GROWTH HORMONE MEDIATED REGULATION OF OSMOREGULATION IN EURYHALINE TELEOSTS

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

MASTER OF SCIENCE

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ABSTRACT

Within the multitude of fish species that exist on our planet, there are a certain number that possess the unique ability to live in both freshwater (FW) and seawater (SW) environments. This ability, known as euryhalinity, is limited to a relatively small number of species, thus making it a prime target for scientific research into osmoregulation, due to the uniqueness of this ability. It has been shown previously that growth hormone (GH) plays an important role in regulating this ability, and in this work, Atlantic salmon (*Salmo salar*) and Rainbow trout (*Oncorhynchus mykiss*) were used as models to examine the expression of specific osmoregulatory genes in response to SW transfer and GH exposure, and to examine the signaling mechanisms used by GH to facilitate any changes. We found that GH utilizes specific cell signaling pathways to facilitate the transition between FW and SW in both Rainbow trout and Atlantic salmon.

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DEDICATION

This thesis is dedicated to my parents, Ron and Joy Martin. Without their constant support and encouragement, I don't think this would have been possible. Thank you for everything that you have done to make this possible.

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LIST OF ABBREVIATIONS

ACTH.....Adrenocorticotropic Hormone

Akt..... Protein-kinase B

ANOVA..... Analysis of Variance

cDNA..... Complementary DNA

CFTR.....Cystic Fibrosis Transmembrane Regulator

DNA..... Deoxyribonucleic Acid

ERK..... Extracellular Signaling-related Kinase

FW..... Freshwater

GH..... Growth Hormone

GHR..... Growth Hormone Receptor

GHSR..... Ghrelin Receptor

GR..... Glucocorticoid Receptor

IGF..... Insulin-like Growth Factor

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

JAK..... Janus Kinase

MAPK..... Mitogen-activated Protein Kinases

MR..... Mineralocorticoid Receptor

mRNA..... Messenger RNA

NCC..... Sodium/Chloride Cotransporter

NKA.....Sodium/Potassium ATPase

NKCC.....Sodium/Potassium/Chloride Cotransporter

PCR..... Polymerase Chain Reaction

PI3K..... Phosphoinositide 3-kinase

PKC..... Protein-kinase C

PL..... Prolactin

RNA..... Ribonucleic Acid

SEM..... Standard Error of the Mean

STAT..... Phosphostat

SW..... Seawater

SWX.....Seawater Challenge

CHAPTER 1: INTRODUCTION AND BACKGROUND

Osmoregulation

For euryhaline teleosts, osmoregulation, along with the ability to survive in both freshwater (FW) and seawater (SW), plays a major role in their lifecycle. In FW, they must cope with the passive loss of ions to the environment, along with passive intake of water. This is accomplished by excreting large amounts of dilute urine, partnered with the active uptake of ions, typically in the gill and intestine. In SW, they must deal with essentially the reverse of FW, that is, passive uptake of ions and passive loss of water. To counter this, fish actively secrete ions, while actively drinking SW.

Survival in either FW or SW is very common, but what makes euryhaline teleosts special is their ability to transition between the two mediums. To facilitate their exceptional ability, these fishes have evolved a number of traits that allow them to survive in varying salinities. Among these traits are various proteins and physiological changes that are specific to certain salinities, but can change when the time comes to transfer. Certain species of teleosts, primarily salmonids, have developed a specific process, known as smoltification, which physically prepares them for the transition to SW.

Briefly, smoltification is a pre-adaptive process that takes place naturally in juvenile salmonids during Feb.-May. Bern (1978), described smolting as a pan-hyperendocrine state, where many hormones with different physiological actions are increasing during development, though not always at the same time or rate. During smoltification, a number of physiological, morphological, and behavioral changes take place. Morphological changes include fin darkening and extensive skin silvering, while the physiological changes involve alteration of transport

proteins. The gill is the primary location for osmoregulation in euryhaline teleosts, and it is there that a number of these changes take place.

The teleost gill is the site of both ion uptake (in FW conditions) and ion secretion (in SW conditions). This ion transport is primarily performed by a type of specialized cell in the gill called ionocytes (also known as chloride cells or mitochondrion-rich cells). Since all teleosts maintain a nearly constant level of internal salinity, regardless of external conditions (Edwards and Marshall, 2013), the ability of the ionocytes to change between ion absorption and ion secretion is crucial in allowing euryhaline teleosts to maintain control of their internal salinity in various environments (Evans et al., 2005). In order to facilitate this change, there are a number of proteins that play key roles, primarily, Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and cystic fibrosis transmembrane regulator (CFTR).

NKA is located within the basolateral membrane of the gill, and provides low levels of Na^+ and a negative charge within the ionocyte, which is used for net Cl^- and Na^+ secretion. Previously it had been shown that an increase in NKA was accompanied by increased salinity tolerance in smolts (Zaugg and McClain, 1970), but it was the discovery of multiple isoforms of NKA, particularly the catalytic α-subunit, in the gill of Rainbow trout (*Oncorhynchus mykiss*) by Richards et al. in 2003 that prompted a new look into the specific role of NKA in osmoregulation.

In Atlantic salmon (*Salmo salar*), it has been shown that in the ionocytes of FW adapted fish, NKAα1A is more abundant, while NKAα1B is more abundant in the ionocytes of SW adapted fish. This, along with data showing an increase in NKAα1B mRNA expression after SW exposure in Atlantic salmon (Nilsen et al., 2007; Tipsmark and Madsen, 2009; Madsen et al., 2009), Rainbow trout (Richards et al., 2003), and Climbing perch (*Anabas testudineus*) (Ip et al.,

2012), would suggest that NKAα1A is the dominant FW form, while NKAα1B is the dominant SW form. Recently, the development of isoform specific antibodies for NKAα1A and NKAα1B in Atlantic salmon has provided further information on the organization of the FW and SW gill.

Research done with these antibodies has shown that in FW adapted Atlantic salmon, filamental and lamellar ionocytes are dominated by NKAα1A, with small amounts of NKAα1B present in small filamental ionocytes. In SW adapted Atlantic salmon, there are a large number of ionocytes containing NKAα1B, with a small number containing NKAα1A. This increase in NKAα1B is coincident with increased salinity tolerance, providing further evidence to support NKAα1B as the SW adapted isoform (McCormick et al., 2013).

Along with the changes in NKA isoforms, there are also changes in NKCC and CFTR in the gill. NKCC is an integral epithelial membrane protein that functions to maintain cell volume and participate in ion transport (Lytle et al., 1995; Wu et al., 1998). Like NKA, NKCC is located on the basolateral membrane, and it uses low Na^+ to transport Cl^- into the ionocyte. This increase in Cl^- will be utilized by CFTR. In vertebrates there are two major isoforms of NKCC, NKCC1, the secretory isoform present on the basolateral membrane, and NKCC2, the absorptive isoform present on the apical surface. In teleosts, it appears that while NKCC1 is present, rather than NKCC2, they possess a Na^+/Cl^- transporter (NCC) on the apical surface (Hiroi et al, 2008). In 2001, Pelis et al. confirmed the presence of NKCC in Atlantic salmon gill, and observed an increase in NKCC and NKCC-positive ionocytes during smolt development. Partial cloning of Atlantic salmon NKCC showed high sequence similarity to the vertebrate NKCC1 isoforms. Later, the T4 human NKCC1 antibody was used to detect a difference in apical and basolateral ionocytes between SW and FW in Mozambique tilapia (*Oreochromis mossambicus*) (Wu et al., 2003).

Wu et al., observed that NKCC immunoreactivity was seen apically in FW ionocytes, while basolateral immunoreactivity was observed in SW ionocytes, implying that there are two NKCC types in tilapia, a FW type and a SW type. Investigating this difference, in 2008 Hiroi et al. identified four cDNAs homologous with human NKCC, NKCC1a, NKCC1b, NKCC2, and NCC (Na^+/Cl^--cotranspoter). FW to SW and SW to FW transfer experiments demonstrated that NKCC1a was highly upregulated in SW and downregulated in FW (Hiroi et al, 2008). These same experiments showed that NCC was upregulated in FW and downregulated in SW, providing evidence that NKCC1a is the SW type cotransporter, while NCC is the FW type.

There are also at least two isoforms of apical CFTR expressed in the Atlantic salmon gill, CFTR1 and CFTR2. CFTR functions as a "downhill" Cl^- channel, allowing secretion of the Cl^- ions brought into the cell by NKCC. It has been shown that during smolt development in Atlantic salmon there is an increase in the mRNA levels of CFTR1, with no change being shown in CFTR2 (Nilsen et al., 2007). This same pattern has also been seen after direct SW exposure over two weeks (Singer et al., 2002). Current immunocytochemistry of SW adapted European sea-bass (*Dicentrarchus labrax*) shows localization of CFTR in the apical pit of inter-lamellar ionocytes (Bodinier et al., 2009). All of this points to CFTR1 being the major gill isoform associated with salinity tolerance. Together, NKA, NKCC, and CFTR function to maintain a constant ion gradient against the external ionic environment.

Accompanying these specific protein changes are changes in the ionocytes themselves. Increased numbers of ionocytes in the gill has been observed in both Atlantic and Coho (*Oncorhynchus kisutch*) salmon during smolt development (Lubin et al., 1989; Richman et al., 1987). It has also been observed that during smolt development the number of NKAα1B ionocytes that also contain NKCC and CFTR increases, and that SW exposure increases both

size and abundance of NKAα1B ionocytes (McCormick et al., 2013). While an increase in total ionocyte number has been found, the current hypothesis is that it is the changing of NKAα1A ionocytes to NKAα1B ionocytes that provides the majority of salinity tolerance, rather than the overall increase in ionocyte concentration.

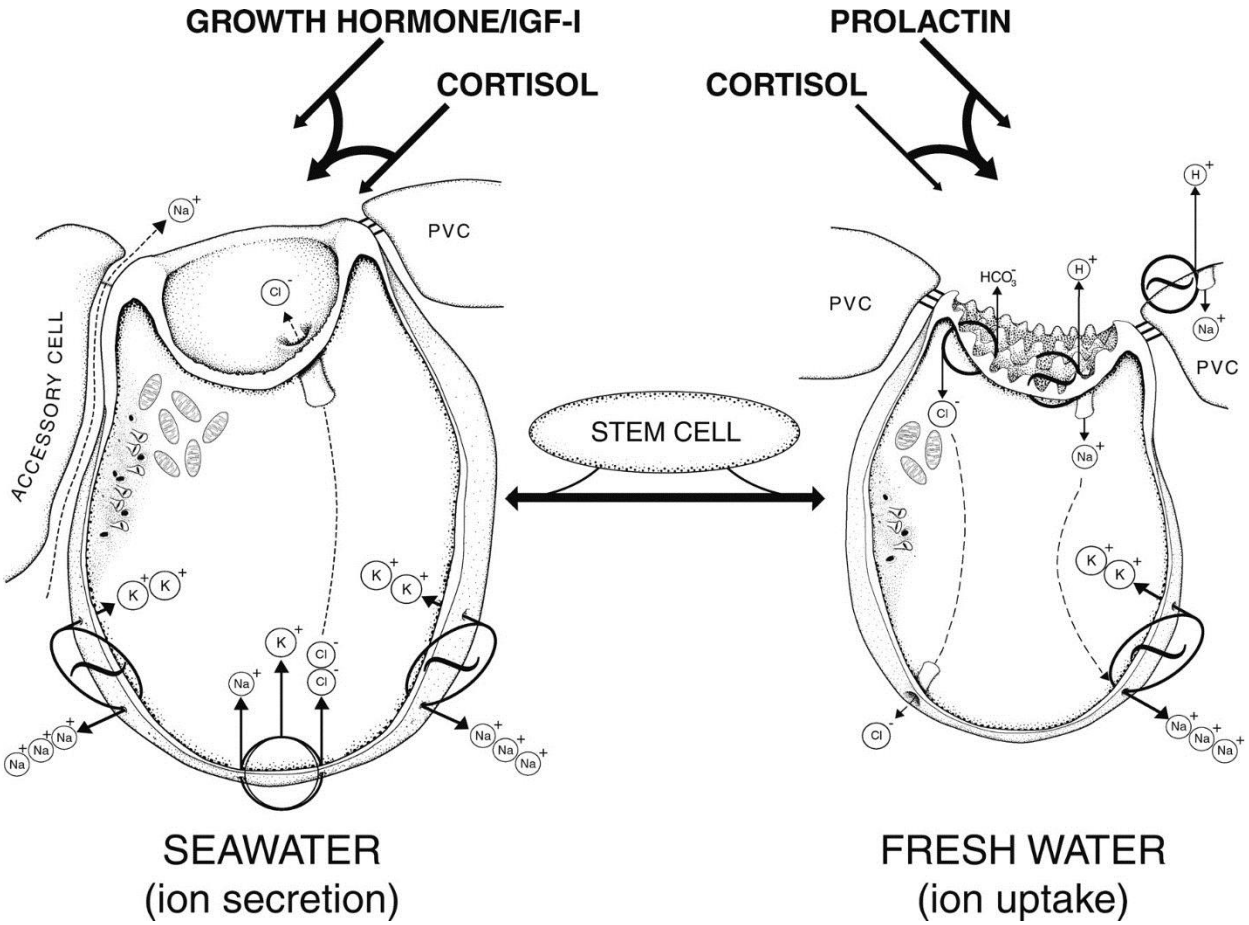

Figure 1. Examples of FW and SW ionocytes and their differences. (Evans et al., 2005)

Endocrine regulation

Growth hormone and insulin-like growth factor 1

Regulating all these osmoregulatory changes are a number of hormones, including prolactin (PL) (Nishioka et al., 1982; Young et al., 1989), cortisol (Nilsen et al., 2008; Shrimpton and McCormick, 1998), and the focus of this research, growth hormone (GH). It has been shown previously that GH increases gill ionocyte numbers, increases gill NKA activity, and decreases plasma osmolality in Atlantic and Coho salmon, all of which are indicators of increased salinity tolerance (Richman, 1987; McCormick, 1996). This GH mediated increase in NKA activity and decrease in plasma osmolality has also been observed in Rainbow trout and Gilthead sea bream (*Sparus auratus*) (Sangiao-Alvarellos et al., 2005; 2006). It has also been shown that exogenous treatment with GH and IGF1 (insulin-like growth factor 1) provides a direct increase in salinity tolerance in juvenile trout and salmon (Takei and McCormick, 2013).

GH, along with IGF1, has also been shown to play a key role in the smoltification process. GH appears to be the first hormone to increase due to photoperiod cues, which are a key factor in the timing of smoltification, suggesting that GH is key to the initiation of the smoltification process. It has been shown that during smoltification, there is an increase in levels of circulating GH in Atlantic and Coho salmon (Björnsson et al., 2011). This increase in circulating GH has been found to be absent in a landlocked population of Atlantic salmon, and is associated with poor salinity tolerance (Nilsen et al., 2008). Accompanying this increase in GH is an increase in plasma IGF1. This increase has been observed in Atlantic salmon parrs and smolts in the spring, with smolts exhibiting more of an increase than parrs (McCormick et al., 2007), however this increase was not observed in all studies (Nilsen et al., 2007).

Local target tissue production of IGF1 is also likely to be involved in osmoregulation and smoltification. It has been shown that both liver and gill IGF1 mRNA levels increase during smoltification in Coho salmon (Sakamoto et al., 1995), and increased transcription of IGF1 has been observed in the gill of smolting anadromous Atlantic salmon (Nilsen et al., 2008). This increase in transcription was absent in a landlocked population of Atlantic salmon. Combined with the increase in circulating GH during smoltification, an observed increase in the transcription of branchial GH receptor (Kiilerich et al., 2007a) may explain the increases in branchial production of IGF1 that have been observed. It has also been shown that GH increases the transcription and/or protein abundance of three major gill osmoregulatory proteins (NKAα1B, NKCC1, and CFTR) (Tipsmarck and Madsen, 2009; McCormick et al., 2013), suggesting that GH plays a large role in the differentiation of ionocytes in the gill. All of these data clearly indicate that GH plays a major role in the control of smoltification in certain species, and overall osmoregulation in euryhaline teleosts.

Cortisol

Cortisol also plays a key role in teleost osmoregulation, and has been particularly well studied with respect to smoltification. An increase in circulating cortisol was first shown in Atlantic salmon smolts by Fontaine and Hatey (1954), and later in Coho salmon by Specker and Schreck (1982). In 1969 Hirano showed that FW to SW transfer increased cortisol levels in Japanese eel (*Anguilla japonica*), and that this increase was not seen in hypophysectomized eel, or during SW to FW transfer. Plasma levels of cortisol remain constant and low in Atlantic salmon parr in spring, but show a 10-fold increase in smolts under the same conditions (McCormick et al., 2007). This spring increase in plasma cortisol is absent in a landlocked strain on Atlantic salmon (Nilsen et al., 2008). In Atlantic salmon, large increases in gill cortisol

receptors were exhibited in both parr and smolts in the spring (Shrimpton and McCormick, 1998). Higher levels of cortisol receptors in ionocytes have also been shown, when compared to other cell types in the gill, using immunocytochemistry and *in situ* hybridization (Uchida et al., 1998).

Cortisol treatment can increase the major transport proteins involved in salt secretion, NKAα1B, NKCC1, and CFTR, along with their abundance in ionocytes (Pelis and McCormick, 2001), and it has been shown that exogenous treatment with cortisol stimulates increased salinity tolerance, along with many of the underlying mechanisms involved in SW osmoregulation (McCormick, 2001). Recently, molecular data have indicated that two "glucocorticoid" receptors (GRs) and one "mineralocorticoid" receptor (MR) are present in most teleosts (Takei and McCormick, 2013), and increased gill GR transcription has been observed in smolting Atlantic and Masu salmon, with no change in gill MR transcription being observed (Mizuno et al., 2001; Nilsen et al., 2008). These data indicate that it is likely that most of the actions of cortisol to promote salt secretion are signaled by one of both GRs.

Also of import is the interaction between cortisol and the GH-IGF axis. GH increases the responsiveness of the interrenal gland to adrenocorticotropic hormone (ACTH), which increases the amount of cortisol released at any given level of ACTH. The abundance of corticosteroid binding sites and transcription of GR in the gill are also upregulated by GH (Shrimpton et al., 1995; Kiilerich et al., 2007b). Cortisol does much the same for GH, increasing the transcription of gill GH and IGF1 receptors (Tipsmark and Madsen, 2009).

Prolactin

While cortisol and GH are promoters of SW adaptation, PL functions as a promoter of FW adaptation. Pickford et al. (1966) showed that hypophysectomized killifish (*Fundulus*

hetereoclitus) failed to survive in FW. However, when exposed to purified PL, survival in FW was achievable. Pickford et al. (1965) also showed that treatment with GH rather than PL was ineffective at prolonging FW survival of hypophysectomized killifish. This was the first evidence for PL playing a major role in FW survival. This ability of PL to enable FW survival was also shown in hypophysectomized *Poecilia latipinna* by Ball and Ensor (1965). It has been shown previously that exposure to FW increases secretion, plasma levels, synthesis, and gene expression of PL (Manzon, 2002). It has been observed that PL affects ionocytes in two ways, by promoting the morphology of FW type ionocytes (Pisam et al., 1993), and by inhibiting the development of SW type ionocytes (Herndon et al., 1991). It has also been shown that ion uptake can be stimulated by treatment with exogenous PL (Zhou et al., 2003). Plasma PL is elevated in Coho and Atlantic salmon in winter and early spring, but decreases in April and May during the peak of smolting (Prunet et al., 1989; Young, 1989).

Treatment with PL has been shown to decrease NKA activity and increase plasma osmolality and ions in SW adapted gilthead seabream exposed to brackish water (Mancera et al., 2002). In pufferfish (*Takifugu rubripes*) it has been shown that following movement from full strength SW to 25% SW, pituitary expression of PL increased, while pituitary expression of GH decreased (Lee et al., 2006). This same expression pattern was also observed in pufferfish raised in 25% SW for 8 weeks as compared to those in 100% SW (Lee et al., 2006). All of this evidence supports PL functioning as a promoter of FW adaptation, though much work is yet to be done.

Growth hormone signaling

Growth hormone is one of the key players in the growth and development of teleost fish. It regulates a variety of physiological processes, including growth, metabolism, reproduction,

immune function, and osmoregulation. With its wide variety of targets and functions, it has, out of necessity, developed a very extensive and varied cell signaling system. This system is initiated by the direct binding of GH, the only natural ligand of the growth hormone receptor (GHR), to GHR. In teleosts, there are multiple isoforms of GHR, derived from separate genes and various gene duplication events. Several studies have shown that the pattern of GHR expression can be regulated by numerous factors (Reinecke et al., 2005; Norbeck et al., 2007; Reinecke, 2010; Reindl et al., 2012).

GHR is a member of the cytokine receptor superfamily, and functions as a membrane bound homodimer. GHR itself does not have any intrinsic kinase activity, but rather functions by activation and recruitment of other cytoplasmic protein kinases (see Zhu et al., 2001 for review). This recruitment of various protein kinases, combined with the multiple subtypes of GHR, allows for the multiplicity of GH effects that have been observed in teleosts. To date, GH signaling has been heavily researched (Zhu et al., 2001; Waters et al., 2006), and various studies have demonstrated that different downstream signaling elements are used for different processes. For example, it has been shown that the different teleost GHR subtypes differentially activate ERK, Akt, and STAT5 (Kittilson et al., 2011), that GH-stimulated lipolysis is mediated by PKC and ERK (Bergan et al., 2013), and that GH stimulated hepatic IGF expression is mediated by ERK, PI3K-Akt, and JAK-STAT (Reindl et al., 2011). The following research seeks to improve our knowledge of GH signaling by examining the mechanism utilized by GH to perform osmoregulatory actions in euryhaline teleosts.

CHAPTER 2: ALTERATIONS IN GROWTH HORMONE SIGNALING PATHWAYS ASSOCIATED WITH SMOLTIFICATION AND SEAWATER ADAPTATION IN ATLANTIC SALMON

Abstract

In has been previously shown that smoltification in Atlantic salmon (*Salmo salar*) is accompanied by a large suite of physiological changes that allow for movement of these fish into SW. It has also previously been shown that GH plays a major role in mediating these changes, and that GH can directly affect expression of key osmoregulatory genes. The molecular mechanism by which GH accomplishes this however, is unknown. In this study, we used Atlantic salmon to identify the cellular signaling pathways activated by GH during smoltification and SW adaptation. Fish were sampled over a period of months (Feb.-May, and July) from a FW group, and were also sampled from two groups (Feb. and May) exposed to a SW challenge. Both FW and SW smolts showed an increase in phosphorylated JAK, STAT, and Akt in the gill, suggesting that activation of JAK-STAT and Akt is the mechanism utilized by GH to mediate changes during smoltification.

Introduction

Euryhaline fishes, including salmonids such as Atlantic salmon (*Salmo salar*), acquire salinity tolerance utilizing a variety of hormonal controls. For Atlantic salmon, an anadromous species that begins life in freshwater, migrates to seawater, then returns to freshwater to spawn, salinity tolerance is acquired through a series of behavioral, physiological, and morphological changes known as smoltification (Hoar, 1988). Considerable research has previously shown that the smoltification process is accompanied by changes in numerous hormones, including growth hormone (GH) and insulin-like growth factor-1 (IGF-I) (Ágústsson et al., 2003;

Stefansson et al., 2012). It has been previously shown that the natural smoltification process is accompanied by increased levels of GH and IGF-I in Atlantic salmon (Björnsson et al., 2011; McCormick et al., 2007). It has also been shown that the increase in GH is not present in a landlocked strain of Atlantic salmon over the same developmental period (Nilsen et al., 2008). This lack of an increase in GH is associated with poor salinity tolerance, which suggests that GH plays a key role in the smoltification process. This is further supported by the fact that GH appears to be the first hormone to increase due to photoperiod, a major trigger in the timing of the smoltification process (McCormick et al., 1995).

This increased salinity tolerance is accomplished through modification of ion transport. Primarily, this ion transport is performed by a specialized cell type in the gill called ionocytes (also known as chloride cells or mitochondrion-rich cells). This ability to move between ion uptake and ion secretion is what allows euryhaline teleosts to maintain control of their internal ion concentration (Evans et al., 2005). It has been previously shown that treatment with GH increases salinity tolerance (Richman, 1987; McCormick, 1996), however, the mechanism by which GH accomplishes this has yet to be determined. The aim of this study was to examine the effects that GH has on salinity tolerance in Atlantic salmon, and to elucidate the mechanism by which GH accomplishes these changes.

Materials and methods

Animals

Atlantic salmon parr were obtained from the White River National Fish Hatchery (Bethel, VT., USA) and transferred to the Conte Anadromous Fish Research Center (Turners Falls, MA., USA). Fish were maintained in 1.6 m diameter tanks supplied with Connecticut River water at a

flow rate of 6–8 L min−1 under natural photoperiod and ambient temperature, and fed to satiation twice daily.

Experiments were conducted in two parts. First, samples were taken on a monthly basis from both parrs and smolts kept in FW. These samples were taken from February through May, as well as July. Second, two groups of parrs and smolts were exposed to a SW challenge. The first group was exposed to full strength SW in February, while the second group was exposed to full strength SW in May. Samples were taken from these fish at 0, 6, 24, and 48 hours after SW exposure.

RNA extraction and analysis

Total RNA was extracted using RNAzol® (Molecular Research Center, Inc., Cincinnati, OH, USA) as specified by the manufacturer's protocol. Each RNA pellet was resuspended in 35–200 µl RNase-free deionized water and quantified by NanoDrop1000 spectrophotometry (A260) (Thermo Scientific, West Palm Beach, FL, USA). RNA samples were then stored at -80° C until further analysis. mRNA was reverse transcribed in 10 µl reactions using 200 ng total RNA and qScript cDNA Synthesis Kit reagents (optimized buffer, magnesium, oligo(dT)primers, random primers, dNTPs, reverse transcriptase) according to the manufacturer's protocol (Quanta Biosciences, Gaithersburg, MD, USA). 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. mRNA levels of IGF-I, IGF-II, IGFR, GHR1, GHR2A, GHR2B, CFTR1, CFTR2, NKA-α1A, NKA-α1B, GHSR, and βActin were determined by quantitative real-time PCR. Briefly, real-time reactions were carried out for samples and no-template controls using PerfeCta SYBR Green Supermix, Low Rox (Quanta Biosciences, Gaithersburg, MD, USA). Reactions contained 2 µl cDNA from the reverse transcription

reactions and 8 µl SYBR Green reaction mix. Cycling parameters were set as follows: 95° C for 10 min and 45 cycles of 95° C for 30s and 55-60° C, depending on primers, for 1 min.

