Introduction

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 [1]. In the United States, shellfish are the highest valued farmed marine animal behind salmon [2]. Bivalve mollusks such as oysters and clams, are the primary shellfish species produced. And, even though oyster production has fallen off in the past decade due to disease, clam production was up 379% from 1998 to 1999 and continues to expand [1]. Recently, increased consumer demand and advances in culture technology have peaked interest in another bivalve species, the **bay scallop** (*Argopecten irradians*) [3-5].

Bivalves are in the phylum Mollusca and include species such as clams, oysters, mussels and scallops. All bivalve mollusks share certain common morphological characteristics. For example, bivalves have a calcareous shell with two valves that are

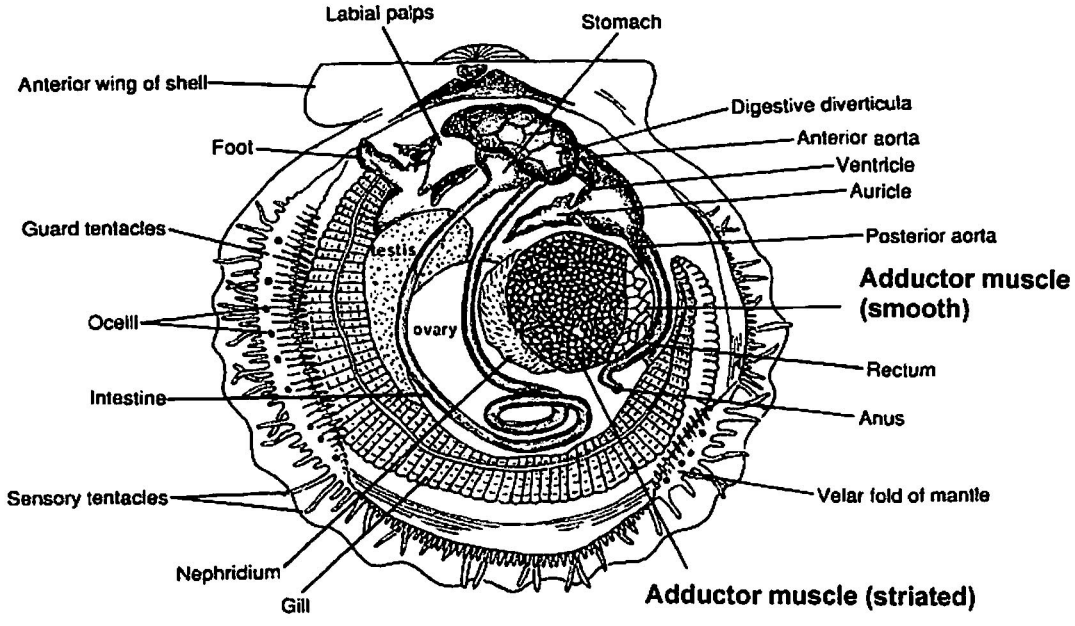


Figure 1. Diagram of the internal organs of a scallop. Modified from *Invertebrate Zoology 6th Ed.* [6]

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 intestines, gonads, and gills as seen in figure 1. Gills in bivalves are large and in most species function to exchange gas and collect food.

While various bivalve species are morphologically similar, bay scallops have characteristics that set them apart. For example, bay scallops have a higher growth rate, can propel themselves through the water as adults, are hermaphroditic, and have a single large adductor muscle (figure 1). In scallops, the anterior adductor muscle has been lost through evolution and the posterior adductor muscle has migrated centrally. The muscle is composed of two different types of muscle fibers. The cross-striated muscle, also

known as the phasic adductor, is the most obvious structure when examining the insides of a scallop. The major function of this muscle is the quick action necessary for opening and closing the shell, the major means of locomotion. In contrast, the smooth, tonic muscle provides sustained contractions for long-term closure. In addition, there is a transition area between the two muscle types where both type of fibers are found.

Bay Scallop - Natural Life History **(Fig. 2)**

Life history patterns are also similar for many bivalves. Embryos develop into planktonic larvae that eventually metamorphose into the adult form. Bay scallops spawn in response to increases in water temperature in the spring or early summer by releasing millions of microscopic sperm and eggs into the water. **Trochophore** embryos develop 24-48 hours after fertilization. A characteristic velum develops when the organism is ~70μm. The velum is an organ used for feeding and locomotion and has rows of tiny hair-like structures known as cilia along its outer margin. At this point the organism is considered to be in the **sub-veliger** stage. On the third day after fertilization, larvae become **straight hinged veligers** with a prodissoconch-I shell.

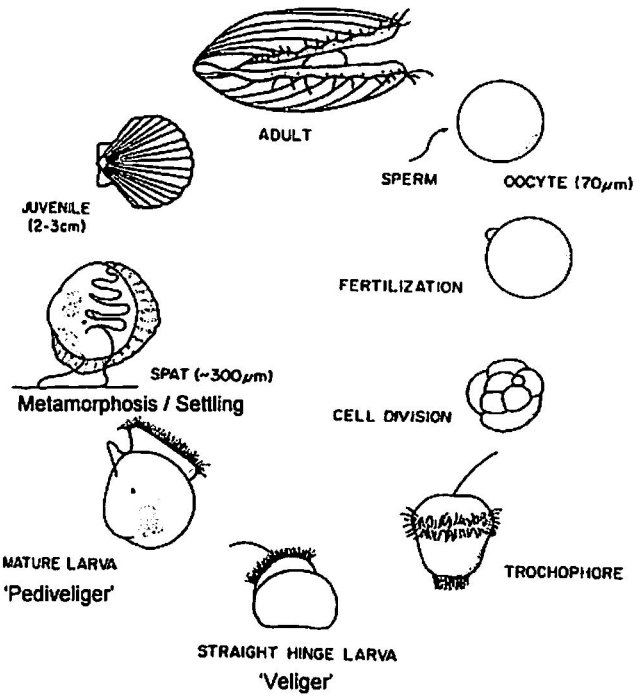


Figure 2. Schematic diagram of the scallop's basic life history. Modified from Sorgeloos *et al.* [7].

