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

OMB Approved 0524-0039
Expires 03/31/2004

PROPOSAL COVER PAGE

1. LEGAL NAME OF ORGANIZATION TO WHICH AWARD SHOULD BE MADE	3. NAME AND TITLE OF AUTHORIZED ORGANIZATIONAL REPRESENTATIVE (AOR)
Marine Biological Laboratory	Richard J. Mullen, Ph.D Manager, Research Administration

2. ADDRESS (Give complete mailing address and Zip Code)	4. a. Telephone No.:	b. Fax Number:	c. E-mail Address:
Marine Biological Laboratory 7 MBL St. Woods Hole, MA 02543-1015	508-289-7691	508-289-7931	rmullen@mbl.edu

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
04 ☐ 1890 Land-Grant University (including Tuskegee Univ.)
05 ☐ 1994 Land-Grant University
06 ☐ Private University or College
07 ☐ Non-Land-Grant Public University or College
08 ☐ Private For-Profit
09 ■ Private Non-Profit
10 ☐ Public Secondary School
11 ☐ State, Local or Tribal Government
12 ☐ Individual
13 ☐ Other _____

6b. In addition, PLEASE CHECK ANY OF THE FOLLOWING THAT APPLY:

☐ Alaska Native-Serving Institution
☐ Cooperative Extension Service
☐ Native Hawaiian-Serving Institution
☐ Hispanic-Serving Institution
☐ Historically Black College or University (other than 1890)
☐ School of Forestry
☐ State Agricultural Experiment Station
☐ Tribal College (other than 1994)
☐ Veterinary School or College

7. TITLE OF PROPOSED PROJECT (140-character maximum, including spaces)

The role of myostatin in shellfish growth

8. PROGRAM TO WHICH YOU ARE APPLYING (Include Program Area and Number: Refer to Federal Register announcement or program solicitation where applicable)	9. TAX IDENTIFICATION NO. (TIN)	10. CONGRESSIONAL DISTRICT NO.
Animal Growth and Nutrient Utilization 42.0	04-2104690	10th

11. DUNS NO. (Data Universal Numbering System)	12. PROPOSED START DATE	13. DURATION REQUESTED (No. of months)
001933779	10/01/05	36

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)

$241,207

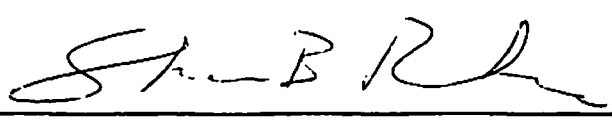
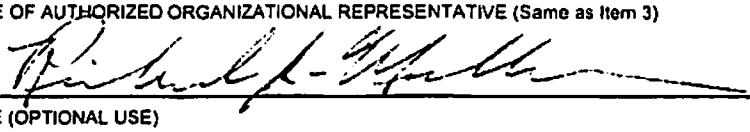
16. PROJECT DIRECTOR (PD)	17. PD BUSINESS ADDRESS (INCLUDE DEPARTMENT/ZIP CODE)
Steven Beyer Roberts	Marine Biological Laboratory 7 MBL St. Woods Hole, MA 02543-1015

18. a. PD Phone No.:	b. PD Fax No.:	c. PD E-mail Address:
508-289-7686	508-289-7900	sroberts@mbl.edu

19. CO-PD(s) NAME	TELEPHONE NUMBER	E-MAIL ADDRESS

20. IF THIS IS A RESEARCH PROJECT, WILL IT INVOLVE RECOMBINANT DNA, HUMAN SUBJECTS, OR LIVING VERTEBRATE ANIMALS?	21. WILL THIS PROJECT BE SENT OR HAS IT BEEN SENT TO OTHER FUNDING AGENCIES, INCLUDING OTHER USDA AGENCIES?
☐ No ■ Yes (If yes, complete Form CSREES-2008)	■ No ☐ Yes (If yes, list Agency acronym(s) & program(s))

By signing and submitting this proposal, the applicant is providing the required certifications set forth in 7 CFR Part 3017, as amended, regarding Debarment and Suspension and Drug-Free Workplace; and 7 CFR Part 3018 regarding Lobbying. Submission of the individual forms is not required. (Please read the Certifications included in this booklet before signing this form.) In addition, the applicant certifies that the information contained herein is true and complete to the best of its knowledge and accepts as to any award the obligation to comply with the terms and conditions of the Cooperative State Research, Education and Extension Service in effect at the time of the award.

SIGNATURE OF PROJECT DIRECTOR(S) (All PDs listed in blocks 16 or 19 must sign if they are to be included in award documents.)	DATE
	May 16, 2005
SIGNATURE OF AUTHORIZED ORGANIZATIONAL REPRESENTATIVE (Same as Item 3)	DATE
	May 16, 2005
SIGNATURE (OPTIONAL USE)	DATE
	May 16, 2005

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE
NATIONAL RESEARCH INITIATIVE COMPETITIVE GRANTS PROGRAM

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UNITED STATES DEPARTMENT OF AGRICULTURE

OMB Approved 0524-0039
Expires 03/31/2004

COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

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
  [ ] Standard Strengthening

For Higher Education Program Proposals Only:

Need Area: _____

Discipline: _____

Project Director(s) (PD):

PD Steven Roberts Institution Marine Biological Laboratory

CO-PD _____ Institution _____

CO-PD _____ Institution _____

CO-PD _____ Institution _____

Project Title: The role of myostatin in shellfish growth

Key Words: bay scallop, myostatin, shellfish, muscle, metamorphosis, growth

(Approximately 250 words)

PROJECT SUMMARY

Rising demand for seafood products along with the inability of wild fisheries to meet demand has resulted in increased reliance on aquaculture. Production of marine shellfish is one of the fastest growing segments of the aquaculture industry and includes some of the highest valued farmed marine animals, yet there is relatively little known concerning the genetic and endocrine mechanisms controlling growth. Increased growth rates in shellfish would have significant impacts on the industry by reducing resources required to obtain market size animals. Recently, myostatin, a member of the transforming growth factor-beta superfamily, was identified in a commercially important shellfish, the bay scallop (Argopecten irradians). In vertebrates, myostatin is clearly an important regulator of growth and development. Animals in which functional myostatin is either downregulated or inactivated demonstrate dramatic increases in growth rates and muscle mass. Myostatin activity in vertebrates appears to be highly regulated at the level of post-translational modifications. Mature myostatin binds non-covalently to its propeptide following proteolytic cleavage, producing an inactive complex incapable of binding to responsive cells, thus increasing growth. While myostatin has been initially characterized in the bay scallop, there is limited information on transcript expression and nothing is known about the myostatin protein. The long-term goal of the current research proposal is to characterize the role of myostatin in regulating shellfish growth. The specific objectives associated with this goal include 1) characterizing the expression of myostatin transcript and protein in larval and adult bay scallops and 2) treating developing and adult scallops with recombinant myostatin propeptide. In order to characterize protein and transcript expression patterns, Western blot analysis and quantitative real-time RT-PCR will be carried out. Expression will be analyzed during metamorphosis and seasonally in adults to examine correlations with environment, growth, and reproduction. Recombinant myostatin propeptide will be used to treat larvae and adults to begin evaluating the functional role of myostatin in scallops. Upon completion of these combined objectives, we will have a better understanding of myostatin's role in growth regulation and other parameters influencing scallop growth. This information will be essential for future development of products and aquaculture practices that could alter myostatin expression and increase production. Given the relatedness of scallops with other commercially important shellfish, it is likely the results of the current proposal will have broad application for species such as oysters, clams, and mussels.

Project Description: Introduction

In the United States, mariculture will provide the most likely avenue for expanding the aquaculture industry. In particular, the culture of shellfish such oysters, clams, mussels, and scallops has increased and has the potential to make significant economic impacts. In the United States, revenue from combined shellfish production has bypassed that from the salmon industry and shellfish are one of the highest valued farmed marine products (FAO 2001). However, for shellfish there is relatively little information on genetic and endocrine factors involved in regulating commercially important traits such as growth.

One growth factor that has received considerable attention as an important regulator of development and growth in mammals is myostatin (McPherron et al. 1997; Lee and McPherron 1999; Lee and McPherron 2001). Myostatin is a member of the transforming growth factor-β (TGF- β) superfamily, and was first characterized in mice (developing somites), where disruption of this gene resulted in a significant increase in muscle mass (McPherron et al. 1997). Myostatin negatively regulates muscle cell proliferation by inhibiting cell cycle progression (Thomas et al. 2000). Overexpression of myostatin down-regulates muscle regulatory proteins, like MyoD and myogenin (Rios et al. 2002) while the removal or inactivation of myostatin results in increased cell proliferation (Thomas et al. 2000). Myostatin knock-out mice were shown to have increased skeletal muscle mass due to both hyperplasia and hypertrophy (McPherron et al. 1997). Naturally occurring mutations in myostatin have been attributed to the 'double muscle' phenotype observed in some breeds of cattle (i.e. Belgian Blue; (McPherron and Lee 1997)). Originally, it appeared that myostatin was only expressed in skeletal muscle. Though there are limited reports of myostatin in other tissues including cardiomyocytes (Sharma et al. 1999) and porcine mammary glands (Ji et al. 1998).

Myostatin activity in vertebrates appears to be highly regulated at the level of post-translational modifications. Members of the TGF-β superfamily, including myostatin, are synthesized as pre-propeptides that are activated by two proteolytic cleavages. The removal of the signal sequence is followed by cleavage at a tetrabasic processing site resulting in a biologically active C-terminal domain. Myostatin's action on target cells is ultimately carried out once the C-terminal, mature ligand binds to a receptor and initiates a downstream signal transduction pathway (Figure 1). Mature myostatin binds non-covalently to its propeptide following proteolytic cleavage, producing an inactive complex incapable of binding to responsive cells (Lee and McPherron 2001).

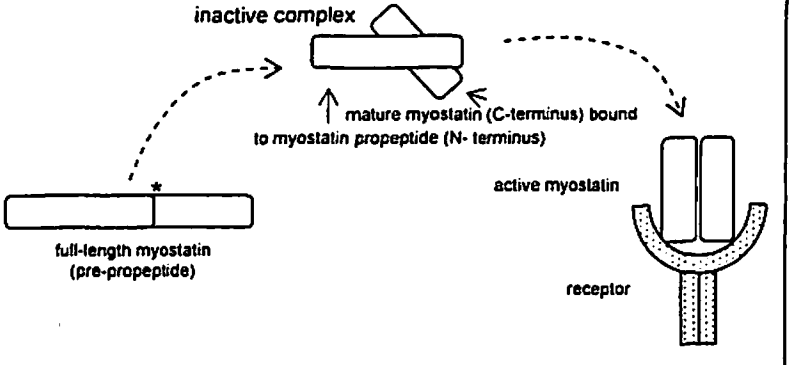


Figure 1. Schematic showing myostatin protein processing. Myostatin pre-propeptide is proteolytically cleaved at processing site (indicated by asterisk). Mature myostatin (white) can non-covalently bind to propeptide (grey), producing an inactive complex. Ultimately, mature myostatin binds to a receptor, initiating signal transduction pathway.

