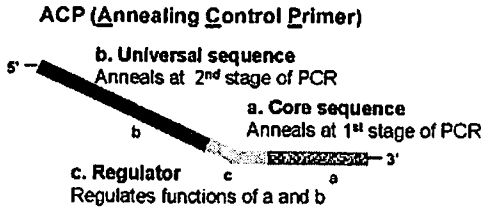

Figure 6. Diagram of ACPs used in the GeneFishing DEG System (Seegene).

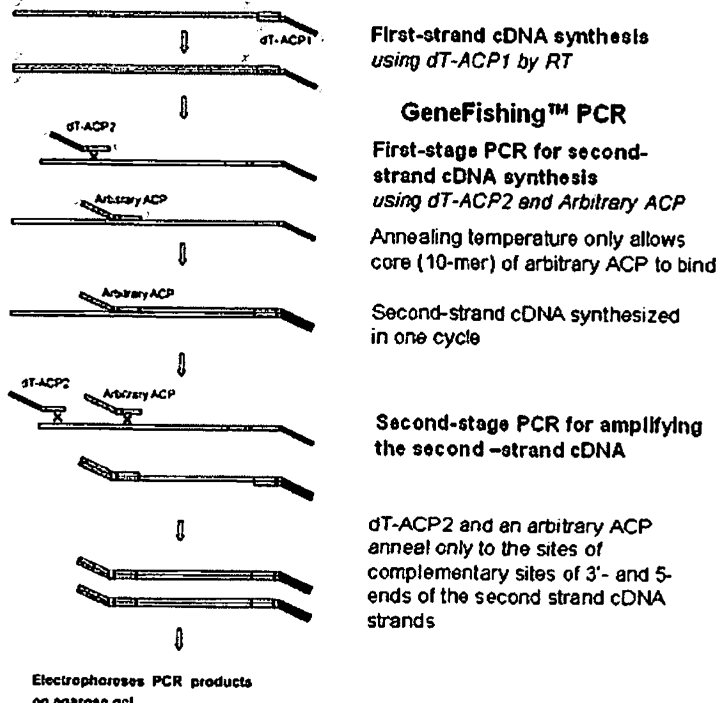

Figure 7. Schematic outlining the two-stage PCR required for the ACP technology used in the GeneFishing DEG kits.

display methods. The principle of ACP technology is based on the tripartite structure of a specific oligonucleotide primer (ACP) having 3'- and 5'- end distinct portions separated by a regulator and the interaction of each portion during two-stage PCR (Figure 6). The ACP system requires a two-stage PCR protocol. A schematic of the two-stage PCR and the corresponding description of each step can be seen in Figure 7. The resulting PCR products will be run on an agarose gel, and differentially expressed bands removed (i.e. Figures 3 & 5). Over 100 different arbitrary primers are available (Seegene) and will be used for analysis. Initial work done in the lab of the PD has demonstrated that for each 10 primers used, a minimum of 5 differentially expressed products will be identified. To compare treated and non-treated scallops, total RNA will be extracted from the adductor muscle and neuronal tissue as previously described (Chomczynski, 1993, Chomczynski, 1987). The RNA from each respective group will be pooled separately in equal concentrations. This pooled RNA will be used for reverse transcription and differential display PCR. PCR products will be cloned into TOPO TA pCR 2.1 (Invitrogen) and positive colonies grown for plasmid DNA. Templates will be prepared in a Rev Prep Orbit (GeneMachines) and the resulting cDNAs sequenced using a modified dideoxy chain termination method with Big Dye Terminator (Applied Biosystems). Sequencing reactions will be precipitated and pellets resuspended in Hi-Di Formamide with EDTA (Applied Biosystems) and analyzed using a 3730 Sequencer (Applied Biosystems). All sequences will be analyzed by NCBI Blast programs for similarity to known genes (Altschul, 1997). ClustalW (MacVector 7.2) analysis will be used for sequence pair-wise and multiple protein alignments. Once gene products have been identified through DNA sequencing, quantititative RT-PCR will be used to confirm differential expression across individual bay scallops.

Methods: Quantitative Real-Time RT-PCR

To examine expression of genes identified from differential display RNA from individual bay scallops will be analyzed quantitatively using real time RT-PCR (Brilliant SYBR Green QRT-PCR Master Mix Kit, 1-Step, Stratagene) in the Opticon Continuous Fluorescence Detection System (MJ Research). The PD has significant experience using this approach for quantifying gene expression in bay scallops (Kim H *et al.* 2004) (attached as preprint) as well as in different fish species (Roberts and Goetz 2003; Roberts *et al.* 2004). The specific methods are described in these studies and are based on the manufacturer's protocols (i.e. Stratagen). For all real time assays, melting curves will be analyzed to verify that no primer dimers were formed and that C_T values represented the desired amplicon. C_T values will be converted to relative RNA abundance levels based on their respective standard curves and will be normalized to the corresponding 18S RNA values.

Methods: Analysis, expected results and potential pitfalls

We expect that enough sequence will be generated using differential display and sequence to comparisons for RT-PCR analysis but recognize generating full-length cDNA clones is necessary to fully characterize gene function and expression. Three basic methods will be used to obtain full sequences; 1) alignments with known sequences generated by PD or others 2) Rapid Amplification of cDNA Ends (RACE) - BD Biosciences SMART technology and 3) screening cDNA libraries already constructed by the PD. The PD has extensive experience using both RACE (i.e Roberts et al. 2004a, Kim H et al. 2004) and screening cDNA libraries (i.e. Roberts and Goetz 2001, Roberts *et al.* 2000)

Based on our preliminary results (Figure 5) we do expect to detect differentially expressed genes in treated versus control scallops. We expect that treatment with the dietary supplement will contribute to an increase in muscle size and expect that genes involved in protein synthesis and possibly genes involved in hormonal control of growth.

One potential concern would be that treatment with the MyoZap compound will not positively effect growth in scallops. While this is a possibility, we will know this well before the start date of the current proposal and are currently investigating the use of other compounds (i.e. growth hormone). Regardless, our preliminary data suggests that we will have a better understanding of genes involved in muscle physiology. For example, cyclin T indentified as part of the preliminary differential gene expression work, has a putative role in muscle growth (Sano and Schneider 2003). To our knowledge this gene has not been cloned in any other bivalve mollusk. The PDs of the current proposal feel strongly that this artificial treatment experiment is an appropriate complement to using SAGE to analyze gene expression profiles in scallops selected for growth over multiple generations (see next section)

Specific Research Objective I.B: Identify gene expression profiles in scallops selected for rapid versus slow growth

We have chosen to exploit the availability of cultured lines of scallops that differ in growth rate for our study of differential gene expression, with the goal of identifying genes that may be associated with rapid or slow growth. At the National Marine Fisheries Laboratory, Milford, CT, our collaborator (Stiles, see letter page 28) has been for several years culturing lines of bay scallops than have been selected for rapid and slow growth, originating from mass spawning events. Currently, they have several lines available that have been selected for a single generation of rapid or slow growth. These lines were created by multiple mass spawnings using non size-selected broodstock from the Stonington, CT natural population. For each line, larvae were pooled from several days of mass spawnings, and each mass spawning used 25-100 broodstock in order to minimize inbreeding. Each broodstock scallop is used once for establishing a line. After progressing from sieves to upwellers, where scallops are fed a mixed algal diet, these animals were held in flow-through raceways in ambient, unfiltered seawater without additional dietary supplementation. Upon reaching reproductive age (1 year), scallops were sorted by size, and truncation selection was used to establish a size-selected line. The exact threshold for selection varied because of differences in the numbers of scallops available, but generally this is based on large and small individuals greater than 1.5 standard deviations from the mean size. This is consistent with size-selection trial by Crenshaw *et al* (1991). These selected scallops were mass-spawned, and larvae pooled to establish an F_1 generation. Three fast and slow growth lines are being maintained to ensure availability. Slow growth and rapid growth selected lines are otherwise maintained under identical hatchery conditions, and grown out using standard hatchery practices.

Serial analysis of gene expression (SAGE) will be the primary techniques used for comparing fast and slow lines of scallops cultured at the Milford Laboratory. The SAGE technique is an enormously powerful high-throughput, comprehensive, sequence-based approach for determining gene expression patterns for the entire transcriptome (Velculescu et al, 1995, 1997; Zhang et al., 1997; Yu et al., 1999). The SAGE analysis provides a unique strength to this proposal in that it will allow us to detect subtle changes in transcript abundance in rapid and slow growth-selected lines, with far greater resolution than RDA or even ESTs (Sun et al. 2004). The

power of SAGE is that it provides a qualitative and *quantitative* measure of gene expression by creating a catalog of gene expression. The basic approach relies on three principles: 1) a short oligonucleotide sequence (tag) isolated from a defined location within a transcript encodes sufficient complexity to identify an expressed gene, 2) serial and parallel sequence analysis of tags maximizes throughput, and 3) PCR-mediated amplification bias is minimized by deferring PCR until amplicons are equivalent in size and roughly equivalent in composition. The experimental design of SAGE facilitates acquisition of longer and full-length cDNAs (van den Berg et al., 1999; Chen et al., 2003). Creating a SAGE library is relatively costly, labor-intensive and limits the number of samples that can be analyze, however the power and efficiency of the method is that many SAGE tags can be read serially in the DNA sequence and that the ***frequency of the SAGE tags reflects transcript abundance in the starting material.***

Methods: Selected Scallop Lines:

In the event the proposal is funded, Stiles (collaborator) has agreed to establish an F_2 size-selected generation for both rapid and slow growth in the summer of 2004 produced using the same techniques as described above in the event this proposal is funded. These scallops will be maintained in the raceways at the Milford lab through the winter of 2004 / 2005. In the spring of 2005, we will take tissue samples of adults the F_2 generation for our differential expression analysis (see below). Stiles will also use the F_2 generation to establish F_3 size-selected lines (at least 2 fast and 2 slow growth lines), which we will sample as late juveniles (see below).

Methods: Long SAGE

We propose here to apply a modified SAGE method that produces relatively long 21 bp tags to profile gene expression between fast and slow growth selected scallop lines.
After experience constructing SAGE tags libraries from a variety of eukaryotic sources, the co-PD (Krause) along with a collaborator on the current proposal (J. Dunn – Brookhaven National Laboratory; see Collaborative Arrangements page 28) adopted a new Long SAGE protocol (Velculescu 2001; Saha et al. 2002) to increase tag length, avoid ligation bias and problems deliminating tags. As detailed below, the new method increases SAGE tag length from 14 bp to 21 bp, which increases the probability that a tag identifies a unique transcript in a genome from approximately 2.7×10^8 to 4.4×10^{12}! Krause and Dunn further modified the protocol to improve yield and efficiency, (see Dunn *et al.* 2002, and an application in Gnatenko *et al.* (2003)

We will synthesize and compare long SAGE libraries for four samples of scallops: rapid and slow growth selected lines sampled as approximately one-year old adults in the F_2 generation (survived overwintering, are initiating gametogenesis, but not yet spawned), and as late juveniles (approximately 4 months old) that have not yet initiated gametogenesis from the F_3 generation. We have chosen to focus on expression contrasts in **adductor muscle**, because ***this is the final commercial product***, but also because ***muscle mass is strongly correlated with overall shell size*** (Thompson and MacDonald 1991), and the ***adductor muscle is an important site lipid and carbohydrate energy storage and utilization*** *(Epp et al. 1988, Bricelj and Krause 1992, Bricelj and Shumway 1991, Ibarra et al. 1999a).* To minimize the effects of individual variability, we will pool tissues from 15 individuals per line. Adductor muscle (smooth and striated) will be dissected and stored in RNA Later (Ambion). We will also dissect and archive samples of neuronal, digestive, gonadal, and gill / labial palp tissue in RNA later (Ambion). RNA will be isolated from adductor muscle using oligo d(T) coated magnetic beads and the Dynal mRNA Direct kit. Following isolation of mRNA on the magnetic beads, first strand cDNA is

synthesized directly on the beads using the cDNA synthesis system with Superscript II or III reverse transcriptase (Invitrogen). At this point, the cDNA is bound to strepavidin beads, and exists as a stable, immobilized library that can be stored at 4°C. cDNA fragments (21 bases each, referred to as a SAGE tag) are isolated from the 3' end from each transcript present in a cell population. cDNA bound to strepavidin beads will be digested with a 4 bp recognizing "anchoring enzyme", *Nla*III, such that a positionally defined fragment of the 3' end of each cDNA is retained on magnetic beads. To this fragment an adaptor is ligated which includes a recognition size for a "tagging enzyme", a type IIS restriction endonuclease, in this case *Mme*I. This step generates a target for a restriction enzyme to cut 17 base pairs *past* the recognition sequence and within the cDNA of interest, generating an isolated 21 bp cDNA "tag". The tags are then isolated, ligated together to form concatemers, and the concatemers (100 to several thousand bp in length) are cloned into a BNL-modified pZero vector and sequenced using standard protocols on an ABI 3100 Analyzer. Each tag in the concatemer is separated by 4 bp of punctuation sequence, which allows identification of the constituent tags. Coverage and sensitivity of SAGE is dependent on the number of tags sequences and their length.

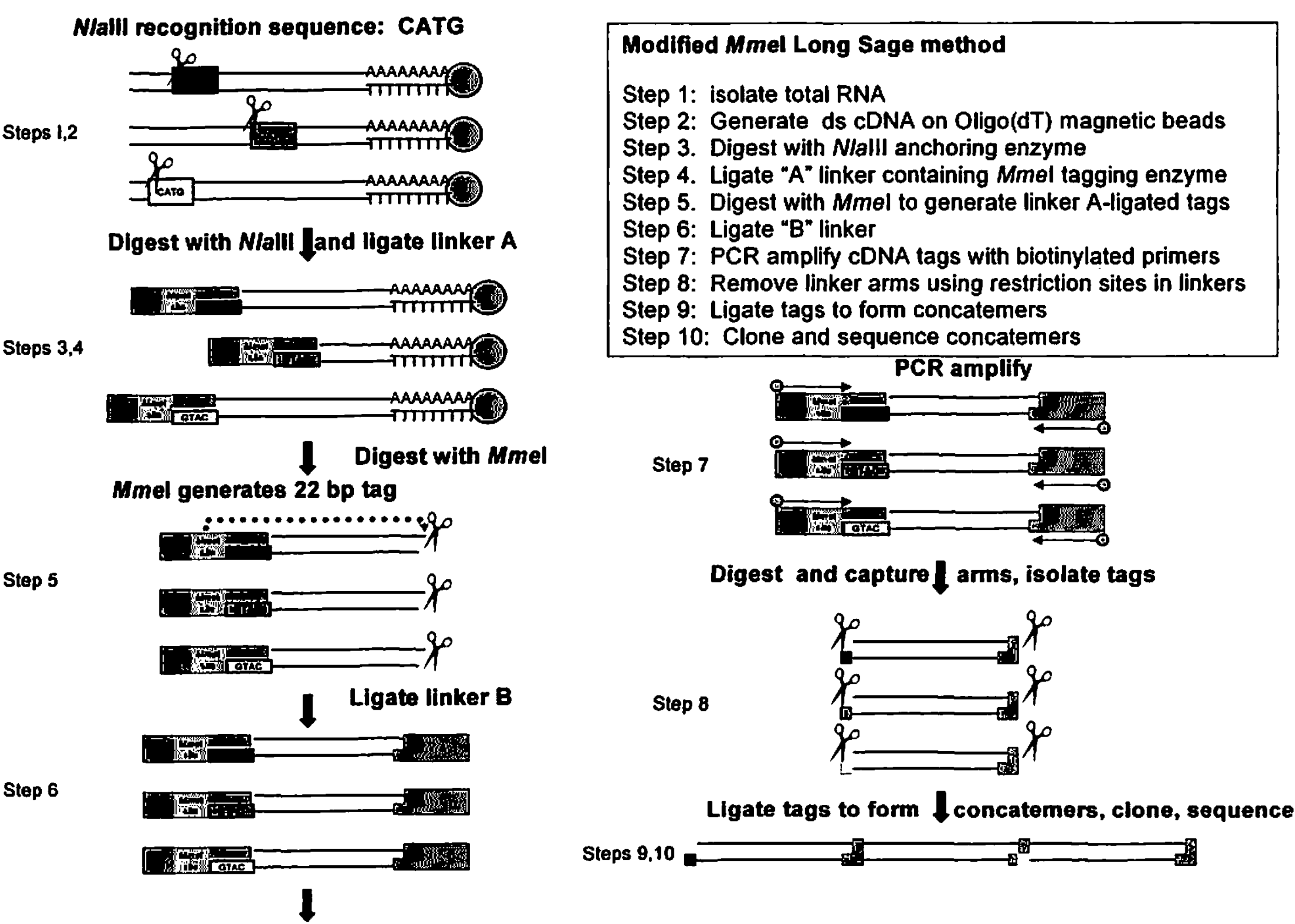

Figure 8. Schematic of Long SAGE technique to be used by Krause (co-PD) and Dunn (collaborator)

On the ABI 3100, 24 runs / day on 36 cm capillaries generates > 7500 tags. Krause will construct SAGE libraries at Hofstra, and assess their quality and concatemer length at BNL. Once high-quality SAGE libraries are verified, approximately 50,000 tags will be sequenced from each of the tissue pools of interest at the MBL by Roberts (PD). In-house (Brookhaven National Laboratory) bioinformatics programs extract the tag sequences and records them in appropriate databases, and can compare frequencies between libraries, here between rapid and slow growing lines as juveniles and reproductive adults.

Methods: Analysis, expected results and potential pitfalls

SAGE is a labor-intensive and technically difficult technique, but its potential is tremendous. Since Krause (co-PD) and Dunn (collaborator) have already developed SAGE libraries for a variety of eukaryotes, we are confident that the construction will be successful. Because we do not have a good estimate of genome size, we cannot estimate the coverage of the complete *A. irradians* transcriptome by our method, but by sampling a large number of tags (50,000 per library) are confident that we have suitable coverage for quantitative and qualitative comparisons. One obvious issue arises with the use of SAGE is the short (21 bp) tags are generated, that are often hard to identify. As part of currently USDA funded research, Roberts (PD) has generated over 2000 ESTs from 4 bay scallop cDNA libraries including developmental stages (see Introduction pg 3) and adductor muscle tissue (Roberts and Goetz 2003). These sequences will provide an easy source of information to identify these tags. If this does not work more ESTs could easily be generated by the PD. In addition, the SAGE technique being used by Krause and Dunn inherently lends itself as a technology for generating full-length clones. This SAGE technique isolates a positionally defined tag at 3' most *Nla*III restriction endonuclease restriction site, and because tags are generated from *Nla*III digests of oligo-dT bound cDNA, all that is required to generate a the 3' end of the transcript is to amplify that cDNA with the single SAGE primer in a linear fashion for approximately 10 rounds of PCR, and then use both the oligo dT and SAGE primer in combination for exponential amplification (see Dunn *et al.* 2002). Once these longer transcripts are isolated, differential expression in growth-selected lines can be analyzed by quantitative RT-PCR as described above (pg 10).

We expect this project will identify a gene or genes directly associated with growth rate differences in cultured bay scallops. Even if the gene products do not appear to be directly linked to growth differences, they will assist in genome mapping. SAGE sequence libraries can be exploited for further characterization of *Argopecten* transcriptomes and will be an invaluable resource. The possible extensions of our analyses of differential gene expression are tremendous. *The relationship between changes in key environmental factors (nutrients, temperature, stressors, and disease) and expression of transcripts that appear to be associated with growth should help us understand how natural genetic variation and its expression can be exploited for aquaculture to a far greater extent than possible before.*

Specific Research Objective II.A: Measure feed conversion efficiency for individual scallops from rapid and slow growth lines

The two most important parameters defining the relationship between a livestock animal and its feed are growth and feed conversion (Church, 1991). Feed conversion can be expressed as feed-conversion rate (FCR), the mass of feed required to produce one unit of mass in growth, or as conversion efficiency (CE), the percentage of feed mass that is incorporated in animal

mass. Both terms are in dry-weight units. Conversion efficiency (CE) will be used in this work because it is more intuitive to think of a large number being "better," in terms of selecting for higher-producing lines.