The "D" shaped larvae begin to feed, and at about 2 weeks the straight hinge is lost and umbos, the early beginnings of the adult shell, develop (prodissoconch-II). When an individual is ~200 μm, a ciliated foot and eyespot develop and the larvae has entered the **pediveliger** stage. For about 3 weeks the developing foot and the velum are both present and the scallop can use either for locomotion. Once a suitable substrate is found, metamorphosis occurs and the velum is lost and a gill feeding system develops. Because metamorphosis and settling behavior basically occur simultaneously, these events will often be discussed as a single event. The dissoconch shell is now apparent and the growth rate begins to increase. During these early stages of juvenile development, the bay scallop will attach to eel grass or algae by using secreted byssal threads. The eel grass provides some natural protection from predators. At this point the developing scallop is called a **spat** that alternatively attaches itself to eel grass or moves along the bottom with its foot. The bay scallop will stay associated with the eel grass for about 3 months. By four to five months from the time of fertilization, the spat will drop to the

estuary bottom and grow into a mature adult. Initially, the scallop is still susceptible to predation until the shell thickens. Bay scallops will live one to two years and can reach sexual maturity in 6 months. The majority of scallops will spawn only once during their lifetime. A few late-season animals may sometimes reach sexual maturity twice over two years.

Bay Scallops - Aquaculture

In the mid 1960s, scientists first identified the bay scallop as a good candidate for culture, and established many of the techniques [8-11] behind the current culture methodology [4,12]. Current technology is primarily focused on getting a quality market sized scallop to the consumer in reduced time with minimum financial expenditure. The culture of scallops as well as other bivalves can be broken into three major phases; 1) hatchery rearing of fertilized gametes; 2) nursery production (usually post-metamorphosis to spat size; 2-4 mm) and 3) growing out in open water to bring the bivalve to market size (see figure 3).

Mature broodstock are obtained from the wild and spawning is initiated by alternate warming and cooling of the holding water. Water temperature is raised and lowered over a period of several weeks to 17°C and a sufficient quantity of algae is provided for food. If the animals are maintained at 17°C, then raising the temperature to 25°C in a few hours will induce spawning. In attempts to shift and compress the time frame necessary to reach maturity and thus lengthen the growing season, bay scallops are spawned as early as the late winter [4]. Eggs and sperm are allowed to mix naturally or are mixed by culturists to obtain desired genetic offspring. Larval culture is usually conducted in conical bottom tanks that are gently aerated. About 1 ½ to 2 weeks after hatching, the larvae enter the **competent phase**. This is a significant change since they are physiologically able to **metamorphose** and **settle** (concurrent events) at this point. Larvae can delay metamorphosis until properly cued.

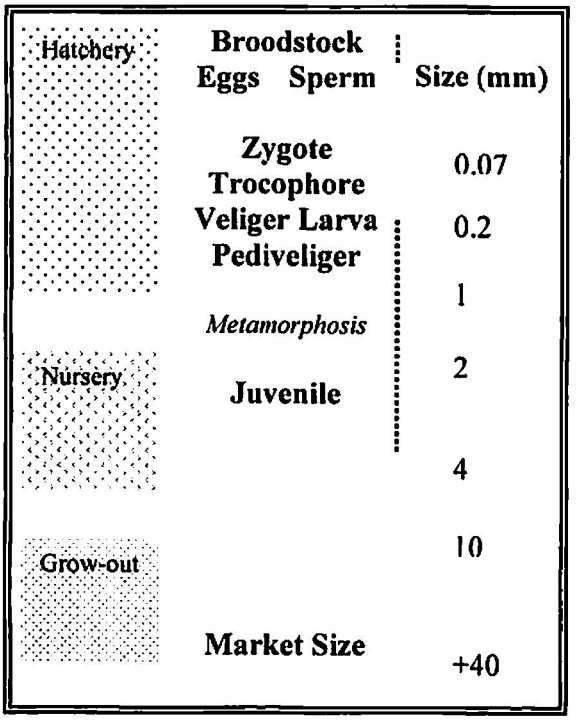


Figure 3. Flow chart outlining the stages of bay scallop development. Culturing facilities are noted at the left and approximate size to the right. Dashed lines indicate when algae is used to supplement the food supply.

Several methods are used for setting competent larvae, including plastic mesh netting, plastic inserts in troughs, and directly in down-wellers (tanks with a gravity fed water supply). One common method is for competent larvae to metamorphose on down-welling sieves with low water flow or static conditions. The low flow of water through a mesh screen provides larvae with

nutrients without crushing them. Once set, it takes a scallop about 1 month to reach 1 mm. Juvenile bay scallops are commonly moved to an up-weller where the gentle flow of water up and out of the tank provides suspended nutrients and removes waste products. Care has to be taken so that scallops do not escape out of the top. Raceways and pearl nets suspended in the water are methods used to raise scallops to a size large enough for final grow out (10mm). Because of the mobile nature of adult scallops, individuals cannot be spread or allowed to settle on natural substrates as oysters and clams are. Methods used for adult grow out include containment in plastic mesh cages on racks, suspended mesh cages, lantern nets, or individually suspended on long lines.

Rationale and Applied Justification

Why Aquaculture ?

Even if the rate of population growth does not increase, the world population will double by the year 2040. This growing global population affects the welfare of communities and ecosystems around the world. To feed this growing population there will be a greater need for protein sources such as seafood. As the population increases so has the technology to increase the efficiency of harvesting fish from the ocean, even for those species that are in low abundance. This has led to overfishing of many species, and in some cases, the complete decimation of others. Since the mid 1990s, the total world fisheries catch has plateaued [1]. This has occurred even with increased fishing pressure. Thus, there has actually been a decrease in the catch per unit effort, a grim foreshadowing for the future of the natural fisheries. As a result of the dwindling natural fisheries, aquaculture has the potential to play a major role in global food production in the future.

Global aquatic production has increased more than ten percent over the past decade to a value of 52.5 billion dollars in 1998 [1]. However, the U.S. aquaculture industry has not been as prolific compared to the rest of the world. Even though the U.S. ranks third in the national consumption of seafood and fourth in total fisheries catch [13], the U.S. only ranks 11^{th} in aquaculture production. Instead of relying on fish farming to meet their seafood demand, the U.S. relies on seafood imports, being the world's second largest importer of fisheries products. In 1999, U.S. consumers spent an estimated $52.3 billion for fisheries products, importing $9.0 billion of edible seafood. 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 [14]. This, in part, has caused the U.S. Department of Commerce to call for a 5-fold increase in aquaculture production by 2025.

Mariculture currently makes up 1/3 of global seafood farming by weight [1] and is the fastest growing segment of the aquaculture industry. In the U.S., mariculture will provide the most likely avenue for expanding the aquaculture industry as inland resources are being used [15]. In the U.S., shellfish are the highest valued farmed marine animal behind salmon [2], and bivalve mollusks such as oysters and clams are the most common shellfish produced. For example, in 1999 U.S. aquaculture of clams and oysters was an 80 million dollar industry [2].

Why Bivalve Mollusks ?

