Introduction

In the U.S., mariculture will provide the most likely avenue for expanding the aquaculture industry as inland resources are in short supply. In particular, the culture of bivalve molluscs such oysters, clams, mussels, and scallops has increased and has the potential to make significant environmental and economic impacts. In the U.S., revenue from combined bivalve production has bypassed that of the salmon industry and is the highest valued farmed marine product (FAO 2001). The research proposed here is designed to ***further promote the aquaculture of all marine bivalves by characterizing the reproductive biology of bivalves, focusing on aspects that could be used in the future to generate sterile organisms***. The ability to induce sterility will facilitate improved growth rates and insure that exotic or manipulated organisms will not have detrimental effects on other species and the environment. Oysters and clams are two of the mostly widely produced and most profitable bivalves cultured in the U.S. (FAO 2001). Therefore much of the proposal will focus on these bivalves, specifically the eastern oyster (*Crassostrea virginica*) and surf clam (*Spisula soliddisma*). However, other bivalves will be examined such as sea scallops (*Plactopecten magellanicus*) because their gonad anatomy provides an excellent model system. Information obtained about the reproductive biology of one species could easily apply to all others. In the remainder of this section, the general life history and reproductive biology of bivalves will be outlined followed by a discussion of current issues facing bivalve aquaculture.

Life History

Unlike animals that are commonly produced for consumption (cows, pigs, and fish) bivalves undergo a complete metamorphosis during production. In bivalves, free-swimming larvae metamorphose into juvenile shellfish that resemble what most consumers are familiar with (two shells encapsulating soft tissue). Figure 1 illustrates the life history of a scallop. While the appearance and anatomy of adult bivalves can be different, development from unfertilized egg to spat is the same across bivalves. Initially, eggs and sperm are released into the water column. Usually within 5 hours, cilia appear and swimming can be observed. Trochophore embryos develop 24-48 hours after fertilization. Normally about 1 week post fertiliaztion, larvae exhibit a straight hinge that has a "D" shape. When an individual is ~200 μm, a ciliated foot and eyespot develop and the larvae are considered mature, entering the pediveliger stage. Once a suitable substrate is found, the planktonic larvae will settle and metamorphose to become suited for a benthic life. As the larvae settle they are referred to

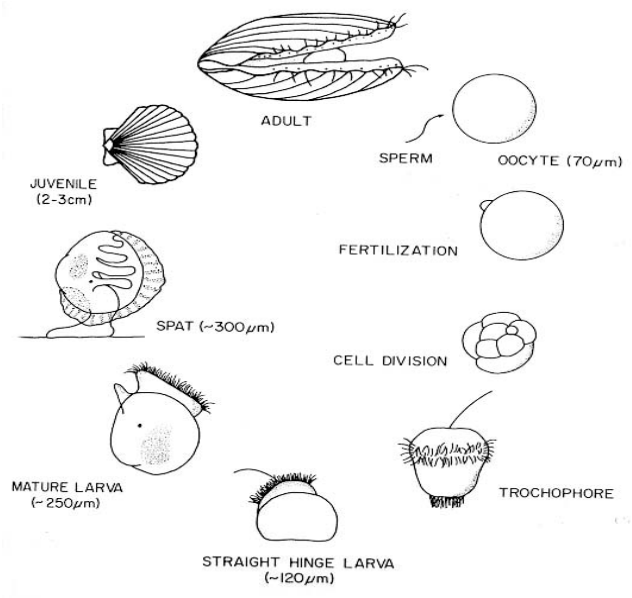


Figure 1. Life cycle of a representative bivalve. Figure taken from (Sorgeloos 1999).

as spat. Settlement and metamorphosis basically occur simultaneously with metamorphosis marked by the loss of the velum and development of a gill feeding system. The dissoconch shell (two valves) is now apparent and the growth rate begins to increase. During early stages of juvenile development gonads and other internal organs begin to develop.

Biology

Adult bivalves have several morphological characteristics that are that are the same across species. For example, bivalves have a calcareous shell with two valves that are hinged dorsally. The two valves are attached by an elastic hinge ligament that allows the two valves to open and close via the action of adductor muscles. The body and foot of bivalves are compressed laterally, and in most cases the spade-like foot is well adapted for borrowing. The shell encloses and protects the internal organs including the digestive system, gonads, and gills. Figure 2 compares the internal anatomy of two different bivalve species, a scallop (left) and oyster (right).

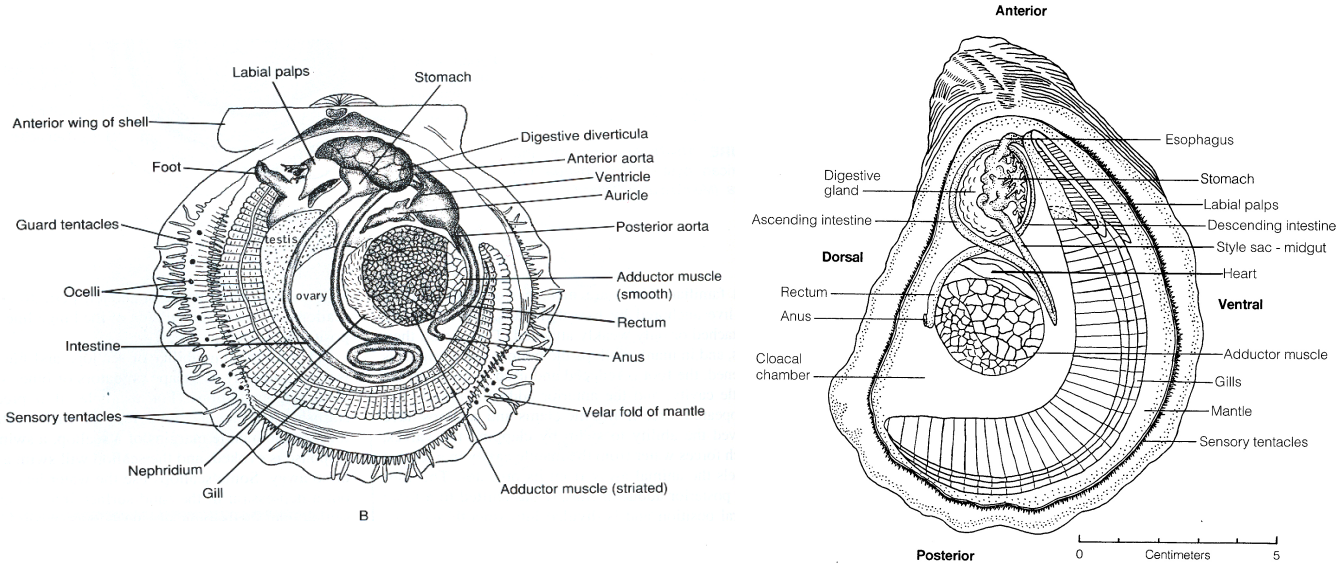


Figure 2. Internal anatomy of two bivalve species. Diagram of scallop (left) taken from (Ruppert and Barnes 1994). Diagram of oyster (right) taken from (Eble and Scro 1996).

While there are several characteristics that are similar across bivalves such as the filter feeding system, there are aspects of gonad morphology that differ. In general, gonads are paired and contain branching tubules that form ducts terminating in a gonoduct. Gametes are budded off the epithelial lining of the tubules. Gametes proliferate prior to spawning (gametogenesis) usually resulting in the enlargement of the gonad at this time. Most bivalves are dioecious, meaning the sexes are separate. Mussels are an example of a dioecious species with the gonad developing within the mantle tissue. In ripe mussels, the mantle is typically orange if female gametes are present and white if male gametes are present. In the sea scallop, another dioecious species, the female gonad is pink and the male gonad white. The gonad in the sea scallop does not develop in the mantle but is located adjacent to the adductor muscle. Most other scallop species are hermaphrodites. This is illustrated in figure 2 (left) in the diagram of a bay scallop, a simultaneous hermaphrodite. The gonad is divided into a dorsal testis and ventral ovary.

Synchronous hermaphrodites usually stagger gamete release to decrease self-fertilization. Other bivalves are sequential hermaphrodites, meaning that the can alter the sex of the gonad but commonly will have either an ovary or testis. An example of this is the oyster (Figure 2 - right). In this species the gonad is in the visceral mass between the digestive gland and the mantle, which is not clearly shown in the figure. When young oysters reach sexual maturity the gonad usually develops as a male. After one spawning cycle a portion of the individuals become females (protandry), while the others will remain as males their entire life. Quahog clams (*Mercenaria*) are also protandric hermaphodites with the sex ratio of a population normally reaching 1:1 by year two (Menzel 1989). The mechanisms that regulate sex in bivalves have not been fully characterized; however there is evidence to suggest environmental (Galtsoff 1964) and genetic factors (Guo, Hedgecock et al. 1998) are involved.