In addition to mammals and birds, myostatin has been isolated from many fish species, including zebrafish (McPherron et al 1997), salmonids (Rescan et al. 2001, Ostbye et al. 2001, Roberts and Goetz 2001), tilapia, white bass (Rodgers et al. 2001), catfish (Kocabas et al 2002),

and sea bream (Maccatrozzo et al. 2001). Myostatin distribution in fish appears to be more varied compared to mammals. For example, using RT-PCR, Rodgers et al. (2001) showed that myostatin is expressed in multiple tissues including eyes, gill filaments, ovaries, gut, brain and testes of tilapia. The PD of the current proposal (Roberts) was one of the first researchers to characterize myostatin expression in salmonids (Roberts and Goetz 2001) and has since examined transcript and 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). Without the ability to knock-out genes in fish, a phenotype similar to what has been observed in mice and cattle has not been observed in fish. However, correlative data associating increased growth (attributed to growth hormone overexpression) with decreased myostatin expression (e.g. Roberts et al. 2004) along with results from other research using transgenic zebrafish overexpressing the myostatin propeptide (Xu et al. 2003) suggests that myostatin does inhibit muscle growth in fish.

As part of a recently completed USDA-NRI award, Roberts (PD) along with colleagues from Colorado State University described a myostatin homologue from the bay scallop (*Argopecten irradians*) (see Progress Report, pg 5). Bay scallops are hermaphroditic, bivalve molluscs with an enlarged adductor muscle, which constitutes the primary consumer food product. While scallops do not make up the largest percent of the shellfish aquaculture industry, they have a significant potential for expansion given the high demand and fast growth rates (see Rationale, pg 8, for more details). The bay scallop myostatin sequence is most similar to mammalian myostatin, containing the conserved tetrabasic proteolytic cleavage site (RXXR) and conserved cysteine residues in the C-terminus. Based on quantitative RT-PCR, the myostatin gene is predominantly expressed in the adductor muscle.

While these combined results suggest the function of myostatin is conserved through evolution, there is little known about myostatin in shellfish. In particular, there is nothing known about the myostatin protein or the function of myostatin in shellfish. Therefore, the overall goal of the proposed research is to determine role of myostatin in shellfish growth. In the long-term, this information could provide the basis for improved hatchery practices and selection programs that will ultimately advance the shellfish aquaculture industry. Given that there are specific differences in the biology and culture practices of shellfish compared to the majority of commercially important agricultured animals, the remaining part of the *Introduction* will discuss information on scallop aquaculture.

Unlike other animals that are commonly produced for consumption (cows, pigs, and fish) shellfish undergo a complete metamorphosis during production. *It should be pointed out that for the current proposal the term shellfish specifically refers to bivalve molluscs.* In bivalves, free-swimming larvae metamorphose into juvenile shellfish that resemble what most consumers are familiar with (two shells encapsulating soft tissue). Bay scallops go through several major changes during development. A free swimming trochophore larva develops into a veliger larvae. The veliger is somehow triggered to enter the competent phase and will then go through metamorphosis approximately 2 weeks after hatching and transform into the body form of an adult scallop. In general, metamorphosis in scallops is marked by a change in shell shape and structure. In addition, major organs become more developed and are relocated, while others, such as the velum, velar retractor muscle, and the anterior adductor muscle are lost entirely. The organs that are maintained through metamorphosis migrate relative to the axes of the shell. For example, the mouth moves from a posterior-ventral location in the larvae to an anterio-dorsal

position in the adult and the foot migrates to a more ventral position. As the anterior adductor muscle is lost, the posterior adductor muscle moves to a more central position and enlarges.

The culture of shellfish has to accommodate for 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). A flow chart describing the different stages of bay scallop aquaculture is shown in Figure 2. 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 shellfish production is relatively new. It has only been in the past decade that many of the physical constraints faced in all phases of shellfish culture have been overcome, including optimization of environmental parameters in hatcheries and engineering of structures to hold adult shellfish (Gosling 2003). While some species of shellfish 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 shellfish to reach a harvestable size is a critical goal for the aquaculture industry.

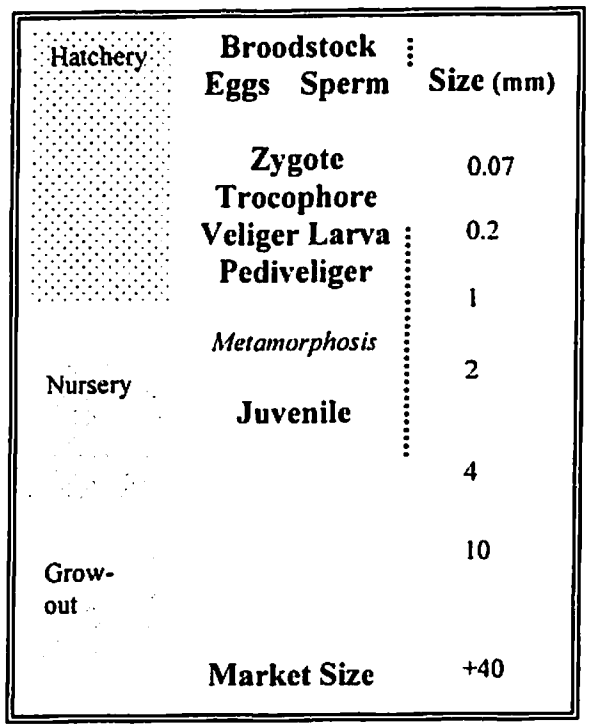


Figure 2. Flow chart outlining the stages of bay scallop development. Culturing facilities are noted at the left and approximate size to the right. Dashed lines indicate when algae is used to supplement the food supply.

Project Description: - Progress Report

The PD on the current proposal has recently completed research on an award from the USDA-NRI Animal Growth and Nutrient Utilization program entitled: ***Isolation and characterization of factors regulated during larval competence and metamorphosis in the bay scallop, Argopecten irradians*** (Grant no. 2003-35206-12834). The overall goal of this post-doctoral fellowship was to identify genes and molecular pathways involved in development and growth of the bay scallop. The specific objectives of this award were to 1) use a subtractive cloning approach to isolate upregulated and downregulated genes during development and 2) use a RT-PCR approach with degenerate primers to isolate candidate genes based on sequence homology.

Suppression-subtraction hybridization was one of the first techniques carried out, uncovering genes regulated in scallops following metamorphosis, including a novel elongation factor homologue. In analysis of the data, high levels of non-coding transcripts containing significant poly(A) regions were identified. This phenomenon would likely affect discovery of factors regulated at low levels. Therefore a second approach was taken that involved arbitrary isolation of differentially expressed genes (DEGs). Developing bay scallops samples were taken throughout development ("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.

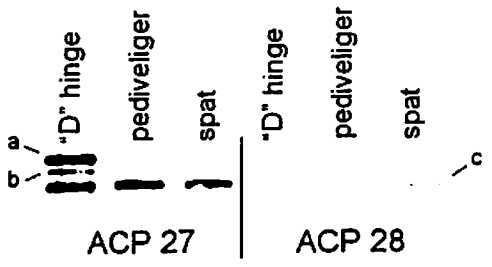


Figure 3. PCR products run out on agarose gel, showing differentially expressed genes (lower case letters) in developing bay scallops (N=~100k).

The PCR products representing regulated genes are indicated with lower case letters and have been sequenced. The proteins with the highest degree of similarity (BlastX) with the deduced amino acid sequence of each band are; "a" - heat shock protein 70 [AAO38780], "b" - Chymotrypsin-like serine proteinase precursor [P35003], and "c" - pheromone receptor Rcb3 B47 [AAQ96349]. A short non-peer reviewed paper describing these data and associated techniques has been published; ***Roberts SB (2004) Lab Studies: Genes Involved with Growth, Development of Bay Scallops. Global Aquaculture Advocate. 7(3): 55-56***

While not specifically described in the post-doctoral grant proposal, an Expressed Sequence Tag (EST) approach was carried out in order to further identify genes expressed during different stages of bay scallop development. Thousands of ESTs were generated from three cDNA libraries from whole body tissue taken at different developmental stages (prior to, during, and following metamorphosis). Two additional cDNA libraries were generated from adductor muscle and gonad tissue and partially sequenced. Nucleotide sequences have been annotated and released in NCBI's GenBank. The ESTs identified from the adductor muscle were described in a publication that came out during the first year of the award: ***Roberts SB, Goetz FW. (2003) Expressed sequence tag analysis of genes expressed in the bay scallop, Argopecten irradians. Biological Bulletin. 205: 227-228.***

A large number of novel transcripts were identified from the developing larvae cDNA libraries including genes involved in organogenesis (e.g. glutathione peroxidase), growth (e.g. tax-responsive element binding protein) and metamorphosis signaling (e.g. gamma-aminobutyric acid receptor-like protein, type II thyroxine deiodinase). The identification of a thyroxine deiodinase is of particular importance as it suggests a role for thyroid hormone in shellfish metamorphosis. Thyroxine deiodinase is a selenoprotein that is the key enzyme responsible for conversion of thyroxine (T4) to triiodothyronine (T3). Selenoproteins contain selenocysteine insertion sequence (SECIS) elements that direct cells to translate UGA (normally a stop codon) as selenocysteine. The bay scallop thyroxine deiodinase has a perfect SECIS element and quantitative real-time RT-PCR analysis verified that levels of this thyroxine deiodinase homologue were elevated just prior to metamorphosis. Along with colleagues from the NOAA Northeast Fisheries Science Center – Milford, CT (Wikfors and Dixon), we were able to

demonstrate that metamorphosis could be induced 4 days earlier in larvae switched from a strict *Isochysis* diet to a diet supplemented with *Pavlova* (CCMP 459). Interestingly, *Pavlova* micro-algae contains about three time more (%) phenylalanine than *Isochysis* (control diet). Phenylalanine is the essential amino acid necessary for production of triiodothyronine (T3). Currently we are preparing a manuscript characterizing thryoxine dieodinase in the bay scallop; ***Roberts S, Dixon M, Wikfors G, Romano C (2005) Characterization of a thyroxine dieodinase from the bay scallop (Argopecten irradians) and the role of thyroid hormone in controlling metamorphosis. In preparation.***

Another substantial product from the bay scallop EST project is a suite of simple sequence repeat (SSR) or microsatellite markers. Eight polymorphic SSRs were identified from the bay scallop ESTs and include one dinucleotide repeat, six trinucleotide repeats, and one compound dinulceotide repeat. These molecular markers are the first ones to be described for the bay scallop (***Roberts SB, Romano C, Gerlach G. (2005) Characterization of EST derived SSRs from the bay scallop, Argopectens irradians. Molecular Ecology Notes. In press).*** To date, these molecular markers have successfully demonstrated the contribution of hatchery reared bay scallop seed in regional enhancement efforts. These markers will also be essential tools for managing hatchery strains and the genetic mapping of commercially important traits.

