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Goals and Strategies for Breeding in Fisheries

Proceedings of the Twenty-seventh UJNR Aquaculture Panel Symposium
Ise, Mie, Japan
November 11 - 12, 1998

Edited by Mamoru Kato, Kunihiko Fukusho, Kazumasa Ikuta,
James P. McVey, Paul Kilho Park, B. Jane Keller

Under the U.S.-Japan Cooperative Program in Natural Resources (UJNR)

National Research Institute of Aquaculture
Fisheries Agency

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Panel Chairmen:
Mamoru Kato, Japan
James P. McVey, United States

Under the U. S. - Japan Cooperative Program in Natural Resources (UJNR)

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PREFACE

A joint United States and Japanese panel on aquaculture was formed in 1969 under the United States-Japan Cooperative Program in Natural Resources (UJNR). The panel currently includes specialists drawn from the government agencies, laboratories, industry, academia, some of which are parts of the U.S. Sea Grant Program in the field of aquaculture. Charged with exploring and developing bilateral cooperation, the panel has focused its efforts on exchanging information related to aquaculture which could be of benefit to both countries.

The UJNR was begun during the Third Cabinet-Level Meeting of the Joint United States-Japan Committee on Trade and Economic Affairs in January 1964. In addition to the aquaculture panel, current subjects in the program include desalination of seawater, toxic microorganisms, air pollution, energy, forage crops, national park management, mycroplasmosis, wind and seismic effects, protein resources, forestry, and several joint panels and committees in marine resources research, development, and utilization.

Accomplishments include: Increased communication and cooperation among technical specialists; exchanges of information, data, and research findings; annual meetings of the panels, a policy-coordinative body; administrative staff meetings; exchanges of equipment, materials, and samples; several major technical conferences; and beneficial effects on international relations.

The 27th US-Japan Aquaculture Panel Symposium was held in Ise, Mie, Japan, from November 11 to 12, 1998, focusing on "Goals and Strategies for Breeding in Fisheries." Two special sessions were also held during field trip from Ise to Kyoto: (1) Mini Symposium held in NRIA on November 13 and (2) Satellite Meeting for Cooperative Research on flounder stock enhancement held in Fisheries Station of Kyoto University in Maizuru on November 20. The symposium was organized by program chair Kunihiko Fukusho and other UJNR Aquaculture Panel staff members of the Japanese side. Thirty-nine papers were totally presented in the symposium. Editorial work of this proceedings has been assisted by the NRIA staff.

Chairmen:
Mamoru Kato, Japan
James P. McVey, United States

This Supplemental Bulletin is published by National Research Institute of Aquaculture (NRIA), of the Fisheries Agency in Japan as a symposium volume of the United States-Japan Cooperative Program in Natural Resources (UJNR) Aquaculture Panel Meeting. It was edited by the Japanese UJNR Aquaculture Panel in accordance with the agreement between US and Japan Panel at the 23rd business meeting that the proceedings should be produced by the nation which hosts the meeting. The proceedings before the 20th symposium edited by the US UJNR Aquaculture Panel have been published as the series of NOAA Technical Report (NMFS). It has been renamed as UJNR Technical Report since the 22nd symposium in Homer, Alaska, 1993.

Participants in the 27th UJNR Aquaculture Panel Meeting

Identification list

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127. S. Kawai
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LINE-PRESERVATION OF EDIBLE SEAWEEDS FOR CULTURE IN JAPAN

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ABSTRACT

For the purposes of scientific study, many edible strains of seaweeds are maintained in various organizations. However, for the purposes of culture, line-preservation is conducted only for *Porphyra* spp., *Undaria pinnatifida* and *Laminaria religiosa*. At present, line preservation of *U. pinnatifida* and *L. religiosa* is a temporary means. In order to solve some serious problems which occur under subculture, successful methods to cryopreserve conchocelis cells of *Porphyra* in liquid nitrogen have been developed recently. Gametophytes of *U. pinnatifida* are also amenable to cryopreservation using the same method employed for *Porphyra*.

INTRODUCTION

The Japanese people have utilized seaweeds for food purposes since ancient times. At present, many species of seaweed are cultured in Japan, and the annual production has risen to 120 billion yen, or 1 billion dollars. Fishermen who have traditionally engaged in seaweed culture, based on empirical experience, have come to use good, reliable strains of seaweeds.

For the purposes of scientific study, many edible strains of seaweeds are maintained in universities, national and prefectural research institutes, as well as at other organizations. However, for the purposes of culture, line-preservation is conducted only for *Porphyra* spp., *Undaria pinnatifida* and *Laminaria religiosa*. At present, line-preservation of *U. pinnatifida* and *L. religiosa* is a temporary means which can be conducted for one year only and is restricted in Iwate Prefecture. In contrast, line-preservation of *Porphyra* is very wide spread.

PORPHYRA

Porphyra is the component of the seaweed food "nori", which takes the form of a black, compressed dry sheet and is used in making sushi, one of Japan's representative dishes. Nori culture constitutes one of Japan's most important fisheries industries. There are many cultivars of *P. tenera* and *P. yezoensis*.

Porphyra have two different stages. One is a filmy macrothallus, or edible gametophyte. The other is a microscopic filamentous thallus, or sporophyte, called conchocelis. Conchocelis grow in shells in the sub-littoral zone. Conchocelis are able to grow without shells and make spherical colonies, called free-living conchocelis. Free-living conchocelis grow slowly and can be divided easily. Therefore, they are good for line-preservation. Many strains of free-living conchocelis are subcultured in many fisheries organizations and are sold to fishermen.

UNDARIA PINNATIFIDA AND LAMINARIA RELIGIOSA

The life cycle of *U. pinnatifida* consists of an alternation between macroscopic diploid sporophytes which are edible and microscopic haploid gametophytes. *L. religiosa* also has the same life-cycle. In industrial culture, fishermen seed the collectors directly with zoospores released by matured sporophylls (Akiyama 1992). Gametophytes grow on the collectors. And in autumn gametophytes produce eggs and sperms. Fertilized eggs develop into edible sporophytes. This traditional method needs many sporophylls, i.e. many sporophytes.

Iwate's fishermen have adopted the another seeding method which had been developed by Kyoto Prefectural Fishery Experimental Station (Akiyama 1992). This method consists in the production of free-living gametophytes in the laboratory from selected one sporophyte. It allows them to obtain a great quantity of homogeneous gametophytes in one year. The gametophytes are cut into small pieces, and then planted on the

collectors. Using this method, the harvested sporophytes have uniform quality because they originated from one selected sporophyte. Therefore they are sold at a higher price. Line-preservation of *L. religiosa* is conducted in the same way as *U. pinnatifida*.

CRYOPRESERVATION

Line-preservation of *Porphyra* is carried out by the conventional subculture method. The subculture method is time-consuming, takes up space and has a risk of genetic change. In order to solve these problems, we tried to cryopreserve conchocelis cells of *Porphyra* in liquid nitrogen (Fujiyoshi 1997a, b).

The protocol of the cryopreservation is as follows. Conchocelis cells of *P. yezoensis* were exposed to the cryoprotective solution which contains 1.5M dimetyl sulfoxide and 0.5 M sorbitol. They were cooled down to −40℃ at the cooling rate of 1℃/min, and then were plunged into liquid nitrogen. After thawing, about half of the cells were alive. And preculturing the cells with sorbitol before freezing improved the survival rate after thawing (Fujiyoshi and Umezawa 1997, Fujiyoshi 1998).

Using the same method employed for *Porphyra*, gametophytes of *U. pinnatifida* are also amenable to cryopreservation, The survival rate was lower than the rate of *Porphyra* yezoensis. However, the sporophytes originated from cryopreserved gametophytes. The sporophytes grew up and matured (Figure 1).

Many species of seaweed have ever been cryopreserved in liquid nitrogen successfully (Arbault and Delanoue 1994, Sakanishi and Saga 1994, Kono et al. 1997, Vigneron et al. 1997, etc.). Some problems still remain. However, cryopreservation is expected to replace subculture in the line-preservation of seaweeds.

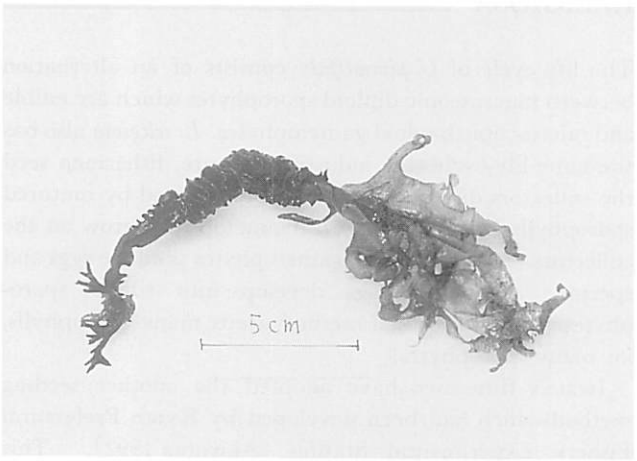

Figure 1. A matured sporophyte of *Undaria pinnatifida* origenated from cryopreserved gametophytes. Cultured with the PES I medium in 1-L spherical aeration-flask for about 6 months after germinating. The upper area was cut off for *in vitro* culture. Bar represents 5 cm.

ACKNOWLEDGMENT

I wish to thank Dr. Y. Ishikawa, Iwate Prefectural Government, who have taught me *Undaria* and *Laminaria* culture in Iwate Prefecture.

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CRYOPRESERVATION OF PACIFIC OYSTER SPERM AND LARVAE

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ABSTRACT

To develop a successful cryopreservation technique for sperm and larvae of Pacific oyster, *Crassostrea gigas*, several conditions were examined.

For the cryopreservation of sperm, the combination of a diluent made of 2/3 strength seawater containing dimethyl sulfoxide (DMSO), sucrose and reduced glutathione (each final concentration is 8 % (v/v), 50 mM and 6 mM, respectively) with cooling in liquid nitrogen vapor showed the highest relative larval yield (45.9%) among three diluents and three cooling methods. For long-term (4 yr) cryopreservation of the sperm, the ratio of normal D-shaped larvae and their survival rate at 6 days after fertilization using the sperm were 78.0 and 77.4%, respectively.

For cryopreservation of larvae, three critical conditions of a two-step method were studied. Under the conditions of cooling at − 1 ℃/min (seeding at − 8 ℃ for 15 min) before plunging into liquid nitrogen at −35℃ and using 1.5 M DMSO and 250 mM trehalose as cryoprotectants, the larvae 15 hr after insemination (trochophore before formation of shell gland, at 21℃) were most tolerant in terms of post-thaw motility (35.8%) and appearance in external morphology among the larvae 9, 12, 15, 18 and 21 hr after insemination. Among 1/4～1/30 concentrations of seawater as the solvent of the cryopreservative suspension containing 1.0 M DMSO and 250 mM trehalose, the rate of shell larvae 4 days after thawing was highest (4.5%) at 1/4 concentration though motilities immediately after thawing did not differ remarkably among concentrations. Among rearing of trochophore larvae at 26 ℃ after cryopreservation for 8 months followed by the embryonic development at 21, 25 or 29℃, the rates of D-shaped larvae at 2 days after thawing significantly decreased inversely proportional to temperature during development (3.6%, 2.1% and 0.6%, respectively), but the rate of normal larvae at 6 days didn't correspond with this tendency. Only one larva developed at 25℃ had settled and become adult. However, some other larvae fed on the diet. This indicates a possibility of further developments in the technique for cryopreservation of oyster larvae.

INTRODUCTION

Cryopreservation of sperm, oocytes, embryos or larvae of marine animals is a useful technique not only for preservation of valuable strains or endangered species, but also for reducing labor for aquaculture rearing, supplying standard samples for biological assay. We have made several attempts to develop successful methods for the cryopreservation of sperm and larvae of the Pacific oyster because of its importance in aquaculture and practicability in artificial insemination.

MATERIALS AND METHODS

Sperm

Three diluents and three cooling methods were examined in combination. Adjusted concentration of sperm was mixed with diluents made of 2/3 strength seawater containing cryoprotectant in a ratio of 1 : 4 (v/v). Two hundred μL of mixed suspension was set in a plastic straw and heat-sealed. One straw contained 10^8 spermatozoa. After cooling, straws were kept in liquid nitrogen more than 1 hr. Straws were thawed in stirred tap water at room temperature. Viability of spermatozoa was determined by the dye exclusion test using 0.3% trypan blue. Motility of spermatozoa was observed microscopically

after addition of a few drops of seawater on a slide glass. Relative larval yield was calculated as the rate of larvae from thawed sperm against the control larvae from fresh sperm. Larvae obtained from ova inseminated with long-term cryopreserved sperm were fed with *Pavlova* sp. in 3-L glass beakers.

Larvae

For the study of larval stage, ova and sperm collected from cultured oyster were inseminated in seawater (21℃) filtered by 0.2 μm hollow fiber membrane and rinsed 3 times. Every 9, 12, 15, 18 and 21 hr after insemination, larvae were sipped and adjusted to be 3,000~4,000 larvae/ml, and then supplied for each experiment. One fifth volume of cryoprotective suspension containing 3.0 M dimethyl sulfoxide (DMSO) and 500 mM trehalose in seawater were added to larval suspension five times every minute. Finally, the same volume of cryoprotective suspension was mixed with the larval suspension. Each 300 μL of mixed suspension was put into plastic straw and heat-sealed. After 20 min equilibrium, straws were set in a programmable freezer (Taiyo Sanso Co. Ltd. model CM-21, equipped with automatic ice seeding system), and cooled at the speed of −1℃/min to − 8 ℃, seeded at the temperature for 15min and cooled at the speed of −1℃/min again. A couple of straws were taken out from the freezer and thawed rapidly in running tap water (24℃) immediately after ice-seeding and after achieving −20, −35 and −40℃. Moreover, each two straws at −35 and −40℃ were plunged into liquid nitrogen and kept for more than 1 hr and they were thawed in stirred tap water. After the thawed suspensions were diluted gradually with seawater to 10 times of volume, larval morphology was observed and the percentage of moving (swimming, turning or rotating) larvae in the post-thaw larvae was calculated as motility.

For the study of the optimum concentration of seawater, ova and sperm were inseminated as mentioned above, except water temperature was 26℃. About 9 hr after insemination, larval development was checked occasionally under a microscope and trochophore larvae just before formation of the shell gland were supplied for the experiment. Several volumes of the cryoprotective suspension which is made of DMSO, trehalose and distilled water were added gradually to larval suspension to make the several concentrations of the seawater between 1/4 and 1/30. Concentrations of DMSO and trehalose after mixture were 1.0 M and 250 mM respectively for all seawater concentrations. The mixed suspension was put into straw, heat-sealed and cooled in the freezer after equilibrium. The cooling program was almost the same as described above but the temperature at seeding was set at −5℃ and straws were kept at −35℃ for 5 min before plunging them into liquid nitrogen. After keeping them in liquid nitrogen for more than 24hr, some straws were thawed and the motility of larvae was estimated. Post-thaw larvae from other straws were reared in filtered seawater after removal of cryoprotectants by filtering through 20 μm Muller gauze, followed by gradual dilution with filtered seawater. The ratio of the number of larvae with shell to that of cryopreserved larvae was calculated as shell larval ratio.

For the rearing of thawed larvae developed at different temperature, inseminated ova were developed to the trochophore stage in seawater of 21, 25 and 29℃. Obtained larval suspensions were gradually mixed with seawater of 1/4 concentration containing cryoprotectants. The final concentrations of cryoprotectant were 1.0 M for DMSO and 250 mM for trehalose, and the density of the larvae were $2.7\sim6.5 \times 10^4$ individuals per ml. Mixed suspension was cooled by the same method as given in the concentration experiment for seawater. After preservation in liquid nitrogen for 8 months, straws were thawed and cryoprotectants were removed. The post-thaw larvae were reared in 2.5-L filtered seawater at 26℃ and fed with *Pavlova* sp. and *Chaetoceros* sp. Post-thaw larvae were reared before the spawning season of natural oysters to avoid contamination.

RESULTS AND DISCUSSION

Sperm

The result of the combination of diluents and cooling methods is shown in Table 1. The combination of a diluent

Table 1. Effect of diluent type and cooling method on the viability and motility of cryopreserved spermatozoa and relative larval yield.

Diluent	Cooling method	Viability of sperm (%)	Motility	Relative larval yield (%)
A	1	61.4	±	34.4
	2	65.4	±	3.8
	3	55.3	±	1.9
B	1	72.7	±	45.9
	2	57.7	±	6.7
	3	52.2	±	1.9
C	1	77.5	+	12.4
	2	72.5	+	1.0
	3	75.5	±	0.0

Diluent
A: 8 %DMSO
B: 8 %DMSO, 50 mM sucrose, 6 mM reduced glutathione
C: 8 %DMSO, 36 mM sucrose, 4.3 mM reduced glutathione, 20% fetal bovine serum
Cooling method 1: nitrogen vapor, 2 : methanol-dry ice, 3 : deep freezer (−80℃)

B containing 8% dimethyl sulfoxide (DMSO), 50mM sucrose and 6mM reduced glutathione with cooling in liquid nitrogen vapor showed good viability and the highest relative larval yield (45.9%). Condensation of thawed sperm at diluent B was slight. Though the viabilities of sperm with diluent C was high enough, relative larval yields were extremely low. Fetal bovine was thought to be harmful for fertilization or development of the embryos. Viabilities of short-term cryopreserved sperms were about 60~70% (data not shown), but long-term preserved sperm showed less than 50% (Figure 1). Fertility of the long-term preserved sperm was inferior to fresh sperm though that of short-term preserved sperm was equal to fresh sperm in this condition. This possibly indicates deterioration of quality of sperm by long-term cryopreservation. The ratio of normal D-shaped larvae and survival rate at 6 days after fertilization of the long-term preserved sperm were slightly lower than those of fresh sperm, but they were as high as 80% (Figure 2). From these results, long-term cryopreserved sperm in this study is thought to have little toxicity to development of embryos and larvae. Further experiments to avoid deterioration of viability and fertility in long-term preservation are needed.

Larvae

Motilities of post-thaw larvae accompanied by hours after insemination and cooling steps are shown in Figure 3. For all cooling steps after seeding, the larvae at 15 hr always achieved the highest motility. In addition, post-thaw larvae at 15 hr showed the best form, direction of swimming and external appearance among five stages under the observations by optical microscope. Toledo *et*

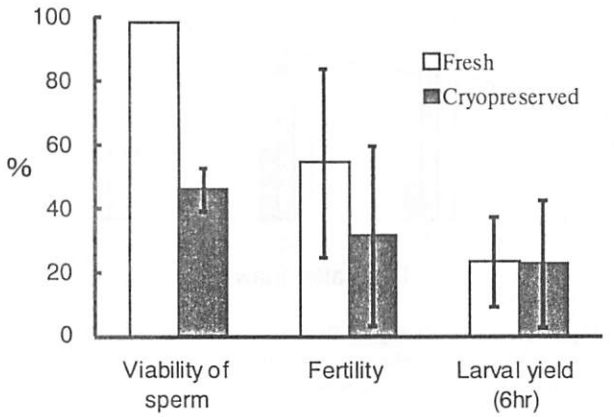

Figure 1. Viability of sperm, fertility and rate of larval yield obtained with fresh and long-term (4 years) cryopreserved sperm

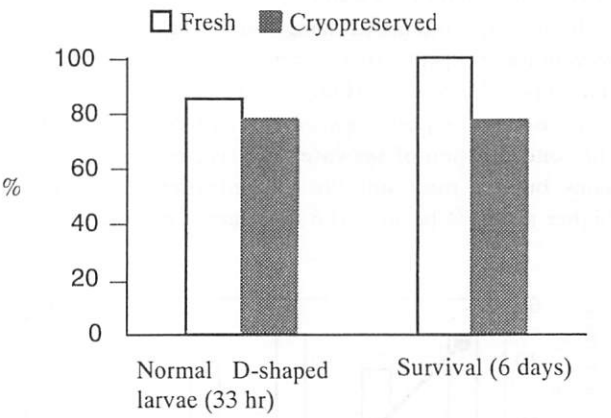

Figure 2. Early larval development rate obtained with fresh and long-term cryopreserved sperm.

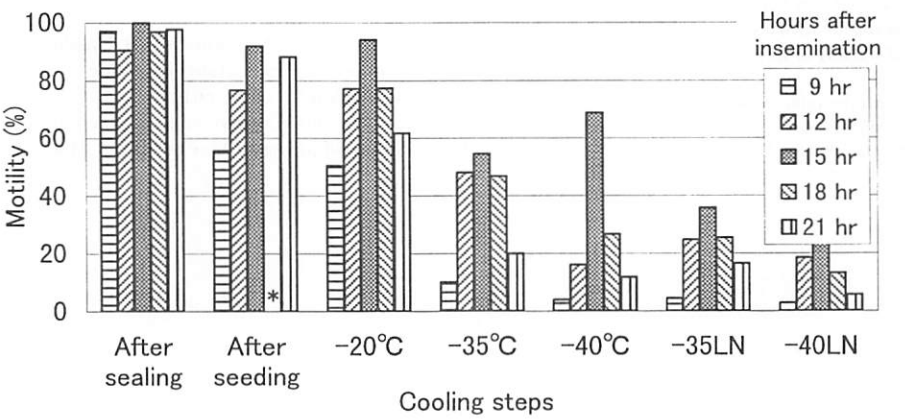

Figure 3. Change of motility of post-thaw larvae accompanied by hours after insemination and cooling steps

All of tested larvae were obtained from the same batch at 21℃. −35LN and −40LN mean keeping more than 1 hr in liquid nitrogen after reaching −35℃ and −40℃, respectively.

* Data was lost by an accident

al. (1989) indicated that the blue mussel *Mytilus edulis* had a higher tolerance (48.8% in survival rate) to freezing at trochophore stage than at cleavage cell stage. In Chinese scallop, *Chlamys farreri*, veliger can survive after thawing from liquid nitrogen (65.1% in survival rate) but trochophore can not (Xue 1994). In our study, larvae at 15 hr after fertilization corresponded to the trochophore before formation of the shell gland, and we concluded that this stage of the Pacific oyster is most tolerant to freezing under the presence of DMSO and trehalose between morula and early shell larvae. Motilities of post-thaw larvae and shell larval ratio 4 days after thawing under different concentrations of seawater are shown in Figure 4. Though the mean value of motility ranged from 25.8 to 47.1%, statistical difference in mean values among concentrations was not found. In contrast, the shell larval ratio 4 days after thawing decreased rapidly according to the drop of concentration of seawater.

In our experiments utilizing full strength seawater as a solvent for cryoprotective suspensions, post-thaw larvae did not make shells even though advantageous 15 hr larvae were used. The post-thaw larvae made shells by reducing the concentration of seawater in cryopreservative suspensions, but the most suitable concentration level might be higher than 1/4 because the shell larval ratio is highest at the maximum concentration level. However, concentration levels higher than 1/4 were not able to be cooled at a time with levels lower than 1/4 because the temperature at ice seeding in the cooling process differed mutually.

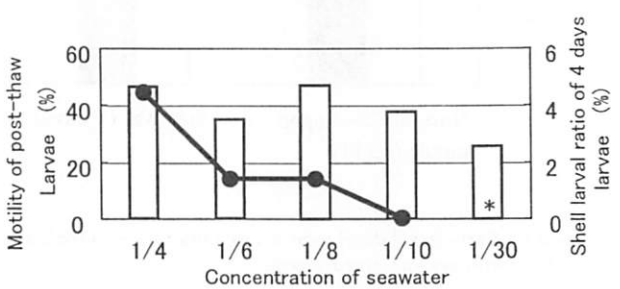

Figure 4. Motility and shell larval ratio of larvae cryopreserved various concentrations of seawater
Bars; mean value of motility of three straws
Line; shell larval ratio
Shell larval ratio means the ratio of the number of larvae with shell to that of cryopreserved larvae.
* Post-thaw larvae were not reared.

Results of the early rearing of post-thaw larvae are shown in Figure 5. The rates of D-shaped larvae at 2 days after thawing were significantly different varying with the temperatures during embryonic development. At 6 days after thawing, survival rate and rate of normal larvae of 25 ℃ were highest among the three groups, and the survival rate was not inferior to that at day 1. Though the scale of experiment was too small to analyze statistically, 29℃ was thought to be hopeless condition for rearing successfully the cryopreserved larvae.

Although algae fed were seen in some larvae, only one larvae of 25℃ settled and grew to adult size.

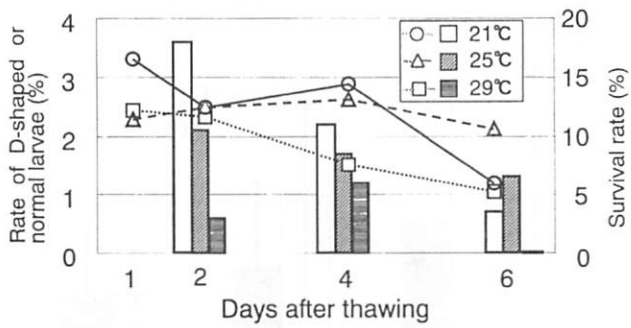

Figure 5. Early rearing of Post-thaw larvae according to temperatures at development of embryo
Bars; rate of D-shaped larvae (day 2) and normal larvae (day 4 and 6) against cryopreserved larvae
Lines; survival rate against cryopreserved larvae

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Bull. Natl. Res. Inst. Aquacult., Suppl. 1 : 7－11 (1999)

NUTRITIONAL REGULATION OF INSULIN-LIKE GROWTH FACTOR-I PLASMA LEVELS IN SMOLTING MASU SALMON *ONCORHYNCHUS MASOU*

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ABSTRACT

Changes in nutritional status lead to changes in the growth rate of salmon, the same as in mammals. The present study investigated the changes in insulin-like growth factor-I (IGF-I) plasma levels of fed and starved smolting masu salmon *Oncorhynchus masou* in fresh water. Plasma IGF-I levels were compared with the changes in the growth rate, condition factor, and plasma growth hormone (GH) levels. Food deprivation for 8 weeks resulted in cessation of growth and a significant decrease in condition factor. Starved masu salmon had significantly lower plasma IGF-I levels after 4 weeks of starvation than the fed fish, and these levels were maintained until 8 weeks. Two weeks of refeeding to starved masu salmon significantly increased its plasma IGF-I levels, and they reached similar levels to the control fish at the last sampling time (12 weeks). In spite of declined plasma IGF-I levels, plasma GH levels significantly increased after 2 weeks of starvation as compared with the control fish. After refeeding, the plasma GH levels declined gradually and became comparable to the control fish at the last sampling time. These results suggest that, in salmon, food deprivation primarily reduces circulating IGF-I levels, as well as IGF-I mRNA expression (Duan and Plisetskaya 1993) in the liver and consequently leads to the delayed growth of salmon.

INTRODUCTION

Insulin-like growth factor (IGF-I) is a mitogenic polypeptide of 70 amino acids that plays an important role in the regulation of development and somatic growth of vertebrates, mainly by mediating growth hormone (GH) action. Recent studies on molecular biology, biochemistry, and physiology of IGFs, IGF-I and IGF-II, have been extended to fishes (Chan and Steiner 1995, Chen *et al.* 1995). IGF-I and IGF-II are present in fish, and their structures are highly conserved between fish and mammals. Biological potency of fish IGF-Is is similar to mammalian IGF-I in bioassay systems (Duan 1998). In fish, the synthesis and production of IGF-I is under positive regulation by GH, the same as in mammal (Duan *et al.* 1994, Moriyama 1995). In salmon, hepatic IGF-I mRNA expression and circulating IGF-I levels increase following elevation of plasma GH levels during smoltification (Duguay *et al.* 1994, Duan *et al.* 1995, Moriyama *et al.* 1997). The IGF-I increase in response to elevated GH levels indicates that regulatory system of IGF-I is highly conserved during evolution from fish to mammal, and that the GH and IGF-I endocrine axis is also involved in regulation of growth in fish.

In mammals, nutritional status is an important systemic modulator of IGF-I production. Deprivation of food resulted in decreased hepatic IGF-I mRNA levels and plasma IGF-I levels in rat, while refeeding of the starved rat led to a rise in them (Clemmons and Underwood 1991). In fish, starvation also decreased the hepatic IGF-I mRNA expression, whereas refeeding of the starved fish led to a rise in hepatic IGF-I mRNA in coho salmon Oncorhynchus kisutch, the same as in mammals (Duan and Plisetskaya 1993). In our previous study, starvation caused the reduction of circulating IGF-I levels in coho salmon (Moriyama *et al.* 1994). Niu *et al.* (1993) also showed that starvation decreased IGF-like peptide (s) levels in plasma of rainbow trout *Oncorhynchus mykiss*. However, there is a lack of detailed information on the fluctuation of plasma IGF-I levels in salmon in association with food deprivation. The present study investigated the effect of food deprivation and refeeding on circulating IGF-I, as well as GH in plasma of smolting masu salmon *Oncorhynchus masou*.

MATERIALS AND METHODS

Animals

The eggs of masu salmon *O. masou* were fertilized using sperm of two year old males obtained in November 1994 at the National Research institute of Aquaculture, Inland Station, Mie, Japan. After hatching in December 1994, fish were reared in an indoor tank with running fresh water at 10-15 ℃ under natural photoperiod until April 1995. Fishes were fed to satiation once daily with commercially prepared pellets by an automatic feeder.

Experiment design and plasma collection

In April 1995, five hundred yearling masu salmon (approximate mean body weight 20 g) were transferred to outdoor tanks with running fresh water at 12-16 ℃. They were maintained under natural photoperiod and were fed 3% of the total body weight once daily with commercially prepared pallets that were topped with 5% feed oil. One hundred fifty smolted masu salmon (approximate mean body weight, 100 g) were transferred to two outdoor tanks in July 1994. Fish were left for two weeks to recover from the effect of handling stress. At the beginning of the experiment, 10 fishes were sampled from each tank (day 0). Food was then withdrawn from one tank (starved group), and fishes in the other tank (fed group) were fed 3% of the body weight by an automatic feeder. Further samples of 10 fishes, caught at random from each tank, were taken at 2, 3, 4, 6 and 8 weeks, respectively. After 8 weeks of starvation, the starved group was refed at a rate of 3% of the total body weight. Subsequent samples were taken at 10 and 12 weeks from the start of the experiment.

Blood sampling

Sampling was carried out between 10 : 00 a.m. and 12 : 00 p.m. (noon). After rapid anesthesia with tricaine methanesulfonate (MS-222, 75 mg/L) solution with $NaHCO_3$ (5 mg/L), blood was collected from 10 fish in each group by a syringe from the caudal vessels. Plasma samples were separated by centrifugation (1,000 g × 5 min) at 4 ℃ immediately after sampling and stored at −80 ℃ until measured. At each sampling time, the length and body weight of sampled fishes were measured. The condition factor of the fish was calculated as follows : 100 ×body weight/lenght^3.

Hormone measurement

The IGF-I levels in plasma were measured in a homologous radioimmunoassay (RIA) using the method of Moriyama *et al.* (1994). Plasma GH was measured in a homologous RIA according to the method of Swanson (1995) with some modifications. Briefly, antibody-bound hormone complexes were separated from free tracer by addition of 100 μL of 0.5% Pansorbin. All the samples were measured in single assays with intra-assay variation of less than 5%.

Statistical analysis

Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by the Fishers Protected Least Significant difference test using 512+ (Brain Power, Inc.) program. Difference at the $p<0.05$ (*) and p, 0.01 (**) levels were considered to be significant.

RESULTS

Changes in body weights, plasma GH and IGF-I levels of masu salmon sampled from January to November 1995 are shown in Figure 1. A slight increase in the plasma GH levels was observed in March, which reached a maximum in April, and then declined in May. On the other hand, plasma IGF-I levels were low in March and gradually increased from April. Subsequently, a peak of IGF-I levels in plasma was observed in May, which slightly declined in June, and then maintained similar levels until November. Silvering molts were first observed in March, and all fish were smolts in May. Therefore, the experiment started in July using smolting fish, as indicated by the arrow.

Masu salmon in the fed group grew rapidly during the experiment. Food deprivation resulted in cessation of growth and decrease in condition factor of fish (Figure 2a, b). After 4 weeks, the body weight and condition factor of starved fish were significantly lower than those of the fed fish ($P<0.01$). At the end of 8 weeks starvation, the body weight and condition factor of the starved fish were 2.1-fold ($P<0.01$) and 1.2-fold ($P<0.01$) less than the fed group, respectively. Refeeding of the starving fish for 4 weeks increased the body weight and condition factor.

Plasma GH levels was significantly elevated as a result of food deprivation (Figure 3a). After 2 weeks of starvation, higher plasma GH levels were observed in the starved fish as compared to the fed fish ($P<0.05$), and these levels were maintained until the end of the starvation period. At 8 week of starvation, the plasma GH levels were 2.9-fold higher than the fed fish ($P<0.01$). After refeeding, plasma GH levels declined gradually and were comparable to the control fish levels at the last sampling time. In spite of elevation of the plasma GH levels, plasma IGF-I levels significantly declined after 4 weeks of starvation, and these levels were maintained until the end of the starvation period (Figure 3b). After 8 weeks starvation, plasma IGF-I levels were 2.1-fold lower than the fed fish ($P<0.01$). Two weeks of refeeding of starved masu salmon

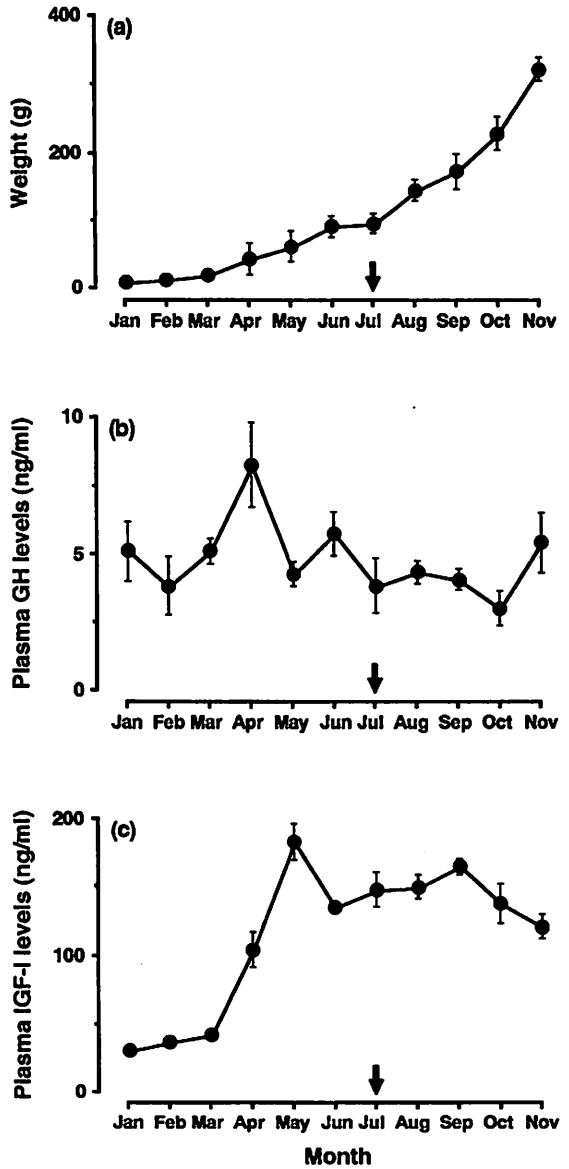

Figure 1. Changes in body weight (a), plasma GH levels (b) and plasma IGF-I levels (c) of masu salmon at each sampling time from January to December 1995. Each symbol with vertical bars represents mean ± SEM (n=10). Arrow indicates the starting time of this experiment.

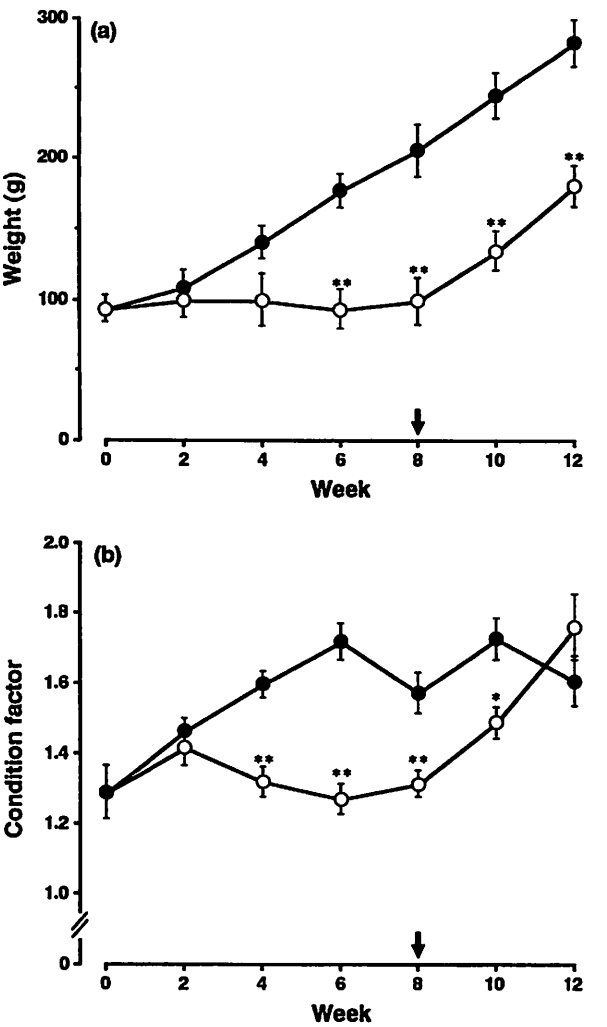

Figure 2. Changes in body weight (a) and condition factor (b) of masu salmon during the study. Fish in the control group (●) were fed once daily during the experiment. Fish in the test group (○) were starved from 0 to 8 weeks and then refed once daily until the end of this experiment. Arrow indicates the refeeding time. Each symbol with vertical bar represents ± SEM (n=10). * $P<0.05$ and * * $P<0.01$ are compared with the fed control group.

significantly increased the plasma IGF-I levels and reached similar levels to the control fish at the last sampling time (12 weeks).

DISCUSSION

In fish, nutritional status is an important systemic modulator of IGF-I production, as well as in mammal. In our previous study, plasma IGF-I levels in the 25-day-starved coho salmon were significantly lower than the fed fish (Moriyama *et al.* 1994). The present study demonstrated that deprivation of food resulted in a significant decrease in circulating IGF-I levels in masu salmon, and that refeeding increased IGF-I to similar levels as the fed fish. In rainbow trout, starvation of 4 weeks caused a significant decrease in plasma IGF-like peptide (s) (Niu *et al.* 1993). A similar decrease in bioactive IGF-I in plasma, examined by cartilage sulfation bioassay, was shown in starved coho salmon (McCormick *et al.* 1992). Perez-Sanchez *et al.* (1995) reported a positive correlation between dietary protein contents and plasma IGF immunoreactive levels in the gilthead seabream *Sparus auratus*. Duan and Plisetskaya (1993) also reported that

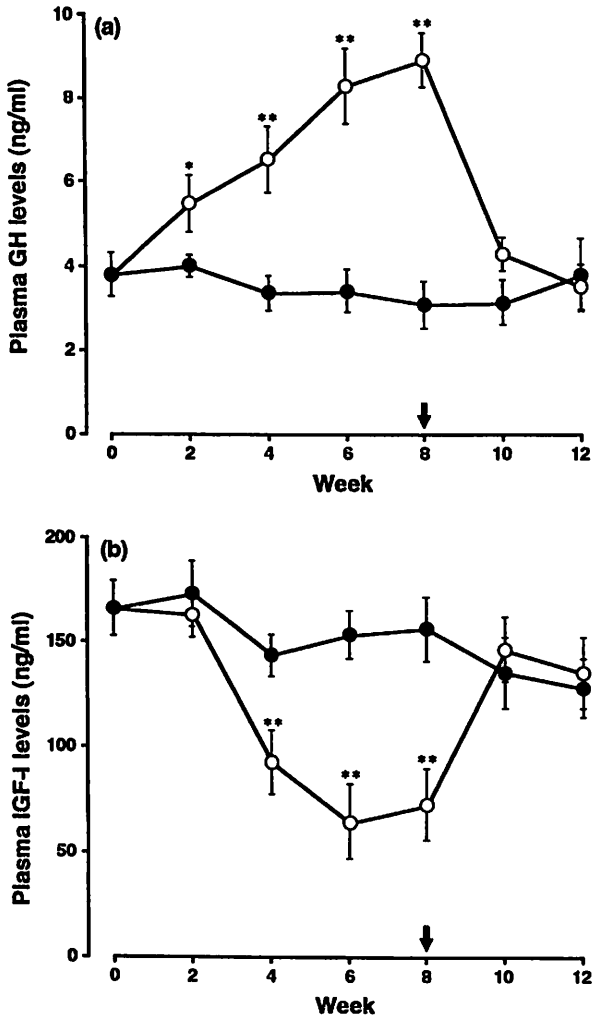

Figure 3. Changes in plasma GH levels (a) and plasma IGF-I levels (b) of masu salmon during the study. Fish in control group (●) were fed once daily during the experiment. Fish in the test group (○) were starved from 0 to 8 weeks and then refed once daily until the end of this experiment. Arrow indicates the refeeding time. Each symbol with vertical bar represents mean ± SEM (n=10). * $P<0.05$ and * * $P<0.01$ are compared with the fed control group.

prolonged starvation resulted in a significant decrease in the hepatic IGF-I mRNA levels in coho salmon, and that refeeding returned the levels on hepatic IGF-I mRNA expression to those of the fed fish. These results indicate that nutritional status regulated production of IGF-I in salmon as well as the hepatic IGF-I mRNA expression.

In salmonid fishes, plasma IGF-I levels were elevated during the parr-smolt transformation (smoltification) association with the changes in plasma thyroid hormone and GH levels (Moriyama *et al.* 1997, Beckman *et al.* 1998). The hepatic IGF-I mRNA levels were also increased in coho salmon during smoltification (Duguay *et al.* 1994, Duan *et al.* 1994). In the present study, an increase in plasma GH levels were observed from March to April 1995, followed closely by the plasma IGF-I levels observed between April and May. Masu salmon used in this experiment became silvering smolts in March, and all of experimental fish became smolts in June. It is indicated that IGF-I may be involved in smoltification of masu as well as other salmonid species. This study, therefore, was conducted from July onwards, since the plasma GH and IGF-I levels decreased to average normal levels and the developmental variations were similar for all the sampled fish in this season.

It has been shown that in salmonids starvation causes an elevation of plasma GH levels (Sumpter *et al.* 1991, Duan and Plisetskaya 1993). The present study confirmed these results of increased plasma GH levels in starved fish. In sharp contrast plasma IGF-I levels were increased in starved fish, and refeeding increased them but reduced plasma GH levels. In coho salmon, starvation causes a decline in the binding of hepatic GH receptor to GH (Gray *et al.* 1992). In rats, starvation leads to a decline in numbers of GH receptor in liver (Baxter *et al.* 1981) and in the hepatic GH receptor mRNA expression (Bornfeldt *et al.* 1989). It was also show that food deprivation resulted in decreased hepatic IGF-I mRNA levels and plasma IGF-I levels (Goldstein *et al.* 1991). These results, taken together, suggest that the decrease in the number of GH receptor in starved fish causes lower binding of circulating GH to its receptor in the liver, which lead to reduction of synthesis and production of IGF-I and consequently leads to the retarded growth of salmon.

In salmon, IGF-I mRNA expression has been found in virtually all tissues, with the highest level in liver (Duan *et al.* 1993). They reported that the hepatic IGF-I expression significantly increased following the administration of GH, but no changes were found in nonhepatic tissues, such as brain, muscle, and ovary. Moreover, the hepatic IGF-I expression in starved salmon was reduced, but no changes were found in nonhepatic tissues (Duan 1998). It is likely that changes in circulating IGF-I levels by GH administration or starvation maybe limited to the liver and further it is possible that IGF-I of liver origin is important for somatic growth of salmon. This study demonstrated that food deprivation reduces circulating IGF-I levels, as well as IGF-I mRNA expression in the liver (Duan and Plisetskaya 1993) and consequently leads to the delayed growth of salmon.

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TRANSIENT β-GLUCURONIDASE (GUS) GENE EXPRESSION UNDER CONTROL OF CaMV 35S PROMOTER IN *PORPHYRA TENERA* (RHODOPHYTA)

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ABSTRACT

To date, much progress has been made in the successful fusion and regeneration of *Porphyra* protoplasts. On the other hand, experiments on transformation are few, and very little is known about appropriate promoters and foreign gene expression in *Porphyra*. We demonstrated the electroporation-mediated DNA transfer of *P. tenera* in this study. Small foliose thalli were collected from"nori-net"at the sea farms and were digested with papain and abalone acetone powder to obtain suitable protoplasts for transformation. At first, adequate conditions of electroporation were investigated at two parameters (voltage DC and pulse width). Then, various kinds of plasmid carried on the cauliflower mosaic virus 35S (CaMV 35S) promoter and the gene encoding β-glucuronidase (GUS) as a reporter were introduced into protoplasts by electroporation. The protoplasts which received the CaMV 35S promoter and GUS construct showed positive GUS expression in results of histochemical and fluorometric assays. Therefore, the CaMV 35S promoter seems to act effectively in *Porphyra* protoplasts and the GUS gene can be used as a marker for the transformation of various genes.

INTRODUCTION

Over the past 10 years, considerable progress has been made in the transfer and expression of exogenous DNA into higher plant cells. The techniques are used not only in genetics and breeding studies, but also in physiological ones. In contrast to higher plants, the number of phycological studies using gene transfer techniques is very few, and little is known about suitable promoters and reporter genes for seaweed. To date, the transient expression of a foreign gene in the protoplasts of *Porphyra miniata* (Kübler *et al.* 1994) and *Ulva lactuca* (Huang *et al.* 1996) has been reported. In these experiments, the bacterial β-glucuronidase (GUS) reporter gene which was controlled by the cauliflower mosaic virus 35S RNA (CaMV 35S) promoter was introduced into protoplasts of both algae. However, its expression was not stable and disappeared in the multicellular bodies; moreover, the transformation rate was lower than 0.5%.

To obtain stable gene expression, a structural modification of the promoter of reporter gene seems to be important. Moreover, to carry out efficient transformation experiments, the selection system to distinguish transgenic cells which depends on reagents such as antibiotics is indispensable. In this study, we tested the sensitivity of *Porphyra* protoplasts to chloramphenicol, kanamycin and hygromycin which seemed to be possible to use as selective reagents. Although we were not successful in cultureing a transgenic *Porphyra* thallus, the transient expression of the GUS reporter gene in electroporated protoplasts and multicellular bodies was detected in this study. Additionally, we selected chloramphenicol as a useful reagent for the transgenic cell selection.

MATERIALS AND METHODS

Algae

Protoplasts were prepared from the thalli of *P. tenera* (strain Sashiki). We supposed that protoplasts which had few or small vacuoles might be appropriate for transformation. As the cells of long thalli usually have big vacuoles, the algae were harvested when they grew to about 2 cm in length and after being dried for about 3 hr in the open air. It was stored at −20℃ until used.

Protoplast isolation

The protoplast isolation was based on the methods of Mizukami *et al.* (1992) with minor modifications. The thalli, less than 2 cm, were washed in sterile seawater. They were preserved in seawater containing 2% papain and 0.5% dextran to digest the proteins around surface cells. After thalli were washed with seawater to remove the papain and the dextran, they were shaken gently in about 10 mL of cell-wall-lysis enzyme solution. The enzyme solution was made from the abalone aceton powder and the bacterial enzyme (main ingredient is alkali hemi-cellulase) at a 7 : 3 ratio at 21-22℃ for 30 to 90 min. The isolated protoplasts were filtered through nylon gauze, and were rinsed several times with sterile seawater. Since the abalone acetone powder contains traces of β-glucuronidase activity (Kübler *et al.* 1994), we rinsed protoplasts carefully. After washing, they were kept in 0.8 M mannitol-water solution under dark and cold conditions until the transformation trials.

Electroporation

Electroporation was carried out with a Shimadzu SSH-1 (Shimadzu Co., Japan) electroporater which can successively radiate square electric pulses. Judging from the survival rate of protoplasts at 24 hr after the electric shock, the optimum electrical conditions, such as voltage DC and pulse duration, were clarified in the preliminary experiment. The number of live protoplasts was counted using a hemocytometer after staining with neutral red. Five kinds of plasmid with the GUS reporter gene and various promoters were introduced into *Porphyra* protoplasts in this experiment. Plasmid pBI 101, pBI 121 and pBI 221 were bought from Clontech Co. (USA). In expectation of the effective and stable GUS reporter gene expression, we additionally tried to introduce the following plasmids which were recently used in the transformation of land plants. One is pBIN 19 with the GUS gene and the upstream sequences of the rice proliferating cell nuclear antigen (PCNA) as its promoter (pBIN 19-PCNA) (Kosugi *et al.* 1991), and the other is pUC 19 with the GUS gene and El_2-CaMV 35S-Ω construction as its promoter and enhancement sequences (pUC19-El_2-CaMV 35S-Ω). El means the upstream sequences of -90 to -417 from transcription start site of CaMV 35S RNA, and El_2 means a repeat of El sequence. The Ω means tabacco mosaic virus mRNA 5' untranslated region. The sequence is reported to be effective to enhance the GUS reporter gene expression (Hayakawa *et al.* 1992).

Assay of GUS reporter gene expression

Histochemical and fluorometric assays were carried out for the GUS gene expression in this experiment. The histochemical assay was performed by Jefferson *et al.* (1987) with minor modification. Protoplasts and regenerating cells directly reacted of 50 mM phosphate buffer (pH 7) containing 1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc) and 20% methanol according to the product described by Kosugi *et al.* (1990). The histochemical GUS reaction with X-Gluc produced the indigogenic blue precipitation. The protoplasts which were electroporated in the absence of plasmids were used as a negative control. Moreover, the GUS reporter gene expression levels of protoplasts which were introduced as pBI 221 or pUC19-El_2-CaMV 35S-Ω were compared using the fluorometric assay (Jefferson *et al.* 1987).

Culture and regeneration of protoplasts

After electropolation, 0.8 M mannitol-water including protoplasts were diluted with the distilled 0.2 M mannitol-seawater to a density of about $1 \times 10^4 \sim 5 \times 10^4$ cells/mL within two or three days. Then, protoplasts were transferred to Guillard F medium (Guillard and Ryther 1962) and cultured in 24-well plates before assay. The plates were kept at 18 ℃ under light and dark control (L: D = 11h :13h) with 25 to 40 μmol $m^{-2}S^{-1}$ in the photochamber. Protoplast regeneration was monitored every 5 days by observing under a light microscope.

Sensitivity tests to chloramphenicol, kanamycin and hygromycin

To select the suitable reagent for selection of transformed *Porphyra* protoplasts, various concentrations of three antibiotics (chloramphenicol, kanamycin and hygromycin) ranging from 10 to 100 μg/mL were tested. The culture of protoplasts was followed the method by Mizukami *et al.* (1995) with minor modifications. Isolated protoplast suspension was mixed with 1 mL agarose medium which consisted of 0.7% low melting point agarose in Guillard F and was pre-warmed at about 35℃ (top agarose). The top agarose containing protoplasts was spread on a flat surface of 1% agarose in the medium (bottom agarose) in a 9-cm-diameter petri dish. Agarose was sterilized under the high pressure and high temperature condition using an autoclave. From which temperature down to about 50℃, these antibiotics were mixed into top and bottom agaroses at various concentrations (0-100 μg/mL). The survival rate of protoplasts in the top agarose was calculated as follows. One hour after the top agarose spreading, 100 normal protoplasts were randomly selected from four fields of view under microscope and surrounded by dots with a sterilized needle on the surface of agarose. Cultures were continued for 30 days under the condition mentioned above, and the number of multicellular bodies regenerated from the marked protoplasts was counted.

RESULTS AND DISCUSSION

Optimum electric conditions for transformation

Field strength, DC voltage and pulse duration are important parameters of electroporation. The DC voltage is directly related to the field strength in the Cell Porator which was used in this study. Therefore, we tested various ranges of DC voltage and pulse duration. Most of *Porphyra* protoplasts given one electric impulse with more than 150 μs of pulse duration and 400V DC bursted, and a few protoplasts that kept their original form could not be regenerate (Figures 1, 2). We assumed that more plasmids with the GUS gene would be introduced into the protoplasts by a hard electric impulse so long as protoplasts can live. Thus, the electric pulse with a voltage of 300V DC (field strength is calculated 3.0 kV/cm) and a pulse duration of 100 μs was recognized as an optimum condition for the gene transformation.

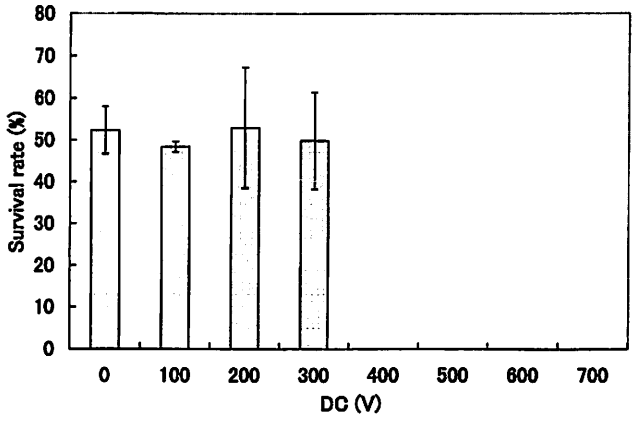

Figure 1. Effect of the various DC voltage on the survival rate of protoplasts of *Porphyra tenera* (Sashiki strain). Each trial was initiated by the application of one electrical pulse with a duration of 50 μs. Values are means±S.D. of four replicates. The survival rate of protoplasts given an impulse with more than 150 μs of pulse duration was near zero.

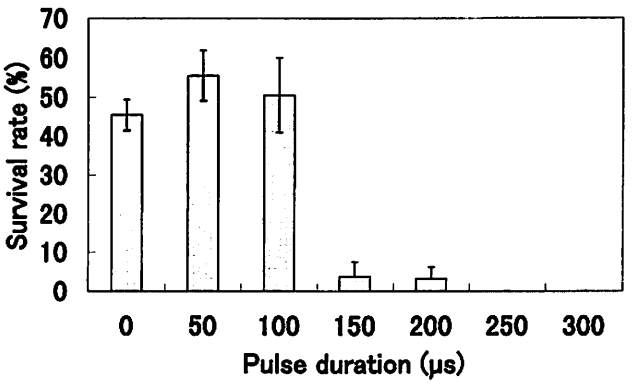

Figure 2. Effect of the pulse durations on the survival rate of protoplasts of *P. tenera*. Each trial was done by the application of one pulse at 300V DC. Values are means±S.D. of four replicates. Protoplasts given an impulse with more than 400V DC were burst.

GUS reporter gene expression

When we introduced three kinds of plasmids, pUC 19-El_2-CaMV 35S-Ω, pBI 121 and pBI 221, into *Porphyra* protoplasts, the expression of the GUS reporter gene was remarkably observed well in histochemical and fluorometric assays. Figure 3 shows GUS stain in the protoplasts and regenerated cells into which the plasmid pUC 19-El_2-CaMV 35S-Ω was introduced. The frequency of stained cells were usually less than 0.5% of all protoplasts given in an electric pulse. The highest one was about 1% (Figure 3-F), while no GUS expression was detected in the protoplasts into which two kinds of plasmids, pBIN 19-PCNA and pBI 101 were introduced. The difference between pBI 121 and pBI 101 is the existence of CaMN 35S sequences which pBI 121 has (Jefferson *et al.* 1987). Moreover, pBI 221 is the plasmid in which CaMV 35S sequences have been introduced into the cloning site of pUC 19. Therefore, CaMV 35S sequences seemed to play a role as the effective promoter of the GUS gene in *Porphyra* protoplasts. This is entirely consistent with the results of the gene transformation experiments in *Ulva lactuca* and *P. miniata* (Kübler *et al.* 1994, Huang *et al.* 1996). Furthermore, higher levels of the GUS reporter gene expression were detected in the protoplasts into which the plasmid pUC 19-El_2-CaMV 35S-Ω were introduced, in comparison with pBI 221, in fluorometric assay (Figure 4). Therefore, El and Ω sequences, which were mentioned about their meanings, seemed to be effective to enhance the GUS reporter gene expression in *Porphyra* protoplasts. However, the PCNA upstream sequences, which is widely used as a promoter of the GUS reporter gene in higher plants (Kosugi *et al.* 1991), was not effective in the protoplasts. In this experiment, however, even if we introduced the plasmid pUC 19-El_2-CaMV 35S-Ω, the transformation rate was usually lower than 0.5% and not stable. The lack of stability of the expression seems to be due to either the integration of a foreign gene into the *Porphyra* genome or the loss of activity of the promoter sequences following cell division and growth. In this experiment, we could detect the slight expression of the GUS reporter gene in multicellular bodies cultured for 40 days (Figure 3-E). However, the number of such bodies was few and we only observed the faint expression just as traces of indigogenic blue precipitation in a partial body. The multicellular bodies released monospores, but we could not detect the GUS expression in the thalli germinated from the spores. To date, no stable transformed *Porphyra* thalli expressing the GUS reporter gene were

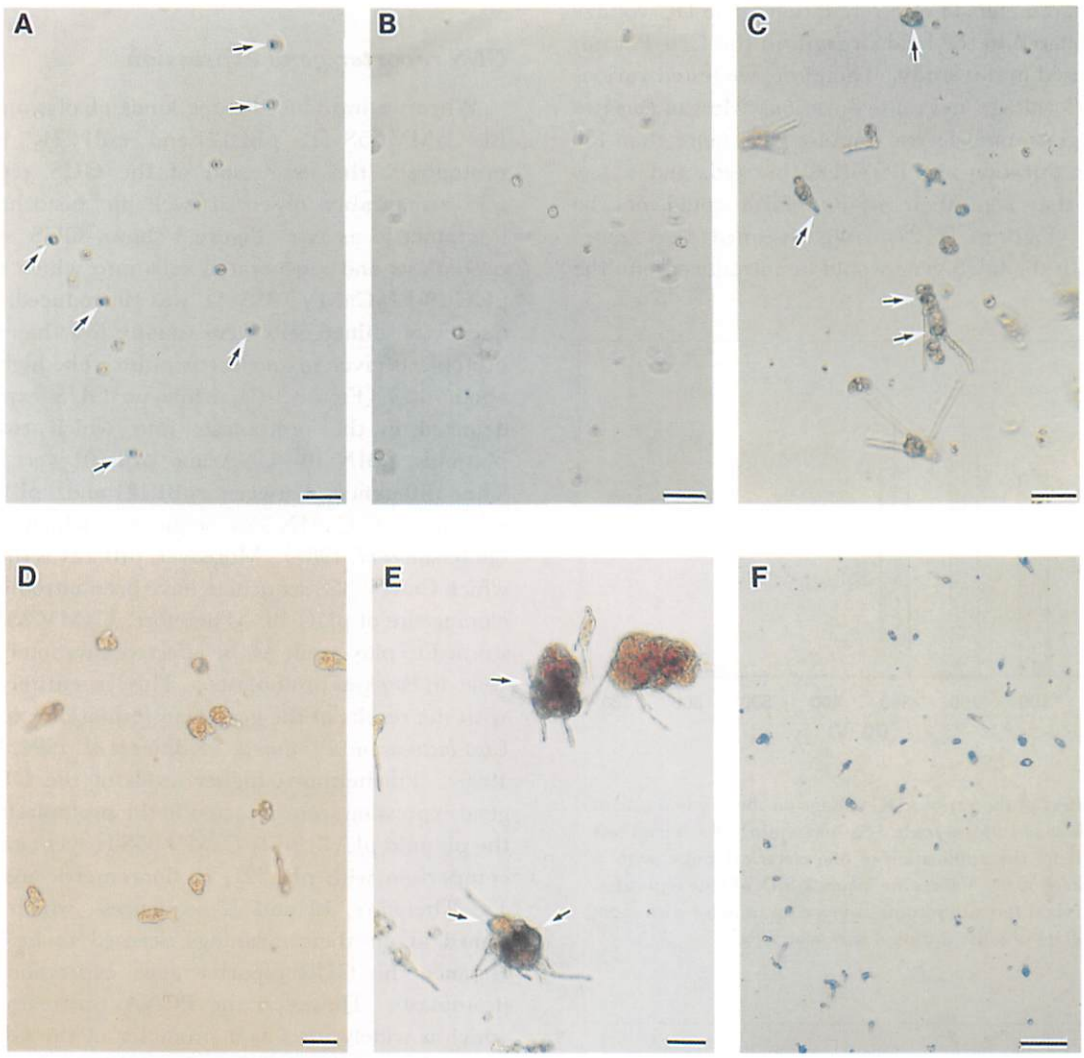

Figure 3. Staining for GUS activity of the electroporated protoplasts and the multicellar bodies germinate from the protoplasts. Arrows show positions of GUS expression. Histochemical GUS reaction with β-D-glucuronide produced indigogenic blue precipitation. A: GUS activity of protoplasts electroporated with the plasmid pUC 19 with El_2-CaMV 35S-Ω construction as a promoter and enhancement sequences (pUC19-El_2-CaMV 35S-Ω). Protoplasts were cultured for 2 days after electroporation. Scale bar shows 50 μm. B: Control protoplasts that were electroporated without any plasmids. No GUS activity was detected in them. Scale bar shows 50 μm. C: Multicellular bodies germinated from protoplasts into which the plasmid pUC19-El_2-CaMV 35S-Ω was introduced. They were cultured for 10 days in Guillard F medium after the introduction treatment. Scale bar shows 50 μm. D: Control multicellular bodies germinated from protoplasts that were electroporated without any plasmids. Scale bar shows 50 μm. E: Multicellular bodies into which the plasmids introduced were cultured for 40 days. Scale bar shows 50 μm. F: An example of the highest-rate expression of the GUS gene in multicellular bodies. About 1% of protoplasts into which the plasmids were introduced showed GUS expressions. Scale bar shows 100 μm.

