THE REGULATION OF INSULIN-LIKE GROWTH FACTORS IN BARRAMUNDI, *Lates calcarifer.*

Thesis submitted by
Sue Janet MATTHEWS BSc (Hons.) (Deakin University)
October 1997

for the degree of Doctor of Philosophy
in the School of Biological Sciences AQUACULTURE
at
James Cook University of North Queensland

STATEMENT OF ACCESS

I, the undersigned, the author of this thesis, understand that James Cook University of North Queensland will make it available for use within the University Library and, by microfilm or other means, allow access to users in other approved libraries. All users consulting this thesis will have to sign the following statement:

> In consulting this thesis I agree not to copy or closely paraphrase it in whole or in part without the written consent of the author; or make proper public written acknowledgment for any assistance which I have obtained from it.

Beyond this, I do not wish to place any restrictions on access to this thesis.

10:10:1997

(Sue Janet Matthews) (Date)

STATEMENT ON SOURCES
DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

10:10:1997

(Sue Janet Matthews) (Date)

All research work involving experimental animals described in this thesis was conducted within the guidelines of "The Australian Code of Practice for the care and use of Animals for Scientific Purposes". The research project undertaken received ethical clearance from the James Cook University of North Queensland Animal Ethics Committee (Approval number A250).

To my parents,
Paul and Janet Matthews,
for always believing in my dreams
and for their encouragement and support,
enabling me to fulfill them.

ACKNOWLEDGEMENTS

I am indebted to all who contributed their time and effort to this project and to those who helped me to complete this thesis. In particular, I would like to thank:

* Dr Trevor Anderson, my supervisor, for introducing me to IGFs and for his encouragement and advice throughout this project. I am particularly grateful for his belief in students with *el fuelgo*, despite their tendency to break his 3 major rules!

* Professor Vicki Sara, Queensland University of Technology (QUT), Brisbane, for her scientific expertise which broadened my knowledge of the IGF field and for her enthusiasm in supporting up and coming scientists.

* Dr Peter Hoeben for supporting collaborative work between James Cook University (JCU) and the IGF phylogeny group at the Centre for Molecular Biotechnology (CMB), QUT and for his continual encouragement and advice from the planning stages to the completion of the manuscript.

* The Australian Postgraduate Award Scheme for providing a generous stipend for the entire duration of the project.

* The School of Biological Sciences at JCU for providing me with the opportunity to undertake this degree and for supporting the project financially with a combined Merit Research Grant (1995) and annual Internal Research Awards (1994-1996).

* The Kirwan Women's Hospital, Townsville, and the Molecular Science Division at JCU for supplying the biological tissues required for receptor purification.

* Dr Anne Kinhult and Gillian Walker for introducing me to molecular biology, answering endless questions and for their sincere friendship. Special thanks to Anne for the provision of anti-sense probes used in Chapter 4.

* My colleagues at QUT, Kharen Doyle, Dr Neil Richardson, Bronwyn O'Brien, Andrea McCracken, Dr Axel Ståhlbom, Dr Chris Collet and others in the CMB for their welcoming friendships and assistance. Thanks also extend to Kharen Doyle for her assistance with rt-PCR in Chapter 4 and also to the Plaisted family for providing a 'home away from home' during my stay.

* My colleagues at JCU, but particularly, Dr Adrian Collins, Dr Jens Knauer, Shaun Smith and Kevin Kane, for their sense of humour and for sharing the office, lab or other day to day experiences of my PhD. Thanks extend to Kevin for measuring the crude energy of the five experimental diets used in Chapter 5.

* Dr Jens Knauer and Don Booth for their technical assistance in establishing a functional aquarium system and Warren Haydon for his practical experience which improved and kept the facilities operational.

* My parents, Janet and Paul Matthews for their patience and care in supporting my goals - no matter what, why, when, or where they may be - and for their willingness to feed fish on holidays in Townsville.

* My entire family and extended family friends in Victoria for their continual support and kindness and for reminding me that even when the experiments weren't working, Melbourne was still wet and cold.

* All my friends, but particularly Lorrine Finlay, Janet McLeod, Paula Stiles, Phil Hocking, Courtney Goldsmith, Andrew Bond, Graham Murdoch and Yvette Williams for their encouragement and support both academically and personally over the past years and for always listening over a fine beverage or expensive phone call.

* And finally, my special thanks to Dr Peter Appleford for his guidance, support and patience throughout my academic pursuits. And lastly, but most importantly and for sharing and loving the 'non-scientific' side of life with Shaka, Zana and I.

ABSTRACT

A knowledge of the factors which regulate insulin-like growth factors (IGFs) in teleost fish is important for understanding the physiological process of growth and thus devising strategies to improve growth in culture systems. In mammals, two IGF molecules have been identified. Whilst IGF-I is predominantly regulated by growth hormone (GH) and the nutritional status of the animal, GH does not appear to regulate IGF-II which is thought to be important for fetal development. Although the IGF system has been well characterised in mammals, there is a paucity of data on IGFs in teleost fish.

The detection of IGFs in teleost species has generally been determined using non-homologous competitive binding assays, often without complete assay validation. In addition, there are few studies which investigate the mechanisms of IGF regulation. The aim of the present study was to determine the effect of nutritional status and water temperature on the growth and regulation of IGFs in juvenile barramundi, *Lates calcarifer*.

Following acidic size exclusion chromatography, circulating IGF-I and IGF-II were detected in the serum of juvenile barramundi using type I and type II radioreceptor (RRA) assays, respectively. Both RRA were rigorously validated using the recommended protocol for the measurement of IGFs in biological fluids (Bang *et al.*, 1994). Peaks containing the IGF molecules were serially diluted and demonstrated parallelism to human IGF-I and IGF-II standard reference curves, in the type I and type II RRA, respectively. IGF binding proteins were identified as a false peak of immunoreactivity, ranging in molecular size from 12.3 - 66 kDa. The IGF binding proteins (IGFBPs) were further characterised using an IGF binding protein assay with subsequent neutral size exclusion chromatography and Western ligand blots. High quantitative recovery was demonstrated in the type I (99 ± 2.7 %) and type II (97 ± 3.4 %) RRA by the addition of unlabelled IGF-I or IGF-II respectively to the serum prior to analysis. Intra- and inter-coefficients of variation were within acceptable literature ranges being 1.7 ± 0.3 % and 8.2 ± 2.1 % respectively for the type I RRA and 3.9 ± 0.6 % and 8.3 ± 2.8 % respectively for the type II RRA. These findings satisfied the requirements of IGF assay validation and thus provided a method for detecting both IGF-I and IGF-II in the serum of juvenile barramundi.

The present study demonstrated that ration size regulates the growth, level of circulating IGF-I and expression of hepatic IGF-I mRNA in juvenile barramundi. In contrast, the expression of IGF-I mRNA in the brain, the ratio of the alternatively spliced Ea-4 : Ea-2

IGF-I mRNA transcripts and the concentration of circulating IGF-II were not significantly affected by ration size. As hepatic IGF-I mRNA and circulating levels of IGF-I were reduced during starvation and there was an accompanying decreased growth, it is likely that systemic IGF-I of hepatic origin is important for somatic growth in this species. The response of IGF-I, both pre- (hepatic only) and post-translational, but not IGF-II, to ration size in juvenile barramundi is similar to findings in gilthead seabream, thereby providing further evidence for the general principle of regulation of the GH:IGF-I axis in fish by nutritional status.

Dietary protein and energy regulated the growth and level of circulating IGF-I in juvenile barramundi, providing support for the theory of nutritional regulation of IGF-I, but not IGF-II, in this species. Since decreased dietary protein and energy caused a reduction in the concentration of circulating IGF-I, which was accompanied by decreased growth, it is likely that systemic IGF-I is affected by protein and energy restriction in this species. Although the mechanisms of this regulation remain unknown, results from studies conducted in other teleost fish demonstrate that protein and energy restriction result in an insensitivity of the liver to GH. Although the role of IGF-II remains somewhat uncertain in teleost fish, it is reasonable to suggest that IGF-II in fish, as in mammals, is not directly influenced by nutrition or GH.

Although water temperature affected growth in both trials there was no clear effect of temperature on circulating IGF-I or IGF-II.

The results of this study provide a technique for detecting changes in circulating IGF levels and indicate that environmental parameters, including ration size, dietary protein and energy content and water temperature affect the growth and the synthesis of insulin-like growth factor-I in juvenile barramundi, *Lates calcarifer*.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

Abbreviation used	Full name
ATP	2-Deoxy adenosine triphosphate
ANOVA	Analysis of variance
B_{max}	Binding capacity
bp	base pair
BPB	Bromophenol blue
BP Ins	Bovine Insulin from pancreas
BSA	Bovine serum albumin
BWI	Body weight increase
CA	Carbonic anhydrase
CAPS	3-[Cyclohexlamino]-1-propanesulfonic acid
cDNA	Complementary DNA
CF	Condition factor
CHISAM	Chloroform Isoamylalcohol buffer
Cpm	Counts per minute
CTP	2-Deoxy cystidine triphosphate
CYT	Cytochrome C
DB	Dextran blue
DEPC	Diethylpyrocarbonate
DNA	Deoxyribose nucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetate
EtBr	Ethidium bromide
FCR	Apparent food conversion ratio
GTC	Guanidium thiosulphate
GTP	2-Deoxy guanosine triphosphate
HM Ins	Human Monocomponent Insulin
HPLC	High performance liquid chromatography
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IGFBP	Insulin-like growth factor binding protein
J	Joule
K	Dissociation constant
kb	kilobase
kDa	kilodalton
kJ	kilojoule
LMWS	Low molecular weight standards
MJ	millijoule
MOPS	3-(N-Morpholino) propane sulphonic acid
ng	nanogram
nm	nanometer
Nonidet® P40	Aethylphenylpolyaethylenglycol
NPU	Net protein utilisation
NSB	Non-specific binding
OV	Ovalbumin
PER	Protein efficiency ratio
PCR	Polymerase chain reaction
pg	picogram
PSL	Phospho-stimulated luminescence
RIA	Radioimmunoassay
RES	Restricted ration

RNA	Ribonucleic acid
RPA	RNA protection assay
RRA	Radioreceptor assay
rt-PCR	Reverse transcriptase PCR
SAT	Satiety ration
SDS	Sodium doecyl sulphate
SDS-PAGE	SDS Polacrylimide gel electrophoresis
SGR	Specific growth rate
STV	Starved
TAE	Tris-acetate EDTA
TB	Total binding
TBE	Tris-borate EDTA
TC	Total counts
TMA	Trimethylamine
Tween-20	Polyoxyethylenesorbitan monolaurate
UV	Ultraviolet

GLOSSARY OF COMMON AND SCIENTIFIC NAMES

Common name	Scientific name
American eel	*Anguilla rostrata*
Atlantic bluefish	*Pomatomus salteris*
Atlantic halibut	*Hippoglossus hippoglossus*
Atlantic salmon	*Salmo salar*
Barramundi	*Lates calcarifer*
Brook trout	*Salvelinus fontinalis*
Brown trout	*Salmo trutta*
Channel catfish	*Ictalurus punctatus*
Chinook salmon	*Oncorhynchus tshawytscha*
Cichlid	*Haplochromis burtoni*
Coho salmon	*Oncorhynchus kisutch*
Common carp	*Cyprinus carpio*
Couch's seabream	*Pagrus pagrus*
European eel	*Anguilla anguilla*
Flounder	*Parlichthys olivaceus*
Gilthead seabream	*Sparus aurata*
Goby	*Gillichthys mirabilis*
Golden perch	*Macquaria ambigua*
Goldfish	*Carrassius auratus*
Japanese eel	*Anguilla japonica*
Mossambique tilapia	*Oreochromis mossambicus*
Nile tilapia	*Oreochromis nilotus*
Rabbitfish	*Siganus guttatus*
Rainbow trout	*Oncorhynchus mykiss*
Red Drum	*Scianepnops ocellatus*
Red seabream	*Pagrus major*
Sheatfish	*Silurus glanis*
Sockeye salmon	*Oncorhynchus nerka*
Spiny dogfish shark	*Squalus acanthias*
Sting ray	*Raja clavata*
Striped bass	*Morone saxatilis*
Tilapia	*Tilapia andersoni*
Tilapia	*Tilapia aurea*

CHAPTER 1:

GENERAL INTRODUCTION

1.1 Animal Growth : An Introduction

The term growth is most simply defined as an increase in size by natural development (Atkinson, 1991). The growth of an animal represents a series of defined events that begin with food intake and terminate in the deposition of proteins or alternative substances. Between these events, the rates of digestion, absorption, assimilation, synthesis and degradation, energy expenditure and excretion interact to regulate the phenomenon of growth.

Growth can be analysed quantitatively by monitoring the expansion of physical dimensions of the organism representing the overall manifestations of the growth process. Such measurements are usually expressed as simple length-weight relationships, relative growth or as growth rates. The perceived ease and readiness with which growth can be observed and measured often implies that growth is a simple, regular process. However, when the variables associated with the regulation of growth are considered, growth is viewed as a more complex, irregular event (Brett, 1979).

Animal growth is influenced by a wide range of extrinsic and intrinsic parameters (Brett, 1979; Donaldson *et al.*, 1979). Such parameters, broadly classified as environmental, genetic and hormonal factors, play significant and often interactive roles in regulating growth. Accordingly, disturbances in one or more growth determinants may result in dramatic changes to normal body growth (McLean and Donaldson, 1993). Environmental factors such as food availability, diet composition and seasonal water temperature all influence the metabolic rate and availability of nutrients for growth (Brett, 1979). The nutritional status of the animal, as well as other environmental parameters, including photoperiod and salinity, affect the synthesis of growth-promoting hormones such as thyroid hormone, growth hormone (GH), insulin and insulin-like growth factors (IGFs) (Donaldson *et al.*, 1979; Sumpter, 1992). In addition, social factors such as predation and competition, and intrinsic genetic traits may also influence the scope for growth. When the opportunity for interactions among these factors is considered, it is evident that the mechanisms

of growth regulation are extremely complex. It is therefore difficult to discuss all factors regulating the growth process. As such, this chapter will briefly discuss growth regulation in teleosts, with emphasis on food availability, diet type, water temperature and the physiological actions of the growth-promoting hormones, namely GH and the IGFs.

1.2 Environmental Regulation of Teleost Growth

1.2.1 Temperature

The environment, both natural or captive, has the potential to influence the growth of an animal in many ways. Of particular influence on the growth of teleost fish is the external temperature, since the body temperature of these animals is reliant upon the temperature of the external environment (Brett, 1979; Sumpter, 1992; Jobling, 1994). In teleosts, fluctuations in water temperature affect growth by changing the metabolic rate and thus the mechanisms of energy partitioning within the animal (Elliot, 1979; Forster and Wieser, 1990; Sumpter, 1992; Jobling, 1994). During exposure to higher water temperatures, the amount of energy required to maintain the standard metabolic rate increases. Under such conditions, the excess energy which was available at a lower temperature for processes such as growth is now required to fulfill the basal metabolic energy requirements (Elliot, 1979; Sumpter, 1992; Jobling, 1994). Therefore, in circumstances where the availability of food remains constant but water temperature increases, the growth rate of teleosts will decrease (Jobling, 1981; Sumpter, 1992). The fluctuations in water temperature in natural environments are usually associated with seasonal transition, and providing the temperature remains within the tolerance level of the species, often the animal will slowly acclimatise and the effect of the energy consumed by the metabolic rate is minimal (Bosclair and Tang, 1993). In artificial conditions, such as those used in aquaculture practices, the environmental temperature may vary significantly over much shorter periods of time causing the effect on the metabolic rate to be quite significant (Jobling, 1994). A more detailed discussion of the changes in energy partitioning during temperature changes has been compiled by Jobling (1994).

1.2.2 Food Availability

The availability of food may also significantly affect teleost growth by altering energy partitioning (Brett, 1979; Storebakken *et al.*, 1991). The relationship between ration and growth is curvilinear (Brett, 1979; De Silva and Anderson, 1995). Growth is negative until the energy supplied by the diet equals the energy required to maintain the standard metabolic energy and the then animal no longer loses weight (Brett, 1979; Jobling, 1994; De Silva and Anderson, 1995). The amount of food required to satisfy the basal metabolic rate is often referred to as the maintenance ration, since at this point, the animal will not lose or gain weight (De Silva and Anderson, 1995). From this point, the growth rate continues to increase gradually with increasing ration size, until the maximal feeding rate is achieved and growth plateaus (Brett, 1979; De Silva and Anderson, 1995). The identification of the optimal feeding rate is therefore of great benefit for commercially farmed animals. Brett (1979) provides a more detailed review of food availability and growth of teleost fish.

1.2.3 Diet Composition

The growth of teleost fish is reliant upon the synthesis and accretion of various tissue types, predominantly muscle and fat, but also including connective and epithelial tissue. The proportion of protein that can be synthesised or fat deposited is highly dependent upon the diet composition. Fish require relatively high levels of dietary protein, compared to other commercially reared animals (Millikin, 1982), thus many studies of teleosts have aimed to identify the amount of dietary protein required for maximum growth. An early study defined the minimum dietary protein level producing optimal weight gain in chinook salmon, *Onchorhynchus tshawtscha* (Delong *et al.*, 1958). Subsequently, the determination of optimal dietary protein has been investigated in many fish species including rainbow trout, *Oncorhynchus mykiss* (Lee and Putnam, 1973), channel catfish, *Ictalurus punctatus* (Garling and Wilson, 1976), striped bass, *Morone saxatilis* (Millikin, 1983), red drum, *Scianenops ocellatus* (Daniels and Robinson, 1986), tilapia, *Tilapia aurea* (Winfree and Stickney, 1981), hybrid tilapia, *Oreochromis niloticus x O. aurea* (Shia and Huang, 1989) and

rabbitfish, *Siganus guttatus* (Parazo, 1990). The growth produced by the dietary protein is reliant upon factors other than crude protein level. Firstly the amino acid composition of the protein source must be considered. Fish require certain essential amino acids (Millikin, 1982) and limitation in one of these essential amino acids results in the deamination of the remaining dietary amino acids and fat deposition (Jobling, 1994; De Silva and Anderson, 1995). Secondly, the amount of growth produced is not only dependent on the provision of the essential amino acids, but also upon the amount of non-protein energy, since the process of protein synthesis requires energy which is additional to that of the basal metabolic rate (Millikin, 1982). Ideally, this energy is provided in the diet in a non-protein form, such that the fish can optimise the dietary protein for tissue accretion (Jobling, 1994; De Silva and Anderson, 1995). If a non-protein source is not supplied, or is not adequate, the fish will deaminate dietary amino acids to produce energy, thus limiting the amount of protein available for growth (Jobling, 1994; De Silva and Anderson, 1995). Increasing the non-protein energy content of a diet can, therefore, result in a greater proportion of the dietary protein being used for protein deposition than for energy. This process is known as protein-sparing and has been reported in several species of fish (De Silva and Anderson, 1995). Both carbohydrate and lipid can be included in the diet as alternative energy sources. Lipid is extremely useful as an alternative energy source in fish diets as it contains high levels of energy, can be effectively utilised by fish, often acts as a binding agent and increases the palatability of the diet (Jobling, 1994; De Silva and Anderson, 1995). Unlike the situation in mammals, carbohydrates are less efficiently used by fish as an energy source (Jobling, 1994; De Silva and Anderson, 1995). However, since carbohydrates are considerably cheaper than lipids, they are often used in fish diets.

To optimise the growth of fish, the diet should be formulated to meet the optimal protein-energy ratio. Optimal protein-to-energy ratios vary significantly among fish species and are significantly affected by water temperature and other parameters affecting energy partitioning (Jobling, 1994).

1.3 Hormonal Regulation of Teleost Growth

Although environmental parameters affect the growth of fish, many hormonal systems also have direct and important roles in the regulation of growth (Donaldson *et al.*, 1979; Sumpter, 1992). The endogenous control of growth in fish is very complex and involves a considerable degree of interaction between a variety of hormones such as GH, thyroid hormones, insulin, IGFs and sex steroids (Donaldson *et al.*, 1979; Sumpter, 1992). In mammals, GH plays a significant role in growth regulation, a fact adequately illustrated by growth cessation in many animals following removal of the pituitary gland (Sara and Hall, 1990). These growth-promoting actions of GH are mediated by insulin-like growth factor-I (IGF-I), a small polypeptide hormone with potent mitogenic effects (Sara and Hall, 1990). Structurally related, insulin-like growth factor-II (IGF-II) also plays a role in regulating growth, but in earlier stages of development and it is not as stringently regulated by GH as IGF-I (Sara and Hall, 1990).

Growth hormone and IGFs also play roles in the regulation of growth of teleost fish (Donaldson *et al.*, 1979; Bern *et al.*, 1991; Sumpter, 1992; Siharath and Bern, 1993). The pronounced role that GH has in controlling growth in fish has been clearly demonstrated by growth cessation following hypophysectomy (Ball, 1969; Donaldson *et al.*, 1979) and the increase in growth following GH administration (Sumpter, 1992). The detection of IGF molecules in teleosts is more recent (Bern *et al.*, 1991; Siharath and Bern, 1993), hence our understanding of these molecules remains relatively limited in comparison with mammals. Therefore, where necessary, this review will refer to mammalian IGF literature to enable a complete discussion of the GH:IGF-I axis.

Although this chapter focuses on IGFs and the GH:IGF-I axis, the importance of other growth regulatory hormones or the synergistic actions of hormone combinations on body growth of teleosts should not be readily dismissed. Reviews by Donaldson *et al.* (1979), Weatherley and Gill (1987), Sumpter (1992) and Peter and Marchant (1995) provide detailed information regarding hormonal regulators of growth in bony fish.

1.4 Growth Factors

Growth factors are molecules produced by a cell or isolated from a tissue, which display growth-regulatory actions upon other cell types. Early attempts to elucidate the regulatory processes of growth in higher vertebrates resulted in the discovery of many growth factors with mitogenic activity (Sara and Hall, 1990). However, in many cases specialised glands which synthesize and release these growth factors were not identified, thus making the classic endocrine ablation experiments impossible to perform. For this reason, the application of *in vitro* bioassays, performed on cell cultures or tissue slices were primarily used to determine the biological activity of these factors.

1.4.1 Insulin-like Growth Factors (IGFs)

The separate findings of three types of biological activity in serum led to the initial discovery of the IGFs.

Salmon and Daughaday (1957) found that hypophysectomised rats had a defect in the synthesis of cartilage matrix protein, chondroitin sulphate, which was rapidly corrected by the administration of GH *in vivo*. This impairment could not be restored by the administration of GH to cartilage segments in primary tissue culture (Salmon and Daughaday, 1957). In addition, serum from intact rats stimulated *in vitro* [^{35}S]-sulphate uptake into cartilage segments, whereas serum from hypophysectomised rats provided no such stimulation (Salmon and Daughaday, 1957). These findings led to the development of the classic 'somatomedin hypothesis' which proposed that the growth-promoting action of GH was mediated by a serum factor defined as 'sulphation activity' (Salmon and Daughaday, 1957).

Subsequent to the investigations of Salmon and Daughaday (1957), serum factors with insulin-like metabolic actions were identified in rat and human serum (Froesch *et al.*, 1963). Approximately 90% of the insulin-like activity in the serum was not suppressed by anti-insulin serum from guinea pigs and it was therefore termed non-suppressible insulin-like activity (NSILA). NSILA detected in both rat and human

serum (Froesch *et al.*, 1963) demonstrated approximately forty fold higher basal levels than that of insulin (Froesch *et al.*, 1967; Gliemann, 1968; Poffenbarger *et al.*, 1968). As NSILA in serum was not immunologically identical with insulin, the identification of the molecule(s) exhibiting this activity was sought.

The search for serum constituents necessary to sustain cell growth in culture was the basis for the third line of research. Studies by Dulak and Temin (1973) demonstrated that cell proliferation in certain cell lines was dependent upon the presence of specific factors in serum, whereas other cell lines were capable of producing their own growth-promoting substances. One such activity, termed multiplication-stimulating activity (MSA), was purified from serum-free medium conditioned by a Buffalo rat cell line (BRL-3A). This activity was observed to stimulate deoxyribose nucleic acid (DNA) synthesis and multiplication of normal fibroblasts *in vitro*.

As further work was completed, the similarities between these activities suggested that all three activities represented a similar, if not identical group of molecules, with a much broader range of biological activity than originally anticipated. In 1972, the term 'somatomedin' (mediating the actions of somatotropin) was introduced to describe serum factors which stimulated sulphate uptake into cartilage, had non-suppressible insulin-like activity in adipose and diaphragm tissue, and caused increased thymidine incorporation into DNA in various tissues (Daughaday *et al.*, 1972).

1.4.2 Characterisation of IGFs

Following the pioneering work through which these growth-promoting, insulin-like substances were identified, these molecules were purified and characterised. The isolation of the somatomedins proved difficult as their concentration was not particularly high in any particular tissue. Using a method including acid/ethanol extraction, total NSILA (200 kDa) was found to be easily removed from native serum Poffenbarger *et al.* (1968). Three different forms of NSILA were subsequently discovered; a soluble 7.5 (kDa) portion (NSILA-s), a larger portion (NSILA-P) which was found to denature and remain within the precipitate and a protein (NSILP) which was detected following Dowex-chromatography (Poffenbarger *et al.*, 1968). Of the

isolated forms, NSILA-s was observed to exhibit potent growth-promoting effects in chick embryo and other fibroblast cells and to stimulate the sulphation of cartilage in various animals (Zing and Froesch, 1973). The growth-promoting action, molecular size and the high abundance of NSILA-s in serum compared to other tissues, raised the possibility of this substance being a circulating hormone.

Two biologically active peptides showing similar growth-promoting properties to NSILA-s were purified from human blood products (Rinderknect and Humbel, 1978a; 1978b). The amino acid sequence of these activities was found to be 48% homologous with human pro-insulin and this peptide was renamed insulin-like growth factor-I (IGF-I) (Rinderknect and Humbel, 1978a). The second bioactive peptide was structurally similar, but not identical to IGF-I, and was named IGF-II (Rinderknect and Humbel, 1978b). The subsequent sequencing of somatomedin-C (Klapper *et al.*, 1983) and somatomedin-A (Endberg *et al.*, 1984) also showed that they were structurally identical to IGF-I, and the IGF-I and IGF-II nomenclature became universal. Subsequently, IGF-I and IGF-II have been extensively studied in mammals and information on the structure, regulation, function and actions of these molecules has been well documented.

1.4.3 Structure of IGFs

Insulin-like growth factor-I and IGF-II are single chained polypeptide molecules comprised of 70 (7646 kDa) and 67 (7471 kDa) amino acids, respectively and each contains three intra-chain disulphide bridges (Rinderknect and Humbel, 1978a; 1978b). The structural homology between these molecules in humans is 62%, suggesting that the genes coding for these molecules were derived from a common ancestral gene. The structural characteristics of IGFs are similar to pro-insulin, demonstrating 50% homology. Insulin-like growth factors are comprised of an amino-terminal B domain which is separated from the A domain by a C domain which consists of 12 amino acids. In contrast to pro-insulin, the IGF molecules also contain a D domain extension peptide at the carboxy terminus and an E domain. Mature IGF peptides are synthesised by proteolytic cleavage of the E domain, which contrasts to

the synthesis of insulin which is produced by cleavage of the signal peptide and removal of the C domain (Sara and Hall, 1990).

The structure of the IGF molecules appears to be highly conserved throughout vertebrate evolution. Cao *et al.* (1989) cloned IGF-I mRNA from coho salmon, *Oncorhynchus kisutsch*, and reported sequence homology with human IGF-I of approximately 80%. Subsequently, recombinant DNA techniques have been used to identify the nucleotide sequences of the IGF prohormones in Atlantic salmon, *Salmo salar* (Duguay *et al.*, 1992), rainbow trout (Shamblott and Chen, 1992) and chinook salmon (Wallis and Devlin, 1993), and it appears that the structure of the mature IGF-I molecule in these species is identical to that of coho salmon (Cao *et al.*, 1989).

The presence of cDNA coding for an IGF molecule similar in structure to mammalian IGF-II has been reported in rainbow trout (Shamblott and Chen, 1992). In addition, findings of distinct IGF-II-like peptides in the liver of spiny dogfish shark, *Squalus acanthias* (Duguay *et al.*, 1995) and in the endocrine pancreas of the sting-ray, *Raja clavata* (Reineke *et al.*, 1994) suggest that the prototypical IGF molecule duplicated and diverged in an ancestor of the extant gnathostomes (Reineke *et al.*, 1994), thus providing support for the existence of structurally distinct IGF-I and IGF-II molecules in teleost fish.

1.4.4 IGF-I Gene

The IGF-I gene in rats and humans consists of six exons and is distributed over more than 100 kilobases (kb) of genomic DNA (Sussenbach *et al.*, 1992). The exons have been shown to contain the same coding information in both species. Exon 1 and 2 contain multiple sites of transcription initiation, with either exon 1 or 2 being spliced to exon 3. Exons 3 and 4 encode the mature peptide, whilst exons 5 and 6 encode alternate E peptides and 3' untranslated regions (UTRs) (Sara and Hall, 1990). The complexity of the mammalian genes analysed so far explains the presence of multiple mRNA transcripts, which range from 0.8 to 7.5 kb in length. Two cDNAs for IGF-I, known as IGF-Ia and IGF-Ib, have been detected in humans and consist of 153 and 195 amino acids, respectively (Jansen *et al.*, 1983; Rotwein *et al.*, 1986). Upon

characterisation, the first 134 amino acids are identical, with the difference in structure relating to the carboxyl-terminal E domain (Etherton, 1993). The mRNAs which encode for these proteins are the result of alternative splicing of the single primary RNA transcript.

Similarly, two IGF-I genes which produce four alternatively spliced IGF-I mRNA transcripts, known as Ea-1, Ea-2, Ea-3 and Ea-4 have been characterised in chinook and coho salmon (Duguay *et al.*, 1994). Using an RNA protection assay to determine the expression level, the Ea-1 and Ea-3 transcripts were found in abundance in the liver, but were barely detectable in other non-hepatic tissues. Conversely, the Ea-4 transcript was present in both hepatic and non-hepatic tissues (Duguay *et al.*, 1993). Recently, two alternatively spliced transcripts, corresponding in structure to salmonid Ea-2 and Ea-4 were reported in barramundi, *Lates calcarifer* (Kinhult, 1997). The Ea-2 and Ea-4 transcripts appeared to be widely distributed with detection in the liver, kidney, gill, brain, heart and spleen (Kinhult, 1997). Although the function of the multiple IGF-I transcripts is not understood, the expression of alternatively spliced transcripts in rats is associated with development and tissue distribution. A similar role has therefore been suggested for these transcripts in teleost fish (Wallis and Devlin, 1993). Little is known of the IGF-II gene in bony fish.

1.4.5 IGF Receptors

In mammals there are two types of specific IGF receptors. The type I (IGF-I) receptor is structurally similar to the insulin receptor and has been reported as the only IGF receptor to have definite IGF-mediated signaling functions (Jones and Clemmons, 1995). Type I IGF receptors and insulin receptors are homologous in structure and both are down-regulated by IGFs and insulin (Rechler and Nissley, 1985). The type I receptor has a molecular weight of > 300,000 and is composed of disulphide linked 130,00 Da alpha (α) and 90,000 beta (β) subunits (Rechler and Nissley, 1985). The α subunit binds the hormone and the β subunit appears to have intrinsic tyrosine

kinase activity and to be autophosphorylated. Type I receptors bind IGF-I and IGF-II with equal affinity and insulin weakly (Daughaday *et al.*, 1981).

The type II (IGF-II) receptor is different from the type I and insulin receptor in both structure and function (Rechler and Nissley, 1985). Comprised of a 250,00 Da protein that contains internal disulphide bonds but is not linked to other membrane components, the type II receptor demonstrates structural homology with the cation-independent mannose 6-phosphate receptor and is thought to function as a 'clearance' receptor, moving lysosomal enzymes (Jones and Clemmons, 1995). Type II receptors bind IGF-II with 100-fold greater affinity than IGF-I, but do not interact with insulin, even at high concentrations (Scott and Baxter, 1987). Furthermore, they are not down-regulated and although type II receptors appear phosphorylated in intact cells, they do not possess intrinsic protein-kinase activity (Rechler and Nissley, 1985).

In addition to binding with their own receptors, the IGFs also bind to the insulin receptor, but with low affinity. Furthermore, receptor hybrids, composed of insulin and IGF-I subunits have been reported in NIH-3T3 and HepG2 cell lines (Moxham *et al.*, 1989). The hybrid receptors bind the IGFs and reportedly signal cytoplasmically *in vitro* (Jones and Clemmons, 1995).

Receptors for IGF-I have recently been identified in teleost fish. Using wheat germ agglutination-agarose affinity chromatography, IGF-I receptors were detected in the ovary of carp, *Cyprinus carpio*, (Gutierrez *et al.*, 1993), cardiac and skeletal muscle of brown trout, *Salmo trutta*, coho salmon and carp (Gutierrez *et al.*, 1995) and brain of carp and trout (Leibush *et al.*, 1996). A recent study also found IGF-I receptors in the testis of trout (Le Gac *et al.*, 1996). These receptors are presumably type I-like. The specific binding of IGF-I in the skeletal and cardiac muscle of the various teleost species differed from patterns observed in higher vertebrates. In the skeletal muscle, specific IGF-I binding was 2-6 fold higher than insulin binding, contrasting with the prevalent nature of insulin receptors in mammals (Gutierrez *et al.*, 1995). In addition, the specific binding of IGF-I to purified cardiac muscle IGF-I receptors was 20% higher (44 - 68%) in fish heart when compared to rat heart (Gutierrez *et al.*, 1995).

These findings suggest the possibility of functional and structural differences between the IGF-I receptors of teleost fish and mammals. Furthermore, the presence of IGF-I receptors in the reproductive tissues of teleosts may provide support for the role of IGF-I in teleost reproduction (Section 1.4.7.4).

1.4.6 IGF Binding Proteins (IGFBPs)

The likely existence of insulin-like growth factor binding proteins (IGFBPs) was recognised during early attempts to extract IGFs (NSILA) from serum. During attempts to purify IGFs, discrepancies in the molecular size of the whole serum NSILA activity (200 kDa; Poffenbarger *et al.*, 1968) and that of the soluble extract (7.5 kDa; Froesch *et al.*, 1967) led to the proposal that specific carrier proteins existed. Although acidic treatment of the NSILA did not yield the soluble NSILA-s extract, binding proteins which demonstrated high affinity for the IGFs were later characterised. Subsequently, six different IGFBPs have been cloned and sequenced in mammals (Rechler, 1993). As much as 90% of the circulating IGFs in mammals are bound with IGFBP-3 and an acid-labile subunit (ALS), forming the 150 kDa IGF complex (Rechler, 1993). The remaining IGFs either bind with IGFBP-1, -2 or -4 though a small amount (5%) circulates as free peptide (Ketelslegers *et al.*, 1996). The 150 kDa complex stabilises the IGF-I in circulation, preventing the movement to the extravascular compartment and prolonging the half life of the molecule (Clemmons and Underwood, 1991). The IGFBPs are also thought to aid in targeting tissues, since they regulate the transport of IGFs from the vascular to the extracellular compartment and regulate their interaction with the IGF receptors (Clemmons and Underwood, 1991). Details of the regulation and specific functions of the IGFBPs have been detailed by Clemmons and Underwood (1991).

Multiple forms of IGFBPs have recently been reported in teleost serum, however information on their regulation and functional significance remains limited. Three major IGFBPs were detected using Western ligand blotting in the serum of coho salmon, striped bass, the goby, *Gillichthys mirabilis* and Mossambique tilapia, *Oreochromis mossambicus* (Kelley *et al.*, 1992; Siharath *et al.*, 1992; Siharath and

Bern, 1993). Based on the molecular weights of these molecules, the three bands were characterised as fish IGFBP-1 (29 kDa), IGFBP-2 (31 kDa) and IGFBP-3 (40-50 kDa) (Kelley *et al.*, 1992). IGFBPs have also been reported in rainbow trout (Niu and Le Bail, 1993), channel catfish (Delahunty *et al.*, 1993) and golden perch, *Macquariaambigua* (Anderson *et al.*, 1993). The regulation of the IGFBPs in teleosts has recently been reviewed in Siharath and Bern (1993), and generally appears to parallel some of the regulatory mechanisms reported in mammals.

1.4.7 Actions of IGFs

The actions of the IGFs in mammals have been reviewed in detail (Daughaday and Rotwein, 1989; Sara and Hall, 1990) and generally include mitogenic, metabolic and growth-related actions in a variety of cell and tissue types. Insulin-like growth factors have also been shown to be important for skeletal growth, larval development, osmoregulation and reproduction in teleosts (Bern *et al.*, 1991; Siharath and Bern, (1993).

1.4.7.1 Skeletal Tissue

Although many tissues are known to be responsive to the mitogenic actions of IGFs, the hormonal regulation of skeletal tissues is of particular significance in understanding entire body growth. The effect of GH was earlier thought to be mediated by circulating IGF-I of hepatic origin (Daughaday and Rotwein, 1989), however there is recent evidence suggesting that GH also stimulated paracrine and autocrine production of IGF-I in both cartilage and skeletal muscle in mammals (Holly and Cwyfan-Hughes, 1994). This theory is referred to in the current literature as the dual (Green *et al.*, 1985) or multiple (Siharath and Bern, 1993) effector theory of GH action.

The effects of GH on the expression of IGF-I mRNA have also been investigated. Hepatic IGF-I mRNA was observed to increase in a dose-dependent manner following intraperitoneal GH injections in coho salmon (Duan *et al.*, 1994). In salmon, the expression of the alternatively spliced, Ea-1 and Ea-3, IGF-I transcripts in the liver was also found to be GH responsive (Duguay *et al.*, 1994). In contrast, the

expression of the alternatively spliced, Ea-2 and Ea-4, transcripts was not regulated by GH in salmonids (Duguay *et al.*, 1994) or barramundi (Kinhult, 1997).

The effect of IGF-II was investigated in only one study, with rat IGF-II reportably 50 times less potent than IGF-I in stimulating sulphate uptake in intact eels (Duan and Hirano, 1990).

Substantial evidence supports coordinated roles of GH and IGF-I in the regulation of body growth of teleost fish. Ash (1977) reported bovine GH directly increased sulphate incorporation in branchial cartilages of rainbow trout and tilapia (*Tilapia andersoni*). In contrast, porcine GH, thyroxine and somatotropin, alone, or in combination had no effect on rainbow trout gill arch bone, unless liver slices were added (Komourdjian and Idler, 1978). The conflicting results were attributed to the use of cartilage versus bone, with the suggestion of local IGF-I production in cartilage (Siharath and Bern, 1993). Subsequently, Duan and Inui (1990a) found sulphate uptake was restored in hypophysectomised Japanese eel, *Anguilla japonica*, injected with eel GH. The stimulation of sulphate uptake in cartilage by IGF-I has now been reported in coho salmon (McCormick, *et al.*, 1992; Tsai, 1993), Japanese eel (Duan and Hirano, 1990; Duan and Inui, 1990b), the goby (Gray and Kelley, 1991; Kelley, 1993) and the goldfish, *Carrassius auratus* (Marchant and Moroz, 1993). The results from these studies provide clear evidence that the stimulation of cartilage matrix protein synthesis is one mechanism by which GH and IGF-I promote skeletal tissue growth in teleosts.

The administration of GH has been shown to increase the growth rate of teleost fish (Bern *et al.*, 1991). Juvenile brook trout, *Salvelinus fontinalis*, treated with human GH were significantly heavier and longer at three weeks than controls (Skyrud *et al.*, 1989).

As in mammalian studies, the treatment of teleost fish with IGF-I *in vivo* has produced variable growth effects. Recombinant human IGF-I administered weekly for three weeks to juvenile brook trout did not stimulate growth, and the higher doses, 1.0 and 3.0 μg/g body weight, resulted in decreased growth and profound insulin-like effects,

causing hypoglycemia and hypoaminoacidemia (Skyrud *et al.*, 1989) In contrast, McCormick *et al.* (1992) observed an increased growth rate in coho salmon which were treated using bovine IGF-I via mini-pumps and fed a limited ration, however, increased growth rate was not observed in fish given an unlimited food supply. The discrepancy between the two studies has recently been attributed to the method of IGF-I administration (Siharath and Bern, 1993) since IGF-I infusion but not injection stimulates growth in adult rats (Lowe, 1991). A comparison between the studies is difficult since nutritional status, which varied between studies, has profound effects on the *in vivo* effects of IGF-I and may result in differing responses to IGF-I administration.

1.4.7.2 Larval Development

Recent studies have indicated that IGF-I is present during differentiation of juvenile fish. In salmonid species, IGF-I is expressed during embryonic development (Duan *et al.*, 1994; Funkenstein *et al.*, in press) and all tissues were reported to produce IGF-I mRNA transcripts (Duguay *et al.*, 1992; Duan *et al.*, 1994). Funkenstein *et al.* (in press) found IGF-I and IGF-I receptor mRNA in gilthead seabream, *Sparus aurata*, larvae with high expression in the gill arch chondrocytes, skeletal muscle, brain, pancreas and in the epithelial cells surrounding the lens. In barramundi, IGF-I immunoreactivity was found to be age and tissue specific with IGF-I immunoreactive cells found in the retina (42 hr), pectoral fin musculature (57 hr), renal tubule epithelia (9 days), gill cytoplasmic structures (13-28 days), endocrine pancreas (14 days), brain (22 days) and perikaraya (13-28 days) (Richardson *et al.*, 1995). These results indicate that IGF-I plays an important role in regulating cell growth and differentiation of juvenile fish in a variety of tissues.

The presence of IGF-I immunoreactive cells associated within the eye indicates IGF-I may play a role in its development (Richardson *et al.*, 1995; Funkenstein *et al.*, in press). The stimulation of rod-precursor cell proliferation in retinal slices prepared from eyes of 12-15 day juvenile cichlid fish, *Haplochromis burtoni* by both bovine

IGF-I (20 ng/ml) and bovine insulin (5 μg/ml) supported this hypothesis of a role of IGF-I in eye development (Mack and Fernald, 1993).

1.4.7.3 Osmoregulation

Osmoregulation in mammals is achieved largely through the kidney, however in fish the gill also plays a major role. The importance of GH and IGF-I in regulating kidney function in mammals was originally indicated by observations of renal hypertrophy in human patients with acromegaly, and low serum IGF-I in patients with chronic renal failure (Takano *et al.*, 1979). In hypophysectomised rats, GH treatment increases both the kidney weight and the level of IGF-I mRNA expression in the kidney (Rotwein *et al.*, 1989). Similarly, the direct infusion of IGF-I increases kidney weight in rats (Flyvberg *et al.*, 1991; Martin *et al.*, 1991). In humans, both GH and IGF-I stimulate renal plasma flow and glomerular filtration rate and reduce total renal vascular resistance (Guler *et al.*, 1989). These results support the role of GH and IGF-I as regulators of renal function in mammals (Cohick and Clemmons, 1993). There is evidence that GH and IGF-I also play a role in osmoregulation in teleost fish. Growth hormone increased the hypoosmoregulatory ability of juvenile salmonids. One day after transfer to seawater immature rainbow trout GH levels were significantly elevated (Sakamoto *et al.*, 1993). Similarly, IGF-I mRNA expression increased in the gill and kidney of immature rainbow trout following transfer to 80% seawater. Increased levels of IGF-I mRNA were also observed in the liver and gill of coho salmon maintained in freshwater during smoltification.

The administration of IGF-I was also found to effectively enhance saltwater adaptation. Rainbow trout treated with a single dose of bovine IGF-I (0.01, 0.05, 0.2 μg/g) demonstrated a greater ability to maintain plasma osmolarity and sodium following transfer to 80% seawater (McCormick *et al.*, 1991). In a later study, single injections of GH or IGF-I improved ion regulation in Atlantic salmon transferred from 12 ppt to higher salinities (McCormick, 1995). Interestingly, the administration of GH or IGF-I did not effect ion regulation in Atlantic salmon kept in fresh water

(McCormick, 1996). The presence of high levels of circulating prolactin was suggested to cause the inefficiency of GH and IGF-I enhancing freshwater adaptation (McCormick, 1996). Antagonism between prolactin and GH effects on salinity tolerance of rainbow trout and brown trout supports this hypothesis (Madsen and Bern, 1992).

Although the exact mechanisms of GH and IGF-I in seawater adaptation in teleosts are not understood, there is some evidence to suggest that these hormones affect chloride cell and gill Na^+, K^+-ATPase production. The administration of growth hormone to juvenile Nile tilapia, using single (0.025 or 2.5 μg/g) or repetitive (3 x 0.25 μg/g) doses, enhanced seawater adaptability and induced the differentiation of chloride cells towards seawater adaptation (Xu *et al.*, 1996). In brown trout, both GH and cortisol increased gill Na^+, K^+-ATPase, chloride cell numbers and salinity tolerance; the actions of the hormones were synergistic (Madsen, 1990). Growth hormone and cortisol, both alone and in combination, stimulated gill Na^+, K^+-ATPase in Atlantic salmon (McCormick, 1996). An increase in gill cortisol receptors in response to GH in coho salmon (Shrimpton *et al.*, 1995), provides an explanation for the actions of GH alone and in combination with cortisol. Following *in vivo* GH treatment, IGF-I was found to stimulate *in vitro* gill Na^+, K^+-ATPase activity of coho salmon (Madsen and Bern, 1993) suggesting that the effects of GH on gill Na^+, K^+-ATPase are mediated by IGF-I. McCormick (1995) demonstrated that IGF-I and cortisol, in combination, stimulated Na^+, K^+-ATPase in Atlantic salmon, although the effect was less stimulatory than GH and cortisol. In contrast, IGF-I alone did not stimulate Na^+, K^+-ATPase in striped bass (Madsen and Bern, 1993) or in coho salmon gill filaments *in vitro* (McCormick, 1995) with the lack of response attributed to an inappropriate dose of IGF-I and the interference by IGFBPs. The effect of IGF-I on Na^+, K^+-ATPase in teleosts thus remains unclear.

1.4.7.4 Reproduction

IGF-I and IGF-II are potent *in vivo* stimulators of mammalian oocyte maturation (Feng *et al.*, 1988) and evidence is emerging to suggest a similar role in teleosts.

IGF-I (10 mM) induced germinal vesicle breakdown (GVBD) in red seabream, *Pagrus major* (Kagawa *et al.*, 1994), appearing to act directly on oocytes rather than via maturation-inducing hormones. IGF-II (13 mM) also induced GVBD (Kagawa *et al.*, 1994).

IGF-I mRNA is expressed in the ovaries of coho salmon (Duan and Plisetskaya, 1993) and IGF-I immunoreactvity is localised in ovaries of red seabream (Kagawa *et al.*, 1994). Intense IGF-I immunoreactivity appears in the peripheral region of the ooplasm at the primary vitellogenic stage, in the granulosa cells at the lipid stage and the outer layer of the zona radiata, suggesting that the granulosa cell layer is the major site of IGF-I production.

1.5 Nutritional Regulation of IGFs

In mammals, nutritional status causes an insensitivity of the liver to GH thereby altering the normal production of IGF-I, which is mainly derived from the liver in response to GH (Clemmons and Underwood, 1991). Accordingly, low serum IGF-I bioactivity is observed in children with protein-calorie malnutrition, despite elevated GH concentrations (Grant *et al.*, 1973). A significant reduction in IGF-I bioactivity was found in 72 hour fasted rats, compared to fed rats (Phillips and Young, 1976). IGF-I bioactivity was restored by feeding (Phillips and Young, 1976). Similar changes were observed in a variety of species using more definitive IGF-I assay techniques. During periods of nutrient deprivation, IGF-I plasma concentrations were found to be significantly decreased, with respect to fed controls, in man (Musey *et al.*, 1993; Kiddy *et al.*, 1989), growing and pre-weaning rats (Maiter *et al.*, 1988; Phillips *et al.*, 1989; Donovan *et al.*, 1990), sheep (Hua *et al.*, 1993), weaning calves (Breier *et al.*, 1988), steers (Breier *et al.*, 1986), chickens (Morishita *et al.*, 1993), maternal and fetal guinea pigs (Dwyer and Strickland, 1992), dogs (Eigenmann *et al.*, 1985) and teleost fish (Perez-Sanchez *et al.*, 1994; Duan and Plisetskaya, 1993).

The mechanisms by which nutrient deprivation decreases IGF-I is poorly understood but appears rapid and complex. Following 12 and 24 hours of protein restriction,

IGF-I levels were reduced by 58 and 66% respectively in rats (Maiter *et al.*, 1988). Serum GH was not affected by low protein intake and injections of 5 to 100 μg rat GH failed to raise to IGF-I serum concentrations in the protein deficient animals. A 15 to 20% reduction in the number of GH binding sites was observed indicating regulation occurs through post-GH receptor processes. However, regulation occurs upstream of gene expression as reduced levels of hepatic IGF-I mRNA expression (Emler and Schalch, 1987; Straus and Takemoto, 1990) and hypoinsulinemia (Merimee *et al.*, 1982; Cheetham *et al.*, 1994) during fasting, protein and energy deficiency have been reported.

In mammalian systems, IGF-II appears to be less closely regulated by GH and concentrations of IGF-II in serum have been reported to be unchanged by short-term fasting (Davenport *et al.*, 1988) or during restricted feeding (Dwyer and Strickland, 1992). However, due to the limited information, it remains difficult to propose a role for IGF-II in mediating metabolic responses to changes in nutrient intake.

The general principles described above also apply to teleost fish. Short or long-term fasting was found to significantly increase GH levels in tilapia (Rodgers *et al.*, 1992), rainbow trout (Sumpter *et al.*, 1991), juvenile coho salmon (Duan and Plisetskaya, 1993) and gilthead sea bream (Perez-Sanchez *et al.*, 1995). In gilthead seabream fed protein deficient diets for 6 weeks, GH levels increased above those of fish fed diets of higher protein content (Perez-Sanchez *et al.*, 1995). Despite elevated GH levels, total GH binding in gilthead sea bream (Perez-Sanchez *et al*, 1995) and hepatic GH binding in coho salmon (Gray *et al.*, 1992) decreased during periods of food restriction.

Serum IGF-I bioactivity, measured by sulphate incorporation in bone or oral cartilage, was significantly decreased by short-term starvation in coho salmon (Komourdjian and Idler, 1978; McCormick *et al.*, 1992). Impairment of sulphate incorporation into cartilage by liver slices from Japanese eel starved for 2 weeks suggests that the liver is

important in regulating systemic IGF activity (Duan and Inui, 1990b). In addition, serum IGF-I immunoreactivity was significantly reduced by feeding limited ration sizes and restricted protein diets to gilthead sea bream for 6 weeks (Perez-Sanchez *et al.*, 1995). In contrast, Drakenberg *et al.* (1989) found no effect of starvation on IGF-I receptor binding activity in whole serum of tilapia, however this may be attributed to the relatively short period of fasting.

Available information suggests nutritional factors affect IGF-I synthesis at least at the translational level in teleosts. Hepatic IGF-I mRNA, 4 kb, decreases following two weeks starvation in the Japanese eel (Duan *et al.*, 1994). In coho salmon, a reduction in the levels of the hepatic, alternatively spliced, IGF-I mRNA transcripts Ea-1 and Ea-3 was found following two weeks starvation with re-feeding restoring levels to control values (Duan *et al.*, 1994). However, fasting did not affect the levels of the universally distributed Ea-4 transcript (Duan and Duguay, 1994).

1.6 Measuring IGFs

In view of the complexity of the IGF system in mammals with two peptides, two receptors- one of which shows cross-reactivity in binding between the two peptides, and at least six different binding proteins described, measurement of IGFs requires considerable care. Care must also be shown in the interpretation of data generated by the assays. A number of methods have been employed to measure IGFs, developing as the knowledge of the molecules has expanded.

1.6.1 Bioassays

Originally, bioassays were used to detect the IGFs. These were based on sulfate or thymidine incorporation into cartilage (Daughaday *et al.*, 1975; Almquist, 1961), insulin-like activity in non-skeletal tissues (Labhart, 1963) or growth-promoting activity in cultured cells (Frykland *et al.*, 1974). As the bioactivity of IGF in the tissues results from the net interactions between IGF, IGFBPs and other components such as proteases (Holly and Cwyfan-Hughes, 1994) the interpretation of the bioassay results was complex and this was recognised as the major disadvantage of these

assays. It is also difficult to mimic the IGF system *in vitro*, since other serum constituents may be foreign to the target cells *in vivo* and cultured cells can be made to produce abnormal patterns of IGFBPs and proteases (Holly and Cwyfan-Hughes, 1994). The problems with the interpretation of IGF bioassays and the characterisation and production of purified mammalian IGFs led to the development of competitive binding assays, such as radioreceptor (RRA) and radioimmunoassays (RIA).

1.13.2 Radioreceptorassays for IGF-I and IGF-II

The discovery of distinct type I and type II IGF receptors in various mammalian tissues and cell types enabled the development of specific radioreceptor assays (RRA) for IGF-I and IGF-II (Marshall *et al.*, 1974; Megeysi *et al.*, 1975). The RRAs allowed routine and precise measurements of the IGF molecules, overcoming the problems associated with bioassays.

