# REGULATION OF INSULIN-AND INSULIN RECEPTOR-ENCODING mRNAS IN RAINBOW TROUT, *Oncorhynchus mykiss*

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**Title**

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

**DOCTOR OF PHILOSOPHY**

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## ABSTRACT

In this work, rainbow trout were used as a model system to examine the regulation of insulin (INS)- and insulin receptor (IR)-encoding mRNA expression profiles. INS- and IR-encoding mRNAs were isolated, cloned, and sequenced; and shown to be differentially expressed within and among multiple tissue types. Regulation was examined through various nutritional and hormonal treatments (*in vivo and in vitro*). A real-time quantitative-PCR assay was developed to measure the respective levels of mRNA expression. Fasting, growth hormone (GH), and somatostatin (SS) differentially regulated INS and IR mRNAs within selected tissues, *in vivo*. Glucose, GH, SS, and insulin-like growth factor-1 (IGF-I) differentially regulated INS and IR mRNAs within selected tissues, *in vitro*. The results of this dissertation research display the identification and differential regulation of multiple INS- and IR-encoding mRNAs and suggest that independent mechanisms may serve to regulate the various isoforms in a tissue-specific manner. Future studies are also suggested.

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## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

# GENERAL INTRODUCTION

Research on fish has provided essential information about the structure, biosynthesis, evolution, and function of insulin (INS) as well as about the structure, evolution, and mechanism of action of insulin receptors (IR). INS, insulin-like growth factor (IGF)-1, and IGF-2 share a common ancestor; INS and a single IGF occur in Agnathans, whereas INS and distinct IGF-1 and IGF-2 appear in Chondrichthyes. Some but not all teleost fish possess multiple INS genes, but it is not clear if they arose from a common gene duplication event or from multiple separate gene duplications. INS is produced by the endocrine pancreas of fish as well as by several other tissues, including brain, pituitary, gastrointestinal tract, and adipose tissue. INS regulates various aspects of feeding, growth, development, and intermediary metabolism in fish. The actions of INS are mediated by the insulin receptor (IR), a member of the receptor tyrosine kinase family. IRs are widely distributed in peripheral tissues of fish, and multiple IR subtypes that derive from distinct mRNAs have been described. The IRs of fish link to several cellular effector systems, including the ERK and IRS-PI3k-Akt pathways. The diverse effects of INS can be modulated by altering the production and release of INS as well as by adjusting the production/surface expression of IR. The diverse actions of INS in fish as well as the diverse nature of the neural, hormonal, and environmental factors known to affect their INS signaling system reflects the various life history patterns that have evolved to enable fish to occupy a wide range of aquatic habitats.

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Fish have been important experimental models from the dawn of research on pancreatic function. In 1904, the effects of extracts of the pancreatic islets from anglerfish and scorpion fish on carbohydrate metabolism were examined (Diamare and Kuliabko, 1904). Slightly later it was shown that the hyperglycemia following pancreatectomy of Selachians could be attributed to insufficient endocrine function and that extracts of fish islet tissue could temporarily alleviate diabetic symptoms in humans (Rennie and Fraser, 1906). Fish insulin was one of the first vertebrate insulins to be isolated (McCormick and Noble, 1924), and the yield of this hormone from fish pancreata has remained better than that from any other group, including mammals, despite sophisticated extraction techniques (Humbel et al., 1972).

Since the appearance of the first issue of *General and Comparative Endocrinology*, which this paper commemorates, the study of pancreatic physiology and of insulin (INS) in particular in fish, has grown steadily. Much of this interest stems from the pivotal phylogenetic position of fish as a vertebrate stem group. In addition, fish are the most numerous and diverse group of vertebrates. They occupy a wide range of aquatic habitats, and have evolved elaborate life history patterns involving morphological, physiological and behavioural adaptations in order to adjust to the vast array of gaseous, thermal, osmotic and other conditions presented in their environments. Lastly, the endocrine pancreas of many fish is represented by an anatomically distinct Brockmann body consisting primarily of islet cells—a system that lends itself to the study of pancreatic function. Indeed, INS from the Brockmann body of tilapia acts as a surrogate to endogenous INS in diabetic mice that have undergone xenotransplantation (Alexander et al., 2006).

Many excellent treatises and reviews have illustrated how fish have provided essential information about the structure, biosynthesis, evolution, and function of INS as well as about the structure and evolution of insulin receptors (IR) (Epple and Brinn, 1987; Mommsen and Plisetskaya, 1991; Chan and Steiner, 2000; Navarro et al., 2002; Stone, 2003; Navarro et al., 2006). Since the appearance of these works, however, numerous studies in fish regarding the structure and biosynthesis of INS and IRs as well as of the physiological functions of INS and signal transduction processes mediated through IRs have been reported. In this review, then, we provide a comprehensive overview of the insulin signaling system of fish that includes a discussion of these most recent advances.

## Insulin

Insulin is a peptide hormone that plays a fundamental role in the regulation of somatic growth, cell differentiation during development, and metabolism of all vertebrates (Hernandez-Sanchez et al., 2006). The ability of INS to regulate anabolic aspects of intermediary metabolism is well known, and INS deficiency can lead to pathological disorders. The pancreas has long been known to be the primary source of INS in vertebrates; however, emerging evidence suggests that select extrapancreatic tissues also produce INS.

### *Biosynthesis*

INS biosynthesis begins at the transcriptional level, where promoter activation is triggered as a consequence of demand for the hormone. The overall structure of the INS gene is well conserved across all known species. The gene consists of three-exons and two-introns; the majority of conservation is across the location of exons, and introns are of

variable length (Steiner et al., 1985). This genomic organization also is present in fish, such as in chum salmon (Kavson et al., 1993) and tilapia (Mansour et al., 1998). Exon 1 encodes for the 5' untranslated region (UTR); exon 2 encodes the signal-peptide, B-chain (ca. 30 amino acids), and a portion of the C-peptide; exon 3 encodes the remainder of the C-peptide, the A-chain (ca. 21 amino acids), and the 3' UTR. Interestingly, most of the variability in the length of the C-peptide is due, in part, to the differential expression of the second intron, which has the potential to add an additional six or seven amino acid residues onto the C-peptide (Chan et al., 1981).

INS promoter activation and initiation has been best characterized in non-piscine species. This body of work has established that the INS promoter is critically important for INS expression, as it determines the transcription start site for RNA polymerase II and regulates the rate of transcription initiation. The 5' end of the promoter is not well defined beyond a few hundred base pairs, but is thought to extend as far as 4kb upstream from the start site (Fromont-Racine et al., 1990). The INS promoter contains multiple sequence elements, short nucleotide regions (6-7 bases), including critical A, E, and C elements, which act as recognition sites for DNA binding proteins (i.e., transcription factors). These cis-acting elements of the INS promoter by themselves do not possess significant transcriptional activity; instead, they must be activated synergistically in order to elicit a dramatic transcriptional response (Ohneda and German, 2000).

To date, there have only been a few reports on the INS cis-elements in fish. A TATA box and transcriptional start site (24-29 bases downstream of TATA) has been identified in a few fish, including tilapia (Mansour et al., 1998), trout (Argenton et al., 1997), and chum salmon (Koval et al., 1989), all of which display some similarities to the

mammalian INS promoter. Tilapia also possess C, E, and A elements, and microsatellite CA repeats similar in sequence to known mammalian cis-elements up to ~400 bp upstream from the transcriptional start site (Mansour et al., 1998; Pohajdak et al., 2004). While this suggests that the structure of INS gene in tilapia may be similar to that of mammals, it may be differentially regulated. This notion has been corroborated by the observation that tilapia Brockmann bodies transplanted in diabetic-induced mice display normolycemia and mammalian-like glucose tolerance (Alexander et al., 2006). By contrast, the rainbow trout INS promoter lacks several E and A elements, which contain conserved six-nucleotide sequence (CTAATG) motifs known to bind PDX-1, a putative transcription factor. Despite the lack of sequence conservation and some known PDX-1 binding motifs found in higher vertebrates, three PDX-1 binding sites were indicated in trout (Argenton, et al., 1997). Moreover, mammalian PDX-1 but not Insulin Enhancer Binding Factor-1 (IEF-1) was shown to bind the rainbow trout INS promoter and activate transcription (Argenton et al., 1997). Tilapia display several E elements that may bind IEF-1 and activate INS gene transcription similar to what is observed in mammals. PDX-1 also binds to elements of the zebrafish INS promoter and is essential for gene transcription (Huang et al., 2001; Milewski et al., 1998). Deletion analysis of the trout INS promoter indicated the region from -160 to -280 to be the most critical in enhancing INS gene transcription (Argenton et al., 1997), whereas, deletion analysis of the tilapia INS promoter suggested further upstream sequence from -535 to -1575 is necessary for enhanced gene transcription (Mansour et al., 1998). Although, additional deletion of the lower proximal promoter region (-1 to -396) in the tilapia INS gene demonstrated a necessity for initiating

transcription, implying cooperative interactions between upper and lower promoter regions (Hrytsenko et al., 2007).

In tilapia, the INS mRNA contains a splice donor (GU) and acceptor (AG) site, and a branch site (CUAAC), for each of the two introns, similar to other known INS genes (Mansour et al., 1998). Although the positions of introns are conserved in tilapia, the extent and variability of the intron sequence compared to chum salmon and mammals are quite different. Since these introns are known to regulate, in part, INS gene transcription within mammals, the variability found within tilapia introns may point to differential regulatory roles.

Translation of the INS-encoding mRNA results in a large-chain precursor, preproinsulin. Typically, preproinsulin contains a ca. 24-amino acid signal sequence, followed by a B-chain (ca. 30 amino acids), a C-peptide of variable length (ca. 28-42 amino acids), and an A-chain (ca. 21 amino acids); however, variations in the lengths of the A- and B-chains have been observed (Dodson and Steiner, 1998; Conlon, 2000, 2001). Preproinsulin is processed in a stepwise manner, by first removing the signal sequence to form proinsulin, then by the removal of the C-peptide to form INS. The C-peptide appears to be important in the formation of the A/B-chain disulfide linkages (e.g., the A/B-chains are connected with two inter-chain disulfide bonds and there is one intra-chain bond on the A-chain). The cysteines involved in disulfide formation are highly conserved and are required for bioactivity of the molecule (Conlon, 2001). To a finite extent, proinsulin is packaged in secretory granules and co-secreted along with its endopeptidases, INS, and C-peptide. (Dodson and Steiner, 1998). Distinct physiological actions of proinsulin or of C-peptide have been difficult to determine, but recent research suggests a role for proinsulin

during vertebrate development (Hernandez-Sanchez et al., 2006; Plisetskaya, 1998; Wicksteed et al., 2003).

*Structure*

Since the elucidation of the primary sequence of porcine INS (Sanger, 1959), the primary structure of INS has been characterized in numerous groups of vertebrates, including ca. 35 species of fish (including cyclostomes, elasmobranchs, and teleosts). Much of the information in fish was derived from direct peptide sequencing, but deduced sequences from full-length cDNAs have been reported for nearly all species for which INS is characterized. Analysis of the peptide sequences of fish reveals a high degree of conservation; however, there are many notable amino acid substitutions, additions, and deletions. As a result, the INSs of fish may contain anywhere from 51-58 amino acids between the A- and B-chains. For example, exceptions in length of the A- and B-chains are seen in dogfish, which has an extra glutamine on the C-terminal A-chain along with glycine and valine on the C-terminal B-chain (Bajaj et al., 1983). Carp INS also contains two extra amino acids on the N-terminal B-chain; and anglerfish INS, and toadfish INS I and INS II contain an extra amino acid on the N-terminal B-chain (Dodson and Steiner, 1998). Interestingly, differential proteolytic processing has been observed in some elasmobranchs, which gives rise to a truncated form of the C-peptide (Conlon, 2001; Conlon and Thim, 1986). A critical component for INS's tertiary structure and bioactivity is the disulfide linkage, which typically occurs between A20-B19, A7-B7, and A6-A11 (Conlon, 2001); however, there are exceptions, but all known INS variants maintain two inter-chain disulfide bonds and one intra-chain disulfide bond.

The ability of INS to interact with the insulin receptor (IR) to evoke a biological response is conferred by the 3-dimensional conformation of the INS molecule. Unfortunately, the crystal structure of INS has only been solved for one Agnathan, Atlantic Hagfish (Cutfield et al., 1979), and one species of Gnathostome fish, silver carp (Li et al., 1991; Zhao et al., 1985). Despite this limited information, it appears that with the exception of specific amino acid substitutions and few additions/deletions, INS molecules of fish and mammals have similar structure. For example, despite 19 out of the 51 amino acid residues differing between hagfish and porcine insulin, they share strong tertiary structural similarity. The primary sequence of silver carp differs by 16 and 19 amino acid residues, respectively, from porcine and hagfish INS. However, silver carp, like hagfish, retains major structural features of porcine INS, but with some notable differences (Fig. 1). The main structural differences between the tertiary structures of porcine and silver carp INS are located in the flexible parts of the structure, such as the N-terminal segments of the A- and B-chain, the A-Chain loop, and the C-terminus of the B-chain (Zhao et al., 1985). The peptide segments forming secondary structures, B9-B19, A12-A19 and B24-B26, have relatively stable conformations. Notably, region A6-A11 of carp INS displays differential protrusion compared to porcine INS, mostly due to the A10Pro substitution in silver carp. Interestingly, although there are three amino acids substitutions ranging from A12-A19, the basic framework occupies the same positions as porcine insulin. Because specific amino acids that are known for the three-dimensional assembly (e.g., Cys residues) and receptor binding (e.g., terminal residues of the A-chain; B9, B10, B13, B21) in porcine insulin, it is speculated that silver carp INS also possesses similar biological potency to porcine INS,

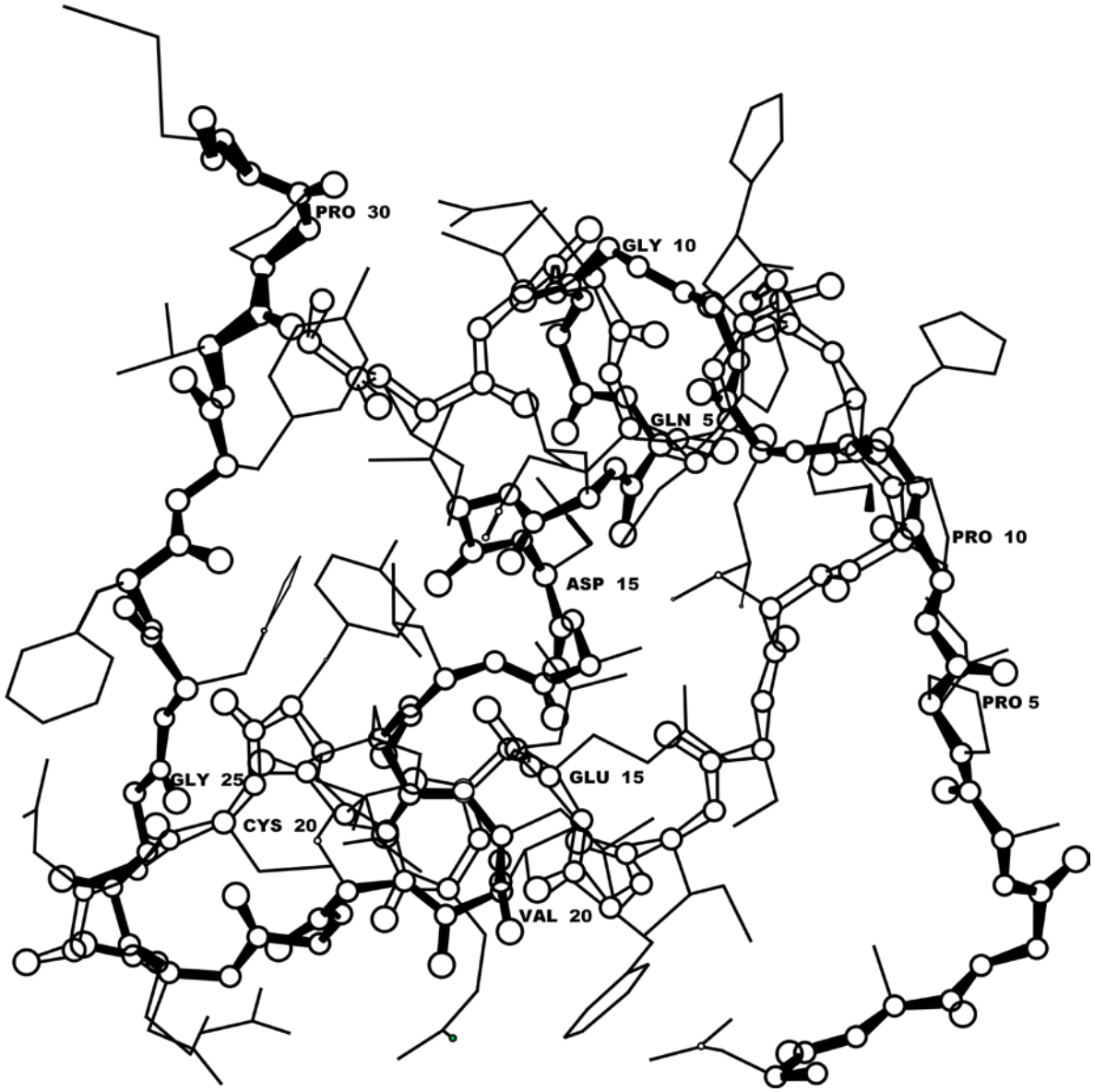


Figure 1. Tertiary structure of insulin from silver carp. Silver carp insulin molecule 4 viewed along the c axis. The A-chain is shown with the open line, and the B-chain with the solid line. (Adapted from Li et al., 1991)

whereas hagfish INS would have reduced potency compared to porcine INS (Zhao et al., 1985). Additionally, a structural model of a holocephalan species, elephant fish, has been proposed that possesses notable amino acid substitutions and deletions. In particular, a A14Val substitution and a termination at B31Ile, which differ from other holocephalans, would extend the B-chain to at least 37 amino acid residues, giving elephant fish a monobasic cleavage rather than the dibasic cleavage seen in most vertebrates (Berks et al., 1989).

To increase the stability and half-life of INS, it may take on the ornate form of a dimer or, in the presence of two $Zn^{2+}$ ions, a hexamer. Dimerization takes place with the interaction between two INS monomers due to hydrogen bonding and hydrophobic interactions between the C-terminus of the B-chains, in turn, providing increased stability. Additionally, in the presence of two $Zn^{2+}$ ions, porcine and silver carp INS form asymmetric hexamers (Cutfield et al., 1979); however, an INS hexamer in hagfish has not been observed (Peterson et al., 1974). The inability of hagfish INS to form hexamers is thought to be a result of an amino acid substitution at B10, where His in porcine and silver carp is thought to be critical for $Zn^{2+}$ binding is substituted with Asp in hagfish.

The INS hexamer diffuses into systemic circulation much more gradually than the INS monomer or dimer; thus, increasing the half-life of the molecule. Additionally, proinsulin is able to cocrystallize with insulin, capable of forming dimer and hexameric units (Steiner, 1973).

The structure of INS is not wooden and unmovable, but instead, displays plasticity in order to have the potential to bind efficiently with the IR. The conformational flexibility of the INS molecule includes the A-chain N-terminal segment, A-chain loop, and B-chain

terminal segments. There are a number of critical INS residues necessary for IR binding and activation. Only one molecule of INS is necessary to provoke a biological response through the binding to the binding to the IR; however, a second INS molecule may also bind to a lower affinity site, but this homotropic allosteric interaction is most likely only observed at pharmacological concentrations of INS (De Meyts and Whittaker, 2002). Conserved INS surface residues involved in site-1 binding are determined to include GlyA1, IleA2, ValA3,GlnA5, TyrA19, AsnA21, CysA20, AsnA21; ValB12, TyrB16, GlyB23, PheB24, PheB25, and TyrB26 (De Meyts, 2004). Site-2 conserved residues involved in binding include SerA12, LeuA13, GluA17, His B10, GluB13, and LeuB17 (De Meyts, 2004). The N-terminus of the A-chain is highly conserved across species and through mutational analysis has been determined to be critical for IR binding. For example, the positive charge from GlyA1 N-terminus forms a salt bridge with the C-terminus of the B-chain. Mutation or deletion of one these flanking residues affects the overall structure and therefore binding potential. Specifically, PheB24 and PheB25 have been determined to be essential for IR binding and the ability of the INS monomer to transition between the T and R state (De Meyts, 2004). B23-26 also have been shown to be partly responsible for the negative cooperatively. Consequently, the C- terminus of the B-chain is perhaps the most important region of the INS molecule for IR binding.

*Evolution*

INS is a member of a family of peptides that includes INS, insulin-like growth factor (IGF)-1 and -2, insulin-like peptides (ILPs), and relaxin peptides (Chan and Steiner, 2000; Wilkinson et al., 2005). The greatest homology among fish INS family members occurs at the site of the B-chain, with notable high conservation of the two cysteine

residues within this region. Beyond this minute commonality, the relaxin peptide sequence displays little resemblance to other INS family members. INS, IGF1, and IGF2 all exhibit similarity within the A- and B-chain regions, while the most obvious dissimilarity between INS and IGF members occurs at the location of the C-peptide and the additional C-terminus (D and E domains). The distinction between INS and IGF peptide sequence remains consistent throughout the fish lineage, from hagfish to fugu. These structural features are consistent with the phylogenetic relationships among all cloned members of INS family of peptides in fish. Phylogenetic analysis reveals five distinct clades that most likely arose through a series of gene duplication events over the course of early vertebrate evolution: a relaxin clade, an IGF-3 clade, an INS clade, an IGF-2 clade, and an IGF-1 clade (not shown).

It appears that the relaxins diverged early from the other members of the INS family. The relaxin subfamily has been best characterized in mammals, and includes relaxins 1-3 and ILPs 3-6 (Halls et al., 2007). Relaxins are structurally similar to INS and IGF displaying highly conserved cysteine residues in their A- and B-chains and subsequent disulfide linkage; however, the sequence similarity is not as well conserved (Wilkinson et al., 2005). Despite structural similarity to INS and IGF, relaxins bind leucine-rich G-protein coupled receptors (LGRs) and not receptor tyrosine kinases (RTK). This suggests that relaxins take on different physiological roles and may not be involved in mitogenesis and/or metabolism (Halls et al., 2007). Relaxin-3 (aka ILP-7) is highly conserved among vertebrates and may serve as the ancestral relaxin that emerged from the divergence of fish (Wilkinson et al., 2005). Multiple relaxin-3 sequences have been identified in fugu (*Takifugu rubipes*) and zebrafish (*Danio rerio*), and a single relaxin has been found in

rainbow trout (*Oncorhynchus mykiss*) that may be specific to the fish lineage (Wilkinson et al., 2005). The multiple relaxin-3 molecules in fish are most homologous with relaxin-2, relaxin-3, and ILP5.

Although the two IGF-3s that have been described in fish appear in a distinct clade, their sequence closely resembles that of relaxin. Clearly, more information is needed to better understand the structure and function of these peptides, and it may turn out that their name will need to be revised.

Given the similarity in the structures of INS, IGF-1, and IGF-2, it is not surprising that they share a common ancestor. The difficultly in differentiating proinsulin and IGF in radioimmunoassays (RIA) (Plisetskaya, 1998) is noteworthy as is the ability of each ligand to bind each other's receptors, but do so with 10- to 100-fold reduced affinity compared to binding their cognate receptors (Hussain et al., 1995). Our analysis supports the notion that IGF-1 first diverged from a common INS/IGF-2 ancestor, then INS and IGF-2 diverged afterward. This is supported by the observation that hagfish, a representative of one of the oldest of vertebrate groups, possess INS and a single IGF, the latter of which clades with the IGF-1s of other fish groups. It appears that the divergence of INS and IGF-2 arose in conjunction with the emergence of the Gnathostome lineage as members of Chondrichthyes possess a distinct IGF-1 and IGF-2 (Duguay et al., 1995). Our model is a somewhat different from that of Chan and Steiner (2000), who proposed that INS diverged from a common IGF-1/IGF-2 ancestor; however, considerably more sequence information on INSs and IGFs has become available in the last 10 years.

There have been several reports of multiple INSs in various groups of vertebrates, including mammals, amphibians, and teleost fish. In some cases, such as in cow, pig, and

flounder, the structural heterogeneity arises from differential processing of a single amino acid precursor (Andoh and Nagasawa, 1998; D'Agostino et al., 1987; Snel and Damgaard, 1998). In other instances, such as in chum salmon, puffer fish, zebrafish, *Xenopus*, and rodents, the heterogeneity arises from the existence of two distinct INS-encoding genes (Deltour et al., 1993; Irwin, 2004; Kavsan et al., 1993; Shuldiner et al., 1991). Whether or not there were one or more such duplication events in the teleost fish lineage is not clear. Irwin (2004) notes that the presence of two INS genes in zebrafish and fugu suggests the duplication occurred prior to the divergence of these groups, and possibly prior to the radiation of all bony fish. On the other hand, multiple INSs have not been characterized in all teleosts, and the similarities among the salmonid INSs suggests a more recent duplication, possibly associated with the tetraplodization of this taxon (Meyer and Van de Peer, 2005). Additional research is needed to determine if multiple INS genes are more wide spread among teleosts and whether or not the polygenic origins of INSs can be traced back to the duplication event associated with the emergence of this group ca. 350 million years ago.

*Distribution*

INS is synthesized, processed, and distributed in multiple forms and in multiple tissue types within vertebrates. Two non-allelic forms (INS1, chromosome 19; INS2, chromosome 7) of INS have been indicated within rats and mice; although, this is unlike the two INS variants found in humans, which are derived from alternative splicing of a single gene (Bunzli et al, 1972). Tissue-specific INS levels have been measured well in excess of systemic plasma INS levels within mammals and fish, implying the possibility of localized INS production (Plisetskaya, 1998; Rosenzweig et al., 1980). Within normal

(non-diabetic/obese) mice, INS mRNA has been detected in the pancreas, brain, and thymus (Chen et al., 2001; Kojima et al., 2004; Rosenzweig et al., 1980). Whereas mice that are diabetic, obese (ob/ob), or fed high-fat diets, proinsulin and insulin production appears more widespread, extending to the liver, adipose, spleen, and bone marrow (Kojima et al., 2004). The reprogramming of tissues following induction of diabetes with strepzotocin [STZ]) predominately results in the production of proinsulin instead of insulin; thus, helping explain the insensitivity to hyperglycemic conditions in STZ treated mice. INS production in the brain of rodents had long been debated (Le Roith et al., 1989; Plisetskaya et al., 1993) because of varying results utilizing radioimmunoassay (RIA). It was not until the INS mRNA could be verified that, indeed, INS production was conclusively found to occur in the brain; albeit, in small quantities relative to pancreatic INS production (Devaskar et al., 1994). INS production in the brain has not only detected in rodents but also in chicken and rabbit (Devaskar et al., 1994).

Fish also possess variant forms of INS. Non-allelic forms of INS are present within teleost fish, as a consequence of multiple genome duplication events (Zhou et al., 2001). Rainbow trout, chum salmon, fugu, channel catfish, greater amberjack, and zebrafish have all been shown to possess multiple non-allelic INS encoding mRNAs (Caruso et al., 2008; Irwin, 2004; Kavson et al., 1993; Papasani et al., 2006). Another report on tilapia speculated on the existence of multiple INS forms, but only a single INS construct was identified (Mansour et al., 1998). However, similar to the two variant forms in humans, barfin flounder display two forms of INS derived from a single gene (Andoh and Nagasawa, 1998).

As was the case in mammals and other vertebrates, the primary source of INS production in fish appears to be pancreatic islet tissue (Youson and Al-Mahrouki, 1999). However, there was early speculation about extrapancreatic INS production in the brain of fish, and the presence of INS was detected by RIA in the brain of salmon and hagfish (Plisetskaya, 1993; Thorndyke et al., 1989). A determination about whether the INS was transported to the brain or was synthesized there had to await the advent of molecular approaches. The presence of INS mRNA in several lobes of the brain has only recently been uncovered in fish (Caruso et al., 2008; Hrytsenko et al., 2007). Under normal physiological conditions, levels of INS1 mRNA were greater than those of INS2 mRNA for all INS producing tissues within rainbow trout (Caruso et al., 2008). Within the brain, INS1 mRNA was detected in several brain regions, including telencephalon, optic tectum, cerebellum, and hypothalamus. The highest levels of INS1 were seen in the cerebellum; however, levels of INS2 mRNA were below the limit of detection within each of the specific regions of the brain (Caruso et al., 2008). INS was also measured in several regions of the brain in tilapia, including cerebellum, hypothalamus, visual cortex, thalamus, and olfactory cortex (Hrytsenko et al., 2008). The hypothalamus and cerebellum exhibited the greatest abundance. Other INS producing extrapancreatic tissue included the pituitary, which displayed significantly higher levels than that found in the brain of tilapia (Hrytsenko et al., 2008). While INS1 mRNA was detected in the pituitary in rainbow trout, it was at reduced levels compared to the brain and other INS- producing tissues (Caruso et al., 2008).

Unfortunately, due to an ill-defined thymus within fish, the presence of thymic INS production has yet to be demonstrated as in mammals. Besides extrapancreatic nervous and

endocrine tissue, INS mRNA has been detected in mesenteric adipose tissue within carp (Roy et al., 2003) and rainbow trout (Caruso et al., 2008). Additionally, INS has been detected in several fish throughout the gut. INS1 mRNA in rainbow trout is expressed in the upper and lower intestine, as well as INS1 and INS2 expression in pyloric cecum (Caruso et al., 2008). This is consistent with reports of INS immunoreactive cells along the intestine of several species, including African lungfish, silver arawana, butterflyfish, and elephant nose; however, several other species were unable to detect the presence of INS along the GI tract (Al-Mahrouki and Youson, 1999; El-Salhy, 1984; Tagliafierro et al., 1996; Youson et al., 2001). It should be noted that the presence of INS-producing cells found in either adipose, pyloric cecum, or intestine could perhaps be a result of endocrine satellite islets that are dispersed throughout the mesentery (peritoneum) (Al-Mahrouki and Youson, 1999).

*Secretion*

Under varying physiological conditions (e.g., season, temperature), INS in the plasma of fish has been measured in the range of 0.2-5 nmol/l or higher following a meal; however, such levels do not approach those seen in postprandial mammals and they take up to 24 h to return to basal levels in carnivorous species (Navarro et al., 2002; Stone, 2003). In addition, localization or "zonation" of INS may contribute to variability of measured levels. For example, levels of INS in the hepatic portal veins were substantially higher than in the systemic circulation (Plisetskaya and Sullivan, 1989; Mommsen and Plisetskaya, 1991). After glucose or arginine injection into brown trout, INS levels in the hepatic portal vein were over twice those in peripheral veins (e.g., caudal vein) (Carneiro et al., 1993).

The secretion of INS in fish is influenced by numerous nutritional, hormonal, neural, and other environmental factors. Depending on the species of fish and their native diet composition; carbohydrates, amino acids, and/or fatty acids may act synergistically or individually as INS secretagogues. For example, in many carnivorous fish species (e.g., salmonids), amino acids possess a greater insulintropic ability than carbohydrates (Gutierrez and Plisetskaya., 1991). Correspondingly, due to the inefficient ability of carnivorous fish to utilize carbohydrates, they require high levels of dietary protein (Stone, 2003). The potency of the insulinotropic action varies with the specific amino acids in the diet profile. Amino acids such as glycine, arginine, lysine, leucine, alanine, and phenylalanine have exhibited the greatest insulinotropic potential *in vivo* and *in vitro* (Navarro et al., 2002; Pliseskaya et al., 1991; Rojas et al., 2009). Conversely, histidine injection inhibited INS secretion from coho salmon in vivo (Plisetskaya et al., 1991). Interestingly, even in tilapia, which are omnivorous but can be maintained on a primarily vegetarian diet, glucose and arginine administration treatment of isolated Brockmann bodies each induced INS secretion in a dose-dependent manner. However, INS mRNA expression in tilapia Brockmann bodies only increased in the presence of supraphysiological levels of both glucose and arginine simultaneously. Moreover, glucose treatment at all doses failed to elicit an increase in tilapia INS promoter constructs (Mansour et al., 1998). This is in contrast to what has been observed in mammals, where the presence of glucose strongly increases the rate of INS transcription (German and Wang, 1994). In addition, glucose and arginine treatment over a 24 h incubation period were able to maintain INS storage and secretion without an increase in INS mRNA, suggesting that INS is not regulated at the transcriptional level in tilapia but instead more so at the level of

translation (Hrytsenko et al., 2008). The regulation and subsequent rise in INS mRNA in mammals is time-dependent; translation of INS mRNA in response to glucose relies primarily on the mRNA pool instead of increasing the transcriptional rate over an acute response. During prolonged periods of stimulation resulting in decreases in the INS mRNA pool, rates of transcriptional and mRNA stability increase (Wicksteed, 2003).

Numerous hormones affect the release of INS in fish. *In vivo* administration of secretin, a member of the glucagon family, increased plasma INS in eels; however, glucagon–like peptide-1 (GLP-1) does not appear to stimulate INS secretion in fish as it does in mammals (Ince, 1983; Plisetskaya and Mommsen, 1996). Growth hormone implantation of rainbow trout increased the expression of INS mRNAs in Brockmann bodies (Caruso and Sheridan, 2010). Injection of somatostatins (SS) into salmonids, including SS-14 and SS-25, resulted in reduced plasma levels of INS depending on time after administration (Sheridan et al., 1987; Eilertson and Sheridan, 1993). In vivo administration of SS-14 in trout also reduced the expression of INS-encoding mRNAs within the Brockmann body (Caruso and Sheridan, 2010). SSs also anatagonize other growth-promoting hormones such as GH and IGF-I as well as their cognate receptors (Sheridan and Hagemeister, 2010).

The autonomic nervous system also affects INS secretion in fish. All islet types possess muscarinic and adrenergic receptors that are capable of receiving parasympathetic and sympathetic modulators, respectively (Epple and Brinn, 1975; Tilzey et al., 1985). For example, anglerfish pancreatic islets subjected to methacoline, which binds muscarinic receptors, induced the secretion of INS (Milgram et al., 1991). Similar to mammals, which display preabsorptive INS release (Strubbe and Bauman, 1978), striped bass were reported

to secrete INS within a short time frame before nutrient absorption could occur (Papatryphon et al., 2001). Catecholamines display contrasting effects on INS release in fish, which seem to be dose dependent. Supraphysiological levels of epinephrine either induce or attenuate INS release in lamprey depending on the previous concentration of INS before administration (Plisetskaya et al., 1977). In rainbow trout Brockmann bodies, low levels of epinephrine inhibited the release of INS while high doses stimulated the release of INS (Tilzey et al., 1985). Catecholamines also inhibited INS release within the eel pancreas *in vitro* and *in situ* (Ince and Thorpe, 1977; Ince, 1980). However in mammals, epinephrine has been indicated to be an antagonist to glucose-induced INS secretion within rat islets (Zawalich et al., 2007).

Other factors such as environmental temperature may be important for INS secretion. Huth and Rapoport (1982) suggested that temperature may be more important than nutritional factors in influencing plasma INS in carp. However, it is not clear if temperature has direct effects on INS or if the elevated response is a consequence of enhanced growth, nor is it clear how widespread temperature effects may be in other species.

*Function*

Insulin has a wide spectrum of actions. In fish, INS coordinates various aspects of feeding, growth, development, and intermediary metabolism, including glucoregulatory actions in the brain.

*Feeding/Fasting/Satiety*

Plasma levels of INS increase following a meal and, in turn, INS promotes peripheral tissue uptake of glucose and amino acids, and activates synthesis of glucose glycogen and triacylglycerols (TG) (Taha and Klip, 1999). It is notable that fish, particularly carnivorous fish, do not utilize carbohydrate well and they display marked glucose intolerance (Moon, 2001). Adaptation of carnivorous fish to a high carbohydrate diet improves their INS response and reduces their glucose intolerance (Mazur et al., 1992). Our lab found that there was an inverse correlation between plasma somatostatin-14 (SS-14) and plasma INS levels in the rainbow trout adapted to high carbohydrate diets such that the most glucose intolerant fish displayed the lowest INS levels attended by the highest SS-14 levels and that the least glucose intolerant fish displayed the highest INS levels attended by the lowest SS-14 levels (Eilertson and Sheridan, unpublished). Such observations are consistent with our finding that hypersomatoatinemia is accompanied by a blunted INS response in glucose challenged trout and that SS inhibits INS secretion and lowers plasma INS (Harmon et al., 1991). Given that glucose is a potent stimulator of SS synthesis and secretion in trout (Ehrman et al., 1999), it is reasonable to suggest that the glucose intolerance of carnivorous fish can be explained, at least in part, by the SS-secreting cells of the pancreatic islets being more sensitive to glucose than the INS-secreting cells. Glucose intolerance also may be promoted from glucagon-like peptide-1 (GLP-1), which also antagonizes pancreatic INS release in teleosts and promotes hyperglycemia following glucose administration (Plisetskaya and Mommsen, 1996; Polakof et al., 2010). However, other reports indicate an adequate response in INS secretion following glucose administration (Blasco et al., 1996; Furuichi and Yone, 1983; Mazur et al., 1992).

Therefore, the glucose intolerance of carnivorous fish remains somewhat enigmatic (Moon, 2001). Several mediators of carbohydrate metabolism have been identified in carnivorous fish, such as glucose sensors, glucose transporters, and regulatory signaling molecules. Further study investigating the regulation of GLUT-4 in INS sensitive tissues such as skeletal muscle, adipose, and liver may provide additional insight into the glucose intolerance of certain fish species. Future studies need to examine species-specific regulatory mediators involved in glucose turnover between glucose-tolerant and glucose-intolerant fish.

In fasting fish, plasma INS titers decline, and the levels observed in fasting fish are similar to those in fasting mammals (Stone, 2003). Alterations in plasma INS in fish during fasting are accompanied by a general shift from an anabolic state to a catabolic state that involves glycogenolysis, lipolysis, and proteolysis (Albalat et al., 2005; Blasco et al., 1992a, 1992b; Larsen et al., 2001; Navarro et al., 1993). The activation of catabolic processes that occurs during fasting is similar to that observed in fish that are INS deficient (Foster and Moon, 1989; Plisetskaya and Duan, 1994; Plisetskaya et al., 1983; Plisetskaya et al., 1989). Interestingly, fish appear to have evolved several strategies for adjusting to food deprivation, and some species of fish are remarkably resistant to fasting. For example, some species rapidly deplete glycogen reserves, while others partially protect such reserves for up to several weeks or others yet that fully protect glycogen for up to several months (Sheridan and Mommsen, 1991). The different strategies appear to be related to evolutionary history as those species that have periods of natural food deprivation (e,g., fasting associated with spawning migrations) as part of their life history appear most resistant to fasting. Notably, during the spawning migration of lamprey and salmon,

plasma INS levels are similar to those during periods of feeding (Mommsen and Plisetskaya, 1991).

Appetite in fish is regulated by a multitude of factors that can stimulate (orexigenic) or inhibit (anorexigenic) food intake (Volkoff et al., 2005). Intracerebroventricular (ICV) injection of 2-deoxy-D-glucose into rainbow trout resulted in an orexigenic response, which indicated that the nervous system detects and responds to varying glycemic levels (Soengas and Aldegunde, 2004). The observation that INS is present and synthesized in the brain of fish (cf. Caruso et al., 2008) suggests that it may play some role in appetite regulation. Plisetskaya (1991) suggested that INS promotes postprandial satiety in fish (Plisetskaya, 1991). INS injected ICV and intraperitonneally (IP) into rainbow trout resulted in reduced food intake more than 1-3 days post-injection; however, it is not clear whether INS promotes anorexigenic signals directly or indirectly via inhibition of orexigenic factors such as NPY (Silverstein and Plisetskaya, 2000). By contrast, ICV injection of INS into channel catfish did not decrease in food intake (Silverstein and Plisetskaya, 2000). The basis(es) for the differing responses is(are) not known, but further work is needed to see if they can be attributed to species-specific, life history-specific, or other factors.

*Growth*

Body size is often correlated with plasma INS levels, especially in salmonids (Sunby et al., 1991; Mommsen and Plisetskaya, 1991). Moreover, during periods of increased growth INS is detected at elevated levels correlating with seasonal and reproductive changes (Plisetskaya, 1989). For example, brown trout (Navarro et al., 1991)

and Atlantic salmon (Dickoff et al., 1989) display nearly double the concentration of basal plasma INS from summer to winter months. During the annual cycle of Sea Bass (*Dicentrarchus labrox*) and Dogfish (*Scyliorhinus canicula*), an elasmobranch, plasma INS remains relatively reduced during spawning and post-spawning periods of the year and then increased during pre-spawning with a concomitant increase in growth (Aug.-Nov.) (Gutiérrez et al., 1987a; Gutiérriz et al., 1988). In some anadromous species, plasma INS levels are also sex dependent at the time of spawning. Males maintain a steady level of plasma INS, promoting spermatogenesis, whereas females INS levels diminish at the point of ovulation (Sower et al., 1985; Plisetskaya et al., 1987).

Arginine, which is a known INS secretagogue in salmonids, was supplemented in the diets of three salmonid species only indicated increased growth within fingerlings, suggesting nutrient utilization pertaining to growth may be specific to the developmental stage (Plisetskaya et al., 1991). Interestingly, GH transgenic coho salmon, which displayed increased growth and maturation did not show a difference in plasma INS compared to size controls; however, GH transgenic salmon had increased levels of INS compared to nontransgenic siblings (Devlin et al., 2000). Streptozotocin-induced diabetes in rats is attended by reduced levels of INS and of IGF-1, and by increased levels of GH, similar to observation seen in diabetic humans (Hussain et al., 1995). Fasting also induces a reduction in INS and IGF-I in fish with corresponding normal or increased levels of GH (Norbeck et al., 2007).

INS appears to have both direct and indirect effects on growth. INS has been shown to increase skeletal growth through sulfate [$^{35}S$] incorporation into gill cartilage (Duan and Hirano, 1990; Marchant and Moroz, 1993). The growth-promoting actions of

INS are like those of IGF-1 and may share a common mechanism (Barbieri et al., 2003). Independently, INS and IGF-1 stimulate $^{35}SO_4$ incorporation within gill cartilage, suggesting their involvement in the regulation of cartilage matrix synthesis (Duan et al., 1990; Chan et al., 1997). The expression of multiple IRs within gill cartilage and INS-induced $^{35}SO_4$ uptake have been measured in other salmonid species (Chan et al., 1997). INS increases growth indirectly by increasing IGF-1 production from the liver when it is synergized with GH (Plisetskaya 1998; Wood et al., 2005). INS also regulates IGF-2, a known anabolic hormone similar to IGF-1; however, IGF-2 elicits its effects through a non-RTK receptor. INS directly stimulated IGF-2 mRNA expression in isolated coho salmon hepatocytes and indirectly stimulated expression through the enhancement of GH stimulation on IGF-2 mRNA levels (Pierce et al., 2010).

INS also initiates several feedback regulatory mechanisms in fish (Table 1). At the levels of the pancreas, INS increased the expression of SS-encoding mRNAs (Ehrman et al., 2005), which was reminiscent of the same feedback effect displayed by GH (Melroe et al., 2004). The increased biosynthesis of SS would lead to reduced INS production and release as discussed above. In the periphery, INS increased the expression of somatostatin receptors (SSTRs) in tissues such as the liver (Nelson and Sheridan, 2006). Increased SSTRs in peripheral tissues would enable/enhance the inhibitory effects of SS on GH and IGF-1 sensitivity and action is such peripheral tissues (Sheridan and Hagemeister, 2010). In mammals, INS has been shown to modify gene transcription and translation. A number of genes pertaining to known mediators of growth and metabolism contain *cis*-acting DNA sequences or insulin-responsive elements (IRE) located upstream to their start site. For instance, phosphoenolpyruvate carboxykinase (PEPCK), c-fos, amylase, liver pyruvate

Table 1. Major actions of insulin in fish*

| Tissue | Action |
|---|---|
| Adipose | ↑ GLUT4 translocation [27]<br>↑↓ lipogenesis Polakof et al., 2011 lipids<br>↑ Lipoprotein lipase activity [5]<br>↓ lipolysis [4] |
| Blood | ↓ fatty acids [195,207]<br>↓ glucose [18, 54, 84, 90, 106, 142, 179, 180, 187, 189, 207, 211,212] |
| Brain | ↑ glycogenesis [77, 217]<br>↓ or no Δ appetite [231,235] |
| Cardiac Muscle | ↑ 2-deoxyglucose uptake [84] |
| Gill cartilage | ↑ $^{35}SO_4^{2-}$ incorporation [65, 153] |
| Liver | ↓↑ β oxidation [207]<br>↑ glycogenesis [76, 78, 81]<br>↓ glycogenolysis [46,50,81]<br>↑ gluconeogenesis [78,81]<br>↓ gluconeogenesis [46, 50,137,193]<br>↑ glycolysis [82, 163]<br>↓ glycolysis [81]<br>↑ IGF1/2 mRNA expression [192,201,252]<br>↑ lipogenesis [47,55,127,149,207,222,245]<br>↓ lipolysis [104,105,207,228]<br>↑ somatostatin receptor subtype 1/2 mRNA expression [175] |
| Pancreas | ↑ preprosomatostatin expression [68] |
| Skeletal Muscle | ↓↑ β oxidation [207]<br>↑ glucose utilization [21]<br>↑ GLUT4 expression [61]<br>↑ glycogenesis [76, 78]<br>↑ lipogenesis [1,127]<br>↑ or no Δ GLUT4 translocation [61,111,194,218]<br>↑ protein synthesis [42] |

*Reference numbers are indicated in superscript

kinase (PK), glyeraldehyde-3-phosphate dehydrogenase (GAPDH), glucagon, and insulin-like growth factor binding protein-1 (IGFBP-1) (Granner et al., 1986; Kimball et al., 1994). INS may mediate gene activation through the initiation of transcription factor cAMP-responsive element-binding protein (CREB) downstream of adenylyl cyclase. CREB has been shown to bind several promoter regions to various hormones and/or metabolic enzymes (Johannessen et al., 2004). INS also increases protein synthesis indirectly via mRNA stability, rRNA synthesis, and activation of elongation factors in rodent myocytes and hepatocytes (Kimball et al., 1994). Whether or not such actions occur in fish remain to be investigated.

*Development*

Only recently have studies on the patterns of INS and IR expression during fish embryonic development been reported. INS mRNAs are expressed during the ontogeny of trout and zebrafish, and precede the differentiation of β cells and the development of the endocrine pancreas (Caruso et al., 2008; Papasani et al., 2006). These findings are also consistent with reports of INS in developing mammals (Hernandez-Sanchez et al., 2006). In zebrafish, INS gene expression occurred as early as 44 hours postfertilization (hpf ) (Milewski et al., 1998); however, zfINSa and zfINSb mRNA have been detected one hpf, but these are thought to be of material origin (Papasani et al., 2006). zfINSa and zfINSb were identified in the head region of developing zebrafish, along with IGF-2 (Papasani et al., 2006). IGF-1 and IGF-2 are also potent mitogenic regulators in zebrafish embryos inducing cell proliferation and DNA synthesis (Pozios et al., 2001). Insulin mRNAs (INS1 and INS2) in rainbow trout were detected in various body regions (head, body, tail) during embryonic development. Both rtINS 1 and rtINS 2 mRNAs appear early in development

(29 days post-fertilization), but decline at later development (through 90 days post-fertilization) (Caruso et al., 2008).

The role of proinsulins during the development of fish is not clear, but they may serve as selective growth, differentiation, and/or maturation factor similar to IGF-1 and IGF-2, as is the case for mammals (Hernandez-Sanchez et al., 2006). Recently, the IR in zebrafish has been shown to be critical for proper development during embryogenesis. IRa and IRb morphants in developing zebrafish resulted in an altered phenotype and depressed INS signaling (e.g., Akt). Interestingly, each IR morphant demonstrated a modified phenotype: morphant zfIRa illustrated growth retardation and an underdeveloped midbrain and eye; morphant zfIRb displayed pericardial edema and a dysmorphic jaw (Toyoshima et al., 2008). Combination morphants (IRa + IRb) resulted in a summation of both aberrant phenotypes. The role of proinsulin in these or other actions during the development of fish is not clear, but it may serve as a selective growth, differentiation, and/or maturation factor similar to IGF-1 and IGF-2 as is the case for mammals (Hernandez-Sanchez et al., 2006).

*Intermediary Metabolism: Carbohydrates*

Exogenous INS treatment *in vivo* and *in vitro* regulates glucose uptake in a tissue- and species-specific manner (Table 1). Acute INS administration *in vivo* reduces plasma glucose levels in fed and fasted teleost fish species for 6 - 10 h, including rainbow trout (Harmon and Sheridan, 1992; Palmer and Ryman, 1972; Plagnes-Juan et al., 2008; Polakof et al., 2010a; Polakof et al., 2010b) carp, red sea bream, yellow tail (Furuichi and Yone, 1983), sea bass (Perez, 1989), freshwater catfish (*Clarias batrachus*) (Bhatt et al., 1980), and catfish (*Ictalurus melas*) (Ottolenghi et al., 1982). Red and white muscle of rainbow

trout increased the rate of glucose utilization following IP injection of INS (Blasco et al., 1996). Chronic INS treatment via implanted mini osmotic pumps over 4 and 21 days also significantly reduced plasma glucose levels in rainbow trout (Polakof et al., 2010a; Slagter et al., 2005). Despite a constant supply of INS administration, rainbow trout were able to tolerate hypoglycemic levels over a prolonged period of time. In nature, this has been observed during periods of spawning in lamprey and salmon, notably in males (Sower et al., 1985; Plisetskaya et al., 1987). INS administration decreases glucose oxidation in fed fish; however, glucose oxidation in fasted fish occurs only in the presence of alanine (Pereira et al., 1995). Although studies on elasmobranchs have been scarce, species such as the spiny dogfish, ratfish, and little skate also respond to INS injection, displaying decreased plasma glucose (deRoos and deRoos, 1979; Grant et al., 1969; Leibson and Plisetskaya, 1968; Patent, 1967).

Adipocytes isolated from rainbow trout subjected to INS treatment did not display glucose uptake, contrary to what is observed in mammals (Christiansen et al., 1987). However, INS treatment within physiological range was able to regulate the uptake of 2-deoxyglocose (2DG) in trout cardiomyocytes (Gallardo et al., 2001). It is noteworthy that IGF-1 was more potent in stimulating glucose and alanine uptake in muscle cells of rainbow trout than INS (Castillo et al., 2004). Only at increased levels (1000 nM) of INS administration were 2DG and alanine incorporated in to muscle cells above basal levels. However, just as in adipose tissue, skeletal muscle displays an increase abundance of IGF-1 receptors compared to IRs in trout (Parrizas et al., 1995; Mendez et al., 2001). Furthermore, IGF-1 administration caused an increase of [$H^3$] thymidine uptake, and consequently DNA synthesis, whereas INS treatment failed to stimulate [$H^3$] thymidine

uptake in rainbow trout muscle cells despite high concentrations (Castillo et al., 2004). Several other reports have also indicated that IGF-1 administration promotes glucose uptake and synthesis of glycogen in fish skeletal muscle (Degger et al., 2000; Drakenberg et al., 1997). Therefore, INS and IGF-1 may possess distinct and overlapping functions within skeletal muscle of fish.

INS promotes glycogenesis and is antagonistic to glycogenolysis in fish in a manner similar to that observed in mammals (Barthel and Schmoll, 2003). For example, postprandial elevated glucose and successive elevated INS stimulated hepatic and muscle glycogen synthesis in Red-tailed Brycon (Figueiredo-Garutti et al., 2002). Or For example, INS stimulated glycogen synthesis in hepatocytes of American eel (Foster and Moon, 1989). In addition, INS inhibited total glycogen phosphorylase a activity in eel liver concomitant with inhibition of *de novo* synthesis of glucose from alanine and lactate in eel or from alanine and glutamate in rainbow trout (Cowley et al., 1977; De la Higuera and Cardenas, 1986; Foster and Moon, 1990). INS increased the rate of gluconeogenesis from serine and alanine in the sea raven with a subsequent rise in glycogen (Foster and Moon, 1987), which suggested that in order for INS to stimulate liver glycogen content *in vitro*, INS must first stimulate gluconeogenesis from selected amino acids to glucose, then shift to glycogenesis. In addition, INS treatment in isolated hepatocytes of American eel increased lactate and alanine to glycogen but interestingly was dependent on seasonal changes (Foster and Moon, 1989).

In mammals, INS was shown to inhibit glycogen phosphorylase (GP), PEPCK, and G6Pase through the activation of Akt/PKB (Aiston et al., 2006; Liao et al., 1998; Schmoll, 2000). Akt/PKB activation has recently been shown to respond to INS in the liver of

rainbow trout (Bouraoui et al., 2010; Plagnes-Juan et al., 2008). *In vivo* and *in vitro* treatment of rainbow trout with INS decreased mRNA expression of hepatic gluconeogenesis, including glucose-6-phosphotase (G6Pase), fructose 1,6 bisphosphatase (FBPase), and phosphoenolpyruvate carboxykinase (PEPCK) (Plagnes-Juan et al., 2008). In addition, rainbow trout fed a high carbohydrate diet did not increase the expression or activity of G6Pase, FBPase, and PEPCK, suggesting that carbohydrate intake poorly regulates gluconeogenic genes (Panserat et al., 2000; Panserat et al., 2001). Amino acids, on the other hand, regulated gluconeogenic enzymes including, G6Pase, PEPCK, PK, 6-phospho-fructo-1-kinase (PFK-1), and serine dehydratase (Lansard et al., 2010). Hepatic treatment of INS with amino acids activated the TOR signaling pathway (see section 3.6 below for more detail on TOR signaling), which resulted in further activation of glycolytic enzymes, including GK, 6-phospho-fructo-1-kinase (6PF1K), and PK, and inhibition of the gluconeogenic enzyme, G6Pase (Lansard et al., 2010). The role of INS in hepatic glycolysis seems to be species-specific. In American eel hepatocytes, INS enhanced hepatic PFK-1 activity via desensitization to ATP and an increase in concentration of fructose-2,6-diphosphate (Fru-2,6-P2) (Foster et al., 1989). Rainbow trout isolated heptatocytes subjected to elevated levels of INS displayed increased activity of pyruvate kinase (PK) (Mommsen et al., 1991). However, in the sea raven, INS decreased Fru-2,6-P2 and had no effect on PK (Foster and Moon, 1990).

Ligands that promote gluconeogeneis (e.g., catecholamines, glucagon) primarily bind G-protein-coupled receptors (GPCR) that in turn stimulate adenylate cyclase (AC) and result in the activation of glycogenolysis within liver and muscle, as well as lipolysis in adipose. In eel heptocytes, intracellular cAMP was diminished following INS

administration, indicating that INS may inhibit AC or activate phosphodiesterase (PDE) (Foster and Moon, 1990). However, the specific role of cAMP or other signaling elements on mediating the effect of INS on the synthesis of production or activity of gluconeogenic enzymes in fish needs further study.

The glucose-sensing mechanism in mammalian β cells is regulated through glucokinase (GK), which determines the rate of glycolysis and subsequent rise in ATP eliciting INS secretion (Polakof et al., 2007). GK has been detected in several species of fish, including tilapia, sea bream, carp, and rainbow trout; however, reports vary regarding its abundance (Panserat et al., 2000; Plagnes-Juan et al., 2008; Polakof et al., 2007). GK levels in carnivorous fish such as trout, sea bream, and perch were higher compared to carp, an omnivorous fish (Navarro et al., 2002). However, Legate et al., 2001, indicated that GK levels were most prominent in bullhead, an omnivore, while American eel and rainbow trout had reduced GK levels post glucose injection. Varying diets with low and high carbohydrate content both elevated glucose and INS levels in trout, but animals fed the high carbohydrate diet had significantly greater hepatic GK expression and activity than fish fed the low carbohydrate diet (Capilla et al., 2003). Similar results were seen in common carp with respect to GK activity (Capilla et al., 2004). GK expression was increased following a combination of glucose and INS treatment but was not affected by INS treatment alone (Legate et al., 2001). This is in agreement with other studies that have failed to induce GK expression or activation following INS treatment in fasted fish, and suggests that glucose is necessary in order to regulate GK, unlike mammals that rely on INS (Plagnes-Juan et al., 2008).

