ISSN 1996-3351

Asian Journal of Biological Sciences

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Asian Journal of Biological Sciences 3 (2): 55-67, 2010
ISSN 1996-3351

Cloning of IGF-I, IGF-II and IGF-IR cDNAs in Mullet (*Mugil cephalus*) and Grouper (*Epinephelus coioides*): Molecular Markers for Egg Quality in Marine Fish

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Abstract: The putative cDNA sequence of insulin like growth factor (IGF)-I, IGF-II and IGF-IR (receptor) was determined from the tissues of mullet, *Mugil cephalus* and grouper *Epinephelus coioides*. The deduced partial coding sequences were deposited at Gene Bank (Accession No. AY427954, AY427955, AY772254, AY776158, AY776159, AY772255). These basic data were used to determine messenger ribonucleic acid (mRNA) levels during embryogenesis in the two species. Results showed that IGF-II has the highest expression ratio and makes it a potential marker for egg quality in the two cultured species.

Key words: IGF-I, IGF-II, IGF-IR, mullet, grouper

INTRODUCTION

Studies in the last decade have shown that the vertebrate egg contains a broad representation of different classes of maternal hormones and growth factors that could be vital to egg development and influence egg quality. The maternal ribonucleic acid (RNA) that codes for hormones and growth factors are stored in translationally inactive form until they are activated and turn on protein expression during oocyte maturation, fertilization or early embryonic development before the endocrine glands develop and become functional (Elies *et al*., 1999). Growth factors required by the cell for cell-cycle progression (LeRoith *et al*., 1995) include Insulin-like Growth Factors (IGF), nerve growth factor and epidermal growth factor among others. The IGFs mediate the action of growth hormone and in effect stimulate deoxyribonucleic acid (DNA) synthesis and cell replication, causing the cell to traverse the successive phases of cell cycle (Jones and Clemmons, 1995). The IGF system consists of insulin, IGF-I and II that are ligands for their corresponding receptors, insulin receptor, IGF-IR and IGF-IIR and the IGF binding proteins.

Recent studies have suggested an important role for IGFs in fish reproduction where expression in ovary, oocytes, fish embryos and testicular cells indicated their involvement during the reproductive process (Kagawa and Moriyama, 1995; Funkenstein *et al*., 1996; LeGac *et al*., 1996; Ayson *et al*., 2002).

The mullets are a family of near-shore catadromous fishes of considerable economic importance. The mature ovaries and the dried roe is a gourmet food that fetches a very high price. Mullets have also been used as bioremediators in fish farms where they contribute to

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the control of macroalgal biomass in shrimp farm effluent (Erler *et al*., 2004). Mullets are also used as efficient means for improving the quality of sediments underneath intensive net-cage fish farms (Lupatsch *et al*., 2003). Groupers belong to the family Serranidae that are among the most highly valued marine finfish for food. In Southeast Asia, more than 20 species have been raised commercially by growing out captured wild juveniles. Hatchery technology has been established in some countries (Pomeroy *et al*., 2002) but egg survival has been variable (Sugama *et al*., 2003).

This study aimed to determine cloned DNA (cDNA) sequences of the three genes, IGF-I, IGF-II and IGF-IR in mullet *M. cephalus* and grouper *E. coioides* to provide basic molecular tools for egg quality determination. A real-time Quantitative Polymerase Chain Reaction (QPCR) assay has been developed to determine gene expression levels in developing embryos, hatched larvae and in non-viable eggs.

MATERIALS AND METHODS

Tissue Collection

Liver, muscle and gonad tissues of mullet (>300 g) were obtained from three tank-reared and wild caught female mullet at Bribie Island, Queensland, Australia while muscle, brain and liver tissues from wild caught mature female grouper (400-700 g) were collected from Cairns, Queensland, Australia (February, 2003). Gonad tissues were collected from the mullet during the spawning season ensuring that the fish were vitellogenic or at maturing stage (May-July 2003).

Tissues were quickly dissected, cut into small pieces (<0.5 inch thick), placed in 1.5 mL tubes (Eppendorf, USA), snapped frozen in liquid nitrogen and stored at -80°C. When liquid nitrogen was not available, tissues were placed in RNA Later (Invitrogen), placed overnight at 4°C and stored at -80°C until used.

RNA Extraction and First Strand cDNA Synthesis

Total RNA was extracted from the tissues with a battery powered homogenizer (Astral, USA) using Trizol (Invitrogen) following the manufacturer's protocol for fatty samples. Resulting RNA pellets were resuspended in 22-25 μL autoclaved distilled water and aliquots were used for RNA measurement and cDNA synthesis. The RNA concentration was measured at 260 nm (spectrophotometer, UV/VIS Gene Quant, Amersham Biosciences Pty. Ltd.) and the quality and integrity of extracted RNA was confirmed by running RNA samples in 1.2% denaturing formaldehyde agarose gels (Qiagen). Only samples that showed ribosomal ribonucleic acid (rRNA) bands and valid absorbance of 260/280 ratio (>2.0) were used for cDNA synthesis.

Three microliters of total RNA extract were used for 5' and 3' RACE first strand cDNA synthesis (Progen). The first strand reaction product was diluted with Tricine EDTA buffer, incubated in a heating block at 72°C for 7 min, aliquoted in tubes and stored at -20°C. These served as cDNA templates for PCR.

