PERIPHERAL HORMONE INTERACTIONS WITH THE GROWTH HORMONE-INSULIN-LIKE GROWTH FACTOR (GH-IGF) SYSTEM IN RAINBOW TROUT

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Program:
Zoology

May 2019

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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

DOCTOR OF PHILOSOPHY

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ABSTRACT

The growth of vertebrates is primarily regulated by the growth hormone-insulin-like growth factor (GH-IGF) system, but not in isolation. The central question of this dissertation was how do other hormones peripheral to the GH-IGF system interact with the system, including feedbacks by GH and IGF themselves on various tissues in rainbow trout (*Oncorhynchus mykiss*)? The representative hormones selected were thyroxine, cortisol, and the sex steroids testosterone and estrogen, along with GH and IGF. These hormones were chosen because they are known to affect overall growth and development during specific life events, but exactly what target genes and what mechanisms are involved are only at the early stages of being delineated in fish. Liver and gill tissues were selected as representative tissues to assess the *in vitro* effects on growth-related genes of the GH-IGF system. A total of more than thirty experiments were conducted, including time- and concentration-response, inhibitory studies, hormone combination studies, and radio-receptor binding assays. Hormones were applied to whole tissue cultures and real-time quantitative-PCR was used to measure hormonal effects on GHR, IGF, and IGFR1 genes. Microsomal preparations were treated with selected hormones and radio-labeled GH or IGF. A gamma counter was used to measure receptor-ligand activity. GH and IGF were found to possess autocrine and/or paracrine actions in self-regulating target growth genes. Thyroxine had no direct effects on targeted growth genes but may interact with other molecules or hormones to elicit its effects on growth and development. Cortisol directly influenced target growth genes in a tissue-specific and isoform-specific manner. Finally, sex steroids differentially regulated the growth genes: estradiol inhibited growth genes while testosterone directly stimulated growth genes. These findings contribute to understanding how hormones peripheral to the GH-IGF system interact with the growth system.

ACKNOWLEDGEMENTS

I would like to thank many people without whom this research and dissertation work would not have been accomplished. First, I thank Dr. Mark Sheridan for giving me the opportunity to become a part of his research lab when I had no idea what scientific research entailed as an undergraduate. From the beginning he provided me with exceptional scientific advice and learning opportunities and experiences that I could not have gained anywhere else. I also want to thank my current major advisor Dr. Katie Reindl who, from the very beginning, has been nothing but supportive and encouraging and my constant cheerleader in reminding me that I can accomplish anything. I thank my committee members Dr. Gary Clambey and Dr. Jane Schuh for their valuable ideas and input and for sticking by my side through the many years of my graduate career. I thank former committee members Dr. Peggy Biga and Dr. Wendy Reed for sharpening my interpretation skills in data analysis. Finally, I absolutely must thank Dr. James Grier for first taking on the role as an unofficial advisor and then as an official committee member. He has helped me more than he will ever know, and I can genuinely say I would not have been able to finish my degree without his guidance and support.

The Department of Biological Sciences at North Dakota State University provided many years of support through research and teaching assistantships plus office space. I thank our lab technician and fellow graduate and undergraduate students for their invaluable assistance toward the completion of my projects. Specifically, thanks to Jeff Kittilson whose knowledge, technical guidance and services were instrumental in assuring my experiments were complete and successful, as well as thanks for friendship and always pushing me to do more. I am grateful for the fellow graduate and undergraduate students, but not limited to: Dr. Chad Walock, Dr. Andrea Cosgrove, Dr. Heather Bergan-Roller, Dr. Michael Caruso, Lincoln Martin, Dillion Marquart,

Alison Hagemeister, Sarah Danielson, Heidi Albers, Alicia Ickstadt, Evan Jones and Tara Slominski for not only help but friendship that has made my graduate career more enjoyable and fun. I want to also thank Wendy Leach for frequent assistance in departmental program concerns. I thank Graduate Studies Dean Claudia Tomany for the approved extension in order to complete my graduate degree.

Lastly, I would like to thank my family for their unconditional support and love. Each one had a pivotal role in my decision to pursue graduate school. Thank you to my parents, Bob and Lynette, for the love, emotional support and encouragement over all the years that has given me the strength and energy needed to finish my degree. Finally, to my husband, Jeremy, who has given me nothing but love and support from day one which has given me the confidence to pursue our future dreams and aspirations.

This research was primarily funded by a grant from the National Science Foundation (IOS 0920116 to M.A. Sheridan).

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^{125}I-GH .....Radiolabeled salmonid Growth Hormone

^{125}I-IGF-1 .....Radiolabeled salmonid Insulin-like Growth Factor 1

ANOVA .....Analysis of Variance

BSA.....Bovine serum albumin

cDNA .....Complementary Deoxyribonucleic Acid

C.....Control

E2 .....17β-Estradiol

ERK.....Extracellular Signal-Regulated Kinase

F .....Cortisol

GH.....Growth Hormone

GHR .....Growth Hormone Receptor

IGF .....Insulin-like Growth Factor

IGFBP .....Insulin-like Growth Factor Binding Protein

IGFR .....Insulin-like Growth Factor Receptor

JAK .....Janus Tyrosine Kinase

K_d .....Dissociation constant

MAPK.....Mitogen-activated Protein Kinases.

mRNA.....Messenger ribonucleic acid

MMI .....Methimazole

PI3K .....Phosphoinositide 3-Kinase

RT-QPCR.....Real-Time Quantitative Polymerase Chain Reaction

RU486.....Mifepristone

SEM .....Standard Error of the Mean

Spiro.....Spironolactone

STAT.....Signal Transducers and Activators of Transcription

T.....Testosterone

T3.....Triiodothyronine

T4.....Thyroxine

CHAPTER 1: GENERAL INTRODUCTION & BACKGROUND

Organismal growth encompasses a series of biological processes involving changes in cell size, number, formation and function are all influenced during the life cycle of an organism. The regulation of somatic growth is an intricate interplay in communication between external environmental cues, internal physiological processes and the nutritional status of the organism. The main coordinator in regulating somatic growth is the growth hormone-insulin-like growth factor (GH-IGF) system. This system is composed of the three hormones [growth hormone (GH), insulin-like growth factor I (IGF-I/IGF1) and insulin-like growth factor II (IGF-II/IGF2)], four cell-surface receptors [growth hormone receptor 1 (GHR1), growth hormone receptor 2 (GHR2), insulin-like growth factor receptor 1A (IGFR1A) and insulin-like growth factor receptor 1B (IGFR1B)] and two sets of plasma binding proteins [growth hormone binding protein (GHBP) and insulin-like growth factor binding proteins (IGFBP)].

Growth hormone (GH) is a member of the prolactin/ growth hormone superfamily. In fish, GH genes are composed of six exons that form a single polypeptide chain of 20-22kDa and are produced by somatotropic cells of the anterior pituitary (Agellon et al., 1988; Yang et al., 1997). GH was denominated after its actions were discovered to stimulate somatic growth. Since it has been discovered, GH has shown to elicit a variety of biological effects that include catabolic actions in lipolysis, anabolic actions in amino acid incorporation and protein synthesis of muscle tissue, regulation in carbohydrate metabolism, and seawater acclimation in fish (Bergan et al., 2013; Fauconneau et al., 1996; Sangio-Alvarellos et al., 2005; Young et al., 1989). Biological actions of GH are initiated when GH binds to growth hormone receptors (GHRs) found on the cell membrane of the liver and other target tissues (e.g. heart, pancreas, spleen).

GH receptors belong to the class 1 cytokine receptor family and consist of a single transmembrane protein that contains three domains (extracellular, transmembrane, and intracellular). The classical view is upon the binding of GH, dimerization of the GH receptor occurs and a ligand-receptor complex forms, which causes a cascade of signaling events to occur through the JAK/STAT, MAPK/ERK, or PI3K pathways. However, recent studies have demonstrated GHRs exist as inactive dimers of the cell membrane in the absence of a ligand, with the transmembrane domains being parallel to each other (Brooks et al., 2014). Activation occurs when GH binds to the first high affinity binding site of the receptor, allowing it to bind to the second binding site of the receptor (Brooks et al., 2014). This evokes a small conformational change in the transmembrane domain, which results in the transmembranes to crossover, causing a cascade of signaling events (Brooks et al., 2014; Brooks and Waters, 2015; Waters, 2016).

A component of the GH-IGF system that can influence the physiological process of GH effects involves growth hormone binding proteins (GHBPs), which are specific, high-affinity, soluble binding proteins. Among fish, GHBPs have been identified in rainbow trout (*Oncorhynchus mykiss*, Sohm et al., 1998), goldfish (*Carassius auratus*, Zhang & Marchant, 1999), and Chinese sturgeon (*Acipenser sinensis*, Liao & Zhu, 2004). However, much of what is known about GHBPs come from studies done in mammals. A large portion of GH circulating within the plasma of mammals has been found bound to GHBP (Baumann et al., 1988; Edens & Talamantes, 1998; Baumann, 2002). The specific function of GHBP is not entirely clear among vertebrates. When GH is bound to GHBPs it increases the biological half-life of GH and prevents GH from proteolytic degradation (Tzanela et al., 1997; Turyn et al., 1997). Yet consequently, when GHBPs are bound to GH, they limit GH's ability to bind to its receptor,

which in turn, diminishes the biological activity and growth-promoting effects of GH (Lim et al., 1990; Mannor et al., 1991; Barnard & Waters, 1997).

In mammals, GHBPs are produced by two disparate mechanisms, either alternative splicing of the pre-mRNA gene for GHR or through proteolytic cleavage of the GHR. More specifically, studies demonstrated rodents produce GHBP through alternative splicing of the mRNA that encodes for GHR. Of the three domains that make up the GHR, the transmembrane and intracellular domains are lost while the extracellular domain is retained and spliced with an additional hydrophilic carboxyl terminus (Baumbach et al., 1989; Edens & Talamantes, 1998). For most mammals, GHBP is produced by a posttranslational modification process to the GHR gene known as ectodomain shedding (Schantl et al., 2004). In this process, the metalloprotease tumor necrosis factor-α converting enzyme (TACE) proteolytically cleaves a portion of the extracellular domain of a single polypeptide membrane-bound GHR (Peschon et al., 1998; Zhang et al., 2000). GH must be bound to the first high-affinity binding site of the GHR for TACE to have the ability to proteolytically cleave the extracellular domain; protection from proteolytic cleavage occurs once a conformational change has occurred with the GHR (Schantl et al., 2004).

In rainbow trout, two distinct molecular size bands relating to GH-GHBP complexes were detected in studies by Sohm and colleagues (1998). Characterization of the GH-GHBP complexes by an antibody directed against the GHR suggested GHBPs represent the extracellular domain of the trout GHR. In addition, a study utilizing Chinese sturgeon GHR-expressing Chinese hamster ovary (CHO) cells demonstrated that fish GHBPs were proteolytically cleaved from the extracellular cell surface domain of the membrane-bound Chinese sturgeon GHR. Therefore, in fish as well as most mammals that are not rodents, a strong parallelism exists in the mechanism for producing GHBPs (Liao et al., 2009). Similarly, a series of studies performed on

salmonids (i.e. salmon and rainbow trout) support GHBPs being derived from the extracellular domain of the GHRs in fish (Einarsdottir et al., 2014).

Regulation of the release of GHBP in fish is not known, but recent studies in salmonids have suggested that GHBP may be regulated through physiological processes such as salinity and nutritional state independently of the primary GH-endocrine system components (Einarsdottir et al., 2014; Bjornsson et al., 2018). Originally, it was suggested GHR expression and plasma GH levels were responsible in regulating plasma GHBP levels. A relatively recent radioimmunoassay (RIA) method established within salmonids to quantitatively measure GHBP levels will be a useful tool to elucidate possible mechanisms responsible in modulating GHBPs found in vertebrates (Einarsdottir et al., 2014).

After GH was discovered researchers began performing various experiments (*in vivo* and *in vitro*) to elucidate the growth-promoting actions of GH. Through various experiments measuring radiolabeled sulfate incorporation, it was proposed that an unknown substance mediated the growth-promoting actions of GH (Denko and Bergenstal, 1955; Salmon and Daughaday, 1957). This unknown substance became known as “sulfation factor”, which was later named “somatomedin” (SM) because of its apparent regulation of GH actions on somatic growth, even though evidence demonstrated a diversity of biological effects (Salmon and Daughaday, 1957; Daughaday et al., 1972; Salmon and DuVall, 1970; Hall and Uthne, 1971). Further research into purifying SM, lead to the discovery of three forms; two of which had the ability to stimulate glucose uptake into adipocytes and muscle tissue (Froesch et al., 1963; Pierson and Temin, 1972; Hall and Uthne, 1971; Van Wyk et al., 1974). Due to these actions and the high homology in structure to insulin and its precursor, proinsulin, two forms of SM

were renamed insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-2 (IGF-2) (Rinderknecht and Humbel, 1978a, b; Klapper et al., 1983; Marquardt et al., 1981).

Insulin-like growth factors (IGFs) belong to the insulin superfamily and consist of 4 domains (B-C-A-D) that encode for a single 70 amino acid polypeptide (IGF-1) or a single 67 amino acid polypeptide (IGF-2) (Moriyama et al., 2000). The majority of circulating IGFs in fish are produced and released from the liver (Shamblott & Chen, 1993). IGF's primary actions are stimulation of protein synthesis, inhibition of proteolysis, and feedback inhibition of GH production (Fryburg, 1994; Fryburg et al., 1995; Blaise et al., 1995). Other actions include augmentation of glucose and free fatty acid uptake, while promoting lipogenesis (Jacob et al., 1989). Unique to fish species specifically, IGF influences osmoregulatory acclimation (McCormick et al., 1991). Increasing evidence has shown that IGF may have independent actions from GH (Rius-Francino et al., 2011; Butler and Roith, 2001). Furthermore, evidence indicates IGF can be locally produced by many nonhepatic tissues to promote cell proliferation and/or differentiation by autocrine or paracrine actions (Berishvili et al., 2006; Franz et al., 2016; Zygar et al., 2005).

IGF biological functions are influenced by two factors: high-affinity binding proteins known as insulin-like growth factor binding proteins (IGFBPs) and insulin-like growth factor receptors (IGFRs); in turn, these two factors can be impacted by environmental and biological cues (Norbeck et al., 2007; Hanson et al., 2014; Chen et al., 2016; Davis and Peterson, 2006; Breves et al., 2014). IGFs within vascular circulation are predominantly found bound to one of the members in a family of high-affinity binding proteins known as IGFBPs.

A common function of IGFBPs is to protect IGFs from proteolytic degradation. The half-life of IGF not bound to an IGFBP is approximately 10 minutes, however, when IGF is bound to

an IGFBP the half-life is significantly increased to multiple hours (Guler et al., 1989; Rajaram et al., 1997).

In mammals, a total of six core IGFBPs have been identified and characterized (Rajaram et al., 1997; Clemmons 2001). The origins of these 6 different IGFBPs have been hypothesized to be a pair of IGFBP genes that were produced when an ancestral IGFBP gene was duplicated and added adjacently within the same chromosome during an early stage of vertebrate evolution, otherwise known as tandem duplication (Ocampo Daza et al., 2011). Next, two whole new genome duplication events (2R) occurred with the chromosome containing the IGFBP paired genes, thus producing 6 IGFBP genes after one gene containing an IGFBP pair was lost (Ocampo Daza et al., 2011). Extending our knowledge into fish, a further round of whole genome duplication (3R) occurred causing two copies (paralogs) in each of the 6 core IGFBPs, except for IGFBP-4, to be retained among many teleost fish (Taylor et al., 2001; Sundstrom et al., 2008; Ocampo Daza et al., 2011; Allard & Duan, 2018; Garcia de la Serrana & Macqueen, 2018). Furthermore, a fourth whole genome duplication (4R) among salmonids has resulted in having up to 4 copies of each of the 6 core IGFBPs previously identified within the mammalian system, again, apart from IGFBP-4 (Macqueen et al., 2013; Allard & Duan, 2018; Garcia de la Serrana & Macqueen, 2018).

Vertebrate IGFBPs are a single chain polypeptide containing a signal peptide along with 3 distinct domains known as amino (N) -terminal domain, a linker domain (L-domain), and a carboxyl (C) -terminal domain (Firth & Baxter, 2002). Of the three domains, the N-terminal and C-terminal domains contain cysteine-rich residues (12 in the N-terminal domain and 6 in the C-terminal domain), which are highly conserved among species and are required for binding IGFs (Firth & Baxter, 2002; Wood et al., 2005). Much of the variability among vertebrate IGFBPs are

found within the central hinge region (L-domain), since no structural conservation is found among any of the 6 core IGFBPs (Firth & Baxter, 2002; Wood et al., 2005). A portion of the many functions of IGFBPs are found to stem from the linker region since this domain can contain functional motifs that mediate physiological functions and also contain sites of posttranslational modifications (Clemmons, 2001; Firth & Baxter, 2002).

IGFRs belong to the tyrosine kinase superfamily and exist as stable inactive heterotetrameric dimers. The extracellular region is composed of two α subunits linked through a disulfide bond forming the ligand-binding region; additionally, a β subunit is linked to each α subunit by a disulfide bond which forms a small portion of the extracellular region of the receptor (Wood et al., 2005). The transmembrane region is composed of only β subunits that pass through the transmembrane (Wood et al., 2005). The intracellular region is composed of β subunits containing tyrosine residues that become phosphorylated upon ligand binding allowing for the recruitment of substrate molecules to bind to their corresponding effector-binding sites (Wood et al., 2005). In turn, docking of substrates recruits second-messenger molecules and further phosphorylation to occur, leading to signaling pathways to be activated (Schlessinger, 2000; Adams et al., 2000). The evolution in which the family of receptors that IGFRs belong to has been extensively reviewed by Caruso and Sheridan (2011).

With observations made over 60 years ago, the original somatomedin hypothesis established GH as the hormone responsible for promoting somatic growth but was dependent on liver-derived IGF hormone mediating its actions (Salmon and Daughaday et al., 1957). Later researchers discovered IGF was produced in many tissues in addition to just the liver (e.g. brain, intestine, muscle, etc.). Similarly, this was also found to be true for fish (Duan et al., 1993; Vong et al., 2003; Wood et al., 2005; Reinecke, 2006). Therefore, another hypothesis was

proposed by Green and collaborators in the 1980s, suggesting GH stimulates growth by promoting precursor cells to differentiate, which causes cells to be responsive to IGF. Furthermore, GH stimulates the local production of IGF in these cells and IGF, in turn, promotes clonal expansion of differentiated cells; this is known as the dual effector hypothesis (Green, Morikawa & Nixon, 1985). More recently, growth research has implemented the techniques of gene knockouts to help explain the mechanisms of GH-IGF actions. Ohlsson and collaborators (2000), found liver-derived IGF is not required for postnatal growth but that autocrine and paracrine IGF is a major determinant of postnatal growth. Yet, within the 21st century, another hypothesis has been proposed to modify the original somatomedin hypothesis. The new hypothesis, known as the dual somatomedin hypothesis, suggests autocrine/paracrine IGF is responsible for postnatal body growth and liver-derived, endocrine-acting, IGF functions in negative-feedback effects on GH secretion and may have other effects on other physiological processes (Ohlsson et al., 2000).

Regulation of growth through the GH-IGF system may be affected by numerous other factors. External environmental and internal physiological cues, potentially including other hormones, could modify the actions of growth hormone (GH) and insulin-like growth factor (IGF). Important external environmental factors, specifically towards fish, have been found to influence growth including ambient water temperatures, the amount of time spent in light, the availability of oxygen, salinity, and endocrine disrupting chemicals. The environmental factors known to effect growth have been investigated previously by our lab (Poppinga et al., 2007; Hanson et al., 2012; Hanson et al., 2014; Martin, 2014; Hanson et al., 2017).

Another major factor affecting growth in all vertebrates is the nutritional status and related availability of nutrients to sustain growth-promoting actions. However, similar to external

environmental factors, interactions among and regulation of growth with the GH-IGF system are beyond the scope of this dissertation and previous work done by our lab can be found in Norbeck et al., 2007, Kittilson et al., 2011, Bergan et al., 2012, Walock et al., 2014, Walock 2017, Bergan et al., 2015, and Bergan-Roller & Sheridan, 2018.

The regulation of organismal growth is, thus, dependent on the GH-IGF system and the internal and external cues surrounding this system. Among vertebrates, the components of the GH-IGF systems are highly conserved. However, the peripheral regulation of this system is very complex, confusing, and remains poorly understood. Reindl and Sheridan (2012) reviewed the state of our understanding, including variation among species, larger taxonomic groupings, different tissues, research techniques, and different researchers. The complexity is compounded in fish due to the presence of many isoforms of different receptors, ligands and binding proteins involved. Despite a considerable amount of research into organismal growth, the understanding of how peripheral hormones outside of the central GH-IGF system influence growth are still being elucidated.

The overall aim of my research was to examine how some of the internal, extra-pituitary hormonal signals are involved in influencing the growth hormone insulin-like growth factor system. The more specific objectives for this research were to: (1) evaluate the effects that GH and IGF *in vitro* treatments have on the transcription of their own receptors and hormonal production, and (2-4, respectively) evaluate the effects of various hormones including *in vitro* thyroxine, cortisol, and reproduction-related steroids (e.g., estrogen and testosterone) on the transcription of the central components for the GH-IGF system including GHRs, IGFs and IGFR1s. Thyroxine and cortisol were chosen because of their broad ranges of metabolic and other effects. Reproduction-related steroids were predicted to affect growth in relation to their

importance in maturation and redirecting resources and physiological processes away from growth and toward reproduction.

The findings of this research can help establish which hormones outside of the GH-IGF system influence transcriptional regulation of the central growth genes that lead to cellular mechanisms promoting growth in rainbow trout. Even though this work pertains to rainbow trout specifically, due to the components of the GH-IGF system being highly conserved among vertebrates, the findings should have broader relevance to understanding growth processes among other vertebrates in general. Also, these findings should help in efforts to improve growth efficiency of vertebrate aquaculture and terrestrial livestock species.

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CHAPTER 2: EFFECTS OF GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR ON THE EXPRESSION OF GROWTH HORMONE RECEPTORS, INSULIN-LIKE GROWTH FACTORS AND INSULIN-LIKE GROWTH FACTOR TYPE 1 RECEPTORS IN RAINBOW TROUT

Abstract

The key coordinators in regulating growth are growth hormone (GH) and insulin-like growth factor (IGF) hormones. Together these two hormones make up the central growth hormone-insulin-like growth factor (GH-IGF) system. However, increasing evidence suggests GH and IGF may have independent physiological functions other than in growth. To understand GH and IGF, it is important to know what adjustments GH and IGF might impose on their own hormone sensitivity and production as well as baseline self-regulation. A series of studies were carried out using liver and gill tissue *in vitro* from sexually immature rainbow trout to evaluate the extra-pituitary self-regulatory effects of GH and IGF on transcriptional mRNA of growth hormone receptors (GHRs), insulin-like growth factors 1 and 2 (IGF1, IGF2) and type 1 IGF receptors (IGFR1). Transcription was evaluated using real-time quantitative polymerase chain reactions (RT-QPCR) after selected tissues were treated by GH or IGF. In liver, both GH and IGF treatments overall significantly decreased GH sensitivity of GHR1 in a time- and concentration-dependent manner and GHR2 in a concentration-dependent manner. Also, hepatic GH treatments significantly decreased the production of IGF1 and IGF2 over a period of time. Hepatic IGF treatments differentially regulated the production of IGFs. Both IGF1 and IGF2 production were significantly reduced over a period of time, yet with higher physiological and pharmacological concentrations of IGF, IGF2 production significantly increased. In gill, both GH and IGF treatments significantly elevated GH sensitivity of GHR1 over a period of time,

while GH sensitivity of GHR2 was significantly reduced only after a physiological concentration treatment of GH. In addition, both GH and IGF treatments on gill filaments significantly increased IGF sensitivity of IGFR1A over a period of time. Specifically, a physiological concentration of GH significantly reduced IGF sensitivity of both IGFR1A and IGFR1B. Finally, IGF production by gill filaments was differentially affected in a concentration-related manner. IGF2 production only significantly increased with physiological or pharmacological concentration treatments of GH, while IGF1 production was significantly reduced only after a pharmacological concentration of IGF was applied. However, IGF1 and IGF2 production reduced over a period of time with either treatment of GH or IGF. All experimental results indicate that GH and IGF do possess autocrine and/or paracrine actions in self-regulating hormone sensitivity and production.

Introduction

The process of growth is complex and intricately regulated through the cross-communication of internal and external cues in the central growth hormone (GH)/ insulin-like growth factor (IGF) system. The GH-IGF system has been studied in a vast array of vertebrate species, and the components of this system seem to be well conserved among lower vertebrates through higher vertebrates (Reinecke et al., 2005). Experiments performed within an array of fish species have shown that different types of growth hormone (e.g. bovine, salmonid, etc.), when administered, result in an increase in growth-promoting activities (i.e. weight and body length) (Agellon et al., 1988; Gill et al., 1985; Clarke et al., 1977; Moriyama & Kawauchi, 1990; Higgs et al., 1977; Dubowsky & Sheridan, 1995). Similar to other vertebrates, growth hormone is produced by the pituitary gland and the neuroendocrine regulation of its secretion involves both stimulatory and inhibitory factors in fish (Peng & Peter, 1997; Canosa, et al., 2007; Dai et

al., 2015). Among fish, GH mRNA expression has been detected also in tissues other than the pituitary such as the gills, heart, liver, kidneys, pyloric ceca, ovaries and lymphoid cells (Yang et al., 1999; Mori & Devlin, 1999; Calduch-Giner & Perez-Sanchez, 1999; Jeay et al., 2002). The central function of GH is to promote growth, but the detection of GH transcripts found in extra-pituitary tissues provides evidence that GH is involved in other physiological functions than just growth (e.g. osmoregulation, reproduction, and immune functions).

The primary mediator for the growth-promoting effects of GH are the insulin-like growth factors (IGFs) that are highly conserved among vertebrates, including fish (Le Roith et al., 2001; Wood et al., 2005). The majority of circulating IGFs in fish are produced and released from the liver (Shamblott & Chen, 1993). However, the expression of IGFs can be found in many extrahepatic tissues throughout all stages of development (Duguay et al., 1992; Shamblott & Chen, 1993; Duan & Plisetskaya, 1993; Tse et al., 2002; Biga et al., 2004; Pierce et al., 2004).

Treatments with GH have demonstrated an increase of circulating IGF plasma concentrations (Moriyama, 1995; Peterson et al., 2005). In order for IGFs to carry out their physiological functions they must bind to cell surface receptors of IGFR1 that belong to the receptor tyrosine kinase family (Caruso & Sheridan, 2011). Specifically, in salmonids, two isoforms (IGFR1A and IGFR1B) have been characterized (Chan et al., 1997; Greene & Chen, 1999). Similar to the vast distribution of IGFs found among extrahepatic tissues, the isoforms of IGFR1 are also widely expressed in a variety of tissues (Duguay et al., 1992; Shamblott & Chen, 1993; Duan & Plisetskaya, 1993; Parrizas et al., 1994; Tse et al., 2002; Maures et al., 2002; Biga et al., 2004) with gill tissue containing the highest abundance of IGFR1s in trout (Greene & Chen, 1999). The many tissues found to express and produce IGFs, in addition to the presence of IGFRs expressed in various tissues, strongly suggests that IGF has growth-promoting actions

that could act in both paracrine and autocrine organ-specific functions that are independent of GH.

The growth-related genes of rainbow trout are of interest because multiple forms of GHRs, IGFs and IGFR1s exists within the genome, due to a series of evolutionary whole genome duplication events (Meyer et al., 2005; Berthelot et al., 2014). In addition, within the rainbow trout species, the growth-related genes have previously been characterized (Yang et al., 1997; Greene and Chen, 1999; Very et al., 2005).

I experimentally manipulated the GH-IGF rainbow trout system *in vitro* to examine and clarify the effects of endogenous GH and IGF on receptor (GHR and IGFR1) sensitivity and hormone (IGF) production on the GH-IGF axis. The specific hypothesis for these studies is that GH and IGF directly modulate the expression of GHRs, IGFs, and IGFR1s.

Materials and Methods

Animals

Juvenile rainbow trout were obtained from Dakota Trout Ranch near Carrington, ND, and transported to North Dakota State University. Fish were maintained in 800-L circular tanks supplied with recirculated (10% replacement volume per day), dechlorinated, municipal water at 14 °C under a 12L:12D hour photoperiod. Fish were fed twice daily to satiation with AquaMax® Grower (PMI Nutrition International Inc., Brentwood, MO) semi-floating trout grower; except for 24-36 hours before initiating experimental manipulations. Fish were acclimated to laboratory conditions for at least two weeks prior to experimentation.

Experimental Conditions for *in vitro* mRNA Expression

For sampling, fish were anesthetized in 0.05% (v/v) 2-phenoxyethanol, measured and weighed (body length and body weight), and bled from the severed caudal vessels. All fish were

juvenile and sexually immature. Liver and gill arches were removed from fish, perfused ex vivo with 0.75% (v/v) saline solution, placed in Hank's medium (in mM: 137 NaCl, 5.4 KCL, 4 $NaHCO_3$, 1.7 $CaCl_2$, 0.8 $MgSO_4$, 0.5 KH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, and 4 of glucose, pH 7.6), and prepared for culture as previously described (Harmon & Sheridan, 1992; Hanson et al., 2010). Briefly, liver tissue was dissected into $1mm^3$ pieces and individual gill filaments were separated from the arches. Gill filaments and liver pieces from individual fish were pooled (i.e. one pool of each tissue of all fish) and washed three times with Hank's medium with 0.24% (w/v) bovine serum albumin. Tissues were dispersed in 24-well culture plates (containing 8-10 liver pieces and 12-15 gill filaments per well) containing 1-ml of Hank's medium with 0.24% (w/v) bovine serum albumin that included essential and non-essential amino acids. A preincubation period was performed at 14 °C under 100% O_2 while shaken at 100 rpm in a gyratory shaker. Replicate wells for a given experiment each came from different fish.

After a 3-hour preincubation period, the medium was removed and tissues received fresh Hank's medium with 0.24% (w/v) bovine serum albumin and essential and non-essential amino acids. Tissue treatments consisted of either salmonid GH (sGH), which was provided by Professor Akiyoshi Takahashi and Dr. Shunsuke Moriyama (Kitasato University, Japan) or salmonid IGF (sIGF) obtained from GroPep, Ltd. (Adelaide, Australia). Concentrations of sGH or sIGF ranged from 0-1000 ng/ml for 6 hours, or physiological levels of 100 ng/ml of sGH or sIGF for various time periods (0, 3, 6, 12 or 24 hours). Incubation of treatments was maintained at the same conditions as the preincubation period. Immediately following completion of treatments, incubation media was removed from the well and tissue samples were placed into 2.0-ml microcentrifuge tubes and frozen on dry ice; tissues were stored at -80 °C for later mRNA analysis.

Steady-state mRNA expression levels of GHRs and IGFs were measured in liver and gill filaments because the relative high levels of mRNAs encoding for the receptors and genes were previously observed in these tissues (Very et al., 2005; Duan et al., 1993; Vong et al., 2003; Wood et al., 2005). Steady-state expression of IGFR1- encoding mRNAs was measured in gill filaments due to the prevalence of IGFR1 mRNAs in this tissue (Nakao et al., 2002).

Quantitative Real-time PCR

Frozen tissues were homogenized and total RNA was isolated using RNAzol® reagent (Molecular Research Center, Cincinnati, OH, USA) as specified by the manufacturer's protocol. Each RNA pellet was re-dissolved with RNAse-free deionized water and total RNA was quantified by ultraviolet (UV A_{260}) spectrophotometry using the Nanodrop 1000 Spectrophotometer (Thermo Scientific, West Palm Beach, FL, USA). mRNA was reverse transcribed in 5-ul reactions using 200 ng total RNA and qScript™ cDNA synthesis kit reagents (qScript Reaction Mix, qScript RT, and nuclease-free water) according to the manufacturer's protocol (Quanta Biosciences™, Inc., Gaithersburg, MD, USA). To eliminate the possibility of contamination with genomic DNA, reactions without reverse transcriptase were included as negative controls; amplification was not detected in negative controls.

Steady-state mRNA levels of GHR1, GHR2, IGF-1, IGF-2, IGFR1A, and IGFR1B were determined by quantitative real-time PCR using the Stratagene MX 3000 detection system (Stratagene, La Jolla, CA, USA). The gene-specific nucleotide primers and probes used to quantify the genes can be found in Table 1. In short, real-time PCR reactions were carried out for samples, standards, and controls in 10-ul total volume reactions. The reactions contained 2 ul of cDNA from the reverse transcription reactions, 5 ul of 2X Brilliant® II QPCR Master Mix, 1 ul of gene-specific probe, 0.85 ul of β-actin probe, 0.5 ul of gene-specific forward and reverse

primers as well as β-actin forward and reverse primers, 0.15 ul of reference dye (Stratagene, Agilent Technologies). Real-time PCR reactions were multiplexed. Cycling parameters were set as follow: an initial denaturation step of 95 °C for 10 minutes followed by 50 cycles of replication, each consisting of 95 °C for 15 seconds and then finally an extension step of 58 °C for 1 minute. Sample copy number was calculated from the threshold cycle number (CT), and the CT was related to the gene-specific standard curve followed by normalization to β-actin.

Statistics

Data are presented as means ± SEM; n represents the number of replicates for each treatment group. Statistical differences were analyzed by one-way ANOVA followed by the Tukey-Kramer test. An α value of < 0.05 was used to indicate significant differences between treatment groups. Statistics were performed with JMP®, Version 12 (SAS Institute Inc., Cary, NC, USA). Outliers of replicates were determined using Dixon's test with an α value of < 0.05 indicating required significance level.

Table 1. Primers and probes used for quantitative real-time PCR

Gene Target	*Description*	*Sequence*	*Concentration (nM)*
GHR1	Forward Primer	5’ TGAACGTTTTTGGTTGTGGTCTA 3’	600
	Reverse Primer	5’ CGCTCGTCTCGGCTGAAG 3’	600
	Probe	5’ ^{FAM}-CAAATGCAAGGATTCC-^{MGBNFQ} 3’	100
GHR2	Forward Primer	5’ CATGGCAACTTCCCACATTCT 3’	600
	Reverse Primer	5’ GCTCCTGCGACACAACTGTTAG 3’	600
	Probe	5’ ^{FAM}-TTCATTTGCCTTCTCC-^{MGBNFQ} 3’	100
IGF-1	Forward Primer	5’ GTGGACACGCTGCAGTTTGT 3’	900
	Reverse Primer	5’ CATACCCCGTTGGTTTACTGAAA 3’	900
	Probe	5’ ^{FAM}-AAAGCCTCTCTCTCCA-^{MGBNFQ} 3’	150
IGF-2	Forward Primer	5’ ACGTGTCGGCCACCTCTCTA 3’	600
	Reverse Primer	5’ TGGGACATCCTGTTTGATTGTG 3’	600
	Probe	5’ ^{FAM}-AGATCATTCCCATGGTGC-^{MGBNFQ} 3’	150
IGFR1A	Forward Primer	5’ AGAGAACACATCCAGCCAGGTT 3’	600
	Reverse Primer	5’ TCCTGCCATCTGGATCATCTT 3’	600
	Probe	5’ ^{FAM}-TGCCCCCGCTGAA-^{MGBNFQ} 3’	150
IGFR1B	Forward Primer	5’ CCTGAGGTCACTACGGGCTAAA 3’	600
	Reverse Primer	5’ TCAGAGGAGGGAGGTTGAGACT 3’	600
	Probe	5’ ^{FAM}-ATCCGTCCCAGTCCT-^{MGBNFQ} 3’	150
β-actin	Forward Primer	5’ GGCTTCTCTCTCCACCTTCCA 3’	600
	Reverse Primer	5’ AGGGACCAGACTCGTCGTACTC 3’	600
	Probe	5’ ^{VIC}-TGCTTGCTGATCCACAT-^{MGBNFQ} 3’	150

Abbreviations: GHR, growth hormone receptor; IGF, insulin-like growth factor; IGFR, insulin-like growth factor receptor.

Results

Growth Hormone *in vitro* Experiments

GHRs

As shown in previous work from our lab (Very et al., 2005), GHR1 and GHR2, outside of the brain components, are abundantly expressed both in liver tissue and gill filaments. Peripheral GH sensitivity was evaluated by quantifying mRNA expression of GHRs within liver tissue. Treatment with GH significantly decreased hepatic GHR1 mRNA transcription levels in a concentration- and time-related manner. At a GH concentration of 10 ng/ml steady-state GHR1 mRNAs fell below control levels; similar decreases in GHR1 mRNA levels were observed with GH concentrations of 100 ng/ml and 1000 ng/ml (Fig. 1A). GHR1 mRNAs rapidly decreased in expression after a 3-hour treatment with GH when compared to control levels; similar declines in GHR1 mRNA expression levels were also seen after 12 hours of treatment (Fig. 1B). GH treatment had a significant effect controlling hepatic GHR2 mRNA expression levels in a concentration- and time-related manner. A significant decrease in GHR2 mRNAs resulted only with a treatment concentration of 1000 ng/ml when compared to control levels (Fig. 1C). Yet, a significant increase in steady-state GHR2 mRNA levels was seen after 6 hours of GH treatment (Fig. 1D); all other time points remained at control levels.

GH sensitivity was also evaluated by quantifying mRNA expression of GHRs in gill filaments. Under GH treatment, mRNAs encoding GHR1 were not significantly regulated in a concentration-related manner (Fig. 2A). However, GH treatment significantly regulated GHR1 mRNA expression in a time-related manner with an increase in GHR1 mRNA expression levels observed at 12 hours (Fig. 2B). GH treatment significantly influenced steady-state GHR2 mRNA expression levels in a concentration-related, but not time-related manner (Fig. 2C and

2D). A reduction in steady-state GHR2 mRNA levels was seen at a concentration of 10 ng/ml when compared to gill filaments treated with 1 ng/ml of GH, while no differences were seen when compared to control levels.

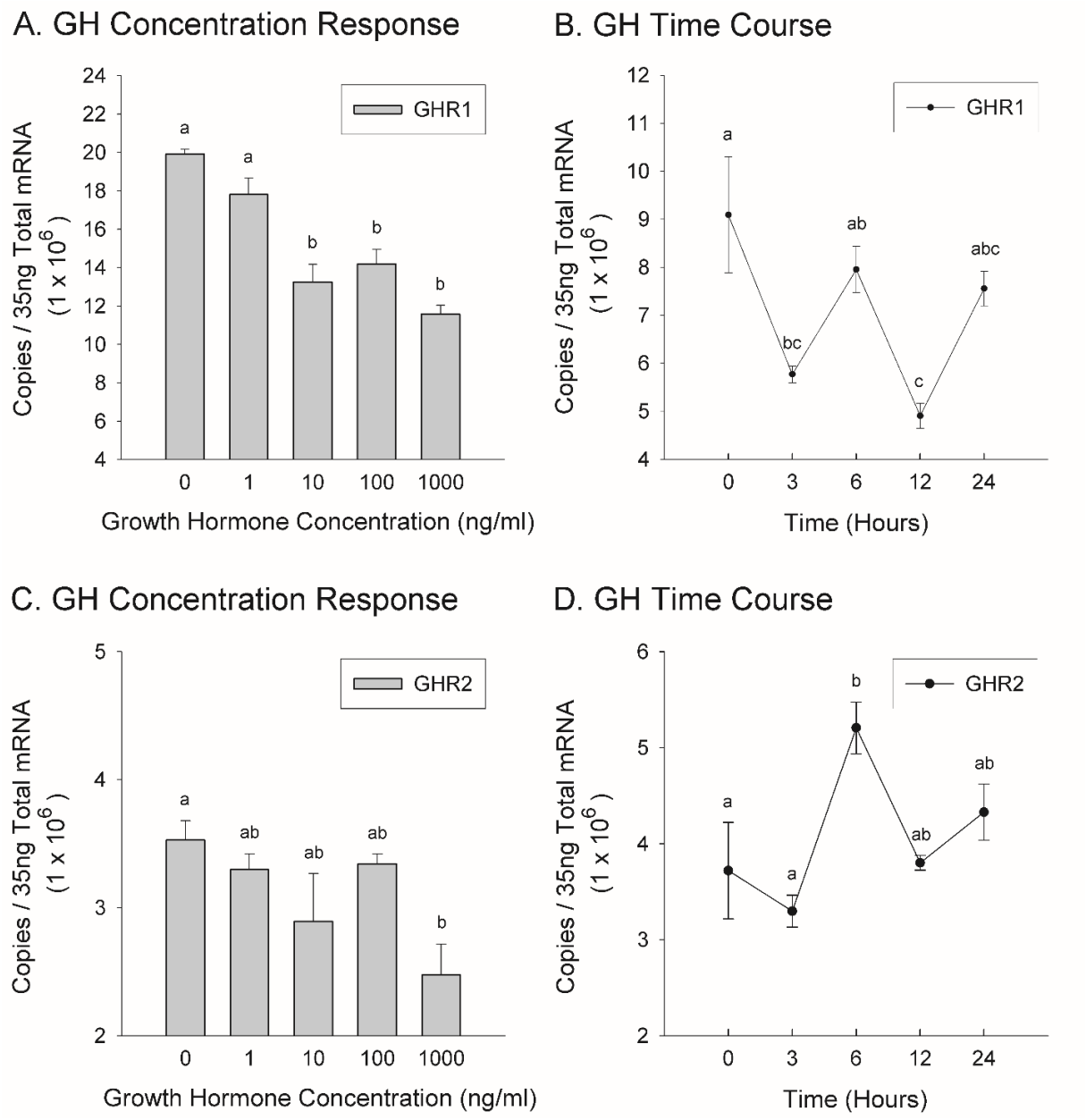

Fig. 1. Effects of growth hormone (GH) treatments on mRNA expression of growth hormone receptors (GHRs), GHR1 (A and B) or GHR2 (C and D), in liver of rainbow trout incubated *in vitro*. Liver tissue was incubated with various concentrations of GH for 6 h (A and C) or were incubated for various times with 100 ng/ml GH (B and D). Data are presented as mean ± SEM. (n = 4-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

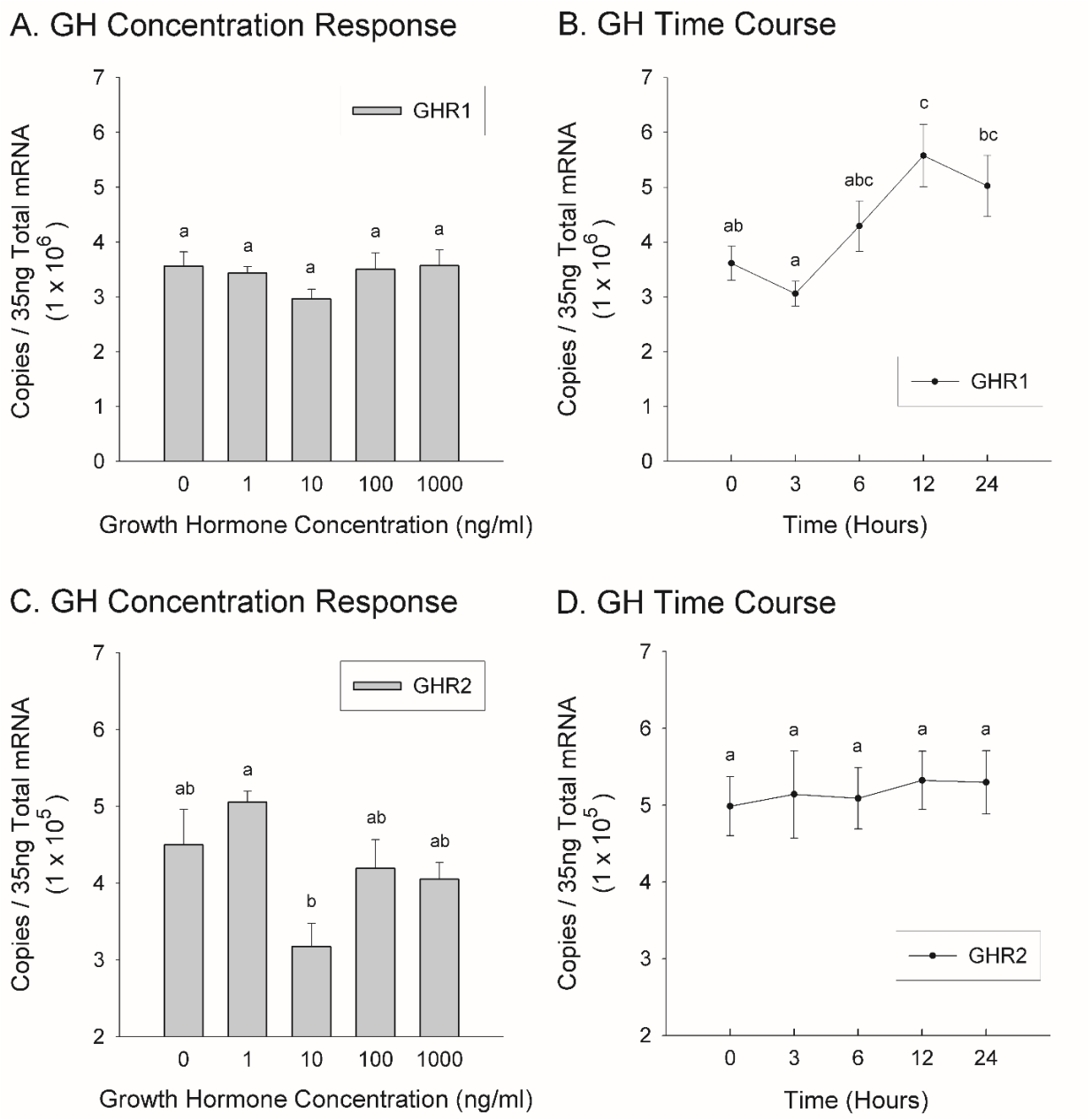

Fig. 2. Effects of growth hormone (GH) treatments on mRNA expression of growth hormone receptors (GHRs), GHR1 (A and B) or GHR2 (C and D), in gill of rainbow trout incubated *in vitro*. Gill filaments were incubated with various concentrations of GH for 6 hours (A and C) or were incubated for various times with 100 ng/ml GH (B and D). Data are presented as mean ± SEM. (n = 5-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFs

IGF production was evaluated by quantifying mRNA expression of IGFs in hepatic tissue following *in vitro* treatments of GH. IGF1 mRNA expression levels were not significantly regulated in a concentration-related manner (Fig. 3A), though the duration in how long liver tissue was treated significantly influenced IGF mRNA expression levels (Fig. 3B). Steady-state IGF1 mRNAs quickly decreased mRNA expression with a 3-hour GH treatment when compared to control levels; furthermore, treatment with GH for 12 and 24 hours brought about the greatest decline in IGF1 mRNA expression. GH treatments on liver tissue significantly influenced IGF2 mRNA expression levels in a concentration- and time-related manner. Among the different concentrations in GH, a dose of 1000 ng/ml significantly decreased steady-state IGF2 mRNAs when compared to hepatic tissue treated with 1 ng/ml of GH; however, neither of these concentrations produced a significant difference from control levels (Fig. 3C). IGF2 mRNA expression levels were reduced after 3 hours and remained at this level thereafter, with a 100 ng/ml treatment of GH, when compared to control levels (Fig. 3D).

In addition, peripheral IGF production was evaluated with gill filaments following *in vitro* treatments of GH by measuring mRNA expression of IGFs. Differing concentrations of GH had no effect in controlling IGF1 mRNA expression levels for gill filaments (Fig. 4A), although, duration of treatment did have a significant effect in controlling IGF mRNA expression levels. Inhibition of IGF1 mRNA production was observed after 3 and 6 hours of treatment; further attenuation of steady-state IGF1 mRNAs occurred at 12- and 24-hour treatments (Fig. 4B). Treatments with GH were significant in modulating IGF2 mRNA expression level in a concentration- and time-related manner. An increase in mRNAs encoding for IGF2 occurred with a concentration of 100 ng/ml of GH when compared to the control group; similar mRNA

expression levels at 100 ng/ml were seen with a treatment at 1000 ng/ml GH (Fig. 4C). On the contrary, decreases in IGF2 mRNA expression levels were observed after 12 and 24 hours of GH treatment when compared to control levels (Fig. 4D).

