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MOLECULAR SIGNALS, RECEPTORS AND GENES CONTROLLING REPRODUCTION, DEVELOPMENT AND GROWTH: PRACTICAL APPLICATIONS FOR IMPROVEMENTS IN MOLLUSCAN AQUACULTURE[1]

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D. E. Morse and A. N. C. Morse (1991) Molecular signals, receptors and genes controlling reproduction, development and growth: Practical applications for improvements in molluscan aquaculture. *Bull. Inst. Zool., Academia Sinica, Monograph* **16**: 441-454. Molecular signals, receptors and signal transducers that regulate reproduction, development and growth can be used as control points to overcome limitations to production at these stages, and thus increase the economic efficiency and yield of molluscan aquaculture. The cloned genes and cDNAs also provide useful tools both for analysis of the regulatory mechanisms controlling reproduction, development and growth, and for the manipulation of these processes. Examples from research with abalone (*Haliotis*), and needs for future research in molluscan and other animal systems, are discussed.

Prostaglandins control spawning in abalones and certain other commercially valuable species. Spawning can be induced by addition of prostaglandins to the surrounding seawater, or, more reliably and inexpensively, by activation of the endogenous enzymatic synthesis of prostaglandin-related spawning triggers by addition of hydrogen peroxide, which serves as obligatory co-substrate for the rate-limiting biosynthetic enzyme. Induction with peroxide has proved widely useful for obtaining synchronous and copious release of fully competent gametes in a large number of abalones, oysters, scallops, mussels, clams and other valuable molluscs; several of these species had not been successfully spawned by other methods.

[1] Portions of this report have been published previously (Morse, 1984a, 1984d and 1991).

Settlement and metamorphosis of *Haliotis* larvae are controlled by at least 2 convergent chemosensory pathways regulated by chemical signals from the environment. One of the most potent natural morphogenetic inducers is a GABA-mimetic peptide found on the surfaces of crustose red algae associated with natural recruiting substrates. The requirement for this inducer can be met by addition of GABA, which binds to the same larval chemosensory receptors that recognize the natural peptide inducer. This procedure can be used simply, safely, and inexpensively to induce rapid larval settlement and metamorphosis—with minimal mortality—in many commercially important abalone species. Peroxide induction of spawning and GABA induction of metamorphosis are now routinely used in commercially successful abalone aquaculture industries in the USA; similar inducers are in routine use for production of other molluscs as well.

The post-receptor pathway of signal transduction provides additional clues for development of inducers of larval settlement and metamorphosis. In *Haliotis* and other larvae, chemosensory receptor binding of the morphogenetic cue triggers post-receptor changes in cyclic AMP, calcium ion and protein phosphorylation, culminating in an induced ion flux across the chemosensory membrane; the resulting excitatory depolarization transduces the chemical signal from the environment to one propagated by the nervous system. A wide variety of effectors that duplicate these post-receptor events also can be used to induce larval metamorphosis, even in species for which the natural inducer may not be known. Induction of metamorphosis by depolarization with K^+ salts has proved most widely useful for a number of aquaculture species.

We discovered that abalone growth can be stimulated by exogenous growth hormone (GH). This finding has been independently verified in three different laboratories in Japan, Australia and the USA. Professor Kawauchi and his colleagues in Japan have obtained evidence for endogenous GH production in abalones, and Professor Joosse, Dr. Geraerts and their colleagues in Holland have characterized other classes of growth-regulating peptides and their genes from related gastropods. In an international collaborative effort now being developed, the cloned cDNAs for these peptides will be used as probes to identify factors regulating their expression, and as templates for enhanced synthesis of the growth regulators, in efforts to further augment molluscan aquaculture production and efficiency without the need for transgenosis.

Key words: Molecular signals, Receptor, Genes, Reproduction, Development, Growth, Mollusca.

Many commercal and developing aquaculture industries face serious economic inefficiencies and losses resulting from insufficient

control of reproduction, development, growth, and survival of cultivars; reliance on inefficient, capital-intensive, energy-intensive and/or labor-intensive technologies; and reliance on increasingly unavailable large bodies of unpolluted water. To improve the economic efficiency and yield of marine aquaculture industries, and to adapt these industries to present economic and environmental constraints, significant intensification of production, modernization of technology, and improvement of cultivars and needed. Recently developed methods of biochemical and genetic engineering can be adapted for the improved control of those biological processes which intrinsically limit the efficiency and yield of marine aquaculture production. As illustrated in recent research and development with abalones and a number of other commercially valuable marine molluscs, such critical processes including reproduction, development, larval settlement, metamorphosis, and the acceleration of growth have proved amenable to improve control by these new methods (Morse, 1984a, 1984b; 1990, 1991; Hooker and Morse, 1985).

