AN ABSTRACT OF THE DISSERTATION OF

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Title: The Use of Molecular Biological Methods to Assess the Effects of Endocrine Disrupting Chemicals and Natural Hormones on Growth in the Sheepshead Minnow (*Cyprinodon variegatus*).

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Carl B. Schreck

The work presented in this dissertation examines possible modes of action for growth inhibition by anthropogenic endocrine disrupting chemicals (EDCs) as well as endogenous hormones associated with growth in fish. Using the sheepshead minnow (SHM) (*Cyprinodon variegatus*) as a model, I developed methods to examine perturbations in the endocrine axis controlling fish growth, and also examined effects of EDCs on the whole fish.

I used two relatively new techniques to study the endocrine growth axis, quantitative real-time PCR (TaqMan) and differential display analysis. TaqMan analysis is a highly sensitive method to measure specific sequences from a small amount of total RNA using a fluorescent probe and specific primer pairs. I optimized a TaqMan assay for SHM IGF-I to measure hepatic IGF-I mRNA concentrations. in fish injected with hormones known to influence fish growth (GH, T_3, E_2, insulin, or a carrier control). IGF-I mRNA levels increased in fish injected with GH, T_3 and insulin, peaking at 12 h post-injection. IGF-I

mRNA levels decreased significantly at 8 h and 12 h post-injection in fish injected with E_2, suggesting that pharmacological levels of E_2 may affect the GH/IGF-I axis and could have consequences for fish living in waters polluted by EDCs.

Differences in growth were observed in fish exposed for 18 weeks to E_2 or chlorpyrifos (an organophsophate). Fish exposed to the highest dose of E_2 grew larger than controls only during the last week of the experiment. Fish exposed to the lower dose of E_2 were not significantly different from controls. The fish exposed to all doses of chloryprifos grew significantly less than controls in a dose-dependent manner. No significant differences were found in hepatic IGF-I mRNA levels in any treatments.

To establish patterns of gene up- or down-regulation, I performed differential display analysis on livers of several fish from the previous two experiments. Several genes were identified as being similar to fish including a microsatellite sequence, a choriogenin (vitelline envelope) protein mRNA sequence, a transferrin mRNA sequence and several ribosomal RNA sequences. This technique to evaluate gene expression will become more useful when more fish genes are added to the data bases.

The Use of Molecular Biological Methods to Assess the Effects of Endocrine Disrupting Chemicals and Natural Hormones on Growth in the Sheepshead Minnow (*Cyprinodon variegatus*)

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Iris Knoebl, Author

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CONTRIBUTION OF AUTHORS

Dr. Leroy C. Folmar provided guidance in the experimental design and assisted in the interpretation of the data and the writing of Chapters 3, 4 and 5. Dr. Nancy D. Denslow provided guidance, laboratory space and equipment for Chapters 3 and 5. Dr. Leah D. Gillis isolated and cloned the IGF-I sense strand sequence used in Chapters 1 and 2.

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1. INTRODUCTION

Increasing evidence suggests that many chemicals in the environment can alter reproduction, growth and survival of wildlife by changing normal endocrine system function. The endocrine system has a complex and interrelated organization that plays a key role in regulating essential physiological and morphological processes. The term "endocrine disrupting chemical" (EDC) has evolved to describe environmental agents that alter the endocrine system in an intact organism (Ashby, 1997). There are many possible EDCs and endocrine pathways that may be affected, but most of the attention has focused on chemicals that mimic estrogens (Stone, 1995; Colborn, 1996; Colborn, 1995; Colborn, 1992). These "xenoestrogens" are structurally diverse molecules that have been linked to developmental and reproductive abnormalities in terrestrial (Crain, 2000; Crain, 1997; Colborn, 1996; Fry, 1995) and aquatic (Oberdoerster, 2000; Sumpter, 1998) wildlife. With respect to the effects of EDCs on fish, the focus has largely been on the induction of female specific proteins in males (vitellogenin and egg shell proteins) and altered sexual development (Cheek, 2001; Oberdoerster, 2000; Folmar, 1996; Folmar, 2001a; Sumpter, 1995; Sumpter, 1998).

Other physiological processes may also be affected by EDCs in fish. The effect of EDCs, especially xenoestrogens, on fish growth is an area of concern. During normal reproductive maturation in female fish, plasma estradiol increases and somatic growth is curtailed as

energy is diverted from somatic growth to gonadal growth. The mechanism that triggers that energy diversion is unknown. A possible consequence of continuous exposure to xenoestrogens may be the redirection of energy toward gonadal growth at the expense of somatic growth during life stages when somatic growth would normally occur. Reduced somatic growth was reported in channel catfish (*Ictalurus punctatus*) fed the synthetic estrogen diethylstilbestrol (Bulkley, 1972), in rainbow trout (*Oncorhynchus mykiss*) fed 17β-estradiol (Sower, 1983; Johnstone, 1978; Ostrowski, 1986) and in rainbow trout exposed to alkylphenolic compounds (Ashfield, 1998), which are all known to have estrogenic effects. Other environmental chemicals known to inhibit growth in fish are organophosphate pesticides such as chlorpyrifos (Cripe, 1986). Although not known to be estrogenic, chlorpyrifos has been implicated as an EDC in mammals by altering reproductive development and stimulating neuroendocrine GnRH mRNA *in vitro* (Gore, 2001). The mechanisms by which the afore-mentioned compounds affected somatic growth in fish were not elucidated, however studies in mammals suggest that chronic exposure to estrogens may inhibit synthesis of hepatic insulin-like growth factors (IGFs) (Borski, 1996; Murphy, 1988).

Growth inhibition may have negative effects not only on individuals but on populations as well. Rapid growth may have a selective benefit for some fish species because they are subjected to size-specific predation, thereby increasing the likelihood of predation for smaller fish. Larger fish may also have a competitive advantage in feeding within a population of fish. Reduced growth in fish populations exposed to EDCs could have serious effects on survival, especially if growth-inhibition is also related to reproductive

dysfunction. Reduced growth in some species may also affect fish as a resource to anglers.

Somatic growth in vertebrates is regulated primarily by an endocrine pathway, known as the somatotropic axis, which involves the brain neuroendocrine system, pituitary growth hormone (GH) and peripheral insulin-like growth factors (IGFs). Insulin-like growth factors (IGF-I and IGF-II) are mitogenic peptide hormones that act through autocrine, paracrine and endocrine actions to regulate body growth in vertebrates (Daughaday, 1989). Because of the complexity of the somatotropic axis, the potential for disruption by EDCs exists at many levels. However, no research exists as to whether, or at what level, chemicals in the environment may disrupt this axis. Basic research, as well as new laboratory techniques are required to elucidate regulatory mechanisms of somatic growth at the molecular level and help define specific sites of possible endocrine disruption in fish.

My research explores some of the molecular mechanisms by which endogenous hormones and EDCs may affect the somatotropic axis. I have focused particularly on changes in IGF-I mRNA synthesis in response to both endogenous hormones, estrogenic chemicals and the pesticide chlorpyrifos. My hypothesis is that estrogenic chemicals and chlorpyrifos may adversely regulate somatic growth by decreasing hepatic IGF-I synthesis and thereby inhibiting growth in sheepshead minnow (*Cyprinodon variegatus*). Several endogenous hormones affect IGF-I synthesis, however little is known about how quickly these hormones elicit a response in this species. To measure changes in the rate of hepatic IGF-I synthesis in response to endogenous hormones, I injected fish with estradiol (E_2), triiodothyronine (T_3), insulin and growth hormone and measured IGF-

I mRNA at several timepoints. I developed a quantitative real-time PCR assay to measure hepatic IGF-I mRNA in this species (Chapter 3). To test whether long-term exposure to E_2 and chlorpyrifos inhibits growth and hepatic IGF-I mRNA synthesis, I exposed fish to those chemicals from hatching until sexual maturity. I measured growth weekly, and determined the level of IGF-I gene induction by measuring IGF-I mRNA at the conclusion of the experiment (Chapter 4). In addition to IGF-I gene induction, other genes may be involved in growth regulation. Most physiological processes involve the induction or suppression of specific genes. To examine genes up- or down-regulated in response to EDC exposure or endogenous hormones, I optimized a method to identify differentially expressed genes (Chapter 5). By identifying differentially expressed genes, a better understanding of the physiological mechanisms involved in response to EDCs can be attained.

The work presented here begins to elucidate an area of research that is important for fish populations exposed to EDCs as well as presents new laboratory techniques to define and measure possible mechanisms of endocrine disruption of the somatotropic axis. To provide a better understanding of the complexity of the somatotropic axis, a review of the current literature is presented (Chapter 2) followed by an overview of EDCs and their possible mechanisms of action.

2. LITERATURE REVIEW

GROWTH

Animal growth can be defined as an increase in size or cell number over time, or as an increase in calories stored as somatic tissue. Growth has also been described as the positive flow of energy into macromolecules and their subsequent organization into cell structures and tissues in excess of the normal replacement rates (Mommsen, 1998).

The energy component of growth is important to understand the factors that affect growth in fishes. Calories ingested through food consumption are used either as energy for metabolism or growth. Metabolic expenditures include body maintenance and repair (standard metabolism), food digestion (specific dynamic action) and movement (active metabolism). Metabolic byproducts are excreted as ammonia, carbon dioxide, urea and feces. A positive energy balance in metabolism is associated with growth (Ware, 1980). The "scope for activity" is a measure of the relative amount of non-maintenance energy reserves in fish and is defined as the difference between active metabolism and standard metabolism (Fry, 1971). Fish with a higher scope for activity, or more energy reserves, are better able to move, grow, reproduce and resist diseases and parasites.

Since metabolism is the sum of anabolic plus catabolic processes, the rate of anabolism exceeds catabolism in growing fish. Anabolic processes are regulated primarily by hormones, however, growth rate in fish can be highly variable because the actions of those

hormones are attenuated by a variety of interacting environmental factors. Most important are food (availability and quality) and tempera-ture (increased temperature will increase standard metabolic rate, thereby reducing energy available for growth if ration is held constant). Other factors affecting fish growth include salinity, dissolved oxygen, rearing density, social interactions, inter- and intraspecific competition, predation, pathogens, and water quality (cf. Moyle, 2000). Any increase in metabolic expenditure to maintain homeostasis in physically or chemically unfavorable environments decreases energy allocation to growth.

The genetic makeup of a fish species determines its ultimate size. Likewise, that species' genes determine whether the male or the female will become larger. Fish differ from other vertebrates in two unique aspects of growth. First, growth is indeterminate, continuing throughout the life of the fish, although the relative growth rates decrease with age. Second, unlike other vertebrates, fish muscle tissue grows by both hyperplasia and hypertrophy long after hatching and past the juvenile stages (Mommsen, 1998). These and other genetically controlled variations in fish growth are a result of the integration of environmental and endogenous cues by the brain. The brain signals the endocrine system to begin the cascade of events that regulate genetic actions resulting in growth. The major endocrine axis involved in growth is the growth hormone releasing factor (GRF) – growth hormone (GH) – insulin-like growth factor (IGF) axis.

ENDOCRINE CONTROL OF GROWTH IN FISH

Historical Perspective

IGF-I and IGF-II are important factors in controlling both growth and metabolism. IGFs in mammals were first identified as factors that stimulated cartilage growth, and as factors having non-suppressible insulin-like activity (NSILA) on adipose tissue metabolism. GH was known to stimulate synthesis of cartilage matrix in rats when measured by cartilage sulfate uptake assays (Salmon, 1957). However, in assays using hypophysectomized rats, sulfate uptake was greatly reduced compared to control rats (Murphy, 1956). *In vivo* injections of pituitary extracts and purified bovine GH restored sulfate uptake into cartilage (Denko, 1955) (Salmon, 1957), but bovine GH alone could not stimulate cartilage synthesis in experiments using cartilage slices *in vitro* (Salmon, 1957). The addition of diluted rat serum restored cartilage synthesis. This observation suggested that a mediating factor, or "sulfation factor" induced by GH, stimulated cartilage growth (Daughaday, 1966). The term "somatomedin" was later given to the sulfation factor, which was able to mediate the effects of GH (Daughaday, 1972).

Meanwhile, other researchers demonstrated that certain serum factors expressed insulin-like metabolic actions (Froesch, 1963) and stimulated the replication of rat embryo fibroblasts in tissue culture (Dulak, 1973). It is now known that two related peptides, similar in structure to proinsulin and probably evolving from a common ancestral gene, mediated these biological effects. The GH-dependent somatomedin (also known as somatomedin C) is now known as IGF-I and the factor that stimulated replication of cultured fibroblasts is

now called IGF-II. While the term somatomedin is still accepted as synonymous with IGF-I, this is not necessarily the case with IGF-II, which is not under strict GH control (Daughaday, 1987).

The "somatomedin hypothesis" became the accepted model of IGF-I action. The original somatomedin hypothesis postulated that GH stimulated hepatic IGF-I production, with IGF-I acting in an endocrine manner on peripheral tissues. Subsequent research led to a re-evaluation of the original somatomedin hypothesis, demonstrating that many tissues expressed IGF-I (D'Ercole, 1980). This discovery expanded the hypothesis to include an autocrine/paracrine function for IGF-I, although it was still believed that this local production of IGF-I was GH dependent and that all GH effects were mediated by IGF-I. Further research raised questions about the role of circulating IGF-I in mediating the actions of GH. Studies using direct injection of GH into the cartilage growth plate of rats resulted in longitudinal bone growth (Isaksson, 1982) and concluded that circulating IGF-I was not required for stimulation of longitudinal bone growth but that GH itself directly stimulated the cartilage. The "dual effector hypothesis" was later proposed (Green, 1985) to explain the roles that GH and IGF-I play in growth and differentiation. This hypothesis suggested that in addition to GH-stimulated hepatic and local IGF-I production, GH had direct effects on peripheral tissues not mediated by IGF-I.

Today it is clear that the GRF-GH-IGF axis is considerably more complicated and involves multiple levels of interactions and controls. The regulation of somatic growth includes both endocrine functions of IGF-I, modulated by GH-induced binding proteins, and a local mode of action involving direct effects of both GH and IGF-I (and possibly IGF-II) at the growth plate.

Investigations into the endocrine control of growth in teleosts revealed that the GRF-GH-IGF axis was conserved among vertebrates. Early studies by Komourdjian and Idler (1978) first revealed that a hepatic factor was involved in the *in vitro* uptake of sulfur in rainbow trout bone. Later researchers found that hypophysectomy of Japanese eel (*Anguilla japonica*) decreased sulfate incorporation in ceratobranchial cartilage (Duan, 1990). Injection with recombinant eel GH caused an increase in plasma GH with a peak after 6 hours, but cartilage sulfate incorporation reached a maximum after 12 hours and remained high for 48 hours. Addition of eel GH alone to culture medium of eel cartilage did not affect sulfate uptake. Moreover, serum from hypophysectomized eels did not stimulate cartilage sulfate uptake whereas serum from intact eels did, suggesting, as in mammals, a somatomedin effect for GH action (Duan, 1990). Furthermore, bovine IGF-I was highly effective in stimulating sulfate uptake in eels while IGF-II was less effective (Duan, 1990). In coho salmon (*Oncorhynchus kisutch*) (McCormick, 1992) and goby (*Gillichthys mirabilis*) (Gray, 1991), similar indirect GH actions were reported, and it was shown that mammalian IGF-I increased sulfate uptake cartilage. The first nucleotide sequence of fish IGF-I mRNA was reported by Cao, *et al.* (1989) in coho salmon. That study also showed that injection with bovine GH increased liver IGF-I mRNA levels. A recombinant coho salmon IGF-I was later produced, purified and characterized (Moriyama, 1993) and a homologous RIA for coho salmon IGF-I was developed (Moriyama, 1994).

Early studies indicated the presence of both IGF-I and IGF-II in mammalian serum as well as serum of turtles and chickens, but only IGF-I was detected in amphibians and fish (Daughaday, 1985) leading

to speculation that the gene duplication giving rise to two IGFs occurred during reptilian evolution. Later, using both radioimmuno-assay and radioreceptor assays, it was revealed that both IGF-I and IGF-II were present in skeletal tissues of 10 vertebrates including frog, trout and shark (Bautista, 1990). Subsequent research demonstrated the presence of both IGF-I and IGF-II in rainbow trout (*O. mykiss*) (Shamblott, 1992) providing evidence that a divergence of IGFs occurred earlier in vertebrate evolution than previously believed.

Overview of the GRF-GH-IGF Axis

A description of the general components for the endocrine growth axis in fish is presented here. Detailed information will be addressed in subsequent sections. The hypothalamus synthesizes and releases neurohormones that regulate GH (somatotropin) secretion from the somatotroph cells of the adenohypophysis. The principal regulatory neurohormones for GH secretion are antagonistic, growth hormone-releasing hormone (GRF) and somatotropin release-inhibiting factor or somatostatin (SRIF). The GH secretagogue (GHS) grehlin may also exist in fish. Many other factors such as neuropeptide Y (NPY) and gonadotropin releasing hormone (GnRH) also regulate GH release. The major effect on growth promotion by GH is through activation of the GH receptors, primarily in the liver, which induce hepatic insulin-like growth factor (IGF-I) synthesis as well as synthesis of several IGF binding proteins (IGFBP). The liver also produces and secretes IGF-II. The IGFs and the IGFBPs are then released into the circulation to mediate growth-promoting effects at peripheral target tissues. The IGFs are also produced locally in many tissues where they act in an autocrine or paracrine manner. The

actions of IGF-I and IGF-II are mediated by the IGF-I receptor, which is expressed by a wide variety of cell types. Figure 2.1 presents a diagram of the endocrine axis regulating growth in fish.

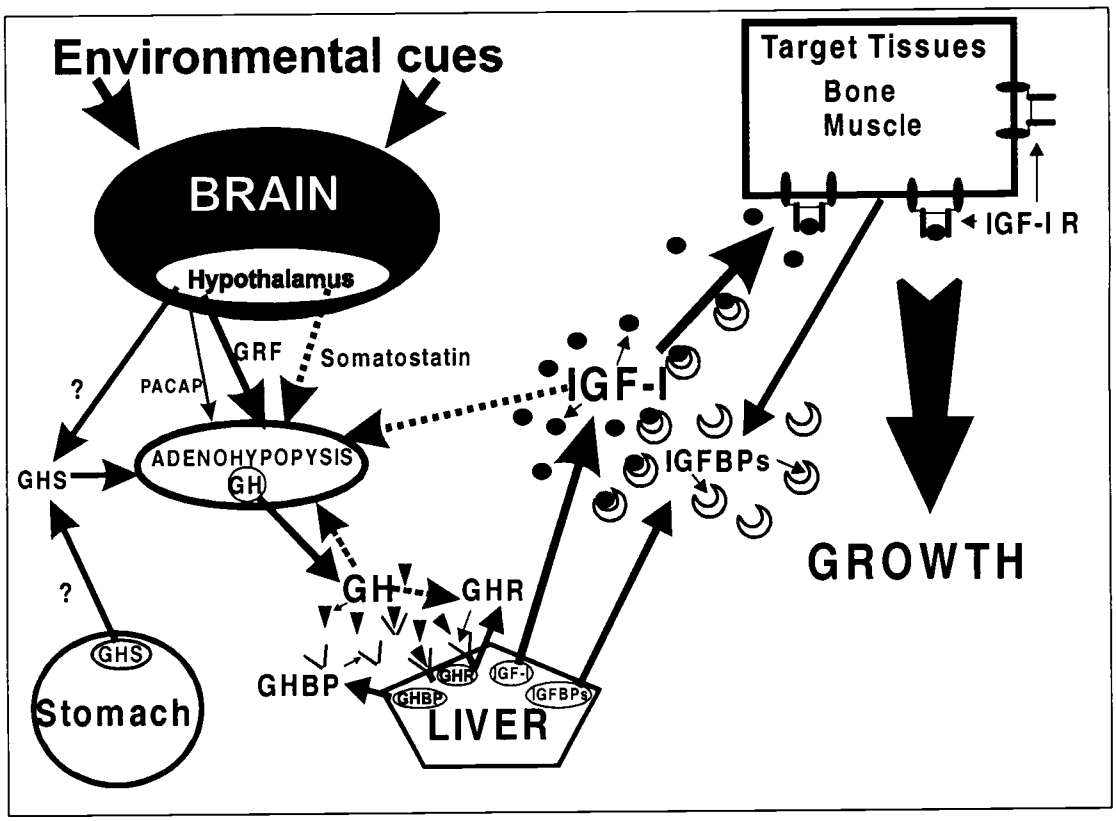

Figure 2.1. An overview of the endocrine axis controlling growth in fish. Growth Hormone (GH) circulates in the blood bound to binding proteins (GHBP). Insulin-like growth factor I (IGF-I) circulates bound to one of six specific binding proteins (IGFBPs). Growth hormone secretagogue (GHS, possibly Grehlin) is produced in both the stomach and hypothalamus, but the source for control of GH release is unknown. Actions of growth hormone releasing factor (GRF), somatostatin, GH and IGF-I are mediated by membrane-bound receptors (GHR and IGF-I R respectively). Solid lines indicate stimulation. Dashed lines indicate inhibition.

SPECIFIC COMPONENTS OF THE SOMATOTROPIC AXIS

The Role of the Hypothalamus

The control of GH release is mediated primarily through the hypothalamus of the brain. The hypothalamus acts as the final integrator of multiple environmental and endogenous cues. The integration of these signals causes the differential release of GH-stimulating or GH-inhibiting factors. These factors, neurohormones, are synthesized within hypothalamic neurons and are released directly into the adenohypophysis by neurosecretory fibers in most teleost fish. In contrast, tetrapods release neurohormones directly into the hypothalamo-hypophysial blood portal system which does not exist in most teleosts (cf. Harvey, 1993).

The summation of numerous stimulatory and inhibitory messages on the hypothalamus results in release of growth hormone releasing factor (GRF) and somatostatin (SRIF) to elicit a positive or negative effect on GH synthesis and secretion. Stimulatory and inhibitory neural information is transmitted to the hypophysiotropic neurons by a variety of factors such as amines (e.g., epinephrine [E], norepinephrine [NE], dopamine [DA], serotonin [5-HT]), neuropeptides (e.g., GHF, SRIF, cholycystokinine [CCK] corticotropin-releasing hormone [CRH], thyrotropin-releasing hormone [TRH], neuropeptide Y [NPY], and activin) and hormones (e.g., GH, glucocorticoids, estrogens, androgens, thyroid hormones and insulin) (Harvey, 1995). In addition to acting within the hypothalamus, many of the neural modulators may be released into the adenohypophysis to directly regulate somatotrope function or to modulate responsiveness to GRF or SRIF stimulation.

GRF is a peptide hormone of approximately 46 amino acids in fish and is released from nuclei in the hypothalamus. The prohormone that yields GRF in fish also produces a 39 amino acid peptide that is similar to pituitary adenylate cyclase-activating polypeptide (PACAP) in mammals (Montero, 2000; Norris, 1997; Small, 2001). In mammals, these two peptides are produced on separate genes. GRF and PACAP belong to the vasoactive intestinal polypeptide (VIP)-glucagon-secretin family and have a number of structural and functional similarities. PACAP structure is well conserved between vertebrates while the structure of GRF is more variable (Montero, 2000). In fish, GRF and PACAP immunoreative neurons are localized in the same regions of the fish diencephalon and directly innervate the pars distalis. Both peptides stimulate pituitary GH release in fish but act through different receptors (Chan, 1998; Parker, 1997; Small, 2001; Wong, 1998; Wong, 2000). Therefore, both GHRH and PACAP may be involved in the stimulatory control of pituitary GH secretion (cf. Montero, 2000). While PACAP could be an important regulator of GH release in fish, more research to isolate its specific action is needed (Small, 2001).

SRIF inhibits GH secretion from somatotropes of the adeno-hypophysis. It is a tetradecapeptide produced and secreted in the brain and several peripheral tissues. SRIF has been identified and cDNAs have been cloned from a number of fish, however the physiology of SRIF has not been extensively studied in fish. Several forms of SRIF exist (SRIF-14 and SRIF-25), however SRIF-14 is the chief GH inhibitor in fish (cf. Mommsen, 1998). Regulation of SRIF release from the fish brain has not been determined, however some evidence implicates dopamine, testosterone and estradiol in SRIF regulation (cf. Lin,

2001). In mammals, SRIF is influenced by a number of factors such as neurotransmitters, hormones and growth factors. GH, thyroid hormones, IGF-I, insulin, dopamine, CRF, CRH, neurotensin, glucagon, and bombesin stimulate SRIF, while opiates, gamma-aminobutyric acid (GABA), leptin and transforming growth factor inhibit SRIF (cf. Lin, 2001).

The GH Family

Growth Hormone, along with prolactin (PRL), somatolactin (SL) and Placental Lactogen (PL), belongs to the GH/PRL family of hormones. This family of hormones is characterized by a close structural relationship and a likely common ancestor (cf. Mommsen, 1998). GH is a large single-chain polypeptide produced by and stored in the somatotrope cells of the pars distalis (adenohypophysis). Two disulfide bonds are responsible for folding of the polypeptide chain. The number of amino acid residues varies by species (187-188 in fish, 191 in humans) and the molecular mass is about 22 kDa in fish and about 23 kDa in humans. The half-life of GH is about 20 to 45 minutes in rainbow trout, and about 15 minutes in Japanese eel (Duan, 1991), while GH has a biological half-life of 20 to 40 minutes in human plasma (Norris, 1997).

Comparative GH structure and genomic organization

Structural information for GH is available for a wide variety of vertebrate species (cf. Chen, 1994). Sequence comparisons and amino acid alignments have provided evidence of two conserved disulfide bonds and four highly conserved domains among all vertebrate GHs

(Kawauchi, 1989). These domains are at positions 13-33 (Domain A_{GH}), 54-94 (B_{GH}), 113-132 (C_{GH}) and 157-187 (D_{GH}) and are separated by variable regions and deletions.

The GH genomic sequences for several fish species have been determined and differences in both GH gene size and structure have been found. The rainbow trout (Agellon, 1988), atlantic salmon (*Salmo salar*) (Johansen, 1989) and chinook salmon (*O. tshawytscha*) (Du, 1993) GH genes have six exons and five introns and span about 4 to 5 kb, which is nearly twice the size of the mammalian GH gene. The tilapia (*Oreochromis niloticus*) GH gene also has six exons and five introns, however the gene spans only about 1.7 kb (Ber, 1992). Interestingly, the tilapia species *O. mossambicus* has a GH gene spanning 5.6 kb, with the same exon/intron number as *T. nilotica* (Sekkali, 1999). The grass carp (*Ctenopharyngodon idellus*) (Zhu, 1992) and common carp (*Cyprinus carpio*) (Chiou, 1990) GH genes have 5 exons and four introns and span about 3 kb. The chum salmon (*O. keta*) (Sekine, 1985), rainbow trout (Agellon, 1988), common carp (Chao, 1989; Koren, 1989) and goldfish (*Carassius auratus*) (Law, 1996) have two GH genes (GH1 and GH2) due to their tetraploid genome (Larhammar, 1994). The grass carp (Zhu, 1992) and tilapias (Ber, 1992; Sekkali, 1999) have only one GH gene. The female chinook salmon also has two GH genes, however male chinook salmon have an additional GH pseudogene, which probably resides on the Y chromosome (Du, 1993). A pseudogene has close homology to the functional gene, but has been disabled by several mutations, as is the case with the chinook salmon GH pseudogene. Humans and other mammals have a single GH gene which spans about 2.6 kb and is composed of five exons and four introns (cf. Harvey, 1995).

The 5' region of the GH gene of all species contain multiple regulatory sequences. The expression of pituitary GH genes is partially regulated by Pit-1 (or GHF-1), a pituitary-specific DNA binding protein capable of activating the growth hormone gene promoters (Bodner, 1987). Pit-1 binds to certain motifs in the 5' flanking region of the GH gene. Sequences homologous to the mammalian Pit-1 motif have been located in the rainbow trout GH gene and experiments indicate that the basic mechanisms regulating GH gene transcription have been conserved between fish and mammals (Argenton, 1993). Pit-1 binding sites have also been described in the tilapia (*O. mossambicus*) GH gene (Sekkali, 1999). Studies to elucidate the mechanism of differential expression for rainbow trout GH1 and GH2 mRNAs revealed the existence of several response elements on the GH genes. The 5'-flanking region and some introns and exons of the rainbow trout GH1 gene contained consensus sequences related to the cAMP response element, thyroid hormone response elements, retinoic acid response elements, estrogen response elements (ERE), and glucocorticoid response elements in addition to the Pit-1 binding sequences. With the exception of the ERE, the GH2 gene contained the same response elements as GH1 (Yang, 1997). A cAMP response site also exists in the 5' region of the GH gene in mammals (Dana S, 1989). Glucocorticoid and thyroid hormones, insulin, and GRF (which acts via cAMP) also control mammalian GH gene transcription through response elements on the GH gene (cf. Harvey, 1995).

GH binding protein

Information about GH binding proteins (GHBP) in fish is scarce. A specific, high-affinity GHBP was identified in goldfish serum (Zhang,

1999). A GHBP was also identified in rainbow trout, however this GHBP had a higher affinity for human GH than for homologous rainbow trout GH (Sohm, 1998). It is likely that further research will reveal similarities in structure and function between fish GHBPs and those of mammals, which have been extensively studied. In mammals, GHBP is identical to the extracellular domain of the growth hormone receptor and is produced by either alternative GHR mRNA splicing (in rodents) or by proteolytic cleavage from the GHR (in humans, rabbits and several other species) (cf. Bauman, 2001). Proteolytic cleavage from the GHR may also be the case with the rainbow trout GHBP, although this has not been shown conclusively (Sohm, 1998). The high affinity GHBP is conserved through vertebrate evolution and is produced in many tissues, especially the liver. In mammals, GHBP is found in varying concentrations in many biological fluids (cf. Bauman, 2001). About half of the circulating GH under basal conditions in humans is bound to GHBPs, and the binding proteins are easily saturated at high GH levels. In mammals, the GHBPs act as a buffer to regulate free and bound GH, prolong GH half-life and modulate GH bioactivity by competing with GH receptors (GHRs) for their ligand. There is species variation in the regulation of GHBP with sexual dimorphism and large pregnancy-related changes in some species. Another circulating GHBP of low affinity and not related to the GHR also exists (Bauman, 1995). The low affinity GHBP binds only a small fraction of circulating GH (5 – 8%) and has only been characterized in humans and rodents to date (cf. Bauman, 2001).

GH receptor

The biological action of GH is initiated by activation of a membrane-bound GH receptor (GHR). The presence of GHR in fish has been demonstrated and the full-length cDNA sequence of GHR from two species, the turbot (*Scophthalmus maximus*) (Calduch-Giner, 2001) and the goldfish (Lee, 2001), have recently been reported. Although the overall homology between the fish GHRs and other GHRs tested was found to be low (32% to 40% amino acid identity) the sequence motifs and overall architecture of the receptor have the characteristics of the class I cytokine/ erythropoietin receptor superfamily to which mammalian GHRs belong. Thus, the GHRs are both structurally and functionally conserved throughout vertebrate evolution.

The receptors in the cytokine/erythropoietin superfamily have a similar structure and include receptors for GH, prolactin, hematopoietin, erythropoietin, interleukins and interferons. The receptors are characterized by an extracellular N terminus, a single transmembrane domain and an intracellular domain. As with the GHR, the extracellular domains of the majority of the receptors in this family also act as soluble binding proteins (cf. Harvey, 1995). The goldfish GHR has 602 amino acids and consists of an extracellular domain of 220 amino acids, a single membrane-spanning segment of 24 residues, and a cytoplasmic region of 334 amino acids. This structure is similar to the GHRs of other species (Lee, 2001).

GH exerts its biological actions in both fish (Calduch-Giner, 1997) and mammals (Carter-Su, 1996) by binding to two GHRs, causing dimerization of the GHR. This dimerization initiates a JAK-

STAT pathway of signal transduction in fish (Lee, 2001) as well as mammals (cf. Carter-Su, 1996). The signal transduction begins with the activation of a cytoplasmic tyrosine kinase (Janus kinase or JAK2). The activated JAK2 in turn phosphorylates tyrosines within itself and the associated GH receptor (which lacks intrinsic tyrosine kinase activity), forming high-affinity binding sites for a variety of signaling molecules. These signaling molecules include the signal transducers and activators of transcription (STAT) proteins, which are activated through phosphorylation. These proteins dimerize and trans-locate to the cell nucleus where they bind to specific DNA sequences and regulate the expression of a variety of GH-dependent genes (cf. Carter-Su, 1996). Conserved regions and structural features in the fish GHR intracellular domain indicate that this signal transduction pathway occurred early during vertebrate evolution (Lee, 2001).

Although not yet elucidated in the fish GHR, other signal transduction mechanisms are known for the mammalian GHR. These pathways regulate cellular function including gene transcription, metabolite transport, and enzymatic activity that result in the actions of GH in body growth and metabolism (Argetsinger, 1996; Finidori, 2000). Receptor activation by GH is transient and several mechanisms are involved in down-regulation of GHR action. These include internalization and degradation of the receptor and recruitment of phosphatases or of specific inhibitors of the JAK/STAT pathway (Finidori, 2000).

The highest concentration of GHR in fish is in the liver (Sakamoto, 1991; Sun, 1997), with much lower concentrations of GHR in such tissues as the brain (Perez-Sanchez, 1991), gonads (Gomez, 1999), gills, intestine and kidney (Sakamoto, 1991).

GH Regulation

The control of GH synthesis and release is complex and involves the interactions of numerous stimulatory and inhibitory factors. Most of these factors are neurohormones produced in the brain. The somatotropes integrate the signals of these various regulatory factors and respond by modulating intracellular signal transduction pathways that lead to GH synthesis or secretion.

GH release is primarily under inhibitory control by SRIF (Norris, 1997), which has a "gating" effect on the pituitary response to GRF (Robinson, 2000) and other stimulatory factors. This effect was demonstrated in rainbow trout pituitaries in which GH synthesis and secretion increased during incubation in serum-free culture without the inhibitory effect of SRIF (Yada, 1991; Yada, 1992). SRIF inhibits GH secretion in many teleosts both *in vitro* (Yada, 1992; Luo, 1990; Luo, 1991; Marchant, 1987) and *in vivo* (Cook, 1984; Sweeting, 1986; Diez, 1992; Melamed, 1995). Details regarding the exact mechanism of SRIF action in fish are scarce, however in mammals SRIF acts by inhibiting cAMP accumulation and thereby decreasing the likelihood of activating voltage-dependent Ca^{2+} channels which trigger exocytosis of stored GH (cf. Bluet-Pajot, 2001). Other inhibitors of GH release in fish are IGF-I, norepinephrine, epinephrine and serotonin. Norepinephrine, epinephrine and serotonin suppress basal GH release from goldfish pituitary cells in a reversible and dose-dependent manner (Lee, 2000; Somoza, 1991). IGF-I inhibits GH gene expression as part of a negative feedback loop (Blaise, 1995), providing a mechanism for regulating GH and IGF-I. A substantial amount of GHR exists in the hypothalamus of fish (Lee, 2001), suggesting the possibility of negative

feedback by GH on its own secretion and receptor number as is demonstrated in mammals (Scacchi, 1997).

GRF stimulates GH synthesis and secretion. These actions of GRF have been demonstrated in several fish species (cf. Bjornsson, 1997). However, a more important function of GRF may be as a long-term trophic factor stimulating somatoptrope proliferation and GH synthesis (cf. Robinson, 2000). The transcriptional action of GRF is mediated primarily by increasing cAMP, which activates a cAMP-responsive transcription factor (CREB) to induce expression of the GH gene. Unlike the GH release mechanism, the GH synthesis pathway activated by CREB cannot be reversed by SRIF in fish (Melamed, 1996; Yada, 1992) or in mammals (Barinaga, 1985). Increasing cAMP levels trigger the release of stored GH allowing a pulse of GH release during the episodic decline in SRIF levels that occur in most species. The pulsatile release of GH will be discussed in more detail later. Recently, GRF has also been shown to activate mitogen-activated protein (MAP) kinase in pituitary cells in mammals (Pombo, 2000) adding another mechanism by which GRF influences somatotroph function. Another peptide, PACAP, produced from the same gene as GRF in fish also stimulates GH release. The GH-releasing action of PACAP is mediated by PACAP receptors (PAC1 receptors), which are coupled to the adenylate cyclase-cAMP-protein kinase A and phospholipase C-IP3-protein kinase C pathways. Activation of the Ca2+ - calmodulin protein kinase II appears to be the mechanism mediating PACAP –stimulated GH release in goldfish (Wong, 2000).

Recent evidence suggests another potent secretagogue of GH may be present in tilapia (*O. mossambicus*) in which a "grhelin-like" substance has been identified (Shepherd, 2000). Grhelin is a peptide

recently found predominately in the stomach and intestinal tract and to a lesser extent in the hypothalamus, placenta, kidney and osteoblast cells in mammals. Ghrelin acts by a third independent pathway to regulate GH release and appears to be more potent than GRF in stimulating GH secretion (cf. Kojima, 2001). In humans, ghrehlin may be the most potent GH release stimulator known (Pombo, 2001). It is unknown whether hypothalamic or stomach ghrelin secretion in mammals is responsible for control of GH secretion from the somatotrophs. Ghrelin may be a physiological mediator of feeding and may regulate growth by stimulating feeding and release of GH in mammals. Ghrelins were also recently identified in the amphibian, *Rana catesbeiana* (Kaiya, 2001).

Factors that regulate GH secretion and synthesis in fish have been studied primarily in carp (cf. Peter, 1995) and to a lesser extent in salmonids (cf. Bjornsson, 1997) and tilapia hybrids (Melamed, 1995). Factors that stimulate GH release in carp and salmonids include gonadotropin-releasing hormones (GnRH and GnRH-II) and dopamine. These factors directly stimulate GH release through different pathways in an additive manner in goldfish pituitaries (Kwong, 1997), however their GH-releasing actions are inhibited by simultaneous treatment with norepinephrine *in vitro* (Lee, 2000). GnRH is a potent stimulator of GH secretion both *in vivo* and *in vitro* in tilapia, however dopamine was not a potent stimulator of GH in this species (Melamed, 1995). Neuropeptide Y (NPY) stimulates GH release from pituitary fragments in goldfish, primarily by stimulating GnRH release (Peng, 1993). Estradiol stimulates GH secretion throughout the reproductive cycle of female goldfish, but testosterone has no effect. In pituitaries from sexually regressed goldfish, pretreatment

with estradiol (E_2) results in a greater GH release in response to thyrotropin-releasing hormone (TRH) than from pituitaries not pre-treated with E_2, thus suggesting a potentiating or positive feedback action of E_2 (Trudeau, 1992). TRH is a potent stimulator of GH release, especially from sexually mature goldfish (Trudeau, 1992). However, in tilapia hybrids, TRH does not cause GH release from isolated pituitary fragments (Melamed, 1995) but does increase plasma GH, indicating a non-pituitary mode of action. The presence of a thyroid hormone response element on the rainbow trout GH gene indicates an effect of thyroid hormone on GH synthesis. Indeed, in rainbow trout, T_3 increases transcription of GH mRNA in the somatotropes, but an increase of GH peptide is not seen in the plasma (Moav, 1992). T_3 increases GH synthesis in isolated tilapia pituitaries (Melamed, 1995) while in carp pituitaries, T_3 and retinoic acid increase the steady state levels of GH mRNA (Farchi-Pisanty, 1995). The response to retinoic acid is not surprising since a retinoic acid response element exists on the GH gene of rainbow trout (Yang, 1997). Cholecystokinin-8 (CCK-8s), probably secreted from CCK/gastrin-like immunoreactive nerve fibers near the somatotropes, stimulates GH release from goldfish pituitaries (Himick, 1993) especially in sexually regressed fish. Recent evidence suggests that nitric oxide (NO) stimulates GH secretion and this action is blocked by SRIF. NO may also regulate GnRH and dopamine stimulated GH secretion in the goldfish pituitary (Uretsky, 2000).

Taken together, the information available on stimulatory and inhibitory factors, as well as environmental factors, demonstrates the complexity of GH regulation in fish. It is also evident that responses to some regulatory factors differ between species. Figure 2.2 provides a

model for some known factors affecting GH synthesis and release in fish.

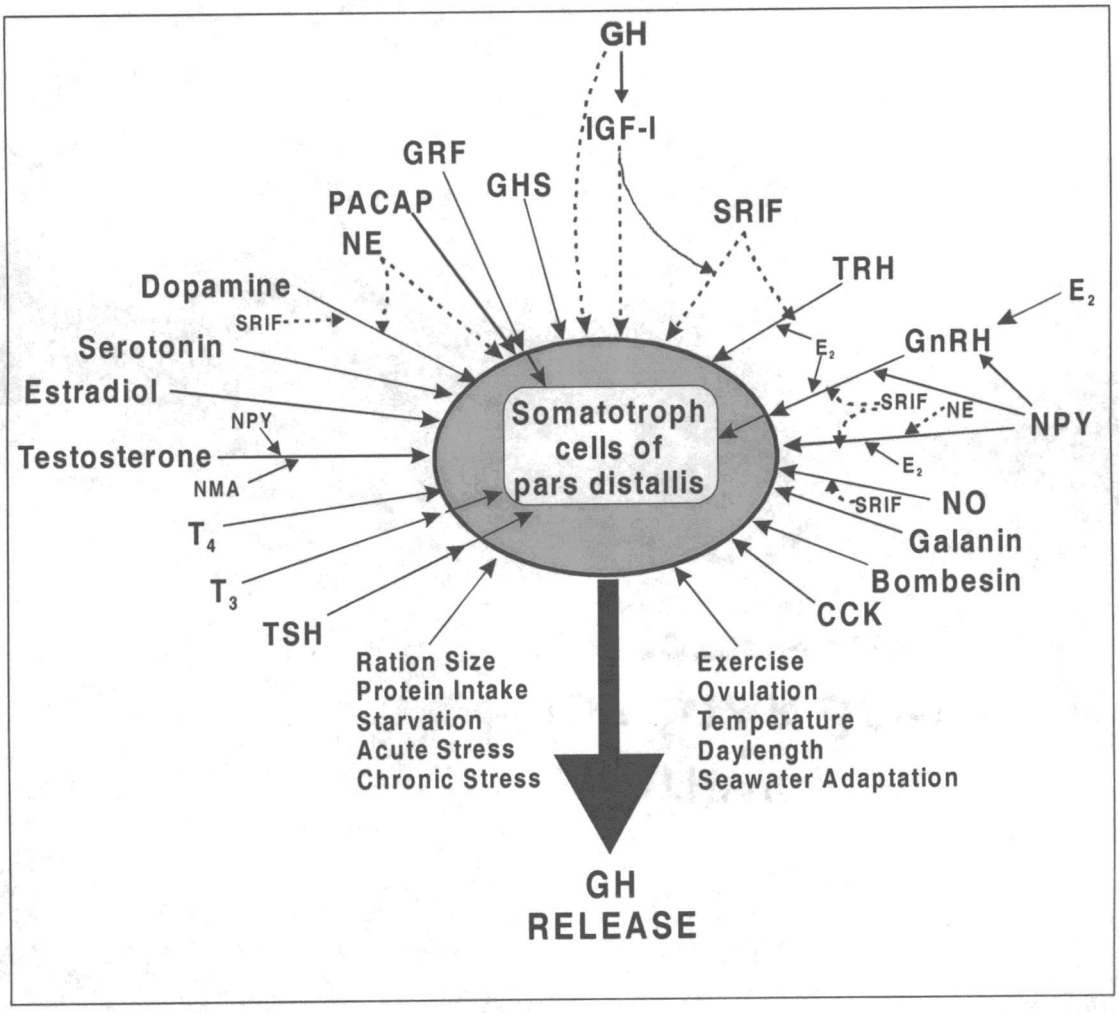

Figure 2.2. Factors controlling the synthesis and release of GH in somatotroph cells of the fish adenohypophysis. Solid lines indicate activating pathways. Dashed lines indicate inhibitory pathways. Arrows extending into the core of the somatotroph indicates regulation of synthesis. Abbreviations: CCK, cholecystokinin; GHS, GH secretagogue (possibly Ghrelin); GRF, Growth hormone releasing factor; GnRH, gonadotropin releasing hormone; IGF-I, insulin-like growth factor-I; NE, Norepinephrine; NMA, N-methyl-aspartate; NO, Nitric Oxide; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase activating polypeptide; SRIF, somatostatin; TRH, thyrotropin releasing hormone; TSH, thyroid stimulating hormone; T_3, triiodothyronine; T_4, thyroxine. (Data modified and updated from Mommsen, 1998)

Biological action of GH

Growth hormone promotes anabolic actions by stimulating incorporation of amino acids into proteins especially in skeletal muscle (cf. Mommsen, 2001). GH can influence muscle protein synthesis through its receptors in muscle tissues, or by stimulating local IGF-I production. GH, in its "dual effector" role, can also act synergistically with IGF-I to mediate effects on target tissues such as bone and cartilage. Studies on the regulation of ceratobranchial cartilage growth in coho salmon support a dual effector role for GH in fish. Injection of GH *in vivo* induced higher thymidine and sulfate uptakes than control cartilage exposed to IGF-I alone (Tsai, 1994). In mammals, GH has been shown to stimulate differentiation of prechondrocytes, causing them to express IGF-I receptors and secrete IGF-I. A local paracrine effect of IGF-I then leads to clonal expansion and further differentiation (Nilsson, 1986).