The second research objective of the post-doctoral fellowship award was to use a RT-PCR approach with degenerate primers to isolate candidate genes involved in development and growth. The effort associated with this approach was reduced somewhat given the number of novel genes identified as part of the EST project. However, RT-PCR was successfully used to identify a myostatin homologue from the bay scallop. ***Kim H-W, Mykles DL, Goetz FW, Roberts SB. (2004) Characterization of a myostatin-like gene from the bay scallop,(Argopecten irradians) BBA – Gene Structure and Expression. 1679(2):174-9 (attached as reprint)*** This gene codes for a 382 amino acid myostatin-like protein. The myosatin 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 gene is predominantly expressed in the adductor muscle, with limited expression in other tissues (Figure 4). It is unclear what function myostatin has in other tissues such as the mantle. One possibility is that myostatin acts as a chalone to synchronize tissue and shell growth. Using the bay scallop myostatin sequence, a *Ciona* myostatin-like gene was virtually cloned from the *Ciona intestinalis* genome and described (Kim et al. 2004). 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.

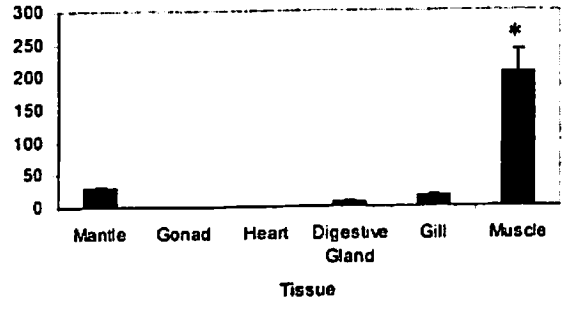


Figure 4. Relative RNA abundance of scallop myostatin in various tissues from bay scallops. Vertical bars represent the mean ±S.E. (N = 3) for each tissue. Significant differences across tissue are indicated with an asterisk at $P \leq 0.05$.

Project Description: Additional Preliminary Data

Following the characterization of a myostatin homologue in the bay scallop, the PD's lab has continued to examine growth and myostatin in bay scallops. One experiment that was carried out by a student, Adam Bissonnette (Saint Anselm College, NH), was treating scallops with a dietary supplement advertised as a "Myostatin Neutralizing Growth Factor Complex". The main ingredient is 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 was hypothesized that since the scallop's diet is primarily algae, this compound would affect growth. After five days of treatment (600 mg MyoZap CSP3 / day via 4 hour immersion feeding) MyoZap exposed and control scallops 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 5. Bands "b" and "d" have been putatively identified based on DNA sequencing and are arginine kinase and cyclin T, respectively. Even though this data is preliminary, the possibility that myostatin decreased in the treated samples and a downregulation of cyclin T occurred is interesting as it coincides with what has been shown in mammalian systems. One suggested mechanism of action of myostatin is 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 be important in muscle growth (Sano and Schneider 2003)) suggest that myostatin's role in shellfish is similar to that in other organisms.

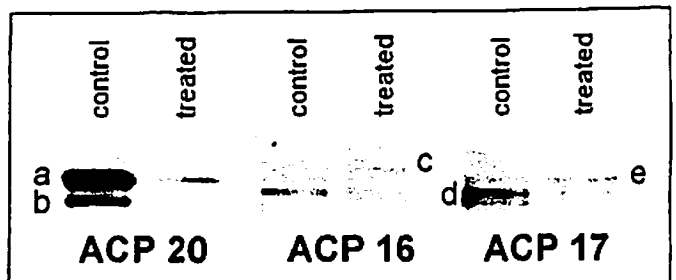


Figure 5. PCR products from RNA from adult scallops (N=2) treated with MyoZap and not treated (controls). Differentially expressed genes are indicated with lower case letters.

This experiment was carried for an additional 6 weeks (two treatments / week) with the remaining scallops. Throughout the experiment morphometric data was documented and at the end of the experiment, weights for individual adductor muscles were recorded. At the end of the experiment there was no significant difference in adductor muscle mass between scallops treated with MyoZap and controls. In the event that any phenotypic effects might be observed at the cellular level, muscle samples were taken for histological analysis (Figure 6). No statistical analysis was done given the low sample size (n=5) and the absence of dramatic gross differences across treatments. Interestingly, samples from treated scallops did appear to have more muscle fibers (T1) and larger fibers (T4) than controls. Instead of repeating similar experiments the PD has decided to focus more on endogenous proteins that could regulate growth in bay scallops.

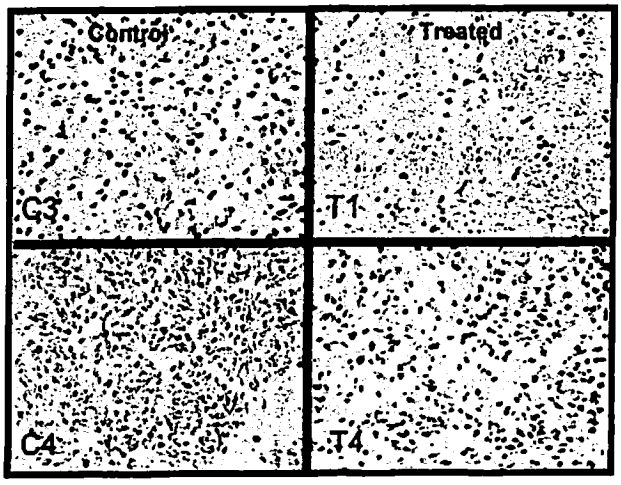


Figure 6. Photomicrographs (630x) of hematoxylin and eosin stained adductor muscle tissue from bay scallops treated with MyoZap (T1, T4) and controls (C3,C4).

Currently, Roberts (PD) is actively producing and purifying recombinant bay scallop myostatin proteins that encode for the propeptide and the mature peptide. Recombinant protein is produced using the pBAD TOPO system (Invitrogen). In this system, a V5 epitope and a polyhistidine region is expressed in addition to the recombinant protein. A His-Patch (HP) thioredoxin leader sequence is expressed for increased translation efficiency and solubility. Protein production is induced by the addition of arabinose. The myostatin propeptide was generated using two primers, mLAP_F (CAC CAT TGA ATA TGA GTA TGA CAT G) and mLAP_R (CCT CTT AGA GCG TGT AGA CTT CCT) to PCR amplify nucleotides 275–1087 of bay scallop myostatin (GenBank accession no. AY553362). The mature, active myosatin (aa: 266-382 bay scallop myostatin GenBank accession no. AAT36326) was generated using two primers, m_F (CAC CTC GAC GGA ACT CTA CTG TGA C) and m_R (CGC ACA ACC ACA TCT GTC TAC T) to PCR amplify nucleotides 1088-1438 of bay scallop myostatin (GenBank accession no. AY553362). Both plasmids were sequenced to verify inserts were in frame. Recombinant proteins were initially produced on a small scale (i.e. Figure 7.B) followed by large scale production (i.e. Figure 7.A). An antibody to the V5 epitope is being used to validate and quantify expression. Figure 7.A includes a coomassie stained protein gel and Western blot of the myostatin propeptide harvested at different times post-induction. Figure 7.B represents a coomassie gel and Western blot of small scale cultures of the mature myostatin peptide, pre (lanes g) and post (lanes f) induction. Using a ProBond Purification System (Invitrogen) we have been able to purify the myostatin propeptide under both hybrid and native conditions according to the manufacturer's protocol (Invitrogen). During May and June of 2005 we plan on continuing purification of both bay scallop myostatin recombinant peptides. In the event that the current proposal is funded these proteins will be available for antibody production and for treatment of developing and adult bay scallops (see Approach, pg. 10).

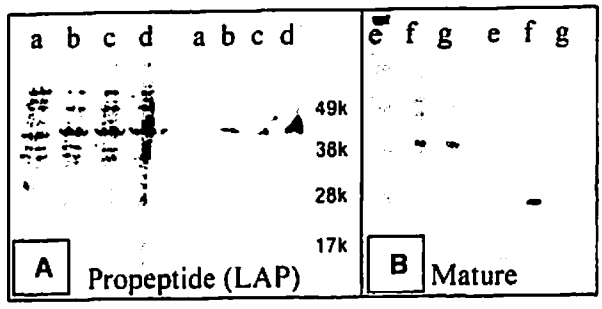


Figure 7. Coomassie stained protein gels and corresponding Western blots (V5 epitope antibody) of recombinant scallop myostatin peptides. Plate A represents propeptide taken pre (a), 1 hr post (b), 2 hr post (c) and 4 hr post (d) induction. Plate B represents small scale cultures of mature myostatin peptide taken prior to (g) and following (f) induction. Lane e is the protein size standard.

Project Description: Rationale and Significance

The global demand for seafood is on the rise. Under the assumption that global seafood demand will increase at a constant rate as the population increases, it is projected that the seafood demand by 2025 would be 156.5 million metric tons, in comparison with 97.4 metric tons in 1990 (US Joint Subcommittee on Aquaculture 2000). Unfortunately, the United States is dependent 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 $10.1 billion of edible seafood in 2002. In contrast, the U.S. only exported $3.3 billion in fisheries products for a deficit of $6.8 billion (Vannuccini 2004). 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 shellfish. One reason for focusing on shellfish are that they 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 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. Shellfish 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 shellfish in open waters can have a substantial effect by removing harmful nutrients.

From an economic standpoint, it is clear that the culture of shellfish 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 shellfish production has bypassed that of the salmon industry and is the highest valued farmed marine animal (FAO 2001). Another important distinction concerning shellfish that has both environmental and economic implications is their diet. Shellfish consume phytoplankton, either cultured during hatchery stages or from the wild during grow-out. Therefore, in contrast to other farmed animals, there is not a reliance on expensive commercial grain and fish meal based diets susceptible to price fluctuations.

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. One dramatic example of demand for scallops is the current import / export figures. According to the Department of Commerce, $162 million in scallops (all species) were imported to the United Stated compared to $55 million in exports (NMFS 2004). 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.

Recently there has been increased attention towards bay scallop research that will facilitate the interpretation of any new findings. For example, since 2001 the number of nucleotide sequences available in NCBI's GenBank (http://www.ncbi.nlm.nih.gov/) has increased from less to 20 to over 7,000. This is largely a result of the USDA funded EST project (described above, pg 4) and by a similar effort in China (NCBI website and personal communication, Ximing Guo). Both groups have also developed a number of molecular markers that will prove to be valuable for aquaculture production.

Why focus on growth?