There are several advantages and benefits in culturing bivalves as opposed to finfish. In marine and estuarine aquatic systems, a major cause of poor water quality is eutrophication. Excess organic waste from finfish aquaculture, including uneaten food, feces, and dead fish, can contribute significantly to eutrophication. Sedimentation of such waste negatively affects benthic communities under and surrounding net pens. In addition, nitrogenous waste (mainly in the form of ammonia) in high quantities can be toxic to fish and shrimp. In contrast, bivalve mollusks are filter feeders and can remove excess organics, nutrients, and particulates [16-18]. Simply reintroducing bivalves into the wild can have a substantial effect by removing harmful nutrients. Filter-feeding mollusks can also be raised in conjunction with finfish to improve water quality.

One rationale that is frequently used to support the advancement of finfish aquaculture is that it takes fishing pressure off wild fish stocks. However, this is not always the case. Many cultured marine species such as striped bass, eel, and salmon have diets high in fishmeal and fish oil; products themselves of wild caught fish. Between 1986 and 1997, 4 of the top 5, and 8 of the top 20 captured fish species were used in feed production for aquaculture and livestock industries [1]. These species include European anchovy, anchoveta, Chilean jack mackeral, Atlantic herring, chub mackeral, Japanese anchovy, round sardinella, Atlantic mackeral, and Atlantic menhaden. The reason fishmeal and fish oil are used in diets is to supply essential amino acids and fatty acids to the fish. In contrast, marine bivalves do not require any supplemental fishmeal and oil for production and, therefore, do not put additional pressure on global natural resources.

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 reach sexual maturity in six months. Bay scallops reach market size in 1-2 years in the wild, or nine months under laboratory conditions [19]. 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 suspended nets as opposed to bottom culture like oysters and clams. Therefore, the use of valuable submerged land is not necessary, grow-out is not limited to inland waters, and culture methods can easily be developed so they do not interfere with the remaining commercial shellfish industry.

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

However: ***The Problem***

One of the primary bottlenecks with bay scallop culture is **the high rate of mortality**. Mortality rates begin to rise during early development and the greatest loss of investment occurs during winter. The solution involves the **control of settlement** in conjunction with an early spawn, yielding a longer growing season that allows harvest in the same year. Increasing adductor muscle mass could also increase survival and production.

As described earlier, after a scallop egg hatches, the embryos progress through several developmental forms, including trochophore, veliger, and pediveliger larval stages. At these pre-metamorphic stages, the larvae are very delicate. This delicate nature, in conjunction with the fact that the larvae are usually excessively handled at this time, results in increased mortality. As spat settle and begin to develop protective shells mortality rates begin to decrease. 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. Actually, during winter, little to zero growth occurs and a significant number of scallops will die [4,5]. 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. The reason for this phenomenon, also termed "winter mortality syndrome", remains unknown. However, there is anecdotal evidence that suggests that first season larval/juvenile growth and size of a young scallop plays a role. Mortality is probably more common among individuals with small body mass since fewer resources are available for defense against disease and environmental conditions.

A solution to overwintering mortality involves harvesting market size scallops prior to the onset of winter. Before this can be done more information on the regulation of growth in scallops is needed. Thus, the long-term goal of the research proposed here is to better understand the factors that control the growth and development of the bay scallop, concentrating particularly on competence and metamorphosis. This complex transformation during the life history of scallops is not fully understood. It is known that first a larvae must enter a competent phase before it is physiologically able to metamorphose and settle. Additionally, it has been documented that growth rates increase significantly after metamorphosis [5]. Thus it would be highly desirable to accelerate the progression of scallops through some of the early development, particularly the metamorphosis of larvae to juvenile scallops. Unfortunately, very little is known about the factors that signal a larvae to become competent to metamorphose. **Thus, the immediate goal of the research proposed here is to isolate and characterize factors that are regulated during larval competence and metamorphosis.** The hypothesis is, that if the levels of these factors are correlated with specific developmental processes such as competence and metamorphosis, then some of them may be important in the control of these processes. Elucidation of factors regulated during development and growth could lead to the development of culture systems that efficiently move the scallop through its life stages, decreasing handling and resulting in the economical production of market size scallops within the first year. In addition, the results of this research should easily be applicable to other important shellfish such as oysters, clams, and mussels.

Research Objectives

Many invertebrate animals go through dramatic morphological changes during development. In genetic research models such as the fruit fly and the nematode worm, *Caenorabditis elegans,* this process has been studied in great detail. Many of the factors that are related to development are now known. However, this is not the case with marine mollusks. 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.

Much of the research on what cues competence and metamorphosis in mollusks has concentrated on external environmental cues such as compounds released from other organisms (for review see [20]). The effects of several physical parameters such as water flow and temperature on metamorphosis have been examined [21]. Pharmacological compounds have also been used to artificially induce metamorphosis in certain mollusks. Some of the most common inducers are gamma aminobutyric acid (GABA), catecholamines, L-beta-3,4 dihydroxyphenylalanine (L-DOPA), and potassium ions [22-27].

Recently, several genes have been isolated from mollusks primarily using a candidate gene approach. Tentative identities based on sequence homology and expression patterns, suggest that these genes could play a role in development and metamorphosis. For example, a Pax-6 homologue, a gene necessary for eye development in many animals, has been reported in multiple tissues in squid, *Loligo opal*escens [28]. In vertebrates and classic invertebrate models, homeobox genes are necessary for proper development, and recently, homeobox homologues have been isolated in several mollusks [29-32]. Finally, several members of the TGF-β superfamily have been reported in species of *Sepia, Crassostrea*, and *Haliotis* [33,34]. While some aspects of the structural changes and genes that occur at metamorphosis in mollusks have been reported (red abalone; [35]), there really is very little known about the **internal, physiological control of competence and metamorphosis in mollusks**. It is our hypothesis that by understanding the gene expression and signal pathways involved in these developmental processes that settling may be managed more efficiently.

Many of the external cues that have been examined do not work across species lines or even efficiently in individuals within a species. In addition, the time it takes to observe an effect following an external stimulus is sometimes unpredictable. In contrast, by knowing what specific genes products or stimulatory pathways are involved in the control of metamorphosis, larvae could be more specifically and directly stimulated to settle and begin growing at the faster, adult growth rate. Elucidating the internal

mechanisms controlling metamorphosis and development might also allow for the genetic selection of a preferred strain of scallops. For example, scallops could be selected and bred on the basis of certain characteristics pertaining to the enhancement of pathways involved in metamorphosis and settling. Knowing what genes are involved in development and what their function is, could be useful in future genetic research. In a laboratory setting, genes could be over or under expressed to clearly identify phenotypic effects of the genes.

