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IMPROVEMENT OF GROWTH AND SURVIVAL IN CULTURED RABBITFISH (*SIGANUS GUTTATUS*)

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Executive Summary

The purpose of this project is to improve the growth and survival of the rabbitfish, *Siganus guttatus,* cultured in Southeast Asia, by understanding the rate-limiting component(s) in the growth hormone-insulin-like growth factor system, which might explain the slow growth rate during the early juvenile stage and use this information to enhance growth rate by administration of growth hormone using various methodologies.

During the period of the report, the growth hormone (GH) from rabbitfish was purified and antiserum was raised in rabbits. This will enable the development of a quantitative assay for the determination of circulating hormone levels. The native protein purified at the start of the project was used in growth enhancement experiments of rabbitfish kept under various salinities. The cDNAs for rabbitfish GH and insulin-like growth factors I and II (IGF-I and IGF-II) were also cloned. Studies were initiated to produce the GH protein in bacteria in large quantities in order to have sufficient amounts needed for growth enhancement experiments. Production of IGF-I peptide in bacteria was likewise attempted. Experiments were also carried out (by using molecular biology methods) in which the level of expression of two genes for growth factors (IGF-I and IGF-II) was determined during early stages of development of the rabbitfish. The results obtained during this period suggest that administration of a little exogenous GH to the fry significantly improved its growth and rearing fry in dilute seawater likewise improved its growth performance.

The collaboration during this period included a meeting between the Israeli and the Philippine PIs during the Fish Endocrinology Meeting in Seattle in which planning of the project was discussed, exchange of materials between the three laboratories, exchange of information by using the electronic mail.

Section I

A. Research Objectives:

1. Purification of *S. guttatus* GH polypeptide and raising antibodies.
2. Molecular cloning of *S. guttatus* GH and IGF-I cDNAs.
3. Cloning of GH and IGF-I in expression vectors for production of large quantities.
4. Effect of exogenous native *S. guttatus* GH on growth;
5. Effect of exogenous bovine GH and low salinity on growth.

B. Research Accomplishments:

1. Isolation of growth hormone (GH) from rabbitfish

GH was extracted from pituitary glands under alkaline conditions, fractionated by gel filtration chromatography on Sephadex G-100, and purified by high performance liquid chromatography (see enclosed reprint in Appendix A).

2. Cloning of rabbitfish GH cDNA

A combination of RT-PCR and RACE (rapid amplification of cDNA ends) resulted in a cDNA of 860 nt, excluding the poly(A) tail. The untranslated regions are 94 and 175 bp in the 5' and 3' ends, respectively. It has an open reading frame of 588 bp coding for a signal peptide of 18 amino acids and a mature protein of 178 amino acid residues (see enclosed reprint in Appendix A).

3. Cloning of rabbitfish IGF-I cDNA

Rabbitfish IGF-I cDNA was successfully cloned from the liver. The preproIGF-I is composed of 186 amino acids (aa); this is composed of 44 aa for the signal peptide, 68 aa for the mature peptide and 74 aa for the E domain. Only one form of E-domain is found in rabbitfish IGF-I (see enclosed manuscript in Appendix B).

4. Cloning of rabbitfish IGF-II cDNA (not in the original proposal)

Rabbitfish IGF-II cDNA was also successfully cloned from the liver. The preproIGF-II is 215 aa long; this is composed of 47 aa for the signal peptide, 70 aa for the mature peptide and 98 aa for the E domain (see enclosed manuscript in Appendix B).

5. Developmental expression of IGFs in rabbitfish

In order to examine if there is differential regulation of both IGFs during early development in rabbitfish, primers were designed for both rabbitfish IGF-I and II and used for RT-PCR of samples from different stages of embryogenesis and different larval stages. IGF-II transcript was expressed in unfertilized eggs (although very weak) and a very strong expression was already observed during morula stage (about 3 hours after fertilization) and this was

maintained until hatching. On the other hand, IGF-I transcript was observed only in embryos near hatching. In the larvae, however, both IGFs were more or less equally expressed (see enclosed manuscript in Appendix B).

6. Experiments to enhance growth in rabbitfish

a) Treatment with bovine GH to improve growth in rabbitfish fry:

Rabbitfish fry were injected with bovine GH at doses of 0.1 and 0.01 μg/g BW. GH injection was given weekly for 4 weeks and the experiment lasted for 8 weeks. Significant increase in growth was observed starting on the 5th week in the treated groups (see enclosed manuscript in Appendix C).

b) Rabbitfish fry grow faster in dilute SW than in full-strength SW. An experiment was conducted to find out whether rearing fry in dilute SW and treating them with bovine GH would have an additive effect on growth. Fry were reared for 8 weeks in dilute SW (20 ppt) and bovine GH (0.01 μg/g BW) was injected weekly in the first 4 weeks. Rearing the fry in dilute SW and treating them also with bGH did not further improve the growth (see enclosed manuscript in Appendix C).

c) Preliminary experiments were performed to test the effect of the purified GH to promote growth in rabbitfish fry. Weekly intraperitoneal injections of the GH significantly accelerated growth. The effect was evident 3 weeks after the start of the treatment, and was still significant 2 weeks after the treatment was terminated (see enclosed reprint in Appendix A).

7. Production of recombinant rabbitfish GH

a) Rabbitfish GH cDNA was used as template to amplify the mature GH polypeptide using appropriate primers and PCR amplification. The amplified fragment was purified by gel electrophoresis, and cloned in pGEM-T Easy vector. DNA from clones containing the insert was sequenced to verify that no errors occurred during PCR amplification. The insert of the mature GH was cut out from this plasmid using NcoI and BamHI restriction enzymes and cloned in the expression vector pET-3d digested also with Nco I and BamHI and used to transform JM109 bacteria (Fig. 1). For expression, the plasmid pET-3d containing rfGH from four clones, which were obtained, was used to transform the host bacteria BL21DE3.

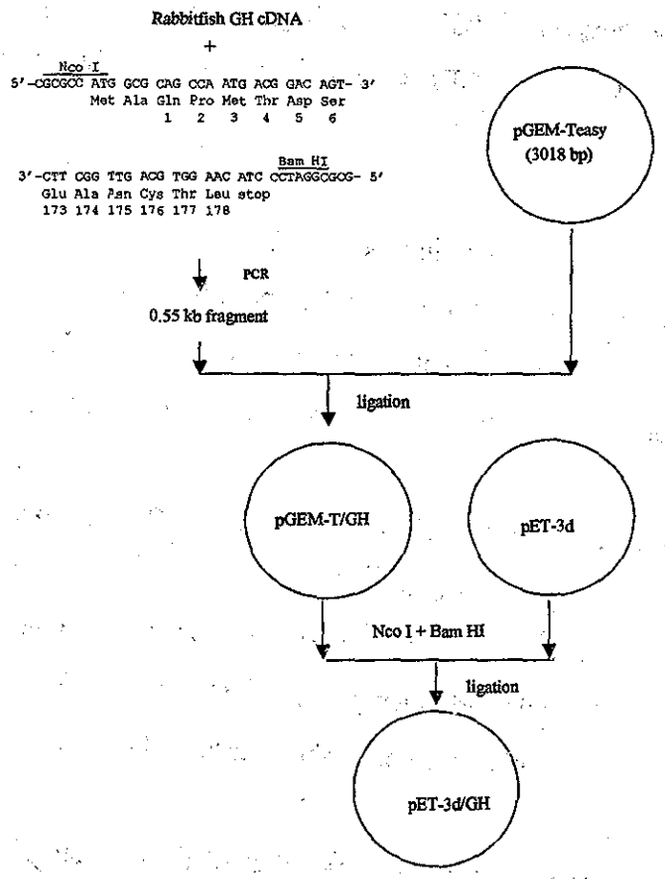

Figure 1. Schematic illustration of the different steps involved in the construction of the recombinant rfGH expression vector pET/GH.

b) Expression of GH was tested by using small-scale cultures of 5 ml and induction with IPTG (0.4mM). One ml aliquots were centrifuged, washed with TE and run on an SDS-reduced polyacrylamide gradient gel of 5%-15% after being boiled for 10 min in sample buffer or after being lyzed using a short sonication. The proteins were transferred to nitrocellulose and immunoreacted with antiserum raised against native rabbitfish GH purified from pituitaries. Three bacterial cultures were found to express rabbitfish GH, two of which express large amounts, as judged from small-scale induction experiments. Upon induction, followed by SDS-PAGE and Western blot analysis, a band similar in its mobility to human GH or pituitary extract from *Sparus aurata* reacted with anti-GH antibodies raised against native rabbitfish GH, indicating that indeed the two bacteria are producing recombinant rabbitfish GH.

c) Clone #2 was used for a large-scale production of rabbitfish GH. Fermentation of ten liters of bacteria was carried out including induction for 4 hours with IPTG, resulting in a pellet of 30 g bacteria. Inclusion bodies containing the recombinant GH were purified from the bacteria

by initial treatment with lysozyme for 30 min in the cold room in a solution containing 10 mM Tris pH 8 and 10 mM EDTA. Then repeated washings in cold water containing 0.1% Triton X-100, sonication and centrifugation were performed until a white pellet of inclusion bodies was obtained. The final washings were done without Triton X. The inclusion bodies were aliquoted into 4 batches of 20 ml each and frozen at –20°C. A 500 microliter aliquot was taken for SDS-PAGE analysis in order to quantify the amount of GH present in the inclusion bodies. From calculations based on a known amount of recombinant ovine GH in an SDS-PAGE analysis suggest that the yield is about 500 mg of GH-containing inclusion bodies (Fig. 2).

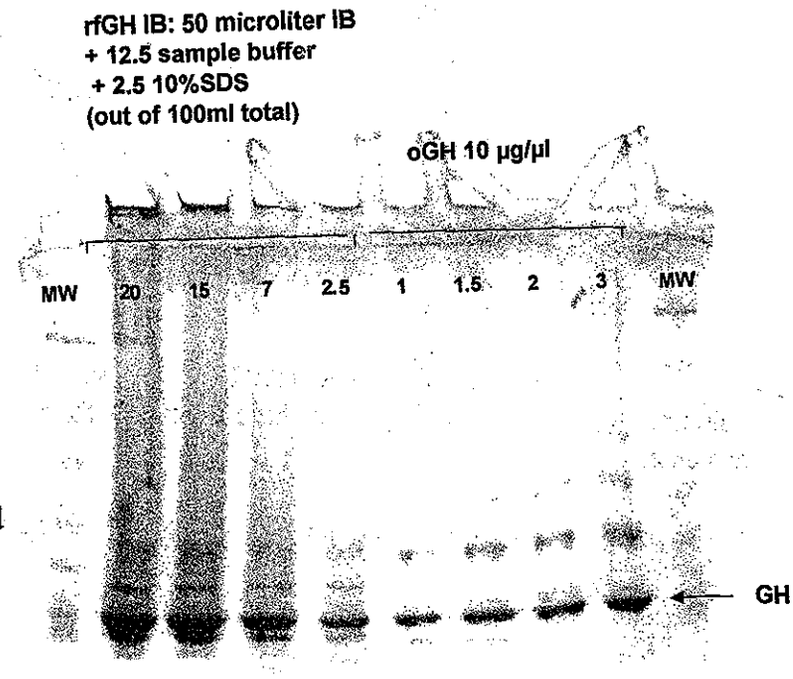

Figure 2. SDS-PAGE analysis of purified inclusion bodies containing rfGH. The numbers above each lane represents amount in microliters loaded onto the gel.

d) Four protocols of refolding were carried out: 0.1 mM, 0.2 mM, 1mM and 5 mM cysteine. Following incubation in the cold for 1 hour the refolded protein was dialyzed against 10 mM Tris pH 9 and loaded onto a Q sepharose mini column. The protein was eluted by using increasing concentrations of NaCl, from 50 mM to 300 mM. Protein content in the fractions was determined at absorbance of 280 nm. Peak fractions were analyzed by HPLC on Superdex columns. The monomer fraction of the refolded hormone was eluted with 100 mM NaCl. Fractions were pooled and dialyzed against 0.05% $NaHCO_3$ and lyophilized. Aliquots were tested under non-reducing conditions of SDS-PAGE. A clean band of about 21 kDa was observed, indicating the presence of monomer GH (Fig. 3).

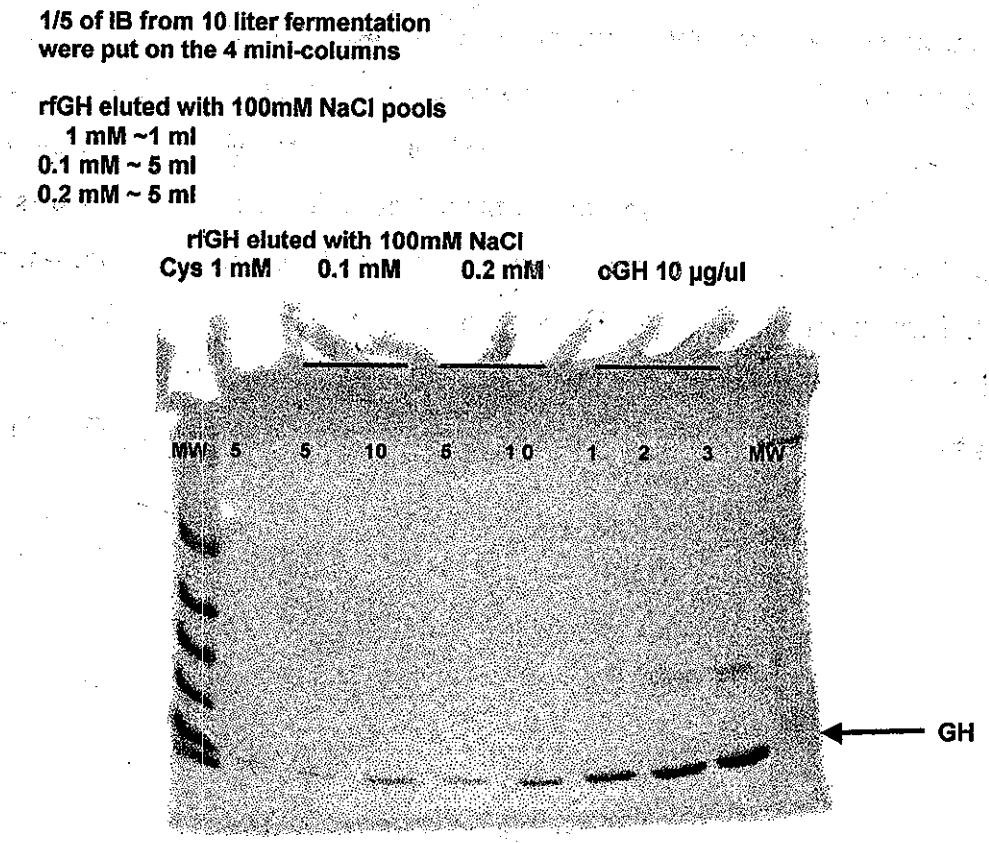

Figure 3. SDS-PAGE of purified rfGH, eluted with 100mM NaCl from Q sepharose mini columns, following refolding using three protocols for re-folding: 0.1 mM, 0.2 mM and 1 mM cysteine.

SDS-PAGE of the purification steps from crude inclusion bodies to purified rabbitfish GH is shown in Fig. 4.

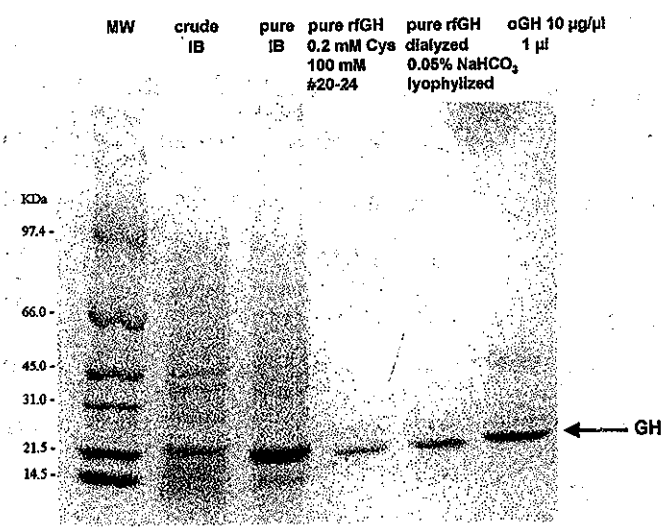

Figure 4. SDS-PAGE of various steps of purification of rfGH.

8. Production of recombinant rabbitfish IGF-I:

Rabbitfish IGF-I was used as template to amplify the mature IGF-I polypeptide as done for GH (Fig. 5).

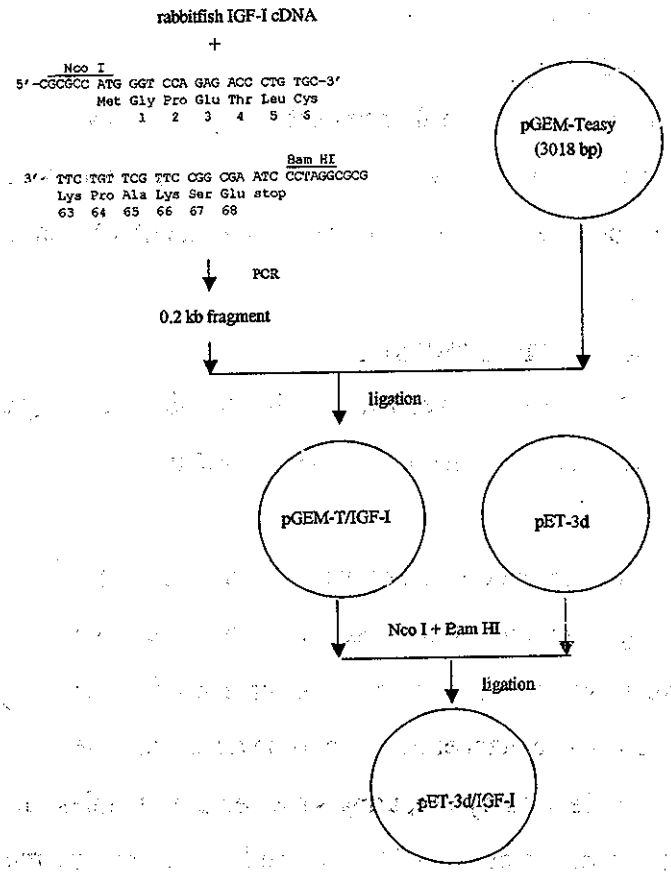

Figure 5. Schematic illustration of the different steps involved in the construction of the recombinant rfIGF-I expression vector pET/IGF.