Rapid growth of scallops is one the primary goals of selective aquaculture breeding programs for several reasons. Decreasing time to harvest would have significant impacts at both the juvenile and adult states. If the length of time larvae are maintained in a hatchery is shorten this would dramatically decrease the high cost of algae culture. Once juvenile scallops are moved from the hatchery to the field, size is a threshold from predation (Tettelbach 1988, Pohl et al. 1991). Along the same lines, the ability to remove adult scallops from open waters sooner will decrease potential exposure to severe environmental conditions and disease, factors that contribute to high rates of mortality. Under current practices a limited number of scallops can reach market size by late fall but a majority of the scallops have to continue to "grow-out" during winter months. During winter, little to zero growth occurs and a significant number of scallops will die (Oesterling 1998, Wikfors et al. 1998). While the actual number of individuals that are lost during the winter might not be as great as early in development, the financial loss is far greater as a considerable investment (facilities, equipment, time) has been put into each scallop by that point. 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 Shumway 1991, Thompson and MacDonald 1991, Gosling 2003). Variability for growth within a single population and observations from full-sib and mass spawning in the hatchery also indicates a strong internal component. However, there has been limited research examining the endocrine and physiological regulation of growth in shellfish. Thus, the overall goal of the current proposal is to better understand the role of the recently identified myostatin homologue in bay scallop growth. The specific research objectives of the current proposal are to:

1) Determine myostatin transcript and protein expression patterns in bay scallops and
2) Characterize effects of recombinant myostatin propeptide treatments

This fundamental information regarding the role of myostatin in scallop growth will lay the groundwork for the possible development of products and husbandry practices to increase production. Specific examples of efforts that could benefit from the research proposed here include the development of marker assisted selection programs and efforts to identify myostatin interacting proteins in common micro-algae strains. However it is essential that we have a complete understanding of myostatin expression in scallops prior to initiating such efforts. Given the relatedness of scallops with other commercially important shellfish, it is likely the results of the current proposal will have broad application for species such as oysters, clams and mussels.

Project Description: Approach

1) Myostatin transcript and protein expression profiling in developing and adult bay scallops

Myostatin has been established as an important regulator of development and growth in vertebrates and has demonstrated significant implications for increasing growth in farmed animals. Based on research results from a recently completed USDA-NRI award, myostatin's structural and general tissue expression patterns have been shown to be conserved in shellfish (Kim et al. 2004, attached as reprint). However, there is no information regarding transcript expression during development and in regards to reproductive maturation. This information is necessary when considering manipulating conditions associated with myostatin to improve growth. There is also nothing known about myostatin protein expression in shellfish. Given the apparent importance of post-translational modification on myostatin action (based on mammalian systems) it is essential that we have a better understanding of this phenomenon in shellfish.

Myostatin transcript and protein expression will be characterized in developing larvae. This will be the first characterization of myostatin during invertebrate metamorphosis. Based on expression in mammals and finfish, it is expected that expression will correspond to significant developmental events. In cattle, myostatin is expressed from Day 16 of gestation throughout development (Bass et al 1999). Expression patterns in cattle directly correlate to the gestational period at which primary myoblasts are starting to fuse and differentiate into myofibers and the secondary myoblasts are initially proliferating and fusing. In chickens, myostatin mRNA was first detected at the blastoderm stage, embryonic day (E) 0 and highest levels were seen on E1 (Kocamis et al 1999). Myostatin mRNA levels declined about 5 fold by E2 and then increased 3-fold at E7, remaining at high levels through E19. In brook trout, significant increases in myostatin mRNA and protein were observed just prior to yolk-sac resorption (Roberts and Goetz 2003). In trout, the period of yolk-sac resorption corresponds to the formation of adult red muscle fiber and hyperplasia in white muscle. Roberts and Goetz proposed that the expression of myostatin at this time is related to when the embryo first begins to use the somatic musculature for locomotion. Regarding the bay scallop, it is expected that transcript and protein expression will be regulated during development events such as when the anterior adductor muscle is lost and the posterior adductor muscle moves to a more central position and enlarges.

In order to evaluate how environment, growth and reproductive status influence myostatin, adductor muscle samples will be taken at three time points for analysis. Scallops will be sampled in December, April and June during the first year of the proposed research. Thus, samples will be taken from scallop prior to, during and at peak reproductive maturation. A primary dogma concerning biological resource flux suggests that somatic growth is influenced by gonad development, and vice versa. For example, growth rates in salmonid fish decrease during spawning (Tveiten et al. 1998). One theory is that during reproductive maturation, energy resources are diverted from somatic growth to meet requirements of gonad maturation. Therefore one would expect higher expression of myostatin during sexual development. In trout, where a similar sampling regime was carried out, this was the case for protein expression, however myostatin transcripts decreased during spawning. Without an antibody to the propeptide in trout it has been difficult to fully characterize myostatin regulation. In the current proposal, for protein analysis in adults (and developing larvae) Western analysis will be performed using polyclonal antibodies directed against the propeptide and mature peptide. By simultaneously quantifying the various forms of myostatin, we will have a more complete

understanding of post-translational regulation. In order to determine cellular expression of myostatin in scallops, immunocytochemistry will be performed.

Methods: Adult Sampling

To characterize seasonal changes in myostatin transcript and protein expression in shellfish, muscle tissue will be analyzed 1) prior to sexual maturation, 2) during gonad maturation and 3) at spawning. Collection of adult scallops will take place early in year one (Fall 2005) of the proposed project. Scallops will be maintained in the Marine Resources Center on ambient water throughout the winter. In early December, 30 scallops will be harvested with adductor muscle tissue snap frozen and stored at -80°C for later analysis. At this first sampling, gonads will be immature. In March, broodstock conditioning will begin so that a controlled spawning event can be carried out to facilitate larval sampling (see following section). In April gonad tissue will be maturing and a second sample will be taken (n=30). Gonadal maturation can easily be monitored in the bay scallop by the color of the gonad. As previously mentioned bay scallops are hermaphrodites with testicular and ovarian tissue surrounded by a common epithelium. When the single gonad is immature this epithelium is black and opaque. As the gonad matures, the male portion of the tissue becomes white whereas the female portion slowly increases in orange intensity. The clasping action of a scallop's shell makes it easy to document this maturation. By approximately 8 weeks bay scallops will be sexually mature and ready for spawning. During spawning the third sampling (n=30) will occur, with adductor muscle tissue stored at -80°C for later transcript and protein analysis.

Table 1. Description of adult and larval bay scallops taken for transcript and protein analysis. Adductor muscle tissue will be taken at three times from 30 individuals at each sampling. For developing larvae a "sample" will include approximately 1500 individuals pooled in order to get enough biological material. Samples will be taken from 3 separate systems at each sampling to account for any tank specific effects (x3).

	Sampling Time	Development Stage	N
Adult	December	sexually immature	30
	April	during maturation	30
	May-June	spawning	30
Larvae	48 hr pf (x3)	pre-metamorphosis	1500
	4 day pf (x3)	pre-metamorphosis	1500
	6 day pf (x3)	pre-metamorphosis	1500
	8 day pf (x3)	pre-metamorphosis	1500
	10 day pf (x3)	metamorphosis	1500
	12 day pf (x3)	metamorphosis	1500
	14 day pf (x3)	metamorphosis	900
	16 day pf (x3)	post-metamorphosis	900
	18 day pf (x3)	post-metamorphosis	900

Methods: Larval Sampling

In order to obtain samples for larvae analysis, a controlled spawn will be carried out at the Marine Biological Laboratory. This will insure a high quality supply of samples for myostatin characterization. As described above, in March of Year 1, broodstock conditioning will begin to facilitate successful spawning by early June. Broodstock conditioning involves

gradually increasing the water temperature in the holding systems approximately 0.5°C/day until a temperature of 18°C is reached. In addition, throughout conditioning, the diets will be supplemented with uni-cultured micro-algae (i.e. *T. isochysis*, *T. chui*) maintained year-round at the Marine Resource Center. Gonad development will be monitored by visual inspection. Based on the progress of sexual development, a mass spawning event will take place in late May – early June. Release of gametes will be induced by successive fluctuations of water temperature (~22-15°C). Based on prior experience we expect to produce 2-20 million eggs per spawning event. The number of eggs is commonly the limiting factor in fertilization. Gametes will be separated and mixed in proper ratios and raised in static systems at 23°C. The fertilized gametes will be divided into at least three systems. Every two days larvae will be transferred to a new tank. At each transfer, samples will be taken for transcript and protein analysis. Because of the size of individuals approximately 1500 individuals will be taken per sample. Identical, pooled samples will be taken from three systems to account for inter-tank variability. Samples will be taken up until setting which will take place between day 10 and 14 post fertilization. Two additional samples will be taken following metamorphosis. All samples will be snap frozen in liquid nitrogen and stored at -80°C for transcript and protein analysis

Methods: Transcript Expression

Quantitative RT-PCR will be used to characterize myostatin gene expression in developing larvae and adductor muscle tissue. Messenger RNA will be extracted and analyzed quantitatively using real time RT-PCR in an Opticon Continuous Fluorescence Detection System (Bio-Rad / MJ Research). The PD has significant experience using this approach for quantifying myostatin expression in bay scallops (Kim et al. 2004; attached as reprint) as well as in different fish species (Roberts and Goetz 2003; Roberts et al. 2004). The specific methods are described detail (e.g. Kim et al. 2004) and are based on the manufacturer's protocols (i.e. Invitrogen). Briefly, dual-labeled probe technology will be used to asses levels of myostatin and corresponding 18s RNA values. Primer and probes used for RT-PCR are shown in Table 2. Respective plasmid preparations will be run to verify that C_T values represent 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.

Table 2. Primers and probes to be used for quantitative RT-PCR

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

Methods: Recombinant protein and antibody production

Recombinant bay scallop myostatin propeptide and mature peptide will continue to be produced and purified as described above (see Additional Preliminary Data, pg 7). Briefly, following elution from Pro-bond columns (Invitrogen) the two samples with highest concentration of recombinant protein as determined by Western anlaysis (V5 epitope), will be dialyzed for 48 hours with 20 mM Tris, ph 7.5. Samples will the lyophilized and stored at -20°C.

Once sufficient protein has been produced, samples will be sent out to a commercial lab (i.e. Rockland Immunological) for polyclonal antibody production. Generally, a rabbit will be immunized with an initial 0.5 mg of myostatin recombinant protein, followed by four booster injections at 2 week intervals. Two weeks following the last injection, the rabbit will be anesthetized and exsanquinated. Immunoglobins (IgGs) will be purified from the immune serum using DEAE Affi-Gel blue columns (BioRad).