Human placenta was the first tissue shown to have receptors for IGFs (Marshall *et al.*, 1974). Two structurally dissimilar types of IGF binding sites were found in particulate human placental preparations (Kasuga *et al.*, 1981). The type I receptor bound IGF-I preferentially and was thought to resemble the insulin receptor structurally. Studies using affinity labelling and SDS-PAGE demonstrated that the type I receptor, like the insulin receptor, was composed of two α and two β subunits linked together by disulphide bonds. The type I receptor from human placenta bound IGF-I and IGF-II with equal affinity (Daughaday *et al.*, 1981). The second receptor found in human placental membranes, the type II receptor, differed in structure and function from the type I and insulin receptor (Daughaday *et al.*, 1981; Kasuga *et al.*, 1981). The human placenta type II receptor consisted of a single polypeptide chain containing interlinking disulphide bonds. Functionally, the type II receptor bound IGF-II preferentially and exhibited low affinity for IGF-I, approximately 10 % of that of IGF-II. Insulin did not bind the type II receptor at any concentration.

Type II receptors were also found in rat placental and rat liver membranes, however their abundance differed in comparison with human placental membrane preparations

(Daughaday *et al.*, 1981; Pilistine *et al.*, 1984; Scott and Baxter, 1987). Using competitive binding techniques, Daughaday *et al.* (1981) reported that the specific binding of IGF-I in rat placental membranes did not exceed 3.3%, but displayed a high abundance of type II receptors. Similarly, Scott and Baxter (1987) found rat liver microsomal membranes to be a very abundant source of high affinity IGF-II receptors. The type II receptors found in rat placental and rat liver membranes displayed high specificity for IGF-II, a lower affinity for IGF-I and no affinity for insulin.

The detection and characterisation of IGF receptors in various membranes preparations was integral to the development of RRA. Daughaday *et al.* (1981) used human placental membrane preparations as a matrix in RRA by developing the first RRA used to measure IGFs. Since the ligand [^{125}I]hIGF-I was displaced by both IGF-I and IGF-II, this assay was considered capable of detecting both IGF-I and IGF-II in serum samples. The high abundance of the type II receptors in different rat tissues allowed the development of RRA specific to IGF-II. Using [^{125}I]hIGF-II as a ligand and type II receptors from rat placental membranes as a matrix, a RRA specific for IGF II activity was developed (Daughaday *et al.*, 1981). In this assay IGF-I was less than 1% effective in displacing [^{125}I]hIGF-II. Similarly, a specific IGF-II RRA was also developed using rat liver membranes as the matrix (Scott and Baxter, 1987).

1.6.3 Radioimmunoassays for IGFs

Following purification of IGFs in sufficient amounts, radioimmunoassays for measuring IGFs were developed. The development and validation of radioimmunoassays for IGF-I have been reviewed by Breier *et al.* (1991) and Blum and Breier (1994).

1.7 Competitive Binding Assays for IGFs - Problems and Pitfalls

A number of problems resulting from the complexity of the IGF system require considerable care to avoid artifacts in IGF assays. Two problems warranting attention in this review are IGFBP interference and heterogeneity.

1.7.1 IGFBP Interference

Inaccurate measurement of IGFs by competitive binding assays occurs when IGFBPs are present in samples. Binding proteins bind IGFs with high affinity (Kd 10^{-10} to 10^{-11} mol/l) and, if present during the assay, cause severe interference by competing for the tracer with the antibody or receptor. Dissociation of the endogenous IGF from the IGFBPs and removal of the IGFBPs from the sample are therefore considered essential for pre-assay preparation (Bang *et al.*, 1994).

Parallel displacement curves are not, in themselves, satisfactory evidence of the lack of interference by IGFBPs. Parallel displacement curves were obtained for extracted and non-extracted serum in both the IGF-I (Moriyama *et al.*, 1994) and IGF-II RIA (Gentil *et al.*, 1996). These results suggest that the presence of IGFBPs, under the conditions in which the fish were held, did not interfere in the assay systems or interfered only weakly, and IGFBP extraction was not required. These authors concluded that the antibody used bound to a part of the IGF which was different from that which bound IGFBPs. An alternative explanation may be that the conditions of the fish from which the samples were drawn resulted in an absence (or substantial decline) of IGFBPs. It is well documented that the IGF:IGFBP ratio changes under different physiological and pathological conditions, causing the interference by IGFBPs in the RIA to also differ (Breier *et al.*, 1991; Bang *et al.*, 1994; Blum and Breier, 1994). Whilst Gentil *et al.* (1996) reported significant differences between IGF-II levels in extracted and unextracted serum from fasted rainbow trout, extraction had no effect on IGF-I concentrations from serum taken from parr, smolt and adult coho salmon (Moriyama *et al.*, 1994). Further work is therefore required to fully determine the effect of IGFBPs under different physiological states in homologous RIA systems.

Interference by IGFBPs is dependent on the amount, type, concentration and affinity of the IGFBPs with respect to the free IGF-I and the amount and affinity of the antiserum or receptor being used (Holly and Cwyfan-Hughes, 1994). Since these parameters vary markedly with physiological conditions, species and type of body

fluid or conditioned cell media from which the sample was obtained, it is considered essential to perform assay validation with the actual experimental samples and the particular assay procedure involved (Bang *et al.*, 1994; Blum and Breier, 1994; Holly and Cwyfan-Hughes, 1994).

Techniques to reduce the interference of IGFBPs in competitive binding assays were based upon the discovery that the IGF:IGFBP interaction is dissociable under acidic conditions (Hintz and Liu, 1977). Extraction therefore relies upon IGFBPs being dissociated from the IGFs under acidic conditions and then being physically separated from each other prior to assay. The most commonly reported methods are size exclusion chromatography under acidic conditions (Zapf *et al.*, 1981; Powell *et al.*, 1986), solid-phase extraction including SEP-PAK columns (Daughaday *et al.*, 1987), acid ethanol extraction (Daughaday *et al.*, 1980), acid-ethanol cryo-precipitation (Breier *et al.*, 1991), formic acid extraction (Bowsher *et al.*, 1991) and sephacryl extraction (Moore *et al.*, 1993). Of these methods, size exclusion chromatography under acidic conditions has been accepted as the most reliable and effective extraction procedure. However, the time-consuming and laborious nature of the extraction and the cost of an effective column are considered to make it unsuitable for routine assays (Blum and Breier, 1994; Holly and Cwyfan-Hughes, 1994). Alternative methods which are based primarily on the use of organic solvents to precipitate the IGFBPs have been developed to allow multiple samples to be extracted easily and quickly. The acid-ethanol procedure reported by Franklin *et al.* (1976) and validated for use in human sera by Daughaday *et al.* (1980) utilised ethanol to precipitate the IGFBPs. Other methods which relied upon other combinations of acid and solvent were also adopted and reportedly improved the original IGFBP removal technique (Kaufmann *et al.*, 1979; Mesiano *et al.*, 1988; Moore and Mylek, 1993). Unfortunately, co-precipitation of IGF-I with the IGFBPs resulted in underestimation of IGF concentrations (Blum and Breier, 1994). In addition, acid-ethanol and/or formic acid extraction left considerable amounts of residual IGFBPs in sera obtained from different pathological states, biological fluids, species or developmental stages

(Mesiano *et al.*, 1988; Blum and Breier, 1994; Holly and Cwyfan Hughes, 1994). Acid gel chromatography is therefore considered the most rigorous extraction method.

1.7.2 IGF Heterogeneity

As IGFs have species specific structure, competitive binding assays for IGFs are also complicated when a heterologous competitive binding assay is used. Heterologous IGF-I RIAs have been used to measure IGF-I in a wide range of species (Dawe *et al.*, 1988; Hansson *et al.*, 1988; Ballard *et al.*, 1990; Bautista *et al.*, 1990; McGuiness and Cogburn, 1990; Bern *et al.*, 1991; Harding *et al.*, 1994). The cross-reactivity in these heterologous assay systems was found to be variable for different species. Furlanetto *et al.* (1977) measured circulating IGF-I in rats using a human IGF-I RIA but chicken IGF-I cross-reacted by only 50% in a similar human IGF-I RIA (Dawe *et al.*, 1988; Ballard *et al.*, 1990). The discrepancy in the cross reactivity was attributed to the heterogeneity of the IGF-I molecule, since rat and chicken IGF-I differ from human IGF-I by 3 (4.3%) and 8 (14.3%) amino acids, respectively. The influence of low immunological cross-reactivity of IGF-I has also been attributed to sequence differences in particular domains of the IGF molecule (Wilson and Hintz, 1982).

Variability in assay response using heterologous IGF assays for measurement in teleost plasma confounded early studies. Using mammalian RRA or RIA, circulating IGF-I-like activities were detected in tilapia (RRA only) (Drakenberg *et al.*, 1989), gilthead seabream (Funkenstein *et al.*, 1989), Atlantic salmon (Lindahl *et al.*, 1985), rainbow trout and golden perch (Anderson *et al.*, 1993). However using a similar RIA protocol, but different antibodies, no significant activity was detected in the serum of Atlantic bluefish, *Pomatomus salteris* (Furlanetto *et al.*, 1977) or common carp (Wilson and Hintz, 1982). IGF-II like activities were detected in rainbow trout using a human IGF-II RIA (Daughaday *et al.*, 1985; Bautista *et al.*, 1990), but not in golden perch (Anderson *et al.*, 1993) using similar methodology. It would appear that structural differences result in lowered cross-reactivity and, in extreme cases, a lack of

measurable IGF-like activity. However, heterologous RRA seem more robust than heterologous RIA for undertaking heterologous assays (Anderson *et al.*, 1993).

1.8 Barramundi, *Lates calcarifer*

Barramundi, *Lates calcarifer* (Bloch) is a popular recreational fish and highly valued food source distributed throughout the coastal rivers of the Indo-pacific region (Grey *et al.*, 1986). Demonstrating a complex life history, males migrate downstream during the monsoonal season to spawn with resident females in estuaries. Following the first or subsequent spawning seasons, most males undergo sex inversion to become breeding females. The ability of this species to tolerate salinity changes and its rapid growth rate makes it an excellent model for growth studies. These features, along with its popularity as a table fish have also been recognised by commercial culturists and led to the rapid expansion of the aquaculture industry for barramundi. In Queensland alone, barramundi production increased 56% during the early 1990's (Lobegeiger, 1994). However, increased production has depressed the market value of this species, making strategies to optimise growth rates and thereby reduce production costs of increasing importance for the economic survival of many barramundi farmers.

Increasing growth rates in culture is limited by a deficiency in the fundamental understanding of growth regulation in barramundi. An investigation of barramundi in five major rivers systems in Northern Australia demonstrated marked differences in growth rate within and between rivers (Davis and Kirkwood, 1984). Although the variable growth rates were attributed to differing environmental conditions, the information obtained from this study provided only preliminary data to understanding the impact of exogenous factors on growth regulation in this species. Research into barramundi has focused on the reproductive cycle (Moore, 1979; Davis, 1982), fecundity (Davis, 1984; Davis, 1985), distribution (Renolds, 1978; Grey, 1986), feeding habits (Russell and Garett, 1983), migration (Renolds, 1978; Davis, 1986; Moore, 1980), diet (Williams, pers. comm.) and only recently IGFs (Richardson *et al.*, 1995; Drakenberg, 1996; Upton *et al.*, 1996; Kinhult, 1997; Matthews *et al.*,

1997). There remains a need for detailed investigation into the physiological mechanisms underlying growth in this species.

Most studies of IGFs have been conducted in temperate species, predominantly the salmonids. Barramundi differ from salmonid species as they are endemic to tropical waters, demonstrate a very rapid growth rate and have diploid chromosomal arrangement. They occupy a more advanced phylogenetic position (Romer, 1966), belonging to the spiny rayed order Acanthopterygii, rather than the soft rayed order of Isopondyli (salmonids). Although recent studies have provided evidence for the presence of IGFs in barramundi, the mechanisms regulating these molecules remain poorly understood in most fish and have not been documented for this species. Understanding the impact of environmental factors on IGF production and regulation will provide information into the growth regulatory mechanisms in this species and also extend the current literature documenting the GH:IGF axis in teleost fish.

1.9 Aims

The aims of the present study were:

a) To detect circulating IGF-I and IGF-II-like activities in barramundi using heterologous RRAs and to validate the assays to allow routine measurement.

b) To determine the effect of food availability (ration size) on IGFs in barramundi at the pre- and post-translational level by measuring circulating IGF-I and IGF-II-like activities and IGF-I messenger RNA in the liver and a non-hepatic tissue (brain).

c) To determine the effect of dietary protein and energy content on the growth, proximate composition and regulation of circulating IGF-I and IGF-II-like activities in barramundi.

CHAPTER 2:

GENERAL MATERIALS AND METHODS

2.1 INTRODUCTION

The methods described in this chapter refer to those generally used throughout the study. Additional methodology or modifications to general techniques, which are specific to the work described in a particular chapter, have been described within that chapter.

Chemical or scientific names and suppliers have been stated in full when first used in a chapter and thereafter appear abbreviated. All abbreviations have been listed on page xvii, preceeding Chapter 1. Buffer solutions used throughout the study have been cited by the buffer name only. The buffer constituents of cited buffers have been listed in Appendix 1.

2.2 Experimental Animals

Juvenile barramundi (~100 g) were obtained from commercial barramundi culturists (Hart Fisheries, Townsville, Australia; Barramundi Waters, Innisfail, Australia or North Queensland Barramundi, Townsville, Australia) and transferred to aquaculture facilities at James Cook University of North Queensland, Townsville, Australia. Fish were held individually in 70 litre tanks supplied with re-circulated fresh water at approximately 60 litres/hr and continous aeration. Unless otherwise stated, water temperature was maintained at 28 ± 1 °C. Light was supplied by overhead fluorescent lamps, regulated to provide a 12 hr light: 12 hr dark photoperiod. Water quality was maintained by particulate and biological filtration, with ammonia, nitrite and nitrate levels routinely measured using standard test kits (Sanbrook Distributors, Townsville, Australia). Fish were fed twice daily to satiety using commercial barramundi pellets (Aquafeed, Brisbane, Australia), unless otherwise stated. All experimental regimes were conducted within the guidelines of "The Australian code of practice for the care and use of animals for scientific purposes" and received ethical clearance by James Cook University of North Queensland animal ethics committee (Approval number A250).

2.2.1 Growth Parameters

Percent body weight increase (% BWI), specific growth rate (SGR), condition factor (CF) and apparent food conversion ratio (FCR) were calculated using equations 2.1, 2.2, 2.3 and 2.4 respectively (Winfree and Stickney, 1981; Hanley (1991).

$$\text{Equation 2.1} \quad \text{Percent body weight increase (\%BWI)} = 100 \times \frac{\text{BW final (g)} - \text{BW initial (g)}}{\text{BW initial (g)}}$$

$$\text{Equation 2.2} \quad \text{Specific growth rate (SGR) (\%)} = \frac{\ln \text{BW final (g)} - \ln \text{BW initial (g)}}{\text{Time (days)}}$$

$$\text{Equation 2.3} \quad \text{Condition Factor (CF)} = \frac{10^5 \times \text{BW (g)}}{\text{Length (mm)}^3}$$

Note: BW refers to the body weight of an individual fish.

$$\text{Equation 2.4} \quad \text{Apparent Food Conversion Ratio (FCR)} = \frac{\text{Wet weight gain (g)}}{\text{Dry food offered (g)}}$$

2.2.2 Anaesthesia

Barramundi were anaesthetised by immersion in a 70 litre tank containing 150 parts per million (ppm) benzocaine (ethyl-p-amino benzote-E1501, Sigma Chemical Company, Castle Hill, Australia). Benzocaine was initially dissolved in approximately 50 ml of absolute ethanol (AR Grade, BDH Chemicals, Kilsyth, Australia) and was injected below the surface of the water using a 20 ml syringe to prevent precipitation. Anaesthesia was achieved within 2-5 min and was considered to be adequate when opercular and fin movements ceased. Following sampling, fish were returned to a 70 litre aquarium containing fresh water, where complete recovery occurred within 5 min.

2.2.3 Blood Sampling and Serum Preparation

Unless otherwise stated, sampling was carried out between 0800 and 1200 hr, and blood was taken following anaesthesia. Blood was obtained from the caudal sinus with a 23 gauge needle and 1 ml syringe and stored on ice in a 1.5 ml microtube for 2-4 hr to allow clotting. Following clotting, serum was prepared by centrifugation in a

bench centrifuge (Eppendorf, Hamburg, Germany) at 14,000 g for 10 min. The serum was transferred into a clean 1.5 ml eppendorf tube and unless otherwise stated, processed immediately for assay use.

2.3 Chromatography

Serum samples were fractionated by size exclusion high performance liquid chromatography (HPLC) at pH 2.8. The apparatus consisted of an automatic injector (WISP 710B, Waters, Milford, USA), pump (Waters 510), Ultraspherogel-SEC 2000 molecular sieve column (Beckman, Melbourne, Australia), ultraviolet (UV) detector (Waters 486) and FC203 fraction collector (Gilson, Middleton, USA). The system was controlled by a programmable system controller (Waters Maxima 280) adapted for use on an IBM compatible, Microbee 386SX computer.

2.3.1 Size Exclusion Chromatography of Serum

Size exclusion HPLC of serum samples was conducted at pH 2.8 using minor modifications of the methods described by Owens *et al.* (1990). Serum samples (150 µl) were diluted in 300 µl double distilled water (dd H_2O), acidified using 150 µl 4x trimethylamine (4x TMA) buffer (Appendix 1) at pH 2.8 and incubated for 20 min at room temperature. Acidified samples were defatted by the addition of an equal volume (600 µl) of Freon (1, 1, 2 trichlorofluroethane, Ajax Chemical Company, Queensland, Australia), vortexed and centrifuged at 14,000 g for 10 min. An aliquot (200 µl) of the aqueous layer was applied to an Ultraspherogel-SEC 2000 molecular sieve column equilibrated at a flow rate of 1 ml/min with trimethylamine (TMA) buffer (Appendix 1) at pH 2.8. Fractions (0.5 ml) were collected every 0.5 min for 30 min and neutralised by the addition of 150 µl neutralised TMA buffer (Appendix 1) and 300 µl of 0.4 M Tris. Aliquots (300 µl) of each fraction were assayed in triplicate for IGF-like activities using type I and type II radioreceptor assays (Section 2.6). A chromatogram of the absorbance peaks at 280 nm of each sample was also recorded.

2.3.2 Calibration of Size Exclusion Chromatography Column

Molecules of known molecular weight were used to calibrate the size exclusion column. The substances used were bovine serum albumin (BSA) (Sigma Chemical Company), carbonic anhydrase (CA) (Sigma Chemical Company), cytochrome C (CYT) (Sigma Chemical Company), iodinated human insulin-like growth factor-I ([^{125}I]hIGF-I) (Amersham, Castle Hill, Australia), iodinated human insulin-like growth factor-II ([^{125}I]hIGF-II) (Amersham) and iodinated human insulin ([^{125}I]insulin) (ICN Biomedicals, Seven Hills, Australia). The molecular weights of BSA, CA, CYT, [^{125}I]hIGF-I, [^{125}I]hIGF-II and [^{125}I]insulin were 66 kDa, 29 kDa, 12.4 kDa, 7.65 kDa, 7.47 kDa and 5.3 kDa, respectively. Dextran blue (DB) (Sigma Chemical Company) was used to determine the void volume of the column. The molecular weight of DB was 2000 kDa.

2.3.2.1 Calibration With Pigmented Molecules

The elution volumes of 200 µl aliquots of 5 mg/ml solutions of CYT and DB were determined by coloration in the fractions. The delay between the UV detector and fraction collector was corrected using the equation (Equation 2.5) described in the Gilson Fraction Collector User's Guide. The delay time was calculated as 0.4 min.

$$\text{Equation 2.5} \quad \text{Delay time (min)} = \frac{\text{Area of tubing (cm}^2\text{)} \times \text{Length of tubing (cm)}}{\text{Flow rate (ml/min)}}$$

2.3.2.2 Calibration With Radioactive Peptides

Radioactive peptides were dissolved in RRA buffer (Appendix 1) at pH 7.4 to obtain working solutions containing approximately 4000 counts per minute (Cpm) in 200 µl. The elution volumes of 200 µl aliquots of [^{125}I]hIGF-I, [^{125}I]hIGF-II, and [^{125}I]insulin were determined by counting the amount of radioactivity in each fraction. Following collection, fractions were quantitatively transferred into 5 ml tubes and counted for 1 min/tube using a gamma counter (Packard Series 5000, Packard, USA).

The allowance of 0.4 min was used to correct the elution volume for the fraction collector delay.

2.3.2.3 Calibration With Ultraviolet Detection

The elution profile of 200 µl aliquots of 1 mg/ml (w:v) solutions of carbonic anhydrase and BSA were determined by ultraviolet (UV) detection at 280 nm. The chromatograms were recorded and the elution volume of the molecule was determined from the peak UV absorbency.

2.4 Microsomal Membrane Preparation

Microsomal membranes prepared from human placenta and rat liver were used as the source of IGF receptors for the RRAs. The placental and liver microsomal membranes were prepared using the methods described by Marshall *et al.* (1974) and Cuatrecasas (1972).

2.4.1 Preparation of Human Placental Membranes

Term placentae (349 ± 50 g) were collected immediately following delivery from the Kirwan Women's Hospital (Townsville, Australia) and transported on ice to James Cook University. Placentae were washed in unbuffered, chilled 250 mM sucrose and prepared according to Marshall *et al.* (1974). Briefly, the trophoblast was dissected from the chorion and amnion, placed into fresh 250 mM sucrose and cut into small pieces with dissection scissors. Placental pieces were blended in three, 20 sec bursts with an upright blender (Phillips HR1376 Blender, Phillips, Sydney, Australia) at low speed. An equal volume of chilled 250 mM sucrose was added to the blended trophoblast prior to homogenization using fifteen, 10 sec bursts at high speed with an Ultra-Turrax homogeniser (Janké and Kunkel, Germany). The membranes were then purified according to a differential centrifugation method described by Cuatrecasas (1972). The crude homogenate was filtered through two layers of nylon gauze, split evenly into two centrifuge buckets and centrifuged at 4° C for 10 min at 600 g using a Beckman J2-MC centrifuge with JA-20 rotor (Beckman, USA). The 600 g

supernatant was decanted and centrifuged at 12,000 g for 40 min using the above centrifuge, rotor and temperature. Following centrifugation, the 12,000 g supernatant and loose white interface between the supernatant and pellet were decanted, combined and brought to 100 mM NaCl and 0.2 mM $MgSO_4$ in a total volume of approximately 350 ml. The mixture was stored at 4° C for 9 hr prior to ultracentrifugation at 105,000 g for 60 min at 4° C using a Beckman L3-50 ultracentrifuge and type 50 Ti rotor (Beckman, USA). The 105,000 g supernatant was discarded and the pellet was resuspended on ice in approximately equal volumes of 50 mM Tris, pH 7.4, using a Dounce homogeniser. The resuspended pellet was re-centrifuged at 105,000 g as previously described. The pellets containing the particulate microsomal membranes were resuspended as above and pooled to make a total volume of approximately 100 ml. This resuspension was divided into 0.5 ml aliquots, frozen at -80 °C, lyophilised using a Dynavak FD6 Freeze Drying Unit (Dynavak, Sweden) and stored at -80 °C until use.

2.4.2 Preparation of Rat Liver Membranes

Rats of the Wistar strain (220 - 280 g) were kindly provided by the Department of Molecular Sciences, James Cook University of North Queensland, Townsville, Australia. Rats were housed at 2 - 4 rats per cage at an ambient temperature of 24 ± 5° C, 50-70 % humidity and a 12 hr light: 12 hr dark photoperiod. Animals were fed a standard rat chow *ad libitum* and were allowed free access to water. Rats received a sharp blow to the head and were sacrificed by either immediate decapitation or cervical dislocation. The livers were immediately dissected, weighed and placed into a chilled, unbuffered 250 mM sucrose solution on ice.

The preparation of the rat liver microsomal membranes was performed as described in Section 2.4.1, with the following modifications. The homogenization of the hepatic tissue was performed using an upright blender at high speed for three, 2 min bursts only. The homogenate was not filtered through nylon gauze prior to centrifugation.

The final pellet was resuspended in 3 ml of 10 mM Tris-HCl, pH 7.4, per 10 g of original tissue.

2.4.3 Microsomal Membrane Protein Determination

The microsomal membrane preparations were analysed for protein using a modified Folin-Lowry method, described in Clark and Switzer (1977). Standards (50-1000 µg/ml) were prepared using lysozyme from chicken egg white (Sigma Chemical Company). Lyophilised aliquots (0.5 ml) of placental or rat liver microsomal membranes were resuspended in 1.5 ml of RIA buffer (Appendix 1) at pH 7.4 and vortexed. Ten 150 µl aliquots were placed into individual vials and increased to 1 ml total volume with dd H_20. Distilled water and RIA buffer blanks were prepared using 1 ml dd H_20 or 150 µl RIA buffer respectively in a total volume of 1 ml dd H_20. Alkaline copper reagent (Appendix 1) was freshly prepared and 6 ml was added to each tube whilst stirring with a vortex mixer. The reaction was allowed to proceed 10-15 min at room temperature following which, 0.3 ml of Folin-Ciocalteu reagent (Sigma Chemical Company) was added to each tube, which was immediately vortexed. The absorbance of the protein standards and assay tubes was measured at 500 nm using a Milton Roy 1201 Spectrophotometer (FSE, USA) and the protein content of the samples were derived from regression equations of the standard curve.

2.5 Preparation of Peptides and Radioligands

Recombinant human IGF-I (hIGF-I) and recombinant human IGF-II (hIGF-II) were purchased from Gropep (Adelaide, Australia). The lyophilised peptides (100 µg) were individually reconstituted in 0.1 M acetic acid (6.665 ml) and stored as 50 µl aliquots at -20 °C until use. Prior to use, one 50 µl aliquot was diluted to a working assay concentration (0.5 ng/µl) using a total volume of 1500 µl of RRA buffer (Appendix 1) at pH 7.4.

Iodinated (3-[^{125}I]iodotyrosyl), human IGF-I ([^{125}I]hIGF-I (285 Ci/g, Cat. no. IM172)) and IGF-II ([^{125}I]hIGF-II (285 Ci/g, Cat. no. IM238)) were obtained from Amersham Life Sciences (Castle Hill, Australia) or Dupont-NEN® (Sydney, Australia). The lyophilised peptides were resuspended in 500 µl of 0.1 M acetic acid, vortexed and stored as 20 µl aliquots at -20° C until use. Prior to assay use an appropriate amount of RIA buffer was added to produce a working concentration ranging between 4.2 x 10^3 and 6.5 x 10^3 Cpm/100 µl.

2.6 Radioreceptor assays

Type I and type II RRAs were used for the detection of circulating IGF-like activities.

2.6.1 Type I RRA

The type I RRA was performed using human placental membranes as the matrix and [^{125}I]hIGF-I as the ligand. Following a method described previously by Anderson *et al.* (1993), 100 µl of [^{125}I]hIGF-I, 100 µl human placental membranes (50 µg/100 µl protein) resuspended in chilled RRA buffer (Appendix 1) and 300 µl standard unlabelled hIGF-I (0.037-80 ng IGF-I/ml) diluted in chilled RRA buffer or 300 µl column eluent were incubated for 16 - 20 hr at 4 °C. Three additional tubes, total counts (TC), total binding (TB) and non-specific binding (NSB) were prepared in triplicate. The total counts tubes contained 100 µl of [^{125}I]hIGF-I only. TB and NSB tubes contained either zero or excess amounts of hIGF-I, respectively. Volume differences in TB and NSB tubes were corrected by the addition of neutralised TMA buffer (Appendix 1), prior to the addition of 100 µl of [^{125}I]hIGF-I, 100 µl human placental membranes and incubated as described previously. Following incubation, receptor bound [^{125}I]hIGF-I in all assay tubes, except TC, was precipitated by the

addition of 300 µl chilled RRA buffer and centrifuged at 4000 rpm (Beckman G6-6R centrifuge, Beckman, Australia) for 30 min at 4 °C. The supernatant was aspirated and the pellet was resuspended in 300 µl chilled RRA buffer and re-centrifuged as detailed above. Following the second aspiration the amount of radioactivity in each tube was measured using a gamma counter (Packard Series 5000, Packard, USA). Specific binding (R) was calculated according to Equation 2.6 (Channing-Rodgers, 1981).

$$\text{Equation 2.6} \quad \text{Specific Binding (R)} = \frac{\text{Sample (Cpm)} - \text{NSB}}{\text{TB} - \text{NSB}}$$

The concentration of IGF-like activity in each fraction was calculated according to Channing-Rodgers (1981). Specific binding values were converted to logit values according to Equation 2.7 (Channing-Rodgers, 1981) and concentrations derived using the regression equation of a log-logit transformed hIGF-I standard curve.

$$\text{Equation 2.7} \quad \text{Logit Value} = \ln\left(\frac{R}{1-R}\right)$$

2.6.2 Type II RRA

The type II RRA utilised rat liver microsomal membranes as the matrix and [^{125}I]hIGF-II as the ligand. The type II RRA was performed identically to the type I RRA detailed in Section 2.6.1, with the substitution of [^{125}I]hIGF-II, hIGF-II and rat liver membranes for [^{125}I]hIGF-I, hIGF-I and human placental membranes, respectively.

CHAPTER 3:

DETECTION AND VALIDATION OF IGF ACTIVITY AND IGF-BINDING PROTEINS IN BARRAMUNDI.

3.1 INTRODUCTION

Insulin-like growth factors play important roles in regulating proliferation, differentiation and specific functions in a variety of cell types (Section 1.4.7). Although other growth factors may be involved in these processes, the IGFs are particularly interesting since they elicit their actions via endocrine, paracrine and autocrine pathways (Section 1.4.7). The IGFs are also considered relatively easy to measure as their concentrations in biological fluids are higher than most other growth factors under normal physiological conditions (Sara and Hall, 1990). The combination of these traits supports the measurement of IGFs in biological fluids as a tool for understanding the regulatory mechanisms of animal growth and development. The techniques used for measuring IGFs and the validation of these methods will be discussed in this introduction.

3.1.1 Measurement of Circulating IGFs

Circulating IGF peptides are most commonly measured on a routine basis using competitive binding techniques such as RRA and RIA. Whilst the details of RRA and RIA methods for detecting IGF-I and IGF-II have been previously discussed (Sections 1.6.2 and 1.6.3), the advantages and disadvantages of RRA and RIA, and of heterologous and homologous assays will be addressed.

The methodologies for RRA and RIA are similar. Both assays require unlabelled IGF to be bound with a specific matrix and the subsequent displacement of the unlabelled IGF by a radioligand, labelled IGF (Channing-Rodgers, 1981). The major difference between RRA and RIA is the use of receptors or antibodies, respectively, as the binding matrix. The major advantages and disadvantages of heterologous RRA and RIA are therefore based on the ease by which the binding matrix can be prepared and the specificity of the binding matrix. The preparation of IGF receptors from microsomal membranes for RRA is easily achieved with minimal cost. Type I and type II receptors can be prepared easily and reliably from a range of tissues or cell lines (Section 1.6.2). In contrast, the preparation of specific antibodies against a species-specific IGF molecule for RIA is often a lengthy and costly process, reliant upon the

prior purification or sequencing of the IGF molecule. However, the cost of the antibodies for RIA, rather than the preparation time, is probably more frequently encountered by the modern researcher, since antibodies for a range of mammalian IGF-I and IGF-II are now commercially available. Another major difference between RRA and RIA is the sensitivity of the assay. RIA are generally considered to be more sensitive than RRA, as the antibodies used are raised against a specific, purified IGF molecule of known sequence (Breier *et al.*, 1991; Blum and Breier, 1994). The high sensitivity of the RIA is advantageous as it enables reliable quantification of the IGF molecule subject to validation. As a result of the specificity of an antibody, its use in heterologous RIA is not generally reliable as small sequence differences in IGF structure may result in the matrix being unable to bind to the IGF molecules of different species (Wilson and Hintz, 1982; Daughaday *et al.*, 1985; Dawe *et al.*, 1988; Ballard *et al.*, 1990). Receptors are not as sensitive to changes in molecular sequence and therefore heterologous RRA can be used for the detection of IGF from a wide range of species, although the sensitivity of the assay may be compromised.

The nature of the binding system of RIA and RRA also offers certain advantages and disadvantages. Heterologous IGF assays are complicated by the unknown affinity of the IGF molecule for the receptor or antibody, due to the heterologous nature of the competitive binding system. This can be overcome by the use of a homologous tracer and standard, allowing accurate and quantitative measures (Ballard *et al.*, 1990; Moriyama *et al.*, 1994; Gentil *et al.*, 1996; Upton *et al.*, 1996). The major disadvantage of homologous IGF assays is the requirement for the IGF molecule of the species of interest to be purified or produced by recombinant techniques. Therefore, heterologous IGF RRA and RIA, post-validation, remain extremely important tools for the measurement of circulating IGF levels in species where purification of the IGF-I or IGF-II molecule is unlikely or incomplete. In all cases, both homologous and heterologous assays require rigorous validation before results can be considered reliable (Bang *et al.*, 1994).

3.1.2 Measuring IGFs in Teleost Serum

Since the quantification of circulating IGF-I and IGF-II in homologous RIA for IGF-I and IGF-II is more sensitive and provides absolute values post-validation, the use of homologous assays for the teleost species of interest is preferred. However, IGF-I or IGF-II purification or recombinant production has been reported in few teleost species (Moriyama *et al.*, 1993; Gentil *et al.*, 1996; Upton *et al.*, 1996). Only two homologous assays for IGFs have been reported in fish, RIA for coho salmon IGF-I (Moriyama *et al.*, 1994) and rainbow trout IGF-II (Gentil *et al.*, 1996). Moriyama *et al.* (1994) reported 100-fold higher concentrations of IGF-I using a homologous salmon RIA when compared to those measured with a human IGF-I RIA. This is perhaps not surprising, since human RIA have failed to detect IGF-I in a number of teleost species (Furlanetto *et al.*, 1977; Wilson and Hintz, 1982).

A lack of cross-reactivity has been reported when human IGF peptides are used in RIA for teleost IGFs or when teleost sera are applied to RIA for mammalian IGFs. The sequence homology between salmon and human IGF-I was determined to be approximately 80% (Cao *et al.*, 1989). The structural difference between these molecules meant that recombinant salmon IGF-I did not cross-react in a RIA for human IGF-I (Moriyama *et al.*, 1994). Similarly, human IGF-I and IGF-II did not cross-react in the rainbow trout IGF-II RIA (Gentil *et al.*, 1996). In an attempt to explain the specificity of the salmon IGF-I RIA, Moriyama *et al.* (1994) attributed the sensitivity to structural variation in particular domains of the IGF molecule. This hypothesis was supported by the large sequence differences between salmon and human IGF-I in the C (41.7%) and D (50%) domains, compared with smaller sequence differences in the A (3.4%) and B (4.8%) domains.

The salmon IGF-I RIA was not sensitive to structural differences between IGF molecules of some other teleost species. Using the salmon IGF-I RIA, serial dilutions of plasma from coho salmon, Atlantic salmon, sockeye salmon, *Onchorhynchus nerka*, rainbow trout, Mossambique tilapia, common carp, Atlantic halibut,

Hippoglossus hippoglossus and the American eel, *Anguilla rostrata* were parallel to the binding curve of the salmon IGF-I (Moriyama *et al.*, 1994). It should be noted that the IGF-I molecules of all salmonids studied to date are highly homologous. These results suggest that the salmon IGF-I antibodies demonstrated similar affinity the IGF-I molecule of these other teleost species. Similar results were documented using the rainbow trout IGF-II RIA (Gentil *et al.*, 1996). The displacement curves of plasma from Couch's seabream, *Pagrus pagrus* and common carp were parallel to that of rainbow trout IGF-II (Gentil *et al.*, 1996) although serial dilutions of plasma from Nile tilapia and sheatfish, *Silurus glanis,* were not parallel to rainbow trout IGF-II and plasma from the European eel, *Anguilla anguilla* did not cross-react (Gentil *et al.*, 1996).

The sensitivity to structural variations of heterologous RIA relative to that of RRA has also been previously reported. Using a human IGF-I RIA, Drakenberg *et al.* (1989) were unable to detect IGF-I in serum from Mossambique tilapia. The inability to detect IGF-I in this study was attributed to the sequence differences between the tilapia and human IGF-I molecules. In the same study, however, IGF-I was detected in tilapia serum using a human IGF-I RRA (Drakenberg *et al.*, 1989). Similarly, Anderson *et al.* (1993) reported IGF-like activity from golden perch in a type I RRA but much lower levels in a human IGF-I RIA. Thus, heterologous RRA may be considered more robust than the heterologous RIA. In this regard, heterologous RRA are extremely useful for assays in species from which IGF molecules have not previously been detected or characterised.

Although heterologous assays for IGFs show certain limitations it is unlikely that their use in teleost studies will be extinguished rapidly. This is due to the abundant availability of commercial mammalian IGF peptides, the lack of teleostean species-specific recombinant IGF peptides and the limited availability of commercial or other piscine IGF antibodies. Furthermore, it is unlikely that recombinant IGFs will be produced for more than a few teleost species. Provided strict validation procedures are

used to determine the appropriateness of the assay protocol for the species of interest, heterologous IGF assays may be used for detecting changes in circulating levels of IGFs or investigating the presence of IGF molecules in teleost species where the sequence of the IGF molecule has not been determined.

3.1.3 Requirements of IGF Assay Validation

The major problems associated with the use of competitive binding assays, both heterologous and homologous are the specificity of the assay system and interaction by IGFBPs (Section 1.7.1). Assay validation needs to be performed in all IGF assays to determine the appropriateness of the assay system for measuring IGF molecules in a particular species (Bang *et al.*, 1994).

Stringent mechanisms for validating assays for IGF measurements in biological fluids have been documented and five requirements outlined (Bang *et al.*, 1994). The first requirement is the demonstration of parallelism between serial dilutions of plasma samples and the IGF standard curve. Although parallelism is not an absolute indication that IGFBPs are not causing interference it does provide a strong indication and is routinely performed (Bang *et al.*, 1994). Parallelism is also used in heterologous IGF RRA to demonstrate the ability of the heterologous IGF peptide to compete with the ligand for the binding matrix. The second stage of the validation is acidification of the samples followed by size exclusion chromatography at acidic pH. Fractions collected during the chromatography are neutralised and assayed. Residual IGFBPs appear as false peaks of immunoreactivity at an elution volume corresponding to the molecular weight of the IGFBPs (24 - 50 kDa) and indicate dissociation and separation of the binding proteins from the circulating peptides. The addition of unlabelled peptide to samples, known as spiking samples, prior to chromatography and the subsequent recovery of the peptide in the assay is the third requirement for validation. A high recovery of the unlabelled peptide and a low variability are considered essential to demonstrate that the chromatography is reliable. As the ratio of IGF to IGFBPs changes under different physiological conditions the fourth

requirement of validation is that IGF levels are determined in samples which represent the variable IGF:IGFBP ratios. The final stage of assay validation requires demonstration of complete removal of IGFBPs from the extracted sample using Western ligand blots or other suitable techniques. Once all five requirements are satisfied, the RRA or RIA is considered valid for determining circulating IGF levels. However the validation is only applicable for the species and under the physiological conditions specified during validation (Bang *et al.*, 1994).

3.1.4 Assay Selection for Barramundi

At the commencement of the present study, information regarding IGFs in barramundi was extremely limited. The presence of IGF-I or IGF-II in serum or any tissue of barramundi had not been reported and as such, no assays for measuring IGF-I and IGF-II-like activities had been validated for this species. Although this information has widened substantially in the last couple of years (Section 1.8) and recombinant barramundi IGF-I is now comercially available (See note below) (Upton *et al.*, 1996), no homologous RIA for barramundi IGF-I or IGF-II was available at the commencement of this study. Therefore, the application and validation of heterologous IGF RRA or RIA were the only feasible options for measuring IGF-I and IGF-II in the present study. Two heterologous RRA were selected since they were considered more robust to structural differences in the IGF molecules than RIAs for mammalian IGFs and were thought more likely to be able to differentiate between the two peptides.

(Note: Although recombinant barramundi IGF-I was produced in milligram quantities (Upton *et al.*, 1996) during the later stages of the present study, the initial stock was not available to the author (Upton, pers. comm.). Subsequently, during the final preparation of this thesis, recombinant barramundi IGF-I was made commercially available (Gro-Pep, Adelaide, Australia) and experiments are now being conducted by the author to determine the cross-reactivity of barramundi IGF-I in the type I and type II RRA used in the present study).

3.1.5 Aims

The aims of the present study were:

1. To detect IGF-I and IGF-II-like activities in the serum of juvenile barramundi using two heterologous IGF RRA.

2. Upon detection, to validate both heterologous IGF RRA according to the validation protocol described by Bang *et al.* (1994), enabling routine measurement of circulating IGF-I and IGF-II-like activities from barramundi.

3. To determine the presence of IGFBPs in barramundi serum using a heterologous IGF-binding protein assay and Western ligand blot techniques.

3.2 MATERIALS AND METHODS

3.2.1. Fish, Sampling and Serum Preparation

Barramundi were held according to conditions described in Section 2.2. Fish were anaesthetised and blood samples were taken as described in sections 2.2.2 and 2.2.3. Serum was prepared and fractionated using size exclusion HPLC at pH 2.8 (Sections 2.2.3 and 2.3.1 respectively).

3.2.2 Detection of IGF Activity

IGF-like activities in all fractions collected were measured using type I and type II RRA as described in sections 2.6.1 and 2.6.2, respectively. Modifications to both RRA were used to enable validation of the detection procedure. In the modified RRAs, 200 µl aliquots of a serum sample from individual fish were fractionated using size exclusion HPLC at pH 2.8 (Section 2.3.1). Fractions were collected every 0.5 min for 30 min and a 158 µl aliquot of each fraction neutralised by the addition of 50 µl TMA and 100 µl of 0.4 M Tris-HCl. Neutralised aliquots were assayed for IGF-like activity using a type I RRA as described in Section 2.6.1 except that the incubation was performed at 25 °C for 2 hr. The shortened incubation time allowed the fractions containing IGF-like activity to be identified prior to pooling. The elution profiles from each fractionation were subsequently aligned according to peaks of IGF-like activity. The remainder of each fraction was pooled for all separations. Pooled fractions (final volume 1710 µl) were neutralised by the addition of 500 µl neutralised TMA buffer (Appendix 1) and 100 µl 0.4 M Tris-HCl. Pooled neutralised fractions (2310 µl) were assayed in triplicate for IGF-I (3 x 300 µl) and IGF-II (3 x 300 µl) using type I and type II RRA as described in sections 2.6.1 and 2.6.2. Fractionation and assaying were repeated for 30 fish.

3.2.3 Cross-Reactivity

Microsomal membranes were prepared as described in sections 2.4. The cross-reactivity of hIGF-I and hIGF-II was determined in both the type I and type II RRA. The displacement of either [^{125}I]hIGF-I or [^{125}I]hIGF-II by hIGF-I, hIGF-II, bovine pancreatic insulin (BP Ins) (Sigma Chemical Company, Sydney, Australia) and human monocomponent insulin (HM Ins) (Commonwealth Serum Laboratories CSL-Novo, NSW, Australia) was determined for both the human placental and rat liver membranes. Standard concentrations (0.037 - 180 ng/ml) of hIGF-I, hIGF-II, BP Ins and HM Ins were prepared with chilled RRA buffer (Appendix 1).

3.2.3.1 Scatchard Analysis

Scatchard analysis was used to estimate the apparent dissociation constant (K) for the binder-ligand reaction and the binding capacity (B_{max}) in the assay systems (Scatchard, 1949). Bound and free fraction data for Scatchard analysis were calculated and plotted according to Channing-Rodgers (1981). The molecular weights used in the Scatchard calculations were 7.649 kDa and 7.471 kDa for hIGF-I and hIGF-II, respectively (Rinderknecht and Humbel, 1978a; 1978b). The specific activity of both [^{125}I]hIGF-I and [^{125}I]hIGF-II was 285 Curie/g. The counting efficiency of the Packard Series 5000 gamma counter was assumed to be 0.7. Scatchard analysis was conducted for hIGF-I and hIGF-II in the type I RRA and hIGF-II in the type II RRA.

3.2.4 Parallelism

Parallelism between human IGF reference curves and barramundi serum was determined. Fourteen 200 µl defatted serum samples were fractionated using size exclusion HPLC at pH 2.8 (Section 2.3.1). An 158 µl aliquot of each fraction was neutralised by the addition of neutralised TMA buffer (50 µl) and 0.4 M Tris (100 µl). Each neutralised aliquot was assayed for IGF-like activity using the modified type I RRA as described in section 3.2.2.

The elution volumes of fractions demonstrating IGF activity were determined and pooled according to the bars (1 - 4) indicated on Fig. 3.1. Pools 3 and 4 (final volume 7 ml) were subsequently used for determining parallelism. Pools 3 and 4 were stored at -80 °C for 24 hr prior to lyophilisation. The two pools and a blank sample, containing 7 ml TMA buffer (pH 2.8), were lyophilised using a Dynavac FD6 Freeze Drying Unit (Dynavak, Sweden) and resuspended in 6780 µl of 3:2 (v:v) TMA buffer : 0.4 M Tris solution. Four serial dilutions of pools 3 and 4 and the blank were prepared with neutralised TMA buffer and assayed in triplicate for IGF-like activities using type I and type II (Sections 2.6.1 and 2.6.2).

3.2.4.1 Statistical Testing of Parallelism

Linear transformations of the hIGF-I and hIGF-II reference curves and the serially diluted pooled serum samples were derived using log-logit plots (Section 2.6.1). The slopes of the log-logit plots were determined using regression analysis using SPSS Version 4.0 (Language Systems Corporation, Chicago, USA). Parallelism between the dilution series and the IGF reference curves was tested using a t-test of slopes (Zar, 1984).

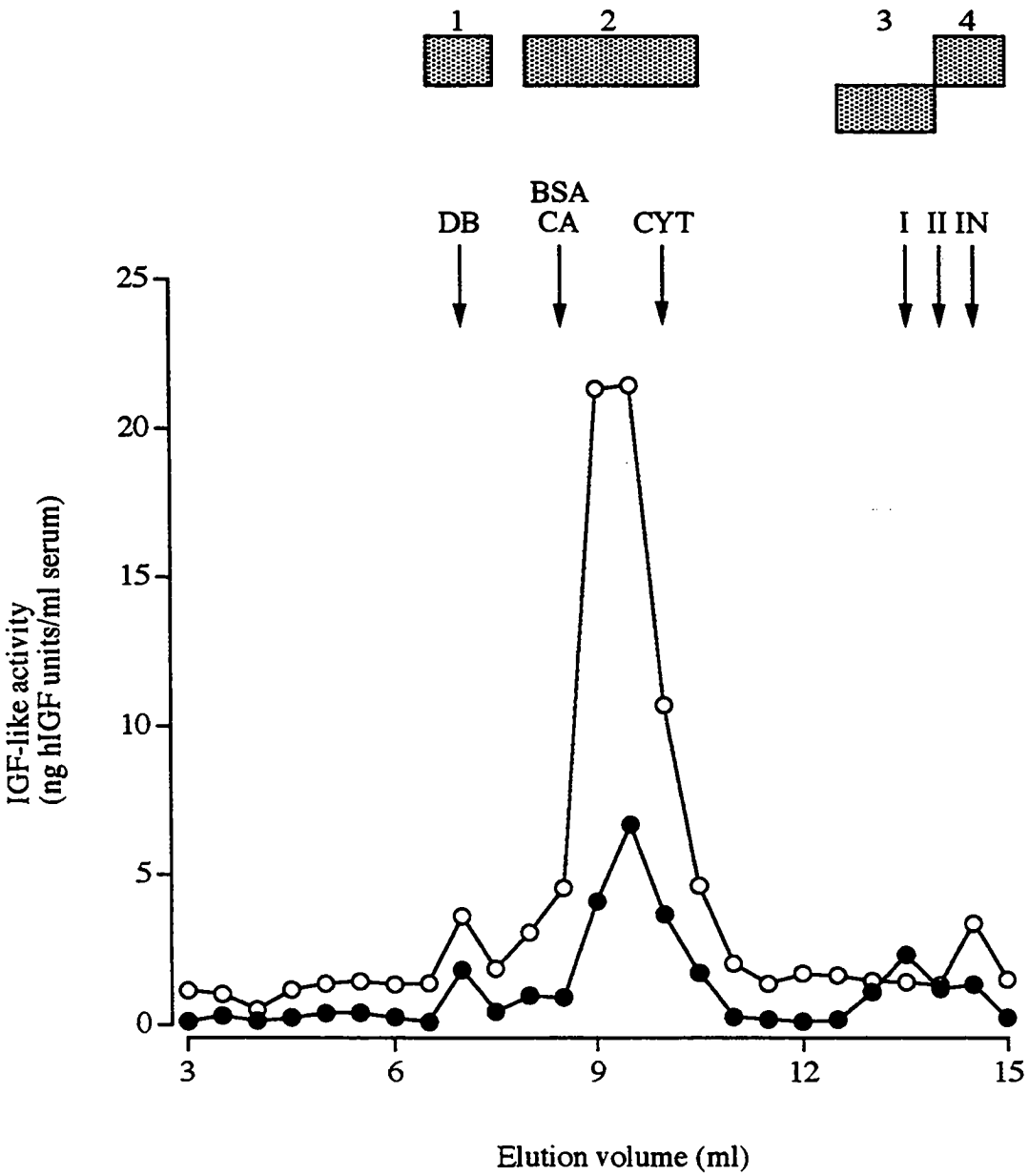
3.2.5 Quantitative Recovery

To determine quantitative recovery, 40 ng/ml of either hIGF-I or hIGF-II were added to 200 µl barramundi serum samples and incubated at 4 °C for 2 hr. Samples were acidified and subjected to size exclusion HPLC (Section 2.3.1) and assayed using type I and type II RRA (Sections 2.6.1 and 2.6.2). Inter- and intra-assay coefficients of variation were calculated using triplicate tubes containing 5 ng/ml of either hIGF-I or hIGF-II for type I and type II RRA, respectively.

3.2.6 Storage

Serum samples were stored at -80 °C for 7 or 30 days to assess the stability of the IGF-like activities. Serum samples were prepared and fractionated (Sections 2.2.3 and

Figure 3.1 IGF-I and IGF-II-like activity detected in 0.5 ml fractions of fresh barramundi serum using a type I (●) or type II (○) RRA. Fractionation was achieved using size exclusion chromatography at pH 2.8. Mean IGF activity values (n=3) are expressed as ng of human IGF-I or IGF-II equivalents, for type I and type II RRA, respectively. The bars marked 1, 2, 3 and 4 indicate the fractions pooled for storage and parallelism testing. The arrows indicate the elution volumes for dextran blue (DB), bovine serum albumin (BSA), carbonic anhydrase (CA), cytochrome C (CYT), [^{125}I]hIGF-I (I), [^{125}I]hIGF-II (II) and human [^{125}I]insulin (IN).


1
2
3
4
DB
BSA
CA
CYT
I
II
IN
25
20
15
10
5
0
3
6
9
12
15
IGF-like activity
(ng hIGF units/ml serum)
Elution volume (ml)

2.3.1) and fractions were pooled according to Fig. 3.1. The amount of activity in pools 1 - 4 was determined using the type I RRA (Section 2.6.1). Data were analysed using a one-way analysis of variance (ANOVA) using SPSS Version 4.0.

3.2.7 IGF Binding Protein (IGFBP) Characterisation

3.2.7.1 IGF Binding Protein Assay

The presence of IGFBPs in barramundi serum was determined using a binding protein assay described by Anderson *et al.* (1993). Serum was prepared for IGFBP analysis either as detailed in section 2.2.3 or, alternatively, following the addition of 7 mg/ml of aprotinin (Sigma Chemical Company, Sydney, Australia), a known serine protease inhibitor, thought to stabilise IGFBPs in biological fluids (Fielder *et al.*, 1990; Binoux *et al.*, 1991). Serum samples prepared with (+A) or without (-A) aprotinin were defatted prior to the IGFBP assay by the addition of equal amounts of Freon. Defatted serum samples were vortexed, centrifuged at 14,000 g for 10 min and the aqueous serum layer retained for the IGFBP assay. A 45 µl aliquot of the aqueous serum layer was incubated with either [^{125}I]hIGF-I (~5000 Cpm/100 µl) or [^{125}I]hIGF-II (~5000 Cpm/100 µl) and 100 µl RRA buffer (pH 7.4) for 12 hr at 4 °C. Following incubation, the serum mixture was fractionated as described in Section 2.3.1 except the pH of the mobile phase used was 7.4. Fractions were collected every 0.5 min for 30 min and later transferred quantitatively into 5 ml tubes for counting. Triplicate aliquots of 100 µl [^{125}I]hIGF-I or [^{125}I]hIGF-II were measured in order to quantify total counts (TC). Radioactivity was measured using a Packard Series 5000 (Packard, USA) gamma counter. Data were expressed as a percentage of the counts per minute (Cpm) recovered per TC added to the incubation (% Cpm recovered).