9. Biological activity of recombinant rabbitfish GH

Several biological assays are currently underway to test the biological activity of the rfGH: injections into rabbitfish fry, addition to cultures of rainbow trout hepatoma cell line RTH149 in order to induce IGF-I gene expression and addition to HEK392 cells that have been stably transfected with mammalian GH receptor cDNA for the determination of GH binding.

C. Scientific Impact of the Collaboration:

During the reported year, the research involved a close collaboration between the scientists participating in the project. Purification of the rabbitfish GH protein and raising antibodies was accomplished by the Philippine partner as were the cloning of rfGH, rfIGF-I and rfIGF-II cDNAs. These cDNAs were transferred to the Israeli partners for cloning in expression vectors and producing recombinant proteins in bacteria. Dried recombinant rfGH was sent back to the

Philippines for testing its ability to enhance growth in rabbitfish fry. The anti-rfGH prepared by the Philippine partner was sent to the Israeli partners and was used to identify the recombinant rfGH.

D. Description of Project Impact:

The recombinant rfGH will be used for enhancement of growth and survival of cultured rabbitfish and for development of quantitative assays for the determination of circulating GH. The recombinant IGF-I will be used as well for the development of quantitative assays to measure its levels in the plasma.

E. Strengthening of Developing Country Institutions:

Dr. Felix Ayson will come to Israel in the second year for training in methods of expression, refolding and purification of recombinant GH and IGF-I polypeptides.

F. Future Work:

Part of the planned work has been accomplished prior to the commencement of the project, during Dr F. Ayson's training in Japan, which enabled us to make much progress beyond the planned schedule. The future work will include large-scale production of rfGH for growth enhancement experiments, growth enhancement experiments by intraperitoneal injections, by incorporation of rfGH in microdiet and by preparing slow release capsules. In order to increase the half-life of GH rabbitfish serum albumin will be cloned. Once the preliminary experiments for expressing rfIGF-I are completed, large amounts will be produced and tested for biological activity.

Section II

A. Managerial Issues: The project started immediately after signing the contract, as planned. First payment was transferred to the Grantee Institution and to the Collaborating Institutions. A second financial report is being prepared for both Institutions. No problems have arisen so far.

B. Budget: No changes were made.

C. Special Concerns: No Changes with respect to original proposal.

D: Collaboration, Travel, Training and Publications: The three partners are continuously communicating through e-mail. Results and information obtained at any of the sites are shared by sending the data like figures, as attached files. Molecular and biochemical tools developed during the project are being transferred according to the need (cDNAs, antibodies, recombinant protein). The first visit of Dr. Ayson to Israel is planned for year 2002.

E. Request for American Embassy Tel-Aviv or A.I.D. Actions: No problems were encountered, thus no need for special assistance.

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Isolation, cDNA Cloning, and Growth Promoting Activity of Rabbitfish (*Siganus guttatus*) Growth Hormone[1,2]

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We report the isolation, cDNA cloning, and growth promoting activity of rabbitfish (*Siganus guttatus*; Teleostei; Perciformes; Siganidae) growth hormone (GH). Rabbitfish GH was extracted from pituitary glands under alkaline conditions, fractionated by gel filtration chromatography on Sephadex G-100, and purified by high-performance liquid chromatography. The fractions containing GH were identified by immunoblotting with bonito GH antiserum. Under nonreducing conditions, the molecular weight of rabbitfish GH is about 19 kDa as estimated by SDS–PAGE. The purified hormone was potent in promoting growth in rabbitfish fry. Weekly intraperitoneal injections of the hormone significantly accelerated growth. This was evident 3 weeks after the start of the treatment, and its effect was still significant 2 weeks after the treatment was terminated. Rabbitfish GH cDNA was cloned to determine its nucleotide sequence. Excluding the poly (A) tail, rabbitfish GH cDNA is 860 base pairs (bp) long. It contained untranslated regions of 94 and 175 bp in the 5′ and 3′ ends, respectively. It has an open reading frame of 588 bp coding for a signal peptide of 18 amino acids and a mature protein of 178 amino acid residues. Rabbitfish GH has 4 cysteine residues. On the amino acid level, rabbitfish GH shows high identity (71–74%) with GHs of other perciforms, such as tuna, sea bass, yellow tail, bonito, and tilapia, and less (47–49%) identity with salmonid and carp GHs. © 2000 Academic Press

Key Words: rabbitfish; growth hormone; growth promotion

Growth hormone (GH) therapy is commonly used to enhance growth in domestic animals and it has also become increasingly popular in fish in aquaculture. In fish, approaches to accelerate growth by GH administration vary from injection and oral administration of the hormone to transgenics. Results from several of these trials were, however, inconsistent. Except for some cases of transgenics, where the fish produced were extraordinarily large, albeit few (Devlin *et al.*, 1994), intraperitoneal injection of either natural or recombinant GH yielded significant results only if the weekly injections were for more than 5–9 weeks (Kawauchi *et al.*, 1986; Sakata *et al.*, 1993; Guillen *et al.*, 1998). Oral administration of the hormone would be the most practical for mass application in fish husbandry, although the question of the effective delivery of the hormone without it being degraded in the stomach should be carefully assessed (Moriyama *et al.*, 1993; McLean *et al.*, 1999).

Slow-growing and economically important fish spe-

[1] The nucleotide sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank databases under Accession No. AB031298.

[2] A patent is pending (IPO 1-1999-01457) for the cDNA sequence of rabbitfish GH reported in this paper.

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cies are good candidates for growth enhancement by GH therapy. Such is the case for rabbitfish, *Siganus guttatus* (Perciformes; Siganidae), an important aquaculture species in Southeast Asia and the Arab Gulf region. A full understanding of the factors that regulate growth in this species is necessary if we are to design strategies to accelerate its growth. As a first step to understanding how growth is regulated in rabbitfish, we purified rabbitfish GH, examined its growth promoting activity in the fry, and cloned its cDNA.

MATERIALS AND METHODS

Fish

Immature rabbitfish (*Siganus guttatus;* 150–200 g body wt) were purchased from fishpond owners in the northern Philippines. Pituitary glands were collected from freshly killed fish and immediately frozen in liquid nitrogen and stored at −80°C until use.

Isolation and Purification

Pituitary glands (1 g) were homogenized in 5 ml of 50 mM ammonium acetate buffer (pH 9.0) containing 5 mM phenylmethylsulfonyl fluoride. The homogenates were stirred for 1 h at 4°C and centrifuged at 12,000*g* for 30 min. The supernatant was applied on a Sephadex G-100 column (1.9 × 100 cm). Proteins were eluted with 50 mM ammonium bicarbonate buffer (pH 9.0) at a flow rate of 15 ml/h. Three-milliliter fractions were collected and the absorbance was measured at 280 nm. Appropriate fractions were pooled and lyophilized, and 10 µg of each fraction was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970). Resolving and stacking gel concentrations were 15 and 7.5%, respectively. The gel was stained with 0.25% Coomassie brilliant blue (R-250) and the protein bands in the gel were visualized after destaining in methanol:acetic acid:water (2.5:1:6.5).

For immunoblotting, protein bands from SDS–PAGE were transferred into nitrocellulose membrane and incubated overnight at 4°C with bonito GH antiserum (diluted 2000×). Immunoreaction was detected using the ABC kit (Vector Labs, CA). Two immunoreactive fractions (G-3 and G-4) were further purified by reversed-phase high-performance liquid chromatography (HPLC).

Five hundred micrograms of G-3 and G-4 fractions was dissolved in 0.1% trifluoroacetic acid (TFA) and was analyzed by HPLC (LC800, Jasco, Tokyo, Japan) on a TSK gel ODS-120T column (0.46 × 25 cm, particle size 5 µm) with a linear gradient of 20–80% acetonitrile in 0.1% TFA for 60 min at a flow rate of 1 ml/min and column temperature of 40°C. Absorbance was monitored at 220 nm. Individual peaks were collected, lyophilized, and analyzed by SDS–PAGE and immunoblotting.

The N-terminal sequence of rabbitfish GH was determined by automatic sequencing on a Shimadzu PSQ1 gas-phase protein sequencer as described by Rand-Weaver and Kawauchi (1992).

cDNA Cloning

Total RNA was extracted from 100 mg of frozen pituitary with Isogen (Nippon Gene, Tokyo, Japan) following the manufacturer's protocol. The concentration of total RNA was estimated by measuring the absorbance at 260 nm (conversion factor: 1 OD = 40 µg RNA/ml). First-strand cDNA was reverse-transcribed from total RNA using the First Strand cDNA Synthesis kit (Pharmacia Biotech, Sweden). Reverse transcription was carried out in a 15-µl reaction mixture containing 2.8 µl of total RNA (5 µg), 5 µl of bulk first-strand cDNA reaction mix, 1 µl of 15 mM dithiothreitol, 1 µl (5 µg/µl) of *Not*I-d(T)$_{18}$ bifunctional primer, and 5.2 µl of DEPC-treated water.

Six degenerate sense primers and two anti-sense primers were synthesized (Espec Oligo Service, Tsukuba, Japan) based on the most conserved regions of the amino acid sequence of known teleost GHs to clone the internal region of rabbitfish GH cDNA.

During PCR, the 50-µl reaction mixture [1 µl first-strand cDNA (template), 2 µl each of sense and anti-sense primers (final conc. 0.4 µM), 1 µl nucleotide mixture (0.2 mM), 5 µl of 10× PCR buffer (final conc.: 10 mM Tris HCl, pH 8.8; 50 mM KCl; 1.5 mM $MgCl_2$), 1.25 units of Taq DNA polymerase] was subjected to 35 cycles of amplification; each cycle consisted of 1 min and 30 s denaturation (94°C), 1 min primer annealing (40°C), and 1 min and 30 s primer extension (72°C). Final extension (72°C) was for 7 min. Among the 12 primer combinations tested, the sense primer GH-8

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[5′-CC(ACGT) AT(ACT) GA(CT) AA(AG) CA(CT) GA-3′] and the anti-sense primer GH-2R [5′-TC(AGTC) AC(TC) TT(AG) TGC AT(AG) TC-3′] yielded a PCR product of the expected size after gel electrophoresis.

The rapid amplification of cDNA ends (RACE) method of Frohman (1990) using the 5′ RACE system kit, Ver. 2.0 (Gibco BRL, Gaithersburg, MD), was used to clone the 5′ end. Two anti-sense primers; RF-GH-1 (5′-AGA CTC AAC CAG TCG ATA GG-3′) and RF-GH-2 (5′-GAC AGC AGC TTC ATC ACA GA-3′), were prepared based on the nucleotide sequence of the internal region of the rabbitfish GH cDNA. These two gene-specific primers and an abridged anchor primer provided in the kit were used for reverse transcription and PCR. PCR conditions were the same as described above.

Two sense primers from the 5′ end of rabbitfish GH cDNA were designed, RF-GH-3 (5′-ATC ACA GCT GAA CTG CAC AA-3′) and RF-GH-4 (5′-AGC AAG GAC TAG GAC CAG AA-3′), and used with *Not*I-$d(T)_{18}$ bifunctional primer as the antisense primer in cloning the full-length rabbitfish GH cDNA. Multiple clones were examined to guard against PCR errors.

PCR products were electrophoresed on agarose gels and visualized by ethidium bromide staining. The DNA was extracted and purified from agarose gels using QIAEX II gel extraction kit (Qiagen, Germany), inserted and ligated into pT7 Blue T-Vector, and transformed in NovaBlue competent cells (Novagen, WI). The plasmid DNA was then extracted from the bacterial cells using QIAprep Spin Plasmid Kit (Qiagen). Nucleotide sequencing was performed using an ABI Prism DNA Sequencer 373 (Perkin–Elmer, CA).

Biological Activity

The growth promoting activity of purified rabbitfish GH was tested in the rabbitfish fry. Forty-five fry (3–4 g BW) were randomly divided into three groups (15 fish/group) and stocked in separate 250-liter fiberglass tanks. Two groups were given weekly intraperitoneal injections of rabbitfish GH at doses of 0.01 and 0.1 μg/g body wt. The hormone was first dissolved in a small volume of 0.01 N NaOH and diluted with saline (0.9% NaCl). The third group was injected with saline solution and served as a control. Fish in each group were individually marked by a small cut in the operculum to follow growth in each fish. The hormone injection was given only until the fifth week and the fish were reared for 4 more weeks. Feeding and water managements were the same in all groups. Changes in fish body weight and fork length were measured every week. Significant differences in body weight and fork length between the control and the hormone-treated groups at each sampling time were determined by *t* test after one-way ANOVA (Sokal and Rohlf, 1995). Percentage data were transformed by arc sine or square root transformations, where appropriate, before the analysis.

RESULTS

Isolation and Purification

The elution pattern of the alkaline extracts of rabbitfish pituitary glands on Sephadex G-100 column has already been reported in a separate paper (Ayson *et al.*, 1999). From the elution profile, fractions G-3 and G-4 contained protein bands of molecular weights between 18 and 22 kDa, and these bands were immunoreactive with bonito GH antiserum. The HPLC profiles of fractions G-3 and G-4 are shown in Fig. 1. When stained with Coomassie's brilliant blue after SDS–PAGE, the peak eluting at 32 min contained a band with molecular weight of about 19 kDa (Fig. 2A); this band cross-reacted with bonito GH antiserum (Fig. 2B). No immunoreaction was detected with anti-salmon PRL and anti-flounder SL (data not shown). The yields of pure GH from G-3 and G-4 fractions were 0.9 and 1.0 mg/g of pituitary (wet weight), respectively.

N-terminal amino acid sequencing of intact protein gave no sequence indicating that the amino terminal is blocked.

cDNA Cloning

A cDNA fragment of 267 bp was amplified in the first PCR using the degenerate primers, GH-8 and GH-2R. This fragment corresponds to nucleotides 347–613 of the cDNA (Fig. 3). The second PCR using primers RF-GH-2 and 5′ RACE abridged anchor primer yielded a product of 354 bp which spans from the 5′ end and overlaps with the known sequence of the

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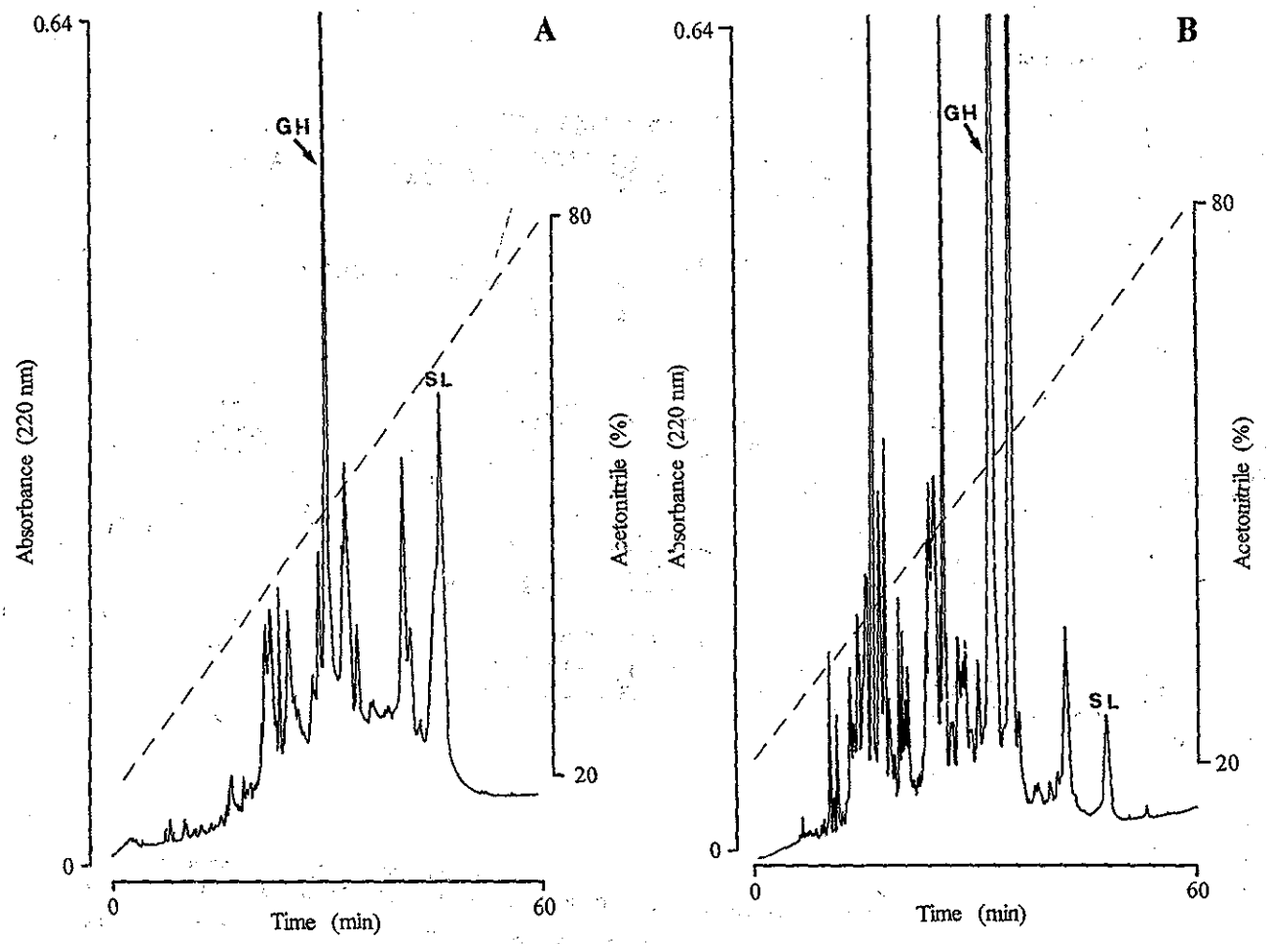

FIG. 1. HPLC chromatograms of fractions G-3 (A) and G-4 (B). The fractions (0.5 mg) were dissolved in 0.1% TFA. The elution position of GH and somatolactin (SL) is indicated. G-3 fraction was first subjected to affinity chromatography to remove the glycoproteins.