Primer Design

Primers for IGF-I, IGF-II and IGF-IR were designed based from published sequences from other fish species. Amino acid and nucleotide sequences were downloaded from National Center for Biotechnology Information (NCBI) (http:///www.ncbi.nlm.nih.gov) and European Bioinformatics Institute (EBI) website (http:///www.ebi.ac.uk) and aligned using the Clustal program from EBI. A consensus sequence was identified among the fish species and specific

Table 1: Primer pairs used to amplify IGF-I, IGF-II and IGF-IR sequences in the respective mullet and grouper tissues

Forward primer 5'-3'	Reverse primer 5'-3'	Annealing temperature (°C)	Expected product size (bp)	Tissue taken
Mullet IGF-IR				
Gtcgccatccaaactgt	gttgtgaagacgccatc	52.4	~485	Gonad
Tacctgaacgccaacaa	ccgttcatgtgtgcgta	53	~818	Gonad
Ttygtcttctcgagvac	acagtttggatggcgac	55	~660	Liver
Cacaactactgctcyaa	acagtttggatggcgac	55	~1173	Liver
Ccsttcachgtbtaccg	gcattbccwgcbagvga	55	~363	Liver
Grouper IGF-IR				
gtcgccatccaaactgt	tggtaaggctgctctgc	52.4	~818	brain
ccsttcachgtbtaccg	ttgaccacgcccttcgc	55	~699	brain
gtggtttctcagggaca	ccgttcatgtgtgcgga	53	~1008	brain
Grouper IGF-I				
gcggagacccrwggggatgtctagcg	tacatkckrtarttyckkcccccyghryt	64	~585	brain
Grouper IGF-II				
catggaracccagmaaagayaacggac	ggmagsstgatsagrggcckgtggwrg	65	~570	brain

All primers are presented 5' to 3' letter(s) corresponding to the nucleotide, a: Adenine; c: Cytosine; g: Guanine; t: Thymine; y: c/t; w: a/t; v: a/c/g; s: c/g; b: c/g/t; r: a/g; k:: g/t; h-a/c/t; m: a/c

and degenerate primer sequences were designed. Selected primer sequences were checked for self-complementarities, primer dimer formation, self-hybridization and melting temperature using NarOligo Program (Oligo ver. 2 Nar). Table 1 shows the sequence of primer pairs that were used to amplify the three genes in mullet and grouper, the corresponding tissues where source of the cDNA template was taken, the annealing temperature and the expected product size. Synthesized primers were purchased from Proligo or Sigma Aldrich.

PCR and Gene Cloning

A 12.5 μL total reaction volume for PCR amplification was used. The reaction consisted of 1 uM each of forward and reverse primers, 0.2 mM dNTPs, 2.5 mM MgCl, 1.25 μL of 10x PCR buffer reaction mix, 0.3 units of Taq polymerase and 1 μL of synthesized 5' or 3' cDNA for template. The PCR reactions were carried out in 0.2 mL PCR tubes and thermal cycling was done in a 0.2 mL block module (Hybaid Limited). Different primer combinations were first tested using the thermal gradient block. Annealing temperatures tested range from 52.4-64°C and basic cycle of pre PCR heat step at 94°C for 1-2 min, followed by 30 cycles of 94°C for 30 sec denaturation, 54-64°C annealing for 30 sec, extension at 72°C for 1 min and final extension of 72°C for 10 min. The PCR products were electrophorosed in 1.5% agarose gel at 140 volts for 25-30 min.

The optimal temperature that produced a positive band was used to do a PCR for gel excision and cloning. Band sizes were visualized in the UV light and the correct band size was excised with a single use carbon steel surgical blade (Swann-Morton). The excised gel band was purified (Perfect Gel Cleanup Kit, Eppendorf) and the purified DNA was ligated into pGEMT Easy vector (Promega). The ligated plasmids were used to transform XL1-Blue subcloning Grade competent cells (Stratagene). The transformation reactions were plated in LB Amp agar plates and incubated at 37°C for 12 h. The positive white colonies were screened by PCR using the universal SP6 and T7 primers. The PCR products were electrophoresed in 1.5% agarose gel to confirm which colonies have the correct DNA insert size. Isolates from the colonies that have the correct inserts were selected and inoculated in LB media. Cultured cells were harvested and the plasmid purified (Wizard Plus SV Minipreps DNA Purification Systems, Promega). Clones were digested with EcoRI enzyme (New England Biolabs) to confirm size. Positive clones were used as template for PCR sequencing reaction.

The sequence reaction mix was 3 μL ABI mix dye, 1.0 μL ABI sequencing buffer, 3.2 pmol of M13 (either forward or reverse), 200-400 ng of purified DNA and autoclaved distilled water to a volume of 10 μL. The mixture was subjected to PCR condition of: pre PCR heat step at 94°C for 5 min, followed by 27 cycles of 94°C denaturation for 10 sec, 50°C annealing for 15 sec and extension of 60°C for 4 min. The PCR reaction was precipitated using ethanol and sodium acetate. The resulting supernatant was carefully aspirated and discarded and the pellet was dried in a vacuum centrifuge at 40°C. Dried samples were sent to Australian Genome Research Facility (AGRF) for sequencing. Resulting nucleotide sequence was analyzed using Sequencher 4.0 (Gene Codes Corporation). The chromatograms were examined for clear and single peaks, nucleotide of the vector trimmed out and nucleotide sequence examined for homology with reported sequences from other species at NCBI using the BLAST Tool (Altschul *et al*., 1997). The different clone sequences were aligned to establish a continuous sequence and overlapping sequences in the clones were connected to obtain the maximum cDNA sequence. Comparison of sequence homology among selected teleost species was done using ClustalW program accessed at EBI.

