FLINDERS UNIVERSITY
ADELAIDE • AUSTRALIA

Queensland University of Technology

Cooperative Research Centre for
Tissue Growth and Repair

FISHERIES RESEARCH & DEVELOPMENT CORPORATION

Development of immunoassays to measure markers of growth and stress in farmed fish

Compiled by : Dr. Anthony Dyer
Phone: 08 8201 5248
Fax: 08 8201 3015
Email: Anthony.Dyer@flinders.edu.au

Project number: 97/316

Principal investigators: Dr. Zee Upton (July 1997-Feb 2000), Dr. John Carragher (March 2000-June 2001).

Development of immunoassays to measure markers of growth and stress in farmed fish

Compiled by : Dr. Anthony Dyer
Phone: +61 8 8201 5248
Fax: +61 8 8201 3015
Email: Anthony.Dyer@flinders.edu.au
CRC for Tissue Growth and Repair
School of Biological Sciences, Flinders University
GPO Box 2100, Adelaide, SA 5001

Principal investigators:
Dr. Zee Upton
Phone: +61 7 3864 2342
Fax: +61 7 3864 1534
E-mail: z.upton@qut.edu.au
School of Life Sciences, Queensland University of Technology, GPO Box 2434, Brisbane, QLD 4001

Dr. John Carragher
Phone: +61 8 8201 3041
Fax: +61 8 8201 3015
Email: John.Carragher@flinders.edu.au
CRC for Tissue Growth and Repair
School of Biological Sciences, Flinders University
GPO Box 2100, Adelaide, SA 5001

ISBN 0-7258-1111-0

11 September 2001

Table of Contents

NON TECHNICAL SUMMARY

97/316 Development of immunoassays to measure markers of growth and stress in farmed fish

PRINCIPAL INVESTIGATOR: Dr John Carragher
ADDRESS: CRC for Tissue Growth and Repair
School of Biological Sciences,
Flinders University
GPO Box 2100
Adelaide SA 5001
Telephone: 08 8201 3041 Fax: 08 8201 3015

OBJECTIVES:

1. To produce reagents and develop assays for measuring plasma IGF concentrations in commercially significant fish species.
2. To use these immunoassays to measure plasma IGF concentrations in fish used in studies examining changes in growth, development, health, stress or nutritional status.
3. To determine if there is any correlation between these measurements indicating that plasma IGF measurements can be used as a diagnostic marker of growth performance or response to stress in farmed fish.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED

This project has successfully developed an assay for measuring blood levels of fish IGF-I in several species of aquacultured finfish (including Atlantic salmon, rainbow trout, barramundi, southern bluefin tuna and silver perch). One of our commercial partners, GroPep Limited, is now using the reagents and assay we jointly developed to market a kit for measuring fish IGF-I. The kit was launched in Sydney at World Aquaculture '99 and has been used by fish researchers worldwide. Sales of the kit and other reagents generated from this research have been particularly successful and sold around the world.

We have demonstrated that this IGF-I assay is a useful indicator of growth performance and could potentially be used to evaluate aquaculture diets or monitor the stress of aquacultured finfish populations. We believe that the benefits we have generated for the scientific community in aiding the analysis of IGF's will have a cascade effect to many aquaculturists as the role of IGF's in finfish growth is further understood. We also believe that with continued effort we may be able to use IGF-I levels as a tool to improve diets and husbandry practices in a range of aquaculture species.

The important role of hormones in regulating growth and development has been extensively documented in mammals. One particular group of hormones is the insulin-like growth factors (IGFs). There are two forms of IGFs, IGF-I and IGF-II, both of which exert not only insulin-like effects, but also, growth promoting effects. While there is growing evidence suggesting the importance of IGF-I in growth and development in fish, interpretation of these studies is hampered by the use of research reagents based on mammals. At the time this project commenced we had preliminary evidence suggesting that the assays used to measure the circulating levels of IGF-I in fish have been underestimating concentrations by up to 200 fold. The lack of sensitivity of the assays may not have permitted detection of subtle, yet important changes in IGF-I levels in fish. Thus the use of fish research reagents may be essential for correctly interpreting results of the biological actions of IGF-I in fish. If IGF-I plays as an important role in fish as has been found in mammals, the benefits of homologous (ie. same species) IGF research reagents to the aquaculture industry could be substantial. Moreover, the development of an assay for fish IGF-I as a reliable marker of growth performance, or response to environmental stress, could in turn impact significantly on fish farm management and nutrition.

As a result of the FRDC funding provided for this project we have achieved these objectives, and are developing and marketing a range of fish IGF's, and antibodies raised against them, to fish researchers around the world. A kit to measure fish IGF-I concentrations was launched at World Aquaculture'99 in Sydney, and has been used successfully by researchers in Australia and overseas. The assay we have developed measures IGF-I in several species of aquacultured finfish (including Atlantic salmon, rainbow trout, barramundi, southern bluefin tuna, silver perch, red sea-bream (snapper) and tilapia. We have measured IGF-I in fish sampled from several nutritional and stress related trials and we have identified very encouraging correlations of IGF-I levels with growth rate. In barramundi and Atlantic salmon fed different diets, IGF-I levels correlated well to growth rate, suggesting that taking a blood sample and measuring IGF-I may be a method to shorten the time required for lengthy growth trials. In addition we demonstrated that IGF-I levels correlated to handling and confinement stress in black bream, handling stress in silver perch, towing stress in southern bluefin tuna, and 'stunting' in Atlantic salmon. The time frame of some of these changes in IGF-I levels is yet to be fully established, however, we hope that evaluation of diets and handling practices may be achieved in just a few days by taking a blood sample and measuring IGF-I concentration.

KEYWORDS: **Barramundi** - *Lates calcarifer,* **Bluefin tuna** - *Thunnus maccoyii***, Atlantic salmon** - *Salmo salar,* **Silver perch** - *Bidyanus bidyanus,* **Black bream** - *Acanthopagrus butcherii***, insulin-like growth factor, IGF-I, growth hormone, aquaculture, immunoassay**

1. Background

1.1 Insulin-like growth factors in mammals

The important role of endocrine hormones in regulating growth and development, as well as in eliciting responses to external factors such as light or environmental stress have been extensively documented in mammals. Our Cooperative Research Centre (CRC) has focused its investigations into tissue growth and repair on the involvement of one particular group of endocrine factors, the insulin-like growth factors (IGFs). There are two forms of IGFs, IGF-I and IGF-II, both of which exert not only insulin-like metabolic effects, but also growth-promoting effects. No separate role has been assigned to IGF-II, though recent research suggests it may play an important part in fetal development. More is known, however, about the function of IGF-I and its role in promoting and maintaining postnatal growth in mammals.

IGF-I acts on muscle, cartilage, and in fact on most cell types to increase growth by stimulating nutrient uptake, protein synthesis and eventually cell division. IGF-I also inhibits protein breakdown. Thus, IGF-I is an anabolic factor, increasing the 'food conversion efficiency' of the cell as well as promoting cell division. IGF-I also stimulates other cellular processes such as calcium influx and transferrin receptor regulation. In addition, IGF-I is involved in the regulation of endocrine hormones particularly growth hormone. IGF-I mediates many of the growth promoting actions of this hormone, as well as regulating its production through a negative feedback mechanism. Clearly, IGF-I has many important biological actions that participate at different levels in growth and development. Indeed, it has been suggested that IGF-I is the ultimate endocrine link in the chain of hormones regulating cell growth.

The biological actions of IGF-I are reflected *in vivo* in blood IGF-I concentrations during development and in a number of growth disorders. IGF-I levels are increased during childhood and during the pubertal growth

spurt, generally higher in females than in males, elevated in acromegaly and following growth hormone administration, low in catabolic states such as starvation and diabetes, and are also low in dwarfs.

The CRC for Tissue Growth and Repair has successfully correlated the changing levels of IGF-I with growth and development in pigs and in conjunction with the Pig Research and Development Corporation and Bunge Meat Industries, Australia's largest pig producer, has developed a diagnostic assay to aid pig industry breeding programs. A patent on this diagnostic technology has been granted and analytical assay services to pig breeders are offered by PrimeGro in Australia, and internationally by the patent owners.

1.2 Insulin-like growth factors in fish and aims of the project

Compared to the situation in mammals, little is known about IGFs in non-mammalian species. This is primarily due to the fact that only small amounts of the purified growth factors have been isolated, thus hampering investigations into their biological effects. Indeed, we were the first to purify IGF-I and IGF-II from a nonmammalian species, namely the chicken. We have continued to pursue our investigations into the structure and function of non-mammalian IGFs using DNA technologies to produce large amounts of recombinant chicken IGF-I and IGF-II. More recently we also produced recombinant salmon and barramundi IGF-I, thus we were in a unique position to closely examine the roles of IGF-I in fish. In particular, we wanted to raise antibodies against these proteins and develop immunoassays to measure IGF-I concentrations in fish plasma. Specifically, we wished to investigate whether IGF-I concentrations in fish plasma could be correlated with growth performance or with responses to nutritional or environmental stress in farmed fish. We also proposed to determine whether these measurements could be used as a diagnostic marker to influence decisions made with respect to fish farm management. In essence we aimed to develop a diagnostic IGF-I immunoassay which could be used to help maximise outputs from fisheries in a similar manner to the assay we have developed for the pig industry.

There is sufficient evidence in the literature to indicate important roles for IGF-I in fish growth and development including:

- human IGF-I stimulates sulfate uptake in salmon and trout cartilage (Takagi and Bjomsson, 1996; Moriyama *et al*., 1993);
- bovine IGF-I induces increased gill Na(+)-K(+)-ATPase activity in brown trout and coho salmon (Madsen *et al*., 1995; Madsen and Bern, 1993);
- plasma IGF-I immunoreactivity increases following oral and intraperitoneal administration of growth hormone to rainbow trout (Moriyama, 1995);
- human IGF-I has been demonstrated to directly control growth hormone secretion by pituitary calls cultured from rainbow trout (Blaise *et al*., 1995);
- IGF-I gene expression is increased in osmoregulatory organs during seawater adaptation of salmonids (Sakamoto and Hirano, 1993);
- IGF-I receptors have been demonstrated in heart and skeletal muscle of carp, coho salmon and brown trout (Gutierrez *et al*., 1995; Parrizas *et al*, 1995);
- ration size and protein intake affect plasma IGF-I immunoreactivity in the gilthead sea bream (Perez-Sanchez *et al*., 1995);
- seasonal increases in IGF-I mRNA levels precede the rapid growth period coinciding with the springtime increases in temperature and photoperiod observed in juvenile coho salmon (Duan *et al*., 1995);
- diminished IGF-I mRNA expression is observed in growth-retarded salmon resulting from premature transfer to seawater (Duan *et al*., 1995);
- fasting or injection of streptozotocin leads to a significant decline in systemic IGF-I in salmon (Moriyama *et al*., 1994);
- growth of coho salmon is stimulated by exogenous administration of bovine IGF-I (McCormick *et al*., 1992).

Thus we believed it was highly likely that plasma IGF-I levels would correlate with growth, development, nutritional or health status in fish. Moreover, we were uniquely placed to investigate this hypothesis, as we were the only research group in the world to have successfully produced milligram quantities of recombinant fish IGF-I. Accordingly, we had the advantage of adequate supply of protein to use not only in the production of antibodies, but also as fish IGF-I reference standards in immunoassays. Furthermore, the CRC has considerable experience in developing strategically directed research to successful commercial outcomes. We also wanted to produce recombinant fish IGF-II and to develop a specific immunoassay for use in investigating the role of this growth factor in fish. This may ultimately prove to be particularly valuable since essentially nothing is known about IGF-II in fish.

2. Need

While there is growing evidence suggesting the importance of IGF-I in growth and development in fish, interpretation of these studies is hampered by the use of heterologous research reagents. Indeed, we have preliminary evidence suggesting that the heterologous immunoassays used to investigate the circulating levels of IGF-I in fish have been underestimating concentrations by up to 200 fold. Moreover the lack of sensitivity of the assays may not have permitting detection of subtle, yet important changes in IGF-I levels in fish resulting from growth, developmental, nutritional or environmental factors. In addition, we have recently ascertained that recombinant human IGF-I is cleared from the circulation of juvenile barramundi almost twice as quickly as recombinant barramundi IGF-I, providing the first *in vivo* evidence that there are functional differences between mammalian and fish IGFs. Thus the use of homologous research reagents may be essential for correctly interpreting results from not only *in vivo*, but also *in vitro* investigations into the biological actions of IGF-I in fish.

If IGF-I plays as an important role in fish as has been found in mammals, the benefits of homologous IGF research reagents to the aquaculture

industry could be substantial. The development of a diagnostic assay for IGF-I as reliable, reproducible marker of growth performance, or response to environmental stress could in turn impact significantly on fish farm management. Clearly, the over-riding aim of every commercial fish farm is to obtain maximum output of a quality product in the most cost efficient manner. Hence the development of an endocrinological assay as an indicator of growth potential or as an early marker of changing health status, may significantly aid achieving this goal. Likewise, the production of recombinant fish IGF-II and the development of a specific fish IGF-II immunoassay may prove to be valuable tools for defining the role of IGF-II in fish growth and development.

3. Objectives

1. To produce reagents and develop immunoassays for measuring plasma IGF concentrations in commercially significant fish species.
2. To use these immunoassays to measure plasma IGF concentrations in fish used in studies examining changes in growth, development, health, stress or nutritional status.
3. To determine if there is any correlation between these measurements indicating that plasma IGF measurements can be used as a diagnostic marker of growth performance or response to stress in farmed fish.