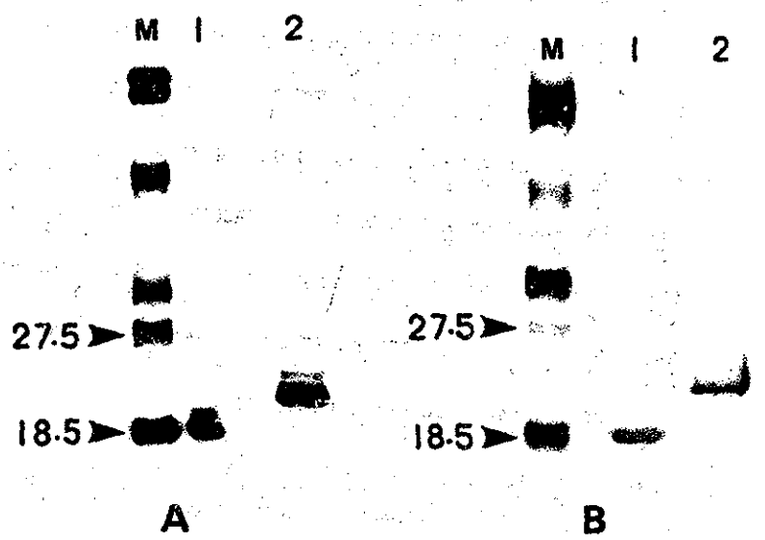

FIG. 2. SDS–PAGE pattern of rabbitfish GH after staining with Coomassie's brilliant blue (A) and after immunostaining with bonito GH antiserum (B). M, molecular weight marker (kDa); 1, nonreduced; 2, reduced with β-mercaptoethanol.

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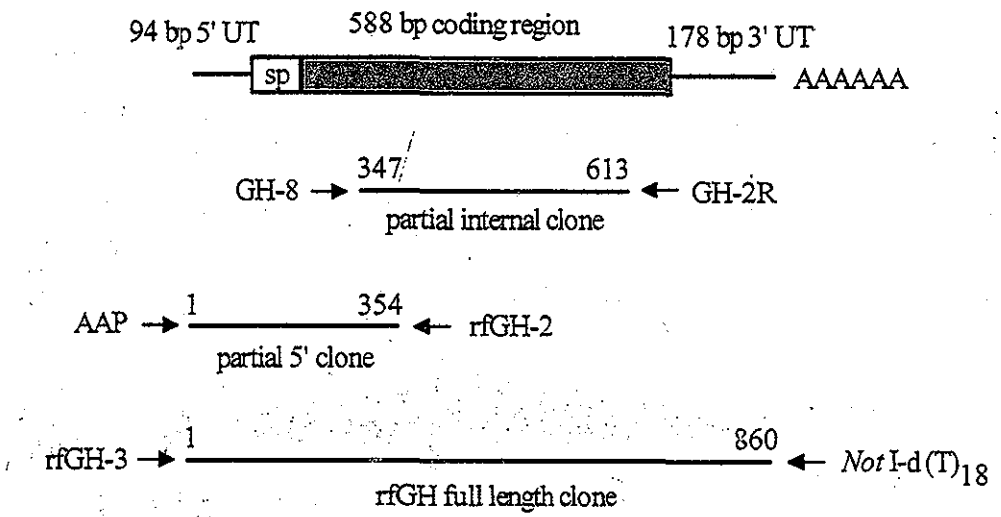

FIG. 3. Schematic diagram showing the position of the three PCR products (partial and full-length clones). The filled box represents the coding region for the mature peptide and the open box, the signal peptide (sp). The untranslated (UT) regions in the 5′ and 3′ ends are shown. The primers used in every PCR are also indicated. AAP is 5′ RACE abridged anchor primer.

```
  1                                   ATCACAGCTGAACTGCACAACAATCATA    28
 29   TCAGCAAGGACTAGGACCAGAACGAGGGCCAGGACCAAGAACAAGAACAAAAGCCACTGA    88

 89   AGAACCATGGAAAAAGTGGTCCTCCTGTTGTCGGTGCTGTCTCTGGGTGTGGTCTGTCCT   148
-18         M  E  K  V  V  L  L  L  S  V  L  S  L  G  V  V  C  P     -1

149   CAGCCAATGACGGACAGTCAGCGTTTCTCCATTGCCGTCAGCAGGATTCATTATCTCCAC   208
  1    Q  P  M  T  D  S  Q  R  F  S  I  A  V  S  R  I  H  Y  L  H     20

209   CAGGTTGCTCAGAGAAGCTTCTTCACCTTTGAGAGTTCTCTGTCAGCAGAGGACCAGCGT   268
 21    Q  V  A  Q  R  S  F  F  T  F  E  S  S  L  S  A  E  D  Q  R     40

269   CAACTCAACAAGATCTTCCTGCAAGATTCCTGCAACTCCGATTACATCCGGAGCCCAATA   328
 41    Q  L  N  K  I  F  L  Q  D  S  C  N  S  D  Y  I  R  S  P  I     60

329   GACAAGCACGAGACACAGCGCAGCTCTGTGATGAAGCTGCTGTCTATCTCCTATCGACTG   388
 61    D  K  H  E  T  Q  R  S  S  V  M  K  L  L  S  I  S  Y  R  L     80

389   GTTGAGTCTTGGGAGTATCCCAGCCGTGCTTTGATCGGTGGTTCAACGAACCAGATTTCC   448
 81    V  E  S  W  E  Y  P  S  R  A  L  I  G  G  S  T  N  Q  I  S    100

449   AACAAACTGTCTGAGCTAAAGCTGGGGATCAGGCTTCTGATGGAGGCCAATCAGGACGGA   508
101    N  K  L  S  E  L  K  L  G  I  R  L  L  M  E  A  N  Q  D  G    120

509   GCAGAGATCTTTCCGGAGAGCTCTGCCTTTCAGCTGGATTATCAGAGTCTAGGCACAGAT   568
121    A  E  I  F  P  E  S  S  A  F  Q  L  D  Y  Q  S  L  G  T  D    140

569   GATCCGAGACAGATGTACGAACTGCTGGCCTGTTTCAAGAAGGACATGCACAAGGTGGAG   628
141    D  P  R  Q  M  Y  E  L  L  A  C  F  K  K  D  M  H  K  V  E    160

629   ACTTATCTGACAGTGGCAAAGTGTCGCCTCTCTCCAGAAGCCAACTGCACCTTGTAGCCC   688
161    T  Y  L  T  V  A  K  C  R  L  S  P  E  A  N  C  T  L  *       178

689   CACCTCTGTGCTGGTAGACCACGCCCACTGTTGATGATGCAATGTGTTGCACCACCACTC   748
749   ACTGCACTAGCCAGCATTTTCACTTGTCTGTTTTAGCTTGTTGAGTGTCTGAGGGACGAA   808
809   GAGGTTGTTTTCTGAAGAGGAAGTGATTCTGTGAATAAAGAGTGTTGCATTC-poly (A)  860
```

FIG. 4. The nucleotide sequence of rabbitfish GH cDNA and its deduced amino acid sequence. The signal peptide (18 amino acids) is underlined. The amino acid residues are numbered beginning from the mature peptide. The termination codon is indicated by an asterisk. The four cysteine residues and the polyadenylation signal are in bold.

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```
                    10         20         30         40         50
rabbitfish   QPMTDSQR*F SIAVSRIHYL HQVAQRSFFT FESSLSAEDQ RQLNKIFLQD
tuna         --I-----L- ------VQH- -LL---L-SD -----QT-E- ----------
Asian seabass --I-----L- ------VQH- -LL---R-SE -----QT-E- -HV-------
yellow tail  --I----HL- -------QN- -LL---L-SN ---T-QT--- ----------
bonito       --I-E---L- ------VQN- -LL---L-SD -----QTQE- ----------
tilapia      -QI-----L- ----N-VTH- -LL---L-SD -----QT-E- ----------
flounder     --I-EN--L- ----G-VQ-- -L--KKL-SD --N--QL--- -L----ASKE
chum salmon I ***IEN--L- N-----VQH- -LL--KM-ND -DGT-LPDER --------L-
rainbow trout ***IEN--L- N-----VQH- -LL--KM-ND -DGT-LPDER --------L-
carp         ***SEN--L- NN--I-VQH- -QL-AKMIND --D--LP-ER ---S---PLS

                    60         70         80         90        100
rabbitfish   SCNSDYIRSP IDKHETQRSS VMKLLSISYR LVESWEYPSR ALIG*GS***
tuna         F------I-- ---------- -L-------- ------F--- S-S-*--***
Asian seabass F------I-- ---------- -L-------- ------FS-- S-S-*--***
yellow tail  F------I-- ---------- -L-------- ------FS-- F-S-*--***
bonito       F------I-- ---------- -L-------- ------F--- S-S-******
tilapia      F------I-- ---------- -L-------G ------F--- S-S-*--***
flounder     F-H--NFL-- -------G-- -Q----V--- -I----FF-- F-VA*SF***
chum salmon I F----S-V-- V------K-- -L---H--F- -I-------Q T--**I-NSL
rainbow trout F----S-V-- V------K-- -L---H--F- -I-------Q T--**I-NSL
carp         F------EA- AG-D---K-- ML---R--FH -I----F--Q S-S-TV-NSL

                   110        120        130        140        150
rabbitfish   ****TNQISN KLSELKLGIR LLMEANQDGA EIFPESSAFQ LD*****YQS
tuna         **APR----P ------T--H --IR-----D -M-AD---L- -APYGNY---
Asian seabass **APRD---P ------T--L --IR------ -M-SD---L- -APYGNY---
yellow tail  --ALR----P R-----T--Q --IT------- -M-SDV--L- -APYGNF---
bonito       **AQR----E ---D--M--Q --IR------- -M-AD---L- -APYGNY---
tilapia      **SLR----P R-----T--L --IR----E- -NY-DTDTL- HAPYGNY---
flounder     **AVRT-VTS ------M-LL K-I------- GG-S---VL- -TPYGN****
chum salmon I MVRNA----E ---D--V--N --ITGS---V LSLDDNDSQ- -PPYGNY--N
rainbow trout MVRNA----E ---D--V--N --ITGS---V LSLDDNDSQ- -PPYGNY--N
carp         TVGNP--LTE --AD--M--S V-IQ-CL--Q PNMDDNDSLP -*PFEDF-LT