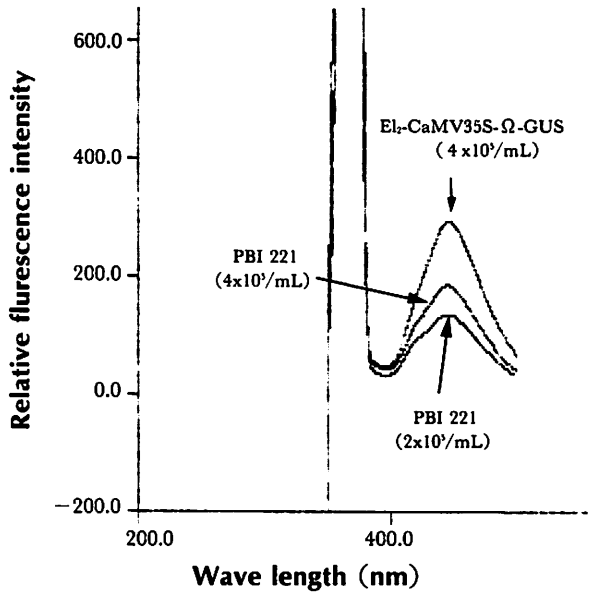

Figure 4. Fluorometric GUS assay of the protoplasts into which the plasmid pBI 221 and the plasmid pUC19-El$_2$-CaMV 35S-Ω were introduced. The density of protoplasts used for the assay is about 2×10^5cells/mL or about 4×10^5cells/mL. The X axis shows emission wave length (nm), and Y axis shows fluorescence intensity.

obtained. Moreover, the transformation rate would differ due to the quality of protoplasts used. The protoplasts that have few or small vacuoles seemed to be appropriate for transformation. They could be made from small and fine thalli.

A suitable reagent to select transformation cells

Figures 5, 6 and 7 show the effect of different concentrations of chloramphenicol, kanamycin and hygromycin. From the results of these sensitivity tests, protoplasts could not survive in the agarose containing chloramphenicol ranging from 40 to 100 μg/mL. Therefore, chloramphenicol seemed to be the reagent to use to select transformation cells. As we have to confirm the expression of the chloramphenicol-acetyl transferase (CAT) gene in *Porphyra* protoplasts, the plasmid vector which includes both the GUS reporter gene and the CAT gene would be effective for various gene transformation tests of the alga. On the other hand, the neomycin-phospho-transferase (NPT II) gene and hygromycin-phosoho-transferase (HPT II) gene may be expected to have little effect.

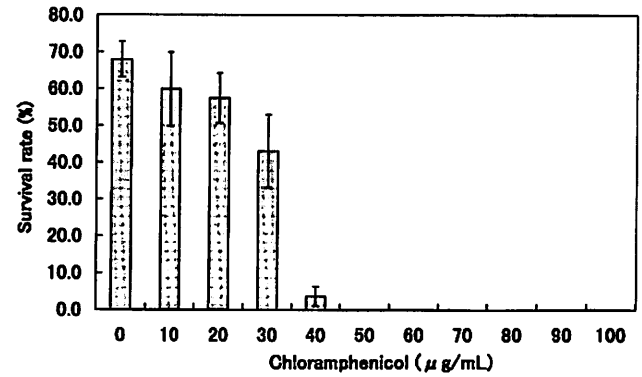

Figure 5. Effects of concentration of chloramphenicol on the survival of protoplasts. Values are means of four fields of view in the culture petri dish under microscope of three replicate treatments. Protoplasts could not survive in the medium containing chloramphenicol ranging from 40 to 100 μg/mL.

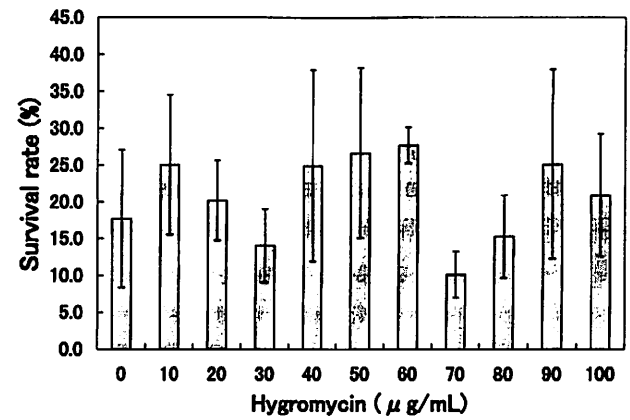

Figure 6. Effects of concentration of kanamycin on the survival of protoplasts. Values are means of four fields of view in the culture petri dish of three replicate treatments.

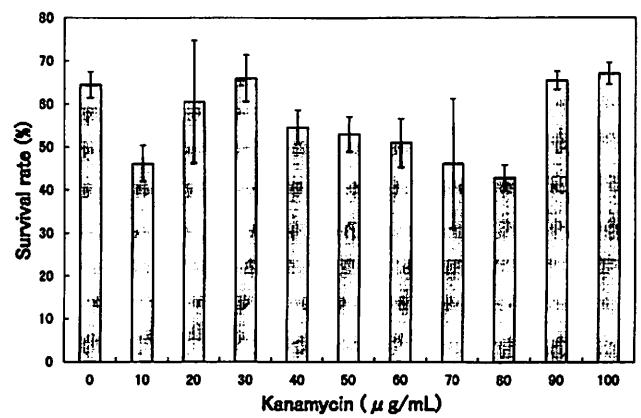

Figure 7. Effects of concentration of hygromycin on the survival of protoplasts. Values are means of four fields of view in the culture petri dish of three replicate treatments.

ACKNOWLEDGMENTS

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GENETIC STRUCTURE IN AUKE CREEK PINK SALMON AND ITS ROLE IN PRODUCTIVITY

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ABSTRACT

More than 20 years of studying a naturally producing run of pink salmon in Auke Creek, near Juneau, Alaska, has provided insight into the role of genetics in the success of anadromous populations. A genetic marking program initiated in 1979 showed that returning Auke Creek pink salmon had strong genetic structure relating to return timing, which persisted in subsequent generations. Population genetic (allozyme) studies of population structure in Auke Creek, as well as of other local pink salmon populations, demonstrated significant effects of return timing on allozyme frequencies in both even- and odd-year (genetically isolated and independent) broodlines. Breeding studies that examined migration timing as a quantitative genetic trait showed that variation in timing of return had a significant basis in additive genetic variance, V_A. Searching for determinants of timing, we studied development rate in early- and late-returning fish in both thermal environments encountered by the population segments. Development rates differed between the segments in ways that can be generally explained as adaptation to environmental conditions. For instance, there were significant additive genetic effects on variation of time to hatch; and there were significant genotype by environment effects on development time to epiboly, eye pigmentation, and mid-hatch. Analysis of family sizes of pink salmon returning to Auke Creek indicated a significant additive genetic effect on marine survival that is more accentuated in large returns than small returns. The implications are that a small portion of the population contributes disproportionately to the next generation; and, as a consequence of interannual environmental variation, the more successful portion varies unpredictably from generation to generation. It is likely that some of the genetic variability in life history traits is maintained as a bet-hedging strategy. Maintenance of genetic variation is a key to the long-term success of a population; however, our studies of interbroodyear hybrids suggest that outbreeding depression may also occur. The source and nature of genetic variation must be factored into aquacultural and harvest management decisions.

INTRODUCTION

In 1979 we began a genetic marking experiment that had a profound influence on much of our subsequent research. Our original objective was to mark one segment of a pink salmon *Oncorhynchus gorbuscha* population, by altering the allele frequencies at an allozyme locus creating an allozyme marker (Lane *et al.* 1990). The altered allele frequencies would make that segment distinct from the other segment of the population and from other local pink salmon populations. Our reasons were to determine the feasibility of this method of marking, to identify shortcomings of the genetic tagging method, and to observe the stability of the mark in subsequent generations.

In the following generations the allozyme marker appeared only in the portion of the run that had been marked, which strongly indicated a population structure based on return timing and spawning location. In other words, the population had substructure resulting from partial reproductive isolation in space and time.

To understand that observation more fully, we have since conducted a series of studies including breeding experiments to examine quantitative genetic implications, an intensive allozyme study to resolve genetic structure that would be expected to result from isolation, and hybridization experiments to examine the integrity of the underlying genetic processes. Here, we present a summary of that work and consider the implications for productivity of pink salmon, and by extension, other salmonids.

THE EXPERIMENTAL SYSTEM

The pink salmon is a convenient species for genetic work and in many respects serves as a model for Pacific salmon. Pink salmon have a fixed two-year life cycle over their natural range. Consequently, there are two genetically distinct lineages, one spawning in even years (referred to as the even broodyear) and the other spawning in odd years (the odd broodyear). Most drainages, such as Auke Creek, support both broodyears, and offer an opportunity to replicate experiments using populations that have two different genetic backgrounds, but share the same average environmental history, at least for the freshwater portion of their life histories.

Most of our work has been conducted on pink salmon from Auke Creek or other streams near Juneau, Alaska (Figure 1). Auke Creek is located about 17 km north of Juneau, Alaska near the University of Alaska Campus and the National Marine Fisheries Service Auke Bay Laboratory (ABL). Auke Creek is about 350m long and drains from Auke Lake into the estuarine waters of Auke Bay. Auke Lake is fed primarily by Lake Creek, and the number of pink salmon that spawn in Lake Creek varies from year to year. But, in most years since 1977, the number has been small relative to the number spawning in Auke Creek (Gharrett, unpublished observations; but see Fukushima and Smoker 1997). ABL operates a weir near tidewater that enumerates both emigrating fry and returning adults and a research hatchery that had been used for pink salmon culture research. However, except for our genetic marking experiments, hatchery production has not contributed to the productivity in Auke Creek since the 1970s. Since 1971, returns of pink salmon to Auke Creek have varied between 1,548 and 26,317 fish, averaging about 6,000 (S. G. Taylor and J. L. Lum, National Marine Fisheries Service, Juneau, AL, unpubl. data 1998). Clearly, spawning habitat limits productivity in streams as small as Auke Creek.

Although Auke Creek is a small stream, marking experiments indicate that there is fidelity of homing to particular spawning areas within the creek both in time and space (Taylor 1980). The return of fish is bimodal in timing. The first peak (early run) occurs in August whereas the second peak (late run) returns after the first of September. The appearance of fish with sea-bright scales marks the beginning of the late run. Auke Creek is also divided between spawning habitat that is influenced by saltwater (intertidal) and habitat that has no saltwater influence (upstream). The weir sits just above tidewater and its position accentuates the natural division. Both intertidal and upstream spawning grounds are used by early-and late-run fish. Lake Creek also serves as a spawning ground, but in most years it is not heavily used and its temporal structure is not as obvious and may vary from year to year.

The life history of the pink salmon is strongly influenced by stream temperature. Early-run fish may encounter temperature above 15 ℃ on their return in August, whereas in mid September, when late-run fish spawn, the temperature is about 12℃. During embryogenesis, the water temperature declines steadily to 8℃ in mid-October, 4℃ in mid-November, and 1 to 2℃ in mid-December, where it remains until March (Hebert *et al.* 1998). Fry begin to emigrate seaward in late March when the temperature begins to rise and they complete their emigration before mid May before the temperature reaches 9℃. Interannual variation in temperature and precipitation may influence survivals and, consequently, the pink salmon productivity of the Auke Lake system (Fukushima 1996).

GENETIC MARKING

The original intent of genetically marking a pink salmon run was to determine the potential for artificially tagging a salmon population so that fish from the population could be detected in a mixed-stock fishery. Subsequent use of the marker to learn about the biology of the species was an acknowledged, but secondary, purpose of the research. At

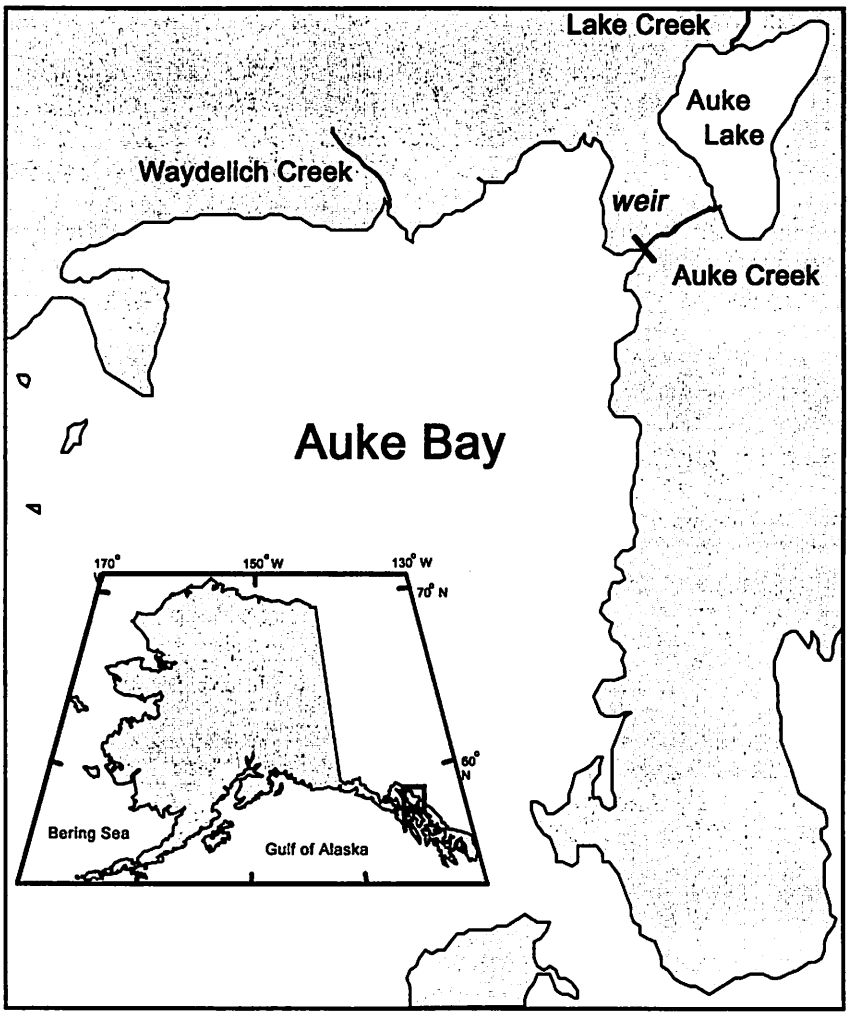

Figure 1. Location of the Auke Creek system.

the time this work was done, allozyme variants resolved using protein electrophoresis provided the only reasonable choice for genetic marking. To choose an appropriate marker, we conducted an allozyme survey of local streams. The survey revealed variability at a number of allozyme loci, but relatively little variability among streams (McGregor 1982).

The choice of a marker allele was based on: (1) the extent of variation within and between populations; (2) the number of fry that would be released to mark the stream; (3) the number of parents necessary to produce that number of fry and avoid passing the population through a demographic bottleneck; and (4) the amount of effort (people and expenditures) we could devote to the marking process. The Alaska Department of Fish and Game guideline for minimum broodstock size was 400, 200 males and 200 females. For pink salmon, 200 females produce about 240,000 eggs. Even with moderate mortalities from handling and culture loss, we could expect to release about 200,000 fry. At a 1% return rate, we would expect about 2,000 adults to return.

Our resources allowed us to discard 9 of 10 fish we screened, that is, a marking effort of 10 : 1. We constructed a statistical model to determine what magnitude of allele frequency changes could be accomplished by that marking effort, and from what initial allele frequency we could create the most detectable marker (Gharrett and Seeb 1990). For a marking effort of 10 : 1, a naturally occurring frequency of about 0.05 provides the strongest (most detectable marker). Conveniently, isoloci *sMDH-B1, 2** (previously called *Mdh-3, 4*) had a slow allele (**70*) with a frequency of about 0.05 as well as a fast allele (**130*) with a frequency of nearly 0.05. We decided to breed only individuals carrying the **70* allele, but not the **130* allele.

Theoretically, that would be about 9.25% of the fish.

The marking procedure involved taking small skeletal muscle sample from a fish, tagging the fish with a unique number, and processing the sample electrophoretically to determine whether to keep the fish as a breeder for our experiment or to discard it.

Because earlier pink salmon marking experiments (Taylor 1980) indicated that there was temporal and spatial structure within Auke Creek, we focused our marking effort on the late, upstream segment of the Auke Creek population. To ensure that we only marked late-returning fish, we did not begin the screening process until mid-September, well after the time any early-returning fish would still be alive. In fall 1979, we screened 3906 late returning Auke Creek pink salmon adults; and of those we retained 390 for use as broodstock. The *sMDH-B1, 2*70* frequency in the 390 spawners was 0.508 (as compared to 0.055 in the screened returning fish), and the *sMDH-B1, 2*130* frequency was 0.000 (as compared to 0.046 in the returning fish). In spring 1980, 178,219 fry (60,000 of which were fin-marked for evaluation purposes) were released (Lane 1984, Lane et al.1990).

In fall 1981, 1,469 fin-marked fish returned to the Auke Creek weir. The *sMDH-B1, 2** allele frequencies in a sample of 1,048 of those fish was 0.510 for *sMDH-B1, 2*70* and 0.000 for *sMDH-B1, 2*130*. There was no evidence for changes in the frequencies of alleles at *sMDH-B1, 2** during either the freshwater or marine portion of their life cycle. After their return in 1981, the experimental fish interbred with wild Auke Creek fish. As a consequence, the frequencies of alleles at the marker loci in the late-returning Auke Creek fish changed; the frequency of *sMDH-B1, 2*70* to 0.253, and the frequency of *sMDH-B1, 2*130* to 0.020 (Lane 1984, Lane *et al.* 1990).

In the next four generations, the frequency of the marker allele (*sMDH-B1, 2*70*) remained high in the late segment (Figure 2). In fact, by examining the profile of returning fin-marked fish, it was apparent that salmon bearing the marker returned during the late part of the late segment, which was the same segment of the parent population that had been altered. This observation has several implications. First, there was no strong selection against genotypes carrying the *sMDH-B1, 2*70*. Second, Auke Creek pink salmon probably have a temporally based structure that exists within both of the more apparent early and late population segments. We think this fine scale temporal structure probably exists because we marked only the later part of the late-returning segment, and the marker allele did not appear in abundance until well after the beginning of the late returning segment (Figure 3). The frequencies of the *sMDH-B1, 2** alleles in the early-returning segment remained similar to the pre-marking frequencies in Auke Creek and increased little over the next several generations. In addition, Waydelich Creek, which is about 1 km from the mouth of Auke Creek, showed no significant changes in *sMDH-B1, 2** frequencies during this time. We infer from these observations that, at least in some instances, pink salmon stray very little from their home stream, and that temporal structure may be an important life history characteristic.

Another observation made possible by the genetic

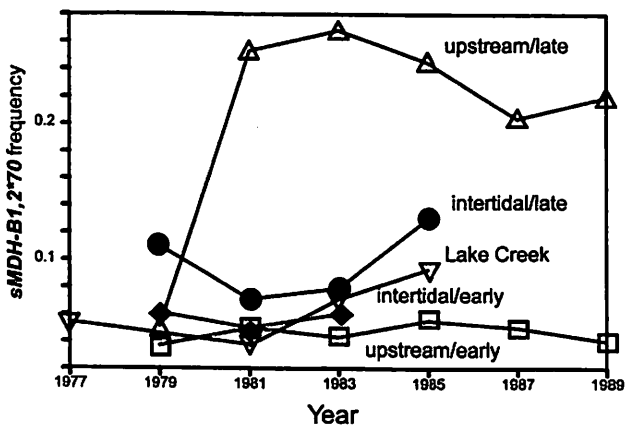

Figure 2. Frequency of the *sMDH-B1, 2*70* marker allele in run segments of odd-year Auke Creek pink salmon following the marking event in 1979.

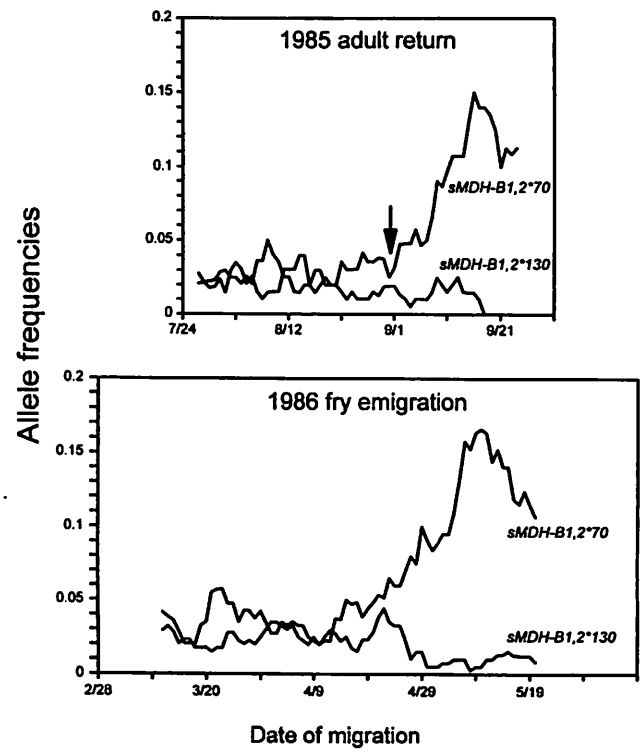

Figure 3. Frequency *sMDH-B1, 2** for pink salmon sampled at the Auke Creek weir during their spawning migration (upper) and seaward emigration (lower). Ten adults or 20 fry were sampled each day. Profiles were smoothed by 5 day running averages. The vertical arrow in the upper figure is the boundary between early and late runs.

marker was that the offspring of early-run parents emigrate earlier than the offspring of late-run parents (Figure 3). In fact, the span of time separating the mid-emigration of early and late-segment fry is approximately the same as that separating the mid-return times of the early- and late-segment parents. At first glance, this observation would seem to be expected. However, salmon are poikilothermic and their development rate is determined in large part by the incubation temperature (Alderdice and Velsen 1978). Early run embryos experience warmer conditions during their early development than do late-run embryos and, by the time they emigrate, have accumulated as much as 10-20% more degree-days than the late-run fish. Degree-days is a standard approximation of the influence of temperature on development in poikilotherms and is the sum of the products of the temperatures and the duration of exposure, [see Hebert *et al.* (1998) for a summary of seasonal temperature patterns in Auke Creek]. The surprising observation is not that fry from early segment parents emigrate first, but that the separation between their mid-emigration times is not greater. This compensatory development time variation within Auke Creek pink salmon is similar to the variance between sockeye salmon *O. nerka* populations demonstrated by Brannon (1987) and has been the subject of our further studies (reviewed below).

GENETICS OF TEMPORAL POPULATION STRUCTURE

The genetic marking results coupled with Taylor's (1980) tagging results strongly indicated a genetic component of the variance of anadromous return timing in the population structure of Auke Creek. Because it is probable that that a life history characteristic such as run timing would be inherited as a quantitative genetic trait rather than a simple Mendelian trait, we conducted a breeding study that observed 120 full-sib families, 60 from each of the early- and late-returning segments, to estimate the genetic components of variability of return timing (Smoker *et al.* 1998). We used a nested, half-sib design to analyze variance of return date to Auke Creek in order to estimate a sire component, that is, the variation attributable to a having a shared sire. The sire component in this analysis is an estimate of one-quarter of the additive genetic variance (V_A). The return timing-segment (early or late) had a significant effect on day of return of offspring ($P<0.0001$); and there was a significant sire component in the variation of return date of both sons ($P<0.045$) and daughters ($P<0.040$). Both of these effects (parent timing segment and sire effect) are probably caused by genetic variation at loci affecting return (Smoker *et al.* 1998). Smoker (unpublished) has also conducted empirical response-to-selection experiments and confirmed that the additive genetic variation can serve as a basis for artificial selection of run timing.

From these breeding experiments, it was clear that genetic variation plays a role in the temporal structure of Auke Creek pink salmon and probably of other populations. If so, temporal structure should also occur in the biochemical genetic structure of Auke Creek pink salmon and of other pink salmon populations. Difficulty in detecting such structure would be expected because, for selectively neutral alleles (as many allozyme loci presumably are), theory demonstrates that it takes very little gene flow to obscure structure. Therefore, a test of structure would have to be a strong, statistically powerful test. We conducted such a test by applying log-linear analysis (a categorical data analog of analysis of variance) to several years' observations of a number of allozyme loci, surveyed in Auke Creek and other pink salmon streams in the Juneau vicinity. Tests for genetic components in population structure in odd-year Auke Creek pink salmon resolved significant effects of return timing ($P<<0.001$) and year of return ($P<0.01$), but did not demonstrate effects of the location of spawning within the creek (upstream or intertidal). We extended the analysis to include other local streams for one year's returns and observed that timing ($P<<0.001$) was more important than stream ($P<0.001$), but that location within the stream was not significant. For even broodyear pink salmon, significance of effects was observed in the order : timing ($P<<0.001$), stream ($P<<0.001$), and year ($P<0.001$); again location within stream was not significant (McGregor et al. 1998).

These results confirm the importance of temporal structure in populations, but in this instance could be resolved only in results of multivariate analysis. Locus by locus, the structure is not as apparent, and a cursory examination, such as we made in choosing a marker allele for the genetic tagging work, revealed very little. However, these results demonstrated the existence of temporal structure at the population genetic level and in doing so completed the connection between a life history characteristic and the resultant population genetic structure, as interpreted using biochemical genetic characters.

GENETIC EVIDENCE OF ADAPTATION IN A TEMPORALLY STRUCTURED POPULATION

One of the observations we made of the genetic marker is that emigration timing of fry is temporally structured, and that timing is related to the anadromous return timing of their parents, in which we demonstrated a genetic component. We also noted that the emigration timing difference between early- and late-segment fry does not conform to the temperature regime differences they experienced (Gharrett and Smoker 1993). We used laboratory experiments to demonstrate differences in the development rate

profiles of early- and late-segment pink salmon (Joyce 1986). Early-segment embryos, which began development in warmer water, developed relatively quickly through early development, but then slowed down to the extent that the developmental stage of late-segment embryos approached the stage of early-segment embryos as development progressed.

The development rate of a pink salmon is a critical life-history character. A successful fry will emerge into the saltwater during the narrow window of opportunity when the marine temperature is suited to predatory activity - but not so warm as to drain the fry's energy, the fry has ample energy reserves to make the transition, and prey is abundant. The strong vernal variation of the marine environment of Auke Creek pink salmon is reviewed in Smoker *et al.* (1998). Given that return timing of adults has genetic components, it is likely that development timing of fry also has genetic components.

We examined the genetic determinants of development rate more closely in a series of quantitative genetic studies. Using a hierarchical half-sib design, we studied development rate in early and late-segment fish in a series of thermal environments that included natural temperature regimes or regimes simulating temperatures encountered by both the early and late segments (Joyce 1986, Hebert 1994, Goddard 1995, Hebert *et al.* 1998). In each of these studies, development rates differed between the return-timing segments in ways that could be generally explained as adaptation to annual environmental variation. In several studies, we observed significant additive genetic effects on development rate measured as elapsed time between fertilization and hatching. We also observed significant genotype by environment interaction effects on development rate, measured as elapsed time between fertilization and completion of epiboly, eye pigmentation, or hatching.

These experiments were conducted on both even- and odd-year fish. The results indicate that compensation in development rate occurs in both brood years and indicate that both Auke Creek pink salmon populations (even and odd years) have adapted to the seasonally different temperature regimes, but that development rates differ between the broodlines (Goddard 1995).

IMPORTANCE OF INTEGRITY OF THE ADAPTED GENOME

Both even- and odd-broodyear salmon exhibit variation of development rate, and genotype by environment (GxE) interactions. However, there were significant differences between broodyears in the nature and extent of variation and (GxE) interactions (Goddard 1994). The simple explanation is that the two broodlines have found two different genetic solutions in adapting to the local environment of Auke Creek. Although the two broodlines have experienced similar average environments, the different sequences of environments (selection regimes) and random drift have generated different filters for the large number of loci and alleles involved in fitness.

A question that follows from the observation of these genetic differences is whether or not the organization and integrity of the adapted genomes is important. That is, would fitness be reduced by disruption of the genome, say by hybridization ? If individuals from genetically different populations interbreed, theory and growing evidence predicts the genetic health and subsequent productivity of a resulting population may be impaired. This phenomenon is called outbreeding depression (Shields 1982).

Two conceptual models can explain how outbreeding depression might occur. In reality, elements from both models can be at work simultaneously, depending on the trait (s) in question. The first model is based on additive genetic effects. It explains that selection works on a collection of independently working genes. In this case, hybrids of disparate populations that have adapted to different local environments would be most fit in an intermediate environment and probably not as fit in either of the parental environments. This model predicts out breeding depression would occur in either parental environment in the F_1 and later generations until natural selection reduces the number of deleterious alleles.

According to the second model, phenotypes are derived from a complex of genes, which interact. In the "coadapted genome model" (Shields 1982), selection favors individuals whose gene complexes work well together. F_1 hybrids between individuals from disparate populations do not necessarily exhibit outbreeding depression because each individual receives a complete copy of a coadapted genome from each parent. However, a reduction in fitness would be expected in F_2 and later generations from the disruption of coadapted allele complexes as a consequence of independent assortment of alleles at gametogenesis (Mendel's second law) (Shields 1982).

We used hybrids between even- and odd-broodline, Auke Creek pink salmon to test for outbreeding depression. We made F_1 hybrid crosses in 1983 and 1992 fertilizing eggs with cryopreserved milt from 1982- and 1991-broodyear males, respectively. F_1 hybrids and controls returned in 1985 and 1994. In both experiments, hybrids and controls had similar return rates, i.e., survival during the marine life phase. From those returns, we made F_2 crosses. Few F_2 hybrids returned in 1987 ; but because we had been unable to release controls, it was not possible to fully evaluate the effect (Gharrett and Smoker 1991). However, F_2 hybrids returning in 1996 had significantly lower survivals than returning F_2 controls (Gharrett *et al.* 1999).

Our experiments indicate that outbreeding depression can occur in Pacific salmon. The test, however, involved

hybrids that would occur rarely in nature. We are now conducting similar experiments using spatially separated populations.

IMPLICATIONS OF AUKE CREEK GENETIC STUDIES

In a variety of experiments, we have demonstrated genetically based temporal population substructure in Auke Creek pink salmon. We also have demonstrated that variations of several life history traits have genetic components, including timing of anadromous migration and embryo development rate. Two other life history traits that are influenced by significant additive genetic variation in Auke Creek pink salmon are size at maturity (Smoker *et al.* 1994) and fecundity (but not egg size) (Smoker *et al.* unpublished). Our interest in the genetics of life history traits stems in part from our curiosity about the genetic determinants of biological systems, but the information does have practical implications because survival and productivity are intimately related to such life history traits.

Key to understanding the implications of a heritable trait is that the heritability (h^2) of a trait is a population parameter measured as the proportion of total phenotypic variation (V_P) in population that is attributable to additive genetic variance (V_A). Detecting a sire component in the variance of anadromous timing, for example, requires that there be variation in timing and that some of the variation has a genetic basis. Many life history traits contribute to the fitness of the population. If the biotic and abiotic conditions were constant over time for pink salmon, they would rapidly adapt to those conditions. Natural selection would winnow out the less fit genotypes; and, because additive effects are directly reflected in phenotypes, reduce the genetic variation.

The existence of additive genetic variation for a fitness-related trait in a population is strong evidence that the environment is not constant, but that selective forces vary from generation to generation (reviewed, e.g., by Mousseau and Roff 1987). By way of illustration, consider anadromous return timing and development rates in Auke Creek pink salmon. The series of freshwater and marine environments encountered by salmon fluctuate interannually. When Auke Creek pink salmon spawn and when they emerge to begin feeding are important to fitness of the population (e.g., Gharrett and Smoker 1993). If adults return too early or too late, they will miss optimum conditions to spawn (McGregor *et al.* 1998, Smoker *et al.* 1998). Likewise, if marine conditions are not optimal for downstream-migrating fry, survival is reduced (Taylor 1980). Variability in return timing means that a portion of the population will encounter optimal conditions and will successfully reproduce. Genetic variability in life history traits is a bet-hedging strategy and is a critical element for ensuring persistence of a population. Since the environment in which salmon develop fluctuates over generations, genetic variability is important to maximizing long-term fitness of a population. In the short-term, mechanisms important to maintaining variability in pink salmon include bimodal run timing, adaptation to local conditions, straying, and sexual reproduction.

The implications of such a "bet-hedging" use of genetic variation are that the variation itself is important, and that an unpredictable portion of the population will produce the next generation. An extrapolation of this concept arrives at the conclusion that in a particular generation many, or in some years most, of the progeny may arise from relatively few parents. This result creates the anomalous situation of survival being a heritable character. Consistent with the prediction, genetic analysis of family size of pink salmon returning to Auke Creek indicates a significant additive genetic effect for marine survival that is more accentuated in generations of large returns than generations of small returns (Geiger *et al.* 1997). This means that a small portion of the population contributes disproportionately to the next generation; but because of interannual environmental variation, the genotypes of the successful portion vary unpredictably from generation to generation. In this situation the variation itself is important to population fitness.

Clearly, maintenance of genetic variation is key to long-term success of a population; however, our studies of interbroodyear hybrids suggest that outbreeding depression may also occur. The hybrids carried much of the variation from both broodlines, but had poorer survival. Hence, maintaining variation *per se* is not an adequate answer to the success of a population, the nature of the variation is also important. For aquacultural and fisheries management strategies to be successful over the long term, it is critical to factor in biological knowledge of the species, particularly with respect to life history variation and population structure. The source and nature of genetic variation are critical elements of long-term productivity of populations.

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ON THE GENETIC DIVERSITY OF WILD AND CULTURED POPULATIONS OF THE JAPANESE PEARL OYSTER *PINCTADA FUCATA*

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ABSTRACT

The pearl oyster is a commercially important marine bivalve species, as it is used for the production of pearls Recently, tremendous deaths of the pearl oyster were reported in Japan. Although the main cause of the deaths has not yet been clarified, the loss of genetic diversity in the cultured and natural populations may have contributed farther damages. It is therefore necessary to establish a method to evaluate the genetic diversities of pearl oyster populations, possibly by the use of DNA fingerprinting techniques. The DNA samples were purified from 24 individuals each of the two populations: a wild population from Noto Peninsula, Japan, and a cultured population of the "white pearl oyster." The RAPD (random amplified polymorphic DNA) analyses were performed with the use of the primer OPA10 (Operon Tech.). Then the intra-population genetic variations were evaluated using the software RAPDistance, with Excoffier distance metric. Student's *t*-test indicated that the mean dissimilarity among individuals of the cultured population was significantly lower than that among individuals of the wild Noto population. However, the degree of the decrease in the genetic variance of the cultured population was only at 5.3 %

INTRODUCTION

The Japanese pearl oyster *Pinctada fucata* is a commercially important marine bivalve species, as it is used for the production of pearls (Wada 1991, Gervis and Sims 1992). Recently, tremendous deaths of the Japanese pearl oyster were reported, mainly in Ehime Prefecture, Japan. Although the main cause of the deaths has not yet been clarified, the loss of the genetic diversities in the cultured and natural populations may have contributed farther damages. It is important to note that natural populations do not necessarily mean wild populations, because the larvae from cultured populations are easily mixed in wild populations and contribute to natural populations. It is therefore necessary to establish the method to evaluate the genetic diversities of the pearl oyster populations by the use of DNA fingerprinting techniques, such as RAPD (random amplified polymorphic DNA) analyses (Welsh and McClelland 1990, Williams *et al.* 1990).

MATERIALS AND METHODS

For the preparation of genomic DNA samples from the Japanese pearl oyster populations, the biggest problem was the difficulty in purifying DNAs without any con-

tamination of mucopolysaccharides, which occur abundantly in this species and inhibit enzyme reactions. A new method was developed, by which mucopolysaccharides were removed from DNAs, which were then served for restriction enzyme treatment and polymerase chain reactions (PCRs) (pending for a Japanese domestic patent).

As to the two pearl oyster populations to be compared, we chose a wild population from Noto Peninsula, Japan, and a cultured population of the "white pearl oyster." The color of the nacre of the latter was significantly whiter than the former, as reported by Wada and Komaru (1990). DNA samples (80 mg per individual) were purified from 24 individuals of each population (Figure 1). Although microsatellite DNA fingerprinting (Stephens *et al.* 1992) was also performed in the present study, only RAPD analyses are described in this report.

Primer OPA10 (Operon Tech., 5'-GTGATCGCAG-3') was used to do the RAPD analysis. The PCR products were separated with 1.5% agarose gel electrophoresis. In order to determine each band position precisely, the molecular size standard (AmpliSize molecular ruler, 50-2,000 bp ladder, Bio-Rad) was loaded at both sides of each PCR-product lane. Intra-population genetic variations were evaluated with the use of the software by Armstrong *et al.* (1994).

RESULTS AND DISCUSSION

To evaluate the intra-population genetic variance from the RAPD data (Table 1), the Excoffier distance metric (Excoffier *et al.* 1992) was used for the pairwise comparison in the dissimilarity of band patterns among individuals of each population (Table 2). Student's *t*-test (Table 3) indicates that the mean dissimilarity among individuals of the "white" population is significantly lower than that among individuals of the wild Noto population.

These results show that the cultured "white" population is genetically a little but significantly less variable than the wild Noto population. This may be caused by a limited number of parents when the "white" population is reproduced (less than 200). The degree of the decrease in the genetic variance, however, is only at 5.3 %.

In case an ideal environment is available for the cultured

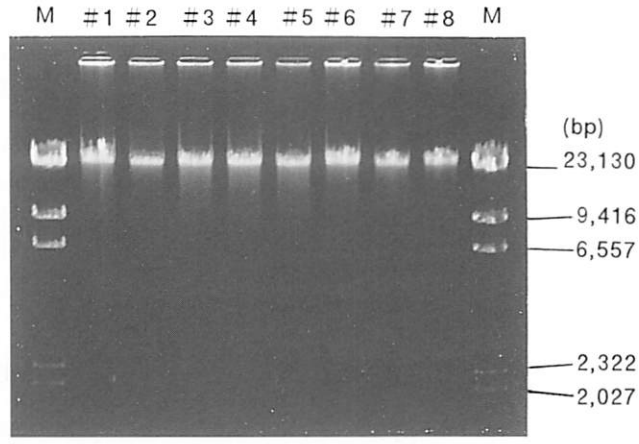

Figure 1. Examples of the purified DNA from the pearl oyster. The lane numbers correspond to the individual numbers from the "white" population. M : λ-*Hin*dIII marker.

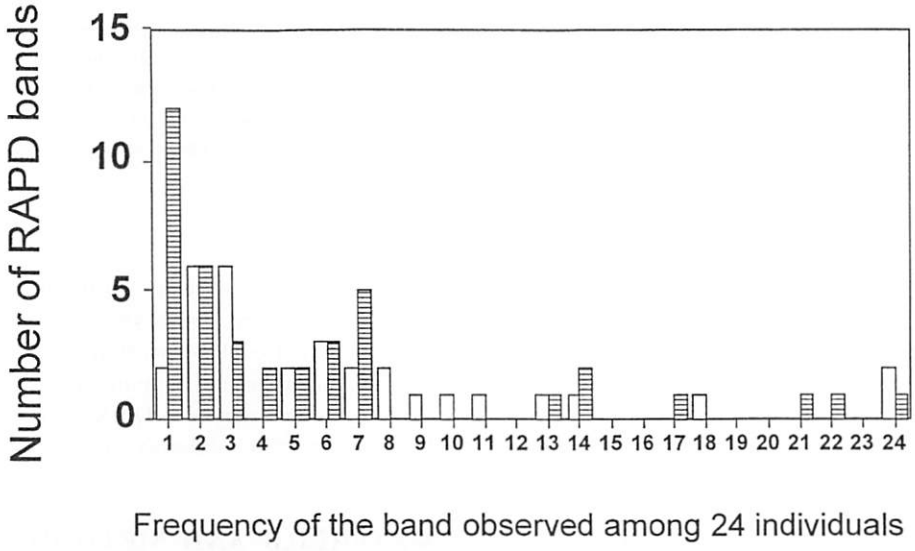

Figure 2. Histogram of RAPD bands with respect to their frequencies of occurrence among 24 individuals of each population. White columns correspond to the "white" population, while striped columns represent Noto population.

Table 1. The occurrence of RAPD bands in 24 individuals of the two populations.

	Individual	RAPD bands[a]
"White" population	# 1	000100001000000001001000010000011000100101000
	# 2	000100001000000001001000000000001001000010100 0
	# 3	000100001000000001000010000000000010000010000 0
	# 4	000100001000000000000001000001000010000001000 0
	# 5	000100001000000001000000010000010100000001000 0
	# 6	000100001000000001000010000010000100000001000 0
	# 7	100100001000000001001001111000001010000010100 0
	# 8	100100010100101000010101000000000100000010000
	# 9	000100001000001010001000000001000010010000 1000
	#10	000100001000000000001000000000000010000010100 0
	#11	100100010010100000010000000000000100000101000
	#12	000100010100000000001000000000001010000001000 0
	#13	100100010010100001000101010000100100010001000
	#14	100100010000000001000010000100101000000010000
	#15	100100010000000001000100000100100010000010000
	#16	000100010000000001001000000100101010000010000
	#17	100100010000000000001000000000000100000101000
	#18	100100010100000100001010100100001000001010000
	#19	100100011001010000001000010000001000000001000
	#20	100100010000000010000100000000000010000010100 0
	#21	100100010100010001000000000000010100000010000
	#22	100100011001010000001000100000000000000010000
	#23	100100010100010001001100000000101000100011000
	#24	100100011001010000000100000000000000000001000
Noto population	# 1	001100010000000000010000100000101000000000000
	# 2	100100010100101100000100000001000100000001000
	# 3	100100010010000010010000000000010010000001001
	# 4	100100010000000000000100000000000010000010000 0
	# 5	101100010000000001000101000100001000000000000
	# 6	100100010000010001000100100100000101000000010000
	# 7	001100010100001000000010000000001010000000001000
	# 8	101100010000100100100100100000101000000001000
	# 9	100100010000000010010010000000000010001000001 0
	#10	100100010000000000000010000000010000100001000
	#11	100100010000000010010010010000010100000001000
	#12	100100010000000000000010001000001010000000001000
	#13	100100110000000011001100000000001010001000 1000
	#14	100100010000000001001011000010010010010001000
	#15	100100101100001101001100010001000100000000000
	#16	100100101010001001010100100000101000000001000
	#17	110100110000000001000100000000100100000000000
	#18	100100110000000000100000000000000010000000001000
	#19	101100110000100110101000000000101000000001000
	#20	100100110000000000000001000100010001000100010 0
	#21	100100010000000000100100001001001000000000000
	#22	100110010000000000000100000000010010000001000
	#23	101101010000000001000000000000100100001000000
	#24	101100010000000000000100000000100010000001000

[a] "1" denotes presence, and "0" denotes absence of a band in an individual.

Table 2. Genetic distances among individuals of each population with the use of Excoffier *et al.* (1992) metric.

"White" population

	# 1	# 2	# 3	# 4	# 5	# 6	# 7	# 8	# 9	# 10	# 11	# 12	# 13	# 14	# 15	# 16	# 17	# 18	# 19	# 20	# 21	# 22	# 23
#2	4																						
#3	8	6																					
#4	12	10	4																				
#5	7	7	5	5																			
#6	11	9	3	1	4																		
#7	6	6	8	12	7	11																	
#8	15	13	9	7	10	8	11																
#9	9	9	9	7	10	8	11	12															
#10	6	4	4	6	7	7	6	9	5														
#11	9	7	7	9	10	10	7	8	8	3													
#12	9	7	7	5	4	6	9	6	8	5	8												
#13	15	11	13	13	14	14	13	12	16	13	10	14											
#14	13	11	9	5	6	6	9	10	10	9	10	6	12										
#15	13	11	9	5	8	6	13	10	12	11	12	8	10	4									
#16	13	11	9	5	6	6	13	12	10	9	12	6	14	4	4								
#17	9	7	5	7	8	8	7	10	8	3	4	8	12	8	10	8							
#18	12	12	10	10	11	11	8	13	9	8	9	11	17	9	3	11	5						
#19	14	12	10	10	11	11	12	13	9	8	9	11	13	11	13	11	5	10					
#20	10	8	6	8	9	9	8	11	7	4	5	9	13	9	11	9	1	4	6				
#21	13	11	9	7	6	8	11	8	10	9	10	4	12	4	6	6	8	11	9	9			
#22	13	13	11	9	8	10	13	12	12	11	12	10	16	10	10	10	8	11	5	9	8		
#23	14	12	10	10	11	11	12	11	11	10	11	9	9	9	9	9	7	10	8	8	5	11	
#24	12	10	8	8	11	9	12	11	9	8	9	11	11	11	9	13	7	12	4	8	9	5	8

Noto population

#2	12																						
#3	9	13																					
#4	6	10	11																				
#5	8	10	13	8																			
#6	7	11	12	7	7																		
#7	6	6	11	8	8	9																	
#8	7	7	12	11	9	8	7																
#9	10	8	13	8	8	9	10	7															
#10	8	8	7	8	8	7	6	7	8														
#11	7	7	10	9	9	6	7	2	5	5													
#12	5	9	8	5	9	6	7	6	9	5	4												
#13	9	11	8	9	13	12	11	10	9	9	8	6											
#14	11	13	6	13	11	12	11	12	11	7	10	10	8										
#15	18	12	17	14	14	15	14	17	14	14	15	15	15	17									
#16	10	12	11	14	12	9	10	11	14	10	9	9	11	13	12								
#17	10	10	13	8	6	7	10	11	8	8	9	9	11	13	10	10							
#18	8	8	9	6	6	7	8	9	8	6	7	5	7	11	12	8	4						
#19	10	10	11	10	12	13	10	5	10	10	7	7	5	13	16	14	12	8					
#20	13	13	12	11	11	12	13	14	11	9	12	12	14	12	9	15	7	9	15				
#21	9	9	12	7	7	8	9	8	5	7	6	8	12	12	13	13	5	7	11	8			
#22	9	11	6	7	11	10	9	10	11	5	8	4	8	8	13	13	11	7	9	10	10		
#23	8	12	13	8	6	9	10	11	10	10	11	9	13	15	14	14	6	6	12	9	7	11	
#24	9	11	8	7	9	12	9	10	11	7	10	6	10	10	11	15	9	7	9	8	8	4	7

Table 3. Student's *t*-test of the significance of the difference of the mean intra-population distances.

	mean		SEM
Noto population	9.59	±	0.17[a]
"White" population	9.08	±	0.17[a]

[a] The difference of the mean distances is significant ($P < 0.05$).

"white" population, it is suggested that the genetic weakness of this population is not prominent compared with the wild Noto population. Once some environmental factors drastically change, for example, by the occurrence of infectious diseases, red tides and abnormal seawater temperature fluctuations, then the wild Noto population may have more advantages than the cultured "white" population, because the former has more genetic variances than the latter, and thus has more possibilities to adjust to the new environments.

The higher genetic variability of the wild population is consistent with the existence of more RAPD bands of low occurrence in the wild than in the cultured population (Figure 2). Though polymorphic alleles of low occurrence do not contribute much to the measures of genetic diversity, their abundance would be quite important for the population to adjust to variable environments.

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PERSPECTIVE ON GENETIC ANALYSIS OF FISH POPULATIONS

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ABSTRACT

It is of fundamental importance to understand genetic structure of natural populations for reasonable conservation and management of fisheries resources. Protein analyses have been mostly employed for assessing genotype of individuals and genetic difference among populations of aquatic organisms for nearly two decades, having provided invaluable genetic information for them. In recent years, molecular genetic analysis has become much more powerful, as nucleic acid level techniques have been introduced in this field. The use of such techniques not only promote detailed analysis of population structure but also broaden applications of genetic investigation in fisheries biology. Technological improvements in DNA methods are still ongoing. Therefore, geneticists concerned in fisheries biology will have much to be studied by utilizing those new DNA methods.

INTRODUCTION

Molecular genetic markers have become very important in these years in the field of fisheries biology. Genetic analysis with molecular markers is one of the most useful approaches to examination of population structure of a species. Knowledge of population structure of a species is of essential importance for appropriate management of species stocks. Molecular markers are also useful for many other purposes, such as genetic variability assessment of hatchery population, trace of released population, species or stock identification of planktonic eggs and larvae, analysis of hybridization, and so on. In this note, 1) outline of rationale and methods for the molecular-genetic approach are described, 2) some examples of our research with molecular markers are presented, and 3) prospects of genetic analysis of fish populations with molecular markers are discussed briefly.

MOLECULAR MARKERS

Rationale

Molecular data are genetic. This is the reason why molecular approaches are so important. Living organisms are genetic entities whose essential information for development and daily life is coded on genetic materials, or DNA. The genetic materials are mixed during sexual reproduction within a species, but not mixed between different species. Accordingly, much more difference in genetic materials is observed usually between different species than within a species when examined with molecular techniques. A species is distributed in some spatial range so that sometimes it has geographic population structure. In such a species, geographic populations are expected to differ from one another in genetic composition, which can be most effectively examined through molecular genetic analyses. Genetic materials are transmitted through parents to offsprings, thus generation to generation. The transmitting route is "phylogeny". Therefore, phylogeny estimation should be most effective on the basis of molecular analysis of the transmitted materials themselves.

It may be appropriate to add some comments on the significance of molecular approaches. First, molecular techniques make us possible to pursue genetic examination of any organisms we concern. Until the introduction of molecular approaches about three decades ago, genetic studies were mostly confined to a handful of laboratory organisms such as the house mouse *Mus musculus*, the fruitfly *Drosophila melanogaster*, and so on. Nowadays, however, we are able to make intensive and extensive genetic studies on any organisms even living in wide open

oceans by using molecular techniques.

Secondly, with molecular methods, we can examine virtually unlimited source of genetic information. A genome, a whole set of DNA in a signal cell of an organism, has huge amount of genetic information in it; a typical vertebrate genome consists of a few thousand million nucleotide pairs. To have virtually infinite amount of genetic information provides us the great advantage to take a sound scientific procedure of the cycle of hypothesis-making and its testing.

The third point is that molecular data can provide common frameworks of genetic differentiation, phylogenetic relationships, and evolutionary time. This is because most of molecular variation is selectively neutral and molecular evolution appears to proceed approximately proportional to time (Kimura, 1983). Such characteristics of molecular data facilitate comparisons of various organismal features and syntheses of a wide array of biological findings. Furthermore, the selective neutrality is also an important feature of molecular makers for population studies. An essential assumption in genetic analysis of population structure is that markers are selectively neutral and subject to random genetic drift during population differentiation (Nei, 1987).

Molecules and techniques

Molecular genetic data are obtained mainly from DNA and proteins. Proteins are products synthesized according to genetic information coded on DNA, being useful for genetic examination. Some proteins have catalytic activity as enzymes and exist in considerable amounts in an organism body. These properties of proteins can be of help in detecting and examining them. This is the reason why proteins were targeted in the first stage of the developments in molecular approaches to population and evolutionary genetic problems. Multiple forms of enzymes encoded by one or more loci are termed isozymes, and those of different alleles at the same locus are called allozymes. Fish population geneticists and systematists have been using allozyme electrophoresis as a primary method for their studies for some twenty years. Up to now, much allozyme data has been compiled for many fish species. The allozyme database, therefore, should be a valuable source of genetic information on them.

In recent years, DNA techniques have been increasingly introduced in this field, and accordingly genetic analysis has become much more powerful. It is one of the largest advantages of DNA as target molecules for genetic markers that DNA, as the self-replicating heritable molecules, can be amplified artificially, unlike proteins. This means that even if you only have a small amount of a DNA segment you can make detailed examination via its amplification. Though origination of the recombinant DNA technology in the 1970s has opened the possibility of DNA amplification, it has been impractical to utilize it in population-level studies that require examination of a large numbers of individuals, because the technology is laborious and time-consuming. Recent development of the polymerase chain reaction (PCR) has been revolutionizing this situation.

The PCR is a simple and rapid method of amplifying a particular DNA segment of interest. The template DNA is mixed with two specific primers and the four nucleotides in the presence of the DNA polymerase. Some thirty reaction cycles of denaturing-annealing-extension result in a million-fold increase in the target DNA segment within a few hours. This amount of DNA copies is far sufficient for direct sequencing, restriction analysis, and other ways of analysis (Figure 1). Therefore, the PCR has been becoming a basic methodology of DNA-level studies in systematic and population biology as well as cell and molecular biology. In addition to its simplicity and rapidity, relaxed sample requirement is another important characteristic of it. The fact that even a minute amount of samples preserved in ethanol will be enough for successful PCR amplification makes DNA approaches more powerful. Now a number of molecular methods are available for individual- or population-level studies in relation to fisheries. Note that most DNA techniques are those based on the PCR.

Though we have a variety of useful molecular methods, each has its own advantages and disadvantages depending upon problems to be solved. It should be noted that we have two options as a target genome. In eukaryote cells, DNA exists mostly in the nucleus (nuclear DNA), but some small parts are in the cytoplasm; the mitochondrial DNA (mtDNA) and/or chloroplast DNA (cpDNA, in plants). The nuclear DNA is a very complex entity with coding and non-coding regions, the latter including introns, repetitive regions (e.g. microsatellites, minisatellites, SINEs, and LINEs), and non-repetitive spacer regions. By contrast, the mtDNA is a small, simple circular DNA mostly consisting of coding regions. While the nuclear DNA is diploid and biparentally inherited with recombination, mtDNA is haploid and maternally inherited without recombination. Genes in the latter have variability an order of magnitude higher than single-copy genes in the former. These differences in the two genomes may provide useful clues to examine reproductive structure of animal populations (Wilson *et al.*, 1985).

More detailed information on the rationale and methods for the molecular genetic markers in fisheries biology and natural history is to be found in some recent publications such as those by Beaumont (1994), Avise (1994), Carvalho and Pitcher (1995), and Hillis *et al.* (1996).

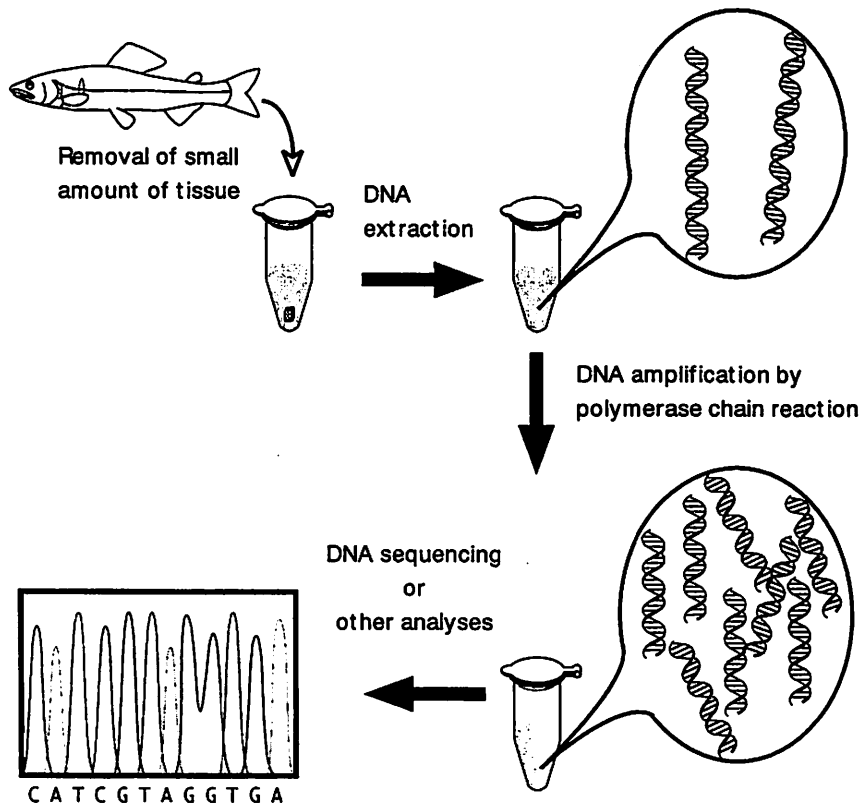

Figure 1. Diagram of flow of PCR-based DNA methods.

SOME EXAMPLES OF GENETIC ANALYSIS OF FISH POPULATIONS

In this section, some examples of genetic analysis of fish populations are reviewed, being exemplified mostly by researches on the ayu-fish in our laboratory. The ayu (*Plecoglossus altivelis*) is an osmeroid fish, being one of the most important species in the inland water fisheries in Japan. This fish occurs throughout Japan, Korea, and some part of China. The ayu has an amphidromous life-cycle, spending its early life in the coastal marine waters and ascending streams after growing young, where it becomes adult and spawns. Hatched larvae directly go down to the sea with water flow.

Population structure

Geographical populations were examined by using allozyme electrophoresis in order to investigate the population structure of this fish along the Japanese Islands. The allozyme analyses revealed that amphidromous populations of the main islands are rather homogeneous genetically, probably due to extensive gene flow by larval dispersal along the coast (Nishida and Takahashi, 1978; Taniguchi *et al.*, 1983; Seki and Taniguchi, 1985). These researches also showed a landlocked population in Lake Biwa to be somewhat differentiated from the other amphidromous populations. The landlocked population of the ayu in Lake Biwa has been known to have morphological and ecological characteristics (Shiraishi and Suzuki, 1962; Kawanabe, 1972; Komada, 1977; Azuma, 1981; Seki *et al.*, 1984; Nishida, 1986, 1988b; Niahida and Sawashi, 1987; Tabata and Azuma, 1986; Iguchi, 1993; Iguchi and Yamaguchi, 1994). The allozyme data confirmed that this population has genetic uniqueness with a relatively independent history.

Further electrophoretic examinations uncovered the existence of very differentiated amphidromous populations in the Ryukyu Islands (Nishida, 1985, 1986; Seki *et al.*, 1988; Sawashi et al., 1993). These populations were found to have some unique morphology as well (Nishida, 1986; Nishida and Sawashi, 1987), so that they were described as a new subspecies endemic to the Ryukyus, *Plecoglossus altivelis ryukyuensis* (Nishida, 1988a). The level of genetic divergence observed through allozyme analysis led an estimation that this Ryukyu subspecies may have been evolving in that area for more than one million years independent from Japanese main island populations

(Nishida, 1986). This scenario is consistent with the proposed geological history of the Ryukyus (Kizaki and Oshiro, 1977).

The Ryukyu subspecies is regarded as a unique genetic resource of the ayu. They once occurred on Amami-oshima and Okinawa Iss. in the Ryukyus, but about 20 years ago the Okinawa populations were extinct (Nishida *et al.*, 1992). The remaining stock on Amami-oshima Is. was examined for their structure and was found to be composed of two subpopulations with low genetic variability (Sawashi and Nishida, 1994; Takagi and Taniguchi, 1994). Such genetic information has been utilized in conservation activities.

Korean populations were shown to be very similar genetically to the Japanese main island populations by later allozyme analyses (Seki *et al.*, 1988; Sawashi *et al.*, 1998). However, some populations of *Plecoglossus altivelis altivelis* on islands around Japan were observed to have somewhat unique allele frequencies (Sawashi *et al.*, 1993; Sawashi *et al.*, 1998). Such genetic differentiation were considered to be primarily due to random genetic drift on smaller islands but to have accumulated during a considerable length of time, say several thousand years (Sawashi *et al.*, 1998). Chinese populations have not been analyzed genetically so far, and thus we have not reached a definite conclusion about their subspecific assignment.