Methods: Protein quantification

To determine levels of myostatin in bay scallop muscle and developing larvae, Western analysis will be used with the antibodies produced against scallop recombinant propeptide and mature myostatin. For Western analysis, muscle extract or whole larvae protein extract will be electrophoresed on NuPage 4–12% Bis–Tris gels (Invitrogen). Gels will be transferred to nitrocellulose membranes, blocked, and incubated with the diluted primary myostatin antibodies. The dilution of the primary antibodies will be determined empirically for each antibody. After rinsing in 1× TBS-T, the membranes will be incubated with a horseradish peroxidase labeled secondary antibody. For detection, the ChemiGlow detection kit (Alpha Innotech) will be used in conjunction with a SuperChemiNova12-bit CCD camera (ChemiImager, Alpha Innotech). Detection level linearity will be verified by image saturation analysis software. Integrated density values will be calculated, background subtracted, and all data normalized with a pooled muscle sample run in duplicate on each gel.

Methods: Immunocytochemistry.

Adductor muscle tissue will be treated for histology and immunocytochemistry. Cross-sectional samples will be taken from adult scallops and fixed for no longer than 24 hours in 10% buffered formalin (Sigma). Fixed tissue will be processed for routine paraffin embedding and 7-8 μm sections cut. Immunocytochemistry will be performed using the ABC Staining System (Santa Cruz Biotechnology) according to the manufacturer's instructions and as described in detail elsewhere (Bobe and Goetz, 2001). Briefly, sections will be deparaffinized, dehydrated and rinsed with water. Following blocking, the primary antibody will be applied and incubated overnight. Primary antibody will be washed and slides incubated with biotinylated anti-rabbit secondary antibody. Staining will be visualized by adding peroxidase substrate. Adjacent sections will be incubated with both myostatin antibodies. In addition, adjacent sections will be incubated without primary antibody as control. We will also attempt to neutralize the antibodies with recombinant myostatin and use it as another control. Adjacent sections will be stained with hematoxylin/eosin to look carefully at cytology.

Expected Outcomes and Potential Pitfalls.

Based on preliminary results demonstrating differential myostatin expression during the time of metamorphosis we expect quantitative RT-PCR will provide a comprehensive expression profile for myostatin that can be correlated with specific developmental changes. The current PD's other research has included the production of a polyclonal antibody directed against the trout mature myostatin protein. The trout antibody recognizes both the mature (14kDa) and full-length (pre-propeptide) (42kDa) myostatin. In the current proposal, two antibodies will be produced, directed against the propeptide and mature peptide. It is expected each antibody will recognize their respective post-proteolytic cleavage peptide and full-length (pre-propeptide) myosatin. The predicted molecular weights for the bay scallop myostatin pre-propeptide, propeptide and mature peptide are 45kDa, 32kDa, and 13kDa, respectively. The use of these two

antibodies will provide a better understanding of how environment, growth and reproductive status influence myostatin expression while facilitating the monitoring of post-translational processing.

One potential problem that could be encountered is the inability to detect myostatin protein expression in developing larvae. Based on protein expression patterns in salmonid embryos, protein is first detected much later than mRNA and at significantly lower levels (Roberts and Goetz 2003, Roberts et al. 2004). In addition, the fact that samples of developing larvae will have to be pooled, there is the potential that substances that could interfere with protein extraction could be present. Special consideration will be paid to adequate rinsing with sterile seawater prior to processing. At minimum, based on past research we are confident that mRNA levels can be detected throughout bay scallop development.

2) Characterizing effects of recombinant myostatin propeptide treatments

The long-term goal of the proposed research is to improve shellfish aquaculture production by optimizing growth. In order to characterize the functional role of myostatin in shellfish and begin evaluating the use of myostatin technology, the second objective of the proposed research involves treating bay scallops with the myostatin propeptide. As previously described, myostatin is proteolitically cleaved to form the mature protein (C-terminus) and propeptide (N-terminus). Binding of the mature peptide to a specific receptor initiates a cascade of events that ultimately leads to inhibition of muscle growth in vertebrates. Several proteins have been shown to interact with the mature, bioactive form of myostatin and thereby inhibit receptor activation (Figure 1). As a result, growth inhibition is decreased and progenitor muscle cells are allowed to grow and proliferate, increasing overall muscle mass. There has considerable research on myostatin interacting proteins in mammals. For example, transgenic mice over-expressing follistatin (shown to bind mature myostatin) exhibited increased muscle mass (Lee and McPherron 2001). Myostatin also interacts with follistatin-like related gene (FLRG) (Hill et al. 2002), titin-cap protein (T-cap) (Nicholas et al. 2002), growth and differentiation factor-associated serum protein-1 (GASP-1) (Hill et al. 2003), and human small glutamine-rich tetratricopeptide repeat-containing protein (hSGT) (Wang et al.2003). It is unclear to date how important these myostatin interacting proteins are *in vivo* for the regulation of myostatin activity and skeletal muscle growth.

One protein that has clearly been shown to interact with the mature myostatin ligand and effect muscle growth in mammals is the myostatin propeptide (Lee and McPherron 2001, Zimmers et al. 2002, Wolfman et al. 2003, Hill et al. 2002, Hill et al. 2003, Xu et al. 2003, Thies et al. 2001. Roberts (PD) is currently a co-PD on a recently awarded USDA-NRI grant (*Production of myostatin gene knockouts in zebrafish, and the effects of specific myostatin interacting proteins on salmonid muscle growth*). One of the goals of this proposal is to identify myostatin interacting proteins in rainbow trout to be used to enhance growth in this species. A primary candidate protein to be characterized is the trout myostatin propeptide.

As previously described, Roberts (PD) is actively producing and purifying bay scallop recombinant propeptide (pg 7). This protein will be used to treat developing larvae and adult bay scallops. There are a limited number of reports by other researchers treating molluscs with recombinant growth promoting proteins. Most of this work involves using finfish growth hormone preparations. For example, Paynter and Chen (1991) exposed juvenile oysters to seawater containing 10^{-9}, 10^{-8} and 10^{-7} *M* trout growth hormone once a week (5 hours) for 5 weeks. Oysters treated with the two highest concentrations of hormone were larger and were 50% heavier (dry tissue weight) (Paynter and Chen 1991). In the abalone (genus: *Haliotis*), a

gastropod mollusc, bovine growth hormone and insulin has been shown to enhance growth (Morse 1984). More recently, Moriyama and Kawauchi (2004) have characterized the effect of recombinant salmon growth hormone, compared to salmon prolactin, and bovine serum albumin on abalone growth. In Moriyama and Kawauchi's study, abalone were treated with growth hormone via immersion and by intramuscular injection. Abalone immersed in concentrations of 30 mg/l (30 min) growth hormone at 4-day or 7-day intervals were larger in body mass compared to controls (Moriyama and Kawauchi 2004). Shell length and body mass were also increased following injections at doses between 2 and 20 ng/g body mass (BM) at 7 day intervals (Moriyama and Kawauchi 2004). Growth hormone has not been isolated from any shellfish. To our knowledge there are no reports of the effects of a endogenous, recombinant binding protein on shellfish growth. For the current proposal we plan on treating larvae and adults with recombinant bay scallop myostatin propeptide via immersion and intramuscular injection. Morphometric and cellular effects will be compared to controls.

Methods: Larval Treatment

Developing bay scallops will be treated via immersion during the later part of Year 2. Spawning will be induced in Year 2 as described above (pgs 11-12) and fertilized gametes will be cultured in static systems at 48 hour intervals. Following the first drain down at 48 hours, larvae will be evenly distributed into 30 liter systems. Initial stocking density will be 10 larvae/ml. A minimum of six systems will be used to allow for two doses of propeptide (10mg/l and 40mg/l) and controls (no treatment) in duplicate systems. Treatments will start on the second drain down, four days post-fertilization and continue every 48 hours until metamorphosis. Normal metamorphosis will usually occur between day 9 and 14 post-fertilization. Treatments will involve immersion in small aerated tanks with recombinant protein or sterile seawater for 3 hours. During treatment larvae will be at higher densities in order to efficiently administer recombinant proteins. Following treatments, sub samples will be taken and photographed to evaluate development and growth using a digital-imaging equipped dissecting scope. Individuals will then be returned to respective tanks. Larval counts will be taken at each drain down, following treatments, to evaluate mortality. Samples will be taken at metamorphosis for each treatment to be used for differential expressed gene analysis (pg 16).

Methods: Adult Treatment

At the end of year two of the proposed research adult bay scallops will be intramuscularly injected with either recombinant propeptide (2ng/g BM, 20ng/g BM) or sterile seawater. Each of the three treatments will be carried out on bay scallops at 5 months of age (n=30). These scallops will be from the spawning event in the second year to produce larvae for immersion treatment. Injections will occur on 7-day intervals for 8 weeks. Twice weekly, shell measurements will be recorded to follow growth. Scallops will be maintained on ambient water at the Marine Resources Center and fed standard micro-algal diets. At the end of the experiment 15 individuals from each treatment will be harvested to assess tissue weight, muscle morphology and genetic differences. Any remaining scallops will be cultured on ambient water until early fall to monitor the effect of recombinant protein treatment on long-term growth.

Methods: Muscle Morphology

Cross-sections of adductor muscle tissue will be collected from scallops and fixed in 10% buffered formalin (Sigma) for 12 hours and then embedded in paraffin and processed for histology. Microtome sections (7 μm) from the fixed/paraffin embedded muscle, will be stained

in hematoxylin and eosin, and then mounted in Permount (Fisher). In general, the same techiniques used in analysis of adductor muscle tissue from MyoZap treated bay scallops will be used (see Figure 6, pg. 6). A suite of muscle fiber measurements will be taken and compared between treated (both dosages) and control scallops intramuscularly injected with recombinant propeptide. Parameters to evaluated include total cross-sectional area, total number of fibers within a given area, mean fiber area, and mean of fiber widths determined in area. Specific attention will be directed towards relative muscle fiber size as an indicator of relative influence of hyperplastic versus hypertrophic muscle growth.