4. Methods

4.1 Production of reagents and development of Immunoassays

4.1.1 Production of recombinant IGFs

Salmon and tuna IGF-II DNA sequences were generated from polymerase chain reaction products using primers designed from existing sequence information. Tuna IGF-I and barramundi, salmon and tuna IGF-II DNA sequences were subcloned into GroPep Limited's patented gene fusion expression vector and the constructs then used to transform *E. coli* cells. Following fermentation of the bacteria the expressed recombinant fish proteins were isolated and processed to homogeneity using downstream processing methods routinely used in the CRC and in GroPep Limited.

4.1.1.1 In vitro characterisation

The functional properties of recombinant barramundi IGF-I were compared to human IGF-I (hIGF-I; GroPep Limited, Adelaide, SA, Australia) in a number of *in vitro* characterisation systems. Stimulation of protein synthesis in L6 rat myoblasts was performed according to Ballard *et al*., (1986). Assessment of binding to type-1 IGF receptors in cultured cells was performed according to Ross *et al*., (1989), while binding to the type-2 IGF receptors in ovine placental membranes was measured as described by Read *et al*., (1986). This same publication describes the method used for comparing the cross-reactivities of barramundi and human IGF-I in a radioimmunoassay using a polyclonal antibody prepared against hIGF-I (GroPep Limited, Thebarton, SA, Australia). Radiolabels used in the *in vitro* characterisation assays and in the *in vivo* clearance study described below were prepared using the chloramine-T procedure (Francis *et al*., 1988).

4.1.1.2 In vitro characterisation

Juvenile barramundi (5-6 months old) were used to assess the effects of IGF-I on glucose and leucine metabolism. Twenty-four hours before

experimental use, food was withdrawn from barramundi stocks and no further food given for the duration of the experiment. IGF-I was administered as a single injection. At the end of the experimental period, barramundi were anaesthetised by immersion in MS222. Liver or flank muscle (100-200 mg) were then excised and stored at –70°C until analysis. The effect of growth factor treatment on glycogen synthesis was determined as described by Ottolenghi *et al*., (1984). The effect of growth factor treatment on protein synthesis was determined using methods based on those described by Freifelder (1982).

4.1.2 Production of antibodies against fish IGFs and development of immunoassays

Polyclonal antibodies against recombinant barramundi and salmon IGF-I and IGF-II were raised in rabbits following standard procedures. A homologous radioimmunoassay (RIA) was developed using the fish IGFs as both radiolabelled ligands and reference standards. IGFs present in fish plasma samples were separated from RIA-interfering IGF binding proteins using acid-ethanol extraction. Parallelism of serial dilutions of the processed plasma samples with the fish IGF reference standards as well as intra- and inter-assay coefficients of variation for each RIA, were determined.

4.2 Correlation of plasma IGF-I levels with growth, nutritional status and stress of farmed fish.

4.2.1 Finfish nutrition and IGF-I

4.2.1.1 Diet and ration size in barramundi

In the first nutritional trial juvenile barramundi (mean weight 12 g ± 0.3 SE, n = 17-19 per diet) were maintained on a commercial pellet diet at rations of 2%, 4% and 10% body weight per day for 7 weeks. Fish were netted and weighed at weekly intervals. At the termination of the trial barramundi were killed by pithing and blood samples obtained. In the second trial, barramundi (mean weight 80g ± 1.1 SE, n = 15 fish per diet)

were fed once daily to satiation on one of 12 different diets. These diets contained crude protein levels between 45.8 and 69.8% and crude fat levels between 13.6 and 22.1%. Barramundi were maintained on these diets for 6 weeks. At the end of the experiment 4 fish from each group were blood sampled and IGF-I levels determined.

4.2.1.2 Diet variation in Atlantic salmon

Six diets were fed to Atlantic salmon for a six-week growth trial. Three experimental southern bluefin tuna feeds were used, a commercial northern bluefin tuna feed (Com.NBT), a commercial salmon feed (Pivot Aquaculture) and Western Australian pilchards. Atlantic salmon were obtained from stock held at the School of Aquaculture, University of Tasmania, and had an initial mean weight (± standard deviation) of 161.4 g (± 26.0 g). Salmon were randomly allocated to one of twelve tanks until a total of 18 fish were in each tank. Salmon were fed twice daily at 2% tank biomass. At the conclusion of the trial, 4 fish from each tank were anaesthetised and blood samples obtained for IGF-I analysis.

4.2.1.3 Diet variation in southern bluefin tuna

This study was carried out on the Southern Bluefin Tuna Aquaculture sub-programme experimental research farm in Port Lincoln over the 1998 farming season. The fish came into the farm at approximately 16kg and were divided between 7 small (12m) pontoons and one large (32m) pontoon. The fish were fed twice daily to satiation with one of 5 diets that were being trialled as part of the SBT feed development project. The diets included 3 formulations of moist pellet that differed in nutrient composition, one extruded dry pellet and a bait-fish fed group. After 16 weeks (ie. in mid-July) fish were harvested, weighed and 10 fish randomly chosen from each pontoon were blood sampled.

4.2.2 Finfish stress and IGF-I

4.2.2.1 Handling and confinement stress in black beam (Acanthopagrus butcherii)

Juvenile black bream (mean weight 50.2 g ± 2.2 SE, n = 65) were captured by hook and line on the Glenelg River, Victoria, Australia. Bream were killed immediately by pithing (unstressed controls), confined in 20L buckets (10, 30 min, 1, 3, 24 hr) or transported back to the laboratory (7 days confinement). At the completion of each of these confinement times a blood sample was obtained from the caudal vessels. Each fish was only sampled once.

4.2.2.2 Handling and isolation stress in silver perch (Bidyanus bidyanus)

Hatchery reared silver perch (Mean weight 48.7g, n = 48) were transferred from 200L cages containing 12 fish (after acclimatisation for 1 month) to isolation in a 200L cage. Blood samples were obtained immediately (unstressed control) or after increasing periods of isolation (1, 3, 6, 12, 24, 48 and 72 hours).

4.2.2.3 Stress effects of long distance towing in southern bluefin tuna (Thunnus macoyii)

Tuna were captured on a commercial fishing vessel in waters offshore from Pt Lincoln, South Australia. For the control 'wild' samples tuna were brought on-board by poling with hook and line, killed immediately with a spike through the brain and spinal chord and a blood sample taken. 'Towed' tuna were captured using a purse seine net and towed for 3 weeks into harbour at 1-2 knots before sampling. Tuna that had recovered for 3 weeks after towing, and tuna that had been kept for 3 months to harvest time were also sampled. Caged tuna were poled from floating pontoons using a baited hook and line for sampling. Blood was collected in a beaker after severing the lateral vessels and a sub-sample drawn into a heparinised syringe.

4.2.2.4 Investigation of 'stunting' in Atlantic salmon (Salmo salar)

Blood samples were obtained from Atlantic salmon smolts which were classified into 3 groups (n = 30) termed normal, intermediates and stunts. This subjective classification was based on the appearance of salmon. Normal fish were full bodied with silver colouration, stunts were thin and darkly coloured (Duan *et al*., 1995). Intermediates were small, poorly conditioned fish that were not displaying the complete stunt morphology but were expected to become stunts. Fish were also sampled from two different pens. The first pen was termed 'pre-bath' which referred to a brief freshwater bath to remove parasites (gill amoebae). The second pen sampled was of fish that had been bathed two days previously. These salmon were termed 'post bath'.

5. Results/Discussion

5.1 Progress against milestones

#	Projected completion date	Milestone	Completed	Refer to section
1	31/12/97	Antibodies to barramundi and salmon IGF-I produced	4	5.3.2
2	31/21/97	DNA sequences for tuna IGF-I and IGF-II obtained	4	Appendix 3
3	30/6/98	Fish immunoassays using antibodies against salmon and barramundi IGF-I developed	4	5.3.3
4	30/6/98	Study on the effect of acute administration of barramundi IGF-I to juvenile barramundi completed	4	5.3.1.3
5	31/12/98	Nutritional studies in barramundi examining the effects of serum IGF-I levels completed	4	5.4.1.1
6	31/12/98	Study examining hepatic IGF-I gene expression and circulating IGF-I levels in snapper during development and following nutritional stress completed	4	Data not shown
7	30/6/99	Statistical analysis of results from milestones 4 and 5 completed and reports and publications written as required	4	5.2.1 & 5.2.2
8	30/6/99	Statistical analysis of results from milestone 6 completed and reports and publications written as required	4	5.2.1 & 5.2.2
9	30/6/99	Recombinant tuna IGF-I, as well as barramundi, salmon and tuna IGF-II produced	4	5.3.1
10	31/12/99	Antibodies to fish IGF-II produced	40%	5.3.2
11	30/6/00	Fish IGF-II immunoassays developed	10%	5.3.2 & 5.3.3
12	30/6/00	Correlation of IGF-I from fish experimental trials to measure growth and stress in farmed fish	4	5.4
13	30/6/00	Statistical analysis of results from milestone 12 completed and reports and publications written as required	4	5.2
14	30/6/00	Analysis of all results completed and final report with recommendations prepared	4	

5.2 Extension of results

5.2.1 Publications

Degger, B., Upton, Z., Soole, K., Collet, C. and Richardson, N. (2000). Comparison of recombinant barramundi and human insulin-like growth factor-I in juvenile barramundi (*Lates calcarifer*): *in vivo* metabolic effects, association with circulating IGF-binding proteins and tissue localisation. General and Comparative Endocrinology **117**: 395-403.

Degger, B.G., Richardson, N., Collet, C., Ballard, F.J. and Upton, Z. (1999). *In vitro* characterization and in vivo clearance of recombinant barramundi (*Lates calcarifer*) IGF-I. Aquaculture **177**: 153-160.

Degger, B., Richardson, N., Collet, C. & Upton, Z. (2000) Production, *in vitro* characterization, *in vivo* clearance and tissue localisation of recombinant barramundi IGF-II. General and Comparative Endocrinology (Submitted).

Dyer, A.R., Soole, K.L. and Matsumoto, G.I. (2000). Quantitative TaqMan PCR without a real time thermal cycler: An assay for fish insulin-like growth factor-I mRNA. Marine Biotechnology (In Press).

Dyer, A.R., Carragher, J.F., Stone, D., Thomas, P.M., Higgs, N., Quinn, K. and Upton, Z. (2000). Effects of acute and chronic stress on plasma IGF-I concentrations in several finfish species. Aquaculture (Submitted).

Pozios K.C., Ding, J., Degger, B., Upton, Z. and Duan, C. (2000) Insulin-like growth factors stimulate zebrafish embryonic cell proliferation through activation of the mitogen-activated protein kinase and phosphatidylinositol 3-kinase signalling pathways. American Journal of Physiology (submitted).

Upton, Z., Yandell, C. A., Degger, B.G., Chan, S.J., Moriyama, S., Francis, G.L. and Ballard, F.J. (1998) Review: Evolution of insulin-like growth factor-I action: *in vitro* characterization of vertebrate IGF-I proteins. Comparative Biochemistry and Physiology **121**: 35-41.

Upton, Z., Quinn, K., Lucas, A., Degger, B., Ward, H., Richardson, N., Soole, K. and Carragher, J. (2000). Development of a homologous fish IGF-I radioimmunoassay and its use for measuring true concentrations of IGF-I in serum from a range of fish species. Aquaculture (In preparation)

5.2.2 Conference presentations

Degger, B. (1999). *In vitro* and *in vivo* characterization of recombinant barramundi insulin-like growth factor-I. World Aquaculture (1999). Sydney, Australia. Oral Presentation.

Degger BG, Richardson N, Collet C, Ballard FJ, and Upton Z. (1997). *In vitro* characterization and *in vivo* clearance of recombinant barramundi (*Lates calcarifer*) IGF-I. Third International Symposium on Research for Aquaculture: Fundamental and Applied Aspects. Barcelona, Spain.

Dyer, A.R. and Soole, K. (1998). The development of a biochemical indicator to monitor the health status and growth rate of the snapper (*Pagrus auratus*). Australian Marine Sciences Association National Conference. Adelaide, South Australia. Oral Presentation.

Dyer, A.R. and Soole, K. (1999). The development of a biochemical indicator to monitor the health status and growth rate of the snapper (*Pagrus auratus*). World Aquaculture '99. Sydney, Australia. Oral Presentation.

Dyer, A.R., Soole, K. and Matsumoto, G.I. (1997). Levels of IGF-I mRNA as a biochemical indicator of the health status and growth rate of the snapper. *Pagrus auratus* (Bloch and Schneider) World Aquaculture '97. Seattle, Washington. Poster Presentation.

Lucas, A., Finch, J., Dyer, A.R., Soole, K.L. and Carragher, J. (2000). Investigation of growth peptides in abalone. The 7th Annual Abalone Aquaculture Workshop. Dunedin, New Zealand.

Upton, Z. (2000). Evolution of insulin-like growth factors. invited by Dr. Cunming Duan to present a seminar to the 'Growth Factor Club'; University of Michigan, Ann Arbor, MI, USA.

Upton Z, Degger BG, Richardson, N, Collet C, and Carragher JC (2000). Evolution of IGFBP structure and function: a review of recent studies investigating non-mammalian IGFBPs. To be presented in a plenary session at IGFBP2000, the 5th International Workshop on IGFBPs at Terrigal, NSW, Australia in October.

Upton, Z, Quinn, K., Lucas, A., Degger, B., Ward, H., Richardson, N., Soole, K. and Carragher, J. (1998). Insulin-like growth factors in aquaculture. 14th Australasian Biotechnology Conference, Adelaide, SA, Australia .