Western blotting

Tissues were homogenized in 250 µl 1x cell lysis buffer (Cell Signaling Technology, Beverly, MA) with 1x protease inhibitor (Calbiochem, San Diego, CA, USA), and 1x phosphatase inhibitor (G-Biosciences, St. Louis, MO, USA). The homogenate was incubated on ice for 5 min then centrifuged at 16,000g for 10 min at 4° C. The protein concentration of the supernatant was determined by the Bio-Rad (Hercules, CA, USA) dye-binding method for microplates. Proteins (typically 50 µg) and molecular weight marker (Cell Signaling Technology, Beverly, MA; Catalog No. 7727) were separated by SDS–PAGE (7.5% running gel) and transferred to 0.45 µm nitrocellulose (Bio-Rad Laboratories) for western analysis. Membranes were washed and visualized with chemiluminescence according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK); chemiluminescence was detected directly and the bands quantified with a FluorChem FC2 imager (Alpha Innotech Corp, San Leandro, CA, USA). The abundance of phosphorylated ERK 1/2, Akt, JAK2, STAT5, and PKCα/β II was normalized to total ERK 1/2, Akt, JAK2, STAT5, and β-actin, respectively. All antisera were obtained from Cell Signaling Technology (Beverly, MA).

Data analysis

Data are expressed as means ± S.E.M. Statistical differences are estimated by one-way or two-way ANOVA, as appropriate, followed by Duncan's multiple range test using SigmaStat (SPSS, Chicago, IL). A probability level of 0.05 is used to indicate significance.

Results

NKA activity levels

Na^+/K^+-ATPase (NKA) activity levels increased from March to May in FW smolts, then decreased to near February levels in July in FW smolts, as compared to parrs (Fig. 1). The highest level of NKA activity was observed in May.

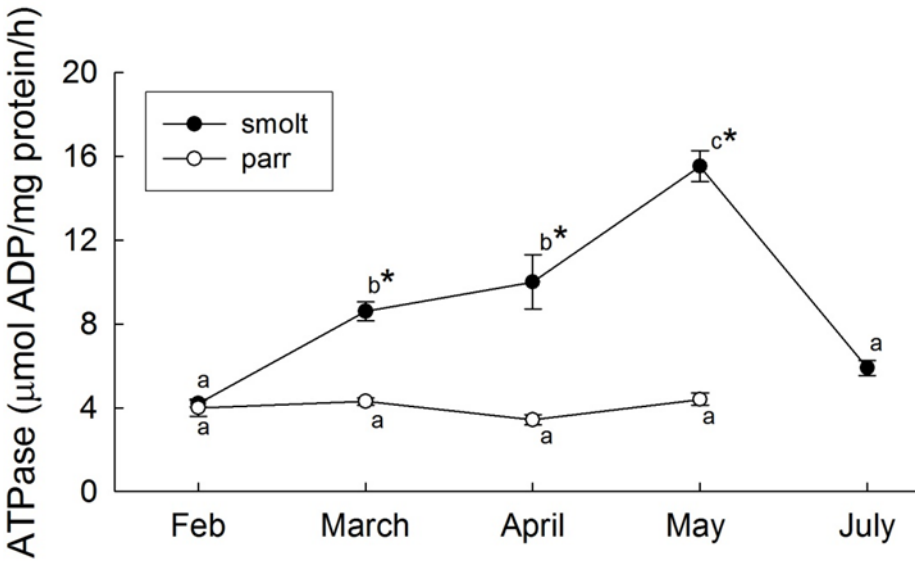

Figure 2. Gill NKATPase activity levels over the normal smoltification period. Parrs and smolts were kept in FW over the course of the normal smoltification period (Feb.-July). Fish were sampled once a month. Data are expressed as mean ±s.e.m. (n=6). Within a group (parrs or smolts), months with different letters are significantly different ($p<0.05$) from each other; * indicates a significant difference ($p<0.05$) between parrs and smolts within the same month. Assay performed as described by McCormick 1993.

GHR mRNA expression levels

Freshwater (FW) levels of GH receptor 2a (GHR2a), and GH receptor 2b (GHR2b) mRNA in the gill increased significantly in smolts from February to May, as compared to parrs, and then decreased slightly in July (Fig. 2A). GH receptor 1 (GHR1) also showed significant increase in the gill, though only from March to May, then a slight decrease in July (Fig. 2A).

GHR2a showed the highest increase of the GHRs, and all three GHRs exhibited highest expression in May in the gill. FW levels of GHR2a in the liver increased significantly in smolts from February to May as compared to parrs, while GHR2b and GHR1 increased significantly from March to May (Fig. 2B). All three GHRs showed a slight decrease from May to July in the liver (Fig. 2B). In the liver, all three GHRs showed their highest expression in May, with GHR2a showing the highest expression of the three. FW levels of GHR2a and GHR2b in white muscle increased significantly in smolts from February to May when compared to parrs, with GHR1 increasing significantly from April to May (Fig. 2C). All three GHRs showed a slight decrease from May to July in white muscle (Fig. 2C). All three GHRs showed their highest expression in May, in white muscle, with GHR2a showing the highest increase of the three.

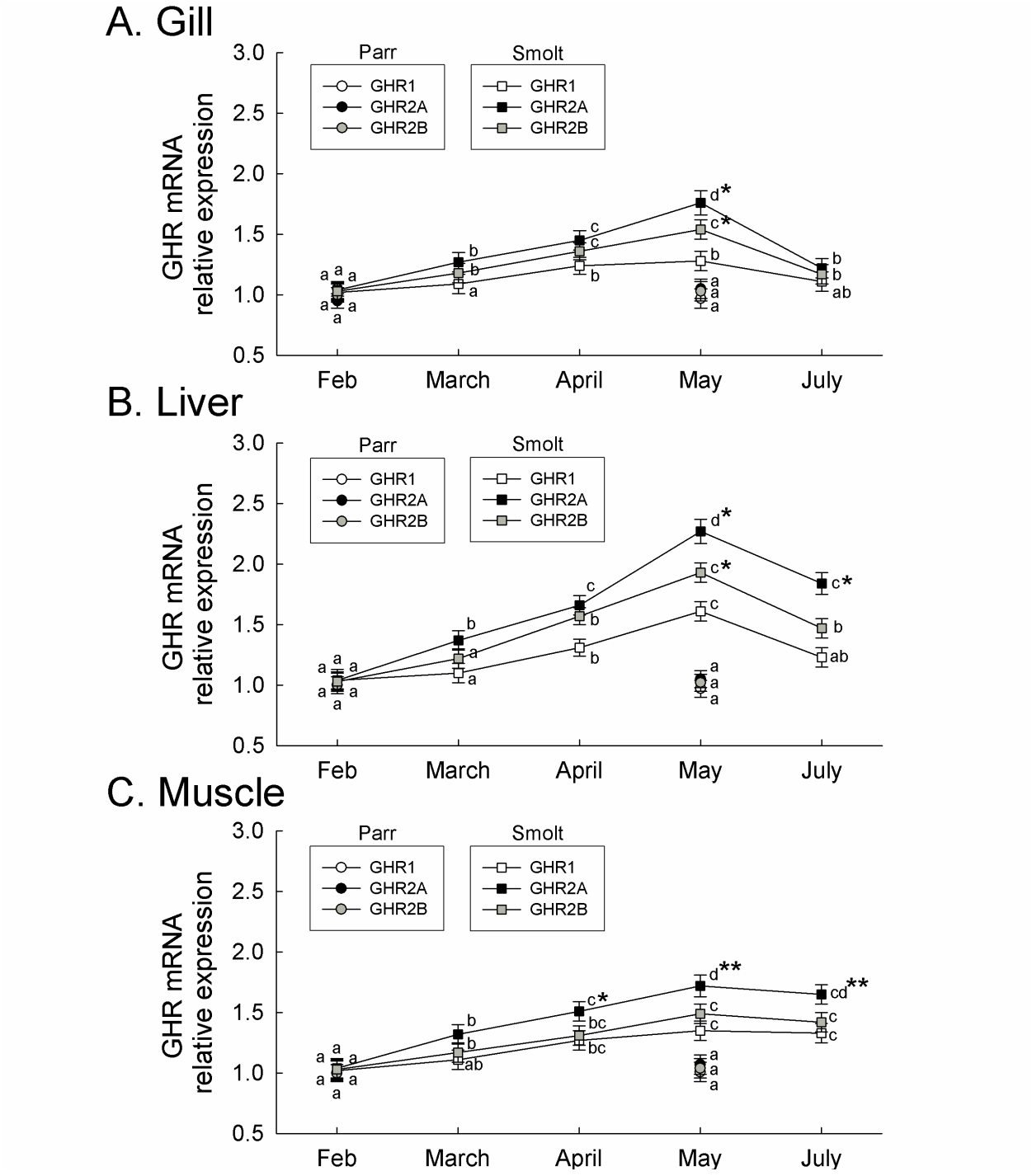

Figure 3. GHR mRNA expression levels in the gill (A), liver (B), and muscle (C) over the normal smoltification period. Parrs and smolts were kept in FW over the course of the normal smoltification period (Feb.-July). Fish were sampled once a month. Data are expressed as mean ±s.e.m. (n=6). Within a group (parrs or smolts), months with different letters are significantly different ($p<0.05$) from each other; * indicates a significant difference (*=$p<0.05$, **=$p<0.01$) between GHR subtypes within the same month.

Seawater (SW) levels of GHR2a were elevated after 6, 24, and 48 hours in the gill in February smolts, while GHR2b was elevated at 24 and 48 hours (Fig. 3A). GHR1 did not show significant increase in gill in February. In May smolts, SW levels of GHR2a and GHR2b were elevated at 6, 24, and 48 hours in gill (Fig. 3B). GHR1 showed no significant increase in May in the gill. In both February and May, GHR2a and GHR2b showed their highest expression at 24 hours. SW levels of GHR2a, GHR2b, and GHR1 in the liver showed no significant increase in February smolts (Fig. 3C). May SW smolts exhibited elevated GHR2a at 24 and 48 hours, along with elevated GHR2b at 24 hours (Fig. 3D). GHR1 showed no significant increase in May SW smolts. May SW levels of GHR2a and GHR2b were at their highest at 24 hours in liver. SW levels of GHR2a in white muscle were elevated at 24 hours in February smolts, with GHR2b and GHR1 showing no significant increase (Fig. 3E). In May SW smolts, GHR2a was elevated at 24 hours in white muscle, with GHR2b and GHR1 showing no significant increase (Fig. 3F).

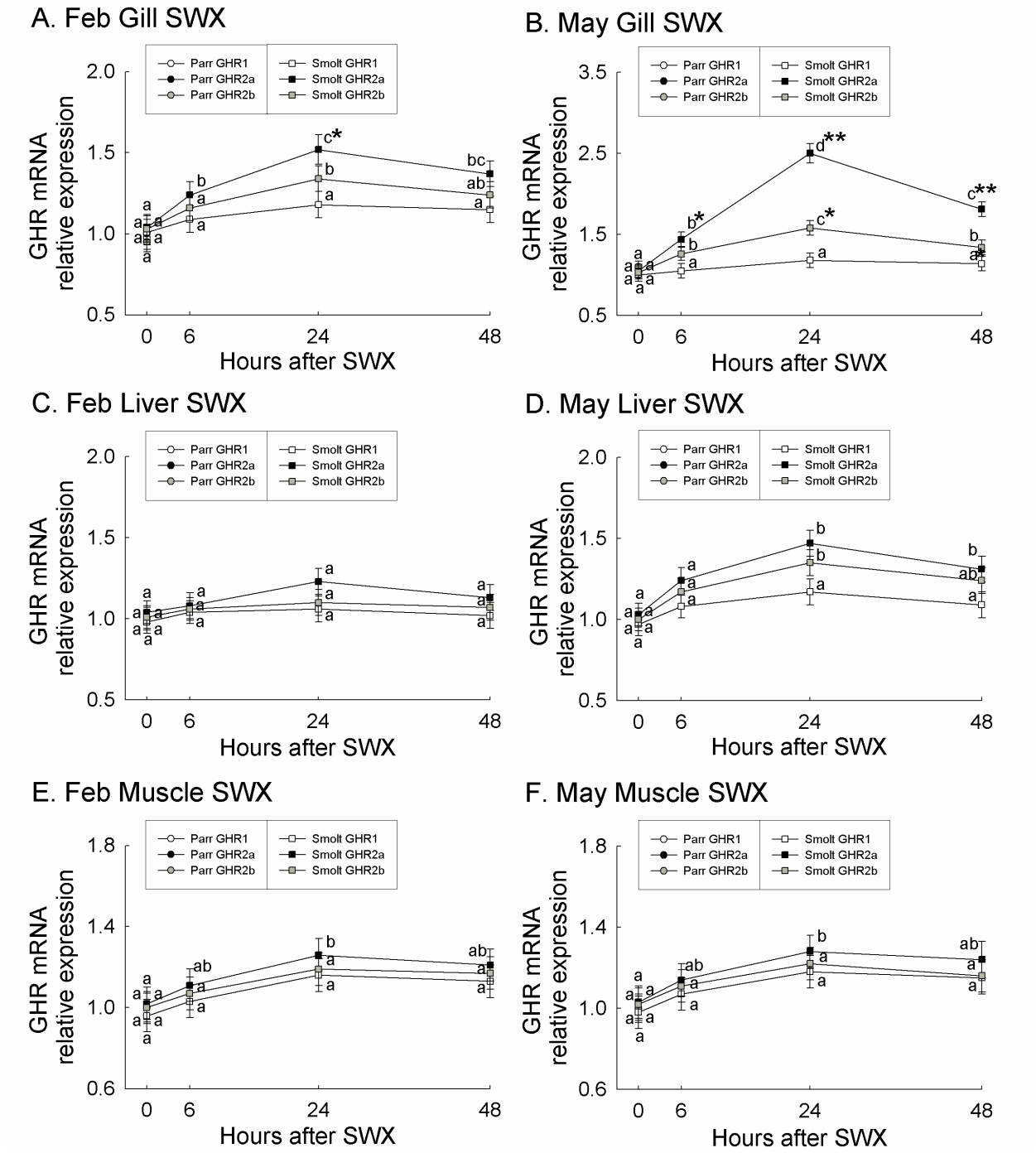

Figure 4. GHR mRNA expression levels in gill (A, B), liver (C, D), and muscle (E, F) during 48 hour SW challenge (SWX). In two separate trials, one in Feb. and one in May, parrs and smolts were exposed to full strength SW for 48 hours. Fish were sampled at 0, 6, 24, and 48 hours. Data are expressed as mean ±s.e.m. (n=6). Within a group (parrs or smolts), times with different letters are significantly different ($p<0.05$) from each other; * indicates a significant difference (*=$p<0.05$, **=$p<0.01$) between GHR subtypes within the same time.

NKA α1A/1B mRNA expression levels

FW levels of NKAα1B mRNA in the gill increased from March to May in smolts, as compared to parrs (Fig. 4C). Conversely, FW levels of NKAα1A in the gill decreased from March to May in smolts (Fig. 4C). Both NKAα1A and NKAα1B mRNA levels had returned to near February levels in July. February SW smolts showed decreased levels of NKAα1A mRNA at 24 and 48 hours, as well as increased levels of NKAα1B at 24 hours in the gill (Fig. 5E). May SW smolts showed decreased levels of NKAα1A mRNA at 48 hours, as well as increased NKAα1B mRNA levels at 6, 24, and 48 hours, with the highest expression peaking at 24 hours (Fig. 5F).

CFTR and NKCC mRNA expression levels

FW levels of cystic fibrosis transmembrane conductance regulator 1 (CFTR1) mRNA in the gill increased from February to May in smolts, as compared to parrs, then decreased from May to July (Fig. 4A). FW levels of CFTR2 mRNA showed no significant change (Fig. 4A). SW mRNA levels of CFTR1 in the gill were elevated at 24 hours in February smolts (Fig. 5A). SW mRNA levels of CFTR2 were elevated at 6 and 24 hours in February smolts (Fig. 5A). In May SW smolts, CFTR1 mRNA levels were elevated at 6, 24, and 48 hours, with the highest elevation present at 24 hours (Fig. 5B). May SW smolts exhibited no significant change in CFTR2 mRNA levels.

FW levels of $Na^+/K^+/2Cl^-$ cotransporter (NKCC) mRNA in the gill increased from March to May in smolts, as compared to parrs, followed by a decrease from May to July (Fig. 4B). SW mRNA levels of NKCC in the gill were elevated at 24 hours in February smolts (Fig. 5C). In May SW smolts, NKCC mRNA levels were elevated at 6, 24, and 48 hours, with the highest

elevation present at 24 hours (Fig. 5D).

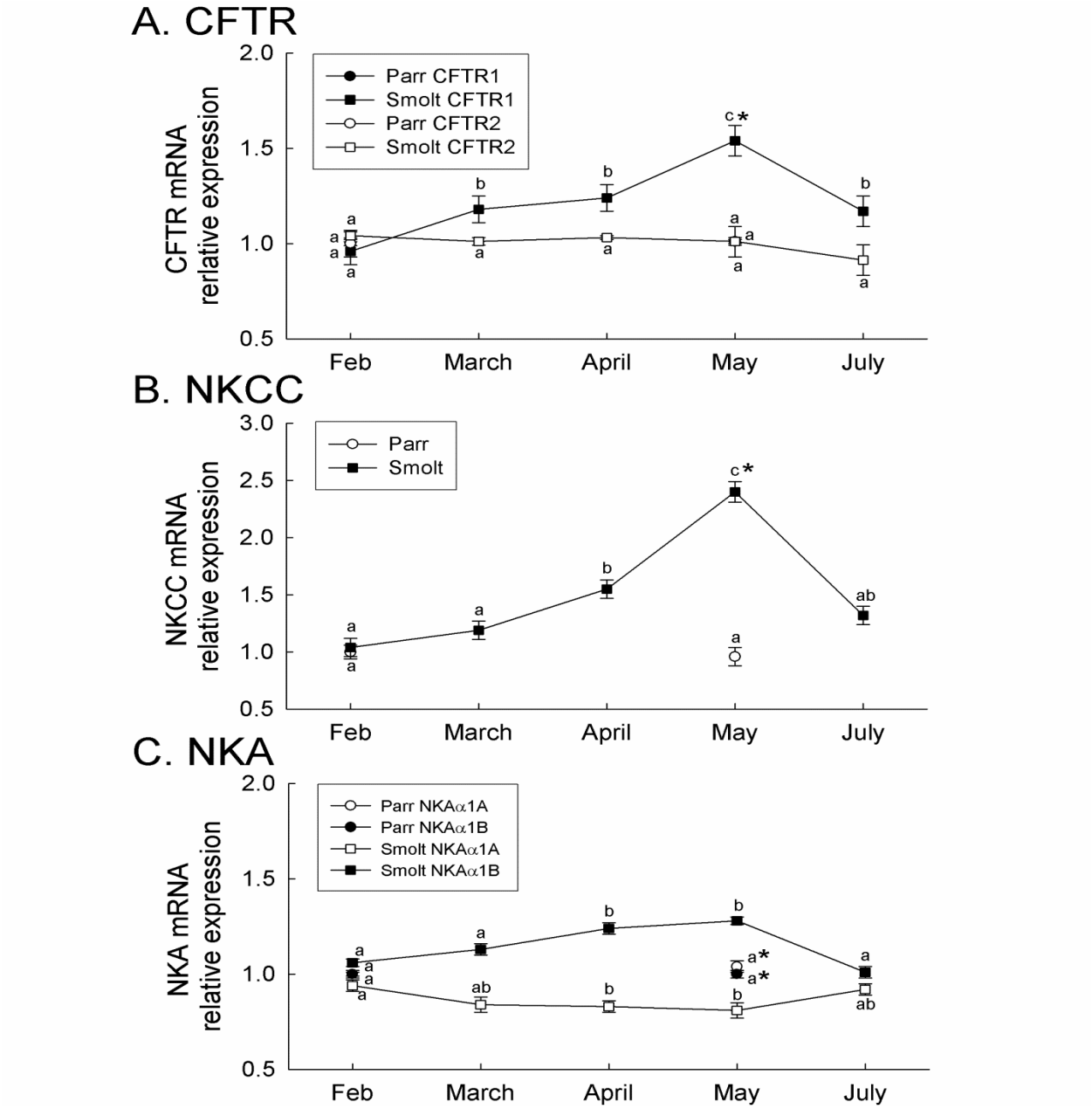

Figure 5. CFTR (A), NKCC (B), and NKA (C) mRNA expression levels in the gill over the normal smoltification period. Parrs and smolts were kept in FW over the course of the normal smoltification period (Feb.-July). Fish were sampled once a month. Data are expressed as mean ±s.e.m. (n=6). Within a group (parrs or smolts), months with different letters are significantly different ($p<0.05$) from each other; * indicates a significant difference ($p<0.05$) between parrs and smolts within the same month.

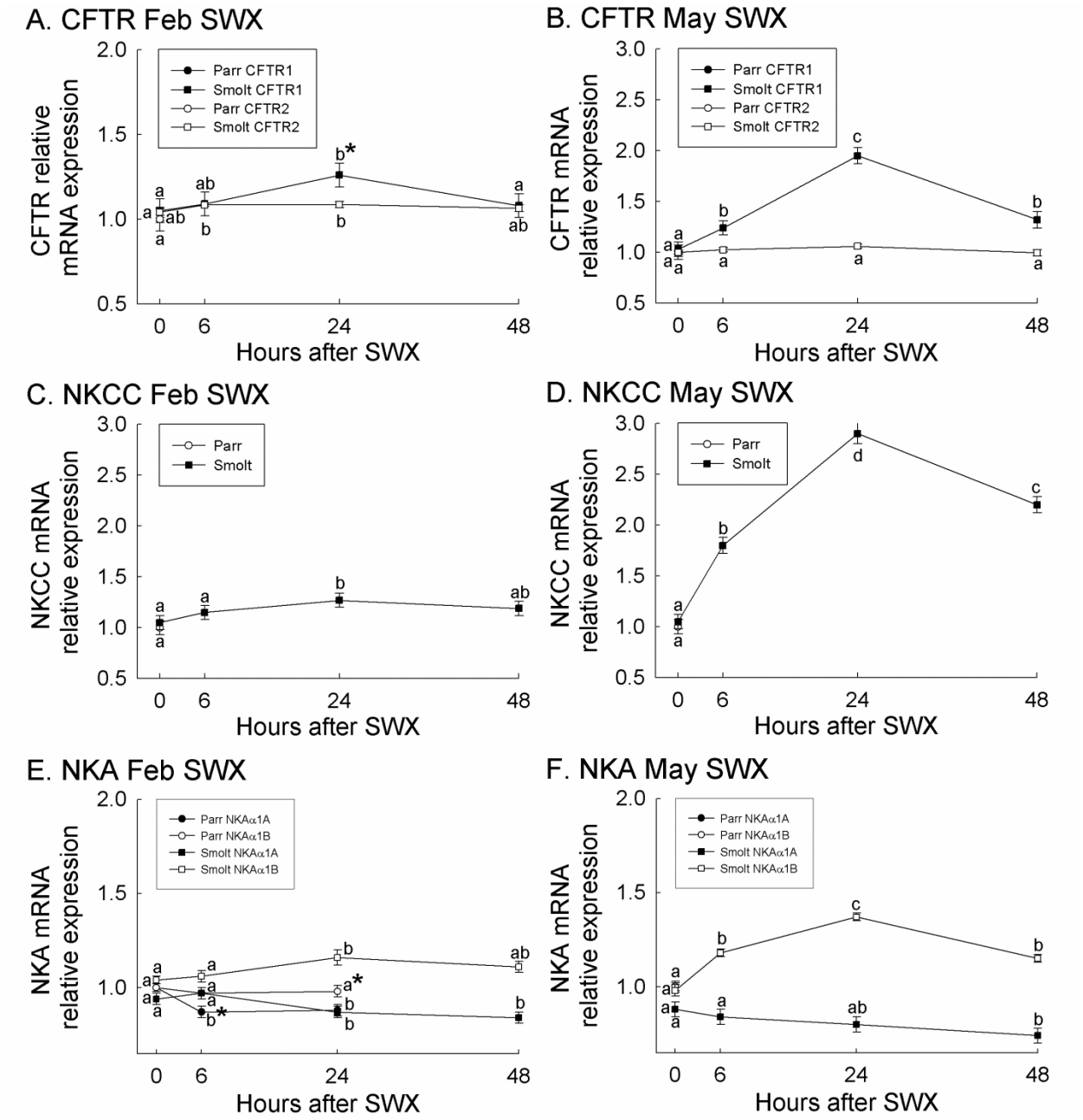

Figure 6. CFTR (A, B), NKCC (C, D), and NKA (E, F) mRNA expression levels in the gill during 48 hour SW challenge (SWX). In two separate trials, one in Feb. and one in May, parrs and smolts were exposed to full strength SW for 48 hours. Fish were sampled at 0, 6, 24, and 48 hours. Data are expressed as mean ±s.e.m. (n=6). Within a group (parrs or smolts), times with different letters are significantly different ($p<0.05$) from each other; * indicates a significant difference ($p<0.05$) between parrs and smolts within the same time.

GHSR mRNA expression levels

FW levels of ghrelin receptor (GHSR) mRNA in the gill increased from March to May in smolts, as compared to parrs, followed by a decrease from May to July (Fig. 6A). FW levels of GHSR mRNA in the liver increased from February to May in smolts, as compared to parrs, and also showed a decrease from May to July (Fig. 6B). FW levels of GHSR mRNA in white muscle increased from March to May, followed by a decrease from May to July (Fig. 6C). All three tissues exhibited highest expression of GHSR mRNA in May.