Because carnivorous fish display glucose intolerance (see section 2.6.1 above), it was predicted that they may lack glucose transporters (GLUT) necessary for cellular glucose uptake. As it turns out, contradicting reports exist regarding GLUT abundance in fish. Although GLUT1 and GLUT4 mRNA and protein were not detected in muscle tissue (white, red, and cardiac) of rainbow trout, American eel, and black bullhead, some form of glucose transporter was detected in white muscle (Legate et al., 2001). Notwithstanding, the failure to detect GLUT1 or GLUT 4 in this study may be due to the use of mammalian probes. However, other studies reported that rainbow trout possess GLUT1 (cardiac muscle), GLUT2 (liver; Brockmann Body; Brain), and GLUT4 (cardiac muscle) (Krasnov et al., 2001; Planas et al., 2000; Teerijoki et al., 2000). In addition, GLUT1, GLUT2, GLUT3, and GLUT4 were detected in Atlantic cod (Hall et al., 2004; Hall et al., 2005; Hall et al., 2006). Moreover, GLUT2 mRNA expression was reported in the liver of sea bass (Terova et al., 2009), and tilapia were found to express GLUT1 in brain, pancreatic islet, and heart tissue but GLUT4 mRNA was not detected within any tissue (Alexander et al., 2006; Hrytsenko et al., 2010).

The abundance of the several GLUTs is variably influenced by INS in fish. GLUT1 was detected in red and white muscle of rainbow trout, and their expression was insensitive to alterations in INS levels (Teerijoki et al., 2000). This is in contrast to mammals, where GLUT1 is responsive to INS (Ducluzeau et al., 2002). The expression of GLUT2 in Brockmann bodies and several regions of the brain in tilapia were regulated by INS and plasma glucose (Polakof et al., 2007). GLUT4 was detected in trout muscle and adipose , and, similar to mammals, INS promotes GLUT4 translocation to the plasma membrane of muscle cells (Diaz et al., 2007 Planas et al., 2000; Rubin and Bogan, 2009). In brown

trout, GLUT4 expression in white muscle was insensitive to INS but was INS sensitive in red muscle (Capilla et al., 2002). GLUT4 has also been identified in salmon and is INS responsive in adipose tissue and isolated adipocytes (Capilla et al., 2004). Developing trout muscle cells *in vitro* displayed increased GLUT4 mRNA expression in response to INS and glucose, whereas, IGF-I increased GLUT1 mRNA expression (Diaz et al., 2007). The expression of GLUT4 in cardiac muscle and of GLUT2 in liver decreased in fasting Atlantic cod, and the expression of both GLUTs increased upon refeeding—a pattern that correlated with plasma glucose levels and suggestive of INS sensitivity (Hall et al., 2006). By contrast, hepatic GLUT4 expression increased during fasting and decreased upon re-feeding of Atlantic cod (Hall et al., 2006). The lack of a GLUT4 response to INS in tilapia skeletal muscle, which makes up nearly 50% of the body mass, may help explain the glucose intolerance observed some species (Hrytsenko et al., 2010). In addition, GLUT2 and GLUT4 display low affinity for glucose in peripheral tissues, which also may help to explain glucose intolerance in carnivorous fish (Capilla et al, 2004). Life history-related difference in the expression and regulation of GLUTs also may help to explain differences in glucose uptake in fish.

*Intermediary Metabolism: Lipids*

Fish utilize lipids as a main energy source to support physiological processes. Unlike mammals, fish store lipids within several tissues including mesenteric adipose tissue, liver, and red muscle (Sheridan, 1988). In general, INS promotes free fatty acid (FFA) and cholesterol uptake, reduces lipolysis, and increases lipogenesis in fish (Table 1) (Perez et al., 1989; Ablett et al., 1981; Ince, 1983). Such effects of INS on lipid metabolism are supported by studies that experimentally induced INS deficiency. For

example, acute INS deficiency induced by anti-INS antiserum resulted in elevated plasma FFA in lamprey and reduced hepatic triacylglycerol lipase in coho salmon (Plisetskaya, 1980). Antilipolytic effects of INS have been demonstrated in rainbow trout *in vivo* and in isolated hepatocytes and adipocytes *in vitro* (Harmon and Sheridan, 1992; Harmon et al., 1993; Albalat et al., 2005). Additionally, INS enhanced lipogensis in skeletal muscle and/or liver of spotted catfish (Machado et al., 1988), lamprey (Kao et al., 1999), toadfish (Tashima and Cahill, 1968), the cyprinid *Notemigonus crysoleucas* (de Vlaming and Pardo, 1975), and rainbow trout (Ablett et al., 1981; Cowley and Sheridan, 1993). The direct influence of INS on lipogenesis was demonstrated in isolated hepatocytes from rainbow trout in which INS stimulated the rate of [$^{14}$C] acetate incorporation into FFA over short- and long-term incubation (Segner et al., 1994). Recent studies in rainbow trout confirm the action of INS *in vivo* on lipogenesis as manifested by reduced plasma FFA concentration and enhanced hepatic lipogenesis (Polakof et al., 2010).

INS regulates several enzymes involved in lipogenesis and lipolysis as well as transcription factors regulating the expression of such enzymes. INS treatment in chinook salmon induced hypolipidemia and decreased hepatic triacylglycerol lipase (TG lipase) activity (Sheridan and Plisetskaya, 1988). TG lipase was also shown to decrease upon INS administration in lamprey larvae and increase the rate of lipogenesis in muscle (Kao et al., 1999). Coho salmon injected with an anti-INS antiserum displayed elevated TG lipase activity in liver (Plisetskaya et al., 1989). Lipogenic enzymes such as acetyl-coenzyme A (acetyl-CoA) carboxylase (ACC) or fatty acid synthase (FAS) were unaffected following IP injection of INS into spotted catfish (Warman and Bottino, 1978). Stimulation of FAS by INS was shown directly in rainbow trout liver *in vitro* (Cowley and Sheridan, 1993). Lipid

storage is influenced by *de novo* lipid synthesis and by deposition from various plasma lipoproteins. The enzyme lipoprotein lipase (LPL) hydrolyzes the lipid components of lipoproteins (e.g., triacylglycerols, phospholipids), thereby allowing the uptake of the lipolytic products into adjacent tissues (Lindberg and Olivecrona, 2002). INS stimulates LPL expression and activity in adipose tissue but not in red muscle of gilthead sea bream (Albalat et al., 2007).

The mRNA expression of enzymes within the liver and skeletal muscle involved in lipogenesis [ATP cirate lyase (ACLY); FAS; and glucose 6-phosphate dehydrogenase (G6PDH)], β oxidation [carnitine palmitoyl transferase 1 (CPT1 A and B); hydroxyacyl-CoA dehydrogenase (HOAD), and insulin-sensitive transcription factors such as sterol regulatory element binding protein 1-like (SREBP1-like), which is known to bind cis-acting DNA lipogenic elements] has been examined in fish. Following INS IP injection, hepatic expression of mRNA encoding FAS, G6PDH, and HOAD diminished, whereas mRNAs encoding CPT1A and SREBP1-like were upregulated (Polakof et al., 2010). Similarly, in muscle tissue, FAS, CPT1A, and HOAD mRNA expression was down regulated and G6PDH was upregulated (Polakof et al., 2010). Rainbow trout implanted with mini-osmotic pumps over a four-day period experienced enhanced lipolysis, displaying increased plasma FFA concentration. Following chronic INS administration, mRNA expression of FAS and SREBP1-like in the liver were downregulated, whereas CPT1B expression was enhanced (Polakof et al., 2010). In muscle tissue, CPT1A, CPT1B, HOAD, and SPEBP1-like mRNAs were downregulated, whereas ACYL and FAS expression were upregulated. Additionally, FAS protein was reduced with acute INS treatment but was unaffected following chronic INS exposure (Polakof et al., 2010). These

results suggest that INS action on mRNA expression of various lipid metabolic enzymes is both time-dependent and tissue-specific. The difference in the action of INS in liver due to the duration of exposure may result from a counterregulatory response against INS-induced hypoglycemia; however, plasma glucose levels were not measured. It also was recently shown that INS treatment combined with amino acids significantly up-regulated lipogenic FAS, ACYL, and SREBP1-like (Polakof et al., 2010). Both the presence of glucose and INS were necessary for increased expression of FAS in the liver of rainbow trout, similar to what is observed in mammals (Lansard et al., 2010). INS injection of rainbow trout also reduced the mRNA expression of carnitine palmitoyltransferase 1 (CPT1), a rate-limiting enzyme of fatty acid β oxidation (Plagnes-Juan et al., 2008).

*Nervous Tissue*

Several fish, from cyclostomes to teleost, have the capacity to withstand supraphysiological doses of INS that result in a hypoglycemic state; yet, they are still able to maintain physical and nuerological function (Mommsen and Plisetskaya, 1991). One possible adaptation to this hypoglycemic state is to draw upon glycogen, which is storage extensively within the brain (Leibson and Plisetskaya, 1968). The fact that INS is produced within the brain (see section 2.3 above) and that INS may cross through the blood brain barrier, which has been demonstrated in mammals (Woods et al., 2003), suggests that fish may be able to self-regulate metabolism in nervous tissue during periods of fasting. In addition, due to the low utilization of glucose in many fish (Moon, 2001), amino acids and/or fatty acids may act as the primary nutrient reserves for other tissues during periods of nutrient deprivation.

The function of brain INS has been studied in mammals and birds but not in fish. In mammals, brain INS has been shown to play a role in memory, cognition, and feeding (Zhao et al., 2004). INS mRNA has also been localized in specific areas of the rabbit brain dealing with olfaction and higher association of the limbic system (Devaskar et al., 1994). In chicken brain cells, INS promoted neuronal growth and protected cells from apoptosis (Morales et al., 1997).

Perhaps the most important function of INS in the brain is to regulate carbohydrate metabolism. INS has been shown to induce glycogen storage in the brain that may be adaptive to meeting the metabolic demands of neuronal cells that are heavily reliant on glucose (Brown, 2004). In fish, large amounts of glycogen are stored within the brain and may be utilized during periods of fasting (Table 1) (Mommsen and Plisetskaya, 1991; Soengas and Aldegunde, 2002). INS *in vitro* treatment in spawning and prespawning lamprey increased brain glycogen synthesis from glucose but not from lactate (Foster et al., 1993). As far as we are aware, the effects of INS on other metabolites in the brain of fish have not been studied.

## Insulin Receptor

The IR executes the biological actions of INS within target cells. The IR is a member of the RTK family that are known to bind growth factors, cytokines, and hormones (DeMeyts, 2004). The IR is a transmembrane glycoprotein that binds INS at its extracellular domain and initiates biological activity at the intracellular domain (Youngren, 2006). IRs have been detected in fish ranging from cyclostomes to elasmobranchs and teleosts (Gutiérrez et al., 1993; Muggeo et al., 1979)

*Biosynthesis*

Although the IR gene and/or cDNA has been characterized from several vertebrates, including a handful of piscine species, most of what is known about IR biosynthesis has been deduced from non-piscine species. Synthesis of IR begins at the level of transcription initiation and post-transcriptional processing of 21-22 exons. Exons 1-12 encode the α subunit and exons 13-21/22 encode the β subunit. The human IR promoter extends over 1800 bp upstream of the start codon, containing numerous CpG rich regions, which provide binding sites for transcription factors (TF) such as Sp1. ApT rich regions (-674/-874 and -1662/-1823) of the IR promoter also provide cis-elements for TF activation (Cameron et al., 1992).

Following translation, the signal sequence (ca. 27-30 amino acids) is recognized by a signal recognition particle in the ER, which is cleaved before the proreceptor is glycosylated, folded, and dimerized under the assistance of chaperones calnexin and calreticulin (Bass et al., 1998). Further processing includes the attachment of high mannose chains to complex chains and amide-linked fatty acids to both the α and β subunits occurs in the Golgi (Bass et al., 1998). The pro-IR is heavily N-glycosylated, especially the α subunit, and forms disulfide linkage between the α and β subunit before undergoing proteolytic cleavage at a tetrabasic site (R-X-R-R), bearing the mature form of IR. The cleavage site is highly conserved among vertebrates, including all known fish IRs. Multiple IR constructs have been identified in mammals as a consequence of alternative processing of a single gene (Belfiore, 2007). In humans, for example, exon 11 is differentially processed; therefore, giving rise to two-IR isoforms, IR-A (without exon 11) and IR-B (with exon 11). IR-A and IR-B differ only in their α subunit, where IR-A lacks

12 amino acid residues from the C-terminal end due to differential splicing of exon 11 (Seino and Bell, 1989).

IR expression is regulated by several factors in fish. Recently, SSs were found to decrease the expression of IR mRNAs in rainbow trout (Caruso and Sheridan, 2010); however, whether this resulted from decreases in transcription or alterations in mRNA stability were not determined. Interestingly, SS also down regulated the expression of type 1 IGF receptor mRNAs in rainbow trout via the ERK and Akt/PKB signaling pathways (Hanson et al., 2010), suggesting the possibility that SSs downregulate IR expression via similar mechanisms. The abundance of IR also appears to be dependent of the native diet of the fish species. For example, carnivorous fish display fewer IRs than herbivorous and omnivorous fish (Planas et al., 2000).

*Structure*

The mature IR molecule is composed of two subunits, designated as α and β that are joined by disulfide bridges. However, the two subunits of the IR are not synthesized as separate polypeptides but rather are generated by specific proteolytic processing of a single larger precursor (Belfiore et al., 2009). Each α/β pair dimerize with another α/β pair creating a symmetric homodimer; however, the four-subunit complex is often referred to as a heterotetramer, $\alpha_2\beta_2$ (De Meyts, 2008). A stable dimer is formed between two αβ-pairs linked via disulfide bonds. Crystal structures of vertebrate IR, ectodomain and/or tyrosine kinase domain, have been reported for human and rodents, which confirm the organization of the receptor (Hubbard et al., 2002; Hu et al., 2003). Interestingly, heterodimers between an IR αβ-pair and an insulin-like growth factor-1 receptor (IGFR1) αβ-pair also exist

(Lawrence et al., 2007). These hybrid receptors are prevalent among most mammalian tissues and preferentially bind IGF-I over INS (Pessin and Frattali, 1993).

Although only a few studies on IR have been conducted in fish, it appears that fish IRs share the basic structural features of other IRs, possessing an extracellular domain with two cysteine-rich regions, a cleavage site that would yield an extracellular α subunit and a membrane-spanning β subunit, and an intracellular domain containing an intact kinase region (Elies et al., 1999). The α subunit ectodomain is composed of four putative regions designated large domain-1 [aka, leucine-rich repeat domain] (L1), CR, large domain-2 (L2), and three-fibronectin type III (FnIII-1, FnIII-2, FnIII-3) domains (Vashisth and Abrams, 2010). The FnIII-2 domain is split by an insert domain (ID) of ~120 amino acid residues that contains the IR proreceptor cleavage and three disulfide bridges that link the α-subunits. The molecular weight (MW) of the α subunit in fish has been indicated to be 120-125 kDa in carp ovaries (Maestro et al., 1997) and 130 kDa in lamprey liver (Leibush and Lappova, 1995), while the MW of the intracellular portion of the β subunit in fish is ~97kDa (Drakenberg et al., 1997). The total MW of α and β subunit from fish heart was determined to be 350 kDa (Gutiérrez et al., 1995). The extracellular component of the β subunit contains FnIII domains that interact with the FnIII domains of the α subunit, which are linked via disulfide bridges and noncovalent interactions (Youngren, 2007). The remainder of the β subunit contains transmembrane region (TM), as well as the intracellular components such as the juxtamembrane (JM), tyrosine kinase (TK), and C-terminal (CT) regions (Fig. 3).

Fish also possess multiple subtypes of IRs encoded by distinct mRNAs derived from multiple genes. For example, two distinct IR-encoding mRNAs have been

characterized in goldfish (Hitchcock et al., 2001), Japanese flounder (Nakao et al., 2002), and zebrafish (Maures et al., 2002). While in salmonids, four distinct IR-encoding mRNAs have been characterized in rainbow trout (Caruso et al., 2010; Chen and Greene, 1999) and coho salmon (Chan et al., 1997). Rainbow trout IR1-4 (Caruso et al., 2010; Greene and Chen, 1999), goldfish IR1 and IR2 (Hitchcock et al., 2001), Japanese flounder IR1 and IR2 (Nakao et al., 2002), turbot IR (Elies et al., 1999), zebrafish (Maures et al., 2002) show complete conservation of the NPXY sequence in the JM domain. This suggests that the JM region of fish IRs may interact with adaptor proteins for signal propagation or may be inactivated through O-glycosylation similar to the JM domain of mammals (Youngren, 2007). The TK domain of mammals contains tyrosine residues necessary for activation of the receptor complex, including specific tyrosine and lysine residues, required for ATP binding (Jensen and De Meyts, 2009); all of which are completely conserved among known IRs of fish.

*Evolution*

IR is a member of a rather heterogeneous family of receptors that includes insulin-related receptor (IRR), relaxin receptor, type 2 IGF receptor (IGFR2), and the type 1 IGF receptor (IGFR1). Although a ligand has not yet been identified for the IRR, knock out of IRR in mice has been shown to be important in male sexual development (Kitamura et al., 2003). Relaxin-like peptides (RLF) bind to a very different receptor than that of RTKs. RLF bind a leucine-rich repeat containing G-protein-coupled receptors (LGFs), which is primarily involved in the development of reproductive organs (Kong et al., 2010; Wilkinson and Bathgate, 2007). IGFR2 also is not a member of the TKR subfamily but rather a member of the mannose-6-phosphate receptor family (El-Shewy and Luttrell,

2009). By contrast, IR and IGFR1 are both members of the TKR subfamily; and while ancient in the animal lineage, they appear to have emerged from a common ancestor as only a single insulin-like receptor has been identified in a number of invertebrates such as *Drosophila sp*. and *Caenorhabditis elegans* (Gronke et al., 2010). Interestingly, IRs display higher conservation throughout evolution compared to INS (Hernandez-Sanchez et al., 2008). It also should be noted that despite the structural dissimilarities between IGFR1 and IGFR2, both have been shown to bind to IGF-1 (El-Shewy and Luttrell, 2009).

Figure 2 depicts the phylogenetic relationships of all known members of the insulin-related receptor family. The analysis reveals two major clades: an IR/IGFR1 clade and a relaxin-related receptor/IGFR2 clade. The divergence of the IGFR2s from relaxin-related receptors and of the IGFR1s from IR appeared to come later during the course of vertebrate evolution. The pattern that emerges from this analysis is consistent with the structural features of the various members of the insulin-related receptor family.

The emergence of variant forms of IR appears to have resulted from several independent duplication events. The IRs of teleosts branch into two major clades, an observation which is consistent with a fish-specific genome duplication event associated with this group (Meyer and Van de Peer, 2005). Although not shown on the tree because complete sequence information was not available for the analysis, the four IR types in salmonids probably arose as a result of a more recent tetraplodization event associated with the evolution of this taxon (Taylor et al., 2003). It is clear from the phylogenetic analysis that additional research is needed to clarify the polygenic origins of IRs in fish and to determine when the various duplication events took place.

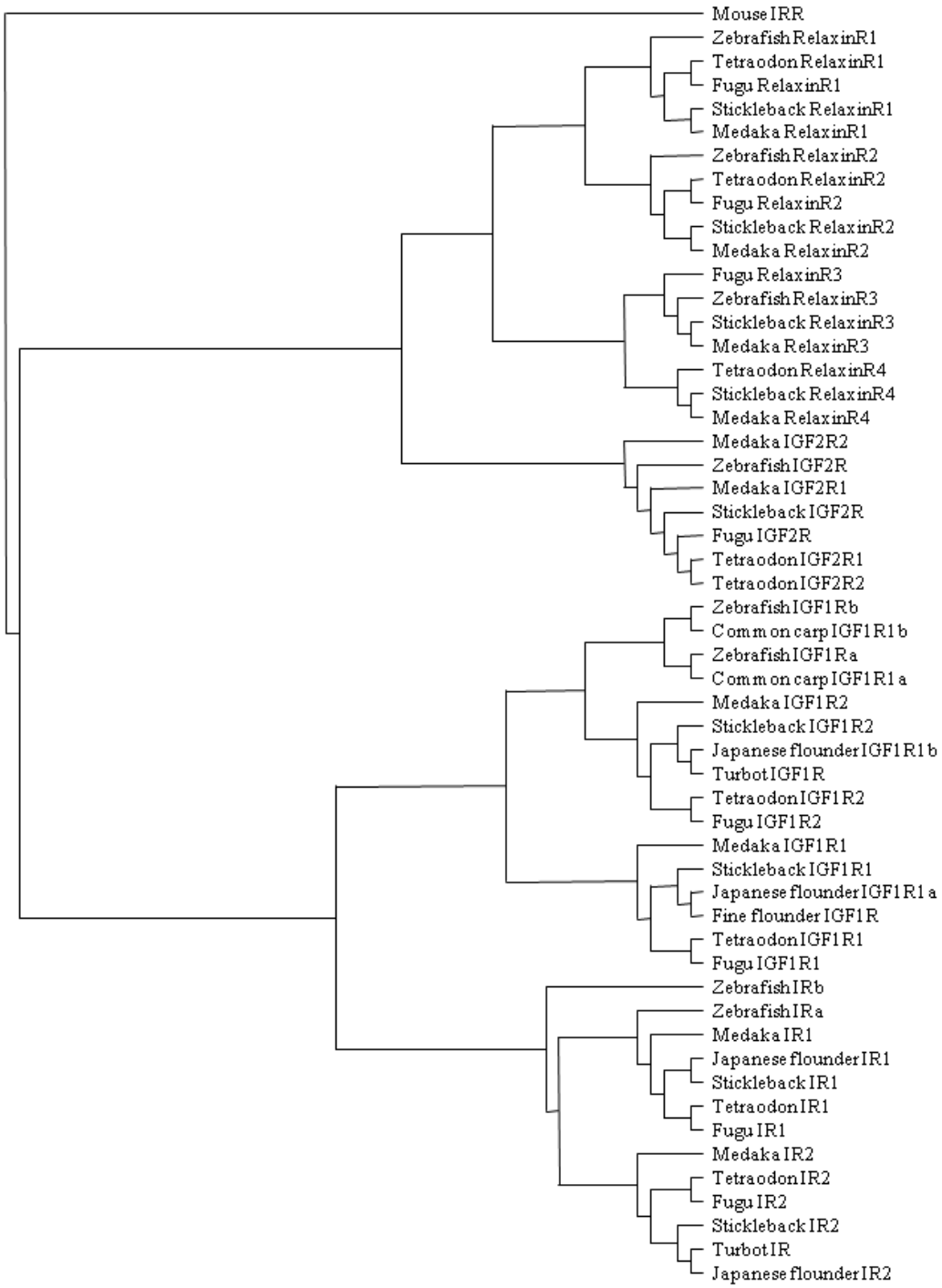


Figure 2. Phylogenetic tree all known members of the insulin receptor family in fish.

The tree was based on alignment of complete amino acid sequences using the N-J bootsrap method in ClustalX 2.0 and rooted using the mouse IRR (BAA77835.1) as an out group; the tree was visualized with TreeView 4.0. The branch lengths represent amino acid substitutions per site from a common ancestor are proportional to the estimated elapsed time since divergence occurred; the bootstrap values were mitted for clarity; however, most values fell within the range of 800-1000, scaled to a maximum of 1000, at each node. Sequences obtained from either GenBank (accession numbers are in parentheses) or e! Ensembl (protein IDs are in parentheses) are as follows: Zebrafish Relaxin R1 (ENSDARP00000111657), Tetraodon Relaxin R1 (ENSTNIP00000016017), Fugu Relaxin R1 (ENSTRUP00000041237), Stickleback RelaxinR1 (ENSGACP00000021895), Medaka Relaxin R1 (ENSORLP00000025093), Zebrafish Relaxin R2 (ENSDARP00000117313), Tetraodon Relaxin R2 (ENSTNIP000000012805), Fugu Relaxin R2 (ENSTRUP0000005617), Stickleback Relaxin R2 (ENSGACP00000027169), Medaka Relaxin R2 (ENSORLP00000004291), Tetraodon Relaxin R4 (ENSTNIP00000012022), Stickleback Relaxin R4 (ENSGACP00000005152), Medaka Relaxin R4 (ENSORLP00000004002); Medaka IGF2R1 (ENSORLP00000025245), Zebrafish IGF2R (ENSDART00000061100), Medaka IGF2R2 (ENSORLP00000025243), Stickleback IGF2R (ENSGACP00000007895), Fugu IGF2R (ENSTRUP00000023173), Tetraodon IGF2R1 (ENSTNIP00000012620), Tetraodon IGF2R2 (ENSTNIP00000012621), Zebrafish IGF1Rb (ENSDARP00000046541), Common Carp IGF1Rb (AAN52152.1), Zebrafish IGF1Ra (ENSDARP00000017066), Common Carp IGF1Ra (AAN52151.1), Medaka IGF1R2 (ENSORLP00000018466), Medaka IGF1R1 (ENSORLP00000010716), Stickleback IGF1R2 (ENSGACP00000019455), Japanese Flounder IGF1Rb

(BAB83670.1), Turbot IGF1R (CAA12278.1), Tetraodon IGF1R2 (ENSTNIP00000012621), Fugu IGF1R1 (ENSTRUP00000012392), Stickleback IGF1R1 (ENSGACP00000017416), Japanese Flounder IGF1Ra (BAB83669.1), Fine Flounder IGF1R (ACJ66864.1), Tetraodon IGF1R1 (ENSTNIP00000014880), Fugu IGF1R2 (ENSTRP00000039547), Zebrafish IRb (EU447178), Zebrafish IRa (NM_001142672), Medaka IR1 (ENSORLP00000022517), Japanese Flounder IR1 (AB065097), Stickleback IR1 (ENSGACP00000022371), Tetraodon IR1 (ENSTNIP00000017949), Fugu IR1 (ENSTRUP00000031110), Medaka IR2 (ENSORLP00000023219), Tetraodon IR2 (ENSTNIP00000002029), Fugu IR2 (ENSTRUP00000018834), Stickleback IR2 (ENSGACP00000013853), Turbot IR (AJ224994), Japanese Flounder IR2 (AB065097).

*Distribution*

In fish as in other vertebrates, IRs are widely distributed in various regions of embryos. In mammals, interestingly, the IR-A isoform predominates over the IR-B isoform in fetal tissue, whereas the reverse is observed in post natal tissues, except within the CNS where IR-A dominates in adults; and an altered IR-A:IR-B ratio is associated with metabolic dysfunction (e.g., peripheral INS resistance, increased risk of type II diabetes) in adults (Belfiore at al., 2009; Kosaki et al., 1998). In fish that display multiple IR subtypes, the relative abundance of IR subtypes differ at a given stage of development and the abundance of a particular IR subtypes may change over the course of development. In rainbow trout embryos for example, four distinct IR subtypes (IR1-IR4) were detected, and of these IR2 and IR3 were more abundant than that IR1 and IR4; a pattern that persisted

only in the brain and pituitary of post-embryonic animals (Caruso et al., 2010; Chen and Greene, 1999; Toyoshima et al., 2008).

IRs also widely distributed in the tissues of juvenile and adult fish, including adipose, brain, cardiac and skeletal muscle, endocrine pancreas, gill, gut, kidney, liver, stomach, intestine, and spleen (Caruso et al., 2010; Chen and Greene, 1999; Leibush et al., 1996; Nakao et al., 2002). In fish with multiple IR subtypes, there is an overlapping but distinct pattern of expression among tissues. In juvenile rainbow trout, for example, IR1 mRNA was most abundant in spleen, liver, kidney, and muscle (white, red and cardiac), but least abundant in adipose (Caruso et al., 2010). IR3 mRNA was most abundant in liver, spleen, kidney, and pancreas; in other tissues, levels of IR3 mRNA were uniformly abundant. By contrast, levels of IR2 and IR4 mRNA were uniformly abundant in most tissues, except in spleen where levels of IR4 were significantly lower (Caruso et al., 2010). In Japanese flounder, IR1 was most abundant in liver, cardiac muscle, gill and ovary; while IR2 was most prominent in liver, kidney, cardiac muscle, gill, and ovary (Nakao et al., 2002).

*Binding*

The binding characteristics of IRs across species of fish are fairly similar in that they (1) exhibit comparable binding affinities, (2) possess both high and low affinity binding sites, and (3) display negative cooperativity. IR binding in fish has been examined in several tissues, including liver, skeletal muscle, cardiac muscle, red muscle, brain, and gonads, as well as in cardiomyocytes, erythrocytes and heptatocytes (Banos et al., 1997; Gutiérrez et al., 1995; Gutiérrez et al., 1993; Gutiérrez and Plisetskaya, 1991; Leibush et

al., 1996; Leibush et al., 1997; Montserrat et al., 2007a, 2007b; Moon et al., 1996; Nowak and Mackowiak, 1993), and the distribution of IRs based on binding studies is in good agreement with that based on molecular analysis (e.g., presence of IR-encoding mRNAs) (Gutiérrez et al., 2006). Some differences in binding features have been observed among tissues. For example, rainbow trout displayed higher INS binding in red muscle and cardiac muscle compared to that in white muscle (Baños et al., 1998; Gutiérrez et al., 1995). Within particular tissues, binding features are relatively similar even across species. For example, affinity values reported in fish muscle tissue range from 0.2-0.5 nM (Kd) (Gutiérrez et al., 2006). It should be noted that mammalian INSs bind with higher affinity to several known piscine IRs than with their native ligands (Mommsen and Plisetskaya, 1991). This may account for some of the discrepancy among studies, some of which used homologous hormone and others of which used heterologous hormone. It also should be noted that studies in fish support an evolutionary trend toward increased IR number, particularly in muscle and liver (Gutiérriz and Plisetskaya, 1991).

IR binding characteristics in fish are regulated by nutritional, hormonal, and environmental factors. The specific binding of INS in skeletal muscle of sea bream decreased during fasting, but recovered to levels comparable to control after a period (1-4 wk) of refeeding (Montserrat et al., 2007). *In vivo* arginine injection induced hyperinsulinemia in both brown trout and common carp, which correspondingly decreased specific binding of INS in red muscle and increased specific binding of INS in skeletal muscle (Banos et al., 1997; Parrizas et al., 1994). Coho Salmon smolts transferred from fresh water to sea water increased INS specific binding in the liver (Gutiérrez and Plisetskaya, 1991). Also, trout fed on high carbohydrate diets induces up-regulation of IRs

in white muscle (Baños et al., 1998; Párrizas et al., 1994). It should be noted that due to the spawning cycle of most teleosts, their nutritional intake and utilization varies by season; therefore, depending on the time of year tissues are collected for binding studies, differences in binding characteristics (i.e., affinity, capacity) may be observed (Mommsen and Plisetskaya, 1991).

*Signal Transduction*

The actions of INS proceed from the binding of the hormone to the receptor and the subsequent activation of several effector pathways. Most of what is known about INS signaling comes from studies in non-piscine species. From these studies, INS binding induces a conformational change in the IR complex, leading to activation of endogenous tyrosine kinase activity and rapid autophosphorylation of several tyrosine residues on the β subunit; such autophosphorylation results in the movement of the activation loop away from the kinase catalytic site, which provides open access to ATP and adaptor proteins in the cytoplasm (Youngren, 2007). Multiple intracellular adaptor proteins including INS receptor substrates (IRS1-4), Shc, Gab1, Cbl, APS, and $P60^{dok}$ are recruited to phosphorylated tyrosine residues and bind via src homology 2 (SH2) domains, altering their activity and thereby initiating numerous downstream cellular responses through several effector pathways, including the PI3K/Akt, ERK, and STAT/Jak-STAT pathways (Youngren, 2007). The PI3K/Akt pathway is essential for the metabolic and mitogenic actions of INS (Cheng et al., 2010). PI3K consists of a p85 regulatory subunit and a p110 catalytic subunit that phosphorylates phosphoinositide (PI), resulting in PI(3)P, $PI(3,4)P_2$, and $PI\ (3,4,5)P_3$. These secondary messengers bind to the pleckstrin homology domain (PH) of PI3K-dependent serine/threonine kinases (PDK1) and Akt, and recruit them to the

plasma membrane. Upon plasma membrane binding, Akt is activated through a conformational change and is phosphorylated by PDK1. Akt [also known as protein kinase B (PKB)] directly phosphorylates components of the GLUT-4 complex, protein kinase C (PKC), and glycogen synthesis kinase 3 (GK3); all necessary constituents of INS-mediated metabolic effects.

INS, along with other hormone mitogens, stimulate protein synthesis in mammals via the mammalian target of rapamycin (mTOR) signaling, a serine/threonine protein kinase related to PI3K that subsequently phosphorylates a number of downstream proteins, including Akt/PKB (Cheng et al., 2010). mTOR regulates somatic growth, cell survival, protein accretion, catabolic inhibition, and overall transcription. There are two major complexes formed that constitute mTOR signaling, mTORC1 and mTORC2. Both mTORC1 and mTORC2 associate with mLST8/GβL and deptor, the latter being the inhibitor of the complexes (Foster and Fingar, 2010). mTORC1 binds raptor and PRAS40 exclusively, where raptor is essential to promote mTORC1 signaling. mTORC2 exclusively binds rictor, mSin1, and PRR5/protor, where rictor and mSin1 promotes mTORC2 signaling. Raptor and mTORC1 also associate with ribosomal S6 protein kinase 1 (S6K1) and eukaryotic initiation factor (eIF) 4E-binding protein 1 (4EBP1). S6K1 is an activator of cell growth and proliferation, whereas 4EBP1 inhibits these processes. Upon mTORC1 phosphorylation of S6K1 and 4EBP1, they are activated and inhibited, respectively. In addition to mTORC1 responding to growth factors, the serine/theonine kinase also senses and is regulated by nutritional factors, specifically branched-chain amino acids (Kim, 2009). mTORC2 on the other hand is primarily regulated by growth factors. mTORC2 directly phosphorylates downstream Akt/PKB, SGK1, and PKCα, which affect

cytoskeleton organization, cell proliferation, and cell survival (Zhou and Huang, 2010). Other molecules activated in mTOR signaling include eukaryotic initiation factors (e.g., eIF4F; p70-S6 Kinase 1) that result in the overall promotion of mRNA translation (Zhou and Huang, 2010). It is not surprising that disregulation of mTOR signaling may lead to pathologies such as cancer (Wu and Hu, 2010). Negative regulation of mTOR signaling includes stress hormones (e.g., cortisol) and lack of nutrients. Activation of S6K1 also acts as a negative feedback mediator directly inhibiting the activation of mTORC2 rictor and IRS-1 (Foster and Fingar, 2010).

Despite that IR and IGFR share substantial structural similarity as well as the ability to bind to each other's cognate ligands and to form hybrid receptors, differences in the biological response transduced through each has begun to emerge. Interestingly, hybrid receptors may compromise 40-90% of total IGF-I binding sites (Soos et al., 1993) and IR and IGFR are both ubiquitously expressed on most cells and activate similar signaling pathways, such as PI3K/Akt (Genua et al., 2009). Because of the evolutionary relatedness of INS and IGF-I, they do share common functions; however, the metabolic role of INS is distinctive and cannot be compensated through IGF alone in mammals (Adamo et al., 1992). Mutations to the IR or cellular components of the INS signaling pathways decrease INS responsiveness and lead to alterations in IR synthesis and degradation, which are associated with pathologies such as diabetes mellitus (Makino et al., 1992). However, the distinct function of these related hormones in fish has just begun to emerge and it appears that INS and IGF possess overlapping metabolic functions (Planas et al., 2000).

Only recently has IR signaling been investigated in piscine species, and what we know comes from a variety of different sources. Based upon available sequence

information of fish IRs, the β-subunits contain several conserved structural motifs, including a transmembrane domain (TM), juxtamembrane domain (JM), tyrosine kinase domain (TK), and a carboxyl-terminus domain (CT) (Caruso et al., 2010; Chen and Greene, 1999; Elies et al., 1999; Hitchcock et al., 2001; Maures et al., 2002; Nakao et al., 2002;). Amidst the JM domain of fish, several important signaling molecules, including IRS-1 and Shc, are known to bind to activated phospho-tyrosine residues via their Sh2 domains and elicit mitogenic and metabolic cell signaling pathways in mammals (Wood et al., 2005). The amino acid sequence, NPXY, within the JM is characteristic and appears essential for functional association between IR and these adaptor proteins. Rainbow trout IRs (Caruso et al., 2010) along with all other fish IR subtypes with characterized JM domains [e.g., goldfish IR1 and IR2 (Hitchcock et al., 2001); Japanese flounder IR1 and IR2 (Nakao et al., 2002); rainbow trout IR 1, IR3 and IR4 (Chen and Greene, 1999); turbot IR (Elies et al., 1999); zebrafish (Maures et al., 2002)] show complete conservation of the NPXY sequence. This suggests that the JM of fish IRs may interact with adapter proteins for signal propagation or be inactivated through O-glycosylation similar to the JM domain of mammals (Youngren, 2007). The TK domain of mammals contains tyrosine residues necessary for activation of the receptor complex, including $Tyr^{1158}$, $Tyr^{1162}$and $Tyr^{1163}$, and a vital lysine residue, $Lys^{1030}$, required for ATP binding (De Meyts and Jensen, 2009); all of which are completely conserved among known the TK domain of fish IRs (Caruso et al., 2010; Chen and Greene, 1999; Elies et al., 1999; Hitchcock et al., 2001; Maures et al., 2002; Nakao et al., 2002;). Although the functional significance of the multiple IR subtypes of fish is not clear, there are several lines of evidence to suggest that they may have distinct roles, including variation among the CT domains of subtypes with regard to

position and extent of serine, threonine, tyrosine residues, and differential expression of subtypes during embryonic development and among tissues of juveniles/adults (Caruso et al., 2010, Nakao et al., 2002; Toyoshima et al., 2008). It also should be noted that the observed pattern of IR subtype expression suggests that independent mechanisms serve to regulate IR biosynthesis in a subtype- and tissue-specific manner.

Analysis of (m)TOR signaling was corroborated in rainbow trout skeletal muscle through the activation of TOR and subsequent phosphorylated downstream effector proteins (Seiliez et al., 2008). Specifically, feeding enhanced phosphorylation of Akt/PKB, p70 S6 kinase, and eIF4E-binding-protein-1 *in vivo* as INS did *in vitro*. Most interestingly, in contrast to mammals, S6K1 and 4EBP1 were long-term stimulated (5 - 8 h) after feeding, most likely due to a delayed rise in plasma amino acid and INS concentration (Seilez et al., 2008). INS also stimulated activation of downstream transcription factors FOXO1 and FOXO4, known to be activated through PI3K and PKB promoting protein accretion (Cleveland and Weber, 2010). INS may have a permissive effect in conjunction with amino acids and only synergistically elicit TOR signaling (Plagnes-Juan et al., 2008; Kim, 2009).

INS and IGF-1 signaling studies conducted in preadipocytes and adipocytes of rainbow trout revealed particular roles for each ligand. INS and IGF-I were found to independently regulate phosphorylation of Akt/PKB and MAPK; however, IGF-I was more potent than INS in stimulating 2-deoxyglucose (2DG) uptake (Bouraoui et al., 2010). It should also be noted that IGF-1 receptors display greater specific binding compared to IR due to increased number and affinity in numerous fish tissues analyzed to date, including adipocytes (Navarro et al., 1999). IGF-1 significantly increased MAPK phosphorylation in

preadipocytes but not differentiated adipocytes. By contrast, IGF-1 increased phosphorylation of Akt in adipocytes but not in preadipocytes. INS had little to no effect on MAPK-P or Akt-P in preadipocytes but did display a slight decrease in MAPK-P and increase in Akt-P in differentiated adipocytes. The significant increase in 2DG uptake via IGF-I administration suggests that IGF-I plays a significant metabolic role in nutrient uptake within fish adipocytes (Bouraoui et al., 2010).

Rainbow trout skeletal muscle cells treated with INS and IGF-1 displayed regulation of mitogenic and metabolic signaling transduction pathways including MAPK/ERK, Akt/PKB, and PI3K (Castillo et al., 2006). Other reports have indicated the activation of Akt/PKB following INS treatment, such as in zebrafish embryonic cells (Pozios et al., 2001). INS IP injection in conjunction with amino acids also stimulated the activation of Akt/PKB in liver of rainbow trout (Plagnes et al., 2008). Therefore, the activation of Akt/PKB by INS may be dose- and tissue-specific.

IR signaling also is critical during embryogenesis. Transfected hepatocytes overexpressing zfIR isoforms, subjected to INS administration, directly activated (i.e., phosphorylation) MAPK and Akt in a dose-dependent manner (Toyoshima et al., 2008). A model that emerges for INS signaling in fish is depicted in Fig. 3. In this model, INS binding to the IR activates the phosphorylation of various Tyr residues on the β-subunits; such phosphorylation could result in the recruitment and binding of various adapter proteins and the subsequent propagation of signal through various effector pathways, including the PI3K/Akt, ERK, and mTOR pathways. Due to the paucity of literature pertaining to adapter proteins in fish, we conducted searches of the Ensembl and GenBank databases. Zebrafish possess predicted sequence for multiple insulin receptor substrates,

denoted as IRS-1 [ENSDARP00000117614], IRS-2 [ENSDARP00000053923], and IRS-4 [ENSDARP00000022483], Tetraodon sp. and stickleback also exhibit an annotated IRS-4 [ENSTNIP00000004014; ENSGACP00000024720, respectively]. Identified IRSs contain a highly conserved IRS phosphotyrosine binding site (PTB domain) and a high degree of sequence similarity among vertebrates (Jarymowycz and Stone, 2008). Zebrafish and tetraodon also contain sequence similarity identified as Shc2 and Shc1, respectively. Shc2 and Shc1 contain conserved regions of sequence denoting a PTB domain and SH2 domain similar to other vertebrates (van der Geer et al., 1999). Zebrafish [ENSDARP00000122829], Tetraodon [ENSTNIP00000006587], Atlantic salmon [NP_001158778], stickleback [ENSGACP00000009150], medaka [ENSORLP00000019097], and fugu [ENSTRUP00000020659] possess sequence encoding the growth factor receptor-bound protein 2 (Grb), which all contain two conserved SH3 (proline-rich ligand binding site) domains and a single SH2 domain, in correlation with other vertebrates (Giorgettiperaldi et al., 1995). Our model is supported by the presence of various adapter proteins (e.g., IRS, Shc, Grb) that possess regions predicted to interact with IR or with elements of effector pathways (e.g., P13K, Akt/PKB, SOS-Ras-Raf-MEK-ERK) and by studies that demonstrated the activation of ERK, Akt/PKB, and mTOR by INS. Continued work on identifying the signaling and their interactions is needed to provide a complete understanding of INS signaling in fish.

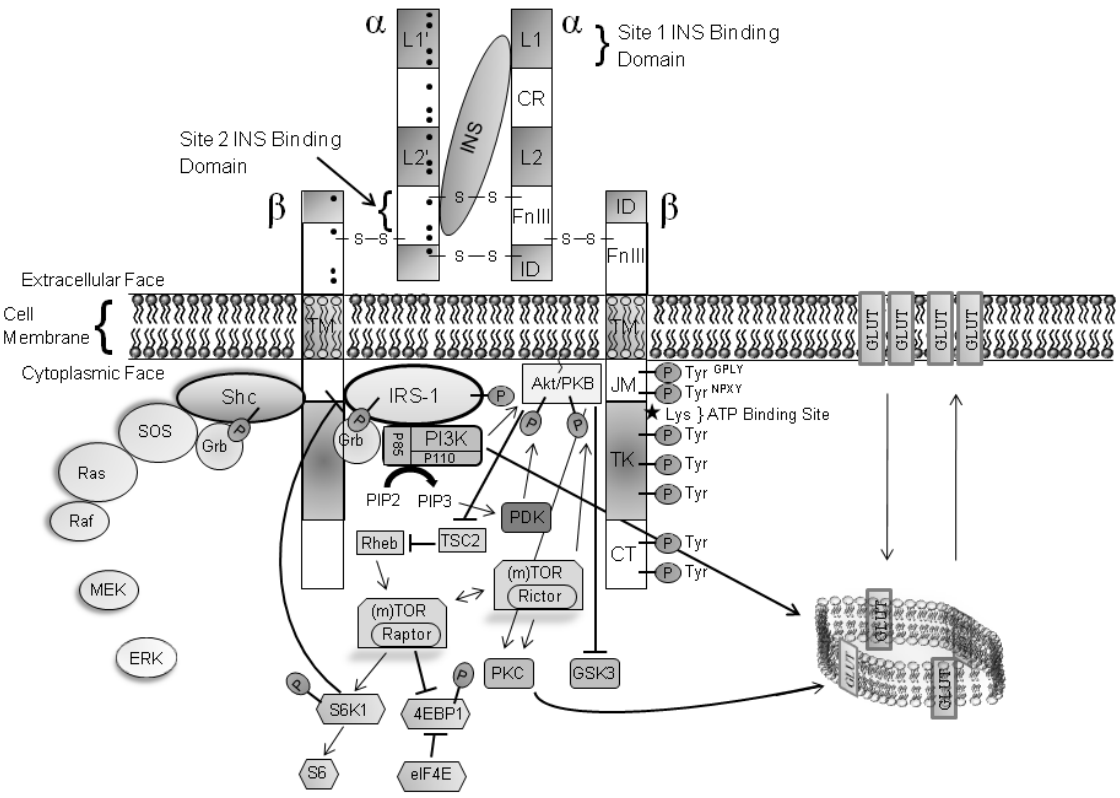


Figure 3. Model of insulin signaling in fish. The model illustrates a dimerized insulin receptor ($\alpha_2\beta_2$) with insulin bound between the binding domains of the two α subunits as well as the various molecular associated with post receptor binding and insulin action in fish. Dots on the on the α subunit as well as on the extracellular domain of the β subunit represent potential N-glycosylation sites (shown only on one α/β pair). Abbreviations: 4EBP-1, eIF4E-binding protein-1; Akt/PKB, protein kinase B; CR, cysteine rich domain; CT, C-terminal domain; eIF4E, eukaryotic translation initiation factor 4E; ERK, extracellular signal-regulated kinase; FnIII, fibronectin domain; GLUT, glucose transporter; Grb, growth factor receptor-bound protein 2; GSK3, glycogen synthase kinase

3; ID, insert domain; IRS-1, insulin receptor substrate-1; JM, juxtamembrane domain; L1, large/leucine-rich repeat domain-1; L2, large/leucine-rich repeat domain-2; MEK, mitogen-activated protein kinase kinase; P, inorganic phosphate group; p85 and p110, regulatory subunits of PI3K; PDK, phosphoinositide-dependent kinase; PI3K, phosphoinositide 3-kinase; PIP2, phosphotidylinositol-3', 4-bisphosphate; PIP3, phosphotidylinositol-3', 4, 5-triphosphate; PKC, protein kinase C; Raf, rapidly accelerated fibrosarcoma; Ras, RAt Sarcoma; Rheb, Ras homolog-enriched in brain; S6K1, ribosomal protein S6 kinase-1; Shc , Src homology 2 domain-containing; SOS, Son of Sevenless; TK, tyrosine kinase domain; TM, transmembrane domain; TOR target of rapamycin; TSC, tuberous sclerosis complex.

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## OBJECTIVES

Insulin regulates a wide array of processes in fish, including appetite regulation, growth, development, and intermediary metabolism. While most INS is produced by the endocrine pancreas of fish, it also is produced in the brain as well as in various regions of the gut and adipose tissue. Localized INS production within these tissues raises the intriguing possibility of distinct functional roles. Insight into how the INS gene is regulated in these extra-pancreatic tissues will broaden our general tissue-specific understanding of transcriptional activation. The overall objective of this dissertation is to examine the regulation of INS- and IR-encoding mRNAs in rainbow trout. The specific objectives of this dissertation are as follows: (1) identify and characterize INS and IR mRNA(s), (2) determine the patterns of INS and IR mRNA expression, (3) evaluate the regulation of INS and IR mRNAs by nutritional state, (4) evaluate the regulation of INS and IR mRNAs by growth hormone and insulin-like growth factor-1, and (5) evaluate the regulation of INS and IR mRNAs by somatostatin. While this work pertains specifically to fish, it will have a broad relevance to understanding INS and IR expression patterns in vertebrates, and provide insight into the evolution of INS and IR. Due to the debilitating disease, diabetes mellitus, in humans, there has been a considerable amount of research focused on the regulation of INS and IR in both mammals and fish; however, little has focused on the regulation of mRNA expression. Given that carnivorous fish, such as rainbow trout, are glucose intolerant, they provide a good model and bring insight for a non-insulin dependent diabetes mellitus (NIDDM) phenotype. The findings may affect applications such as improving growth and feed efficiency in farm-raised animals, notably fish, and provide insight into the treatment of diabetes mellitus in mammals via xenographs from Brockmann bodies or adipocytes.

# CHAPTER ONE. RAINBOW TROUT (*Oncorhynchus mykiss*) POSSESS TWO INSULIN-ENCODING mRNAS THAT ARE DIFFERENTIALLY EXPRESSED

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## Abstract

Insulin (INS) plays a critical role in the growth, development, and metabolism of vertebrates. In this study, two unique cDNAs that encode preproinsulin were isolated, cloned and sequenced from the endocrine pancreas (Brockman body) of rainbow trout. One 592-bp cDNA (INS 1) encodes a 105-amino acid protein and the other 587-bp cDNA (INS 2) encodes a 107-amino acid protein. The sequences share 93% nucleotide identity and 91.4% deduced amino acid identity. Quantitative real-time PCR revealed that the two INS-encoding mRNAs were differentially expressed, both in terms of distribution among tissues as well as in terms of abundance within selected tissues of juvenile trout. Both INS 1 and INS 2 mRNAs were detected in pancreas, adipose tissue, pyloric cecum, and brain; however, only INS 1 mRNA was detected in upper and lower intestine and pituitary. In all cases where INS 1 and INS 2 were co-expressed, INS 1 was more abundant. INS 1 and INS 2 also were differentially expressed in various body regions (head, body, tail) during embryonic development. Both INS 1 and INS 2 mRNAs were detected early in development (29 days post-fertilization), but their expression declined as development proceeded (through 90 days post-fertilization); in most cases, unlike the situation in

juveniles, INS 2 mRNA was more abundant than INS 1 mRNA in embryos. These findings contribute to our understanding of the evolution, distribution, and function INS.

## Introduction

Insulin (INS) is one of the most studied regulatory peptides in vertebrates and plays key roles in the regulation of their growth, development, and metabolism (McMurtry, 1993; Plisetskaya and Duguay, 1993; Hernandez-Sanchez et al., 2006). In mammals, INS is produced in the β-cells of the endocrine pancreas; however, INS production also has been observed in neuronal and other extrapancreatic tissues (Smit et al., 1998; Hernandez-Sanchez et al., 2006). In teleost fish, INS is found in large principal islets or Brockmann bodies, which are collections of endocrine cells largely distinct from exocrine tissue (Youson and Al-Mahrouki, 1999); the extrapancreatic production of INS in fish is still a matter of contention (Plisetskaya et al., 1993).

Insulin is synthesized from a large-chain precursor, preproinsulin. Typically, preproinsulin contains a ca. 24-amino acid signal sequence, followed by a B-chain (30aa), a C-peptide of variable length (~28-42), and an A-chain (21aa); however, variations in the lengths of the A- and B-chains have been observed (Dodson and Steiner, 1998; Conlon, 2000, 2001). Because proinsulin is packaged in secretory granules along with its endopeptidases, INS as well as C-peptide and proinsulin to some extent are co-released and enter the circulation (Dodson and Steiner, 1998). Distinct physiological actions of proinsulin or of C-peptide have been difficult to determine, but recent research suggests a role for proinsulin during vertebrate development (Plisetskaya, 1998; Hernandez-Sanchez et al., 2006)

Insulin is a member of a family of peptides that includes INS, insulin-like growth factor (IGF) I and II, insulin-like peptides (ILPs), and relaxin (Chan and Steiner, 2000; Wilkinson et al. 2005). Phylogentic analysis indicates that there was an ancestral insulin-like gene that gave rise to invertebrate ILPs and, ultimately, to the ILP/relaxin and INS/IGF subfamilies in vertebrates through a series of independent gene duplication events (Smit et al., 1998; Chan and Steiner, 2000; Olinski et al., 2006). Separate INS and IGF genes appear to have arisen after the emergence of vertebrates, and distinct IGF I and IGF II genes emerged with the advent of gnathostomes (McRory and Sherwood, 1997; Chan and Steiner, 2000). Chan and Steiner (2000) proposed that such duplication events enabled the evolution of new functions, suggesting that the mitogenic role of invertebrate ILPs was subsumed by IGFs and that INS acquired the primary function of a metabolic hormone. Interestingly, multiple INSs have been described in mammals, amphibians, and teleost fish. Such structural heterogeneity results from the differential processing of a single INS gene or from the existence of multiple INS genes. For example, polymorphisms of a single insulin gene or differential posttranscriptional modifications have been proposed to underlie bovine, porcine, and flounder INS variants (D'Agostino et al., 1987; Snel and Damgaard, 1988; Andoh and Nagasawa, 1998). By contrast, two separate non-allelic INS genes have been described in rats, mice, *Xenopus*, catfish, chum salmon, puffer fish, and zebrafish (Lomedico et al., 1979; Bunzli et al., 1972; Shuldiner et al., 1991; Kavson et al., 1993; Mommsen et al., 2002; Irwin, 2004; Dr. Geoffrey Waldbieser, personal communication). No information has been reported about differences in the roles of the INS variants.

The aim of this study was to further characterize the polygenic origins of INS and to determine the patterns of variant INS expression in order to provide additional insight into the evolution and function of INS. Our specific hypothesis was that rainbow trout possess two preproINS-encoding mRNAs and that these mRNAs are differentially expressed.

## Materials and methods

*Experimental Animals*

For most experiments, juvenile rainbow trout (*Oncorhynchus mykiss;* approximately 12-18 months of age) of both sexes and obtained from Dakota Trout Ranch near Carrington, ND, USA were used. Following transport to North Dakota State University, the fish were maintained in well-aerated, 800-L circular tanks supplied with recirculated (10% replacement volume per day) dechlorinated municipal water at 14$^{o}$C under a 12 light:12 darkness photoperiod. Fish were fed twice daily with AquaMax™ Grower (PWI Nutrition International. Brentwood, MO, USA). Animals were acclimated to laboratory conditions for at least 6 weeks prior to experiments.

Rainbow trout embryos at several stages of development also were used in this study. Fertilized rainbow trout eggs from 3-year old fall spawning broodstock were obtained from, and raised at the Alma Aquaculture Research Station (Alma, ON, Canada). Embryos were reared in a Heath incubator until a week after hatch and then transferred to raceways. Both the Heath incubator and raceways were supplied with constantly running, aerated well-water at an average temperature of 8.5°C throughout the experimental period. The embryos were collected at specific ages based upon staging determined in previous studies (Raine et al., 2004): eyed-egg (29 days post-fertilization, dpf; 246.5 degree-days),

post-hatch (53 dpf; 450.5 degree-days), swim-up (68 dpf; 578 degree-days), and complete yolk-absorbed (90 dpf; 765 degree days), where one degree-day is the average daily water temperature x the number of days reared at his temperature. Food was first introduced at the swim-up stage, and embryos were fed with Silver Cup starter diet (Nelson and Sons, Murray, Utah), at a ration of 5.0% of biomass per day (to excess).

All experimental protocols were reviewed and approved by the North Dakota State University Animal Care and Use Committee or by the University of Guelph Animal Care Committee, as applicable.

*RNA Extraction*

Juvenile rainbow trout were anaesthetized by immersion in 0.05% (v/v) 2-phenoxyethanol. Selected tissues (e.g., pancreas, brain, etc.) dissected from juveniles were placed into 2-mL microcentrifuge tubes, immediately frozen on dry ice, and stored at –90$^{o}$C for later analysis. Rainbow trout embryos were first dissected free of the chorion (29 dpf) and/or yolk (29, 53 and 68 dpf) and immediately divided in to body segments, placed into 2 mL microcentrifuge tubes and flash frozen in liquid nitrogen, prior to storage at -70ºC.

Total RNA was extracted using TRI-Reagent® as specified by the manufacturer (Molecular Research Center, Cincinnati, OH, USA). Isolated RNA was dissolved in 50-100 µL Rnase-free deionized water. Total RNA was quantified by ultraviolet ($A_{260}$) spectrophotometry and diluted to 100 ng/µL in RNase-free deionized water. RNA samples were then stored at –90$^{o}$C until further analysis.

*Oligonucleotide Primers And Probes*

Gene-specific primers used for isolation of cDNAs were designed by examining homologous regions of known non-mammalian INS sequences using GeneTool software (BioTools, Edmonton, AB, Canada) and custom synthesized by Sigma-Genosys (The Woodlands, TX, USA). Additional primers for reverse transcription were provided in the iScript™ cDNA Synthesis kit (BioRad, Knightdale, North Carolina, USA) or in the SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA). Gene- specific oligonucleotide primers and probes used for real-time PCR were designed using ABI Primer Express® Version 2 software based upon our determined sequences for INS 1 and INS 2 (accession numbers EF416285 and EF416286, respectively) and on the previously published sequence for β-actin (accession number AF157514) and custom synthesized by Applied Biosystems. The probes were minor-groove binding probes labeled with either FAM (INS 1 and INS 2 probes) or VIC (β-actin probe). Primers and probes were used for reverse transcription and PCR without further purification.