Methods: Isolation of Differentially Expressed Genes (DEGs)

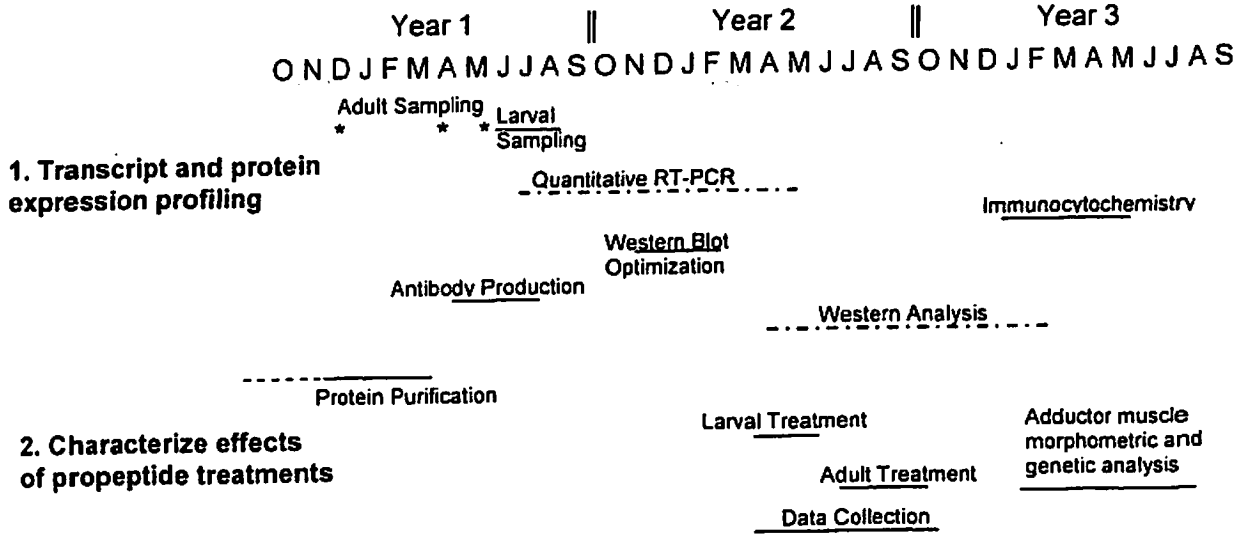
To complement shell / body mass measurements and histological analysis, differentially expressed transcripts will be identified. In the event significant variations in mass and/or growth rates are not observed, these results will also provide insight into other physiological process affected by exposure to mysostatin recombinant proteins. In order to compare the differentially expressed genes in the treated and non-treated scallops a differential display approach will be used (GeneFishing DEG Kits – Seegene). This is the same approach described above (see Progress Report, pg 4; Additional Preliminary Data, pg 6). The reason this approach is being used is that it is economical, fast, and easily managed to compare a large number of different samples. Unlike conventional subtraction, using DEG analysis will allow simultaneous comparision of all samples (e.g. larvae, muscle, treated, controls). Initially, total RNA will be extracted from larvae (pooled) and adductor muscle (individual) 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 et al. 1997). ClustalW (MacVector 7.2) analysis will be used for sequence pair-wise and multiple protein alignments. Quantitative RT-PCR will be carried out to validate differential expression.

Expected Outcomes and Potential Pitfalls

It is expected that treatment of bay scallops with recombinant myostatin propeptide will alter growth in bay scallops by inactivating the mature myostatin ligand. In developing larvae metamorphosis is likely to occur at an earlier time. In adductor muscle tissue of treated bay scallops both hypertrophy and hyperplasia is expected to increase. As a result, total tissue mass and shell size would be larger than controls. One potential pitfall in this experiment is that treatment of larvae could result in undesired secondary effects including elevated mortality. The bay scallop is the only species in which myostain has been characterized that undergoes a complete metamorphosis. Metamorphosis is a precise and complex process involving complete restructuring and generation of new tissue and organs. Propeptide treatment during metamorphosis could negatively impact timing of biological events resulting in mortality. However it should be noted that this information will be useful in determining how myostatin technologies would be developed in the long-term.

Timeline

In order to be prepared in the event that the current proposal is funded, the PD will continue to produce recombinant propeptide and mature myostatin protein during the summer of 2005. This protein will be available for antibody production and bay scallop treatments. Broodstock scallops will be maintained at the Marine Biological Laboratory prior to the proposed start date. During Year 1, the primary focus will be on sampling adult and larval scallops for transcript and protein analysis. Two antibodies will be produced by a commercial research company. By Year 2, quantitative RT-PCR analysis will have begun. During the summer of Year 2, larvae and adults will be exposed to recombinant myostatin propeptide and monitored. Western analysis is expected to be finished by the middle of the final year of the proposed research. Immunocytochemistry associated with research objective 1 as well as morphometric and genetic analysis associated with research objective 2 will be carried out in the third and final year. A schematic diagram describing when major milestones are expected to take place is shown below.



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Wikfors G, Widman J, Stiles S, Robohm R and Calabrese A 1998. New England bay scallops as a one-season aquaculture crop. Marine Aquaculture: Emerging Technologies and Global Opportunities, University of Connecticut, Stamford, Connecticut Sea Grant College Program.
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Facilities and Equipment – Steven Roberts

Laboratory: Dr. Roberts has an 800 sq.ft. research laboratory in the Marine Resources Center (MRC) at the Marine Biological Laboratory (MBL). The lab is equipped with routine laboratory furniture, plumbing, chemical and biohazard hoods, and other utilities.

Animal Holding Facility: The aquatic holding facility at the Marine Biological Laboratory is located on the first floor of the MRC; a 32,000 ft^2 state-of-the-art facility capable of housing and culturing a number of vertebrate and invertebrate marine organisms. The PD's laboratory and office are also located in this facility. The life support systems of the MRC can deliver a continuous supply of raw, ambient-temperature, as well as filtered, tempered flow-through seawater. The system's capabilities also include eight recirculating seawater systems. Seawater in the recirculating and filtered-tempered systems is ozone-sanitized to control fouling and facultative pathogens. To safeguard the local environment and its indigenous animal and plant populations from incursions by exotic laboratory or exogenous species, effluent seawater from selected regions of the building is also ozone sterilized. To insure reliability and safeguard animals being cultured or maintained under husbandry conditions, all incoming seawater supply lines, filtration systems, and treated-water heat exchangers are redundant. All pumps, temperature sensors, and the ozone distribution system are computer controlled and alarmed. The entire system is further supported by routine night watchman security checks as well as a 500 kW emergency generator in event of electrical outages.

Animals: All scallops used in this research will be maintained in the aquatic holding facility at the Marine Biological Laboratory, located on the first floor of the Marine Resources Center. In addition, the MRC has a staff of collectors that can obtain marine scallops throughout the year. Scallops will be spawned and juveniles cultured on the first floor of the MRC. The 3rd floor of the MRC houses a micro-algae production facility that produces several strains of micro-algae for bivalve rearing year-round. There is direct plumbing from this facility to the tanks scallops will be raised in.

Computers: : In the laboratory are 2 Power Mac G4s for data analysis. There is a single copy license for MacVector that can be run on all Macs. A local "Blast" on the Mac G5 along with complete NCBI nonredundant protein, nucleotide and EST databases is maintained. Programs for "phred" and "crossmatch" are maintained locally on a G4 for EST analysis. There are also 3 Dell Optiplex PCs for analysis and word processing. All computers in the lab are directly connected to the internet and they are also networked to one another and to computers running several pieces of laboratory equipment (e.g., ChemiImager 5500 and Opticon). The library also provides comprehensive and up-to-date database searching and electronic journals via the MBL internet site.

Equipment:
ALF Express Sequencer
Alpha Innotech ChemiImager 5500 (SuperChemiNova12-bit CCD camera)
MJ Research Opticon 2 (includes a DNA thermal gradient PCR engine)
Sorval Super T21 highspeed centrifuge
Perkin-Elmer EZ201 spectrophotometer
Lab-Line benchtop orbital bacterial shaking incubator
Fisher Biotech hybridization incubator
2-Boekel incubator shakers
3 thermocyclers (MJ-PTC-200, PTC-100, PTC-150)
2-Road Runner Owl electrophoretic mingel systems for SDS PAGE
Xcell SureLock Mini-Cell System
Trans-Blot SD-Biorad transfer system
2- EC 600 and 1-EC 452 power supplies
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 - Cahn C-30 microbalance; 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
2 dissecting microscopes, 2 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 an ABI 3700 sequencer, automated arrayer and reader and ancillary robotics.

CURRICULUM VITAE – STEVEN BEYER ROBERTS (PD)

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 · Assistant Research Scientist
Marine Biological Laboratory, Woods Hole, MA

2002-2003 · Postdoctoral Scientist
Program in Scientific Aquaculture
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 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

Refereed Publications (past 4 years)

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

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

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

Refereed Publications (past 4 years, continued)

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. 32(3):921-34

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. Vol 138(1):32-41

Kim H-W, Mykles DL, Goetz FW, Roberts SB. (2004) Characterization of an invertebrate myostatin homologue from the bay scallop, *Argopecten irradians*. BBA – Gene Structure and Expression. 1679(2):174-9

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. 207(Pt 21):3741-8

Jentoft S, Topp N, Seeliger M, Malison JA, Barry TP, Held JA, Roberts SB, Goetz FW. (2005) Lack of growth enhancement by exogenous growth hormone treatment in yellow perch (*Perca flavescens*) in four separate experiments. Aquaculture. *In press*

Roberts SB, Romano C, Gerlach G. (2005) Characterization of EST derived SSRs from the bay scallop, *Argopectens irradians*. Molecular Ecology Notes. *In press*

Biga PR, Roberts SB, Iliev DB, McCauley LA, Moon JS, Collodi P, Goetz FW. (2005) The isolation, characterization, and expression of a novel GDF11 gene and a second myostatin form in zebrafish, *Danio rerio*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology . *In press*

Roberts S, Dixon M, Wikfors G, Romano C (2005) Characterization of a thyroxine dieodinase from the bay scallop (*Argopecten irradians*) and the role of thyroid hormone in controlling metamorphosis. *In preparation*

Selected Non-refereed Publications

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

Ettensohn K, Biga PR, Romano C, Devlin RH, and Roberts SB. (2004) Genes Differentially Expressed in Growth Hormone Transgenic Salmon (Abstract). Biological Bulletin 2004 207: 168

Roberts SB, Goetz FW. (2004) Quantitative real-time PCR methods for disease detection in marine fish. Proceedings of Aquaculture 2004, March 1-5, Honolulu, HI. pg 503 (Abstract)

Roberts SB (2004) Lab Studies: Genes Involved with Growth, Development of Bay Scallops. Global Aquaculture Advocate. 7(3): 55-56

Ongoing Research Support

03-17/556808 11/01/03 – 10/31/05
Northeast Regional Aquaculture Center
Development of diagnostic and management techniques to select cod broodstocks and hatchery stocks free from nodavirus.
$124,612

2004-04533 02/02/05 – 02/02/08
United States Department of Agriculture
Production of myostatin gene knockouts in zebrafish, and the effects of specific myostatin interacting proteins on salmonid muscle growth.
$426,000

04-1-3 02/01/05 – 01/01/07
Northeast Regional Aquaculture Center
Development of genetic markers to assess disease resistance in the Eastern oyster
$125,486

Past Research Support

2003-35206-12834 11/01/02 – 10/31/04
United States Department of Agriculture
Isolation and characterization of factors regulated during larval competence and metamorphosis in the bay scallop, *Argopecten irradians*.
$89,934

Barnstable County, Massachusetts 06/31/04 – 12/31/04
The use of microsatellite markers to improve bay scallop stock enhancement efforts
$20,000

Professional Activities

Pan American Marine Biotechnology Association
World Aquaculture Society
National Shellfish Association
Sigma Xi Scientific Research Society
American Fisheries Society