Quantitative PCR (QPCR)

Samples

Samples (approx. 0.1 g) were collected from spawned eggs of mullet (n = 2) and grouper (n = 7) at late embryo and newly hatched larval stage. From each batch of spawn, samples of eggs that sank were also collected. For pelagic spawners like grouper and mullet, eggs that remain suspended in the water column are considered good quality eggs while sunken eggs are considered to be of poor quality. Microscopic examination of the sunken eggs herein referred to as non-viable eggs showed that they did not exhibit development compared to the eggs that float in the water column. The samples were stored and RNA extracted following the procedure as in tissues used for isolating cDNA sequences of the genes. Total RNA extract in the samples were diluted in nuclease-free water to a uniform concentration of 1 μg uL^{-1}, DNAase treated (Promega) and used as template for first strand cDNA synthesis (Invitrogen).

QPCR Primers

From the determined cDNA sequences, specific primers for IGF-I, IGF-II and IGF-IR in both mullet and grouper were designed to amplify fragment of target genes for QPCR assays (Table 2). The primers designed for mullet ß-actin were the same primers used to amplify β-actin in grouper that was used as the housekeeping gene for this study.

QPCR Optimization

Optimization assays for QPCR were tested for each gene and reaction components for a 25 μL master mix was established. The PCR reaction was carried out in 0.1 mL strip tubes

Table 2: Primer sequence used for QPCR assay to amplify fragment of IGF-I, IGF-II and IGF-IR in mullet and grouper

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Expected size (bp)
β-actin	ccacgagaccacctacaaca	ctctggtggggcaatgat	181
Mullet			
IGF-I	agccacaccctctcaactact	aagcagcactcgtccacaat	105
IGF-II	ctgtgccaaacccgccaagt	ctccgcctgcctccgaaact	215
IGF-IR	ctccttccacccagcagtta	ttggctgaaacatgtcctga	152
Grouper			
IGF-I	ctgtgcacctgccaagacta	tgtgctgtcctacgctctgt	153
IGF-II	aaatagcgtcggcagaga	cctctgccacacctcgta	260
IGF-IR	ttagcagaacagccttacca	acatcctcatcagctcgaat	121

using Rotor Gene (Corbett). The PCR conditions were as follows: hold at 50°C for 2 min, denature at 95°C for 2 min, 40 cycles of denature, annealing and extension at 95°C for 20 sec, 62°C for 20 sec and 72°C for 15 min. Melting curve analysis was conducted with ramping rate from 72-99°C rising at 1°C at each step, with a 5 sec gap on first step and for each step afterwards. The determined calibrator for both mullet and grouper assays were the cDNA template from respective gonads taken from wild caught fish at pre spawning stage with oocyte diameter of >450 μm. Standard curves were established using the synthesized cDNA of the calibrator in serial dilution and reaction efficiency determined.

QPCR Assay

Four amplification reactions, one each for IGF-I, IGF-II, IGF-IR and β-actin mRNA expression in every sample were carried out in triplicates in different tubes at the same time. A negative reverse-transcriptase cDNA template for each sample was included to check for any genomic contamination using β-actin as primer.

Statistical Analysis

Relative expression ratios (RER) were transformed (log10) and subjected to normality and homogeneity of variance test. Homogenous data sets were subjected to analysis of variance (ANOVA) while non homogenous data sets were subjected to Kruskall Wallis Test to determine significant difference among the means. Interaction effect between genes and samples were done by factorial design ANOVA. All statistical tests were conducted using Statistical Program for Social Sciences (SPSS ver. 16, Windows).

RESULTS AND DISCUSSION

cDNA Sequences

Specific and degenerate primer pairs designed were able to amplify the expected size in the templates from gonad, liver and brain. No amplification reaction resulted in template prepared from the muscle tissues.

cDNA Sequence

IGF-I and IGF-II

The deduced amino acid sequences of IGF-I and IGF-II in grouper and mullet in comparison with siganid are shown in Fig. 1 and 2. The IGF-I and IGF-II mRNA sequence from grouper liver have been deposited immediately before our sequence results and comparison with the sequence from grouper brain in this study showed minor differences in amino acid residues as shown in the boxed letter. Single base differences were located in B, C, A and E domain (http://www.gropep.com.au). Identity sequence of IGF-I in grouper brain and liver was 97% and between grouper and mullet was 93%. The range of homology of IGF-I among grouper, mullet and siganid is 94-97%. The known coding sequence of teleost IGF-I including the signal peptide was 186 amino acid (aa) and the length of coding sequence in grouper from this study was also 186 residues. Sequence identity of IGF-II between grouper brain and liver was 97% with three aa differences, two located in the signal peptide region and one in the E region. Sequence homology between grouper, mullet and rabbitfish was 92%. The coding sequence for teleost IGF-II including the signal peptide and the E domain was 215 bp and this study had sequenced 92% in grouper brain.