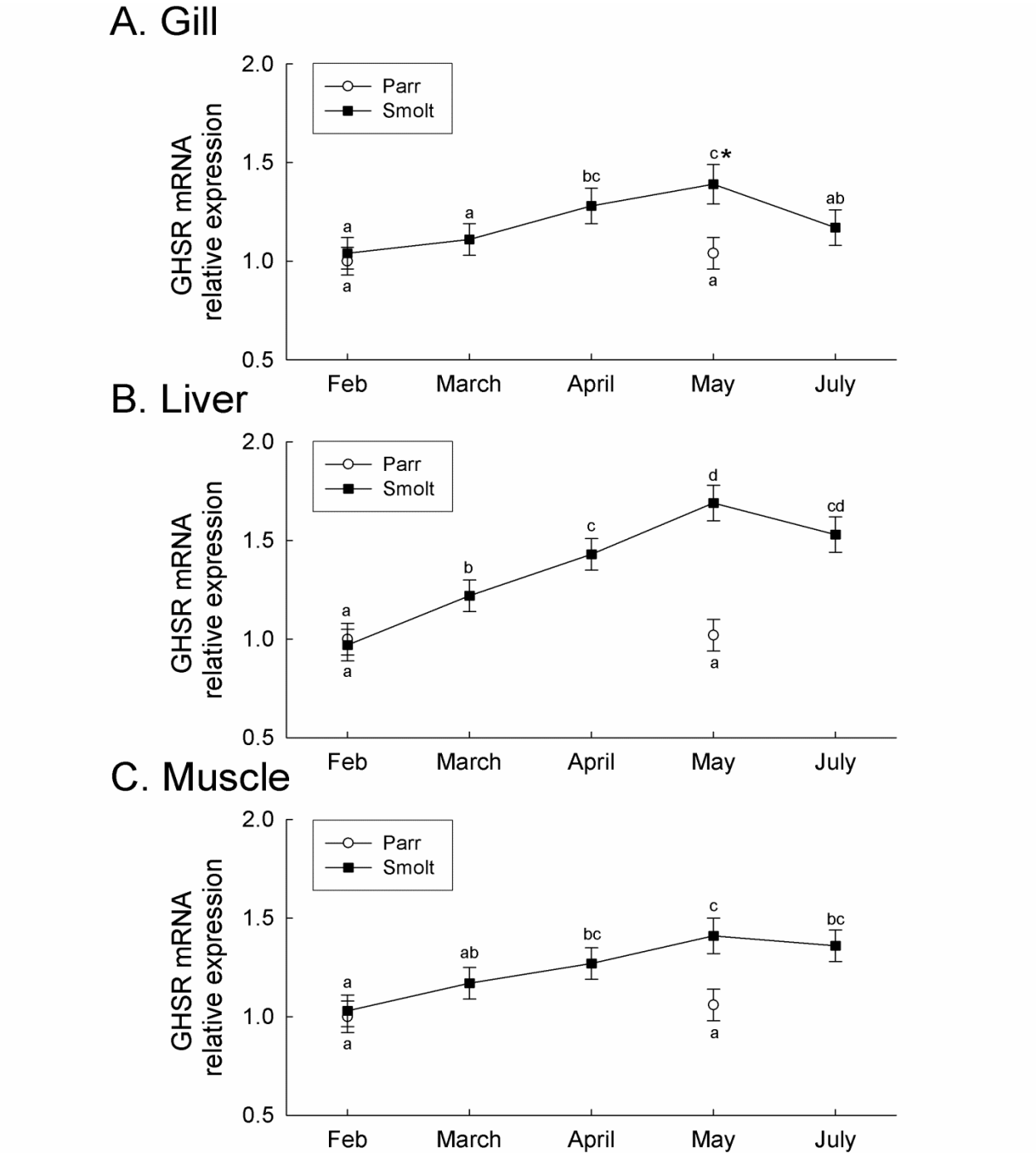

Figure 7. GHSR mRNA expression levels in gill (A), liver (B), and muscle (C) over the normal smoltification period. Parrs and smolts were kept in FW over the course of the normal smoltification period (Feb.-July). Fish were sampled once a month. Data are expressed as mean ±s.e.m. (n=6). Within a group (parrs or smolts), months with different letters are significantly different ($p<0.05$) from each other; * indicates a significant difference ($p<0.05$) between parrs and smolts within the same month.

SW levels of GHSR mRNA in the gill were elevated at 24 and 48 hours in February smolts (Fig. 7A). In May SW smolts, GHSR mRNA levels were elevated at 6, 24, and 48 hours (Fig. 7B). Both February and May smolts exhibited highest expression at 24 hours. In the liver, GHSR mRNA levels showed no significant increase in February SW smolts (Fig. 7C). In May, SW smolts showed elevated levels of GHSR mRNA at 24 hours in the liver (Fig. 7D). In both February and May SW smolts, white muscle showed elevated levels of GHSR mRNA at 24 hours (Fig. 7E, F).

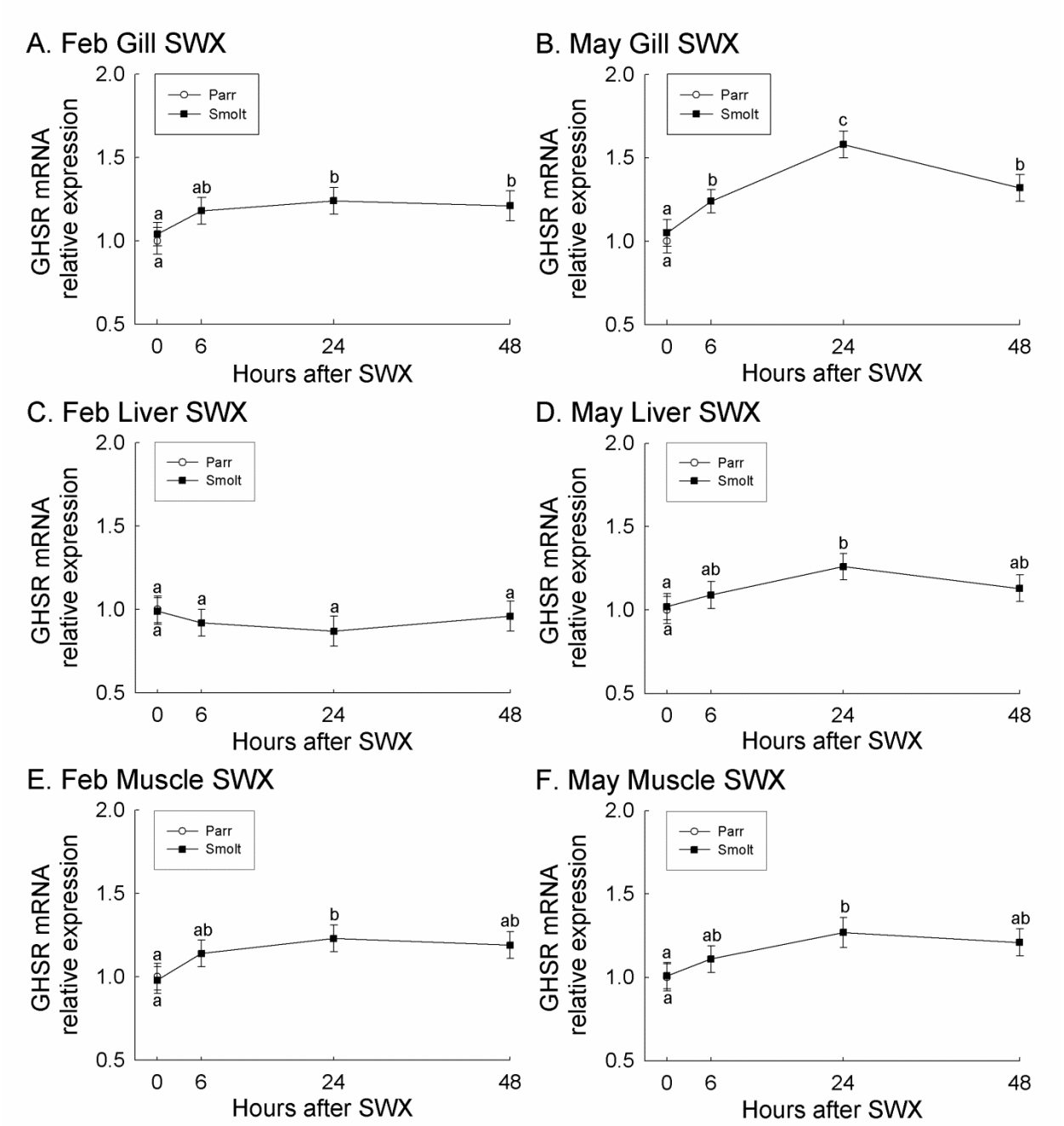

Figure 8. GHSR mRNA expression in gill (A, B), liver (C, D), and muscle (E, F) during 48 hour SW challenge (SWX). In two separate trials, one in Feb. and one in May, parrs and smolts were exposed to full strength SW for 48 hours. Fish were sampled at 0, 6, 24, and 48 hours. Data are expressed as mean ±s.e.m. (n=6). Within a group (parrs or smolts), times with different letters are significantly different ($p<0.05$) from each other.

IGF-I and II mRNA expression levels

FW levels of IGF-I mRNA in the gill, liver, and white muscle increased from March to May in smolts, as compared to parrs, then decreased from May to July (Fig. 8A ,B, C). FW levels of IGF-II mRNA in liver and white muscle increased from March to May, while IGF-II mRNA in the gill increased from April to May (Fig. 8B, C, A). IGF-II expression in all three tissues showed a decrease from May to July. IGF-I and II both showed the highest levels of expression in May in all tissues.

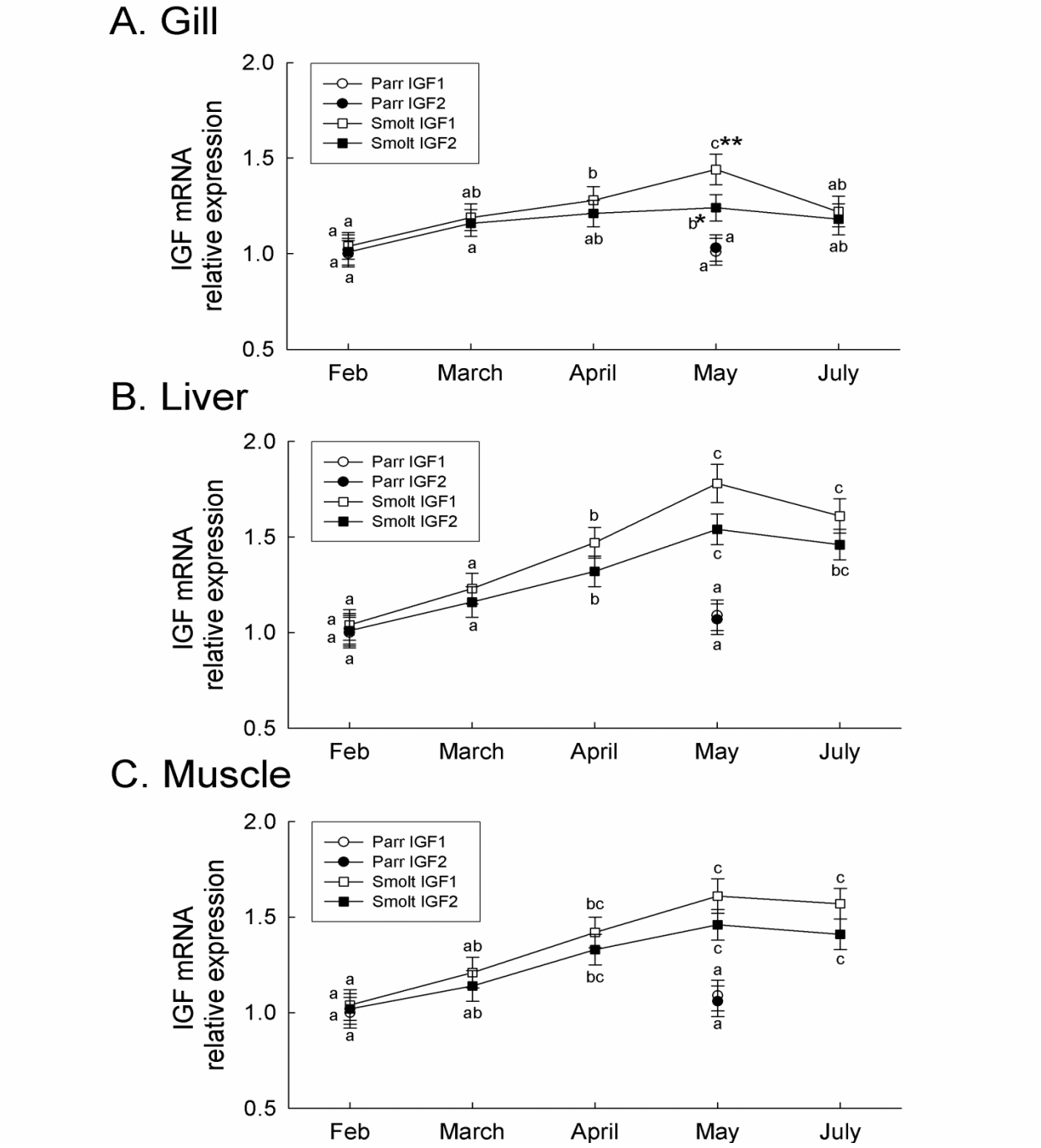

Figure 9. IGF mRNA expression in gill (A), liver (B), and muscle (C) over the normal smoltification period. Parrs and smolts were kept in FW over the course of the normal smoltification period (Feb.-July). Fish were sampled once a month. Data are expressed as mean ±s.e.m. (n=6). Within a group (parrs or smolts), months with different letters are significantly different ($p<0.05$) from each other; * indicates a significant difference (*=$p<0.05$, **=$p<0.01$) between parrs and smolts within the same month.

SW levels of IGF-I mRNA in white muscle were elevated at 24 and 48 hours in February smolts (Fig. 9E). February SW smolts also showed a decrease in IGF-II mRNA levels at 24 hours in white muscle (Fig. 9E). May SW smolts showed decreased levels of both IGF-I and II in white muscle at 24 and 48 hours (Fig. 9F). In the gill, February SW smolts showed elevated levels of IGF-I mRNA at 24 hours, with no change in levels of IGF-II mRNA (Fig. 9A). May SW smolts showed elevated levels of IGF-I mRNA at 6, 24, and 48 hours in the gill, along with elevated levels of IGF-II mRNA at 24 hours (Fig. 9B). In the liver, February SW smolts showed no increase in either IGF-I or IGF-II mRNA levels (Fig. 9C). In May SW smolts, there was an increase in IGF-I mRNA levels at 24 hours in the liver, with no significant change in levels of IGF-II mRNA (Fig. 9D).

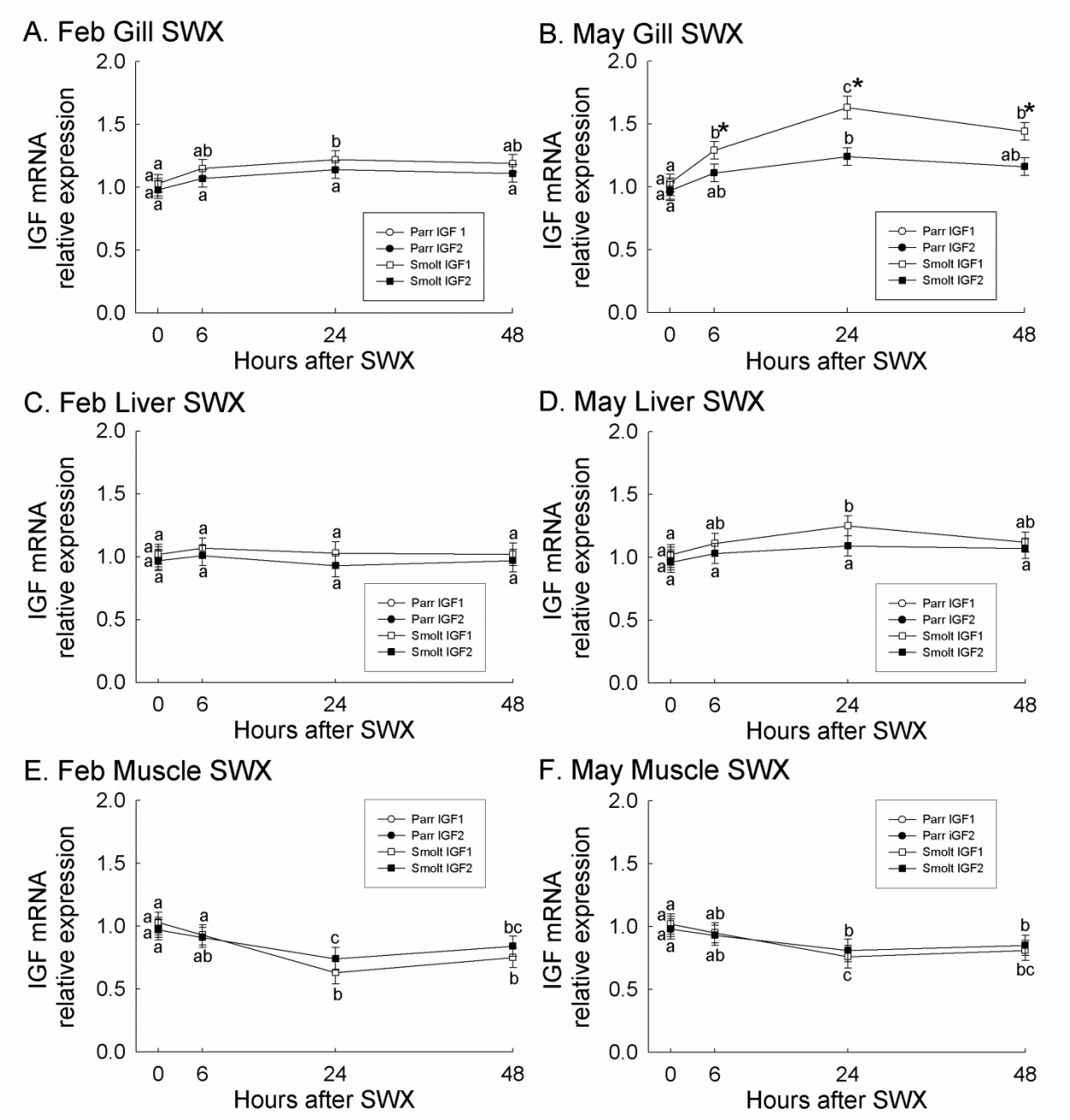

Figure 10. IGF mRNA expression in gill (A, B), liver (C,D), and muscle (E,F) during 48 hour SW challenge (SWX). In two separate trials, one in Feb. and one in May, parrs and smolts were exposed to full strength SW for 48 hours. Fish were sampled at 0, 6, 24, and 48 hours. Data are expressed as mean ±s.e.m. (n=6). Within a group (parrs or smolts), times with different letters are significantly different ($p<0.05$) from each other; * indicates a significant difference ($p<0.05$) between IGF subtypes within the same time.

IGFR mRNA expression levels

FW levels of insulin-like growth factor receptor (IGFR) mRNA increased from March to May in both white muscle and gill, as compared to parrs, then decreased from May to July (Fig. 10B,A). Both gill and white muscle showed the highest levels of IGFR mRNA expression in May.

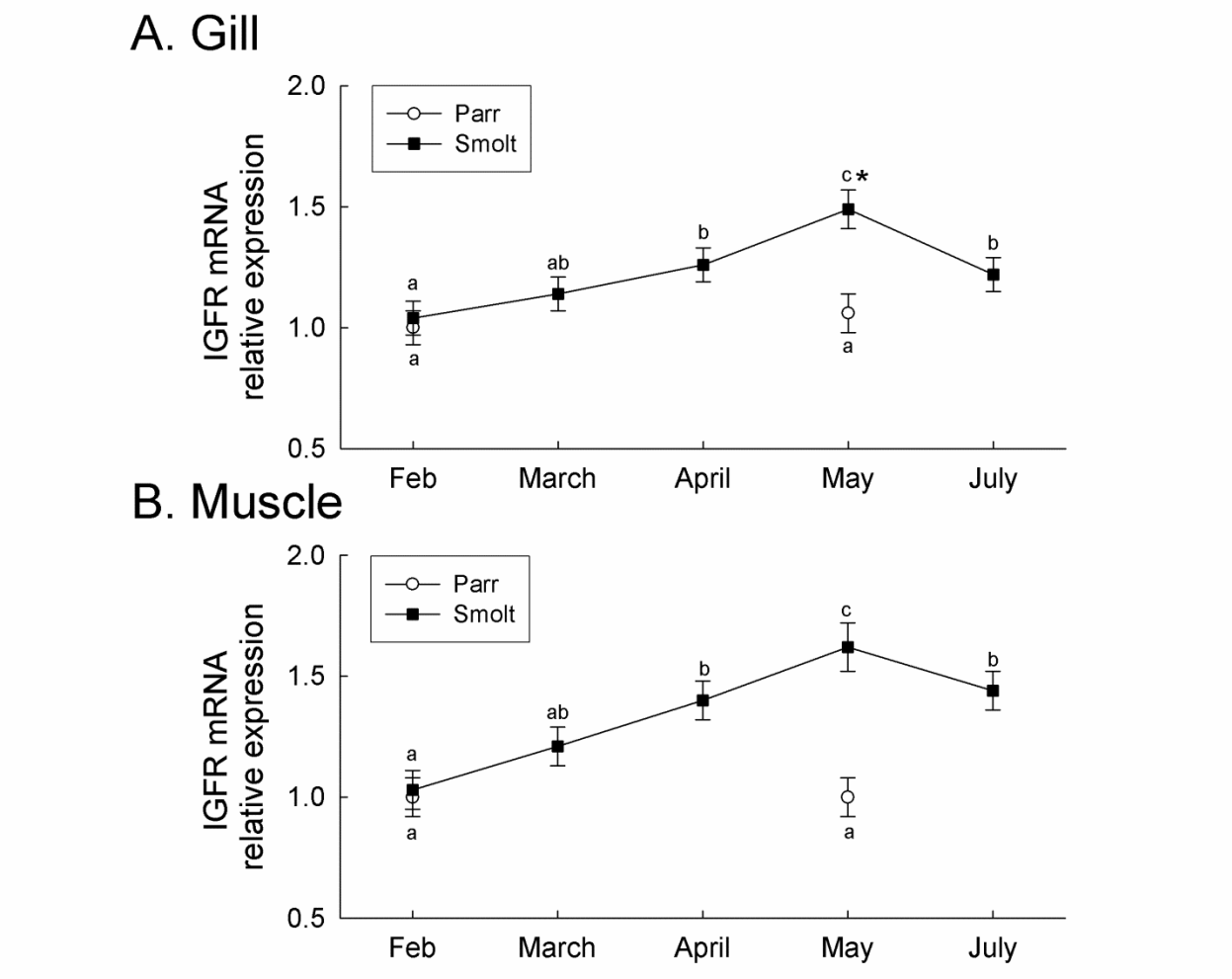

Figure 11. IGFR mRNA expression in gill (A) and muscle (B) over the normal smoltification period. Parrs and smolts were kept in FW over the course of the normal smoltification period (Feb.-July). Fish were sampled once a month. Data are expressed as mean ±s.e.m. (n=6). Within a group (parrs or smolts), months with different letters are significantly different ($p<0.05$) from each other; * indicates a significant difference ($p<0.05$) between parrs and smolts within the same month.

SW levels of IGFR mRNA in gill and white muscle were elevated at 24 hours in February smolts (Fig. 11A, C). In May SW smolts, gill showed elevated levels of IGFR mRNA at 6, 24, and 48 hours, with the highest elevation showing at 24 hours (Fig. 11B). May SW smolts also showed elevated levels of IGFR mRNA in white muscle at 24 hours (Fig. 11D).

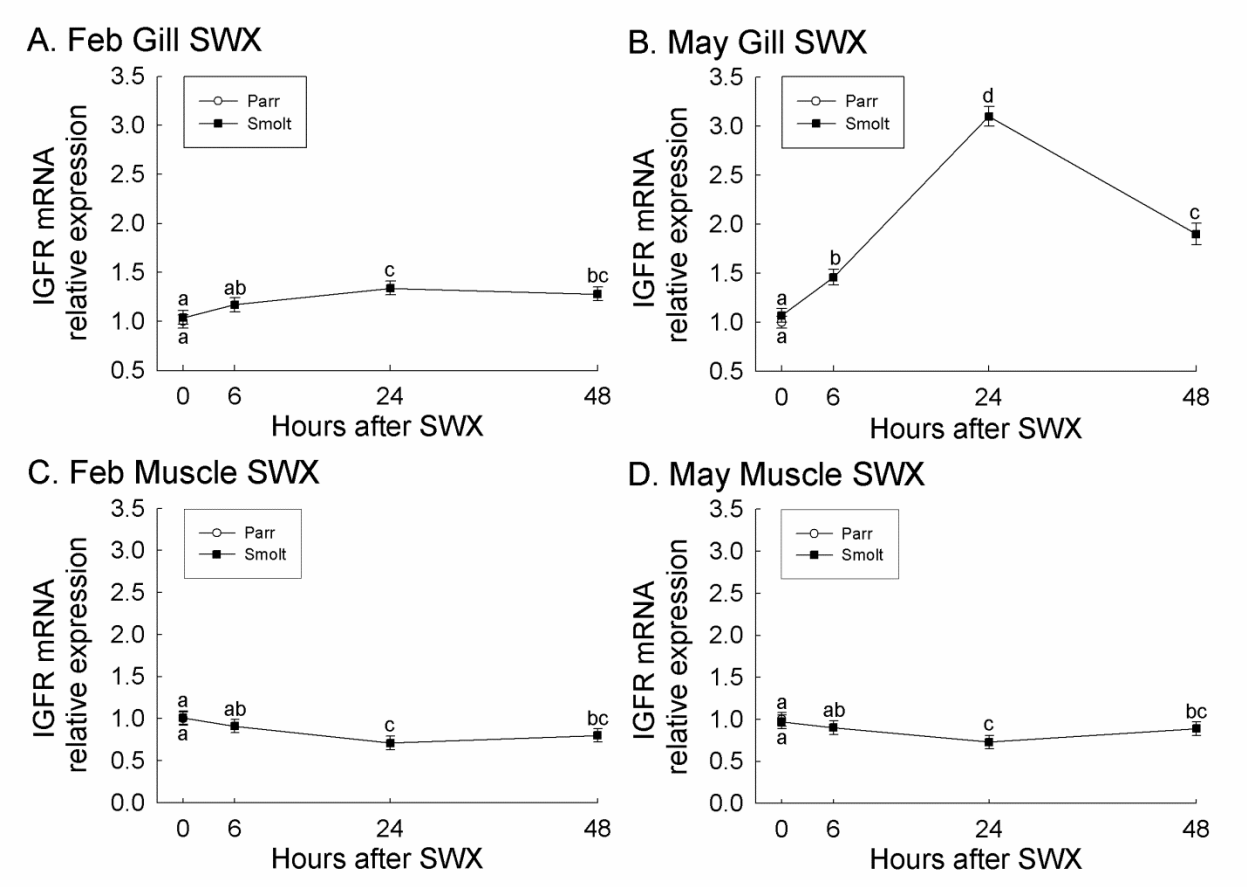

Figure 12. IGFR mRNA expression in gill (A, B) and muscle (C, D) during 48 hour SW challenge (SWX). In two separate trials, one in Feb. and one in May, parrs and smolts were exposed to full strength SW for 48 hours. Fish were sampled at 0, 6, 24, and 48 hours. Data are expressed as mean ±s.e.m. (n=6). Within a group (parrs or smolts), times with different letters are significantly different ($p<0.05$) from each other.