Two scallops, living side-by-side in the same environment, often will grow at different rates. The reasons for differences in growth under identical conditions can be 1) **higher rate of feed consumption 2) more rapid filtration of phytoplankton or 2) more effective use of phytoplankton ingested to produce growth.** All of these will contribute to higher CE. Selection for faster growth attributable to higher feed consumption has the potential to shorten time to market, but does not necessarily increase the carrying capacity of the culture system, either land-based or in natural waters. Selection for faster growth attributable to higher CE would be preferred because lines thus selected will improve both time-to-market and carrying capacity of the system. Previous livestock-improvement programs for bivalve mollusks have focused mainly on growth without consideration of the physiological basis for this selection. Our study will be unique in considering both growth and conversion efficiency in individual bay scallops. A second unique feature of our study will be that scallops will be reared under conditions of optimized ration and regime so that food limitation will not depress an individual's capacity to express superior growth of CE characteristics.

Methods: Measuring Feed Conversion Effieciency

Twenty scallops from each line and 10 wild scallops will be grown in automated molluscan rearing chambers (Smith and Wikfors 1998) on a dietary ration and regime that optimizes growth rate. Chambers will be programmed to feed each population 5% of scallop live weight in dry weight of *Tetraselmis spp.* per day, as 16 small aliquots delivered every 90 minutes (Wikfors *et al.* 1996). Algae will be cultured aseptically in semi-continuous carboy assemblies (Ukeles, 1973). Weekly, scallops will be removed from the chambers, their live weight will be measured, and feeding will be increased according to new live-weight values. Feeding will proceed for approximately 4-8 weeks, depending upon growth rates and variance, so that any differences in growth rate between lines will be apparent statistically. At the end of the feeding experiment, clearance rate of each individual scallop will be determined, using the experimental *Tetraselmis* diet, with standard methods (Hildreth and Crisp, 1976). Finally, dry weight of each scallop (soft tissue) will be determined gravimetrically to calculate conversion efficiency of each scallop, based upon growth and clearance-rate data. Tissue samples from all individuals will be taken and stored in RNAlater (Ambion) for molecular analysis.

Specific Research Objective II.B: Identify genes differentially expressed in digestive tissues associated with increased feed efficiency

As mentioned above, the reasons for differences in growth under identical conditions can be a higher rate of feed consumption, more rapid filtration of phytoplankton, or more effective use of phytoplankton ingested to produce growth, including particle selection. The research associated with this specific research objective will complement the previous experiment by contributing to our understanding of the genes responsible for these important aspects of CE. Tissues that will be taken for molecular analyses will be gills, labial palp, and digestive tissues. Small tissue samples of equal mass will be taken to complement the CE measurements. The digestive tissues; stomach, gland, partial intestine and crystalline style sac will be taken as a single samples as they are embedded in one another. The reason that gills, labial palp and

digestive tissues will be taken separately is so that genes having specific biological role in higher filtration (gill and labial palp) and effective use (digestive tissues) can be easily identified.

Methods: Isolation of Differentially Expressed Genes (DEGs)
Quantitative Real-Time RT-PCR

The specific methods used for the identification of differentially expressed genes will be the same as described for analysis of muscle and neuronal tissue for Research Objective I.1 (page 9 & 10) Total RNA will be extracted from gills, labial palp, and digestive tissues from all individuals examined for CE (N=30). Total RNA from 6 individuals with the highest CE (overall) and from the 6 individuals with the lowest CE (overall) will be pooled separately in equal concentrations to be used for initial GeneFishing DEG analysis (Seegene). PCR products identified will be sequenced and differential expression will be verified using quantitative RT-PCR. Full-length sequences of selected genes that appear to have a biological role in reed conversion efficiency will be obtain with a bioinformatics approach, cDNA library screening or Rapid Amplification of cDNA ends (RACE).

Methods: Analysis, expected results and potential pitfalls

For the experiments carried out in the automated molluscan rearing chambers, we expect to observe a measurable difference in CE across all individuals. The data from the rearing chamber will provide information on the relationship between CE and the genotype of growth selected lines. Wikfors will be overseeing these experiments and has extensive experience carrying out similar feeding experiments, therefore we do not foresee any problems in experimental design. Data generated from the rearing chamber experiments will help us understand whether bigger scallops are eating more, more efficiently or combination of both.

The molecular analysis of genes associated with increased feed efficiency will primarily provide information on what genes play a role in the different aspects of CE for all bivalves. This information could eventually be used for to assess CE for other bivalves (adults or larvae) or used as markers for selection. While not expected, the identification of genes involved CE, could elucidate different genetic mechanisms by which increased CE is obtain in rapid- versus slow-growth selected individuals.

Specific Research Objective II.C: Compare over-wintering mortality rates in rapid and slow growing scallop lines.

Bivalve life history and culture techniques used in aquaculture makes the industry prone to high mortality rates. For the bay scallop one of the primary causes of this is what is known as overwintering mortality. During winter, little to zero growth occurs and a significant number of scallops will die (Gutsell 1930, Sastry 1968, Oesterling 1998, Wikfors *et al.* 1998). While the actual number of individuals that are lost during the winter might not be as great as in early development, the financial loss is far greater as a considerable investment (facilities, equipment, time) has been put into each scallop by that point. The exact reasons for this phenomenon remains unknown, however, there is anecdotal evidence that suggests that first season larval/juvenile growth and size of late juveniles plays a role. Mortality is probably more common among individuals with small body mass since fewer resources are available for defense against disease and environmental conditions. In order to understand the relationship of grow rate and susceptibility to winter mortality, the rapid and slow growing scallop lines generates at Milford (along side wild scallop populations will be overwintered in cages used by the bivalve industry.

Methods: Overwintering Survival Trial.

During the winter of Year 1 of the proposed research, scallops that have been selected for fast and slow growth (F3) will be evaluated in commercial, rigid mesh cages. The cages are similar to one used by industry and are commonly used by Stiles (collaborator) to asses survival of scallops that have been selected for shell phenotype. The cages are made of plastic-coated wire with a 7.5 cm mesh. Each cage measures 56 x 56 x 94 cm and is divided horizontally into three sections or tiers. Two ballast areas below the bottom tier proved an offset from the sea floor approximately 15 cm. Cage inserts of smaller mesh (15 mm) measuring 41 x 10 x 81 cm will be used to hold the scallops. Fifty individuals from the line selected for rapid growth in the beginning of Year 1 (F3) and fifty individuals selected for slow growth, will be randomly chosen to go into 1 of 4 cages to be place out in local waters. Scallops will be individually measured (shell height, width, and depth). The siblings for each line (>100 individuals) will remain in the land based tank facility at the Milford Laboratory with measurements taken corresponding to measurements of caged scallops. The cages will be deployed in late October and recovered in March (start of year two). Percent survival will be assessed and over-winter growth rates will be determined.

Methods: Analysis, expected results and potential pitfalls

While we recognize that this component of the proposed research will not likely provide as much significant genetic information, we feel that it is important in interpreting the sequence information generated from DEG and SAGE analysis. In addition, minimal additional effort will be required to carry out this trial as similar studies are ongoing at the Milford Laboratory. We would expect that growth will be minimal for both the rapid and slow-selected lines during winter months due to decreased metabolism. We would also expect that the larger scallops (rapid growth line) would enter winter with more energetic reserves (based on size) and have a greater likelihood to survive.

In order to guard against this possibility of 100% mortality and increase chances of identify differences in survival, a subset (N=50) of animals from each selected line (F3) will be maintained by Roberts at the Marine Biological Laboratory's Marine Resource Center (see Roberts' Facilities and Equipment Statement) in similar cages, in indoor tanks with flowing ambient water. This will allow easy overwintering monitoring. If marked differences in survival are observed, tissue samples will be taken from all survivors and stored in RNALater. If time permits at the end of year three, samples will be compared by either DEG (Roberts) or a modified representational difference analysis (Krause) for differential gene expression in order to better understand the role of specific gene sequences in survival and examine any functional correlation with selection.

Project Description: Timeline

Prior to the start date of the current proposal, the PD will be involved in experiments designed to provide tissue samples for DEG analysis (Research Objective I.1) as described. At the start of Year 1, Stiles will have bay scallops selected for rapid and slow growth (F2) and will spawn these lines early in year 1 to produced a F3 generation. The feed conversion efficiency experiments will be carried out during Year 1, with the majority of the molecular work performed during Year 2. By the end of Year 1, Krause and Dunn will begin to construct SAGE libraries from adductor muscle samples taken from rapid and slow growing selected lines. Libraries will be constructed with tissue from adult (1+ yr) and late juvenile (4 m) bay scallops. The majority of Year 2 will be spent sequencing products obtained for DEG and SAGE analysis. The final year will be spent primarily isolating full-length transcripts and correlating gene expression with growth and feed conversion efficiency (quantitative RT-PCR). Any remaining time will be used for *in situ* and temporal gene characterization. However it should be noted that this will only be done if preceding tasks are completed. Below is a chart outlining the tasks to be performed.

Year 1 Year 2 Year 3

M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D J F

Research Goal I

1. Analyze genes differentially expressed in muscle tissue from scallop treated with growth promoting compounds

2. Identify gene expression profiles in scallops selected for rapid versus slow growth

DEG Analysis (Roberts)
qRT-PCR verification (Roberts)
DNA Sequencing (Roberts)
Scallop Culture / Spawn (Stiles)
SAGE Library Construction (Krause & Dunn)
(Krause & Dunn)
Isolation of full-length transcripts (Roberts & Krause)

Research Goal II

1. Measure feed conversion efficiency for individual scallops from rapid and slow growth lines

2. Identify genes differentially expressed in digestive tissues associated with increased feed efficiency

3. Compare overwintering mortality rates in rapid and slow growing scallop lines

FCE Trials (Wikfors)
DEG Analysis (Roberts)
qRT-PCR verification (Roberts)
In situ and temporal characterization (Roberts & Krause)
Cage Set (Stiles)
Data analysis (Roberts & Stiles)

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Fisher 550 ultrasonic tissue processor
Various horizontal gel systems for RNA/DNA gels
TL-2000 translinker
Gel drying system (Savant SGD4050)
Thelco high performance above ambient incubators for bacteria and hybridization
Balances - top loading balances (O-Haus); semi-analytical balance -Mettler AC-100
microcentrifuges
So-Low ultralow freezer (U85-22)
2 - 45.0 ft^3 sliding glass door refrigerators
XCell Surelock Mini-Cell Protein Electrophoresis System
2 dissecting microscopes, 1 compound microscopes

Shared Facilities: There is a core microscopy facility at MBL (http://www.mbl.edu/inside/what/services/serv_micro.html) containing various microscopes and histological processing equipment. There is a genome facility in the *Josephine Bay Paul Center* at MBL that has a GeneMachines Rev Prep Orbit, ABI 3730 sequencer, automated arrayer and reader and ancillary robotics.

Facilities and Equipment – Maureen Krause – Co-PD
Hofstra University

The co-PD's molecular biology laboratory is equipped with one Eppendorf 96-well Mastercycler, a _80EC freezer, two -20 freezers, refrigerators, several water baths, microfuges, and instruments for PAGE and agarose gel electrophoresis. She has shared access to an electroporator, photodocumentation equipment and software, refrigerated microfuges, low speed table-top centrifuges, refrigerated high speed and ultra-centrifuges, UV and visible light spectrophotometers, hoods, incubators and shakers, well as autoclaves and four dedicated bioinformatics computer workstations.

Facilities and Equipment – John Dunn - Collaborator
Brookhaven National Laboratory

Brookhaven National Laboratory (BNL) is operated under contract to the U.S. Department of Energy by Brookhaven Science Associates, a consortium between The State University of NY at Stony Brook and Battelle Memorial Corporation, Columbus, OH. Co-PI Dunn has a fully equipped laboratory in the Biology Department for recombinant DNA studies, creation and characterization of SAGE libraries, *in vitro* transcription and cloning of cDNAs, and expression of recombinant proteins. A laminar flow_hood equipped with UV sterilization, two Eppendorf gradient 96_well PCR machines, _80EC freezer, high and low speed centrifuges, electroporator, instruments for gel electrophoresis, photodocumentation system, and spectrophotometers are available. Co-PI Dunn oversees the Biology Department's genome sequencing center with two ABI 373's and two 377 flourescent DNA sequencers with 96_lane upgrades, and an ABI 3100 Capillary Analyzer, Real-time PCR capabilities, and, most recently, a Pyrosequencer. In house bioinformatics expertise is also available.

Facilities and Equipment – Northeast Fisheries Center, Milford Laboratory
NOAA, National Marine Fisheries Service, Milford, CT
Sheila Stiles – Collaborator
Gary Wikfors - Collaborator

Laboratory: The original facility, a one-room wooden building supported on pilings, was replaced in 1940 by a brick building having about 4800 square feet of floor space. This structure is still in use. In addition, a three-story laboratory-office building containing more than 28,000 square feet of floor space was completed in 1966. The two buildings house 30 laboratories and offices. The laboratory is well-equipped for field studies and for collecting the animals necessary for laboratory tests.

Animal Holding Facilities: A seawater system pumps water from Milford Harbor into the laboratory where it is heated, cooled, or filtered, as needed. This temperature-controlled water is supplied to 13 wet labs. Forty-five outdoor raceway tanks are available for holding marine animals. Seventeen raceway tanks and nine circular tanks are enclosed in a heated building to allow year-round use. Recently a 960 square foot greenhouse was completed to allow mass production of algae needed for the aquaculture of scallops. In addition, a facility was established for the culture of bay scallops using recirculating seawater systems, taking advantage of the large quantities of algae grown in the greenhouse.

Microalgae: Several laboratories are devoted to the culture of microalgae. A highly regarded collection of about 200 pure cultures of different species or strains of algae is maintained; these cultures have been provided to laboratories throughout the world. Certain types of algae are produced in large quantities to provide food for the shellfish maintained in the laboratory. The staff tests various types of algae and schedules of feeding to determine the best conditions for each type of shellfish.

Equipment: The laboratory is equipped for a variety of experimental work. Transmission and scanning electron microscopes are available for ultrastructural work, as are several high-resolution phase-contrast microscopes and the necessary preparatory equipment . Equipment for chemical and biochemical analyses is available, including a dual-beam spectrophotometer, an atomic absorption spectrophotometer, a spectrofluorometer, electrophoresis equipment, flow-cytometer and a high pressure liquid chromatograph.

Field Research: The 49-foot research vessel, *R/V Victor Loosanoff*, is equipped with many types of hydrographic and biological sampling gear. Small boats are available when the larger boat is too large for the job. SCUBA divers can perform field experiments and specialized sampling. Milford scientists participate in offshore fisheries cruises along the Atlantic Coast on NOAA's larger research vessels.

COLLABORATIVE ARRANGEMENTS

There will be two project directors on this proposal; Steven Beyer Roberts (PD), and Maureen Krause (Co-PD). There will be three Collaborators on this proposal; Sheila Stiles, Gary Wikfors and John Dunn. Letters from the CoPD and Collaborators follow. The specific responsibilities of each participant are:

Steven Beyer Roberts (PD): The PD will oversee all of the aspects of the project and will be specifically responsible for treating bay scallops with growth promoters, differential display, quantitative RT-PCR, DNA sequencing. and obtaining full-length cDNA clones.

Maureen Krause (Co-PD): Will be responsible for SAGE analysis, DNA sequencing, and generating full-length cDNA clones

Sheila Stiles (Collaborator): Will be responsible culturing rapid and slow growing selected lines of bay scallops over 3 generations. Her participation will be primarily in year 1 of the proposed research.

Gary Wikfors (Collaborator): Will be responsible for overseeing the feed conversion efficiency trial for bay scallops in customized automated molluscan rearing chambers during year 1.

John Dunn (Collaborator): Will collaborate with Krause in the application of Long SAGE technology to analyze gene expression in rapid and slow growing selected lines of bay scallops.

UNITED STATES DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
NATIONAL MARINE FISHERIES SERVICE
Northeast Fisheries Science Center
Milford Laboratory
212 Rogers Avenue
Milford, CT 06460-6499

June 8, 2004

Dr. Steven Beyer Roberts
Marine Biological Laboratory
7 MBL Street
Woods Hole, MA 02536

Dear Steven,

This letter is sent to inform you of my interest and willingness to participate as a collaborator in the study entitled "*Functional genomic analyses of production-related traits in cultured bivalves*" being submitted to the USDA-NRI Functional Genomics program. As a research geneticist in the Biotechnology Branch at the United States Department of Commerce, National Marine Fisheries Service, Milford Aquaculture Laboratory, my research experience and expertise include the areas of genetics, breeding, cytogenetics, cytology and aquaculture of shellfish. The Milford Laboratory houses facilities for conditioning, spawning and maintaining adult broodstock, a hatchery for culturing larvae, a nursery for post-set, and a tank system for grow-out and maintenance of juvenile scallops.

My major role in this particular project is to provide bay scallops from genetic lines developed through selective breeding primarily for fast and slow growth. While I have not requested funds for my professional services and time, I do understand that you have requested funds from the USDA during year one to cover materials and supplies required to maintain the lines of bay scallops that have been developed at our facility. In addition, I understand that funds are requested to provide compensation for a technician to assist in scallop culture for a portion of year one.

I greatly look forward to being involved in this very important and worthwhile project.

Sincerely,

Sheila Stiles

Sheila Stiles. Ph.D.
Research Geneticist

UNITED STATES DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Northeast Fisheries Science Center
166 Water Street
Woods Hole, MA 02543-1026

June 14, 2004

Dr. Steven Roberts, Ph.D.
Principal Investigator
Marine Biological Laboratory
7 MBL Street
Woods Hole, MA 02543

Dear Dr. Roberts:

This letter is to confirm my enthusiastic willingness to participate in your proposal to USDA, "Functional analysis of gene expression related to important production traits in marine shellfish," as an unfunded collaborator.

The specific objectives of this research focus on bay scallop and include 1) examining gene expression profiles of scallops that have been selected for fast and slow growth over multiple generations, 2) identifying genes associated with improved feed conversion efficiency, and 3) characterizing genes associated with increased over-wintering survival. Once factors associated with these important production traits are identified, they could be used in a selected breeding process. As described in the proposal, I will conduct feeding trials with genetic lines of bay scallops to compare their growth and conversion efficiency on optimized dietary rations and regimes. These feeding trials will be conducted in our unique, computer-controlled, molluscan rearing chambers using microalgal cultures from our bacteria-free carboy-culture facility. We will make our facility available to your technician, who will conduct clearance-rate measurements with individual scallops from these feeding trials. We will participate as unfunded collaborators because we have identified determination of improved production characteristics in domesticated bay scallops as a priority research topic in our program and view your grant as an enhancement to our on-going research.

I very much look forward to working with you and the rest of the impressive team you have organized for this important and timely research.