*Isolation And Sequence Analysis Of Insulin-Encoding cDNAs*

A two-phase approach was adopted for the isolation of selected cDNAs using RT-PCR and rapid amplification of cDNA ends (RACE)-PCR. Isolation of the 3' cDNA sequence was accomplished using the SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA) following the manufacturer's protocol. Briefly, RNA was reverse transcribed into RACE-ready cDNA containing the sequence tags necessary for SMART™ chemistry, and 3'-RACE was carried out using a degenerate INS primer (INS: 5'CAGCACCTGTGTGGMTCTCA 3'). After an initial denaturation at

94°C for 5 min, a 35-cycle PCR was preformed with each cycle consisting of denaturation (94°C for 1 min), annealing (65°C for 1 min), and extension (72°C for 1 min) phases. In the last cycle, the extension time was increased to 10 min. The PCR product was identified by electrophoresis on an agarose gel containing 1% of each OmniPur (EMD chemicals, Gibbstown, NF, USA) and NuSieve GTG agarose (Bio-Wittaker Molecular Applications, Rockland, ME, USA) in 1 x Tris-borate-EDTA (TBE) buffer followed by ethidium bromide staining. The resulting PCR products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). Purified plasmids (75 fmol) were sequenced using the CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's protocol.

After determining that portions of two distinct INS cDNAs (denoted INS 1 and INS 2) of high homology had been isolated by the initial PCR, 5'-RACE was preformed for each form using a SMART™ RACE cDNA Amplification Kit under manufacturer's suggested conditions with INS gene-specfic primers (INS 1: 5' CACCAGATAGAGGGCGTCCA 3'; INS2: 5' CGTTCTCCTGGGACGATTTTG 3'). The resulting PCR products were visualized, cloned into the pGEM-T Easy Vector, and sequenced as described previously.

*Preparation Of cDNA Standards*

Approximately 1 μg of each of the 3'-RACE-ready INS 1 and INS 2 cDNA products was used as template for PCR with forward (INS 1: 5'-AGCTTCTTCACCACCACAAACTC; INS 2; 5'-AACCAACTTTTCCTTCAGCATTTC) and reverse (INS 1 5'-CTGGGGAAAAATAAAACACGTCTAG; INS 2 5'-

GGAAAAATAAAACATGTCTATGCCA) gene-specific primers under the same conditions as described above. The resulting PCR products were visualized, cloned into the pGEM-T Easy Vector, and their sequences verified as described previously.

*Real-Time Reverse Transcription PCR*

From 200 ng total RNA, endogenous poly(A)$^+$ RNA was reverse transcribed in a 10 μL reaction using a TaqMan Reverse Transcription Reagent Kit containing MultiScribe Reverse Transcriptase and oligo d(T)$_{17}$ as a primer (Applied Biosystems) according to the manufacturer's instructions. Reactions without reverse transcriptase were included as negative controls; no amplification was detected in negative controls.

mRNA levels of the two INS-encoding forms were determined by real-time RT-PCR using iQ™Supermix chemistry and a STRATAGENE MX3000P® Detection System (Stratagene). Real-time PCR reactions were carried out for samples, standards, and no-template controls in a 10 μL reaction, containing 1 μL (20 ng) cDNA, 1μL each forward primer [INS 1: 5'- ACTGCCACGCTCAAATTCTCA 3' (600 nM); INS 2: 5'-TCCCCTAACAGGGTTT CTCTTTC (600 nM); β-actin: 5'-GGCTTCTCTCTCCACCTTCCA 3' (900 nM)], reverse primer [INS 1: 5'-TTTGCACTCCATGTTTGCAAA (600 nM); INS 2: 5'-GAACGGGTACTCGGCAACTTC (600 nM); β-actin 5'-AGGGACCAGACTGTCGTAACTC 3' (900 nM)] and probe [INS 1: 5'- FAM-GCATCCTCCTTC 3' (150 nM); INS 2: 5' FAM-TCGTCCCAGGAG 3' (150 nM); β-actin 5'-VIC-TGCTTGCTGATCCACAT 3' (150 nM)] at optimized concentrations for the mRNA species to be measured, and 5 μL TaqMan Universal PCR Master Mix. Cycling

parameters for real-time PCR were as follows: 95°C for 10 min, and 45 cycles for 92°C for 15 s plus 60°C for 1 min. PCR efficiency for the INS 1 assay ranged from 94% to 103% and that for the INS 2 assay ranged from 98% to 101%. To verify the specificity of the assays, cross hybridization was assessed by substituting alternate primer/probe sets in Taqman reactions for each standard. No amplification was observed with mismatched primer/probe sets.

*Data Analysis*

Copy number calculations were based on threshold cycle number ($C_T$). The $C_T$ for each sample was determined by the MX3000P™ real time analysis detection software after manually setting the threshold. Sample copy number was determined by relating $C_T$ to a gene-specific standard curve, followed by normalization to β-actin. No difference ($P>0.05$) was observed in β-actin expression among the various treatment groups. No-template control samples reached a maximum ΔRn of 0.03 at 40 cycles. Therefore, copy numbers of mRNA were considered non-significant if $C_T$ exceeded 44 cycles; this value corresponds to a detection limit of less than 100 mRNA copies. Intra-assay variation was 0.20% and 0.25% for INS 1 and INS 2, respectively; inter-assay variation was 1.08% and 0.94% for INS 1 and INS 2, respectively.

*Statistical Analyses*

Nucleotide and deduced amino acid sequences were aligned and analyzed with GeneTool and PepTool sequence analysis programs, respectively (BigTools Inc., Edmonton, Alberta, Canada). ClustalX (default parameters, except pairwise aligment was set at 30 for gap opening and 13 for gap extension) was used in conjunction with the

neighbor-joining method (Saitou and Nei, 1987) to generate the phylogenetic tree; the tree was visualized with TreeView (Page, 1996). Quantitative data are expressed as means ± S.E.M. Statistical differences were estimated by ANOVA followed by Duncan's multiple range test; a probability level of 0.05 was used to indicate significance. All statistics were preformed using SigmaStat (SPSS, Chicago, IL, USA).

## Results

### *Characterization Of Two Insulin cDNAs*

Two distinct cDNA fragments, each approximately 500 bp in size, were amplified by 3'-RACE PCR from total RNA isolated from the pancreas of rainbow trout using a degenerate INS primer. Sequence analysis of these fragments revealed a portion of the INS coding region, and suggested the successful isolation of two INS-encoding cDNAs, denoted INS 1 and INS 2. Further investigation using 5'-RACE PCR with gene-specific primers revealed products of approximately 280 bp and 340 bp, respectively, for INS 1 and INS 2. Assembly of the cDNA 3'-RACE and 5'-RACE fragments resulted in a 592-bp INS 1 cDNA and a 587-bp INS 2 cDNA, which have an overall nucleotide identity of 93% (Fig. 4). The INS 1 sequence contains an initiation site 88 bases from the most 5' end, a 315-bp coding region, and a 187-bp 3' untranslated region (UTR). The INS 2 sequence contains an initiation site 84 bases from the most 5' end, a 321-bp coding region, and a 182-bp 3' UTR.

The INS 1 and INS 2 cDNAs encode 103- and 105- amino acid putative proteins, respectively. The INS 1 protein contains a putative signal peptide of 22 amino acids, a 28-amino acid B-chain, a 30-amino acid C-peptide, and a 21-amino acid A-chain. The INS 2

protein contains a putative signal peptide of 22 amino acids, a 28-amino acid B-chain, a 32-amino acid C-peptide, and a 21-amino acid A-chain. The rainbow trout INSs share 91.4% amino acid identity.

We took advantage of two regions of the nucleotide sequence within the C-peptide domains of INS 1 and INS 2 to design primers and probes that could distinguish between the two transcripts by real-time RT-PCR. Real-time RT-PCR of pancreatic total RNA showed significant amplification of INSs above no-template controls. Standard curves showed a linear relationship between log of the RNA copy number and $C_T$, with correlation coefficients of 0.99 for both INS forms.

*Differential Expression Of INS mRNAs Among And Within Tissues*

RNA from various tissues of juvenile trout was extracted and reverse transcribed. The resulting cDNAs encoding INS 1 and INS 2 were quantified by real-time RT-PCR, which enabled an assessment of the distribution of INS mRNAs among tissues (Fig. 5). Insulin 1 and INS 2 mRNAs were found in pancreas, adipose, pyloric cecum, and whole brain. By contrast, INS 1 mRNA but not INS 2 mRNA was detected in upper intestine, lower intestine, and pituitary (Fig. 5A). Neither INS 1 or INS 2 mRNA was detected in heart, kidney, liver, muscle, spleen, or gill. Overall, the highest levels of INS mRNA expression were in the pancreas, followed by adipose tissue.

The abundance of the two insulin mRNAs within tissues also was determined by real-time RT-PCR. Under normal physiological conditions, levels of INS 1 mRNA were greater than those of INS 2 mRNA for all tissues. Within the brain, where INS 1 mRNA predominated, INS 1 mRNA was detected in each of the major brain regions, including

telencephalon, optic tectum, cerebellum, and hypothalamus, but was in highest abundance in the cerebellum (Fig. 5B); levels of INS 2 mRNA were below the limit of detection within each of the specific regions of the brain.

*Patterns Of INS mRNA Expression During Embryonic Development*

Quantitation of INS mRNAs by real-time RT-PCR also allowed for comparison of the two insulin mRNAs within body segments of rainbow trout during embryonic development (Fig. 6). In general, INS mRNA levels varied based upon stage of development (days post fertilization, dpf) and body segment. In head and tail, INS mRNA levels were high initially (29 dpf), then declined and remained low from 53 dpf to 90 dpf. Levels of INS 2 mRNA were higher than those of INS 1 at 29 dpf in both head and tail; similarly, INS 2 mRNA levels were higher than INS 1 mRNA levels at 90 dpf in tail. In body, which was not distinct until 53 dpf and included tissue that would give rise to the endocrine pancreas, INS mRNA levels remained relatively high from 53 dpf to 90 dpf; INS 2 mRNA levels were consistently higher than those of INS 1 throughout all observed stages of body development.

## Discussion

In this study, we isolated, cloned, and sequenced two cDNAs that encode for distinct preproinsulins from the endocrine pancreas (Brockmann body) of rainbow trout, designated INS 1 and INS 2. We also demonstrated that the two insulin-encoding mRNAs are differentially expressed, both in terms of distribution among tissues as well as in terms of abundance within tissues. These findings confirm our starting hypothesis and provide new insight into the evolution and function of insulin.

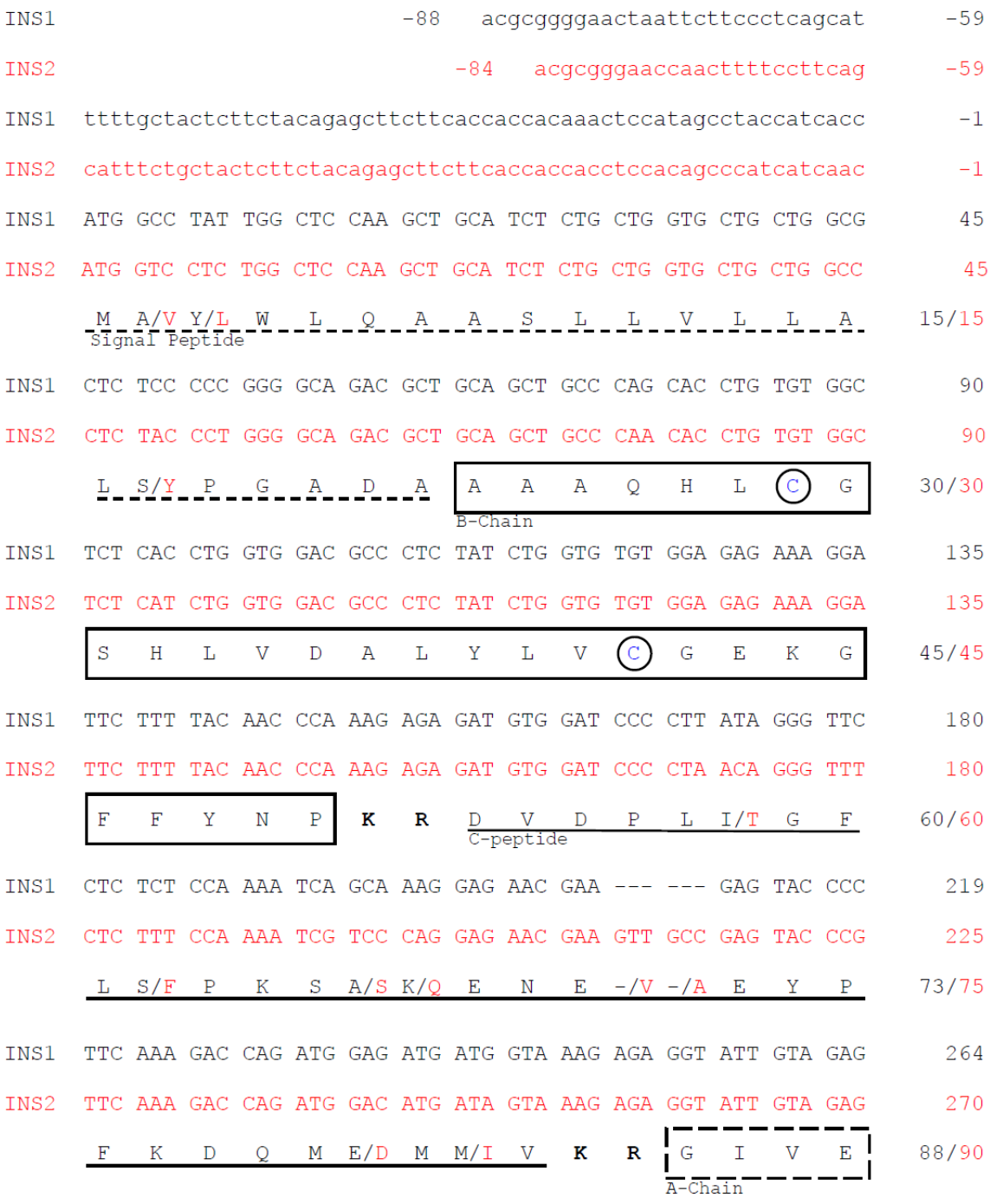
INS1 -88 acgcggggaactaattcttccctcagcat -59
INS2 -84 acgcgggaaccaacttttccttcag -59
INS1 ttttgctactcttctacagagcttcttcaccaccacaaactccatagcctaccatcacc -1
INS2 catttctgctactcttctacagagcttcttcaccaccacctccacagcccatcatcaac -1
INS1 ATG GCC TAT TGG CTC CAA GCT GCA TCT CTG CTG GTG CTG CTG GCG 45
INS2 ATG GTC CTC TGG CTC CAA GCT GCA TCT CTG CTG GTG CTG CTG GCC 45
M A/V Y/L W L Q A A S L L V L L A 15/15
Signal Peptide
INS1 CTC TCC CCC GGG GCA GAC GCT GCA GCT GCC CAG CAC CTG TGT GGC 90
INS2 CTC TAC CCT GGG GCA GAC GCT GCA GCT GCC CAA CAC CTG TGT GGC 90
L S/Y P G A D A A A A Q H L C G 30/30
B-Chain
INS1 TCT CAC CTG GTG GAC GCC CTC TAT CTG GTG TGT GGA GAG AAA GGA 135
INS2 TCT CAT CTG GTG GAC GCC CTC TAT CTG GTG TGT GGA GAG AAA GGA 135
S H L V D A L Y L V C G E K G 45/45
INS1 TTC TTT TAC AAC CCA AAG AGA GAT GTG GAT CCC CTT ATA GGG TTC 180
INS2 TTC TTT TAC AAC CCA AAG AGA GAT GTG GAT CCC CTA ACA GGG TTT 180
F F Y N P K R D V D P L I/T G F 60/60
C-peptide
INS1 CTC TCT CCA AAA TCA GCA AAG GAG AAC GAA --- --- GAG TAC CCC 219
INS2 CTC TTT CCA AAA TCG TCC CAG GAG AAC GAA GTT GCC GAG TAC CCG 225
L S/F P K S A/S K/Q E N E -/V -/A E Y P 73/75
INS1 TTC AAA GAC CAG ATG GAG ATG ATG GTA AAG AGA GGT ATT GTA GAG 264
INS2 TTC AAA GAC CAG ATG GAC ATG ATA GTA AAG AGA GGT ATT GTA GAG 270
F K D Q M E/D M M/I V K R G I V E 88/90
A-Chain


Figure 4. Comparison of the cDNA and deduced amino acid sequences of rainbow trout insulins (INS1, INS2). Gaps inserted by GeneTool v. 1.0 for maximum alignment are indicated by dashes. The signal peptide is underlined by dashes. The B-chain is denoted by the solid box. The C-peptide is underlined. The A-chain is denoted by the dashed box. Conserved cysteine residues are denoted by a ○ around the amino acid. The stop sequence is denoted by *.

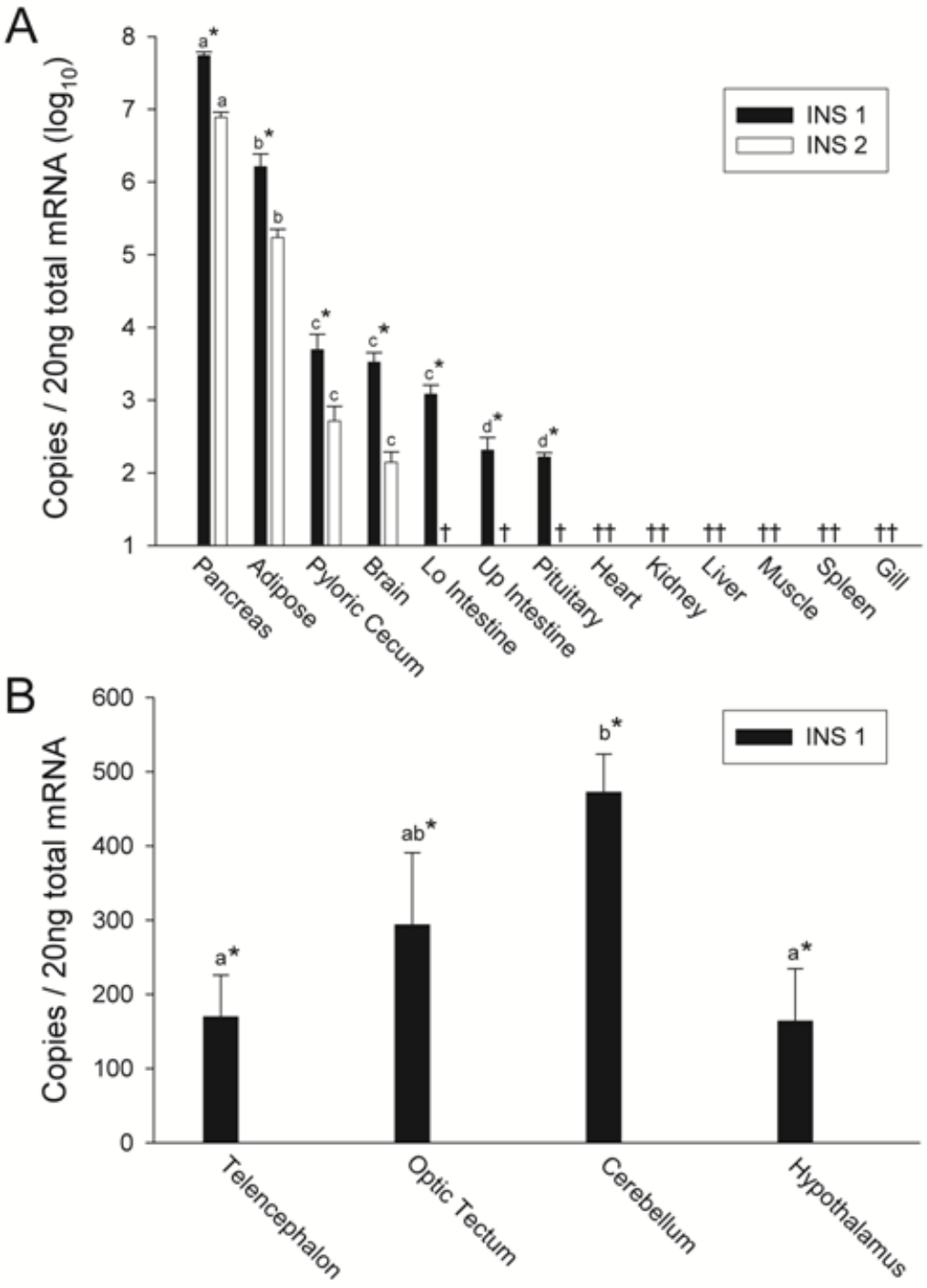


Figure 5. Abundance of insulin 1 (INS1) and insulin 2 (INS2) mRNAs in selected tissues (A) and brain regions (B) of rainbow trout. mRNAs were quantified by real-time RT-PCR;

the limit of detection was 100 mRNA copies. Data are presented as mean ± SEM (n=7). For a given insulin mRNA type, groups with different letters are significantly ($P<0.05$) different; * indicates a significant ($P<0.05$) difference between insulin mRNA types within the same tissue; † indicates not detected.

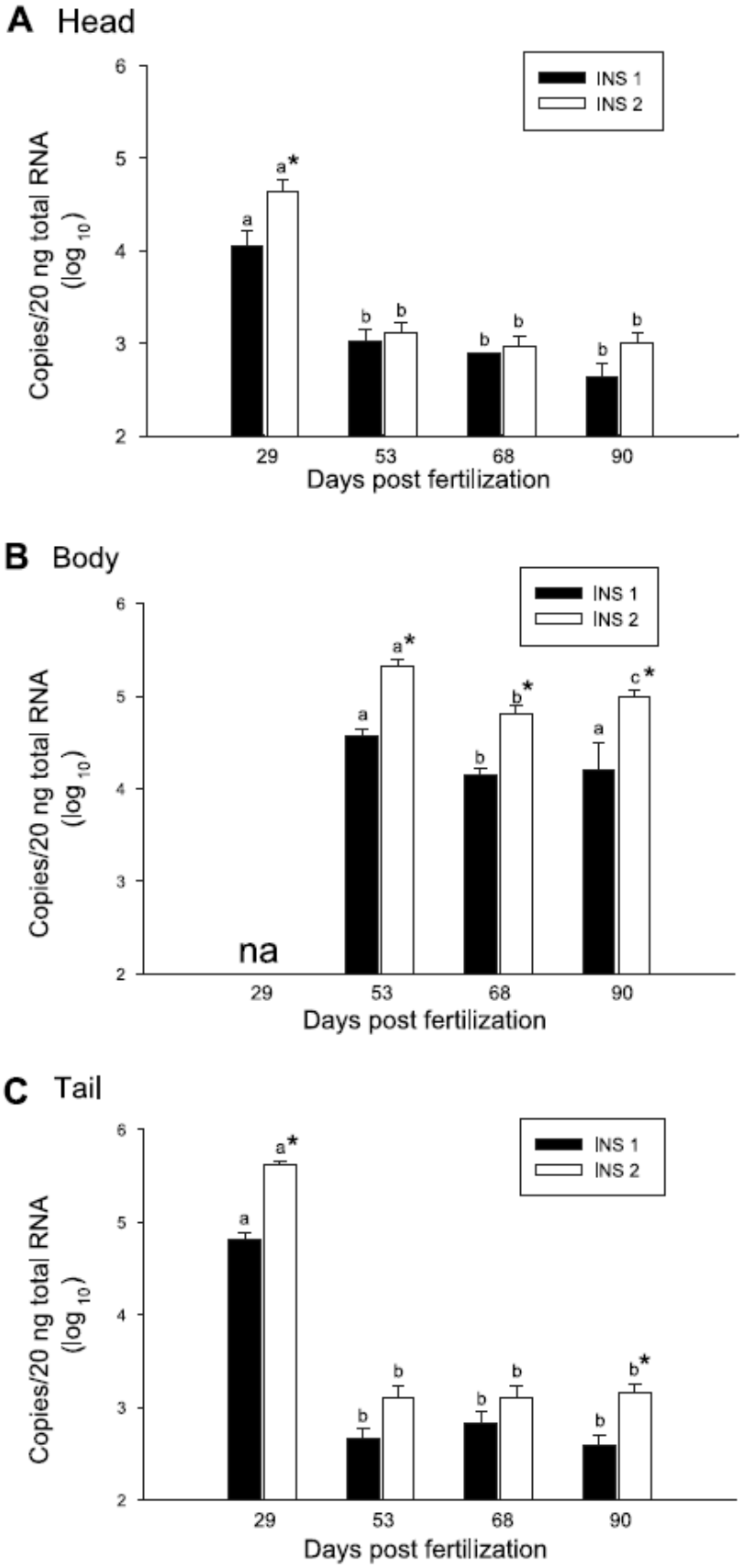
A Head
INS 1
INS 2
Copies/20 ng total RNA (log 10)
Days post fertilization
29
53
68
90
B Body
na
C Tail

Figure 6. Expression of insulin 1 (INS1) and insulin 2 (INS2) mRNAs in the head (A), body (B), and tail regions of rainbow trout during embryonic development. Samples were taken at early-hatch (29 days post fertilization, dpf), mid-development (53 dpf), yolk sac-absorbed (68 dpf), and late-development (90 dpf); because there was no distinct body region at 29 dpf, it was not analyzed (na) at this stage of development. mRNAs were quantified by real-time RT-PCR; the limit of detection was 100 mRNA copies. Data are presented as mean ± SEM (n=4-8). For a given insulin type, groups with different letters are significantly ($P<0.05$) different; * indicates a significant ($P<0.05$) difference between insulin types at a given stage of development.

The two rainbow trout INS-encoding cDNAs shared 93% nucleotide identity; INS 1 would encode a 105-amino acid preproinsulin and INS 2 would encode a 107-amino acid precursor. Comparison of INS cDNAs revealed a high level of conservation among salmonids (Table 2).

Rainbow trout INS 1 shared an approximately 98% nucleotide identity with chum salmon INS 1; and rainbow trout INS 2 shared an approximately 91% nucleotide identity with chum salmon INS 2. Identities of approximately 91-97% were observed when comparing type 1 INSs with type 2 INSs (e.g., trout INS 1 and chum salmon INS 2). Interestingly, rainbow trout INS cDNAs were only 66-83% identical at the nucleotide level and 51-78% at the amino acid level compared to non-salmonid fish species. Nucleotide identities with non-piscine vertebrates were only slightly lower at 63-69%, and amino acid identity was 47-69% between trout and non-piscine INSs (Table 2). Most of the variability between INS 1/INS 2 was within the C-peptide region.

The deduced peptides of trout contained numerous features conserved among vertebrate INSs examined to date. In the A/B-chains, these characteristics included conserved cysteines necessary for disulfide linkage and bioactivity of the molecule. Of the 6 conserved cysteines, 4 are involved in inter-disulfide linkages between the B-chain and A-chain, and 2 are involved in an intra-disulfide linkage within the A-chain. In trout INS 1/INS 2, the inter-disulfide bonds are probably formed between a Cys at position $B_7$ and $CysA_7$, and between $CysB_{19}$ with Cys $A_{20}$; while the intra-disulfide bond is probably formed between $A_6$ and $A_{11}$. In addition, rainbow trout INS 1 and INS 2 possess all 10 of the amino acids that have been conserved in vertebrates INS ($GlyA_1$, $IleA_2$, $ValA_3$, $TyrA_{19}$, $LeuB_6$, $GlyB_8$, $LeuB_{11}$, $ValB_{12}$, $GlyB_{23}$ and $PheB_{24}$). Using alanine-scanning mutageneis, residues $IleA_2$, $ValA_3$, $TyrA_{19}$, $GlyB_{23}$, and $Phe_{24}$ have been found to interact directly with the INS receptor (Kristensen et al., 1997). Five other residues ($LeuB_6$, $GlyB_8$, $LeuB_{11}$, $GluB_{13}$ and $PheB_{25}$) have been found to maintain the receptor-binding conformation of INS (Conlon, 2001), and rainbow trout INSs possess four of these ($LeuB_6$, $GlyB_8$, $LeuB_{11}$, and $PheB_{25}$) as well as a conserved substitution at $B_{13}$(Glu → Asp). Rainbow trout INS 1 and INS 2 also contain a $LeuA_{13}$ and $LeuB_{17}$ that may form part of the second receptor-binding site (Conlon, 2001). The 2 sets of basic amino acid residues (Lysine, Arginine) that separate the A/B-chain from the C-peptide are necessary for proteolytic cleavage and also are highly conserved throughout vertebrates (Conlon, 2001). In trout INS 1/INS 2, proteolytic cleavages sites are located at positions $K_{29}$-$R_{30}$ and $K_{83}$-$R_{85}$/$K_{85}$-$R_{86}$, respectively. The resulting C-peptides of trout INS 1 and INS 2 differed in amino acid length and sequence, differing in 8 out of 32 positions.

Despite the high degree of similarity between the two rainbow trout INS-encoding mRNAs, the position and extent of the differences suggest the existence of two non-allelic INS genes. There have several reports of multiple INSs in various groups of vertebrates, including mammals, amphibians, and teleost fish. In some cases, such as in cow, pig, and flounder, the structural heterogeneity arises from differential processing of a single amino acid precursor (D'Agostino et al., 1987; Snel and Damgaard, 1988; Andoh and Nagasawa, 1998). In other instances, such as in catfish, chum salmon, puffer fish, zebrafish, *Xenopus*, mice, and rats, the heterogeneity arises form the existence of two distinct INS-encoding genes (Lomedico et al., 1979; Bunzli et al., 1972; Shuldiner et al., 1991; Kavson et al., 1993; Mommsen et al., 2002; Irwin, 2004; Dr. Geoffrey Waldbieser, personal communication). The current findings extend our understanding of the polygenic origins of insulin.

The phylogenetic relationships of cloned trout INSs compared with other vertebrate INSs are shown in Fig. 7. The branching of the tree is consistent with the phylogeny of vertebrates, with teleost fish forming a clade distinct from that of the tetrapods. The emergence of variant forms of INS appears to have resulted from several independent duplication events. Whether or not there was one or more such duplication events in the teleost fish lineage is not clear. Irwin (2004) notes that the presence of two INS genes in zebrafish and fugu suggests the duplication occurred prior to the divergence of these groups, and possibly prior to the radiation of all bony fish. On the other hand, multiple INS have not been characterized in all teleosts, and the similarities among the salmonid INSs suggests a more recent duplication, possibly associated with the tetraplodization of this taxon (Meyer, 2005). Additional research is needed to determine if multiple INS genes are

more wide spread among teleosts and whether or not the polygenic origins of INSs can be traced back to the duplication event associated with the emergence of this group ca. 350 million years ago.

The distribution of INS mRNA expression among tissues is wide spread and helps to resolve numerous questions about insulin biosynthesis. The abundance of INS mRNAs in the pancreas is in agreement with data for other vertebrates, including fish (Duguay and Mommsen, 1994; Chan and Steiner, 2000). To the present, it was unclear whether or not INS peptide found in the brain of fish was synthesized there or transported via blood/cerebrospinal fluid (Plisetskaya et al., 1993). The current findings suggest that INS is biosynthesized in various regions of the brain of fish, in a manner similar to that noted previously for humans, rabbits, rats, and chickens (Devaskar et al, 1994; Morales et al, 1997; Rosenzweig et al, 1997). Whether or not the source of INS mRNAs in the pituitary of trout is epithelial cells or remnants of neurosecretory cells remains to be determined. The finding of INS-encoding mRNAs in the gut including pyloric cecae appears to be unique; however, the possibility of the distribution of endocrine islets throughout the gut region during teleost development has been described (Youson, 2006) and immunoreactive INS has been detected in the gut of fish and mammals (Larsson, 1977; El-Salhy, 1984). The occurrence of INS mRNAs in adipose also is unusual. Recently, carp adipocytes were found to secrete immunoreactive insulin *in vitro*; two distinct INS-encoding mRNAs were isolated from these cells and there expression was increased by glucose (Roy et al., 2003).

The current results also indicate that INS mRNAs are expressed during the ontogeny of trout and precede the differentiation of beta cells and the development of the endocrine pancreas.

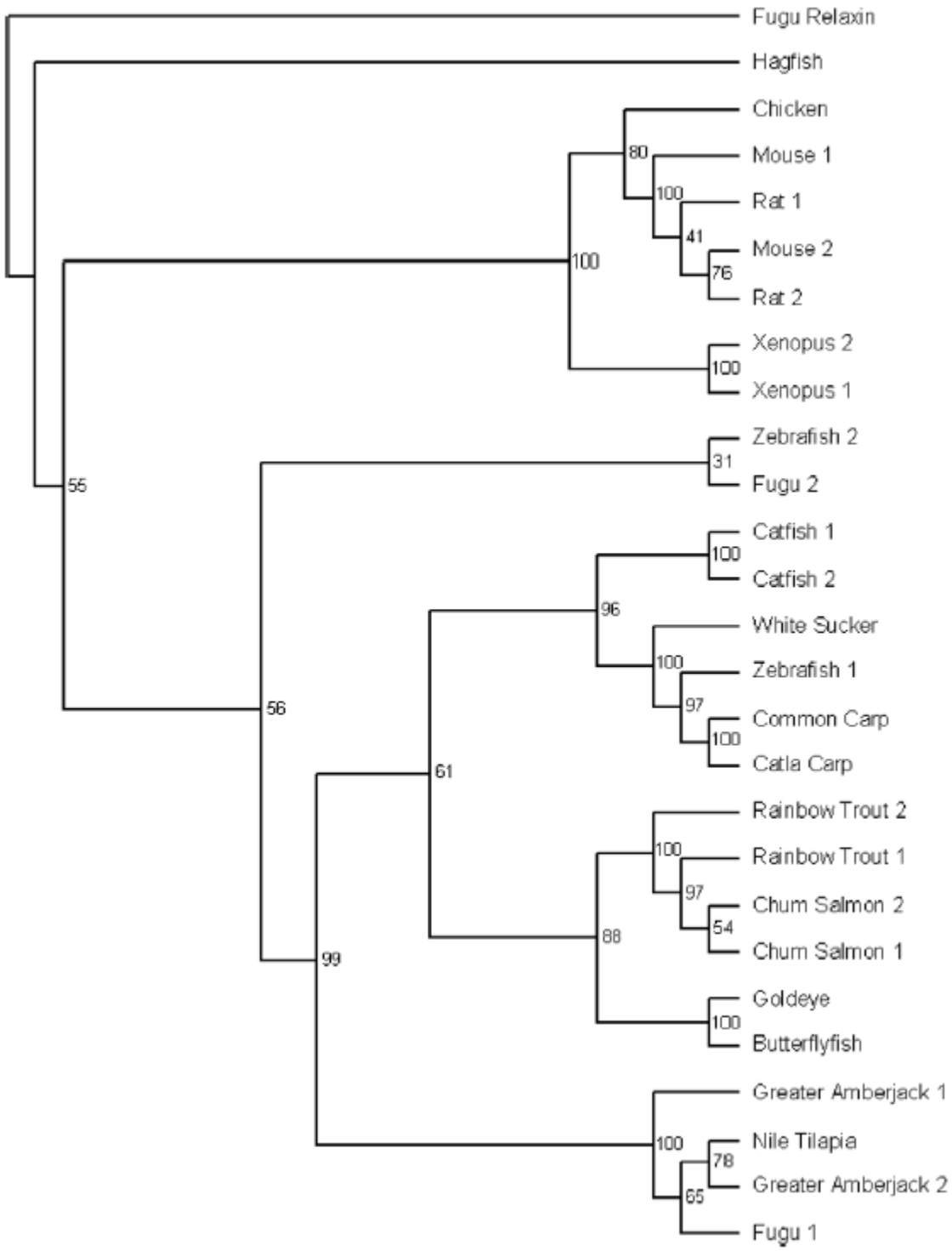
Fugu Relaxin
Hagfish
Chicken
Mouse 1
Rat 1
Mouse 2
Rat 2
Xenopus 2
Xenopus 1
Zebrafish 2
Fugu 2
Catfish 1
Catfish 2
White Sucker
Zebrafish 1
Common Carp
Catla Carp
Rainbow Trout 2
Rainbow Trout 1
Chum Salmon 2
Chum Salmon 1
Goldeye
Butterflyfish
Greater Amberjack 1
Nile Tilapia
Greater Amberjack 2
Fugu 1
80
100
41
76
100
100
31
55
100
96
100
97
100
56
61
100
97
54
88
99
100
100
78
65

Figure 7. Phylogenetic tree of preproinsulins of fish and of selected other vertebrates. The tree was based upon the alignment cDNA sequences of the insulin coding region and was derived using the N-J bootstrap method in Clustal X; the tree was visualized using TreeView. All known species with multiple full-length cDNA sequences encoding preproinsulin were included. Branch lengths represent amino acid substitutions per site from a common ancestor; the numbers at each node represent the bootstrap values. Sequences were obtained from GenBank (accession numbers are in parentheses) as follows: rainbow trout (this study; EF416285, EF416285), chum salmon 1 (X00148), chum salmon 2 (X13559), butterflyfish (AF199588), goldeye (AF282408), nile tilapia (AF038123), catla carp (AF373021), hagfish (V00649), common carp (X00989), white sucker (AF199585), chicken (NM_008387), rat 1 (NM_019129), rat 2 (NM_019130), mouse 1 (NM_008386), mouse 2 (NM_008387), Xenopus 1 (M24443), Xenopus 2 (M24442), fugu 1 (Irwin,2004), fugu 2 (Irwin, 2004), zebrafish1 (AF036326), zebrafish 2 (DQ156497), greater amberjack 1 (AB262770), greater amberjack 2 (AB262771), and fugu relaxin 3 (DQ462200); the full open reading frames for both catfish insulin genes was obtained from Dr. Geoffrey C, Waldbieser (USDA-ARS-Catfish Genetics Research Unit, Stoneville, MS).

Table 2. Amino Acid and cDNA nucleotide identities among insulin of teleost fish and other representative vertebrates. Percentage identities were calculated on sequences aligned using GeneTool v. 1.0. Sequences were obtained from this study and from GenBank (accession numbers are in parentheses) as follows: rainbow trout (this study; EF416285, EF416285), chum salmon 1 (X00148), chum salmon 2 (X13559), butterflyfish

Table 2 (continued).

(AF199588), goldeye (AF282408), barfin flounder (AB029318), anglerfish (V00634), nile tilapia (AF038123), catla carp (AF373021), human (AY890083), hagfish (V00649), common carp (X00989), white sucker (AF199585), chicken (NM_008387), rat 1 (NM_019129), rat 2 (NM_019130), mouse 1 (NM_008386), mouse 2 (NM_008387), Xenopus 1 (M24443), Xenopus 2 (M24442), fugu 1 (Irwin 2004), fugu 2 (Irwin 2004), zebrafish 1 (AF036326), zebrafish 2 (DQ156497), greater amberjack 1 (AB262770), and greater amberjack 2 (AB262771).

| Organism | Insulin 1 | | Insulin 2 | |
|---|---|---|---|---|
| | Amino Acid identity (%) | Nucleotide identity (%) | Amino Acid identity (%) | Nucleotide identity (%) |
| Rainbow Trout 1 | -- | -- | 91.4 | 93.0 |
| Rainbow Trout 2 | 91.4 | 93.0 | -- | -- |
| Chum Salmon 1 | 96.2 | 97.8 | 88.6 | 90.6 |
| Chum Salmon 2 | 94.3 | 96.5 | 87.6 | 90.9 |
| Greater Amberjack 2 | 82.9 | 79.4 | 80.4 | 77.0 |
| Anglerfish | 81.9 | 79.1 | 78.5 | 76.3 |
| Goldeye | 81.0 | 81.0 | 78.5 | 78.5 |
| Butterflyfish | 78.1 | 82.9 | 74.8 | 80.7 |
| Nile Tilapia | 78.1 | 77.8 | 73.8 | 75.1 |
| Fugu 1 | 75.2 | 81.0 | 72.0 | 77.9 |
| Catla Carp | 74.3 | 75.9 | 72.9 | 74.8 |
| White Sucker | 74.3 | 74.9 | 72.0 | 74.1 |

Table 2 (continued).

| | | | | |
|---|---|---|---|---|
| Common Carp | 73.3 | 74.9 | 72.0 | 73.5 |
| Zebrafish 1 | 71.4 | 75.2 | 70.1 | 74.1 |
| Chicken | 59.1 | 69.2 | 56.1 | 67.3 |
| Greater Amberjack 1 | 58.1 | 71.1 | 55.1 | 68.5 |
| Fugu 2 | 58.1 | 70.5 | 54.2 | 67.6 |
| Xenopus 2 | 53.3 | 66.0 | 51.9 | 66.4 |
| Zebrafish 2 | 52.4 | 70.2 | 51.4 | 66.4 |
| Xenopus 1 | 52.4 | 65.1 | 50.9 | 65.1 |
| Hagfish | 50.5 | 65.1 | 48.6 | 62.9 |
| Rat 2 | 50.5 | 67.3 | 48.6 | 66.7 |
| Rat 1 | 49.5 | 68.6 | 47.7 | 66.7 |
| Mouse 1 | 48.6 | 66.4 | 46.7 | 65.1 |
| Mouse 2 | 48.6 | 63.5 | 46.7 | 62.6 |

These findings are consistent with reports of INS in developing mammals (DePablo et al, 2005). Recently, two insulin variants were identified in the head region of developing zebrafish (Papasani et al., 2006). The role of proinsulins/INSs during the development of fish is not clear, but they may serve as selective growth, differentiation, and/or maturation factors as is the case for mammals (Hernandez-Sanchez et al., 2006). For example, mice deficient in insulin and/or insulin receptor have reduced body size at birth compared to normal individuals (Hales et al, 1991). Future studies on the pattern of INS receptor

expression should provide added insight into the role of INS action in during the development of fish.

The pattern of expression of INS mRNAs observed among and within juvenile trout tissues as well as among body regions of trout embryos suggests differential roles for proINS 1 and INS 2. While the sequence of the INS A/B chain encoded by trout proINS 1 and proINS 2 is the same, several possibilities exist to explain differential function. One possibility is that the mRNAs encode precursors that assume different shapes that potentially influence their subsequent processing by prohormone convertases and, ultimately, the proportion of INS, proINS and C-peptide that are produced and secreted. Another possibility is differential function of segments other than the INS A/B chain. For example, the deduced proinsulin and their component c-peptide sequences for trout INS 1 and INS 2 are distinct. The C-peptide of mammalian INS has been shown to possess bioactivity and to interact with the INS receptor (Ohtomo et al, 1998; Henriksson et al, 2005). In either event, the divergence of preproINS 1/preproINS 2 structure and function in fish appears to be reminiscent of the past divergence of INS/IGF (Chan and Steiner, 2001).

In summary, we found that rainbow trout possess two INS-encoding mRNAs that are differentially expressed, both among tissues as well as within tissues. The differential expression of INS mRNAs suggests that independent mechanisms serve to regulate INS production in a type-and tissue-specific manner. While this pattern of expression suggests differential roles for proINS 1 and proINS 2, detailed functional studies of the protein products need to be performed. In addition, future studies should examine receptor

specificity of the various proinsulins/INSs for the multiple forms of INS receptors observed in fish.

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# CHAPTER TWO. ISOLATION AND CHARACTERIZATION OF A mRNA ENCODING A NOVEL INSULIN RECEPTOR (IR) SUBTYPE, IR2, FROM RAINBOW TROUT (*Oncorhynchus mykiss*) AND PATTERNS OF EXPRESSION OF THE FOUR IR SUBTYPES, IR1-IR4, IN TISSUES AND DURING EMBRYONIC DEVELOPMENT

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## Abstract

Insulin (INS) plays a critical role in the growth, development, and metabolism of vertebrates. In this study, a cDNA encoding a novel insulin receptor (IR) subtype was isolated, cloned, and sequenced from the liver of rainbow trout. A 1525-bp cDNA encoding a partial amino acid sequence of the β-subunit including the transmembrane domain, the tyrosine kinase domain, and the 3' untranslated region (UTR) was obtained and designated IR2 based on comparison with known IR subtypes, including the three previously reported IR subtypes of trout. Trout IR2 shares 90.0%, 82.8%, and 84.3% nucleotide identity with previously characterized trout IR1, IR3 and IR4, respectively. Quantitative real-time PCR revealed that the four IR mRNAs were differentially expressed, both in terms of distribution among tissues as well as in terms of abundance within selected tissues of juvenile trout. IR1 mRNA was most abundant in spleen, liver, kidney, and muscle (white, red and cardiac), but least abundant in adipose. IR3 mRNA was most

abundant in liver, spleen, kidney, and pancreas; in other tissues, levels of IR3 mRNA were uniformly abundant. By contrast, levels of IR2 and IR4 mRNA were uniformly abundant in most tissues, except in spleen where levels of IR4 were significantly lower. All IR subtypes were detected over the course of embryonic development. In head and tail regions, levels of IR2 and IR3 mRNA declined from pre-hatch (29 days post-fertilization, dpf) to post-hatch (68-90 dpf), whereas levels of IR1 and IR4 remained relatively unchanged. These findings contribute to our understanding of the evolution, distribution, and function of insulin receptors.

## Introduction

Insulin (INS) plays an essential role in the growth, metabolism and development of vertebrates. Insulin initiates its biological actions by binding to the membrane-associated insulin receptor (IR) that is widely distributed in many tissues (Joost, 1995). Most of what is known about IR structure and signal transduction comes from studies in mammals. The IR is a member of the receptor tyrosine kinase (RTK) family, and possesses a N-terminal extracellular domain with a cysteine-rich binding region followed by a cleavage site that gives rise to an exclusively extracellular α subunit attached by disulfide bonds to a membrane-spanning β subunit that contains an intact kinase region in its intracellular domain. A stable dimer is formed between two αβ-pairs linked via disulfide bonds; interestingly, heterodimers between an IR αβ-pair and an insulin-like growth factor-1 receptor (IGFR1) αβ-pair also exist (Lawrence et al., 2007). Upon INS binding, several regions on the intracellular domain of the β subunit are autophosphorylated which, in turn, initiates signal transduction through a number of effector pathways, including the PI3K/AKT, ERK, and STAT/Jak-STAT pathways (Castillo et al., 2006; Youngren, 2007).

To date, only a few studies on IR have been conducted in fish; however, from these studies a couple of patterns emerge. First, fish IRs share the basic structural features of other IRs, possessing an extracellular domain with two cysteine-rich regions, a cleavage site that would yield an extracellular α subunit and a membrane-spanning β subunit, and an intracellular domain containing an intact kinase region (Elies et al., 1999). Second, fish possess multiple subtypes of IRs encoded by distinct mRNAs derived from multiple genes. The multigenic origins of IRs in fish likely resulted from a fish-specific genome duplication event associated with the evolution of this group (Hoegg et al., 2004). For example, two distinct IR-encoding mRNAs have been characterized in goldfish (Hitchcock et al., 2001), Japanese flounder (Nakao et al., 2002), and zebrafish (Maures et al., 2002). In addition, three distinct IR-encoding mRNAs were previously characterized in rainbow trout (Greene and Chen, 1999a) and four distinct IR-encoding mRNAs were characterized in coho salmon (Chan et al., 1997). Additional sequence information is needed to provide greater insight into the evolution of IRs and into the functional significance of multiple IR subtypes and the mechanisms controlling their distribution and expression.

The aim of this study was to further characterize the IRs of fish. We used rainbow trout to enhance our understanding of the polygenic origins of IRs and to examine patterns of IR expression among tissues and during embryonic development.

## Materials and methods

### *Experimental Animals*

For most experiments, juvenile rainbow trout (*Oncorhynchus mykiss*) of both sexes (ca. 100g) were obtained from Dakota Trout Ranch near Carrington, ND, USA. Following

transport to North Dakota State University, the fish were maintained in well-aerated, 800-L circular tanks supplied with recirculated (100% replacement volume per day) dechlorinated municipal water at 14$^{o}$C under a 12h light:12h darkness photoperiod. Fish were fed twice daily with AquaMax™ Grower (PWI Nutrition International. Brentwood, MO, USA). Animals were acclimated to laboratory conditions for at least 6 weeks prior to experiments.

Rainbow trout embryos and larvae at several stages of development were also used in this study. Fertilized rainbow trout eggs from 3-year old fall spawning broodstock were obtained from, and raised at the Alma Aquaculture Research Station (Alma, ON, Canada). Embryos and larvae were reared and collected at the following stages as described previously (Raine et al., 2004): eyed-egg (29 days post-fertilization, dpf; 246.5 degree days), post-hatch (53 dpf; 450.5 degree days), swim-up (68 dpf; 578 degree days), and complete yolk-absorbed (90 dpf; 765 degree days). One degree-day is the average daily water temperature x the number of days reared at this temperature. Food was first introduced at the swim-up stage, and embryos were fed with Silver Cup starter diet (Nelson and Sons, Murray, Utah), at a ration of 5.0% of biomass per day (to excess).

All experimental protocols were reviewed and approved by the North Dakota State University Animal Care and Use Committee or by the University of Guelph Animal Care Committee, as applicable.

*RNA Extraction*

Juvenile rainbow trout were anaesthetized by immersion in 0.05% (v/v) 2-phenoxyethanol and euthanized by transection of the spinal cord. Selected tissues (e.g., pancreas, liver, etc.) dissected from juveniles were placed into 2-mL microcentrifuge tubes,

immediately frozen on dry ice, and stored at –80°C for later analysis. Rainbow trout embryos were first dissected free of the chorion (29 dpf) and/or yolk (29, 53 and 68 dpf) and immediately divided into body segments. The body segments were quickly placed into 2-mL microcentrifuge tubes and flash frozen in liquid nitrogen, prior to storage at -80ºC.

Total RNA was extracted using TRI-Reagent® as specified by the manufacturer (Molecular Research Center, Cincinnati, OH, USA). Isolated RNA was dissolved in 50-100 µL Rnase-free deionized water. Total RNA was quantified by ultraviolet ($A_{260}$) spectrophotometry and diluted to 100 ng/µL in RNase-free deionized water. RNA samples were then stored at –80°C until further analysis.

*Oligonucleotide Primers And Probes*

Gene-specific primers used for isolation of cDNAs were designed by examining conserved regions of the three previously published rainbow trout IR subtype sequences (Greene and Chen, 1999a) using GeneTool software (BioTools, Edmonton, AB, Canada) and custom synthesized by Sigma-Genosys (The Woodlands, TX, USA). Additional primers for reverse transcription were provided in the iScript™ cDNA Synthesis kit (BioRad, Knightdale, North Carolina, USA) or in the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories Inc., Mountain View, CA, USA). Gene-specific oligonucleotide primers and probes used for real-time PCR were designed using ABI Primer Express® Version 2 software based upon the sequence of our novel IR subtype, designated IR2 (FJ769224) and on previously published sequences for IR1 (previously known as IRa; AF062496), IR3 (previously known as IRc; AF062498) and IR4 (previously known as IRb; AF062497) and for β-actin (AF157514), custom synthesized by Applied

Biosystems. The probes were minor-groove binding probes labeled with either FAM (IR1, IR2, IR3 and IR4 probes) or VIC (β-actin probe). Primers and probes were used for reverse transcription and PCR without further purification. The sequences for the various primers and probes are shown in Table 3.

*Isolation And Sequence Analysis Of Insulin Receptor-Encoding cDNAs*

A three-phase approach was adopted for the isolation of selected cDNAs using RT-PCR and rapid amplification of cDNA ends (RACE)-PCR. Isolation of the cDNA sequence was accomplished using the SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA) following the manufacturer's protocol. In phase I, RNA was reverse transcribed into cDNA containing the sequence tags necessary for SMART™ chemistry, and remaining cycles of PCR were carried out using a set of IR non gene specific primers (Table 3). After an initial denaturation at 94$^{o}$C for 5 min, a 35-cycle PCR was preformed with each cycle consisting of denaturation (94$^{o}$C for 1 min), annealing (65$^{o}$C for 1 min), and extension (72$^{o}$C for 1 min) phases. In the last cycle, the extension time was increased to 10 min. The PCR product was identified by electrophoresis on an agarose gel containing 1% of each OmniPur (EMD chemicals, Gibbstown, NF, USA) and NuSieve GTG agarose (Bio-Wittaker Molecular Applications, Rockland, ME, USA) in 1 x Tris-borate-EDTA (TBE) buffer followed by ethidium bromide staining. The resulting PCR products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). Purified plasmids (75 fmol) were sequenced using the CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's protocol.

In phase II, after determining that the initial PCR reaction resulted in an unique cDNA (designated IR2) containing regions with high sequence identity with known IR-encoding cDNAs, 3'-RACE was preformed using a SMART™ RACE cDNA Amplification Kit under manufacturer's suggested conditions with a IR2 gene-specific primer (Table 1). The resulting PCR product was visualized, cloned into the pGEM-T Easy Vector, and sequenced as described previously.

In phase III, 5'-RACE for IR2 was also carried out using SMART™ RACE cDNA Amplification Kit under the manufacturer's suggested conditions with a second IR2 gene-specific primer (Table 1). The PCR product was visualized, cloned into the pGEM-T Easy Vector, and sequenced as described previously.

*Preparation Of cDNA Standards*

Standards for each of the IR subtypes (IR1, IR2, IR3, and IR4) of rainbow trout were synthesized by PCR. Approximately 1 μg of the 5'-RACE-ready IR cDNA product was used as template for PCR with forward gene-specific primers (Table 1) under the same conditions as described above. The resulting PCR products were visualized under ultraviolet light, cloned into the pGEM-T Easy Vector, and their sequences verified as described previously.

*Real-Time Reverse Transcription PCR*

From 200 ng total RNA, endogenous poly(A)$^+$ RNA was reverse transcribed in a 10 μL reaction using a iScript™ cDNA Synthesis kit (BioRad, Knightdale, North Carolina, USA) containing a RNase H$^+$ reverse transcriptase and a blend of oligo(dT) and random hexamer primers according to the manufacture's instructions. Reactions without reverse

transcriptase were included as negative controls; no amplification was detected in negative controls.

Levels of the four IR-encoding mRNA subtypes were determined by real-time RT-PCR using Stratagene Brilliant II and a STRATAGENE MX3000P® Detection System (Stratagene, La Jolla, CA, USA). Real-time PCR reactions were carried out for samples, standards, and no-template controls in a 10 μL reaction; each reaction contained 1 μL cDNA, 1μL each of forward primer and reverse primer (at a final concentration of 600 nM for IR gene-specific primers; 900 nM for β-actin primers; Table1), 1μL probe (at a final concentration of 150 nM for all probes; Table 1), 0.85 μL RNase-free deionized water, 0.15 μL Reference Dye (at a final concentration of 30 nM)and 5 μL Brilliant II® QPCR Master Mix. Cycling parameters for real-time PCR were as follows: 95$^{o}$C for 10 min, and 45 cycles for 95$^{o}$C for 15 s plus 63$^{o}$C for 30 s plus 72$^{o}$C for 45 s. Under these conditions, mean PCR efficiencies were 102.2%, 102.8%, 102.0% and 104.8%, respectively, for the IR1, IR2, IR 3 and IR 4 assays. To verify the specificity of the assays, cross hybridization was assessed by substituting alternate primer/probe sets in Brilliant II reactions for each standard. No amplification was observed with mismatched primer/probe sets.

*Data Analysis*

Copy number calculations were based on threshold cycle number ($C_T$). The $C_T$ for each sample was determined by the MX3000P™ real time analysis detection software after manually setting the threshold. Sample copy number was determined by relating $C_T$ to a gene-specific standard curve, followed by normalization to β-actin. No difference ($p>0.05$) was observed in β-actin expression among the various treatment groups. No-template

control samples did not exceed a maximal increase of 300 (ΔRn) fluorescence units over 45 cycles. Therefore, copy numbers of mRNA were considered non-significant if $C_T$ exceeded 45 cycles; this value corresponds to a detection limit of less than 100 mRNA copies.

*Sequence analyses*

Nucleotide sequences were aligned and analyzed with GeneTool sequence analysis program (BioTools Inc., Edmonton, AB, Canada). Only completely overlapping segments greater than 300nt in length were considered in the analysis. The best nucleotide substitution model was determined using AIC values obtained from MrModeltest v2 (provided by J. Nylander, Uppsala University). This substitution model was used to develop Bayesian phylogentic trees in MrBayes 3 (Ronquist and Huelsenbeck, 2003). Four-chains were utilized at a default temp of 0.2, and 2000000 MCMC sampling at every 100 trees; the first 2500 trees were considered as "burn in" and discarded. The tree was visualized and formatted with PhyloWidget (Jordan and Piel, 2008). Insulin receptor sequences for fugu, medaka, stickleback, and tetraodon were obtained from the Ensembl project website (http://www.ensembl.org) utilizing a basic search for insulin receptor and by using a BLAST search utilizing cDNA sequence for rainbow trout IR1 (AF062496), IR2 (FJ769224), IR3 (AF062498), and IR4 (AF062497) within their respective genomes. All other sequences were obtained from a BLAST search of GenBank, utilizing the cDNA sequence for rainbow trout IR1, IR2, IR3, and IR4.