Upton, Z., Yandell, C.A., Degger, B.G., Chan, S.J., Moriyama, S., Francis, G.L. and Ballard, F.J. Evolution of IGF function: *in vitro* characterization of vertebrate IGFs. Insulin and IGF superfamily peptides and related molecules: structure, function and evolution, Satellite Symposium, Barcelona, Spain.

5.2.3 Reports to industry

Dyer, A.R. (1999). Investigation of insulin-like growth factor-I (IGF-I) in Atlantic salmon: A possible link of IGF-I with the ability of salmon to thrive after transfer to seawater. Submitted to TASSAL as a summary of results obtained from collaborative research.

Dyer, A.R. (2000). Insulin-like growth factor-I (IGF-I) as a predictor of Atlantic salmon smoltification: Determining the time to transfer Atlantic salmon to sea-cages using the salinity challenge test and IGF-I levels.

5.3 Production of reagents and development of Immunoassays

5.3.1 Fish IGFs

Fish insulin-like growth factors produced included:

- *Barramundi, *Snapper, *Salmon/Trout, *Tilapia, *Tuna IGF-I
- Barramundi, Salmon, Tuna IGF-II (DNA sequence information is supplied in Appendix 3: Tuna and salmon IGF-II DNA sequences)

(*Indicates now commercially available as research reagents from GroPep Limited)

5.3.1.1 Demonstration of recombinant IGF-I purity

A 10 L fermentation of *E.coli* transformed with the construct for expression of the barramundi IGF-I (bIGF-I) protein yielded 20 mg of recombinant bIGF-I. The growth factor recovered from the final step of the downstream processing eluted as a single peak on an analytical HPLC and migrated as a single band with the expected size of approximately 7.3 kDa, demonstrating the purity of the recombinant protein (Figure 1).

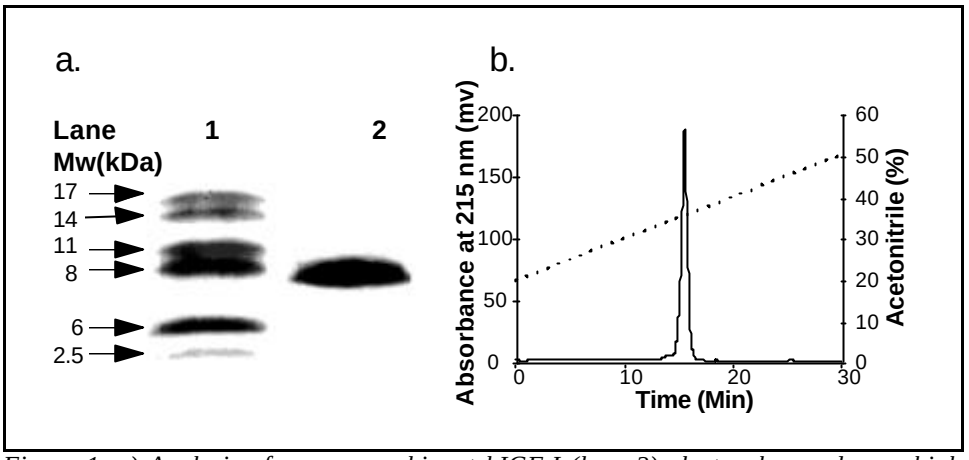


Figure 1. a) Analysis of pure recombinant bIGF-I (lane 2) electrophoresed on a high-density SDS-polyacrylamide gel under reducing conditions. Arrows indicate the size of the molecular mass markers (lane 1). b) Analysis of pure recombinant bIGF-I on a microbore C4 reverse-phase HPLC column. Elution of protein, monitored as absorbance at 215 nm, was achieved by a gradient of acetonitrile (dotted line) in 0.1% (v/v) trifluoroacetic acid.

5.3.1.2 In vitro characterisation of recombinant IGF-I

The functional properties of recombinant bIGF-I were compared with human IGF-I (hIGF-I) in a number of *in vitro* characterisation systems. The abilities of the two peptides to compete for binding of radiolabelled IGF-I to IGF receptors were similar. The two proteins competed for binding to the IGF-I receptors on rat (Figure 2 (a)) and salmon cells (b) to a similar extent.

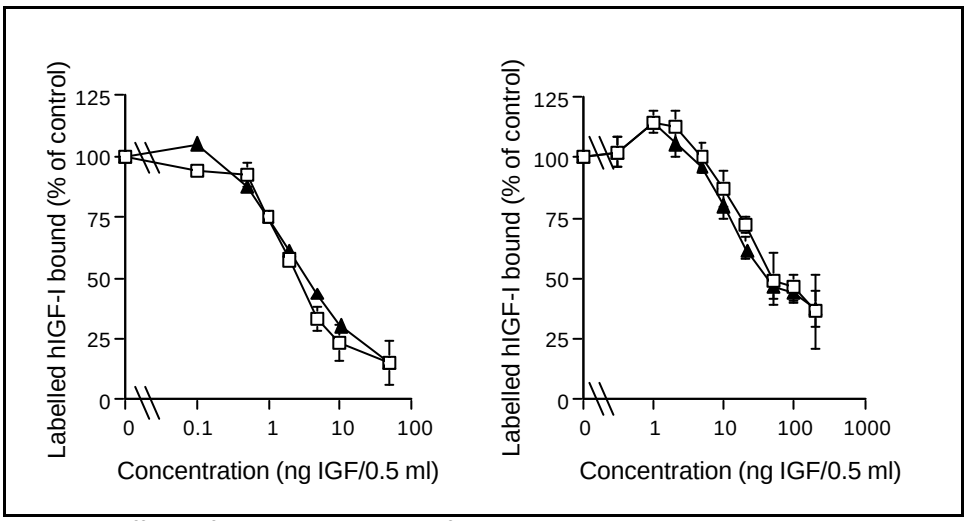


Figure 2. Effects of IGF-Is on binding of labelled hIGF-I to a) L6 rat myoblasts and b) CHSE-214 salmon embryo fibroblasts. The proteins tested were hIGF-I (n) and bIGF-I (ð). Values are the means of triplicate determinations at each peptide concentration. S.E.M. values are indicated by vertical bars where they are larger than the symbols.

In vitro characterisation revealed that there were striking similarities between hIGF-I and bIGF-I in their biological and receptor interactions. In addition to the data presented here *in vitro* activity has been demonstrated in other collaborative studies (Pozious *et al*., 2000). To confirm that the recombinant form of the protein retained all functional activity it was necessary to establish if bIGF-I remained active *in vivo.* Hence the effect of recombinant bIGF-I on glucose incorporation into muscle glycogen reserves and incorporation of leucine into protein in the liver were assessed in juvenile barramundi.

5.3.1.3 In vivo characterisation of recombinant IGF-I

Recombinant bIGF-I significantly increased the incorporation of glucose into flank muscle glycogen, when compared with controls (Figure 3). No significant increase was observed with bIGF-I treatment until the dose was increased to 0.3 µg/g body weight ($P = 0.0002$). Interestingly, injection with bIGF-I at a dose of 1.0 µg/g body weight was fatal to barramundi in all cases tested. Similarly, there were no significant changes in 1eucine incorporation into liver protein of barramundi treated with bIGF-I until the growth factor dose was increased to 0.3 µg/g body weight ($P = 0.012$) (Figure 3). Under these conditions, the uptake of 1eucine was 1.4- to 1.8-fold higher than that observed in controls. Administration of IGF-1 at a dose of 1.0 µg/g body weight was not attempted since the analysis of glycogen metabolism described above had demonstrated that IGF-I at this dose was fatal to barramundi.

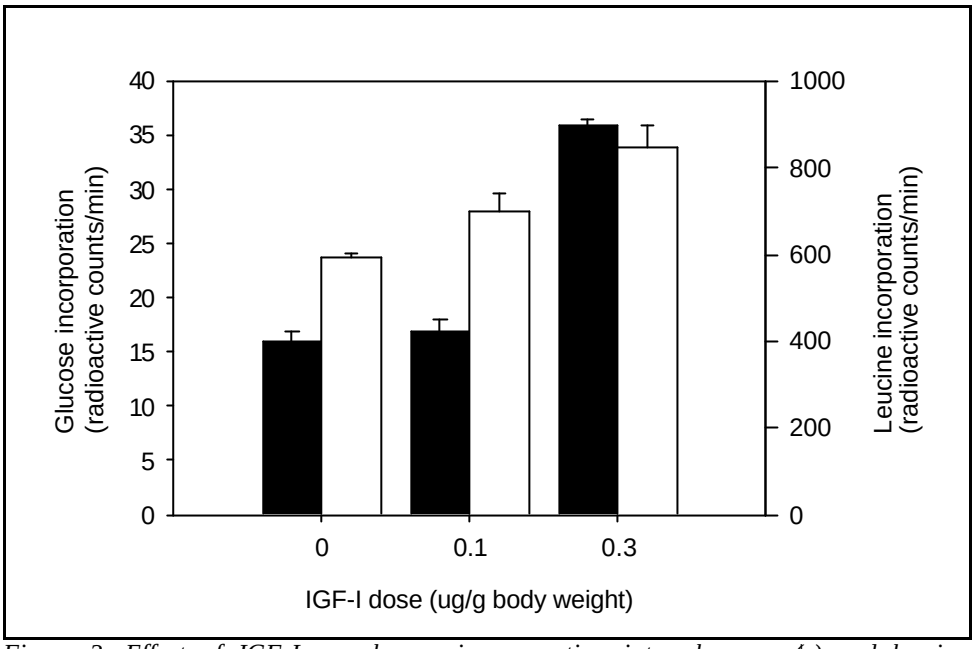


Figure 3. Effect of IGF-I on glucose incorporation into glycogen (▮) and leucine incorporation into barramundi liver protein (□). Each bar represents the mean values (± SEM) of incorporation as determined from 9 to 17 individual fish treated with 0.1 and 0.3 pg IGF-I/g bodyweight.

The *in vitro* and *in vivo* data suggested that the recombinant form of IGF-I was biologically active and was used to raise antibodies for the development of an immunoassay for fish IGF-I. Similar characterisation studies were carried out for all recombinant fish IGF's produced to confirm biological activity.

5.3.2 Antibody production

Polyclonal rabbit antibodies were raised against barramundi and salmonid IGF-I and are commercially available from GroPep Limited. At the time of writing antibodies against tuna IGF-II were still in preparation. Rabbits have been immunised, however initial titrations for anti-tuna IGF-II antibodies were low. GroPep Limited is continuing this portion of the research and will complete the antibody production in the coming months. Unfortunately, the low antibody titres have prevented the development of an immunoassay for fish IGF-II; this is the only aspect of this research program that has not achieved the established milestone.

5.3.2.1 Demonstration of rabbit anti-barramundi IGF-I antibody binding

A radioimmunoassay (RIA) was conducted to demonstrate the ability of the anti-barramundi IGF-I antibody to bind recombinant barramundi IGF-I. In this assay the % of bound radioactively-labelled-IGF-I is measured in the presence of increasing amounts of competing unlabelled recombinant bIGF-I. The antibody was shown to be sensitive, with 50% binding of radioactive IGF-I obtained in the presence of only 1mg/ml bIGF-I (Figure 4). The antibody was also shown to be specific for bIGF-I and showed little or no binding to hIGF-I, hIGF-II and salmon insulin (Figure 5).

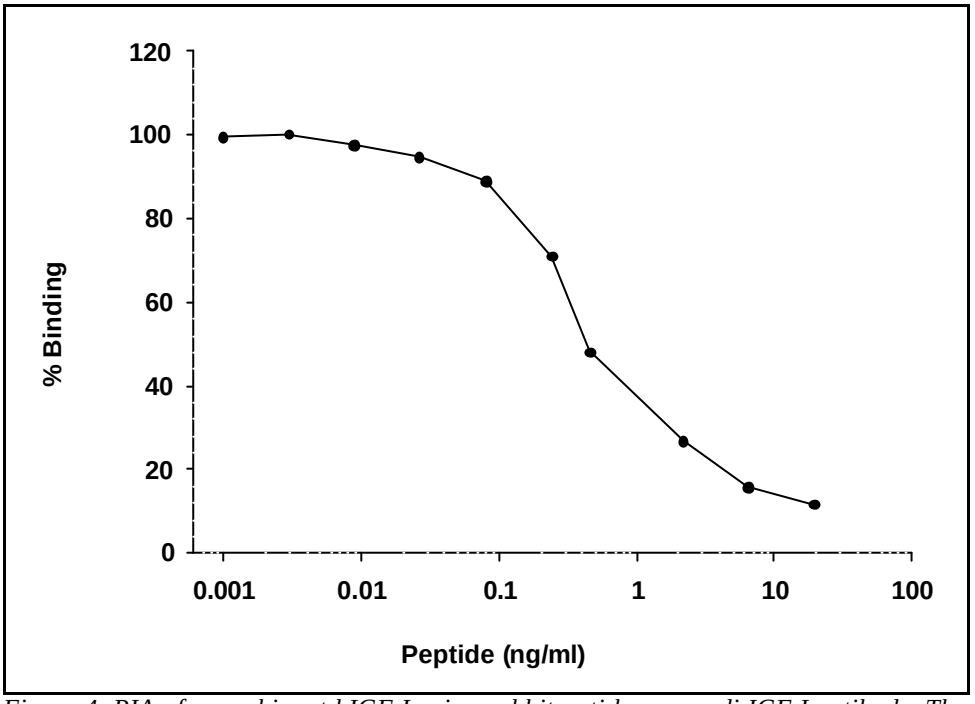


Figure 4. RIA of recombinant bIGF-I using rabbit anti-barramundi IGF-I antibody. The antibody is shown to bind recombinant bIGF-I.