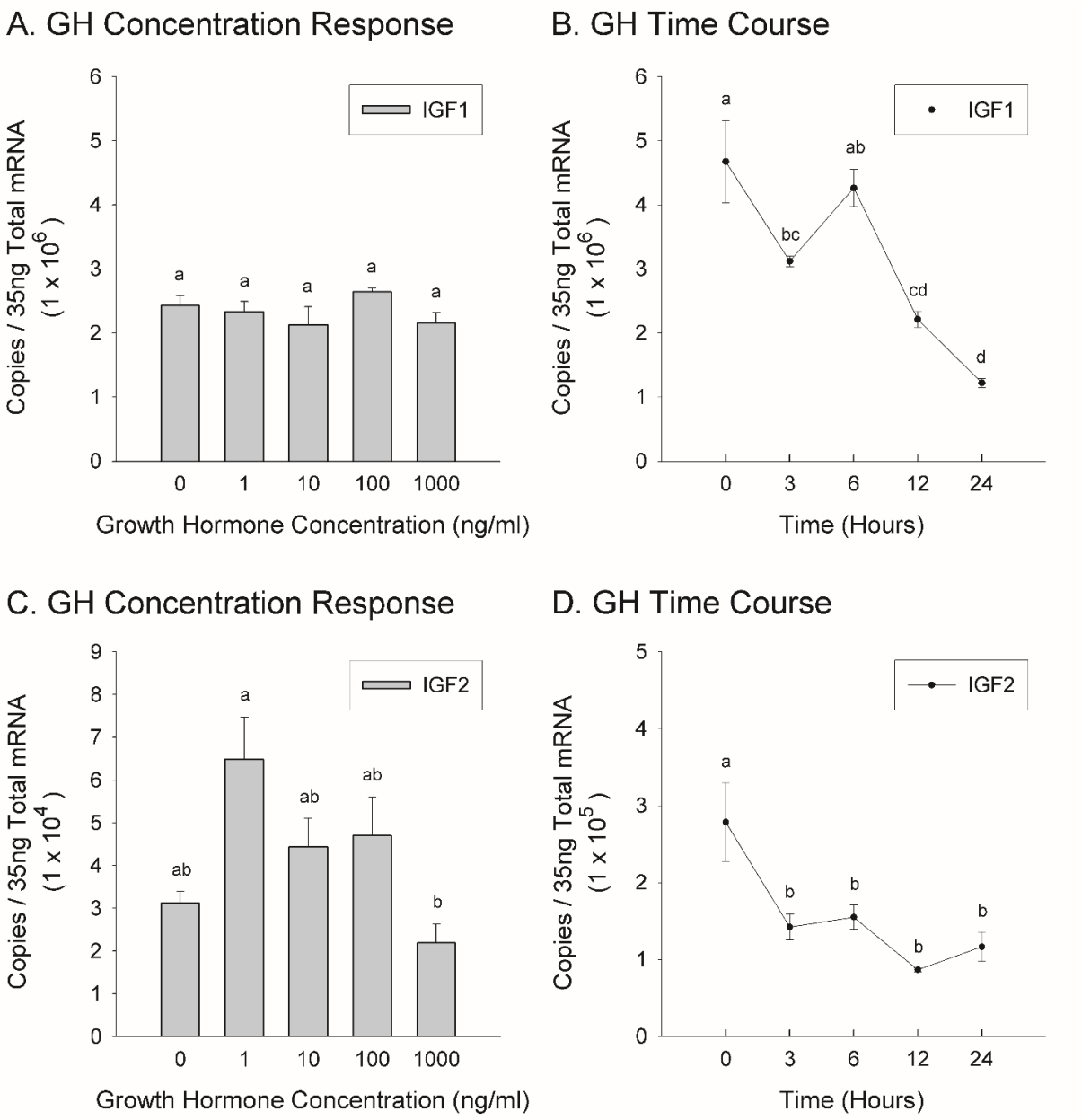

Fig. 3. Effects of growth hormone (GH) treatments on mRNA expression of insulin-like growth factors (IGFs), IGF1 (A and B) or IGF2 (C and D), in liver of rainbow trout incubated *in vitro*. Liver tissue was incubated with various concentrations of GH for 6 hours (A and C) or were incubated for various times with 100 ng/ml GH (B and D). Data are presented as mean ± SEM. (n = 4-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

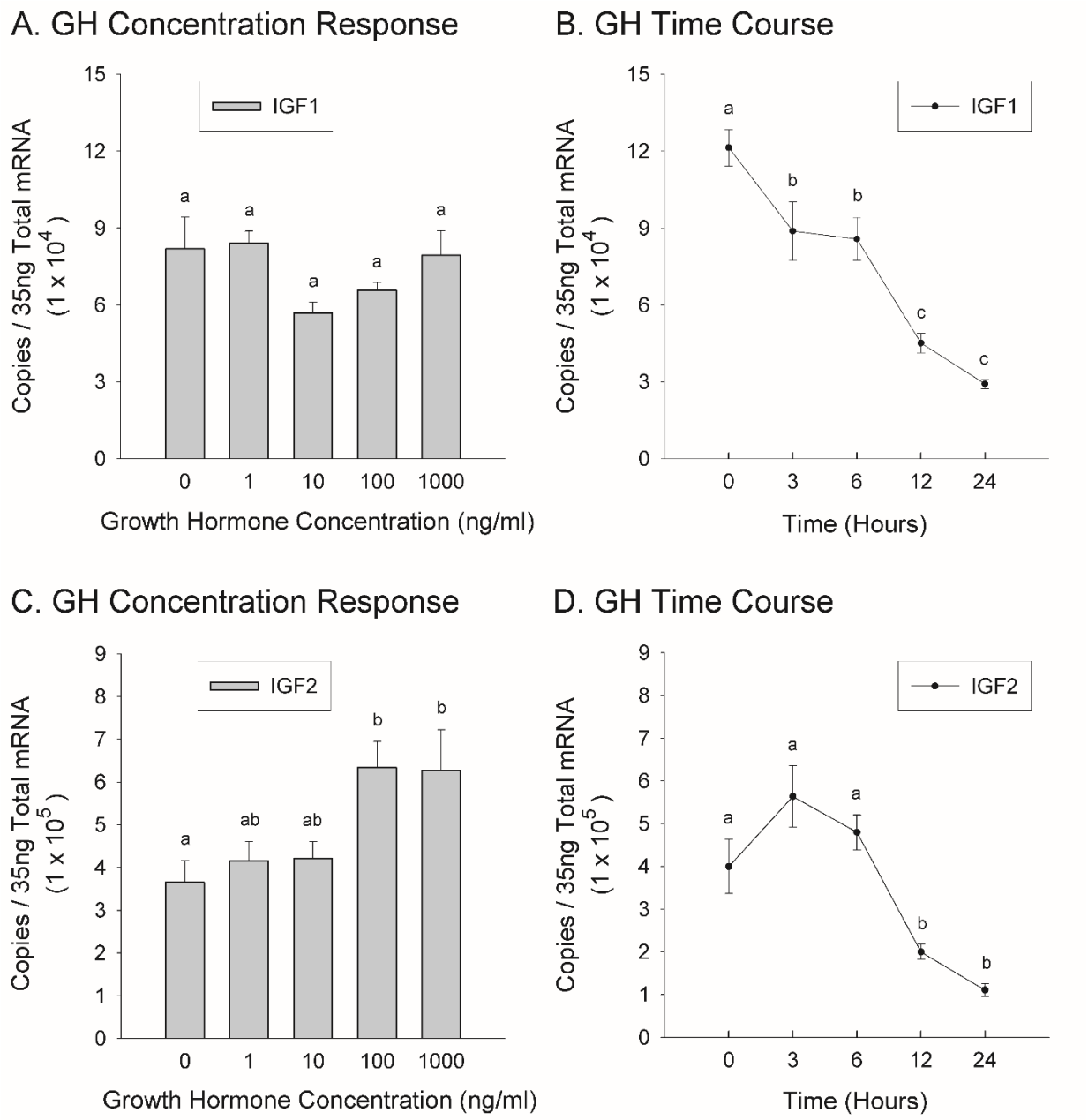

Fig. 4. Effects of growth hormone (GH) treatments on mRNA expression of insulin-like growth factors (IGFs), IGF1 (A and B) or IGF2 (C and D), in gill of rainbow trout incubated *in vitro*. Gill filaments were incubated with various concentrations of GH for 6 hours (A and C) or were incubated for various times with 100 ng/ml GH (B and D). Data are presented as mean ± SEM. (n = 5-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFRs

Peripheral IGFR sensitivity was evaluated by quantifying mRNA expression levels of IGFRs in gill filaments after *in vitro* GH treatments. IGFR1A encoding mRNAs were significantly regulated in a concentration- and time-related manner with GH treatments. A decrease in IGFR1A mRNA expression levels was seen only with a concentration of 10 ng/ml of GH when compared to control expression levels (Fig. 5A). However, an increase in IGFR1A mRNAs resulted after 12 and 24 hours of GH treatment when compared to control levels (Fig. 5B). After a 10 ng/ml GH treatment, IGFR1B mRNA expression levels significantly declined when compared to the no treatment group (Fig. 5C). GH treatments were not significantly effective in regulating IGFR1B mRNA expression levels in a time-related manner (Fig. 5D).

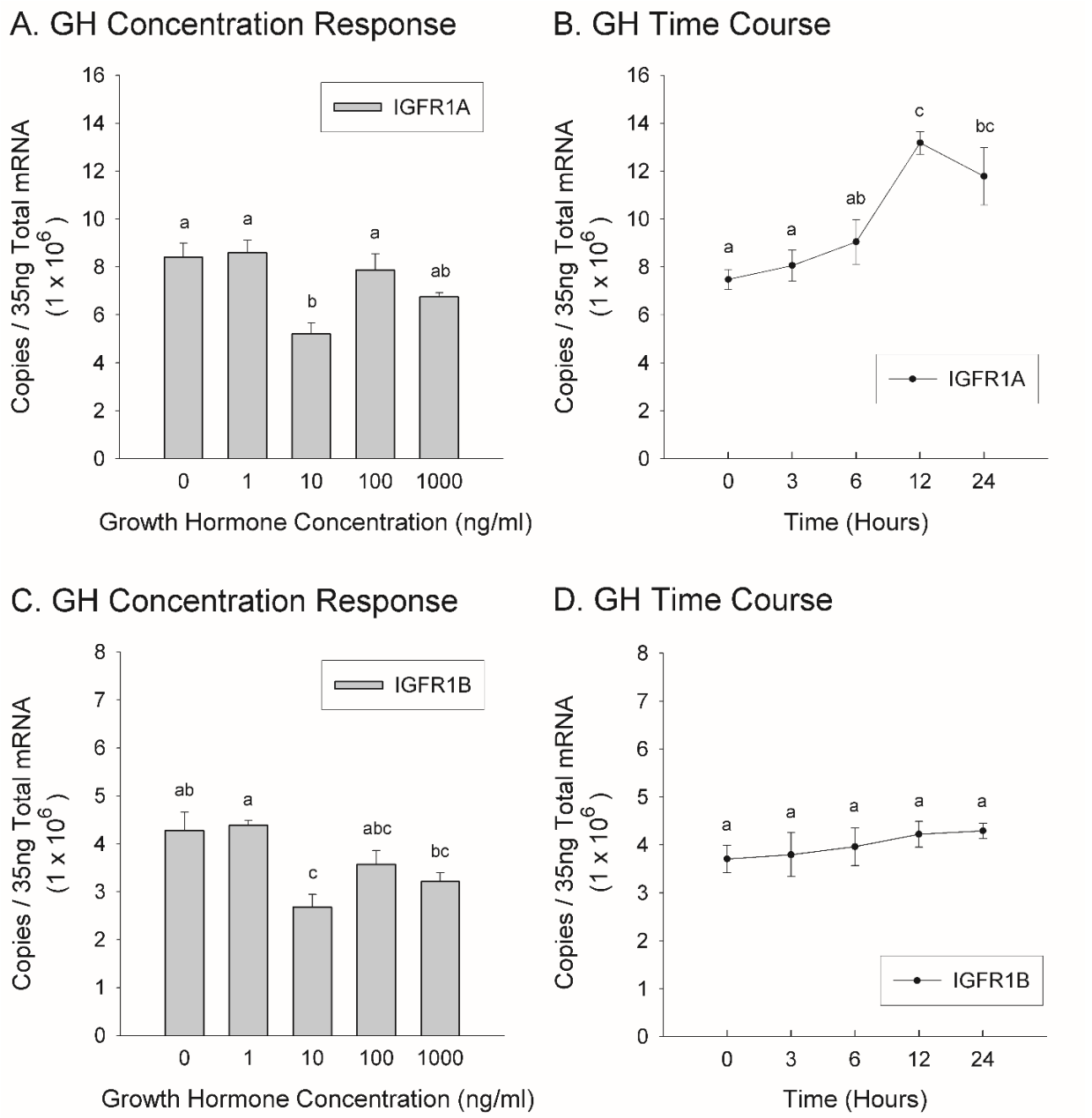

Fig. 5. Effects of growth hormone (GH) treatments on mRNA expression of insulin-like growth factor receptors (IGFRs), IGFR1A (A and B) or IGFR1B (C and D), in gill of rainbow trout incubated *in vitro*. Gill filaments were incubated with various concentrations of GH for 6 hours (A and C) or were incubated for various times with 100 ng/ml GH (B and D). Data are presented as mean ± SEM. (n = 5-6). For a given IGFR1 subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

Insulin-like Growth Factor Hormone *in vitro* Experiments

GHRs

GH sensitivity was assessed by measuring mRNA expression levels of GHRs within liver tissue after *in vitro* IGF experiments. Treatments with IGF diminished steady-state GHR1 mRNA expression levels in a concentration- and time-related manner. Significant changes in GHR1 mRNA levels from the controls were seen first with a dose of 1 ng/ml and levels remained diminished to the same extent with all other treatment concentrations (Fig. 6A). After 12 and 24 hours of treatment with IGF a significant decrease occurred in GHR1 mRNA expression levels when compared to the control (Fig. 6B). Steady-state GHR2 mRNAs were also regulated by IGF in a concentration- and time-related manner. Expression levels of GHR2 mRNAs were significantly reduced with all treatment concentrations of IGF to the same extent when compared to control levels (Fig. 6C). On the contrary, GHR2 mRNAs increased in expression compared to control mRNAs in a time-related manner. Significant increases in hepatic GHR2 mRNA expression were seen to peak at 6- and 12-hour treatments, with mRNA expression levels returning to control levels at 24 hours (Fig. 6D).

Gill filaments were implemented in evaluating peripheral GH sensitivity by quantifying GHR mRNA expression. IGF treatments were significant in regulating GHR1 mRNA expression levels in concentration- and time-related manner. A reduction in GHR1 mRNA levels was seen at a concentration of 1000 ng/ml when compared to gill filaments treated with 10 ng/ml of IGF, while no differences were observed when compared to control levels (Fig. 7A). IGF significantly increased steady-state GHR1 mRNAs by 6 hours; maximum stimulation was observed at 12 and 24 hours (Fig. 7B). IGF treatments were not significantly effective in

regulating GHR2 mRNA expression levels in a concentration- or time-related manner (Fig. 7C and 7D).

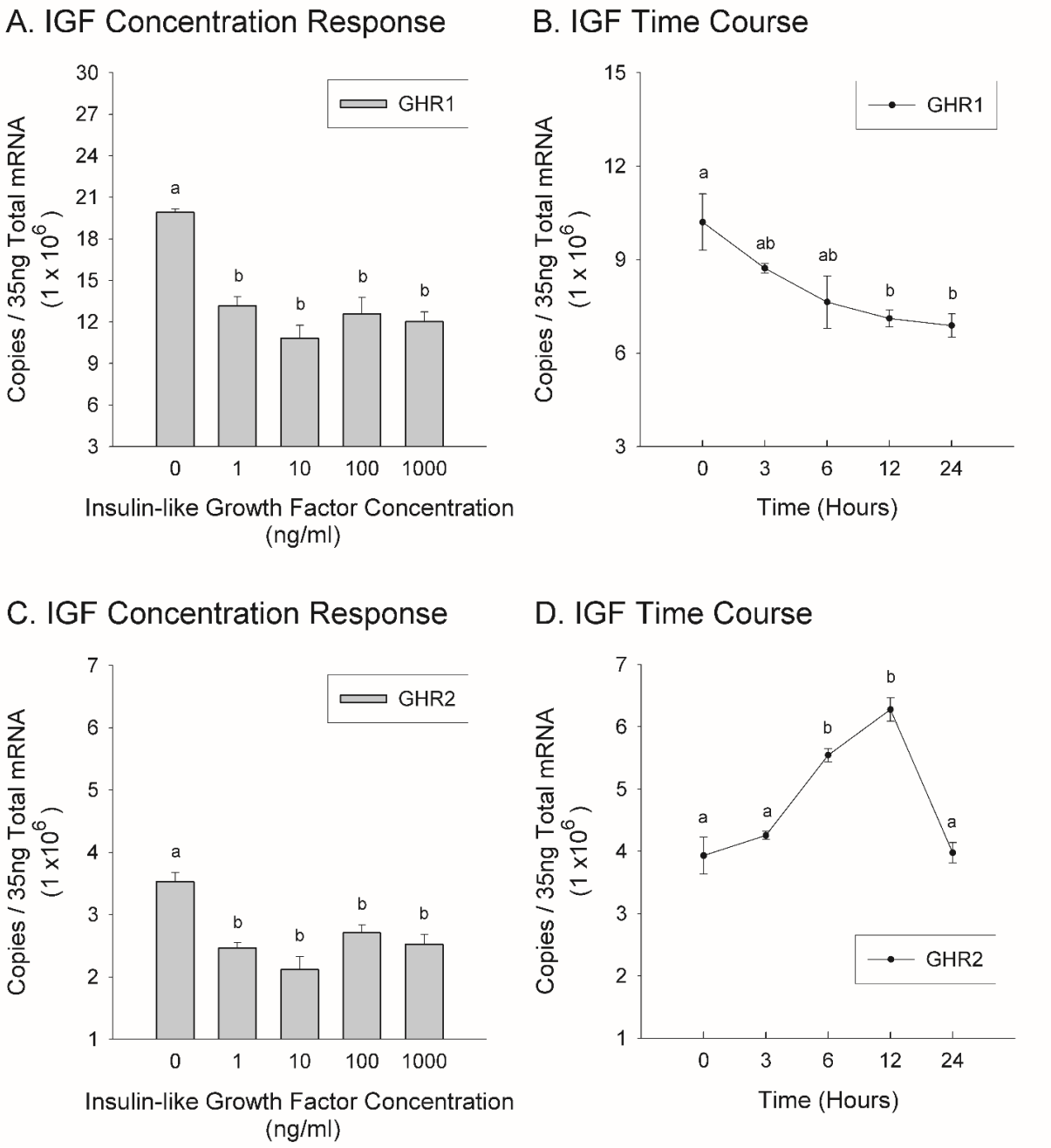

Fig. 6. Effects of insulin-like growth factor (IGF) treatments on mRNA expression of growth hormone receptors (GHRs), GHR1 (A and B) or GHR2 (C and D), in liver of rainbow trout incubated *in vitro*. Liver tissue was incubated with various concentrations of IGF for 6 hours (A and C) or were incubated for various times with 100 ng/ml IGF (B and D). Data are presented as mean ± SEM. (n = 4-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

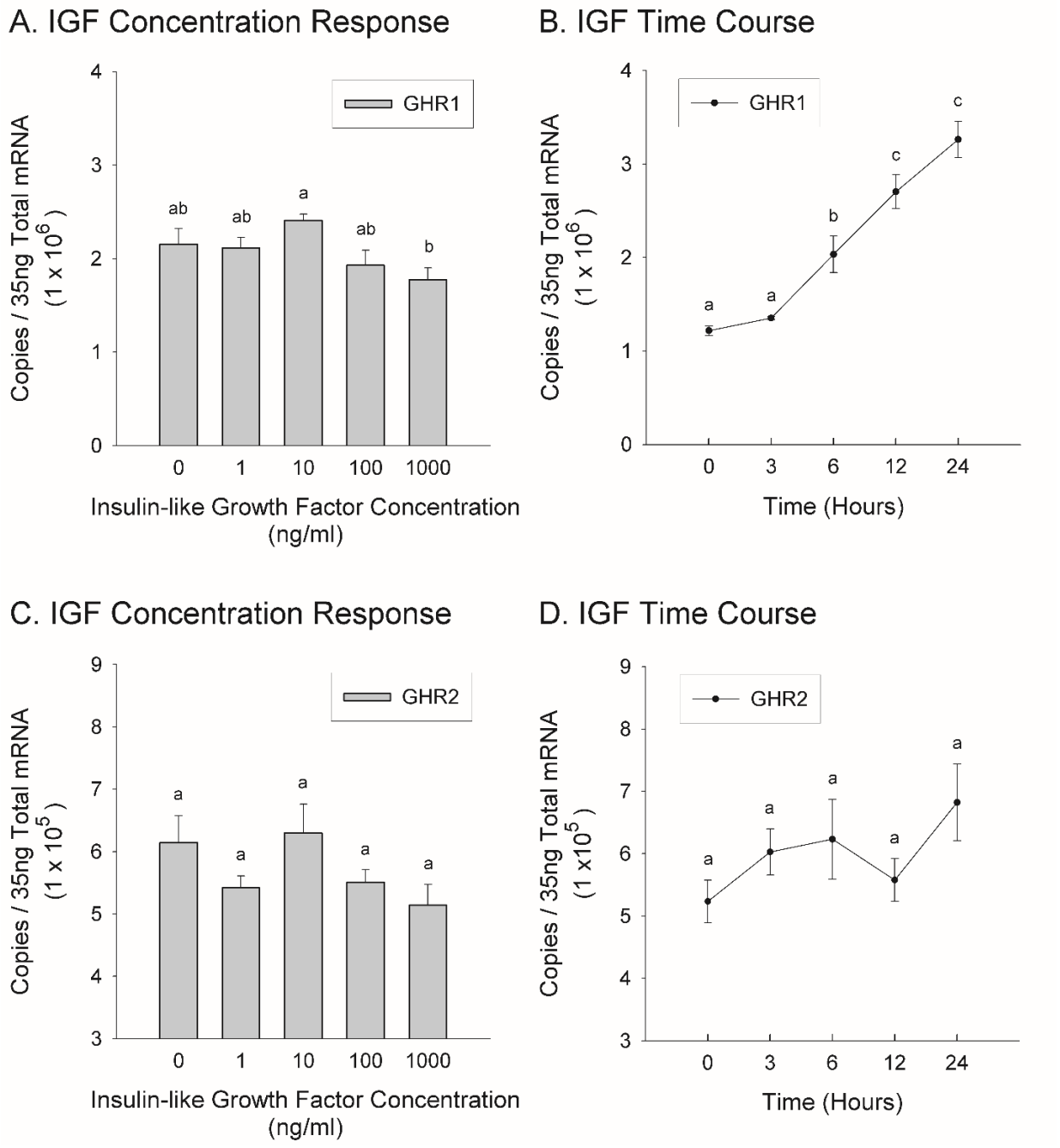

Fig. 7. Effects of insulin-like growth factor (IGF) treatments on mRNA expression of growth hormone receptors (GHRs), GHR1 (A and B) or GHR2 (C and D), in gill of rainbow trout incubated *in vitro*. Gill filaments were incubated with various concentrations of IGF for 6 hours (A and C) or were incubated for various times with 100 ng/ml IGF (B and D). Data are presented as mean ± SEM. (n = 4-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFs

IGF production was evaluated by measuring IGF1 and IGF2 mRNA expression in hepatic tissue following IGF *in vitro* treatments. Treatments of IGF were significant in regulating IGF1 mRNA expression levels in a time-related (Fig. 8B), but not concentration-related, manner (Fig. 8A). At 3 hours, a peak in steady-state IGF1 mRNAs was observed over control levels; yet, IGF1 mRNA expression levels fell below control levels at 12 hours. Maximum suppression in production of IGF1 mRNAs was detected at 24 hours. Treatments of IGF were significant in regulating IGF2 mRNA expression levels in a concentration- and time-related manner. Lower doses of 1 ng/ml and 10 ng/ml of IGF reduced IGF2 mRNA expression levels when compared to concentrations of 100 ng/ml and 1000 ng/ml; however, all treatment groups were not significantly different from the control group (Fig. 8C). A reduction in IGF2 mRNA expression levels were also observed after all treatment times (3, 6, 12 and 24 hours) of IGF treatments when compared to control levels (Fig. 8D).

Peripheral IGF production was evaluated by measuring IGFs mRNA expression of gill filaments following *in vitro* treatments of IGF. The mRNA expression levels of IGF1 were significantly controlled by IGF treatments in a concentration- and time-related manner. A concentration of 1000 ng/ml induced a decline in IGF1 mRNA expression levels from the control (Fig. 9A). In addition, a decline in IGF1 mRNA expression was observed after 12 and 24 hours of IGF treatment (Fig. 9B). Various IGF concentrations were not significant in regulating IGF2 mRNA expression levels (Fig. 9C); however, IGF2 mRNA levels were regulated in a time-related manner. A significant decline in IGF2 mRNA expression was seen after 3 and 6 hours of treatment, with maximal reduction occurring at 12 and 24 hours post IGF treatment (Fig. 9D).

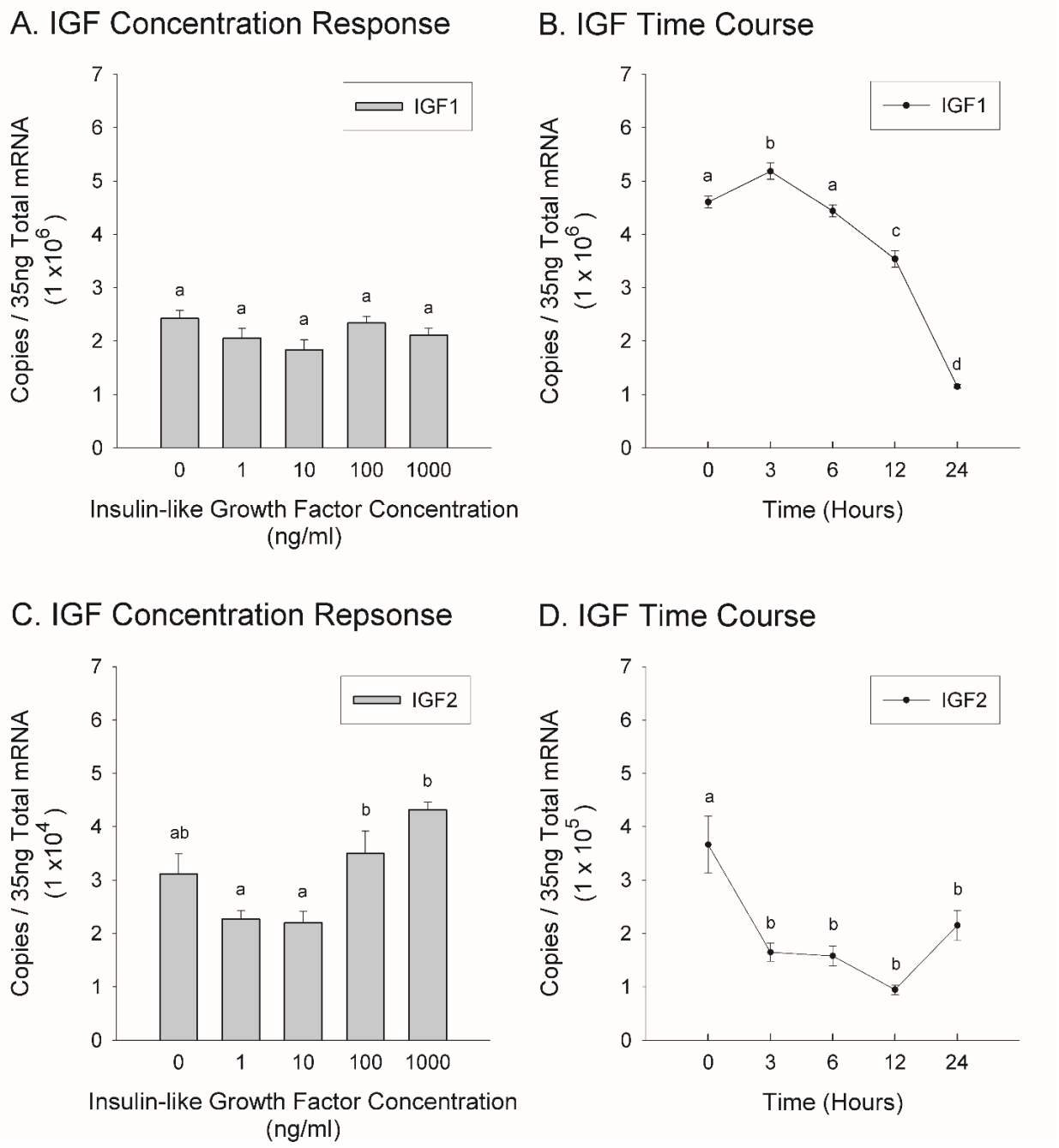

Fig. 8. Effects of insulin-like growth factor (IGF) treatments on mRNA expression of IGFs, IGF1 (A and B) or IGF2 (C and D), in liver of rainbow trout incubated *in vitro*. Liver tissue was incubated with various concentrations of IGF for 6 hours (A and C) or were incubated for various times with 100 ng/ml IGF (B and D). Data are presented as mean ± SEM. (n = 4-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

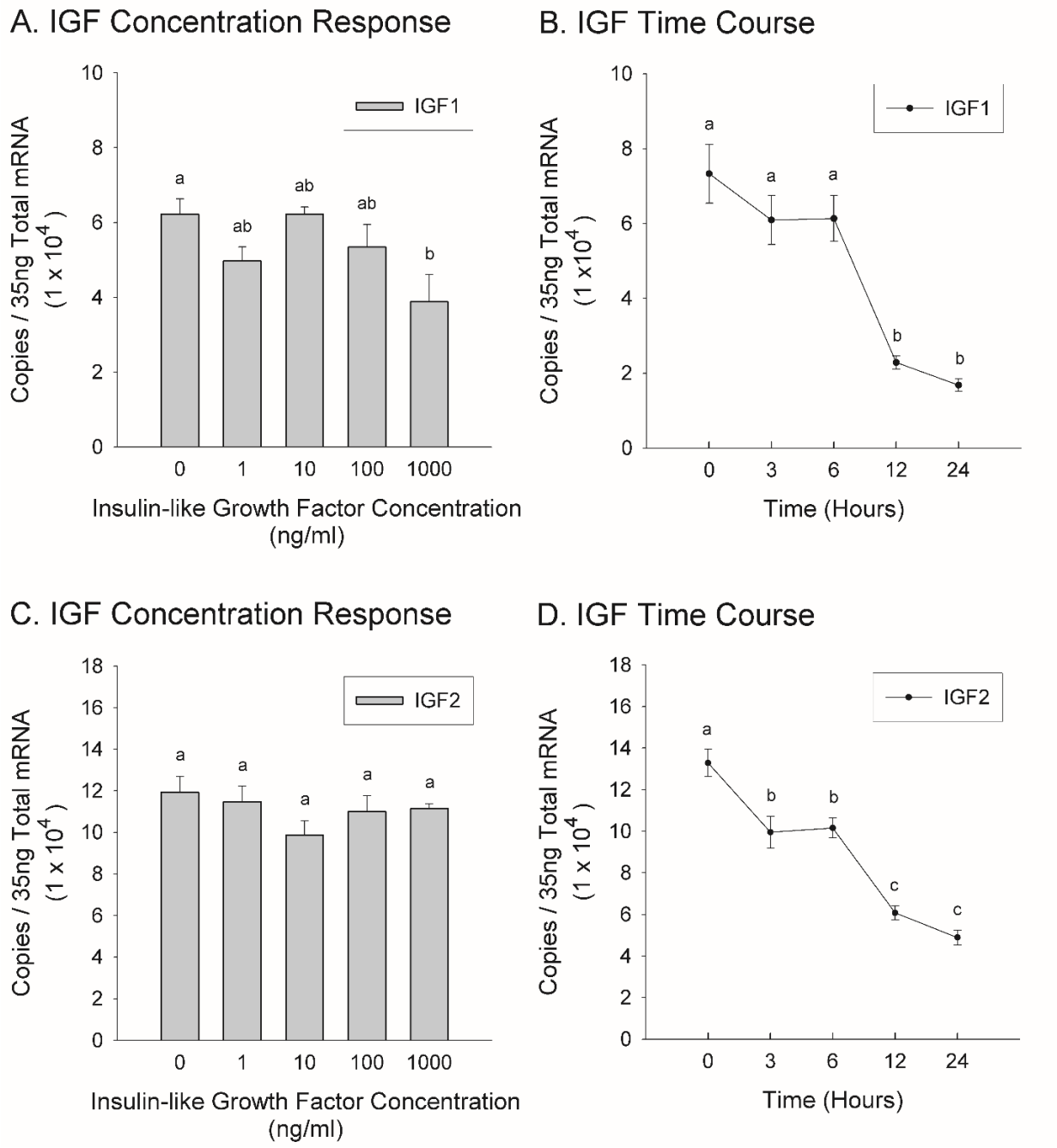

Fig. 9. Effects of insulin-like growth factor (IGF) treatments on mRNA expression of IGFs, IGF1 (A and B) or IGF2 (C and D), in gill of rainbow trout incubated *in vitro*. Gill filaments were incubated with various concentrations of IGF for 6 hours (A and C) or were incubated for various times with 100 ng/ml IGF (B and D). Data are presented as mean ± SEM. (n = 4-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFRs

Peripheral IGF sensitivity was evaluated by quantifying IGFRs mRNA expression levels following *in vitro* IGF treatments of gill filaments. Treatments with IGF were significant in regulating IGFR1A mRNA expression levels in a time-related, but not concentration-related, manner (Fig. 10A and 10B). Steady-state expression levels of IGFR1A mRNAs increased after a 24-hour treatment with IGF. Steady-state mRNA expression levels of IGFR1B were not significantly regulated in a concentration- or time-related manner (Fig. 10C and 10D).

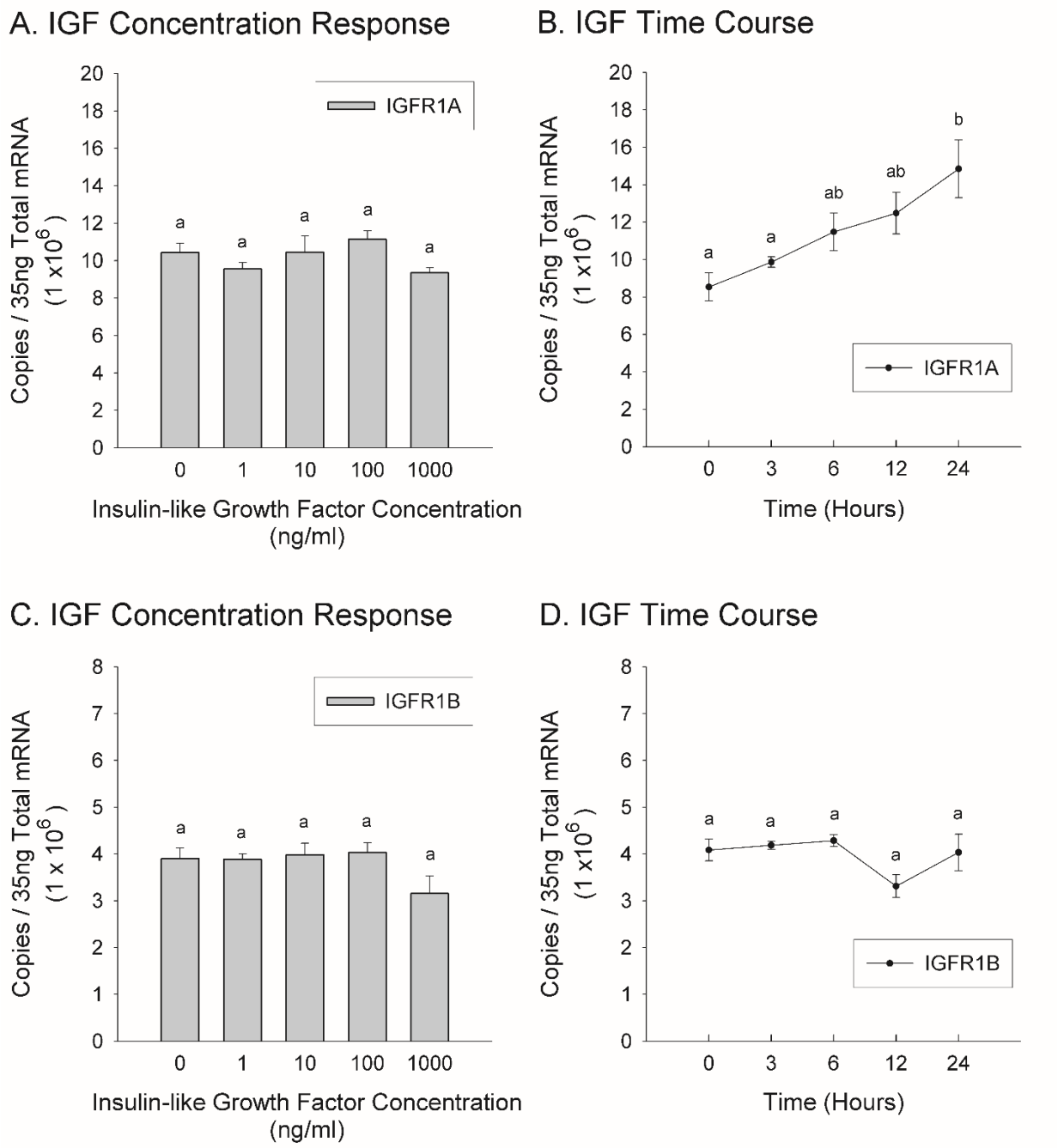

Fig. 10. Effects of insulin-like growth factor (IGF) treatments on mRNA expression of insulin-like growth factor receptors (IGFRs), IGFR1A (A and B) or IGFR1B (C and D), in gill of rainbow trout incubated *in vitro*. Gill filaments were incubated with various concentrations of IGF for 6 hours (A and C) or were incubated for various times with 100 ng/ml IGF (B and D). Data are presented as mean ± SEM. (n = 4-6). For a given IGFR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

Discussion

Growth hormone and insulin-like growth factor are well known for stimulating cell growth and proliferation. My experiments provide an in-depth examination of how GH and IGF peripherally influence steady-state mRNA transcription levels of receptor sensitivity and hormone production on components of the GH-IGF system for a teleost fish. The results of these studies indicate components of the GH-IGF system are differentially regulated directly by GH and IGF treatments in rainbow trout hepatic and gill filament tissue cultures; these results support our starting hypothesis that GH and IGF modulate the expression of GHRs, IGFs and IGFR1s. From these studies the effects of GH and IGF on gene transcription for receptor sensitivity (GHR and IGFR) and hormone production (IGF) suggest GH and IGF feedback mechanism(s) at the hepatic and gill filament mRNA expression levels and suggest GH and IGF possess autocrine/paracrine actions.

The ability of GH to peripherally regulate aspects of the GH-IGF system was demonstrated by several findings. First, GH directly inhibited GHR1-encoding mRNAs in the liver. However, the same effect was not seen on all tissue examined; a stimulation in GHR1-encoding mRNAs was observed in gill filaments. Second, over a period of time GH stimulated GHR2 mRNA expression levels in the liver but had no effect on GHR2-encoding mRNAs in gill over a period of time. Third, over a period of time GH directly inhibited the mRNA expression of both IGF1 and IGF2 in all tissues examined. Fourth, GH directly stimulated mRNAs encoding for IGFR1A but had no effect on mRNA expression levels for IGFR1B in gill. Encompassing all results, these findings indicate that GH differentially regulates peripheral responsiveness for GHR1 and GHR2 in an isoform-specific and tissue-specific manner, reduces

peripheral production of IGFs and stimulates peripheral responsiveness to IGFRs in an isoform-specific manner.

An interesting observation from these studies indicates when you treat liver tissue with GH it causes the GHR1 mRNA expression levels to decrease in a time- and concentration-related manner. In a study with vertebrates, a time- and dose-dependent down-regulation in GHRs was also observed after a single injection of bovine GH (bGH) was administered to rats (Maiter et al., 1988). In fish, an intraperitoneal injection of recombinant bream GH (rbGH) significantly down-regulated GHR mRNA expression on liver treated (Shved et al., 2011). Other studies, however, produced conflicting results with either no change or up-regulation in mRNA expression levels of growth hormone receptors after GH treatments. In another study performed on black seabream, *in vivo* GH treatments resulted in no change in GHR mRNA expression (Jiao et al., 2006).

Turning to studies that utilized *in vitro* cell-seeded cultures of hepatocytes, both GHR1 and GHR2 mRNA expression levels significantly increased in a dose-dependent manner after treatment with recombinant grouper GH (rgGH). However, GHR1 mRNA expression levels were only noticeably increased after a high concentration dose of rgGH, while a significant increase in GHR2 mRNA expression levels was noted over a physiological and supra-physiological concentration (Wang et al., 2016).

Even within our own lab we have contradictory results. When isolated rainbow trout hepatocytes were treated with trout GH (tGH), a 2.5- to 3-fold increase in transcription of GHR2 and GHR1 mRNAs, respectively, resulted (Very & Sheridan, 2007). Also, cell surface mRNA expression of GHR1 and GHR2 increased with increasing salmonid GH (sGH) concentration treatments, utilizing a Chinese hamster ovarian cell line (CHO-K1) to express already

characterized rainbow trout GHR1 and GHR2 receptors. A reduced surface expression of GHRs in CHO-K1 cells occurred only after GH treatments were removed from a 24-hour induction period (Reindl et al., 2009). In my experiments, a differential regulation in GHR1 and GHR2 mRNA expressions were observed. GHR1 mRNA transcription displayed a down-regulation after GH treatment in a concentration- and time-dependent manner, while GHR2 mRNA transcription displayed an up-regulation in a time-dependent manner. Down-regulation occurred only at a supra-physiological concentration.

Similarly, differential regulation was also observed among isolated Mozambique tilapia hepatocytes treated with tilapia GH. From a 2-hour treatment to a 4-hour treatment of GH, Pierce and colleagues (2012) observed an increase in GHR2 mRNA expression levels that remained elevated after a 6-hour treatment, but soon after declined toward control GHR2 mRNA levels in tilapia not treated with GH. In the same experiments performed by Pierce (2012), GH treatment of tilapia hepatocytes at any time point did not significantly affect GHR1 mRNA levels when compared to tilapia hepatocytes not treated with GH. Pierce (2012) suggested that the reason for the differential regulation in receptor mRNA expression levels among GHRs was due to specific functions these receptors may hold. GHR2 in fish may be the functional receptor involved in growth-promoting actions, while GHR1 in fish may be involved in regulating metabolic effects by interpreting the nutritional status of the fish and regulating GH actions through this specific receptor.

Post-transcriptional modifications to the GHR extracellular domain might also be important in regulating GHR mRNA expression levels after GH treatment. For instance, GHBP are formed in most non-rodent mammals and in fish by the process of shedding a portion of the extracellular domain of the GHR gene (Einarsdottir et al., 2014). Schantl et al. (2004) stated that

for GHBP to form in non-rodent mammals, GH must be present and bound to the first high-affinity binding site of the GHR. This might in part explain the decrease in GHR1 mRNA expression observed in my experiments. When an abundance of GH is present to bind to the first high-affinity binding site of the GHR, it may allow for the proteolytic cleavage to the extracellular domain of the GHR causing the formation of a complex that consist of GH bound to the extracellular domain of the GHR, or otherwise known as the GH-GHBP complex. When GH is bound to GHBP it could cause a decrease in the turn-over rate of GHR mRNA expression levels observed in some tissues.

Another explanation for the decrease in GHR1 mRNA expression during my experiment might be from a binding study previously done by our lab. In that study we utilized a transfected Chinese hamster ovarian cell line (CHO-K1) to express rainbow trout GHRs. It was observed that short term exposure of radio-labeled isotope sGH by CHO-K1 cells expressing trout GHRs caused a down-regulation in GHRs expressed at the surface level. This decrease in cell surface expression was by internalizing the ligand-receptor complex into the cells within 15 minutes of GH exposure (Reindl et al., 2009). Experimental conditions were carried out for a 90-minute duration, however, time points beyond 90 minutes were not tested. Perhaps this internalization of the ligand-receptor complex can last up to 3 hours, explaining the decrease in steady-state mRNA expression observed in my experiments for GHR1. This decrease might prime the tissue only to reset the cell so it responds to new information, which may explain the increase of GHR1 mRNA expression toward control levels at 6 hours. However, liver tissue may favor the internalization of ligand-receptor complex for GHR1 since GHR1 mRNA expression levels decrease even further with a 12-hour treatment.

In gill filaments, GHR1 mRNA expression levels increased over a period of time while no change in GHR2 mRNA expression levels were observed. Euryhaline fish gills play a vital role in adaptation to differing salinity environments. Studies performed on Atlantic salmon showed that plasma GH levels increased with day length and that increased plasma GH levels also strongly correlated with an increase in gill Na+, K+ -ATPase activity (McCormick et al. 2000). Also, when temperatures increase during spring season plasma GH levels positively correlate with a rise in temperatures (McCormick et al., 2002). It is logical that GHR1 mRNA expression levels increase for the high GH concentration levels found consistently in all studies of euryhaline fish prior to entering seawater, in preparation for the changing parameters.

The classic view is that GH release from the anterior pituitary stimulates the production of IGF by the major target organ of the liver in fish (Wood et al., 2005). However, my results demonstrated liver tissue treated by GH over a time displayed a decrease of IGF1 and IGF2 mRNA expression levels. IGF2 mRNA expression levels in particular decreased in a concentration-related manner when liver tissue was treated with 1 ng/ml of GH versus 1000 ng/ml GH. An overwhelming number of studies with many species of fish have demonstrated that hepatocytes treated with GH increase in IGF mRNA expression, whether treatments were performed *in vitro* or *in vivo* (Shamblott et al., 1995; Schmid et al., 2000; Kajimura et al., 2001; Tse et al., 2002; Vong et al., 2003; Pierce et al., 2004; Peterson et al., 2005; Pierce et al., 2005; Leung et al., 2008; Pierce et al., 2010; Reindl et al., 2011; Shved et al., 2011). Parallel to the results seen with GH treatments increasing hepatic IGFs mRNA expression, injection of GH treatment on extrahepatic tissues have shown to stimulate IGF1/IGF2 mRNA expression levels (Sakamoto & Hirano, 1993; Shamblott et al., 1995; Vong et al., 2003). In my experiments, GH treatments at concentrations of 100 ng/ml and 1000 ng/ml significantly increased gill IGF2

mRNA expression levels. These results correlate to what has been observed in other studies that have shown an increase in mRNAs encoding for IGFs after GH treatments have been administered in gill tissue (Sakamoto & Hirano, 1993; Vong et al., 2003).

In euryhaline fish a major event that occurs during their life history is the ability to transfer from freshwater to saltwater environments, known as the smoltification process or the parr-smolt transformation. The ability for euryhaline fish to be able to transition from freshwater mediums to saltwater medium or vice versa is a unique trait even among certain species of teleost fish and they utilize their ability to maintain the balance between fluid water content at a homeostatic state through osmoregulation. The primary structures in fish that play a vital role in osmoregulation are the gills. Osmoregulation studies in fish have shown GH to play a vital role in the smoltification process. Specifically, plasma GH levels increase during transfer from freshwater to saltwater environments (Young et al., 1989; Sakamoto & Hirano, 1993). GH actions have also shown to reduce the plasma sodium levels (Collie et al., 1989) and also increase gill Na+/K+-ATPase activity in salmonids (Bjornsson et al., 1987; McCormick et al., 2000). A study performed *in vivo* on rainbow trout examined how different treatments of IGF1 and GH prior to a seawater transfer affected measurements of plasma osmolarity. Results indicated GH and IGF had the ability to effectively improve the ability to maintain plasma osmolarity, furthermore, IGF treatments acted in a dose-dependent manner and had a greater effect in maintaining plasma osmolarity than GH (McCormick et al., 1991). These results indicated action of GH known for seawater adaptation once again might be mediated through IGF1. My results indicate IGF2 might play a role in mediating homeostatic regulation in changing environments for our rainbow trout line.

On the other hand, two different studies in two different species of fish (Rainbow trout and Mozambique tilapia) indicated there was no change in IGF mRNA expression of gill tissue when treated with GH (Shamblott et al., 1995; Kajimura et al., 2001). However, I observed that IGF1 and IGF2 mRNA expression levels significantly decreased with GH treatment over longer time periods. The only plausible explanation for the observed decrease in both IGF1 and IGF2 mRNA expression levels over a period of time in liver and gill tissue with these experiments may lie in how cells/tissues are cultured. Many of the *in vitro* experiments displaying an increase in IGFs involved isolated hepatocyte cells cultured on plates with surfaces designed to provide suitable conditions for cell lines to grow. Whereas in my experiments, whole tissue pieces cultured onto culture plates may not be as suitable for the hormone to penetrate through membranes of the tissue to reach the target cells. Whole tissue cultures may require more time for the hormone to reach the cells that contain the genes I examined for analysis of growth.