3.2.7.2 Acid-Ethanol Extraction

The use of an organic solvent to precipitate IGFBPs following dissociation of the IGF/IGFBP complex with acid has been documented as an alternative method to

HPLC for the dissociation and removal of IGFBPs from biological fluids (Section 1.7.1). The efficiency of this method was tested by acid-ethanol extraction of serum samples prior to assaying for IGFBPs (Section 3.2.7.1). Acid ethanol extraction was conducted using a method described by Daughaday *et al.* (1980). Briefly, 100 µl serum samples were mixed thoroughly with 400 µl of an acidic solution containing 87.5% (v:v) ethanol and 12.5% (v:v) 2 M HCl. The tubes were incubated at room temperature for 30 min and centrifuged at 4 °C for 15 min at 1500 g. A 100 µl aliquot of the supernatant was neutralised with 40 µl 0.855 M Tris, stored at 4 °C for 2 hr and re-centrifuged at 4 °C for 5 min at 11,000 g. The IGFBP assay was performed as described in section 3.2.7.1, using 45 µl of the acid-ethanol extracted aqueous layer.

3.2.8 Electrophoresis and Western Ligand Blots

The molecular weight of barramundi serum IGFBPs was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western ligand blotting using methods described by Laemmli (1970), Hossenlopp *et al.* (1986) and Towbin *et al.* (1979).

3.2.8.1 SDS-PAGE

SDS-PAGE was performed using a modified method of Laemmli (1970) using 10% acrylamide separating gels and 3.5 % acrylamide stacking gels, under non-reducing conditions. Each separating gel (7.9 cm x 5.0 cm) consisted of 4x Tris buffer, pH 8.8 (1250 µl), 10 % SDS (50 µl), distilled H_2O (2002 µl), 30 % acrylamide (29.2 % (w:v) acrylamide and 0.8 % (w:v) N, N'-*bis*-methylene acrylamide, 1666.5 µl), tetramethlethylenediamine (TEMED, 6.25 µl) and 10 % (w:v) ammonium persulphate (APS, 25 µl). Each stacking gel (7.9 cm x 4.0 cm) consisted of 4x Tris buffer, pH 6.8 (625 µl), 10 % SDS (25 µl), distilled H_2O (1525 µl), 30 % acrylamide (29.2 %

(w:v) acrylamide and 0.8 % (w:v) N, N'-*bis*-methylene acrylamide, 325 µl), TEMED (3 µl) and 10 % (w:v) APS (13 µl). Gels were cast using Mini-PROTEAN® Electrophoresis Casting Stand (Bio-Rad, North Ryde, Australia).

A human blood sample, collected from a healthy male aged 27 years, was used as the control biological fluid. ^{14}C-Bovine serum albumin (^{14}C-BSA) (24.0 mCi/g, NEN-Dupont, North Ryde, Australia), ^{14}C-ovalbumin (^{14}C-OV) (3.8 mCi/g, NEN-Dupont) and ^{14}C-carbonic anhydrase (^{14}C-CA) (0.02 mCi/mg, NEN-Dupont) were used as molecular weight markers on the autoradiograph. The molecular weights of ^{14}C-BSA, ^{14}C-OV and ^{14}C-CA, were 66 kDa, 45 kDa and 29 kDa, respectively. In addition to the radioactive molecular weight markers, low molecular weight, pre-stained standards (LMWS, Biorad, North Ryde, NSW, Australia) were used as transfer controls during Western blotting. Human or barramundi serum samples (15 µl) were denatured by the addition of 85 µl sample buffer (Appendix 1) followed by immersion in a boiling waterbath for 5 min. The ^{14}C-labelled molecular weight markers were denatured using the same process, except 100 µl of sample buffer (Appendix 1) was added to 10 µl of each marker. The LMWS (10 µl) were placed in a 40 °C waterbath for 1 min. Human (20 µl) or barramundi (25 µl) samples, ^{14}C-labelled molecular weight markers (20 µl) and LMWS (10 µl) were added to individual sample wells and electrophoresis was performed with electrode buffer (Appendix 1) as the running buffer and a constant current of 60 mA/gel for approximately 45 min.

3.2.8.2 Western Ligand Blotting

Western ligand blotting was performed using a modified method of Towbin *et al.* (1979). Following electrophoresis, gels were removed from the electrophoresis apparatus, soaked in 3-[cyclohexlamino]-1-propanesulfonic acid (CAPS) buffer

(Appendix 1) for 5 min and placed on a Protran BA85, 0.45 μm nitrocellulose membrane (Medos Company, Victoria, Australia) equilibrated with CAPS buffer. The gel-nitrocellulose membrane assembly was sandwiched between two sheets of Whatman 3MM filter paper (Medos Company, Burwood, Australia) and two Scotch-Brite pads (Biorad, North Ryde, NSW, Australia) saturated with CAPS buffer. Proteins were transferred from the gel to the nitrocellulose membrane using a Mini-PROTEAN® Trans-Blot Cell (Biorad, North Ryde, NSW, Australia) under constant voltage of 30 V for 12 hr.

3.2.8.3 Autoradiography

Following blotting, the nitrocellulose sheet was washed by sequential immersion in Tris-saline-N buffer (Appendix 1) for 10 min, followed by 2 hr in Tris-saline-B buffer (Appendix 1) and finally 10 min in Saline-T buffer (Appendix 1). The membranes were incubated with 4 x 10^6 Cpm of [^{125}I]hIGF-I overnight at 4 °C in sealed plastic bags with 3 ml of 0.15 M NaCl at pH 7.4. After incubation, the membranes were washed with 3 x 15 min immersions in Saline-T buffer (Appendix 1). The sheets were air dried for 1 hr, exposed to Fuji-mex autoradiographic film between two Kodak X-Omatic intensifier screens for 10 days at -80 °C then developed.

3.3 RESULTS

3.3.1 Detection of IGF Activity

Following acidic size-exclusion chromatography, various forms of IGF-like activity were identified in barramundi serum using type I and type II RRA. Using a type I RRA, four IGF-like activities were detected in size-excluded barramundi serum (Fig. 3.1). Three of these peaks were also detected by the type II RRA (Fig. 3.1).

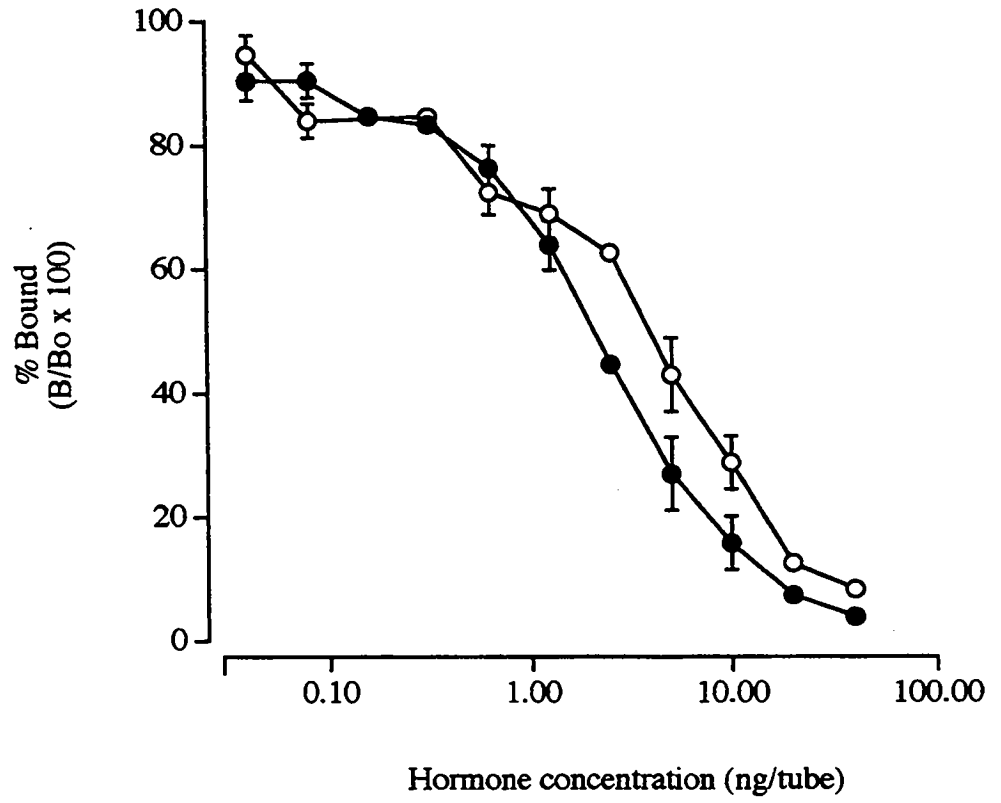
The largest molecular weight activity, detected by both assays eluted at a void volume corresponding to a molecular weight of > 66 kDa. This activity was equivalent to 1.9 ng hIGF-I/ml serum in the type I RRA or 3.7 ng hIGF-II/ml serum in the type II RRA. The major peak of IGF-like activity eluted at a volume corresponding to 12.3 - 29 kDa and was equivalent to 6.6 ng hIGF-I/ml serum in the type I RRA and 21.2 ng hIGF-II/ml serum in the type II RRA. The third peak of IGF-like activity, which was only detected in the type I RRA, eluted at the same volume as [^{125}I] hIGF-I ($\approx$ 7.8 kDa) and displayed 2.4 ng hIGF-I/ml serum. A fourth peak of IGF-like activity, equivalent to 3.7 ng hIGF-II/ml serum using the type II RRA was similar in molecular size to hIGF-II ($\approx$ 7.6 kDa). This peak displayed 1.3 ng hIGF-I equivalents/ml serum in the type I RRA.

3.3.2 Cross-Reactivity of Peptides

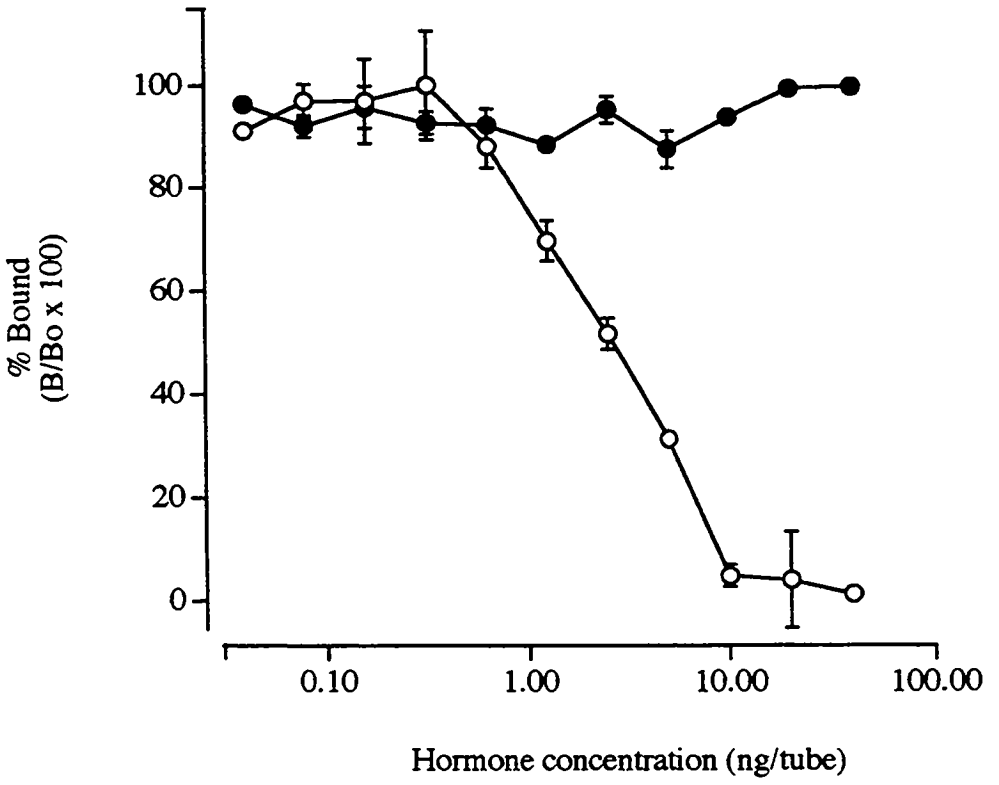
The cross-reactivity of human placental and rat liver microsomal membrane preparations by hIGF-I and hIGF-II are shown in Fig. 3.2a and Fig. 3.2b respectively. At concentrations ranging from 0.78-80 ng/tube, hIGF-II was found to be equipotent to hIGF-I in the type I RRA (Fig. 3.2a). In contrast, hIGF-I did not cross react in the type II RRA (Fig. 3.2b). Furthermore, no cross reactivity was observed by either BP Ins or HM Ins in either the type I or type II RRA (Data not shown). Unfortunately, recombinant barramundi IGF-I was not available (Note: section 3.1.4) for use in the present study (Upton, pers. comm.).

Figure 3.2 The displacement of (a) [^{125}I]hIGF-I from human placental membranes (50μg protein) and (b) [^{125}I]hIGF-II from rat liver membranes (50μg protein) by IGF-I (●), IGF-II (○). Values are expressed as mean (n=3) ± standard deviation.

(a)


100
80
60
40
20
0
% Bound
(B/Bo x 100)
0.10
1.00
10.00
100.00
Hormone concentration (ng/tube)

(b)


100
80
60
40
20
0
% Bound
(B/Bo x 100)
0.10
1.00
10.00
100.00
Hormone concentration (ng/tube)

3.3.3 Scatchard Analysis

Scatchard analysis showed the equipotent displacement of [^{125}I]hIGF-I by hIGF-I and hIGF-II in the type I RRA (Fig. 3.3a). The apparent dissociation constants (K), for hIGF-I and hIGF-II in the type I RRA were within the same order of magnitude, being 3 x 10^{-9} M and 2 x 10^{-9} M, respectively (Fig. 3.3a). Furthermore, the binding capacities (B_{max}) for hIGF-I and hIGF-II in the type I RRA were estimated as 2.9 pg/μg protein and 3.1 pg/μg protein, respectively. The scatchard plot of hIGF-II in the type II RRA revealed a linear relationship (Fig. 3.3b). The apparent dissociation constant and estimated B_{max} for hIGF-II in the type I RRA were 0.8 x 10^{-9} M and 6.5 pg/μg protein, respectively.

3.3.4 Parallelism

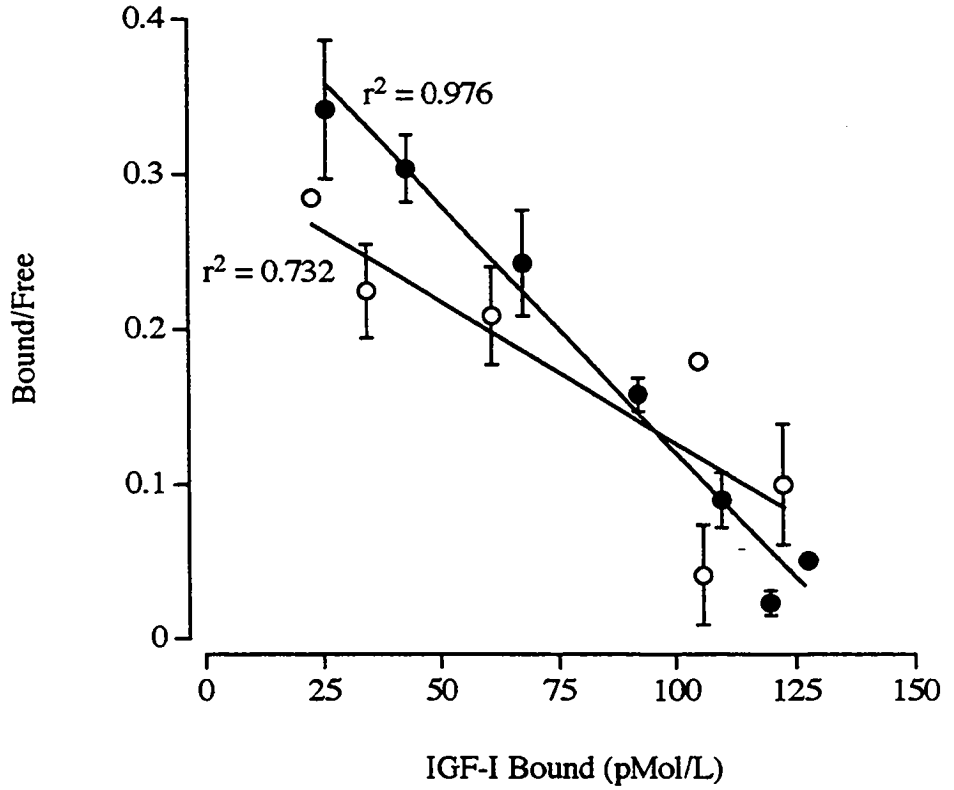
The slope of the hIGF-I and hIGF-II reference curves and the binding curves for the diluted serum samples were not significantly different ($p \geq 0.05$) in both the type I (Fig. 3.4a) and type II RRA (Fig. 3.4b).

3.3.5 Quantitative Recovery

In the type I RRA the recovery of hIGF-I and hIGF-II was found to be 99 ± 2.7 % and 97 ± 3.4 % respectively (Table 3.1). The recovery of IGF-II in the type II RRA was 89 ± 3.9 % (Table 3.1). Estimates of intra- and inter-assay variability were respectively 1.7 ± 0.3 % and 8.2 ± 2.1 % for the type I RRA, and 3.9 ± 0.6 % and 8.27 ± 2.8 % for the type II RRA (Table 3.1). The limit of sensitivity for measurement of IGF-I and IGF-II-like activities in size-excluded barramundi serum was 0.315 ng IGF-I/tube for the type I RRA and 0.625 ng IGF-II/tube for the type II RRA.

Figure 3.3 Scatchard plot of the binding of (a) hIGF-I (●) and IGF-II (○) by human placental membranes (50μg protein) using [^{125}I]hIGF-I as the ligand (b) IGF-II (○) by rat liver membranes (50μg protein) using [^{125}I]hIGF-II as the ligand. Values are expressed as mean (n=3) ± standard deviation.

(a)


0.4
0.3
0.2
0.1
0
Bound/Free
r² = 0.976
r² = 0.732
0
25
50
75
100
125
150
IGF-I Bound (pMol/L)

(b)

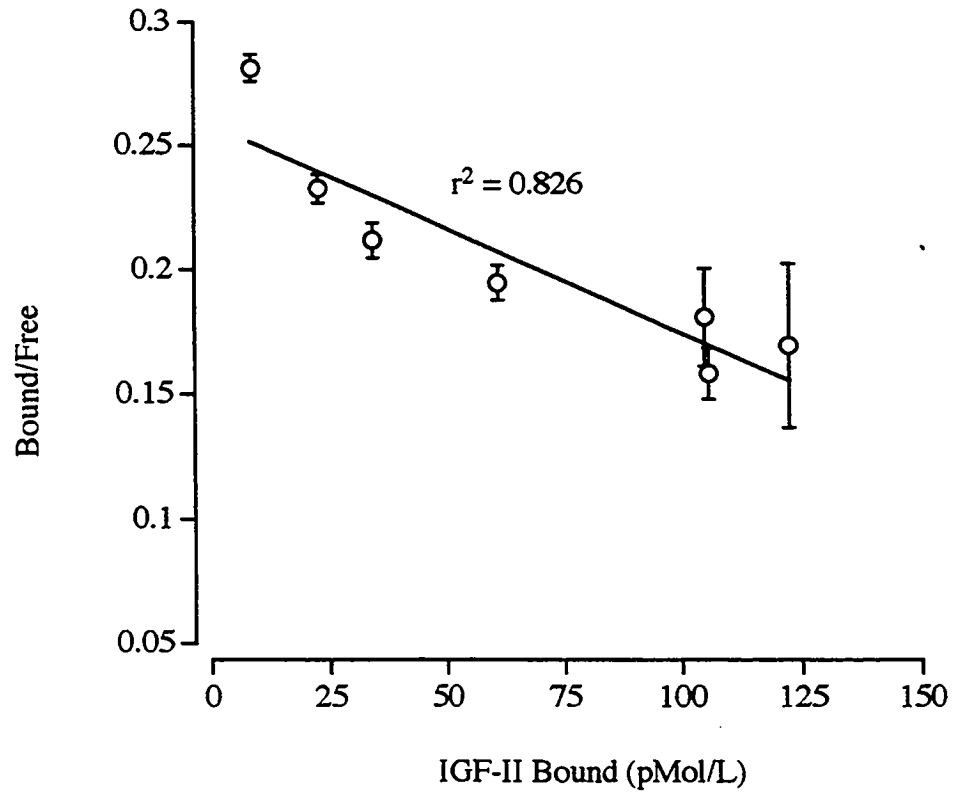
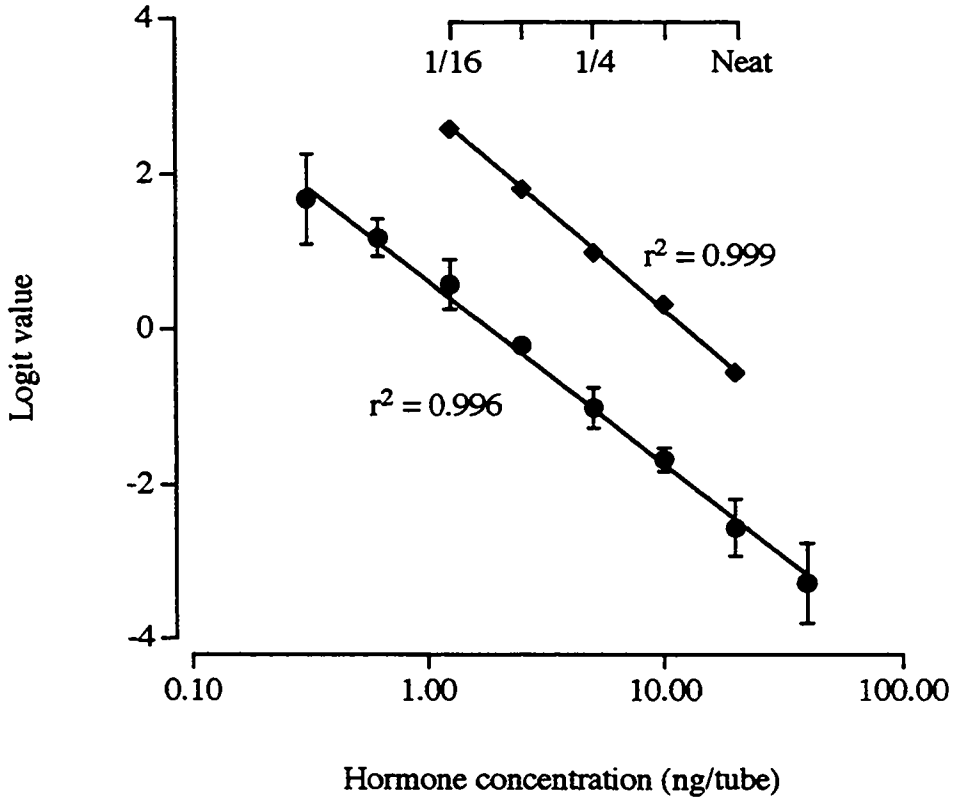

0.3
0.25
0.2
0.15
0.1
0.05
Bound/Free
r² = 0.826
0
25
50
75
100
125
150
IGF-II Bound (pMol/L)

Figure 3.4. Parallelism between a dilution series of (a) hIGF-I (●) and IGF-like activity in pool 3 (◆) from barramundi serum determined by the type I RRA and (b) hIGF-II (○) and IGF-like activity in pool 4 (▲) from barramundi serum determined by the type II RRA. The scale bar indicates the serial dilution of the serum pools 3 or 4, ranging from undiluted (Neat) to 4-fold serially diluted (1/16). Values are expressed as mean (n=3) ± standard deviation.

(a)


4
2
0
-2
-4
Logit value
1/16
1/4
Neat
r2 = 0.999
r2 = 0.996
0.10
1.00
10.00
100.00
Hormone concentration (ng/tube)

(b)

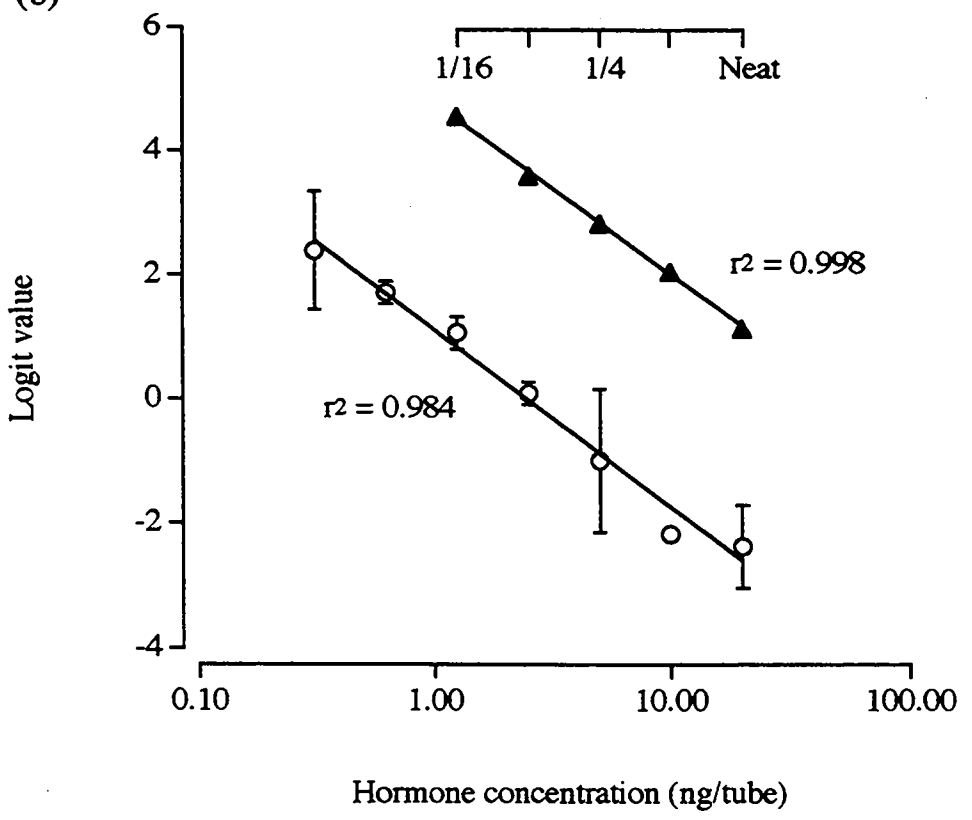

6
4
2
0
-2
-4
Logit value
1/16
1/4
Neat
r2 = 0.998
r2 = 0.984
0.10
1.00
10.00
100.00
Hormone concentration (ng/tube)

Table 3.1 Recovery of hIGF-I and hIGF-II and intra- and inter-assay variability for type I and type II RRA. Values for recovery and assay variability parameters are expressed as means (n=9) ± SEM. Values for the limit of assay variability represent IGF activity levels expressed in nanograms (ng) of hIGF-I or hIGF-II equivalent units, for the type I and type II RRA, respectively.

Parameter	Type I RRA	Type II RRA
Recovery of hIGF-I (%)	99 ± 2.7	
Recovery of hIGF-II (%)	97 ± 3.4	89 ± 3.9
Intra-assay variability (%)	1.7 ± 0.3	3.9 ± 0.6
Inter-assay variability (%)	8.2 ± 2.1	8.3 ± 2.8
Limit of assay sensitivity (ng/ml)	0.315	0.625

3.3.6 Storage

The activities in pooled serum stored at -80 °C for 7 or 30 days, were not significantly ($p > 0.05$) different from that of fresh serum when measured by the type I RRA (Table 3.2).

3.3.7 IGF Binding Protein Assay

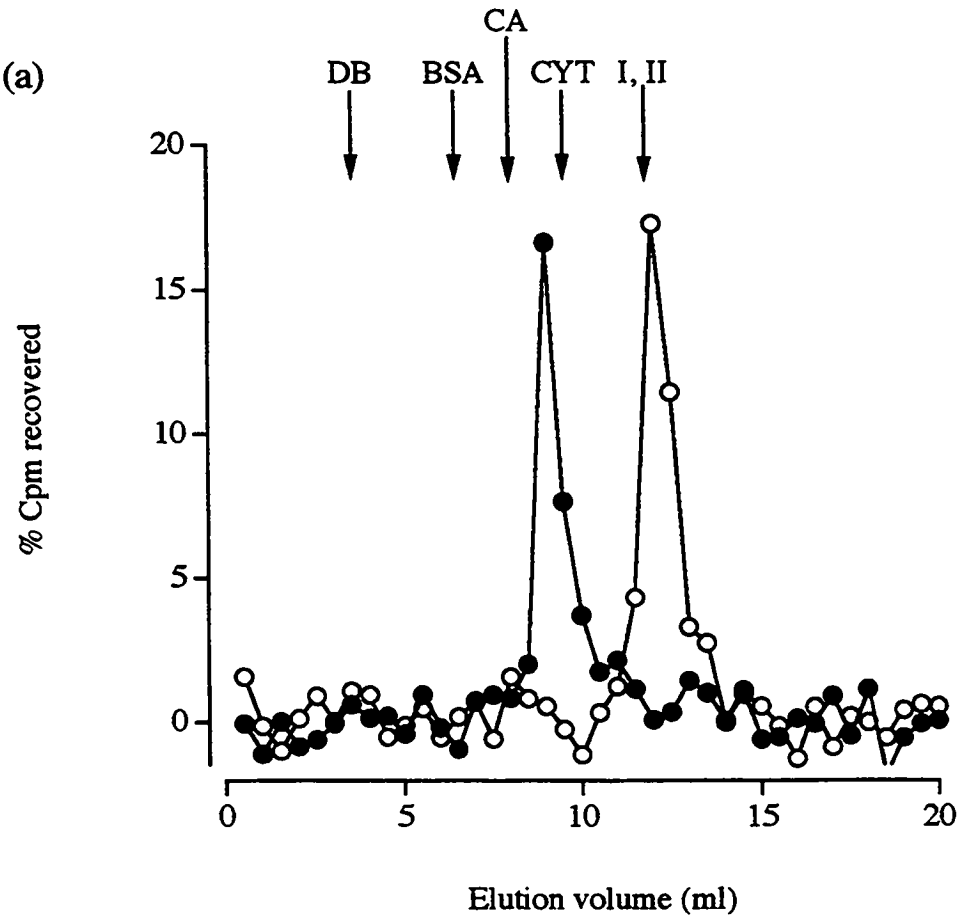
Incubation of fresh barramundi serum with [^{125}I]hIGF-I, under conditions of incubation of the IGFBP assay and subsequent size exclusion chromatography at pH 7.4, resulted in an elution profile with one large peak at 9 ml (Fig. 3.5a). At pH 7.4, the elution volume of this peak corresponded to a molecular weight ranging between 12.3 and 29 kDa. Following acid-ethanol extraction of serum samples prior to incubation with [^{125}I]hIGF-I a peak eluting at 12 ml was detected (Fig. 3.5a). The elution volume of the peak eluting at 12 ml corresponded to that of free [^{125}I]hIGF-I and [^{125}I]hIGF-II. The percentage of total counts recovered was 16.6 ± 0.8 and 17.3 ± 1.1 (mean, n=3 ± sd) for the 9 and 12 ml peaks respectively.

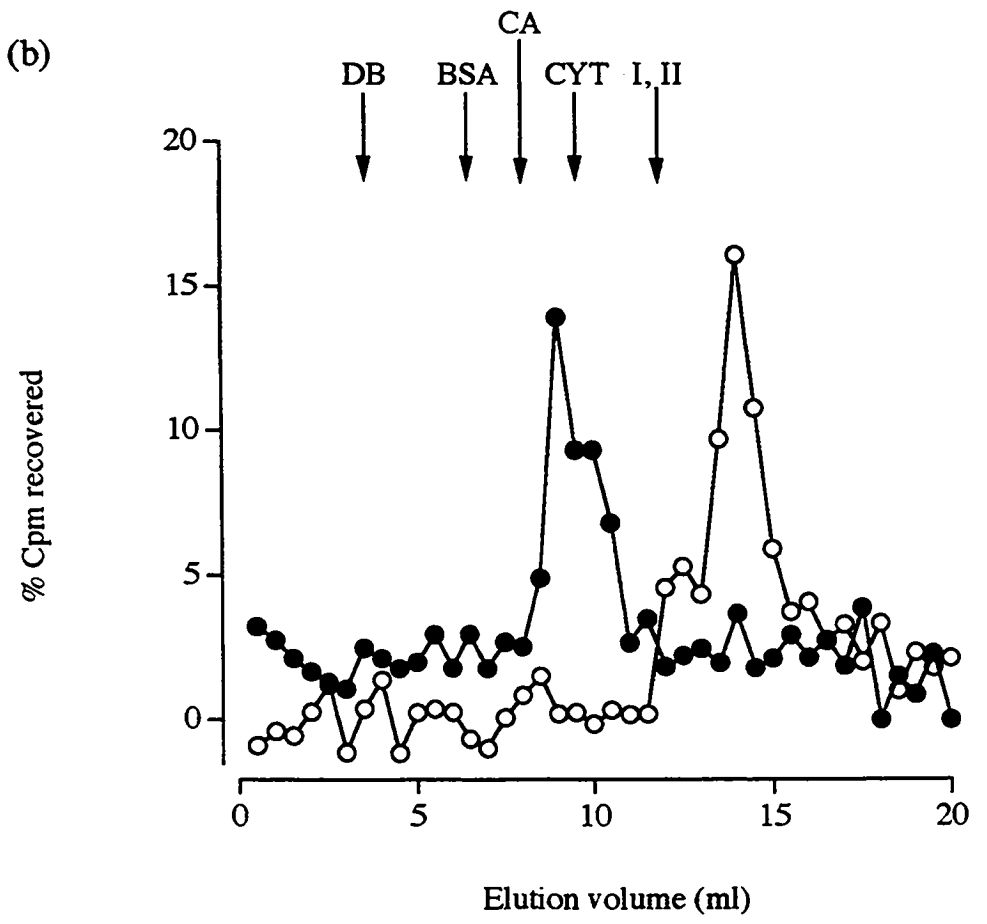
A single peak eluting at 9 ml was detected following chromatography (pH 7.4) of fresh barramundi serum incubated with [^{125}I]hIGF-II in the IGFBP assay (Fig. 3.5b). Following acid-ethanol extraction of the serum a peak of activity eluted at 14 ml at pH 7.4 (Fig. 3.5b). The molecular weight of the 14 ml peak was less than 7.5 kDa, that of [^{125}I]hIGF-I and [^{125}I]hIGF-II which eluted at 12 ml. The percentage of total counts recovered (mean, n=3 ± sd) in the 9 and 14 ml peaks from the IGF-II IGFBP assay were 13.9 ± 0.9 and 16.1 ± 0.2 respectively.

Table 3.2 The amount of activity detected in pooled barramundi serum fractions following storage at -80 °C for 0, 7 and 30 days by type I RRA. Fractions were pooled according to Fig. 3.1. Values mean activity levels (n=9) expressed in nanograms (ng) of hIGF-I equivalent units ± SEM.

	Pool 1	Pool 2	Pool 3	Pool 4
Fresh	1.89 ± 0.17	6.61 ± 0.26	2.43 ± 0.11	1.30 ± 0.11
7 Days	1.88 ± 0.20	6.60 ± 0.27	2.41 ± 0.08	1.29 ± 0.41
30 Days	1.86 ± 0.36	6.60 ± 0.31	2.42 ± 0.05	1.30 ± 0.22

Figure 3.5 Elution profiles of fresh (●) or acid-ethanol extracted (○) barramundi serum incubated with (a) [^{125}I]hIGF-I or (b) [^{125}I]hIGF-II under conditions of the IGFBP assay and obtained using size exclusion chromatography at pH 7.4. Values are expressed as the mean (n=3) percentage of counts per minute (% Cpm) recovered. The arrows indicate the elution volumes of dextran blue (DB), bovine serum albumin (BSA), carbonic anhydrase (CA), cytochrome C (CYT), [^{125}I]hIGF-I (I) and [^{125}I]hIGF-II (II).


(a)
CA
DB
BSA
CYT
I, II
20
15
10
5
0
% Cpm recovered
0
5
10
15
20
Elution volume (ml)

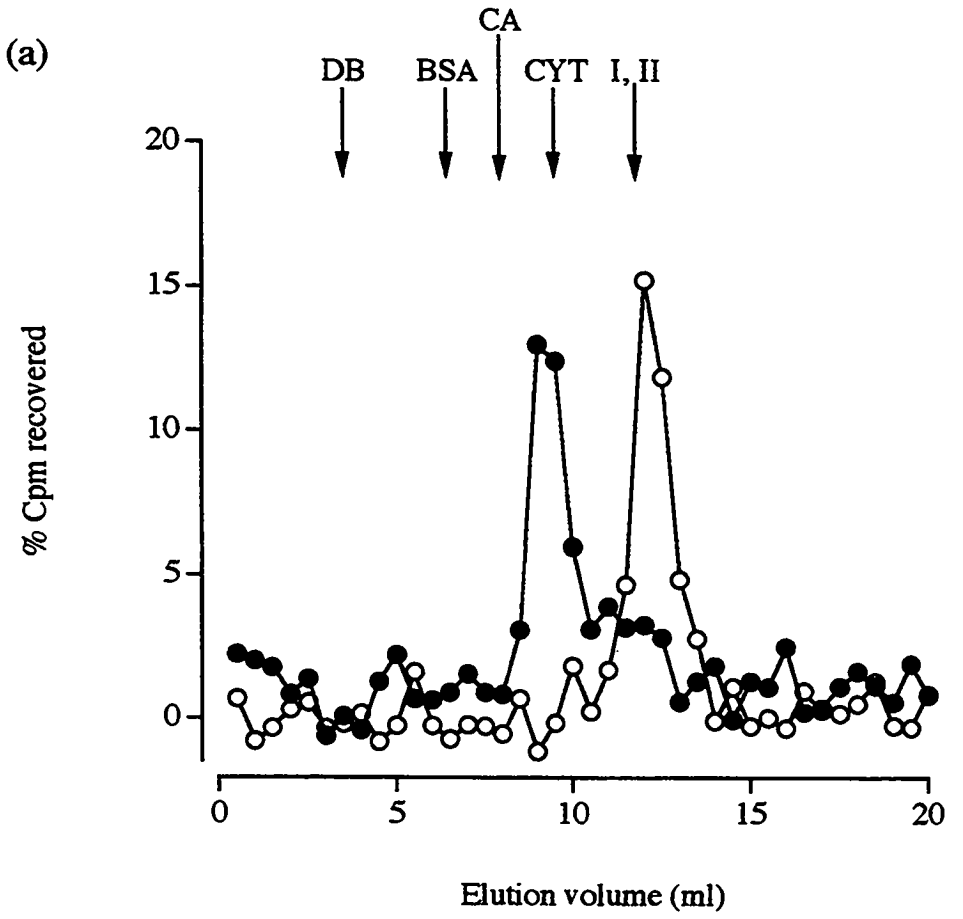

(b)
CA
DB
BSA
CYT
I, II
20
15
10
5
0
% Cpm recovered
0
5
10
15
20
Elution volume (ml)

The elution profiles of fresh and acid-ethanol extracted sera to which aprotinin had been added at the time of blood collection incubated with [^{125}I]hIGF-I in the IGFBP assay remained identical to profiles for samples without the addition of aprotinin (Fig. 3.6a). The percentage of counts recovered (mean, n=3 ± sd) for the 9 and 12 ml peaks were slightly lower than those of the non-treated samples, being 13.0 ± 1.4 and 15.2 ± 0.8 respectively. The elution volume of the peaks also remained unchanged in aprotinin-treated fresh and acid-ethanol extracted sera incubated with [^{125}I]hIGF-II in the IGFBP assay (Fig. 3.6b). The percentage of counts recovered (mean, n=3 ± sd) for the 9 ml peak in the aprotinin-treated fresh serum sample incubated with [^{125}I]hIGF-II increased to 57.1 ± 2.7 (Fig. 3.6b). In contrast the percentage of counts recovered for the 14 ml peak in the aprotinin-treated acid-ethanol extracted sample incubated with [^{125}I]hIGF-II was slightly lower (12.9 ± 0.4) than that of the non-treated acid-ethanol extracted sample incubated with [^{125}I]hIGF-II (16.1 ± 0.9).

3.3.8 SDS-PAGE and Western Ligand Blot

The autoradiograph of the Western ligand blot demonstrated the presence of at least one IGFBP in barramundi serum. A major band at approximately 45 kDa and two minor bands at approximately 37 kDa and 22 kDa were detected in the barramundi serum (Fig. 3.7). In human serum three bands at approximately 43 kDa, 35 kDa and 20 kDa were identified (Fig. 3.7).

Figure 3.6 Elution profiles of fresh (●) or acid-ethanol extracted (○) barramundi serum treated with aprotinin at the time of blood collection and incubated with (a) [^{125}I]hIGF-I or (b) [^{125}I]hIGF-II under conditions of the IGFBP assay. Elution profiles were obtained using size exclusion chromatography at pH 7.4. Values are expressed as the mean (n=3) percentage of counts per minute (% Cpm) recovered. The arrows indicate the elution volumes of dextran blue (DB), bovine serum albumin (BSA), carbonic anhydrase (CA), cytochrome C (CYT), [^{125}I]hIGF-I (I) and [^{125}I]hIGF-II (II).


(a)
CA
DB
BSA
CYT
I, II
20
15
10
5
0
% Cpm recovered
0
5
10
15
20
Elution volume (ml)

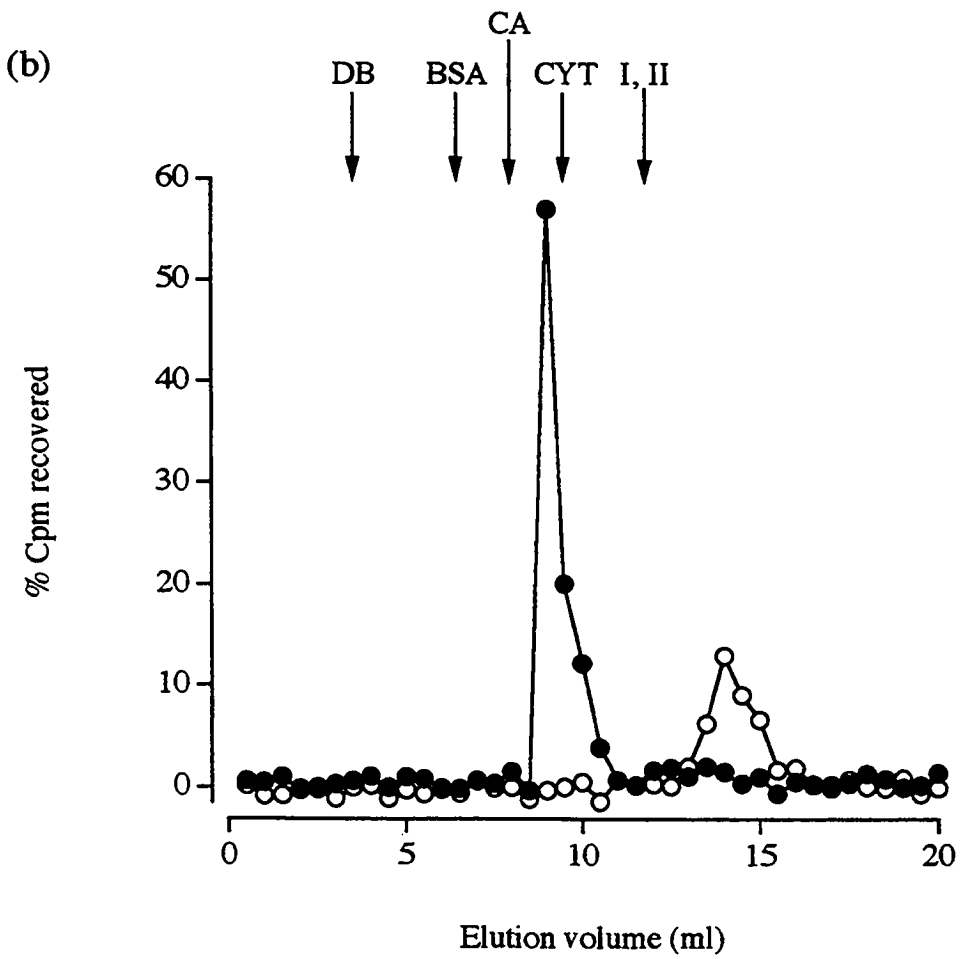
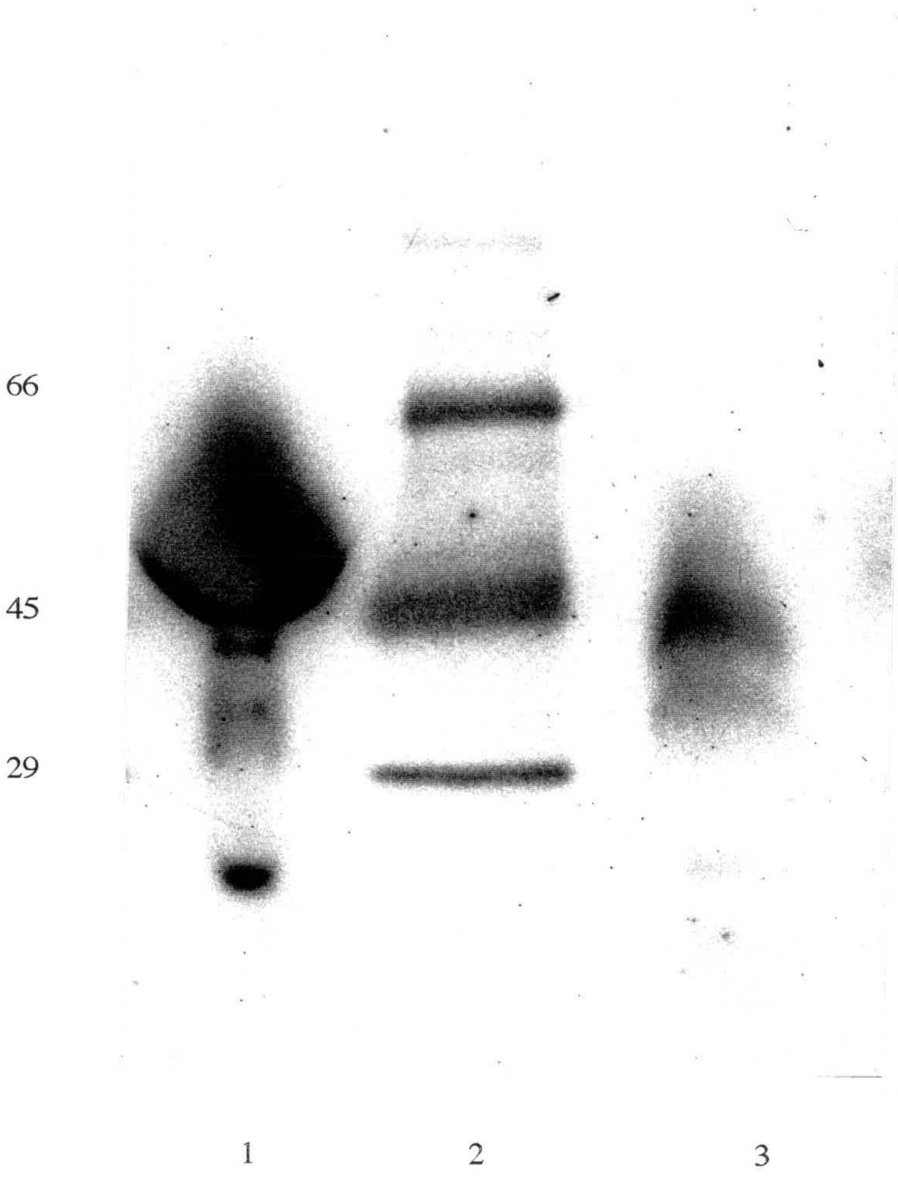

(b)
CA
DB
BSA
CYT
I, II
60
50
40
30
20
10
0
% Cpm recovered
0
5
10
15
20
Elution volume (ml)

Figure 3.7 Autoradiograph of a Western ligand blot for IGFBPs. Protein standards, ^{14}C-Bovine serum albumin (^{14}C-BSA), ^{14}C-Ovalbumin (^{14}C-OV) and ^{14}C-Carbonic anhydrase (^{14}C-CA) with indicated molecular weights of 66kDa (66), 45kDa (45) and 29kD (29) respectively, are shown in Lane 2. Lane 1 contains IGFBPs detected in a human serum sample incubated with [^{125}I]hIGF-I. Lane 3 contains IGFBPs detected in a barramundi serum sample incubated with [^{125}I]hIGF-I.


66
45
29
1
2
3

3.4 DISCUSSION

3.4.1 Membrane Specificity

The membrane preparations used for the type I and type II RRA in the present study allowed differentiation between hIGF-I and hIGF-II as described previously (Daughaday *et al.*, 1981 Scott and Baxter, 1987). Van Wyk *et al.* (1980) found that mammalian IGF-I and IGF-II were equipotent in a RRA using human placental membranes. In the present study, the binding curves and binding capacities for hIGF-I and hIGF-II were similar in the type I RRA, supporting the earlier findings that both peptides equally displace [^{125}I]hIGF-I. The apparent dissociation constants (K) for hIGF-I and hIGF-II in the type I RRA were also similar and were consistent with the K values of 1 x 10^{-9}M, reported by Daughaday *et al.* (1981). The binding capacities estimated for the type I RRA were higher than those reported by Daughaday *et al.* (1981), however this may be attributed to differences in membrane preparation.

The specificity of the rat liver membranes for the IGF-II peptide (or IGF-II variants) reported in previous studies (Megyesi *et al.*, 1975; Rechler *et al.*, 1980; Scott and Baxter, 1987) was again observed in rat liver preparations used in the present study. Human IGF-I was not able to displace [^{125}I]hIGF-I from the rat liver binding matrix at any of the concentrations used in the present study. Similarly, the cross-reactivity of IGF-I in a type II RRA was previously reported as less than 1% (Megyesi *et al.*, 1975; Rechler *et al.*, 1980). The scatchard plot of hIGF-II in the type II RRA indicated the presence of single order of binding sites. Binding sites for IGF-II, but not IGF-I, were previously reported in rat liver (Scott and Baxter, 1987) and rat placental membranes (Daughaday *et al.*, 1981; Perdue *et al.*, 1983; Pilistine *et al.*, 1984). The apparent dissociation constant of hIGF-II using rat placental membranes reported in the present study, 0.8 x 10^{-9}M, was similar to that reported by Perdue *et al.* (1983), 0.6 x 10^{-9}M. The binding capacity reported in the present study was also lower (6.5 pg/μg protein) than that reported by Daughaday *et al.* (1981), 5 pmol/mg protein, suggesting differences in the preparation procedures.

3.4.2 Detection of IGF Activity

Two activities approximating the size of human IGF-I and IGF-II were detected in barramundi serum by mammalian type I RRA. Two factors indicate that the activity found at ≈ 7.8 kDa is barramundi IGF-I (bIGF-I). Firstly, this activity was active only in the type I RRA and not the type II RRA, indicating that this molecule behaves in these assays as does mammalian IGF-I. Subsequent to the present study, Upton *et al.* (1996) have shown that recombinant barramundi IGF-I (rbIGF-I) has the same affinity for mammalian type I receptors as does mammalian IGF-I but does not react with the type II receptor. Secondly, the activity found in this study was of a size consistent with that predicted for the mature barramundi IGF-I peptide based on the mRNA sequence data of 66 amino acids (Kinhult, 1997).

Recently, an activity with a M_r of approximately 8 kDa was detected in barramundi serum using a mammalian IGF-I RIA (Drakenberg, 1996). The concentration (13 ng/ml) of that activity was considerably higher than that found in the present study (2.4 ng/ml). The discrepancy between these values may indicate differences in the physiological state of the animal or in the relative reactivities of hIGF-I and bIGF-I under conditions of the IGF-I RIA and the type I RRA. As recombinant bIGF-I and hIGF-I have equal affinity for type I receptors (Upton, *et al.*, 1996), it is likely that the values reported in this and following studies are similar to the absolute values for IGF-I in barramundi.

The peak of activity found at ≈ 7.6 kDa was active in both the type I and type II RRA. As this molecule behaved in the RRAs in a manner similar to mammalian IGF-II and had a similar molecular weight to hIGF-II, it was concluded that the molecule was barramundi IGF-II (bIGF-II). The molecular size of the molecule also corresponded with recent information of the mature barramundi IGF-II peptide, derived from the characterisation of the barramundi IGF-II gene (Collet, pers. comm.) and with IGF-II immunoreactivities (7.5 - 8 kDa) reported in the serum, liver and muscle of barramundi using a mammalian IGF-II RIA (Drakenberg, 1996). Recombinant bIGF-II is not

available and thus it is not possible to determine how the measured values described in this study relate to true absolute values of bIGF-II in serum. However, a dilution series of barramundi serum fractions resulted in a relationship parallel to a similar dilution series of hIGF-II. Thus it was concluded that this assay was able to provide reliable data about the relative levels of bIGF-II in barramundi serum.

Although early attempts to measure IGF-II in fish blood using a variety of assays were unsuccessful (Daughaday *et al.*, 1985; Anderson *et al.*, 1993), there is now substantial evidence to support the existence of IGF-II in teleosts. A homologous IGF-II RIA for rainbow trout was recently described (Gentil *et al.*, 1996), IGF-II-like peptides (7 kDa) were detected in tilapia serum (Drakenberg, 1996), IGF-II mRNA transcripts were detected in various tissues of barramundi (Collet, pers. comm.), molecules derived from trout skeletal muscle demonstrated binding to type II IGF receptors (Bautista *et al.*, 1990) and messenger RNA coding for IGF-II was described for a salmonid (Shamblott and Chen, 1992; Shamblott *et al.*, 1995). IGF-II-like molecules (6 - 8 kDa) have also been detected in the gut and pancreas of the sting-ray, *Raja clavata*, (Drakenberg, 1996) and in the liver of the spiny dogfish shark (Duguay *et al.*, 1995).