Recent years, we have been examining genetic population structure of this species much in detail by using mtDNA sequence analysis. The control region (D-loop region), only a non-coding region of the mtDNA known to be most variable in it, was chosen to be sequenced. Our sequencing results (Iguchi *et al.*, 1997) showed that this region is highly variable indeed, suggesting their utility for the analysis of intra-specific divergence. The sequence data revealed substantial, though not large, genetic divergence between amphidromous and Lake Biwa populations, roughly 80% of fish being distinguishable by the nucleotide substitutions. The data also showed that genetic variability was lower in the Lake Biwa population than in amphidromous ones (Figure 2).

Among amphidromous populations of *P. a. altivelis*, largest net nucleotide divergence was observed between Korean and Japanese populations (Iguchi *et al.*, 1999a). Within the Japanese Archipelago, though no obvious correspondence was found between genetic and geographical distances, minute but significant heterogeneities among samples were observed in the frequency of nucleotides at specific sites. The results imply that a single large

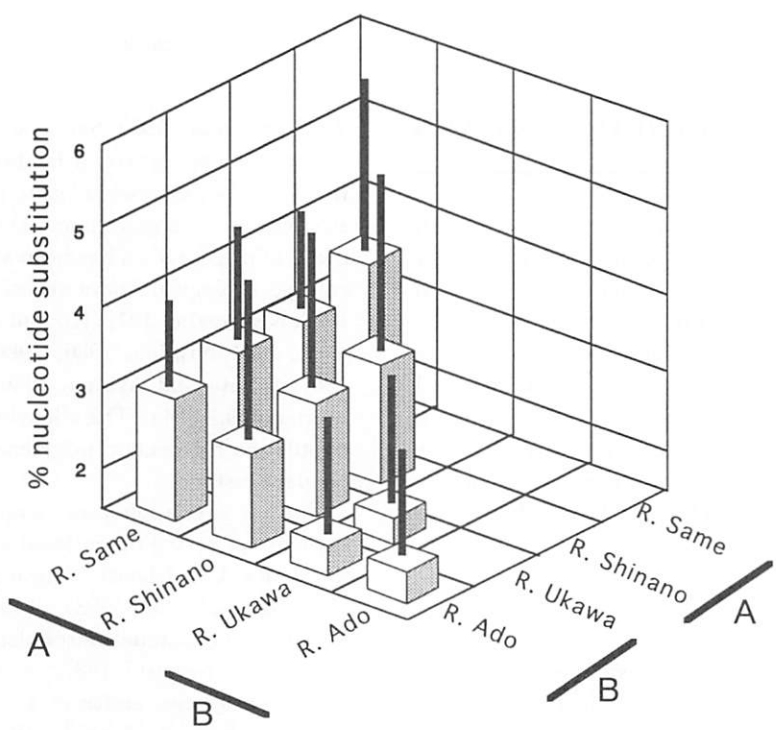

Figure 2. Mean sequence differences within and between populations of the ayu. Bold vertical lines denote standard deviation. A: amphidromous population, B: Lake Biwa population. (Modified from Iguchi *et al.*, 1997)

amphidromous population exists along the Japanese Archipelago, but the population is substructured as a metapopulation. These mtDNA analyses basically confirmed the above-mentioned picture of population structure of the ayu from allozyme analyses and gave more fine scale resolution. For much detailed analysis, microsatellite DNAs have begun to be surveyed.

Genetic monitoring of transplanted stocks

For many years, transplantation of young ayu of the Lake Biwa population to various rivers in Japan has been conducted. In the light of the above-mentioned finding that this lake population is somewhat diverged genetically from amphidromous populations, genetic monitoring of transplanted stocks is needed. Some attempts have been made by using allozyme markers (Seki and Taniguchi, 1988; Seki *et al.*, 1994), suggesting that transplanted stocks from Lake Biwa almost disappeared from transplanted rivers before spawning. Another research utilizing both allozyme and mtDNA-RFLP markers showed that some part of a transplanted Lake Biwa stock remained until the spawning season (Pastene *et al.*, 1991). In view of the lack of evidence for introgression of the genetic markers of the transplanted stock into the native one, it was concluded that the transplanted stock hardly contributed to reproduction of the river population.

However, details of this disappearing process of genetic markers of transplanted fish have not been clarified yet. One important point is to examine genetic markers in samples of larvae. Allozyme markers can not be used for such minute amount of samples like as hatched larvae of the ayu. PCR-based DNA technologies should be suitable for this kind of research in particular. Such a study utilizing PCR-based DNA markers, the mtDNA and microsatellite DNA, is now being conducted in our laboratory.

Genetic variability in hatchery populations

Recently, attempt for stock enhancement through the release of cultured fish has become widespread. Although large-scale release of cultured fish has an immediate effect on stock abundance, it also has the potential of leading to alteration in the genetic make-up of wild populations. Differential reproductive histories of hatchery and wild populations may generate genetic differences in various traits. Depending upon the extent of the differentiation, cultured-fish released into natural waters might have genetic effects on wild populations through interbreeding and competitive exclusion, which usually appear to be negative (Hinder *et al.*, 1991).

Many hatchery populations of ayu had been established before genetic management was recognized to be important. In some hatchery stocks, breeding has persisted for multiple generations without the introduction of exotic broodstocks. The DNA sequence variation was examined in the mtDNA control region of three wild and six hatchery populations of ayu (Iguchi *et al.*, 1999b). Compared to wild populations, hatchery populations showed considerably lower genetic variation. Haplotype and nucleotide diversities decreased with successive hatchery generations, indicating that genetic drift and/or adaptation to culture conditions could be responsible for the reduction in genetic variation (Figure 3). It is concluded that, if released extensively, the hatchery populations have a potential impact on wild populations by reducing the genetic variability through interbreeding. Serious attention should be payed to reduce the genetic impact of cultured fish on wild populations.

PROSPECTS AND CONCLUSIONS

As shown above, recent molecular techniques, those of PCR-mediated DNA methods in particular, are promising in resolving various problems in fisheries biology. It should be noted that requirement of only a minute amount of DNA as material for these methods leads a variety of important applications in research. For example, specific DNA sequences can be amplified and examined from minimal quantities of tissue such as scales or fin-clips taken from living fish without killing. This is ideal for conservation genetics investigation of endangered populations. Furthermore, it may be possible to apply DNA analysis to dried tissue adhering to historical collections of scales or otoliths, enabling a comparison of present and past genetic structure of populations.

The extremely relaxed sample requirement for assessing

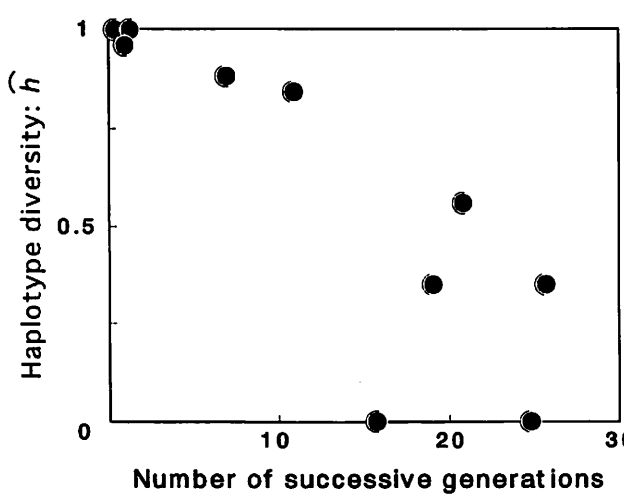

Figure 3. Relationships between successive generations and haplotype diversity in cultured populations of the ayu. (After Iguchi *et al.*, 1999b)

DNA markers has opened another interesting possibility of application to the field of the molecular ecology for marine organisms. For instance, we successfully applied mtDNA markers to species identification of eggs and larvae of eels in plankton samples (Aoyama *et al.*, in press). The DNA-based species identification enabled us to clarify early life-history and distribution of concerned fish species.

DNA study is still in its infancy. New DNA methods are still emerging one after another. Among them, the DNA micro-array (DNA chip) technology appears to have high potential for various aspects of future genetic analysis of individuals and natural populations. Among the existing methods, the accumulation of DNA sequence data is strongly needed, because DNA sequence is the universal, ultimate genetic information. Once determined, it is of permanent use as a part of the DNA database, which provides information necessary for comparisons or further studies to improve understanding of organisms of our concern. DNA data in aquatic organisms are still very limited, though increasing rapidly. Molecular genetic researches should be executed much more actively in fisheries biology.

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ARTIFICIAL FERTILIZATION OF FOUR SPINE SCULPIN, *COTTUS KAZIKA*

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ABSTRACT

Four spine sculpin *Cottus kazika* is well distributed in Japan, from Akita to Fukuoka Prefectures on the Japan Sea side, and from Kanagawa to Miyazaki Prefectures on the Pacific Ocean side. But it is necessary to increase artificial preservation and seed stocking because resources of the four spine sculpin have decreased. In Fukui Prefecture, they mostly live in the middle of Kuzuryu River and the area where they live has become a natural preserve. But the four spine sculpin has become a special regional dish of high quality fish. Examinations of seed production targeted for culturing of the four spine sculpin had begun in 1988. One of these examinations was on the artificial fertilization. In the seed production of this species, there were problems, e. g. difficulties in using wild fish as parents for they have decreased in number, and unstable eyeing rates of fertilized eggs from cultured fish. In the present study, therefore, relationship between egg quality and eyeing rates, and changes in the motility of diluted sperm were observed in order to develop useful techniques to stabilize the artificial fertilization.

INTRODUCTION

The gill cover of the four spine sculpin *Cottus kazika* has four spines and its largest is 30 cm. From January to February they come down the river to spawn. The male and female pair off and the eggs are spawned at near the river mouth. It is well known that the male fish takes care of the eggs.

Because wild fish have decreased in number, it has become necessary to use cultured parental fish. Because eggs that are stripped from 0-year-old female fish (maturation begins at age 0) are so small that the quality of eggs is not good and the female parental fish usually dies at age 2. Therefore 1-year-old parental fish have been used in seed production.

Natural spawning and artificial egg collection were examined. Natural spawning yields unstable amount of egg collection and with the cases of unfertilized eggs. Artificial egg collection offers the egg collection possible, but the eyeing rates are unstable in artificial fertilization.

The eggs that adhere to the net have low hatching rate, thus the egg management still needs to be improved.

Feeding scheme and schedule is not established firmly. Furthermore fish deformity and cannibalism occur in seed production. Further improvement is needed to rear the fish successfully.

To prepare sperm solution, the stomach is opened and the testes are taken out, cut and diluted one hundred times with two-thirds artificial seawater. Parallel to this work, eggs are stripped, spread thinly on the net and fertilized. Then the remaining sperm are washed in two-thirds artificial seawater. But eyeing rates are unstable in this way.

Therefore the present study focuses on examining changes in the motility of diluted sperm and the relationship between egg quality and eyeing rates, in order to develop the techniques to stabilize the artificial fertilization.

MATERIALS AND METHODS

Parental fish

One-year-old fish, which was cultured from artificial larvae and fed formula food for flounder, was used as parental fish.

Sperm dilution

Testes were taken out from two male fishes (T.L. 149 mm, B.W. 68.1 g, S.W. 3.58 g; T.L. 170 mm, B.W. 98.2 g, S.W. 4.70 g), and the sperm were diluted to 2% with four kinds of solution, i.e.(1) artificial seminal plasma for rainbow trout *Oncorhynchus mykiss* (NaCl 7.60 g; KCl 2.98 g; $CaCl_2$ 0.37 g; $MgCl_2 2H_2O$ 0.31 g; $NaHCO_3 6H_2O$ 0.21g / L), (2) isotonic solution for *O. mykiss* (NaCl 9.04 g; KCl

0.24 g; $CaCl_2$ 0.26 g/ L), (3) 66.6% artificial seawater, (NaCl 18.7 g; KCl 0.51 g; $CaCl_2$ 0.80 g; $MgCl_2$ 1.70 g; $MgSO_4$ 2.33 g; $NaHCO_3$ 0.15 g/ L) and (4) 0.11 M sodium citrate. Rates of sperm motility were shown as the percentages of advancing sperm in the all moving sperm under microscope(× 200). The observation of sperm motility was conducted once every 10 min for 50 min after dilution.

Egg quality

The egg quality was examined during seed production. It was estimated by the condition of stripped eggs which was gel-like or dumpling-like, and by the location of oil droplets in eggs, also by the dispersion or maldistribution and by the diameter size and by the number. Then the oil droplets of stripped eggs were examined and the stripped eggs were artificially fertilized by the sperm diluted with the 66.6% artificial seawater. They were adhered to the slide glass. When the adhered eggs were seen, all of the fertilized eggs on slide glass were counted. Then they were put in a cage made of nylon, incubated in 13℃ artificial seawater. To determine eyeing rates, the percentages based on the number of eyed eggs divided by all of the adhered fertilized eggs were measured after 10 days.

RESULTS

Sperm dilution

Percent of sperm motility in the artificial seminal plasma showed that it was 64% at the start, but 20 and 40 min later it decreased to between 30 and 40%; and 10, 30 and 50 min later it was from 70 to 80%. The isotonic solution showed that it was 67% at the start, which was almost the same as the artificial seminal plasma, but 40 min later it moved up higher between 80 and 90%, 50 min later it went down to 54%. The two-thirds artificial seawater that used in artificial fertilization showed that it was a low 21% at the start, and 20 min later it was between 20 and 30%, but 30 min later it was 11% and 40 min later 5% and 50 min later no moving sperm were observed. The 0.11 M sodium citrate solution showed that no moving sperm were observed from the start, and 40 min later no sperm were revived when the diluted sperm solution was placed in the two-thirds artificial seawater (Table 1). Therefore, according to sperm dilution, it was found that the artificial seminal plasma or the isotonic solution were better than the two-thirds artificial seawater which have been used to dilute sperm.

Egg quality

The author classified egg quality into four grades, which were judged by the number, size and condition of oil droplets and the existence of small eggs, such as immature, mature, overmature and abnormal eggs. Regarding the immature egg, the oil droplets, 0.01 to 0.09 mm, were at dispersed innumerably. The nature of stripped eggs was dumpling-like and the eggs which had been spread on the net failed to stick to the net and the eyed eggs were failed to be obtained. Regarding the mature eggs, the oil droplets maldistributed and there were sizes from small to large and the diameter was from 0.02 to 0.2 mm and their numbers reached more than 4. The nature of stripped eggs was gel-like. The eggs which had been spread on the net stuck to the net and the eyeing rates showed between 20 to 80%. Regarding the overmature eggs, the oil droplets maldistributed and their diameter was from 0.2 to 0.4 mm; their number was only 1 to 3, and the nature of stripped eggs was gel-like. The eggs that had been spread on the net stuck to the net, but the eyeing rates showed from 0 to 2%. Regarding the abnormal eggs, the oil droplets either dispersed or maldistributed and the diameter size and the number of them were the same as mature and overmature eggs. Although the regular eggs had only the diameter of roughly 1.7 mm, abnormal eggs had small eggs that had the diameter of 0.3 to 0.5 mm. The nature of stripped eggs was dumpling-like. The eggs that had been spread on the net failed to stick to the net and the eyed eggs were not obtained (Table 2). Therefore when the nature of stripped

Table 1. Changes of sperm motility diluted with some solution

Elapsed time (min.)	Artificial seminal plasma for *O. mykiss* (%)	Isotonic solution for *O.mykiss* (%)	※Artificial seawater (%)	0.11M Sodium citrate (%)
0	63. 6	66. 7	20. 6	0
10	76. 8	88. 4	21. 7	0
20	37. 5	81. 5	24. 6	0
30	71. 7	80. 3	11. 3	0
40	38. 6	83. 7	4. 8	★
50	70. 2	54. 1	0	

※ The composition is NaCl 18. 7 g; KCl 0. 51 g; $CaCl_2$ 0. 80 g; $MgCl_2$ 1. 70 g; $MgSO_4$ 2. 33 g; $NaHCO_3$ 0. 15 g/L

★ No motility of sperm in the 66. 6% artificial seawater

Table 2. Influences of egg quality on eyeing rates

Egg maturation	Oil droplets in eggs			Egg quality on stripping	Eyeing rates (%)
	Location	Size in diameter (mm)	Number		
Immature	Dispersion	0.01 - 0.09	Many	Dumpling	0♯
Mature	maldistribution	0.02 - 0.2	4 or more	Gel	20-80
Overmature	maldistribution	0.2 - 0.4	1-3	Gel	0-2
Abnormal※	Dispersion or maldistribution	0.02 - 0.4	1 or more	Dumpling	0♯

※ Including small eggs whose diameter range 0.3-0.5 mm
♯ Failed to stick to a net

eggs was dumpling-like, it was found that eyed eggs were not obtainable. Even if the nature of stripped eggs were gel-like, it is necessary to investigate the location of the oil droplets.

DISCUSSION

Sperm dilution

Although the sperm are not active during dilution, it is necessary to revive sperm motility on fertilization. But in this experiment, these solutions were not found. Therefore we would like to analyze the ionic composition to make the solution of sperm dilution that will be used in artificial fertilization.

Egg quality

Good parental fish are essential for seed production and egg collection. But although wild fish has been thought best, the cultured fish has to be used because the number of wild fish has decreased. Because the quality of eggs stripped from the cultured parental fish has not been good, it is deduced that eyeing rates and survival rates of larvae become low. The study on the relationship between the nature of oil droplets and eyeing rates on rainbow trout showed that eggs that have the dispersed oil-droplets come to the highest eyeing rates. But eggs that have many oil-droplets of maldistribution yielded the highest eyeing rates for four spine sculpin. It will be necessary to subdivide the egg-maturation further because the range of eyeing rates that were obtained from the mature eggs in this examination was large.

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RELATIONSHIPS BETWEEN GROWTH AND SMOLTIFICATION IN AMAGO SALMON *ONCORHYNCHUS MASOU ISHIKAWAE* (BASIC STUDIES TOWARDS ESTABLISHING A PARR STRAIN)

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ABSTRACT

In order to clarify the relationships between individual growth and smoltification, two kinds of clone of amago salmon *Oncorhynchus masou ishikawae* group were used. In Clone1-group, regardless of feeding condition, fish larger than 12 g on September 30 became smolt in the following winter. After this time, the specific growth rate of potential smolt was getting higher than that of parr. From this result, It was considered on Clone1-group that the timing to initiate smoltification was around September 30 and fish larger than about 12 g would be candidates of smolt. In Clone3-group, the timing to initiate smoltification was around August 29 and the critical size was about 4 g. Therefore, the body weight (critical size) seemed to be one of the factors influencing phase differentiation in each clone which might start form late August through late September. The differentiation was not influenced by the specific growth rate of parr or potential smolt.

In addition, in order to obtain the marketable size of parr, we tried the breeding of a parr strain by gynogenesis. We selected larger parr from the fish groups and reared these fish to the adult stage. And then, we produced the next generation of each individual by gynogenesis. We produced three generations by the gynogenesis , using the fish lot in which the larger size of parr appeared. Finally we have succeeded in establishing a strain which contained a high ratio of marketable parr.

INTRODUCTION

Amago salmon, *Oncorhynchus masou ishikawae*, is one of the most popular freshwater farming fish in Japan growing up to 50-100g (marketable size) in about one year from hatching. The remarkable characteristics of amago salmon are parr-marks and red spots on their body surface. Fish farmers do prefer parr type fish, which has clear parr-marks and better market acceptance rather than smolt type, which shows silver color on the body surface.

The growth rate between smolt and parr types is different. Smolt type grows faster than parr type. It is well known that smoltification ratio depends on growth conditions, such as feeding and temperature, and is influenced by genetic factors, being different between distinct strains. Previous experiments conducted by us had indicated that the smoltification process takes place much earlier in life history, however, its visual differentiation can only be done by winter, when fish is around twelve months old. It is still unknown how much body weight or growth rate of fish (originated from different strains), at a particular time, would influence the smoltification. In order to investigate these aspects we have examined groups of cloned fishes from two different strains.

MATERIALS AND METHODS

Experiment 1: Relationships between smoltification and individual body weight or growth rate before the smolting period

This experiment was conducted at Gifu Prefectural Fisheries Research Institute in1994. We used two kinds of clone fish groups, Clone1 and Clone3, composed by 200 individuals each. Both clones are the result of a third generation of homozygous gynogenetic strains, produced according to Kobayashi *et al.*, 1994. An all-female group, used as control, was inseminated by a sex-reversal male, following Usuda (1989) protocol. This group was proved

to exhibit genetic variation by fingerprintings analysis (data not shown).

We marked all individuals by inserting a pit-tag into the abdomen of each fish. Three groups (Clone1, 3, all-female fish) were mixed and then separated into the two groups. One group was fed abundantly with commercial compound diet, and the other group was fed a half amount of that. We reared the fish in an outdoor pond under the natural day-length condition by using river water. We began the experiment in August, and we measured body weight of each individual fish monthly. In December and January (smoltification period), all fish were clarified into smolt or parr, the former also included mid-smolt (Kubo, 1974). And then, the relationships between smoltification and individual body weight or specific growth rate were examined. Specific growth rate during sampling intervals was calculated as $\{\ln(W_2) - \ln(W_1)\} \times 100/d$, in which W_2 was the individual weight (g) at the end of the interval period, W_1 was the individual weight (g) at the beginning of the interval period, and d meant days of the interval.

Experiment 2: the breeding of parr strain

We began the breeding of the parr strain from 1991, using the fish group containing a large parr. This group was introduced from a fish farmer at Gero Town in Gifu Prefecture. Large parr were selected in winter, and the first generation was produced on each individual large parr by the gynogenesis with the suppression of the second maturation division, according to Usuda's method (Usuda, 1989). In the following winter, the smoltification was checked, and large parr were selected from a particular lot. Subusequently, the alteration of generations were repeated in the large parr group two times by gynogenesis.

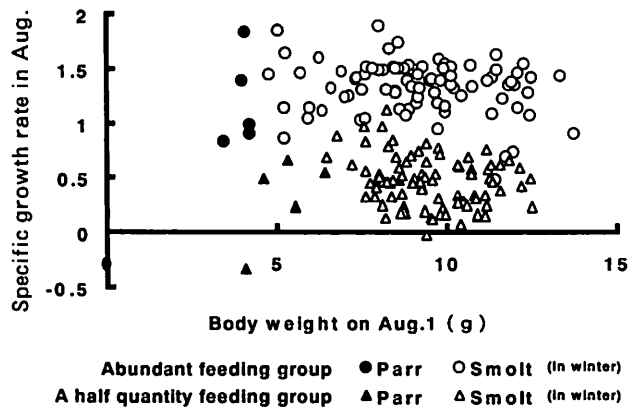

Figure 1. The relationships between smoltification in winter and the individual body weight on August 1 or specific growth rate in August in Clone 1.

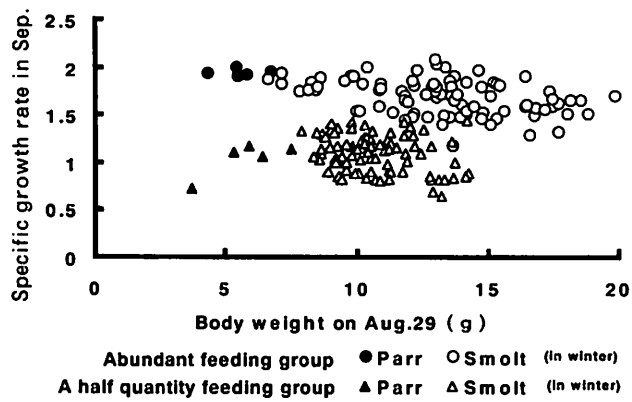

Figure 2. The relationships between smoltification in winter and the individual body weight on August 29 or specific growth rate in September in Clone 1.

RESULTS AND DISCUSSION

Experiment 1

Figure 1 shows the relationships between smoltification and individual body weight on August 1 or the specific growth rate in August in Clone1. In the abundant feeding group, fish larger than 5 g on August 1 became smolt in the following winter, and the boundary in body weight between parr and potential smolt was very clear. This tendency was also recognized in the half quantity feeding group, but the boundary was 7 g. This value was larger than that of the abundant feeding group. The difference in the specific growth rate in August between parr and potential smolt was not recognized in both feeding groups.

Figure 2 shows the relationships between smoltification and the individual body weight on August 29 or the specific growth rate in September in Clone 1. The tendency of the relationships was the same as that in August. As the boundary in body weight between parr and potential smolt was 7 g in the abundant feeding group, and 8 g in the half quantity feeding group, the difference between both groups became smaller.

Figure 3 shows the relationships between smoltification and the individual body weight on September 30 or the specific growth rate in October in Clone1. On September 30, the body weight boundary between parr and potential smolt became about the same value (12 g) in both feeding groups. This result suggests that regardless of feeding conditions, fish larger than 12 g on September 30 become smolt. After this time, the specific growth rate of potential smolt tended to be getting higher than that of parr.

Figure 4 shows the relationships between smoltification and the individual body weight on October 31 or the specific growth rate in November in Clone1. The difference of the specific growth rate in November between parr and potential smolt was very clear in both feeding groups.

From the above results, it is considered that the timing

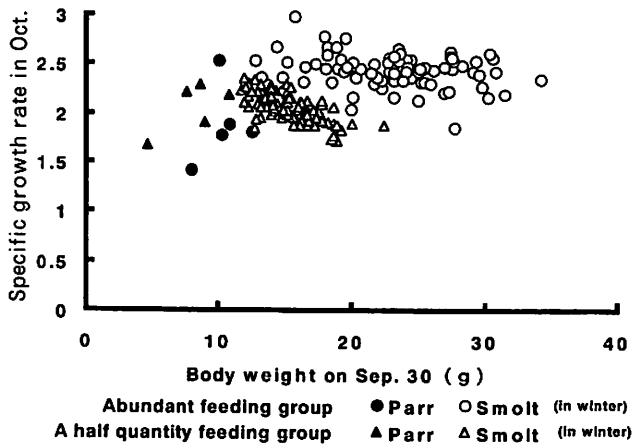

Figure 3. The relationships between smoltification in winter and the individual body weight on September 30 or Specific growth rate in October in Clone 1.

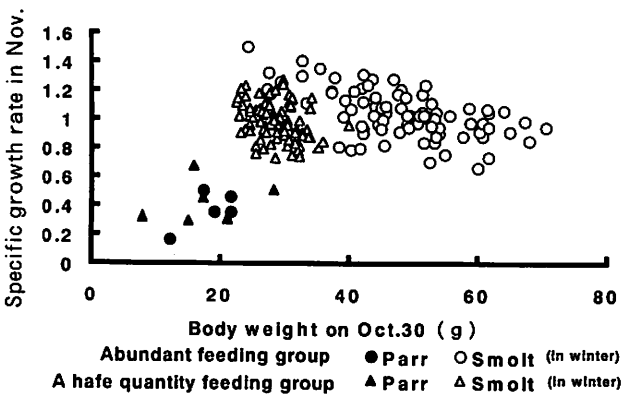

Figure 4. The relationships between smoltification in winter and the individual body weight on October31 or specific growth rate in November in Clone 1.

to initiate the smoltification is around September 30, and fish larger than about 12 g at the time are candidates to be smolt in Clone1.

Table 1 shows the minimum body weight of potential smolt on each measuring day. These values of body weight indicate the boundary between parr and potential smolt. It seemed that in Clone3, its boundary of both feeding groups became about the same value (4 g) on August 29. However, the body weight of parr and potential smolt of all-female fish group was overlapped, and the boundary was obscure (Figure 5). This is considered to be caused by genetic variations of all-female fish. These results suggest that the timing to initiate the smoltification and the body size to determin the phase differentiation are influenced by genetic factors.

Judging from the above results, even if the feeding conditions are changed as seen in this experiment in the amago salmon, the timing to initiate the smoltification

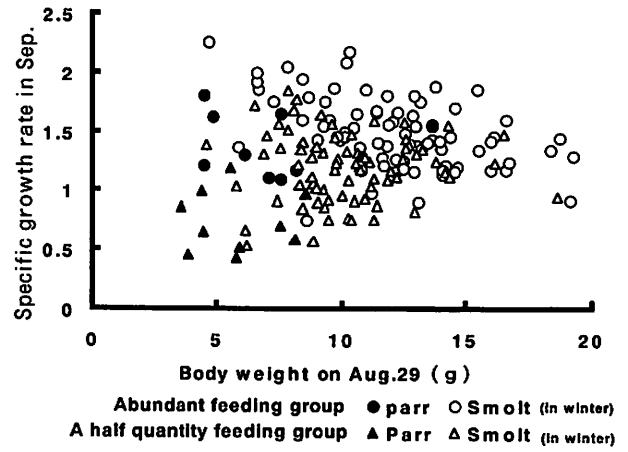

Figure 5. The relationships between smoltification in winter and the individual body weight on August 29 or specific growth rate in September in the all-female fish group.

exists from August through September. Without regard to the specific growth rate of parr or potential smolt, fish larger than the critical size at a particular time transformed to smolt, and the critical size changed by the difference in genetic factors. After initiate of the smoltification, the specific growth rate of potential smolt became higher than that of parr. As a result of these phenomena, it was probably observed that the ratio of smolt was higher when the growth of fish group was better (Tashiro *et al.*, 1983 a,b). Thus the body weight frequency-distribution changes from one mode to two modes of parr and smolt (Kuwada, 1995), and the minimum body size of smolt and the ratio of smolt are different among fish stocks (Kumazaki and Tashiro, 1987; Goto, 1997). In masu salmon, it is thought that the growth before winter has a relation with the ratio of smoltification in spring (Hirata *et al.*, 1988). The growth before smoltification in amago salmon is so important as well as in masu salmon.

The critical size at a particular time may be also an important factor for the transformation from parr to smolt. This suggests that we should pay attention to the critical size influencing smoltification, if in the case of the breeding of marketable parr type fish.

Experiment 2

Figure 6 shows relationships between the phase differentiation and the composition of body weight in each generation of Gero4 strain. By repeating the selection, the ratio of parr became higher, and the small size of smolt disappeared in the strain. In the third generation, the marketable size of fish (50-100g) were almost parr, though a small part of the large fish group over about 80 g was smolt. Thus, we succeeded in establishing a strain which contained a large number of marketable size of parr.

Table 1. The minimum body weight of potential smolt on each measuring day

Experimental group	Feeding conditions	August 1	August 29	September 30	October 31	December 2
		g	g	g	g	g
Clone1	Abundant	4.78	6.63	12.07	24.6	39.68
	A half quantity	6.49	7.88	11.69	22.51	31.18
Clone3	Abundant	3.5	4.05	6.44	13.67	22.57
	A half quantity	4.08	4.17	5.35	10.99	16.24

However, this strain has never been examined under the different environmental condition from that of the Gifu Pref. Fish. Res. Ins. Therefore, in order to put this strain to the practical use, we intend to try a rearing experiment of this strain at some fish farms.

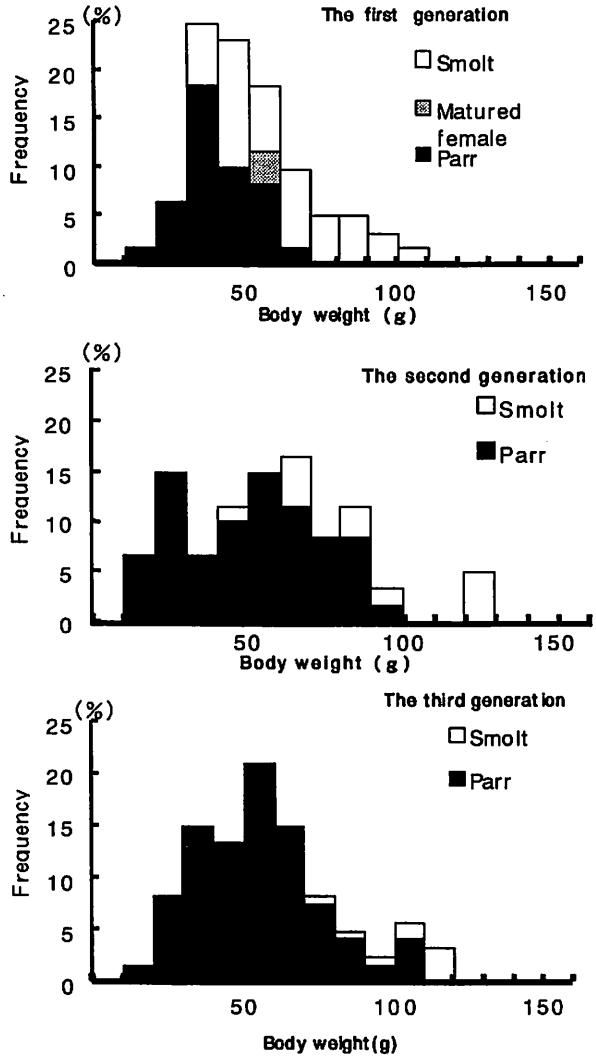

Figure 6. The relationships between phase differentiation and the composition of body weight in each generation of the Gero4 strain.

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SEX CONTROL IN HONMOROKO *GNATHOPOGON CAERULESCENS* AND NIGOROBUNA *CARASSIUS CARASSIUS GRANDOCULIS*

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ABSTRACT

We attempted monosex female production in honmoroko *Gnathopogon caerulescens* and nigorobuna *Carassius carassius grandoculis* by the combination of gynogenetic procedures and sex reversal. Gynogenetic diploid honmoroko and nigorobuna were induced by a 40-min cold shock (0 ℃) or 1-min heat shock (40℃). In almost groups of gynogenetic diploids, female ratios were significantly higher than those of controls, but various proportions of gynogenetic males appeared. A portion of these males produced only females as F_1 progeny, but the other males produced males in various proportions. High water temperature during the larval developments affected the sex differentiation and induced sex reversal from genetic female to phenotypic male. Hormone treatment with 17-methyltestosterone also could induce the sex reversal from genetic female to phenotypic male, and the optimum concentration for the treatment was at 0.1 μg/L water. Using those sex-reversed males, we produced successfully all female progeny in nigorobuna at a commercial scale, but 27% of males included progeny were produced in honmoroko. At present, we aim to establish a 100% all-female honmoroko production from inbred lines based on the application of a cloned fish approach.

INTRODUCTION

Honmoroko *Gnathopogon caerulescens* and nigorobuna *Carassius carassius grandoculis* are cyprinid fishes, endemic to Lake Biwa in Honshu Island, Japan. Honmoroko mature 1 year after hatching and die at between 2 and 5 years of age (Nakamura 1954, Maki 1966). Nigorobuna begin to spawn from the 2nd spring after birth and live for more than 10 years. These species are economically important fisheries resources in the lake and have been materials used for the traditional dishes around the lake. People prefer the mature females with developed eggs for these dishes. Although the necessity for appropriate stock maintenance has long been emphasized, the natural populations have decreased drastically in the last 10 years. In order to enhance the stocks, a million-order release of the juveniles in those species has been carried out in the lake.

Recently, many investigators have noted that artificial gynogenesis is useful for rapid establishment of inbred lines as well as for production of monosexual populations, since gynogenesis results in progeny with an all-maternal inheritance without genetic contributions of spermatozoa. These techniques are relatively simple and generally employ ultraviolet-ray (UV) irradiation to genetically inactivate sperm and physical shock treatment for duplication of the haploid set of egg chromosomes. We have elucidated the conditions for production of all female progeny of these species by using gynogenetic procedures and sex-reversed males in order to improve artificial propagation and aquaculture on honmoroko and nigorobuna (Fujioka 1993a,b, 1997, 1998, Fujioka *et al.* 1995).

In this paper, we introduce and summarize some conditions of gynogenetic procedures and several characteristics of gynogenetic diploids in honmoroko and nigorobuna, and also describe the present status of the sex control in these fishes.

PRODUCTION OF THE GYNOGENETIC DIPLOIDS

The breeding season of honmoroko coincides with that of nigorobuna. If UV-irradiated sperm of honmoroko is inseminated with the eggs of this fish, there is a possibility of male occurrence due to incompletely UV-treated sperm. To prevent this, we used the UV-irradiated sperm of nigorobuna to activate the development of eggs of honmoroko for gynogenesis and alternately the sperm of honmoroko to activate the eggs of nigorobuna. The sperm of both species were diluted 100 times with physiological

Table 1. Conditions for the production of gynogenetic diploids in honmoroko and nigorobuna * (Ueno 1992, Fujioka 1993a, Nemoto 1994, Fujioka 1997)

	Honmoroko	Nigorobuna
UV dose for inactivation of sperm	2000-3000 erg/mm^2	2000-3000 erg/mm^2
Duration of cold shock (0℃)	40 min	30-50 min
Duration of heat shock (40 or 41℃)	60 sec.	60-90 sec.
The best timing to start the shock G2 *1	5-7 min	8-9 min
The best timing to start the shock G1 *2	30-40 min	40-50 min

* Eggs were incubated at 20℃
*1 Prevention of 2nd meiotic division.
*2 Prevention of 1st mitotic division.

saline solution (7.5 g NaCl, 2.0 g KCl, 2.0 g $CaCl_2$, 0.2 g $NaHCO_3$, 1000 ml distilled water, adjusted to pH 7.0). The conditions for the production of gynogenetic diploids in honmoroko and nigorobuna are summarized in Table 1. A UV dose of 2000-3000 erg/mm^2 was necessary to genetically inactivate the sperm of both species. The highest yield of gynogenetic diploids caused by prevention of the 2nd meiosis (G2) or 1st mitosis (G1) was produced after 40-50 min duration of cold-shock (CS ; 0℃) or 60-90 sec duration of heat-shock (HS; 40 or 41℃). When eggs are incubated at 20℃, the best timing to start the shock-treatment for preventing the 2nd meiotic division was 5 to 7 min and 8 to 9 min after insemination in honmoroko and in nigorobuna, respectively. The egg nucleus advanced to the anaphase at this time (Figure 1A), and the shock induced the chromosome duplication caused by the prevention of formation of the second polar body (Figure 1B). To prevent the 1st mitotic division was around 40 min after insemination in both species. By the use of these procedures, gynogenetic diploids were normally obtained in 3-30% for G2 and 0.1-5% for G1.

PERFORMANCE OF THE GYNOGENETIC DIPLOIDS

Sex ratios of 10 gynogenetic groups of G2 and those controls from each single female in honmoroko are shown in Table 2. In gynogenetic diploids, female ratios were 61.8-100%, and 3 to 35% males appeared in eight groups. In only two groups, no males appeared. In most groups, the female ratio of gynogenetic diploids was significantly higher than that of the control, and the mean ratio was 87.2% and this value was twice that of the control. Sex ratios of the gynogenetic diploid nigorobuna from five different mothers are represented in Table 3. Female ratios significantly ($P<0.01$) increased in two groups, but in others several numbers of males appeared. In the families of mother no. 3 and 4, female ratios were 50% or less. These results suggest that the proportion of females in gynogenetic diploids differ in each maternal parent.

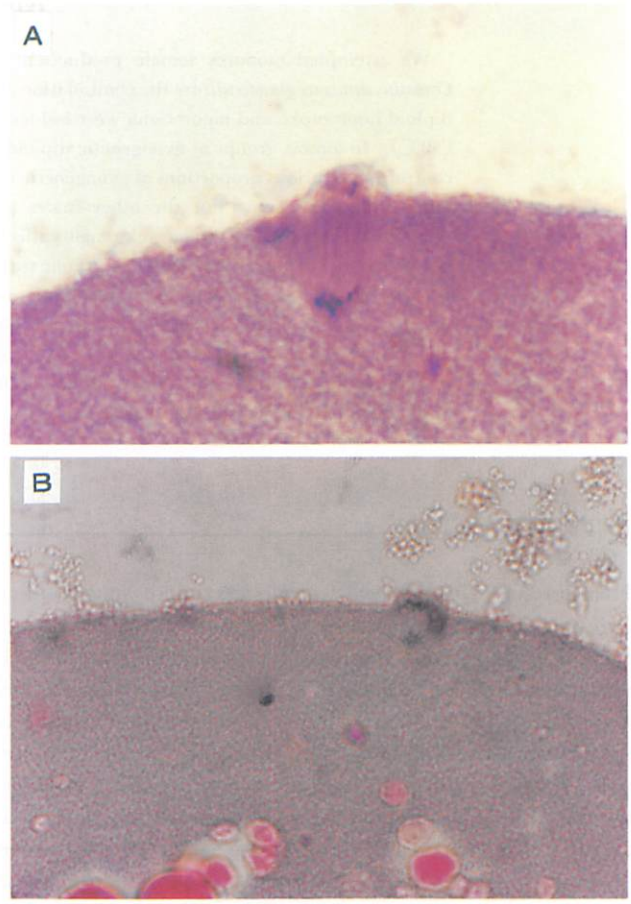

Figure1. Cytological observations on fertilization in the honmoroko. eggs inseminated with UV-irradiated nigorobuna sperm.
A : The egg nucleus 7 min after insemination.
B : The aperm head and prevented daughter chromosome sets of 2nd meiosis after the cold shock.

Table 4 shows sex ratios in the gynogenetic diploid honmoroko of G2 from four different females, and results in progeny tests of gynogenetic males, which appeared in these families, mated with normal females. When males from A, B and C were crossed with normal females, the sex ratios of their progeny were all or almost females. But in 5 different gynogenetic males from D, males were produced

Table 2. Sex ratios of control and gynogenetic diploids in honmoroko (Fujioka 1998)

Experiment No.	Control				Gynogenetic diploid			
	Female	Male	IU*	Female (%)	Female	Male	IU*	Female (%)
1	23	29	0	44.2[c]	19	1	0	95.0[a,d]
2	31	22	0	58.5[c]	8	1	0	88.9[b,f]
3	11	31	8	22.0[a]	23	6	1	76.7[c,d]
4	55	18	2	73.3[a]	21	1	0	95.5[a,c]
5	21	30	0	41.2[c]	34	0	1	97.1[a,d]
6	20	41	1	32.3[a]	9	1	1	81.8[c,d]
7	24	40	0	37.5[c]	21	10	3	61.8[b,d]
8	25	37	1	39.7[c]	64	2	0	97.0[a,d]
9					10	0	0	100[a]
10					18	4	1	78.3[b]
Total	210	248	12	44.7[c]	227	26	7	87.2[a,d]

* No. of fish with intersex and undifferentiated gonads.
[a,b] Significantly different from one female: one male at $P<0.01$ and $P<0.05$, respectively.
[c] Not significantly different from one female: one male ($P>0.05$).
[d,e] Significantly different from the sex ratio of individual control at $P<0.01$ and $P<0.05$, respectively.
[f] Not significantly different from the sex ratio of individual control ($P>0.05$).

Table 3. Sex ratios of gynogenetic diploids in nigorobuna (Fujioka 1997)

Mother no.	Female	Male	IU*	Female (%)
1	9	1	0	90.0[b]
2	10	0	0	100[a]
3	10	10	0	50.0[c]
4	9	14	1	37.5[c]
5	7	3	1	63.6[c]

* No. of fish with intersex and undifferentiated gonads.
[a,b] Significantly different from one female: one male at $P<0.01$ and $P<0.05$, respectively.
[c] Not significantly different from one female: one male ($P>0.05$).

in F_1 progeny in various proportions. Sex ratios in progenies between 1 gynogenetic male and 5 different normal females are shown in Table 5. The proportion of males in 4 families was lower than 19% and indicated approximately equal ratios. But the family from female NF-2 had about 50% males. These results suggested that some factors in the maternal parent can affect the sex ratio of their offspring occasionally, and also this agreed with the sex ratios observed in gynogenetic diploids depending on the maternal parents.

Responses of sex ratios to two constant temperatures among four families from 4 different mothers of nigorobuna are shown in Table 6. The fry were reared under constant low (19.0-21.0 ℃) and high (28.0-31.0 ℃) temperature during 60 days post-hatching. In the families from mother no. 2, the proportion of males in both gynogenetic and control diploid significantly ($P<0.01$) increased in high temperature than that in low temperature, but the proportion of males in gynogenetic diploid of the families from mother no. 1 did not increase in high temperature. In control diploids from mother no. 2-4, proportion of males in high temperature slightly increased than those in low temperature. In honmoroko, sex ratios were intensively affected by temperature during early development, too (Fujioka, unpublished). These results indicate that honmoroko and nigorobuna have an environmental and genetic basis for determining sex similar to the Atlantic silversides *Menidia menidia* reported by Conover and Kynard (1981) and to hirame flounder *Paralichthys olivaceus* mentioned by Yamamoto (1995).

PRODUCTION OF SEX-REVERSED MALES

In honmoroko, sex differentiation is known to be complete by 50th day (15 mm in standard length) after hatching (Fujioka, unpublished). When 17-methyltestosterone (17-MT) was administered to gynogenetic diploid honmoroko at 0.1-100 μg/L water (17 hr per day) for 30 days from 15th day after hatching, proportion of matured males significantly increased at a dose of 0.1 μg/L (Table 7). However, no males appeared at the concentration of more than 1.0 μg/L, 17-MT of 0.1 μg/L treated throughout a day for 50 days from 10th day after hatching could performed more complete masuculinization in room

Table 4. Sex ratios in gynogenetic diploids and in progeny test of gynogenetic males mated with normal females in honmoroko (Fujioka 1998).

Female	Gynogenetic diploids			Gynogenetic Males *	Progenies of gynogenetic males and normal females		
	Female	Male	Male (%)		Female	Male	Male (%)
A	32	1	3.0[a]	a-1	20	0	0[a]
B	33	2	5.7[a]	b-1	17	3	15.0[a]
C	4	6	60.0[c]	c-1	20	0	0[a]
				c-2	20	0	0[a]
				c-3	20	0	0[a]
				c-4	20	0	0[a]
D	29	5	14.7[a]	d-1	26	4	13.3[a]
				d-2	14	16	53.3[c]
				d-3	6	24	80.0[a]
				d-4	4	26	86.7[a]
				d-5	1	29	96.7[a]

* Gynogenetic males produced from each female.
[a] Significantly different from one female: one male at $P<0.01$.
[c] Not significantly different from one female: one male at $P>0.05$.

Table 5. Sex ratios in progenies between one gynogenetic male and five different normal females (Fujioka 1998).

Female no.	No. of initial fry	Survival (%)	Sex ratios		
			Female	Male	Male (%)
NF-1	150	75.3	68	12	15.0[a]
NF-2	150	93.3	41	39	48.8[c]
NF-3	150	94.0	72	8	10.0[a]
NF-4	150	58.0	71	9	11.3[a]
NF-5	150	96.7	65	15	18.8[a]

[a] Significantly different from one female: one male at $P<0.01$.
[c] Not significantly different from one female: one male at $P>0.05$.

Table 6. Sex ratios of gynogenetic diploids and control diploids reared at low and high temperature in nigorobuna (Fujioka 1997).

	Temperature	Sex	Mother no.				Total
			1	2	3	4	
Gynogenetic diploid	20±1℃	Male	0	0	1		1
		Female	20	8	9		37
	29.5±1.5℃	Male	1	8		1	10
		Female	19	7		4	30
Control diploid	20±1℃	male	1	6	19	23	49
		Female	25	6	12	6	49
	29.5±1.5℃	male	0	22	16	17	55
		Female	24	6	4	3	37

Table 7. Dosege of 17-methyltestosterone and appearance of mature male in honmoroko (Fujioka 1993b).

Dosage (μg/L)	No. of initial fish *	Survival at 11 months after hatching (%)	No. of fish observed	No. of mature males (%)
0	80	36.3	29	7 (24.1)
0.1	80	32.5	26	14[a] (53.8)
1	80	22.5	18	0[a]
10	80	17.5	14	0[a]
100	80	15.0	12	0[b]

* 15 days after hatching.
[a] Significantly different from the value of control ($P<0.05$).
[b] Not significant ($P>0.05$).

Table 8. Dosege of 17-methyltestosterone and appearance of mature male and female in nigorobuna (Fujioka *et al.* 1995).

Dosage (μg/L)	No. of initial fish *	Survival 2 years after hatching (%)	No. of mature male	No. of mature female (%)
0	30	53.3	2	14
1	30	30.0	7[a]	2

[a] Significantly different from the value of control.

temperature (about 24℃). On the other hand, at high temperature (about 28 ℃) the proportion of males in control and 0.01 μg/L were higher than those at room temperature (Figure 2).

Sex differentiation in nigorobuna was observed during 30-80 days after hatching (Fujioka, unpublished). In nigorobuna, administration of 17-MT for the sex reversal from female to male could be made at the dose of 1.0 μg/L (8 hr per day) for 60 days from 25 days after hatching at room temperature (about 24℃) (Table 8). Since these males produced almost of all female offspring, they were regarded as sex-reversed males. From these results, it is suggested that adequate concentrations of 17-MT are 0.1 μg/L in honmoroko and 1.0 μg/L in nigorobuna, and high temperature treatment can induce sex-reverse of genetic female to phenotypic male without hormone treatment.

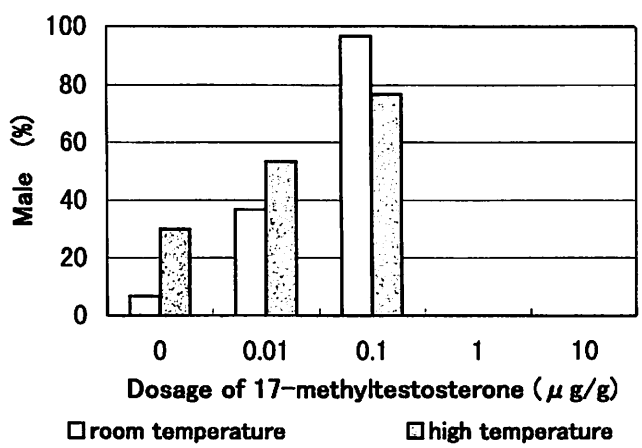

Figure. 2 Dosage of 17-methyltestosterone and proportion of males in each group reared at room and high temperature.

MONOSEX FEMALE PRODUCTION IN COMMERCIAL SCALE

Using possible sex-reversed males of honmoroko, three thousands of offspring were produced by normal females. They contained 73% females of which ratio was 34% higher than that of the control (Figure 3). The males, 27% of the monosex brood stock, were thought as sex-reversed males due to high temperature (25-29℃) during their early development. In nigorobuna, all female offspring were completely produced by sex-reversed males mating with normal females at room temperature (18-25 ℃) (Figure 4). The growth and survival of these all-female brood stocks showed almost the same as those in normal control. These sex-reversed males could be allowed to use for the production of all-female seedlings at a commercial scale under the controlled temperature during sex differentiating periods.

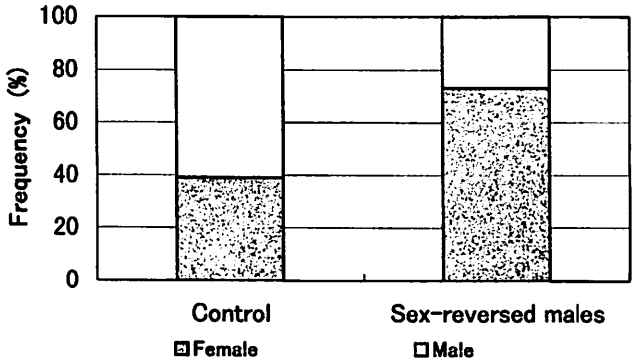

Figure. 3 Sex ratios of the broodstocks produced by normal and sex-reversed males in honmoroko on a commercial scale.

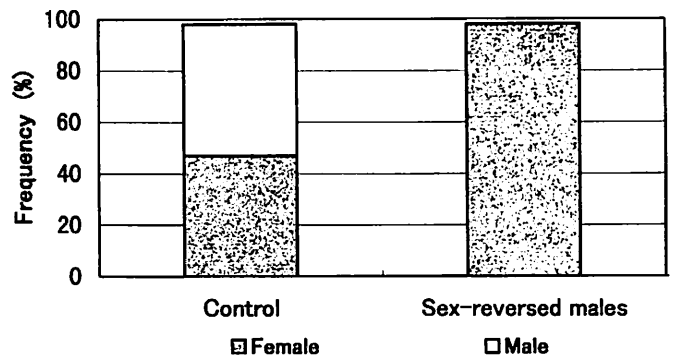

Figure. 4 Sex ratios of the broodstocks produced by normal and sex-reversed males in nigorobuna on a commercial scale.

CONCLUSION AND PROGRESS

The first object of monosex female production in honmoroko and nigorobuna has almost been achieved. In the course of these studies, we elucidated that the sex determining system is basically female homogamety in these species, but genetic components and environmental conditions such as high temperature during early stages affect sex ratio also. At present, we aim to establish a 100% female honmoroko production from inbred lines based on the application of a cloned fish approach.

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PRESENT STATE OF RED SEA BREAM *PAGRUS MAJOR* CULTURE AND BREEDING EXPERIMENTS IN NAGASAKI PREFECTURE

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ABSTRACT

Red sea bream *Pagrus major* is an important species for mariculture in Japan with a total production of 77 thousand tons in 1996. The present state of mariculture of the fish is greatly due to the advancement of seed production technology. In 1996, a total of 119 million juveniles were produced in Japan. Of these, 30 million juveniles were used for stocking and 89 million juveniles were used for mariculture seed. Nagasaki is one of the most productive prefectures in seed production and mariculture of the fish with a total production of 7.7 million juveniles and 9 thousand tons of harvest in 1996. Triploidy, gynogenesis and crossbreeding (red sea bream with crimson sea bream *Evynnis japonica*) were conducted at the Nagasaki Prefectural Institute of Fisheries in the 1980s. We could not produce valuable breeds for commercial mariculture, but characteristic phenotypes such as albinos were obtained through gynogenesis.

PRESENT STATE OF RED SEA BREAM CULTURE IN JAPAN

Mariculture

Red sea bream *Pagrus major* is distributed from the coastal waters around Japan to the East China Sea and Southeast Asian waters. The fish is called the "king of fish" in Japan. The brilliant red body color as well as the good taste of its white meat is a favorite of the Japanese. It is often used at various festive events such as weddings and new year's celebration. The fish grows to nearly 1 m in body length, although commercial size is 30～40 cm in body length and about 1 kg in body weight.

The annual wild catch of the fish has decreased, from about 20 thousand tons in the 1950-1960s to about 15 thousand tons in the 1980-1990s. On the other hand, the mariculture production has been increasing, from 450 tons in 1970 to 77 thousand tons in 1996. Since 1981 when the mariculture production surpassed the wild catch for the first time in Japan, the difference has been increasing year after year. The mariculture production of the fish (77.0 thousand tons) was about 5 times that of the wild catch (16.5 thousand tons) in 1996 (Figure 1) (Department of Statistics and Information 1998).

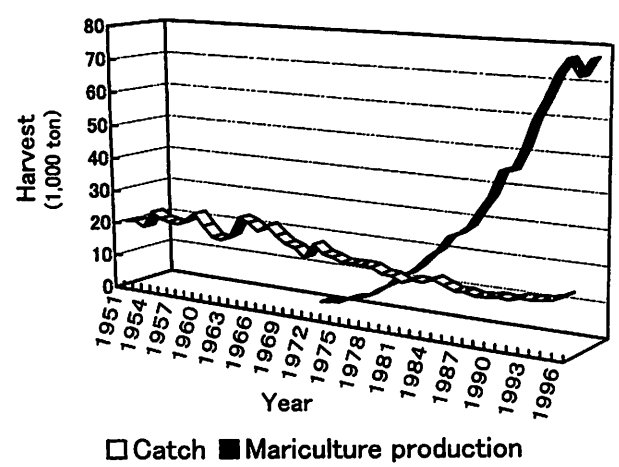

Figure 1. Wild catch and mariculture production of red sea bream in Japan from 1951 to 1996.

Seed production

The development of mariculture of the fish is greatly due to the advancement of seed production technology. Since 1962 when Yamashita (1963) produced 7 juveniles for the first time in Japan, the technology of seed production has been advancing steadily.

The mariculture production has been increasing in proportion to the increase in the number of produced

juveniles (Figure 2). In all, 119 million juveniles were produced in 1996. Of these, 89 million juveniles were used for mariculture and 30 million juveniles were used for stocking into coastal waters around Japan (Fisheries Agency and Japan Sea Farming Association 1998).

The growth of artificially produced juveniles of red sea bream has been improved through broodstock selection and other methods at Kinki University (Kato *et al.* 1998) and several commercial hatcheries. At the time when the wild-caught juveniles were used for mariculture seed, 3 to 4 years was necessary to reach the commercial size of 1 kg. Nowadays, the period has been shortened to 2 to 3 years by using artificially produced juveniles.

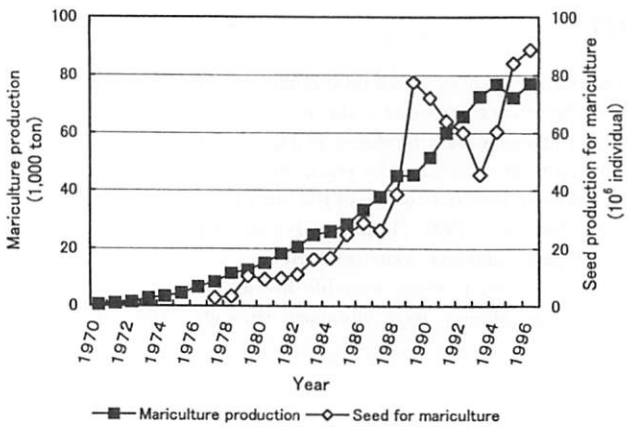

Figure 2. Artificially produced juveniles (seed) for mariculture and mariculture production of red sea bream from 1970 to 1996.

PRESENT STATE OF RED SEA BREAM CULTURE IN NAGASAKI PREFECTURE

In 1996, Nagasaki Prefecture ranked third in mariculture production and fifth in seed production of red sea bream in Japan. It produced 9.2 thousand tons of harvest by mariculture and 7.7 million juveniles by seed production.

Total output of mariculture in Nagasaki Prefecture was 47.5 billion yen in 1996. The components of maricultural output were 15.1 billion yen for yellowtail, 12.8 billion yen for pearl, 9.1 billion yen for red sea bream, 7.1 billion yen for other finfishes such as flatfish, puffer and striped jack, and 3.4 billion yen for prawn, shell, algae, and others. Thus, red sea bream culture yielded about 20 % of the total output of mariculture. It occupies an important position in mariculture in Nagasaki.

BREEDING EXPERIMENTS ON RED SEA BREAM IN NAGASAKI PREFECTURE

Triploidy

Most of 2-year-old red sea bream mature in spring and some of them start to spawn before they reach the commercial size of 1 kg (Kitajima 1978). Generally, fish growth is delayed during sexual maturation, so production of sterile fish is desired for mariculture, because it is expected that a proportion of the energy normally used in gonad growth may be diverted to flesh production. Triploidy has been of interest because triploid fish are supposed to be sterile. Therefore, the experiments to induce triploid red sea bream were conducted in 1986 and 1987 (Arakawa *et al.* 1987).

Figure 3 shows the method for triploid induction of red sea bream by second polar body retention in our experiments. The milt was collected in a dry syringe and the eggs were collected in a bowl. The eggs were inseminated with enough milt (about 0.2 ml for 1g eggs), and at 30 seconds after a little seawater was added to activate the sperm, and at 30 seconds after the eggs were washed with a lot of seawater. Inseminated eggs were subjected to 10 to 15 minutes cold treatment at 1 to 7 ℃ (usually 1 to 3 ℃), starting at 1.5 to 3.0 minutes after insemination. The induction rates of triploidy by this method were judged almost 100% by the comparison of the major axis of erythrocytes. The average length of erythrocytes of triploids (13.1 μ m) was greater than that of control diploids (10.2 μ m).

The development of gonads of triploid red sea bream was studied histologically during the spawning season (Kitamura *et al.* 1991). The gonadal development was classified into four stages : I , immature ; II , developing

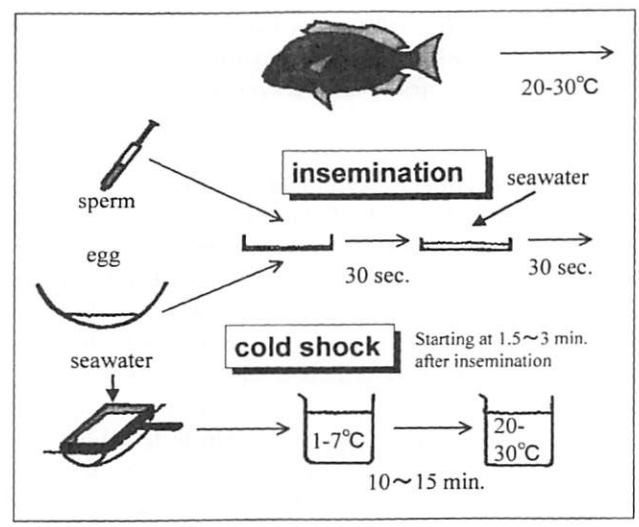

Figure 3. Method of insemination and cold shock treatment for triploid induction of red sea bream (from Arakawa *et al.* 1987).

immature or atretic ; Ⅲ, developing ; Ⅳ, mature, according to the criteria by Matsuyama *et al.* (1987) and Matsuura *et al.* (1987).