The ability of IGF to peripherally regulate aspects of the GH-IGF system was demonstrated by several findings. First, IGF directly inhibited GHR1-encoding mRNAs in the liver, but directly stimulated GHR1-encoding mRNAs in gill filaments. Secondly, over time IGF treatments directly increased expression levels for GHR2 in liver but had no effect on GHR2 mRNA expression in gill. Third, IGF at first stimulated IGF1-encoding mRNAs in the liver, but over longer periods of time directly inhibited IGF1 mRNA expression. In addition, IGF directly inhibited IGF1 mRNA expression in gill. Fourth, IGF directly inhibited IGF2 mRNA expression in both liver and gill tissues. Lastly, IGF directly stimulated IGFR1A mRNA expression, but had no effect on IGFR1B mRNA expression in gill. All together, these findings indicate IGF differentially regulates peripheral responsiveness to GHR1 and GHR2 tissue-specific and isoform-specific manner, differentially regulates IGF1 and IGF2 production in a isoform-specific

manner in liver, overall reduces IGF production in the gill, and stimulates peripheral IGF responsiveness to IGFRs in a isoform-specific manner.

One of the primary functions of insulin-like growth factor is to stimulate somatic growth, but it also known to have functions in regulating the amount of GH being released by the anterior pituitary, resulting in IGF also possessing a negative feedback mechanism in controlling growth. GH plasma levels were shown to significantly and rapidly decrease when hIGF1 has been injected into rainbow trout. This attenuation of GH plasma levels remains up to at least 2 hours after trout have been injected with hIGF1 (Blaise et al., 1995). Also, cultured pituitaries from tilapia and gilthead seabream displayed a decrease in GH released in a dose-dependent manner (Kajimura et al., 2002; Mohammed-Geba et al., 2016). In agreement with these previous studies, Fruchtman et al. (2000) showed that isolated pituitary cells from many teleost species had suppressed GH secretion. Additionally, in gilthead seabream IGF treatments significantly diminished mRNAs encoding for GH in a dose-dependent manner (Mohammed-Geba et al., 2016). Fruchtman and colleagues (2001; 2002) demonstrated a localization of high-affinity IGFR1s within fish pituitary cells; when IGF binds to its receptors in these cells IGF elicits it actions in two distinct cellular signaling pathways of the phosphatidylinositol 3-kinase (PI 3-K) and mitogen-activated protein kinase (MAPK) in controlling GH production and release. In my experiments, GHR1 and GHR2 were down-regulated after IGF treatments in a dose-dependent manner. Also, mRNAs encoding for GHR1 diminished in a time-dependent manner whereas mRNAs encoding for GHR2 increased at 6-12 hours of IGF treatment but returned to control levels after 24 hours. In comparing GH production and release at the pituitary related to extra-pituitary tissues such as the liver, it is plausible that not only does IGF regulate GH production

and release at the pituitary level, it may also diminish GHR sensitivity at the hepatic level as part of it negative feedback mechanism in controlling growth.

My studies indicated a rapid, significant decrease in hepatic IGF2 mRNA expression levels in a time-related manner. Hepatic IGF1 mRNA expression levels were found to significantly increase rapidly, but with and after a 12-hour treatment IGF1 mRNA expression levels fell dramatically. I know of only two other studies examining hepatic tissue and measuring expression levels encoding for IGF1 mRNAs. In both studies, hepatocytes that were treated with no hormone and just growth medium showed a significant decrease in IGF1 mRNA expression after approximately 12-18 hours of cells being cultured (Schmid et al., 2000; Pierce et al., 2004). Schmid and colleagues (2000) also examined the *in vitro* effects of IGF1 treatment on IGF1 mRNA expression levels. Similar to my experiments, they found that over a time-course, which extended beyond my treatment times, IGF1 mRNA expression levels decreased with treatments of IGF1. Within the same set of experiments, Schmid and colleagues (2000) also noted no change in IGF1 mRNA expression. They suggested that this may mean IGF1 does not possess any autocrine or paracrine functions within tilapia hepatocytes.

I cannot explain why my studies show an increase in gill GHR1 mRNAs after treatment with IGF over time. In my GH treatment I didn't see an increase in the transcription of IGFs, whereas the majority of researches observe either an increase or no change in IGF transcription. However, my IGF treatment might have caused what most other researchers observed with increased IGF transcription if it were to circulate either in a paracrine/autocrine fashion in the liver (since IGF is primarily produced by the liver) or perhaps could act in an endocrine manner at the mRNA level of the gill.

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CHAPTER 3: EFFECTS OF THYROXINE ON THE EXPRESSION OF GROWTH HORMONE RECEPTORS, INSULIN-LIKE GROWTH FACTORS AND INSULIN-LIKE GROWTH FACTOR TYPE 1 RECEPTORS IN RAINBOW TROUT

"Of the numerous types of hormones known in vertebrates, thyroid hormones (THs) are among the most puzzling." Holzer and Laudet (2015)

Abstract

In fish, thyroid hormones, thyroxine (T4) and triiodothyronine (T3), are important for embryonic growth and larval development. Early studies show that maternal-derived thyroid hormone concentrations decrease as embryogenesis and larval development proceed. Also, many *in vivo* immersion T4 and T3 experiments have shown improved or increased growth, development, and survival rates of several fish species. However, the exact genes and mechanisms in which thyroid hormones influence juvenile growth and development stages are unclear. It has been shown that the main thyroid hormone produced by thyroid follicles in fish is T4 and that it is deiodinated to T3 in peripheral tissues, such as the liver. A few studies have focused on the direct effects of T3 on growth genes with little attention paid to whether T4 has any direct effects on growth genes. Therefore, a series of time and concentration studies, as well as, an inhibitory study utilizing the thioamide inhibitor, methimazole, and a combination treatment study of T4 and GH were conducted to identify if direct effects of T4 exist in liver or gill filament tissues. Real-time quantitative polymerase chain reaction (RT-QPCR) was used to evaluate various treatments with T4 on target growth genes in the growth hormone-insulin-like growth factor (GH-IGF) system. T4 generally decreased GH and IGF sensitivity and IGF production over time in both liver and gill tissues. However, T4 did not have consistent effects on transcriptional mRNA levels. Therefore, I deduced that T4 does not directly affect

transcription of genes controlling for growth, but instead, could work in concert with many different hormones or molecules to bring about its effects on growth and development.

Introduction

Although growth is generally controlled through the growth hormone (GH)/insulin-like growth factor (IGF) system in vertebrates, with GH and IGF being the primary hormones through the GH-IGF axis (Woods et al., 2005), other hormones such as thyroid hormones [thyroxine (T4) and triiodothyronine (T3)] also have functions in regulating growth in fish. It is well known that thyroid hormones are necessary for normal growth and development in vertebrates such as fish (Power et al., 2001). It has been shown that there are surges of T4 and T3 production at certain stages of the fish life cycle that are important for fish development and growth. Without these surges there are dramatic faults in the development of a fish (Inui and Miwa, 1985; Miwa and Inui, 1987).

Like other vertebrates, teleost fish produce and release T4 from thyroid follicles. However, unlike mammals, teleost fish do not possess a thyroid gland, but have scattered thyroid follicles in the subpharyngeal region along the afferent artery (Yamano, 2005) and sometimes within the regions of the heart and head of the kidney (Geven et al., 2007). T4 is the main hormone produced by the thyroid follicles in fish and is deiodinated to T3 in peripheral tissues, predominantly the liver (Blanton & Specker, 2007). Interestingly, GH can stimulate the conversion of T4 to T3 by increasing the 5' monodeiodinase enzyme responsible for removing an iodide unit from the outer ring of T4 (De Luze and Leloup, 1984; MacLatchy and Eales, 1990; MacLatchy et al., 1992; Leatherland and Farbridge, 1992).

Much of the work on how thyroid hormones influence growth has been on the production and secretion of GH at the pituitary level. Isolated pituitaries with a treatment of T3 were found

to increase the synthesis of GH (Melamed et al., 1995). Administration of thyroid hormones produced an increase in mRNA steady-state levels of GH (Moav and McKeown, 1992) as well as a concentration-dependent response in the secretion of GH (Luo and McKeown, 1991; Farchi-Pisanty et al., 1995) from pituitaries of rainbow trout and carp. However, not all studies agree that thyroid hormones increase the production and release of GH from the pituitary. Nishioka and colleagues (1985) found that administration of T3 had no effect on the secretion of GH from tilapia pituitaries. In eel injected with T4 or T3 an inhibition of GH synthesis and secretion occurred (Rousseau et al., 2002).

In my experiments rainbow trout (Oncorhynchus mykiss) were used to investigate the influence of thyroxine on the GH-IGF system. An *in vitro* tissue culture system was used to elucidate and gain further understanding of the endogenous actions that thyroxine has in peripherally influencing somatic growth. A supplementary study examining different antagonist to thyroxine were conducted to validate specific effects from treatments of thyroxine. The genes responsible for controlling growth (GHRs, IGFs, and IGFR1s) were examined by mRNA expression levels after various treatments with thyroxine. I postulate thyroxine can directly modulate the expression of the genes controlling for growth.

GHR mRNA expression levels were measured in liver and gill filaments because of high ligand binding and high levels of mRNA encoding the receptors observed previously in these tissues. (Gray et al., 1992; Very et al., 2005). IGFR1 mRNA were measured in gill filaments due to the prevalence of IGF-I binding and expression of IGFR1-encoding mRNAs in this tissue. (Leibush et al., 1996; Nakao et al., 2002) Furthermore, mRNA levels of IGF were measured in liver and gill filaments because of their relative abundance in these tissues. (Duan et al., 1993; Vong et al., 2003; Wood et al., 2005; Reinecke, 2006).

Materials and Methods

Animals

Juvenile rainbow trout were obtained from Dakota Trout Ranch near Carrington, ND, and transported to North Dakota State University where they were maintained 800-L circular tanks supplied with recirculated (10% replacement volume per day), dechlorinated, municipal water at 14 °C under a 12L:12D hour photoperiod. Fish were fed twice a day daily to satiation with AquaMax® Grower (PMI Nutrition International, Inc., Brentwood, MO) semi-floating trout grower, except for 24-36 hours before initiating experimental manipulations. Fish were acclimated to laboratory conditions for at least two weeks prior to experimentation.

Experimental Conditions for mRNA Expression

Fish were anesthetized in 0.05% (v/v) 2-phenoxyethanol, measured and weighed (body length and body weight), and bled from the severed caudal vessels. All fish were juvenile and sexually immature. Liver and gill arches were removed from fish, perfused ex vivo with 0.75% (v/v) saline solution, placed in Hank's medium (in mM: 137 NaCl, 5.4 KCL, 4 $NaHCO_3$, 1.7$CaCl_2$, 0.8 $MgSO_4$, 0.5 KH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, and 4 of glucose, pH 7.6), and prepared for culture as previously described (Harmon and Sheridan, 1992; Hanson et al., 2010).

Liver tissue was dissected into 1mm^3 pieces and individual gill filaments were separated from the arches. Gill filaments and liver pieces from individual fish were pooled (i.e. one pool of each tissue of all fish) and washed three times with Hank's medium with 0.24% (w/v) bovine serum albumin. Tissues were placed in 24-well culture plates (containing 8-10 liver pieces and 12-15 gill filaments) containing 1-ml of Hank's medium with 0.24% (w/v) bovine serum albumin that included essential and non-essential amino acids. A preincubation period was

performed at 14 °C under 100% O_2 while shaken at 100 rpm in a gyratory shaker. Replicate wells for a given experiment each came from different fish.

After a 3-hour preincubation period, the medium was removed, and tissues received fresh Hank's medium with 0.24% (w/v) bovine serum albumin and essential and non-essential amino acids. Tissues were treated with thyroxine (T4) (Sigma), ranging in concentrations from 0-250 ng/ml for 6 hours, or at a specified physiological concentration of 25 ng/ml for various periods of time (0, 3, 6, 12, 24 hours), while maintaining the same conditions as during preincubation. T4 was initially dissolved in Hank's culture media without bovine serum albumin (BSA) and essential and non-essential amino acids. The concentrations of T4 used were based upon the range of concentrations measured in plasma of rainbow trout and Atlantic salmon before, during, and after sexual maturation (Laidley and Leatherland, 1988; Holloway et al., 1999; Ebbesson et al., 2000; Ebbesson et al., 2008). Following treatment completion, the incubation media was removed from the tissue samples. Samples were placed in 2.0-ml microcentrifuge tubes and immediately frozen on dry ice. The tissues were stored at -80 °C for later mRNA and binding analysis.

Experimental Conditions for Thyroxine Inhibition

Conditions were identical to mRNA expression experimental conditions up to the 3-hour preincubation period. After a 3-hour preincubation period was completed, Hank's media was removed, and tissues received fresh media with 0.24% BSA and essential and non-essential amino acids. Tissues were treated with either 10-ul of methimazole (Sigma) inhibitors at 100 ng/ml or 320 ng/ml; 50-µl of T4 (Sigma) at 25 ng/ml; or T4 with methimazole (10-µl of inhibitor and 50-µl of hormone). Earlier, I tried to use propylthiouracil (PTU) as an inhibitor for T4 treatment because the literature, at that time, suggested that it was a potent inhibitor. However,

when I did preliminary experiments with PTU, all I saw was an increase in my expression levels. So, I searched for another inhibitor and came up with methimazole. I used two different doses of methimazole because Crane and colleagues (2006) used both low- and high-doses of methimazole and I wanted to do the same.

Tissue samples that received only inhibitor were treated for 4 hours whereas tissue samples that received T4 were treated for 6 hours, and samples that received both inhibitor and hormone treatment were treated first for 4 hours with inhibitor and then an additional 6 hours once the hormone T4 was added. All samples were maintained under the same preincubation period conditions as the mRNA expression experimental conditions. T4 and methimazole were initially dissolved in hank's media without BSA or essential and non-essential amino acids. A control group was implemented that contained only hank's culture media. The concentrations of methimazole were established from a previous study in fathead minnows (Crane et al., 2006). Following the completion of treatments, incubation media was removed from the samples, and tissues were placed in 2.0-ml microcentrifuge tubes and immediately frozen on dry ice. Tissues were stored at -80 °C until processed for mRNA analysis. The same genes were analyzed as in the mRNA expression study.

Experimental Conditions for Combination Study of GH and Thyroxine

This study consisted of liver and gill tissues being collected similarly to the mRNA expression study. Tissues were preincubated for 3 hours with 1-ml of Hank's media with 0.24% (w/v) BSA that included essential and non-essential amino acids at 14 °C under 100% O_2 shaken at 100 rpm on a gyratory shaker in 24-well plates. After completion of the preincubation period, new Hank's media was replaced, and samples received various individual hormones or combination of hormones. The treatment groups were Hank's media control, growth hormone

(100 ng/ml), thyroxine (25 ng/ml) and growth hormone + thyroxine. Hormone treatments were added simultaneously to the samples and were treated for 6 hours. With fulfillment of the study, treatment media was removed, and tissues were stored at -80 °C in 2.0-ml microcentrifuge tubes until further processing for mRNA analysis.

Quantitative Real-time PCR

Frozen tissues were homogenized and total RNA was isolated using RNAzol® reagent (Molecular Research Center, Cincinnati, OH, USA) as specified by the manufacturer's protocol. Each RNA pellet was re-dissolved in 60-100 µl of RNase-free deionized water and total RNA was quantified by ultraviolet (UV A_{260}) spectrophotometry using the Nanodrop 1000 Spectrophotometer (Thermo Scientific, West Palm Beach, FL, USA). mRNA was reverse transcribed in 5-µl reactions using 200 ng total RNA and qScript™ cDNA synthesis kit reagents (qScript Reaction Mix, qScript RT, and nuclease-free water) according to the manufacturer's protocol (Quanta Biosciences™, Inc., Gaithersburg, MD, USA). To eliminate the possibility of contamination with genomic DNA, reactions without reverse transcriptase were included as negative controls. Amplification was not detected in negative controls.

Steady-state mRNA levels of GHR1, GHR2, IGF-1, IGF-2, IGFR1A, and IGFR1B were determined by quantitative real-time PCR using Stratagene Mx 3000 detection system (Stratagene, La Jolla, CA, USA). The gene-specific nucleotide primers and probes used for experiments can be found in Table 1 of Chapter 2. In short, real-time PCR reactions were carried out for samples, standards, and controls in 10-µl total volume reactions. The reactions contained 2 µl of cDNA from the reverse transcription reactions, 5 µl of 2X Brillant® II QPCR Master Mix, 1 µl of gene-specific probe, 0.85 µl of β-actin probe, 0.5 µl of gene-specific forward and reverse primers as well as β-actin forward and reverse primers, and 0.15 µl of reference dye

(Stratagene, Agilent Technologies). Real-time PCR reactions were multiplexed. Cycling parameters were set as follow: an initial denaturation step of 95 °C for 10 minutes followed by 50 cycles of replication, each consisting of 95 °C for 15 s and then finally an extension step of 58 °C for 1 minute. Sample copy number was calculated from the threshold cycle number (CT), and the CT was related to a gene-specific standard curve followed by normalization to β-actin.

Statistics

Data are presented as means ± SEM; n represents the number of replicates for each treatment group. Statistical differences were analyzed by one-way ANOVA followed by Tukey-Kramer method for post-hoc analysis. An alpha value of < 0.05 was used to indicate significant differences between and among treatment groups. Statistics were performed on β-actin corrected copies of mRNA using JMP®, Version 11 (SAS, Cary, NC, USA). Outliers were determined using Dixon's test with an alpha value of < 0.05 indicating significant differences.

Results

Concentration- and Time-related Studies

GHRs

Sensitivity to GH was evaluated by quantifying mRNA expression of GHRs in hepatic and gill filaments after *in vitro* treatments of T4. Outside the brain, two distinct GHR-encoding mRNAs, GHR1 and GHR2, are abundantly present in liver and gill filaments (Very et al., 2005). Thyroxine had no significant effect on hepatic GHR1 or GHR2 mRNAs in a concentration-related manner (Fig. 11A and 11C). However, T4 had a significant effect in influencing hepatic GHR1 and GHR2 mRNA levels in a time-related manner. Expression levels of GHR1 rapidly decreased at 3 hours and gradually rebounded back toward control levels after 24 hours of

treatment (Fig. 11B). Similar to GHR1, expression levels of GHR2 rapidly decreased at 3 hours, but rebounded back toward control levels much quicker after 6 hours of treatment (Fig. 11D).

Thyroxine treatment in gill had no significant effect on steady-state GHR1 mRNAs in a concentration- and time-related manner (Fig. 12A and 12B). Further, T4 had no significant effect in influencing GHR2 mRNA expression levels in a concentration-related manner (Fig. 12C). Yet, T4 had a significant effect on GHR2 mRNA expression levels in a time-related manner (Fig. 12D). Thyroxine significantly inhibited GHR2 mRNA expression levels at 12 hours when compared to GHR2 mRNA expression levels at 3 hours.

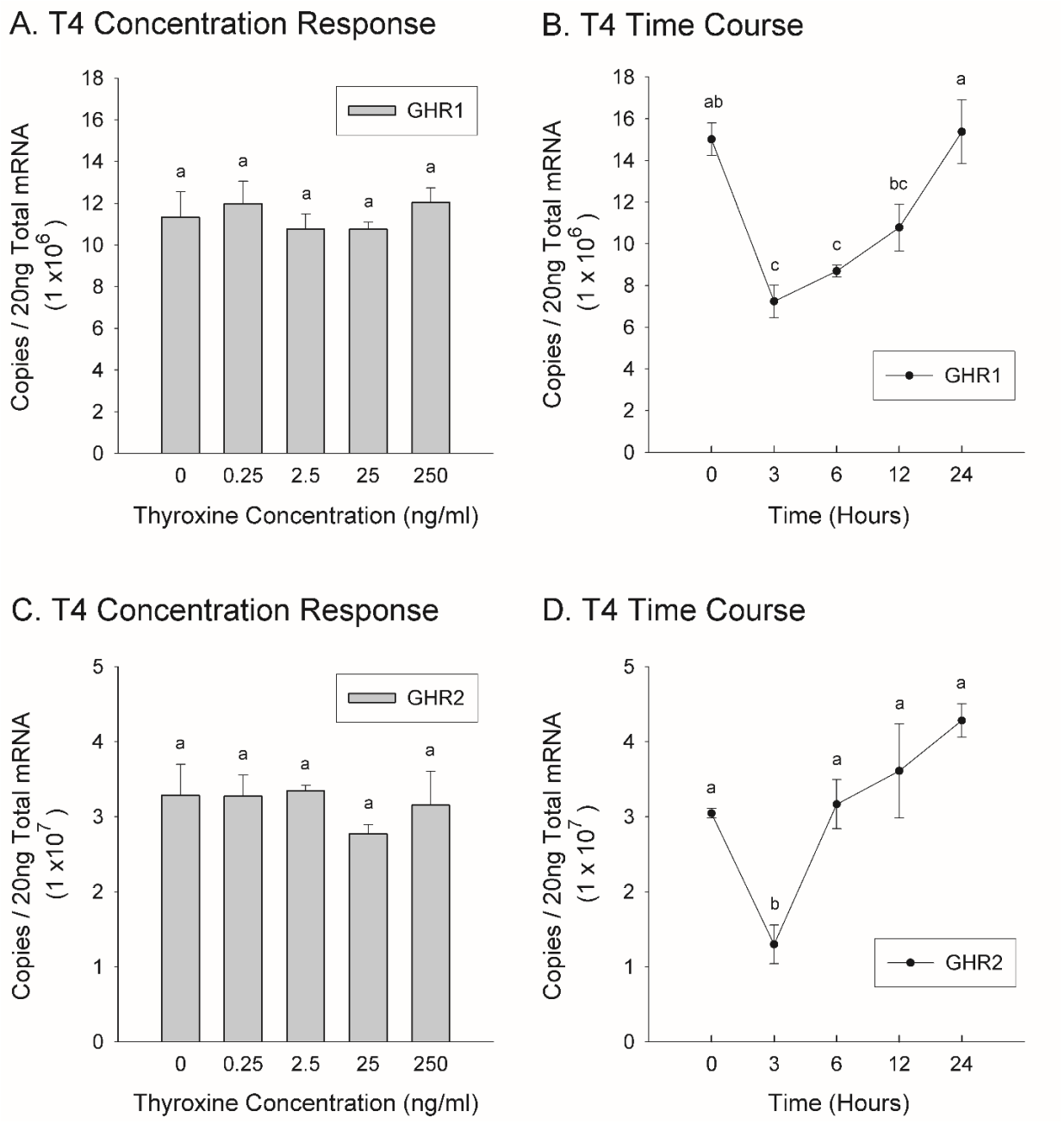

Fig. 11. Effects of thyroxine (T4) treatments on mRNA expression of growth hormone receptors (GHRs), GHR1 (A and B) or GHR2 (C and D), in liver of rainbow trout incubated *in vitro*. Liver tissue was incubated with various concentrations of T4 for 6 hours (A and C) or were incubated for various times with 25 ng/ml T4 (B and D). Data are presented as mean ± SEM. (n = 4-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

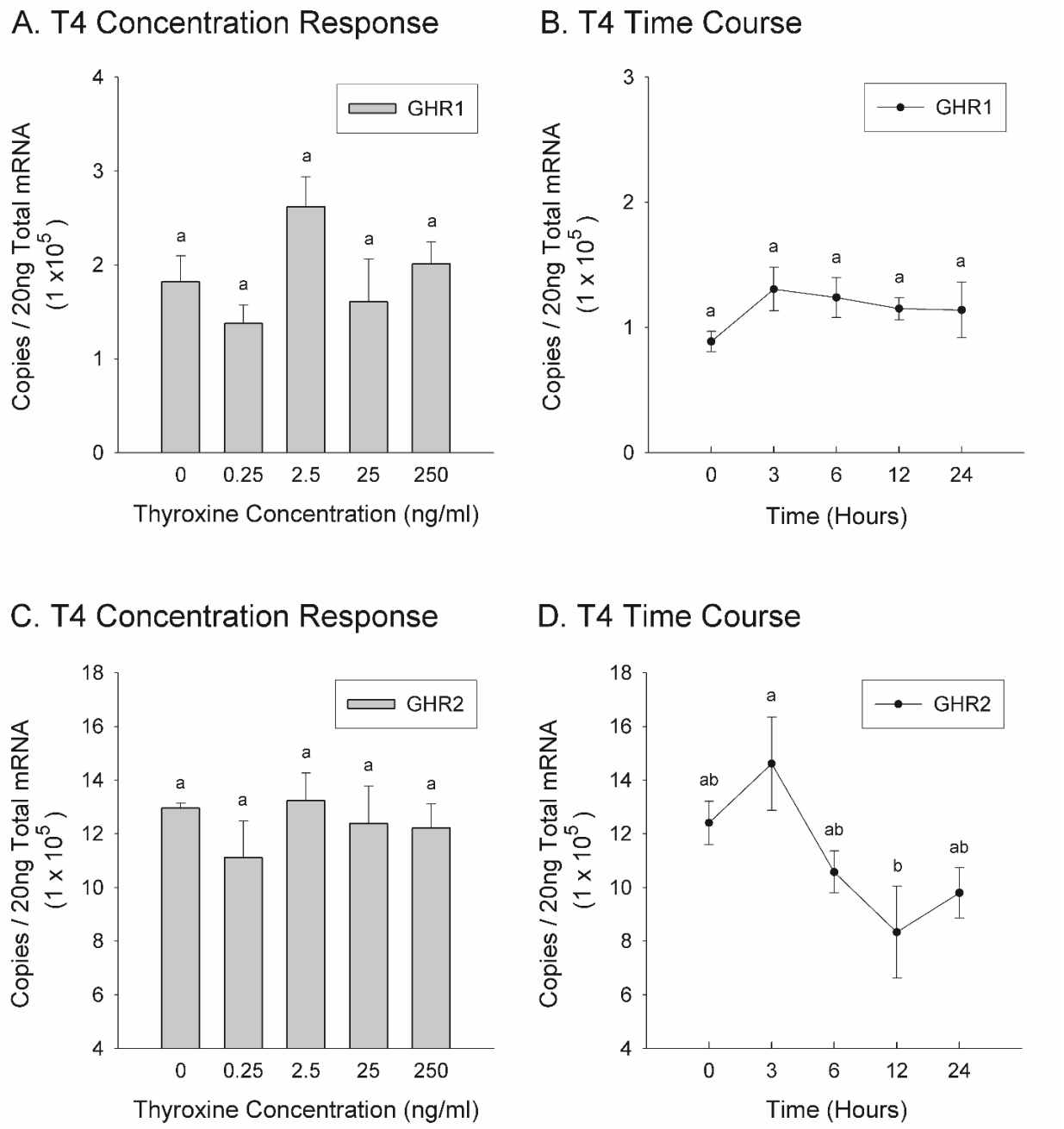

Fig. 12. Effects of thyroxine (T4) treatments on mRNA expression of growth hormone receptors (GHRs), GHR1 (A and B) or GHR2 (C and D), in gill of rainbow trout incubated *in vitro*. Gill filaments were incubated with various concentrations of T4 for 6 hours (A and C) or were incubated for various times with 25 ng/ml T4 (B and D). Data are presented as mean ± SEM. (n = 5-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFs

Peripheral IGF production was evaluated by quantifying mRNA expression of IGFs in hepatic and gill filaments following *in vitro* treatments of T4. Thyroxine had no significant effect on hepatic IGF1 or IGF2 mRNAs in a concentration-related manner (Fig. 13A and 13C). Duration of treatment with T4 had a pronounced effect in influencing expression levels for both hepatic IGF1 and IGF2 mRNAs. Steady-state IGF1 mRNAs significantly diminished after 3 hours of treatment; with minimum levels reached at 24 hours (Fig. 13B). Levels of mRNA encoding IGF2 significantly diminished at a rapid rate (3 hours); IGF2 mRNA expression levels remained diminished in all other time points at the same level (Fig. 13D).

In gill, T4 had no significant effect in regulating IGF1 mRNAs in a concentration-related manner (Fig. 14A), while T4 had a significant effect in regulating IGF1 mRNAs in a time-related manner (Fig. 14C). Expression levels of IGF1 mRNAs increased rapidly after 3 hours of treatment, but then returned to control levels by 6 hours. Further treatment of T4 at 12 hours and 24 hours resulted in a significant decrease of IGF1 mRNA expression levels relative to IGF1 mRNA expression levels in the control. The effects of T4 on the expression of IGF2 mRNAs in gill filaments were significant in a concentration- and time-related manner. An increase in expression levels for IGF2 mRNAs transpired at a T4 concentration of 2.5 ng/ml; mRNA expression levels returned toward control mRNA expression levels with concentrations of 25 ng/ml and 250 ng/ml (Fig. 14C). Steady-state IGF2 mRNAs were steadily inhibited by 12 hours of T4 treatment, with maximum inhibition observed after 24 hours (Fig. 14D).

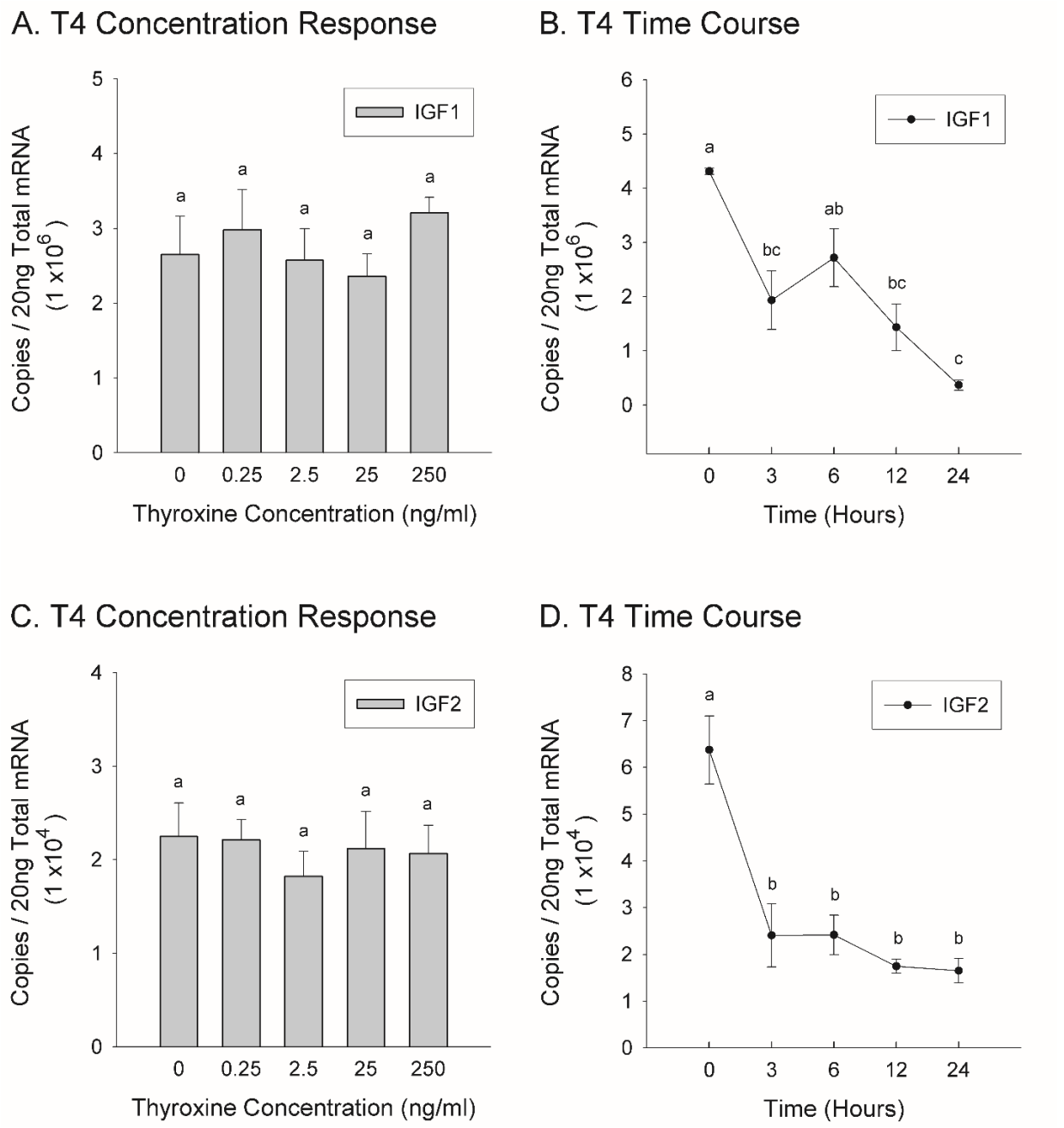

Fig. 13. Effects of thyroxine (T4) treatments on mRNA expression of insulin-like growth factors (IGFs), IGF1 (A and B) or IGF2 (C and D), in liver of rainbow trout incubated *in vitro*. Liver tissue was incubated with various concentrations of T4 for 6 hours (A and C) or were incubated for various times with 25 ng/ml T4 (B and D). Data are presented as mean ± SEM. (n = 4-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

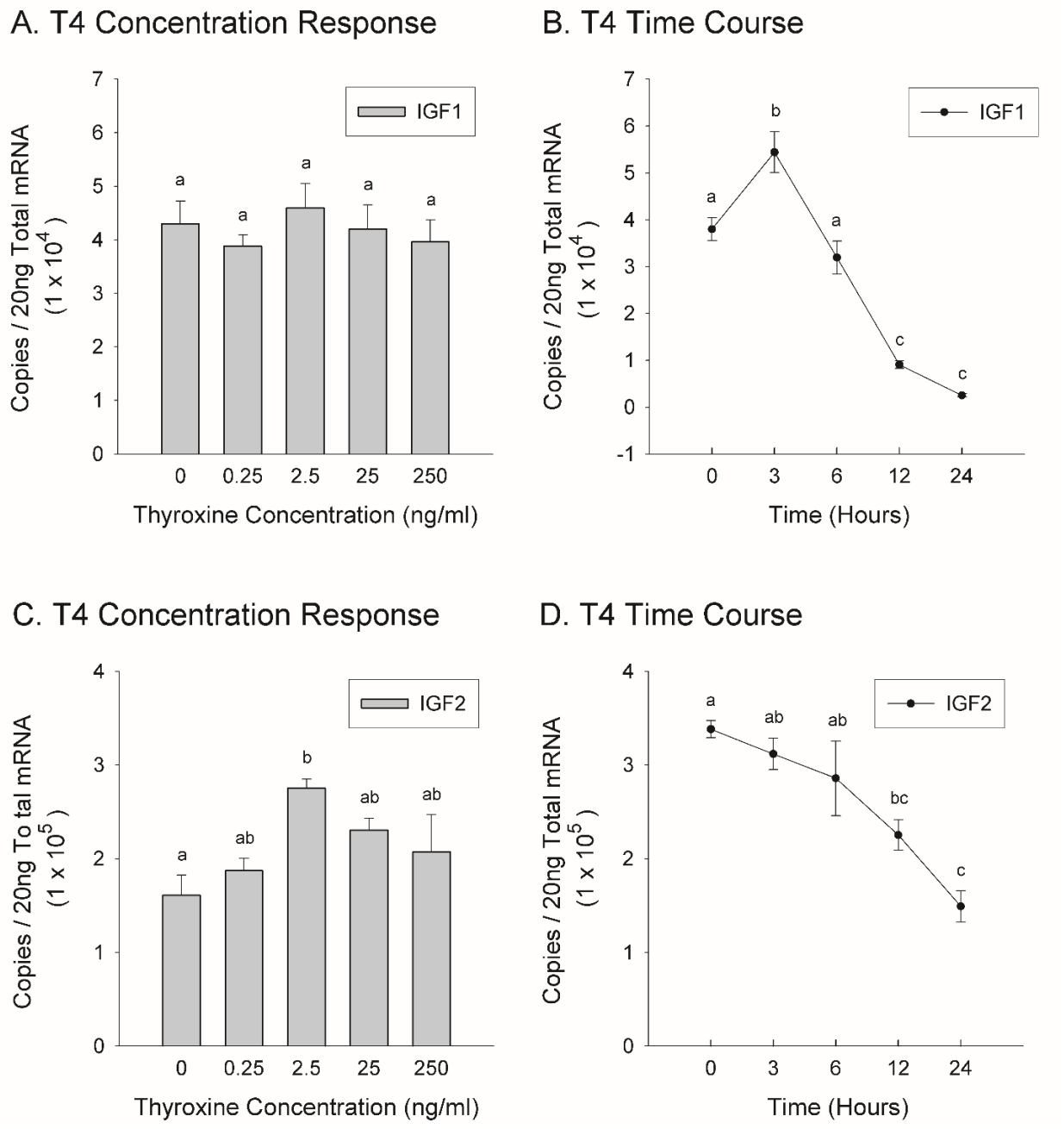

Fig. 14. Effects of thyroxine (T4) treatments on mRNA expression of insulin-like growth factors (IGFs), IGF1 (A and B) or IGF2 (C and D), in gill of rainbow trout incubated *in vitro*. Gill filaments were incubated with various concentrations of T4 for 6 hours (A and C) or were incubated for various times with 25 ng/ml T4 (B and D). Data are presented as mean ± SEM. (n = 5-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFRs

Peripheral IGF1 sensitivity was assessed by quantifying mRNA expression of IGFRs in gill filaments after *in vitro* treatments of T4. Thyroxine had no significant effect on IGFR1A or IGFR1B mRNAs in a concentration-related manner in gill filaments (Fig. 15A and 15C). However, T4 at a concentration of 25 ng/ml had a significant effect in altering IGFR1A and IGFR1B in a time-related manner. Compared to control expression levels, a significant reduction of mRNAs encoding for IGFR1A were detected after 24 hours of T4 treatment (Fig. 15B). Significant reductions in steady-state IGFR1B mRNAs were observed at 12 hours and 24 hours of T4 treatment (Fig. 15D). The greatest reduction occurred at 24 hours, with expression levels approximately 5-fold below those of control expression levels.

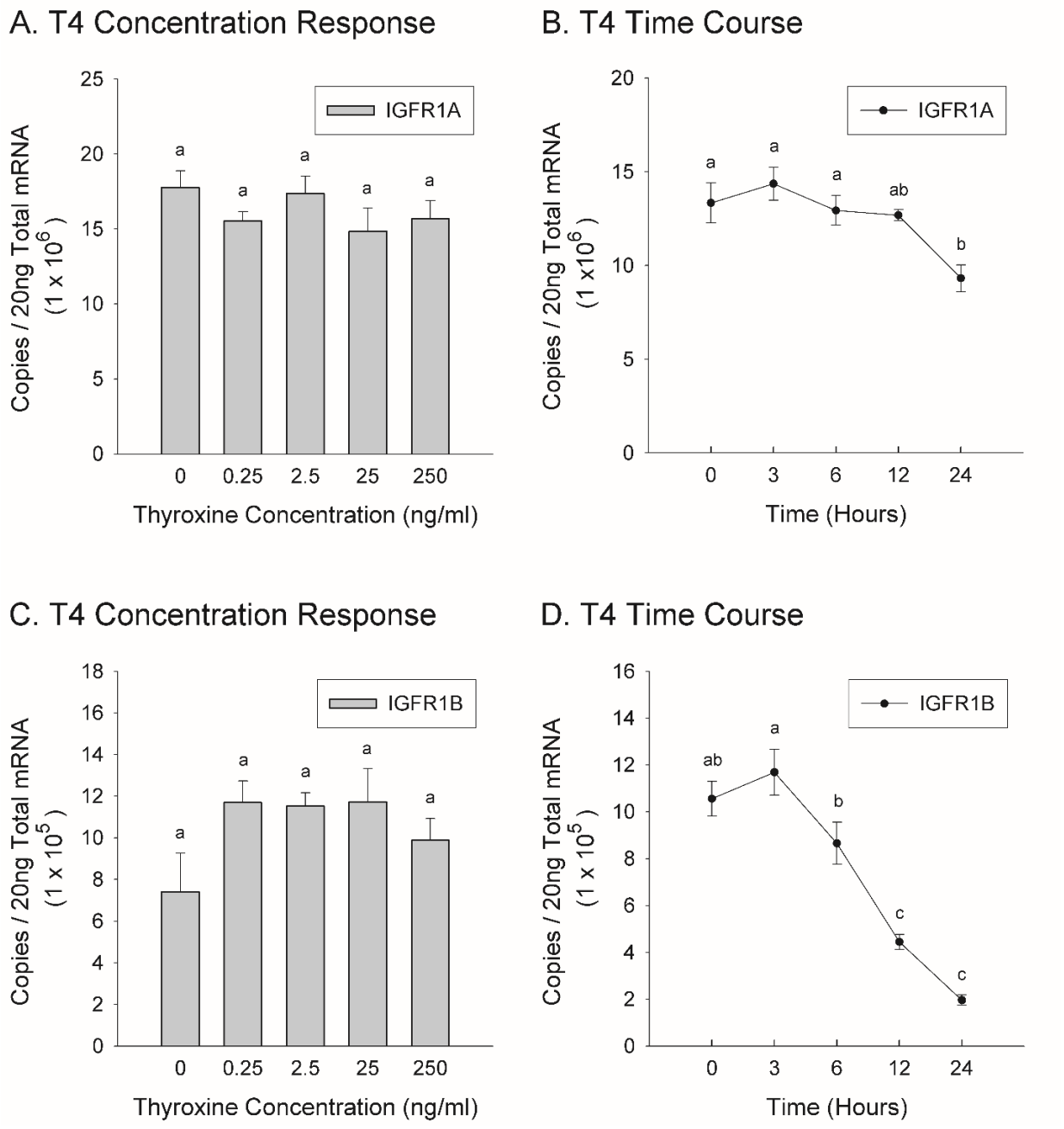

Fig. 15. Effects of thyroxine (T4) treatments on mRNA expression of insulin-like growth factor receptors (IGFRs), IGFR1A (A and B) or IGFR1B (C and D), in gill of rainbow trout incubated *in vitro*. Gill filaments were incubated with various concentrations of T4 for 6 hours (A and C) or were incubated for various times with 25 ng/ml T4 (B and D). Data are presented as mean ± SEM. (n = 4-6). For a given IGFR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

Inhibition Study

GHRs

Experiments were also conducted with T4 and the antithyroid drug methimazole (MMI) at various concentrations in hepatic tissue and gill filaments. In hepatic tissue, different treatments of T4 and MMI were significant in controlling GHR1 and GHR2 mRNA expression levels. Treatment with T4 resulted in similar levels seen with control GHR1 mRNA levels. A low-dose (100 ng/ml) treatment of MMI produced a significant decrease in transcription of GHR1 mRNAs relative to hepatic tissue treated with T4 (Fig. 16A). However, when a treatment of T4 + MMI 100 was delivered, no significant change relative to the control or T4-treated groups was seen in GHR1 mRNA expression. A high-dose (320 ng/ml) treatment of MMI resulted in steady-state GHR1 mRNAs to decline relative hepatic tissue treated with T4 (Fig. 16A). When comparing all treatment groups from the control or T4-treated groups, the greatest decrease in hepatic GHR1 mRNA expression occurred when a treatment of a high-dose of MMI in conjunction with T4 were administered (Fig 16A). Expression levels of GHR2 mRNAs remained comparable to the control group when treated with T4. Analogous to results seen with GHR1 mRNA expression, GHR2 mRNA expression significantly decreased with a high-dose treatment of MMI relative to those treated with T4; yet, these mRNA expression levels were comparable to control mRNA expression levels (Fig 16B). Unlike results observed with mRNAs encoding for GHR1, treatments with a combination of either a high-dose (320 ng/ml) or low-dose (100 ng/ml) of MMI + T4 resulted in no observable change for GHR2 mRNA expression levels when compared to the T4-treated and the no treatment control groups (Fig. 16B).

In gill filaments, T4 or MMI treatments did not have a significant effect in inhibiting GHR1 mRNA expression (Fig. 17A). Treatments with T4 did not significantly alter GHR2

mRNA expression levels compared to the control mRNA expression levels (Fig. 17B). Also, MMI treatments with either a high-dose (320 ng/ml) alone, a low-dose (100 ng/ml) in combination with T4 or a high-dose (320 ng/ml) in combination with T4 did not significantly alter GHR2 mRNA expression levels when compared to the T4-treated or the control groups . However, a significant decrease in transcription of GHR2 mRNAs did occur. A high-dose (320 ng/ml) of MMI alone or in combination with T4 or a low dose (100 ng/ml) of MMI in combination with T4 produced a significant decline in GHR2 mRNA expression compared to gill filaments that were treated with just a low-dose (100 ng/ml) of MMI (Fig. 17B).

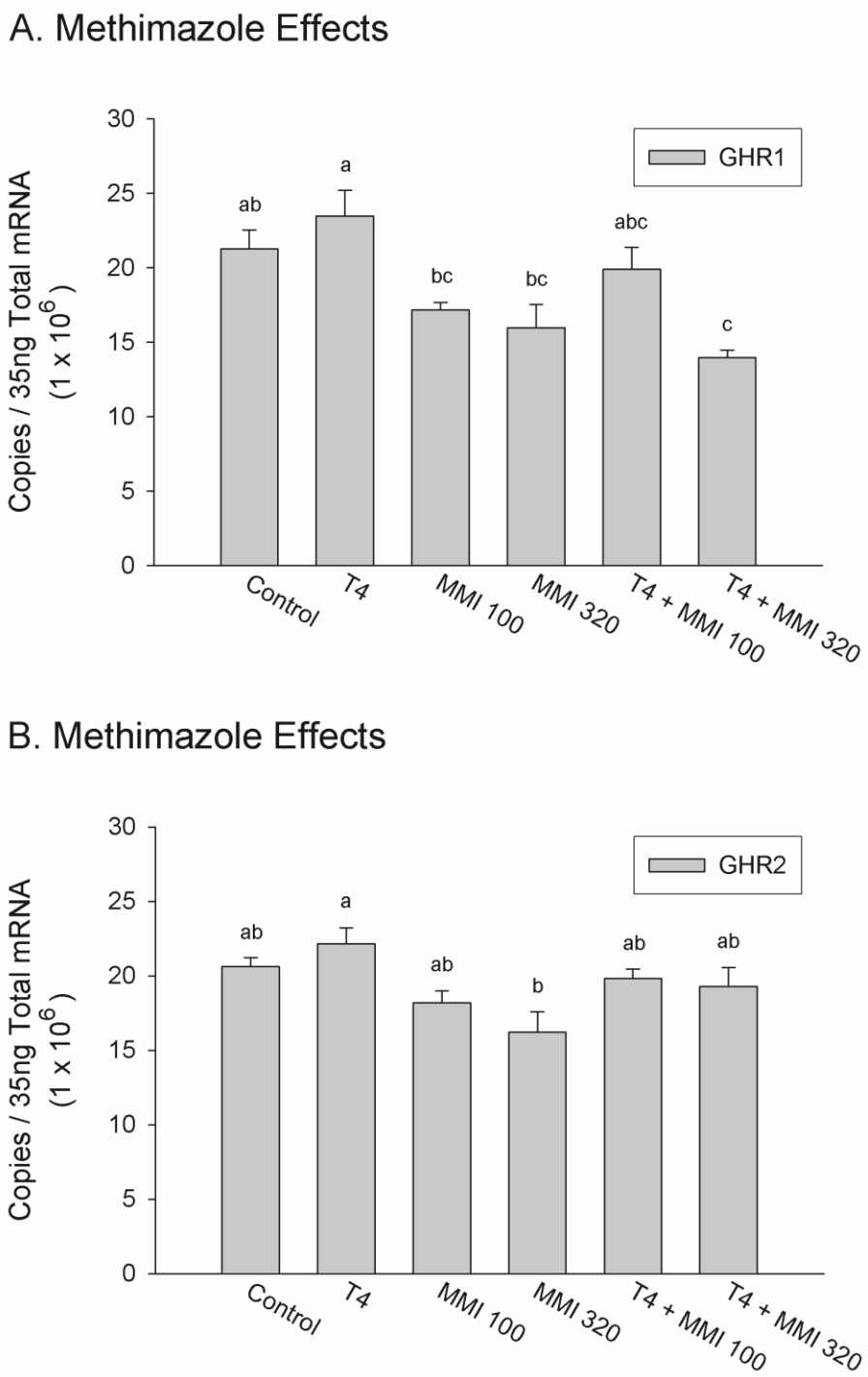

Fig. 16. Effects of thyroxine (T4) and antithyroid drug methimazole (MMI) on the transcription of growth hormone receptors (GHRs), GHR1 (A) and GHR2 (B), mRNAs in liver tissue of *in vitro* cultures isolated from rainbow trout. Liver tissue pieces were pretreated with or without MMI at concentrations of 100 ng/ml (MMI 100) or 320 ng/ml (MMI 320), for 4 hours, then treated with or without 25 ng/ml T4 for 6 hours. Data are presented as mean ± SEM. (n = 4-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

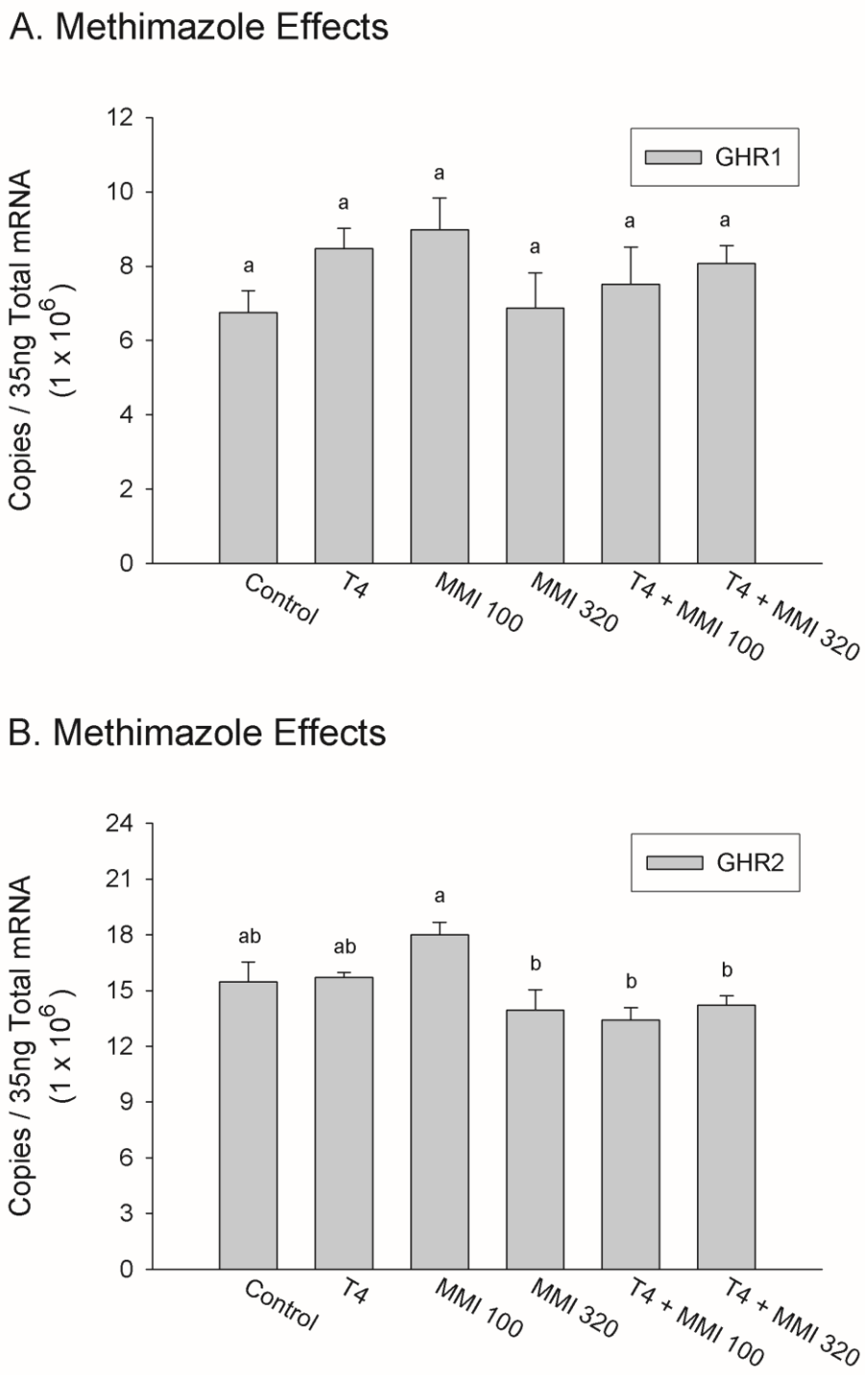

Fig. 17. Effects of thyroxine (T4) and antithyroid drug methimazole (MMI) on the transcription of growth hormone receptors (GHRs), GHR1 (A) and GHR2 (B), mRNAs in gill filaments of *in vitro* cultures isolated from rainbow trout. Gill filaments were pretreated with or without MMI at concentrations of 100 ng/ml (MMI 100) or 320 ng/ml (MMI 320), for 4 hours, then treated with or without 25 ng/ml T4 for 6 hours. Data are presented as mean ± SEM. (n = 5-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFs

Experiments were also conducted with T4 and different concentrations of MMI in hepatic tissue and gill filaments to examine effects on hormonal IGF production. Expression levels of IGF1 and IGF2 in hepatic tissue were not significantly affected after various treatments with either T4 or MMI (Fig, 18A and 18B).