GH regulates somatic growth indirectly by inducing hepatic production of IGF-I and thereby enhancing growth systemically. Substantial evidence that the GH-IGF-I axis has been highly conserved in fish and other vertebrates now exists (cf. Bjornsson, 1997; Moriyama, 2000; Mommsen, 2001). Research in mammals suggests that in addition to stimulating IGF-I expression, GH regulates gene expression of other mitogenic factors. Among these factors are hepatocyte growth factor, fibroblast growth factor in cartilage, and epidermal growth factor (EGF) in kidney (cf. LeRoith, 2001). This suggests that some effects of GH may be IGF-I independent and may involve regulation of factors other than IGF-I. However, IGF-I gene knockout mice, which have high levels of circulating GH levels due to the loss of negative feedback from IGF-I, are smaller than controls,

suggesting that the major effects of GH on growth are IGF-I dependent (LeRoith, 2001).

GH also has a catabolic action in stimulating triacylglycerol lipase activity in the liver resulting in lipolysis. This activity has been demonstrated in salmonids (Sheridan, 1986) and results in elevated circulating fatty acid levels in rainbow trout *in vivo* (Leatherland, 1981) and a stimulation of fatty acid and glycerol release from liver *in vitro* (O'Connor, 1993; Leatherland, 1981).

In fishes, GH has functions beyond the regulation of somatic growth. GH is important in smoltification and seawater adaptation in salmonids (McCormick, 1996). This action of GH is independent of its growth promoting effects (Bolton, 1987). Plasma GH levels and clearance rates are significantly higher in seawater-adapted fish (Sakamoto, 1991). In tilapia (*O. mossambicus*), transfer to seawater increases GH and cortisol levels and improves hypoosmoregulatory ability, and treatment with GH increases chloride cell density and stimulates gill Na^+K^+ ATPase activity (cf. Mancera, 1998). Also, GH plays an important role in reproductive function in fish. In goldfish ovarian follicles, GH enhances the actions of gonadotropic hormone (GtH) on steroid production (Van Der Kraak, 1990) while in eel (*Anguilla anguilla L.*), GH acts on the liver to potentiate E_2 induction of hepatic vitellogenin (egg yolk precursor) synthesis (Peyon, 1996). GH has been shown to increase both hepatic estrogen receptor mRNA and estrogen receptor levels in rats (Freyschuss, 1994). GH may alter aggression, physical activity or appetite in fish (cf. Mommsen, 2001), thereby influencing behavior favorable to growth. GH plays a role in intestinal growth and increased digestive capacity (Mommsen, 1998), providing a mechanism for increased food consumption and

assimilation. Figure 2.3 provides a model of targets for growth hormone action in fish.

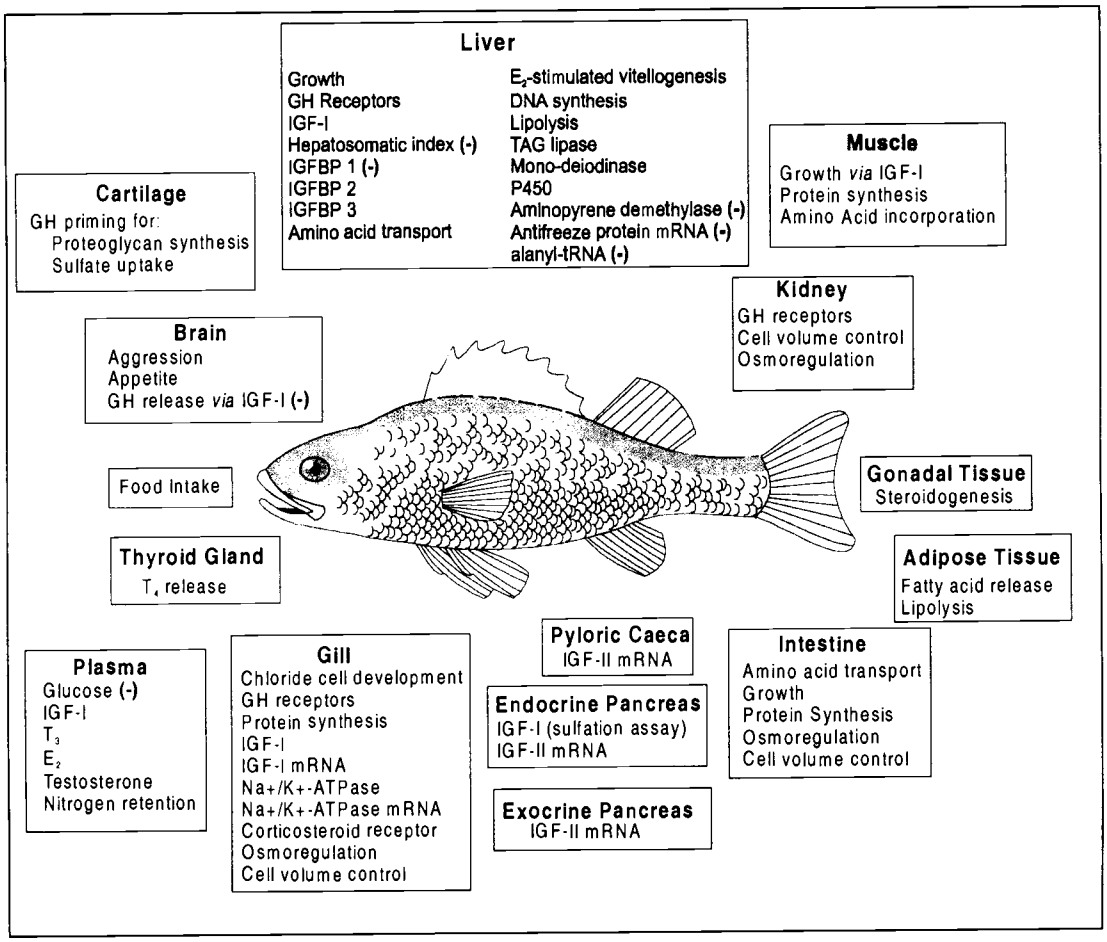

Figure 2.3. Model of the targets for GH action in fish. Target tissues for GH and specific actions of GH in these tissues are included. All variables are increased by GH unless signified by (-). (Data modified and updated from Mommsen, 1998)

Growth hormone release in teleosts follows an intrinsic rhythm with both daily and seasonal fluctuations. Grass carp have either a single cluster of GH pulses, or two pulses separated by a 2.6 hour interval of baseline release (Zhang, 1994). Likewise, rainbow trout have asynchronous fluctuations in GH with approximately two peaks every 24 hours (Gomez, 1996). In addition to a daily rhythm of GH

release, goldfish have a seasonal cycle of plasma GH levels. The highest concentrations are in the spring, pre-spawning and early post-spawning. The lowest concentrations are in autumn in sexually regressed fish (Marchant, 1986; Marchant, 1989).

GH concentrations change in fish plasma according to the physiological state of the animal. Nutritional status, especially the dietary protein:energy ratio, plays a major role in plasma GH levels and in the sensitivity of the liver to GH stimulated IGF-I release (Thissen, 1994; Marti-Palanca, 1996; Perez-Sanchez, 1995). Paradoxically, plasma GH in starved rainbow trout is higher than in fed fish (Sumpter, 1991; Farbridge, 1992) although starved fish do not grow. Starvation also causes a decrease of hepatic IGF-I mRNA and circulating IGF-I levels (Duan, 1993). An increase in dietary protein levels resulted in decreased plasma GH concentration and increased growth rates in the gilthead sea bream (Perez-Sanchez, 1995). The same study reported an increase in hepatic GH binding sites and plamsma IGF-I-like immunoreactivity associated with higher dietary protein levels. Plasma GH was found to be higher in fish with lower growth rates due to protein deprivation (Perez-Sanchez, 1995; Marti-Palanca, 1996). A possible reason for increased plasma GH concentration in starved and slow-growing fish may be the result of a diminished negative feedback effect of IGF-I on GH. The rising GH levels during starvation may also trigger the catabolic effect of GH to stimulate release of glycerol and fatty acids from the liver (cf. Bjornsson, 1997). In humans, GH and IGF-I levels are also regulated by nutritional factors (Thissen, 1994). Starvation increases plasma GH, while in rats, plasma GH is suppressed by starvation (Pombo, 2001). A low growth rate in humans (but not in rats) is associated with higher GH levels

(Thissen, 1994). Stress disturbs the balance of GH and cortisol with an increase in cortisol and a decrease in GH in both starved and fed fish (Pickering, 1991). In coho salmon, premature transfer to seawater will cause abnormal development in some fish. These fish, called stunts, grow poorly and eventually die. Some stunts will resume normal growth if placed back into fresh water. While the plasma GH levels in these stunted fish are elevated, the hepatic IGF-I expression is significantly reduced (Duan, 1995), as are the number of hepatic GH receptors (Gray, 1992). The low level of hepatic GH receptors, along with the diminished plasma IGF-I levels probably decreases the negative feedback effect of IGF-I similar to the effect in starved fish. Stunted fish have a functional GH resistance that leads to abnormal development and retarded growth (Duan, 1995) (Gray, 1990).

GH is not essential for intra-uterine growth and development in humans (Laron, 1993). Postnatally, the circulating levels of GH are highest during the period of maximal growth (ages 2 to 17 years) (Norris, 1997). Beginning at about 4 years of age, a daily rhythm of GH secretion is established. This rhythm of GH secretion continues throughout adult life, and depending on the physiological state of the individual, is irregular and spontaneous. The greatest GH release is usually during deep sleep. In adult male rats, GH secretion is pulsatile. Large peaks of GH occur approximately every three hours with serum GH concentrations falling to nearly zero between peaks. In females, GH secretion is more continuous and serum growth hormone profiles show only small peaks. The sexually dimorphic pattern appears to be imprinted by a surge of testosterone during a critical neonatal period (Jansson, 1985). In most animals studied, the

frequency of the GH secretions are as important as the magnitude of the GH secretions in determining the biological response to GH (Robinson, 2000). Additionally, the pattern of GH secretion into the circulation increases the expression of GH receptor (GHR) and GH binding proteins (GHBP), which in turn determine the response of peripheral tissues to GH.

The IGF family

The major elements regulating IGF function in fish include ligands (IGF-I, IGF-II and insulin), cell-surface receptors (the IGF-I, IGF-II/mannose-6-phosphate and the insulin receptors) and a number of IGF binding proteins (IGFBPs). Several other proteins have recently been identified in mammals as potential components of the IGF system. These include two additional receptors, the insulin-receptor-related receptor (IRR) and the insulin-IGF-I hybrid receptor as well as an increasing number of IGFBP-related proteins and proteases (Werner, 2000).

The insulin-like growth factors of fish belong to a family of similar peptide hormones including IGF-I, IGF-II, insulin and relaxin (primarily a mammalian peptide unique to pregnancy and birth, but also found in oviparous sharks (Norris, 1997). Structural similarity exists between IGF-I, IGF-II and proinsulin and all three are translated into preprohormones. PreproIGF, beginning at the N-terminal, consists of a signal peptide followed by B-, C-, A-, D- and E-domains. The signal peptide and E-domain are proteolytically removed to produce mature IGFs. Although not part of the mature protein, the E-domain has some mitogenic activity in rainbow trout (Tian, 1999). Preproinsulin consists only of the signal peptide and the B-, C- and A- domains. The signal peptide and the C and E-domains are

removed to form mature insulin. Three disulfide bonds are formed during processing in IGFs and insulin, two between the B- and A-domains and one within the A-domain.

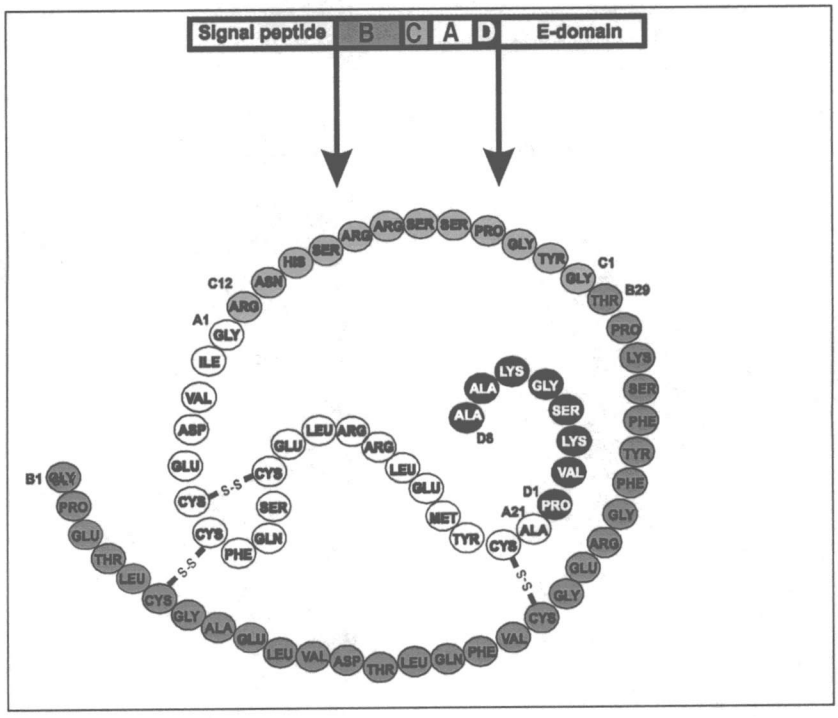

Figure 2.4. Structure of preproIGF-I and amino acid sequence of salmon IGF-I.

Comparative IGF Peptide Structure

Both of the mature IGFs (IGF-I and IGF-II) in vertebrates are single-chain peptides of about 7500 Daltons. IGF-I, which has 70 amino acids in most species examined, is basic, with an NH_2-terminal B domain consisting of 29 amino acids, a C domain of 12 residues, an A domain of 21 amino acids and a COOH-terminal D region comprised of 8 amino acid residues. In fish species examined; eel (*Anguilla japonica*), carp (*Cyprinus carpio*), coho salmon (Cao, 1989), chinook salmon (Wallis, 1993) grass carp (Bai, 2000), bluntsnout bream (*Megalobrama amblycephala*) (Bai, 2000) and goldfish IGF-I have 70

amino acids. Known exceptions to this structure in fish IGF-I are tilapia (*O. mossambicus*) (Reinecke, 1997), flounder (*Paralichthys olivaceus*) (Tanaka, 1998; Kim, 1998) and sculpin (*Cottus scorpius*) (Loffing-Cueni, 1998) IGF-Is with 68 residues (residue10 and 11 of the C region are absent). Gilthead seabream (*Sparus aurata*) (Duguay, 1996) and barramundi (*Lates calcarifer*) (Kinhult, 1996) IGF-Is have 67 amino acids (the final residue of the B domain as well as residue10 and 11 of the C region are absent). Black seabream (*Acanthopagrus schlegeli*) also has 67 amino acids (residues 1, 11 an12 are absent) (Chen, 1997). Catfish (*Clarias macrocephalus*) have both a brain-specific and a ubiquitous IGF-I form (McRory, 1994). Both forms have 70 amino acids but differ in 25 of the 70 residues, especially in the D domain in which all amino acids differ. Shark (*Squalus acanthias*) IGF-I also has 70 amino acids but has only 11 residues in the C region and 9 residues in the D domain (Duguay, 1995).

IGF-II, a slightly acidic peptide with 70 amino acid residues in most fish examined, has an N-terminal B domain of 28 amino acids, a C domain of 15 amino acids, an A region of 21 residues, and a C-terminal D region of 6 residues. Fish in which IGF-II has been identified include coho salmon (Shamblott, 1992), chum salmon (Palamarchuk, 1997), Japanese eel (Yamaguchi, 1999), gilthead seabream (Duguay, 1996), barramundi (Collet, 1997), tilapia (Chen, 1997), sculpin (Loffing-Cueni, 1999), pufferfish (*Fugu rubriceps*) (Clark, 1998), shark (*S. acanthias*) (Duguay, 1995) and common carp (Tse, 2001). The eel IGF-II has 69 residues with only 14 in the C domain (Yamaguchi, 1999), while the shark (*S. acanthias*) IGF-II has 68 residues and a C domain containing 13 amino acids (Duguay, 1995).

The IGF-II molecule in most mammals has 67 amino acids. The N-terminal B domain consists of 28 amino acids, the C region has 12, the A domain has 21 and the D domain has 6 residues (cf. Rotwein, 1999). Two variant human IGF-II proteins have been characterized: a 70 and a 69 amino acid variant. The additional residues are contained in the C domain in both variants. The 70 amino acid variant comprises approximately 25% of serum IGF-II in humans, but has only one third of the potency of the 67-residue IGF-II for the IGF-I receptor (Hampton, 1989). The biological significance of the 69-residue IGF-II is unknown. In the mink (*Mustela vison*), the IGF-II molecule has 68 amino acids with an extra residue in the C domain compared to humans (Ekstrom, 1993).

Only one form of IGF cDNA has been identified in Atlantic hagfish (*Myxine glutinosa*) and sea lamprey (*Petromyzon marinus*) (Nagamatsu, 1991; Yamaguchi, 1999) while elasmobranchs and teleosts have both IGF-I and IGF-II This suggests the possible evolutionary divergence of IGF-I and IGF-II between Agnatha and Gnathostomata (Moriyama, 2000).

While the mature IGF-I peptide is highly conserved among mammals, some authors maintain that considerable variability exists among nonmammalian IGF-I molecules (Rosenfeld, 1999; Rotwein, 1999). Many residues differ in all domains. Only the six cysteine residues are conserved across the nonmammalian species sequenced. These cysteine residues are involved in the formation of three disulfide bonds in the mature protein, and it is therefore likely that all IGF-I molecules have a similar three-dimensional shape (Rosenfeld, 1999). The tyrosine residues at positions analogous to tyrosines 24, 31 and 60 in mammalian IGF-I also are conserved in fish. These residues are

thought to play an important role in the affinity of mammalian IGF-I to the IGF-I receptor (Bayne, 1990) and may play similar role with non-mammalian IGF-I. In contrast, tyrosines 24 and 31 are not conserved in the single IGF protein identified in Atlantic hagfish (Nagamatsu, 1991). It is unlikely that IGF from the primitive vertebrate binds to mammalian IGF-I receptors.

Conversely, Moriyama (2000) maintains that the amino acid sequences of IGF-I and IGF-II are highly conserved among all vertebrates. The sequence identity of IGF-I among fishes is 67% to 97% and fish IGF-Is are 60% to 83% identical to human IGF-I. Sequence identity of IGF-II among fishes is 66% to 95% and the identity of fish IGF-IIs to human IGF-II are 64% to 78%. The amino acid sequences in the C-and D-domains of both IGFs are less conserved than those in the B- and A-domains (Moriyama, 2000).

The basic endocrine mechanism of growth regulation by IGFs has been highly conserved among vertebrates (Duan, 1998; Chan, 1993). Portions of the A and B regions have amino acid sequences that are important for binding to the IGF-I receptor or to IGF binding proteins. Such structural conservation of a ligand molecule supports the hypothesis that binding domains of the IGF-I receptor may also be highly conserved and the IGF binding proteins are phylogenetically old proteins (Rotwein, 1991). The least conserved sections, the C and D domains, probably have non-critical biological functions.

IGF genomic organization

IGF-I and IGF-II in both fish (Shamblott, 1992) and mammals (Dull, 1984) are encoded by different genes. The IGF-I gene structures

in several fish species have been described with the salmonids receiving considerable attention. Pacific salmon have two non-allelic IGF-I genes reflecting their tetraploid genome and the subsequent divergence of each pair of genes (Kavsan, 1994; Shamblott, 1992; Wallis, 1993). However, only a single mature IGF-I peptide with identical sequences is produced in salmonids. In chum salmon, each gene is approximately 17 kb in length and probably contains five exons and four introns, but this has not been determined conclusively (Kavsan, 1993; Kavsan, 1994; Rotwein, 1999). The eventual mature IGF-I peptide along with the first part of the E domain in chum and chinook salmon is encoded in exons 2 and 3 (Kavsan, 1993; Wallis, 1993). Two differentially used splice donor sites exist at the 3' end of exon 3 which code for the beginning of the E domain (Kavsan, 1993; Kavsan, 1994; Wallis, 1993). RNA processing at the more distal splice site adds 81 nucleotides to the E domain. Additionally, subsequent alternative splicing can include both exons 4 and 5, or just exon 5. This differential splicing can result in the generation of four distinct types of IGF-I mRNAs with different lengths of E peptides from each of the two nonallelic genes in chinook salmon (cf. Rotwein, 1999). In chum salmon however, a mutation has eliminated the proximal splice site in exon 3 of one of the nonallelic IGF-I (IGF-I1) genes (Kavsan, 1994).

Evidence suggests that the zebrafish (*Danio rerio*) may have more than one IGF-I gene (Chen, 2001). The IGF-I gene examined, named IGF-Ia, spans about 15 kb and is divided into five exons. The 81 nucleotide segment located at the 3' end of exon 3 in salmonids, and which codes for 27 amino acids of the E domain, is missing in zebrafish (Chen, 2001). Meanwhile, researchers investigating the

Japanese flounder IGF-I gene have described a gene spanning about 17.5 kb and having five exons and four introns (Tanaka, 1998).

The human IGF-I gene spans approximately 100 kb with six exons and five introns (cf. Rotwein, 1999). The mature IGF-I peptide is encoded in exons 3 and 4, which correspond to salmonid exons 2 and 3. The chicken IGF-I gene spans 50 kb and has five exons and four introns (Kajimoto, 1991) while the rat IGF-I gene spans 80kb and has six exons and five introns (cf. Rotwein, 1999).

Information on the organization of the IGF-II gene in fish is limited; however evidence collected thus far suggests that only a single mRNA is produced for IGF-II. A second nonallelic IGF-II gene has not been reported for the salmonid species. The coding region of the rainbow trout was determined to be approximately 5.3 kb and has four exons and three introns (Shamblott, 1998). The coding region of the tilapia IGF-II gene has four exons and three introns and spans a region of about 12.9 kb (Chen, 1997). In both species, the signal peptide is encoded in exons 1 and 2, the mature IGF-II peptide on exons 2 and 3 along with a portion of the E domain, and the remainder of the E domain is encoded on exon 4. The barramundi (Collet, 1997) and chum salmon (Palamarchuk, 1997) IGF-II genomic sequences have also been determined. Both are similar in structure with four exons and three introns and span about 9 kb. In all fish for which the IGF-II gene structure is known, the signal peptide of the IGF-II propeptides is located in two exons rather than one as in mammals and chicken (Collet, 1997). One promoter has been mapped in chum salmon (Palamarchuk, 1997) and barramundi (Collet, 1997) to date, indicating that differentially expressed 5' untranslated regions

and differential splicing do not occur in the fish IGF-II genes (Collet, 1997; Duguay, 1996; Shamblott, 1992).

Like the fish IGF-II gene, the mammalian IGF-II gene is a single-copy gene. However, unlike the fish gene (Collet, 1997), the IGF-II gene in humans, rats and mice is contiguous with the insulin gene (Bell, 1985). The IGF-II gene ranges in size from 15 kb in the rat to more than 28 kb in humans. The human IGF-II gene consists of 10 exons while the rat and mouse genes have six exons, however the coding region for prepro-IGF-II is found within the last three exons (cf. Rotwein, 1999). Exons 1 to 7 in humans (1 to 3 in rodents) are non-coding and form multiple IGF-II mRNAs due to alternative promoters (four in humans, three in rodents) and multiple transcription start sites. The various promoters are expressed in a tissue- and developmentally-specific way (Vu, 1994). In adult human liver, transcription of IGF-II mRNA is driven by promoter P1 rather than P3 and P4 of fetal liver. It is speculated that in humans, promoter 1 (which is absent in rodents) may be an important factor in IGF-II synthesis in adults (Jin, 1995).

The IGF-II gene is an imprinted gene in mammalian fetal liver, but biallelic expression occurs in adult liver (Vu, 1994). The imprinting of the IGF-II gene in fish has not been examined. Genomic imprinting is a mechanism whereby only one of the two parental alleles is expressed. Although the mechanism of imprinting is unknown, differential methylation of the parental alleles may be involved (O'Dell, 1998).

IGF mRNA structure

Multiple forms of proIGF-I have been found in salmonids (Duguay, 1992; Wallis, 1993; Shamblott, 1993) and Japanese flounder (Tanaka M., 1998). The multiple prohormone forms in these species result from alternative RNA splicing generating different size carboxyl-terminal E-domains. Salmonids have four different E domains designated as Ea-1 (35 aa), Ea-2 (47 aa), Ea-3 (62 aa), and Ea-4 (74 aa) (Shamblott, 1992; Duguay, 1992; Duan, 1998). The physiological significance of the different E peptides is unknown (Kavsan, 1994). Japanese flounder has two preproIGF-I forms designated preproIGF-I-1 and preproIGF-I-2. The two forms have identical signal peptides and result in the same mature IGF-I peptide, but they contain different E domains as a result of alternative splicing in exon 3 (Tanaka M., 1998). Zebrafish (Chen, 2001) also have two IGF-I mRNA forms, Ea-1 and Ea-2. However, evidence suggests that the two forms in zebrafish are controlled not by alternative splicing, but by alternative gene expression (Chen, 2001).

Humans have two IGF-I prohormones designated ProIGF-IA and proIGF-IB as a result of differential splicing. The resulting mature peptides are identical. The E domain of human, rat and mouse IGF-Ia is generated through splicing of exons 3 and 5 and yields a 35 amino acid residue E domain, which is the predominant IGF-I mRNA species in human liver, hepatoma, and fibroblasts (Nagaoka, 1991). The E domain of rat and mouse IGF-Ib is generated through splicing of exons 3, 4 and 5 and yields a 41 amino acid residue domain, however, human IGF-IB uses exons 3 and 4 and yields a 78 amino acid E domain (cf. Rotwein, 1991).

IGF Binding Proteins

IGF-I and IGF-II circulate in vertebrate blood tightly bound to any of several distinct but structurally similar binding proteins (IGFBP). The IGF/IGFBP/IGF receptor system is well conserved between mammalian and non-mammalian vertebrates (Kelley, 2001) and different classes of circulating IGFBPs may also be functionally conserved between fish and mammals. The IGFBPs mediate the physiological actions of the IGFs. These proteins differ in their site of origin and in their biological functions. As carriers of IGFs in the plasma, the IGFBPs regulate metabolic clearance, prolong half-lives of IGFs, control the flux of IGFs from the vascular space and modulate the interaction of the IGFs with their receptors. The IGFBPs also prevent the insulin-like activity of IGFs by maintining the free IGF-I concentrations lower than the insulin concentrations in plasma (insulin does not bind to carrier proteins). The binding proteins have different affinities for IGF-I and IGF-II. Multiple cell types produce specific forms of IGFBPs and give tissue specificity to the actions of the IGFs. The mixture of binding proteins produced by a cell or tissue type may be important in determining the responsiveness of those cells and tissues to IGF-I and IGF-II (cf. Lowe, 1991; Kelley, 2001).

Substantial data exist for mammalian IGFBPs, however data on fish IGFBPs are just beginning to accumulate. Based on binding studies and estimated protein molecular weights, at least three major forms of IGFBP have been detected in plasma and some tissues of rainbow trout, coho salmon, golden perch (*Macquaria ambigua*), striped bass (*Morone saxatilis*), tilapia (*O. mossambicus*) and longjawed mudsucker (*Gillichthys mirabilis*) (Kelley, 1992; Anderson, 1993; Niu, 1993; Fukazawa, 1995; Shimizu, 1999; Bern, 1991; Siharath, 1993;

Park, 2000). These proteins include a 40-50 kDa IGFBP, and two IGFBPs that are 31 kDa or less (24-31 kDa). The IGFBPs are both hormonally and nutritionally regulated. The 40-50 kDa form shows positive regulation by GH and is directly correlated with somatic growth rate. It may be the fish version of mammalian IGFBP-3, which is in the same size range and is also GH-regulated (cf. Kelley, 2001). The two smaller forms may be the fish versions of mammalian IGFBP-1 and IGFBP-2, as they are in the same size range and appear to be negatively regulated by insulin, up-regulated in catabolic states and inversely correlated with somatic growth as are their mammalian counterparts. Other forms of binding proteins have been identified in a few fish. A large protein (80-85 kDa), which binds to IGFs has been identified in striped bass (Siharath, 1995). In hypophysectomized tilapia a 20 kDa IGFBP has been detected that may be either a proteolytic fragment of a known IGFBP, or a novel IGFBP (Park, 2000). Additional sequence data for fish IGFBPs are required before a clear identification and comparison with the mammalian IGFBPs can be made. Recently, a 31 kDa IGFBP was cloned and sequenced in zebra-fish (Duan, 1999). Sequence comparison showed a high sequence identity with mammalian IGFBP-2 and was found to inhibit IGF-I stimulated cell proliferation. In addition, a 30 kDA IGFBP was recently purified in rainbow trout (Bauchat, 2001). Partial sequence analysis as well as functional and biochemical analysis suggests that it is similar to human IGFBP-1.

The 40 – 50 kDa IGFBP-3-like protein is the most abundant IGFBP in the fish circulation while the two smaller IGFBPs circulate at much lower levels. In coho salmon plasma, most IGF-I exists as a 40kDa binary complex with IGFBP and only about 0.3% circulates as

free (unbound) IGF-I (Shimizu, 1999), which is the biologically active form.

In mammals, six distinct (IGFBP-1 – 6) high affinity IGF binding proteins have been sequenced and cloned. These six IGFBPs share functional and structural characteristics including a conserved gene organization, three structural domains in the mature protein and the ability to bind IGF-I and IGF-II with binding affinities in the range of 1-100 nanomolar (Collet, 1998; Baxter, 2000). IGFBP-6 differs slightly from the other IGFBPs in the number of disulfide bonds (8 rather than 9) and it has a much higher affinity for IGF-II than for IGF-I. In recent years, several new candidate proteins with partial structural similarity to IGFBPs have been proposed for inclusion in the IGFBP family, however these proteins have a much lower affinity for the IGFs and some have a high affinity for insulin (Collet, 1998). By international consensus, these similar proteins have been designated as IGFBP-related proteins (Baxter, 1998).

In human serum, most IGF-I and IGF-II (75% - 90%) circulate in a 150 kDa ternary complex formed by an IGF, IGFBP-3 and a glycoprotein known as the acid labile subunit (ALS) (cf. Jones, 1995; Ferry, 1999). A ternary binding complex does not appear to exist in coho salmon (Shimizu, 1999), which is the only fish that has been examined to date. When the IGFs are bound in the ternary complex, they are unable to cross the capillary barrier. The ternary complex may be a storage pool for circulating IGFs serving to prolong the half-lives of IGF-I and IGF-II. Both IGFBP-3 and ALS synthesis are stimulated by GH. Approximately 1% or less of the IGFs circulate as free IGF, while the IGFs not bound in the ternary complex circulate bound to the other IGFBPs, especially IGFBP-2 and IGFBP-5 (Martin,

1999). IGFBPs are produced by a variety of tissues and are found in various biological fluids where they appear to have discrete functions. The different biding proteins may modulate IGF action differently and the same binding protein can have either an IGF-inhibiting or potentiating role under different conditions. Another important role of the IGFBPs may be to transport IGFs to the extracellular matrices of certain tissue types (cf. Rajaram, 1997). A complex picture is emerging with regard to the regulation of IGFs by IGFBPs in mammals. It is likely that a similarly complex picture will emerge for regulation of IGFs in fishes.

IGF receptors

The actions of IGF-I and IGF-II are also mediated by the IGF-I receptor, which is structurally and functionally similar to the insulin receptor. The IGF-I and insulin receptors are heterotetrameric glycoproteins of 350 kDa which function as tyrosine kinases. The fish IGF-I receptor can be stimulated by both IGF-I and to a lesser extent by insulin (Leibush, 1996). In fish muscle, heart, ovary and brain, IGF-I receptors are more abundant and more specific than insulin receptors (Mendez, 2001). This is different from adult mammals where insulin receptors are more abundant. The IGF-I receptor seems to be conserved both structurally and functionally in fish. Ligand binding studies using human and salmon IGF-I showed high affinity binding sites on membranes of fish heart, ovary, brain and skeletal muscle (Gutierrez, 1993; Gutierrez, 1995; Parrizas, 1995; Leibush, 1996). Specific binding sites for IGF-I were detected in brown trout (*Salmo trutta*) at several stages of development (5 weeks to 2 ½ years old) indicating that IGF-I receptors are expressed in multiple tissues at

several different developmental stages in fish. The IGF-I receptor in carp and trout is approximately 350 kDA, which is similar to the size of the human IGF-I receptor (350 kDa) (Parrizas, 1995; Leibush, 1996).

Little is known about the structure of the IGF-I receptors in teleosts; however current research is beginning to characterize their structure and expression. Sequence data from the tyrosine kinase domain of two coho salmon IGF-I receptors (SIR-5 and SIR-6) have been reported (Chan, 1997) as well as a portion of the tyrosine kinase domains of turbot and rainbow trout (Elies, 1996). Recently, complete sequences for some species have been characterized. cDNA sequences for distinct IGF-I and insulin receptors from turbot (Elies, 1999) and Japanese flounder (Nakao, 1999) have been identified. The sequences are similar to the mammalian IGF-I and insulin receptors. Two IGF-I receptor cDNAs (rtIGFR Ia and rtIGFR Ib) were isolated from rainbow trout (Greene, 1999). The amino acid sequences were reported to be 85% identical to each other in the tyrosine kinase domain and more similar to mammalian IGF-I receptors than to insulin receptors. The highest mRNA levels of rtIGFR Ia were found in juvenile gill and adult heart while the highest levels of rtIGFR Ib were in adult pyloric caeca. The lowest levels of both mRNAs were in juvenile heart, liver, muscle and spleen, and in adult liver (Greene, 1999). The IGF-I receptor is widely distributed, appearing in gill cartilage, skin, kidney, heart, pyloric caeca and brain of adult gilthead seabream (Perrot, 1999). The complete cDNA and mRNA expression for the turbot (*Psetta maxima*) IGF-I receptor has also been recently reported (Elies, 1999).

The cellular actions of IGF-I and IGF-II and IGF-I receptor characteristics are poorly understood in fish. Recent work in zebrafish has revealed that, as in mammals, the IGF-I receptor in fish has high affinities for both IGF-I and IGF-II. Both IGF-I and IGF-II stimulate embryonic cell proliferation and DNA synthesis through activation of the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3 – kinase (PI3 kinase) pathways, which are also major pathways in the mammalian IGF-I receptor (Pozios, 2001). This research provides evidence that the IGF-I receptor in fish has signal transduction pathways similar to mammalian receptors (Pozios, 200).

The IGF-I receptor in mammals is expressed as a single precursor molecule with a signal peptide and α- and β-subunits. The α- and β-subunits are separated by proteolytic cleavage and then linked by primary disulfide bonds. The mature receptor has a α2β2-configuration where two α- and β-subunits are joined by disulfide bonds (Jones, 1995; LeRoith, 1995). Ligand binding occurs in the cystine-rich region of the extracellular α-subunit domain. The β-subunit has a hydrophobic transmembrane domain with a short extracellular region. The tyrosine kinase region is within the β-subunit intracellular domain.

Activation of the intracellular tyrosine kinase is triggered by ligand binding to the α-subunit. This activation may be due to triggering of a transformational change in β-subunit. The activation leads to receptor autophosphorylation (Stewart, 1996), which in turn generates motifs containing phosphotyrosines that are recognized by cytosolic proteins that initiate signal transduction. Members of at least five major families of protein substrates interact directly with the

cytoplasmic domain of the IGF-I receptor (Blakesley, 1999). The specificity of signaling from the IGF-I receptor is dictated at each downstream step in the signal cascade. For example, docking proteins, called insulin receptor substrates (IRS) are phosporylated on tyrosine residues and create recognition sites for other signaling proteins resulting in specific signaling cascades. Other signaling pathways lead to the activation of MAPK by the MAPK cascade, which in turn initiate other transcription factors. The phosphatidyl-inositol 3-kinase (PI3 kinase) pathway is also activated. The activation of multiple pathways result in cellular proliferation, differentiation, protein synthesis, inhibition of cell death, among other functions (Butler, 1998).

The IGF-II receptor (IGF Type II receptor) is a single-chain glycoprotein identical to the mammalian cation-independent mannose-6-phosphate (M6-P) receptor. The IGF-II/M6-P receptor is structurally unrelated to the IGF-I and insulin receptor and does not appear to have any cell signaling mechanism. Some conflicting data exist as to whether the IGF-II/M6-P receptor can mediate a biological response (cf. Lowe, 1991). This receptor, involved in transport of lysosomal enzymes, is a trans-membrane receptor located in the Golgi network. Previous research with chickens and amphibians reported that the M6-P receptors in these species did not possess IGF binding capacity (Clairmont, 1989). However, a putative IGF-II receptor that was highly specific for IGF-II and did not bind insulin was recently identified in brown trout embryos (Mendez, 2001). The molecular mass was approximately 250 kDa, which is similar in size to the mammalian IGF-II receptor. The highest level of IGF-II binding was detected during organogenesis. The identification in a teleost species disputes the

suggestion that IGF-II binding ability of the M6-P receptor and any physiological function mediated by IGF may have been acquired at a later evolutionary stage than the divergence of IGF-I and IGF-II (Duan, 1998).

IGF-II exerts its metabolic and growth-promoting effects by interacting with the IGF-I receptor (Kiess, 1987; Furlanetto, 1987), therefore the most likely role of the IGF-II/M6-P receptor is to bind and degrade IGF-II and thereby regulate its mitogenic effects. Deletion of the IGF-II/M6-P receptor results in fetal overgrowth and death in mammals (Lau, 1994).

IGF tissue distribution in fish

The liver is the primary source for IGF-I and IGF-II in fish circulation, however IGF-I and IGF-II are also produced locally in most tissues. In rainbow trout, both IGF-I and IGF-II mRNA is expressed in all tissues and developmental stages examined (Liver, gill, pyloric caeca, spleen, kidney, heart, muscle and brain of both juveniles and adults, testes and ovary of adults) (Shamblott, 1993). The greatest amounts of IGF-I and IGF-II mRNA are in the liver and the amounts are greater in the adult liver than in juvenile liver for both IGF-I and IGF-II. There are relative differences in IGF-I versus IGF-II mRNA in some tissues between adults and juveniles (Shamblott, 1993). In coho salmon, IGF-I mRNA was also found in whole embryos, intestine and adipose tissue (Duguay, 1992; Duan, 1993). In the gilthead seabream, the tissue distribution of IGF-I and IGF-II was similar to the rainbow trout with the exception that IGF-II mRNA expresion in the heart was as high as that in liver (Duguay, 1996). The expression of both IGF-I

and IGF-II in all tissues and development stages thus far examined suggests that both IGF-I and IGF-II have distinct roles and are important to development and growth in fish.

Regulation of IGF expression

IGF-I expression is GH dependent in teleosts. Exogenous GH increases hepatic IGF-I mRNA and plasma IGF-I levels in a dose-dependent manner in salmonids (Cao, 1989; Moriyama, 1994; Duan, 1994; Moriyama, 1995; Shamblott, 1995). IGF-I also controls its own release by negative feedback, inhibiting GH synthesis (Perez-Sanchez, 1992; Blaise, 1995), which in turn inhibits hepatic IGF-I expression.

The regulation of the IGF-II gene in fish is poorly understood (Palamarchuk, 1999). GH treatment increases hepatic IGF-II mRNA expression in salmonids (Shamblott, 1995), but not in gilthead seabream (Duguay, 1996). Additional research is necessary to understand the regulation of this hormone.

Biological action of IGFs

Experimental evidence in fish supports the role of IGF-I and IGF-II in somatic growth stimulation. Exogenous IGF-I increases linear growth in coho salmon (McCormick, 1992) and higher plasma IGF-I levels are observed in fast growing chinook salmon than in slow growing fish (Beckman, 1998). Manipulation of growth rates by fasting and feeding in gilthead seabream and salmon show a positive correlation between plasma IGF-I levels and growth rate (Perez-Sanchez, 1995; Duan, 1993). Hepatic levels of IGF-I mRNA in juvenile coho

salmon increase following a rise in plasma GH and insulin during a rapid growth period that corresponds to increases in springtime temperature and photoperiod. These seasonal changes in GH and IGF-I may be associated with environmental cues (Duan, 1995). IGF-I has a stimulatory effect on DNA synthesis in fish condrocytes (Duan, 1992; Tsai, 1994) and stimulates protein synthesis in gulf killifish (*Fundulus grandis*) muscle (Negatu, 1995). IGF-I and IGF-II both stimulate proteoglycan synthesis by cultured fish cartilage (Duan, 1990; Gray, 1991; Kelley, 1993; McCormick, 1992; Marchant, 1993; Tsai, 1994; Takagi, 1996).

The conserved structure and function of the IGFs between fish and mammals suggests that the biochemical and cellular actions of both IGF-I and IGF-II are also conserved, although specific research in fish is scarce. Recent work in zebrafish provides evidence that IGF-I and IGF-II are potent mitogens for embryonic cells and stimulate DNA synthesis (Pozios, 2001).

The IGFs, especially IGF-I, have been extensively studied in mammals and have been found to play essential roles in growth. Both peptides elicit a mitogenic response in concentrations in the nano-molar range *in vitro*. IGF-I has broad specificity and promotes cell differentiation and proliferation resulting in body growth. IGF-I is also involved in regulation of protein, lipid, carbohydrate and mineral metabolism in cells. IGF-I regulates cell survival by inhibiting apo-ptosis and stimulates neurite outgrowth. One of the more widely studied effects of the IGFs *in vitro* is their function as progression factors, causing cells to progress through the cell cycle thereby stimulating DNA synthesis and cell replication (cf. Cohick, 1993; Sara, 1990; Humbel, 1990; Daughaday, 1989).