Early Gonad Formation

There has been significant amount of microscopic analysis of developing embryos that dates back to beginning of the century in a variety of molluscs (reviewed in Sastry 1979). From these combined works, several cytological events leading to the formation of the gonad have been described. Gonads form from groups of mesodermal cells in proximity to the visceral ganglion and the ventral side of the pericardium (Wada 1968). These primordial germ cells multiply and aggregrate in two groups. In the clam the gonad first develops as a thin layer of cells adjacent to the kidney (Loosanoff 1937). The primordial germ cells spread through the connective tissue and are surrounded by a thin membrane. Branching tubules develop and can be observed in oysters approximately 8-12 weeks after spat settle. As the follicles enlarge and develop more branching tubules are present. As the follicles are established, primordial germ cells stop multiplying. In the germinal epithileum, deviratives of germ cells, gonial cells, begin to develop into oocytes and spermacytes. In oyster, this process, known as gametogenesis, will have already started with the first year of life (Galtsoff 1964).

Gametogenesis

The next step in sexual development is the maturation of the gametes from primitive gonial cells to mature sperm or eggs. This process is the same among bivalve species and has been previously described (Sastry 1979) (Figure 3).

In spermatogenesis, primary spermatogonia mitotically divide to produce secondary spermatogonia. Meiosis follows to produce

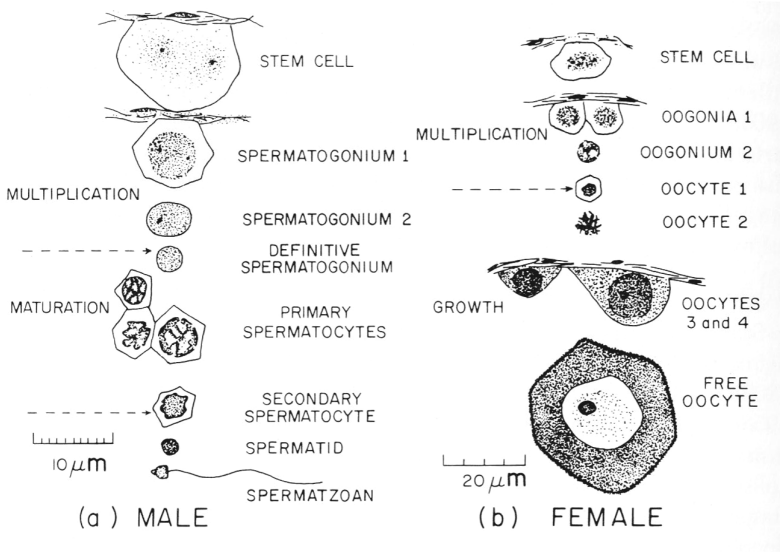


Figure 3. The sequence of gametogenic stages in bivalves. (a) Spermatogenesis. (b) Oogenesis. Figure from (Sastry 1979).

spermocytes which lie in the follicle as a uniform band. Spermocytes rapidly give rise to secondary spermocytes, followed by spermatids that differentiate into spermatozoa. Spermatozoa are located in the core of the follicular lumen. In the female gonad, gonial cells undergo mitotic division and give rise to primary oogonia. These primary oogonia attach to the wall of the follicle and a subpopulation will undergo mitotic divisions giving rise to secondary oogonia. Meiosis is initiated; however it is arrested at the prophase stage of meiosis until fertilization. Just as in lower vertebrates, oocytes begin to accumulate nutrients in a process known as vitellogenesis. Vitellogenesis involves the uptake of primarily lipid globules and yolk platelets into the cytoplasm and an enlargement of the nucleus. Oocytes continue to grow until they are spawned during the breeding season or resorbed into the follicle (atresia). Spawning, or the release of gametes into the water, is also controlled by a combination of environmental factors that presumably encourage high survivability of offspring (i.e. temperature, adequate food supply). Gametogenesis reoccurs each reproductive cycle, which is usually on the order of 1-2 times a year.

Factors influencing reproduction

The control of differentiation and gametogenesis involve endogenous factors (i.e. genes, neuro-hormones) that are often affected by a complex interaction of exogenous factors including temperature, food, light, and salinity. The effects of exogenous factors are easy to study in a laboratory setting, however the internal pathways of reproductive regulation have yet to be fully characterized. There has been increasing evidence to suggest that bivalves have a pathway for reproductive control similar to what is observed in lower vertebrates such as fish. A basic hypothesis is that sensory receptors are stimulated by the surrounding conditions which in turn **signal the nervous and gonadal tissue to release factors** (hormones) that directly control gonad differentiation and gametogenesis. A simplified model of this hypothesis is shown in figure 4.

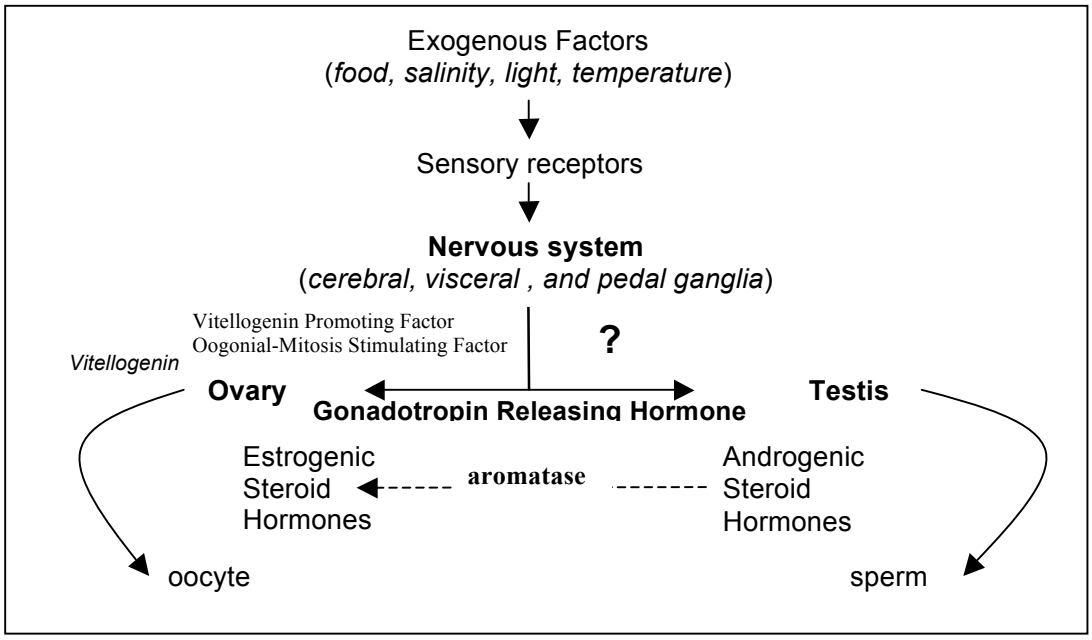


Figure 4. Possible pathways controlling reproduction in bivalves.

While bivalves do not have a central nervous system similar to higher vertebrates, they do have 3 major ganglia that are essentially a small mass of neuronal tissue containing neurosecretory cells. These ganglia are referred to as the cerebral, visceral and pedal ganglia. The majority of neurosecretory cells are located in the cerebral ganglia. Researchers have shown that the activity of neurosecretory cells in the mussel increases with the developing gonad (De Zwann and Mathieu 1992). A peptide isolated from the cerebral ganglia (oogonial-mitosis stimulating factor) has been shown to stimulate gonad mitosis, re-initiation of meiosis in males, and vitellogenesis in female mussels (Lubet and Mathieu 1982; Mathieu, Robbins et al. 1991). There is evidence to suggest gonadotropin-releasing hormone (GnRH) is present in bivalves and controls gonad development. GnRH neuropeptides are pivotal components in the regulation of reproduction in vertebrates. In vertebrates GnRH stimulates gonadotropin release from the pituitary into the blood where they travel to the gonads to regulate steroidogenesis and gametogenesis. Multiple forms of GnRH have been isolated in all vertebrates examined. GnRH has not been isolated from bivalves however the genes encoding the peptide have been isolated in other invertebrates (urochordates - Powell, Reska-Skinner et al. 1996; Adams, Tello et al. 2003; octopus - Iwakoshi, Takuwa-Kuroda et al. 2002). The presence of GnRH immunoreactive neurons and fibers have been reported in the cerebral and pedal ganglia of mussels (Pazos and Mathieu 1999). In the same study, researchers showed that several vertebrate GnRHs increased DNA synthesis in gonial cells of oysters (Pazos and Mathieu 1999). Taken together these data suggest that GnRH is most likely present in bivalves and could have a similar function as observed in vertebrates.