However, in gill filaments, treatment with T4 and MMI had significant effects on IGF1 and IGF2 mRNA expression levels. While any treatment groups that received MMI inhibitor and or hormone did not significantly differ from IGF1 mRNA levels relative to the control group, treatment groups of MMI 320 and T4 + MMI 320 significantly reduced IGF1 mRNA levels relative to those treated with only T4 (Fig. 19A). Significantly reduced mRNA expression levels of IGF2 were observed with a high-dose (320 ng/ml) of MMI relative to both T4-treated and control groups (Fig. 19B). Treatments with T4, MMI 100 or T4 + MMI 320 had no significant effect in regulating IGF2 mRNAs relative to the control group. Gill filaments treated with T4 in combination with a low-dose (100 ng/ml) of MMI significantly diminished IGF2 mRNA expression levels relative to those treated with T4 alone or the control groups (Fig. 19B).

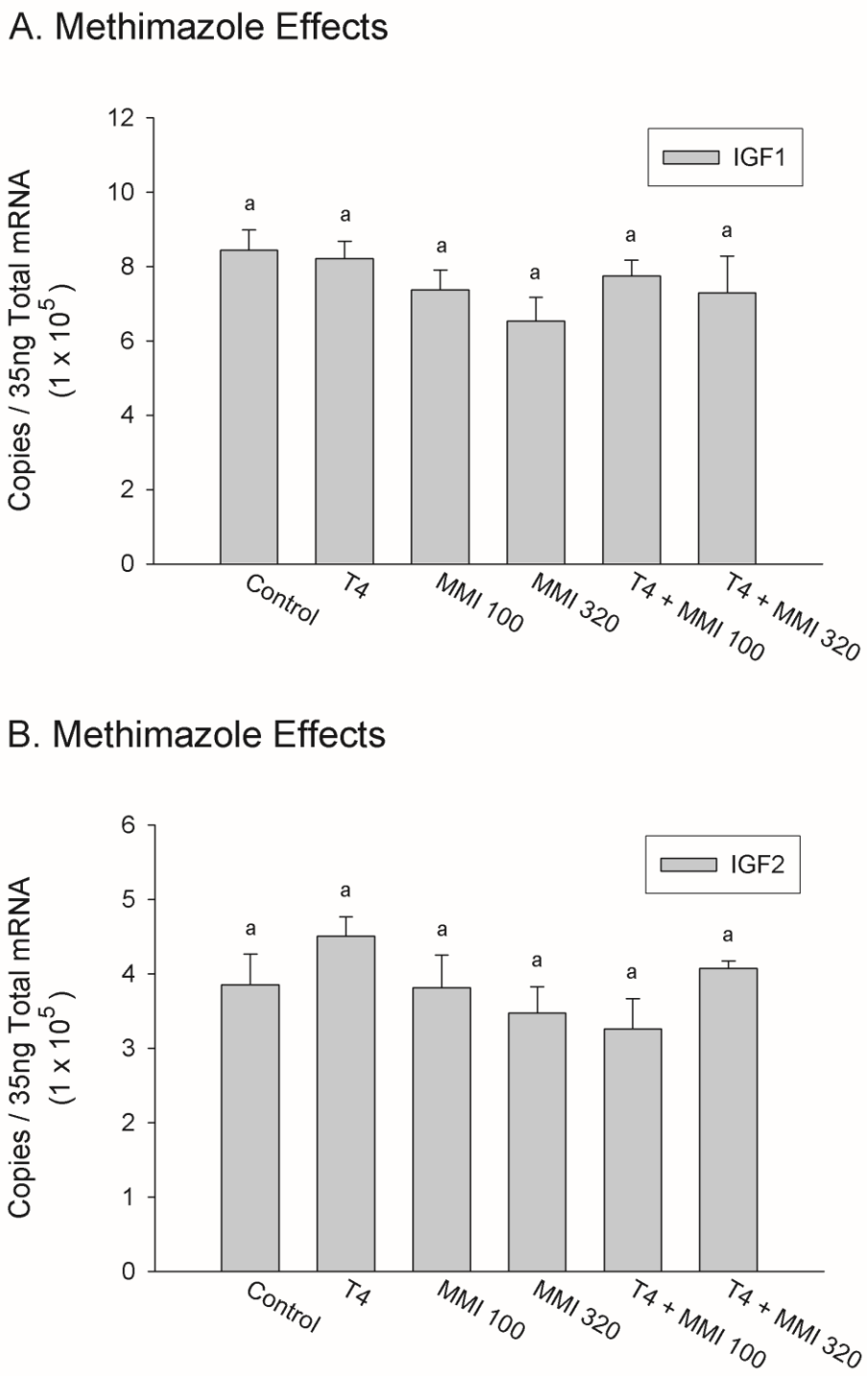

Fig. 18. Effects of thyroxine (T4) and antithyroid drug methimazole (MMI) on the transcription of insulin-like growth factors (IGFs), IGF1 (A) and IGF2 (B), mRNAs in liver tissue of *in vitro* cultures isolated from rainbow trout. Liver tissue pieces were pretreated with or without MMI at concentrations of 100 ng/ml (MMI 100) or 320 ng/ml (MMI 320), for 4 hours, then treated with or without 25 ng/ml T4 for 6 hours. Data are presented as mean ± SEM. (n = 4-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

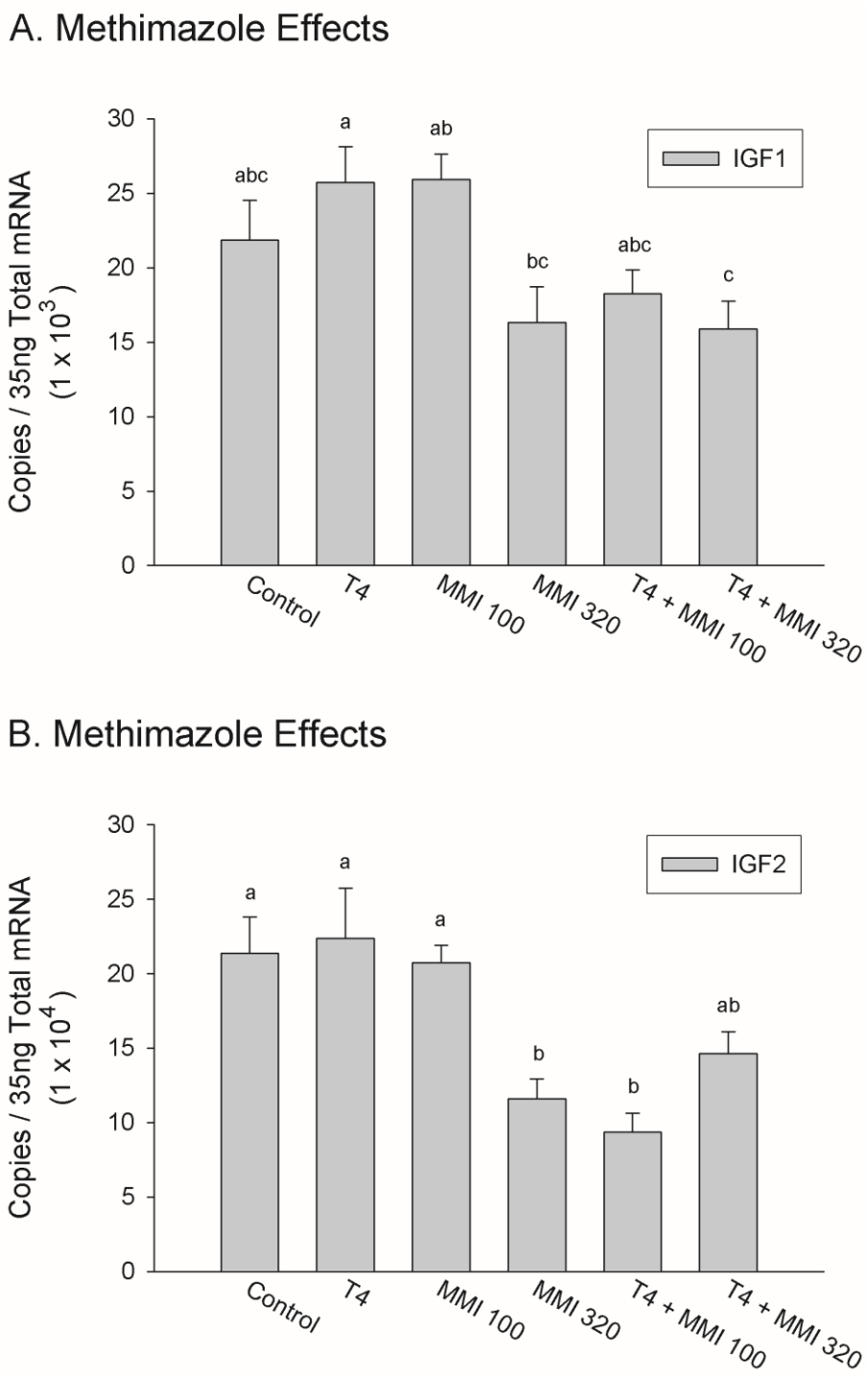

Fig. 19. Effects of thyroxine (T4) and antithyroid drug methimazole (MMI) on the transcription of insulin-like growth factors (IGFs), IGF1 (A) and IGF2 (B), mRNAs in gill filaments of *in vitro* cultures isolated from rainbow trout. Gill filaments were pretreated with or without MMI at concentrations of 100 ng/ml (MMI 100) or 320 ng/ml (MMI 320), for 4 hours, then treated with or without 25 ng/ml T4 for 6 hours. Data are presented as mean ± SEM. (n = 5-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFRs

Experiments examining IGF hormone sensitivity were conducted by treating gill filaments with T4 and different concentrations of MMI inhibitor. Various treatments of T4 and MMI significantly affected IGFR1A and IGFR1B mRNA expression levels. IGFR1A mRNA expression levels significantly increased with T4 treatment compared to treatment groups that received either MMI 320 or T4 + MMI 320 (Fig. 20A). Also, treatment with low-dose (100 ng/ml) of MMI resulted in a significant increase in IGFR1A mRNA expression relative to all other treatment groups except for the T4-treated group. When gill filaments were treated with T4 + MMI100, IGFR1A mRNA expression levels were significantly diminished relative to gill filaments treated with a low-dose of MMI (Fig. 20A). IGFR1A mRNA expression levels remained around control levels with a treatment of either MMI 320 or T4 + MMI320, although IGFR1A mRNA expression levels were significantly less when compared to gill filaments that were treated with T4 alone (Fig 20A). Like IGFR1A mRNA expression levels, IGFR1B mRNA expression levels increased with a low-dose (100 ng/ml) treatment of MMI relative to the control group (Fig 20B). All other treatment groups produced no significant change in IGFR1B mRNA expression levels and remained around control expression levels.

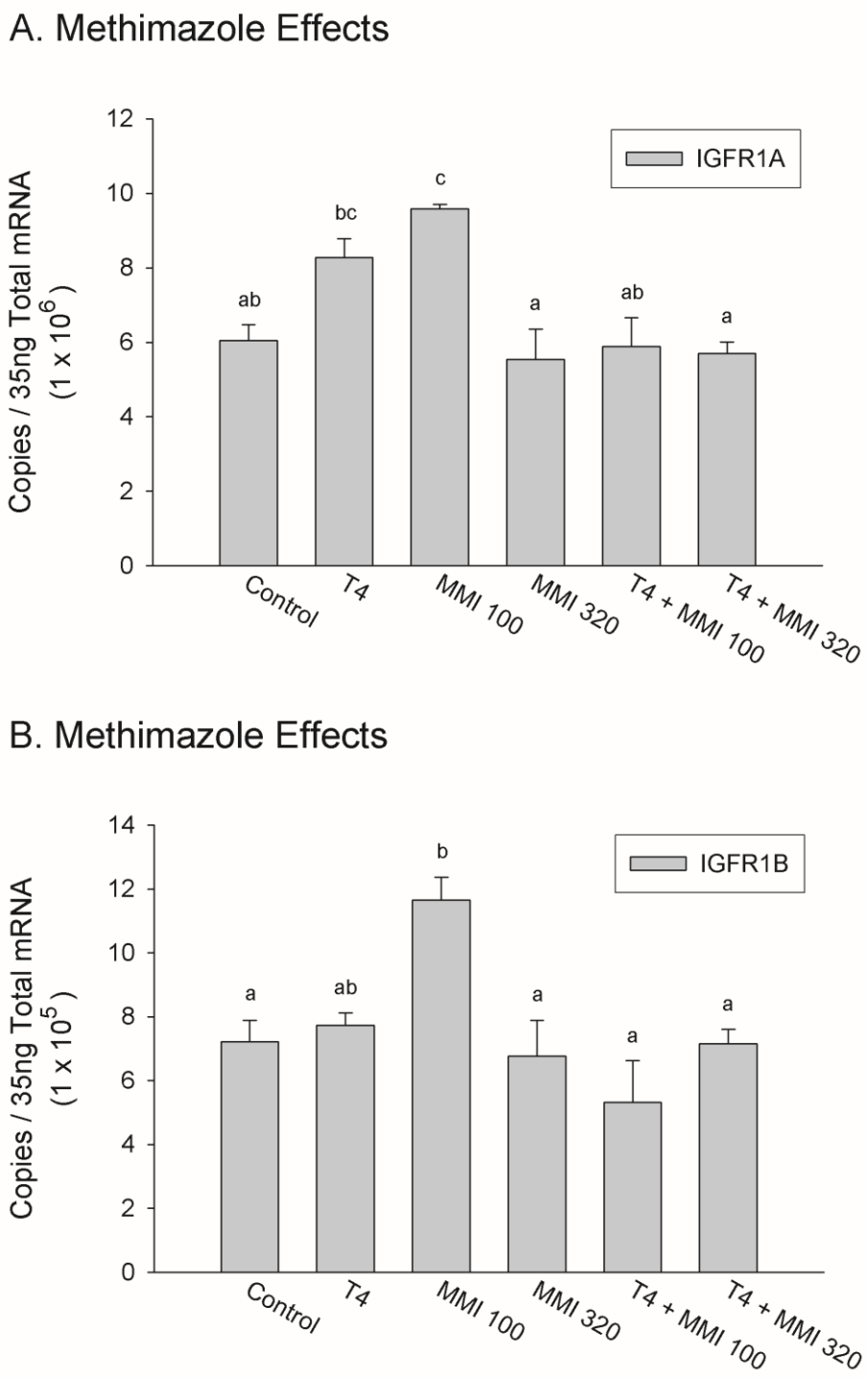

Fig. 20. Effects of thyroxine (T4) and antithyroid drug methimazole (MMI) on the transcription of insulin-like growth factor receptors (IGFRs), IGFR1A (A) and IGFR1B (B), mRNAs in gill filaments of *in vitro* cultures isolated from rainbow trout. Gill filaments were pretreated with or without MMI at concentrations of 100 ng/ml (MMI 100) or 320 ng/ml (MMI 320), for 4 hours, then treated with or without 25 ng/ml T4 for 6 hours. Data are presented as mean ± SEM. (n = 4-6). For a given IGFR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

Combination Study of GH and Thyroxine

GHRs

Hepatic tissue and gill filament culture experiments were also implemented to observe the direct effects of T4 and GH alone and in combination on GH sensitivity. Various treatments administered had significant effects in influencing hepatic GHR1 and GHR2 mRNA expression levels. GH stimulated GHR2 mRNA expression levels when compared to hepatic tissue treated with T4 alone (Fig. 21B). Thyroxine treatment alone had no significant effect on basal GHR1 and GHR2 mRNAs when compared to control levels (Fig. 21A and 21B). Yet, treatment effects seen among GHR1 and GHR2 were comparable with a combination treatment of GH + T4. A combination of GH + T4 resulted in a significant decrease in GHR1 and GHR2 mRNA expression levels relative to their respective control groups and all other treatments (Fig 21A and 21B).

In gill filaments, various T4 and GH treatments had similar significant effects influencing hepatic GHR1 and GHR2 mRNA expression levels. Neither GH nor T4 alone had a significant effect on steady-state GHR1 or GHR2 mRNAs relative to their respective control groups (Fig. 22A and 22B). In addition, a combination of GH + T4 significantly diminished both GHR1 and GHR2 mRNA expression levels when compared to all other respective treatment groups.

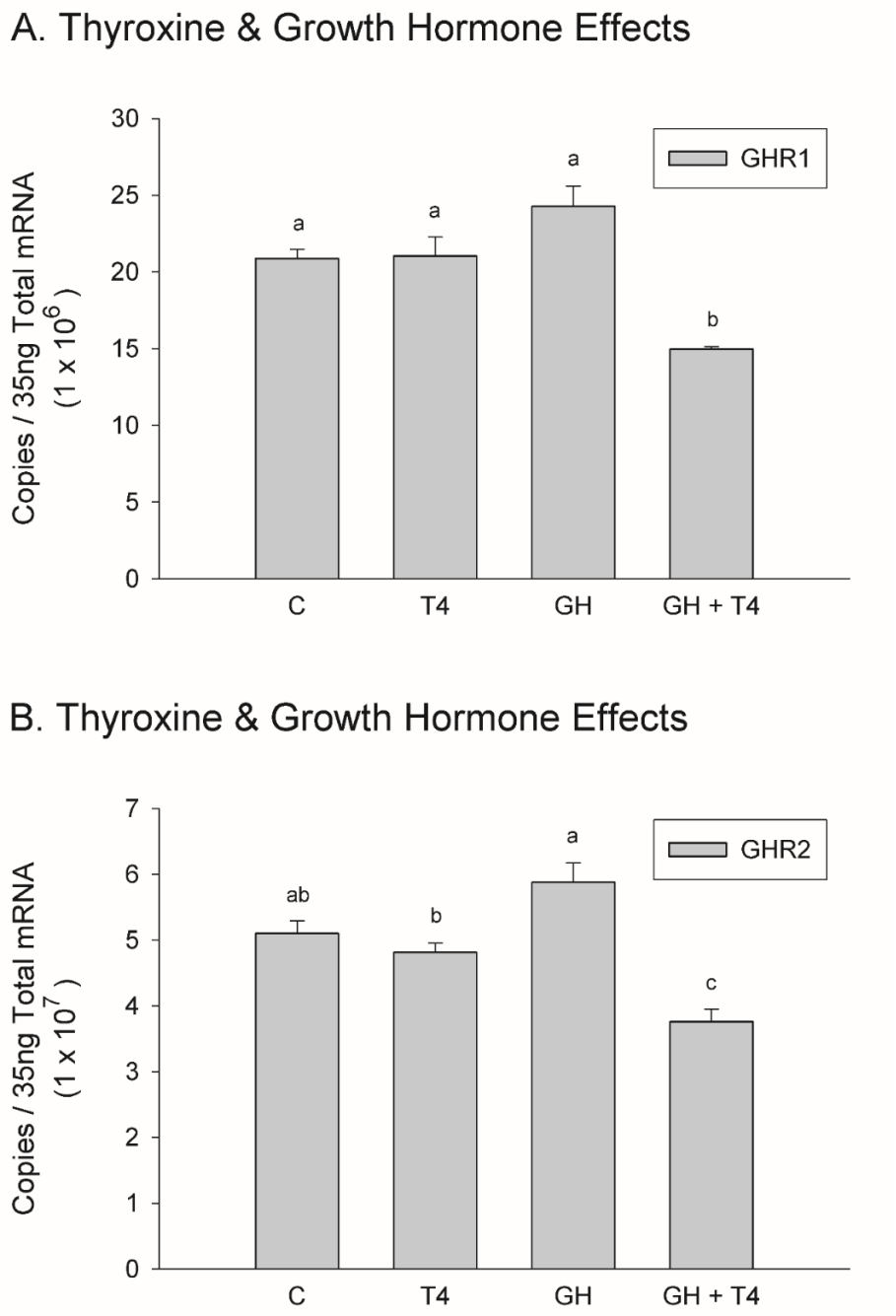

Fig. 21. Effects of thyroxine (T4), growth hormone (GH) or a combination of GH and T4 hormone treatments on growth hormone receptors (GHRs), GHR1 (A) or GHR2 (B), mRNA expression in liver tissue of *in vitro* cultures isolated from rainbow trout. Liver tissue pieces were incubated for 6 hours in medium alone (no hormone, control; C), medium containing T4 (25 ng/ml), medium containing salmonid growth hormone (sGH; 100 ng/ml), or medium containing sGH (100 ng/ml) and T4 (25 ng/ml); in combination treatments, sGH and T4 were added simultaneously. Data are presented as mean ± SEM. (n = 4-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

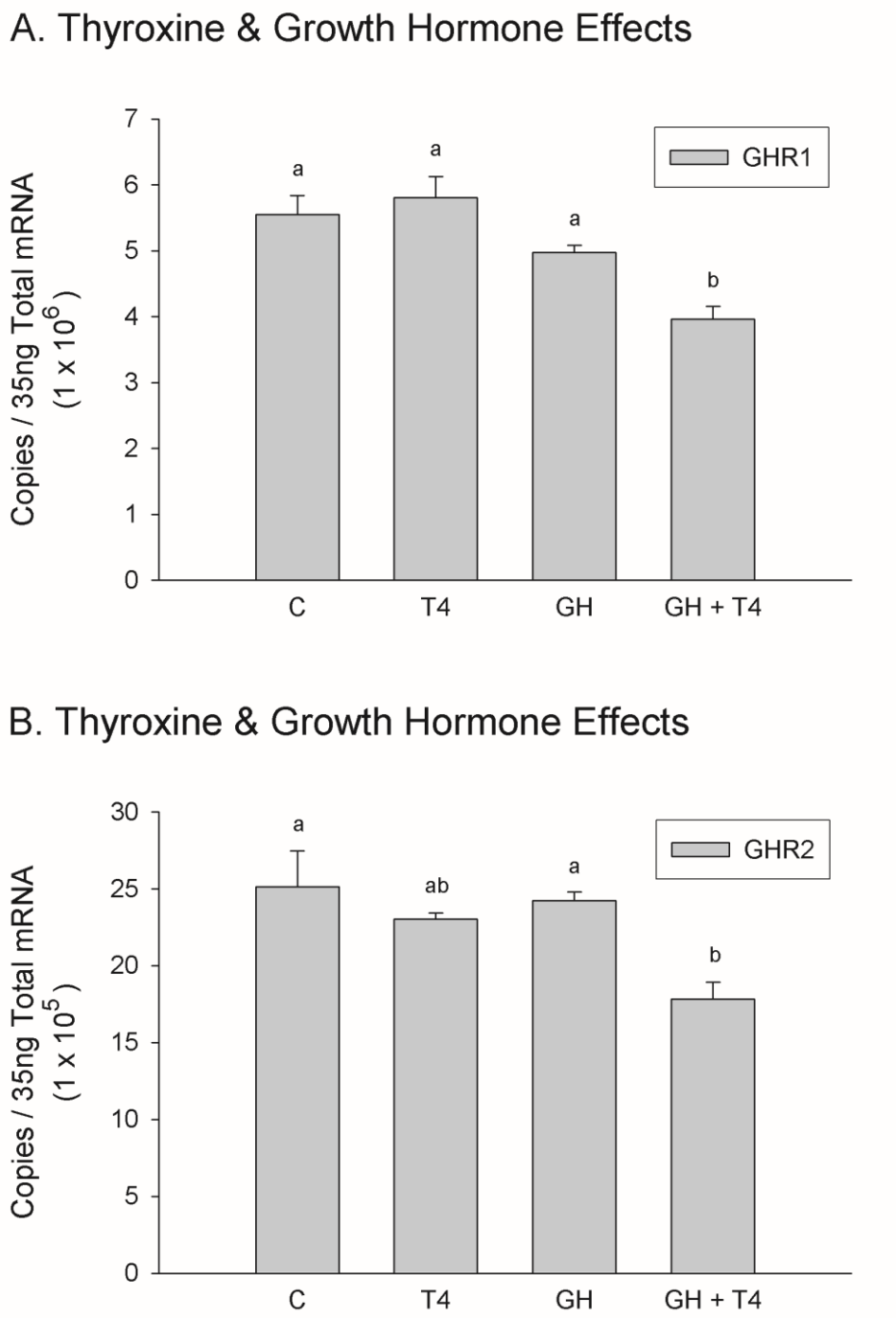

Fig. 22. Effects of thyroxine (T4), growth hormone (GH), or a combination of GH and T4 hormone treatments on growth hormone receptors (GHRs), GHR1 (A) or GHR2 (B), mRNA expression in gill filaments of *in vitro* cultures isolated from rainbow trout. Gill filaments were incubated for 6 hours in medium alone (no hormone, control; C), medium containing T4 (25 ng/ml), medium containing salmonid growth hormone (sGH; 100 ng/ml), or medium containing sGH (100 ng/ml) and T4 (25 ng/ml); in combination treatments, sGH and T4 were added simultaneously. Data are presented as mean ± SEM. (n = 5-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFs

Hepatic tissue and gill filament culture studies were conducted to observe the direct effects on IGF production with treatments of T4 and GH alone and in combination. Hepatic IGF1 and IGF2 mRNA expression levels were significantly affected by various treatments with T4 and GH. GH stimulated IGF1 mRNA levels over control levels, while T4 treatment produced similar IGF1 mRNA levels relative to the control group (Fig. 23A). Relative to all other treatment groups, the combination treatment of GH + T4 produced a significant decrease in IGF1 mRNA expression levels (Fig 23A). Neither GH nor T4 treatments alone had a significant effect on hepatic IGF2 mRNA expression levels relative to the control group (Fig 23B). Yet, GH + T4 were significant in reducing IGF2 mRNA expression levels relative to all treatment groups (Fig. 23B).

Various GH and T4 treatments with gill filament cultures significantly affected steady-state IGF1 and IGF2 mRNA levels. Treatments with either T4 or GH alone resulted in steady-state IGF1 mRNA levels similar to control group levels. A significant reduction in IGF1 mRNA expression occurred following treatment with GH + T4 (Fig. 24A). Similar to effects observed with IGF1, steady-state IGF2 mRNA levels remained around control levels when treated with T4 or GH alone. A significant reduction in IGF2 mRNA levels was observed when GH + T4 were administered to gill filaments (Fig 24B).

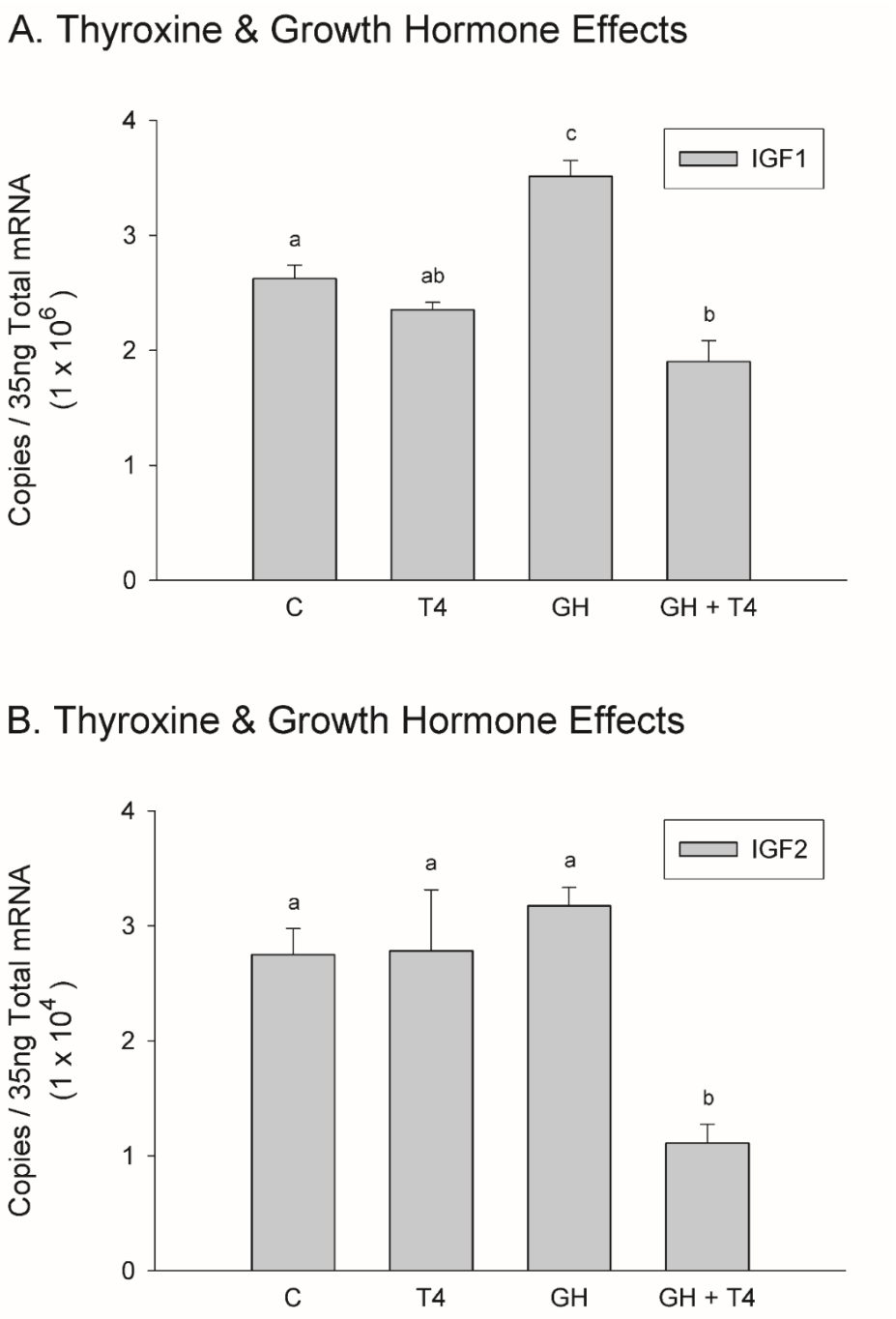

Fig. 23. Effects of thyroxine (T4), growth hormone (GH), or a combination of GH and T4 hormone treatments on insulin-like growth factors (IGFs), IGF1 (A) or IGF2 (B), mRNA expression in liver tissue of *in vitro* cultures isolated from rainbow trout. Liver tissue pieces were incubated for 6 hours in medium alone (no hormone, control; C), medium containing T4 (25 ng/ml), medium containing salmonid growth hormone (sGH; 100 ng/ml), or medium containing sGH (100 ng/ml) and T4 (25 ng/ml); in combination treatments, sGH and T4 were added simultaneously. Data are presented as mean ± SEM. (n = 5-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

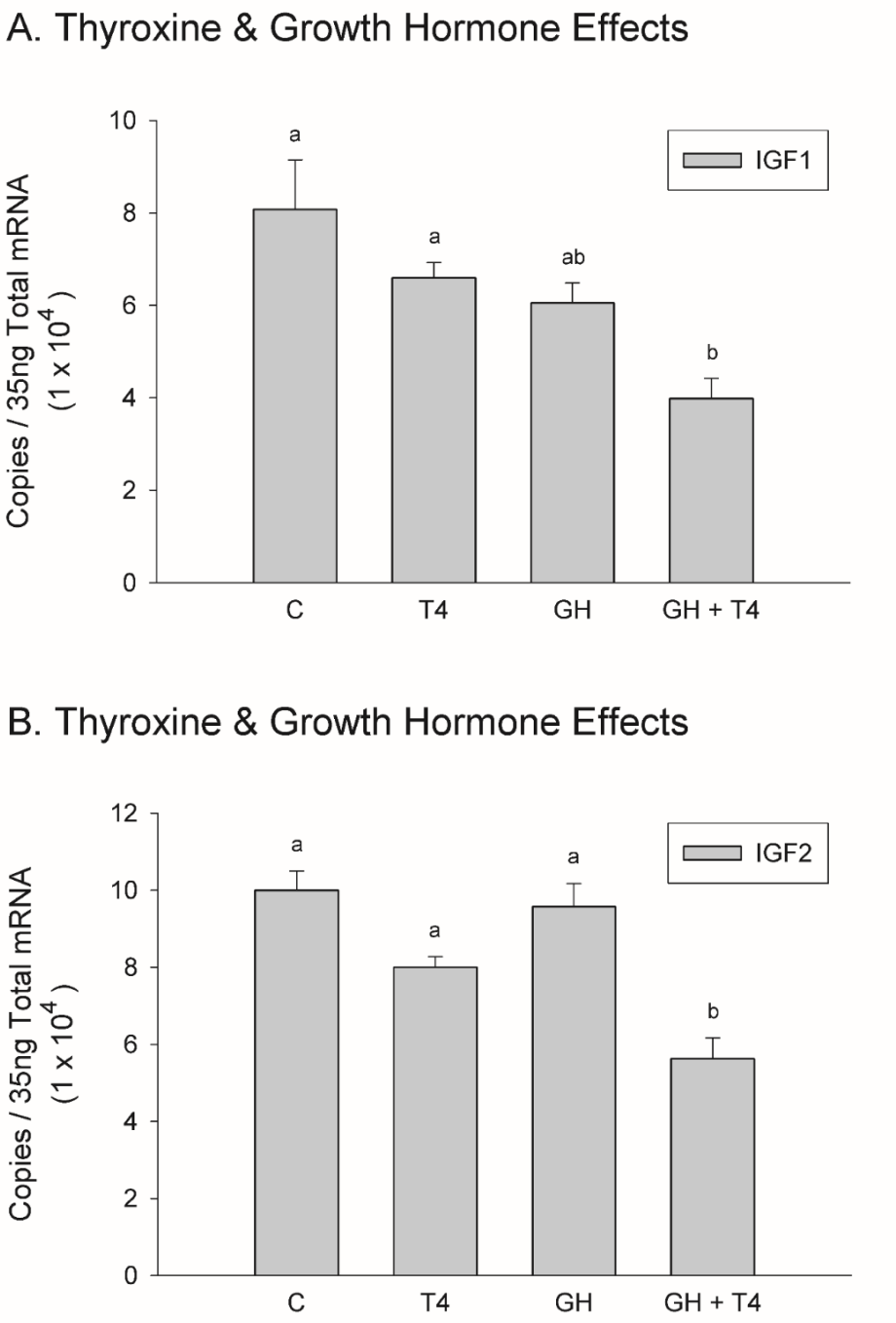

Fig. 24. Effects of thyroxine (T4), growth hormone (GH), or a combination of GH and T4 hormone treatments on insulin-like growth factors (IGFs), IGF1 (A) or IGF2 (B), mRNA expression in gill filaments of *in vitro* cultures isolated from rainbow trout. Gill filaments were incubated for 6 hours in medium alone (no hormone, control; C), medium containing T4 (25 ng/ml), medium containing salmonid growth hormone (sGH; 100 ng/ml), or medium containing sGH (100 ng/ml) and T4 (25 ng/ml); in combination treatments sGH and T4 were added simultaneously. Data are presented as mean ± SEM. (n = 5-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFRs

Insulin-like growth factor receptor 1 experiments were conducted on gill filaments to identify the direct effects of T4 and GH alone and in combination. Various treatments had significant effects on IGFR1A and IGFR1B mRNA expression. A significant decrease in IGFR1A mRNA expression, relative to T4-treated and control groups, was observed after treatment with GH (Fig. 25A). Furthermore, a treatment consisting of GH + T4 resulted in the most significant decline in IGFR1A mRNA expression compared to all other treatments (Fig 25A). Effects seen on IGFR1A mRNA expression levels were similar to effects observed on IGFR1B mRNA levels. GH treatment significantly reduced IGFR1B mRNA expression relative to the control group (Fig. 25B). Once more, the decrease in IGFR1B mRNA expression was observed to be significantly different among all treatment groups the treatment of GH + T4 (Fig. 25B).

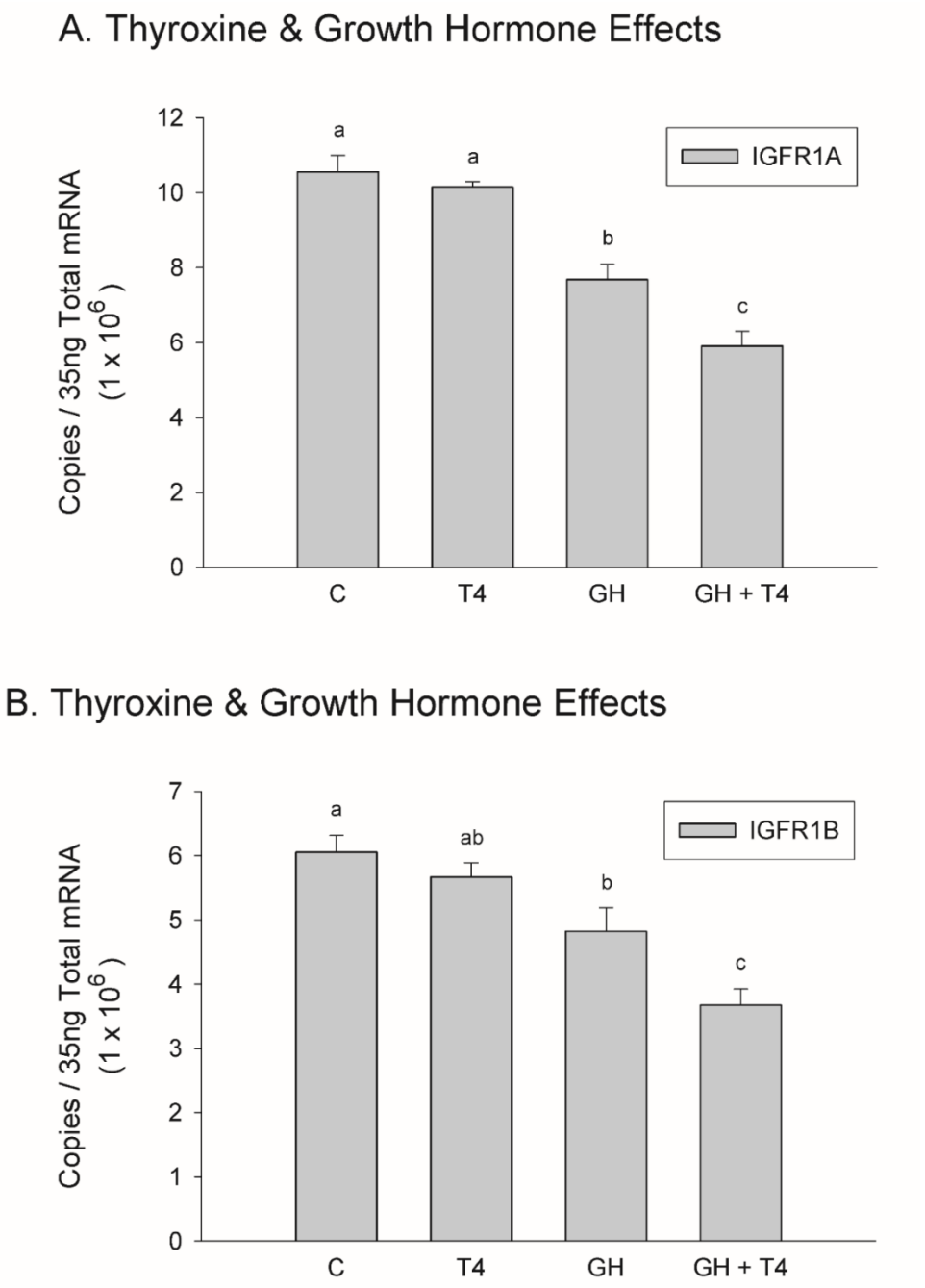

Fig. 25. Effects of thyroxine (T4), growth hormone (GH), or a combination of GH and T4 hormone treatments on insulin-like growth factor receptors (IGFRs), IGFR1A (A) or IGFR1B (B), mRNA expression in gill filaments of *in vitro* cultures isolated from rainbow trout. Gill filaments were incubated for 6 hours in medium alone (no hormone, control; C), medium containing T4 (25 ng/ml), medium containing salmonid growth hormone (sGH; 100 ng/ml), or medium containing both sGH (100 ng/ml) and T4 (25 ng/ml); in combination treatments, sGH and T4 were added simultaneously. Data are presented as mean ± SEM. (n = 5-6). For a given IGFR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

Discussion

This study was an examination of the influence of thyroxine treatment on the GH-IGF system in a teleost fish. Previous studies with thyroid hormone treatment have primarily focused on T3 actions, not T4, at the extra-pituitary level and mostly in mammals. However, because of the differences between mammal and fish, including that T4 is converted to T3 mostly within the liver of fish, and little to no experiments of T4 treatment examining direct effects on extra-pituitary tissues, I conducted my experiments using T4.

The findings reveal that thyroxine may not directly affect elements of the GH-IGF system but may have indirect effects on the GH-IGF system related to the surrounding internal environment. These findings did not support our initial starting hypothesis that T4 can directly modulate the expression of GHRs, IGFs and IGFR1s.

The lack of T4 direct effects on the GH-IGF system was supported by observations in the concentration-related experiments. First, there was no change overall in steady-state mRNA expression levels of selected growth genes when various concentrations of T4 were administered to either liver or gill tissue. There was one isolated instance with a specific gene (IGF2) in one specific tissue (gill) where a rise in steady-state mRNA expression levels was observed. That increase occurred at a concentration of 2.5 ng/ml which, depending on the time of the season and life-history status, is either within normal total thyroxine plasma concentration levels or a factor of 10 below the plasma concentration levels usually observed in salmon or trout (Laidley & Leatherland, 1988; Holloway et al., 1999; Ebbesson et al., 2000; Ebbesson et al., 2008). Second, T4 treatments did not significantly affect steady-state mRNA expression levels for any of the selected growth genes when compared to the control groups during the inhibition experiments with either liver or gill filament tissue. Finally, along the same lines in which T4 did not

significantly affect steady-state mRNA expression levels for the selected growth genes in the inhibition studies, T4 also did not significantly affect steady-state mRNA expression levels for the selected growth genes when compared to the control groups during the combination studies with GH in liver or gill tissue.

Other studies have used T3 on fish. A T3 dose of 10^{-7} M on Mozambique tilapia (*Oreochromis mossambicus*) had no significant effect on GHR1 or GHR2 steady-state mRNA expression in isolated hepatocytes (Pierce et al., 2012). In addition, a 100 nM T3 treatment on primary Coho salmon (*Oncorhynchus kisutch*) hepatocytes resulted in no significant change in GHR mRNA expression from control mRNA expression levels (Pierce et al., 2005). Furthermore, Pierce and colleagues (2005) indicated a T3 dose of 100 nM did not significantly alter IGF1 mRNA expression levels when compared to control levels in Coho salmon. A study done with silver sea bream (*Sparus sarba*) hepatocytes indicated T3 had no effect in a concentration-related manner on IGF1 mRNA expression levels (Leung et al., 2008). These results are in co-ordinance with T4 results observed with my current studies. These results may indicate that thyroid hormones, T3 and T4, are not significant in "regulating" GHRs sensitivity to GH or in the mRNA production of IGF1 directly in these fish species.

However, not all studies are congruent regarding effects involving T3 or T4. In one Coho salmon study, a 10^{-7} M dose of T3 significantly reduced IGF2 mRNA expression levels when compared to controls (Pierce et al., 2010). Yet, in a study done with Mozambique tilapia hepatic IGF1 mRNA expression levels increased with an *in vivo* treatment of a 6 µg/g of body weight injection of T3, as well as, an *in vitro* treatment of 100 nM of T3 (Schmid et al., 2003). In addition, T3 treatments administered to zebrafish (*Danio rerio*) liver organ cultures and amphioxus hepatic caecum organ cultures produced an increase in IGF1 mRNA expression in a

dose-dependent manner (Wang & Zhang, 2011). Contrary to my current results, a concentration of 1000 ng/ml of T4 was found to be significant in elevating IGF1 mRNA expression levels compared to all treatment groups in hepatocytes of silver sea bream (Leung et al., 2008). The dose that produced a stimulatory response on IGF1 mRNA expression in silver sea bream was noted to be of pharmacological importance, as the normal range of circulating T4 plasma levels is usually between 46-87 ng/ml (Leung et al., 2008). The normal range of circulating total T4 plasma levels found within Atlantic salmon and rainbow trout were between the ranges of 3-25 ng/ml. No physiological or pharmacological dose produced a significant change in steady-state mRNA expressions for the growth genes examined in my experiments.

Contradictory effects of T3 and T4 can also be seen in mammals. Ikeda et al. (1991) observed that treatment with T3 perfused through rat liver produced a direct stimulatory effect on IGF1 mRNA expression, but when porcine hepatocytes were treated with T3 no direct influence was observed on IGF1 mRNA expression (Brameld et al., 1995). However, hepatocytes treated with a 100 nM concentration of T4 resulted in IGF1 mRNA expression levels to significantly diminish (Brameld et al., 1995). All results taken together, indicate that direct effects of T3 or T4 on IGF1 or IGF2 mRNA production may be species-specific. Further studies are needed to gain a better understanding in other fish species to examine and understand whether T3 or T4 have direct effects on extra-pituitary tissues that contain genes involved with growth.