Table 1 shows the maturity stages of gonads of control diploid red sea bream. Both the ovaries and testes of the 2-year-old control fish reached the highest maturity stage Ⅳ, and most of the 3-year-old control fish were mature (stage Ⅲ or Ⅳ).

Table 2 shows the maturity stages of gonads of triploid red sea bream. There were no females in the triploids. Triploid red sea bream did not reach maturity stages Ⅲ or Ⅳ throughout the spawning season. Some of the 2-year-old and most of the 3-year-old triploid males reached maturity stage Ⅱ (developing immature), but there were some histological differences from those of the control fish. The testis of the triploid fish had spermatogonia, spermatocytes and spermatid-like cells, but spermatozoa was absent or few and abnormal. (This stage is defined as Ⅱ * in this table.) The rest of the triploid fish were completely immature (stage Ⅰ), and we histologically observed many macrophages in these testes, especially in the 2-year-old triploids produced in 1986.

Figure 4 shows the gonadosomatic index (GSI) of triploid and control male red sea bream. The GSI reached the highest value in April. The values of control males were 6 to 14. On the contrary, the GSI of triploid males was extremely low. The values were only about 1/5 of control fish.

Only a few 3-year-old triploid fish produced milt when stripped, but there were some spermatozoa with two heads and two or three flagella, and the density of these cells in the stripped fluid was very low. From these results, we concluded that the triploid red sea bream were sterile.

Figure 5 shows the growth of triploid and control red sea bream. The growth of the triploid fish produced in 1986 was the same as that of the control fish from beginning to 2 years old, but after that, it became slower than that of the control fish. The growth of the triploid fish produced in 1987 was similar to that of the control fish throughout the experiment period.

Triploid red sea bream are supposed to be sterile, but their growth was similar or inferior to that of the control fish and did not accelerate during spawning season. Therefore, we consider that the triploid red sea bream

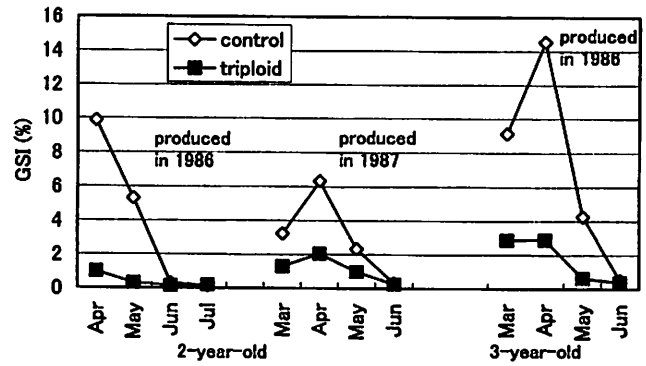

Figure 4. Gonadosomatic index (GSI) of triploid and control male red sea bream during spawning season (from Kitamura *et al.* 1991).
GSI = GW/{BW − (GW + VW)} × 100, where
BW is body weight ; GW is gonad weight ;
VW is visceral weight.

Table 1. Maturity stages of gonads of 2- and 3-year-old control red sea bream (from Kitamura *et al.* 1991)

Fish age	Month	Maturity stage			
		Ovary	Testis	Bisexual gonad	
				(Ovary) *	(Testis) *
2-year-old (produced in 1986)	Apr	Ⅳ (1)	Ⅳ (4)	−	−
	May	Ⅰ (4)	Ⅲ (1)	−	−
	Jun	Ⅰ (3)	Ⅰ (2)	Ⅰ (1)	−
	Jul	Ⅰ (3)	Ⅰ (3)	−	−
2-year-old (produced in 1987)	Mar	Ⅰ (1) Ⅲ (1)	Ⅳ (1)	−	Ⅳ (1)
	Apr	Ⅲ (1)	Ⅳ (4)	−	−
	May	Ⅰ (1)	Ⅳ (2)	−	Ⅳ (2)
	Jun	−	Ⅰ (4) Ⅳ (1)	−	−
3-year-old (produced in 1986)	Mar	Ⅰ (1) Ⅲ (1)	Ⅳ (3)	−	−
	Apr	Ⅱ (1) Ⅲ (1)	Ⅳ (3)	−	−
	May	Ⅰ (1) Ⅱ (1) Ⅲ (1)	Ⅰ (2) Ⅳ (3)	−	−
	Jun	Ⅰ (1)	Ⅰ (5) Ⅳ (2)	−	−

(Ovary) * : Ovary with an outer layer of testicular tissue
(Testis) * : Testis with rudimentary oocytes
Parentheses denote number of fish
Maturity stage is classified according to Matsuyama *et al.* (1987) and Matsuura *et al.* (1987)

Table 2. Maturity stages of gonads of 2- and 3-year-old triploid red sea bream (from Kitamura *et al.* 1991)

Fish age	Month	Maturity stage			
		Ovary	Testis	Bisexual gonad	
				(Ovary) *	(Testis) *
2-year-old (produced in 1986)	Apr	–	I (2) II * (3)	–	–
	May	–	I (5)	–	–
	Jun	–	I (4)	–	I (1)
	Jul	–	I (4)	–	I (1)
2-year-old (produced in 1987)	Mar	–	II * (5)	–	–
	Apr	–	II * (6)	–	–
	May	–	II * (5)	–	–
	Jun	–	I (2) II * (3)	–	–
3-year-old (produced in 1986)	Mar	–	II * (5)	–	–
	Apr	–	II * (5)	–	–
	May	–	I (2) II * (3)	–	–
	Jun	–	I (1) II * (4)	–	–

(Ovary) * : Ovary with an outer layer of testicular tissue
(Testis) * : Testis with rudimentary oocytes
Parentheses denote number of fish
Maturity stage is classified according to Matsuyama *et al.* (1987) and Matsuura *et al.* (1987)

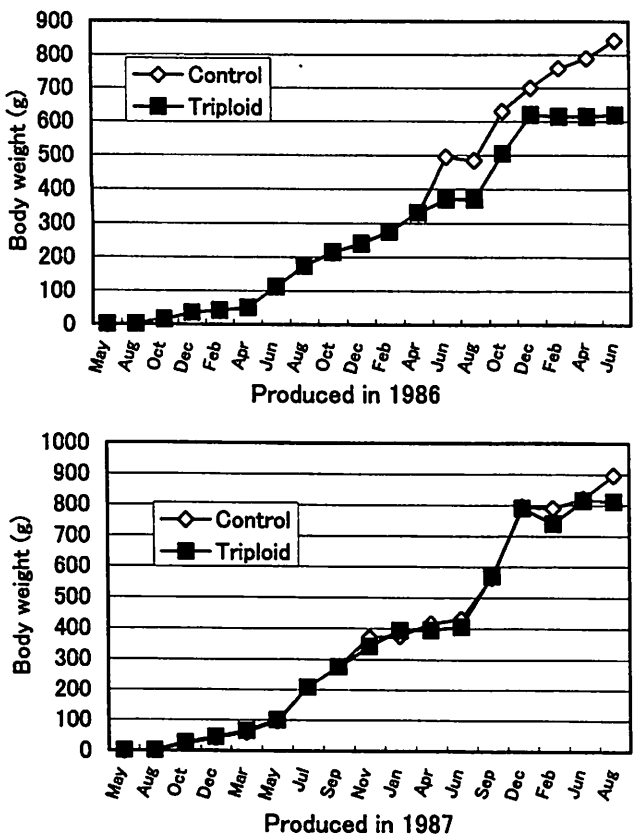

Figure 5. Growth of triploid and control red sea bream.

produced from our experiment are not valuable for commercial mariculture.

Gynogenesis

The body color of male red sea bream darkens during spawning season. Japanese prefer the red body color of red sea bream, so females are desirable for mariculture. Gynogenesis has been of interest because it may bring all females. Therefore, the experiments to induce gynogenetic diploid red sea bream were conducted in 1986 and 1987 (Arakawa and Miyahara 1988).

Milt of black or red sea breams was diluted with ringer solution for *Pleuronectes platessa* in a volume ratio of 1 : 50, and irradiated with a sterilizing lamp at an intensity of 1,000~1,500 erg/mm^2. Eggs were inseminated with the irradiated milt, and subjected to cold treatment. The condition of cold treatment was the same as the triploid induction. The induction rate of gynogenetic diploid red sea bream produced in 1987 inseminated with UV-irradiated black sea bream milt was judged 100% by isozyme expression (Sugama *et al.* 1988).

Table 3 shows the maturity stages of gynogenetic diploid red sea bream. Gynogenetic diploid red sea bream produced from our experiments were not all females, contrary to our expectation. Both ovary and testis developed to the highest maturity stage IV.

We could not produce all female red sea bream by gynogenesis. However, albinos of red sea bream were induced through gynogenesis in 1986 (photo 1). They have white or cream body color and red eyes. The body color of the albinos does not turn dark even when they were cultured under direct sunbeam. The black line, which appears in the flesh of cultured fish thus reducing the value of flesh especially when we eat it as sashimi, was not apparent in the flesh of the albinos (photo 2). The body color of some albinos became pink when they were fed a

Table 3. Maturity stages of gonads of 2- and 3-year-old gynogenetic diploid red sea bream

Fish age	Month	Maturity stage			
		Ovary	Testis	Bisexual gonad	
				(Ovary) *	(Testis) *
2-year-old (produced in 1986)	Apr	I (3) III (1)	–	I (1)	–
	May	I (1)	I (1)	I (1)	I (1) II (1)
	Jun	I (2)	I (1)	–	I (2)
	Jul	I (3)	–	I (1)	I (1)
2-year-old (produced in 1987)	Mar	III (4)	–	–	IV (1)
	Apr	III (1) IV (1)	I (1)	–	IV (3)
	May	I (2) IV (3)	–	–	–
	Jun	I (3)	I (1)	–	I (1)
3-year-old (produced in 1986)	Mar	III (1)	I (2) II (1) IV (1)	–	–
	Apr	III (3)	IV (2)	–	–
	May	I (1) III (1)	IV (2)	–	I (1)
	Jun	I (3)	I (1)	–	I (1)

(Ovary) * : Ovary with an outer layer of testicular tissue
(Testis) * : Testis with rudimentary oocytes
Parentheses denote number of fish
Maturity stage is classified according to Matsuyama *et al.* (1987) and Matsuura *et al.* (1987)

Photo 1. Albino of red sea bream.

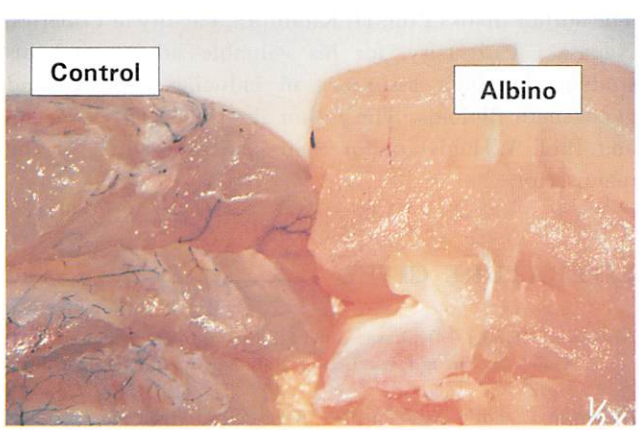

Photo 2. Flesh of albino and control red sea bream.

carotenoid-containing diet (photo 3).

Today, the albino red sea bream is not used for mariculture because the growth and survival were lower than those of normal fish. Commercial use of the albino

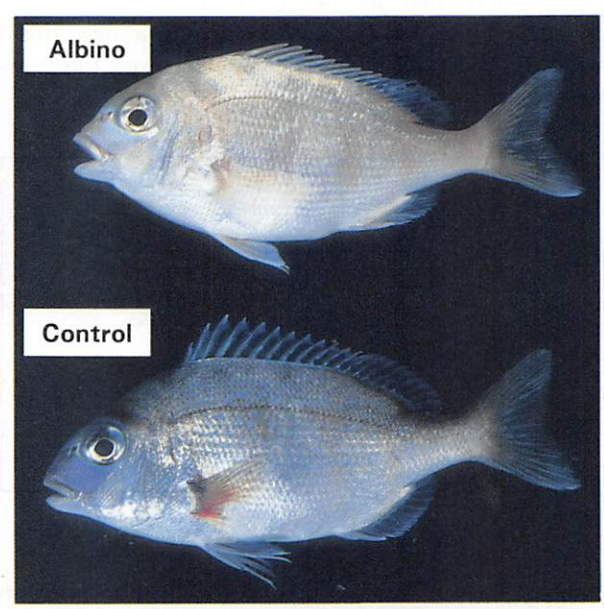

Photo 3. Albino and control red sea bream fed carotenoid-containing diet.

red sea bream is still for future study, especially in the possibility for utilizing them as breeding material.

Crossbreeding

The body color of wild red sea bream is brilliant red, but turns dark when cultured under direct sunbeam. Therefore, to improve the body color of the cultured fish, crossbreeding with crimson sea bream *Evynnis japonica* was conducted in 1985, expecting to produce a new breed with a bright red body color like the crimson sea bream and

good growth like the red sea bream (Arakawa and Yoshida 1986). The crossbred was reared for 22 months and the growth and body color was compared with those of the control red sea bream (Arakawa *et al.* 1988). At the end of this experiment, average body weight of the crossbred (239 g) became less than that of the control fish (273 g) (Figure 6).

Figure 7 shows the body color classification of the 22-month-old crossbred and the control red sea bream. Body color was classified into 4 classes (bright red, red, reddish-black, blackish). Almost all red sea bream were classified into reddish black (20%) or blackish (76%), while 32 % of the crossbred were classified into bright red and 38 % into red.

Figure 8 shows the distribution of body weight of the crossbred classified by body color. The body weight of the crossbred differed in body color. The lighter group had bright red or red body color; the heavier group was almost reddish-black.

These results show that the crossbred could not be used directly for mariculture, but there were some crossbreds with red body color and heavy weight (Figure 8). This may suggest the possibility of producing a new breed, having red body color and good growth, from this crossbred through selective breeding.

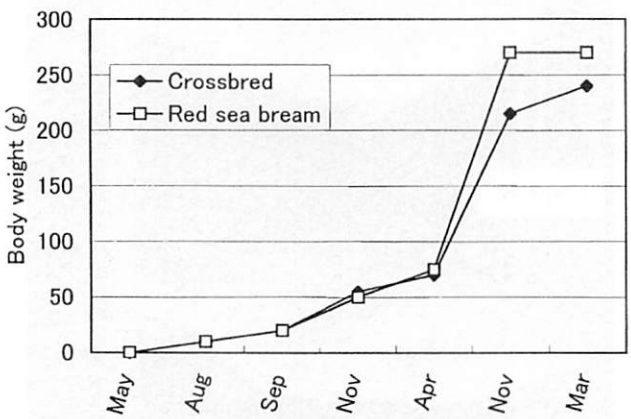

Figure 6. Growth of crossbred (red sea bream with crimson sea bream) and control red sea bream (from Arakawa *et al.* 1988).

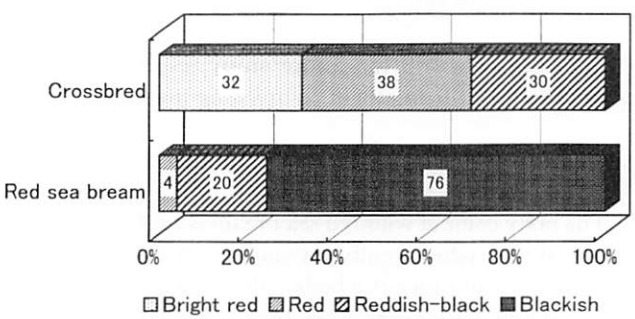

Figure 7. Ratio of body color of 22-month-old crossbred (red sea bream with crimson sea bream) and control red sea bream.

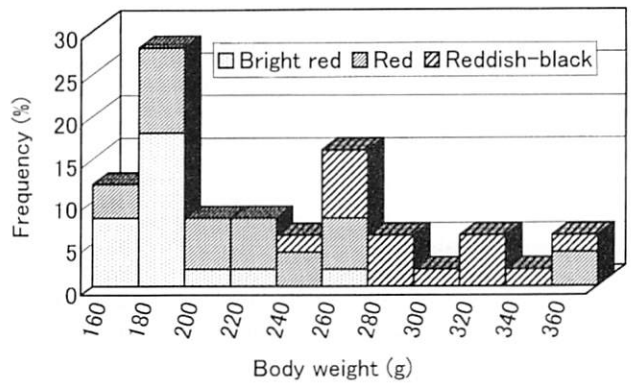

Figure 8. Distribution of body weight of 22-month-old crossbred (red sea bream with crimson sea bream) classified by body color (from Arakawa *et al.* 1988).

CONCLUSION

We could not produce the valuable breeds for commercial mariculture through our chromosome manipulation and crossbreeding. However, some individuals with characteristic phenotype, for instance the albino and the crossbred with red body color and good growth, were obtained through these experiments.

We consider that chromosome manipulation and crossbreeding are useful technologies to produce a new breed, especially when they are combined with other breeding technologies, for instance selective breeding.

ACKNOWLEDGEMENT

The author thanks Prof. H. Kitamura, Faculty of Fisheries, Nagasaki University, for his valuable advice and his students for their assistance in inducing triploids and gynogenetic diploids. The author also thanks Dr. T. Yotsui and Prof. A. Hagiwara for their critical reading of the manuscript.

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COHO SALMON BROODSTOCK DEVELOPMENT－ 1977 TO 1998

Ten Generations of Systematic Selective Breeding

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ABSTRACT

The Domsea coho salmon broodstock program, since its inception in 1977, has relied on a systematic selective breeding protocol to enact genetic improvements in production traits of coho salmon *Oncorhynchus kisutch*. The protocol has involved establishment of pedigree lines or families coupled with intensive between- and within-family selection and a rotational mating scheme among selected individuals. The protocol has remained essentially unchanged over the past 20 years. Since these broodstock mature on a 2-year cycle, the broodstock have undergone 10 generations of intensive selective breeding. In this report, we focus on the effects that the selection program may have had on the stock. We will report estimates of inbreeding based on pedigree analysis, genetic changes reflected by allozyme data, and overall effects on viability and fitness of the broodstock. Preliminary analysis indicates that traits related to reproductive fitness (embryonic and juvenile survival and growth), which are normally sensitive to inbreeding, have not undergone any decline. In fact, improvements in growth and survival due to selection gains and/or improvements in husbandry have not only compensated for any inbreeding effects, but resulted in an overall improvement in these economically important traits.

INTRODUCTION

Genetic selection programs represent a balance between the accumulation of "positive" alleles for quantitative traits and the avoidance of the accumulation of deleterious recessive alleles. Strong selection pressure can often only be accomplished at the risk of high levels of inbreeding. Furthermore, most breeding programas involve "closed" populations (i. e., migration into the population is eliminated) having a restricted breeding population size. Concerns of fish and shellfish breeders about inbreeding depression are generally based on estimates derived from a single generation of intensive inbreeding (Aulstad and Kittelsen 1971, Kincaid 1976, 1983). As estimated inbreeding levels rise, breeders often respond to a decrease in growth performance (under the assumption that it is due to inbreeding depression) by introducing unrelated individuals into the breeding program, outbreeding. Unfortunately, outbreeding can slow or reverse selection gains through the disruption of gene complexes, dilution of positive alleles, or introduction of new recessive deleterious alleles. There are few long-term breeding studies with aquatic organisms to provide culturists with an expectation of possible selection gains or inbreeding losses in phenotypic traits. Understanding the relationship between phenotypic traits, total inbreeding, and rate of inbreeding is important in the design and implementation of a selection program.

Hershberger *et al.* (1990a) reported on the study of inbreeding levels in captive coho salmon *Oncorhynchus kisutch* broodstocks selected for growth in marine net-pens. The study concluded that decreases in body size gains after five generations of selection were not due to inbreeding, but were related to environmental factors. Although

cumulative inbreeding levels were approaching 10%, it was apparent that the incremental increase in inbreeding over several generations had not resulted in any detectable depression in growth, survival, or reproductive traits. The phenotypic effects of inbreeding levels accumulated over several generations, as would occur in most selection and captive rearing programs, may not be similar to those observed after a single generation of intense inbreeding (Pirchener 1969). This study reports on estimates of inbreeding in coho salmon broodstock after 10 generations of selection using allozyme and pedigree analysis in conjunction with analysis of reproductive and phenotypic traits.

METHODS

Broodstock Selection Program

The University of Washington, Domsea Farms, Inc., and the Washington Sea Grant Program initiated a selection and breeding program in 1977 with coho salmon to develop a broodstock for the marine net-pen industry in the state of Washington. The program's initial focus, in keeping with Domsea Farms' business strategy, was on producing pan-sized (350-400gm) subadults. The founding population had been derived from the Washington Department of Fish and Wildlife's Skykomish Hatchery, Skykomish, Washington, in 1971 and 1972, and had been subjected to three generations of mass selection (by mating several hundred adults) before the initiation of the Domsea Farms' broodstock program (Novotny 1975). Utilizing estimated genetic values, and considering that the facilities available to the program would only allow raisig 40 families of 600 individuals or less, a selection scheme was designed to yield maximum response and to be useful in a commercial operation (Figure 1). This scheme involved several different types of concurrent selection (e. g., family and individual) and utilized a selection index that incorporated heritability estimates, relative economic values, genetic correlations, and mean values on all the traits of interest. Breeding was conducted by a circular mating procedure (Figure 2) to minimize the possibility of crossing wihtin lines. On a theoretical basis, these steps should limit the change in inbreeding to about 1% per generation (Hershberger and Iwamoto 1984). Because of the 2-year life cycle of coho salmon under captive culture, odd- and even-year spawning broodstock were developed as independent broodlines. Internal control lines were eatablished for each of the year lines during the first generation, but were terminated in 1992 because of their poor performance and limitations in rearing apace.

As part of the selection program, a number of reproductive traits (spawner weight and length, fecundity, egg size (at the eyed stage), survival to the eyed stage, and survival to ponding) and growth traits (length and weight at 1800 temperature units (TUs) post ponding, and weight after 4 and 8 months of saltwater rearing) were monitored. In 1986, the program was modified to rear broodstock in freshwater throughout their life cycle. This change in rearing strategy substantially reduced mortality following transfer to saltwater and during the summer months prior to maturation. The timing of the measurements previously made in saltwater was adjusted accordingly to reflect the different temperature regime experienced by the all-freshwater-reared broodstock.

Inbreeding Estimates

Estimates of inbreeding (Wright's inbreeding coefficient) for each of the families sampled were computed from the family pedigree using the CompuPed v 4.0 program (RCI Software, Loveland, Colorado). The inbreeding level of the founding generation, F3BR73 (Figure 3), was assumed to be 0.00 for the purposes of the calculation.

Allozyme Analysis

For the current study, 159 coho salmon representing 40 full-sib families from the F12BR73 broodline (the 12th generation of the broodline founded in 1973) were examined for allozyme variation sing methods outlined by Aebersold *et al.* (1987). The following 67 loci were resolved (locus nomenclature follows Shaklee et al. 1990): mAAT-1*, sAAT-1, 2*, sAAT-3*, sAAT-4*, ADA-1*, ADA-2*, mAH-1*, mAH-2*, mAH-3*, sAH*, ALAT*, AK*, CK-A1*, CK-A2*, CK-B*, CK-C1*, CK-C2*, EST-1*, EST-4*, FBALD-3*, FBALD-4*, FDHG*, FH*, bGALA*, GAPDH-2*, GAPDH-3*, GAPDH -4*, GAPDH-5*, GPI-A*, GPI-B1*, GPI-B2*, GR*, bHEX*, IDDH1*, mIDHP-1*, mIDHP-2*, sIDHP-1*, sIDHP-2*, LDH-A1*, LDH-A2*, LDH-B1*, LDH-B2*, LDH-C*, aMAN*, mMDH-2*, mMDH-3*, sMDH-A1, 2*, sMDH-B1, 2*, MPI*, PEPA*, PEPB-1*, PEPC*, PEPD-2*, PEPLT*, PGDH*, PGK-1*, PGK-2*, PGM-1*, PGM-2*, PK-2*, PNP-1*, PNP-2*, sSOD-1*, TPI-1*, TPI-2*, TPI-3*, and TPI-4*.

Since no baseline exists for the genetic content of the founding population, the results of this analysis were compared to existing data from five coho salmon populations (Little Pilchuck (N=120), Harris Creek (N=120), Grizzly Creek BY91 (N=100), Grizzly Creek BY93 (N=100), and Lewis Creek (N=67)) which are found in the same watershed (Snohomish and Skykomish River Basins) as the founding population for the Domsea Farms broodstock. Allozyme data from the populations were analyzed using the program BIOSYS (Swofford and Selander 1981).

SELECTION SCHEME FOR COHO SALMON STOCK DEVELOPMENT

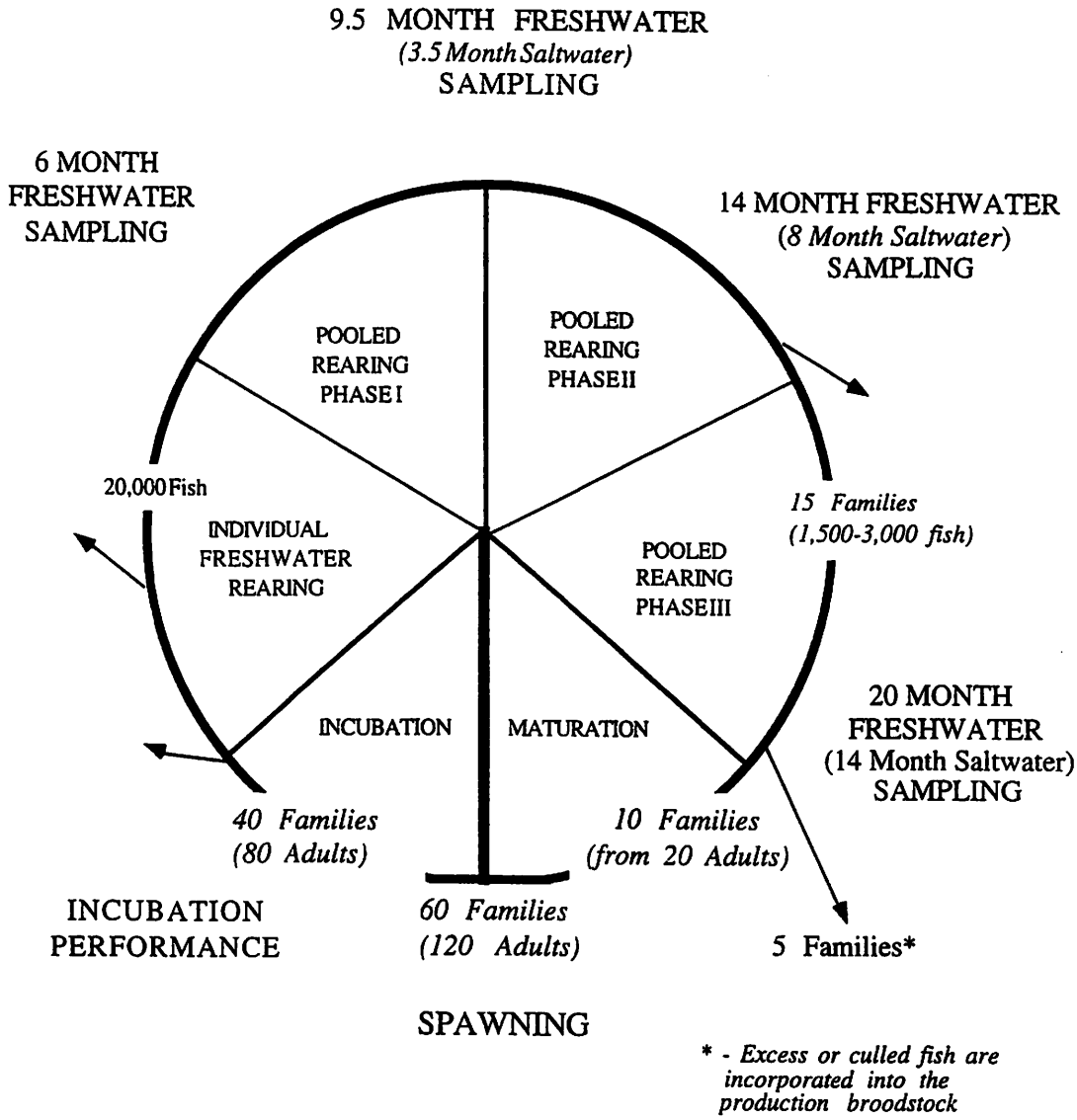

Figure 1. The Domsea coho salmon broodstock selection scheme. The entire cycle represents a 2-year interval between generations. Since 1986, the post-smolt grow-out phases have been conducted in freshwater. Sampling periods for both the marine and freshwater selection programs are presented. Arrows pointing away from the cycle indicate the culling of individuals from all families or the selection of specific families.

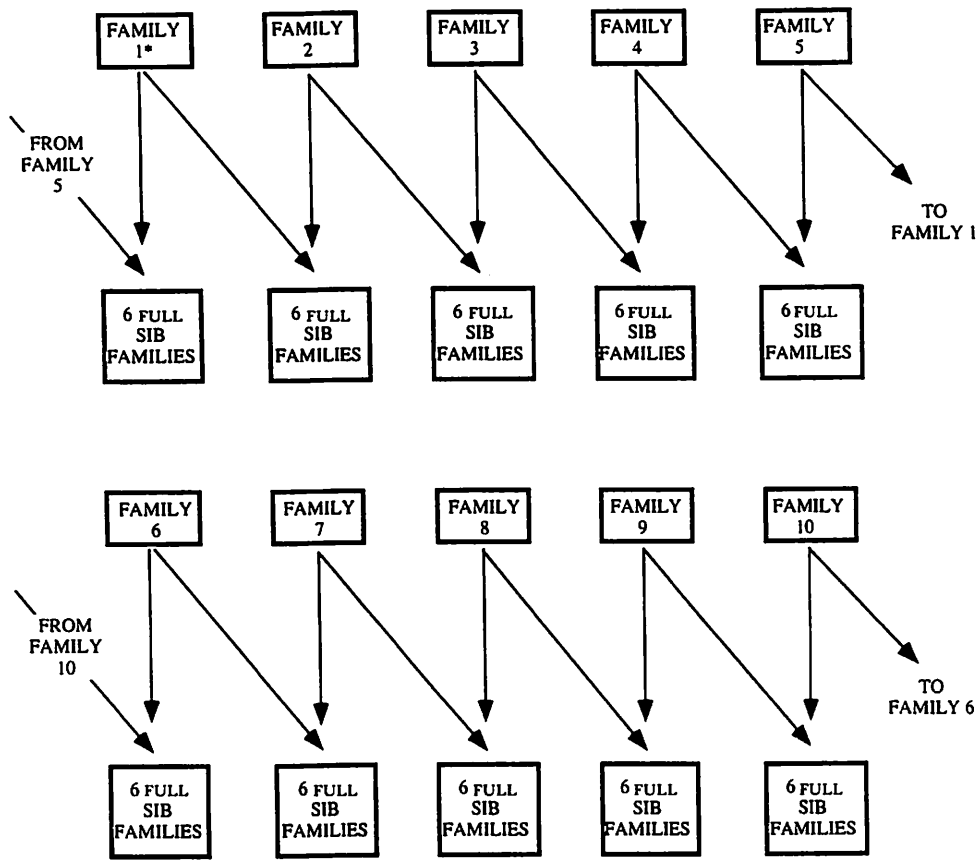

Figure 2. Circular mating scheme used in crossing individuals from selected families. Under ideal conditions, each family cross was composed of six single-pair matings to form six double first cousin families. Matings were generally assortative, and matings involving a familial relationship of first cousin or closer were not allowed.

RESULTS AND DISCUSSION

In the 20 years since the program's inception, significant progress has been made in determining the extent of genetic involvement in several production-related traits and the relationships among those traits (see for example Iwamoto *et al.* 1982, 1990; Saxton *et al.* 1984). Corresponding confirmation of the genetic basis underlying those traits has been obtained through substantially realized gains for pre-smolt and post-smolt growth and survival under production conditions (Hershberger *et al.* 1990 b). There was an average 60% improvement in weight at "harvest" (8 months of saltwater rearing) arter the first four generations of selection.

That genetic improvement was rapid and significant was in part due to the high level of selection intensity imposed upon the population. In every generation, severe truncations in the breeding population occurred --at most, 6 males and 6 females from each of 10 full-sib families served as the nucleus for the succeeding generation. The relationships among families for the 10 generations are shown in the pedigree in Figure 3 where the lines indicate connections between parents and progeny. Because of the limited numbers of spawners per generation, it was inevitable that mating of close relatives would occur. By the fourth generation of the selection program (BY83), it was obvious that all individuals had become related to some degree.

The inbreeding level after 10 generations was calculated to be 22.8% $[F=1/(2N+4)]$, based on population size and excluding self- and sib-mating (Falconer 1981). On

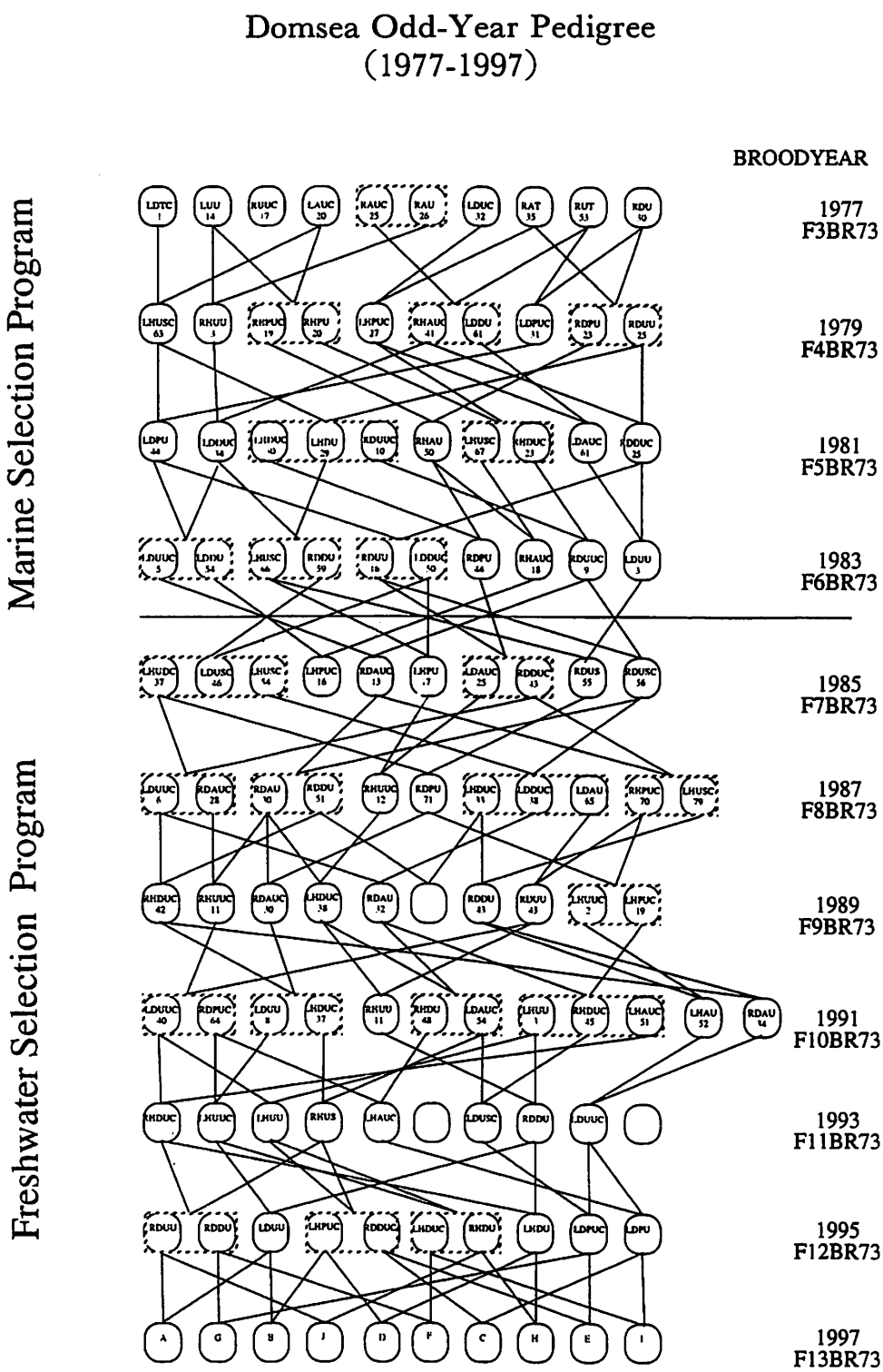

Figure 3. Pedigree for Domsea broodstock from broodyears 1977 to 1997. Letter designations refer to freeze brand marks given to each family during communal rearing, and number designations refer to family number. Families enclosed within hatched boxes are double-first cousins.

the other hand, the average inbreeding estimate for the current generation, F14BR73 (Figure 3), based upon pedigree analysis was considerably lower--14.95 ± 0.21%. These estimates are well within the theoretical range where inbreeding effects should become evident. For example, at a level of 10% inbreeding for rainbow trout, Kincaid (1976, 1983) found a 4.7% reduction in weight at 2 years, Gjerde *et al.* (1983) showed that fingerling growth decreased by 3.0%, Aulatad and Kittelsen (1971) observed decreases in hatch rate and fry survival of 13.7 and 12.2%, respectively, and Su *et al.* (1996) reported decreases in female and male spawning weight of 3.92% and 5.77%, respectively, as well as significant decreases in fecundity and hatchability.

Major shifts have apparently occurred with the allozyme profile of the Domsea broodstock over the course of 20 years of the selection program. The allozyme analysis for the five reference populations indicated that 24 to 45% (mean=31%) of the 67 loci examined were variable (Table 1). By contrast, only 9% (6 of 67) of the loci were polymorphic in the Domsea stock, a decrease of approximately 71% after 10 generations of the selection program. The average number of alleles per locus was also lower in the Domsea stock (1.1) than in the reference populations (range=1.3 to 1.5)(Table 1). Mean heterozygosity for the Domsea stock, however, did not show a corresponding decrease, but was higher (0.032) than estimates for the reference populations (Table 1) --that is, although the absolute number of polymorphic loci has decreased, heterozygosity at several of the loci has increased. For example, the frequencies of variant alleles at EST-1*, GAPDH-1*, PEPC*, and PGM-1* are higher in the Domsea population than in any of the reference populations (Table 2).

As the previous results indicate, the Domsea broodstock has sustained considerable inbreeding and shifts in electrophoretically variable allozyme loci as byproducts of genetic selection and drift. We examined three reproductive traits--female spawner weight, egg weight, and survival to ponding--to determine whether corresponding changes in fitness had occurred. Figure 4 displays the data for female spawner weight and egg weight over the 20-year period. Female weight has varied considerably depending on the maturation environment, and year-to-year variations are more likely due to rearing conditions. The apparent decrease in female weight after BY87 was largely attributed to changes in management and husbandry that occurred during that period. Similar declines did not occur with subsequent broodyears. Egg weight showed a similar decrease after BY87 but has since remained approximately constant. Survival to ponding (Figure 5), with the one exception of BY87, has not indicated any significant negative trends. The traits under direct selection, presmolt and post-smolt growth and survival, continue to show positive response to selection (data not shown).

Table 1. Heterozygosity, mean number of alleles per locus, and percentage of loci showing allozyme variability at 67 loci for Domsea broodstock and five reference populations (naturally spawning Snoqualmie River coho salmon populations).

Populaiton	Alleles per locus	Loci variable (%)	Heterozygosity
Little Pilchuck (1)	1.3	23.9	0.024
Harris Gr. J (2)	1.5	44.8	0.029
Grizzly Cr. A (3)	1.3	26.9	0.026
Grizzly Cr. A (4)	1.3	26.9	0.026
Lewis Cr. A (5)	1.3	31.3	0.027
Domsea (6)	1.1	9.0	0.032

Table 2. Frequencies of variant alleles at six loci polymorphic in the Domsea broodstock. Frequency ranges of these alleles are also presented for five reference populations (naturally spawning Snoqualmie River coho salmon populations).

Allele	Frequency in Domsea broodstock	Range of frequencies in reference populations
EST-1*90	0.350	0.112-0.210
bGALA*118	0.403	0.277-0.431
GAPDH-2*50	0.084	0.000-0.035
PEPC*95	0.365	0.146-0.278
RGM-1*147	0.261	0.013-0.034
PNP-1*93	0.127	0.054-0.143

The 10 generations of systematic genetic selection and substantial inbreeding have not led to perceptible decreases in performance of the Domsea broodstock. However, the estimated inbreeding level is high and there is evidence of significant changes in the genetic profile of the population as evidenced by electrophoretic data. Theory, substantiated by experimental matings, would indicate that inbreeding depression should be evident. It may be that inbreeding effects observed in the progeny of sib or other matings of close relatives are indicative of but not highly correlated with the performance of progeny resulting from generations of mating. As Pirchener (1969) proposed, in domesticated animals, selection can balance an increase in inbreeding of approximately 2% per generation. An alternative explanation may be that this coho salmon strain, and perhaps other Pacific salmon species and strains, do not conform well to conventional thinking regarding inbreeding and may be substantially more robust than theory would dictate. On the other hand, under "good" husbandry conditions some deleterious conditions may not express themselves because of a lack of environmental perturbation, or improvements in husbandry techniques may be compensating for a general degradation

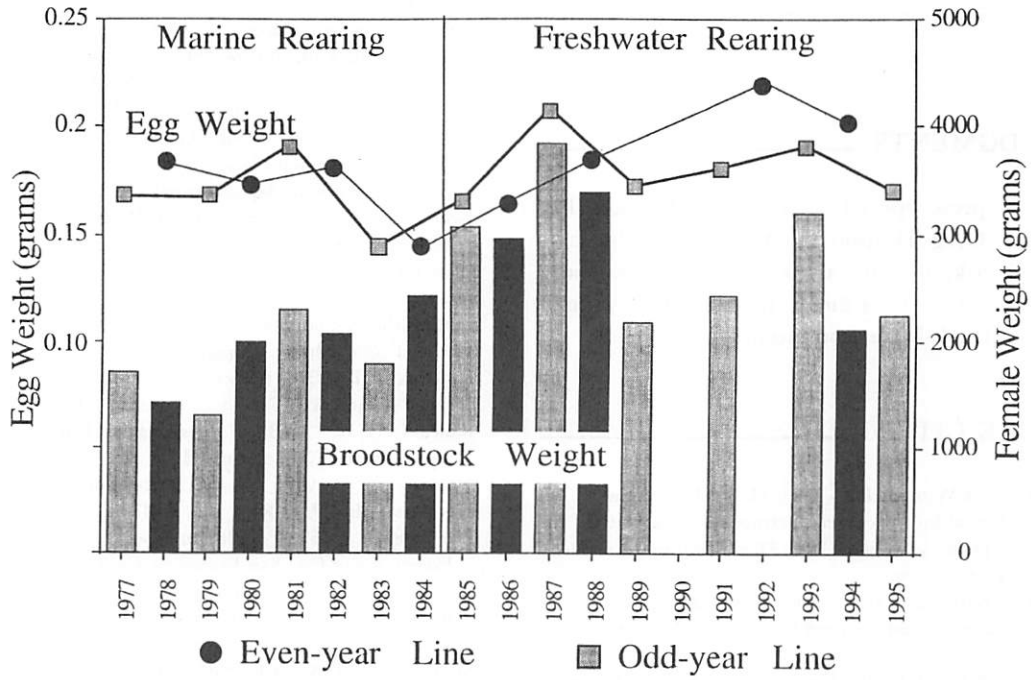

Figure 4. Female spawner weight (grams) and eyed egg weight (grams) from broodyears 1977 to 1995. N=60-100 females for each generation.

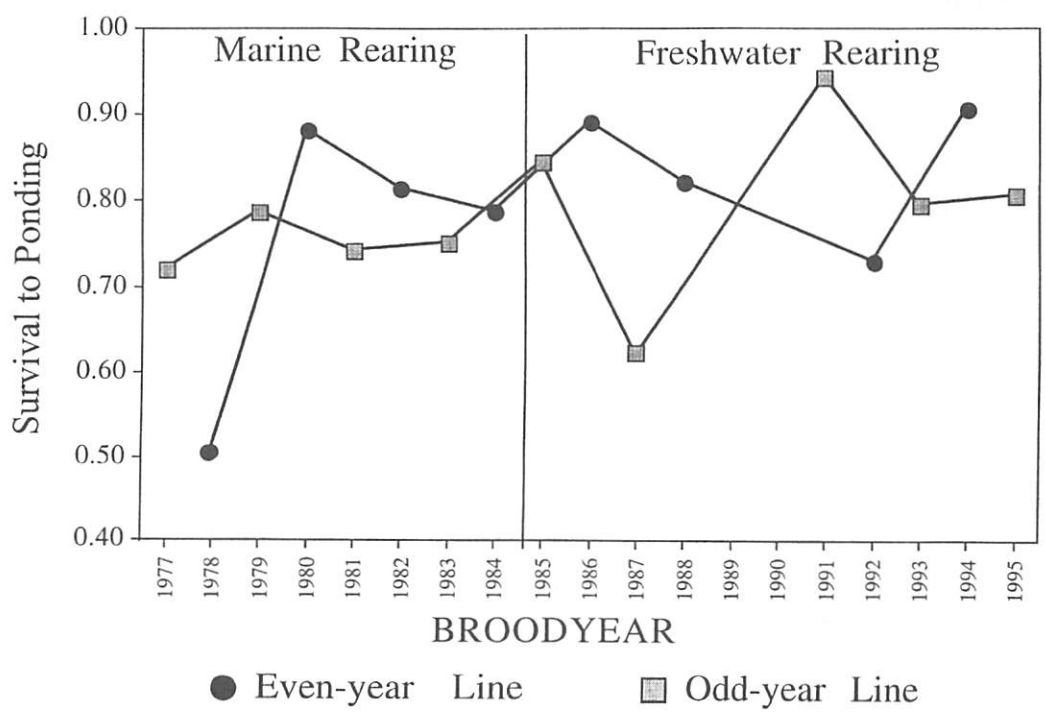

Figure 5. Survival-to-ponding for broodyears 1977 to 1995. Survivals are calculated from 60-70 families from each generation.

in the traits observed. With the proliferation of captive broodstock programs and the current controversy surrounding hatcheries, this topic certainly warrants further investigation.

ACNOWLEDGMENTS

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RECENT TRENDS IN SOME PACIFIC RIM SALMON POPULATIONS

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ABSTRACT

Salmon catches in some Pacific Rim regions have declined dramatically from recent historic high levels. For example, in 1995 the commercial salmon catch in Alaska reached a record high level of 218 million fish. Two years later, in 1997, the Alaska commercial catch had declined by 45% to 121 million fish. In Japan, the record high salmon harvest of 89 million fish in 1996 declined 15% to 76 million in 1997. In both 1997 and 1998, Bristol Bay sockeye salmon runs in Alaska were so depressed the area was declared an economic disaster by government officials. These dramatic declines in abundance may add support to the idea of a long-term interdecadal climatic shift in oceanic environmental conditions leading to reduced marine survival of salmonids. Many salmonid stocks in the Pacific Northwest and throughout much of British Columbia that were already at low levels have remained depressed or have declined further during this recent period. In spite of these sharp declines in commercial catches of Alaska salmon, prices paid to fishermen participating in capture fisheries have remained depressed due to global competition from farmed salmon.

INTRODUCTION

In 1996 at the 25th U.S.-Japan Natural Resources (UJNR) Aquaculture Panel Symposium in Yokohama, I entertained the prospect that some capture fisheries for Pacific salmon were, at that time, in a crisis of abundance (Heard 1997). Throughout a two-decade period from the mid-1970s to the mid-1990s, salmon returns in Alaska and Japan continued to increase and record catches were occurring regularly in both areas. Following a careful review of related issues, the postulate for a crisis of salmon abundance was rejected. However, it was clearly evident the amount of salmon available in world markets, especially due to the rapid and continued growth in farmed salmon, was having major economic and policy influences on salmon fisheries throughout the Pacific Rim. An underlying question was why were some fisheries for salmon experiencing such high levels of stock abundance ?

Pacific salmon runs have always been characterized by fluctuations. Changes in abundance occur at irregular intervals without obvious cause and these shifts may only involve a one or two year variation from longer-term trends. Due to anadromous life histories, significant abundance fluctuations can result from factors originating in freshwater, in estuarine waters, or in marine waters. Fluctuations may originate from completely natural or from anthropogenic events. Combinations of factors can interact causing major changes in abundance with only the vaguest of clues as to underlying causes. Although short-term interannual fluctuations often occur, much recorded history of Pacific salmon is highlighted by longer-term cyclic trends of either declining or increasing abundance (Figure 1). Even before fisheries management began keeping records of salmon runs, cyclic patterns in prehistoric abundance are readily documented from analyses of sediment cores and stable marine-origin nitrogen isotopes derived from returning adult salmon hundreds of years earlier (Finney 1998).

In Alaska, record-high salmon harvest were recorded in the mid-1990s: 193 million, 196 million, and 218 million fish respectively in 1993, 1994, and 1995 (Savikko 1997). In Japan, three years of consecutive record-high salmon harvest also occurred within this same general time frame: 69 million, 77 million, and 89 million fish respectively in 1994, 1995, and 1996 (Kaeriyama *et al.* 1998). In both Alaska and Japan, however, a 15% to 20% decline in the commercial harvest of salmon occurred in the year following record catches.

It may only be a coincidence these similar event sequences were not synchronized but were offset by one year. In Alaska, both the peak return year and following first-year decline occurred one year earlier than the same sequence pattern in Japan. Alaska salmon catches in 1997, the second year following the peak year, also declined an

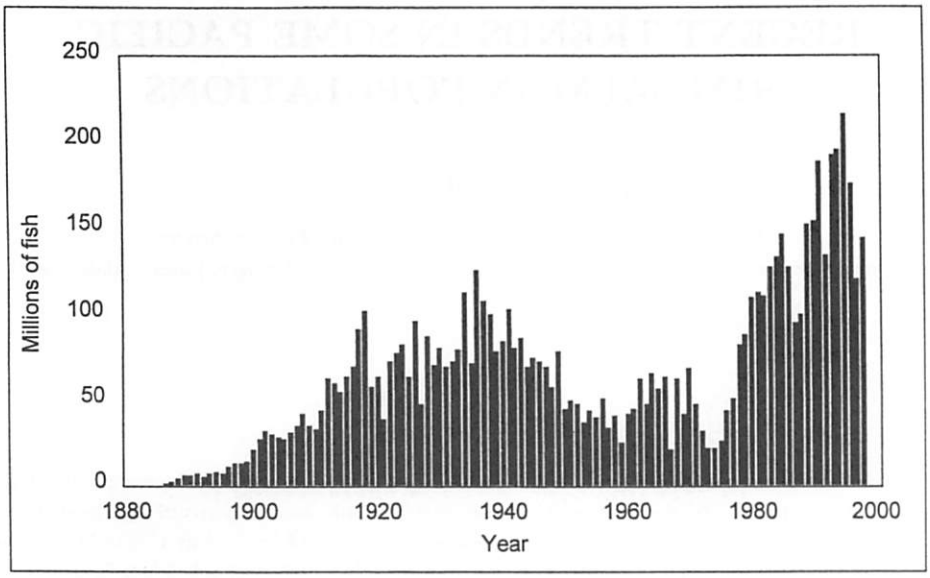

Figure 1. Alaska commercial salmon harvest, 1886-1998.

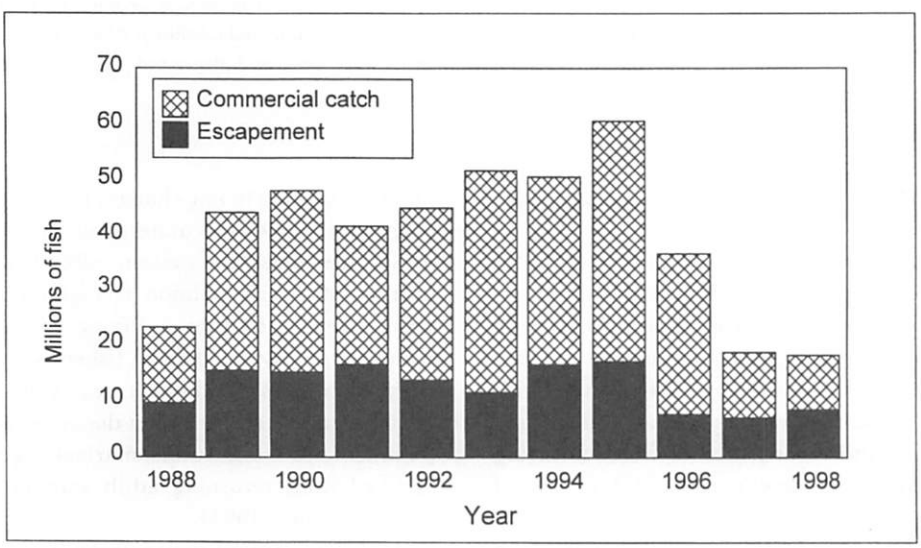

Figure 2. Commercial catch and escapement of Bristol Bay sockeye salmon, 1988-1998.

additional 30% over the 1996 level. And, in Japan early indications are that the 1998 salmon harvest, the second year following the peak, will decline an additional 20-25% over 1997 levels.

This report looks at possible causes of these sudden downturns in abundance from recent record levels in both Alaska and Japan and considers some of the consequences of these changes.

STOCK-GROUP VARIATIONS WITHIN REGIONS

While record salmon catches were occurring in Alaska and Japan in the mid-1990s, many stocks in the Pacific Northwest and throughout parts of British Columbia that were already at low levels generally remained depressed or declined further (Welch *et al.*1998a). Several Pacific Northwest stocks are either already listed or are have been petitioned for listing under the Endangered Species Act (ESA) of the United States (Kope and Wainwright 1998). Listings under ESA laws require development of specific

recovery plans for restoration that place restrictions on fishing and other activities likely to impact the endangered stock(s) or hinder their recovery efforts.

Pacific salmon fisheries are often characterized by multiple kinds of fishing gears that focus on one or more species within a district or other subunit of a region. While average salmon harvest across broad regional areas are useful surrogates for monitoring interannual variations and longer-term trends, these data sets do not necessarily reflect localized events within districts. In Alaska, for example, although there were significant declines in statewide catches of salmon in 1996 and 1997, the severity of these declines varied among species and across districts.

The Bristol Bay sockeye salmon *Oncorhynchus nerka* return, in both 1997 and 1998, was the most seriously impacted district and fishery in Alaska. Forecast for total returns in Bristol Bay in both years fell well below expectations: in 1997 the forecast was for 36 million fish but the actual run was 19 million; in 1998 the forecast was for 30 million but the actual run was 18.4 million fish (Figure 2). In both years the commercial harvest of sockeye salmon in Bristol Bay was roughly half or less the anticipated preseason forecast.

Returns in other districts and of other species in Western Alaska were also very low in 1997 and 1998, especially chinook salmon *O. tshawytscha* and chum salmon *O. keta* in the Yukon River. The commercial catch of summer chum salmon returning to the Yukon River, which had exceeded one million fish twice within the last decade, was less that 30 thousand fish in1998 (Kruse 1998). Because of severe social and economic consequences of weak salmon runs in Bristol Bay and Western Alaska, the State of Alaska and U.S. Government determined these areas qualified for Federal disaster relief funds similar to areas with catastrophic losses due to floods, fires, hurricanes, or other natural disasters.

The weak 1997 runs throughout Western Alaska also extended to districts south of the Alaska Peninsula including parts of Kodiak Island. In 1998, however, with low returns again the pattern in Bristol Bay and Western Alaska, the South Peninsula and Kodiak Districts had unexpectedly large catches of pink salmon *O. gorbuscha*. A record even-year pink salmon catch was recorded in 1998 from Kodiak Island (Savikko 1998). Pink salmon fishermen in both Kodiak and Prince William Sound during 1998 were placed on catch limits and considerable numbers of fish went unharvested (Buckley 1998). Unanticipated strong returns in these districts as well as better than average returns of some species in other districts brings into question the causes of the sharp declines in Bristol Bay and Western Alaska. Are factors that affect depressed runs in Bristol Bay and Bering Sea drainages unique to these areas or are basin-wide factors beginning to influence salmon abundance throughout the Pacific Rim ?

FACTORS AFFECTING ABUNDANCE

Following the dismal 1997 sockeye salmon return to Bristol Bay, a series of conferences in Alaska and elsewhere began examining possible causes for the decline. Many hypotheses were considered including, among others, the following: (1) changes in freshwater conditions causing reduced survival in juvenile stages; (2) increased predation of juveniles or adults; (3) starvation or reduced growth at sea due to reduced productivity from lower trophic levels of the food web; (4) high seas interceptions of adults or sub-adults while at sea; (5) high mortality of adults entering Bristol Bay due to unusually high sea temperatures; (6) increased disease prevalence or changes in migratory behavior due to a stressful marine environment; and (7) overall changes in ocean conditions caused by a new interdecadal climatic shift in the marine environment.

Analyses of available data allowed many of these potential causes to be discounted. There is ample evidence, however, that unique environmental conditions existed in Bristol Bay and the Bering Sea in both years and it is likely these conditions influenced salmon returns those years (Kruse 1998). Notwithstanding the importance of regional influences on salmon abundance, a growing consensus among many scientists is that recent declines may be due to major interdecadal climatic changes in the North Pacific Ocean. These changes may already be in place or are, at least, beginning to occur (Beamish and Bouillon 1993, Hare and Francis 1995, Mantua *et al.* 1997, Noakes 1998, Welch *et al.* 1998a). This belief is not necessarily based on the failure of the recent Bristol Bay sockeye salmon returns but on a broader array of related factors including shifts in a number of long-term environmental indices, declines in other fish populations, and changes in available food resources in the North Pacific Ocean (Brodeur and Ware 1992, 1995, Kawasaki and Omori 1995, Beamish *et al.* 1998, Ingraham *et al.* 1998, Noakes *et al.* 1998, Klyashtorin and Rukhlov 1998).

When the 1998 sockeye salmon return to Bristol Bay was again unexpectedly weaker than predicted along with record low chum salmon and chinook salmon returns to other Western Alaska river systems, the question of causal factors gained a new level of urgency. Were these dramatic declines in abundance due to basin-wide changes in the North Pacific Ocean ecosystem or due to restricted environmental conditions in the Bering Sea and Bristol Bay ? More specifically, do the recent downturns in overall salmon catches both in Alaska and Japan portend a longer-term decline in abundance ? And, if so, what are the possible causes and consequences of this trend ?

Kruse (1998) examined many of these questions in some detail, and reviewed physical and biological factors that influence environmental variability in Bristol Bay and Bering Sea. He reviewed possible effects of a series of unprecedented environmental anomalies in this region in

1997 and 1998 including the 1997-1998 El Niño Southern Oscillation (ENSO) event, the first-recorded occurrence of aquamarine waters in eastern Bering Sea due to coccolithophore phytoplankton blooms, large scale die-offs of several species of sea birds due to starvation caused by lowered marine productivity, possible altered migratory patterns of salmon, unusually high sea temperatures, and changes in the atmospheric Aleutian Low Pressure System. He concluded that more information was needed to determine the impacts of the full suite of potential factors that may have led to the poor salmon runs in Bristol Bay and Western Alaska in 1997 and 1998. This belief was based on the many unique regionally-based environmental factors and ecosystem changes in Bristol Bay and Bering Sea together with the relatively strong runs of salmon in other parts of Alaska, especially in 1998 (Kruse 1998).

CLIMATIC FLUCTUATIONS AND PACIFIC RIM SALMON

Basin-wide correlations between climatic-driven environmental factors and trends in salmon populations are well documented (Pearcy 1992, Beamish 1993, Beamish and Bouillion 1993, Rogers and Ruggerone 1993, Francis and Hare 1994, Klyashtorin and Smirnov 1995, Mantua *et al.* 1997, Klyashtorin and Rukhlov 1998, Noakes *et al.* 1998). The methodology generally used for these correlations is to compare various long term-climatic indices and long-term trends in salmon production. Three such climate indices, Aleutian Low Pressure Index (ALPI), North Pacific Index (NPI), and Atmospheric Circulation Index (ACI), show good coherence with changes in salmon abundance (Noakes *et al.* 1998). Each index measures different aspects of climate-based environmental influences that, as reviewed by Noakes and colleagues, represent surrogate measures of complex biological and physical processes that influence primary production in the ocean and subsequently fish production. The ALPI and NPI indices are derived within in the North Pacific Ocean while the ACI index, as reviewed by Klyashtorin and Rukhlov (1998), is based on wind patterns across the Northern Hemisphere.