The presence of sex steroid hormones such as estrogens and testosterone has been reported in several invertebrates (Botticelli, Hisaw et al. 1960; Schoenmakers and Dieleman 1981; Hines, Bryan et al. 1996). In bivalves, sex steroids appear to be important in the control of reproduction and have been identified in the gonads of a few species (Reis-Henriques and Coimbra 1990; Matsumoto, Osada et al. 1997). In sea scallops, injection of sex steroids into undifferentiated larvae promoted gonadal differentiation and shifted sex ratios (Wang 2000). Estradiol-17β has been shown to be associated with sexual maturation in the oysters and scallops (Matsumoto, Osada et al. 1997) Recently, Osada et al. (2003) has demonstrated that estradiol-17β, in conjunction with a vitellogenesis promoting factor (VPF) from the cerebral and pedal ganglion, stimulate vitellogenesis in the ovary of scallops. These results are consistent to what has been described for vertebrates (Nagahama 1994) and it is likely sex steroids have a role in reproduction across bivalves. This would mean that enzymes responsible for the metabolism of sex steroids (17β-hydroxysteroid dehydrogenases, aromatase) could indirectly regulate reproductive development by controlling the levels of sex steroids. Specifically, aromatase is required for the synthesis of estrogens from androgens, therefore down regulation of aromatase would decrease estrogen synthesis. Since estrogen has been shown to be involved in differentiation and gametogenesis, these processes could ultimately be affected.

Bivalve Culture

The culture of bivalves can be broken into three major phases; 1) hatchery rearing of fertilized gametes; 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. Compared to production of land based animals, commercial bivalve production is relatively in new. It has only been in the past decade that many of the physical constraints faced in all phases of bivalve culture have been overcome. This includes the optimization of environmental parameters in hatcheries and engineering of structures to hold adult bivalves (Gosling 2003). While some species of bivalves can grow out on natural bottoms, other species such as scallops are grown in mesh cages on racks, suspended mesh cages, lantern nets, or individually suspended on long lines. In almost all instances adult bivalves are raised in open waters, exposed to severe weather conditions, predators, and disease.

Improvement in management practices has significantly increased production of bivalves over the past 25 years (FAO 2001). The growth of the industry has resulted in a need to overcome new problems that are affecting the quality and quantity of production. Specifically, there is demand for improved growth and increased disease resistance. Increased growth rates would decrease the time it takes to get a product to market thus decreasing cost and probability of mortality. The desire to obtain faster growing bivalves is also evident by the significant amount of scientific research related to growth in bivalves (i.e.Gaffney and Allen 1993; Guo and Allen 1994; Guo, DeBrosse et al. 1996; Favrel, Lelong et al. 1998; Wang, Guo et al. 1999; Ruiz-Verdugo, Ramirez et al. 2000; Que and Allen 2002; Gricourt, Bonnec et al. 2003). Disease is also a problem, particularly in the artificial, high density environments experienced by larvae in a hatchery. Dermo (*Perkinsus*) and MSX (multi-nucleated sphere unknown) are both single-cell parasites that invade the oyster's soft body resulting in death of the individual. The combination of these two diseases has devastated the oyster industry in the Chesapeake Bay area and now is slowly moving up the Atlantic coast (Andrews 1996; Ford 1996). These diseases have crippled both the culture and capture fisheries for these species. Another disease known as QPX (quahog parasite unknown) acts similar to dermo and MSX and results in the death of the hard clams in hatcheries (Ford, Kraeuter et al. 2002) and in open waters (Whyte, Cawthorn et al. 1994; Calvo, Walker et al. 1998)

To overcome issues concerning growth and disease several approaches have been undertaken including intensive breeding programs on the east and west coast of the U.S. (Allen 1998) and the consideration of introducing of new species such as the suminoe oyster (*Crassostrea ariakensis*) that appear to have natural disease resistivity (Dew, Berkson et al. 2003; Grabowski, Powers et al. 2003; Zhou and Allen 2003). Other approaches to increase growth and increase disease resistivity include the use of transgenesis. This technology has been applied to several commercially important fish species (Devlin 1997) and could be applied to bivalves. **Without debating the numerous environmental and ethical issues associated with these approaches, it would be prudent to have the ability to culture 100% sterile bivalves to increase growth rates and prevent the introduction of non-native genotypes or species into an environment.**

Rationale and Significance

Why bivalves?

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

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

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

Why sterility?

With increasing efforts to improve bivalve culture, several approaches have been taken including intensive breeding programs, introduction of new species, and genetic manipulation. This expansion of bivalve aquaculture has now demanded a need for production of sterile organisms. There are three major reasons why the ability to induce sterility would be beneficial:

1) Nutrient reserves normally utilized in gonad development could be directed to somatic tissue, **increasing growth and flesh quality**.
2) Sterility will prevent accidental introduction/establishment of **non-native species** into the environment.
3) Sterility will eliminate **genetic contamination** of native bivalve populations.

In bivalves, as in most animals, nutrients or energy are required for two major physiological processes, reproduction and growth. By eliminating the development of the gonad,

more resources can be used for somatic growth. This has been demonstrated as sterile, triploid oysters grow larger than their counterparts. Reproduction is often accompanied by the deterioration in taste and quality of the flesh as glycogen stores are used up (Gosling 2003). Induced sterility would therefore result in a better (sweeter) tasting product similar to what is found in non-reproductive oysters.

In an attempt to rebuild the oyster industry in the U.S. the suminoe oyster (*Crassotera ariekensis*) has been considered for production in the Chesapeake Bay. Native populations of *Crassotera virginica* have been reduced to minimal levels as a result of dermo and MSX. The suminoe oyster is apparently not as susceptible to these diseases as the eastern oysters. There are several examples of non-native species brought into an area (i.e. zebra mussel). Whether introduction is on purpose or by accident, detrimental effects have been realized (Khalanski 1997). The culture of non-native bivalves could be the answer to some of the industries problems, however sterility must be ensured to prevent negative effects on native species.

The third reason sterility is necessary for the development of the bivalve aquaculture industry is to contain manipulated genetics material. It is likely that as technology develops, genetically modified organisms will be used for scientific research and potentially for production purposes. Just as with the introduction of new species; the effects of accidental introduction into the environment would have unknown consequences, possibly detrimental ones. Therefore, any genetically modified bivalve that has the potential to be exposed to the environment needs to be sterile.

Currently, techniques such as induced polyploidy are available to generate sterile bivalves. Polyploidy can be induced be inhibiting meiosis I, meiosis II, or first cleavage. Inhibition can be attained with shocking the cells with temperature, pressure or chemicals. In bivalves, chemicals such as Cytochalasin B (CB) and 6-dimethylaminopurine are used to induce triploidy. Triploids are commonly sterile because homologous chromosomes cannot pair during meiosis. Triploid induction does not guarantee sterility and there are numerous reports of slow growing gonads and abnormal gametogenesis or gonads that are actually fertile (Guo and Allen 1994). Due to the continued growth of bivalve aquaculture there is a need to develop new approaches to inhibit reproductive development. Unfortunately, very little is known about mechanisms involved in the control of reproductive development in marine bivalves. **Therefore, the long term goal of the proposed research is to characterize the reproductive biology in marine bivalves, concentrating particularly on aspects that could be used to develop novel approaches to induce sterility**.

Research Approach

From the research that has been done in bivalves along with the extensive research on the reproductive biology in fish (reviews; Redding and Patino 1993; Nagahama 1994; Devlin and Nagahama 2002), it is clear that there are two crucial physiological events in sexual development. These include initial gonad differentiation and gametogenesis. In fish, hormonal induction of sterility is very sensitive to the state of sexual differentiation. In addition, the inhibition of specific factors regulating gametogenesis, have been shown to induce sterility in fish (see page 13). The reason fish are a good model for comparison even though they are phylogenetically distant from bivalves, is that there are several aspects of their life history and reproductive biology that are similar. Thus, the specific objectives of the research proposed here are to:

1) Characterize early gonad differentiation in juvenile bivalves
2) Isolate factors involved in gametogenesis in adult bivalves.

In order to characterize early gonad development in bivalves, two specific approaches will be taken. First, conventional histological techniques and cellular localization of a germ-cell specific marker will be used to document the progression of cytological events. In order to elucidate any differences across bivalves, two species will be examined; oysters and sea scallops. The reason these two bivalves will be used is because their sexual anatomy represents the two most common forms in commercially important bivalves (protandric hermaphrodite and dioecious). These different sexual strategies could be control via different mechanisms. Based on when major changes occur in gonad differentiation, cDNA libraries will be made from at least two developmental stages of juvenile oysters and sea scallops. Expressed Sequence Tag (EST) analysis will be done on each library to identify transcripts. It is expected that the EST approach will identify reproductive genes (i.e. hormone receptors, enzymes) important to early gonad differentiation, based on sequence homology. These genes could eventually be used as targets to inhibit gonad differentiation.