                   160        170        180        190        200
rabbitfish   LGTDDP*RQM YELLACFKKD MHKVETYLTV AKCRLSPEAN CTL
tuna         --A-ESL-RS ---------- ---------- ---------- ---
Asian seabass --A-ESL-RT ---------- ---------- ---------- ---
yellow tail  --GEELL-RN ---------- ---------- ---------- ---
bonito       --G-ESL-RN ---------- --------M- ---------- ---
tilapia      --GNESL--T ---------- ---------- ---------- ---
flounder     ********** S--F------ ---------- -----F---- ---
chum salmon I --G-GNV-RN ---------- ---------- ----K-L--- ---
rainbow trout --G-GNV-R- ---------- ---------- ----K-L--- ---
carp         M-*ENNL-ES FR-------- --------R- -N--R-LDS- ---
```

FIG. 5. Alignment of the amino acid sequence of rabbitfish GH with other teleost GHs. Gaps (*) are introduced to maximize sequence alignment. Dashes (-) indicate amino acid residues identical to those of rabbitfish GH. The location of the five amino acid deletion unique to rabbitfish GH is boxed.

internal region (nucleotides 1–354) (Fig. 3). To obtain the full sequence, a third PCR was carried out using newly designed primers (RF-GH-3 and RF-GH-4) and *Not*I-d(T)$_{18}$ bifunctional primer. Excluding the poly (A) tail, the rabbitfish GH cDNA consisted of 860 bp. This was composed of 94 bp in the 5′ untranslated region, 54 bp for the signal sequence, 534 bp coding for the hormone, and 175 bp in the 3′ untranslated region including the hexanucleotide AATAAA, a typical polyadenylation site located 13 nucleotides upstream of the poly (A) tail (Fig. 4). Thus, an open reading frame of rabbitfish GH cDNA encodes a prehormone of 196 amino acid residues with a signal peptide of 18 amino acids and a mature protein of 178 amino acid residues.

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TABLE 1
Nucleotide and Amino Acid Sequence Identity of Rabbitfish GH Compared with Those of Other Fish Species

Fish species	Sequence identity (%)	
	Nucleotides	Amino acids
Tuna (*Thunnus thynnus*)	73.40	74.09
Asian seabass (*Lates calcarifer*)	73.40	72.53
Yellow tail (*Seriola quinqueradiata*)	72.88	72.53
Bonito (*Katsuwonus pelamis*)	72.53	71.50
Tilapia (*Oreochromis niloticus*)	70.63	69.94
Flounder (*Paralichtys olivaceus*)	60.27	57.51
Rainbow trout (*Salmo gairdneri*)	59.06	58.03
Chum salmon (*Oncorhynchus keta*)	58.37	58.03
Carp (*Cyprinus carpio*)	54.57	47.15

Note. Tuna (Sato *et al.*, 1988); Asian seabass (Knibb *et al.*, 1991); yellow tail (Watahiki *et al.*, 1988); bonito (Noso *et al.*, 1988); tilapia (Rentier-Delrue *et al.*, 1989); flounder (Watahiki *et al.*, 1989); rainbow trout (Agellon and Chen, 1986); chum salmon (Sekine *et al.*, 1985); carp (Koren *et al.*, 1989).

Sequence Comparison

The deduced amino acid sequence of rabbitfish GH was compared with those of other fish species (Fig. 5). The sequence identity is listed in Table 1. Rabbitfish GH has four cysteine residues located at highly conserved positions; aa 51, 151, 168, and 176 (Fig. 4). Rabbitfish GH is highly identical (>70%) with GHs of more advanced teleosts, such as sea bass, tuna, yellow tail, and bonito, and less identical with those of salmonids and carp GHs.

Biological Activity

Rabbitfish fry injected with the hormone at a dose of 0.1 μg/g body wt showed significantly higher increase in body weight and fork length compared to the control starting on the third week of treatment (Fig. 6). The fry injected with 0.01 μg/g body wt of the hormone showed a significantly higher increase after the fifth week. Although the treatment was stopped after the fifth week, the significant increase in body weight and fork length in the hormone-treated groups was maintained until the seventh week or for 2 weeks after the last injection.

DISCUSSION

We have isolated and purified GH from rabbitfish pituitary glands by extraction under alkaline condition followed by gel filtration chromatography on Sephadex G-100 and rp-HPLC. In each step in the purification process, rabbitfish GH was monitored by SDS–PAGE and immunoblotting with bonito GH antiserum. The immunoreaction with bonito GH antiserum was specific; no immunoreaction was detected with anti-salmon PRL and anti-flounder SL, and the intensity of the immunoreaction was strong, indicating a high concentration of GH in the extract. This can be expected since GH cells are major constituents of the pituitary gland. The purified protein was also confirmed to be GH based on its molecular weight. Under nonreducing conditions, rabbitfish GH has a molecular weight of about 19 kDa, as estimated from SDS–PAGE. This agrees with the molecular weight of 20.4 kDa, calculated from the cDNA clone. The yield of GH was 1.9 mg/g wet wt of pituitary gland. In contrast, the yield of PRL was only half that of GH (unpublished results).

We tried to determine the amino acid sequence of the N-terminal region of the intact protein but found it blocked, indicating that the first amino acid from the N-terminus is pyroglutamine. This was confirmed with the deduced amino acid sequence of the GH cDNA.

We have also cloned the rabbitfish GH cDNA. The prehormone is composed of 196 amino acids with a signal peptide of 18 amino acids. The mature hormone has 178 amino acids, slightly larger (by 5 amino acids) than flounder GH, which is the smallest teleost GH (173 amino acids) known so far (Watahiki *et al.*, 1989). In rabbitfish GH, there is a deletion of 5 amino acids (Pro-Tyr-Gly-Asp-Tyr) in position 143–147 immediately preceeding the 14 amino acid deletion in flounder GH. This deletion is unique to rabbitfish GH since no such deletion was observed in any known fish GHs (Fig. 5). This stretch of amino acid residues is outside the five conserved domains of GHs as described by Watahiki *et al.* (1989). The biological significance of this deletion is presently unknown. Single or double amino acid deletions are also found in rabbitfish GH; amino acids 9, 103–104, and 157 (Fig. 5). As in the other teleost GHs (Rand-Weaver *et al.*, 1993), rabbitfish GH has four cysteine residues located in highly conserved positions.

Rabbitfish GH is closer in identity to GHs of other perciforms like tuna, sea bass, yellow tail, bonito, and tilapia than to those of salmonid and carp GHs. As is evident in Fig. 5, except for the highly conserved amino acids in 170–180, the sequences of salmonids

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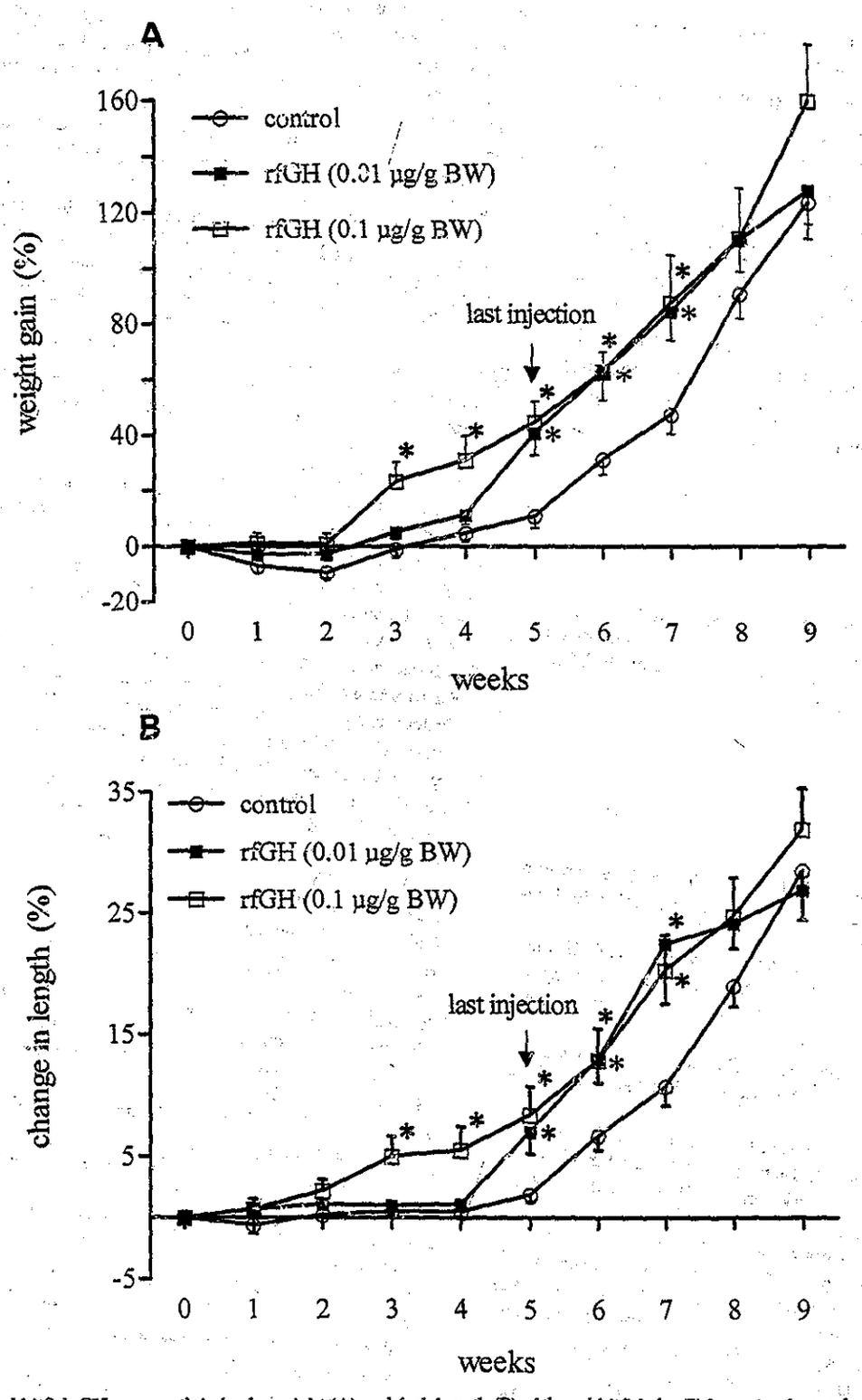

FIG. 6. Effects of rabbitfish GH on growth in body weight (A) and fork length (B) of the rabbitfish fry. Fish received a total of six intraperitoneal injections every 7 days. Vertical lines represent SEM. *Significant differences ($P < 0.05$) compared to the control.

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and carp GHs are clearly different from that of perciforms where rabbitfish is classified.

The purified rabbitfish GH was effective in promoting growth in rabbitfish fry. Although the hormone was given only every 7 days, its growth promoting effect was already evident after the third week in the fry treated with 0.1 μg/g body wt and starting on the fifth week in those treated with 0.01 μg/g body wt. Interestingly, the significant effect of the hormone was still observed 2 weeks after the final injection. Considering that the hormone was given only once a week, administering the hormone daily using appropriate routes such as feeding would probably translate into much larger growth increments.

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Differential Expression of IGF-I and II mRNAs during Embryogenesis and Early Larval Development in Rabbitfish, *Siganus guttatus*

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ABSTRACT

In rodents, the expression of IGF-II is higher than IGF-I during fetal life while the reverse is true after birth. We wanted to examine if this is also true in fish and whether IGF-I and IGF-II are differentially regulated during different stages of embryogenesis and early larval development in rabbitfish. We first cloned the cDNAs of rabbitfish IGF-I and IGF-II from the liver. Rabbitfish IGF-I has an open reading frame of 558 base pairs (bp) that codes for a signal peptide of 44 amino acids (aa), a mature protein of 68 aa, and a single form of E domain of 74 aa. Rabbitfish IGF-II, on the other hand, has an open reading frame of 645 bp that codes for a signal peptide of 47 aa, a mature protein of 70 aa, and an E domain of 98 aa. On the amino acid level, rabbitfish IGF-I shares 68% similarity with IGF-II. We then examined the relative expression of the two IGFs in unfertilized eggs, during different stages of embryogenesis, and early larval stages of rabbitfish by a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Primers that amplify the mature peptide region of both IGFs were used and PCR for both peptides was done simultaneously allowing identical PCR conditions for both. The identity of the PCR products was confirmed by direct sequencing. Contrary to published reports in seabream and rainbow trout, IGF-I mRNA was not detected in rabbitfish unfertilized eggs; it

was first expressed in larvae soon after hatching. IGF-II mRNA however, was expressed in unfertilized eggs, albeit weak, and was already strongly expressed during the cleavage stage. mRNAs for both peptides were strongly expressed in the larvae although IGF-II mRNA expression was higher than IGF-I.

Key words: rabbitfish, IGF-I, IGF-II, cDNA cloning, mRNA expression

INTRODUCTION

Insulin-like growth factors I and II (IGF-I and II) are two highly homologous mitogenic peptides that are expressed ubiquitously and show diverse effects on development, growth and metabolism (reviewed by Rosenfeld and Roberts, 1999; Werner and LeRoith, 2000). The central role of IGF-I is that it regulates post-natal growth and mediates the growth promoting action of growth hormone (GH), although studies also show that it is important for normal embryonic growth (Baker *et al.*, 1993; Powell-Braxton, 1993). The physiological function of IGF-II, on the other hand, is not yet definitely known although it is considered as a fetal growth factor based on the evidence in mice where a significant growth retardation was observed in pups born with the IGF-II gene disrupted or knocked-out (De Chiara *et*

al., 1990). The biological actions of IGFs are mediated by IGF type I (IGF-I-R) and II (IGF-II/mannose 6-phosphate-R) cell surface receptors. Interaction with IGF-I-R is mainly responsible for the mitogenic effects of IGF (LeRoith *et al.*, 1995) and there is enough consensus at present that much of the biological action of IGFs on growth and differentiation is mediated by this receptor. The role of type II IGF receptor in IGF signal transduction remains controversial (Kiess, 1999; Nissley, 1999). In addition, there is the insulin receptor (IR)/IGF-I-R hybrid which seems to be widely expressed and even appear to be the most abundant form of receptor in certain tissues (Baiyles *et al.*, 1997) but the specific differences in signaling characteristics between the IGF-I-R and the IR/IGF-I-R hybrids have not yet been established. Furthermore, at least six insulin-like growth factor binding proteins (IGFBPs) are already found in circulation and they modulate the biological activity of IGFs by controlling the availability of free IGFs and by prolonging their half-life (Hwa *et al.*, 1999). These IGFBPs show contrasting effects on IGF function. IGFBP-1 (Van Buul-Offers *et al.*, 2000), IGFBP-2 (Duan *et al.*, 1999; Hoeflich *et al.*, 199; Wolf *et al.*, 2000) and IGFBP-3 (Hochscheid *et al.*, 2000) have inhibitory effects on IGF actions whereas there is evidence that IGFBP-5 functions as a growth factor (Miyakoshi *et al.*, 2001).

To accelerate development and improve growth of rabbitfish (*Siganus guttatus*), an important aquaculture species in the Southeast Asian region, we have been studying the hormones in the pituitary-liver axis of this fish. We have isolated and cloned rabbitfish GH and found it potent in accelerating growth in the fry (Ayson *et al.*, 2000). In this study, we cloned the cDNAs for rabbitfish IGF-I and II from the liver and examined the expression of the mRNA of both IGFs during different stages of embryogenesis and during early larval development. We report here that IGF-I mRNA was not detected in unfertilized eggs but was first expressed in the larvae soon after hatching. IGF-II mRNA, however, was detected in unfertilized eggs, although very weak, and was already strongly expressed starting from the cleavage stage. Similar to those observed in other vertebrates, IGF-II is expressed more than IGF-I in rabbitfish embryos.

MATERIALS AND METHODS

Cloning of rabbitfish IGF-I and IGF-II cDNAs

The procedure of Ayson *et al.* (2000) in cloning rabbitfish GH was followed with some modifications. Briefly, total RNA was extracted from the liver with Isogen (Nippon Gene, Tokyo) and first strand cDNA was reverse-transcribed from 5 μg of total RNA using First strand cDNA synthesis kit (Amersham Pharmacia Biotech, Sweden).

Degenerate sense and antisense primers (Tables 1 & 2) designed based on the conserved sequences of the B and A domains of known IGFs were synthesized and used to clone the internal regions of rabbitfish IGF cDNAs. A 50-µl PCR reaction mixture using GeneAmp's AmpliTaq Gold kit (Perkin-Elmer, CA) was subjected to 15 min hot start at 95°C and 40 cycles of amplification; each cycle consisted of 1 min 30 s denaturation (94°C), 1 min primer annealing (45°C), 1 min 30 s primer extension (72°C) and a final extension of 7 min at 72°C.

The 5'RACE method of Frohman (1990) using 5'RACE system kit, ver 2.0 (Gibco BRL, Gaithersburg, MD) was used to clone the 5' end of the cDNAs. Antisense primers (Tables 1 & 2) designed based on the known nucleotide sequence of the internal region of rabbitfish IGF-I and II were synthesized. These gene-specific antisense primers were used together with the abridged anchor primer provided in the 5'RACE kit in reverse transcription and PCR. PCR conditions were the same as described except for the annealing temperature (50°C).

Sense primers (Tables 1 & 2) were designed based on the known nucleotide sequences of the 5' end of rabbitfish IGF-I and II, and these were used with *Not*-I adapter primer as the antisense primer in cloning the 3' end of IGF cDNAs. Takara's LA Taq kit(Takara, Japan) was used during PCR. PCR conditions were the same as described except that the 15 min hot start

(95°C) was omitted and the annealing temperature was raised to 55°C.

PCR products were electrophoresed in agarose gels and visualized by ethidium bromide staining. The DNA was extracted and purified from agarose gels using QIAEX II gel extraction kit (Qiagen, Germany), inserted and ligated into pT7 Blue T-vector (Novagen, WI - for partial clones) or TOPO XL cloning vector (Invitrogen, CA - for full length clones) and transformed in JM 109 competent cells (Takara - for partial clones) or TOP 10 chemically competent cells (Invitrogen - for full length clones). The plasmid DNA was then extracted from the bacterial cells using QIAprep Spin Plasmid kit (Qiagen). Nucleotide sequencing was performed using an ABI Prism 377 DNA Sequencer (Perkin-Elmer, CA).

Expression of IGF-I and II mRNAs during embryogenesis and early larval development in rabbitfish

Unfertilized eggs, embryos at different stages of development (cleavage (32-cell stage), gastrula (50% epiboly), neurula, during tail bud elongation, and embryos with heart beat), and larvae from hatching until 6 days old were collected. Unfertilized eggs were removed from the ovary by cannulation one day before the expected date of spawning. Because of the very small size of the eggs and larvae of rabbitfish, samples from

each developmental stage were pooled and immediately put in dry ice and later stored at -80°C until analysis. Total RNA from the pooled samples (100-300 mg) was extracted and first strand cDNA was reverse-transcribed from a fixed amount of total RNA (5 μg). During PCR, one μl each of first strand cDNA was used as the template in all samples. Primers (Tables 1 & 2) that amplify the mature peptide region of rabbitfish IGF-I and II were synthesized and used. PCR conditions were as described except for the annealing temperature (58°C). A PCR using first strand cDNA from the liver as the template, and one without a template, were also included as positive and negative control, respectively. The identity of the PCR products was confirmed by direct sequencing.

Rabbitfish β-actin was used as the internal standard during quantification of IGF-I and II mRNA expression. Using degenerate primers designed based on the sequences of known teleosts β-actin, rabbitfish β-actin was first cloned and from its partial sequence, sense (5′-GCC-AAC-AGG-GAA-AAG-ATG-AC- 3′) and antisense (5′ -TTG-CCA-ATG-GTG-ATG-ACC-TG- 3′) primers were designed. PCR conditions were optimized by first checking the linearity of the PCR reaction and based on the results of the linearity check, a PCR of 40 cycles was used for all samples. PCR products were loaded into the gel and stained with ethidium

bromide. Quantification of PCR products was done using GeneGenius gel documentation and analysis system (Syngene, U.K.). Values for IGF-I and II were normalized with that of β-actin.

RESULTS

Structure of rabbitfish IGF-I and II cDNAs

The nucleotide sequence of rabbitfish IGF-I cDNA was obtained in three steps. The sequence of the B and A domains was determined using degenerate primers based on the highly conserved region of the peptide. The unknown sequences in the 5′ and 3′ regions were determined by 5′ and 3′ RACE, respectively. By connecting the sequences of the overlapping clones, the nucleotide sequence from the start of the 5′ end until some portion of the 3′ untranslated region of rabbitfish IGF-I cDNA was obtained. From this sequence, rabbitfish preproIGF-I was found to consist of 186 amino acids (aa) (558 bp) (Fig. 1). This is composed of 44 aa for the signal peptide, 68 aa for the mature peptide comprising B,C,A, and D domains, and 74 aa for the E domain, when compared with the IGFs from 10 fish species (Fig. 3).

Similarly, we found out that rabbitfish preproIGF-II is 215 aa long (Fig. 2). The 645 bp open reading frame is composed of 47 aa for the signal peptide, 70 aa for the mature peptide

(B,C,A,D domains), and 98 aa for the E domain, when compared with the IGF-IIs from 6 fish species (Fig. 4).

Expression of IGF-I and II mRNAs during embryogenesis and early larval development in rabbitfish

mRNA for IGF-I was first detected in larvae soon after hatching but not in unfertilized eggs and during embryogenesis (Fig 5A). In contrast, IGF-II mRNA was already detected in unfertilized eggs, albeit low, and the expression was already strong starting at the cleavage stage and remained so throughout embryonic development (Fig. 5A,B). In larvae until 6 days old, both IGFs were strongly expressed although IGF-II is expressed more than IGF-I (Fig. 6A,B).

DISCUSSION

Rabbitfish proIGF-I and II cDNAs contained the B,C,A,D and E domains, typical of IGFs. The E domain is proteolytically removed as a post-translational modification to yield the mature peptides. The mature peptide of rabbitfish IGF-I and II have the conserved six Cys residues; 2 in the B domain and 4 in the A domain.

As shown in Fig. 3, the B and A domains of IGF-I have the highest sequence homology in several fishes. Except for a single amino acid substitution in tilapia, the A domain of the

10 fish species in Fig. 3 has 100% sequence homology. The B domain of rabbitfish IGF-I contains the last amino acid threonine that is lacking in other advanced fish species (Order Perciformes). The C domain of rabbitfish IGF-I lacks histidine and asparagine, similar to the C domains of the perciforms. Amino acid # 9 in the C domain of rabbitfish IGF-I is proline; this is serine in all other fish species.

The mature region of rabbitfish IGF-II (70 aa) is longer by 2 amino acids than rabbitfish IGF-I (68 aa). After aligning the 6 conserved Cys residues of both IGFs, the sequence homology of the mature peptide region of rabbitfish IGF-I to IGF-II is 68% on the amino acid level. Similar to IGF-I, the B and A domains of IGF-II in most species are also highly conserved (Fig. 4) but in addition, the D domain of IGF-II is also highly conserved. The amino acid sequence of both A and D domains of IGF-II in fish species known to date has 100% homology.

The E domain of IGF-I is, however, highly variable and more than one form can be found in some species; 2 in humans (Rotwein, 1986), 4 in salmonids (Cao *et al.*, 1989; Wallis and Devlin, 1993), 2 in barramundi and flounder corresponding to salmon Ea2 and Ea4 (Kinhult Stahlbom, 1999; Tanaka *et al.*, 1998). Only one form of E domain is found in rabbitfish and this has 74 aa residues. This E-domain is cleaved from the rest of the peptide. Prior to its cleavage, it is hypothesized that

this domain may be important for intracellular transport, processing or correct folding (Duguay *et al.*, 1996). They also possess some biological activity. Siegfried *et al.* (1992) have shown that a synthetic peptide of the E domain of human IGF-Ib is mitogenic when amidated, and Tian *et al.* (1999) reported that recombinant peptides of rainbow trout's 3 forms of E domain possess mitogenic activity even in heterologous systems.

There are no reports of multiple forms of E-domains for IGF-II. The E domain of the 6 species listed in Fig. 4 constitutes only a single form. Likewise, there are no reports of the E domain of IGF-II having any biological activity after it is cleaved from the mature peptide.

The role of IGF-I in regulating post-natal growth and in mediating the growth promoting effect of GH has been confirmed in many ways not only in mammals but in fish as well. Because of the dependence of GH on hepatic IGF-I in realizing this effect, although a recent study show that IGF-I from non-hepatic tissues is sufficient for normal growth and development in mouse (Yakar *et al.*, 1999), it was thought that IGF-I is not important during embryogenesis until it was shown that mice lacking IGF-I had impaired fetal growth and development (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993). It appears now that IGF-I is also important for normal embryonic development although its concentration is generally lower in the embryos than in the

adults. In the present study, we examined the presence of IGF-I transcript in unfertilized eggs, during different stages of embryogenesis, and in the larvae of rabbitfish. Unlike in gilthead seabream (Funkenstein *et al.*, 1996) and rainbow trout (Greene and Chen, 1997) where IGF-I was detected in unfertilized eggs, IGF-I mRNA was not detected in unfertilized eggs of rabbitfish and in the different stages of embryogenesis. Probably, the level of IGF-I mRNA in rabbitfish unfertilized eggs and embryos was very low that it was not detected after 40 cycles of amplification. IGF-I mRNA was first detected in rabbitfish larvae soon after hatching. In their studies, Funkenstein *et al.*(1996) and Greene and Chen (1997) used a higher concentration of total RNA during the reverse transcription step - 20 μg which is 4-fold higher than that used in the present study.

On the other hand, IGF-II has been postulated as a fetal growth factor. Support for this hypothesis came when targeted disruption of the IGF-II gene resulted in fetal growth retardation - mice lacking the IGF-II gene were 40% smaller at birth than their normal counterparts (De Chiara *et al.*, 1990). The reduction in the growth rate in mice lacking the IGF-II gene is confined only *in utero* clearly demonstrating the dependence of embryonic development on IGF-II. In addition, post-natal overexpression of IGF-II does not result in increased somatic

growth whereas mice overexpressing IGF-I are larger than the normal type and showed signs of overgrowth after 3-4 weeks of post-natal life (Matthews *et al.*, 1988). These findings and the generally higher levels of IGF-II in the embryos compared to adults in many species argue for its importance in early life.

The observation that IGF-II expression is higher than IGF-I during early development seems to be also true in fish as shown in our present results. IGF-II mRNA was strongly expressed in rabbitfish embryos during the cleavage stage whereas IGF-I mRNA was first detected only in the larvae soon after hatching. Furthermore, IGF-II mRNA was also detected in rabbitfish unfertilized eggs, albeit weak, whereas IGF-I was not. Similar observations were obtained by Greene and Chen (1999) in rainbow trout embryos. Nevertheless, the absence of PCR products for IGF-I in unfertilized eggs and during embryogenesis in rabbitfish does not imply poor quality of RNA from these samples or low efficiency in reverse transcription because a PCR product for rabbitfish β-actin was amplified in all samples and there was no apparent difference in their expression in all stages. In the larvae, although IGF-II mRNA levels were also higher than IGF-I during the first 6 days after hatching, the reverse is true in the adult as shown in the higher level of IGF-I mRNA in the liver. Overall, the findings of the present study seem to agree with the concept that IGF-II is probably more important

than IGF-I during embryogenesis and IGF-I exerting more influence on growth and development in later life stages.

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Figure Legends

Figure 1. Nucleotide and deduced amino acid sequences of rabbitfish preproIGF-I. The signal peptide (44 aa) is underlined, the mature region (68 aa) consisting of B,C, A,D domains is in bold letters, and the six Cys residues of the mature peptide are double underlined. The E domain is composed of 74 aa. The amino acids are numbered starting from the mature peptide.

Figure 2. Nucleotide and deduced amino acid sequences of rabbitfish preproIGF-II. The signal peptide (47 aa) is underlined, the mature region (70 aa) is in bold letters (B,C,A,D domains), and the 6 Cys residues of the mature peptide are double underlined. The E domain is composed of 98 aa. The amino acids are numbered starting from the mature peptide.

Figure 3. Alignment of the amino acid sequence of rabbitfish IGF-I with the IGF-Is of 10 fish species. Gaps (*) are introduced to maximize the sequence alignment. Dashes (-) indicate amino acid residues identical to those of rabbitfish IGF-I.

Figure 4. Alignment of the amino acid sequence of rabbitfish IGF-II with the IGF-IIs of 6 fish species. Gaps (*) are introduced to maximize the sequence alignment. Dashes (-)

indicate amino acid residues identical to those of rabbitfish IGF-II.

Figure 5. IGF-I and II mRNA expression during rabbitfish embryogenesis determined by semiquantitative RT-PCR. (**A**) Agarose gel electroporesis of RT-PCR products for IGF-II and β-actin. No PCR product for IGF-I. m-marker, uf-unfertilized eggs (very faint band for IGF-II), c-cleavage stage (32-cell stage), g-gastrula, n-neurula, tbe-tail bud elogation, hb-embryo with heart beat, nh-newly hatched larvae. (**B**) IGF-II mRNA levels expressed as the ratio of IGF-II and β-actin. Labels are similar as that of Figure 5A.

Figure 6. IGF-I and II mRNA expression during early larval development in rabbitfish determined by semiquantitative RT-PCR. (**A**) Agarose gel electroporesis of RT-PCR products for IGF-I, IGF-II and β-actin. nc-negative control, liver-positive control. (**B**) IGF-I and II mRNA levels expressed as the ratio with β-actin.

Table 1. Primers used in cloning rabbitfish IGF-I cDNA

I.	**Degenerate primers for cloning the internal region**	
	IGF-1F	5′ -GG(CTG)AT(CT)GTGGA(TC)GAGTGCTGC- 3′
	IGF-2F	5′ -GAGAC(CGT)CT(CG)TG(CT)GGGGC(GTA)- 3′
	IGF-1R	5′ -GC(AG)CA(GA)TACAT(CT)TC(CGT)AG- 3′
II.	**Primers for cloning the 5′ end**[1]	
	rfIGF1-1R	5′ -CAAACTGCAGCGTGTCGACC- 3′
	rfIGF1-2R	5′ -AAAGCCTCTCTCTCCACACAC- 3′
III.	**Primers for cloning the 3′ end**[2]	
	rfIGF1-1F	5′ -GATGTCTAGCGCTCTTTCCTTTC- 3′
	rfIGF1-2F	5′ -GTGATGTCTTCAAGAGTGCG- 3′
	rfIGF1-3F	5′ -ACGAGTGCTGCTTCCAAAGCTG- 3′
IV.	**Primers used for RT-PCR** (PCR product size = 454 bp)	
	rfIGF1fl-F	5′ -TCTCACTACTGCTGTGCATC- 3′
	rfIGF1fl-R	5′ -GTGTTGCCTCGACTTGAGTT- 3′

[1]The abridged anchor primer provided in the 5′RACE kit was used as the forward primer

[2]*Not* I-d$(T)_{18}$ bifunctional primer was used as the reverse primer

Table 2. Primers used in cloning rabbitfish IGF-II cDNA

I. Degenerate primers for cloning the internal region		
IGF2-1F	5′	-GA(AG)ACGCT(AG)TGTGG(AG)GGA- 3′
IGF2-1R	5′	-GGCACAGTACTGTCCA(AG)- 3′
IGF2-2R	5′	-ACAACACTCCTC(TC)ACGAT(AC)CC- 3′
II. Primers for cloning the 5′ end[1]		
rfIGF2-1R	5′	-TTGAGGTCACAGCTACGGAA- 3′
rfIGF2-2R	5′	-CTGCTGGTTGGCCTACTGAA- 3′
III. Primers for cloning the 3′ end[2]		
rfIGF2-1F	5′	-TGACTACTGCCATCTGACATG- 3′
rfIGF2-2F	5′	-ACTCACTTTGCCACACCTGCCG- 3′
rfIGF2-3F	5′	-CAGAACCGTGGGATTGTAGAAG- 3′
IV. Primers used for RT-PCR (PCR product size = 452 bp)		
rfIGF2fl-F	5′	-TGACGCTCTACGTTGTGGAA- 3′
rfIGF2fl-R	5′	-CTGTGGAAGATTGCCTGCTC- 3′

[1]The abridged anchor primer provided in the 5′RACE kit was used as the forward primer

[2]*Not* I-d$(T)_{18}$ bifunctional primer was used as the reverse primer