Table 3. Primers and probes used for sequence analysis and for real-time Quantitative PCR

| Target | Description | Sequence |
|---|---|---|
| IR | Phase I. non gene-specific | |
| | Forward primer | 5’-GGACACGCGTGTGGCGGTGAA-3’ |
| | Reverse primer | 5’-TCCCCCGCCATCTGAATCATCTCCT-3’ |
| IR2 | Phase II. | |
| | IR2 gene-specific 3' RACE primer | 5’-TGCGCTGCCTCCGACCA-3’ |
| IR2 | Phase III. | |
| | IR2 gene-specific 5' RACE primer | 5’-CCCCGCCATCTGAATCATCTCCTT-3’ |
| IR1 | cDNA Standard | |
| | Forward primer | 5’-CCCAGGCTGCAGGCTTCGTGTG-3’ |
| | Reverse primer | 5’-GCCATGCCGTCTGCTATCTCTCCTG-3’ |
| IR2 | cDNA Standard | |
| | Forward primer | 5’-TTCGTAGGAGGTGGAGTCTTCATGATCTTC-3’ |
| | Reverse primer | 5’- CCCCCGCCATCTGAATCATCTC-3’ |
| IR3 | cDNA Standard | |
| | Forward primer | 5’-GAAACGGCTCCTGGACTGACCCC-3’ |
| | Reverse primer | 5’-GCCGTCTGCTATCTCTGCTGCCATC-3’ |
| IR4 | cDNA Standard | |

Table 3 continued.

| | | |
|---|---|---|
| | Forward primer | 5’-CCGCGTGGGTGGCAGGAAAC-3’ |
| | Reverse primer | 5’- GCCATGCCGTCTGCTATCTCTGCTG-3’ |
| | Q-PCR Product | |
| IR1 | Forward primer | 5’-GTGGGAGCGGAGTCTTCA-3’ |
| | Reverse primer | 5’-TCGCCACCTCCCAGTCAT 3’ |
| | Probe | 5’$^{FAM}$- CGCCAGCGACGTATA -$^{MGBNFQ}$3’ |
| | Q-PCR Product | |
| IR2 | Forward primer | 5’- CTCTGATCGCCTCGTCCAA-3’ |
| | Reverse primer | 5’- CTCCCAGTCATCAGGCACGTA -3’ |
| | Probe | 5’$^{FAM}$- AGAGTACCTGAGCGCCAA-$^{MGBNFQ}$3’ |
| | Q-PCR Product | |
| IR3 | Forward primer | 5’-CCAGCCTACTCTTGTTGTCATG-3’ |
| | Reverse primer | 5’-GAGTCAGGGCGGAGGCA-3’ |
| | Probe | 5’$^{FAM}$- TTCAAATCACCGTGT -$^{MGBNFQ}$3’ |
| | Q-PCR Product | |
| IR4 | Forward primer | 5’-GAGGTGGGCAGGGATAAGAT-3’ |
| | Reverse primer | 5’- CGATGCCCTCGTAGACCAT-3’ |
| | Probe | 5’$^{FAM}$- GACCCAGCTCTCGTAG -$^{MGBNFQ}$3’ |
| | Q-PCR Product | |

Table 3 continued.

| | | |
|---|---|---|
| β-Actin | Forward primer | 5'-GGCTTCTCTCTCCACCTTCCA-3' |
| | Reverse primer | 5'-AGGGACCAGACTCGTCGTACTC-3' |
| | Probe | 5'$^{VIC}$-TGCTTGCTGATCCACAT-$^{MGBNFQ}$3' |

Abbreviations: IR, insulin receptor; Q-PCR, Real-time quantitative PCR.

*Statistical analyses*

Quantitative data are expressed as means ± S.E.M. There were 6-8 individual replicates (different tissues came from different animals) for the tissue distribution experiment and 4-8 individual replicates for the embryonic development experiment. Statistical differences were estimated by the Generalized Linear Mixed Model (GLIMMIX) approach using a split plot design. Whole plot treatments were tissue or embryonic stage and the sub plot treatments were IR subtype. Insulin receptor subtype was treated as repeated measure (as all four were measured in the same tissue or embryonic stage) and an unstructured variance-covariance structure was used when fitting the model. A probability level of 0.05 was used to indicate significance. In all cases, the main effects were found to be significant. Comparisons of simple effects were made by the least squares means method using a slicing approach in GLIMMIX; the maximum experiment-wise Type I error rate was controlled at 0.05 using a Bonferroni adjustment. Notations on the face of the figures denote simple effects comparisons; details of the main effects are given in the legends of the figures. All statistics were performed using SAS v 9.2 (Cary, NC, USA).

## Results

*Characterization Of Novel Insulin Receptor-Encoding cDNA*

A distinct cDNA fragment, approximately 300 bp in size, was amplified by traditional PCR from total RNA isolated from the liver of rainbow trout using IR primers. Sequence analysis of the fragment revealed a portion of the IR coding region, and suggested the successful isolation of a IR-encoding cDNA subtype not previously characterized in rainbow trout. Further investigation using 3'-RACE PCR with gene-specific primers revealed a product of approximately 1200 bp, including the polyA+ tail. A 400bp fragment was revealed using 5'-RACE PCR with gene-specific primers. Assembly of the initial cDNA portion along with the 3'-RACE and 5'-RACE fragments resulted in a unique 1525-bp IR-encoding cDNA. This unique IR-encoding cDNA was designated rainbow trout IR subtype 2 (IR2) based upon comparison with IR subtypes previously characterized in coho salmon (Chan et al., 1997) and rainbow trout (Greene and Chen, 1999a). The IR2 sequence contains a 1248-bp coding segment of the β-subunit, including part of the transmembrane domain and the intracellular domain with an intact tyrosine kinase segment, and a 255-bp 3' untranslated region (Fig. 8). Trout IR2 shares 91.1%, 82.5%, and 83.7% nucleotide identity with previously characterized trout IR1 (previously known as IRa; AF062496), IR3 (previously known as IRc; AF062498), and IR4 (previously known as IRb; AF062497), respectively (Table 4).

We took advantage of four regions of the nucleotide sequence within the overlapping segments of rainbow trout IR1, IR2, IR3, and IR4 to design primers and probes that could distinguish among the various transcripts using real-time RT-PCR.


GCG GGC CTG CTG CTA TTC GTA GGA GGT GGA GTC TTC ATG ATC TTC AAG AAG 51
A G L L L F V G G G V F M I F K K 17
AAA CAA ACT GAG GGA CCC ACT GGA CCT CTG ATC GCC TCG TCC AAC CCA GAG 102
K Q T E G P T G P L I A S S N P E 34
TAC CTG AGC GCC AAT GAC ATG TAC GTG CCT GAT GAC TGG GAG GTG GCG AGG 153
Y L S A N D M Y V P D D W E V A R 51
GAG AAG ATC AAC ATC TTG AAG GAG CTG GGT CAG GGC TCA TTT GGC ATG GTC 204
E K I N I L K E L G Q G S F G M V 68
TAC GAG GGC ATC GCC AAG GAC ATT GTG AAG GGC GAG CCG GAC ACG CGT GTG 255
Y E G I A K D I V K G E P D T R V 85
CGC GTG AAG ACG GTC AAC GAG TCG GCC AGT CTG AGA GAG AGG ATA GAG TTC 306
A V K T V N E S A S L R E R I E F 102
CTC AAC GAA GCG TCG GTC ATG AAG GCC TTT AGC TGC CAC CAC GTG GTG CGT 357
L N E A S V M K A F S C G G V V R 119
CTG CTG GGG GTG GTG TCT AAA GGC CAG CCC ACT CTG GTG GTG ATG GAG CTG 408
L L G V V S K G Q P T L V V M E L 136
ATG ACC CAC GGG GAT CTG AAG AGC TTC CTG CGC TGC CTC CGA CCA GAC TCT 459
M T H G D L K S F L R C L R P D S 153
GAG AAT AAC CCC ACA GGG AAG CCC CCT CCC ACA CTG AAG GAG ATG ATT CAG 510
E N N P T G K P P P T L K E M I Q 170
ATG GCG GGG GAG ATA GCA GAC GGC ATG GCC TAC CTC AAC GCC AAA AAG TTT 561
M A G E I A D G M A Y L N A K K F 187
GTT CAC AGA GAT CTG GCC GCC AGG AAC TGC ATG GTG GCT GAG GAC AAC ACC 612
M H R D L A A R N C M V A E D N T 204

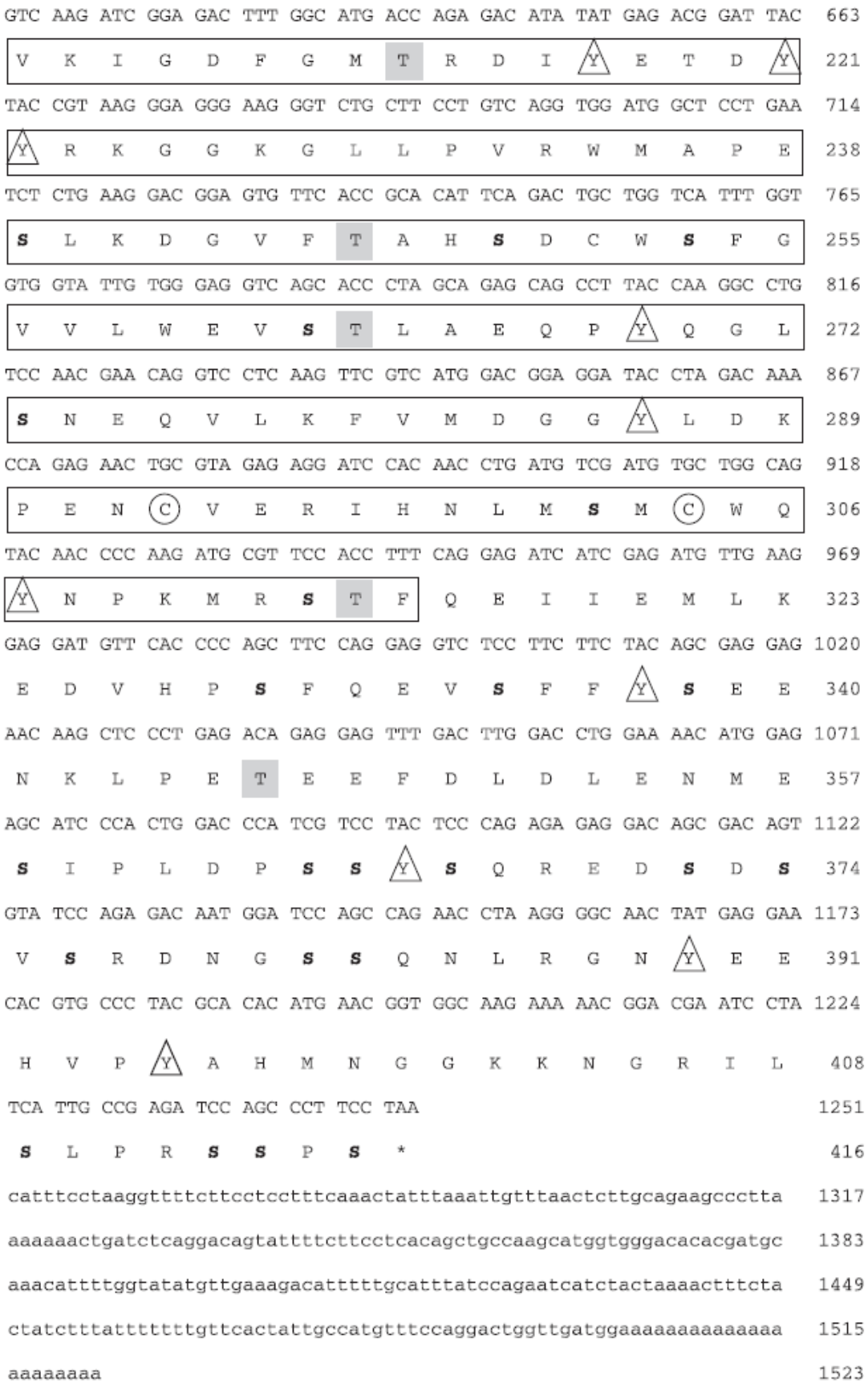
GTC AAG ATC GGA GAC TTT GGC ATG ACC AGA GAC ATA TAT GAG ACG GAT TAC 663
V K I G D F G M T R D I Y E T D Y 221
TAC CGT AAG GGA GGG AAG GGT CTG CTT CCT GTC AGG TGG ATG GCT CCT GAA 714
Y R K G G K G L L P V R W M A P E 238
TCT CTG AAG GAC GGA GTG TTC ACC GCA CAT TCA GAC TGC TGG TCA TTT GGT 765
S L K D G V F T A H S D C W S F G 255
GTG GTA TTG TGG GAG GTC AGC ACC CTA GCA GAG CAG CCT TAC CAA GGC CTG 816
V V L W E V S T L A E Q P Y Q G L 272
TCC AAC GAA CAG GTC CTC AAG TTC GTC ATG GAC GGA GGA TAC CTA GAC AAA 867
S N E Q V L K F V M D G G Y L D K 289
CCA GAG AAC TGC GTA GAG AGG ATC CAC AAC CTG ATG TCG ATG TGC TGG CAG 918
P E N C V E R I H N L M S M C W Q 306
TAC AAC CCC AAG ATG CGT TCC ACC TTT CAG GAG ATC ATC GAG ATG TTG AAG 969
Y N P K M R S T F Q E I I E M L K 323
GAG GAT GTT CAC CCC AGC TTC CAG GAG GTC TCC TTC TTC TAC AGC GAG GAG 1020
E D V H P S F Q E V S F F Y S E E 340
AAC AAG CTC CCT GAG ACA GAG GAG TTT GAC TTG GAC CTG GAA AAC ATG GAG 1071
N K L P E T E E F D L D L E N M E 357
AGC ATC CCA CTG GAC CCA TCG TCC TAC TCC CAG AGA GAG GAC AGC GAC AGT 1122
S I P L D P S S Y S Q R E D S D S 374
GTA TCC AGA GAC AAT GGA TCC AGC CAG AAC CTA AGG GGC AAC TAT GAG GAA 1173
V S R D N G S S Q N L R G N Y E E 391
CAC GTG CCC TAC GCA CAC ATG AAC GGT GGC AAG AAA AAC GGA CGA ATC CTA 1224
H V P Y A H M N G G K K N G R I L 408
TCA TTG CCG AGA TCC AGC CCT TCC TAA 1251
S L P R S S P S * 416
catttcctaaggttttcttcctctttcaaactatttaaattgtttaactcttgcagaagcccttа 1317
aaaaaactgatctcaggacagtattttcttcctcacagctgccaagcatggtgggacacacgatgc 1383
aaacattttggtatatgttgaaagacatttttgcatttatccagaatcatctactaaaactttcta 1449
ctatctttatttttttgttcactattgccatgtttccaggactggttgatggaaaaaaaaaaaaaa 1515
aaaaaaaa 1523

Figure 8. Partial cDNA and deduced amino acid sequence of rainbow trout insulin receptor 2 (IR2). The transmembrane domain is denoted by a dashed box. Amino acids underlined by dashes denote the potential IRS-1 binging domain. Amino acid underlined by a solid line denotes potential ATP binding. The tyrosine kinase domain is denoted by a solid box. Conserved cysteine residues are denoted by a ○ ; conserved tyrosine residues are denoted by a △ ; conserved threonine residues are denoted by gray shading; conserved serine residues are denoted by bold lettering; the stop sequence is donated by an *.

Real-time RT-PCR of liver total RNA showed significant amplification of IRs above no-template controls (Fig. 9). Standard curves showed a linear relationship between log of the RNA copy number and $C_T$, with correlation coefficients of 0.99 for all IR forms.

*Differential Expression of IR-Encoding mRNAs Among And Within Tissues*

RNA from various tissues of juvenile trout was extracted and reverse transcribed. Steady-state levels of the mRNAs encoding IR1, IR2, IR3, and IR4 were quantified by real-time RT-PCR, which enabled an assessment of the distribution of IR mRNAs among

Table 4. Amino Acid identities among insulin receptor of teleost fish and other representative vertebrates. Percentage identities were calculated on sequences aligned using PepTool v. 1.0. Sequences were obtained from this study and from GenBank (accession numbers are in parentheses) as follows: rainbow trout 1(formerly IRa) (AF062496), rainbow trout 4(formerly IRb) (AF062497), rainbow trout 3(formerly IRc) (AF062498), rainbow trout 2 (this study; FJ769224), coho salmon 1 (AF021040), coho salmon 2 (AF021041), coho salmon 3 (AF021042), coho salmon 4 (AF021043), japanese flounder 1

Table 4 continued. (AB065097), japanese flounder 2 (AB065097), fugu 1 (scaffold_122 618894 to 619280), fugu 2 (scaffold_90 208360 to 224156), goldfish 1 (AF218355), goldfish 2 (AF321225), zebrafish 1(a) (XM 685442), zebrafish 2(b) (XM 685972), tilapia (AF493794), turbot (AJ224994), and sole (FJ515913).

| Organism | Insulin Receptor 2 Amino Acid Identity (%)[1] |
|---|---|
| Rainbow Trout 1 | 91.1 |
| Coho Salmon 1 | 93.7 |
| Rainbow Trout 2 | - |
| Coho Salmon 2 | 100.0 |
| Rainbow Trout 3 | 82.5 |
| Coho Salmon 3 | 91.0 |
| Rainbow Trout 4 | 83.7 |
| Coho Salmon 4 | 91.0 |
| Japanese Flounder 1 | 90.1 |
| Japanese Flounder 2 | 85.3 |
| Fugu 1 | 88.7 |
| Fugu 2 | 79.8 |
| Zebrafish 1 | 86.4 |
| Zebrafish 2 | 88.1 |
| Goldfish 1 | 84.4 |
| Goldfish 2 | 82.2 |
| Sole | 93.5 |
| Turbot | 89.2 |

Table 4 continued.

| | |
|---|---|
| Tiliapia | 95.5 |

tissues. Insulin receptors were widely distributed, and all subtypes were detected in each of the tissues examined (Fig. 10). IR1, IR2, IR3, and IR4 mRNAs were found in liver, muscle (white, red and cardiac), pancreas, adipose, pyloric cecum, pituitary, whole brain, intestine (upper and lower), gill, spleen and kidney. Within the brain, IR subtypes were detected in all of the regions examined, including the telencephalon, optic tectum, cerebellum and hypothalamus (Fig.10B).

The pattern of IR mRNA expression varied among tissues (Fig. 10A). Insulin receptor 1 mRNA was most abundant in spleen, liver, kidney, and muscle (white, red and cardiac), but least abundant in adipose. Insulin receptor 3 mRNA was most abundant in liver, spleen, kidney, and pancreas; in other tissues, levels of these mRNA subtypes were uniformly abundant. By contrast, levels of IR4 mRNA were uniformly abundant in most tissues, except in spleen where levels were significantly lower.

The relative abundance of IR-encoding mRNAs also varied within tissues (Fig. 10A). However, the pattern of expression of the various mRNAs varied. For example, for whole brain and pituitary, IR2 and IR3 mRNAs were more abundant than wither IR1 or IR4 mRNAs. In liver, upper and lower intestine, and kidney, IR1 and IR3 mRNAs were more abundant than IR2 or IR4 mRNAs. In cardiac and red muscle, IR 1 mRNA levels were greater than those of IR2, IR4, or IR4. Within regions of the brain, IR2 and IR3 were

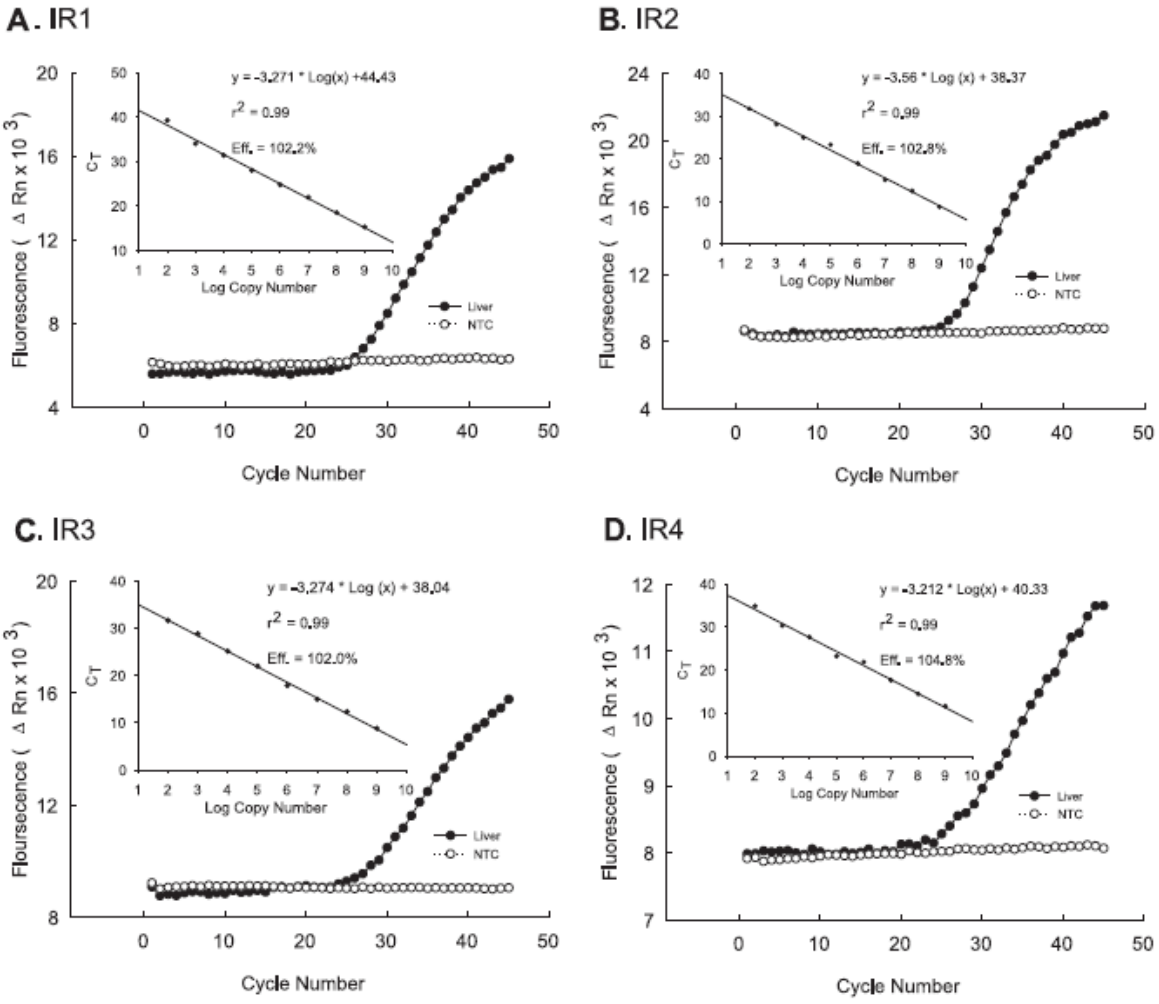


Figure 9. Quantitative real-time PCR of mRNAs encoding (A) insulin receptor subtype 1 (IR1), (B) IR2, (C) IR3, and (D) IR4 obtained from rainbow trout. The threshold cycle number ($C_T$) for each sample was determined from its amplification plot (representative sample plot is shown for each IR subtype). Sample copy number was determined by relating $C_T$ to a standard curve (inset) comprised of serial dilutions of known amounts of each IR cDNA, then normalized to β-actin levels. Abbreviation: NTC, no-template control.

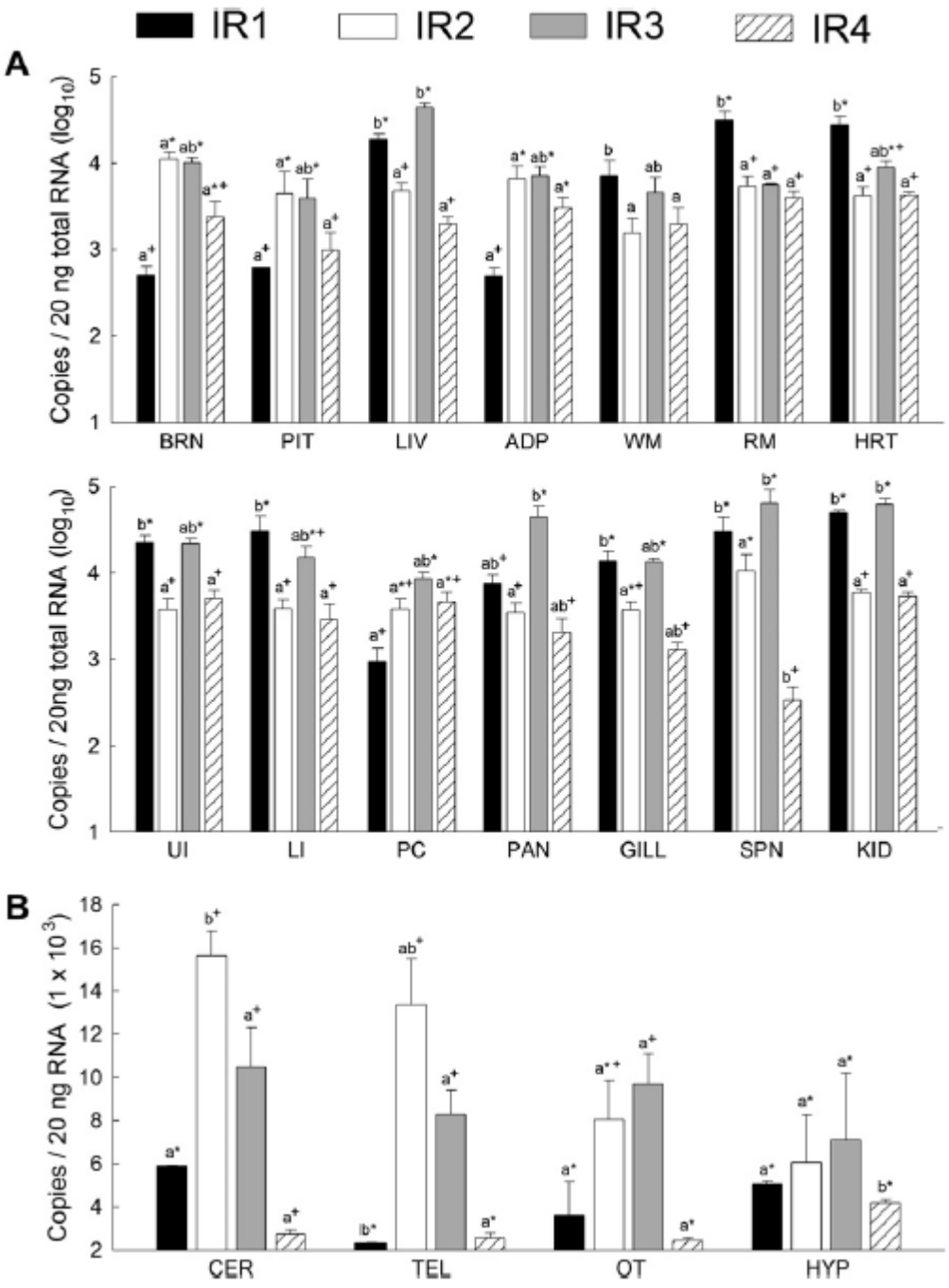


Figure 10. Expression of mRNAs encoding insulin receptor subtype 1 (IR1), IR2, IR3, and IR4 in (A) selected tissues and (B) brain regions of rainbow trout. mRNAs were quantified by real-time RT-PCR. Data are presented as mean ± SEM (6-8). There were significant main effects of tissue ($F=2.45$, $p=0.014$)/brain region ($F=4.84$, $p=0.044$) as well as a

significant main effect of IR subtype among tissues ($F=3.71$, $p<0.002$) or brain regions ($F=6.23$, $p=0.02$); and there no significant interaction between tissue and IR subtype ($F=1.71$, $p=0.22$) or between brain region and IR subtype ($F=2.16$, $p=0.17$). For a given IR subtype, groups with different letters are significantly ($p<0.05$) different; within a tissue or brain region, IR subtypes with a different symbol are significantly ($p<0.05$) different from each other.

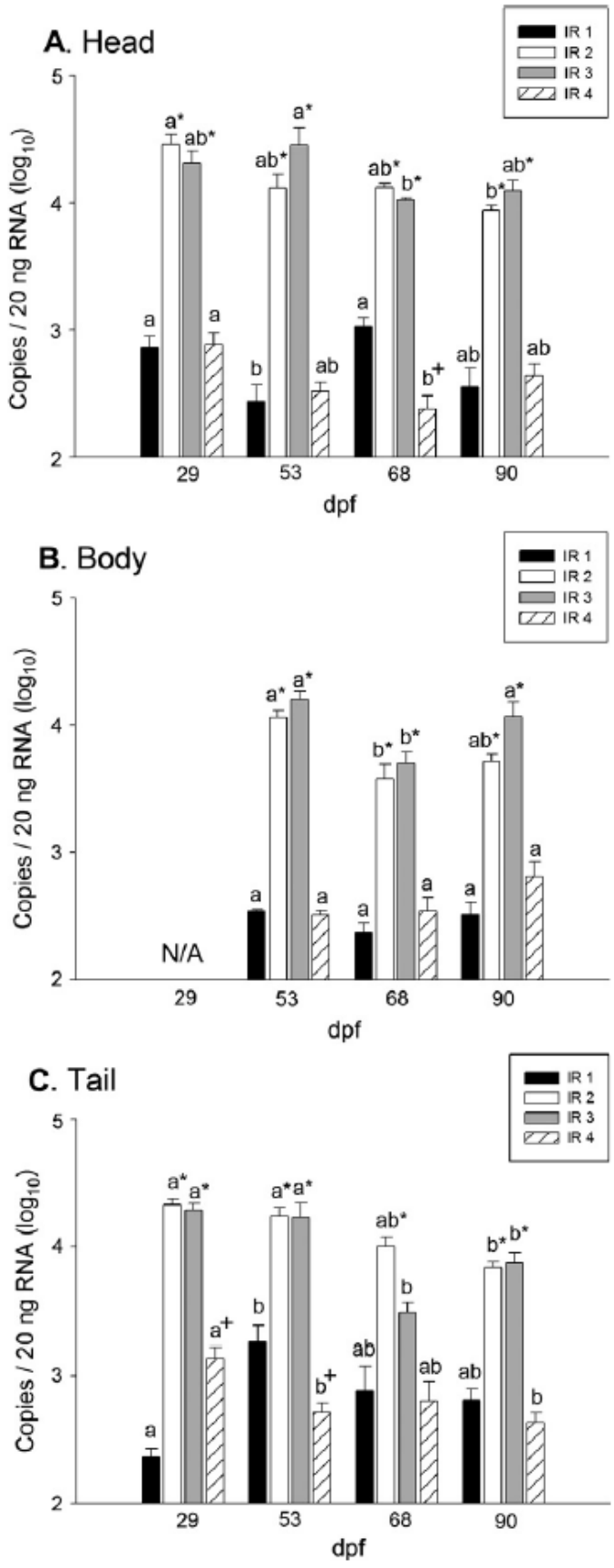
A. Head
B. Body
C. Tail
IR 1
IR 2
IR 3
IR 4
Copies / 20 ng RNA (log10)
dpf
29
53
68
90
N/A

Figure 11. Expression of mRNAs encoding insulin receptor subtype 1 (IR1), IR2, IR3, and IR4 in (A) head, (B) body, and (C) tail regions of rainbow trout during embryonic development. Samples were taken at early-hatch (29 dpf), mid-development (53 dpf), yolk sac-absorbed (68 dpf), and late-development (90 dpf); because there was no distinct body region at 29 dpf, it was not analyzed (n/a) at this stage of development. mRNAs were quantified by real-time RT-PCR. Data are presented as mean ± SEM (n=4-8).
There was a significant main effect of stage of development ($F=6.08$; $p=0.0036$) as well as a significant main effect of IR subtype within body region ($F=120.04$, $p<0.0001$); there also was a significant interaction between stage of development and IR subtype ($F=2.48$, $p=0.024$), but the main effects are interpretable because the order of the means for the second factor appear the same in each level of the first factor. For a given IR subtype, groups with different letters are significantly ($p<0.05$) different; within a tissue or brain region, IR subtypes with a different symbol are significantly ($p<0.05$) different from each other.

generally most abundant, whereas IR1 and IR4 were least abundant, except hypothalamus, in which all IR subtypes were present in equal abundance (Fig. 10B).

*Patterns Of IR-Encoding mRNA Expression During Embryonic Development*

Quantification of IR mRNAs by real-time RT-PCR also allowed for comparison of the four IR-encoding mRNAs within body segments of rainbow trout during embryonic development (Fig. 11). All IR subtypes were detected over the course of embryonic development. In general, IR mRNA levels varied based upon stage of development (dpf)

and body segment (head, body and tail). In head and tail regions, mean levels of IR2 and IR3 mRNAs declined over the course of development, displaying the lowest abundance in post-hatch embryos (at either 68-90 dpf, depending on IR subtype). By contrast, levels of IR1 and IR4 in the head and tail regions fluctuated right after hatch (53 dpf), but by 90 dpf the levels were similar to those in pre-hatch embryos (29 dpf), except IR 4 in the tail region which remained reduced. In the body segment, levels of all of IR 1 and IR 4 remained unchanged from 53 dpf (when a distinct body region was collected) to 90 dpf, whereas levels of IR2 and IR 3declined from 53 dpf to 68 dpf, then increased from 68 dpf to 90 dpf. IR2 and IR3 mRNA levels were consistently higher than those of IR1 and IR4 throughout all observed stages of development.

## Discussion

In this study, we isolated, cloned, and sequenced a unique cDNA that encodes for a distinct IR subtype from the liver of rainbow trout, designated IR2. Together with the three IR subtypes previously characterized—IR 1, IR3, and IR4 (Greene and Chen, 1999a) — this brings the total number of IR subtypes known for rainbow trout to four. We also found that the four IR subtypes of rainbow trout are differentially expressed both among and within tissues of juvenile fish as well as during embryonic development.

The rainbow trout IR2-encoding cDNA shared 91.1%, 82.5%, and 83.7% nucleotide identity with IR1, IR3, IR4 respectively. Comparison of IR cDNAs revealed a high level of conservation among salmonids (Table 4). Rainbow trout IR2 shared an approximately 90.8%, 99.3%, 88.2%, and 89.1% nucleotide identity with coho salmon

IR1, IR2, IR3, and IR4, respectively. All other IR fish species also displayed a high level of conservation, ranging from approximately ca 60-90% (Table 4).

Rainbow trout IR2 coding region contains several structural motifs representative of an IR β-subunit, including a partial transmembrane domain (TM), juxtamembrane domain (JM), tyrosine kinase domain (TK), and a carboxyl-terminus domain (CT). Amidst the JM domain, several important signaling molecules, including IRS-1 and Shc, are known to bind to activated phospho-tyrosine residues (e.g., $Tyr^{972}$) via their Sh2 domains and elicit mitogenic and metabolic cell signaling pathways in mammals (Youngren, 2007). The amino acid sequence, NPXY, within the JM is characteristic and appears essential for functional association between IR and these adaptor proteins. Rainbow trout IR2 along with all other fish IR subtypes with characterized JM domains [e.g., goldfish IR1 and IR2 (Hitchcock et al., 2001); Japanese flounder IR1 and IR2 (Nakao et al., 2002); rainbow trout IR 1, IR3 and IR4 (Greene and Chen, 1999a); turbot IR (Elies et al., 1999); zebrafish (Maures et al., 2002)] show complete conservation of the NPXY sequence. This suggests that the JM of fish IRs may interact with adapter proteins for signal propagation or may be inactivated through O-glycosylation similar to the JM domain of mammals (Youngren, 2007). The TK domain of mammals contains tyrosine residues necessary for activation of the receptor complex, including $Tyr^{1158}$, $Tyr^{1162}$and $Tyr^{1163}$, and a vital lysine residue, $Lys^{1030}$, required for ATP binding (Jensen and De Meyts, 2009); all of which are completely conserved among known IR-TK fish species, including rainbow trout IR1-4.

There have been several reports of multiple IRs among mammals and teleost fish. In mammals, the structural heterogeneity arises from alternative splicing of a primary transcript, and two isoforms have been described, IRA and IRB (Seino and Bell, 1989;

Kosaki et al., 1998). In fish, the molecular heterogeneity results from the existence of multiple distinct transcripts. Two distinct IR-encoding mRNAs have been characterized in goldfish (Hitchcock et al., 2001), Japanese flounder (Nakao et al., 2002), and zebrafish (Maures et al., 2002). Taking into account the present findings, four distinct IR-encoding mRNAs have been characterized in the two salmonid species examined thus far: rainbow trout (this study; Greene and Chen, 1999a) and coho salmon (Chan et al., 1997). That the distinct mRNAs of fish derive from separate genes is supported by a couple of observations. First, despite the high degree of similarity between the four rainbow trout IR-encoding mRNAs, the position and extent of the differences suggests the existence of four non-allelic IR genes. Second, our analysis of genomic sequences on the Ensembl website revealed two separate IR-encoding genes each for fugu, (fugu 1 ENSTRUT00000018912, fugu 2 ENSTRUT00000031228), medaka (medaka 1 ENSORLT00000022518, medaka 2 ENSORLT00000023220), stickleback (stickleback 1 ENSGACT00000022413, stickleback 2 ENSGACT00000013878), and tetraodon (tetraodon 1 ENSTNIT00000002544, tetraodon 2 ENSTNIT00000018173).

The phylogenetic relationships of cloned trout IRs compared with other teleost IRs are shown in Fig. 12. The emergence of variant forms of IR appears to have resulted from several independent duplication events. The IRs of teleosts branch into two major clades, an observation which is consistent with a fish-specific genome duplication event (Meyer and Van de Peer, 2005). The four IR types in salmonids probably arose as a result of a more recent tetraplodization event associated with the evolution of this taxon (Taylor et al., 2003); in such a scheme, trout IR 1 and IR 2 are paralogs and trout IR 3 and IR 4 are paralogs. The analysis also suggests that the IRs in goldfish and zebrafish derive from a

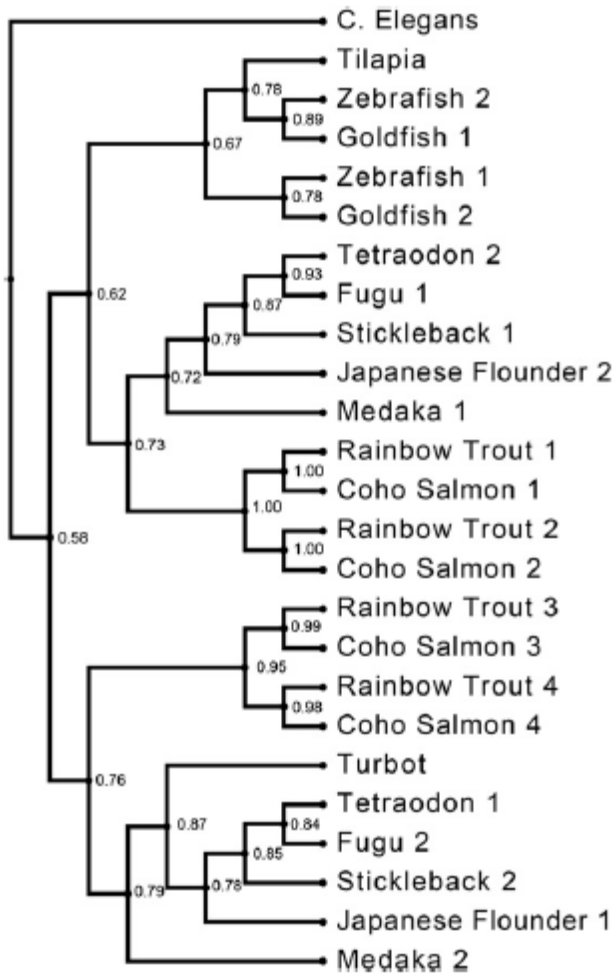


Figure 12. Phylogenetic tree of insulin receptors of fish. The tree was based upon the alignment of cDNA sequences and considered only completely overlapping segments greater than 300nt in length; the numbers at each node represent posterior probability values. Sequences were obtained from either GenBank (accession numbers in parentheses) or e! Ensembl (transcript ID numbers in parentheses) as follows: rainbow trout 1( formerly IRa) (AF062496), rainbow trout 4(formerly IRb) (AF062497), rainbow trout 3(formerly IRc) (AF062498), rainbow trout 2 (this study; FJ769224), coho salmon 1 (AF021040), coho salmon 2 (AF021041), coho salmon 3 (AF021042), coho salmon 4 (AF021043), Japanese flounder 1 (AB065097), Japanese flounder 2 (AB065097), fugu 1

(ENSTRUT00000018912), fugu 2 (ENSTRUT00000031228), goldfish 1 (AF218355), goldfish 2 (AF321225), medaka 1 (ENSORLT00000022518), medaka 2 (ENSORLT00000023220), stickleback 1 (ENSGACT00000022413), stickleback 2 (ENSGACP00000022371), tilapia (AF493794), turbot (AJ224994), zebrafish 1(a) (AF400271), zebrafish 2(b) (AF400272), rat (AY566293), mouse (NM 010568), (human (BC117172), chicken (AF111857), xenopus (NM 001088233), and C. Elegans (AC 084196).

secondary loss of genes orthologous to trout IR3/IR4 and from a separate duplication event giving rise to the paralogs, goldfish IR1/zebrafish IR2 and goldfish IR2/zebrafish IR1; although, the order and timing of such events are not certain. It is clear from the phylogenetic analysis, however, that further revision of the naming scheme of IR subtypes is needed. Additional research also is needed to clarify the polygenic origins of IRs in fish and to determine when the various duplication events took place.

Insulin receptors are differentially expressed in rainbow trout. This is supported by the widespread distribution of IR mRNA subtypes and the observation that IR mRNAs subtypes are differentially expressed among as well as within tissues of juvenile rainbow trout. Such widespread distribution is similar to previous reports on the patterns of IR expression in mammals and fish (Watanabe et al., 1992; Greene and Chen, 1999a; Nakao et al., 2002; Serrano et al., 2005) and is consistent with the broad roles of INS on growth, development, and metabolism (Plisetskaya and Duguay, 1993; Hernandez-Sanchez et al., 2006). Insulin receptors also are expressed during embryonic development. Notably, the relative abundance of IR subtypes differ at a given stage of development and the abundance

of a particular IR subtypes change over the course of development. These findings are consistent with reports of IR in developing mammals and fish (Accili et al., 1996; Greene and Chen, 1999b). In mammals, INS plays a critical role in development, and mice deficient in insulin and/or insulin receptors have reduced body size at birth compared to normal individuals (Hales et al, 1991; de Pablo and de la Rosa, 1995). The specific role(s) of INS in the development of fish is not clear, but INS is expressed both pre- and posthatch and during which organogenesis is occurring (Papasani et al., 2006; Caruso et al., 2008).

Although the functional significance of the multiple IR subtypes of rainbow trout is not clear, there are several lines of evidence to suggest distinct roles. First, the IR subtypes are expressed in a tissue-specific manner. Notably, levels of IR1 and IR3 are highest in tissues that play a central role in metabolism (e.g., liver, muscle, pancreas) as well as in those involved with nutrient absorption (e.g., upper and lower intestine). Second, the pattern of expression of IR subtypes changes over the course of embryonic development. In particular, levels of IR 2 and IR3 are higher than those of IR 1 and IR 4, and levels of IR 2 and IR3 are typically higher in pre-hatch embryos than in post-hatch embryos, at least in the head and tail regions. Third, despite high conservation of the JM domain of rainbow trout IR1-4, there is substantial variation among the CT domains with regard to position and extent of serine, threonine, tyrosine residues. The CT regions plays a role in kinase activation and signal propagation in mammals (Cheatham and Kahn, 1995), and variations in this region among the trout IR subtypes may have important consequences on receptor activation, cell signaling, and receptor activation. Given these observations, it is reasonable to suggest that in trout IR1 and IR3 are important for nutrient absorption and metabolism and that IR1 and IR2 may be important for processes associated with early

development (e.g., proliferation, differentiation, migration, etc.). The absence of IR subtypes in goldfish and zebrafish that are orthologous to trout IR3/IR4 may lead to differences in insulin action among fish species. In mammals, interestingly, the IRA isoform predominates over the IRB isoform in fetal tissue, whereas the reverse is observed in postnatal tissues, and an altered IRA:IRB ratio is associated with metabolic dysfunction (e.g., peripheral INS resistance, increased risk of type II diabtes) in adults (Belfiore at al., 2009). It also should be noted that the observed pattern of IR expression in trout suggests that independent mechanisms serve to regulate IR biosynthesis in a subtype- and tissue-specific manner. Further studies are needed to examine the ligand specificity of fish IRs and linkage between IRs and specific physiological processes.

In summary, we found that rainbow trout possess a fourth IR-encoding mRNA, IR2. Moreover, we found that the mRNAs encoding the four IR subtypes, IR1-4, are differentially expressed. These findings extend our understanding of the evolution and potential function of insulin receptors.

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# CHAPTER THREE. EXPRESSION OF INSULIN AND INSULIN RECEPTOR mRNAS ARE REGULATED BY NUTRITIONAL STATE AND GLUCOSE IN RAINBOW TROUT (*Oncorhynchus mykiss*)

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Portions of this chapter have been published as follows: Caruso, M.A., Sheridan, M.A. 2012. The expression of insulin and insulin receptor mRNAs is regulated by nutritional state and glucose in rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 175, 321-328. Caruso, M.A. was the primary contributor to this publication.

## Abstract

Many species of fish, including rainbow trout, possess multiple INS- and IR-encoding mRNAs. In this study, rainbow trout (*Oncorhynchus mykiss*) were used as a model to study the regulation of INS (INS1, INS2) and IR (IR1, IR2, IR3, and IR4) mRNA expression by nutritional state and glucose. In the nutritional state study, fish were either fed continuously, fasted (4 or 6 weeks), or fasted 4 weeks, then refed for 2 weeks. Nutritional state regulated INS and IR mRNA expression in a subtype- and tissue-specific manner. A 4-week fast reduced INS1 expression in endocrine pancreas (Brockmann body) and of INS1 and INS2 in brain, whereas a 6-week fast reduced the expression of both INS1 and INS2 in pancreas but only of INS1 in brain. Refeeding only restored INS2 levels in pancreas. In adipose tissue, by contrast, a 4-week fast increased INS1 expression, and a 6-week fast increased the expression of both INS1 and INS2. Nutritional state also modulated the pattern of IR mRNA expression. Fasting for 4 weeks resulted in no significant change in IR expression except decreased levels of IR3 mRNA in the pancreas and increased levels of IR4 mRNA in cardiac muscle. Prolonged fasting (6 weeks)

increased the expression of IR4 mRNA in the pancreas as well as of IR2 and IR4 mRNAs in liver, IR3 and IR4 mRNAs in cardiac muscle, and IR4 mRNA in gill; and decreased levels of IR3 and IR4 mRNAs in adipose. Refeeding increased IR4 in pancreas, and IR1 and IR4 in liver compared to fasted counterparts. Glucose differentially regulated the expression of INS and IR mRNAs in Brockmann bodies and liver pieces incubated *in vitro*, respectively. Low glucose (1 mM) reduced pancreatic expression of both INS1 and INS2 mRNAs compared to levels observed at 4 or 10 mM glucose. In the liver, IR1 and IR2 mRNA expression was insensitive to glucose concentration, whereas expression of IR3 and IR4 was attenuated at 1 and 10 mM compared to 4 mM glucose. These findings indicate that the pattern of INS and IR expression in selected tissues is regulated by nutritional state and glucose.

## Introduction

INS is a member of a class of peptide hormones that exert their biological actions through the binding of tyrosine kinase receptors (RTK), which commence physiological responses involved in nutrient homeostatis and mitogenesis within target tissues. The nutritional state of a fish is a critical factor regulating INS secretion (Navarro et al., 1992). Postprandial INS secretion occurs in all fish examined to date; however, the extent of INS response is lower than that of higher vertebrates and displays significant variability within each species (Stone, 2003). Fasting is natural part in the life cycle of some piscine species (e.g., salmon), which undergo durations of self-induced starvation (i.e., spawning), causing a shift in energy utilization and tissue storage. Some mammals will also undergo periods of self-induced anorexia during cyclic periods of suspended animation (i.e., hibernation) and rely upon stored nutrients for energy. Unlike mammals, hypoglycemia is well tolerated

in piscine species in response to fasting, or when provided an exogenous INS administration (Harmon and Sheridan, 1992). During intermittent periods of nutrient deficiencies, INS basal secretion occurs somewhat in constitutive pulsatile manner (Navarro et al., 1992). INS levels in fish are also similar to mammals during periods of acute and chronic fasting (Gutierrez et al., 1987; Plisetskaya et al., 1989). In fish, nutrient deprivation typically depletes liver glycogen, then mesenteric adipose tissue, all while conserving skeletal muscle tissue (Blasco et al., 1992). Fasting steadily lowers plasma INS levels, which may be restored after a short period of re-feeding (Montserrat et al., 2007; Shimizu et al., 2009). However, a significant decrease in plasma INS is more pronounced several weeks after fasting, as seen in juvenile carp (Blasco et al., 1992), brown trout (Navarro et al., 1992), goldfish (Patent and Foa, 1971), salmon (Thorpe and Ince, 1976), sea bass (Gutiérriz et al., 1991), cod (Hemre et al., 1990), and rainbow trout (Moon et al., 1989). Although, not all reports observe a steady drop in plasma INS over time, where depressed, INS levels remain nearly constant at lower levels (Shimizu et al., 2009). In addition, the variable decrease in INS levels may be dependent upon season and reproductive status (Sower et al., 1985). The diet composition fed upon carnivorous fish (e.g., rainbow trout), specifically protein-to-carbohydrate ratio, alters INS secretion (Navarro et al., 1992) and INS responsive signaling pathways (e.g., Akt/TOR) involving glucose, lipid, and amino acid metabolism (Seiliez et al., 2011). Although plasma INS levels are known to fluctuate due to nutritional status, diet composition, and environmental factors, mRNA levels of INS and IR have not been measured in response to a truancy of nutrients in fish.

INS secretion in fish responds to oral administration of nutrients but also through intraperitoneal (IP) and intravenous (IV) injection of glucose *in vivo* (Perez et al., 1989). Interestingly, glucose metabolism may not be necessary to cause INS secretion in fish. Catfish pancreas treated *in vitro* with 2-deoxyglucose (2DG), a non-metabolizable glucose analogue, displayed INS secretion, unlike mammals, which require the oxidation of glucose for INS secretion (Ronner, 1991). In addition, artificial administration of glucose in rainbow trout and American eel using intravenous glucose tolerance test (IGTT), the most rapid way to induce hyperglycemia, displayed a maximal increase in INS almost immediately following injection (Legate et al., 2001). Although INS secretion has been measured *in vivo* and *in vitro* utilizing various treatments of glucose administration, mRNA levels of INS and IR have not been measured in response to various doses of glucose.

Gene duplication events throughout the teleost lineage have resulted in partial tetraploidy, leading to the formation of non-allelic INS and IR genes, many of which have been characterized in salmonid species (Chan et al., 1997). Previously, we characterized two INS mRNAs from the endocrine pancreas (Brockmann body) and a fourth IR mRNA from the liver of rainbow trout (*Oncorhynchus mykiss*) (cf. Caruso et. al, 2008; Caruso et. al., 2010). Extrapancreatic production of INS has been speculated upon, but only recently has evidence been provided to indicate that INS is synthesized in the brain and adipose tissue of rainbow trout (Plisetskaya, 1989; Caruso et al., 2008), whereas IR expression is ubiquitous expressed throughout all tissues in rainbow trout (Caruso et al., 2010; Greene and Chen, 1999). The existence of multiple IR isoforms may underlie tissue-specific actions of INS. Despite the increasing information on differential expression of INS and IR subtypes, little has been reported about the regulation of these elements. In the present

study, we used rainbow trout to test the hypothesis that INS and IR mRNA expression patterns are differentially regulated according to nutritional state and glucose.

## Materials and Methods

### *Animals*

Juvenile rainbow trout (*Oncorhynchus mykiss*) of both sexes were obtained from Dakota Trout Ranch near Carrington, ND, USA. Following transport to North Dakota State University, the fish were maintained in well-aerated, 800-L circular tanks supplied with recirculated (100% replacement volume per day) dechlorinated municipal water at 14$^{o}$C under a 12 light:12 darkness photoperiod. Fish were fed to satiety twice daily with AquaMax™ Grower (PWI Nutrition International, Brentwood, MO, USA). Animals were acclimated to laboratory conditions for at least 6 weeks prior to experiments.

### *Nutritional State Experiment*

Fish were randomly assigned to one of six treatment groups (initial, fed continuously for 4 weeks, fasted for 4 weeks, fed continuously for 6 weeks, fasted for 6 weeks, and fasted for 4 weeks then refed for 2 weeks) and transferred to group-dependent (fed, fast, and refed) 100 L circulator tanks with a flow-through water supply at 14$^{o}$C under a 12h:12h light:dark photoperiod. Fish were allowed to acclimate for two weeks in their experimental tank prior to beginning the experiment. Animals were fed twice daily to satiety for the duration of the study, except 24h before sampling. At the time of sampling, fish were anesthetized in 0.05% (v/v) 2-phenoxyethanol, measured (body weight and length), euthanized by transection of the spinal cord, and selected tissues (e.g., Brockmann body, brain, adipose, gill, cardiac muscle, white skeletal muscle, liver) were removed and

immediately placed in 2-ml microcentrifuge tubes, frozen on dry ice, and stored at -80$^{o}$C until further analysis. These tissues were selected because of their central importance in metabolism and because of the high abundance and pattern of differential expression of INS and IR mRNAs determined previously [4, 5].

*In Vitro Glucose Challenge Experiments*

The influence of glucose on the expression of INS-encoding mRNAs was studied in isolated Brockmann bodies incubated *in vitro*. Brockmann bodies were removed from anesthetized [0.05% (v/v) 2-phenoxyethanol] fish and placed in a chilled (14$^{o}$C) petri dish containing a modified Hanks' medium [in mM: 137 NaCl, 5.4 KCl, 4 $NaHCO_3$, 1.7 $CaCl_2$, 0.8 $MgSO_4$, 0.5 $KH_2PO_4$, 0.3 $Na_2HPO_4$, 10 HEPES, and 0.24% bovine serum albumin (BSA) without glucose, pH 7.6; referred to as basal medium]. Individual organs were dissected further to remove surrounding connective tissue and then longitudinally bisected (to yield hemi-islets) and rinsed twice with fresh basal medium. Two or three hemi-islets were placed into each well of a 24-well polystyrene culture plate (Falcon 3407) containing 1 ml of basal medium, and preincubated with gyratory shaking (100 rpm) for 2 h at 14$^{o}$C and gassed under 100% $O_2$. After preincubation, basal medium was removed, and 1 mL of experimental medium (containing 1, 4, or 10 mM glucose) was added, and the incubation was initiated for 6 hours (14$^{o}$C, gassed with 100% $O_2$). The concentrations of glucose were based on previous studies with rainbow trout hemi-islets, representing hypo-, normo-, and hyper-glycemic conditions, respectively; the NaCl concentrations of the experimental medium were adjusted so that all solutions were isosmotic [11]. After incubation, experimental medium was removed and hemi-islets were placed in 2-mL microfuge tubes

and immediately frozen on dry ice and stored at -80°C until later analysis, completed within 2 weeks.

The influence of glucose on the expression of IR-encoding mRNAs was studied on liver pieces incubated *in vitro*. Livers were removed from anesthetized fish as described above and immediately perfused with ice cold 0.75% (w/v) saline to remove blood. Livers were cut into ca. 1 mm cubes and placed into ice-cold Hank's medium (in mM: 136.9 NaCl, 5.4 KCl, 0.81 $MgSO_4$, 0.44 $KH_2PO_4$, 0.33 $Na_2HPO_4$, 10 HEPES, 1.5mM $CaCl_2$, 5 $NaHCO_3$; pH7.6 ); pieces from an individual liver were pooled, and the pools were kept separated for replicate treatments. Pieces were washed three times with Hank's medium, followed by two times with Incubation Medium [Hank's medium with 0.24% (w/v) bovine serum albumin]. Six to eight pieces were then placed into each well of 24-well culture plate contain 1 ml of incubation medium, and preincubated with gyratory shaking (100 rpm) for 2 h at 14°C and gassed under 100% $O_2$. After preincubation, medium was removed, and 1 ml of incubation medium containing either 1, 4, or 10 mM glucose was added. After 6 h of incubation (14°C, 100% $O_2$), liver pieces were removed, placed into 2-mL microfuge tubes, immediately frozen on dry ice, and stored at -80°C until further analysis, completed within 2 weeks.