Identification of the underlying molecular mechanisms controlling reproduction, development, metamorphosis and growth in abalones and other species has revealed several essential processes that can be controlled easily and efficiently by provision of the required biochemical regulatory substances or their inexpensive natural analogs (Morse *et al.*, 1977a; 1979a). These biochemical methods are reliable, safe and convenient, making possible the low-cost improvement of mass-production and efficiency (Morse *et al.*, 1977b, 1978 1979b, 1990, 1991; Morse, 1984a). Such methods have proved widely applicable to the improved production of a large number of species of abalones, oysters, scallops, mussels, clams and other valuable molluscs under cultivation for food and other products worldwide (Morse, 1984a).

Other methods for the acceleration of growth, improvement of food-conversion efficiency, and enhancement of the resistance of cultivars which are presently under development involve the use of recombinant DNA probes to analyze signals and mechanism controlling gene expression, and the use of genetic engineering for the amplified production of hormones and related growth-regulating molecules, production of vaccines, manipulation of

genetic content, and clonal production. These and similar methods, including techniques for controlled gene amplification and transfer, are under investigation for the enhancement of production of a number of important marine plant and animal-species.

CONTROL OF REPRODUCTION

Control of reproduction is one of the most central and basic processes required for efficient aquaculture. Analyses conducted in our laboratory revealed that reproduction in abalones and a large number of other commercially valuable molluscs is controlled by hormones known as prostaglandins (Morse *et al.*, 1977a, 1977b, 1978; Morse, 1984a). In a number of these species, prostaglandins released in the spawn of one animal will trigger the spawning of of other gravid animals nearby. Taking advantage of this observation, we found that we could induce spawning in gravid male or female abalones simply by adding very low concentrations of prostaglandins to the surrounding seawater (Morse *et al.*, 1977a). However, this procedure was dependent upon the use of rather expensive hormones, and proved less than perfectly reliable.

In searching for a more reliable and less expensive method that could be used for the control of reproduction in a large number of species, we found that the enzymatic synthesis of prostaglandins in the reproductive tissues of molluscs (and certain other species) was naturally dependent upon—and limited by—the rate of synthesis of minute amounts of hydrogen proxide (Morse *et al.*, 1977a, 1977b). This hydrogen peroxide is made naturally, in the reproductive tissues, by prostaglandin-endoperoxide synthetase, the enzyme that catalyzes the rate-limiting reaction in the biosynthesis of the prostaglandins. The peroxide then is used by the cupric copper-containing active site of this enzyme as an obligatory or required co-substrate in the prostaglandin biosynthetic reaction.

Based upon this finding, we developed a convenient inexpensive method for reliably inducing spawning, simply by adding a small amout of hydrogen peroxide to the seawater surrounding the animals to be reproduced (Morse *et al.*, 1977a, 1977b, 1978). This method conveniently and reliably activates the synthesis of the endogenous prostaglandin spawning trigger, and thence induces the animals to spawn. Copious spawning of both males and

females is induced synchronously. The eggs and sperm obtained by this method prove fully competent for normal fertilization and development (Morse *et al.*, 1977b, 1978).

This method for the direct chemical activation of spawning trigger synthesis has proved to be rapid, reliable, inexpensive and widely applicable to many species (including a growing number which could not reliably be spawned by other methods). As a result, the peroxide method has been widely adopted in research and industrial applications, for improved control of reproduction of a large number of abalones, oysters, scallops, mussels, clams, the tridacnid giant clams, and other commercially valuable molluscs under cultivation or development (Morse, 1984a).

The induction of spawning with hydrogen peroxide appears to be mechanistically similar to the method developed earlier by Drs. Kikuchi and Uki (1974), in which UV-irradiated seawater is used to induce spawning. It is likely that UV photolysis of seawater generates species of electronically active oxygen similar or identical to those resulting from the direct addition of hydrogen peroxide to alkaline seawater (Morse *et al.*, 1977b). We and others have found, however, that the peroxide method appears to be somewhat more reliable and rapid, and capable of induciug spawning in species which had proved to be refractory to induction with UV-irradiated seawater. Also, the addition of hydrogen peroxide generally can induce spawning in specimens in which gravity is not advanced sufficiently to make induction by the UV method possible.