Two high molecular weight peaks of activity were also identified in the type I and type II RRA. The activity having an M_r between 12.3 - 66 kDa corresponds to [^{125}I] hIGF-I and [^{125}I] hIGF-II binding activity in barramundi serum. This activity is probably due to IGFBPs competing with the receptors for the ligand in the RRA. The size of the indicated molecules must be considered approximate as only limited resolution is afforded at these molecular sizes by the column used in the present study. Similar high molecular weight activities (≥ 25 kDa) were observed in the serum and liver of barramundi using a mammalian IGF-II RIA, although they were not detected using a mammalian IGF-I RIA (Drakenberg, 1996). The 12.3 - 66 kDa peak was approximately 7-fold greater in the IGF-II RRA than in the type I RRA. It would appear, therefore, that this activity is a binding protein with higher affinity for hIGF-II.

Previous studies have also reported the presence of high molecular weight (17 - 90 kDa) activities in blood products of various teleost species using heterologous IGF RIA or RRA (Drakenberg *et al.*, 1989; Ng *et al.*, 1991; Anderson *et al.*, 1993; Pérez-Sánchez *et al.*, 1994; Drakenberg, 1996). In these studies the activity was also attributed to the presence of residual IGFBPs.

The suggestion that the 12.3 - 29 kDa peak was IGFBPs was further supported by the binding protein characterisation studies and Western ligand blotting. A peak ranging between 12.3 and 29 kDa was detected in barramundi serum using an IGFBP assay with either [^{125}I]hIGF-I or [^{125}I]hIGF-II. These results suggest the presence of IGFBPs in barramundi which bind both bIGF-I and bIGF-II. The presence of multiple IGFBPs for bIGF-I was determined by Western ligand blotting under non-reducing conditions. Three bands were identified in barramundi serum, a major band at approximately 43 kDa and two minor bands at approximately 35 kDa and 20 kDa. In view of the capacity of the column used in this study to differentiate between molecules of this size, these data are not inconsistent with the reported 12.3 - 29kDa peak. Multiple IGFBPs of similar molecular weight have previously been reported in a variety of teleost species. Kelley *et al.* (1992) reported two major IGFBPs (29 and 31 kDa) and a minor IGFBP (31-31.5 kDa) in coho salmon, striped bass, Mossambique tilapia and a goby. Multiple IGFBPs ranging from 23-120 kDa were also reported recently in the striped bass (Siharath *et al.*, 1995; Fukazawa *et al.*, 1995). Similarly, Niu and Le Bail (1993) reported a major IGFBP of 41.5 kDa and two minor IGFBPs (34 and 23 kDa) in the serum of rainbow trout. Only one IGFBP was reported in the serum of golden perch (Anderson *et al.*, 1993) and in channel catfish (Delahunty *et al.*, 1993). The size of the barramundi IGFBPs reported in the present study also appears consistent with recent sequence data for IGFBPs in this species (Collet, pers. comm.).

As the 12.3 - 29 kDa peak was not detected in acid-ethanol extracted serum incubated with either [^{125}I]hIGF-I or [^{125}I]hIGF-II it would support the presence of barramundi IGFBPs. Furthermore, the peak of radioactivity in the extracted serum incubated with

[^{125}I]hIGF-I eluted at a volume corresponding to free [^{125}I]hIGF-I or [^{125}I]hIGF-II at pH 7.4, indicating the dissociation of IGF and IGFBPs.

Since aprotinin increased the recovery of the 12.3 -29 kDa complex in serum incubated with [^{125}I]hIGF-II by 43 %, but had no effect on the sample incubated with [^{125}I]hIGF-I it indicates that either the IGFBP:[^{125}I]hIGF-II complex was more susceptible to degradation by serine protease activity than IGFBP:[^{125}I]hIGF-I complex or that the sample was contaminated. The protection of IGFBPs from serine protease degradation by aprotinin has previously been reported in humans (Binoux *et al.*, 1991), mice (Fielder *et al.*, 1990) and fish (Yandell, 1992). The complete characterisation and stability of IGFBPs in barramundi, therefore, requires further study.

The identity of the activity eluting at the void volume corresponding to a M_r of greater than 66 kDa, remains uncertain. This activity was present in both RRA but did not bind either [^{125}I] hIGF-I or [^{125}I] hIGF-II. As this activity did not bind [^{125}I] hIGF-I or [^{125}I] hIGF-II it is unlikely to be an IGFBP, however since it reacted in both RRA it would appear to compete directly with the ligand for the receptor. Similar high molecular weight activities have appeared in IGF assays in Mossambique tilapia (Drakenberg *et al.*, 1989) and golden perch (Anderson *et al.*, 1993). In both tilapia (Drakenberg *et al.*, 1989) and barramundi, this activity was observed in both type I and type II assays, indicating it to have some structural similarities to mammalian IGF-II. As these molecules can also be detected in hIGF-I RIA (Drakenberg *et al.*, 1989; Anderson *et al.*, 1993) they are competing with the ligand for the binding site, rather than affecting the integrity of the receptor by binding to the membrane in the RRA. This activity is also probably too large in size to be an IGF precursor which would be 15 - 20 kDa based on fish IGF cDNA sequence data (Cao *et al.*, 1989; Duguay *et al.*, 1992; Shamblott and Chen, 1992; Wallis and Devlin, 1993). In addition, a previous attempt to demonstrate processing of this activity to produce molecules of a similar size

to IGF-I or IGF-II was unsuccessful (Anderson *et al.*, 1993). Further studies will be required to fully characterise this activity.

The range of molecular size of IGF-like activities detected in teleost species remains variable. In addition to the range of molecules described above, a small (2 kDa) molecular weight activity was detected in the liver of barramundi using a mammalian IGF-II RIA (Drakenberg, 1996). Although no such activity was detected in this study, the possibility that proteolytically cleaved portions of IGF molecules are detected in some IGF assays should not be dismissed.

3.4.3 Validation

The concentrations of IGF-like activities derived using heterologous assays are generally considered quantitative following complete validation. However the subsequent development of homologous assays indicate underestimation of IGF activities by heterologous assays. The advent of homologous RIA for coho salmon insulin (Plisetskaya *et al.*, 1986) and IGF-I (Moriyama *et al.*, 1994) and rainbow trout IGF-II (Gentil *et al.*, 1996) demonstrated that plasma concentrations of these peptides were greatly underestimated by heterologous methods. Rigorous IGF assay validation therefore provides assays capable of detecting changes in IGF, even if absolute values cannot be determined. As no validated homologous assay for either barramundi IGF-I or IGF-II has been documented, the method described in this study provides a means for investigating the physiology of IGFs in this species. In addition, as it is likely that IGFs will be purified from only relatively few teleost species, the present methods may be applied to investigate IGF physiology in other teleost species, post-validation.

The validation procedures described in this chapter followed rigorous protocols for the validation of IGF measurements in biological fluids (Bang *et al.*, 1994). Samples were acidified and subjected to acid gel chromatography, parallelism between bIGF-I and bIGF-II and IGF reference curves was demonstrated and high quantitative recoveries and low coefficients of variation, which fell within accepted literature values, were obtained for both RRA. In addition, the type I and type II RRA seem

appropriate for detecting IGF-like activities in barramundi serum under different physiological conditions (Chapter 4 and 5).

3.4.4 Conclusion

In conclusion, this chapter describes the detection of IGF-I, IGF-II and IGFBPs in barramundi serum. The RRA used to detect circulating IGF-I and IGF-II-like activities were rigorously validated according to Bang *et al.* (1994). The method described attempts to address the major limitation of heterologous RRA, that of intermediate affinity between ligand and receptor, by satisfying all requirements of IGF assay validation. The methods described in this chapter therefore provide a reliable and accurate means to detect and measure circulating IGF-I and IGF-II levels in barramundi serum from a single sample.

CHAPTER 4:

THE EFFECT OF RATION SIZE AND WATER TEMPERATURE ON GROWTH AND PRE- AND POST-TRANSLATIONAL IGF LEVELS IN BARRAMUNDI.

4.1 INTRODUCTION

Food availability and water temperature directly affect the growth of fish (Weatherley and Gill, 1987; De Silva and Anderson, 1995) and accordingly, food restriction and non-optimal water temperatures have been reported to inhibit fish growth (Danzmann *et al.*, 1990; Sumpter *et al.*, 1991; Sumpter, 1992; Fine *et al.*, 1996). The profile of growth regulating hormones, such as GH (Sumpter *et al.*, 1991; Fine *et al.*, 1996) and IGF-I (Perez-Sanchez *et al.*, 1994), have recently been reported to fluctuate during periods of food deprivation or changes in water temperature in teleosts, however the mechanisms of regulation and relationship with body growth have not been fully elucidated. An investigation into the effect of ration size and water temperature on pre- and post-translational IGFs in barramundi may therefore provide valuable information on the role of these hormones in the regulation of growth in a tropical teleost.

4.1.1 Regulation of Energy Mobilisation During Food Deprivation in Teleosts

Many species of fish naturally undergo substantial periods of food restriction, reducing the energy available for growth after the metabolic requirements have been fulfilled. Studies on a variety of teleost species under natural and experimental conditions indicate that although energy mobilisation strategies differ among species there is a general tendency for fish to conserve body protein at the expense of stored lipid and glycogen (De Silva and Anderson, 1995). Upon the depletion of non-protein energy stores, protein mobilisation occurs (Love, 1980; Weatherley and Gill, 1987; Collins, 1996). Energy mobilisation in teleosts has been comprehensively reviewed by Jobling (1994).

A complex variety of mechanisms has been implicated in the regulation of energy mobilisation by hormones during food deprivation in fish and the process remains poorly understood. Thyroid hormones (Higgs and Eales, 1977), corticosteroids (Donaldson *et al.*, 1979), sex steroids (Fagerlund *et al.*, 1977), insulin (Ince and Thorpe, 1976; Inui and Ishioka, 1983; Ablett *et al.*, 1981), prolactin and growth

hormone (Donaldson *et al.*, 1979; Sumpter *et al.*, 1991; Perez-Sanchez *et al.*, 1994) have all been reported to regulate protein, lipid and carbohydrate metabolism in a variety of teleost species. Due to the nature of the present study, this review will focus the effects of nutrition on the GH:IGF-I axis. However, the actions of other hormones and the possibility of synergistic actions of hormone combinations should not be dismissed.

4.1.2 Ration Size and Growth

The relationship between growth and ration size is curvilinear (De Silva and Anderson, 1995). The ration at which the animal neither gains nor loses weight is termed the maintenance ration and occurs when the energy consumed in the ration equals the standard metabolic energy required for the animal to maintain itself. Below this ration endogenous energy stores are mobilised by the animal, and weight loss ensues. Increasing ration size above the maintenance ration results in growth or energy storage by the animal. Initially growth increases rapidly with increasing ration size until the maximum ration size is approached and growth plateaus.

As water temperature has a significant effect on the metabolic rate of teleosts, the growth response to ration size in fish is influenced by water temperature (Fine *et al.*, 1996). Metabolic rate increases with elevations in water temperature, increasing the ration that is required to supply standard metabolic energy. Thus, a larger ration is required in order to produce growth. A similar ration size at a lower temperature will allow greater potential for growth. The nature of the growth, that is whether it results from accretion of lipid or protein, will also vary at different temperatures for given amounts of excess energy (Jobling, 1994).

4.1.2.1 Effect of Food Restriction on the GH:IGF-I Axis and IGF-II

In vertebrates, the effect of nutrient deprivation on the GH:IGF axis has been documented (Section 1.5). During periods of starvation, plasma GH is elevated despite depressed growth (Clemmons and Underwood, 1991). As there is substantial evidence demonstrating the growth-promoting actions of GH under normal conditions (Sara and Hall, 1990), these results may intially appear paradoxical. However, the

significant reduction in the number of GH binding sites, post receptor resistance to GH and decreased plasma IGF-I levels in many species following starvation (Section 1.5) suggest that although circulating GH concentrations are elevated, certain GH actions are down-regulated at a later stage in the hormonal cascade (Clemmons and Underwood, 1991). It therefore appears that the circulating levels of IGF-I, the molecule mediating the action of GH in the GH:IGF-I axis, and not the concentration of circulating GH more accurately indicates the nutritional, and thus growth, status of the animal.

In mammals, the effects of nutrition on IGF-II levels have not been studied as extensively as the effects on IGF-I. In studies where the nutritional regulation of IGF-II was investigated, serum IGF-II levels in adult humans were been reported to decrease (Merimee *et al.*, 1982) or remain unchanged (Davenport *et al.*, 1988) during short-term fasting. Similarly, there was a minimal decrease in serum IGF-II in fetal rats which were subject to intrauterine nutritional deprivation by maternal starvation (Donovan *et al.*, 1991). In these animals there was no change in IGF-II mRNA (Donovan *et al.*, 1991). The proposal that IGF-II responds to nutritional changes indirectly (Clemmons and Underwood, 1991) has been hypothesised from the presumed net movement of IGF-I, IGF-II and IGFBPs under such conditions. In mammals, there is presumably an acceleration in the movement of IGF-II from the vascular compartment during periods of food deprivation in response to severe changes in IGF-I levels which result in the saturation of IGFBPs and/or modification of the binding of IGF-II to cell surface receptors (Clemmons and Underwood, 1991).

4.1.2.2 Ration Size and IGFs Levels in Fish

In fish, the regulatory effects of feeding level on the GH:IGF-I axis are poorly described. As in mammals, serum GH was elevated during fasting in tilapia (Rodgers *et al.*, 1992), rainbow trout (Sumpter *et al.*, 1991), coho salmon (Gray *et al.*, 1992; Duan and Plisetskaya, 1993) and gilthead seabream (Perez-Sanchez *et al.*, 1995). Hepatic GH binding was significantly reduced in coho salmon (Gray *et al.*, 1992) and

gilthead seabream (Perez-Sanchez *et al.*, 1995) following starvation, suggesting that there is GH insensitivity in the liver. There was decreased IGF-I bioactivity during periods of food deprivation in trout (Komourdjian and Idler, 1978), coho salmon (McCormick *et al.*, 1992), goby (Gray and Kelley, 1991) and Japanese eel (Duan and Inui, 1990a) and decreased plasma IGF-I in gilthead seabream (Perez-Sanchez *et al.*, 1995) and flounder, *Parlichthys olivaceus* (Nam *et al.*, 1996). The expression of hepatic IGF-I mRNA was also significantly reduced by fasting in Japanese eel (Duan and Plisetskaya, 1993). Similarly, the alternatively spliced IGF-I mRNA Ea-1 and Ea-3 transcripts in coho salmon (Duguay *et al.*, 1994) declined with food restriction and were restored to those of control values by re-feeding (Duguay *et al.*, 1994). These results suggest that nutritional status regulates IGF-I synthesis in teleosts by modifying the sensitivity of the liver to circulating GH, which reduces the production of systemic IGF-I, however the exact mechanisms of regulation are not fully understood.

Whilst the effect of food deprivation on IGF-I has been investigated in some teleost species, the effects on IGF-II in fish has not been characterised.

4.1.3 Aims

Environmental parameters have been shown to have a significant effect on the growth of barramundi. The natural tropical estuarine environment which barramundi inhabit is prone to a range of environmental fluctuations, including periods of food deprivation and fluctuating water temperature and salinity. In addition to the natural habitat, this species is grown for commercial aquaculture, where environmental parameters, particularly nutritional factors, are often manipulated to optimise growth. It is therefore appropriate to investigate the effects of both ration size and water temperature on circulating IGFs in barramundi. The subsequent determination of IGF-I mRNA expression may allow insight into the mechanisms underlying IGF regulation and hence growth regulation in this species.

The aim of the studies described in this chapter was to determine the effect of ration size and water temperature on juvenile barramundi growth, circulating IGF-I and IGF-II levels, total hepatic and brain IGF-I mRNA expression and the expression of the alternatively spliced (Ea-2 and Ea-4) hepatic IGF-I mRNA transcripts.

4.2 MATERIALS AND METHODS

4.2.1 Experimental Animals

Fifty-four juvenile barramundi (132.03 g ± 3.14 g, mean ± SEM) were obtained from Barramundi Waters (Innisfail, Queensland, Australia) and were held in individual tanks as described in Section 2.2, except for water temperature. Fish (n=27) were held in water at either 21 ± 2 °C or 28 ± 3 °C and were acclimated for 4 weeks to laboratory conditions prior to the commencement of the experiment.

4.2.2 Experimental Design

Following acclimation, fish at each temperature were allocated randomly to one of three experimental groups (n=9/group). Experimental groups were assigned satiety (SAT), restricted (RES) or starved (STV) ration sizes. The amount of food designated to each ration was based on growth data from barramundi feeding charts (Department of Primary Industries, Queensland, Australia). At 28 °C the SAT ration was 4 g wet weight diet/100g body weight per day (% $BW.day^{-1}$) and RES ration was 2% $BW.day^{-1}$. At 21 °C the SAT ration was 2% $BW.day^{-1}$ and the RES ration was 1% $BW.day^{-1}$. Fish in the STV group received no food. The trial was conducted for a period of 42 days. The diet used was a commercial barramundi diet (Aquafeed, Brisbane, Australia) containing approximately 48 % crude protein and 20 MJ/kg crude energy. Fish were weighed weekly, without anaesthesia, and the proportion of food adjusted for changes in body weight. The amount of food consumed by each fish per day was recorded throughout the experiment. The treatment groups will be cited using the temperature (21 or 28) followed by the abbreviated ration name (SAT, RES or STV) throughout the chapter.

4.2.3 Measurement of Growth and Sampling

At 21 and 42 days, all fish were anaesthetised (Section 2.2.2), weighed and measured. Growth parameters, including % BWI, SGR, CF and FCR were determined using formulae described in section 2.2.1. Fish were blood sampled and serum prepared

(Section 2.2.3) at 21 and 42 days. Serum samples were stored at -80 °C until IGF analysis.

Liver and brain samples were collected at 21 and 42 days. Three fish from each treatment group were sacrificed at each timepoint using anaesthesia followed by immediate cervical dislocation. Liver and brain tissue were quickly dissected from each fish, immediately frozen using liquid nitrogen and stored at -80 °C until IGF-I mRNA analysis.

4.2.4 Circulating IGF Analysis

Two aliquots (200 µl) of each serum sample were acidified and fractionated using size exclusion HPLC at pH 2.8 (Section 2.3.1), and fractions (0.5 ml) were collected every 0.5 min for 30 min. Fractions eluting between 10 and 15 ml were neutralised (Section 2.3.1) and assayed in triplicate for either IGF-I or IGF-II using the type I (Section 2.6.1) or type II (Section 2.6.2) RRA, respectively. The concentrations of IGF-I and IGF-II were determined using the regression equation of a log-logit transformed IGF standard curve (Section 2.6.1). The total concentrations of circulating IGF-I or IGF-II were determined by summing the concentrations in the fractions corresponding in molecular size to IGF-I and IGF-II in the type I and type II RRA, respectively. Values were expressed as nanograms (ng) of human IGF equivalent units per ml of serum.

4.2.5 IGF-I Messenger RNA Analysis

Insulin-like growth factor-I messenger RNA (mRNA) analysis was determined using a RNA protection assay (RPA) following the extraction of total RNA from the tissue samples.

The following terminology is used throughout the chapter. 'DEPC water' refers to water containing 0.1 % (v:v) diethlpyrocarbonate (DEPC, BDH Chemicals) which was autoclaved at 121° C for 90 min prior to use. 'Sterile water' refers to water which was autoclaved only. 'Saturated phenol' refers to phenol (Ajax chemicals, Acacia

Ridge, Australia) saturated using DEPC water prior to use. All restriction enzymes used for the molecular procedures were purchased from Boehringher Mannheim (Stones Corner, Australia), unless otherwise indicated. All glassware, centrifuge tubes and other equipment used for the RNA extraction was soaked overnight at room temperature in DEPC water and pipette tips were autoclaved only in order to prevent RNase contamination.

4.2.5.1 Extraction of Total RNA

Total RNA was extracted from tissue samples using a guanidium thiosulphate (GTC) phenol-chloroform procedure modified from Chomczynski and Sacchi (1987). Tissue samples were homogenised in 2 ml of Solution D (Appendix 1) using multiple 10 sec pulses at 12,000 rpm with a Polytron homogeniser (Kinematica, Switzerland) until a smooth paste was obtained. To the homogenate, 0.2 ml of 2 M sodium acetate, 2 ml of saturated phenol and 0.4 ml of chloroform:isoamylalcohol (CHISAM) buffer (Appendix 1) were added, with tubes being mixed by inversion after the addition of each reagent. Tubes were subsequently incubated for 15 min on ice and centrifuged at 9500 rpm for 20 min at 4 °C using a Beckman J2-21M/E centrifuge (Beckman, USA). The upper aqueous layer was reconstituted with one volume of CHISAM buffer and re-centrifuged as above. The upper aqueous layer was again recovered and precipitated by thorough mixing with 2.5 volumes of 99 % ethanol and incubation for at least 60 min at -20 °C. The precipitate was recovered by centrifugation as described above and resuspended in 360 µl of DEPC water. A second precipitation step was then conducted using 40 µl of 2 M sodium acetate and 2.5 volumes of 99 % ethanol with incubation conditions described above. The second precipitate was recovered by centrifugation at 14,000 g for 15 min at 4 °C using a Microfuge E (Beckman, UK). The pellets were recovered and 3 or 4 additional precipitations using 2.5 volumes of 99 % ethanol were conducted to remove residual GTC salt. Following the final precipitation, pellets were resuspended briefly in 0.5 ml of 75 % ethanol and recovered

by centrifugation at 14,000 rpm for 15 min at 4 °C. The pellets were air-dried for 10 - 15 min at room temperature and resuspended in 360 µl of DEPC water.

4.2.5.2 Estimation of Total RNA Concentration

An aliquot (1 µl) of each resuspended RNA sample was diluted sixty fold using sterile water and the absorbency (A) over wavelengths ranging from 230 to 280 nanometers (nm) were determined using a Beckman DU-68 spectrophotometer with Soft Pac Module (Beckman, USA). The purity of the RNA sample was assessed by the determination of the amount of residual protein (Equation 4.1) and GTC salt (Equation 4.2), as described by Chomczynski and Sacchi (1987).

Equation 4.1 $$\text{Amount of residual protein} = \frac{A_{260}}{A_{280}}$$

Equation 4.2 $$\text{Amount of residual GTC salt} = \frac{A_{260}}{A_{230}}$$

Purity was accepted if the values for residual protein were between 1.7 and 2.0 and were greater than 2.0 for residual GTC salt. If the purity was not acceptable, a further precipitation step was performed. The concentration of total RNA in the pure samples was then calculated using Equation 4.3 (Chomczynski and Sacchi, 1987) and the sample stored at - 80 °C.

Equation 4.3 $$\text{Total RNA Concentration } (\mu g / \mu l) = \frac{A_{260} \times 40 \mu g\, ml^{-1} (\text{RNA constant}) \times 60 (\text{Dilution factor})}{1000}$$

4.2.5.3 RNA Gel Electrophoresis

The integrity of the 28s and 18s ribosomal bands in the extracted RNA was assessed using electrophoresis. Aliquots (2 µl) of each RNA sample were diluted 3.25 fold with sterile water followed by the addition of 1 µl of 0.45 µm filtered, 10x 3-(N-Morpholino) propane sulphonic acid (MOPS) buffer (Appendix 1), 3.5 µl of 37% (v:v) formaldehyde solution and 10 µl of re-distilled formanide (Progen Industries,

Darra, Queensland, Australia). Samples were incubated for 5 min at 60 °C, cooled on ice for 5 min and 2 µl RNA loading buffer (Appendix 1) added.

Gels were prepared by boiling a 1 % (w:v) sterile agarose (High-pure low agarose, AGP Technologies, Upper Mt. Gravatt, Australia) solution for 3 min, cooling the mixture to 60 °C and adding 10 % (v:v) 10x MOPS buffer, 0.7 % (v:v) formaldehyde and 0.2 µg/ml ethidium bromide (EtBr). Gels were cast using either LKB GNA-100 (Pharmacia LKB, Sweden) or DNA Sub (Bio-Rad, North Ryde, Australia) apparatus, allowed to set for approximately 30 min and placed into the electrophoresis apparatus with 1x MOPS buffer (Appendix 1). RNA samples and a RNA marker solution containing 0.36 - 9.49 kilobase (kb) bands (Promega, Sydney, Australia) were loaded and separated for 30 min at 100 V using a Gene Power Supply (GPS200/400, Pharmacia LKB, Sweden). The 28s and 18s ribosomal bands were visualised using a Macrovue UV lamp (Pharmacia LKB, Sweden) and recorded by either polaroid or digital photography (Fig. 4.1). The concentration of RNA in the samples derived spectrophotometrically (Equation 4.1) was verified using the known concentrations of the bands of the RNA marker. Volumes corresponding to 2 µg of total RNA for each sample were recorded for the RNA protection assay (RPA).

4.2.6 Barramundi IGF-I Complementary DNA Templates

The plasmid stocks containing the barramundi IGF-I complementary DNA (cDNA) templates and methods were developed by Anne Kinhult (School of Life Sciences, Queensland University of Technology, Brisbane, Australia). The protocol for the reverse transcriptase polymerase chain reaction (rt-PCR), subcloning of PCR products, electroporation of competent bacterial cells and sequencing protocols used in the preparation of the plasmids stocks have been described in detail by Kinhult (1997). Three plasmids containing different barramundi IGF-I cDNA fragments were used in the present study. Plasmids were pGEM-T vectors (Promega, Sydney, Australia) with a 140, 220 or 333 base pair (bp) barramundi IGF-I cDNA fragment inserted at the T/A cloning site (Fig. 4.2a). The 5' - 3' orientation of the 140 and 220 bp

Figure 4.1 Total RNA extracted from the liver of juvenile barramundi. The 28s (28s) and 18s (18s) ribosomal bands of the hepatic RNA samples (20μg) are located in Lanes 1 - 9. An RNA marker (M) is located in Lane 10.

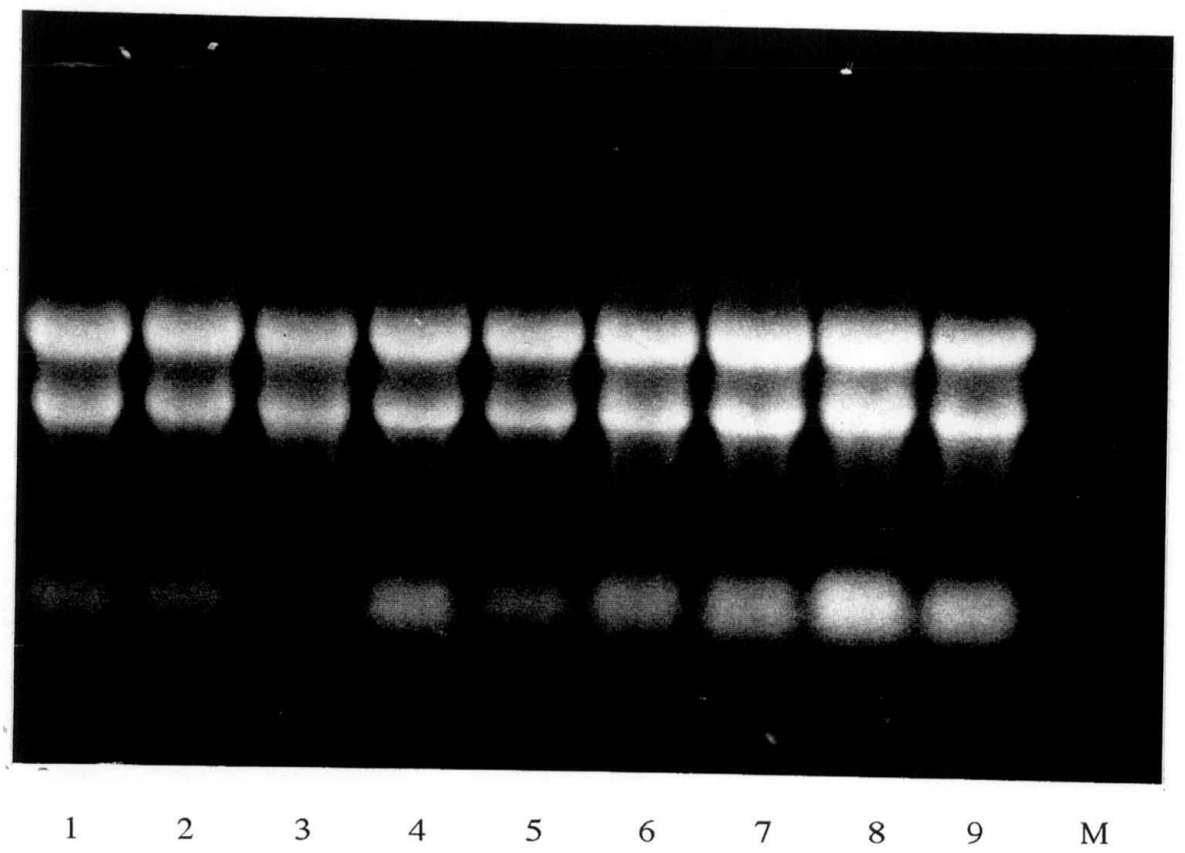
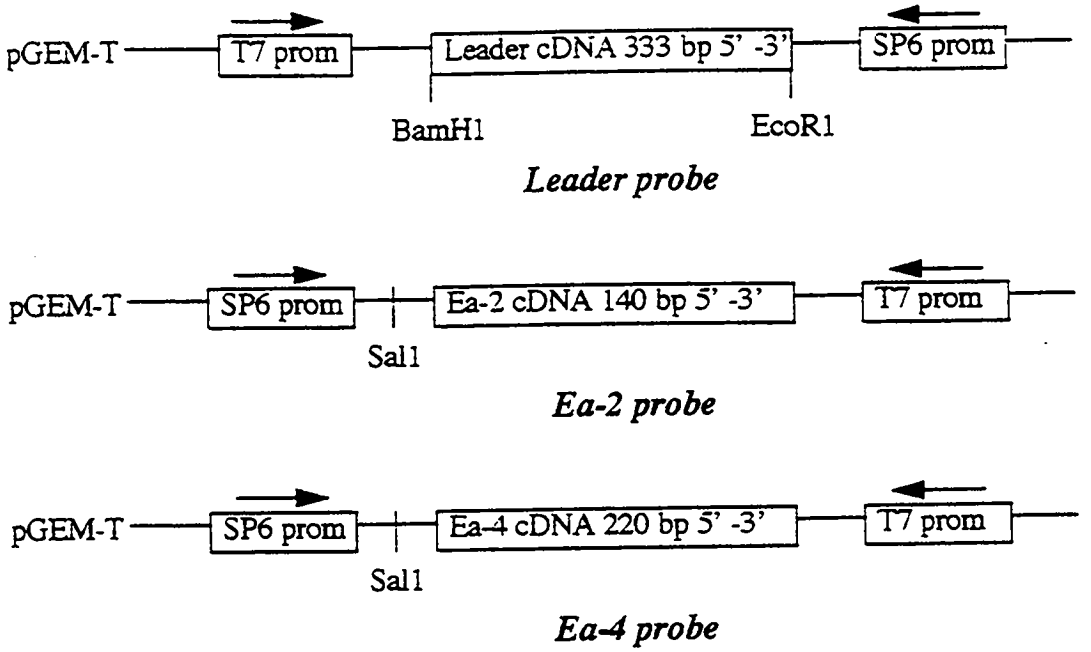
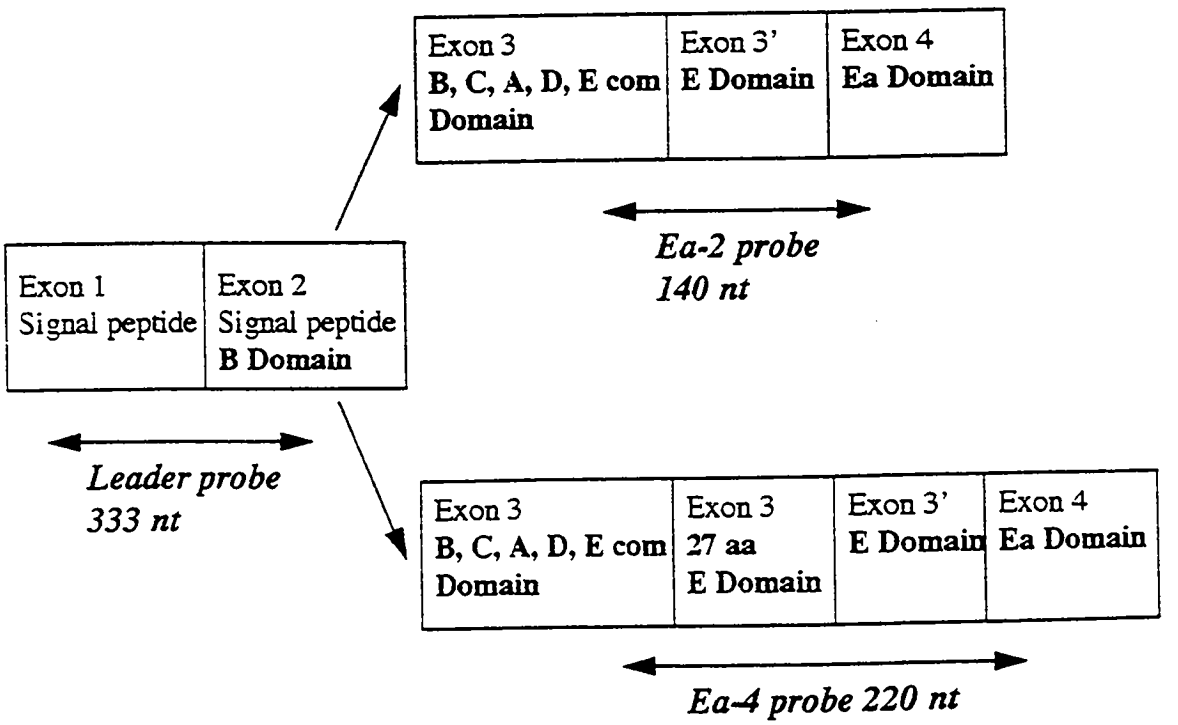

28s
18s
1
2
3
4
5
6
7
8
9
M

Figure 4.2 Barramundi IGF-I specific probes. (a) The insertion sites for the three cDNA fragments are indicated in relation to the orientation of the flanking RNA promotors. The promotors are drawn as boxes with overlying arrows that indicate the 5' to 3' direction. The vectors that carry the cDNA inserts were pGEM-T. (b) The complementary regions in barramundi IGF-I mRNA to the three probes. The mRNAs are drawn as boxes that represent the exons, exon number and the encoded domians are indicated. "E com" is equivalent to the first 16 encoded amino acids in the E domain. The mRNAs diverge after exon 2 into the upper Ea-2 and lower Ea-4 cDNA. The probes are indicated with doublehead arrows. Figure reproduced with permission from Kinhult (1997).

(a)


pGEM-T
T7 prom
Leader cDNA 333 bp 5' -3'
SP6 prom
BamH1
EcoR1
Leader probe
pGEM-T
SP6 prom
Ea-2 cDNA 140 bp 5' -3'
T7 prom
Sal1
Ea-2 probe
pGEM-T
SP6 prom
Ea-4 cDNA 220 bp 5' -3'
T7 prom
Sal1
Ea-4 probe

(b)


Exon 3
B, C, A, D, E com Domain
Exon 3'
E Domain
Exon 4
Ea Domain
Ea-2 probe
140 nt
Exon 1
Signal peptide
Exon 2
Signal peptide
B Domain
Leader probe
333 nt
Exon 3
B, C, A, D, E com Domain
Exon 3
27 aa
E Domain
Exon 3'
E Domain
Exon 4
Ea Domain
Ea-4 probe 220 nt

fragments was the same as the SP6 RNA polymerase promoter positioned outside the polylinker in pGEM-T (Fig 4.2a). In contrast, the 333 bp fragment had the same 5' - 3' orientation as the T7 RNA polymerase promoter positioned outside the polylinker in pGEM-T (Fig 4.2a).

The resulting plasmids were transformed onto the bacterial strain DH5-α and stored at -70 °C as a 15 % (v:v) glycerol stock. The three anti-sense probes that were used in the present study and their complementary regions in barramundi IGF-I mRNA are illustrated in Fig. 4.2b. For convenience the probes are referred to as the Ea-2 (140 bp), Ea-4 (220 bp) and leader (333 bp) probes.

4.2.6.1 Plasmid Preparation and Extraction

Bacterial stocks carrying the inserted pGEM-T vectors were spread onto agar plates treated with 50 µg/ml ampicillin and incubated overnight at 37 °C. Single colonies were inoculated into vials containing 10 ml LB-broth (Appendix 1) containing 50 µg/ml ampicillin and incubated at 37 °C with continuous shaking for 10 - 15 hr. Bacteria were harvested by centrifugation at 14,000 g using a Microfuge E™ centrifuge (Beckman, USA) for 3 min and the pellet was retained.

Plasmids were extracted on a mini-prep scale by resuspending the pellet in 200 µl of lysis buffer (Appendix 1) and incubating for 5 min at room temperature followed by the addition of 400 µl of freshly prepared alkaline solution (Appendix 1) and further incubation on ice for 5 min. The proteins, high molecular weight RNA and chromosomal DNA were precipitated by incubation in 300 µl of 7.5 M ammonium acetate solution on ice for 10 min. The supernatant (800 µl) was recovered by centrifugation for 3 min at 14,000 g and plasmid DNA precipitated using 0.6 volumes of isopropanol. The precipitate was recovered by centrifugation at 14,000 g for 10 min and pellets were resuspended briefly using 70 % ethanol, air-dried for 15 min and

resuspended in 50 µl Tris-EDTA (TE) buffer (Appendix 1) with 2 µl RNaseA (Boehringer Mannheim).

4.2.6.2 Characterisation, Linearisation and Transcription

Plasmid DNA was characterised by digestion using restriction enzymes which cut the plasmids at sites flanking the cDNA inserts indicated in Fig. 4.2a. Resulting DNA fragments were visualised by resolution in agarose gels. Five µl of each dissolved plasmid was digested in a 200 µl sterile solution consisting of 1x One-Phor-All buffer (Pharmacia-Upjohn, Stockholm, Sweden), 5 µl Sac I (15,000 U/ml) and 5 µl Sac II (5,000 U/ml) and incubated at 37 °C for 1 hr. As a control for the digestion efficiency, undigested plasmids (2 µl) were prepared using 3 µl sterile water. Digested (20 µl) and undigested (2 µl) plasmids were prepared for electrophoresis by the addition of 0.2 volumes of 6x loading buffer (Appendix 1). Lamda DNA Hind III Digest (500 µg/ml, Boehringher Mannheim) (4 µl), and DNA marker ϕ 174 Hind II (50 µg, Boehringher Mannheim) (2 µl) were used as known molecular weight markers. All samples were loaded onto to an agarose gel containing 0.8 % (w:v) agarose, 50 ml 1x Tris acetate EDTA (TAE) buffer (Appendix 1) and 0.5 g EtBr and run at 100 V for 30 min using 1x TAE as the running buffer. Bands were visualised under UV light and recorded by polaroid or digital photography. Plasmid DNA containing the cDNA inserts of the correct size were linearised for anti-sense riboprobe production.

Plasmids were linearised for anti-sense riboprobe production using a restriction enzyme that cut the polylinker 5' end of the 5' cDNA insert (Fig. 4.2a). Thus plasmids containing the Ea-2 and Ea-4 inserts were cut with Sal1 and the plasmid containing the leader insert was cut with BamH1 (Fig. 4.2a). Plasmids (40 µl) were incubated with 200 µl of 2x One-Phor-All buffer and 10-15 U of the appropriate

restriction enzyme for 60 min at 37 °C. Linearisation was confirmed by visualising an aliquot of the incubated solution by agarose gel electrophoresis (Section 4.2.9.5). The remaining plasmid solution was treated with 400 µl of proteinase K buffer (Appendix 1), incubated for 45 min at 37 °C and made up to 0.45 M sodium acetate (pH 5.2). The extraction was conducted by the addition of 400 µl of a 1:1 (v:v) saturated phenol: chloroform solution, followed by centrifugation for 5 min at 14,000 g at room temperature. The aqueous layer was retained, treated with 1 volume of chloroform, centrifuged as detailed above and subsequently retained again and precipitated by the addition of 2.5 volumes 99 % (v:v) ethanol with incubation at -20 °C for at least 60 min. The pellet was recovered by centrifugation at 14,000 g for 15 min at 4 °C and resuspended briefly in 75 % (v:v) ethanol prior to centrifugation as described above. The pellets were air-dried for approximately 15 min at room temperature, resuspended in 20 µl DEPC water and stored at 4 °C until further use.

Linearised plasmids served as templates for riboprobe synthesis and were heated at 65°C for 10 min prior to transcription. Linearised plasmids (2 µl) were diluted three fold using sterile water and transcribed *in vitro* in the presence of 10 mM dithiothreitol (DTT, Promega, USA), 0.5 mM 2-Deoxy adenosine triphosphate (ATP), 0.5 mM 2-Deoxy guanosine triphosphate (GTP), 0.5 mM 10 mM 2-Deoxy cystidine triphosphate (CTP) (Pharmacia LKB, Sweden), 1x Transcription buffer (Boehringer Mannheim), 5 µM UTP, 40 U RNase inhibitor from human placenta (Boehringer Mannheim), 50 mCi ^{32}P-UTP (3000 Ci/mM, Breasatec, Adelaide, Australia) and 40 U of the appropriate RNA polymerase at 37 °C for 10 min. The RNA polymerase used for each linearised plasmid was determined by the promoter preceding the cDNA insert (Fig. 4.2a). The RNA polymerases used were SP6 (Boehringer Mannheim) for the leader probe and T7 (Boehringer Mannheim) for Ea-2 and Ea-4 probes (Fig 4.2a). Following the first incubation, 20 U of the appropriate RNA polymerase was added and the incubation continued for a further 30 min. Subsequently, 0.1 µl of DNase I

(5000 U/ml) (Pharmacia-Upjohn) was added and the incubation continued for another 15 min. Finally, 20 µl of loading buffer (Appendix 1) was added and the mixture incubated at 90 °C for 2 - 3 min.

4.2.6.3 Purification of Antisense Riboprobe

Full-length riboprobes were purified by separation using a 5 % polyacrylimide/ 8 M urea gel as described in the ribonuclease protection assay RPA II ™ kit instruction manual (Ambion, Texas, USA), with the following modifications. The entire solution was separated at 40 Watts (W) for approximately 5 min using 10x Tris-borate EDTA (TBE) buffer (Appendix 1) as the running buffer. The full-length probe was visualised using autoradiography, excised, placed into 350 µl of elution buffer (Appendix 1) and incubated at 37 °C overnight. Following incubation, 1 µl of the eluted probe solution was placed onto a glass-microfibre filter pad (GF/C, 2.4 cm diameter, Whattman, Maidstone, England), in a 10 ml vial with 5 ml of Ready Safe scintillant (Beckman, USA) and counted using a Beckman LS 5000 TA beta counter (Beckman, USA). The volume of probe solution containing 1 x 10^5 Cpm was determined. The probe was stored at -20 °C until use in the ribonuclease protection assay (RPA).

4.2.6.4 Transcription of Sense RNA

Sense RNA from the plasmid carrying the leader cDNA fragment was linearised using EcoR1 (Boehringher Mannheim) and transcribed *in vitro* on a five-fold larger scale as described by Kinhult (1997). The concentration of sense RNA was determined by spectrophotometry and electrophoresis (Sections 4.2.5.1 and 4.2.5.3 respectively).

4.2.7 RNA Protection Assay (RPA)

Thirty µg of brain total RNA or 20 µg of hepatic total RNA was incubated with 1 x 10^5 Cpm anti-sense riboprobe and 0.5 M ammonium acetate. Two control tubes containing 10 µg of torulla yeast RNA (Ambion) were also prepared. RNA in all tubes was precipitated using 2.5 volumes of 99 % ethanol and incubation at -20 °C for

15 min. The pellets were recovered by centrifugation at 14,000 g for 15 min at 4 °C, resuspended in 20 µl of hybridisation buffer (Appendix 1) and incubated at 90 °C for 3 - 4 min to denature and solubilise the RNA. Samples were then vortexed thoroughly, centrifuged at 14,000 g for 1 - 2 min and hybridised overnight at 45 °C using a hybridisation oven (Hybaid, United Kingdom).

Unprotected single-stranded RNA in the hybridised samples was digested with 0.5 U RNaseA (Ambion) and 20 U RNase T1 (Ambion). One of the yeast control tubes was RNase treated, whilst the other received only digestion buffer. RNase treated tubes were mixed thoroughly, centrifuged briefly and incubated at 37 °C for 30 min to digest unprotected single-stranded RNA. Samples were precipitated using 300 µl of RNase inactivation/precipitation buffer (Appendix 1) and incubated for approximately 30 min at -20 °C.

The molecular size marker was a Sau 3A digested pBluescript (Stragene, La Jolla, USA) end-labelled using T4 DNA polymerase (New England Biolabs, UK) and α-^{32}P-deoxyribose cytosine 5'-triphosphate (Breasatec, Adelaide, Australia) according to protocol described by Sambrook *et al.* (1989). End-labelled markers were stored for use in the RPA for one month at -20 °C.

Precipitates resulting from the RNase digestion were pelleted by centrifugation at 14,000 g for 15 min at 4 °C. Pellets were denatured and solubilised by resuspension in 8 µl of loading buffer (Appendix 1) and by heating at 90 °C for 3-4 min. An aliquot (5 µl) of the endlabelled RNA marker was also heat treated. Protected fragments were separated using a 5 % polyacrylimide/ 8 M urea gel with 1 x TBE buffer (Appendix 1) as the running buffer. The separation was performed on a gel sequencing system (Pharmacia) using 40 Watts for 70 min. Gels were dried onto filter paper using a SGD 4050 slab gel dryer (Savant, Farmingdale, UK) and the protected fragments were visualised by exposure to phosphoimager plates for approximately 48 hr and then to X-ray film for 2-7 days at -80 °C (Figs. 4.3 and 4.4).

Figure 4.3 An autoradiograph of an RPA gel using the leader (5') probe to detect total IGF-I mRNA in the liver of juvenile barramundi. The position of the IGF-I 333 bp protected fragement is indicated (arrow). Liver samples (20μg) from satiety (Lanes 1 - 4), restricted (Lanes 7 - 9) and starved fish (Lanes 5 - 6) are indicated. An end-labelled Sau 3A digested pBluescript plasmid (Section 4.2.7) used as the marker is indicated (M) and the molecular sizes (bp) appear to the left of the marker. The yeast control hybridisations (Section 4.2.7) treated with RNase (Y+) or digestion buffer only (Y-) are also indicated.

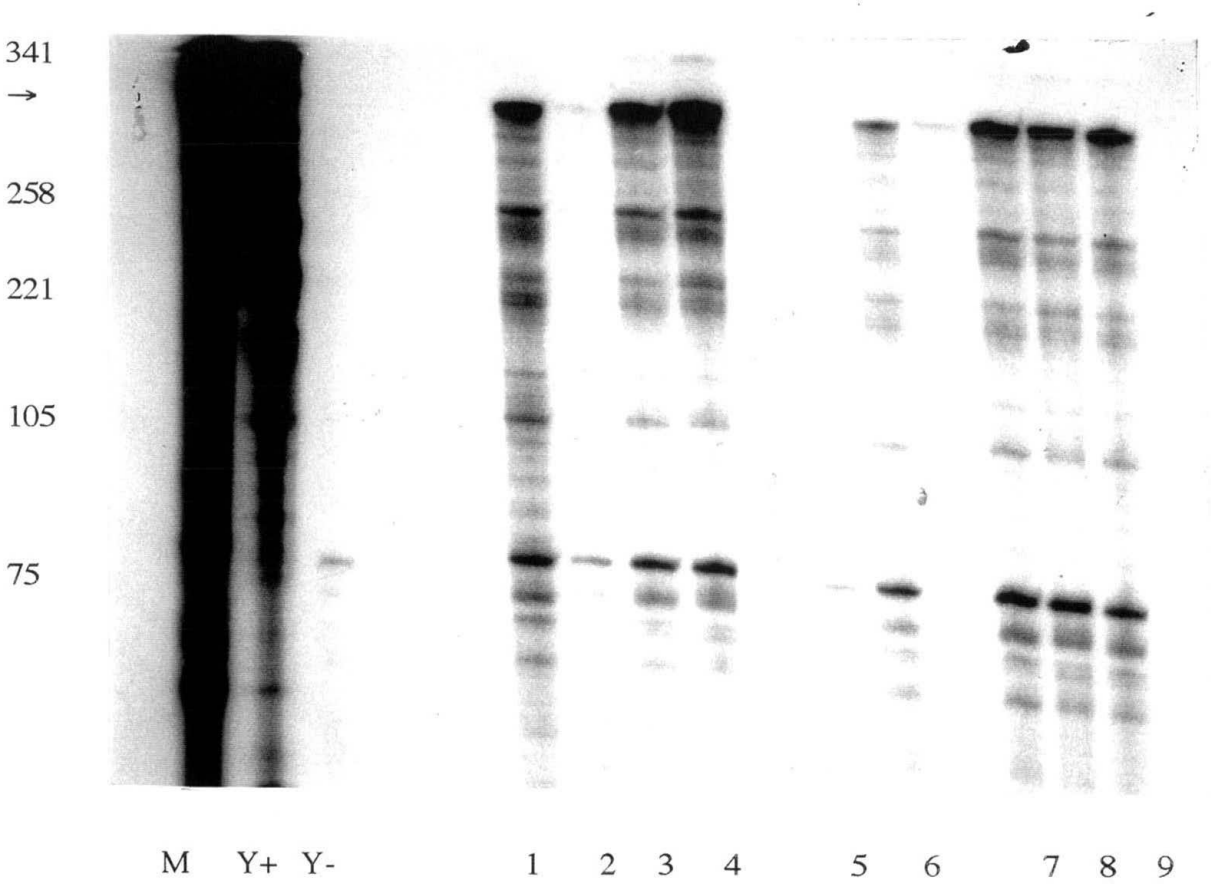
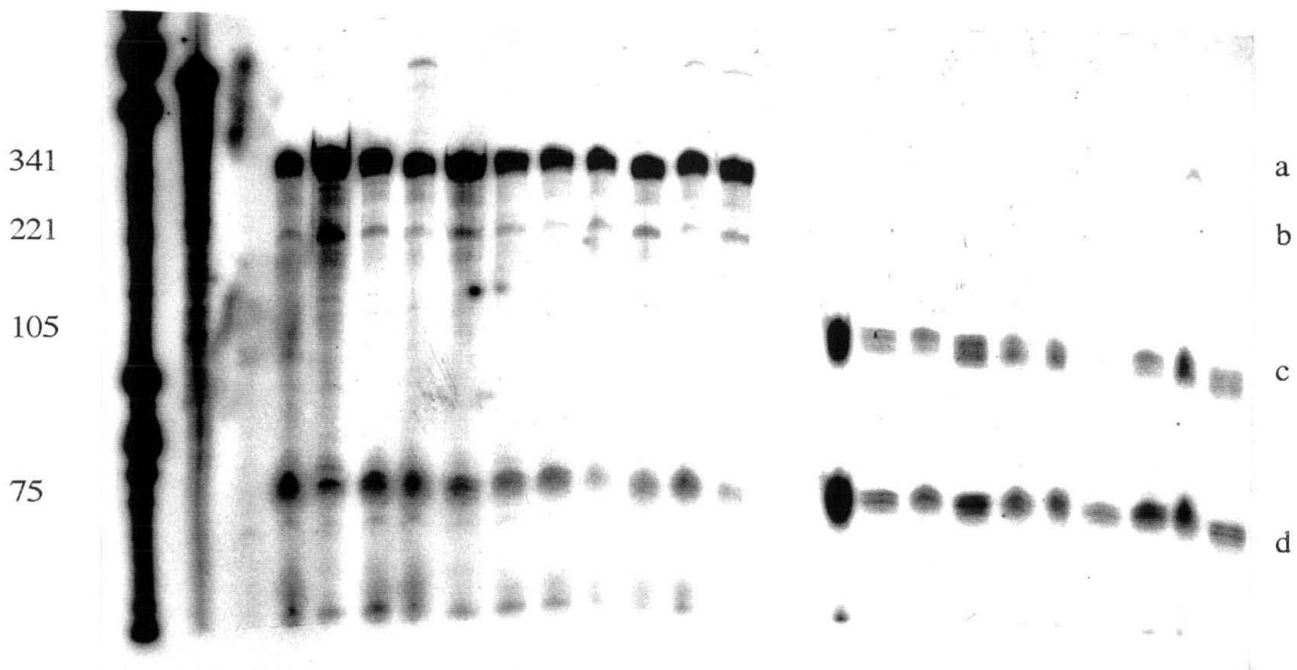

341
→
258
221
105
75
M
Y+
Y-
1
2
3
4
5
6
7
8
9

Figure 4.4 An autoradiograph of an RPA gel using the Ea-2 and Ea-4 probes to detect IGF-I mRNA transcripts in the liver of juvenile barramundi. The positions of the full-length Ea-4 220 bp protected fragment (a), Ea-2 140 bp protected fragment (b), presumptive Ea-3 156 bp fragment (c) and the 75 bp protected fragment (d) by both Ea-4 and Ea-2 are indicated. An end-labelled Sau 3A digested pBluescript plasmid (Section 4.2.7) used as the marker is indicated (M) and the molecular sizes (bp) appear to the left of the marker. The yeast control hybridisations (Section 4.2.7) treated with RNase (Y+) or digestion buffer only (Y-) are also indicated.