Finally, the techniques used in the proposed research will focus on the isolation of genes at the whole body level. However, the formation of the adductor muscle also occurs during metamorphosis and, therefore, it is possible that genes specific to the development of the scallop muscle will also be isolated. Understanding more about the genes that control the development of muscle tissue in these organisms has the potential for increasing adductor muscle yield in scallops and other pectinids. There is also the potential to enhance taste, as muscle fiber size and density are correlated with firmness [36,37], a major component of taste.

In order to isolate genes that may be important for larval competence and the induction of metamorphosis in the bay scallop, two specific objectives will be carried out in the proposed research:

1) A subtractive cloning approach will be used to isolate upregulated and downregulated genes during development.

2) A reverse transcriptase polymerase chain reaction (RT-PCR) approach with degenerate primers will be used to isolate candidate genes based on sequence homology.

Specific Objectives

1. Isolation of regulated genes using subtraction.

To isolate genes that are regulated during development and metamorphosis, subtractive cloning technology will be used. Most of the previous work on molluscan gene expression has used a candidate gene approach. Though this technique can be successful, there is an assumption that these genes are highly conserved, sometimes across divisions as broad as phyla. In contrast, subtraction is based on an inclusive approach that will isolate conserved as well as novel genes. The general premise behind molecular subtraction is that one can have two different pools of individuals; for example, the same mollusc species but at two different developmental stages. Between these two stages, many genes will be equally expressed, while a few genes will be novel or differentially expressed. By subtracting the two pools, genes that are differentially regulated (either up or down) can be isolated.

There are several types of subtractive technologies that are available such as differential display PCR (DDPCR), library subtraction, chemical crosslinking subtraction, and suppression subtraction hybridization (SSH). There are advantages and disadvantages for each of these subtraction methods. The most sensitive approach is DDPCR that uses random primer pairs to isolate cDNA fragments of messages that may

be differentially expressed. However, from a global perspective, the major disadvantage with DDPCR is that it will only isolate cDNAs that happen to be amplified by the particular random primers used. This might be acceptable if one could use a very large number of random primers, thus amplifying regions of all possible messages. However, because DDPCR also generates a large number of false positives, it means that there is an incredible input of labor to clone and verify all potential clones when the analysis is done with many primer pairs. Also, it is virtually impossible to test the number of primer pairs that would ensure that all messages would be covered. A more directed and global approach is SSH [38,39] (figure 4).

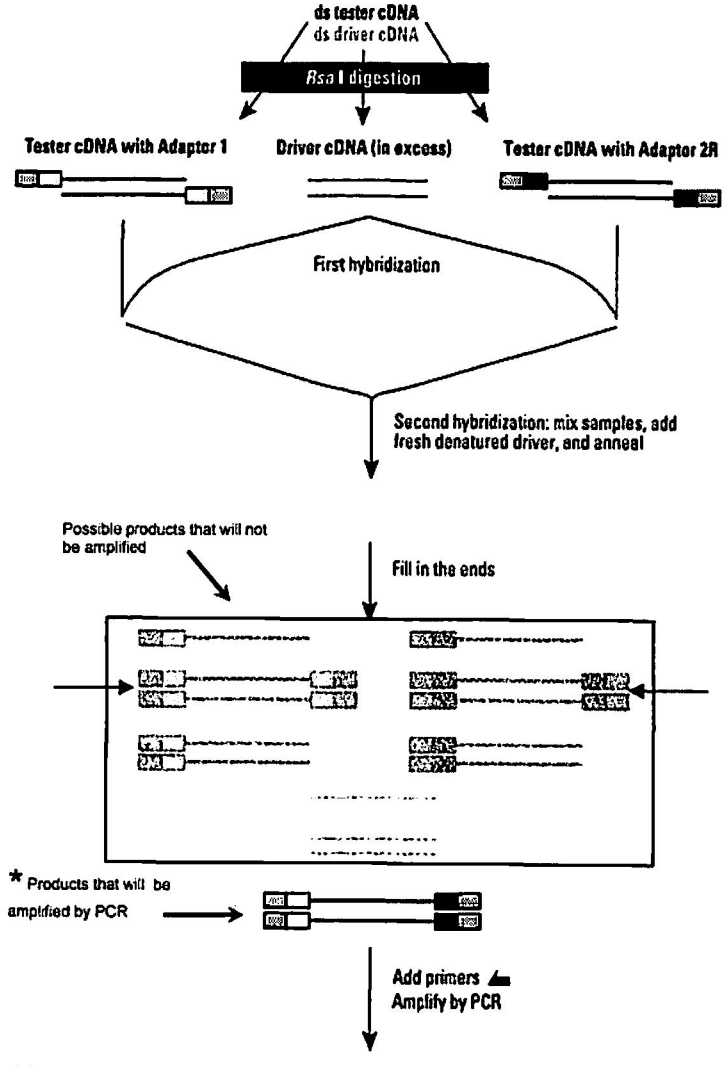


Figure 4. Schematic of Clontech PCR-Select™ subtraction method. Modified from Clontech User Manual PT1117-1.

This method was used originally to isolate genes that were regulated during metamorphosis in frogs and resulted in the successful isolation of 16 genes [40]. In fact, it was considered to be so inclusive that the authors described it as a method that took the place of a saturation mutagenesis screen to isolate metamorphic genes. SSH has also been used successfully in isolating genes involved in ascidian metamorphosis [41]. The other advantage of this method is that the basic reagents and methodologies are available in a kit from Clontech ("PCR Select"). SSH involves digesting the cDNAs obtained from two sources (e.g., scallops prior to metamorphosis and scallops at or during metamorphosis), and ligating two different adaptors to one group of cDNA (**tester**) and hybridizing it with an excess of the other cDNA (**driver**). Genes that are common to both tester and driver cDNAs will hybridize (products not shown in figure 4) and cannot be PCRed. However, cDNAs unique to tester cDNA will not hybridize to driver cDNA. The unique single strand tester cDNA is eventually hybridized together (second hybridization) and now contains both of the adaptors that are used for PCR. The adaptors are engineered to prevent undesirable amplification during PCR by means of a method called suppression PCR. Suppression occurs when complementary sequences are present on each end of a single stranded cDNA (indicated by red arrows in figure 4). During each primer annealing step, the hybridization kinetics strongly favor the formation of a "pan"-like secondary structure that prevents primer annealing. When occasionally a primer anneals and is extended, the newly synthesized strand will also have the inverted terminal repeats and form another pan-like structure. Nonspecific amplification is suppressed such that only the tester cDNA containing different adapters will be amplified (marked by * in figure 4). This will isolate genes that are upregulated in the tester cDNA. The tester and driver cDNAs can be switched to isolate genes that are downregulated as well.