Abundance of phosphorylated JAK

FW smolts exhibited elevated levels of phosphorylated JAK from February to July in the gill, and from March to July in the liver and white muscle, as compared to control (Fig. 12A, B, C). February SW smolts showed an increase in phosphorylated JAK at 24 hours in the gill (Fig. 13A). February SW smolts showed no increase in phosphorylated JAK in the liver, and white muscle showed a decrease in phosphorylated JAK at 24 and 48 hours (Fig. 13C, E). May SW smolts showed an increase in phosphorylated JAK after 6, 24, and 48 hours in the gill, an increase at 24 hours in the liver, and a decrease at 24 and 48 hours in white muscle (Fig. 13B, D, F).

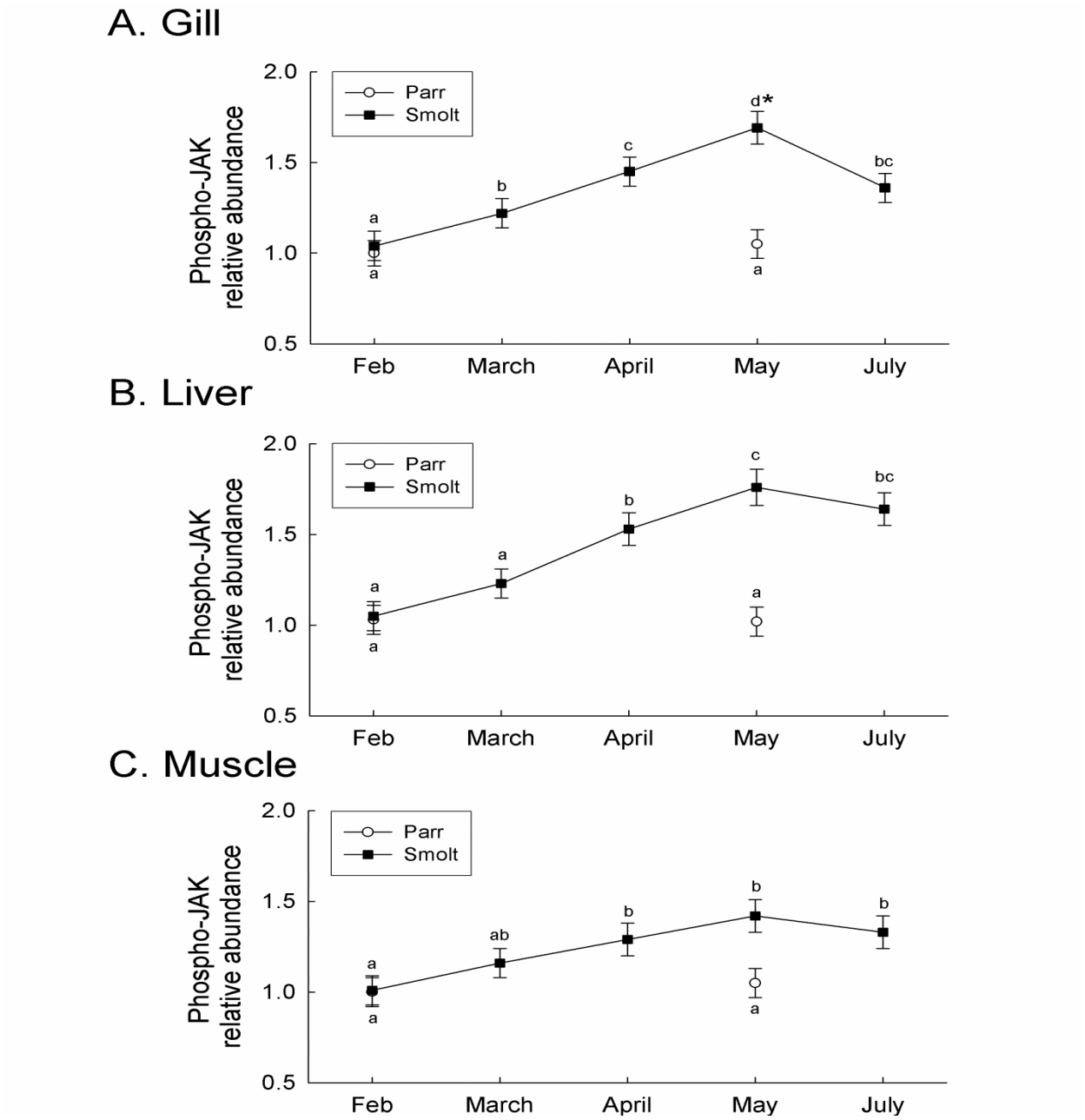

Figure 13. Phospho-JAK2 abundance in gill (A), liver (B), and muscle (C) over the course of the normal smoltification period. Parrs and smolts were kept in FW over the course of the normal smoltification period (Feb.-July). Fish were sampled once a month. Data are expressed as mean ±s.e.m. (n=4). Within a group (parrs or smolts), months with different letters are significantly different ($p<0.05$) from each other; * indicates a significant difference ($p<0.05$) between parrs and smolts within the same month.

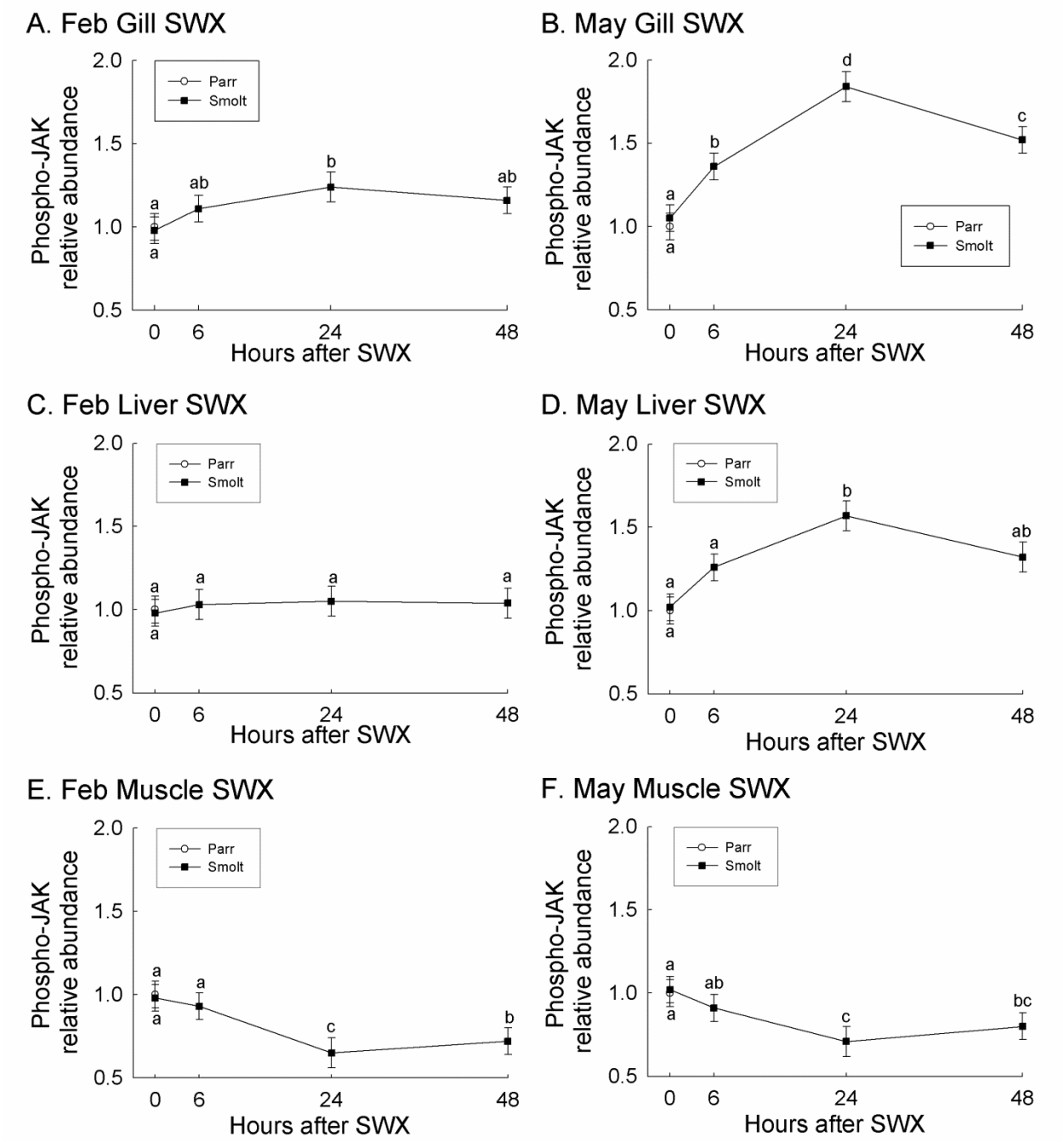

Figure 14. Phospho-JAK2 abundance in gill (A, B), liver (C, D), and muscle (E, F) during 48 hour SW challenge (SWX). In two separate trials, one in Feb. and one in May, parrs and smolts were exposed to full strength SW for 48 hours. Fish were sampled at 0, 6, 24, and 48 hours. Data are expressed as mean ±s.e.m. (n=4). Within a group (parrs or smolts), times with different letters are significantly different ($p<0.05$) from each other.

Abundance of phosphorylated Akt

FW smolts exhibited elevated levels of phosphorylated Akt from March to July, as compared to control, in the liver and white muscle (Fig. 14B, C). FW gill exhibited an increase in phosphorylated Akt in May (Fig. 14A). February SW smolts showed an increase in phosphorylated Akt at 24 hours in the gill, with liver showing no change, and white muscle showing a decrease at 24 and 48 hours (Fig. 15 A, C, E). May SW smolts showed an increase in phosphorylated Akt at 24 and 48 hours in gill, an increase at 24 hours in liver, and a decrease at 24 and 48 hours in white muscle (Fig. 15 B,D,F).

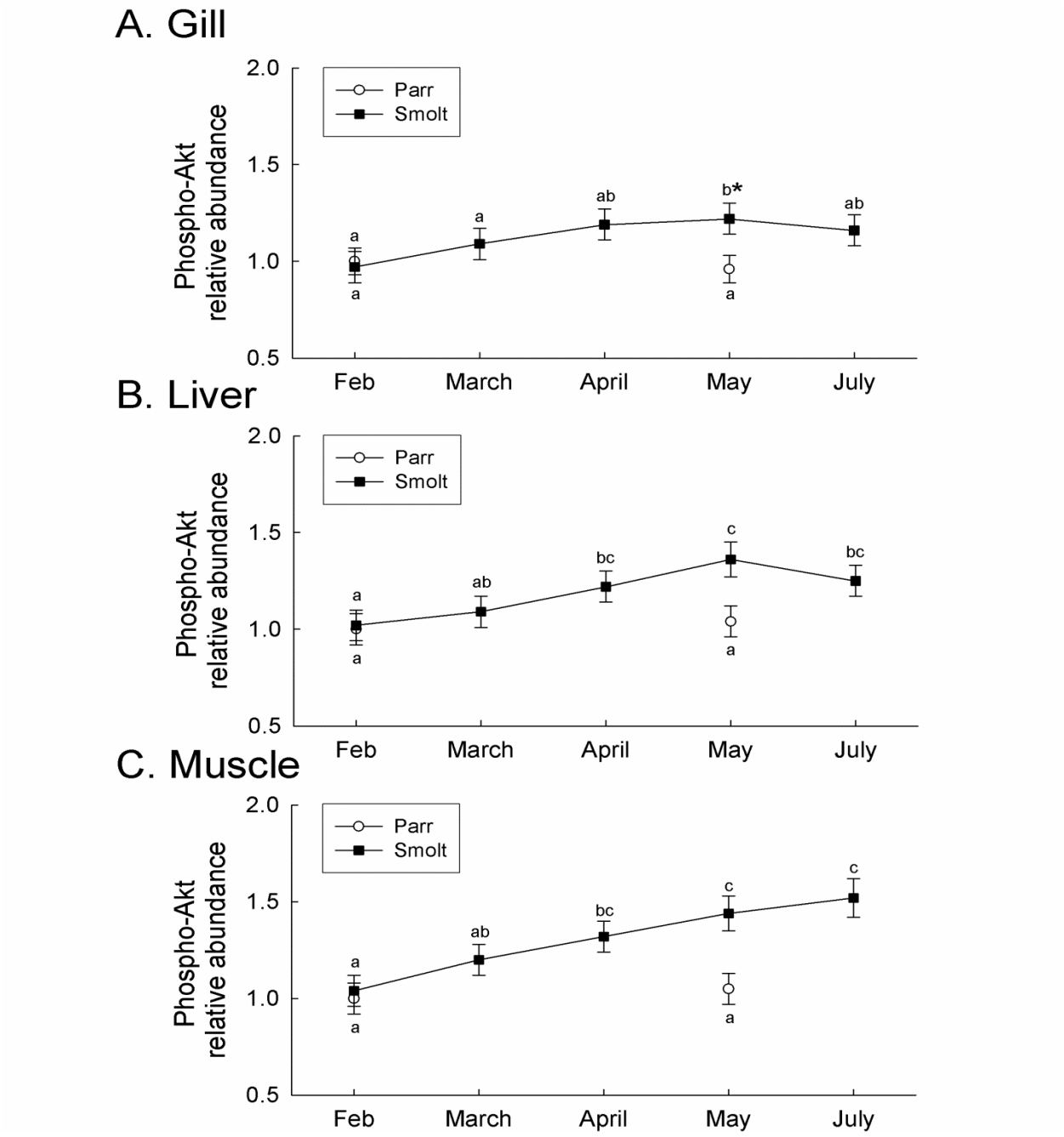

Figure 15. Phospho-Akt abundance in gill (A), liver (B), and muscle (C) over the course of the normal smoltification period. Parrs and smolts were kept in FW over the course of the normal smoltification period (Feb.-July). Fish were sampled once a month. Data are expressed as mean ±s.e.m. (n=4). Within a group (parrs or smolts), months with different letters are significantly different ($p<0.05$) from each other; * indicates a significant difference ($p<0.05$) between parrs and smolts within the same month.

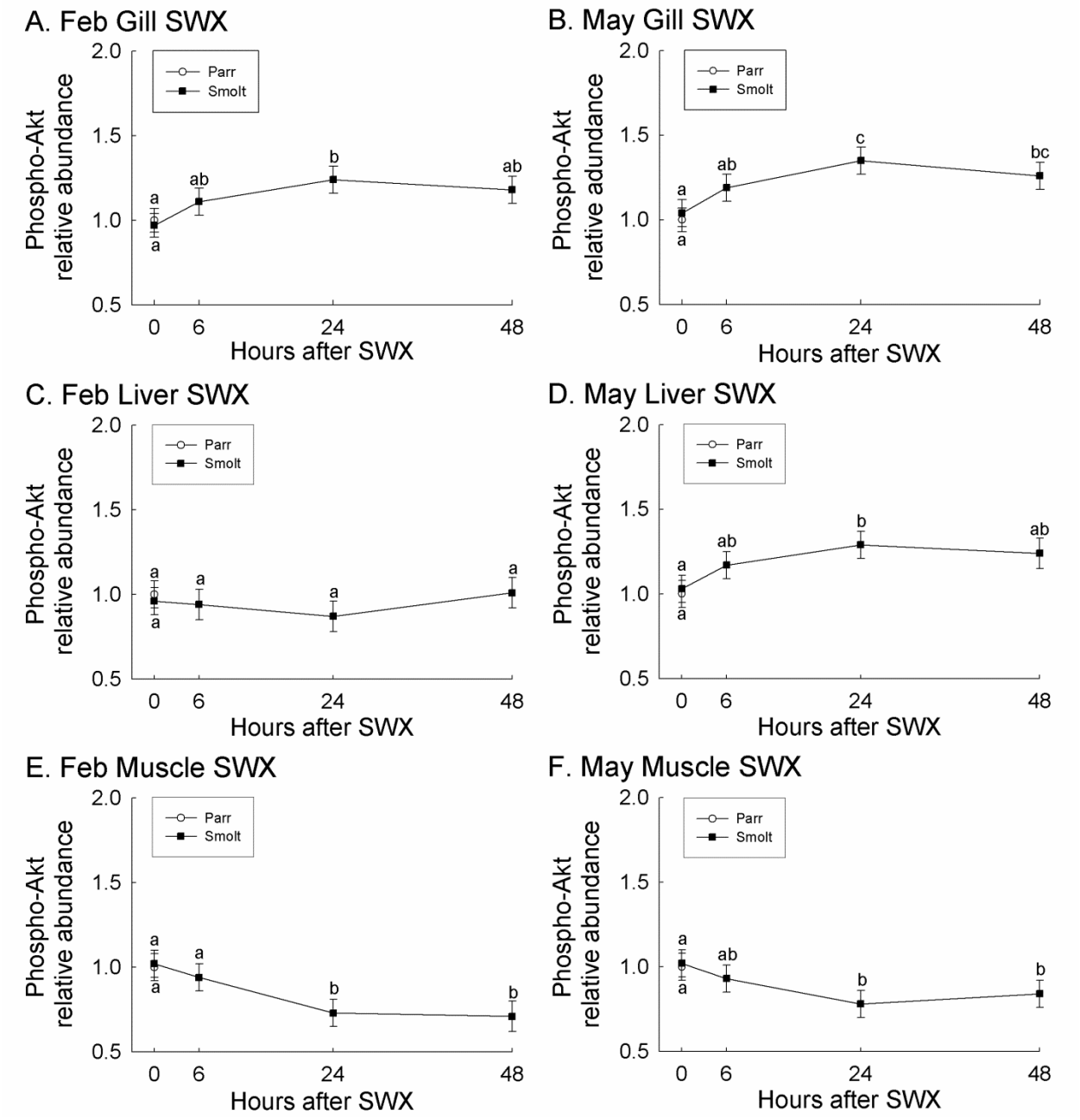

Figure 16. Phospho-Akt abundance in gill (A, B), liver (C, D), and muscle (E, F) during 48 hour SW challenge (SWX). In two separate trials, one in Feb. and one in May, parrs and smolts were exposed to full strength SW for 48 hours. Fish were sampled at 0, 6, 24, and 48 hours. Data are expressed as mean ±s.e.m. (n=4). Within a group (parrs or smolts), times with different letters are significantly different ($p<0.05$) from each other.

Abundance of phosphorylated ERK

FW smolts exhibited an increase in phosphorylated ERK from March to July in white muscle, as compared to control (Fig. 16C). FW smolts also exhibited an increase in phosphorylated ERK in May in the liver, while gill exhibited no significant change (Fig. 16 B, A). February SW smolts showed elevated levels of phosphorylated ERK at 24 hours in the liver, increased levels at 24 and 48 hours in the white muscle, and no significant change in the gill (Fig. 17C,E,A). May SW smolts showed an increase in phosphorylated ERK at 24 and 48 hours in the liver and white muscle, while gill showed no significant change (Fig. 17D, F, B).

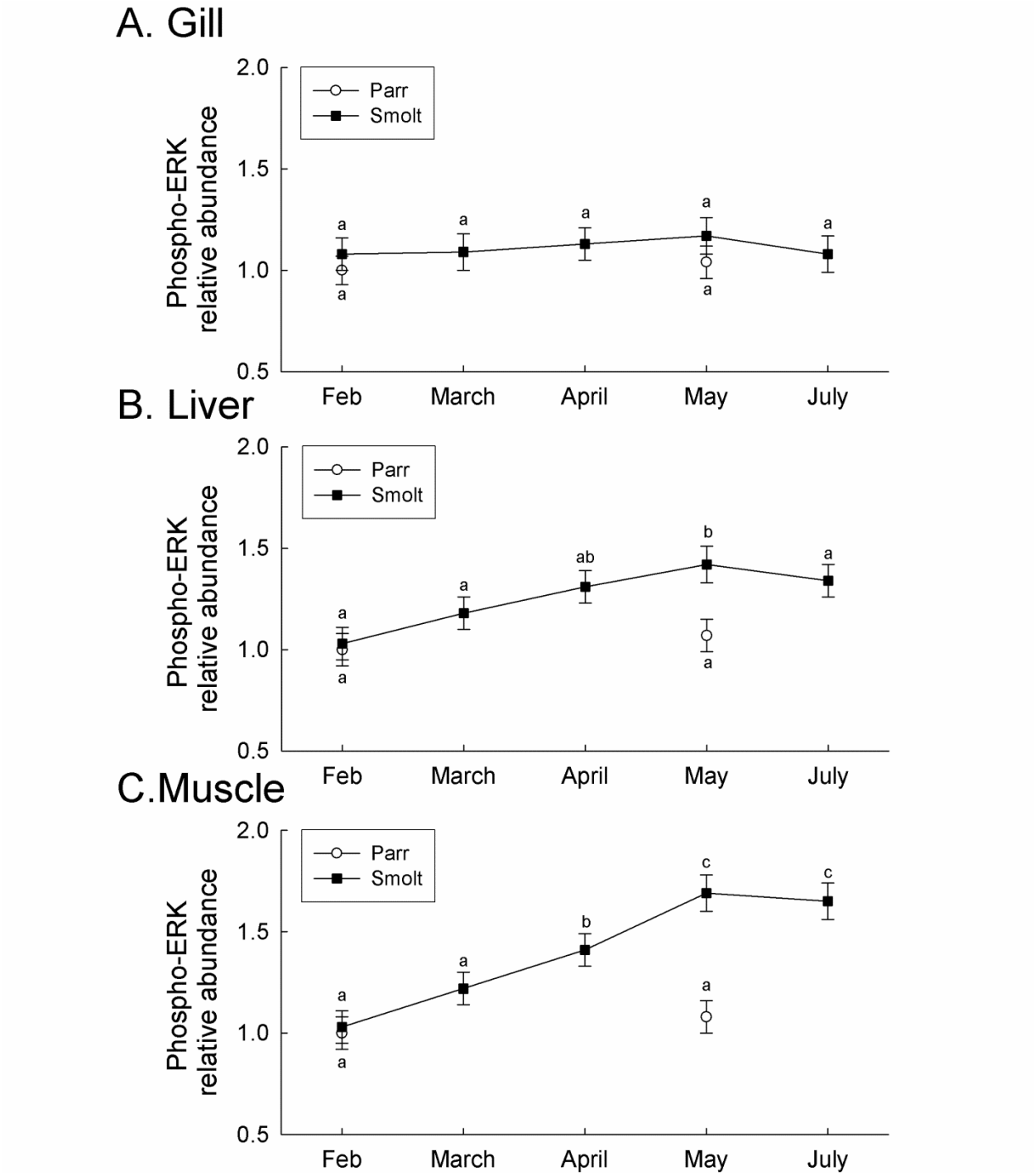

Figure 17. Phospho-ERK abundance in gill (A), liver (B), and muscle (C) over the course of the normal smoltification period. Parrs and smolts were kept in FW over the course of the normal smoltification period (Feb.-July). Fish were sampled once a month. Data are expressed as mean ±s.e.m. (n=4). Within a group (parrs or smolts), months with different letters are significantly different ($p<0.05$) from each other.

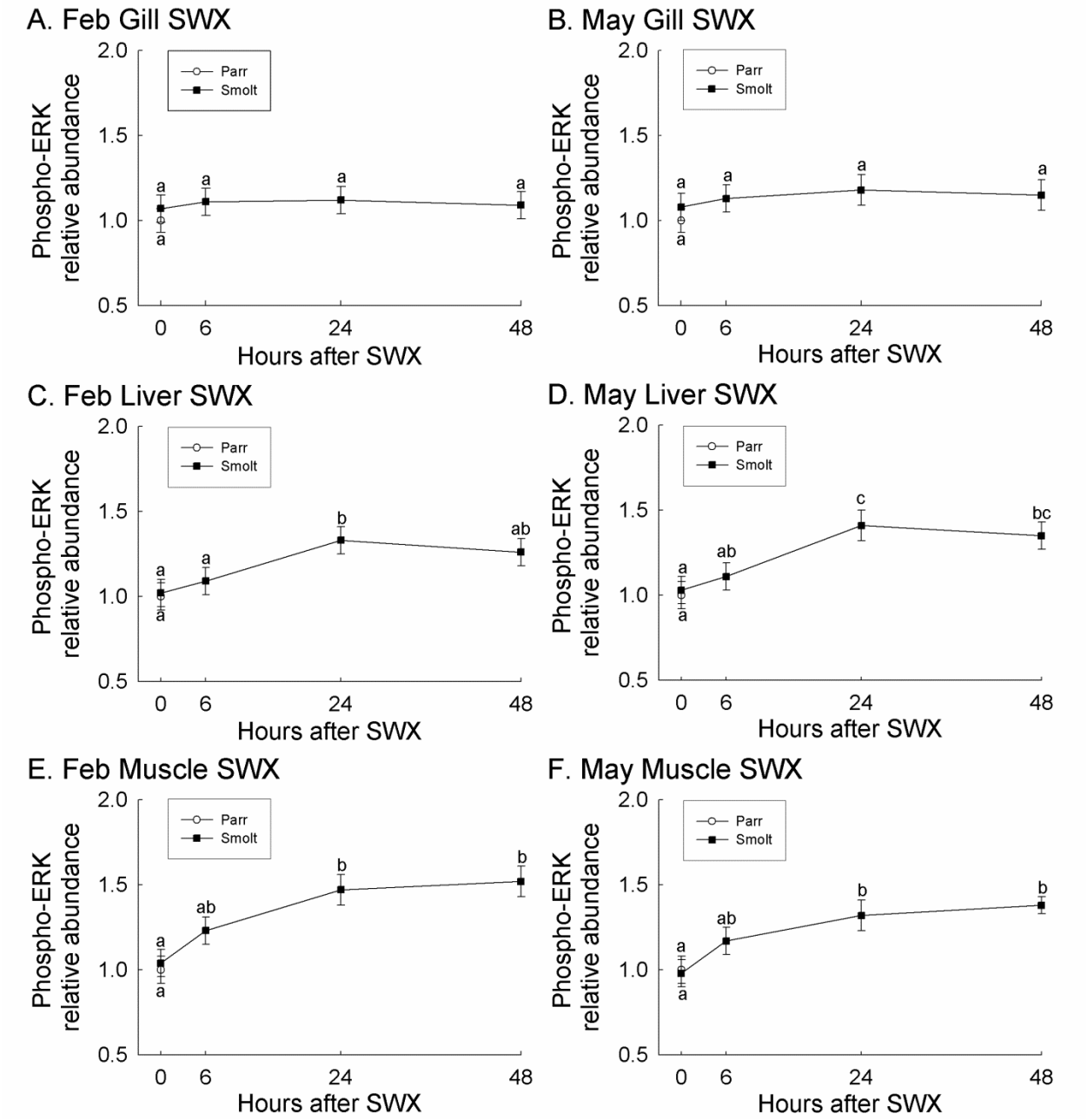

Figure 18. Phospho-ERK abundance in gill (A, B), liver (C, D), and muscle (E, F) during 48 hour SW challenge (SWX). In two separate trials, one in Feb. and one in May, parrs and smolts were exposed to full strength SW for 48 hours. Fish were sampled at 0, 6, 24, and 48 hours. Data are expressed as mean ±s.e.m. (n=4). Within a group (parrs or smolts), times with different letters are significantly different ($p<0.05$) from each other.

