NUTRITIONAL AND NEUROENDOCRINE CONTROL OF APPETITE IN TRANSGENIC COHO SALMON

by

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ABSTRACT

The increased feeding motivation and enhanced growth found in growth hormone (GH) transgenic coho salmon, *Oncorhynchus kisutch*, raises questions regarding the hormonal changes regulating growth and feed intake. These fish exhibit increased circulating GH and insulin-like growth factor I (IGF-I) and GH mRNA expression resulting from the GH transgene, but the role of the other hormones in the GH-axis and appetite control are unknown. Transgenic and non-transgenic coho salmon pre-smolts were fed diets with 15, 17, 19 and 21 MJ of digestible energy/kg for 84 days and they were analyzed for growth, feed intake and protein utilization. Gene mRNA expression of the major regulators in the GH-axis: growth hormone, growth hormone receptor (GHR), insulin-like growth factor I, growth hormone-releasing hormone (GHRH) and somatostatin (SS) as well as the appetite hormone, cholecystokinin (CCK), were analyzed with quantitative PCR in the brain of fish from this experiment and in six brain regions (telencephalon, hypothalamus, pituitary, optic tectum, midbrain, cerebellum) and muscle and liver of size-matched (55g) full-ration transgenic, restricted-ration transgenic (pair-fed to non-transgenic fish) and full-ration non-transgenic fish. Transgenic fish had greater feed intake and growth which were respectively negatively and positively correlated with the digestible energy content of the diet. Protein use was more efficient in transgenic fish when fed the 15 and 17 MJ/kg diets only and diet had no effect on gene expression. Plasma IGF-I was greater in transgenic fish but did not correlate with weight as in the non-transgenic coho salmon. In the size-matched study, GH expression was highest in transgenic fish, except in the pituitary where there were no differences. GHR expression generally followed that of GH but was tissue specific. Transgenic fish has higher IGF-I expression but hepatic IGF-I levels in ration-restricted transgenic fish were reduced to that of non-transgenic fish. GHRH, SS or CCK expression did not differ between groups. It is concluded that transgenic fish, with enhanced growth, feed intake and protein utilization, ingested feed to meet a caloric demand that was possibly set by increased GH, GHR and IGF-I whereas the other GH regulating hormones and the CCK satiety signal showed little change.

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DEDICATION

To Anna

CO-AUTHORSHIP STATEMENT

The following research projects were designed by Peter A. Raven, Dr. Robert H. Devlin and Dr. David A. Higgs. The nutrition experiments in Chapter 2 involved contributions by Dr. David A. Higgs in the form of diet design and extensive manuscript revision. The experiment was constructed and performed, data analyzed, and manuscript researched and written by Peter A. Raven.

The gene analysis experiment in Chapter 3 was partially performed by other members of the Genetics Lab at the Department of Fisheries and Oceans/UBC Center for Aquaculture and Environmental Research. The fish were raised and sampled by other members of Dr. Devlin's lab. Quantitative PCR assays for insulin-like growth factor I, growth hormone receptor, myosin, albumin, ß-actin and growth hormone were designed by a Department of Fisheries and Oceans technician, Mitchell Uh. Furthermore, Mitchell Uh measured expression of these genes in liver and muscle tissue. Gene expression assays for growth hormone-releasing hormone, somatostatin and cholecystokinin were designed by Peter Raven and, in addition to the assays noted above, were used to measure gene expression in the brain. Analyses of the fish from the nutrition experiment were performed by Peter A. Raven. Data analysis and manuscript research and writing were conducted by Peter A. Raven with revisions by Dr. Robert H. Devlin.

CHAPTER 1

Nutrition, growth hormone and appetite control and thesis objectives.

1.1 Introduction

The growth of the aquaculture industry and its modern economic importance has led to the suggestion that endocrinologically or genetically modified salmonids may be used to increase the maximum size and feed utilization, and decrease the growth duration of farmed fish (Mayer et al., 1994; Devlin et al., 1995). Indeed, many species of commercially-raised fish have now been modified for increased growth performance and feed efficiency including Arctic char (Pitkanen et al., 1999), carp (Hinits and Moav, 1999), tilapia (Rahman and Maclean, 1999), mud loach (Nam et al., 2001) and Atlantic salmon (Cook et al., 2000). Coho salmon, *Onchorynchus kisutch*, transgenic for chinook salmon growth hormone (GH) production is another of these genetically modified species (Devlin et al., 1994; Devlin et al., 1995). Transgenic coho salmon can grow at approximately 2-3 times the daily rate of wild stocks as a result of centrally uncontrolled GH production which can ultimately result in transgenic and non-transgenic siblings being very different sizes (from several fold to 20 fold depending on the species and strain). Associated with increased growth is a greater food consumption in which the fish feed more aggressively (Devlin et al., 1995; Devlin et al., 1999; Sundstrom et al., 2003; Sundstrom et al., 2004b).

Growth hormone transgenesis has many pleiotropic effects. For example, pituitary size decreases in GH transgenic coho salmon with age, due to the lack of endogenous GH production (Mori and Devlin, 1999). In contrast the intestinal surface area of these fish is 2.2 fold greater than that of controls (Stevens and Devlin, 2000). Increased intestinal surface area has also been seen in transgenic Atlantic salmon (Stevens et al., 1999). Changes to the shape of the head, caudal peduncle and abdominal region have resulted from enhanced cartilage and bone growth stimulated by increased GH producton (Ostenfeld et al., 1998). Parr-smolt transformation also occurs sooner in transgenic salmon than controls and there is an attendant increase in the osmoregulatory ability of the transgenic fish (Devlin et al., 1995; Devlin et al., 2000). Due to the structure of the muscle in transgenic coho salmon, they may have a higher glycolytic and aerobic requirement for muscle use (Hill et al., 2000). Indeed, these fish have greater oxygen consumption and lower swimming efficiency when starved and they consume more oxygen than controls after feeding (Lee et al., 2003; Leggatt et al., 2003). Furthermore, during smolting,

transgenic coho salmon are more susceptible to bacterial disease although no differences are seen at the fry stage (Jhingan et al., 2003).

However, before this study was undertaken it was unknown how transgenic coho salmon utilized their enhanced feed intake for growth related to non-transgenic counterparts. Furthermore, there was a scarcity of knowledge in respect to the way in which increased growth hormone titres and feed intake modified, or was modified by, the physiology of both the GH axis and the associated hormones that control feeding. Therefore, the thesis had three major goals: 1) To add to our understanding of growth physiology and nutrient utilization in transgenic coho salmon. 2) To investigate the manner in which appetite regulation is modified by changes in the GH axis in vertebrates; specifically elevations in circulating GH. 3) To use these changes in physiology to help predict the potential impacts of transgenic coho salmon on the natural ecosystem if these fish were to escape and interact with wild non-transgenic fish. The latter is a major concern when developing genetically modified fish and farming non-transgenic salmon in sea pens (Johnstone et al., 1978; Kapuscinski and Hallerman, 1991; Devlin, 1997).

1.2 Dietary Energy Utilization

In order to elucidate the hormonal mechanisms that result in an increased desire for fish to feed, it is critical to determine the manner in which transgenic fish utilize the nutrients they consume for growth. The energy-yielding fraction of feed includes protein, lipid, and carbohydrate and the non-energetic fraction is comprised of vitamins and minerals.

The protein component of feed is composed of about 20 amino acids of which 10 are essential for proper fish growth. The latter include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (NRC, 1993). Proteins are enzymatically cleaved to amino acids and di- and tripeptides in the intestine before being absorbed and catabolized (NRC, 1993). Many protein sources contain different amino acid compositions that include limitations in some amino acids that cannot be sufficiently synthesized by fish. Thus, the proper abundance of each amino acid must be provided through the use of protein sources that are complementary in digestible amino acid levels and possibly some supplemental amino acids such as lysine and/or methionine. The protein content required for maximum growth efficiency differs between species and reduced growth or increased susceptibility to disease appears in the absence of proper protein balance (NRC, 1993).

Dietary lipid is required to provide 1) sufficient dietary energy, 2) essential fatty acids of the omega-3 (n-3) and/or omega 6 (n-6) families, and 3) fat soluble vitamins. Lipids are digested

to free fatty acids or 2-monoglycerides before being absorbed and incorporated into tissues or metabolized for energy (NRC, 1993). Various fish species require a range of dietary lipid for different growth rates, but excessive lipid will result in fat deposition (NRC, 1993; Higgs and Dong, 2000). Carbohydrates may be added to feed to provide energy and allow the use of protein and lipid for tissue or chemical synthesis rather than metabolic energy. Fish possess the enzymes to digest carbohydrates with the exception of complex carbohydrates such as cellulose and fibre (NRC, 1993). Carbohydrate digestibility varies and will be discussed below.

Minerals are essential for proper physiological function through hormone and enzyme synthesis, osmoregulation and electron transfer (NRC, 1993). Many essential minerals can be absorbed from the water through the gills, skin and gut including calcium, magnesium, sodium, potassium, iron, iodine, zinc, copper and selenium but these minerals, in addition to phosphates, sulphates and chlorine, can also be absorbed from the feed (NRC, 1993).

Vitamins are essential for proper metabolism through their role as coenzymes. The water-soluble vitamins choline, myoinositol, riboflavin, thiamin, pantothenic acid, niacin, biotin, folate and vitamins B and C may be made by microorganisms in some fish intestines but in most cases must be supplied in the diet (NRC, 1993). The fat-soluble vitamins A, D, E and K are dependent on factors that alter fat absorption by the gut. An excess of fat soluble vitamins may result in toxic vitamin storage, and an extreme deficiency in any vitamin group results in disease (NRC, 1993). Additional feed components include the necessary pigments (astaxanthin and canthaxanthin) for fish colouration, antioxidants to prevent naturally oxidizing lipids from damaging other feed components, pellet binders, and in some cases hormones and feed stimulants (NRC, 1993).

Few studies have been conducted on the nutrient utilization in GH transgenic fish (Chatakondi et al., 1995b; Fu et al., 1998; Farmanfarmaian and Sun, 1999; Krasnov et al., 1999b; Cook et al., 2000; Fu et al., 2000; Martinez et al., 2000; Rahman et al., 2001; Dunham et al., 2002), but research on unmodified farmed salmon species is abundant. The effects of varying digestible protein, lipid, carbohydrate, and total energy on the growth and nutrient composition of fish have often been assessed in nutrition studies. Furthermore, the ratios of dietary lipid to protein, and lipid to total digestible energy, have strong effects on the growth of farmed fish. Other dietary parameters such as feeding frequency, feeding time and diet pellet size may also affect fish growth (Brett and Groves, 1979; Higgs et al., 1995). These nutrition variables will be discussed in relation to both farmed and transgenic fish.

1.2.1 Protein

Fish growth and the deposition of new tissue are greatly affected by total protein intake. The ratio of protein to total energy in the diet can affect feed intake and protein utilization, resulting in differences in growth (mass or length) and differences in protein levels in the proximate composition of the fish. In cases where excessive protein is available, protein must be de-aminated and excreted. When total energy is limiting in diets, protein may be used as an energy source, potentially at the expense of protein available for growth (Millikin, 1982; Higgs et al., 1995). Alternatively, when adequate non-protein dietary energy is available a protein sparing effect may occur, in which protein deposition efficiencies increase in fish on lower protein diets. In Atlantic salmon this has occurred in studies where there has been an increase in dietary carbohydrate (Hemre et al., 1995; Grisdale-Helland and Helland, 1997; Sveier et al., 1999; Hillestad et al., 2001), lipid (Hillestad and Johnsen, 1994) or total energy (Bendiksen et al., 2003; Azevedo et al., 2004; Krogdahl et al., 2004). Protein sparing has also been observed in masu salmon with an increase in dietary energy (Lee and Kim, 2001) and in coho salmon with an increase in dietary lipid (Millikin, 1982; Chan et al., 2002). Conversely, Azevedo et al. (2004) did not find a protein sparing affect in lake trout under the conditions of their study.

The protein content of fish tissues is dependent on fish species, age, size, and diet. Protein content increases in Atlantic salmon (Shearer et al., 1994), and rainbow trout (Reinitz, 1983) as they grow and this effect must be accounted for when analyzing the effect of dietary treatment on protein concentration. Coho salmon maintained on a high ration had increased muscle protein content (Markert et al., 1977) whereas starved fish lose protein in their muscle and liver (Einen and Thomassen, 1998; Einen et al., 1998). In cases where too much dietary energy is present, protein content can decrease whereas lipid storage increases (Weathercup et al., 1997; Johansen et al., 2002).

The presence of GH greatly affects fish growth and protein utilization. Similarly, increased protein synthesis and accretion has been seen compared to controls in GH-injected rainbow trout (Forster et al., 1988; Fauconneau et al., 1996) and bass (Farmanfarmaian and Sun, 1999). In contrast to the general trend of increased body protein, bovine growth hormone injection caused a decrease in coho salmon muscle protein (Markert et al., 1977). This may have occurred as a result of insufficient total energy intake and the use of protein for energy. GH transgenic carp were found to have increased protein retention and content (Fu et al., 1998; Dunham et al., 2002) and transgenic tilapia were observed to have both greater protein synthesis and efficiency (Martinez et al., 2000; Rahman et al., 2001).

1.2.2 Lipid

Most stored energy in Pacific salmon is in the form of protein, but because this energy is needed for activities such as swimming, most of the energy that is mobilizable for metabolism is stored as lipid (Hendry et al., 2000). Lipid, like protein, proportionally increases in tissues with fish size (Vanstone and Markert, 1968; Shearer et al., 1994; Torstensen et al., 2001; Nordgarden et al., 2003) and with certain types of diets. Higher energy intake (due to increased energy content in the diet or an increase in ration (Shearer, 1994)), has been found to raise lipid content in masu salmon (Lee and Kim, 2001), coho salmon (Chan et al., 2002), rainbow trout (Bolliet et al., 2000) and Atlantic salmon (Grisdale-Helland and Helland, 1997). High fat diets also have been found to increase fat content in Atlantic salmon (Bjerkeng and Berge, 2000; Torstensen et al., 2001; Bendiksen et al., 2003) and rainbow trout (Jobling et al., 1998). Alternatively, both starvation and increased activity lower muscle and liver lipid stores (Reinitz, 1983; Einen and Thomassen, 1998; Einen et al., 1998; Morris et al., 2003; Azevedo et al., 2004). High dietary lipid can cause enhanced growth on high protein diets, but when lipid is increased at the cost of protein, as in low protein diets, growth can be reduced (Weathercup et al., 1997; Chan et al., 2002). This effect is similar to an increased protein sparing effect with a reduction in protein until the point at which protein becomes growth limiting.

As excess energy is often stored as lipid, lipid content may be an indicator of energy use. Indeed, the lipid content of fish can be altered by changing the protein to energy ratio of the feed (Morris et al., 2003; Azevedo et al., 2004), allowing for greater growth and energy storage. Lipid storage in fish tissues is a tradeoff with tissue moisture content and the two have been noted to be inversely related in coho (Nettleton and Exler, 1992; Chan et al., 2002), Atlantic (Foda, 1974; Jonsson et al., 1996; Einen et al., 1998; Bjerkeng and Berge, 2000; Simpkins et al., 2003; Solberg, 2004), and sockeye (Hendry et al., 2000) salmon, as well as rainbow trout (Reinitz, 1983; Weathercup et al., 1997; Jobling et al., 1998) and GH-injected coho salmon (Markert et al., 1977).

Because lipid content is related to energy intake, fish may be using a lipostat to regulate growth and body weight. Thus, they may increase feed intake to increase growth when fat stores are too low and this continues until the body lipid content reaches normal levels (Jobling and Johansen, 1999; Johansen et al., 2001; Johansen et al., 2002). Lipostat control is the expected mechanism in catch-up growth where feed has been restricted. Consequently, growth-restricted fish drastically increase growth rate upon feeding to satiation until normal size for the age group

is reached. This model for feed intake control does not agree with some studies on trout and chinook salmon, in which fish were shown to eat to a protein demand (Azevedo et al., 2004).

Growth hormone has a marked effect on lipid storage and use. Growth hormone transgenic Atlantic salmon (Cook et al., 2000), and GH transgenic carp (Chatakondi et al., 1995a) have lower lipid content than controls which is concordant with the known lipolytic functions of GH (Higgs et al., 1975; Higgs et al., 1976; Markert et al., 1977). Lipid is mobilized and recruited for the increased energy needs and this resuls in a lower body lipid content.

1.2.3 Carbohydrate

Carbohydrates are biologically available to varying degrees in different fish species due to both the species' physiological ability to digest carbohydrate and the form of carbohydrate in the diet. Herbivorous and omnivorous fish are more effective at metabolically utilizing a wide range of carbohydrates than carnivorous salmon (reviewed by Krogdahl et al., 2005). Also, rainbow trout are better at digesting starch than Atlantic salmon (Krogdahl et al., 2004), although previous studies have shown that carbohydrate absorption is low in the latter species as well (Austreng, 1978). Glucose itself is efficiently absorbed by salmon but the degree to which starch is biologically available is dependent on the degradation of the cellulose layer surrounding the starch granule and the extent of gelatinization (Higgs et al., 1995).

As carbohydrate is not known to be required in the diet of wild salmon, the presence of high dietary carbohydrate quantities generally reduces feed utilization and growth (Higgs et al., 1995). For example, a protein sparing effect of low carbohydrate can be seen in Atlantic Salmon (Hemre et al., 1995; Grisdale-Helland and Helland, 1997; Hillestad et al., 2001; Krogdahl et al., 2004) but in cases where starch is 17% of the diet or higher, growth and feed efficiency are reduced (Grisdale-Helland and Helland, 1997; Hemre et al., 2002). Furthermore, a high carbohydrate diet that is low in lipid content may cause lower growth and feed utilization (Torstensen et al., 2001).

Coho salmon cannot effectively use carbohydrates in the form of raw starch and fibre in their feed (Sugiura et al., 1998) as increased fibre may reduce amino acid absorption rates (Millikin, 1982; Sugiura et al., 1998). High fibre also reduces lipid use in Atlantic salmon (Hemre et al., 1995). Despite the poor use of carbohydrates by salmon and the negative impact that high carbohydrate diets have on nutrient absorption in general, transgenesis may be able to improve carbohydrate use. For example, some indication of enhanced carbohydrate utilization

has been observed in rainbow trout that are transgenic for human glucose transporter I and rat hexokinase II (Krasnov et al., 1999a).

1.3 Growth Hormone Axis

Growth hormone is important in the control of growth, ion balance, and smolting in many fish (Peng et al., 1984; Prunet et al., 1989; Devlin et al., 1995; Devlin et al., 2000). The hormones responsible for controlling GH levels include growth hormone-releasing hormone, somatostatin, and neuropeptide Y, and their interactions are complex (Fig. 1.1)(Hurley and Phelps, 1992; Uchiyama et al., 1994; Flavell et al., 1996; Pellegrini et al., 1997; Minami et al., 1998). Feeding regulation is linked to GH control since neuropeptide Y is important in modulating both GH and appetite hormone systems. Other hormones involved in feed intake include cholecystokinin and leptin, and these may also interact with the GH axis (Peter, 1997; Silverstein and Plisetskaya, 2000; Gelineau and Boujard, 2001; Volkoff and Peter, 2001). These linkages may explain in part why increased feeding responses have been observed in GH transgenic fish. Transgenic mice have been extensively studied and as a result will be discussed in this review to address the evidence for GH control of appetite (Szabo et al., 1995; Flavell et al., 1996; Pellegrini et al., 1997; Peng et al., 2001). The recent research in fish will also be considered in comparison to the research on transgenic mice to reveal similarities and differences that may exist. Growth hormone transgenic salmon now permit examination of the effect that GH transgenesis has on the GH axis in fish.