In the current proposal, three separate pools of cDNA will be isolated from developing bay scallops, though only two are used at a time for subtraction. The three stages at which cDNA will be obtained are (**A**) planktonic straight hinge larvae, approximately 1 week after hatching; (**B**) larvae at approximately 2 weeks that have not settled; and (**C**) larvae at approximately 2 weeks that are in the process of settling, see table 1. By using three developmental stages for SSH, genes involved in competence and metamorphosis can be individually isolated. This experimental design (table 1) incorporates a redundancy that increases the odds of isolating novel genes. For example, when B=tester and A=driver the product(s) is likely upregulated during larval competence. When C=tester and A=driver the product(s) is likely upregulated in relationship either to larval competence and/or metamorphosis (see table 1). Using Northern analysis the differential expression of a given cDNA can be verified and a more specific temporal expression pattern can be obtained.

A	planktonic veliger larvae (not competent)
B	pediveliger larvae that have not settled (presumed to be competent)
C	pediveliger larvae that have settled (competent and metamorphosing)

Tester	Driver	Possible characteristics of isolated gene
A	B	larval competence; downregulated
B	A	larval competence; upregulated
A	C	induction of metamorphosis and/or competence; downregulated
C	A	induction of metamorphosis and/or competence; upregulated
B	C	larval competence; upregulated - induction of metamorphosis; downregulated
C	B	larval competence; downregulated - induction of metamorphosis; upregulated

Table 1. Description of the three stages of development taken for cDNA subtraction. Potential characteristics of genes isolated is described corresponding to choice of cDNA used as Tester and Driver.

Protocol:

Subtraction: Scallops at appropriate stages will be provided by Dr. Dale Leavitt through a research shellfish hatchery, at the Massachusetts Maritime Academy (Buzzards Bay, MA-please see letter Dr. Leavitt). At the three stages of development described above, 1 gram of whole body tissue will be placed into ice-cold Tri-Reagent (Molecular Research Center Inc.). Tissue will be homogenized with a TissueTearor (Biospec) and RNA isolated as previously described [42,43]. The mRNA isolation will be performed using the Poly-A-Tract mRNA Isolation System (Promega). SSH will be performed using a kit (PCR Select, Clontech) according to the manufacturer's instructions with modifications as previously described [44]. For each developmental stage, double strand cDNA will be made from 2.0 ug of mRNA template and will then be digested with *Rsa*I. For simplicity, in the description of the remaining processes, the terms "tester" and "driver" will be used to designate the two pools of cDNA being subtracted (figure 4). The tester cDNA pool will be equally divided and an adaptor (adaptor 1) will be ligated on both ends of tester cDNA in one half of the pool. This fraction will be referred to as 'tester 1'. A different adaptor (adaptor 2R) will be ligated on the other half of digested tester cDNA (now referred to as 'tester 2'). Driver cDNA will be also digested with *Rsa*I. Initially, two separate hybridizations will be performed at 68°C for 8 h using an excess of driver and tester 1 and 2. These two hybridization mixtures will then be pooled in the presence of an excess of driver and incubated at 68°C overnight. After hybridization, the mixture will be diluted and used for PCR. After incubation at 75°C for 5 min and 94°C for 25 s, a primary PCR will be performed (94°C, 10 s (denature); 66°C, 30 s (anneal); 72°C, 1.5 min (extend); 27 cycles) using Advantage Polymerase Mix (Clontech) with a primer that will be complementary to both adaptors 1 and 2R. A secondary PCR will then be performed (94°C, 10 s (denature); 68°C, 30 s (anneal); 72°C, 1 min and 30 s (extend); 11 cycles) using a diluted aliquot of the primary PCR as a template, and primers complementary only to adaptor 1 (primer 1) and only to adaptor 2R (primer 2R).

This PCR will be performed in presence of ^{32}P dATP so that it can be used immediately as a probe. The resulting PCR mixture will be then applied to a Centrisep Column (Princeton Separation) to remove unincorporated reagents.

Reverse Northern: A major obstacle with SSH has been the analysis of the subtracted product. This product contains a mix of presumably regulated cDNAs that can be redundant in terms of their presence in the mix. To address this, a procedure involving a **Reverse Northern** strategy will be used that will determine which cDNAs isolated by SSH actually represent mRNAs that are regulated, and at the same time provides the technical means to isolate them from the mixture and to clone them for further analysis [44].

After denaturation, the labeled PCR mixture will be used to probe a Northern blot on which total RNA obtained from scallops at the same stages of development as that used for subtraction, is run in separate lanes. Since the labeled PCR mixture is a collection of different cDNAs representing multiple mRNAs that are potentially activated, it is possible that the labeled PCR probe will hybridize to a number of bands, of which some may be close together and/or may be close to some bands that are not regulated (i.e., false positives). Thus, to increase the resolution of the bands, the RNA gel will be run longer than normal so that the bands are spread over a greater length of the gel. In addition, if further resolution is needed, a different percentage agarose can be used in the gel. After washing under stringent conditions, the Northern blot will be exposed to X-ray film for 48 h. The film and original Northern membrane will be aligned and differentially regulated bands between larvae and juvenile scallops will be excised, soaked in water for 10 min and boiled for at least 10 min. After spinning, the supernatant will be removed and the probe cDNA will be ethanol- precipitated (37°C, overnight) in the presence of glycogen using 3 M sodium acetate and absolute ethanol. The samples will be centrifuged to pellet the DNA and washed. An aliquot of the DNA will be reamplified by PCR) using primers 1 and 2R. The reamplified PCR products will then be separated on an agarose gel, the bands will be cut and cloned in TOPO pCR 2.1 (Invitrogen,) and the ligated plasmid will be used to transform competent TOP 10 cells (Invitrogen). Inserts will be sequenced as previously described [45] and will be used to probe Northerns to substantiate that the band truly represented a differentially regulated cDNA as observed during the original Northern capture. In addition, samples from additional developmental stages will also be obtained and run on Northerns to get a more accurate temporal expression pattern for selected cDNAs that are regulated.