Abundance of phosphorylated STAT

FW smolts exhibited an increase in phosphorylated STAT from April to July, as compared to control, in the liver and white muscle (Fig. 18B,C). The gill of FW smolts showed an increase in phosphorylated STAT in May (Fig. 18A). February SW smolts showed an increase in phosphorylated STAT at 24 hours in the gill, with the liver showing no significant changes, and the white muscle showing a decrease at 24 and 48 hours (Fig 19 A, C, E). May SW smolts showed an increase in phosphorylated STAT at 24 hours in the gill, no significant change in the liver, and a decrease at 24 hours in white muscle (Fig. 19B, D, F).

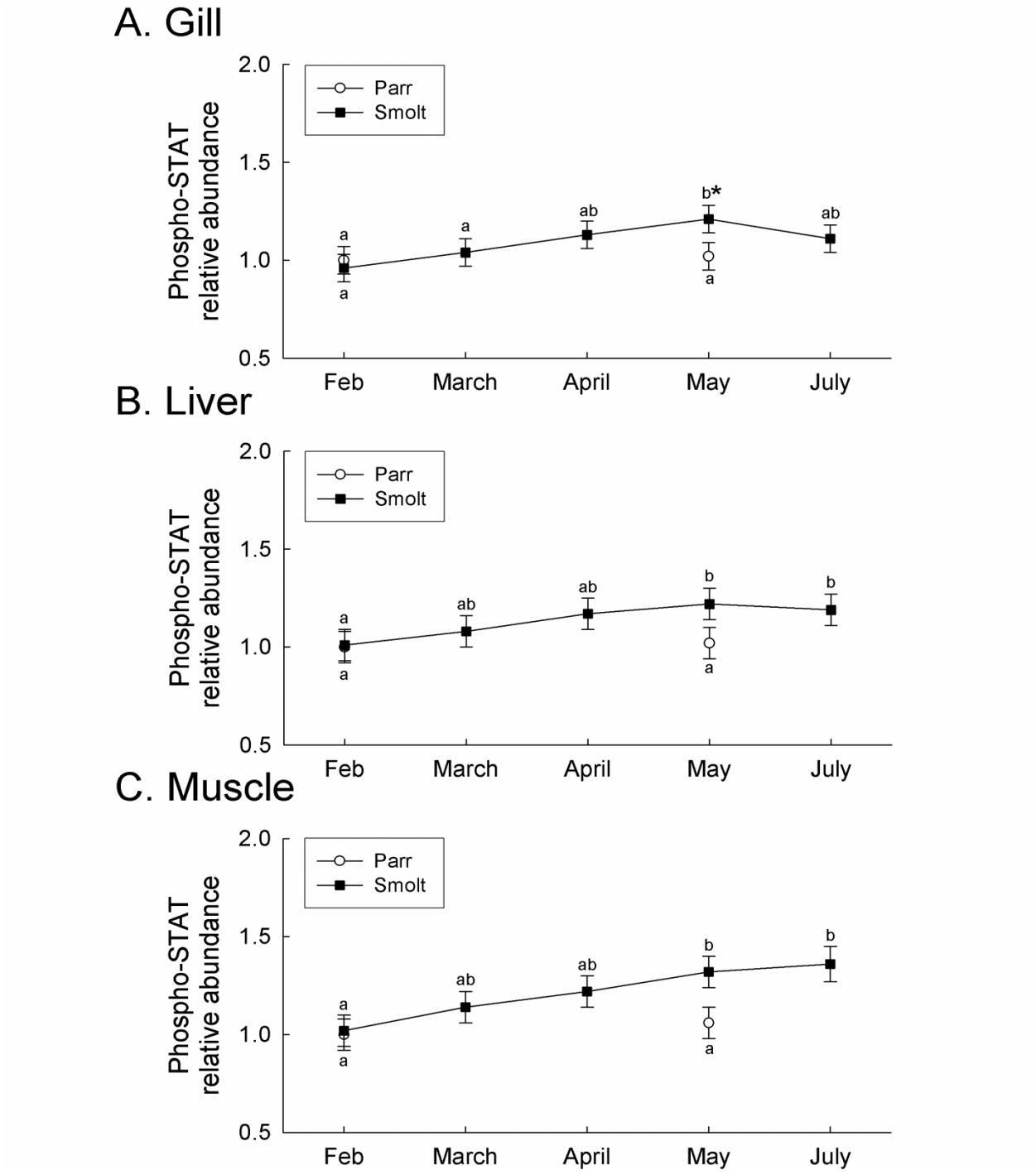

Figure 19. Phospho-STAT5 abundance in gill (A), liver (B), and muscle (C) over the course of the normal smoltification period. Parrs and smolts were kept in FW over the course of the normal smoltification period (Feb.-July). Fish were sampled once a month. Data are expressed as mean ±s.e.m. (n=4). Within a group (parrs or smolts), months with different letters are significantly different ($p<0.05$) from each other; * indicates a significant difference ($p<0.05$) between parrs and smolts within the same month.

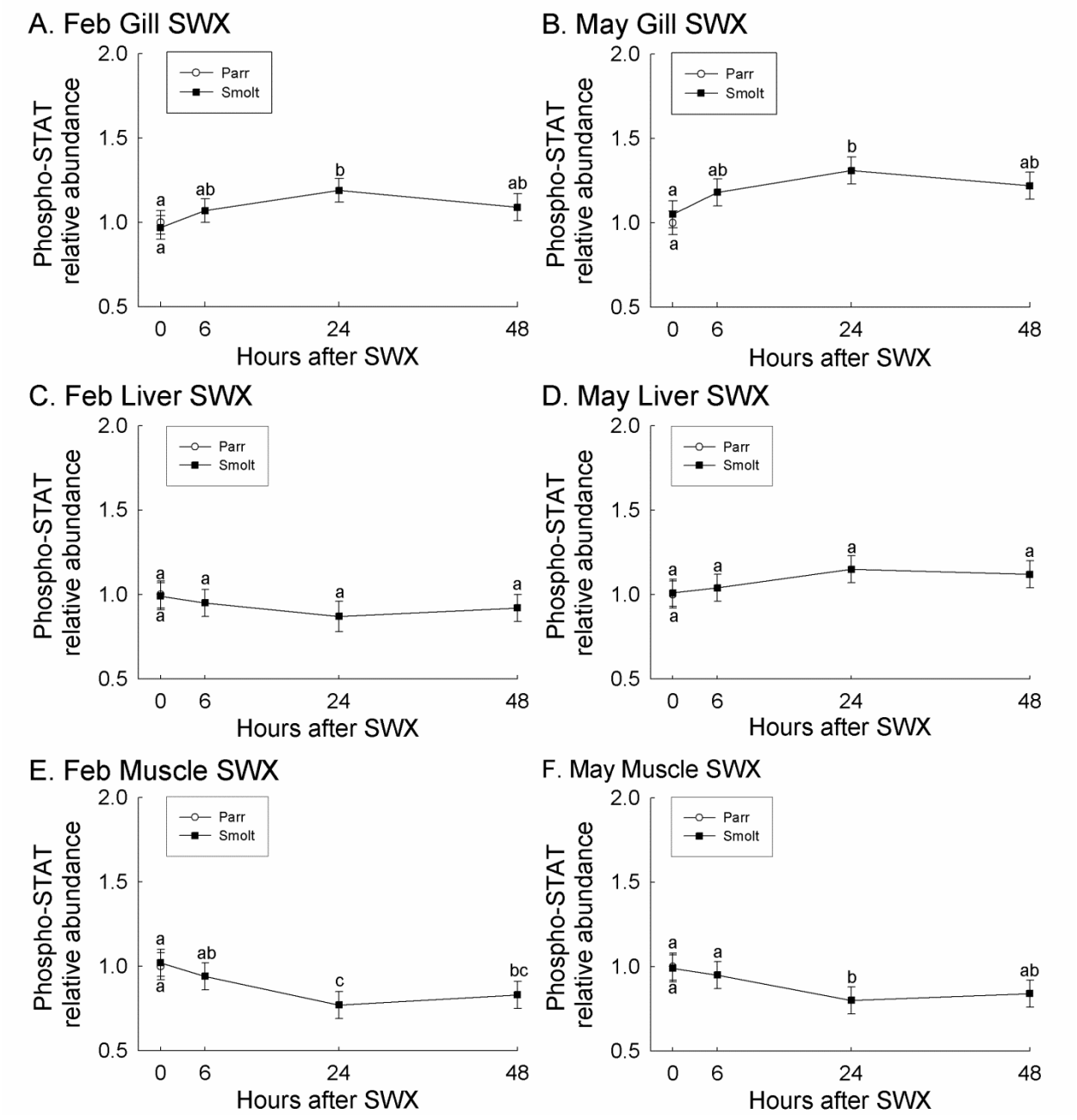

Figure 20. Phospho-STAT5 abundance in gill (A, B), liver (C, D), and muscle (E, F) during 48 hour SW challenge (SWX). In two separate trials, one in Feb. and one in May, parrs and smolts were exposed to full strength SW for 48 hours. Fish were sampled at 0, 6, 24, and 48 hours. Data are expressed as mean ±s.e.m. (n=4). Within a group (parrs or smolts), times with different letters are significantly different ($p<0.05$) from each other.

Abundance of phosphorylated PKC

FW smolts exhibited an increase in phosphorylated PKC from March to May in white muscle, as compared to control (Fig. 20C). FW smolts also exhibited an increase in phosphorylated PKC from March to May in the liver, with no significant change observed in the gill (Fig. 20B, A). February SW smolts showed an elevated level of phosphorylated PKC at 6, 24, and 48 hours in the liver and white muscle, with gill showing no change (Fig. 21C, E, A). May smolts showed an elevated level of phosphorylated PKC at 24 and 48 hours in the liver and white muscle, with no change shown in gill (Fig. 21D, F, B).

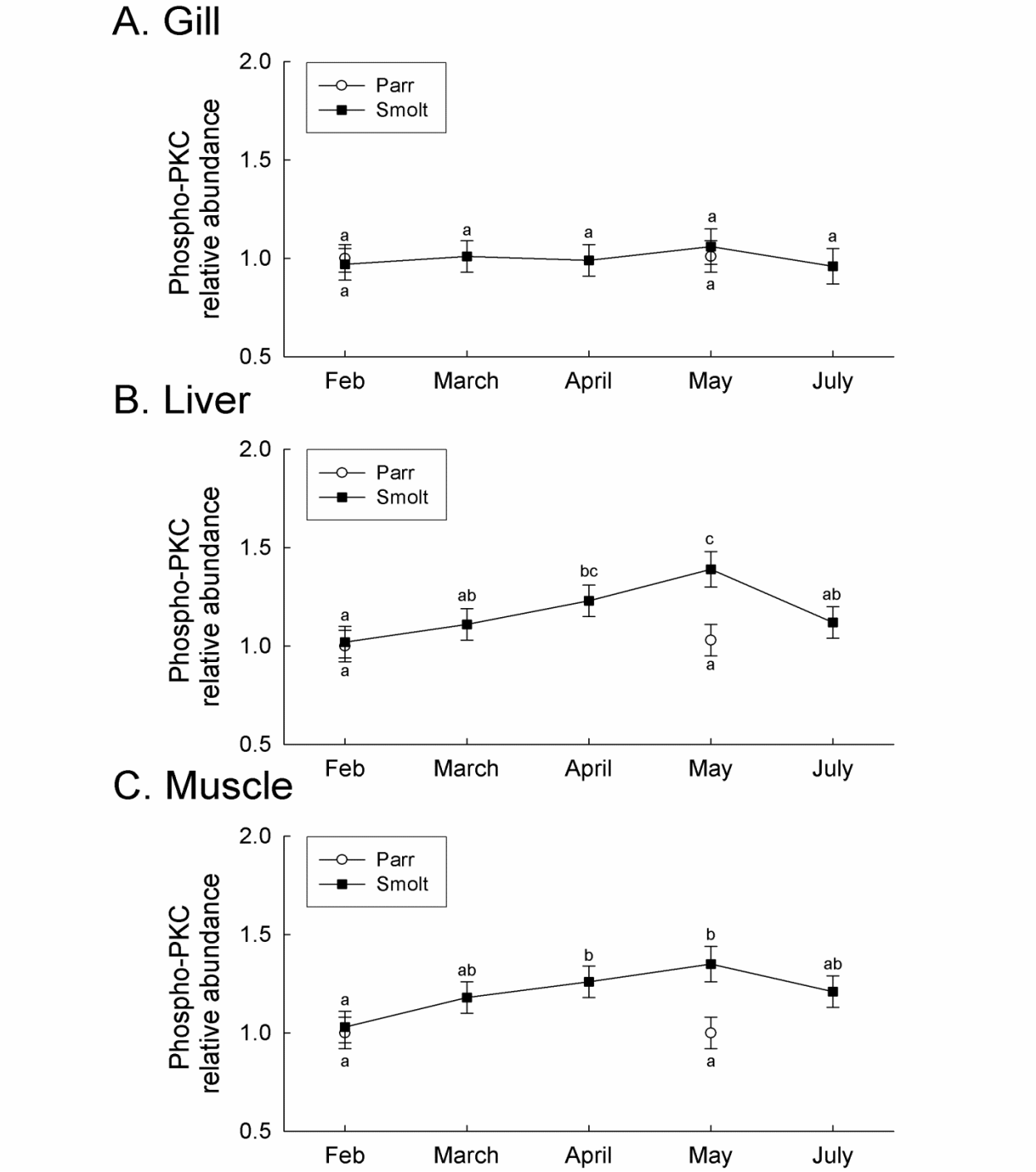

Figure 21. Phospho-PKC abundance in gill (A), liver (B), and muscle (C) over the course of the normal smoltification period. Parrs and smolts were kept in FW over the course of the normal smoltification period (Feb.-July). Fish were sampled once a month. Data are expressed as mean ±s.e.m. (n=4). Within a group (parrs or smolts), months with different letters are significantly different ($p<0.05$) from each other.

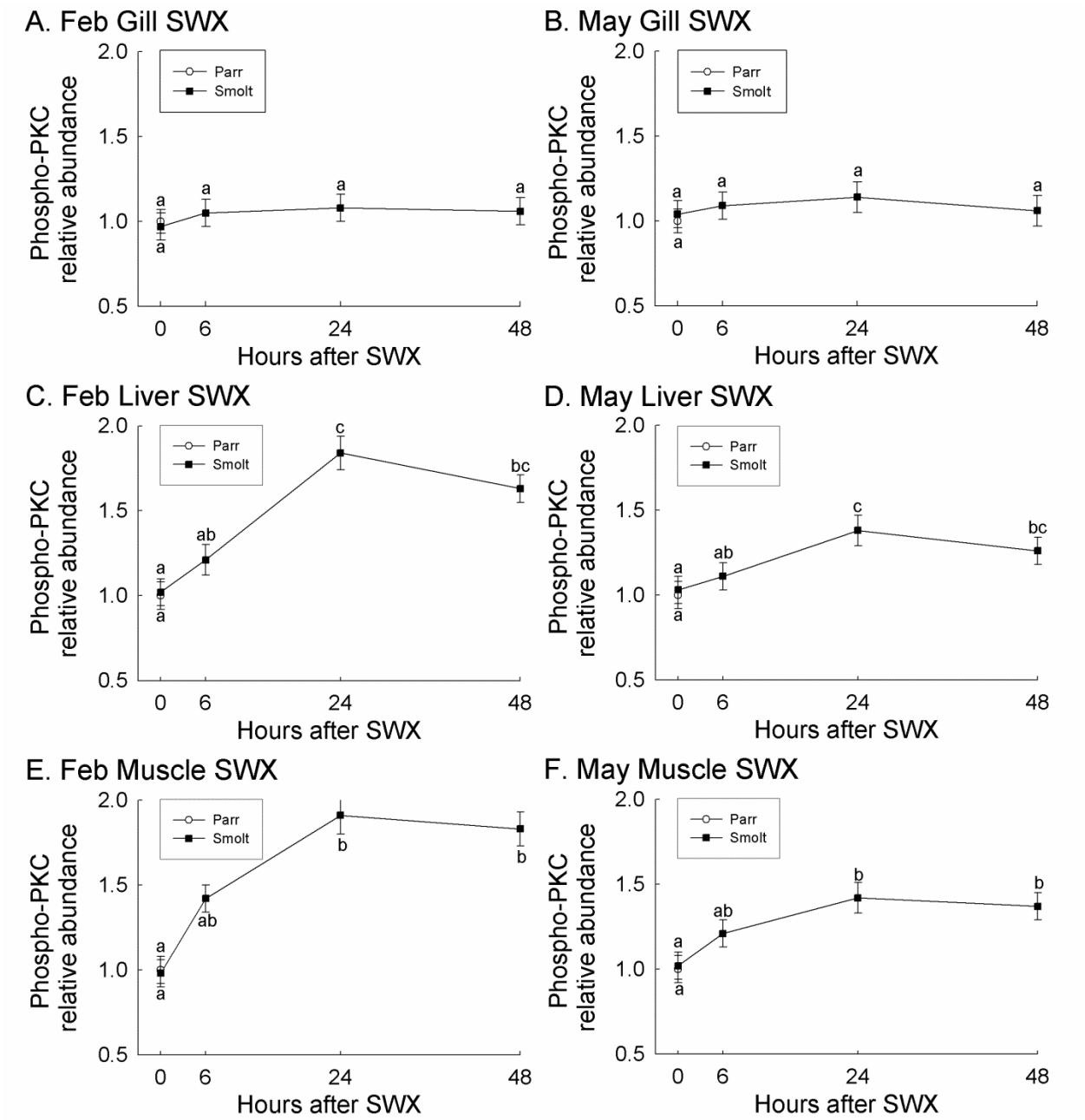

Figure 22. Phospho-PKC abundance in gill (A, B), liver (C, D), and muscle (E, F) during 48 hour SW challenge (SWX). In two separate trials, one in Feb. and one in May, parrs and smolts were exposed to full strength SW for 48 hours. Fish were sampled at 0, 6, 24, and 48 hours. Data are expressed as mean ±s.e.m. (n=4). Within a group (parrs or smolts), times with different letters are significantly different ($p<0.05$) from each other.

Discussion

Both the parr-smolt transformation and SW exposure induce changes in the mRNA expression levels of characteristic osmoregulatory proteins, namely NKAα1a, NKAα1b, and NKCC, in Atlantic salmon. Here we provide evidence that these changes in expression levels are mediated by the GH/IGF axis, and that GH mediates these changes through activation of the JAK/STAT and PI3K/Akt signaling pathways.

Our findings show that during both the normal course of smolt development in February through May, and during SW exposure, there is an increase in expression of NKAα1b in the Atlantic salmon smolt gill, along with a concurrent decrease in expression of NKAα1a. This suggests that in Atlantic salmon, NKAα1a serves as the dominant FW isoform, while NKAα1b serves as the dominant SW isoform. This is consistent with findings in Atlantic salmon (Nilsen et al., 2007; Tipsmark and Madsen, 2009; Madsen et al., 2009), Rainbow trout (*Oncorhynchus mykiss*) (Richards et al., 2003), and Climbing perch (*Anabas testudineus*) (Ip et al., 2012). Our findings also show a marked increase in the expression of NKCC mRNA in smolts in both SW exposure and over the natural smolting period. These findings are also consistent with previous findings in Atlantic salmon (Nilsen et al., 2007).

Consistent with the findings of Nilsen et al. (2007), we found that CFTRI mRNA expression increases over the course of the normal FW smolting period from March to May, followed by a decrease in July. We also saw increased CFTRI expression in smolts exposed to SW challenge, with our highest expression point being 24 hrs post exposure. This is consistent with findings in European sea bass (*Dicentrarchus labrax*) (Bodinier et al., 2009). We also saw increases in the mRNA expression of GHSR that coincided with the expression increases of the typical osmoregulatory genes, namely NKA, NKCC, and CFTR. These increases (increase from

April to May in FW smolts, increased expression after SW challenge) would suggest that GHSR also plays a role in the smoltification process of Atlantic salmon.

In our examination of the elements of the GH/IGF axis during smoltification, we found evidence that the GH/IGF axis plays a key role in mediating smoltification. This is unsurprising, as it is know that during smoltification there is a surge in plasma GH and IGF-I (see Hoar, 1988; Björnsson, 1997 for review), and that the development and differentiation of the SW type chloride cell in the branchial epithelia is regulated by GH, IGF-I, and cortisol (see Sakamoto and McCormick, 2006 for review). It has also previously been shown that injections of GH increased NKA activity and reduced plasma osmolality in tilapia (*Oreochromis mossambicus*) (Sakamoto et al., 1997), and that GH stimulates mRNA expression of NKAα1b and NKCC in the gill of Atlantic salmon (Tipsmark and Madsen, 2009). Our results show that there are increases in mRNA expression of GHRs, IGF-I, and IGFR consistent with the increases seen in the typical osmoregulatory genes we examined, specifically, increased expression levels in April and May FW smolts, and increased expression following SW challenge. These increases in GHRs and IGF-I in response to SW challenge have also been shown previously in Rainbow trout (Poppinga et al., 2007). These results support previous evidence that the GH/IGF axis plays a major role in the smoltification of Atlantic salmon.

In examining the molecular mechanisms of GH action during smoltification, we have found evidence that suggests that GH's actions during smoltification are mediated via the JAK/STAT and PI3K/Akt signaling pathways. We found that during the period of increased expression in April and May consistent with the osmoregulatory genes we examined (NKA, NKCC, CFTR), there was also an increase in the phosphorylation of JAK2, STAT5 and Akt, all

of which are signaling elements know to be activated by the GH/IGF axis (Reindl et al., 2011; Bergan et al., 2012).

Our results support previous evidence showing the changes in mRNA expression of key osmoregulatory genes during the course of normal Atlantic smoltification and during SW exposure. They also support previous work that provides evidence of the key role played by the GH/IGF axis in mediating the smoltification process. This work is the first to propose a possible mechanism for these actions of GH, namely by activation of the JAK/STAT and PI3K/Akt signaling pathways.

CHAPTER 3: GROWTH HORMONE SIGNALING PATHWAYS IN OSMOREGULATION IN RAINBOW TROUT

Abstract

It has been known for some time that GH plays an important role in the facilitation of osmoregulatory adaptation. Despite this, the cellular signaling mechanism utilized by GH to coordinate these functions is unknown. In this study Rainbow trout were used as a model to examine the direct effects of GH on the expression of key osmoregulatory genes, as well as the cell signaling pathways utilized to carry out these changes. Treatment with GH was performed in both a time and dose manner. Changes in key osmoregulatory genes were observed after GH treatment, and treatment with GH showed an increase in phosphorylated Akt and MAPK. This would suggest that these are the pathways utilized by GH to facilitate these changes.

Introduction

For euryhaline teleosts, the ability to maintain a constant internal salinity in the face of varying external conditions is essential to survival. The ability to move between ion uptake and secretion is what allows euryhaline teleosts to maintain control of their internal ion concentration (Evans et al., 2005). The primary site of ion exchange is the gill, and this exchange is carried out primarily by specialized cells called ionocytes. Within these specialized cells are key proteins involved in ion regulation, Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and cystic fibrosis transmembrane regulator (CFTR). Changes in ionocyte abundance, changes in expression levels, and changes in expressed isoforms of these key proteins play a large role in transitioning between ion uptake and ion secretion (Zaugg and McLain, 1970; Singer et al., 2002; Wu et al., 2003; Richards et al., 2003; McCormick et al., 2009).

It has been shown that NKA possesses multiple isoforms in teleosts (Richards et al., 2003), with NKAα1A being more prevalent in freshwater (FW) ionocytes and NKAα1B being more prevalent in seawater (SW) (McCormick et al., 2009). It has also been shown that NKCC possesses multiple isoforms in teleosts, and that these isoforms are differentially expressed in FW and SW (Wu et al., 2003; Hiroi et al., 2008). CFTR also has multiple isoforms, with CFTR1 being upregulated in SW (Singer et al., 2002), though work is yet to be done to definitively determine the FW and SW isoforms of CFTR.

Behind all these changes lie a number of endocrine controls, most notably cortisol, prolactin (PL), and growth hormone (GH). Prolactin functions to promote FW adaptation, promoting the morphology of FW type ionocytes (Pisam et al., 1993), and inhibiting SW type ionocytes (Herndon et al., 1991). GH and cortisol function to promote SW adaptation. It has been shown previously that treatment with exogenous cortisol stimulates increased salinity tolerance (McCormick, 2001), as does treatment with exogenous GH (Takei and McCormick, 2013). GH has also been shown to increase gill ionocyte numbers, increase gill NKA activity, and decrease plasma osmolality in Atlantic (*Salmo salar*) and Coho salmon (*Oncorhynchus kisutch*) (Richman, 1987; McCormick, 1996), Rainbow trout (*Oncorhynchus mykiss*) (Sangiao-Alvarellos et al., 2005), and gilthead sea bream (*Sparus auratus*) (Sangiao-Alvarellos et al., 2006).