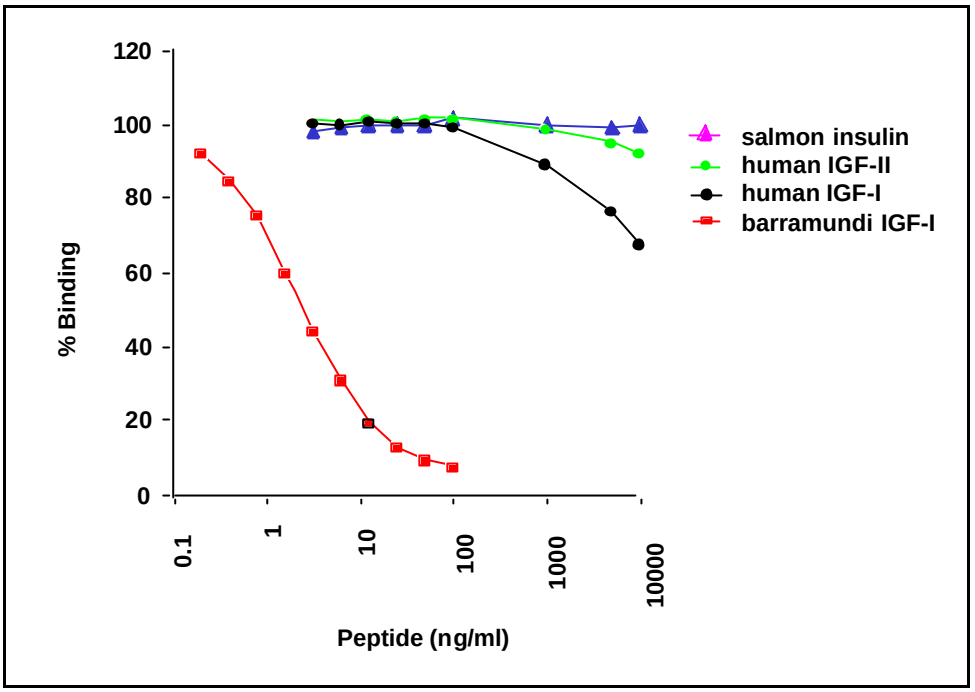


Figure 5. RIA using rabbit anti-barramundi IGF-I antibody and recombinant IGF's. The antibody is shown to be specific by showing minimal binding to human IGF-I and IGF-II or salmon insulin.

5.3.3 Immunoassays

Using the anti-barramundi IGF-I antibody a fish RIA for IGF-I was developed. Figure 6 demonstrates that this assay recognised a number of recombinant fish IGF-I's, including barramundi, tuna, tilapia, salmonid and bream suggesting that this assay was generic and probably capable of measuring IGF-I in many fish species.

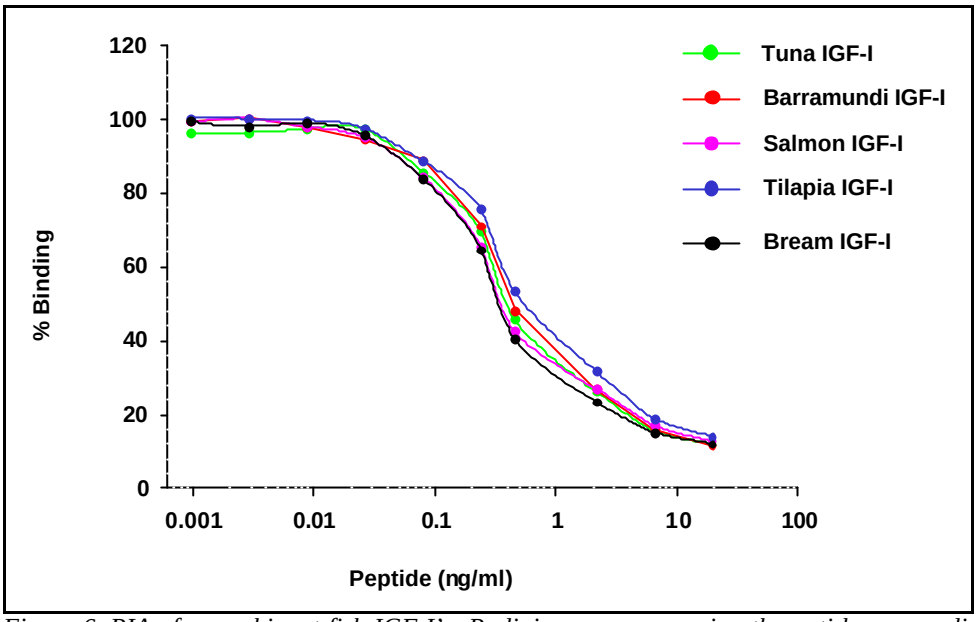


Figure 6. RIA of recombinant fish IGF-I's. Radioimmunoassay using the anti-barramundi IGF-I showed parallel binding curves for a number of fish species and suggested this assay may be capable of measuring IGF-I in a range of fish species.

To measure circulating IGF-I levels in fish plasma it was necessary to include an extraction step remove IGF binding proteins (Table 1). In the absence of binding protein extraction little or no IGF-I is detected. For example, samples of bream plasma that were not extracted resulted in a mean RIA value of 12ng/ml IGF-I, in comparison to 37 ng/ml after acid-gel chromatography and 42 ng/ml after acid ethanol extraction. Acid-gel chromatography and acid-ethanol extraction both enabled the measurement of IGF-I in variety of fish species and provided similar RIA measurements. The acid-ethanol extraction is the more simple technique and was adopted for routine sample analysis.

Species	Acid-Gel Chromatography	Acid-Ethanol Extraction
*Bream	37 ± 0.6	42 ± 2.5
Barramundi	157 ± 10	166 ± 11
Tilapia	73 ± 2.0	67 ± 3.8
Salmon	34 ± 1.0	25 ± 1.1
Tuna	88 ± 3.8	87 ± 2.3

*Table 1. Comparison of IGF-I RIA values obtained from fish plasma samples after acid-gel chromatography and acid-ethanol extraction. *Indicates an IGF-I RIA value of 12 ng/ml from bream plasma which did not have binding proteins extracted. Values are mean IGF-I (ng/ml) ± SE.*

Prior to measuring IGF-I concentration in fish plasma it was necessary to establish that extracted plasma diluted parallel to the recombinant tuna IGF-I (tIGF-I) standard used in this assay. Serial dilutions of extracted finfish plasma were demonstrated to dilute parallel to recombinant tIGF-I, suggesting that the IGF-I in plasma samples bound to the anti-barramundi IGF-I antibody at similar affinity (Figure 7). Parallel curves were obtained for all fish plasma's investigated suggesting that this RIA could be used as a generic assay for measuring finfish IGF-I.

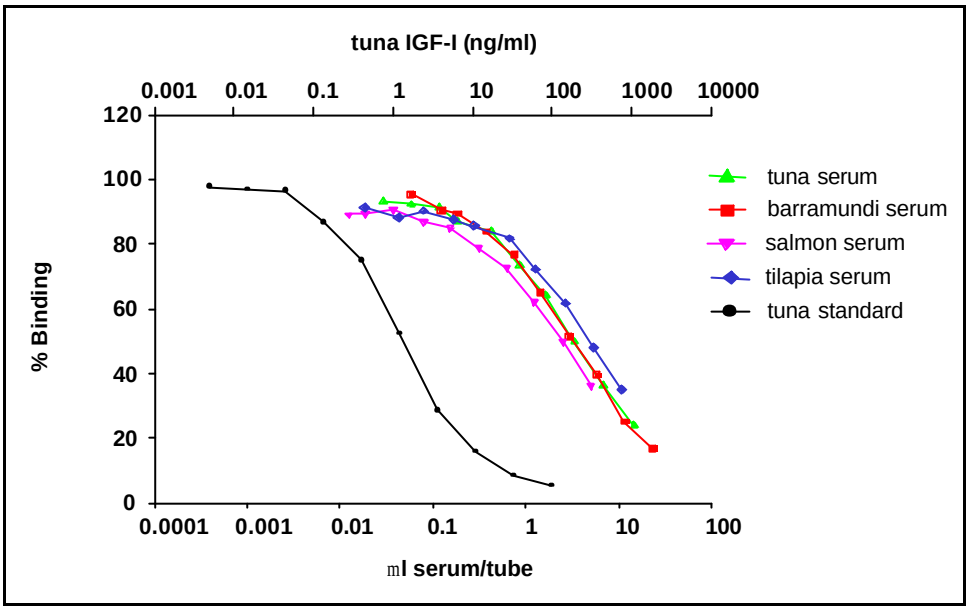


Figure 7. IGF-I RIA of various serially diluted finfish plasma's. Parallel curves for tuna, barramundi, salmon and tilapia serum with the recombinant tuna IGF-I standard demonstrate that this assay can be used to measure IGF-I in a range of finfish.

5.3.4 Discussion

In summary, we have produced 8 recombinant fish IGF's and antibodies against two of these peptides. The polyclonal antibodies raised against barramundi IGF-I proved to be capable of binding to all fish IGF-I's investigated and were used to produce a generic fish IGF-I assay. In addition to the reagents listed we have produced a kit for measuring fish IGF-I concentrations. This kit has been sold to finfish researchers throughout the world and has made a significant impact on investigating changes in fish IGF's in the scientific community.

5.4 Correlation of plasma IGF-I levels with growth, nutritional status and stress of farmed fish

5.4.1 Finfish nutrition and IGF-I

Nutritional status is a major regulator of growth. A diet lacking in energy or protein content causes a cessation of growth in juvenile animals. In fish, circulating IGF-I peptide levels have been shown to be nutritionally regulated (Moriyama *et al.*, 1994, Niu *et al.*, 1993, Perez-Sanchez *et al.*, 1995) and hepatic IGF-I mRNA and IGF-I peptide levels were shown to decrease upon fasting in the eel (Duan and Hirano, 1992), and in salmon (Duan and Plisetskaya, 1993). Thus the effect of nutritional status on growth provides an opportunity to characterise the RIA developed by the CRC for Tissue Growth and Repair and determine the ability of this technique to monitor the growth performance of commercially important aquaculture species. A number of trials investigating different nutritional regimes by external collaborators were used to provide data on the correlation of IGF-I to fish growth.

5.4.1.1 Diet and ration size in barramundi

In a nutritional trial carried out with juvenile barramundi, IGF-I levels increased in response to diet ration size and were also an indication of weight gain (Figure 8). Of particular interest was that the growth rate of the fish fed 4% and 10% body weight was very similar in the last week of the trial. This was reflected by IGF-I concentrations, which were not significantly different in these two groups. This suggested that IGF-I could be used as a recent indicator of growth performance and provided early evidence that IGF-I levels could be used to monitor the growth of fish under different nutritional regimes.

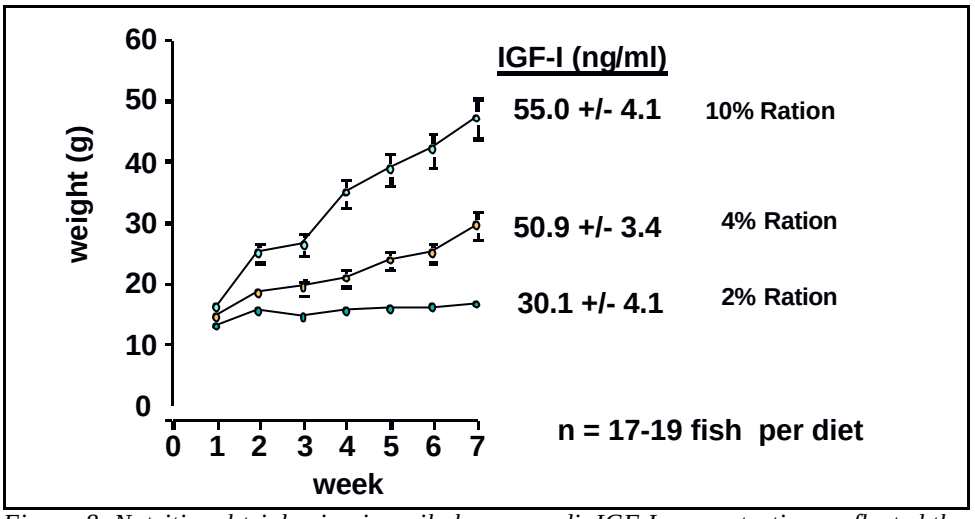


Figure 8. Nutritional trial using juvenile barramundi. IGF-I concentrations reflected the growth rates obtained with different diet rations of 2%, 4% and 10% body weight/day. Values are means ± SE.

In a second nutritional trial, juvenile barramundi IGF-I levels reflected growth rates obtained using 12 different diets (Figure 9). Barramundi fed diets containing different levels of crude protein and fat showed a strong correlation ($r^2 = 0.65$) between average daily growth and IGF-I levels. This positive correlation between growth rate and IGF-I levels provided further evidence that IGF-I may be a biochemical predictor of growth performance and a useful tool in evaluating aquaculture diets. It should be noted that although 15 fish per treatment were used in the growth trial, only 4 fish per treatment were blood sampled.