341
221
105
75
a
b
c
d
M Y+ Y-

The amount of phospho-stimulated luminescence (PSL/mm^2) in each protected fragment was determined using a Fujifilm BAS-1500 Phosphoimager and Microcomputer imaging device (MCID). PSL units were converted to picograms (pg) of IGF-I mRNA using a standard curve generated by hybridisation of the leader probe with known amounts (0.6 - 314 pg) of synthetic sense RNA. Regression analysis demonstrated a significant ($p < 0.001$) relationship between concentration of sense RNA (pg) and PSL/ mm^2 (Kinhult, 1997).

4.2.8 Normalisation of Total RNA

The derived concentration of IGF-I mRNA was normalised using the actual amount of total RNA used in the RPA by electrophoresis. Aliquots of each RNA sample were separated on a denaturing formaldehyde gel (Section 4.2.5.3) and bands were visualised and recorded using digital photography under UV light. Digitised photos were scanned using a Scan Jet IIcx (Hewlett Packard, USA) and densitometry was performed using the Microcomputer imaging device (MCID, Imaging Research Inc., USA). The density of the bands was used to calculate the proportion of the 30 μg (brain) or 20 μg (liver) used in the RPA.

4.2.9 Reverse Transcriptase Polymerase Chain Reaction

All reagents used in the cDNA synthesis and reverse transcriptase polymerase chain reaction (rt-PCR) procedures were from the Superscript™ Pre-amplification System (Boehringher Research Laboratories, Melbourne, Australia), with the exception of Amplitaq™ polymerase which was obtained from Perkin Emler Applied Biosystems (Melbourne, Victoria). First strand cDNA was synthesized from total RNA using 200 U of reverse transcriptase superscript combined with 2.5 ng/ml of random oligonucleotide hexamers (Kinhult, 1997) in 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$ and 0.1 mM BSA at 42 °C for 50 min. Single strand cDNA was treated for 20 min at 37 °C using 2 U of RNase H. The reaction was subsequently

amplified in a polymerase chain reaction (PCR) using a DNA thermal cycler (Perkin Elmer-Cetus, Scoresby, Victoria). Reaction conditions were previously described by Erlich (1989) and the oligonucleotide primer sequences were described by Kinhult (1997). PCR products were visualised using gel electrophoresis.

4.2.10 Statistics

Data for fish that consumed less than 10 % of the designated ration for the entire trial or which died during the trial were removed. The actual sample sizes for the growth data for each treatment group at 21 and 42 days are shown in Table 4.1.

The experimental design included three fixed factors, time, water temperature and ration size. A fully crossed 3-way ANOVA was not appropriate since ration size was nested within temperature. A 3-level (time, water temperature and ration) nested ANOVA was also inappropriate as the nested level, ration, must be random (Zar, 1984) and in this instance was fixed (selected). Data for each measured parameter from each temperature and timepoint was therefore analysed independently using a one-way ANOVA using SPSS Version 4.0 (Language Systems Corporation, Chicago, USA). Homogeneity of variance was tested by Cochran's C and Bartlett's-box F using SPSS Version 4.0. Data which demonstrated heterogeneous variances ($p < 0.05$) were transformed using $\log_{10}(X + 1)$. Treatment groups were considered significantly different at $p < 0.05$. Where the ANOVA showed a significant ($p < 0.05$) result, pair-wise comparisons of biological relevance were conducted using Tukey's honestly significant difference (HSD) test for unequal sample size (Zar, 1984). The comparisons of different ration sizes at each temperature, as well as a particular ration size across temperatures were considered biologically relevant for the present study. The results of Tukey's HSD pair-wise comparisons for each measured parameter have been tabulated in Appendix 2. Superscripts (a-c) have been used throughout the chapter to demonstrate significant ($p < 0.05$) differences between ration sizes at a particular temperature, whereas superscripts (x,y) demonstrate significant ($p < 0.05$) differences between a particular ration size across temperatures. Pair-wise comparisons between treatment groups were considered significantly different at $p <$

0.05. In addition to the comparisons mentioned, parameters for the 21/SAT and 28/RES treatments were also compared to demonstrate the effect of temperature at a fixed amount of food, since both groups were allocated 2% BW.day^{-1}. To avoid confusion, this comparison has not been indicated by superscript but is mentioned in the text only.

4.3 RESULTS

4.3.1 Growth

Ration size and water temperature both significantly affected the growth of juvenile barramundi throughout the 42 day trial (Table 4.1a and 4.1b).

At day 21 % BWI increased significantly ($p < 0.05$) with increasing ration size at both 21 °C and 28 °C. Increased temperature significantly ($p < 0.05$) increased % BWI for fish fed the SAT and RES rations. The % BWI of the 28/SAT group at day 21 was significantly ($p < 0.05$) higher than the 21/SAT group. The % BWI of the 28/RES group at day 21 was also significantly ($p < 0.05$) higher than the 21/RES group. The % BWI of the 28/RES group was significantly ($p < 0.05$) greater than the 21/SAT group, both of which were fed 2% $BW.day^{-1}$. There was no significant ($p > 0.05$) difference in % BWI between the 28/STV and 21/STV groups.

Specific growth rate was significantly ($p < 0.05$) affected at day 21 by ration size and water temperature (Table 4.1a). SGR increased significantly ($p < 0.05$) with increasing ration size at 21 °C. At 28 °C, the SGR for the 28/SAT and 28/RES groups were significantly ($p < 0.05$) higher than the 28/STV group, however there was no significant ($p > 0.05$) difference between 28/SAT and 28/RES groups. The SGRs for SAT and RES groups at 28 °C were significantly ($p < 0.05$) higher than those at 21 °C. The SGR for the 28/RES group was significantly ($p < 0.05$) higher than the 21/RES group. The SGR for the 28/STV and 21/STV groups were not significantly ($p > 0.05$) different.

Condition factor of fish at day 21 was significantly ($p < 0.05$) affected by ration size but not water temperature (Table 4.1a). The effect of ration seen at both 21 °C and 28 °C was similar. At both temperatures, the CF of the SAT and RES groups were significantly ($p < 0.05$) higher than that of the STV group at day 21. The CF of the SAT groups was slightly higher than the RES groups at both temperatures, but these differences were not significant ($p > 0.05$). There was also no significant ($p > 0.05$) difference between the CF of the 21/SAT and 28/RES groups at day 21.

Table 4.1 Body weight increase (% BWI), specific growth rate (SGR), condition factor (CF), apparent food conversion ratio (FCR) and sample size (n) of juvenile barramundi for different ration/temperature treatments at (a) day 21 or (b) day 42. Values are expressed as mean ± SEM. Values for ration sizes at the same water temperature with different primary superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar ration sizes at different water temperatures with different secondary superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.

(a)

Treatment	BWI (%)	SGR (%)	CF	FCR (g/g)	n
21/SAT	$15.86 \pm 2.11^{a,x}$	$0.70 \pm 0.09^{a,x}$	$1.43 \pm 0.02^{a,x}$	1.86 ± 0.31^{a}	7
21/RES	$7.05 \pm 1.37^{b,x}$	$0.32 \pm 0.06^{b,x}$	$1.32 \pm 0.08^{a,x}$	2.47 ± 0.98^{a}	6
21/STV	$-5.19 \pm 0.72^{c,x}$	$-0.25 \pm 0.04^{c,x}$	$1.14 \pm 0.02^{b,x}$	Not applicable	9
28/SAT	$28.66 \pm 2.04^{a,y}$	$1.20 \pm 0.08^{a,y}$	$1.41 \pm 0.03^{a,x}$	1.54 ± 0.13^{a}	9
28/RES	$20.13 \pm 1.67^{b,y}$	$0.87 \pm 0.07^{a,y}$	$1.35 \pm 0.04^{a,x}$	1.13 ± 0.09^{a}	6
28/STV	$-4.14 \pm 0.81^{c,x}$	$-0.20 \pm 0.04^{b,x}$	$1.12 \pm 0.03^{b,x}$	Not applicable	8

(b)

Treatment	BWI (%)	SGR (%)	CF	FCR (g/g)	n
21/SAT	$5.13 \pm 1.27^{a,x}$	$0.24 \pm 0.06^{a,x}$	$1.46 \pm 0.02^{a,x}$	8.01 ± 4.63^{a}	5
21/RES	$9.39 \pm 1.07^{a,x}$	$0.43 \pm 0.05^{a,x}$	$1.43 \pm 0.02^{a,x}$	1.29 ± 0.12^{a}	4
21/STV	$-8.67 \pm 4.68^{b,x}$	$-0.45 \pm 0.26^{b,x}$	$1.16 \pm 0.01^{b,x}$	Not applicable	4
28/SAT	$8.64 \pm 1.63^{a,x}$	$0.39 \pm 0.07^{a,x}$	$1.45 \pm 0.02^{a,x}$	4.19 ± 0.83^{a}	6
28/RES	$7.85 \pm 2.29^{a,x}$	$0.36 \pm 0.10^{a,x}$	$1.43 \pm 0.03^{a,x}$	2.74 ± 0.64^{a}	3
28/STV	$-5.11 \pm 0.57^{b,x}$	$-0.25 \pm 0.03^{b,x}$	$1.07 \pm 0.01^{b,x}$	Not applicable	3

There were no significant differences in the apparent FCR of the SAT and RES groups at either 21 °C or 28 °C at day 21 (Tables 4.1a).

The growth of fish, both % BWI and SGR, was generally lower at day 42 than at day 21. Apparent FCRs were also generally lower over days 21 - 42 suggesting a reduced food intake.

By day 42, % BWI was significantly affected by ration size but not temperature (Table 4.1b). The % BWI for the STV groups at both 21 °C and 28 °C was significantly ($p < 0.05$) lower than that of the SAT and RES at the respective temperatures. The % BWI of the SAT and RES groups at both 21 °C and 28 °C was not significantly ($p > 0.05$) different. There were no significant ($p > 0.05$) differences in % BWI for similar ration sizes at different temperatures using pair-wise comparisons. There was no significant ($p > 0.05$) difference in % BWI for the 21/SAT and 28/RES groups, which both received 2% $BW.day^{-1}$.

At day 42, the SGRs of the STV groups at both 21 °C and 28 °C were significantly ($p < 0.05$) lower than those of the respective SAT and RES groups (Table 4.1b). The SGRs of the SAT and RES groups were not significantly ($p > 0.05$) different at either temperature. There was also no significant ($p > 0.05$) difference in SGR for similar ration sizes at different temperatures for the 21 - 42 day period using pair-wise comparisons. There was no significant ($p > 0.05$) difference in SGR for the 21/SAT and 28/RES groups, which both received 2% $BW.day^{-1}$.

Condition factor of fish at day 42 was again significantly ($p < 0.05$) affected by ration size but not water temperature (Table 4.1b). The CFs of the SAT and RES groups at both 21 °C and 28 °C were significantly ($p < 0.05$) higher than that of the STV groups at the respective temperatures. The CF of the SAT group was not significantly ($p > 0.05$) different to that of the RES groups at either temperature. There was again no significant ($p > 0.05$) difference in the CF of the 21/SAT or 28/RES groups, which both received 2% $BW.day^{-1}$.

There was a tendency for the FCR for the SAT groups to be greater than for the RES groups both at 21 °C and at 28 °C by day 42, however the differences were not statistically significant ($p > 0.05$) (Table 4.1b).

4.3.2 Circulating IGF-I and IGF-II Activities

The concentration of circulating IGF-I was significantly ($p < 0.05$) affected by ration size and water temperature and was generally related to changes observed in % BWI and SGR. At day 21 the circulating IGF-I concentration had decreased significantly ($p < 0.05$) with decreasing ration size at both temperatures (Fig. 4.5). There was also a significant ($p < 0.05$) difference between the circulating concentration of IGF-I in the SAT group at 21 °C (13.67 ± 2.36 ng hIGF-I/ml serum) when compared to the SAT group at 28 °C (18.64 ± 3.20 ng hIGF-I/ml serum). In contrast, there was no significant ($p > 0.05$) difference in circulating IGF-I concentration between the RES (10.17 ± 2.74 ng hIGF-I/ml serum, 11.23 ± 2.10 ng hIGF-I/ml serum) or STV (0.33 ± 0.21 ng hIGF-I/ml serum, 0.15 ± 0.08 ng hIGF-I/ml serum) rations at 21 or 28 °C, respectively. There was no significant ($p > 0.05$) difference between the concentrations of circulating IGF-I between the 21/SAT and 28/RES groups, which both received 2% BW.day^{-1}.

In contrast to circulating IGF-I, the concentration of circulating IGF-II was not significantly ($p < 0.05$) affected by ration size or water temperature at day 21 (Fig. 4.6). The circulating concentrations of IGF-II were 3.88 ± 0.83 ng hIGF-II/ml serum, 3.58 ± 0.77 ng hIGF-II/ml serum and 2.80 ± 1.01 ng hIGF-II/ml serum, for the 21/SAT, 21/RES and 21/STV treatment groups, respectively and 4.04 ± 1.24 ng hIGF-II/ml serum, 3.78 ± 1.07 ng hIGF-II/ml serum and 2.64 ± 0.91 ng hIGF-II/ml serum for the 28/SAT, 28/RES and 28/STV treatment groups, respectively.

Figure 4.5 Circulating IGF-I levels in juvenile barramundi held at either 21 °C (semi-solid bars) or 28 °C (hatched bars) fed a satiety (SAT) or restricted (RES) ration size or starved (STV) for 21 days. Values are expressed as mean ± SEM. Sample sizes are listed in Table 4.1a. Values for ration sizes at the same water temperature with different primary superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar ration sizes at different water temperatures with different secondary superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.

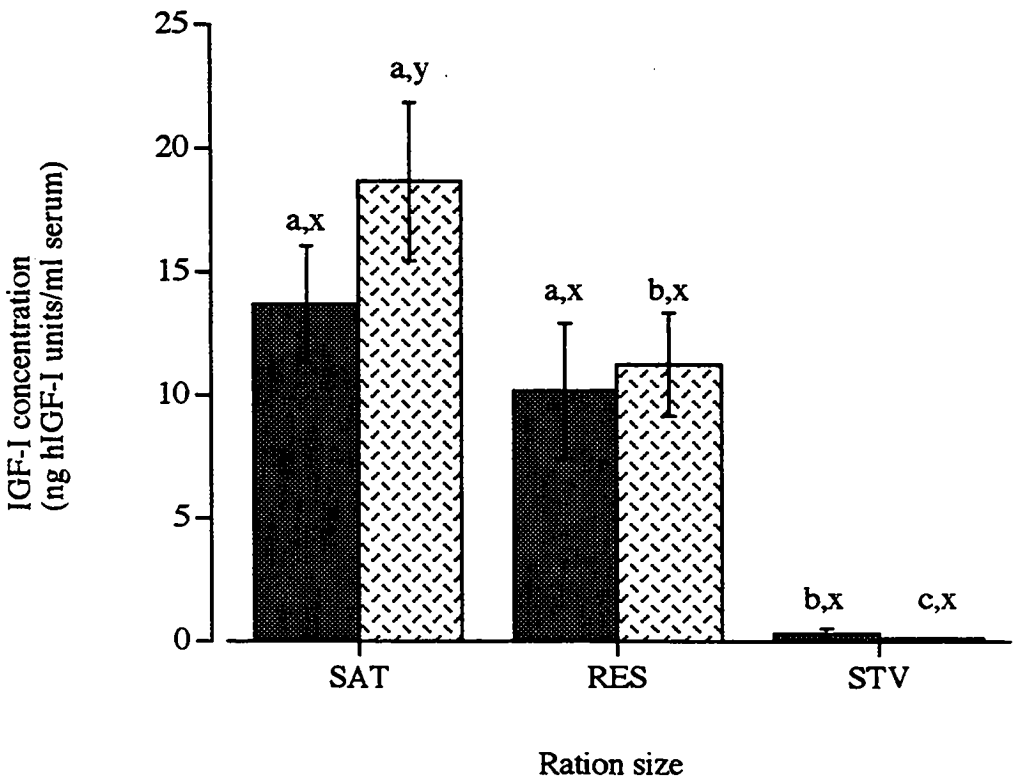
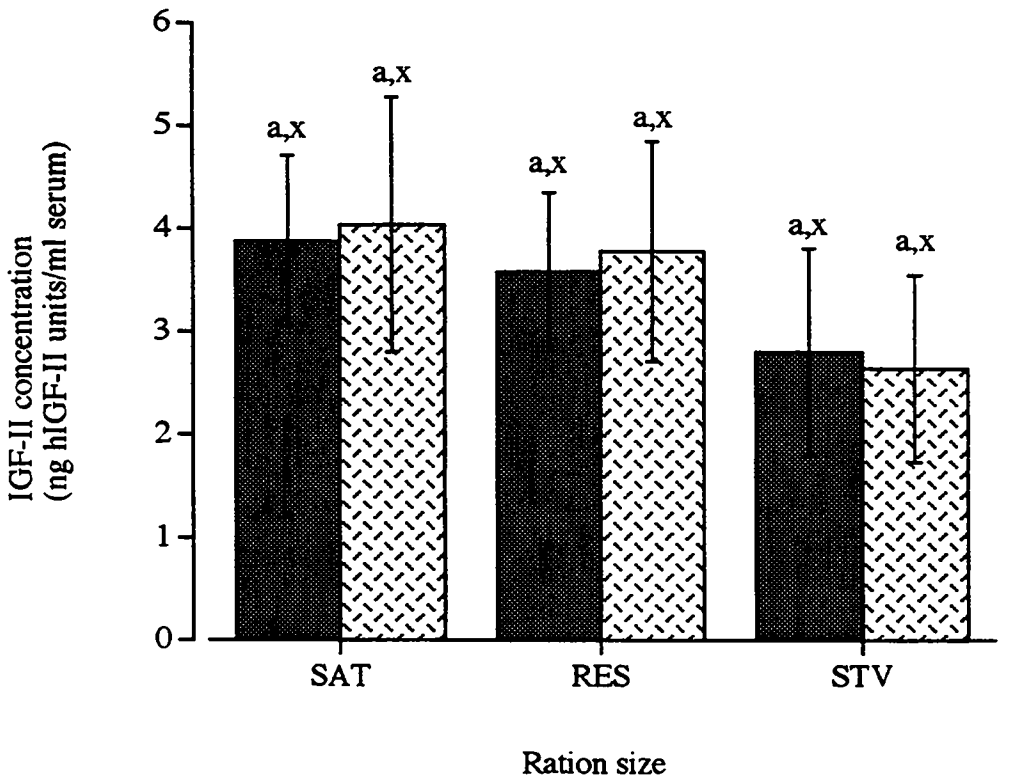

IGF-I concentration
(ng hIGF-I units/ml serum)
25
20
15
10
5
0
a,y
a,x
a,x
b,x
b,x
c,x
SAT
RES
STV
Ration size

Figure 4.6 Circulating IGF-II levels in juvenile barramundi held at either 21 °C (semi-solid bars) or 28 °C (hatched bars) fed a satiety (SAT) or restricted (RES) ration size or starved (STV) for 21 days. Values are expressed as mean ± SEM. Sample sizes are listed in Table 4.1a. Values for ration sizes at the same water temperature with different primary superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar ration sizes at different water temperatures with different secondary superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.


IGF-II concentration
(ng hIGF-II units/ml serum)
6
5
4
3
2
1
0
a,x
a,x
a,x
a,x
a,x
a,x
SAT
RES
STV
Ration size

At day 42, the concentration of circulating IGF-I continued to reflect trends observed in % BWI and SGR, with significantly ($p < 0.05$) lower values in STV groups when compared to fed, both SAT and RES, groups at both temperatures (Fig. 4.7). There was no significant ($p > 0.05$) difference in circulating IGF-I concentration between the SAT and RES groups at either temperature. Nor was there a significant difference in serum IGF-I concentration for a particular ration size across temperatures.

The concentration of circulating IGF-II was again not significantly ($p < 0.05$) affected by ration size and water temperature at day 42 (Fig. 4.8). The concentrations of IGF-II were 3.73 ± 1.44 ng hIGF-II/ml serum and 4.04 ± 0.58 ng hIGF-II/ml serum for the SAT ration, 3.18 ± 1.07 ng hIGF-II/ml serum and 4.02 ± 0.80 ng hIGF-II/ml serum for the RES ration and 3.34 ± 0.78 ng hIGF-II/ml serum and 3.04 ± 0.99 ng hIGF-II/ml serum for the STV ration at 21 °C and 28 °C respectively. Again, there was no significant ($p > 0.05$) difference in the concentrations of circulating IGF-II for a particular ration size across temperatures at day 42.

4.3.3 Expression of IGF-I mRNA

4.3.3.1 Total IGF-I mRNA

The expression of total hepatic IGF-I mRNA after 21 days was affected by ration size. The expression of total hepatic IGF-I mRNA was approximately 10-fold lower in the STV groups at 21 °C (0.20 ± 0.05 pg/μg total RNA) and 28 °C (0.15 ± 0.04 pg/μg total RNA) when compared to the SAT and RES groups at 21 °C (1.62 ± 0.18 pg/μg total RNA and 2.24 ± 0.08 pg/μg total RNA, respectively) and 28 °C (1.97 ± 1.25 pg/μg total RNA and 1.56 ± 0.11 pg/μg total RNA, respectively) (Fig. 4.9). Despite the 10-fold reduction in hepatic IGF-I mRNA in starved fish, statistical interpretation of the data was restricted due to the limited sample size.

Figure 4.7 Circulating IGF-I levels in juvenile barramundi held at either 21 °C (semi-solid bars) or 28 °C (hatched bars) fed a satiety (SAT) or restricted (RES) ration size or starved (STV) for 42 days. Values are expressed as mean ± SEM. Sample sizes are listed in Table 4.1b. Values for ration sizes at the same water temperature with different primary superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar ration sizes at different water temperatures with different secondary superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.

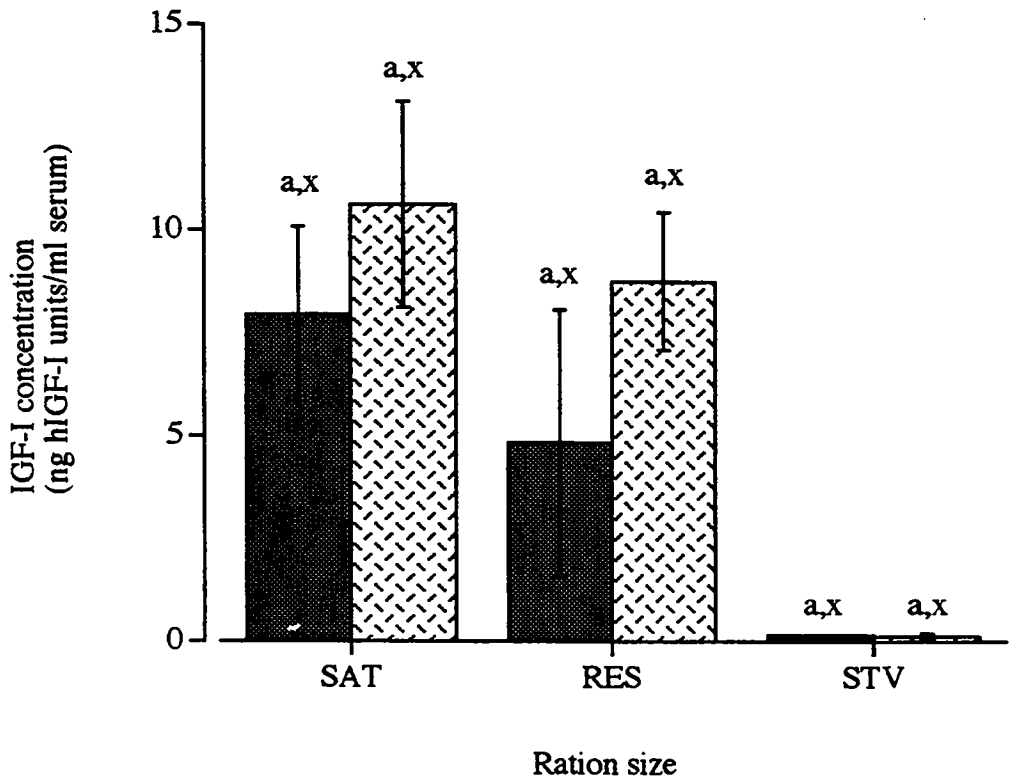

15
10
5
0
IGF-I concentration
(ng hIGF-I units/ml serum)
a,x
a,x
a,x
a,x
a,x
a,x
SAT
RES
STV
Ration size

Figure 4.8 Circulating IGF-II levels in juvenile barramundi held at either 21 °C (semi-solid bars) or 28 °C (hatched bars) fed a satiety (SAT) or restricted (RES) ration size or starved (STV) for 42 days. Values are expressed as means ± SEM. Sample sizes are listed in Table 4.1b. Values for ration sizes at the same water temperature with different primary superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar ration sizes at different water temperatures with different secondary superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.

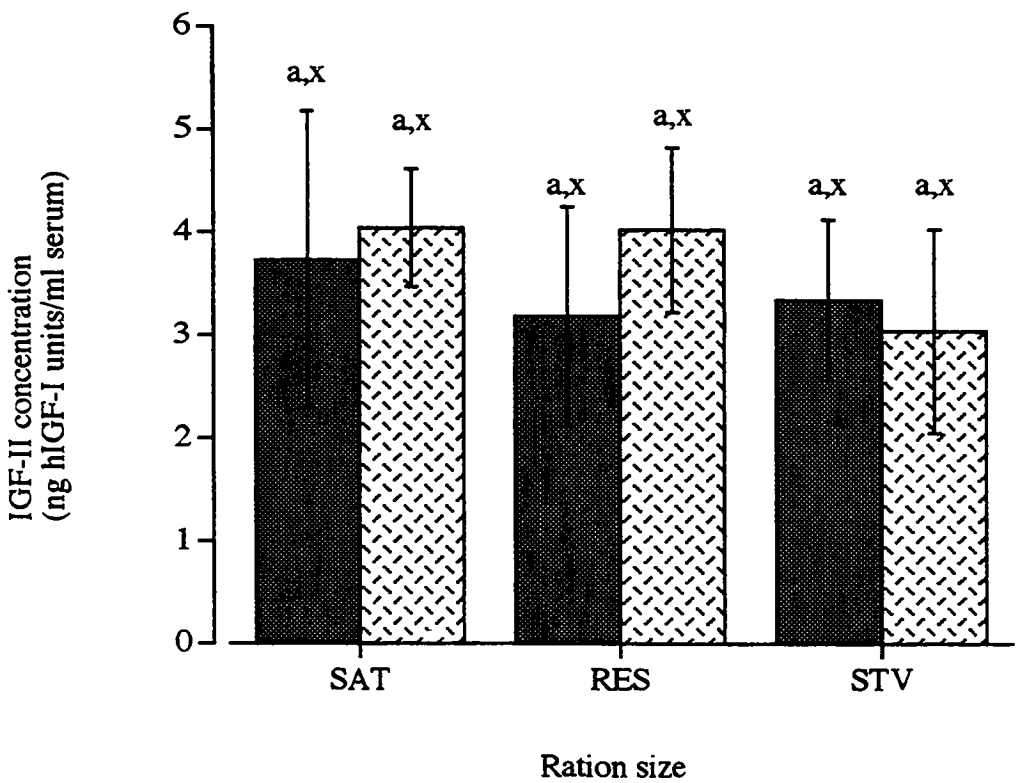
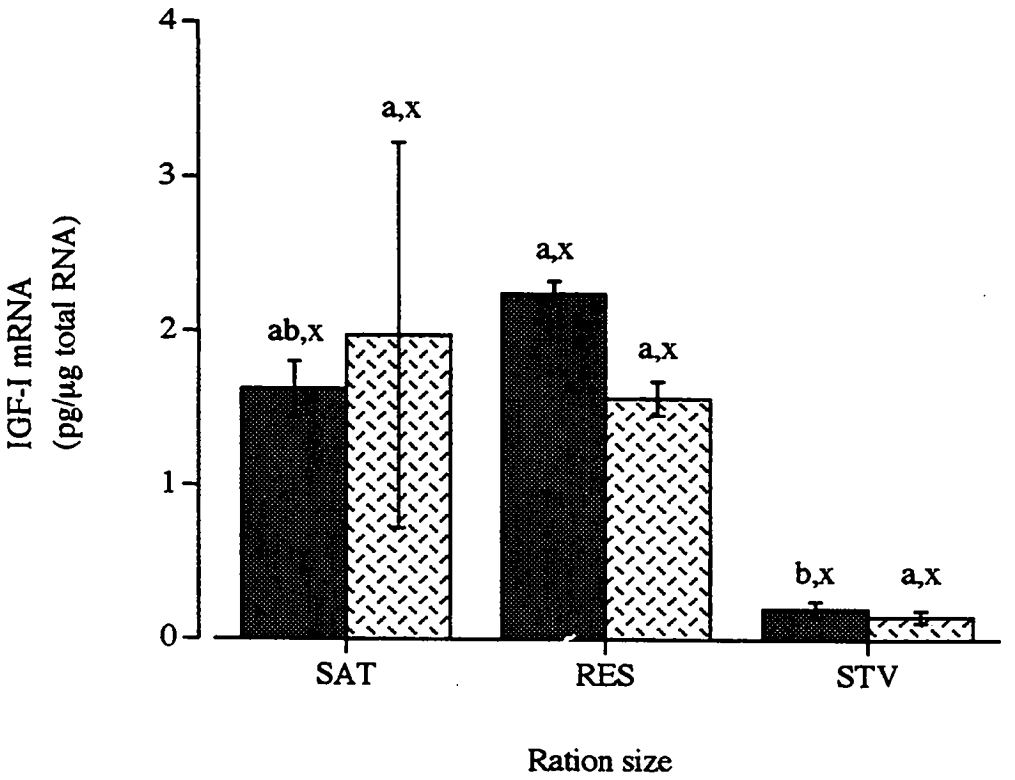

IGF-II concentration
(ng hIGF-I units/ml serum)
6
5
4
3
2
1
0
a,x
a,x
a,x
a,x
a,x
a,x
SAT
RES
STV
Ration size

Figure 4.9 Hepatic levels of total IGF-I mRNA expression in juvenile barramundi held at either 21 °C (semi-solid bars) or 28 °C (hatched bars) fed a satiety (SAT) or restricted (RES) ration size or starved (STV) for 21 days. Values are mean (n=3) ± standard deviation. Values for ration sizes at the same water temperature with different primary superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar ration sizes at different water temperatures with different secondary superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.


IGF-I mRNA
(pg/µg total RNA)
4
3
2
1
0
a,x
ab,x
a,x
a,x
b,x
a,x
SAT
RES
STV
Ration size

At the 0.05 significance level, the only statistically higher expression of hepatic IGF-I mRNA was found when the 21/RES group was compared to the 21/STV group. When compared to the appropriate STV groups the expression of hepatic IGF-I mRNA in the 21/SAT group and in the 28/SAT group neared significance with p values between 0.05 and 0.1.

The expression of IGF-I mRNA in the brain was not significantly ($p > 0.05$) altered by ration size or temperature at 21 days (Fig 4.10). The average level of IGF-I expression in the brain for the 0 -21 day period was 0.12 ± 0.01 pg/μg total RNA. The level of IGF-I expression in the brain was therefore approximately 10-fold lower than the hepatic levels of fed fish at 21 days.

The expression of IGF-I mRNA at day 42 was similar to that reported at day 21, with the STV groups at 21 °C (0.10 pg/μg total RNA) and 28 °C (0.29 ± 0.02 pg/μg total RNA) again displaying approximately 10-fold lower levels when compared to the SAT and RES at 21 °C (2.83 ± 1.57 pg/μg total RNA and 1.71 ± 0.15 pg/μg total RNA, respectively) and 28 °C (1.39 ± 0.33 pg/μg total RNA and 2.20 ± 0.23 pg/μg total RNA, respectively) (Fig. 4.11). Despite the 10-fold reduction in the expression of hepatic IGF-I mRNA in starved fish at 42 days, the ANOVA was not significant at the 0.05 level ($p = 0.051$).

The expression of IGF-I mRNA in the brain was not significantly affected ($p > 0.05$) by ration size or temperature at 42 days (Fig 4.12). The average level of IGF-I expression in the brain was 0.16 ± 0.03 pg/μg total RNA at day 42. The level of IGF-I expression in the brain therefore remained at approximately 10-fold lower levels than in the liver of fed fish.

Figure 4.10 Levels of total IGF-I mRNA in the brain of juvenile barramundi held at either 21 °C (semi-solid bars) or 28 °C (hatched bars) fed a satiety (SAT) or restricted (RES) ration size or starved (STV) for 21 days. Values are mean (n=4) ± standard deviation. Values for ration sizes at the same water temperature with different primary superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar ration sizes at different water temperatures with different secondary superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.

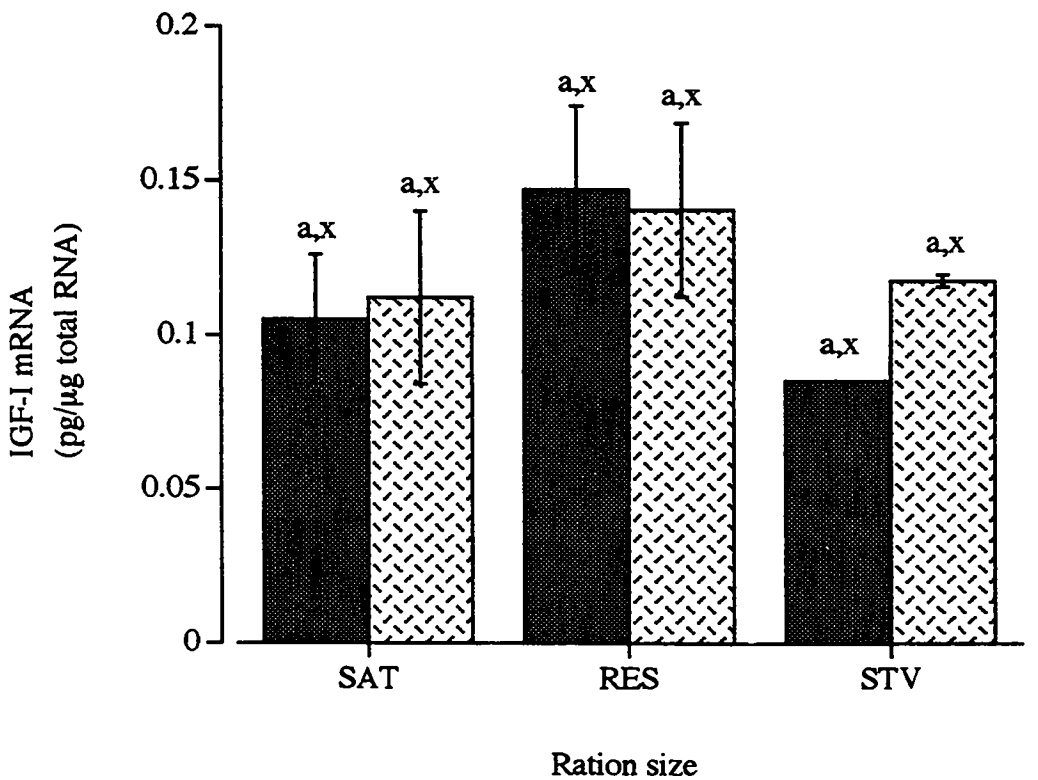

0.2
0.15
0.1
0.05
0
IGF-I mRNA
(pg/µg total RNA)
a,x
a,x
a,x
a,x
a,x
a,x
SAT
RES
STV
Ration size

Figure 4.11 Hepatic levels of total IGF-I mRNA expression in juvenile barramundi held at either 21 °C (semi-solid bars) or 28 °C (hatched bars) fed a satiety (SAT) or restricted (RES) ration size or starved (STV) for 42 days. Values are means (n=3) ± standard deviations. Values for ration sizes at the same water temperature with different primary superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar ration sizes at different water temperatures with different secondary superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.

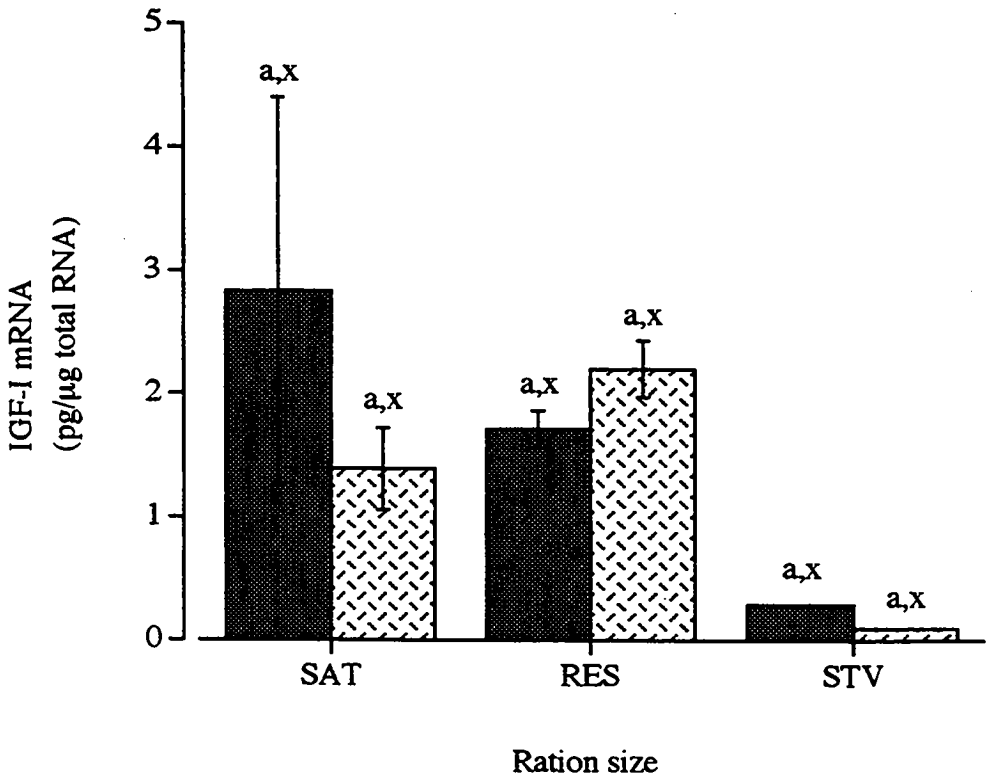
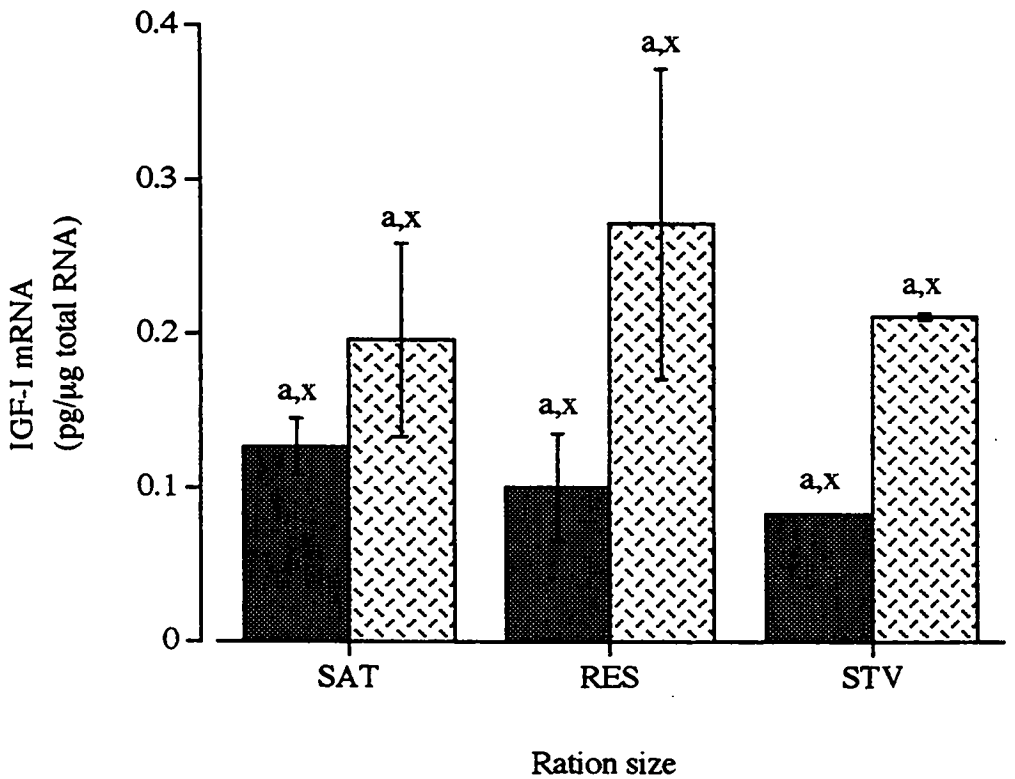

IGF-I mRNA
(pg/µg total RNA)
5
4
3
2
1
0
a,x
a,x
a,x
a,x
a,x
a,x
SAT
RES
STV
Ration size

Figure 4.12 Levels of total IGF-I mRNA in the brain of juvenile barramundi held at either 21 °C (semi-solid bars) or 28 °C (hatched bars) fed a satiety (SAT) or restricted (RES) ration size or starved (STV) for 42 days. Values for ration sizes at the same water temperature with different primary superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar ration sizes at different water temperatures with different secondary superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.


0.4
0.3
0.2
0.1
0
IGF-I mRNA
(pg/µg total RNA)
a,x
a,x
a,x
a,x
a,x
a,x
SAT
RES
STV
Ration size

4.3.3.2 Ea-2 and Ea-4 IGF-I mRNA Transcripts

In addition to the total mRNA, two alternatively spliced IGF-I transcripts, Ea-2 and Ea-4, were detected in the liver of juvenile barramundi. Values were calculated as the ratio of Ea-4 : Ea-2 in units of PSL/ μg total RNA, since sense RNA for these transcripts could not be successfully synthesised. Although this ratio of Ea-4 : Ea-2 expression fluctuated, there was no significant ($p > 0.05$) effect of ration or water temperature (Table 4.2). The ratio of Ea-4 : Ea-2 varied from 1.16 ± 0.25 (21/RES at day 21) to 3.66 ± 4.30 (21/STV at day 21) and the mean ratio was 1.76 ± 0.69 (Table 4.2).

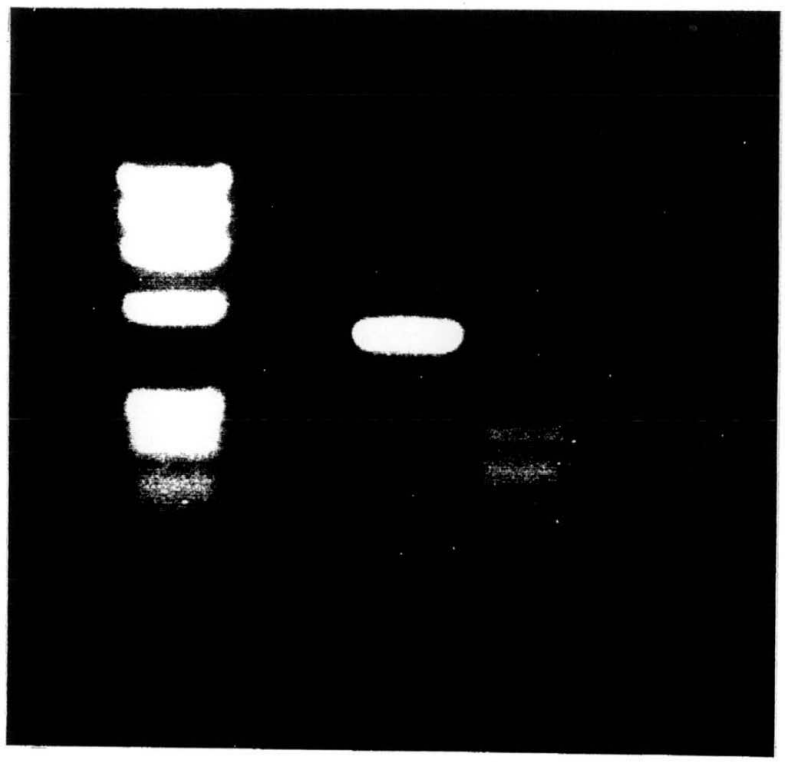
4.3.3.3 Another Alternatively Spliced IGF-I mRNA Transcript

In addition to the Ea-2 and Ea-4 alternatively spliced mRNA transcripts, a band corresponding to the size of salmon Ea-3 (~156 bp) was also detected in liver using the Ea-4 antisense probe (Fig. 4.4). However an attempt to amplify this band using rt-PCR was not successful and only the Ea-2 and Ea-4 bands were detected using rt-PCR and electrophoresis (Fig. 4.13).

Table 4.2 The proportion of Ea-4 : Ea-2 alternatively spliced transcripts in the liver of juvenile barramundi held at 21 °C or 28 °C and fed a satiety (SAT) or restricted (RES) ration or starved (STV) for 21 or 42 days. Values are expressed as the ratio of the amount of Ea-4:Ea-2 IGF-I mRNA alternatively spliced transcript in phosphostimulated luminescence (PSL)/ μg total RNA. Unless otherwise indicated, three replicates are shown for each treatment group (Fish #1- #3). "D" represents total RNA which degraded during sample storage or preparation. No significant ($p > 0.05$) differences between treatment groups were found using one-way ANOVA on transformed data.

Time	Treatment	Ea-4 : Ea-2			
(days)		Fish # 1	Fish # 2	Fish # 3	Mean ± sd
21	21/SAT	1.44	1.65	1.82	1.64 ± 0.20
21	21/RES	1.00	1.01	1.46	1.16 ± 0.26
21	21/STV	1.64	8.60	0.75	3.66 ± 4.30
21	28/SAT	1.07	1.81	D	1.44 ± 0.52
21	28/RES	1.20	1.16	1.35	1.24 ± 0.10
21	28/STV	1.30	1.55	1.36	1.40 ± 0.13
42	21/SAT	1.30	1.04	1.90	1.42 ± 0.44
42	21/RES	2.56	2.21	D	2.38 ± 0.25
42	21/STV	1.73	2.28	D	2.00 ± 0.39
42	28/SAT	1.90	1.97	D	1.93 ± 0.05
42	28/RES	1.52	2.13	2.11	1.92 ± 0.35
42	28/STV	1.21	1.45	D	1.33 ± 0.17
				Mean ± sd	**1.79 ± 0.69**

Figure 4.13 Electrophoresis of rt-PCR products using oligonucleotide hexamers on cDNA originating from barramundi liver. Amplification of the alternatively spliced, Ea-2 and Ea-4, IGF-I mRNA transcripts from the liver of satiety fed barramundi appear in Lane 2. The lane containing the Hae III digested ϕX174 marker (M) is indicated. The molecular sizes (base pairs) of the bands in the marker are situated to the left. Lane 1 represents the control.


1078
872
603
310
281
234
118
M
1
2

4.4 DISCUSSION

4.4.1 Food Deprivation, Growth and IGFs

The present study demonstrated that ration size and water temperature significantly effect the growth and synthesis of IGF-I, but not IGF-II, in juvenile barramundi.

By day 21, the growth of barramundi decreased with decreasing ration size at both temperatures, as has been observed in a wide range of teleost species (Brett, 1979; Weatherley and Gill, 1987; Storebakken *et al.* 1991, Sumpter *et al.*, 1991; Jobling, 1994). The negative growth rate and reduction in the condition factor, an indicator of body shape, of starved fish at both temperatures suggested that starved fish were catabolising body stores for energy. The growth of fish fed the RES ration and the similar condition factor of SAT and RES rationed fish at either temperature suggest the RES ration adequately fulfilled the metabolic energy requirements of the fish and that the SAT ration did not result in the accretion of excess body fat/condition. Both the 28/RES and the 21/SAT groups received 2% $BW.day^{-1}$ of feed. The 28/RES group demonstrated higher growth, both % BWI and SGR, than the 21/SAT groups.

The FCR in the present study were subject to large error. This may be attributed to the fact that it was calculated as apparent FCR, where the amount of food offered, rather than the amount of food eaten, was used. Due to the difficulty in getting fish to eat the entire ration and due to reduced feed consumption after day 21 sampling, it is unlikely that the amount of food offered accurately represented the actual food consumed, thus resulting in overestimation and inaccuracies in FCR.

The decreases in % BWI and SGR with decreasing ration size at day 21 were reflected in circulating IGF-I concentrations and the expression of hepatic of total IGF-I mRNA within each temperature. These results are in accordance with previous investigations of the effect of food deprivation on plasma IGF-I immunoreactivity in gilthead seabream (Perez-Sanchez *et al.*, 1994; Perez-Sanchez *et al.*, 1995) and flounder (Nam *et al.*, 1996) and IGF-I bioactivity in rainbow trout (Komourdjian and Idler, 1978), coho salmon (McCormick *et al.*, 1992), Japanese eel (Duan and Inui, 1990a), striped

bass (Siharath *et al.*, 1996) and the goby (Gray and Kelley, 1991). Whilst the levels of circulating IGF-I in juvenile barramundi reported in the present study are approximately two-fold lower than plasma IGF-I immunoreactivity levels measured in gilthead seabream fed similar ration sizes by heterologous RIA, it is impossible to compare values when different competitive binding assays and conditions have been used.

The regulation of hepatic IGF-I mRNA by nutritional status has also been reported previously in teleosts (Duan and Plisetskaya, 1993). Similar to the results of the present study, where the expression of hepatic IGF-I mRNA was reduced ten-fold in starved barramundi, Duan and Plisetskaya (1993) found that four weeks of starvation significantly lowered the expression of hepatic IGF-I mRNA in coho salmon. In addition, they found that the expression of hepatic IGF-I mRNA was restored to control levels following two weeks of re-feeding. Two weeks of starvation also significantly decreased hepatic IGF-I mRNA expression in the Japanese eel (Duan and Plisetskaya, 1993).

Starvation and the accompanying decrease in growth did not effect the expression of IGF-I in the brain of juvenile barramundi. Four weeks of starvation was also found not to affect the expression of IGF-I mRNA in the kidney, spleen, ovary, gill, gut, muscle or brain tissues of coho salmon (Duan and Plisetskaya, 1993). Furthermore, in the present study, the level of total IGF-I mRNA expression in the liver was approximately 10 fold higher than in the brain of juvenile barramundi. These results are consistent with those previously reported for barramundi, where total IGF-I expression in the liver was higher than in the brain, muscle or kidney (Kinhult, 1997) and with those reported in salmonid species (Duan and Plisetskaya, 1993). The liver of freshwater coho salmon smolts was found to have the highest expression of IGF-I mRNA when compared to muscle, brain, kidney, spleen, gill, gonad, fat tissue and intestine (Duan and Plisetskaya, 1993) and the expression of both IGF-I and IGF-II mRNA was highest in the hepatic tissue of juvenile and adult rainbow trout (Shamblott and Chen, 1993; Shamblott *et al.*, 1995). The absolute concentrations of total IGF-I

mRNA in salmonids and barramundi are not directly comparable due to differences in riboprobe structure and the method of total RNA normalisation. These results therefore provide substantial evidence that circulating IGF-I in teleosts is primarily derived from the liver and that food deprivation down-regulates IGF-I synthesis.

Although circulating GH levels or hepatic GH binding were not investigated in the present study, the number of GH binding sites in the liver decreased with decreasing ration size in gilthead seabream, despite elevations in circulating GH concentrations (Perez-Sanchez *et al.*, 1994; 1995). Elevated serum GH concentrations were also observed in fasting rainbow trout (Sumpter *et al.*, 1991) and coho salmon (Gray *et al.*, 1992; Duan and Plisetskaya, 1993). It therefore appears that the mechanisms of IGF-I regulation by food deprivation in teleost species are due to reductions in GH receptors in the liver thus inducing GH insensitivity, similar to that described in mammals.

No significant differences in % BWI, SGR and concentration of IGF-I were observed between the 28/RES and 21/SAT groups, which both received 2% $BW.day^{-1}$, however the 21/SAT group grew approximately 20 % less than the 28/RES group. The lack of statistical significance in this difference may be attributed to the small sample size.

The decline in circulating IGF-I with ration size was also generally associated with a decrease in the expression of IGF-I mRNA. However, due to the restricted sample sizes used in the present study, the relationship between levels of IGF-I mRNA and circulating IGF-I for the SAT and RES groups were not emphasised as the absolute quantification of mRNA using RNA protection assays is not considered achievable without substantially larger sample sizes.

Although the expression of total hepatic IGF-I mRNA was ten-fold lower in starved barramundi, ration size and water temperature did not significantly affect the ratio of the alternatively spliced, Ea-4 : Ea-2 IGF-I mRNA transcripts in the liver of juvenile barramundi. Similarly, Duan and Plisetskaya (1993) found no effect of starvation on the expression of Ea-4 transcript in salmon. In contrast to the expression of the Ea-4 transcript, fasting lead to decreased expression of both the Ea-1 and Ea-3 alternatively

spliced IGF-I mRNA transcripts in the liver of salmon which was restored by re-feeding (Duan and Plisetskaya, 1993). Unfortunately, as the Ea-1 and Ea-3 transcripts were not measured in the present study as they are yet to be characterised in barramundi.