The GH axis consists of growth hormone-releasing hormone (GHRH) which stimulates GH production or release by the pituitary gland, and somatostain (SS, or SRIF (somatotropin release inhibitory factor)) which inhibits it (Fig. 1.1)(Hurley and Phelps, 1992; Uchiyama et al., 1994; Peng et al., 2001). Both hormones act on the GH secreting cells, the somatotrophs in the pituitary gland to maintain levels of GH necessary for appropriate physiological function. GH is part of a negative feedback loop which acts through GH stimulation of SS neurons and neuropeptide Y (NPY) neurons in the hypothalamus. NPY stimulates SS production and inhibits GHRH release (Minami et al., 1998). Another hormone, insulin-like growth factor I (IGF-I) is stimulated by GH and has direct endocrine and paracrine growth effects on target tissues. IGF-I also inhibits GH production, feeding back at the level of the pituitary and hypothalamus (Mathews et al., 1988b; Wallenius et al., 2001). NPY is also known to stimulate feeding (Peter, 1997; Silverstein and Plisetskaya, 2000), while the digestive tract hormone cholecystokinin

(CCK) has a satiating effect (Silver et al., 1989; Silverstein and Plisetskaya, 2000; Jensen et al., 2001).

1.3.1 Growth Hormone Receptor

The physiological actions of GH are dependent on the affinities and densities of membrane bound growth hormone receptors (GHRs). GHR expression is seen in most tissues, with the greatest expression in the liver at the major site of IGF-I synthesis. GHR densities are influenced by circulating GH and are correlated with plasma IGF-I concentrations in transgenic mice. GHR knockout mice have low or absent circulating IGF-I (Bartke et al., 1994; Peng et al., 2001; Al-Regaiey et al., 2005) and the level of GHR mRNA expression in the liver is comparable to that of IGF-I in response to GH (Iida et al., 2004). Mutated bovine GH (bGH) binds to GH receptors in the liver causing upregulation of GHR (Chen et al., 1991) and in bGH and human GH transgenic mice, overexpression of GH increases hepatic GH binding (Bartke et al., 1994; Chen et al., 1995). Furthermore, transgenic GHRH-producing mice (increased GH production) have a three to four fold increase in hepatic GHR expression and nine fold increase in an alternatively spliced GHR transcript encoding for GH binding protein (Gonzalez et al., 2001).

GHR expression may be tissue specific. Iida et al. (2004) found that bGH, GH antagonist and control mice showed no differences in pituitary GHR mRNA expression and Chen et al. (1997) found that bGH mice had decreased GH binding in the kidney. Despite the absence of GHR differences found by Iida et al. (2004), GHR dependent feedback control by GH does occur at the level of the pituitary as somatotrophs of GHR deficient mice (in the absence of GH feedback) show histological features characteristic of hyperactive secretion (Asa et al., 2000).

Growth hormone receptor has been characterized in many fish species, including black sea bream (Tse et al., 2003), gilthead sea bream (Calduch-Giner et al., 2003; Saera-Vila et al., 2005), sturgeon (Liao and Zhu, 2004), turbot (Calduch-Giner et al., 2000), salmon (Fukada et al., 2004; Wargelius et al., 2005), goldfish (Lee et al., 2001), Japanese flounder (Nakao et al., 2004), snake head (Sun et al., 1997), and rainbow trout (Very et al., 2005). As in mice, GHR mRNA is present in most tissues with the highest expression found in the liver (Calduch-Giner et al., 2000; Lee et al., 2001; Calduch-Giner et al., 2003; Liao and Zhu, 2004). Different levels of GHR expression exist in various tissues, likely due to the presence of two GHR forms, GHR I and II which are present in black but not gilthead sea bream (Calduch-Giner et al., 2003; Tse et al., 2003; Saera-Vila et al., 2005), in coho salmon (GenBank #: AF403539, AF403540) and in

rainbow trout (Very et al., 2005). GHR I is the predominant form found in the liver and brain while pancreas and spleen have a greater expression of GHR II (Saera-Vila et al., 2005; Very et al., 2005).

The reduction of IGF-I expression through fasting occurs with changes in GHR density (Wargelius et al., 2005). Food restricted rats have lower GH binding and decreased GHR mRNA and circulating IGF-I than fed rats (Maniar et al., 1994). Fasting decreases GHR density in fish as well (Fukada et al., 2004). Coho salmon and sea bream show a decrease in hepatic GHR mRNA expression after fasting but these changes are tissue specific (Fukada et al., 2004; Saera-Vila et al., 2005). In sea bream muscle GHR I expression did not change with fasting while GHR II expression increased three fold (Saera-Vila et al., 2005). Although GHR expression changes with fasting, a threshold in energy deficiency appears to exist before which changes in GHR do not occur. Requeni et al. (2005) found that although growth rate and energy retention of Atlantic salmon decrease with an increase in the energy from plant protein that is used to replace fish oils in the feed, no changes in liver GHR binding or plasma IGF-I concentrations were seen. GHR expression may also be species specific as differences were seen in a similar experiment on sea bream (Gomez-Requeni et al., 2004).

1.3.2 Insulin-Like Growth Factor I

Insulin-like growth factor I (IGF-I) is produced predominantly in the liver in response to circulating GH. The growth effects of GH are mediated to a large extent by IGF-I, but both hormones may be needed for proper development. Mice with increased IGF-I production that are GH deficient grow larger than control GH deficient mice but not larger than mice with both intact IGF-I and GH genes (Behringer et al., 1990). The growth effects differed in these mice and this experiment suggested that proper liver growth is dependent on GH, and brain growth is stimulated by IGF-I. In addition, organomegaly is seen in mice with uncontrolled human IGF-I production without changes to skeletal growth (Mathews et al., 1988b). The lack of growth in GH transgenic mice before body tissues are IGF-I responsive further demonstrates the role of IGF-I in somatic growth (Mathews et al., 1988a).

Transgenic mice with alterations to the GH axis provide evidence for hormonal mechanisms regulating IGF-I production. In mice over-producing human growth hormone-releasing hormone (GHRH), plasma GH and IGF-I are increased and this response can be reduced with GHRH antagonists (Kovacs et al., 1997). Similarly, mice with increased GH or GH agonist production have higher plasma IGF-I (Mathews et al., 1988a; Blackburn et al., 1997;

Sotelo et al., 1998) and liver IGF-I mRNA expression (Mathews et al., 1988a), while those with decreased pituitary GH production have reduced plasma IGF-I (Camacho-Hubner et al., 1991; Szabo et al., 1995). Dwarf, GH deficient mice have decreased GHRH and increased SS mRNA expression when intracerebroventricularly injected with IGF-I (Sato and Frohman, 1993). Growth hormone stimulates IGF-I production and IGF-I may feedback on GHRH neurons in the hypothalamus or on somatotrophs in the pituitary to suppress further GH release (Mathews et al., 1988b; Wallenius et al., 2001). One strain of mice with a mutated GH transgene, and resultant low plasma IGF-I, has increased pituitary GH production because GHR binding of the mutated GH in the liver blocks active GH binding and IGF-I production is not activated. The absence of negative feedback by IGF-I in this case may explain the increase in pituitary GH protein production (Chen et al., 1991).

The growth effects of IGF-I are also seen in fish. Plasma IGF-I positively correlates with weight gain in fish such as Atlantic salmon (Dyer et al., 2004), coho salmon (Duan et al., 1995; Myers et al., 1998; Beckman et al., 2004a), chinook salmon (Pierce et al., 2002), and rainbow trout (Gabillard et al., 2003), but not barramundi (Nankervis et al., 2000). The regulatory mechanisms of IGF-I in fish are similar to those in mice. GH addition increased IGF-I liver mRNA expression in coho salmon hepatocyte cultures (Duan et al., 1992; Pierce et al., 2004; Pierce et al., 2005a) and GH injected coho salmon had four-fold greater plasma IGF-I concentrations (Shimizu et al., 1999). GH injection increased plasma IGF-I in rainbow trout (Foucher et al., 1992) and GH transgene expression may also cause IGF-I expression in the liver through autocrine and paracrine mechanisms in tilapia (Caelers et al., 2005).

Differential expression of IGF-I mRNA transcripts is seen in fish. GH addition caused increased Ea-1 and Ea-3 IGF-I mRNA expression in the liver but not the Ea-4 transcript in other tissues of coho (Duguay et al., 1994) and Atlantic salmon (Duan, 1998). Furthermore, hepatic IGF-I mRNA decreased in fasted fish but no changes were seen in other tissues in both coho salmon (Duan and Plisetskaya, 1993) and carp (Hua and Lin, 2001).

Due to the importance of IGF-I for proper somatic growth, gene expression is influenced by energy intake. Reduced feed intake, or starvation causes decreases in plasma IGF-I or liver IGF-I mRNA expression in chinook salmon (Pierce et al., 2005b), coho salmon (Duan and Plisetskaya, 1993; Shimizu et al., 1999; Pierce et al., 2001), carp (Hua and Lin, 2001), flounder (Nam et al., 1996), barramundi (Matthews et al., 1997), sea bream (Meton et al., 2000; Gomez-Requeni et al., 2004), and in most studies on tilapia (Drakenberg et al., 1989; Uchida et al., 2003). This decrease with starvation often coincides with an increase in plasma GH due to the

development of GH resistance by the liver and the uncoupling of IGF-I responsiveness from GH expression (Narnaware and Peter, 2001a; Valente et al., 2003; Pierce et al., 2004). Salmon with stunted growth have low IGF-I mRNA in the liver and low IGF-I protein, which may cause the apparent increases in GH due to the lack of negative feedback control (Duan et al., 1995; reviewed by Dickhoff et al., 1997). Indeed, human IGF-I decreases GH secretion from pituitary cultures in arctic char (Cameron et al., 2005). In most cases refeeding will restore plasma IGF-I and IGF-I mRNA expression (Duan and Plisetskaya, 1993; Meton et al., 2000).

Some exceptions to the preceding trends are seen. Meton et al. (2000) found that growth of sea bream responded to changes in ration size and feed composition while plasma IGF-I did not. Two weeks of fasting decreased plasma IGF-I in tilapia, but did not affect GH (Uchida et al., 2003) and the characteristic increase in plasma GH that accompanies IGF-I decreases during fasting does not occur in Atlantic salmon (Bjornsson et al., 1994). Also, the correlation of growth and ration to IGF-I was found to be uncoupled with a change in temperature (Beckman et al., 2004b). Further studies on the time course of these hormonal interactions may help explain these discrepancies.

1.3.3 Growth Hormone-Releasing Hormone

Growth hormone-releasing hormone (GHRH, or growth hormone-releasing factor (GRF)) is produced by neuronal cells in the arcuate nucleus of the medial basal hypothalamus (Hurley and Phelps, 1993). GHRH feedback pathways have been extensively studied in the rat, in which transgenic individuals allow study of altered hormonal physiological states (Szabo et al., 1995; Flavell et al., 1996; Pellegrini et al., 1997; Peng et al., 2001). In transgenic growth retarded rats, human GH (hGH) is constantly expressed, resulting in high levels of hGH in the rat at all times (Flavell et al., 1996). The hGH causes a decrease in GHRH mRNA expression which in turn decreases GH mRNA as well as GH protein in the pituitary (Flavell et al., 1996). The effects of this decrease is a reduced size, or dwarf phenotype due to the inability of hGH to promote somatic growth in this rat. Pellegrini et al. (1997) used these same rats to analyze the relationship of GHRH to GH through the addition of GH producing tumor cells to the pituitary. The stunted growth resulting from a lack of GH production was countered by the addition of GH in this way and GHRH cell number and cell expression were reduced (Pellegrini et al., 1997).

This negative feedback control by GH is also seen in other types of transgenic rodents (Hurley and Phelps, 1993). In non-transgenic rats, inactivation of circulating GHRH caused a decrease in GH production in the pituitary and an increase in GHRH mRNA production in the

hypothalamus, which could be reversed by addition of GH (Uchiyama et al., 1994). Furthermore, GH injection or intracerebroventricular infusion into GH deficient dwarf rats decreased hypothalamic GHRH mRNA expression (Sato and Frohman, 1993). These results support the presence of GHRH in the hypothalamus and the importance of GHRH in GH feedback regulation.

Growth hormone also affects the density of GHRH receptors in somatotrophs (Szabo et al., 1995; Peng et al., 2001). A transgenic giant mouse that overproduces GH shows a characteristic drop in GHRH mRNA expression as well as a drop in GHRH receptor RNA (Szabo et al., 1995). GHRH receptors are normally inhibited by GH yet in another transgenic giant mouse no changes in pituitary GHRH receptors were seen (Peng et al., 2001). Growth hormone receptors may be present in GHRH secreting neurons as GH receptors are seen in the arcuate nucleus of the rat hypothalamus, but evidence for direct feedback by growth hormone on GHRH neurons has yet to be observed (Minami et al., 1998). It is likely that GH has an indirect effect on GHRH secretion by acting on other neurons; these will be discussed later.

Although most GH regulation work is done in transgenic mice, GHRH is also present in fish in which it is encoded with another GH-stimulating gene, pituitary adenylate cyclase-activating polypeptide (PACAP) (Parker et al., 1993). GHRH-containing neurons have been found in the brain of the green molly (Batten et al., 1990), as well as in the brain of the sea bass (Moons et al., 1989), pejerry (Miranda et al., 2002), eel (Montero et al., 1998) and salmon (Parhar and Iwata, 1996) where they are associated closely with somatotrophs. In addition to immunoreactive studies, the GHRH/PACAP cDNA has been characterized in many fish species in which mRNA expression is found in most tissues, including the brain (Parker et al., 1993; McRory et al., 1995; Fradinger and Sherwood, 2000; Small and Nonneman, 2001; Jiang et al., 2003).

The mechanisms regulating GHRH action in fish are thought to be similar to those of mammals. In tilapia, injection or implantation of human, bovine and carp GHRH increased circulating GH levels and somatic growth (Melamed et al., 1995; Kelly et al., 1996; Shepherd et al., 2000). Furthermore, GHRH induces GH secretion from pituitary cells of carp (Kagabu et al., 1998), rainbow trout (Luo et al., 1990; Luo and McKeown, 1991), and salmon (Parker et al., 1997). In contrast, GHRH had no effect on GH release by either eel or turbot pituitary cells in culture, possibly because basal release is at the maximum physiological level in these species (Montero et al., 1998; Rousseau et al., 2001). Interperitoneal injection of human GH and GHRH stimulated an increase in nutrient uptake by the blood in rainbow trout, indicating increased

growth and metabolism (Hernandez Llorente et al., 2004). GH regulation may vary among species, especially in fish where PACAP is equipotent, or more biologically active than GHRH (Parker et al., 1997). Analyzing the effect of GH transgenesis on GHRH regulation in fish is now possible with GH transgenic coho salmon.

1.3.4 Somatostatin

In contrast to the stimulatory effects of GHRH, inhibitory control of GH secretion arises through a decrease in GHRH, mediated by effects of increased somatostatin (SS). SS neurons are present in the medial basal hypothalamus and in the periventricular nucleus (PeV) of the brain (Hurley et al., 1994). They synapse with GHRH neurons in the median eminence of the hypothalamus and inhibit the secretion of GHRH and decrease GH production (Hurley and Phelps, 1992; Uchiyama et al., 1994; Peng et al., 2001).

In transgenic mice that produce human GHRH in the brain and pituitary, there is an increase in SS mRNA expression relative to controls (Hurley and Phelps, 1992). A similar increase is seen with GH injections although the response is not as great (Hurley and Phelps, 1992). Hurley et al. (1994) used three types of transgenic mice that produced either bovine or human GH to look at SS expression in the hypothalamus. In all mice there was an increase in SS neuron number and SS mRNA expression until a plateau was reached. They also hypothesized that not only could SS mRNA expression be increased, but that GH could also cause the observed increase in the number of SS neurons during development. An increase in SS cell number and mRNA expression is seen in GH transgenic rats, and the addition of GH secreting tumor cells further increases the SS cell number and mRNA expression (Pellegrini et al., 1997). Human GH-producing rats show a similar increase in SS mRNA expression (Szabo et al., 1995) and human GHRH-producing mice show a 128% increase in SS mRNA with associated increases in SS receptor mRNA in the pituitary (Peng et al., 2001).

A state of GH deficiency demonstrates the importance of SS in reducing GHRH expression. In dwarf rats with a destroyed pituitary and a lack of GH production, a 40% decrease in SS expression is observed compared to controls (Hurley and Phelps, 1992). Similarly, GH deficient rats show a increase in SS mRNA expression when injected with GH (Sato and Frohman, 1993). Deactivation of circulating GHRH molecules causes a decrease in SS mRNA in some areas of the brain but not in others, such as the periventricular nucleus of the hypothalamus (PeV) (Uchiyama et al., 1994). In mice lacking GH receptors, GH cannot act to increase SS expression and there is a 46% drop in SS mRNA (Peng et al., 2001). Direct

injection of GH into the PeV reduces the amplitude and duration of GH pulsatile secretion, but does not change the frequency (Minami et al., 1998). This observation along with the identification of GH receptor mRNA expression in SS neurons and other corroborative evidence for decreases in GH secretion with GH injections in the PeV (Minami et al., 1998), suggest that GH has a direct action on SS neurons.

Somatostatin is present in fish as well. SS immunoreactivity is found in fish such as the green mollie (Batten et al., 1990), sea bream (Power et al., 1996), sea bass (Moons et al., 1989) and other teleosts (Olivereau et al., 1984; Tsuneki and Nozaki, 1989). Complementary DNA has been characterized from the rainbow trout (Moore et al., 1995) and three forms of differentially expressed somatostatin mRNAs have been characterized from the goldfish brain (Lin et al., 1999; Yunker et al., 2003; Canosa et al., 2005). SS functions in the same way as described in mice and rats. In vitro, it inhibits GH production by pituitary cultures in the European eel (Olivereau et al., 1984; Rousseau et al., 1998; Rousseau et al., 2001), turbot, (Rousseau et al., 2001), tilapia (Melamed et al., 1995), catfish (Lescroart et al., 1996), carp (Lin et al., 1993; Kagabu et al., 1998), arctic char (Cameron et al., 2005), goldfish (Kwong and Chang, 1997) and rainbow trout (Luo et al., 1990; Luo and McKeown, 1991). SS also reduces the increases in GH secretion stimulated by dopamine (Wong et al., 1993; Agustsson et al., 2000), or ghrelin (Unniappan and Peter, 2004). Likewise, SS inhibitors cause a rise in GH secretion in pituitary cultures containing an intact hypothalamus (Xiao and Lin, 2003). Melamed et al. (1996) and Ran et al. (2004) found that the decrease in GH production by cultured pituitary cells was caused by a decrease in GH release without a decrease in GH mRNA transcription. Differences in intracellular responses to various forms of the SS peptide may explain this discrepancy (Yunker et al., 2003; Yunker and Chang, 2004).

In vivo, an inverse relationship is seen between SS immunoreactivity and serum GH over different seasons in goldfish (Marchant et al., 1989; Lin et al., 1999) but not in rainbow trout (Holloway et al., 1994), although a correlation is seen during the maturation period (Holloway et al., 1999). SS injections reduce serum GH in goldfish (Cook and Peter, 1984), tilapia (Melamed et al., 1995), and rainbow trout (Very et al., 2001; Peterson et al., 2003). Immunization against serum SS resulted in elevated circulating GH levels in rainbow trout (Peterson et al., 2003) and a growth enhanced chinook salmon was produced through the injection of an anti-SS compound (Mayer et al., 1994). Interestingly, long term starvation of eel and carp reduces SS immunoreactivity in the pituitary despite the fact that starvation often causes an increase in circulating GH (Olivereau et al., 1984). GH resistance similar to that seen in the liver may be

occurring in the brain as well. The mechanism of SS action seems to be similar between mammals and fish, and the previous data can be corroborated with research on transgenic coho salmon in which increased GH production should have marked effects on SS expression.

1.4 Appetite Regulators

Many hormones are involved in regulating the hunger and satiation responses in fish. Feed intake is stimulated by β-endorphin (de Pedro et al., 1995a), galanin (de Pedro et al., 1995b), melanin concentrating hormone, agouti-related peptide and orexins A and B (reviewed by Tritos and Maratos-Flier, 1999). Conversely, serotonin is important in short term satiation (Halford and Blundell, 2000) and feed intake is decreased by bombesin (Himick and Peter, 1994), coticotrophin-releasing factor, neurotensin, and glucagon-like peptide 1 (reviewed by Tritos and Maratos-Flier, 1999). Leptin is a major regulator of feed intake, GH and lipid metabolism (Cai and Hyde, 1998). In goldfish, leptin injections at low levels that would not normally have an effect on feeding, enhance the inhibitory effect of cocaine and amphetamine-regulated transcript (CART) peptides on NPY and thereby decrease appetite (Volkoff and Peter, 2001). Furthermore it attenuates the effect of CCK (Volkoff et al., 2003). Another important hormone in both GH control and appetite is the gut-derived hormone, ghrelin. Ghrelin increases GH release and stimulates feed intake (reviewed by Unniappan and Peter, 2005). Both the orexigenic hormone, NPY, and anorexigenic hormone, CCK, are also important in appetite control and have roles in GH regulation.