*Analysis Of Insulin And Insulin Receptors mRNAs*

Total RNA was extracted using TRI-Reagent® as specified by the manufacturer (Molecular Research Center, Cincinnati, OH, USA). Isolated RNA was dissolved in 50-100 µL RNase-free deionized water. Total RNA was quantified by ultraviolet ($A_{260}$) spectrophotometry and diluted to 100 ng/µL in RNase-free deionized water. RNA samples

were then stored at – 80$^{o}$C until further analysis. From 200 ng total RNA, endogenous poly(A)$^{+}$ RNA was reverse transcribed in a 10 μL reaction using an iScript™ cDNA Synthesis kit (BioRad, Knightdale, North Carolina, USA) containing a RNase H$^{+}$ reverse transcriptase and a blend of oligo(dT) and random hexamer primers according to the manufacturer's instructions. Reactions without reverse transcriptase were included as negative controls; no amplification was detected in negative controls.

Quantification of INS and IR mRNAs by real-time RT-PCR using Stratagene Brilliant II and the Stratagene MX3000P detection System (Stratagene, La Jolla, CA) were performed as described previously [4, 5]. Briefly, real-time reactions were carried out for samples, standards, and no-template controls in a 10 μl volume, containing 1 μl cDNA from the reverse transcription reactions, 5 μl Brilliant II QPCR Master Mix, and 1 μl of each forward primer (900 nM), reverse primer (900 nM), and probe (150 nM). Cycling parameters were set as follows: 95 °C for 10 min and 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Sample copy number was calculated from the threshold cycle number ($C_T$), and the $C_T$ was related to a gene-specific standard curve followed by normalization to β-actin. No difference ($P > 0.05$) was observed in β-actin expression among the various treatment groups. No-template control samples did not exceed a maximal increase of 300 (ΔRn) fluorescence units over 45 cycles. Therefore, copy numbers of mRNA were considered non-significant if $C_T$ exceeded 45 cycles; this value corresponds to a detection limit of less than 100 mRNA copies.

*Statistical Analysis*

Quantitative data are expressed as means ± S.E.M. Statistical differences were estimated by the Generalized Linear Mixed Model (GLIMMIX) approach using a split plot design. For the nutritional state experiment, whole plot treatments were time and the sub-plot treatments were INS/IR subtype. For *in vitro* culture experiments, whole plot treatments were dose and sub-plot treatments were INS/IR subtype. Insulin and IR subtype were treated as repeated measures, and an unstructured variance–covariance structure was used when fitting the model. A probability level (p-value) of 0.05 was used to indicate significance. In all cases, the main effects were found to be significant. Comparisons of simple effects were made by the least squares means method using a slicing approach in GLIMMIX; the maximum experiment-wise Type I error rate was controlled at 0.05 using a Bonferroni adjustment. Notations on the face of the figures denote simple effects comparisons; details of the main effects are given in the legends of the figures. All statistics were performed using SAS v 9.2 (Cary, NC, USA).

## Results

*Nutritional State Experiment*

Fish that were fed continuously for either 4 or 6 weeks displayed significant increases in growth, whereas the growth of animals that were continuously fasted was retarded. For continuously fed fish, body length, body weight, and condition factor were significantly increased over initial measures (Table 5). Fasted fish (4 weeks or 6 weeks) possessed the same body length as fish at the beginning of the experiments. In addition, fish fasted for 4 weeks or for 6 weeks displayed significantly lower body weight and

condition factor than their continuously fed counterparts. Fish that were refed for 2 weeks following a 4-week fast resumed growth, and exhibited body weight and condition factors that were significantly greater than their fasted counterparts (Table 5).

Two distinct INS-encoding mRNAs and four distinct IR-encoding mRNAs isoforms were detected by real-time quantitative RT-PCR. Steady-state levels of INS1 mRNA were more abundant than those of INS2 mRNA (Fig. 13) in Brockmann bodies; however, INS subtype mRNA abundance varied in adipose tissue and brain depending on time and experimental condition (e.g., fed; fast). Levels of both INS-encoding mRNAs were more abundant in Brockmann bodies than in adipose tissue or brain (data not shown). Expression of IR mRNAs were tissue dependent (Fig. 14). IR1 mRNA was found in greater abundance than that encoding IR2, IR3, and IR4 for all selected tissues, except skeletal muscle. IR2 mRNA was expressed to similar levels among liver, muscle (skeletal and cardiac), and gill--levels that were greater than those observed in Brockmann bodies and adipose tissue. IR3 mRNA was most abundant adipose tissue, followed by liver, gill, skeletal muscle, cardiac muscle, and Brockmann bodies. IR4 mRNA was most abundant in gill, cardiac muscle, and skeletal muscle, and least abundant in adipose, liver, and Brockmann bodies (data not shown).

Nutritional state modulated the expression of INS mRNAs. In Brockmann bodies, fasting reduced the expression of both INS1 and INS2 mRNAs, an effect that was most prominent after 6 weeks (Fig. 13A). Notably, fasting influenced the expression of both INS mRNAs to a similar extent, and the pattern observed in fed fish in which INS1 mRNA was more abundant that INS2 mRNA also was observed in fasted fish. Fasting had a similar effect on the expression of INS mRNAs in the brain, with levels if INS1 and INS2

significantly reduced at both 4 weeks and 6 weeks compared to their fed counterparts (Fig. 13C). By contrast, fasting increased or had no effect on expression of INS mRNAs in adipose tissue. Levels of the INS1 mRNA isoform were significantly elevated over those in fed fish at both 4 and 6 weeks, whereas levels of the INS2 mRNA isoform were significantly elevated only after 6 weeks of fasting (Fig. 13B). Refeeding fish that had been previously fasted generally reversed fasting-associated changes in INS expression. In Brockman bodies, levels of both INS1 and INS2 returned to levels observed in fed fish (Fig. 1A). In adipose tissue, levels of INS1 mRNA also trended toward returning to levels observed in fed fish, but INS2 expression did not rebound (Fig. 13B). Refeeding also failed to cause a rebound in the levels of INS1 and INS 2 mRNAs in brain (Fig. 13C). Insulin receptor mRNA levels also were affected by nutritional state. In Brockmann bodies, fasting significantly increased IR4 at 6 weeks (Fig. 14A). Refeeding reversed fasting-associated changes in IR4 mRNA expression in Brockmann bodies. In adipose tissue, a 4-week fast had no effect on IR expression. Extended fasting for 6 weeks increased the mRNA expression of IR4; refeeding reversed this fasting-associated change (Fig. 14B). Fasting for 4 weeks also had no effect on IR mRNA expression in liver; however, long-term fasting for 6 weeks decreased hepatic expression of IR3 mRNA (Fig. 14B). Refeeding did not return IR3 mRNA expression in liver to levels observed in continuously fed fish. Although fasting had no effect on IR expression in skeletal muscle, 6 weeks of fasting increased levels of IR4 mRNA in cardiac muscle, a change that tended to be reversed by refeeding (Fig. 14D and 14E). In gill, fasting had no effect on mRNA expression of IR subtypes after 4 weeks, but a 6-week fast increased levels of IR4 mRNA

Table 5. Effects of nutritional state on selected body characteristics of rainbow trout.[a]

| Characteristic | Initial | 4 weeks | | |
|---|---|---|---|---|
| | 6 weeks | | | |
| | **Fed** | **Fast** | **Fed** | **Fast** | **Fasted/refed** |
| Body weight (g) | 146.3 ± 5.8[ab] | 217.9 ± 11.3[c] | 119.0 ± 7.2[a] | 307.2 ± 15.3[d] | 132.3 ± 4.2[a] |
| 175.1 ± 19.0[b] | | | | | |
| Body length (cm) | 22.57 ± 0.3[a] | 25.06 ± 0.4[b] | 22.16 ± 0.4[a] | 27.51 ± 0.43[c] | 23.34 ± 0.23[a] |
| 24.03 ± 0.81[ab] | | | | | |
| Condition factor[b] | 1.27 ± 0.03[b] | 1.38 ± 0.02[c] | 1.09 ± 0.03[a] | 1.47 ± 0.02[d] | 1.04 ± 0.03[a] |
| 1.24 ± 0.04[b] | | | | | |

a

Fish were fed or fasted continuously for the period indicated; fasted/refed animals were fasted for 4 weeks, then refed for 2 weeks. Data are presented as means ± S.E.M. ($n$ = 7–9). For a given characteristic, groups with different letters are significantly different ($P < 0.05$) from one another.

b

Calculated as [body weight/(body length)$^3$] × 100.

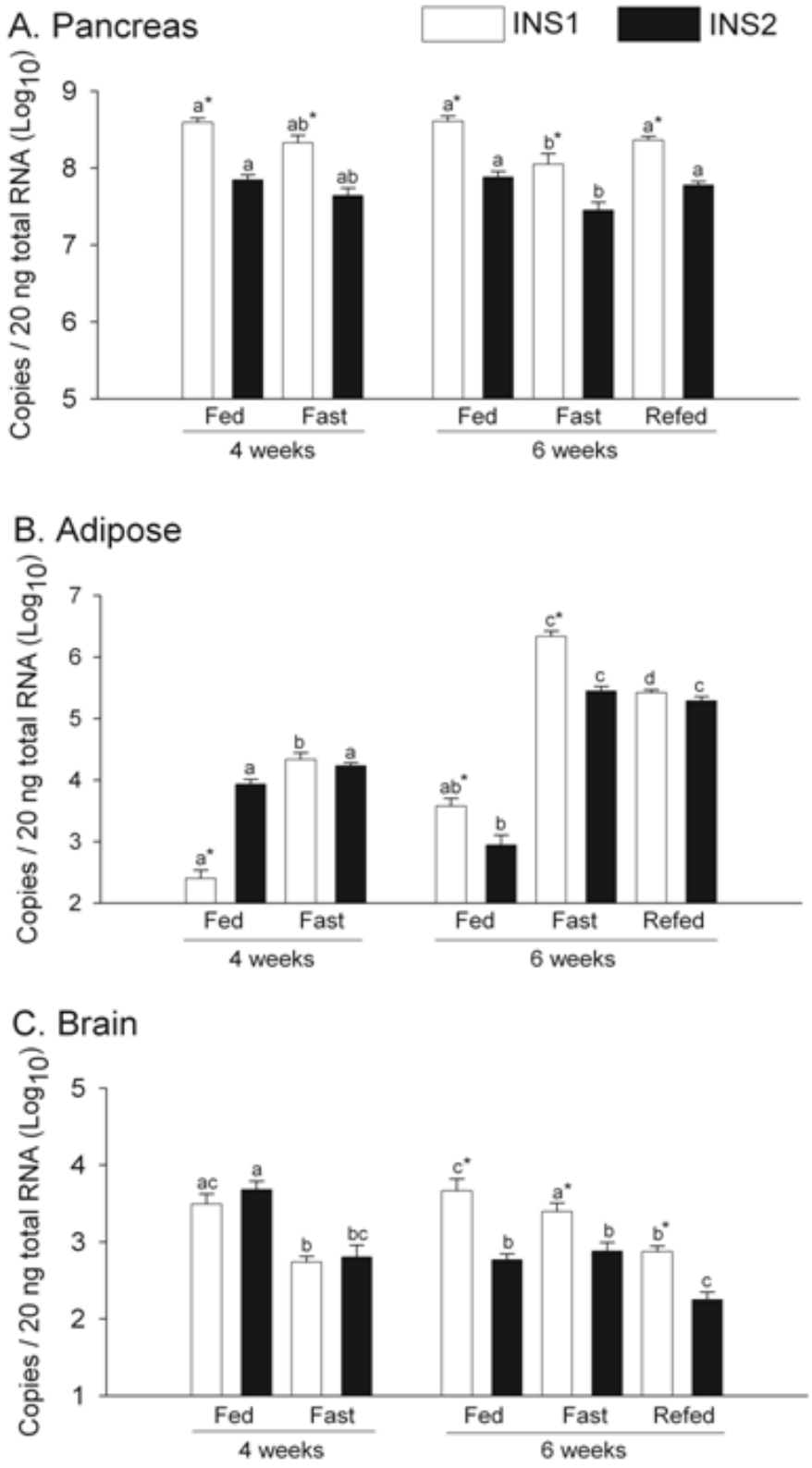
A. Pancreas
INS1
INS2
Copies / 20 ng total RNA (Log10)
9
8
7
6
5
Fed
Fast
Fed
Fast
Refed
4 weeks
6 weeks
B. Adipose
Copies / 20 ng total RNA (Log10)
7
6
5
4
3
2
Fed
Fast
Fed
Fast
Refed
4 weeks
6 weeks
C. Brain
Copies / 20 ng total RNA (Log10)
5
4
3
2
1
Fed
Fast
Fed
Fast
Refed
4 weeks
6 weeks

Figure 13. Changes in insulin subtype (INS1, INS2) mRNA levels in the pancreas (A), adipose (B), and brain (C) of rainbow trout associated with feeding, fasting, and refeeding. Data are presented as means ± SEM. For a particular tissue, letters denote differences for a given subtype; * denotes a difference in subtype. Differences between tissues are described only in text.

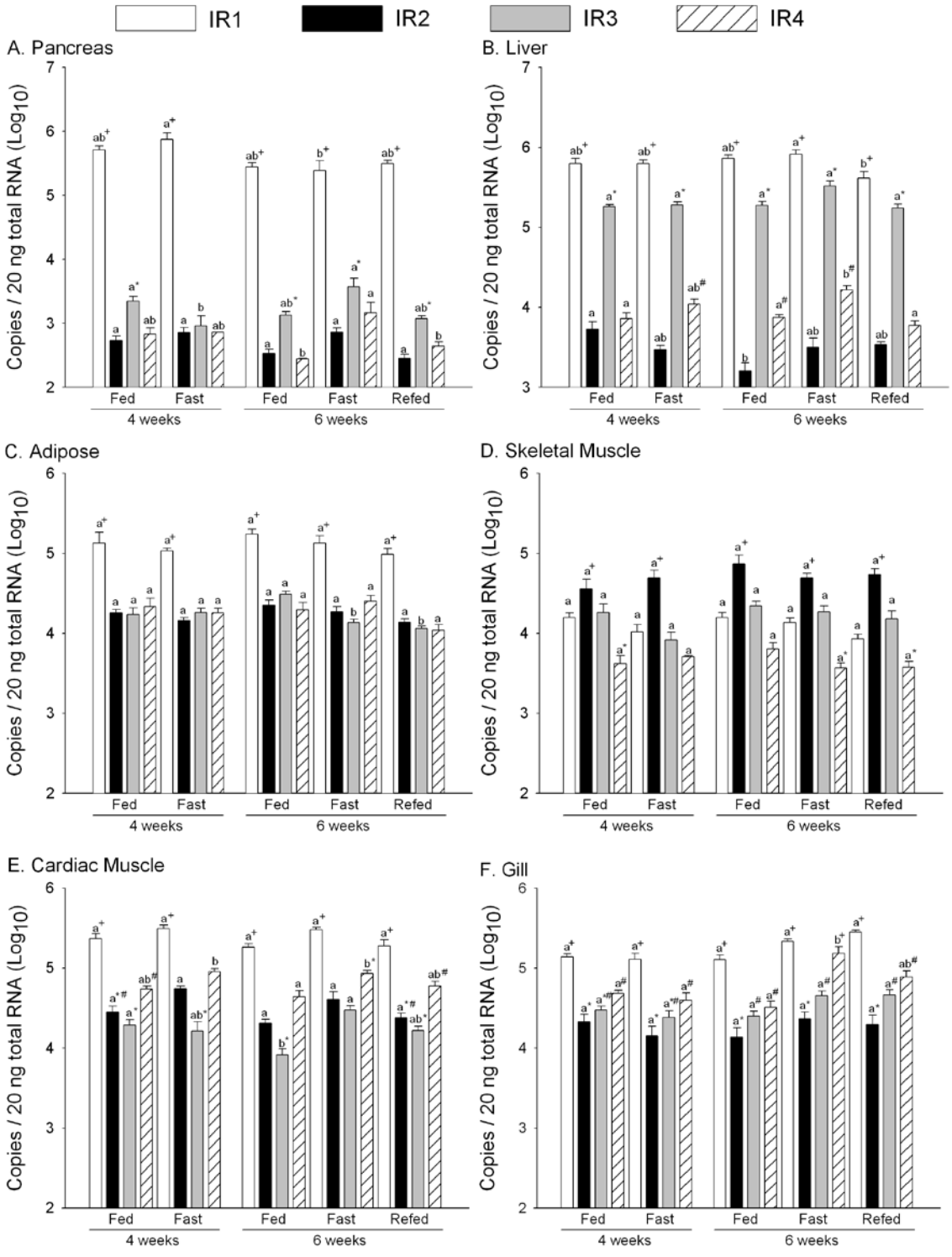
IR1
IR2
IR3
IR4
A. Pancreas
B. Liver
C. Adipose
D. Skeletal Muscle
E. Cardiac Muscle
F. Gill
Copies / 20 ng total RNA (Log10)
Fed
Fast
Refed
4 weeks
6 weeks

Figure 14. Changes in insulin receptor isoform (IR1, IR2, IR3, IR4) mRNA expression in the pancreas (A), adipose (B), liver (C), cardiac muscle (D), skeletal muscle (E), and gill (F) of rainbow trout associated with feeding, fasting, and refeeding. Data are presented as means ± SEM. For a particular tissue, letters denote differences for a given isoform; typographical symbols denote a difference in isoform. Differences between tissues are described only in text.

(Fig. 14F). Refeeding tended to return IR4 mRNA expression in gill to levels observed in fed fish.

*In vitro glucose challenge experiments*

Two INS-encoding mRNAs and four IR-encoding mRNAs were detected, respectively, in Brockmann bodies and liver pieces incubated *in vitro*. Glucose regulated the expression of INS mRNAs in Brockmann bodies (Fig. 15). The expression of INS mRNAs was lower at 1 mM glucose than at 4 mM glucose. Interestingly, at the highest glucose concentration (10 mM), levels of INS mRNAs were similar to those observed at both 1 and 4 mM glucose. Despite the changes induced in INS mRNA expression in Brockmann bodies, the pattern of expression was such that INS1 mRNA was more abundant that INS2 mRNA at all concentrations of glucose.

Glucose also regulated the expression of IR-encoding mRNAs in liver, but in an IR subtype-specific manner (Fig. 16). The expression of IR1 and IR2 mRNAs were insensitive to variations in glucose concentration. By contrast, the levels of IR3 and IR4 mRNAs increased when medium glucose increased from 1 to 4 mM. However, at high

glucose (10 mM), the abundance of IR3 and IR4 mRNAs declined to levels observed at 1 mM in a manner similar to the influence of glucose on INS mRNA expression. Across all concentrations of glucose, expression of IR4 mRNA was less than that of IR1, IR2, and IR3 mRNAs.

## Discussion

Previous research shows that fish possess multiple INS- and IR-encoding mRNAs (Caruso and Sheridan, 2011). Rainbow trout, for example, possess two distinct INS mRNAs and four distinct IR mRNAs that are differentially expressed in terms of distribution among tissues as well as in terms of abundance within specific tissues (Caruso et al., 2008; Caruso et al., 2010; Green and Chen, 1998). As noted previously, despite the fact that the two trout INS mRNAs encode the same INS A/B-chain, the C-peptides, and consequently, the proINS molecules differ (Caruso et al., 2008). Because proINS, INS, and C-peptide are co-secreted, the pattern of INS mRNA expression potentially influences the relative abundance of each of these bioactive peptides. Also as noted previously, the four trout IR mRNAs encode distinct IRs that possess differences in position and number of Ser, Thr, and Tyr residues that may influence receptor activation and cell signaling; therefore, the complement of IR receptor subtypes expressed on target cells may influence the response to INS (Caruso et al., 2010). The present study confirms that pattern of differential expression of mRNAs that encode INS isoforms and IR subtypes and supports our starting hypothesis that nutritional state regulates the expression of INS and IR mRNAs.

That nutritional state regulates the expression of INS-encoding mRNAs was supported by several observations. First, fasting decreased the expression both INS1 and INS2 mRNAs in Brockmann bodies, an effect that was most pronounced after 6 weeks. Notably, refeeding resulted in a rebound in the expression of both INS1 and INS2 to levels observed in fed fish.

Second, a 4-week fast increased the expression of INS1 mRNA but not of INS2 mRNA in adipose tissue; however, after 6 weeks, the expression of both INS1 and INS2 mRNAs in this tissue increased. Third, 4 weeks of fasting reduced the expression of both INS mRNAs in the brain. Collectively, these findings indicate for the first time to our knowledge that nutritional state regulates INS mRNA expression in both a tissue- and isoform-specific manner. Changes in INS expression in the Brockmann body--the largest contributor to the plasma INS pool based upon the abundance of INS mRNA and protein (Caruso et al., 2008)--are consistent with reports in several species of fish, including carp, brown trout, goldfish, Atlantic salmon, sea bass, cod, and rainbow trout, that fasting lowers plasma levels of INS which may be restored after a short period of re-feeding (cf. Caruso and Sheridan, 2011).

Nutritional state also regulated the expression of IR-encoding mRNAs in a tissue- and subtype-specific manner. This contention is supported by the observations that fasting either increased the expression of IR subtypes (liver IR3) or had no effect (white skeletal muscle) or increased the expression of IR subtypes (adipose tissue IR4, Brockmann body IR4, cardiac muscle IR4, gill IR4). Although refeeding was not able to reverse the deceased expression of IR3 mRNA associated with fasting in liver, the fasting-associated increases observed in non-hepatic tissues tended to be reversed. Similar observations have

been reported in chickens, which display a natural INS resistance akin to fish (Caruso and Sheridan, 2011), whereby a 48-h fast had no effect on IR mRNA and protein expression in muscle but decreased hepatic IR mRNA and protein expression, an effect restored by

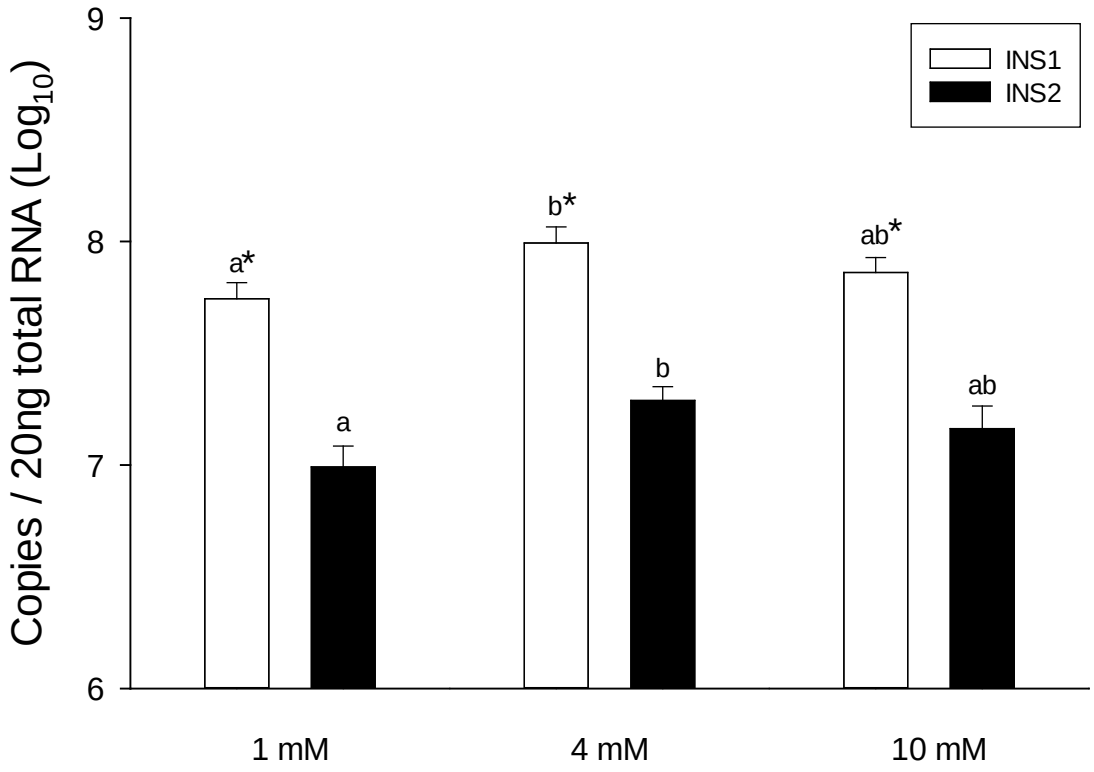


Figure 15. Effects of glucose on insulin subtype (INS1, INS2) mRNA expression from rainbow trout hemi-islets incubated *in vitro*. Glucose (at 1mM, 4mM, and 10mM) was dissolved in Hanks' buffered medium with differing NaCl concentrations to maintain osmolality. Data are presented as mean ± SEM. For a given INS subtype, groups with different letters are significantly ($P<0.05$) different; * denotes a significant ($P<0.05$) difference between INS subtypes.

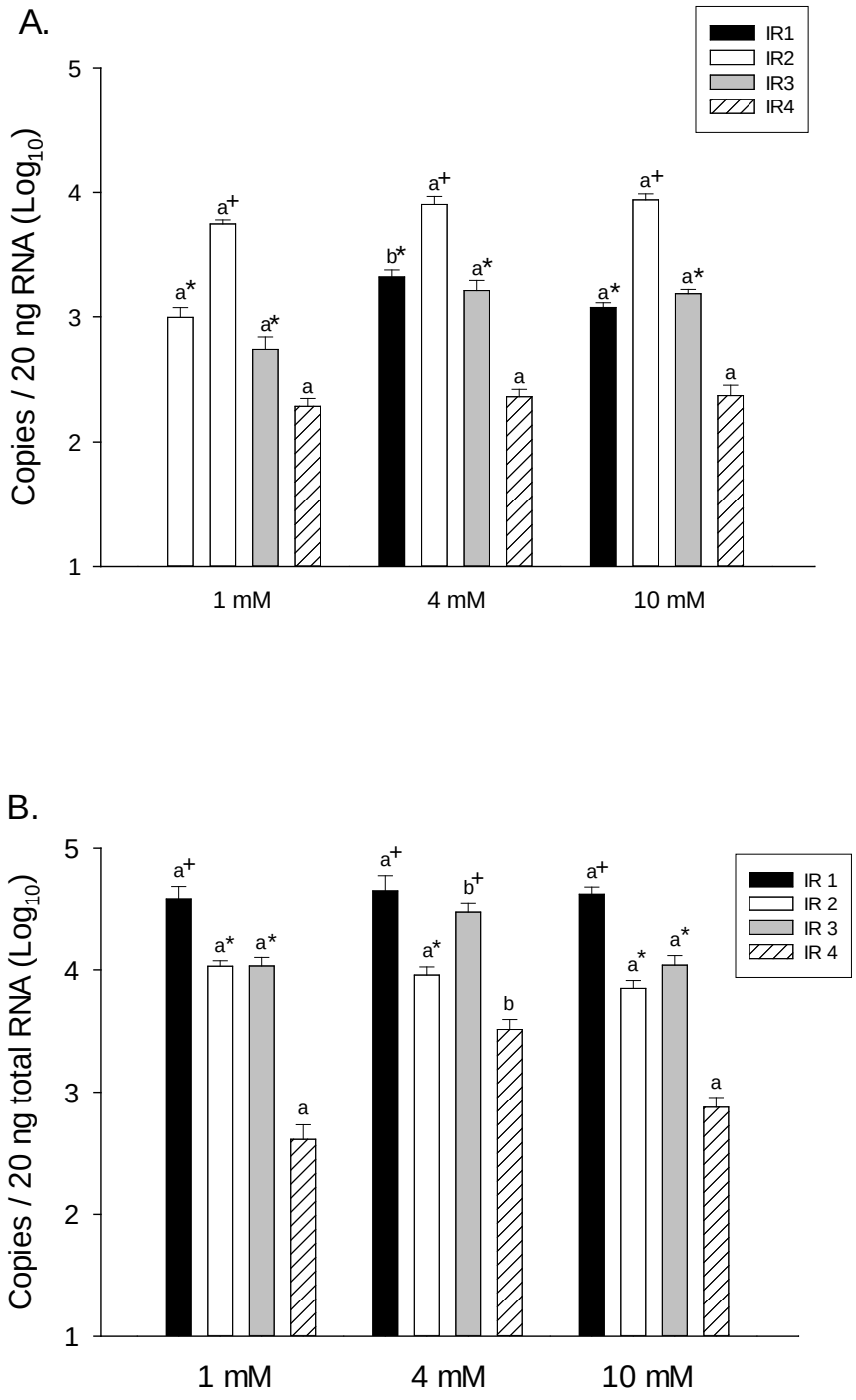


Figure 16. Effects of glucose on insulin receptor isoform (IR1, IR2, IR3, IR4) mRNA expression from rainbow trout pancreas (A) or liver (B) incubated *in vitro*. Glucose (at

1mM, 4mM, and 10mM) was dissolved in Hanks' buffered medium with differing NaCl concentrations to maintain osmolality. Data are presented as mean ± SEM. For a given IR isoform, groups with different letters are significantly ($P<0.05$) different; typographical symbols indicate a significant ($P<0.05$) difference between IR isoforms within the same tissue.

refeeding (Dupont et al., 1998). By contrast, fasting decreased the number of apparent IRs in white skeletal muscle of rainbow trout and sea bream, although it was not clear if this resulted from alterations in IR localization, synthesis, and or degradation [15, 26].

Nutritional regulation of INS and IR mRNAs may have several important roles. Following periods of feeding in fish, nutrients (e.g., glucose, amino acids, fatty acids) stimulate INS synthesis and secretion and, in turn, INS promotes the uptake of nutrients into peripheral tissues as well as the synthesis of macromolecules [e.g., glycogen, protein, triacylglycerol (TG)] in those tissues (Cowley and Sheridan, 1993; Figueirdo-Garutti et al., 2002; Mommsen and Plisetskaya, 1991; Perez et al., 1989; Plisetskaya, 1989). INS also inhibits the breakdown of stored TG (Harmon and Sheridan, 1992). The current findings support feeding-stimulated production of INS not only in the pancreatic tissue, but in adipose tissue and brain as well. The significance of extrapancreatic INS is unknown, but it may act locally (in a paracrine/autocrine fashion) to influence nutrient metabolism as adipose tissue and brain store significant amounts of TG and glycogen, respectively (Mommsen and Plisetskaya, 1991). Local production of INS also may be important for regulating appetite and feeding behavior as intracerebraoventricular (ICV) injection of INS promotes anorexia in rainbow trout (Soengas and Aldegunde, 2004). The current findings

also demonstrate widespread distribution of IRs among tissues that are necessary for the peripheral actions of INS during times of feeding.

During periods of food deprivation in fish, there is a shift from an anabolic state to a catabolic state in which stored macromolecules (e.g., glycogen, TG, protein) are broken down, gluconeogenesis and ketogenesis, primarily in the liver, is initiated, and the resulting products (e.g., glucose, fatty acids, ketones) are mobilized into the blood (Mommsen and Plisetskaya, 1991; Moon et al., 1989; Sheridan and Mommsen, 1991). Fasting-associated catabolism is accompanied by reduced mesenteric fat mass, reduced liver mass, reduced body condition, and reduced body growth (Kittilson et al., 2011). The anabolic to catabolic shift in fish is mediated by the aforementioned decline in plasma levels of INS (cf. Caruso and Sheridan, 2011) as well as by increased levels of several hormones including glucagon and somatostatin (Moon et al., 1989; Sheridan and Kittilson, 2004; Sheridan and Mommsen, 1991). The present findings of reduced pancreatic INS mRNA expression would underlie the reduced levels of plasma INS observed during fasting and would be adaptive for facilitating catabolic processes. The significance of the fasting-associated changes observed in brain (decreased INS expression) and adipose tissue (increased INS expression) are not clear, but they may be related to enhanced glycogen/ketone body utilization and lipid/lipoprotein processing, which are adaptive during periods of food deprivation. Although daily brain INS mRNA expression levels have not been observed in rainbow trout, brain glycogen has been shown to correlate with daily feeding regimes (Figueroa et al., 2000; Polakof et al., 2007).

The observed prolonged (6 weeks) fasting-associated decrease in specific IR subtype expression in liver that, in turn, could contribute to reduced sensitivity to INS and

potentially favor the increased lipolytic, glycogenolytic, gluconeogenetic, and ketogenetic processes that mobilize stored nutrients from this tissue for use by peripheral tissues during periods of food deprivation. Our findings further suggest that IR subtype 4 may be preferentially linked to hepatic catabolic processes. By contrast, the failure to decrease IR expression or to increase IR expression in peripheral tissues such a skeletal muscle, cardiac muscle, and gill would be adaptive for maintaining/increasing the sensitivity of the these tissues to INS so as to facilitate the uptake of nutrients in the face of declining INS and/or to contribute to conservation of protein ("protein sparing"), particularly crucial in muscle tissue, when food is not available. The significance of the increase in IR expression in adipose tissue is unclear, but it may be related to heightening the processing of lipids and lipoprotein mentioned above. The increase in IR expression Brockmann bodies during fasting also is unknown, but it may be related to intraislet regulation. Notably, all fasting-associated alterations in IR expression in extrahepatic tissues involve IR3.

Glucose modulated the expression of INS-encoding mRNAs in Brockmann bodies and of IR-encoding mRNAs in the liver of rainbow trout. Although an increase in glucose from 1 mM to 4 mM increased the expression of both INS1 mRNA and INS2 mRNA, expression of IR mRNAs at 10 mM glucose decreased to levels observed at 1 mM. These findings are consistent with previous studies that show that plasma levels of INS increase in carnivorous fish following a glucose challenge, but their response is blunted, resulting in glucose intolerance (Harmon et al., 1991; Moon, 2001; Plisetskaya and Duan, 1994). Interestingly, however, the promoter of the INS gene from the omnivorous tilapia has been characterized and glucose was unable to increase reporter activity in promoter constructs (Monsour et al., 1998). Glucose also affected the expression of IR as manifested by an

increase in the expression of IR3 and IR4 at 4 mM glucose compared to 1 mM; however, at 10 mM glucose levels of IR3 and IR4 were similar to those at 1 mM. The subtle changes in IR subtype mRNA expression may be due in part to the glucose intolerance of rainbow trout, in which amino acids are more widely utilized than glucose (Koricanac et al., 2004). Additionally, the absence of INS in the experimental medium may retard glucose uptake by the liver, subsequently diminishing regulatory feedbacks. It should be noted, however, that glucose uptake may have occurred despite the absence of INS via the glucose transporter, GLUT-2. In mammals, uptake of glucose by the liver and pancreas is mediated by the GLUT-2 independently of INS (Thorens et al., 1992). Glucose transporter-2 has been identified in the liver of zebrafish (Castillo et al., 2009) and sea bass (Terova et al., 2009), and in the Brockmann bodies of rainbow trout (Krasnov et al., 2001) and Atlantic cod (Hall et al., 2004). Moreover, nutritional status does not appear to mediate the expression of GLUT-2 within the liver of Atlantic cod (Hall et al., 2004) or rainbow trout (Panserat et al., 2001). However, GLUT-2 expression increases in Brockmann bodies of rainbow trout fed a high-carbohydrate diet (Polakof et al., 2008). Regardless, an increase in glucose has shown to potentiate IR mRNA expression in mammalian liver tissue and hepatocyte cell cultures (Briata et al., 1989). More similar to carnivorous fish, INS resistance in mammals is marked by decreased glucose utilization, especially in the liver, which in some cases is correlated to decreased IR protein and mRNA expression (Koricanac et al., 2004).

In summary, we demonstrated that the expression of INS-encoding and IR-encoding mRNAs are regulated by nutritional state and glucose. Furthermore, the findings suggest that independent mechanisms exist to regulate expression of INS and IR mRNAs in a tissue- and isoform/subtype-specific manner.

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# CHAPTER FOUR. GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR REGULATE THE EXPRESSION OF INSULIN AND INSULIN RECEPTOR mRNAS IN RAINBOW TROUT (*Oncorhynchus mykiss*)


## Abstract

Insulin (INS) is a critical peptide hormone that coordinates various aspects of growth, development, and metabolism through interactions with multiple insulin receptor (IR) isoforms on various target tissues. In this study, we used rainbow trout as a model system to survey the effects of growth hormone (GH) and insulin-like growth factor-I (IGF-I) on the expression of INS1, INS2, IR1, IR2, IR3, and IR4 mRNAs. GH and IGF-I regulated the expression of INS and IR in a isoform- and tissue-specific manner. GH-implanted animals displayed increased INS1 and INS2 in the pancreas; reduced INS1 and INS2 in adipose; no change within the brain; reduced IR1, IR2, IR3, and IR4 expression in the cardiac muscle; reduced IR1 in the gill; increased IR3 in the liver; and increased INS1, INS2, IR2, IR3, and IR4 in the pancreas. GH treatment *in vitro* displayed no change in INS1 and INS2, decrease IR4 over time, and decrease in IR2 with increasing dose in pancreas; decreased IR2 with an increasing dose in the liver; and decreased IR1, IR2, and IR3 over time in the gill. IGF-I treatment *in vitro* increased the expression of INS1 and INS2, and decreased IR3 and IR4 over time in the pancreas; decreased IR1, IR2, and IR4 over time in the liver; and decreased IR1, and increased IR3 and IR4 over time in the gill. These results demonstrate that GH and IGF-I modulate the expression of INS1, INS2, IR1, IR2, IR3, and IR4, and the results imply that independent mechanisms may serve to regulate the various isoforms in a tissue specific manner.

## Introduction

Growth and metabolism in vertebrates amalgamates a myriad of physiological processes, from infancy, juvenile, and adult, which are guided by the meticulous scheme of biochemical mediators. The growth hormone (GH) - insulin-like growth factor-1 (IGF-I) axis is a basic model used to describe the mode of growth in vertebrates (Holzenberger et al., 2006). IGF-I synthesis and secretion is stimulated by the presence of GH in the liver and other peripheral tissues (Bjornsson et al., 2002). Additionally, IGF-I may promote its own production within peripheral tissues in an autocrine and paracrine positive regulatory feedback manner (Duan et al., 1994). INS is known to interact with GH and IGF-I in a synergistic manner, promoting anabolism within several tissues (Wood et al., 2005). GH initiates growth by means of protein anabolism and indirectly by promoting the secretion of both INS and IGF-I (Dominici et al., 2005). INS directly increases tissue accretion in fish by promoting lipogenesis, glucose uptake, $^{35}SO_4^{2-}$ incorporation, and protein synthesis (cf. Caruso and Sheridan, 2011). INS also increases growth indirectly by increasing IGF-I production from the liver when it is synergized with GH (Plisetskaya 1998).

INS's metabolic repercussions affect nearly all tissue types, providing a signal for an incoming hoard of nutrients. Although certain species of fish have a limited ability to utilize dietary carbohydrates, INS will induce the stimulation of glucose uptake and glycogen synthesis (Novoa et al., 2004; Rojas et al., 2009). Despite similarities in the growth promoting actions of INS and GH, they display disparate metabolic functions. GH treatment *in vitro* has been shown to stimulate hepatic lipid mobilization in rainbow trout

(O'Connor et al., 1993). Conversely, INS counteracts GH effects on the liver and promotes lipogenesis (Sheridan and Kittilson, 2004). INS also down regulates gluconeogenic enzymes in the liver and primary hepatocytes of rainbow trout, similar to mammals (Plagnes-Juan et al., 2008; Ramnanan et al., 2010). GH, on the other hand, decreases glycogenic potential and increases gluconeogenic enzymes in rainbow trout (Sangiao-Alvarellos et al., 2005; Sheridan, 1986).

The actions of INS and IGF-I are mediated by tyrosine kinase (TK) receptors, affecting the transduction of intracellular signals, including Akt/PKB, which modulate mitogenic and metabolic responses within target tissues (Plagnes-Juan et al., 2008). IRs are known to be ubiquitous in most vertebrates; consequently, INS's mitogenic effects span several tissue types. INS's growth-promoting abilities are like those of IGF-I and may share a common intracellular mechanism as well as the ability to bind each other's receptors (Barbieri et al., 2003). GH is known to interact with two receptor subtypes in rainbow trout, GHR1 and GHR2 (Very et al., 2005). When bound, GH activates a number of cellular effectors including STAT5, PI3K, and ERK, which have the potential to directly cross talk with similar insulin signaling cellular effectors (Xu et al., 2009).

Recently, two INS mRNAs and four IR mRNAs were isolated and characterized from rainbow trout, which are differently expressed among and within tissues (Caruso et al., 2007; Caruso et al., 2010; Greene and Chen, 1999). Therefore, the INS signaling system in rainbow trout consists of multiple INS products and multiple IR isoforms, which encompass the capacity to elicit multiple responses within a single target tissue. Despite the importance of INSs and IRs in the orchestration of essential biological processes, little has been reported about the other hormonal factors that may serve to regulate their

production. In this study, we used rainbow trout to evaluate the effects of GH and IGF-I on the expression of INS1, INS2, IR1, IR2, IR3, and IR4. The rationale for this study stemmed from the known interactions between GH, IGF-I, and INS on the regulation of growth in vertebrates.

## Materials and methods

*Experimental Animals*

Juvenile rainbow trout (*Oncorhynchus mykiss*) of both sexes were obtained from Dakota Trout Ranch near Carrington, ND, USA. Following transport to North Dakota State University, the fish were maintained in well-aerated, 800-L circular tanks supplied with recirculated (10% replacement volume per day) dechlorinated municipal water at 14$^{o}$C under a 12h light:12h darkness photoperiod. Fish were fed to satiation twice daily with AquaMax™ Grower (PWI Nutrition International. Brentwood, MO, USA), except 24-36 h before experimental manipulations. Animals were acclimated to laboratory conditions for at least 6 weeks prior to experiments. All experimental protocols were reviewed and approved by the North Dakota State University Animal Care and Use Committee.

*Experimental Treatment In Vivo*

The effects of growth hormone (GH) on the patterns of insulin(s) and insulin receptor(s) mRNA expression were evaluated by implanting fish with Alzet® mini-osmotic pumps (Alza; Palo Alto, CA) containing either 0.75% (w/v) NaCl (control) or 200 ng/ml ovine growth hormone (obtained from NIH). Mini-pump flow rate was established to be 0.135 μl $h^{-1}$, which at 14°C should provide sustained release for 29 days. Fish were first anesthetized with 0.05% (v/v) 2-phenoxyethanol, and their body length and mass were

determined. Mini-pumps were inserted into the peritoneal cavity through a 1.0-cm incision that was made ca. 0.5 cm right of the ventral midline and ca. 2.0 cm rostral of the pelvic fins. The incision was closed with two stitches and antibiotic ointment (Neosporin®) was applied topically to the incision area. Fish were placed into 100-L tanks (one tank for each treatment group, n=6) under the same conditions as acclimation, except that each tank was treated with 250 mg erythromycin with no water turnover for 30 min while supplemented with pure oxygen and feeding was suspended. The entire implantation procedure took about 1 min per fish. Seventy-two hours after tagging and transfer, fish were fed twice daily at a ration of 2% of their initial body mass per feeding (ca. 20.4 g) and continued for the duration of the experiment, except 24h prior to sampling. One hour post feeding excess food was siphoned, collected, removed of moisture in an oven until a constant mass was determined (ca. 2 hours at 100$^{o}$C), and weighed in order to calculate food intake (7% increase to dried food was added to adjust for moisture content). Selected tissues [brain, gill, cardiac muscle, skeletal muscle, adipose, liver and endocrine pancreas (Brockmann body)] were sampled 21 days after implantation from anesthetized fish as before. Tissues were immediately frozen on dry ice for later analyses. The selection of tissues was based on their central importance in growth and metabolic processes and because of high abundance and pattern of differential expression of INS/IR mRNAs determined previously (Caruso et al., 2008; Caruso and Sheridan, 2010).

*Preparation Of Pancreatic Islets*

For *in vitro* experiments, the fish were anesthetized and their Brockmann bodies removed and prepared for culture. In each study, trout Brockmann bodies were removed from anesthetized (0.05% (v/v) 2-phenoxyethanol) fish and placed in a chilled (14$^{o}$C) petri

dish containing a modified Hanks' medium [in mM: 137 NaCl, 5.4 KCl, 4 $NaHCO_3$, 1.7 $CaCl_2$, 0.8 $MgSO_4$, 0.5 KH2$PO_4$, 0.3 $Na_2HPO_4$, 10 N-2-hydroxyehtylpiperazine-N'-2-ethanesulfonic acid, and 0.24% bovine serum albumin (BSA) supplemented with 5 mM glucose, pH 7.6; referred to as basal medium]. Individual organs were dissected further to remove surrounding connective and exocrine tissues and then longitudinally bisected (to yield hemi-islets) and placed in fresh basal medium for a 2-h preincubation at 14$^oC$ and gassed with 100% $O_2$. *In vitro* cultures were preformed in 24-well polystyrene culture plates (Falcon 3407) containing 1 ml of experimental medium (GH or IGF-I) or control (basal) medium at 14$^oC$. Two to three hemi-islets were placed in each well and incubation was initiated for a period of up to 120 min *14$^oC$, gassed with 100% $O_2$). Incubation proceeded under the same conditions as preincubation for up to 24h, after which the medium was removed and the islets were immediately frozen on dry ice. Islets were stored at -80$^o$ until RNA extraction and quantification.

*Preparation Of Liver Samples*

Rainbow trout were anesthetized with 0.05% (v/v) 2-phenoxy ethanol immediately before each experiment. A minimum of 6 livers were used for each experiment; each liver is used as an individual replicate. Livers were removed and immediately perfused with ice-cold Medium A (in mM: 136.9 NaCl, 5.4 KCl, 0.81 $MgSO_4$, 0.44 $KH_2PO_4$, 0.33 $Na_2HPO_4$, 10 HEPES, 5 $NaHCO_3$; pH7.6) to remove blood. In a petri dish on ice, livers were diced into 1 mm cubes using razor blades. Each diced liver was washed 3 times with Medium A, followed by 2 times with Incubation Medium [In Medium A: 0.24% deffated BSA, 1.5mM $CaCl_2$, 3 mM glucose, 2 mL Gibco MEM amino acid mix (50X) per 100mL, 1 mL Gibco nonessential amino acid mix (100X) per 100 mL; pH 7.63]. Six to eight liver cubes were

placed in each well of 24-well culture plates. One-milliliter incubation medium was added to each well and liver pieces were allowed to recover for 1h at 14$^{o}$C with orbital shaking and 100% oxygen. After recovery, medium was removed and liver pieces were rinsed with 1 mL Incubation Medium. 900 μL incubation medium was added to each well containing liver samples for quantification of INS and IR mRNAs. Experimental treatments are applied to the liver samples in medium as described below.

*Preparation Of Gill Samples*

Rainbow trout were anesthetized with 0.05% (v/v) 2-phenoxy ethanol immediately before each experiment. A minimum of 6 fish were used for each experiment. Fish were bled, and gill arches removed and stored in ice-cold Dulbecco's Modified Eagle's Medium Base (DMEM) (Sigma D-5030 plus in mM: 27 NaCl, 4 L-glutamine, 4 glucose, 10 HEPES, 4 $NaHCO_3$, 0.24% BSA; pH 7.6). In a petri dish on ice, individual gill filaments were removed from the arches of DMEM using razor blades. Individual filaments were pooled and washed 3 times with DMEM. Nine to eleven gill filaments were placed in each well of 24-well culture plates. One-milliliter DMEM was added to each well and gill filaments were allowed to recover for 1 h at 14$^{o}$C with shaking and 100% oxygen. After recovery, medium was removed and gill was rinsed with 0.5mL DMEM before addition of 700 μL DMEM plus antibiotics (100 U/mL Penicillin; 100 μg/mL Streptomycin; 0.1% (v/v) Fungizone, pH 7.6) to each well. Experimental treatments were applied to the gill samples in medium as described below.

*Dose Experiment*

Hormone (GH, IGF-I) diluted in Incubation Medium was added in a 50 μL volume to each well, giving final incubation concentration of 1000 ng/mL, 100ng/mL, 10 ng/mL, and 1 ng/mL. Incubation Medium is added to 0 ng/mL control wells. Samples to be measured are incubated for 6h at 14$^{o}$C with shaking and 100% $O_2$. Liver samples were collected, frozen on dry ice, and stored at -80$^{o}$C for later RNA extraction.

*Time Course Experiment*

Based upon results from the dose experiment, an appropriate and effective dose is chosen for the time course experiment. Liver samples were prepared as described above. Hormone (e.g. GH, IGF, etc.) was added at the appropriate dose to each well in a 50 μL volume (n=8). For measurement of insulin receptor mRNA(s), liver samples for time=0 group were immediately collected, frozen on dry ice, and stored at -80$^{o}$C for later analysis by real-time PCR. All other samples were collected and frozen at the appropriate times (t=1,3, 6, 12, 24 h).

*RNA Extraction*

Total RNA was extracted using TRI-Reagent® as specified by the manufacturer (Molecular Research Center, Cincinnati, OH, USA). Isolated RNA was dissolved in 50-100 μL Rnase-free deionized water. Total RNA was quantified by ultraviolet ($A_{260}$) spectrophotometry and diluted to 100 ng/μL in RNase-free deionized water. RNA samples were then stored at –80$^{o}$C until further analysis.

*Real-Time PCR Assay For Insulin And Insulin Receptor-Encoding mRNAs*

Quantification of rainbow trout INS1, INS2, IR1, IR2, IR3, and IR4 mRNAs were measured as described previously (Caruso et al., 2008; Caruso et al., 2010). Briefly, from 200 ng total RNA, endogenous poly(A)$^+$ RNA was reverse transcribed in a 10 μL reaction using a iScript™ cDNA Synthesis kit (BioRad, Knightdale, North Carolina, USA) containing a RNase H$^+$ reverse transcriptase and a blend of oligo(dT) and random hexamer primers according to the manufacturer's instructions. Reactions without reverse transcriptase were included as negative controls; no amplification was detected in negative controls.

mRNA levels of the four IR-encoding subtypes were determined by real-time RT-PCR using Stratagene Brilliant II and a STRATAGENE MX3000P® Detection System (Stratagene, La Jolla, CA, USA). Real-time PCR reactions were carried out for samples, standards, and no-template controls in a 10 μL reaction; each reaction contained 1 μL cDNA, 1μL each of forward primer, reverse primer, and probe at concentrations optimized for the mRNA species to be measured, 1 μL RNase-free deionized water, and 5 μL TaqMan Universal PCR Master Mix. Cycling parameters for real-time PCR were as follows: 95$^o$C for 10 min, and 45 cycles for 95$^o$C for 15 s plus 63$^o$C for 30 s plus 72$^o$C for 45 s.

*Data Analysis*

Copy number calculations were based on threshold cycle number ($C_T$). The $C_T$ for each sample was determined by the MX3000P™ real time analysis detection software after manually setting the threshold. Sample copy number was determined by relating $C_T$ to a gene-specific standard curve, followed by normalization to β-actin. No difference ($P>0.05$)

was observed in β-actin expression among the various treatment groups. No-template control samples did not exceed a maximal increase of 300 (ΔRn) fluorescence units over 45 cycles. Therefore, copy numbers of mRNA were considered non-significant if $C_T$ exceeded 45 cycles; this value corresponds to a detection limit of less than 100 mRNA copies.

*Statistical Analyses*

Quantitative data are expressed as means ± S.E.M. There were 6 individual replicates for the implantation experiment and the tissue culture experiment. For the implantation experiment, statistical differences were estimated by ANOVA, followed by Duncan's multiple range test; a probability level of 0.05 was used to indicate significance. Statistics for the implantation experiment were performed using SigmaStat v. 1.0 (SPSS, Chicago, IL, USA). Statistical differences for the tissue culture experiment were estimated by the Generalized Linear Mixed Model (GLIMMIX) approach using a split plot design. Whole plot treatments were either time or dose and the sub-plot treatments were INS/IR isoforms. Insulin and IR subtype were treated as repeated measure (both INS1 and INS2 or all IR1, IR2, IR3, and IR4 were measured in the same time/dose group) and an unstructured variance–covariance structure was used when fitting the model. A probability level (p-value) of 0.05 was used to indicate significance. In all cases, the main effects were found to be significant. Comparisons of simple effects were made by the least squares means method using a slicing approach in GLIMMIX; the maximum experiment-wise Type I error rate was controlled at 0.05 using a Bonferroni adjustment. Notations on the face of the figures denote simple effects comparisons; details of the main effects are given in the

legends of the figures. Statistics for tissue culture experiments were performed using SAS v 9.2 (Cary, NC, USA).

## Results

*Food Conversion*

Although food intake was similar between saline- and GH-implanted fish, food conversion was 37% higher in GH treated animals compared to controls (Fig. 17).

*Relative Growth*

Growth Hormone implantation resulted in significant growth, in which the most pronounced effect was also on body length. Relative growth in mass was increased by 42%, whereas relative growth in length was increased by 140% in GH treated fish compared to controls. The condition factor of GH implanted fish was also significantly increased compared to saline-implanted fish (Fig. 18).

*Effects Of GH-Implantation On INS Expression*

The effects of GH on INS expression varied by isoform as well as by tissue. Two isoforms of INS mRNA were detected: INS1 and INS2. GH implantation increased steady-state levels of both INS1 by 46% and INS2 by 205% in the pancreas (Fig. 19A). In adipose, GH treatment decreased the expression of INSs; INS1 decreased by 82% and INS2 decreased by 63% compared to controls (Fig. 19B). In the brain, GH implantation had no effect on INS1/2 expression (Fig. 19C).

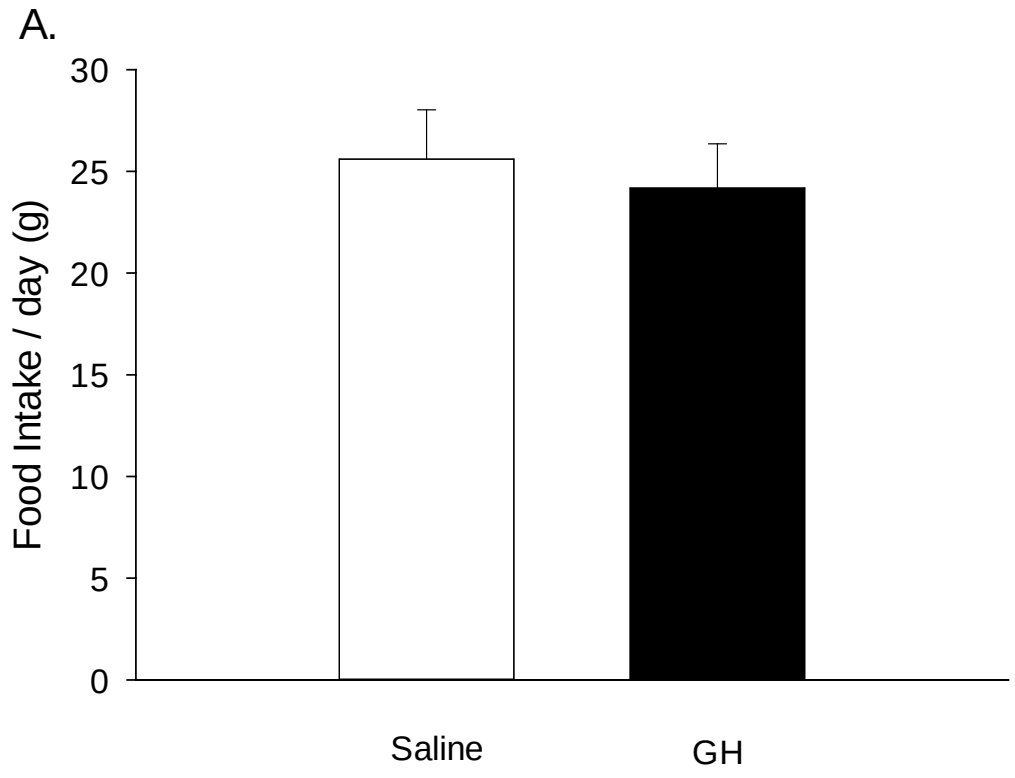
*Effects Of GH-Implantation On IR Expression*

The effects of GH implantation on IR expression also varied by receptor isoform as well as by tissue. Four isoforms of IR mRNA were detected: IR1, IR2, IR3, and IR4. In pancreas, GH had no effect on IR1; and increased IR2 by 52%, IR3 by 98%, and IR4 by 165% (Fig. 20A). In adipose, GH decreased IR2 by 43% but other IR mRNAs remained unchanged (Fig. 20B). In liver, GH did not affect the expression of IR1, IR2, and IR4 but did increase IR3 by 96% (Fig. 20C). In cardiac muscle, GH decreased the expression of all IRs; GH declined IR1 by 59%, IR2 by 59%, IR3 by 77%, and IR4 by 30% (Fig. 20D). In skeletal muscle, GH had no effect on IR mRNAs except an increase of IR3 by 350% (Fig. 20E). In gill, GH implantation decreased the expression or had no to little effect on all IRs. GH decreased IR1 by 35% while IR2, IR3, and IR4 had no effect relative to control (Fig. 20F).