In the present study, the Ea-4 IGF-I mRNA transcript was predominant over the Ea-2 transcript, with the mean ratio of Ea-4 : Ea-2 being 1.76: 1. The higher expression of Ea-4 in the liver of barramundi, of the same order of magnitude as that reported in the present study, was also reported by Kinhult (1997). These data are different to those described in the liver of the common carp where the Ea-2 transcript predominates (Liang *et al.*, 1996). There is no apparent reason for this inter-species difference.

Although the Ea-2 and Ea-4 were found to be the only types of IGF-I transcripts that varied in the region encompassed by the oligonucleotides E1 and E2, described by Kinhult (1997), the Ea-4 antisense probe also protected a fragment of approximately 156 bp of unknown identity in the present study. Although Ea-1 and Ea-3 alternatively spliced IGF-I mRNA transcripts have not been sequenced in barramundi, based on the structure of the salmonid Ea-3 IGF-I mRNA transcript (Duan and Plisetskaya, 1993) the Ea-4 probe used in the present study should also protect an Ea-3 transcript in the RPA. However, the additional 156 bp band identified in the RPA was not successfully amplified using rt-PCR.

Although there were reductions in the amount of food consumed by fish over the 21-42 day period, the % BWI, SGR and CF of juvenile barramundi were significantly ($p < 0.05$) reduced in the STV groups when compared to the SAT and RES groups at 21 °C or 28 °C at day 42. The observed decline in food consumption was probably related to the recovery of fish from the stress of blood sampling at day 21.

The levels of circulating IGF-I and total hepatic IGF-I mRNA were also reduced in STV fish at day 42, indicating that starvation affected the IGF-I axis throughout the trial. In contrast, the expression of total IGF-I mRNA in the brain, the ratio of the IGF-I alternatively spliced IGF-I mRNA transcripts, Ea-4: Ea-2 and the concentration

of circulating IGF-II remained unaffected by ration size and water temperature at day 42, as compared with that reported at day 21.

In contrast to circulating IGF-I and total hepatic IGF-I mRNA, the concentration of circulating IGF-II was not significantly affected by ration size or water temperature at day 21 or 42. These results clearly indicate that circulating levels of IGF-II are not regulated by ration size in barramundi. The effect of nutritional status, even in mammals, remains unclear. Serum IGF-II levels in adult humans were been reported to decrease (Merimee *et al.*, 1982) or remain unchanged (Davenport *et al.*, 1988) during short-term starvation. Similarly, there was a minimal decrease in serum IGF-II, and no decrease in IGF-II mRNA, in fetal rats which were subject to intrauterine nutritional deprivation by maternal starvation (Donovan *et al.*, 1991). Thus, in view of the mammalian data available, the unaltered circulating IGF-II levels found in juvenile barramundi fed different ration sizes reported in the present study appear consistent.

The actions of IGF-II during periods of nutritional deficiency are thought to result from changes in extravascular IGF-I concentrations (Clemmons and Underwood, 1991). With decreased levels of circulating IGF-I, the interaction between IGF-II, IGFBPs and type I receptors assumes greater importance (Clemmons and Underwood, 1991). The response of IGF-II to nutritional deprivation include the acceleration of IGF-II from the vascular compartment due to the saturation of IGFBPs (themselves down-regulated during food deprivation) and changes in the binding of IGF-II to cell surface receptors (Clemmons and Underwood, 1991). However, the exact role that IGF-II plays in mediating changes in the anabolic response to nutritional status remains unknown in mammals. The regulation of IGF-II by nutritional regulation in teleosts is also unknown with serum IGF-II not commonly measured in teleost species. Gentil *et al.* (1996) measured IGF-II in fasted rainbow trout and reported a significant difference in the concentration of IGF-II from extracted and non-extracted serum, although it seems likely that the decrease resulted from interference by IGFBPs (Gentil *et al.*, 1996) rather than reflecting a true decline with fasting.

Further studies investigating the impact of nutritional status on IGF-II are therefore required in fish.

4.4.2 Conclusion

The present study clearly demonstrated that ration size and water temperature regulate the growth, level of circulating IGF-I and expression of hepatic IGF-I mRNA in juvenile barramundi. In contrast, the expression of IGF-I mRNA in the brain, the ratio of Ea-4 : Ea-2 IGF-I transcripts and the concentration of circulating IGF-II were not significantly affected by ration size or water temperature. Since starvation caused a reduction in hepatic IGF-I mRNA and circulating levels of IGF-I which was accompanied by decreased growth, it is likely that systemic IGF-I of hepatic origin is the most important for somatic growth in this species. The responses of IGF-I, both pre- (hepatic only) and post-translational, but not IGF-II, to ration size in juvenile barramundi are similar to those reported for other teleost and mammalian species, thereby providing further evidence for the general principle of regulation of the GH:IGF-I axis in fish by nutritional status.

CHAPTER 5:

THE EFFECT OF DIETARY PROTEIN AND ENERGY AND WATER TEMPERATURE ON GROWTH AND CIRCULATING IGFs IN BARRAMUNDI.

5.1 INTRODUCTION

The scope for growth in fish is influenced not only by availability of food, but also by the composition of the diet. Due to the importance of protein in tissue construction, protein has long been considered the fundamental unit of growth (Brody, 1945) and studies investigating protein utilisation have been conducted frequently in teleosts (Cowey, 1975; Millikin, 1982; Tacon and Cowey, 1985). Similarly, the fundamental role of dietary energy in growth has provided the impetus for many investigations (Millikin, 1982; Degani and Viola, 1987; Lie *et al.*, 1988; De Silva *et al.*, 1991; Hanley, 1991). The majority of studies seek to determine optimal protein/energy ratios for commercial purposes. The mechanisms by which growth-regulating hormones mediate the interaction between dietary protein and energy and growth have attracted less attention until recently (Sumpter *et al.*, 1991; Perez-Sanchez *et al.*, 1994; Perez-Sanchez *et al.*, 1995).

An investigation into the effect of dietary protein and energy on IGFs in barramundi will therefore provide valuable information on the role of these hormones in regulating growth in response to protein and energy levels in teleosts.

5.1.1 Dietary Requirements for Growth

The growth of fish is dependent upon the construction of new tissues, predominantly muscle, fat and connective and epithelial tissue. The proportion of protein or fat laid down during tissue construction is highly reliant upon diet (De Silva and Anderson, 1995). There are many metabolic processes occurring within a fish, the balance of which determines the fate of nutrients consumed by the animal. The regulation of these metabolic processes occurs in response to substrate concentration and the impact of a variety of hormones, of which the IGFs are two.

Protein synthesis will occur only if the essential amino acids are supplied in the correct ratio and there is energy available (De Silva and Anderson, 1995). Most fish species demonstrate relatively high dietary requirements for protein and essential amino acids,

which in some cases are more than twice those of rat, chicken and pig (Mertz, 1975; Cowey, 1975; Cowey, 1979; Ogino, 1980). If dietary energy is not provided in a non-protein form, amino acids will be catabolised to produce energy via gluconeogenesis, thereby reducing the amount of essential amino acids available for protein synthesis and restricting growth (Jobling, 1994). Non-protein energy can be provided in diets using carbohydrates and lipids. Since the inclusion of these reduces the amount of protein utilised for energy, this process is known as 'protein-sparing' (Jobling, 1994; De Silva and Anderson, 1995). However, carbohydrates are poorly utilised by many fish, especially carnivores, and evidence suggests that proteins and lipids are the major sources of energy (Millikin, 1982; De Silva *et al.*, 1991; Jayaram and Beamish, 1992; De Silva and Anderson, 1995).

Imbalances in the protein-energy ratio result in either wasted protein or the production of fatty fish. The optimal protein-energy ratio varies with both species and environment. Ratios ranging from 17 mg/kJ of protein for tilapia (De Silva *et al.*, 1991) to 28 mg/kJ for channel catfish (Reis *et al.*, 1989) have been reported. Dietary protein and energy specifications for optimal growth of barramundi are 40 - 42 % protein and 14 - 15 MJ/mg energy and 33 -35 % protein and 17 - 18 MJ/kg energy, for temperature conditions greater than 26 °C or less than 23 °C respectively (Williams, pers. comm.). Water temperature and other environmental factors which affect energy partitioning in fish also alter the optimal protein-energy ratio (Hecht and McEwan, 1992).

5.1.2 Effect of Dietary Protein on IGFs

Although some of the effects of protein-energy ratios on the GH:IGF axis have been reported in mammals, the regulatory mechanisms are not completely understood (Bornfelt *et al.*, 1989; Clemmons and Underwood, 1991). Dietary protein restriction was reported to enhance GH release and lower plasma IGF-I in humans (Soliman *et al.*, 1986) and lower serum IGF-I in rats (Maiter *et al.*, 1988). Maiter *et al.* (1988)

was unable to restore serum IGF-I concentrations to normal levels using GH injections in protein-deficient rats. While the level of serum IGF-I was significantly reduced in protein-deprived rats, only a modest reduction in the number of GH binding sites was observed (Maiter *et al.*, 1988). Since the reduction in serum IGF-I levels did not correlate with GH receptor loss, post-receptor regulation, in addition to GH receptor loss, was suggested as a mechanism for the GH resistance occurring in the early stages of protein deficiency (Bornfelt *et al.*, 1989; Clemmons and Underwood, 1991). However, further studies are necessary to fully understand the regulation of IGF-I by dietary protein and energy levels in mammals.

5.1.3 Dietary Protein, Water Temperature and the GH:IGF-I Axis in Fish

In bony fish, there is evidence indicating that the actions of administered GH are modified by both diet quality and water temperature. Bovine GH increased the growth rate of common carp fed low-protein diets, but not high protein diets (Fine *et al.*, 1996) and the growth-promoting effect of bovine GH was significantly enhanced in carp held at lower temperature (Fine *et al.*, 1996). Similarly, but using an elevated water temperature, Danzmann *et al.* (1990) reported limited growth enhancement by GH, a depressed hepatosomatic index (HSI) and increased GH mRNA levels in rainbow trout in response to GH administration. These results demonstrate that when conditions are less than optimal it is likely that attenuation of growth is, at least partially, caused by modifications to the GH:IGF-I axis, as reported in higher vertebrates. However, under optimal conditions of dietary protein and water temperature, growth is not attenuated and GH administration had little or no effect on fish growth.

There has been only one study reporting the effects of dietary protein restriction on the GH:IGF-I axis in a teleost species (Perez-Sanchez *et al.*, 1995). In this study, increases in dietary protein (35, 45 or 55 %) resulted in an increase in the specific

growth rate (SGR) of gilthead seabream but a significant and progressive decline in plasma GH levels. In contrast, plasma IGF-I immunoreactivity and hepatic GH binding increased significantly as dietary protein levels increased (Perez-Sanchez *et al.*, 1995). However, the role of both protein restriction and water temperature on the GH:IGF-I axis in other species and the actual mechanisms of regulation remain to be studied.

5.1.4 Aims

Dietary protein and energy content have been shown to affect the growth of barramundi, and optimal protein and energy requirements of barramundi are thought to vary with changes in water temperature (Williams, pers. comm.). The effect of different diet types on growth-promoting hormones has not been investigated.

The aim of the studies described in this chapter was to determine the effect of dietary protein and energy ratios and water temperature on juvenile barramundi growth and circulating IGF-I and IGF-II levels.

5.2 MATERIALS AND METHODS

5.2.1 Experimental Animals

Seventy-two juvenile barramundi (98.73g ± 1.89g, mean ± SEM) were obtained from North Queensland Barramundi (Rassmussen, Queensland, Australia) are were held in individual tanks as described in Section 2.2, except for water temperature. Fish (n=36) were held in water at either 21 ± 2 °C or 28 ± 3 °C are were acclimated for 4 weeks to laboratory conditions prior to the commencement of the experiment.

5.2.2 Base Ingredient Analysis and Diet Formulation

Diets were formulated to obtain a series of isonitrogenous compositions with varying crude energy and a series of isoenergetic compositions with varying crude protein (Fig. 5.1). The proximate composition of dietary ingredients, as determined by analysis (Section 5.2.7) are detailed in Table 5.1. Diets were either isonitrogenous, 45 % protein, with variable crude energy, 15, 18 or 21 MJ/kg, or isoenergetic, 18 MJ/kg, with variable crude protein, 35, 45 or 55 %. Dietary formulations and proximate composition are detailed in Table 5.2.

Diets were prepared by mixing dry ingredients and fish oil, using a Model A120 Hobart mixer (Hobart Corporation, Troy, Ohio, USA), then adding adequate water to allow pelleting. The mixture was extruded using a Hobart #12 chopper attachment with 1/8 inch die hole diameter (Hobart Corporation, Troy, Ohio, USA) powered by a Model A120 Hobart mixer. Pellets were dried overnight at 50 °C and stored at -20 °C until use.

5.2.3 Experimental Design

Following acclimatisation, six fish at each temperature were sacrificed as time zero controls. The remaining 30 fish at each temperature were allocated randomly an experimental diet (n=6). Fish were fed the respective diets at 2 % BW.day^{-1} at 21 °C

Figure 5.1 Block design showing the two-way relationship between the isonitrogenous and isoenergetic dietary treatments. Crude energy is expressed as kJ/g and crude protein as a percentage.

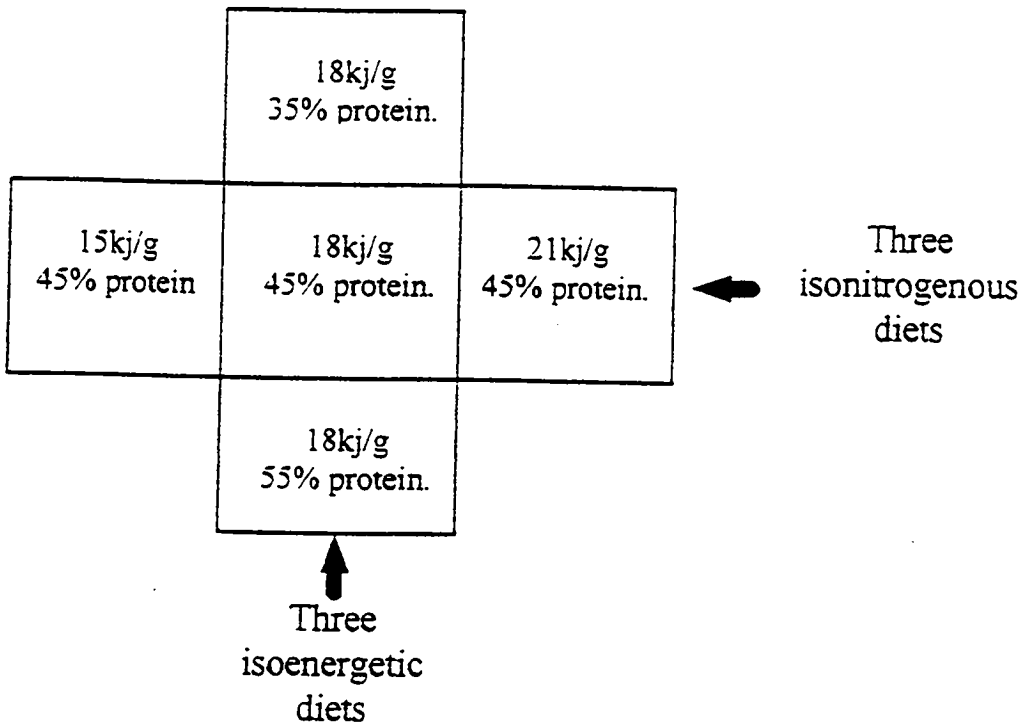

18kj/g
35% protein.
15kj/g
45% protein
18kj/g
45% protein.
21kj/g
45% protein.
Three
isonitrogenous
diets
18kj/g
55% protein.
Three
isoenergetic
diets

Table 5.1 Moisture (%), protein (%) and energy (MJ/kg) in the base ingredients used for the experimental diets. Values are expressed as ranges (n=2), except moisture, where n=1.

Base ingredient	Moisture	Protein	Energy
Corn starch [1]	10.37	0.32 ± 0.30	17.6 ± 1.2
Casein [2]	3.61	89.23 ± 0.40	22.5 ± 0.0
Fishmeal [3]	7.08	66.82 ± 0.42	19.8 ± 0.2
Fish oil [4]	Not conducted	Not conducted	38.1 ± 2.2
Cellulose [5]	6.49	0.22 ± 0.46	16.4 ± 0.2

[1]Corn starch (Goodman Fielder, NSW, Australia) was hydrolysed by autoclaving at 126°C for 20 minutes prior to use in the diets.
[2]Casein was obtained from Bonlac Food Limited, Victoria, Australia.
[3]Fish meal (Causeway Produce, Townsville, Australia) was ground in a hammer mill prior to use in the diets.
[4]Fish Oil (Peruvian) was obtained from Causeway Produce, Townsville, Australia.
[5]α-cellulose (Solka Floc) was obtained from James River Corporation, Berlin, New Hampshire.

Table 5.2 Formulations, protein (% dry weight) and energy (MJ/kg) content and the protein/energy (P/E) ratio of the experimental diets. The amount of each base ingredient in each diet is expressed as grams per kg of dry diet mixture. Values for protein and energy are expressed as ranges (n=2).

Diet	15/45	18/35	18/45	18/55	21/45
Cornstarch	55.8	200.9	133.9	55.80	200.9
Casein	130.7	101.7	130.7	159.8	130.7
Fish meal	542.5	422.0	542.5	663.1	542.5
Fish oil	30.0	130.0	83.0	40.0	130.0
Cellulose	272.7	176.5	141.2	112.3	26.7
Vitamins[1]	30.0	30.0	30.0	30.0	30.0
Minerals[1]	5.0	5.0	5.0	5.0	5.0
Crude Protein	45.3 ± 0.4	35.7 ± 0.6	45.6 ± 0.1	55.4 ± 0.1	45.79 ± 0.3
Energy	16.5 ± 0.2	18.2 ± 0.2	18.1 ± 0.7	18.6 ± 0.1	20.52 ± 1.1
P/E Ratio	2.74	1.97	2.51	2.98	2.23

[1]Vitamin and mineral premixes were obtained from Kevin Williams (QDPI, Bribie Island, Queensland, Australia).

or 4 % BW.day^{-1} at 28 °C (Section 4.2.2) for a period of 42 days. At 21 and 42 days, fish were weighed and the ration adjusted for changes in body weight. The amount of food consumed by each fish per day was recorded throughout the trial. For convenience, the treatment groups will be cited throughout the chapter using the temperature (21 or 28) followed by the abbreviated energy (15, 18 or 21) and protein content (35, 45 or 55) of the diet.

5.2.4 Growth, Sampling and Hepato-Somatic Index

At 21 and 42 days, fish were anaesthetised, weighed and measured (Section 2.2.2). Growth parameters, % BWI, SGR, CF and FCR, were determined using formulae described in section 2.2.1. Diet utilisation was also determined by the calculation of the protein efficiency ratio (PER) and net protein utilisation rate (NPU). PER and NPU were calculated according to equations 5.1 and 5.2 (De Silva and Anderson, 1995), respectively.

$$\text{Equation 5.1} \quad \text{Protein Efficiency Ratio (PER)} = \frac{\text{Wet weight gain (g)}}{\text{Dry weight of protein in diet (g)}}$$

$$\text{Equation 5.2} \quad \text{Net Protein Utilisation (NPU)} = 100 \times \frac{\text{Protein gain in fish carcass (g)}}{\text{Protein intake in food (g)}}$$

Blood samples were taken and serum prepared (Section 2.2.3) from each fish from each diet at 21 and 42 days. Serum was stored at -80 °C until IGF analysis.

At the completion of the trial, all fish were anaesthetised and then sacrificed by cervical dislocation. The liver was dissected and weighed. The liver tissue and carcass of each fish were stored in individual sealed plastic bags at -20 °C until analysis.

Hepato-somatic indices (HSI) for individual fish were calculated using equation 5.3 (De Silva and Anderson, 1995).

$$\text{Equation 5.3} \quad \text{Hepato Somatic Index (HSI)} = 100 \times \frac{\text{Liver weight (g)}}{\text{Body weight (g)}}$$

5.2.5 Circulating IGF Analysis

The levels of circulating IGF-I and IGF-II were determined as described in section 4.2.4.

5.2.6 Preparation of Fish Homogenates

Fish carcasses were homogenised individually using the autoclaving method described by Williams *et al.* (1995), with the following modifications. Frozen carcasses were weighed into individual 1 litre glass jars and autoclaved at 126 °C for 4 hr. The jars were then re-weighed and the differences in weight attributed to water movement. The content of each jar was homogenised *in situ* using the high speed setting on a Braun Multiquick 300 hand blender (Braun, Sydney, Australia) for 5 min. The homogenates were subsequently dried to constant weight at 50 °C, ground using the food processor attachment of the Braun Multiquick 300 blender and ground finely with a mortar and pestle, subsequent to proximate analysis.

5.2.7 Proximate Analysis

The moisture, protein and lipid content of base ingredients and fish homogenates were determined using standard techniques. The ash content was also determined by standard methodology, but was conducted only on the fish homogenates.

5.2.7.1 Moisture

Samples of known weight were placed into individual weigh trays and dried to constant weight at 50 °C. Moisture content (%) was determined gravimetrically.

5.2.7.2 Total Crude Protein

Total crude protein was determined in duplicate for base ingredients and fish

homogenates from nitrogen using the Kjeldahl method. Samples (0.2 g) were weighed into Kjeldahl tubes and digested with one high selenium catalyst tablet (3.5 g, Ajax Chemicals, Killsyth, Australia) and 6 ml of concentrated sulphuric acid (Ajax Chemicals) by heating at 420 °C in a Tectator Digestion System 40, 1016 Digestor (Tectator, Sweden). Digestion was considered complete when the solution became transparent (~30 min). Samples were cooled to room temperature, diluted with equal volumes of water and distilled in excess sodium hydroxide for 3 min using a Tectator Kjeltec System 1002 distilling unit (Tectactor, Sweden). Boric-acid (20 ml) was used in the receiving flask. Following distillation, the receiver flask was titrated with 0.5 M sulphuric acid to endpoint. Nitrogen (%) and crude protein (%) were calculated according to equations 5.4 and 5.5 respectively (Crooke and Simpson, 1971).

$$\text{Equation 5.4} \quad \%\text{Nitrogen} = \frac{\left(\text{Normality of } H_2SO_4 \times \left(\text{Sample titrant (ml)} - \text{Sample blank (ml)}\right) \times 14.01\right)}{\text{Dry weight of sample (g)} \times 10}$$

Equation 5.5 % Crude Protein = % Nitrogen x 6.25

Protein gain (g) in the fish was calculated by subtracting the initial proportion of body weight protein (g) from the final proportion of body weight protein (g). The initial body weight protein of each fish was derived using the average % crude protein of the time 0 controls.

5.2.7.3 Total Lipid

Lipid was determined gravimetrically by an adapted method of Folch *et al.* (1957). Samples (500 mg) were weighed into pre-weighed 10 ml glass vials and suspended in 5 ml of 2:1 (v:v) chloroform/methanol mixture. Suspended samples were sonicated for 15 min, vortexed and left overnight. Samples were subsequently centrifuged at 2500 g for 10 min and the organic layer aspirated. This process was repeated three times to ensure complete extraction of the lipid. Following the final extraction,

samples were dried overnight at 50 °C and re-weighed. Lipid content (%) in the fish was determined by the difference between the initial and final sample weight.

Lipid gain (g) was calculated by subtracting the initial proportion of body weight lipid (g) from the final proportion of body weight lipid (g). The initial body weight lipid of each fish was derived using the average % lipid of the time 0 controls.

5.2.7.4 Ash

Samples (500 mg) were weighed into clean, dry, pre-weighed ceramic crucibles and heated in a muffle furnace at 540 °C overnight. Ash (%) was determined as the difference between the initial and final sample weights.

5.2.7.5 Gross Energy

The gross energy of samples was determined by bomb calorimetry using a Parr Semi-micro Calorimeter (Parr Instrument company, Moline, Illinois, USA). Prior to sample oxidation, the bomb was allowed to reach temperature equilibrium. Dry samples (50-220 mg) were pelleted, weighed and ignited in the presence of excess oxygen (Industrial grade, Class 020, BOC Gases, Chatswood, NSW, Australia). The ignition fuse was alloy wire of known specific calorific value (9.6 kJ/mm). The amount of heat generated was measured and recorded using a Series 4500 microscribe strip chart recorder (Houston Instruments, Austin, Texas, USA). The bomb calorimeter was calibrated using pelleted benzoic acid (energy content 26.6 kJ/g).

5.2.8 Statistical Analysis

Data for fish that consumed less than 10 % of the designated ration for the entire trial or which died during the experiment were removed. The actual sample sizes for the growth data for each treatment group at 21 and 42 days are shown in Table 5.3.

The experimental design included three fixed factors, dietary protein/energy ratio, water temperature and time. For reasons explained in section 4.2.10, data for each measured parameter from each temperature and timepoint were analysed independently

by one-way ANOVA. Means were considered significantly different if $p < 0.05$. Homogeneity of variance was tested by Cochran's C and Bartlett's-box F. Data which demonstrated heterogeneous variances ($p < 0.05$) were transformed using $\log_{10}(X + 1)$. Where ANOVA showed a significant ($p < 0.05$) effect of treatment, pair-wise comparisons of biological relevance were conducted using Tukey's HSD test for unequal sample size ($p < 0.05$) (Zar, 1984). The comparison of different protein and energy diets at each temperature, as well as particular protein and energy diets across temperatures were considered biologically relevant for the present study. The results of Tukey's HSD pair-wise comparisons for each measured parameter have been tabulated in Appendix 3. Superscripts (a-c) have been used throughout the chapter to demonstrate significant ($p < 0.05$) differences between protein and energy diets at a particular temperature, whereas, superscripts (x-y) demonstrate significant ($p < 0.05$) differences between particular protein and energy diets across temperatures. Pair-wise comparisons between treatment groups were considered significantly different if $p < 0.05$.

Correlation analysis was conducted to determine the relationship between carcass moisture and lipid content. The p value was considered significant at the 0.05 level. All analyses were performed using SPSS Version 4.0.

5.3 RESULTS

5.3.1 Growth

There was a significant ($p < 0.05$) effect of both diet and water temperature on the growth of juvenile barramundi during the first 21 days of the trial (Table 5.3). Following 21 days, there was a general tendency for % BWI and SGR to increase with increasing dietary protein (with constant energy). Increased dietary protein resulted in a % BWI increase from 6.43 ± 1.06 to 11.22 ± 3.00 and from 9.18 ± 5.64 to 20.08 ± 3.43, at 21 °C and 28 °C respectively. SGR increased with increasing dietary protein from 0.30 ± 0.05 to 0.50 ± 0.13 and 0.41 ± 0.24 to 0.86 ± 0.14, at 21 °C and 28 °C respectively. However, due to large variances these changes were not significant. The % BWI and SGR both increased significantly ($p < 0.05$) with increasing dietary energy (with constant protein) at both temperatures. At 21 °C, fish fed the 21/45 diet for 21 days demonstrated significantly ($p < 0.05$) higher % BWI and SGR than fish fed the 15/45 and 18/35 diets (Table 5.3a). The % BWI and SGR of fish fed the 21/45 diet at 28 °C for 21 days were significantly ($p < 0.05$) higher than for fish fed the 15/45, 18/35 and 18/45 diets (Table 5.3b). There was also a trend for % BWI and SGR to be higher at 28 °C than at 21 °C, with the % BWI and SGR of the 28: 21/45 group significantly ($p < 0.05$) higher than the 21: 21/45 after 21 days.

There was a tendency for the condition factor (CF) to increase with increasing dietary protein and energy content at day 21, with the exception of protein at 28 °C. However, the differences were not significant ($p > 0.05$) between groups at 21 °C or 28 °C (Table 5.3). There was also a trend for CF at 21 °C to be higher than the corresponding diet at 28 °C, with the 18/55 group at 21 °C demonstrating a significantly ($p < 0.05$) higher CF than the 18/55 group at 28 °C.

Table 5.3 Body weight increase (BWI), specific growth rate (SGR), condition factor (CF), apparent food conversion ratio (FCR) and sample size (n) at 21 days of juvenile barramundi fed different protein energy diets at (a) 21 °C or (b) 28 °C. Values are expressed as mean ± SEM. Values for diets at the same water temperature with different superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar diets at different water temperatures with different superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.

(a)

Treatment	BWI (%)	SGR (%)	CF	FCR (g/g)	n
21: 15/45	$5.23 \pm 0.83^{a,x}$	$0.24 \pm 0.04^{a,x}$	$1.26 \pm 0.01^{a,x}$	$5.98 \pm 1.03^{a,x}$	6
21: 18/35	$6.43 \pm 1.06^{a,x}$	$0.30 \pm 0.05^{a,x}$	$1.27 \pm 0.05^{a,x}$	$4.94 \pm 0.93^{ab,x}$	4
21: 18/45	$10.08 \pm 1.03^{ab,x}$	$0.46 \pm 0.04^{ab,x}$	$1.37 \pm 0.03^{a,x}$	$2.88 \pm 0.24^{ab,x}$	3
21: 18/55	$11.22 \pm 3.00^{ab,x}$	$0.50 \pm 0.13^{ab,x}$	$1.38 \pm 0.03^{a,x}$	$3.08 \pm 0.75^{ab,x}$	5
21: 21/45	$17.05 \pm 1.41^{b,x}$	$0.75 \pm 0.06^{b,x}$	$1.41 \pm 0.06^{a,x}$	$1.67 \pm 0.10^{b,x}$	5

(b)

Treatment	BWI (%)	SGR (%)	CF	FCR (g/g)	n
28: 15/45	$7.83 \pm 2.52^{a,x}$	$0.36 \pm 0.11^{a,x}$	$1.15 \pm 0.02^{a,x}$	$6.95 \pm 1.48^{ab,x}$	4
28: 18/35	$9.18 \pm 5.64^{a,x}$	$0.41 \pm 0.24^{a,x}$	$1.12 \pm 0.01^{a,x}$	$14.22 \pm 8.85^{a,x}$	3
28: 18/45	$15.52 \pm 3.64^{a,x}$	$0.68 \pm 0.15^{ab,x}$	$1.22 \pm 0.02^{a,x}$	$3.98 \pm 1.22^{ab,x}$	5
28: 18/55	$20.08 \pm 3.43^{ab,x}$	$0.86 \pm 0.14^{ab,x}$	$1.18 \pm 0.07^{a,y}$	$2.66 \pm 0.63^{ab,x}$	5
28: 21/45	$38.18 \pm 7.78^{b,y}$	$1.50 \pm 0.28^{b,y}$	$1.25 \pm 0.03^{a,x}$	$2.11 \pm 0.91^{b,x}$	6

Food conversion ratio was significantly ($p < 0.05$) lower in the 21/45 group at both temperatures at day 21 (Table 5.3). FCR showed little change with dietary protein at 21 °C, however, FCR decreased with increasing dietary energy at 21 °C and with both dietary protein and energy at 28 °C. There was no significant ($p > 0.05$) difference in FCR for the same diet across temperatures at day 21.

The growth of barramundi was again significantly ($p < 0.05$) affected by diet and water temperature at day 42, however, the food intake and growth rate of fish at 21 °C decreased substantially during this stage of the trial (Table 5.4).

At day 42, % BWI and SGR were significantly ($p < 0.05$) affected by the composition of the diet (Table 5.4). There was again a tendency for % BWI and SGR to increase with increasing dietary protein at 28 °C but not at 21 °C, while % BWI and SGR increased significantly ($p < 0.05$) with energy content at both temperatures. The significant ($p < 0.05$) effects were identical to those reported at 21 days. At 21 °C, fish fed the 21/45 diet demonstrated significantly ($p < 0.05$) higher % BWI and SGR than did fish fed the 15/45 and 18/35 diets (Table 5.4a). The % BWI and SGR of fish fed the 21/45 diet at 28 °C were significantly ($p < 0.05$) higher than those of fish fed the 15/45, 18/35 and 18/45 diets (Table 5.4b). Again % BWI and SGR tended to be higher at 28 °C than 21 °C with % BWI and SGR of the 18/55 and 21/45 groups held at 28 °C significantly ($p < 0.05$) higher than those of fish fed the same diets but held in water of 21 °C.

Condition factor (CF) was significantly ($p < 0.05$) affected by diet composition at both temperatures at day 42 (Table 5.4). At 21 °C, CF significantly increased with increased dietary energy, with the 21/45 group being significantly higher than the 18/45 and 15/45 groups. Dietary protein also affected CF with the 18/55 group being significantly higher than the 18/35 group at 21 °C. At 28 °C increased dietary energy again resulted in significant changes in CF, with the 21/45 group being significantly

Table 5.4 Body weight increase (BWI), specific growth rate (SGR), condition factor (CF), apparent food conversion ratio (FCR) and sample size (n) at 42 days of juvenile barramundi fed different protein energy diets at (a) 21 °C or (b) 28 °C. Values are expressed as mean ± SEM. Values for diets at the same water temperature with different superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar diets at different water temperatures with different superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.

(a)

Treatment	BWI (%)	SGR (%)	CF	FCR (g/g)	n
21: 15/45	$2.24 \pm 0.63^{a,x}$	$0.11 \pm 0.03^{a,x}$	$1.28 \pm 0.02^{a,x}$	$26.32 \pm 17.01^{a,x}$	6
21: 18/35	$3.74 \pm 0.58^{a,x}$	$0.17 \pm 0.03^{a,x}$	$1.24 \pm 0.03^{a,x}$	$6.20 \pm 1.03^{a,x}$	4
21: 18/45	$2.39 \pm 1.50^{a,x}$	$0.11 \pm 0.07^{a,x}$	$1.39 \pm 0.04^{ab,x}$	$32.00 \pm 24.97^{a,x}$	3
21: 18/55	$5.50 \pm 1.96^{a,x}$	$0.25 \pm 0.09^{a,x}$	$1.38 \pm 0.03^{b,x}$	$5.95 \pm 2.01^{a,x}$	5
21: 21/45	$7.06 \pm 2.26^{a,x}$	$0.32 \pm 0.10^{a,x}$	$1.41 \pm 0.05^{bc,x}$	$6.10 \pm 2.59^{a,x}$	5

(b)

Treatment	BWI (%)	SGR (%)	CF	FCR (g/g)	n
28: 15/45	$6.59 \pm 2.36^{a,x}$	$0.30 \pm 0.11^{a,x}$	$1.17 \pm 0.03^{a,x}$	$16.33 \pm 11.20^{a,x}$	4
28: 18/35	$5.67 \pm 3.43^{a,x}$	$0.26 \pm 0.15^{a,x}$	$1.22 \pm 0.02^{ab,x}$	$13.43 \pm 5.39^{a,x}$	3
28:18/45	$9.98 \pm 1.47^{a,x}$	$0.45 \pm 0.06^{a,x}$	$1.30 \pm 0.02^{ab,x}$	$4.29 \pm 0.84^{a,x}$	5
28: 18/55	$21.10 \pm 4.00^{ab,y}$	$0.90 \pm 0.16^{ab,y}$	$1.26 \pm 0.04^{ab,x}$	$2.34 \pm 0.76^{a,x}$	5
28: 21/45	$26.41 \pm 4.47^{b,y}$	$1.10 \pm 0.17^{b,y}$	$1.37 \pm 0.04^{b,x}$	$1.62 \pm 0.32^{a,x}$	6

higher than the 15/45 group. There was no significant effect of dietary protein at 28 °C. There was a tendency for CF to be lower for the corresponding diets at 21 °C than at 28 °C, however these changes were not significant at day 42.

Food conversion ratio showed no particular trend in relation to dietary protein or energy at 21 °C at day 42 (Table 5.4). At 28 °C, however, FCR tended to decrease with increasing dietary protein and energy, but the results were not significant ($p > 0.05$) as the errors were very large in most treatment groups. Again FCR values tended to be lower at 28 °C than at 21 °C for similar diets, however no significant ($p > 0.05$) differences in FCR for a particular diet across temperatures were detected.

5.3.2 Proximate Composition

The proximate composition of juvenile barramundi following 42 days was significantly ($p < 0.05$) affected by diet and water temperature (Table 5.5).

The HSI was significantly ($p < 0.05$) affected by diet (Table 5.5). At 21 °C, dietary energy significantly ($p < 0.05$) elevated HSI, increasing with increased dietary energy. Although dietary protein increase also tended to increase HSI, differences were not significant ($p > 0.05$) at 21 °C. At 28 °C, increasing dietary energy again resulted in an increased HSI, with the HSI of the 21/45 group being greater than that of the 15/45 group (Table 5.5b). Again HSI tended to increase with increasing dietary protein at 28 °C, however the differences were not significant ($p > 0.05$). Although HSI was lower at 21 °C for all diets when compared to the corresponding diet at 28 °C, again no significant ($p > 0.05$) differences were found.

The moisture content of the 21/45 group was significantly ($p < 0.05$) lower than that of the 15/45 group at 21 °C (Table 5.5a). At 28 °C, the 21/45 group demonstrated a lower moisture content than all other groups, but the differences were not significant ($p = 0.0585$) (Table 5.5b). The moisture content remained relatively constant with

Table 5.5 Hepato-somatic index (HSI) and carcass moisture (%), protein, (%), lipid (%) and ash (%) content of juvenile barramundi at time = 0 and after 42 days of feeding with experimental diets at (a) 21°C and (b) 28°C. Values are expressed as mean ± SEM. Actual sample sizes are shown in Tables 5.4. Values for diets at the same water temperature with different superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar diets at different water temperatures with different supercripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.

(a)

Treatment	HSI	Moisture	Protein	Lipid	Ash
Time 0	$1.49 \pm 0.10^{a,x}$	$68.63 \pm 0.81^{ab,x}$	$61.46 \pm 0.57^{ab,x}$	$22.10 \pm 1.05^{ab,x}$	$16.19 \pm 0.69^{ab,x}$
21: 15/45	$1.45 \pm 0.15^{a,x}$	$71.47 \pm 1.27^{a,x}$	$64.88 \pm 1.27^{a,x}$	$17.45 \pm 1.55^{a,x}$	$18.14 \pm 0.50^{a,x}$
21: 18/35	$1.74 \pm 0.10^{ab,x}$	$69.16 \pm 1.23^{ab,x}$	$59.18 \pm 0.36^{b,x}$	$23.32 \pm 0.75^{b,x}$	$16.88 \pm 0.19^{ab,x}$
21: 18/45	$1.76 \pm 0.16^{ab,x}$	$68.22 \pm 1.48^{ab,x}$	$59.50 \pm 1.43^{b,x}$	$22.33 \pm 2.55^{ab,x}$	$14.35 \pm 2.29^{b,x}$
21: 18/55	$2.30 \pm 0.19^{b,x}$	$69.83 \pm 0.72^{ab,x}$	$61.27 \pm 1.17^{ab,x}$	$22.06 \pm 1.19^{ab,x}$	$15.93 \pm 0.49^{ab,x}$
21: 21/45	$2.49 \pm 0.12^{c,x}$	$66.37 \pm 0.72^{b,x}$	$54.40 \pm 0.76^{c,x}$	$30.13 \pm 0.93^{c,x}$	$14.36 \pm 0.32^{b,x}$

(b)

Treament	HSI	Moisture	Protein	Lipid	Ash
Time 0	$1.54 \pm 0.11^{ab,x}$	$71.57 \pm 0.44^{a,x}$	$64.13 \pm 1.13^{a,x}$	$19.05 \pm 1.42^{a,x}$	$17.25 \pm 0.63^{ab,x}$
28: 15/45	$1.19 \pm 0.12^{a,x}$	$71.63 \pm 0.51^{a,x}$	$67.21 \pm 0.80^{a,x}$	$14.77 \pm 1.53^{a,x}$	$19.55 \pm 0.25^{a,x}$
28: 18/35	$1.11 \pm 0.05^{a,x}$	$70.44 \pm 1.97^{a,x}$	$62.70 \pm 0.44^{a,x}$	$18.65 \pm 0.93^{a,x}$	$18.07 \pm 0.39^{ab,x}$
28: 18/45*	$1.42 \pm 0.13^{ab,x}$	$70.56 \pm 1.54^{a,x}$	$62.70 \pm 0.41^{a,x}$	$19.61 \pm 0.41^{a,x}$	$17.73 \pm 0.71^{a,x}$
28: 18/55	$1.73 \pm 0.07^{ab,x}$	$71.43 \pm 3.60^{a,x}$	$63.97 \pm 0.60^{a,x}$	$17.60 \pm 1.10^{a,x}$	$17.23 \pm 0.99^{ab,x}$
28: 21/45	$1.94 \pm 0.20^{b,x}$	$67.62 \pm 2.68^{a,x}$	$57.27 \pm 1.49^{b,x}$	$26.86 \pm 1.97^{b,x}$	$13.94 \pm 1.16^{b,x}$

increasing dietary protein at both temperatures. Although moisture content was generally higher at 28 °C than at 21 °C, there were no significant ($p > 0.05$) differences.

Ash content was also significantly ($p < 0.05$) affected by dietary energy (Table 5.5). At 21 °C, the ash content of the 18/45 and 21/45 groups was significantly ($p < 0.05$) lower than that of the 15/45 group (Table 5.5a). At 28 °C, the ash content of the 21/45 group was significantly ($p < 0.05$) lower than that of the 15/45 and 18/45 groups (Table 5.5b). Ash content tended to decrease with increasing dietary protein at 21 °C but not at 28 °C (Table 5.5). There were no significant ($p > 0.05$) differences in ash content for a particular diet across temperatures.

Carcass protein content was significantly affected by dietary energy at both temperatures, with protein content decreasing significantly ($p < 0.05$) with increasing dietary energy (Table 5.5). Carcass protein content tended to increase with increasing dietary protein, although differences were not significant ($p > 0.05$) at either temperature. Carcass protein also appeared to be higher at 28 °C than at 21 °C, although the differences were not significant ($p > 0.05$).

Increased dietary energy resulted in significant ($p < 0.05$) increases in carcass lipid at both temperatures, whilst dietary protein had no significant ($p > 0.05$) effect. Carcass lipid content also appeared higher at 21 °C than at 28 °C, although the differences were not significant ($p > 0.05$).

5.3.3 Protein and Lipid Deposition

Protein and lipid deposition were significantly ($p < 0.05$) affected by diet (Table 5.6). At 28 °C, lipid gain increased significantly ($p < 0.05$) with increasing dietary energy, with the 21/45 group having greater lipid gain than the 15/45 and 18/45 groups at both temperatures (Table 5.6). There was a trend for lipid gain to increase with dietary energy increases at 28 °C, however differences were not significant ($p > 0.05$).

Table 5.6 Lipid and protein gain (g), protein efficiency ratios (PER, g/g) and net protein utilisation (NPU, %) of juvenile barramundi fed experimental diets at (a) 21°C or (b) 28°C. Values are expressed as mean ± SEM. Samples sizes are shown in Tables 5.4. Values for diets at the same water temperature with different superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar diets at different water temperatures with different superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.

(a)

Treatment	Lipid gain (g)	Protein gain (g)	PER		NPU
			0-21 days	21-42 days	0-42 day
21: 15/45	$-3.21 \pm 1.63^{a,x}$	$8.35 \pm 1.26^{a,x}$	$0.42 \pm 0.06^{a,x}$	$0.26 \pm 0.07^{a,x}$	$38.68 \pm 1.37^{ab,x}$
21: 18/35	$3.80 \pm 0.97^{a,x}$	$4.05 \pm 0.72^{a,x}$	$0.62 \pm 0.09^{a,x}$	$0.49 \pm 0.08^{a,x}$	$18.73 \pm 4.21^{a,x}$
21: 18/45	$2.61 \pm 0.07^{a,x}$	$4.94 \pm 1.61^{a,x}$	$0.77 \pm 0.07^{a,x}$	$0.30 \pm 0.19^{a,x}$	$31.17 \pm 2.96^{ab,x}$
21: 18/55	$3.31 \pm 0.18^{a,x}$	$9.29 \pm 1.08^{a,x}$	$0.75 \pm 0.18^{a,x}$	$0.52 \pm 0.17^{a,x}$	$42.78 \pm 5.74^{b,x}$
21: 21/45	$16.01 \pm 1.94^{b,x}$	$6.51 \pm 2.12^{a,x}$	$1.33 \pm 0.09^{b,x}$	$0.74 \pm 0.23^{a,x}$	$25.95 \pm 8.46^{ab,x}$

(b)

Treatment	Lipid gain (g)	Protein gain (g)	PER		NPU
			0-21 days	21-42 days	0-42 day
28: 15/45	$-1.66 \pm 1.86^{a,x}$	$11.54 \pm 2.93^{a,x}$	$0.40 \pm 0.13^{a,x}$	$0.37 \pm 0.13^{a,x}$	$33.56 \pm 8.31^{a,x}$
28: 18/35	$2.01 \pm 1.51^{a,x}$	$6.80 \pm 4.96^{a,x}$	$0.49 \pm 0.29^{a,x}$	$0.45 \pm 0.30^{a,x}$	$23.44 \pm 18.27^{a,x}$
28: 18/45	$5.77 \pm 1.63^{a,x}$	$14.91 \pm 3.51^{ab,x}$	$0.74 \pm 0.16^{ab,x}$	$0.58 \pm 0.10^{a,x}$	$37.95 \pm 7.17^{a,x}$
28: 18/55	$7.52 \pm 3.12^{a,x}$	$30.07 \pm 5.27^{ab,y}$	$0.79 \pm 0.13^{ab,x}$	$1.00 \pm 0.19^{ab,x}$	$58.51 \pm 9.01^{a,x}$
28: 21/45	$28.44 \pm 7.25^{b,x}$	$33.57 \pm 6.04^{b,y}$	$1.63 \pm 0.30^{b,x}$	$1.66 \pm 0.34^{b,y}$	$79.22 \pm 17.35^{a,x}$

At 21 °C, dietary energy appeared to have no effect on lipid gain (Table 5.6). Lipid gain tended to be higher at 28 °C than 21 °C, however no significant ($p > 0.05$) differences were found.

There was a significant ($p > 0.05$) correlation between carcass moisture and lipid content (data not shown).

At 28 °C, protein gain increased significantly ($p < 0.05$) with dietary energy increases, with the 21/45 group gaining significantly ($p < 0.05$) more protein than the 15/45 group. There was also a trend for increased protein gain with increased dietary protein, however these differences were not significant ($p > 0.05$) (Table 5.6b). At 21 °C protein gain also tended to increase with increasing dietary protein, however differences were not significant ($p > 0.05$). However, at 21 °C there was no effect of dietary energy on protein gain. Protein gain was greater at 28 °C than 21 °C for all diets, with significant ($p < 0.05$) differences being found for the 18/55 and 21/45 groups.

PER increased with increasing dietary energy content at both temperatures (Table 5.6). The PER of the 21/45 group was significantly ($p < 0.05$) higher than for the 15/45 and 18/45 groups at 21 °C at day 21 (Table 5.6a). Similar trends in PER were observed at day 42 at 21 °C, however there was no significant difference at the 0.05 level (Table 5.6a). At 28 °C, the 21/45 group was significantly ($p < 0.05$) higher than the 15/45 group at day 21 (Table 5.6b). At 28 °C, the PER of the 21/45 group was significantly ($p < 0.05$) higher than that of the 15/45 and 18/35 groups at day 42 (Table 5.6b). At 21 °C there was no effect of dietary protein on PER, while at 28 °C there tended to be an increase in PER with increasing dietary protein, although the differences were not significant ($p > 0.05$).

There were no significant differences in PER for a particular diet across temperatures at day 21, however at day 42, the PER of the 21/45 group at 28 °C was significantly ($p < 0.05$) higher than that of the 21/45 group at 21 °C.

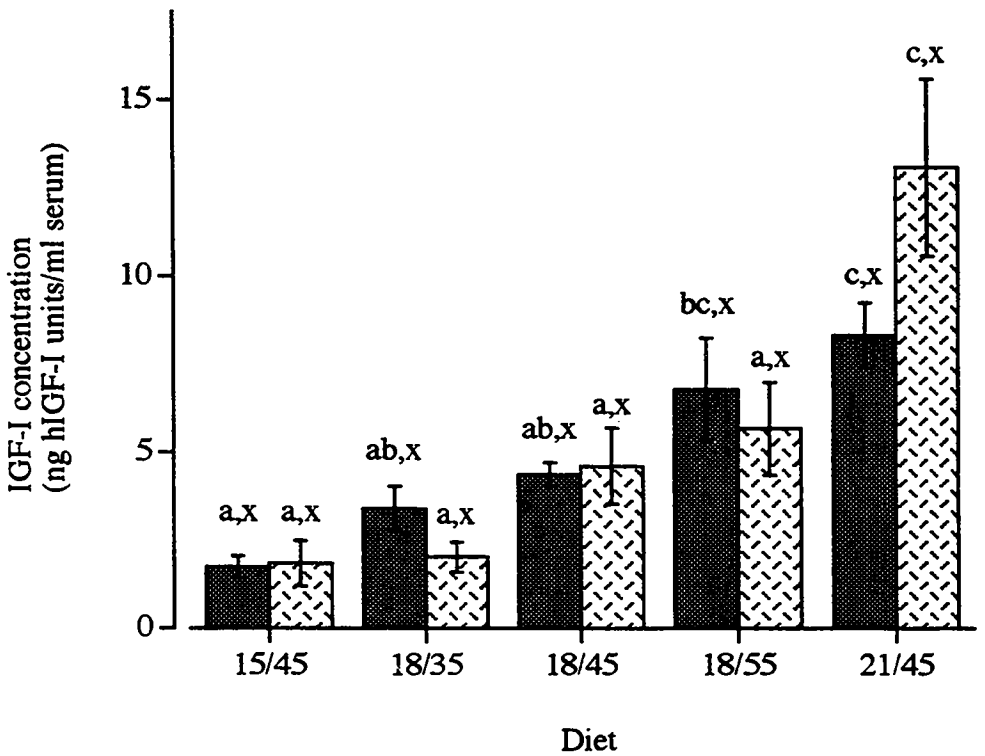
There was a tendency for NPU to increase with increasing protein content at day 42 at both temperatures (Table 5.6), with values significantly ($p < 0.05$) higher in the 21/45 group than in the 18/35 group at 21 °C (Table 5.6a). NPU tended to increase with increasing dietary energy at 28 °C, but tended to decrease with dietary energy at 21 °C (Table 5.6). There was a general tendency for NPU to be higher at 28 °C than at 21 °C and the NPU of the 21/45 group at 28 °C to be significantly ($p < 0.05$) higher than the same diet at 21 °C.

5.3.4 Circulating IGF-I and IGF-II Activities

The concentration of circulating IGF-I was significantly ($p < 0.05$) affected by dietary protein and energy content. At day 21, the serum concentration of IGF-I showed increases with increasing dietary energy at both 21 °C and 28 °C, with the 21/45 treatment group demonstrating significantly higher levels than the 15/45 and 18/45 groups (Fig. 5.2). There was also a tendency for circulating IGF-I to increase with increasing dietary protein at both temperatures, although the differences between the 18/35, 18/45 and 18/55 treatment groups were not significant at day 21 (Fig. 5.2). The effect of dietary protein and energy content on circulating IGF-I level was also observed at 28 °C. There was no significant ($p > 0.05$) effect of water temperature on the circulating IGF-I levels between groups of fish fed the same diet type across temperatures (Fig. 5.2) at day 21.

The concentrations of IGF-I were slightly lower at day 42, however the trends associated with changes in the dietary protein and energy content were similar to those

Figure 5.2 Circulating IGF-I levels in juvenile barramundi held at either 21 °C (semi-solid bars) or 28 °C (hatched bars) fed diets differing in protein and energy content (Section 5.2.2) for 21 days. Values are expressed as mean ± SEM. Sample sizes are listed in Table 5.3. Values for different diet compositions at the same water temperature with different primary superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar diet composition at different water temperatures with different secondary superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.


IGF-I concentration
(ng hIGF-I units/ml serum)
15
10
5
0
a,x
a,x
ab,x
a,x
ab,x
a,x
bc,x
a,x
c,x
c,x
15/45
18/35
18/45
18/55
21/45
Diet

observed at day 21 (Fig. 5.3). At 21 °C, there was a tendency for circulating IGF-I levels to increase with increasing dietary energy, however differences were not significant ($p > 0.05$). At 28 °C, the 21/45 groups demonstrated significantly ($p < 0.05$) higher levels of circulating IGF-I than the 18/45 and 15/45 groups. Dietary protein showed a significant ($p < 0.05$) effect on circulating IGF-I concentration at both temperatures, with the 18/55 groups being significantly ($p < 0.05$) higher than the 18/35 groups. Circulating IGF-I levels generally tended to be higher at 28 °C, with the 21/45 group at 28 °C demonstrating significantly ($p < 0.05$) higher circulating IGF-I concentrations than the same treatment group at 21 °C (Fig. 5.3).

In contrast to circulating IGF-I, the concentration of circulating IGF-II was not significantly ($p < 0.05$) affected by dietary protein or energy or water temperature at day 21 (Fig. 5.4) or day 42 (Fig. 5.5).