To isolated factors involved in gametogenesis two specific approaches will be taken. A reverse transcription polymerase chain reaction (RT-PCR) approach will be used to isolate candidate genes based on sequence homology. For this technique several species will be used including oysters, scallops, clams, and mussels. In addition, EST analysis will be used to examine cDNA libraries constructed from the cerebral and pedal ganglia of the surf clam (*Spisula*) and from male and female gonadal tissue of oysters. The surf clam will be used because of the prominent ganglia and oysters are chosen because of their commercial importance.

1) Characterization of gonad differentiation

It has been clearly demonstrated in fish that sex determination and differentiation is a labile process that can be altered by treatment of sex steroids (i.e. estradiol-17β and 17α-methyltestosterone) to manipulate sexual develop or cause sterility (reviews; Pandian and Sheela 1995; Devlin and Nagahama 2002). Timing of the treatment in relation to sex determination was

shown to be pivotal to the ultimate status of the gonad (i.e. fertile or sterile) (Devlin and Nagahama 2002). Treatment too early, or too late will not generate the desired effect. In addition to determining the timing of sex differentiation in bivalves, identify factors that control this gonad differentiation could lead to better approaches to induce sterility. For example, if a protein critical for the formation of a functional gonad was inhibited, (i.e. antisense RNA, dominant negatives, transgenesis) the animal is likely to be sterile.

To obtain baseline information concerning germ cell migration and gonad formation in bivalves, juvenile oysters (*Crassostrea*) and sea scallops (*Plactopecten*) will be hisotologically characterized. Samples will be taken on a weekly basis beginning shortly after metamorphosis and embedded for histological analysis. Dr. F.W. Goetz has done similar histological analysis of gonadal development in fish and has offered to assist in analysis (see attached letter from F.W. Goetz). This histological analysis will be performed during the first year. During year 2, samples will be taken again for *in situ* analysis. The techniques for *in situ* analysis will be worked out during the first year on sub-samples, but to ensure correct sampling timing and tissue viability, samples will be taken that correspond to sequential events during development (i.e. germ cell migration) following basic histological analysis. These samples will be analyzed for the expression of vasa (VAS). Vasa is germ-cell specific marker that will localize the cells destined to form gametes and specifically follow there migration. When samples for *in situ* analysis are obtained during year 2, corresponding samples will be taken to generate at least 2 cDNA libraries for EST analysis in order to identify putative genes that are involved in gonad formation during year 3. This will be done by comparing expressed transcripts with known sequences in the NCBI database as well as characterizing differential expression across libraries. If time remains at the end of year 3, cDNA fragments obtained from ESTs will be further characterized by obtaining full-length sequences and examining expression patterns using PCR techniques. ***Experiments proposed here to characterize gonad differentiation will provide information on appropriate times to manipulate sex in bivalves and putative targets for inducing sterility.***

Specific Protocols

Sampling

Oysters and sea scallops will be spawned at the MBL and raised through juvenile stages of development. Juveniles will be allowed to set on crushed oyster shells. The PD has experience spawning and raising bivalves (bay scallops, sea scallops). This has been a significant component of his post-doctoral research: "Isolation and characterization of factors regulated during larval competence and metamorphosis in the bay scallop, *Argopecten irradians*". Following metamorphosis and the setting of the spat, samples will begin to be taken for analysis. During year 2 corresponding samples will also taken and placed at -80°C for EST analysis. In the unlikely event mass mortality occurs during hatchery culture, juvenile seed could be obtained from Aquaculture Research Corporation (ARC) (Dennis, MA) or the Massachusetts Maritime Academy Aquaculture Hatchery (Bourne, MA).

Histology

Juvenile bivalves will be fixed by placing the oyster shells with attached juveniles into large bottles containing 1% glutaraldehyde/4% formalin in 28‰ NaCl (1G4F) (Howard and Smith 1983). After a minimum of one week, fixed juveniles will be removed from large oyster

shells and decalcified in formic acid. After decalcification, animals will be rinsed and processed in paraffin by conventional methods. Briefly, the bodies will be dehydrated in a series of increasing alcohol, cleared in toluene, and then infiltrated with paraffin. Processed bodies from individuals sampled at a given time will be mass embedded in parallel in paraffin blocks (~5 bodies/block; 3.0 x 2.5 cm block). Blocks will be initially trimmed and placed in tap water for 2 days to soften. Sections will be taken and slides (75 x 50 mm) will be made from sequential regions, stained with hematoxylin/eosin, and visualized under a compound microscope. Characterization of germ cell differentiation and early gonad development will be based on descriptions published for several bivalve species (Sastry 1979) and published work specific to oysters (Elston 1980).

In situ analysis

In situ analysis will be performed as has been previously described in detail by a researcher in the lab where the PD did his graduate training (Bobe and Goetz 2000; Bobe and Goetz 2001). After processing, tissue sections will be hybridized with a ~400-500 bp biotinylated vasa cDNA probe (BioPrime, Gibco). Hybridization and the detection of transcript will be completed using the *In situ* Hybridization and Detection System from Gibco. This system utilizes the binding of a streptavidin-alkaline phosphatase conjugate to the hybridized biotinylated probe and detection with NBT (nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3 indolylphosphate p-toluidine salt). Controls will consist of slides incubated without the primary probe and with a probe made to lambda DNA.

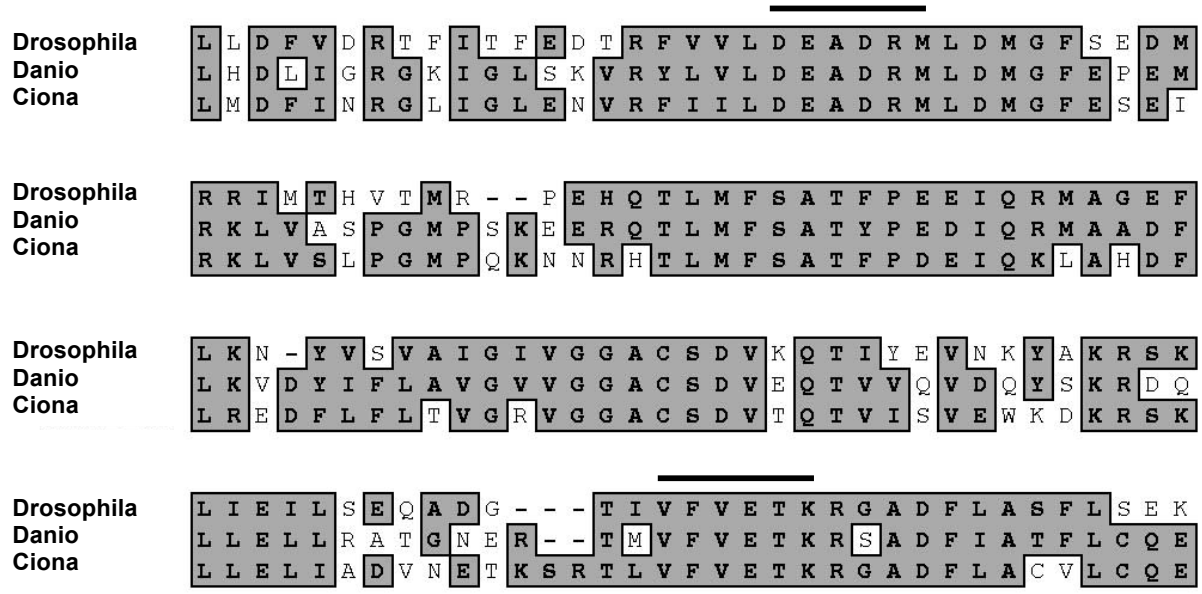


Figure 5. Amino acid alignment of vasa protein from the fruitfly (Drosophila – GenBank # P09052), zebrafish (Danio – GenBank # BAA22535), and sea squirt (Ciona – GenBank # BAB12216). Location of primers is denoted with solid lines.