IGF-IR

The partial deduced amino acid sequence of mullet, grouper and turbot for comparison is shown in Fig. 3. In teleosts, turbot has the longest deposited IGF-IR sequence at Gene

```
grouperbrain   MSSALSFQWHLCDVFKSAMCCISCSHTLSLLLCVLTLTPTATGAGPETLCGAELVDTLRF 60
grouperliver   MSSALSFQWHLCDVFKSAMCCISCSHTLSLLLCVLTLTPTATGAGPETLCGAELVDTLQF 60
mullet         MSSAKSFQWHLCDVFKSAMCCISCSHTLSLLLCVLTLTPTATGAGPETLCGAELVDTLQF 60
rabbitfish     MSSALSFQWHLCDVFKSAMCCISCSHTLSLLLCILTLTPTATGAGPETLCGAELVDTLQF 60
               **** ********************************:************************:**

grouperbrain   VCGERGFYFSKPTGYGPNARRSRGIVDECCFQSCELRRLEMYCAPAKTSKAARSVRAQRH 120
grouperliver   VCGERGFYFSKPTGYGPNVRRSRGIVDECCFQSCELRRLEMNCAPAKTSKAARSVRAQRH 120
mullet         VCGDRGFYFSKPTGYGPNARRSRGIVDECCFQSCELRRLEMYCAPAKTNKSVRSVRSQRH 120
rabbitfish     VCGERGFYFSKPTGYGPNSRRPRGIVDECCFQSCELRRLEMYCAPAKTSKAARSVRAQRH 120
               ***:************** **.****************** ******.*:.*****:***

grouperbrain   TDMPRAPKVSTAGHKVDKGTERRTAQQPDKTKNKKRPLPGHSHSSFKEVHQKNSSRGNSG 180
grouperliver   TDMPRAPKVSTAGHKVDKGTERRTAQQPDKTKNKKRPLPGHSHSSFKEVHQKNSSRGNTG 180
mullet         TDMPRTPKVSTAGHKVDKGAERRTAQQPDKTKNKKRPISGHSHSSFKEVHQKNSSRGSTG 180
rabbitfish     TDMPRTPKVSAAGQKVDKGTERRTAQQPDKTKSKKRPLSGHSHSSFKEVHQKNSSRGNTG 180
               *****:****:**:*****:***********.****:.*****************.:*

grouperbrain   GRNYRM 186
grouperliver   GRNYRM 186
mullet         GTNYRM 186
rabbitfish     GRNYRM 186
                 * ****
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Fig. 1: Partial deduced amino acid sequence of grouper brain IGF-I in comparison with other fish species (NCBI accession number for the following: grouper liver-AAS01183, mullet-AY427954, rabbitfish-AY198184). Boxed letters shows aa difference between liver and brain in grouper

```
grouperbrain   METQQRYGHHSLCHTCRRTESSRMKKVKMSSSSRALLFALALTLYVVEIASAETLCGGEL 60
grouperliver   METPQRYGHHSLCHTCRRTESSRMKVKKMSSSSRALLFALALTLYVVEIASAETLCGGEL 60
mullet         METQQRYGHHTLCHTCRRTESSRMKVKKMSSSSRALLFALALTLYVVEMASAETLCGGEL 60
rabbitfish     METQQRHGHHSLCHTCRRAESSRMKVRRMSASSRALLFALALTLYVVEIASAETLCGGEL 60
               ***  **:***:*******:******   :**:******************:************

grouperbrain   VDALQFVCEDRGFYFSRPTSRGSNRRNQNRGIVEECCFRSCDLNLLEQYCAKPAKSERDV 120
grouperliver   VDALQFVCEDRGFYFSRPTSRGSNRRNQNRGIVEECCFRSCDLNLLEQYCAKPAKSERDV 120
mullet         VDALQFVCGDRGFYFSRPTSRGNNRRTQSSGIVEECCFRSCNLHLLEQYCAKPAKSERDV 120
rabbitfish     VDALQFVCDDRGFYFSRPTSRGNSRRPQNRGIVEECCFRSCDLNLLEQYCAKPAKSERDV 120
               ******** ************..** *. ***********:*:*****************

grouperbrain   SATSLQVIPVMPALKPEVPRKPHVTVKYSKYEVWQRKAAQRLRRGVPAILRAKKFRRQAE 180
grouperliver   SATSLQVIPVMPALKPEVPRKPHVTVKYSKYEVWQRKAAQRLRRGVPAILRAKKFRRQAE 180
mullet         SATSLQVIPVMPALKQEITRKQHVTVKYSKYEVWQRKAAQRLRRGVPAILRAKKFRRQAE 180
rabbitfish     SATSLQVIPVMPAPKPEVSRKPHVTVKYSKYEVWQRKAAQRLRRGVPAILRAKKFRRQAE 180
               ************* *  *:.** ****************************************