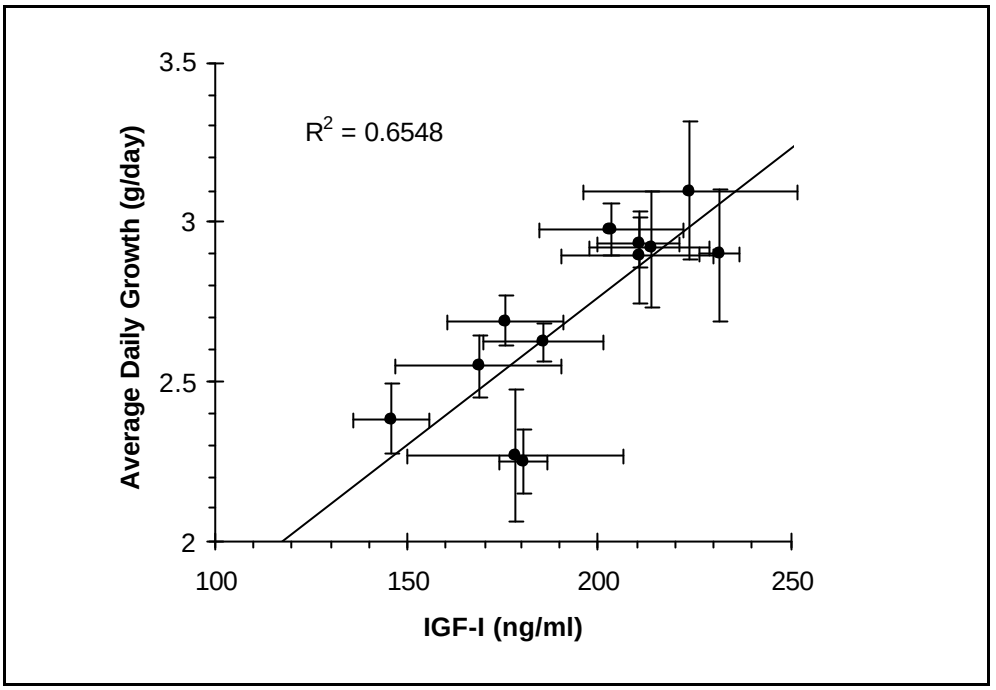


Figure 9. Nutritional trial using juvenile barramundi. Barramundi were maintained on 12 different diets containing from 45.8 to 69.8% crude protein and 13.5 to 21.6% crude fat demonstrated a strong correlation between IGF-I concentration and growth rate at the end of the 6 week trial.

5.4.1.2 Diet variation in Atlantic salmon

In a nutritional study evaluating six different diets in Atlantic salmon, mean IGF-I levels correlated to growth rates obtained using the different diets (Figure 10). However, when the IGF-I levels of individual fish are compared to growth rate the correlation is not so obvious (Figure 11). At this stage we are uncertain why the correlation to these individual fish is not clear. It must be noted however that in studies we have conducted investigating stress in finfish (Section 5.4.2) we have demonstrated that IGF-I declines in response to an external stress. It is possible that towards the end of this trial some of these fish were stressed (by handling, confinement or some other variable) which resulted in a shift in IGF-I concentration in some of these salmon. Perhaps this was enough to prevent individual correlations to growth rate while maintaining a reasonable correlation to the means.

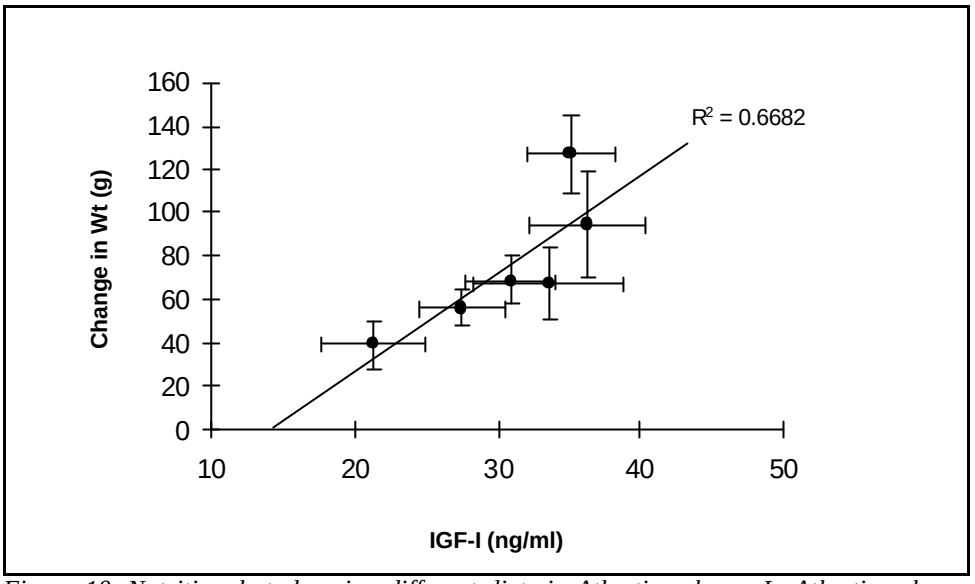


Figure 10. Nutritional study using different diets in Atlantic salmon. In Atlantic salmon maintained on 6 different diets mean IGF-I concentration was positively correlated to growth rate.

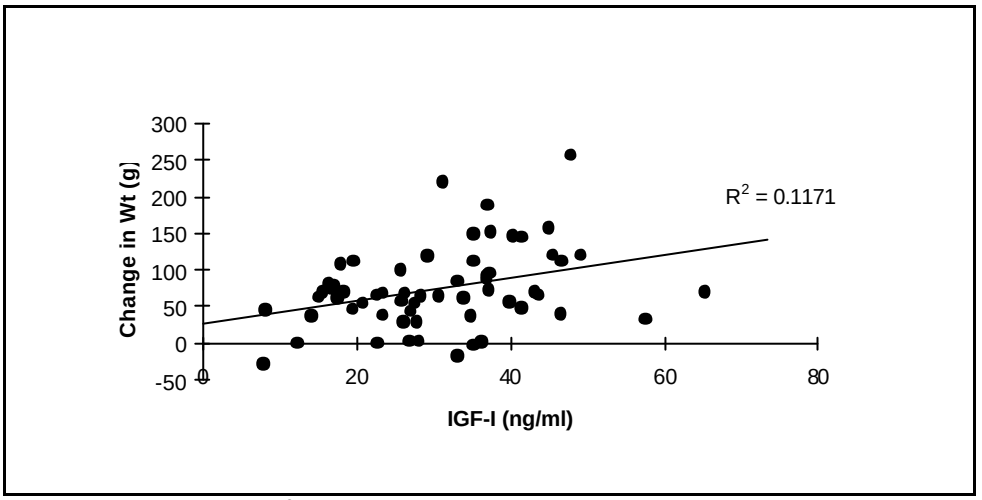


Figure 11. Correlation of individual growth rate and IGF-I levels. In Atlantic salmon maintained on 6 different diets IGF-I values for individual fish were not correlated to growth rate.

5.4.1.3 Diet variation in southern bluefin tuna

A nutritional trial was also carried out in bluefin tuna. However, there was no obvious correlation between growth rate and IGF-I level (Figure 11). This was probably due to the facts that only a single plasma sample was taken at the end of the trial and that the trial was terminated in winter when fish were not growing rapidly. IGF levels were thought to have declined by this part of the season and therefore did not accurately reflect the growth rate of the tuna during the grow-out period. This result illustrates

some of the limitations of using IGF-I to monitor growth rates as this endocrine indicator is expected to change in response to external signals over relatively short periods of time. It also demonstrated that in some cases collaborations with other industry bodies which were carrying out nutritional trials was not ideal as being able to sample fish more frequently would be more likely to provide an indication of fish growth.

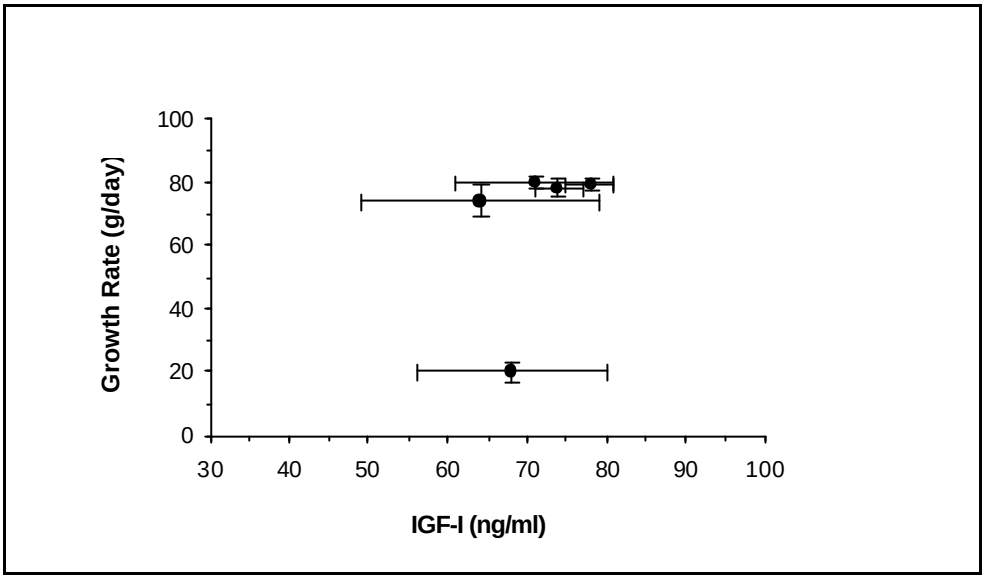


Figure 11. Nutritional trial using different diets in tuna. Bluefin tuna maintained on 5 different diets did not show a correlation between IGF-I concentration and growth rate. At the termination of this trial during winter the tuna growth rate had slowed. Since IGF-I concentration is expected to change over a relatively short time frame a single sample at the termination of this trial was not informative.

5.4.2 IGF-I and finfish stress

Stress has been shown to have profound effects on fish growth (Pickering, 1990). Each of which may have associated endocrine changes for which the magnitude and type of response can vary depending on a wide range of factors (Sumpter, 1997). For example, plasma GH concentration has been shown to increase (Cook and Peter, 1984), decrease (Pickering *et al.*, 1991; Farbridge and Leatherland, 1992) and remain unchanged (Wagner and McKeown, 1986) in response to stress in fish. These apparently contradictory results demonstrate the wide variety of responses and technical difficulties encountered when assessing stress in fish (Sumpter, 1997). Since IGF-I is known to mediate the action of GH, variable

responses in IGF-I levels may also be expected. Indeed, IGF-I has been shown to respond to stress in salmon, with levels increasing shortly after a chasing stress in Atlantic salmon parr (3 and 7 hours, McCormick, 1998). The data presented here demonstrates stress-related changes in IGF-I levels in four commercially important aquaculture species in response to handling, confinement and other husbandry practices. In addition, we investigate the possible role of IGF-I in 'stunting', a condition that results in retarded growth and mortality of salmon after transfer to seawater (Clarke and Nagahama, 1977).

5.4.2.1 Handling and confinement stress in black beam

To investigate the time frame over which IGF-I levels can change, wild black bream were captured and confined for increasing periods of time before sampling. IGF-I levels remained relatively stable for up to 1 hour after capture and confinement (Figure 12). After 3 hours confinement IGF-I concentration had begun to decline and by 24 hours was significantly decreased. After 1 week in captivity circulating IGF-I concentration appeared to be recovering to normal levels and suggested that bream were acclimatising to these conditions. These observations were reflected by cortisol concentrations, which increased with confinement time but were reduced after a week acclimatisation.

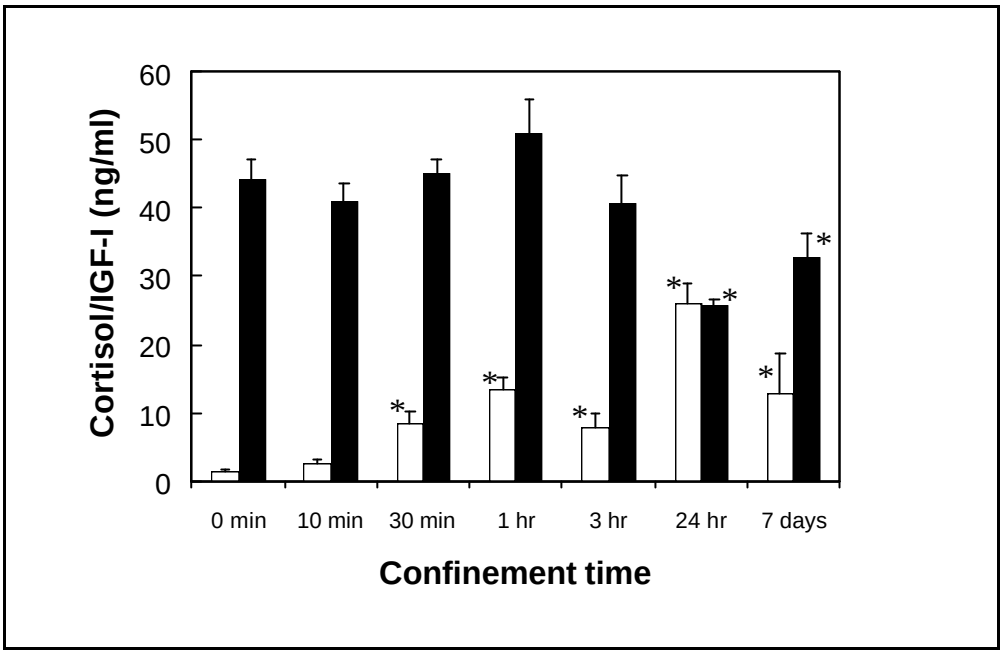


*Figure 12. The effect of handling and confinement on IGF-I (¡) and cortisol (?) in juvenile black bream. IGF-I levels decreased after 1-hour confinement and began to recover after 7 days. Cortisol levels reflect this stress response and increase with confinement time up to 24 hours before decreasing by day 7. * Indicates significant difference by ANOVA from 0 min, P < 0.05.*

5.4.2.2 Handling stress in silver perch

In a stress related trial in silver perch the effect of handling and isolation on IGF-I levels was investigated. IGF-I levels remained relatively stable in fish 1 hour after transfer and isolation (Figure 13). Approximately 6 hours after transfer, IGF-I concentration began to decline and was lowest after 12 hours. After 24 hours IGF-I levels began to recover and had returned to initial levels by day 2. These results demonstrate that after application of a stress, short-term changes (< 1 hour) in IGF-I levels do not occur, although longer-term changes were detectable.