cDNA Library Screening: At this point, because the cDNA used for SSH was initially cut randomly by *Rsa*I (to facilitate hybridization), the pieces of regulated cDNA obtained from the Reverse Northern represent only partial cDNAs of the regulated mRNA transcript. Thus, for selected cDNAs (ones that are interesting based on the degree of regulation), full-length transcripts will be obtained by cDNA library screening. During the time that the initial subtractions are being carried out, cDNA libraries of bay scallop larvae and juveniles will be generated from the same stage mRNA used for SSH. In the laboratory in which the PD received his doctoral training, over 20 cDNA libraries of various tissues from trout and zebrafish were constructed in ZAP Express (Stratagene),

and these libraries have been used successfully to obtain a number of reproductive genes e.g. [44,46-49]. The same system will be used to generate cDNA libraries of bay scallop tissue at different developmental stages. Libraries will be plated at 30,000 plaques/plate and lifted to nylon membranes. Plasmid preparations of upregulated cDNAs cloned in TOPO will be restriction enzyme digested and the resulting pieces purified on agarose gels. The appropriate cDNA bands will be excised, purified from the gel and labeled using ^{32}P dATP. Lifts will be hybridized with labeled cDNAs overnight and washed the following day under stringent conditions (0.1 X SSPE, 0.5% SDS, 65°C) and exposed to X-ray film. Positive plaques will be rescreened once to homogeneity and in vivo excised to phagemids. Plasmid preparations of phagemids will be sequenced completely on both strands and the sequences analyzed against known sequences in GenBank for preliminary identification based on homology.

Finally, cDNAs of transcripts that are regulated and have the potential to be important in development and metamorphosis based on sequence homology and the degree of expression, will be further characterized if time permits. For example, in situ analysis may be used for tissue localization at stages that have high levels of expression.

2. Isolation of candidate genes using RT-PCR

It is possible that the subtractive approach proposed above may not elucidate transcripts that can be clearly implicated in development. For example, only cDNAs that exhibit homology to structural genes might be isolated by SSH. Therefore, a second approach involving RT-PCR will also be used simultaneously to try and isolate genes that could potentially have a role in the control of development and metamorphosis. Using RT-PCR, candidate genes will be isolated based on sequence homology with genes from other organisms. The basic principle behind this technique is that conserved regions among candidate gene families are used for designing degenerative primers to be used for PCR. In this case, a specific gene can be targeted or more commonly, a family of genes with similar functions and common structural domains can be used.

A few interesting developmental genes, such as homeobox genes, have been isolated in several **non-bivalve** invertebrate groups. Homeobox genes are transcription factors that control development in many species. Mox homeobox genes are one class in this group and HruMOX has been isolated in the mollusk (gastropod), *H. rufescens* [30]. This gene is differentially expressed during metamorphosis. The high homology of the deduced homedomain sequence within vertebrate species, strongly suggests that if it is present, a transcript could be isolated from scallops.

A great deal of work as been done on asicidian (subphylum Urochordata) metamorphosis and the genes involved. The research suggests that epidermal growth factor (EGF) signaling plays a major role in this process. In the ascidian, *Herdmania curvata,* the EGF-like protein "Hemps" is a key regulator of metamorphosis [50] [51]. This was shown by expression patterns and the fact that metamorphosis was blocked by the addition of an antibody to "Hemps" [50]. In *Boltenia villosa,* SSH was used to isolate genes expressed during metamorphic competance [41]. One of the genes isolated was a cornichon homolog. In *Drosophila*, cornichon is thought to play a role in EGF signalling during embryogenesis [52]. Cornichon is highly conserved across species and could also be targeted for RT-PCR analysis in scallops. Finally, using differential screening, Nakayama *et al.* isolated two genes, Ci-meta1 and Ci-meta2 from *Ciona intestinalis* that

are expressed after metamorphosis but not prior [53]. Both of these genes have protein characteristics (repeats, signal sequences, and binding domains) that implicate them in EGF signally. Specifically, Ci-metal has 13 calcium-binding EGF-like repeats that are highly conserved across species and thus makes it a good starting point for designing degenerative primers.

Another group of genes that has the potential of being involved in the development of scallops is the transforming growth factor (TGF)-β superfamily. This superfamily is made up of different subgroups including bone morphogenetic proteins (BMP), growth differentiation factors (GDF), activins, inhibins, and TGF-β. There are several structural characteristics that all members of the TGF-β superfamily possess including conserved cysteine residues that support the "knot" structure, a RXXR proteolytic cleavage site and a highly conserved carboxy-terminal region. Many of these genes have been shown to be important in the development of both invertebrates and vertebrates.

Recently TGF-β superfamily homologues have been isolated in mollusks, including oysters [34] [33]. Therefore for the proposed research those sequences and other newly isolated genes will be used to design primers. Since the adductor muscle of the adult scallop is the primary component of the body and the marketable product, genes related to muscle development will also be targeted. One member of the TGF-β superfamily involved in the control of vertebrate muscle development is GDF-8, also known as myostatin. Myostatin was first characterized in the mouse where it is expressed during embryogenesis in developing somites [54]. Adult mice that did not express the myostatin gene were 25-30% heavier than wild-type littermates and the individual muscles of the myostatin null mice were 2-3 times heavier. This increase in muscle mass was attributed to an increase in fiber number (hyperplasia) and size (hypertrophy). Subsequently, the double muscling phenotype found in Belgian Blue and Peidmontese cattle was attributed to mutations in the myostatin gene [55-57]. If a myostatin homologue is present in bay scallops there is the potential that muscle yield could eventually be improved by manipulation of this protein.

Protocol:

Scallop tissues and RNA samples will be obtained as described above at different developmental stages. Messenger RNA (0.25ug) will be reverse transcribed to cDNA using AMV reverse transcriptase (Promega) following manufacturers instructions. At least two sets of primers will be designed based on the TGF-β superfamily and EGF-like domains.

TGF-β superfamily: PCR primers will be generated by aligning a number of TGF-β family sequences (figure 5). Special attention will be paid to mGDF [34] and GDF-8 [55]. Since mGDF was isolated from oysters, this sequence would theoretically be the most closely related in general to scallops, and since GDF-8 is myostatin, it has the potential to be important based on its function in controlling muscle growth in other animals. Within the conserved regions indicated by the solid lines, two primers have

been developed: *Forward1* {GGN TGG GA(C,T) TGG AT(A,C,T) (A,T)TN GCN CCN} and *Reverse1* {(A,G)CA NCC (A,G)CA NC(C,G,T) NTC NAC NAC CAT} that will be used for PCR (see below).

Proteins with EGF-like domains: PCR primers will be generated by aligning Ci-meta1 with related sequences all containing EGF domains. Ci-meta1 can be aligned with genes containing conserved regions from zebrafish, *Xenopus*, a cnidarian, and humans (figure 6). Within the conserved regions indicated by the solid lines, two primers have been developed: *Forward2* {TG(C,T) AAN CCN GGN T(T,A)(C,T) CAN GGN} and *Reverse2* {NGT (A,G)CA NGT (A,G)(T,A)A (A,T)(C,G)N N(C,T)(C,T) NAC} that will also be used for PCR (see below).