In general, each of these indices indicate that a shift in climatic patterns affecting the North Pacific Ocean either has occurred or is in early stages of development. Some scientists believe that such a shift to a new level of lower ocean productivity occurred in the late1980s or early 1990s (Noakes 1998, Welch *et al.* 1998a). Prior coherence between these indices and salmon abundance suggest that a long-term downward trend in many Pacific Rim salmon populations may be imminent (Figure 3). Klyashtorin (1998) takes this premise one step further. Using the ACI index as a conceptual tool for long-term forecasting of abundance, he suggests a probable declining catch trend for American salmon over the next 25-year period that bottoms out at roughly 40 to 50% below the peak catches of the early to mid-1990s (Figure 4).

It is instructive to view temporal climatic events affecting Pacific salmon under three scenarios: (1) interannual fluctuations, year-to-year variations in localized or regional weather patterns including periodic El Niño events; (2) interdecadal climate oscillations that reflect longer term trends, the so-called regime shifts in ocean productivity, that may cause major cyclic patterns in salmon abundance; and (3) global warming that may involve longer-range global or hemispheric changes due either to anthropogenic

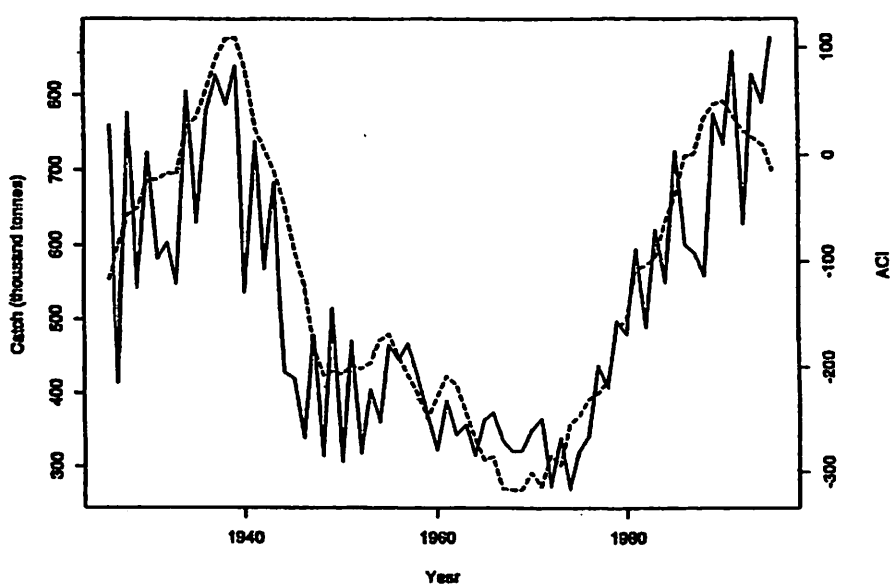

Figure 3. All nation catch of sockeye, pink, and chum salmon (solid line) and Atmospheric Circulation Index (dashed line) for the period 1926-1995. From Noakes *et al.* 1998.

effects such as increases in greenhouse gases and losses of ozone layers and to natural post-glacial warming episodes. Each of these three levels may affect salmon populations differently. In the short run, however, it is all but impossible to sort out which events are causing what affect. Assuming that an interdecadal regime shift in the North Pacific Ocean ecosystem is already underway, as exemplified by current declines in salmon production, a paramount issue now concerns how the confounding effects of global warming may influence prior patterns of cyclic abundance.

One characteristic of the previous regime shifts in the eastern North Pacific Ocean has been an inverse pattern of salmon production between Alaska and the Pacific Northwest. In the mid-1970s when Alaska stocks were at record low levels, Pacific Northwest chinook salmon and coho salmon stocks were in a period of relatively high productivity. Beginning with the 1977 regime shift and continuing throughout the next two decades, Alaska stocks began experiencing high survivals and rapid improvements while Pacific Northwest stocks entered a period of low survivals and declining run strength (Hare *et al.* in press). With a new regime shift potentially underway and indications of reduced salmon production occurring in Alaska stocks, is a significant improvement in the status of Pacific Northwest stocks likely to follow this earlier inverse production pattern ? This may not occur due to compounding influences of recent El Niño events, the potentially more serious impacts of the current broader-range global warming trend, and the degradation of freshwater habitats in the Pacific Northwest.

Proponents of the concept that a major ocean climatic shift occurred early in the current decade have presented data on British Columbia steelhead *O. mykiss* and Oregon coho salmon *O. kisutch* showing a sudden decline, not an increase, in marine survivals of both species beginning in1990 (Welch *et al.* 1998a). Oregon coho salmon survivals were already at historical low levels before the additional declines in1990. Welch *et al.* (1998b) examined ocean temperatures and monthly migration patterns and found that sharp thermal boundaries limit the marine distribution of sockeye salmon throughout the ocean phase of their life history. Furthermore, these researchers speculated that given the predicted ocean temperature increases at current rates of greenhouse gas emissions, sockeye salmon could be excluded from the entire North Pacific Ocean and be restricted to the Bering Sea by the middle of the next century. It, therefore, is not a foregone conclusion that a new regime shift with declining trends in salmon production in Alaska will be accompanied by increased production in the Pacific Northwest or other areas.

PRICES PAID TO FISHERMEN WITH REDUCED SALMON RUNS

A traditional pattern of supply and demand economics in capture fisheries generally produce higher prices for fishermen when catches are low. Commercial salmon catches in both Alaska and Japan, however, have not always followed this pattern, especially in recent years. Exvessel prices paid to fishermen in Alaska have fluctuated widely over the past decade with significant socio-economic impacts throughout the industry (Figure 5). A decade-long reduction in exvessel value of the commercial salmon harvested in Alaska, exacerbated by record catches in the mid-1990s, has declined even further with lower

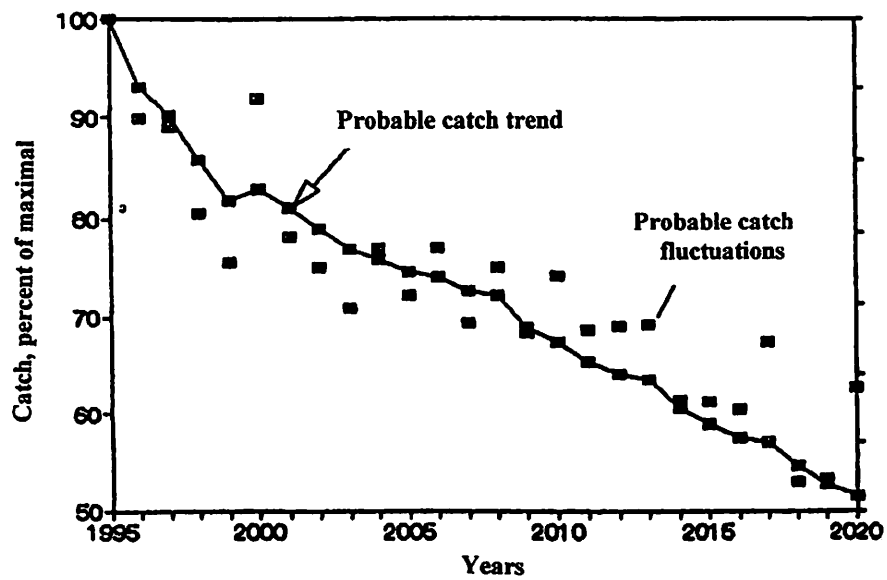

Figure 4. Possible dynamics of American salmon harvests in percent of maximal catch, 1995-2020. From Klyashtorin 1998.

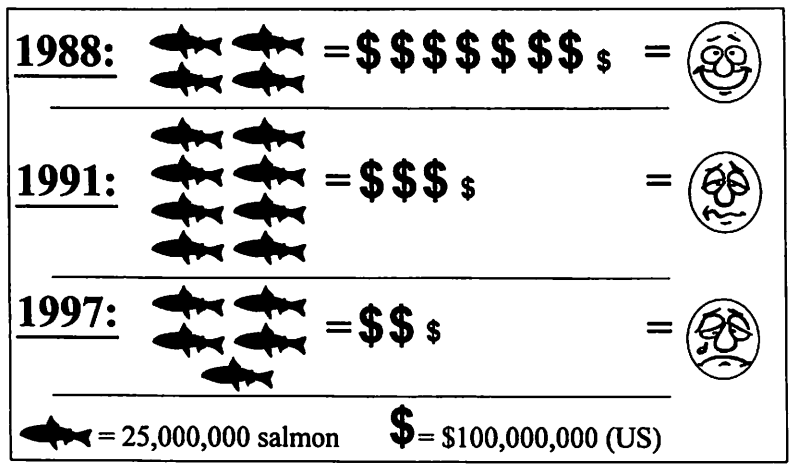

Figure 5. Supply and demand patterns illustrating commercial catch, exvessel values, and general outlook of Alaska salmon fisheries in 1988, 1991, and 1997.

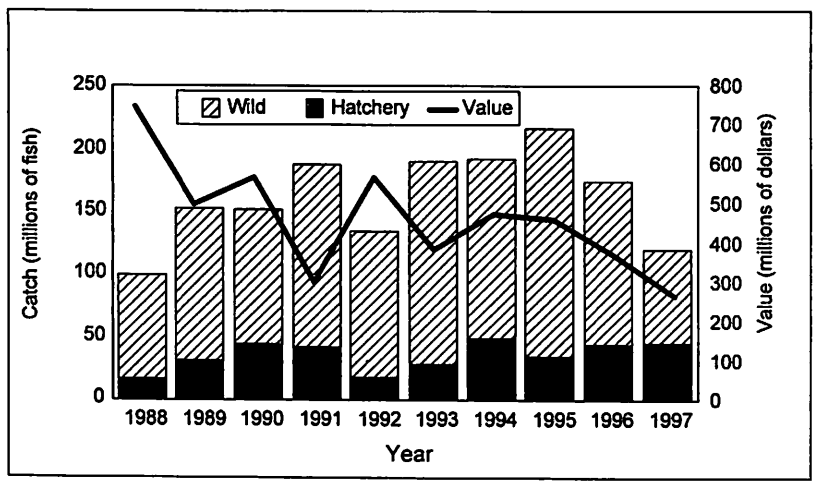

Figure 6. Total numbers of commercially caught wild and hatchery Alaska salmon, 1988-1997, and exvessel value of catch. Hatchery salmon include both common property and cost recovery harvested fish. Data source, Alaska Department of Fish and Game.

catches in1996 and 1997 (Figure 6). If this trend continues, especially with the prospect of future declines in salmon production caused by ecosystem changes, further socio-economic hardships for the commercial salmon fishing industry in Alaska and elsewhere appear likely.

The total world supply of salmon including production from farmed salmon and capture fisheries continues to increase and currently is at a record 1.5 million mt annual level. While production from capture fisheries has fluctuated between 600 thousand and 900 thousand mt annually over the past decade, production from farmed salmon has shown a steady increasing trend, growing from about 100 thousand mt in 1987 to 700 thousand mt in 1997 (Figure 7). The continued growth of farmed salmon production has become the dominant factor in determining the value of salmon caught in capture fisheries. Also, indications are that farmed salmon production will continue to grow, that the cost for producing farmed salmon will continue to decline, that capture fisheries for salmon, of necessity, will undergo significant changes, and that the salmon industry will become dominated by salmon farmers (Knapp 1998). The current status of capture fisheries for salmon in Alaska is perhaps best illustrated by how it has changed in recent years relative to total world salmon production. As recently as 1982 Alaska salmon held a dominate 45% share of the total world supply. However, 15 years later in 1997, Alaska's share of world salmon production had dropped to only 19% (Figure 8) in spite of a decade-long period of record high commercial catches (Spiess 1998).

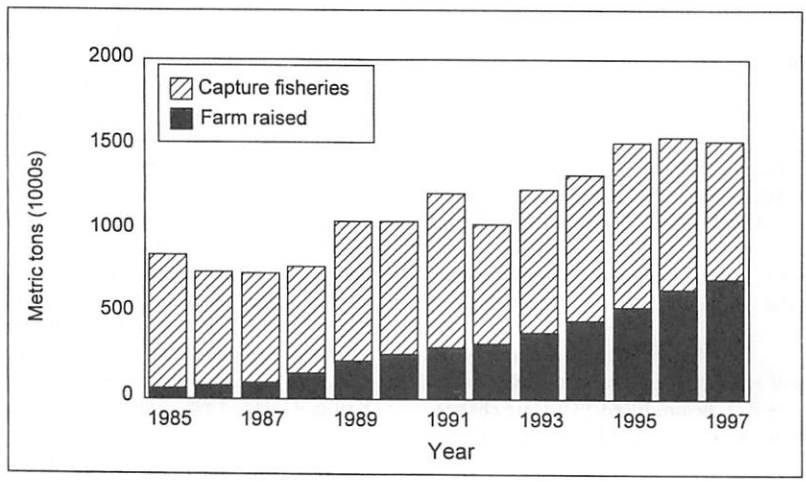

Figure 7. Total world salmon production, 1985-1997. (Capture fisheries for anadromous salmon include both naturally spawned and hatchery produced fish.) Data source, SMIS 1998.

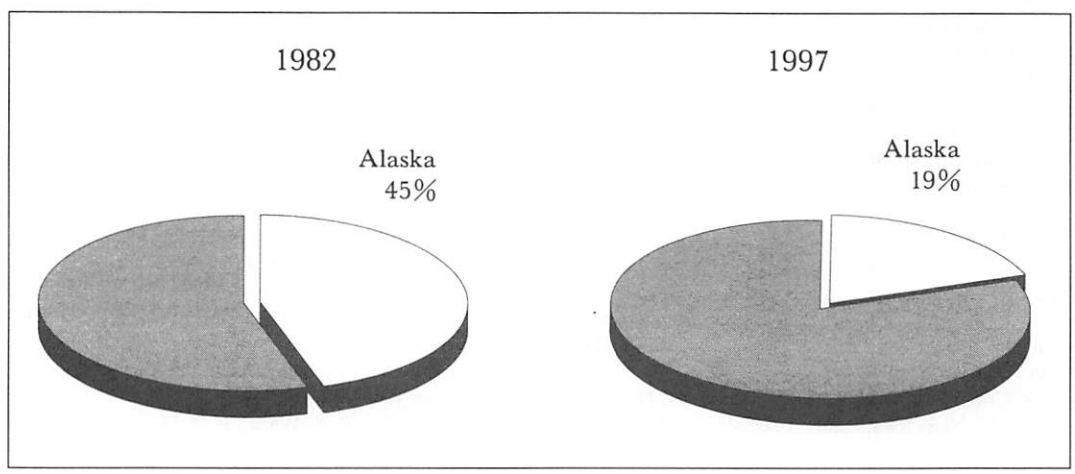

Figure 8. Alaska percentage of total world salmon production in 1982 and 1997. From Spiess 1998.

SUMMARY

Recent declines in commercial catches of anadromous salmon in Alaska and Japan, from record levels for over a decade indicate that a new period of reduced marine productivity and salmon survival may be underway. The downward trend in salmon productivity appears consistent with previous coherence in abundance cycles and changes in environmental factors. Differences in run strength between species in different regions and districts, however, also suggest it may be too soon to conclude that a climatic-driven regime shift in the North Pacific Ocean ecosystem is underway and portends smaller salmon runs in most areas. Complicating effects of the recent strong ENSO events and the unknown long-range consequences of increases in greenhouse gasses and global warming trends increase uncertainty about the future productivity of anadromous runs around the Pacific Rim.

With near certainty, however, there surely will be further dynamic interactions between capture fisheries and the continued dramatic growth in worldwide farmed salmon production. These interactions, together with continued increases in availability of salmon products in world markets, likely will influence the future outlook of capture fisheries for salmon to a greater extent than climate-based cyclic changes in the abundance of anadromous runs.

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POPULATION STRUCTURE OF TWO SPECIES TARGETED FOR MARINE STOCK ENHANCEMENT IN THE GULF OF MEXICO

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ABSTRACT

Enhancement of overfished stocks of harvested marine species through supplementation with hatchery-raised individuals is a viable option in the Gulf of Mexico (Gulf), where virtually all U. S. Gulf Coast states have active programs supplementing wild stocks or are carrying out research designed to that end. Two targeted species are red drum *Sciaenops ocellatus* (Sciaenidae) and red snapper *Lutjanus campechanus* (Lutjanidae). Both support important fisheries and are overfished. Life-history observations and mark-recapture experiments are consistent with the hypothesis that red drum comprise a single population (stock) in the Gulf. Red snapper, alternatively, appear to be sedentary, non-migratory, and typically associated with specific substrates or structures. We employed molecular genetic tools to assess population structure across the northern Gulf in both species. Analysis of mitochondrial (mt) DNA genotypes of red drum revealed a significant isolation-by-distance effect, where female migration is inversely related to distance from the estuary or bay of natal origin. Trajectories of correlograms in spatial autocorrelation analysis suggest an upper limit to geographic neighborhood size of roughly 700-750 km. Enhancement of red drum should thus be based on the premise that supplementation of a given bay or estuary will impact geographically proximal bays or estuaries more than geographically distant ones. Analysis of mtDNA and microsatellite DNA loci of red snapper indicated that red snapper from the northern and western Gulf comprise a single population. This finding is not consistent with expectations based on life history and adult movement. Our studies demonstrate that population structure is not necessarily concordant with the perceived biology of species. Analysis of population structure should be examined thoroughly prior to implementation of enhancement programs for marine species.

INTRODUCTION

Knowledge of the population structure of economically important marine fish species is critical to several issues pertinent to management of a fishery, including stock enhancement. Identification of subpopulations (stocks) provides biologically meaningful geographic boundaries for assessing a number of parameters, including genetic diversity. The latter is important because subpopulations (stocks) may possess novel genetic, physiological, behavioral, and/or other characters that promote distinct differences in life-history traits such as growth rates, fecundity, abundance, and disease resistance (Stepien 1995). In addition, subpopulations subjected to environmental or other stress (e. g., overexploitation) that reduce effective population size may be prone to "mutational meltdown" where slightly deleterious, recessive mutations accumulate and erode fitness (Lynch *et al.* 1995). Knowledge of stock structure also is important for conservation and management of a fishery because assessment and allocation decisions can be tailored to the unique needs of both resource and resource users within a subregion. Finally, knowledge of stock structure is critical to stock enhancement or supportive breeding (*sensu* Ryman and Laikre 1991) relative to defining geographic boundaries for monitoring post-supplementation effects on genetic effective population size and/or assessing supplementation (enhancement) success (Blankenship and Leber 1995).

Over the past decade, studies in our laboratory have been directed in large part towards assessing population structure of economically important marine fish species in U.S. waters of the Gulf of Mexico (hereafter, northern

Gulf). We have employed genetic markers, including allozymes (Gold *et al.* 1994), mitochondrial (mt)DNA (Richardson and Gold 1997, Gold and Richardson 1998, Gold *et al.* 1997a, b, 1999), and microsatellite DNA loci (Broughton and Gold 1997, Heist and Gold 1999), to test genetic homogeneity and quantify gene flow among geographic localities for several species. In a general way, population structure occurs when gene flow is restricted for any of a number of reasons, including oceanographic parameters (e.g., current patterns), diffusion effects, behavioral characteristics, natural selection, or past history (Palumbi 1994).

In this paper, we synopsize several years work on population structure of two species of marine fishes in the northern Gulf for which supportive breeding or supplementation programs are either operational or in the planning stage. The first is the red drum *Sciaenops ocellatus*, a member of the family Sciaenidae. Red drum is an important recreational species in bays and estuaries of the northern Gulf and it is managed intensively by various coastal states (Pattillo *et al.* 1997). The species has considerable name recognition in the restaurant trade (viz., "blackened" redfish), and at one time, supported a viable commercial fishery as well (Pattillo *et al.* 1997). Hatchery technology (husbandry) of red drum is well developed, and the life cycle is effectively "closed" (McCarty 1990). Stock enhancement programs are being considered by several Gulf Coast states, and a long-standing program exists in the state of Texas where hatchery-raised red drum fingerlings have been stocked in various bays and estuaries since the 1970s (McEachron *et al.* 1995). The second species is the red snapper *Lutjanus campechanus*, a member of the family Lutjanidae. Red snapper is arguably the most important recreational and commercial marine fish species in offshore waters of the northern Gulf, and is managed intensively by the U.S. federal government (GMFMC 1989, 1991). Red snapper also have considerable name recognition in Gulf Coast states, both among fishermen and as a food item in restaurants. Hatchery (husbandry) technology for red snapper is in its infancy, and no stock enhancement programs are yet operational. However, a major stock-enhancement effort for red snapper in the northern Gulf is currently in the planning stage (Pruder and Hawkins in press).

Consideration of the life history and other aspects of the two species yields different predictions regarding population structure (gene flow) in the northern Gulf. Red drum spawn near passes and inlets to bays and estuaries, and the pelagic eggs and larvae move into nursery areas on incoming tides (Matlock 1987). Juveniles remain in bays and estuaries for three to four years, and then move offshore, typically forming large schools (Matlock 1987). Mark-recapture studies have demonstrated little to no movement of juveniles between bays and estuaries; adults, however, can migrate extensively across the northern Gulf (Matlock 1987, Matlock and Weaver 1979). Because adults appear capable of spawning throughout their >30 year life-span (Murphy and Taylor 1990), the potential for gene flow throughout the northern Gulf appears high, leading to the prediction that little to no population (stock) structure in red drum should exist in the northern Gulf.

Red snapper, alternatively, spawn offshore on the continental shelf, where eggs and larvae remain pelagic for approximately 30 days (Leis 1987). After settlement, post-larvae are generally sedentary and are associated with low or high relief bottoms (Bradley and Bryan 1975). Mark-recapture and sonic-tracking experiments (Fable 1980, Szedlmayer 1997, Bradley and Bryan 1975) indicate there is little to no movement of adults. Based primarily on the latter, the potential for gene flow among red snapper in the northern Gulf would appear to be limited, leading to the prediction that population (stock) structure of red snapper could exist in the northern Gulf.

The primary genetic markers employed in our studies of population structure have been restriction-enzyme sites in mitochondrial (mt)DNA. Use of mtDNA in studies of population structure is well documented in a number of vertebrate species, including several fishes (Ovenden 1990, Avise 1992). Major benefits of using mtDNA markers, relative to analogous, nuclear-encoded sequences, include (i) a lower genetic effective population size because of genetic haploidy and maternal inheritance (Birkey et al. 1983), and (ii) a higher rate of nucleotide substitution (Brown 1983). Because of the former, mtDNA is expected to be four times more sensitive in detecting population structure, should it exist; because of the latter, mtDNA is capable, in theory, of detecting population structure that has occurred in recent evolutionary time.

MATERIALS AND METHODS

Localities in the northern Gulf where fish have been obtained, along with sample sizes at each locality, are given in Figures 1 and 2. Information regarding methods, dates of collection, tissue removal, and preservation are given in the primary papers: red drum (Gold *et al.* 1993, 1999), and red snapper (Gold *et al.* 1997b). Red drum were sampled as juveniles from bays and estuaries; adult red snapper were sampled in offshore waters. The total number of fish assayed from the northern Gulf was 1,371 for red drum and 707 for red snapper. Samples of red drum from four consecutive cohorts (year classes) were obtained at several localities (listed in Gold *et al.* 1999).

Assay of mtDNA restriction sites followed methods outlined in Gold and Richardson (1991). Specific restriction enzymes and mtDNA probes used to digest whole mtDNA molecules are given in Gold *et al.* (1999) for red drum and Gold *et al.* (1997b) for red snapper. A total of 104 red drum and 93 red snapper restriction sites,

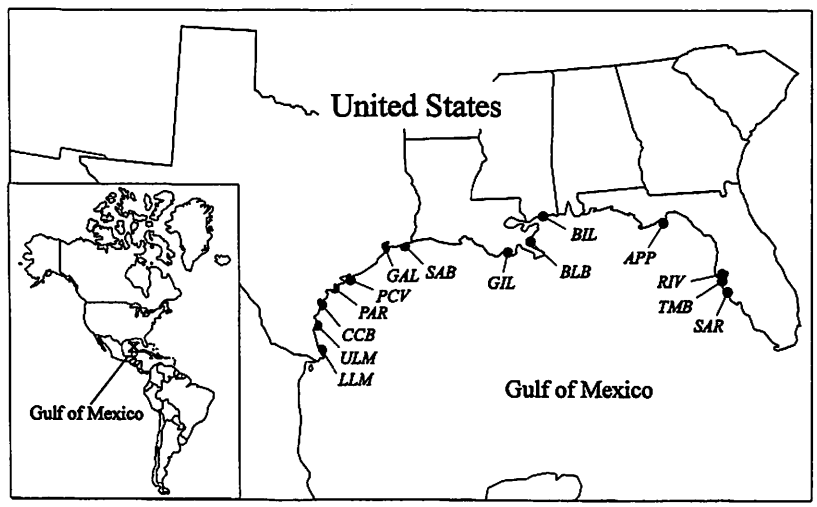

Figure 1. Localities in the Gulf of Mexico where samples of red drum were obtained. Acronyms are as follows (sample sizes in parentheses): *LLM*-Lower Laguna Madre, Texas (147); *ULM* - Upper Laguna Madre, Texas (94); *CCB* - Corpus Christi Bay, Texas (104); *PAR* - Redfish Bay, Texas (38); *PCV* - Pass Cavallo, Texas (90); *GAL* - Galveston Bay, Texas (117); *SAB* - Sabine Pass, Texas (106); *GIL* - Grand Isle, Louisiana (121); *BLB* - Black Bay, Louisiana (20); *BIL* - Biloxi Bay, Mississippi (117); *APP* - Apalachicola Bay, Florida (154); *RIV* - Riviera Bay, Florida (69); *TMB* - Tampa Bay, Florida (83); and *SAR* - Sarasota Bay, Florida (111).

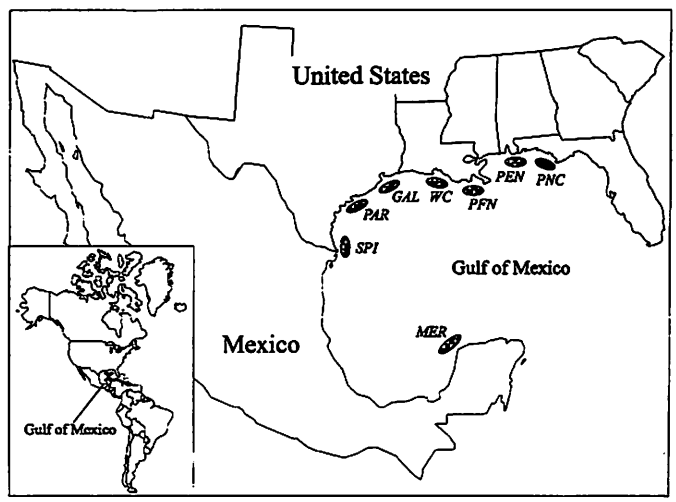

Figure 2. Localities in the Gulf of Mexico where offshore samples of red snapper were obtained. Acronyms are as follows (sample sizes in parentheses): *MER* - Merida, Mexico (44); *SPI* - South Padre Island, Texas (95); *PAR* - Port Aransas, Texas (112); *GAL* - Galveston, Texas (97); *WC* - West Cameron, Louisiana (54); *PFN* - Port Fourchon, Louisiana (86); *DIL* - Dauphin Island, Alabama (103); *PEN* - Pensacola, Florida (25); and *PNC* - Panama City, Florida (91).

distributed essentially randomly in each mtDNA genome (Figure 3), were assayed. The number of unique mtDNA haplotypes identified was 145 for red drum and 92 for red snapper. Statistical approaches, including molecular analysis of variance (Excoffier *et al.* 1992) and spatial autocorrelation analysis (Wartenberg 1989), used to examine the spatial distribution of mtDNA haplotypes and their frequencies are given in Gold *et al.* (1999) for red drum and Gold *et al.* (1997b) for red snapper.

RESULTS AND DISCUSSION

Red drum: Prior to examining the distribution of mtDNA

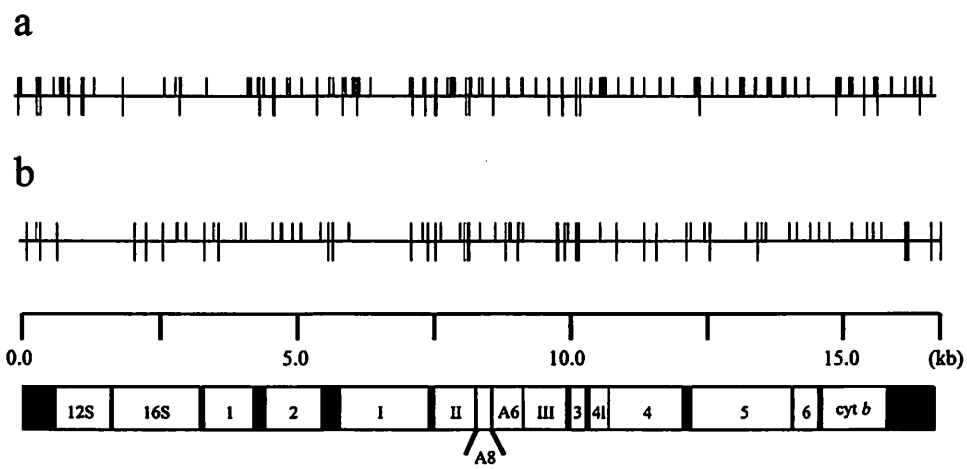

Figure 3. Restriction-enzyme maps for mtDNA of red drum (a) and red snapper (b). Restriction sites that extend below the line were found in all individuals surveyed. Maps are oriented to the human mtDNA gene map: clear boxes refer to the small and large rRNA genes (12S and 16S), NADH dehydrogenase subunits (1-6, 4L), cytochrome oxidase subunits (I-III), ATPase subunits (A6, A8), and cytochrome *b* (b); transfer RNA genes and spacers appear as black areas and the D-loop or control region is shaded.

haplotypes across localities, we first tested homogeneity of mtDNA haplotype frequencies across year classes within localities. All tests of temporal homogeneity (i.e., homogeneity of mtDNA haplotype frequencies between or among year classes at individual localities) were non-significant, permitting pooling of temporal samples at each locality. Analysis of molecular variation (AMOVA) revealed significant spatial heterogeneity (Table 1) both among localities ($P=0.014$) and among regions ($P=0.008$). In the latter, localities were pooled into three regions (East, Central, and West Gulf), permitting a test of homogeneity among localities within regions. This test was non-significant ($P=0.135$), indicating the absence of significant genetic divergence in mtDNA haplotype frequencies among proximate geographic localities. These results were corroborated by tests of homogeneity of mtDNA frequencies: significant spatial heterogeneity ($P<0.001$) was found both among localities and among regions (i.e., East, Central, and West Gulf); whereas tests between adjacent localities in different regions were non-significant (Gold *et al.* 1999). Taken together, these results indicate that significant genetic divergence exists among red drum in the Gulf, and moreover, that the degree of genetic divergence between localities increases over geographic distance. A final point to note is that over 99% of the variance in mtDNA haplotypes frequencies was distributed within sampling localities.

Spatial autocorrelation analysis (SAAP) of mtDNA haplotypes was used to examine further whether genetic divergence in red drum in the Gulf was largely a function of geographic distance between individual bays and estuaries. SAAP runs employed mtDNA haplotypes that occurred in seven or more individuals (27 haplotypes total). Seventeen significant ($P<0.05$) Moran's I values were generated when equal distances between distance classes were used. Six of these (five positive) occurred in the first two distance classes, while 11 (nine negative) occurred in the last two distance classes. Four of the positive values in the first distance class, and four of the negative values in the last distance class, were highly significant ($P<0.01$). Identical results were obtained in SAAP runs that used equal numbers of pairwise comparisons in each distance class. These results demonstrate a significant isolation-by-distance effect (Sokal and Oden 1978), where frequencies of mtDNA haplotypes are positively correlated in geographically proximate localities and negatively correlated in geographically distant ones. This is exemplified by correlograms of mtDNA haplotypes exhibiting significant Moran's I values (Figure 4). In both cases (i.e., equal distances between distance classes and equal frequencies per distance class), there was a fairly regular decline from significant, positive autocorrelation at 300 km between localities to negative autocorrelation at 1000 to 1500 km between localities (Figure 4). The geographic distance where no correlation exists is between 700-750 km, suggesting perhaps an upper geographic limit to gene flow from natal bays or estuaries (Gold *et al.* 1999).

Our finding of significant genetic heterogeneity among spatial samples of red drum is counter to the prediction, based on mark-recapture data and life history of adults, that little to no stock structure in red drum should exist in the northern Gulf. It also differs from our previous studies of red drum from the northern Gulf where no significant differences were found in frequencies of both mtDNA haplotypes and alleles at several allozyme loci (Gold *et al.* 1993, 1994). We did, however, identify a weak isolation-

Table 1. Analysis of molecular variance (AMOVA) among mitochondrial DNA haplotypes of red drum (*Sciaenops ocellatus*) from the Gulf of Mexico.

Variance component	Observed partition		Φ_{ST} values	P[a]
	Variance	% total		
Gulf of Mexico				
Among localities	0.00100	0.21	0.002	0.014
Within localities	0.47443	99.79	–	–
East vs Central vs West Gulf[b]				
Among regions	0.00072	0.15	0.002	0.008
Among localities within regions	0.00052	0.11	0.001	0.135
Within localities	0.47443	99.74	–	–

[a]Probability of more extreme variance component by chance (1,000 permutations)
[b]East Gulf localities (Sarasota Bay, Tampa Bay, Riviera Bay, Apalachicola Bay); Central Gulf localities (Biloxi Bay, Black Bay, Grand Isle): West Gulf localities (Sabine Pass, Galveston Bay, Pass Cavallo, Redfish Bay, Corpus Christi Bay, Upper Laguna Madre, Lower Laguna Madre)

by-distance effect in the distribution of mtDNA haplotypes (Gold *et al.* 1993). The difference between this and our previous studies is the almost twofold difference in the number of fish sampled from individual localities, from an average of 50-60 fish per locality to nearly 100. This increase in sample size appears to have amplified the isolation-by-distance effect to the point where the differences in mtDNA haplotype frequencies between samples from geographically distant localities have become statistically significant. What this demonstrates is that the significant genetic heterogeneity observed among red drum in the northern Gulf does not necessarily identify discrete subpopulations or stocks with definable and discrete geographic boundaries but rather the existence of genetic units defined by geographic distance from a bay or estuary of natal origin. This "stock" concept differs from that typically encountered in most studies of stock structure in marine fishes where subpopulations (stocks) are assumed to occupy areas defined by geographic boundaries. In the case of red drum, we suggest the upper limit to geographic boundaries from natal bays or estuaries could be approximately 700-750 km.

Two final points are as follows. First, we have hypothesized elsewhere (Gold *et al.* 1999) that the isolation-by-distance effect observed in red drum is likely a function of behavior and could stem from either natal site philopatry (homing) or limited offshore movement relative to a natal bay or estuary. Genetic data alone do not discern between these alternatives, and it is necessary to point out that in red drum, the hypothesis is restricted to females, as the effect is based on the maternally inherited mtDNA molecule. The second point is that the degree of genetic divergence among red drum in the northern Gulf, although significant, is small. The Φ_{ST} values of 0.002, obtained from AMOVA (Table 1), differed significantly from zero but are consistent with relatively high levels of genetic

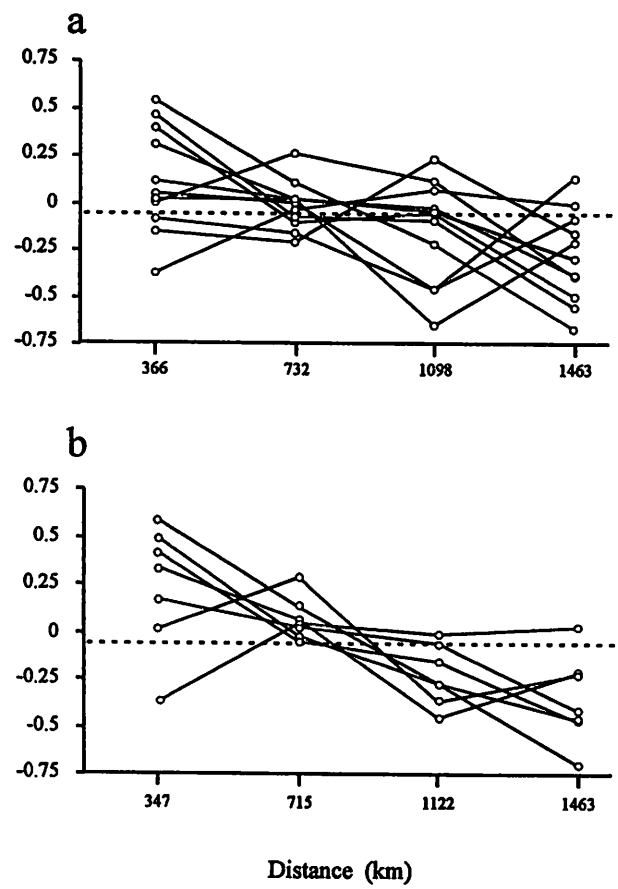

Figure 4. Spatial autocorrelation correlograms of mtDNA haplotypes of red drum with a significant Moran's I value in one or more distance classes. Abscissa: distance classes, with equal frequencies per distance class (a) and equal distances between distance classes (b). Ordinate: Moran's I values. Dashed line is Moran's I value expected when no correlation exists.

effective migration throughout the northern Gulf. Using the island model of Wright (1943), and substituting Φ_{ST} for F_{ST}, Gold *et al.* (1999) estimated the number of genetic effective migrants (N_em) to be approximately 250 individuals per generation. This dramatically underscores the principle noted by Wright (1969) that significant genetic divergence can occur in the face of high gene flow and points to the use of caution in employing N_em estimates as an index of population subdivision.

Red snapper: Results of our analysis of mtDNA variation among samples of red snapper from the Gulf are reported fully in Gold *et al.* (1997b) and Gold and Richardson (1998). Briefly, analysis of the distribution of mtDNA haplotypes both between or among samples of red snapper taken at different years from the same localities, and among localities from northern Florida to the Yucatan Peninsula in Mexico, revealed little evidence of temporal or spatial population structure. Analyses included tests of homogeneity in mtDNA haplotype frequency, estimates of Θ (the F_{ST} analog of Weir and Cockerham 1984), and *V* tests (DeSalle *et al.* 1987) of the spatial distribution of individual haplotypes found in four or more individuals. We also constructed a minimum-parsimony network (Figure 5.) by connecting composite haplotypes based on single restriction-site gains or losses. The most common haplotype (*a*), occurring in >48% of all individuals surveyed, formed a central "hub" that was connected by single restriction-site differences to other "hub" haplotypes (*b-e*), occurring in frequencies of 13%, 5.5%, 4.1%, and 2.4%, respectively. None of the other "hub" haplotypes or their derivative haplotype groupings were restricted geographically, i.e., each occurred occurring at multiple localities across the northern Gulf. The same pattern, i.e., absence of geographic cohesion or restriction of a haplotype or haplotype grouping, also was observed for haplotypes occurring in 2% or fewer of the individuals assayed (Table 2). Our finding that both haplotype groups and rare haplotypes do not exhibit spatial partitioning is consistent with results of homogeneity testing in demonstrating the absence of detectable population structure in red snapper from the northern Gulf.

We also carried out spatial autocorrelation analysis of mtDNA haplotypes occurring in nine or more individuals (10 haplotypes total). Three significant ($P<0.05$) Moran's I values (all negative) were generated in SAAP runs when equal distances between distance classes were used: two occurred in the penultimate distance class and one occurred in the last distance class (Gold *et al.* 1997b). Nearly identical results were obtained in SAAP runs that used equal numbers of pairwise comparisons in each distance class. Overall, mean Moran's I values were negative in all distance classes and did not differ significantly ($P > 0.05$) from expected values of I in the absence of autocorrelation (Gold *et al.* 1997b).

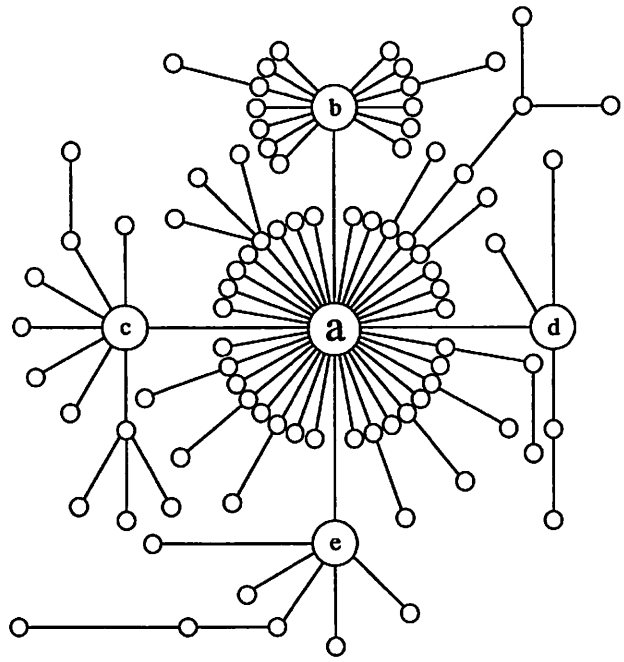

Figure 5. Minimum-length parsimony network of mtDNA haplotypes of red snapper sampled from the Gulf of Mexico. Except for "hub" haplotypes (*b-e*), branch lengths between haplotypes are proportional to the number of (inferred) restriction-site changes. "Hub" mtDNA haplotypes b-e differ by one restriction-site difference from the most common (*a*) haplotype.

Heist and Gold (1999) recently examined the distribution of alleles at five, nuclear-encoded microsatellite DNA loci among four of the samples of red snapper examined previously for variation in mtDNA. Briefly, microsatellites are short stretches of nuclear DNA composed of di-, tri-, and tetranucleotide arrays that are distributed throughout chromosomes and embedded in unique DNA sequences (Weber 1990, Wright 1993). Because variants (new alleles) at microsatellite loci are believed to arise more rapidly than variants in mtDNA or nuclear sequences encoding proteins, microsatellites have the potential of being more effective in detecting population structure than either mtDNA or allozymes. This has been demonstrated recently in both Atlantic cod (Bentzen *et al.* 1996, Ruzzante *et al.* 1997) and Atlantic salmon (McConnell *et al.* 1997).

We examined a total of 194 individuals from the four geographic localities: Panama City (Florida), Dauphin Island (Alabama), Galveston (Texas), and Merida (Mexico). Allele distributions at each locus were similar; each locus possessed one or two common alleles and a number of low-frequency alleles (data not shown). Genotype frequencies at each locus, whether considered by sample or pooled across samples, did not differ significantly ($P>0.05$) from expected genotype proportions based on Hardy Weinberg equilibrium. Tests of (spatial)

Table 2. Geographic distribution of low frequency mitochondrial (mt)DNA haplotypes in red snapper (*Lutjanus campechanus*) from the Gulf of Mexico.

Locality	MtDNA haplotype number													
	4	6	7	13	14	15	17	19	27	29	30	38	55	68
Merida, Mexico	1	–	2	1	–	–	–	–	1	–	1	–	–	–
South Padre Island, TX	–	3	1	3	1	2	–	2	3	–	1	1	–	–
Port Aransas, TX	1	–	1	3	2	1	–	1	2	2	–	2	4	1
Galveston, TX	–	4	1	–	–	1	1	–	4	1	2	–	–	1
West Cameron, LA	3	–	–	1	–	–	2	–	1	1	–	–	–	2
Port Fourchon, LA	–	–	–	–	–	1	5	1	1	1	2	1	–	–
Dauphin Island, AL	1	–	2	3	–	1	3	–	1	–	–	–	1	–
Pensacola, FL	1	1	1	1	1	–	–	–	–	–	–	–	–	–
Panama City, FL	3	1	3	1	1	1	6	1	–	1	–	1	1	1

homogeneity of allele frequencies at each of the five loci, and tests of whether estimated Θ (after Weir and Cockerham 1984) and R_{ST} (another analog of Wright's F_{ST}, after Slatkin 1995) values differed from zero, were uniformly non-significant (Table 3). Estimates of Θ and R_{ST} between pairs of samples at each locus (and over all loci) also did not differ significantly from zero, nor did pairwise values of either Θ or R_{ST} vary with geographic distance between localities (data not shown).

Patterns of genetic variation observed to date in red snapper are consistent with the hypothesis of a single population (stock) in the northern Gulf. Frequencies of both common and rare mtDNA haplotypes and of alleles at five microsatellite loci appear randomly distributed across the northern Gulf and there appears to be no phylogeographic structure to mtDNA haplotypes or haplotype groupings (lineages). Existence of a single, Gulf-wide population of red snapper is counter to the prediction, based largely on mark-recapture and sonic-tracking experiments involving adults (Bradley and Bryan 1975, Fable 1980, Szedlmayer 1997), that population structure could exist because of limited potential for gene flow. Assuming that adults are sedentary, the simplest hypothesis (based on the observed genetic data) is that gene flow occurs via hydrodynamic transport of pelagic eggs and larvae (Goodyear 1992).

There are, however, caveats to the hypothesis of a single red snapper population in the northern Gulf. The first is that one cannot prove a null hypothesis. Red snapper could be subdivided in the northern Gulf yet have the same parametric allele frequencies for the loci thus far examined. A second caveat is that under a hydrodynamic-transport hypothesis one would expect more extensive movement of pelagic eggs and larvae between geographically contiguous localities than between geographically distant ones. Surface current patterns in the northern Gulf are not particularly strong and often go in reverse directions (Shulman and Bermingham 1995). This would be expected to generate a "stepping-stone" pattern of egg and larval transport, that in turn should produce an isolation-by-distance effect where correlations in mtDNA haplotype frequencies vary from positive values between geographically proximal localities to negative values between geographically distant localities. Spatial autocorrelation analysis of red snapper mtDNA, however, revealed no overall correlation (positive or negative) among haplotypes based on geographic distance between localities. In addition, the relationship between genetic distance and geographic distance at microsatellite loci was non-significant. The absence of patterns indicating an isolation-by-distance effect in red snapper from the northern Gulf remains puzzling. Finally, one of the assumptions underlying use of genetic data to assess population structure is that populations are at genetic equilibrium with respect to genetic drift and migration. We have hypothesized elsewhere (Gold *et al.* 1997b) that red snapper in the northern Gulf may represent immigrant subpopulations that expanded northward following the last glacial retreat. Waters on the reduced continental shelf in the northern Gulf were much cooler during the Pleistocene (Rezak *et al.* 1985), and may not have provided suitable habitat for red snapper. Very possibly, there has been insufficient time for present-day red snapper subpopulations to reach the genetic equilibrium.

CONCLUSIONS

Our genetic studies to date on red drum and red snapper in the Gulf of Mexico (Gulf) demonstrate the following. First, predictions regarding population structure that are based on approaches such as mark-recapture (tagging) or are inferred from life history may be misleading. Red drum form large schools in offshore waters of the Gulf and

Table 3. Tests of spatial homogeneity of five microsatellite loci among samples of red snapper (*Lutjanus campechanus*) from the Gulf of Mexico. Sample localities were Panama City (Florida), Dauphin Island (Alabama), Galveston (Texas), and Merida (Mexico).

Locus	[a]P_{RB}	Θ	[b]P	R_{ST}	[b]P
Lca-20	0.32	− 0.001	0.87	− 0.007	0.76
Lca-22	0.21	− 0.003	0.77	− 0.009	0.90
Lca-43	0.08	0.012	0.08	0.008	0.26
Lca-64	0.28	0.001	0.27	0.008	0.12
Lca-91	0.43	0.004	0.22	− 0.014	0.85

[a] P_{RB} is probability based on the randomization procedure (1,000 replicates) of Roff and Bentzen (1989)
[b] P is probability of the null hypothesis that Θ or $R_{ST}=0$, based on random permutation (1,000 trials per comparison).

are known to move considerable distances yet gene flow appears to be related inversely to the bay or estuary of natal origin. Red snapper, alternatively, exhibit sedentary behavior as adults yet gene flow appears to be unrestrained across the northern Gulf. Thus, contrary to prediction, there is limited gene flow in red drum but not red snapper. Programs for stock enhancement for the two species accordingly should be designed differently. Enhancement of red drum should be based on the premise that supplementation of a given bay or estuary will impact geographically proximal bays or estuaries more than geographically distant ones. Second, stock structure *per se* does not necessarily take the form of discrete, geographic units where gene flow is abruptly limited. Gene flow among red drum in the northern Gulf appears to be limited not by geography but by other factors that are perhaps behavioral in nature. Finally, there are constraints regarding inferences about population structure that are based on genetic studies. These constraints largely relate to whether a population is, or subpopulations are, in genetic equilibrium and whether historical or contemporaneous genetic signatures have been uncovered. At present, the issue of stock structure of red snapper in the northern Gulf may be constrained by this problem.

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U.S. GULF OF MEXICO MARINE STOCK ENHANCEMENT FIVE-YEAR PROGRAM: 1999 - 2004

PROGRAM MANAGEMENT FOR PLANNING, EXECUTION, REVIEW, and EVALUATION

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ABSTRACT

Many marine fisheries in the United States are under stress from overexploitation and/or habitat degradation; in response, Congress has mandated the restoration of depleted U.S. fisheries, within a given time period. Preliminary estimates indicate that conventional restrictive management protocols alone cannot satisfy these congressional mandates. The tools and options of marine fishery management need to be expanded to include proactive actions and activities. One such potentially proactive option is stock enhancement. A decision has been made to fund a research stock enhancement program to better evaluate its potential value. The U.S. Gulf of Mexico Marine Stock Enhancement Program is a multi-institutional, multi-year and multi-million dollar integrated scientific effort to develop and demonstrate sound and broad-based stock enhancement methods to augment conventional management strategies. The authors identify and characterize the critical components of an integrated, scientifically sound investigation of a highly complex stock enhancement system. It initiates use of a computerized management approach which enhances planning, review, and evaluation efforts by identifying and tracking critical activities, institutional assignments and responsibilities, developing time line and completion projections, and managing resource allocation.

INTRODUCTION

The United States, through the National Oceanic and Atmospheric Administration (NOAA), Regional Fisheries Management Councils and Interstate Marine Fisheries Commissions (IMFC), manages, conserves, and protects living marine resources within its Exclusive Economic Zone. Their aim is to maximize benefits to the nation from these living resources without compromising the long-term health of coastal and marine ecosystems. The Magnuson-Stevens Fishery Conservation Act, as amended by the Sustainable Fisheries Act, requires that fishery management plans contain both conservation and management measures that prevent overfishing while achieving optimum yield from each fishery.

Many factors, both natural and human-related, affect the status of stocks and ecosystems. Although it is virtually impossible to control or even alter most factors, scientific information concerning trends and relationships has supported the development and utilization of sophisticated fishery management tools and practices. Nonetheless, many marine fisheries are under stress from over-exploitation and/or habitat degradation. One-third of all fish stocks, for which there is population information, are overutilized, and nearly half are below optimum population size. Congress mandated the restoration of U.S.

fisheries, within a given time period, through the elimination of overfishing and habitat destruction. Preliminary estimates indicate that conventional restrictive management protocols (restricting harvest and preventing habitat loss) alone cannot satisfy these congressional mandates.

The tools and options of marine fishery management need to be expanded to include proactive actions and activities. One such potentially proactive option is stock enhancement. Historically, massive releases of fish stocks did not produce the desired results. Stock enhancement, as a means to replenish fisheries, has been largely ignored for the last 30-40 years. It appears, however, that previous efforts in stock enhancement were without scientific merit as they were essentially limited to the production and release of fish. Recent, and more sophisticated small scale efforts to replenish salmonids, scallops, and Hirame flounder fisheries through stock enhancement have shown clear promise.

A decision has been made to fund a research stock enhancement program to better evaluate its potential value. The U.S. Gulf of Mexico Marine Stock Enhancement Program (USGMMSEP) is a multi-institutional, multi-year, and multi-million dollar integrated scientific effort to develop and demonstrate sound and broad-based stock enhancement methods to augment conventional management strategies. This stock enhancement program is based upon solid scientific principles and utilizes many recent advances in aquaculture and stock and ecosystem assessment. This paper does not extend the discussion of the potential value of stock enhancement. Rather, it begins subsequent to a decision to proceed. The question addressed is how to best manage, execute, review, and evaluate effective management of a complex program. In part, the success of USGMMSEP will depend upon the quality of program planning, review, and evaluation efforts.

This paper identifies and characterizes the critical components of an integrated, scientifically sound investigation of a highly complex system. It initiates use of a computerized program management approach which enhances planning, review, and evaluation efforts by identifying and tracking critical activities, institutional assignments and responsibilities, developing timeline and completion projections, and managing resource allocation. The computerized program management must be maintained in real time, being updated and modified as new and better information becomes available.

The paper describes "*Cornerstone Issues*" outlining the approach, justification, and strategies for this stock enhancement initiative. The paper suggests an interface between conventional stock management activities by NOAA and others and this stock enhancement initiative; it follows by identifying the *critical projects* upon which the success or failure of the stock enhancement initiative will depend. These critical activities and their subsets are organized in project manager format. From that format, *institutional assignments, a five-year timeline*, and *resource allocation* are addressed. The plan provides a sound basis for *review and evaluation* of efforts expended, technical highlights, cost and/or institutional problem areas - if and when they develop. The plan also reflects corrective actions as taken to resolve any problems.

CORNERSTONE ISSUES

The mission of the U.S. Gulf of Mexico Marine Stock Enhancement Program (USGMMSEP) is the refinement, field testing, and demonstration of successful marine fishery management for the Gulf of Mexico that blends aquaculture technology with traditional fishery management practices. Responsible stock enhancement will be developed to supplement wild stocks production without significantly altering the genetic diversity of resident populations. Upon development and validation, responsible stock enhancement can be used to augment traditional management strategies of catch restriction and habitat restoration.

The USGMMSEP is solidly based upon scientific principles, innovative technologies, and a sophisticated approach. It intends to augment and ease, but not replace, conventional restrictive fisheries management strategies. Successful stock enhancement to restore certain fish populations could simultaneously speed restoration and minimize the need for the most draconian restrictions. There are many factors which strongly influence both the structure and approach of this initiative. The "Cornerstone and Long-Term" issues that must be recognized and honored for this program to succeed are:

1. Dramatic advances in marine aquaculture, stock enhancement and fisheries management technologies have made possible a new integrated, proactive, and holistic approach to fisheries restoration and management.
2. Congress has mandated the restoration of U.S. fisheries, within a given time period, through the elimination of both overfishing and habitat destruction (Magnuson-Stevens Act as amended by the Sustainable Fisheries Act); however:
   A. Existing restrictive fisheries management strategies alone cannot satisfy the congressional mandate.
   B. Stock enhancement activities alone cannot restore fisheries and satisfy the congressional mandate.
   C. The combination of selected fisheries management strategies and stock enhancement could satisfy the congressional mandate for at least some fisheries.
3. The design and execution of cost-effective stock enhancement requires a thorough understanding of the selected fishery and its existing limitations, including the rationale underlying existing and planned fisheries

management restrictions.

4. Only those fisheries constrained by inadequate spawning populations, or such constraints that can be overcome by the release of fish, will be selected for enhancement activities.
5. There are downside health, genetic, and ecosystem risks associated with the production and release of unhealthy and/or genetically compromised fish. These may include, but not be limited to, introduction of disease, reduction of genetic diversity, and the alteration of communities and ecosystems.
6. There is a paucity of data on the health status, genetic status, and community and ecosystem structures of important Gulf of Mexico fisheries. Data must be collected, analyzed, and used as selection criteria among candidate fish species being considered for this stock enhancement program.
7. Only high health (specific pathogen-free) fish, with appropriate genetic characteristics should be considered for release.
8. Preliminary stock enhancement releases must be undertaken under suitable experimental designs which address release sites, release periods, size at release, etc., in order to determine appropriate release strategies.
9. Physical, chemical, and biological tags must be developed and utilized to track released fish, including future generations, to obtain quantitative estimates of the impact of the fishery and support cost per benefit analyses.
10. The stock enhancement program must develop and utilize a broad and all-inclusive integrated scientific approach. It is the only responsible means for the development and integration of marine stock enhancement in fisheries management strategies.
11. No single institution is capable of addressing all critical issues; therefore, a multi-institution consortium was formed where each institution contributes specialities within a coordinated effort.
12. Current consortium institutions include the Gulf Coast Research Laboratory (GCRL), Mississippi; The Oceanic Institute (OI), Hawaii; and Mote Marine Laboratory (MML), Florida. Together, they have the necessary expertise in fish maturation and reproduction, fry production, fry transport, fish health, fish genetics, fisheries ecology, fishery biology, fish behavior, fish tagging, risk assessment, mathematical modeling, fishery economics, and fishery management. Additional institutions will be invited to participate to meet program requirements.
13. With demonstrated success in the Gulf of Mexico, a U.S.-wide stock enhancement program will be developed in multiple geographic areas.

CRITICAL PROJECTS AND SUBPROJECTS

Conventional Fishery Management Program

It is essential that the stock enhancement initiative interface closely with the conventional fishery management programs. Conventional fishery management programs are guided and funded by NOAA's National Marine Fisheries Service (NMFS), and the Gulf of Mexico Fisheries Management Council (GMFMC). It is generally understood that successful stock enhancement efforts must be an integral part of, and are dependent upon, comprehensive fishery management activities. Essential fishery management activities include, but are not limited to, stock assessment, the identification of overfished stocks, factors contributing to the overfished condition, and subsequent regulations to restore or sustain a fishery. Decisions guiding the stock enhancement initiative and monitoring its long-term results are and will be based upon the information provided by, and controls imposed by, fisheries managers. Figure 1 shows conventional stock management activities (solid lines) augmented by stock enhancement (dotted lines) as an additional management tool.

Stock Enhancement Program

Institutional principal investigators (K. Leber, J. Lotz, and D. Ziemann) individually analyzed the overall stock enhancement initiative, and identified and recommended consideration of certain critical technical activities and subactivities. Their input was integrated then subdivided into ten categories as follows: (A) Species Selection; (B) Fishery Demographics and Ecology; (C) Disease and Parasites; (D) Fish Culture for Release; (E) Genetics; (F) Tags and Tagging Technologies; (G) Cost and Benefit Analysis; (H) Release and Recovery Strategies; (I) Fish Behavior and Conditioning; and (J) Multiple Experimental Releases and Evaluation (Figure 2). Note that the purpose of this paper is to describe a management program for enhancing the planning, review, and evaluation functions. These functions require a clear picture of specific activities to be undertaken and their interactions. The categories selected above, while rational, do not represent the only possible breakdown or even the ultimate breakdown of activities. However, they do provide a point of departure.

It is noted here that the first activity required under each critical project is a review paper generated from a comprehensive workshop. This stock enhancement program will publish a book reviewing the status of all critical activities in this stock enhancement research program, targeting "Red Snapper" in the Gulf of Mexico.