Sincerely,

~~Frank Almeida, Deputy Center Director, NEFSC~~

Gary H. Wikfors, Ph.D., Research Microbiologist

Biology Department

Bldg. 463, 50 Bell Avenue
Upton, NY 11973
Telephone: 631-344-3012
Facsimile: 631-344-3407
E-Mail: jdunn@bnl.gov

June 10, 2004

Dr. Maureen K. Krause
Biology Department
114 Hofstra University
Hempstead NY 11549

Dear Maureen,

This letter is to confirm my enthusiastic willingness to collaborate with you on your studies aimed at "Functional genomic analyses of production-related traits in cultured bivalves." My laboratory would be particularly interested in helping you use our Long SAGE technology to help you examine gene expression profiles of scallops that have been selected for fast and slow growth over multiple generations, identifying genes associated with improved feed conversion efficiency and in characterization of genes associated with increased over-wintering survival, as outlined in your proposal. Our interest in SAGE-based expression analysis is a long-standing one and your studies would complement several ongoing projects in my laboratory. I would be particularly interested working with you to develop an integrated system which serially combines SAGE analysis with techniques of Representational Difference Analysis (RDA) and Suppressive Subtractive Hybridization (SSH) to obtain stage specific SAGE tags and associated DNA fragment representations from genes that influence production related traits in your cultured bivalves.

For this work we would be ready to provide you with all the needed reagents (vectors, enzymes, oligonucleotide cassettes, primers, etc.) for constructing cDNA and SAGE libraries and also assist you in their initial characterization at the nucleotide sequence level using our ABI capillary and slab gel instruments.

I look forward to working with you and the other members of the team you have assembled to work on this project.

Sincerely,

John J. Dunn, Ph. D
Sr. Scientist

CURRICULUM VITAE – STEVEN BEYER ROBERTS – Project Director

Academic Experience	B.S. – North Carolina State University (Raleigh, NC) – 1997 Natural Resources - Concentration in Marine and Coastal Resources Minor in Zoology - Honors Program - Magna Cum Laude
	Ph.D. – University of Notre Dame (Notre Dame, IN) – 2002 Integrative Cell and Molecular Physiology *"Characterization of Growth Hormone in Yellow Perch and Myostatin in Several Teleost Species"* Ph.D. Advisor: Dr. Frederick Goetz
Professional Experience	2003-Present • Staff Scientist II Program in Scientific Aquaculture Marine Biological Laboratory, Woods Hole, MA
	2002-2003 • Postdoctoral Scientist Marine Biological Laboratory, Woods Hole, MA
	2000-2002 • Research Assistant and Graduate Student University of Notre Dame
	1998-2000 • Teaching Assistant and Graduate Student General Biology Laboratories - University of Notre Dame
	1997-1998 • Research Assistant – Center for Marine Science Research University of North Carolina at Wilmington
	1994-1997 • Lab / Aquaculture Technician – Zoology Department North Carolina State University
	1994-1995 • Field Technician – NOAA / EPA Environmental Monitoring and Assessment Program of Estuaries University of North Carolina at Wilmington
Professional Societies	American Fisheries Society World Aquaculture Society National Shellfish Association Sigma Xi Scientific Research Society

Refereed Publications (past 4 years)

Roberts SB, Langenau DM, Goetz FW. (2000) Isolation through cloning of fish prostaglandin endoperoxide synthase (cyclooxygenase) *in* Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish; B Norberg, OS Kjesbu, GL Taranger, E Andersson, and SO Stefansson, editors. Bergen, Norway. July 4-9, 1999. p 197.

Moser ML, Roberts SB. (2000) Effects of nonindigenous ictalurids and recreational electrofishing on the ictalurid community of the Cape Fear River drainage, North Carolina. *in* Catfish 2000: Proceedings of the International Ictalurid Symposium; ER Irwin, WA Hubert, CF Rabeni, HL Schramm, Jr., and T Coon, editors. Davenport, IA. June 23-25, 1998. pp 479-485.

Roberts SB, Langenau DM, Goetz FW. (2000) Cloning and characterization of prostaglandin endoperoxide synthase-1 and -2 from the brook trout ovary. Mol Cell Endocrinol.160(1-2):89-97.

Roberts SB, Goetz FW. (2001) Differential skeletal muscle expression of myostatin across teleost species, and the isolation of multiple myostatin isoforms. FEBS Lett. Vol 491, No. 3, pp. 212-216.

Roberts SB, Goetz FW. (2003) Myostatin protein and mRNA transcript levels in adult and developing brook trout. Mol Cell Endocrinol. 210 (1-2): 9-20.

Roberts SB, Goetz FW. (2003) Expressed sequence tag analysis of genes expressed in the bay scallop, *Argopecten irradians*. Biol Bull. 205: 227-228.

Roberts SB, Barry T, Malison J, Goetz FW. (2004) Production of a recombinantly-derived growth hormone antibody and the characterization of growth hormone levels in yellow perch. Aquaculture. Vol. 232/1-4: 591-602

Hollis DM, Goetz FW, Roberts SB, Boyd SK. (2004) Acute neurosteroid modulation and subunit isolation of the GABAa receptor in the bullfrog, *Rana catesbeiana*. Journal of Molecular Endocrinology. Vol. 32, 921–934

Biga PR, Cain KD, Hardy RW, Schelling GT, Overturf K, Roberts SB, Goetz FW, Ott TL. (2004) Growth hormone differentially regulates muscle myostatin1 and -2 and increases circulating cortisol in rainbow trout (*Oncorhynchus mykiss*). General and Comparative Endocrinology. *In press*

Kim H-W, Mykles DL, Goetz FW, Roberts SB. (2004) Characterization of a myostatin-like gene from the bay scallop, *Argopecten irradians*" Biochimica et Biophysica Acta – Gene Structure and Expression. *In press*

Biga PR, Roberts SB, Iliev DB, McCauley LAR, Goetz FW. (2004) The isolation, characterization, and expression profile of a novel GDF11 gene in zebrafish. Gene. *In review*

Roberts SB, McCauley LAR, Devlin RH, Goetz FW. (2004) Transgenic salmon over-expressing growth hormone exhibit decreased myostatin transcript and protein expression. Journal of Experimental Biology. *In final revision*

Selected Non-refereed Publications

Roberts SB, Goetz FW. (2003) Genes involved with growth and development in the bay scallop (Extended Abstract) Proceedings of the 14th International Pectinid Workshop, April 23-29, St. Petersburg, FL, USA. Pg 137

Mebane B, Roberts SB, Lindell S, Goetz FW. (2003) Researchers develop low-tech recirculating culture system for quahog clams. Global Aquaculture Advocate. 6: 35-36

CURRICULUM VITAE – MAUREEN KAY KRAUSE – Co-Project Director

Academic Experience

B.S. – University Of North Carolina At Wilmington (Wilmington, N.C.) - 1985
Marine Biology
summa cum laude with Honors in Biology

Ph.D. – State University of New York at Stony Brook (Stony Brook, N.Y.) - 1992
Ecology and Evolution
Phenotypic expression of glucose-6-phosphate isomerase genotype in the bay scallop, *Argopecten irradians*, and the blue mussel, *Mytilus edulis*.
Ph.D. Advisor: Dr. Richard K. Koehn

Professional Experience

2001-Present • Assistant Professor of Biology
Department of Biology
Hofstra University, Hempstead, New York.

2000 – present • Guest Scientist, Genomics Group,
Biology Department
Brookhaven National Laboratory, Upton, New York.

2000 - 2001 • Adjunct Assistant Professor of Biology and Research Scientist
Department of Biology
Hofstra University, Hempstead, New York.

1999 – 2000 • Assistant Professor of Biology
Department of Biological Sciences
Mary Washington College, Fredericksburg, Virginia.

1994 – 1999• Assistant Professor of Biology and Marine Science
Southampton College of Long Island University, Southampton, New York.

1995 – 1997 • Consultant, New York, Bay Scallop Restoration project
Cornell Cooperative Extension, Riverhead, New York

1992 - 1994 • Nat'l Inst. of Environmental Health Sciences Postdoctoral Research Fellow
Integrated Toxicology Program, Duke University Marine Laboratory, Beaufort, North Carolina.

1985 - 1992 • Research and Teaching Assistant
State University of New York at Stony Brook, Stony Brook, New York

1987 • Consultant
Applied Biomathematics, Incorporated, Setauket, New York.

1986 - 1988. New York State Sea Grant Scholar
State University of New York at Stony Brook, Stony Brook, New York

Professional Societies

National Shellfisheries Association
Sigma Xi Scientific Research Society
Genetics Society of America
Phycological Society of America
Society for Molecular Biology and Evolution
Society for the Study of Evolution

Additional Professional Activities and Honors	Panel Member, National Science Foundation Population Biology Program, 1997, 1999, 2003. Hofstra University team for FIRST II: National Science Foundation's Faculty Institutes for Reforming Science Teaching (ongoing). Ad Hoc Grant Proposal Review: National Science Foundation, National Sea Grant, N.Y. and N.J. Sea Grant Nominee, David Newton Teaching Award, Southampton College, 1998, 1999.

Grants and Awards (Past 10 years)

2003 Co-PI, National Science Foundation
Course Curriculum and Laboratory Improvement Program Grant, "Combining Successful Models to Improve Learning in Biology Core Courses", $199,941. Dr. Beverly Clendening, PI, Dr. Peter Daniel and Dr. Robert Seagull, Co-PIs.

2002, 2003 Presidential Research Awards, Hofstra University.

2002, 2003 Faculty Research and Development Grants,
Hofstra College of Liberal Arts and Sciences,.

2000 Faculty Professional Activity Grant, Mary Washington College, $4000 (declined due to move).

2000 Mary Washington College Jesse Ball Dupont Summer Science Research Award, Mary Washington College (Competitive award supports the research training of two undergraduates by providing monies for research materials, stipends, student housing and summer support for faculty mentors)

1995-1999 Southampton College Research Release Time Award

1997 National Science Foundation Research Grant, Division of Environmental Biology, Program in Systematics and Population Biology / Research in Undergraduate Institutions, for "Molecular evolution of the glucose phosphate isomerase (*Gpi*) locus in bay scallops, *Argopecten irradians.*" (J.H. McDonald, Co-PI).

Refereed Publications

Dunn, J.J., S.R. McCorkle, L.A. Praissman, G. Hind, D van der Lelie, W.F. Bahou, D. V. Gnatenko, and M.K. Krause, 2002. Genomic Signature Tags (GSTs): A System for Profiling Genomic DNA. Genome Research. 12: 1756-1765.

Van Beneden, R.J, M.K. Krause, L.D. Rhodes and H.S. Gardner. 1998. Molecular analysis of medaka tumors: New models for carcinogenicity testing. *Proceedings of Research Review: US Army Biomedical Research and Development Laboratory.*

Krause, M.K., L.D. Rhodes, and R.J. Van Beneden. 1997. Cloning of the p53 tumor suppressor gene from the Japanese medaka (Oryzias latipes) and evaluation of mutational hotspots in MNNG-exposed fish. Gene 189:101-106.

Marelli, D.C., M.K. Krause, W.S. Arnold and W.G. Lyons. 1997. Systematic relationships among Florida populations of Argopecten irradians (Lamarck, 1819) (Bivalvia: Pectinidae). The Nautilus 110:31-41.

Marelli, D.C., W.G. Lyons, W.S. Arnold and M.K. Krause. 1997. Subspecific status of Argopecten irradians concentricus (Say, 1822) and of the bay scallops of Florida. The Nautilus 110:42-44.

Krause, M.K. and V.M. Bricelj, 1995. Gpi genotypic effects on quantitative characters in the bay scallop, Argopecten irradians. Marine Biology 123: 511-522.

Krause, M.K. 1995. The role of Gpi polymorphism in glycolytic flux variations and its effect on genotype-dependent viability in the bay scallop. 8th International Pectinid Workshop. IFREMER, Actes de Colloques - No. 17, pp 243-247.

Krause, M.K., W.S. Arnold and W.G. Ambrose, Jr. 1994. Morphological and genetic variation among three populations of calico scallops, Argopecten gibbus. Journal of Shellfish Research 13: 529-537.

Bricelj, V.M. and M.K. Krause. 1992. Resource allocation and population genetics of the bay scallop, Argopecten irradians: effects of age and allozyme heterozygosity on reproductive output. Marine Biology 113: 253-261.

Selected Non-refereed Publications

Krause, M.K. and S.E. Shumway. 1998. A preliminary study of the effects of initial culture density on winter mortality and growth in the northern bay scallop, Argopecten irradians irradians. Final report submitted to the NYSUDC and Cornell Cooperative Extension. 14 pp.

Patents

Dunn, J.J, G. Hind, D.van der Lelie, M.K. Krause *(application in progress)* Genomic Signature Tags (GSTs): A System for Profiling Genomic DNA.

CURRICULUM VITAE - SHEILA STILES - Collaborator

USDOC/NOAA/National Marine Fisheries Service
Northeast Fisheries Science Center
Biological Laboratory
212 Rogers Ave., Milford, CT 06460

Phone: (203) 882-6524
Fax: 203-882-6570
E-mail: sheila.stiles@noaa.gov

Academic Experience

B.S. Xavier University, New Orleans, LA, Biology
M.S. University of Connecticut, Storrs, CT, Zoology/Ecology
Ph.D. University of Massachusetts, Amherst, MA, Fish Genetics

Professional Experience

Research Geneticist
Leader of Genetics Unit in the Biotechnology of Aquacultured Animals Branch.
Currently, plan, direct and conduct research on genetics and breeding of commercial bay scallops which consists of selection, inbreeding, as well as population genetics. Member of several professional societies and author/co-author of several scientific articles, reports and abstracts. Reviewer of a number of proposals and technical articles.

Professional Societies

Genetics Society of America
Genetics Society of Canada
American Fisheries Society, Genetics Section
Sigma Xi, Membership Committee
ICES (International Council for the Exploration of the Sea) Genetics Working Group

Selected Publications (past 4 years)

Brown, M.V., L. Strasbaugh and S. Stiles. 2000. Methodology for the generation of molecular tags in *Placopecten magellanicus* (sea scallop) and *Argopecten irradians* (bay scallop). Journal of Shellfish Research 19 (1): 569.

Picozza, E., J. Crivello, M. Brown, L. Strasbaugh and S. Stiles. 2000. Status report for the characterization of the *Argopecten irradians* genome. Journal of Shellfish Research 19 (1): 578.

Choromanski, J. and S. Stiles, 2003. Evaluation of genetic lines of bay scallops for grow-out, overwintering survival and stock enhancement. International Pectinid Workshop Proceedings, Florida.

Stiles, S., J. Choromanski and D. Jeffress. 2003. Genetic strategies for culture and stock enhancement of bivalves. Journal of Shellfish Research 22 (1): 301.

Stiles, S., J. Choromanski and D. Jeffress. 2004. A review of genetic studies on commercial species of bivalves. Journal of Shellfish Research (*in press*).

CURRICULUM VITAE - GARY H. WIKFORS – Collaborator

Research Microbiologist
Team Leader, Phytoplankton Trophic Interactions Project
Biotechnology Branch (Acting Branch Chief)
Aquaculture and Enhancement Division
Northeast Fisheries Center, Milford Laboratory
NOAA, National Marine Fisheries Service
Milford, CT 06460

Phone: 203-882-6525
Fax:203-882-6517
E-mail:Gary.Wikfors@noaa.gov

Academic Experience

B.S., Biology, University of Maine at Orono, Orono, ME, 1976
M.S., Marine Biology, University of Bridgeport, Bridgeport, CT, 1980
Ph.D., Phycology, University of Connecticut, Storrs, CT, 1996

Professional Experience

Assistant Instructor
University of Bridgeport, Bridgeport, CT, 1977-1978

Biological Laboratory Technician
NOAA, NMFS, Milford, CT, 1978-1979

Biologist
Olin Research, New Haven, CT, 1979

Microbiologist - Research Microbiologist
NOAA, NMFS, Milford, CT, 1980-present

Assistant Professor of Marine Science (Adjunct)
University of Connecticut, 1997-present

Assistant Professor of Marine Science (Adjunct)
CIBNOR, La Paz, Mexico, 2000-present

***Research Interests*:**

Microalgal culture, including engineering of aquaculture-feed production systems.
Phytoplankton physiological ecology.
Nutritional requirements of commercially-important bivalve mollusks as related to microalgal morphological and biochemical characteristics.
Effects of harmful algae upon invertebrates.
Immune response of bivalve mollusks to environmental and microbiological stresses.

Professional Activities

Member at Large, National Shellfisheries Association and appointed NMFS representative to ICES Working Group on Harmful Algal Bloom Dynamics and Long Island Sound Management Committee
Editorial Board: Aquaculture, Journal of Shellfish Research, Bulletin of Environmental Contamination and Toxicology.
Technical and Industry Advisory Committee, Northeast Regional Aquaculture Center, USDA, 1992-8.
Co-Chair of US-France Bilateral Agreement Project, "Domestication of Bivlalve Mollusks," 2000-present.
NOAA representative to EPA Long Island Sound Management Committee (and Science and Technical Advisory Committee) 2000-present.

Awards:

1998 NOAA Technology Transfer Award.
1999 NOAA Fisheries Bronze Award
2001 Judith Brennan-Hoskins Award (NEFSC Service Recognition)

Grants Received:

UCONN Hard Clam Research Initiative, 1988
NOAA Oyster Disease Initiative, 1990
NRAC Work Group on remote setting of oyster larvae, 1992
UCONN Marine Science and Technology Center, Bay scallop aquaculture, 1992.
Connecticut DEP Long Island Sound Research Fund, Microbial Food Web Structure in Long Island Sound, 1994.
NOAA OAR Aquaculture Initiative, Recirculating systems for scallop nursery, 2000.
ECOHAB Brown Tide Research Initiative, 1998.
ECOHAB Effects of HAB's upon grazers, 1999.
ECOHAB Sterol Metabolism, 2002.
State of Massachusetts, Lagoon Pond phytoplankton successions, 2002.

Selected Publications (past 4 years):

Hégaret, H., Wikfors, G.H., Soudant, P., Delaporte, M., Alix, J.H., Quére, C., Le Coz, J.R., Paillard, C., Moal, J. & Samain, J.-F. 2004. Immunological competence of eastern oysters, *Crassostrea virginica*, fed different microalgal diets and challenged with a high-temperature stress. Aquaculture 234, 541-560.

Hégaret, H., Wikfors, G.H. & Soudant, P. 2003a. Flow-cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica,* subjected to a sudden, high-temperature stress: I. Haemocyte types and morphology. Journal of Experimental Marine Biology and Ecology 293, 237-248.

Hégaret, H., Wikfors, G.H., & Soudant, P. 2003b. Flow-cytometric analysis of hemocytes from eastern oysters, *Crassostrea virginica,* subjected to a sudden, high-temperature stress: II. Hemocyte functions: aggregation, viability, phagocytosis and respiratory burst. Journal of Experimental Marine Biology and Ecology 293, 249-265.

Capriulo, G.M, Smith, G., Troy, R., Wikfors, G.H., Pellet, J., Yarish, C. 2002. The planktonic food web structure of a temperate zone estuary, and its alteration due to eutrophication. Hydrobiologia, 475/476, 263-333.

Wikfors, G.H. and M. Ohno. 2001. Minireview: Impact of algal research in aquaculture. Journal of Phycology, 37:968-974. (invited).

Wikfors, G.H. 2000. Microalgal Culture. Pages 520-525 in, Stickney, R. (Ed.), The Encyclopedia of Aquaculture. John Wiley & Sons, Inc., New York, NY. (invited).