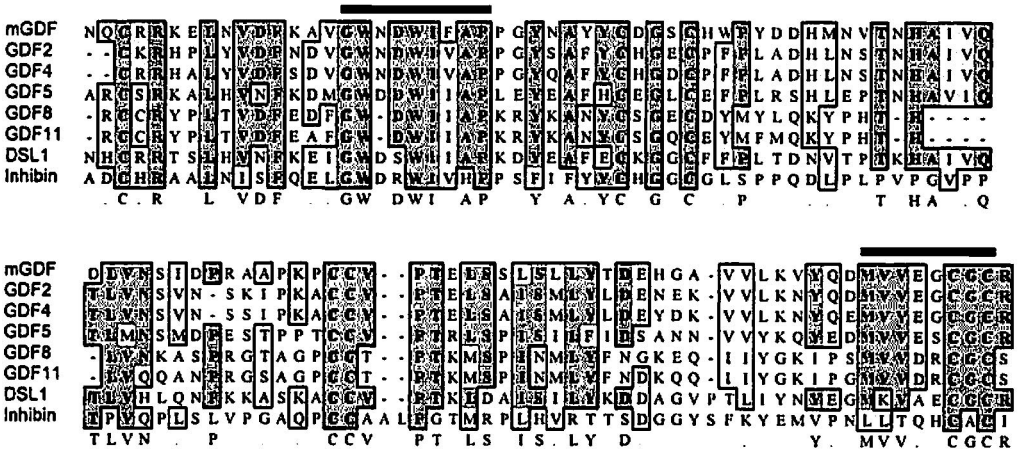


Figure 5. Amino acid alignment of TGF-β superfamily members from different species used to design degenerative primers. Solid bars indicate region where degenerative primers were derived. Sequences seen are: mGDF oyster GB Ac# AJ130967; BMP2 chicken GB Ac# Q90751; BMP4 chicken GB Ac# Q90752; GDF5 mouse GB Ac# P43027; GDF8(myostatin) zebrafish GB Ac# O42222 ; GDF11 mouse GB Ac# AAF21633; DSL1(Dorsalin) chicken GB Ac# P34822; Inhibin cow GB Ac# P07994.

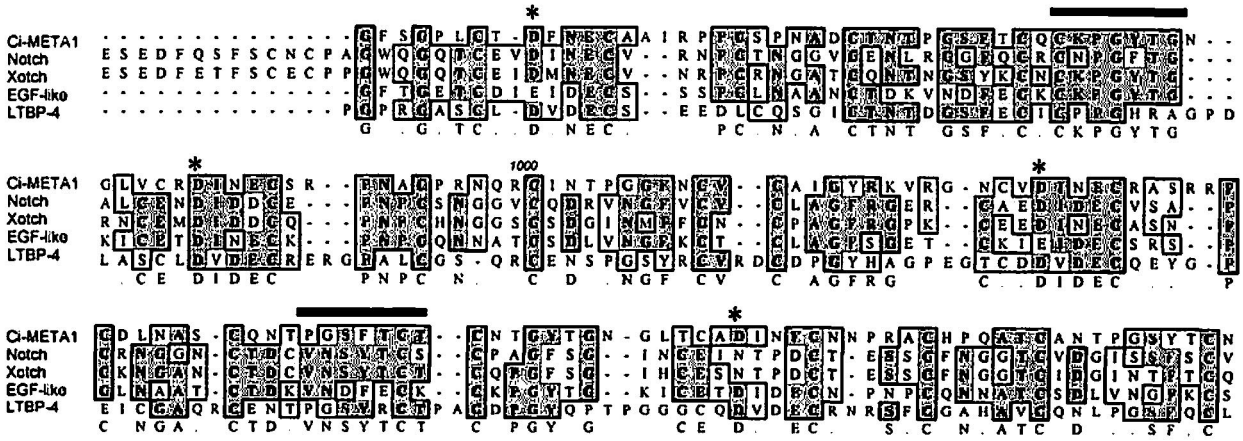


Figure 6. Amino acid alignment of Ci-meta1 and proteins with EGF domains from different species used to design degenerative primers. Solid bars indicate region where degenerative primers were derived. Asterisks indicated the start of the conserved, repeated calcium-binding EGF-like domain. Sequences seen are: CI-META1 *C. intestinalis* GB Ac# BAB40596; Notch zebrafish GB Ac# P46530; Xotch *Xenopus* GB Ac# A35844; EGF-like *P. carnea* (cnidarian) GB Ac# AAK92130; LTBP-4 (latent TGF binding protein) Human GB Ac# AAC39879.

Based on maximum homology and minimum degeneracy, the regions chosen for TGF-β and EGF-like domain aligned sequences have good potential to be active with RT-PCR in scallops, however, other areas can also be used to generate primers if these are not successful. The cDNA will be incubated at 95°C for 1 min followed by PCR (94°C, 10 s (denature); 50-65°C, 30 s (anneal); 72°C, 1 min (extend); 30 cycles) then incubated at 72°C for 10 min. The annealing temperature will be empirically determined using a gradient thermocycler. Polymerase chain reactions will be separated on agarose gels and visualized under UV light looking for a band at the appropriate size. If appropriate bands are visualized they will be gel purified, and cloned in pCR 2.1 (Invitrogen). Positive clones will be grown for plasmid preparation and the cDNAs will be sequenced.

While cDNAs obtained by RT-PCR might be involved in developmental processes in scallops, they will not have been isolated by a differential screening process as with SSH. Thus, these cDNAs will be used to probe Northerns of RNA from scallops taken through the entire developmental process. This will determine if there is any regulation, and therefore, correlation with development and metamorphosis. Transcripts that are regulated and have the potential to be important in development and metamorphosis based on their sequence homology will be further characterized as with SSH. This will involve obtaining the full-length cDNA by screening cDNA libraries as described for SSH.