A. *Species Selection*

Species selection involves a primary and secondary

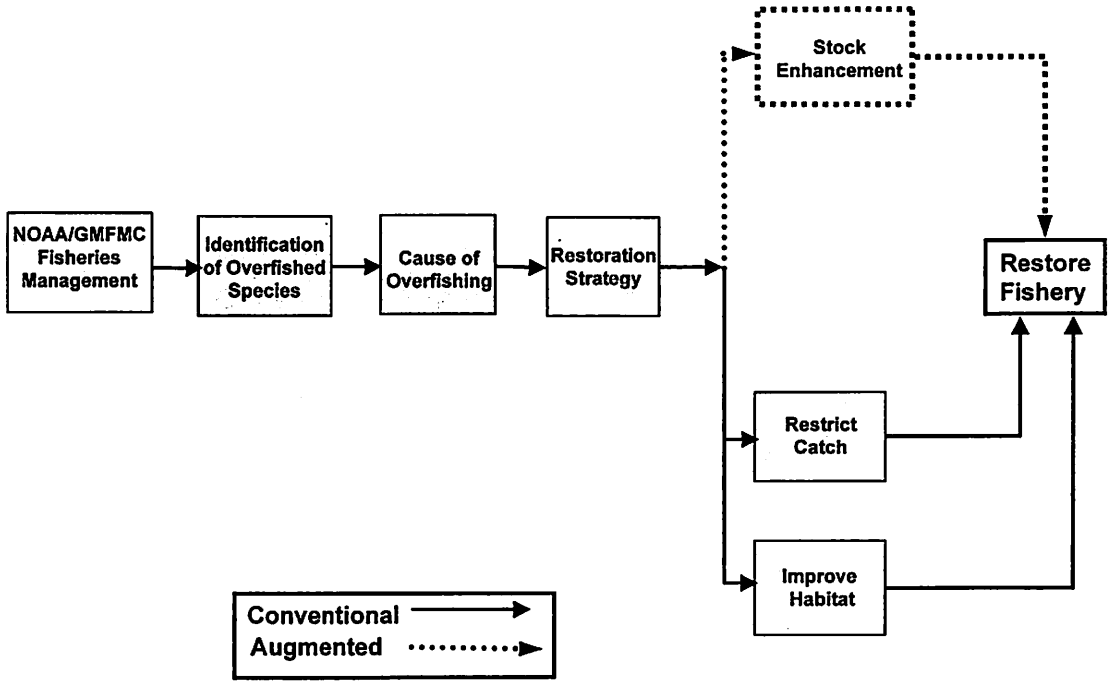

Figure 1. Augmented Fisheries Management

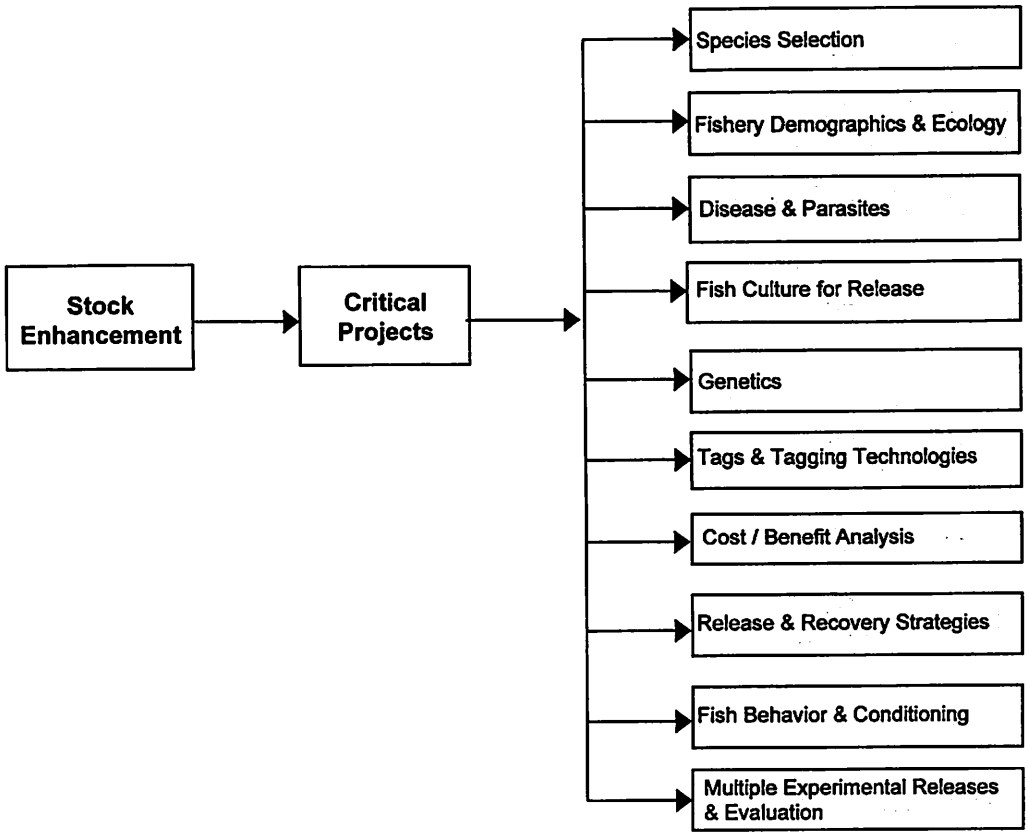

Figure 2. Critical Projects for Stock Enhancement

selection process, which are:

a. Primary selection: the species must be designated as overfished by NOAA, GMFMC or IFMC. In the case of the Gulf of Mexico, there are four fisheries that are designated as overfished; these are Red Snapper, Nassau Grouper, Jewfish and Red Drum. Therefore, the final species selection for this stock enhancement initiative must be Red Snapper, Nassau Grouper, Jewfish or Red Drum.

b. Secondary Selection: Leber (1994) organized and executed a species selection workshop titled "Species Prioritization of Marine Finfish for Stock Enhancement in Hawaiian Waters." In planning sessions, a consensus was reached on both the prioritization and relative weights given to various selection criteria; the most significant issues in ranking and weight are extracted as follows:

Selection Criteria for Stock Enhancement Species Selection

Rank	Criterion for Selection	Weight	Importance
1	Commercial / Recreational Demand	108	12.6
2	Ease of Maturation and Reproduction	104	12.2
3	Releasing Juveniles Should Increase Population	90	10.5
4	Ease of Larval Rearing	83	9.7
5	Cost-Effectiveness of Stock Enhancement	64	7.5
6	Ease of Juvenile Rearing	61	7.1
7	Ease of Experimental Design and Monitoring	58	6.8
8.5	Extent of Recruitment Limitation	51	6.0
8.5	Likelihood of Rapid Success	51	6.0
10	Impact of Resident Biota	29	3.4
11	Low Mortality : Growth Ratio	24	2.8
12	Documented Decline in Fish Stock Landings	23	2.7
13.5	Availability of Habitat	19	2.2
13.5	Residential versus Migratory	19	2.2

Also listed, in descending order, were: socioeconomic attractiveness; inshore seasonal availability; fishing pressure; facilities; ease of protection until market size; reproduction in a habitat that is limited or degraded; availability of food; ease of transport and distribution; cost of monitoring effect; seasonal/environmental factors; mitigation issues; nonconsumptive uses; and size at capture.

This study serves to illustrate the potential complexities involved in species selection. It is important to note that in addition to being complex, there is a paucity of data on the criteria identified. As the information base is expanded, more precise species selection will be possible.

In early 1998, USGMMSEP convened a meeting of stakeholders to select a candidate species, among Red Snapper, Nassau Grouper, Jewfish and Red Drum. Red Snapper was virtually the unanimous choice. The selection was based primarily on the importance of the fishery to both commercial and recreational interests. When time allows, a more formal selection process will be revisited, for information purposes only, using a full set of selection criteria.

B. *Fishery Demographics and Ecology*

The first critical activity in this category is completion of a review paper "Demographics and Ecology of the Gulf of Mexico Reef Fish: Red Snapper." This manuscript will be produced as part of a book "Stock Enhancement of Red Snapper" produced by a workshop to be held in 1999.

Other critical activities and subactivities included in this category are:

a. Habitat Assessment
   - Habitat assessment surveys and experimental site selection
   - Determine essential fish habitat - SCUBA video, reef rugosity

b. Population Structure
   - Wild population demographics: stock substructure, distribution, age structure, sex ratio, fecundity, variation
   - Populations surveys - SCUBA, hook and line, baited video, trapping and tagging
   - Population model

c. Ecology of Resident Population
   - Growth and reproduction
   - Dietary requirements, preferences, feeding rates, feed Availability at candidate release sites
   - Mortality (age dependent)
   - Prey-predator relationships
   - Wild fish behavior-aggregation, schooling, horizontal movement, circadian rhythm
   - Wild juvenile behavior
   - Juvenile recruitment patterns and abundance
   - Ecophysiological requirements

C. *Disease and Parasites*

The first critical activity in this category is completion of a review paper "Diseases and Parasites of the Gulf of Mexico Reef Fish: Red Snapper." This manuscript will be produced as part of a book "Stock Enhancement of Red Snapper" produced by a workshop to be held in 1999.

Other critical activities and subactivities in this category are:

a. Assess the Disease and Parasite Status of Wild Populations

b. Develop Health Specification for Fish to be Released

c. Develop Health Management and Quarantine Guidelines
   - Broodstock acquisition and holding
   - Maturation/reproduction

Larval production
Fry culture

d. Monitor Disease and Parasite Status of Fish Across All Culture Stages

e. Assess the Disease and Parasite Status of Recovered Fish (Environmental Indicator).

D. *Fish Culture for Release*

The first critical activity in this category is completion of a review paper "Fish Culture of the Gulf of Mexico Reef Fish: Red Snapper." This manuscript will be produced as part of a book "Stock Enhancement of Red Snapper" produced by a workshop to be held in 1999.

Other critical activities and subactivities in this category are:

a. Broodstock Acquisition
Establish primary broodstock acquisition site
Establish alternative broodstock acquisition sites
Develop broodstock quarantine, screening, and holding facilities
Develop alternative quarantine, screening, and holding facilities
Collect broodstock, screen, hold, and condition
Produce healthy, mature, and genetically diverse broodstock

b. Maturation and Reproduction Operations
Develop maturation and spawning protocols
Produce sufficient numbers of fertilized eggs

c. Hatchery Operations
Develop hatchery protocols including live feed production
Produce sufficient number of healthy and genetically diverse fry

d. Nursery Operations
Develop nursery protocols to improve fish fitness and tolerance
Improve growth rate and survival
Condition fish for wild feeds
Condition fish for predator avoidance
Tag individuals
Produce sufficient numbers of healthy, genetically diverse, conditioned, and tagged fingerlings for release purposes.

E. *Genetics*

The first critical activity in this category is completion of a review paper "Genetics of the Gulf of Mexico Reef Fish: Red Snapper." This manuscript will be produced as part of a book "Stock Enhancement of Red Snapper" produced by a workshop to be held in 1999.

Other critical activities and subactivities in this category are:

a. Wild Stock Variability
Genetic structure or structures
Develop genetic analytical protocols

b. Broodstock Genetic Structure
Establish reliable sources for broodstock acquisition
Establish stock genetic diversity
Develop a broodstock selection and management plan
Monitor genetic structure of broodstock acquired

c. Develop Genetic Protocols and Guidelines for Fish-for-Release

d. Monitor and Screen the Genetics of Fingerlings for Release Purposes

e. Monitor and Screen the Genetics of Recaptured Fish

f. Seek Cooperation and Inclusion in NMFS Red Snapper Genetics Program

F. *Tags and Tagging Technologies*

The first critical activity in this category is completion of a review paper "Individual Tags for the Gulf of Mexico Reef Fish: Red Snapper." This manuscript will be produced as part of a book "Stock Enhancement of Red Snapper" produced by a workshop to be held in 1999.

Other critical activities and subactivities in this category are:

a. Technology Development
Adapt existing tag and tagging technology
Determine tag reliability
Evaluate tagging effect on hatchery fish
Adapt mass marking technologies
Establish quality control for tag implant performance
Develop tag coding management
Develop tagging facilities
Computerize tag decoding capabilities

b. Genetic Tags
Identify genetic markers
Broodstock - molecular tags
Fingerling - molecular tags

G. *Cost/Benefit Analyses*

The first critical activity in this category is completion of a review paper "Cost/Benefit Analysis for Stock Enhancement of the Gulf of Mexico Reef Fish: Red Snapper." This manuscript will be produced as part of a book "Stock Enhancement of Red Snapper" produced by a workshop to be held in 1999.

Other critical activities and subactivities in this category are:

a. Culture Costs
Adapt hatchery-cost model
Analyze laboratory scale process in use by USGMMSEP and scale to full production and preparation of fingerlings for release
Complete sensitivity studies to identify areas for cost reduction

b. Release Costs
Analyze laboratory scale releases and expand to estimate full scale Release costs
Complete sensitivity studies to identify areas for cost reduction

c. Monitoring and Recovery Costs Projected for Inclusion in Fisheries Management protocols

H. *Release and Recovery Strategies*

The first critical activity in this category is completion of a review paper "Release and Recovery Strategies for Stock Enhancement of the Gulf of Mexico Reef Fish: Red Snapper." This manuscript will be produced as part of a book "Stock Enhancement of Red Snapper" produced by a workshop to be held in 1999.

Other critical activities and subactivities in this category are:

a. Release Strategies
- Site Selection Guidelines
- Microhabitat, feed, predator refuge
- Fish size and release number guidelines
- Seasonal/chronological guidelines
- Site, fish size, release number, and seasonal interaction guidelines
- Transport, delivery, and injection methods and materials
- Acclimation in release habitat
- Experimental releases

b. Recovery Strategies
- Random sampling in test and control sites
- Random stratified sampling in test and control sites
- Guidelines for recovery from recreational fishery
- Guidelines for recovery from commercial fishery
- Recovery of experimental releases

I. *Fish Behavior (hatchery and wild) and Conditioning*

The first critical activity in this category is completion of a review paper "Fish Behavior and Conditioning for Stock Enhancement of the Gulf of Mexico Reef Fish: Red Snapper." This manuscript will be produced as part of a book "Stock Enhancement of Red Snapper" produced by a workshop to be held in 1999.

Other critical activities and subactivities in this category are:

a. Compare Behavior of Hatchery and Wild Fish
- Nursery aggregation, schooling, horizontal movement, circadian
- Rhythm, predator avoidance, and feeding activities

b. Characterize Learning Ability of Red Snapper

c. Develop Conditioning Methods for Predator Avoidance

d. Develop Conditioning Methods for Natural Feeds

e. Condition Fingerlings for Release Purposes

J. *Multiple Experimental Fish Releases and Evaluation*

The first critical activity in this category is completion of a review paper "Multiple Experimental Research Releases for Stock Enhancement of the Gulf of Mexico Reef Fish: Red Snapper." This manuscript will be produced as part of a book "Stock Enhancement of Red Snapper" produced by a workshop to be held in 1999.

Other critical activities and subactivities in this category are:

a. Assessment and Improvement of Techniques

b. Assessment of the Impact on Wild Stock
- Demographics
- Genetics
- Disease and parasites
- Predators
- Prey
- Carrying capacity
- Ecological community

c. Assessment of Release/Recovery
- Survival rate after release
- Growth rate after release

d. Assessment of Stock Enhancement Success
- Contribution to juvenile recruitment
- Contribution to adult recruitment
- Contribution of released fish to reproduction and recruitment
- Contribution to fishery landings
- Contribution of released fish to wild stock abundance.

PROJECT MANAGER FORMAT DEVELOPMENT

The first task of identifying the critical projects and subprojects is complete. It is clear that the program is technically broad-based and complex with many internal and external interdependencies. Additional complexities arise from the fact that the program is both multi-year and multi-institutional. Fundamental questions arise, including:

- What needs to be done ?
- What are the interdependencies between needs ?
- Who will do it ?
- When will it be done ?
- What happens if something is not done ?
- What happens if it is not completed in time ?
- How much will it cost ?
- How much did it cost ?
- How does one review and evaluate accomplishments ?

From the outset, it is important to understand that effective review and evaluation starts with effective planning. There needs to be clarity among the investigators and administrators by identifying and addressing these questions in advance. The program management effort previously established a relationship between conventional and research activities and identified the critical projects.

Figure 3 depicts the critical projects and subprojects and how they related to research trials in an interactive way through multiple research releases and recoveries leading to the final goal of a transferrable stock enhancement technology package. It is envisioned that full scale stock enhancement releases will fall under the purview of state and federal agencies. It is recognized that there are many

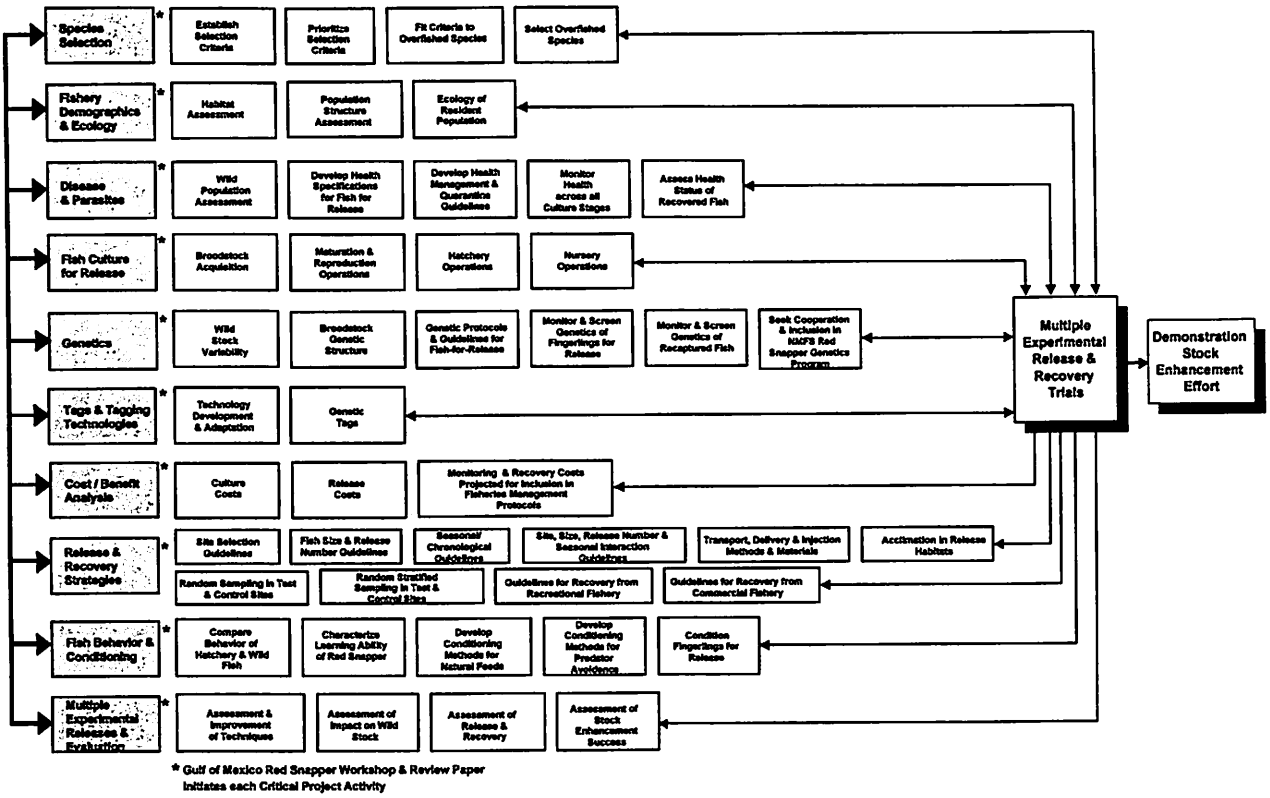

Figure 3. Critical Projects & Sub-projects for Stock Enhancement

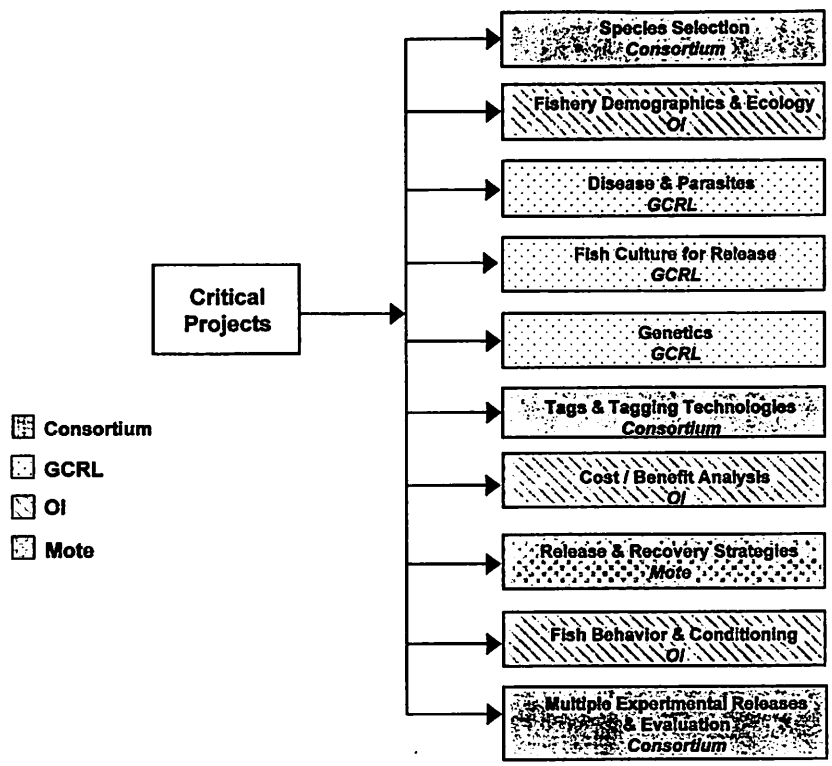

Figure 4. Principal Institutional Assignments

interdependencies between critical projects and sub-projects. Some projects must be started before others can be started. Other projects must be completed before other projects are started. The fundamental message is that none of the critical projects stands alone. The process of identifying these interdependencies is underway.

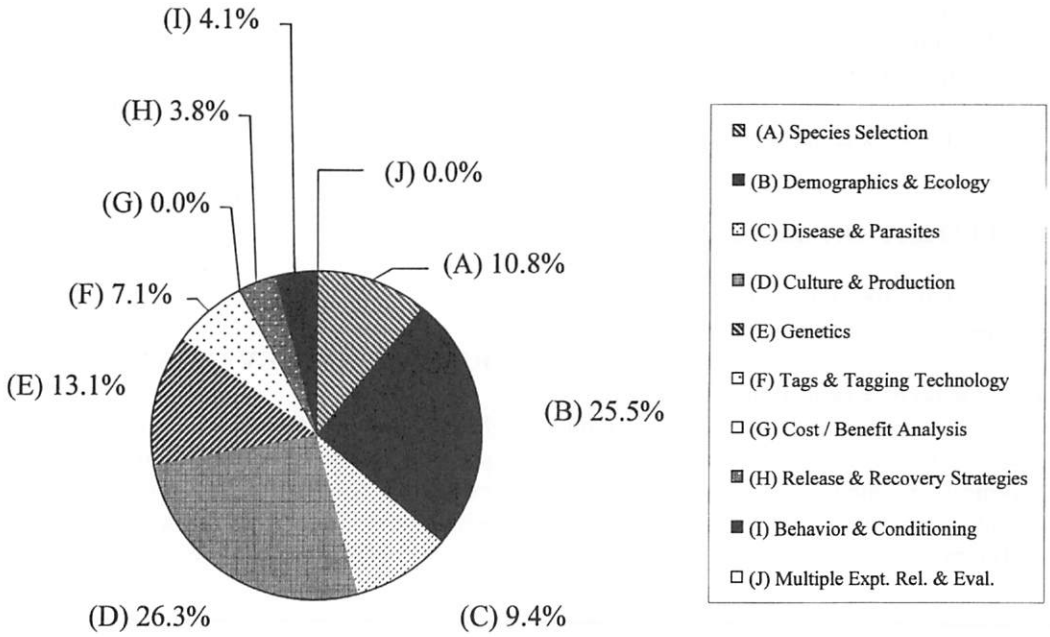

Figure 5. Resource Allocation by Project Activity-Year 1

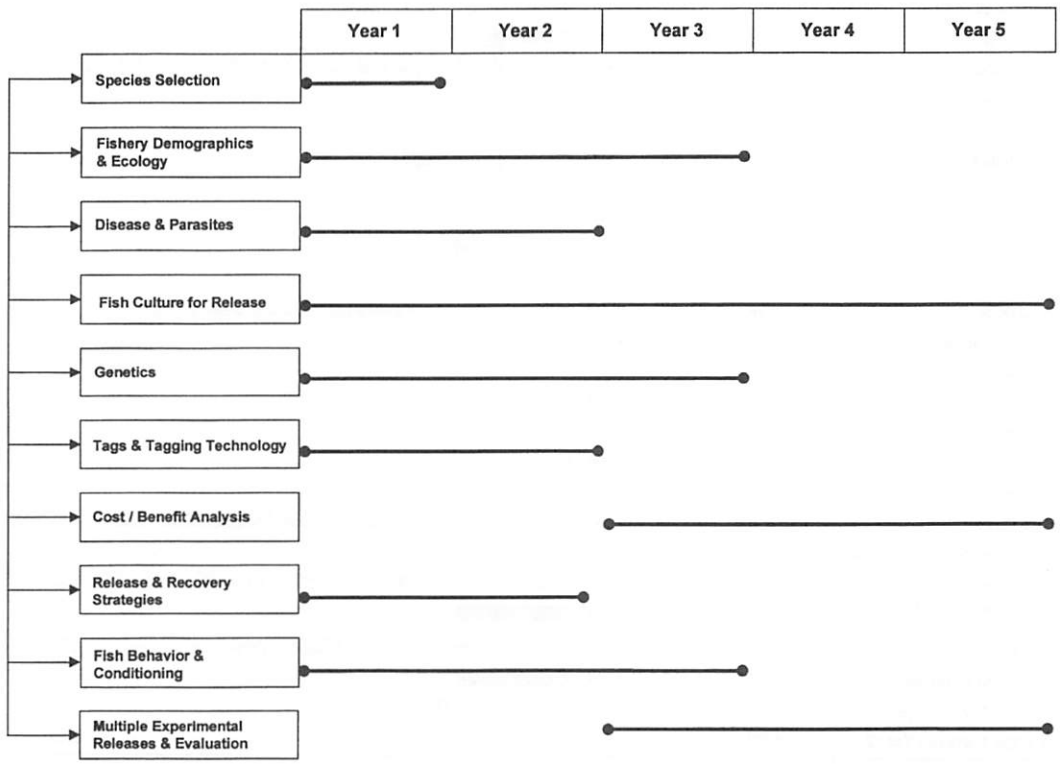

Figure 6. Timeline of Major Project Activities for Stock Enhancement

INSTITUTIONAL ASSIGNMENTS

This program currently involves three institutions, and may be expanded, and each institution has multiple investigators. Only institutional assignments of critical projects is completed (Figure 4). Responsibilities are designated as GCRL, OI, MML, or Consortium for the purposes of clarity. There are many subprojects and many more sub-subprojects that should be covered by institutional assignments. This work is in progress.

RESOURCE ALLOCATION

Resource allocation estimates for each of the critical projects are expected to change from year to year as some projects are completed and others are initiated. The percent allocation for each critical project for Year 1 are shown in Figure 5. The percentage allocations and funds available are expected to change with time as projects are completed and projects initiated. Unanticipated difficulties may require allocation changes to be implemented as the program continues.

FIVE-YEAR TIME LINE

It is difficult to estimate activities in outyears of complex projects. However, planning for outyears is essential. Programs such as this operate both in parallel and part in series. It is not effective to delay planning for subsequent steps until current steps are completed. Therefore, the program management plan focuses on a five-year period. This requires continual recognition of the ultimate goal, which in this instance is the transfer of stock enhancement technologies to suitable state or federal agencies for full scale exploitation and fisheries restoration. Year-by-year projections of times required for projects and subactivities are shown in Figures 6 and 7.

REVIEW AND EVALUATION PROCESS

Maximum value from this approach to management planning is derived by periodically (yearly) reviewing and evaluating project performance and accomplishments within the overall or long term picture (Figure 8). Especially important is the recognition of what must be

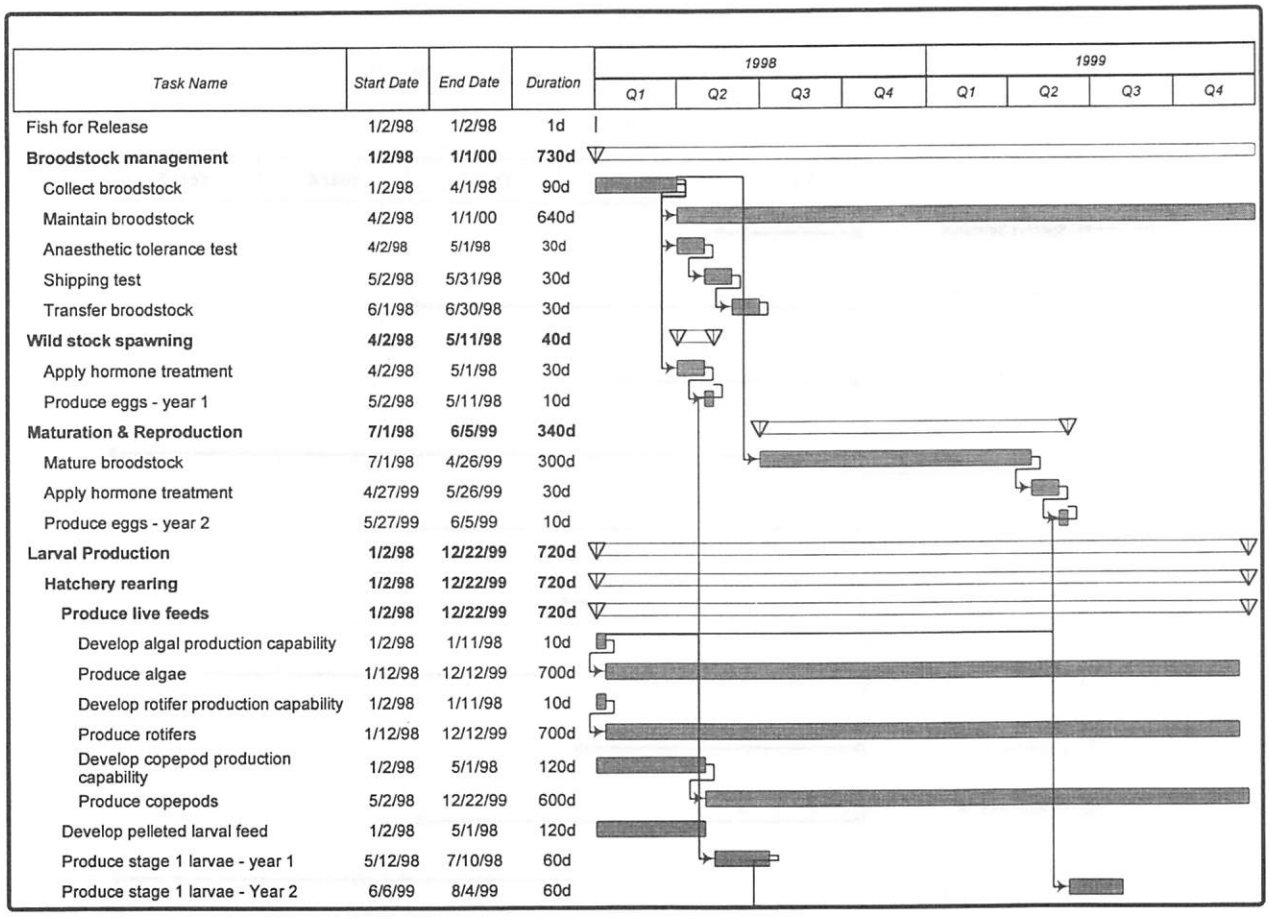

Figure 7. Detailed Timeline for Stock Enhancement Project Activities - Year 1

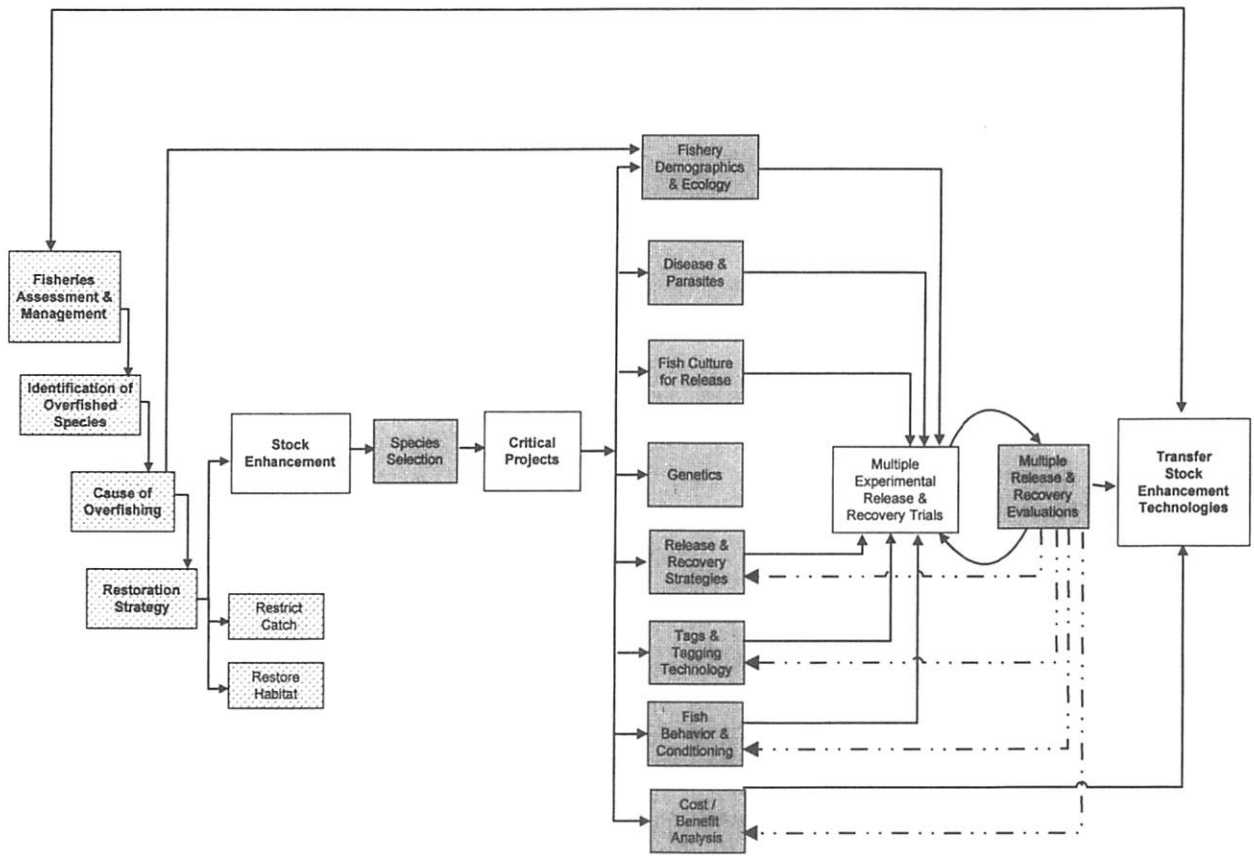

Figure 8. Major Stock Enhancement Program Actibities and Interactions

done differently to meet the long term objectives. The review and evaluation process must be followed by corrective actions which include: modification of assignment responsibility, modification of allocations, and recognition of alternative pathways to accomplish critical points.

These program management plans should be updated and used to guide the effort, review, and evaluation efforts for the next time period. The plan itself must be continually updated following rigorous review of the issues. Merely stretching timelines while failing to make corrective action renders the activity as useless.

ACKNOWLEDGMENTS

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CULTURE AND STOCK ENHANCEMENT OF SHORTNOSE AND ATLANTIC STURGEONS

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ABSTRACT

As early as 1875, the high value of Atlantic sturgeon *Acipenser oxyrinchus* flesh and eggs (caviar) prompted attempts to restore declining fisheries through artificial propagation. There was very limited success in producing fry and efforts were abandoned around the turn of the century. The first production of advanced juveniles occurred in South Carolina in 1981. However, due to the difficulty of capturing and handling the large broodstock, attention shifted to the much smaller shortnose sturgeon *A. brevirostrum* which was more readily available. Since the mid-1980s, a culture technology has been developed which includes spawning, larval rearing, and growout. The life cycle has been closed, and domestic broodfish are routinely spawned. A pilot scale stock enhancement study was conducted with shortnose sturgeon during 1984-1992 using juveniles produced from wild broodstock. Recent sampling suggests that some shortnose sturgeon stocked at an advanced age have failed to imprint on the target river. Some of these fish appear to have colonized a more northerly river. Life history studies on wild stocks of Atlantic and shortnose sturgeons have produced evidence that suggests these sturgeons imprint on their natal river like anadromous salmonids. Success of future stock enhancement efforts may depend on the ability to imprint sturgeon on target rivers. A study of the molecular genetics of wild and cultured shortnose sturgeon is underway with a limited number of specimens. Preliminary mtDNA analyses indicate that approximately 5% of the total genetic variation can be ascribed to differences among drainages, indicating a modest level of population structuring. Results of recent studies suggest that several populations of both Atlantic and shortnose sturgeon have been extirpated within the last 1-2 decades, and that other populations are in danger of extirpation, increasing the probability that stock enhancement will occur. In the past few years, research on culture of Atlantic sturgeon has resumed and small scale stocking efforts have been initiated. Information from genetic studies is essential for effective management and to guide stock enhancement efforts. In addition to potential use in fisheries enhancement, there is growing interest in commercial aquaculture of Atlantic coast sturgeons, similar to that of the Pacific coast white sturgeon *A. transmontanus*. Current fishery regulations limit commercial culture of both the Atlantic and shortnose sturgeons. However, as genetic information is developed and culture techniques are refined, there may be opportunity to farm these species under controlled conditions.

INTRODUCTION

Sturgeons (Acipenseridae) are distributed circumglobally in temperate waters of the northern hemisphere. In general, their eggs (caviar) and flesh are highly prized. Historically, several species in the United States supported substantial fisheries. Heavy fishing pressure and anthropogenic impacts to habitats have resulted in substantial decline in abundance in nearly all species. On the east coast, sturgeons were harvested at least as early as 2198 B.C. (Ritchie 1969). Exploitation of the Atlantic *Acipenser oxyrinchus*, shortnose *A. brevirostrum*, and lake *A. fulvescens* sturgeons intensified with the arrival of European settlers. By about 1900, most major fisheries had collapsed (Harkness and Dymond 1961, Smith 1985, 1990), and commercial harvest is now prohibited for these species throughout their U.S. range. On the west coast, white sturgeon *A. transmontanus* were heavily exploited but harvests are now strictly controlled.

Attempts at artificial propagation of U.S. sturgeons began with the large (ca 4 m) Atlantic sturgeon as early as 1875, but production of fry was limited (Ryder 1890, Cobb 1900). During 1906-1909, hatchery efforts were focused on the smaller (ca 1.5 m) shortnose sturgeon but success was

limited until recently when hormones were used to aid in spawning (Smith *et al.* 1985). Since then, substantial progress has been made in the areas of spawning, larval rearing, growout, and stock enhancement (Smith *et al.* 1986, 1995, Smith 1990, Smith and Jenkins 1991). Similarly, during the 1980s, a culture technology was developed for the white sturgeon (Conte *et al.* 1988). Today, this latter species is commercially farmed on the west coast with products used domestically as well as in international commerce. Research on culture of Atlantic sturgeon resumed (Smith *et al.* 1981), and recently small stocking studies have been initiated. Although current regulations prohibit commercial harvesting of this species in the United States, the regulations do allow aquaculture under certain conditions (ASMFC 1998). There is also substantial interest in the aquaculture of shortnose sturgeon, but current regulations prohibit commercial culture.

This manuscript provides an overview of the sturgeon research conducted and underway in South Carolina. Information related to biology, life history, fisheries, population status, culture, and stock enhancement of Atlantic and shortnose sturgeons is summarized, and major research needs are identified.

METHODS

Information contained herein is based on studies of Atlantic and shortnose sturgeons in South Carolina from 1980 to present. In most cases, the specific methods have been described in previous publications and reports. The objectives of these studies varied widely and included field, laboratory, and hatchery work. In the field, sturgeons were collected by directed sampling by staff, from bycatch of commercial American shad *Alosa sapidissima* gill net fishermen, and from commercial (and ex-commercial after fishery closure) Atlantic sturgeon fishermen. Fish were used variously as broodstock, in mark-recapture studies, in biotelemetry research, or held in captivity for use as experimental animals. Tissue samples were also collected for ageing (pectoral fin rays), for sex and maturity determination (gonads), and for molecular genetics analyses (barbels).

Ecological and life history aspects were determined primarily by collection of specimens in gill nets, trammel nets, and trawls, and through radio and acoustic telemetry studies. Salinity, temperature, and dissolved oxygen (DO), and often substrate type, were determined for most capture and telemetry locations.

Radio and acoustic telemetry studies were conducted on shortnose sturgeon in the Savannah River during 1985-1991 (Hall *et al.* 1991, Collins and Smith 1993). Shortnose sturgeon broodfish from this river were spawned and about 92,000 progeny of various ages were stocked into the river at several locations in a pilot stock enhancement study conducted during 1984-1992 (Smith *et al.* 1995, Smith and Collins 1996). Collaborative propagation research on Savannah River strain shortnose sturgeon has been conducted at several locations in South Carolina, including U.S. Fish and Wildlife Service (USFWS) facilities [e.g., Orangeburg National Fish Hatchery (ONFH), Orangeburg; Bears Bluff National Fish Hatchery (BBNFH), Wadmalaw Island] and South Carolina Department of Natural Resources (SCDNR) facilities (e.g., Marine Resources Research Institute, Charleston; Waddell Mariculture Center, Bluffton). Cultured juveniles have been used in a variety of studies, including efforts to delineate environmental tolerances (salinity and dissolved oxygen; Jenkins *et al.* 1993) ; to identify optimal tagging techniques (Smith *et al.* 1990, Collins *et al.* 1994); and to determine the biological effects of removing pectoral fin rays for ageing (Collins and Smith 1996).

A mark-recapture study of adult shortnose sturgeon was conducted on the Savannah River population during 1988-1992 (Collins and Smith 1993, Smith *et al.* 1993) and similar studies are underway in the lower Edisto River focused on juvenile shortnose and Atlantic sturgeons. During 1997-1998, adult Atlantic sturgeon were captured in the Edisto and adjacent Combahee Rivers and used in telemetry studies to identify movement and habitat use of the adults. In 1998, adult shortnose sturgeon were added to the study.

Adult shortnose sturgeon are known to occur in the Santee-Cooper system, a reservoir/canal/river system formed by the damming of the Santee and Cooper Rivers. In the Cooper River, adult shortnose sturgeon were captured during the spawning season in 1995-1998 below the Pinopolis Dam at river kilometer (rkm) 80 and fitted with radio transmitters to assess spawning and movements. In addition, adults have been captured by commercial catfish trotline fishermen in the upper freshwater reservoir, Lake Marion. One adult shortnose was also captured in the lower lake, Lake Moultrie, by an SCDNR biologist. A telemetry study was initiated in 1998 to determine the movement and reproduction of this apparently landlocked population.

During 1970-1985, the primary sturgeon fishery in the southeastern United States was associated with the Winyah Bay system. Here, fishermen set gill nets in January-April in the ocean adjacent to Winyah Bay to intercept adults migrating along the coast and/or into adjacent spawning rivers. During 1978-1982, the fishery was surveyed to collect fisheries and biological data (Smith *et al.* 1984). In recent years, fisheries managers have become concerned about the impact of bycatch mortality of sturgeons on stock recovery. Thus, sturgeon bycatch in the commercial shad gill net fishery was documented in Winyah Bay during 1994-1996 (Collins *et al.* 1996).

RESULTS AND DISCUSSION

Both sturgeons inhabit a number of South Carolina rivers. However, for some rivers it is unknown whether reproducing populations are present. A compilation of available information on the distributions of the two species in South Carolina has been published (Collins and Smith 1997).

Shortnose sturgeon

Propagation techniques

Attempts to culture and spawn shortnose sturgeon began in the early 1900s at the Torresdale Hatchery in Philadelphia, Pennsylvania. From 1906 to 1909, adult sturgeon were captured and placed in hatchery ponds and checked in the spring for sexual development (Meehan 1909). On several occasions, eggs were obtained but typically no ripe males were available. From this effort, only about 400 sturgeon fry were produced with the primary problem being the inability to obtain simultaneously ripe males and females.

The next spawning work occurred in 1981 on the Connecticut River where 62 eggs were taken from a captured female and fertilized with a simultaneously captured male. A dozen larvae hatched but most died during the first week (Buckley and Kynard 1981). At that time, efforts were also focused on shortnose sturgeon by a private consulting firm (Washburn and Gillis Associates, Ltd.). They spawned broodstock from the St. John River, New Brunswick, Canada, but results were similar with most of the 20 larvae produced dying shortly after hatching.

In the early 1980s, the USFWS and SCDNR initiated a program focused on development of hatchery and nursery techniques for Atlantic and shortnose sturgeons (Smith and Dingley 1984). In 1983, success occurred using recently captured wild fish obtained from the Cooper River. In contrast to previous efforts, hormones were used to induce final maturation and ovulation. This approach was based on results obtained by Smith and Dingley (1984) from spawning the Atlantic sturgeon.

Techniques for the capture and spawning of wild shortnose sturgeon have been provided (Smith *et al.* 1985). However, some modifications have occurred and thus the general techniques are presented here. Potential broodstock are captured in the rivers during the natural spawning migrations (January-March) using gill and trammel nets fished by commercial shad fishermen and by research staff. The sturgeon are visually inspected for abdominal distention (females) and expression of milt (males) and selected fish transported by truck to the ONFH or to BBNFH. At the hatchery, fish are placed in tanks receiving flow-through freshwater and prophylactically treated to prevent fungus infections. Within several days to a few weeks, a sample of eggs is surgically removed from each female and examined after boiling and sectioning as described by Conte et al. (1988). Females with extensive migration of the germinal vesicle are selected for hormone treatment.

Females are typically injected with common carp pituitary (CCP) at a rate of 4 mg/kg body weight with males receiving up to 3 mg/kg. A 10% "primer" shot is given intramuscularly to females followed 12 h later with a 90% "resolving" dose. Males receive the entire dose about 24 h prior to the females' resolving dose. Ovulation occurs within about a day of administering the final injection to the female. Eggs are taken from an abdominal incision or by strip spawning. The incision technique allows removal of more eggs with less labor. The strip spawning technique yields small batches of eggs every 1-2 h over an extended period (up to 26 h). Milt from several males is mixed together and added to the eggs. After about 30 sec, water is added to the egg/milt mixture. After 5 min, pond mud or diatomaceous earth is added and stirred for 15-20 min to prevent sticking and clumping of eggs. Eggs are then placed in McDonald hatching jars or Heath Techna trays and incubated in flow-through water at 18-20°C. Eggs hatch in 136 (18°C) to 111 (20°C) h. Hatching success ranged from 0-86% in early spawning attempts with wild caught broodstock. From 1984-1992, a total of 416,166 fry were produced (Smith et al. 1995). Maximum production occurred in 1989 when 120,480 fry were produced from 8 females (Smith *et al.* 1995). Spawning of wild broodstock ceased in 1992 as hatchery work became focused on production and use of cultured broodstock.

Five year classes of sexually mature broodstock have been produced at the BBNFH, a division of the Warm Springs Regional Fisheries Center. Some females mature at 4 yr of age at a size of 83 cm TL, weight 4.7 kg, while most mature by 5 yr of age. Males also matured at 4 yr of age but at a smaller size (74 cm TL, 4.3 kg). Males and females grow at a similar rate for the first 2-3 yr (approximate size at 2 yr - 68 cm TL, 2 kg) under culture conditions and then exhibit sexual dimorphism with the females growing more rapidly. Average size at first maturity for wild broodfish appeared to be approximately 56 cm TL.

For spawning purposes, cultured broodfish are placed in temperature-controlled recirculating systems beginning in October at a temperature of 24-26°C. Temperature is gradually decreased to 8°C by mid-January and slowly increased to 12-13°C by 1 March. Eggs are surgically removed and examined and suitable females are injected with CCP at a rate of 4 mg/kg body weight while males are injected at a rate of 1 mg/kg using the procedures for wild fish. Females are stripped spawned and the eggs are divided into different batches and fertilized with milt from a single male. Each batch of eggs is de-adhesed using Fuller's earth and placed in a separate McDonald hatching jar at 15°C. Hatching begins in 6-8 days. Hatching success typically ranges from 30-70% for each batch. Fry

production from the cultured broodstock was 15,000 fry in 1996, 56,000 in 1997, and 115,000 in 1998.

Feeding of fry typically begins at 10-12 days posthatch. Fry are fed live brine shrimp nauplii until 30 days posthatch. At this time, they are gradually switched to chopped black worms (*Tubifex* spp.). Commercial feed (semi-moist trout starter) is mixed with the black worms beginning approximately 40 days posthatch. The amount of semi-moist feed is increased daily until fish are converted (approximately 70 days posthatch) to the manufactured ration.

A variety of nursery and environmental studies have been conducted. It was shown that use of black worms during the transition diet phase was highly beneficial and that soft-moist rations (e.g. Biodiet, Biokyowa) were satisfactory for growing juveniles to adults (Smith *et al.* 1995). Tolerance to lower oxygen concentrations and higher salinities increased with age. Small juveniles (28-38 days old) exhibited 20-35% mortality at dissolved oxygen levels of 3.0-3.5 mg/L while 10-month-old fish (310-365 mm TL) tolerated levels of 2.5-3.0 mg/L for up to 6 h with no mortality. Similarly, mortality of 22-day-old fish was 60 and 95% at salinities of 9 and 11 g/L, respectively. At 11 months of age, shortnose sturgeon were able to tolerate salinities to 25 g/L with no mortality after 18 h (Jenkins *et al.* 1993). Adults can tolerate full strength seawater (35 g /L salinity) and dissolved oxygen concentrations as low as 2.0 mg/L at least for a short period.

In summary, domestic broodstock can be spawned and the progeny reared for various research purposes, including further domestication studies. This spawning technology should be useful for production of juveniles for various research purposes and for future stock enhancement/ restoration work.

Life history, ecology, and bycatch information

The area used by juveniles as nursery habitat is the vicinity of the fresh/brackish water interface. During months of high water temperature, adults also appear restricted to the area at and just above the interface. However, there may be behavioral differences among sturgeon in different rivers. Preliminary analyses of recent telemetry data suggest that in some rivers, adults concentrate in certain deep holes near the interface. In other rivers, the adults move upriver on flood tides and downriver on ebb tides within the interface, thus minimizing the range of salinities encountered (0-5 g/L).

Spawning takes place upriver (up to several hundred rkm) in freshwater during winter-early spring at water temperatures of about 10-16℃ (Collins and Smith 1993). Exceptions to this pattern may occur where dams block the spawning migration, such as in the Cooper River. In this case, spawning occurs at the base of the dam at rkm 80. Many females do not spawn in consecutive years and may not migrate upriver. This characteristic makes population estimation during the spawning season difficult. However, it appears that most populations are small (100-1,000 adults). The highly adhesive eggs attach to hard substrates (e.g., rocks, submerged trees), and recent studies in artificial streams suggest that larvae and early juveniles may require several months to move downriver from the spawning area (B. Kynard, USGS/BK, pers. commun.). The smallest juveniles collected in the downriver nursery habitat has been about 25 cm TL, age estimated to be 7-9 months. Such juveniles are believed to remain in the downriver nursery habitat until they mature (estimated time 5-9 yr). Once mature, adults may then participate in upriver spawning migrations (Smith and Dingley 1984, Hall *et al.* 1991, Collins and Smith 1993).

Due to their external bony plates, shortnose sturgeon are especially vulnerable to gill and trammel nets. The greatest impact occurs in the American shad gill net fishery which is often conducted throughout entire river systems, from the ocean to upriver areas (Collins *et al.* 1996). Fishing occurs at the time (winter-spring) when adult sturgeon exhibit movements associated with spawning. In one river, the estimated annual bycatch was several hundred adults. Bycatch mortality increases with time in the net and increasing water temperature. Besides the incidental captures, illegal directed fishing (i.e., poaching) is believed to occur in some rivers. For example, in the Cooper River a group of poachers was arrested with about 50 adult shortnose sturgeon in 1995. In this river, the adult population on the spawning ground is estimated to be between 100 and 300 individuals (D. Cooke, SCDNR, pers. commun.).

Tagging and tissue removal studies

Several studies were conducted to identify suitable tags and to determine the biological effect of tissue removal from live sturgeon. The best externally visible tag was an abdominally attached, plastic, streamer-type tag with a T-shaped anchor. The best tag overall was an internal PIT (passive integrated transponder) tag (Smith *et al.* 1990, Collins *et al.* 1994). However, both tags required use of advanced juveniles (~ 300 cm TL) for proper attachment/insertion. Reading of the PIT tag requires specialized equipment.

Due to their protected status, shortnose sturgeon should not be killed. However, biological tissues are required for genetic studies and ageing of fish. Controlled studies were conducted to test whether the removal of a barbel or a marginal pectoral fin ray would have any adverse impacts. Results showed that clipped barbels regenerated over a period of months (Collins *et al.* 1994) and pectoral fins healed completely after removal of the anterior spine (Collins and Smith 1996). Thus, these tissues can be taken for ageing and genetic studies without causing major health problems for the fish.

Stock enhancement research

A pilot scale shortnose sturgeon stock enhancement study was conducted during 1984-1992 in the Savannah River (Smith and Jenkins 1991, Smith *et al.* 1995). Wild shortnose sturgeon from that river were spawned and their offspring reared to various sizes. Of the 92,000 fish released, 18,000 were tagged and stocked at various sites from rkm 54 to 273 and at various times of year to identify suitable stocking parameters. Stocking at middle and upriver sites provided higher survival than at downriver sites, and stocking during late fall appeared to be more beneficial than other seasons (Smith and Collins 1996). Regardless of time or area stocked, juveniles moved to the lower river nursery habitat previously identified for wild fish. After stocking, over 35% of juveniles captured from the river were identifiable as stocked fish. The 35% contribution can be considered a minimum because of tag loss and the large number of untagged fish that were stocked. Thus, cultured shortnose sturgeon made a substantial contribution to the juvenile population.

Recent sampling efforts indicate that stocked fish have survived and reached the size and age at which wild fish become sexually mature (Smith and Collins 1996). It is not yet known, however, whether stocked fish are successfully spawning. In the Edisto River, about 65 km north of the Savannah River, over 10% of the shortnose sturgeon captured during 1996-1997 were identifiable as fish stocked into the Savannah River as advanced juveniles (ca 1 yr). These fish had been at large for as long as 7 yr.

Genetics

A study is underway to identify the sequence analysis of the mitochondrial DNA (mtDNA) control region of shortnose sturgeon from four major river systems in South Carolina (Edisto, Cooper, Savannah, and the Winyah Bay system). This preliminary study is the basis of a larger survey of genetic diversity within and among shortnose sturgeon populations throughout the southeastern United States. To date, approximately 150 individuals have been assayed for control region diversity. Two major classes of mtDNA sequence variation have been observed: variation in the number of a tandemly duplicated 83 base pair (bp) repeat and base substitutions in regions flanking these repeats. Base substitutions are rare within the repeated regions, presumably due to a high rate of duplication and deletion (and resulting homogenization) of individual repeat units. The preliminary analyses have uncovered at least nine base substitutions (primarily transitions) outside of the repeat region that define a minimum of 13 unique mtDNA haplotypes. No length variation has thus far been observed outside of the tandem repeat region. Sample sizes are too small from some river systems to draw firm conclusions regarding differentiation among drainages. However, our preliminary analyses show that approximately 5% of the total genetic variation can be ascribed to differences among drainages, indicating a modest level of population structuring. The major portion (approximately 95%) of the total genetic variation resides among individuals within river drainages. Parsimony analyses describing the relationships among these mtDNA variants reveal no evidence for phylogenetic structuring of lineages by river basin. Thus, our results suggest that differentiation among river drainages is not consistent with long-term evolutionary separation, but is most likely influenced by recent contact via coastal movement among drainages. This movement is probably not of sufficient magnitude to alter demographic processes within drainages, such that each river most likely represents a separate management unit. It is cautioned, however, that the analyses are incomplete at this stage, and many more samples and more complex permutations of these basic analyses are required before firm conclusions can be drawn.

Another interesting finding to date is the existence of several individuals harboring highly divergent mtDNA haplotypes. Comparisons of these sequences with other sturgeon mtDNA sequences in databases indicate that these individuals have an mtDNA type identified previously in Atlantic sturgeon. However, these animals were identified by field examination as shortnose sturgeon. It is not known if these individuals were misidentified, are morphologically aberrant Atlantic sturgeons, or are products of hybridization. Hybridization among sturgeons is known. Development of nuclear gene assays are underway to address the possibility of hybridization.

Atlantic Sturgeon

Propagation techniques

During 1875-1900, a number of attempts were made to artificially propagate Atlantic sturgeon (Ryder 1890, Dean 1894, Leach 1920). Although success was limited, these early studies did identify major problems to be solved prior to successful culture: acquisition of ripe broodfish of both sexes at the same time; clumping of the adhesive eggs; and fungal infections of eggs. In 1979, South Carolina researchers were able to obtain broodfish from around the Winyah Bay jetties, inject them with sturgeon pituitary, obtain viable eggs through abdominal incision, and successfully fertilize them (Smith *et al.* 1980). Clumping of the eggs was solved through treatment with diatomaceous earth, but fungal infection was still a problem. Acceptable feeds were identified and juveniles were reared for 130 days. The following year, Smith *et al.* (1981) were able to again spawn wild adults and grow the offspring to 204 days of age.

The next successful spawning of Atlantic sturgeon occurred in 1993 by the USFWS. Researchers obtained wild Hudson River broodfish (Kahnle *et al.* 1998) and injected them with LHRHa on the schedule recommended for white sturgeon (Conte *et al.* 1988). Offspring obtained

in 1993 as well as in 1994, 1995, and 1996 are being reared as part of a broodstock development program because acquisition of wild broodfish of both sexes is still a major problem. Excess juveniles have been sent to 18 organizations for use in a wide variety of studies, demonstrating the great interest in the biology and culture of this species.

Some cultured juveniles were used in field trials in the Hudson River, New York, and the Chesapeake Bay, Maryland. Preliminary results indicate that fish stocked into both systems have survived and that they make up a large proportion of the juvenile populations. However, information to date suggests that they may not be behaving identically to wild juveniles. Further, because the juveniles were stocked at relatively advanced ages, there is concern that the cultured fish may not imprint on the system into which they were stocked.

Life history, ecology, and bycatch

Information on the fishery, biology, and management of Atlantic sturgeon in the United States has been reported (Smith *et al.* 1984, Smith 1985, Smith and Clugston 1997). This section summarizes recent findings and ongoing activities with subadult and adult Atlantic sturgeon in South Carolina. Excellent seasonal growth data for juveniles of various ages have been collected, suggesting that growth is most rapid during spring and fall. During summer, growth in length is negligible, and weight generally decreases. Recent data suggest that while many juveniles spend the summer near the fresh/brackish water interface, some older juveniles move many rkms upriver, possibly in search of thermal refugia. During spring and fall, the juveniles primarily inhabit the upper estuary in the vicinity of the fresh/brackish water interface. In winter, juveniles move into higher salinity portions of the estuary, with older juveniles actually moving onto the nearshore continental shelf. Bycatch mortality in both the shad fishery, as noted for shortnose sturgeon, and in the penaeid shrimp trawl fishery may be substantial during this season.

Spawning sites have not been identified in any southern river, but a number of rivers/systems in South Carolina contain age 1 juveniles, suggesting successful spawning. Movements of adults tracked with telemetry equipment and capture locations of developing and ripe fish suggest that most spawning takes place well upriver from the brackish/freshwater interface. For example, a running ripe male was captured at rkm 300 in the Savannah River at the base of the first dam. However, in spring 1998 two larval (including one yolksac larva) sturgeon were captured at rkm 41, just above the interface area, in the lower Savannah River. Although damage to the specimens prevented positive identification to species, the sizes, capture dates, water temperatures, and developmental stages suggest that they were very young Atlantic sturgeon larvae.

The Winyah Bay system supported the major fishery with adults also taken from more southern rivers (Smith *et al.* 1984). During 1998, efforts were focused on capture of adults in the ACE basin (Ashepoo, Combahee, and Edisto Rivers). Ripe adults of both sexes (including females that had ovulated) were captured in the Combahee and Edisto Rivers during spring 1998, suggesting that this system contains a reproducing population. During fall 1998, a number of running ripe males have again been captured in the Edisto and Combahee Rivers. Telemetry studies indicate that these fish are making extensive movements up and down the rivers. Information obtained from these fish will be used to address the hypothesis that a distinct segment of the population spawns in the fall rather than spring (Smith *et al.* 1984). Availability of adults may be useful to support future Atlantic sturgeon culture and stock enhancement research.

Genetics

Ongoing molecular genetics studies of Atlantic sturgeon have been hampered by a scarcity of valid reference samples. Because subadults and older juveniles frequently leave their natal rivers and move long distances to other systems, only samples of age ≤ 1 juveniles and of adults on the spawning grounds can be used to characterize fish from a particular river. Collection efforts have been targeting these two sizes of fish, which are much more difficult to obtain than the larger juveniles. Preliminary results suggest that northern populations are genetically very similar while southern populations tend to be much more distinct (I. Wirgin, New York University, pers. commun.). Based on current recommendations relative to stock enhancement, this information suggests that fish stocked into a southern river should be spawned from broodstock taken from that river, or perhaps from an adjacent river if the target river's population has been extirpated (ASMFC 1996).

CONCLUSIONS

Substantial progress has been made over the past two decades in identifying the life history characteristics and ecological requirements of Atlantic and shortnose sturgeons in South Carolina. However, knowledge gaps still exist pertaining to spawning sites, larval nursery areas, population size and health, population structure, and anthropologic effects. Such areas are being addressed in current and planned research activities.

The spawning technology for shortnose and Atlantic sturgeons still lacks full predictability and is in need of refinement. However, hatching and nursery systems are developed to the point that juveniles can be reliably produced for various research and stocking purposes. Domestication of shortnose sturgeon broodstock provides

the means for continued spawning research and production of experimental specimens without impacting wild populations.

Genetic studies are underway to address the critical need for information on stock structure. However, acquisition of sufficient samples of sturgeons from the various river systems is problematic. Once these are acquired, genetic analyses can provide the information needed for appropriate management of remaining stocks and for the possibility of success in restoring depleted and extant populations.