CURRICULUM VITAE - JOHN J. DUNN - Collaborator

Academic Experience	A.B. – Biology – West Chester University – 1966 Ph.D. – Microbiology – Rutgers University – 1970
Professional Experience	1970 – 1972 Postdoctoral Scientist Molecular Biology Department University of Heidelberg, Germany 1972 – 1974 Assistant Microbiologist Biology Department Brookhaven National Laboratory 1974 – 1977 Associate Microbiologist Biology Department Brookhaven National Laboratory 1974 – 1981 Adjunct Assistant Professor Department of Microbiology SUNY, Stony Brook 1977 – 1988 Microbiologist Biology Department Brookhaven National Laboratory 1981 – present Adjunct Professor Department of Microbiology SUNY, Stony Brook 1987 – present Adjunct Professor Department of Microbiology and Immunology SUNY, Stony Brook 1987 – present Adjunct Professor Department of Biochemistry and Molecular Genetics SUNY, Stony Brook 1988 – present Senior Microbiologist Biology Department Brookhaven National Laboratory
Awards	1984 – Ernest Orlando Lawrence Memorial Award 1992 – Honorary Degree: Doctor of Science from Long Island University, Southampton Campus 1999 – Waksman Medal, Waksman Institute, Rutgers University

Selected Publications (past 4 years)

Ding, W., Huang , X., Yang, X., Dunn, J. J., Luft, B. J., Koide, S., and Lawson, C. L. Structural identification of a key protective B-cell epitope in Lyme disease antigen OspA. J. Mol. Biol. **302**:1153-1164 (2000)

Gomes-Solecki, M. J. C., Dunn, J. J., Luft, B. J., Castillo, J., Dykhuizen, D. E., Yang, X., Glass, J. D., and Dattwyler, R. J. Recombinant Chimeric Borrelia Proteins for Diagnosis of Lyme Disease. J Clinical Microbio.38: 2530–35 (2000)

Anderson, C. W., Dunn, J. J., Freimuth, P. I., Galloway, A. M., and Allalunis-Turner, J. Frameshift mutation in *PRKDC*, the gene for DNA-PKcs, in the DNA repair-defective, human, Glioma-derived cell line M059J. Radiation Res. 156:2-9 (2001).

Kumaran, D., Eswaramoorthy, S., Luft, B. J., Lawson, C. L., Dunn, J. J., and Swaminathan, S. Crystal structure of outer surface protein C (OspC) from the Lyme disease spirochete, *Borrelia burgdorferi*. EMBO J. **20**:971-978 (2001)

Rithidech, K., Dunn, J.J., Roe, B.A., Gordon, C. R., and Cronkite, E.P. Evidence for two commonly deleted regions on mouse chromosome 2 in gamma ray-induced acute myeloid leukemic cells. Experimental Hematol. 30(6):564-570 (2002).

Dunn, J.J., McCorkle, S.R., Praissman, L.A., Hind, G., van der Lelie, D., Bahou, W.F., Gnatenko, D.V., and Krause, M.K. Genomic signature tags (GSTs): A new system for profiling genomic DNA. Genomic Research 12:1756-1765 (2002).

Gnatenko, D.V., Dunn, J.J., McCorkle, S.R., Weissmann, D., Perrotta, P.L., and Bahou, W.F. Transcript profiling of human platelets using microarray and serial analysis of gene expression. Blood 101: 2285-2293 (2003).

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CONFLICT OF INTEREST LIST FOR COMPETITIVE PROGRAMS ONLY

Name: **Steven Roberts (PD)**

For each project director (PD) and other personnel that are required based on the specific program guidelines, list alphabetically by last name (and with last name first), the full names of individuals in the following categories and mark each category which applies with an 'x'. Additional pages may be used as necessary. A conflict of interest list for each PD must be submitted before a proposal is considered complete. Inclusion of a C.V. or publication list in the proposal is not sufficient.

- All co-authors on publications within the past four years, including pending publications and submissions
- All collaborators on projects within the past four years, including current and planned collaborations
- All thesis or postdoctoral *advisees/advisors*
- All persons in your field with whom you have had a consulting/financial arrangement/other conflict-of-interest in the past four years

Note: Other individuals working in the applicant's specific area are not in conflict of interest with the applicant unless those individuals fall within one of the listed categories.

Name	Co-Author	Collaborator	Advisees/ Advisors	Other – Specify Nature
Barry, Terry	x			
Berlinsky, David		x		
Biga, Peggy	x	x		
Bouchard, Deborah		x		
Boyd, Sunny	x			
Brown, Nick		x		
Cain, Kenneth	x			
Collodi, Paul		x		
Devlin, Robert	x			
Goetz, Frederick	x	x	x	
Grier, Harry	x			
Hardy, Ronald	x			
Hollis, Dave	x			
Iliev, Dimitar	x	x		
Jackson, Leslie	x			
Johnson, Stewart		x		
Karney, Richard		x		
Kim, Hyun-Woo	x			
King, William	x			
Langenau, David	x			
Lindell, Scott		x		
Malison, Jeff	x			
McCauley, Linda	x	x		
Mebane, Bill	x	x		
Moser, Mary	x			

Mykles, Donald	x			
Nardi, George		x		
Ott, Troy	x			
Overturf, Kenneth	x			
Smolowitz, Roxanna		x		
Sullivan, Craig	x			
Sunila, Inke		x		
Taylor, Ron	x			
Walton, William		x		

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CONFLICT OF INTEREST LIST
FOR COMPETITIVE PROGRAMS ONLY

Name: _Maureen K. Krause___________

For each project director (PD) and other personnel that are required based on the specific program guidelines, list alphabetically by last name (and with last name first), the full names of individuals in the following categories and mark each category which applies with an □x□. Additional pages may be used as necessary. A conflict of interest list for each PD must be submitted before a proposal is considered complete. Inclusion of a C.V. or publication list in the proposal is not sufficient.

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- All persons in your field with whom you have had a consulting/financial arrangement/other conflict-of-interest in the past four years

Note: Other individuals working in the applicant's specific area are not in conflict of interest with the applicant unless those individuals fall within one of the listed categories.

Name	Co-Author	Collaborator	Advisees/ Advisors	Other – Specify Nature
Burke, Russell	x	x		
Burkholder, JoAnn		x		
Clendening, Beverly		x		
Daniel, Peter		x		
Dunn, John	x	x		
Ford, Susan		x		
Gnatenko, Dmitri V.	x	x		
Hind, Geoffrey	x	x		
Htyte, Nay	x	x	x	
McCorkle, Shawn	x	x		
McDonald, John H.		x		
McGuire, Beth M.	x	x	x	
Milkie, Robyn C.	x	x	x	
Oldach, David		x		
Parrow, Matthew	x	x		
Praissman, Laura A.	x	x		
Pumo, Dorothy E.		x		
Roberts, Steven		x		
Rawson, Paul		x		
Reece, Kimberly				X (personal conflict)
Sanford, Chris		x		
Shumway, Sandra E.		x		
Stiles, Sheila		x		
Van der Lelie, Daniel	x	x		
Wikfors, Gary		x		

According to the Paperwork Reduction Act of 1995, an agency may not conduct or sponsor, and a person is not required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0524-0039. The time required to complete this information collection is estimated to average .5 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information.

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CONFLICT OF INTEREST LIST
FOR COMPETITIVE PROGRAMS ONLY

Name: SHEILA STILES

For each project director (PD) and other personnel that are required based on the specific program guidelines, list alphabetically by last name (and with last name first), the full names of individuals in the following categories and mark each category which applies with an 'x'. Additional pages may be used as necessary. A conflict of interest list for each PD must be submitted before a proposal is considered complete. Inclusion of a C.V. or publication list in the proposal is not sufficient.

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- All persons in your field with whom you have had a consulting/financial arrangement/other conflict-of-interest in the past four years

Note: Other individuals working in the applicant's specific area are not in conflict of interest with the applicant unless those individuals fall within one of the listed categories.

Name	Co-Author	Collaborator	Advisees/ Advisors	Other – Specify Nature
Brown, M.V.	X			
Crivello, J.	X			
Choromanski, J.	X	X		
Cooper, C	X			
Jeffress, D.	X			
Krause, M		x		
Longwell, AD	X			
Picozza, E.	X			
Xue, Q-Z	X			
Roberts, S		X		
Scheweitzer, D	X			
Strasbaugh, L.	X			
Wikfors, G	X	X		

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CONFLICT OF INTEREST LIST
FOR COMPETITIVE PROGRAMS ONLY

Name: Gary H. Wikfors

For each project director (PD) and other personnel that are required based on the specific program guidelines, list alphabetically by last name (and with last name first), the full names of individuals in the following categories and mark each category which applies with an 'x'. Additional pages may be used as necessary. A conflict of interest list for each PD must be submitted before a proposal is considered complete. Inclusion of a C.V. or publication list in the proposal is not sufficient.

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- All collaborators on projects within the past four years, including current and planned collaborations
- All thesis or postdoctoral *advisees/advisors*
- All persons in your field with whom you have had a consulting/financial arrangement/other conflict-of-interest in the past four years

Note: Other individuals working in the applicant's specific area are not in conflict of interest with the applicant unless those individuals fall within one of the listed categories.

Name	Co-Author	Collaborator	Advisees/ Advisors	Other – Specify Nature
Alix, J.H.	x			
Capriulo, G.M.	x			
Delaporte, M.	x			
Hégaret, H.	x			
Krause, M		x		
Le Coz, J.R.	x			
Moal, J.	x			
Ohno, M.	x			
Paillard, C.	x			
Pellet, J.	x			
Quére, C.	x			
Roberts, S		x		
Samain, J.F.	x			
Smith, G.	x			
Soudant, P.	x			
Stiles, S	X	x		
Troy, R.	x			
Yarish, C.	x			

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CONFLICT OF INTEREST LIST FOR COMPETITIVE PROGRAMS ONLY

Name: Dunn, John J.

For each project director (PD) and other personnel that are required based on the specific program guidelines, list alphabetically by last name (and with last name first), the full names of individuals in the following categories and mark each category which applies with an □x□. Additional pages may be used as necessary. A conflict of interest list for each PD must be submitted before a proposal is considered complete. Inclusion of a C.V. or publication list in the proposal is not sufficient.

- All co-authors on publications within the past four years, including pending publications and submissions
- All collaborators on projects within the past four years, including current and planned collaborations
- All thesis or postdoctoral *advisees/advisors*
- All persons in your field with whom you have had a consulting/financial arrangement/other conflict-of-interest in the past four years

Note: Other individuals working in the applicant's specific area are not in conflict of interest with the applicant unless those individuals fall within one of the listed categories.

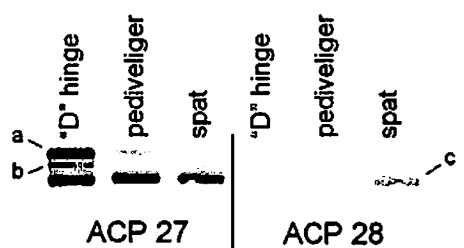
Name	Co-Author	Collaborator	Advisees/ Advisors	Other – Specify Nature
Allalunis-Turner, M.J	X			
Anderson, C.W.	X	X		
Bahou, W.F	X			
Cronkite, E.P	X			
Ding, W	X			
Eswaramoorthy, S	X			
Freimuth, P.	X			
Galloway, A.M	X			
Gnatenko, D.V	X	X		
Gordon, C. R	X			
Hind, G	X			
Huang, X	X			
Koide, S	X			
Kumaran, D	X			
Lawson, C.L	X	X		
Luft, B.J	X	X		
McCorkle, S.R	X	X		
Perrotta, P.L	X			
Praissman, L.A	X			
Rithidech K.	X	X		
Roe, B.A	X			
Swaminathan, S	X	X		
van der Lelie, D	X	X		
Weissmann, D	X			
Yang, X.,	X			

ccording to the Paperwork Reduction Act of 1995, an agency may not conduct or sponsor, and a person is not required to respond to a collection of information unless it splays a valid OMB control number. The valid OMB control number for this information collection is 0524-0039. The time required to complete this information collection is stimated to average .5 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and mpleting and reviewing the collection of information.

Results from Prior NRI Support *Investigator*: **Steven Beyer Roberts (PD)**
"Isolation and characterization of factors regulated during larval competence and metamorphosis in the bay scallop, *Argopecten irradians*"
Proposal # 2002-03633; Start Date: 11/1/02; End Date 10/31/04

The purpose of this study has been to identify the internal factors that control the growth and development of the bay scallop. By understanding what specific factors are involved in the control of development, scallop larvae could be stimulated to settle and begin to grow faster, potentially decreasing mortality rates and decreasing the time needed to get a bay scallop to market size. Additionally, genes homologous to growth factors in other organisms were targeted.

Several approaches are being used to identify important factors. Differential display is being used to isolate upregulated and downregulated genes during development. Specifically, cDNA from "D"-hinge larvae, pediveligers, and spat haven been compared using the GeneFishing DEG system (Seegene). Selected differentially expressed genes are seen in the adjacent figure. These genes have putatively been identified based on sequence homology as; **"a" - heat shock protein 70, "b" - Chymotrypsin-like serine proteinase precursor and "c" - pheromone receptor Rcb3 B47.** Research is currently underway to fully characterize differentially expressed genes and generate full-length clones.



Expressed sequence tag (EST) analysis has also been performed on 4 cDNA libraries. This includes cDNA libraries generated from bay scallop larvae taken at 3 different developmental stages and one adductor muscle library. ***To date 3,192 sequences have been generated*** (960-"D"hinge; 384-metamorphosing larvae; 1394-set spat; 454-muscle tissue). Initial analysis of the ESTs from developing larvae as indicated the presence of regulated transcripts. To date over 2000 sequences generated from this research are available to the public as part of the NCBI's EST database (dbEST). **A description of ESTs from adductor muscle tissue has been published; *Roberts SB, Goetz FW. (2003) "Expressed sequence tag analysis of genes expressed in the bay scallop, Argopecten irradians". Biol Bull. 205: 227-228***

Reverse transcription polymerase chain reaction (RT-PCR) was used to isolate a myostatin homologue from the adductor muscle of the bay scallop and sea scallop. Myostatin is a member of the transforming growth factor-β (TGF- β) superfamily, and has been established as a regulator of development and growth in several vertebrates. Specifically, myostatin has been shown to inhibit skeletal muscle growth as bovine species with natural mutations in this gene demonstrate a "double muscle" phenotype. This is the first time myostatin has been isolated from any invertebrate. The identification of this growth factor could have significant implications for bivalve aquaculture as inhibited expression could potentially result in increase growth rates. A full-length bay scallop myostatin has been obtained using the SMART RACE cDNA Amplification kit (Clontech) and tissue expression has been analyzed with quantitative real-time RT-PCR. **A manuscript is in press: *H.-W. Kim, D.L. Mykles, F.W. Goetz, S.B. Roberts. "Characterization of a myostatin-like gene from the bay scallop, Argopecten irradians" Biochemica et Biophysica Acta – Gene Structure and Expression***

YEAR 1 - S. Roberts

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0039
Expires 03/31/2004

BUDGET

ORGANIZATION AND ADDRESS				USDA AWARD NO.			
Marine Biological Laboratory 7 MBL Street Woods Hole, MA 02543				DURATION PROPOSED MONTHS: 12	DURATION PROPOSED MONTHS: 12	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/Matching Funds Approved by CSREES (If Different)
PROJECT DIRECTOR(S) Steven Beyer Roberts				Funds Requested by Proposer	Funds Approved by CSREES (If different)		
A. Salaries and Wages	CSREES-FUNDED WORK MONTHS						
1. No. Of Senior Personnel	Calendar	Academic	Summer	24,250			
a. 1 (Co)-PD(s)	6						
b. ___ Senior Associates							
2. No. of Other Personnel (Non-Faculty) a. ___ Research Associates/Postdoctorates							
b. ___ Other Professionals							
c. ___ Paraprofessionals							
d. ___ Graduate Students							
e. ___ Prebaccalaureate Students							
f. ___ Secretarial-Clerical							
g. 1 Technical, Shop and Other				32,500			
Total Salaries and Wages				56,750	0	0	0
B. Fringe Benefits (If charged as Direct Costs)				19,068			
C. Total Salaries, Wages, and Fringe Benefits (A plus B)				75,818	0	0	0
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)							
E. Materials and Supplies				20,000			
F. Travel				2,000			
G. Publication Costs/Page Charges							
H. Computer (ADPE) Costs							
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)							
J. All Other Direct Costs (In budget narrative, list items and dollar amounts, and provide supporting data for each item.)				131,440			
K. Total Direct Costs (C through J)				229,259	0	0	0
L. F&A/Indirect Costs (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				31,080			
M. Total Direct and F&A/Indirect Costs (K plus L)				260,339	0	0	0
N. Other							
O. Total Amount of This Request				260,339	0	0	0

P. Carryover -- (If Applicable) Federal Funds: $ Non-Federal funds: $ Total $ 0

Q. Cost-Sharing/Matching (Breakdown of total amounts shown on line O)		
Cash (both Applicant and Third Party)		
Non-Cash Contributions (both Applicant and Third Party)		

NAME AND TITLE (Type or print)	SIGNATURE (required for revised budget only)	DATE
Project Director Steven Beyer Roberts		June 10, 2004
Authorized Organizational Representative Richard J. Mullen; Manager, Research Administration		June 10, 2004
Signature (for optional use)		

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Form CSREES-2004 (12/2000)

YEAR 2 - S. Roberts

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0039
Expires 03/31/2004

BUDGET

ORGANIZATION AND ADDRESS				USDA AWARD NO.			
Marine Biological Laboratory 7 MBL Street Woods Hole, MA 02543				DURATION PROPOSED MONTHS: 12	DURATION PROPOSED MONTHS: 12	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/Matching Funds Approved by CSREES (If Different)
PROJECT DIRECTOR(S) Steven Beyer Roberts				Funds Requested by Proposer	Funds Approved by CSREES (If different)		
A. Salaries and Wages	CSREES-FUNDED WORK MONTHS						
1. No. Of Senior Personnel	Calendar	Academic	Summer				
a. 1 (Co)-PD(s)	6			25,220			
b. ___ Senior Associates							
2. No. of Other Personnel (Non-Faculty) a. ___ Research Associates/Postdoctorates							
b. ___ Other Professionals							
c. ___ Paraprofessionals							
d. ___ Graduate Students							
e. ___ Prebaccalaureate Students							
f. ___ Secretarial-Clerical							
g. 1 Technical, Shop and Other				19,717			
Total Salaries and Wages				44,937	0	0	0
B. Fringe Benefits (If charged as Direct Costs)				15,099			
C. Total Salaries, Wages, and Fringe Benefits (A plus B)				60,036	0	0	0
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)							
E. Materials and Supplies				15,000			
F. Travel				1,000			
G. Publication Costs/Page Charges				1,000			
H. Computer (ADPE) Costs							
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)							
J. All Other Direct Costs (In budget narrative, list items and dollar amounts, and provide supporting data for each item.)				127,047			
K. Total Direct Costs (C through J)				204,083	0	0	0
L. F&A/Indirect Costs (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				19,634			
M. Total Direct and F&A/Indirect Costs (Kplus L)				223,716	0	0	0
N. Other							
O. Total Amount of This Request				223,716	0	0	0

P. Carryover -- (If Applicable) .... **Federal Funds: $** **Non-Federal funds: $** **Total $** 0

Q. Cost-Sharing/Matching (Breakdown of total amounts shown on line O)		
Cash (both Applicant and Third Party)		
Non-Cash Contributions (both Applicant and Third Party)		

NAME AND TITLE (Type or print)	**SIGNATURE** (required for revised budget only)	**DATE**
Project Director Steven Beyer Roberts		June 10, 2004
Authorized Organizational Representative Richard J. Mullen; Manager, Research Administration		June 10, 2004
Signature (for optional use)		

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YEAR 3 - S. Roberts

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0039
Expires 03/31/2004

BUDGET

ORGANIZATION AND ADDRESS	USDA AWARD NO.			
Marine Biological Laboratory 7 MBL Street Woods Hole, MA 02543	DURATION PROPOSED MONTHS: 12	DURATION PROPOSED MONTHS: 12	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/Matching Funds Approved by CSREES (If Different)
PROJECT DIRECTOR(S) Steven Beyer Roberts	Funds Requested by Proposer	Funds Approved by CSREES (If different)		