Knowing that GH plays such an important role in the control of osmoregulation, we know surprisingly little about the underlying signaling mechanism utilized by GH, despite extensive knowledge of GH signaling (see Zhu et al., 2001 for review). Briefly, GH functions through binding of the growth hormone receptor (GHR), which recruits or activates various cytoplasmic tyrosine kinases to function as downstream signaling elements. It has been shown

previously that GH utilizes a variety of downstream signaling elements to facilitate a number of unique functions, such as lipolysis (Bergan et al., 2013) and hepatic insulin-like growth factor (IGF) expression (Reindl et al., 2011). In this study, Rainbow trout were utilized to examine the direct effects of exogenous GH on various osmoregulatory elements, as well as elucidate the cell signaling elements utilized by GH to carry out these actions. Rainbow trout were chosen for this study due to their natural euryhalinity and their extensively well characterized GH-IGF system (Reindl and Sheridan, 2012).

Materials and methods

Animals

Juvenile rainbow trout (*O. mykiss*) of both sexes (with no macroscopically discernable gonads) were obtained from Dakota Trout Ranch near Carrington, ND, USA. Following transport to North Dakota State University, the fish were maintained in well-aerated, 800-L circular tanks supplied with recirculated (10% replacement volume per day) dechlorinated municipal water at 14 °C under a 12 h light: 12 h dark photoperiod. Fish were fed to satiation twice daily with AquaMax™ Grower 400 (45% crude protein, 16% crude fat, 3% fiber)(PWI Nutrition International, Brentwood, MO, USA), except 24–36 h before experiments. Animals were acclimated to laboratory conditions for at least 6 weeks prior to experiments. All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council, Washington, DC) and approved by the North Dakota State University Institutional Animal Care and Use Committee.

For experiments, fish were anesthetized by immersion in 0.05% (v/v) 2-phenoxyethanol, bled, and the gill arches removed. Gill arches were stored in ice cold Hank's solution. Individual filaments were dissected from the arches while immersed in Hank's on ice. Isolated

filaments from individual fish were pooled and washed three times with Hank's. Twelve to 15 gill filaments from a given pool were placed in 24-well culture plates containing 0.5 ml of Hank's; filaments in replicate wells for a given experiment came from different fish. The medium was removed and 1.0 ml of Hank's plus BSA was added to each well. Filaments were allowed to recover in the incubation medium for 3 h at 14° C with gyratory shaking (100 rpm) under 100% oxygen. After recovery, the medium was removed and the filaments were washed with 1.0 ml of Hank's. Filaments were then treated with incubation medium alone (control) or with varying concentrations of GH for various times as noted in the figure legends. Time treatments were performed with a constant GH dose of 100 ng/ml. Treatment was also done with a constant dose of GH (100ng/ml) in combination with GH pathway inhibitors. Inhibitors used were as follows: 20 µM LY294002, 25µM Carb, 10 µM U0126, 50 µM Hex, 200 µM Nico, and 10 µM CC. Treatment was stopped by removing the incubation medium and immediately freezing the filaments on dry ice. Filaments were stored at -80° C for later analysis.

RNA extraction and analysis

Total RNA was extracted using RNAzol® (Molecular Research Center, Inc., Cincinnati, OH, USA) as specified by the manufacturer's protocol. Each RNA pellet was resuspended in 35–200 µl RNase-free deionized water and quantified by NanoDrop1000 spectrophotometry (A260) (Thermo Scientific, West Palm Beach, FL, USA). RNA samples were then stored at -80° C until further analysis. mRNA was reverse transcribed in 10 µl reactions using 200 ng total RNA and qScript cDNA Synthesis Kit reagents (optimized buffer, magnesium, oligo(dT)primers, random primers, dNTPs, reverse transcriptase) according to the manufacturer's protocol (Quanta Biosciences, Gaithersburg, MD, USA). 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. mRNA levels of NKCC, CFTR, NKA-α1A, NKA-α1B, GlinR, and βActin were determined by quantitative real-time PCR. Briefly, real-time reactions were carried out for samples and no-template controls using PerfeCta SYBR Green Supermix, Low Rox (Quanta Biosciences, Gaithersburg, MD, USA). Reactions contained 2 µl cDNA from the reverse transcription reactions and 8 µl SYBR Green reaction mix. Cycling parameters were set as follows: 95° C for 10 min and 45 cycles of 95° C for 30s followed by 55° C for 1 min.

Western blotting

Tissues were homogenized in 250 µl 1x cell lysis buffer (Cell Signaling Technology, Beverly, MA) with 1x protease inhibitor (Calbiochem, San Diego, CA, USA), and 1x phosphatase inhibitor (G-Biosciences, St. Louis, MO, USA). The homogenate was incubated on ice for 5 min then centrifuged at 16,000g for 10 min at 4° C. The protein concentration of the supernatant was determined by the Bio-Rad (Hercules, CA, USA) dye-binding method for microplates. Proteins (typically 50 µg) and molecular weight marker (Cell Signaling Technology, Beverly, MA; Catalog No. 7727) were separated by SDS–PAGE (7.5% running gel) and transferred to 0.45 µm nitrocellulose (Bio-Rad Laboratories) for western analysis. Membranes were washed and visualized with chemiluminescence according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK); chemiluminescence was detected directly and the bands quantified with a FluorChem FC2 imager (Alpha Innotech Corp, San Leandro, CA, USA). The abundance of phosphorylated ERK 1/2, Akt, JAK2, STAT5, and PKCα/β II was normalized to total ERK 1/2, Akt, JAK2, STAT5, and β-actin, respectively. All antisera were obtained from Cell Signaling Technology (Beverly, MA).

Data analysis

Data are expressed as means ± S.E.M. Statistical differences were estimated by one-way or two-way ANOVA, as appropriate, followed by Duncan's multiple range test using SigmaStat (SPSS, Chicago, IL). A probability level of 0.05 was used to indicate significance.

Results

mRNA expression

The results of our qPCR data indicate that NKAα1A decreases in a time dependent manner (Fig. 22A) and increases in a dose dependent manner (Fig. 22B) in response to GH. This time dependent decrease agrees with previous data, however the dose related increase was unexpected. Our results also showed the NKAα1B responded to GH with a dose dependent increase (Fig. 22B) and no change in response to time (Fig. 22A). NKCC and CFTR showed no significant change in response to dose or time (Fig. 23 and 24, respectively), while GHSR showed no significant change in response to dose (Fig. 25B), but exhibited a time dependent response (Fig. 25A).

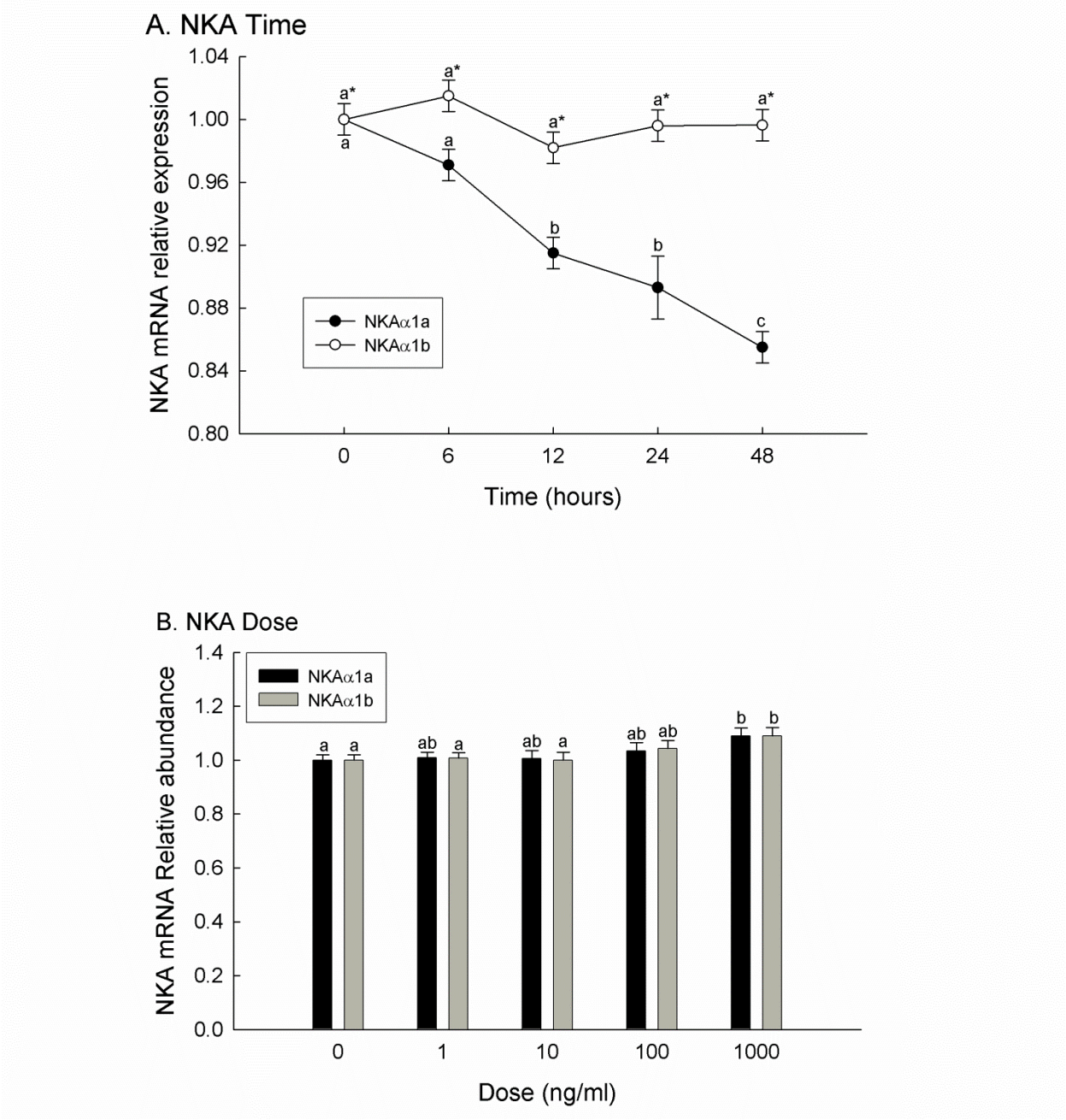

Figure 23. Effects of time and dose treatment of GH on NKA expression in trout gill. Trout gill filaments were treated with GH over a time (100 ng/ml GH over varying times) (A) and dose (varying concentration for 24 hrs) (B) course. Data are expressed as mean ±s.e.m. (n=6). Within a group (α1A or α1B), treatments with different letters are significantly different ($p<0.05$) from each other; * indicates a significant difference ($p<0.05$) between α1A and α1B within the same treatment.

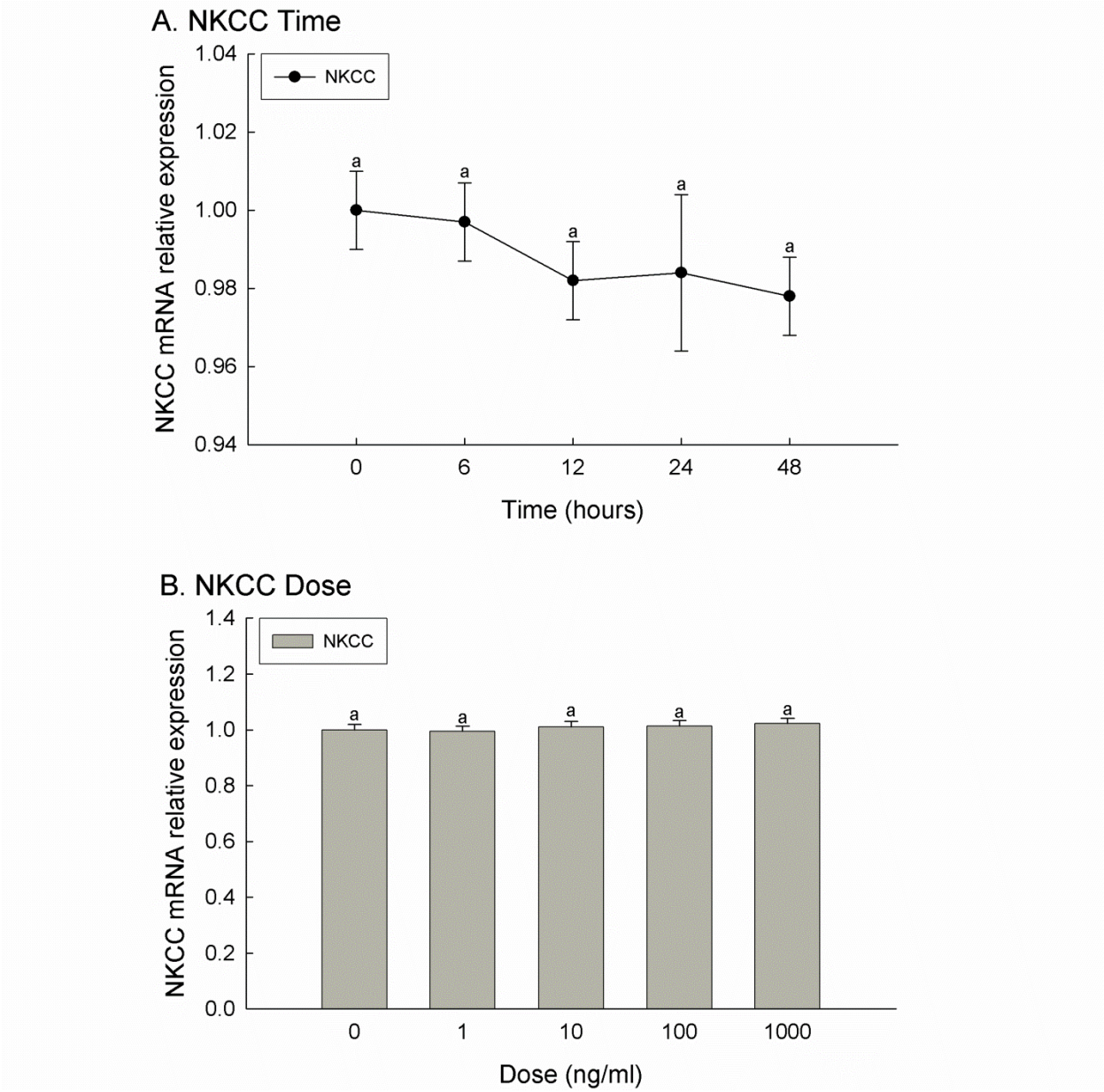

Figure 24. Effects of time and dose treatment of GH on NKCC expression in trout gill. Trout gill filaments were treated with GH over a time (100 ng/ml GH over varying times) (A) and dose (varying concentration for 24 hrs) (B) course. Data are expressed as mean ±s.e.m. (n=6). Treatments with different letters are significantly different ($p<0.05$) from each other.

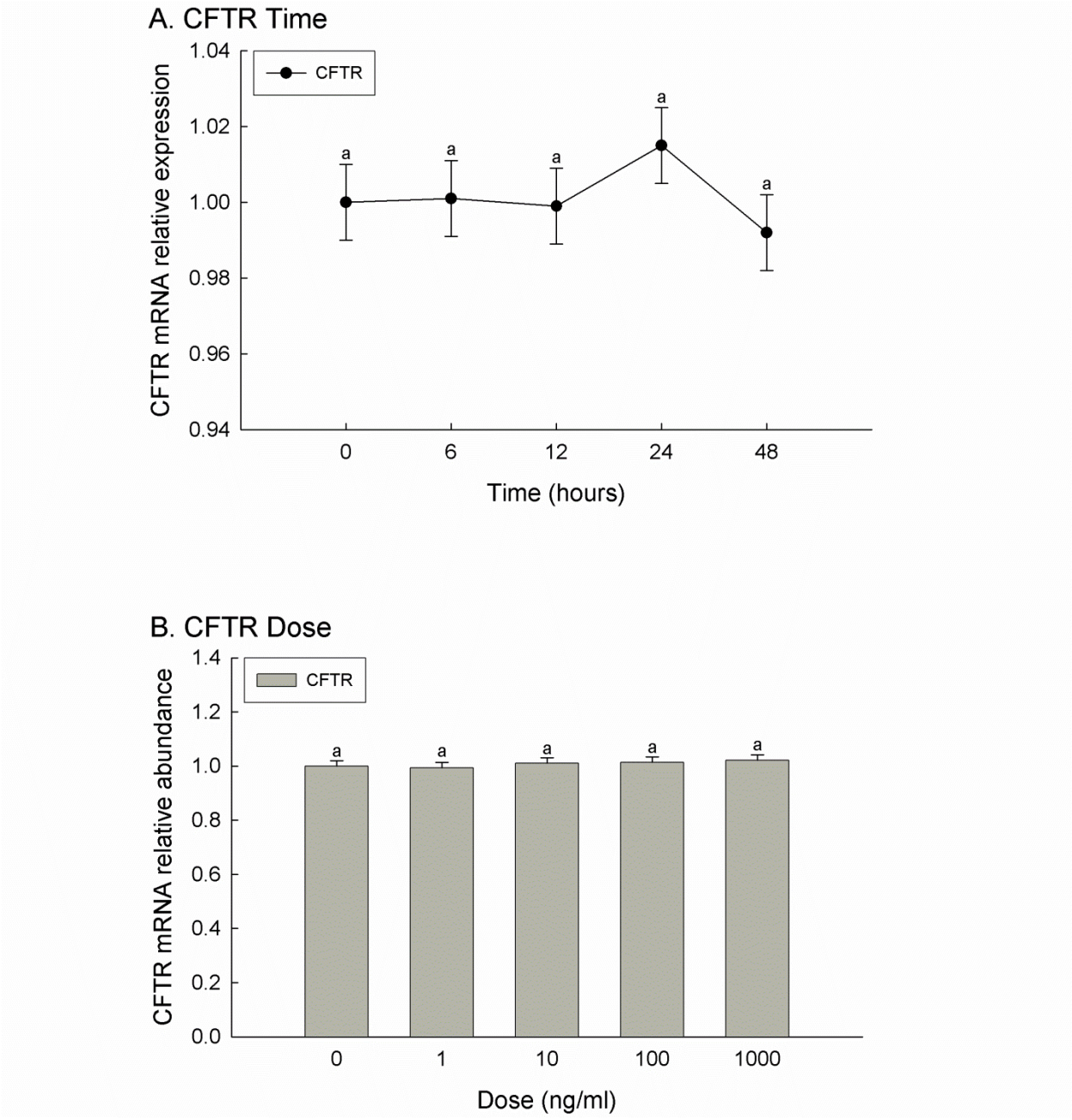

Figure 25. Effects of time and dose treatment of GH on CFTR expression in trout gill. Trout gill filaments were treated with GH over a time (100 ng/ml GH over varying times) (A) and dose (varying concentration for 24 hrs) (B) course. Data are expressed as mean ±s.e.m. (n=6). Treatments with different letters are significantly different ($p<0.05$) from each other.

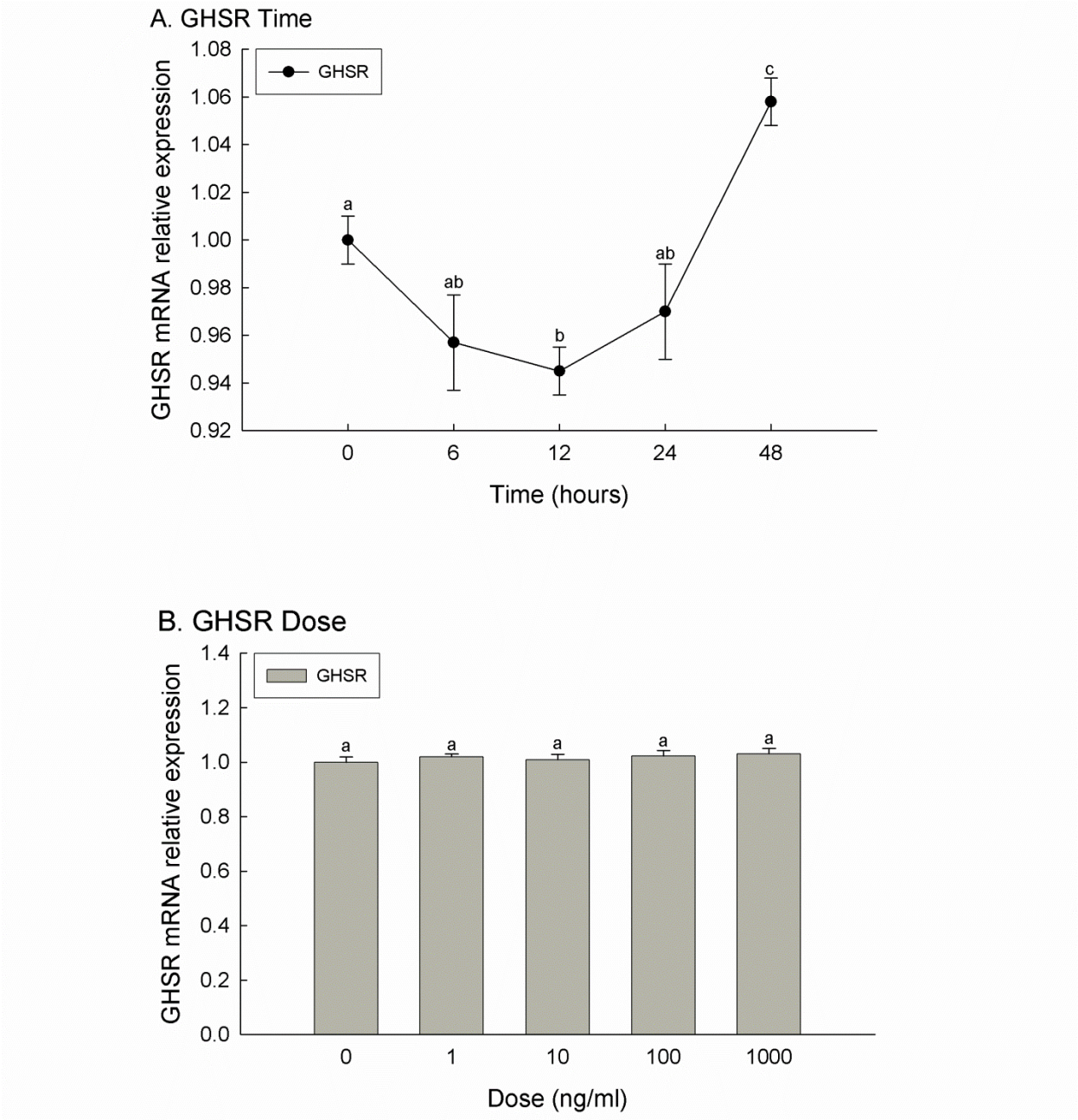

Figure 26. Effects of time and dose treatment of GH on GHSR expression in trout gill. Trout gill filaments were treated with GH over a time (100 ng/ml GH over varying times) (A) and dose (varying concentration for 24 hrs) (B) course. Data are expressed as mean ±s.e.m. (n=6). Treatments with different letters are significantly different ($p<0.05$) from each other.

Activation of Akt and MAPK

Analysis of the Western blots performed in this experiment show activation of two distinct signaling elements. Akt demonstrated a time dependent increase in activation in response to GH, peaking at an interval of 30 min (Fig. 26A). Akt also demonstrated a decrease in activation when treated with 1000 ng/ml GH (Fig. 26B). MAPK also exhibited a time dependent increase in activation in response to GH, peaking at 60 min (Fig. 27A). This time dependent increase was not accompanied by a dose dependent increase, with no significant activation observed in response to a dose course of GH (Fig. 27B). Other signaling elements tested were JAK (Fig. 28), STAT (Fig. 29), and PKC (Fig. 30), none of which showed significant change in response to a time or dose course of GH.

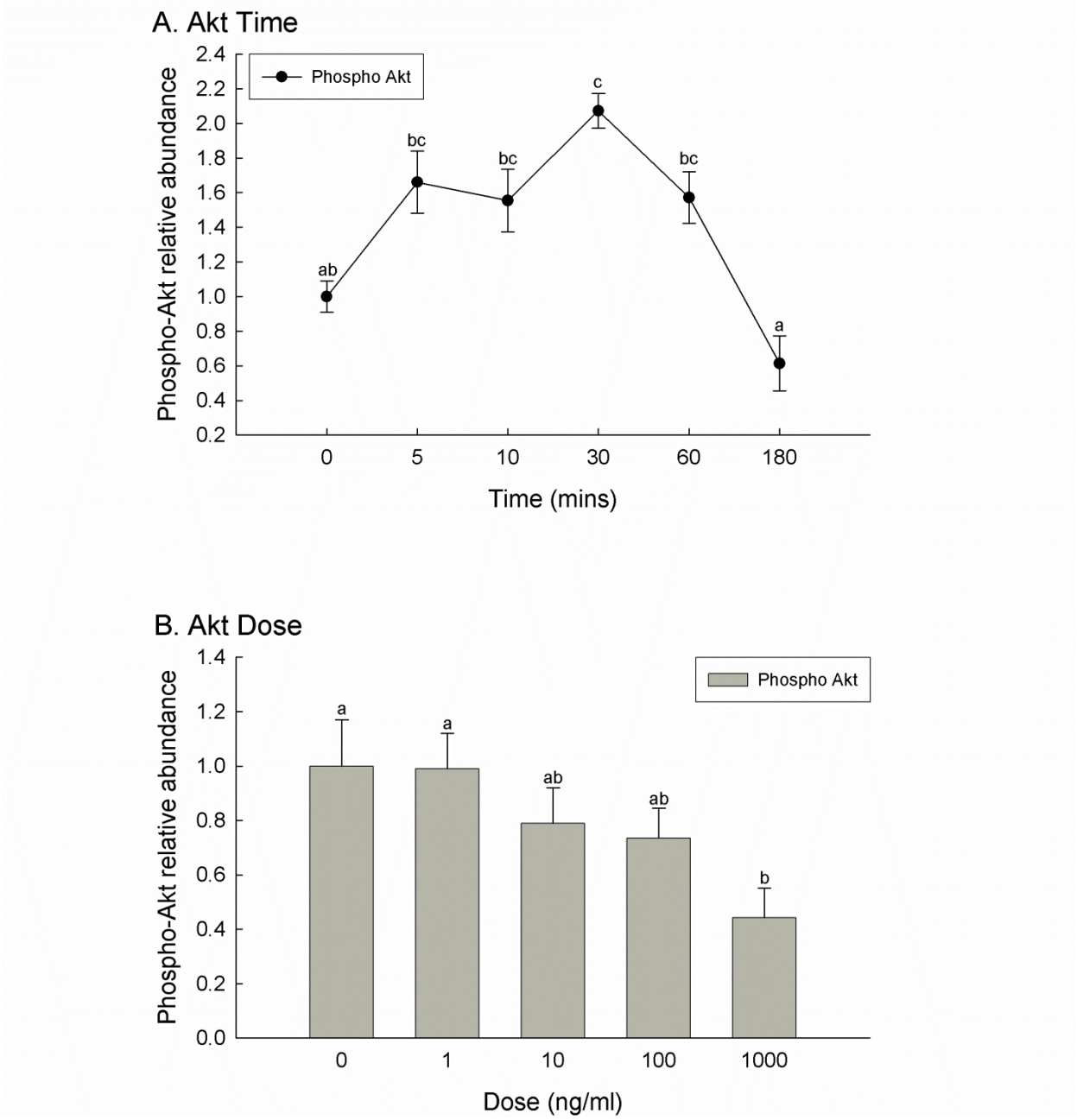

Figure 27. Effects of time and dose treatment of GH on phosphorylated Akt abundance in trout gill. Trout gill filaments were treated with GH over a time (100 ng/ml GH over various times) (A) and dose (varying concentrations of GH for 30 min.) (B) course. Data are expressed as mean ±s.e.m. (n=4). Treatments with different letters are significantly different ($p<0.05$) from each other.