Vasa is a germ-cell specific marker that will facilitate the identification and localalizaion of cells that are destined to form gametes. Recently, there has been the report of a vasa-like gene in oysters with a expression pattern consistent with what has been observed in other organisms (Fabioux, Huvet et al. 2003). An attempt to contact the authors of this abstract has been made in order to obtain either cDNA or sequence information. If this information cannot be obtained from these researchers, degenerative PCR will be used to clone the vasa homologue from oyster tissue. By aligning the amino acid sequence of two invertebrates and one vertebrate, initially two primers will be designed to regions of high homology
[primer a: GA(C/T)GA(A/G)GCNGA(C/T)(A/C)GNATG]
[primer b: (C/T)TCNGT(C/T)TCNAC(A/G)AANAC] (Figure 5).

cDNA library construction and EST analysis

In the laboratories in which the PD received his doctoral training and at his current location, over 30 cDNA libraries of various tissues from fish and bivalves have been constructed in ZAP Express (Stratagene), including libraries from adductor muscle and different developmental stages of the bay scallop for EST analysis (Roberts and Goetz 2003) (see – Results From Current NRI Support, page 30). The same system will be used to generate cDNA libraries of juvenile bivalves at different developmental stages determined from histological analysis in year 1.

Messenger ribonucleic acid (5.0 ug) from the soft tissue of approximately 20 individuals from a single sampling time will be used to construct a cDNA library in Zap Express (Stratagene). Based on histological analysis samples will taken at 2 time periods for each species (scallop and oysters). Sampling will occur 1) during prominent migration of primordial germ cells and 2) just prior to when spermatogonia / oogonia are first identified. Complementary DNA produced for library construction will be fractionated using sephacryl SF500, and two cDNA size classes are then ligated in separate reactions with the Zap Express vector. These ligations will be packaged together to produce one cDNA libraries of different average sizes. Both libraries are then mass excised to pBK-CMV phagemids and plated at low density. Individual colonies will be randomly picked and plasmid preparations made using the RevPrep Orbit (GeneMachines). Plasmid preparations will be sequenced from the 5' end using the dideoxy chain termination method with "Big Dye Terminator" (Applied Biosystems) and the BK reverse vector primer. The reactions are precipitated and resuspended in "Hi-Di Formamide with EDTA" (Applied Biosystems) and run on an ABI Prism 3730 automated sequencer (Applied Biosystems). Initially, 20,000 sequences will be generated (5,000/species/library).

Sequence chromatogram files are trimmed for quality using phred (http://www.phrap.org/phrap.docs/phred.html), vector screened using cross match (http://www.phrap.org/phrap.docs/phrap.html) and analyzed locally using 1) blastx against the NCBI nonredundant (nr) protein database, 2) blastn against the NCBI nucleotide (nt) database and 3) blastn against the NCBI EST (dbEST) database. All sequences will be grouped by category and tentative identified based initially on a blastx similarity score of $<10^{-3}$ or, in the case of blastx scores of $>10^{-3}$, a blastn score of $<10^{-5}$. All ESTs will be analyzed for redundancy

using CAP3 (Huang and Madan 1999). Occurrence of redundant sequences across developmental stages will be analyzed to detect differential expression.

2) Isolation of factors involved in gametogenesis in adult bivalves

Gametogenesis refers to the growth and development of gametes (eggs and sperm). In the future, molecular techniques could be used to inactivate factors or hormones vital for gametogenesis, thereby inducing sterility. Two approaches will be used to isolate endogenous factors important in gametogenesis: 1) A reverse transcription polymerase chain reaction (RT-PCR) approach to isolate candidate genes based on sequence homology and 2) EST analysis of tissues involved in the control of reproduction. Both of these approaches will focus on neuro-endocrine and gonadal control of gametogenesis. The basic principle behind the first approach is that conserved regions among candidate gene families are used for designing degenerative primers to be used for PCR. At least two genes will be targeted including GnRH and aromatase. The reason that these two genes have been selected is partially because there is significant evidence to suggest that they are present (See Introduction – page 5). In addition, examination of current research underway in the field of induced fish sterility has indicated these two genes as important targets. Scientists in Europe are investigating the feasibility of inhibiting GnRH to induce sterility in trout (Smith, Breton et al. 2003). The methodology used involves genetic engineering of fish to express antisense mRNA to GnRH thus inhibiting normal translation of GnRH. In addition to the successful generation of transgenically sterile fish, fertility was able to be regained upon injection of GnRH at time of sexual maturation (Smith, Breton et al. 2003). In the U.S., GnRH is also the focus of different approach to induce sterility, by disrupting the GnRH system in fish. Maryland Sea Grant (NOAA) is funding a research project entitled: A novel approach to induce sterility in farmed fish: disrupting the early establishment of the GnRH System (R/A-03) (Maryland Sea Grant 2003). Aromatase is the target for induced sterility in zebrafish in research funded by Illinois-Indiana Sea Grant entitled: "Targeted inactivation of the fish aromatase gene." According to the description of the project, the aromatase gene will be inactivated to produce a sterile line of fish (Illinois-Indiana Sea Grant 2003). Specifically, a primordial germ cell line will be used to transfer foreign DNA in the germ line of fish embryo. While the genetic manipulation techniques used in these fish studies have not been developed for bivalves, it is clear that isolation and characterization certain genes is important for the development of this approach.

The second approach that will be used to isolate factors important in gametogenesis is EST analysis. The reason an EST approach is used, is to identify other factor that are important that are difficult to target. The expected result from this approach is to identify other reproductive genes (i.e. hormone receptors, sex steroid metabolism enzymes, neuro-hormones, cyclins, vitellogenin, glycogen transporters) based on sequence homology with other organisms. These factors could eventually be used as targets to induce sterility in bivalves. As opposed to targeting specific genes, this approach will provide sequence for thousands of expressed genes in a specific tissue at a specific time. cDNA libraries will be constructed late in year 1 from the nervous and gonad tissue from sexually mature adult bivalves undergoing gametogenesis. Reproductive status will be determined by morphological examination. By the end of year 2 approximately 15,000 ESTs will have been generated for gonadal and nervous tissue of oysters and surf clams respectively. Year 3 will be spent obtaining full-length sequences and

characterizing expression of genes deemed to be important in gametogenesis based on sequence homology. ***The use of both a targeted and random approach will ensure identification of several putative targets that could be manipulated to induce sterility in bivalves.***

Specific Protocols

Tissue and RNA extraction

Nervous and gonadal tissue will be taken from sexually mature oyster, clams, scallops and mussels. Tissue will be homogenized with a TissueTearor (Biospec) and RNA isolated as previously described (Chomczynski 1987; Chomczynski 1993). These organisms are readily available in the facility the PD is located (see Facilities and Equipment – page 21). RNA from all species will be used in the RT-PCR approach to isolate candidate genes based on sequence homology. Cerebral and pedal ganglia RNA from the surf clam and both male and female gonad RNA from oysters will be used for EST analysis.

Targeting of candidate gene - GnRH

Unfortunately, there is little sequence homology of the prepro GnRH peptide across species, except the mature decaptide. This region is too small to develop two primers for conventional degenerative RT-PCR. To overcome this, a RACE-based degenerative PCR approach will be taken similar to the technique used for the isolation of GnRH from octopus (Iwakoshi, Takuwa-Kuroda et al. 2002). The template for initial PCR will be 5' and 3' RACE ready cDNA from the cerebral and pedal ganglia of *Spisula* generated using SMART RACE cDNA Amplification Kit (Clontech). This system provides a novel method for 5'and 3' RACE that has integrated the old Marathon cDNA amplification kit (Clontech) with the SMART (Switching Mechanism at 5' end of RNA Transcription). Unlike other systems ligation of and adapter is not needed. The mRNA isolation will be performed using the Poly-A-Tract mRNA Isolation System (Promega). First-strand cDNA synthesis is primed using a modified oligo (dT) primer. Once the reverse transcriptase reaches the 3' end of the mRNA, it adds multiple dC residues. The SMART II A oligonucleotide anneals to this end and provides an extended template for reverse transcription. After the PowerScript RT switches templates from the mRNA molecule to the SMART oligo, a complete cDNA a copy of the mRNA is made. This first-strand cDNA is ready to be used directly in PCR. The first round of 3' RACE PCR will be performed with the primer specific to sequence added to the end of the cDNA by the SMART oligo and a degenerative primer corresponding to Glu^{1}- Gly^{6} of the mature GnRH peptide [primer b: CA(A/G)AA(C/T)TA(C/T)CA(C/T)TT(C/T)NNNAA(C/T)CC] (Figure 6). PCR cycling conditions will follow manufactures instruction except that the reaction will be run on a gradient thermocycler (MJ Research) in order to obtain an optimized annealing temperature for the degenerative primer. If there is not a clear band a second round of PCR will be done, otherwise the band will be removed from the gel, extracted for DNA and cloned into the TOPO 2.1 vector (Invitrogen). If a second round of PCR is needed then the PCR product will be reamplified with a nested primer based on the SMART oligo sequence and a degenerative primer based on the amino acid residues Ser^{4}-Gly^{10} [primer c: (T/A)(C/G)NAA(C/T)GGNTGGCA(C/T)CCNGG)] (Figure 6). All PCR products will be cloned, purfied and sequenced on an ABI Prism 3730 automated sequencer as described above. Once sequence information is obtained gene-specific primers will be designed and used in 3' and 5' RACE to obtain full-length cDNA.