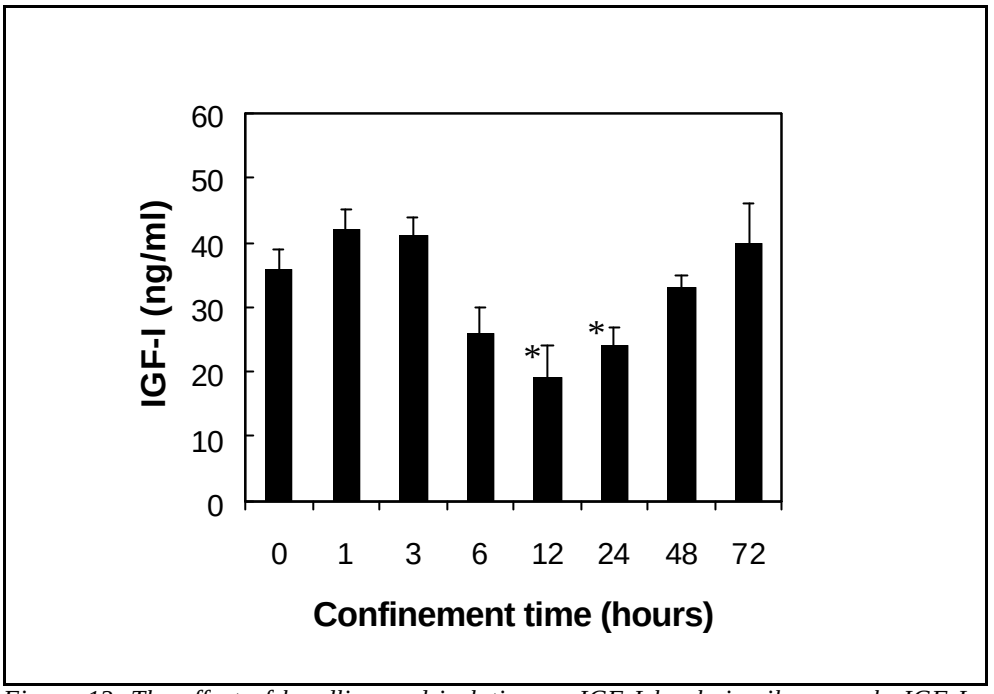


*Figure 13. The effect of handling and isolation on IGF-I levels in silver perch. IGF-I levels decreased after isolation for 3 hours and continued to decline up to 12 hours after transfer. After 24 hours in isolation IGF-I levels were increasing and had returned to starting values by 72 hours. * Indicates significant difference by ANOVA from 0 min, P < 0.05.*

5.4.2.3 Stress effects of long distance towing in southern bluefin tuna

The aquaculture of southern bluefin tuna aquaculture involves the capture of wild fish stocks, which are then on grown to increase value. After capture these fish are gradually towed to inshore waters for grow-out. The effect of this potentially stressful procedure on IGF-I levels was investigated. Tuna were caught by poling and blood sampled immediately to provide unstressed or control levels of IGF-I and cortisol in wild fish (Figure 14). In tuna towed from wild capture sites and blood sampled 24 hours after arriving in Boston Bay, Pt Lincoln, IGF-I levels had declined. After a 3-week recovery period, circulating IGF-I had returned to control (wild fish) levels. In addition, IGF-I levels appear to be inversely related to cortisol levels, which demonstrate that levels of IGF-I could possibly be used to monitor stress in tuna.

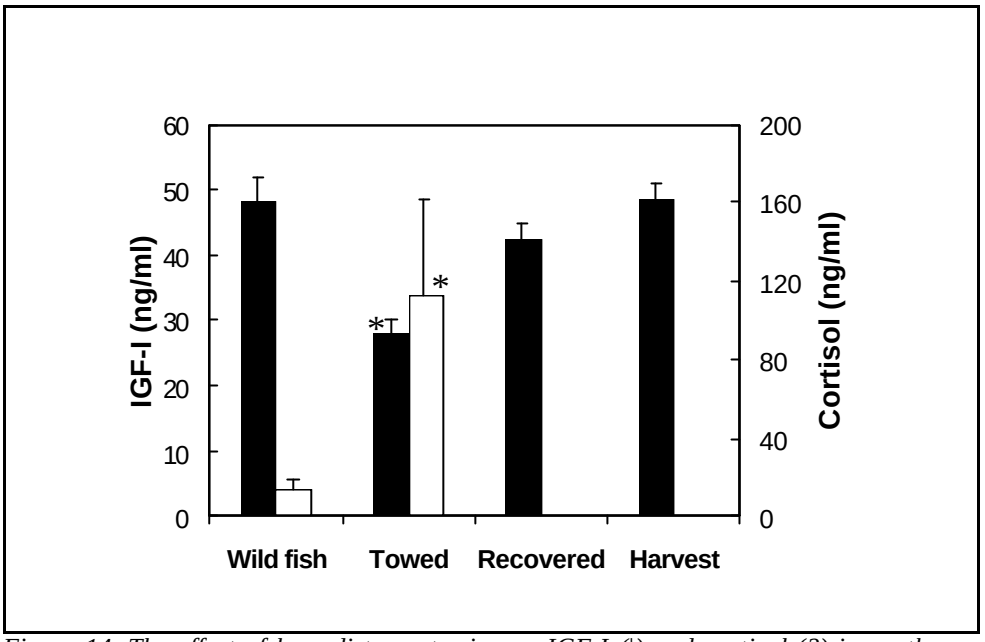


*Figure 14. The effect of long distance towing on IGF-I (¦) and cortisol (?) in southern bluefin tuna. IGF-I levels in tuna, which were towed, demonstrated a stress response characterised by an increase in cortisol and decrease in circulating IGF-I. After a 3-week recovery period IGF-I had increased and had reached control values 3 months after capture. * Indicates significant difference by ANOVA from 0 min, P < 0.05*

5.4.2.4 Investigation of 'stunting' in Atlantic salmon

The objectives of this study were to investigate a possible link between the levels of plasma IGF-I and the occurrence of 'stunting', a condition that results in the mortality of Atlantic salmon after transfer to seawater, primarily due to malnutrition. The failure of these fish to thrive in seawater is probably due stress induced by an inability to osmoregulate sufficiently under these conditions. Since IGF-I has a demonstrated role in osmoregulation and smoltification, the determination of IGF-I levels in these fish may provide a better understanding of and potential solutions to the significant number of mortalities encountered at this time.

The mean (± SE) weight, length and condition factor ($SGR = \frac{\ln W_t - \ln W_0}{t} \times 100$) of pre-bath and post bath fish are shown in Tables 2 and 3. In both sets of data the salmon that were categorised as normal were larger fish and had a higher condition index than stunting fish. There was some difficulty (and subjectivity) in categorising fish in the intermediate or stunt group and weight, length and condition measurements were not always significantly different between these groups.

	Weight (g)	Length (mm)	Condition (k)
Normal	162.5 ± 15.25^{a}	228.3 ± 6.61^{a}	1.32 ± 0.03^{a}
Intermediate	54.2 ± 2.48^{b}	182.9 ± 3.17^{b}	0.88 ± 0.02^{b}
Stunts	45.2 ± 3.94^{b}	177.2 ± 4.44^{b}	0.79 ± 0.02^{c}

Table 2. Mean weight, length and condition index of salmon sampled prior to the freshwater bath..Letters indicate significant difference (P < 0.05) by ANOVA.

	Weight (g)	Length (mm)	Condition (k)
Normal	137.6 ± 12.72^{a}	216.5 ± 5.53^{a}	1.32 ± 0.02^{a}
Intermediate	61.9 ± 3.85^{b}	190 ± 3.92^{b}	0.89 ± 0.01^{b}
Stunts	42.1 ± 1.39^{b}	172.1 ± 2.71^{c}	0.83 ± 0.02^{c}

Table 3. Mean weight, length and condition index of salmon sampled 2 days after the freshwater bath. Letters indicate significant difference (P < 0.05) by ANOVA.

Plasma IGF-I levels of pre-bath and post-bath fish are shown in Figure 15. IGF-I levels reflected salmon health status. Fish in the normal group had levels significantly higher ($P<0.05$) than those of the stunting groups. Additionally, the normal fish which had been bathed 2 days previously showed very low levels of IGF-I, probably as a stress response from the bathing procedure.

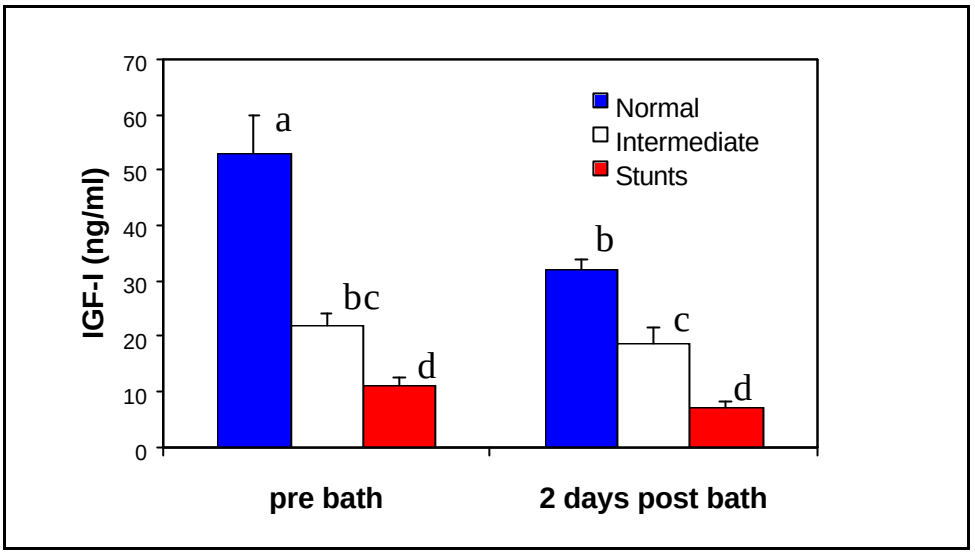


Figure 15. Plasma IGF-I levels in Atlantic salmon separated into normal, intermediate and stunt groups. Plasma IGF-I levels reflected the health status of salmon, with high growth factor levels in healthy fish and much lower in the stunt groups. After a freshwater bath to remove parasites, healthy salmon possessed lower levels of IGF-I than fish that were not bathed. Letters indicate significant difference ($P < 0.05$) by ANOVA.

For individual salmon, IGF-I concentrations correlate well to fish condition in salmon prior to the freshwater bath (Figure 16 (a)). In the absence of serial data to determine growth rate the assumption made here is that fish condition is also an indication of fish growth or performance. Thus, prior to the bath IGF-I concentration is a good indicator of fish growth. For individual salmon 2 days after the fresh water bath there is no correlation between fish condition and IGF-I levels (Figure 16 (b)). This is most likely due to the dramatic decrease in IGF-I levels after this stress and demonstrates that stresses of this type can alter the effectiveness of using IGF-I levels as an indicator of fish growth. Perhaps the inability to effectively correlate IGF-I levels to individual growth rates can be attributed to short term IGF-I changes that are stress induced.

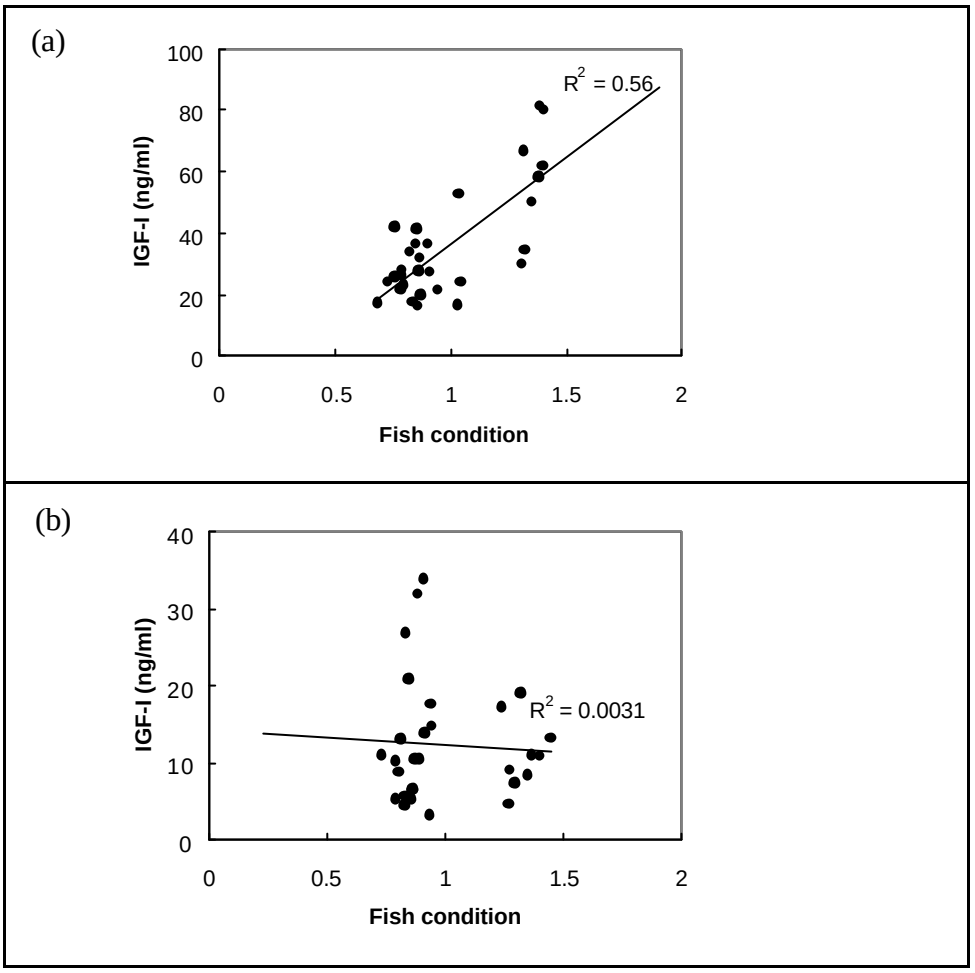


Figure 15. Correlation of IGF-I levels and fish condition in Atlantic salmon. In salmon sampled prior to a freshwater bath to remove parasites, IGF-I values correlate well to fish condition. Two days after the bath this correlation is no longer observed.