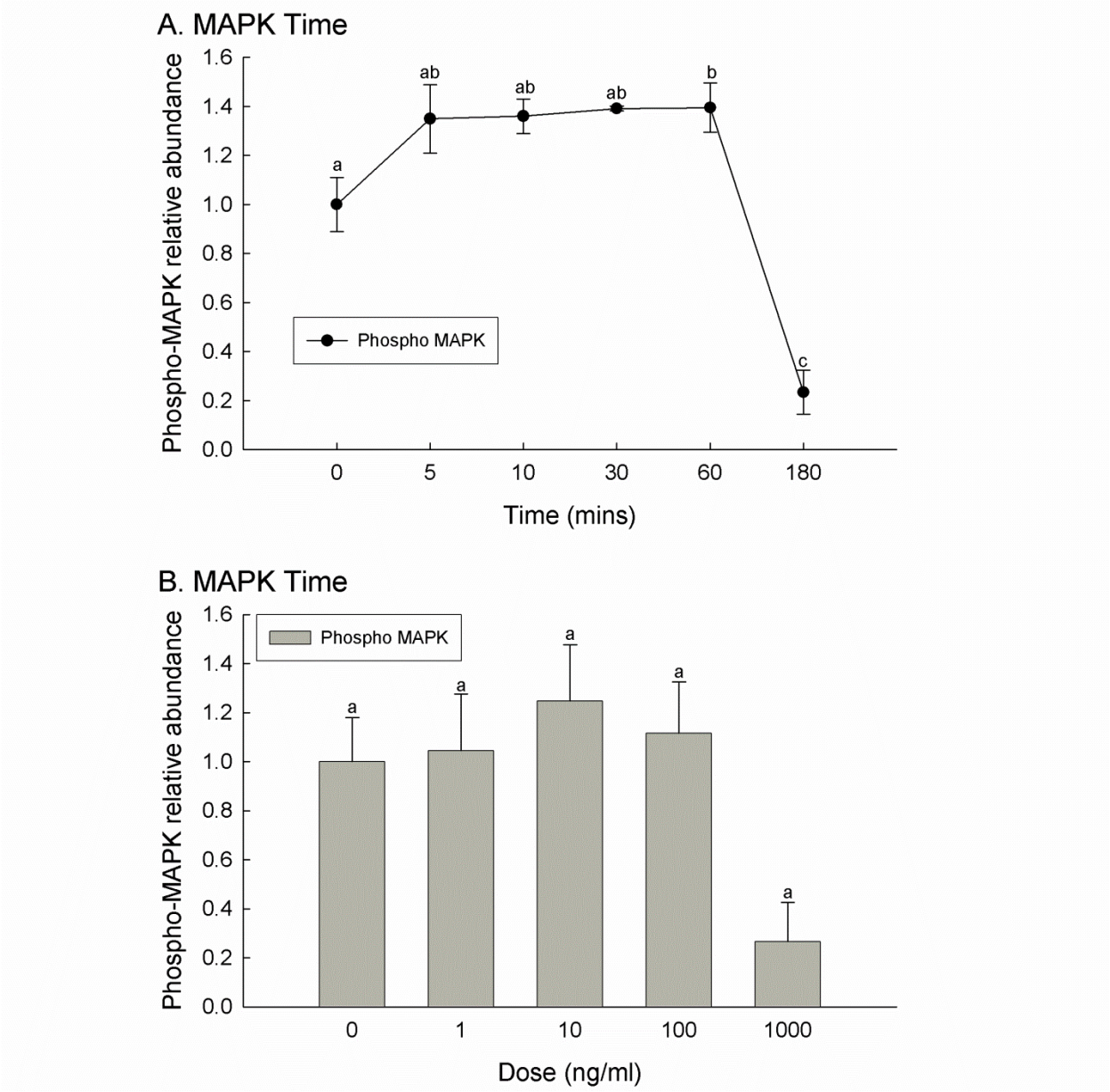

Figure 28. Effects of time and dose treatment of GH on phosphorylated MAPK abundance in trout gill. Trout gill filaments were treated with GH over a time (100 ng/ml GH over various times) (A) and dose (varying concentrations of GH for 30 min.) (B) course. Data are expressed as mean ±s.e.m. (n=4). Treatments with different letters are significantly different ($p<0.05$) from each other.

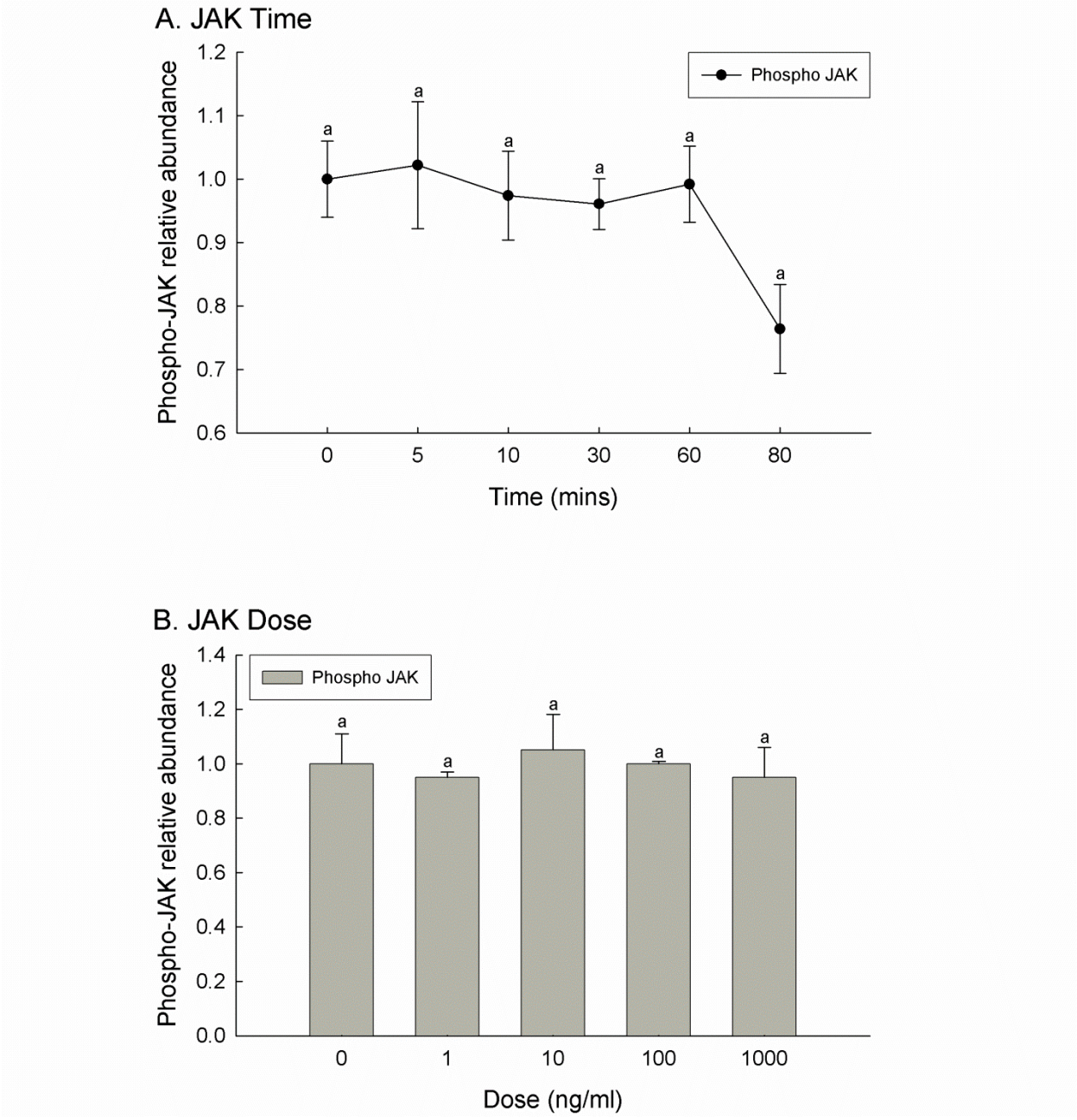

Figure 29. Effects of time and dose treatment of GH on phosphorylated JAK abundance in trout gill. Trout gill filaments were treated with GH over a time (100 ng/ml GH over various times) (A) and dose (varying concentrations of GH for 30 min.) (B) course. Data are expressed as mean ±s.e.m. (n=4). Treatments with different letters are significantly different ($p<0.05$) from each other.

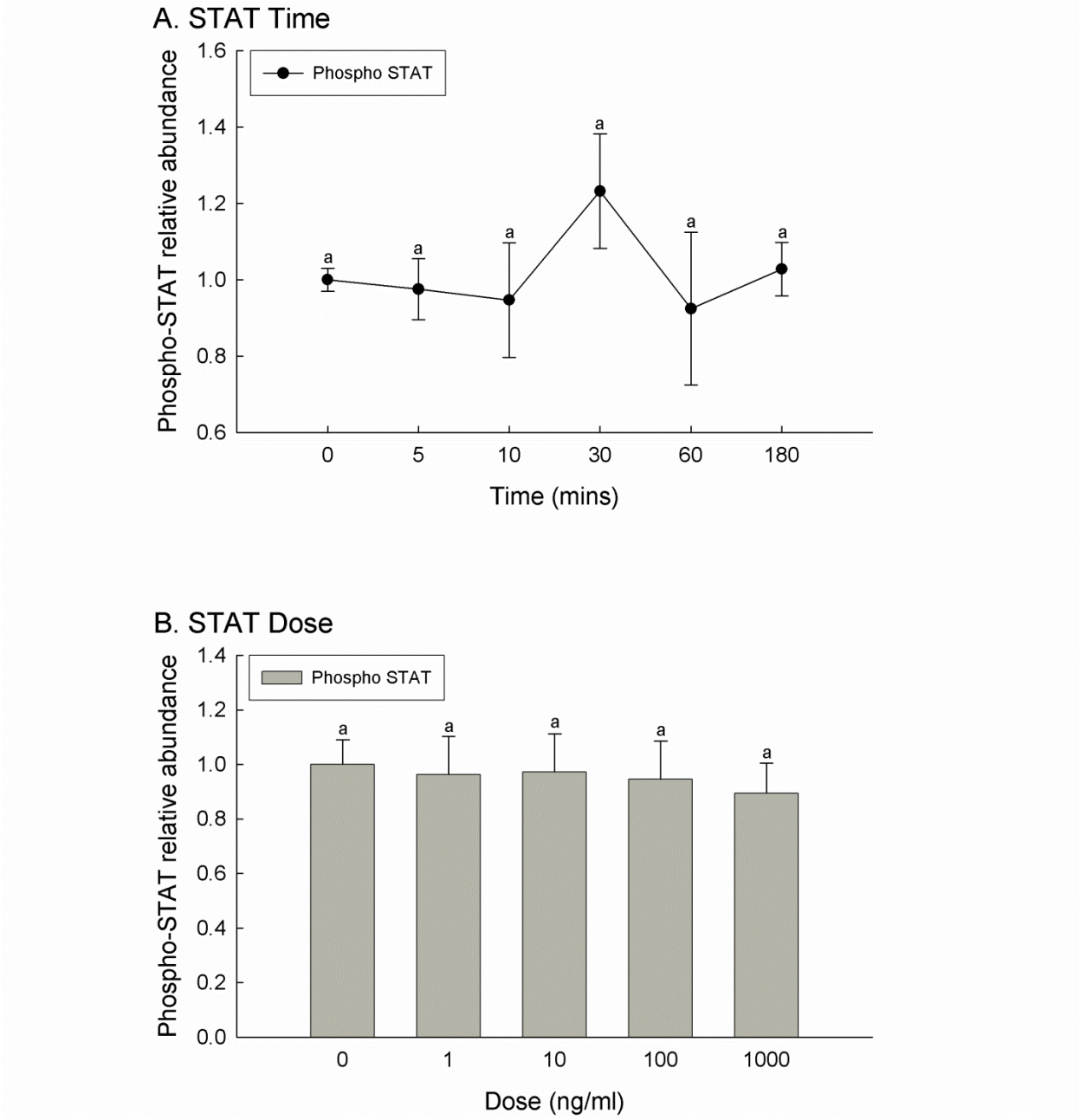

Figure 30. Effects of time and dose treatment of GH on phosphorylated STAT abundance in trout gill. Trout gill filaments were treated with GH over a time (100 ng/ml GH over various times) (A) and dose (varying concentrations of GH for 30 min.) (B) course. Data are expressed as mean ±s.e.m. (n=4). Treatments with different letters are significantly different ($p<0.05$) from each other.

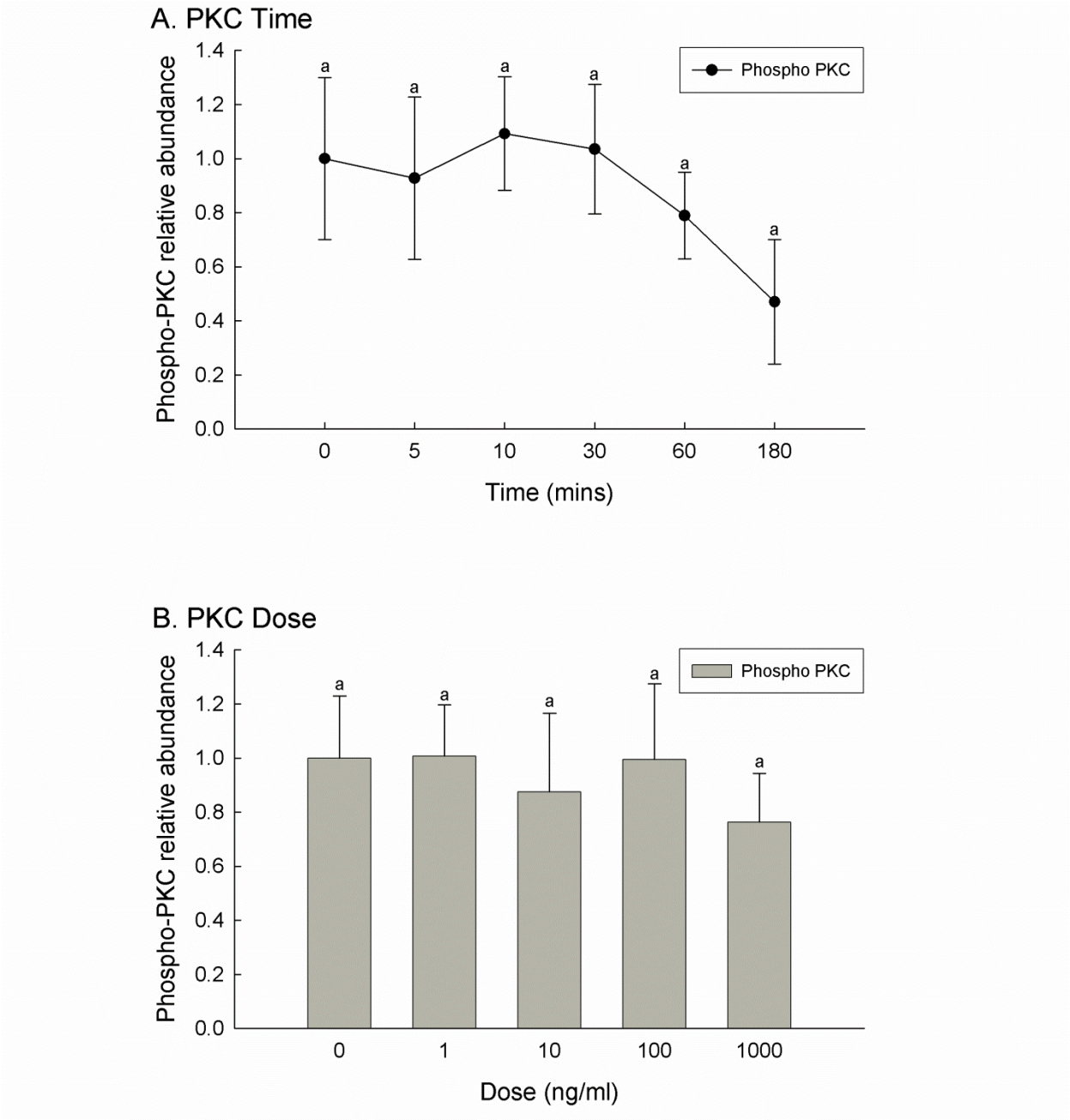

Figure 31. Effects of time and dose treatment of GH on phosphorylated PKC abundance in trout gill. Trout gill filaments were treated with GH over a time (100 ng/ml GH over various times) (A) and dose (varying concentrations of GH for 30 min.) (B) course. Data are expressed as mean ±s.e.m. (n=4). Treatments with different letters are significantly different ($p<0.05$) from each other.

Inhibition of activated pathways

Expression levels of our genes of interest were examined when treated with specific inhibitors for our signaling elements of interest, along with a GH treatment (controls used were media and media+GH (100 ng/ml for 24 hrs), treatments were inhibitor or inhibitor+GH (100 ng/ml GH for 24 hrs). None of our examined genes showed any significant change in response to the inhibitor treatment (Figs. 31 & 32).

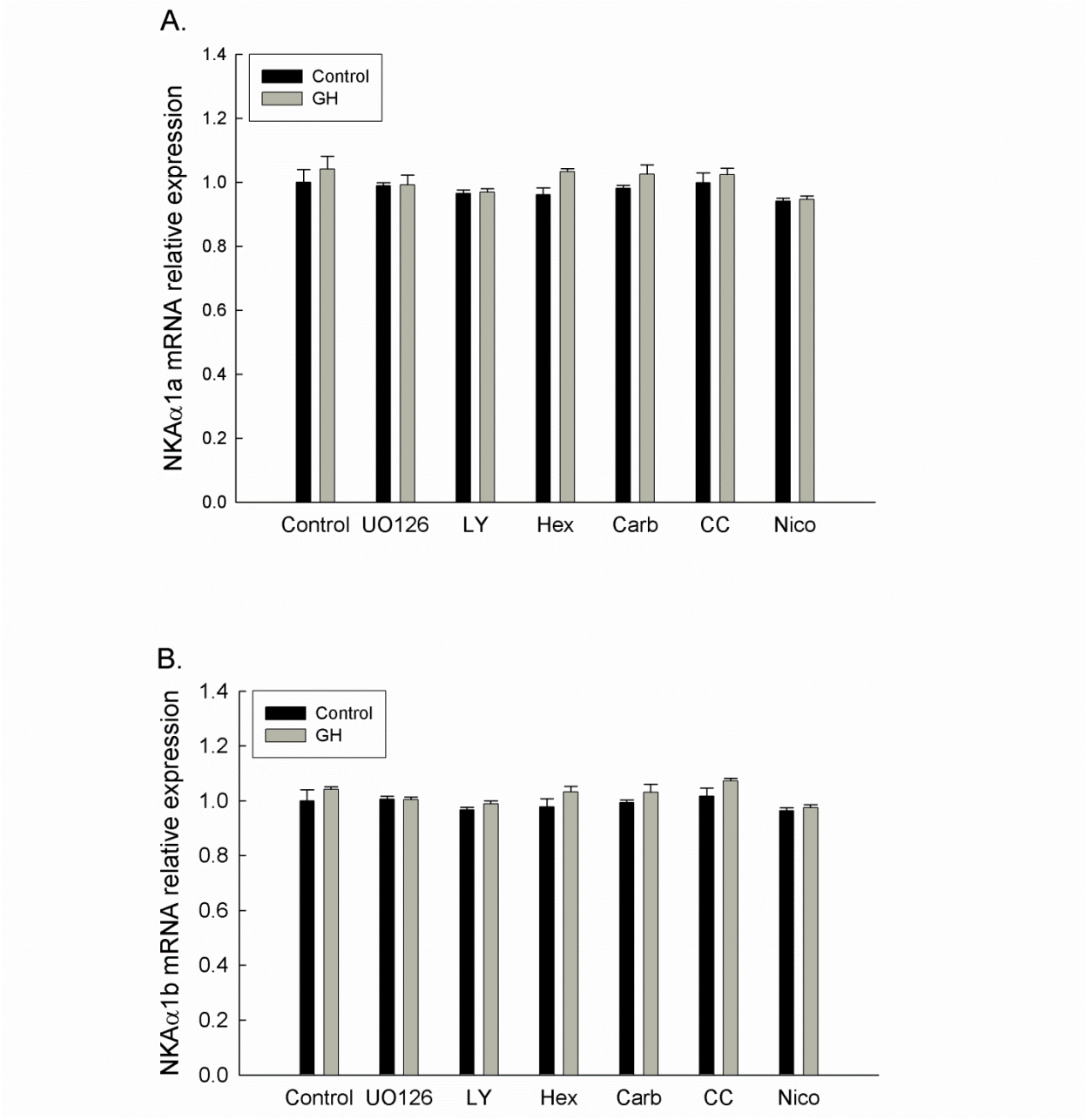

Figure 32. Effects of time and dose treatment of GH combined with specific pathway inhibitors on NKA expression in trout gill. Trout gill filaments were pretreated for 2 hours with or without specific cell signaling inhibitors for the following signaling elements: PI3K (10 µM LY294002), Akt (10 µM Carb), MEK (10 µM U0126), JAK2 (10 µM Hex), and PKC (10 µM CC); followed by treatment with 100 ng/ml GH for 24 hours. Data are expressed as mean ±s.e.m. (n=6). Treatments with different letters are significantly different ($p<0.05$) from each other.

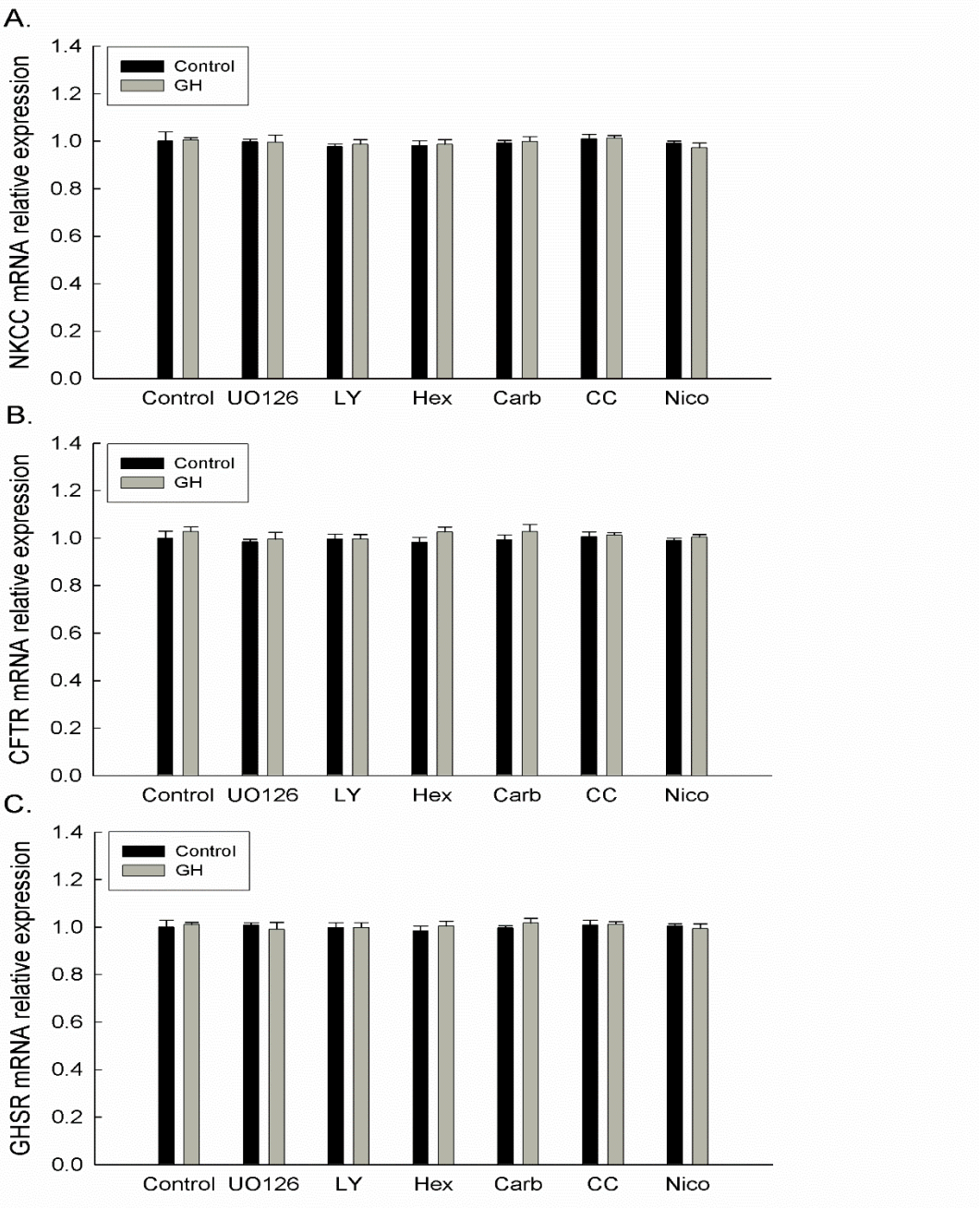

Figure 33. Effects of time and dose treatment of GH combined with specific pathway inhibitors on NKCC (A), CFTR (B), and GHSR (C) expression in trout gill. Trout gill filaments were pretreated for 2 hours with or without specific cell signaling inhibitors for the following signaling elements: PI3K (10 µM LY294002), Akt (10 µM Carb), MEK (10 µM U0126), JAK2 (10 µM Hex), and PKC (10 µM CC); followed by treatment with 100 ng/ml GH for 24 hours. Data are expressed as mean ±s.e.m. (n=6). Treatments with different letters are significantly different ($p<0.05$) from each other.