A. Salaries and Wages	CSREES-FUNDED WORK MONTHS: Calendar	Academic	Summer	Funds Requested by Proposer	Funds Approved by CSREES (If different)	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/Matching Funds Approved by CSREES (If Different)
1. No. Of Senior Personnel				26,229			
a. 1 (Co)-PD(s)	6						
b. ___ Senior Associates							
2. No. of Other Personnel (Non-Faculty) a. ___ Research Associates/Postdoctorates							
b. ___ Other Professionals							
c. ___ Paraprofessionals							
d. ___ Graduate Students							
e. ___ Prebaccalaureate Students							
f. ___ Secretarial-Clerical							
g. 1 Technical, Shop and Other				20,505			
Total Salaries and Wages				46,734	0	0	0
B. Fringe Benefits (If charged as Direct Costs)				15,703			
C. Total Salaries, Wages, and Fringe Benefits (A plus B)				62,437	0	0	0
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)							
E. Materials and Supplies				15,000			
F. Travel				1,500			
G. Publication Costs/Page Charges				1,000			
H. Computer (ADPE) Costs							
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)							
J. All Other Direct Costs (In budget narrative, list items and dollar amounts, and provide supporting data for each item.)				129,509			
K. Total Direct Costs (C through J)				209,446	0	0	0
L. F&A/Indirect Costs (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				20,359			
M. Total Direct and F&A/Indirect Costs (K plus L)				229,805	0	0	0
N. Other							
O. Total Amount of This Request				229,805	0	0	0

P. Carryover -- (If Applicable) Federal Funds: $ Non-Federal funds: $ Total $ 0

Q. Cost-Sharing/Matching (Breakdown of total amounts shown on line O)		
Cash (both Applicant and Third Party)		
Non-Cash Contributions (both Applicant and Third Party)		

NAME AND TITLE (Type or print)	SIGNATURE (required for revised budget only)	DATE
Project Director Steven Beyer Roberts		June 10, 2004
Authorized Organizational Representative Richard J. Mullen; Manager, Research Administration		June 10, 2004
Signature (for optional use)		

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Form CSREES-2004 (12/2000)

TOTAL (3 YEARS)
S. Roberts

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0039
Expires 03/31/2004

BUDGET

ORGANIZATION AND ADDRESS	USDA AWARD NO.			
Marine Biological Laboratory 7 MBL Street Woods Hole, MA 02543	DURATION PROPOSED MONTHS: 36	DURATION PROPOSED MONTHS: 36	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/Matching Funds Approved by CSREES (If Different)
PROJECT DIRECTOR(S) Steven Beyer Roberts	Funds Requested by Proposer	Funds Approved by CSREES (If different)		

A. Salaries and Wages	CSREES-FUNDED WORK MONTHS						
1. No. Of Senior Personnel	Calendar	Academic	Summer	75,699			
a. 1 (Co)-PD(s)	18						
b. ____ Senior Associates							
2. No. of Other Personnel (Non-Faculty) a. ____ Research Associates/Postdoctorates							
b. ____ Other Professionals							
c. ____ Paraprofessionals							
d. ____ Graduate Students							
e. ____ Prebaccalaureate Students							
f. ____ Secretarial-Clerical							
g. 1 Technical, Shop and Other				72,722			
Total Salaries and Wages				148,421	0	0	0
B. Fringe Benefits (If charged as Direct Costs)				49,869			
C. Total Salaries, Wages, and Fringe Benefits (A plus B)				198,290	0	0	0
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)							
E. Materials and Supplies				50,000			
F. Travel				4,500			
G. Publication Costs/Page Charges				2,000			
H. Computer (ADPE) Costs							
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)							
J. All Other Direct Costs (In budget narrative, list items and dollar amounts, and provide supporting data for each item.)				387,996			
K. Total Direct Costs (C through J)				642,786	0	0	0
L. F&A/Indirect Costs (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				71,073			
M. Total Direct and F&A/Indirect Costs (K plus L)				713,860	0	0	0
N. Other							
O. Total Amount of This Request				713,860	0	0	0

P. Carryover – (If Applicable) ..... **Federal Funds: $** **Non-Federal funds: $** **Total $** 0

Q. Cost-Sharing/Matching (Breakdown of total amounts shown on line O)		
Cash (both Applicant and Third Party)		
Non-Cash Contributions (both Applicant and Third Party)		

NAME AND TITLE (Type or print)	**SIGNATURE** (required for revised budget only)	**DATE**
Project Director Steven Beyer Roberts		June 10, 2004
Authorized Organizational Representative Richard J. Mullen; Manager, Research Administration		June 10, 2004
Signature (for optional use)		

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UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0039

YEAR 1

BUDGET

ORGANIZATION AND ADDRESS	USDA AWARD NO.			
Hofstra University	DURATION PROPOSED MONTHS: 12 Funds Requested by Proposer	DURATION PROPOSED MONTHS: ____ Funds Approved by CSREES (If different)	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/Matching Funds Approved by CSREES (If Different)
PROJECT DIRECTOR(S) Dr. Maureen Krause				

	CSREES-FUNDED WORK MONTHS: Calendar	Academic	Summer	Funds Requested by Proposer	Funds Approved by CSREES (If different)	Non-Federal Proposed Cost-Sharing/ Matching Funds	Non-federal Cost-Sharing/Matching Funds Approved by CSREES
A. Salaries and Wages							
1. No. Of Senior Personnel							
a. 1 (Co)-PD(s)			1.50	$9,399			
b. ____ Senior Associates							
2. No. of Other Personnel (Non-Faculty)							
a. 1 Research Associates/Postdoctorates	12			36,000			
b. ____ Other Professionals							
c. ____ Paraprofessionals							
d. ____ Graduate Students							
e. 1 Prebaccalaureate Students				3,840			
f. ____ Secretarial-Clerical							
g. ____ Technical, Shop and Other							
Total Salaries and Wages →				49,239			
B. Fringe Benefits (If charged as Direct Costs)				11,641			
C. Total Salaries, Wages, and Fringe Benefits (A plus B) →				60,880			
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)				6,500			
E. Materials and Supplies				11,050			
F. Travel				2,000			
G. Publication Costs/Page Charges				500			
H. Computer (ADPE) Costs							
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)							
J. All Other Direct Costs (In budget narrative, list items and dollar amounts, and provide supporting data for each item.)				24,712			
K. Total Direct Costs (C through J) →				105,642			
L. F&A/Indirect Costs (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				24,298			
M. Total Direct and F&A/Indirect Costs (K plus L) →				129,940			
N. Other →							
O. Total Amount of This Request →				129,940			

P. Carryover -- (If Applicable)Federal Funds: $ Non-Federal funds: $ Total $

Q. Cost-Sharing/Matching (Breakdown of total amounts shown on line O)		
Cash (both Applicant and Third Party) →		
- Non Cash Contributions (both Applicant and Third Party)		

NAME AND TITLE (Type or print)	SIGNATURE (required for revised budget only)	DATE
Project Director		
Authorized Organizational Representative		
Signature (for optional use)		

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Form CSREES-2004 (12/2000)

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0039

YEAR 2

BUDGET

ORGANIZATION AND ADDRESS Hofstra University	USDA AWARD NO.			
PROJECT DIRECTOR(S) Dr. Maureen Krause	DURATION PROPOSED MONTHS: 12 Funds Requested by Proposer	DURATION PROPOSED MONTHS: ____ Funds Approved by CSREES (If different)	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/Matching Funds Approved by CSREES (If Different)

	CSREES-FUNDED WORK MONTHS						
A. Salaries and Wages	Calendar	Academic	Summer				
1. No. Of Senior Personnel							
a. 1 (Co)-PD(s)			1.50	$9,869			
b. ___ Senior Associates							
2. No. of Other Personnel (Non-Faculty) a. 1 Research Associates/Postdoctorates	12			37,440			
b. ___ Other Professionals							
c. ___ Paraprofessionals							
d. ___ Graduate Students							
e. 1 Prebaccalaureate Students				3,994			
f. ___ Secretarial-Clerical							
g. ___ Technical, Shop and Other							
Total Salaries and Wages →				51,303			
B. Fringe Benefits (If charged as Direct Costs)				12,114			
C. Total Salaries, Wages, and Fringe Benefits (A plus B) →				63,417			
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)							
E. Materials and Supplies				10,513			
F. Travel				2,000			
G. Publication Costs/Page Charges				500			
H. Computer (ADPE) Costs							
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)							
J. All Other Direct Costs (In budget narrative, list items and dollar amounts, and provide supporting data for each item.)				25,641			
K. Total Direct Costs (C through J) →				102,071			
L. F&A/Indirect Costs (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				23,476			
M. Total Direct and F&A/Indirect Costs (K plus L) →				125,547			
N. Other →							
O. Total Amount of This Request →				125,547			

P. Carryover – (If Applicable)Federal Funds: $ Non-Federal funds: $ Total $

Q. Cost-Sharing/Matching (Breakdown of total amounts shown on line O)		
Cash (both Applicant and Third Party) →		
- Non Cash Contributions (both Applicant and Third Party)		

NAME AND TITLE (Type or print)	SIGNATURE (required for revised budget only)	DATE
Project Director		
Authorized Organizational Representative		
Signature (for optional use)		

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Form CSREES-2004 (12/2000)

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0039

YEAR 3

BUDGET

ORGANIZATION AND ADDRESS Hofstra University				USDA AWARD NO.			
PROJECT DIRECTOR(S) Dr. Maureen Krause				DURATION PROPOSED MONTHS: 12 Funds Requested by Proposer	DURATION PROPOSED MONTHS: ____ Funds Approved by CSREES (If different)	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/Matching Funds Approved by CSREES (If Different)
A. Salaries and Wages	CSREES-FUNDED WORK MONTHS						
1. No. Of Senior Personnel	Calendar	Academic	Summer				
a. 1 (Co)-PD(s)			1.50	$10,289			
b. ____ Senior Associates							
2. No. of Other Personnel (Non-Faculty) a. 1 Research Associates/Postdoctorates	12			38,938			
b. ____ Other Professionals							
c. ____ Paraprofessionals							
d. ____ Graduate Students							
e. 1 Prebaccalaureate Students				4,154			
f. ____ Secretarial-Clerical							
g. ____ Technical, Shop and Other							
Total Salaries and Wages →				53,379			
B. Fringe Benefits (If charged as Direct Costs)				12,600			
C. Total Salaries, Wages, and Fringe Benefits (A plus B) →				65,979			
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)							
E. Materials and Supplies				8,476			
F. Travel				2,000			
G. Publication Costs/Page Charges				1,000			
H. Computer (ADPE) Costs							
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)							
J. All Other Direct Costs (In budget narrative, list items and dollar amounts, and provide supporting data for each item.)				26,615			
K. Total Direct Costs (C through J) →				104,071			
L. F&A/Indirect Costs (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				23,936			
M. Total Direct and F&A/Indirect Costs (K plus L) →				128,009			
N. Other →							
O. Total Amount of This Request →				128,009			

P. Carryover -- (If Applicable)Federal Funds: $ Non-Federal funds: $ Total $

Q. Cost-Sharing/Matching (Breakdown of total amounts shown on line O)		
Cash (both Applicant and Third Party) →		
- Non Cash Contributions (both Applicant and Third Party)		

NAME AND TITLE (Type or print)	SIGNATURE (required for revised budget only)	DATE
Project Director		
Authorized Organizational Representative		
Signature (for optional use)		

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Form CSREES-2004 (12/2000)

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0039

CUMULATIVE

BUDGET

ORGANIZATION AND ADDRESS Hofstra University				USDA AWARD NO.			
PROJECT DIRECTOR(S) Dr. Maureen Krause				DURATION PROPOSED MONTHS: 12 Funds Requested by Proposer	DURATION PROPOSED MONTHS: ____ Funds Approved by CSREES (If different)	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/Matching Funds Approved by CSREES (If Different)
A. Salaries and Wages	CSREES-FUNDED WORK MONTHS						
1. No. Of Senior Personnel	Calendar	Academic	Summer				
a. 1 (Co)-PD(s)			4.50	$29,557			
b. ____ Senior Associates							
2. No. of Other Personnel (Non-Faculty) a. 1 Research Associates/Postdoctorates	36			112,378			
b. ____ Other Professionals							
c. ____ Paraprofessionals							
d. ____ Graduate Students							
e. 1 Prebaccalaureate Students				11,988			
f. ____ Secretarial-Clerical							
g. ____ Technical, Shop and Other							
Total Salaries and Wages →				153,923			
B. Fringe Benefits (If charged as Direct Costs)				36,356			
C. Total Salaries, Wages, and Fringe Benefits (A plus B) →				190,279			
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)				6,500			
E. Materials and Supplies				30,039			
F. Travel				6,000			
G. Publication Costs/Page Charges				2,000			
H. Computer (ADPE) Costs							
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)							
J. All Other Direct Costs (In budget narrative, list items and dollar amounts, and provide supporting data for each item.)				76,968			
K. Total Direct Costs (C through J) →				311,786			
L. F&A/Indirect Costs (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				71,710			
M. Total Direct and F&A/Indirect Costs (K plus L) →				383,496			
N. Other →							
O. Total Amount of This Request →				383,496			

P. Carryover – (If Applicable) Federal Funds: $ Non-Federal funds: $ Total $

Q. Cost-Sharing/Matching (Breakdown of total amounts shown on line O)		
Cash (both Applicant and Third Party) →		
- Non Cash Contributions (both Applicant and Third Party)		

NAME AND TITLE (Type or print)	SIGNATURE (required for revised budget only)	DATE
Project Director		
Authorized Organizational Representative		
Signature (for optional use)		

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UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0039

YEAR 1

BUDGET

ORGANIZATION AND ADDRESS Brookhaven Science Associates, LLC/Brookhaven National Lab				USDA AWARD NO.			
PROJECT DIRECTOR(S) John Dunn				DURATION PROPOSED MONTHS: _12_ Funds Requested by Proposer	DURATION PROPOSED MONTHS: ____ Funds Approved by CSREES (If different)	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/Matching Funds Approved by CSREES (If Different)
A. Salaries and Wages	CSREES-FUNDED WORK MONTHS						
1. No. Of Senior Personnel	Calendar	Academic	Summer	$4,586			
a. _1_ (Co)-PD(s)	.5						
b. ____ Senior Associates							
2. No. of Other Personnel (Non-Faculty) a. ____ Research Associates/Postdoctorates							
b. _1_ Other Professionals	1.5			$6,241			
c. ____ Paraprofessionals							
d. ____ Graduate Students							
e. ____ Prebaccalaureate Students							
f. ____ Secretarial-Clerical							
g. ____ Technical, Shop and Other							
Total Salaries and Wages →				$10,827			
B. Fringe Benefits (If charged as Direct Costs)				$4,190			
C. Total Salaries, Wages, and Fringe Benefits (A plus B) →				$15,017			
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)							
E. Materials and Supplies				$5,000			
F. Travel							
G. Publication Costs/Page Charges							
H. Computer (ADPE) Costs							
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)							
J. All Other Direct Costs (In budget narrative, list items and dollar amounts, and provide supporting data for each item.)							
K. Total Direct Costs (C through J) →				$20,017			
L. F&A/Indirect Costs (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				$4,695			
M. Total Direct and F&A/Indirect Costs (K plus L) →							
N. Other →							
O. Total Amount of This Request →				$24,712			

P. Carryover -- (If Applicable)Federal Funds: $ **Non-Federal funds: $** **Total $** 0

Q. Cost-Sharing/Matching (Breakdown of total amounts shown on line O)		
Cash (both Applicant and Third Party) →	0	
- Non Cash Contributions (both Applicant and Third Party)		

NAME AND TITLE (Type or print)	**SIGNATURE** (required for revised budget only)	**DATE**
Project Director		
Authorized Organizational Representative		
Signature (for optional use)		

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UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0039

YEAR 2

BUDGET

ORGANIZATION AND ADDRESS Brookhaven Science Associates, LLC/Brookhaven National Lab				USDA AWARD NO.			
PROJECT DIRECTOR(S) John Dunn				DURATION PROPOSED MONTHS: 12 **Funds Requested by Proposer**	DURATION PROPOSED MONTHS: ____ **Funds Approved by CSREES (If different)**	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/Matching Funds Approved by CSREES (If Different)
A. Salaries and Wages	CSREES-FUNDED WORK MONTHS						
1. No. Of Senior Personnel	Calendar	Academic	Summer	$4,816			
a. 1 (Co)-PD(s)	.5						
b. ____ Senior Associates							
2. No. of Other Personnel (Non-Faculty) a. ____ Research Associates/Postdoctorates							
b. 1 Other Professionals	1.5			$6,553			
c. ____ Paraprofessionals							
d. ____ Graduate Students							
e. ____ Prebaccalaureate Students							
f. ____ Secretarial-Clerical							
g. ____ Technical, Shop and Other							
Total Salaries and Wages →				$11,369			
B. Fringe Benefits (If charged as Direct Costs)				$4,400			
C. Total Salaries, Wages, and Fringe Benefits (A plus B) →				$15,769			
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)							
E. Materials and Supplies				$5,000			
F. Travel							
G. Publication Costs/Page Charges							
H. Computer (ADPE) Costs							
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)							
J. All Other Direct Costs (In budget narrative, list items and dollar amounts, and provide supporting data for each item.)							
K. Total Direct Costs (C through J) →				$20,769			
L. F&A/Indirect Costs (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				$4,872			
M. Total Direct and F&A/Indirect Costs (K plus L) →							
N. Other →							
O. Total Amount of This Request →				$25,641			

P. Carryover -- (If Applicable)Federal Funds: $ **Non-Federal funds: $** **Total $** 0

Q. Cost-Sharing/Matching (Breakdown of total amounts shown on line O)		
Cash (both Applicant and Third Party) →	0	
- Non Cash Contributions (both Applicant and Third Party)		

NAME AND TITLE (Type or print)	SIGNATURE (required for revised budget only)	DATE
Project Director		
Authorized Organizational Representative		
Signature (for optional use)		

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BUDGET

YEAR 3

ORGANIZATION AND ADDRESS Brookhaven Science Associates, LLC/Brookhaven National Lab				USDA AWARD NO.			
PROJECT DIRECTOR(S) John Dunn				DURATION PROPOSED MONTHS: 12 **Funds Requested by Proposer**	DURATION PROPOSED MONTHS: ____ **Funds Approved by CSREES (If different)**	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/Matching Funds Approved by CSREES (If Different)
A. Salaries and Wages	CSREES-FUNDED WORK MONTHS						
1. No. Of Senior Personnel	Calendar	Academic	Summer	$5,057			
a. 1 (Co)-PD(s)	.5						
b. ____ Senior Associates							
2. No. of Other Personnel (Non-Faculty) a. ____ Research Associates/Postdoctorates							
b. 1 Other Professionals	1.5			$6,881			
c. ____ Paraprofessionals							
d. ____ Graduate Students							
e. ____ Prebaccalaureate Students							
f. ____ Secretarial-Clerical							
g. ____ Technical, Shop and Other							
Total Salaries and Wages →				$11,938			
B. Fringe Benefits (If charged as Direct Costs)				$4,620			
C. Total Salaries, Wages, and Fringe Benefits (A plus B) →				$16,558			
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)							
E. Materials and Supplies				$5,000			
F. Travel							
G. Publication Costs/Page Charges							
H. Computer (ADPE) Costs							
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)							
J. All Other Direct Costs (In budget narrative, list items and dollar amounts, and provide supporting data for each item.)							
K. Total Direct Costs (C through J) →				$21,558			
L. F&A/Indirect Costs (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				$5,057			
M. Total Direct and F&A/Indirect Costs (K plus L) →							
N. Other →							
O. Total Amount of This Request →				$26,615			