CONTROL OF LARVAL SETTLEMENT, METAMORPHOSIS AND EARLY POSTLARVAL SURVIVAL

Serious economic and operational inefficiencies and high losses typically are found in conventional aquaculture industries resulting from poor control of processes dependent upon larval metamorphosis (Morse, 1984a, 1984b; Hooker and Morse, 1985). By analyzing the natural requirements for the induction of larval settlement and metamorphosis in a number of abalone species, we found that these processes are efficiently induced by the chemosensory recognition of GABA-mimetic peptide inducers that the larvae normally find on the surfaces of certain crustose red algae in their natural environment (Morse

et al., 1979a; Morse, A. and Morse, 1984; Morse, A., 1988; Morse, 1990, 1991). This process ensures the substratum-specific recruitment of abalone larvae to suitable habitats in coastal waters. In cultivation, however, if inducing molecules are not adequately provided, the larvae exhibit lower success at metamorphosis, with subsequently lower survival and growth as juveniles (Morse *et al.*, 1979a, 1979b; Morse, 1984a, 1984b, 1990, 1991; Hooker and Morse, 1985).

Although the crustose red algae proved to be unsuitable for direct use in aquaculture operations (specimens collected from the field frequently carry many small predators, and the algae grow too slowly for large-scale growth of predator-free surfaces), characterization of the biochemical inducer from these algae has made direct biochemical control feasible (Morse *et al.*, 1979b). From the characterization of the natural GABA-mimetic peptide inducer that the larve encounter on the algal surface (Morse, A., 1988), a number of simple and inexpensive biochemical analogs capable of satisfying the learval requirement for an inducer was found. Most efficiently and inexpensively, the natural biochemical requirement for induction of settlement and metamorphosis in abalones can be met by providing the larvae in culture with γ-aminobutyric acid (GABA) (Morse *et al.*, 1979a, 1979b), an analog of the natural inducing peptide that is recognized by the same externally accessible chemosensory receptors on the larvae that normally detect the algal signal molecule (Trapido-Rosenthal and Morse, 1986; Morse, 1990, 1991). Thus, GABA can be used simply, safely, and inexpensively to induce complete and rapid larval settlement and metamorphosis—with minimal mortality—in many commercially important abalone species (Morse, 1984a, 1990, 1991; Hooker and Morse, 1985). Provision of GABA or similar neuro-transmitter-related, amino acid-derived compounds (such as DOPA) also is proving useful for improving the efficiency of induction of settlement and metamorphosis of a number of other valuable molluscan species (including various oysters and clams) (Morse *et al.*, 1979a; Morse, 1984a).

As found by a number of researchers in several different countries, the results discussed above prove applicable to a number of abalone species. At last count, 13 different species of temperate and tropical *Haliotis* have been found in which

the larvae are induced to settle (attach to substratum), metamorphose, and commence normal post-metamorphic growth, in response to crustose coralline red algae and in response to GABA (Morse, 1984a, 1991). A few years ago, Akashige *et al.* (1981) reported that *H. discus hannai* failed to show such results in response to GABA, and that after induction, evidence of toxicity was seen. However several differences in the conditions employed in the experiments of Akashige *et al.* (1981) and those of Morse *et al.* (1979a, 1979b, 1980), which apparently account for the different results have been identified (Morse, 1984a, 1990, 1991). The principal differences appear to include the degree and manner of bacterial prophylaxis. Recently, *H. discus hannai* larvae were shown to be efficiently induced to settle and metamorphose (>90%), with little or no mortality, when exposed to GABA under the originally described conditions (Morse, 1990, 1991). GABA is now used routinely for the induction of larval settlement and metamorphosis in the largest and most successful commercial abalone producing companies in the United States (Morse, 1991).

Abalone mucus also has been found to induce the settlement and metamorphosis of abalone larvae (Seki and Kanno, 1981; Akashige *et al.*, 1981), and this method is used in Japan. A recent quantitative experimental comparison of the effectiveness of GABA and abalone mucus for hatchery-scale production concluded, however, that higher efficiency and higher survival resulted when GABA was used (Searcy-Bernal *et al.*, 1991).

IONIC DEPOLARIZATION AND OTHER SIGNAL TRANSDUCERS ALSO CAN BE USED FOR METAMORPHOSIS

The post-receptor pathway of signal transduction provides additional clues for development of inducers of larval settlement and metamorphosis. In *Haliotis* and other larvae, chemosensory receptor binding of the morphogenetic cue triggers post-receptor changes in cyclic AMP, calcium ion and protein phosphorylation, culminating in an induced ion flux across the chemosensory membrane; the resulting excitatory depolarization transduces the chemical signal from the environment to one propagated by the larval nervous system (Morse, 1990). A wide variety of effectors that

duplicate these post-receptor events also can be used to induce larval metamorphosis, even in species for which the natural inducer may not be known (Morse, 1991). Induction of metamorphosis by depolarization with K^+ salts has proved most convenient, inexpensive, and widely useful for a number of aquaculture species (Baloun and Morse, 1984; Morse, 1990, 1991).

