The US Gulf of Mexico Marine Stock Enhancement Program (USGMSEP): The Use of Aquaculture Technology in "Responsible" Stock Enhancement

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The USGMSEP is investigating the use of aquaculture technology as an additional tool for the restoration of depleted marine stocks, particularly the red snapper, *Lutjanus campechanus*. Molecular genetics, feed production technology, and larval production technology are used to address three critical stock enhancement issues: 1) understanding the nature of the system to be enhanced, 2) producing robust, compatible individuals for release, and 3) critically evaluating the effects of releases. The program has developed 1) microsatellite and mitochondrial DNA markers that allow population analysis, broodstock analysis, and offspring analysis; 2) a method of copepod culture that allows for mass production of red snapper; and 3) a manageable, repeatable procedure for larval rearing of large numbers of red snapper, a species heretofore unculturable in large numbers.

Introduction

Worldwide, marine fish populations are in decline. Some predict that the current capture fisheries (while relatively stable over the last 15 years) are unsustainable past the year 2040.[1] Population trends for the 15 most commercially important species in the US indicate that about half of those are declining.[2, 3] Five fish species in the Gulf of Mexico, including the red snapper (*Lutjanus campechanus*), are listed as overfished by the National Marine Fisheries Service (NMFS).

The Magnuson-Stevens and Sustainable Fisheries Act require that plans for restoration of overfished species be put in place. Historically, there have been four approaches to deal with the problem. Two methods (fishing regulation and habitat protection) form the basis for most of the current approaches. Production aquaculture is a third approach. Stock enhancement constitutes a fourth approach. Fishing regulation has had limited success.[4] Habitat management/restoration has shown some promise,[5, 6] but is yet unproven as a long-term, large-scale solution. Production aquaculture has been hugely successful on a worldwide basis and now accounts for about 25% of the world's food fish production.[1] Stock enhancement, a combination of aquaculture technology and release of cultured fish into the wild, was, in fact, the technique of choice in marine fisheries management in the nineteenth and early twentieth centuries. However, a century of enhancement activities produced little evidence of effectiveness.[7] Further, concerns over maladaptive behaviors, artificial genetic selection, and disease problems in cultured fish created skepticism about the desirability of stock enhancement.

In the face of growing concern over the continued decline of managed stocks, Blankenship and Leber,[7] citing studies in Japan,[8, 9] Norway,[10] and Hawaii,[11] revived the idea that marine stock enhancement was possible through carefully planned research. Their paper outlined ten essential components of a "responsible" enhancement program that, once the species in question and management goals are determined, can be distilled into three critical issues: 1) understanding the nature of the system, 2) producing robust, compatible individuals for release, and 3) evaluating the effects of releases.

The U.S. Gulf of Mexico Marine Stock Enhancement Program (USGMSEP) is a research consortium consisting of the Gulf Coast Research Laboratory, Ocean Springs, MS; Mote Marine Laboratory, Sarasota, FL; and the Oceanic Institute, Waimanalo, HI. It is investigating the use of aquaculture technology as a tool that could operate in conjunction with traditional fisheries management techniques to restore depleted marine stocks, particularly the red snapper (*Lutjanus campechanus*), the primary foodfish in the Gulf of Mexico.[12] In this paper, we will focus on how our program uses aquaculture technology, specifically molecular genetics, feed production, and larval production to produce red snapper that