grouperbrain   KIKAQEQAVHHRPLITLP----------------- 198
grouperliver   KIKAQEQAVFHRPLISLPSKLPPVLLATDNYVNHK 215
mullet         KIKAQEQS---------------------------- 188
rabbitfish     KIKAQEQAIFHRPLISLPSKLPPILLTTDNYVSHK 215
               *******:
```

Fig. 2: Partial deduced amino acid sequence of grouper brain IGF-II in comparison with other fish species (NCBI Accession number for the following: grouper liver-AAS58520, mullet-AY427955, rabbitfish-AY198185). Boxed letters shows aa difference between liver and brain in grouper

Bank and this was used to provide a good comparison with the sequence results obtained from this study. Sequence corresponding to 711 amino acids of IGF-IR in mullet was isolated and it corresponded to 50% of the coding region in turbot IGF-IR. In grouper, sequences corresponding to 594 aa were isolated and they corresponded to 42% of the coding region in turbot. Homology between mullet and grouper was 82%, between mullet and turbot was 72% and between grouper and turbot was 75%. The transmembrane domain in turbot spans from aa positions 946 to 964 and the tyrosine kinase domain spanned aa positions 1007 to 1134 (Elies *et al*., 1996). Alignment of grouper and mullet sequences with the identified transmembrane domain in turbot showed 45% homology across the three species and alignment with the tyrosine domain showed 87% homology.

```
grouperigf-ira  ----------------------PFTVYRIDIHACNRQVQRCSAAEFVFSRTKPAEKADDI 38
grouperigf-1rb  ------------------------------------------------------------
mulletigf-ir    ADR--------------------KFDFMRIDIHACNRQVQRCSAAEFVFSRTKPAEKSDDI 162
turbotigf-ir    ADREFEFMEQAVSERVQIFDLQPFTVYRIDIHACNRQVQRCSAAEFVFSRTKPAEKADDI 840

grouperigf-ira  P---------------------GPVTWEGHEDWVFLRWPEPPHPN----------------- 62
grouperigf-1rb  ------------------------------------------------------------
mulletigf-ir    PGKVTWVFSRTKPAEKSDDIPGKVTWEGHEDWVFLRWPEPRHPNGLEGHEDWVFLRWPEP 222
turbotigf-ir    P---------------------GQVTWEGHEDWVFLRWPEPPHPN----------------- 864

grouperigf-ira  ----GLILMYEIKFKLAAETEKHECVSGQMYHTQRGVRLSNLSPGNYSVRVRATSLAGNG 118
grouperigf-1rb  ------------------------------------------------------------
mulletigf-ir    RHPNGLILMYEIKFKLATETEKHECVSGQTYQAQRDVRLSNLSPGNYSVRVRATSLAGNG 282
turbotigf-ir    ----RLVLMYEIKFKLAAETEKHECVSGQTYQNQRGVRLSNLSPGHYSVRVRATSLAGNG 920

grouperigf-ira  SWTHALDLYVAERYENVLYAMIFVPIVIILVICLLVSMLVVLSRKRNSDRLGNGVLYASV 178
grouperigf-1rb  ------------------------------------------------------------
mulletigf-ir    SWTQSS--------------------AIIVLICCLAVMLVFFNRKRNSDRLGNGVLYASV 322
turbotigf-ir    SWTNAVDLYVAERYENVLYAMIFIPIAIILFICLLVTMLVVLNKKRNSDRLGNGVLYASV 980

grouperigf-ira  NPEYFSAAEMYVPDEWEVAREKITLSRELGQGSFGMVY---EGLAKGVVKITSEFAAACK 235
grouperigf-1rb  ----------------------------------------AKGVVKDEPETRVAIK 16
mulletigf-ir    NPEYFSAAEMYVPDEWEVAREKITLSRELGQGSFGMVYAREEGVAKGVVKDEPEMRVAIQ 382
turbotigf-ir    NPEYFSAAEMYVPDEWEVAREKIALSRELGQGSFGMVY---EGLAKGVVKDEPETRVAIK 1037
                                                  ******  .*  .*:

grouperigf-ira  TVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTLVIMELMTRGDLKSYLRS 295
grouperigf-1rb  TVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTLVIMELMTRGDLKSYLRS 76
mulletigf-ir    TVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTLVIMELMTRGDLKSHLRS 442
turbotigf-ir    TVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTLVIMELMTRGDLKSYLRS 1097
                ************************************************************:***

grouperigf-ira  --------------------QMAGEIADGMSYLNANKFVHRDLAARNCMVAEDFTVKIGD 335
grouperigf-1rb  LRPKEQQWSSLSLPPLKKMLQMAGQIADGMAYLNANKFVHRDLAARNCMVAEDFTVKIGD 136
mulletigf-ir    LR--KENSTTQVLPPLKKMIQMAGEIADGMAYLNANKFVHRDLAARNCMVAEDFTVKIGD 500
turbotigf-ir    LRPKEQQWSSLSLPPLRKMLQMAGQIADGMAYLNANKFVHRDLAARNCMVADDFTVKIGD 1157
                        ****:*****:********************:********

grouperigf-ira  FGMTRDIYETDYYRKGGKGLLPVRWMSPESLKNGVFTTMSDVRSFGVVLWEIATLAEQPY 395
grouperigf-1rb  FGMTRDIYETDYYRKGGKGLLPVRWMSPESLKDGVFTTTSDVWSFGVVLWEIATLAEQPY 196
mulletigf-ir    FGMTRDIYETDYYRKGGKGLLPVRWMSPESLKDGVFTTMSDVWSFGVVLWEIATLAEQPY 560
turbotigf-ir    FGMTRDIYETDYYRKGGKGLLPVRWMSPESLKDGVFTTHSDVWSFGVVLWEISTLAEQPY 1217
                ********************************:***** *** *********:********

grouperigf-ira  QGMSNEQVLRFVMEGGLLDKPDNCPDMLFELMRMCWQYNPKMRPSFLEIISSIKDELDPP 455
grouperigf-1rb  QGLSNEQVLRFVMEGGLLEKPQNCPDMLFELMRMCWQYNPKMRPSFVEIISSLKDELEPA 256
mulletigf-ir    QGMSNEQVLRFVMEGGLLDKPDNCPDMLFELMRMCWQYNPKMRPSFLEIISSIKDDLDPP 620
turbotigf-ir    QGLSNEQVVRFVMEGGLLEKPQNCPDMLFELMRMCWQFNPKMRPAFVEIISSLKDELEPS 1277
                **:*****:*********:**:***************:******:*:*****:**:*:*.