1.4.1 Neuropeptide Y

The complexities of GH regulation become important when considering the interactions between the GH axis and the hormones that are involved in feeding control, such as neuropeptide Y (NPY). NPY is produced by neurons in the arcuate nucleus (ARC) and is structurally related to the gut peptides PP and PYY (Larhammer, 1996). NPY is thought to stimulate food intake in general, and specifically stimulate SS expression in the ARC and PeV to reduce pituitary GH release (Minami et al., 1998). Studies of food intake often do not consider the effects of NPY on the GH axis, and studies concerned with GH interactions do not normally consider the role of NPY in feeding (Chan et al., 1996; Silverstein et al., 1996; Peter, 1997; Silverstein and Plisetskaya, 2000).

Some evidence explains the effects of NPY on the GH axis. In transgenic mice without GH receptors, NPY mRNA in the hypothalamus is only 86% that of controls (Peng et al., 2001).

This suggests that GH receptors are necessary for NPY to interact with GH and maintain its expression level. In rats with pituitaries removed, somatotrophs are removed and GH cannot be produced. This causes a decrease in NPY mRNA levels that can be alleviated through the addition of rat GH (Chan et al., 1996). In mice that over-produce human GH no changes in NPY mRNA are seen (Peng et al., 2001), suggesting that there is a maximum physiological expression in NPY that cannot be exceeded with increasing additions of GH to these mice (Peng et al., 2001). Injections of NPY into the third ventricle of the rat brain caused a 3 to 4 hour drop in blood GH (Minami et al., 1997). Similarly, injections of rat GH into the ARC cause a drop in GH peak concentrations and secretion duration (Minami et al., 1997). This is hypothesized to be the result of GH-induced NPY stimulation of SS neurons to inhibit GHRH secretion (Minami et al., 1997). This mode of action is supported by the presence of GH receptor genes in 95% of the NPY expressing cells in the ARC of the rat (Kamegai et al., 1996), and by other independent identifications of GH receptors in NPY neurons (Chan et al., 1996; Peng et al., 2001). Minami et al. (1998) found that 65% of cells showing GH receptor expression in the ARC were NPY cells and 60% of cells with GH receptor expression in the PeV were SS cells. Kamegai et al. (1994) obtained the same percentages by monitoring c-*fos* expression as a proxy for neuronal activity after the administration of recombinant human growth hormone to the ARC and PeV. It is likely that GH directly stimulates NPY neurons in addition to SS secreting cells.

There is considerably more research on the role of NPY than on other feeding hormones in fish. NPY cells are found in a variety of fish tissues, including the brain (Peng et al., 1994; Ebbesson et al., 2000; Weng and Fang, 2003; Chen et al., 2005; Sakharkar et al., 2005). It is also associated with GH cells in sea bass (Moons et al., 1989) and pejerry (Traverso et al., 2003). NPY is associated with the pituitary in the grouper (Chen et al., 2005), sole (Rodriguez-Gomez et al., 2001), catfish (Gaikwad et al., 2004) and zebra fish (Mathieu et al., 2002) but not in one species of Antarctic fish (Mathieu et al., 2001) or in the goldfish (Peng et al., 1994). NPY mRNA expression is found in various regions of the rainbow trout brain with the greatest expression in the telencephalon (Doyon et al., 2003). The exact role that NPY plays in GH control is assumed to be similar to that seen in mammals but most studies on NPY in fish concentrate on feeding behavior. Indeed, NPY immunoreactivity has been detected in the gustatory regions of many fish species (Farrell et al., 2002; Pirone et al., 2004).

Generally, NPY causes an increase in feeding and suppresses energy expenditure (Silverstein and Plisetskaya, 2000). Fasting increases NPY expression in mammals, chinook salmon, and gold fish (Peter, 1997). During starvation, NPY peptide and mRNA increases in

salmon (Silverstein et al., 1996) and intracerebroventricular (ICV) injections of NPY increase food intake by 45% to 100% in the catfish (Silverstein and Plisetskaya, 2000). Fasting increases brain NPY mRNA expression in the gold fish while refeeding returns expression to a lower level (Narnaware and Peter, 2001a; Volkoff et al., 2003). How the feeding effects of NPY are linked to GH levels and function in fish needs to be determined. GH is seen to increase immediately after feeding, yet it is thought to stimulate NPY secretion that would then increase appetite further after feeding has occurred (Peter, 1997). It is evident that there may be unaddressed interactions, and it is possible that the overall hormonal or neuronal state of fish after feeding dampens the orexigenic effects of NPY. Neuronal responses to gut distension may also influence the control of appetite and the effect of NPY and other hormones. Appetite in many fish has been found to follow gastric evacuation and some teleost species eat to a specific volume (Grove et al., 1985; Singh and Srivastava, 1985; Seyhan et al., 1998; Tekinay and Davies, 2002).

1.4.2 Cholecystokinin

Cholecystokinin (CCK) is released by 1) the intestinal tract in response to the presence of digesta (Koven et al., 2002) and 2) by different portions of the brain following feeding (Peyon et al., 1999) to cause satiation (Peter, 1997; Jensen et al., 2001). CCK is also important in other functions such as gallbladder discharge and trypsin and chymotrypsin secretion (Einarsson et al., 1997). CCK has not been studied in transgenic mice, but in other mice and rats CCK administration shortens feeding or prevents feeding initiation (Silver et al., 1989), and can act with leptin to decrease feeding and reduce body weight (Barrachina et al., 1997; Matson et al., 2000). Interestingly, CCK does not change due to starvation in some strains of rats (Schneider et al., 1979). CCK may also be related to the GH axis in some cases as it can inhibit GH release in particular types of rats (Karashima et al., 1984).

Although mammals have one form of CCK, it is present in 3 preprohormone forms in the rainbow trout (designated CCK-N, CCK-L and CCK-T according to amino acid differences) and in three forms in the goldfish (Jensen et al., 2001), likely due to a gene duplication event in both cases (Jensen et al., 2001). CCK-N mRNA is found in the brain, upper and middle intestine and pyloric caeca of the goldfish, while CCK-L and CCK-T mRNA are found in the brain and gut (Jensen et al., 2001). At the peptide level, only CCK-N and CCK-L are present in the brain (Jensen et al., 2001). CCK immunoreactivity and mRNA expression have been found in the intestine and brain of many other teleost species including Japanese flounder (Suzuki et al.,

1999), herring (Kamisaka et al., 2005), halibut (Kamisaka et al., 2001), pufferfish (Kurokawa et al., 2003) and bluefin tuna (Kamisaka et al., 2002).

The localization of CCK neurons with the pituitary and hormonal responses to CCK suggest that it interacts with the GH axis. CCK is found in the telencephalon of the brain as well as the neurohypophysis and proximal pars distalis of the pituitary in the green molly (Batten et al., 1990). CCK neuronal terminals have been found to synapse with somatotrophs in the proximal pars distalis of the pituitary in goldfish brains (Peter, 1997), although no pituitary CCK gene expression was found by Jensen et al. (2001). Similarly, Moons et al. (1989) found that CCK neurons directly innervate somatotropic cells in the proximal pars distalis in the sea bass. Thus, CCK is likely synthesized in CCK cell bodies in the hypothalamus and transported through the cell to the pituitary for use. Hormonal changes also support an association with GH. In goldfish, after natural feeding, there is an increase in plasma GH followed by a decrease. This trend can be replicated through the addition of CCK (Peter, 1997). Furthermore, central and peripheral administration of CCK reduces preprosomatostatin-I mRNA expression in the brain (Canosa and Peter, 2004). This reduction may contribute to the increase in GH after feeding. How CCK interacts with GH and what the consequences are to the other hormones involved in GH control requires further study.

The satiating effect of CCK in many fish species may be achieved through intestinal or neural production. Indeed, CCK fibres have been found in the gustatory centers of the goldfish brain (Farrell et al., 2002) and CCK mRNA expression increases two hours after feeding (Peyon et al., 1999). When CCK_A receptor antagonists were added to the food of rainbow trout, food intake increased significantly compared to the untreated control (Gelineau and Boujard, 2001). Thus, CCK released from the gut likely acts on CCK_A receptors to stop feeding as CCK applied through the food would enter the trout through the gut (Gelineau and Boujard, 2001). ICV injections of the sulfated CCK octapeptide in the catfish caused a decrease in food consumption after thirty minutes (Silverstein and Plisetskaya, 2000). Conversely, CCK receptor antagonists remove the satiety signal achieved through CCK injection in goldfish, although in this study no differences in brain CCK mRNA expression were seen after fasting (Volkoff et al., 2003).

1.5 Objectives and Hypotheses

The first objective of this thesis research was to examine the potential regulatory changes in the GH axis that are occurring in GH transgenic coho salmon as well as how the GH axis responds to changes in dietary energy. To accomplish this, I first sought to determine how these

fish utilize the dietary components of their feed and how they respond to changes in dietary digestible energy while maintaining similar ratios of the energetically contributing components: protein, lipid and carbohydrate. Previous studies have shown that increased GH in vivo causes increases in growth, protein utilization and lipid mobilization. In this study it was hypothesized that GH transgenic coho salmon would have greater growth than controls as well as increased protein and decreased lipid content. Further, it was hypothesized that these fish would eat to volumetric satiation due to uncontrolled regulation of appetite through changes to the GH axis and that maximum gut distension would determine digestible nutrient and energy intake regardless of the energetic content of the feed. Alternatively, it was postulated that growth-enhanced salmon may eat to satisfy a new, higher level of energy requirement.

The second objective was to determine what changes, if any, have occurred to the hormones of the GH axis in the GH transgenic coho salmon. In this regard, the mRNA expression levels (as an indicator of protein production) of GH, IGF-I, GHRH, SS, GHR and CCK were measured in six brain regions of GH transgenic and non-transgenic coho salmon. These regions included the telencephalon, optic tectum, mid brain, cerebellum, hypothalamus and pituitary. It was postulated that the increased exogenous GH production in transgenic coho salmon would cause a decrease in endogenous GH production by the pituitary, and that brain IGF-I mRNA levels would not change due to the unresponsive nature of the Ea-4 transcript to GH (Duguay et al., 1994; Duan, 1998). Moreover, it was thought at the outset that GHRH expression may decrease and SS expression increase in the hypothalamus of transgenic coho salmon, while GHR expression may increase in the pituitary and hypothalamus to allow for the negative feedback of GH on its own production. Due to the increased appetite observed in transgenic coho salmon it was hypothesized that CCK expression would be reduced in the brain, and that this would result in a decreased satiation response.

The third objective was to examine the changes to the previously described hormone and neuropeptide mRNA levels occurring between GH transgenic coho salmon on high and low energy diets. In addition plasma IGF-I concentrations were analyzed. It was hypothesized that GH production would be decreased in the brain of fish on the high energy diet due to the increased feedback by circulating IGF-I, and that CCK would be higher in transgenic fish due to an increased satiation response. Since decreases in feed and energy intake cause a decrease in GH production, it was also postulated that GHRH may increase and SS decrease in fish ingesting diets of higher energy content.

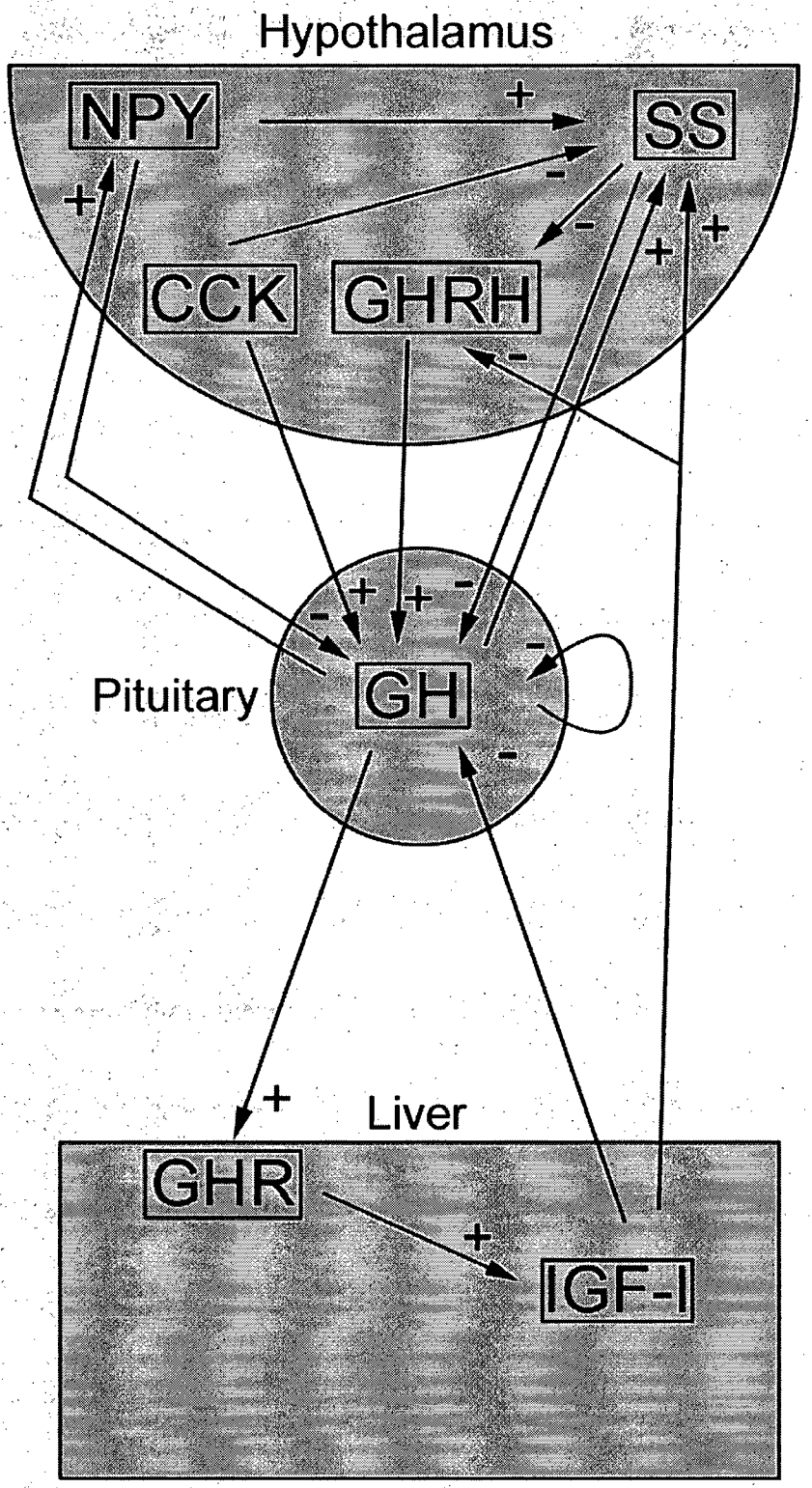

Figure 1.1: Hormonal interactions of the GH axis. The effects that are shown have mostly been observed in mammalian studies but are expected to operate in fish. NPY: neuropeptide Y, SS: somatostatin, CCK, cholecystokinin, GHRH: growth hormone-releasing hormone, GH: growth hormone, GHR: growth hormone receptor, IGF-I: insulin-like growth factor 1. Both NPY and CCK have roles in appetite regulation.

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CHAPTER 2[1]

Influence of dietary digestible energy content on growth, protein and energy utilization and body composition of growth-hormone transgenic and non-transgenic coho salmon (*Oncorhynchus kisutch*).

2.1 Introduction

Growth hormone (GH) transgenic (T) coho salmon, *Oncorhynchus kisutch,* were generated through the insertion of a gene construct consisting of a sockeye (*O. nerka*) GH gene fused to a metallothionein-B promoter sequence (Devlin et al., 1994). Subsequently, it was shown that the T coho salmon grew much more rapidly and matured in a shorter period than wild coho salmon (Devlin et al., 1994; Devlin et al., 2004a), primarily due to their markedly enhanced appetite (feed intake) without any loss in their ability to convert feed into flesh. The extreme desire for food during growth of T coho salmon, however, appears to increase their risk of predation because of reduced predator awareness (Devlin et al., 1999; Sundstrom et al., 2003; Sundstrom et al., 2004a), but little is known regarding their energy metabolism and nutritional requirements.

The increased growth performance of T coho salmon using commercially-available diets that are optimal in composition for growth of non-transgenic (NT) coho salmon has been well documented (Devlin et al., 1994; Devlin et al., 1995; Devlin et al., 2000; Devlin et al., 2004a; Devlin et al., 2004b). However, little is known about the nutrient and energy needs of T coho salmon or other aquatic finfish species relative to their non-transgenic counterparts and whether there is much scope to enhance the growth of T fish through improvements in the composition of extant diet formulations for fish (Fu et al., 1998; Cook et al., 2000; Rahman et al., 2001). The nutritional requirements and growth responses of many commercially important NT salmonid species have been determined and some examples are as follows: Atlantic salmon, *Salmo salar* (Austreng et al., 1987; Shearer et al., 1992; Hillestad et al., 2001); rainbow trout, *O. mykiss* (Weathercup et al., 1997; Chan et al., 2002; Glencross et al., 2004); chinook salmon, *O. tshawytscha* (McCallum and Higgs, 1989; Hajen et al., 1993a); coho salmon (Forster et al., 1988; Haard et al., 1996); masu salmon, *O. masu* (Lee and Kim, 2001). Many of these studies have been directed at optimizing the dietary concentrations of the energy yielding nutrients i.e.,

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protein, lipid, and carbohydrate and the ratio of digestible protein to energy for cost efficient growth and/or improvement of flesh quality. Moreover, emphasis has also been placed on optimization of various abiotic (e.g., water temperature) and biotic (e.g., fish size), factors to maximize fish growth and feed utilization (Brett and Groves, 1979).

The development of genetically modified transgenic fish to improve the cost efficacy of aquaculture has led to concerns regarding the potential impacts that these fish may have on the environment and, in the possible case of T escapes, their potential effects on the survival and sustainability of wild populations of salmon and other fish species. Many fish species have now been modified for various traits, especially for increased growth performance (Du et al., 1992; Devlin et al., 1994; Devlin et al., 1995; Hinits and Moav, 1999; Pitkanen et al., 1999; Rahman and Maclean, 1999; Morales et al., 2001; Nam et al., 2001; Rahman et al., 2001; Lu et al., 2002). However, concomitant changes in fish behaviour and physiology have resulted from these modifications, which conceivably could not only influence the success of the genetically-modified species in the natural environment but also, as indicated above, lead to undesirable interactions with other NT wild fish species (Rahman et al., 1998; Devlin et al., 1999; Martinez et al., 2000; Stevens and Devlin, 2000; Devlin et al., 2004b; Sundstrom et al., 2004b). Examination of the physiology, growth, and nutrition of T fish in culture can facilitate our understanding of what potential adverse impacts the growth-enhanced fish may have on natural fish populations, particularly if it is known how susceptible they are to predation and infectious disease agents, what their food (energy and nutrient) needs are, and how well they metabolically utilize diets of varying available energy and non-nutritive content (e.g., chitin or fibre-enriched diets) relative to NT counterparts.