P. Carryover -- (If Applicable)Federal Funds: $ **Non-Federal funds: $** **Total $** 0

Q. Cost-Sharing/Matching (Breakdown of total amounts shown on line O)		
Cash (both Applicant and Third Party) →	0	
- Non Cash Contributions (both Applicant and Third Party)		

NAME AND TITLE (Type or print)	**SIGNATURE** (required for revised budget only)	**DATE**
Project Director		
Authorized Organizational Representative		
Signature (for optional use)		

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UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0039

BUDGET SUMMARY

ORGANIZATION AND ADDRESS				USDA AWARD NO.			
Brookhaven Science Associates, LLC/Brookhaven National Lab				DURATION PROPOSED MONTHS: __36__ Funds Requested by Proposer	DURATION PROPOSED MONTHS: ____ Funds Approved by CSREES (If different)	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/Matching Funds Approved by CSREES (If Different)
PROJECT DIRECTOR(S) John Dunn							
A. Salaries and Wages	CSREES-FUNDED WORK MONTHS						
1. No. Of Senior Personnel	Calendar	Academic	Summer	$14,459			
a. __1__ (Co)-PD(s)	1.5						
b. ____ Senior Associates							
2. No. of Other Personnel (Non-Faculty) a. ____ Research Associates/Postdoctorates							
b. __1__ Other Professionals	4.5			$19,675			
c. ____ Paraprofessionals							
d. ____ Graduate Students							
e. ____ Prebaccalaureate Students							
f. ____ Secretarial-Clerical							
g. ____ Technical, Shop and Other							
Total Salaries and Wages →				$34,134			
B. Fringe Benefits (If charged as Direct Costs)				$13,210			
C. Total Salaries, Wages, and Fringe Benefits (A plus B) →				$47,344			
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)							
E. Materials and Supplies				$15,000			
F. Travel							
G. Publication Costs/Page Charges							
H. Computer (ADPE) Costs							
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)							
J. All Other Direct Costs (In budget narrative, list items and dollar amounts, and provide supporting data for each item.)							
K. Total Direct Costs (C through J) →				$62,344			
L. F&A/Indirect Costs (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				$14,623			
M. Total Direct and F&A/Indirect Costs (K plus L) →							
N. Other →							
O. Total Amount of This Request →				$76,968			

P. Carryover -- (If Applicable)Federal Funds: $ **Non-Federal funds: $** **Total $** 0

Q. Cost-Sharing/Matching (Breakdown of total amounts shown on line O)		
Cash (both Applicant and Third Party) →	0	
- Non Cash Contributions (both Applicant and Third Party)		

NAME AND TITLE (Type or print)	SIGNATURE (required for revised budget only)	DATE
Project Director		
Authorized Organizational Representative		
Signature (for optional use)		

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Form CSREES-2004 (12/2000)

BUDGET JUSTIFICATION - Steven Roberts; Marine Biological Laboratory - PD

A. **Salaries and Wages** (note: a 4.0 % salary raise was calculated between years for all personnel and a fringe rate of 33.6 % was applied to all salaries for each year)

Project Director: As of October, 2003, the PD was promoted to Staff Scientist at the Marine Biological Laboratory (MBL) in Woods Hole, MA. If funded, the PD will devote approximately half of the calendar year towards this project. MBL is a "soft money" institution and, therefore, scientists are expected to recover their salary from grants. Thus, the PD is requesting 6 months of salary/year for three years to cover a portion of his contribution to the project.

Technician: Funds are requested to partially cover a technician position for three years. Specifically, funds are requested to cover 12 months of salary/year for the first year of the proposal and 6 months of salary for the second and third year. During year one, approximately 7 months of salary will be used to provide technical assistance to NMFS collaborators (Sheila Stiles and Gary Wikfors; see Collaborative Arrangements, page 28). Five months of salary in year one and 6 months of salary/year in years two and three is requested to cover a technician position to work with the PD, and is an essential position because of the recombinant DNA work, high volume of DNA sequencing, and quantitative real-time RT-PCR.

E. **Materials and Supplies**

Funds are requested for the purchase materials and supplies need to complete the proposed research. During year one, $13,000 is requested to partially cover the cost of supplies required for culturing selected bay scallop lines, culturing micro-algae and carrying out feed efficiency experiments (i.e. hollow core purification filters, glassware, bag filters, overwintering cages, mooring apparatus, data loggers). Approximately $7,000 in year one and $15,000 / year in years two and three are specifically requested for materials and supplies required during the proposed research and are listed below.

A. *Specific molecular biology supplies*

1) RNA/mRNA isolation preparations (e.g., Tri Reagent and PolyAtract)
2) RNAlater (Ambion)
3) PCR reagents
4) reagents for in-house sequencing
5) plasmid preparation kits (e.g., Wizard) and gel extraction systems
6) GeneFishing DEG kits (Seegene)
7) cloning kits (e.g., TOPO)
8) Rapid Amplification of cDNA Ends (RACE) kits (e.g., BD SMART RACE; Clontech)
9) primers and dual-labeled probes (IDT)
10) Brilliant SYBR Green QRT-PCR Master Mix kits, (Stratagene)

B. *General molecular supplies*

1) general plasticware (e.g. pipette tips, petri dishes)
2) RNAse free plasticware and tips
3) agarose
4) components for bacteria media
5) miscellaneous reagents and plasticware/glassware

F. Travel

Funds are requested to partially cover the costs of travel for the PD to travel to Milford, CT, to transport samples and meet with collaborators, particularly in year one. Funds are also requested to partially cover the costs of airfare and minimal accommodations for the PD to travel to international/national (i.e. World Aquaculture Association. National Shellfisheries Association) and regional (i.e. NMFS-Milford Shellfish Industry Conference, NRAC-Northeast Regional Aquaculture Expo) scientific meetings to present research results pertaining to the proposed project.

J. All Other Direct Costs

Roberts – MBL: Grand total = $4,500

1) Funds are requested specifically for maintenance of these pieces of equipment including low temperature freezers, bacterial incubators, shakers, power supplies, and high speed centrifuges that need maintenance.
2) Funds are also requested for the yearly inspection and calibrations of pipettors and balances.
3) Funds are specifically requested for telephone and shipping costs directly related to the grant research.

Subcontract- Hofstra University: Grand total $383,496
$383,496 (total) $129,940 (year 1) $125,547 (year 2) $128,009 (year 3)
Subcontract to Dr. Maureen Krause at Hoftra University for SAGE library construction, preliminary DNA sequencing, and generation of full-length cDNA clones. (See M. Krause budgets for cost breakdown and specific budget narrative)

M. Indirect Costs

Indirect costs were calculated as follows:
Year 1: 25% of $99,318 (research costs at MBL) + $25,000 (Subcontract)
Year 2: 25% of $78,535 (research costs at MBL)
Year 3: 25% of $81,437 (research costs at MBL)

(MBL's normal, federally approved rate is 59%).

BUDGET JUSTIFICATION - Maureen K. Krause, Hofstra University, Co-PD

A. Salaries and Wages

A 4.0 % salary raise was calculated between years for all personnel, except for year one, in which the PD receives a 5% raise (in agreement with faculty contract). A fringe rate of 30.0% was applied to the postdoctoral salary for each year, and 7.65% FICA was applied to the PD's summer salary and to the undergraduate student monies during the summer.

***Co-Project Director*:** M. K. K. is a full-time tenure-track Assistant Professor (9 month salary) at Hofstra University. If funded, the Co- PD will devote her summer months and January intersession to this project, although only 1.5 summer months of salary is requested each year because of budget limitations. During the entire of the year, she will supervise the construction of SAGE libraries, generate SAGE extension products, and will help coordinate and assist in the overall activity for grant with the PD and collaborators.

***Post-doctoral Fellow*:** Funds are requested to cover a full-time postdoctoral position for three years. Because of the Co-PD's teaching load (9 contact hours / semester) and the high volume of molecular work required, it is essential to have this position to complete the lab work. This also presents an excellent opportunity for cross-disciplinary training and education: a shellfish biologist can become skilled in molecular techniques, or vice versa.

Undergraduate students: Funds are requested for part-time undergraduate assistance in the laboratory. Hofstra University is, according to the USDA, a mid-size institution with limited institutional success, and our mission is primarily education. We also qualify as an NSF RUI institution; hence, training of students is consistent with our mission. Undergraduate involvement in faculty-led research is strongly encouraged and an integral part of the undergraduate education in biology. All Biology B.S. majors are required to conduct independent research, and these funds will help recruit students to this particular project.

E. Materials and Supplies

Funds are requested for the purchase materials and supplies need to complete the proposed research. During year one, $6,500 is requested to cover the cost of a refrigerated table-top centrifuge, as the PD currently shares one with four other labs and none have microplate capacity. This will be a frequently-needed piece of equipment.

Molecular biology supplies

	Year 1	Year 2	Year 3	Total
General biochemicals (bacteriological media, RNA storage and purification reagents, cloning kits, buffer reagents, etc.)	2,300	2,263	1,826	6,389
SAGE/RDA enzymes	4,000	4,000	1,000	9,000
Other enzymes (for PCR)	1,000	1,000	2,300	4,300
Oligonucleotide synthesis (SAGE-tag specific primers, biotinylated primers for SAGE cassettes)	1,500	2,500	2,600	6,600
Glass and plasticware	2,000	500	500	3,000
computer software and storage media	250	250	250	750

F. Travel

Funds are requested to partially cover the costs of travel for the Co-PD to travel to Milford, CT, and Woods Hole, MA, to transport samples and meet with collaborators. Funds are also requested to partially cover the costs of airfare and minimal accommodations for the PD to travel to international/national (i.e. World Aquaculture Association. National Shellfisheries Association) and regional (i.e. NMFS-Milford Shellfish Industry Conference, NRAC-Northeast Regional Aquaculture Expo) scientific meetings to present research results pertaining to the proposed project.

J. All Other Direct Costs

Subcontract-Brookhaven National Laboratory: Grand total 76,968
$24,712 (year 1) $25,641 (year 2) $26,615 (year 3)
Subcontract to Dr. John Dunn at Brookhaven National Laboratory for SAGE library verification, initial screening and sequencing, and for sequencing of SAGE extension products on identified genes of interest. (See BNL budget for cost breakdown and specific budget narrative).

M. Indirect Costs

Indirect costs were calculated as follows:
23% of total direct costs.

Note: Hofstra University's normal, federally-approved rate is 63% of salary and wages, which would have been approximately 30% of our direct costs.

BUDGET JUSTIFICATION - Brookhaven National Laboratory: John Dunn, Collaborator, Maureen Krause, Hofstra University, Co-PD

A. Salaries and Wages

A 5.0 % salary raise was calculated between years for all personnel and a fringe rate of 38.7 % was applied to all salaries for each year.

Collaborator: John J. Dunn will devote approximately 3% effort to this project, providing advice and input regarding SAGE libraries, bioinformatics, and data interpretation and analyses. This investigator currently oversees the genome sequencing facility at BNL.

***Technician*:** Funds are requested to partially cover a technician position for three years. Specifically, funds are requested to cover 12.5% effort for Judith Romeo, Sr. Laboratory Specialist. This technician will be responsible for DNA sequencing for initial screening of SAGE libraries and for the sequencing of candidate genes as isolated.

E. Materials and Supplies

Funds are requested for the purchase materials and supplies for high-throughput plasmid purification and for automated DNA sequencing to verify quality and concatemer efficiency of SAGE libraries in years one and two and to examine longer sequences of candidate genes in years two and three.

Specific molecular biology supplies:

	Yr 1	Yr2	Yr3
Sequencing supplies and reagents:	$2,500	$2,500	$2,000
General biochemicals:	$1,000	$1,000	$1,000
Oligonucleotides:	$500	$500	$1,500
General supplies/disposables:	$1,000	$1,000	$500

M. Indirect Costs

Indirect costs were calculated as follows:
23.456% of total direct costs. The resulting IDC is 19% of the total award.

(BNL's normal, federally-approved rate would have been approximately 89% of total costs for this proposal).

S. Roberts (PD)

UNITED STATES DEPARTMENT OF AGRICULTURE
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OMB Approved 0524-0039
Expires 03/31/2004

CURRENT AND PENDING SUPPORT

Instructions:

1. Record information for active and pending projects, including this proposal. (Concurrent submission of a proposal to other organizations will not prejudice its review by CSREES.)
2. All current efforts to which project director(s) and other senior personnel have committed a portion of their time must be listed, whether or not salary for the person involved is included in the budgets of the various projects.
3. Provide analogous information for all proposed work which is being considered by, or which will be submitted in the near future to, other possible sponsors including other USDA programs.

NAME (List/PD #1 first)	SUPPORTING AGENCY AND AGENCY ACTIVE AWARD/PENDING PROPOSAL NUMBER	TOTAL $ AMOUNT	EFFECTIVE AND EXPIRATION DATES	% OF TIME COMMITTED	TITLE OF PROJECT
	Active:				
Roberts, S.B.	USDA-NRICGP 2002-03633	$89,934	11/01/02 - 10/31/04	90%	Isolation and characterization of factors regulated during larval competence and metamorphosis in the bay scallop, Argopecten irradians
Roberts, S.B. Lindell, S. Johnson, S Bouchard, D. Nardi, G Berlinsky, D. Brown, N.	NRAC-USDA 02-5-7	$124,612	10/01/03 - 10/01/05	5%	Development of diagnostic and management techniques to select cod broodstocks and hatchery stocks free from nodavirus
	Pending:				
Roberts, S.B Smolowitz, R. Karney, R. Sunila, I. Leavitt, D. Walton, W. Goetz, F.W.	NRAC-USDA 04-1-3	$128,486	04/01/05 - 04/01/07	25%	Development of genetic markers to assess disease resistance in the Eastern oyster
Goetz, F.W. Roberts, S.B. Collodi, P	USDA-NRICGP	$472,840	10/01/04 - 10/01/07	25%	Production of myostatin gene knockouts in zebrafish, and the effects of specific myostatin interacting proteins on salmonid muscle growth
Roberts, S.B. Krause, M.K.	USDA-NRICGP (current proposal)	$713,860	03/01/05 - 03/01/08	50%	Functional genomic analyses of production-related traits in cultured bivalves

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M. Krause
(Co-PD)

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OMB Approved 0524-0039
Expires 03/31/2004

CURRENT AND PENDING SUPPORT

Instructions:

1. Record information for active and pending projects, including this proposal. (Concurrent submission of a proposal to other organizations will not prejudice its review by CSREES.)
2. All current efforts to which project director(s) and other senior personnel have committed a portion of their time must be listed, whether or not salary for the person involved is included in the budgets of the various projects.
3. Provide analogous information for all proposed work which is being considered by, or which will be submitted in the near future to, other possible sponsors including other USDA programs.

NAME (List/PD #1 first)	SUPPORTING AGENCY AND AGENCY ACTIVE AWARD/PENDING PROPOSAL NUMBER	TOTAL $ AMOUNT	EFFECTIVE AND EXPIRATION DATES	% OF TIME COMMITTED	TITLE OF PROJECT
	Active:				
	Pending:				
Roberts, SB Krause, MK	USDA-NRICGP (current proposal)	$713,860	03/01/05 - 03/01/08	12%	Functional genomic analyses of production-related traits in cultured bivalves

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OMB Approval 0524-0039
Expires 03/31/2004

ASSURANCE STATEMENT(S)

STATEMENT OF POLICY - Institutions receiving CSREES funding for research are responsible for protecting human subjects, providing humane treatment of animals, and monitoring use of recombinant DNA. To provide for the adequate discharge of this responsibility, CSREES policy requires an assurance by the institution's Authorized Organizational Representative (AOR) that appropriate committees in each institution have carried out the initial reviews of protocol and will conduct continuing reviews of supported projects. CSREES also requires AOR certification by citing a timely date that an appropriate committee issued an approval or exemption.

NOTE: Check appropriate statements, supplying additional information when necessary.

1. INSTITUTION	2. CSREES PROJECT NUMBER OR AWARD NUMBER (if known)
Marine Biological Laboratory	3. PROJECT DIRECTOR(S) Steven Beyer Roberts

4. TITLE OF PROJECT

A. BIOSAFETY OF RECOMBINANT DNA

- ☐ Project does not involve recombinant DNA.
- ■ Project involves recombinant DNA and was either approved ( ) or determined to be exempt ( ) from the NIH Guidelines by an Institutional Biosafety Committee (IBC) on Pending (Date).

This performing organization agrees to assume primary responsibility for complying with both the intent and procedures of the National Institutes of Health (NIH), DHHS Guidelines for Research Involving Recombinant DNA Molecules, as revised.

B. CARE AND USE OF ANIMALS

- ■ Project does not involve vertebrate animals.
- ☐ Project involves vertebrate animals and was approved by the Institutional Animal Care and Use Committee (IACUC) on ________ (Date).

This performing organization agrees to assume primary responsibility for complying with the Animal Welfare Act (7 USC, 2131-2156), Public Law 89-544, 1996, as amended, and the regulations promulgated thereunder by the Secretary of Agriculture in 9 CFR Parts 1, 2, 3, and 4. In the case of domesticated farm animals housed under farm conditions, the institution shall adhere to the principles stated in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, Federation of Animal Science Societies, 1999.

C. PROTECTION OF HUMAN SUBJECTS

- ■ Project does not involve human subjects.
- ☐ Project involves human subjects and
  - ☐ Was approved by the Institutional Review Board (IRB) on ________ (Date). Performing Institution holds a Federalwide assurance number ________; if not, a Single Project Assurance is required.
  - ☐ Is exempt based on exemption number ________.
  - ☐ Specific plans involving human subjects depend upon completion of survey instruments, prior animal studies, or development of material or procedures. No human subjects will be involved in research until approved by the IRB and a revised Form CSREES-2008 is submitted.

This performing organization agrees to assume primary responsibility for complying with the Federal Policy for Protection of Human Subjects as set forth in 45 CFR Part 46, 1991, as amended, and USDA regulations set forth in 7 CFR 1c, 1992. All nonexempt research involving human subjects must be approved and under continuing review by an IRB. If the performing organization submits a Single Project Assurance, supplemental information describing procedures to protect subjects from risks is required.

SIGNATURE OF AUTHORIZED ORGANIZATIONAL REPRESENTATIVE	TITLE	DATE
[signature]	Manager; Research Administration	June 14, 2004

According to the Paperwork Reduction Act of 1995, an agency may not conduct or sponsor, and a person is not required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0524-0039. The time required to complete this information collection is estimated to average .50 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information.

CSREES-2008 (12/02/00)

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB approved 0524-0039

ASSURANCE STATEMENT(S)

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NOTE: Check appropriate statements, supplying additional information when necessary.

1. INSTITUTION	2. CSREES PROJECT NUMBER OR AWARD NUMBER (if known)
Hofstra University	
	3. PROJECT DIRECTORT(S) Dr. Maureen Krause

4. TITLE OF PROJECT
Functional genomic analyses of production-related traits in cultured bivalves

A. BIOSAFETY OF RECOMBINANT DNA

- [x] Project does not involve recombinant DNA.
- [ ] Project involves recombinant DNA and was either approved ( ) or determined to be exempt ( ) from the NIH Guidelines by an Institutional Biosafety Committee (IBC) on ____________________ (Date).

This performing organization agrees to assume primary responsibility for complying with both the intent and procedures of the National Institutes of Health (NIH), DHHS Guidelines for Research Involving Recombinant DNA Molecules, as revised.