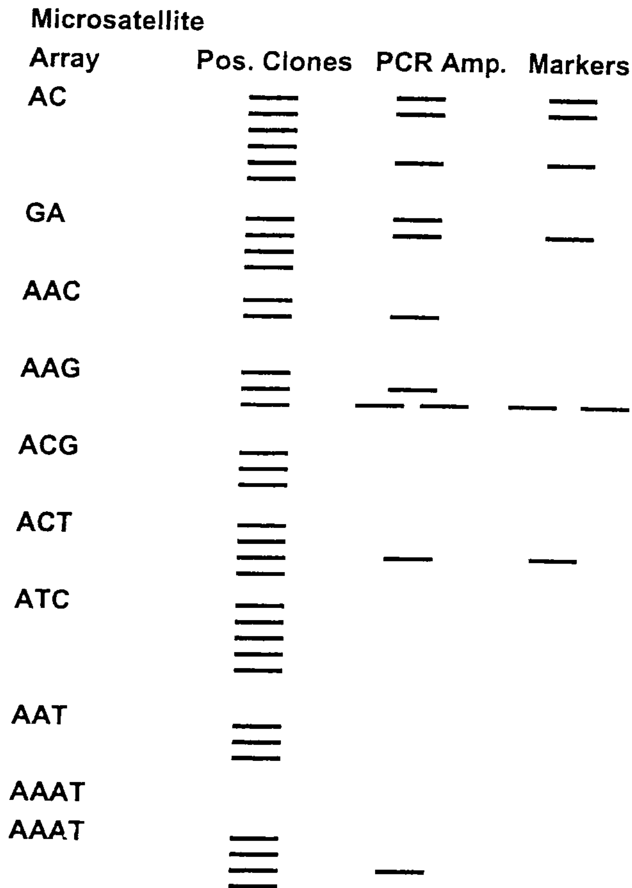

Figure 1. Microsatellite DNA arrays in red snapper, *Lutjanus campechanus*. Clones positive for the various arrays are represented in the first column, successfully amplified arrays are represented in the second column, and clones with polymorphisms sufficient to serve as population markers are represented in the third column.

will be used to address Blankenship and Leber's three critical stock enhancement issues.

The Program

Molecular genetics

Enhancement programs have come under increasing scrutiny during the last 10-15 years due to concerns over artificial or domestication selection in cultured animals and the resulting genetic risks to wild stocks.(13) Busack and Currens(14) concluded that some degree of selection is inevitable in cultured animals; therefore, potential impacts must be identified and, if possible, mitigated through proper selection of broodstock, robust mating protocols, natural rearing conditions, and "wild-fish- friendly" release strategies.(15) Our genetics program is designed to develop genetically sound breeding and release procedures to minimize insofar as possible the genetic impacts on receiving populations. This can be accomplished through the development of molecular markers that are tracked in both donor and recipient populations. We have developed two kinds of molecular markers that will allow us to acquire baseline data on the wild population, characterize broodstock, and conduct parental analysis of offspring.

Microsatellite DNA

Microsatellite markers consist of repeated arrays of non-coding nucleotides that exist as distinct alleles at a single locus in the nuclear genome and are inherited in a Mendelian fashion.(16) A size-selected genomic li-

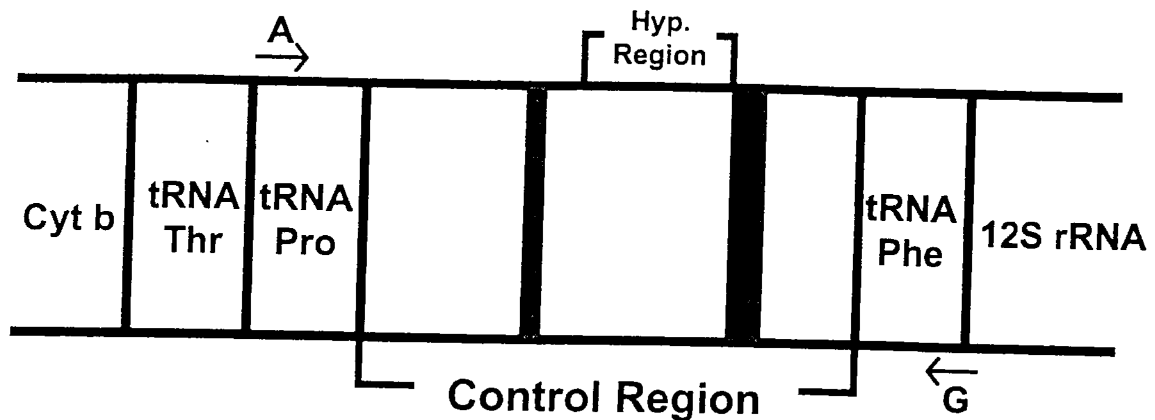

Figure 2. Representation of the mitochondrial DNA control region in red snapper, *Lutjanus campechanus*. Previously published primers used to amplify the region are shown. The hypervariable region, which is sufficiently variable to serve as a population marker, is indicated.

brary was screened for the presence of ten di-, tri-, and tetra-nucleotide microsatellite arrays. The most frequently occurring clones were those that contained the di-nucleotide repeat AC (6 clones) and the tri-nucleotide repeat ATC (5 clones) (Fig. 1). Thirty four clones were sequenced, 10 of which (1 clone contained 2 microsatellites) yielded sufficient flanking sequence to design PCR primers that successfully amplified the entire array (Fig. 1). The primers were then used to amplify each of the 11 microsatellites from 20 fish collected from several locations in the northern Gulf and Atlantic Florida coast. Six of the 11 loci examined displayed polymorphisms at a level to be considered useful as a genetic marker. Using the combination of the microsatellite markers and recently published PCR primer sequences,[17] we now have a sufficient number of single-locus microsatellites available to use for population structure analysis, broodstock characterization, and parental analysis of offspring.