In this study my main goal was to determine whether size- and age-matched T and NT coho salmon differed in their abilities to ingest and metabolically utilize feed that varied widely in digestible energy (DE) content while other aspects of their diet composition remained constant, e.g., the ratio of digestible protein to available energy and essential amino acid balance. In this regard, I was particularly interested in determining whether the physical distention of the stomach and other aspects of the digestive physiology of T coho salmon would allow these fish to maintain similar available protein and energy intake and consequently fish growth across a wide range of dietary DE content. The latter was accomplished by progressively increasing dietary fibre content at the expense of the energy yielding nutrients viz., protein, lipid and carbohydrate. It was of further interest to ascertain how well the T coho salmon utilized the fibre-enriched diets. It was felt that the findings from this study could have potential ecological

implications especially in cases where rises in sea surface temperatures result in suboptimal nutrient and energy concentrations in prey for maximum salmon growth (e.g., increased contribution of prey of higher chitin content and/or prey of reduced DE content; (Higgs et al., 1995)). Also, I thought that the results could be of interest for improving the cost effectiveness of aquaculture, especially if I obtained indications that the T coho salmon would grow well on the fibre-enriched diets without much loss in protein and energy utilization. For example, such a finding may indicate that T coho salmon are able to grow well on diets extensively based on fibre-rich plant protein sources such as inexpensive canola meal and sunflower meal, although this would have to be confirmed in subsequent studies.

2.2 Materials and Methods

2.2.1 Diet Composition

Four test dry diets of different DE content were formulated with constant ratios of digestible protein to available energy i.e., 23 MJ/kg on a dry weight basis as well as essential amino acid balance. This was accomplished by proportionately reducing the levels of the dietary protein sources as the concentration of DE was lowered in equal steps from 21 MJ/kg to 15 MJ/kg (Table 1), decreasing the supplemental levels of anchovy oil and pre-gelatinized wheat starch while simultaneously maintaining a constant ratio between the available dietary concentrations of lipid and carbohydrate (1.8 g/g) and concomitantly raising dietary concentrations of α-cellulose (non-nutritive bulk). Care was taken to ensure that all diets contained adequate levels of essential amino acids, vitamins and minerals in relation to the known essential amino acid, vitamin and mineral needs of Pacific salmon and other salmonids (NRC, 1993). Further, all diets were supplemented with 0.5% chromic oxide as an indigestible indicator. All diets were prepared and steam pelleted (2.5 mm pellets were fed to NT coho salmon between day 0 and 84 and to T coho salmon between day 0 and 63, and 4.0 mm pellets thereafter) as described by Higgs et al. (1979). Moreover, all diets were stored under cool (5°C) and dry conditions in air-tight containers throughout the study. During the first 3 weeks of the experiment only, one kilogram amounts of each of the pelleted diets were supplemented with an additional 10 ml of anchovy oil containing 50 mg of hydrolyzed krill powder in an attempt to improve the palatability of the lowest DE diet, since this was not accepted well by the NT coho salmon (unlike the T coho salmon).

2.2.2 Growth Experiment

Triplicate groups of 25 size matched (initial mean weight 12.9 ± 1.2 g (SD)) and age matched (1.5 year-old) NT and T coho salmon were fed each of the aforementioned test diets by hand twice daily (0800 and 1300 h) to satiation for 84 days using a randomized block design. At each feeding time, the pellets were added at a slow to modest rate depending upon fish appetite and the point of satiety was deemed to be reached when 3 consecutive pellets were not consumed and therefore reached the bottom of the tank. Unconsumed pellets were counted and this number multiplied by the mean air-dry weight of the pellets for a given diet provided an estimate of feed wastage. This, in turn, was deducted from the daily ration dispensed to give the actual ration consumed by each group. The fish were reared in 180 L opaque blue fibreglass tanks supplied with running (8L/min), 8-11 °C, aerated (dissolved oxygen, 8.4-10.6 mg/L; total gas pressure, 96.6-100.3%) well water and maintained on a simulated natural photocycle (Vancouver, Canada, 49^{0} 15' N 123^{0} 10' W from January to March) using daylight fluorescent lights regulated by a photocell. Each of the tanks was covered by a hinged plexiglass sheet whose back half was darkened to afford the fish some cover. Also, the water was introduced into each tank in a manner that forced the fish to swim at about 0.5 body lengths per second to minimize aggressive behavior between the fish and discourage the fish from resting on the tank bottom. Before the start of the experiment the fish were fed commercially available extruded dry pelleted diets of appropriate composition for NT Pacific salmon at this life history stage (Skretting Canada, Vancouver, BC). During the study, the 8 different treatment groups were distinguished on the basis of their genotype (NT or T) and the digestible energy content of their prescribed diet (i.e., 15, 17, 19 or 21 MJ/kg). Thus, for example, NT coho salmon fed the diet with 17 MJ DE/ kg were denoted as NT-17.

2.2.3 Fish Weighing, Sampling and Treatment

The fish in each group were not fed for at least 21hr before they were anesthetized in tricaine methane sulphonate (MS 222; 150 mg/L supplemented with 300 mg of sodium bicarbonate/L for 1.5 min) and then at 28-day intervals individually weighed to the nearest 0.01 g and measured (fork lengths) to the nearest 0.1 cm.

On day 0, a total of 10 NT and 10 T fish that were common to their respective genotypes were sampled and euthanized (0.2 g MS 222/L) for individual determinations of their initial whole body proximate compositions. Each group of fish was stored in a vacuum-packed, oxygen impermeable food-grade bag at -20°C pending analysis. On day 84, 6 fish were sampled

randomly from each group for individual determinations of whole body proximate composition and these samples were treated and stored as described above for the initial samples. Subsequently, 10 fish were removed from each of the 12 T groups of coho salmon and these were placed into twelve 150 L digestibility tanks for assessment of the bioavailable levels of protein, organic matter, and energy in each of the test diets as described below.

It should be mentioned that on day 5 of the study, all groups were treated with 4 mg of Chloramine T/L for a 15 min period followed by 0.2 ml of formalin/L for 20 min to eliminate a low incidence of suspected bacterial infection that was noted in some of the T groups. This protocol was repeated 10 days later.

2.2.4 Digestibility Study

Because the final sizes of the NT coho salmon were too small for proper assessment of their respective diet digestibilities (low fecal output) and these fish had poor appetite, diet digestibility determinations were conducted only using the T coho salmon. In this regard, estimates of the apparent digestibility coefficients for protein, organic matter (ash-free dry weight), and gross energy were obtained using the procedures and digestibility tanks described by Hajen et al. (1993a; 1993b). The same water supply that was used for the growth trial was employed and all groups of fish were fed their prescribed diet to satiation twice daily for 10 days and then feces were collected for 5 additional consecutive days. The feces that were collected from each of the fecal collection columns each morning before the first daily feeding were centrifuged, freeze-dried and then pooled (n = 3 per diet treatment) for the 5-day period for subsequent analyses of their respective moisture, ash, protein, gross energy and chromic oxide concentrations. The dietary concentrations of the preceding proximate constituents, energy and chromic oxide were also determined (see below).

2.2.5 Chemical Analyses

All diets as well as the initial and final NT and T fish and fecal samples were analyzed in duplicate for moisture (16 hr at 100 °C in a drying oven), ash (2 hr at 600 °C in a muffle furnace; AOAC Official Method 942.05 (Horwitz, 2000)), lipid (chloroform/methanol procedure of Bligh and Dyer (1959)), and protein (Technicon industrial method No. 334-74W/B, revised March, 1977, Technicon Industrial Systems, Tarrytown, N.Y.; percent nitrogen in each sample was multiplied by 6.25 to obtain percent protein). The gross energy concentrations in the diets and feces were determined by adiabatic bomb calorimetry (IKA Calorimeter System C5001 duo

control, Germany). The gross energy concentrations in the fish samples were estimated by ascribing 0.02364 MJ/g protein and 0.03954 MJ/g lipid. Dietary and fecal chromic oxide concentrations were determined using the procedures of Fenton and Fenton (1979)).

2.2.6 Performance Parameters

Fish performance during this study was assessed by the following:

(1) Final mean weight (g)

(2) SGR; Specific growth rate in weight or length (SGR = [(ln final weight or fork length-ln initial weight or fork length)/number of days] x 100

(3) Percent survival (number of fish remaining on day 84 expressed as a percentage of initial number of fish in each group)

(4) CF; Condition factor [CF = (fish weight (g)/fork length $(cm)^3$) x 100]

(5) DFI; Dry feed intake. DFI was expressed either as g/fish (DFI_T calculated as the total weight of dry feed consumed per fish in each interval or in the total experiment) or as % body weight/day (DFI%). The latter was calculated for a given time period (t, t +1) according to the following formula of Richardson et al. (1985), DFI_D x 100/ $logw_{t+1} + logw_t /2)^{exp}$ where DFI_D was the mean daily dry feed intake per fish (t, t + 1), and $logw_t$ and log_{t+1} were the averaged log wet weights at the start (t) and the end (t +1) of the period.

(6) TEI; Total energy intake (TEI expressed as MJ/g body weight/day). TEI = DFI_D x gross energy content of diet (MJ/g)/geometric mean weight of the fish (g/fish) for a given time period as calculated in 5 above.

(7) AEI: Available energy intake (AEI expressed as MJ/g body weight/day). AEI = DFI_D x available energy content of diet (MJ/g)/ geometric mean weight of the fish (g/fish) for a given time period as calculated in 5 above.

(8) TPI; Total protein intake (TPI expressed as g/body weight/day). TPI = DFI_D x crude protein content of diet (g/g)/geometric mean weight of the fish (g/fish) for a given time period as calculated in 5 above.

(9) API; Available protein intake (API expressed as g/g body weight/day). API = DFI_D x available protein content of diet (g/g)/geometric mean weight of the fish (g/fish) for a given time period as calculated in 5 above.

(10) FER; Feed Efficiency Ratio (FER = wet weight gain (g)/ DFI_T).

(11) PER; Protein efficiency ratio (PER = wet weight gain (g)/ protein intake (g))

(12) PPD; Percent protein deposited (PPD = protein gain (g) x 100/ protein intake (g))

(13) APPD; Available percent protein deposited (APPD = protein gain (g) x 100/available protein intake)

(14) GEU; Gross energy utilization (GEU = gross energy gain (MJ/fish) x 100/ gross energy intake (MJ/fish))

(15) AEU; Available energy utilization (AEU = energy gain (MJ/fish) x 100/available energy intake (MJ/fish))

(16) ADC; Apparent digestibility coefficients (ADC, %) for dietary protein (ADC_p), energy (ADC_{en}) and organic matter (ADC_{om}) were determined according to Cho et al (1985) as follows:

$ADC = [\ 1- (F/D \times D_{cr}/F_{cr})] \times 100$ where F = % nutrient (p or om) or energy content (MJ/g) of feces, D = % nutrient (p or om) or energy content (MJ/g) of diet, D_{cr} = % chromic oxide in diet and F_{cr} = % chromic oxide in feces.

(17) Digestible protein (DP, g/kg) and energy (DE, MJ/kg) concentrations in the diets on a dry weight basis;

DP = (diet protein content, g/kg dry diet x ADC_p)

DE = (diet gross energy content (MJ/kg) x ADC_{en})

2.2.7 Statistics

Due to the similar variability in each genotype at the start of the experiment, statistical comparisons were carried out using a two-way analysis of variance in Sigma Stat (v. 3.0.1, Build 3.01.0, SPSS Inc.) for initial tank means (tanks were treated as replicates, three tanks per treatment). Subsequently, large differences in growth and other performance values at the end of the experiment prevented combined analysis of both diet treatments and genotypes as the data could not be normalized by any transformation. As a result, a two-way ANOVA was run with diet and block effects within each genotype along with Tukey multiple comparison post hoc tests. In DFI, TEI, AEI, TPI and API the means for each point and significant differences between the digestible energy of the diets over the whole experiment (tot) were determined by a two-way ANOVA with diet and interval as the variables and Tukey multiple comparison post hoc tests (for the non-transgenics, values were log transformed before analysis). Further, an ANOVA (on ranks in cases without normality) was performed between the genotypes, ignoring the effects of the diet, or a student's t-test was performed within each treatment between

genotypes (if any comparison between the genotypes was made at all) to provide an indication of the degree to which the genotypes differed.

Comparisons between end proximate compositions were made using an ANCOVA (general linear model) in SYSTAT (v. 10.2.05, SYSTAT Corporation Inc.) on 5 fish from each tank (60 fish total) with the variables of genotype, diet, block and the covariate weight. Fish weight has been found to affect proximate composition, especially percentages of protein and lipid (Vanstone and Markert, 1968; Foda, 1974; Shearer et al., 1994; Johansen et al., 2001; Torstensen et al., 2001). Therefore, it was important in this study to remove the effect of this confounding variable to properly determine the effect of diet treatment on the whole body proximate composition of both genotypes of fish. Comparisons of proximate compositions between genotypes were not performed due to the non-normal distribution of the weight covariate and resultant violation of ANCOVA assumptions. Comparisons between start compositions were made using an ANOVA on arcsine square root transformed percentiles. All differences were considered significant at $P \leq 0.05$.

2.3 Results

2.3.1 Composition and Digestibility of Test Diets

The determined concentrations of crude and digestible protein and gross and digestible energy in the test diets were close to those expected. In this regard, DP levels varied between 355 and 433 g/kg and DE levels ranged from 15.1-20.7 MJ/kg and ratios of DP to DE extended from 20.9-23.6 g/MJ (mean of 22 g/MJ across all diets; Table 2.1). Crude lipid levels were also noted to be close to expected values (ranged from 144-191 g/kg). As expected the ADC_{om} and ADC_{en} values were found to be strongly positively correlated with dietary DE ($r^2 = 0.99$) due to progressive reduction of indigestible crude fibre and the attendant inclusion of more digestible protein, lipid and carbohydrate as the dietary DE content was increased (Table 2.1). Interestingly, ADC_p values showed the opposite trend since the diet with 15 MJ DE/kg had the highest value (92.5%) and those containing 17-21 MJ DE/kg had values of 91-91.5% (Fig. 2.1).

2.3.2 Fish Growth, Feed Intake, Diet Utilization and Percent Survival

All diets supported excellent growth in T coho salmon but only diets with $\geq$ 17 MJ DE/kg supported growth in NT coho salmon (Table 2.2; Fig. 2.2). Values for SGR for T coho salmon fed diets with 17-21 MJ DE/kg were observed to be 6-13 fold higher than those of NT counterparts (Table 2.2). Within T fish, SGR values for weight and final mean weights and

lengths were not significantly depressed in T-17 and T-19 groups relative to T-21 groups whereas T-15 groups exhibited significantly reduced growth with respect to the foregoing parameters relative to T-21 fish. In comparison to T-21 fish, SGR values for length were lower in T fish when the diets contained ≤ 17 MJ DE/kg. Collectively the foregoing growth results indicated that T fish at this stage of their life history required > 17 MJ DE/kg diet for maximum growth under our experimental conditions. In general, the values for all of the preceding growth performance parameters in NT fish indicated that these fish too required > 17 MJ DE/kg diet and preferably ≥ 19 MJ DE/kg for maximum growth, especially in weight (Table 2.1). Terminal CF values were uninfluenced by diet treatment in both T and NT fish (Fig. 2.2) and in the former groups, they rose throughout the study especially until day 56. Alternatively, in NT fish terminal CF values remained similar to initial values (T-17, T-19 and T-21 fish) or declined during the study (NT-15 fish).

The excellent growth of T fish relative to NT fish was associated with much higher dry feed intake (DFI_T Fig. 2.3, DFI% Fig. 2.4), TEI (Fig. 2.5), TPI (Fig. 2.5), and protein utilization (particularly PPD values and in two situations, i.e., at DE 15 and 17 MJ/kg, PER values; Fig. 2.6) than noted in NT fish and, in some cases, GEU (T-15 fish versus NT-15 fish; T-21 fish versus NT-21 fish; Fig. 2.7). Within T fish, overall values for DFI% (% body weight/day basis) and TEI showed an inverse relationship to diet DE (DFI% values for T-15 and 17 fish significantly higher than those for T-19 and 21 fish), but those for AEI, TPI, and API were directly related to diet DE (r^2 = 0.68 for AEI, 0.49 for TPI, and 0.32 for API and AEI was significantly higher in T-21 fish versus T-15 fish). Hence, it was apparent that although T fish attempted to maintain similar available (digestible) energy intake as the dietary DE was lowered, they were unable to do so, especially when diet DE was 15 MJ/kg (Fig. 2.5). Regardless of the dietary DE content, T fish exhibited equivalent abilities to convert dietary protein and energy into body protein and energy (no significant effects of diet treatment noted for PER, PPD, APPD, GEU and AEU values). By contrast, FER values were significantly increased with each increment of DE probably as a consequence of the marked decreases in ration (fibre intake) of the T fish consuming the diets of progressively higher DE content.

The progressively increased growth of NT fish in weight and length as the dietary DE content was raised from 15 to 21 MJ/kg was associated with marked elevations in dry feed intake (the overall mean values for DFI_T observed for NT-19 and NT-21 fish were significantly higher than that noted for NT-15 fish in Fig 2.3., and the value for DFI% for NT-21 fish was higher than that for NT-15 fish in Fig. 2.4) , TEI (overall mean value observed for T-21 fish

significantly higher than found for all other groups and that for T-15 fish was significantly lower than observed for all other groups), FER (FER values significantly increased when DE ≥ 17 MJ/kg relative to NT-15 fish and were significantly higher for NT-21 fish than observed for NT-17 coho salmon) and protein utilization (significantly higher PER and PPD values noted in all NT treatment groups in comparison to NT-15 fish when DE ≥ 17 MJ/kg).

The percent survival of all NT groups of fish during the study was 100% and the percent survival of T fish was generally high (> 94%) except for fish consuming the diet with 17 MJ DE/kg where 84% survived (Table 2.2). The poorer survival of T-17 fish was observed especially in two of the replicate groups (80% survival in each case) whereas percent survival in the third replicate group was 92%.

It should be noted that significant block effects were found during some intervals for the final weight, percent protein, and FER, PER, DFI, and TEI values for non-transgenic fish and for FER, DFI and AEI values for transgenic fish. These variables were dependant on feed intake and therefore exhibited block effects that were present in the feed intake data. As each block contained a group fed each diet treatment, an increase in one block would not affect the treatment comparisons, especially in instances where no significant differences were seen.

2.3.3 Whole Body Proximate Composition

On day 0 of the study, T and NT fish were not found to be significantly different in mean percentages of body protein, but T fish had markedly reduced percentages of body lipid and significantly higher percentages of body moisture and ash relative to NT fish (Table 2.3).

In general, on day 84, whole body percentages of protein and ash were unaffected by dietary DE content in both T and NT fish (Table 2.3). The exceptions to the foregoing generalization were found within T fish, where the mean percentage of body protein in T-21 fish was significantly lower than observed in T-19 fish and the percentage of body ash in T-21 fish was significantly lower relative to fish given the other test diets, which showed no differences amongst themselves. Percentages of lipid and moisture were not significantly affected in NT fish by diet treatment. However, in T fish, the mean terminal percentage of body lipid was directly related to dietary DE (significantly elevated in T-19 and 21 fish versus T-15 fish) and body water content in general was found to be inversely related to dietary DE (significantly higher in T-15 fish than observed in T-19 and 21 fish).

As general trends, T fish had higher percentages of body protein and lipid and lower percentages of body moisture and ash than observed in NT fish across all diet treatments, which is consistent with the improved nutritional status of the T fish relative to the NT fish.

2.4 Discussion

The present findings indicate that growth hormone-transgenic coho salmon had markedly increased rates of growth relative to non-transgenic coho salmon over the experimental period, irrespective of the range of the digestible energy content of the diet employed, namely, 15-21 MJ/kg. The former fish achieved the higher growth rates relative to the latter through dramatic increases in their feed, protein and energy intake as well as by more efficacious conversion of dietary protein into body protein and to a lesser degree, improved feed efficiency (when dietary DE 15 and 17 MJ/kg) and gross energy utilization (when dietary DE 15 or 21 MJ/kg). The aforementioned findings agree with and extend those of previous studies that have compared growth of growth hormone-transgenic coho salmon with non-transgenic coho salmon (Devlin et al., 1994; Devlin et al., 2000; Devlin et al., 2004a).