grouperigf-ira  FREMSFFYSADNKPPD----------------------------TITEE-----NKPPDT 482
grouperigf-1rb  FREVSFFYSADNKPPD----------------------------TITEE-----NKPPDT 283
mulletigf-ir    FREMSFFYSEENKPPDTEELDMEVEN-MENIPLDPVSTRQPCSAVLSPSGCAGGVLPPST 679
turbotigf-ir    FKDSSFFYSADNKPVDDPQVHQDKMDSVDDVPLDPPSSTQPQQSPVPQQ-----TPPPPS 1332
                *:: ***** :*** *                                  :. .       ** :

grouperigf-ira  EELD--------------MEVENMENPSSGCTG-GTPPP---------------------- 506
grouperigf-1rb  EELD--------------MEVENMENPSSGCTG-GTPPP---------------------- 307
mulletigf-ir    QQLSPMQGPSTPVLLGP-MSPSPPGHVASALASPGQTLDKHSGHVSANGPVVVVLRPNFD 738
turbotigf-ir    SEAPPAPSLSPSSPSSPCTSTAAMDKQASGASGNGLSGPSHAAGSGLGTSAGVAMCPSLE 1392
                .:                    .     : :*. :. * .

grouperigf-ira  --------------------------
grouperigf-1rb  --------------------------
mulletigf-ir    DMQPYAHMNGIT-------------- 750
turbotigf-ir    ELPPYAHMNGGRKNERAMPLLQSSAC 1418
```

Fig. 3: Partial deduced amino acid sequence of the mullet and grouper IGF-IR (NCBI Accession nos AY772256, AY772254, AY772255) in comparison with turbot (AJ224993). Underlined letters were identified transmembrane domain and letters in dashed lined were the tyrosine kinase domain. ClustalW results obtained from EBI website

Quantitative PCR (QPCR)

Primers for IGF-I and IGF-II were designed from the D and E domains which are absent in insulin ensuring that only the target sequences were amplified. The QPCR primers to amplify IGF-IR were designed in the region towards the 3' end of the transcript as this has been established to have high variability compared with other gene receptors that also exhibit a tyrosine kinase domain.

Table 3: Volume of reaction components in QPCR assay per triplicate to amplify target gene fragments in the eggs of experimental animals

Components	β-actin	IGF-I	IGF-II	IGF-IR
Mullet				
Rnase free water	6.5	6.5	5.5	6.5
Platinum SybrGeen QPCR supermix UDG*	12.5	12.5	12.5	12.5
MgCl (50 mM)	-	-	1.0	-
Forward primer (10 μmole)	0.5	0.5	0.5	0.5
Reverse primer (10 μmole)	0.5	0.5	0.5	0.5
Template	5.0	5.0	5.0	5.0
Grouper				
RNAse free water	6.5	7.58	7.58	6.5
Platinum SybrGeen QPCR supermix UDG *	12.5	10.42	10.42	12.5
Forward primer (10 μmole)	0.5	1.0	1.0	0.5
Reverse primer (10 μmole)	0.5	1.0	1.0	0.5
Template	5	5.0	5.0	5.0

*Invitrogen

Optimization assays showed that for each gene, different ratios of the reaction components were required to eliminate formation of non-specific products during amplifications (Table 3). Computed reaction efficiencies of the standard curve of calibrator for mullet IGF-I, IGF-II and IGF-IR were 2.18, 1.98 and 1.94, respectively and the reference gene was 2.08 with correlation coefficient ranging from 0.91 to 0.99. In grouper, the computed PCR efficiency for the reference gene was 1.94 while values for IGF-I, IGF-II and IGF-IR were 2.13, 1.94 and 1.95, respectively and correlation coefficient range of 0.91 to 0.98. The mean Ct values were taken at a set thresh hold line of 0.0524 for all samples analyzed. Due to the different reaction efficiencies, Pfaffl (2001) method was used to calculate for the relative expression ratio (RER) and the final data were expressed as a ratio of the target gene normalized to the reference genes in the samples relative to the calibrator.

β-actin was present and expressed at similar levels in each of the samples analyzed and was valid as the housekeeping gene for this assay. The target genes were detected in all samples analyzed and were differentially expressed (Fig. 4, 5).

Grouper

Expression of IGF-I was 2.5 fold higher in late embryo than in hatched larvae and 13 fold higher in non-viable eggs compared to that in hatched larvae ($p<0.05$). The IGF-II was also 3 fold higher in late embryo and non-viable eggs than in hatched larvae ($p<0.05$). Compared to hatched larvae, mean RER of IGF-IR is 2 fold higher in late embryo and 19 fold higher in non-viable eggs. IGF-IR is higher than IGF-I (1.6-2.7 fold) in all samples examined ($p<0.05$). Highest mean expression of all three genes was found in non-viable eggs while lowest expression was in hatched larvae (Fig. 4).

Mullet