Mitochondrial DNA

The mtDNA genome of fish is a 16.5 kb, closed-circular piece of DNA containing 13 genes coding for proteins, 2 genes coding for ribosomal RNAs (small 12s and large 16s RNA), 22 genes coding for tRNAs, and a major non-coding AT-rich region that contains the initiation sites for mtDNA replication and RNA transcription. This region, often called the control region or D-loop, has a high mutation rate compared to the nuclear genome as well as other mtDNA regions, and it has proven suitable for many population genetics studies with fish.[18]

The control region is immediately flanked by sequences encoding two tRNAs (threonine-proline) and cytochrome b, and by additional sequences encoding tRNA (phenylalanine) and the small 12s rRNA (Fig. 2). The entire control region was amplified using primers located in tRNA-Pro and tRNA-Phe, respectively. The appropriate PCR product was gel-purified, quantified, and cloned. Blue/white selection was employed to screen for inserts. Plasmids from white colonies were purified and screened for inserts by EcoRI-digestion followed by agarose gel electrophoresis. Clones were quantified and sequenced. To obtain flanking tRNA sequences, species-specific primers were designed in the control region and coupled with other published primers in adjacent genes[19] to amplify DNA fragments that contained either the tRNA-Phe or the tRNA-Thr and tRNA-Pro. Appropriate PCR products were electrophoresed, purified, cloned, and sequenced. Sequencing was conducted either at the University of Maine DNA Sequencing Facility or at the Gulf Coast Research Laboratory using automated sequencers. To verify that specific target mtDNA was being amplified rather than nuclear pseudogenes, nested PCR with mtDNA primers published in a previous study[20] were also used to successfully amplify a portion of the control region. Secondary structures of the tRNAs Thr, Pro, and Phe were elucidated and DNA sequences were imported into a multiple sequence editor and aligned.

Using the control region sequence obtained from 27 red snapper collected from several geographically separated sites from the northern Gulf and Atlantic coast of Florida, we determined a consensus sequence for the control region. From this information, a ~300

Table 1. The effect of the inoculation of filtered copepodids and adult copepods into ponds as they are refilled. Treatments consisted of either a ½ water change using ambient "brown" water or a ½ water change with the inoculation.

Container / Experiment	Treatment	Lag Time (days)	Nauplius Density (#/L)
Pond 2	Water change	15	140
Pond 4	Water change	12	245
Pond 3	Water change, inoculation	5	424
Pond 1	Water change, inoculation	3	263
Pond 1 (2nd exp.)	Water change, inoculation	2-5	126-140
Pond 2 (2nd exp.)	Water change, inoculation	4	209

base-pair segment of the control region, which contains a high proportion of variable sites (hypervariable region), was identified. PCR primers flanking this hypervariable site were constructed and used to produce a PCR fragment that could be easily direct sequenced. Preliminary results on more than 100 fish from the target population have shown that the hypervariable region contains sufficient polymorphisms to be useful as a genetic tag.

Feed production

Culture of members of Lutjanidae (more generally known locally as snappers) has been limited by the ability to determine and supply appropriate larval foods. Doi and Singhagraiwan[21] showed that copepod nauplii were important for the culture of lutjanid species. Schipp et al.[22] reported 40% survival (at day 21) of *L. argentimaculatus* fed on cultured copepod nauplii. Bootes[23] successfully grew 283 red snapper juveniles only after rearing in a tank of bloomed zooplankton supplemented with wild zooplankton. Therefore, it seems that lutjanids, including the red snapper, require copepod nauplii rather than easily cultured brine shrimp and rotifers as an initial food.[22,24] Copepod mass culture technology, unfortunately, is a young and inexact science. Copepod culture systems previously had required complicated, labor intensive tank systems, which included separate algal culture facilities or fertilization.[22,25] Further, many systems required terminal harvesting[22,26] and were susceptible to population crashes.[25] Ogle[27,28] showed that copepods could be cultured in our facility using a simple "brown-water" method; therefore, we investigated how to "scale-up" production of nauplii to accommodate continuous mass production of fish while minimizing cost and labor using modifications of the "brown-water" method. We investigated closed system culture as well.