Digestible energy content of the diet was found to be a major factor that influenced the growth responses of non-transgenic coho salmon in this study. Indeed, non-transgenic coho salmon ingesting the diet with 15 MJ of digestible energy/kg actually lost weight during the study even though they had unlimited feeding opportunity. Best growth of non-transgenic fish was observed when the determined dietary DE content was 18.5-20.7 MJ/kg and this was coincident with high digestible dietary protein concentration i.e., 405- 433 g/kg, 180-191 g of crude lipid/kg, 21-22 g of digestible protein/available MJ and alpha-cellulose less than 100g/kg. These ranges for the dietary nutrient and energy levels are similar to those recommended by Higgs et al. (1995) for optimum growth of juvenile non-transgenic coho salmon and are in accord with the findings of Fagerlund et al. (1983). The poorer growth responses noted for the control coho salmon given the diets of lower digestible energy and protein content were likely due to elevated dietary concentrations of crude fibre and reduced concentrations of the protein sources that are known to contribute to overall diet palatability in salmonids i.e., fishmeal, squid meal, and krill meal. With respect to the feeding behavior of the non-transgenic coho salmon during this study, it was noted that all groups had far less interest in the feed than their transgenic counterparts and this disinterest increased progressively with each stepwise decrease in the digestible energy content of the diet. Other factors that may have lowered the feeding responses of non-transgenic coho salmon in this study during this stage of the life history may have

included being more sensitive to the extent of cover, lighting and other disturbances. Also, wild non-transgenic coho salmon and other salmon species are known to have slower rates of growth in winter (when two thirds of this study was conducted) relative to spring and summer (Fagerlund et al., 1983; Markert et al., 1984; Metcalfe and Thorpe, 1992) which has not been observed in growth-hormone transgenic salmon (Devlin et al., 1995; Mori and Devlin, 1999; Devlin et al., 2004a). In the non-transgenic coho salmon, growth and dry feed intake increased throughout the experiment with increasing photoperiod, but did not reach the intake seen in the transgenic salmon. Certainly, the non-transgenic coho salmon ingesting the diets containing reduced protein and energy and high fibre content did not overcome the risk of feeding exposure to the same degree as those given the other test diets and this contributed to the observed differences in growth rate between the genotypes given the various diets (Metcalfe and Thorpe, 1992; Sundstrom et al., 2003). Although the non-transgenic coho salmon did not feed well in comparison to their transgenic counterparts, comparisons between the genotypes are still important as an indicator of the natural differences that exist between these fish.

The initial condition factors of the transgenic coho salmon were found to be lower than noted in the non-transgenic coho salmon. This resulted from the pair feeding of the former fish to the ration level consumed by the non-transgenic fish so that the initial weights of both age-matched genotypes would be similar or equivalent at the start of the study. Bone growth in salmon has been found to be independent of muscle and tissue growth with length increasing even when energy intake is insufficient to support a desired weight, as in starved fish (Einen et al., 1998). In this case, the transgenic fish at the start of the experiment were longer in fork length than the non-transgenic fish even though they had similar weights. Consequently, the initial CF values were lower in the former fish than the latter, but they improved rapidly as the transgenic fish were allowed to feed to satiation and realize their maximum growth potential. The observed dissimilar concentrations of whole body lipid on day 0 between the genotypes (body lipid content significantly lower in the transgenic fish relative to non-transgenic fish) probably arose from insufficient energy availability prior to the onset of the experiment. The differences in initial rate of growth in weight and length coupled with the subsequent increases in body lipid may have arisen simply from provision of adequate energy resources but could have also involved compensatory growth mechanisms (Johansen et al., 2001; Johansen et al., 2002). However, transgenic fish continued to grow quickly relative to the non-transgenic coho salmon throughout the whole experimental period.

In general, other studies have shown that salmonids consume palatable diets of high nutritive value to satisfy their daily energy needs. For instance, Atlantic salmon feed intakes have been noted to increase when their diets have lower energy content (Shearer et al., 1992; Johansen et al., 2002). Also, feed intake in coho salmon has been observed to decrease when they have been fed low protein diets of increased digestible energy content (Chan et al., 2002). Hormones that can increase metabolism, such as growth hormone in the transgenic fish, can also increase feed intake (Markert et al., 1977). Our findings for the transgenic fish therefore agree with those of previous studies with respect to their need to satisfy daily energy needs and the dramatically enhanced appetite of these fish, which characteristically have high circulating titres of growth hormone, relative to non-transgenic counterparts (Devlin et al., 1999; Cook et al., 2000). Our results, however, contrast with those of Martinez et al. (2000) in which transgenic tilapia were found to have reduced feed consumption in relation to the controls, although it is important to note that these fish contained a different gene construct.

Although the transgenic coho salmon in this study consumed progressively more feed and total energy on a body weight basis as the digestible energy content of the diet was lowered, it is apparent that the groups given the diet of lowest digestible energy content (15 MJ/kg) were unable to ingest as much available energy as those given the diets where the digestible energy content was $\geq$ 17 MJ/kg. This may have occurred because their stomachs were not able to accommodate any more of the fibre-rich 15 MJ/kg diet (stomach reached maximum physical distention) and consequently their growth rates declined relative to the fish given the other test diets. At the outset of this study, we hypothesized that the transgenic fish would likely consume more of the low energy diets to meet their metabolic demands and thereby offset a slower growth rate until the point of maximum gut distension was reached. Here, further feed intake would be prevented and hence the fish would no longer be able to increase their growth rate in comparison to the fish consuming the diets of higher digestible energy content. Maximum growth occurred when the diet concurrently contained 22 grams of digestible protein per available MJ. Interestingly, the available protein intake of all groups of transgenic coho salmon regardless of diet treatment was not significantly different. Thus the reduced growth of transgenic coho salmon fed the diet with 15 MJ of digestible energy was due to inadequate intake of energy, not energy and protein. Further, we observed that there were no significant differences between the transgenic coho salmon given the different diets in terms of their abilities to convert dietary protein into flesh and to utilize dietary gross and available energy for growth.

Feed efficiency values in transgenic coho salmon were significantly improved with each stepwise increment in dietary digestible energy content. This likely reflected the progressive reduction of crude fibre and concurrent increases in digestible protein contents as the dietary digestible energy content was increased. It is noteworthy that the transgenic fish were no better at utilizing the feed for growth than the non-transgenic fish until the dietary digestible energy content dropped below 19 MJ/kg. The latter situation likely was caused by the low feed intakes of the non-transgenic coho salmon coupled with the high indigestible carbohydrate contents of the diets containing 15 and 17 MJ of digestible energy/kg. The trends in feed efficiency in relation to the growth rates of both transgenic and non-transgenic coho salmon found in this study generally agreed with those seen in other studies on salmonids and non-salmonids. For instance, one would expect a higher feed efficiency with rapid growth as less energy is needed for maintenance (Jobling, 1994; Cook et al., 2000). In this regard, Cook et al. (2000) found a 10% increase in feed efficiency in growth hormone-transgenic Atlantic salmon that were growing 2.62 to 2.85 fold faster than control fish. Similarly, coho salmon injected with bovine growth hormone showed increases in both growth and feed efficiency (Markert et al., 1977). Moreover, higher feed efficiency has also been observed in other genera of transgenic fish, such as growth hormone-transgenic tilapia (Rahman et al., 1998; Martinez et al., 2000). Also, increases in feed efficiency in direct relation to dietary energy content have been noted in non-transgenic masu (Lee and Kim, 2001), Atlantic (Morris et al., 2003), and coho salmon (Chan et al., 2002) as well as rainbow trout (Bolliet et al., 2000). By contrast, rainbow trout consuming low amounts of a high protein and high lipid diet were observed to have reduced feed efficiency compared to those ingesting a low protein and lipid diet, but this difference was not apparent at a higher feeding rate (Reinitz, 1983). The amount of fibre in the diet is also known to adversely affect nutrient absorption and feed utilization by decreasing the transit time of ingesta through the gut (Brett and Higgs, 1970; Millikin, 1982). Once again, this likely is one of the main reasons why the non-transgenic coho salmon that consumed the two diets of lowest digestible energy content (highest α-cellulose contents) had markedly reduced values for feed efficiency. Moreover their feed (energy and nutrient) intakes may have been only sufficient to satisfy their maintenance energy needs. Consequently, less of their available dietary energy would have been available for growth. In contrast, the transgenic fish did not show a sudden drop in feed efficiency with reduction of dietary digestible energy (elevation of dietary fibre content) which suggests that they were very efficient at absorbing nutrients such as protein (e.g., amino acids as revealed by our findings for apparent digestibility coefficients for protein). Further, our results

for growth and protein utilization in transgenic fish suggest that they were able to maintain ratios of available protein and energy levels within an optimal range, especially when the dietary digestible energy content was ≥ 17 MJ/kg. One of the reasons why the transgenic coho salmon were so efficient at nutrient absorption likely relates to their previously observed greater anterior intestinal surface area compared to controls (Stevens et al., 1999; Stevens and Devlin, 2000; Stevens and Devlin, 2005).

Diet treatment did not influence protein efficiency ratios or percentages of protein deposited in transgenic coho salmon. Non-transgenic coho salmon, however, had similar protein efficiency ratios when dietary digestible energy content was ≥ 17 MJ/kg but not when the diet contained 15 MJ/kg. Values for percent protein deposited followed a similar trend and were more meaningful since they reflected differences in the abilities of the fish to convert dietary protein into body protein rather than gain in weight due to shifts in body protein, lipid and water accretion. Protein retention has been observed to vary between 47% and 56% in Atlantic salmon (Grisdale-Helland and Helland, 1997), which is similar to the range observed for the available percentages of protein deposited in the transgenic coho salmon in the present experiment. Protein efficiency ratios or percentages of protein deposited are affected by the amount of dietary protein in relation to lipid (Chan et al., 2002), starch (Hemre et al., 1995) or total energy content of the diet (Hillestad and Johnsen, 1994; Higgs et al., 1995; Lee and Kim, 2001). In cases where excessive protein is available, protein is de-aminated and excreted. When energy is limiting, protein must be used as an energy source at the expense of protein available for growth (Higgs et al., 1995). In non-transgenic coho salmon fed low protein diets, protein utilization has been observed to increase in direct relation to dietary lipid content until an optimum lipid concentration is reached (Higgs et al., 1995). Furthermore, at the same digestible lipid level, protein efficiency ratios in coho salmon have been found to increase as dietary protein content is lowered (Chan et al., 2002). The preceding findings suggest that coho salmon utilize dietary protein for growth more efficiently as dietary protein is reduced provided that there is sufficient dietary energy available from non-protein energy sources, i.e., lipid and carbohydrate. In transgenic coho salmon the dietary digestible energy content as well as digestible concentrations of lipid and carbohydrate appeared to be sufficient even at 15 MJ/kg for optimal protein utilization. However, in non-transgenic coho salmon ingesting the diet with 15 MJ of digestible energy/kg, the diet was likely too low in digestible lipid and carbohydrate content to spare protein for growth.

The observed improved ability of growth hormone-transgenic coho salmon to convert dietary protein into body protein even when the diet concurrently contained high indigestible carbohydrate (fibre) content suggests that these fish may be able to perform well on diets based on high fibre plant protein or carbohydrate sources such as inexpensive sunflower meal (Gill, 2002) as well as on natural diets high in chitin or ash content (e.g., marine Mysidacea and Mollusca as well as Gastropods, and fresh water amphipods and gammarids (Higgs et al., 1995)). Improvements in the cost effectiveness of salmon aquaculture could be realized by de-emphasizing the use of expensive premium quality fishmeal to meet a significant portion of the daily protein needs, whereas in nature, the fish conceivably could grow and survive well if there were shifts in the daily diet composition that involve the inclusion of more prey of reduced digestible energy content. If high fibre plant derived diets are adopted, care should be taken to ensure that the dietary fibre contents should be less than 10% to minimize the adverse effects of formulated diets for *Oncorhynchus* salmonid species on the environment (Higgs et al., 1995).

The whole body proximate compositions of the transgenic and non-transgenic coho salmon in this study followed expected trends. In this regard, the transgenic fish at the start of the experiment had lower lipid but greater moisture content than the non-transgenic fish. These results are concordant with the known lipolytic functions of growth hormone in coho salmon and they also reflect the reduced plane of nutrition of the transgenic fish to achieve similar initial weights between the transgenic and non-transgenic coho salmon (Higgs et al., 1975; Higgs et al., 1976; Markert et al., 1977). Within the transgenic fish at the end of the study, lipid was observed to increase in direct relation with the digestible energy content of the diet (excess energy was deposited as lipid) while percentages of moisture generally followed the reverse trend. The foregoing moisture-lipid relationship and increased lipid content agrees well with data from studies on non-transgenic salmon (Nettleton and Exler, 1992; Hendry et al., 2000; Lee and Kim, 2001; Torstensen et al., 2001; Chan et al., 2002; Bendiksen et al., 2003; Solberg, 2004).

In other transgenic and growth hormone-treated fish, there tends to be an increase in feed conversion efficiency, protein synthesis and retention, amino acid transport and quantities, muscle growth, and lipid metabolism (Sun, 1990; Foster and Houlihan, 1991; Chatakondi et al., 1995a; Fauconneau et al., 1996; Fauconneau et al., 1997; Fu et al., 1998; Farmanfarmaian and Sun, 1999; Krasnov et al., 1999b; Cook et al., 2000; Fu et al., 2000; Martinez et al., 2000; Rahman et al., 2001; Dunham et al., 2002). The trend of increased feed efficiency and ability to incorporate protein (possible lipid mobilization) are also seen in growth hormone-transgenic

coho salmon. However, in contrast to the findings in the present experiment, other studies have reported decreased or unaffected feed intake and reduced feeding motivation in transgenic fish (Sun, 1990; Guillen et al., 1999; Martinez et al., 2000). Such effects may have resulted from characteristics of the species, the strain or the gene construct utilized.

2.5 Conclusions

My results suggest that juvenile growth-hormone transgenic salmon require > 17 MJ of digestible energy /kg diet on diets containing about 22 g of digestible protein/ available MJ for maximum growth. Further, the non-transgenic coho salmon were found to need at least this amount of digestible dietary energy and preferably 19 MJ or more/kg diet for best growth. Marked differences were noted between the genotypes in their abilities to not only grow but also convert dietary protein into new body protein with the transgenic fish being far more effective in regard to both performance parameters. Limitations in stomach distention likely prevented the transgenic coho salmon from meeting their daily energy requirements when the dietary digestible energy content was below 17 MJ/kg i.e., at 15 MJ/kg. The excellent ability of transgenic coho salmon to utilize dietary protein and energy for growth across a wide range in dietary digestible energy content may indicate that these fish are efficient at utilizing diets high in fibre (e.g., some sources of inexpensive plant protein) and chitin content and to metabolically utilize digestible carbohydrate for growth. If these hypotheses are proven to be correct in subsequent studies, there could be important benefits such as more cost effective culture of salmon. These findings may also have important ecological implications since the transgenic salmon conceivably could survive better than non-transgenic coho salmon in the ocean environment in situations of suboptimal prey nutrient composition and energy density.

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Table 2.1
Ingredients and proximate compositions of the test diets[1]

	Experimental Diet			
Ingredients (g/kg)	15 MJ/kg	17 MJ/kg	19 MJ/kg	21 MJ/kg
LT-anchovy meal	315.63	350.71	389.68	432.97
Blood flour; spray- dried	31.69	35.22	39.13	43.47
Squid meal	46.15	51.28	56.98	63.32
Krill meal	62.93	69.90	77.68	86.32
Wheat gluten meal	45.97	51.09	56.77	63.07
Pre-gelatinized wheat starch	67.84	76.54	86.17	96.87
Vitamin supplement[2]	20.00	20.00	20.00	20.00
Mineral Supplement[3]	30.00	30.00	30.00	30.00
Anchovy oil; stabilized	90.75	101.95	114.37	128.20
Soybean lecithin	10.00	10.00	10.00	10.00
Choline chloride (60%)	5.00	5.00	5.00	5.00
Vitamin C, monophosphate (42%)	3.57	3.57	3.57	3.57
α-cellulose	253.86	177.95	93.66	-
Permapell (lignin-sulphonate binder)	10.00	10.00	10.00	10.00
DL-methionine	1.61	1.79	1.99	2.21
Chromic oxide	5.00	5.00	5.00	5.00
Total	**1000**	**1000**	**1000**	**1000**
Calculated proximate constituents (g/kg)				
Protein	364.5	405.0.	450.0	500.0
Lipid	145.8	162.0	180.0	200.0
Carbohydrate	92.1	101.4	111.8	123.4
Determined proximate constituents (g/kg)				
Protein[4]	384.8	404.9	445.4	476.2
Digestible protein[5]	355.8	372.2	405.2	433.0
Lipid	144.4	160.6	180.3	191.0
Dry Matter	918.8	919.1	918.9	915.2
Ash	83.4	91.9	101.7	111.4
Gross Energy (MJ/kg)	21.6	22.1	22.6	23.3
Digestible energy (MJ/kg) [6]	15.1	16.8	18.5	20.7
Digestible protein / digestible energy (g/MJ)	23.6	22.1	21.9	20.9

[1] All values are on a dry weight basis.

[2] Vitamin supplement consisted of (amount/kg dry matter): Ca D-pantothenate, 168.4 mg; pyridoxine-HCl, 49.3 mg; riboflavin, 60 mg, folic acid, 15 mg; thiamine mononitrate, 56 mg; biotin, 1.5 mg; vitamin B_{12}, 0.09 mg; vitamin K (as menadione sodium bisulphte), 18 mg (menadione); vitamin E, 300 IU; vitamin D_3, 2400 IU; vitamin A, 5000 IU; inositol, 400 mg; niacin, 300 mg; BHT, 22 mg.

[3] Mineral supplement consisted of (mg/kg dry matter): Mn (as $MnSO_4 \cdot H_2O$), 75 ; Zn (as $ZnSO_4 \cdot 7H_2O$), 100; Co (as $CoCl_2 \cdot 6H_2O$), 3; Cu (as $CuSO_4 \cdot 5H_2O$), 5; Fe (as $FeSO_4 \cdot 7H_2O$), 100; I (as KIO_3 and KI, 1:1) 10; F (as NaF), 5; Na (as NaCl), 1000; Se (as Na_2SeO_3), 0.1; Mg (as $MgSO_4 \cdot 7H_2O$), 400; K (as K_2SO_4 , 1055 and as K_2CO_3, 1055).

[4] Protein as determined by multiplying % nitrogen x 6.25

[5] Digestible protein calculated using the apparent protein digestibility coefficients (ADC_p) determined for each diet i.e., ADC values of 92.45, 91.53, 90.96, and 90.96 for the 15, 17, 19 and 21 MJ/kg diets, respectively.

[6] Digestible energy calculated using the apparent energy digestibility coefficients (ADC_{en}) values determined for each diet i.e., 60.94, 76.36, 81.79 and 88.93 for the 15, 17, 19, and 21 MJ/kg diets, respectively.

Table 2.2
Growth and percent survival of transgenic and non-transgenic coho salmon on diets with different digestible energy[1]

Diet	Initial Weight[2] (g)	Final Weight (g)[3]	Initial Length (g)	Final Length (g)[3]	SGR Weight (%/day)[3]	SGR Length (%/day)[3]	Percent Survival[4]
Transgenic							
15MJ/kg	12.84 ± 0.04^{AB}	130.62 ± 0.97^{A}	11.1 ± 0.02^{A}	21.4 ± 0.2^{A}	2.76 ± 0.01^{A}	0.783 ± 0.010^{A}	94.7
17 MJ/kg	13.13 ± 0.08^{A}	145.44 ± 4.76^{AB}	11.1 ± 0.05^{A}	22.2 ± 0.1^{AB}	2.86 ± 0.04^{AB}	0.825 ± 0.006^{AB}	84.0
19 MJ/kg	13.10 ± 0.02^{A}	151.95 ± 4.58^{B}	11.0 ± 0.02^{A}	22.6 ± 0.2^{B}	2.92 ± 0.03^{B}	0.855 ± 0.011^{BC}	96.0
21 MJ/kg	13.11 ± 0.08^{A}	159.29 ± 1.34^{B}	11.1 ± 0.05^{A}	23.0 ± 0.1^{B}	2.97 ± 0.01^{B}	0.873 ± 0.004^{C}	98.7
Non-transgenic							
15 MJ/kg	12.64 ± 0.11^{AB}	12.55 ± 0.46^{a}	10.5 ± 0.02^{B}	10.8 ± 0.1^{a}	-0.010 ± 0.033^{a}	0.034 ± 0.006^{a}	100
17 MJ/kg	12.65 ± 0.07^{B}	15.43 ± 1.44^{b}	10.5 ± 0.02^{B}	11.4 ± 0.2^{ab}	0.227 ± 0.101^{ab}	0.095 ± 0.024^{ab}	100
19 MJ/kg	12.75 ± 0.07^{B}	17.19 ± 1.21^{bc}	10.6 ± 0.02^{B}	11.6 ± 0.1^{ab}	0.350 ± 0.084^{b}	0.109 ± 0.014^{ab}	100
21 MJ/kg	12.72 ± 0.12^{B}	19.24 ± 0.72^{c}	10.5 ± 0.03^{B}	12.3 ± 0.3^{b}	0.491 ± 0.037^{b}	0.182 ± 0.027^{b}	100

[1] All values are means of three tank means ± SEM. Significant differences are indicated by letter superscripts.
[2] Significant differences are determined by a two-way ANOVA in which genotypes differed within the 17, 19 and 21 but not the 15 MJ/kg treatment groups. Diet treatments within each genotype and overall did not differ.
[3] Comparisons between genotypes could not be made due to drastic differences in values. The greater sizes or growth rates of the transgenic fish are obvious, and when tested between genotypes (ignoring previous test and diet differences), are significant.
[4] Total percent survival of all fish in the diet treatment group after pooling replicate tanks.