The use of denerative primers often results in amplification of non-specific products. In

addition the selection of amino acid residues used to design the primers might not be highly conserved in bivalves. If using the conditions outlined here for RACE of GnRH in *Spisula* is unsuccessful then different degenerative primers can be designed altering which residues are targeted and the degree of degeneracy. Other RACE ready cDNAs could also be generated from ganglia in other bivalves and at multiples stages of reproductive maturation in order to isolate a full-length. I expect to obtain a full-length GnRH cDNA of approximately 800 bps based on the prepro GnRH cDNA sequence of invertebrates found in the NCBI (octopus GenBank # AB037165-770bp; ciona I GenBank # AY204706 -1063 bp; ciona II GenBank # AY204708 -866 bp).

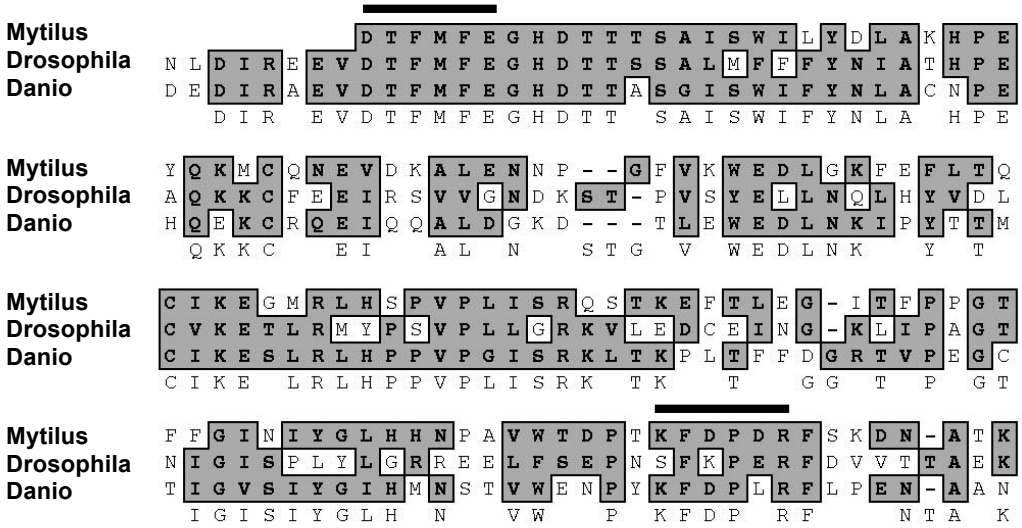
GnRH Family of Peptides

	1			2	3	4	5	6	7	8	9	10
Vertebrate												
Mammal	pGlu			His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	GlyNH2
Guinea Pig	pGlu			Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	GlyNH2
Chicken - I	pGlu			His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	GlyNH2
Rana	pGlu			His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	GlyNH2
Seabream	pGlu			His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	GlyNH2
Salmon	pGlu			His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	GlyNH2
Whitefish	pGlu			His	Trp	Ser	Tyr	Gly	Met	Asn	Pro	GlyNH2
Medaka	pGlu			His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	GlyNH2
Catfish	pGlu			His	Trp	Ser	His	Gly	Leu	Asn	Pro	GlyNH2
Herring	pGlu			His	Trp	Ser	His	Gly	Leu	Ser	Pro	GlyNH2
Chicken -II	pGlu			His	Trp	Ser	His	Gly	Trp	Tyr	Pro	GlyNH2
Dogfish	pGlu			His	Trp	Ser	His	Gly	Trp	Leu	Pro	GlyNH2
Lamprey - III	pGlu			His	Trp	Ser	His	Asp	Trp	Lys	Pro	GlyNH2
Lamprey - I	pGlu			His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	GlyNH2
Invertebrate												
Octopus	pGlu	Asn	Tyr	His	Phe	Ser	Asn	Gly	Trp	His	Pro	GlyNH2
Tunicate - I	pGlu			His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	GlyNH2
Tunicate - II	pGlu			His	Trp	Ser	Leu	Cys	His	Ala	Pro	GlyNH2
Tunicate-III	pGlu			His	Trp	Ser	Tyr	Glu	Phe	Met	Pro	GlyNH2
Tunicate-IV	pGlu			His	Trp	Ser	Asn	Gln	Leu	Thr	Pro	GlyNH2
Tunicate-V	pGlu			His	Trp	Ser	Tyr	Glu	Tyr	Met	Pro	GlyNH2
Tunicate-VI	pGlu			His	Trp	Ser	Lys	Gly	Tyr	Ser	Pro	GlyNH2
Tunicate-VII	pGlu			His	Trp	Ser	Tyr	Ala	Leu	Ser	Pro	GlyNH2
Tunicate-VIII	pGlu			His	Trp	Ser	Leu	Ala	Leu	Ser	Pro	GlyNH2
Tunicate-IX	pGlu			His	Trp	Ser	Asn	Lys	Leu	Ala	Pro	GlyNH2

Figure 6. Primary structures of GnRH in vertebrates with location of denerative primers used to PCR GnRH from bivalves denoted with solid lines. Figure from (Gorbman and Sower 2003).

Targeting of candidate gene - Aromatase

The full-length cDNA of aromatase has been cloned in a number of vertebrates and in an invertebrate, Drosophila GenBank # P33269 (Gandhi, Varak et al. 1992). An incomplete cDNA from the bivalve *Mytilus galloprovincialis* with homology to aromatase has also been isolated Genbank # AAC32835.1 (Snyder 1998). By aligning the corresponding amino acid sequences from these two invertebrate species along with a representative vertebrate sequence (zebrafish), regions of high homology can be observed (Figure 7).



(Mytilus – GenBank # AAC32835.1) fruitfly (Drosophila – GenBank # P33269) and zebrafish (Danio – GenBank # AAH56734). Location of primers is denoted with solid lines.

Two degenerative primers will be design in regions of high homology, a forward [primer c: GA(C/T)ACNTT(C/T)ATGTT(C/T)GA(A/G)] and reverse primer [primer d: C(G/T)(A/G)TCNGG(A/G)TC(A/G)AA(C/T)TT] (Figure 7). Total RNA will be used from the gonad of several bivalves. The mRNA isolation will be performed using the Poly-A-Tract mRNA Isolation System (Promega). First-strand cDNA synthesis will be synthesized using AMV reverse transcriptase (Promega). This cDNA will be used as a template for PCR using a gradient thermocycler to optimize degenerative primer annealing. PCR products will be sequenced as described above. Once cDNA fragments of the aromatase gene are isolated full-length cDNA sequence could be obtain by using the RACE technology described above. The mRNA starting material used would be from the same source as the initial fragment was cloned and gene-specific primers could be designed from the same fragment.

Expressed Sequence Tag (EST) analysis

cDNA libraries from surf clam ganglia and oyster male and female gonad tissue will be used to contruct cDNA libraries and ESTs generated as described for juvenile oysters and scallops (page12-13). Specifically, RNA will be combined from cerebral and pedal ganglia of 2 male and 2 female clams to generate one library and RNA from the gonads of 4 male and 4 female oysters to generate 2 cDNA libraries, respectively. A total of 15,000 sequences will be generated; 5,000 – clam ganglia, 5,000 – male oyster, 5,000 – female, oyster. Sequences will be analyzed as described above and with a putative identification given to each sequence. It is expected that a portion of these transcripts will be able to be identified as genes involved in reproductive development (based on sequence homology) and thus could eventually be used to develop techniques to induce sterility.

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Cooperative State Research, Education, and Extension Service

Washington, DC 20250-2200

JUL 12 2004

Proposal Number: 2004-01567
Proposal Title: Characterization of Gonadal Differentiation and Isolation of Factors Involved in Reproductive Development in Marine Bivalves

Dr. Steven Beyer Roberts
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Woods Hole, MA 02543-1015

Dear Steve ~~Dr. Roberts,~~

Thank you for submitting your proposal to the National Research Initiative (NRI) Competitive Grants Program. The NRI's Animal Reproduction Program was able to fund less than 15% of the standard research and strengthening proposals submitted during the current funding cycle. Although the costs of research are constantly rising, the availability of funds for agricultural research has not matched these increases. We regret that the program is unable to provide funding for your proposal this year.

We are continuing efforts to make awards that are meaningful in terms of duration and funds awarded. However, the overall quality of proposals has also increased and the competition is extremely keen for the limited funds available. Because of the limited funds, not all proposals that have been recommended for funding will be funded.