Regarding my time-course experiments on T4 effects on selected growth genes, I observed several things. First, except for GHR1 in the gill which had no effect, all growth genes examined had a significant decrease in mRNA expression levels during the times selected for these experiments. Second, specific genes in the liver, consisting of both GHRs and both IGFs, had a rapid decrease in mRNA expression with T4 treatments. Third, specific genes in the gill,

consisting of both IGFs and both IGFRs, had a gradual decrease in mRNA expression with T4 treatments. Fourth, in gill tissue, GHR2 mRNA expressions significantly decreased between two specific time-points of 3 hours and 12 hours, however, the decrease in GHR2 mRNA expression at those times were not significantly different from GHR2 mRNA expression levels observed in the control. To my knowledge, this is the first study to examine T4 treatments over a time course.

Keeping in mind that the majority of T4 is converted into T3 in liver of fish (Blanton & Specker, 2007), it is still reasonable to compare the T4 treatments in my studies to T3 treatments in other studies. If T4 is being converted into T3, a change in mRNA expression of the selected growth genes could be due to the biological actions of either hormone. Only two other labs have examined T3 thyroid hormone effects in a time-course manner in fish, involving longer and shorter time-courses than in my experiments. Mozambique tilapia primary hepatic cells were treated with 100 nM concentration of T3 in both a long time-course and short-time course experiments. Schmid and colleagues (2003) noticed a significant increase in IGF1 mRNA expression at 6 hours during a long time-course. After the 6-hour increase in hepatic IGF1 mRNA expression, a significant decrease occurred for the remaining time points up to 42 hours. However, by the end of the long time-course, T3 treated hepatocytes remained at approximately three times higher in IGF1 mRNA expression relative to the corresponding control IGF1 mRNA expression levels (Schmid et al., 2003). In a short time-course study of the time in which IGF1 mRNA expression levels increased, Schmid and colleagues (2003) observed a significant increase of approximately 2-fold in IGF1 mRNA expression levels to occur between 4 and 6 hours when compared to untreated hepatic cells. Like my current studies, Wang and Zhang (2011) utilized whole organ liver cultures to examine IGF1 mRNA expression in zebrafish and the cephalochordate amphioxus. Similar results were found to occur among both organisms and

those compared to previously mentioned T3 treatment in tilapia that were treated during longer time-courses (Wang & Zhang, 2011; Schmid et al., 2003). During short time-course treatments it was found IGF1 significantly increased after 30 minutes of 100 nM T3 treatment in both zebrafish and amphioxus and remained at these elevated IGF1 mRNA expression levels over control mRNA expression levels for the 8-hour time-course (Wang & Zhang, 2011). In zebrafish and amphioxus hepatic cells that received T3 treatments IGF1 had mRNA levels that remained approximately two-fold higher than IGF1 mRNA levels observed in the control group (Wang & Zhang, 2011).

With all current results considered, it may indicate that T4 alone does not produce biologically active direct effects in rainbow trout. Yet, with consistent and noticeable time-dependent effects observed in my experiments, there may be something, either by the liver tissue in general or specifically by the hepatocytes, that is being produced which T4 is interacting with, to cause the observed rapid or gradual decreases in mRNA expression for the selected growth genes. However, because direct T4 effects were observed with a pharmacological concentration at 24 hours of treatment in silver sea bream (Leung et al., 2008), it would be worth studying whether T4 can potentially influence steady-state mRNA expression levels of growth genes in other fish species.

When comparing my studies with others examining thyroid effects on genes involved with the GH-IGF system, the methodology involving which type of cell or tissue is used may lead to specific responses occurring at certain time-points, shorter time-points for hormone interaction with cells versus interacts with cells in a whole organ tissue cultures due to surface area to volume ratio.

In fish, both GH and T4/T3 hormones are key regulators of growth. Studies based on plasma thyroid hormone levels in fish, when GH is intraperitoneally injected into rainbow trout, have shown that it aides in the conversion of T4 into T3 (de Luze & Leloup, 1984; MacLatchy & Eales, 1990; MacLatchy et al., 1992; Leatherland and Farbridge, 1992). The single deiodination of T4 and increased plasma T3 levels observed after an injection of GH is believed to occur due to the increased activity of the hepatic deiodinase of trout (MacLatchy & Eales, 1990; MacLatchy et al., 1992).

Also, to consider, studies examining the GH gene and its chemical amino acid sequence make-up have indicated certain consensus sequences to be retained within the promoter region across vertebrates. In rainbow trout two GH genes exist and their amino acid sequence relating to the thyroid hormone response element is conserved within the promotor region and within introns and exons throughout the GH genes (Yang et al., 1997). In order for thyroid hormones to elicit a response in influencing steady-state mRNA expression this consensus sequence needs to be present within the GH gene to interact with the thyroid hormones.

In my combination experiments, T4 treatments did not have any significant direct effect on selected growth genes in either liver or gill filament tissues. This was also what was observed in my concentration-related studies. In line with the original somatomedin hypothesis, when GH treatment was administered to liver tissue a significant increase in IGF1 mRNA expression levels were observed when compared to control expression levels. This is not in agreement with what was previously observed in concentration- and time-related studies in Chapter 2. Also, in the combination studies of GH and T4 when GH was administered to gill filaments, a significant decrease in IGFR1A and IGFR1B mRNA expression occurred when compared to their respective control groups. This observation is also contrary to what was observed during the

concentration-related and time-related studies performed in chapter 2. Surprisingly, within the current combination studies of GH and T4 a significant decrease in steady-state mRNA expression levels was observed for all selected growth genes. This is opposite to what was thought would be observed when GH and T4 were added simultaneously, due to GH having direct actions in aiding the conversion of T4 into T3 by increasing deiodinase activity (MacLatchy & Eales, 1990; MacLatchy et al., 1992.). Also, the GH gene contains a consensus sequence for the thyroid hormone response element within its amino acid make-up (Yang et al., 1997). Also relating back to the original somatomedin hypothesis that GH has a general conserved action in vertebrates to increase IGF mRNA expression levels, my thoughts were an increase in steady-state mRNA expression for selected growth genes would be observed.

In other studies, done with a different fish species of coho salmon, Pierce et al. (2005) found that T3 did not have a significant effect in increasing IGF1 mRNA expression levels. In addition, when T3 and GH treatments were administered no effect was seen to occur with hepatic IGF mRNA expression levels in coho salmon (Pierce et al., 2005). However, when hepatocytes were treated with T3 and IGF2 mRNA expression levels were examined a significant decrease was observed (Pierce et al., 2010). Also, when GH was administered, IGF2 mRNA expression significantly rose above control levels (Pierce et al., 2010). Finally, when GH and T3 were administered together, IGF2 mRNA expression levels were found to be significantly expressed at a higher level than hepatocytes treated with T3 alone, but IGF2 mRNA expression levels were also significantly lower than hepatocytes treated with just GH treatment (Pierce et al, 2010). IGF1 mRNA expression is of most interest in studies that utilize GH with another hormone such as T3. My thoughts are because this relates back to the somatomedin hypothesis that GH treatment stimulates the production of hepatic IGF1 or IGF2 production.

In mammals, specifically porcine, it was found that T4 treatments significantly decreased exon 1 of IGF1 mRNA expression but had no effect on exon 2 of IGF1 mRNA expression, while T3 treatments had no effect on IGF1 (exon 1 or exon 2) mRNA expression. T4 treatments were also found to stimulate the extracellular domain region of GHR mRNA expression in pigs (Brameld et al., 1995). However, when GH + T4 were used to treat hepatocytes an increase of IGF1 (exons 1 and 2) mRNA expression were significantly increased over control levels. Furthermore, an increase in mRNA expression levels for the extracellular domain receptor region of GHR was observed after a GH + T4 treatment (Brameld et al., 1995). With GH + T3 treatment, IGF1 (exon 1 and exon 2) mRNA expression was significantly elevated over control levels (Brameld et al., 1995). In primary rat hepatocytes that were treated with either GH or T3 treatments, IGF1 mRNA expression levels were greatest in GH-treated than T3-treated, but both individual hormone treatments were greater than the control, non-treated group (Tollet et al., 1990). However, when a combination of GH and T3 treatment were treated on rat hepatocytes a substantially significant increase in IGF1 mRNA expression resulted. Overall, in mammals it seems that T3 or T4 have a noticeable stimulatory effect, but T3 may be more important to potentiate GH-induced IGF1 mRNA production.

In my inhibition study, thyroxine treatment did not significantly influence steady-state mRNA expression for the growth genes selected. Yet, effects were observed when an antithyroid drug, methimazole (MMI) was administered. Overall, I observed that the high-dose (320 ng/ml) of MMI had significant effects in diminishing steady-state mRNA expression, while the low-dose (100 ng/ml) of MMI had no change in effects or increased steady-state mRNA expression of the growth genes selected. When MMI effects were observed to diminish steady-state mRNA expression levels of the selected growth genes, it was always compared to the T4

treated groups. A significant decrease in IGF1 mRNA expression was observed in the gill after a high-dose (320 ng/ml) of MMI treatment was administered.

Treatments with both MMI and T4 used the inhibitor for 4 hours. Following the 4-hour inhibitor treatment, T4 was added for 6 hours to see if thyroxine could elicit previously expected stimulatory actions. When the T4-treated groups were compared to groups that received inhibitor, a significant decrease in steady-state mRNA expression levels of IGF1 and IGFR1A were observed with a MMI 320 and T4 as a single treatment in gill filaments; these steady-state mRNA expression levels were also equal to the steady-state mRNA expression levels observed with just a high-dose of MMI administered. Because (1) MMI is a known inhibitor of T4 conversion to T3, (2) steady-state mRNA expression levels of T4-treated groups did not change relative to the control group in my experiment but treatment with MMI 320 was able to decrease IGF1 mRNA expression levels, and (3) that those IGF1 mRNA expression levels did not return to control levels or higher than control levels, we could logically say the observed diminished IGF1 mRNA expression levels might be due to possible blockade of thyroid hormone(s) functions.

Similar mRNA expression level profiles were observed with IGFR1A in gill with a high-dose (320 ng/ml) methimazole treatment. On another note, even though I saw an overall diminished IGF2 mRNA expression in the gill after only a high-dose of MMI, a treatment with MMI 320 + T4 produced different results. It seems T4 was able to stimulate IGF2 at mRNA levels between those observed in the control and those treated with MMI 320. However, another conundrum was that IGF2 gill filaments treated with a low-dose (100 ng/ml) MMI + T4, the same diminished IGF2 mRNA expression level was observed as in the group that received only a high-dose (320 ng/ml) of MMI. So, it looks as though T4 in the presence of a low-dose of MMI

was not able to exert its effects in bringing IGF2 mRNA expression levels back toward control levels, but with T4 in the presence of a high-dose of MMI, T4 was able to raise mRNA expression levels back towards control levels slightly.

Taken all together, it seems MMI has a stronger potential to inhibit thyroid hormone actions in gill filaments than in liver tissue. This could be due to the surface area to volume ratio in the hormone having a shorter time to cross the cell-membrane in gill filaments versus liver tissue. Another problem I may have with the design of my experiments is the length of time the inhibitor has in order to inhibit the response of T4 effects. Crane (2006) exposed fathead minnows for a number of days to specific concentrations of methimazole and noticed there was a reduced T4 synthesis, but the body mass was unaffected by inhibitor treatments. They suggested compensatory growth mechanism were able to overcome the decrease in T4 synthesis in fathead minnows.

The takeaway message across all experiments performed with thyroxine is that T4 does not have direct effects on genes with the GH-IGF system but may partially interact with other molecules or hormones to potentiate its influence on selected growth genes of the GH-IGF system. All in all, the variability in both my results and those of other researchers demonstrates the puzzling nature of thyroxines in relation to growth.

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CHAPTER 4: EFFECTS OF CORTISOL ON THE EXPRESSION OF GROWTH HORMONE RECPTORS, INSULIN-LIKE GROWTH FACTORS AND INSULIN-LIKE GROWTH FACTOR TYPE 1 RECEPTORS IN RAINBOW TROUT

Abstract

Cortisol in vertebrates plays significant roles in intermediary metabolism, osmoregulation, growth, stress responses, and immune functions to name a few. In fish, cortisol is the main corticosteroid produced by the interrenal cells. It is generally accepted that during times of stress cortisol is a negative regulator of somatic growth, yet some studies have shown an increase in the production and release of GH from the pituitary. Despite this knowledge, only a handful of researchers have explored the direct effects cortisol might have on growth genes. Using real-time quantitative polymerase chain reaction, the transcriptional expression of growth hormone receptors (GHR1 and GHR2), insulin-like growth factors 1 and 2 (IGF1, IGF2) and type 1 IGF receptors (IGFR1) were measured after various *in vitro* cortisol treatments to indicate if endogenous cortisol directly affects genes involved with growth in rainbow trout (*Oncorhynchus mykiss*). The results from my experiments with liver tissue indicated a significant increase in GH sensitivity with GHR1 but not GHR2 after cortisol treatment. Also, cortisol directly stimulated the production of mRNAs encoding of hepatic IGF2. Stimulatory effects on hepatic IGF1 mRNA expression were only observed after a supraphysiological concentration of cortisol was applied, however, both IGF1 and IGF2 transcriptional mRNA levels decreased over longer periods of cortisol treatments. However, when cortisol and GH were used in combination, hepatic GH sensitivity and hepatic IGF production significantly decreased. In gill filament cultures, cortisol increased GH sensitivity of GHR1 over longer periods of time. GHR2 showed an increase with only a narrow range of physiological

concentrations of cortisol normally found in salmonids. Additionally, IGF1 production significantly increased after doses representative of those seen in salmonids after a stressed-state. However, longer periods of time significantly decreased the production of IGF1 and IGF2 mRNAs in gill filaments. Finally, IGF sensitivity was significantly increased after physiological and stressed-state concentrations of cortisol were applied to gill filaments. Overall, these results demonstrate cortisol directly influences the transcriptional regulation of genes involved with growth in vertebrates.

Introduction

Growth incorporates a series of changes that take place in a continuous fashion bringing about adult formation and function; maintenance of adult form and function are continued through the end of life. Maintenance and regulation of growth can be shaped through internal factors (e.g., genetics) or external factors (e.g., environmental and nutritional states). These internal and external factors play an intricate role in regulating the somatic growth of fish and can induce developmental processes such as hatching, metamorphosis, smoltification, sexual maturation, and spawning (Reinecke, 2010; Pankhurst and Munday, 2011; Bjornsson et al., 2011; McMenamin and Parichy, 2013). Fish are unique in that they have the ability to grow throughout their life (indeterminate growth), however, the majority of somatic growth occurs in a large surge just after their first emergence up until just prior to sexual maturity (Mommsen, 2001; Enberg et al., 2008).

Cortisol (F) is known to affect growth in fish but not in stimulating growth in fish. Rather, cortisol acts as a stress hormone in suppressing growth and redistributing energy toward metabolic processes needed for homeostasis (Sheridan, 1986; Mommsen et al., 1999; Laiz-Carrion et al., 2002). In fish, cortisol plays important physiological roles in ionic and osmotic

regulation, immune functions and intermediary metabolism (McCormick, 2011; Tort, 2011; Faught & Vijayan, 2016).

Glucocorticoids (cortisol, corticosterone and cortisone) and mineralocorticoids (aldosterone) are two main classes of corticosteroid hormones synthesized among vertebrates. Glucocorticoids function to influence metabolism, cell growth and development, while mineralocorticoids function to regulate electrolyte and water balance. Depending on which teleost fish is being examined, aldosterone is synthesized and found in small amounts within the plasma (Chavin and Singley, 1972; Whitehouse and Vinson, 1975; Greenwood et al., 2003) or aldosterone is not synthesized at all, due to the lack of the aldosterone synthase enzyme needed to convert 11-deoxycorticosterone to aldosterone (Jiang et al., 1998, Baker, 2003; Bury and Strum, 2007). Although minuscule levels of aldosterone are found in the plasma of some teleost fish, aldosterone is not considered to act as a mineralocorticoid in teleosts (Bern, 1967; Wendelaar Bonga, 1997; Prunet et al., 2006). Cortisol is the main corticosteroid produced in teleost fish and is known to function as a glucocorticoid and mineralocorticoid (Wendelaar Bonga, 1997; Mommsen et al., 1999; McCormick et al., 2008).

Much of the work on how cortisol influences growth has been on the production and secretion of GH at the pituitary level. The effects of cortisol on the production and secretion of GH are enigmatic. In eel, there was no significant difference in the release of GH after treatment with differing concentrations of cortisol (Rousseau et al., 2002). Contradictory results in the same species of fish, Mozambique tilapia, are found with the release of GH after cortisol administration. Kajimura and colleagues (2003) found no significance, while Nishokia et al. (1985) and Uchida et al. (2004) found a significant increase in the release of GH. Furthermore,

Peterson and Small (2005) found a high dose of cortisol to stimulate pituitary GH mRNA expression.

In the current study, rainbow trout (*Oncorhynchus mykiss*) were used to investigate the influence of cortisol on the GH-IGF system. An *in vitro* tissue culture system was implemented to elucidate and gain further understanding of the endogenous actions that cortisol has in the peripheral influences on somatic growth. Supplementary, a study examining different antagonist to cortisol were conducted to validate specific effects observed with cortisol treatment. The genes responsible for controlling growth (GHRs, IGFs, and IGFR1s) were examined by mRNA expression levels after various treatments with cortisol. I postulate that cortisol can directly modulate the expression of the genes controlling growth.

Materials and Methods

Animals

Juvenile rainbow trout were obtained from Dakota Trout Ranch near Carrington, ND, and transported to North Dakota State University where they were maintained 800-L circular tanks supplied with recirculated (10% replacement volume per day), dechlorinated, municipal water at 14 °C under a 12L:12D hour photoperiod. Fish were fed twice a day to satiation with AquaMax® Grower (PMI Nutrition International, Inc., Brentwood, MO) semi-floating trout grower, except for 24-36 hours before initiating experimental manipulations. Fish were acclimated to laboratory conditions for at least two weeks prior to experimentation.

Experimental Conditions for mRNA Expression

During the time of sampling, fish were anesthetized in 0.05% (v/v) 2-phenoxyethanol, measured and weighed (body length and body weight), and bled from the severed caudal vessels. All fish were juvenile and sexually immature. Liver and gill arches were removed from fish,

perfused ex vivo with 0.75% (v/v) saline solution, placed in Hank's medium (in mM: 137 NaCl, 5.4 KCL, 4 $NaHCO_3$,1.7$CaCl_2$, 0.8 $MgSO_4$, 0.5 KH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, and 4 of glucose, pH 7.6), and prepared for culture as previously described (Harmon and Sheridan, 1992; Hanson et al., 2010).

Liver tissue was cut into $1mm^3$ pieces and individual gill filaments were separated from the arches. Gill filaments and liver pieces from individual fish were pooled (i.e. one pool of each type of tissue per fish) and washed three times with Hank's culture media with 0.24% (w/v) bovine serum albumin. Tissues were placed in 24-well culture plates (containing 8-10 liver pieces and 12-15 gill filaments) containing 1-ml of Hank's medium with 0.24% (w/v) bovine serum albumin that included essential and non-essential amino acids and preincubated at 14 °C under 100% O_2 while being shaken at 100 rpms in a gyratory shaker; replicate wells for a given experiment came from different fish.

After a 3-hour preincubation period, the medium was removed, and tissues received fresh Hank's medium with 0.24% (w/v) BSA and essential and non-essential amino acids. Tissues were then treated with either cortisol (F) (Sigma, St. Louis, MO) ranging in concentration from 0-500 ng/ml for 6 hours, or physiological levels of 100 ng/ml of F for various periods of time (0, 3, 6, 12, 24 hours), while maintaining under the same conditions as preincubation procedure. F was initially dissolved in 95% (v/v) ethanol, with the final concentration of ethanol in the culture wells not exceeding 0.0028% (v/v); control incubations contained the same final concentration of ethanol. The concentrations of F used were based upon the range of F concentrations measured in plasma of brown trout and rainbow trout during an experiment were they were exposed to an environmental stressor, as well as, a comprehensive report summarizing documentation of plasma cortisol changes that occur to various types of aquaculture-related stressors (Pickering &

Pottinger, 1989; Barton & Iwama, 1991). Following the completion of study treatments, incubation media was removed from the samples, and tissues were placed in 2.0-ml microcentrifuge tubes and immediately frozen on dry ice. The tissues were stored at -80 °C for later mRNA processing and analysis.

GHR mRNA expression levels were measured in liver and gill filaments because of high levels of mRNA encoding the receptors observed previously in these tissues. (Gray et al., 1992; Very et al., 2005; Walock et al., 2014). IGFR1 mRNA were measured in gill filaments due to the prevalence in the expression of IGFR1-encoding mRNAs in this tissue (Leibush et al., 1996; Nakao et al., 2002). Furthermore, mRNA levels of IGF were measured in liver and gill filaments because of their relative abundance in these tissues (Duan et al., 1993; Vong et al., 2003; Wood et al., 2005; Reinecke, 2006).

Experimental Conditions for Cortisol Inhibition

Conditions for these experiments were analogous to mRNA expression experimental conditions up to the 3-hour preincubation period. After a 3-hour preincubation period was completed, Hank's media was removed, and tissues received fresh media with 0.24% BSA and essential and non-essential amino acids. Tissues were either treated with 10 ul of RU486 (Sigma) or spironolactone (Sigma) inhibitors at 1,000 ng/ml, 10,000 ng/ml, respectively; 50 µl of F (Sigma) at 100 ng/ml; or F with either RU486 or spironolactone (10 µl of inhibitor and 50 µl of hormone). Tissue samples that received only the inhibitors were treated for 4 hours, tissue samples that received F were treated for 6 hours, and samples that received both inhibitor and hormone treatment were first treated for 4 hours with inhibitor and then an additional 6 hours once the hormone F was added, while all samples were maintained under the same preincubation period conditions as the mRNA expression experimental conditions. F, RU486, and

spironolactone were initially dissolved in 95% (v/v) ethanol with the final concentration of ethanol in the wells not exceeding 0.0028% (v/v). The control group contained the same final concentrations of ethanol in which F, RU486, and spironolactone were dissolved. The concentrations of RU486 and spironolactone were established from previous studies that were completed with rainbow trout and Atlantic salmon (Aluru and Vijayan, 2007; Tipsmark et al., 2009). Following the completion of study treatments, incubation media was removed from the samples and tissues were placed in 2.0-ml microcentrifuge tubes, and immediately frozen on dry ice. Tissues were stored at -80 °C until processed for mRNA analysis. The same genes were analyzed as in the mRNA expression study.

Experimental Conditions for Combination Study of GH and Cortisol

This study consisted of liver and gill tissues being collected similarly to the mRNA expression study. Tissues were preincubated for 3 hours with 1-ml of Hank's media with 0.24% (w/v) BSA that included essential and non-essential amino acids at 14 °C under 100% O_2 shaken at 100 rpm on a gyratory shaker in 24-well plates. After completion of the preincubation period, new Hank's media was replaced, and samples received various individual hormones or combination of hormones. The treatment groups were ethanol control (final concentration < 0.0028%), growth hormone (100 ng/ml), cortisol (100 ng/ml) and growth hormone + cortisol. Hormone treatments were added simultaneously to the samples and were treated for 6 hours. With fulfillment of the study, tissues were stored at -80 °C until further processing for mRNA analysis.

Quantitative Real-time PCR

Frozen tissues were homogenized and total RNA was isolated using RNAzol® reagent (Molecular Research Center, Cincinnati, OH, USA) as specified by the manufacturer's protocol.

Each RNA pellet was re-dissolved in 60-100 µl of RNAse-free deionized water and total RNA was quantified by ultraviolet (UV A_{260}) spectrophotometry using the Nanodrop 1000 Spectrophotometer (Thermo Scientific, West Palm Beach, FL, USA). mRNA was reverse transcribed in 5-µl reactions using 200 ng total RNA and qScript™ cDNA synthesis kit reagents (qScript Reaction Mix, qScript RT, and nuclease-free water) according to the manufacturer's protocol (Quanta Biosciences™, Inc., Gaithersburg, MD, USA). To eliminate the possibility of contamination with genomic DNA, reactions without reverse transcriptase were included as negative controls; amplification was not detected in negative controls. Steady-state mRNA levels of GHR1, GHR2, IGF-1, IGF-2, IGFR1A, and IGFR1B were determined by quantitative real-time PCR using Stratagene Mx 3000 detection system (Stratagene, La Jolla, CA, USA). The gene-specific nucleotide primers and probes used for the experiments can be found in table 1 of chapter 2. In short, real-time PCR reactions were carried out for samples, standards, and controls in 10-µl total volume reactions. The reactions contained 2 µl of cDNA from the reverse transcription reactions, 5 µl of 2X Brillant® II QPCR Master Mix, 1 µl of gene-specific probe, 0.85 µl of β-actin probe, 0.5 µl of gene-specific forward and reverse primers as well as β-actin forward and reverse primers, and 0.15 µl of reference dye (Stratagene, Agilent Technologies). Real-time PCR reactions were multiplexed. Cycling parameters were set as follow: an initial denaturation step of 95 °C for 10 minutes followed by 50 cycles of replication, each consisting of 95 °C for 15 s and then finally an extension step of 58 °C for 1 minute. Sample copy number was calculated from the threshold cycle number (CT), and the CT was related to a gene-specific standard curve followed by normalization to β-actin.

Statistics

Data are expressed as a means ± SEM; n represents the number of replicates for each treatment group. Statistical differences were analyzed by one-way ANOVA followed by Tukey-Kramer method for post-hoc analysis. An alpha value of < 0.05 was used to indicate significant differences between and among treatment groups. Statistics were performed on β-actin corrected copies of mRNA using JMP® version 11 (SAS Institute Inc., Cary, NC, USA). Outliers of replicates were determined using Dixon's test with an alpha value of < 0.05 indicating required significance level.

Results

Concentration- and Time-related Studies

GHRs

Peripheral sensitivity to GH was evaluated by quantifying mRNA expression of GHRs in hepatic and gill filaments after *in vitro* treatments of cortisol (F). Outside the brain, two distinct GHR-encoding mRNAs, GHR1 and GHR2, are abundantly present in liver and gill filaments (Very et al., 2005; Walock et al., 2014). In liver, F increased GHR1 mRNAs in a concentration- and time-related manner (Fig. 26A & 26B); while F had no significant effect on steady-state GHR2 mRNAs, although a general trend towards an increase in mRNA expression was noted (Fig. 26C & 26D). Increases in hepatic GHR1 mRNA expressions were observed at 100 ng/ml, with significant maximal stimulation occurring at a dose of 500 ng/ml (Fig. 26A). After 24 hours of treatment with F, steady-state levels of GHR1 mRNAs significantly increased when compared to control levels (Fig. 26B).

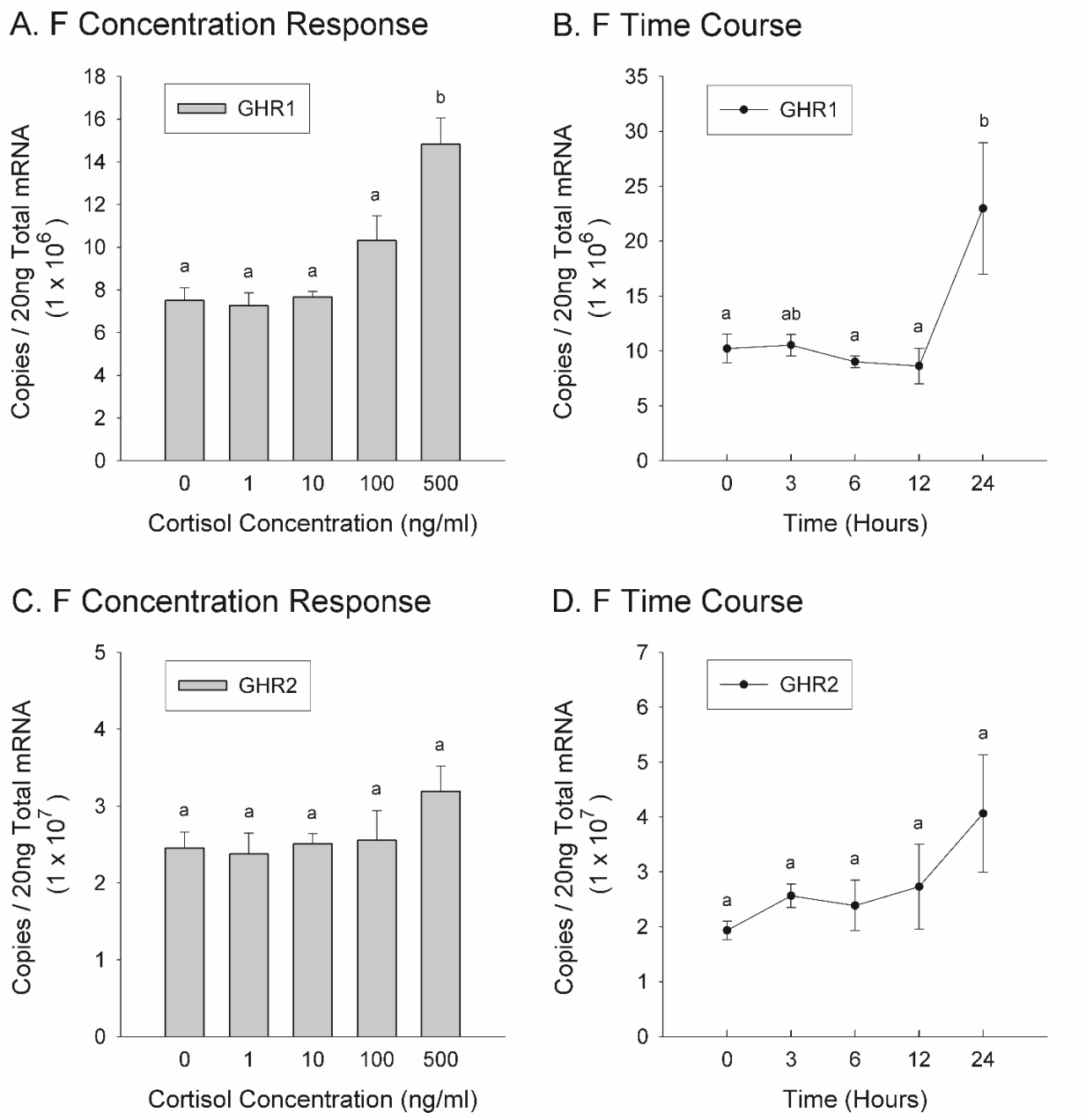

Fig. 26. Effects of cortisol (F) treatments on mRNA expression of growth hormone receptors (GHRs), GHR1 (A and B) or GHR2 (C and D), in liver of rainbow trout incubated *in vitro*. Liver tissue was incubated with various concentrations of F for 6 hours (A and C) or were incubated for various times with 100 ng/ml F (B and D). Data are presented as mean ± SEM. (n = 5-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

Cortisol treatment of gill caused disparate effects in a concentration- and time-related manner in GHR1 and GHR2. In regard to GHR1, F significantly increased mRNA expression levels in a time-related manner (Fig. 27B), but not in a concentration-related manner (Fig. 27A). A general increase of GHR1 mRNA levels was first seen at 6 hours when compared to control levels; maximal stimulation peaked through 12 hours and 24 hours (Fig. 27B). Concerning GHR2, F significantly increased mRNA expression levels in a concentration-related manner (Fig. 27C), but not in a time-related manner (Fig. 27D). Concentrations of 10 ng/ml and 100 ng/ml increased steady-state GHR2 mRNAs over control levels; a concentration of 500 ng/ml brought GHR2 mRNA expression levels back to control levels (Fig. 27C).

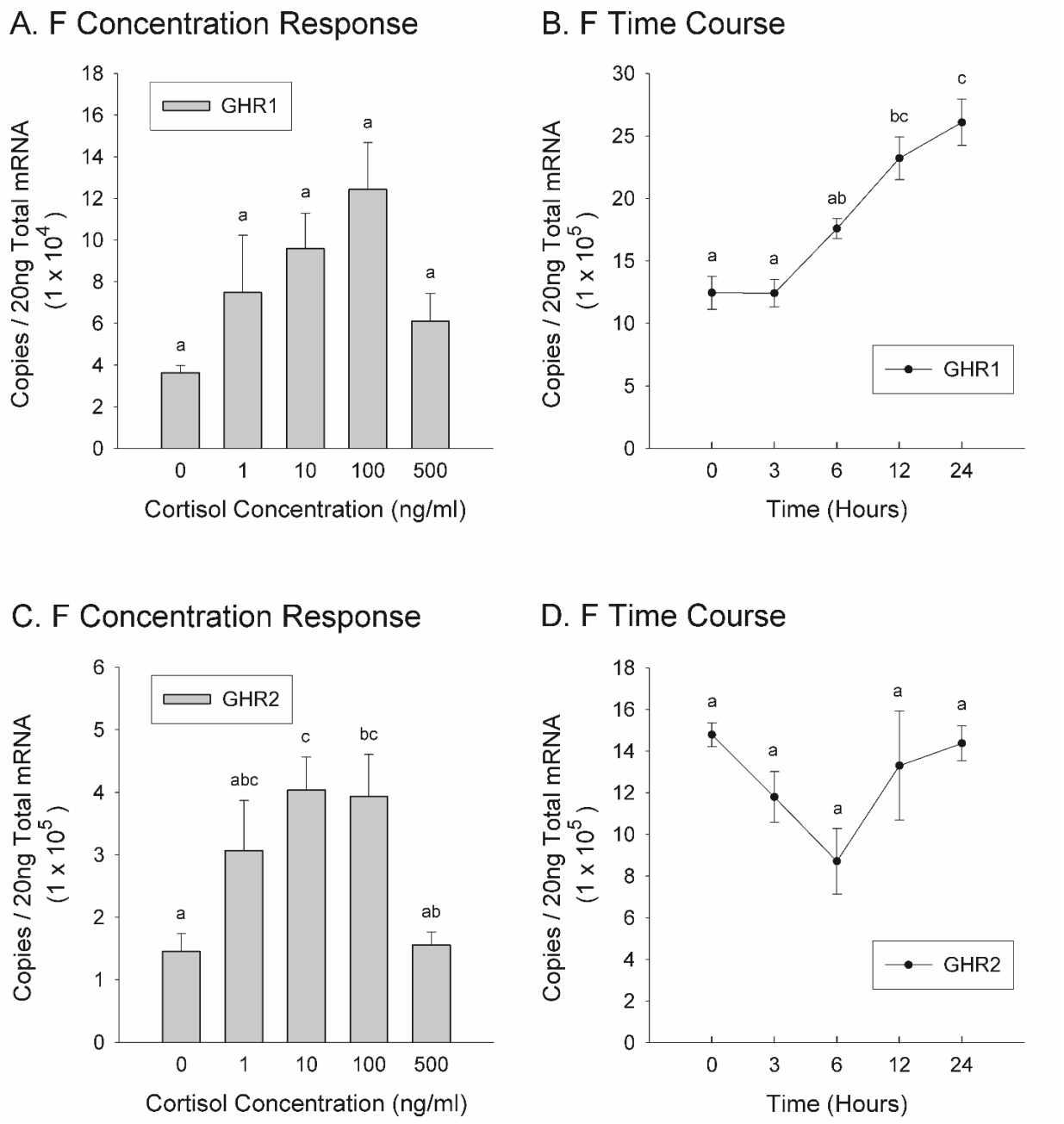

Fig. 27. Effects of cortisol (F) treatments on mRNA expression of growth hormone receptors (GHRs), GHR1 (A and B) or GHR2 (C and D), in gill of rainbow trout incubated *in vitro*. Gill filaments were incubated with various concentrations of F for 6 hours (A and C) or were incubated for various times with 100 ng/ml F (B and D). Data are presented as mean ± SEM. (n = 4-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFs

Peripheral IGF production was evaluated by quantifying mRNA expression of IGFs in hepatic and gill filaments following *in vitro* treatments of F. In liver, F elevated expression levels of IGF1 in a concentration- and time-related manner. Significant amplification of IGF1 steady-state mRNAs only occurred after a supraphysiological concentration of 500 ng/ml was applied (Fig. 28A). A non-significant elevated expression in IGF1 mRNAs was observed following a 3-hour F treatment. More importantly, IGF1 mRNA expression levels significantly decreased with a longer treatment of F after 24 hours (Fig. 28B). Cortisol had a significant effect in influencing steady-state IGF2 mRNAs in a concentration- and time-related manner. Similar to IGF1 mRNA expression levels, IGF2 mRNA expression levels were amplified over controls only at a supraphysiological concentration of 500 ng/ml (Fig. 28C). Like hepatic IGF1 mRNA expression levels, longer durations in treatment (12 hours and 24 hours) produced a reduction in IGF2 mRNA expression levels (Fig. 28D).

In gill filaments, F had a significant effect in influencing IGF1 mRNAs in a concentration- and time-related manner. A significant increase in IGF1 mRNA expression levels occurred only at a concentration of 100 ng/ml (Fig. 29A). Similar to IGF1 and IGF2 mRNA expression levels in the liver, steady-state IGF1 mRNAs in gill were depressed with longer durations (12 hours and 24 hours) of treatment with F (Fig. 29B). Concentrations of F treatment had no effect in influencing IGF2 mRNA expression in gill filaments (Fig. 29C). However, F had significant effects in influencing IGF2 mRNAs in a time-related manner. A progressive decline in IGF2 mRNAs occurred up to 12 hours of treatment with 100 ng/ml; thereafter, mRNA expression levels were maintained at similarly depreciated levels for the remainder of the experiment (Fig. 29D).

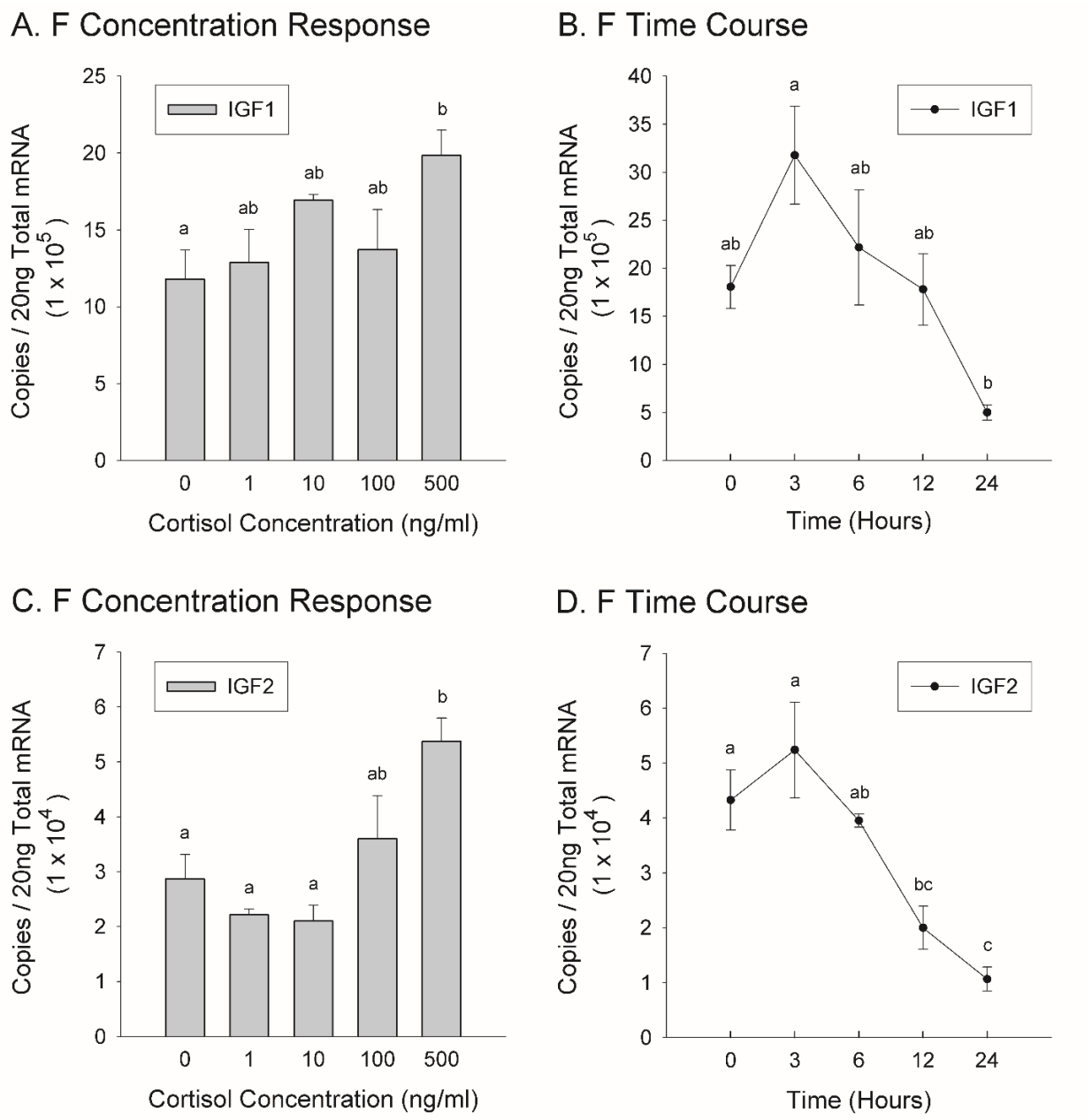

Fig. 28. Effects of cortisol (F) treatments on mRNA expression of insulin-like growth factors (IGFs), IGF1 (A and B) or IGF2 (C and D), in liver of rainbow trout incubated *in vitro*. Liver tissue was incubated with various concentrations of F for 6 hours (A and C) or were incubated for various times with 100 ng/ml F (B and D). Data are presented as mean ± SEM. (n = 5-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

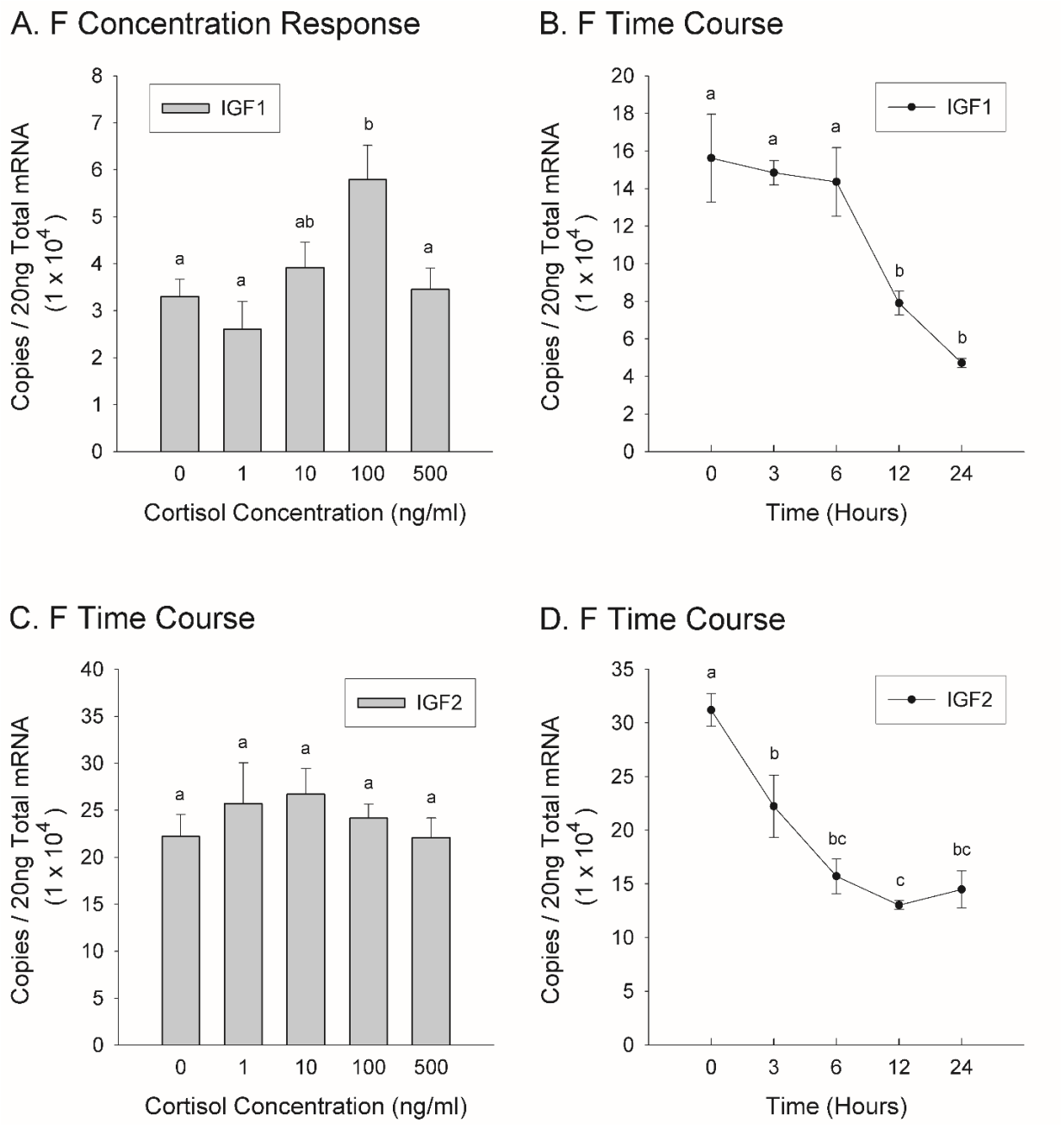

Fig. 29. Effects of cortisol (F) treatments on mRNA expression of insulin-like growth factors (IGFs), IGF1 (A and B) or IGF2 (C and D), in gill of rainbow trout incubated *in vitro*. Gill filaments were incubated with various concentrations of F for 6 hours (A and C) or were incubated for various times with 100 ng/ml F (B and D). Data are presented as mean ± SEM. (n = 4-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFRs

Peripheral IGF1 sensitivity was assessed by quantifying mRNA expression of IGFRs in gill filaments after *in vitro* treatments of F. The effects of F on IGFR1A mRNA expression were significant in a concentration-related manner, but not in a time-dependent manner (Fig. 30B). A significant increase in gill IGFR1A mRNAs was first observed at a F concentration of 10 ng/ml. Maximal stimulation of IGFR1A mRNA expression occurred at 100 ng/ml, with levels approximately 3-fold over the controls (Fig. 30A). Furthermore, a supraphysiological concentration (500 ng/ml) of F returned steady-state mRNA expression of IGFR1A toward control levels. Cortisol did not have significant effects in influencing steady-state mRNA expression levels of IGFR1B in a concentration-related manner (Fig. 30C). Contrarily, F had a significant effect in influencing IGFR1B mRNAs in a time-related manner. After 6 hours of F treatment, steady-state IGFR1B mRNAs declined; similar steady-state mRNA expression levels were maintained through the remainder of experimental time points (Fig. 30D).