```
  1                                                   TTTGCTCTCTAAATC   15
 16  CGTCTCCTGTTCGCTAAATCTCACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTCG   75
 76  GAATATTGAGATGTGACATTGCCCGCATCTCATCCTCTTTCTCCCCGTTTTTAATGACTT  135

136  CAAACAAGTTCATTTTCGCCGGGCTTTGTCTTGCGGAGACCCGTGGGGATGTCTAGCGCT  195
-44                                                   M  S  S  A   -41

196  CTTTCCTTTCAGTGGCATTTATGTGATGTCTTCAAGAGTGCGATGTGCTGTATCTCCTGT  255
-40   L  S  F  Q  W  H  L  C  D  V  F  K  S  A  M  C  C  I  S  C   -21

256  AGCCACACCCTCTCACTACTGCTGTGCATCCTCACCCTGACTCCGACAGCAACAGGGGCG  315
-20   S  H  T  L  S  L  L  L  C  I  L  T  L  T  P  T  A  T  G  A   -1

316  GGTCCAGAGACCCTGTGCGGGGCGGAGCTGGTCGACACGCTGCAGTTTGTGTGTGGAGAG  375
 1    G  P  E  T  L  C  G  A  E  L  V  D  T  L  Q  F  V  C  G  E   20
     [---------------------------- B domain ---------------------

376  AGAGGCTTTTATTTCAGTAAACCAACGGGCTATGGGCCGAACTCGCGGCGACCACGAGGC  435
21    R  G  F  Y  F  S  K  P  T  G  Y  G  P  N  S  R  R  P  R  G   40
     -------------------------][---------- C domain --------][--

436  ATCGTGGACGAGTGCTGCTTCCAAAGCTGCGAGCTGCGGCGTCTGGAGATGTACTGTGCA  495
41    I  V  D  E  C  C  F  Q  S  C  E  L  R  R  L  E  M  Y  C  A   60
     ----------------------- A domain -------------------------]

496  CCCGCCAAGACAAGCAAGGCCGCTCGCTCCGTGCGTGCACAGCGCCACACGGACATGCCC  555
61    P  A  K  T  S  K  A  A  R  S  V  R  A  Q  R  H  T  D  M  P   80
     [------ D domain ------][-----------------------------------

556  AGAACTCCCAAGGTTAGTGCCGCAGGCCAGAAGGTGGACAAGGGCACAGAGCGCAGGACG  615
81    R  T  P  K  V  S  A  A  G  Q  K  V  D  K  G  T  E  R  R  T  100
     ------------------------ E domain --------------------------

616  GCACAGCAGCCAGACAAGACAAAAAGCAAGAAGAGACCTTTATCTGGACATAGTCATTCA  675
101   A  Q  Q  P  D  K  T  K  S  K  K  R  P  L  S  G  H  S  H  S  120
     ------------------------------------------------------------

676  TCCTTCAAGGAAGTGCATCAGAAAAACTCAAGTCGAGGCAACACGGGGGGCAGAAATTAC  735
121   S  F  K  E  V  H  Q  K  N  S  S  R  G  N  T  G  G  R  N  Y  140
     ------------------------------------------------------------

736  AGAATGTAGGGGAGGAGTGAATGGACAAATGCCCAGCGACTTGGGAAGAGAGAAGGGAGT  795
141   R  M ***
     -----]

796  GGCCTTACCTGGTACCCCTGTGGAATGGTTCACTGTAAAACAAAACTAAGAGGAGGCTAA  855
856  AAGAGCGGCAGTCTGAAAACAAAGCTCTTCAAAATGATCCAAACCTAAAGAGCTAAGTGG  915
916  TGTTTTTTTTTAAGGGGTTATGATGAA - rest of 3′ untranslated region
```

Fig 1 IGFI

```
  1                                                GCCTGGTCTTTGGGACAGCCTCTC   24
 25  ACACATCATCTCACCGCACCAACTGGGAACCTAACTCACCTGCAATCTCTCCAACCAAAT   84

 85  AACCCCCACCCCCAGACGTTTTGACTACTGCCATCTGACATGGAGACCCAGCAAAGACAC  144
-47                                              M  E  T  Q  Q  R  H   -41

145  GGACACCACTCACTTTGCCACACCTGCCGGAGAGCGGAGAGCAGCAGAATGAAGGTCAGG  204
-40   G  H  H  S  L  C  H  T  C  R  R  A  E  S  S  R  M  K  V  K   -21

205  AGGATGTCTGCGTCCAGTCGTGCGCTGCTGTTTGCACTGGCCCTGACGCTCTACGTTGTG  264
-20   R  M  S  A  S  S  R  A  L  L  F  A  L  A  L  T  L  Y  V  V   -1

265  GAAATAGCCTCGGCAGAGACGCTGTGTGGGGGAGAGCTGGTGGACGCGCTGCAATTTGTC  324
  1   E  I  A  S  A  E  T  L  C  G  G  E  L  V  D  A  L  Q  F  V   20
     [-------------------------------- B domain ----------------

325  TGTGACGATAGAGGCTTCTATTTCAGTAGGCCAACCAGCAGGGGAAACAGCCGACGCCCC  384
 21   C  D  D  R  G  F  Y  F  S  R  P  T  S  R  G  N  S  R  R  P   40
     ----------------------------------][----------- C domain --

385  CAGAACCGTGGGATTGTAGAAGAGTGTTGTTTCCGTAGCTGTGACCTCAACCTGCTGGAG  444
 41   Q  N  R  G  I  V  E  E  C  C  F  R  S  C  D  L  N  L  L  E   60
     --------][-------------------------- A domain --------------

445  CAGTACTGTGCCAAACCAGCCAAATCCGAAAGGGACGTGTCGGCCACCTCTCTACAGGTC  504
 61   Q  Y  C  A  K  P  A  K  S  E  R  D  V  S  A  T  S  L  Q  V   80
     -----------][--- D domain ---][----------------------------

505  ATACCCGTGATGCCCGCACCAAAACCGGAAGTCTCAAGGAAGCCGCATGTGACCGTGAAG  564
 81   I  P  V  M  P  A  P  K  P  E  V  S  R  K  P  H  V  T  V  K   100
     ------------------------------------------------------------

565  TATTCCAAATACGAGGTGTGGCAGAGGAAGGCGGCCCAGCGGCTCCGGAGGGGTGTCCCC  624
101   Y  S  K  Y  E  V  W  Q  R  K  A  A  Q  R  L  R  R  G  V  P   120
     ------------------------- E domain -------------------------

625  GCCATCCTGAGGGCCAAAAAGTTTCGGAGGCAGGCGGAGAAGATCAAAGCCCAGGAGCAG  684
121   A  I  L  R  A  K  K  F  R  R  Q  A  E  K  I  K  A  Q  E  Q   140
     ------------------------------------------------------------

685  GCAATCTTCCACAGGCCCCTGATCAGCCTGCCCAGTAAACTGCCTCCCATCTTGCTCACC  744
141   A  I  F  H  R  P  L  I  S  L  P  S  K  L  P  P  I  L  L  T   160
     ------------------------------------------------------------

745  ACGGACAACTATGTCAGCCACAAATGAGCCCGCTGCCAGCCCTTTGCACAGACAAGAGTT  804
161   T  D  N  Y  V  S  H  K ***                                   168
     -----------------------]

805  TGGAGGGAGAAAAAAAGACTAGGGGATTATAGCTTTGTCTCTGACGTCATTTCTGTGGCA  864
865  GTCCTCTTTGACCTCCCTGC - rest of 3′ untranslated region
```

Fig 2 IGF-II

```
                    domains[1]      B                        C                A                D
rabbitfish          GPETLCGAELVDTLQFVCGERGFYFSKPT  GYGPNSRRP**R  GIVDECCFQSCELRRLEMYCA  PAKTSKAA
gilthead seabream   S---------------------------*  -----A--S**-  --------------------  --------
black seabream      S---------------------------*  -----A--S**-  --------------------  --------
barramundi          ----------------------------*  ---S-A--S**-  --------------------  -P--*---
sculpin             ----------------------------*  -----A--S**-  --------------------  -P------
flounder            ----------------------------   -----A--S**-  --------------------  --------
tilapia             -------------------------N---  ----SA--S**-  -------------Q------  -V--P-IS
salmon              ----------------------------   ----S---SHN-  --------------------  -V-SG---
rainbow trout       ----------------------------   ----S---SHN-  --------------------  -V-SG---
carp                -------------------D---------  ----S---SHN-  --------------------  -V-PG-TP
goldfish            -------------------D---------  ----S---SHN-  --------------------  -V-PG-TP

                                                                E
rabbitfish          RSVRAQRHTDMPRTPKVSAAGQKVDKGTERRTAQQPDKTKSKKRPLSGHSHSSFKEVHQKNSSRGNTGGRNYRM
gilthead seabream   -------------A----T--H-----------------N-----P-----------P-------A----***
black seabream      -------------A----T--H-----------------N-----P-----------P-------A-------
barramundi[2]       ----------------***************************---P--------D--E---------------
sculpin             ----------**-A-------H----S---------G---N-----P--------D---------TM-------
flounder[2]         -------------A----T-VH-----------------N-----P---------------------------
tilapia             ----S--------A----SRAN***--------P------N----SP---*--------------SS-------
salmon[2]           ------------------T-V-N--R--------H-----P-***********-------------------
rainbow trout[2]    ------------------T-V-S--R--------H-----P-***********-------------------
carp                ----------S---A*************************--*--P-Q----Y-------------------I
goldfish            --L-------****************--**---********--*-IC------C--------------------
```

Source: gilthead seabream, *Sparus aurata* (Duguay *et al.*, 1996); black seabream, *Acanthopagrus schlegeli* (Genbank Acc. No. AF030573); barramundi, *Lates calcarifer* (Kinhult Stahlbom *et al.*, 1999); sculpin, *Cottus scorpius* (Loffing-Cueni *et al.*, 1998); flounder, *Paralichthys olivaceus* (Tanaka *et al.*, 1998); tilapia, *Oreochromis mossambicus* (Reinecke *et al.*, 1997); salmon, *Oncorhynchus kisutch* (Cao *et al.*, 1989); rainbow trout, *Oncorhynchus mykiss* (Shamblott and Chen, 1992); carp, *Cyprinus carpio* (Liang *et al.*, 1996); goldfish, *Carassius auratus* (Kermouni *et al.*, 1998)

[1]domains B,C,A,D constitute the mature peptide; [2]more than one form of E domain is found

Fig 3 IGF-I

```
                    domains[1]        B                    C                A              D
rabbitfish          EIASAETLCGGELVDALQFVCDDRGFYFSRPT  SRGNSRRPQNR  GIVEECCFRSCDLNLLEQYCA  KPAKSE
gilthead seabream   -V-------------------E----------  ----N------  ---------------------  ------
barramundi          -M-------------------E----------  ---SN------  ---------------------  ------
tilapia             -M-------------------E----------  ----N----T-  ---------------------  ------
sculpin             --T------------------E----------  ----N--N---  ---------------------  ------
chum salmon         -V-------------------E----------  --S----S---  ---------------------  ------
rainbow trout       -V-------------------E----------  --S----S---  ---------------------  ------