In mullet, due to limited samples (2 hatchings) the data was not subjected to statistical tests. Mean values however showed that IGF-II RER was also highest compared to IGF-I (1-3 fold higher) and IGF-IR (6-50 fold higher). The IGF-I was higher in hatch larvae than in late embryo (2 fold) or non-viable eggs (4 fold). The IGF-II expression was similar between late embryo and hatch larvae but lower in non-viable eggs (2 fold) while IGF-IR was highest in non-viable eggs compared to late embryo (48 fold) and hatched larvae (6 fold). The IGF-IR was lower than in IGF-IR in late embryo (13 fold) and hatch larvae (3 fold) but higher in non-viable eggs (6 fold), however these are considered as preliminary results only (Fig. 5).

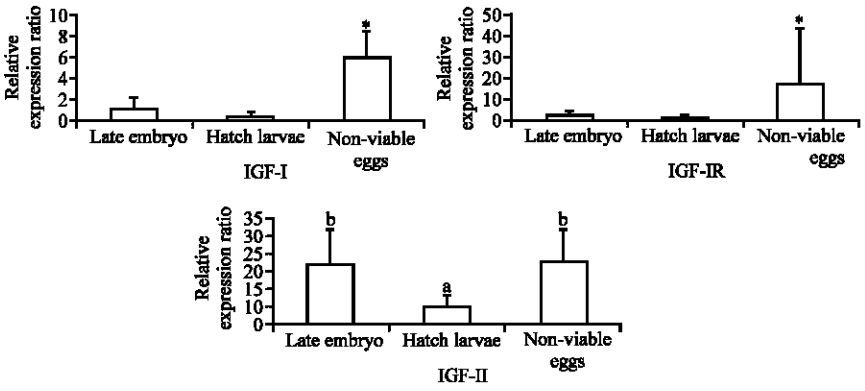

Fig. 4: Relative expression ratio of IGF-I, IGF-II and IGF-IR in grouper at late embryo, non-viable eggs and hatched larvae Error bars indicate mean SEM (n = 7). Asterisks (Kruskall Wallis test) and different letters (ANOVA) indicate significant difference at $p<0.05$

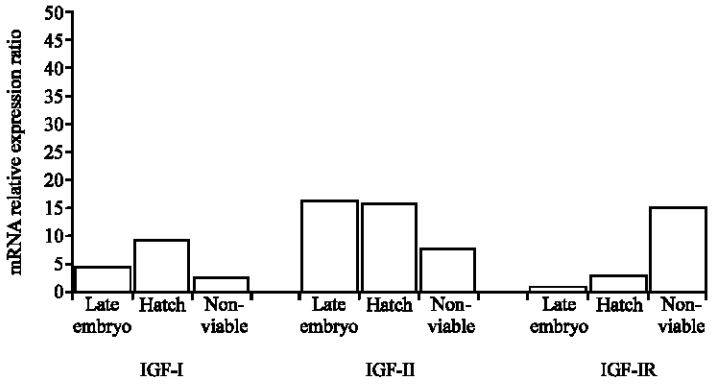

Fig. 5: Mean relative expression ratio of IGF-I, IGF-II and IGF-IR in mullet. (n = 2) at late embryo, non-viable eggs and hatched larvae

In marine teleosts with pelagic spawning, sunken eggs are most often presumed to be unfertilized and detection of high levels of the growth factor genes suggests they were maternally inherited (Greene and Chen, 1997, 1999; Perrot *et al*., 2000). The identification of a strong relative expression level of IGF-II expression compared to IGF-I and IGF-IR transcripts in the samples analyzed supported the concept that IGF-II represents the fetal growth factor (Humbel, 1990) and makes it a potential marker in further studies during embryonic development in fish. From an economic point of view, it is important to effectively evaluate the quality of hatchery production as early as possible to avoid wasting resources on what may turn out to be poor quality eggs (Planas and Cunha, 1999).

A high sequence homology between the putative coding regions of the IGFs exists across fish species and the degenerate primers designed according to the regions of high homology were successful in isolating IGF-I, IGF-II and IGF-IR in both grouper and mullet.

A single form of both IGF-I and IGF-II was cloned from grouper and mullet while several studies on teleosts reported multiple forms. Five forms were reported for IGF-I in goldfish

(Kermouni *et al*., 1998), four in rainbow trout (Greene and Chen, 1997) and tilapia (Schmid *et al*., 1999) and two in gilthead seabream (Perrot *et al*., 2000). Only one form of IGF-II was reported in daddy sculpin (Loffing-Cueni *et al*., 1999), rainbow trout (Greene and Chen, 1997), rabbitfish (Ayson *et al*., 2002) and chum salmon (Palamarchuk *et al*., 2002) while three forms have been isolated in tilapia (Schmid *et al*., 1999). The occurrence of multiple forms of IGFs in different species was reported to be either due to alternative RNA splicing of sequence domains (Duguay *et al*., 1992) or as products of different distinct genes (Greene and Chen, 1999; Nakao *et al*., 2002). The different forms that exhibited temporal expression patterns made distinct functional contributions to growth regulation and development (Maures *et al*., 2002). Comparison of IGF-I and IGF-II sequence in grouper brain from this study and the sequence available from GenBank (NCBI Accession No. AAS01183 and AAS58520) showed a sequence identity of 97% due to minor amino acid changes. These differences could be attributed to different tissue source, variations in locality or simply PCR sequencing error. It could also be likely due to different forms. However, the different forms of IGF-I in goldfish ovary showed 28 aa mismatches in the coding sequence between them (Kermouni *et al*., 1998) while IGF-I isoforms in rainbow trout showed different size of cDNA bands on the agarose gel.

The alignment of IGF-II sequences revealed that this hormone is highly conserved among vertebrate animals with the overall sequence identity of 84% between rainbow trout and human (Duan, 1997). The IGF-II transcripts have been detected at all stages of human pre-implantation development including unfertilized oocytes and embryo samples (Lighten *et al*., 1997). The same results have been found in teleosts (Greene and Chen, 1997; Palamarchuk *et al*., 2002).