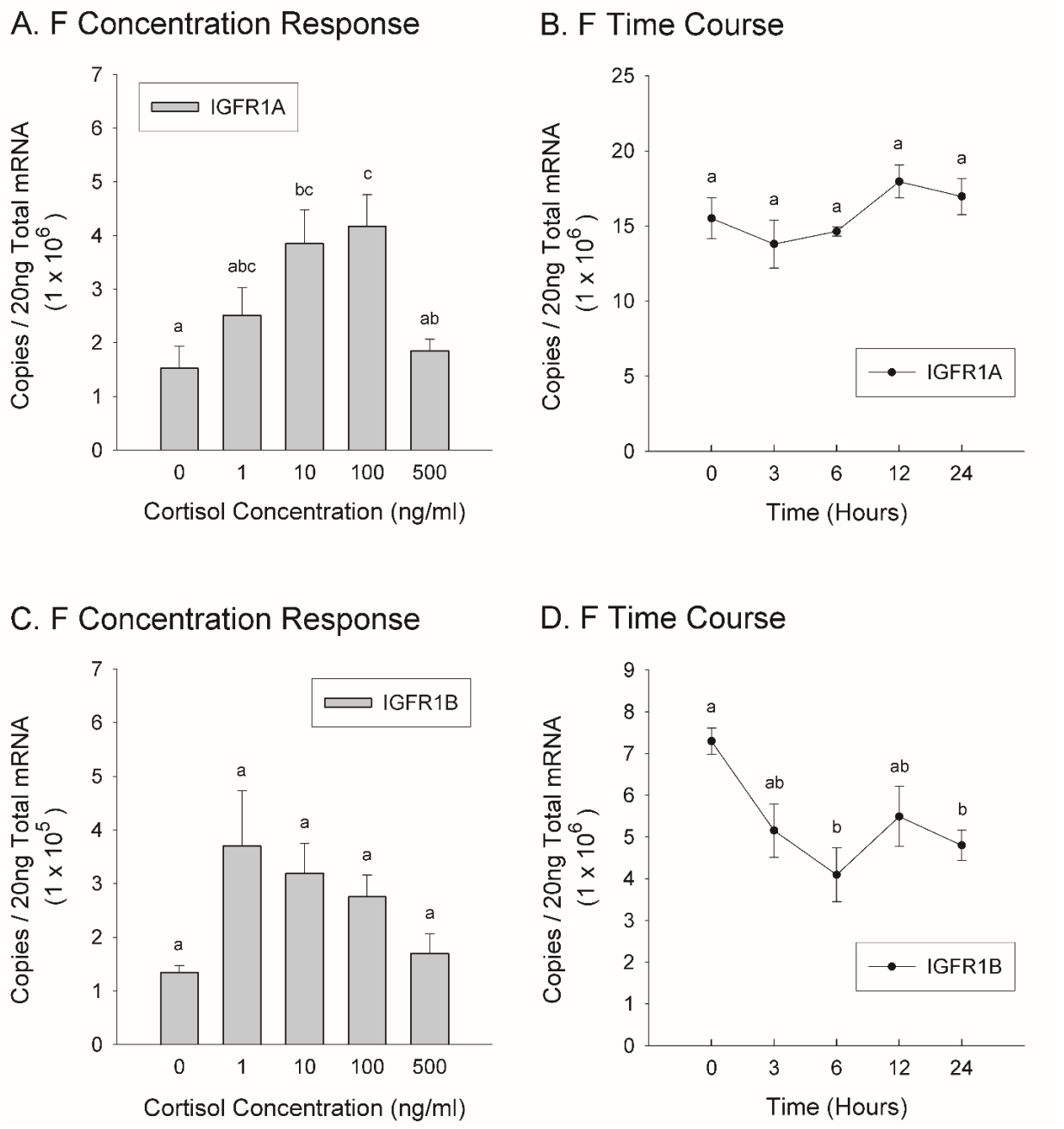

Fig. 30. Effects of cortisol (F) treatments on mRNA expression of insulin-like growth factor receptors (IGFRs), IGFR1A (A and B) or IGFR1B (C and D), in gill of rainbow trout incubated *in vitro*. Gill filaments were incubated with various concentrations of F for 6 hours (A and C) or were incubated for various times with 100 ng/ml F (B and D). Data are presented as mean ± SEM. (n = 4-6). For a given IGFR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

Inhibition Study

GHRs

To substantiate that F treatments induced steady-state GHR mRNAs in hepatic tissue and gill filaments, we examined various treatments of F and two disparate antagonists. The type of treatment administered had significant effects on expression levels of GHR1 and GHR2 in hepatic tissue. GHR1 mRNAs increased over controls with treatment of F, as seen previously (Chapter 2) in the concentration- and time-related experiments (Fig. 31A). Treatment with either a glucocorticoid antagonist mifepristone (RU486) or a mineralocorticoid antagonist spironolactone (Spiro) resulted in significantly suppressed GHR1 mRNA expression levels relative to all other treatment groups, with Spiro having had the greatest impact in suppressing expression of GHR1 (Fig. 31A). Furthermore, treatment of F in conjunction with either RU486 or mineralocorticoid antagonist Spiro resulted in significantly lower levels of expression for GHR1 mRNAs relative to hepatic tissue treated with F; the expression levels observed in both of these treatment groups that had returned toward control levels (Fig. 31A). Cortisol did not significantly influence hepatic GHR2 mRNAs relative to control mRNAs (Fig. 31B). In addition, treatments with RU486 or Spiro did not alter GHR2 mRNA expression relative to the control group. A significant reduction in steady-state GHR2 mRNAs was observed with a joint treatment of F and Spiro relative to hepatic tissue treated with F (Fig. 31B).

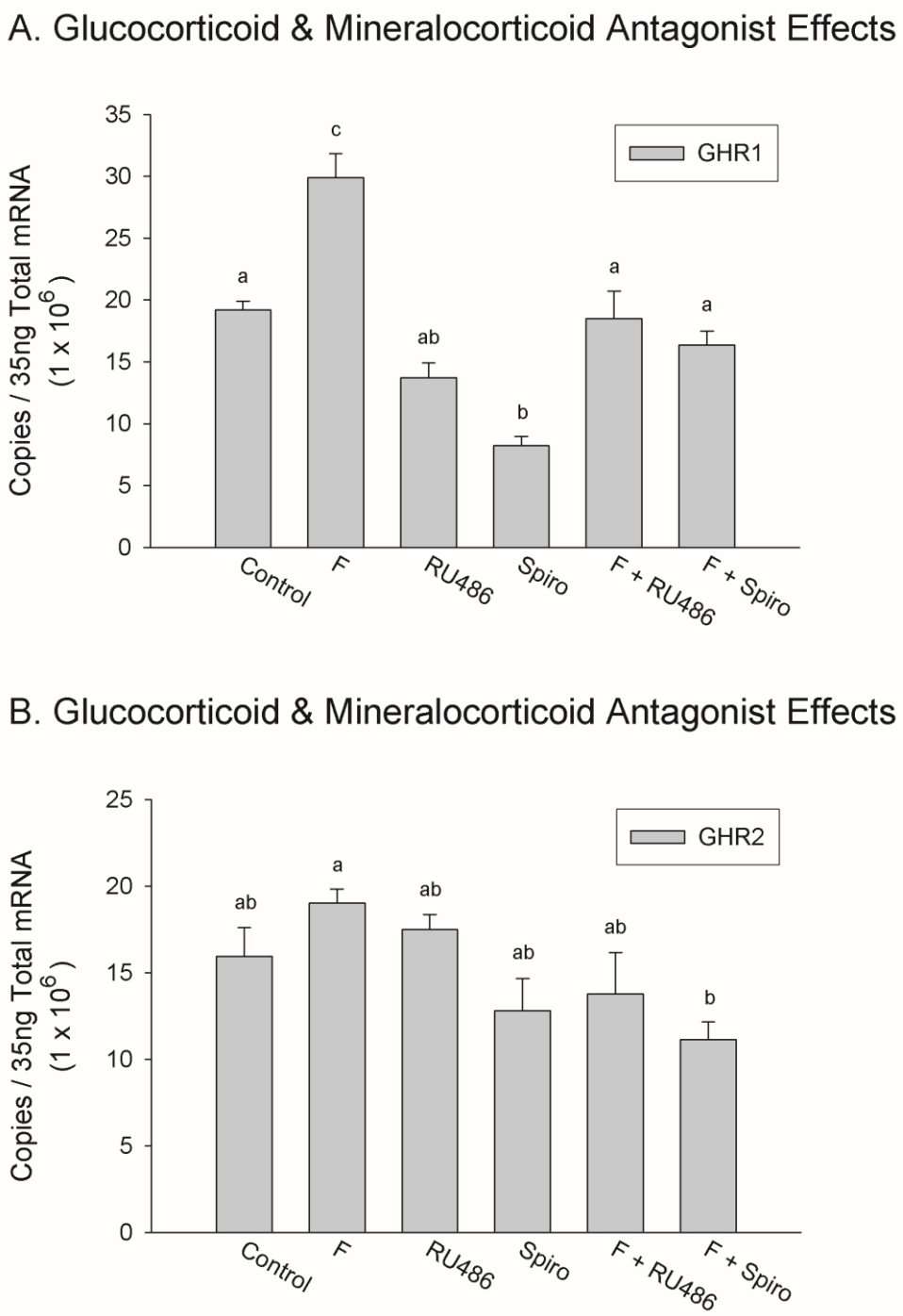

Fig. 31. Effects of cortisol (F), glucocorticoid antagonist drug mifepristone (RU486), mineralocorticoid antagonist drug spironolactone (Spiro) or combinations of either F and RU486 or F and Spiro treatments on transcription of growth hormone receptors (GHRs), GHR1 (A) and GHR2 (B), mRNAs in liver tissue of *in vitro* cultures isolated from rainbow trout. Liver tissue pieces were pretreated with or without RU486 (1,000 ng/ml) or Spiro (10,000 ng/ml), for 4 hours, then treated with or without F (100 ng/ml) for 6 hours. Data are presented as mean ± SEM. (n = 5-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

Inhibition experimentations with cortisol and cortisol antagonists were also conducted in gill filaments and found to influence GHR1 and GHR2 transcription of mRNAs. Cortisol treatment did not produce a significant difference in mRNA expression levels relative to control levels in either GHR1 or GHR2 (Fig. 32A & 32B). A joint treatment of F and Spiro resulted in GHR1 mRNA levels significantly diminished relative to untreated gill filaments (Fig. 32A). However, a Spiro treatment alone did not produce a difference in expression of GHR1 mRNAs relative to any treatment group. Similarly, a significant depression of steady-state GHR2 mRNAs was noted with a treatment of F + Spiro when compared to control levels or those treated with F (Fig. 32B). Treatment with RU486 alone or in conjunction with F did not significantly modify GHR1 or GHR2 mRNA expression levels relative to their respective control groups (Fig. 32A & 32B).

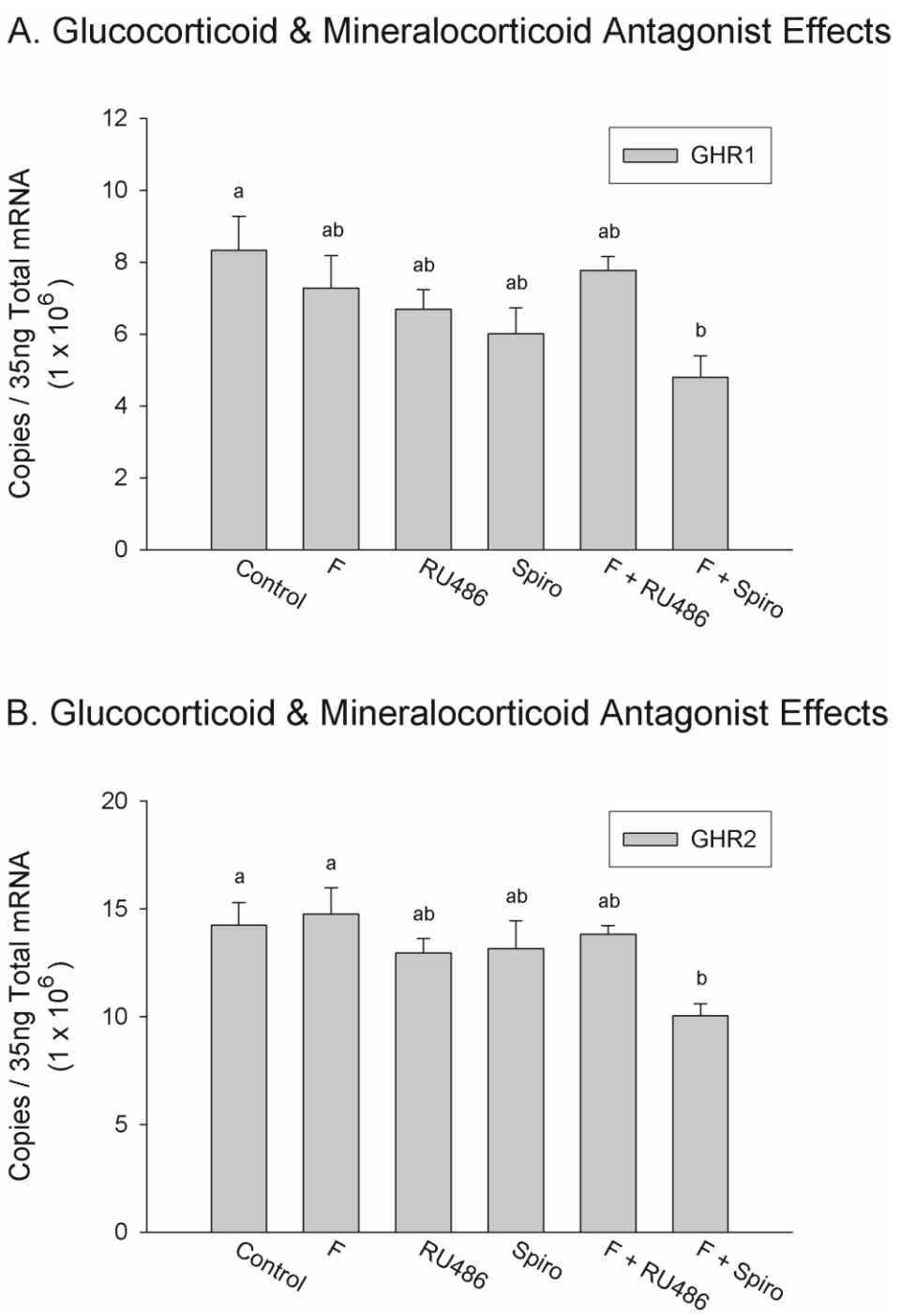

Fig. 32. Effects of cortisol (F), glucocorticoid antagonist drug mifepristone (RU486), mineralocorticoid antagonist drug spironolactone (Spiro) or combinations of either F and RU486 or F and Spiro treatments on transcription of growth hormone receptors (GHRs), GHR1 (A) and GHR2 (B), mRNAs in gill filaments of *in vitro* cultures isolated from rainbow trout. Gill filaments were pretreated with or without RU486 (1,000 ng/ml) or Spiro (10,000 ng/ml), for 4 hours, then treated with or without F (100 ng/ml) for 6 hours. Data are presented as mean ± SEM. (n = 5-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFs

Various treatments with F and two disparate antagonists were implemented to validate that F has the capability to regulate IGF production in hepatic and gill filament tissues. Administration of treatments with F and selected antagonists were significant in influencing IGF1 and IGF2 mRNA expression levels in hepatic tissue. Specifically, F treatment did not alter expression in IGF1 mRNAs (Fig. 33A). In addition, no observable changes in steady-state IGF1 mRNAs resulted after treatment with antagonists. A significant inhibition of hepatic IGF1 mRNA expression did occur with a joint treatment of F and Spiro when compared to F-treated and untreated groups (Fig. 33A). Contrary to results seen with IGF1 mRNAs, treatment with F produced an increase in expression of hepatic IGF2 mRNAs relative to control levels (Fig. 33B). However, all remaining treatment groups used to examine influences on steady-state IGF2 mRNAs resulted in no significant differences between F-treated or untreated control groups (Fig. 33B).

IGF1 or IGF2 production was not significantly influenced in gill filaments by F or any combination of antagonistic treatments (Fig. 34A & 34B).

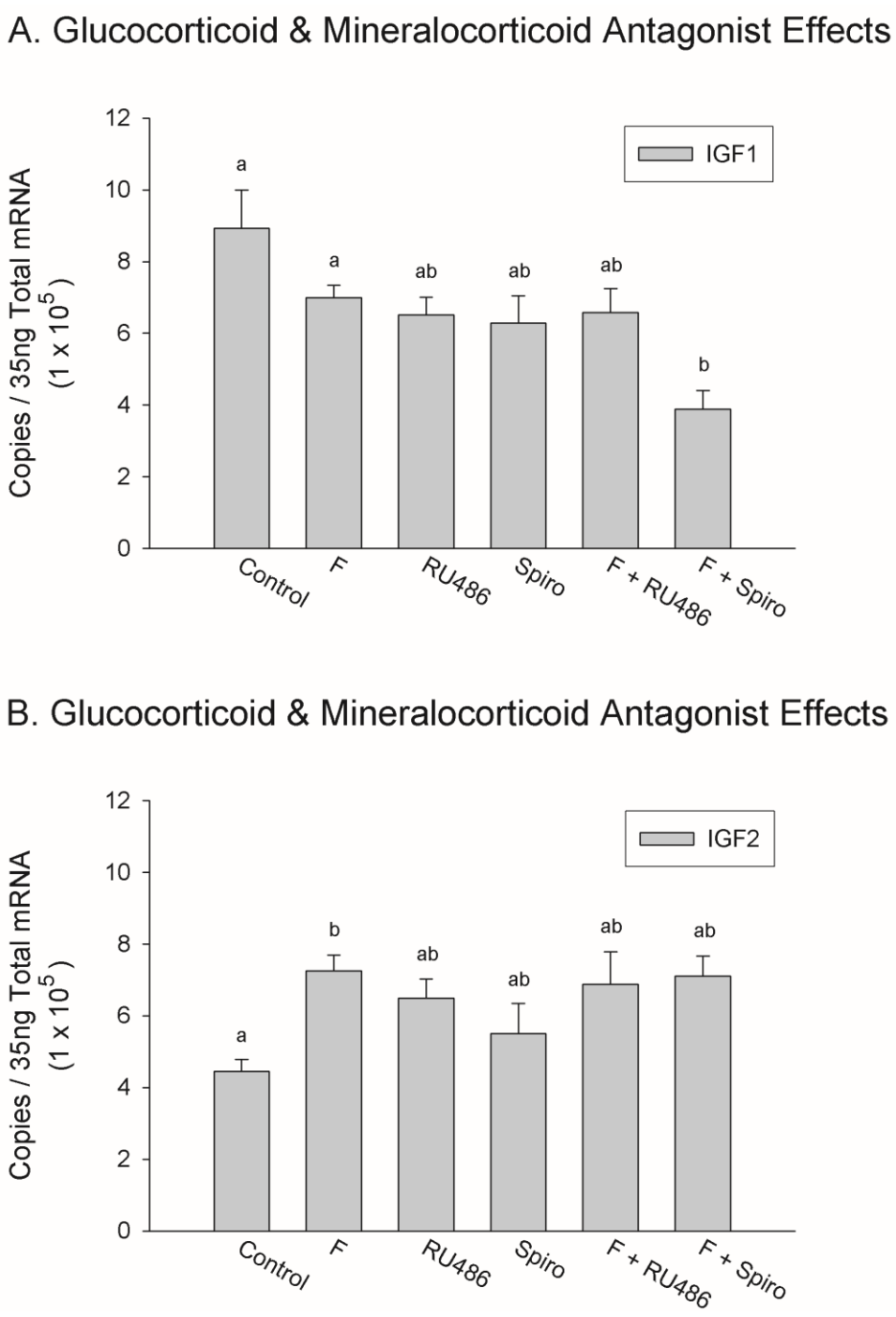

Fig. 33. Effects of cortisol (F), glucocorticoid antagonist drug mifepristone (RU486), mineralocorticoid antagonist drug spironolactone (Spiro) or combinations of either F and RU486 or F and Spiro treatments on transcription of insulin-like growth factors (IGFs), IGF1 (A) and IGF2 (B), mRNAs in liver tissue of *in vitro* cultures isolated from rainbow trout. Liver tissue pieces were pretreated with or without RU486 (1,000 ng/ml) or Spiro (10,000 ng/ml), for 4 hours, then treated with or without F (100 ng/ml) for 6 hours. Data are presented as mean ± SEM. (n = 5-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

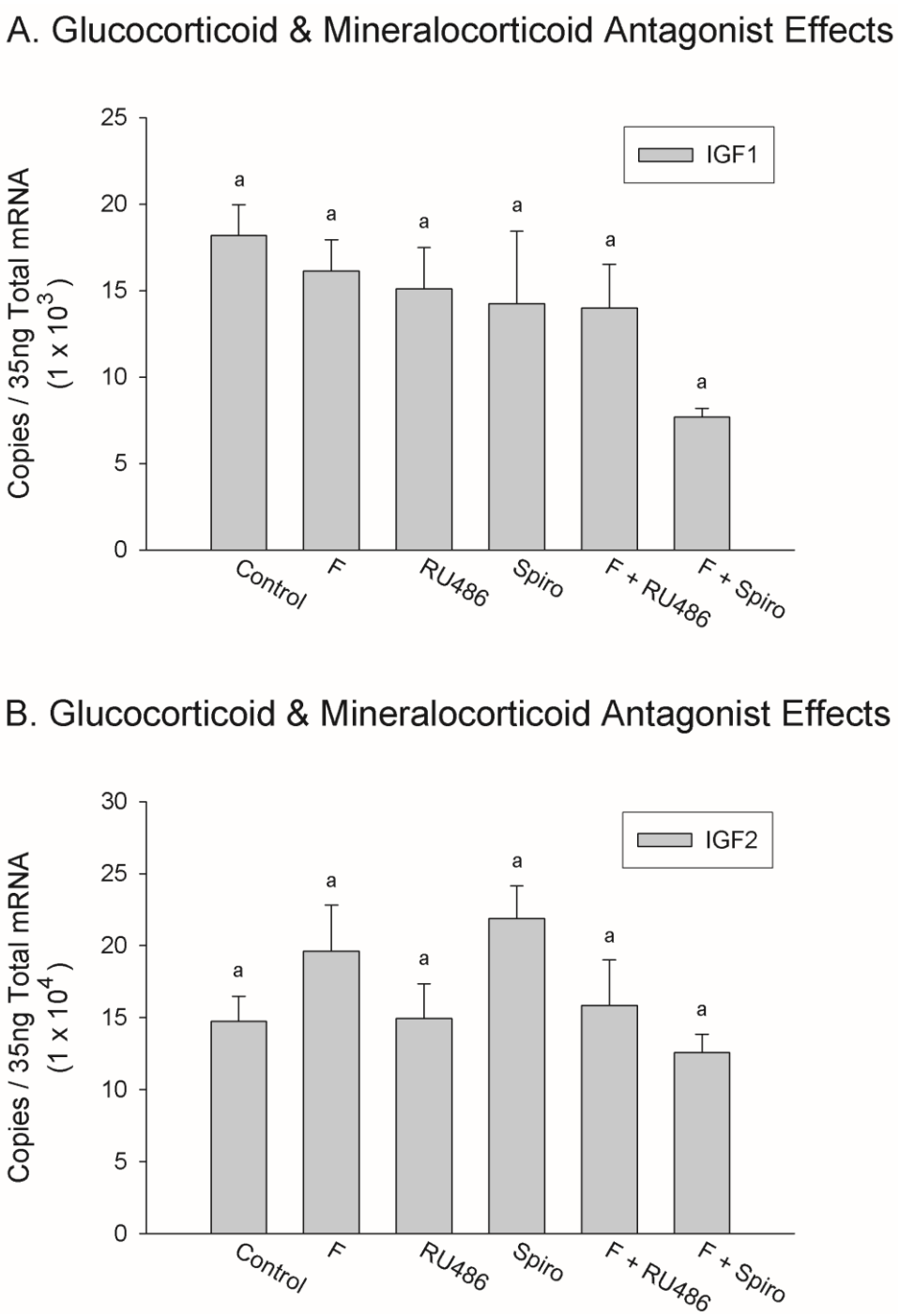

Fig. 34. Effects of cortisol (F), glucocorticoid antagonist drug mifepristone (RU486), mineralocorticoid antagonist drug spironolactone (Spiro) or combinations of either F and RU486 or F and Spiro treatments on transcription of insulin-like growth factors (IGFs), IGF1 (A) and IGF2 (B), mRNAs in gill filaments of *in vitro* cultures isolated from rainbow trout. Gill filaments were pretreated with or without RU486 (1,000 ng/ml) or Spiro (10,000 ng/ml), for 4 hours, then treated with or without F (100 ng/ml) for 6 hours. Data are presented as mean ± SEM. (n = 5-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFRs

Gill filaments were utilized to confirm F and two disparate antagonists in their ability to influence IGF sensitivity. Various treatments with F and antagonists resulted in significant effects with IGFR1A and IGFR1B mRNA expression levels. Cortisol and RU486 treatment did not significantly modify IGFR1A mRNA levels relative to control levels. Although, a Spiro treatment significantly reduced IGFR1A mRNA expression levels relative to the F-treated group (Fig. 35A). An even greater significant decrease in IGFR1A mRNA expression was seen with a joint treatment of F plus Spiro relative to untreated or F-treated gill filaments (Fig. 35A). Like IGFR1A mRNAs, F did not have a significant effect in modifying steady-state IGFR1B mRNAs. Suppression of IGFR1B mRNA expression resulted when filaments were treated with either RU486 or Spiro relative to untreated or F-treated groups (Fig. 35B). Treatments with F + RU486 or F + Spiro also significantly diminished IGFR1B mRNA expression levels relative to control and F-treated groups. However, these treatment groups were not significantly different from their corresponding inhibitors without F (Fig. 35B).

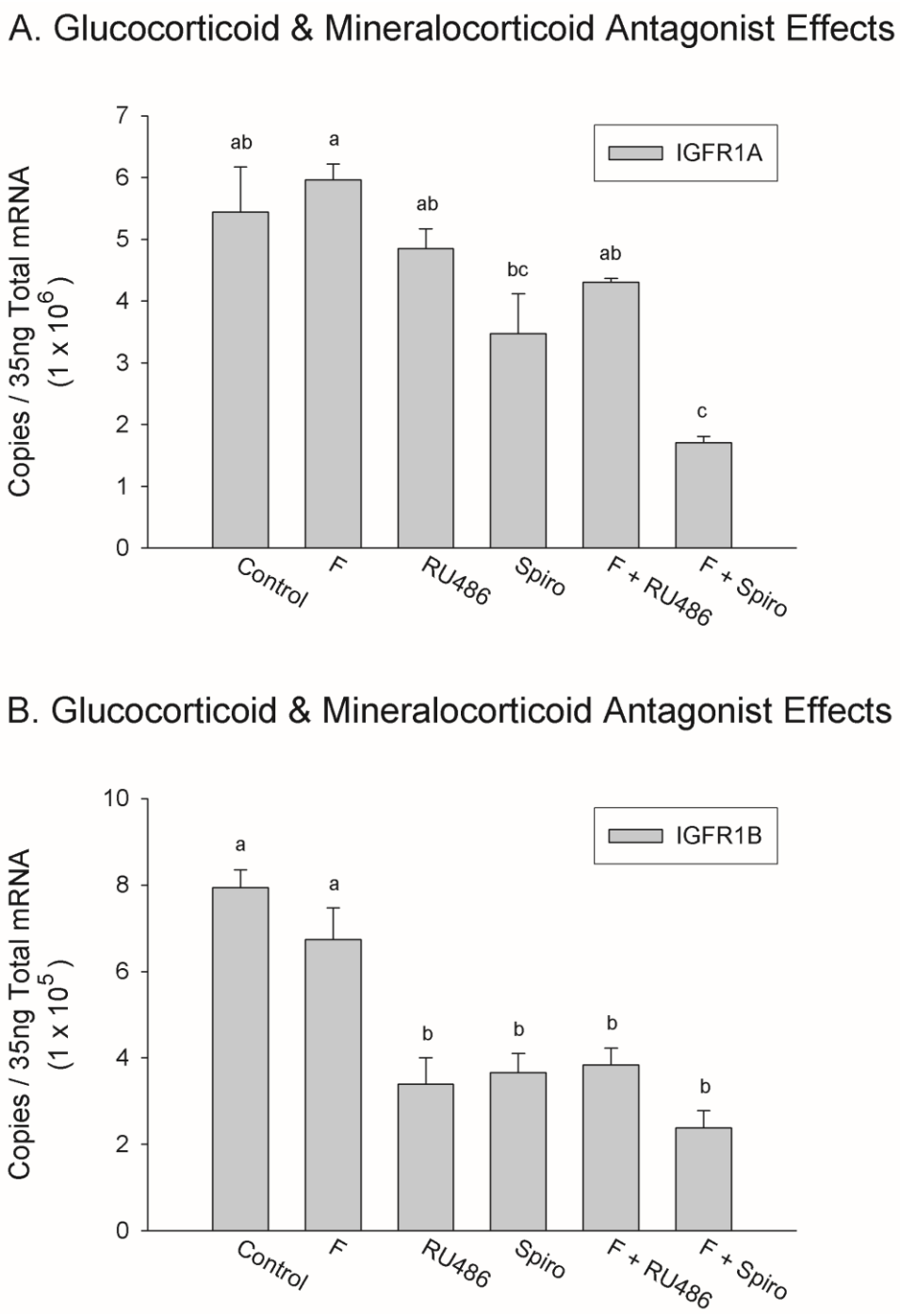

Fig. 35. Effects of cortisol (F), glucocorticoid antagonist drug mifepristone (RU486), mineralocorticoid antagonist drug spironolactone (Spiro) or combinations of either F and RU486 or F and Spiro treatments on transcription of insulin-like growth factor receptors (IGFRs), IGFR1A (A) and IGFR1B (B), mRNAs in gill filaments of *in vitro* cultures isolated from rainbow trout. Gill filaments were pretreated with or without RU486 (1,000 ng/ml) or Spiro (10,000 ng/ml), for 4 hours, then treated with or without F (100 ng/ml) for 6 hours. Data are presented as mean ± SEM. (n = 4-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

Combination Study of GH and Cortisol

GHRs

GH sensitivity to various treatments of GH and F were examined in hepatic and gill filament tissue cultures. Treatments applied to hepatic tissue were significant in influencing GHR1 and GHR2 mRNA expression levels. GH stimulated the expression of GHR1 mRNAs relative to the control group (Fig. 36A). Also, an equivalent increase in GHR1 mRNAs was seen after a treatment with F (Fig. 36A). Even though an increase in GHR1 mRNA expression was seen after only GH or F treatment, a significant reduction in GHR1 mRNA expression occurred after an amalgamation of GH + F (Fig. 36A). Like GHR1, GH stimulated the hepatic expression of GHR2 mRNAs relative to the control group (Fig. 36B). However, a treatment with F resulted in similar levels seen by the control group. An amalgamation with GH + F brought about a significant reduction in GHR2 mRNAs relative to all treatment groups (Fig. 36B).

Experiments with gill filaments and various treatments with GH and F had no effect on GHR1 or GHR2 mRNA expression (Fig. 37A & 37B).

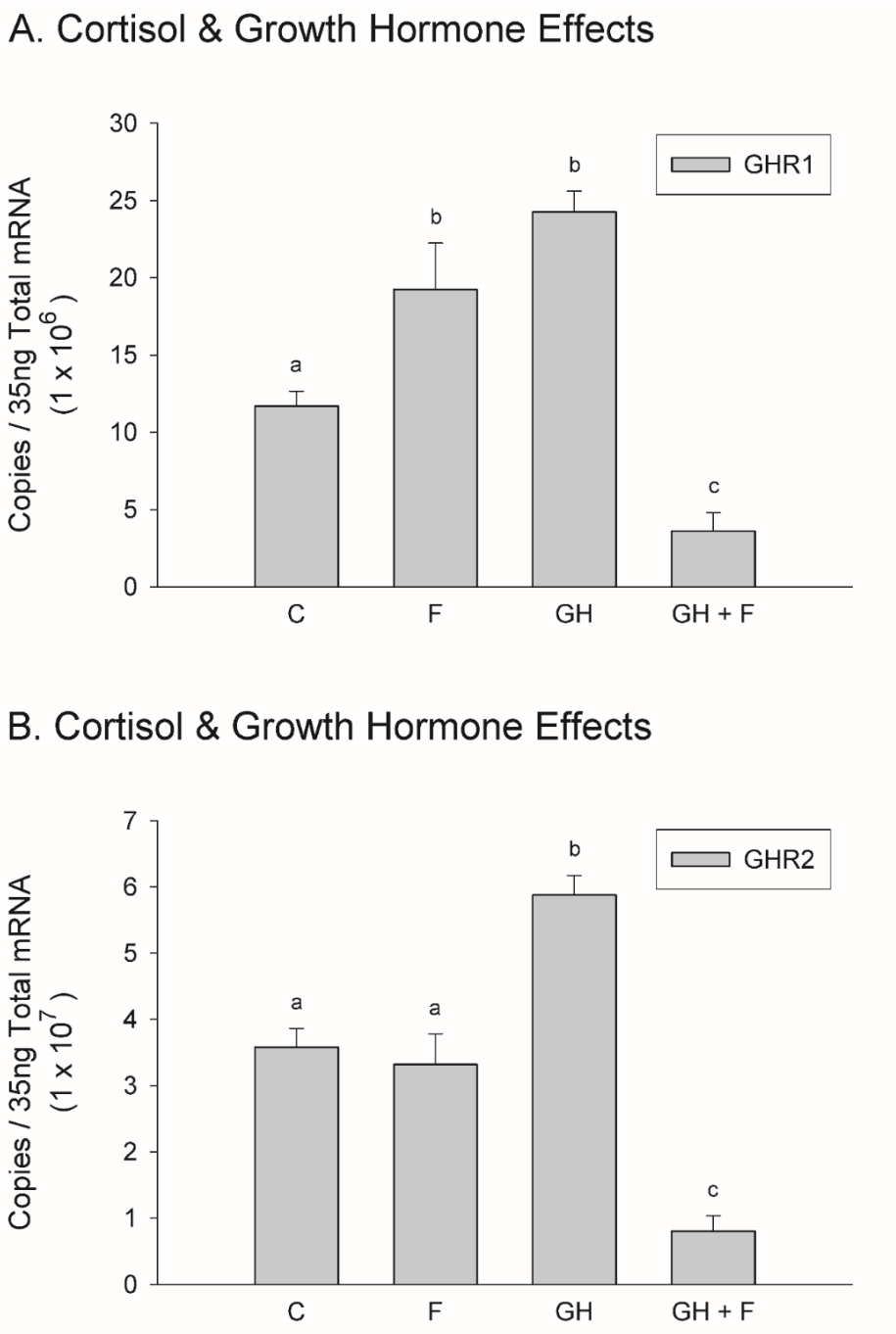

Fig. 36. Effects of cortisol (F), growth hormone (GH) or a combination of GH and F hormone treatments on growth hormone receptors (GHRs), GHR1 (A) or GHR2 (B), mRNA expression in liver tissue of *in vitro* cultures isolated from rainbow trout. Liver tissue pieces were incubated for 6 hours in medium alone (no hormone, control; C), medium containing F (100 ng/ml), medium containing salmonid growth hormone (sGH; 100 ng/ml), or medium containing sGH (100 ng/ml) and F (100 ng/ml); in combination treatments, sGH and F were added simultaneously. Data are presented as mean ± SEM. (n = 5-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

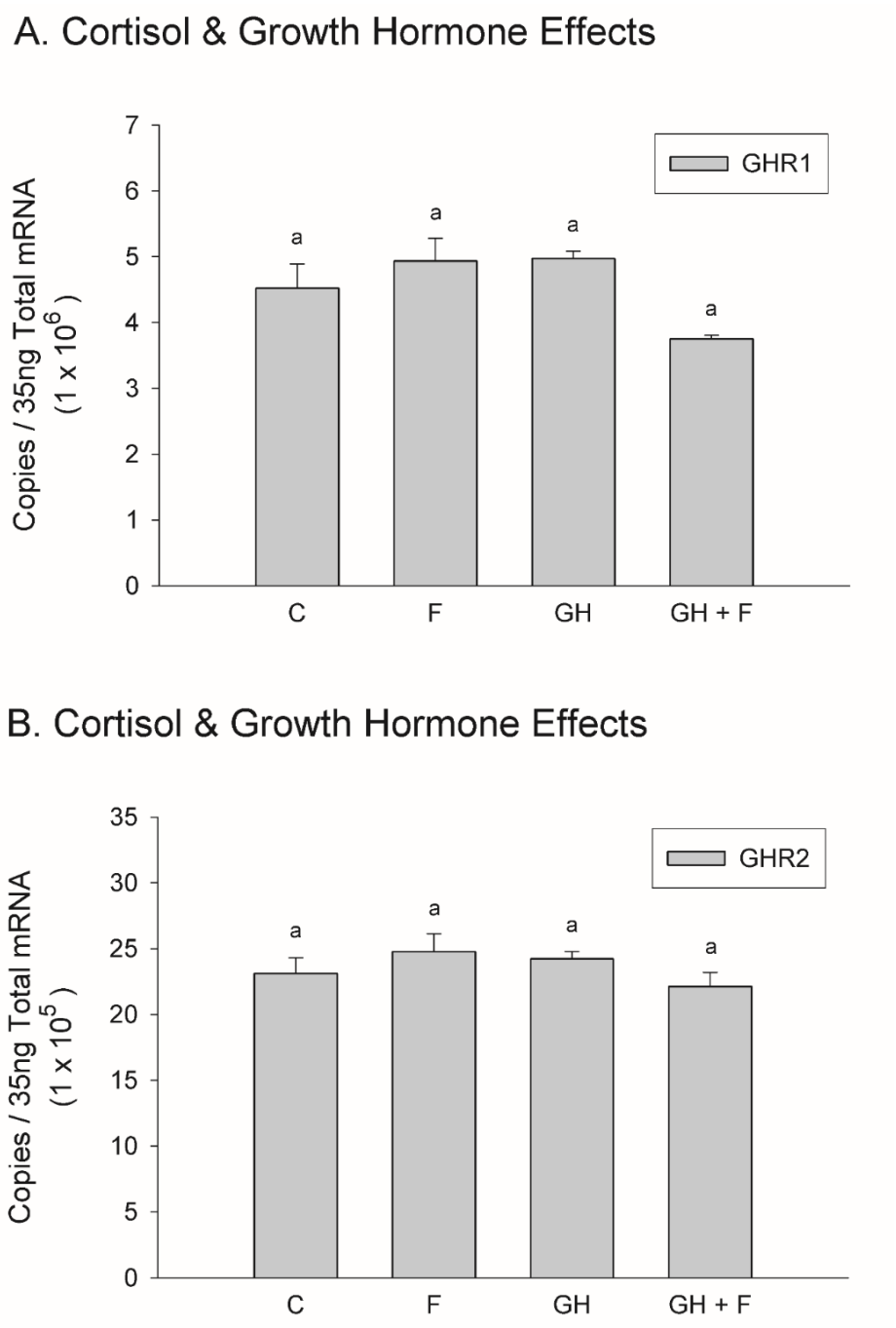

Fig. 37. Effects of cortisol (F), growth hormone (GH) or a combination of GH and F hormone treatments on growth hormone receptors (GHRs), GHR1 (A) or GHR2 (B), mRNA expression in gill filaments of *in vitro* cultures isolated from rainbow trout. Gill filaments were incubated for 6 hours in medium alone (no hormone, control; C), medium containing F (100 ng/ml), medium containing salmonid growth hormone (sGH; 100 ng/ml), or medium containing sGH (100 ng/ml) and F (100 ng/ml); in combination treatments, sGH and F were added simultaneously. Data are presented as mean ± SEM. (n = 4-6). For a given GHR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFs

Production of IGFs were evaluated with various *in vitro* treatments of GH and F in hepatic and gill filament tissue cultures. Hepatic tissue dosed with different treatments of GH and F were statistically significant in influencing steady-state IGF1 and IGF2 mRNA levels. GH stimulated the mRNA expression levels of IGF1 over control levels, while a F treatment kept mRNA expression levels of IGF1 relative to the control group (Fig. 38A). In addition, IGF1 mRNA expression levels were extremely suppressed when treated with an amalgamation of GH and F (Fig. 38A). Unlike IGF1 mRNA expression levels, hepatic IGF2 mRNA expression levels significantly increased over control levels after a treatment with F (Fig. 38B). Yet, steady-state IGF2 mRNAs remained around control levels after a treatment with GH (Fig. 38B). An amalgam of GH and F treatment produced comparable IGF2 mRNA expression levels seen by the control group. However, IGF2 mRNA levels were significantly reduced when compared to hepatic tissue treated with F or GH alone (Fig. 38B).

In gill filaments, various treatments with GH and F were significant in influencing IGF1 mRNA expression levels but was not significant in regulating IGF2 mRNA expression levels (Fig. 39B). Single treatments with F or GH resulted in comparable IGF1 mRNA levels to those seen by the control group. Gill filaments treated with an amalgam of GH and F produced a significant decrease in steady-state IGF1 mRNAs relative to untreated and GH-treated groups (Fig. 39A).

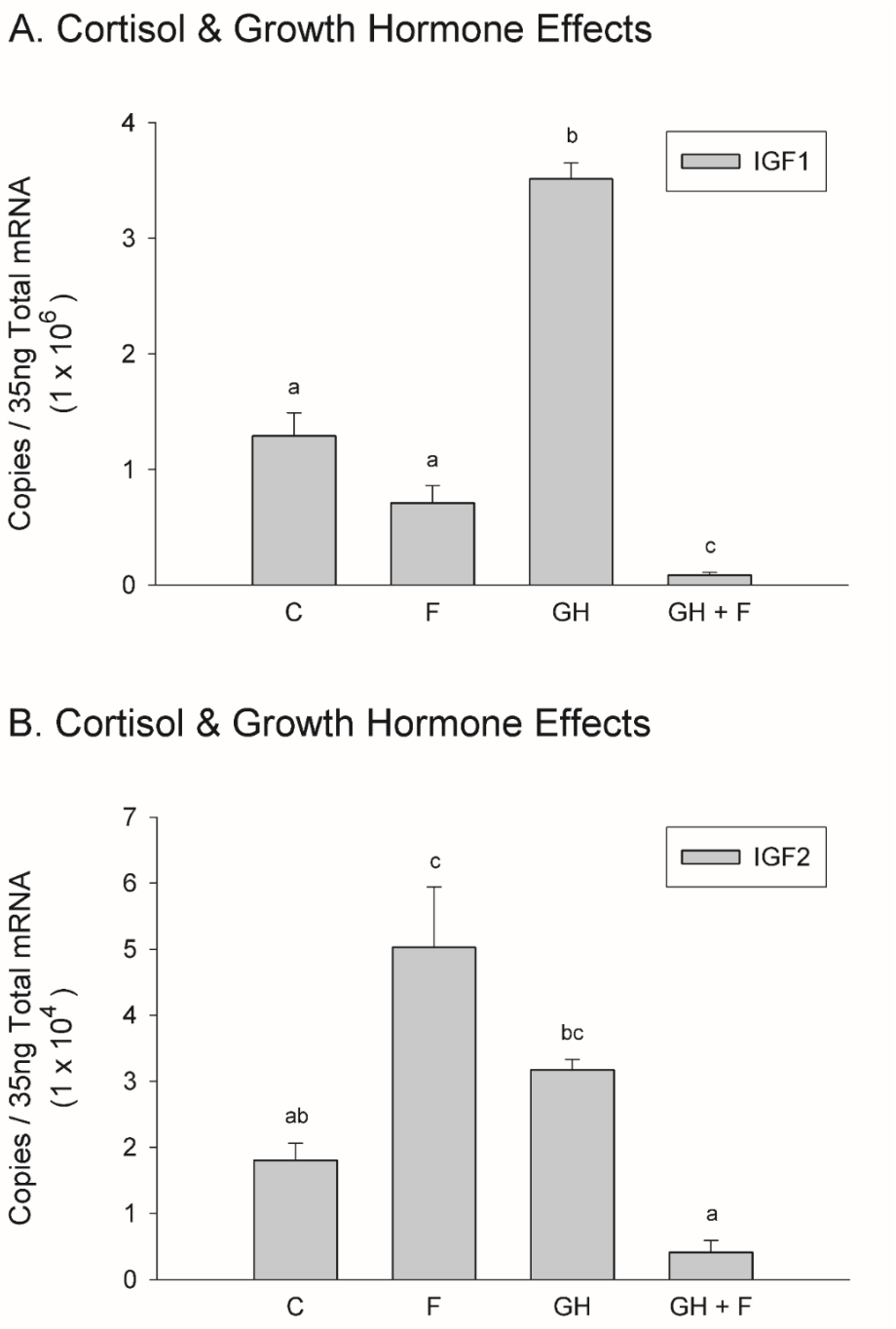

Fig. 38. Effects of cortisol (F), growth hormone (GH) or a combination of GH and F hormone treatments on insulin-like growth factors (IGFs), IGF1 (A) or IGF2 (B), mRNA expression in liver tissue of *in vitro* cultures isolated from rainbow trout. Liver tissue pieces were incubated for 6 hours in medium alone (no hormone, control; C), medium containing F (100 ng/ml), medium containing salmonid growth hormone (sGH; 100 ng/ml), or medium containing sGH (100 ng/ml) and F (100 ng/ml); in combination treatments, sGH and F were added simultaneously. Data are presented as mean ± SEM. (n = 5-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

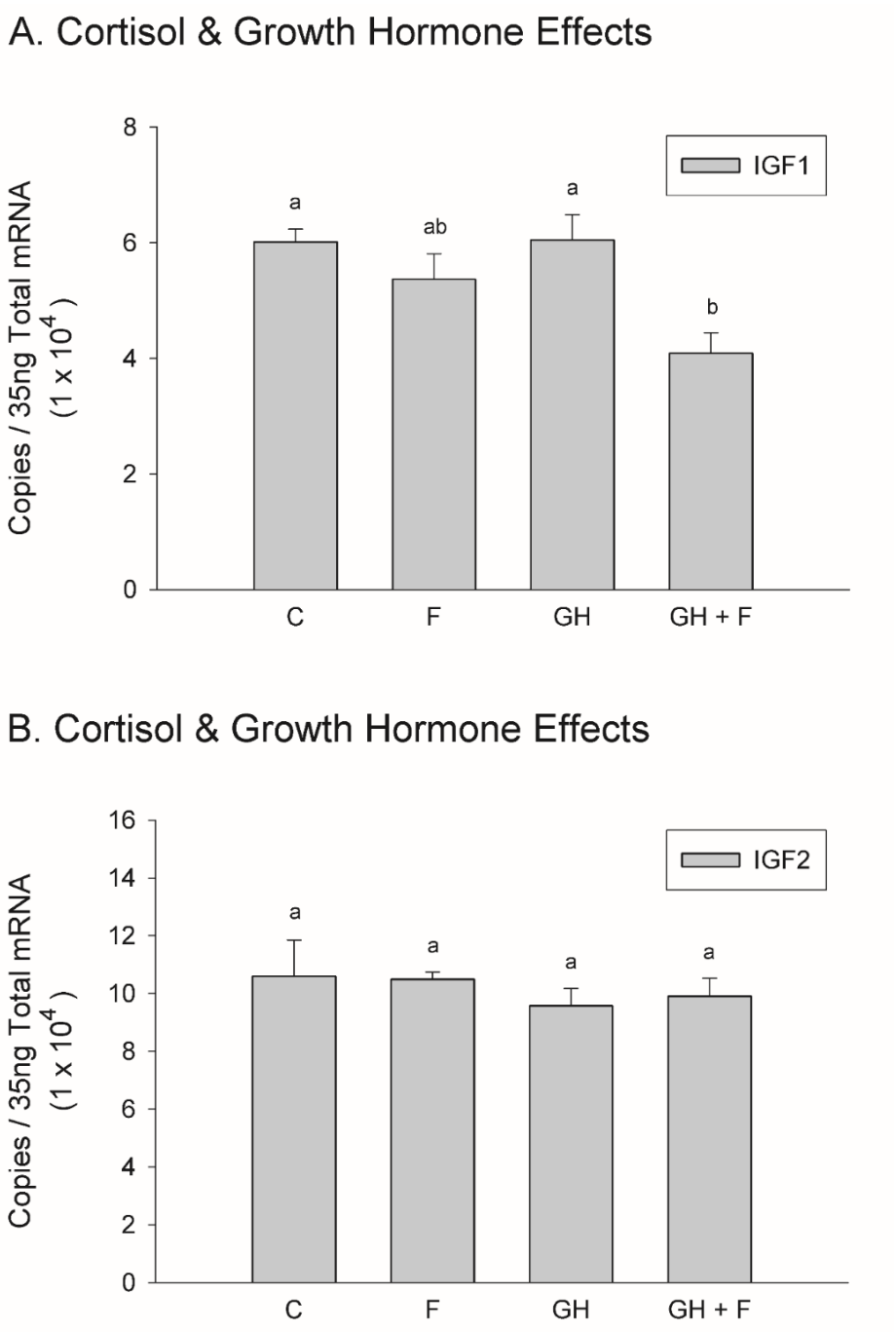

Fig. 39. Effects of cortisol (F), growth hormone (GH) or a combination of GH and F hormone treatments on insulin-like growth factors (IGFs), IGF1 (A) or IGF2 (B), mRNA expression in gill filaments of *in vitro* cultures isolated from rainbow trout. Gill filaments were incubated for 6 hours in medium alone (no hormone, control; C), medium containing F (100 ng/ml), medium containing salmonid growth hormone (sGH; 100 ng/ml), or medium containing sGH (100 ng/ml) and F (100 ng/ml); in combination treatments, sGH and F were added simultaneously. Data are presented as mean ± SEM. (n = 4-6). For a given IGF subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

IGFRs

Direct effects on IGF sensitivity with various treatments of GH and F were investigated with *in vitro* gill filament tissue cultures. Treatments delivered to gill tissue were not significant in influencing IGFR1A mRNA expression levels (Fig. 40A) but were significant in influencing IGFR1B mRNA expression levels. Steady-state IGFR1B mRNAs were significantly reduced relative to the control group after an amalgamation treatment of GH and F (Fig. 40B). Single treatments of F or GH produced comparable IGFR1B mRNA expression levels seen by the control and GH + F groups.