5.4.3 Discussion

5.4.3.1 IGF-I and finfish nutrition

In the nutritional studies described here IGF-I concentration was positively correlated to growth rate and provided significant evidence to suggest measuring IGF-I concentration may provide a short method for evaluating aquaculture diets. Indeed, we assessed 12 diets in barramundi and 6 diets in Atlantic salmon using IGF-I and daily growth measurements. In both trials IGF-I levels were excellent predictors of growth performance and provided a method to assess diet formulation comparable to growth rate data. These nutritional trials were conducted through collaborative efforts and provided us with the opportunity to obtain samples from fish species in growth trials that would be impossible maintain in the necessary numbers at the facilities available to this project. Although the data

obtained was invaluable, limitations were apparent. This was demonstrated in the trial using Atlantic salmon that were maintained on 6 different diets. Although mean IGF-I concentrations correlated to growth rate, individual values did not. Since we now know IGF-I concentration is affected by stress we believe this was due to a stress which may have significantly impacted on the IGF-I concentrations of some of the fish in the final days of the trial. Further limitations for analysing the link between IGF-I and nutritional status were highlighted in the nutritional trial conducted in bluefin tuna. Fish were sampled once at the conclusion of the growth period. Since IGF-I is known to fluctuate with environmental cues such as season and temperature, obtaining a single sample after an extensive grow-out period was not informative. These factors aside, the successful correlation of IGF-I concentrations to growth rate suggest there is considerable potential to use this technique as a tool to evaluate aquaculture diets. Future collaborations will be structured to allow us access to more frequent sampling and input into experimental design and sampling strategies to gain information linking the concentration of IGF-I to individual fish growth rate over short periods of time.

5.4.3.2 IGF-I and finfish stress

In some of the first studies of this kind IGF-I concentration was correlated to finfish stress. Wild black bream provided evidence regarding the time scale in which IGF-I concentrations are changed, and demonstrated that sampling within 1 hour of the initial stress is an appropriate sampling technique for accurate IGF-I determinations. Bluefin tuna showed a decrease in IGF-I in response to towing, and Atlantic salmon responded to the stress of a freshwater bath and also showed that IGF-I levels were indicative of stunting. These results demonstrated that IGF-I concentration responded to a wide variety of stressors and could be useful in monitoring stress in aquacultured finfish populations and/or as a tool for optimising husbandry practices.

6. Benefits

The biotechnology company GroPep Limited is a direct beneficiary of this project by the production and marketing of IGF products and reagents developed during this research. Fish IGF products developed from this research have been sold to researchers worldwide (a list of these reagents is provided in Appendix 4). The development of the IGF-I assay and reagents will also benefit scientists investigating the endocrinology of growth and development of fish, as this project has produced homologous research reagents to support their studies. In fact a publication has already appeared in which fish researchers have used our IGF-I kit to investigate growth in coho (*Oncorhynchus kisutch*) and chinook (*Oncorhynchus tshawytscha*) salmon (Shimizu *et al*., 2000). Our studies demonstrate that this assay is a useful predictor of finfish growth and stress. The development of a diagnostic IGF immunoassay that correlates with growth performance or health status will directly benefit the aquaculture industry by enabling the scientific monitoring of management practices for farmed fish. To date, the IGF-I assay developed here has been applicable to all finfish species examined and thus may provide benefits to almost all finfish aquaculturists. As has been illustrated with the immunoassay that our CRC has developed for the pig industry, it is feasible that the technology developed could be exploited worldwide, with Australians, the owners of the Intellectual property, being the beneficiaries.

7. Further Development

We successfully developed an assay for measuring blood levels of fish insulin-like growth factor-I (IGF-I). We then measured IGF-I in a number of nutrition trials and obtained very encouraging correlations of IGF-I levels and growth rate and/or stress. To further develop the use of these reagents and technologies we hope that our continued research, and hopefully that of other researchers, will help to establish IGF-I as a tool to increase aquaculture productivity. This may be achieved by the use of

IGF-I levels to maximise survival and growth by improving nutrition and/or minimising stress as a result of improved diets and husbandry systems becoming marketable earlier. A further application of this research lies in broodstock selection. The CRC for Tissue Growth and Repair and PrimerGro has successfully correlated the changing levels of IGF-I with growth and development in pigs and in conjunction with the Pig Research and Development Corporation and Bunge Meal Industries has developed a diagnostic assay to aid pig industry breeding programs. This method of broodstock selection has resulted in a significant increase in growth rate for already highly selected pig strains and holds similar potential for the aquaculture industry. We have also demonstrated a significant link between circulating IGF-I levels and stunting in Atlantic salmon. Indeed, we have continued our efforts investigating the role of IGF-I in smoltification and have demonstrated a close relationship between the levels of this growth factor and the ability of smolt to survive the transfer to seawater. With further research we believe the application of IGF-I in the finfish industry may provide a useful method to minimise the time required for growth trials and also as a method for optimising seawater transfer time in Atlantic salmon to minimise smolt mortality during this period.

8. Conclusion

In conclusion, this research project has been successful in generating a range of products for the analysis of fish IGF's. We have also demonstrated that IGF-I is a useful indicator of growth performance and could potentially be used to evaluate aquaculture diets or monitor the stress of aquacultured finfish populations. We believe that the benefits we have generated for the scientific community in aiding the analysis of IGF's will have a cascade effect to many aquaculturists as the role of IGF's in finfish growth is further understood. We also believe that with continued effort we may be able to commercialise methods for the use of IGF-I to improve diets and husbandry practices in a range of aquaculture species.

9. References

Ballard, F.J., Read, L.C., Francis, G.L., Bagley, C.J. and Wallace, J.C. (1986). Binding properties and biological potencies of insulin-like growth factors in L6 myoblasts. Biochem. J. **233**: 223-230.

Blaise, O., Weil, C., Le Bail, P.Y. (1995). Role of IGF-I in the control of GH secretion in rainbow trout (*Oncorhynchus mykiss*). Growth Reg. **5**: 142-150.

Clarke, W.C. and Nagahama, Y. (1977). Effects of premature transfer to seawater on juvenile coho salmon (Oncorhynchus kisutch). Can. J. Zool. 55: 1620-1630.

Cook, A.F. and Peter, R.E. (1984). The effects of somatostatin on serum growth hormone levels in the goldfish, *Carassius auratus*. Gen. Comp. Endocrinol. **54**: 109-113.

Duan, C. and Hirano, T. (1992). Effects of insulin-like growth factor I and insulin on the *in vitro* uptake of sulphate by eel branchial cartilage: Evidence for the presence of independent hepatic and pancreatic sulphation factors. J. Endocrinol. **133**: 211-219.

Duan, C. and Plisetskaya, E.M. (1993). Nutritional regulation of insulin-like growth factor-I mRNA expression in salmon tissues. J. Endocrinol. **139**: 243-252.

Duan, C., Plisetskaya, E.M. and Dickhoff, W.W. (1995). Expression of insulin-like growth factor I in normally and abnormally developing coho salmon (*Oncorhynchus kisutch*). Endocrinol. **136**: 446-452.

Farbridge, K.L. and Leatherland, J.F. (1992). Plasma growth hormone levels in fed and fasted rainbow trout (*Oncorhynchus mykiss*) are decreased following handling stress. Fish Physiol. Biochem. **10**: 67-73.

Francis, G.L., Upton, F.M., Ballard, F.J., McNeil, K.A. and Wallace, J.C. (1988). Insulin-like growth factors 1 and 2 in bovine colostrum: Sequences and biological activities compared with those of a potent truncated form. Biochemical J. **251**: 95-103.

Freifelder, D. M. (1982). Physical Biochemistry, 2nd ed., pp. 129-155. Freeman, New York.

Gutierrez, J, Parrizas, M., Maestro, M.A., Navarro, I. And Plisetskaya, E.M. (1995). Insulin and IGF-I binding and tyrosine kinase activity in fish heart. J. Endocrinol. **146**: 35-44.

Madsen, S.S. and Bern, H.A. (1993). *In-vitro* effects of insulin-like growth factor-I on gill Na^+, K^+-ATPase in coho salmon, *Oncorhynchus kisutch*. J. Endocrinol. **138**: 23-30.

Madsen, S.S., Jensen, M.K. and Kristiansen, K. (1995). Expression of Na(+)-K(+)-ATPase in the brown trout, *Salmo trutta*: *in vivo* modulation by hormones and seawater. Amer. J. Physiol. **269**: R1339-Rl3345.

McCormick, S.D., Kelley, K.M., Young, G., Nishioka, R.S. and Bern, H.A. (1992). Stimulation of coho salmon growth by insulin-like growth factor I. Gen. Comp. Endocrinol. **86**: 398-406.

McCormick, S.D., Shrimpton, J.M., Carey, J.B., O'Dea, M.F., Sloan, K.E., Moriyama, S. and Bjornsson, B.Th. (1998). Repeated acute stress reduced growth rate of Atlantic salmon parr and alters plasma levels of growth hormone, insulin-like growth factor I and cortisol. Aquaculture **168**: 221-235.

Moriyama, S. (1995). Increased plasma insulin-like growth factor-I (IGF-I) following oral and intraperitoneal administration of growth hormone to rainbow trout, *Oncorhynchus mykiss*. Growth Reg. **5**: 164-167.

Moriyama, S., Duguay, S.J., Conlon, J.M., Duan, C., Dickhoff, W.W. and Plisetskaya, E.M. (1993). Recombinant coho salmon insulin-like growth factor-I expression in Escherichia coli, purification and characterisation. Eur. J. Biochem. **218**: 205-212.

Moriyama, S., Swanson, P., Nishii, M., Takahashi, A., Kawauchi, H., Dickhoff, W.W. and Plisetskaya, E.M. (1994). Development of a homologous radioimmunoassay for coho salmon insulin-like growth factor-I. Gen. Comp. Endocrinol. **96**: 149-161.

Niu, P.D., Perez-Sanchez, J., and Le Bail, P.Y. (1993). Development of a protein binding protein assay for teleost insulin-like growth factor (IGF)-like: Relationships between growth hormone (GH) and IGF-like inthe blood of rainbow trout (*Oncorhynchus mykiss*). Fish. Physiol. Biochem. **11**: 1-6.

Ottolenghi, C., Puviani, A. C., Baruffald, A., and Brighenti, L. (1984). Effect of insulin on glycogen metabolism in isolated catfish hepatocytes. Comp. Biochem. Physiol. A **78**: 705-710.

Parrizas, M., Plisetskaya, E.M., Planas, J. and Gutierrez, J. (1995). Abundant insulin-like growth factor-1 (IGF-1) receptor binding in fish skeletal muscle. Gen. Comp. Endocrinol. **98**: 16-25.

Perez-Sanchez, J., Marti-Palanca, H. and Kaushik, S.J. (1995). Ration size and protein intake affect circulating growth hormone concentration, hepatic growth hormone binding and plasma insulin-like growth factor-I immunoreactivity in a marine teleost, the gilthead sea bream. J. Nutr. **125**: 546-552.

Perez-Sanchez, J., Plisetskaya, E.M., Planas, J. and Gutierrez, J. (1995). Abundant insulin-like growth factor-I (IGF-I) receptor binding in fish skeletal muscle. J. Nutrition **125**: 546-552.

Pickering, A.D. (1990). Stress and the suppression of somatic growth in teleost fish. Prog. Clin. Biol. Res. **342**: 473-479.

Pickering, A.D., Pottinger, T.G, Sumpter, J.P., Carragher, J.F. and Le Bail, P.Y. (1991). The effects of acute and chronic stress on the levels of circulating growth hormone in the rainbow trout, *Oncorhychus mykiss*. Gen. Comp. Endocrinol. **83**: 86-93.

Pozios, K.C., Ding, J., Degger, B., Upton, Z. and Duan, C. (2000). Insulin-like growth factors stimulate zebrafish embryonic cell proliferation through activation of the mitogen-activated protein kinase and a phophphatidylinositol 3-kinase signalling pathways.

Read, L.C., Ballard, F. J., Francis, G.L., Baxter, R.C., Bagley, C.J. and Wallace, J.C. (1986). Comparative binding of bovine, human and rat insulin-like growth factors to membrane receptors and to antibodies against human insulin-like growth factor-I. Biochem. J. **233**: 215-221.

Ross, M., Francis, G.L., Szabo, L., Wallace, J.C. and Ballard, F.J., 1989. Insulin-like growth factor (IGF)-binding proteins inhibit the biological activities of IGF-I and IGF-2 but not des-(1-3)-IGF-I. Biochem. J. **258**: 267-277.