Both IGF-I and IGF-II appear to have important roles in fish development. In larval gilthead seabream, both IGF-I and IGF-II mRNA were detected in 1-day-old larva, and IGF-I mRNA increased through day 16 of larval development. However, IGF-II mRNA decreased after day 3 of larval development and stayed at lower levels (Duguay, 1996). Both IGF-I and IGF-II mRNA are maternally transferred to rainbow trout eggs (Greene, 1997), and IGF-I mRNA is present in unfertilized teleost eggs (Funkenstein, 1996; Greene, 1997). These observations suggest an autocrine/paracrine mode of action for IGFs exists prior to the development of the circulatory system (Greene, 1997). In addition, three forms of IGF-I mRNA (Ea-1, Ea-3 and Ea-4) are differentially expressed during rainbow trout development. Although IGF-I and IGF-II mRNA was detected during the various stages of embryonic development, the presence of mature IGF peptides was not confirmed (Greene, 1997).

Both IGF-I and IGF-II are essential for both fetal and postnatal growth in mammals (cf. LeRoith, 2001). During postnatal life, most of the growth-promoting effects of GH are mediated by IGF-I. During prenatal growth, both IGF-I and IGF-II are important, however their actions are not dependent on GH (Jones, 1995). IGF-II appears to be under the control of placental lactogen (Gray, 1987) in mammals. In rodents, IGF-II is more predominant in prenatal tissues, with IGF-I predominating postnatally. In humans, significant levels of IGF-II can be detected in adults (Sara, 1990).

Development of an organism involves cell differentiation, cell growth and cell death. Growth factors such as IGF-I and IGF-II play a role in these processes. Development begins with differentiation and

proliferation of embryonic stem cells. Each lineage of stem cells then progress further into more specific progenitor cells and finally into terminally differentiated cells. During organ development, a subset of stem cells undergoes terminal differentiation into the specific cell types required while another subset of cells remain as progenitors to permit continued growth during development and in response to cell loss. Terminally differentiated cells eventually die either by apoptosis (programmed cell death) or by necrosis. These events are part of normal organ development, cell turnover and in response to injury (cf. LeRoith, 2001).

IGF-I in both the liver and gills may be involved in salmonid seawater adaptation, as IGF-I mRNA increases during smoltification (Sakamoto, 1995). Injection of IGF-I improves the ability of rainbow trout to maintain plasma osmolarity and sodium levels (McCormick, 1996) and may mediate the osmoregulatory action of GH. Injection of GH into salmon and tilapia increased IGF-I mRNA expression in the gills (Sakamoto, 1993; Shepherd, 1997), however this effect did not occur in the gilthead seabream (Duguay, 1996). Immature rainbow trout transferred to 80% seawater had increased plasma GH after 1 day and an increase in IGF-I mRNA in gills by day 1 and in kidneys by day 8. However, IGF-I mRNA was not significantly increased in the liver, indicating that the hypoosmoregulatory action of GH may be mediated by local synthesis of IGF-I in these organs (Sakamoto, 1995). No specific role for IGF-II in fish osmoregulation has been established.

IGFs also play a role in fish reproduction. Fish ovaries have IGF-I receptors (Maestro, 1997; Maestro, 1997) and produce both IGF-I and IGF-II mRNA and mature peptides (Schmid, 1999; Kagawa, 1995). Both IGF-I and IGF-II appear to be involved in the maturation

of fish oocytes and in follicle development in an autocrine/ paracrine manner exerting their effects at different stages of oocyte development (Schmid, 1999). In the tilapia, IGF-I expression in the granulosa and theca cells was highest during vitellogenesis and declined in the later stages of oocyte development. IGF-II mRNA occurred only in the granulosa cells during the late follicle stage of oocyte development (Schmid, 1999). IGF-I induces final oocyte maturation in red sea-bream (*Pagrus major*), possibly by inducing meiotic cell division through the IGF-I receptor on the oocytes (Kagawa, 1994). In addition IGF-I may contribute to the regulation of ovarian steroidogenesis in salmonids (Maestro, 1997).

IGF-I and IGF-II are both expressed in fish testes and bind to an IGF-I receptor (Le Gac, 1995; Le Gac, 1996; Loffing-Cueni, 1999). IGF-I in combination with 11-ketotestosterone (11-KT, the spermatogenesis inducing hormone) *in vitro*, stimulates spermatogenesis in the Japanese eel (Nader, 1999). In the testicular cells of the rainbow trout, both IGFs stimulated DNA synthesis by interacting with the IGF-I receptor (Loir, 1994). The mechanisms by which IGF-I and IGF-II act on fish gonads to elicit their actions have not been demonstrated.

It is evident from the complexity of the somatotropic axis that environmental chemicals have the potential to interfere with the normal physiology of this axis through a number of mechanisms. The following section summarizes some of the evidence for endocrine disrupting chemicals in the environment as well as possible mechanisms of action for these chemicals.

ENDOCRINE DISRUPTING CHEMICALS

Substantial evidence now exists for the presence of endocrine disrupting chemicals, especially estrogenic chemicals, in the aquatic environment (De Guise, 2001). Chemicals such as pesticides, industrial wastes and many other pollutants may affect wildlife due to their environmental persistence, bioaccumulation in tissues and ability to elicit toxicity at very low concentrations (cf. Fry, 1995). The number and range of possible estrogenic compounds in the environment is only recently becoming known. Sources of chemical pollutants in the environment range from sewage effluents and industrial wastes to runoff from roads as well as animal feed lots. Regardless of their initial source, most of these pollutants eventually enter the aquatic environment. These chemicals include pesticides (organochlorines, organophosphates), industrial chemicals (polychlorinated biphenyls (PCBs), petroleum hydrocarbons), plastics (bisphenol A) and nonionic surfactants (nonylphenol and octylphenol) used as industrial and household detergents (Sumpter, 1998). In addition, natural and synthetic estrogens such as 17β-estradiol (E_2) and ethinyl estradiol (EE_2) and chemicals such as alkylphenols from sewage treatment plant effluents have elicited estrogenic effects in aquatic wildlife (Folmar, 2001a; Folmar, 1996; Jobling, 1993; Jobling, 1996; White, 1994).

One of the effects of xenoestrogens observed in aquatic wildlife includes feminization of male fish. Wild male fish near sewage treatment plants in both the United States and United Kingdom have been found with reduced serum testosterone levels and the presence of vitellogenin (a yolk protein produced in females in response to E_2 and not normally found in males) in the bloodstream (Folmar, 1996; Folmar, 2001a; Jobling, 1993; Jobling, 1996; Sumpter, 1995). Male

roach (*Rutilis rutilis*) exposed to treated sewage effluent in rivers throughout the United Kingdom had irreversible intersexuality (development of oocytes in the testis) (Rodgers-Gray, 2001). Wild male Mediterranean swordfish (*Xiphias gladius*) have high levels of vitellogenein and zona radiata proteins (Fossi, 2001).

Compounds with anti-estrogenic properties have also been identified. PCBs, polyaromatic hydrocarbons (benzo(a)pyrene and b-naphthoflavone) and some insecticides (Mirex and endosulfan) inhibit vitellogenesis and ovarian growth in female fish. Some of these chemicals also decrease circulating E_2 levels and fecundity in wild female fish (cf. Oberdoerster, 2000).

Incidences of masculinized female fish have been reported. In populations of wild chinook salmon in the Columbia river, a large percentage (84%) of phenotypic female fish sampled have a genetic marker for the Y chromosome (Nagler, 2001). This genetic marker was not observed in hatchery raised fish. The authors speculate that the wild fish may have been sex reversed, possibly due to EDCs that are present in the Columbia river. Masculinized wild female mosquito fish (*Gambusia affinis*) have also been reported downstream from a paper mill (Howell, 1980). Analysis revealed that the plant sterol, stigmastanol, was metabolized by an aquatic mycobacterium to an androgen, possibly androstenedione (Denton, 1985). However, androgen disruption may be possible in fish exposed to organochlorines. Androgen receptors identified in the kelp bass (*paralabrax clathratus*) and the Atlantic croaker (*Micropogonias undulatus*) bind hydroxylated PCBs, DDT isomers and metabolites of vinclozolin, although at very low affinities (Sperry, 1999; Sperry, 1999). The significance or

functional response to these chemicals was not determined and the incidence of androgen disruption in wild fish is unknown.

In addition to disturbances in sex steroid physiology, disruption of thyroid hormone function has also been documented in fish. Coho salmon fed Mirex and PCBs had reduced circulating T_3 and T_4 levels (Leatherland, 1978). Coho salmon injected with PCB Aroclor 1254 had alterations in normal patterns of T_4 during development (Folmar, 1982). Freshwater catfish (*Clarias batrachus*) had altered T_3 and T_4 levels after aquatic exposure to the pesticides endosulfan, malathion and carbaryl (Sinha, 1991; Sinha, 1991). Xenobiotic chemicals may compete with thyroid hormones for binding sites on transport proteins, thereby increasing the rate at which the thyroid hormones are excreted (cf. Oberdoerster, 2000). Thyroid hormones are critical for metabolism, growth and reproduction in fish. Thyroid hormone levels increase prior to vitellogenesis in some seasonally breeding fish (Cyr, 1996), possibly to mobilize energy toward egg production. Metamorphosis in some fish species is also dependent on increasing thyroid hormone levels. For example, smoltification in anadromous salmonids (Folmar, 1979; Dickhoff, 1993) and flounder metamorphosis from bilateral symmetry to asymmetry in the adult (Inui, 1994) are dependent on thyroid hormones.

Because the endocrine system is complex and regulates all responses and functions of the body, identification of the specific mechanisms through which EDCs elicit their response is difficult. Another confounding factor is that EDCs often do not structurally resemble the naturally occurring endogenous hormones (McLachlan, 1996), making it difficult to predict the mode of action based on

structure alone. The major mechanisms by which EDCs can disrupt endocrine function are through alterations in:

1) the hypothalamic-pituitary control of an endocrine axis,
2) the activity of proteins (e.g., $P450_{arom}$ enzyme) involved in synthesis of hormones or availability of cholesterol for steroid synthesis,
3) the function of plasma hormone binding (transport) proteins,
4) the hormone receptor (e.g., acting as an agonist or antagonist or affecting synthesis or stability), and
5) the metabolism and clearance of the hormone from the site of action (Crain, 1997).

EDCs can alter the neuroendocrine regulation of hormone secretion by interfering with or altering the normal function of the hypothalamus. Although no data are available in fish or wildlife species, research in mammals has found several examples of disruption in normal hypothalamic physiology due to pesticides. Rats injected with the pesticide chlordimeform had desensitization of hypothalamic alpha-adrenergic receptors which resulted in a decrease in normal GnRH and TSH secretion in response to norepinephrine (Goldman, 1990). Other research has shown that the pesticides chlorpyrifos and methoxychlor affect hypothalamic GnRH gene expression, cell survival and neurite outgrowth in cell culture, and chlorpyrifos also substantially increases GnRH mRNA levels altering the timing of reproductive maturation in female rats (Gore, 2001). In addition to these direct effects on the neuroendocrine system, it seems plausible that EDCs may also have indirect effects by interfering with normal feedback loops acting at the level of the hypothalamus. An increase or decrease in endogenous hormones resulting from disruption of peripheral hormone pathways may alter hypothalamic responses.

Because of the complexity of signaling cascades involved in some hormone synthesis pathways, a number of opportunities exist for an EDC to interfere with normal hormone synthesis. EDCs may interfere with normal transcription of genes encoding proteins and enzymes involved in hormone synthesis. The steroidogenic pathway, for example, begins with cholesterol, which is converted to intermediate steroids by a number of steroidogenic enzymes including cytochrome P450 17, 20-lyase (a rate-limiting enzyme that converts C21 to C19 steroids) and aromatase ($P450_{arom}$, the enzyme that converts testosterone to E_2). Few studies have examined the effects EDC exposure on steroidogenesis in fish or other wildlife. In perch (*Perca fluviatilis*) living in a lake near a public refuse dump, 75% of the female fish were sexually immature compared to a reference lake (Noakjsson, 2001). Physiological measurements revealed low $P450_{arom}$ activity and reduced circulating levels of steroids associated with the impaired sexual maturation of the majority of female fish. The chemicals responsible were not identified. *In vitro* ovarian steroidogenesis studies using polycyclic aromatic hydrocarbons (PAHs) resulted in decreased E_2 secretion in coho salmon (Afonso, 1997) and flounder (*Platichthys flesus* L.) (Rocha Monteiro, 2000) ovarian follicles. In addition to decreased E_2 secretion, androstenedione and testosterone were inhibited in flounder ovaries due to inhibited cytochrome P450 17, 20-lyase and $P450_{arom}$ activity (Rocha Monteiro, 2000).

After a hormone is synthesized and released, its biological availability is regulated by its ability to bind to hormone binding or transport proteins. Binding proteins protect the hormone from degradation and excretion. They also act as a reservoir to maintain an

equilibrium of free and bound hormone and thereby regulate the amount of free hormone available for receptor binding. For example in plasma, E_2 binds to the sex hormone binding globulin (SHBG) produced by the liver. Sex hormone binding globulins have been identified and characterized in arctic char (*Salvelinus alpinus L.*), rainbow trout, black bream, and greenback flounder (*Rhombosolea tapirina*) (Ovrevik , 2001; Hobby, 2000). SHBGs are stimulated by E_2, and binding capacity of the SHBG was greater in vitellogenic rainbow trout and black bream than in non-reproductive fish (Hobby, 2000). The effects of EDCs on binding protein concentrations in fish are unknown. However xenoestrogens have the capacity to regulate SHBG levels, as demonstrated in cell cultures where phytoestrogens increased production of SHBG (Loukovaara, 1995). Therefore, EDCs could increase plasma SHBG levels, and inhibit hormone action by decreasing the amount of free hormone available to interact with receptors. In addition to affecting the concentration of binding or transport proteins, EDCs may not themselves bind to SHBGs. The synthetic estrogens diethylstilbestrol (DES) and EE_2 (Crain D.A., 1998) and the EDCs octylphenol and o,*p*'-DDT (Arnold, 1996) do not bind appreciably to SHBG. The lack of binding affinity of these chemicals for the SHBG increases their availability to the target cells compared to the endogenous hormone. The ability of EDCs to interact with binding proteins could have a major effect on their relative potency.

EDCs could also disrupt normal endocrine function by binding to hormone receptors. An EDC could act as an agonist by activating the receptor to initiate the signal or action normally induced by the natural hormone. Conversely, an EDC may act as an antagonist by binding to the receptor to inhibit the natural hormone from binding,

thereby blocking the normal response. While hormone receptors have a high affinity for the natural hormone ligand, they are not completely specific and can bind other ligands, particularly if a high concentration of other ligands exists. Many EDCs known to bind to the estrogen receptor do so at much lower affinity than E_2, however due to alterations in hormone function discussed previously, an EDC may be more potent than E_2 at activating the receptor. For example, increased bioavailability of an EDC due to disruption of binding protein physiology may increase its potency to the receptor. Just as binding affinities for the receptors differ between EDCs and natural hormone ligands, dissociation rates may differ. An EDC may interfere with normal receptor activation by prolonging receptor activation and thereby increasing the physiological response (cf. Crain, 2000).

Endocrine disruption at the receptor level typically involves the classic nuclear receptor mechanism. Steroids, or EDCs, diffuse through the membrane and bind to intracellular nuclear receptors, which, upon activation bind, to hormone response elements to alter gene induction. For example, the organochlorine chlordecone binds to the estrogen receptor in immature rainbow trout livers (Donohoe, 1996). Although the binding affinity is approximately 1000-fold lower than estradiol, chlordecone induces vitellogenin synthesis, indicating a genomic response. Recent evidence, however, indicates that many EDCs could also bind to steroid receptors on cellular membranes and thereby interfere with nongenomic actions of steroids (Thomas, 2000). This was demonstrated in spotted seatrout where Kepone and *o,p'*-DDD bind to the ovarian maturation inducing steroid (MIS) membrane receptor. These chemicals act as antagonists by inhibiting MIS induced oocyte maturation *in vitro* (Das, 1999).

The final mechanism by which EDCs could disrupt normal endocrine function is by interfering with hormone degradation and excretion. Alterations in the normal hormone excretion rates can alter the hormone concentrations within an animal. Most steroids are excreted after conversion to hydrophilic compounds, usually by Phase II conjugation in the liver. Some EDCs have been found to stimulate the hydroxylation of steroids and thereby increase excretion. For example, DDT, chlordane and dieldrin increased both androgen and estrone metabolism in mammals, resulting in decreased gonadal weight (Levin, 1968; Welch, 1971). PCBs caused an increase in hepatic P450 enzyme activity and a decrease in circulating steroid concentrations in fish (Sivarajah, 1978). Likewise, bleached kraft pulp mill effluent causes elevated hepatic P450IA activity and decreases plasma sex steroid concentrations in lake whitefish (*Coregonus clupeaformis*) and white sucker (*Catostomus commersoni*) (Munkittrick, 1992; Munkittrick, 1994).

It is clear that greater understanding of the physiological mechanisms involved in endocrine system function will help elucidate the modes of action for EDCs. For example, until the basic mechanism of steroid hormone-receptor interactions were understood, the possibility of a xenobiotic acting on a receptor were unknown. When the dynamics of other regulatory aspects of the endocrine system in fish are understood, further actions of EDCs will become apparent.

One area in need of exploration is the possible interaction of EDCs with the endocrine somatotropic axis in fish. Chronic exposure to environmental estrogens or other EDCs may inhibit growth in fish. Evidence is mounting implicating gonadal steroids in somatic growth

regulation. Chronic exposure to estrogens inhibits somatic growth in rats (Borski, 1996; Murphy, 1988), while chronic exposure to dihydrotestosterone enhanced growth (Borski, 1996). The growth regulation in rats appeared to be at the level of hepatic IGF-I gene expression by reducing IGF-I mRNA. In laboratory studies, estrogens impaired growth in several fish species (Bulkley, 1972; Ostrowski, 1986; Donaldson, 1979). The effect of estrogenic chemicals or other EDCs on hepatic IGF-I and IGF-II mRNA levels in fish is unknown. However, during reproductive maturation, elevated levels of gonadal steroids coincide with diminished somatic growth.

Persistent environmental pollutants also have an effect on the growth rate of fish (Hammar, 1993; Jensen, 1982; Connolly, 1991). PCBs, for example, inhibit growth in wild arctic char (Hammar, 1993). The organophosphate pesticide chlorpyrifos inhibits fish growth in laboratory studies (Cripe, 1986). Although not known to be estrogenic, chlorpyrifos has been implicated as an EDC in mammals by altering reproductive development and stimulating neuroendocrine GnRH mRNA *in vitro* (Gore, 2001). It is unknown whether the growth inhibiting effects of chlorpyrifos in fish are related to endocrine disruption. Possible mechanisms for growth inhibition by these chemicals were not examined.

EDCs may affect somatic growth through a number of mechanisms including modulation of hepatic IGF mRNA levels either by affecting mRNA stability or transcription rate. EDCs may disrupt the synthesis of hepatic IGF-I or IGF-II by occupying or down-regulating receptors for GH or by interfering with normal signal transduction initiated by the activated receptors. Estrogenic chemicals may act

through the hepatic estrogen receptor to alter transcription of genes important in growth regulation.

With the growing recognition that pollutants can affect living organisms through the endocrine system, clarification of the mechanisms of action of these chemicals is necessary. The endocrine system responds to incredibly small amounts of signal chemicals and often a cascade of various interrelated responses and hormones result from a single chemical signal. Because of the number and complexity of mechanisms by which EDCs could affect growth, basic research of the somatotropic axis in relation to EDCs is important to begin to understand the possible consequences of EDC exposure. Studies in mammals have demonstrated a complex interrelationship between GH/IGF-I and estrogen (Murphy, 1988; Borski, 1996) that may affect both somatic and gonadal growth. The research presented here attempts to begin exploring the relationship of endogenous hormones, estradiol and chlorpyrifos with IGF-I and the somatotropic axis in fish.

3. QUANTIFICATION OF SHEEPSHEAD MINNOW (*CYPRINODON VARIEGATUS*) IGF-I MRNA LEVELS USING REAL-TIME PCR (TAQMAN) TECHNOLOGY FOLLOWING TREATMENT WITH GROWTH REGULATING HORMONES

INTRODUCTION

The endocrine control of growth in vertebrates is regulated primarily by pituitary growth hormone (GH) and its subsequent stimulation of insulin-like growth factor (IGF) synthesis. Insulin-like growth factors (IGF-I and IGF-II) are peptide hormones that induce multiple effects including cell growth, metabolism and differentiation (Daughaday, 1989). Although the major source of circulating (endocrine) IGFs is the liver, many tissues produce IGFs, which in turn act through autocrine and paracrine actions to regulate somatic growth (Daughaday, 1989).

There is now substantial evidence that this GH-IGF-I axis is highly conserved in fish and other vertebrates (cf. Bjornsson, 1997; Moriyama, 2000; Mommsen, 2001). Administration of GH elevates hepatic IGF-I mRNA and plasma IGF-I levels in a dose-dependent manner in salmonids (Cao, 1989; Moriyama, 1994; Duan, 1994; Moriyama, 1995; Shamblott, 1995). Although GH is the primary regulatory hormone for IGF-I synthesis in fish, other hormones also have a stimulatory effect on IGF-I levels. Insulin plays a significant role in metabolism and growth of fish and mammals. In mammals, insulin regulates the expression of a large number of genes including the stimulation of the GH receptor and IGF-I genes (O'Brien, 1996; O'Brien, 2001). Coho salmon injected with streptozotocin to induce diabetes and impair insulin production had lower growth rates and

hepatic IGF-I mRNA levels than control fish (Plisetskaya, 1994). *In vitro* studies with primary salmon hepatocyte cultures indicate that insulin acts synergistically with GH to stimulate hepatic IGF-I gene expression (Duan, 1992). In addition to insulin and GH, Triiodothyronine (T_3) also potentiates GH induced IGF-I synthesis in rats (Rotwein, 1991). T_3 increases IGF-I mRNA in coho salmon hepatocyte cultures (Duan, 1992) while adding thyroxine (T_4) to food increases growth in juvenile coho salmon (Higgs, 1976). In contrast, 17β-estradiol (E_2) appears to inhibit somatic growth and down-regulate IGF-I synthesis in mammals (Borski, 1996; Murphy, 1988). The effect of E_2 on hepatic IGF-I mRNA synthesis in fish has not been explored, however elevated levels of E_2 coincide with diminished somatic growth during reproductive maturation. An inhibitory effect of E_2 on hepatic IGF-I synthesis or somatic growth in fish is of particular interest due to the presence of estrogenic chemicals in natural waters (cf. De Guise, 2001).

To evaluate the effects of GH, insulin, T_3 and E_2 on hepatic IGF-I mRNA concentrations in a commonly used marine test species, the sheepshead minnow (*Cyprinodon variegatus)* (SHM), we injected fish with these hormones and measured IGF-I mRNA at four time points up to 24 h. To measure IGF-I mRNA, we developed and optimized a real-time quantitative PCR (TaqMan) assay and used an *in vitro* transcribed SHM IGF-I RNA (cRNA) strand as a standard. Using this relatively new, highly sensitive technique, we were able to quantify SHM IGF-I mRNA from nanogram amounts of total RNA.

MATERIALS AND METHODS

Test Animals

Adult offspring of laboratory raised SHM weighing 1.4g (± 0.6) and 43 mm (± 5.6) in standard length were used. The fish were maintained at the U.S. Environmental Protection Agency, Gulf Ecology Division Wet Lab in 100 L glass aquaria supplied with flowing, aerated seawater (20‰ salinity, 25-26°C)with a 16L:8D photoperiod. Stocking density was 25 to 32 fish per tank, and fish were acclimated to their tanks for three weeks prior to experimental treatment. Fish were fed to satiation with Tetramin flake fish food twice per day except on weekends when they were fed once per day.

Chemicals

Hormones (E_2, T_3, GH [bovine], and insulin [bovine]) were purchased from Sigma (St. Louis, MO). RNase - free chemicals and reagents, RNA*secure* Resuspension Solution and DNA-*free* DNase Treatment & Removal Reagents were purchased from Ambion (Austin, TX). RNA STAT-60 Total RNA/mRNA isolation reagent was purchased from Tel-Test (Friendswood, TX).

Hormone Treatment

Fish were injected with either 17β-estradiol (E_2), growth hormone (GH), insulin or triiodothyronine (T_3). E_2 and T_3 were dissolved in triethylene glycol (TEG) and GH and insulin were dissolved in 0.15 M saline. The fish received two injections, one in the afternoon of the first day and a second in the morning of the following day. Injection

concentrations shown to have a biological response by other researchers were given as follows: GH - 10 ug/g body weight (B.W.) in saline (Shamblott, 1995), T_3 - 1.0 ug/g B.W. in TEG (Takagi, 1996), E_2 - 5 ug/g B.W. in TEG (Folmar, 1995), insulin - 1 I.U./kg B.W. in saline (Ablett, 1981). The hormones were dissolved in the carrier solution at a concentration which allowed delivery of the dose in a 25 ul injection volume. Test fish were anesthetized for injection in a sleep dose of tricaine methanesulfonate (MS222, 20 mg/L) buffered with sodium bicarbonate (50 mg/L). The hormones were injected into the peritoneum (*ip*) of 32 fish per hormone treatment using a 25 gauge 5/8" tuberculin syringe. Due to insufficient fish availability, only 24 fish were injected with 25 ul TEG alone to serve as controls. Each tank of fish was randomly chosen for the hormone treatment, and the fish were returned to the same tank after the injections. The fish were not fed during 48 h experiment.

Sampling Procedure

Eight fish (both male and female) from each hormone treatment were sampled 4 h, 8 h, 12 h and 24 h after the second injection. Control fish were sampled at 0 h, 12 h and 24 h after the second injection. At each sampling, fish were randomly netted and placed into a lethal dose of MS222 (40 mg/L) buffered with sodium bicarbonate (50 mg/L). Each fish was measured (total length) and weighed (to the nearest 0.01g). The blood was removed by severing the caudal peduncle. Livers were quickly removed, snap frozen in liquid nitrogen and stored at -70° C until processed.

RNA Preparation

Total RNA was isolated from livers using RNA-STAT 60 Total RNA/mRNA isolation reagent with a modification of the manufacturer's protocol as follows: Frozen livers were homogenized using a Polytron PT1200 hand-held homogenizer (Kinematica A.G., Switzerland) in 1 ml RNA-STAT 60 after which 200 ul chloroform was added. Samples were vortexed at maximum speed for 30 sec followed by centrifugation at 4°C for 15 min. The aqueous layer was removed to a clean tube and the extraction was repeated by adding 1 ml RNA-STAT 60 and 200 ul chloroform to the aqueous layer and vortexing for 30 sec. After centrifugation at maximum speed for 15 min the aqueous layer was removed to a clean tube. The pellet was precipitated by adding 600 ul isopropanol and centrifuging at 7000 X G for 30 min at 4°C. The supernatant was removed and the pellet washed twice in 75% ethanol. After air drying the pellet for several minutes at room temperature, the pellet was resuspended in RNA Secure Resuspension Reagent (Ambion, Inc.) following the manufacturer's protocol. Genomic DNA was removed using a kit (DNA-Free, Ambion, Inc.) following the manufacturer's protocol. The purity and concentration of each RNA sample was determined by measuring the absorbance at A260 and A280 in a UV plate reader (Spectra MAX 190, Molecular Devices). The quality of the total RNA was checked by denaturing-agarose gel electrophoresis and ethidium bromide staining. The remaining RNA was stored at -70° C until needed.

Preparation of cDNA

cDNA was prepared from 2 ul diluted IGF-I cRNA standard or 2 ug total RNA from the liver samples in a 20 ul reaction volume. The

RNA was first incubated at 65°C with 500 uM deoxynucleotide (dNTP) mix, 250 nM IGF-I gene-specific reverse primer and nuclease-free water to a volume of 12 ul for 5 min. The mixture was cooled to 50°C for 2 min after which 8 ul of a pre-warmed (50°C) buffer/enzyme mixture [4 ul 5X first-strand buffer (Gibco/BRL), 10 mM dithiothreitol (DTT), 40 units ribonuclease inhibitor (RNaseOUT, Gibco/BRL), and 200 units SuperScript II (Gibco/BRL) reverse transcriptase] was added. The mixture was incubated at 50°C for 50 min. The reaction was stopped by heating to 70°C for 15 min. The resulting cDNA was stored at -20°C.

Preparation of cRNA Standard

An *in vitro* transcribed sense RNA transcript was used to generate a standard curve for hepatic SHM IGF-I mRNA quantitation. Sense RNA was prepared from the plasmid vectors (pGEM-T Easy Vector, Promega) with a cloned SHM IGF-I sequence behind an SP6 RNA polymerase promoter. The plasmid was first treated with RNase to remove all RNA contamination and was then linearized by overnight digestion with a restriction enzyme (NCO1) downstream of the polymerase binding site and the cloned product. After restriction enzyme digestion, the cDNA template was purified using a PCR purification column (Qiagen QiaQuick). The eluted samples containing the IGF-I cDNA template were treated with Proteinase-K, extracted with phenol-chloroform-isoamyl alcohol, precipitated and then resuspended in 10 mM Tris-Cl (pH 8.3). A sample of the resuspended cDNA template was electrophoresed on a 1.4% agarose/tris-acetate EDTA buffer (TAE) gel to confirm a single band of the anticipated size.

In vitro transcription was performed using a Maxi Script kit (Ambion, Inc.) and 1 μg of the purified cDNA template according to the kit manufacturer's protocol. The *in vitro* transcription reaction was purified using a Chroma-spin 100 column (Clontech, Inc.) to remove nucleotides and incomplete transcripts.

The concentration of the resulting sense-strand cRNA was determined by absorbance at 260 nm. Ten-fold serial dilutions were made in nuclease-free water (Ambion) containing carrier tRNA (transfer RNA from wheat, 1 μg /ul: Sigma, St. Louis, MO). The cRNA standard dilutions were aliquoted and frozen at -70°C until assayed.

Quantitative Real-Time RT-PCR Analysis of mRNA

The quantitative real-time PCR (TaqMan) assay uses a fluorogenic probe designed to hybridize with the target sequence between the forward and reverse primers. The probe is labeled at the 5' end with a reporter dye and with a quencher dye at the 3' end. When the probe is intact, the fluorescence is quenched due to the proximity of the quencher to the reporter. During the PCR reaction, the probe is cleaved by the 5' nuclease activity of the Taq Polymerase enzyme, resulting in increasing fluorescence of the reporter dye during each PCR cycle. By measuring the fluorescent signal during each PCR cycle, the amount of specific PCR product (SHM IGF-I mRNA) can be monitored during the log-linear phase of the reaction. During this phase of the PCR reaciton, the increasing fluorescence at each PCR cycle is directly proportional to the initial amount of target mRNA in the sample. The fluorescence is monitored at each cycle by the ABI

PRISM 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA).

Oligonucleotide Primers and TaqMan Probe Design

The sequence for SHM IGF-I cDNA (Figure 3.1) was analyzed by Primer Express software (PE Applied Biosystems, Foster City, CA) to select forward and reverse primers and a fluorogenic probe. This software selects primer and probe sequences with optimal melting temperatures, secondary structure, base composition and amplicon length for the TaqMan reaction. Primer sequences in adjacent exons were chosen to prevent amplification of any remaining genomic DNA fragments in the samples. The fluorogenic probe was labeled at the 5' end with the reporter dye FAM (6-carboxyl-fluorescein) and at the 3' end with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine). The selected primers and probe were purchased from ABI Prism (PE Applied Biosystems, Foster City, CA). The specificity of the primers was tested under normal PCR conditions using a thermalcycler (Perkin-Elmer 9700, Foster City, CA). A single band with the expected size was observed on a 2.5% agarose gel after ethidium bromide staining (data not shown).

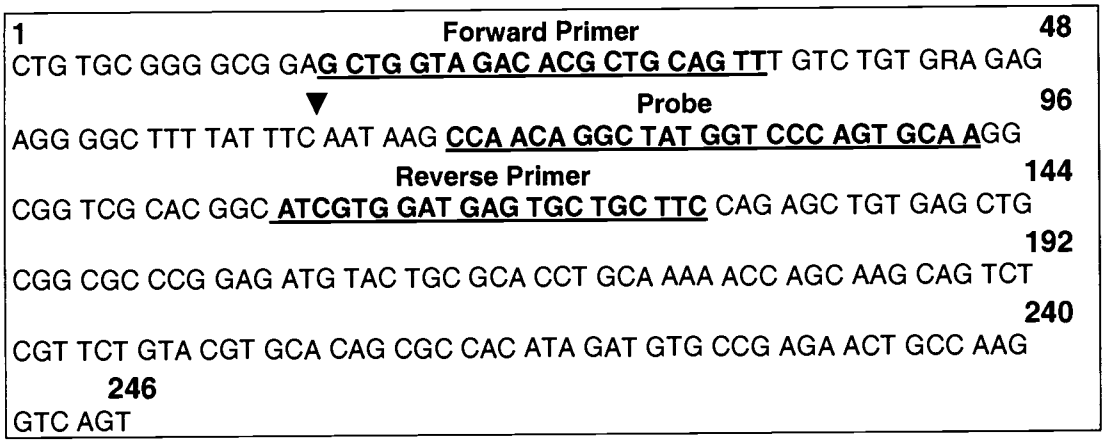

Figure 3.1. Partial nucleotide sequence of sheepshead minnow IGF-I cDNA depicting forward and reverse primers and fluorogenic probe. ▼ indicates intron 2 splice site.

Before performing assays on the experimental samples, the primer and probe concentrations were optimized. The concentrations of these components were tested empirically for each primer/probe combination by testing concentrations of 50, 300 and 900 nM for each primer. The primer concentrations giving the lowest C_t value (PCR cycle at which the fluorescent signal reaches a pre-determined threshold) were chosen for subsequent assays. The optimal concentration for SHM IGF-I was 300 nM for the forward primer and 900 nM for the reverse primer. The optimum probe concentration was determined by testing 25 nM increments from 25 – 225 nM using the optimized forward and reverse primer concentrations. The SHM IGF-I probe concentration resulting in the lowest C_t value was 200 nM and was used in subsequent assays.

Quantitative Real-time PCR

Quantitative (TaqMan) PCR was performed in duplicate for each sample except for the standard curve which was constructed using

triplicate samples of IGF-I cDNA at each concentration ($10^{-10} \rightarrow 10^{-16}$ g/ul). A TaqMan PCR Core Reagent Kit (PE Applied Biosystems, Foster City, CA) was used. The real-time PCR was performed in MicroAmp Optical 96-well reaction plates (ABI Prism, Foster City, CA). A standard curve was included on each 96-well plate and all samples were randomly assigned to each reaction plate. The reaction mixture (25 ul total volume) for the IGF-I assays consisted of the core components according to the manufacturer's instructions (200 uM dNTPs [dATP, dCTP, dGTP] and 400 uM dUTP, 0.01 unit/ul of AmpErase uracil-N-glycosylase [UNG], 0.05 unit/ul of AmpliTaq Gold, 1X TaqMan Buffer) plus 5.5 mM MgCl2, 200 nM probe, 300 nM of the forward primer, 900 nM of the reverse primer, and 2 ul of sample cDNA or standard cDNA. The PCR was run at 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C 15 sec, 60°C 1 min in an ABI PRISM 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The increasing fluorescence signals were measured during each PCR cycle by the ABI Prism 5700 and amplification plots and standard curves were construction by the ABI PRISM software.

To minimize variability in RNA quantitation and integrity, the samples were normalized to 18S ribosomal RNA. The 18S ribosomal primers and a labeled probe were purchased as a kit (PE Applied Biosystems, Foster City, CA). The RT and PCR reactions were carried out using the same quantity of cDNA as the IGF-I assays and with the identical core reagents except for the primers and probes. The ribosomal RNA forward and reverse primers and the probe concentrations were 50 mM in accordance with the manufacturer's recommendation.

Data Analysis

The normalized results of the quantitative real-time PCR assay were analyzed by one-way analysis of variance (ANOVA) with a Tukey post-test using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). The data were square-root transformed to correct for unequal variances prior to analysis.

RESULTS

Quantitative Real-Time PCR

The standard curve was generated using 10-fold dilutions of *in vitro* transcribed SHM sense IGF-I cRNA ($10^{-10} \rightarrow 10^{-16}$ g/ul). The amplification plots of the IGF-I cRNA standards are presented in Figure 3.2. The IGF-I cRNA standard plots were used to determine the threshold cycle (C_t). The C_t value represents the PCR cycle where reporter fluorescence (ΔRn) reaches a threshold value. This C_t value corresponds to the initial amount of target (IGF-I mRNA) in a sample of total RNA. Total RNA containing more of the target (IGF-I) mRNA will reach the threshold cycle before an RNA sample containing less of the target mRNA. A standard curve is generated by plotting the C_t values versus the log of the dilution factor of the IGF-I cRNA standards (Figure 3.3). Using the cloned IGF-I cRNA sequence as a template, linear standard curves were consistently generated.

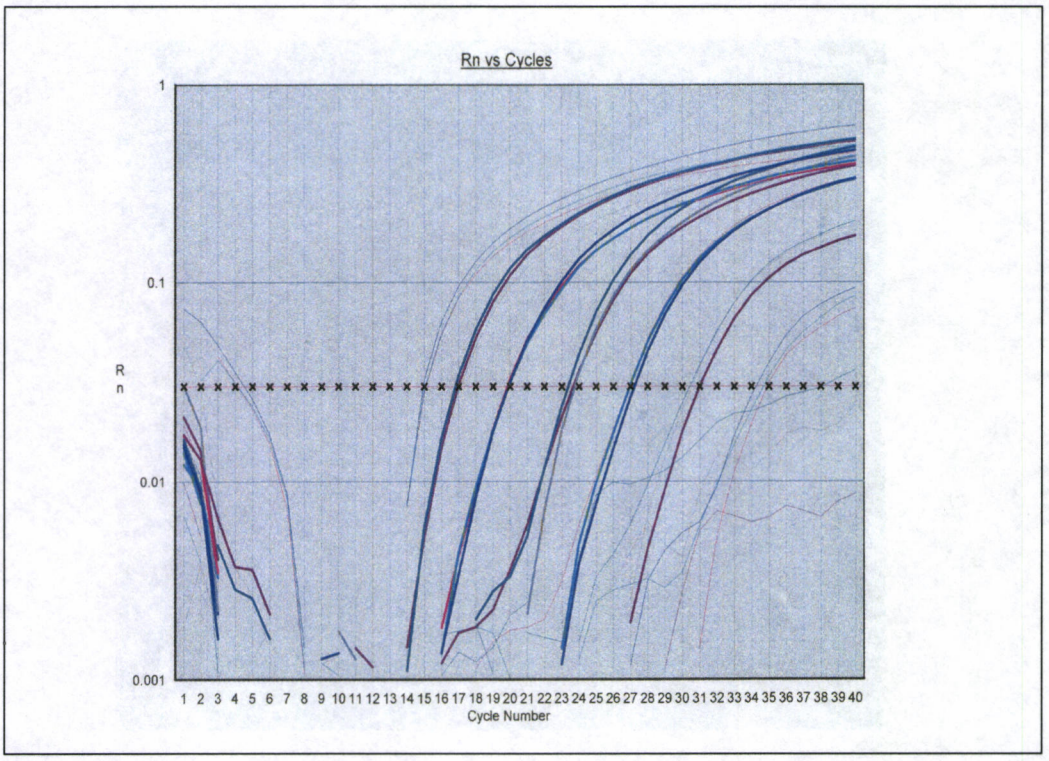

Figure 3.2. Representative amplification plot of SHM IGF-I standards. Ten-fold dilutions of sheepshead minnow IGF-I sense strand (cRNA) standards were assayed in triplicate. Each plot represents the increasing fluorescent signal (Rn) of the probe at each PCR cycle number. The horizontal line at Rn = 0.05 represents the value chosen to calculate the threshold value (Ct) in the log-linear phase of PCR amplification. The C_t value represents the PCR cycle at which an increase in reporter fluorescence (ΔRn) above the threshold value was first detected. This C_t corresponds to the initial amount of template in a sample of RNA.

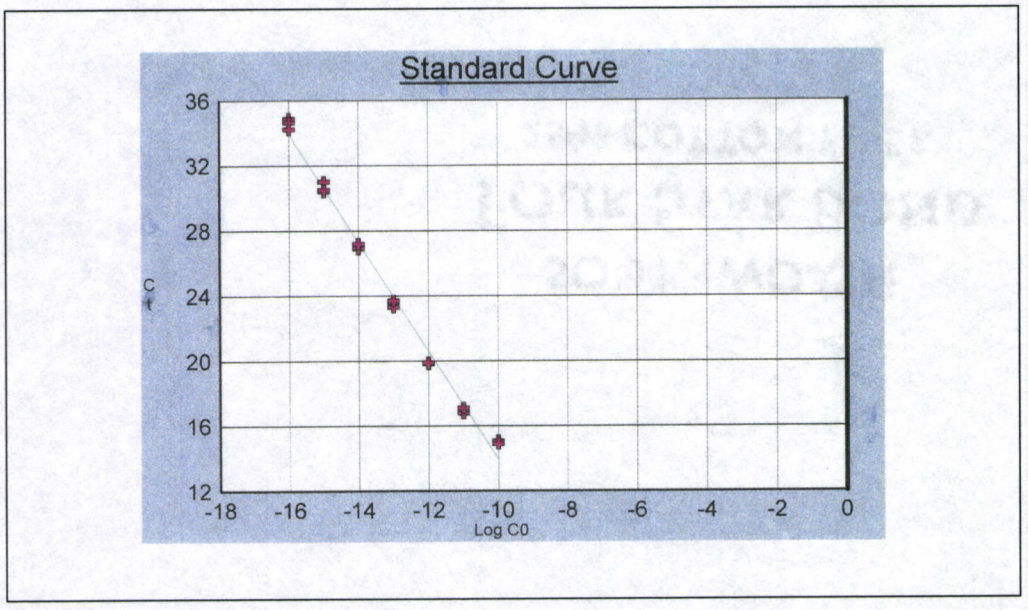

Figure 3.3. Representative standard curve of IGF-I standards. Ten-fold dilutions of sheepshead minnow IGF-I sense strand standards were assayed in triplicate. The standard curve was generated by plotting the threshold values (Ct) determined from the amplification plots (fig. 1.2) versus the log of the dilution factors (CO) of the IGF-I standards ($10^{-10} \rightarrow 10^{-16}$ g/ul). N = 2 except for CO = -16 (N = 3) andCO = -12 (N = 1).

The amplification plots for the RNA samples of the injected fish are presented in Figure 3.4. All of the sample RNA plots fall within the Ct range of the amplification plots of the SHM IGF-I standard dilutions (Fig. 3.2). The amount of IGF-I mRNA in each sample is determined by comparing the Ct value of each sample to the standard curve values. The calculations were performed by the ABI Prism software at the completion of each PCR run. Samples duplicates that varied by more than 15% were re-run. The inter-assay variability was 10.1% and control wells (no-template) generated no fluorescent signal, indicating no cross-well contamination. A cloned SHM IGF-II cRNA sequence did not generate a fluorescent signal, indicating that the primers and probes used in this assay were specific for SHM IGF-I and

did not amplify IGF-II mRNA (data not shown). The Taqman assay for the 18S ribosomal assay generated similar amplification plots (data not shown).