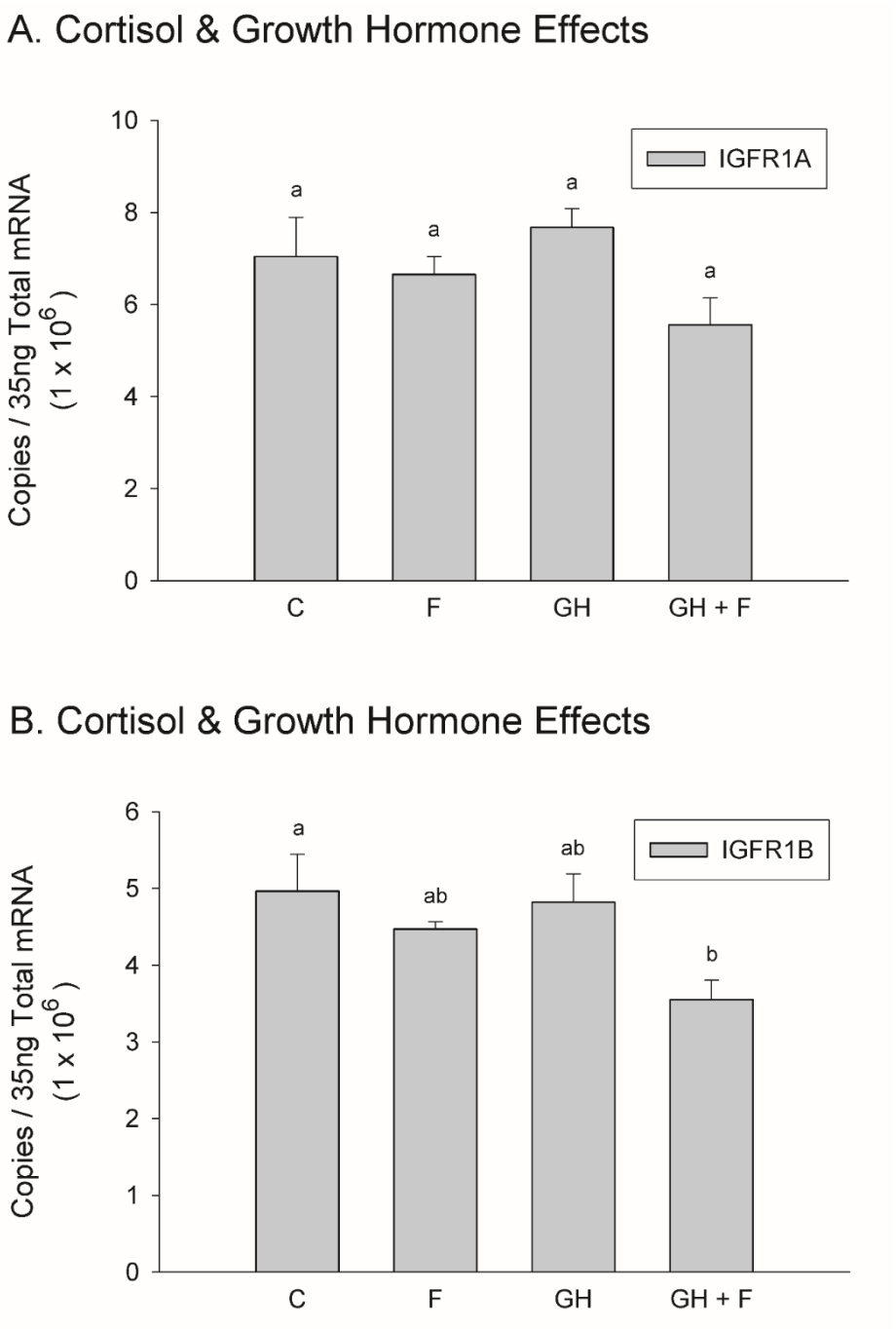

Fig. 40. Effects of cortisol (F), growth hormone (GH) or a combination of GH and F hormone treatments on insulin-like growth factor receptors (IGFRs), IGFR1A (A) or IGFR1B (B), mRNA expression in gill filaments of *in vitro* cultures isolated from rainbow trout. Gill filaments were incubated for 6 hours in medium alone (no hormone, control; C), medium containing F (100 ng/ml), medium containing salmonid growth hormone (sGH; 100 ng/ml), or medium containing sGH (100 ng/ml) and F (100 ng/ml); in combination treatments, sGH and F were added simultaneously. Data are presented as mean ± SEM. (n = 5-6). For a given IGFR subtype, treatment groups with different letters are significantly ($P < 0.05$) different from each other.

Discussion

These studies provided an examination of the cortisol influences on the GH-IGF system in a teleost fish. The findings reveal that cortisol has direct effects in differentially influencing specific elements of the GH-IGF system and support my starting hypothesis that cortisol can directly modulate the expression of genes controlling for growth. Peripheral cortisol influences on GH and IGF sensitivity and on peripheral IGF production provide an important mechanism in integrating growth with the reallocation or energy toward intermediary metabolism events.

The ability of F to influence peripheral aspects of the GH-IGF system was supported by a few observations. First, F directly increased the expression of GHR1 mRNA expression but had no direct influence on GHR2 mRNA expression in liver tissue. Secondly, F directly increased expression of IGF2-encoding mRNAs but had no influence on IGF1-encoding mRNAs in the liver tissue. Finally, in general, F did not have consistent direct influences on GHRs or IGFs or IGFR1s mRNAs in gill filament tissue. Taken together, these findings indicate F increases peripheral responsiveness to GH as well as peripheral production of IGFs. However, such effects appear to be tissue-specific (liver having an increased response but gill no response) and isoform-specific (increased mRNA expression for hepatic GHR1 and hepatic IGF2 but no change in mRNA expression of hepatic GHR2 and hepatic IGF1).

My findings are consistent with what has previously been reported in the field. Previous research has shown no difference in mRNAs encoding GHR2 but a significant increase in GHR1 mRNA abundance after black sea bream and rainbow trout hepatic tissues were treated with F (Jiao et al., 2006; Philip & Vijayan, 2015). It was also reported that a synthetic glucocorticoid agonist, dexamethasone, significantly increased GHR mRNA expression levels, however, which specific GHR isoform was not indicated (Pierce et al., 2005). As mentioned earlier, it was

reported that a high dose of cortisol treatment resulted in an increase of pituitary GH mRNA expression (Peterson & Small, 2005). Utilizing a rainbow trout pituitary cell-line, dexamethasone treatment also stimulated the accumulation of GH mRNA (Yang et al., 1994). Also, plasma GH levels were found to significantly rise after cortisol treatments (Nishioka et al., 1985; Uchida et al., 2004). This is plausible since a consensus sequence for the glucocorticoid response element (GRE) has been characterized within the promotor region of both rainbow trout GH genes (Yang et al., 1997).

Why cortisol would cause an increase in the production and release of GH is not entirely clear. Also, I observed in my experiments that the steady-state mRNA expression levels of IGF1 did not significantly change relative to the control groups after a cortisol treatment, except for the supraphysiological dose of 500 ng/ml in the dose experiment. Previous experiments with catfish (*Ictalurus punctatus*; Peterson & Small, 2005), silver sea bream (*Sparus sarba*; Leung et al., 2008), tilapia (*Oreochromis mossambicus*; Pierce et al., 2011) and rainbow trout (*Oncorhynchus mykiss*; Philip & Vijayan, 2015) have stated that treatment with cortisol either significantly decreased or did not change mRNAs-encoding for IGF1 relative to the control groups. In the GH-IGF system, IGF1 is a primary mediator that induces GH effects. However, I and others have shown that GH actions in producing IGF1 are hindered when F treatment is applied. This could be due to 1) GH being bound to GHBP (Einarsdottir et al., 2014) or 2) the knockdown of GH's signaling pathway, halting the production of IGF1 (Reindl et al., 2011). Philip and Vijayan (2015) make a compelling proposal for the mechanism by which F attenuates hepatic GH signaling. During stress, cortisol plasma levels rise and the increased concentrations have been correlated to a reduction in growth (McCormick et al., 1998) as well as suppression of the immune response (Tort, 2011). The proposed mechanism of F action on growth links the stress

effects on the growth system in cross-talk communication with the immune system (Philip & Vijayan, 2015). The key protein(s) that link the two systems together are collectively known as suppressors of cytokine signaling (SOCS). It was shown by them, that cortisol upregulates the transcription of SOCS-1 and SOCS-2 (cf. Philip et al., 2012), which in turn prevents STAT5 phosphorylation and decreases the total protein expression of JAK2 (Philip & Vijayan, 2015). Previously, from our lab, GH's signaling mechanism in trout was found to activate three different pathways [extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt), and signal transducer and activator of transcription 5 (STAT5) pathways] once GH bound to its receptor and recruited and activated the required tyrosine kinase known as Janus kinase 2 (JAK2) (Reindl et al., 2011).

In addition, in my studies, GH produced a notable increase of IGF1 mRNA expression but when GH and F were combined as a treatment, IGF1 mRNA expression was nearly blocked completely. Significant decreases in mRNAs encoding for IGF1 after a treatment of GH and F were also observed previously with *in vivo* and *in vitro* studies with hepatocytes or hepatic tissue (Kajimura et al., 2003; Pierce et al., 2011).

Even though IGF2 has been identified in fish for some time, it was thought (since IGF2 mRNA expression levels were high during embryogenesis) that IGF2 actions were strictly involved in embryonic tissue growth and not postnatal growth (Jones & Clemmons, 1995; Wood et al., 2005). In my experiments IGF2 mRNA expression levels consistently increased with treatment of F. To the best of my knowledge, this is the first report of a direct increase in hepatic IGF2 mRNA expression with a treatment of F. However, treatment with the glucocorticoid agonist Dex (dexamethasone) produced a significant increase in hepatic IGF2 transcripts within coho salmon (Pierce et al., 2010). Contrary to my results, it was shown that hepatic IGF2

mRNA expression of tilapia decreased with increasing concentrations of F treatment (Pierce et al., 2011). In my time-course study, hepatic IGF2 mRNA expression levels did steadily decrease with longer treatment times.

A couple of reasons Pierce and colleagues saw a decline in tilapia hepatic IGF2 mRNA expression might have been due to the culture techniques and amount of time for preparation before implementing pre-incubation and selected hormone incubation times. Or, it could be merely due to a difference seen among different species of fish. Pierce et al.'s (2011) culturing technique was to cannulate the liver, dissect and digest the tissue, mash the cut up liver pieces through a filter, a 1-2 hour settling period, 4 hours of pre-incubation, and a 6 hour treatment with hormone, thus, equaling around ~14 hours total. My technique was to dissect liver out of fish, cut them up into smaller pieces, preincubate for 3 hours, and treat in most cases for 6 hours (except for the time course experiments which were longer) for a total of ~11-12 hours. Could a difference of only around 2 hours be enough to cause a difference increase in steady-state mRNA expression versus a decrease?

Along other lines, in my combination experiment F treatment induced hepatic IGF2 mRNA expression. Also, within the combination study GH treatment induced hepatic IGF2 mRNA expression, but slightly although not significantly, less than F treatment. Surprisingly, treatment together with both of these hormones significantly and dramatically diminished IGF2 transcript levels. In a close relative of rainbow trout (*Oncorhynchus mykiss*), Coho salmon (*Oncorhynchus kisutch*), a treatment of Dex or GH alone stimulated IGF2 mRNA expression levels to the same degree (Pierce et al., 2010). Furthermore, a synergistic significantly induced effect in IGF2 mRNA expression levels to a greater extent was noted during a treatment of Dex + GH (Pierce et al., 2010). In another fish species, Mozambique tilapia F treatment did not have

any effect on hepatic IGF2 encoding mRNAs, while GH treatment significantly elevated IGF2 encoding mRNAs (Pierce et al., 2011). Again, when F and GH were used in treating as a combination, they acted in a synergistic manner to significantly elevate IGF2 mRNA expression levels even more over the elevated levels observed by GH treatment alone (Pierce et al., 2011). My results are the first results to report an attenuation in hepatic IGF2 mRNA expression after a combination treatment of GH + F. Faught and Vijayan, (2016) proposed in a review paper that the upregulation of hepatic GHR1 transcripts by F is important for IGF2 signaling. My results confirm that interpretation. I further propose that the upregulation of GHR1 mRNAs is through a cell signaling pathway independent of the JAK-STAT pathway in the absence of GH. However, there might be more occurring when GH and F are present at the same time. I believe there are many open-ended points that need to be further tested.

Regarding the gills, I am unsure of what is occurring except that during my inhibitory experiments glucocorticoid or mineralocorticoid antagonists were equally potent in significantly diminishing IGFR1B transcripts in gill filaments. Since this was the only case in which this was observed I can say these antagonists have a direct effect specifically on this receptor of the GH-IGF system. However, what it means I have no clue.

Regarding the inhibitory experiments, my thoughts for conducting it were that maybe I there would be differences in how cortisol action is downregulated between the two tissues, glucocorticoid antagonist downregulating F actions more strongly in the liver and mineralocorticoid antagonist downregulating F actions more strongly in the gill. However, this was not the case. The only times I saw any significant antagonistic action by the glucocorticoid was in the gill and influences on the transcription rate for IGFR1B. On the other hand, a significant antagonistic action by the mineralocorticoid antagonist was observed in

downregulating 1) hepatic GHR1 mRNA expression and 2) transcription of IGFR1B mRNAs in the gill filaments, with only a slight insignificant decrease in IGFR1A mRNA expression in gill filaments. In addition, decreases in receptor genes (GHRs and IGFRs) in the gill filaments were noted (with GHR1 being slightly insignificant) with a combined treatment of F plus the mineralocorticoid Spiro. Interaction between F and Spiro in specifically inhibiting receptor transcription levels and not hormone transcription levels (IGFs) of gill tissue specifically is an interesting find, even though I cannot interpret it at this point. In liver tissue, I saw a significant decrease in mRNA transcripts in both receptor genes for GH (GHRs) and with IGF1 gene when F and Spiro were used as a conjunct-treatment. Overall, to me these results indicate that a mineralocorticoid might be the main target for cortisol's diminishing of growth gene effects. However, this has to be taken with caution due to at least three factors or reasons: 1) The different doses for RU486 and Spiro were 1,000 ng/ml and 10,000 ng/ml, respectively. Thus, Spiro has a 9-fold greater potency if you compare concentrations alone. 2) The preferential binding affinities of F to a glucocorticoid receptor versus a mineralocorticoid receptor are not clear, since cortisol could possibly act as a mineralocorticoid or a glucocorticoid. 3) Spiro has been used to examine possible mineralocorticoid receptor (MR) function in fish only recently. Further work on the receptor-ligand kinetics of Spironolactone acting as a specific blocker of corticosteroid receptor needs to be done in fish.

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CHAPTER 5: AN *IN VITRO* MODEL FOR EVALUATING PERIPHERAL REGULATION OF GROWTH IN FISH. EFFECTS OF 17β-ESTRADIOL AND TESTOSTERONE ON THE EXPRESSION OF GROWTH HORMONE RECEPTORS INSULIN-LIKE GROWTH FACTORS, AND INSULIN-LIKE GROWTH FACTOR TYPE 1 RECEPTORS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)[1]

Abstract

A central component of growth coordination in vertebrates is the growth hormone (GH)-insulin-like growth factor-1 (IGF-1) system. To date, most studies on the control of vertebrate growth have focused on regulation of pituitary GH production and release. In this study, we used liver, muscle, and gill tissue from sexually immature rainbow trout incubated *in vitro* to evaluate the extrapituitary effects of 17β-estradiol (E2) and testosterone (T) on mRNA and functional expression of growth hormone receptors (GHR), insulin-like growth factors 1 and 2 (IGF-1, IGF-2), and type 1 IGF receptors (IGFR1). E2 significantly decreased steady-state levels of GHR1, GHR2, and IGF-1 mRNAs in liver as well as of GHR1 and GHR2 mRNAs in muscle and of IGF-1 and IGF-2 mRNAs in gill in a time- and concentration-dependent manner. E2 had no effect on levels of IGFR1 mRNAs in muscle or on GHR and IGFR1 mRNAs in gill. Functional expression of GHRs as assessed by ^{125}I-GH binding capacity was reduced by E2 in liver and muscle; however, E2 did not affect ^{125}I-IGF-1 binding capacity in muscle or ^{125}I-GH and ^{125}I-IGF-1 binding capacity in gill. By contrast, T increased steady-state levels of GHR1, GHR2, IGF-1, and IGF-2 mRNAs in liver, of GHR1, GHR2, IGFR1A, and IGFR1B in muscle,

[1] This chapter has been previously published as follow: Norbeck, L.A., & Sheridan, M.A., 2011. *General and Comparative Endocrinology* 173, 270-280. As lead author, I wrote the manuscript, performed all the experiments, analyzed the data, and made all the figures for this chapter.

and of GHR1, GHR2, IGF-1, IGF-2, IGFR1A, and IGFR1B mRNAs in gill in a time- and concentration-dependent manner. Binding capacity of ^{125}I-GH in liver and of ^{125}I-GH and ^{125}I-IGF-1 in both muscle and gill also was increased by T. These data indicate that E2 and T directly affect peripheral aspects of the GH-IGF system, and suggest, at least in immature rainbow trout, that E2 reduces hepatic sensitivity to GH as well as reduces peripheral production of IGFs and that T increases peripheral sensitivity to GH and IGF as well as increases peripheral production of IGFs.

Introduction

Animal growth is influenced by genetic, environmental, and nutritional factors. Extrinsic factors, such as temperature, photoperiod and food availability, are particularly important in the growth of fish, including the triggering of developmental processes such as hatching, metamorphosis, smoltification, sexual maturation, and spawning (Bjornsson, 1997; McLean and Donaldson, 1993; Reinecke, 2010). Although most species of fish have the capacity to grow throughout their life (indeterminate growth), their rate of growth typically slows over time, especially just prior to sexual maturity when energy is channeled to gonad growth and gamete production (Enberg et al., 2008; Mommsen, 2001).

Integration of intrinsic and extrinsic factors results in modulation of the growth hormone (GH)-insulin-like growth factor (IGF) system. The GH-IGF system of fish is particularly complex given the existence of multiple receptors for GH (GHRs) and multiple type 1 IGF receptors (IGFR1), IGF-2 production that, unlike the situation in mammals, is sensitive to GH in postembryonic animals, and in some species a gonad-specific IGF (IGF-3) that also is regulated by GH (Berishvili et al., 2010; Pierce et al., 2010; Reinecke et al., 2005; Very et al., 2005).

Accumulating evidence suggests that sex steroids may influence the growth of fish by modulating components of the GH-IGF system (Carnevali et al., 2005). The observation that plasma levels of sex steroids increase during sexual maturation of fish, ranging from nondetectable to 60 ng/ml for E2 or to 160 ng/ml for T, and that these changes correlate with changes in plasma GH suggest that sex steroids regulate GH secretion (Baynes and Scott, 1985; Holloway et al., 1999; van Bohemen and Lambert, 1981). This notion is supported by some studies that showed that 17β-estradiol (E2) increased plasma GH in goldfish, rainbow trout, and tilapia (Holloway and Leatherland, 1997; Melamed et al., 1995; Trudeau et al., 1992); however, this response appears variable as E2 decreased plasma GH salmon in Atlantic salmon (Lerner et al., 2007). The effect of androgens also appears variable. Testosterone (T) increased plasma GH in goldfish, an effect that required aromatization of T to E2 (Canosa et al., 2002), as well as in fasted but not fed rainbow trout (Holloway and Leatherland, 1997); however, T and 11-ketotestosterone (11KT) had no effect on plasma GH in coho salmon (Larsen et al., 2004).

Data also indicate that sex steroids may act peripherally on aspects of the GH-IGF system. For example, in tilapia, elevated E2 levels did not correlate with high hepatic GHR mRNA expression (Kajimura et al., 2004). Furthermore, E2 *in vivo* inhibited hepatic expression GHR mRNAs in black sea bream (Jiao et al., 2006) as well as hepatic expression IGF-1 and IGF-2 mRNAs in gilthead sea bream (Carnevali et al., 2005). Moreover, plasma levels of IGF-1 were reduced by E2 immersion of Atlantic salmon (Lerner et al., 2007). In tilapia, the expression of GHR and IGF-1 was reduced in the liver but not in the testes of E2-treated males (Davis et al., 2008). Testosterone injection decreased the expression of GHR2 but not GHR1 mRNA in black sea bream (Jiao et al., 2006). In addition, T and 11KT implantation increased the expression of IGF-1 mRNA in coho salmon (Larsen et al., 2004). The variability of the response of GH-IGF

system components to sex steroids may result from differences in the stage of sexual maturation, nutritional status, or other environmental influence as well as from tissue- and species-specific differences reflective of the animal's evolutionary/life history.

In this study, we used an *in vitro* rainbow trout system to examine the influence of sex steroids on the GH-IGF system in order to clarify their endogenous actions and to provide greater insight into the coordination of growth and reproduction. The specific hypothesis of the study was that E2 and T directly modulate the expression of GHRs, IGFs, and of IGFR1s. Our focus was on the primary source of peripheral IGF-1/2 (liver) and the major growth responsive tissues (muscle, gill) [Gray et al., 1990; Parrizas et al., 1995; Poppinga et al., 2007; Very et al., 2005; Wood et al., 2005).

Materials and Methods

Animals

Juvenile rainbow trout of both sexes (91.4 ± 5.3 g) were obtained from Dakota Trout Ranch near Carrington, ND, and transported to North Dakota State University where they were maintained in 800-l circular tanks supplied with recirculated (100% replacement volume per day), dechlorinated, municipal water at 14º C under a 12L:12D photoperiod. Fish were fed to satiation twice daily with AquaMax® Grower (PMI Nutrition International, Inc., Brentwood, MO), except 24-36 h before initiating experimental manipulations. Animals were acclimated to laboratory conditions for at least two weeks prior to experimentation.

Experimental Conditions

At the time of sampling, animals were anesthetized in 0.05% (v/v) 2-phenoxyethanol, measured (body length and body weight), and bled from the severed caudal vessels. Gonads were removed and sex was determined by microscopic examination of fresh mounted gonadal

tissue; all fish were sexually immature (gonad somatic index, GSI < 0.2). Livers and gill arches were removed from animals, perfused ex vivo with 0.75% (w/v) saline solution, placed in Hank's medium (in mM: 137 NaCl, 5.4 KCl, 4 $NaHCO_3$, 1.7 $CaCl_2$, 0.8 $MgSO_4$, 0.5 KH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, and 4 glucose, pH 7.6), and prepared for culture as previously described (Hanson et al., 2010; Harmon and Sheridan, 1992). White muscle was similarly removed, placed into Hank's medium, and prepared for culture. Briefly, liver and muscle were cut into 1 mm^3 pieces and individual gill filaments were dissected from the arches; liver and muscle pieces as well as gill filaments from individual fish of known sex were pooled (i.e., one pool of each type of tissue per fish) and washed three times with Hank's medium with 0.24% (w/v) bovine serum albumin. Tissues were placed in 24-well culture plates (8-10 liver pieces, 35-40 muscle pieces, or 12-15 gill filaments) containing 1-ml of Hank's medium with 0.24% (w/v) bovine serum albumin and preincubated at 14° C under 100% O_2 while being and shaken at 100 rpm in a gyratory shaker; replicate wells for a given experiment came from different fish. After 3 h, the medium was removed and the tissues were washed twice with 1.0 ml of Hank's medium with 0.24% (w/v) bovine serum albumin. The tissues were then treated with E2 (Sigma, St. Louis, MO), ranging in concentration from 0 to 250 ng/ml, or T (Sigma), ranging in concentration from 0 to 500 ng/ml, incubated under the same conditions as preincubation for various periods of time (0, 3, 6, 12 or 24 h). 17β-estradiol and testosterone were initially dissolved in 95% (v/v) ethanol, with the final concentration of ethanol in the culture wells not exceeding 0.001% (v/v); control incubations contained the same final concentration of ethanol. The concentrations of E2 and T used in these experiments were based on the range of concentrations measured for these sex steroids in the plasma of rainbow trout before, during, and after sexual maturation (Holloway et al., 1999). Following incubation, the tissues were rinsed

with Hank's medium, placed in 2.0-ml microfuge tubes, and immediately frozen on dry ice. The tissues were stored at -80 °C for later mRNA and binding analyses. Functional and mRNA expression of GHRs were measured in liver, white muscle and gill filaments because of the high ligand binding and high levels of mRNAs encoding the receptors observed previously in these tissues, whereas functional and mRNA of IGFR1 were measured in white muscle and gill due to the prevalence of IGF-1 binding and expression of IGFR1-encoding mRNAs in these tissues (cf. Gray et al., 1990; Parrizas et al., 1995; Poppinga et al., 2007; Very et al., 2005). Similarly, mRNA levels of IGFs were measured only in liver and gill filaments because of their relative abundance in these tissues (cf. Poppinga et al., 2007; Wood et al., 2005). As this was the first time we incubated muscle tissue *in vitro*, preliminary studies were conducted to assess the viability of the tissue pieces under culture conditions; tissues remained viable for up to 24h as assessed by O_2 consumption.

GH and IGF-I Binding

Binding of ^{125}I-labeled salmonid GH (sGH) or ^{125}I-labeled salmonid IGF-1 (sIGF-1) to microsomes prepared from liver, muscle, and/or gill tissue were performed as described previously (Gray et al., 1990; Parrizas et al., 1995). Salmonid GH and sIGF-I were obtained from GroPep, Ltd. (Adelaide, Australia) and iodinated according to the supplier's protocol. Protein was determined by the Bio-Rad, Inc. (Hercules, CA) Bradford dye-binding method for microplates using bovine serum albumin as a standard.

Quantitative Real-time PCR

Frozen tissues were homogenized and total RNA was extracted using TRI reagent® (Molecular Research Center, Cincinnati, OH, USA) as specified by the manufacturer's protocol. Each RNA pellet was redissolved in 60-100 µl RNase-free deionized water and total RNA was

quantified by ultraviolet (UV A_{260}) spectrophotometry. Total RNA was diluted with RNase-free deionized water to 100 ng/µl. RNA was reverse transcribed in a 10-µl reaction using 200 ng total RNA with an AffinityScript QPCR cDNA synthesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Reactions without reverse transcriptase were included as negative controls to exclude the possibility of contamination with genomic DNA; no amplification was detected in negative controls.

Steady-state mRNA levels of GHR1, GHR2, IGF-1, IGF-2, IGFR1A, and IGFR1B were determined by quantitative real-time PCR using a Stratagene Mx 3000p detection system (Stratagene, La Jolla, CA) as previously described (Malkuch et al., 2008; Poppinga et al., 2007; Very et al., 2005). Briefly, real-time PCR reactions were carried out for controls, standards, and samples in a 10-µl total volume (1 µl cDNA; 5 µl 2X Brillant II Master Mix; 1 µl of each gene-specific probe, forward primer, and reverse primer at concentrations optimized for each RNA species; 1 µl RNase-free deionized water). Cycling parameters were set as follows: an initial denaturation of 95 ºC for 10 min followed by 50 cycles, each consisting of 95 ºC for 15 sec then 58 ºC for 1 min. Sample copy number was calculated from the threshold cycle number (CT), and the CT was related to a gene-specific standard curve followed by normalization to β-actin.

Statistics

Data are expressed as a means ± SEM; n represents the number of animals. Statistical differences were analyzed by ANOVA followed by Duncan's multiple range test using SigmaStat (SPSS, Chicago, IL). A probability of < 0.05 was used to indicate significance.

Results

Effects of Sex Steroids on mRNA and Functional Expression of GHRs

The direct effects of E2 and T on the expression of GHR mRNAs was determined in liver, muscle, and gill filaments incubated *in vitro*. Consistent with our previous observations (Very et al., 2005), two distinct GHR-encoding mRNAs, GHR1 and GHR2, were detected in all three tissues. In liver, E2 treatment deceased steady-state levels of GHR1 mRNA and GHR2 mRNA in a concentration- and time-related manner. Significant changes in mRNA levels were observed 3 h after E2 treatment for GHR2 and after 6 h for GHR1; by 6 h, levels of mRNAs encoding GHR1 and GHR2 were depressed to the same extent and there was no further change in the expression of either subtype for the remainder of the experiment (Fig 41A). Maximum inhibition of GHR expression was observed at an E2 concentration of 250 ng/ml; and there was no apparent difference between GHR1 and GHR2 in their responsiveness to E2 (Fig. 41C). 17β-estradiol treatment also reduced steady-state levels of GHR1 and GHR2 mRNAs in muscle in a concentration- and time-related manner. 17β-estradiol significantly reduced GHR mRNA levels within 3-6 h, depending on GHR subtype, and continued to decline up to 12 h after E2 treatment (Fig 42A). As was the case in liver, maximum inhibition of GHR expression in muscle by E2 occurred at 250 ng/ml, and there was no apparent difference between GHR1 and GHR2 in the responsiveness to E2 (Fig. 42C). In gill, E2 had no significant influence on steady-state levels of GHR1 or GHR2 mRNAs (Fig. 43A, 43C).

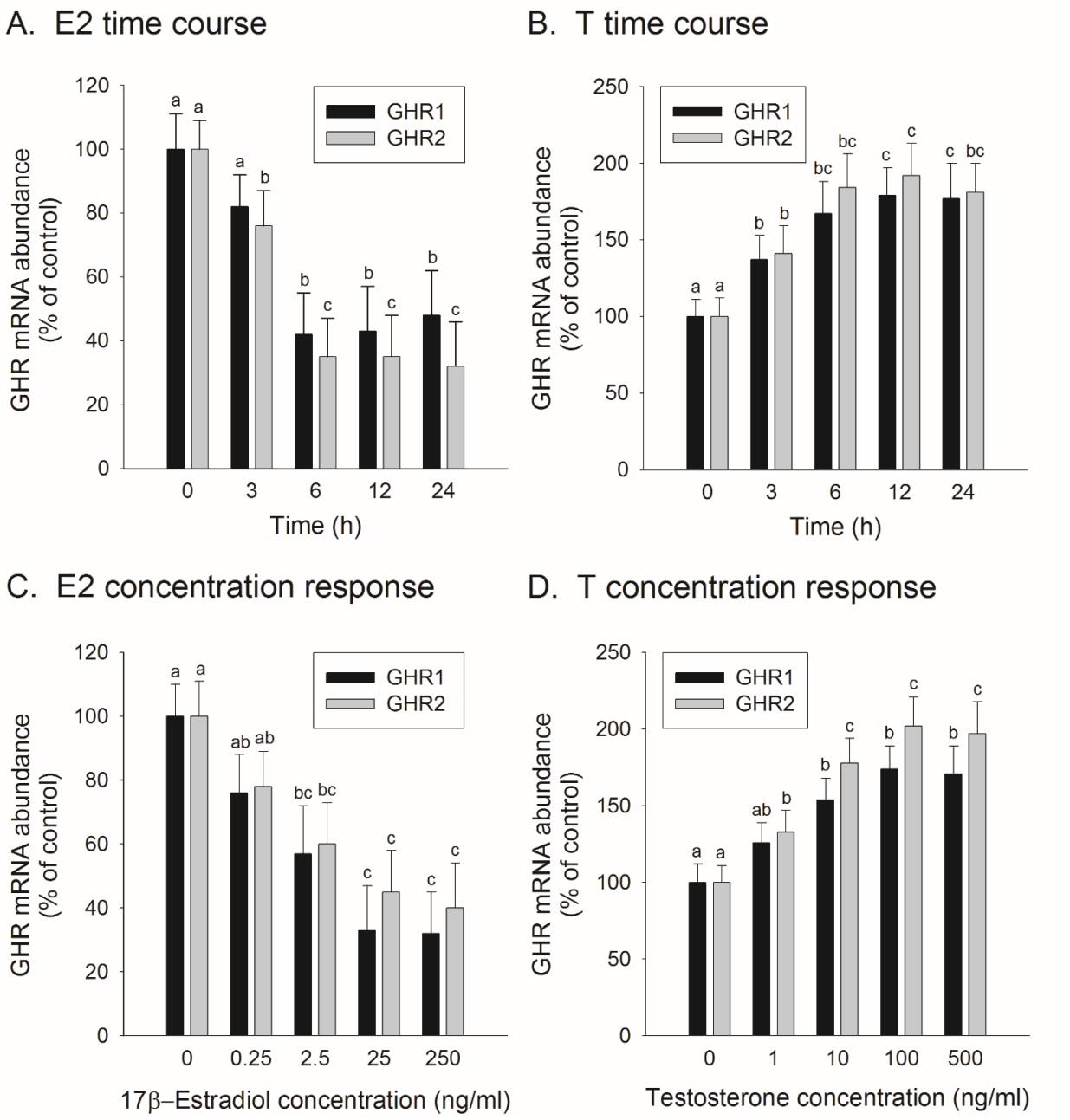

Fig. 41. Effects of 17β-estradiol (E2) and testosterone (T) on mRNA expression of growth hormone receptors in liver of rainbow trout incubated *in vitro*. Liver pieces were incubated for various times with 25 ng/ml E2 (A) or 100 ng/ml T (B) or with various concentrations of E2 (C) or T (D) for 6 h. Data are presented as mean ± SEM. (n = 6-8). For a given GHR subtype, groups with different letters are significantly ($P < 0.05$) different from each other.

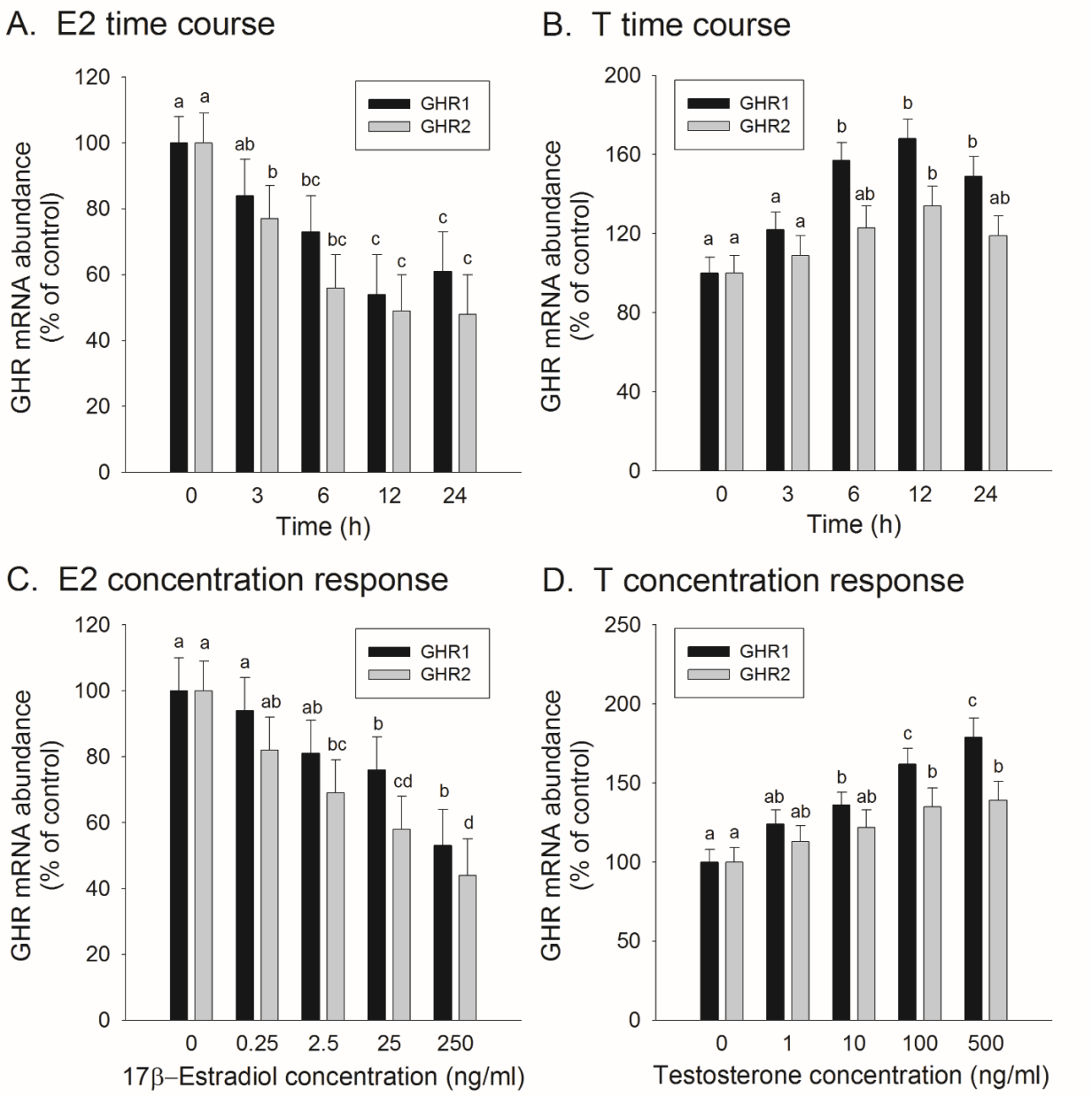

Fig. 42. Effects of 17β-estradiol (E2) and testosterone (T) on mRNA expression of growth hormone receptors in muscle of rainbow trout incubated *in vitro*. White muscle pieces were incubated for various times with 25 ng/ml E2 (A) or 100 ng/ml T (B) or with various concentrations of E2 (C) or T (D) for 6 h. Data are presented as mean ± SEM. (n = 6-8). For a given GHR subtype, groups with different letters are significantly ($P < 0.05$) different from each other.

Testosterone, by contrast, increased steady-state levels of GHR1 and GHR2 mRNAs in a time- and concentration-related manner. In liver, T increased the expression of GHR1 and GHR2 mRNAs rapidly and to a similar extent, reaching a plateau by 6 h (Fig 41B). Significant increases in hepatic GHR expression were observed at a concentration as low as 1 ng/ml (GHR2), with maximal stimulation occurring at 100 ng/ml (Fig. 41D). In muscle, T significantly increased GHR1 mRNA by 6 h; thereafter, GHR1 expression leveled off (Fig 42B). Testosterone significantly stimulated GHR1 mRNA expression over controls at 10 ng/ml; maximum stimulation was observed at 500 ng/ml (Fig. 42D). The effects of T on GHR2 expression were less pronounced, with significant expression observed after 12 h (Fig. 42C) and at concentrations of 100-500 ng/ml (Fig. 42D). A pattern similar to that observed in liver also was observed in gill filaments. Testosterone stimulated a rapid increase in the steady-state levels of both GHR mRNAs that peaked by 24 h (Fig. 43B). A maximum response in GHR expression in gill filaments was achieved with a T concentration of 500 ng/ml (Fig. 43D).

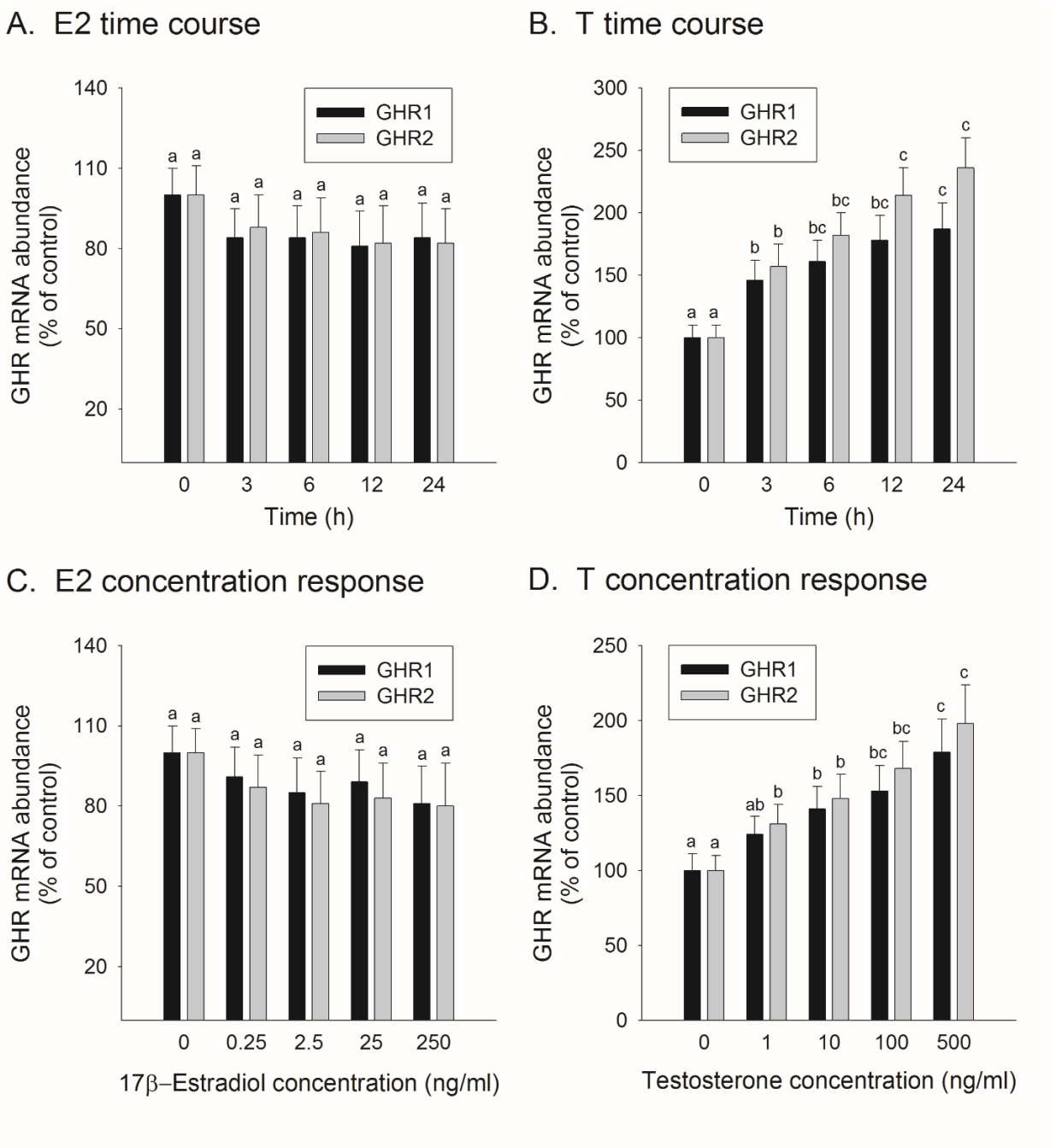

Fig. 43. Effects of 17β-estradiol (E2) and testosterone (T) on mRNA expression of growth hormone receptors in gill filaments of rainbow trout incubated *in vitro*. Gill filaments were incubated for various times with 25 ng/ml E2 (A) or 100 ng/ml T (B) or with various concentrations of E2 (C) or T (D) for 6 h. Data are presented as mean ± SEM. (n = 6-8). For a given GHR subtype, groups with different letters are significantly ($P < 0.05$) different from each other.

Functional expression of GHR was determined by ^{125}I-GH binding to microsomes. 17β-estradiol reduced the capacity of ^{125}I-GH binding by 57% in liver and by 33% in muscle; however, E2 had no effect on ^{125}I-GH binding capacity in microsomes prepared from gill filaments (Fig. 44A). 17β-estradiol had no effect on binding affinity in liver, muscle, or gill (liver control K_d=1.77±0.23 nM, liver E2 K_d=1.84±0.34 nM, $p > 0.05$; muscle control K_d=2.41±0.37 nM, muscle E2 K_d=2.39±0.35 nM, $p > 0.05$; gill control K_d=4.02±0.41 nM, gill E2 K_d=4.13±0.46 nM, $p > 0.05$). As was the case with mRNA expression, T stimulated functional expression of GHR as assessed by ^{125}I-GH binding. Testosterone stimulated ^{125}I-GH binding capacity in microsomes isolated from liver by 113%, from muscle by 83%, and from gill filaments by 133% (Fig. 44B), but had no effect on binding affinity (liver control K_d=1.69 ±0.26 nM, liver T K_d=1.77 ±0.30 nM, $p > 0.05$; muscle control K_d=2.82±0.42 nM, muscle T K_d=2.74±0.39 nM, $p > 0.05$; gill control K_d=4.26±0.47 nM, gill T K_d=3.98±0.43 nM, $p > 0.05$).

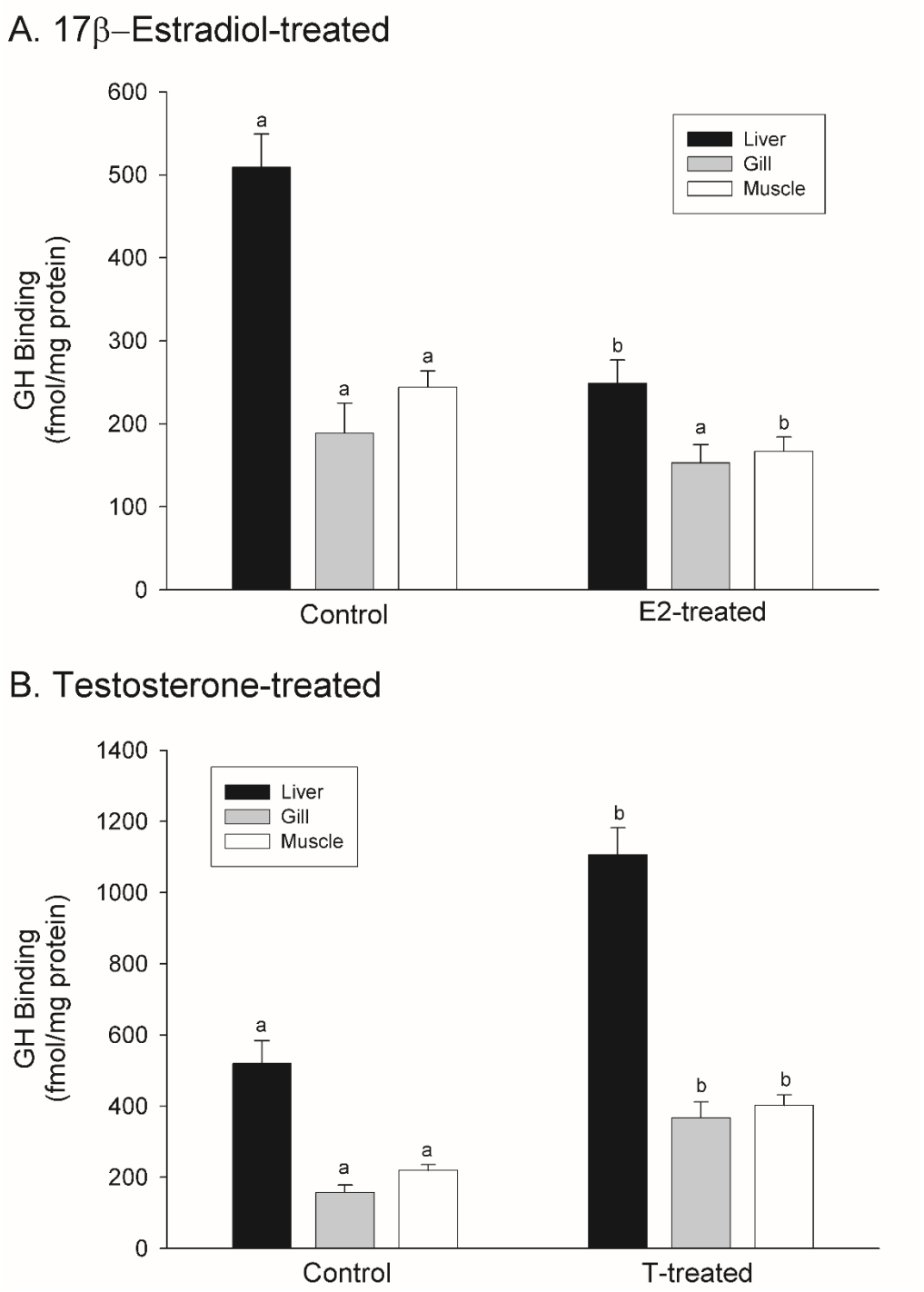

Fig. 44. Effects of 17β-estradiol (A) and testosterone (B) on binding of ^{125}I-salmonid growth hormone (GH) to microsomes prepared from liver, muscle and gill filaments of rainbow trout. Liver pieces, white muscle pieces or gill filaments were incubated *in vitro* with 25 ng/ml 17β-estradiol (E2) or 100 ng/ml testosterone (T) for 6 h; after which time, microsomes were prepared and binding assays were conducted as described in the Materials and Methods section. The data shown illustrate binding capacity (Bmax), and are presented as mean ± SEM. (n = 6-8). For a given tissue, groups with different letters are significantly ($P < 0.05$) different from each other.

Effects of Sex Steroids on mRNA Expression of IGFs

The direct effects of E2 and T on the expression of IGF-1 and IGF-2 mRNAs also were determined in liver pieces and gill filaments incubated *in vitro*. In liver, E2 treatment deceased steady-state levels of IGF-1 and IGF-2 mRNAs in a concentration- and time-related manner. The expression of both IGF forms declined rapidly, reaching minimum levels following 6 h of E2 exposure (Fig. 45A). Significantly reduced IGF-1 expression was observed at an E2 concentration of 2.5 ng/ml, with maximum inhibition achieved with E2 concentrations of 25 ng/ml (IGF-1) to 250 ng/ml (IGF-2); however, there was no difference in the pattern of response of either IGF-1 or IGF-2 to E2 (Fig. 45C). 17β-estradiol significantly inhibited the expression of IGF-1 and IGF-2 mRNAs in gill filaments. Steady-state levels of both IGF mRNAs were significantly depressed after 6 h of E2 treatment (25 ng/ml) and remained at those levels for the remainder of the treatment period; however, mRNA levels of IGF-2 did not change over the course of the experiment when treated with this same concentration (25 ng/ml) of E2 (Fig. 46A). 17β-estradiol treatments at 2.5, 25, and 250 ng/ml were effective at reducing IGF-1 expression in gill filaments; whereas, IGF-2 expression was only affected at the supraphysiological concentration of 250 ng/ml (Fig. 46C).