Table 2.3
Whole body proximate composition of transgenic and non-transgenic coho salmon in relation to diet treatment

Component (%)	Transgenic				Non-transgenic			
	15 MJ/kg Diet	17 MJ/kg Diet	19 MJ/kg Diet	21 MJ/kg Diet	15 MJ/kg Diet	17 MJ/kg Diet	19 MJ/kg Diet	21 MJ/kg Diet
Fish Day 0[1]								
Protein			17.66 ± 0.22^{A}				17.94 ± 0.12^{A}	
Lipid			2.08 ± 0.30^{A}				8.11 ± 0.39^{B}	
Moisture			78.72 ± 0.41^{A}				72.40 ± 0.32^{B}	
Ash			2.92 ± 0.11^{A}				2.46 ± 0.06^{B}	
Fish Day 84[2]								
Protein	16.47 ± 0.27^{ab}	16.41 ± 0.27^{ab}	16.57 ± 0.29^{a}	16.11 ± 0.50^{b}	15.39 ± 0.29^{A}	15.52 ± 0.38^{A}	15.30 ± 0.30^{A}	15.23 ± 0.43^{A}
Lipid	10.78 ± 0.52^{a}	11.37 ± 0.48^{ab}	12.40 ± 0.28^{bc}	12.43 ± 0.48^{c}	6.57 ± 0.64^{A}	7.65 ± 0.76^{A}	8.20 ± 0.40^{A}	8.55 ± 0.16^{A}
Moisture	71.85 ± 0.48^{a}	70.26 ± 0.37^{ab}	69.40 ± 0.39^{b}	69.78 ± 0.32^{b}	73.78 ± 0.54^{A}	73.75 ± 0.80^{A}	73.15 ± 0.37^{A}	73.23 ± 0.19^{A}
Ash	1.85 ± 0.06^{a}	1.86 ± 0.09^{a}	$1,97 \pm 0.06^{a}$	1.64 ± 0.08^{b}	2.51 ± 0.12^{A}	2.53 ± 0.05^{A}	2.48 ± 0.07^{A}	2.48 ± 0.11^{A}

[1] Day 0 proximate constituents are determined for each genotype before feeding different diets and displayed as mean ± SEM, n=5. Each of the five replicates used to determine the mean was a composite sample of two fish. Significant differences are shown with letter superscript differences within each proximate component. Significant differences were not determined between day 0 and day 84 fish proximate constituents.

[2] All day 84 proximate constituents are displayed as means ± SEM, n = 3. Differences is letter superscripts along a row but within a genotype (transgenic or control) denote a significant difference at $P \leq 0.05$ determined from an ANCOVA with weight as a covariate. Comparisons between genotypes were not performed due to the non-normal nature of the weight distribution, and consequently the ANCOVA assumptions were violated. All percentages are on a wet weight basis.

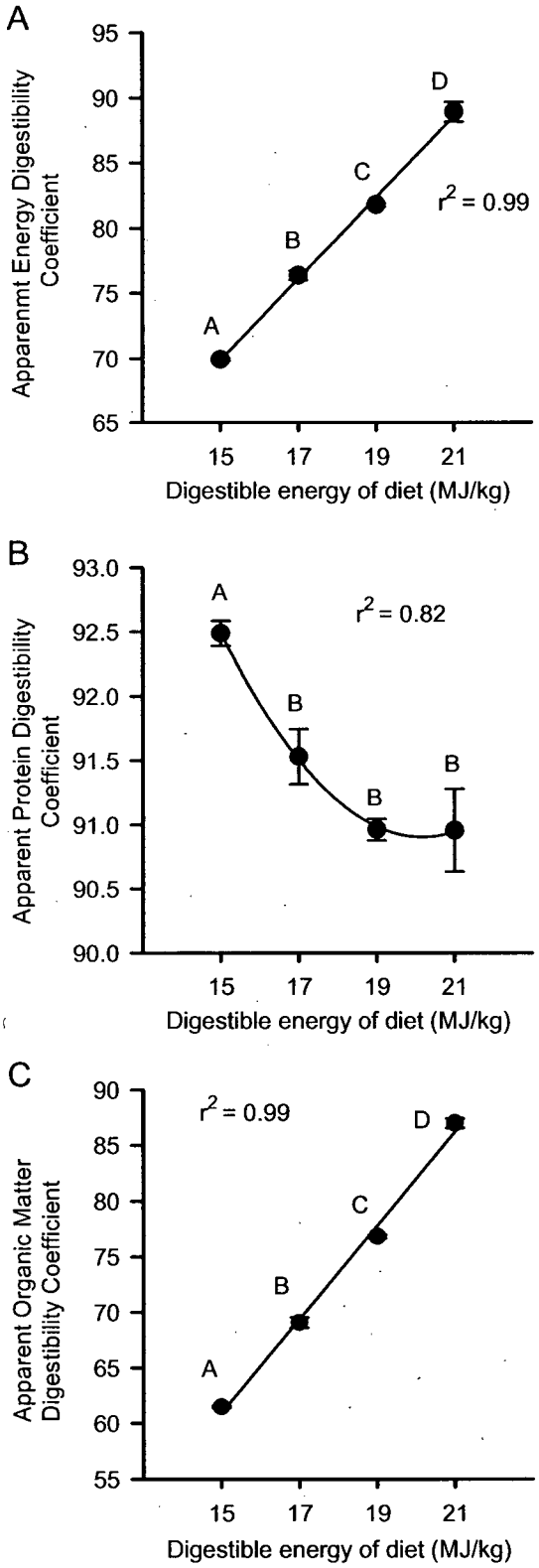

Fig. 2.1. Apparent digestibility coefficients for energy (A), protein (B) and organic matter (C) of fish on diets of different digestible energy. Letter differences denote significant differences. All points are means of three replicate tank means with standard error.

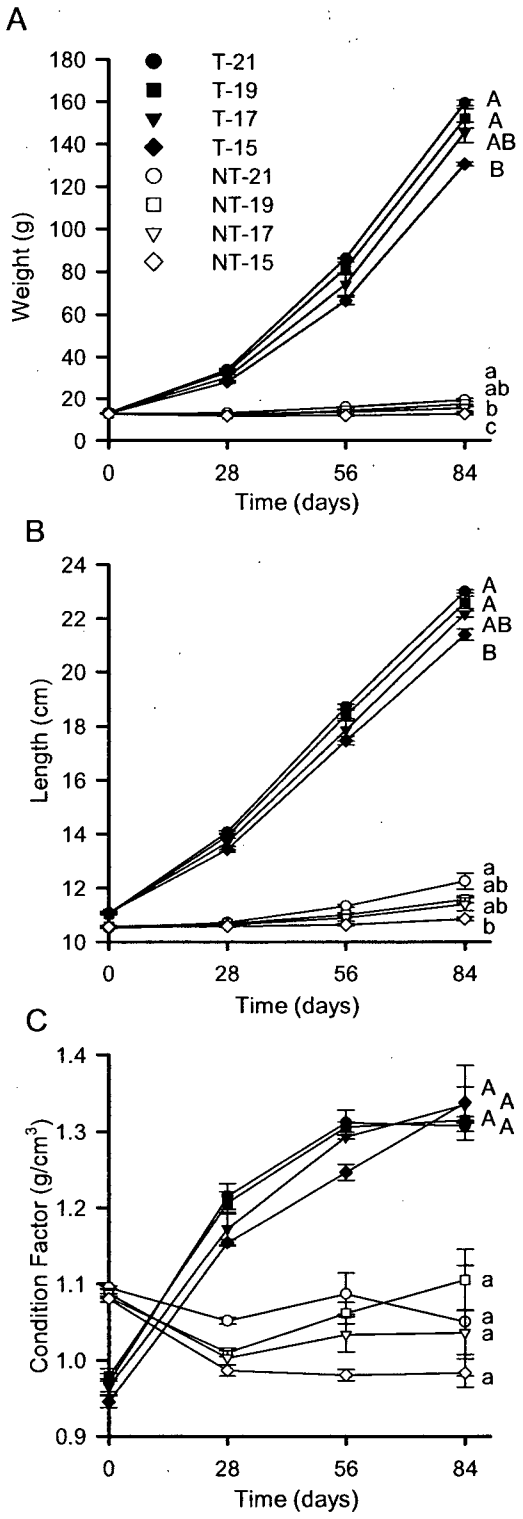

Fig. 2.2. Growth measures. (A) Fish weight over the course of the experiment on the 21 MJ/kg diet (21), 19 MJ/kg diet (19), 17 MJ/kg diet (17) and 15 MJ/kg diet (15) for transgenic (T) and non-transgenic (NT) fish. (B) Fish length. (C) Condition factor. Significant differences in values at experiment end, 84 days, are denoted by letter differences.

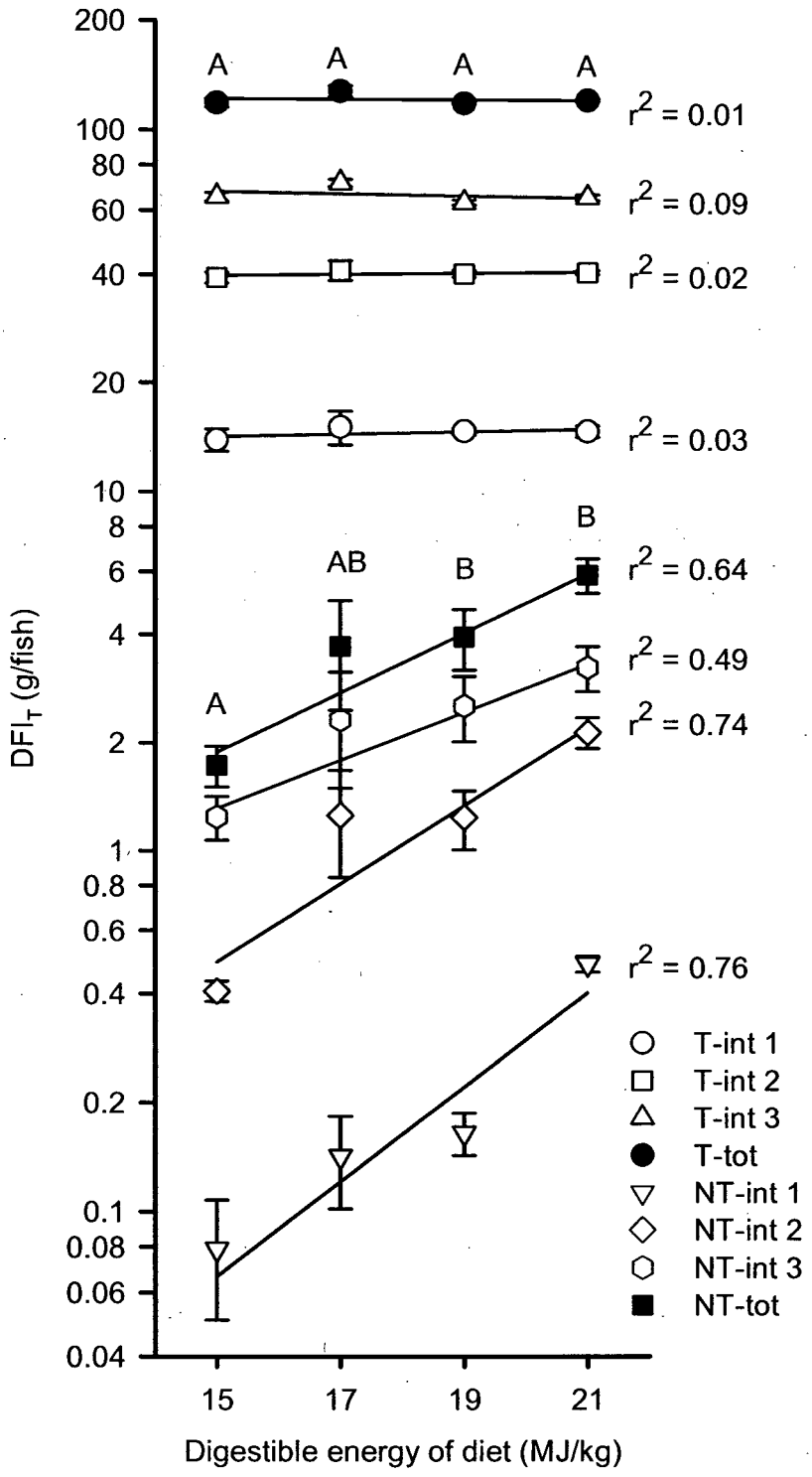

Fig. 2.3. Total dry feed intake (DFI_T) during each interval (int 1-3) and over the entire experiment (tot) for transgenic (T) and non-transgenic (NT) fish on diets of different digestible energy. Each data point is a mean of three replicate tank means with associated standard error. Significant differences in total DFI are denoted by letter differences. The intervals are displayed to emphasize trends.

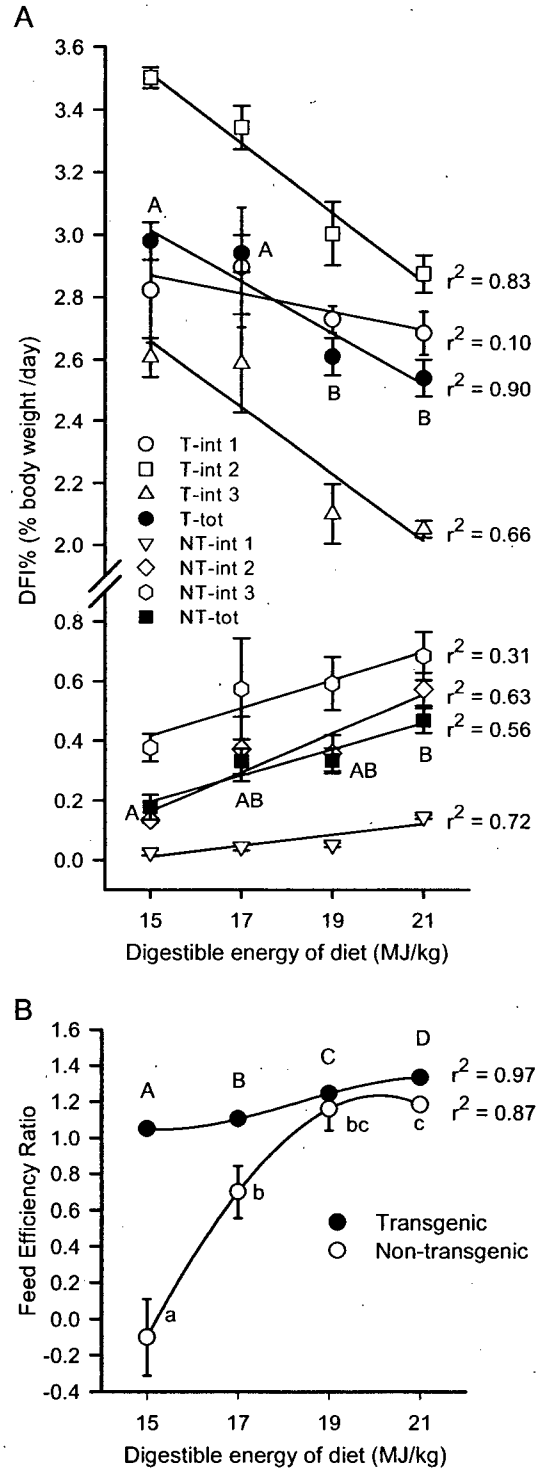

Fig. 2.4. (A) Dry feed intake expressed as % body weight/day (DFI%) during each interval (int 1-3) and over the entire experiment (tot) for transgenic (T) and non-transgenic (NT) fish on each diet. Significant differences between diets are denoted by letter differences over the experiment (tot) with other intervals included to emphasize trends. Points are means of three replicate tank means with standard error for int 1-3. Plotted points for the total (tot) are least squares means from a two-way ANOVA with diet and interval as variables with the LS standard errors of the diet variable. (B) Feed efficiency. Significant differences between diets are denoted by letter differences within each genotype. Comparisons between genotypes are not shown.

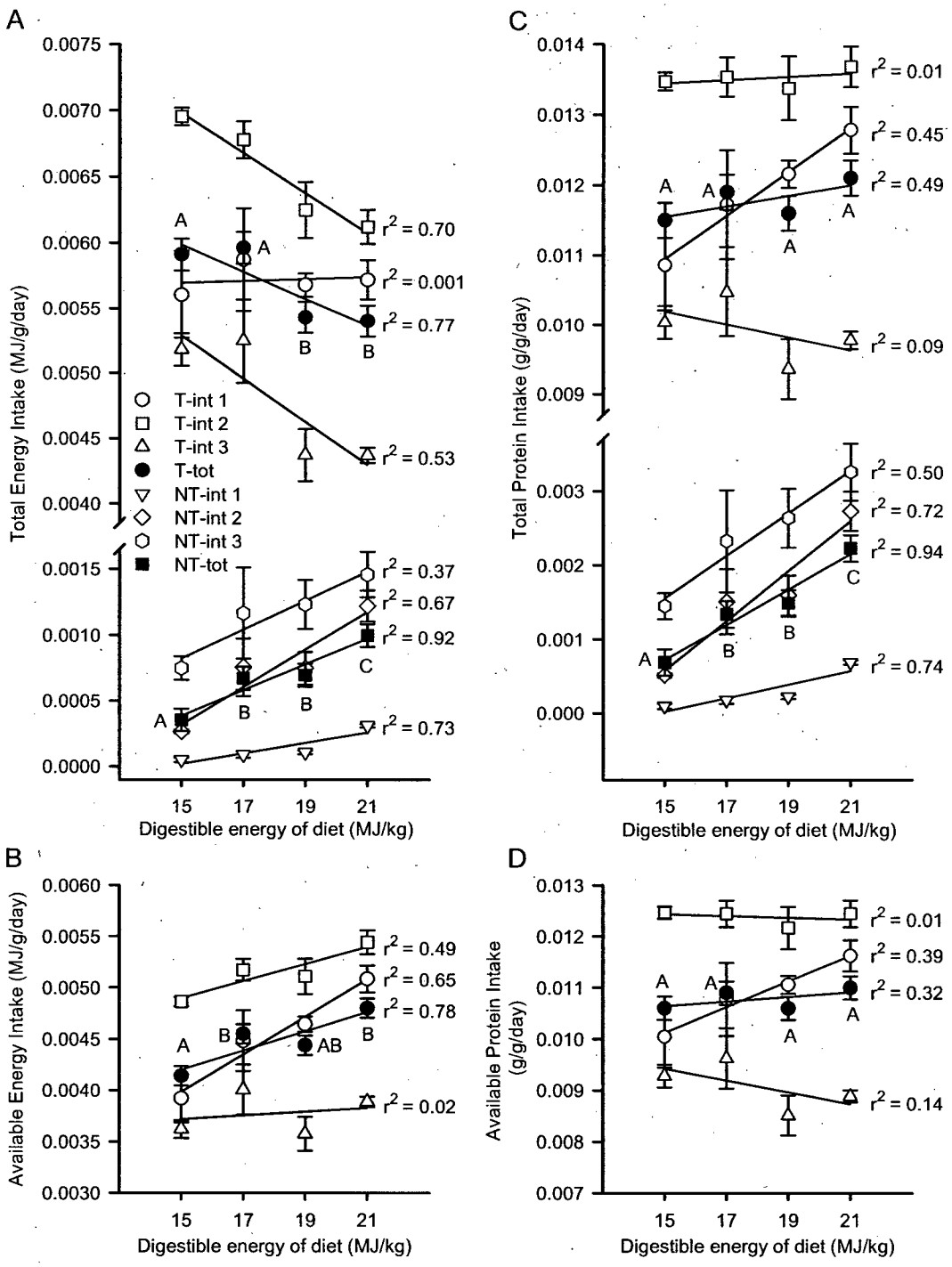

Fig. 2.5. Energy intake (total (A) and available (B)) and protein intake (total (C) and available (D)) during each interval (int 1-3) and over the entire experiment (tot) for transgenic (T) and non-transgenic fish (NT). Significant differences over the experiment (tot) are denoted by letter differences and determined from a two-way ANOVA with diet and interval as variables. Other intervals are shown to emphasize trends. Points are means of three tank means with associated standard error for int 1-3, and LS means with LS standard errors of diet for the total (tot).