                                                 E
rabbitfish          RDVSATSLQVIPVMPAPKPEVSRKPHVTVKYSKYEVWQRKAAQRLRRGVPAILRAKKFRRQAEKIKAQEQAIFHRP
gilthead seabream   ----------L----PL-Q-----Q-------------------------------Y------------------
barramundi          ----------------L-Q--P--Q-------------------------------------------------V-----
tilapia             ----------------L-Q--PK-Q-------------------------------YK-H------K--------
sculpin             ------------M---L-Q--P--Q-G-----------------------------------------------GV-N--
chum salmon         ----------I--MV-TI-QD-P--*----------A-------------------R------V--------M----
rainbow trout       ----------I--MV-TI-QD-P--*----------A-------------------R------V--------M----

rabbitfish          LISLPSKLPPILLTTDNYVSHK
gilthead seabream   ----G-----V--A-----N--
barramundi          ----------V--------N--
tilapia             ----------V------F----
sculpin             ----------V--A----LN--
chum salmon         --T-------V-PP-------N
rainbow trout       --T-------V-PP-------N
```

Source: gilthead seabream, *Sparus aurata* (Duguay *et al.*, 1996); barramundi, *Lates calcarifer* (Collet *et al.*, 1997); tilapia, *Oreochromis mossambicus* (Chen *et al.*, 1997); sculpin, *Cottus scorpius* (Loffing-Cueni *et al.*, 1999); chum salmon, *Oncorhynchus keta* (Genbank Acc. No. X97225); rainbow trout, *Oncorhynchus mykiss* (Shamblott and Chen, 1992);

[1]B,C,A,D domains constitute the mature peptide

Fig 4 IGF-II

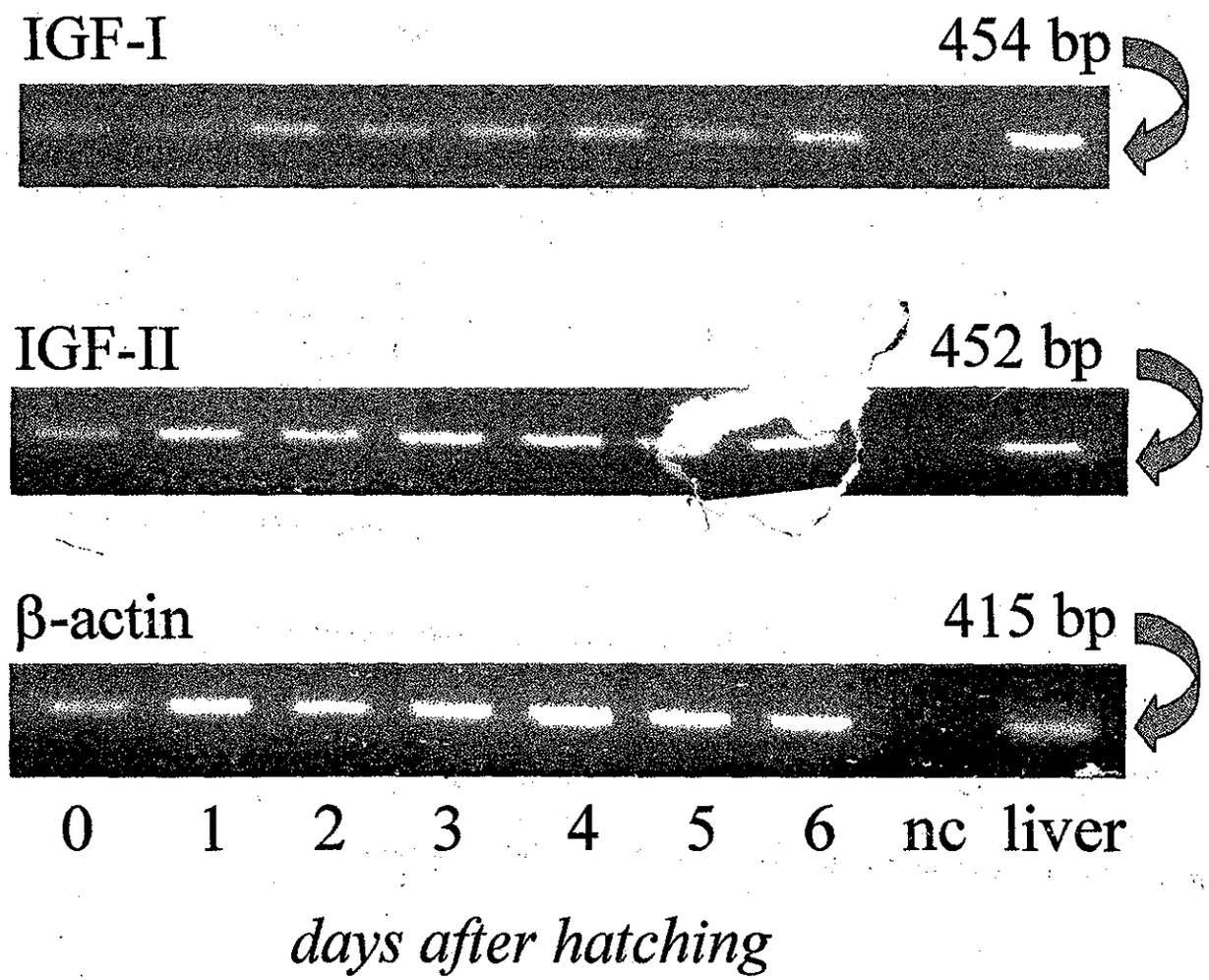
IGF-I
454 bp
IGF-II
452 bp
β-actin
415 bp
0 1 2 3 4 5 6 nc liver
days after hatching

Fig 5A

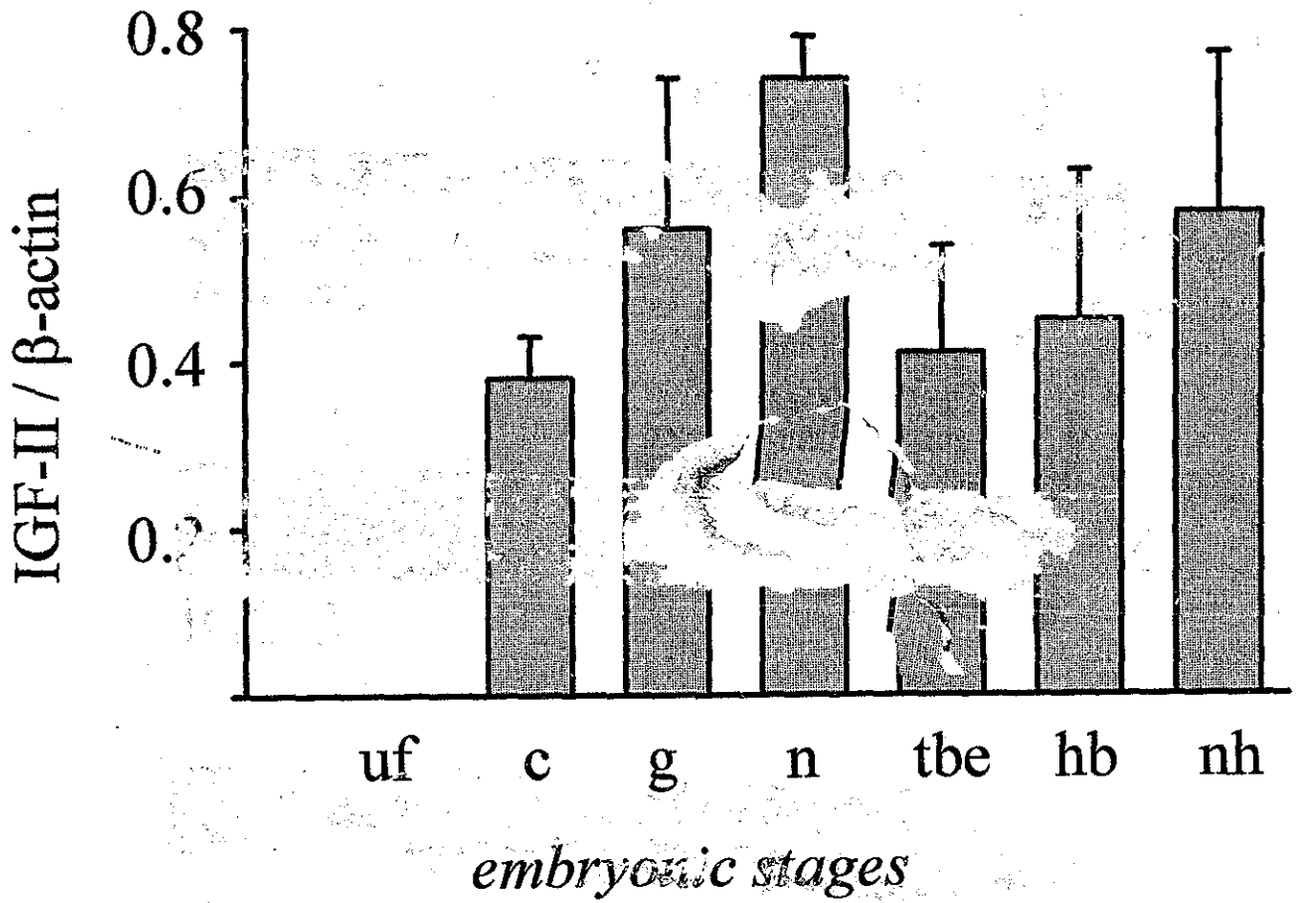

Fig 5B

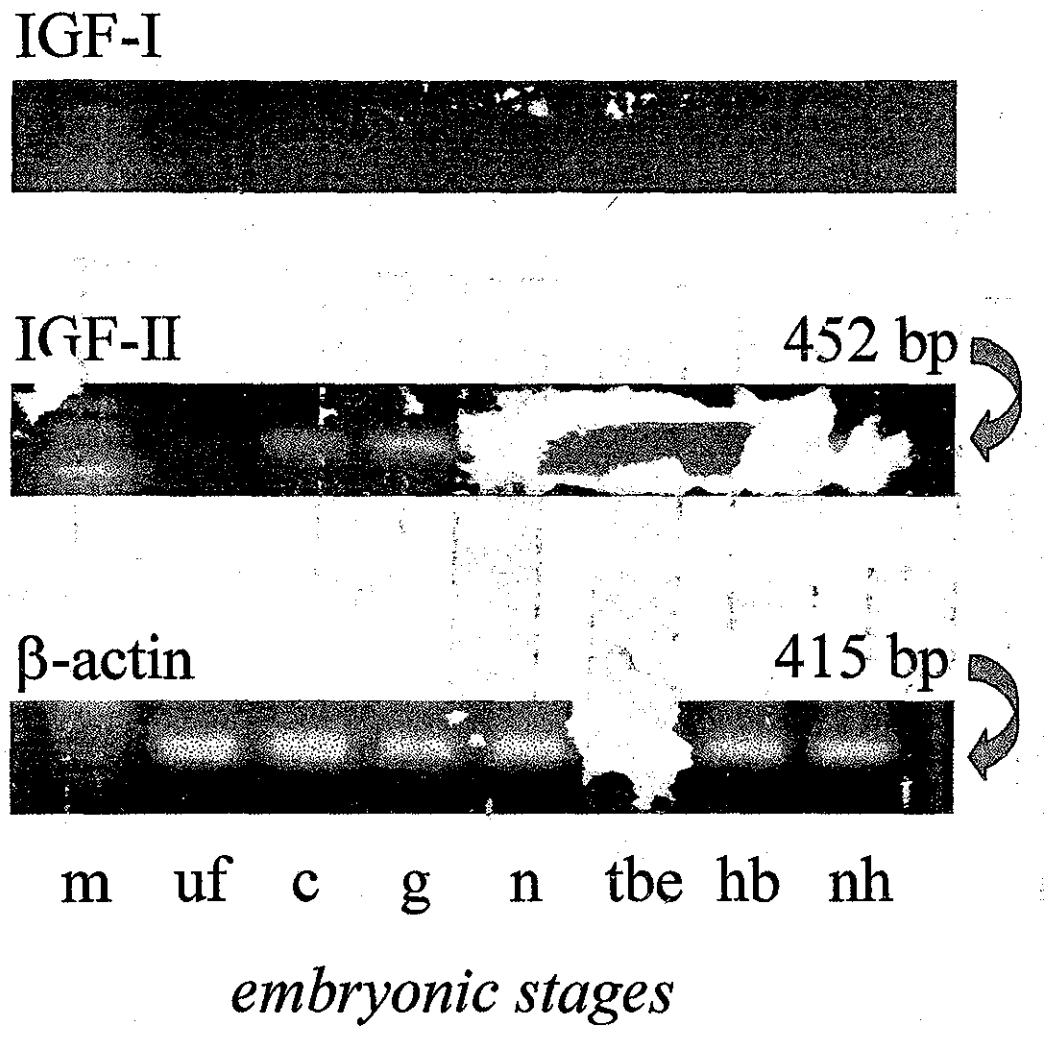
IGF-I
IGF-II
452 bp
β-actin
415 bp
m uf c g n tbe hb nh
embryonic stages

Fig 6A

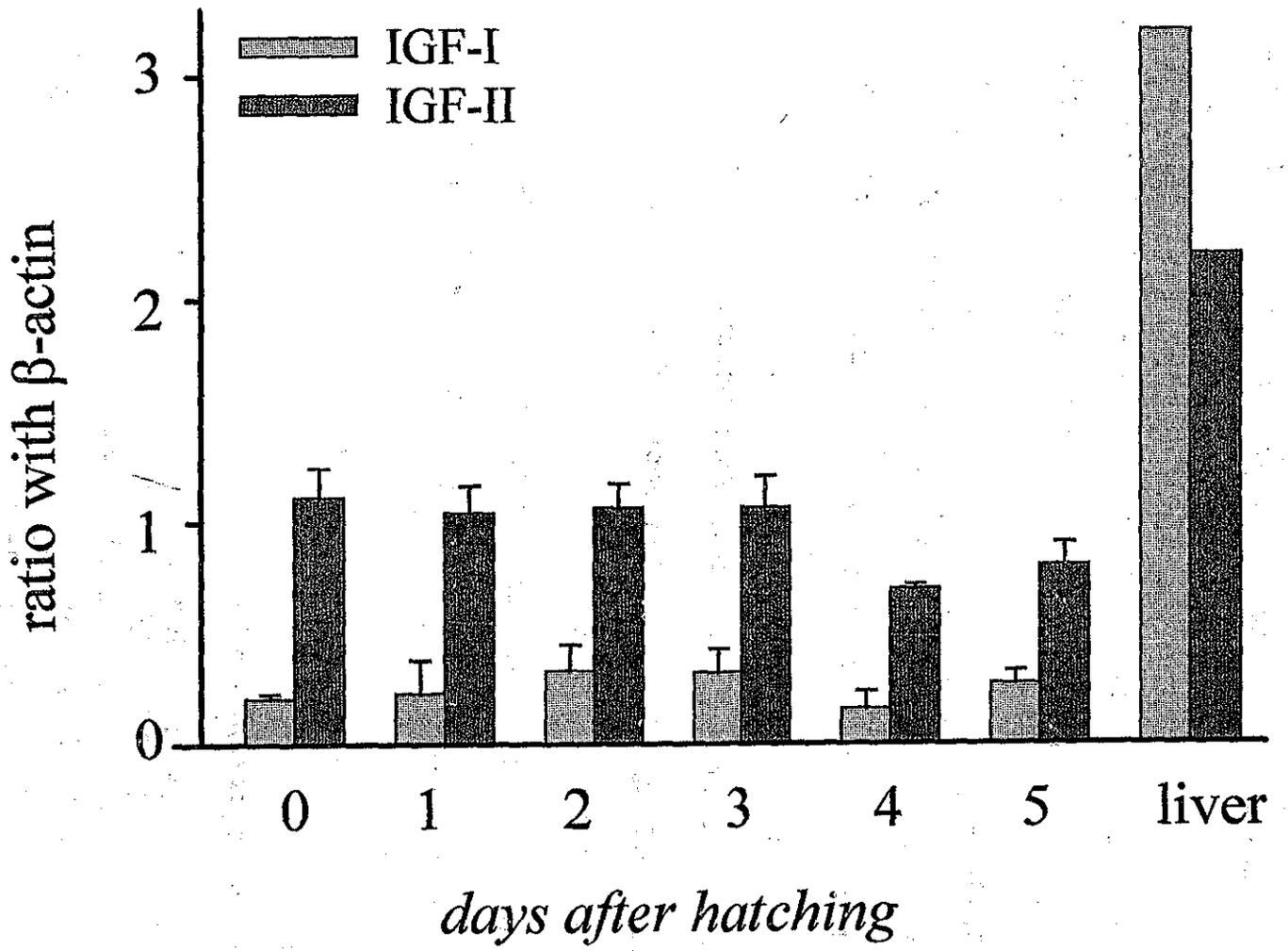
IGF-I
IGF-II
ratio with β-actin
3
2
1
0
0
1
2
3
4
5
liver
days after hatching

Fig 6B

Exogenous Growth Hormone and Low Water Salinity Accelerate Growth of Rabbitfish (*Siganus guttatus*) Fry

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Abstract

Complains by fish farmers about the slow growth of rabbitfish (*Siganus guttatus*) under culture conditions is one of the major hindrances for its mass culture in the Philippines. We examined whether rearing rabbitfish fry in dilute seawater (SW) and treating them with exogenous growth hormone (GH) by intraperitoneal injection would enhance their growth. Rearing the fry for 3 months in 10 and 20 ppt significantly improved their growth. Treating the fry with bovine GH at 0.01 μg/g BW resulted in significantly faster growth compared with the saline-treated controls. However, combining low water salinity and GH treatment did not further improve the growth since rabbitfish fry reared in 20 ppt and injected with bovine GH at doses of 0.01 and 0.1 μg/g BW did not result in faster growing fish compared to the fry reared in 20 ppt but without GH treatment. These results suggest that rabbitfish is best reared in dilute SW for faster growth. For fish farmers who have problems with the availability of freshwater supply, rearing rabbitfish fry in SW would need additional rearing intervention like the use of some growth promoting agents such as GH to improve its growth.

Keywords: salinity effects, GH effects, growth, rabbitfish

Introduction

In the northern part of the Philippines, the rabbitfish (*Siganus guttatus*) is cultured in brackishwater earthen ponds or in fish pens and cages set in coastal waters or near the rivers. Since *S. guttatus* are herbivores, they are sometimes reared together with milkfish in brackishwater ponds where they are fed natural food (filamentous algae) in the early part of culture and later with artificial feeds when the supply of natural food is already depleted. In fish pens and cages however, artificial feeds is the main food throughout the culture period.

Fish farmers complain of the slow growth of this species. Despite this, however, farmers still would like to grow them because they command a better price compared to milkfish. Since production inputs of these two species are more or less the same and since the market price of *S. guttatus* is more than twice the price of milkfish, fish farmers get higher profit with *S. guttatus*.

It is generally accepted that growth in fish is under the control of growth hormone (GH) and insulin-like growth factor I (IGF-I). Increased growth is usually accompanied by increased levels of circulating GH and IGF-I. Thus, approaches to enhance growth in fish include, among others,

exogenous GH administration. The GH given could either be homologous or heterologous and native or recombinant. We have isolated GH in *S. guttatus* and used the purified native hormone for growth enhancement trials (Ayson et al., 2000). Several studies have shown that heterologous GH preparations are also effective in enhancing growth in several fish species (reviewed by Donaldson et al., 1979). Among the available non-piscine GHs, bovine GH is the most commonly used.

Although many of the studies were done in euryhaline species and only few in marine species, rearing fish in low saline waters sometimes results in faster growth compared to rearing in full-strength seawater (SW) (Lambert et al., 1994; Woo and Kelly, 1995; Woo et al., 1997). *S. guttatus* is also found in mangrove areas and was found to tolerate abrupt transfer to 10 ppt. We tested whether they would grow faster in dilute SW than in full-strength SW. We report here that rabbitfish fry grew better in dilute SW than in full-strength SW and that treatment with bovine GH also improved its growth.

Materials and Methods

Fish

The rabbitfish fry used in all experiments were produced from the Fish Hatchery of SEAFDEC AQD.

Experiment 1. Effect of low water salinity on growth of rabbitfish fry

Although rabbitfish inhabits and spawns purely in marine waters, they are sometimes caught in brackishwater environments like in mangrove areas and near river mouths. We wanted to examine whether growth of rabbitfish fry will be improved when reared in dilute SW, as was observed in other fish species. About 300 pcs. rabbitfish fry (BW=5-6 g and reared exclusively in SW from hatching) were randomly divided into 3 groups (100 fry/group). Two groups were directly transferred to one-ton tanks containing water with salinities of 10 and 20 ppt, and the third group was transferred to another tank containing full-strength SW (33-35 ppt). Fish were reared in these water salinities for 3 months. Low salinity water was prepared by diluting SW with FW, put in a reservoir and allowed to stay overnight before use. All groups were fed with artificial pellet at a feeding rate of 4-5% BW per day. The feed ration was given in the morning and in the afternoon.