*Effects of IGF-I And GH Treatment On INS Expression In Pancreatic Hemi-Islets*

To determine whether or not the effects of GH on INS expression *in vivo* were direct, INS mRNAs were measured in isolated pancreatic hemi-islets. Because GH is known to mediate and promote IGF-I secretion, IGF-I also was administered *in vitro*. In general, the effects of GH on INS isoform expression did not vary within pancreatic hemi-islets incubated *in vitro*. Two isoforms of INS mRNA were detected: INS1 and INS2. GH treatment resulted in no significant change in INS expression across time or dose (Fig. 21A).

The effects of IGF-I on INS expression varied by isoform within pancreatic hemi-islets incubated *in vitro*. The dose course of IGF-I-stimulated (0, 1, 10, 100, 1000 ng/ml)


A.
Food Intake / day (g)
30
25
20
15
10
5
0
Saline
GH

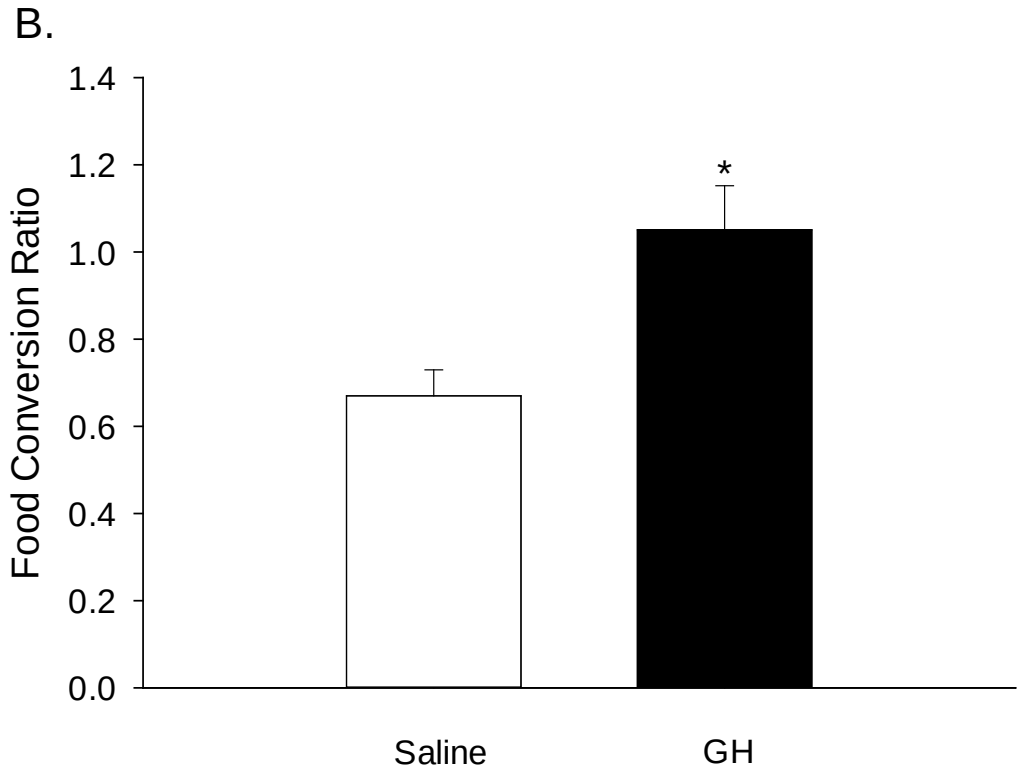
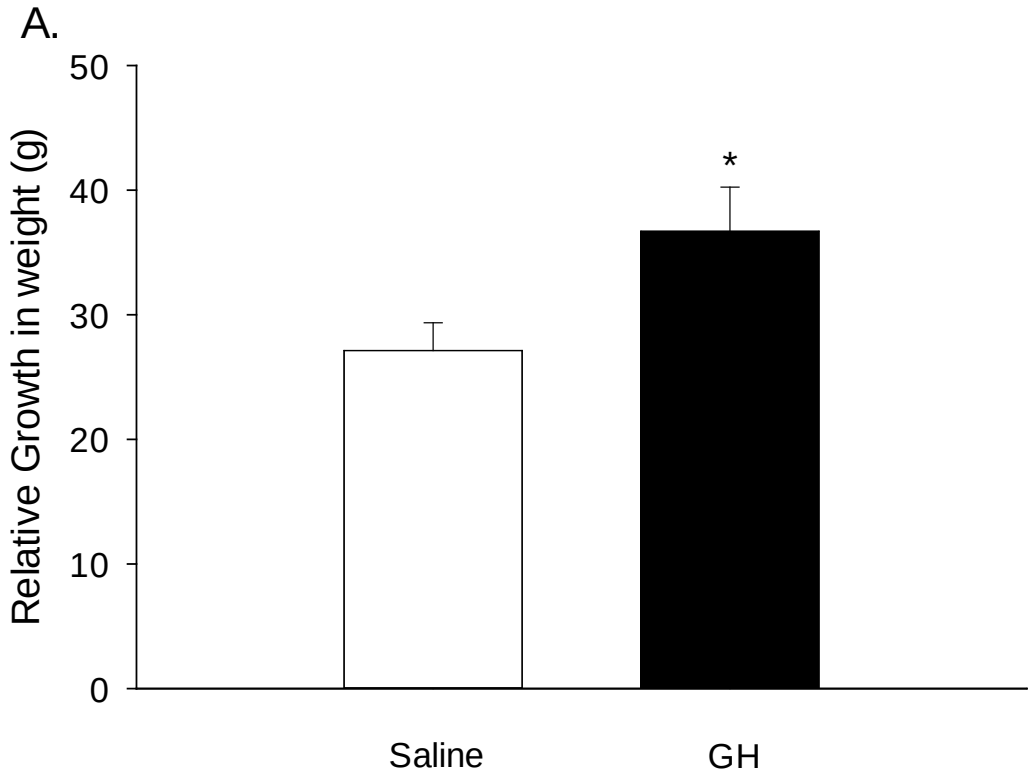
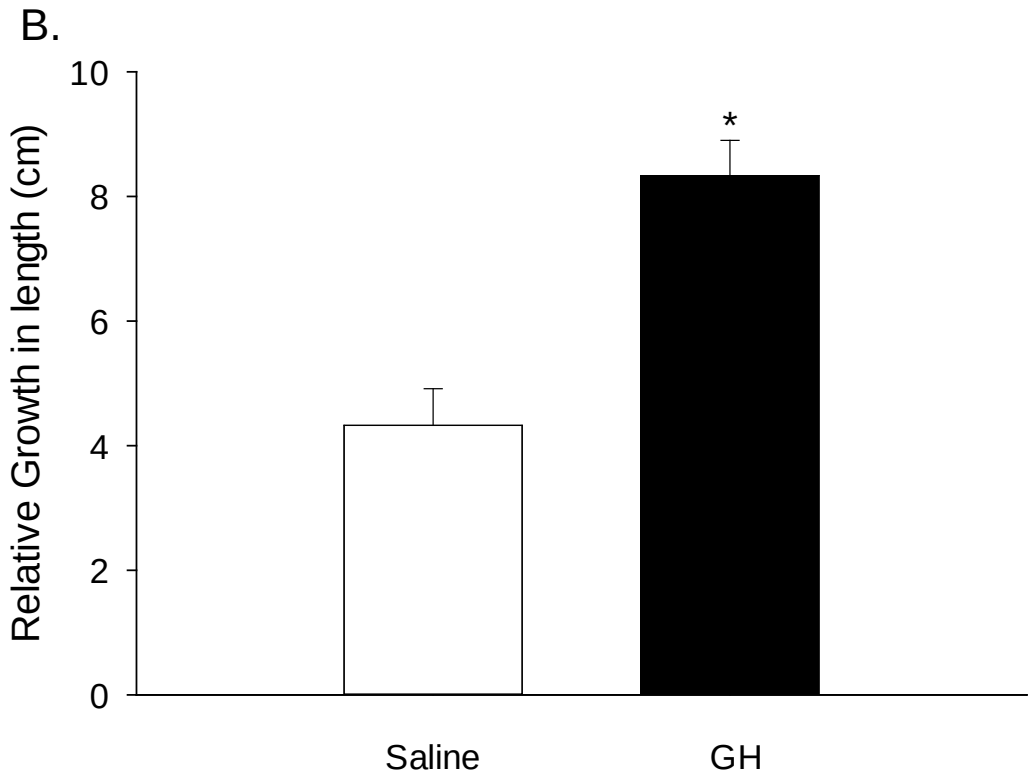

B.
Food Conversion Ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Saline
GH

Figure 17. Average daily food intake (A) and food conversion ratio (B). Food conversion ratio was calculated as (body wet mass gain) (dry weight food intake per fish)$^{-1}$. Data are presented as mean ± SEM. For a given treatment, groups with different letters are significantly ($P<0.05$) different.


A.
Relative Growth in weight (g)
50
40
30
20
10
0
*
Saline
GH


B.
Relative Growth in length (cm)
10
8
6
4
2
0
*
Saline
GH

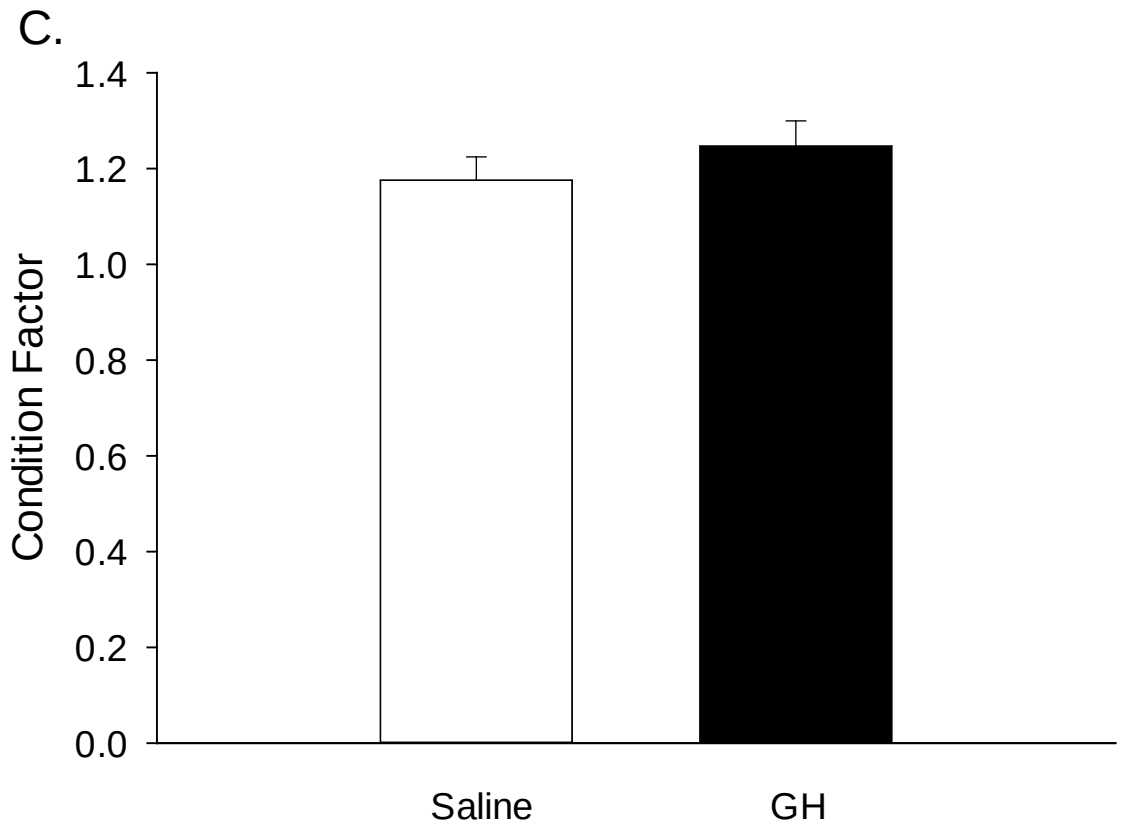
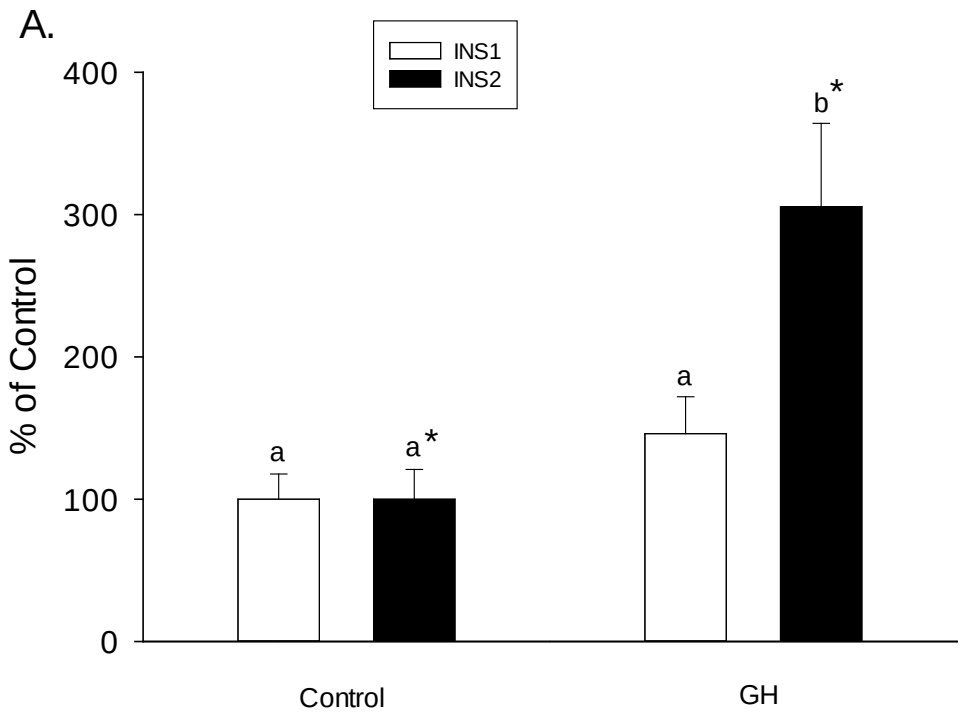
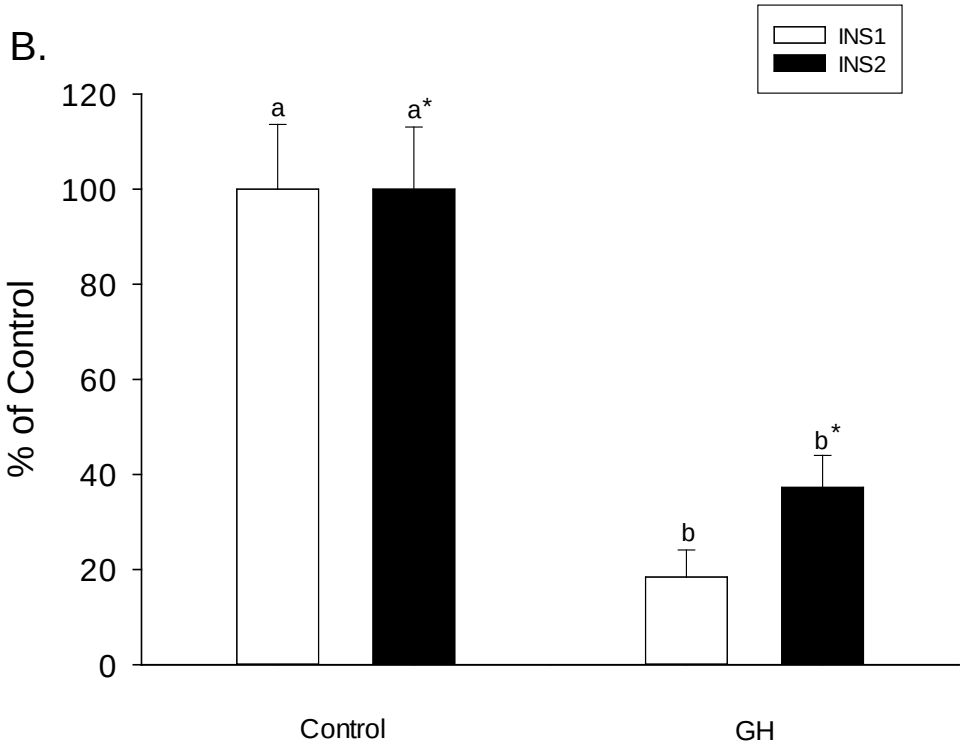



Figure 18. Changes of growth characteristics in weight (A), length (B), and condition factor (C) of rainbow trout implanted with oGH and 0.75% saline over a 21-day trial. Relative growth and length were calculated as (100*[final body mass (or length) - initial body mass (or length)]) / [initial body mass (or length)]. Condition factor was calculated as [weight/(length)$^3$]*100. Data are presented as mean ± SEM. For a given treatment, groups with different letters are significantly ($P<0.05$) different.


A.
INS1
INS2
400
300
200
100
0
% of Control
a
a*
a
b*
Control
GH


B.
INS1
INS2
120
100
80
60
40
20
0
% of Control
a
a*
b
b*
Control
GH

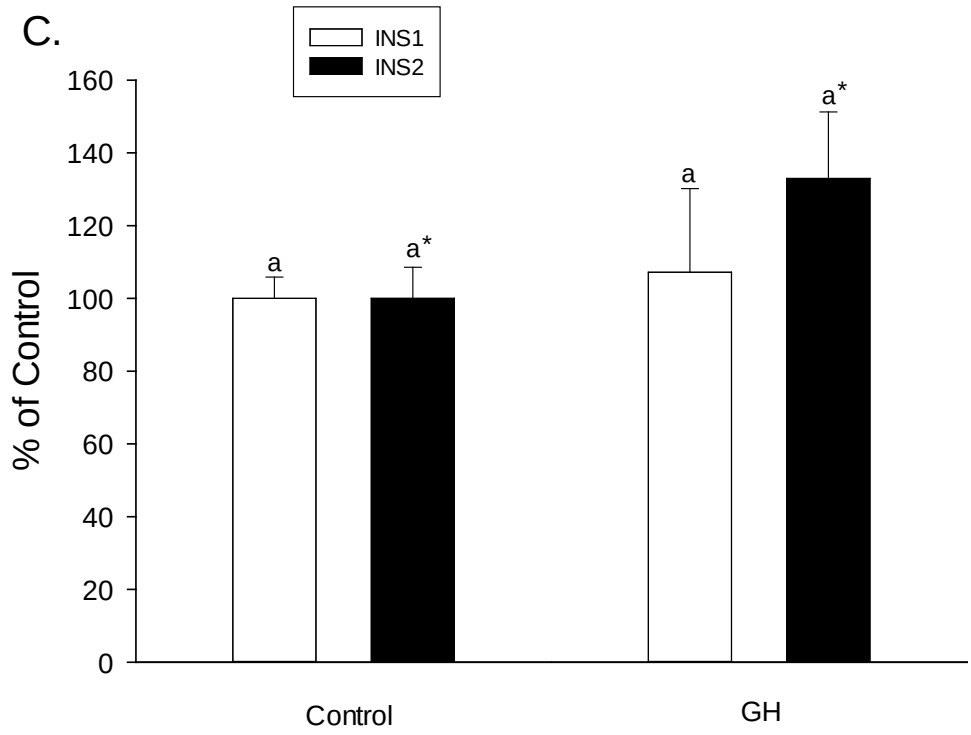
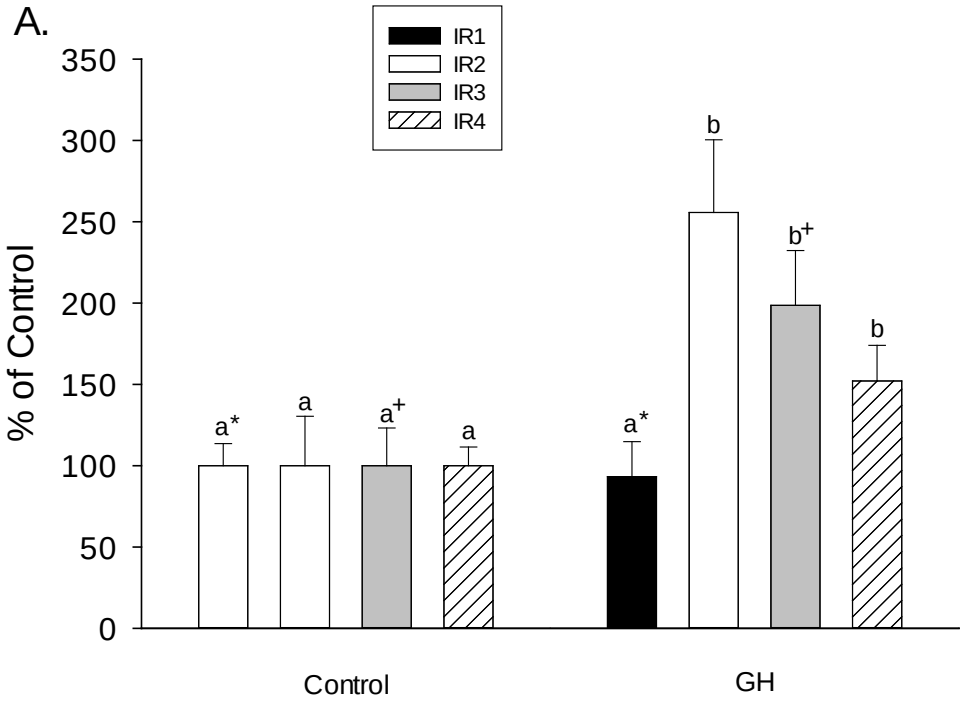
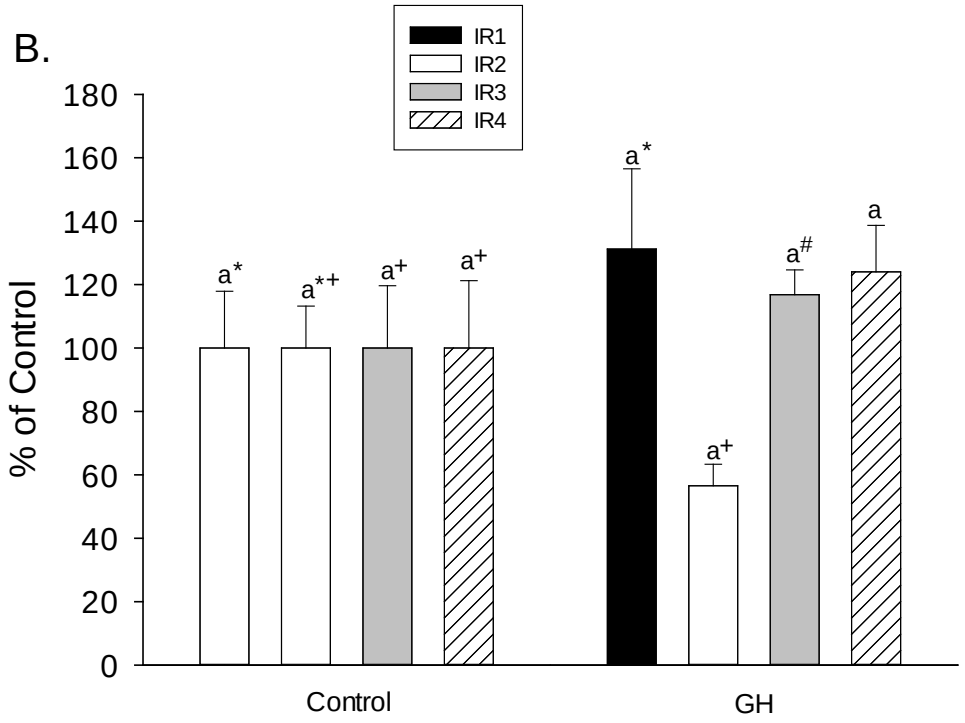
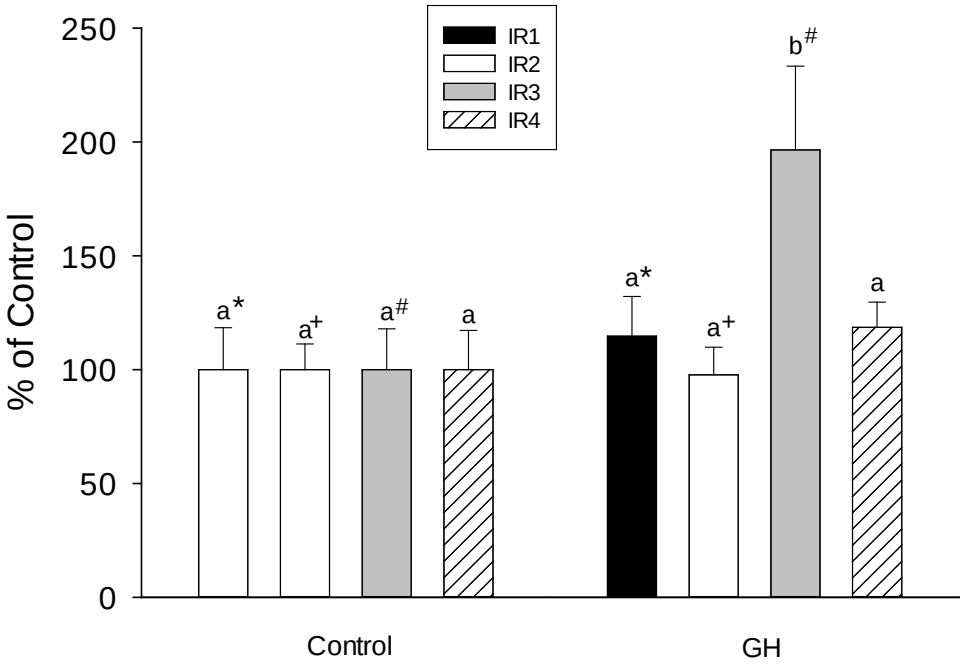



Figure 19. Changes in insulin subtype (INS1, INS2) mRNA levels in the pancreas (A), adipose (B), and brain (C) of rainbow trout associated with GH implantation. Data are presented as means ± SEM. For a particular tissue, letters denote differences for a given subtype across treatments; * denotes a difference in subtypes within a given treatment. Differences between tissues are described only in text.

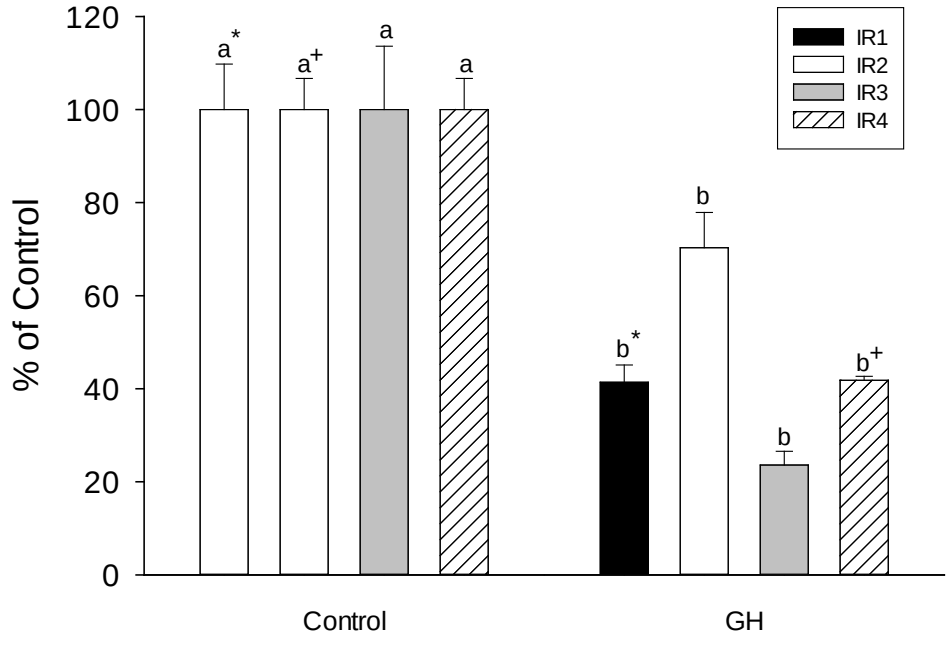

A.
IR1
IR2
IR3
IR4
% of Control
350
300
250
200
150
100
50
0
a*
a
a+
a
a*
b
b+
b
Control
GH


B.
IR1
IR2
IR3
IR4
% of Control
180
160
140
120
100
80
60
40
20
0
a*
a*+
a+
a+
a*
a+
a#
a
Control
GH

C.


IR1
IR2
IR3
IR4
% of Control
250
200
150
100
50
0
a*
a+
a#
a
a*
a+
b#
a
Control
GH

D.


IR1
IR2
IR3
IR4
% of Control
120
100
80
60
40
20
0
a*
a+
a
a
b*
b
b
b+
Control
GH

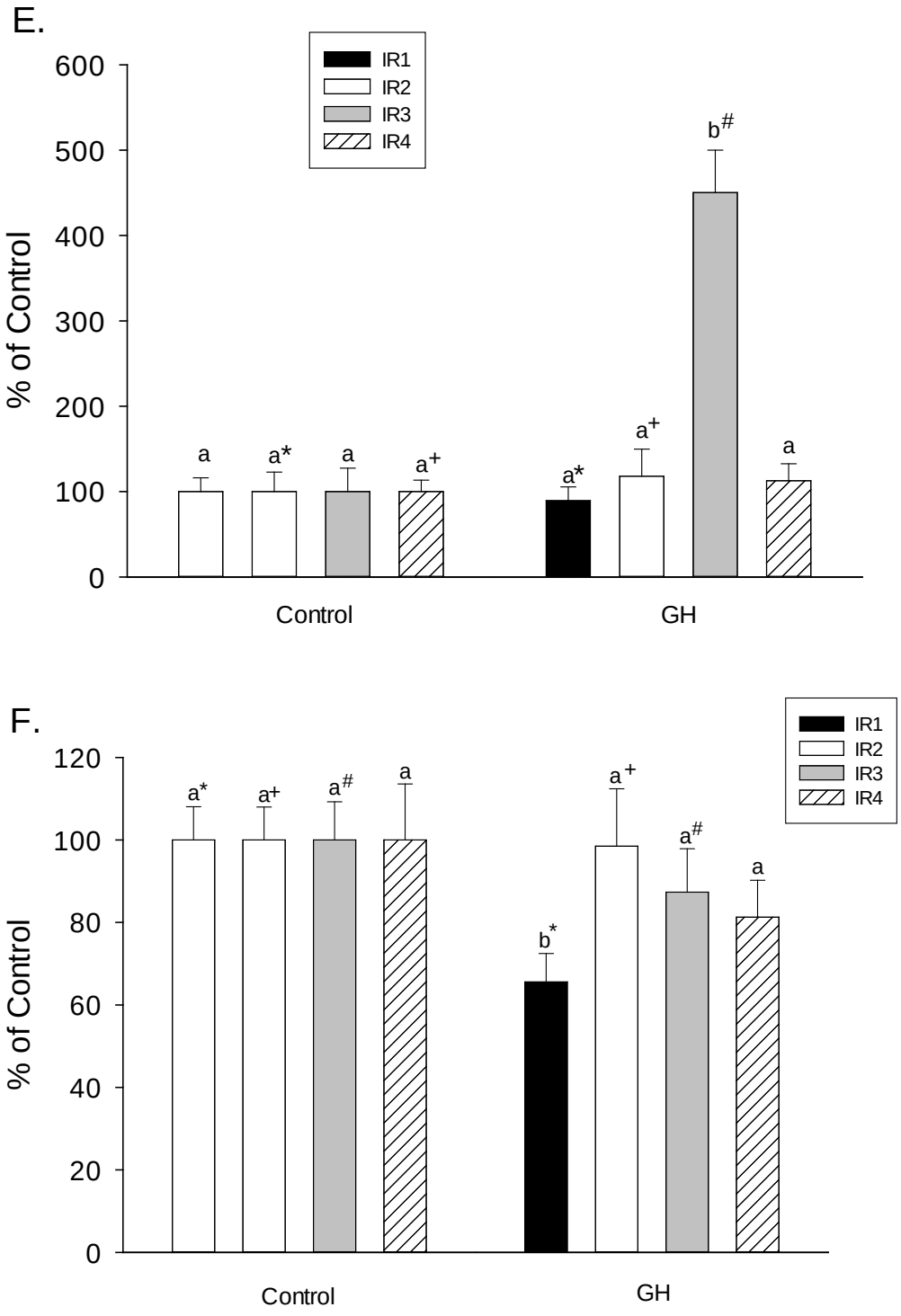



Figure 20. Changes in insulin receptor isoform (IR1, IR2, IR3, IR4) mRNA expression in the pancreas (A), adipose (B), liver (C), cardiac muscle (D), skeletal muscle (E), and gill (F) of rainbow trout associated with GH implantation. Data are presented as means ±

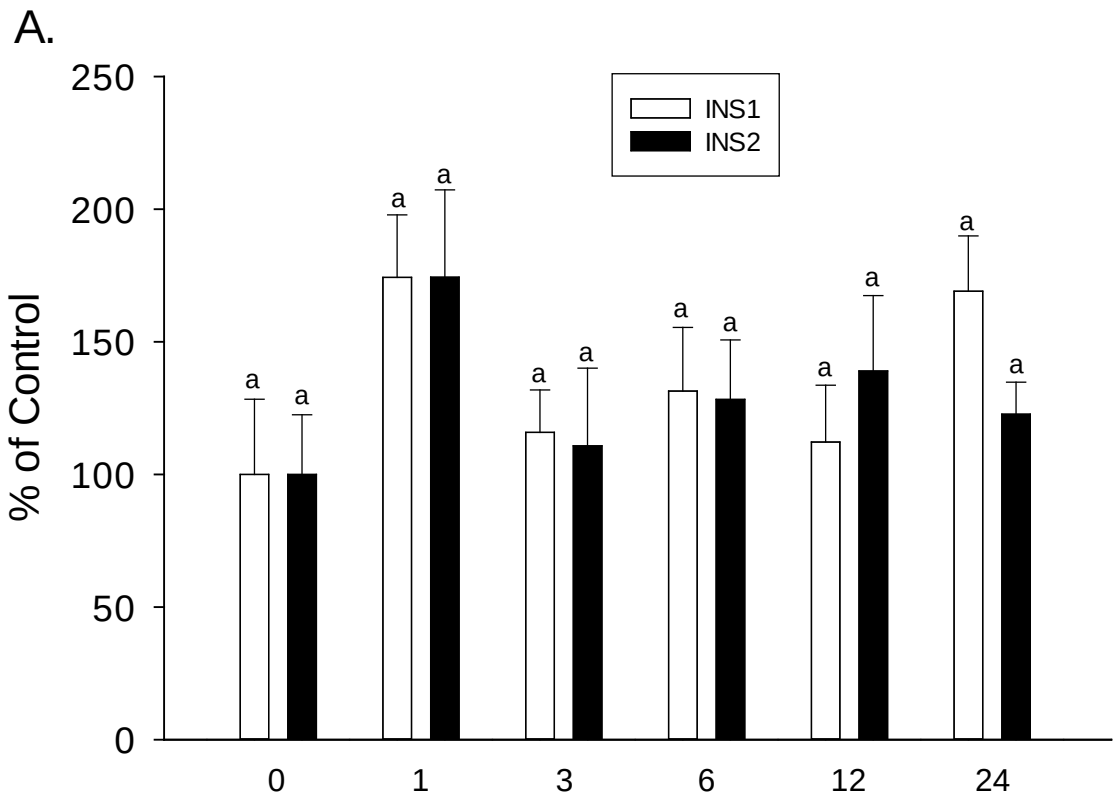
SEM. For a particular tissue, letters denote differences for a given isoform across treatments; typographical symbols denote a difference in isoform within a given treatment. Differences between tissues are described only in text.

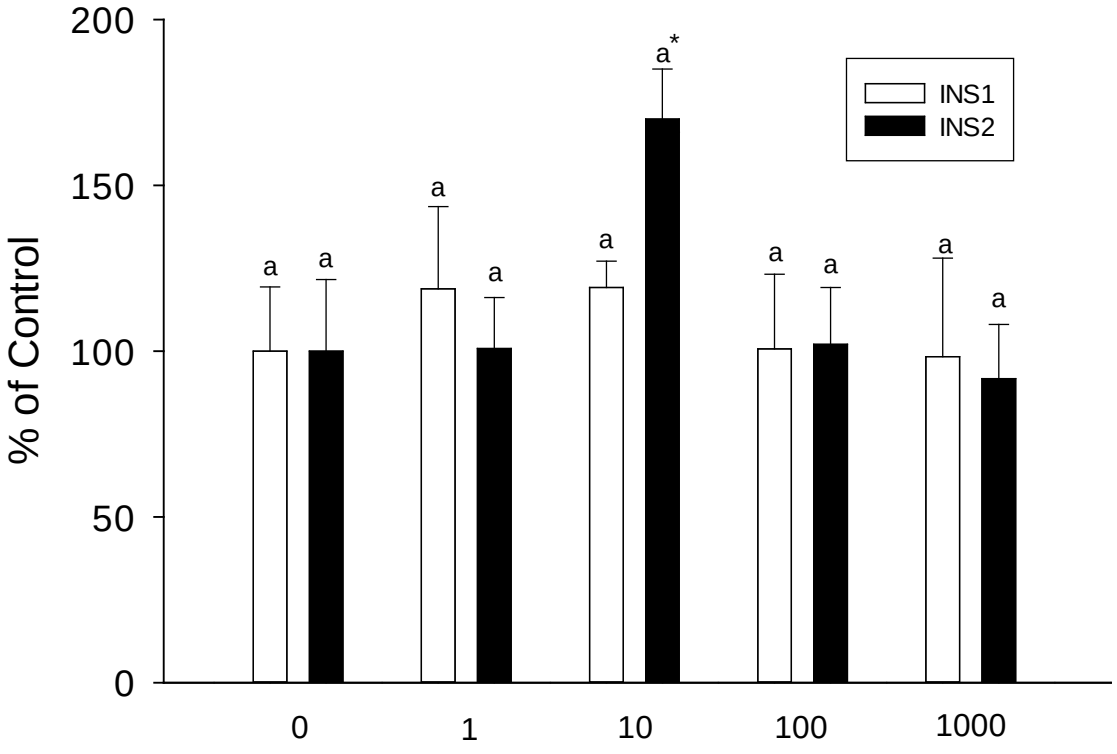
INS expression is shown in Fig 21. In general, an increase in IGF-I dose increased the expression of INS1 and INS2, with the exception of 1ng/ml, which reduced the expression of both isoforms. Maximum expression for each mRNA species was observed at a dose of10ng/ml, which increased INS1 and INS2 expression ca. 50- and 80- fold, respectively. IGF-I attenuated incremental reductions in both INS mRNA species over time with the lowest INS expression levels at 24h (Fig. 21B).

*Effects of IGF-I And GH Treatment On IR Expression In Pancreatic Hemi-Islets*

The effects of IGF-I and GH on IR expression varied by isoform within pancreatic hemi-islets incubated *in vitro*. All IR isoforms (IR1, IR2, IR3, IR4) were detected. Over the time course, GH had no effect on the expression of IR2 and IR3; IR1 expression was reduced at hours 12 and 24; and expression of IR4 steady declined from 1 - 24h. GH dose treatment had no effect on IR1 expression; IR2 expression was reduced at an elevated dose of 1000ng/ml; and expression of IR3 and IR4 peaked at 10ng/ml then reduced at elevated concentrations (Fig. 22A).

Time course IGF-I treatment reduced IR1 expression at 1, 12, and 24h; reduced IR at 1, 3, 6, 12, and 24h; reduced IR3 at later time points 6, 12, and 24h; and reduced IR4 at 24h. Across varying IGF-I treatment doses, IR1 was unaffected; IR2 was reduced at


A.
% of Control
250
200
150
100
50
0
INS1
INS2
a
a
a
a
a
a
a
a
a
a
a
a
0
1
3
6
12
24


% of Control
200
150
100
50
0
INS1
INS2
a
a
a
a
a
a*
a
a
a
a
0
1
10
100
1000

B.

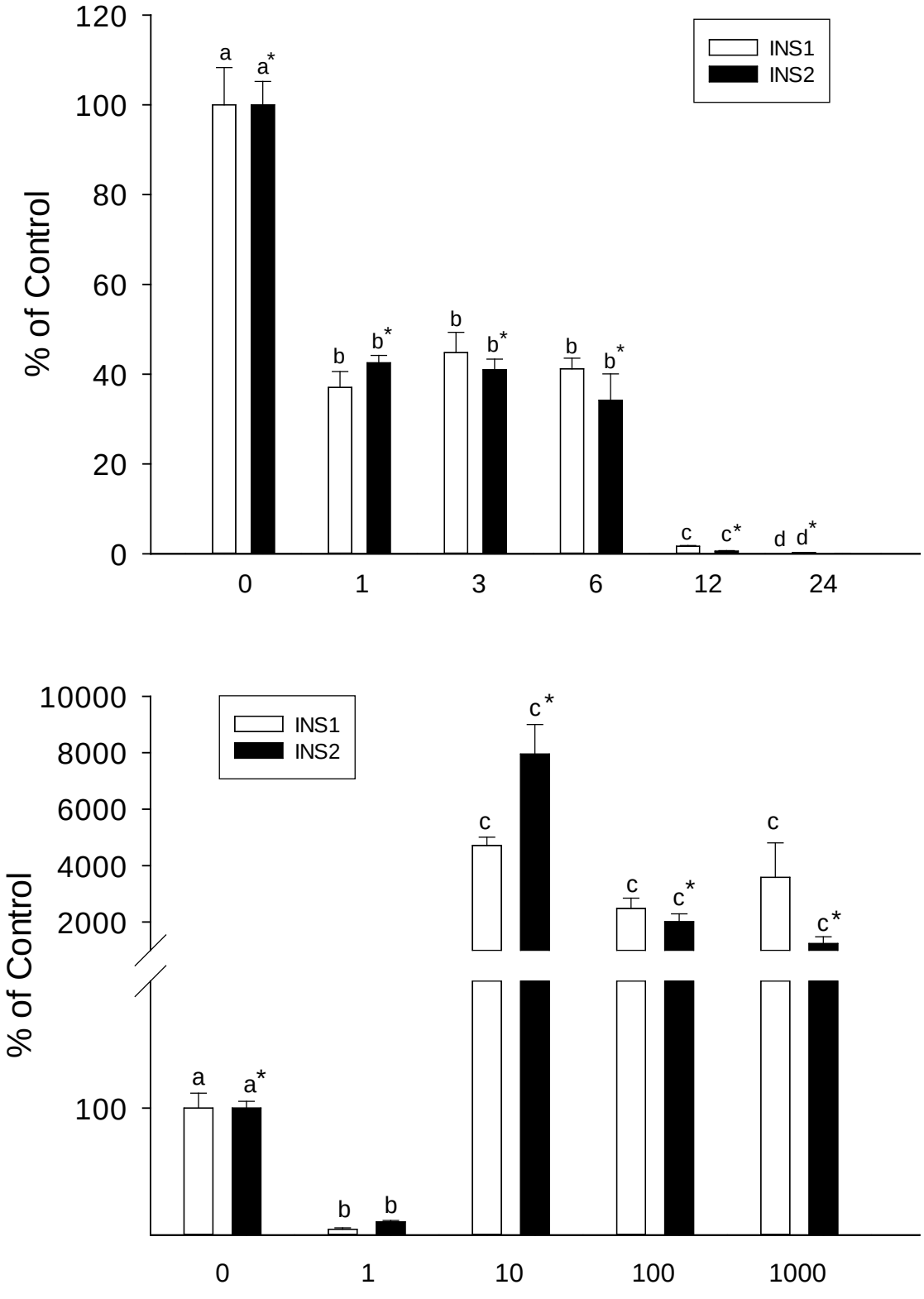

% of Control
INS1
INS2
0
20
40
60
80
100
120
a
a*
b
b*
b
b*
b
b*
c
c*
d
d*
0
1
3
6
12
24
% of Control
INS1
INS2
2000
4000
6000
8000
10000
100
a
a*
b
b
c
c*
c
c*
c
c*
0
1
10
100
1000

Figure 21. Expression of insulin subtype (INS1, INS2) mRNA from rainbow trout pancreatic hemi-islets after exposure to, GH (A) or IGF-I (B), dose (at 1, 10, 100, and 1000 ng [at 6h]) and time (1, 3, 6, and 24h [at 100ng] treatments. Data are presented as mean ± SEM. For a given INS subtype, groups with different letters are significantly ($P<0.05$) different; * denotes a significant ($P<0.05$) difference between INS subtypes.

1ng/ml but then remained elevated at and above 10ng/ml; IR3 was reduced at 100ng/ml; and IR4 was increased at 1ng/ml and reduced at 1000ng/ml (Fig. 22B).

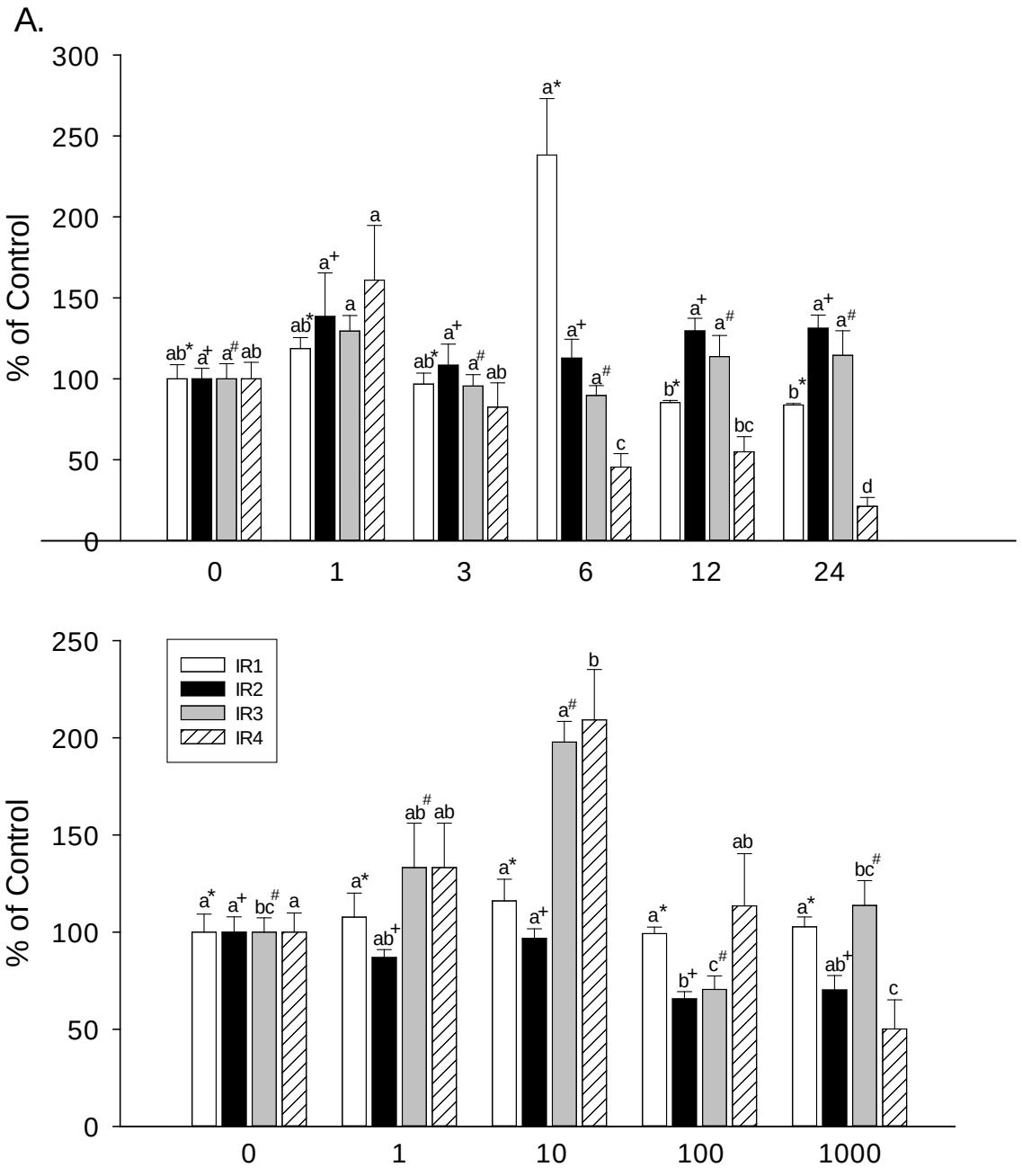
*Effects Of IGF-I And GH Treatment On IR Expression In Liver*

The effects of IGF-I and GH on IR expression varied by isoform within liver pieces incubated *in vitro*. All IR isoforms (IR1, IR2, IR3, IR4) were detected. In general, GH treatment over time had no effect on IR expression. Across varying doses, GH increased IR1at 10 and 100ng/ml; IR2 reduced at 1000ng/ml; and IR3 and IR4 remained unchanged (Fig. 23A).

IGF-I treatment over time reduced IR1 at 12 and 24; increased IR2 at 1, 6, and 12h and was reduced at 24h; and IR3 and IR4 were reduced at 24h. Administering IGF-I dose treatment, IR1 was increased at 1, 10, and 1000ng/ml; and the remaining IR isoforms were unchanged (Fig. 23B).

*Effects Of IGF-I And GH Treatment On IR Expression In Gill*

The effects of IGF-I and GH on IR expression varied by isoform within gill filaments incubated *in vitro*. All IR isoforms (IR1, IR2, IR3, IR4) were detected. Over a


A.
% of Control
300
250
200
150
100
50
0
0
1
3
6
12
24
IR1
IR2
IR3
IR4
% of Control
250
200
150
100
50
0
0
1
10
100
1000

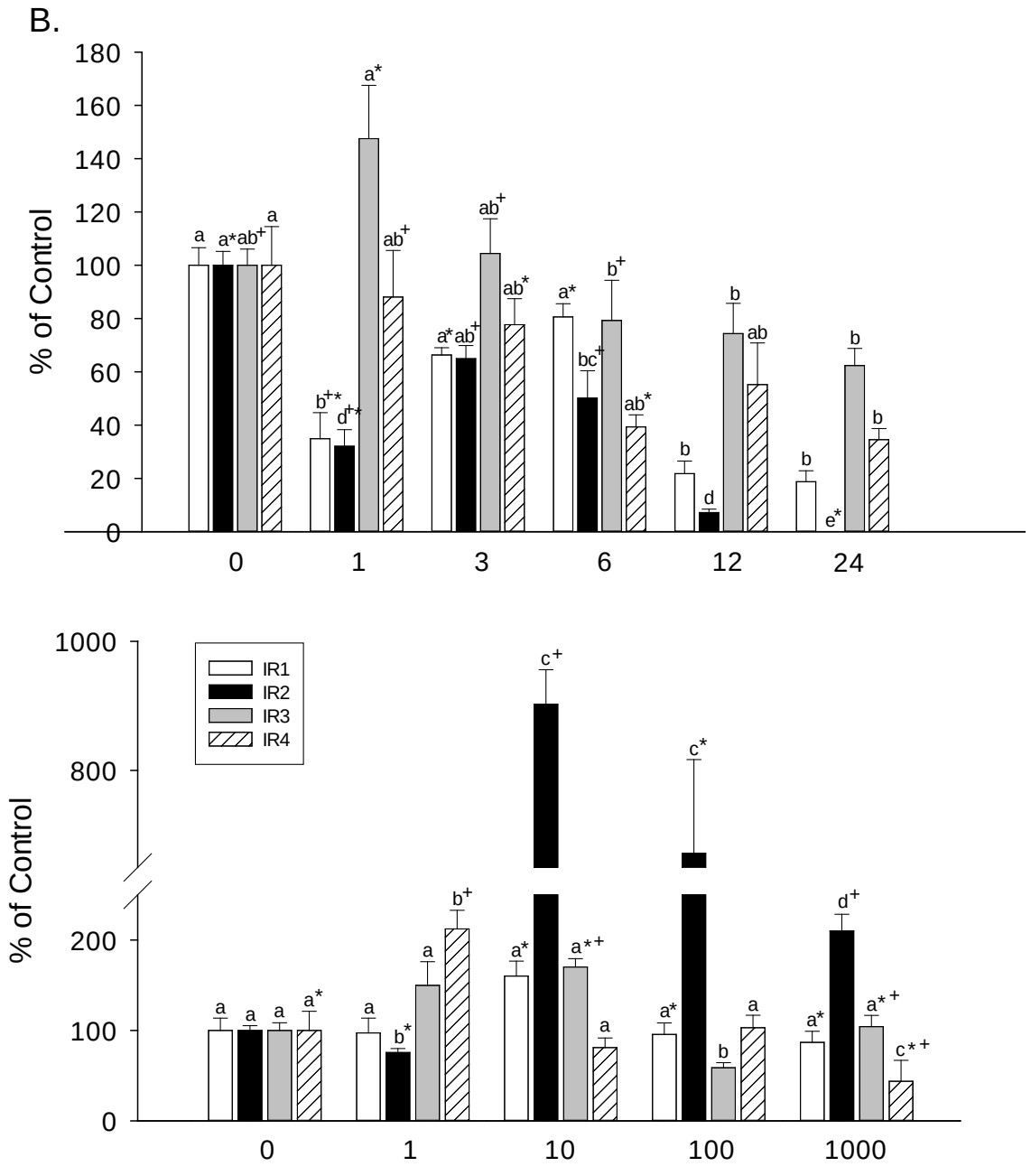
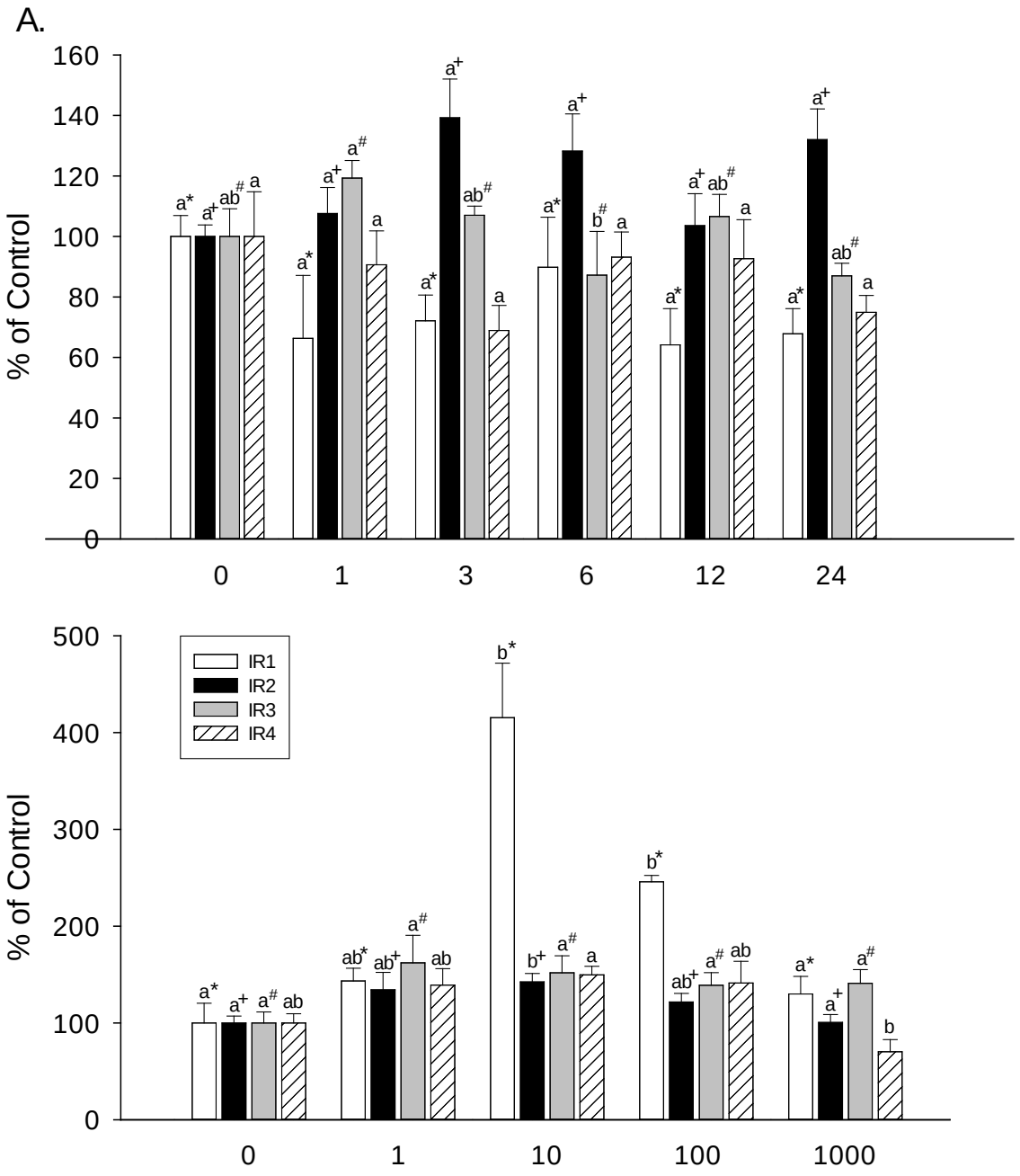



Figure 22. Expression of insulin receptor isoform (IR1, IR2, IR3, IR4) mRNA from rainbow trout pancreatic hemi-islets after exposure to, GH (A) or IGF-I (B), dose (at 1, 10,

100, and 1000 ng [at 6h] and time (1, 3, 6, 12, and 24 h [at 100ng] treatments. Data are presented as mean ± SEM. For a given IR isoform, groups with different letters are significantly ($P<0.05$) different; typographical symbols indicate a significant ($P<0.05$) difference between IR isoforms.