Figure 5.3 Circulating IGF-I levels in juvenile barramundi held at either 21 °C (semi-solid bars) or 28 °C (hatched bars) fed diets differing in protein and energy content (Section 5.2.2) for 42 days. Values are expressed as mean ± SEM. Sample sizes are listed in Table 5.3. Values for different diet compositions at the same water temperature with different primary superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar diet composition at different water temperatures with different secondary superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.

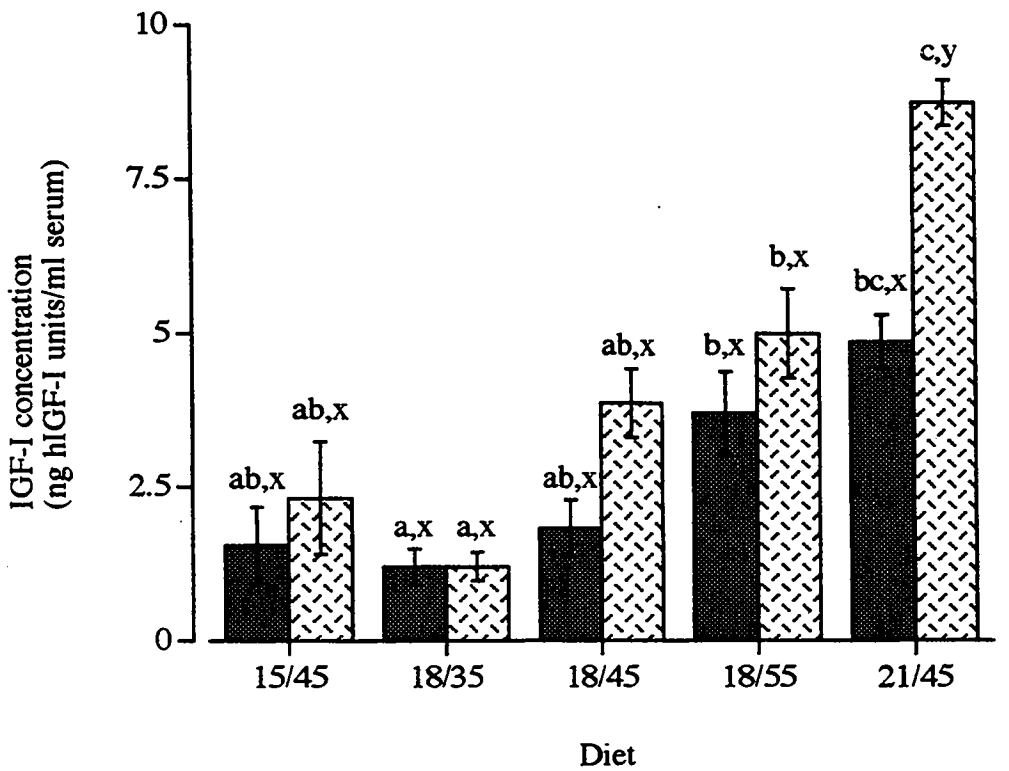

IGF-I concentration
(ng hIGF-I units/ml serum)
10
7.5
5
2.5
0
ab,x
ab,x
a,x
a,x
ab,x
ab,x
b,x
b,x
bc,x
c,y
15/45
18/35
18/45
18/55
21/45
Diet

Figure 5.4 Circulating IGF-II levels in juvenile barramundi held at either 21 °C (semi-solid bars) or 28 °C (hatched bars) fed diets differing in protein and energy content (Section 5.2.2) for 21 days. Values are expressed as mean ± SEM. Sample sizes are listed in Table 5.3. Values for different diet compositions at the same water temperature with different primary superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar diet composition at different water temperatures with different secondary superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.

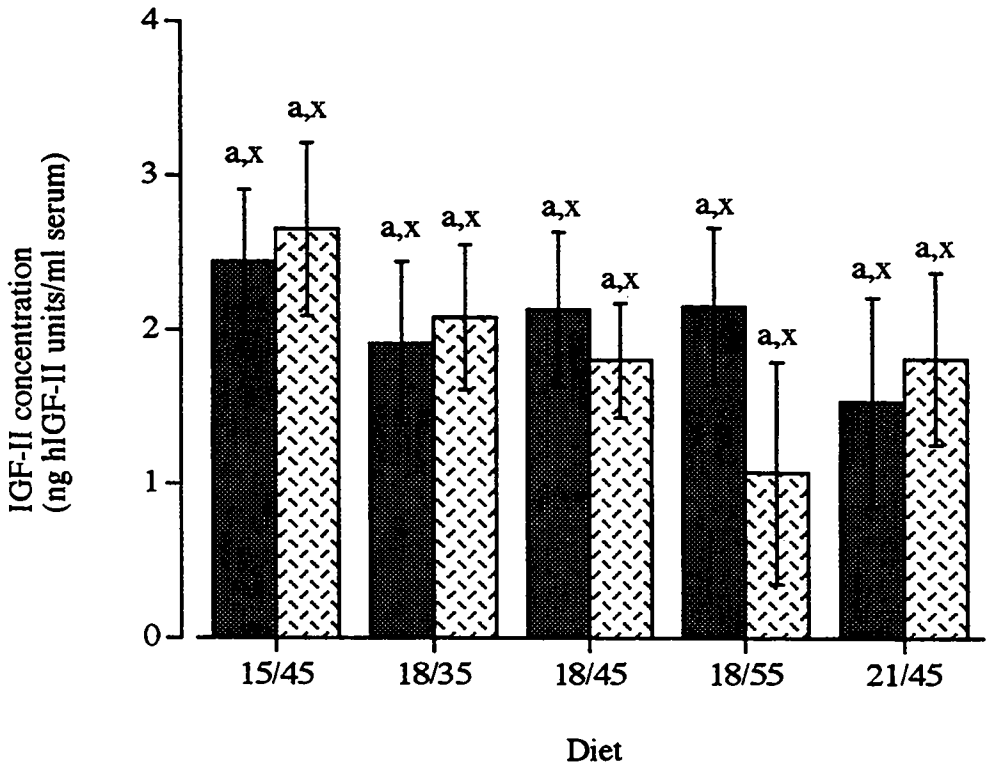
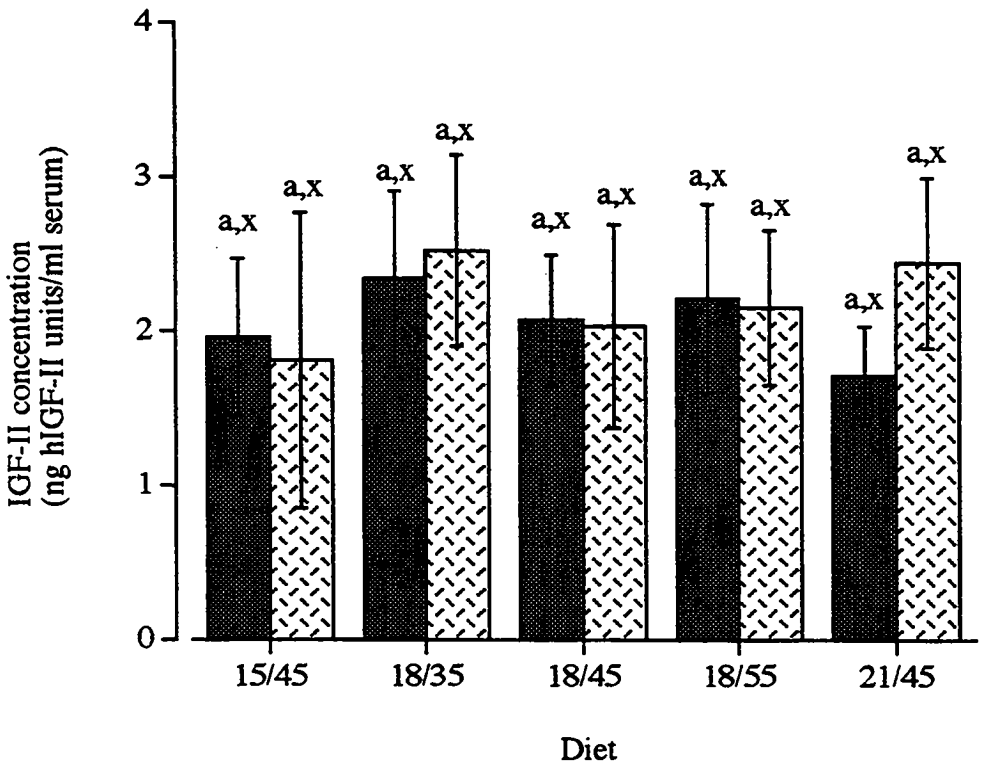

IGF-II concentration
(ng hIGF-II units/ml serum)
4
3
2
1
0
a,x
a,x
a,x
a,x
a,x
a,x
a,x
a,x
a,x
a,x
15/45
18/35
18/45
18/55
21/45
Diet

Figure 5.5 Circulating IGF-II levels in juvenile barramundi held at either 21 °C (semi-solid bars) or 28 °C (hatched bars) fed diets differing in protein and energy content (Section 5.2.2) for 42 days. Values are expressed as mean ± SEM. Sample sizes are listed in Table 5.3. Values for different diet compositions at the same water temperature with different primary superscripts (a-c) are significantly ($p < 0.05$) different. Values for similar diet composition at different water temperatures with different secondary superscripts (x-y) are significantly ($p < 0.05$) different using pair-wise comparisons.


IGF-II concentration
(ng hIGF-II units/ml serum)
4
3
2
1
0
a,x
a,x
a,x
a,x
a,x
a,x
a,x
a,x
a,x
a,x
15/45
18/35
18/45
18/55
21/45
Diet

5.4 DISCUSSION

5.4.1 Growth

The present study indicates that % BWI and SGR in juvenile barramundi increased as dietary protein increased from 35 to 55 % (with 18 MJ/kg energy) and as dietary energy increased from 15 MJ/kg to 21 MJ/kg (with 45% protein). The highest % BWI and SGR was observed in fish fed the 21/45 diet at both temperatures. The other diets, in order of the decreasing % BWI and SGR were ranked as 18/55, 18/45, 18/35 and 15/45. Whilst the amount of weight gain varied with diet type, all fish grew, demonstrating that the diet formulation and ration sizes selected for the study were adequate to support the growth of juvenile barramundi. Apparent FCR was generally inversely related to the changes in % BWI and SGR, however large errors probably associated with the failure of fish to consume the entire ration, masked any statistical differences.

Whilst the 21/45 diet can be easily recognised as producing the highest % BWI and SGR at both water temperatures, the type of growth obtained from a particular diet type, in terms of lipid or protein deposition, provides much information about the metabolism of the barramundi in response to dietary protein and energy.

5.4.2 Effects of Isonitrogenous Diets on Body Lipid and Protein Deposition

In the present study, lipid deposition increased both in absolute terms (Table 5.6) and in relative terms (Table 5.5) in juvenile barramundi held at both temperatures and fed isonitrogenous diets with increasing energy. The lipid gain indicates that as dietary energy increased, the amount of excess energy also increased. The lipid loss by fish fed diets having 15 MJ/kg and 45 % protein indicates that these animals were utilising stored lipid to provide energy for growth. The variation in protein gain, PER and NPU amongst groups of fish fed isonitrogenous diets at 21 °C demonstrates that the excess energy at this temperature is stored as fat. Since the PER indicates the wet

weight gain per weight protein consumed, it does not differentiate between protein or lipid deposition. PER generally increased as dietary energy increased. In contrast, the protein gain remained largely unchanged. The NPU, which measures the proportion of protein consumed by the fish that is laid down as tissue protein, decreased with increasing dietary energy at 21 °C, suggesting less protein gain and greater lipid deposition by fish utilising the high energy diets at lower temperatures. Similar results have been previously found in barramundi fed high energy diets at low water temperatures (Williams, pers. comm.).

At 28 °C, however, a different pattern was observed. Whilst PER increased with increasing dietary energy, so too did the total protein gain and NPU. However, the latter parameters did not vary greatly between the groups fed the 15 MJ/kg and 18 MJ/kg diets. These results reflect a move from a negative to positive protein energy balance with increasing dietary energy at this level.

The substantial increase in NPU with the increase in dietary energy to 21 MJ/kg reflects the shift in metabolism that allows non-protein energy to provide for the needs of basic metabolism and of growth and protein synthesis. Such protein sparing effects of non-protein energy have also been observed in tilapia (Winfree and Stickney, 1981; El-Sayed, 1987), yellowtail (Takeda *et al.*, 1975), channel catfish (Garling and Wilson, 1976), mullet (Perera and De Silva, 1978) and rainbow trout (LeGrow and Beamish, 1986).

The difference observed in the overall response to temperatures is probably due to a shut down of protein accretion at the lower temperature (Fauconneau, 1985). Such a shut down would allow any energy consumed that is in excess to basic metabolic requirements to be stored as fat at the lower temperature.

5.4.3 Effects of Isoenergetic Diets on Body Lipid and Protein Deposition

There was a trend for protein content to increase with increased dietary protein content of isoenergetic diets at both temperatures. Although differences were large at 28 °C, these differences were not found to be significant. The large increase in protein and lipid deposition with the 21/45 diet at 28 °C may be a result of the high lipid content of the diet, as energy in the form of lipid is thought to be more available to fish than carbohydrate (Millikin, 1982). Thus, the level of dietary energy available to the fish from this diet may be much greater than the dietary composition indicated, hence more protein may be deposited and the additional energy stored as fat. Although there was a slight increase in lipid deposition with increasing dietary protein level at 28 °C, this increase was not significant and was completely absent at 21 °C. The protein gain in animals fed the 18/55 diet was nearly equivalent to that of animals fed the 21/45 diet at 28 °C. NPU also increased with the increase in dietary protein (58.51 %), but did not achieve the levels of the animals fed the 21/45 diet (79.22 %). This reflects the need of the animals fed the 18/55 diet to provide energy for growth by hydrolysis of amino acids, a need reduced by feeding a diet higher in non-protein energy (Cowey, 1979; 1980). This conclusion is supported by the work of Kane (1996), who found an increase in ammonia excretion by juvenile barramundi fed a diet of 18 MJ/kg and 55 % protein over that of fish fed a diet of 21 MJ/kg and 45% protein (same diets prepared for use in this study).

PER also increased in response to increasing dietary protein at 28 °C, but to a lesser extent than in response to diets containing increasing dietary energy, as there was no increase in total body lipid to accompany the increase in total body protein.

The increased growth observed due to increased protein content of the diet, whilst maintaining dietary energy at 18 MJ/kg, suggests that the energy supplied to the animal from the protein is more available to it than the non-protein energy. In the diets used for this study, protein replaced both carbohydrate and lipid as the energy source in the isoenergetic diets. Since it is known that protein is more available to most fish than carbohydrate (Millikin, 1982), it is likely that the effect observed in this study was due to the use of protein by the animal for energy in preference to the carbohydrate.

The response of barramundi fed isoenergetic diets at 21 °C reflects the relative shut-down of protein synthesis at this temperature. There was no increase in growth, total body lipid or PER. There was a slight, but not significant ($p > 0.05$), increase in total body protein indicating that only a limited amount of protein synthesis was occurring. However, NPU or protein retention increased significantly from 18.73 % to 42.78 % with an increase in dietary protein from 35 % to 55 %. Thus, even with low rates of protein synthesis at the lower temperature, energy provided by protein hydrolysis proved to be more available to the barramundi.

5.4.4 Response of Other Measures of Growth to Isonitrogenous or Isoenergetic Diets

Other measures of growth such as HSI, CF and percentage composition responded as would be predicted based on the above discussion of the responses of protein and lipid deposition. HSI increased with increases in both dietary energy and protein at 21 °C and 28 °C, reflecting a change in the amount of excess energy with increasing dietary energy and the provision of protein energy. The values obtained for HSI for fish at 21 °C were marginally higher than values at 28 °C as would be expected of fish in a greater positive energy balance (Collins and Anderson, 1995).

Body water tended to decrease with decreasing dietary energy, being significantly ($p <$

0.05) correlated with carcass lipid content. The inverse relationship between carcass lipid and moisture has been reported in other fish species (Winfree and Stickney, 1981; Hanley, 1991).

5.4.5 Response of Circulating IGF-I and IGF-II to Isonitrogenous and Isoenergetic Diets

In the present study the concentration of circulating IGF-I, but not IGF-II, increased with increases in dietary protein and energy at both temperatures. The pattern of the response of serum IGF-I resembled those of % BWI and SGR more closely than those of lipid and protein gain, NPU and PER. As % BWI and SGR are indicators of growth and do not differentiate between lipid and protein gain, it would appear that circulating levels of IGF-I are related to the growth of the animal. This has previously been reported in gilthead seabream (Perez-Sanchez *et al.*, 1995).

Circulating IGF I levels increased with both increasing dietary protein and energy, although the response due to dietary energy appeared greater. With increasing dietary energy levels in isonitrogenous diets there becomes a greater proportion of non-protein energy and therefore more dietary protein becomes available for growth. Once the maximal usage of dietary protein for growth has occurred excess energy is converted to fat and stored. It would appear in the present study that as a greater amount of dietary protein is utilised for growth the levels of circulating IGF-I also increase. This is reflected in the NPU with the exception of the 21/45 diet at 21°C. The lesser response of IGF-I to dietary protein level may be complicated by the reduction in non-protein energy with increased dietary protein in isoenergetic diets. As the level of dietary protein increased the levels of both dietary lipid and carbohydrate, relative to dietary protein, decreased. At a dietary protein level of 35 % there was insufficient protein to support growth to an extent that would result in a high level of circulating IGF-I, even though there was sufficient non-protein energy. As the level of protein increased in the diet there was a corresponding decrease in the amount of dietary lipid

and carbohydrate, resulting in a large proportion of the additional protein being catabolised to provide energy for growth. Thus, the growth response to increasing levels of dietary protein was limited as a large proportion of the additional dietary protein would be required to produce energy. This was reflected in smaller increases in the circulating levels of IGF-I. A significant increase in plasma IGF-I immunoreactivity in fish with an increase in dietary protein has previously been reported (Perez-Sanchez *et al.*, 1995). In that paper, the increase in IGF-I immunoreactivity was more marked than in the previous study and probably reflects the fact that it was dietary carbohydrate that was replaced by dietary protein. As dietary energy derived from carbohydrate is generally less available to fish than protein- or lipid-derived energy, such replacement may in fact increase the level of available energy in the diet, resulting in a more marked growth enhancement. In the present study, both lipid and carbohydrate energy were replaced to significant levels by protein, resulting in a maintenance of dietary energy available for the isonitrogenous diets and therefore restricting growth enhancement to levels less than those observed in the study of Perez-Sachez *et al.* (1995).

In mammals, dietary protein and energy balance affect the normal production of IGF-I, which is mainly derived from the liver in response to GH (Pell and Bates, 1990; Clemmons and Underwood, 1991). Serum IGF-I in growing rats was correlated with growth rate and appeared to be primarily influenced by dietary protein (Prewitt *et al.*, 1982; Rosebrough *et al.*, 1996), whereas in adult humans both dietary energy and protein appeared to be important in regulating IGF-I (Underwood *et al.*, 1986). Thus, the exact mechanisms by which dietary protein and energy regulate IGF-I synthesis remain to be fully characterised.

Whilst the results of the present study indicate that both dietary protein and energy affect IGF-I production, it is not possible to further identify the mechanism of regulation without investigating other aspects of the GH:IGF-I cascade in this species.

There was no response of IGF-II to variation in the protein or energy content of the diet. To the author's knowledge the effect of dietary nutrient levels on IGF-II concentrations has not been previously reported in fish. These data are further supported by the lack of an effect of growth changes due to food availability on circulating IGF-II in barramundi reported in Chapter 4 and also appear similar to those reported in mammals, where the levels of circulating IGF-II appear much less sensitive to changes in nutrient deprivation than IGF-I (Section 4.4.1, p118).

5.4.6 Conclusion

The results of the present study clearly demonstrate that dietary protein and energy regulate the growth, proximate composition and level of circulating IGF-I in juvenile barramundi, again providing support for the nutritional regulation of IGF-I, but not IGF-II, in this species. Since decreases in dietary protein and energy caused a reduction in the concentration of circulating IGF-I, which was accompanied by decreased growth, it is likely that systemic IGF-I is affected by protein and energy restriction in this species. Although the mechanisms of this regulation remain unknown, results from studies conducted in other teleost fish demonstrate that protein and energy restriction result in an insensitivity of the liver to GH. It may be possible that the regulation of IGF-I in barramundi also occurs via such mechanisms. In contrast to IGF-I, the concentration of circulating IGF-II was not significantly affected by dietary protein and energy. Although the role of IGF-II remains somewhat unknown in teleost fish, it may be reasonable to suggest that, like in mammals, IGF-II in fish is not directly regulated by nutrition or GH.

CHAPTER 6:

GENERAL DISCUSSION

6.1 Discussion

The present study developed and validated methods for the measurement of IGF-I and IGF-II in a tropical teleost species, the barramundi *(Lates calcarifer)*, and investigated the regulatory effect of nutrition and water temperature on the concentration of circulating IGF peptides, IGF-I mRNA and body growth of juvenile fish.

Initially IGF activities were detected and characterised using heterologous type I (IGF-I) and type II (IGF-II) IGF RRAs. These assays were subsequently validated for use in barramundi. The membrane preparations used for the type I RRA and type II RRA in the present study allowed differentiation between hIGF-I and hIGF-II. The equipotent binding of IGF-I and IGF-II to type I IGF receptors has been previously documented (Daughaday *et al.*, 1981). Therefore, as expected, the binding curves and binding capacities of hIGF-I and hIGF-II in the type I RRA were also similar in the present study, demonstrating that both peptides equally displaced [^{125}I]hIGF-I. In contrast, human IGF-I was not able to displace [^{125}I]hIGF-II from in the type II RRA at any of the concentrations tested. These finding are also in accordance with previous characterisation studies of the type II receptor from rat liver membranes (Megyesi *et al.*, 1974; Rechler *et al.*, 1980; Scott and Baxter, 1987).

Using the type I RRA, two activities approximating the size of human IGF-I and IGF-II were detected in barramundi serum. The $\approx$ 7.8 kDa activity was detected only in the type I RRA and not the type II RRA, indicating that this molecule behaved as does mammalian IGF-I. Two factors provided further support for this molecule being barramundi IGF-I (bIGF-I). Firstly, Upton *et al.* (1996) have shown that recombinant barramundi IGF-I (bIGF-I) has the same affinity for mammalian type I receptors as does mammalian IGF-I, but does not react with the type II receptor. Secondly, the activity found in the present study was of a size consistent with the mature barramundi IGF-I peptide predicted from mRNA sequence data (Kinhult,

1997). Experiments are being currently performed to determine the cross-reactivity of recombinant barramundi IGF-I in the type I and type II RRA used in the present study (Section 3.1.4).

In contrast, the peak of activity found at ≈ 7.6 kDa was active in both the type I and type II RRA. The molecular weight and behaviour of this molecule in the RRAs were similar to mammalian IGF-II, suggesting that the molecule was barramundi IGF-II. Furthermore, the molecular size of the molecule corresponded with recent information of the size of the mature barramundi IGF-II peptide, derived from the characterisation of the barramundi IGF-II gene (Collet, pers. comm.) and with IGF-II activity (7.5 - 8 kDa) reported in the serum, liver and muscle of barramundi using a mammalian IGF-II RIA (Drakenberg, 1996).

High molecular weight peaks of activity ranging from 12.3 - 66 kDa were also identified in the type I and type II RRA. Previous studies have also reported the presence of high molecular weight (17 - 90 kDa) activities in blood products of various teleost species using heterologous IGF RIA or RRA and have attributed such activity to IGFBPs (Drakenberg *et al.*, 1989; Ng *et al.*, 1991; Anderson *et al.*, 1993; Perez-Sanchez *et al.*, 1994; Drakenberg, 1996). In the present study, the suggestion that the 12.3 - 29 kDa peak was IGFBPs was supported by the 20 kDa, 35 kDa and 43 kDa bands detected by Western ligand blotting and binding protein characterisation studies. Although the molecular size of the activity detected in the RRA differs from those detected in the IGFBP studies, in view of the capacity of the size exclusion column used in this study to differentiate between molecules of this size, these data were not inconsistent. Multiple IGFBPs of similar molecular weight have previously been reported in a variety of teleost species (Kelley *et al.*, 1992; Anderson *et al.*, 1993; Delahunty *et al.*, 1993; Niu and Le Bail, 1993; Siharath *et al.*, 1995; Fukazawa *et al.*, 1995). Acid-ethanol extraction also provided support for the

idea that the 12.3 - 29 kDa peak in barramundi serum was IGFBPs as the peak was not detected in extracted serum incubated with either [^{125}I]hIGF-I or [^{125}I]hIGF-II. Whilst these results collectively supported the existence of IGFBPs in barramundi serum, their complete characterisation requires further investigation.

The procedures conducted to validate the type I and type II RRA followed a rigorous protocol described for the validation of IGF measurements in biological fluids (Bang *et al.*, 1994). Serum samples were acidified and subjected to acid gel chromatography prior to analysis. Furthermore, IGF reference curves and serial dilutions of fractions containing bIGF-I or bIGF-II were parallel and unlabelled IGF peptide added to serum prior to chromatography was recovered in high quantities. Intra- and inter-assay coefficients of variation were also obtained for both RRA and were below the limits generally accepted in the literature. The application and appropriateness of the type I and type II RRA was also demonstrated by the capacity of the assays to measure IGFs under different physiological conditions.

The concentrations of IGF-like activities derived from barramundi serum in the present study were considered quantitative following validation of the heterologous assays. Recent studies using homologous RIA for coho salmon IGF-I (Moriyama *et al.*, 1994) and rainbow trout IGF-II (Gentil *et al.*, 1996) have reported plasma concentrations of IGF-I or IGF-II up to 100-fold higher when compared to their heterologous assay counterparts. Although this may highlight a limitation of the assays used in the present study, no homologous assays for either barramundi IGF-I or IGF-II are available (See note, Section 3.1.4). In addition, since it is likely that IGFs will only be purified from relatively few teleost species, a prerequisite for developing an homologous assay, the method developed herein, is likely to enable the detection of changes in circulating IGFs in other teleost species, following validation. The effect of nutrition and water temperature on circulating IGF-I and IGF-II was also investigated.

Ration size significantly affected fish growth and synthesis of IGF-I, but not IGF-II, in juvenile barramundi. The growth of barramundi predictably decreased with decreasing ration size and was accompanied by a reduction in the condition factor of starved fish at both water temperatures, suggesting that the starved fish were catabolising body stores for energy. Starved fish had significant reductions in the concentration of circulating IGF-I. Similarly, reductions in plasma IGF-I immunoreactivity were reported in gilthead seabream fed different ration sizes (Perez-Sanchez *et al.*, 1994; Perez-Sanchez *et al.*, 1995) and in a variety of nutritionally deprived mammalian species (Clemmons and Underwood, 1991). Further supporting the regulatory effect of ration size on IGF-I in teleosts are previous reports that IGF-I bioactivity is reduced during starvation in fish (Komourdjian and Idler, 1978; Duan and Inui, 1991; Gray and Kelley, 1991; McCormick *et al.*, 1992; Siharath *et al.*, 1996).

In mammals, the reduction in systemic IGF-I associated with periods of starvation is associated with reduced levels of hepatic IGF-I mRNA (Clemmons and Underwood, 1991). In the present study, the expression of total hepatic IGF-I mRNA, but not brain IGF-I mRNA, was significantly reduced in starved fish after three weeks. The regulation of hepatic IGF-I mRNA by short-term starvation has been previously reported in coho salmon (Duan and Plisetskaya, 1993) and the Japanese eel (Duan and Hirano, 1992), suggesting that as in mammals, systemic IGF-I of hepatic origin is regulated by the nutritional status of the animal.

In contrast to IGF-I, starvation for 42 days had no effect on circulating IGF-II levels in juvenile barramundi. At present, there is a paucity of data regarding the role and regulation of IGF-II in teleost fish. In mammals, however, the regulation of IGF-II is thought to be independent of nutritional status (Clemmons and Underwood, 1991) with reports of minimal (Donovan *et al.*, 1991) or unchanged plasma IGF-II levels, during short-term starvation (Davenport *et al.*, 1988). Furthermore, the expression of

IGF-II mRNA in fetal rats was not altered by maternal starvation (Donovan *et al.*, 1991). The data obtained for circulating IGF-II in barramundi in the present study appear consistent with that known in mammals, and until further studies are conducted in fish, the role of IGF-II in teleosts and its regulation will remain uncertain.

The manipulation of dietary protein and energy also significantly affected the growth and the concentration of circulating IGF-I in juvenile barramundi. The % BWI and SGR of juvenile barramundi generally increased with increases in dietary protein and energy levels. The highest % BWI was observed in fish fed the 21/45 diet at both temperatures, whilst the other diets, in order of the decreasing influence on % BWI and SGR were 18/55, 18/45, 18/35 and 15/45. Although the 21/45 diet provided the highest % BWI and SGR at both temperatures, the type of growth, in terms of lipid or protein deposition, is fundamental to our understanding in this species. In the present study, the deposition of lipid and protein in juvenile barramundi increased with increasing dietary energy content, with a constant dietary protein level of 45 %. These results are in agreement with previous studies in fish (Takeda *et al.*, 1975; Garling and Wilson, 1976; Perera and De Silva, 1978; Winfree and Stickney, 1981; LeGrow and Beamish, 1986; El-Sayed, 1987) and evinced the protein-sparing effect of non-protein energy in juvenile barramundi.

In contrast, the growth due to increasing protein content of the diet whilst maintaining dietary energy at 18 MJ/kg was largely a result of increased protein accretion and suggested that the energy supplied to the barramundi from the protein is more available. This suggestion is further supported by previous findings which document the preferential use of dietary protein for energy in teleost fish (De Silva and Anderson, 1995).

Associated with changes in weight gain and proximate composition were significant changes in the concentration of circulating IGF-I, but not IGF-II, in juvenile

barramundi. Circulating IGF-I increased with increasing dietary protein (with constant energy) and increasing dietary energy (with constant protein) at both water temperatures. This supports the only other published report of dietary protein effects on circulating IGF-I in teleosts (Perez-Sanchez *et al.* 1995), where significant increases in specific growth rate and plasma IGF-I immunoreactivity of gilthead seabream occurred with increases in dietary protein at constant dietary energy.

In mammals, dietary protein and energy ratios affect systemic IGF-I production, which predominantly occurs in the liver in response to GH action (Clemmons and Underwood, 1991). Although GH levels were not investigated in the present study, there is some evidence suggesting that the regulation of IGF-I by dietary protein and energy in fish occurs via similar mechanisms to those reported in mammals. Perez-Sanchez *et al.* (1995) reported an increase in the number of hepatic GH binding sites and a decline in plasma GH levels with increasing dietary protein. Other studies conducted in teleosts also report increases in plasma GH concentration during periods of nutritional deficiency (Sumpter *et al.*, 1991; Gray *et al.*, 1992; Duan and Plisetskaya, 1993). These results presumably reflect an insensitivity of the liver to GH action and thereby a lack of the negative feedback effect of IGF-I on pituitary GH release (Perez-Sanchez *et al.*, 1995).

IGF-II was not affected by dietary protein and energy content in juvenile barramundi, again providing support for the suggestion that, like in mammals, IGF-II is not stringently regulated by nutrition or GH in fish.

Although water temperature significantly affected growth for both trials, there was no clear effect of temperature on circulating IGF-I or IGF-II.

6.2 Conclusion

In concluding, the present study documented the validation of two heterologous RRA which were used for detecting changes in circulating IGF-I and IGF-II levels in juvenile barramundi, *Lates calcarifer*. The study also demonstrates the regulation of circulating IGF-I and total hepatic IGF-I mRNA by ration size and of circulating IGF-I by dietary protein and energy content in barramundi. The regulation of IGF-II did not appear to be controlled by nutritional factors in this species, and further work is necessary to determine the function and regulation of IGF-II in teleosts.

REFERENCES

REFERENCES

Ablett, R.F., Simhuber, R.D. and Selvonhick, D.P. (1981) The effect of bovine insulin on ^{14}C-glucose metabolism and ^{3}H-leucine incorporation in fed and fasted rainbow trout *(Salmo gairdneri). General and Comparative Endocrinology*, **44**, 418-427.

Anderson, T.A., Bennett, L.R., Conlon, M.A. and Owens, P.C. (1993) Immunoreactive and receptor active insulin-like growth factor-I and IGF-binding protein in blood plasma from the freshwater fish, *Maquariaambigua* (golden perch). *Journal of Endocrinology*, **136**, 191-198.

Almquist, S. (1961) Studies on sulfation factor (SF) activity in human serum. *Acta Endocrinologica*, **36**, 31.

Ash, P.J. (1977) Incorporation of [^{35}S]sulphate into mucopolysaccaride by teleost cartilage *in vitro*: the influence of mammalian growth hormone, teleost plasma and mammalian plasma. *General and Comparative Endocrinology*, **32**, 187-194.

Atkinson, A. (1991) The Macquarie Dictionary. Maquarie University, New South Wales, Australia.

Ball, J.N. (1969) Prolactin (fish prolactin or paralactin) and growth hormone. In: Fish Physiology (W.S. Hoar and D.J. Randell, Eds.), Volume 2, Academic Press, New York, pp. 207-240.

Ballard, J.F., Johnson, R.J., Owens, P.C., Francis, G.L., Upton, F.M., McMurty, J.P. and Wallace, J.C. (1990) Chicken insulin-like growth factor-I: amino acid sequences, radioimmunoassay and plasma levels between strains during growth. *General and Comparative Endocrinology*, **79**, 459-468.

Bang, P., Baxter, R.C., Blum., W.F., Breier, B.H., Clemmons, D.R., Hall, K., Hintz, R.L., Holly, J.M., Rosenfeld, J.M. and Zapf, J. (1994) Valid measurements of total insulin-like growth factor concentrations in biological fluids. Recommendations from the 3rd International Symposium of Insulin-like Growth Factors. *Journal of Endocrinology*, **143**, C1-C2.

Barrows, F.T., Sell, J.S. and Nickun, J.G. (1988) Effects of dietary protein and energy levels on weight gains, body composition and RNA:DNA ratios of fingerling walleyes. *The Progressive Fish Culturist*, **50**, 211-218.

Bautista, C.M., Mohan, S. and Baylink, D.J. (1990) Insulin-like growth factors I and II are present in the skeletal tissues of ten vertebrates. *Metabolism*, **39**, 96-100.

Bern, H.A., McCormick, S.D., Kelley, K.M., Gray, E.S., Nishioka, R.S., Madsen, S.S. and Tsai, P.I. (1991) Insulin-like growth factors "underwater": role in growth and function in fish and other poikilothermic vertebrates. In: Modern Concepts of Insulin-like Growth Factors (E.M. Spencer, Ed.). Proceedings of the Second International Symposium on Insulin-like Growth Factors/Somatomedins, January 12-16, San Francisco, USA, Elsevier Science Publications, Amsterdam.

Binoux, M., Roghani, M., Hossenlopp, P., Hardouin, S. and Gourmelen, M. (1991) Molecular forms of human IGF binding proteins: Physiological implications. *Acta Endocrinologica (Copenhagen)*, **124**, 41-47.

Blum, W.F. and Breier, B.H. (1994) Radioimmunoassays for insulin-like growth factors and insulin-like growth factor binding proteins. *Growth Regulation*, **4**, 11-19.

Bornfelt, K.E., Arnqvist, H.J., Endberg, B., Matthews, L.S. and Norstedt, G. (1989) Regulation of insulin-like growth factor-I and growth hormone receptor gene expression by diabetes and nutritional state in rat tissues. *Journal of Endocrinology*, **122**, 651-656.

Bosclair, D. and Tang, M. (1993) Empirical analysis of the influence of swimming pattern on the net energy cost cost of swimming in fishes. *Journal of Fish Biology*, **42**, 169-183.

Bowsher, R.R., Lee, W.H., Apathy, J.M., O'Brien, P.J., Ferguson, A.L. and Henry, D.P. (1991) Measurement of insulin-like growth factor-II in physiological fluids and tissues. I An improved extraction proceedure and radioimmunoassay for human and rat fluids. *Endocrinology*, **128**, 805-814.

Breier, B.H., Bass, J.J., Butler, J.H. and Gluckman, P.D. (1986) The somatotrophic axis in young steers: influence of nutritional status on pulsatile release of growth hormone and circulating concentrations of insulin-like growth factor-I. *Endocrinology* **111**, 209-205.

Breier, B.H., Gluckman, P.D. and Bass, J.J. (1988) Plasma concentrations of insulin-like growth factor-I and insulin in the infant calf: ontogeny and influence of altered nutrition. *Journal of Endocrinology*, **199**, 43-50.

Breier, B.H., Gallagher, B.W. and Gluckman, P.D. (1991) Radioimmunoassay for insulin-like growth factor-I: solutions to some potential problems and pitfalls. *Journal of Endocrinology*, **128**, 347-357.

Brett, J.R. (1979) Environmental factors and growth. In: Fish Physiology. (W.S. Hoar, D.J. Randell and J.R. Brett, Eds.). Academic Press, New York, pp.599-675.

Brody, S. (1945) Bioenergetics and Growth. Reinhold, New York, pp. 1023.

Cao, Q., Duguay, S.J., Plisetskaya, E., Steiner, D.F. and Chan, S.J. (1989) Nucleotide sequence and growth hormone-regulated expression of salmon insulin-like growth factor I mRNA. *Molecular Endocrinology*, **3**, 2005-2010.

Channing-Rodgers, R.P. (1981) Quality control and data analysis in binder-ligand assay, including radioimmunoassay, fluroroimmunoassay, enzymeimmunoassay and other immunoassay methods (Volumes 1 & 2). Scientific Newsletters, Anaheim, California, USA.

Cheetham, T.D., Taylor, A., Holly, J.M.P., Clayton, K., Cwyfan-Hughes, S. and Dunger, D.B. (1994) The effects of recombinant human insulin-like growth factor-I (IGF-I) administration on the levels of IGF-I, IGF-II and IGF-binding proteins in adolescents with diabetes mellitus. *Journal of Endocrinology*, **142**, 367-374.

Chomczynski, P. and Sacchi, N. (1987) Single step method of RNA isolation by guanidium thiosulphate-phenol chloroform extraction. *Analytical Biochemistry*, **162**, 156-159.

Clark, J.M. Jr. and Switzer, R.L. (1977) Photometric assay of color developed by a chemical reaction. In: Experimental Biochemistry, 2nd Edition. WH Freeman and Company, New York, USA.

Clemmons, D.R. and Underwood, L.E. (1991) Nutritional regulation of insulin-like growth factor-I and insulin-like growth factor binding proteins. *Annual Review of Nutrition*, **11**, 393-412.

Cohick, W.S. and Clemmons, D.R. (1993) The insulin-like growth factors. *Annual Review of Physiology*, **55**, 131-153.

Collins, A.L. (1996) Physiological changes associated with feeding frequency in the golden perch, *Macquariaambigua* (Richardson) (Pisces: Percichthyidae). PhD Thesis, James Cook University of North Queensland, Townsville, Australia.

Collins, A.L. and Anderson, T.A. (1995) The regulation of endogenous energy stores during starvation and refeeding in the somatic tissues of golden perch. *Journal of Fish Biology*, **47**, 1004-1015.

Cowey, C.B. (1975) Aspects of protein utilisation by fish. *Proceedings of the Nutrition Society*, **34**, 57-63.

Cowey, C.B. (1979) Protein and amino acid requirements of finfish. In: Finfish Nutrition and Fishfeed Technology (J.E. Halver and K. Tiews, Eds.), Volume I. Heenemann, Berlin, pp. 3-16.

Cowey, C.B. (1980) Protein and amino acid requirements in fish. In: Proceedings of the European Aquaculture Association. Symposium on Protein Metabolism and Nutrition (H.J. Oslage and K. Rohr, Eds.). EAAP Publication no. 27, pp. 729-774.

Crooke, W.M. and Simpson, W.E. (1971) Determination of ammonium in Kjeldahl digest of crops by automated procedure. *Journal of Scientific Agricultural Research*, **22**, 9-10.

Cuatrecasas, P. (1972) Isolation of the insulin receptor of liver and fat cell membranes. *Proceedings of the National Academy of Science USA*, **69**, 318-322.

Daniels, W.H. and Robinson, E.H. (1986) Protein and energy requirements of juvenile red drum *(Sciaenops ocellatus)*. *Aquaculture*, **53**, 243-252.

Danzmann, R.G., Van der Kraak, G.J., Chen, T.T. and Powers, D.A. (1990) Metabolic effects of bovine growth hormone and geneticlly engineered rainbow trout *(Oncorhynchus mykiss)* reared at a high temperature. *Canadian Journal of Fisheries and Aquatic Science*, **47**, 1292-1301.

Daughaday, W.H., Hall, K., Rabin, M.S., Salmon, W.D., Van den Brande, J.L. and Van Wyk, J.J. (1972) Somatomdein: proposed designation for sulphation factor. *Nature*, **235**, 107.

Daughaday, W.H., Phillips, L.S. and Herington, A.C. (1975) Measurement of somatomedin by cartilage *in vitro*. *Methods in Enzymology*, **37**, 93.

Daughaday, W.H., Mariz, I.K. and Blethen, S.L. (1980) Inhibition of access of bound somatomedin to membrane receptor and immunobinding sites: A comparison of radioreceptor and radioimmunoassay of somatomedin in native and acid-ethanol extracted serum. *Journal of Clinical Endocrinology and Metabolism*, **4**, 781-788.

Daughaday, W.H., Mariz, I.K. and Trivedi, B. (1981) A preferential binding site for insulin-like growth factor-II in human and rat placental membranes. *Journal of Clinical Endocrinology and Metabolism* **14**, 282-288.

Daughaday, W.H., Kapadia, M., Yanow, C.E., Fabrick, K. and Mariz, I.K. (1985) Insulin-like growth factors I and II of non-mammalian sera. *General and Comparative Endocrinology*, **59**, 316-325.

Daughaday, W.H., Kapadia, M. and Mariz, I. (1987) Serum somatomedin binding proteins: physiologic significance and interference in radioligand assay. *Journal of Laboratory and Clinical Medicine*, **109**, 355-363.

Daughaday, W.H. and Rotwein, P. (1989) Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum and tissue concentrations. *Endocrine Reviews*, **10**, 68-91.

Davenport, M.L., Svoboda, M.E., Koerber, K.L., Van Wyk, J.J., Clemmons, D.R. and Underwood, L.E. (1988) Plasma concentrations of insulin-like growth factor-II (IGF-II) are not changed by short-term fasting and re-feeding. *Journal of Clinical Endocrinology and Metabolism*, **67**, 1231-1236.

Davis, T.L.O. (1982) Maturity and sexuality in barramundi, *Lates calcarifer* (Bloch), in the Northern Territory and south-eastern Gulf of Carpentaria, Australia. *Copeia*, 44-149.

Davis, T.L.O. (1984) Estimation of the fecundity in barramundi, *Lates calcarifer* (Bloch), using an automatic particle counter. *Australian Journal of Marine and Freshwater Research*, **35**, 111-118.

Davis, T.L.O. (1985) Seasonal changes in gonad maturity, and abundance of larvae and early juveniles of barramundi, *Lates calcarifer* (Bloch), in the Van Diemen Gulf and Gulf of Carpentaria. *Australian Journal of Marine and Freshwater Research*, **36**, 177-190.

Davis, T.L.O. (1986) Migration patterns in barramundi, *Lates calcarifer* (Bloch), in the Van Diemen Gulf, Australia with estimates of fishing mortality in specific areas. *Fisheries Research*, **26**, 669-682.

Davis, T.L.O. and Kirkwood, G.P. (1984) Age and growth studies on barramundi, *Lates calcarifer* (Bloch) in Northern Australia. *Australian Journal of Marine and Freshwater Research*, **35**, 673-689.

Dawe, S.R., Francis, G.L., McNamara, P.J., Wallace, J.C. and Ballard, F.J. (1988) Purifiaction, partial sequences and properties of chicken insulin-like growth factors. *Journal of Endocrinology*, **117**, 173-181.

Degani, and Viola, A. (1987) The protein sparing effects of carbohydrates in the diets of eels *(Anguilla anguilla)*. *Aquaculture*, **64**, 283-291.

Delahunty, G., Jedrick, C. and Jedlicka, A. (1993) Insulin-like growth factor I and insulin-like growth factor binding protein in the channel catfish, *Ictalurus punctatus*. Second International Symposium on Fish Endocrinology, St Malo, France, pp.L-58.

Delong, D.C., Halver, J.E. and Mertz, E.T. (1958) Nutrition of salmonid fishes. VI Protein requirements of chinook salmon at two water temperatures. *Journal of Nutrition*, **65**, 589-599.

De Silva, S.S. (1991) Interactions of varying dietary protein and lipid levels in young red tilapia: evidence of protein sparing. *Aquaculture*, **95**, 305-318.

De Silva, S.S. and Anderson, T.A. (1995) Fish Nutrition in Aquaculture. Chapman and Hall, New York.

Donaldson, E.M., Higgs, D.A. and McBride, J.R. (1979) Hormonal Enhancement of Growth. In: Fish Physiology: Bioenergetics and Growth. (W.S. Hoar, D.J. Randell and J.R. Brett, Eds.), Volume VIII, Academic Press, New York, USA, pp. 445-595.

Donovan, S.M., Atilano, L.C., Hintz, R.L., Wilson, D.M. and Rosenfield, R.G. (1991) Differential regulation of the insulin-like growth factors (IGF-I and -II) and IGF binding proteins during malnutrition in the neonatal rat. *Endocrinology*, **129**, 149-157.

Drakenberg, K. (1996) Comparative studies on the IGF system in vertebrates: studies in rats, and in bony, cartilaginous, and jawless fish. PhD Thesis, Karolinska Hospital and Institute, Stockholm, Sweden.

Drakenberg, K., Sara, V.R., Lindahl, K.I. and Kewish, B. (1989) The study of insulin-like growth factors in tilapia, *Oreochromous mossambicus*. *General and Comparative Endocrinology*, **4**, 173-180.

Duan, C., Duguay, S.J., Swanson, P., Dickhoff, W.W. and Plisetskaya, E.M. (1994) Tissue-specific expression of insulin-like growth factor-I messenger ribonucleic acids in salmonids: developmental, hormonal and nutritional regulation. In: Perspectives in Comparative Endocrinology. National Research Council of Canada, pp. 365-372.

Duan, C. and Hirano, T. (1990) 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. *Journal of Endocrinology*, **133**, 211-219.

Duan, C. and Inui, Y. (1990a) Effects of recombinant eel growth hormone on the uptake of [^{35}S]sulphate by ceratobranchial cartilages of the Japanese eel, *Anguilla japonica*. *General and Comparative Endocrinology*, **79**, 320-325.

Duan, C. and Inui, Y. (1990b) Evidences for the presence of a somatomedin-like plasma factor (s) in the Japanese eel, *Anguilla japonica*. *General and Comparative Endocrinology*, **79**, 326-331.

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

Duguay, S.J., Chan, S.J., Mommsen, T.P. and Steiner, D.F. (1995) Divergence of insulin-like growth factors I and II in the elasmobranch, *Squalus acanthias*. *FEBS letters* **371**, 69-72.

Duguay, S.J., Park, L.K., Samadpour, M. and Dickhoff, W.W. (1992) Nucleotide sequence and tissue distribution of three insulin-like growth factor I prohormones in salmon. *Molecular Endocrinology*, **6**, 1202-1210.

Duguay, S.J., Swanson, P. and Dickhoff, W.W. (1994) Differential expression and hormonal regulation of alternatively spliced insulin-like growth factor-I transcripts in salmon. *Journal of Molecular Endocrinology*, **12**, 25-37.

Dulak, N.C. and Temin, H.M. (1973) A partially purified polypeptide fraction from rat cell liver conditioned medium with multiplication-stimulating activity for chicken embryo fibroblasts. *Journal of Cellular Physiology*, **81**, 153-160.

Dwyer, C.M. and Strickland, N.C. (1992) The effects of maternal undernutrition on maternal and fetal serum insulin-like growth factors, thyroid hormones and cortisol in the guinea pig. *Journal of Developmental Physiology*, **18**, 303-313.

Eigenmann, J.E., de Bruijne, J.J. and Froesch, E.R. (1985) Insulin-like growth factor-I and growth hormone in canine starvation. *Acta Endocrinologica*, **108**, 161-166.

Elliot, J.M. (1979) Energetics of freshwater teleosts. In: Fish Phenology: anabolic adaptiveness in teleosts (P.J. Miller Ed.). Academic Press, London, pp. 29-61.

El-Sayed, A.M. (1987) Protein and energy requirements of *Tilapia zillii* fingelings. PhD Thesis, Michigan State University, East Lansing, MI, USA.

Emler, C.A. and Schalch, D.S. (1987) Nutritionally-induced changes in hepatic insulin-like growth factor I (IGF-I) gene expression in rats. *Endocrinology*, **120**, 832-834.

Endberg, G., Carlquist, M., Jornvall, H. and Hall, K. (1984) The characterisation of somatomedin A, isolated by microcomputer-controlled chromatography, reveals an apparent identity to insulin-like growth factor-I. *European Journal of Biochemistry*, **143**, 117-124.

Erlich, R.B. (1989) PCR Techonology: Priciples and application for DNA amplification. Stocton Press, New York.

Etherton, T.D. (1993) Insulin, insulin-like growth factors, and growth factors. In: The Endocrinology of Growth, Development, and Metabolism in Vertebrates. Academic Press, New York, USA, pp. 197-217.

Fagerlund, U.H.M. and McBride, J.R. (1977) Effect of 17α-methyltestosterone on growth, gonad development, external features and proximate composition of muscle of steelhead trout, coho and pink salmon. *Fish Pathology*, **716**, 305-314.

Fauconneau, B. (1985) Protein synthesis and protein deposition in fish. In: Nutrition and Feeding in Fish (C.B. Cowey, A.M. Mackie and J.G. Bell, Eds.). Academic Press, Orlando, pp 17-45.

Feng, P., Catt, K.J. and Knecht, M. (1988) Transforming growth factor-β stimulates meiotic maturation of the rat oocyte. *Endocrinology*, **122**, 181-186.

Fielder, P.J., Thordarson, G., Talamantes, F. and Rosenfield, R.G. (1990) Characterisation of insulin-like growth factor binding proteins (IGFBPs) during gestation in mice: effects of hypophysectomy and IGFBP-specific serum protease activity. *Endocrinology*, **127**, 2270-2280.

Fine, M., Zilberg, D., Cohen, Z., Degani, G., Boaz, M. and Gertler, A. (1996) The effect of dietary protein level, water temperarute and growth hormone administration on growth and metabolism of common carp *(Cyprinus carpio). Comparative Biochemistry and Physiology*, **114A**, 35-42.

Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissue. *Journal of Biological Chemistry*, **226**, 497-509.

Flyvberg, A., Bornfeldt, K.E., Orskov, H., Arnqvist, H.J. (1991) Effect of insulin-like growth factor I infusion on renal hypertrophy in experimental diabetes mellitus in rats. *Diabetologia*, **34**, 715-720.

Forster, R.P. and Wieser, W. (1990) Patterns of routine swiming and metabolic rate in juvenile cyprinids at three temperatures: analysis with respirometer-activity-monitoring system. *Journal of Comparative Physiology*, **B160**, 71-76.

Franklin, R.C., Rennie, G.G., Burger, H.G. and Cameron, D.P. (1976) A bioassay for NSILA-s in individual serum samples and its relationship to somatotropin. *Journal of Clinical Endocrinology and Metabolism*, **43**, 1164-1169.

Froesch, E.R., Burgi, H., Ramseier, E.B., Bally, P. and Labhart, A. (1963) Antibody-suppressible and non-suppressible insulin-like activities in human serum and their physiological significance: An insulin assay with adipose tissue of increased precision and specificity. *Journal of Clinical Investigation*, **42**, 1816-1834.

Froesch, E.R., Burgi, H., Muller, W.A., Humbel, R.E., Jakob, A. and Labhart, A. (1967) Non-suppressible insulin-like activity of human serum: purification, physiochemical and biochemical properties and its relation to total serum ILA. *Recent Progressions in Hormones Research*, **23**, 565-605.

Froesch, E.R. and Zapf, J. (1985) Insulin-like growth factors and insulin: comparative aspects. *Diabetologia*, **28**, 485-493.

Frykland, L., Uthne, K. and Sievertsson, H. (1974) Isolation and characterisation of polypeptides from human plasma enhancing the growth of human normal cells in culture. *Biochemical and Biophysical Research Communications*, **61**, 950.

Fukazawa, Y., Siharath, K., Iguchi, T. and Bern, H.A. (1995) *In vitro* secretion of insulin-like growth factor binding proteins from liver of striped bass, *Morone saxatilis*. *General and Comparative Endocrinology*, **99**, 239-247.

Funkenstein, B., Silbergeld, A., Cavari, B. and Laron, Z. (1989) Growth hormone increases plasma levels of insulin-like growth factor (IGF-I) in a teleost the gilthead sea bream *(Sparus aurata)*. *Journal of Endocrinology*, **120**, R19-R21.

Funkenstein, B., Amuly, R. and Chan, S.J. (in press) Localization of IGF-I and IGF-I mRNA in *Sparus aurata* larvae. *General and Comparative Endocrinology*.

Furlanetto, R.W., Underwood, L.E. Van Wyk, J.J. and D'Ercole, A.J. (1977) Estimation of somatomedin-C levels in patients with pituitary disease by radioimmunoassay. *Journal of Clinical Investigation*, **60**, 648-657.