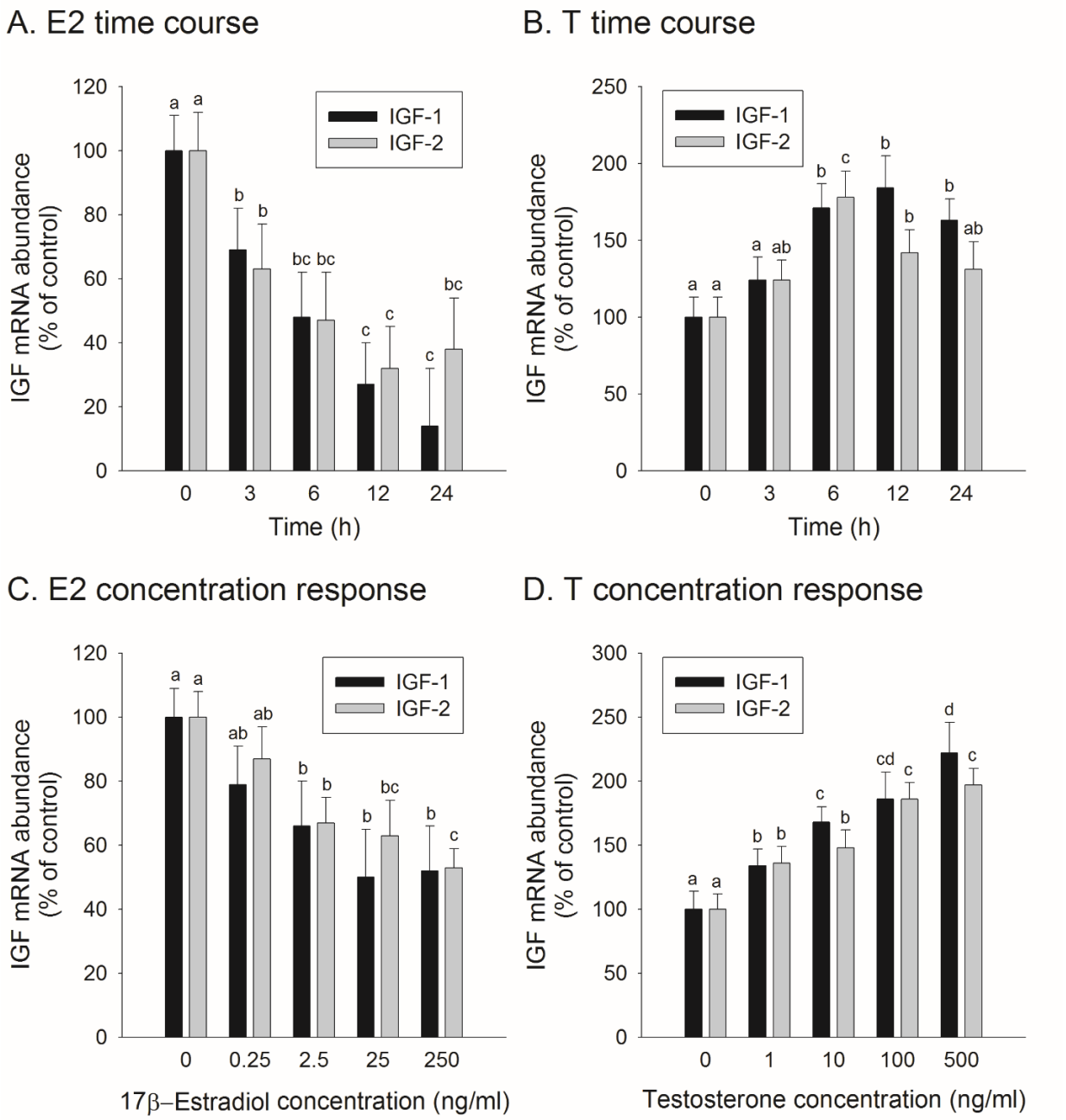

Fig. 45. Effects of 17β-estradiol (E2) and testosterone (T) on mRNA expression of IGFs in liver of rainbow trout incubated *in vitro*. Liver pieces were incubated for various times with 25 ng/ml E2 (A) or 100 ng/ml T (B) or with various concentrations of E2 (C) or T (D) for 6 h. Data are presented as mean ± SEM. (n = 6-8). For a given IGF type, groups with different letters are significantly ($P < 0.05$) different from each other.

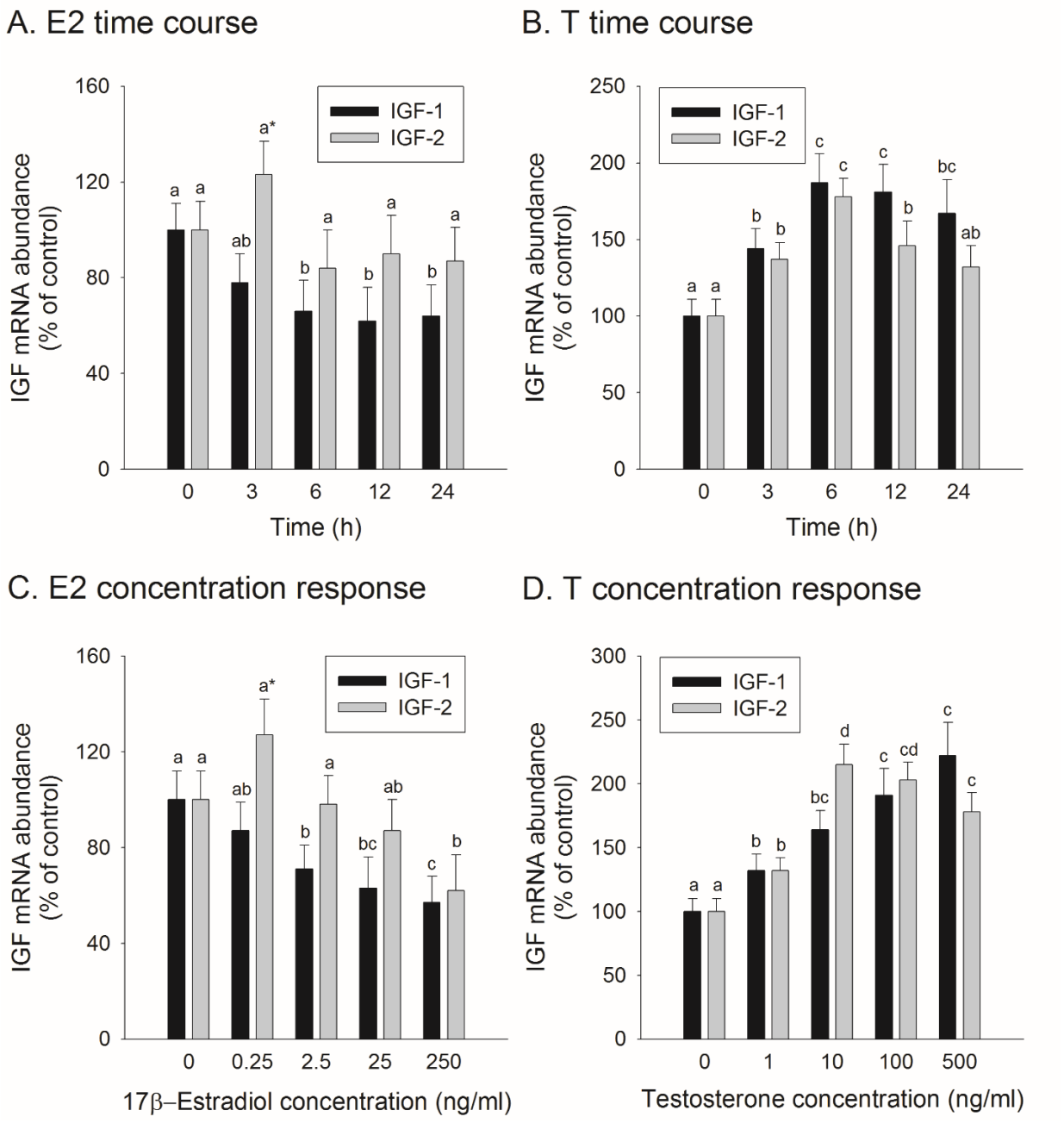

Fig. 46. Effects of 17β-estradiol (E2) and testosterone (T) on mRNA expression of IGFs in gill filaments of rainbow trout incubated *in vitro*. Gill filaments were incubated for various times with 25 ng/ml E2 (A) or 100 ng/ml (B) or with various concentrations of E2 (C) or T (D) for 6 h. Data are presented as mean ± SEM. (n = 6-8). For a given IGF type, groups with different letters are significantly ($P < 0.05$) different from each other. * indicates significant difference ($P < 0.05$) between IGF subtypes at corresponding incubation time or E2 concentration.

Similar to the situation for GHR expression, T increased steady-state levels of IGF-1 and IGF-2 mRNAs in a time- and concentration-related manner. In liver, T increased the expression of GHR1 and GHR2 mRNAs to a similar extent, reaching maximum levels with 6-12 h of T exposure (Fig 45B). Testosterone concentrations as low as 1 ng/ml significantly increased hepatic expression of both IGF-1 and of IGF-2 mRNAs, with maximal stimulation occurring at 500 ng/ml (Fig. 45D). In gill filaments, T stimulated a rapid increase in the steady-state levels of GHR mRNAs that peaked by 6 h, and then declined (Fig. 46B). A maximum response in IGF-1 expression in gill filaments was observed at a concentration of 500 ng/ml; whereas, the maximum response of IGF-2 was achieved at 10 ng/ml, followed by a slightly reduced response at 500 ng/ml (Fig. 46D).

Effects of Sex Steroids on mRNA and Functional Expression of IGFRs

The direct effects of E2 and T on the expression of IGFR1 mRNAs was determined in muscle pieces and gill filaments incubated *in vitro*. Consistent with our previous observations, two distinct IGFR1-encoding mRNAs, IGFR1A and IGFR1B, were detected gill [30] as well as in muscle. The effects of E2 on the expression of IGFR1 mRNAs in muscle were slight; only after 24h of E2 exposure was the decline in steady-state levels of IGFR1A significant compared to control (Figs. 47A, 47C). 17β-estradiol had no effect on steady-state levels of mRNAs encoding either IGFR1A or IGFR1B in gill (Fig. 48A, 48C).

By contrast, T significantly increased steady-state levels of IGFR1A and IGFR1B mRNAs in a time- and dose-related manner in both muscle and gill. In muscle, T significantly elevated mRNA levels of both IGFR1A and IGFR1B by 6 h; there after expression of IGFR1A remained elevated through 12 h, then declined or, in the case of IGFR1B declined immediately (Fig. 47B). Steady-state levels of both IGFR1s in muscle were significantly elevated over

control levels at a T concentration of 10 ng/ml; maximal promotion of IGFR1 mRNA expression was observed with 500 ng/ml (Fig. 47D).

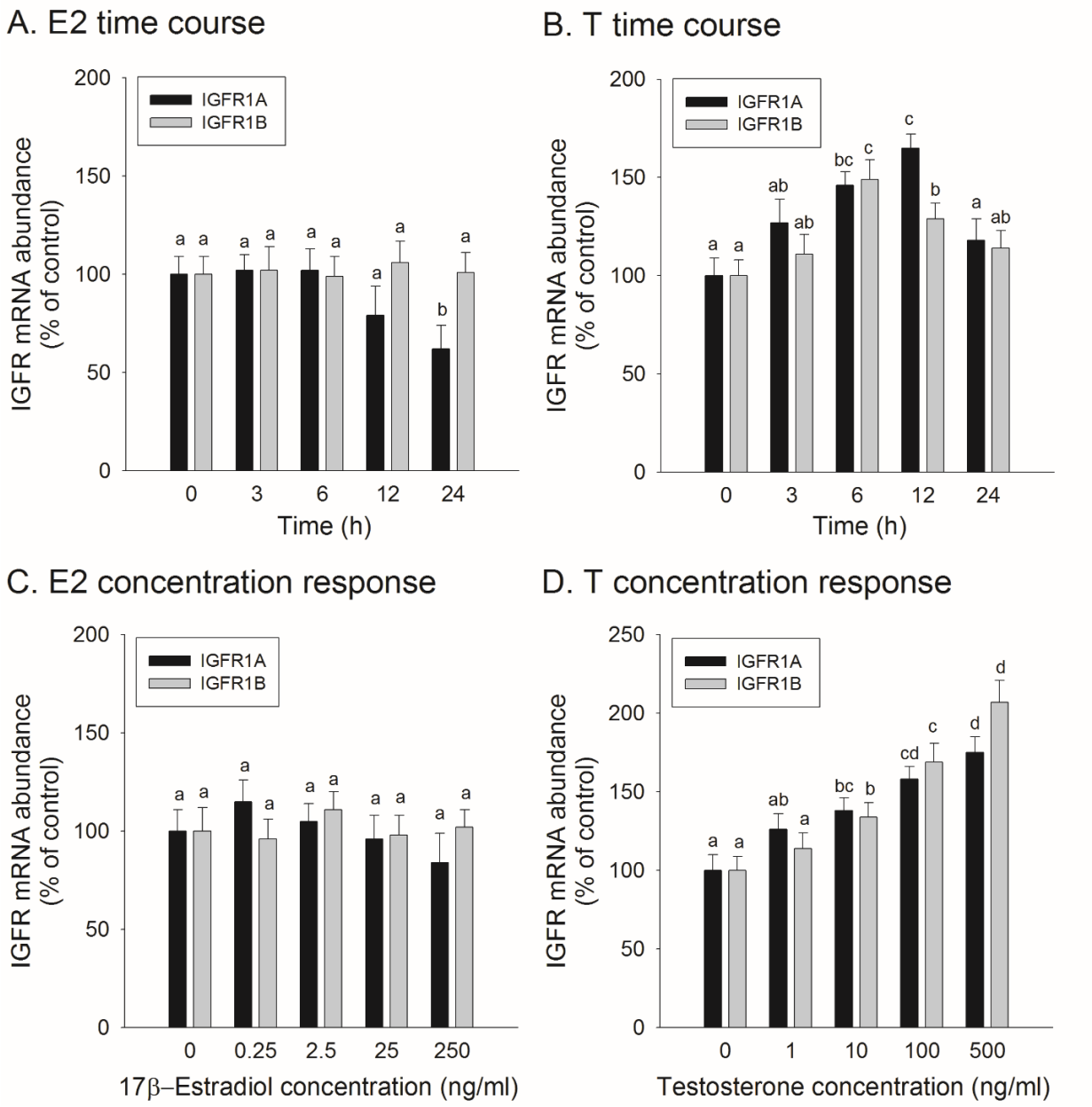

Fig. 47. Effects of 17β-estradiol (E2) and testosterone (T) on mRNA expression of type 1 insulin-like growth factor receptors (IGFR1) in muscle of rainbow trout incubated *in vitro*. White muscle pieces were incubated for various times with 25 ng/ml E2 (A) or 100 ng/ml T (B) or with various concentrations of E2 (C) or T (D) for 6 h. Data are presented as mean ± SEM. (n = 6-8). For a given IGFR1 subtype, groups with different letters are significantly ($P < 0.05$) different from each other.

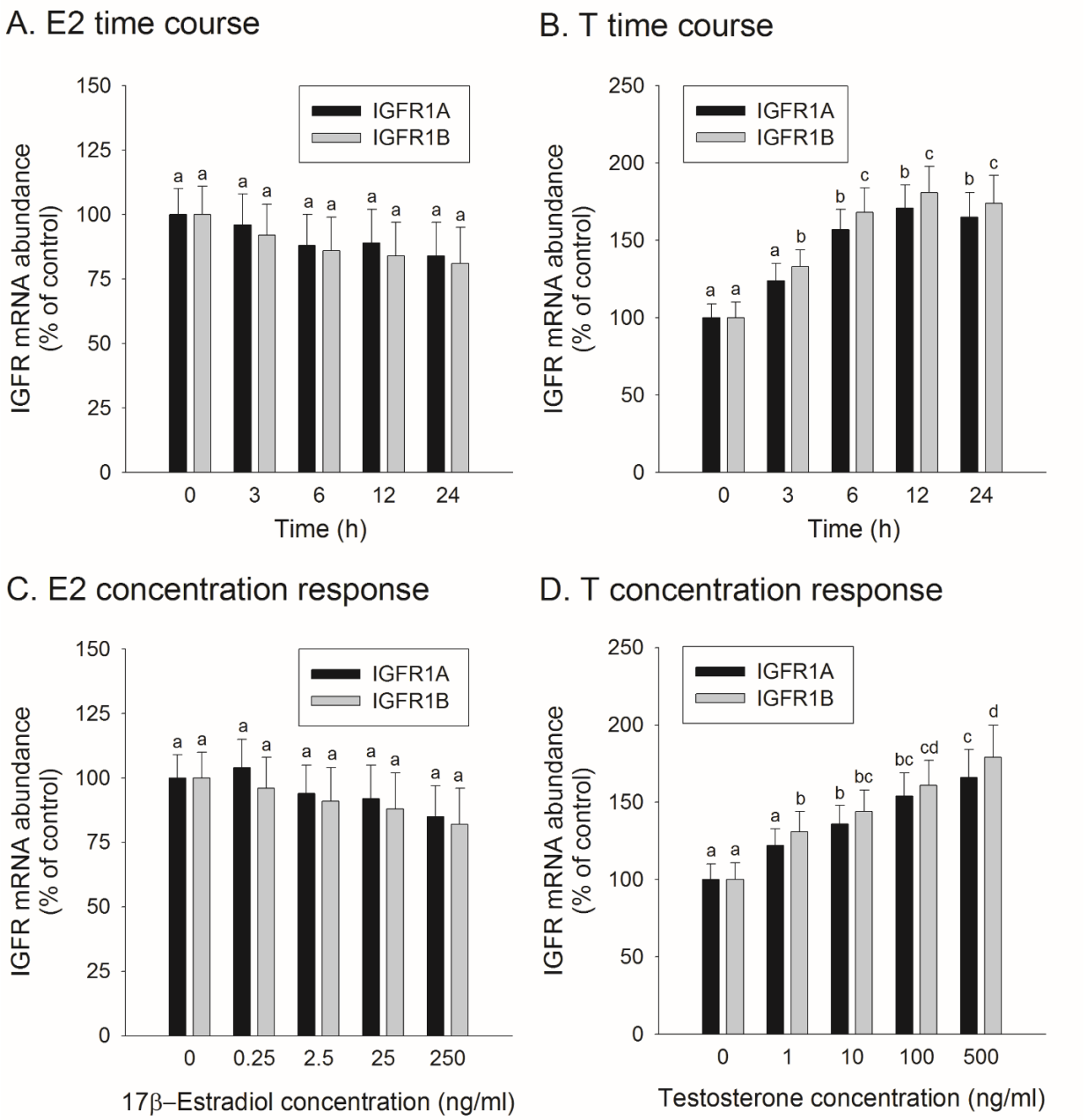

Fig. 48. Effects of 17β-estradiol (E2) and testosterone (T) on mRNA expression of type 1 insulin-like growth factor receptors (IGFR1) in gill filaments of rainbow trout incubated *in vitro*. Gill filaments were incubated for various times with 25 ng/ml E2 (A) or 100 ng/ml T (B) or with various concentrations of E2 (C) or T (D) for 6 h. Data are presented as mean ± SEM. (n = 6-8). For a given IGFR1 subtype, groups with different letters are significantly ($P < 0.05$) different from each other.

In gill, T stimulated expression of IGFR1 mRNAs in a progressive manner, with maximum levels reached after 6 h; thereafter, levels of both IGFR1 mRNAs remained at similarly elevated levels for the duration of the experiment (Fig. 48B). Significant increases in mRNA expression were observed at a T concentration as low as 1 ng/ml for IGFR1B and as 10 ng/ml for IGFR1A; maximal expression for both IGFR1 subtypes was achieved at a T concentration of 500 ng/ml (Fig. 48D).

Functional expression of IGFR1 was determined by ^{125}I-IGF-1 binding to microsomes prepared from white muscle and gill filaments. 17β-estradiol had no effect on ^{125}I-IGF-1 binding capacity (Fig. 49A) or binding affinity in either muscle or gill (muscle control K_d=0.87±0.24 nM, muscle E2 K_d=0.93±0.27 nM, $p > 0.05$; gill control K_d=1.22±0.32 nM, gill E2 K_d=1.16±0.30 nM, $p > 0.05$). By contrast, T stimulated functional expression of IGFR1 as assessed by ^{125}I-IGF-1 binding. Testosterone increased the binding capacity of ^{125}I-IGF-1 by 79% and 88% in microsomes prepared from muscle and gill filaments, respectively (Fig. 49B). Testosterone, like E2, had no effect on binding affinity in either muscle of gill (muscle control K_d=0.79±0.25 nM, muscle E2 K_d=0.84±0.31 nM, $p > 0.05$; gill control K_d=1.09±0.29 nM, gill E2 K_d=1.21±0.34 nM, $p > 0.05$).

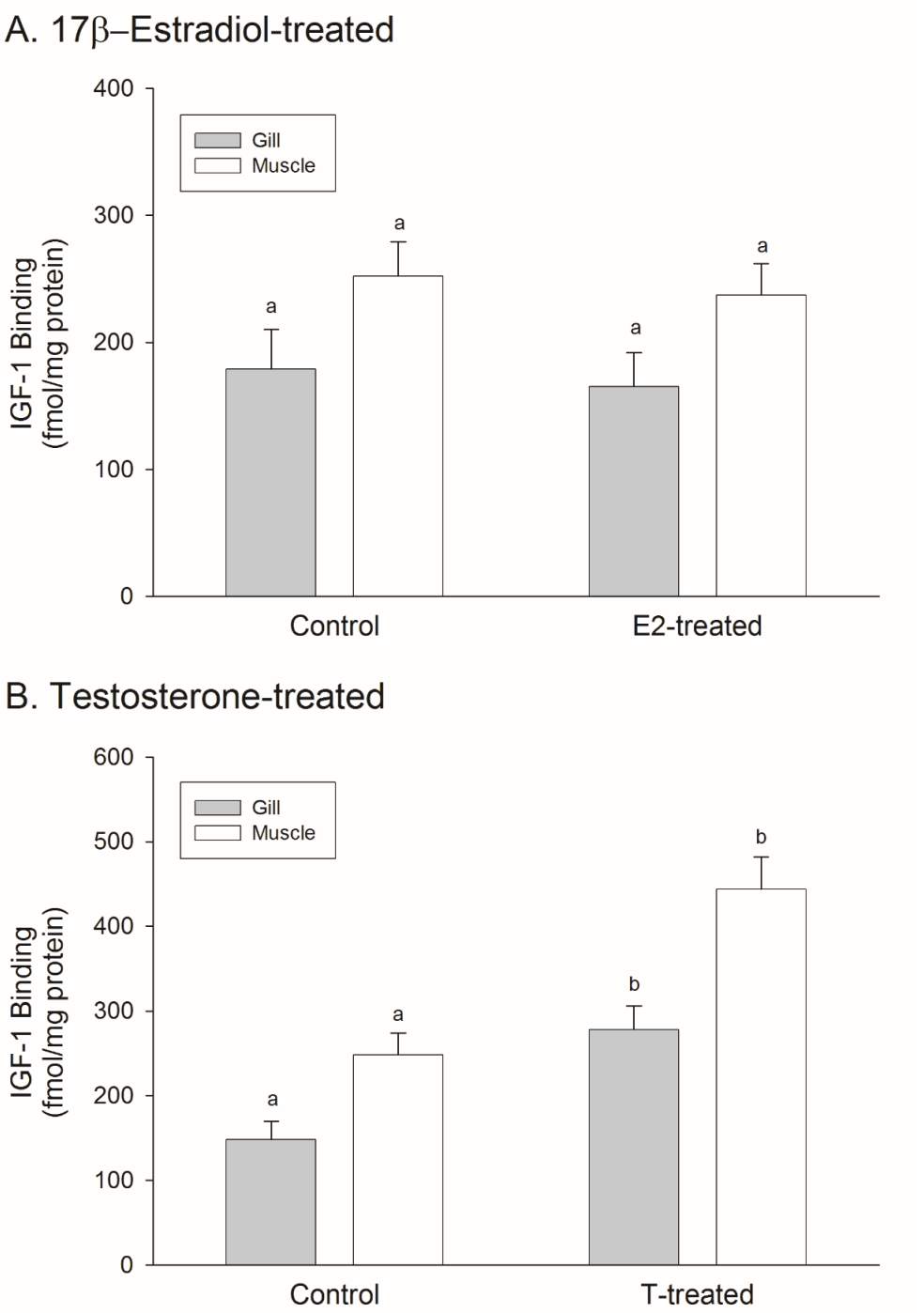

Fig. 49. Effects of 17β-estradiol (A) and testosterone (B) on binding of ^{125}I-salmonid insulin-like growth factor-1 (IGF-1) to microsomes prepared from muscle or gill filaments of rainbow trout. White muscle pieces or gill filaments were incubated *in vitro* with 25 ng/ml 17β-estradiol (E2) or 100 ng/ml testosterone (T) for 6 h; after which time, microsomes were prepared and binding assays were conducted as described in the Materials and Methods section. The data shown illustrate binding capacity (Bmax) and are presented as mean ± SEM. (n = 6-8). Groups with different letters are significantly ($P < 0.05$) different from each other.

Discussion

This study was a comprehensive examination of the influence of the sex steroids, E2 and T, on the GH-IGF system in a teleost fish. The findings reveal for the first time to our knowledge that E2 and T act directly to differentially regulate elements of the GH-IGF system and support our starting hypothesis that E2 and T modulate the expression of GHRs, IGFs, and of IGFR1s. Peripheral regulation of GH and IGF sensitivity and of peripheral IGF production by E2 and T provide an important mechanism of integrating organismal growth with reproductive events.

The ability of E2 to regulate peripheral aspects of the GH-IGF system was supported by several current observations. First, E2 directly inhibited steady-state levels of GHR-encoding mRNAs in liver and muscle. Notably, the effects of E2 were not uniform on all tissues as E2 had no effect on expression of GHR mRNAs in gill. Second, E2 significantly reduced functional expression of GHR in liver and muscle as assessed by ^{125}I-GH binding but had no effect on ^{125}I-GH binding in gill, consistent with findings on mRNA expression. Third, E2 directly inhibited the expression of IGF-1 and of IGF-2 mRNAs in liver. In gill, E2 reduced the expression of IGF-1 mRNA but not of IGF-2 mRNA except at supraphysiological concentrations. Lastly, E2 had slight to no effects on mRNA or functional expression of IGFR1s in muscle or gill. Taken together, these findings indicate that E2 reduces peripheral responsiveness to GH (by inhibiting mRNA and functional expression of GHRs) as well as peripheral production of IGFs; however, such effects appear to be tissue- (in the case of GHR expression) and isoform- (in the case of IGF expression) specific.

Interactions between E2 and aspects of the GH-IGF system were suggested by previous studies that showed that plasma GH levels increase during sexual maturation of several species

of teleosts (Bjornsson et al., 1994; Holloway et al., 1999; Kakizawa et al., 1995; Stacey et al., 1984; Trudeau et al., 1992) and that exogenous E2 increases plasma levels of GH in goldfish, rainbow trout, and tilapia (Holloway and Leatherland, 1997; Melamed et al., 1995; Trudeau et al., 1992). Such an effect of E2, however, appears variable as studies in Atlantic salmon showed that E2 reduced plasma GH (Lerner et al., 2007). The basis for the variable response among these studies is not clear, but it may be due to the differences in experimental methods (mode of administration, concentration, duration of exposure), stage of development, or species. Estrogens have been shown to influence pituitary release of GH by directly stimulating GH secretion and/or indirectly by modulating the responsiveness of the pituitary to hypothalamic releasing factors (Chang and Wong, 2009). Paradoxically, estrogens do not consistently promote organismal growth. For example, the synthetic estrogen, diethylstilbestrol (DES), only stimulated growth of Chinook salmon when given with the anti-estrogen clomiphene citrate (Schreck and Fowler, 1982), and tilapia treated with ethinylestradiol (EE2) had a higher proportion of females that grew more slowly than their untreated counterparts (Meredith et al., 1999). The inability of E2 to promote growth in the face of elevated plasma GH may be due to desensitization of peripheral aspects of the GH-IGF system in immature fish such as those described in the current study (e.g., reduced responsiveness to GH and IGF, reduced synthesis of IGF-1 and IGF-2). Such a notion is consistent with previous *in vivo* findings in tilapia, black sea bream, and gilthead sea bream that showed that E2 injection reduced hepatic expression of GHR and of IGF-1 and IGF-2 mRNAs (Carnevali et al., 2005; Davis et al., 2008; Jiao et al., 2006). *In vivo* exposure of maturing fathead minnow or developing tilapia (10-40 days postfertilization) with EE2 also reduced hepatic expression of GHR and IGF-1 mRNAs as well as plasma levels of IGF-1 (Filby and Tyler, 2007; Shved et al., 2007). Contrary to our current findings is the report

that *in vivo* exposure of juvenile yellow perch with E2 increased hepatic IGF-1 mRNA levels in both male and female fish (Goetz et al., 2009). It is not clear if this difference is due to the dose or manner (in feed) E2 treatment or to species variation. One pattern that does emerge is that E2 effects vary by tissue. For example, consistent with our current findings was the observation that E2 injection reduced GHR expression in a tissue-specific manner such that mRNA levels in liver were inhibited by E2 but those in gonads were unaffected by E2 (Davis et al., 2008). Tissue and sex differences in E2 response on expression GHR, IGF-1 and IGFR1 mRNAs in adult fathead minnow and in EE2 response on expression of IGF-1 mRNA in developing tilapia also have been reported (Filby et al., 2006; Shved et al., 2008). Tissue-specific effects of E2 also have been observed *in vitro*. For example, EE2 and DES inhibited growth of tilapia gill cartilage *in vitro* as assessed by thymidine uptake (Ng et al., 2001), whereas E2 promoted the expression of IGF-1 and IGF-2 mRNA in gonads removed from sexually mature tilapia (Haung et al., 2007).

The ability of T to regulate peripheral aspects of the GH-IGF system also was supported by several observations of this study. First, T directly increased the expression of GHR1 and GHR2 mRNAs in liver, muscle, and gill. Second, T increased the functional expression of GHR in liver, muscle, and gill as measured by ^{125}I-GH binding. Third, T directly increased steady-state levels of mRNAs encoding IGF-1 and IGF-2 in liver and gill; and there was no difference in the effects of T on IGF form in either tissue. Fourth, T directly increased the expression of IGFR1A- and IGFR1B-encoding mRNAs in muscle and gill, with the stimulatory effects on both receptor subtypes being similar. Lastly, T stimulated functional expression of IGFR in muscle and gill as assessed by^{125}I-IGF-1 binding. Taken together, these findings indicate that T increases peripheral responsiveness to GH (by stimulating mRNA and functional expression of GHRs), increases peripheral production of IGFs, and increases peripheral responsiveness to IGFs

(by stimulating mRNA and functional expression of IGFR1s). Unlike E2, the effects of T appear to be uniform on tissues and subtype/isoform element in the GH-IGF system.

Previous studies also demonstrate an interaction between androgens and the GH-IGF system. Plasma levels of T increase during the course of sexual maturation (Holloway et al., 1999) and exogenous treatment of goldfish and rainbow trout with T increase plasma levels of GH (Canosa et al., 2002; Holloway and Leatherland, 1997); however, T and 11-ketotestosterone (11KT) had no effect on plasma GH in coho salmon (Larsen et al., 2004). Numerous reports in several species indicated that androgens (e.g., T, 17-methyltesterone) promote organismal growth (McBride and Fagerlund, 1973; Riley et al., 2002; Schreck and Fowler, 1982). By contrast, T *in vivo* was reported to inhibit growth of perch, an effect that was associated with reduced food intake (Mandiki et al., 2005). As with the E2, the bases of such disparate effects of T are not known, but they may be due to differences in experimental methods, stage of development, and/or species. The growth-promoting effects of androgens may result from effects operating at the level of the pituitary (Chang and Wong, 2009) and/or effects in the periphery. The current findings support a role of T in sensitizing peripheral aspects of the GH-IGF system, including a heightened responsiveness to GH and IGF and augmented synthesis of IGF-1 and IGF-2. Previous work in coho salmon showing that T and 11KT increased plasma IGF-1 without affecting plasma GH is consistent with T acting peripherally (Larsen et al., 2004). By contrast, T injection reduced hepatic expression of GHR2 but not of GHR1 in black sea bream (Jiao et al., 2006); however, it is not clear if this effect of T was direct or indirect. Dihydrotestosterone (DHT) increased IGF-1 mRNA expression in isolated hepatocytes from mature male tilapia; however, the opposite effect of DHT was observed on hepatocytes isolated from mature female tilapia (Riley et al., 2004).

The observed sex steroid-induced alterations in GH and IGF sensitivity and IGF production may play important physiological roles in energy allocation and coordinating growth with metabolic and reproductive (i.e., sexual maturation) processes. Prior to sexual maturation, fish undergo a phase of rapid somatic growth predominated by anabolic process (Mommsen et al, 2001). At the onset of sexual maturation, somatic growth declines and increasing energy is devoted to gonad development and a host of sex-specific processes such as repackaging and de novo synthesis of lipid for assembly of lipoproteins such as vitellogenin in females and increased nucleotide synthesis associated with testicular growth in males (Holloway et al., 1999). The energetics during gonadal recrudescence is complex and involves simultaneous activation of catabolic and anabolic processes, and the metabolic events that occur in anadromous species may be particularly complex given the energy demands of migration and adaption to an altered osmotic environment. During the rapid growth phase of immature fish, GH resulting from the GH-IGF system promotes organismal growth via increased amino acid uptake, increased RNA synthesis, increased protein synthesis, increased muscle growth, and increased cartilage growth (Mommsen, 2001). As sexual maturation commences, there is a shift in GH action that appears to be mediated by sex steroids. Part of this shift may involve a positive feedback between GH and sex steroids in which GH stimulates gonadal steroidogenesis which results in elevated plasma sex steroids, and sex steroids, in turn, stimulate GH release, with the net result being elevated plasma GH and increased gonad development (Chang and Wong, 2009). Another part of this shift may involve two additional feedback loops that are sex steroid-specific. One of these proposed additional feedback loops involves positive feedback of T onto peripheral elements of the GH-IGF system. In this feedback, T enhances the sensitivity of peripheral tissues to GH and IGF and increases the production of IGF. Such a positive feedback helps

explain why maturing males grow faster than non-maturing males (Larsen et al., 2004). A negative feedback is proposed between E2 and peripheral elements of the GH-IGF system. In this feedback system, E2 decreases the sensitivity to GH and decreases the production of IGF. Such a negative feedback helps to explain decreased somatic particularly of females during sexual maturation (Holloway et al., 1999). By decreasing peripheral sensitivity to GH and reducing IGF production, E2 may attenuate the growth-promoting actions of GH and enhance the reproductive and possibly metabolic (lipid catabolic, protein sparing) actions of GH (Sheridan, 1994). The exact cues that underlie changes in peripheral aspects of the GH-IGF system and initiate the proposed shift in GH action are not known, but they undoubtedly involve the interplay of numerous signals. The possibility that a critical threshold in gonad development (perhaps reflected by GSI) or body condition (perhaps reflected by condition factor) should be examined.

The observed influence of sex steroids on GH and IGF sensitivity and IGF production also has implications for endocrine disruption of growth. For example, environmental exposure of immature fish to synthetic and natural estrogens could reduce peripheral sensitivity to GH and inhibit IGF production that potentially leading to growth retardation and/or an altered developmental trajectory. This notion is supported by studies that showed that E2, 4-nonylphenol (NP), or β-sitosterol retarded growth of rainbow trout and that NP decreased plasma levels of IGF-1 in Atlantic salmon (Hanson et al., 2012; Lerner et al., 2007). Seawater adaptability of both Atlantic salmon and rainbow trout, which also relies upon the GH-IGF system, was similarly retarded by estrogen exposure (Hanson et al., 2012; Lerner et al., 2007), suggesting that E2 may attenuate the osmoregulatory actions of GH as well as the growth-promoting actions of GH.

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CHAPTER 6: CONCLUSIONS

Growth of vertebrates is regulated through the central GH-IGF system. However, this system interacts peripherally with surrounding external environments and internal physiological processes that influence how somatic growth proceeds. My work focused on some of the internal extra-pituitary hormonal signals that might be affecting the growth hormone insulin-like growth factor system. This also included direct effects observed by GH and IGF on their own growth-related genes. The goal of assessing peripheral hormone effects on growth-related genes of the GH-IGF system was conducted by hormone treatments on whole tissue cultures. Hormonal treatment effects were then quantified through real-time quantitative-PCR to indicate if the hormones directly interacted with the transcriptional processing of the growth-related genes.

An important concept in discussing the effects observed by GH and IGF treatments on liver and gill tissue is how cells communicate to each other. Cell communication is termed cell signaling. Different types of cell signaling are referred to as either endocrine, paracrine or autocrine. Endocrine signaling refers to proteins or molecules that are released by a cell and travel a far distance through the bloodstream to reach the target cells. Paracrine signaling refers to proteins and molecules released by a cell that travel a short distance to nearby target cells. Finally, autocrine signaling refers to proteins and molecules that are released by the cell which can trigger a response on the same cell that produced that specific protein or molecule.

It has been suggested IGF could work in an autocrine or paracrine fashion (Berishvili et al., 2006; Zygar et al., 2005; Franz et al., 2016). My *in vitro* studies produced observations where IGF treatments on liver tissue were able to overall negatively influenced GH sensitivity and IGF production by the liver. In this scenario, cell signaling could be through autocrine or

paracrine cell communication. However, without specific experimentation, autocrine and paracrine signaling cannot be determined for sure, but only suggested. Also, GH treatments on liver and gill filament tissue were able to increase GH sensitivity through GHR2 and GHR1, respectively. Since GH transcripts have been detected in adult liver and gill tissue of rainbow trout (Yang et al., 1999), the GH treatment effects observed on GH sensitivity could be due to autocrine or paracrine signaling. But, as stated above, without specific experimentation, autocrine and paracrine signaling cannot be distinguished.

GH treatments on liver overall negatively influenced GH sensitivity in my studies. The down-regulation of GH sensitivity was also observed in two other studies, one in rats (Maiter et al., 198) and the other in seabream fish (Shved et al., 2011). However, within the field (Pierce et al., 2012; Wang et al., 2016) and even our own lab there are conflicting results (Very & Sheridan, 2007; Reindl et al., 2009), where an up-regulation of hepatic GH sensitivity was observed after GH treatments were applied. The apparent discrepancies might be related to different techniques. The experiments with increases in GH sensitivity used *in vitro* primary hepatocytes, while those showing down-regulation of GH sensitivity used *in vivo* tissues or whole organs in culture techniques. So a possible reason why conflicting results are observed within the field and even within the same labs could be due to differences in methodologies.

Another possible reason a decrease in GH sensitivity was observed by the liver is in the nature of post-transcriptional modifications to the GHR extracellular domain, which can be cleaved after GH binds to its receptor to form GHBP (Einarsdottir et al., 2014). This process in the formation GHBP and the possible function of the GHBP is to extend the half-life of GH and compete with the GHR in binding and activating cellular responses could be a factor in the decrease of cellular response to activate transcription of GHRs (Reindl & Sheridan, 2012).

GH treatments over time negatively influenced IGF production by the liver. I cannot explain those results. In many other studies involving a number of different species of fish, an increase in the production of IGF was found to occur regardless of technique (Shamblott et al., 1995; Schmid et al., 2000; Kajimura et al., 2001; Tse et al., 2002; Vong et al., 2003; Pierce et al., 2004; Peterson et al., 2005; Pierce et al., 2005; Leung et al., 2008; Pierce et al., 2010; Reindl et al., 2011; Shved et al., 2011). However, I showed that higher physiological and pharmacological doses of GH were able to stimulate IGF2 production in the gill. Increases of IGF production by the gill after GH treatment were also observed by others (Sakamoto & Hirano, 1993; Vong et al., 2003). Since the majority of circulating IGFs in fish are produced and released from the liver (Shamblott & Chen, 1993) and GH is known to stimulate production of hepatic IGF (Wood et al., 2000) a significant down-regulation in IGF production currently observed after a GH treatments with my studies is unexplained at this time.

In all of my experiments with thyroxine in both tissues, I concluded that thyroxine had no direct effects on growth-related genes of the GH-IGF system. In the time- and concentration-response experiments not a single concentration elicited an effect among the growth genes tested. Also, when additional experiments were conducted that contained a treatment group with only T4, no effects were observed to be consistently different from the control group. The only observable influences on growth genes after a T4 treatment was applied were during the time course treatments. Also, during my combination study when tissues were treated with T4 and GH, a significant decrease in growth genes were observed. Again, I cannot explain that result. But it led me to postulate that T4 may need to interact with other molecules or hormones to indirectly influence growth during juvenile development. Because significant T4 effects were seen only over longer periods of time during the time course treatments, it is possible the right

time frame of a 6-hour treatment was not the ideal time to observe significant effects. However, other researchers in the field have observed significant direct effects of T4 treatments on growth-related genes, but only at pharmacological concentrations (Leung et al., 2008). Since a direct effect of T4 treatment occurred at pharmacological concentrations in one species, further studies are merited on other fish species, at varying concentrations, and in the presence of other molecules and hormones to better understand if T4 has direct (or indirect) effects on growth-related genes.

Hepatic GHR1 and hepatic IGF1 are differentially regulated after cortisol treatments. I observed in the current time- and concentration-response experiments that mRNAs encoding for hepatic GHR1 were significantly upregulated. mRNAs encoding for hepatic IGF1 at a higher physiological concentration (100 ng/ml) and during the time course studies resulted in either no significant differences or significant declines in mRNA expression levels over time, respectively.

Time- and concentration-response studies alone did not lead to the conclusion that a consistent trend in hepatic GHR1 and IGF1 mRNA expression levels occurred. It was not until additional inhibitory and hormone combination studies were conducted that significant increases in GHR1 mRNA expression levels and no change in IGF1 mRNA expression levels clarified a consistent trend in the same growth genes as consistently causing effects after only F treatments. Similar F effects on GHR1 (Jiao et al., 2006; Philip & Vijayan, 2015) and IGF1 (Peterson & Small, 2005; Pierce et al., 2011; Philip & Vijayan, 2015) were observed by others in the field. It is generally accepted that cortisol is a stress hormone and acts in suppressing growth and redistributing energy toward metabolic processes needed for homeostasis (Sheridan, 1986; Mommsen et al., 1999; Laiz-Carrion et al., 2002). This would be consistent with my studies in which the upregulation of hepatic GHR1 and no significant change in hepatic IGF1 mRNA

expression levels occurred. Furthermore, Philip and Vijayan (2015) have linked the effects seen by cortisol on growth. In the mechanism they described, GH signaling is shut down by inactivating the JAK2-STAT5 pathway in which hepatic IGF1 typically is produced. Instead an upregulation in suppressors of cytokine signaling (SOCS) occurs, due to increased plasma cortisol levels, which in turn inhibits the necessary phosphorylation of STAT5 leading to the decreased hepatic production of IGF1 (Philip & Vijaya, 2015).

Hepatic IGF2 mRNA expression levels were consistently observed to be upregulated with F treatments. A trend in an upregulation of hepatic IGF2 mRNA expression was recognized after a series of studies were conducted and consistently found to elicit the same effect by F. My observed direct increase of hepatic IGF2 mRNA expression after F treatments are the first to be reported to occur in fish. Although similar significant increases mRNAs encoding for hepatic IGF2 were seen in the field, the significant increase of IGF2 mRNA expression was produced by a synthetic glucocorticoid agonist, dexamethasone (Pierce et al., 2010). In addition, with my results and similar results seen by others (Pierce et al., 2011) in hepatic IGF2 mRNAs being upregulated and the previously mentioned downregulation of hepatic IGF1 mRNAs during representative times of stress suggest that cortisol differentially regulates hepatic IGF mRNAs due to different functions of IGF1 and IGF2 in fish. Finally, my observations of direct effects by F on the upregulation of hepatic IGF2 mRNA expression can add to an already proposed mechanism by Faught and Vijayan (2016) in how cortisol can influence IGF2 functions. It is suggested that upregulated IGF2 mRNA expression, through the upregulation of hepatic GHR1 mRNA expression, could involve cell signaling pathways independent of the JAK-STAT pathway that lead to different physiological functions observed from IGF1 physiological functions (Faught & Vijayan, 2016). However, since cortisol was shown to have direct effects in

upregulating IGF2 mRNA expression, it could also be suggested that IGF2 signaling involves different signaling pathways independent of the JAK2-STAT5 pathway normally implemented for IGF1 functions in growth. In states of stress, cortisol possibly upregulates IGF2 mRNA expression due to unknown functions that are important in activating IGF2 metabolic functions but at the same time cortisol exerts no effects on IGF1 mRNA expression levels, since the known function of IGF1 is to stimulate growth, which is energy demanding and not warranted during times of high stress.

Combination hormone treatments of GH + F resulted in a significant downregulation of mRNAs encoding for hepatic GHRs and hepatic IGFs. During this experiment GH treatment alone significantly elevated hepatic GHRs and hepatic IGFs, while F treatments alone significantly elevated hepatic GHR1 and hepatic IGF2 mRNA expression levels but had no significant effect on hepatic GHR2 and hepatic IGF1 mRNA expression levels. I initially hypothesized, since separate treatments of GH and F both upregulated GHR1 and IGF2 mRNA expression levels, that there would be a synergistic effect in the upregulation of these two growth genes. However, this is not the case. In fact, the exact opposite occurs, a downregulation in GHR1 mRNAs and an almost complete shut down in transcription of IGF2 mRNAs resulted from the combination treatment of GH + F. The reason for observing such a dramatic and significant decrease in the transcription of hepatic GHR mRNAs and hepatic IGF mRNAs is currently unexplained.

Estrogen significantly reduced peripheral responsiveness to GH in liver and muscle tissue. In addition, estrogen also significantly reduced hepatic IGF1 and hepatic IGF2 production and IGF2 production by gill filaments. It has been shown that GH plasma levels increase during sexual maturation when E2 production is elevated. However, my results showed

a reduction in hepatic GH responsiveness. I also saw reduced production of hepatic IGF1 and hepatic IGF2 and IGF2 production by gill filaments. This inability to utilize the elevated levels of GH that are produced in response to the presence of elevated E2 is known as growth hormone resistance. GH resistance was also observed among other fish species that were treated with E2 and saw a reduction in hepatic GHR and hepatic IGF mRNAs (Carnevali et al., 2005; Davis et al., 2008; Jiao et al., 2006). During the time of sexual maturation in females, somatic growth may be reduced in order to divert energy that would have been used for somatic growth toward the more energy demanding gonadal steroidogenesis and reproductive growth. In addition, somatic growth may also shut down with activation of the catabolic mechanism of GH which mediates energy production by lipolysis (Bergan & Sheridan, 2018).

Testosterone significantly enhanced peripheral responsiveness to GH, enhanced peripheral IGF production, and enhanced peripheral IGF sensitivity. In previous studies several fish species were reported to have enhanced somatic growth after treatments with androgens prior to sexual maturation (McBride & Fagerlund, 1973; Schreck & Fowler, 1982; Riley et al., 2002). Yet, when sexual maturation proceeds, the energy that was once used for somatic growth is reallocated toward incorporating increasing energy demands towards male gonadal recrudescence and steroidogenesis.

As my data only focused on the transcriptional levels of the selected growth genes, further studies should be designed to examine mechanisms in which activation of specific cell signaling pathways bring about physiological functions. Previously in our lab it was shown that, when GH mediates its growth-promoting actions, it can activate one of three possible cell signaling pathways (Reindl et al., 2011). A cortisol mechanism in which GH promoting actions are inactivated was only recently proposed in that the phosphorylation of STAT5 is blocked by

SOCS proteins during states of high stress (Philip & Vijayan, 2015). The discovery of mechanisms such as these could lead to understanding why specific transcriptional gene products are activated or deactivated and specific physiological functions occur in specific cells.

There is a need for future studies to understand many aspects of both thyroid hormones (T4 and T3). Because Leung et al. (2008) were able to observe significant direct effects by T4, it would be useful to broaden such studies to other fish species to determine if T4 does in fact have physiological functional roles on the growth-related genes.

Future studies across the various hormones of interest on growth-related genes in sexually mature rainbow trout would be important since somatic growth prior to sexual maturation, as in the juvenile, immature trout I studied, is likely regulated differently than somatic growth after sexual maturation has occurred.

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