A.
% of Control
160
140
120
100
80
60
40
20
0
0
1
3
6
12
24
IR1
IR2
IR3
IR4
% of Control
500
400
300
200
100
0
0
1
10
100
1000

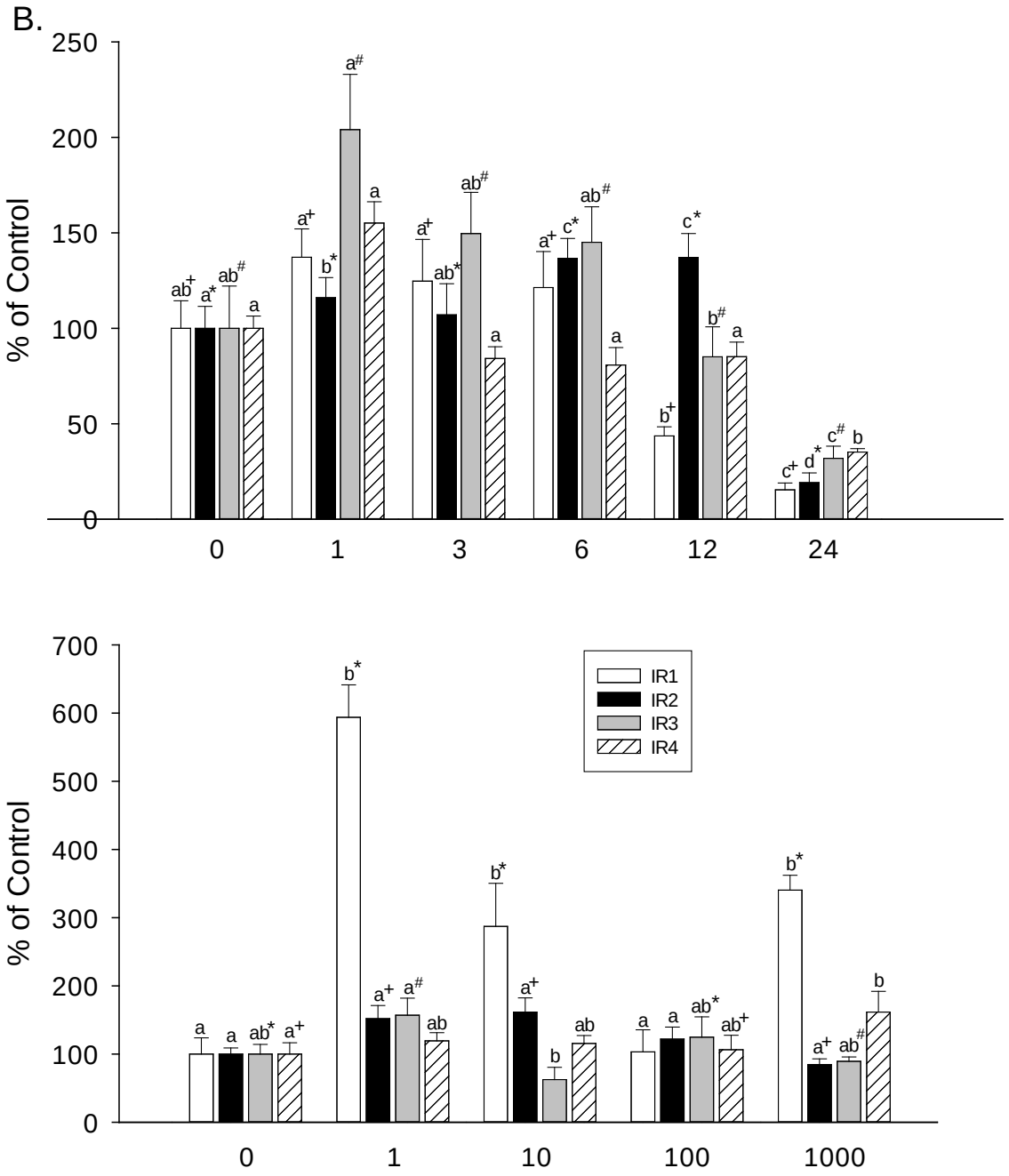



Figure 23. Exression of insulin receptor isoform (IR1, IR2, IR3, IR4) mRNA from rainbow trout liver pieces after exposure to, GH (A) or IGF-I (B), dose (at 1, 10, 100, and

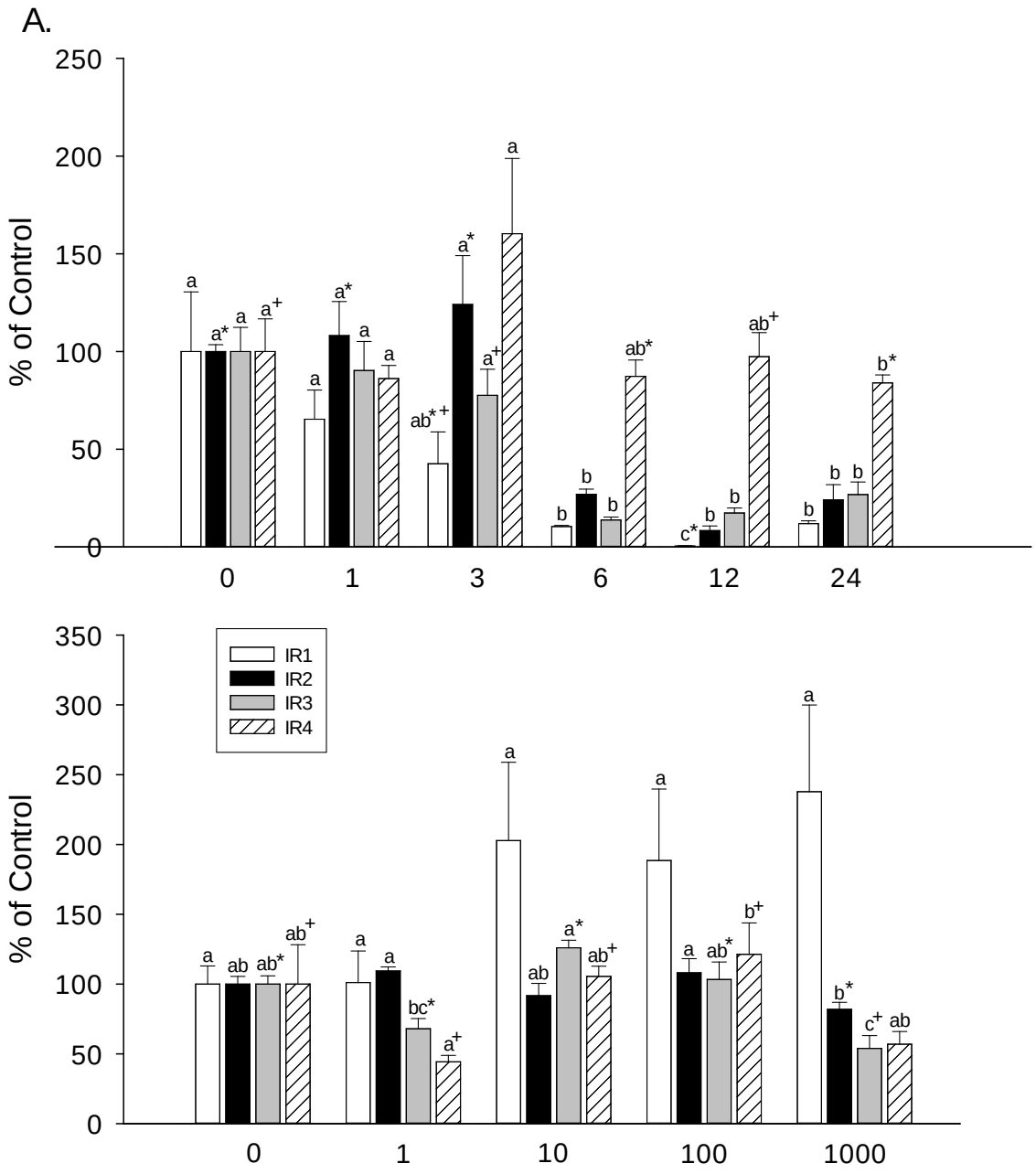
1000 ng [at 6 h]) and time (1, 3, 6, 12, and 24 h [at 100ng]) treatments. Data are presented as mean ± SEM. For a given IR isoform, groups with different letters are significantly ($P<0.05$) different; typographical symbols indicate a significant ($P<0.05$) difference between IR isoforms.

time course, in general, GH treatment reduced all IR isoforms at 6h and remained depressed throughout 24h. During a dose GH treatment, IR1, IR2, and IR4 were relatively unchanged; and IR3 was reduced at 100ng/ml (Fig. 24A).

IGF-I time treatment reduced IR1 at 6 - 24h; IR2 was unchanged; IR3 increased at later time points of 12 and 24h; and IR4 increased at 3h and remained elevated throughout 24h. In general, IGF-I treatment did not affect the expression of IR mRNA species across varying doses (Fig. 24B).

## Discussion

We previously demonstrated that INSs and IRs were differentially expressed, both in terms of distribution among tissues and in terms of relative abundance within tissues (Caruso et al., 2008; Caruso and Sheridan, 2010). The current study demonstrates the effects of chronic *in vivo* administration of GH, which modulates somatic growth consistent with the known actions in mammals and fish (Moriyama et al., 2000; Lichanska and Waters, 2008), and regulates the pattern of INS and IR expression. In addition, the results of this study indicate that GH and IGF-I treatment *in vitro* modulate the expression of multiple INS and IR mRNAs within selected tissues.


A.
% of Control
250
200
150
100
50
0
0
1
3
6
12
24
IR1
IR2
IR3
IR4
% of Control
350
300
250
200
150
100
50
0
0
1
10
100
1000

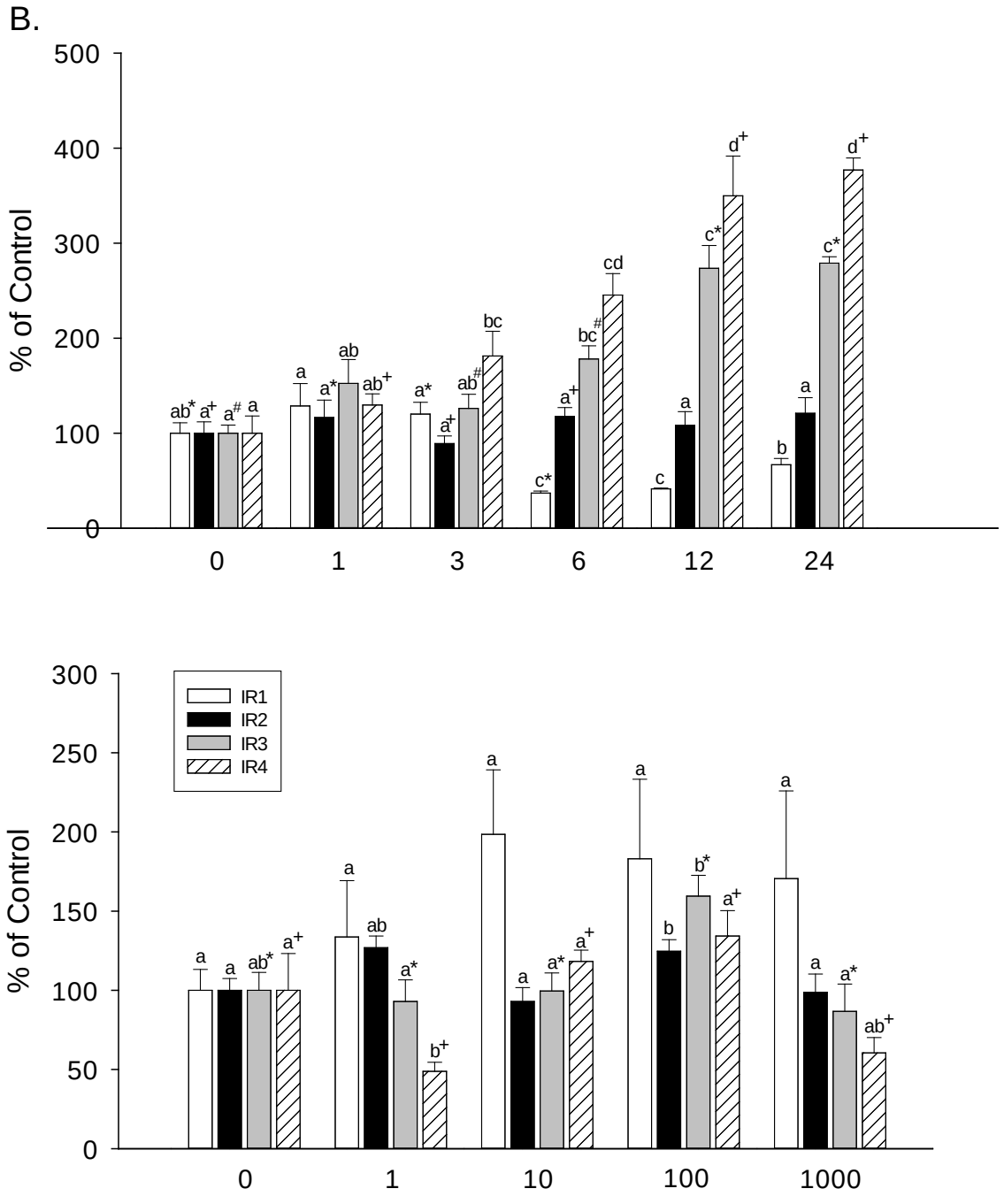



Figure 24. Expression of insulin receptor isoform (IR1, IR2, IR3, IR4) mRNA from rainbow trout gill filaments after exposure to, GH (A) or IGF-I (B), dose (at 1, 10, 100, and 1000 ng [at 6 h]) and time (1, 3, 6, 12, and 24 h [at 100ng] treatments. Data are presented

as mean ± SEM. For a given IR isoform, groups with different letters are significantly ($P<0.05$) different; typographical symbols indicate a significant ($P<0.05$) difference between IR isoforms.

GH implantation resulted in a significant increase in growth, both in terms of body mass and notably body length, which is consistent with previous reports in both mammals and fish (Biga and Meyer, 2009; Dubowski and Sheridan, 1995; Klindt et al., 1996). Relative length exhibited a compelling difference compared to relative growth both in terms of GH delivery. GH administration in mammals has shown to have a lipolytic effect and increase skeletal muscle anabolism (Vijayakumar et al., 2009). This may serve to suggest that the increase in growth does not occur significantly within adipose but instead has a significant impact on bone and skeletal muscle growth.

GH implantation regulated INS mRNA expression in a isoform- and tissue-specific manner. The expression of both INS1 and INS2 mRNA in the pancreas increased with GH implantation. This observation is consistent with reports in transgenic mice, which overexpress GH and correspondingly exhibit hyperinsulinemia (Balbis et al., 1996). Interestingly, GH transgenic coho salmon, which displayed increased growth and maturation, did not show a difference in plasma INS compared to size controls; however, GH transgenic salmon had increased levels of INS compared to nontransgenic siblings (Devlin et al., 2000). GH treatment *in vitro* resulted in no significant change in steady-state levels of INS mRNAs within pancreatic hemi-islets. This observation is in stark contrast to the significant increase in INS1 and INS2 expression following IGF-I dose treatment. Although IGF-I was not measured following GH implantation in this study, previous

reports indicate sustained IGF-I production following prolonged GH administration within rainbow trout (Biga et al., 2004). This suggests that IGF-I may have mediated the increase in INS expression observed following GH implantation. However, in rats, IGF-I treatment *in vivo* had no significant effect on serum INS values or glycemic regulation (Yamaza et al., 2007). Additionally, IGF-I treatment from perfused rat pancreata slightly decreased INS secretion (Guler et al., 1989). Over the IGF-I time course, INS mRNAs were temporally attenuated, which is in contrast to the observation of the dose treatment and is somewhat perplexing. Although, this observation may suggest that IGF-I gradually reduces INS expression over time and/or SS or glucagon countered the effects of IGF-I over time (Melroe, 2004).

The recent authentication of extrapancreatic INS producing tissues in fish has just recently begun to emerge (Caruso et al., 2008; Hyrtsenko et al., 2007; Roy et al., 2003). However, little evidence has emanated regarding the regulation of these INS producing tissues. This is the first known report of INS mRNA regulation commencing an *in vivo* implantation of growth modulating exogenous hormone. GH implantation had no effect on INS expression in the brain and decreased both INS1 and INS2 in adipose. Whether the effects of GH on INS expression on these tissues were direct or mediated through IGF-I is unknown and requires further study. GH (Pan et al., 2005) and IGF-I (Nishijima et al., 2010) have both been shown to be permeable across the blood-brain barrier in rodents and suggests the same maybe true for fish. Therefore, INS expression in nervous tissue is insensitive to GH treatment *in vivo*; however, further study needs to be conducted to authenticate the direct effects of GH and IGF-I treatment on nervous tissue.

The decrease in INS expression within adipose tissue following GH implantation may have been mediated indirectly through one or more adipokines. Mice injected with rGH or transgenic mice that display chronic, constitutively elevated levels of GH, both decrease the production of leptin and adiponectin (Berryman et al., 2011). Correspondingly, rats treated with IGF-I also exhibit decreased leptin and adiponectin synthesis and secretion (Yamaza et al., 2007). However, other adipokines such as resistin have been shown to correlate a direct relationship to circulating GH/IGF-I levels in rats (Chiba et al, 2008). In mammals, INS has been reported to increase leptin synthesis and secretion, whereas leptin attenuates pancreatic INS production and increases INS sensitivity (Yildiz and Haznedaroglu, 2005). However, leptin's nucleotide and amino acid identity is not well conserved among vertebrates, and recent studies suggest a divergent function for leptin in fish, which may not act as an adiposity signal as it does in mammals (Kling et al., 2009). Adiponectin, however, has been shown to display similar expression patterns in zebrafish in accordance with mammals during nutrient deprivation, suggesting a conserved function (Nishio, et al., 2008). The inverse INS-expression pattern observed in adipose relative to pancreas remains equivocal and will require further study.

GH implantation also regulated IR mRNA expression in an isoform- and tissue-specific manner. Increases in expression were observed with IR2, IR3, and IR4 in pancreas, and IR3 in liver and skeletal muscle from GH implantation. The increase in pancreatic IR expression is in contrast to *in vitro* GH treatment, which, in general, displayed no effect or a decrease in IR expression. With the exception of an increase in IR2 at elevated doses, IGF-I treatment also had no effect or decreased IR expression in pancreatic hemi-islets. This suggests that GH or IGF-I may not directly mediate the

expression of IR mRNAs within the pancreas. GH and IGF-I treatment *in vitro* displayed varying effects on IR expression in a time- and dose-dependent manner in liver pieces. Despite increased IR3 expression *in vivo*, this observation was not corroborated following *in vitro* treatment. Transgenic mice overexpressing bGH exhibit reduced heptic IR number as well as increased tyrosine kinase activity (Balbis et al., 1996). Combination studies utilizing glucose and IGF-I administration in humans displayed an increase in INS secretion and sensitivity via upregulation of IR (Hussain et al., 1993). Decreased IR expression was observed for all four IR mRNA isoforms in cardiac muscle, IR1, IR2, and IR3 in the gill, and IR2 in adipose following GH implantation. Gill filaments incubated *in vitro* were insensitive to GH and IGF-I dose treatment. GH having little effect or decrease on the expression of all IRs in gill and cardiac muscle is not immediately obvious. Another study utilizing prolonged GH treatment in rainbow trout indicated down regulation of IGF-IRa in cardiac muscle and had no significant effect in liver or skeletal muscle (Biga et al., 2004).

The notion that INS1, INS2, IR1, IR2, IR3, and IR4 expression are differentially regulated is supported by several lines of evidence. First, the overall pattern of expression of INS and IR mRNAs are differentially affected by GH and IGF-I. Second, the effects of GH and IGF-I on INS and IR is isoform specific. Taken together, these findings suggest that the expression of INSs and IRs are regulated independently and that GH and IGF-I may be operating through different mechanisms in different tissues to regulate INS and IR expression. Other factors, including nutritional state (Caruso and Sheridan, dissertation [Chap. 3]), also have been found to influence INS and IR mRNA expression in rainbow trout. The regulation of INS and IR expression has important consequences on the INS

signaling system and, ultimately, on the physiological actions of INS. The predominate effects of GH increasing INS1, INS2, IR2, IR3, and IR4 expression in the pancreas would be consistent with the growth-promoting effects of this hormone. For example, at the level of the pancreas, an increase in INS production from the β cell would demand to be metabolically invigorated requiring more nutrients and thus correspondingly would require a host of increased insulin receptors. Conversely, tissues that are reduced in sensitivity to INS have limited metabolic and growth potential. However, altered tyrosine kinase activity could have a compensatory effect on reduced sensitivity.

These findings, the first reporting the effects of GH and IGF-I on INS and IR expression, contribute to a broader understanding of the role of GH and IGF-I in regulating the growth and metabolic potential in vertebrates and extend our knowledge of factors regulating target organ sensitivity. In summary, the finding of this study indicate that exogenous GH implantation increased food conversion and augmented growth, while GH treatment *in vitro* modulated the expression of INSs and IRs suggest that independent mechanisms may serve to regulate the various isoforms. The coordinate regulation of INS biosynthesis/secretion and IR biosynthesis/membrane recruitment are important for mediating the various and numerous actions of INS. Continued study of the intracellular mechanisms that serve to regulate INS and IR expression are needed to better understand the tissue- and function-specific responses of INS.

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# CHAPTER FIVE. EXPRESSION OF INSULIN AND INSULIN RECEPTOR mRNAS IS REGULATED *IN VIVO* AND *IN VITRO* BY SOMATOSTATIN IN RAINBOW TROUT (*Oncorhynchus mykiss*)


## Abstract

Insulin (INS) is a critical peptide hormone that coordinates various aspects of growth, development, and metabolism through interactions with multiple insulin receptor (IR) isoforms on various target tissues. In this study, we used rainbow trout as a model system to survey the effects of somatostatin-14 (SS) implantation on the expression of INS1, INS2, IR1, IR2, IR3, and IR4 mRNAs. SS regulated INS and IR expression in an isoform- and tissue-specific manner. SS-14 implanted animals displayed decreased INS1 and INS2 expression in pancreas; increased INS1 and INS2 in adipose; increased INS1 in brain; reduced expression of IR1, IR2, IR3, and IR4 in cardiac muscle; reduced IR3 in gill; reduced IR2 and IR4 in liver; and reduced IR2 and IR3 in adipose. Tissue pieces harvested from rainbow trout were used to survey the direct effects of SS-14 treatment on the expression of aforementioned mRNAs. SS treatment regulated INS and IR mRNAs in a dose- and temporal-dependent manner. SS treatment *in vitro* displayed increased INS1,INS2, and IR4 expression over a 24h period, and in general, reduced INS1, IR1, IR2, IR3, and IR4 at increased SS-14 concentrations in pancreatic hemi-islets; increased IR1, IR2, and IR3 at 24h in liver pieces; and reduced IR2, IR3, and IR4 in gill filaments at elevated SS-14 concentrations. These results demonstrate that SS-14 modulates the expression of INS1, INS2, IR1, IR2, IR3, and IR4, and the results imply that independent mechanisms may serve to regulate the various isoforms in a tissue-specific manner.

## Introduction

The growth of an organism integrates a myriad of biological processes that are influenced by the juxtaposition of intrinsic and extrinsic determinants. The GH-IGF axis is a simplified model used to represent the control of growth since many of the growth-promoting actions of GH in fish and mammals are mediated indirectly via IGF-I (Moriyama et al., 2000; Holzenberger et al., 2006). Amidst the axis, several chemical factors have the potential to influence the synthesis and secretion of GH and IGF-I, shifting a graceful balance between a process of growth promotion and one of growth stagnation. INS is one of the most pivotal hormonal assimilators of growth and metabolism in vertebrates and is known to interact with GH and IGF-I in a synergistic manner, whereas somatostatin (SS) regulates GH and IGF-I as a negative regulatory mediator (Klein and Sheridan, 2008). As an inhibitory feedback mechanism, GH and INS promote the production of pancreatic SS as well as other peripheral tissues (Ehrman et al., 2005; Jain and Lammert, 2009). SS has been shown to directly decrease growth-related processes such as lipid and carbohydrate anabolism and inhibit the secretion of a number of growth-related hormones -- INS, GH, and IGF-I (Very et al., 2002; Sheridan et al., 1987). Along with decreasing the production of GH and IGF-I, SS also decreases the sensitivity and expression of GH and IGF-I on target tissues (Sheridan and Hagemeister, 2010). *In vivo* and *in vitro* administration of INS, GH, and IGF-I in rainbow trout have been shown to increase the expression of pancreatic SS variants (Ehrman et al., 2005; Eilertson et al., 1995; Melroe et al., 2004).

Conventionally, somatostatins (SS) oppose the growth promoting effects of INS and downregulate pancreatic INS expression and secretion in mammals (Philippe et al., 1993).

Although, due to various isoforms of SSs such as SS-14, SS-25, SS-28 found in fish and specific amino acid substitutions, each variant displays differential physiological effects. Both SS-14 and SS-25 treatment inhibit the release of INS in coho salmon (Sheridan et al., 1987); while treatment of SS-25-II but not SS-14 reduced plasma INS in rainbow trout (Eilertson and Sheridan, 1993). Despite INS-induced nutritional stimulation of pancreatic islets, the presence of SS attenuates the release of INS and retards growth in rainbow trout (Very et al., 2001). Carnivorous fish, such as rainbow trout, are putatively glucose intolerant, and maybe so due to SS-induced downregulation of INS expression and/or secretion. For example, glucose injection in rainbow trout initially diminished plasma INS levels and concomitantly raised SS-25 and GLU secretion, suggesting inter-islet inhibition of β cell INS secretion (Harmon et al., 1991).

The actions of INS are transduced through receptors of the tyrosine kinase (TK) family, which catalytically induce auto- and trans-phosphorylation, leading to activation of downstream effector molecules within target cells (Plagnes-Juan et al., 2008). Once nutrients are procured, they are available for lipid, carbohydrate, and protein anabolism (Caruso and Sheridan, 2011). SS not only acts a growth inhibitor, but also functions as a key metabolic regulatory hormone. For example, treatment of SS-14 *in vitro* stimulates hepatic lipid mobilization in rainbow trout (Eilertson and Sheridan, 1993). Conversely, INS counteracts SS's effects on the liver and promotes hepatic lipogenesis (Sheridan and Kittilson, 2004). In rat pancreas, SS expression and secretion is inversely related to INS concentration in vivo (Julien et al., 2004).

Previously, two INS mRNAs (Caruso et al., 2008) and four IR mRNAs (Caruso et al., 2010; Greene and Chen, 1999) were isolated and characterized from rainbow trout,

which are differently expressed among and within tissues. The INS signaling system in rainbow trout consists of multiple preproinsulin-derived products (proinsulin, INS, C-peptide) and multiple IR isoforms, which contain the potential to elicit multiple physiological responses within a single target tissue. Despite the growing body of knowledge on differential expression of INS and IR isoforms, the regulation of these elements has just begun to emerge. In the present study, we used SS implantation and *in vitro* treatment over a temporal- and concentration-dependent manner to evaluate the regulation of INS and IR mRNA expression. Rainbow trout gill, liver, and pancreatic hemi-islets were chosen for this study because of their central importance to growth and metabolic related processes, and the high abundance of IRs present in these tissues are important biological targets for the action of INS.

## Materials and methods

### *Experimental Animals*

Juvenile rainbow trout (*Oncorhynchus mykiss*) of both sexes were obtained from Dakota Trout Ranch near Carrington, ND, USA. Following transport to North Dakota State University, the fish were maintained in well-aerated, 800-L circular tanks supplied with recirculated (10% replacement volume per day) dechlorinated municipal water at 14$^{o}$C under a 12h light:12h darkness photoperiod. Fish were fed to satiation twice daily with AquaMax™ Grower (PWI Nutrition International. Brentwood, MO, USA), except 24-36 h before experimental manipulations. Animals were acclimated to laboratory conditions for at least 6 weeks prior to experiments. All experimental protocols were reviewed and approved by the North Dakota State University Animal Care and Use Committee.

*Experimental Treatment In Vivo*

The effects of SS on the patterns of insulin(s) and insulin receptor(s) mRNA expression were evaluated by implanting fish with Alzet® mini-osmotic pumps (Alza; Palo Alto, CA) containing either 0.75% (w/v) NaCl (control), 500 ng/ml bovine SS-14 (obtained from Sigma). Mini-pump flow rate is established to be 0.135 μl $h^{-1}$, which at 14°C should provide sustained release for 29 days. Fish were first anesthetized with 0.05% (v/v) 2-phenoxyethanol, and their body length and mass were determined. Mini-pumps were inserted into the peritoneal cavity through a 1.0-cm incision that was made ca. 0.5 cm right of the ventral midline and ca. 2.0 cm rostral of the pelvic fins. The incision was closed with two stitches and antibiotic ointment (Neosporin®) was applied topically to the incision area. Fish were placed into 100-L tanks (one tank for each treatment group, n=7) under the same conditions as acclimation, except that each tank was treated with 250 mg erythromycin with no water turnover for 30 min while supplemented with pure oxygen and feeding was suspended. The entire implantation procedure took about 1 min per fish. Seventy-two hours after tagging and transfer, fish were fed twice daily to satiety at a ration of 2% of their initial body mass per feeding (ca. 20.4 g) and continued for the duration of the experiment, except 36h prior to sampling. One hour post feeding excess food was siphoned, collected, removed of moisture in an oven until a constant mass was determined (ca. 2 hours at 100$^{o}$C), and weighed in order to tabulate food intake (7% increase to dried food was added to adjust for moisture content). Selected tissues [brain, gill, cardiac muscle, skeletal muscle, adipose, liver and endocrine pancreas (Brockmann body)] were sampled 21 days after implantation from anesthetized fish as before. Tissues were immediately frozen on dry ice for later analyses. The selection of tissues is based on their

central importance in growth and metabolic processes and because of high abundance and pattern of differential expression of INS/IR mRNAs determined previously (Caruso et al., 2008; Caruso et al., 2010).

*Preparation Of Pancreatic Islets*

For *in vitro* experiments, the fish were anesthetized and their Brockmann bodies removed and prepared for culture. In each study, trout Brockmann bodies were removed from anesthetized (0.05% (v/v) 2-phenoxyethanol) fish and placed in a chilled (14$^{o}$C) petri dish containing a modified Hanks' medium [in mM: 137 NaCl, 5.4 KCl, 4 $NaHCO_3$, 1.7 $CaCl_2$, 0.8 $MgSO_4$, 0.5 KH2$PO_4$, 0.3 $Na_2HPO_4$, 10 N-2-hydroxyehtylpiperazine-N'-2-ethanesulfonic acid, and 0.24% bovine serum albumin (BSA) supplemented with 5 mM glucose, pH 7.6; referred to as basal medium]. Individual organs were dissected further to remove surrounding connective and exocrine tissues and then longitudinally bisected (to yield hemi-islets) and placed in fresh basal medium for a 2-h preincubation at 14$^{o}$C and gassed with 100% $O_2$. *In vitro* cultures were preformed in 24-well polystyrene culture plates (Falcon 3407) containing 1 ml of secretagogue-containing medium or control (basal) medium at 14$^{o}$C. Two to three hemi-islets were placed in each well and incubation was initiated for a period of up to 120 min at 14$^{o}$C, gassed with 100% $O_2$. Preliminary studies on basal and glucose-stimulated release of trout INS were conducted to optimize final culture conditions. Test solutions were prepared by adjusting the NaCl concentration so that all solutions were iso-osmotic. Incubation proceeded under the same conditions as preincubation for up to 24 h, after which the medium was removed and the islets were immediately frozen on dry ice. Islets were stored at -80$^{o}$ until RNA extraction and quantification.

*Preparation Of Liver Samples*

Rainbow trout were anesthetized with 0.05% (v/v) 2-phenoxy ethanol immediately before each experiment. A minimum of 6 livers was used for each experiment. Livers were removed and immediately perfused with ice-cold Medium A (in mM: 136.9 NaCl, 5.4 KCl, 0.81 $MgSO_4$, 0.44 $KH_2PO_4$, 0.33 $Na_2HPO_4$, 10 HEPES, 5 $NaHCO_3$; pH7.6) to remove blood. In a petri dish on ice, livers were diced into 1 mm cubes using razor blades. Each diced liver was pooled individually and washed 3 times with Medium A, followed by 2 times with Incubation Medium [In Medium A: 0.24% deffated BSA, 1.5mM $CaCl_2$, 3 mM glucose, 2 mL Gibco MEM amino acid mix (50X) per 100mL, 1 mL Gibco nonessential amino acid mix (100X) per 100 mL; pH 7.63]. Six to eight liver cubes were placed in each well of 24-well culture plates. One milliliter incubation medium was added to each well and liver pieces are allowed to recover for 120 min at 14$^{o}$C with orbital shaking and 100% oxygen. After recovery, medium was removed and liver pieces were rinsed with 0.5 mL Incubation Medium. 900 μL Incubation Medium were added to each well containing liver samples for quantification of insulin and insulin receptor(s) mRNA. Experimental treatments were applied to the liver samples in medium as described below.

*Preparation Of Gill Samples*

Rainbow trout were anesthetized with 0.05% (v/v) 2-phenoxy ethanol immediately before each experiment. A minimum of 6 fish were used for each experiment. Fish were bled, and gill arches removed and stored in ice-cold Dulbecco's Modified Eagle's Medium Base (DMEM) (Sigman D-5030 plus in mM: 27 NaCl, 4 L-glutamine, 4 glucose, 10 HEPES, 4 $NaHCO_3$, 0.24% BSA; pH 7.6). In a petri dish on ice, individual gill filaments

were removed from the arches of DMEM using razor blades. Individual filaments were pooled from each of the 6 fish and washed 3 times with DMEM. Nine to eleven gill filaments were placed in each well of 24-well culture plates. One-half milliliter DMEM was added to each well and gill filaments were allowed to recover for 1 h at 14$^{o}$C with shaking and 100% oxygen. After recovery, medium was removed and gill was rinsed with 0.5mL DMEM before addition of 700 μL DMEM plus antibiotics (100 U/mL Penicillin; 100 μg/mL Streptomycin; 0.1% (v/v) Fungizone, pH 7.6) to each well. Experimental treatments were applied to the gill samples in medium as described below.

*Dose Experiment*

Hormone (SS-14) diluted in incubation medium was added in a 50 μL volume to each well, giving final incubation concentration of 1000 ng/mL, 100ng/mL, 10 ng/mL, and 1 ng/mL. Incubation medium was added to 0 ng/mL control wells. Samples measured were incubated for 6 h at 14$^{o}$C with shaking and 100% $O_2$. Liver samples were collected, frozen on dry ice, and stored at -80$^{o}$C for later analysis of insulin and insulin receptor mRNA(s) expression levels by real-time PCR.

*Time Course Experiment*

Based upon results from the dose experiment, an appropriate and effective dose was chosen for the time course experiment. Liver samples were prepared as described above. Hormone (SS) was added at the appropriate dose to each well in a 50 μL volume (n=6). For measurement of insulin and insulin receptor mRNA(s), samples for time=0 group are immediately collected, frozen on dry ice, and stored at -80$^{o}$C for later analysis by real-time

PCR. All other samples were collected and frozen at the appropriate times (t=3, 6, 12, 24 h).

*RNA Extraction*

Juvenile rainbow trout were anaesthetized by immersion in 0.05% (v/v) 2-phenoxyethanol. Selected tissues (e.g., pancreas, liver, etc.) dissected from juveniles were placed into 2-mL microcentrifuge tubes, immediately frozen on dry ice, and stored at –80$^{o}$C for later analysis. Total RNA was extracted using TRI-Reagent® as specified by the manufacturer (Molecular Research Center, Cincinnati, OH, USA). Isolated RNA was dissolved in 50-100 µL Rnase-free deionized water. Total RNA was quantified by ultraviolet ($A_{260}$) spectrophotometry and diluted to 100 ng/µL in RNase-free deionized water. RNA samples were then stored at –80$^{o}$C until further analysis.

*Real-Time PCR Assay For Insulin And Insulin Receptor-Encoding mRNAs*

Quantification of rainbow trout INS1, INS2, IR1, IR2, IR3, and IR4 mRNAs were measured as described previously (Caruso et al., 2008; Caruso et al., 2010). Briefly, from 200 ng total RNA, endogenous poly(A)$^{+}$ RNA was reverse transcribed in a 10 µL reaction using a iScript™ cDNA Synthesis kit (BioRad, Knightdale, North Carolina, USA) containing a RNase H$^{+}$ reverse transcriptase and a blend of oligo(dT) and random hexamer primers according to the manufacturer's instructions. Reactions without reverse transcriptase were included as negative controls; no amplification was detected in negative controls.

mRNA levels of the four IR-encoding subtypes were determined by real-time RT-PCR using Stratagene Brilliant II and a STRATAGENE MX3000P® Detection System

(Stratagene, La Jolla, CA, USA). Real-time PCR reactions were carried out for samples, standards, and no-template controls in a 10 μL reaction; each reaction contained 1 μL cDNA, 1μL each of forward primer, reverse primer, and probe at concentrations optimized for the mRNA species to be measured, 1 μL RNase-free deionized water, and 5 μL TaqMan Universal PCR Master Mix. Cycling parameters for real-time PCR were as follows: 95$^{o}$C for 10 min, and 45 cycles for 95$^{o}$C for 15 s plus 63$^{o}$C for 30 s plus 72$^{o}$C for 45 s.

*Data Analysis*

Copy number calculations were based on threshold cycle number ($C_T$). The $C_T$ for each sample was determined by the MX3000P™ real time analysis detection software after manually setting the threshold. Sample copy number was determined by relating $C_T$ to a gene-specific standard curve, followed by normalization to β-actin. No difference ($P>0.05$) was observed in β-actin expression among the various treatment groups. No-template control samples did not exceed a maximal increase of 300 (ΔRn) fluorescence units over 45 cycles. Therefore, copy numbers of mRNA were considered non-significant if $C_T$ exceeded 45 cycles; this value corresponds to a detection limit of less than 100 mRNA copies.

*Statistical Analyses*

Quantitative data are expressed as means ± S.E.M. There were 6 individual replicates for the implantation experiment and the tissue culture experiment. For the implantation experiment, statistical differences were estimated by ANOVA, followed by Duncan's multiple range test; a probability level of 0.05 was used to indicate significance. Statistics for the implantation experiment were performed using SigmaStat v. 1.0 (SPSS,

Chicago, IL, USA). Statistical differences for the tissue culture experiment were estimated by the Generalized Linear Mixed Model (GLIMMIX) approach using a split plot design. Whole plot treatments were either time or dose and the sub-plot treatments were INS/IR isoforms. Insulin and IR subtype were treated as repeated measure (both INS1 and INS2 or all IR1, IR2, IR3, and IR4 were measured in the same time/dose group) and an unstructured variance–covariance structure was used when fitting the model. A probability level (p-value) of 0.05 was used to indicate significance. In all cases, the main effects were found to be significant. Comparisons of simple effects were made by the least squares means method using a slicing approach in GLIMMIX; the maximum experiment-wise Type I error rate was controlled at 0.05 using a Bonferroni adjustment. Notations on the face of the figures denote simple effects comparisons; details of the main effects are given in the legends of the figures. Statistics for tissue culture experiments were performed using SAS v 9.2 (Cary, NC, USA).

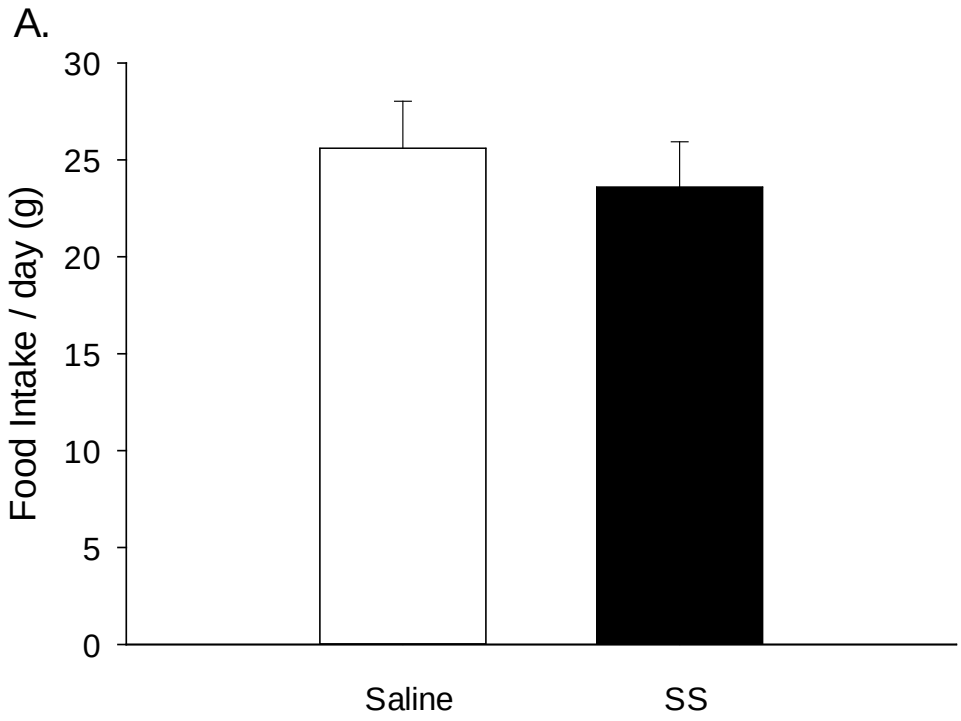
## Results

### *Food Conversion*

Although food intake was similar between saline and SS-14 treated fish, food conversion was 17% lower in SS-14 treated animals compared to controls (Fig. 25).

### *Relative Growth*

Somatostatin-14 treatment resulted in significant growth retardation, in which the most pronounced effect was on body length. Relative growth in mass was reduced by 14%, whereas relative growth in length was reduced by 39% in SS-14 treated fish compared to controls. The condition factor of SS implanted fish was also significantly increased


A.
Food Intake / day (g)
30
25
20
15
10
5
0
Saline
SS

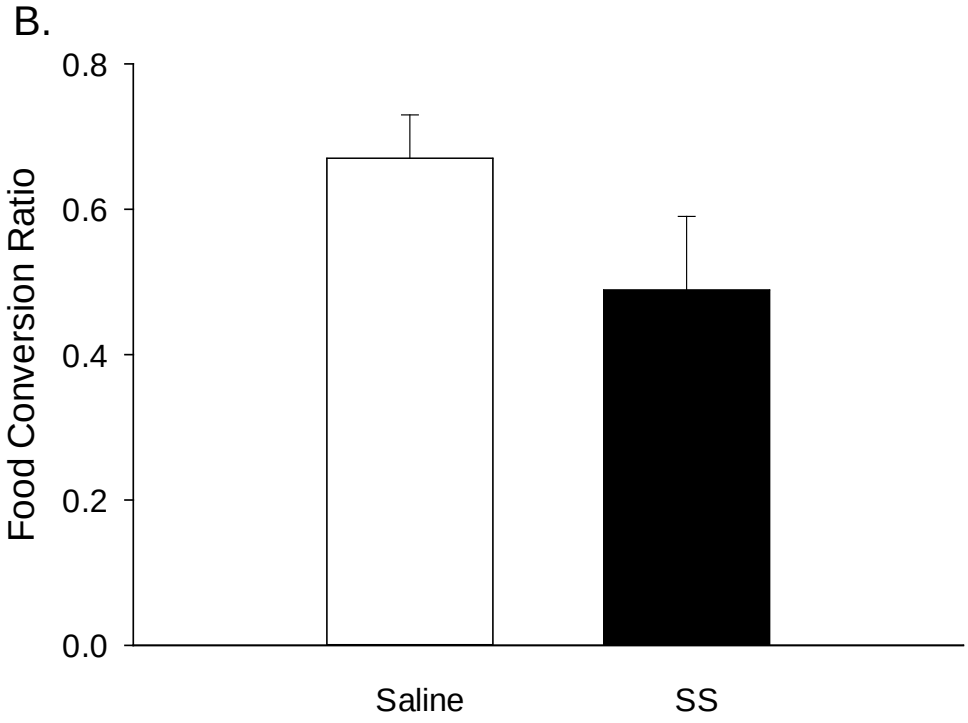

B.
Food Conversion Ratio
0.8
0.6
0.4
0.2
0.0
Saline
SS

Figure 25. Average daily food intake (A) and food conversion (B). Food conversion ratio was calculated as (body wet mass gain) (dry weight food intake per fish)$^{-1}$. Data are presented as mean ± SEM. For a given treatment, groups with different letters are significantly ($P<0.05$) different.

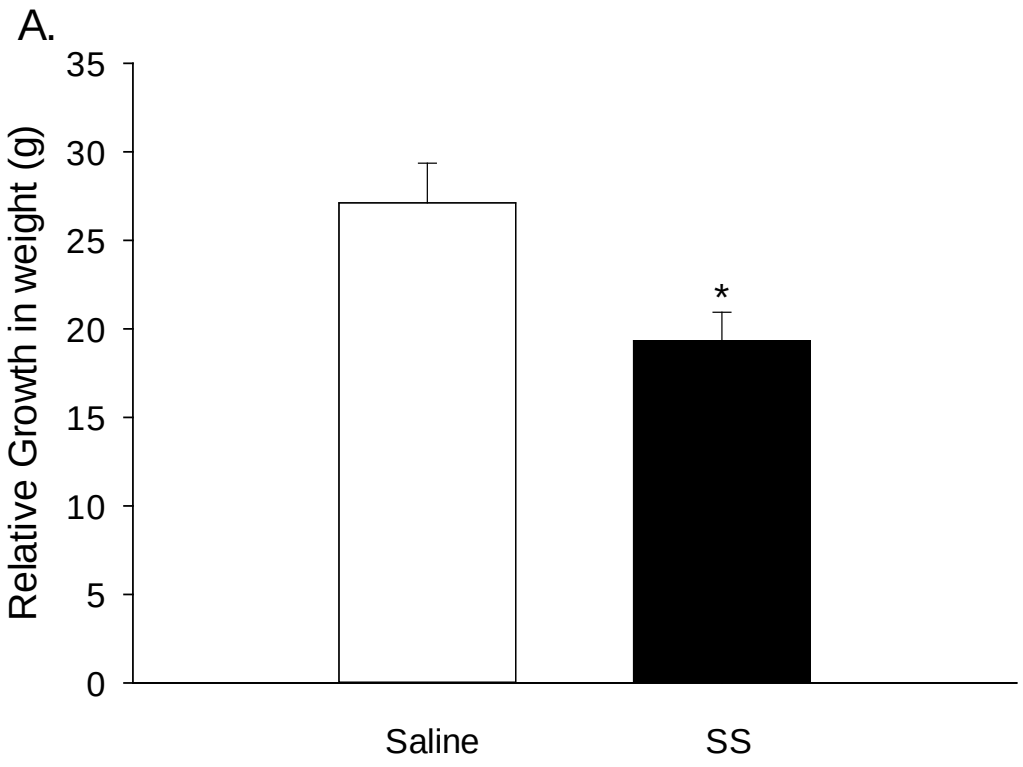
compared to saline-implanted fish due to the pronounced effect on skeletal growth (Fig. 26).

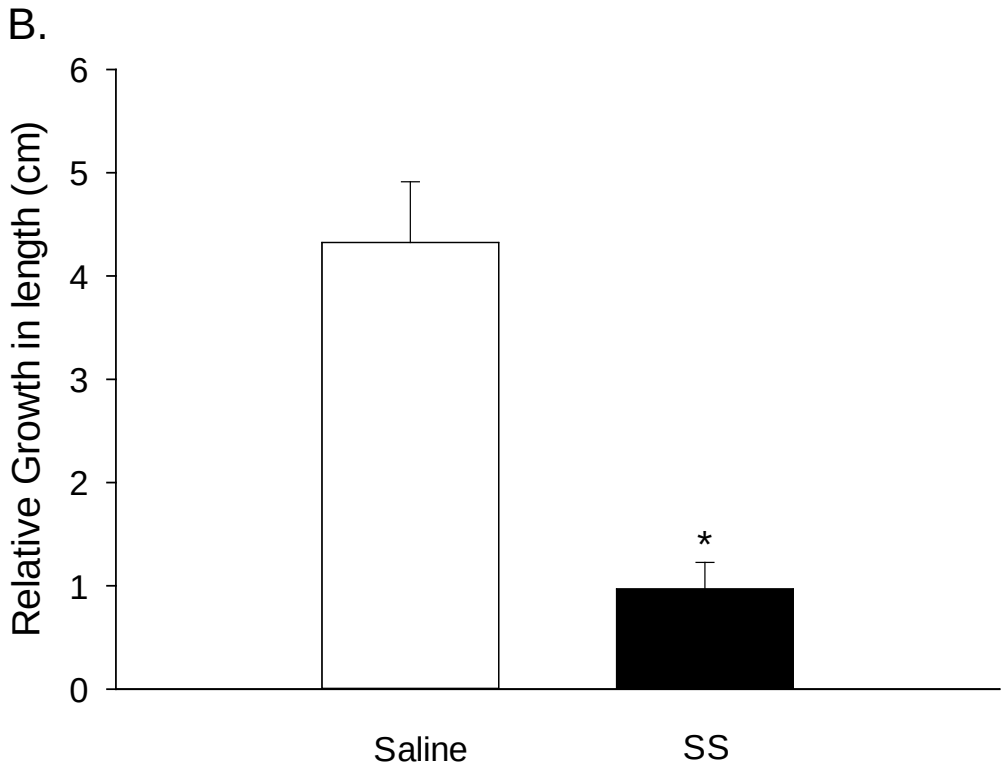
*Effects Of SS On INS Expression*

The effects of SS on INS expression varied by isoform as well as by tissue. Two isoforms of INS mRNA were detected: INS1 and INS2. In pancreas, SS-14 implantation decreased the steady-state level of INS1 by 63% and INS2 by 51% (Fig. 3A). In adipose, SS treatment increased the expression of both INSs; INS1 increased by 159% and INS2 increased by 299% (Fig. 3B). In the brain, INS1 increased by 75% following treatment of SS (Fig. 27C).

*Effects Of SS IR Expression*

The effects of SS on IR expression also varied by receptor isoform as well as by tissue. Four isoforms of IR mRNA were detected: IR1, IR2, IR3, and IR4. In pancreas, SS had no effect on IR mRNA isoforms compared to saline-implanted controls (Fig. 28A). In adipose, SS implantation decreased IR2 and IR3 by 49% and 40% respectively (Fig. 28B). In liver, SS treatment had no effect on IR1 or IR3 and culminated in a decrease of IR2 by 48% and IR4 by 62% (Fig. 28C). In cardiac muscle, SS decreased the expression of all IRs; SS depressed IR1 by 58%, IR2 by 49%, IR3 by 67%, and IR4 by 52% (Fig. 28D). SS


A.
Relative Growth in weight (g)
35
30
25
20
15
10
5
0
*
Saline
SS


B.
Relative Growth in length (cm)
6
5
4
3
2
1
0
*
Saline
SS

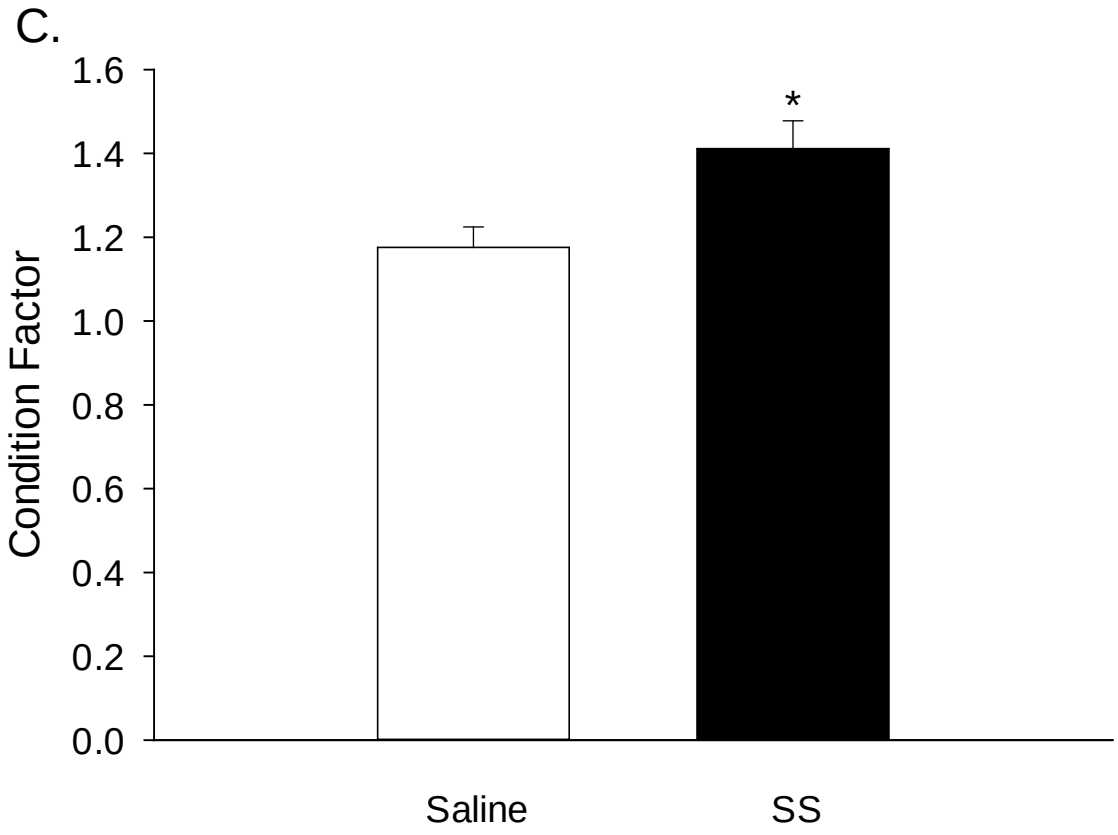
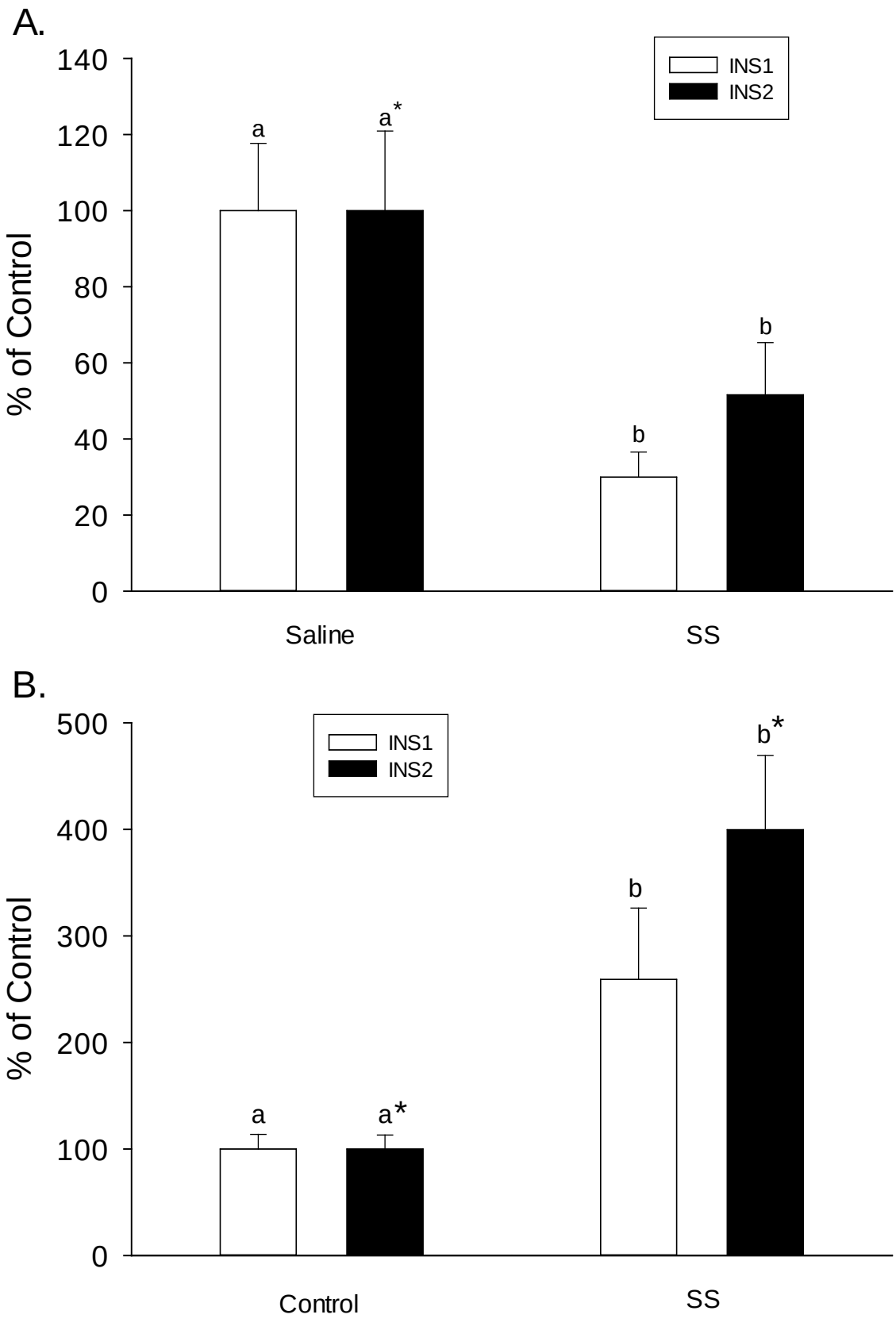



Figure 26. Changes of growth characteristics in weight (A), length (B), and condition factor (C) of rainbow trout implanted with SS-14 and 0.75% saline over a 21-day trial. Relative growth and length were calculated as (100*[final body mass (or length) - initial body mass (or length)]) / [initial body mass (or length)]. Condition factor was calculated as

[weight/(length)$^3$]*100. Data are presented as mean ± SEM. For a given treatment, groups with different letters are significantly ($P<0.05$) different.