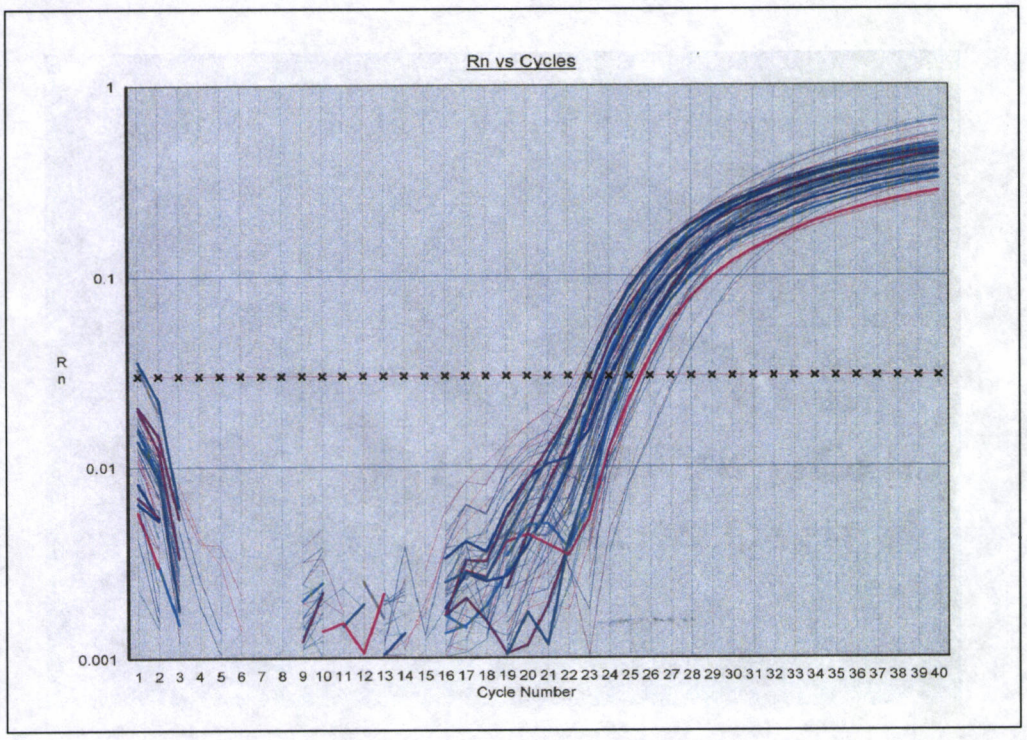

Figure 3.4. Representative amplification plots of IGF-I mRNA of SHM injected with GH, E_2, T_3, insulin or TEG. The amplification plots determine the Ct values (cycle number at which the fluorescent signal [Rn] passes the selected threshold [horizontal line at Rn = 0.05]) for each sample.

IGF-I mRNA Quantitation

Figure 3.5 presents the normalized results of the quantitative real-time PCR assay for IGF-I mRNA. The Ct values of the 18S RNA samples were used to normalize the IGF-I data using the following calculation for each sample:

$$\frac{\left(\frac{1}{\text{Mean 18S } C_t} \times \text{Qty. IGF-I}\right)}{\frac{1}{\text{Sample 18S } C_t}} = \text{Normalized Qty IGF-I}$$

Where: Mean 18S C_t is the mean value for <u>all</u> 18S samples
Qty. IGF-I is the quantitation of IGF-I for that sample
Sample 18S C_t is the value for that sample

All RNA samples from the 0 h controls, 6 samples from the 2 h controls, three from the 24 h controls, three from the 8 h insulin treatment and three samples from the 24 h T_3 treatment group were degraded and therefore not analyzed.

The results of the TaqMan assay (Fig. 3.5) demonstrate that hepatic IGF-I mRNA increased after GH treatment until 12 h post-injection, after which time IGF-I mRNA began to decrease but were still above the 4 h levels. IGF-I mRNA levels of GH injected fish were not significantly different from controls at 12 h. Fish injected with insulin had the highest level of hepatic IGF-I mRNA of all treatments at 4 h, then decreased at 8 h followed by a peak at 12 h and decreased again at 24 h. T_3 injection caused a gradual increase in hepatic IGF-I mRNA levels with a peak at 12 h followed by a gradual decrease throgh the 24 h sampling period. IGF-I mRNA levels for fish injected with GH, T_3 and insulin all reached a peak at 12 h greater than, but not significantly different from, the control levels followed by a decrease at 24 h. Livers from fish injected with E_2 had significantly less IGF-I mRNA than GH ($P < .05$) and insulin ($P < .001$) injected fish at 4 h post-injection. At 8 h post-injection, IGF-I mRNA for E_2 injected fish remained lower than GH ($P < .001$) injected fish, and were also significantly lower than T_3 ($P < .05$) injected fish, but not different from

insulin treated fish. After12 h, the livers of fish injected with E_2 had significantly lower amounts of IGF-I mRNA than all other treatment groups (Controls, $P < .05$; GH, T_3 and insulin, $P < .001$). However, at 24 h, there were no significant differences between any treatments.

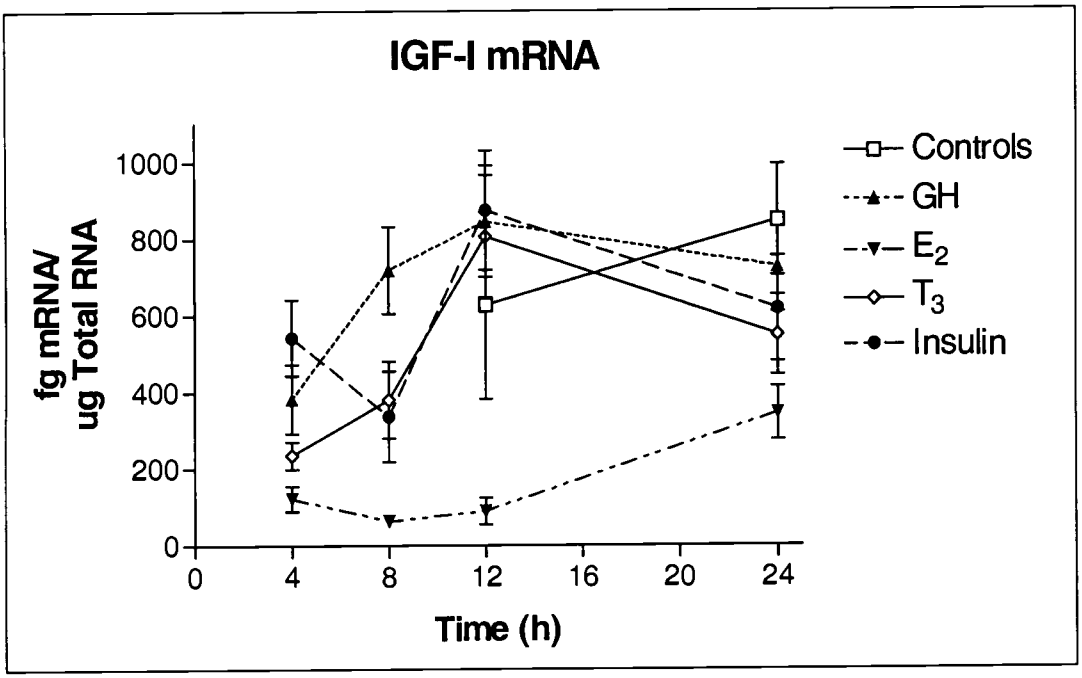

Figure 3.5. Concentration of hepatic IGF-I mRNA determined by quantitative real-time PCR (TaqMan) analysis of fish injected with growth hormone (GH, 10 ug/g body weight (B.W.), estradiol (E_2, 5 ug/g B.W.), triiodothyronine (T_3, 1.0 ug/g B.W.), insulin (1 I.U./kg B.W.) or carrier alone (controls). Each data point represents 8 fish except controls at 12 h (2 fish), insulin at 8 h (5 fish), T_3 at 24 h (5 fish) and controls at 24 h (5 fish). Bars represent standard errors.

DISCUSSION

The purpose of this study was to optimize an assay to measure hepatic SHM IGF-I mRNA levels in response to endogenous hormones (GH, T_3, E_2, insulin) known to influence somatic growth in fish. We were particularly interested in a possible inhibitory effect of E_2 on

hepatic IGF-I mRNA levels. To this end, we used a relatively new real-time PCR (TaqMan) technology, which we optimized to quantify SHM IGF-I mRNA.

We found the TaqMan assay to be a fairly quick and efficient method to quantify mRNA. By comparing values from samples of total RNA to a standard curve constructed from known quantities of *in vitro* transcribed SHM IGF-I mRNA, we were able to quantify the IGF-I mRNA in a given sample. This assay has several advantages over quantitative RPA, Northern blot or slot-blot hybridization, and competitive RT-PCR for measuring gene expression. These advantages include the sensitivity of the assay (theoretically to mRNA copy number), the small amount of total RNA required (100 nanograms to 2 ug), the elimination of the need for post-PCR processing, and the simplicity of the assay, which facilitates processing large sample numbers.

We did not have negative controls for this experiment, so we are not able to verify whether stress due to the injections may have had an effect on the IGF-I mRNA levels, however comparison with fish that never received any injections from other studies did not show any obvious differences in IGF-I mRNA levels. We were primarily interested in the effects of the hormones themselves and therefore chose sham-injection as the controls. The pharmacological levels of the hormones used probably masked any effect of injection stress. Both male and female fish were used in this experiment. In looking at the liver IGF-I mRNA levels between males and females, both in this study, and in Chapter 4, no differences were observed. The results of the TaqMan assay for fish injected with GH, T_3 and insulin indicate that these hormones increase hepatic IGF-I mRNA levels. These

results are consistent with findings in other fish species and support role of these hormones in regulating growth in fish. The increase in hepatic IGF-I mRNA in response to GH in SHM reflects other research which demonstrates that exogenous GH increases hepatic IGF-I mRNA and plasma IGF-I levels in fish (Cao, 1989; Moriyama, 1994; Duan, 1994; Moriyama, 1995; Shamblott, 1995) and supports the role of GH in stimulating hepatic IGF-I synthesis.

The increase in hepatic SHM IGF-I mRNA after 12 h in response to insulin reported here is in agreement with other research. Insulin acts synergistically with GH to increase hepatic IGF-I gene expression in salmon hepatocytes (Duan, 1992) and impaired insulin production results in lower hepatic IGF-I mRNA levels *in vivo* in coho salmon (Plisetskaya, 1994). Insulin is able to bind to IGF-I receptors, although with much lower affinity than the native ligand (Leibush, 1996). The decline in IGF-I mRNA at 8 h may be nothing more than biological variation, as the IGF-I mRNA levels at 4 h and 8 h were not statistically different from each other.

We used T_3 in this study because it is the biologically active form of thyroid hormone (Bres, 1986) and is more effective than T_4 in stimulating fish growth (Higgs, 1979). The results of the Taqman assay indicate a gradual increase in hepatic IGF-I mRNA levels of T_3 injected fish from 4 h to 12 h post injection compared to the more rapid rise of GH injected fish. The differences in the rate of IGF-I mRNA induction observed here may be due to the different mechanisms by which T_3 and GH stimulate IGF-I mRNA synthesis. T_3 increases transcription of the GH gene (Moav, 1992), while injected GH acts directly on the IGF-I gene to induce IGF-I mRNA synthesis. It may be that in sheepshead minnows, GH injections also induced a

rapid, direct stimulation of IGF-I mRNA synthesis, while the exogenous T_3 probably stimulated pituitary GH release. The GH subsequently stimulated IGF-I synthesis, and resulted in a slower IGF-I response supporting previous demonstrations of a functional relationship between thyroid hormones and the GH/IGF-I axis in fish (Moav, 1992; Luo, 1991; Farchi-Pisanty, 1995; Melamed, 1995).

In contrast to the stimulatory effects of GH, T_3 and insulin on hepatic IGF-I mRNA synthesis, injection with E_2 had a significant inhibitory effect. While the effect of E_2 on hepatic IGF-I gene expression in fish has not been reported by other researchers, studies in our lab have shown that chronic aquatic exposure to E_2 (20 and 100 ng/L) from hatching until 18 weeks of age does not significantly alter the IGF-I mRNA content of the liver compared to controls (Chapter 4). Our results are in opposition to mammalian studies in which single injection of E_2 (5 ug/100g BW) had no significant effect on hepatic IGF-I gene expression in rats (Murphy, 1988). However, chronic administration of E_2 (1 or 0.1 ug E_2 daily for 10 days) along with GH (10, 30 or 90 ug/ hGH) resulted in a significant decrease in GH-induced hepatic IGF-I gene expression, serum IGF-I concentration and body weight in mammals (Murphy, 1988). Moreover, liver IGF-I gene expression, plasma IGF-I concentrations and somatic growth were reduced in rats given subcutaneous (*sc*) implants providing physiological E_2 levels for 14 days (Borski, 1996). The difference in our results compared to those in the mammalian studies may be due to the higher injected dose of E_2 administered to the SHM, or to inherent genetic differences in response to E_2 between species.

We were interested in a possible inhibitory role of E_2 in hepatic IGF-I mRNA synthesis. Such a role for E_2 in somatic growth inhibition

may help explain the observed shift from somatic growth to gonadal growth during reproductive maturation in fish. An estrogen response element exists on the rainbow trout GH gene (Yang, 1997) and E_2 is known to increase plasma GH levels in fish, which in turn stimulates ovarian IGF-I gene expression (Holloway, 1997; Holloway, 2000; Zou, 1997). Ovarian IGF-I has been implicated in oocyte maturation in several fish species (Negatu, 1998; Kagawa, 1995; Weber, 2000). The effect of the increased GH levels on hepatic IGF-I gene expression was not examined in those studies. However, the observed decrease in hepatic IGF-I mRNA level in response to E_2 in the present study in conjunction with the increase in plasma GH elicited by E_2 observed by other researchers suggests that, in the presence of E_2, GH may preferentially play a role in fish reproduction rather than in somatic growth. The mechanism by which GH selectively increases ovarian versus hepatic IGF-I synthesis is not known. In mammals, IGF-I gene expression appears to be tissue-specific. Increased uterine IGF-I expression in response to E_2 is direct and not mediated by GH in rats (cf. Murphy, 1991; Murphy, 1987). It is not clear how E_2 inhibits GH induced hepatic IGF-I gene expression while up-regulating uterine IGF-I gene expression. However, differences in IGF-I gene induction are most likely due to differences in the transcriptional elements available in the individual tissues. Murphy (1991) postulated that since E_2 receptors exist in both tissues, other tissue-specific factors such as differences in IGF-I gene promoters or an estrogen-responsive promoter may exist on the IGF-I gene. More recently, two distinct estrogen receptor (ER-α, ER-β) subtypes in mammals (Mosselman, 1996) and three (ER-α, ER-β, ER-γ) in fish (Hawkins, 2000) have been discovered. These distinct ER subtypes have different tissue distribution, affinities for E_2 and separate E_2 mediated effects, leading to

speculation that these multiple estrogen receptor subtypes are involved the differing responses of target tissues to E_2 (Juul, 2001). It is clear that a complex relationship exists between IGF-I and E_2 with differences not only between fish and mammals, but also differences in individual tissue types within an organism. Additional research is necessary to understand this complex relationship in fish, and also to understand how the physiological state, developmental or reproductive stage, and environmental factors influence the interaction of these hormones.

In conclusion, we used quantitative real-time (TaqMan) technology to quantify hepatic IGF-I mRNA in fish injected with endogenous hormones (GH, T_3, E_2, insulin) known to regulate growth in fish. A TaqMan assay was optimized and a cloned sheepshead minnow IGF-I mRNA sense strand was used to construct a standard curve. Hepatic IGF-I mRNA levels increased in fish injected with GH, T_3 and insulin, peaking at 12 h post-injection. The most significant effect was in fish injected with E_2, in which IGF-I mRNA levels decreased at 8 h and 12 h post-injection and gradually increased at 24 h. These results support previous research in which GH, T_3 and insulin increased the IGF-I mRNA concentrations in fish liver. Previous work has shown that the individual hormones act through different pathways to increase IGF-I mRNA levels in the liver. Our research shows that E_2, at least in pharmacological doses, has the opposite effect by decreasing hepatic IGF-I mRNA levels in the sheepshead minnow. Further research is warranted to determine whether the suppressive effect of E_2 on hepatic IGF-I mRNA levels may have consequences to wild fish living in waters contaminated with estrogenic chemicals.

4. EFFECTS ON GROWTH AND HEPATIC IGF-I mRNA OF SHEEPSHEAD MINNOWS EXPOSED TO ESTRADIOL AND CHLORPYRIFOS

INTRODUCTION

The threat of widespread distribution and persistence of endocrine-disrupting chemicals (EDCs) in the environment and the potential for serious effects in human, fish and wildlife populations has warranted a Federal research strategy (National Science and Technology Council report, 1996) to assess environmental effects of these anthropogenic chemicals. Considerable attention has been directed toward pollutants that may mimic estrogens (Colborn, 1995; Colborn, 1996; Stone, 1995), are linked to developmental and reproductive abnormalities in wildlife (Fry, 1995; Guillette, 1994) and alterations in gene expression in fish (Denslow, 2001).

While concern over environmental estrogens and their effects on vertebrate reproduction and development is important, little attention has been directed toward other equally important endocrine systems that may also be affected by anthropogenic chemicals. Chronic exposure to EDCs may inhibit growth in fish. Vertebrate growth is primarily regulated by an endocrine pathway involving the brain neuroendocrine system, pituitary growth hormone (GH), and insulin-like growth factors (IGFs), as well as other chemical and environmental factors. Insulin-like growth factors are mitogenic peptide hormones that play an important role in virtually every organ system (cf. Van Wyk, 1991). The liver, in response to growth hormone (GH) as well as other hormones, is the major source of plasma IGF-I (Daughaday, 1989), and in coho salmon (*Oncorhynchus kisutch*) the

liver contains the highest IGF-I mRNA levels of any tissue tested (Duan, 1993). In addition, evidence implicating gonadal steroids in somatic growth regulation is mounting. Chronic exposure to estradiol inhibits somatic growth in rats (Borski, 1996; Murphy, 1988), while chronic exposure to dihydro-testosterone enhances growth (Borski, 1996). The growth inhibition appears to be regulated at the level of hepatic insulin-like growth factor (IGF-I) gene expression. Hepatic IGF-I mRNA expression was significantly inhibited in rats administered estradiol (Borski, 1996; Murphy, 1988).

Evidence exists for growth inhibition in fish associated with exposure to estrogenic chemicals. Reduced somatic growth was reported in channel catfish (*Ictalurus punctatus*) fed the synthetic estrogen diethylstilbestrol (Bulkley, 1972), in rainbow trout (*O. mykiss*) fed 17β-estradiol (Sower, 1983; Johnstone, 1978; Ostrowski, 1986) and in rainbow trout exposed to alkylphenolic compounds (Ashfield, 1998). These compounds are all known xenoestrogens. Possible mechanisms for growth inhibition by estrogenic chemicals have not been examined in fish. Little is known about the regulation of somatic growth by gonadal steroids in fish; however, during reproductive maturation elevated levels of gonadal steroids coincide with diminished somatic growth as energy is diverted toward gonadal growth.

Some pesticides inhibit growth in fish. For example, chlorpyrifos (Dursban), an organophosphate, reduced growth in the sheepshead minnow (SHM) (*Cyprinodon variegatus*) after chronic exposure (Cripe, 1986). Chlorpyrifos was one of the most widely used insecticides in agriculture and households until it was banned by the EPA in the summer of 2000. Chlorpyrifos, and other organophosphate pesticides,

interfere with normal nervous system function by inhibiting acetyl-cholinesterase activity (Chanda, 1996). Although not known to be estrogenic, chlorpyrifos has been implicated as an EDC in mammals by altering reproductive development and stimulating neuroendocrine GnRH mRNA *in vitro* (Gore, 2001). The mechanism by which chlorpyrifos inhibits fish growth is unknown, however it is possible that chlorpyrifos influences hormones that regulate growth or energy utilization.

Our current understanding of the ability of environmental contaminants to mimic endogenous hormones reflects a need to explore the cellular and molecular mechanisms by which these endocrine disrupting chemicals affect physiological processes. Basic research of the mechanisms of regulation of somatic growth and the somatotropic axis in response to estrogens and other xenobiotics in fish is required to help define areas of possible endocrine disruption. In addition, information as to the possible mode of action of pesticides such as chlorpyrifos on growth inhibition in fish would aid in risk assessment analysis of this pesticide and others with similar structure. Research to determine whether hepatic IGF-I mRNA synthesis is altered in response to estrogenic compounds or pesticides would constitute an initial step to understand these mechanisms. To this end, we conducted a long-term flow-through aquatic exposure (18 weeks) beginning with newly-hatched SHM fry to determine whether exposure to estradiol or chlorpyrifos would inhibit growth. Sheepshead minnows were chosen because they are a commonly used species in aquatic toxicity testing. At the conclusion of the aquatic exposure, hepatic IGF-I mRNA was measured using a quantitative real-time (TaqMan) PCR assay.

MATERIALS AND METHODS

Chemical Exposure

Newly hatched (within 36 h post-hatch) SHM fry were removed from brood tanks a few at a time and were randomly placed into glass bowls (10 cm diameter X 5 cm high) until each bowl contained 50 fry. The fry from each bowl were then placed into randomly chosen chambers situated within 135 L exposure tanks. The chambers were made of 10 cm glass petri dishes to which Nytex sleeves were affixed with silicon sealant. The Nytex sleeves were sufficiently high to be above the water level in the tanks to contain the fry and to prevent dispersal of food. The water depth within the tanks and chambers was 15 cm. Each 135 L exposure tank contained 4 chambers for a total of 200 fry in each exposure tank. An air stone with gentle aeration was placed near the chambers to provide water movement through the chambers. A previous observation using non-toxic dye showed that water freely flowed through the chambers. After 7 days, the fry were released from the chambers into the exposure tanks. The water depth within the exposure tanks was maintained at 15 cm until week 3, at which time the water level was increased to full volume (33 cm deep) and maintained at that level until termination of the experiment.

The SHM fry were exposed to either 17β-estradiol (E_2) or chlorpyrifos using triethylene glycol (TEG) as the carrier solvent or to TEG alone using a six-cell dosing apparatus. The chemicals were delivered by two-channel, Hamilton Microlab 500B/C dispensers fitted with 100 ul Hamilton syringes. With each cycle of the dosing apparatus 50 ul of a stock chemical solution was injected into 1 L of filtered, salinity-controlled seawater and delivered to the exposure tanks. The dosing

apparatus maintained a water flow rate of 18 L per hour. Two concentrations of E_2 (20 and 100 ng/L), three concentrations of chlorpyrifos (12.5, 25 and 50 µg/L), and the TEG carrier control solvent were tested. The exposures were replicated. Seawater in the exposure tanks was maintained at 29° - 30°C with a constant photoperiod of 16L:8D. Salinity (18-21PPT) and temperature were monitored daily while dissolved oxygen (mean 6.1, range 5.2 - 7.4) and pH (range 7.5 - 8.1) were measured once per week. The fish were never crowded during the duration of this experiment and ammonia was not an issue because this was a flow-through system.

Feeding

Fish were initially fed a measured diet of *Artemia* nauplii three times per day. At week three, a measured amount (to the nearest .01g) of finely ground Tetramin flake food replaced one of the *Artemia* feedings each day. At week 6, *Artemia* feedings were discontinued and fish were fed a measured diet of finely ground Tetramin flake food twice per day. The amount of food was increased as the fish grew larger so that the fish were fed to satiation at each feeding. Food was not a limiting factor. The fish in the test aquaria were fed in random order at each feeding and all tanks received the same amount of food.

Fish Sampling

Fish were sampled weekly until week 10 and bi-weekly until test termination at week 18. At each sampling 10 fish were collected from each tank, anesthetized with a lethal dose of buffered MS-222 (50 mg/L MS-222 plus 50 mg/L NHCO3) and weighed to the nearest 0.01

mg. The fish were placed on a light table and the standard length was measured to the nearest 0.01 mm using electronic digital calipers. Livers were collected from each fish at week 18. Sex was determined by secondary sex characteristics (black bar on caudal fin, blue coloration on the head and orange or yellow anal fins) evident in males and by cursory examination of the gonads. Livers were placed into cryovials, frozen in liquid nitrogen and stored at -70 ° C until processed.

Chemical Analysis

Water from each tank was sampled to measure the concentration of the chemicals. Water samples were taken 72 hours after the start of the chemical flow, 48 hours after adding the test fry and once per week thereafter. One L samples were taken from each tank using a siphon. Water was taken from the center of the tank at mid-level and siphoned into clean, 1L glass containers.

Estradiol Determination

Water samples were filtered through E-18 solid phase extraction tubes (Supelco), washed with distilled water and the E_2 eluted from the column with 5.0 mL methanol (MeOH). Samples were evaporated to dryness under nitrogen in an N-Vap (Oranomation Assoc., Inc.) at 55-60°C. The water samples collected from the TEG control tanks were injected with 50 ul of the stock solutions of each estradiol concentration to equal the exposure concentrations to serve as controls and were extracted in the same manner as the E_2 exposure tank samples. The samples were stored at -20°C until analyzed. The samples were analyzed for E_2 concentration by Enzyme-linked

Immunoassay (EIA) using an Estradiol EIA kit (Oxford Biomedical Research, Inc.) following the manufacturer's protocol.

Chlorpyrifos Determination

Water samples were analyzed by Gas Chromatography with Electron Capture Detector (GC-ECD, Hewlett Packard 6890) following U.S. EPA guidelines (Marcovich, U.S.EPA SOP # CHE) (U.S. EPA, Methods SW-846-3500B; U.S. EPA Guidelines). Duplicate water samples were extracted with hexane, followed by GC-ECD analysis based on a three point calibration curve and extraction verification with the use of pentachloronitrophenol (PCNB) as a surrogate. Samples spiked with a known amount of chlorpyrifos were also analyzed to determine extraction efficiency.

RNA Preparation

Frozen livers were weighed and the total weight was recorded. Total RNA was isolated using the method previously described (Chapter 2). Genomic DNA was removed using a kit (DNA-Free, Ambion, Inc.) following the manufacturer's protocol. The purity and concentration of the RNA sample was determined by measuring the absorbance at A260 and A280 and the quality was checked by denaturing-agarose gel electrophoresis and ethidium bromide staining. The RNA was stored at -70° C until assayed.

Quantitative Real-Time RT-PCR

Quantitative (TaqMan) RT-PCR analyses were performed in triplicate for each sample and standard. A standard curve was constructed using sense strand cRNA at each concentration as described in Chapter 3. A single-step quantitative RT-PCR Core Reagent Kit (Stratagene) was used. The single-step procedure allows reverse transcription (RT) of the mRNA and subsequent PCR amplification of the resulting cDNA in the same reaction tube. The real-time PCR was performed in MicroAmp Optical 96-well reaction plates (ABI Prism). A standard curve was included on each plate and all samples were randomly assigned to each reaction plate. The reaction mixtures (25 ul total volume) for the IGF-I assays consisted of the core components according to the manufacturer's instructions plus 5.5 mM $MgCl_2$, 200 nM probe, 300 nM forward primer, 900 nM reverse primer, 40 units of Rnase-Out ribonuclease inhibitor (Gibco), 1.5 ul 0.1 mM dithiothreitol (DTT) and 200 ng of total RNA or cRNA standard. The RT was run at 48°C for 55 min, 95°C for 10 min followed by the PCR of 40 cycles of 95°C 15 sec, 60°C 1 min in an ABI PRISM 5700 Sequence Detection System. The increasing fluorescence signals were measured during each PCR cycle and amplification plots were constructed by the ABI PRISM.

To minimize variability in RNA quantitation and integrity, the samples were normalized to 18S ribosomal RNA.

Statistical Analysis

Chi-square analysis was used to analyze sex ratio differences. Two-way analysis of variance comparing week versus weight or length

for each treatment was used to analyze the overall differences in growth compared to controls. A Dunnet's post-test was then performed to compare each treatment to controls. One-way ANOVA with Tukey's multiple comparison post-test was used to compare treatments within a single week and to compare IGF-I mRNA.

RESULTS

The average measured concentrations of estradiol and chlorpyrifos, taken weekly during the 18 week flow-through exposures are presented in Table 4.1.

Table 4.1

Nominal and mean measured concentrations (± standard deviations) of 17β-estradiol and chlorpyrifos from water samples taken weekly during 18 week flow-through exposures of sheepshead minnows (*Cyprinodon variegatus*)

Chemical	Nominal Concentration	Measured Concentration (± S.D.)
Estradiol	20 ng/L	21.0 ng/L (±8.8)
	100 ng/L	112.5 ng/L (±39.7)
Chlorpyrifos	12.5 ug/L	6.5 ug/L (±3.7)
	25 ug/L	13.3 ug/L (±6.4)
	50 ug/L	24.3 ug/L (±13.0)

Fish Growth

Fish exposed to chlorpyrifos were significantly smaller than control fish in both length and weight (figure 4.1). Significant differences in fish exposed to the highest dose of chlorpyrifos were

evident beginning at week one for both length ($P < 0.001$) and weight ($P < 0.01$). Fish exposed to 12.5 ($P < 0.05$) and 25 ($P < 0.01$) ug/L chlorpyrifos were significantly smaller in length beginning at week one. The fish exposed to chlorpyrifos at 25 ug/L were different in weight ($P < 0.01$) compared to controls beginning at week four. Chlorpyrifos exposure inhibited growth in a dose-dependent manner.

Fish exposed to the higher dose of estradiol weighed more than control fish only at weeks 16 and 18 ($P < 0.05$) and were longer than controls only at week 18 ($P < 0.05$) (figure 4.1). The fish exposed to 20 ug/L estradiol were not significantly different from controls.

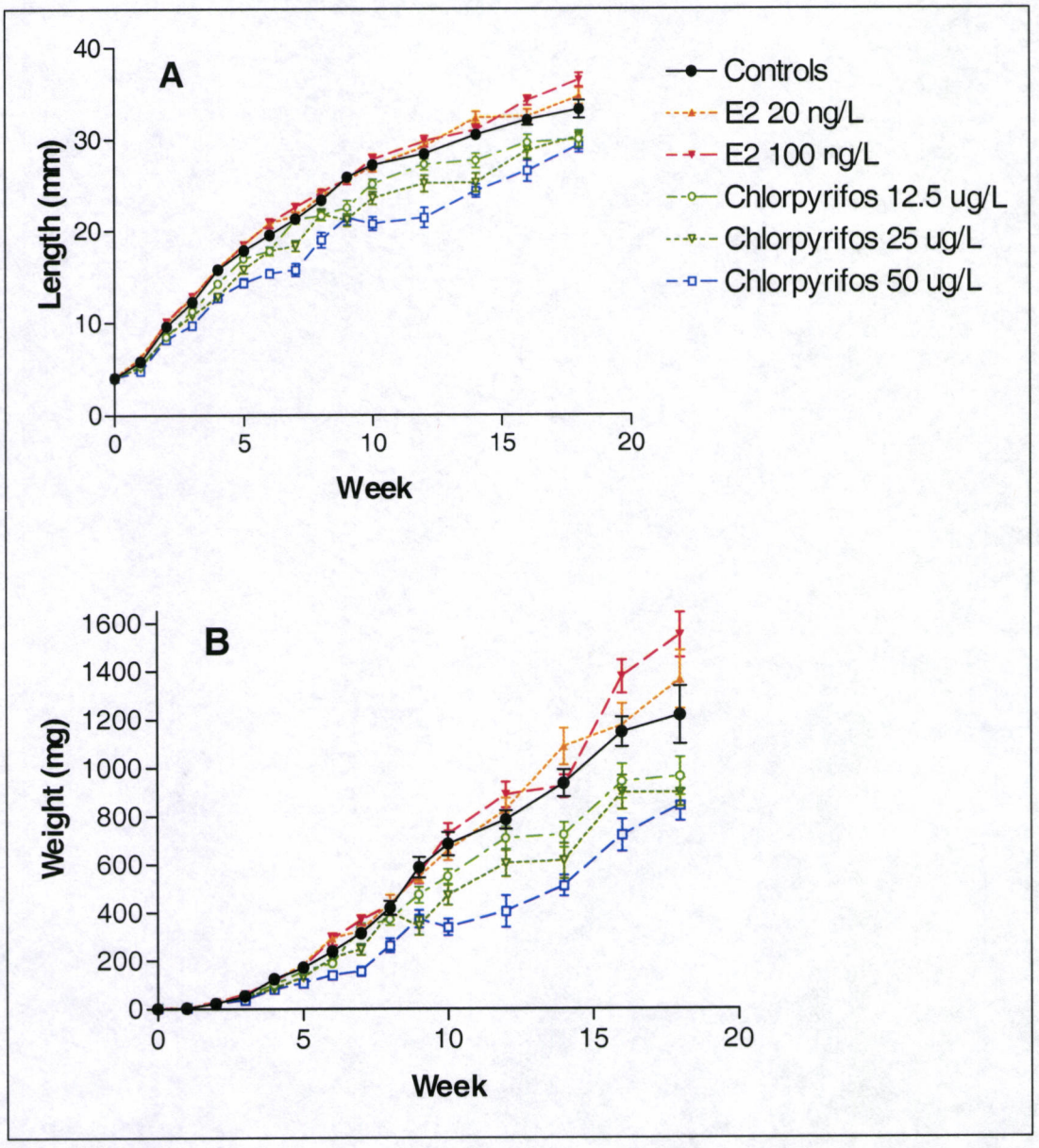

Figure 4.1. Mean (N=20) weights (mg) (A) and lengths (mm) (B) (± standard deviations) of sheepshead minnows sampled weekly until week 10 and bi-weekly until week 18.

The sex ratios for the fish sampled at week 18 are presented in Table 4.2. A total of 20 fish was sampled from each treatment group. Sex was determined only at the last sampling. There was no signficant difference in sex ratios based on Chi-Square analysis. The

hepatosomatic index was calculated for all fish sampled at week 18, but no significant differences were found using ANOVA.

Table 4.2. Sex ratios (determined by secondary sex characteristics) at week 18 for sheepshead minnows exposed to estradiol (10 and 100 ng/L), chlorpyrifos (12.5, 25, and 50 ug/L) and TEG controls.

Treatment	Males	Females
TEG-Controls	9	11
E2 20 ng/L	6	14
E2 100 ng/L	6	14
Chlorpyrifos 12.5 ug/L	8	12
Chlorpyrifos 25 ug/L	8	12
Chlorpyrifos 100 ug/L	10	10

Quantitative Real-Time PCR

Ten-fold dilutions of SHM IGF-I sense strand produced a linear standard curve (data not shown). All of the sample amplification plots fell within the range of the standard curve. The inter-assay variability was 11% and no-template control wells did not generate a fluorescent signal, indicating no cross-well contamination. Samples were normalized to the Ct values of the 18S ribosomal assay as previously described (Chapter 3). Samples with greater than 15% variability among the replicates were re-assayed. Figure 4.2 presents the normalized results of the quantitative real-time PCR assay for IGF-I mRNA. No significant differences were found between treatment groups using one-way ANOVA. A correlation analysis of IGF-I mRNA vs. length or weight revealted no significant correlations.

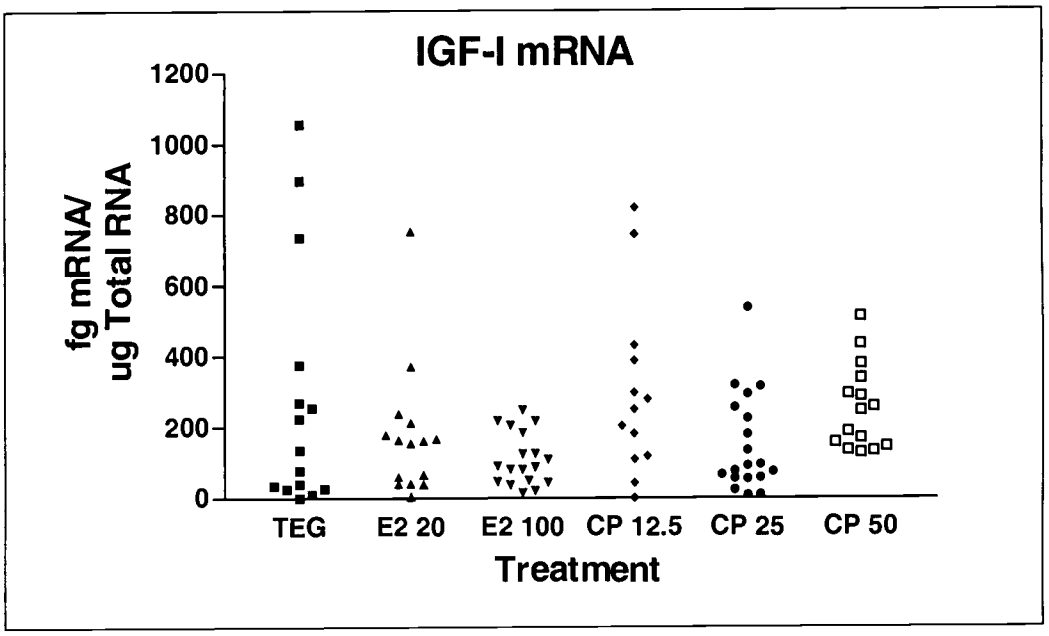

Figure 4.2. Quantity (fg IGF-I mRNA/ug total RNA) of hepatic IGF-I mRNA for sheepshead minnows exposed to TEG controls (TEG), chlorpyrifos at 12.5, 25, or 50 ug/L (CP 12.5, CP 25, CP 50) or 17β-estradiol at 20 or 100 ng/L (E2 20, E2 100). Each data point represents the mean of triplicate assays for one fish.

DISCUSSION

Chlorpyrifos concentrations were selected to be environmentally relevant. A previous study (Cripe, 1986) concluded that a measured concentration of 3.0 μg /L of chlorpyrifos was sufficient to cause a significant decrease in growth in sheepshead minnows. Nominal concentrations sufficiently high to allow for an expected drop in mean measured concentration were chosen.

The estradiol concentrations chosen were within the range of concentrations measured in natural water and therefore environmentally relevant (Desbrow, 1997; Shore, 1993; Tabak, 1981). The

lower estradiol concentration (20 ng/L) was chosen because it is below the threshold concentration required to induce vitellogenin synthesis in male sheepshead minnows (Folmar, 2000), while the 100 ng/L concentration was chosen because it elicited the vitellogenic response (Denslow, 2001).

Sheepshead minnows exposed to the 100 ng/L dose of E_2 grew slightly more than controls both in weight and length. Increased growth was significant during the last two sampling times (week 16 and 18). This is in contrast to several other investigations in which E_2 or estrogenic chemicals reduced growth in several fish species. Previous studies exposing all male SHM to 100 ng/L of E_2 using the identical flow-through system revealed that the fish had significant vitellogenin levels (Folmar, 2000) indicating that E_2 was entering the fish. For example, juvenile rainbow trout exposed to environmentally relevant (1, 10, 50 ug/L) aquatic concentrations of alkylphenols (nonylphenol, octylphenol, nonylphenol diethoxylate and nonylphenol mono-carboxylic acid) from hatching until 22 days, then reared in clean water until day 108 were significantly smaller than controls (Ashfield, 1998). Although alkylphenols are classified as xenoestrogens because they activate the estrogen receptor, these chemicals may influence growth directly by acting elsewhere in the growth axis. Most studies of the effects of estrogenic chemicals on the growth of fish administered the chemical dose by feeding. Juvenile rainbow trout fed a diet containing 30 mg E_2/Kg food for 76 days had significantly reduced weight gain and high mortality compared to controls (Herman, 1988). Rainbow trout fry fed a ration containing 5ug E_2/g food for 175 days (Sower, 1983) had significantly lower weight gains and higher mortality than control fish. Likewise, rainbow trout fed a

diet containing 2.0 mg E_2/kg food for 10 weeks exhibited decreased body weight gain compared to controls (Ostrowski, 1986). Similar results were found in brook trout (*Salvelinus fontinalis*) fed 20 ug E_2/g food (Johnstone, 1978). Channel catfish (*Ictalurus punctatus*) fingerlings fed a diet containing 50 mg E_2/kg food for 12 weeks showed significant weight reduction compared to controls, but those fed a diet of 5 mg E_2/kg food did not (Gannam, 1991). Moreover, sexually immature channel catfish (Bulkley, 1972) fed diethylstilbestrol (DES, a synthetic estrogen) at a concentration of .05, .5, and 5.0 mg DES/kg food for 25 days had significantly lower weight gain than controls as well as renal hypertrophy and appetite suppression at the higher doses. A sex-dependent effect was observed in tilapia (*Oreochromis mossambicus*) yolk-sac fry fed ethynylestradiol (EE_2, a synthetic estrogen) at 0.05 mg EE_2/g feed for 28 days followed by 8 months in clean water. Body weight gain in males was significantly reduced, while females gained significant body weight compared to controls. The sex ratio was also significantly skewed ($P<.01$) toward females with some intersex gonads also observed. In the same study, both male and female tilapia fed *o,p'*-DDD (an estrogenic pesticide) at a concentration of 0.8 mg /kg of food had significantly reduced growth rates. Conversely, in the same experiment, only females had increased weight gain compared to controls when fed 1.6 mg *o,p'*-DDD /kg of food (Meredith, 1999).

Examples of estrogenic growth enhancement include elvers (*Anguilla anguilla*), which had a significant weight gain when fed a diet of 15 mg/kg E_2 for 45 days (Degani, 1986). Common carp (*Cyprinus carpio*) fed a diet incorporated with 800 ppm E_2 for the first 30 days post hatch and then fed a hormone-free diet for 10 months grew better

than control fish, although the authors attribute some of this increased growth to stocking density. A sex ratio skewed toward females was also observed in that study (Nagaraj, 1988). Juvenile coho salmon (*Oncorhynchus kisutch*) fed a diet of 2.5 mg/kg E_2 for 14 weeks had greater weight gain than controls (Yu, 1979). Meanwhile, plaice (*Pleuronectes platessa*) fed 1.2 mg/kg DES grew better than controls (Cowey, 1973). The differences between the reduction in growth in channel catfish fed DES (Bulkley, 1972) and an increase in growth of plaice fed DES (Cowey, 1973) was attributed to a species-specific growth response (Donaldson, 1979). Early work in the guppy (*Lebestes reticulatus*) and platyfish (*Platypoecilus maculatus*) fed estrogenic chemicals showed increased growth in males only (cf. Donaldson, 1979). Donaldson (1979) points out that the males of the guppy and platyfish are normally smaller than females and the increased growth may be a feminizing effect. Higgs et al. (1982) also maintains that differences in growth response to E_2 and other steroid hormones may be attributed to the species, size and nutritional status of the fish as well as the dietary dose, timing of dose and other environmental factors (Higgs, 1982).