Garling, D.L. and Wilson, R.P. (1976) Optimum dietary protein to energy ratio for channel catfish fingelings, *Ictalurus punctatus*. *Journal of Nutrition*, **106**, 1368-1375.

Gentil, V., Martin, P., Smal, J. and Le Bail, P-Y (1996) Production of recombinant insulin-like growth factor-II in the development of a radioimmunoassay in rainbow trout *(Oncorhynchus mykiss)*. *General and Comparative Endocrinology*, **104**, 156-167.

Gliemann, J. (1968) Assay of insulin-like activity by the isolated fat cell method. III Studies of the suppressible and non-suppressible insulin-like activity of serum. *Diabetologia*, **4**, 95-104.

Grant, D.B., Hambley, J., Becker, D. and Pimstone, B.L. (1973) Reduced sulphation factor in undernourished children. *Arcives of Disease in Childhood*, **48**, 596-600.

Gray, E.S. and Kelley, E.M. (1991) Growth regulation in the gobiid teleost, *Gillichthys mirabilis* roles of growth hormone, hepatic growth hormone receptors and insulin-like growth factor-I. *Journal of Endocrinology*, **131**, 57-66.

Gray, E.S., Kelley, K.M., Law, S., Tsai, R., Young, G. and Bern, H.A. (1992) Regulation of hepatic growth hormone receptors in coho salmon *(Oncorhynchus kisutch)*. *General and Comparative Endocrinology*, **88**, 243-252.

Green, H., Morikawa, M. and Nixon, T. (1985) A dual effector theory of growth hormone action. *Differentiation*, **29**, 195-198.

Grey, D.L. (1986) An overview of *Lates calcarifer* in Australia and Asia, In: Management of Wild and Cultured Sea Bass/Barramundi *(Lates calcarifer)*. Australian Centre for International Agricultural Research Proceedings, **20**, 15-21.

Guler, H.P., Eckardt, K.U., Zapf, J., Bauer, C. and Froesch, E.R. (1989) Insulin-like growth factor I increases glomerular filtration rate and renal plasma flow in man. *Acta Endocrinologica (Copenhagan)*, **121**, 101-106.

Gutierrez, J., Parrizas, M., Carneiro, N., Maestro, J.L., Maestro, M.A. and Planas, J. (1993) Insulin and insulin-like growth factor-I receptors and tyrosine kinase activity in carp ovaries: changes with reproductive cycle. *Fish Physiology and Biochemistry*, **11**, 247-254.

Gutierrez, J., Parrizas, M., Maestro, M.A., Navarro, I. and Plisetskaya, E.M. (1995) Insulin and insulin-like growth factor-I binding and tyrosine kinase activity in fish heart. *Journal of Endocrinology*, **146**, 35-44.

Hanley, F. (1991) Effect of feeding supplementary diets containing varying levels of lipid on growth, food conversion, and body composition of Nile tilapia, *Oreochromis niloticus* (L.). *Aquaculture*, **93**, 323-334.

Hansson, H-A, Edwall, D., Lowenadler, B., Norstedt, G., Paleus, S. and Skottner, A. (1988) Insulin-like growth factor I in the pancreas of normal and diabetic adult rats. *Acta Physiologica Scandinavia*, **132**, 569-576.

Harding, J.E., Liu, L., Evans, P C. and Gluckman, P.D. (1994) Insulin-like growth factor-I alters feto-placental protein and carbohydrate metabolism in fetal sheep. *Endocrinology*, **34**, 1509-1514.

Hecht, T. and McEwan, A.G. (1992) Changes in gross protein and lipid requirements of rainbow trout, *Oncorhynchus mykiss* (Walbaum), at elevated temperatures. *Aquaculture and Fisheries Management*, **23**, 133-148.

Higgs, D.A. and Eales, J.G. (1977) Influence of food deprivation on radioiodothyronine and radioactive kinetics in yealing brook trout, *Salvelinus fontinalis*, with consideration of the extent of L-thyroxine conversion to 3, 5, 3'-triiodo-L-thyronine. *General and Comparative Endocrinology*, **32**, 29-40.

Hintz, R.L. and Liu, F. (1997) Demonstration of specific plasma protein binding sites for somatomedin. *Journal of Clinical Endocrinology and Metabolism*, **45**, 988-995.

Holly, J.M.P. and Cwyfan-Hughes, S.C. (1994) Measuring insulin-like growth factors: why, where and how? *Journal of Endocrinology*, **140**, 165-169.

Hossenlopp, P.D., Seurin, B., Sergovia-Quinson, S. and Binox, M. (1986) Analysis of serum insulin-like growth factor binding proteins using Western blotting: use of the method for titration of the binding proteins and competitive binding status. *Analytical Biochemistry*, **154**, 138-143.

Hua, K.M., Ord, R., Kirk, S., Li, Q.J., Hodgkinson, S.C., Spencer, G.S.G., Molan, P.C. and Bass, J.J. (1993) Regulation of plasma and tissue levels of insulin-like growth factor-I by nutrition and treatment with growth hormone in sheep. *Journal of Endocrinology*, **136**, 217-224.

Humbel, R.E. (1990) Insulin-like growth factors I and II. *European Journal of Biochemistry* **190**, 445-462.

Inui, Y. and Ishioka, H. (1983) Effects of insulin and glugagon on the incorporation of ^{14}C-glycine into protein and carbohydrate in the liver and opercula muscle of the eel *in vitro*. *General and Comparative Endocrinology*, **36**, 133-141.

Ince, B.W. and Thorpe, A. (1976) The *in vitro* metabolism of ^{14}C-glucose and ^{14}C-glycine in insulin treated northen pike *(Esox lucius)*. *General and Comparative Endocrinology*, **28**, 481-486.

Ince, B.W. and Thorpe, A. (1977) Glucose and amino acid stimulated insulin release *in vivo* in the European silver eel *(Anguilla aquilla)*. *General and Comparative Endocrinology*, **31**, 249-256.

Jansen, M., Van Shiak, F.M.A., Ricker, A.T., Bullock, B., Woods, D.E., Gabby, K.H., Nussbaum, A.L., Sussenbach, J.S and van Den Brande, J.L. (1983) Sequence of a cDNA encoding human insulin-like growth factor I precursor. *Nature*, **306**, 609-611.

Jayaram, M.G. and Beamish, F.W.H. (1992) Influence of dietary protein and lipid on nitrogen and energy losses in Lake trout, *Salvelinus namaycush*. *Candian Journal of Aquatic Science*, **49**, 2267-2272.

Jobling, M. (1981) Some effects of temperature, feeding and body weight on nitrogenous excretion in young plaice *Pleronectes platessa*. *Journal of Fish Biology*, **18**, 87-96.

Jobling, M. (1994) Fish Bioenergetics. Chapman and Hall, New York.

Jones, J.I. and Clemmons, D.R. (1995) Insulin-like growth factors and their binding proteins:biological actions. *Endocrine Reviews*, **16**, 3-34.

Kagawa, H., Kobayashi, M., Hasegawa, Y. and Aida, K. (1994) Insulin and insulin-like growth factors I and II induce final maturation of oocytes of red seabream, *Pagrus major*, *in vitro*. *General and Comparative Endocrinology*, **95**, 293-300.

Kailola. P.J., Williams, M.J., Stewart, P.C., Reichelt, R.E., McNee, A. and Grieve, C. (1993) Australian Fisheries Resources. Bureau of Resource Sciences, Government of Australia, Canberra, Australia.

Kajimoto, Y. and Rotwein, P. (1990) Evolution of insulin-like growth factor-I (IGF-I): Structure and expression of an IGF-I precursor from *Xenopus laevis*. *Molecular Endocrinology* **4**, 217-225.

Kane, K (1996) The effect of temperature, ration, dietary protein-energy and body size on the partitioning of energy into waste products in barramundi, *Lates calcarifer*. Bsc Honours Thesis, James Cook University, Townsville, Australia.

Kaufmann, U., Zapf, J. and Froesch, E.R. (1979) The effect of different extraction proceedures on two different molecular weight species of serum non-suppressible insulin-like activity (NSILA) and on the carrier protein of small molecular weight NSILA (NSILA-s). *Acta Endocrinologica (Copenhagen)*, **90**, 637-647.

Kelley, K.M. (1993) Experimental diabetes mellitus in a teleost fish. I Effect of complete isletectomy and subsequent hormonal treatment on metabolism in the goby, *Gillichthys mirabilis*. *Endocrinology*, **132**, 2689-2695.

Kelley, K.M., Gray, E.S. Siharath, K., Nicoll, C.S. and Bern, H.A. (1993) Experimental diabetes mellitus in a teleost fish. II Roles of insulin, growth hormone (GH), insulin-like growth factor-I and hepatic GH receptors in diabetic growth inhibition in the goby, *Gillichthys mirabilis*. *Endocrinology*, **132**, 2696-2701.

Kelley, K.M., Siharath, K and Bern, H.A. (1992) Identification of insulin-like growth factor-binding proteins in the circulation of four teleost fish species. *Journal of Experimental Zoology*, **263**, 220-224.

Ketelslegers, J.M. Maiter, D., Underwood, L.E. and Thissen, J-P. (1996) Nutritional regulation of the growth hormone and insulin-like growth factor binding proteins. *Hormone Research*, **45**, 252-257.

Kiddy, D.S., Hamilton-Fairley, D., Franks, S., Tsouros, A., Anyouku, V., Evans, K., James, V.H.T. and Reed, M.J. (1989) Reduction of free testosterone concentration in serum by a low calorie diet in women with normal or polycystic ovaries: the roles of insulin and IGF-I. *Journal of Endocrinology*, **121**, 253.

Kinhult, A.K.K. (1997) Barramundi *(Lates calcarifer)* IGF-I: characterisation of cDNA, genomic sequences, and regulation of mRNA expression. PhD Thesis, Queensland University of Technology, Brisbane, Australia.

Klapper, D.G., Svoboda, M.E. and Van Wyk, J.J. (1983) Sequence analysis of somatomedin-C: confirmation of identity with insulin-like growth factor I. *Endocrinology*, **112**, 2215-2217.

Komourdjian, M.P. and Idler, D.R. (1978) Hepatic mediation of hormonal and nutritional factors influencing the *in vitro* sulfur uptake by rainbow trout bone. *General and Comparative Endocrinology*, **36**, 33-39.

Labhart, A. (1963) Antibody-suppressible and nonsuppressible insulin-like activities in human serum and their physiologic significance. An insulin assay with adipose tissue of increased precion and specifity. *Journal of Clinical Investigation*, **42**, 1816.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature*, **227**, 680-685.

Lee, D.J. and Putnam, G.B. (1973) The response of rainbow trout to varying protein/energy ratios in a test diet. Oregon Agricultural Experiment Station, Corvallis, Oregon, Technical paper 3485.

LeGac, F., Loir, M., Le Bail, P-Y. and Ollitrault, M. (1996) Insulin-like growth factor-I (IGF-I) mRNA and IGF-I receptor in trout testis and in isolated spermatogenic and serotoli cells. *Molecular Reproduction and Development*, **44**, 23-25.

LeGrow, S.M. and Beamish, F.W.H. (1986) Influence of dietary protein and lipid on apparent heat increment of rainbow trout, *Salmo gairdneri*. *Canadian Journal of Fisheries and Aquatic Sciences*, **43**, 19-25.

Leibush, B., Parrizas, M., Navarro, I., Lappovo, Y., Maestro, M.A., Encinas, M., Plisetskaya, E.M. and Gutierrez, J. (1996) Insulin and insulin-like growth factor-I receptors in fish brain. *Regulatory Peptides*, **61**, 155-161.

Liang, Y.H., Cheng, C.H.K. and Chan, K.M. (1996) Insulin-like growth factor I Ea2 is the predominantly expressed form of IGF in common carp *(Cyprinus carpio)*. *Molecular and Marine Biology and Biotechnology*, **5**, 145-152.

Lie, O., Lied, E. and Lambertsen, G. (1988) Feed Optimization in Atlantic cod *(Gadus morhua)*: fat versus protein content in the feed. *Aquaculture*, **69**, 333-341

Lindahl, K.I., Sara, V., Fridberg, G. and Nishimiya, T. (1985) The presence of somatomedin in the Baltic salmon, *Salmo salar*, with special reference to smoltification. *Aquaculture*, **45**, 177-183.

Lobegeiger, R. (1994) Aquaculture Production Survey: Report to Farmers. *Queensland Aquaculture News*, **4**, 5-6.

Love, R.M. (1980) The Chemical Biology of Fishes, Volume 2. Academic Press, New York, USA.

Lowe, W.L. (1991) Biological actions of the insulin-like growth factors. In: Insulin-like Growth Factors: Molecular and Cellular Aspects (D. LeRoith, Ed.). CRC Press, Boca Raton, pp. 49-85.

McCormick, S.D. (1995) Hormonal control of gill Na^+, K^+-ATPase and chloride cell function. In: Fish Physiology, Volume XIV, Iono-regulation: Cellular and Molecular Approaches (C.M. Wood and T.J. Shuttleworth (Eds.). Academic Press, New York.

McCormick, S.D. (1996) Effects of growth hormone and insulin-like growth factor-I on salinity tolerance and gill Na^+, K^+-ATPase in Atlantic salmon *(Salmo salar)*: Interaction with cortisol. *General and Comparative Endocrinology*, **101**, 3-11.

McCormick, S.D., Sakamoto, T., Hasegawa, S. and Hirano, T. (1991). Osmoregulatory actions of insulin-like growth factor-I in rainbow trout *(Oncorhynchus mykiss). Journal of Endocrinology*, **130**, 87-92.

McCormick, S.D., Tsai, P.I., Kelley, P.M., Nishioka, R.S. and Bern, H.A. (1992) Hormonal control of sulfate uptake by branchial cartilage of coho salmon: role of insulin-like growth factor-I. *The Journal of Experimental Zoology*, **262**, 166-171.

McGuiness, M.C. and Cogburn, L.A. (1990) Measurement of developmental changes in plasma insulin-like growth factor-I levels of broiler chickens by radioreceptor assay and radioimmunoassay. *General and Comparative Endocrinology*, **79**, 446-458.

McLean, E. and Donaldson, E.M. (1993) The role of growth hormone in the growth of poikilotherms. In: The Endocrinology of Growth, Development and Metabolism in Vertebrates. Academic Press, New York, USA, pp. 43-71.

Mack, A.F. and Fernald, R.D. (1993) Regulation of cell division and rod differentiation in the teleost retina. *Developmental Brain Research*, **76**, 183-187.

Madsen, S.S. (1990) The role of cortisol and growth hormone in seawater adaptation and development of hypoosmoregulatory mechanisms in sea trout parr *(Salmo trutta trutta). General and Comparative Endocrinology*, **79**, 1-11.

Madsen, S.S. and Bern, H.A. (1992) Antagonism of prolactin and growth hormone: impact on seawater adaptation in two salmonids, *Salmo trutta and Oncorhynchus mykiss. Zoological Science*, **9**, 755-784.

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, *Onchorhynchus kisutch.* Journal of *Endocrinology*, **138**, 23-30.

Maiter, D., Maes, M., Underwood, L.E., Flieson, T., Gerard, G. and Ketelslegers, J.M. (1988) Early changes in serum concentrations of somatomedin-C induced by dietary protein deprivation in rats: contributions of growth hormone receptor and post-receptor defects. *Journal of Endocrinology*, **118**, 113-120.

Marchant, T.A. and Moroz, B.M. (1993) Hormonal influences on *in vitro* [^{35}S]-sulphate uptake by gill arches from the goldfish (*Carassius auratus* L.). *Fish Physiology and Biochemistry*, **11**, 393-399.

Marshall, R.N., Underwood, L.E., Voina, S.J., Foushee, D.B. and Van Wyk, J.J. (1974) Characterisation of the insulin and somatomedin-C receptors in human placental cell membranes. *Journal of Clinical Endocrinology and Metabolism*, **39**, 283-292.

Martin, A.A., Thomas, F.M., Owens, P.C., Knowles, S.E. and Ballard, F.J. (1991) IGF-I and its variant des-(1-3) IGF-I, enhance growth in rats with reduced renal mass. *American Journal of Physiology*, **261**, F626-F633.

Matthews, S.J., Kinhult, A.K.K., Hoeben, P., Sara, V.R. and Anderson, T.A. (1997) Nutritional regulaiton of insulin-like growth factor-I mRNA expression in barramundi, *Lates calcarifer. Journal of Molecular Endocrinology*, **18**: 273-276.

Megeysi, K., Kahn, R., Roth, J., Neville, D.M., Nissley, S.P., Humbel, R.E. and Froesch, E.R. (1975) The NSILA-s receptor in liver plasma membranes: characterisation and comparaison with the insulin receptor. *The Journal of Biological Chemistry*, **250**, 8990-8996.

Merimee, T.J., Zapf, J. and Froesch, E.R. (1982) Insulin-like growth factors in fed and fasted states. *Journal of Clinical Endocrinology and Metabolism*, **55**, 999-1002.

Mertz, E.T. (1975) The protein and amino acid needs. In: Fish Nutrition (J.E. Hlaver, Ed.). Academic Press, New York, pp.106-144.

Mesiano, S., Young, I.R., Browne, C.A. and Thorburn, G.D. (1988) Failure of acid-ethanol treatment to prevent interference by binding proteins in radioligand assays for the insulin-like growth factors. *Endocrinology*, **119**, 453-460.

Millikin, M.R. (1982) Qualitative and quantitative nutrient requirements of fishes: a review. *Fisheries Bulliten*, **80**, 655-686.

Millikin, M.R. (1983) Interactive effects of dieatary protein and lipid on growth and protein utilisation of age-0 striped bass. *Transactions of the American Fish Society*, **112**, 185-193.

Moore, R. (1979) Natural sex inversion in the giant perch *(Lates calcarifer). Australian Journal of Marine and Freshwater Research*, **30**, 801-813.

Moore, R. (1980) Reproduction and migration in the giant perch *(Lates calcarifer).* Ph.D. Thesis, University of London.

Moore, L.G. and Mylek, M.E. (1993) A novel method for the extraction of sheep insulin-like growth factors -I and -II from plasma prior to radioimmunoassay. *Journal of Endocrinology*, **137**, 239-245.

Moriyama, S., Duguay, S.J., Conlon, M.J., 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. *European Journal of Biochemistry*, **218**, 205-211.

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. *General and Comparative Endocrinology*, **96**, 149-161.

Morishita, D., Sasaki, K., Wakita, M. and Hoshino, S. (1993) Effect of fasting on serum insulin-like growth factor-I (IGF-I) levels and IGF-I-binding in cockerels. *Journal of Endocrinology*, **139**, 363-370.

Moxham, C., Duronio, V. and Jakobs, S. (1989) Insulin-like growth factor I receptor beta-subunit heterogeneity: evidence for hybrid tetramers composed of insulin-like and insulin receptor heterodimers. *Journal of Biological Chemistry*, **264**, 13238-13244.

Musey V.C., Goldstein, S., Farmer, P.K., Moore, P.B. and Phillips, L.S. (1993) Differential regulation of IGF-I and IGF-binding protein-1 by dietary composition in humans. *American Journal of Medical Science*, **305**, 131-138.

Nam, T-J., Park, K-Y., Lee, Y-D. and Kim, Y-U. (1996) Serum levels of insulin-like growth factor-I in flounder, *Parlichthys ovilaceus. Journal of the Korean Fisheries Society*, **29**, 150-156.

Ng, T.B., Leung, T.C. and Woo, N.Y.S. (1991) Insulin-like growth factor-I-like immunoreactivity in serum and tissues of the tilapia, *Oreochromis mossambicus. Biochemistry International*, **24**, 359-368.

Niu, P-D. and Le Bail, P-Y. (1993) Presence of insulin-like growth factor binding protein (IGF-BP) in rainbow trout *(Oncorhynchus mykiss)* serum. *Journal of Experimental Zoology* **265**, 627-636.

Ogino, C. (1980) Protein requirement of carp and rainbow trout. *Bulliten of the Japanese Society of Scientific Fisheries*, **46**, 385-388.

Owens, P.C., Johnson, R.J., Campbell, R.G. and Ballard, F.J. (1990) Growth hormone increases insulin-like growth factor-I (IGF-I) and decreases IGF-II in plasma of growing pigs. *Journal of Endocrinology*, **124**, 269-275.

Parazo, M.M. (1990) Effect of dietary protein and energy on growth, protein utilisation and carcass composition of rabitfish, *Siganus guttatus. Aquaculture*, **86**, 41-49.

Pell, J.M. and Bates, P.C. (1990) The nutritional regulation of growth hormone action. *Nutritrition Research Reviews*, **3**, 163-192.

Perdue, J.F., Chan, J.K., Thibault, C., Radaj, P., Millis, B. and Daughaday, W.H. (1983) The biochemical characterisation of detergent-solubilised insulin-like growth factor-II receptors from rat placenta. *The Journal of Biological Chemistry*, **258**, 7800-7811.

Perera, P.A.B. and De Silva, S.S. (1978) Studies on the chemical biology of young grey mullet, *Mugil cephalus* (L.). *Journal of Fish Biology*, **13**, 297-304.

Perez-Sanchez, J., Marti`-Palana, H. and LeBail, P-Y. (1994) Homologous growth hormone (GH) binding in gilthead sea bream *(Sparus aurata)*. Effect of fasting and refeeding on hepatic GH-binding and plasma somatomedin-like immunoreactivity. *Journal of Fish Biology*, **44**, 287-301.

Perez-Sanchez, J., Marti`-Palana, H. and Kaushik, S.J. (1995) Ration size and protein intake effect circulating growth hormone concentration, hepatic growth hormone binding and plasma insulin-like growth factor-I immunoreactivity in a marine teleost, the gilthead sea bream *(Sparus aurata)*. *American Institute of Nutrition* , **6**, 546-551.

Peter, R.E. and Marchant, T.A. (1995) The endocrinology of growth in carp and related species. *Aquaculture*, **129**, 299-321.

Philipps, A., Drakenberg, K., Persson, B., Sjogren, B., Eklof, A.C., Hall, K. and Sara, V. (1989) The effects of altered nutritional status upon insulin-like growth factors and their binding proteins in neonatal rats. *Paediatric Research*, **26**, 128-134.

Phillips, L.S. and Young, H.S. (1976) Nutrition and somatomedin I. Effect of fasting and refeeding on serum somatomedin activity and cartilage growth activity in rats. *Pediatric Research*, **26**, 128-134.

Pilistine, S.J., Moses, A.C. and Munro, H.N. (1984) Insulin-like growth factor receptors in rat placental membranes. *Endocrinology* **115**, 1060-1065.

Plisetskaya, E.M., Dockhoff, W.W., Piquette, T. and Gorbman, A. (1986) The assay of salmon insulin by homologous radioimmunoassay. *Fish Physiology and Biochemistry* **1**, 37-43.

Poffenbarger, P.L., Ensinck,J.W., Hepp, D.K. and Williams, R.H. (1968) The nature of human serum insulin-like activity (ILA): Characterisation of ILA in serum and serum fractions obtained by acid-ethanol extraction and adsorption chromatography. *Journal of Clinical Investigation*, **47**, 301-320.

Powell, D.R., Rosenfield, R.G., Baker, B.K., Liu, F. and Hintz, R.L. (1986) Serum somatomedin levels in adults with chronic renal failure: the importance of measuring insulin-like growth factor I (IGF-I) and IGF-II in acid-chromatographed uremic serum. *Journal of Clinical Endocrinology and Metabolism*, **63**, 1186-1192.

Prewitt, T.E., D'Ercole, A.J., Switzer, B.R. and Van Wyk, J.J. (1982) Relationship of serum immunoreactive somtomedin-C to dietary protein and energy in growing rats. *The Journal of Nutrition*, **112**, 144-150.

Rechler, M.M. (1993) Insulin-like growth factor binding proteins. *Vitamin Hormones*, **47**, 1-114.

Rechler, M.M. and Nissley, S.P. (1985) The nature and regulation of the receptors for insulin-like growth factors. *Annual Review of Physiology*, **47**, 425-442.

Rechler, M.M., Zapf, J., Nissley, S.P., Froesch, E.R., Moses, A.C., Podskalny, J.M., Schilling, E.E. and Humbel, R.E. (1980) Interactions of insulin-like growth factors I and II with multiplication-stimulating activity with receptors and serum carrier proteins. *Endocrinology*, **107**, 1451-1459.

Reinecke, M., Weinar, E., Maake, C., Drakenberg, K., Falkmer, S. and Sara, V.R. (1994) IGF-2-like peptides are present in insulin cells of the elasmobranchian endocrine pancreas: an immunohistochemical and chromatographic study. *Histochemistry*, **102**, 365-371.

Reis, L.M., Reutebuch, E.M. and Lovell, R.T. (1989) Protein-to-energy ratios in production diets and growth, fedding conversion and body composition of channel catfish, *Ictalurus punctatus*. *Aquaculture*, **77**, 21-27.

Renolds, L.F. (1978) Population dynamics of barramundi, *Lates calcarifer* (Pieces: Centropomidae) in Papua New Guinea. M.Sc. Thesis, University of Papua New Guinea.

Richardson, N.A., Anderson, A.J., Rimmer, M.A. and Sara, V.R. (1995) Localisation of insulin-like growth factor-I (IGF-I) immunoreactivity in larval and juvenile barramundi *(Lates calcarifer)*. *General and Comparative Endocrinology*, **100**, 282-292.

Rinderknect, E. and Humbel, R.E. (1978a) The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *Journal of Biological Chemistry*, **253**, 2769-2776.

Rinderknect, E. and Humbel, R.E. (1978b) Primary structure of human insulin-like growth II. *FEBS Letters*, **89**, 283-286.

Rivero, F., Goya, L. and Pascual-Leone, A.M. (1994) Effects of undernutrition and diabetes on serum and liver mRNA expression of IGFs and their binding proteins during rat development. *Journal of Endocrinology*, **145**, 427-440.

Rodgers, B.D., Helms, L.M.H. and Grau, E.G. (1992) Effects of fasting, medium glucose and amino acid concentrations on prolactin and growth hormone release *in vitro* from the pituitary of the tilapia, *Oreochromus mossambicus*. *General and Comparative Biochemistry*, **86**, 344-351.

Romer, A.S. (1966) Vertebrate paleontology. 3rd Edition. The University of Chicago Press, Chicago, pp.66.

Rosebrough, R.W., Mitchell, A.D. and McMurty, J.P. (1996) Dietary crude protein changes rapidly alter metabolism and plasma insulin-like growth factor I concentrations in broiler chickens. *The Journal of Nutrition*, **126**, 2888.

Rotwein, P., DeVol, D., Lajara, P., Bechtel, P. and Hammerman, M. (1989) Physiological regulation of insulin-like growth factor expression. In: Molecular and cellular biology of insulin-like growth factors and their receptors (LeRoith, D. and Raizada, M.K., Eds.). Plemun Press, New York, pp. 117-124.

Russell, L.F. and Garett, R.N. (1983) Use of juvenile barramundi, *Lates calcarifer* (Bloch), and other fishes of temporary supralittoral habitats in a tropical estuary in northern Australia. *Australian Journal of Marine and Freshwater Research*, **34**, 805-811.

Sakamoto, T., Hirano, T., Madsen, S.S., Nishioka, R.S. and Bern, H.A. (1993) Insulin-like growth factor-I (IGF-I) in salmonid gill: a mediator of osmoregulatory action of growth hormone (GH). Proceedings of the XII International Congress of Comparative Endocrinology, Toronto, pp. A-136.

Salmon, W.D. and Daughaday, W.H. (1957) A hormonally controlled serum factor which stimulates sulphate incorporation by cartilage *in vitro*. *Journal of Laboratory and Clinical Medicine*, **49**, 825-836.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a laboratory mannual (2nd Edition). Cold Spring Harbour Laboratory Press, Cold Spring Harbour, USA.

Sara, V.R., Carlsson-Skwirut, C., Andersson, C., Hall, E., Sjögren, B., Holmgren, A. and Jörnvall, H. (1986) Characterisation of somatomedins from human fetal brain; identification of a variant form of insulin-like growth factor-I. *Proceedings of the National Academy of Science USA*, **83**, 4904-4907.

Sara, V.R. and Hall, K. (1990) Insulin-like growth factors and their binding proteins. *Physiological Reviews*, **70**, 591-614.

Scatchard, G. (1949) The attractions of proteins for small molecules and ions. *Annual New York Academy of Science*, **51**, 660.

Scott, C.D. and Baxter, R.C. (1987) Purification and immunological characterisation of the rat liver insulin-like growth factor-II receptor. *Endocrinology*, **120**, 1-9.

Shamblott, M.J. and Chen, T.T. (1992) Identification of a second insulin-like growth factor in a fish species. *Proceedings of the National Academy of Science USA*, **89**, 8913-8917.

Shamblott, M.J. and Chen, T.T. (1993) Age-related and tissue-specific levels of five forms of insulin-like growth factor mRNA in a teleost. *Molecular and Marine Biology and Biotechnology*, **2**, 351-361.

Shamblott, M.J., Cheng, C.M., Bolt, D. and Chen, T.T. (1995) Appearance of insulin-like growth factor mRNA in the liver and pyloric ceca of a teleost in response to exogenous growth hormone. *Proceedings of the National Academy of Science USA* **92**, 6943-6946.

Shia, S-Y. and Huang, S-L. (1989) Optimal dietary protein level for hybrid tilpia *(Oreochromis niloticus x O. aureas)* reared in seawater. *Aquaculture*, **81**, 119-127.

Shrimpton, J.M., Devlin, R.H., McLean, E., Byatt, J.C., Donaldson, E.M. and Randall, D.J. (1995) Increases in gill cytosolic corticosteroid receptor abundance and saltwater tolerance in juvenile coho salmon *(Oncorhynchus kisutch)* treated with growth hormone and placental lactogen. *General and Comparative Endocrinology*, **98**, 1-15.

Siharath, K. and Bern, H.A. (1993) The physiology of insulin-like growth factor-I and its binding proteins in teleost fishes. *Proceedings Zoology Calcutta Haldane Comm.*, 113-124.

Siharath, K., Kelley, K.M. and Bern, H.A. (1992) Insulin-like growth factor binding proteins (IGFBPs) in striped bass. *American Zoology*, **32**, A19.

Siharath, K., Kelley, K.M. and Bern, H.A. (1996) A low molecular weight (25-kDa) IGF binding protein is increased with growth inhibition in the fasting striped bass, *Morone saxatilis. General and Comparative Endocrinology*, **102**, 307-316.

Siharath, K., Nishioka, R.S. and Bern, H.A. (1995) Regulation of IGF-binding proteins (IGFBPs) by growth hormone (GH) in the striped bass, *Morone saxatilis. Aquaculture*, **135**, 241-242.

Skyrud, T., Andersen, O., Alestrom, P. and Gautvik, K. (1989) Effect of recombinant human growth hormone and insulin-like growth factor-I on body growth and blood metabolites in brook trout *(Salvelinus fontinalis). General and Comparative Endocrinology*, **75**, 247-245.

Soliman, A.T., Hassan, A.E.I., Aref, M.K., Hintz, R.L. and Rosenfeld, R.G. (1986) Serum insulin-like growth factor I and II concentrations and growth hormone and insulin responses to arginine infusion in children with protein-energy malnutrition before and after nutrtional rehabilitation. *Pediatric Research*, **20**, 1122-1130.

Storebakken,T., Hung, S.S.O., Calvert, C.C. and Plisteskaya, E.M. (1991) Nutrient partitioning in rainbow trout fed at different feeding rates. *Aquaculture*, **96**, 191-203.

Straus, D.S. and Takemoto, C.D. (1990) Effect of starving on insulin-like growth factor-I (IGF-I) and growth hormone receptor mRNA levels and IGF-I gene transcription in rat liver. *Molecular Endocrinology*, **4**, 91-100.

Sumpter, J.P. (1992) Control of growth of rainbow trout *(Oncorhynchus mykiss)*, *Aquaculture*, **100**, 299-320.

Sumpter, J.P., Le Bail, P-Y., Pickering, A.D., Pottinger, T.G. and Carragher, J.F. (1991) The effect of starvation on growth and plasma growth hormone concentrations of rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology*, **83**, 94-102.

Sussenbach, J.S., Steenbergh, P.H. and Holthuizen, P. (1992) Structure and expression of the human insulin-like growth factor genes. *Growth Regulation*, **2**, 1-9.

Tacon, A.G.J. and Cowey, C.B. (1985) Protein and amino acid requirements, In: Fish Energetics: New Perspectives (P. Tyler and P. Calow, Eds.). Croom Helm, London, pp. 155-183.

Takano, K., Hall, K., Kastrup, K.W., Hizuka, N., Shizume, K., Kawai, K., Akimoto, M., Takuma, T. and Sugino, N. (1979) Serum somatomedin A in chronic renal failure. *Journal of Clinical Endocrinology and Metabolism*, **48**, 371-376.

Takeda, M., Shimeno, S., Hosokawa, H., Kajiyama, H. and Kaisyo, T. (1975) The effects of dietary calorie-to-protein ratio on the growth, feed conversion and body composition of young yellowtail. *Bulliten of the Japanese Society of Scientific Fisheries*, **41**, 443-447.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrlamide gels to nitrocellulose sheets: proceedure and some applications. *Proceedings of the National Academy of Science*, **76**, 4350-4354.

Tsai, P.I. (1993) Cartilage growth response during smoltification in coho salmon: GH influence *in vivo* on the response to IGF-I *in vitro*. M.A. Thesis in Endocrinology, University of California, Berkeley.

Underwood, L.E., Clemmons, D.R. Maes, M., D'Ercole, A.J. and Ketelslegers, J-M. (1986) Regulation of somatomedin C/insulin-like growth factor I by nutrients. *Hormone Research*, **24**, 166-176.

Upton, Z., Moriyama, S., Degger, B.G., Francis, G.L. and Ballard, F.J. (1996) Evolution of insulin-like growth factor-I (IGF-I) function: *in vitro* comparaison of barramundi, salmon, chicken and human IGF-I. Proceedings of the 10th International Congress of Endocrinology, June 12-15, San Francisco, USA.

Van Wyk, J.J., Svoboda, M.E. and Underwood, L.E. (1980) Evidence from radioligand assays that somatomedin-C and insulin-like growth factor-I are similar to each other and different from other somatomedins. *Journal of Clinical Endocrinology and Metabolism*, **50**, 206-215.

Wallis, A.E. and Devlin, R.H. (1993) Duplicate insulin-like growth factor-I genes in salmon display alternative splicing pathways. *Molecular Endocrinology*, **7**, 409-422.

Weatherley, A.H. and Gill, H.S. (1987) The Biology of Fish Growth. Academic Press, London, UK.

Williams, K.C., Barlow, C.G., Brock, I. and Rodgers, L.J. (1995) Use of autoclaving in the preparation of homogenates for determining the proximate chemical and fatty acid composition of fish. *Journal of the Science of Food and Agriculture*, **69**, 451-456.

Wilson, D.M. and Hintz, R.L. (1982) Interspecies comparison of somatomedin structure using immunological probes. *Journal of Endocrinology*, **95**, 59-64.

Winfree, R.A. and Stickney, R.R. (1981) Effect of dietary protein and energy on growth, feed conversion efficiency and body composition of *Tilapia aurea*. *Journal of Nutrition*, **111**, 1001-1012.

Yandell, C.A. (1992) The stabilisation and characterisation of insulin-like growth factor binding protein in the blood products of golden perch, *Macquaria ambigua*. Honours Thesis, Deakin University, Geelong, Victoria.

Xu, B., Miao, H., Zhang, P. and Li, D. (1996) Osmoregulatory actions of growth hormone in juvenile tilapia *(Oreochromis niloticus)*. Proceedings of the 3rd International Symposium on Fish Endocrinology, Japan, May 27-31, pp. 24.

Zapf, J., Walter, H. and Froesch, E.R. (1981) Radioimmunological determination of insulin-like growth factors I and II in normal subjects and in patients with growth disorders and extrapancreatic tumor hypoglycemia. *Journal of Clinical Investigation*, **68**, 1321-1330.

Zar, J.H. (1984). Biostatistical Analysis (2nd Edition). Prentice-Hall, New York.

Zing, A.E. and Froesch, E.R. (1973) Effects of partially purified preparations with non-suppressible insulin-like activity on sulphate incorporation into rat and chicken cartilage. *Diabetologia*, **9**, 472-476.

APPENDIX 1 LIST OF BUFFER SOLUTIONS

This table contains the receipes for buffer solutions used throughout the entire study. Chemical names abbreviated in this table have been listed in full in the introductory pages.

Buffer Name	Consituents
Alkaline copper reagent[a]	(w:v) copper sulphate (w:v) sodium tartrate sodium carbonate sodium hydroxide
Alkaline solution	0.2M sodium hydroxide 1% (w:v) SDS
CAPS buffer	10mM CAPS 10% (v:v) methanol pH 11.0
CHISAM	49:1 (v:v) chloroform: isoamylalcohol
Electrode buffer	0.192M glycine 0.025M Tris-HCl 0.1% (w:v) SDS pH 8.3
Elution buffer[c]	0.5M ammonium acetate 1mM EDTA 0.2% SDS
Hybridisation buffer[c]	80% (v:v) deionised formanide 100mM sodium citrate (pH 6.4) 300mM sodium acetate 1mM EDTA
Inactivation/Precipitation buffer[c]	Ambion patent pending
LB Broth	1% (w:v) tryptone 0.5% (w:v) yeast extract 0.17M sodium chloride pH 7.5
Loading buffer[c]	95% (v:v) formanide 0.025% (w:v) xylene cyanol 0.025% (w:v) BPB 0.025% (w:v) SDS 0.5M EDTA
6x Loading buffer	0.25% (w:v) BPB 0.25% (w:v) xylenecyanol FF 30% glycerol
Lysis buffer	50mM glucose 25mM Tris-HCl (pH 8.0) 10mM EDTA
10x MOPS buffer	0.2M MOPS 10mM EDTA 50mM sodium acetate pH 7.0
Neutralised TMA buffer	1:1 (v:v) TMA buffer: 0.4M Tris-HCl

Appendix 1 cont.

Proteinase K buffer	100µg/ml proteinase K *(Tritirachium album)* 25mM EDTA 2.5% (w:v) SDS 50mM Tris-HCl pH 8.0
RIA buffer	0.3M sodium phosphate 0.2% (w:v) protamine sulphate 0.2% (w:v) soduim azide 0.05% (v:v) Tween-20 pH 7.4
RNA loading buffer	50% glycerol 1mM EDTA 0.4% (w:v) BPB
RRA buffer	0.01M Tris-HCl 0.1M calcium chloride 0.5% (w:v) BSA pH 7.4
Saline-T buffer	0.15M sodium chloride 0.1% (v:v) Tween-20
Sample buffer	50mM Tris-HCl (pH 7.4) 10 % (v:v) glycerol 2.5% (w:v) SDS 0.001% (w:v) BPB
Solution D	4M GTC 25mM sodium citrate (pH 7.0) 0.5% (w:v) N-lauroyl sarcosine 0.7% (v:v) mercaptoethanol
TAE buffer[b]	10mM Tris-HCl 5mM sodium acetate 0.5mM EDTA pH 8.0
10x TBE buffer[b]	0.9M Tris 0.9M boric acid 20mM EDTA
TE buffer[b]	10mM Tris-HCl 1mM EDTA pH 8.0
TMA buffer	200mM HPLC grade acetic acid 50mM Trimethylamine pH 2.8 or pH 7.4
4x TMA buffer	800mM HPLC grade acetic acid 200mM trimethylamine pH 2.8
Tris-saline-N buffer	0.15M sodium chloride 0.01M Tris-HCl (pH 7.4) 3% (v:v) Nonidet® P40

Appendix 1 cont.

Tris-saline-B buffer	0.15M sodium chloride 0.01M Tris-HCl (pH 7.4) 1% (w:v) BSA

[a] Buffer was prepared on the day of use by mixing the constituents in the order listed.

[b] Buffer (or constituent) was autoclaved at 121°C, 15lb/sq for 30 min prior to use.

[c] Buffers were pre-made as part of a commercial RPA kit (Ambion, Texas, USA). The receipes were obtained from the kit protocol mannual, with the exception of the innactivation/precipitation buffer which was not listed due to a patent pending.

APPENDIX 2 TUKEY'S HONESTLY SIGNIFICANT DIFFERENCE TESTS FOR CHAPTER 4 DATA

Tukey's honestly significant difference tests for unequal sample sizes were used in chapter 4 for selected pair-wise comparisons of biological relevance. Pair-wise comparisons were significantly different if $p < 0.05$. Pairs that were not significantly different are indicated by N/S. Experimental groups have been abbreviated according to section 4.2.2.

% BWI	0 - 21 days [1]		21 - 42 days [2]	
Comparison	Tukey's q	Sig. of q (p)	Tukey's q	Sig. of q (p)
28/SAT vs 28/RES	5.40	$p < 0.05$	0.34	N/S
28/SAT vs 28/STV	22.53	$p < 0.05$	5.83	$p < 0.05$
28/RES vs 28/STV	15.00	$p < 0.05$	4.76	$p < 0.05$
21/SAT vs 21/RES	5.29	$p < 0.05$	1.90	N/S
21/SAT vs 21/STV	13.94	$p < 0.05$	6.17	$p < 0.05$
21/RES vs 21/STV	7.75	$p < 0.05$	7.66	$p < 0.05$
28/SAT vs 21/SAT	8.45	$p < 0.05$	1.74	N/S
28/RES vs 21/SAT	2.56	N/S	1.12	N/S
28/RES vs 21/RES	7.56	$p < 0.05$	0.60	N/S
28/STV vs 21/STV	0.72	N/S	1.40	N/S

[1]Tukey's q (0.05, 39, 6) = 4.232
[2]Tukey's q (0.05, 19, 6) = 4.469

CF	0 - 21 days [1]		21 - 42 days [2]	
Comparison	Tukey's q	Sig. of q (p)	Tukey's q	Sig. of q (p)
28/SAT vs 28/RES	1.59	N/S	1.12	N/S
28/SAT vs 28/STV	8.56	$p < 0.05$	17.45	$p < 0.05$
28/RES vs 28/STV	6.16	$p < 0.05$	14.10	$p < 0.05$
21/SAT vs 21/RES	3.01	N/S	1.73	N/S
21/SAT vs 21/STV	8.28	$p < 0.05$	14.79	$p < 0.05$
21/RES vs 21/STV	4.74	$p < 0.05$	12.38	$p < 0.05$
28/SAT vs 21/SAT	0.81	N/S	0.63	N/S
28/RES vs 21/SAT	2.24	N/S	1.66	N/S
28/RES vs 21/RES	0.61	N/S	0.06	N/S
28/STV vs 21/STV	0.13	N/S	3.67	N/S

[1]Tukey's q (0.05, 39, 6) = 4.232
[2]Tukey's q (0.05, 19, 6) = 4.469; Tukey's q (0.10, 19, 6) = 3.966

SGR	0 - 21 days [1*]		21 - 42 days [2*]	
Comparison	Tukey's q	Sig. of q (p)	Tukey's q	Sig. of q (p)
28/SAT vs 28/RES	3.33	N/S	0.46	N/S
28/SAT vs 28/STV	23.48	$p < 0.05$	11.91	$p < 0.05$
28/RES vs 28/STV	17.88	$p < 0.05$	9.92	$p < 0.05$
21/SAT vs 21/RES	4.92	$p < 0.05$	2.99	N/S
21/SAT vs 21/STV	18.31	$p < 0.05$	8.75	$p < 0.05$
21/RES vs 21/STV	12.32	$p < 0.05$	11.14	$p < 0.05$
28/SAT vs 21/SAT	5.84	$p < 0.05$	2.64	N/S
28/RES vs 21/SAT	2.13	N/S	1.74	N/S
28/RES vs 21/RES	6.80	$p < 0.05$	0.96	N/S
28/STV vs 21/STV	1.57	N/S	1.25	N/S

[1]Tukey's q (0.05, 39, 6) = 4.232

[2]Tukey's q (0.05, 18, 6) = 4.495

*Statitics performed on log transformed data to correct homogeneity of variance.

Hepatic IGF-I mRNA	0 - 21 days [1*]		21 - 42 days [2*]	
Comparison	Tukey's q	Sig. of q (p)	Tukey's q	Sig. of q (p)
28/SAT vs 28/RES	4.58	$p < 0.20$	3.17	N/S
28/SAT vs 28/STV	4.29	$p < 0.20$	4.22	$p < 0.20$
28/RES vs 28/STV	1.15	N/S	1.25	N/S
21/SAT vs 21/RES	4.15	$p < 0.20$	4.87	$p < 0.10$
21/SAT vs 21/STV	5.30	$p < 0.10$	3.62	N/S
21/RES vs 21/STV	0.17	$p < 0.05$	2.14	N/S
28/SAT vs 21/SAT	0.12	N/S	0.45	N/S
28/RES vs 21/SAT	1.27	N/S	5.16	$p < 0.10$
28/RES vs 21/RES	0.26	N/S	0.61	N/S
28/STV vs 21/STV	1.65	N/S	0.00	N/S

[1]Tukey's q (0.05, 6, 6) = 5.628; Tukey's q (0.10, 6, 6) = 4.726; Tukey's q (0.20, 6, 6) = 3.85

[2]Tukey's q (0.05, 6, 6) = 5.628; Tukey's q (0.10, 6, 6) = 4.726; Tukey's q (0.20, 6, 6) = 3.85

*Statitics performed on log transformed data to correct homogeneity of variance.

APPENDIX 3 TUKEY'S HONESTLY SIGNIFICANT DIFFERENCE TESTS FOR CHAPTER 5 DATA

Tukey's honestly significant difference tests for unequal sample sizes were used in chapter 5 for selected pair-wise comparisons of biological relevance. Pair-wise comparisons were significantly different if $p < 0.05$. Pairs that were not significantly different are indicated by N/S. Experimental groups have been abbreviated according to section 5.2.3.

% BWI	0 - 21 days [1]		21 - 42 days [1]	
Comparison	Tukey's q	Sig. of q (p)	Tukey's q	Sig. of q (p)
21: 15/45 vs 28: 15/45	0.64	N/S	1.58	N/S
21: 18/35 vs 28: 18/35	0.57	N/S	0.59	N/S
21: 18/45 vs 28: 18/45	1.19	N/S	2.44	N/S
21:18/55 vs 28: 18/55	2.23	N/S	5.78	$p < 0.05$
21: 21/45 vs 28: 21/45	5.55	N/S	7.49	$p < 0.05$

[1]Tukey's q (0.05, 36, 10) = 4.735

SGR	0 - 21 days [1]		21 - 42 days [1]	
Comparison	Tukey's q	Sig. of q (p)	Tukey's q	Sig. of q (p)
21: 15/45 vs 28: 15/45	0.77	N/S	1.71	N/S
21: 18/35 vs 28: 18/35	0.60	N/S	0.68	N/S
21: 18/45 vs 28: 18/45	1.25	N/S	2.70	N/S
21:18/55 vs 28: 18/55	2.36	N/S	5.95	$p < 0.05$
21: 21/45 vs 28: 21/45	5.14	$p < 0.05$	7.46	$p < 0.05$

[1]Tukey's q (0.05, 36, 10) = 4.735

CF	0 - 21 days [1]		21 - 42 days [1]	
Comparison	Tukey's q	Sig. of q (p)	Tukey's q	Sig. of q (p)
21: 15/45 vs 28: 15/45	2.78	N/S	3.41	N/S
21: 18/35 vs 28: 18/35	3.21	N/S	0.52	N/S
21: 18/45 vs 28: 18/45	3.35	N/S	2.46	N/S
21:18/55 vs 28: 18/55	5.16	$p < 0.05$	3.79	N/S
21: 21/45 vs 28: 21/45	4.13	N/S	1.32	N/S

[1]Tukey's q (0.05, 36, 10) = 4.735

FCR	0 - 21 days [1]		21 - 42 days [1]	
Comparison	Tukey's q	Sig. of q (p)	Tukey's q	Sig. of q (p)
21: 15/45 vs 28: 15/45	0.51	N/S	1.10	N/S
21: 18/35 vs 28: 18/35	4.16	N/S	0.67	N/S
21: 18/45 vs 28: 18/45	0.52	N/S	2.69	N/S
21:18/55 vs 28: 18/55	0.23	N/S	0.40	N/S
21: 21/45 vs 28: 21/45	0.25	N/S	0.52	N/S

[1]Tukey's q (0.05, 36, 10) = 4.735

PER	0 - 21 days [1]		21 - 42 days [1]	
Comparison	Tukey's q	Sig. of q (p)	Tukey's q	Sig. of q (p)
21: 15/45 vs 28: 15/45	0.11	N/S	0.54	N/S
21: 18/35 vs 28: 18/35	0.62	N/S	0.17	N/S
21: 18/45 vs 28: 18/45	0.15	N/S	1.21	N/S
21:18/55 vs 28: 18/55	0.23	N/S	2.40	N/S
21: 21/45 vs 28: 21/45	1.82	N/S	4.81	$p < 0.05$

[1]Tukey's q (0.05, 36, 10) = 4.735

NPU: 0 - 42 days

Comparison	Tukey's q	Sig. of q[1] (p)
21: 15/45 vs 28: 15/45	0.52	N/S
21: 18/35 vs 28: 18/35	0.40	N/S
21: 18/45 vs 28: 18/45	0.61	N/S
21: 18/55 vs 28: 18/55	1.62	N/S
21: 21/45 vs 28: 21/45	5.74	$p < 0.05$

[1]Tukey's q (0.05, 36, 10) = 4.735

HSI: 0 - 42 days

Comparison	Tukey's q	Sig. of q[1] (p)
21: T=0 0 vs 28: T=0	0.30	N/S
21: 15/45 vs 28: 15/45	1.83	N/S
21: 18/35 vs 28: 18/35	3.75	N/S
21: 18/45 vs 28: 18/45	2.12	N/S
21: 18/55 vs 28: 18/55	4.09	N/S
21: 21/45 vs 28: 21/45	4.13	N/S

[1]Tukey's q (0.05, 44, 12) = 4.808

Moisture: 0 - 42 days

Comparison	Tukey's q	Sig. of q[1] (p)
21: T=0 0 vs 28: T=0	2.37	N/S
21: 15/45 vs 28: 15/45	0.15	N/S
21: 18/35 vs 28: 18/35	1.05	N/S
21: 18/45 vs 28: 18/45	2.00	N/S
21: 18/55 vs 28: 18/55	1.58	N/S
21: 21/45 vs 28: 21/45	1.29	N/S

[1]Tukey's q (0.05, 43, 12) = 4.808

Protein gain: 0 - 42 days

Comparison	Tukey's q	Sig. of q[1] (p)
21: 15/45 vs 28: 15/45	0.87	N/S
21: 18/35 vs 28: 18/35	0.63	N/S
21: 18/45 vs 28: 18/45	2.40	N/S
21: 18/55 vs 28: 18/55	5.78	$p < 0.05$
21: 21/45 vs 28: 21/45	7.86	$p < 0.05$

[1]Tukey's q (0.05, 36, 10) = 4.735

Lipid gain: 0 - 42 days

Comparison	Tukey's q	Sig. of q[1] (p)
21: 15/45 vs 28: 15/45	0.45	N/S
21: 18/35 vs 28: 18/35	0.44	N/S
21: 18/45 vs 28: 18/45	0.81	N/S
21: 18/55 vs 28: 18/55	1.25	N/S
21: 21/45 vs 28: 21/45	3.84	N/S

[1]Tukey's q (0.05, 38, 10) = 4.735

Ash: 0 - 42 days

Comparison	Tukey's q	Sig. of q[1] (p)
21: T=0 vs 28: T=0	0.69	N/S
21: 15/45 vs 28: 15/45	1.09	N/S
21: 18/35 vs 28: 18/35	0.78	N/S
21: 18/45 vs 28: 18/45	2.32	N/S
21: 18/55 vs 28: 18/55	1.03	N/S
21: 21/45 vs 28: 21/45	0.35	N/S

[1]Tukey's q (0.05, 41, 12) = 4.808

IGF-I	0 - 21 days [1]		21 - 42 days [1]	
Comparison	Tukey's q	Sig. of q (p)	Tukey's q	Sig. of q (p)
21: 15/45 vs 28: 15/45	0.09	N/S	1.97	N/S
21: 18/35 vs 28: 18/35	1.25	N/S	0.00	N/S
21: 18/45 vs 28: 18/45	0.04	N/S	2.24	N/S
21:18/55 vs 28: 18/55	1.08	N/S	2.47	N/S
21: 21/45 vs 28: 21/45	4.55	N/S	7.40	$p < 0.05$

[1]Tukey's q (0.05, 45, 10) = 4.646

IGF-II	0 - 21 days [1]		21 - 42 days [1]	
Comparison	Tukey's q	Sig. of q (p)	Tukey's q	Sig. of q (p)
21: 15/45 vs 28: 15/45	0.09	N/S	0.16	N/S
21: 18/35 vs 28: 18/35	0.20	N/S	0.95	N/S
21: 18/45 vs 28: 18/45	0.12	N/S	0.04	N/S
21:18/55 vs 28: 18/55	0.76	N/S	1.12	N/S
21: 21/45 vs 28: 21/45	0.15	$p < 0.05$	0.04	$p < 0.05$

[1]Tukey's q (0.05, 45, 10) = 4.646