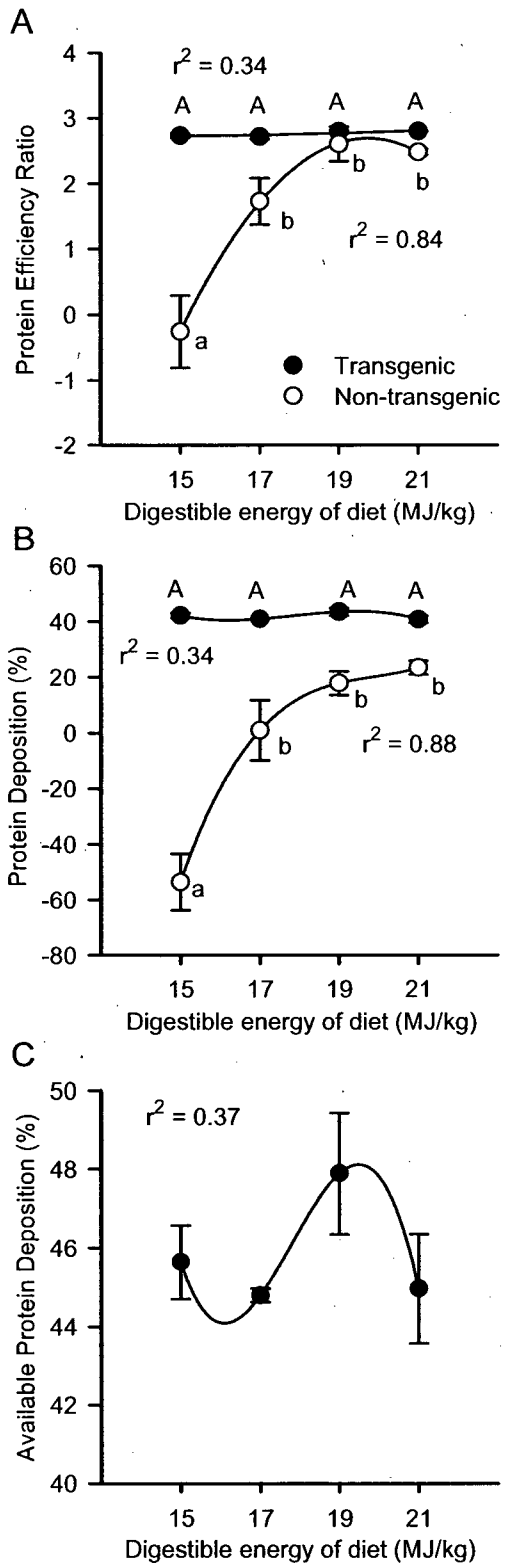

Fig. 2.6. Protein efficiency ratio (A) and percent protein deposition (B) with available percent protein deposition (C). Significant differences between diet treatments within genotypes are denoted by letter differences. Significant differences between genotypes are not shown.

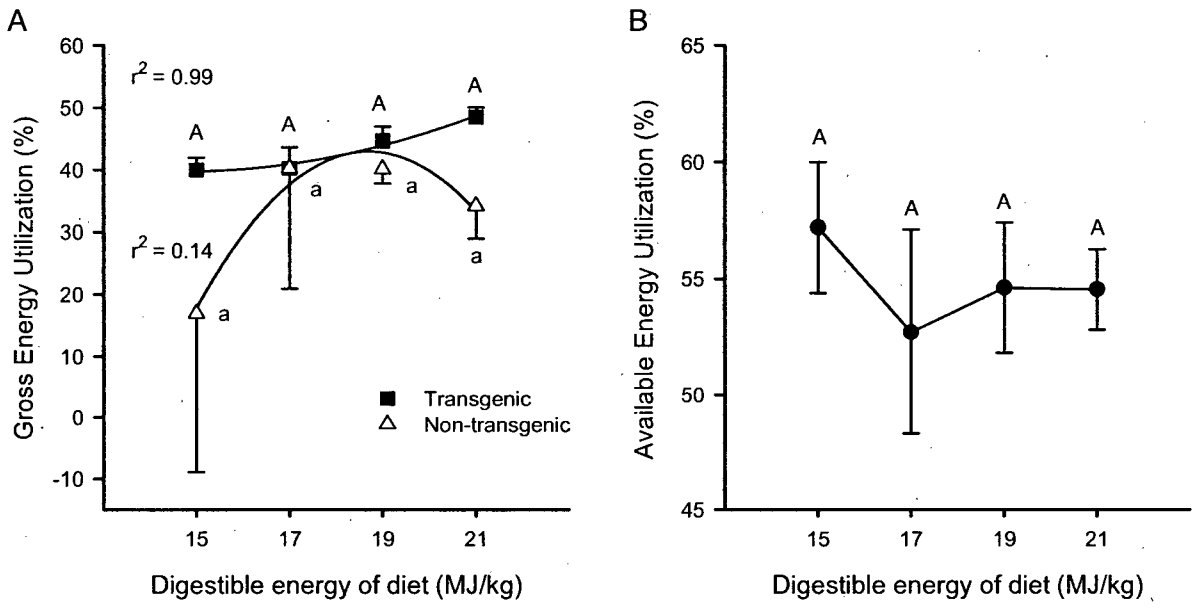

Fig. 2.7. Gross (A) and available (B) energy utilization. Significant differences are denoted by letter differences. Upper error bars only shown for transgenic fish and lower error bars only shown for non-transgenic fish in (A) to avoid obscuring the data points. Differences between genotypes in (A) not shown.

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CHAPTER 3

Gene expression of growth hormone-regulating and appetite-regulating peptides in the brain of growth hormone transgenic and non-transgenic coho salmon, *Oncorhynchus kisutch*.

3.1 Introduction

The increased feed intake and enhanced growth found in growth hormone (GH) transgenic coho salmon, *Oncorhynchus kisutch*, raises questions regarding the hormonal changes that mediate these physiological traits. The enhanced growth rate in these fish results from centrally uncontrolled GH production by an all-fish GH transgene present throughout the body tissues (Devlin et al., 1994; Devlin et al., 2004a). Concurrent increases in appetite and feed intake result in altered feeding behaviour (and other behaviours such as reduced predator awareness) and allow for rapid development and maturation (Devlin et al., 1999; Devlin et al., 2000; Sundstrom et al., 2003; Devlin et al., 2004a; Sundstrom et al., 2004a). However, the precise hormonal and neuroendocrine mechanisms controlling increased GH production and associated appetite in these fish are unknown. Circulating levels of hormones involved in growth hormone control, the GH axis, have been examined (Mori and Devlin, 1999; Devlin et al., 2000), but brain and pituitary expression of genes encoding these hormones, with the exception of GH (Mori and Devlin, 1999), have not been analyzed. Furthermore, it is unknown how the GH-axis in transgenic coho salmon responds to changes in dietary energy and the potential impact this may have on appetite.

Growth hormone production by somatotrophs in the pituitary is regulated both by the hypothalamus and by circulating hormones. Growth hormone-releasing hormone (GHRH), produced by the hypothalamus, is the main stimulator of GH release while somatostatin (SS, or somatotropin-release inhibitory factor, SRIF) and neuropeptide Y (NPY), inhibit it (Hurley and Phelps, 1992; Uchiyama et al., 1994; Minami et al., 1998; Peng et al., 2001). Growth is thought to be mediated primarily by liver-derived insulin-like growth factor I (IGF-I) in response to GH binding to liver growth hormone receptors (GHRs)(Bartke et al., 1994; Al-Regaiey et al., 2005). Both circulating GH and IGF-I can negatively feedback on GHRH and SS neurons in the hypothalamus, and on somatotrophs in the pituitary to reduce endogenous GH production (Mathews et al., 1988b; Wallenius et al., 2001). Gene expression of hormones in the GH axis has been characterized in many fish species, but to my knowledge no studies have analyzed the GH axis in a GH transgenic fish.

In non-transgenic fish, the way the GH axis is regulated is similar to that seen in mammals. GHRH stimulates GH release from teleost pituitary cells (Luo and McKeown, 1991; Parker et al., 1997) and GHRH injection causes an increase in circulating GH (Melamed et al., 1995; Kelly et al., 1996; Shepherd et al., 2000). Unlike mammals, GHRH is encoded in the same gene as another GH-releasing peptide, pituitary adenylate cyclase-activating polypeptide (PACAP) (Parker et al., 1993). Both these peptides have potent GH releasing effects and in some cases PACAP may be more effective at stimulating a GH response (Parker et al., 1997). Somatostatin inhibits pituitary GH release in many fish species (Luo and McKeown, 1991; Lin et al., 1993; Melamed et al., 1995; Lescroart et al., 1996; Kwong and Chang, 1997; Parker et al., 1997) and SS injection reduces plasma GH levels (Cook and Peter, 1984; Melamed et al., 1995; Very et al., 2001). Generally, growth hormone increases GHR expression but the response is tissue specific likely due to the presence of two forms of GHR (Saera-Vila et al., 2005; Very et al., 2005). IGF-1 increases in response to GH addition to teleost hepatocyte cultures, or as in-vivo GH injections (Foucher et al., 1992; Shimizu et al., 1999; Pierce et al., 2004).

Neuropeptide Y (NPY) is also involved in appetite regulation. NPY is a known orexigen (Silverstein and Plisetskaya, 2000), countered in part by the satiety signal from the octapeptide, cholecystokinin (CCK), which is produced by the gut and brain (Peyon et al., 1999; Gelineau and Boujard, 2001). During starvation, NPY mRNA increases in salmon (Silverstein et al., 1996), and intracerebroventricular injections of NPY increase food intake by 45% to 100% in the catfish (Silverstein and Plisetskaya, 2000). Fasting increases brain NPY mRNA expression in the gold fish while refeeding returns expression to a lower level (Narnaware and Peter, 2001a; Volkoff et al., 2003). Conversely, CCK injections caused a decrease in food consumption in the catfish (Silverstein and Plisetskaya, 2000) and CCK receptor antagonists increased feed intake in rainbow trout and removed the satiation affects of CCK addition in the goldfish (Gelineau and Boujard, 2001; Volkoff et al., 2003). Cholecystokinin may also be involved in regulating the GH-axis. CCK neuronal terminals have been found to synapse with somatotrophs in the pituitary of at least three teleost species (Moons et al., 1989; Batten et al., 1990; Peter, 1997). Furthermore, central and peripheral administration of CCK reduces preprosomatostatin-I mRNA expression in the gold fish brain (Canosa and Peter, 2004).

A further connection between the GH-axis and food intake exists through GH, IGF-I and GHR. Reduced feed intake or starvation causes decreases in plasma IGF-I or liver IGF-I mRNA expression in chinook salmon (Pierce et al., 2005b) and coho salmon (Duan and Plisetskaya, 1993; Shimizu et al., 1999; Pierce et al., 2001). This decrease with starvation often coincides

with an increase in plasma GH due to the development of GH resistance by the liver and the uncoupling of IGF-I responsiveness from GH expression (Narnaware and Peter, 2001a; Valente et al., 2003; Pierce et al., 2004). GH resistance results from decreases in hepatic GHR expression after fasting (Fukada et al., 2004; Saera-Vila et al., 2005). Salmon with stunted growth but high circulating GH have low IGF-I mRNA in the liver and low IGF-I protein, which may cause the apparent increases in GH due to the lack of IGF-I feedback (Duan et al., 1995; reviewed by Dickhoff et al., 1997). In most cases refeeding will restore plasma IGF-I, IGF-I mRNA expression and subsequently growth (Duan and Plisetskaya, 1993; Meton et al., 2000).

The objective of my first study was to determine the expression patterns of the hormones responsible for GH regulation in the brains of GH transgenic coho salmon raised in two culture conditions. One group of transgenic coho salmon was fed a full ration and one group was fed a restricted ration equivalent to the full ration of non-transgenic coho salmon (pair-fed). Messenger RNA expression of GH, GHR, GHRH, SS, IGF-I and CCK was measured in six brain regions that consisted of the telencephalon, optic tectum, midbrain, hypothalamus, pituitary and cerebellum, in addition to muscle and liver. Direct comparisons of gene expression with non-transgenic coho salmon allowed me to determine how the GH-axis likely responded to increased levels of GH and if any of the apparent changes coincided with those suspected to influence appetite. As mRNA expression has been found to respond to both hormone and diet manipulation for many of these hormones (Duan et al., 1992; Duguay et al., 1994; Uchiyama et al., 1994; Pellegrini et al., 1997; Peyon et al., 1999; Fukada et al., 2004; Pierce et al., 2004; Saera-Vila et al., 2005), expression analysis will in most cases indicate changes to protein production and subsequently physiology. The second study investigated the expression levels of the above hormones in similar brain regions of transgenic coho salmon from the experiment outlined in Chapter 2; specifically those fish fed the extremes of the energy-varied diets (i.e. 21 MJ/kg and 15 MJ/kg diets). The goal was to determine how the expression patterns of my hormones of interest changed between fish with different dietary energy intake. Using these expression patterns I hoped to determine if GH transgenic coho salmon responded normally to changes in dietary energy intake and whether the hormonal control of appetite through CCK expression was operating properly in these fish.

3.2. Materials and Methods

3.2.1 Fish Culture and Sampling

3.2.1.1 Trial 1: Transgenic, ration-restricted transgenic, and non-transgenic samples

Transgenic (OnMTGH1) coho salmon and non-transgenic Chehalis River broodstock were raised on a simulated natural photoperiod (Vancouver, Canada, 49 ^{0} 15' N 123 ^{0} 10' W) in separate 2935 L tanks supplied with 8-11 ^{0}C well water. One tank of transgenic fish was fed to satiation twice a day with commercial fish feed (T-Full) (Skretting Canada, Vancouver, BC) for 9 months until reaching a mean body weight of approximately 55g. The non-transgenic fish were fed twice a day to satiation for 21 months to reach the same weight (NT-Full). Another group of transgenic coho salmon were pair-fed a ration equivalent to the amount consumed by the non-transgenic fish for 21 months to maintain an equivalent growth rate (T-Restricted). Thus, all fish were size matched at the time of sampling (overall weight 55.5 ± 1.2 g, mean ± SE).

Over three days, 11 randomly-selected fish/day were obtained serially from each group in turn and euthanized in tricane methane sulphonate (0.2 g MS 222/L), weighed, measured, and rapidly team-sampled for muscle, liver and brain tissue. The telencephalon was extracted prior to the remainder of the brain being removed to cooled 0.2 M phosphate buffered saline and divided into the 1) hypothalamus (containing the saculous venosus), 2) optic tectum, 3) cerebellum and 4) remainder of the brain (midbrain). The pituitary was removed from the cavity under the brain, yielding a total of eight tissues for analysis. All tissues were immediately frozen in liquid nitrogen following dissection and stored at -80 ^{0}C. It should be noted that the work prior to this point was conducted by Dr. Devlin and technical personnel associated with his lab prior to initiation of my thesis research.

3.2.1.2 Trial 2: Transgenic samples from fish raised on different energy diets

Fish were raised as described in Chapter 2. During the end of the digestibility study (refer to Chapter 2), 15 of each of the transgenic groups fed the 15 MJ/kg and 21 MJ/kg diets were serially sampled for blood and brain tissue in the same manner as described in Trial 1. All fish were fed the afternoon prior to sampling. Euthanization and brain dissection were performed as described for Trial 1 with the exception that all brain regions were placed in RNAlater (Ambion) and left for one afternoon at room temperature before being stored at -80 ^{0}C. Before the brain regions were placed into Trizol reagent for RNA extraction, part of the midbrain was dissected and added to the hypothalamus so that the hypothalamus samples contained the

same tissue regions as isolated in Trial 1. The brain regions from 12 of the 15 fish sampled for each diet treatment were processed as described below. Plasma IGF-I concentrations were determined using an I^{125} radioimmunoassay (GrowPep IGF-I Fish Kit) as described by the manufacturer and measured in a gamma counter (1272 Clinigamma, LKB Wallac).

3.2.2 RNA Extraction and cDNA Synthesis

All frozen brain tissues (Trial 1) and only transgenic samples from RNAlater (Trial 2) were placed in Trizol reagent (Invitrogen) and immediately homogenized using a micropestle. The recommended Trizol procedure was followed with the addition of 2 μg muscle glycogen as a carrier to the brain and pituitary extractions before isopropanol precipitation. The RNA pellet was dissolved in RNAse free water (Gibco, ultraPURE) and quantified by mass spectrophotometry (Milton Roy, Spectronic 1001 Plus) before being diluted to 500 ng/uL to be used in reverse transcription reactions. RNA samples were stored at -80 ^{0}C and were kept on ice during use to minimize RNA degradation. Complementary DNA was synthesized using the Multiscribe Reverse Transcriptase Kit (Applied Biosystems) with Oligo-d(T) primer and 500 ng RNA. A second set of cDNA was produced using a gene specific reverse primer for salmon growth hormone (GH4R, Table 3.1), and 250 ng RNA. Additional reverse transcription reactions (Oligo-d(T) and GH4R) were performed using randomly selected samples of each brain region to combine and make two cDNA standards. It should be noted that cDNA for the brain regions from 11 fish were available for the NT-Full and T-Restricted groups and 10 fish were available for the T-Full group. Muscle and liver samples were extracted by Mitchell Uh (a technician in the Devlin lab) who analyzed them by quantitative PCR (Q-PCR) for GH, GHR, IGF-1, ß-actin, myosin and albumin mRNA expression prior to brain analysis. The proper tissue specific expression of myosin and albumin confirmed the success of the Q-PCR design and procedure (Fig. 3.2E,F).

3.2.3 Quantitative PCR

3.2.3.1 Assay design

Primers for GHRH, and CCK were determined from alignments of teleost cDNA sequences from GenBank and used to amplify coho salmon cDNA (GenBank # used for alignments: GHRH: #X73233, AF343976, AF343977; CCK: #AJ011846, AJ012056, AJ012055). Prospective amplicons were inserted into pCRII-TOPO vectors (Invitrogen) and incorporated into OneShot chemically competent *E. coli* cells (Invitrogen). Colonies were

plated and screened and vectors with successfully incorporated transcripts were extracted with the QIAprep Spin Miniprep Kit (Qiagen). DNA inserts were sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Gene sequences were then compared to other teleost GenBank sequences to confirm gene identity. Quantitative PCR primers and TaqMan MGB probes (Applied Biosystems) were designed from the newly determined coho salmon gene sequences (Table 3.1). SS primers and probe were designed directly from rainbow trout, *Onchorynchus mykiss*, cDNA (Genbank#: U32471). Furthermore, previously designed quantitative PCR assays for β-actin, GH, IGF-I and GHR were used (Table 3.1).