Sakamoto, T. and Hirano, T. (1993). Expression of insulin-like growth factor I gene in osmoregulatory organs during seawater adaptation of the salmonid fish: possible mode of osmoregulatory action of growth hormone. Proc. Nat. Acad. Sci. (USA) **90**: 1912-1916.

Shimizu, M., Swanson, P., Fukada, H., Hara, A. and Dickhoff, W.W. (2000). Comparison of extraction methods and assay validation for salmon insulin-like growth factor-I using commercially available components. Gen. Comp. Endocrinol. **119**: 26-36.

Sumpter, J.P. (1997). Environmental control of fish reproduction: A different perspective. Fish. Physiol. Biochem. 17 :25-31.

Takagi,Y. and Bjornsson, B.T. (1996). Regulation of cartilage glycosaminoglycan synthesis in the rainbow trout, *Oncorhynchus mykiss* by 3,3'-tri-iodo-L-thyronine and IGF-I. J. Endocrinol. **149**: 357-365.

Wagner, G.F. and McKeown, B.A. (1986). Development of a salmon growth hormone radioimmunoassay. Gen. Comp. Endocrinol. **62**: 452-458.

Appendix 1: Intellectual Property

This research project has generated a number of commercially viable products and reagents for the analysis of fish IGF's, although no intellectual property has arisen that is patentable. Our research focused on the development of methods and tools for the analysis of fish IGF's thus there was little scope for the development of uses of this technology for commercial gain. The generation of intellectual property and uses for the reagents and assays developed during this project will be assessed in future research.

Appendix 2: Staff

Principal investigator:

Dr. Zee Upton, Lecturer in cell biology, Queensland University of Technology
School of Life Sciences Queensland University of Technology, GPO Box 2434, Brisbane, QLD 4001
Phone: +61 7 3864 2342 Fax: +61 7 3864 1534
E-mail: z.upton@qut.edu.au

Co-investigators:

Dr. John Carragher, Lecturer in Aquaculture, Flinders University
School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide SA 5001
Phone: +61 8 8201 3041 Fax +61 8 8201 3015
Email john.carragher@flinders.edu.au

Dr. Neil Richardson, Postdoctoral Fellow, Queensland University of Technology
School of life Sciences, Garden's Point Campus, GPO Box 2434, Brisbane, Qld 4000
Phone: +61 7 3864 2984 Fax: +61 7 3864 1534

Dr. Kathleen Soole, Lecturer, Flinders University
School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide, SA 5001
Phone: +61 8 8201 2030 Fax: +61 8 8201 3015
E-mail: bikls@flinders.edu.au

Research and support staff:

Dr. Helena Ward, Communications Manager, CRC for Tissue Growth and Repair

Brian Degger, PhD candidate, CRC for Tissue Growth and Repair

Dr Anthony Dyer, Research Associate, School of Biological Sciences, Flinders University

Naomi Higgs, Research Assistant, CRC for Tissue Growth and Repair

Kirsty Quinn, Research Assistant, CRC for Tissue Growth and Repair

Adam Takos, Research Assistant, CRC for Tissue Growth and Repair

Product commercialisation staff:

Dr. Geoffrey Francis, Biotechnology Reagents Group Manager, Gropep Limited

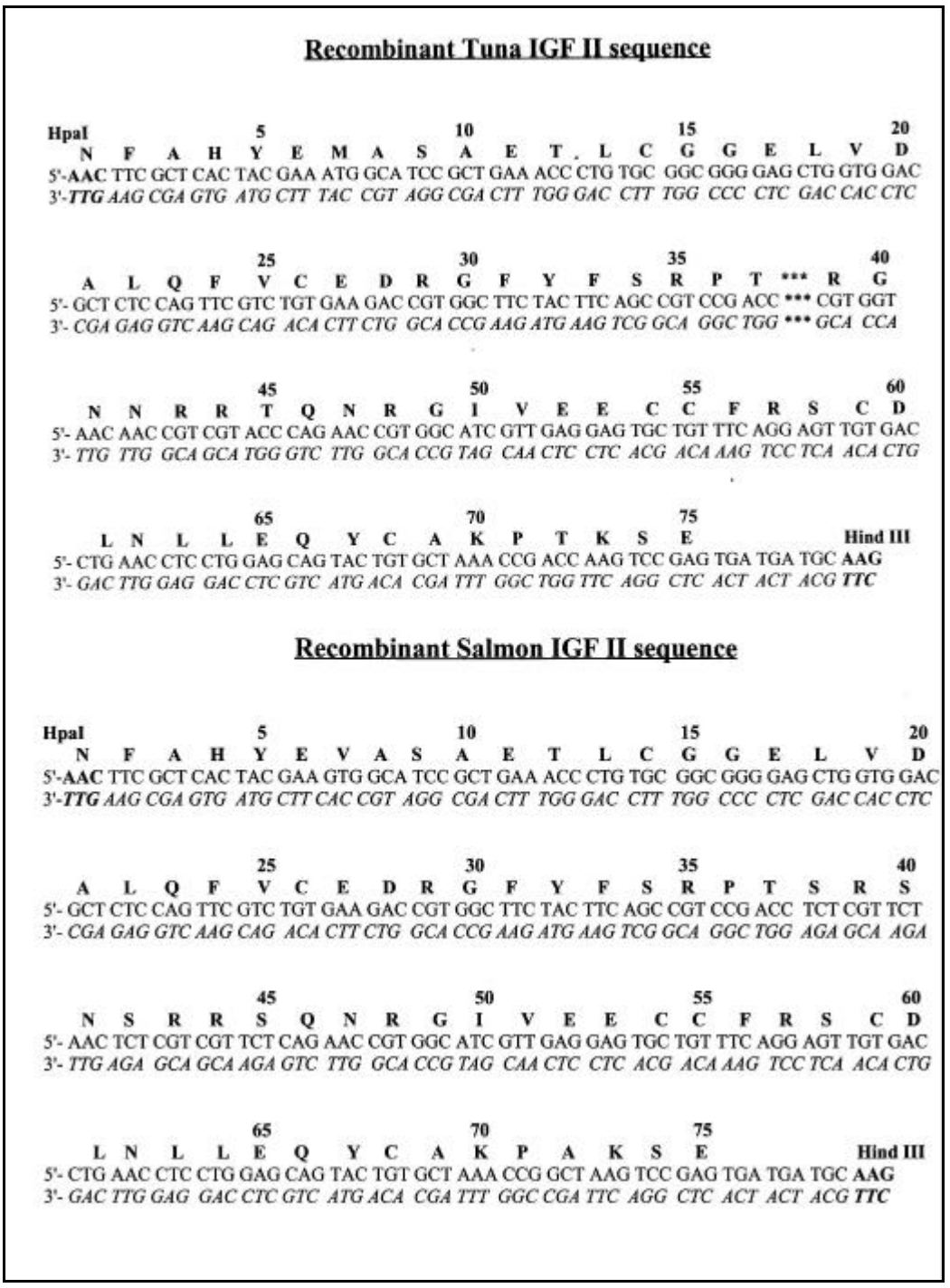
Dr. Stephen Hardy, Sales Manager, Gropep Limited

Dr. Ben Hunt, Research Associate, GroPep Limited

Dr. Pat Wallace, New Research products Manager, GroPep

Appendix 3: Tuna and salmon IGF-II DNA sequences

* Indicates deletions



Appendix 4: Products and reagents

The following is a list of aquaculture products that are available from GroPep Limited. Products and reagents that were generated from this FRDC funded project are indicated with a *.

GroPep

GroPep Ltd
28 Dolgleish Street
Thebarton SA 5031
PO Box 10065 BC
Adelaide SA 5000
Australia
Ph (61 8) 8354 7700
Fax (61 8) 8354 7788
E-Mail:
sales@gropep.com.au
Website
www.gropep.com.au

* GroPep Fish IGF-I RIA Kit

Summary:

For measuring the circulating levels of Insulin-like Growth Factor-I (IGF-I) in the serum or plasma of a number of important aquaculture species.

Background:

Insulin-like growth factor I (IGF-I) is a naturally produced molecule of approximately 70 amino acids that stimulates growth and differentiation in many cell types. Measuring IGF-I is useful for investigating and monitoring a number of biological processes including the actions of growth hormone, many of which are mediated by IGF-I.

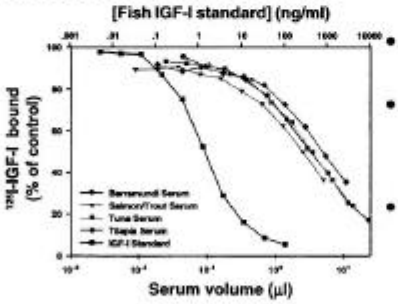
If measurements of IGF-I are to have any practical application, it is essential that an appropriate assay system is used. *GroPep* has therefore developed a kit specifically for the finfish Aquaculture industry and Researchers working in the field.

Suitable for:

- Atlantic salmon (*Salmo salar*)
- Carp (*Cyprinus carpio*)
- Goldfish (*Carassius auratus*)
- Red Sea bream (Snapper) (*Pagrus auratus*)
- Tilapia (*Oreochromis mossambicus*)
- Barramundi (*Lates calcarifer*)
- Coho salmon (*Oncorhynchus kisutch*)
- Rainbow trout (*Oncorhynchus mykiss*)
- Southern bluefin tuna (*Thunnus maccoyii*)

GroPep expects that the kit will also be suitable for measuring serum IGF-I levels in fish species other than those listed. It will be necessary for the user to check that the kit gives accurate and meaningful results by checking for parallelism with the fish IGF-I standards. Please note that quantitative equivalence should not be assumed for non-listed species.

Assay Specificity and Performance:



- **Sensitive:**
  Detects down to 0.15 ng/ml
- **Highly specific for fish IGF-I:**
  < 0.5 % cross-reactivity with human IGF-I
  < 1 % cross-reactivity with human IGF-II
  < 0.01 % cross-reactivity with salmon insulin
- **Accurate and Reproducible:**
  Variation within the same assay: 3 %
  Variation between separate assays: 16 %
  (n=9)

NEW!!

GroPep Fish IGF-I RIA Kit
Product Code: KITA
Cost: US$500 (f.o.b.)

Order Now by FAX: +61 8 8354 7788
Order Now by E-mail: sales@gropep.com.au
Order Now through our Website: www.gropep.com.au

Notes for Use:

IGF-I binding proteins present in the serum actively bind IGF-I and may interfere with IGF-I measurement. To remove these interfering proteins an acid ethanol extraction of the serum is required prior to assay.

The RIA protocol is a two step assay performed over two days.

Day 1: Acid-ethanol extraction of serum and incubation with ^{125}I-labelled fish IGF-I tracer and Antiserum 1.

Day 2: Assay termination and sample counting.

The GroPep kit contains the reagents required for assaying 100 fish serum samples in triplicate. Sufficient standard is included for three standard curves so samples can be assayed in more than one assay run.

Reagents Supplied:

- 3 vials of recombinant fish IGF-I standard
- ^{125}I-labelled fish IGF-I tracer
- Antiserum 1: Produced in rabbit
- Antiserum 2 : Produced in sheep
- IgG for precipitation: Isolated from rabbit serum
- 3 vials of QC serum (known IGF-I concentration)
- RIA buffer (10 x concentrate)
- PEG solution (5 x concentrate)

Reagents required but not supplied:

- Acid ethanol solution
- 0.855 M Tris buffer

Preparation details supplied.

Other Aquaculture Products from GroPep :

	Product	*Quantity*	*Code*	*Price (f.o.b)*
	IGF-I:			
★	Barramundi IGF-I (Receptor Grade)	20 µg	YU020	US$200
	(Lates calcarifer)	100 µg	YU100	US$350
★	Red Sea bream (Snapper) IGF-I (Receptor Grade)	20 µg	AGU020	US$200
	(Pagrus auratus)	100 µg	AGU100	US$350
★	Salmon/Trout IGF-I (Receptor Grade)	20 µg	WU020	US$200
	(Oncorhynchus kisutch & O. mykiss)	100 µg	WU100	US$350
★	Tilapia IGF-I (Receptor Grade)	20 µg	AFU020	US$200
	(Oreochromis mossambicus)	100 µg	AFU100	US$350
★	Tuna IGF-I (Receptor Grade)	20 µg	AEU020	US$200
	(Thunnus maccoyii)	100 µg	AEU100	US$350
	Growth Hormone:			
	Black Bream GH (Receptor Grade)	20 µg	GHAU020	US$200
	(Acanthopagrus butcheri)	100 µg	GHAU100	US$350
	Salmon/Trout GH (Receptor Grade)	20 µg	GHBU020	US$200
	(Oncorhynchus kisutch & O. mykiss)	100 µg	GHBU100	US$350
	Antibodies (Polyclonal Rabbit):			
	Growth Hormone, anti-bream	15 µl	PAG1	US$100
	(Characterized and titred for RIA)	75 µl	5PAG1	US$200
	Growth Hormone, anti-salmon/trout	5 µl	PAN1	US$100
	(Characterized and titred for RIA)	25 µl	5PAN1	US$200
★	IGF-I, anti-barramundi	10 µl	PAF1	US$100
	(Characterized and titred for RIA)	50 µl	5PAF1	US$200
★	IGF-I, anti-salmon/trout	5 µl	PAM1	US$100
	(Not characterized for RIA)	25 µl	5PAM1	US$200
	IGF-I, antibody for immunohistochemistry	200 µg	PABCa	US$250
	(Affinity purified. Recognizes IGF-I in human, rat and barramundi tissues)			

Multi-Product packs of Growth Factors and Antibodies also available. Consult GroPep Website for details on Pack sizes and ordering. www.gropep.com.au