B. CARE AND USE OF ANIMALS

- [x] Project does not involve vertebrate animals.
- [ ] Project involves vertebrate animals and was approved by the Institutional Animal Care and Use Committee (IACUC) on ____________________ (Date).

This performing organization agrees to assume primary responsibility for complying with the Animal Welfare Act (7 USC, 2131-2156), Public Law 89-544, 1996, as amended, and the regulations promulgated thereunder by the Secretary of Agriculture in 9 CFR Parts 1, 2, 3, and 4. In the case of domesticated farm animals housed under farm conditions, the institution shall adhere to the principles stated in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, Federation of Animal Science Societies, 1999.

C. PROTECTION OF HUMAN SUBJECTS

- [x] Project does not involve human subjects.
- [ ] Project involves human subjects and
  - [ ] Was approved by the Institutional Review Board (IRB) on ______________ (Date). Performing Institution holds a Federalwide assurance number ______________; if not, a Single Project Assurance is required.
  - [ ] Is exempt based on exemption number __________.
  - [ ] Specific plans involving human subjects depend upon completion of survey instruments, prior animal studies, or development of material or procedures. No human subjects will be involved in research until approved by the IRB and a revised Form CSREES-2008 is submitted.

This performing organization agrees to assume primary responsibility for complying with the Federal Policy for Protection of Human Subjects as set forth in 45 CFR Part 46, 1991, as amended, and USDA regulations set forth in 7 CFR 1c, 1992. All nonexempt research involving human subjects must be approved and under continuing review by an IRB. If the performing organization submits a Single Project Assurance, supplemental information describing procedures to protect subjects from risks is required.

SIGNATURE OF AUTHORIZED ORGANIZATIONAL REPRESENTATIVE	TITLE	DATE
[signature]	Provost and Senior Vice President for Academic Affairs	6/10/04

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CSREES-2008 (12/02/00)

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB approved 0524-0039

ASSURANCE STATEMENT(S)

STATEMENT OF POLICY - Institutions receiving CSREES funding for research are responsible for protecting human subjects, providing humane treatment of animals, and monitoring use of recombinant DNA. To provide for the adequate discharge of this responsibility, CSREES policy requires an assurance by the Institution's Authorized Organizational Representative (AOR) that appropriate committees in each institution have carried out the initial reviews of protocol and will conduct continuing reviews of supported projects. CSREES also requires AOR certification by citing a timely date that an appropriate committee issued an approval or exemption.

NOTE: Check appropriate statements, supplying additional information when necessary.

1. INSTITUTION Brookhaven Science Associates/ Brookhaven National Lab.	2. CSREES PROJECT NUMBER OR AWARD NUMBER (if known) 3. PROJECT DIRECTORT(S) John Dunn

4. TITLE OF PROJECT
Functional genomic analyses of production-related traits in cultured bivalves

A. BIOSAFETY OF RECOMBINANT DNA

X Project does not involve recombinant DNA.

Project involves recombinant DNA and was either approved ( ) or determined to be exempt ( ) from the NIH Guidelines by an Institutional Biosafety Committee (IBC) on ____________________ (Date).

This performing organization agrees to assume primary responsibility for complying with both the intent and procedures of the National Institutes of Health (NIH), DHHS Guidelines for Research Involving Recombinant DNA Molecules, as revised.

B. CARE AND USE OF ANIMALS

X Project does not involve vertebrate animals.

Project involves vertebrate animals and was approved by the Institutional Animal Care and Use Committee (IACUC) on ____________________ (Date).

This performing organization agrees to assume primary responsibility for complying with the Animal Welfare Act (7 USC, 2131-2156), Public Law 89-544, 1996, as amended, and the regulations promulgated thereunder by the Secretary of Agriculture in 9 CFR Parts 1, 2, 3, and 4. In the case of domesticated farm animals housed under farm conditions, the institution shall adhere to the principles stated in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, Federation of Animal Science Societies, 1999.

C. PROTECTION OF HUMAN SUBJECTS

X Project does not involve human subjects.

Project involves human subjects and

Was approved by the Institutional Review Board (IRB) on ______________ (Date). Performing Institution holds a Federalwide assurance number ______________; if not, a Single Project Assurance is required.

Is exempt based on exemption number ___________.

Specific plans involving human subjects depend upon completion of survey instruments, prior animal studies, or development of material or procedures. No human subjects will be involved in research until approved by the IRB and a revised Form CSREES-2008 is submitted.

This performing organization agrees to assume primary responsibility for complying with the Federal Policy for Protection of Human Subjects as set forth in 45 CFR Part 46, 1991, as amended, and USDA regulations set forth in 7 CFR 1c, 1992. All nonexempt research involving human subjects must be approved and under continuing review by an IRB. If the performing organization submits a Single Project Assurance, supplemental information describing procedures to protect subjects from risks is required.

SIGNATURE OF AUTHORIZED ORGANIZATIONAL REPRESENTATIVE	TITLE	DATE
[signature]	Manager	6/8/04

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CSREES-2008 (12/02/00)

OMB Approved 0524-0039
Expires 03/31/2004

National Environmental Policy Act Exclusions Form

Project Director Name	Institution
Steven Beyer Roberts	Marine Biological Laboratory
Address	

Under 7 CFR Part 3407 (CSREES's implementing regulations of the National Environmental Policy Act of 1969 (NEPA)), environmental data or documentation is required in order to assist CSREES in carrying out its responsibilities under NEPA, which includes determining whether the proposed activity requires the preparation of an environmental assessment or an environmental impact statement, or whether such activity can be excluded from this requirement on the basis of several categories. Therefore, it is necessary for the applicant to advise CSREES whether the proposed activity falls into one of the following Department of Agriculture or CSREES categorical exclusions, or whether the activity does not fall into one of these exclusions (in which case the preparation of an environmental assessment or an environmental impact statement may be required). Even though the applicant considers that a proposed project may or may not fall within a categorical exclusion, CSREES may determine that an environmental assessment or an environmental impact statement is necessary for a proposed project should substantial controversy on environmental grounds exist or if other extraordinary conditions or circumstances are present that may cause such activity to have a significant environmental effect.

Please Read All of the Following and Check All Which Apply

[X] **The proposed activity falls under the categorical exclusion(s) indicated below:**

Department of Agriculture Categorical Exclusions
(found at 7 CFR 1b.3 and restated at 7 CFR 3407.6 (a)(1)(i) through (vii))

[ ] (i) Policy development, planning and implementation which are related to routine activities such as personnel, organizational changes, or similar administrative functions
[ ] (ii) Activities that deal solely with the functions of programs, such as program budget proposals, disbursements, and transfer or reprogramming of funds
[ ] (iii) Inventories, research activities, and studies such as resource inventories and routine data collection when such actions are clearly limited in context and intensity
[ ] (iv) Educational and informational programs and activities
[ ] (v) Civil and criminal law enforcement and investigative activities
[ ] (vi) Activities that are advisory and consultative to other agencies and public and private entities, such as legal counseling and representation
[ ] (vii) Activities related to trade representation and market development activities abroad

CSREES Categorical Exclusions
(found at 7 CFR 3407.6(a)(2)(i) through (ii))

The following categories of CSREES actions are excluded because they have been found to have limited scope and intensity and to have no significant individual or cumulative impacts on the quality of the human environment:

(i) The following categories of research programs or projects of limited size and magnitude or with only short-term effects on the environment:
[X] (A) Research conducted within any laboratory, greenhouse, or other contained facility where research practices and safeguards prevent environmental impacts
[ ] (B) Surveys, inventories, and similar studies that have limited context and minimal intensity in terms of changes in the environment
[ ] (C) Testing outside of the laboratory, such as in small isolated field plots, which involves the routine use of familiar chemicals or biological materials
[ ] (ii) Routine renovation, rehabilitation, or revitalization of physical facilities, including the acquisition and installation of equipment, where such activity is limited in scope and intensity

OR

[ ] **Proposed activity does *not* fall into one of the above categorical exclusions**
(**NOTE**: If checked, please attach an explanation of the potential environmental impacts of the proposed activity. May require completion of an environmental assessment or an environmental impact statement.)

According to the Paperwork Reduction Act of 1995, an agency may not conduct or sponsor, and a person is not required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0524-0039. The time required to complete this information collection is estimated to average .25 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information.

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Date: Thu, 10 Jun 2004 14:43:21 +0100
To: "'sroberts@mbl.edu'" <sroberts@mbl.edu>

Boston, June 10, 2004

Dr. Steven B. Roberts
Marine Biological Lab
Prog. in Scientific Agriculture
7 MBL St.
Woods Hole MA 02543
USA

Ref. No.: BBA RPN 511848
Title: Characterization of a myostatin-like gene from the bay scallop, Argopecten irradians

Dear Dr. Roberts:

We are pleased to inform you that the above mentioned paper has been accepted for publication in Biochimica et Biophysica Acta. The typescript has been forwarded to Elsevier's Production Department in Amsterdam and will be included in the section devoted to Gene Structure and Expression (EXP).

Your article will be published rapidly in electronic form, as well as in the traditional print journal in the first available scheduled issue.

Shortly, you will receive an acknowledgement letter from our Production Department detailing information regarding proofs, reprints and copyright transfer. The BBA Editorial office handles only editorial matters and does not have any information regarding production issues. Should you have any further inquiries regarding this manuscript, please contact our author support department at: authorsupport@elsevier.com <mailto:authorsupport@elsevier.com>

We once again thank you for your contribution to BBA and hope that you will continue to submit your research articles to the journal.

Yours sincerely,
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Elsevier Inc./BBA
Administrative Coordinator

Accepeted Pre-Print – Biochemica et Biophysica Acta- Gene Structure and Expression

Characterization of a myostatin-like gene from the bay scallop, *Argopecten irradians*

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Keywords: myostatin, GDF-8, scallop, muscle, TGF-β, *Argopecten irradians, Ciona*

Nucleotide sequence data reported are available in GenBank databases under the accession number **AY553362**.

Summary

A complete cDNA was cloned from the bay scallop (*Argopecten irradians*) that codes for a 382 amino acid myostatin-like protein (sMSTN). The sMSTN sequence is most similar to mammalian myostatin (MSTN), containing a conserved proteolytic cleavage site (RXXR) and conserved cysteine residues in the C-terminus. Based on quantitative RT-PCR, the sMSTN gene is predominantly expressed in the adductor muscle, with limited expression in other tissues. Using the sMSTN sequence, a *Ciona* MSTN-like gene was also identified from the *Ciona intestinalis* genome. These findings indicate that the MSTN gene has been conserved throughout evolution and suggests that MSTN could play a major role in muscle growth and development in invertebrates, as it does in mammals.

Introduction

The transforming growth factor β (TGF-β) superfamily includes a number of factors that are responsible for growth and development of tissue. One member of this superfamily, originally identified as growth and differentiation factor-8 (GDF-8), was first characterized in mice, where disruption of this gene resulted in a significant increase in muscle mass [1]. Based on the phenotype of the GDF-8 null mice, and the predominant expression of GDF-8 in muscle, the factor has been referred to as myostatin (MSTN) [1]. Naturally occurring mutations in MSTN were soon attributed to the 'double muscle' phenotype observed in some breeds of cattle [2-4].

MSTNs have been cloned from representatives of various vertebrate groups [4]. The cDNAs encode proteins that are generally 373-376 amino acids in length and, as with other TGF-β superfamily members, contain a conserved proteolytic processing site and carboxy-terminal region with a specific pattern of cysteine residues. MSTN cDNAs have now been cloned from a number of phylogenetically diverse fish species [5-11]. A major difference observed between fish and mammals appears to be in the number of MSTNs present within a species. In salmonids, two MSTN isoforms are observed [5,6,9] that are greater than 90% identical within a species and are the products of two separate genes [5]. A second form of MSTN has also been reported in shi drum, fugu and sea bream [11]. However, based on the expression pattern and phylogenetic analysis of the sequence, this form is thought to be different than the second MSTN observed in salmonids.

MSTNs have been well characterized in vertebrates, but to our knowledge, the only invertebrate protein sharing significant sequence homology with MSTN that has been reported is myoglianin; a protein characterized from *Drosophila melanogaster* [12]. While myoglianin is certainly similar to vertebrate MSTNs and is expressed in muscle embryonically, it is a much larger protein (598 amino acids). In contrast, in the present study, we describe a cDNA isolated from the bay scallop (*Argopecten irradians*), that is similar in size and homologous with vertebrate MSTNs. Further, the expression of this mRNA is very high in the skeletal muscle of adult scallops. Taken together, this suggests that the scallop cDNA could be an invertebrate MSTN homologue.

Methods

Cloning Scallop MSTN

Total RNA was extracted from adductor muscle tissue of 2 bay scallops using Tri Reagent (Molecular Research Center) as previously described [13,14]. Messenger ribonucleic acid (mRNA) was isolated from total RNA using the Poly-A-Tract mRNA Isolation kit (Promega). Purified mRNA was reverse transcribed using AMV reverse transcriptase and an anchored oligo-dT primer. The amino acid sequence for a large number of vertebrate MSTNs were compared and three degenerative primers were designed for nested PCR (mstnF1, mstnR1, and mstnR2) (Table 1). The first round of PCR was carried out with mstnF1 and mstnR1 (94°C, 30 sec; 50°C, 20 sec; 72°C, 30 sec; 35 cycles). The resulting PCR product was used as a template for a second round of PCR with mstnF1 and mstnR2 using the same cycling conditions. One prominent 225 bp band was observed that was cut, gel purified, cloned and sequenced. To obtain full length cDNA, Rapid Amplification of cDNA Ends (RACE) technology was used. Specifically, the 3' RACE System (Invitrogen) and SMART RACE cDNA Amplification Kits (BD Biosciences) were employed according to the manufacturers' instructions using gene specific primers based on the sequence of the initial 225 bp fragment. Once the full-length bay scallop sequence was determined by piecing together RACE products, two additional specific primers were developed to obtain a single clone encompassing the complete coding sequence.

During RACE, cDNAs were cloned in TOPO/pCR 2.1 (Invitrogen) and positive colonies were grown for plasmid DNA. Templates were prepared in a Rev Prep Orbit (GeneMachines) and the resulting cDNAs were sequenced using a modified dideoxy chain termination method with Big Dye Terminator (Applied Biosystems). Sequencing reactions were precipitated and pellets resuspended in Hi-Di Formamide with EDTA (Applied Biosystems) and analyzed using a 3730 Sequencer (Applied Biosystems). All sequences were analyzed by NCBI Blast programs

for similarity to known genes [15]. ClustalW (MacVector 7.2) analysis was used for sequence pair-wise and multiple protein alignments.

Quantitative Real-Time RT-PCR

For analysis of scallop MSTN-like (sMSTN) mRNA tissue expression, quantitative real time RT-PCR (Thermoscript One-Step System, Invitrogen) was used with the Opticon Continuous Fluorescence Detection System (MJ Research) using dual-labeled probes designed to specifically hybridize to sMSTN and 18S RNA (Primer Express Software, Applied Biosystems) (Table 1). Total RNA was extracted as described above from mantle, gonad, heart, digestive gland, gill, and adductor muscle tissue from an adult bay scallop. The initial cDNA synthesis and two-step PCR cycling program (40 cycles) were performed consecutively in the same reaction well by incubating samples first at 50°C for 30 min, followed by PCR. For PCR, an initial 5 min 94°C incubation was performed followed by 40 cycles of denaturation (94°C for 15 s) and annealing/extension (66.5°C for 1 min). Fluorescent detection was performed after each annealing/extension step. Each assay (sMSTN and 18 s RNA) was carried out in a separate vessel (25 µl) of a 96-well plate and the concentration of components were: Thermoscript reaction mix, 1×; sense primer, 0.2µM; anti-sense primer, 0.2µM; fluorogenic probe, 0.2µM; $MgSO_4$, 5 mM; RNA, 0.5µg. For all tissues samples, the absence of genomic DNA was verified by running identical RNA samples in real time PCR assays in which Taq DNA polymerase (Platinum Taq DNA polymerase, Invitrogen) was substituted for the dual function enzyme.

All data are given in terms of relative RNA abundance and expressed as means +/- standard errors. One-way ANOVAs were performed followed by Tukey's test. All significance levels were set at $p \leq 0.05$

Results and Discussion

Scallop MSTN-like cDNA sequence

The full-length sMSTN clone (GenBank accession number AY553362) obtained with RACE was 1539 bp with an open reading frame of 1146 bp, presumably coding for a 382 amino acid protein (Figure 1). The characteristic MSTN RXXR cleavage site is present as are the 9 conserved cysteine residues (Figure 2). The 117 amino acids of the conserved, carboxy terminal region of sMSTN are most similar to MSTNs from several mammalian species with an average of 48% identical and 61% similar residues (NCBI Blastp; [15]). Across the entire open reading frame, human MSTN (GenBank accession number NP_005250) is the most similar, being 28% identical with sMSTN (Table 2). The most similar fish MSTNs were 25% identical across the entire protein.

Sequence similarity between sMSTN and all other MSTNs was much higher in the C terminus (Table 2, Figure 2), and this is logical given that this portion represents the biologically active protein. However, in this region sMSTN was still slightly closer to the human MSTN (47% identical) as compared to fish MSTNs (46%; Table 2). In fact, the higher similarity of sMSTN to the entire human MSTN is a result of the higher identity in the prodomain (N-terminus) (Table 2).

Of the sequences in GenBank, the closest invertebrate sequence to sMSTN is myoglianin (GenBank accession number NP_524627) from *D. melanogaster* (Table 2), a TGF-β protein that is expressed throughout the life cycle in *Drosophila* [12]. While myoglianin aligns most closely with MSTNs when compared to NCBI sequences, it is unlikely that this protein is the

invertebrate homologue of MSTN since it is at least 220 amino acids longer. Alternatively, myoglianin (and particularly the N terminus) may have evolved from an ancestral protein that gave rise to vertebrate MSTNs and the MSTN-like gene that we have isolated in scallops. Another TGF-β family protein has been reported from oysters called molluscan growth and differentiation factor (mGDF; [16]). However, mGDF is most similar to bone morphogenetic protein 2 (Table 2) and, therefore, is not a MSTN homologue.

In order to further understand the evolution of this gene, the sMSTN sequence was aligned against the translated *Ciona intestinalis* (Chordata; subphylum: Urochordata) genome (http://aluminum.jgi-psf.org/prod/bin/runBlast.pl?db=ciona4). A gene was identified on Scaffold 533 (contig 1) in the *Ciona* genome that is, like sMSTN, very similar to mammalian MSTNs (Table 2). This *Ciona* MSTN-like gene (ciMSTN) is approximately 2600 bp and has 3 exons that putatively contain coding regions for a 363 amino acid protein (Figure 2). Over the entire protein, the sMSTN protein has greater homology with ciMSTN protein than other invertebrate proteins including myoglianin (Figure 2 and Table 2). This higher homology is a result of greater sequence identity in the N-terminus and was expected given the sequence homology of sMSTN with mammalian MSTNs and the phylogenetic relationship of *Ciona* with vertebrates.

A portion of sMSTN, spanning residues 330-341 (SPTLSQXXXAIS), represents additional amino acids in sMSTN compared to vertebrate MSTNs (Figure 2). Interestingly, a smaller addition (RPDLXXXR) in that area was also observed in the *Ciona* MSTN-like protein (Figure 2). The significance of additional amino acids in this region is not known, however, sequences nearly identical to the additional amino acid sequence in sMSTN were found in the mature peptide of the prolactin precursor in the European eel (GenBank accession number **P33096**) and dystrophin in the zebrafish (GenBank accession number **AF339031**).