We showed that inoculating the ponds with the filtered adults and copepodids (from the nauplius harvest) as the pond is refilled with ambient bay water increased the nauplius density in a considerably shorter time than natural blooming (Table 1). We also showed that by filtering only about half the pond in any given harvest, we could maintain continuous production presumably by maintaining a variety of life history stages.

Our refined technique pumps ambient "brown" water from Davis Bayou, Mississippi Sound, through a 300-µm mesh into pairs of 75 m^3 tanks. After being allowed to settle for 1 day,

Table 2. Closed system production of copepod nauplii compared to pond production.

Container	Maximum Density (#/L)	Mean Density (#/L)
Raceway – aerated	94	47
Raceway – not aerated	123	48
Pond 2	209	69
Pond 4	339	168
Pond 3	424	183
Pond 1	268	143

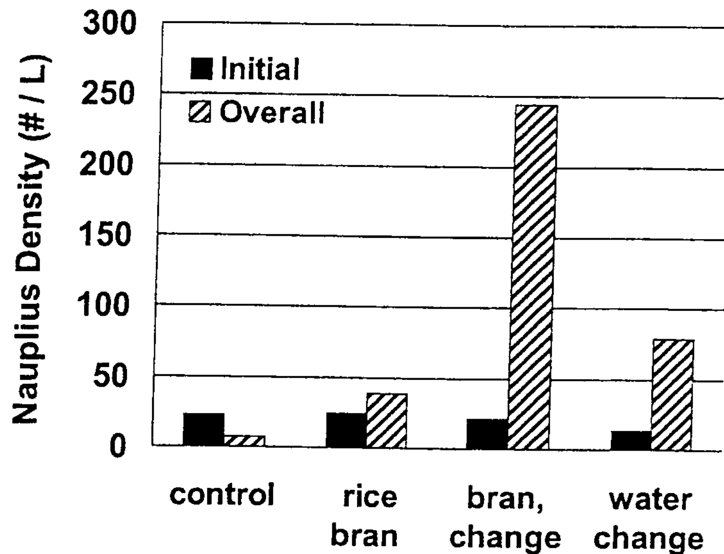

Figure 3. Closed system copepod nauplius production under different feeding regimes.

system produces either *Acartia tonsa* or *Pseudodiaptomus pelagicus*. This system produces enough nauplii to provide 22 500 red snapper larvae with 2000 copepod nauplii/L each day.[29]

Closed system culture (raceways) produced copepod nauplii but at a lower density than the ponds (Table 2). Experience suggests that raceways may not support the nauplius exploitation rate supported by the ponds. Ponds can support as much as 25% daily exploitation. Based on a limited experiment, aeration appears to make no difference in raceway production. Data suggest that water exchange is a critical component of production. Moreover, the data suggest that production might be greatly improved by fertilization with inexpensive rice bran (perhaps in ponds as well) (Fig. 3). So far, the closed system has not worked well, but it deserves further consideration because of potential benefits such as predictable, year-round production and isolation from disease organisms in the wild.

half of each tank is filtered through a mesh designed to retain adult and copepodid stages of copepods. That filtrate is subsequently filtered through a variably sized mesh to retain nauplii of the desired size fraction. Adults and copepodids are returned to the production tanks and the tanks are refilled with ambient "brown" water. Harvested nauplii are fed to red snapper larvae. Four tanks (2 pairs) provide continuous production on alternate days. Production tanks (75 m^3) produced an average of 3.7 million nauplii/day (range 616 000 – 12.5 million) with an average concentration of 139/L (range 4.5 – 424). Typically, the

Larval culture

Production of a new aquaculture species such as the red snapper is constrained by lack of knowledge of larval biology, developmental processes, water quality tolerances, and disease issues. In general, culture of lutjanids has developed in the last 10-15 years only. Previous attempts at culturing other lutjanid species

Table 3. Larval red snapper rearing conditions (1998 data). Recirculation, filtration, and siphoning treatments were fed at the 5 nauplii/mL rate.

Treatment	Number Stocked*	Percent Survival at 26 Days
Recirculation	1000	2.2
Filtration	1000	7.5
Siphoning	1000	7.6
Fed 5 nauplii/mL	1000	12.5
Fed 20 nauplii/mL	1000	30.4

* Stocked into a 200-L tank. Density of larvae had no effect.

Table 4. Mortality and survival of red snapper in 1999 production.