In addition to the direct depolarization with potassium ion, a large number of chemicals known to affect intracellular cyclic AMP and calcium levels, protein phosphorylation, cell membrenes, membrane ion channels, ion translocation, and ionic depolarization of cell membranes also have been found to induce settlement and metamorphosis of abalone and other larvae (Morse, 1991). These include: cyclic AMP analogs, forskolin, substituted xanthines such as isobutylmethyl xanthine and theophylline, calcium ionophores and chelators, a low external chloride concentration, specific and non-specific ion-channel openers (such as ivermectin and organic solvents, respectively), and free arachidonic and palmitoleic acids (Morse *et al.*, 1980a; Baloun and Morse, 1984; Baxter and Morse, 1987; Morse, 1990; Jensen *et al.*, 1990). Possible applications of these inducers of metamorphosis for use in aquaculture have not yet been explored.

The settlement and metamorphosis of abalone larvae are regulated by at least two different kinds of chemical signals from the environment; these signals are recognized and transduced by convergent chemosensory pathways (Baxter and Morse, 1987). We recently have found that cilia purified from *Haliotis* larvae contain the still-functional chemosensory receptors and signal transducing proteins that recognize the environmental molecules, and control larval settlement and metamorphosis in response to this recognition (Morse, 1990). From these cilia, we have isolated messenger RNA coding for two signal transducing G proteins (Wodicka and Morse, 1991). This has permitted us to extend our studies of metamorphic control to the complementary DNA (cDNA) and protein sequence levels. By copying the messenger RNA with the enzyme, reverse transcriptase, then amplifying cDNA with the polymerase chain reaction (PCR), cloning the cDNA and determining its sequence, we have been able to

identify the signal transducing G proteins that are encoded by the messenger RNAs in the cilia, and thus confirm details of their mechanism of action drawn from our studies of the intact larvae (Wodicka and Morse, 1991). The gene sequences for the molluscan control elements also permit us to access a large data base for homologous control elements in many other systems. The G protein cDNAs cloned and sequenced from the *Haliotis* larvae prove to be closely homologous to counterparts recently cloned from the mammalian brain; from what is known already about the function of these proteins and their genes in mammalian brain, we may be able to predict new and more general methods for control of settlement in molluscan larvae.

ACCELERATION OF GROWTH, AND IMPROVEMENT OF NUTRITION AND RESISTANCE, THROUGH GENE REGULATION AND GENETIC ENGINEERING

A major remaining limitation to the efficiency of aquaculture for many animal species is a complex of problems related to their relatively low growth rates and/or low food-conversion efficiency, with consequent high costs of feeding and prolonged sensitivity to trauma, stress and disease (Morse, 1984a, 1984b; Hooker and Morse, 1985). Through the ability to induced synchronous metamorphosis and the start of juvenile growth under defined conditions (as described above), it has become possible to dissect these problems by analysis of the hormonal requirements for optimal growth, nutritional efficiency, and resistance of abalones and other molluscs in cultivation.

Several lines of evidence now indicate that growth hormone (GH) and certain other peptide hormones control the rate of growth in abalones and related gastropod molluscs. Our laboratory first demonstrated that exogenously provided GH purified from mammals can, at very low concentrations (optimum effectiveness at 10^{-9}M), increase the rate of growth and increase nutrient assimilation or food-conversion efficiency in post-metamorphic *Haliotis rufescens* (Morse, 1981, 1984a, 1984b; Hooker and Morse, 1985). These experiments demonstrated that heterologous GH added to the surrounding water produces a rapid and significant acceleration in growth rate, and significantly reduces the heterogeneity of size-distribution of the

treated abalone, relative to their untreated sibling controls (Morse, 1981). This effect is highly specific for GH; of 20 vertebrate peptide hormones tested over a wide range of concentrations, only GH (and, to a lesser extent, insulin) produed this effect.