The IGF-IR has a heterotetrameric structure and a tyrosine kinase domain in the cytoplasmic portion of the beta subunit and has considerable structural and functional similarity to the insulin receptor. It encodes one of the longest 5' untranslated regions (UTR) among the eukaryotic genes (LeRoith *et al*., 1995). Its presence in teleosts was detected in the ovarian follicular cells at all stages of gonadal development (Maestro *et al*., 1997, 1999; Perrot *et al*., 2000), fertilized eggs, throughout the embryonic development and hatched larvae (Maestro *et al*., 1997; Elies *et al*., 1999; Greene and Chen, 1999; Mendez *et al*., 2001; Maures *et al*., 2002). Only one form of IGF-IR fragment was isolated in mullet consistent with the findings of one distinct type of IGF-IR in turbot, trout (Elies *et al*., 1996) and barramundi (Drakenberg *et al*., 1997). In grouper as in salmon (Chan *et al*., 1997), rainbow trout (Greene and Chen, 1999), zebrafish (Maures *et al*., 2002), Japanese flounder (Nakao *et al*., 2002) and gilthead seabream (Perrot *et al*., 2000), two IGF-IR forms were isolated. It still remains to be determined if this pattern reflects genuine differences between species or wether a second IGF-IR is yet to be identified in the species that so far revealed only one form.

In tilapia, that has asynchronous ovarian development, expression pattern of IGF-I in gonads was higher than IGF-II (Schmid *et al*., 1999). A reverse pattern was found in gilthead seabream that has hermaphroditic gonad where lower levels of IGF-I mRNA compared to IGF-II was found in the developing gonads but no comparison was made in testis (Perrot *et al*., 2000). The IGF-II was found to be significantly high in females exhibiting follicular maturational competence (FMC) than those exhibiting medium or low FMC (Bobe *et al*., 2003a, b). Except for the findings by Ayson *et al*. (2002) that did not detect IGF-I during embryonic development in rabbitfish, transcripts of IGF-I, IGF-II and IGF-IR was detected in unfertilized eggs, throughout the embryonic stage and hatched larvae and showed variations in levels of expression pattern in zebrafish and rainbow trout (Greene and Chen, 1999; Ayaso *et al*., 2002; Maures *et al*., 2002). Generally, IGF-I

expression level was lower than IGF-II during embryonic development (Greene and Chen, 1999; Ayson *et al*., 2002) however a study by Maures *et al*. (2002) showed relatively unchanged levels of both IGF-I and II in all embryonic stages examined. The IGF-IR transcripts has shown differential expression patterns in the developing egg and showed a decreasing transcript levels from embryogenesis towards adulthood (Elies *et al*., 1999; Maures *et al*., 2002). It was noted that strong hybridization signals of IGF-IR was found in several fast growing areas of the embryo such as the fin buds (Maures *et al*., 2002).

CONCLUSION

The isolation and the determination of the nucleotide sequence can be used as molecular tools for studying the growth factors involved during embryogenesis in marine fish and can be explored as molecular markers of egg quality. The samples were chosen to serve as preliminary comparison to determine wether the three genes are differentially expressed and be detected with QPCR assay compared to previous published reports. The samples were considered as critical sampling points during hatchery phase in aquaculture. Late embryo where presence of heartbeat can be detected microscopically is the stage where percent fertilization of the spawning batch is most often measured while the newly hatch larvae (~1-2 h after hatching) is the stage when the percent hatching is determined. High percentage values of the two physical parameters indicate good quality spawning batch especially in large-scale hatcheries. The non-viable eggs were assayed to determine and establish wether these growth factors were expressed in a poor quality eggs. Examination of the IGF-II expression levels in a larger number of samples exhibiting different development and hatching performance during the early stages of development could determine if indeed IGF-II expression levels provides a reliable criterion in distinguishing between poor and good quality eggs.

ACKNOWLEDGMENT

The authors would like to acknowledge the assistance of Hazra Thaggard and Dr. Mike Rimmer in the sample collection. This study was part of the John Allwright Fellowship of the Australian Centre for International Agricultural Research with the project collaboration of SEAFDEC/AQD granted to JG (2002-2004). A. Elizur, A. Anderson and N. Richardson provided concept for the work, J. Nocillado helped design primers for amplification of the target genes and assisted in the sample and tissue collection and J. Gonzaga performed gene cloning, mRNA expression assay and statistical analysis.

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