Tissue Expression

Quantitative Real-time RT-PCR was used to evaluate sMSTN expression in different tissues. sMSTN RNA was detected in all tissues sampled, however the highest mean levels were detected in adductor muscle tissue in which levels were over 6-times higher than in all other tissues examined (Figure 3). As with Real-time PCR, less intense bands were observed on Northern blots in mantle and gill tissue (data not shown). If the scallop cDNA is a MSTN homologue, elevated expression in adductor muscle would be expected based on MSTN expression and function in vertebrates. In higher vertebrates, MSTN is predominantly expressed in skeletal muscle, though there have been reports of MSTN protein in cardiomyocytes and Purkinje fibers of the heart [17], as well as MSTN mRNA expression in the mammary gland [18]. Tissue expression of MSTN in fish appears to be more complex and is likely related to the occurrence of two forms of MSTN is some species. In species in which only one form of MSTN has been identified, expression has been observed in lower levels compared to muscle in a variety of tissues including gonad, heart, gut, and gill filaments [7,10]. Transcripts of salmonid MSTN homologous to MSTN1 have been found in multiple tissues suggesting constitutive expression [5,6,9]. On the other hand, RNA expression of the MSTN2 orthologs in rainbow trout and brook trout is limited to brain and muscle tissue [5,6]. In the present study, MSTN mRNA was detected by Real time PCR to some extent in all tissues examined including mantle, gonad, heart, digestive gland, gill, and muscle. While not statistically significant, sMSTN was expressed at somewhat higher levels in mantle and gill tissue compared to other tissues (excluding muscle). While MSTN expression has been observed in a variety of tissues in vertebrates, particularly fish, the function(s) of MSTN in non-muscle tissue has not been fully

characterized. While speculative, one explanation for the presence of sMSTN expression in the scallop mantle is that it could be involved in the relationship of somatic and shell growth as the mantle in bivalve molluscs is associated with shell formation. As MSTN has been detected in the gills of several fish species as well as in bay scallops, MSTN could play a role in respiration. However, MSTN expression in these other tissues, could simply be related to the presence of muscle tissue within these organs.

In conclusion, the present study describes the isolation and characterization of a MSTN-like gene from an invertebrate, the bay scallop. Tentative identification of this cDNA is based on sequence size and homology with vertebrate MSTNs, and the predominant expression in muscle tissue. In addition, a MSTN-like sequence from *C. intestinalis* has been described with sequence similarity to scallop and vertebrate MSTNs. It is possible that MSTN has a major role in muscle growth and development in bivalve molluscs, as it does in vertebrates. However, further research is required to elucidate the evolution and biological function(s) of MSTN in invertebrates.

Acknowledgements

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Figure Legends

Figure 1. The nucleotide and deduced amino acid sequences of bay scallop myostatin (sMSTN). The proteolytic processing site (RXXR) is underlined. Location of degenerative primers (mstnF1, mstnR1 and mstnR2) used to obtain the initial sMSTN fragment denoted with arrows. The nucleotide sequence data of sMSTN appears in the GenBank database under the accession number **AY553362**.

Figure 2. Amino acid alignment of zebrafish (*D. rerio*; accession number **AAP85526**), gilthead sea bream (*S. aurata*; accession number **AAL05943**), human (*H. sapiens*; accession number **NP_005250**), bay scallop (*A. irradians*; accession number **AY553362**) and putative *Ciona intestinalis* MSTNs. A vertical line indicates the site of proteolytic processing and the division of N-terminus and C-terminus of the complete proteins. Conserved cysteine residues are denoted with asterisks. Note: *C. intestinalis* sequence was constructed from sequence located on Scaffold 533 (contig 1) of the *Ciona* genome (http://genome.jgi-psf.org/ciona4/ciona4.home.html).

Figure 3. Relative RNA abundance of sMSTN in various tissues from bay scallops. Vertical bars represent the mean ± SE (N=3) for each tissue. Significant differences across tissue are indicated with an asterisk at $p \leq 0.05$.

Table 1
Primers and probes used for initial cDNA isolation and quantitative real-time RT-PCR. Fluorescent dyes incorporated into probes are italicized.

Primer / Probe	Sequence (5' - 3')
mstnF1	WSNMGNTGYMGNTAY
mstnR1	SWRCANCCRCANCKRTCNAC
mstnR2	GSNSYRCARCANGGNCC
mstnF	GGGATGATGATGGTTATGAACCA
mstnProbe	*FAM*-CTTGATCTTCGCACATCGCTGAGGAAGT-*AbQ*
mstnR	CGTCGACCTCTTAGAGCGTGTA
18sF	CGGAGAGGGAGCCTGAGAA
18sProbe	*VIC*-CTACCACATCCAAGGAAGGCAGCAGG-*TAMRA*
18sR	AGTCGGGAGTGGGTAATTTGC

Table 2. Amino acid sequence identities of TGF-β superfamily members from various organisms. Identities are given in relation to the complete proteins and to the regions upstream and downstream of the proteolytic processing site.

Complete Protein	*A.i*	*C.i.*	*D.m.*	*C.g.*	*S.a. gdf-8b*	*D.r.*	*H.s.*	*H.s.* gdf11	*H.s.* bmp2
A. irradians	100								
C. intestinalis	19	100							
D. melagonaster	14	13	100						
C. gigas	16	12	10	100					
S. aurata gdf-8b	25	22	14	17	100				
D. rerio	25	23	15	16	68	100			
H. sapiens	28	24	15	17	60	67	100		
H. sapiens gdf11	24	23	15	14	53	57	56	100	
H. sapiens bmp2	19	15	11	36	19	20	19	19	100

N-terminus	*A.i*	*C.i.*	*D.m.*	*C.g.*	*S.a. gdf-8b*	*D.r.*	*H.s.*	*H.s.* gdf11	*H.s.* bmp2
A. irradians	100								
C. intestinalis	15	100							
D. melagonaster	7	11	100						
C. gigas	10	8	5	100					
S. aurata gdf-8b	19	19	9	8	100				
D. rerio	18	20	9	8	61	100			
H. sapiens	22	22	10	10	48	57	100		
H. sapiens gdf11	15	20	8	7	42	45	42	100	
H. sapiens bmp2	14	10	6	7	16	17	15	17	100

C-terminus	*A.i*	*C.i.*	*D.m.*	*C.g.*	*S.a. gdf-8b*	*D.r.*	*H.s.*	*H.s.* gdf11	*H.s.* bmp2
A. irradians	100								
C. intestinalis	36	100							
D. melagonaster	40	30	100						
C. gigas	31	27	34	100					
S. aurata gdf-8b	46	39	43	32	100				
D. rerio	46	39	46	30	86	100			
H. sapiens	47	40	44	31	84	87	100		
H. sapiens gdf11	46	37	44	32	81	85	90	100	
H. sapiens bmp2	36	31	33	61	38	36	37	33	100

Sources include: sMSTN (*A. irradians*; GenBank accession number **AY553362**), *Ciona* MSTN (*C. intestinalis*), myoglianin (*D. melagonaster*; GenBank accession number **AAD24472**), mGDF (*C. gigas*; GenBank accession number **CAA10268**), MSTN/gdf-8b (S. *aurata*; GenBank accession number **AAL05943**), MSTN (*D. rerio*; GenBank accession number **AAP85526**), MSTN (*H. sapiens*; GenBank accession number **NP 005250**), gdf11 (*H. sapiens*; GenBank accession number **AAF21630**), and bmp2 (*H. sapiens*; GenBank accession number **NP 001191**).

```
   1  AACCATCTCTAACGTAACCGCTACAGCGGAACAATCAGCCGATAGCTGTCAACGAGTAAGGATGCATCA
  70  CATTTGCCATCCTCTCCTGTTCTCTGTGTTGTTCTCGGCTGTATACGCTGTAGCGATTAACCAGCCAAA
 139  CAATACCTATCAGACATTAGAACAGGTTAATAAGGATCTACCTTCCATTCCAAAGCATGATACTTTAAC
 208  AGATGAAATTTACGATGACGATTATTATTCTGAGTTGGCATTGGAGCGTGCTCGTGCCAAAAACAATAT
 277  TGAATATGAGTATGACATGCTGAATGATACGGCGAATTCGACAGATTATTATTACTATGAAGAAATAGA
                              M  L  N  D  T  A  N  S  T  D  Y  Y  Y  Y  E  E  I  E   18

 346  ACAAGTAAAAAATCCAAAGCAACAAAAGTGTCAGATGTGTACAATCAGAGACGAACAGAAACGGCACCG
        Q  V  K  N  P  K  Q  Q  K  C  Q  M  C  T  I  R  D  E  Q  K  R  H  R   41

 415  TGTGGAAGCAATAAAAAACAGAATTTCACATGTCTTGAAACTGGATGTACTTGGCATGCCAAACACAAC
        V  E  A  I  K  N  R  I  S  H  V  L  K  L  D  V  L  G  M  P  N  T  T   64

 484  AGCAAAACGATTACCAAAGGTTCCGTCGTTTTTACGATTACGTGAAAAATATGAAAATGCTCAAATGCA
        A  K  R  L  P  K  V  P  S  F  L  R  L  R  E  K  Y  E  N  A  Q  M  Q   87

 553  ATCTGATTCACCAAATAGCCGAAAGGAGGAGAAATTGAGATACCAAGATGTGCAGGAGGAATATGGACA
        S  D  S  P  N  S  R  K  E  E  K  L  R  Y  Q  D  V  Q  E  E  Y  G  Q  110

 622  GCCGGAAAGGACATATAGTTTCGCTAGAGAACTTCCAGCTGAAATGGACCAGCAATTCCCTAATACCAT
        P  E  R  T  Y  S  F  A  R  E  L  P  A  E  M  D  Q  Q  F  P  N  T  I  133

 691  ATACTTTGATATGCAAGATTCCCCAGAAAAGGAAACAAACAAGGCTTTACTATGGGTGTATATCAGCCC
        Y  F  D  M  Q  D  S  P  E  K  E  T  N  K  A  L  L  W  V  Y  I  S  P  156

 760  CGATGATATAATAGACAGAAATATGACTGAAATATACGTTTACACCATCGACCCGCCCGGCAAATTTAG
        D  D  I  I  D  R  N  M  T  E  I  Y  V  Y  T  I  D  P  P  G  K  F  S  179

 829  CAAAGTTCCAACCAAACGAGAAATCGGCAGAAGAAAACGACATTATATGAAAGCATCTGGTTGGCATCA
        K  V  P  T  K  R  E  I  G  R  R  K  R  H  Y  M  K  A  S  G  W  H  H  202

 898  CTTTGATATACTTGACGAGGTGCAGAAATGGACCTACCGAACTCATTTGAACCTTGGACTCGTTGTGGA
        F  D  I  L  D  E  V  Q  K  W  T  Y  R  T  H  L  N  L  G  L  V  V  E  225

 967  GGCTTTGGACGAGACAGGCCACAACCTAGTTGTACTTCCACCAACATTTGGGGATGATGATGGTTATGA
        A  L  D  E  T  G  H  N  L  V  V  L  P  P  T  F  G  D  D  D  G  Y  E  248

1036  ACCAATGCTTGATCTTCGCACATCGCTGAGGAAGTCTACACGCTCTAAGAGGTCGACGGAACTCTACTG
        P  M  L  D  L  R  T  S  L  R  K  S  T  R  S  K  R  S  T  E  L  Y  C  271
                                               __________

1105  TGACACACGAGAAGAGACAGCTTGCTGCAGGTATCCCCTAGAAGTTGATTTTGTCGCGTTTGGGTGGGA
        D  T  R  E  E  T  A  C  C  R  Y  P  L  E  V  D  F  V  A  F  G  W  D  294
                       ------------------->
                             mstnF1
1174  CTTTGTGATAGCTCCCCTTACATACGCGGCTTATTACTGCGCTGGGGAATGTAAAGGTGAACAGTTAGA
        F  V  I  A  P  L  T  Y  A  A  Y  Y  C  A  G  E  C  K  G  E  Q  L  D  317

1243  CGATACACTTCATGCCCATGTGATTCAACAAGCACCGTCCCCAACATTGAGTCAGCCGCAGTCAGCCAT
        D  T  L  H  A  H  V  I  Q  Q  A  P  S  P  T  L  S  Q  P  Q  S  A  I  340

1312  AAGTAACGTGGGTCCGTGCTGCACTCCCACTAAAATGTCCGACTTAGCTATGCTTTTCTTCGATCATAA
        S  N  V  G  P  C  C  T  P  T  K  M  S  D  L  A  M  L  F  F  D  H  N  363
                <----------------
                     mstnR1
1381  TTCGAACATAGCATTGACGCGATTGCCCAGGATGAAAGTAGACAGATGTGGTTGTGCGTAAGTGAAAGA
        S  N  I  A  L  T  R  L  P  R  M  K  V  D  R  C  G  C  A  *         382

1450  CAAAACTTCAGAAAGAAAGAGACAGGATGGTGATGTATCTGCGAAATAGTAAAGAATGTTTGTTTGAAC
1519  GGGAAAAAAAAAAAAAAAAAA
```

Figure 1

```
D. rerio        --MHFTQVLISLSVLIACGPVGYGDITAHQQPSTATEESELCSTCEFRQHS 49
S. aurata       -----MLVFLGLTVLLSAG----SSVEMNQTSKLLAESGEQCSACDFREHS 42
H. sapiens      MQKLQLCVYIYLFMLIVAGP---VDLNENSEQKENVEKEGLCNACTWRQNT 48
A. irradians    --------MLNDTANSTDY----YYYEEIEQVK--NPKQQKCQMCTIRDEQ 37
C. intestinalis ---------------------------MDQQS----SCGRHKVCSERREEM 20

D. rerio        KLMRLHAIKSQILSKLRLKQA--PNISRDVVKQLLPKAPPLQQLLDQYDVL 98
S. aurata       KQMRLHSIKSQILSILRLEQA--PNISRDMIRQLLPKAPPLTQLLDQYDPR 91
H. sapiens      KSSRIEAIKIQILSKLRLETA--PNISKDVIRQLLPKAPPLRELIDQYDVQ 97
A. irradians    KRHRVEAIKNRISHVLKLDVLGMPNTT----AKRLPKVRSFLRLREKYENA 84
C. intestinalis RSMRIEKLKQNILSKLRLSSP--PNIIT---EQPLSTDSARIMELMRRYGMD 66

D. rerio        GDD--SKDG-AVEEDDEHATTETIMTMATEPD-----PIVQVDRKPK---C 138
S. aurata       -----------VEEED--HATTETIITMATKHN-----PIAQDELTS----C 121
H. sapiens      RDD--SSDG-SLEDDDYHATTETIITMPTESD-----FLMQVDGKPK---C 137
A. irradians    QMQSDSPNSRKEEKLRYQDVQEEYGQPERTYSFARELPAEMDQQFPN---T 132
C. intestinalis QDRG-TSDAEDQETEDEEVNSERVILTSRRRKNRICLFTAPFSNSPSGAGG 116

D. rerio        CFFSFSPKIQANRIVRAQLWVHLRPAEEATTVFLQISRLM--PVKDGG--R 185
S. aurata       CLFSLSPKIQPKNILRAQLWVHLRPADIVTSVFLQISRLK--PGKEGNNTR 170
H. sapiens      CFFKFSSKIQYNKVVKAQLWIYLRPVETPTTVFVQILRLIK-PMKDGTR-Y 186
A. irradians    IYFDMQDS-PEKETNKALLWVYISPDDIIDRNMTEIYVYTIDPPGKFSKVP 182
C. intestinalis CYFTFSEQQRSFAVHSAFLWYYVRPVNTTETYALVELQRYV-ATANGPPRP 166

D. rerio        HRIRSLKIDVNAG-VTSWQSIDVKQVLTVWLKQPETNRGIEINAYDAKGND 235
S. aurata       IRVRSLKIDTDAG-AGSWQSIDIKSLLQAWLRQPETNYGIEINAYDSKGED 220
H. sapiens      TGIRSLKLDMNPG-TGIWQSIDVKTVLQNWLKQPESNLGIEIKALDENGHD 236
A. irradians    TKREIGRRKRHYMKASGWHHFDILDEVQKWTYRTHLNLGLVVEALDETGHN 233
C. intestinalis VWVRSLRVSLNGSSKGFWRKIRVTGLVNGWLERPSYNLGIRIISRG--LSD 215

                                                       *        **
D. rerio        LAVTSTETGEDG-LLPFMEVKISEGPKRIR-RDS--GLDCDENSSESRCCR 283
S. aurata       LAVTSAEPGEEG-LQPFIEVKILDNPKRSR-RDS--GLNCDEESAETRCCR 268
H. sapiens      LAVTFPGPGEDG-LNPFLEVKVTDTPKRSR-RDF--GLDCDEHSTESRCCR 284
A. irradians    LVVLPPTFGDDDGYEPMLDLRTSLRKSTRSKRST--ELYCDTR-EETACCR 281
C. intestinalis LVMTESRVGEKNNQKPFMDVELVARTRRRRKRDAAEGLRCDANSTETRCCR 266

                                         *   *
D. rerio        YPLTVDFEDFGWDWIIAPKRYKANYCSGECDYMYLQKYPHTHLVNKAS--- 331
S. aurata       YPLTVDFEEFGWDWIIAPKRYRANYCSGECEFMHLQQYPHAHLVNKAN--- 316
H. sapiens      YPLTVDFEAFGWDWIIAPKRYKANYCSGECEFVFLQKYPHTHLVHQAN--- 332
A. irradians    YPLEVDFVAFGWDFVIAPLTYAAYYCAGECKGEQLDDTLHAHVIQQAPSPT 337
C. intestinalis FDLDINFNAAGWNWIIFPRTYNARYCSGECAYLQLSNTREQLIRHMGNRPD 317

                             **                               * *
D. rerio        ---PRG---TAGPCCTPTKMSPINMLYFNGKEQIIYGKIPSMVVDRCGCS- 375
S. aurata       ---PRG---TAGPCCTPTKMSPINMLYFNRKEQIIYGKIPSMVVDHCGCS- 360
H. sapiens      ---PRG---SAGPCCTPTKMSPINMLYFNGKEQIIYGKIPAMVVDRCGCS- 376
A. irradians    LSQPQSAISNVGPCCTPTKMSDLAMLFFDHNSNIALTRLPRMKVDRCGCA- 382
C. intestinalis L--PSSR--HVENCCVPKRMIGISMLYYDNDGNIIFSKVPDMEIVKCDCL- 363
```

Figure 2

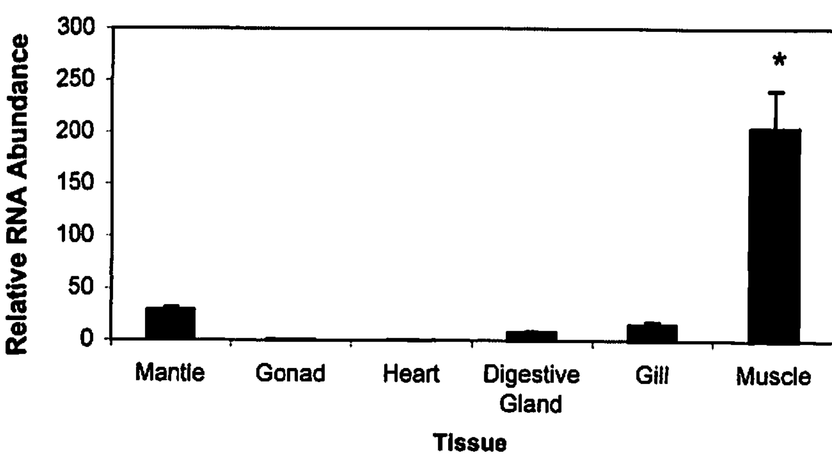

Figure 3