Most early studies of the effects of estradiol on fish growth were conducted in an attempt to find ways in increase fish growth. Most of the attempts proved unsuccessful, and as the previous examples illustrate, most resulted in growth suppression. The dose of the estrogenic chemical may have been a factor in the observed growth reductions. It is difficult to determine the actual dose of a chemical administered through feeding. Pharmacological levels of estrogenic chemicals may have inhibited growth by inducing negative side effects. Liver (hepatocyte hypertrophy and hyperplasia) and kidney (hypertrophy)

anomalies as well as edema were observed in several studies (Bulkley, 1972; Gannam, 1991; Herman, 1988), possibly due in part to accumulation of vitellogenin in the blood (Herman, 1988; Folmar, 2001b). Several studies reported high mortality in fish fed estrogenic chemicals (Sower, 1983; Herman, 1988). We exposed fish to estradiol by aquatic exposure, which is a more likely route of exposure to wild fish. In the present study, we chose estradiol concentrations to be both below (20 ng/L) the level found to elicit an estrogenic response (induction of vitellogenin) and at the lower limit at which an estrogenic response has been observed (100 ng/L) in this species. No mortalities occurred in the present study, and no obvious organ anomalies or edema were observed. In addition, the hepato-somatic index for estradiol exposed fish was not different from control fish, indicating no liver hypertrophy or hyperplasia. It is possible that estradiol did not inhibit growth in this study because of the low concentration to which the fish were exposed. The effect of E_2 concentration was demonstrated in channel catfish, which had growth inhibition when fed a high dose (50 mg E_2/kg food) and no growth inhibition when fed a low dose (5 mg E_2/kg food) of estradiol (Gannam, 1991).

The increased growth of SHM exposed to the higher dose of E_2 observed in the present study may also be due to species differences, as indicated by Donaldson (1979) and Higgs (1982). Males and female SHM are generally equal in size with some sources reporting that females are slightly larger (cf. Jones, 1978). In most mammals, females tend to be smaller than males and there is speculation that estradiol contributes to the smaller size of females (Borski, 1996) as demonstrated in rodents (Borski, 1996; Murphy, 1988). Perhaps growth in fish with sexually dimorphic size differences are more likely

to be influenced by E_2 exposure, as was the case with guppy and platyfish where growth only in males was increased (cf. Donaldson, 1979).

Field investigations of the concentration of chlorpyrifos in aquatic ecosystems has indicated that the concentrations tested in this study were environmentally relevant, especially after episodes of heavy runoff from agricultural fields (Racke, 1993). The concentrations of chlorpyrifos tested in this study were well below the reported 96-hour LC_{50} (the concentration estimated to be lethal to 50% of the test animals after 96 hours) of 136 ug/L (EPA unpublished data) and no mortalities were observed. Chlorpyrifos use was banned in 2000 because of its toxic effect on children (EPA Administrator announcement, June, 2000). Until then, it was commonly used in agriculture and in homes. One of the most important uses for chlorpyrifos was in mosquito control, where it was dispersed into water bodies. Under field conditions, chlorpyrifos has a short half life in water and tends to volatilize or partition into sediment. However, frequent direct or indirect entry (runoff, spray drift) of chlorpyrifos into aquatic systems can result in frequent exposure of fish to low, sublethal concentrations of chlorpyrifos (Racke, 1993). However, a dose-response inhibition of growth, both length and weight, was evident at the concentrations tested in the present study. The reduction in growth observed in SHM exposed to chlorpyrifos is in agreement with previous investigations in which SHM embryos exposed to chlorpyrifos by aquatic exposure for 28 days had significant growth reduction at measured concentrations of 3.0 ug/L (Cripe, 1986). Newly hatched fathead minnow (*Pimephales promelas*) larvae had significantly reduced growth after 30 days of aquatic exposure to 2.68 ug/L of

chlorpyrifos (Jarvinen, 1983). Jarvinen, *et al* (1983) also reported high mortalities and significant deformities at 2.68 ug/L in the fathead minnow, suggesting that the fathead minnow may be more sensitive to toxic effects of chlorpyrifos than SHM. Significant growth reduction was also reported for California grunion (*Leuresthes tenuis*) fry exposed to 1.0 ug/L for 25 days (Goodman, 1985) and in gulf toadfish (*Opsanus beta*) fry exposed to 3.7 ug/L for 49 days (Hansen, 1986).

The chlorpyrifos-exposed fish exhibited behavioral differences compared to control fish. SHM are active fish and aggressive feeders. The fish exposed to all concentrations of chlorpyrifos were lethargic and did not feed aggressively. The reduced food consumption observed in the chlorpyrifos exposed fish was probably an important factor in the decreased growth of those fish. Previous studies of behavioral responses of rainbow trout (Little, 1990) and Atlantic salmon (Symons, 1973) to organophosphate pesticides have documented impaired feeding behavior in those species. One of the main targets of toxicity for organophosphate pesticides such as chlorpyrifos is acetylcholinersterase (AChE), an enzyme important in the cholinergic systems of the central and peripheral nervous system. Inhibition of AChE impairs nerve impulse transmission in SHM (Coppage, 1972) and in other fish (Weiss, 1961; Pavlov, 1992). The cholinergic system in the fish brain has been implicated in controlling feeding behavior in fish. Bream (*Abramis brama*) exposed to sublethal concentrations of an organophosphate pesticide (phosphoric acid) exhibited decreased food consumption and inhibited brain AChE activity (Pavlov, 1992).

This study was undertaken primarily to determine whether the growth inhibition observed in some fish species exposed to estrogenic substances was due to decreased hepatic IGF-I mRNA. I found no

significant differences in hepatic IGF-I mRNA from fish exposed to E_2 as measured by quantitative real-time PCR. In contrast, the hepatic IGF-I mRNA levels in fish injected with pharmacological levels of E_2 (2 injections of 5 ug/g body weight) were lower than controls at 12 hours and remained lower after 24 hours (Chapter 2). Moreover, evidence exists in rats that chronic exposure to physiological levels of E_2 results in growth inhibition and reduced hepatic IGF-I mRNA (Borski, 1996; Murphy, 1988). The lack of growth inhibition by aquatic exposure to E_2 along with the normal levels of hepatic mRNA may be due to concentrations too low to elicit an effect in this species. No previous investigations of the growth regulating effects of E_2 on fish reported either plasma IGF-I or hepatic IGF-I mRNA levels. Neither plasma IGF-I nor E_2 were measured in this study.

While no previous studies on fish have measured IGF-I in response to E_2 treatment, some investigators have found that E_2 increases plasma GH. Immature rainbow trout (Holloway, 1997; Holloway, 2000) and female goldfish (Zou, 1997) implanted with E_2 pellets had increased plasma GH levels. Plasma GH also increases in sexually maturing fish (Bjornsson, 1994). The GH increase may play a role in oocyte maturation, as GH-stimulated ovarian IGF-I has been implicated in oocyte maturation in gulf killifish (*Fundulus heteroclitus*) (Negatu, 1998) and *in vitro* germinal vesicle breakdown of red sea-bream (Kagawa, 1995) and striped bass (*Morone saxatilis*) oocytes (Weber, 2000). These findings suggest that the stimulation of GH by E_2 plays a role in reproduction rather than in somatic growth. Niether hepatic IGF-I mRNA nor plasma levels of IGF-I were examined in those studies.

Although SHM exposed to chlorpyrifos were significantly smaller than controls, the growth inhibition does not appear related to decreased hepatic IGF-I mRNA levels. Although these fish had reduced food intake, they were not starved and therefore did not exhibit decreased hepatic IGF-I mRNA and circulating IGF-I levels along with growth reduction associated with starvation in fish (Duan, 1993). It is likely that the reduced growth seen in the chlorpyrifos treated fish was due to the general toxicity of chlorpyrifos manifested in depressed activity and food consumption.

In conclusion, we tested the hypothesis that exposure to estrogenic chemicals and the organophosphate pesticide chlorpyrifos would decrease growth in sheepshead minnows and that the cause of the growth suppression would be the reduction of hepatic IGF-I synthesis. Newly hatched sheepshead minnow fry were exposed to E2 (20 and 100 ng/L) and chlorpyrifos (12.5, 25 and 50 ug/L) for 18 weeks. Although growth of fish exposed to chlorpyrifos was reduced in a dose-dependent manner, fish exposed to the higher concentration of E_2 grew larger than controls during the last two weeks of the experiment. The results of the quantitative real-time PCR assay indicate that the hepatic IGF-I mRNA levels of the fish exposed to E2 and chlorpyrifos were not significantly lower than in control fish. This is in contrast to fish injected with E_2 in a previous experiment in which IGF-I mRNA was lower than controls (Chapter 3). It is possible that the concentration of E_2 to which the fish were exposed was below the threshold level that would elicit a growth inhibiting response in this species. The inhibited growth of the chlorpyrifos exposed fish may have been due to depressed feeding activity and a general toxic effect of the chemical. Further research using a higher concentration

of E_2 is necessary to determine whether a threshold for growth inhibition exists for this species.

5. DIFFERENTIAL DISPLAY ANALYSIS OF LIVER mRNA FROM FISH TREATED WITH NATURAL HORMONES AND A PESTICIDE

INTRODUCTION

Most physiological processes involve the induction or suppression of specific genes. Recent studies where fish and other wildlife were exposed to anthropogenic chemicals have demonstrated that such exposure regulates certain genes. For example, exposure of fish to estrogenic chemicals induces genes regulating such reproductive processes as eggshell protein and vitellogenin synthesis (Denslow, 2001; Denslow, 2001). Endogenous hormones play a major role in regulating gene expression, and peptide hormones are themselves products of gene induction. For example, pituitary growth hormone (GH) regulates the synthesis of insulin-like growth factor I (IGF-I) in the liver by binding to and activating GH receptors in the cell membrane. This activation initiates a signal transduction pathway resulting in the expression of the IGF-I gene (cf. Carter-Su, 1996). While the GH/IGF-I axis is the major endocrine axis controlling growth, many other factors and genes are involved. Some evidence suggests that estradiol may contribute to the regulation of somatic growth in fish by inhibiting the expression of IGF-I (Chapter 3). Identification of genes regulated by endogenous hormones and anthropogenic chemicals would provide better understanding of physiological responses at the molecular level. Such information would add to the basic knowledge of the endocrine system, as well as provide clearer insight into the actions of anthropogenic chemicals on fish. To this end, I optimized a method using fluorescence differential display technology, to identify differentially expressed genes in fish treated with endogenous

hormones known to regulate growth (GH, T_3, E_2) and a growth-inhibiting pesticide (chlorpyrifos).

MATERIALS AND METHODS

The RNAspectra Green Fluorescent mRNA Differential Display System kit (GenHunter Corp. Nashville, TN) was used for all RT-PCR reactions. Unless otherwise stated, all materials were included in the kit.

Experimental Treatments and Sampling

Fish were treated either by injection or by flow-through aquatic exposure as described in chapters 2 and 3 respectively. Fish treated by flow-through aquatic exposure to the following measured chemical concentrations for 18 weeks: E_2 (112.5 ng/L ±39.7), chlorpyrifos (23.4 ug/L ±13.0). Control fish were exposed to triethylene glycol (TEG 50ul/L) carrier only. Fish treated by injection received either GH (10 ug/g body weight [BW]) or T_3 (1.0 ug/g BW). The fish injected with GH were sampled 6 hr after injection. Fish injected with T_3 were sampled 24 hr after injection. Fish were sampled as previously described (Chapters 3 and 4). Briefly, fish were placed into a lethal dose of tricaine methanesulfonate (MS222, 40 mg/L) buffered with sodium bicarbonate (50 mg/L). The blood was removed by severing the caudal peduncle and the livers were quickly removed, snap frozen in liquid nitrogen and stored at -70° C until processed. The sex of the fish was recorded.

Differential Display Analysis

Differential display (DD) technology was developed by Liang and Pardee (Liang, 1992) as a tool for identifying and cloning differentially expressed genes. Differential display technology involves the reverse transcription of mRNA with fluorescent labeled oligo-dT primers anchored to the beginning of the poly(A) tail, followed by a PCR reaction using a second short primer with an arbitrary sequence. The amplified cDNA products produced by the primer pair are run on a DNA sequencing gel where they are distributed by size. The gels are then scanned on a fluorescence imager. By analyzing mRNA from controls side-by-side with mRNA from treated animals, differences in gene expression can be identified. Visualization and identification of many mRNA species is possible by using different combinations of anchor primers and arbitrary primers. After identifying genes of interest on the gels, the corresponding gel bands are cut out, reamplified and sequenced for identification. Figure 5.1 depicts the DD RT-PCR procedure.

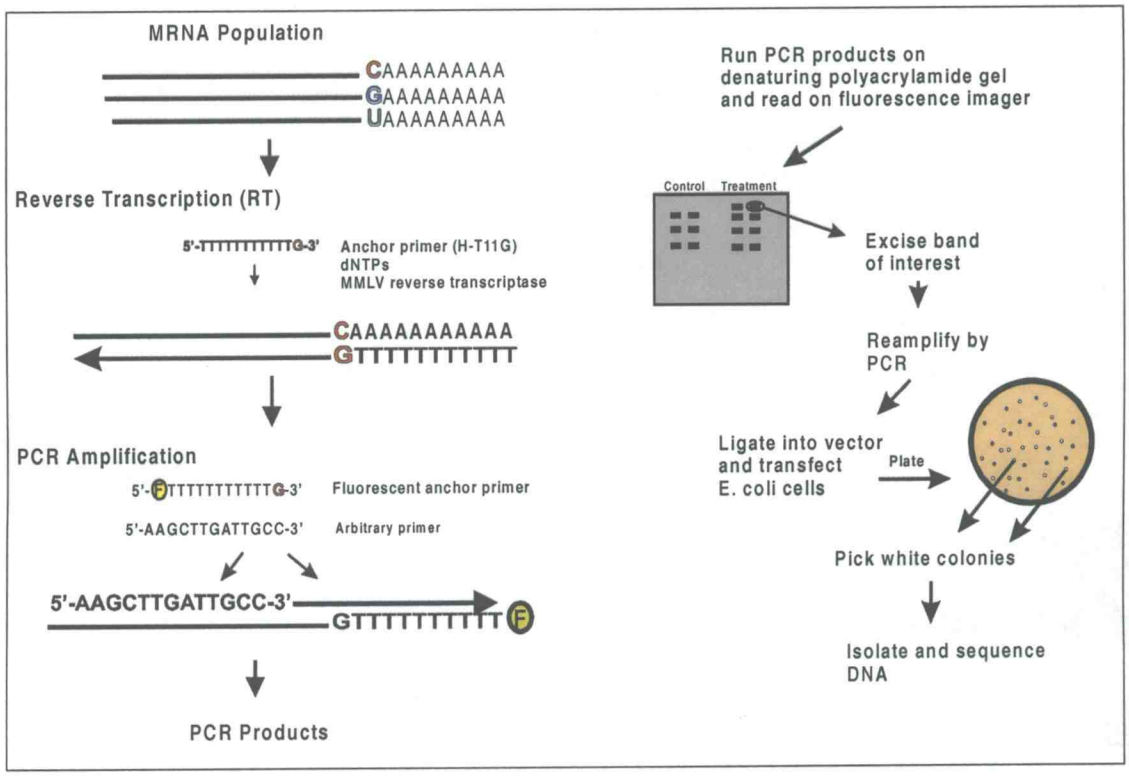

Figure 5.1. Schematic depiction of the differential display (DD) RT-PCR reaction and procedure for cloning differentially expressed bands.

Reverse Transcription (RT) of mRNA

Total RNA was prepared as previously described (Chapter 3). To avoid possible false positives, three samples from each treatment were prepared and run side-by-side on the gels. A separate RT reaction of each RNA sample was performed for each of the three anchor primers. RT reactions (20 μL final volume) were prepared for each RNA sample with the following components: 0.2 ug total RNA; 0.2 uM unlabeled anchor primer (H-T_{11}G, H-T_{11}C, or H-T_{11}A); 100 U MMLV reverse transcriptase, nuclease-free water, 5X RT buffer, deoxynucleotide (dNTP) mix; 40 U RNase inhibitor (RNase Out, Gibco). The RT reaction was carried out as described by the manufacturer.

PCR Reaction

PCR reactions were performed using the GenHunter RNAspectra protocol. A PCR reaction was prepared for each combination of anchor primer and arbitrary primer for each sample. The fluorescent labeled anchor primers were used in this reaction and the reaction mixtures were protected from light as much as possible. Table 5.1 lists the anchor primers and arbitrary primers used. The reaction mixture (20 ul total volume) contained 0.1 vol of the reverse transcription reaction, 0.1 vol 10X PCR buffer, 0.2 uM labeled anchor primer, 0.2 uM arbitrary primer (Gen Hunter), 1 uM dNTP mix, and 1 U AmpliTaq DNA polymerase (PE Applied Biosystems, Foster City, CA). The PCR reaction was run for 40 cycles with the following conditions: 94°C for 30 sec, 40°C for 2 min, 72°C for 60 sec; followed by a 5 min 72°C extension step.

Table 5.1
Primer pairs used in differential display RT-PCR of SHM liver RNA. Anchor primers anneal to the Poly-A tail of mRNA. The arbitrary primers are random sequences that anneal to complementary sequences along the mRNA . Arbitrary primers are designated by numbers (H-AP-1 through H-AP-80) assigned by the manufacturer (GenHunter).

Anchor Primer	Arbitrary Primer (13 bases in length)		
FH-T_{11}**G**	H-AP–6	H-AP-17	H-AP-19
	H-AP-20	H-AP-22	H-AP-23
	H-AP-24		
FH-T_{11}**C**	H-AP-3	H-AP-5	H-AP-21
	H-AP-57	H-AP-59	H-AP-60
FH-T_{11}**A**	H-AP-17	H-AP-18	H-AP-19
	H-AP-58	H-AP-62	H-AP-64

Gel Electrophoresis and Fluorescence Imaging

Each PCR reaction was mixed with 10 ul of DNA sequence loading dye (99% formamide, 1mM EDTA (pH 8.0), 0.009% xylene cyanole, 0.009% bromophenol blue), heated to 80°C for 2 min and then put on ice. Six µl of each sample were loaded onto a 6% denaturing polyacrylamide gel (PreMix Gel Solution for Manual Sequencing, BMA, Cambrex, Inc.) in 1% tris-borate EDTA (TBE) buffer. After all samples were loaded, the gel unit was covered with aluminum foil to protect the fluorescent dye from light. The gel was run at 60 watts constant power (voltage no more than 1700) for 2 to 3 h.

The gel was scanned in a Typhoon 8600 Fluorescence Imager (Molecular Dynamics, Inc.) using a fluorescein setting with a 505 nm filter. After scanning, the image was visualized using ImageQuant software (Molecular Dynamics, Inc.) and an actual-size paper printout was made. The paper printout was examined for differentially expressed messages. To qualify as either a down- regulated or up-regulated message, all three samples within a treatment or control group had to express the same pattern for that gene. Gel bands of interest were marked on the paper printout. The gel was placed on top of the printout and gel bands were cut out using sterile scalpel blades. The cut gel bands were placed into 50 µl nuclease-free water and frozen at –20°C. Three gel slices from each gene of interest were excised.

Reamplification of Gel Bands

Genes of interest were reamplified from the gel bands. The frozen gel bands were thawed, the water was removed, and bands

were washed by adding 50 µl of nuclease-free water and soaking for 10 - 15 min after which the water was removed. The gel slice to be amplified was added directly to a PCR reaction containing the same reagents and primer combination used to generate the bands with the exception of using the unlabeled anchor primer. The reactions were run using the same PCR conditions described previously.

After amplification, the PCR reaction was purified using a PCR purification kit (QiaQuick, Qiagen, Inc.) following the manufacturer's protocol. To verify amplification of the gene, 20 µl of the purified PCR product was run on a 1.0% agarose gel in tris-acetate EDTA (TAE) buffer and stained with ethidium bromide. The size and approximate mass of the DNA fragment were estimated by comparing to a low DNA mass ladder (Life Technologies) that was run on the same gel.

Vector Ligations and Transformations

The reamplified DNA fragments were ligated into pGEM-T Easy Vectors (Promega) following the manufacturer's protocol and using an insert:vector molar ratio of 3:1. The ligation reactions were incubated at 4°C overnight.

Bacterial transformations were carried out in 6 ml polypropylene Falcon tubes. The tubes were placed on ice and 2 µl of the ligation reaction was pipetted into the bottom of the tube. Twenty-five µl of high efficiency competent JM109 cells (Promega) were were pipetted into the tubes and gently swirled. The reactions were incubated on ice for 45 min. The cells were then heat shocked at 42°C for 45 sec, placed back on ice for 2 min after which 450 µl of room temperature nutrient broth containing glucose (SOC medium) (Gibco)

was added. The tubes were incubated at 37°C with shaking (225 rpm) for 1 h after which 50 ul of the transformed cells were spread onto pre-warmed LB/ampicillin/oxacillin treated plates and incubated at 37°C overnight. Five colonies from each DNA insert were picked and streaked onto new LB/ampicillin/oxacillin plates. The plates were incubated overnight at 37°C, chilled at 4°C and shipped to the DNA Sequencing Core Laboratory of the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville for DNA sequencing. After sequences were determined, GenBank was searched using the Basic Local Alignment Search Tool (BLAST) program (Altschul, 1997) to determine the identity of the genes.

RESULTS

The goal of this study was to identify genes that are either up- or down-regulated in response to hormones (GH, T_3, E_2) or pesticide (chlorpyrifos) exposure using a fluorescent differential display RT-PCR (DD) technique. Figure 5.2 presents a segment of a DD gel showing several bands chosen for subsequent cloning and sequence identification. Bands were chosen for further evaluation if all three samples of a treatment group were either darker than the controls or other treatments (up-regulated) or lighter (or did not appear) than controls (down-regulated). The bands labeled IK4 are darker in all three bands representing E_2 treated fish as well in several samples in other treatment groups. The bands were reamplified, cloned and sequenced. The sequences were identified, using the Basic Local Alignment Search Tool (BLAST) program, as being similar to the medaka (*Oryzias latipes*) choriogenin H minor gene. The bands labeled IK6 appeared in all treatment groups. The sequence was identified as being similar to

transferrin mRNA from several fish species. The bands labeled IK3 appear darker in all three lanes representing the chlorpyrifos (CP) treated fish. The gene sequence from those bands was identified as being similar to *Cyprinodon diabolis* micro-satellite C780. Figure 5.3 shows a portion of the BLAST search results for clone IK3 showing the sequence identity.

Twenty-two clones were sequenced and the sequences evaluated by BLAST search of GenBank. We identified 10 clones with sequences similar to genes designated as fish genes (Table 5.2). Of these, two (IK6 and IK 24) had sequence segments matching known fish genes that were more than 250 nucleotides in length. Two (IK4, IK7) had matching segments between 100 and 250 nucleotides in length and another two (IK3, IK25) had sequence matches that were between 50 and 100 nucleotides long. The remaining four clones (IK16, IK17, IK20, IK23) identified as fish genes had matching nucleotide sequences of 20 or less. The remaining clones had short (< 30) nucleotide sequence segments matching human, chicken, bovine or mouse genes.

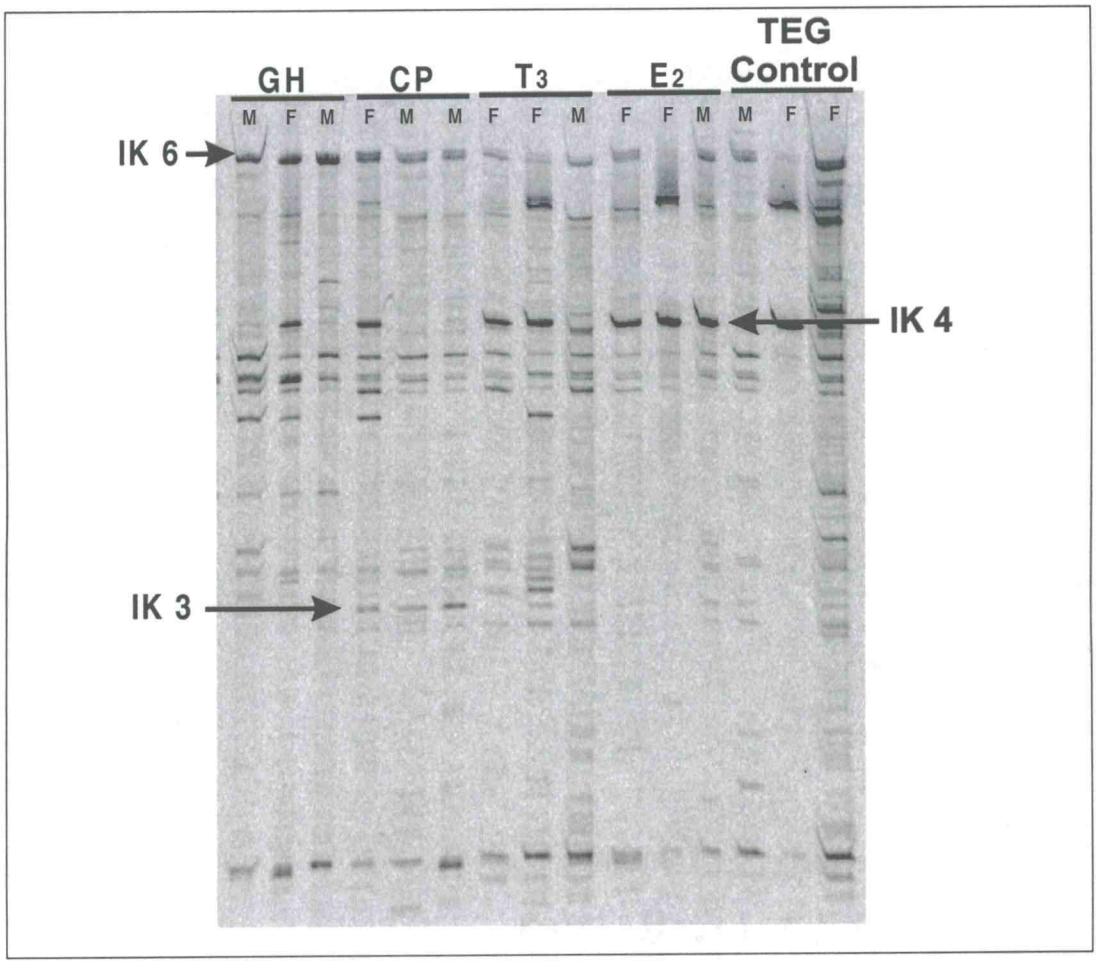

Figure 5.2. Representative segment of a differential display (DD) RT-PCR gel of liver mRNA from fish treated either by injection with growth hormone (GH), triiodothyronine (T_3), or given an aquatic exposure to estradiol (E_2), chlorpyrifos (CP) or triethylene glycol carrier control (TEG Control). Arrows denote gel bands that were subsequently cloned and sequenced. IK3 represents a gene that appears up-regulated in response to chlorpyrifos (CP) treatment. IK4 represents a gene up-regulated by E_2 treatment and is also present in female fish of other treatment groups. IK6 represents a gene that is present in all treatment groups. The sex of the individual fish is denoted (M or F) at the top of each gel lane.

```
>gi|5031225|gb|AF113029.1|AF113029  Cyprinodon diabolis microsatellite C780
sequence Length = 311
Identities = 55/63 (87%)   Strand = Plus / Minus

          323                                                          385
Query:    tgacctttgt  t gagtattaatg aca ttcaggtc ag atgaatg c aacctggccg t tcagacaca
          ||||||||||  ||||||||||     ||||||||  |||||||  ||||||||||  |||||||||
Sbjct:    tgacctttgt  c gagtattaatg gtg ttcaggtc gt atgaatg  c gacctggccg c tcagacaca
          82                                                           20
```

Figure 5.3. Section of BLAST results showing the sequence identity for clone IK3 and *Cyprinodon diabolis* microsatellite C780. The numbers following >gi are the GenBank accession number of the gene sequence. "Strand = Plus/Minus" is the orientation of the two sequences being compared. "Query" denotes the sequence of the unknown clone (IK3). "Sbjct" denotes the sequence of the gene sequence on GenBank. (*Cyprinodon diabolis* C780 RNA). Identities=55/63 indicates that of 63 bases in the sequence, 55 match the sequence from Sheepshead minnow (C. *variegatus*) RNA. Vertical lines between the two sequences show the complementary bases.

Table 5.2
Description of gene sequence clones with identities of known fish sequences from gel bands for differential display RT-PCR of fish treated with endogenous hormones (GH, E_2, T_3) or a pesticide (Chlorpyrifos, CP) or controls. Primer pairs indicate the anchor primer (A, G, or C) and the number of the arbitrary primer assigned by the manufacturer (GenHunter). The expression pattern indicates whether the gene is up-regulated (↑) or down-regulated (↓) by the treatment indicated. ↑ ALL indicates the gene was present in all treatments. Clone sizes are the approximate number of nucleotides. Sequence identity indicates the number of matching nucleotides in a consecutive sequence of the gene identified by the Basic Local Alignment Search Tool (BLAST) program. The BLAST identification given is the one with the highest sequence identity identified by BLAST.

Gel Band IK #	Primer Pair	Expression Pattern	Sequence Identity	BLAST Identification
3	G 23	↑ CP	53/63	*Cyprinodon diabolis* micro-satellite C780
4	G23	↑ E_2	93/113	mRNA for *Oryzias latipes* zona radiata (ZP 2)
6	G 23	↑ ALL	274/333	mRNA for *Ammodytes marinus* transferrin
7	G 23	↑ ALL	127/142	*Ictalurus punctatus* 40S ribosomal protein S17
16	C 5	↑ GH	20/20	*Anguilla* spp. cytochrome b
17	C 5	↑ ALL	18/18	*Salmo salar* odorant receptor ASOR11
20	C 5	↓T_3	20/20	*Danio rerio* DNA sequence on chromosome 3
23	C 21	↑ T_3	19/19	*Poecilia reticulata* clone BsR4 keratin mRNA
24	C 57	↑ ALL	215/253	*Ictalurus punctatus* ribosomal protein L37a
25	C 21	↑ ALL	52/54	*Ictalurus punctatus* ribosomal protein L5b

DISCUSSION

By using differential display RT-PCR we were able to identify several genes from liver mRNA of sheepshead minnows. This procedure allows identification of genes that are differentially expressed in response to a treatment by comparing mRNA fingerprint patterns of the exposed fish to untreated control fish. No prior

knowledge of potential gene expression is required. Out of a possible 240 primer pair combinations, we have examined only 19 to date, representing about 8% of the potentially expressed mRNAs. Many of the bands on the gels were expressed in all treatment groups. These genes were expressed in all samples in relatively equal abundance and did not appear to be regulated by the experimental treatment. Other bands appeared at random and were due to individual variation in the fish or to artifacts introduced in the PCR process. To be considered a valid differentially expressed gene, the band must have been present (or darker) or absent (or lighter) in all three of the samples for a given treatment. Bands that appeared in all three samples of a treatment group but were absent from the controls were considered to be up-regulated. Those absent in all three lanes of a treatment group but appearing in all controls were deemed down-regulated. This condition for determining up- or down-regulated genes was chosen to reduce the possibility of false-positives and as an assurance that differences in expression were actually due to the experimental treatment. Depending on the primer combination, there were multiple up or down- regulated bands, but many had no obvious expression pattern. Each treatment group of three fish included samples of both male and female fish to avoid choosing bands expressed only due to sex differences in gene expression.

The gene for the sheepshead minnow choriogenin protein (clone IK4) was previously identified as being up-regulated by E_2 using DD analysis (Denslow, 2001). The choriogenin protein (also sometimes referred to as a zona radiata or zona pelucida 2 (ZP2) protein) is an egg membrane protein produced in female fish in response to E_2. The bands appearing in gel lanes of treatment groups other than those

exposed to E_2 in the present study were from female fish. Therefore, these fish also expressed the gene due to stimulation by endogenous E_2. We had prior knowledge of the location and identity of these bands (N. Denslow, personal communication), and chose them as an additional control for our DD and cloning technique. The transferrin gene (IK6) was down-regulated by estradiol treatment in SHM in previous work (Denslow, 2001), however in the present study, this gene appears to be expressed in fish from all treatments groups. Transferrin is an iron transporting protein that is important in maintaining iron levels within a cell. A section of the sequence for IK3 was similar to devils hole pupfish *(Cyprinodon diabolis)* microsatellite C780. Microsatellites are highly polymorphic DNA markers comprised of 1 to 6 base pairs that are repeated in tandem arrays and distributed throughout the genome. They are commonly used for genetic mapping. Microsatellites are numerous and spread throughout the eukaryotic genome. They are highly variable and because they are non-functional, they are not subjected to strong selection pressure. The sequence identity of 53/63 between *C. variegatus* and *C. diabolis* may be due to the fish being from the same genus with similarities in their genome. Several ribosomal proteins were expressed in all treatments (clones IK7, IK24, IK25). This is not surprising in that ribosomal mRNA comprises a large portion of total RNA, which is used in the DD RT-PCR procedure. The high sequence identity of two of those genes (> 100) indicate strong similarity to the gene sequences in the GenBank database. The longer a segment of matching nucleotide sequences a clone has, the more likely that it is a gene similar to the sequence identified in GenBank. Many of the sequences we cloned from the DD gels had short sequences (< 30) similar to human, other mammal, or bird genes. These results are most likely due to random sequence

matches and does not necessarily indicate a gene match. One of the drawbacks of DD technology for use in fish is that the number of fish genes thus far identified and recorded in the databases is small compared to those for humans and some other mammals. Therefore, the likelihood of matching and identifying a novel fish gene is lower than for species more commonly used in laboratory studies. Further research directed toward sequencing and identifying fish genes to add to the databases will help resolve this problem and make this technique a more powerful tool for fish research in the future.

Additional work to identify genes that are up-or down-regulated in response to endogenous growth promoting hormones and anthropogenic chemicals is required. DD analysis is qualitative and not quantitative, therefore, the fish gene sequences identified in the present study must still be validated to be sure that they are indeed regulated by the experimental treatment. This will be done either by Northern blot analysis or by quantitative real-time PCR technology. In addition, we hope to run more DD gels to increase the number of primer pair combinations and to identify more gene sequences associated with growth. We examined only a small portion of the possible mRNA population and we did not identify any bands with sequences similar to IGF-I, IGF binding proteins or other genes known to be associated with growth. With no prior knowledge of the genes that may be amplified and visualized by DD RT-PCR, the chance of identifying an important or novel gene may depend on evaluating as many primer pair combinations as possible.

In summary, we performed differential display analysis to identify genes that are up- or down-regulated by endogenous hormones known to regulate somatic growth (GH, T_3, E_2) and to a

growth-inhibiting pesticide (chlorpyrifos). Several genes from the sheepshead minnow were identified as being similar to other fish genes in the GenBank database. Those genes included a microsatellite sequence from the devils hole pupfish (*Cyprinodon diabolis*), a chorio-genin protein mRNA sequence from the medaka (*Oryzias latipes*), and a transferrin mRNA sequence similar to transferrin from several fish species. Many of the clones we examined did not match known fish genes, but had short sequences similar to genes identified as human or other mammals and birds. Differential display RT-PCR analysis can be used to identify genes expressed or inhibited in response to endogenous hormones or to anthropogenic chemicals. This technique will be more valuable as more fish gene sequences are identified and added to the available databases.

6. CONCLUSIONS AND SUMMARY

The advent of molecular biology, and molecular techniques has opened the door for development of new methods to look at the effects of environmental contaminants on humans and wildlife. For the past decade, concern over environmental contaminants that may mimic endogenous hormones has been increasing. The presence of these "endocrine disrupting chemicals" (EDCs) in the environment has resulted in a national mandate to require the U.S. Environmental Protection Agency (EPA) to develop a screening and testing strategy (Patlak, 1996). This mandate, as well as the general importance of evaluating the effects of EDCs, requires the contribution of several areas of biological knowledge.

My work has integrated the fields of endocrinology, toxicology and molecular biology and I believe the assays I developed and describe in my dissertation may contribute to a new paradigm for investigating the issue of EDCs and growth in fish as well as to the development of new screening methods. In addition, looking at the individual components of the endocrine growth axis at the molecular level may help elucidate physiological pathways involved in organismal responses to EDCs.

Both *in vivo* and *in vitro* assays are required to fully understand the effects of both natural hormones and EDCs on an organism. *In vivo* assays measure the whole-organism physiological response to an EDC, but fail to decipher a mechanism of action. On the other hand, *in vitro* assays may pinpoint a particular mechanism of action, but may overlook integrated endocrine responses to other endogenous or environmental conditions.

The work presented in this dissertation provides information on the effects of both endogenous hormones and anthropogenic chemicals on growth in the sheepshead minnow (*Cyprinodon variegatus*). By looking for perturbations in the endocrine axis controlling fish growth (somatotropic axis or GH/IGF-I axis) as well as effects at the level of the whole organism, I investigated possible modes of action for growth inhibition by anthropogenic endocrine disrupting chemicals (EDCs) as well as endogenous hormones associated with growth.

In Chapter 3, I evaluated the effect of several endogenous, growth-promoting hormones on hepatic insulin-like growth factor I (IGF-I) mRNA concentrations. I injected fish with growth hormone (GH), triiodothyronine (T_3), estradiol (E_2) or insulin and sampled the livers over a 24 hour period. In order to accurately measure mRNA, I optimized a method to extract high-quality RNA from fish livers to use in further evaluations of IGF-I mRNA. To measure the concentration of IGF-I mRNA in the liver RNA, I optimized a quantitative real-time (TaqMan) PCR assay. This assay uses relatively new technology to quantify a target mRNA sequence. By using this technology, I was able to show that hepatic IGF-I mRNA levels increased in fish injected with GH, T_3 and insulin, peaking at 12 h post-injection. These results supported previous research in which GH, T_3 and insulin regulated the IGF-I mRNA concentrations in fish liver (Shamblott, 1995; Duan, 1992; Duan, 1992)]. The most significant effect I found was in fish injected with E_2, in which IGF-I mRNA levels decreased significantly at 8 h and 12 h post-injection. These results suggested that pharmacological levels of E_2 may affect the GH/IGF-I axis and could have consequences for fish living in waters polluted by EDCs.

To test the hypothesis that exposure to estrogenic chemicals would decrease growth in sheepshead minnows by reducing hepatic IGF-I synthesis, I conducted a long-term exposure study using E_2 (Chapter 4). I also exposed fish to chlorpyrifos (Dursban), an organophosphate pesticide, also known to suppress growth in this species. I exposed newly hatched sheepshead minnow fry to two concentrations of E_2 and three concentrations of chlorpyrifos for 18 weeks. Although growth was inhibited in a dose-dependent manner in fish exposed to chlorpyrifos, fish exposed to E_2 grew either the same as slightly larger than controls. I measured hepatic IGF-I mRNA from the chemically treated fish using the TaqMan assay to determine whether aquatic exposure to E_2 or chlorpyrifos affected IGF-I synthesis. The results of those assays showed that the hepatic IGF-I mRNA levels of the fish exposed to E_2 and chlorpyrifos were not significantly lower than control fish. These results were in contrast to my previous experiment in which E_2 injections decreased IGF-I mRNA levels in the liver, and suggest that inhibition of IGF-I mRNA was not the likely cause of the growth inhibition in fish exposed to chlorpyrifos. Although the concentrations of E_2 used in this experiment were environmentally relevant (21 and 112.5 ng/L measured concentration) it is possible that they were below the threshold level that would elicit a growth inhibiting response in this species. Therefore, further studies using higher E_2 concentrations are warranted, however, the environmental relevance of higher concentrations must be kept in mind. The exposure concentrations for chlorpyrifos were also environmentally relevant (6.5, 13.3, and 24.3 ug/L measured concentrations). Since IGF-I mRNA levels were not suppressed, the reduced growth of these fish may have been due primarily to depressed feeding activity and a general toxic effect of the chemical.

To further evaluate the molecular effects of endogenous hormones and anthropogenic chemicals, I used differential display RT-PCR analysis to identify differentially regulated genes (Chapter 5). I analyzed RNA from fish injected with endogenous hormones (GH, T_3, E_2) from my first experiment (Chapter 3), as well as RNA from fish exposed to E_2 (112.5 ng/l) and chlorpyrifos (24.3 ug/L) from my second experiment (Chapter 4). By amplifying segments of RNA using PCR with 19 different sets of primer pairs, and then running the PCR products on large sequencing gels, I was able to visualize bands that were either induced or inhibited by the experimental treatments. Several genes were identified as being similar to fish genes in the Gen-Bank database. These included a microsatellite sequence, a chorio-genin (vitelline envelope) protein mRNA sequence, a transferrin mRNA sequence and several ribosomal RNA sequences. Many of the clones I examined did not match known fish genes, but had short sequences similar to genes identified as human or other mammals and birds. This technique will be more valuable as more fish gene sequences are identified and added to the available databases.

In conclusion, pharmacological doses of E_2 appear to down-regulate hepatic IGF-I mRNA levels in the sheepshead minnow. However, aquatic exposure to environmentally relevant concentrations of E_2 did not affect hepatic IGF-I mRNA levels in the sheepshead minnow nor did it inhibit growth at the concentrations tested. To determine whether fish living in water contaminated by estrogenic are at risk for growth inhibition, further studies using higher concentra-tions of E_2 or other estrogenic chemicals may be warranted. While aquatic exposure to chlorpyrifos inhibited growth, the mechanism of action is still unclear. Hepatic IGF-I mRNA levels in these smaller fish

were no different than controls. The depressed feeding activity, as well as a general toxic effect of chlorpyrifos may have contributed to the slow growth observed in these fish.

By using quantitative real-time PCR (TaqMan), a relatively new technique for quantifying mRNA, I measured hepatic IGF-I mRNA levels in response to several endogenous hormones (GH, T_3, insulin) associated with growth. I found that, as expected, IGF-I mRNA levels increased in response to those hormones. The TaqMan assay is a sensitive and fairly simple method to quantify specific mRNA. Finally, I optimized a fluorescent differential display RT-PCR technique to identify differentially expressed genes associated with different experimental treatments. I identified several genes that were similar to other fish genes on the GenBank database. This technique has the potential to be a powerful tool in evaluating the effects of anthropogenic chemical exposure and endogenous hormones at the molecular level.

BIBLIOGRAPHY

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

Afonso, L. O. B., Campbell, P. M., Iwama, G. K., Devlin, R. H. and Donaldson, E.M. (1997). The effect of the aromatase inhibitor fadrozole and two polynuclear aromatic hydrocarbons on sex steroid secretion by ovarian follicles of coho salmon. *General and Comparative Endocrinology* 106: 169-174.

Agellon, L. B., Davies, S. L., Chen, T. T. and Powers, D. A. (1988). Structure of a fish (rainbow trout) growth hormone gene and its evolutionary implications. *Proceedings of the National Academy of Sciences* 85: 5136-5140.

Agellon, L. B., Davies, S. L., Lin, C. M., Chen, T. T. and Powers, D. A. (1988). Rainbow trout has two genes for growth hormone. *Molecular Reproduction and Development* 1: 11-17.

Alexandrides, T., Moses, A. C. and Smith, R. J. (1989). Developmental expression of receptors for insulin, insulin-like growth factor-1 (IGF-I), and IGF-II in rat skeletal muscle. *Endocrinology* 124: 1064-1076.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., *et al.* (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* 25: 3389-3402.

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

Argenton, F., Vianello, S., Bernardini, S., Jacquemin, P., Martial, J., *et al.* (1993). The transcriptional regulation of the growth hormone gene is conserved in vertebrate evolution. *Biochem Biophys Res Commun* 192: 1360-1366.

Argetsinger, L. S. and Carter-Su, C. (1996). Mechanism of signaling by growth hormone receptor. *Physiological Review* 76: 1089-1107.

Arnold, S. F., Robinson, M. K., Notides, A. C., Guillette, L. J., Jr., and McLachlan, J. A. (1996). A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens. *Environmental Health Perspectives* 104: 544-548.