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GENETIC CONSIDERATIONS DURING THE EXPERIMENTAL AND EXPANDED PHASES OF SNOOK STOCK ENHANCEMENT

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ABSTRACT

A responsible approach to stock enhancement requires that negative impacts on the gene pools of wild populations be mitigated by the implementation of genetically sound breeding and release procedures. Such procedures should be readily adaptable as a stock enhancement program evolves from the experimental to the expanded phases of production and release. Common snook *Centropomus undecimalis* is a neotropical estuarine fish that constitutes a socioeconomically valuable recreational fishery in Florida (USA). Experimental releases of hatchery-reared snook are underway in southwest Florida to assess the potential for successful snook stock enhancement. Relevant genetic, demographic, and biological data are available for wild snook stocks. Herein, we apply those data and population genetic principles to develop genetic guidelines for snook stock enhancement. In Florida, snook populations are biologically and genetically divergent between the Atlantic Ocean and the Gulf of Mexico. No transfers should occur between the Atlantic and Gulf snook populations. In the Gulf, snook may be further subdivided into interconnected demes having limited genetic exchange; broodstock sources should be limited to the targeted system or an adjacent estuary. Compared to other marine and estuarine fishes, allozyme and mitochondrial DNA polymorphism is low in *C. undecimalis*. Most allozyme polymorphism is maintained in the form of rare alleles occurring at frequencies of ≤ 0.05. During the expanded phases of stocking, it is recommended that at least 100 wild-caught adults per generation interval (*GI*), ≈ 3 years, be used to found hatchery populations and that the genetic effective sizes of those populations be ≥ 50. This should preserve $>99\%$ of the original heterozygosity and incorporate rare alleles into hatchery populations. We modeled the potential reductive effects of stocking on the effective sizes of enhanced snook populations. Assuming 50 effective hatchery breeders are used, hatchery contributions to Atlantic or Gulf populations should not exceed 31% per *GI*. Conservatively estimating hatchling survivorship and wild spawning stock abundance, we propose stocking guidelines that satisfy this requirement.

INTRODUCTION

The common snook *Centropomus undecimalis* (Bloch) is a semicatadromous, stenothermic, euryhaline species occurring in the tropical and subtropical Western Atlantic Ocean. It is a top predator in estuarine and nearshore environments, attaining weights of up to 27 kg and lengths of up to 1.3 m (IGFA 1996). Throughout its range, the common snook is a valuable game and food fish (Tucker and Campbell 1985). In the United States, common snook occur along the southern half of the Florida peninsula and along the southeastern Texas coast. Although the species has supported commercial and recreational fisheries in Texas coastal lagoons in the past, it is only rarely landed there at this time because of overharvest and adverse environmental factors (Matlock and Osburn 1987). In Florida, common snook continues to represent an important component of the sport fishery, ranking among the top three species specifically targeted by recreational anglers (Muller and Murphy 1998). Declines during the late 1970s and early 1980s resulted in its designation as a species of "special concern" by state fishery managers ; harvest is currently regulated by permit requirements, prohibition of sale, strict bag and size limits, gear restrictions, and seasonal closures. Nonetheless, approximately 1.6 million common snook were caught by Florida anglers during 1997, of which at least 200,000 were harvested. Despite the increasingly stringent regulations, the annual rate of

harvest has increased fourfold during the last decade (Muller and Murphy 1998). Additional harvest controls have recently been proposed by the Florida Marine Fisheries Commission and await approval by the Florida Cabinet. During the early 1980s, the interest in stock enhancement as a potential management tool for the Florida common snook fishery intensified. Mariculture programs were initiated at the University of Miami Rosenstiel School of Marine and Atmospheric Sciences, Harbor Branch Oceanographic Institute (HBOI), Mote Marine Laboratory (MML), and Florida Marine Research Institute (FMRI). Propagation of common snook in captivity proved to be a difficult, stepwise process in which significant problems associated with broodstock handling, egg production, bacterial infection, and larval and juvenile feeding had to be overcome (Anonymous 1993). Recently, through collaborative effort, MML, HBOI, and FMRI were able to refine breeding techniques for common snook (Kennedy *et al.* 1998) and to rear sufficient numbers of hatchlings for controlled release into juvenile nursery habitats (S. Serfling, MML, pers. commun.). This achievement raised the possibility that cultured snook may be used to enhance overexploited stocks in Florida or to offset losses caused by degradation of critical habitat and natural, acyclic perturbations (e.g., cold kills, red tide).

It has been recommended that incipient stocking programs adopt an experimental approach (Leber 1999), predicated upon the involvement of many scientific subdisciplines, adherence to the scientific method, and the use of "active-adaptive" management. Currently, pilot studies involving small-scale releases of common snook are being conducted in southwest Florida to determine optimal release strategies, e.g., size at release, timing of release, stocking densities, critical habitat assessment (Leber *et al.* 1997). During this "experimental" stocking phase, survival, growth, and recruitment of cultured fish to local (or non-local) populations will be assessed (Leber and Arce 1996, Leber *et al.* 1998). If, after completing the experimental phase for common snook, stock enhancement appears to be a useful tool for the overall management of the Florida common snook fishery, the stocking program could progress, rapidly or gradually, into an "expanded" phase of production and release.

A responsible approach to marine stock enhancement requires that potential negative impacts upon the gene pools of wild populations be mitigated through the use of genetically sound breeding and release protocols (Blankenship and Leber 1995). Consequently, researchers and managers at MML and FMRI seek to include genetic considerations into the overall management plan for their developing snook program. Herein, we integrate population genetic principles and baseline information on genetic diversity, population structure, and demographics of wild snook stocks to address genetic hazards and to develop a preliminary genetic risk management strategy for the snook enhancement program. We begin by reviewing the general types of genetic concerns that are most relevant to marine stock enhancement programs.

GENETIC HAZARDS

Some level of genetic exchange must be anticipated between native and hatchery stocks for marine stock enhancement programs. There are numerous ways in which cultured organisms can have a direct genetic impact on recipient stocks (reviewed by Utter 1998). The majority of genetic hazards may be grouped into three categories. We define genetic "Type I " hazards as those that occur by way of hatchery-mediated translocation of exogenous genes into native populations. Hatchery progeny derived from breeders belonging to a genetically divergent stock may, upon release, interbreed with conspecific or even congeneric members of the recipient stock (Leary *et al.* 1995, Sheridan 1995). The admixing of genetically discrete stocks (Altukhov and Salmenkova 1987) can break down local adaptations through introgression of maladapted genes or by disruption of coadapted genomes, thereby affecting the fitness of the native stock (outbreeding depression ; c. f., Templeton 1986, Waples 1995). For example, interrace crosses between even- and odd-year returning pink salmon have resulted in decreased survivorship and increased bilateral asymmetry in F_2 hybrids (Gharrett and Smoker 1991).

If genetic stock structure in a candidate species has been characterized, genetic hazards associated with intraspecies introgression may be minimized through judicious broodstock source selection (Hindar *et al.* 1991, Philipp *et al.* 1993). This approach reduces Type I hazards but does not mitigate all genetic risks.

Genetic hazards in the second category (Type II hazards) may be broadly defined as those stemming from genetic changes in a hatchery population, irrespective of the source of broodstock, that directly result from the processes of broodstock sampling, breeding, and rearing. Typically, the number of breeders selected to found the hatchery stock represents a small percentage of the available breeders in the source population. When insufficient numbers of breeders are used, sampling error can cause large stochastic differences in allelic and genotypic frequencies (Taniguchi and Sugama 1990) or reduced levels of genetic variation in hatchery broods compared to the wild stock (Bartley and Kent 1990). Hatchery populations can also be genetically compromised if the initial broodstock sampling fails to capture a sufficient range of phenotypic variability available in the source population (Leary *et al.* 1986). Other types of genetic changes to hatchery populations include artificial selection and domestication (Kohanne and Parsons 1988) and inbreed

ing depression (Tave 1993). Artificial selection, domestication, stochastic allele frequency changes, and reduced levels of variation can occur in the F_1 generation. However, hatchery populations must usually be propagated over multiple generations without sufficient input of additional wild genotypes before experiencing the deleterious effects of inbreeding.

The third category of genetic hazard (Type Ⅲ) is represented by a singular mechanism --- the possible genetic swamping of natural populations through successful enhancement efforts. This mechanism can lead to post-stocking alterations in the native gene pool even when hatchery populations lack Type I and Type Ⅱ genetic risk factors. Because of the disproportionate contribution of hatchery-derived progeny to the gene pool of a supplemented stock, an inevitable reduction occurs in the genetically "effective" population size of the admixed (enhanced) stock in the following generation (Ryman and Laikre 1991). The effective population size (N_e) represents the hypothetical abundance (number of individuals) in an ideal population (i.e., randomly mating, demographically constant, devoid of selection, migration, and mutation) that would undergo genetic change at the same rate as an actual population of abundance N. The magnitude of N_e in an admixed population composed of hatchery and wild stocks is a function of the original effective population size of the wild stock ($N_{e,w}$), the effective number of breeders in the hatchery stock ($N_{e,h}$), and the relative contribution of reproductively mature hatchery offspring (x) to the admixed population. According to the Ryman/Laikre model

$$N_e = \left[\frac{x^2}{N_{e,h}} + \frac{(1-x)^2}{N_{e,w}} \right]^{-1} \qquad \text{(Eq. 1)}$$

Reductions in N_e, if severe, can result in substantial allelic and genotypic frequency changes over time and, depending upon future population abundance (Waples and Do 1994), excessive loss of genetic diversity. Tringali and Bert (1998) evaluated the sensitivity of the model parameters $N_{e,h}$, $N_{e,w}$, and x over a range of values that may be typical for marine stock enhancement programs. The parameter x, a function of the number of cultured fish stocked, was shown to exert the greatest influence on the effective population sizes of supplemented marine populations. By using the model to quantitatively assess the Type Ⅲ risk level for two marine species having highly disparate population dynamics and genetic structures (i.e., red drum and Atlantic sturgeon), Tringali and Bert (1998) underscored the relationship between species life history and the potential genetic impact of stock enhancement.

BIOLOGICAL AND GENETIC RESOURCES IN FLORIDA COMMON SNOOK

Population Dynamics and Biology

Largely because of its popularity as a game fish and food fish, common snook has been extensively studied in Florida. Consequently, many biological, demographic, and life history traits for the species have been well characterized. Many of these traits differ between common snook from the Atlantic and Gulf of Mexico waters of Florida. From tagging studies (reviewed by Tringali and Bert 1996), extensive movement by adult common snook has been documented along Florida Atlantic nearshore waters --40% of 1,947 individuals recaptured had dispersed 50-350 km from their site of release. In contrast to the extremely vagile members of the Florida Atlantic common snook population, members of the Florida Gulf population exhibit a strong philopatric behavior within natal estuaries -- 99.5% of 2,053 common snook tagged in Gulf estuaries were recaptured<10 km from their release site, regardless of the time interval between tagging and recapture. Important biological differences also occur between Atlantic and Gulf common snook, including growth rate, natural mortality, female longevity, age at maturity (Taylor *et al.* 1998a), and annual reproductive cycle (Taylor *et al.* 1998b). Because these biological traits typically have significant components of additive genetic variation and high heritabilities in fishes (Hard 1995), the inherent differences distinguishing these groups of common snook should be viewed as a genetic resource.

Atlantic and Gulf populations also differ in total abundance and in abundance trends (Muller and Murphy 1998). From 1988-1998, the average total abundance for the exploitable portion of the Atlantic population (ages 3+) was estimated to be 410,000. Annual abundance estimates have declined since 1993 (N =506,000) to a 10-year minimum of 250,000 in 1998. In the Gulf, the average total abundance (age 3+) between 1988 and 1998 was estimated to be 607,000; annual abundances have fluctuated considerably around that mean. Currently, the exploitable Gulf population is thought to be composed of 850,000 snook and the breeding population, which contains a portion of 2-year-old snook (Taylor *et al.* 1998a), may be in excess of 1 million.

Genetic Structure and Diversity

Population structure and genetic diversity in common snook were examined by Tringali and Bert (1996) using mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) analysis and allozyme electrophoresis. Application of neighbor-joining cluster analysis to between-sample mtDNA sequence divergence values revealed that common snook populations are genetically

subdivided in Florida between Atlantic and Gulf waters (Figure 1). Florida Gulf samples showed a high degree of mtDNA similarity to Caribbean samples ; these samples formed a group that was divergent from the Florida Atlantic group. Using the nucleotide divergence values for pairwise comparisons of mtDNA haplotypes from Tringali and Bert (1996), we performed a hierarchical analysis of molecular variance to calculate Φ statistics (analogous to *F* statistics) and to estimate components of genetic variance (Table 1). The majority of mtDNA variance is apportioned within samples. However, a significant amount of the total variance (~10%) is partitioned between the Atlantic group and the Gulf/Caribbean group, providing statistical support for the hypothesis that these groups represent genetically divergent populations. Components of variance among samples within the Atlantic, Gulf, and Caribbean groups, respectively, were not different from zero (negative variances and Φ statistics are allowed by the AMOVA procedure), indicating that

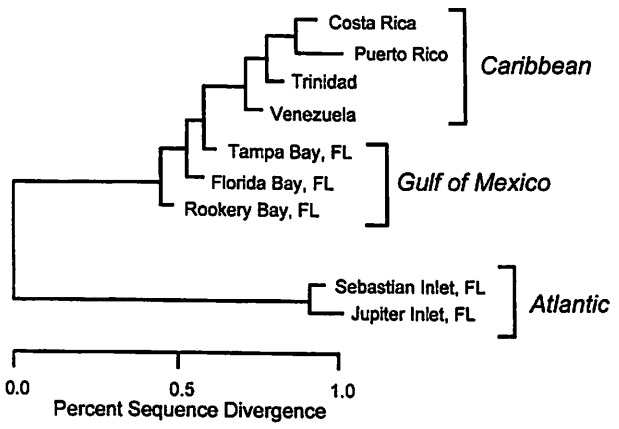

Figure 1. Geographic relationships among common snook *Centropomus undecimalis* based on a neighbor-joining analysis (RESTSITE computer program, version 1.2 ; Nei and Miller 1990) of between-sample mtDNA sequence divergences [tree redrawn from Tringali and Bert (1996)]. See Figure 3 for the collection locations of the Florida samples.

the mitochondrial genomes of common snook are relatively homogeneous on a regional basis. A hierarchical analysis of geographic structure based on allozymes using *F* statistics (Weir and Cockerham 1984) was generally concordant with the mtDNA hypothesis that gene flow between Florida Atlantic snook and those from other regions is restricted, although the sample from Florida Bay could not be assigned with statistical certainty to either the Atlantic or the Gulf population (Tringali and Bert 1996).

Tringali and Bert (1996) observed that allozyme and mtDNA polymorphism is generally low in common snook. In their allozyme survey of 187 Florida common snook (49 Atlantic, 138 Gulf), the average number of alleles per locus for the 31 presumptive genetic loci examined was approximately 1.4 for the Atlantic population and 1.6 for the Gulf population. The average heterozygosity value, $\overline{H_o}$, for all loci was 0.027 (±0.010) for the Atlantic population and 0.033 (± 0.013) for the Gulf population. Measures of allozyme diversity for each sample are given in Table 2. For each locus, the majority of alleles other than the most common allele occurred at very low frequency (<0.05 ; Figure 2A). Because the probability of sampling alleles diminishes as allele frequency decreases (Figure 2B), it is likely that many alleles occurring at frequencies ≤0.01 were not detected. Thus, the actual distribution is most likely U-shaped -- highly skewed toward very rare alleles and very common alleles at the expense of intermediate-frequency alleles (Chakraborty *et al.* 1980).

All measures of mtDNA variability are very low in common snook (Table 2 ; see also Wilson *et al.* 1997). Nucleotide diversity is an order of magnitude below values for the majority of marine and estuarine perciform fishes, e.g., red drum, sheepshead (FMRI, unpublished data), black drum, spotted seatrout, red snapper, and greater amberjacks (Gold and Richardson 1998), but similar to diversity values for other lower percoid fishes that, like snook, are sequential hermaphrodites (Gold and Richardson 1998). Both nucleon (h) and nucleotide sequence diversities (p) were higher in Atlantic samples than in Gulf samples. The disparate p values between these two populations suggests that the effective (female)

Table 1. Hierarchical analysis of molecular variance (AMOVA ; Excoffier et al. 1992) among mitochondrial DNA composite haplotypes of common snook from Florida. Analysis based on data presented in Tringali and Bert (1996). nc=not calculated.

Variance Component	Φ statistic and value	Variance	% of total	P[a]
Between Atlantic and Gulf/Caribbean	Φ_{CT}=0.099	0.006	9.98	0.014
Among samples within Atlantic	Φ_{SC}=-0.251	−0.024	−29.13	0.487
Among samples within Gulf/Caribbean	Φ_{SC}=-0.068	−0.007	−18.87	0.998
Within samples	nc	0.083	138.79	nc

[a] Probability of obtaining a more extreme random value, based on 5,000 permutations.

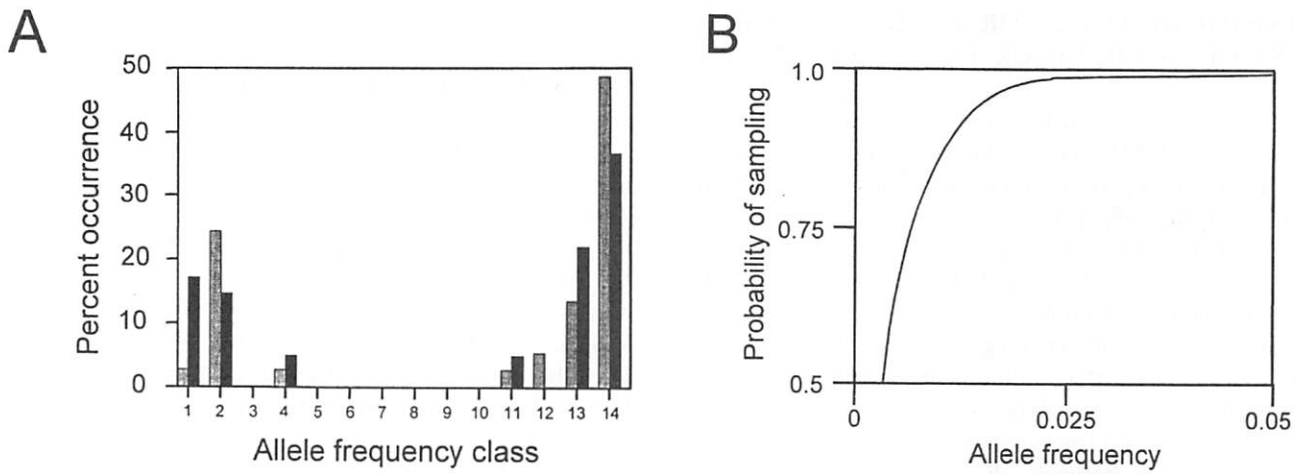

Figure 2. (A) Observed distribution of allozyme allele frequencies in common snook *Centropomus undecimalis* from the Florida Atlantic (light bars; $n=49$ individuals) and Florida Gulf (dark bars; $n=138$ individuals). Alleles were allocated by frequency of occurrence into 14 classes: 0.0-<0.01, 0.01-<0.05, 0.05-<0.1, 0.1-<0.2, 0.2-<0.3, 0.3-<0.4, 0.4-<0.5, 0.5-<0.6, 0.6-<0.7, 0.7-<0.8, 0.8-<0.9, 0.9-<0.95, 0.95-<0.99, 0.99-1.0 (see Chakraborty et al. 1980). Protein electrophoretic procedures and a list of the 31 presumptive loci surveyed may be found in Tringali and Bert (1996).
(B) Single-locus probability of sampling an alternate allele (*y-axis*) when it occurs in a population at a given frequency (*x-axis*) and when 138 individuals are sampled.

Table 2. Summary of allozyme and mitochondrial DNA variation in *Centropomus undecimalis* from Florida. Except for the average number of allozyme alleles per locus ($\overline{n_a}$), results appear in Tringali and Bert (1996). $\overline{H_o}$ = average proportion of heterozygotes per locus; P_{95} = percentage of loci in which the frequency of the most common allele did not exceed 95%; h = mtDNA nucleon (haplotype) diversity; p = percent mtDNA nucleotide sequence divergence. Locations of samples are depicted in Figure 3.

Location	Allozyme			mtDNA	
	$\overline{n_a}$	P_{95}	$\overline{H_o}$	h	p
Atlantic coast					
Sebastian Inlet	1.23	13	0.022	0.83	0.28
Jupiter Inlet	1.29	3	0.024	0.82	0.16
Gulf of Mexico					
Florida Bay	1.24	7	0.023	0.50	0.06
Rookery Bay	1.42	10	0.032	0.63	0.08
Tampa Bay	1.32	7	0.025	0.33	0.08

population size has remained higher in the Atlantic population (~32,000 females) than in the Gulf population (~11,000 females) for an ecologically meaningful period of time.

To summarize the biological and genetic data, common snook in Florida are regionally divided into two populations occurring in Atlantic and Gulf of Mexico waters. Each population contains unique biological and genetic resources that should be preserved. Because common snook in Florida Gulf waters are highly philopatric, the Gulf population may be further subdivided into loosely-connected demes that occasionally exchange migrants among adjacent estuaries. Gene flow among common snook within the respective Atlantic and Gulf populations appears to be sufficiently high to homogenize neutral genetic variation over time. However, the gene pools of localized demes along the Florida Gulf coast may still be temporally affected by a large-scale stock enhancement program. Accordingly, the genetic-management goals for snook stock enhancement in Florida should be focused on the conservation of within-population diversity and between-population divergence.

RECOMMENDATIONS FOR GENETIC MANAGEMENT OF SNOOK STOCK ENHANCEMENT

The common snook stock enhancement program being conducted by MML and FMRI in southwest Florida is currently in the experimental release phase. Between April 1997 and April 1998, approximately 25,000 cultured snook were tagged and released into various juvenile nursery habitats in Sarasota Bay (K. Leber, S. Serfling, and B. Halstead, unpublished data). Assessments of the various release treatments are ongoing. Monitoring studies by MML have shown that cultured snook can contribute significantly to the abundance of juvenile snook (up to 30%) in net samples from stocked nursery habitats 1 year after release (N. Brennan, K. Leber, and S. Serfling, unpublished data). Cultured juvenile snook so far have exhibited strong release-site fidelity, as would be expected for wild Gulf snook. The husbandry and stocking technologies for snook are rapidly progressing to a point at which the large-scale stocking of hatchery-reared snook could be considered an optional management tool for the Florida snook fishery. Because the nature and potential severity of genetic impacts upon enhanced populations change as stocking programs evolve from experimental to expanded phases of production and release, we evaluate the genetic concerns of these phases separately.

Experimental Stocking Phase

During the experimental stocking phase, managers of the common snook enhancement program should avoid transferring genetic material between subdivided stocks (Type I risks). Tringali and Bert's (1996) genetic stock identification for wild snook populations provides the baseline information needed for broodstock source selection. However, there remain two caveats concerning the genetic characterization of common snook (see Grant *et al.* 1999). First, geographic patterns in adaptive traits (e.g., disease resistance, thermal tolerance, timing of spawning) might be masked in assays of presumably neutral markers (Utter *et al.* 1993, Conover 1998) such as those employed in the genetic study of common snook. Second, fine scale stock structure (e.g., among samples within regions) may not always be detected in mtDNA RFLP and allozyme analyses, especially when the sampled genetic diversity is low (Brunner *et al.* 1998). Accordingly, we advocate a conservative approach regarding broodstock source selection.

Therefore, mindful of the genetic, biological, and behavioral differences among common snook, we recommend that the species be divided into multiple conservation units in Florida. Hatchery-mediated genetic exchange between Florida Atlantic and Gulf populations should be strictly avoided. For stock enhancement programs involving Gulf common snook, we recommend that hatchery broodstock be obtained from the recipient spawning stock or collected from systems adjacent to the operational estuary (Figure 3). An exception to this guideline is needed for the southernmost system, i.e., Florida Bay/Florida Keys. Because common snook eggs, larvae, and juveniles are absent from this system (Peters 1993), Tringali and Bert (1996) posited that local adult stocks may be a mixed stock composed of individuals from both the Atlantic and the Gulf. A detailed study of stock composition in this area is ongoing and, until more is known, snook from this area should not be used to stock any other system. Finally, because of the high vagility of Florida Atlantic common snook, it appears that geographic constraints pertaining to broodstock source could be relaxed in that region.

Although it appears that cultured snook released during MML's pilot studies may contribute significantly to localized juvenile abundance in certain nursery habitats, we estimate that contributions of reproductively mature cultured snook to any local breeding subpopulation will be minimal (<<5%) during the course of the experimental stocking phase. Managers of the stocking program currently use wild adults captured from local common snook populations for broodstock. Because only indigenous genotypes are propagated at MML, Type I hazards have been eliminated during the experimental phase. Because newly collected wild snook will be used to produce each generation of hatchery progeny, the Type II hazards relating to inbreeding depression and hatchery adaptation incurred by hatchery fish over multiple generations of captive propagation will also be eliminated (Utter 1998). Type II genetic changes that can occur in the F_1 generation (including artificial selection/domestication, allele frequency shifts, and diversity reductions) remain a possibility for hatchery broods of common snook. However, because of the limited hatchery input to wild stocks (Ryman and Laikre 1995), these changes are unlikely to significantly impact locally adapted gene pools in wild snook unless the experimental phase continues over multiple generation intervals.

Though unlikely to impact the recipient population, fitness reductions in hatchery offspring could affect the outcomes of tests of the various release strategies. Captive propagation imposes very different selection pressures than does natural reproduction (Doyle 1983) and some level of domestication in hatchery broods is almost inevitable (Waples 1999). In studies in which fitness differentials in performance traits have been documented between hatchery-derived and wild fish, hatchery-derived fish typically exhibit poorer performance in the natural environment, suggesting that natural selection has already optimized most genotypic states in those wild populations (Hindar *et al.* 1991). Therefore, culture protocols should be implemented that increase the likelihood that hatchery snook have fitness potentials that are similar to those of

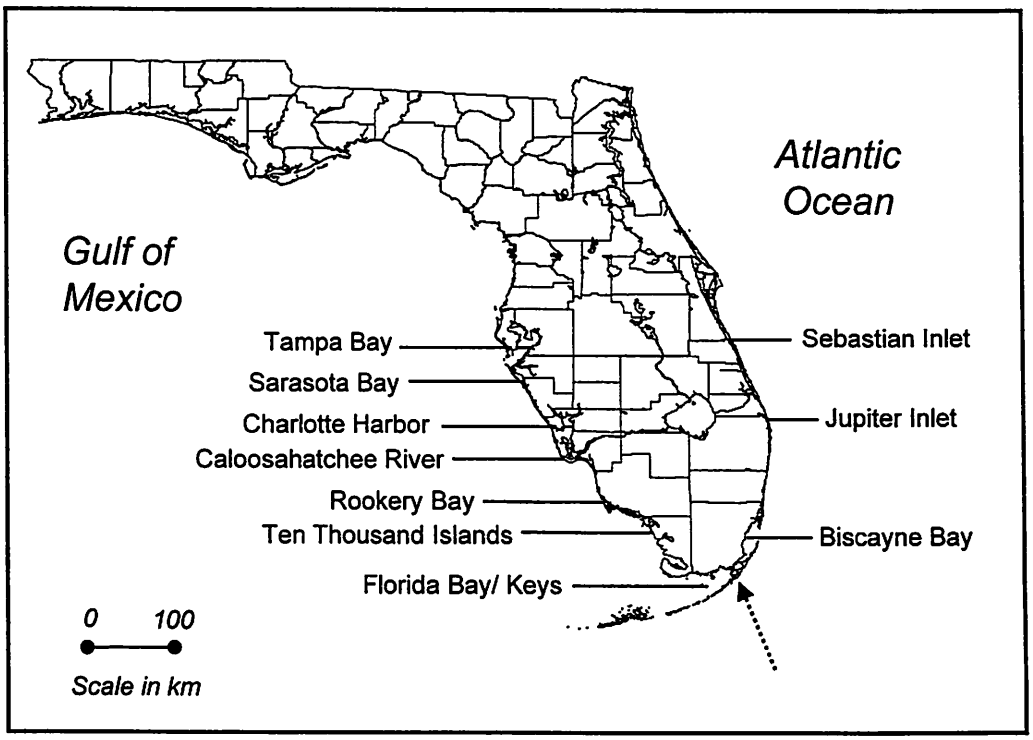

Figure 3. Map of Florida showing the locations of major estuarine systems within the range of common snook. Geographic boundaries for the named systems may be obtained from NOAA's (1985) National Estuarine Inventory Data Atlas. Sebastian and Jupiter Inlets occur within the Indian River estuarine drainage.

wild snook, and these protocols should be continuously evaluated and adjusted, if necessary, during the experimental phase.

Accordingly, we recommend that managers of the common snook stocking program continue using wild-caught adults as broodstock. To capture within-population phenotypic diversity, broodstock should be systematically sampled from the recipient population over the course of the protracted breeding season, April-September (Taylor *et al.* 1998b), and from various spatially and environmentally separated spawning aggregates, e.g., those from barrier island inlets, passes to secondary (within-estuary) embayments, and mouths of coastal rivers (Peters *et al.* 1998). Egg production for common snook is usually accomplished by fertilizing the eggs of strip-spawned females, which may have undergone hormone treatment to induce egg maturation, with the sperm of one or more males (Wallace *et al.* 1993). To minimize the risk of F_1 domestication, family sizes should be equalized (Allendorf 1993) so that hatchery selection will operate only through fitness differentials among different genotypes within families of full- or half-sibs (depending on the mating scheme employed). Sources of potential artificial selection during rearing should be identified and avoided. For example, a particular concern for snook reared at high density may be cannibalism of slower growing individuals by faster growing individuals. Mitigation of this problem may require segregation of progeny by size during rearing or by reducing rearing densities.

Expanded Stocking Phase

Should the common snook stock enhancement program expand to the production phase in Florida, additional captive-propagation and stocking guidelines will be necessary. During breeding and rearing (Type II processes), the objective should be to produce hatchery broods that are similar to wild stocks with respect to both adaptive and selectively neutral variation. Intraspecific genetic variability in common snook is low; therefore, to propagate a sufficient amount of within-population genetic diversity, a minimum of 100 hatchery breeders (N_b) should be used per generation interval (3 years). This strategy should maintain natural allele frequencies and preserve 99.5% of the original heterozygosity and the majority of allelic diversity present in the source population (Allendorf and Ryman 1987).

Due to the potential for genetic swamping in common snook, risks associated with Type III (Ryman/Laikre) hazards should be minimized. To do so, we recommend

that a minimum of 50 *effective* hatchery breeders be used per generation interval. To achieve a ratio of at least 0.5 effective breeders to actual hatchery breeders, attention to parental sex ratio and to family size variance will be required (Crow and Denniston 1988, Kincaid 1995). $N_{e,h}/N_h$ ratios ranging 50-75% have been achieved in other hatchery programs through the use of genetically efficient protocols (Hedrick and Hedgecock 1994).

Stocking guidelines were formulated as follows. Adopting a minimum-allowable N_e value of 500 for the enhanced stock (FAO/UNEP 1981, Tringali and Bert 1998), we first used the Ryman/Laikre model (Eq. 1) to estimate maximum relative contributions (x_{max}) to recipient wild stocks (subpopulations) for values of $N_{e,h}$. We then estimated the maximum-allowable number of juvenile hatchlings (H_{max}) that should be stocked in a subpopulation of known abundance by using the expression

$$x_{max} = \frac{H_{max} \cdot S_r}{(H_{max} \cdot S_r) + N_w} \qquad \text{(Eq. 2)}$$

where S_r is the pre-recruitment survival rate (i.e., the anticipated survival rate of released juvenile cultured snook to reproductive age) and N_w is the spawning stock abundance of the recipient subpopulation. As a conservative measure, the genetic structure model used for broodstock source selection was used to define the range of subpopulation abundances.

For $N_{e,h}$ values of 50 and 75, maximum relative contributions of hatchery-released snook should be limited to 31.5% and 38.7%, respectively, during a generation interval. For these two values of x_{max}, Figure 4 depicts the maximum-allowable stocking limits for values of S_r between 5-15% and values of N_w between 100,000-500,000, based on annual estimates of Muller and Murphy (1998). We anticipate that the parameter ranges modeled in Figure 4 would be applicable to the majority of stocking activities during an expanded phase of snook production. Notably, the relatively small increase in $N_{e,h}$ from 50 to 75 allows a significant increase in the maximum number of hatchlings that could be released. For example, assuming a pre-recruitment survival rate of 15% for hatchery-reared snook, up to 840,000 hatchlings could be propagated from 75 effective breeders and stocked into a wild spawning stock of 200,000 individuals (per *GI*) compared to only 614,000 hatchling snook propagated from 50 effective breeders. This results in a 27% increase in the stocking limit for snook, potentially increasing the rate at which a declining stock could be rebuilt.

Finally, we recommend that a genetic monitoring program for supplemented snook populations be incorporated into the overall management plan during an expanded stocking phase, should it occur, or if small-scale (experimental) releases in particular waterways occur repeatedly over multiple generation intervals. Components of the monitoring program should include the characterization of genetic diversity and composition in hatchery broods and periodic genetic sampling of the recipient stock to evaluate any fluctuations in gene frequencies and reductions in pre-stocking levels of genetic diversity that may be associated with hatchery releases. Available allozyme and mtDNA genotype frequency data for wild snook stocks (Tringali and Bert 1996, Wilson *et al.* 1997) should be useful in this process. However, because of the low level of genetic variation found in those data, additional genetic markers may be required for certain analyses.

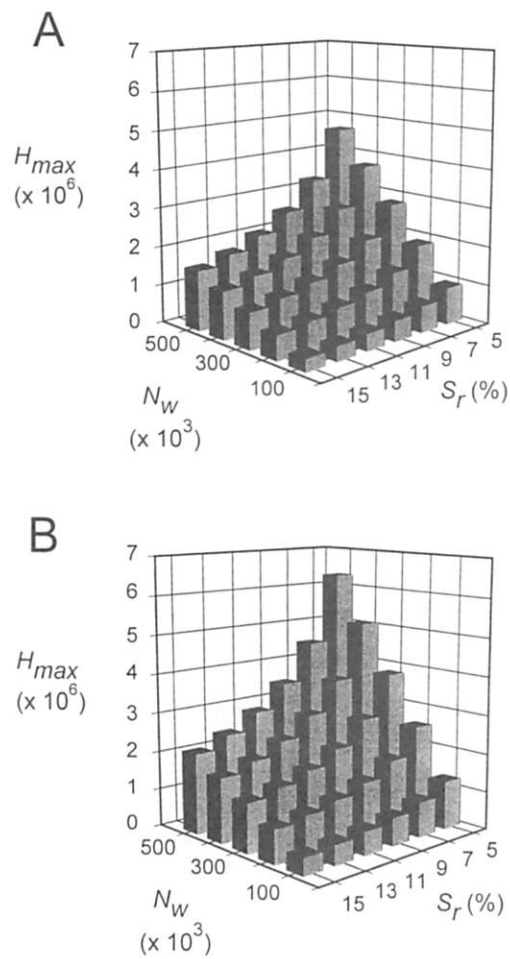

Figure 4. Maximum-allowable number of cultured snook (H_{max}) per generation interval for survival rates (S_r) prior to recruitment to the breeding subpopulation between 5-15% and subpopulation abundances of wild common snook (N_w) between 100,000-500,000. Estimates generated for $N_{e,h}$ values of (A) 50 and (B) 75 breeders.

CONCLUDING REMARKS

A growing body of evidence demonstrates that cultured marine organisms can make substantial contributions to fisheries landings in some coastal marine species (Leber and Arce 1996, Masuda and Tsukamoto 1998, Rimmer and Russell 1998). However, the effectiveness of marine stock enhancement as a resource management tool remains a hotly debated subject in the United States (Radonski and Loftus 1995, Travis *et al.*1998). The potential for negative genetic consequences typically and justifiably ranks high among the list of concerns. We have adopted a conservative approach throughout our assessment of genetic risk and in the formulation of risk-adverse guidelines for snook stock enhancement. We anticipate that genetic risk management will be an ongoing process within the program, subject to refinement and amendment as more information becomes available. Our general conclusion from this preliminary assessment is that cultured snook, propagated and released according to the preceding guidelines, are not likely to have significant short- or long-term impacts on the genetic composition or diversity of wild snook populations in Florida.

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GENETIC CONSERVATION IN STOCK ENHANCEMENT-ASIA AND AMERICA PROSPECTS

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ABSTRACT

Stock enhancement has been considered as a tactic to replenish depleted fish stocks since the late 1800s. Release of millions of hatchery-produced juveniles only began with the rapid development in aquaculture technology during recent years. Since fry production in hatcheries may use only a few brood fish or genetically modified fish to produce juveniles, genetic composition in wild populations can be altered through releasing efforts. This report summarizes the discussion among a group of geneticists from Asia and North America on genetic changes following stock enhancement. While the changes in the gene pool resulting from interactions between cultures and wild stocks were confirmed, the resultant impacts on the future fate of stock enhancement remain unknown. To avoid any unexpected catastrophe in genetic change, it is important to consider the genetic conservation in any stock enhancement activities. Finally, the discussion group proposed guidelines for developing a genetic management plan for the target species, for sampling hatchery broodstock from natural populations, and for management and operation of hatchery and production systems. Genetic resource management should be developed and evaluated in any stock enhancement activity. These guidelines are anticipated to be modified as stock enhancement activities proceed.

INTRODUCTION

In 1996, total world aquaculture production was estimated at 34,116,249 metric tonnes or more than two and one-half times of the amount in 1987 (FAO 1998). The annual growth rate of production during this period was 11%. The impressive growth rate of production has exceeded the prediction by Csavas (1994) but the production has to be doubled by year 2025 to meet the current food fish consumption rate of 13.5 kg per capita (New 1997). Because of the stagnant growth of capture fisheries, demand for seafood has relied on the growth in aquaculture production. New (1991) suggested that stock enhancement will have a major role to play in meeting future fish production needs. Ocean ranching has been considered as one of the feasible methods to increase total aquatic yield. The idea of using aquaculture to restore and enhance the loss from diminished polluted environments and from overfishing was reported earlier by Eschmeyer (1955) but the results were inconclusive (Radonski and Martin 1986). Recent developments in hatchery technology according to the paper presented during Larvi '95 in Belgium and stagnant growth from capture fisheries by Grainger (1998) have encouraged the activities of stock enhancement once more. The attempts to raise fish under artificial conditions and to introduce them to the natural environment pose the risk to affect the genetic resources in natural populations. Most of all, the progress in hatchery techniques has greatly increased survival; consequently, few broodstock are required for juvenile production. This leads to concerns about genetic changes through the recruitment of hatchery-produced fish in stock enhancement activities (Simon *et al.* 1986, Allendorf and Ryman 1987, Waples and Teel 1990).

This paper summarizes the information on enhancement and conservation presented by geneticists from Asia, the United States, and others during the fourth international workshop organized by the Oceanic Institute in Hawaii under funding support from the U.S. Department of Commerce in 1993. Experts from different countries presented their stock enhancement efforts and concerns. Guidelines to conserve the biodiversity of natural stocks and to avoid the genetics changes through stock enhancement efforts are presented.

BREEDING IN AQUACULTURE

Selective breeding has been implemented as one of the tactics to improve fish performance to increase the production in aquaculture. It is estimated that at least 30% of the increase in rate and efficiency of protein production in

agricultural animals has resulted from genetic research and comprehensive industry breeding programs (Dickerson 1970). Many aspects in fish performance such as growth rate, age at maturation, disease resistance, and environment tolerance have been improved through selective breeding. Both traditional and biotechnological methods can be used to amplify the desired gene expression. Either approach will alter the genetic structure of the targeted animals. Those genetically modified organisms can be cultured in an enclosed area and harvested for consumption without affecting the genetic structure of natural stocks. However, the chance of altering the genetic structure of wild populations is high when those animals are released to natural waters.

Depletion of major fish stocks was reported worldwide. Several causes, such as habitat degradation and overfishing, have resulted in population declines of several stocks throughout the world. Three principal tactics to replenish depleted stocks are: regulating fishing efforts; restoring degraded fish habitats; and increasing recruitment through release (stock enhancement). Marine stock enhancement is not a new concept and was implemented in the late 1800s unsuccessfully (National Research Council 1992). Early enhancement efforts ignored the genetic effects of release of hatchery-reared fish on wild populations (Pullin 1992). At that time, survival from fertilized eggs and newly hatched larvae which were released was low and made only minor contributions to the recruitment. Following the recent progress in aquaculture, release of hatchery-reared juveniles into natural environments has been re-identified as one of the top solutions to restore the depleted stocks. Millions of juveniles can be produced in the hatchery and achieve higher post-released survival than previous practices. Consequently, many concerns were raised regarding the conservation of original genetic diversity. Unintentional selection in the hatchery can change the gene pool of natural breeding populations and potentially affect their survival and reproduction in the future. Particularly, genetically modified organisms are becoming a part of modern aquaculture. Releasing genetically modified organisms will cause profound genetic impacts on wild stocks and has to be prohibited completely.

SUCCESSFUL STOCK ENHANCEMENT

Increasing the natural recruitment of a particular stock through releasing hatchery-reared juveniles has been proved as a useful tool as discussed during the first international symposium on stock enhancement and sea ranching in Norway in 1997. With the increasing successful cases of stock enhancement in augmenting existing fish populations and restoring fish populations in environments where they previously existed, enhancement programs must consider gene conservation. From the genetics point of view, a successful stock enhancement must maintain existing patterns of genetic diversity and the health of a natural population during enhancement. Considering the thousands and millions of juveniles entering into natural environments, the genetics of wild stocks are undoubtedly affected. Enhancement will affect the genetics of wild stocks when gene flow occurs between cultured and wild stocks. The potential genetic effects on natural resources from the use of cultured stocks to restore populations were reported by Waples (1991). Those genetic effects include direct and indirect effects as well as genetic changes to hatchery stocks. Each effect is the result of different causative agents as shown in Table 1. The relative importance of each effect varies with the species and has to be addressed.

Two common approaches for stock enhancement are relocation of broodstock from one region to another, and collection of local broodstock to produce juveniles for release within the same region. Genetic risk to the natural stocks will vary depending on which approach is used. The former has the greatest potential to affect genetic variation. Genetic impact could be more significant if a new genotype was brought in from another region to enhance a local population. The latter is recommended to prevent the loss of adaptive genes. Concerns have been expressed about the impact of domestication selection, or the decreased fitness of wild populations because of exposure to the hatchery environment. However, assuming that genetic resources are properly managed, several generations in the hatchery may have a minimal impact on the overall gene frequency. In any case, the genetic risks and benefits of each approach should be evaluated before a final decision is made.

Table 1. Levels of potential genetic effects on natural resources from the use of enhancement culture to restore populations (Waples 1991).

Genetic Effects of Concern from Enhancement	Causative Agents
Direct genetic effects	Hybridization, introgression
Indirect genetic effects	Altered selection regimes or reduction in population size resulting from competition, disease, or other factors.
Genetic changes to hatchery stocks	Selection, genetic drift, stock transfers

EXAMPLES OF ENHANCEMENT ACTIVITIES

Many enhancement activities were reported by the participants of the workshop. In Japan, many different species of fish, crustaceans and molluscs have been targeted for enhancement. Over the years, millions of red sea bream *Pagrus major*, scallop, ayu *Plecoglossus altivelis*, and kuruma shrimp *Penaeus japonicus* were released. Increase in natural stocks resulting from enhancement efforts were documented. The projects were supported by the public both morally and financially. In Norway, enhancement efforts began in the 1800s with salmonids and cod. Large numbers of small fish were released in the 1930s and more recently (1980s), at 15-20 gm, cod *Gadus morhua* were released back into the area where the broodstock were caught. Enhancement efforts were tried to replenish the depleted stocks such as silver carp, bighead carp, mud carp, and tilapia from overfishing in Vietnam's Red River. Positive results are yet to be identified. In China, enhancement programs were carried out to restock the recruitment losses from dam construction or pollution in natural water bodies. Their efforts include hatchery release programs; programs to overcome reproduction dispersal barriers posed by dams; programs to introduce new species to lakes, rivers, and bays; and programs to enhance production from land-based aquaculture. In Thailand, 75 million fry of fish and shrimp species were released in 1989. In Indonesia, undetermined numbers of carp and tilapia were released to natural water bodies for various reasons.

To date, not all of the projects have increased the size of stock populations. Conservation of original genetic structure is not the initial concern by any of the above projects, rather the total population size. Some of the approaches intentionally relocated fish stocks from one region to enhance another region, such as landlocked ayu enhancement programs in Japan, with positive results. In the southern United States, several states near the Gulf of Mexico have initiated enhancement programs to increase the catch of red drum *Sciaenops ocellata* by recreational fishermen. They have to address the public concerns about the number of broodstock used in producing fry. The genetic risks and benefits of each approach should be evaluated before a decision is made.

Actions taken at the State of Washington Department of Fisheries since the 1980s to promote the conservation and health of the diverse salmon resources in Washington were discussed by Shaklee *et al.* (1993). Washington Department of Fisheries' mission is to "....preserve, protect, perpetuate and manage the food fish and shellfish in state waters and offshore waters. The Department shall conserve the food fish and shellfish resources in a manner that does not impair the resource...". Although the mission statement does not explicitly mention genetic conservation, the "preserve and protect" clause implies that policymakers need to embrace genetic conservation objectives. Key features for their genetically-based policies and guidelines are to:

- Develop formal agency policies for salmon genetics and stock management and conservation;
- Generate a comprehensive inventory (identification and characterization) of all naturally spawning salmon and steelhead stocks in the state;
- Review existing spawning guidelines, hatchery practices, stock transfer guidelines, and fisheries management strategies to assure compliance with identified genetic objectives and policies;
- Incorporate genetic risk assessment and management as routine components of future departmental actions;
- Pursue basic and applied research investigations regarding salmon genetics, reproductive and developmental biology, behavioral ecology, and hatchery-wild stock interactions, and
- Design and implement monitoring and evaluation programs to assess the performance of specific actions and programs with respect to identified operational goals.

EVALUATION OF GENETIC IMPACTS FROM STOCK ENHANCEMENT

The negative impacts of cultured stocks on wild stocks were reported during the workshop. The wild stocks were overwhelmed by release of brown trout *Salmo trutta* from a single source in freshwater systems throughout Norway. A subsequent population crash may have resulted from the enhancement effort. In China, many wild carp populations have been destroyed because of stocking or escape of cultured hybrid carp to natural environments. There were no conclusive agreements among participants on the answer to the question "Is genetic change always bad"? There is a need to evaluate the impact of cultured fish on wild stocks through experiments although this puts wild populations at risk. Some risk is required to prevent future disasters.

Monitoring is the key to elucidate the positive impacts from stock enhancement efforts. Several steps are needed to monitor gene flow from hatchery stocks to wild populations and, finally, to evaluate enhancement effects. These steps are:

- Determination of wild stock genetic variability prior to release of hatchery stocks. This information will provide a baseline for comparison after a stock enhancement program is implemented.
- The genetic structure of the breeding and F_1 population must be evaluated to determine the potential impact on wild populations.

After releasing,

- Periodic monitoring of the genetic structure of wild stocks is needed to provide a feedback mechanism,

subsequent to the initiation of a stock enhancement program.

GUIDELINES FOR GENETIC RESOURCE MANAGEMENT

To preserve the original genetic structure of wild populations, guidelines were developed for genetic management plans, for sampling hatchery broodstock material from natural populations, and for management and operation of the hatchery and production systems. The guidelines for developing a genetic management plan for the target species are:

- Define status of the target stock;
- Define harvest and genetic goals of the enhancement program;
- Identify and quantify genetic risks and consequences;
- Define an enhancement strategy;
- Implement a monitoring and evaluation program;
- Outline research needs and objectives;
- Develop a feedback mechanism.

The guidelines for sampling hatchery broodstock from natural populations are:

- Sample the full range of phenotypic diversity within the managed unit;
- Have a geneticist review the sampling plan.

The guidelines for management and operation of hatchery and production systems are:

- Avoid inbreeding except when desired;
- Avoid domestication of stocks;
- Maintain adequate effective population size to minimize random genetic change;
- Consider the use of genetic markers;
- Use selective breeding only when appropriate;
- Monitor and respond to gene flow from hatchery to wild stocks;
- Minimize genetic selection in the hatchery;
- Evaluate how to improve survival and behavior under wild conditions.

Detailed explanations for each guideline can be found in the proceedings entitled "Selective breeding of fishes in Asia and the United States" edited by Main and Reynolds (1993). Kapuscinski and Miller (1993) also gave a more detailed overview of hatchery guidelines for genetic concepts pertaining to enhancement, broodstock collection, spawning, rearing and release procedures.

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UJNR EXPERIMENTAL RELEASE OF JAPANESE FLOUNDER AT WADA BEACH, WAKASA BAY: FEEDING HABITS OF WILD AND RELEASED JUVENILES

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ABSTRACT

Field experiments by mass release of hatchery reared juveniles of Japanese flounder *Paralichthys olivaceus* were conducted at Wada Beach, Fukui Prefecture, Japan under U.S.-Japan cooperation supported by UJNR. Optimal time and size of release was investigated by comparing feeding conditions of wild and recaptured released juveniles. Feeding habits of juveniles in Wada beach was characterized by an early shift of major prey item from mysids to fish and a high diversity of mysids prey. In 1997, 40,000 juveniles were released in the early (29 May) and late (2 July) seasons, expected to higher and lower densities of prey mysids, respectively. There were several differences in feeding condition between the two groups. Main stomach contents of the early group and wild juveniles were mysids and fish, while those of the late group primarily mysids. Stomach contents index (SCI) of the early group was similar to that of wild juveniles, and SCI of the late group was quantitatively less than that of the early release and wild juveniles. Percent empty stomach of the late group was much higher than the other two groups. Suggesting that it is better to release juveniles when mysids are abundant. In 1998 two different size groups (large: 60.7 mm TL, 50,000 fish; small: 37.1 mm TL, 50,000 fish) were released on 21 May. Although the number of large group recaptures was almost twice that of the small group, differences in apparent growth and feeding condition of recaptured fish were not clear between the two groups. Feeding conditions in 1998 were lower than those in 1997, due parhaps to low abundance of mysids caused by high water temperature. Under such conditions, release of 100,000 juveniles may result in the carrying capacity of the study area for Japanese flounder to be exceeded.

INTRODUCTION

The Japanese flounder *Paralichthys olivaceus* is very important in commercial fisheries, stock enhancement, and aquaculture in Japan. Mass releases of artificially raised seedlings have been conducted almost all around Japanese coastal areas since the mid-1980s. However, a rapid reduction of numbers of hatchery raised juveniles after releasing commonly occurs. Several fundamental problems are still remained poorly understood (Tanaka *et al.* 1998). Especially, timing, size of released fish and magnitude of releases are important factors in successful stocking. UJNR cooperative research on juvenile flounder release started to investigated these aspects in relation to prey, predators and wild flounder abundance in 1997. The research of the first 2 years forcused on optimal timing and size of released flounder.

Abundance of mysids, the main prey of Japanese flounder juveniles, increases in early spring and decreases in early summer in south-western areas of Sea of Japan

(Tanaka *et al.* 1997). Timing of release relative to mysids abundance appears to be very important for their post-release feeding (Furuta 1996). In 1997, 2 groups of juveniles were released in different seasons at Wada beach, Wakasa Bay, to see effect of release timing on feeding and growth.

Japanese flounder juveniles commonly exhibit a relatively simple feeding chronology; initially feeding on mysids and switching to fish with growth. Since timing of the shift from mysids to fish varies depending on habitat quality and tolerance to starvation being size-dependent, size of release is very important in post-release survival. In 1998, 2 different size groups were released to test size dependent feeding and growth.

There are many endogenous and exogenous factors involved in the survival of juveniles: nutritional condition, growth potential, food availability and presence of predators. Of these factors, food availability seems to be the most direct and important in feeding and growth. Although the flounder juveniles can survive for more than 10 days without food, starving may increase probability of predation due to behavioral change (Furuta 1998) and growth retardation (Houde 1987). Therefore, feeding is the first concern to be tested. The main purpose of this article is to characterize the feeding habits of released and wild Japanese flounder juveniles at Wada Beach, and to consider the optimal timing size at release from results of stomach-content analyses.

Most of data presented here and more detailed analyses will be appeared in a paper entitled "Food habits of the Japanese flounder juveniles released in Wada Beach, Wakasa Bay, the Sea of Japan, in comparison with those of the wild juveniles" (Tanaka *et al.* in preparation). In order to introduce a part of UJNR cooperative research activities, the authors report a preliminary result in this UJNR proceedings.

Table 1. Size, nember, and date of releases conducted in 1997 and 1998.

	1997	1998
date of release	29 May (early group) 2 July (late group)	21 May
Average size at release	53.5mm TL (early group) 51.8mm TL (late group)	60.7mm TL(large group) 37.1mm TL(small group)
Number of release	40,000 fish (early group) 40,000f ish (late group)	50,000 fish (large group) 50,000 fish (small group)

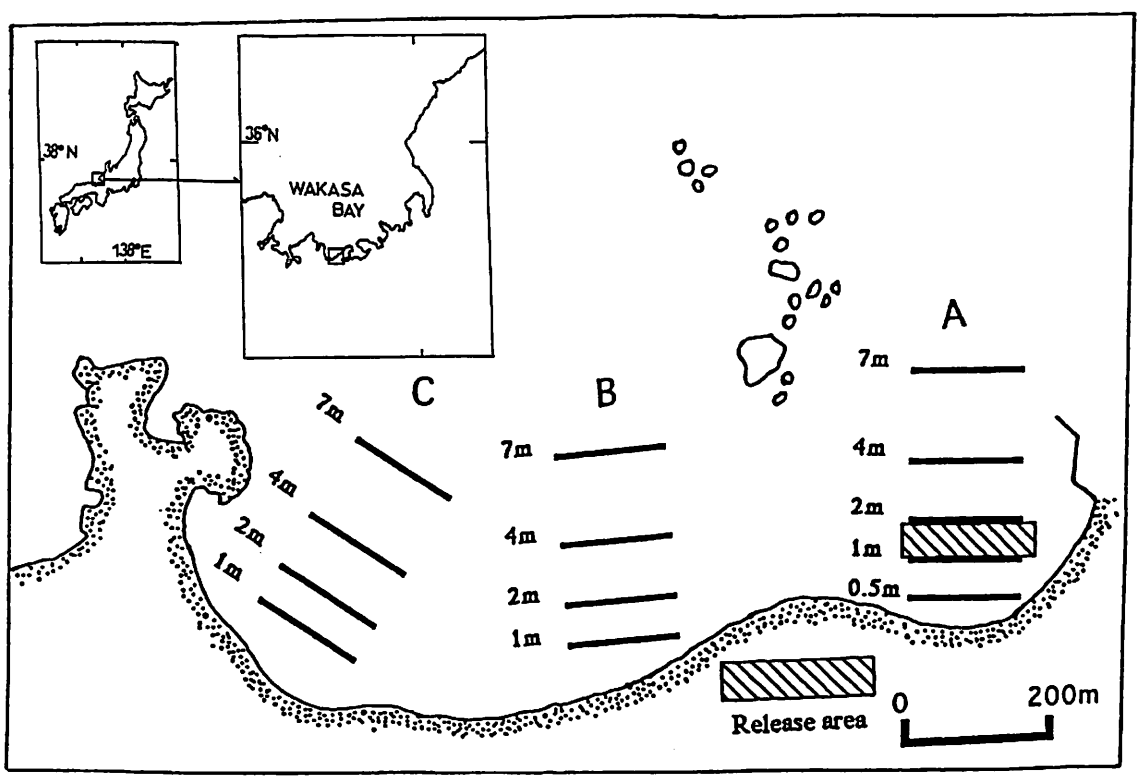

Figure 1. Map of the UJNR experimental field, Wada Beach, with sampling lines.

MATERIALS AND METHODS

The experimental field, Wada Beach is located in Wakasa Bay, Fukui Prefecture, mid coastal area of the Sea of Japan (Figure 1). Flounder juvenile releases were conducted in early (29 May) and late (2 July) seasons in 1997, which were predicted to be correspond to higher and lower densities of mysids, respectively. Average release size of the early group was 53.5 mm TL, and that of the late group was 51.8 mm TL. Both groups had 40,000 fish each. In 1998, two different size groups of juveniles of which mean total length was 60.7 mm TL (large group) and 37.1 mm TL (small group) were released on 21 May to examine size-dependent feeding and growth. Amount of each group released was 50,000 fish (100,000 in total) (Table 1). Releases were made in the eastern subarea (A) at Wada Beach and at 1-2 m depth in both years (Figure 1). Otoliths of all juveniles were marked with alizarin complexone to discriminate each group.

The experimental field was divided into three subareas A, B and C. Two-meter beam trawl tows for the juveniles were conducted at 0.5, 1, 2, 4, 7 m depths in each subarea, 1, 2, 3, 6, 10, 16, 30, 35, 36, 40, 50, 64 days after release of the first group in 1997 and 1, 2, 4, 7, 14, 21, 28, 35, 42, 49, 63 days after release in 1998. Mysids were sampled simultaneously with a sledge net (0.5 m mouth width and 0.7 mm mesh aperture) in 1997.

Most of released juveniles were collected in subarea A in both years, consequently, released and wild juveniles collected in subarea A were used for stomach contents analysis. All fish were frozen in the field. Total length, body length, body weight and body weight excluding the viscera was measured. Dissected stomachs were preserved in 10% formalin. The stomach contents were classified into major taxa and mysids were identified to the genus level. Number of animals eaten were counted and their wet weights measured. The following indices were used to evaluate feeding condition.

1) wet weight composition of stomach contents
2) stomach content index (SCI); juveniles that have empty stomach were excluded.
   $=100\times$ (wet weight of stomach contents/body wet weight excluded the internal organ)
3) percent empty stomachs
   $=100\times$ (number of juveniles that have empty stomach/number of juveniles examined)

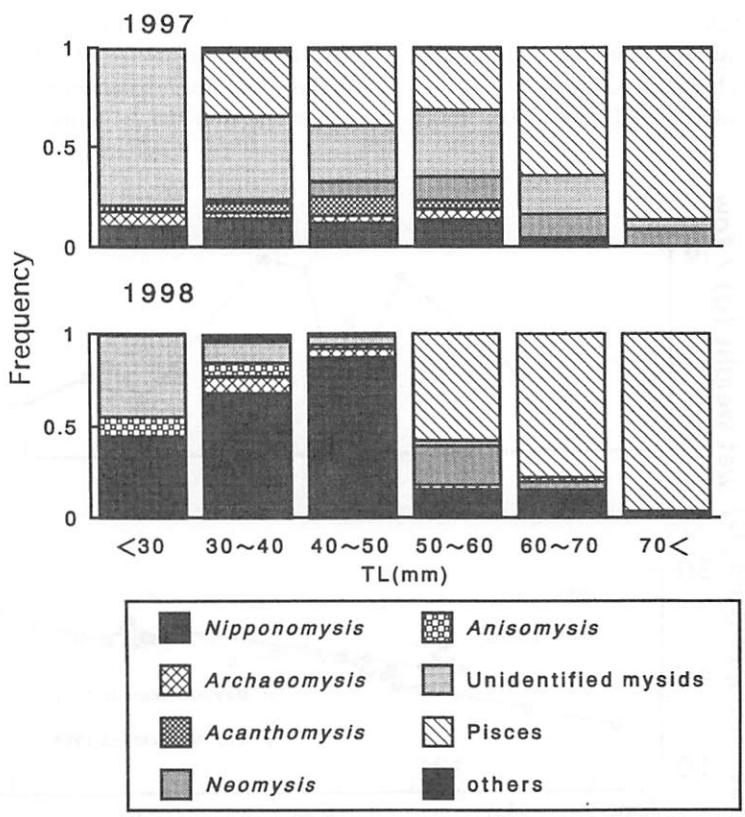

Figure 2. Wet weight composition of stomach contents of wild juveniles at each 10 mm size group in 1997 (upper) and 1998 (lower).

RESULTS

Changes in food habits of released flounder with growth

Stomach contents of recaptured flounder were primarily composed of mysids and fish in both 1997 and 1998, however, the shift from mysids to fish was different between the two years. A relatively high percentage of fish appeared in the 30 mm size class of the flounder and it occupied a large percentage in the 60 mm and 70 mm size classes in 1997. In 1998, stomach contents consisted primarily of mysids in the sizes smaller than 50 mm, and fish predominated in the stomachs of larger size classes (Figure 2). Species composition of mysids in stomachs was diverse including the genera *Nipponomysis*, *Archaeomysis*, *Acanthomysis*, *Neomysis* and *Anisomysis* (Figure 2). In 1997 unidentified mysids were predominant, but in 1998 *Nipponomysis* was the dominant genus. Fish prey consisted primarily of advanced stages of Japanese anchovy arvae (Table 2). One wild flounder juvenile was detected from the stomach of a released flounder juvenile in 1997.