Approximately 70-80% of the water was changed daily in the morning at which time siphoning of the tank bottom to remove wastes was also carried out. Growth measurements were done every 15 days and the feeding rate was adjusted accordingly. The experiment was conducted twice.

Experiment 2. Effect of bovine GH treatment on growth of rabbitfish fry

Administration of bovine GH (bGH) accelerates growth in a number of fish species. Since this is readily available, we tried this in rabbitfish. Three groups of rabbitfish fry were stocked separately in 250-l fiberglass tanks. Each group contained 3 tanks with 15 fish/tank. Fish in the first and second groups were injected with bGH at a dose of 0.01 μg/g BW (low dose) and 0.1 μg/g BW (high dose), respectively. The third group was injected with saline solution and served as the control. The injection was given intraperitoneally every week for 4 weeks. The hormone was first dissolved in small amount of 0.01 N NaOH then with saline solution. Each fish was marked with a small cut in the operculum to be able to follow growth in individual fish. Growth (increments in weight and length) was measured every week. Rearing was done in full-strength SW (33-35 ppt) for 7 weeks; the first 4 weeks with bGH

injection and the last 3 weeks was to observe for some residual effect of the hormone, if any. Fish were fed with fish pellet 3x daily at a feeding rate of 7.5% BW. About 50-80% of the water was changed daily in the morning and siphoning of the tank bottom to remove wastes was also carried out daily. The fish was acclimated to the experimental condition for one week.

Experiment 3. Interaction of low water salinity and GH treatment on growth of rabbitfish fry

We observed in the previous experiments that GH treatment and rearing rabbitfish fry in dilute SW enhanced its growth. We wanted to examine if rabbitfish fry subjected simultaneously to these 2 factors would grow faster or bigger compared to fry subjected separately to these factors. Three groups of fish were used in this experiment with 3 replicate tanks per group. Ten fish were stocked in each 250-l tanks. Fish in group 1 was reared in dilute SW (20 ppt) without GH treatment whereas fish in groups 2 and 3 were reared in dilute SW (20 ppt) but with bGH injection at a dose of 0.01 μg/g BW (low dose) and 0.1 μg/g BW (high dose), respectively. The hormone was injected into individual fish every week for 4 weeks. Growth measurement was done every week. To follow growth in each

fish, all fish were individually marked with small cuts in the operculum. Fish were fed fish pellets 2x daily at a feeding rate of 5% BW and water in the rearing tanks was also changed daily. The experiment lasted for 8 weeks. Acclimation period before the start of the experiment was for one week.

Statistics

Significant differences in body weight and length between the fish reared in dilute SW and full-strength SW, and between the control and the hormone-treated groups at each sampling time were determined by *t* test after one-way ANOVA (Sokal and Rohlf, 1995). Percentage data were transformed by arc sine or square root transformations, where appropriate, before the analysis.

Results

Experiment 1

Rabbitfish fry reared in dilute SW were significantly larger than those reared in full-strength SW (Figs. 1 A & B). Fry reared in 10 ppt and 20 ppt were already significantly larger than the fry reared in full-strength SW starting on the 6th and 8th weeks of rearing and this was maintained until the end of the experiment. The difference

in the growth of the fry reared in dilute SW and in full-strength SW increased as the culture duration progressed.

Experiment 2

The fry reared in full-strength SW and injected with bGH at low dose (0.01 μg/g BW) were significantly larger than the control starting on the 5th week (Figs. 2 A & B). The significant effect on growth was observed during the weeks when the bGH treatment was already stopped. Although larger, the growth of the fry injected with the high dose of the hormone (0.1 μg/g BW) was not significantly different from the control.

Experiment 3

The growth of rabbitfish fry reared in dilute SW (20 ppt) and treated with bGH was not significantly different from the fry reared in dilute SW but without bGH treatment (Figs. 3 A & B).

Discussion

Rabbitfish fry reared in dilute SW grew faster than those reared in full-strength SW. The response of the fry to the different water salinity was also different; they grew best in 10 ppt, about 1/3 SW. As shown in Figure 1A,

the effect of low water salinity on growth of rabbitfish fry became prominent as the culture duration progressed; it became significant starting from 6 weeks of culture and remained so until the end of the 12-week culture period.

It is hypothesized that the effect of salinity on fish growth is due to the fact that the energetic cost spent for osmoregulation is lowest in isosmotic environment and the energy saved is channeled towards growth (Morgan and Iwama, 1991). This, however, is not the general rule since the response of fish to challenges in water salinity is not consistent. The optimal salinity for growth seems to differ among species and in different life stages, and in some species even with season (Morgan and Iwama, 1991). This is particularly true among the tilapias. To mention a few, there are reports of tilapia growing faster in full-strength SW than in lower salinities or FW (Kuwaye et al., 1993) or conversely, growing poorly in SW than in other salinities (Fang and Chiou, 1989).

For marine fish species that can tolerate brackishwater, not much information is available regarding their growth rate when reared in lower salinities. The Atlantic cod (*Gadus morhua*) which is observed to occur also in brackishwater was found to grow better in intermediate salinity (14 ppt)(Lambert et al., 1994). Growth of the

seabream (*Sparus sarba*) which is commonly cultured in floating net cages in coastal waters was also higher in 15 ppt compared with 7 and 35 ppt (Woo and Kelly, 1995). The rabbitfish spawns and spends the entire life cycle in marine waters, are caught by fishermen in seagrass beds and in mangrove areas, places where the salinity can go brackish. In the hatchery, we observed that rabbitfish fry can survive abrupt transfer from SW until 10 ppt but die when abruptly transferred to pure FW. A salinity of about 15 ppt is considered to be isosmotic to the fish blood so that it is not surprising to observe euryhaline marine fish species growing best in salinities of 14-15 ppt as observed in the Atlantic cod and sea bream (Lambert et al., 1994; Woo and Kelly, 1995). For rabbitfish, however, growth is best in 10 ppt, although it also grows better in 20 ppt compared to full-strength SW.