Ashby, J., *et al.* (1997). The challenge posed by endocrine-disrupting chemicals. *Environmental Health Perspectives* 105: 164-169.

Ashfield, L. A., Pottinger, T. G. and Sumpter, J. P. (1998). Exposure of female juvenile rainbow trout to alkylphenolic compounds results in modifications to growth and ovosomatic index. *Environmental Toxicology and Chemistry* 17: 679-686.

Bai, J., Lao, H. and Ye, X. (2000). Molecular cloning and sequence analysis of insulin-like growth factor I cDNA from bluntsnout bream (*Megalobrama amblycephala*). *GenBank*. Baihedong, Guangzhou, Guangdong 510380, China, Biotechnology Department, Pearl River Fishery Research Institute, CAFS.

Bai, J. J., Ye, X., Li, Y. H., Li, X. H., Jian, Q., *et al.* (2000). Isolation and characterization of grass carp (*Ctenopharyngodon idellus*) insulin-like growth factor gene. *GenBank*. Baihedong, Guangzhou, Guangdong 510380, China, Biotechnology Department, Pearl River Fisheries Research Institute.

Barinaga, M., Bilezikjian L.M., Vale, W. W., Rosenfeld, M. and Evans, R. M. (1985). Independent effects of growth hormone releasing factor on growth hormone release and gene transcription. *Nature* 314: 279-281.

Bauchat, J. R., Busby, W. H. J., Garmong, A., Swanson, P., Moore, J., *et al.* (2001). Biochemical and functional analysis of a conserved IGF-binding protein isolated from rainbow trout (*Oncorhynchus mykiss*) hepatoma cells. *Journal of Endocrinology* 170: 619-628.

Bauman, G. (1995). Growth hormone binding to a circulating receptor fragment -- the concept of receptor shedding and receptor splicing. *Experimental and Clinical Endocrinology and Diabetes* 103: 2-6.

Bauman, G. (2001). Growth hormone binding protein 2001. *Journal of Pediatric Endocrinology and Metabolism* 14: 355-375.

Bautista, C. M., Mohan, S. and Baylink, D. J. (1990). Insulin-like growth factors I and II are present in the skeletal tissues of ten vertebrates. *Metab. Clin. Exp* 39: 6-100.

Baxter, R. C. (2000). Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *American Journal of Physiology, Endocrinology and Metabolism* 278: E967-E976.

Baxter, R. C., Binoux, M. A., Clemmons, D. R., Conover, C. A., Drop, S. L. S., *et al.* (1998). Recommendations for nomenclature of the insulin-like growth factor binding protein superfamily. *Journal of Clinical Endocrinology and Metabolism* 83: 3213.

Bayne, M. L., Applebaum, J., Chicchi, G. G., Miller, R. E. and Cascieri, M. A. (1990). The roles of tyrosine 24, 31, and 60 in the high affinity binding of insulin-like growth factor-I to the type 1 insulin-like growth factor receptor. *Journal of Biological Chemistry* 265: 15648-15652.

Beckman, B. R., Larsen, D. A., Moriyama, S., Lee-Pawlak, B. and Dickhoff, W. W. (1998). Insulin-like growth factor-I and environmental modulation of growth during smoltification of spring chinook salmon (*Oncorhynchus tshawytscha*). *General and Comparative Endocrinology* 109: 325-335.

Bell, G. I., Gerhardt, D. S., Fong, N. M., Sanchez-Pescador, R. and Rall, L. B. (1985). Isolation of human insulin-like growth factor genes: Insulin-like growth factor II and insulin genes are contiguous. *Proceedings of the National Academy of Sciences of the U.S.A.* 82: 6450-6455.

Ber, R. and Daniel, V. (1992). Structure and sequence of the growth hormone-encoding gene from *Tilapia nilotica*. *Gene* 113: 245-250.

Bern, H. A., McCormich, S. D., Kelley, D. M., Gray, E. S., Nishioka, R. S., *et al.* (1991). Insulin-like growth factors "under water": role in growth and function of fish and other poikilothermic vertebrates. *Modern Concepts of Insulin-like Growth Factors*. E. M. Spencer, Elsevier Science Publishing Co., Inc: 85-96.

Bjornsson, B. T. (1997). The biology of salmon growth hormone: from daylight to dominance. *Fish Physiology and Biochemistry* 17: 9-24.

Bjornsson, B. T., Taranger, G. L., Hansen, T., Stefansson, S. O. and Haux, C. (1994). The interrelation between photoperiod, growth hormone, and sexual maturation of adult Atlantic salmon (*Salmo salar*). *General and Comparative Endocrinology* 93: 70-81.

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

Blakesley, V. A., Butler, A. A., Koval, A. P., Okubo, Y. and LeRoith, D., (1999). IGF-I Receptor Function. *Contemporary Endocrinology: The IGF System.* R. Rosenfeld and C. J. Roberts. Totowa, NJ, Humana Press, Inc.: 143-163.

Bluet-Pajot, M. T., *et al.* (2001). Growth hormone secretagogues and hypothalamic networks. *Endocrine* 14: 1-8.

Bodner, M. and Karin, M. (1987). A pituitary-specific trans-acting factor can stimulate transcription from the growth hormone promoter in extracts of nonexpressing cells. *Cell* 50: 267-275.

Boeni-Schnetzler, M., Schmid, C., Meier, P. J. and Froesch, E. R. (1991). Insulin regulates insulin-like growth factor I mRNA in rat hepatocytes. *American Journal of Physiology* 260: E846-E851.

Bolton, J. P., Collie, N. L., Kawauchi, H. and Hirano, T. (1987). Osmoregulatory actions of growth hormone in rainbow trout *(Salmo gairneri). Journal of Endocrinology* 112: 63-68.

Borski, R. J., Tsai, W., DeMott-Friberg, R. and Barkan, A. L. (1996). Regulation of somatic growth and the somatotropic axis by gonadal steroids: Primary effect on insulin-like growth factor I gene expression and secretion. *Endocrinology* 137: 3253-3259.

Brameld, J. M., Weller, P. A., Saunders, J. C., Buttery, P. J. and Gilmour, R. S. (1995). Hormonal control of insulin-like growth factor-I and growth hormone receptor mRNA expression by porcine hepatocytes in culture. *Journal of Endocrinology* 146(2): 239-245.

Bres, O. and Eales, J. G. (1986). Thyroid hormone binding to isolated trout (*Salmo gairdneri*) liver nuclei *in vitro*: binding affinity, capacity and chemical specificity. *General and Comparative Endocrinology* 61: 29-39.

Bulkley, R. V. (1972). Diethylstilbestrol in catfish feed. *Transactions of the American Fisheries Society* 101(3): 537-539.

Butler, A. A., Yakar, S., Gewolb, I. H., Karas, M., Okubo, Y., *et al.* (1998). Insulin-like growth factor-I receptor signal transduction: at the interface between physiology and cell biology. *Comparative Biochemistry and Physiology* 121(B): 19-26.

Calduch-Giner, J., Duval, H., Chesnel, F., Boeuf, G., Perez-Sanches, J., *et al.* (2001). Fish growth hormone receptor: molecular characterization of two membrane-anchored forms. *Endocrinology* 142: 3269-3273.

Calduch-Giner, J. A., Sitjà-Bobadilla, A., Alvarez-Pellitero, P. and Pérez-Sánchez, J. (1997). Growth hormone as an in vitro phagocyte-activating factor in the gilthead sea bream (*Sparus aurata*). *Cell and Tissue Research* 287: 535-540.

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

Carter-Su, C., Schwartz, J. and Smit, L. S. (1996). Molecular mechanism of growth hormone action. *Annual Review of Physiology* 58: 187-207.

Chan, D. W., Yu, K. L., Rivier, J. and Chow, B. K. (1998). Identification and characterization of a receptor from goldfish specific for a teleost growth hormone-releasing hormone-like peptide. *Neuroendocrinology* 68: 44-56.

Chan, H. H. and Eales, J. G. (1975). Identification of iodoamino acids in the plasma and bile of brook trout, *Salvelinus fontinalis*. *Canadian Journal of Zoology* 53: 97-101.

Chan, S. J., Cao, Q.-P., Nagamatsu, S. and Steiner, D. F. (1993). Insulin and insulin-like growth factor genes in fishes and other primitive chordates. *Biochemistry and Molecular Biology of Fishes, Volume 2: Molecular Biology Frontiers*. P. W. Hochachka and T. P. Mommsen. Amsterdam, Elsevier: 407-418.

Chan, S. J., Plisetskaya, E. M., Urbinati, E., Jin, Y. and Steiner, D. F. (1997). Expression of multiple insulin and insulin-like growth factor receptor genes in salmon gill cartilage. *Proceedings of the National Acadamy of Sciences of the U.S.A.* 94: 12446-12451.

Chanda, S. M. and Pope, C. N. (1996). Neurochemical and neurobehavioral effects of repeated gestational exposure to chlorpyrifos in maternal and developing rats. *Pharmacology and Biochemistry of Behavior* 53(4): 771-776.

Chao, S. C., Pan, F. W. and Chang, W. C. (1989). Purification of carp growth hormone and cloning of the complementary DNA. *Biochim. Biophy. Acta* 1007: 233-236.

Cheek, A. O., Brouwer, T. H., Carroll, S., Manning, S., McLachlan, J. A., *et al.* (2001). Experimental evaluation of vitellogenin as a predictive biomarker for reproductive disruption. *Environmental Health Perspectives* 109: 681-690.

Chen, J.-Y., Chang, C.-Y., Chen, J.-C., Shen, S.-C. and Wu, J.-L. (1997). Production of biologically active recombinant tilapia insulin-like growth factor-II polypeptides in *Escherichia coli* cells and characterization of the genomic structure of the coding region. *DNA and Cell Biology* 16: 883-892.

Chen, M. H., Lin, G., Gong, H., Weng, C., Chang, C., et al. (2001). The characterization of prepro-insulin-like growth factor-1 Ea-2 expression and insulin-like growth factor-1 genes (devoid 81 bp) in the zebrafish (*Danio rerio*). *Gene* 268: 67-75.

Chen, M. H. C., Chang, C. Y. and Wu, J. L. (1997). Cloning and Characterization of Insulin-like Growth Factor I cDNA from black seabream (*Acanthopagrus schlegeli*). *GenBank*. Taipei, Taiwan 115, R.O.C., Institute of Zoology, Academia Sinica,.

Chen, T. T., Marsh, A., Shamblott, M., Chan, K. M., Tang, Y. L., *et al.* (1994). Structure and evolution of fish growth hormone and insulin-like growth factor genes. *Fish Physiology*, Academic Press, Inc. XIII: 179- 207.

Chiou, C. S., Chen, H. T. and Chang, W. C. (1990). The complete nucleotide sequence of the growth-hormone gene from the common carp (*Cyprinus carpio*). *Biochim Biophys Acta* 1087: 91-94.

Clairmont, K. B. and Czech, M. P. (1989). Chicken and Xenopus mannose 6-phosphate receptors fail to bind insulin-like growth factor II. *Journal of Biological Chemistry* 264: 16390-16392.

Clark, M. S. (1998). "*Fugu rubriceps* cosmid 258N02 containing IGF-II, TH, NAP2 genes". EMBL database. Accession Number AL021880.

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

Colborn, T. (1995). Environmental estrogens: Health implications for humans and wildlife. *Environmental Health Perspectives* 103: 135-136.

Colborn, T. and Clement, C. (1992). *Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection*. Princeton, NJ, Princeton Scientific Publishing Co.

Colborn, T., Dumanoski, D. and Myers, J. P. (1996). *Our Stolen Future*. New York, Penguin Books.

Collet, C. and Candy, J. (1998). How many insulin-like growth factor binding proteins? *Molecular and Cellular Endocrinology* 139: 1-6.

Collet, C., Candy, J., Richardson, N. and Sara, V. (1997). Organization, sequence and expression of the gene encoding IGF-II from barramundi (Teleosteii; *Lates calcarifer*). *Biochemical Genetics* 35: 211-224.

Connolly, J. P. (1991). Application of a food chain model to polychlorinated biphenyl contamination of the lobster and winter flounder food chains in New Bedford Harbour. *Environmental Science and Technology* 25: 760-770.

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

Coppage, D. L. (1972). Organophosphate pesticides: specific level of brain AChE inhibition related to death in sheepshead minnows. *Transactions of the American Fisheries Society* 101: 534-536.

Cowey, C. B., Pope, J. A., Adron, J. W. and Blair, A. (1973). Studies on the nutrition of marine flatfish. The effect of oral administration of diethylstilboestrol and cyproheptadine on the growth of *Pleuronectes platessa*. *Marine biology* 19: 1-6.

Crain D.A., Noreiga, N., Voneir, P. M., Arnold, S. F., McLachlan, J. A., *et al.* (1998). Cellular bioavailability of natural hormones and environmental contaminants as a function of serum and cytosolic binding factors. *Toxicology Ind Health* 14: 261-273.

Crain, D. A. and Guillette, L. J., Jr. (1997). Endocrine-Disrupting Contaminants and Reproduction in Vertebrate Wildlife. *Reviews in Toxicology* 1: 47-70.

Crain, D. A., Rooney, A. A., Orlando, E. F. and Guillette, L. J., Jr. (2000). Endocrine-disrupting contaminants and hormone dynamics: lessons from wildlife. *Environmental Endocrine Disrupters: An Evolutionary Perspective*. L. J. Guillette, Jr., and D. A. Crain. New York, NY, Taylor & Francis: 1-21.

Cripe, G. M., Hansen, D. J., Macauley, S. F. and Forester, J. (1986). Effects of diet quantity on sheepshead minnows (*Cyprinodon variegatus*) during early life-stage exposures to chlorpyrifos. *Aquatic Toxicology and Environmental Fate: ASTM STP 921*. T. M. Poston and R. Purdy. Philadelphia, American Society for Testing and Materials. 9: 450-460.

Cyr, D. G. and Eales, J. G. (1996). Interrelationships between thyroidal and reproductive endocrine systems in fish. *Rev Fish Biol Fish* 6: 165-200.

Dana S, K. M. (1989). Induction of human growth hormone promoter activity by the adenosine 3',5'-monophosphate pathway involves a novel responsive element. *Molecular Endocrinology* 3: 815-821.

Das, S. and Thomas, P. (1999). Pesticides interfere with the nongenomic action of a progestogen on meiotic maturation by binding to its plasma membrane receptor on fish oocytes. *Endocrinology* 140: 1953-1956.

Daughaday, W. H., Hall, K., Raben, M. S., Salmon, W. D. J., van den Brande, J. L., *et al.* (1972). Somatomedin: proposed designation for sulphation factor. *Nature* 235: 107.

Daughaday, W. H., Hall, K., Salmon, W.D.,Jr., Van den Brande, J. L. and Van Wyk, J. J. (1987). On the nomenclature of the somatomedins and insulin-like growth factors. *Journal of Clinical Endocrinology and Metabolism* 65: 1075-1076.

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

Daughaday, W. H. and Reeder, C. (1966). Synchronous activation of DNA synthesis in hypophysectomized rat cartilage by growth hormone. *Journal of Laboratory and Clinical Medicine* 68: 357-368.

Daughaday, W. H. and Rotwein, R. (1989). Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum and tissue concentrations. *Endocrine Reviews* 10: 68-90.

Daughaday, W. H. and Rotwein, R. (1989). Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocrine Reviews* 10: 68-90.

De Guise, S., *et al.* (2001). Consensus statement: Atlantic coast contaminants workshop 2000. *Environmental Health Perspectives* 109(12): 1301-1302.

Degani, G. (1986). Effect of dietary 17-β-estradiol and 17-α-methyltestosterone on growth and body composition of slow-growing elvers (*Anguilla angilla* L.). *Comparative Biochemistry and Physiology* 84A: 243-247.

Denko, C. W. and Bergenstal, D. M. (1955). The effect of hypophysectomy and growth hormone on cartilage sulfate metabolism. *Proceedings of the Society for Experimental Biology and Medicine* 84: 603-605.

Denslow, N. D., Bowman, C. J., Ferguson, R. J., Lee, H. S., Hemmer, M. J., Folmar, L.C. (2001). Induction of gene expression in sheepshead minnows (*Cyprinodon variegatus*) treated with 17β-estradiol, diethylstilbestrol, or ethinylestradiol: The use of mRNA fingerprints as an indicator of gene regulation. *General and Comparative Endocrinology* 121: 250-260.

Denslow, N. D., Lee, H. S., Bowman, C. J., Hemmer, M. J. and Folmar, L. C. (2001). Multiple responses in gene expression in fish treated with estrogen. *Comparative Biochemistry and Physiology Part B* 129: 277-282.

Denton, T. E., Howell, W. M., Allison, J. J., McCollumn, J. and Marks, B. (1985). Masculinization of female mosquitofish by exposure to plant sterols and *Mycobacterium smegmatis*. *Bulletin of Environmental Contamination and Toxicology* 35: 627-632.

D'Ercole, A. J., Applewhite, G. T. and Underwood, L. E. (1980). Evidence that somatomedin is synthesized by multiple tissues in the fetus. *Developmental Biology* 75: 315-328.

Desbrow, C., Routledge, E., Sheahan, D., Waldock, M. and Sumpter, J. P. (1997). The Identification and Assessment of Oestrogenic Substances in Sewage Treatment Works Effluents. Almondsbury, Bristol, U.K., 74 pp., Environment Agency.

Dickhoff, W. W. (1993). Hormones, metamorphosis, and smolting. *The Endocrinology of Growth, Development, and Metabolism in Vertebrates*. M. P. Schreibman, C. G. Scanes and P. K. T. Pang. New York, Academic Press: 519-540.

Diez, J. M., Giannico, G., McLean, E. and Donaldson, E. M. (1992). The effect of somatostatins (SRIF-14, 25 and 28), galanin and anti-SRIF on plasma growth hormone levels in coho salmon (*Oncorhynchus kisutch* Walbaum). *Journal of Fish Biology* 40: 887-893.

Donaldson, E. M., Fagerlund, U. H. M., Higgs, D. A. and McBride, J.R., (1979). Hormonal Enhancement of Growth. *Fish Physiology - Bioenergetics and Growth*. W. S. Hoar, D. J. Randall and J. R. Brett. New York, NY, Academic Press, Inc. VIII: 455-597.

Donohoe, R. M. and Curtis, L. R. (1996). Estrogenic activity of chlordecone, o,p'-DDT and o,p'-DDE in juvenile rainbow trout: induction of vitellogenesis and interaction with hepatic estrogen binding sites. *Aquatic Toxicology* 36: 31-52.

Du, S. J., Devlin, R. H. and Hew, C. L. (1993). Genomic structure of growth hormone genes in chinook salmon*(Oncorhynchus tshawytscha)*: Presence of two functional genes, GH-I and GH-II, and a male-specific pseudogene, GH. *DNA and Cell Biology* 12: 739-751.

Duan, C. (1998). Nutritional and developmental regulation of insulin-like growth factors in fish. *Journal of Nutrition* 128: 306S-314S.

Duan, C., Ding, J., Li, Q., Tasai, W. and Pozios, K. (1999). Insulin-like growth factor binding protein 2 is a growth inhibitory protein conserved in zebrafish. *Proceedings of the National Academy of Sciences USA* 96: 15274-15279.

Duan, C., Duguay, S. J. and Plisetskaya, E. M. (1992). Hormonal regulation of insulin-like growth factor I (IGF-I) mRNA expression in coho salmon. *American Zoologist* 32: 13A.

Duan, C., Duguay, S. J. and Plisetskaya, E. M. (1993). Insulin-like growth factor I (IGF-I) mRNA expression in coho salmon, *Oncorhynchus kisutch*: tissue distribution and effects of growth hormone/prolactin family proteins. *Fish Physiology and Biochemistry* 11: 371-379.

Duan, C., Duguay, S. J., Swanson, P., Dickhoff, W. W. and Plisetskaya, E. M. (1994). Regulation of insulin-like growth factor gene expression in salmon. *Perspectives in Comparative Endocrinology*. K. G. Davey, R. E. Peter and S. S. Tobe. Ottawa, National Research Counsil: 365-372.

Duan, C. and Hirano, T. (1990). Stimulation of ^{35}S-Sulfate uptake by mammalian insulin-like growth factors I and II in cultured cartilages of the Japanese eel, *Anguilla japonica*. *Journal of Experimental Zoology* 256: 347-350.

Duan, C. and Hirano, T. (1991). Plasma kinetics of growth hormone in the Japanese eel, *Anguilla japonica*. *Aquaculture* 95: 179-188.

Duan, C. and Hirano, T. (1992). Effects of insulin-like growth factor-I and insulin on the in vitro uptake of sulphate by eel branchial cartilage: Evidence for the presence of independent hepatic and

pancreatic sulphation factors. *Journal of Endocrinology* 79: 320-325.

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

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

Duan, C., Noso, T., Moriyama, S., Kawauchi, H. and Hirano, T. (1992). Eel insulin: isolation, characterization and stimulatory action on [^{35}S]-sulfate and [^{3}H]-thymidine uptake in the branchial cartilage of the eel *in vitro. Journal of Endocrinology* 133: 221-230.

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

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

Duchamp, C., Burton, K. A., Herpin, P. and Dauncey, M. J. (1996). Perinatal ontogeny of porcine growth hormone receptor gene expression is modulated by thyroid status. *European Journal of Endcrinology* 134: 524-531.

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

Duguay, S. J., Lai-Zhang, J., Steiner, D. F., Funkenstein, B. and Chan, S. J. (1996). Developmental and tissue-regulated expression of IGF-I and IGF-II mRNAs in *Sparus aurata. Journal of Endocrinology* 16: 123-132.

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

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

Dull, T. J., Gray, A., Hayflick, J. S. and Ullrich, A. (1984). Insulin-like growth factor II precursor gene organization in relation to insulin gene family. *Nature* 310: 777-781.

Ekstrom, T.J., Backlin, B. M., Lindqvist, Y. and Engstrom, W. (1993). Insulin-like growth factor II in the mink (*Mustela vision*): determination of a cDNA nucleotide sequence and developmental regulation of its expression. *General and Comparative Endocrinology* 90: 243-250.

Elies, G., Duval, H., Bonnec, G., Wolff, J., Boeuf, G., *et al.* (1999). Insulin and insulin-like growth factor-I receptors in an evoluted fish, the turbot: cDNA cloning and mRNA expression. *Molecular and Cellular Endocrinology* 158: 173-185.

Elies, G., Groigno, L., Wolff, J., Boeuf, G. and Boujard, D. (1996). Characterization of the insulin-like growth factor type 1 receptor messenger in two teleost species. *Molecular and Cellular Endocrinology* 124: 131-140.

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

Farchi-Pisanty, O., Hackett, P. B. J. and Moav, B. (1995). Regulation of fish growth hormone transcription. *Molecular Marine Biology and Biotechnology* 4: 215-23.

Ferry, R.J., Jr., Katz, L. E. L., Grimberg, A., Cohen, P. and Weinzimer, S. A. (1999). Cellular actions of insulin-like growth factor binding proteins. *Hormone and Metabolism Research* 31: 192-202.

Finidori, J. (2000). Regulators of growth hormone signaling. *Vitamins and Hormones* 59: 71-97.

Folmar, L. C., Denslow, N. D., Kroll, K., Orlando, E. F., Enblom, J., *et al.* (2001a). Alterations in serum sex steroids and vitellogenin in feral walleye (*Stizostedion vitreum*) collected near a metropolitan sewage treatment plant. Archives of Environmental Contamination and Toxicology 40:392-398.

Folmar, L. C., Denslow, N. D., Rao, V., Chow, M., Crain, D. A., *et al.* (1996). Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environmental Health Perspectives* 104: 1096-1101.

Folmar, L. C., Denslow, N. D., Wallace, R., LeFleur, G., Bonomelli, S., *et al.* (1995). A highly conserved N-terminal sequence for teleost vitellogenin with potential value to the biochemistry, molecular biology and pathology of vitellogenesis. *Journal of Fish Biology* 46: 255-263.

Folmar, L. C. and Dickhoff, W. W. (1979). Plasma thyroxine and gill Na- K ATPase changes during seawater acclimation of coho salmon (*Onocorhynchus kisutch*). *Comparative Biochemistry and Physiology* 63A: 329-333.

Folmar, L. C., Dickhoff, W. W., Zaugg, W. S. and Hodgins, H. O. (1982). The effects of aroclor and number two diesel fuel on smoltification and sea water adaptation of coho salmon (*Oncorhynchus kisutch*). *Aquatic Toxicology* 2: 291-297.

Folmar, L. C., Gardner, G. R., Schreibman, M. P., Magliulo-Cepriano, L., Mills, L. J., *et al.* (2001b). Vitellogenin-induced pathology in male summer flounder (*Paralichthys dentatus*). *Aquatic Toxicology* 51(4): 431-441.

Folmar, L. C., Hemmer, M., Hemmer, R., Bowman, C., Kroll, K., *et al.* (2000). Comparative estrogenicity of estradiol, ethynyl estradiol and diethylstilbestrol in an *in vivo*, male sheepshead minnow (*Cyprinodon variegatus*), vitellognin bioassay. *Aquatic Toxicology* 49: 77-88.

Forhead, A. J., Li, J., Saunders, J. D., Dauncey, M. J., Gilmour, R. S., *et al.* (2000). Control of ovine hepatic growth hormone receptor and insulin-like growth factor I by thyroid hormones in utero. *American Journal of Physiology, Endocrinology and Metabolism* 278: E1166-E1174.

Fossi, M. C., Casini, S., Ancora, S., Moscatelli, A., Ausili, A., *et al.* (2001). Do endocrine disrupting chemicals threaten Mediterranean swordfish? Preliminary results of vitellogenin and *zona radiata* proteins in *Xiphias gladius*. *Marine Environmental Research* 52: 477-483.

Freyschuss, B., Sahlin, L., Masironi, B. and Eriksson, H. (1994). The hormonal regulation of the oestrogen receptor in rat liver: an interplay involving growth hormone, thyroid hormones and glucocorticoids. *Journal of Endocrinology* 142: 285-298.

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

Fry, D. M. (1995). Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environmental Health Perspectives* 103: 165-171.

Fry, F. E. J. (1971). The effect of environmental factors on the physiology of fish. *Fish Physiology*. W. S. Hoar and D. J. Randall. New York, NY, Academic Press. VI Environmental Relations and Behavior: 1-99.

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

Funkenstein, B., Shemer, R., Amuly, R. and Cohen, I. (1996). Nucleotide sequence of the promoter region of *Sparus aurata* insulin-like growth factor I gene and expression of IGF-I in eggs and embryos. *Molecular Marine Biology and Biotechnology* 5: 43-51.

Furlanetto, R. W., DiCarlo, J. N. and Wisehart, C. (1987). The type II insulin-like growth factor receptor does not mediate deoxyribonucleic acid synthesis in human fibroblasts. *Journal of Clinical Endocrinology and Metabolism* 64: 1142.

Gallo, G., de Marchis, M., Voci, A. and Fugassa, E. (1991). Expression of hepatic mRNAs for insulin-like growth factors-I and -II during the development of hyothyroid rats. *Journal of Endocrinology* 131: 367-372.

Gannam, A. L. and Lovell, R. T. (1991). Effects of feeding 17 α-methyltestosterone, 11-ketotestosterone, 17 β-estradiol, and 3,5,3'-triiodothyronine to channel catfish, *Ictalurus punctatus*. *Aquaculture* 92: 377-388.

Giustina, A. and Wehrenberg, W. B. (1995). Influence of thyroid hormones on the regulation of growth hormone secretion. *European Journal of Endocrinology* 133: 646-653.

Goldman, J. M., Cooper, R. L., Laws, S. C., Rehnberg, G. L., Edwards, T. L., *et al.* (1990). Chlordimeform-induced alterations in endocrine regulation within the male rat reproductive system. *Toxicology and Applied Pharmacology* 104: 25-35.

Gomez, J. M., Boujard, T., Fostier, A. and LeBail, P. Y. (1996). Characterization of growth hormone nycthemeral plasma profiles in catheterized rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Zoology* 274: 171-180.

Gomez, J. M., Mourot, B., Fostier, A. and LeGac, F. (1999). Growth hormone receptors in ovary and liver during gametogenesis in female rainbow trout (*Oncorhynchus mykiss*). *Journal of Reproductive Fertility* 115: 275-285.

Goodman, L. R., Hansen, D. J., Cripe, G. M., Middaugh, D. P. and Moore, J. C. (1985). A new early life-stage toxicity test using the California grunion (*Leuresthes tenius*) and results with chlorpyrifos. *Ecotoxicology and Environmental Safety* 10: 12-21.

Gore, A. C. (2001). Environmental toxicant effects on neuroendocrine function. *Endocrine* 14: 235-246.

Gray, A., Tam, A. W., Dull, T. J., Hayflick, J., Pintar, J., *et al.* (1987). Tissue-specific and developmentally regulated transcription of the insulin-like growth factor 2 gene. *DNA* 6: 283-295.

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

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

Gray, E. S., Young, G. and Bern, H. A. (1990). Radioreceptor assay for growth hormone in coho salmon (*Oncorhynchus kisutch*) and its application to the study of stunting. *Journal of Experimental Biology* 256: 290-296.

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

Greene, M. W. and Chen, T. T. (1997). Temporal expression pattern of insulin-like growth factor mRNA during embryonic development in a teleost, rainbow trout (*Oncorhynchus mykiss*). *Molecular Marine Biology and Biotechnology* 6: 144-151.

Greene, M. W. and Chen, T. T. (1999). Characterization of teleost insulin receptor family members. II. Developmental expression of insulin-like growth factor type I receptor messenger RNAs in rainbow trout. *General and Comparative Endocrinology* 115: 270-281.

Guillette, J., L.J., Gross, T. S., Masson, G. R., Matter, J. M. and Percival, H. F. (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environmental Health Perspectives* 102(8): 680-688.

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

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

Hammar, J., Larsson, P. and Klavins, M. (1993). Accumulation of persistent pollutants in normal and dwarfed arctic char (Salvelinus alpinus sp. Complex). *Canadian Journal of Fisheries and Aquatic Sciences* 50: 2574-2580.

Hampton, B., Burgess, W. H., Marshak, D. R., Cullen, K. J. and Perdue, J. F. (1989). Purification and characterization of an insulin-like growth factor II variant from human plasma. *Journal of Biological Chemistry* 264: 19155-19160.

Hansen, D. J., Goodman, L. R., Cripe, G. M. and Macauley, S. F. (1986). Early life-stage toxicity test methods for Gulf toadfish (*Opsanus beta*) and results using chlorpyrifos. *Ecotoxicology and Environmental Safety* 11: 15-22.

Harvey, S. (1993). Growth hormone secretion in poikilotherms and homeotherms. *The Endocrinology of Growth, Development, and*

Metabolism in Vertebrates. M. P. Schreibman, C. G. Scanes and P. K. T. Pang. New York, Acadmic Press: 151-182.

Harvey, S. (1995). Growth hormone release: integrative hypothalamic control. Growth Hormone. S. Harvey, C. G. Scanes and W. H. Daughaday. Boca Raton, FL, CRC Press, Inc.: 131-162.

Harvey, S. (1995). Growth hormone synthesis. *Growth Hormone*. S. Harvey, C. G. Scanes and W. H. Daughaday. Boca Raton, FL, CRC Press: 55-77.

Harvey, S. and Hull, K. L. (1995). Growth hormone action: growth hormone receptors. *Growth Hormone*. S. Harvey, C. Scanes and W. H. Daughaday. Boca Raton, FL, CRC Press: 303-335.

Hawkins, M. B., Thornton, J. W., Crews, D., Skipper, J. K., Dotte, A., *et al.* (2000). Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. *Proceedings of the National Academy of Sciences* 97(20): 10751-10756.

Herman, R. L. and Kincaid, H. L. (1988). Pathological effects of orally administered estradiol to rainbow trout. *Aquaculture* 72: 165-172.

Hertz, P., Silberman, M., Even, L. and Hochberg, Z. (1989). Effects of sex steroid on the response of cultured rat pituitary cells to growth hormone-releasing hormone and somatostatin. *Endocrinology* 125: 581-585.

Higgs, D. A., Donaldson, E. M., Dye, H. M. and McBride, J. R. (1976). Influence of bovine growth hormone and L-thyroxine on growth, muscle composition, and histological structure of the gonads, thyroid, pancreas, and pituitary of coho salmon (*Oncorhynchus kisutch*). *Journal of the Fisheries Research Board of Canada* 33: 1585-1603.

Higgs, D. A. and Eales, J. G. (1979). The influence of diet composition on radiothyronine kinetics in brook trout, *Salvelinus fontinalis* (Mitchill). *Canadian Journal of Zoology* 57: 396-402.

Higgs, D. A., Fagerlund, U. H. M., Eales, J. G. and McBride, J. R. (1982). Application of thyroid and steroid hormones as anabolic agents in fish culture. *Comparative Biochemistry and Physiology* 73B: 143-176.

Himick, B. A., Golosinski, A. A., Jonsson, A.-C. and Peter, R. E. (1993). CCK/gastrin-like immunoreactivity in the goldfish pituitary:

regulation of pituitary hormone secretion by CCK-like peptides in vitro. *General and Comparative Endocrinology* 92: 88-163.

Hobby, A. C., Geraghty, D. P. and Pankhurst, N. W. (2000). Differences in binding characteristics of sex steroid binding protein in reproductive and nonreproductive female rainbow trout (*Oncorhynchus mykiss*), black bream (*Acanthopagrus butcheri*), and greenback flounder (*Rhombosolea tapirina*). *General and Comparative Endocrinology* 120: 249-259.

Holloway, A. C. and Leatherland, J. F. (1997). Effect of gonadal steroid hormones on plasma growth hormone concentrations in sexuallly immature rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology* 105: 246-254.

Holloway, A. C., Melroe, G. T., Ehrman, M. M., Reddy, P. K., Leatherland, J. F., *et al.* (2000). Effect of 17β-estradiol on the expression of somatostatin genes in rainbow trout (*Onchorhynchus mykiss*). *American Journal of Physiology- Regulatory, Integrative and Comparative Physiology* 279: R389-R393.

Houston, B. and O'Neill, L. E. (1991). Insulin and growth hormone act synergistically to stimulate insulin-like growth factor-I production by cultured chicken hepatocytes. *Journal of Endocrinology* 128: 389-393.

Howell, W. M., Black, D. A. and Bortone, S. A. (1980). Abnormal expression of secondary sex characteristics in a population of mosquitofish, *Gambusia affinis holbrooki*: evidence for environmentally-induced masculinization. *Copeia* 4: 676-681.

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

Inui, Y., Mia, S. and Yamano, K. (1994). Hormonal control of flounder metamorphosis. *Perspectives in Comparative Endocrinology*. K. Davey, R. Peter and S. Tobe. Ottawa, ON Canada, National Research Council of Canada: 408-411.

Isaksson, O. G., Jansson, J. O. and Gause, I. A. (1982). Growth hormone stimulates longitudinal bone growth directly. *Science* 216: 1237-1239.

Jansson, J. O., Ekberg, S., Isaksson, O., Mode, A. and Gustafsson, J. A. (1985). Imprinting of growth hormone secretion, body growth, and hepatic steroid metabolism by neonatal testosterone. *Endocrinology* 117(5): 1881-1889.

Jarvinen, A. W., Nordling, B. R. and Henry, M. E. (1983). Chronic toxicity of Dursban (chlorpyrifos) to the fathead minnow (*Pimephales promelas*) and the resultant acetylcholinesterase inhibition. *Ecotoxicology and Environmental Safety* 7: 423-434.

Jensen, A. L., Spigarelli, S. a. and Thommes, M. M. (1982). PCB uptake by five species of fish in Lake Michigan, Green Bay of Lake Michigan and Cayoga Lake, New York. *Canadian Journal of Fisheries and Aquatic Sciences* 45 (Suppl 1): 170-178.

Jin, I. H., Sinha, G., Yballe, C., Vu, T. H. and Hoffman, A. R. (1995). The human insulin-like growth factor-II promoter P1 is not restricted to liver: evidence for expression of P1 in other tissues and for a homologous promoter in baboon liver. *Horm Metab Res* 27: 447-9.

Jobling, S., Sheahan, D., Osborne, J. A., Mattiessen, P. and Sumpter, J. P. (1996). Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environmental Toxicology and Chemistry* 15(2): 194-202.

Jobling, S. and Sumpter, J. P. (1993). Detergent components in sewage effluent are weakly oestrogenic to fish: An *in vitro* study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic Toxicology* 27: 361-372.

Johansen, B., Johnsen, O. C. and Valla, S. (1989). The complete nucleotide sequence of the growth-hormone gene from Atlantic salmon (*Salmo salar*). *Gene* 77: 317-324.

Johnson, T. R., Blossey, B. D., Denko, C. W. and Ilan, J. (1989). Expression of insulin-like growth factor I in cultured rat hepatocytes: effects of insulin and growth hormone. *Molecular Endocrinology* 3(3): 580-587.

Johnstone, R., Simpson, T. H. and Youngson, A. F. (1978). Sex reversal in salmonid culture. *Aquaculture* 13: 115-134.

Jones, J. I. and Clemmons, D. R. (1995). Insulin-like growth factors and their binding proteins: Biological actions. *Endocrine Reviews* 16(1): 3-34.

Jones, P. W., Martin, F. D. and Hardy, J. D. J. (1978). Development of Fishes of the Mid-Atlantic Bight: An Atlas of Egg, Larval and Juvenile Stages, U.S. Fish & Wildlife Service, Office of Biological Services. II. Anguillidae through syngnathidae: 458.

Juul, A. (2001). The effects of oestrogens on linear bone growth. *Human Reproduction Update* 7(3): 303-313.

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

Kagawa, H. and Moriyama, S. (1995). Effects of insulin-like growth factor-I on final maturation of oocytes of red seabream, *Pagrus major, in vitro. _Reproductive Physiology of fish symposium*, Austin, TX 1995.

Kagawa, H., Moriyama, S. and Kawauchi, H. (1995). Immunocyto-chemical localization of IGF-I in the ovary of the red seabream, *Pagrus major. General and Comparative Endocrinology* 99: 307-315.

Kaiya, H., Kojima, M., Hosoda, H., Koda, A., Yamamoto, K., *et al.* (2001). Bullfrog ghrelin is modified by n-octanoic acid at its third threonine residue. *Journal of Biological Chememistry* 276: 40441-40448.

Kajimoto, Y. and Rotwein, P. (1991). Structure of the chicken insulin-like growth factor I gene reveals conserved promoter elements. *Journal of Biological Chemistry* 266: 9724-97231.

Kavsan, V. M., Grebenjuk, V. A., Koval, A. P., Skorokhod, A. S., Roberts, J., C.T. , *et al.* (1994). Isolation of a second nonallelic insulin-like growth factor I gene from the salmon genome. *DNA and Cell Biology* 13: 555-559.

Kavsan, V. M., Koval, A. P., Grebenjuk, V. A., Chan, S. J., Steiner, D. F., *et al.* (1993). Structure of the chum salmon insulin-like growth factor I gene. *DNA and Cell Biology* 12: 729-737.

Kawauchi, H. and Yasuda, A. (1989). Evolutionary aspects of growth hormones from nonmammalian species. *Advances in Growth Hormones and Growth Factor Research.* E. E. Muller, D. Cocchi and V. Locatelli. Roma-Milano and Berlin, Pythagora Press and Springer-Verlag: 51-68.

Kelley, K. M., Gray, E. S., Siharath, K., Nicoll, C. S. and Bern, H. A. (1993). Experimental diabetes mellitus in a teleost fish. II. Roles of insulin, growth hormone (GH), insulin-like growth factor-I (IGF-I) and hepatic GH receptors in diabetic growth inhibition in the goby, *Gillichthys mirabilis. Endocrinology* 132: 2695-2701.

Kelley, K. M., Haigwood, J. T., Perez, M. and Galima, M. M. (2001). Serum insulin-like growth factor binding proteins (IGFBPs) as markers for anabolic/catabolic condition in fishes. *Comparative Biochemistry and Physiology Part B* 129: 229-236.

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

Kiess, W., Haskell, J. F., Lee, L., Greenstein, L. A., Miller, B. E., *et al.* (1987). An antibody that blocks insulin-like growth factor (IGF) binding to the type II IGF receptor is neither an agonist nor an inhibitor of IGF-stimulated biological responses in L6 myoblasts. *Journal of Biological Chemistry* 262: 12745.

Kim, D. S. (1998). Expression of IGF-Ib cDNA clone isolated from *Paralichthys olivaceus* in mammalian CHO cell line using green fluorescence protein (GFP) tagging: secretory production of big IGFIb-GFP fusion proteins from stable transfected CHO cell culture. *GenBank.* Microbiology Department, Pukyong National University, Korea.

Kinhult, A. (1996). Barramundi (*Lates calcarifer*) IGF-I: characterization of cDNA, genomic sequence and regulation of mRNA expression. Brisbane, Australia, Queensland University of Technology.

Kojima, M., Hosoda, H., Matsuo, H. and Kangawa, K. (2001). Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor. *Trends in Endocrinology & Metabolism* 12: 118-122.

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

Koren, Y., Sarid, S., Ber, R. and Daniel, V. (1989). Carp growth hormone: molecular cloning and sequencing of cDNA. *Gene* 77: 309-315.

Kwong, P. and Chang, J. P. (1997). Somatostatin inhibition of growth hormone release in goldfish: Possible targets of intracellular mechanisms of action. *General and Comparative Endocrinology* 108: 446-456.

Larhammar, D. and Risinger, C. (1994). Molecular genetic aspects of tetraploidy in the common carp *Cyprinus carpio*. *Molecular Phylogenetics and Evolution* 3: 59-68.

Laron, Z. (1993). An update on Laron syndrome. *Arch. Dis. Child.* 68(345-346).

Lau, M. M. H., Stewart, C. E. H., Liu, Z., Bhatt, H., Rotwein, P., *et al.* (1994). Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes and Development* 8: 2953-2963.

Law, M. S., Cheng, K. W., Fung, T. K., Chan, Y. H., Yu, K. L., *et al.* (1996). Isolation and characterization of two distinct growth hormone cDNAs from the goldfish, *Carassius auratus*. *Archives of Biochemistry and Biophysiology* 330: 19-23.

Le Gac, F. and Loir, M. (1995). Insulin like growth factor expression, binding and action in trout testis (*Oncorhynchus mykiss*). *Proceedings of the Fifth International Sumposium on the Reproductive Physiology of Fish*, Austin, Texas: 354- 356.

Le Gac, F., Loir, M., Le Bail, P.-Y. and Ollitrault, M. (1996). Insulin-like growth factor (IGF-I) mRNA and IGF-I receptor in trout testis and in isolated spermatogenic and sertoli cells. *Molecular Reproduction and Development* 44: 23-35.

Leatherland, J. F. (1981). Conversion of L-thyroxine to triiodo-L-thyronine in rainbow trout (*Salmo gairdneri*) liver and kidney homogenates. *Comparative Biochemistry and Physiology* 69B: 311-314.

Leatherland, J. F. and Nuti, R. F. (1981). Effects of bovine growth hormone on plasma free fatty acid concentration and liver, muscle, and carcass lipid content in rainbow trout (*Salmo Gairdneri* Richardson). *Journal of Fish Biology* 19: 487-498.

Leatherland, J. F. and Sonstegard, R. A. (1978). Lowering of serum thyroxine and triiodothyronine levels in yearling coho salmon, *Oncorhynchus kisutch*, by dietary Mirex and PCBs. *Canadian Journal of Fisheries and Aquatic Sciences* 35: 1285-1289.