The review panel members and ad hoc reviewers carefully evaluated your proposal. These panel members and reviewers are fellow scientists selected for their competence in research and knowledge of your field. Your proposal was evaluated on scientific merit, qualifications of project personnel, adequacy of facilities, and relevance to long-range improvements in and sustainability of U.S. agriculture.

Enclosed are the relative ranking sheet, panel summary (or triage information, if applicable) and individual reviews for your proposal. Once you have had sufficient time to evaluate this information carefully, please feel free to contact me (mmirando@csrees.usda.gov, 202-401-4336) if you would like to schedule a telephone consultation regarding the review of your proposal. Information concerning important program information and answers to frequently asked questions can also be obtained on our web site at www.csrees.usda.gov.

In the event that you plan to submit a revised application to the NRI's Animal Reproduction Program, the next deadline for receipt of proposals submitted to this program is tentatively scheduled for December 3, 2004, pending release of the fiscal year 2004 Request for Applications.

Thank you for your interest in the NRI.

Sincerely,

Mark Mirando

Mark A. Mirando, Ph.D., National Program Leader
Animal Reproduction Program

United States Department of Agriculture
Cooperative State Research,Education and Extension Service

National Research Initiative Competitive Grants Program

Animal Reproduction

Proposal 2004-01567 **submitted by** Roberts

Characterization of Gonadal Differentiation and Isolation of Factors Involved in Reproductive Development in Marine Bivalves

The review panel grouped proposals into one of the relative categories below. The percentage indicates the final distribution of proposals in each category.

Recommended for Funding:

Outstanding %	2.2
High Priority %	15.4
Medium Priority %	34.1

Not Recommended for Funding:

Low Priority %	20.9
Some Merit %	23.1
Do Not Fund %	4.4

This proposal was placed in : Low Priority

Animal Reproduction - PANEL SUMMARY

The panel decision regarding your proposal is based on the input provided by the reviews and the collected expertise and judgment of the individual panel members. This panel summary reflects the consensus opinion of the panel regarding your proposal.

Proposal Number: 2004-01567 **Project Director:** Roberts

Proposal Title: Characterization of Gonadal Differentiation and Isolation of Factors Involved in Reproductive Development in Marine Bivalves

Objectives of the Proposal:

The overall goal of the proposed research is to characterize the basic reproductive biology in the bivalves, oysters and scallops, in order to eventually develop methods for sterilization to promote aquaculture in production of these organisms. The specific objectives are to: (1) characterize gonadal differentiation in juvenile bivalves and (2) isolate factors involved in gametogenesis in adult bivalves by using traditional histological analysis, in situ studies and EST analysis.

Positive Aspects of the Proposal:

The strengths of this proposal include that it is a well prepared submission. The background information provided the necessary foundation and rationale for the proposed studies. The Project Director, as a junior investigator, is well trained. The outcome of the proposed study would likely provide basic knowledge on reproduction in oysters and scallops. The area of investigation and model used are very appropriate for the USDA. The emphasis on reproduction is good and critical to understand for future consideration in bivalve aquaculture.

Negative Aspects of the Proposal:

There are several serious weaknesses of this proposal. It suffers from lack of preliminary data, is not focused and is not hypothesis driven. One question that arises is why the Project Director is not extending his previous study on the growth and development of bay scallops, in which he had identified a growth factor that may have significant implications for bivalve aquaculture. This would have been a logical extension instead of the current proposal. Too many species are proposed for study. There was a disconnect between the objectives and the experiments proposed. While the idea of developing sterile lines of bivalves to increase size and, potentially, palatability of the product was sound, there was no discussion of how these populations of sterile individuals would be repopulated for continued production.

Synthesis Comments:

The review panel had low enthusiasm for this premature proposal. The proposal suffers from lack of preliminary data, focus and testable hypotheses. One note of concern brought up in the panel is that Dr. Goetz who had offered his lab and equipment for the proposed study has since left Woods Hole and the question arises on the availability of laboratory facilities and equipment for the proposed research.

USDA, Cooperative State Research, Education, and Extension Service Competitive Programs National Research Initiative (NRI) Competitive Grants Program **PROPOSAL REVIEW SHEET**	Proposal #: 04-01567	Project Director Name: Roberts, SB
	Program Name: Reproductive Efficiency	

See Program Description and Guidelines for Proposal Preparation for Evaluation Factors

Comments: *(If needed, use additional sheets)* **NOTE:** Reviews, with reviewers' names removed, will be sent to applicants.

Characterization of gonadal differentiation and isolation of factors involved in reproductive development in marine bivalves

Grant Summary:
The objectives of this proposal were to: 1) Characterize early gonad differentiation in juvenile bivalves; 2) Isolate factors involved in gametogenesis in adult bivalves. The PD proposes to complete these objectives by obtaining samples of oysters and sea scallops at different stages of development and analyzing them for histology (to determine stage of development) and insitu analysis to detect germ cells with a gene- VASA which stains positively in germ cells. Furthermore RNA will be obtained from each developmental age and cDNA libraries conducted for genes expressed during each of those stages. In the second objective the PD proposes to isolate endogenous factors important in gametogenesis such as GnRH and aromatase using RT-PCR with degenerate primers. A second approach is to utilize EST analysis to identify genes based on sequence homology with other organisms in the oyster and scallop.

Scientific Merit:

General Comments:
The purpose of the current grant appears to be more of an establishment of a resource (cDNA libraries and characterization of gene expression during bivalve --oyster and scallop-- gonadal development and gametogenesis) than the testing of a hypothesis. Some of the initial characterization and hypothesis development needs to be conducted and demonstrated as preliminary data before adequate justification of the proposal can be made. While the idea of developing sterile lines of bivalves to increase size and potentially palatability of the product is sound there is no discussion of how these populations of sterile individuals will be repopulated for continued production.

While the PD has demonstrated that he can make and develop cDNA libraries from bivalve species there does not appear to be any further utilization of the genes that were identified on his postdoctoral research project to actually utilize these for further research or enhancement of production. A major concern is that identification of genes within this grant would not lead to actual pursuit of function or application to improvement of bivalve production.

The PD has the expertise and facilities and equipment to conduct the research. The research is relevant to increasing and improving production of bivalves.

USDA, Cooperative State Research, Education, and Extension Service
National Research Initiative Competitive Grants Program

PROPOSAL REVIEW SHEET

Proposal No.	Project Director
2004-01567	Roberts

Please return to:

Animal Reproduction (41.0)

SEE GUIDELINES FOR REVIEW

Comments: (If needed, use additional sheets) NOTE: Reviews, with reviewers names removed, will be sent to applicants.

Examination of endogenous neurohormonal control of molluscan reproduction is an approach to both enhancing reproductive success and controlling reproduction in individuals (e.g. exotic species). The alternative approaches used in controlling reproduction have involved direct induction of triploidy using CB (not preferred by public health regulatory agencies) or crossing diploid and tetraploids to obtain putatively sterile triploid offspring. The proposed approach of examining the neurohormonal control mechanisms of gonad maturation is elegant in its simplicity and may lead to a host of practical applications for the molluscan aquaculture industry.

The Principal Investigator, Steven Roberts appears to be well-qualified to carry out the proposed studies having recently (2002) studied with Frederick Goetz, a noted teleost fish endocrinologist, and recently completed studies in gene sequencing in bay scallops; skills which put together are a *sine qua non* for project success.

The budget as proposed appears to be reasonable or even a bit modest for the proposed work. The "new-ground" exploratory nature of this project with bivalve mollusks by a relatively young investigator building a career path is an argument that this is USDA money well-spent if awarded.

USDA, Cooperative State Research, Education, and Extension Service
National Research Initiative Competitive Grants Program

PROPOSAL REVIEW SHEET

Proposal No.	Project Director
2004-01567	Roberts

Please return to:

Animal Reproduction (41.0)

SEE GUIDELINES FOR REVIEW

Comments: (If needed, use additional sheets) NOTE: Reviews, with reviewers names removed, will be sent to applicants.

Proposal # - 2004-01567, Roberts

Summary

This proposal investigates basic reproductive biology in the bivalves oysters and scallops. The long-term goal is to characterize the reproductive biology with a focus on developing sterility approaches. The objectives are to characterize gonad differentiation and development, and using an EST library to be developed, identify genes for future analysis. Specific genes, (i.e. GnRH) will also be examined. The increased understanding of the basic reproductive biology is thought to be used in the future to design sterility procedures.

Strengths

The area of investigation, and model used, are very appropriate for the USDA. This is a critical and important agriculture product for the USDA and increasing in economic impact. The focus on reproduction is good, and basic information obtained will be useful for future development of more efficient culture and sterilization procedures. Assessing gonad development is very good and critical to understand for future consideration. The genes selected are appropriate for specific analysis and to assess regulation of reproductive status.