Reviewer for:

BARD, the United States - Israel Binational Agricultural Research & Development Fund
Maryland Sea Grant
National Sea Grant College Program: Oyster Disease Program
USDA National Research Initiative: Animal Growth and Nutrient Utilization
USDA National Research Initiative: Animal Reproduction

Undergraduate Students Mentored:

Patrice Pazar, University of Colorado
Phoenix Becker; University of Maine
Adam Bisonette; St. Anselm College
Kristen Ettensohn; Dartmouth College
Carly Allen; University of Hawaii
Angela Sampson; Massachusetts Maritime Academy
Chris Dickson; University of Colorado

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

OMB Approved 0524-0039
Expires 03/31/2004

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
Anderson, Robert		x		
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			
Ettensohn, Kristen	x			
Gerlach, Gabriele	x	x		
Goetz, Frederick	x	x	x	
Grier, Harry	x			
Hardy, Ronald	x			
Hart, Deborah		x		
Held, James	x			
Hollis, Dave	x			
Iliev, Dimitar	x	x		
Jentoft, Sissel	x			
Johnson, Stewart		x		
Karney, Richard		x		
Kim, Hyun-Woo	x			
King, William	x			
Langenau, David	x			
Leavitt, Dale		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			
Rago, Paul		x		
Romano, Christina	x	x		
Seeliger, Matthew	x			
Smolowitz, Roxanna		x		
Sullivan, Craig	x			
Sunila, Inke		x		
Taylor, Ron	x			
Topp, Nicole	x			
Wade, Terry		x		
Walton, William		x		

Results from Other NRI Supported Research

Production of myostatin gene knockouts in zebrafish, and the effects of specific myostatin interacting proteins on salmonid muscle growth.

USDA-NRICGP 2005-35206-15261; $472,840; 02/01/05 -02/01/08

PD: Goetz, F

co PDs: Collodi P, Roberts S

This grant was initiated 2 months ago therefore work has just begun on the project. A summary of the grant is given below

A crucial step in making aquaculture profitable for many species is the speed and efficiency of getting fish to market size. Thus, how to stimulate and maximize growth is a major interest in aquaculture. There have been attempts to stimulate growth in fish using pituitary growth hormone injections or growth hormone transgenically modified organisms. However, growth hormone does not appear to be an economically (injection) and/or a socially (transgenic) acceptable solution for commercial fish production. This has promoted interest in looking for other growth enhancing substances including a newly discovered gene product called myostatin. Myostatin is a negative regulator of growth that, when removed, results in enhanced muscle growth in mammals. Because it inhibits growth, there is the possibility to develop agents that could be delivered in the diet that would reduce or block myostatin effects on growth. While the effects of myostatin have been unequivocally demonstrated in mammals, the biological effects in fish are still unclear. Fish possess myostatin proteins; however, to develop myostatin further for aquaculture, it is imperative to determine if myostatin is a negative regulator of growth in fish and that is the goal of the present study. Two different approaches will be used to accomplish this goal. It is known from mammalian studies that certain proteins will bind to myostatin making it inaccessible and nonfunctional, resulting in increased growth. We plan to produce similar fish-specific myostatin binding proteins and test their effects on growth by injecting them into trout. The other approach will be to produce zebrafish lines that have been genetically altered such that their myostatin is nonfunctional (i.e., myostatin gene knockout). The development and growth of muscle in these lines will eventually be compared to normal zebrafish. These studies should demonstrate whether myostatin has an effect on muscle growth in fish as it does in mammals. If so, then future studies can be directed at developing tools to block myostatin in aquaculturally important fish species.

A recombinant myostatin propeptide, based on the existing brook trout myostatin protein, will be produced. In addition, novel myostatin interacting proteins will be isolated from brook trout using a yeast 2 hybrid system. Following cloning, these additional interacting proteins will also be produced as recombinant proteins. The effects of the propeptide and several of the novel interacting myostatin proteins will be tested by injecting the proteins into juvenile brook trout and assaying muscle morphometry, histochemistry, and specific gene/protein expression. Specific myostatin gene knockout lines will be produced in zebrafish by 1) homologous recombination of a disrupted myostatin type 1 and myostatin type 2 gene in embryonic stem cells (ES - grown on trout feeder cells), 2) injection of ES cells into zebrafish and 3) selection and breeding of germline chimerids theoretically carrying the disrupted myostatin gene.

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 Funds Requested by Proposer	DURATION PROPOSED MONTHS: 12 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) Steven Beyer Roberts				

A. Salaries and Wages	CSREES-FUNDED WORK MONTHS							
	Calendar	Academic	Summer					
1. No. Of Senior Personnel				16,667				
a. 1 (Co)-PD(s)	4							
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				17,500				
Total Salaries and Wages .. **				34,167	0	0	0	
B. Fringe Benefits (If charged as Direct Costs)				10,831				
C. Total Salaries, Wages, and Fringe Benefits (A plus B) .. **				44,998	0	0	0	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)								
E. Materials and Supplies				16,000				
F. Travel				500				
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.)				3,000				
K. Total Direct Costs (C through J) .. **				64,498	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.)				16,124				
M. Total Direct and F&A/Indirect Costs (K plus L) .. **				80,622	0	0	0	
N. Other .. **								
O. Total Amount of This Request .. **				80,622	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		May 16, 2005
Authorized Organizational Representative Richard J. Mullen; Manager, Research Administration		May 16, 2005
Signature (for optional use)		

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
Marine Biological Laboratory
7 MBL Street
Woods Hole, MA 02543

PROJECT DIRECTOR(S)
Steven Beyer Roberts

USDA AWARD NO.

	CSREES-FUNDED WORK MONTHS			DURATION PROPOSED MONTHS: 12 Funds Requested by Proposer	DURATION PROPOSED MONTHS: 12 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	Calendar	Academic	Summer				
1. No. Of Senior Personnel				17,333			
a. 1 (Co)-PD(s)	4						
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				18,200			
Total Salaries and Wages				35,533	0	0	0
B. Fringe Benefits (If charged as Direct Costs)				11,264			
C. Total Salaries, Wages, and Fringe Benefits (A plus B)				46,797	0	0	0
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)							
E. Materials and Supplies				12,000			
F. Travel				1,000			
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.)				5,000			
K. Total Direct Costs (C through J)				64,797	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.)				16,200			
M. Total Direct and F&A/Indirect Costs (K plus L)				80,997	0	0	0
N. Other							
O. Total Amount of This Request				80,997	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		May 16, 2005
Authorized Organizational Representative Richard J. Mullen; Manager, Research Administration		May 16, 2005
Signature (for optional use)		

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 Funds Requested by Proposer	DURATION PROPOSED MONTHS: 12 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) Steven Beyer Roberts				

A. Salaries and Wages	CSREES-FUNDED WORK MONTHS							
1. No. Of Senior Personnel	Calendar	Academic	Summer					
a. 1 (Co)-PD(s)	4			18,027				
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				18,927				
Total Salaries and Wages .. **				36,954	0	0	0	
B. Fringe Benefits (If charged as Direct Costs)				11,715				
C. Total Salaries, Wages, and Fringe Benefits (A plus B) .. **				48,669	0	0	0	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)								
E. Materials and Supplies				12,000				
F. Travel				1,000				
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.)				2,000				
K. Total Direct Costs (C through J) .. **				63,669	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.)				15,918				
M. Total Direct and F&A/Indirect Costs (K plus L) .. **				79,587	0	0	0	
N. Other .. **								
O. Total Amount of This Request .. **				79,587	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		May 16, 2005
Authorized Organizational Representative Richard J. Mullen; Manager, Research Administration		May 16, 2005
Signature (for optional use)		

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 Funds Requested by Proposer	DURATION PROPOSED MONTHS: 36 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) Steven Beyer Roberts				

A. Salaries and Wages	CSREES-FUNDED WORK MONTHS							
	Calendar	Academic	Summer					
1. No. Of Senior Personnel				52,027				
a. 1 (Co)-PD(s)	12							
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				54,627				
Total Salaries and Wages .. **				106,654	0	0	0	
B. Fringe Benefits (If charged as Direct Costs)				33,810				
C. Total Salaries, Wages, and Fringe Benefits (A plus B) .. **				140,464	0	0	0	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)								
E. Materials and Supplies				40,000				
F. Travel				2,500				
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.)				10,000				
K. Total Direct Costs (C through J) .. **				192,964	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.)				48,243				
M. Total Direct and F&A/Indirect Costs (K plus L) .. **				241,207	0	0	0	
N. Other .. **								
O. Total Amount of This Request .. **				241,207	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		May 16, 2005
Authorized Organizational Representative Richard J. Mullen; Manager, Research Administration		May 16, 2005
Signature (for optional use)		

BUDGET JUSTIFICATION - Steven Roberts (PD)

A. Salaries and Wages (note: a 4.0 % salary raise was calculated between years for all personnel and a fringe rate of 31.7% was applied to all salaries for each year)

Project Director: The PD will devote approximately 33 % of the calendar year during the three years of the proposed project. MBL is a "soft money" institution and, therefore, scientists are expected to recover their salary from grants. Thus, the PD is requesting 4 months of salary/year for three years.

Technician: Funds are requested to partially cover a technician position for three years. Specifically, funds are requested to cover 6 months of salary/year for three years. This technician will work with the PD, and is an essential position because of the large number of sample processing and recombinant DNA work that must be completed.

E. Materials and Supplies

Funds are requested for the purchase of materials and supplies needed to complete the proposed research and are specifically listed below. During year one, an additional $4,000 is requested compared to following years. These funds are specifically requested for materials needed for recombinant protein purification and polyclonal antibody production. Antibody production services will be contracted out (e.g. Rockland Immunological). Below a list of supplies is provided for which funds are specifically requested.

A. *Specific molecular biology supplies*

1) RNA/mRNA isolation preparations (e.g., Tri Reagent and PolyAtract)
2) PCR reagents
3) Dual-labelled probes, primers
4) Platinum One-Step RT-PCR kits (Invitrogen)
5) reagents for in-house sequencing
6) plasmid preparation kits (e.g., Wizard) and gel extraction systems
7) GeneFishing DEG kits (Seegene)
8) cloning kits (e.g., TOPO)
9) ABC Staining System (Santa Cruz)

B. *Protein Purification and Western Analysis supplies*

1) High-Performance Gels for SDS-PAGE (e.g., NuPAGE Novex Gels; Invitrogen)
2) Molecular weight standards
3) Buffers, dyes, and reducing agents
4) Bradford Assay reagents
5) nitrocellulose membranes
6) ProBond Purification Columns (Invitrogen)

C. *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
6) solutions for routine buffer preparations

F. Travel

Funds are requested to partially cover the costs of airfare and minimal accommodations for the PD to travel to international (i.e. International Symposium on Fish Endocrinology) and national (i.e. World Aquaculture Society sponsored – Aquaculture US) scientific meetings to present research results pertaining to the proposed project.