Higher growth rates in lower salinities may come about as a result of increase in food intake (appetite), increase food conversion efficiency, improved digestibility of macromolecules (proteins and lipids) and thus food assimilation, lower metabolic rate, among others (Dutil et al., 1994; Woo et al., 1997). Since this experiment was designed only to find the best water salinity for rearing rabbitfish fry during the nursery and grow-out culture

periods, we were not able to look at any of the factors that may explain the faster growth of rabbitfish fry in 10 ppt. Nevertheless, we observed in the course of the experiment that rabbitfish fry reared in full-strength SW do not eat as much compared with the fry in 10 and 20 ppt. A fixed feeding rate of 5% BW/day and adjusted every 15 days after every sampling was given to the fry and we observed that fish in 10 and 20 ppt were more aggressive in feeding than the fish in full-strength SW. As a consequence, it may be argued at this time, that the faster growth of rabbitfish fry in low water salinity is probably a result of increased appetite and thus increased food intake.

Administration of bGH either by injection, dissolved in the rearing medium, by oral intubation, in cholesterol pellet for implantation or by mini-osmotic pumps resulted in faster growth in most fish species studied. The dose of the hormone used ranged from 0.5 μg/g BW to as high as 40 μg/g BW for injection (Degani and Gallagher, 1984; Weatherley and Hill, 1987; Down et al., 1988; Cavari et al., 1993; Hunt et al., 2000; Silverstein et al., 2000); 12.5 μg/g BW/week for oral intubation (McLean et al., 1990); 100 mg/liter for 4 hours every 5 days for 8 weeks for

immersion (Schulte et al., 1989); 0.5 μg/g/week for mini-osmotic pump (Down et al., 1988); and 0.5-5.0 μg/g/week for cholesterol pellet implant (Down et al., 1988). As shown in Figures 2A and B, bGH treatment at doses of 0.01 and 0.1 μg/g BW enhanced growth in rabbitfish fry as is observed in other species. The group treated with the low dose showed the best response. The hormone was given weekly for 4 weeks but the effect was observed after the injection was already stopped.

As shown in experiments 1 and 2, rearing rabbitfish fry in dilute SW and treating them with bGH resulted in faster growth. We wanted to examine further if rearing rabbitfish fry in 20 ppt and at the same time injecting them with GH would have an additive effect on its growth. We hypothesized that these 2 factors together would confer additive advantage in terms of their growth promoting effect - that the fry reared in low salinity and injected with GH would be bigger than those reared also in low salinity but without GH treatment. Surprisingly, this was not the case. As shown in Figures 3A and B, there was no significant difference in the growth of the fry in all the 3 groups indicating that GH treatment in the fry reared in 20 ppt does not result in faster growing fish compared to

just rearing them in 20 ppt and without GH treatment. This suggests that the exogenous GH given to the fry in dilute SW does not have an added advantage to the fish in terms of growth performance. Why is it that the exogenous GH given to the fry reared in full-strength SW resulted in significantly faster growing fish but do not have an effect when given to fry reared in 20 ppt? We do not have an explanation to this at present. Nevertheless, we suspect the involvement of prolactin (PRL) in this phenomenon. In tilapia in FW, PRL can bind to the GH receptors in the liver and can displace GH from its receptor because of the high similarity in the structure of PRL and GH. Thus, in FW, PRL may possess somatotrophic actions similar to GH (Shepherd et al., 1997). Because of the high levels of PRL that is expected in fish in low salinity waters, it remains possible that the additional exogenous GH given to rabbitfish fry in dilute SW does not have an additional effect on growth because it was not able to bind to its receptor which was probably already saturated with PRL.

From the results of the experiments described here, we can recommend to the fish farmers to rear rabbitfish fry in dilute SW in nursery and grow-out phases. Fish ponds and fish pens intended for rabbitfish rearing should be located in areas where there is access to FW like near the rivers.

However, in areas where FW supply is a problem, rearing rabbitfish in full-strength SW needs additional rearing intervention like treatment with growth-promoting agents such as GH has to improve its growth.

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Figure Legends

Figure 1. Bi-weekly increase in body weight (**A**) and body length (**B**) of rabbitfish fry reared for 3 months in full-strength SW (33 ppt) and dilute SW (10 and 20 ppt). Asterisks denote significant differences ($P<0.01$; $P<0.05$) with the group reared at 33 ppt.

Figure 2. Weekly increase in body weight (**A**) and body length (**B**) of rabbitfish fry reared in full-strength SW (33 ppt) and injected intraperitoneally weekly for 4 weeks with bGH at 0.1 μg/g BW (high dose) or 0.01 μg/g BW (low dose). Asterisks denote significant differences ($P<0.01$; $P<0.05$) with the saline-injected control.

Figure 3. Weekly increase in body weight (**A**) and body length (**B**) of rabbitfish fry reared in dilute SW (20 ppt) and injected intraperitoneally weekly for 4 weeks with bGH at 0.1 μg/g BW (high dose) or 0.1 μg/g BW (low dose).

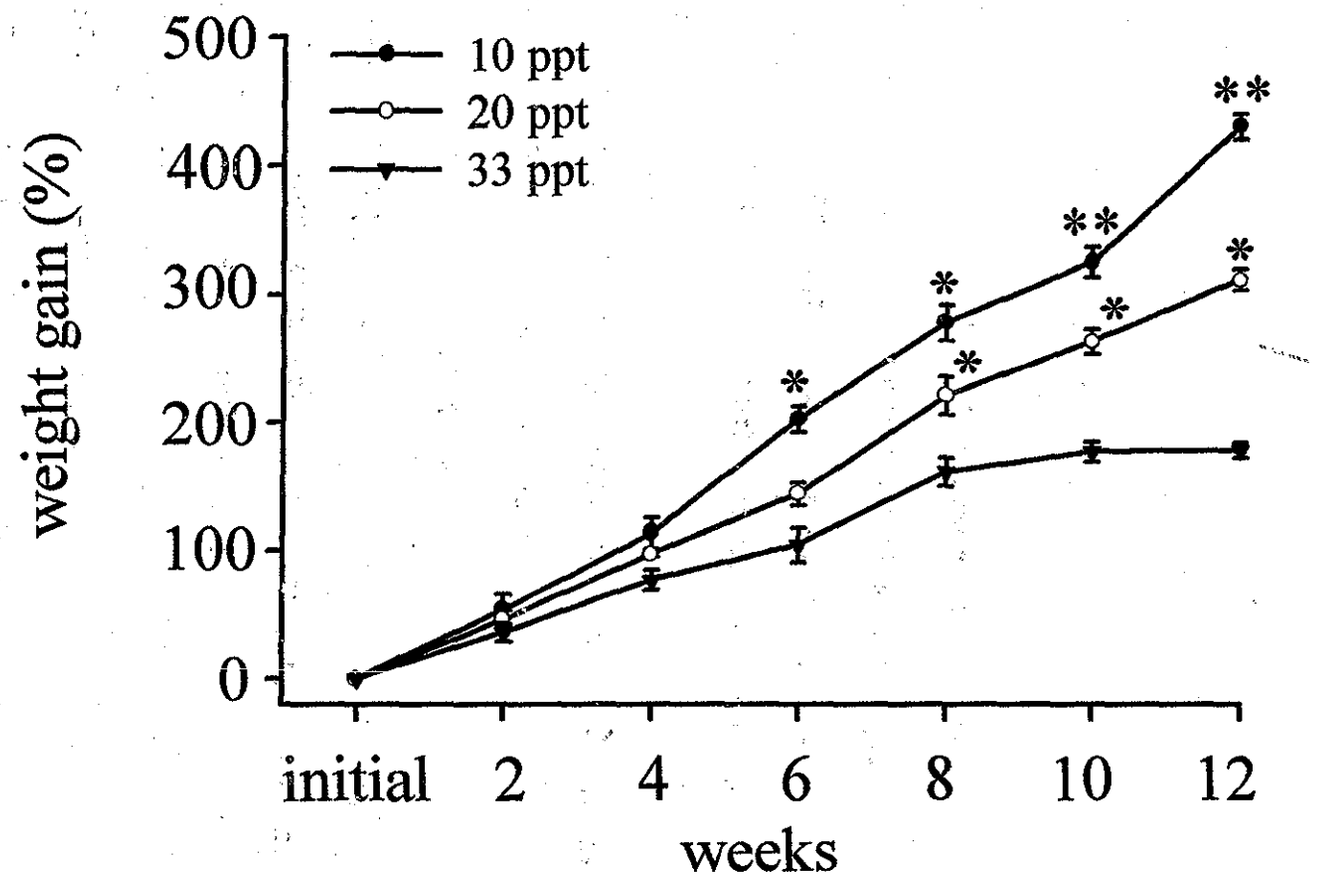
500
400
300
200
100
0
weight gain (%)
10 ppt
20 ppt
33 ppt
*
*
*
*
*
**
**
initial
2
4
6
8
10
12
weeks

Fig 1A

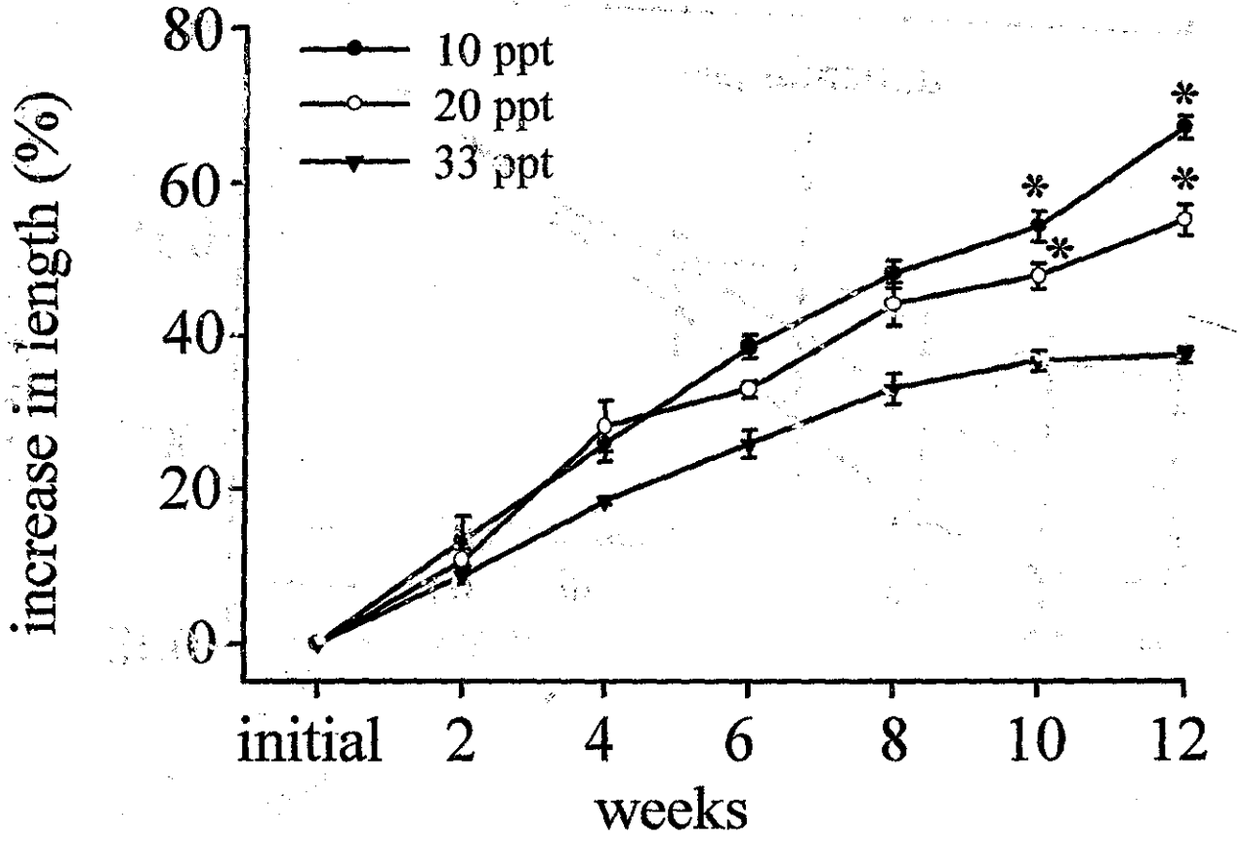
80
60
40
20
0
increase in length (%)
10 ppt
20 ppt
33 ppt
*
*
*
*
initial
2
4
6
8
10
12
weeks

Fig 1B

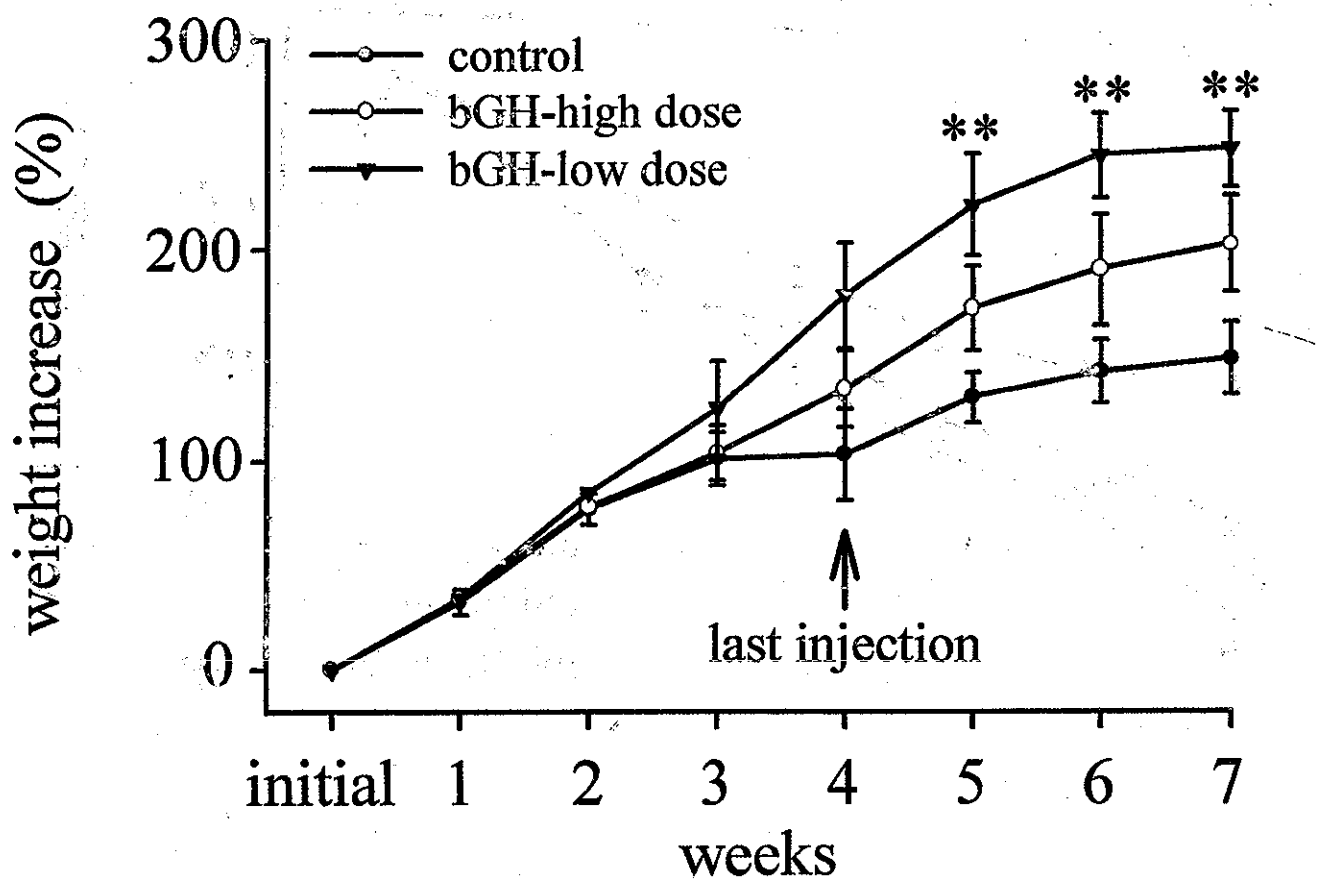
300
200
100
0
weight increase (%)
control
bGH-high dose
bGH-low dose
**
**
**
last injection
initial
1
2
3
4
5
6
7
weeks

Fig 2θ

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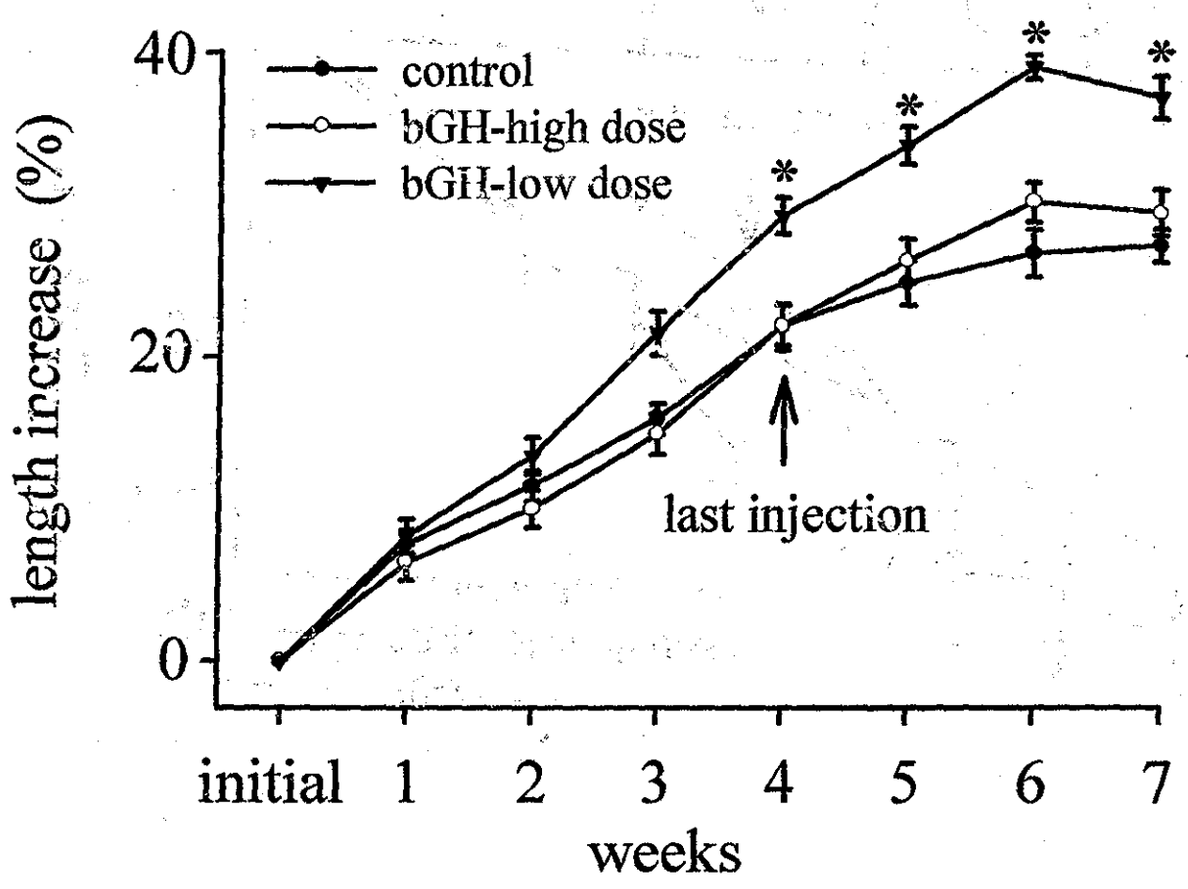
40
20
0
length increase (%)
control
bGH-high dose
bGH-low dose
*
*
*
*
last injection
initial
1
2
3
4
5
6
7
weeks

Fig 2B

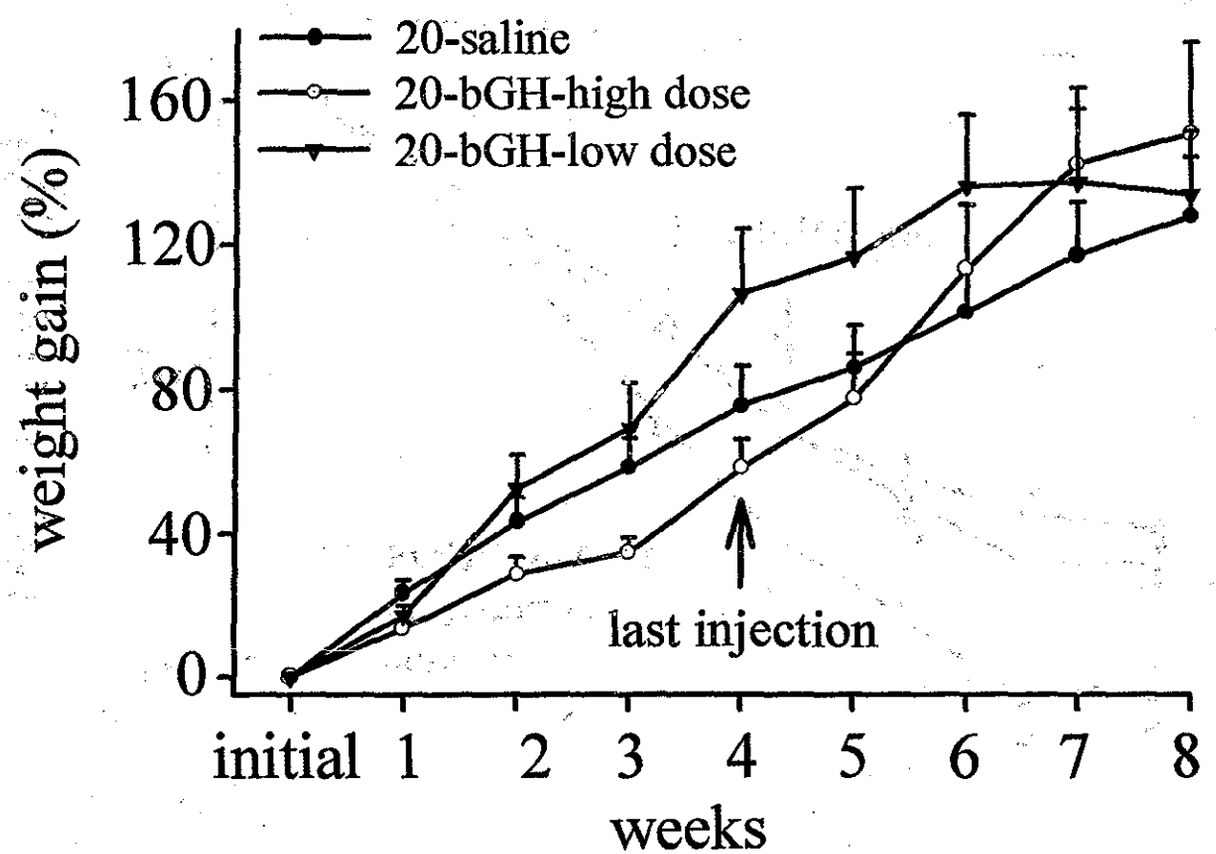
20-saline
20-bGH-high dose
20-bGH-low dose
weight gain (%)
160
120
80
40
0
last injection
initial
1
2
3
4
5
6
7
8
weeks

Fig 3A

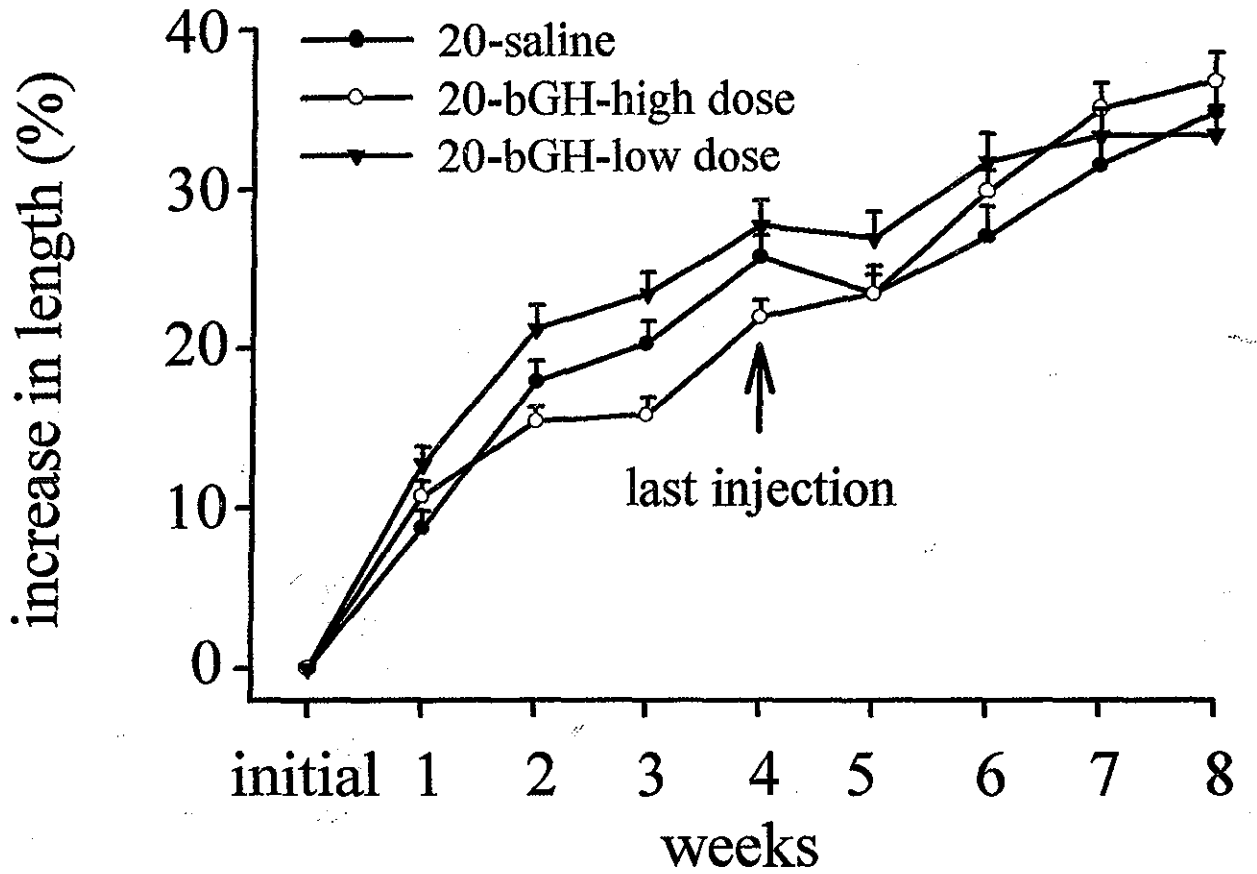
20-saline
20-bGH-high dose
20-bGH-low dose
increase in length (%)
40
30
20
10
0
last injection
initial
1
2
3
4
5
6
7
8
weeks

Fig 3B