Lee, E. K., Chan, V. C., Chang, J. P., Yunker, W. K. and Wong, A. O. (2000). Norepinephrine regulation of growth hormone release from goldfish pituitary cells. I. Involvement of alpha2 adrenoreceptor and interactions with dopamine and salmon gonadotropin-releasing hormone. *Journal of Neuroendocrinology* 12: 311-322.

Lee, L. T. O., Nong, G., Chan, Y. H., Tse, D. L. Y. and Cheng, C. H. K. (2001). Molecular cloning of a teleost growth hormone receptor and its functional interaction with human growth hormone. *Gene* 270: 121-129.

Leibush, B., Parrizas, M., Navarro, I., Lappove, Y., Maestro, M. A., *et al.* (1996). Insulin and insulin-like growth factor-I receptor in fish brain. *Regulatory Peptides* 61: 155-161.

LeRoith, D., Scavo, L. and Butler, A. (2001). What is the role of circulating IGF-I? *Trends in Endocrinology & Metabolism* 12: 48-52.

LeRoith, D., Werner, H., Beitner-Johnson, D. and Roberts, C.T., Jr. (1995). Molecular and cellular aspects of the insulin- like growth factor I receptor. *Endocrine Reviews* 16: 143-163.

Levin, W., Welsch, R. M. and Conney, A. H. (1968). Inhibitory effect of phenobarbital or chlordane pretreatment on the androgen-induced increase in seminal vesicle weight in the rat. *Steroids* 13: 155-161.

Liang, P. and Pardee, A. B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257: 967-971.

Lin, X. and Peter, R. E. (2001). Somatostatins and their receptors in fish. *Comparative Biochemistry and Physiology Part B* 129: 543-550.

Little, E. E., Archeski, R. D., Flerov, B. A. and Kozlovskaya, V. I. (1990). Behavioral indicators of sublethal toxicity in rainbow trout. *Archives of Environmental Contamination and Toxicology* 19: 380-385.

Loffing-Cueni, D., Schmid, A. C., Graf, H. and Reinecke, M. (1998). IGF-I in the bony fish *Cottus scorpius*: cDNA, expression and differential localization in brain and islets. *Molecular and Cellular Endocrinology* 141: 187-194.

Loffing-Cueni, D., Schmid, A. C. and Reinecke, M. (1999). Molecular cloning and tissue expression of the insulin-like growth factor II prohormone in the bony fish, *Cottus scorpius*. *General and Comparative Endocrinology* 113: 32-37.

Loir, M. and Le Gac, F. (1994). Insulin-like growth factor-I and -II binding and action on DNA synthesis in rainbow trout spermatogonia and spermatocytes. *Biological Reproduction* 51: 1154-1163.

Loukovaara, M., Carson, M., Palotie, A. and Adlercreutz, H. (1995). Regulation of sex hormone-binding globulin production by isoflavonoids and patterns of isoflavonoid conjugation in HepG2 cell cultures. *Steroids*: 656-661.

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

Luo, D., and McKeown, B. A. (1991). Interaction of carp growth hormone-releasing factor and somatostatin on in vitro release of growth hormone in rainbow trout (*Oncorhynchus mykiss*). *Neuroendocrinology* 54: 359-364.

Luo, D. and McKeown, B. A. (1991). The effect of thyroid hormone and glucocorticoids on carp growth hormone-releasing factor (GRF)-induced growth hormone (GH) release in rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology* A99: 621-626.

Luo, D., McKeown, B. A., Rivier, J. and Vale, W. (1990). In vitro responses of rainbow trout (*Oncorhynchus mykiss*) somatotrophs to carp growth hormone-releasing factor (GRF) and somatostatin. *General and Comparative Endocrinology* 80: 288-298.

Maes, M., Underwood, L. E. and Ketelslegers, J.-M. (1986). Low serum somatomedin-C in insulin-dependent diabetes: evidence for a post-receptor mechanism. *Endocrinology* 118: 377-382.

Maes, M., Underwood, L. E. and Ketelslegers, J. M. (1983). Plasma somatomedin-C in fasted and refed rats: close relationship with changes in liver somatogenic but not lactogenic binding sites. *Journal of Endocrinology* 97(2): 243-252.

Maestro, M. A., Mendez, E., Parrizas, M. and Gutierrez, J. (1997). Characterization of insulin and insulin-like growth factor-I ovarian receptors during the reproductive cycle of carp (*Cyprinus carpio*). *Biology of Reproduction* 56: 1126-1132.

Maestro, M. A., Planas, J. V., Moriyama, S., Gutierrez, J., Planas, J., *et al.* (1997). Ovarian receptors for insulin and insulin-like growth factor I (IGF-I) and effects of IGF-I on steroid production by isolated follicular layers of the preovulatory coho salmon ovarian follicle. *General and Comparative Endocrinology* 106: 189-201.

Mancera, J. M. and McCormick, S. D. (1998). Osmoregulatory actions of the GH/IGF axis in non-salmonid teleosts. *Comparative Biochemistry and Physiology* 121(B): 43-48.

Marchant, T. A., Dulka, J. G. and Peter, R. E. (1989). Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, *Carassius auratus L. General and Comparative Endocrinology* 73: 458-468.

Marchant, T. A., Fraser, R. A., Andrews, P. C. and Peter, R. E. (1987). The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus L.*) pituitary fragments in vitro. *Regulatory Peptides* 17: 41-52.

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

Marchant, T. A. and Peter, R. E. (1986). Seasonal variations in body growth rates and circulating levels of growth hormone in the goldfish, *Carassius auratus*. *Journal of Experimental Zoology* 237: 231-239.

Marcovich, D. T. Analysis of chlorpyrifos from seawater. Gulf Breeze, FL 32561, US EPA.

Marti-Palanca, H., Martinez-Barbera, J.P., Pendon, C., Valdivia, M.M., Perez-Sanchez, J. and Kaushik, S. (1996). Growth hormone as a function of age and dietary protein: energy ratio in a marine teleost, the gilthead sea bream (*Sparus aurata*). *Growth Regulation* 6:253-259.

Martin, J. L. and Baxter, R. C. (1999). IGF binding proteins as modulators of IGF action. *Contemporary Endocrinology: The IGF System.* R. Rosenfeld and C. J. Roberts. Totowa, NJ, Humana Press, Inc.: 227-255.

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

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

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

McLachlan, J. A., Arnold, S.F. (1996). Environmental Estrogens. *American Scientist* 84: 452-461.

McRory, J. E. and Sherwood, N. M. (1994). Catfish express two forms of insulin-like growth factor-I (IGF-I) in the brain. *Journal of Biological Chemistry* 269: 18588-18592.

Melamed, P., Eliahu, N., Sevani-Sivan, B., Ofir, M., Farchi-Pisanty, O., et al. (1995). Hypothalamic and thyroidal regulation of growth hormone in tilapia. *General and Comparative Endocrinology* 97: 13-30.

Melamed, P., Gur, G., Elizur, A., Rosenfeld, H., Sivan, B., *et al.* (1996). Differential effects of gonadotropin releasing hormone, dopamine and somatostatin and their second messengers on the mRNA levels of gonadotropin II beta subunit and growth hormone in the teleost fish, tilapia. *Neuroendocrinology* 64: 320-328.

Mendez, E., Planas, J. V., Castillo, J., Navarro, I. and Gutierrez, J. (2001). Identification of a type II insulin-like growth factor receptor in fish embryos. *Endocrinology* 142: 1090-1097.

Mendez, E., Smith, A., Figueiredo-Garutti, J., Planas, V., Navarro, I., *et al.* (2001). Receptors of insulin-like growth factor-I (IGF-I) predominate over insulin receptors in skeletal muscle throughout the life cycle of brown trout, *Salmo trutta. General and Comparative Endocrinology* 122: 148-157.

Meredith, H. O., Richman, H. H., Collier, J. T., Seale, A. P., Riley, L. G., *et al.* (1999). Pesticide effects on prolactin release from the Rostral Pars Distalis *in vitro* and their effects on growth *in vivo* in the tilapia (*Oreochromis mossambicus*). *Environmental Toxicology and Risk Assessment: 8th Volume, ASTM STP 1364.* D. Henshel, American Society for Testing and Materials.

Moav, B. and McKeown, B. A. (1992). Thyroid hormone increases transcription of growth hormone mRNA in rainbow trout pituitary. *Hormone and Metabolism Research* 24: 10-14.

Moav, B. and McKeown, B. A. (1992). Thyroid hormone increases transcription of growth hormone mRNA in rainbow trout pituitary. *Hormone and Metabolic Research* 24: 10-14.

Mommsen, T. P. (1998). Growth and Metabolism. *The Physiology of Fishes*. D. H. Evans. Boca Raton, CRC Press: 65-97.

Mommsen, T. P. (2001). Paradigms of growth in fish. *Comparative Biochemistry and Physiology Part B* 129: 207-219.

Mommsen, T. P. and Moon, T. W. (2001). Hormonal regulation of muscle growth. *Muscle Development and Growth*. I. A. Johnston. San Diego, Academic Press. 18: 251-308.

Montero, M., Yon, L., Kikuyama, S., Dufour, S. and Vaudry, H. (2000). Molecular evolution of the growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide gene family. Functional implication in the regulation of growth hormone secretion. *Journal of Molecular Endocrinology* 25: 157-168.

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

Moriyama, S., Duguay, S. J., Conlon, J. M., Duan, C., Dickhoff, W. W., *et al.* (1993). Recombinant coho salmon insulin-like growth factor I expression in *Escherichia coli*, purification and characterization. *European Journal of Biochemistry* 218: 205-211.

Moriyama, S., Swanson, P., Nishi, M., Takahashi, A., Kawauchi, H., *et al.* (1994). Development of a homologous radioimunoassay for coho salmon insulin-like growth factor-I. *General and Comparative Endocrinology* 96: 149- 161.

Moriyama, S. M., Ayson, F. G. and Kawauchi, H. (2000). Growth regulation by insulin-like growth factor-I in fish. *Bioscience, Biotechnology and Biochemistry* 64: 1553-1562.

Morrione, A., Valentinis, B., Xu, S., Ument, G., Louivi, A., *et al.* (1997). Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. *Proceedings of the National Academy of Sciences, USA* 94: 3777-3782.

Mosselman, S., Polman, J. and Dijkema, R. (1996). ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Letters* 392(1): 49-53.

Moyle, P. B. and Cech, J. J., Jr. (2000). Fishes: An introduction to ichthyology. Upper Saddle River, NJ, Prentice-Hall, Inc.: 111-122.

Munkittrick, K. R., Van Der Kraak, G. J., McMaster, M. E. and Portt, C. B. (1992). Response of hepatic MFO activity and plasma sex steroids to secondary treatment of bleached kraft pulp mill effluent and mill shutdown. *Environmental Toxicology and Chemistry* 11: 1427-1439.

Munkittrick, K. R., Van Der Kraak, G. J., McMaster, M. E., Portt, C. B., van den Heuvel, M. R., *et al.* (1994). Survey of receiving-water environmental impacts associated with discharges from pulp mills. 2. Gonad size, liver size, hepatic EROD activity and plasma sex steroid levels in white sucker. *Environmental Toxicology and Chemistry* 13: 1089-1101.

Murphy, L. J. (1991). The uterine insulin-like growth factor system. *Modern Concepts of Insulin-like Growth Factors*. E. M. Spencer, Elsevier Science Publishing Co., Inc.: 275-284.

Murphy, L. J. and Friesen, H. G. (1988). Differential effects of estrogen and growth hormone on uterine and hepatic insulin-like growth factor I gene expression in the ovariectomized hypohysectomized rat. *Endocrinology* 122: 325- 332.

Murphy, L. J., Murphy, L. C. and Friesen, H. G. (1987). Estrogen induces insulin-like growth factor-I expression in the rat uterus. *Molecular Endocrinology* 1(7): 445-450.

Murphy, W. R., Daughaday, W. H. and Hartnett, C. (1956). The effect of hypophysectomy and growth hormone on the incorporation of labeled sulfate into tibila epiphyseal and nasal cartilage of the rat. *Journal of Laboratory and Clinical Medicine* 47(715-722).

Nader, M. R., Miura, T., Ando, N., Miura, C. and Yamauchi, K. (1999). Recombinant human insulin-like growth factor I stimulates all stages of 11-ketotestosterone-induced spermatogenesis in the Japanese, eel, *Anguilla japonica, in vitro*. *Biology of Reproduction* 61: 944-947.

Nagamatsu, S., Chan, S. J., Falkmer, S. and Steiner, D. F. (1991). Evolution of the insulin gene superfamily: Sequence of a preproinsulin-like growth factor cDNA from the Atlantic hagfish. *Journal of Biological Chemistry* 266: 2397-2402.

Nagaoka, I., Someya, A., Iwabuchi, K. and Yamashita, T. (1991). Expression of insulin-like growth factor-IA and factor-IB mRNA in human liver, hepatoma cells, macrophage-like cells and fibroblasts. *Federation of European Biochemical Societies Letters* 280: 79-83.

Nagaraj, C. G. and Rao, G. P. S. (1988). Effect of testosterone acetate and estradiol benzoate on sexuality and growth of *Cyprinus carpio* (Linn.). *The First Indian Fisheries Forum, Proceedings* - December 4-8, 1987. M. M. Joseph: 115-117.

Nagler, J.J., Bouma, J., Thorgaard, G.H. and Dauble, D.D. (2001). High incidence of a male-specific genetic marker in phenotypic female chinook salmon from the Columbia River. *Environmental Health Perspectives* 109: 67-69.

Nakao, Y., Tanaka, M. and Nakashima, K. (1999). Complementary DNA cloning and characterization of two messenger RNA species of flounder insulin receptor family. Japan Society for Comparative Endocrinology, Nagoya.

Negatu, A., Hsiao, S. M. and Wallace, R. A. (1998). Effects of insulin-like growth factor-I on final oocyte maturation and steroid production in *Fundulus heteroclitus*. *Fish Physiology and Biochemistry* 19: 13-21.

Negatu, Z. and Meier, A. H. (1995). In vitro incorporation of [14 C] glycine into muscle protein of Gulf killifish (*Fundulus grandis*) in response to insulin-like growth factor-1. *General and Comparative Endocrinology* 98: 193-201.

Nilsson, A., Isgaard, J., Lindahl, A., Dahlstrom, A., Skottner, A., *et al.* (1986). Regulation by growth hormone of the number of chondrocytes containing IGF-I in rat growth plate. *Science* 233: 571-574.

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

Noakjsson, E., Tjarnlund, U., Bosveld, A. T. and Balk, L. (2001). Evidence for endocrine disruption in perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) in a remote Swedish lake in the vicinity of a public refuse dump. *Toxicology and Applied Pharmacology* 174(2): 160-176.

Norris, D. O. (1997). *Vertebrate Endocrinology*. San Diego, CA, Academic Press.

Oberdoerster, E. and Cheek, A. O. (2000). Gender benders at the beach: Endocrine disruption in marine and estuarine organisms. *Environmental Toxicology and Chemistry* 20: 23-36.

O'Brien, R. M. and Granner, D. K. (1996). Regulation of gene expression by insulin. *Physiological Reviews* 76: 1109-1161.

O'Brien, R. M., Streeper, R. S., Ayla, J. E., Stadelmaier, B. T. and Hornbuckle, L. A. (2001). Insulin-regulated gene expression. *Biochemistry Society Transactions* 29(Pt. 4): 552-558.

O'Connor, P. K., Reich, B. and Sheridan, M. A. (1993). Growth hormone stimulates hepatic lipid mobilization in rainbow trout, *Onchorhynchus mykiss*. *Journal of Comparative Physiology* 163 B(427-431).

O'Dell, S. D. and Day, I. N. (1998). Insulin-like growth factor II (IGF-II). *The International Journal of Biochemistry & Cell Biology* 30: 767-771.

Ostrowski, A. C. and Garling, J., D.L. (1986). Dietary androgen-estrogen combinations in growth promotion in fingerling rainbow trout. *The Progressive Fish-Culturist* 48: 268-272.

Ovrevik, J., Stenersen, J., Nilssen, K. and Tollefsen, K. E. (2001). Partial characterization of a sex steroid-binding protein in plasma from arctic charr (*Salvelinus alpinus L.*). *General and Comparative Endocrinology* 122: 31-39.

Palamarchuk, A. Y., Holthuizen, P. E., Muller, W. E., Sussenbach, J. S. and Kavsan, V. M. (1997). Organization and expression of the chum salmon insulin-like growth factor II gene. *FEBS Letters* 416: 344-348.

Palamarchuk, A. Y., Kavsan, V. M., Sussenbach, J. S. and Holthuizen, P. E. (1999). The chum salmon IGF-II gene promoter is activated by hepatocyte nuclear factor 3β. *FEBS Letters* 446: 251-255.

Park, R., Shepherd, B. S., Nishioka, R. S., Grau, E. G. and Bern, H. A. (2000). Effects of homologous pituitary hormone treatment on serum insulin-like growth factor binding proteins (IGFBPs) in hypophysectomized tilapia , *Oreochromis mossambicus*, with special reference to a novel 20-kDa IGFBP. *General and Comparative Endocrinology* 117: 404-412.

Parker, D. B., Power, M. E., Swanson, P., Rivier, J. and Sherwood, N. M. (1997). Exon skipping in the gene encoding pituitary adenylate cyclase-activating polypeptide in salmon alters the expression of two hormones that stimulate growth hormone release. *Endocrinology* 138: 414-423.

Parrizas, M., Maestro, M. A., Banos, N., Navarro, I., Planas, J., *et al.* (1995). Insulin/IGF-I binding ratio in skeletal and cardiac muscles of vertebrates: A phylogenetic approach. *American Journal of Physiology* 38: R1370-R1377.

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

Patlak, M. (1996). A testing deadline for endocrine disrupters. *Environmental Science and Technology* 30:540-544.

Pavlov, D. D., Chuiko, G. M., Gerassimov, Y. V. and Tonkopiy, V. D. (1992). Feeding behavior and brain acetylcholinesterase activity in bream (*Abramis brama L.*) as affected by DDVP, an organophosphorus insecticide. *Comparative Biochemistry and Physiology* 103C(3): 563-568.

Peng, C. (1993). Role of neuropeptide Y on growth hormone and gonadotropin-II secretion in the goldfish, University of Alberta: 198.

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

Perez-Sanchez, J., Smal, J. and LeBail, P.-Y. (1991). Location and characterization of growth hormone binding sites in the central nervous system of a teleost fish (*Oncorhynchus mykiss*). *Growth Regulation* 1: 145-152.

Perez-Sanchez, J., Weil, C. and Le Bail, P. Y. (1992). Effects of human insulin-like growth factor-I on release of growth hormone by rainbow trout (*Oncorhynchus mykiss*) pituitary cells. *Journal of Experimental Zoology* 262: 287-290.

Perrot, V., Moiseeva, E. B., Gozes, Y., Chan, S. J., Ingleton, P., *et al.* (1999). Ontogeny of the insulin-like growth factor system (IGF-I, IGF-II, and IGF-1R) in the gilthead seabream (*Sparaus aurata*): Expression and cellular localization. *General and Comparative Endocrinology* 116: 445-460.

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

Peyon, P., Baloche, S. and Burzawa-Gerard, E. (1996). Potentiating effect of growth hormone on vitellogenin synthesis induced by 17 β-estradiol in primary culture of female silver eel (*Anguilla anguilla* L.) hepatocytes. *General and Comparative Endocrinology* 102: 263-273.

Phillips, L. S., Goldstein, S. and Pao, C. I. (1991). Nutrition and somatomedin. XXVI. Molecular regulation of IGF-I by insulin in cultured rat hepatocytes. *Diabetes* 40(11): 1525-1530.

Pickering, A. D., Pottinger, T. G., Sumpter, J. P., Carragher, J. F. and Le Bail, P. Y. (1991). Effects of acute and chronic stress on the levels of circulating growth hormone in the rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* 83: 86-93.

Plisetskaya, E. M. and Duan, C. (1994). Insulin and insulin-like growth factor I coho salmon *Oncorhynchus kisutch* injected with streptozotocin. *American Journal of Physiology* 267: R1408- 1412.

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

Pombo, C. M., Zalvide, J., Gaylinn, B. D. and Diegues, C. (2000). Growth hormone-releasing hormone stimulates mitogen-activated protein kinase. *Endocrinology* 141: 2113-2119.

Pombo, M., Pombo, C. M., Garcia, A., Caminos, E., Gualillo, O., *et al.* (2001). Hormonal control of growth hormone secretion. *Hormone Research* 55(Suppl. 1): 11-16.

Pozios, K. C., Ding, J., Degger, B., Upton, Z. and Duan, C. (2001). IGFs stimulate zebrafish cell proliferation by activating MAP kinase and PI3-kinase-signaling pathways. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 280: R1230-R1239.

Racke, K. D. (1993). *Environmental Fate of Chlorpyrifos*. New York, NY, Springer-Verlag.

Rajaram, S., Baylink, D. J. and Mohan, S. (1997). Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. *Endocrine Reviews* 18: 801-831.

Reinecke, M., Schmid, A., Ermatinger, R. and Loffig-Cueni, D. (1997). Insulin-like growth factor I in the teleost *Oreochromis*

mossambicus, the tilapia: Gene sequence, tissue expression, and cellular localization. *Endocrinology* 138: 3613-3619.

Robinson, I. C. A. F. (2000). *Control of growth hormone (GH) release by GH secretagogues*. Novartis Foundation Symposium.

Rocha Monteiro, P. R., Reis-Henriques, M. A. and Coimbra, J. (2000). Polycyclic aromatic hydrocarbons inhibit in vitro ovarian steroidogenesis in the flounder (*Platichthys flesus* L.). *Aquatic Toxocology* 48(4): 549-559.

Rodgers-Gray, T. P., Jobling, S., Kelly, C., Morris, S., Brighty, G., *et al.* (2001). Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. *Environmental Science and Technology* 35(3): 462-470.

Rosenfeld, R. G. and Roberts, C.T., Jr. Eds. (1999). *The IGF System. Molecular Biology, Physiology and Clinical Applications.* Contemporary Endocrinology. Totawa, New Jersey, Humana Press.

Rotwein, P. (1991). Structure, evolution, expression and regulation of insulin-like growth factors I and II. *Growth Factors* 5: 3-18.

Rotwein, P. (1999). Molecular biology of IGF-I and IGF-II. *The IGF System: molecular biology physiology and clinical applications*. R. G. Rosenfeld and C. T. Roberts, Jr. Totowa, NJ, Humana Press: 19-35.

Sakamoto, T. and Hirano, T. (1991). Growth hormone receptors in the liver and osmoregulatory organs of rainbow trout: characterization and dynamics during adaptation to sea water. *Journal of Endocrinology* 130: 425-433.

Sakamoto, T., Hirano, T., Madsen, S. S., Nishioka, R. S. and Bern, H. A. (1995). Insulin-like growth factor I gene expression during parr-smolt transformation of coho salmon. *Zoological Science* 12: 249-252.

Sakamoto, T., Iwata, M. and Hirano, T. (1991). Kinetic studies of growth hormone and prolactin during adaptation of coho salmon, *Oncorhynchus kisutch*, to different salinities. *General and Comparative Endocrinology* 82: 184-191.

Sakamoto, T., McCormick, S. D. and Hirano, T. (1993). Osmoregulatory actions of growth hormone and its mode of action in salmon: a review. *Fish Physiology and Biochemistry* 11: 155-164.

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

Sara, V. R. and Hall, K. (1990). Insulin-like growth factors and their binding proteins. *Physiological Reveiws* 70: 591-607.

Scacchi, M., Danesi, L., Pincelli, A. I., Dubini, A. and Cavagnini, F. (1997). Effect of chronic treatment with biosynthetic growth hormone (GH) in the GH response to double GH-releasing hormone administration in children with short stature. *Journal of Endocrinological Investigation* 20: 72-76.

Schmid, A. C., Naf, E., Kloas, W. and Reinecke, M. (1999). Insulin-like growth factor-I and -II in the ovary of a bony fish, *Oreochromis mossambicus*, the tilapia: in situ hybridisation, immunohistochemical localisation, Northern blot and cDNA sequences. *Molecular and Cellular Endocrinology* 156: 141-149.

Sekine, S., Mizukami, T., Nishi, T., Kuwana, Y., Saito, A., *et al.* (1985). *Proceedings of the National Academy of Sciences* 82: 4306-4310.

Sekkali, B., Brim, H., Muller, M., Argenton, F., Bortolussi, M., *et al.* (1999). Structure and functional analysis of a tilapia (*Oreochromis mossambicus*) growth hormone gene: activation and repression by pituitary transcription factor Pit-1. *DNA Cell Biology* 18: 489-502.

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

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

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

Shamblott, M. J., Leung, S.-M., Greene, M. W. and Chen, T. T. (1998). Characterization of a teleost insulin-like growth factor II (IGF-II) gene: evidence for promoter CCAAT/enhancer-binding protein (C/EBP) sites, and the presence of hepatic C/EBP. *Molecular Marine Biology and Biotechnology* 7: 181-190.

Shepherd, B. S., Eckert, S. M., Parhar, I. S., Vijayan, M. M., Wakabayashi, I., *et al.* (2000). The hexapeptide KP-102 (D-ala-D-beta-Nal-ala-trp-D-phe-lys-NH(2)) stimulates growth hormone release in a cichlid fish (*Ooreochromis mossambicus*). *Journal of Endocrinology* 167: R7-10.

Shepherd, B. S., Sakamoto, T., Nishioka, R. S., Richman, N. H., III, Mori, I., *et al.* (1997). Somatotropic actions of the homologous growth hormone and prolactins in the euryhaline teleost, the tilapia, *Oreochromis mossambicus*. *Proceedings of the National Academy of Sciences of the U.S.A.* 94: 2068-2072.

Sheridan, M. A. (1986). Effects of thyroxin, cortisol, growth hormone, and prolactin on lipid metabolism of coho salmon, *Oncorhynchus kisutch*, during smoltification. *General and Comparative Endocrinology* 64: 220-238.

Shimizu, M., Swanson, P. and Dickhoff, W. W. (1999). Free and protein-bound insulin-like growth factor-I (IGF-I) and IGF-binding proteins in plasma of coho salmon, *Oncorhynchus kisutch*. *General and Comparative Endocrinology* 115: 398-405.

Shore, L. S., Gurevitz, M. and Shemesh, M. (1993). Estrogen as an environmental pollutant. *Bulletin of Environmental Contamination and Toxicology* 51: 361-366.

Siharath, K. and Bern, H. A. (1993). The physiology of insulin-like growth factors (IGF) and its binding proteins in teleost fishes. *Proc. Zool. Soc., Calcutta, Haldane Comm. Volume*: 113-124.

Siharath, K., Nishioka, R. S., Madsen, S. S. and Bern, H. A. (1995). Regulation of IGF-binding proteins by growth hormone in the striped bass, *Morone saxatilis*. *Molecular Marine Biology and Biotechnology* 4: 171-178.

Sinha, H., Lal, B. and Singh, T. P. (1991). Pesticides induced changes in circulating thyroid hormones in the freshwater catfish *Clarias batrachus*. *Comparative Biochemistry and Physiology C* 100: 107-110.

Sinha, N., Lal, B. and Singh, T. P. (1991). Effect of endosulfan on thyroid physiology in the freshwater catfish, *Clarias batrachus*. *Toxicology* 67: 187-197.

Sivarajah, K., Franklin, C. S. and Williams, W. P. (1978). The effects of polychlorinated biphenyls on plasma steroid levels and hepatic microsomal enzymes in fish. *Journal of Fish Biology* 13: 401-409.

Small, B. C. and Nonneman, D. (2001). Sequence and expression of a cDNA encoding both pituitary adenylate cyclase activating polypeptide and growth hormone-releasing hormone-like peptide in channel catfish (*Ictalurus punctatus*). *General and Comparative Endocrinology* 122: 354-363.

Sohm, F., Manfroid, I., Pezet, A., Rentier-Delrue, F., Rand-Weaver, M., *et al.* (1998). Identification and modulation of a growth hormone-binding protein in rainbow trout (*Oncorhyncnus mykiss*) plasma during seawater adaptation. *General and Comparative Endocrinology* 111: 216-224.

Somoza, G. M. and Peter, R. E. (1991). Effects of serotonin on gonadotropin and growth hormone release from in vitro perifused goldfish pituitary fragments. *General and Comparative Endocrinology* 82: 103-110.

Sower, S. A., Schreck, C. B. and Evenson, M. (1983). Effects of steroids and steroid antagonists on growth, gonadal development, and RNA/DNA ratios in juvenile steelhead trout. *Aquaculture* 32: 243-254.

Sperry, T. D. and Thomas, P. (1999). Characterization of two nuclear androgen receptors in Atlantic croaker: Comparison of their biochemical properties and binding specificities. *Endocrinology* 140: 1602-1611.

Sperry, T. S. and Thomas, P. (1999). Identification of two nuclear androgen receptors in kelp bass (*Paralabrax clathratus*) and their binding affinities for xenobiotics: Comparison with Atlantic croaker (*Microgpogonias undulatus*) androgen receptors. *Biology of Reproduction* 61: 1152-1161.

Stewart, C. E. H. and Rotwein, P. (1996). Growth, differentiation, and survival: Multiple physiological functions for insulin-like growth factors. *Physiological Reviews* 76: 1005-1026.

Stone, R. (1995). Environmental estrogens stir debate. *Science* 265: 308-310.

Sumpter, J. P. (1995). Feminized responses in fish to environmental estrogens. *Toxicology Letters* 82/83: 737-742.

Sumpter, J. P. (1998). Xenoendocrine disrupters -- environmental impacts. *Toxicology Letters* 102-103: 337-342.

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

Sun, X., Zhu, S., Chan, S. S. H., Toresson, G. and Cheng, H. K. (1997). Identification and characterization of growth hormone receptors in snakehead fish (*Ophiocephalus argus* Cantor) liver. *General and Comparative Endocrinology* 108: 374-385.

Sweeting, R. M. and McKeown, B. A. (1986). Somatostatin reduces plasma growth hormone levels during seawater adaptation in coho salmon, *Oncorhynchus kisutch*. *Canadian Journal of Zoology* 64: 2062-2063.

Symons, P. E. K. (1973). Behavior of young Atlantic salmon (*Salmo salar*) exposed or force-fed fenitrothion, an organophosphate insecticide. *Journal of the Fisheries Research Board of Canada* 30: 651-655.

Tabak, H. H., Bloomhuff, R. M. and Bunch, R. L. (1981). Studies on the persistence and stability of natural urinary and synthetic ovulation-inhibiting hormones in untreated and treated wastewaters. *Dev. Ind. Microbiol.* 22: 497-519.

Takagi, Y. and Bjornsson, B.Th. (1996). Regulation of cartilage glycosaminoglycan synthesis in the rainbow trout, *Oncorhynchus mykiss*, by 3,3', 5-tri-iodo-L-thyronine and IGF-I. *Journal of Endocrinology* 149: 357-365.

Tanaka, M., Taniguchi, T., Yamamoto, I., Sakaguchi, K., Yoshizato, H., *et al.* (1998). Gene and cDNA structures of flounder insulin-like growth factor-I (IGF-I): Multiple mRNA species encode a single short mature IGF-I. *DNA and Cell Biology* 17: 859-868.

Thissen, J.-P., Ketelslegers, J.-M. and Underwood, L.E. (1994). Nutritional regulation of the insulin-like growth factors. *Endocrine Reviews* 15:80-101.

Thomas, P. (2000). Chemical interference with genomic and nongenomic actions of steroids in fishes: role of receptor binding. *Marine Environmental Research* 50: 127-134.

Thrailkill, K. (2000). Insulin-like growth factor-I in diabetes mellitus: its physiology, metabolic effects, and potential clinical utility. *Diabetes Technology and Therapy* 2(1): 69-80.

Tian, X. C., Chen, M. J., Pantschenko, A. G., Yang, T. J. and Chen, T. T. (1999). Recombinant E-peptides of pro-IGF-I have mitogenic activity. *Endocrinology* 140: 3387-3390.

Trudeau, V. L., Somoza, G. M., Nahorniak, C. S. and Peter, R. E. (1992). Interactions of estradiol with gonadotropin-releasing hormone and thyrotropin-releasing hormone in the control of growth hormone secretion in the goldfish. *Neuroendocrinology* 56: 483-490.

Tsai, P. I., Madsen, S., McCormick, S. D. and Bern, H. A. (1994). Endocrine Control of cartilage growth in coho salmon: GH influence *in vivo* on the response to IGF-I *in vitro*. *Zoological Science* 11: 299-303.

Tse, M. C. L., Chan, K. M. and Cheng, C. H. K. (2001). PCR-cloning and Gene Expression Studies on Common Carp (*Cyprinus carpio*) Insulin-like Growth Factor-II. *GenBank*. Hong Kong, Biochemistry, Chinese University of Hong Kong, Shatin.

Tsukada, A., Ohkubo, T., Sakaguchi, K., Tanaka, M., Nakashima, K., *et al.* (1998). Thyroid hormones are involved in insulin-like growth factor-I (IGF-I) production by stimulating hepatic growth hormone receptor (GHR) gene expression in the chicken. *Growth Hormone and IGF Research* 8: 235-242.

U.S. Environmental Protection Agency. Methods SW-846-3500B (Organic Extraction and Sample Preparation), 3510C (Separatory Funnel Extractions), and 8010B (Analysis of Halogenated Volatile Organics by Gas Cromatography). Washington DC 20460, Office of Solid Waste.

U.S. Environmental Protection Agency. Recommended Guidelines for Measuring Organic Compounds in Puget Sound Water, Sediment, and Tissue Samples. 1200 Sixth Ave., Seattle, WA 98101, Region 10.

Uretsky, A. D. and Chang, J. P. (2000). Evidence that nitric oxide is involved in the regulation of growth hormone-secretion in goldfish. *General and Comparative Endocrinology* 118: 461-470.

Van Der Kraak, G., Rosenblu, P. M. and Peter, R. E. (1990). Growth hormone-dependent potentiation of gonadotropin- stimulated steroid production by ovarian follicles of the goldfish. *General and Comparative Endocrinology* 79: 233-239.

Van Wyk, J. J. (1991). Frontiers of research on the somatomedins/insulin-like growth factors. *Modern Concepts of Insulin-like Growth Factors*. E. M. Spencer, Elsevier Science Publishing Co., Inc.: 691-703.

Vu, T. H. and Hoffman, A. R. (1994). Promoter-specific imprinting of the human insulin-like growth factor II gene. *Nature* 371: 714-717.

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

Ware, D. M. (1980). Bioenergetics of stock and recruitment. *Canadian Journal of Fisheries and Aquatic Sciences* 37: 1012-1024.

Weber, G. M. and Sullivan, C. V. (2000). Effects of insulin-like growth factor-I on in vitro final oocyte maturation and ovarian steroidogenesis in striped bass, *Morone saxatilis*. *Biology of Reproduction* 63: 1049-1057.

Weiss, C. M. (1961). Physiological effect of organic phosphorus insecticides on several species of fish. *Transactions of the American Fisheries Society* 90: 143-152.

Welch, R. M., Levin, R., Kuntzman, M., Jacobson, M. and Conney, A. H. (1971). Effect of halogenated hydrocarbon insecticides on the metabolism and uterotropic action of estrogens in rats and mice. *Toxicology and Applied Pharmacology* 19: 234-246.

Werner, H. and LeRoith, D. (2000). New concepts in regulation and function of the insulin-like growth factors: implications for understanding normal growth and neoplasia. *Cellular and Molecular Life Science* 57: 932-942.

White, R., Jobling, S., Hoare, S. A., Sumpter, J. P. and Parker, M. G. (1994). Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* 135(1): 175-181.

Wolf, M., Ingbar, S. H. and Moses, A. C. (1989). Thyroid hormone and growth hormone interact to regulate insulin-like growth factor-I messenger ribonucleic acid and circulating levels in the rat. *Endocrinology* 125: 2905-2914.

Wong, A. O., Leung, M. Y., Shea, W. L., Tse, L. Y., Chang, J. P., et al. (1998). Hypophysiotropic action of pituitary adenylate cyclase-activating polypeptide (PACAP) in the goldfish: immunohistochemical demonstration of PACAP in the pituitary, PACAP

stimulation of growth hormone release from pituitary cells, and the molecular cloning of pituitary type I PACAP receptor. *Endocrinology* 139: 3465-3479.

Wong, A. O., Li, W. S., Lee, E. K., Leung, M. Y., Tse, L. Y., *et al.* (2000). Pituitary adenylate cyclase activating polypeptide as a novel hypophysiotropic factor in fish. *Biochemistry and Cell Biology* 78: 329-343.

Woo, N. Y. S., Chung, A. S. B. and Ng, T. B. (1991). Influence of oral administration of 2,5,2,-triiodo-L-thyronine on growth, digestion, food conversion and metabolism in the underyearling red seabream, *Chrysophrys major* (Temminck & Schlegel). *Journal of Fish Biology* 39: 459-468.

Yada, T. and Hirano, T. (1992). Inhibition of growth hormone synthesis by somatostatin in cultured pituitary of rainbow trout. *Journal of Comparative Physiology* B162: 575-580.

Yada, T., Kobayashi, T., Urano, A. and Hirano, T. (1992). Changes in growth hormone and prolactin messenger ribonucleic acid levels during seawater adaptation of amago salmon (*Oncorhynchus rhodurus*). *Journal of Experimental Zoology* 262: 420-425.

Yada, T., Urano, A. and Hirano, T. (1991). Growth hormone and prolactin gene expression and release in the pituitary of rainbow trout in serum-free culture. *Endocrinology* 129: 1183-1192.

Yamaguchi, K., Moriyama, S. and Kawauchi, H. (1999). *Molecular cloning of insulin-like growth factor-II cDNA in Japanese eel, Anguilla japonica.* Proceedings of the Annual Meeting of the Japanese Society of Fisheries Science, Sendai, (Abstract 441).

Yamaguchi, K., Moriyama, S., Takahashi, A., Kawauchi, H. and Sower, S. A. (1999). *Cloning of lamprey insulin-like growth factor cDNA.* Japan Society of Comparative Endocrinology, Nagoya.

Yang, B. Y., Chan, K. M., Lin, C. M. and Chen, T. T. (1997). Characterization of rainbow trout (*Oncorhynchus mykiss*) growth hormone 1 gene and the promoter region of growth hormone 2 gene. *Archives of Biochemistry and Biophysics* 340: 359-368.

Yu, T. C., Sinnhuber, R. O. and Hendricks, J. D. (1979). Effect of steroid hormones on the growth of juvenile coho salmon (*Oncorhynchus kisutch*). *Aquaculture* 16: 351-359.

Zhang, W.-M., Lin, H.-R. and Peter, R. E. (1994). Episodic growth hormone secretion in the grass carp, *Ctenopharyngodon idellus* (C. & V.). *General and Comparative Endocrinology* 95: 337-341.

Zhang, Y. and Marchant, T. A. (1999). Identification of serum GH-binding proteins in the goldfish (*Carassius auratus*) and comparison with mammalian GH-binding proteins. *Journal of Endocrinology* 161: 255-262.

Zhu, Z., Ling, H. and Chen, T. T. (1992). Primary structure and evolutionary analysis of the growth hormone gene from grass carp (*Ctenopharyngodon idellus*). *European Journal of Biochemistry* 207: 643-648.

Zou, J. J., Trudeau, V. L., Cui, Z., Brechin, J., Makenzie, K., *et al.* (1997). Estradiol stimulates growth hormone production in female goldfish. *General and Comparative Endocrinology* 106: 102-112.

APPENDIX

APPENDIX
DETAILED METHODS

RNA ISOLATION

Obtaining high-quality, intact total RNA is the first and possibly the most critical step in performing many molecular biology techniques including differential display analysis, quantitative real-time PCR (TaqMan), Northern analysis and in vitro translation. High-quality RNA is not degraded and has no DNA or protein contamination.

Special precautions are necessary in the laboratory because of the sensitivity of RNA to degradation by ubiquitous RNases. RNases are difficult to inactivate. Endogenous RNases are found in tissues and cells, while exogenous sources such as reagents, glassware, hands, etc. can also be sources of RNase contamination. The most common sources of RNase contamination bacteria and molds that may be present in airborne particles and RNases from the hands of the researcher. Gloves should be worn at all times and changed frequently, especially after handling commonly used items such as refrigerator handles and other laboratory equipment. Proper sterile technique should be used when handling reagents and RNA samples. Sterile, individually wrapped disposable plasticware is generally considered RNase-free. Non-disposable glass labware should be baked at 450°C overnight. Autoclaving is not effective at destroying RNases. Water, all solutions and buffers used for RNA isolation should be treated with 0.05% diethyl pyrocarbonate (DEPC) overnight at room temperature and then autocolaved for 30 min to remove trace DEPC.

Tris buffer cannot be treated with DEPC because DEPC adversely affects the buffer. Most commonly used buffers and reagents are available "nuclease free" from various vendors. All reagents and buffers used for RNA should be labeled for "RNA Use Only" and the bottles should only be handled with gloves. Only aerosol- resistant tips (ART tips) should be used for pipetting. A separate set of pipetters should be dedicated to RNA use and cleaned at least weekly with an RNase eliminating solution such as RNase-Zap (Ambion) or RNase Away (Molecular Bioproducts). Counter tops and lab benches as well as any other surfaces that could come in contact with the RNA samples (such as mini gel boxes and gel molds) should also be periodically cleaned with one of those solutions.

Several methods exist for RNA isolation. Each method depends on the quality of the tissues for isolation of intact RNA. Since most of the actual RNA isolation procedures take place in a strong denaturant (e.g. guanidinim thyocyanate (GITC), lithium chloride (LiCl), sodium dodecyl sulphate (SDS), phenol) that renders RNases inactive, it is typically before and after isolation that RNA integrity is at risk. Endogenous RNases can quickly degrade RNA during harvesting of tissues. Tissues should be flash-frozen in liquid nitrogen immediately after harvesting to prevent degradation and stored at –80°C until processed. I have stored tissues for over a year before isolating RNA with good results.

Guanidinium Thiocyanate and Acid Phenol Extraction Method

This method for total RNA isolation was developed by Chomczynski and Sacchi [Chomczynski, 1987 #1093]. In general, the method involves homogenizing tissues or cells in a strong denaturing

solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol). After homogenization, the RNA is extracted by an acid phenol extraction. The low pH (4.5) of the phenol causes the RNA to move to the aqueous phase while DNA and proteins move to the organic phase and interphase. The homogenate is mixed with water saturated phenol, chloroform:isoamyl alcohol (24:1) and sodium acetate by vortexing and subsequent centrifugation. The aqueous supernatant contains the RNA, which is precipitated with isopropanol followed by centrifugation at 4°C. The resulting RNA pellet is washed with 75% ethanol and resuspended in water or buffer. While this method produces high-quality RNA, it is a time consuming method involving several toxic chemicals.

I first attempted RNA isolation from sheepshead minnow livers using a modification of the Chomczynski and Sacchi method as advised by collaborators at the University of Florida's Differential Display Core (personal communication). Several attempts using this method failed to yield significant amounts of RNA. The poor results may have been due to my inexperience, the small amount of starting material, or because the procedure involved several extraction steps. It was important not to disturb the interface between the phases when removing the aqueous layer during phenol/chloroform extraction so that contaminating proteins and DNA would not be transferred with the RNA. This resulted in leaving a substantial quantity of aqueous phase behind. Large percentages of RNA can be lost with each extraction due to inefficient removal of the aqueous phase.