A.
140
120
100
80
60
40
20
0
% of Control
INS1
INS2
a
a*
b
b
Saline
SS
B.
500
400
300
200
100
0
% of Control
INS1
INS2
a
a*
b
b*
Control
SS

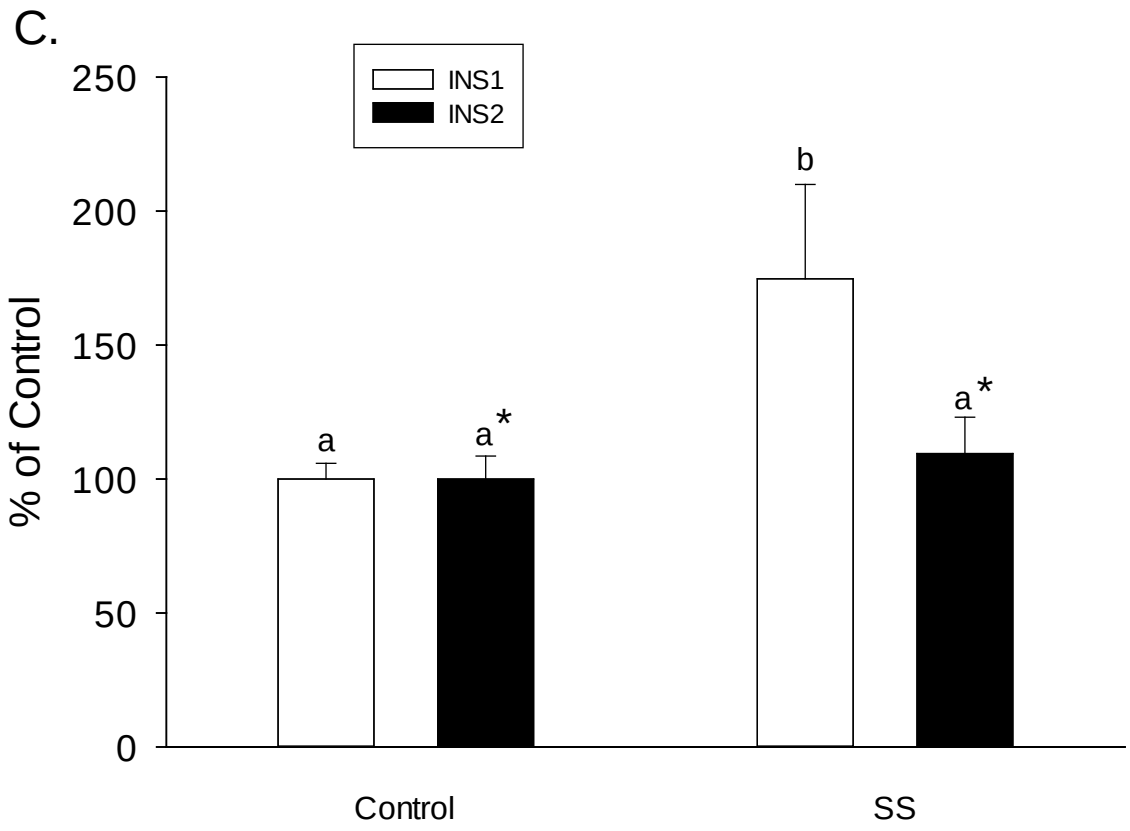
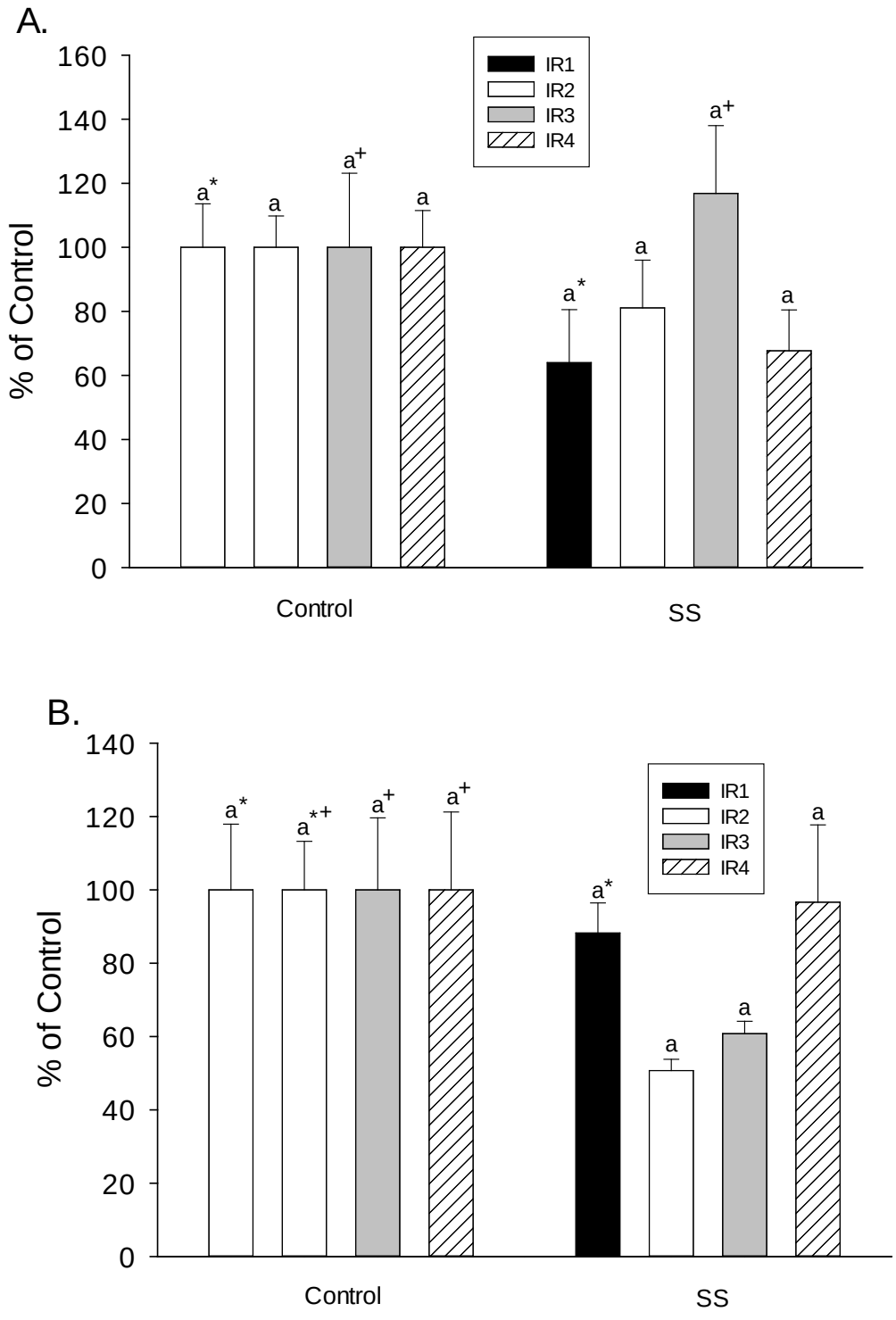
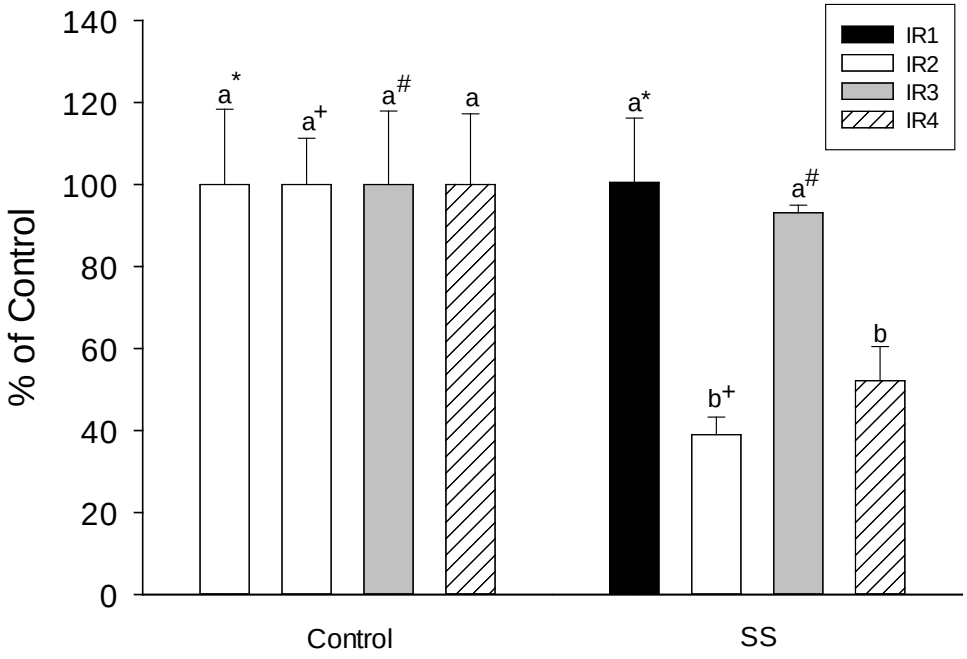


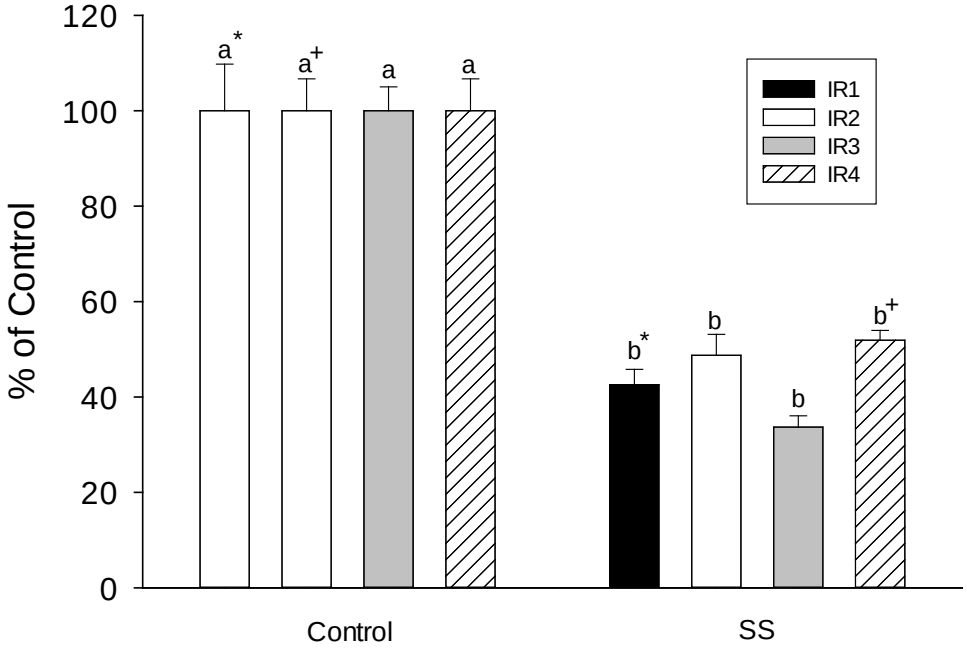

Figure 27. Changes in insulin subtype (INS1, INS2) mRNA levels in the pancreas (A), adipse (B), and brain (C) of rainbow trout associated with SS-14 implantation. Data are presented as means ± SEM. For a particular tissue, letters denote differences for a given subtype across treatments; * denotes a difference in subtypes within a given treatment. Differences between tissues are described only in text.

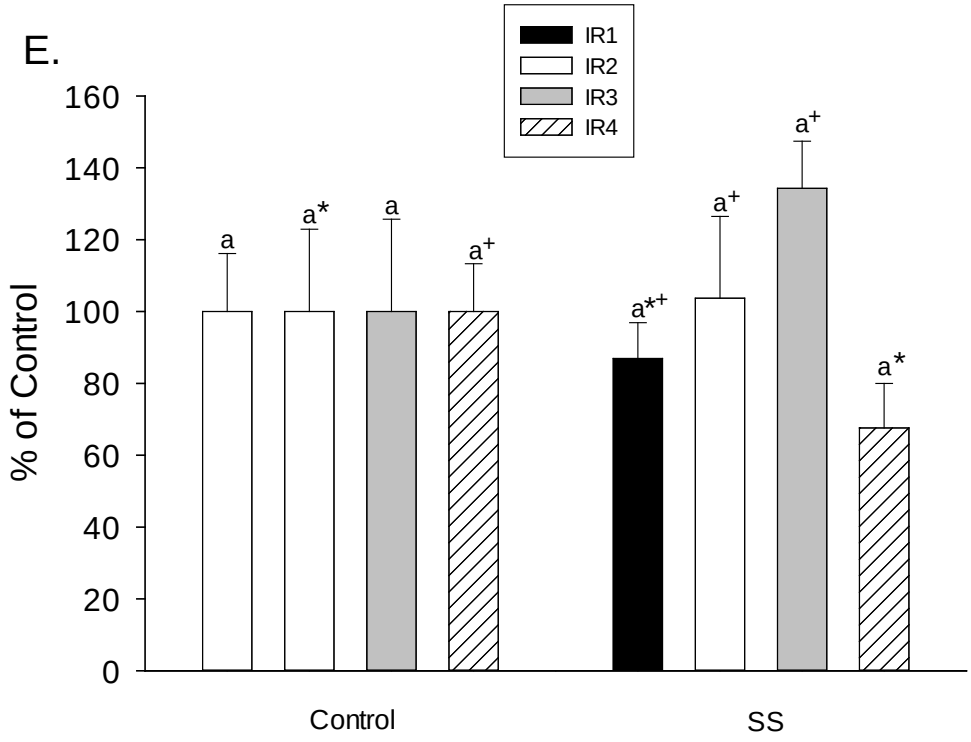

A.
160
140
120
100
80
60
40
20
0
% of Control
IR1
IR2
IR3
IR4
a*
a
a+
a
a*
a
a+
a
Control
SS
B.
140
120
100
80
60
40
20
0
% of Control
IR1
IR2
IR3
IR4
a*
a*+
a+
a+
a*
a
a
a
Control
SS

C.


140
120
100
80
60
40
20
0
% of Control
IR1
IR2
IR3
IR4
a*
a+
a#
a
a*
b+
a#
b
Control
SS

D.


120
100
80
60
40
20
0
% of Control
IR1
IR2
IR3
IR4
a*
a+
a
a
b*
b
b
b+
Control
SS


E.
IR1
IR2
IR3
IR4
% of Control
160
140
120
100
80
60
40
20
0
a
a*
a
a+
a*+
a+
a+
a*
Control
SS

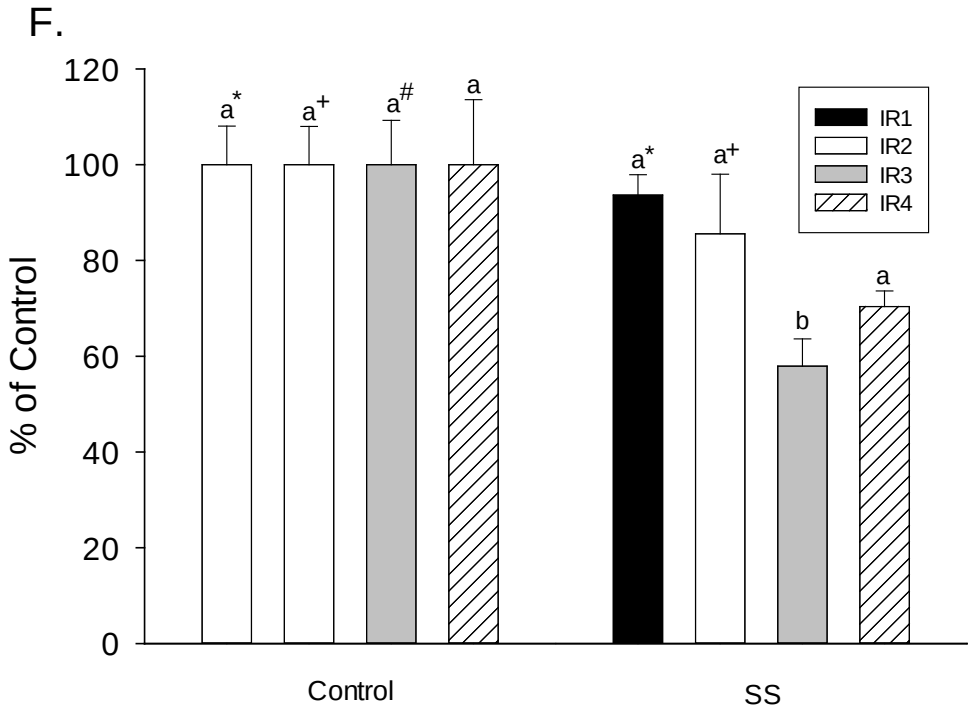

F.
IR1
IR2
IR3
IR4
% of Control
120
100
80
60
40
20
0
a*
a+
a#
a
a*
a+
b
a
Control
SS

Figure 28. Changes in insulin receptor isoform (IR1, IR2, IR3, IR4) mRNA expression in the pancreas (A), adipose (B), liver (C), cardiac muscle (D), skeletal muscle (E), and gill (F) of rainbow trout associated with SS-14 implantation. Data are presented as means ± SEM. For a particular tissue, letters denote differences for a given isoform across treatments; typographical symbols denote a difference in isoform within a given treatment. Differences between tissues are described only in text.

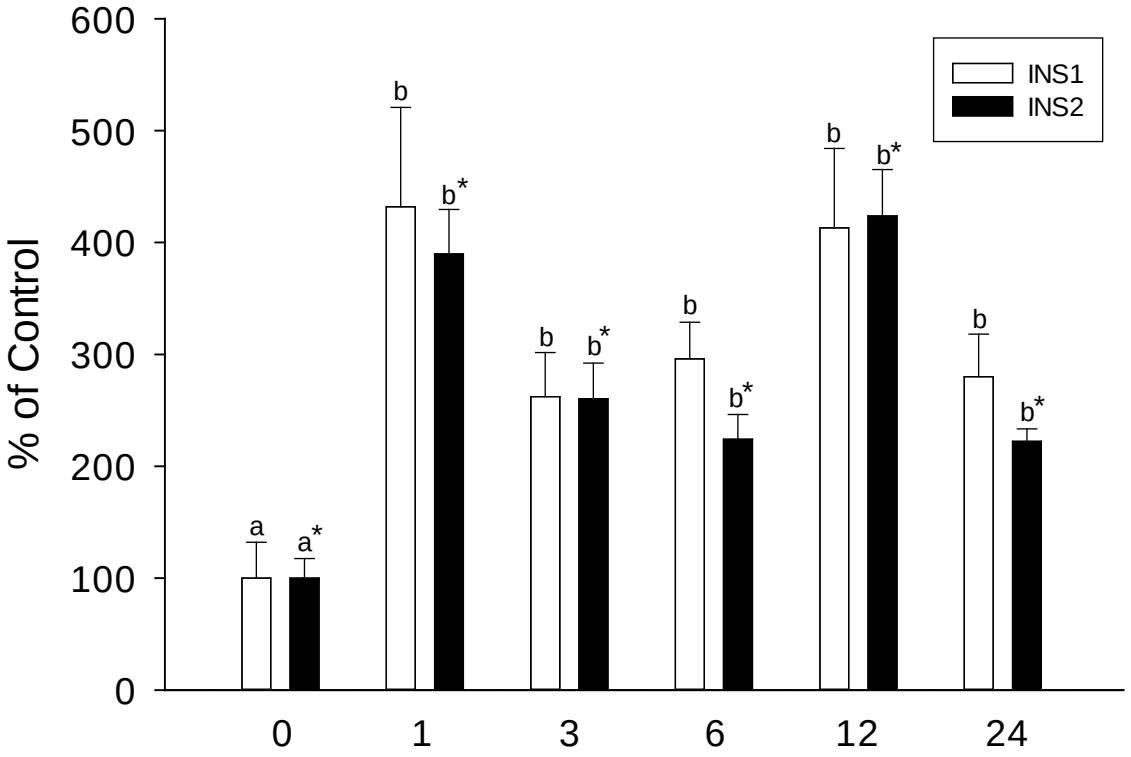
implantation resulted in no significant effect on the expression of all four IR mRNAs in skeletal muscle (Fig. 28E). In gill, SS implantation had no significant effect on IR1, IR2, and IR4; however, IR3 decreased by 42% (Fig. 28F).

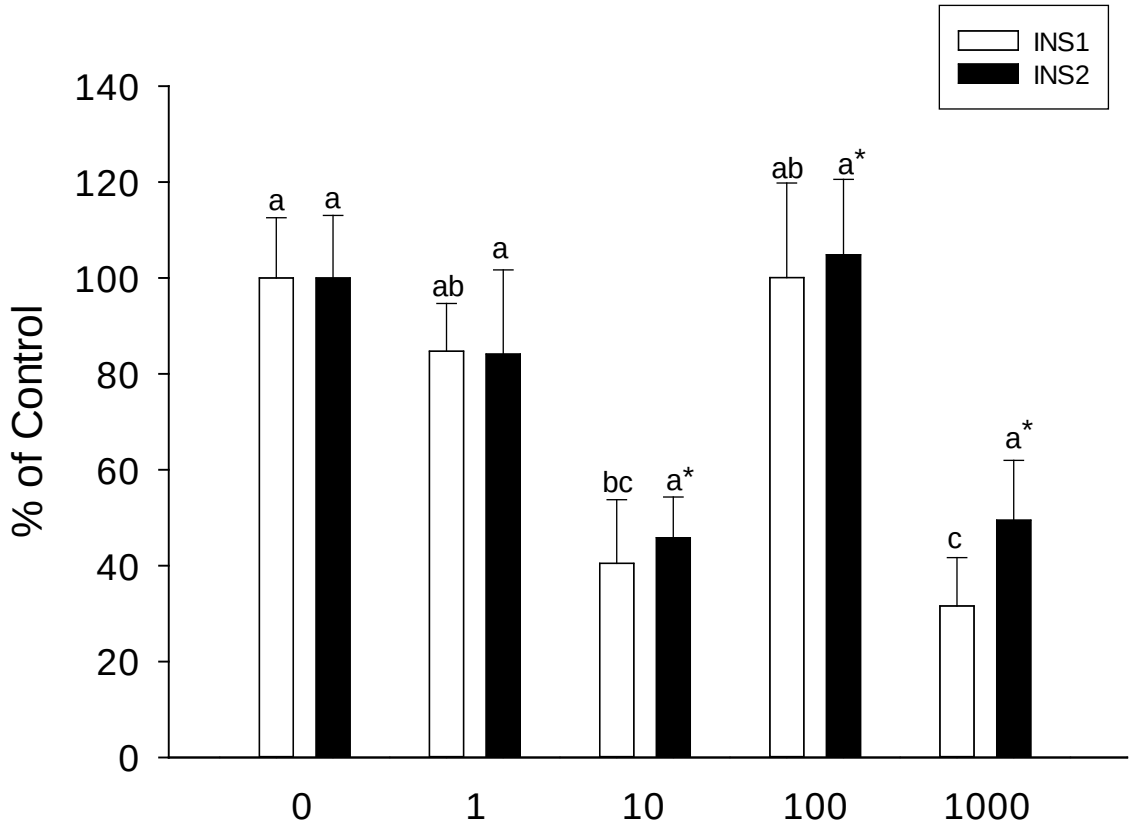
*Effects Of SS-14 Treatment On INS And IR Expression In Pancreatic Hemi-Islets*

Over a 24h time-course treatment, SS increased INS1 and INS2 from 1h to 24h post administration. Expression of INS2 was unaffected by SS dose treatment; INS1 expression was reduced at SS concentrations of 10 and 1000ng/ml (Fig. 29A).

IR mRNA isoforms were regulated by SS treatment in both a temporal- and dose-dependent manner. Throughout the time-course SS assay, IR1 expression did not significantly change; IR2 decreased at 6h; IR3 increased at 24h; and IR4 decreased from 6 to 24h. Over the SS dose course, IR1 decreased at 1000ng/ml; IR2 and IR4 decreased at and above 10ng/ml; and IR3 decreased at 100 and 1000ng/ml (Fig. 29B).

A.


600
500
400
300
200
100
0
% of Control
INS1
INS2
a
a*
b
b*
b
b*
b
b*
b
b*
b
b*
0
1
3
6
12
24


140
120
100
80
60
40
20
0
% of Control
INS1
INS2
a
a
ab
a
bc
a*
ab
a*
c
a*
0
1
10
100
1000

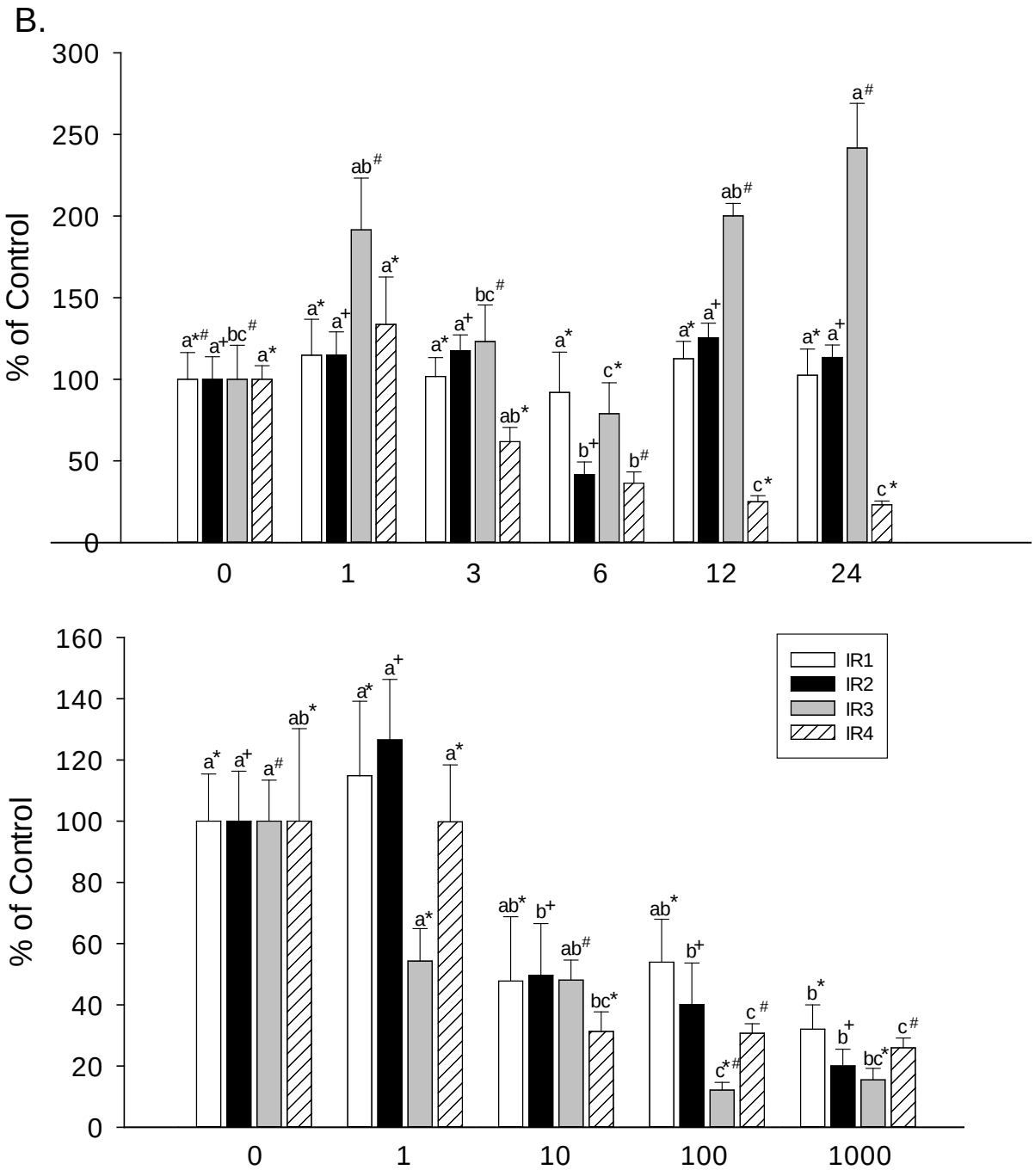



Figure 29. Expression of insulin [INS1, INS2] (A) and insulin receptor isoform [IR1, IR2, IR3, IR4] (B) mRNA from rainbow trout pancreatic hemi-islets after exposure to SS-14 dose (at 1, 10, 100, and 1000 ng [at 6 h]) and time (1, 3, 6, 12, and 24 h [at 100ng])

treatments. Data are presented as mean ± SEM. For a given INS subtype, groups with different letters are significantly ($P<0.05$) different; * denotes a significant ($P<0.05$) difference between INS subtypes. For a given IR isoform, groups with different letters are significantly ($P<0.05$) different; typographical symbols indicate a significant ($P<0.05$) difference between IR isoforms.

*Effects Of SS-14 Treatment On IR Expression In Liver Pieces*

In the time-course assay, SS-14 treatment decreased IR1 at 6 and 12h; IR2 and IR4 expression remained relatively unchanged; and IR3 increased at 24h. Following a SS-14 dose-course treatment, IR1and IR2 were reduced at 1ng/ml; IR3 increased at 10ng/ml; and IR4 reduced at 1000ng/ml (Fig. 30).

*Effects Of SS-14 Treatment On IR Expression In Gill Filaments*

During the time-course SS assay, IR1 expression was unchanged; IR2 decreased at 12h; IR3 decreased at 1h; and IR4 decreased at 3h. Over varying SS concentrations, IR1 expression was remained steady without significant change; and IR2, IR3, and IR4 decreased at elevated concentrations of 100 and 1000ng/ml (Fig. 31).

## Discussion

We previously reported the expression patterns of multiple INS and IR mRNAs in rainbow trout, both in terms of distribution among tissues and in terms of relative abundance within tissues (Caruso et al., 2008; Caruso and Sheridan, 2010). The current study demonstrates the effects of continuous *in vivo* administration of SS-14 over 21 days,

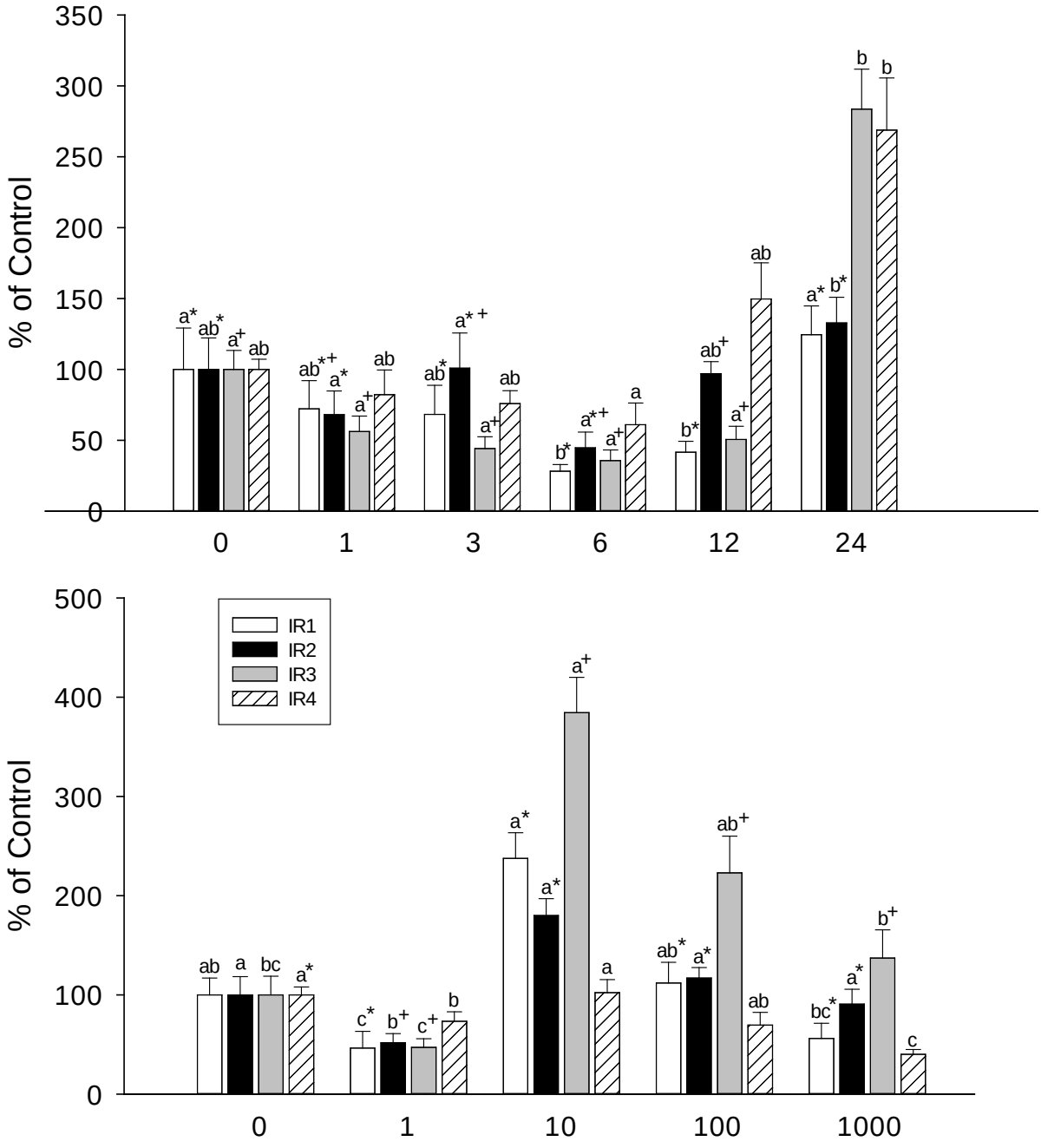



Figure 30. Expression of insulin receptor isoform (IR1, IR2, IR3, IR4) mRNA from rainbow trout liver pieces after exposure to SS-14, dose (at 1, 10, 100, and 1000 ng [at 6 h])

and time (1, 3, 6, 12, and 24 h [at 100ng]) treatments. Data are presented as mean ± SEM. For a given IR isoform, groups with different letters are significantly ($P<0.05$) different; typographical symbols indicate a significant ($P<0.05$) difference between IR isoforms.

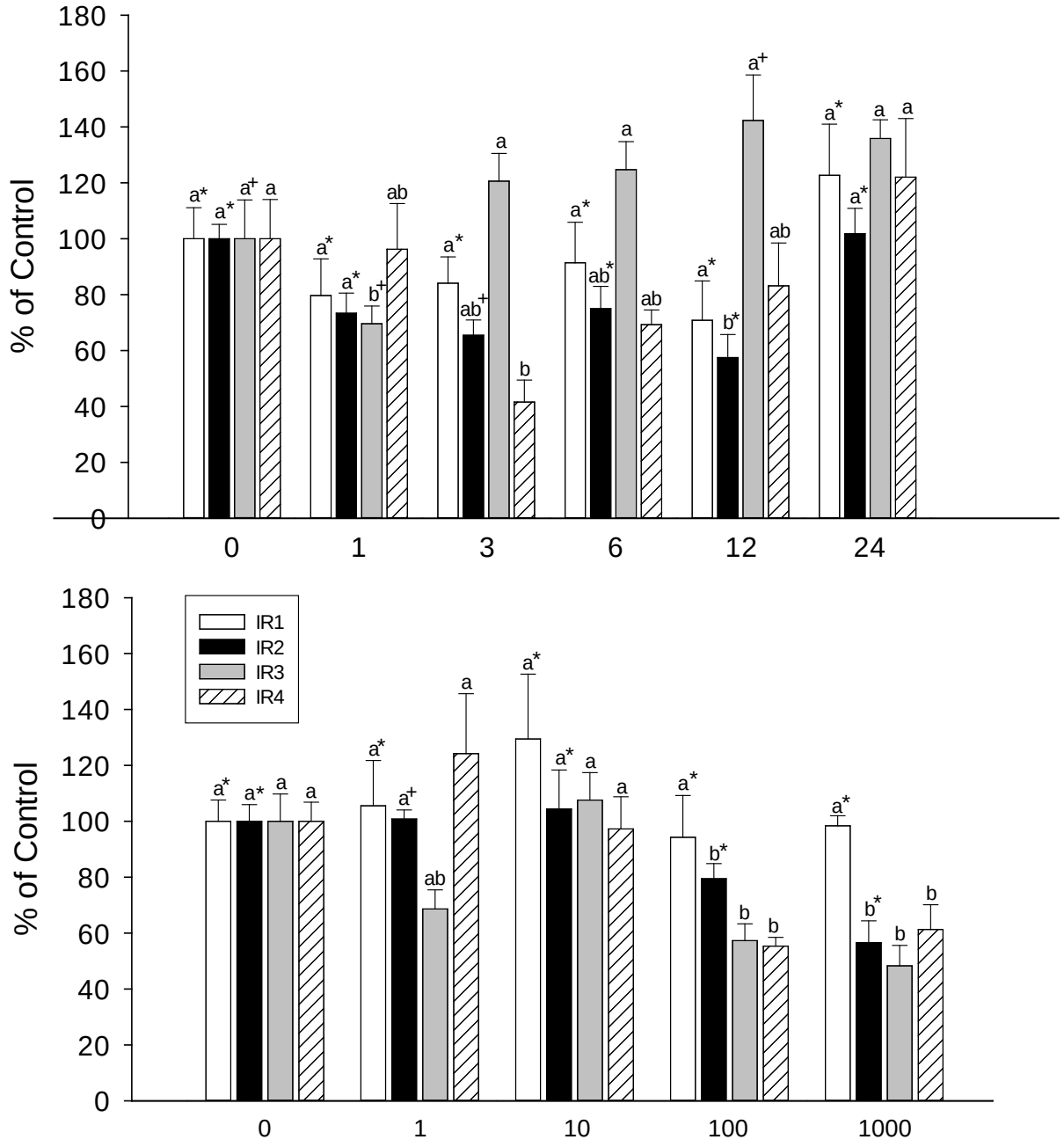



Figure 31. Expression of insulin receptor isoform (IR1, IR2, IR3, IR4) mRNA from rainbow trout gill filaments after exposure to SS-14, dose (at 1, 10, 100, and 1000 ng [at 6 h]) and time (1, 3, 6, 12, and 24 h [at 100ng]) treatments. Data are presented as mean ±

SEM. For a given IR isoform, groups with different letters are significantly ($P<0.05$) different; typographical symbols indicate a significant ($P<0.05$) difference between IR isoforms.

which diminished somatic growth despite sufficient nutrient intake, confirming the known actions of this hormone in mammals and fish (Moaeen-Ud-Din and Yang, 2009; Klein and Sheridan, 2008). This report also suggests that SS-14 treatment does not affect food intake or appetite, as observed in previous experiments (Very et al., 2001). These findings extend our understanding of the actions of SS-14 on the regulation of organismal growth and the INS signaling system in rainbow trout.

Exposure of SS-14 implantation resulted in a significant retardation of growth, in terms of both body mass and body length. This observation is consistent with previous reports on chronic exposure to SS-14 treatment in fish (Very et al., 2001). Relative length exhibited a compelling difference compared to relative weight in SS delivery. The decrease in skeletal growth is in parallel with other reports indicating inhibition of chondrocyte differentiation (Nelson and Sheridan, 2005) and reduced [$^{35}$S] sulfate incorporation into gill cartilage (Very et al., 2001). The antagonist relationship between SS and GH offer insight into their juxtaposing roles on somatic growth. SS is known to inhibit the release of GH and as a corollary inhibit the release and production of IGFs and IGFRs from the liver of fish (Klein and Sheridan, 2008). Although plasma GH or IGF-I was not measured in this study, previous reports of extended SS exposure indicated significantly reduced levels of these growth promoting hormones (Very et al., 2008).

SS regulated INS mRNA expression in a subtype- and tissue-specific manner. The expression of INS mRNAs decreased in the pancreas, increased in adipose, and only INS1 was increased in the brain by SS implantation. The pancreatic expression profile is consistent with reports in mammals and fish, where the putative actions of SS are known to inhibit the release of INS (Gerich et al., 1975; Yao et al., 2005). The *in vitro* SS-14 dose treatment was somewhat consistent with SS implantation, displaying reduced INS1 at concentrations of 10 and 1000ng/ml. However, the time-course SS treatment exhibited increased INS1 and INS2 expression over a 24h period, which is somewhat enigmatic. The effects of SS on INS expression over the time course may been due to an increase in glucagon (GLU) concentration. Although SS has been shown to inhibit the release of pancreatic hormones (GLU, INS, Pancreatic polypeptide [PP]) in fish (Nelson and Sheridan, 2005), GLU has been shown to increase plasma INS in fish and mammals (Perez et al., 1988; Bertuzzi et al., 1995). Extrapancreatic INS producing tissues in fish has recently been identified (Caruso et al., 2008; Hyrtsenko et al., 2007; Roy et al., 2003); however, little evidence has emerged regarding the regulation of these tissues. SS is known to have a lipolytic effect on adipose tissue while INS is known to promote lipogenesis (Dubowsky and Sheridan, 1995). SS lipolytic activity may be regulated in part by the lipogenic effects of INS in adipose tissue, while SS increase INS mRNA in adipose may lead to increased lipid deposition and tissue preservation. Multiple SS variants are synthesized and secreted from various regions of the teleost brain (Nelson and Sheridan, 2005); additionally, systemic SS does cross the blood-brain barrier, as shown in mammals but does so only with a limited ability (Fricker et al., 2009). This indicates that SS may be interacting on INS producing cells within the brain in a similar paracrine manner as in the

pancreas. However, there is a disparate regulation of INS mRNA expression in the brain compared to pancreas. SS implantation affecting only the expression INS1 but not INS2 is consistent with the previous report of INS1 predominant expression within nervous tissue of rainbow trout (Caruso et al., 2008). This is the first known report of INS mRNA regulation commencing an *in vivo* implantation of SS.

SS also regulated IR mRNA expression in a isoform- and tissue-specific manner. SS had no effect on the expression of the four IR mRNAs in the pancreas and skeletal muscle, IR1 and IR3 in the liver, IR1, IR2 and IR4 in the gill, and IR1 and IR4 in adipose. IR mRNA expression decreased in all tissues as follows: IR2 and IR4 in the liver, IR1, IR2, IR3, and IR4 in cardiac muscle, IR3 in gill, and IR2 and IR3 in adipose post SS implantation. There were no increases in IR expression observed following SS treatment *in vivo*. In general, over the SS time- and dose-course treatments, IR expression was either unchanged or reduced at variable time points and/or doses in all tissues examined. However, expression of IR3 increased in both pancreatic hemi-islets and liver pieces at later observed time points and at 10ng within the liver. With the exception of IR3, the general expression pattern was consistent with what was observed in the implantation experiment. This is the first known report of IR expression following SS treatment, both *in vivo* and *in vitro*, which these authors are aware of. The lack of promotion of any of the IR mRNAs suggests that SS may decrease the metabolic potential and eventual somatic growth of the liver, gill, adipose, and cardiac muscle. IR expression may also be regulated by localized INS levels. For example, the decrease in INS from the pancreas may reduce negative feedback of its own receptors within that tissue, similar to what is observed in mammalian β cells (Leibiger et al., 2002). Likewise, the dramatic increase in INS from

adipose tissue may locally negatively regulate IR expression in an autocrine manner. SS decreasing the expression of all IRs in cardiac muscle, while skeletal muscle was unaffected is perplexing and not immediately obvious. Skeletal muscle has been shown to be preserved during periods of nutrient deprivation with a concomitant increase in SS secretion (Ehrman et al., 2002). The lack of response in IR expression within skeletal muscle would be consistent with the preservation of this tissue. Although plasma glucose and free fatty acid concentrations were not measured following SS implantation, other *in vivo* SS treatment reports elevated lipolysis and carbohydrate mobilization (Eilertson and Sheridan, 1993). The hyperlipidemic and hyperglycemic state from chronic SS administration may point to the downregulation of IR expression in tissues that are not primarily regulated by INS. For example, in mammals, because fatty acids are the major metabolic substrate within cardiac muscle, hyperlipidemia would reduce the demand for other metabolites, such as glucose or lactate, which is primarily regulated by INS-stimulated glucose transporter (GLUT)-4 translocation (Abel, 2004). The downregulation of all IR isoforms in cardiac muscle also parallels a similar experiment utilizing GH implantation (Caruso and Sheridan, unpublished results) -- GH, like SS, induces free fatty acid mobilization resulting in hyperlipidemia.

The mechanisms by which SS modulates INS or IR expression are not known. SS-14 is known to interact with numerous SS receptor (SSTR) subtypes; four subtypes have been characterized in fish, some with multiple isoforms (SSTR 1A/1B, 2A/2B, 3A/3B/3C, 5A/5B) (Klein and Sheridan, 2008). SS receptors are G-protein coupled and have been shown to link with numerous cellular effector pathways, including activation of PKA via cAMP, Akt/PKB, PLC, and MAPK (Hagemeister and Sheridan, 2008; Paulssen et al.,

2000). Rainbow trout possess multiple conserved *cis*-DNA binding elements within the INS promoter region (Argenton et al., 1997), which may bind similar transcription factors as those find in mammals. Downstream DNA binding proteins activated from one or more of the aforementioned cell signaling pathways, such as CREB (Han et al., 2011), may have the potential to promote transcription initiation of the INS gene in fish.

In summary, the finding of this study indicate that exogenous SS implantation reduced food conversion and retarded growth, and modulated the expression of INSs and IRs and suggest that independent mechanisms may serve to regulate the various isoforms. The coordinate regulation of INS biosynthesis/secretion and IR biosynthesis/membrane recruitment are important for mediating the various and numerous actions of INS. Continued study of the intracellular mechanisms that serve to regulate INS and IR expression are needed to better understand the tissue- and function-specific responses of INS.

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# GENERAL CONCLUSIONS

Insulin is a pleiotropic peptide hormone, synthesized and secreted in all metazoans, and is produced in single or multiple isoform(s) from one or more tissue types. Previous INS research involving fish species dates back nearly a century and is primarily fixated on INS's nutrient function and regulation. INS initiates its actions when docked to its cognate receptor, the insulin receptor, inducing a conformation change of the IR complex and subsequent catalytic activation, transducing intracellular signaling effector pathways in target tissues. Rainbow trout possess multiple INS products (INS, proinsulin, C-peptide) and multiple IR isoforms, which underlie an elaborate signaling system with the potential to promote a vast array of biological responses. We have shown, for the first time that nutritional state and exogenous hormone treatment independently regulated the expression of INS- and IR-encoding mRNAs.

## Identification And Characterization Of INS- And IR-Encoding mRNAs

Fish possess multiple INS-encoding mRNAs, the expression of which varies during the course of embryonic development as well as among tissues of juveniles and adults. In addition, genome duplication events and/or alternatively splicing has resulted in multiple INS- or IR-mRNA isoforms in both fish and mammals (Andoh et al., 1998; Bunzli et al., 1972; Irwin, 2004). Not until recently has extrapancreatic INS mRNA expressing tissues been identified. Roy et al., 2003 was the first to report INS expression from carp adipocytes. To date, our expression analysis in rainbow trout is the only other known report to measure INS mRNAs in adipose. The expression of INS mRNA in the brain has been observed in mammals and fish (Devaskar et al., 1994; Hrytsenko et al., 2007). Our expression profile confirms the measurement of INS mRNAs in nervous tissue, which are

also shown to be expressed throughout selected brain regions. Further studies could identify the specific cell types that express INS mRNAs within extrapancreatic tissues. Greene and Chen, 1999 were the first to report a semi-quantitative expression profile of three IRs (IR1, IR3, IR4) in rainbow trout in selected tissues. Since then, we isolated and characterized a fourth IR-encoding mRNA in rainbow trout and determined the patterns of all four IR mRNAs across selected tissues with absolute quantification. Besides zebrafish (Papasani et al., 2006), this is the only report to measure INS and IR mRNAs in fish throughout embryonic development. Identification of INS gene cis-elements and transcription factors that bind the INS promoter region may provide further insight into tissue-specific regulation of INS transcription. While these patterns of differential expression are suggestive, further research needs to be conducted to assess the physiological roles of the various isoforms and to elucidate the regulatory mechanisms that underlie their expression.

**Regulation Of INS- And IR-Encoding mRNAs**

INS is a profound nutritional regulator of carbohydrates, lipids, and amino acids in all vertebrates (Chan and Steiner, 2000). To evaluate the nutritional regulation of INS and IR mRNAs, fish were either fed or fasted for 4 and 6 weeks, and a third group was fasted for 4 weeks and then refed for 2 weeks. Fasting decreased the expression of INS mRNAs in pancreas and brain, and increased in adipose compared to fed counterparts. Refed trout displayed restored levels of INS mRNAs in the pancreas, but not in brain or adipose compared to fed animals. In general, fasting for 6 weeks resulted in a greater amount of significant changes in IR expression compared to 4 weeks; increased IR4 in pancreas, IR2 and IR4 in liver, IR3 and IR4 in cardiac muscle, and IR4 in gill; while IR3 and IR4

decreased in adipose. Glucose treatment [at 1, 4, 10 mM] increased IR3 and IR4 in liver pieces, and INS1, INS2, and IR1 in pancreatic hemi-islets at 4mM, in vitro.

We examined the effects of GH, IGF-I, and SS on INS and IR mRNA expression because of their central importance to known growth- and metabolic-related processes in vertebrates. Several studies had already examined the effects of exogenous hormone treatment (e.g., SS) on INS secretion in piscine species (Klein and Sheridan, 2008; Wood et al., 2009). GH increased INS mRNA in pancreas, reduced in adipose, and was unchanged in brain; reduced IR1, IR2, IR3, and IR4 in cardiac muscle, reduced IR1 in gill, increased IR3 in liver, and increased IR2, IR3, and IR4 in pancreas *in vivo*. GH had no effect on INS expression in pancreatic hemi-islets, and, in general, had no effect or decreased IR expression in liver pieces and gill filaments in vitro. IGF-I increased the expression of INS mRNA in pancreas, decreased or had no effect on IR expression in liver, and increased or decreased IR mRNA(s) in gill, *in vitro*. Because GH is a known secretagogue of IGF-I, this suggests that IGF-I, and not GH, induced the increase in INS mRNA expression from the pancreas *in vivo*. Future studies utilizing either treatment or transgenic overexpression of IGF-I, or GH knockdown may elucidate the role of IGF-I on INS and IR mRNA expression. Somatostatin (SS) decreased INS mRNAs in the pancreas, increased in adipose and INS1 in brain, *in vivo*. SS increased INS mRNAs in pancreas and IR mRNAs in liver over time, *in vitro*. It also would be interesting to examine the effects of other pancreatic- (glucagon, pancreatic polypeptide) and adipose-derived hormones (e.g., adipokines) on INS and IR mRNA expression.

## Patterns Of INS And IR mRNA Expression

From our studies examining INS and IR mRNA expression, a few patterns emerge. In juvenile trout, INS1 is always expressed in abundance to INS2 in all INS mRNA expressing tissues. Interestingly, during embryonic development, INS2 is expressed in abundance to INS1 within all body regions. IR1 is predominantly expressed in all muscle tissues (red, white, cardiac); IR1 and IR3 are expressed in abundance within liver, intestine, pancreas, gill, spleen, and kidney; and IR2 and IR3 are predominantly expressed throughout brain segments, pituitary, and body regions during embryonic development. INS expression decreased in pancreas and increased in adipose following fasting and SS treatment, in vivo. Whereas, INS expression increased in the pancreas and decreased in adipose following feeding and GH treatment *in vivo*. Further in vitro studies may utilize metabolites, GH, IGF-I, and SS on adipose tissue or adipocytes to determine their direct effects on INS and IR mRNA expression patterns.

## Future Studies

Further studies should be designed to examine mechanisms, other than the modulation of mRNA expression, from nutritional and hormonal treatments in vivo and in vitro. The possibility remains that the differential regulation of INS and IR mRNAs are not transduced into changes in translated protein products, which could be measured in intracellular pools, and in plasma or plasma membranes, respectively. Studies could examine mRNA stability, transcriptional rates, membrane recruitment, translational regulation, post-translational modifications, and/or binding capacity. Additional INS products should also be investigated. Although a receptor for the C-peptide has not been determined, recent studies in rainbow trout suggest a postprandial metabolic role in the gut

(Polakof et al., 2010). The multiple C-peptide isoforms in rainbow trout may each contain novel functions and may be independently regulated. The role of multiple proinsulins still needs to be further refined. Due to the structural similarity of proinsulin to IGF-I, both molecules have been difficult to measure independently in the plasma of salmonids (Plisetskaya, 1998). Proinsulin also may contain the capacity to bind multiple IR and IGF-IR isoforms. Inhibition or downregulation of prohormone convertases, which convert proinsulin to INS, may assist in the elucidation for the role of proinsulin in vertebrates.

Research in fish indicates that INS secretion is affected by a variety of macronutrients. However, clear patterns of species specificity and the impact of native diet on the responsiveness to each macronutrient have not emerged. Moreover, the explanation for poor glucose clearance in carnivorous fish is still not fully understood. Expanded study of multiple species with disparate native diets will be necessary to elucidate the mechanisms underlying peripheral glucose intolerance. Such expanded studies will enhance our overall understanding of INS, IR, GLUT, and known INS responsive signaling modulators in fish and to establish correlates of these features with life history.

The role of INS in adipose tissue, lipid accumulation, glucose and amino acid uptake, and gluconeogenesis all possess unresolved mechanisms and much of the literature falls short or is contradictory. Shortcomings may exist because of several factors including, species variation, season, native diet, and the use of non-fish specific markers and probes. In addition, much of what we know about INS action in fish comes from studies on muscle and liver, and our knowledge of actions of INS on adipose and brain are somewhat lacking and requires further attention. Further research also needs to be conducted on various transporters responsible for glucose and amino acid uptake in

response to nutritional and biochemical mediators within selected tissues of herbivorous, omnivorous, and carnivorous fish. Most notably, however the critical importance of amino acids in many fish, there is a dearth of literature on amino acid transporters in fish. Once amino acid transporters could be identified, the tissue-specific regulation of amino acid uptake could be more clearly defined, especially in response to INS.

Future research also needs to be conducted on IR and propagation of signal through effector pathways. In particular, information is needed about the regulation of IR expression and interaction between IR and adapter proteins. In addition, little is known about the functional significance of the various IR subtypes. In particular, further studies are needed to examine the ligand specificity of fish IRs and the linkage between IR subtypes and cellular effector pathways as well as between the effector pathways and specific physiological processes.

## Conclusion

Rainbow trout possess multiple insulin- and insulin receptor-encoding mRNAs, which are differentially expressed and independently regulated in a tissue- and isoform-specific manner.

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