Timeline

The normal reproductive season for scallops is spring-summer. Thus, if the present proposal is funded the PD would have to wait a number of months to take scallops in early stages of development. Thus, in anticipation of the start of this project, appropriate larvae samples will be provided by Dr. Dale Leavitt from a research shellfish hatchery, at the Massachusetts Maritime Academy (Buzzards Bay, MA-please see letter Dr. Leavitt). The samples will be homogenized in Tri-Reagent and stored at –70° C for RNA processing. During the first part of year one, tissue will be processed for RNA, and mRNA will be isolated. By the end of year one RT-PCR and SSH will be completed. By the beginning of year 2, using Northern analysis, a more detailed expression pattern will be obtained with multiple developmental stages, and cDNA libraries will have been constructed. Genes that are likely candidates for regulating development and metamorphosis will be isolated from cDNA libraries so that the full length cDNA can be cloned and fully sequenced. As time allows at the end of the second year, genes will continue to be characterized throughout development and at the tissue level using Northern analysis and possibly in situ analysis. An outline of all of the proposed tasks and approximate time of completion can be seen in figure 7.

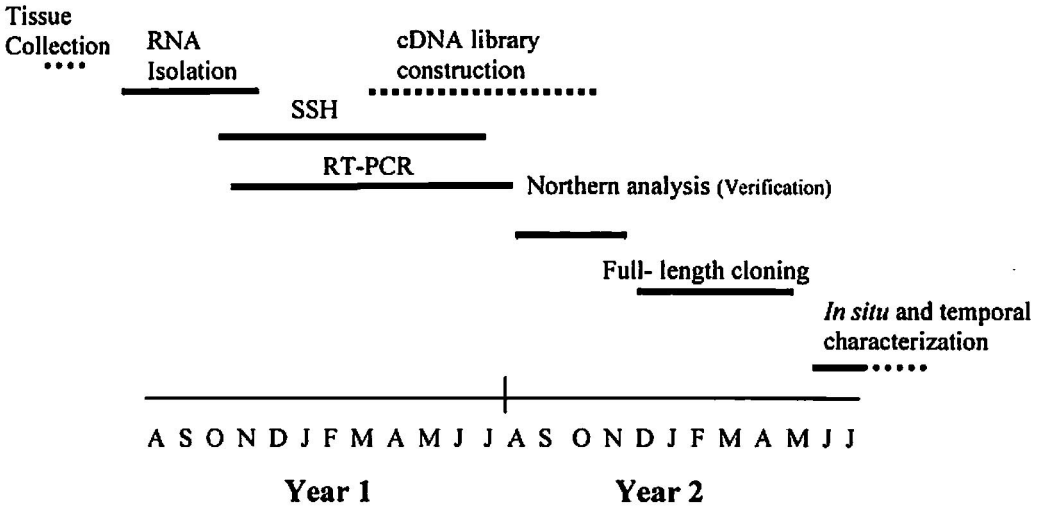
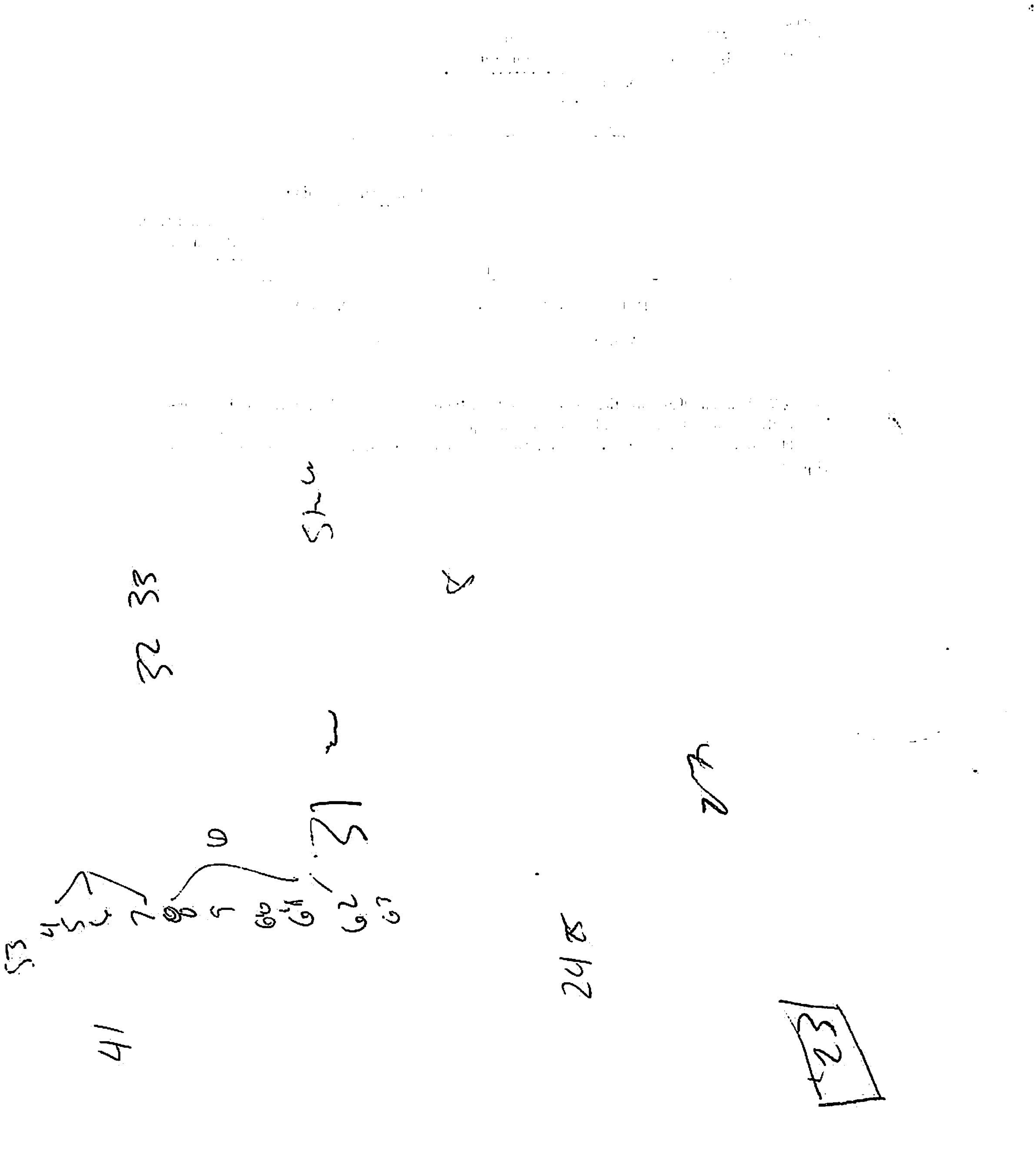


Figure 7. Timeline depicting the approximate time frame each task will require and the when it is to be completed. Note that tissue collection will take place prior to the initiation of funding due to reproductive cycle. The degree to which tissue localization will be done is dependent on completion of preceding tasks.



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