J. All Other Direct Costs

During years 1 and 2, $1000 and $3000 are requested to cover tank space rental charges at the Marine Resources Center. During year 2 more space is required as multiple treatments will be used and systems will be set up in triplicate to allow for accurate analysis. For year one, scallops will be used for mRNA and protein analysis only therefore animals will be easily consolidated. Costs are calculated on the following rate: $6/sq.ft./month from November to April; $12/sg.ft./month from May to October

$2,000 per year is requested to specifically cover costs specifically listed below.

1) Funds are requested for maintenance 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 mailing costs directly related to the grant research.

M. Indirect Costs

Indirect costs are calculated as 25% of total direct costs.
(MBL's normal, federally approved rate is 59%).

S. Roberts

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

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. 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	15%	Development of diagnostic and management techniques to select cod broodstocks and hatchery stocks free from nodavirus
Roberts, S.B Smolowitz, R. Karney, R. Sunila, I. Leavitt, D. Walton, W. Goetz, F.W	NRAC-USDA 04-1-3	$128,486	02/01/05 - 02/01/07	25%	Development of genetic markers to assess disease resistance in the Eastern oyster
Goetz, F.W. Roberts, S.B. Collodi, P	USDA-NRICGP 2005-35206-15261	$472,840	02/01/05 - 02/01/08	33%	Production of myostatin gene knockouts in zebrafish, and the effects of specific myostatin interacting proteins on salmonid muscle growth
	Pending:				
Anderson, R Smolowitz, R Roberts, S Leavitt, D	NRAC-USDA 05-4-2	$189,430	03/01/06- 03/01/08	17%	Assessing resistance to QPX disease: development of cellular and molecular methods
Goetz, F.W Wade, T Roberts, SB Rago, P Hart, D	NOAA-OHHI	$112,193	09/01/05- 09/01/07	20%	Sea scallops as sentinels of deepwater pollution
Roberts, SB	USDA-NRICGP (current proposal)	$241,207	11/01/05- 11/01/08	33%	The role of myostatin in shellfish growth

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

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
Marine Biological Laboratory

2. CSREES PROJECT NUMBER OR AWARD NUMBER (if known)

3. PROJECT DIRECTOR(S)
Steven Roberts

4. TITLE OF PROJECT
The role of myostatin in shellfish growth

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
Richard Vigneau	Manager, Research Administration	5/13/05

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
Expires 03/31/2004

National Environmental Policy Act Exclusions Form

Project Director Name	Institution
Steven Beyer Roberts	Marine Biological Laboratory
Address 7 MBL Street, Woods Hole, MA 02543	

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

[☑] **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:

- [☑] (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.)

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

Hyun-Woo Kim[a], Donald L. Mykles[a], Frederick W. Goetz[b], Steven B. Roberts[b,*]

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[b] *Program in Scientific Aquaculture, Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543, USA*

Received 24 March 2004; received in revised form 26 May 2004; accepted 10 June 2004
Available online 2 July 2004

Abstract

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.

Keywords: Myostatin; GDF-8; Scallop; Muscle; TGF-β; *Argopecten irradians*; *Ciona*

1. 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

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

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Table 1
Primers and probes used for initial cDNA isolation and quantitative real-time RT-PCR

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

Fluorescent dyes incorporated into probes are italicized.

skeletal muscle of adult scallops. Taken together, this suggests that the scallop cDNA could be an invertebrate MSTN homologue.

2. Methods

2.1. Cloning scallop MSTN

Total RNA was extracted from adductor muscle tissue of two 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 sequences 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 s; 50 °C, 20 s; 72 °C, 30 s; 35 cycles). The

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   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
                  mstnR2
1381  TTCGAACATAGCATTGACGCGATTGCCCAGGATGAAAGTAGACAGATGTGGTTGTGCGTAAGTGAAAGA
        S  N  I  A  L  T  R  L  P  R  M  K  V  D  R  C  G  C  A  *            382
                                                mstnR1
1450  CAAAACTTCAGAAAGAAAGAGACAGGATGGTGATGTATCTGCGAAATAGTAAAGAATGTTTGTTTGAAC
1519  GGGAAAAAAAAAAAAAAAAAA
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Fig. 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 appear in the GenBank database under the accession number AY553362.

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 (GenMachines) 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 pairwise and multiple protein alignments.

2.2. 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

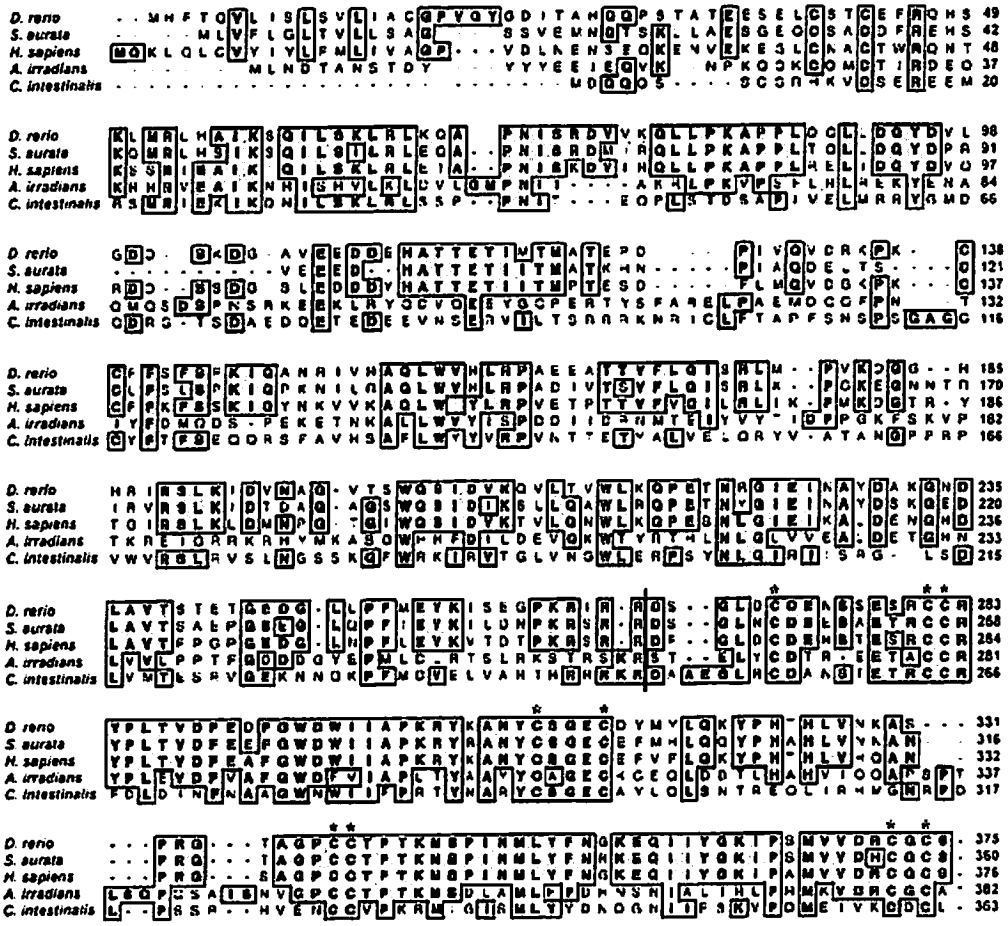

Fig. 2. Amino acid alignment of zebrafish (*D. rerio*; accession number AAP85526), gilthead sea bream (*S. aurata*; accession number AAL05943), human (*H. sapiens*; accession number NP_005250), bay scallop (*A. irradians*; accession number AY553362) and putative *C. intestinalis* MSTNs. A vertical line 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).

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 concentrations of components were: Thermoscript reaction mix, 1 ×; sense primer, 0.2 μM; anti-sense primer, 0.2 μM; fluorogenic probe, 0.2 μM; $MgSO_4$, 5 mM; RNA, 0.5 μg. 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 ± S.E. One-way ANOVAs were performed followed by Tukey's test. All significance levels were set at $P = 0.05$.

3. Results and discussion

3.1. 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 (Fig. 1). The characteristic MSTN RXXR cleavage site is present as are the nine conserved cysteine residues (Fig. 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; Ref. [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, Fig. 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

Table 2
Amino acid sequence identities of TGF-β superfamily members from various organisms

	A.i.	*C.i.*	*D.m.*	*C.g.*	*S.a. gdf-8b*	*D.r.*	*H.s.*	*H.s.* gdf11	*H.s.* bmp2
Complete protein									
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. 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. 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

Identities are given in relation to the complete proteins and to the regions upstream and downstream of the proteolytic processing site.
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).

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; Ref. [16]). However, mGDF is most similar to bone morphogenetic protein 2 (Table 2) and, therefore, is not an 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 (=ciona4" locatortype="URL" >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 three exons that putatively contain coding

regions for a 363-amino-acid protein (Fig. 2). Over the entire protein, the sMSTN protein has greater homology with ciMSTN protein than other invertebrate proteins including myoglianin (Fig. 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 (Fig. 2). Interestingly, a smaller addition (RPDLXXXR) in that area was also observed in the *Ciona* MSTN-like protein (Fig. 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).

3.2. 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 six times higher than in all other tissues examined (Fig. 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 an 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 in 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 an 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, an 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.

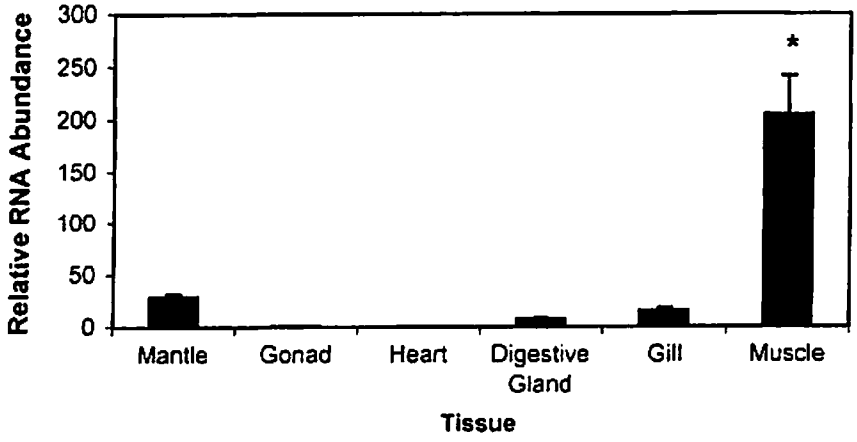

Fig. 3. Relative RNA abundance of sMSTN in various tissues from bay scallops. Vertical bars represent the mean ± S.E. ($N=3$) for each tissue. Significant differences across tissue are indicated with an asterisk at $P \leq 0.05$.

Acknowledgements

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