Discussion

The data gathered in this study shows that GH exhibits a number of effects on the osmoregulatory genes in Rainbow trout, and that these changes are mediated by activation of specific cell signaling elements.

Our results show that in response to GH, NKAα1A shows a time dependent decrease. This was expected, and further supports previous evidence that NKAα1A is the FW isoform of NKA (McCormick et al., 2009), and that GH plays a role in SW adaptation in salmonids (Takei and McCormick, 2013). It has been shown previously that exposure of Rainbow trout to SW causes a decrease in expression of NKAα1A (Richards et al., 2003), further suggesting that GH acts to support SW adaptation when combined with the data presented here. Richards et al. also showed that SW exposure causes an increase in NKAα1B expression, an effect that was not observed in our GH treatments. It has been shown that an increase in NKAα1B expression is facilitated by GH in other euryhaline species (Tipsmark and Madsen, 2009); however, our data suggest that for Rainbow trout that is not the case. This would suggest that while GH facilitates a reduction in NKAα1A expression, it is another hormone that facilitates the increase in NKAα1B. Cortisol would be a likely candidate, as it has been shown that treatment with cortisol increases NKA activity in Rainbow trout (McCormick and Bern, 1989), and that cortisol has extensive interactions with GH when involved in osmoregulatory adaptation (Madsen, 1990).

Also shown in our analysis of mRNA expression is a time-dependent response of GHSR to GH. Our results show that after 12 hrs, GHSR exhibits a decrease in expression as compared to control, followed by an increase at 48 hours. There are few available data on ghrelin and GHSR in Rainbow trout, but it has been shown that in juveniles, ghrelin decreases food intake (Jonsson et al., 2010). It has also been shown that GHSR expression increases in the same

pattern as known osmoregulatory genes (NKA, CFTR, NKCC) in Atlantic salmon smolts (Martin et al., *in preparation)*. These observations seem to indicate that GHSR plays a role in SW adaptation during smoltification, but that GH may not be the hormone that facilitates this increase. Further work would need to be performed to confirm these findings.

Along with the findings from our mRNA expression data, we also observed activation of two distinct signaling elements. Both Akt and MAPK exhibited increased activation in response to a GH time treatment. As Akt and MAPK are both known GH signaling elements (see Zhu et al., 2001 for review), these data support Akt and MAPK as the primary signaling elements that GH activates to facilitate the changes observed in the osmoregulatory genes studied. Previous data show that in Atlantic salmon smolts, there is activation of the PI3K/Akt pathway after exposure to SW (Martin et al., *in preparation*), further supporting GH mediated osmoregulatory changes via activation of Akt. To further validate this hypothesis, we examined the mRNA expression of the main osmoregulatory genes when treated with GH and specific cell signaling inhibitors. These treatments showed no significant results for any of the genes tested. Whether this was due to experimenter error or some other factor is unknown, and more work is needed to further validate these findings.

Taken together, our results support previous findings that support GH playing a key role in the ability of euryhaline teleosts to adapt to a SW environment, based on the expression changes of key osmoregulatory genes. This work is the first to propose a possible mechanism for the actions of GH in facilitating these changes, namely through activation of Akt and MAPK. These findings also support previous findings in Atlantic salmon that support the activation of Akt during SW adaptation.

CHAPTER 4: OVERVIEW, CONCLUSIONS, AND A LOOK FORWARD

Currently, there is a fair amount of research that is investigating the extraordinary ability that euryhaline telesosts possess, namely, the ability to live in both FW and SW, two environments at opposite ends of the habitat spectrum. In FW they must deal with passive loss of ions and uptake of water, while in SW they must combat passive ion gain and the loss of water. In Chapter 1, I discussed a number of key factors that are involved in the facilitation of this ability. I discussed how some species of salmonids have evolved a specific life phase change, known as smoltification, which coordinates the transition from FW to SW as a part of their natural lifecycle. Along with this life phase, I discussed a number of key proteins that are involved in both smoltification and SW adaptation in general.

NKATPase plays a key role, with various isoforms having evolved to adapt to both FW and SW. The discovery of these multiple isoforms in Rainbow trout by Richards et al. in 2003 has led to a vast array of studies to determine the function of each isoform. Currently, evidence supports NKAα1B functioning as the FW isoform, while NKAα1B functions as the SW isoform (McCormick et al., 2013). Coordinating the changes in NKA isoforms, along with changes in other osmoregulatory proteins, are a number of hormones. Specifically, I spoke mostly about cortisol, PL, and GH, which is the focus of this research. Previously it has been shown that each of these three hormones plays a key role in osmoregulation, with PL functioning to promote FW adaptation (Ball and Ensor, 1965), while cortisol (Hirano, 1969) and GH (Richman, 1987; McCormick, 1996) promote SW adaptation. While we know that each of these hormones plays a key role in osmoregulation, there is very little know about the cellular signaling mechanism used to coordinate these functions, which leads to the research presented above.

Chapter 2 presents the data collected from a study of Atlantic salmon parrs and smolts, both over the natural smoltification cycle in FW and after exposure to a SW challenge. Multiple genes that play key roles in osmoregulation were examined, along with genes of the GH/IGF axis. Also examined were known GH signaling elements, in order to provide insight into the GH mechanism utilized during osmoregulation. The expression patterns of the osmoregulatory genes examined followed the expected pattern, with NKAα1A decreasing during smoltification and SW transfer, with NKAα1B increasing in the same situations. Also observed were increases in the expression of NKCC and CFTR, two more key players in osmoregulation. These changes are in agreement with previous findings, and provide further support for the roles of these genes in osmoregulation. Along with these changes in gene expression, activation of GH cell signaling elements was observed. Activation of the PI3K/Akt and JAK/STAT pathways was observed, suggesting that these pathways are being utilized by GH to facilitate its actions during smoltification and SW adaptation.

In Chapter 3, a different approach to gathering data about GHs osmoregulatory actions was taken. Direct treatment of Rainbow trout gill tissue with GH was used to observe the direct causal effects of GH. Both time and dose treatments were used, along with examination of cell signaling elements and a treatment with inhibitors for specific cell signaling elements. Among the genes analyzed, only two exhibited any significant change. A time-dependent decrease in NKAα1a was observed, which supports the role of GH as a promoter of SW adaptation. Also observed was a time-dependent decrease, followed by an increase, in GHSR expression. Little is known about the effects of ghrelin and GHSR in osmoregulation, and further research is needed to determine its role. When the cell signaling elements were analyzed, activation of two signals was observed. Both Akt and MAPK showed increased activation in response to the GH time

treatment, suggesting that these signaling pathways are being utilized by GH to facilitate the changes observed in NKA and GHSR. To further validate the role of these signaling elements, we performed a treatment with GH and specific signaling inhibitors; however, no significant change was observed in any of the genes observed in this treatment.

When taken together, a number of conclusions can be drawn from these two experiments. Firstly, there is now more evidence to support the roles of the various NKA isoforms in osmoregulation, namely NKAα1a and NKAα1b. There is also more evidence to support the importance of NKCC and CFTR in osmoregulation. The changes in GHSR that were observed in both experiments suggest that it plays a role in osmoregulation; however, further research is needed. Finally, the evidence presented suggests that to facilitate these osmoregulatory changes, GH functions through the activation of the PI3K/Akt, JAK/STAT, and MAPK pathways.

Looking forward, there is still much to do. There are still euryhaline species that have yet to be studied, and species with the ability to become euryhaline are still being discovered. The roles of the various NKA isoforms are fairly constant within different species; however, there are exceptions, and these exceptions merit further examination. Further research is also needed to examine the cell signaling elements utilized by the key hormones during adaptation not only to SW, but also adaptation to FW. While the evidence presented above does provide support for the activation of certain GH signaling pathways, the lack of change in the inhibitor treatment prevents the establishment of a direct causal link, opening the door for further research. Also of note is that the cell signaling elements utilized by cortisol and PL during osmoregulation have yet to be examined. With respect to the importance of this research and other research pertaining to osmoregulation, as we go forward the importance will only increase. With climate change and other environmental factors constantly changing habitat conditions, more and more species may

be forced to seek new habitat ranges, which may lead to an increase in species that are considered euryhaline. Along with habitat changes, this research also has large implications for the salmon farming industry. Salmon farming and fishing is a $3 billion industry, and provides food to millions. If they are provided with more accurate information on how they can increase survivability of released salmon, we can move closer to food sustainability. Overall, there has been a large increase in our knowledge of osmoregulation in recent years, but even more is yet to come.

REFERENCES

Ágústsson, T., Sundell, K., Sakamoto, T., Ando, M., Björnsson, B.T. (2003). Pituitary gene expression of somatolactin, prolactin, and growth hormone during Atlantic salmon parr-smolt transformation. *Aquaculture* 222, 229-238.

Ball, J.N., and Ensor, D.M. (1965). Effect of prolactin on plasma sodium in the teleost, *Poecilia latipinna*. *J. Endocrinology* 32, 269-270.

Bergan, H. E., Kittilson, J. D., and Sheridan, M. A. (2012). Nutrition-regulated lipolysis in rainbow trout (*Oncorhynchus mykiss*) is associated with alterations in the ERK, PI3K-Akt, JAK-STAT, and PKC signaling pathways. *Gen. Comp. Endocrinol.* 176, 367-376.

Bergan, H.E., Kittilson, J.D., and Sheridan, M.A. (2013). PKC and ERK mediate GH-stimulated lipolysis. *Journal of Mol. Endocrinol.* 51, 213-224.

Bern, H. A. (1978). Endocrinological studies on normal and abnormal salmon smoltification. In *Comparative Endocrinology* (eds. P. J. Gaillard and H. H. Boer), pp. 77–100. Amsterdam: Elsevier/North Holland Biomedical Press.

Björnsson, B. T., (1997). The biology of salmon growth hormone: from daylight to dominance. *Fish Physiol. Biochem.* 17, 9-24.

Björnsson, B. Th., Stefansson, S. O. and McCormick, S. D. (2011). Environmental endocrinology of salmon smoltification. *Gen. Comp. Endocrinol.* 170, 290–298.

Bodinier, C., Lorin-Nebel, C., Charmantier, G., and Boulo, V. (2009). Influence of salinity on the localization and expression of the CFTR chloride channel in the ionocytes of juvenile *Dicentrarchus labrax* exposed to seawater and freshwater. *Comp. Biochem. Physiol.* 153A, 345-351.

Edwards, S. L. and Marshall, W. S. (2013). Principles and patterns of osmoregulation and euryhalinity in fishes. In *Fish Physiology*, Vol. 32, *Euryhaline Fishes* (eds. S. D. McCormick, C. J. Brauner and A. P. Farrell). Amsterdam: Academic Press, pp. 1–44.

Evans, D. H., Piermarini, P.M. and Choe, K. P. (2005). The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiolog. Rev.* 85, 97–177.

Fontaine, M. and Hatey, J. (1954). Sur la teneur en 17-hydroxycorticosteroides du plasm de saumon (*Salmo salar* L.). *Compt. Rend. Acad. Sci.* 239, 319–321.

Herndon, T.M., McCormick, S.D., Bern, H.A., 1991. Effects of prolactin on chloride cells in opercular membrane of seawater-adapted tilapia. *Gen. Comp. Endocrinol*. 83, 283–289.

Hirano, T. (1969) Effects of hypophysectomy and salinity change on plasma cortisol concentration in the Japanese eel, *Anguilla japonica*. *Endocrinologia Japonica* 16, No. 5, 557-560.

Hiroi, J., Yasumasu, S., McCormick, S. D., Hwang, P. P., and Kaneko, T. (2008). Evidence for an apical Na-Cl cotransporter involved in ion uptake in a teleost fish. *J. Exp. Biol.* 211, 2584-2599.

Hoar, W.S. (1988). The physiology of smolting salmonids. *In* Fish Physiology, Vol. XIB (ed. W.S. Hoar and D. Randall) pp. 275-343. New York: Academic Press.

Ip, Y. K., Loong, A. M., Kuah, J. S., Sim, E.W. L., Chen, X. L., Wong, W. P., Lam, S. H., Delgado, I. L. S., Wilson, J. M., and Chew, S. F. (2012). Roles of three branchial Na^+-K^+-ATPase α-subunit isoforms in freshwater adaptation, seawater acclimation, and active ammonia excretion in *Anabas testudineus*. *Am J Physiol Regul Integr Comp Physiol*. 303, 112-125.

Jönsson, E., Kaiya, H., and Björnsson, T. (2010). Ghrelin decreases food intake in juvenile rainbow trout (*Oncorhynchus mykiss*) through the central anorexigenic corticotropin-releasing factor system. *Gen. and Comp. Endocrinol.* 166, 39-46.

Kiilerich, P., Kristiansen, K. and Madsen, S. S. (2007a). Hormone receptors in gills of smolting Atlantic salmon, *Salmo salar*: Expression of growth hormone, prolactin, mineralocorticoid and glucocorticold receptors and 11 beta-hydroxysteroid dehydrogenase type 2. *Gen. Comp. Endocrinol.* 152, 295–303.

Kiilerich, P., Kristiansen, K. and Madsen, S. S. (2007b). Cortisol regulation of ion transporter mRNA in Atlantic salmon gill and the effect of salinity on the signaling pathway. *J. Endocrinol.* 194, 417–427.

Kittilson, J.D., Jones, E., and Sheridan, M.A. (2011). ERK, Akt, and STAT5 are differentially activated by the two growth hormone receptor subtypes of a teleost fish (*Oncorhynchus mykiss). Frontiers in Endocrinology* 2, 1-7.

Lee, K.M., Kaneko, T., Katoh, F., and Aida, K. (2006). Prolactin gene expression and gill chloride cell activity in fugu Takifugu rubripes exposed to a hypoosmotic environment. *Gen. Comp. Endocrinol.* 149, 285-293.

Lubin, R. T., Rourke, A. W. and Bradley, T. M. (1989). Ultrasturctural alteration in branchial chloride cells of Atlantic salmon, *Salmo salar*, during the parr-smolt transformation and early development in sea water. *J. Fish Biol.* 34, 259–272.

Lytle, C., Xu, J., Biemesderfer, D., and Forbush III, B. (1991). Distribution and diversity of Na-K-Cl cotransport proteins: a study with monoclonal antibodies. *Am. J. Physiol. Cell Physiol.* 269, 1496-1505.

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

Madsen, S. S., Kiilerich, P., and Tipsmark, C. K. (2009). Multiplicity of expression of Na^+, K^+-ATPase α-subunit isoforms in the gill of Atlantic salmon (*Salmo salar*): cellular localisation and absolute quantification in response to salinity change. *The Journal of Experimental Biology* 212, 78-88.

Mancera, J.M., Carrion, R.L., and del Rio, M.D.M. (2002). Osmoregulatory action of PRL, GH, and cortisol in the gilthead seabream (*Sparus aurata L.*) *Gen. Comp. Endocrinol.* 129, 95-103.

Manzon, L.A., 2002. The role of prolactin in fish osmoregulation: a review. *Gen. Comp. Endocrinol*. 125, 291–310.

Martin, L., Kittilson, J, McCormick, S. D., and Sheridan, M. (2014) Alterations in growth hormone signaling pathways associated with smoltification and seawater adaptation in Atlantic salmon (*Salmo salar*). *In preparation*

McCormick, S. D. (1993) Methods for non-lethal gill biopsy and measurement of Na^+,K^+-ATPase activity. *Can. J. Fish. Aquat. Sci.* 50, 656-658.

McCormick, S. D., Björnsson, B. Th., Sheridan, M., Eilertson, C., Carey, J. B., and O'Dea, M. (1995) Increased daylength stimulates plasma growth hormone and gill Na^+,K^+-ATPase in Atlantic salmon (*Salmo salar*). *J. Comp. Physiol.* 165, 245-254.

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

McCormick, S. D. (2001). Endocrine control of osmoregulation in teleost fish. *Am. Zool.* 41, 781–794.

McCormick, S. D., Shrimpton, J. M., Moriyama, S. and Björnsson, B. Th. (2007). Differential hormonal responses of Atlantic salmon parr and smolt to increased daylength: A possible developmental basis for smolting. *Aquaculture* 273, 337–344.

McCormick, S. D., Regish, A. M. and Christensen, A. K. (2009). Distinct freshwater and seawater isoforms of Na^+/K^+-ATPase in gill chloride cells of Atlantic salmon. *J. Exp. Biol.* 212, 3994–4001.

McCormick, S. D., Regish, A. M., Christensen, A. K., and Björnsson, B. Th. (2013). Differential regulation of sodium-potassium pump isoforms during smolt development and seawater exposure of Atlantic salmon. *J. Exp. Biol.* in press.

Mizuno, S., Ura, K., Onodera, Y., Fukada, H., Misaka, N., Hara, A., Adachi, S. and Yamauchi, K. (2001). Changes in transcript levels of gill cortisol receptor during smoltification in wild masu salmon, *Oncorhynchus masou*. *Zool. Sci.* 18, 853–860.

Nilsen, T. O., Ebbesson, L. E., Stefansson, S. O., Madsen, S. S., McCormick, S. D., Björnsson, B. Th. and Prunet, P. (2007). Differential expression of gill Na^+,K^+-ATPase α- and βsubunits, Na^+,K^+,$2Cl^-$ cotransporter and CFTR anion channel in juvenile anadromous and landlocked Atlantic salmon *Salmo salar*. *J. Exp. Biol.* 210, 2885–2896.

Nilsen, T. O., Ebbesson, L. O. E., Kiilerich, P., Björnsson, B. Th., Madsen, S. S., McCormick, S. D. and Stefansson, S. O. (2008). Endocrine systems in juvenile anadromous and landlocked Atlantic salmon (*Salmo salar*): Seasonal development and seawater acclimation. *Gen. Comp. Endocrinol.* 155, 762–772.

Nishioka, R. S., Bern, H. A., Lai, K. V., Nagahama, Y. and Grau, E. G. (1982). Changes in the endocrine organs of coho salmon during normal and abnormal smoltification- an electronmicroscopy study. *Aquaculture* 28, 21–38.

Norbeck, L.A., Kittilson, J.D., and Sheridan, M.A., (2007). Resolving the growth-promoting and metabolic effects of growth hormone; differential regulation of GH-IGF-I system components. *Gen. Comp. Endocrinol.* 151, 332-341.

Pelis, R. M. and McCormick, S. D. (2001). Effects of growth hormone and cortisol on Na+K+2Cl- cotransporter localization and abundance in the gills of Atlantic salmon. *Gen. Comp. Endocrinol.* 124, 134–143.

Pickford, G.E., Robertson, E.E., and Sawyer, W.H. (1965). Hypophysectomy, replacement therapy, and tolerance of the euryhaline killifish, *Fundulus heteroclitus*, to hypotonic media. *Gen. Comp. Endo.* 5, 160-180.

Pickford, G.E., Pang, P.K.T., and Sawyer, W.H. (1966). Prolactin and serum osmolality of hypophysectomized killifish, *Fundulus heteroclitus*, in fresh-water. *Nature* 209, 1040-1041.

Pisam, M., Auperin, B., Prunet, P., Rentierdelrue, F., Martial, J., Rambourg, A., (1993). Effects of prolactin on alpha and beta chloride cells in the gill epithelium of the saltwater adapted tilapia *Oreochromis niloticus*. *Anat. Rec*. 235, 275–284.

Poppinga, J., Kittilson, J., McCormick, S. D., and Sheridan, M. A. (2007). Effects of somatostatin on the growth hormone-insulin-like growth factor axis and seawater adaptation of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 273, 312-319.

Prunet, P., Boeuf, G., Bolton, J. P. and Young, G. (1989). Smoltification and seawater adaptation in Atlantic salmon (*Salmo salar*): plasma prolactin, growth hormone, and thyroid hormones. *Gen. Comp. Endocrinol.* 74, 355–364.

Reindl, K.M., Kittilson, J.D., Bergan, H.E., and Sheridan, M.A. (2011). Growth hormone-stimulated insulin-like growth factor-1 expression in rainbow trout (*Oncorhynchus mykiss*) hepatocytes is mediated by ERK, PI3K-Akt, and JAK-STAT. *Am J Physiol Regul Integr Comp Physiol* 301, 236-243.

Reindl, K.M., and Sheridan, M.A., (2012). Peripheral regulation of the growth hormone/insulin-like growth factor system in fish and other vertebrates. *Aquac. Int.* 163, 231-245.

Reinecke, M., Bjornsson, B.T., Dickhoff, W.W., McCormick, S.D., Navarro, I., Power, D.M., and Gutierrez, J. (2005). Growth hormone and insulin-like growth factors in fish: where we are and where to go. *Gen. Comp. Endocinol.* 142, 20-24.

Reinecke, M. (2010). Influences of the environment on the endocrine and paracrine fish growth hormone-insulin-like growth factor-I system. *J. Fish Biol.* 76, 1233-1254

Richards, J. G., Semple, J. W., Bystriansky, J. S. and Schulte, P. M. (2003). Na^+/K^+-ATPase α isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *J. Exp. Biol.* 206, 4475–4486.

Richards, J. G., Semple, J. W., Bystriansky, J. S., and Schulte, P. M. (2003). Na^+/K^+-ATPase α-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *The Journal of Experimental Biology* 206, 4475-4486.

Richman, N. H., Tai de Diaz, S., Nishioka, R. S., Prunet, P. and Bern, H. A. (1987). Osmoregulatory and endocrine relationships with chloride cell morphology and density during smoltification in coho salmon (*Oncorhynchus kisutch*). *Aquaculture* 60, 265–285.

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

Sakamoto, T., Sheperd, B. S., Madsen, S. S., Nishioka, R. S., Siharath, K., Richman III, N. H., Bern, H. A., and Grau, E.G. (1997). Osmoregulatory actions of growth hormone and prolactin in an advanced teleost. *Gen. Comp. Endocrinol.* 106, 95-101.

Sakamoto, T., and McCormick, S. D. (2006). Prolactin and growth hormone in fish osmoregulation. *Gen. Comp. Endocrinol.* 147, 24-30.

Sangiao-Alvarellos, S., Miguez, J. M., and Soengas, J. L. (2005). Actions of growth hormone on carbohydrate metabolism and osmoregulation of rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* 141, 214-225.

Sangiao-Alvarellos, S., Arjona, F. J., Miguez, J. M., Martin del Rio, M. P., Soengas, J. L., and Mancera, J. M. (2006). Growth hormone and prolactin actions on osmoregulation and energy metabolism of gilthead sea bream (*Sparus auratus*). *Comp. Biochem. Physiol.* 144A, 491-500.

Shrimpton, J. M., Devlin, R. H., Mclean, E., Byatt, J. C., Donaldson, E. M. and Randall, D. J. (1995). Increases in gill corticosteroid receptor abundance and saltwater tolerance in juvenile coho salmon (*Oncorhynchus kisutch*) treated with growth hormone and placental lactogen. *Gen. Comp. Endocrinol.* 98, 1–15.

Shrimpton, J. M. and McCormick, S. D. (1998). Seasonal differences in plasma cortisol and gill corticosteroid receptors in upper and lower mode juvenile Atlantic salmon. *Aquaculture* 168, 205–219.

Singer, T. D., Clements, K. M., Semple, J. W., Schulte, P. M., Bystriansky, J. S., Finstad, B., Fleming, I. A. and McKinley, R. S. (2002). Seawater tolerance and gene expression in two strains of Atlantic salmon smolts. *Can. J. Fish. Aquat. Sci.* 59, 125–135.

Specker, J. L. and Schreck, C. B. (1982). Changes in plasma corticosteroids during smoltification of coho salmon, *Oncorhynchus kisutch*. *Gen. Comp. Endocrinol.* 46, 53–58.

Stefansson, S. O., Haugland, M., Björnsson, B. T., McCormick, S. D., Holm, M., Ebbesson, L. O. E., Holst, J. C., Nilsen, T. O. (2012). Growth, osmoregulation and endocrine changes in wild Atlantic salmon smolts and post-smolts during marine migration. *Aquaculture* 362-363, 127-136.

Takei, Y. and McCormick, S. D. (2013). Hormonal control of fish euryhalinity. In *Fish Physiology*, Vol. 32, *Euryhaline Fishes* (eds. S. D. McCormick, C. J. Brauner and A. P. Farrell), pp. 69–124. Amsterdam: Academic Press.

Tipsmark, C. K. and Madsen, S. S. (2009). Distinct hormonal regulation of Na^+,K^+-ATPase genes in the gill of Atlantic salmon (*Salmo salar* L.). *J. Endocrinol.* 203, 301–310.

Uchida, K., Kaneko, T., Tagawa, M. and Hirano, T. (1998). Localization of cortisol receptor in branchial chloride cells in chum salmon fry. *Gen. Comp. Endocrinol.* 109, 175–185.

Waters, M.J., Hoang, H.N., Fairlie, D.P., Pelekanos, R.A., and Brown, R.J., (2006). New insights into growth hormone action. *Journal of Mol. Endocrinol.* 36, 1-7.

Wu, Q., Delpire, E., Hebert, S. C., and Strange, K. (1998). Functional demonstration of Na^+-K^+-$2Cl^-$ cotransporter activity in isolated, polarized choroid plexus cells. *Am. J. Physiol. Cell Physiol.* 275, 1565-1572.

Wu, Y. C., Lin, L. Y., and Lee, T. H. (2003). Na^+, K^+, Cl^- cotransporter: a novel marker for identifying freshwater- and seawater-type mitochondria-rich cells in gills of the euryhaline tilapia, *Oreochromis mossambicus*. *Zoo. Studies* 42, 186-192.

Young, G. (1989). Cortisol secretion in vitro by the interrenal of coho salmon (*Oncorhynchus kisutch*) during smoltification: relationship with plasma thyroxine and plasma cortisol. *Gen. Comp. Endocrinol.* 63, 191–200.

Zaugg, W. S. and McLain, L. R. (1976). Influence of water temperature on gill sodium,

potassium-stimulated ATPase activity in juvenile coho salmon (*Oncorhynchus kisutch*). *Comp. Biochem. Physiol.* 54A, 419–421.

Zhou, B., Kelly, S.P., Ianowski, J.P., Wood, C.M., 2003. Effects of cortisol and prolactin on Na+ and Cl_ transport in cultured branchial epithelia from FW rainbow trout. *Am. J. Physiol. Regul. Integr. Comp. Physiol*. 285, R1305–R1316.

Zhu, T., Goh, E. L. K., Graichen, R., Ling, L., and Lobie, P. E. (2001). Signaling transduction via the growth hormone receptor. *Cellular Signalling*. 13, 599-616.