	Discrete Mortality (%)	Cumulative Survival (%)
Hatchery	93.3	6.7
Handling	7.8	5.4
Nursery	51.3	3.4

have provided mixed results. Lim et al.[29] (using *L. johnii*) and Emata et al.[31] (using *L. argentimaculatus*) reported 1-5% survival with 2 major periods of mortality, whereas Watanabe et al.[32] reported 10-20% survival, with chronic mortality occurring throughout culture of *L. analis*. Watanabe et al.[32] hypothesized that the differences in survival rates among the studies may be related to the different feeding regimes in the studies. They also noted that innate species-specific differences probably were involved. The only previous successful attempt at red snapper culture[23] produced 283 fish (at 30 days) with an average survival of 1.8%. Our program is working to identify and solve some of the technical problems constraining production of red snapper.

Briefly, we capture wild adult fish, inject them with HCG, and strip the resulting gametes about 24 hr post-injection. Eggs and milt are mixed, and larvae are hatched in a hatching chamber. To date, larvae have been produced at the Claude Poteet Mariculture Center, Alabama Department of Natural Resources, then transported to the Gulf Coast Research Laboratory. Upon arrival, following the methods of Ogle et al.,[33] larvae are stocked into 1000-L rearing tanks containing 250 L of settled, chlorinated, dechlorinated, and salinity-adjusted (35 ppt) water at a density of 40/L. Copepod nauplii (typically *Acartia tonsa* or *Pseudodiaptomus pelagicus*) (28-68 µm in size) are titrated to a density of 2.5/mL (avg. 1.8/mL) by day 3. Beginning on day 5, aliquots of water are added daily to result in a total volume of 1000 L and a larval density of 10/L by day 12. Copepod density is maintained but with increasing size fractions over time (28-125 µm from days 5-7, 68-125 µm from days 7-9, and 68-200 µm from days 9-23). *Artemia* nauplii are offered to the larvae beginning on day 12, titrated to a final density of 10-20/mL by day 14, and maintained until day 35. On day 23, larvae are harvested, counted, and transferred into the nursery where they are maintained until release (3-6 months). In the nursery, they are offered commercial pellets (Moore-Clark mahimahi diet, Moore-Clark, Vancouver, BC) and weaned off *Artemia*.

The primary rule that we have determined is that you must feed the larvae and LEAVE THEM ALONE. Rearing condition experiments indicate greater survival in undisturbed tanks (Table 3). Experience also suggests that larvae do not do well in tanks smaller than 200 liters. We now routinely use 1000-L tanks. The data suggest that high food density may be important (Table 3), but we have never reached the highest densities achieved in the experiments in the mass production program. The lower density of food in the mass production program may partly explain our pattern in mortalities. Some larvae fail to initiate feeding and die early, but because the larvae are so small in such a large volume (and because we have been unwilling to terminate the experiments for sake of production) we can not exactly quantify this mortality event. In general though, larvae do exceptionally well until about day 19, at which point they begin dying. By day 23, over 90% of the larvae are dead (Table 4). We cannot explain this event. Histologically, this appears to be a time of major organogenesis; thus perhaps this is a normal mortality event. On the other hand, perhaps this is related to improper or inadequate nutrition in the early developmental stages. At day 23, the larvae are harvested, counted, and transferred to the nursery. Aggressive behavior then becomes a significant source of mortality. In fact, about half of the larvae entering the nursery will die due to aggressive behavior (Table 4). So far, grading is ineffective in controlling this mortality because the grading process itself produces mortality. So, by the end of 3 months, there is only 3.4% survival (Table 4), but at least we have identified the major problems. Experiments planned for this year may elucidate the cause of the 19-day mortality. We continue to develop an effective grading procedure. This year we will try to reduce the aggressive behavior in the nursery by overcrowding the fish. Some research suggests that overcrowding may reduce aggression.

Other constraints include susceptibility to infection with the parasitic dinoflagellate *Amyloodinium ocellatum* and water quality. In 1998, we lost virtually all our fish to *Amyloodinium* (or the inability to tolerate treatment for *Amyloodinium*). In general, fish pro-

duced in 1998 were intolerant of handling. Although we cannot demonstrate it scientifically, we suspect that the intolerance may have been due to stress resulting from improper nutrition. In 1999, we had problems with *Amyloodinium* as well, but the fish were able to tolerate the treatment (perhaps due to better nutritional status), allowing us to control the outbreak. We had some water quality problems in 1999 due to filter failures, which resulted in some mortality. Generally, however, the routine sampling that is part of our health management program detects problems quickly. Rapid detection allows us the opportunity to deal with the problems. We plan to expand biosecurity to prevent infections.

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