These observations have now been independently confirmed in three different laboratories (two working with abalones; one with oysters). Professor Kawauchi and his colleagues (at Kitasato University, Japan), working with the Ezo abalone, *Haliotis discus hannai*, have observed even more dramatic increases in growth than those we reported, with increases up to 3-fold seen. Kawauchi *et al.* have been using recombinant GH peptide from Pacific salmon; they observed comparabble growth-accelerating effectiveness of this peptide when provided to the abalone either exogenously (as a dip, or applied to the food) as we had done, or when administered by injection (Kawauchi 1989; Kawauchi and Yasuda, 1989). This group also demonstrated that methods of GH administration are effective in accelerating growth of salmonid fishes as well (Moriyama and Kawauchi, 1990). In Australia, Professor Peter Hanna (Deakin University) also has observod that mammalian GH provided exogenously as a dip significantly accelerated growth in juvenile black-lip abalone (*H. ruber*) (Hanna *et al.*, 1991). Professor Thomas Chen (Center of Marine Biotechnology, University of Maryland), recently has observed comparable acceleration of growth in the American oyster, *Crassostrea virginica*, in response to short immersions in recombinant rainbow trout GH (Payner and Chen, unpublished observations).

The above-mentioned studies all demonstrate that mammalian GH peptides are effective, at low concentrations, in stimulating growth in abalones and other molluscs, thus suggesting that growth of these molluscs may be controlled by receptors for GH-like peptides, and that the molluscs themselves may produce such peptides endogenously. This suggestion recently has been confimed by Professor Kawauchi and his group, who have purified and sequenced peptide fragments with some homologies to vertebrate GH, from the abalone *H. discus hannai* (Kawauchi, unpublished observations). These peptide sequences are now guiding the design of matching oligonucleotide probes needed to detect, amplify and clone the

gene sequences corresponding to the endogenous abalone GH.

Pioneering work in Holland with a related gastropod mollusc has identified other peptide hormones (and their genes) involved in the regulation of growth. Studies conducted by Professor Joos Joosse, Dr. Wynand Geraerts, and their colleagues, including classical extirpation and implantation studies, demonstrated that neurosecretory cells of the central nervous system produce peptide hormones that regulate nutrient assimilation and somatic growth in the gastropod molluscs (see review by Joosse, 1988). Of the several families of peptide neurohormones and hormones identified in these studies, the molluscan insulin-related peptides (MIPs), with sequence homologies in their genes and gene-organizations corresponding to the mammalian insulins, insulin-like growth factors and somatomedins (the latter two being growth factors of prime importance in mammals) are most strongly impilcated as peptide regulators of somatic growth in the snails (Smit *et al.*, 1988; Smit, 1990; Geraerts and Joosse, personal communication). The genes coding for four different members of this peptide family from *Lymnaea stagnalis* have been cloned and fully sequenced by this group.

The genes coding for these growth regulating hormones can be used in three ways for the improvement of growth performance under cultivation conditions. First, the cloned genes can be used as probes for the detection and quantitation of the endogenous messenger RNA coding for the synthesis of the growth regulating peptides. Molecular hybridization studies using these cloned genes thus can identify those factors that control transcription of the growth hormone genes. The application of these findings can be used to stimulate the endogenous production of the growth accelerating hormones, without recourse to addition of exogenous hormone or complications from regulatory agency requirements. An international collaborative effort aimed at this objective is now in progress. Secondly, the recombinant DNAs can be used as templates for the economical production of the hormones (in yeast or other microorganisms), to provide supplies sufficient for growth acceleration by incorporation in feeds or other delivery systems. And thirdly, the growth hormone genes may be linked to suitable promoters and vector sequences for the genetic engineering of impoved

cultivars, as already in progress with fish (Zhang *et al.*, 1990). Applications of the latter two approaches face significant regulatory hurdles, however.

CONCLUSION AND PROSPCT: NEED FOR FUTURE RESEARCH

Identification of the molecular signals and their receptors and signal transducers that control reproduction, larval metamorphosis, and growth has led to the development of simple, inexpensive and reliable new techniques that are proving useful to augment the efficiency of control of these processes in molluscs and other aquaculture species. Identification, cloning and characterization of the genes that code for these signals, receptors, and transducers will make possible the coordinated genetic and molecular dissection of the complex regulatory mechanisms, and will provide additional information and tools needed for improvements in aquaculture efficiency. Cloning of the genes for these essential elements will provide new analytical tools (including hybridization and anti-sense probes of gene expression) that can help reveal the molecular mechanisms underlying neuroendocrine control. Cloning of these genes also will provide opportunities for template-directed synthesis of regulatory molecules that can be incorporated in feeds or used in other simple delivery systems. We believe that characterization and analytical use of the cloned genes will provide new insights for improved control of endogenous hormone synthesis and enhanced production, which in many cases (such as discussed here) can be accomplished by relatively simple methods without transgenosis.

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