Table 2. Percentage of occurrence of fish categories detected from the stomachs of wild juveniles in 1997 and 1998.

	1997 (%)	1998 (%)
Japanese anchovy larvae (*Engraulis japonica*)	81.7	90
Japanes flounder	1.8	0
Unidetified fish	16.5	10

1997 field experiment: testing time at release

Figure 3 shows the abundance of mysids (1997) and seasonal changes of water temperature (1997, 1998). The first release in 1997 was done at high abundance of mysids, while the 2nd release at low abundance.

When the early group was released, the mean total length of wild flounder juveniles were about 30 mm and they remained smaller than the early released group throughout the sampling period (Figure 4). Conversely, the late released group was smaller than the wild juveniles until the last sampling date (Figure 4). Juvenile growth (size shift of captured flounder) was nearly the same between wild and released flounder juveniles in the both early and late groups.

Stomach content composition of the early group was similar to wild juveniles. The main prey item of the early group was mysids though they also ate fish just after release (Figure 5). This trend continued until mid-June, when the main prey shifted from mysids to fish. In the case of the late group, the main prey item was mysids with an occasional high percentage of fish (Figure 5). *Archaeomysis*, a shallow burrower, frequently accounted for a high percentage of the stomach contents of released juveniles, but was

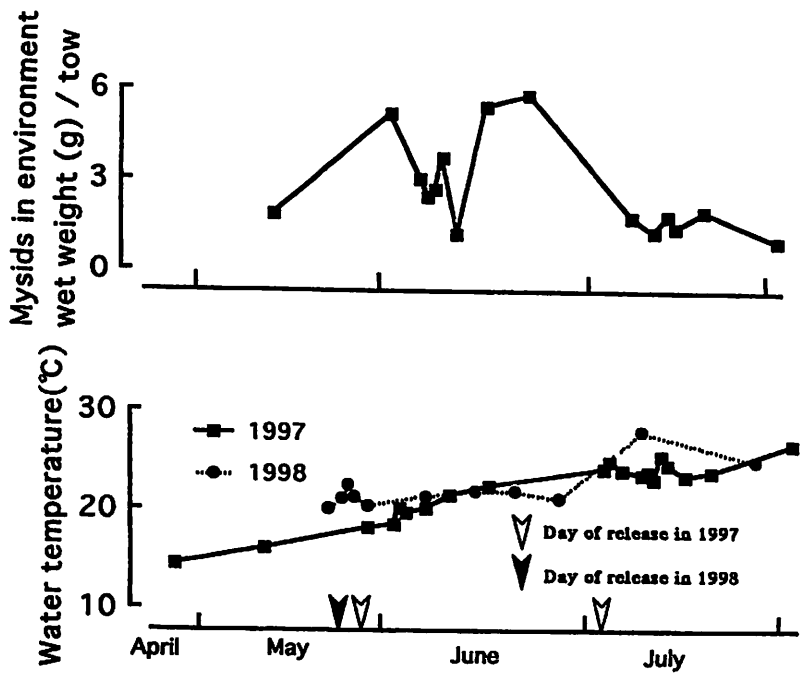

Figure 3. Seasonal changes in bottom water temperature at release site in 1997 and 1998, and mysid abundance in g wet weght per tow in 1997. Arrows show the dates of release.

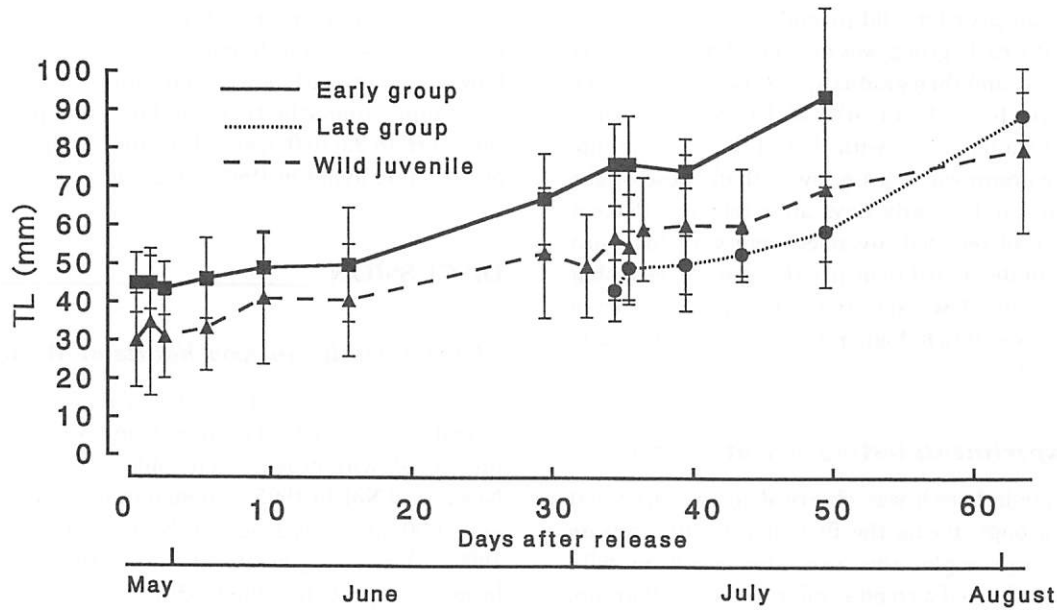

Figure 4. Changes in mean total length of the early and late released flounder, and wild juveniles sampled at Wada Beach in 1997. Vertical bars indicate standard deviation.

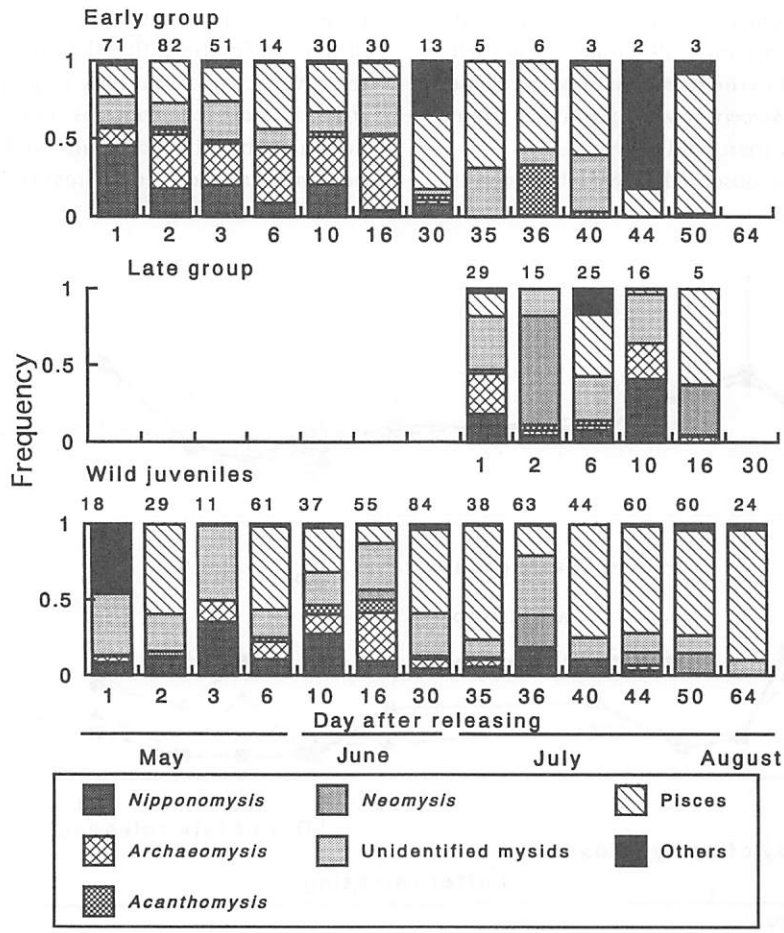

Figure 5. Changes in stomach contents of the early and late released flounder, and wild flounder juveniles sampled at Wada Beach in 1997. Numeral above each column shows number of individual analyzed.

seldam important prey for wild juveniles.

The SCI of the early group was between 3 and 4 at early days after release, and then gradually decreased to between 1 and 2 in early June (Figure 6). SCI of wild juveniles showed almost similar value with that of the early group. SCI of the late group was a little lower than those of the other two groups in the early days after release. Percent empty stomachs of released juveniles were very high just after release, but decreased to nearly the same level as that of wild juveniles in a few days. percent empty stomach of the late group was much higher than that of the early group (Figure 6).

1998 field experiment: testing size at release

Little increase in length was observed in the large and small release groups during the first month after release (Figure 7). Surprisingly, this was also found in wild juveniles, which showed a trend similar to the small group.

Fish were observed in the stomachs of the large group during the early days after release, however mysids occupied major part of their stomach contents even when the juveniles reached 60 mmTL. In the small group, myisids were main prey, and the stomach contents of wild juveniles consisted primarily of mysids till June (Figure 8). *Nipponomysis* predominated in the stomachs of both released and wild flounder, but *Archaeomysis* was again more important in the diet of released than wild juveniles.

Clear difference was not observed in SCI between the large and small groups (Figure 9). Very high percent empty stomach was found in early days after release, however, consitently lower percentage of the small group had empty stomachs than the large group. SCI in 1998 ranged from 2.0 to 0.5, and this value is approximately half of the value found in 1997 (Figure 6, 9).

DISCUSSION

Characteristics in food habits at Wada Beach

A variety of studies have shown that juvenile Japanese flounder typically feed on mysids initially and switch to a diet of fish with growth. This shift occurred at 60 mm in Nagasaki (Noichi 1995) , at 80 mm in Kyoto (Tane 1992) and at 80 mm body length in Niigata (Fujii and Noguchi 1996). Variability in the timing of the shift at different locations is probably due to differences in mysid and fish prey abundance and availability. In our research field area, an early shift in diet from mysids to fish was apparent with making up a large part of the diet of flounder larger than 30 mm in 1997 and representing the dominant prey in flounder greater than 50 mm in 1998 (Figure 2). Results of our study also differed from previous investigations in the diversity of mysid prey of Japanese flounder. Previous studies, stomach contents consisted primarily of one dominant misid species, although the species varied depending on localities: *Acanthomysis* in Ibaragi, Chiba,

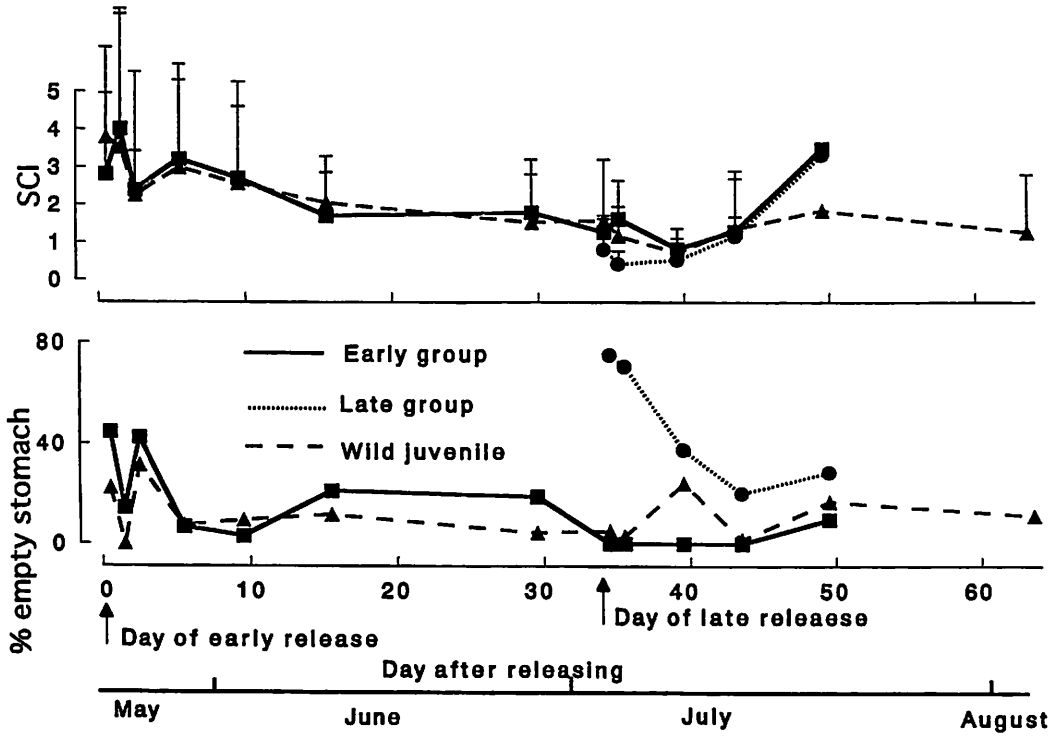

Figure 6. Changes in mean SCI (upper) and percent empty stomach (lower) of the early and late released flounder, and wild juveniles sampled at Wada Beach in 1997. Vertical bars indicate standard deviation.

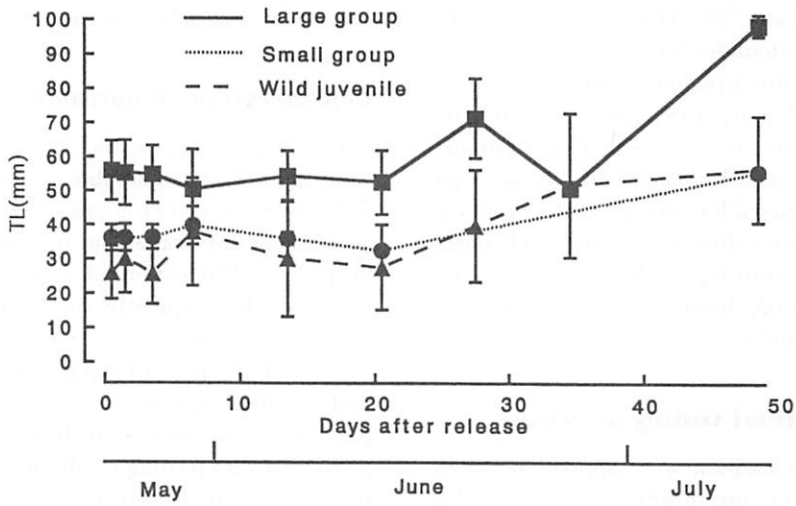

Figure 7. Changes in mean total length of the large and small released flounder, and wild juveniles sampled at Wada Beach in 1998. Vertical bars indicate standard deviation.

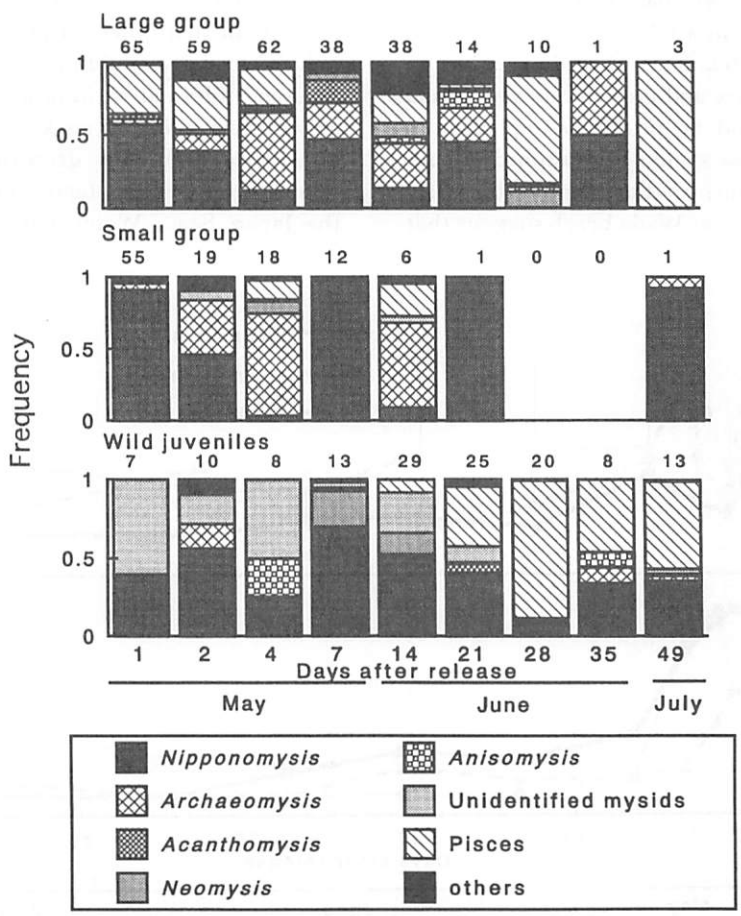

Figure 8. Changes of stomach contents composition of the large and small released flounder, and wild juveniles sampled at Wada Beach in 1998. Numeral above each column shows number of individual analyzed.

Niigata and Kyoto, *Neomysis* in Sizuoka and Fukuoka, *Nipponomysis* in Aomori (Tane 1992, Hirota *et al.* 1990). In constrast at Wada Beach flounder feeding was characterized by a multi-species composition of mysids.

The early shift in the diet from mysids to fish and the high diversity of mysids in the diet suggest that mysids availability is relatively low at Wada Beach. *Archaeomysis*, important in the diet of juveniles released at the beach, is known as a shallow burrower during day-time, and consequently more difficult to capture than the commonly eaten epi-benthic species, suggesting limited availability of more accessable mysids in this field.

Consideration on optimal timing at release

Differences in feeding condition were apprent between the early and late release groups. Feeding condition of the early group was similar to that of wild juveniles, however, the late group had a lower SCI and high percentage of empty stomachs. These results suggest a release coincident with the seasonal peak of mysid abundance is beneficial to flounder feeding. A similar result has been reported by Furuta *et al.* (1997) in Tottori coastal area. There are several factors involving in post-release survival of juveniles. Seasonal changes of abundance of predators (Furuta *et al.* 1998), prey availability and growth rate in relation to water temperature would be primarily important to decide time of release. These should be examined in both field and laboratory experiments. Furthermore the early shift in the diet to fish evident at Wada beach suggests that abundance of fish larvae is also a consideration in determining optimal release timing.

Consideration on optimal size of release

Generally large juveniles have high tolerance for starvation, and are less vulnerable to predators. Yamashita *et al.* (1994) demonstrated larger size had higher percentage of post-release survival along the north Pacific coastal waters of Japan. Production of large juveniles for release is expensive; Consequently, small sizes should be induced in determination of the effect of release size on survival.

Clear difference of feeding condition between the large and small groups were not evident. A slightly higher percent empty stomach in the large release group (Figure 9) suggest that feeding conditions better for the small than the large group. Despite the similarity in feeding condition of small and large groups, almost twice as many of the large group were recaptured than small. This suggest that optimal size for release was determined by the predater present rather than prey available to the groups of flounder.

In 1998 length increase and SCI of released juveniles was lower than that observed in 1997. This may be due to reduced food availability caused by release of one hundred thousand fish, and/or insufficient food abundance due to annual variavility. Tanaka *et al.* (1997) showed that abundance of mysids decreases markedly when water temperature exceeds about 20℃ in the southern parts of the Japan Sea. Water temperature in late May 1998

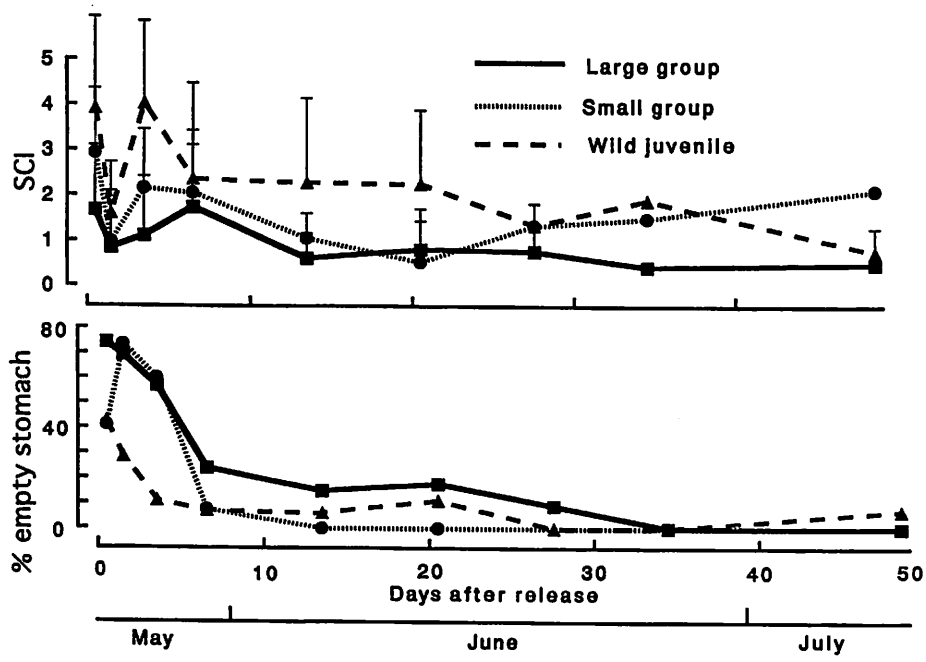

Figure 9. Changes in mean SCI (upper) and percent empty stomach (lower) of the large and small released flounder, and wild juveniles sampled at Wada Beach in 1997. Vertical bars indicate standard deviation.

exceeded 20℃, and was 3℃ higher than that in 1997. The higher water temperature have reduced earlier mysid abundance in May 1998 and the lower feeding condition would be attributed to annual fluctuation of mysid abundance.

ACKNOWLEGEMENTS

The authors express sincere thanks to H. Sudo, Japan Sea National Research Fisheries Institute, for instructing in the identification of mysids. We are grateful to the staffs of Japan Sea Firming Association Miyazu and Obama Stations, students at Fukui Prefectual University and S. Furuta at Tottori Prefecture for thier helpful assistance in producing and releasing juveniles, and field sampling. We also thank to the field assistance of the U. S. participants : J. Hakel, N. King, and K. Park.

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GOAL AND PRESENT STATUS OF THE UJNR COOPERATIVE RESEARCH ON JUVENILE FLOUNDER RELEASE OVERVIEWED FROM THE JAPAN SIDE

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ABSTRACT

The origin, in terms of scientific interests, of the ongoing UJNR cooperative research project can be traced to the 1st Flatfish Symposium held in 1990, where Japanese and American researchers of flounder species met together. After 5 years of involvement with Japanese investigators through the JSPS-NSF joint program for Post-doctor Fellowship and US-JPN Cooperative Research Programs, John Miller (North Carolina State University) proposed cooperative research between USA and Japan which was accepted by the UJNR aquaculture panel at 24th Corpus Christi meeting.

The 1996 Japanese team comprised 3 fields of expertise: mass seedling production, evaluation of released fish through mark and recapture, and eco-physiology of juvenile flounder. The Japan Sea-Farming Association (Miyazu and Obama stations), Fukui Prefectural University and Kyoto University corresponded to these fields, respectively. After discussing goals and approaches, a release experiment was initiated at Wada beach in Fukui Prefecture, the objective of which was to estimate carrying capacity (or productive capacity).

Three factors investigated were release time, size and number. The 1st-year experiment investigated time of releasing relative to prey abundance in the nursery ground, and the 2nd-year experiment investigated optimum size at release. During the next 3 years, excess amount of juveniles at different levels would be released to test density-dependent effects on habitat utilization, feeding, growth and survival. In conjunction with this mass-release experiment, laboratory experiments were conducted to estimate food requirements under different environmental variables, and analyze the production rates of dominant prey mysids. Elements of the field mass-release and laboratory rearing experiments were combined using enclosure experiments.

Previously our understanding was that Japanese and summer flounder display different ecological traits under different nursery environments. Preferred habitats were sandy substratum with stable and clear-water for the former and muddy substratum with highly fluctuating and turbid-water for the latter. Ecological comparisons between these related species should be tested under different fields and artificial environments to generalize patterns of colonization. The challenge of this research enterprise appears to stimulate scientific interests of both US and Japanese graduate students and young researchers, and hopefully being advanced under UJNR great support.

INTRODUCTION

Recently fisheries throughout the world have been declining due mainly to overexploitation and human impacts on marine ecosystem. It has become a consensus that more scientific-based management is needed to maintain sustainable yield. To realize such fisheries management basic information on recruitment mechanisms of fisheries resources is inevitably needed. Among various marine food resources flatfish is a commercially important group in both quality and quantity. This group includes a large variety of species of which life histories are diversified

within continental shelf habitats, and substantial number of flatfish are well adapted to shallow habitats. Metamorphosing larvae of coastal flatfish species generally migrate inshore and settle in shallow habitats, even though the adults inhab is relatively deeper waters (Minami and Tanaka, 1992). This general life cycle implies that human impacts could directly affect recruitment process of the flatfish species. These ecological and fisheries characteristics suggest that flatfish could be a good model in understanding recruitment mechanisms. Based on these biological advantages of flatfish, the International Flatfish Ecology Symposium has been held every 3 years since 1990.

The idea of stock enhancement for Japanese marine fish emerged in late '50's to enhance once decreased wild stocks by means of mass-release of hatchery-raised seedlings in Japan (Tanaka *et al.*, 1998). After 40-years of trial and error, stock enhancement was shown to be a realistic tool for sustaining fisheries, however there appeared to be several basic biological problems which curtail this goal. In recent years substantial reductions in fisheries have occurred in both the United States as well as in Japan. In the U.S. this has led to increased interest in stock enhancement. However, US researchers have considered to test its potential usefulness and risk from basic ecological and/or ecophysiological viewpoints prior to initiating programs of mass releases.

These applied or practical needs combined with basic scientific interests, resulting in US-Japan cooperative research project under support of UJNR Aquaculture panel.

Brief historical backgrounds on the UJNR cooperative research

As described in introduction the background of the cooperative project can be traced to the 1st International Flatfish Symposium held in the Netherlands in 1990, where the 1st author met with Drs. J. Miller and J. Burke. In the early '90's a US-Japan cooperative research project was conducted under support by JSPS-NSF* to compare early life ecophysiological traits between closely related two species, Japanese flounder *Paralicithys olivaceus* and summer flounder *P. dentatus* under collaboration by Kyoto University and NOAA Beaufort Laboratory. The 2nd and 3rd Flatfish Symposia also accelerated collaborative relationship between J. Miller's group and ours. The present cooperative research project was also stimulated at the 24th UJNR meeting held in Corpus Christi in 1995 after a presentation on a hypothetical eco-physiological model by J. Miller. (Miller *et al.*, 1997) Over the following year positive discussions between US and Japan developed cooperative research idea advanced from both US and Japanese scientists. In 1997, the UJNR cooperative research project on the flounder release started (Table 1).

Why Japanese flounder as a model ?

The most interesting aspects on the early life traits must be the flounder's dramatic metamorphosis, during which drastic morphological and physiological changes occur associated with ecological changes which can be characterized by inshore migration and settlement (Minami and Tanaka, 1992). Outlines of these comprehensive aspects have been documented using laboratory-raised and wild fishes (see Minami, 1982; Inui and Miwa, 1985; Seikai *et al.*, 1986; Miwa *et al.*, 1988; Tanaka *et al.*, 1989). Geographic and ecological characteristics of metamorphosis have partly been analyzed (Tanaka et al., 1997; Tanaka et al., 1998), and long-term data on annual abundance of wild juveniles have been accumulated in several geographically different sites along the coasts of the Japan Sea. The other advantage this species has is that broad background information on the larvae and juveniles have been accumulated. A major part of knowledge has been obtained in relation to the stock enhancement project: from both technical developments in mass-production of seedlings in hatcheries and mass-release of juveniles in the sea. These technical developments can provide us with sufficient numbers of high quality juveniles at specific times for *In situ* ecological experiments.

Although the total catch of Japanese flounder is not so large, multi-uses interests have been placed on the flounder in Japan: stock enhancement, aquaculture, fisheries and its management, and sports-fishing. Nurseries for the juveniles are commonly formed in shallow sandy beaches, implying that the early life stages must be influenced by direct and indirect anthropogenic impacts. When we consider environmental management for coastal areas, we could learn from the life history consistency and flexibilities of the local populations. Based on these features the Japanese flounder becomes one of the most important model species in the world through the past 3-times Flatfish Symposia. The Japanese flounder has closely related species such as the summer flounder and southern flounder P. lethostigma in the east coast of the United States of which nursery habitats are largely different from that of the Japanese flounder. Interspecific comparison on life history aspects could provide us more generalized and basic information than when we focus on a single species.

In summary, the Japanese flounder appears to be an excellent model species with which to develop scientifically-sound stock enhancements supported by basic ecological information on recruitment mechanism and

* JSPS: Japan Society for the Promotion of Science, NSA: National Science Foundation

Table 1. Background of UJNR cooperative research on juvenile flounder release

1990 Nov.	1st Flatfish Symposium (Netherlands) John Miller*1, John Burke*2, M. Tanaka
1992	JSPS-NSF Post-doctor Fellowship J. Burke stayed for 1 year at Kyoto Univ. to investigate Japanese flounder eco-physiology at early stages
1993 Oct.	2nd Flatfish Symposium (Netherlands)
1994-95	JSPS-NSF Joint Program: US-JPN Cooperative Researchon comparative ecology of Japanese and summer flounder J. Burke and D. Hoss visited Kyoto T. Seikai, Y. Tanaka, M. Tanaka, T. Maki, I. Kinoshita visited NMFS Beaufort Laboratory
1995 Oct.	24th UJNR meeting at Corpus Christi John Miller proposed Joint Research to test eco-physiological model UJNR Aquaculture panel positively responded
1996 Jan.	1st Planning meeting at NRIA*3 J. McVey, M. Azeta, M. Tanaka
May	2nd Planning meeting at Kyoto University J. Miller, J. Burke, J. Specker*4
Oct.	25th UJNR meeting at NRIFS*5 J. Miller, M. Tanaka presented related papers
Nov.	3rd Flatfish Symposium (Netherlands)
1997 May	1st Release was performed at Wada beach Kilho Park participated
Sep.	26th UJNR meeting at University of New Hampshire UJNR Cooperetive Research was discussed
1998 May	2nd release was performed at Wada beach US graduate students: Nick King, Joanne Harcke
Nov.	27th UJNR meeting in Ise MAFF workshop in Sendai Satellite UJNR meeting at Maizuru Fisheries Station of K.U.

*1 Department of Zoology, North Carolina State University
*2 NOAA Beaufort Laboratory
*3 National Research Institute of Aquaculture
*4 University of Rhode Island
*5 National Research Institute of Fisheries Sciences

ecosystem-level effects.

Carrying capacity: goal and approach

Thirty-years stock enhancement trials for the Japanese flounder demonstrate fundamental biological issues over which curtail further development of this project as follows (Tanaka *et al.*, 1998; Tanaka, 1999). 1) quality of seedlings including genetic diversity, 2) juvenile ecology related to recruitment, 3) stock structure of the wild population, 4) effects of mass release on natural ecosystems. Many researchers have tried to resolve these problems in recent years, however, no research has been attempted on estimating the carrying capacity of the nursery environments which must be of primary importance as the fundamental ecological criterion for stocking.

There seem to be several reasons why estimation of carrying capacity has not yet been approached in spite of its definite importance. It appears to be a quite time-consuming work; analyzing multi-year variations in mortality, growth, distribution area and food consumption in relation to the juvenile abundance. Another reason is that there has been no effective experimental tool for measuring carrying capacity. However, recent advances in rearing techniques enable us to utilize high quality seedling for a large-scale field experiment whenever we need them. Using hatchery-raised juveniles we can manipulate density of juveniles in the nursery.

In situ field experiment could be combined with laboratory experiments on estimation of food requirement of the juveniles and estimation of production rate of prey mysids. A framework of our approach in evaluating carrying capacity is shown in Figure 1.

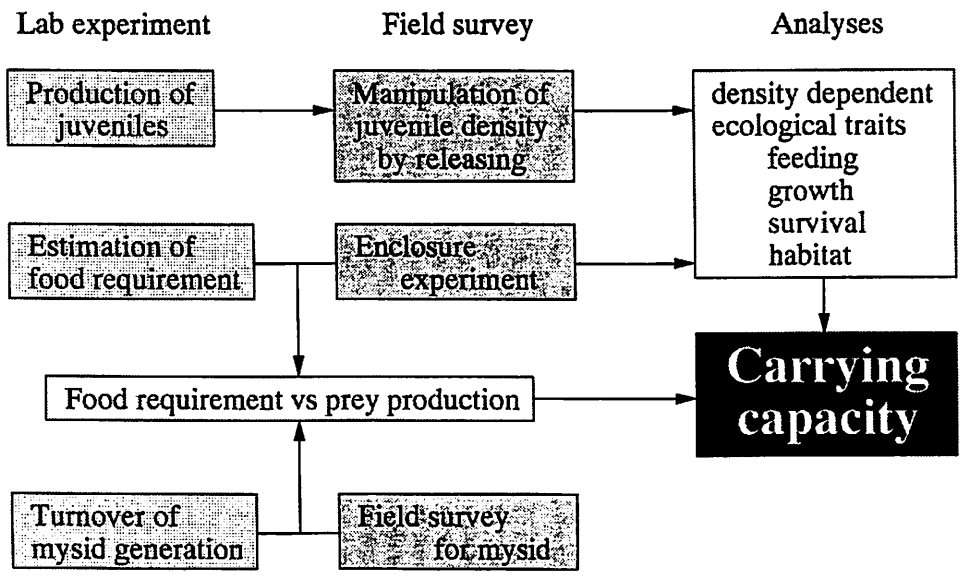

Figure 1. Research framework estimating carrying capacity of a nursery ground, Wada beach, for Japanese flounder juveniles.

Constituents of Japanese team

Previous researches in the stock enhancement project were separated into 3 processes; seedling production, releasing technology and post-release ecological surveys, being done mainly by sea-farming stations, Prefectural Fisheries Experimental Stations, National Fisheries Research Institutes and universities, respectively. This implies the pre-release process has been conducted independently from the post-release process, in which effective feedback function doesn't work. To resolve this discontinuity a research group was organized, which was composed of experts in mass-seedling production and releasing techniques, and experts in fisheries biology and fish ecophysiology. The former corresponds to staffs at JASFA (Japan Sea-Farming Association) and the latter members at two universities (Table 2).

Another critical subject is to set up an experimental system in which we can freely make any kind of *In situ* experiments. Several biotic and abiotic conditions were required related to spatial scale and topography, abundance of wild flounder, prey and predator abundance. A social factor is also important because almost the entire coastal areas of Japan are commonly utilized by fishermen. After one year searching Wada beach, Wakasa Bay, the Sea of Japan was decided as UJNR cooperative research field site (Figure 2).

Table 2. Institutes and members involved in the UJNR cooperative research team

Japan Sea-Farming Association (JASFA)
Miyazu station: T. Tsusaki, Y. Hondo
Obama station: K. Nogami

Fukui Prefectural University
O. Tominaga, D. Kawasaki*, T. Maki*

Kyoto University
Fisheries Station: T. Seikai, I. Kinoshita
Division of Applied Biosciences: M. Tanaka
I. Hayashi, M. Ueno, W.S. Gwak*, Y. Tanaka*,
H. Yamaguchi*, Mostafa Hossain**

Seikai National Fisheries Research Institute
Y. Koshiishi, C. Zeng

<UJNR Secretary>
National Research Institute of Aquaculture
K. Fukusho, I. Nakayama

<Collaborators>
Fukuyama University
H. Fushimi
Japan Sea National Fisheries Research Institute
H. Sudo
Tottori Prefectural Government Office
S. Furuta, N. Tange

*graduate students, **JSPS post-doctor

Annual step approaching the goal

Previous work has revealed three major items limiting the efficiency of marine fish stock enhancement: release time, size and number. Most release to date were performed at the time when hatchery-raised juveniles attained a certain size. However, Furuta (1996) demonstrated that timing of release should be determined in relation to high abundance of prey animals and less abundances of predators. Based on his idea we first investigated a suitable

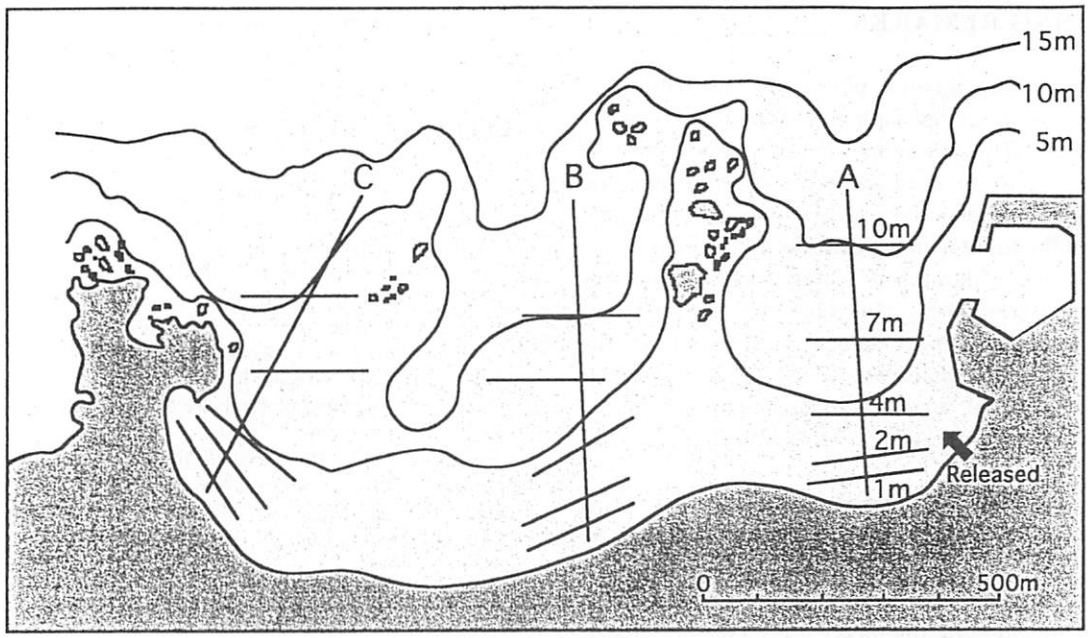

Figure 2. Experimental field for the UJNR cooperative research, Wada beach. Releases were done at 1 and 2 meter zone of the A area. Three sampling lines were set and each 5 depth descrete samplings were conducted on each sampling line.

timing of release relative to prey abundance in 1997. Higher recapture rates (in other words, higher survival rate) were associated with early releases (late May) than with late release (early July). The 2nd experiment was conducted to see suitable size at release: a larger size group (60.7mm TL) and a smaller size group (36.7mm) were simultaneously released in late May 1998. Total number of recaptured juveniles was two-times higher in the large-size group than in the small-size group, whereas there were no significant differences in % empty stomach and % stomach content weight to body weight rates between the two size groups as shown in Figure 3. The two years experiments also revealed that mass-release of hatchery-raised juveniles evidently influenced feeding of wild juveniles: higher percents of empty stomach occurred in wild fish shortly after the mass-release (Tanaka *et al.*, 1999). The next release experimnet will be done to determine whether"over-populated"conditions manipulated by large releases of juveniles will induce enlargement of habitat utilization, low feeding incidence, low growth rate and higher mortality rate. Analysing those density-dependent changes in ecological traits (related to habitat utilization), should permit prediction of maximum environmental capacity of this field site for the juvenile flounder.

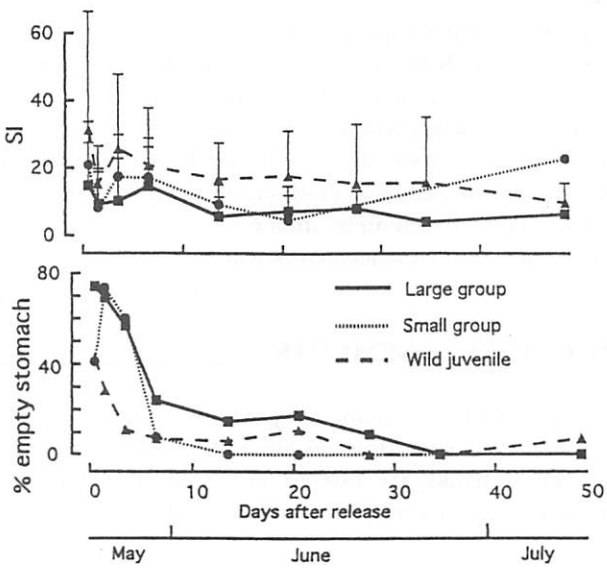

Figure 3. Post-release changes in stomach index (SI) and % empty stomach of wild flounder juveniles, and released large and small group juveniles. Very high percentages of % empty stomach were found in both released groups shortly after release.

CONCLUDING REMARKS

There are three fundamental points of view which are essentially important in developing field sciences as ecology (Tanaka, 1999). This is true in general, and in particular as biology related to stock enhancement. Even in a certain nursery, abundance of a target fish, prey animals and predators can fluctuate widely year to year. Depending on annual variations in numerical components of a nursery area, ecological relationships between the target species and ambient organisms must change. In this context, the most important aspect appears to be long-term continuous data and interannual analyses focused on generalized ecological processes underlying highly variable phenomena.

Another two basic aspects important in enhancing ecological works seem to be comprehensive and comparative viewpoints. When evaluating environmental capacity, we have to analyze various aspects of direct and indirect relationships surrounding the target fish. This requires us to have a more synthetic goal and more comprehensively organized research team. Comparative view points definitely accerelate field science because we can get phenotypic variations among different types of nuseries, and interspecific differences and/or similarities to establish underlying processes associated with species or guilds of species.

In this UJNR Cooperative Research Project, a comprehensive research team was organized. We also tried to promote collaboration with a similar project undertaken by a team at Seikai National Research Institute in Kazusa, Kyushu. In addition objective of the UJNR Cooperative Research is to exchange graduate students between both sides. This is definitely important in general and in advancing stock-enhancement science.

ACKNOWLEDGEMENTS

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COMPARISON OF SUMMER FLOUNDER *PARALICHTHYS DENTATUS* MATURATION, SPAWNING, AND EGG QUALITY BY HORMONAL INDUCTION VS. ENVIRONMENTAL CUES

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In an effort to synchronize ovulation activity, enhance gamete production, and investigate the potential for natural spawning in a commercial hatchery, we examined the use hormones versus environmental conditioning of broodstock Summer flounder. Wild adult fish were induced to spawn after one year of domestication using injections of carp pituitary extract (CPE), injections of salmonid GnRHa+domperidone (Ovaprim®), and by environmental cues only. The experiment was initiated by randomly distributing 30 females of similar maturational status into three treatment groups. Progression of maturational status, incidence of spawning, egg production, and egg quality were measured for the duration of ten days.

All three groups advanced their maturational stage, but not all fish spawned in response to their treatment. Females in Group 1 (CPE) had a 100% incidence of spawning, those in Group 2 (Ovaprim®) had a 40% incidence of spawning, and only 10% spawned in response to environmental cues (Group 3). Of eighteen males, sixteen had reached a mature "running ripe" stage in response to only environmental cues, and six of these males were used to fertilize eggs for this study. On per spawn basis, a similar quantity of eggs was obtained from females who responded to injections of CPE (Group 1), and salmonid GnRHa+domperidone (Group 2). However, total quantity of eggs (per kg of body weight) from females in Group 1 was significantly greater ($p<0.05$) than that from females in Group 2. There was no significant difference ($p>0.05$) in floating percentage, fertilization percentage, and hatching percentage between first batch eggs spawned by females in Groups 1 and 2. Variability in egg batch quality was examined by comparing the fertilization and hatching percentage of eggs from multiple spawns by individuals in Group 1. First batch eggs had a significantly ($p<0.05$) higher percentage of fertilization and hatching than those spawned in the second and third batch, which had no significant difference ($p>0.05$) between them. A high coefficient of correlation and a positive significant ($p<0.05$) relationship was found between the percentage of fertilized eggs and the percentage of hatched eggs for both Groups 1 and 2. There was no significant correlation ($p>0.05$) between the percentage of floating, and the percentage of fertilization or hatching of eggs that were spawned by fish in response to either hormonal treatment.

These results indicate that CPE continues to be the most reliable method of hormonal induction of broodstock Summer flounder with regard to total egg production. However, fish treated with both CPE and salmonid GnRHa+domperidone yielded eggs of similar quality and quantity per spawn. The percentage of fertilized eggs, and not the percentage of floating eggs, was determined to be the best predictor of egg viability (hatching), and should be used as a simple assessment of egg quality in the commercial hatchery. Additional data will be presented which correlates the percentage of fertilization in eggs as a reliable predictor of viable larvae (% feeding after 12 days).

MASS MARKING OF AQUACULTURE FISH FOR SELECTIVE FISHERIES AND MAINTAINING GENETIC INTEGRITY OF NATIVE POPULATIONS

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Recent advances in marine aquaculture have led to several successful programs and a new enthusiasm for marine stock enhancement and sea ranching. To maintain genetic integrity of native stocks and optimize harvest of hatchery fish during the rebuilding phase of enhancement a new management tool, referred to as mass marking and selective fisheries, is being promoted. The basic concept is to mark all hatchery fish with a mark that is easily identified by fishers and implement selective fishery regulations that allow retention of marked hatchery fish and release of unmarked wild fish. Salmonid managers on the West Coast of North America have begun to employ this concept. An automated marking and tagging system has been developed to mark the millions of hatchery salmon necessary for such a program. The system is capable of adipose fin marking and/or coded-wire tagging salmonids without anesthesia or human handling at a rate of 2 fish per second for a size range of 60-150 mm, and at a cost significantly less than what it presently costs to do this marking manually.

MICROSATELLITE DNA LOCI FOR PARENTAGE TESTING IN SUMMER FLOUNDER

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Increasing dependence on captive breeding of marine fishes has created a need to evaluate and manage the genes of broodstock animals. The economic return on hatchery investment can be maximized by developing a program of selective breeding, in which only the best animals are used to produce high-quality fingerlings. We are interested in knowing whether there are differences in breeding value for growth rate, survival or pigmentation among wild-caught broodstock. To that end, we have isolated dinucleotide microsatellites from a summer flounder (*Paralichthys dentatus*) genomic library. The library was constructed by ligating SAU3A I-cut genomic flounder DNA to BamH I-cut pBlueScript vector and transforming into XL-I Blue competent cells. A radioactively labelled (CA) 10 oligonucleotide probe was used to screen the library, and 100 independent clones were sequenced. Six loci (UNHFL 001-006) with repeats lengths of n=15-21 were selected for primer synthesis. Primer sets were designed to produce varying size PCR products and with 3 different fluorescent dye labels such that all six loci could be accomplished in one reaction, producing distinct products when run in one lane of an ABI 373A DNA sequencer. All six loci were successfully multiplex amplified in as single PCR when using standard phenol:chloroform-extracted DNA templates. We are now analyzing complete factorial crosses among 4 males and 4 females to evaluate the performance of different full and half-sib families in a common rearing environment.

POTENTIAL USE OF THE mtDNA CONTROL SEQUENCE AS A GENETIC MARK FOR EVALUATING STOCK ENHANCEMENT EFFORTS FOR RED SNAPPER

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Enthusiasm for stock enhancement is diminished by potential disruption of the genetic integrity of enhanced stocks. The genetic concerns are divided among three areas, (1) increased matings between close relatives accompanied by inbreeding depression (2) inadvertent shifts in allele frequencies resulting in reduced frequency and potential loss of rare alleles, and (3) introduction of nonindigenous alleles accompanied by outbreeding depression.

Inadvertent introduction of nonindigenous alleles can be avoided if broodstock are collected from the stock that is targeted for enhancement. This raises the question, what constitutes a single stock of fish? The geographic area over which appropriate broodstock can be found depends upon the genetic homogeneity and relative isolation of local populations. Genetic markers may be useful in determining the spatial distribution of a particular stock of fish. One source of potentially useful genetic markers is mitochondrial DNA (mtDNA). The utility of mtDNA as a genetic marker depends on the presence of distinct haplotypes in the population and the ability to detect them.

The goal of our study was to characterize the molecular structure of the mitochondrial control region (mtCR) of Red Snapper *Lutjanus campechanus*, and to determine whether enough variation exists in the mtCR to allow its use in as genetic identifiers to support the U.S. Gulf of Mexico Marine Stock Enhancement Program (US-GMSEP) in Mississippi.

Red Snapper were obtained from waters off Cape Canaveral, Mississippi, and Louisiana west of the Mississippi River. DNA was extracted from tail fin clips using a standard phenol:chloroform: EtOH extraction and precipitation, quantified by fluorimetry, and used as template in a PCR reaction with conserved primers to the tRNA sequences flanking the control region. The 916 bp fragment produced was gel purified and ligated into a pGEM®-T easy cloning vector and used to transform JM109 competent cells and plated on LB media. Using blue-white selection, colonies with inserts were identified, and plasmid DNA containing an insert of the proper size was isolated from corresponding minipreps. The entire control region and portions of tRNA-proline and tRNA phenylalanine were sequenced at the DNA sequencing facility at the University of Maine.

The control region of Red Snapper is approximately 824 bp in length, and contains several conserved sequence blocks near its 3' end. The 5' portion contains several inverted repeats and a single TAS element. The entire control region was scanned for the presence of 38 different restriction enzyme sites. Based on restriction site analysis, 5 haplotypes were identified among the 20 fish examined. A hypervariable sequence was identified in the 3' half of the control region. The sequence of this 108 bp region contains 16 variable sites and was unique for each of the 20 fish examined. The sequence of this hypervariable region of individual fish may potentially be different enough that the chance of encountering an identical sequence in an unrelated fish would be low.

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GROWTH OF SUMMER FLOUNDER: WHAT SHOULD WE SELECT FOR AND WHEN?

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As summer flounder (*Paralichthys dentatus*) commercial aquaculture develops in the eastern United States, the industry will certainly want to select for fast-growing individuals. We are gathering basic biological information on growth rates and energy budgets of larval and juvenile stages of this species so that decisions can be made by geneticists about what characteristics should be selected and when the selection should occur.

When summer flounder larvae are reared in a communal tank, metamorphosis occurs over a period of 3-4 weeks and previous research has shown that settling of the fish is highly correlated with growth, so that all the fish are approximately the same size when they settle. When larvae are reared in individual bowls from 8 days after hatch (DAH) to 30 DAH, the differences in growth rate that ultimately lead to different times of metamorphosis begin to appear between 20 and 25 DAH. If the settled fish are not "graded", the larger (earlier metamorphosing) fish exert severe cannibalism on the smaller (later metamorphosing) fish. We grade fish by removing settled individuals from the larval rearing tanks on a weekly basis and growing the juveniles in separate batches.

We conducted an experiment to determine the growth rates of newly settled fish from three gradings taken from one larval rearing cycle. Fish from the rearing cycle settled over a three week period, so three groups of graded fish were used (Grades 1, 2, and 3). Random subsamples of each grade were placed in three replicate 75-L aquaria per grade at a density of 30 juveniles per aquarium. Grade 1 fish were measured beginning at 32 DAH, when they averaged 13.0 mm TL; Grade 2 fish were measured beginning at 39 DAH, when they averaged 14.4 mm TL; Grade 3 fish were measured beginning at 46 DAH, when they averaged 15.4 mm TL. All fish in each aquarium were measured for TL biweekly with an image analysis system. No significant differences in growth rate were observed among the three groups over a six-week period, through the time of weaning to a formulated diet. Thus, although Grade 1 fish were always larger than Grade 2 fish (which in turn were larger than Grade 3 fish) because they had settled earlier, the post-settlement growth rates of the three groups were identical. However, at the end of nine weeks of post-settlement growth by Grade 1 fish, intra-replicate variation in length was increasing and cannibalistic attacks were again becoming a problem. This research will be continued and expanded to determine whether and when selection for fast growth should be conducted.

We are just beginning research on energy budgets of fast-growing vs. slow-growing summer flounder. Our goal is to determine whether some summer flounder grow fast a) simply because they consume more food, b) because they metabolize more efficiently, or c) some combination of the two. We will present our strategy and methods for conducting this research. Our assumption is that, given two types of equally fast-growing summer flounder (voracious food consumers vs. efficient metabolizers), it would be economically more beneficial for the industry to select for the efficient metabolizers.

DIFFERENCES IN GROWTH BETWEEN STRAINS OF STRIPED MULLET *MUGIL CEPHALUS*

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Striped mullet (*Mugil cephalus*) exhibit one of the largest geographic distributions amongst teleosts and have been extensively cultured, supporting major fisheries in many parts of the world. Because it is tolerant to various salinities and feeds at the lowest trophic levels, this species is an attractive candidate for culture. The species is also of cultural significance in Hawaii being one of the main fishes cultivated by the ancient Hawaiians predating the 14th century. The tremendous geographic range of the striped mullet presents obvious barriers to gene flow and one could infer the existence of "races" of striped mullet that potentially have characteristics (e.g., growth, fecundity, resistance to disease) that are variable between these groups. Investigations utilizing protein electrophoresis and mitochondrial DNA analysis clealy indicate discrete groupings throughout its geographic range.

The result of a side-by-side comparison of growth between fry from Taiwan and fry from Hawaii conducted in Guam, indicate that differences in growth do exist and are consistent with the population groupings (Hawaii strain mean S.D. body weight $=201.9 \pm 44.7$g; Taiwan strain $=702.8 \pm 129.5$g after 1 year in culture). The results clearly indicate that the differences in the rate of growth are intrinsic to each group and significant enough to warrant examination as to the whether or not, *Mugil cephalus*, is a single species or a multispecies complex. While it appears that research efforts have apparently resulted in a means to capitalize on a biological trait, that does not necessarily mean that aquafarmers in Hawaii will be able to benefit from the research conducted. In fact, the next obstacle facing the culture of striped mullet in Hawaii underscores one of the many predicaments faced by farmers, politicians, resesrchers, administrators, and extension personnel trying to promote and develop aquaculture in Hawaii. At present, the striped mullet is not allowed to be imported into Hawaii under State Law as it is not on the approved importation species list, in any of its categories. These regulations that are developed and enforced by the Department of Agriculture, Plant Quarintine Divsion, were set up to protect the islands from introductions that are potentially harmful to Hawaii's unique and varied ecosystem. The primary concerns are potential impacts with the environment if the species to be imported somehow escapes, which is assumed as a worse case scenario. Environmental impacts also include possible affects on interbreeding of the introduction with native stocks as is the case with the striped mullet. The classic "battle lines" are formed with environmentalist on one side proclaiming how fragile Hawaii's ecosystem is and "farmers" on the other asserting the need for economic development. Here we have a case of technology developed in Hawaii spanning two decades and millions of dollars invested and still with aquafarmers in Hawaii not being able to profit from the research activities as other factors prevent full exploitation of the results. It is clear that the conversion from research and technology development to commercialization in aquaculture does not appear to take place as one would logically assume.

CURRENT PROGRESS OF THE BREEDING TECHNIQUE USING COMPLETELY HOMOZYGOUS FISHES IN HIRAME *PARALICHTHYS OLIVACEUS*

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To ensure the methodology of breeding using completely homozygous fishes or cloned populations in hirame, practical trials on genetic improvement have been carried out since 1991.

Primary objective of the breeding was to make clonal lines that showed high growth rate. Fifty-seven mitotic gynogenetic diploids of one half a year after the birth with individual growth records were obtained. Then large females were selected to induce completely homozygous cloned populations. Males as well as females were produced in a cloned population, they had been very useful to maintain the clonal line because the third generation was already born by intra-clonal cross. Inter-clonal cross has given more vigorous heterozygous cloned populations since 1995. At the end of 1996, it was revealed that the resultant heterozygous cloned fishes achieved faster growth than corresponding controls (normal feminized fishes). As secondary effects, remarkable examples of some disease resistance did appear in cloned populations. Thus, it is concluded that the breeding technique is very successful and established.

On the basis of these results, the Fisheries Agency of Japan in 1997 made guidelines for the commercial use of progenies of artificially induced completely homozygous hirame. Commercial aquaculture of an improved cloned population has been started in this year.

Populations of genetically homogeneous cloned hirame have been used as experimental animals, especially in the molecular biology. The fruitful results of such research will contribute to realistic aquaculture.

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EFFORTS TO UNDERSTAND STOCK STRUCTURE OF SUMMER FLOUNDER (*PARALICHTHYS DENTATUS*) IN NORTH CAROLINA, USA.

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Understanding the stock structure of the summer flounder is critical to attempts to manage this species. Recent interest in the commercial culture and stock enhancement make research in this area particularly urgent. Research on summer flounder in the coastal waters of North Carolina is of particular relevance to the stock structure of this species due to the existence of a zoogeographic boundary at Cape Hatteras, NC. The importance of this boundary is being investigated through mark-recapture studies of adults, field sampling of larvae and laboratory experiments on larvae and juveniles originating from different brood stocks.

Twenty three thousand summer flounder were marked in coastal waters and movement of recaptured animals relative to season and the zoogeographic boundary analyzed. Seasonal occurrence of larvae relative to this boundary was compared and animals were characterized in terms of fin ray numbers and size and developmental stage at arrival at the coast. In the laboratory we reared larvae from two brood stocks; one originating from the northern extent of the summer flounder's range and the other from North Carolina. These animals were used to determine the importance of temperature to fin ray formation and to compare growth and development of the two groups of larvae relative to temperature. Additional laboratory experiments include comparisons of salinity tolerance and the importance of temperature in sex determination for the two groups of fish. Our results are discussed relative to competing hypotheses regarding the stock structure of this flounder.

POPULATION CONTROL USING TRIPLOID BIVALVES AND THE SPECIAL PROBLEM OF CHROMOSOME SET INSTABILITY

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Triploidy in bivalves represents not only an opportunity for product improvement but also the potential of preventing unwanted reproduction - so-called population control. The advent of tetraploids, especially in oysters, makes population control feasible on a fairly large scale. For example, it is reasonable to think about pilot scale plantings of non-native species if all are triploid. A special problem that has emerged over the last five years has been "reversion" of triploids to a heteroploid mosaic state, e.g., diploid and triploid. We have confirmed this process in three species and it seems to be a regular feature of triploid populations, whether induced during early development or produced from tetraploid × diploid matings. However, the frequency of reversion in cytochalasin induced triploids is about 2-3 times higher than in tetraploid × diploid ones. The frequency of "reversion" varies with population and among grow out sites. Harsh environments seem to exacerbate the problem of reversion. Once reversion begins it is progressive and more and more diploid cells accumulate with time. Surprisingly, the gametes produced in mosaics seldom have haploid cells in them. Some preliminary cytogenetic data suggests that chromosome elimination may be caused by unusual chromosome clumping, as evidenced by mitotic metaphase spreads. Although vexing, there is also evidence that the reversion problem has a genetic basis and "true breeding" lines of triploids may be possible where 100% triploidy is imperative.

STOCK ENHANCEMENT OF PACIFIC THREADFIN (*POLYDACTYLUS SEXFILIS*) IN HAWAII

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The Oceanic Institute in 1993 began a stock enhancement program in Hawaii for Pacific threadfin (*Polydactylus sexfilis*, known locally as moi), building upon the knowledge gained during a previous multi-year program to develop stock enhancement technology for striped mullet (*Mugil cephalus*). Hatchery and nursery techniques were developed for moi to support the production of fingerlings for tag-release-recapture experiments. The objectives of these experiments were to determine the optimal release strategies (size, season, site and their interactions) for subsequent pilot-scale releases. Fingerlings were tagged with coded wire tags (CWT) or CWT's and experimental visible implant elastomer (VIE) tags. Releases of tagged fingerlings occurred during the natural juvenile recruitment period in 1993, 1994, 1996 and 1997. Recapture efforts continued at approximately monthly intervals from January 1994 through May 1995, and at two-week intervals from September1996 to the present.

Recaptured tagged fish were analyzed for relative survival rate, growth rate, and dispersion distance from release site. There was a significant size-at-release effect, with fish released at the largest size (100-130 mm fork length) generally showing the greatest survival and those released at small sizes (50-70 mm FL) showing lowest survival. Dispersal distances along the coast from release sites in a semi-enclosed bay (Kahana Bay) and a larger, more open coastal area (Kailua Bay) were similar. Catch per unit effort (CPUE) analyses revealed strong interannual differences in the abundance of wild fish, and a relationship between number of fish released and CPUE for tagged fish.

Analyses of stomach contents from wild and cultured fish show that there are no significant differences in dietary composition, even very soon after release. Diet items consist primarily of epifaunal and infaunal crustaceans found in the shoreline surf zone. Preliminary analyses do not indicate any significant feeding selectivity.

Preliminary minisatellite DNA analyses have not shown significant genetic differences between threadfin populations on different islands, or between population from several locations on Oahu.

The results of the stock enhancement program to date with mullet and moi in Hawaii show that stock enhancement can play an important role in marine resource management. The challenge that remains is to incorporate stock enhancement into an integrated marine resource management plan.