Detailed Procedure

Frozen livers ranging in size from <10 mg to 50 mg were homogenized in denaturing buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol) using a Polytron PT1200 hand-held homogenizer (Kinematica A.G., Switzerland). The livers were homogenized for 30 – 40 sec. The homogenate was mixed with ½ vol of phenol and ½ vol of chloroform:isoamyl alcohol (24:1) to extract the RNA. The samples were votexed for 3 min, chilled on ice for 15 min and then centrifuged at 10,000 X g for 20 min at 4°C. The aqueous layer was removed, transferred to a fresh, RNase-free tube and the extraction procedure was repeated.

The RNA was precipitated by adding an equal volume of isopropanol, then stored overnight at –20° C followed by centrifugation at 10,000 X g for 20 min at 4° C the next day. The resulting RNA pellet was washed with 0.5 ml ice-cold 75% ethanol and allowed to air dry for about 5 min. The RNA pellet was then treated with Proteinase K (described in 1.2).

Proteinase K treatment

Fish livers tend to have endogenous RNases that are reactivated in subsequent molecular biological reactions such as PCR (N. Denslow, personal communication). To eliminate the RNases, the RNA pellet was treated with Proteinase-K. The dry RNA pellet was resuspended with 750 µl Proteinase-K solution (50 mM Tris-HCl, pH 7.5; 0.5 M EDTA, pH 8; 0.5% SDS; 100 µg/ml Proteinase K in DEPC-treated water). The solution was incubated at 37° C for 30 min. The

RNA was extracted by adding an equal volume of phenol/ chloroform:isoamyl alcohol (5:1), vortexing for 30 sec and centrifuging at 25° C for 5 min. The aqueous layer was removed with a pipette to a clean tube. The RNA was precipitated by adding 1/10 vol 3M sodium acetate, pH 5.2 and then 3 vol of 100% ethanol and allowed to precipitate overnight at -20° C. The sample was centrifuged at 10,000 X g for 20 min at 4°C. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol. After washing, the ethanol was removed and the pellet allowed to air dry. The RNA pellet was resuspended in 50µl DEPC-treated water. The RNA was then treated with DNase.

DNase treatment

A DNase-digestion was performed to eliminate possible genomic DNA contamination. The resuspended RNA was added to a DNase reaction mixture (20 µl 5X Transcription Buffer (Promega, Inc.), 100 mM $CaCl_2$; 10 U RNase inhibitor; 20 U RNase-Free DNase (Promega) and incubated at 37° C for 30 min to 1 h. After incubation, 200 µl DEPC-treated water was added and the RNA was extracted with phenol/chloroform:isoamyl (5:1) and precipitated with ethanol as previously described. After air drying, the pellet was resuspended in 20 – 50 µl of DEPC-treated water.

Silica-gel Spin Column Method

I next attempted to isolate RNA using a silica-gel membrane spin column kit. With this method, aqueous RNA adsorbs to silica in the presence of chaotropic salts, alcohol and low pH conditions. The RNA can be eluted with low salt, high pH buffers or with high temperature.

I used Qiagen RNeasy Mini Kits (Qiagen, Inc.), although several manufacturers offer similar kits. I tried this method after consulting with collaborators at the University of Florida's Protein Core and because the manufacturer claimed the method was quick, easy, provided high yields and did not involve the use of phenol or chloroform. I followed the manufacturer's protocol as follows. Frozen livers were homogenized in the buffer provided (buffer RLT) to which β-mercaptoethanol was added. The manufacturer recommended using no more than 30 mg tissue per spin column, so larger livers were first cut to an appropriate size with a razor blade. The homogenate was centrifuged for 3 min and the lysate transferred to a clean tube and mixed with one volume of 70% ethanol. The mixture was loaded onto a silica-gel spin column and centrifuged for 15 – 30 sec until it passed through the silica-gel. The spin column, containing the RNA, was washed by pipetting the wash buffer (buffer RW1) provided onto the silica-gel in the column and centrifuging for 15 sec. This initial wash eliminates most DNA, according to the manufacturer. To remove any remaining salts, the column was then washed twice with a second wash buffer (buffer RPE) containing ethanol. After the second wash, the column was centrifuged for 2 min to dry the silica gel and eliminate any residual ethanol that would interfere with subsequent reactions. The RNA was eluted from the column by pipetting 25 µl of RNase-free water onto the silica gel and centrifuging for 1 min. The elution step was repeated with an additional 25 µl of RNase-free water.

To eliminate residual RNases, the RNA was treated with Proteinase K as described in 1.2 above, except after incubation at 37° C, the RNA was extracted with another silica-gel spin column using a clean-up procedure recommended by the manufacturer. The sample

was mixed with 350 µl of buffer RLT after which 250 µl of 100 % ethanol was added. The sample was applied to a spin column and centrifuged for 15 sec followed by two washes with buffer RPE. The column was centrifuged until dry and the RNA eluted by pipetting 25 µl RNase-free water onto the silica gel and centrifuging for 1 min. The elution step was repeated with an additional 25 µl of RNase-free water.

The manufacturer of the spin-column kit (Qiagen, Inc.) claimed that the spin columns effectively remove DNA, however I found significant DNA contamination that would interfere with both differential display analysis and quantitative real-time PCR reactions. I therefore DNase treated all samples as previously described with the exception that after incubation at 37°C, the RNA was extracted using another spin column and eluted by performing the clean-up procedure described in the previous paragraph.

The spin column manufacturer later offered a kit for DNA elimination on the silica gel column before elution of the RNA. I altered the DNase procedure in the following manner. After Proteinase-K digestion, the samples were loaded onto the silica-gel spin columns as previously described and washed with RW1 buffer. A buffer and DNase enzyme mixture provided by the manufacturer was pipetted onto the silica-gel and allowed to incubate at room temperature for 15 to 30 min. The DNase and other contaminants were removed by another wash with RW1 buffer, two washes with RPE buffer, and then eluted with water.

The silica gel spin column procedure gave inconsistent results. Most RNA samples were mildly degraded and still had DNA contamination. The procedure for eliminating DNA on the spin

column using the kit offered by the manufacturer failed to eliminate DNA contamination even when the incubation time or enzyme amount was increased. The initial method of performing the DNase digestion in a tube with subsequent column clean up eliminated more DNA, however most samples still had unacceptably high DNA contamination. The yield per mg tissue using this method was low and the number of completely degraded RNA samples was unacceptably high. The low yield was probably not associated with the number of spin column extractions performed. RNA has a high affinity for the silica gel, however RNA less than 100 bases in length are eliminated using the spin column method (personal communication with Qiagen representative). In addition, using three spin columns per RNA sample was time consuming and expensive.

To address the problem of RNA degradation, I later modified the homogenization procedure. Unless tissue samples are in immediate contact with a strong denaturing agent immediately upon thawing, endogenous RNases quickly degrade RNA. Although the liver samples were kept frozen on dry ice, put into lysis buffer and immediately homogenized, some degradation may have taken place. To solve this problem, I ground the liver samples under liquid nitrogen. I placed a liver sample into a cryogenic mortar containing a small amount of liquid nitrogen. The sample was ground to a coarse powder with a pre-chilled pestle and then 600 µl of the RLT homogenization buffer (Qiagen, Inc.) was added to the liquid nitrogen. The sample and homogenization buffer were ground to a fine powder. The remaining liquid nitrogen was allowed to evaporate and the frozen liver and buffer powder was removed from the mortar with a pre-chilled spatula and placed into a pre-chilled cryo-vial. The samples were kept frozen

in liquid nitrogen or stored at -70° C until processed. Samples could be stored at -70° C for several months before processing. To continue with RNA isolation, the samples were placed on ice until slightly thawed and then homogenized with a hand-held homogenizer until completely thawed (30 – 45 sec). The silica-gel spin column method was then used.

Grinding the samples under liquid nitrogen significantly improved the quality of the RNA . However, this method was extremely time consuming as only about six samples could be ground per hour. The number of samples to be processed made this procedure prohibitively slow.

RNA-STAT 60 Method

I next used an RNA isolation reagent. This method proved far superior to the other methods described and was subsequently used for all further RNA isolation. The RNA yields and quality were much higher and the procedure was relatively quick.

I used RNA-STAT 60, (Tel-Test, Inc., Friendsville, TX) although several other similar products are available. This protocol was based on the method of Chomczynski and Sacchi which I initially attempted. The RNA-STAT 60 reagent included phenol and guanidinium thiocyante in a monophase solution. In general, the procedure involved homogenizing the samples in the RNA STAT-60 after which chloroform was added. The homogenate separated into two phases: aqueous and organic. As in the Chomczynski and Sacchi method, the total RNA remained in the aqueous phase while DNA and proteins were in the organic phase and interphase. The reagent contained a

red dye that remained in the organic phase. The color difference made it easier to distinguish the two phases and allowed more efficient recovery of the aqueous phase without disturbing the interphase and organic phase. I also used two other commercially available products (RNA Secure and DNA-Free, Ambion, Inc.) that have recently become available to improve RNA quality and yield.

The specific procedure I used to isolate total RNA from liver samples was the following modification of the protocol provided by the manufacturer. Frozen livers were homogenized (30 – 45 sec) in 750 µl RNA-STAT 60 after which 150 µl chloroform was added and samples vortexed for 30 sec. Samples were centrifuged at 12,000 X g at 4°C for 15 min. The aqueous layer was removed to a clean tube. The extraction was repeated on the aqueous phase by adding 750 µl RNA-STAT 60 and 200 µl chloroform and vortexing for 30 sec. After centrifugation at 12,000 X g for 15 min the aqueous layer was removed to a clean tube. The RNA was precipitated by adding 750 µl isopropanol and centrifuging at 7000 X G for 30 min to 1 h (longer time for smaller tissue samples) at 4°C. Alternatively, for very small tissue samples (< 10 mg), precipitation overnight at -20° C followed by centrifugation for 1 h improved yield. The supernatant was removed by pipetting and the pellet washed twice in 500 µl 75% ethanol. After air drying the pellet for several minutes at room temperature, the pellet was resuspended in 50 – 100 µl RNA Secure Resuspension Reagent (Ambion, Inc.). The RNA-Secure had been pre-warmed 60° C before adding to the RNA pellets, as recommended by the manufacturer. The samples were incubated at 60° C for 10 min. The RNA-Secure reagent eliminates any residual RNases, eliminating the need for Proteinase-K treatment.

Genomic DNA was removed using Ambion's DNA-Free kit following manufacturer's protocol. Briefly, a 1/10 volume of buffer was added to the RNA samples and 1 to 2 µl RNase-free DNase was added (depending on the amount of RNA) and the mixture was incubated at 37° C for 1.5 h. The DNase was removed by the addition of the Inactivation Reagent supplied in the kit. After adding a 1/10 volume of the Inactivatin Reagen, the samples were incubated at room temperature for 2 min, then centrifuged for 2 min at 12,000 X g to pellet the Inactivation Solution. The RNA supernatant was removed to a clean 1.5 ml microcentrifuge tube. Several µl of the RNA was removed for RNA quality analysis as described below. The remainder was stored at -70° C until needed.

RNA Quality Analysis

The quality and quantity of total RNA was determined by a combination of methods. The quantity of total RNA in a sample was measured by spectrophotometry at 260nm, where 1 absorbance unit (A260) equals 40 µg of single-stranded RNA/ml. The ratio of the A260/A280 readings is an indicator of the purity of an RNA sample. RNA with no protein contamination has a ratio of 2.0 if diluted in a buffer at pH8 (e.g. TE buffer). The ratio will be slightly lower if the RNA sample is diluted in water because distilled water tends to be slightly acidic. Due to the variations in starting materials and isolation procedures, a range of A260/A280 ratios between 1.7-2.1 was considered acceptable (Promega Technical Manual).

The quality and integrity of total RNA can best be visualized by denaturing agarose gel electrophoresis and ethidium bromide staining. High-quality, intact total RNA has two brightly stained bands

corresponding to the 28S and 18S eukaryotic ribosomal RNAs. The 28S band (top band) should be approximately twice the intensity of the 18S band. That ratio reverses in degraded samples because the 28S ribosomal RNA degrades to an 18S-like species (Promega Technical Manual). Degraded RNA also appears as a smear below the 18S band. Severely degraded RNA appears as a continuous smear with no bands or only faint bands visible. Figure A.1 gives examples of RNA quality.

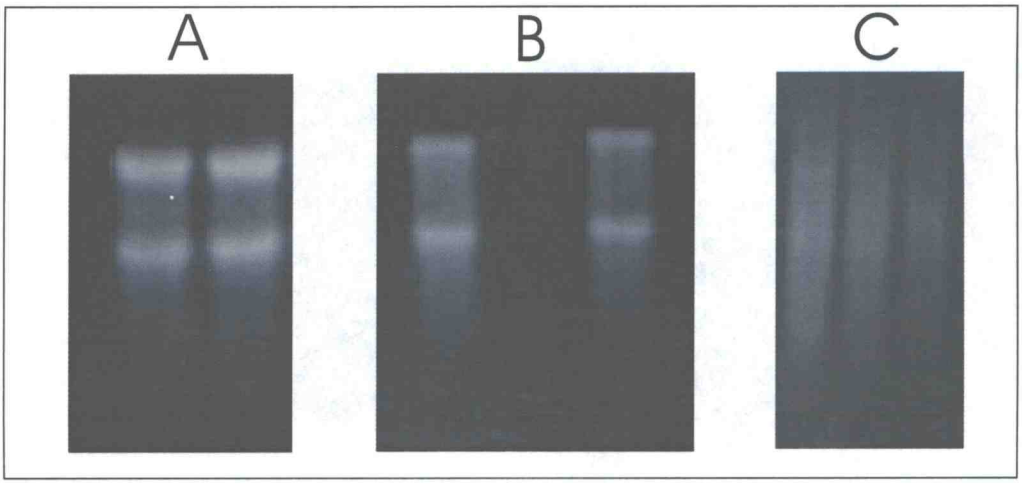

Figure A.1. Example of RNA quality. Total RNA (1 – 2 ug) run on denaturing agarose gel and stained with ethidium bromide. A Good quality RNA. B. Poor quality RNA. C Degraded RNA

Purity and concentration of the RNA sample was determined by measuring the absorbance at 260 nm (A260) and 280 nm (A280) in a UV plate reader (Molecular Dynamics) or in a spectrophotometer (Beckman DU5700). The RNA was diluted to a factor of 1/50 by adding 2 µl of the RNA to 98 µl nuclease-free water. The absorbance reading of the diluted sample at A260 was used to quantify the RNA sample in the following manner. An A260 value of 1 is equal to 40 µg of RNA /ml when the sample is diluted in water; therefore the

concentration of the original RNA sample (μg/ml) = (40 x A260 x dilution factor) / 1000 =μg/μl.

The integrity of the total RNA was checked by denaturing-agarose gel electrophoresis and ethidium bromide staining as follows: A 2 μg sample of the RNA was added to 15 μl RNA loading dye (37% formalde-hyde, 10X MOPS, formamide, 0.5M EDTA, glycerol, bromophenol blue, all chemicals RNase-free) heated at 65° C for 10 min and run on a 1% agarose gel (SeaKem LE agarose, 37% formaldehyde, 10X MOPS, RNase-free water) using a mini-gel electrophoresis apparatus and 1X MOPS running buffer. The gel was run at 80V for 45 – 55 min. The gel was stained for 10 min in 0.5% ethidium bromide and water solution. The gel was then destained overnight in distilled water and then visualized under UV light. Alternatively, 2.0 μl ethidium bromide may be added directly to the gel before it is poured, in which case overnight destaining is unnecessary.

mRNA QUANTITATION BY REAL-TIME QUANTITATIVE PCR (TAQMAN)

Quantitative Real-Time PCR or TaqMan is a method to determine the presence or absence of a specific sequence. This assay uses a fluorogenic probe designed to hybridize with the target sequence between the forward and reverse primers. The probe is labeled at the 5' end with a reporter dye and with a quencher dye at the 3' end. The fluorescence is quenched due to the proximity of the quencher when the probe is intact. During the PCR reaction, the probe is cleaved by the 5' nuclease activity of the Taq Polymerase enzyme, resulting in increasing fluorescence of the reporter dye during each PCR cycle. The fluorescence is monitored by the ABI PRISM 5700 Sequence Detection System and each PCR cycle. The increased fluorescence is directly proportional to the initial amount of target mRNA in the sample. Using this highly sensitive assay, we were able to quantify SHM IGF-I mRNA from nanogram amounts of total RNA.

Oligonucleotide Primers and TaqMan Probe Design

The sequence for sheepshead minnow IGF-I cDNA were analyzed by Primer Express software (Perkin-Elmer) and primer and fluorogenic probe sequences were selected. All primers were designed to lie in adjacent exons to prevent amplification of any larger genomic DNA fragments in the samples. The fluorogenic probe was labeled at the 5' end with the reporter dye FAM (6-carboxyl-fluorescein) and at the 3' end with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine). The selected primers and probe were purchased from ABI Prism (Applied Biosystems). The specificity of the primers was tested under normal PCR conditions using a thermalcycler (Perkin-Elmer 9700,

Foster City, CA). A single band with the expected size was observed on a 2.5% agarose gel after ethidium bromide staining (data not shown).

Preparation of RNA Sense Strand Standards

Standard curves were generated using *in vitro* transcribed sense RNA. The IGF-I sequence was cloned into a plasmid vector (pGEM-T Easy Vector, Promega) with the cloned sequence behind an SP6 RNA polymerase promoter. The plasmid was first treated with RNase to remove all RNA contamination as follows: 1 μg plasmid was added to 50 μl RNase/buffer mix (Ambion) and incubated at 37°C overnight. The RNase was inactivated by adding 75 μl of inactivation/PPT III solution (Ambion), vortexing and incubating 15 min at -20°C. The supernatant was removed and the pellet washed with ice-cold 70% ethanol and then allowed to air dry. The resulting plasmid pellet was linearized by overnight digestion with a restriction enzyme (NCO1) downstream of the polymerase binding site and the cloned product. After digestion, the DNA template was purified using a PCR Purification column (Qiagen QiaQuick). The eluted samples were treated with Proteinase-K (Proteinase-K (20ng/ml), 0.5 M EDTA, 1M Tris, 20% SDS, nuclease-free water) and incubated at 37°C for 1 h. The DNA template was purified by phenol-chloroform-isoamyl alcohol (1:1), vortexed 15 sec and centrifuged at 12,000 X g for 5 min. The aqueous phase was removed and phenol-chloroform extracted again, followed by a final extraction with an equal volume of chloroform only. The DNA template was precipitated from the aqueous phase with 1/10 volume 3MNaOAC (pH 5.2) and 3 volumes of 100% ethanol overnight at -20°C followed by centrifugation at 14,000 X g for 15 min at 4°C. The resulting DNA pellet was resuspended in 10 mM Tris-Cl (pH 8.3).

Three µl of the resuspended DNA template was checked on a 1.4% agarose/ Tris-acetate-EDTA buffer (TAE) gel to confirm a single band of the anticipated size.

In vitro transcription was performed using a Maxi Script kit (Ambion) and 1 µg of the purified DNA template. *In vitro* transcription was performed according to the kit manufacturer's protocol as follows: A reaction mix was assembled containing the 10X buffer, 10mM deoxynucleotides (dNTPs), SP6 polymerase and ribonuclease inhibitor supplied with the kit along with 1 µg of DNA template and water to a volume of 20 µl. The reaction mixture was incubated at 37°C for 1.5 h. After incubation, 0.5 M EDTA and water were added and the *in vitro* transcription reaction was purified using a Chroma-spin 100 column (Clonetech, Inc.) to remove nucleotides and incomplete transcripts.

The concentration of the resulting sense-strand cRNA was determined by absorbance at 260 nm. Ten-fold serial dilutions were carried out in nuclease-free water (Ambion) containing carrier tRNA (1 µg/µl). Transfer RNA from wheat (Sigma) was used as yeast tRNA had significant cross-reactivity with the IGF-I probe. The standard RNA dilutions were aliquoted and frozen at -70°C until needed.

Two-step Quantitative Real-Time PCR

For the two-step TaqMan assay, cDNA was prepared from the RNA in a separate step. The cDNA was then used in subsequent TaqMan PCR reactions. This method was convenient when several sequences were to be quantitated from the same RNA samples.

Preparation of cDNA

cDNA was prepared from 2 µl diluted RNA standard or 2 µg total RNA samples in a 20 µl reaction volume. The RNA was incubated at 65°C with 500 µM dNTP mix, 250 nM gene-specific reverse primer and nuclease-free water to a volume of 12 µl for 5 min. The mixture was cooled to 50°C for 2 min after which 8 µl of a pre-warmed (50°C) buffer/enzyme mixture (4 µl 5X first-strand buffer (Gibco/BRL), 10 mM DTT, 40 units ribonuclease inhibitor (RNaseOUT, Gibco/BRL), and 200 units SuperScript II (Gibco/BRL) reverse transcriptase) was added. The mixture was incubated at 50°C for 50 min. The reaction was stopped by heating to 70°C for 15 min. The cDNA was stored at -20°C until needed.

If random hexamers (100 ng) were used instead of the gene-specific reverse primer (to amplify a variety of mRNAs instead of just a single mRNA species), the reaction was incubated at 25°C for 2 min before adding SuperScript II and then incubated at 25°C for another 10 min. The samples were then incubated at 42°C for 50 min and 70°C for 15 min.

Optimization of Primer and Probe Concentrations

Before performing assays on the experimental samples, the TaqMan assay was optimized for primer and probe concentrations. The concentrations of these components were tested empirically for each primer/probe combination (TaqMan PCR Reagent Kit protocol, Applied Biosystems, Inc.) The optimum primer concentration was determined by testing concentrations of 50, 300 and 900 nM for each primer against each concentration of the second primer. A matrix of

nine conditions was tested using the appropriate sense-strand RNA as the PCR template. The primer concentrations that gave the lowest C_t value (PCR cycle at which the fluorescent signal first reaches a pre-determined threshold) were chosen for subsequent assays. For both IGF-I and IGF-II, the optimal concentrations were 300 nM for the forward primer and 900 nM for the reverse primer. The optimum probe concentration was determined by testing 25 nM increments from 25 – 225 nM using the optimized forward and reverse primer concentrations. The probe concentration that gave the lowest C_t value was chosen. The optimum concentration for IGF-I was 200 nM.

TaqMan Assay

TaqMan PCR was performed in triplicate for each cDNA sample and standard using 96-well optical plates and caps (Applied Biosystems). The reaction mix (25 µl total volume) consisted of 5.5 mM $MgCl_2$, 200 µM of dNTPs (dATP, dCTP, dGTP) and 400 µM dUTP, 0.01 unit/µl of AmpErase UNG, 0.05 unit/µl of AmpliTaq Gold, 200 nM probe, 1X TaqMan Buffer (PE Applied Biosystems), 300 nM of the forward primer, 900 nM of the reverse primer, and 2 µl of cDNA. A master mix was first prepared with all of the components except the cDNA. To avoid variation in reaction mix between 96-well plates, enough master mix was prepared for all samples to be assayed. The master mix was stored at 4°C overnight if not used the first day. Twenty three microliters of the master mix was placed into each well of the plate. To avoid cross contamination between wells, the caps were loosely placed over all the wells immediately after adding master mix. The standard cDNA or the sample cDNA (2 µl) were added to each well and the caps securely fastened immediately afterward.

When each plate was completed, it was stored at -70°C. Storage at -70°C for several weeks does not diminish the quality of the reaction [Smetsers, 1998 #927]. The plates were placed on dry ice for transport to the University of Florida in Gainesville, where the PCR reaction was performed. Each plate was thawed on ice just prior to the PCR reaction. The PCR was run in the ABI PRISM 5700 Sequence Detection System using the following protocol.

50°C for 2 min → 95°C for 10 min
followed by 40 cycles of
95°C for15 sec → 60°C for 1 min.

After each PCR reaction was completed, the results were analyzed using ABI PRISM 5700 software. The amplification plot of the standards was first examined and a threshold was selected that was within the log phase of the PCR reactions. This threshold was subsequently used on all the samples in that assay. A standard curve was generated from the standards. A standard curve with a slope of -3.3 corresponds to 100% efficiency in the PCR reaction (Applied Biosystems Protocol). The cycle number at which the fluorescent signal reaches the threshold (known as the C_t value) for each unknown sample was compared to the C_t value of the standard curve to quantify the amount of the target sequence in that sample. The ABI PRISM software automatically calculates the quantities from the C_t values.

The samples were normalized to 18S ribosomal RNA using primers and a labeled probe purchased as a kit (Applied Biosystems). The PCR reactions were carried out using the same core reagents and the same quantity of total RNA as the IGF-I assays with the exception of the primers and probes. The ribosomal RNA forward and reverse

primers and the probe concentrations were 50 mM in accordance with the manufacturer's recommendation. The PCR parameters were the same as those for the IGF-I assay.

The Ct values of the 18S RNA samples were used to normalize the IGF-I data using the following calculation for each sample:

$$\frac{\left(\frac{1}{\text{Mean 18S } C_t} \times \text{Qty. IGF-I}\right)}{\frac{1}{\text{Sample 18S } C_t}} = \text{Normalized Qty IGF-I}$$

Where: Mean 18S C_t is the mean value for all 18S samples
Qty. IGF-I is the quantitation for that sample
Sample 18S C_t is the value for that sample

Single-Step Quantitative Real-Time PCR

For single-step quantitative real-time PCR, total RNA is added directly to the reaction mix without first reverse transcribing into cDNA. The TaqMan reaction protocol is altered to become a reverse-transcription PCR (RT-PCR) reaction. Because there is only one step involved, this method is convenient when many samples are to be analyzed and only one sequence is being quantitated. Additionally, a much smaller amount of total RNA is required (100-200 ng vs. 1 – 2 μg). There are several drawbacks to this method, one being the vulnerability of RNA to degradation. The RT-PCR assay should be run immediately after loading the 96-well plate. Storing the master mix at 4°C or at –20°C or freezing the plates at –80°C caused deterioration of the reactions.

Quantitative (TaqMan) RT-PCR was performed in triplicate for each sample. A standard curve was constructed using triplicate samples of sense strand at each concentration. A single-step quantitative RT-PCR Core Reagent Kit (Stratagene) was used. The primers and probe were the same as those used in the two-step method described previously. The reaction mixtures (25 µl total volume) for the IGF-I assays consisted of the core components according to the manufacturer's instructions plus 5.5 mM $MgCl_2$, 200 nM probe, 300 nM forward primer, 900 nM reverse primer, 40 units of Rnase-Out ribonuclease inhibitor (Gibco), 1.5 µl 0.1 mM DTT and 200 ng of total RNA. The RT-PCR was run in an ABI PRISM 5700 Sequence Detection System using the following parameters.

48°C for 55 min → 95°C for 10 min
followed by 40 cycles of
95°C 15 sec → 60°C 1 min.

The standards and samples were analyzed as previously described for the two-step TaqMan method.

The samples were normalized to 18S ribosomal RNA using primers and a labeled probe purchased as a kit from Applied Biosystems. The RT-PCR reactions were carried out using the same core reagents and the same quantity of total RNA as the IGF-I assays with the exception of the primers and probes. The ribosomal RNA forward and reverse primers and the probe concentrations were 50 nM as specified by the manufacturer. The ribosomal RT-PCR reactions were performed in duplicate for each sample. The samples were normalized as previously described.

DIFFERENTIAL DISPLAY ANALYSIS

Differential Display technology was developed by Liang and Pardee [Liang, 1992 #1094] as tool for identifying and cloning differentially expressed genes. Differential display technology involves the reverse transcription of mRNA with oligo-dT primers anchored to the beginning of the poly(A) tail, followed by a PCR reaction using a second short primer with an arbitrary sequence. The amplified cDNA products produced by the primer pair are run on a DNA sequencing gel where they are distributed by size. Running mRNA from controls side-by-side with mRNA from treated animals allows examination of differences in gene expression. Visualization and identification of many mRNA species is possible by using different combinations of anchor primers and arbitrary primers. After identifying genes of interest on the gels, the corresponding gel bands are cut out, reamplified and sequenced for further study.

The RNAspectra Green Fluorescent mRNA Differential Display System kit (GenHunter Corp. Nashville, TN) was used for all gels. The manufacturer's protocol with some exceptions was used.

Reverse Transcription (RT) of mRNA

Intact total RNA that was free of DNA contamination (see RNA Isolation Procedure) was used for all differential display reactions. To avoid possible false positives, three samples from each treatment were prepared and run side-by-side on the gels. A separate RT reaction of each RNA sample was performed for each of the three anchor primers.

Total RNA was diluted with nuclease-free water to a 0.1 μg/μL concentration. The RNA was freshly diluted and was not allowed to freeze and thaw. All components except nuclease-free water, RNase inhibitor and total RNA samples were included in the GenHunter kit. The reactions were kept on ice at all times. RT reactions (20 μL final volume) were prepared for each RNA sample with the following:

Nuclease-free water	8.4 μl
5X RT buffer	4.0 μl
dNTP mix	1.6 μl
RNase inhibitor (40 U/μl)	
(RNase Out, Gibco)	1.0 μl
Anchor primer (not labeled)	2.0 μl
Total RNA (diluted)	2.0 μl

A master mix (multiply all components by the number of samples + 1 to allow for pipetting errors) of all components except the total RNA was prepared. After pipetting 17 μl of the master mix into each 0.5 ml microcentrifuge tube, 2 μl of the diluted total RNA sample was added. The reactions were put into a thermalcycler programmed with the following cycling parameters:

60°C for 5 min. → 37°C for 60 min. → 75°C for 5 min. → 4°C soak

After 10 min at 37°C, the thermalcycler was paused and 1 μL reverse transcriptase (MMLV) was added to each reaction. After the RT reaction was complete, the reactions were put on ice or stored at –20°C for later use.

PCR Reaction

A PCR reaction was prepared for each combination of anchor primer and arbitrary primer. All components except nuclease-free water, RT-Mix and Taq DNA polymerase were supplied in the kit. The components were thawed and set on ice. The PCR reactions were set up at room temperature in thin-walled reaction tubes. To avoid pipetting errors, a master mix (multiply all components by the number of reactions + 1) of all components except the RT-mix and the arbitrary primer was prepared. The fluorescently labeled anchor primers were used in this reaction. Since fluorescent dyes are light sensitive, I kept primers and samples in the dark or covered with foil as much as possible.

	Per reaction
Nuclease-free water	10.2 µl
10X PCR buffer	2.0 µl
dNTP mix	1.6 µl
Labeled anchor primer	2.0 µl
AmpliTaq DNA polymerase (5 U/uL) (Applied Biosystems)	0.2 µl
	16.0 µl per reaction

Sixteen µl of the master mix was placed into each thin-walled tube after which 2.0 µl of the RT-mix (from step 1) prepared with the same anchor primer, and 2.0 µl of the selected arbitrary primer were added. The reactions were mixed by pipetting up and down and then put into a thermocycler (Perkin-Elmer 9700) programmed for the following PCR conditions:

40 cycles of

94°C for 30 sec. → 40°C for 2 min. → 72°C for 60 sec.

Followed by 72°C for 5 min. → 4°C soak

The samples were kept covered by foil after the PCR reaction. Just before loading on the gel, the samples were spun down in a microcentrifuge, mixed with 10 µl loading dye (99% formamide, 1mN EDTA, pH 8.0, 0.009% xylene cyanole FF, 0.009% bromophenol blue), heated to 80°C for 2 min. and put on ice. Six µl of each sample was loaded onto a 6% denaturing poly-acrylamide gel in 1% TBE buffer as described below.

Gel Setup

A 6% polyacrylamide gel was poured and set up in a Model S2 sequencing electrophoresis gel apparatus (Whatman Biometra, formerly Gibco-Life Technologies, Inc.) using the following procedure.

Two glass plates, one small (30 cm X 40 cm X .3 cm) and one large (30 cm X 42 cm X 0.3 cm) were thoroughly cleaned as follows: Both sides of the glass were washed with dishwashing detergent, rinsed with distilled water and dried with large Kim-wipe tissues. The surface to be in contact with the gel was further cleaned by pouring 2.0 M NaOH on the surface and scrubbing with a gloved hand for 1 –2 min followed by thorough rinsing with distilled water. The glass was dried with Kim-wipe tissues and then rinsed with isopropanol and dried again with Kim-wipes. On the small plate only, silicone coating (eg. Acrylase, Sigmacote or Rain-off) was applied on the side of the glass plate that would be in contact with the gel. The silicone coating was spread over the entire plate and buffed to a haze with a Kim-wipe

and let dry for a few minutes. This was followed by buffing with a damp Kim-wipe and then dry Kim-wipes until the plate was dry and slick. Then the glass was given a final rinse with isopropanol and thoroughly dried with Kim-wipes. It was necessary to take precautions to clean the glass plates thoroughly and to dry them with isopropanol so that the gel matrix would not stick to any parts of the gel causing bubbles to form.

The gel was assembled by placing mylar (0.4 mm thick, 1 cm wide, 40 cm long) spacers along the outside edges of the large glass plate and making sure that the edges were flush with the sides and bottom of the glass. The small glass plate was placed on top of the spacers with the silicone coated side facing down. The casting clamp provided with the gel apparatus was carefully put in place along one side, then along the bottom edge and then up the remaining side. The glass plates and spacers were checked to be sure everything was aligned and firmly in place in the casting clamp.

The gel matrix was prepared using Long Ranger 6% PreMix Gel Solution for Manual Sequencing (BMA, Cambrex, Inc.) which is supplied in 1X TBE (10.8 g TRIS, 5.5 g boric acid, 4 ml 0.5M EDTA, pH 8, 1 L ddH_2O) buffer and 7 M urea in premeasured single gel (75 ml) amounts in a squeeze bottle. To prepare the gel, 53 µl Tetramethylethylene-diamine (TEMED) and 375 µl 10% ammonium persulfate (APS) and a small amount of bromophenol blue was added. The solution was swirled until mixed and then poured between the glass plates using the squeeze bottle and cap. The glass plates were tipped toward one side to allow the gel matrix to flow down one side, along the bottom and toward the opposite side and then up toward the other side. Care was taken to avoid forming bubbles in the matrix. A

constant excess of gel matrix was kept in the gel-loading area to avoid bubbles. When the gel reached the top of the small glass plate, more gel matrix was added to the gel loading area. A 48 well square-tooth comb was inserted just until the top of the teeth met the top of the small glass plate. This was necessary to prevent samples from spilling over into adjacent wells when samples were loaded. Three large binder clamps were placed on the upper edge of the glass plates to hold the small glass plate down on the comb. The gel was allowed to polymerize 3 h or overnight. When the gel had polymerized, the binder clamps were removed, being careful not to dislodge or move the comb. The silicone casting clamp was removed. The comb was carefully removed by loosening it from the large glass plate with a razor blade and then pulling the comb straight out to avoid tearing any of the wells. To remove any loose gel matrix, the glass plates were rinsed with water and dried with Kim-wipes. The gel sandwich was transferred to the gel running unit with the large plate facing out. The glass plates were clamped into place and enough 1X TBE was poured into the top chamber to cover the small glass plate and wells. The chamber was checked for leaking buffer and adjusted if leaking was evident. The gel contains urea, which leaches out of the gel and interferes with electrophoresis. A fine-tip bulb pipette was used to squirt TBE buffer into the individual wells to wash away any urea leaching from the gel into the loading wells. TBE buffer was added to the lower chamber.

Samples were loaded into the wells in reverse order because the fluorescent scanner produces a mirror image of the gel. Before loading samples, the wells were again washed of urea using a fine-tip bulb pipette. After all samples were loaded, the gel unit was covered

with aluminum foil to protect the fluorescent dye from light. The gel was run at 60 watts constant power (voltage no more than 1700) for 2 to 3 h until the xylene marker dye was about 3 inches from the bottom of the gel.

Fluorescence Imaging of Gel

After removing the gel sandwich from the apparatus, the glass plates were again cleaned with water and isopropanol to remove any buffer or gel matrix which would interfere with fluorescent scanning. The gel (with both glass plates in place) was scanned, placing the large plate down, in a Typhoon 8600 Fluorescence Imager (Molecular Dynamics, Inc.) using a fluorescein setting with a 505 nm filter. After scanning, the image was visualized using ImageQuant software (Molecular Dynamics, Inc.) and a full-size printout was made. The printout was examined for differentially expressed messages. To qualify as either a down- regulated or up-regulated message, all three samples within a treatment or control group had to express the same pattern for that gene. Gel bands of interest were marked on the printout. The gel was scanned again after removing the small glass plate. The plate was removed by gently wedging a thin metal spatula between the two glass plates at one corner and lifting the plate up, leaving the gel in place on the large glass plate. The mylar spacers were removed. To serve as markers to orient the gel on the printout, several small triangular pieces of laboratory tape were placed along the edges and bottom of the large glass plate (on the underside of the plate). The gel was scanned again and an full-size printout made. The gel was placed on top of the printout and the triangular markers were aligned.

Gel bands of interest were cut out by using sterile No. 11 scalpel blades. The cut gel bands were removed using forceps and placed into 50 µl nuclease-free water in labeled 0.5 ml microfuge tubes and frozen at –20°C until processed. Three gel slices from each gene of interest were removed. After all of the desired gel bands were excised, the gel was again scanned to be sure that the bands had been removed. If all the bands had been properly excised, the gel was discarded.

Reamplification of Gel Bands

Genes of interest were reamplified from the gel bands. The frozen gel bands were thawed and the water was removed. The gel bands were washed by adding 50 µl of nuclease-free water to each tube and letting the band soak for 10 – 15 min to remove any urea. The tubes were briefly centrifuged to deposit the gel band at the bottom of the tube and the water was removed by pipetting. For amplification, the gel slice was added directly to a PCR reaction containing the same primer combination used in the initial PCR reaction (except using unlabeled anchor primer). The components from the GenHunter kit were used in following reaction mix:

Nuclease-free water	26.6 µl
10 X PCR buffer	4.0 µl
dNTP mix	1.0 µl
Anchor Primer used in initial PCR reaction (not fluorescein labeled)	4.0 µl
Arbitrary Primer used in initial PCR reaction	4.0 µl
AmpliTaq DNA Polymerase	0.4 µl
Excised gel slice	

The reactions were carried out in thin-walled PCR tubes using the same PCR conditions described previously.

After amplification, the PCR reaction was purified using a PCR purification kit (QiaQuick, Qiagen, Inc.) following the manufacturer's protocol. The protocol purifies single- or double-stranded DNA fragments from primers, nucleotides, polymerases and salts using a spin column and a series of buffer washes. The purified DNA was eluted from the spin column with 40 µl Tris-Cl buffer. To verify amplification of the gene, 20 µl of the cleaned PCR product was run on a 1.0% agarose gel in TAE along with a low DNA mass ladder (Life Technologies) and stained with ethidium bromide. The size and approximate mass of the DNA fragment was estimated. Using this method of reamplification, I was consistently able to reamplify the gel band of interest.

Vector Ligations

Reamplified DNA fragments were ligated into pGEM-T Easy Vectors (Promega) following the manufacturer's protocol. Initial experiments showed that an insert:vector molar ratio of 3:1 was most successful. The following formula was used to calculate the appropriate amount of PCR product (insert) to add to the ligation reaction:

$$\frac{(50 \text{ ng of vector}) \times (\text{kb size of insert})}{3.0 \text{ kb vector}} \times \frac{3}{1} = \text{ng of insert}$$

Ligation reactions were set up for each DNA fragment using the pGEM-T Easy vector system components (Promega) as follows:

2X ligation buffer	5 µl
pGEM-T Easy vector (50 ng)	1 µl
PCR product (see calculation)	X µl
T4 DNA Ligase (3 Weiss units/µl)	1 µl
Nuclease-free water to a final volume of 10 µl	

A positive control reaction using a BlueScript (PBSII) control vector in place of the pGEM-T Easy vector, and a background control with no PCR product were also set up. The ligation reactions were incubated at 4°C overnight.

Transformations

Plates (plastic petri dishes) were prepared with ampicillin and oxacillin using the following procedure:

Luria broth agar was prepared (0.5 L Luria broth, 7.5 g granulated agar), autoclaved and cooled to 50°C. Next, 50 mg X-Gal (5-bromo-4-chloro-3-indolyl-b-D-glactoside) in dimethylformamide (Promega), 2.5 ml 0.1M IPTG (isopropyl-beta-D-thiogalactopyranoside, dioxane free) (Promega), 40 mg oxacillin (Sigma) and 25 mg ampicillin (Sigma) were added. The mixture was swirled to mix and 30 ml were pipetted into each sterile plate. The plates were covered and allowed to harden in a dark room. Plates were stored up to 1 week at 4°C.

Bacterial transformations were carried out in 6 ml polypropylene Falcon tubes. The tubes were placed on ice and 2 µl of the ligation reaction was pipetted into the bottom of the tube. One tube with no ligation reaction served as a control for cells with no insert. High efficiency competent cells [JM109 (Promega)cells or DH

5α (Gibco)] were removed from -70°C storage and placed on ice until just thawed, about 5 min. Twenty five µl of the competent cells were pipetted into the tubes and gently swirled. The reactions were incubated on ice for 45 min. The cells were then heat shocked at 42°C for 45 sec, placed back on ice for 2 min after which 450 µl of room temperature SOC medium (nutrient Luria broth containing glucose) (Gibco) was added. The tubes were incubated at 37°C on a shaker set to 225 rpm for 1 h. The transformed cells (50 µl per plate) were plated onto two pre-warmed LB/ampicillin/oxacillin treated plates and incubated at 37°C overnight.

The control plates were examined for the following results: control for cells with no insert should have no colonies; positive controls should have all white colonies, background control should have only blue colonies. Using sterile technique, five white (containing the DNA insert) colonies from each DNA insert were picked by touching a sterile toothpick to the colony, streaking a small section of a new LB/ampicillin/oxacillin plate and then breaking the tip of the toothpick into a sterile 12 ml culture tube containing 3 ml sterile LB broth and 3 µl ampicillin (50 mg/ml). The culture tubes and plates were incubated overnight at 37°C. After overnight incubation, the plates were chilled at 4°C and the clones shipped to the University of Florida, Gainesville for DNA sequencing.

A sterile loop was used to transfer some of the overnight cultures into fresh 12 ml culture tubes with 3 ml sterile LB broth and 3 µl ampicillin. The fresh cultures were incubated overnight. The cells from the previous overnight cultures were pelleted by slow (6,000 rpm) centrifugation. The plasmid DNA was purified using a Qiagen mini-prep kit according to the manufacturer's instructions. Briefly,

the pelleted cells were resuspended and then lysed with the buffers provided in the kit. The lysate was centrifuged to pellet cellular debris and the supernatant was applied to a spin column. The column was centrifuged for 30 – 60 sec to allow DNA to adhere to the membrane. The DNA was washed with buffers and eluted with 50 µl of Tris-HCl buffer.

To screen the plasmids for the proper insert size, 1.5 µl of the eluted DNA was digested with a restriction enzyme (ECO R1), run on a 1.4% agarose gel and stained with ethidium bromide. The insert DNA was compared to a DNA ladder to be sure the insert was the expected size. The remaining plasmid DNA was stored at -70°C.

To make glycerol stocks of the transformed cells, the second overnight cell cultures were centrifuged at 4,000 rpm for 30 – 60 sec to concentrate the cells. All but 1.7 ml of the supernatant was removed and discarded. Sterile glycerol (300 µl) was added to 1.7 ml of the cell culture, vortexed briefly to mix and frozen in an ethanol and dry ice bath for 20 min. The stocks were then stored at –70°C.