Weaknesses

No preliminary data is presented to address the feasibility of the procedures proposed, utility of model or assessment of outcomes. Basic experiments on histology would have been useful and shown the ability to collect at the various developmental periods. Expertise with the molecular procedure is not extensive and preliminary data would increase enthusiasm.

Why do the EST library since have a number of specific genes could start with such as GnRH. Feasibility of getting sequencing and using library major project likely not feasible in grant period. Suggest getting preliminary data before proposal.

To get more information on gonad development, could isolation of gonad for RNA preparation and PCR be done? This may improve data interpretation. Otherwise, original data normalization is difficult.

Objective was to develop sterilization procedure, yet no direct experiments to address this topic. Modifying steroid levels, temperature or other culture factors should have been discussed. A disconnect between objectives and experiments proposed.

USDA, Cooperative State Research, Education, and Extension Service
National Research Initiative Competitive Grants Program

PROPOSAL REVIEW SHEET

Proposal No.	Project Director
2004-01567	Roberts

Please return to:
Animal Reproduction (41.0)

SEE GUIDELINES FOR REVIEW

Comments: (If needed, use additional sheets) NOTE: Reviews, with reviewers names removed, will be sent to applicants.

The research described in this proposal is, basically, a molecular biological investigation of the control of reproduction in two bivalves used to "typify" this rather broad group of aquaculture organisms; one (oyster) is a dioecious protandric hermaphrodite and the other (sea scallop) is a simultaneous hermaphrodite. In addition, a baseline histological study will be undertaken to characterize germ cell migration and gonad formation in bivalves. This will provide a set of guidelines on the correct time for sampling for specific events in gonad formation and, perhaps, for induction of sterility.

There is little doubt that much more information is needed with respect to the control of gonad formation and the mechanisms by which the reproductive cycles, and even sex, are controlled in these species. Such information would make a multitude of factors important to the successful aquaculture of these organisms more controllable/understandable and provide the mechanisms through which these controls could be exercised. However, the approach taken in this proposal appears somewhat diffuse and seems to include a very large sampling/synthesis of molecules controlling the functions and mechanisms involved in the process of reproduction without delving much into their activities or interactions. It may be beneficial to develop more information in one or a couple of areas and use those as bases from which to develop more in depth understanding of some of the control mechanisms.

As the proposal now stands it seems that the major result from the 3 years of research will be an extensive histological examination gonadal development in the two bivalves and a wide array of ESTs and cDNA libraries about which information will be garnered on relationships to genes in other animals based on sequence homology. This approach, as well as the more targeted one of analyzing the control of genes that play a role in reproduction in higher vertebrates (e.g., aromatase and GnRH) will yield valuable data if the mechanisms/genes involved are similar to other animals. There is little mention made in the proposal about contingency plans if these two factors are quite divergent and how the experimental approach may have to be altered.

The proposal would have benefited immensely by inclusion of a timeline and a set of milestones so that the relationship between the different types of experiments would be better illustrated.

Proposal 04-01567
PI: Steven Roberts

Characterization of gonadal differentiation and isolation of factors involved in reproductive development in marine bivalves

This proposed research project will determine the genes associated with gonadal determination, differentiation, and post-pubertial gametogenesis. He will do this by utilizing molecular methods such as random sequencing of whole animal EST libraries, cloning of specific genes that are know to be important in vertebrate reproductive endocrinology, and *in situ* hybridizations to examine primordial germ cell (PGC) migration. He has identified **five (5)** models species, multiple tissues to investigate, and 2 life stages. The future goal is to identify key gene(s) which can be targeted for disruption in order to generate populations of sterile bivalves with 100% confidence.

The PD is very young (PhD awarded in 2002) and naïve (as we all were before we got old and jaded). His demonstrable experience with bivalves is a single report in the local lab's journal describing genes represented in a scallop EST library. To his credit, he has identified an increasingly important aspect of US aquaculture and is trying to get on the ground floor. Funding of this proposal would provide the needed resource to initiate this worthy effort and the goal stated in this proposal would likely fill his career.

The goal is not novel nor is the strategy (at least in fish) but this proposal represents an important effort to apply successful molecular strategies to address a huge problem in the bivalve aquaculture industry.

Although there are aspects of this proposal that is worthy, the proposal suffers from the lack of preliminary data, the lack of probes to be used for several of the tasks, and the huge assumption that genes of the vertebrate endocrine system (specifically, GnRH and aromatase) are present in his model organisms and function as critical genes in the reproductive system of bivalves. Even if a GnRH-like molecule is found in bivalves, it certainly could simply be one of many neurotransmitters that have been found in both verts and inverts. In fact, GnRH and its receptors are being found in multiple vertebrate tissues (e.g., fish gonads).

There is a good chance that the EST survey will provide needed probes and insights into the general molecular physiology of the model bivalves, I would be very surprised if any information was derived that would be useful to attack the proposed problem. This proposal is truly a "fishing expedition" with no compass.

The PD must focus on one or two model species, at least in these early years. Two tissues from five models (oysters, scallops, clams, mussels and surf clam) is too much. Also, I was not clear which oyster the PD was planning to use (C.v.or C.a.?). Rather than do a broad survey-type of project, I would prefer an in-depth examination of one species. In fact, I would also suggest focusing on one life stage for the first proposal.

Proposal 04-01567
PI: Steven Roberts

The PD has the facilities to conduct this work when the proposal was submitted but his mentor (Dr Goetz) and lab will be moving from WHOI in the near future. If the PD stays at WHOI, will he continue to have the facilities necessary for these studies.

There are no preliminary data to support any of the assumptions (fish are not bivalves and presumed similarities need to be supported). Although vasa is certainly a marker for PGCs in bivalves, the PD should have at least generated the PCR amplicons he proposes to generate in the proposal. In fact, I would highly suggest that the PD obtain portions of all cDNA probes and demonstrate the usefulness of heterologous reagents (e.g., antisera and cDNAs).

The methods are well-established and certainly routine for the PD, his mentor, or someone else at WHOI.

Specific points:
Provide supporting data for your model organism if you are going to make large leaps of faith, especially when there is little support in the literature. The PD is heavily leaning on his background in fish endocrinology.

The EST libraries will be constructed from whole larvae and adult nervous tissues and gonads. Although there are no doubts that the library can be successfully constructed and screened, I question if 5,000 ESTs are sufficient or if simply looking for homologs in published databases will recognize key genes.

The proposal is not focused.

Keep in mind that once one can "see" gonadal differentiation, the "determining" genes and factors have already induced their effects and are likely no longer present.

The PD will need to clone the aromatase and GnRH cDNAs, express recombinant enzyme to appropriate demonstrate activity, and/or demonstrate that blocking gene expression or function (inhibitors or knock-down RNAi technologies) affects reproduction. Otherwise, he should not include it in future proposals unless it is for an "exploratory grant".

PROJECT DIRECTOR: S.B. Roberts
Proposal # 04-01567

Scientific: The overall goal of the proposed research is to characterize the reproductive biology in marine bivalves in order to eventually develop methods for sterilization to promote aquaculture production of these organisms. The specific objectives are 1) to characterize gonad differentiation in juvenile bivalves and 2) to isolate factors involved in gametogenesis in adult bivalves. The research will use oysters and scallops. The approach will include traditional histological analysis and in situ studies using a germ specific marker. A second approach will include EST analysis to identify transcripts expressed during early gonad maturation.

Overall, this is a very well prepared submission. The background information provides the necessary foundation and rationale for the proposed studies. The PI has presented a series of logically designed experiments that are appropriate to the objectives of the proposal. The PI has a high probability of achieving at least the first objective within the proposed time period of this grant. The production of 15,000 ESTs could take at least two years at a high cost. There will likely be much difficulty in identifying genes of interest. Yet this approach will likely yield at least a few genes of interest. This is not a small undertaking. The merit of the proposed research is that the results will add significant information to our knowledge on the basic aspects of the reproduction in two important aquaculturally raised bivalves.

There is no question that the routine histological analysis can be done successfully. The in situ analysis will rely on the successful cloning of the germ specific marker, VAS. The PI has had experience with using RACE-based degenerative PCR approach. The EST techniques will be new in the PD's laboratory. The former advisor and now collaborator at Woods Hole, Dr. Goetz, has offered the use of his laboratory since some of the equipment necessary for these studies is not available in the PD's laboratory.

Investigator: The principal investigator is a young well-trained investigator. The PD has nine publications (some in proceedings) since 1999 representing a solid publication record.

Relevance to US Agriculture: The rationale of the proposed research is extremely sound in that the information from the proposed studies would add significant information to our knowledge on the basic reproduction in bivalves. This proposal is a timely proposal for one of the fastest growing segments within the aquaculture industry.

Other Considerations: None