3.2.3.2 Trial 1

Samples were run on 96 well plates in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with the proper cDNA standards (Oligo-d(T) for all assays except GH which uses the GH4R standard). Average PCR efficiencies were as follows: ß-actin, 97.6%; GH, 99.2%; GHR, 97.4%; IGF-I, 97.4%; GHRH, 98.9%; SS, 96.5%; CCK, 97.7%. Three samples from one plate were run on all plates used in the same gene assay to standardize between plates. Relative quantities determined on a plate were corrected for changes in the standardized samples before analysis to reduce inter-plate variability (standard sample quantity on plate of interest / standard sample quantity from initial plate · experimental sample on plate of interest). Following correction for interplate variability, quantities were divided by the β-actin quantity for that sample to correct for variation between cDNA reactions and to yield a relative ratio of gene expression. Because all tissue types were run together on each plate, comparisons can be made between treatment groups within each brain region and between brain regions. The exception to this was the GH assay on pituitaries in which expression was too high to standardize to that of other brain regions. A new cDNA Oligo-d(T) standard was reverse transcribed from the same samples as the previous standard for use in 9 plates in assays using the original standard (5 CCK, 1 IGF-I, 1 GHR and 2 B-actin plates). Brain totals were determined by assigning the gene expression ratio of the NT-Full group to 1 (0.01 for GH) and the expression of the T-Full and T-Restricted groups as a multiple of NT-Full for each tissue before averaging all tissues within an assay (not including the pituitary).

3.2.3.3 Trial 2

The procedure in Trial 2 was similar to Trial 1 with the exception that all samples for each brain region within each gene assay were run on 1, or in some cases 2, plates to reduce

inter-plate variability. Therefore, comparisons between tissues cannot be as reliably made. Furthermore, only selected brain regions that showed differences in Trial 1 were analyzed in Trial 2. Average PCR efficiencies for Trial 2 were as follows: ß-actin, 99.4%; GH, 99.2%; GHR, 99.4%; IGF-I, 98.7%; GHRH, 99.5%; SS, 98.8%; CCK, 99.3%.

3.2.4 Statistics

Statistical differences were determined in Trial 1 using a one-way analysis of variance (ANOVA, Sigma Stat v. 3.0, SPSS Inc.) on treatment group within each brain region and gene assay. Non-normally distributed data were Ln or Log transformed. Two-way ANOVA was not possible in most cases due to the inability of the expression data to be normalized between tissues with any transformation and the resultant violation of ANOVA assumptions. Correlations were performed with a Pearson product moment correlation analysis or a non-parametric Spearman rank correlation analysis. In Trial 2 mRNA expression and plasma IGF-I concentrations were compared using t-tests and Mann Whitney U tests. Samples with low RNA expression at the level in which repeats within a 0.5 Ct difference could not be obtained were removed from statistical analysis. Significance was determined at $p \leq 0.05$.

3.3 Results

3.3.1 Trial 1

Within each brain region some samples showed expression that was too low to accurately quantify and were removed from analysis, although amplification was seen in brain regions for all the genes tested using the present assays. As anticipated from the known pituitary-dominant expression of GH, expression was low for GH in all NT-Full fish brain regions except the hypothalamus, but these results are included in Fig. 3.1 for comparison. In the GH assay, no amplification was seen in the liver of NT-Full fish, 5 samples were removed from analysis in the T-Full cerebellum and 1 cerebellum sample, 1 muscle and 2 liver samples were removed from the T-Restricted group. The GHR assay had 1 liver sample removed from the NT-Full group and 1 muscle and 2 liver samples removed from the T-Restricted group. In IGF-1, 2 pituitary, 1 liver and 1 cerebellum samples were removed from NT-Full, 1 liver was removed from T-Full and 1 pituitary, 2 liver and 1 cerebellum were removed from the T-Restricted group. All pituitary samples showed low expression in the GHRH assays in addition to 3 cerebellum samples from T-Full fish and 1 from NT-Full fish. Only the hypothalamus and telencephalon could be accurately quantified from the SS assay. Similar to the GHRH assay, CCK expression

was too low in pituitaries of all fish as well as in 1 cerebellum sample from each of the NT-Full and T-Full groups. Despite the absence of these samples, each tissue analyzed within each group and assay had 9-11 replicates (with the exception of one group of 5 replicates in the GH assay and one group of 7 replicates in the GHRH assay).

The gene used for normalization, ß-actin, was lower in the pituitary tissues than the other brain regions (Fig. 3.1A). T-Full fish had greater ß-actin expression than NT-Full fish in the hypothalamus, optic tectum and telencephalon and in the brain overall (total). In all tissues T-Restricted fish had similar expression to both the T-Full and NT-Full fish and no differences between any groups were seen in the cerebellum, midbrain, pituitary, liver or muscle (Fig. 3.2D).

Growth hormone expression was highest in the hypothalamus and similar between T-Full and T-Restricted fish (Fig 3.1B). In all brain regions NT-Full fish had significantly lower GH expression than T-Restricted fish and was lower than the T-Full group in the midbrain and optic tectum only. Pituitary GH expression did not change between the groups, although there was a slightly lower expression in T-Full fish. In muscle and liver tissue both transgenic groups had greater GH expression than the NT-Full fish, the latter of which did not show any expression in the liver (Fig. 3.2A). T-Restricted fish had reduced hepatic GH expression compared to T-Full fish.

Growth hormone receptor (GHR) expression was highest in the hypothalamus but differences between groups varied between brain regions. T-Restricted fish had the greatest GHR expression in the hypothalamus, midbrain and telencephalon but were similar to T-Full fish in the cerebellum, optic tectum and overall (Fig. 3.3A). NT-Full fish showed similar expression to T-Full fish in some tissues but were significantly lower in the hypothalamus and cerebellum. Contrary to other tissues, T-Full fish had lower GHR expression in the pituitary in which there was no difference between T-Restricted or NT-Full fish. Similar to the trends in the brain, the transgenic groups had significantly higher GHR expression in muscle and liver (Fig. 3.2B).

The differences in insulin-like growth factor I (IGF-I) expression varied (Fig. 3.3B). Neither the cerebellum nor the optic tectum showed differences between groups. T-Restricted fish had greater expression than T-Full fish in the hypothalamus but this difference disappeared in the midbrain and telencephalon. In the pituitary this difference switched with greater expression in the T-Full fish. In half of the brain regions, T-Restricted fish had greater IGF-I than NT-Full fish while no differences between the two are seen in the remaining brain regions despite a trend to elevated levels in T-Full fish. Overall, transgenic fish had greater IGF-I expression than non-transgenic fish in the brain. IGF-I expression was highest in T-Full fish in

both the muscle and liver although it was only significantly so in the latter (Fig. 3.2C). NT-Full fish had expression lower than, and similar to, that of T-Restricted fish in muscle and liver tissue respectively.

There were no differences observed between groups in growth hormone-releasing hormone expression within any of the brain regions examined (Fig. 3.4A). Expression between regions differed with the highest expression seen in the hypothalamus and telencephalon, followed by lower expression in both the midbrain and optic tectum, and extremely low expression in the cerebellum. Somatostatin expression in the telencephalon was higher in NT-Full fish than T-Full fish but both did not differ significantly from the intermediate expression in T-Restricted fish (Fig. 3.4B). The hypothalamus and total brain showed no differences between groups. Cholecystokinin (CCK) also showed similar expression between groups in all brain regions and overall (Fig. 3.5). The telencephalon had the highest CCK expression followed by the optic tectum while the remaining tissues, had lower CCK expression levels.

Correlation analysis between the expression of GH and GHR, GH and IGF-I, and GHR and IGF-I in the telencephalon, hypothalamus, pituitary, muscle, and liver showed significant relationships in only seven instances (Table 3.2). There was a strong positive correlation between GHR and IGF-I in the telencephalon and hypothalamus of NT-Full fish, and in the hypothalamus, pituitary and liver of T-Restricted fish. Furthermore, significance was almost reached in the pituitary of T-Full fish and the telencephalon of T-Restricted fish. GH was positively correlated with IGF-I in T-Full muscle and with GHR in the T-Restricted telencephalon.

3.3.2 Trial 2

In each graph the expression ratio is shown for all brain regions within each assay. Comparisons between the gene expression ratios of each tissue cannot be made as samples from each brain region were run on separate plates. Expression did not differ between transgenic fish fed the 21 MJ/kg diet and those fed the 15 MJ/kg diet for any of the genes tested or in any tissue with the exception of pituitary GHR expression in which fish on the 21 MJ/kg diet had significantly lower expression (Fig. 3.6).

Plasma concentrations of IGF-I were significantly higher in transgenic (T) than non-transgenic (NT) fish (T: 37.4 ±1.6 ng/mL; NT: 6.4 ±1.1 ng/mL)(Fig 3.7A). There was a strong positive correlation between plasma IGF-I and mass in the non-transgenic fish only ($p = 0.001$) (Fig. 3.7C). Diet treatment had no effect on plasma IGF-I in both genotypes (T fed 21 MJ/kg

diet: 36.7 ± 2.1 ng/mL; T fed 15 MJ.kg diet: 38.1 ± 2.6 ng/mL; NT fed 21 MJ/kg diet: 8.5 ± 1.8 ng/mL; NT fed 15 MJ/kg diet: 4.3 ± 1.1 ng/mL) but the non-transgenic fish fed the 21 MJ/kg diet almost reached a plasma IGF-I concentration significantly higher than that of the 15 MJ/kg diet group ($p = 0.058$)(Fig. 3.7A).

3.4 Discussion

3.4.1 Trial 1

A general trend of increased ß-actin expression was seen in T-Full fish but only reached significance in three brain regions. Because ß-actin is not normalized to any other gene these differences may have arisen from technical variation (i.e. varying reverse transcription efficiencies causing differences in total cDNA quantities between samples). However, since the samples were processed in random order, the persistent trend across tissues suggests that there may have been an alternative biological explanation. T-Full fish had a much greater growth rate than T-Restricted and NT-Full fish due to increased feed intake. ß-actin is an important structural protein and may have been increased in rapidly developing cells, although muscle and liver did not show this trend. Furthermore, ß-actin expression appeared to be much lower in the pituitary than other tissues (Fig. 3.1), possibly due to increased expression of other genes in pituitary cells and the decrease in the relative abundance of ß-actin expression. The differences that may have existed in ß-actin expression between the NT-Full and T-Full groups is small, approximately 10 to 20 %, depending on the tissue, and there was no difference between the NT-Full and T-Restricted groups. Thus, normalization to this expression as a proxy for total RNA quantities would have a small impact on gene expression ratios and would not affect mutifold differences in expression.

Growth hormone expression followed trends that were expected in an assay that detects both endogenous GH and GH transgene expression. Detectable, but unquantifiable, levels were present in all non-transgenic tissues (except the pituitary, hypothalamus and muscle), whereas expression was abundant in all transgenic tissues. GH expression in non-pituitary tissues of non-transgenic coho salmon has been found previously (Mori and Devlin, 1999). In addition, trace amounts of cellular cross-contamination during sampling and trace amounts of genomic DNA in the cDNA samples may have minutely contributed to the apparent GH expression. In the present study, pituitary GH expression was high and similar in all groups. This is in contrast to a previous study by Mori and Devlin (1999) on transgenic coho salmon. They found that transgenic fish had reduced pituitary GH RNA compared to that of non-transgenic fish.

However, they noted that the magnitude of this difference decreased with the size of the fish being compared. The present study was conducted on 55g fish, a size that may well be expected to lack differences in pituitary GH expression when extrapolating the trend found by Mori and Devlin (1999).

There was no evidence for an increase in pituitary GH expression by feed-restricted fish as was seen by Valente et al. (2003) and Pierce et al. (2004). Despite this difference, my results are in agreement with two studies on tilapia and Atlantic salmon in which a difference in feed intake greater than that in this experiment (i.e. fasting) did not increase circulating GH (Bjornsson et al., 1994; Uchida et al., 2003). The difference in feed intake between these two groups may not have been great enough for the formation of GH resistance by the liver, a concomitant decrease in plasma IGF-I, and an increase in GH production (Maniar et al., 1994; Duan et al., 1995; Wargelius et al., 2005). Indeed, I found no differences in hepatic GHR expression in T-Restricted fish. Within other tissues, the similar GH expression between the transgenic groups agrees with expected trends as the GH transgene is driven by a metallothionein-B promoter sequence and is not known to respond to signals for endogenous GH regulation (Devlin et al., 2004a).

The general increase in growth hormone receptor expression across tissues in both full and restricted ration transgenic groups coincided with the increase in GH seen in these fish. In transgenic mice, increased GH stimulated increased GH binding and GHR mRNA expression which in turn increased GH receptor abundances to both facilitate IGF-I production and to allow for negative feedback to both the hypothalamus and pituitary from circulating GH (Chen et al., 1991; Bartke et al., 1994; Chen et al., 1995; Asa et al., 2000). This appears to be happening in transgenic fish as well. There is considerable variability in the significance of the observed increases which may be in part due to the expression of multiple forms of GHR. Black sea bream, coho salmon and trout have two forms of GHR, designated GHR I and GHR II, which show differential expression in body tissues (Fukada et al., 2004; Saera-Vila et al., 2005). GHR I is the predominant form in the liver and brain while GHR II is dominant in the pancreas and spleen. My assay did not distinguish between the two forms of GHR and therefore, my expression values reflected the net changes in these two transcripts. The two forms of GHR may be changing in distinct ways among the brain regions analyzed. Such changes would explain the sharp decrease in pituitary, and increase in hypothalamic, GHR expression in T-Full fish. Among the brain regions, the hypothalamus showed the greatest GHR expression. This region contains neuropeptide Y, and somatostatin neurons involved in the negative regulation of GH

production in many fish species (Olivereau et al., 1984; Moons et al., 1989; Batten et al., 1990; Power et al., 1996; Lin et al., 1999; Rodriguez-Gomez et al., 2001; Mathieu et al., 2002; Traverso et al., 2003; Chen et al., 2005). An upregulation in GHR expression in the hypothalamus would increase the sensitivity of SS and NPY neurons to changes in the chronically high circulating GH by allowing for greater GH binding and preventing receptor saturation.

IGF-I is important for proper somatic growth and is a major mediator of the growth effects stimulated by GH in vertebrates. The increased GH and GHR mRNA expression noted in this study was accompanied by an increase in IGF-I expression in transgenic fish over the non-transgenic controls in muscle and brain (total) tissue. This result does not agree with studies by Duguay et al. (1994) and Duan (1998) who found that the IGF-I transcript present in all non-hepatic tissues, transcript Ea-4, did not increase with the hepatic transcripts, Ea-1 and Ea-3, after GH addition to coho and Atlantic salmon. Conversely, a study by Iida et al. (2004) on GH transgenic mice suggested that IGF-I mRNA expression in the pituitary may increase when exposed to chronically higher GH and that IGF-I is regulated differently between the pituitary and liver. It is clear from this study that there are region specific expression patterns of IGF-I in non-hepatic tissues of transgenic coho salmon, but the manner by which various IGF-I transcripts may have been involved in this observation needs to be determined. In support of the above studies, and in addition to studies on teleost hepatocyte cultures (Duan et al., 1992; Pierce et al., 2004; Pierce et al., 2005a), hepatic IGF-I expression in our experiment increased in the T-Full treatment. In the T-Restricted group, feed restriction reduced the increase in hepatic IGF-I expression to that of NT fish. The difference in IGF-I expression in the liver but lack thereof in the brain is consistent with fasted coho salmon and carp in which liver IGF-I mRNA expression responded to starvation while other tissues did not (Duan and Plisetskaya, 1993; Hua and Lin, 2001). Interestingly, the decrease in IGF-I expression by the liver in the feed-restricted group is not accompanied by an obvious decrease in GHR expression even though these two variables were positively correlated in most of the T–Restricted tissues. These two variables were not related in NT-Full or T-Full treatments suggesting that other factors (e.g. food intake or appetite satiation) may be controlling GHR and IGF-I expression in these groups.

GHRH expression did not differ significantly between groups in any of the brain regions. This is surprising as I hypothesized that increased GH production in transgenic fish would negatively feedback on GHRH expression. There is a slight trend in the hypothalamus, optic tectum and telencephalon for increased GHRH expression in NT-Full and T-Restricted fish.

This may be due to a reduction in GHRH expression in T-Full fish, as hypothesized above, combined with an increased GHRH expression in T-Restricted fish due to decreased circulating IGF-I (Mathews et al., 1988a; Wallenius et al., 2001), but these results are inconclusive. The highest GHRH expression was found in the hypothalamus and telencephalon. Consistent with my data, GHRH cells have been detected in many fish tissues with expression in areas where GHRH neurons extend to the pituitary (Parhar and Iwata, 1996; Montero et al., 1998; Miranda et al., 2002).

SS expression was measurable in two tissues, the hypothalamus and telencephalon, of which only the telencephalon showed a significant difference. There was a trend in the hypothalamus for reduced SS in the full-ration transgenic fish but contrary to our hypotheses, SS expression was not significantly different. Increases in circulating GH due to transgenesis and injection positively feedback on SS neurons in order to suppress GH production by the pituitary in rodents (Hurley and Phelps, 1992; Sato and Frohman, 1993; Hurley et al., 1994; Szabo et al., 1995; Pellegrini et al., 1997). GH transgenic coho salmon can have as much as a 40-fold increase in circulating GH (Devlin et al., 1994) and yet SS expression in this experiment did not change. Three transcripts of preprosomatostatin, encoding for three SS proteins, have been found in goldfish in which they may elicit different intracellular pituitary responses (Lin et al., 1999; Yunker et al., 2003; Yunker and Chang, 2004). My assay did not distinguish between these forms and further studies are needed to determine if there are multiple SS mRNA transcripts in coho salmon and the manner in which they may respond to changes in GH production.

To understand the increased feeding motivation in GH transgenic coho salmon we analyzed the expression of one of the appetite regulating hormones, CCK, that may interact with the GH axis. However, CCK expression did not differ between groups in any of the brain regions analyzed despite the very large difference in feeding behaviour observed between transgenic and non-transgenic salmon. The lack of a difference suggests that the CCK satiation signal in GH transgenic fish was operating in a similar way as non-transgenic fish, at least at the time of sampling. My data do not support the initial hypothesis that the low level of feed provided to the T-Restricted group would cause a chronic decrease in CCK expression. One explanation is that CCK expression may have been low in all groups as they were sampled at least 24 hours after last feeding, allowing diurnal CCK fluctuations to mask any long term changes in expression. CCK mRNA expression rises post-feeding (Peyon et al., 1999), and our fish were not fed immediately prior to sampling. Studies on the time course of CCK expression

in GH transgenic coho salmon is therefore warranted. Furthermore, three forms of CCK are found in the rainbow trout and goldfish, designated CCK-N, CCK-L and CCK–T (Jensen et al., 2001). These transcripts have different expression patterns throughout the brain (Jensen et al., 2001) but my assay is designed to detect the combined expression of all three forms. In order to confirm my conclusions, expression of the different CCK transcripts in the brain and CCK protein in other tissues, especially the gut, should be analyzed as the gut is the first area to respond to changes in energy intake (Jensen et al., 2001; Kamisaka et al., 2001; Kamisaka et al., 2002). Differences in expression between transcripts in the brain and differences in CCK protein production in the gut are likely occurring in these fish and should be examined as they were not specifically examined in my analysis.

3.4.2 Trial 2

Comparisons between the transgenic fish fed a 21 MJ/kg and 15 MJ/kg diet showed no differences in any of the tissues examined, with the exception of GHR in the pituitary which is likely not biologically significant as the increase was not accompanied by any changes to GH mRNA expression. Possibly, the difference in energy intake between fish in this study was not enough to affect neuronal expression of any of the genes of interest. During starvation, hepatic IGF-I expression (Duan and Plisetskaya, 1993) and plasma IGF-I concentrations (Shimizu et al., 1999; Pierce et al., 2001) are expected to decline. Although we did not measure liver IGF-I mRNA expression in this trial, plasma IGF-I concentrations did not change in response to dietary energy intake. Consistent with studies on GH injection in salmon and rainbow trout (Foucher et al., 1992; Shimizu et al., 1999) and the resultant increases in growth, the GH transgenic coho salmon did have a 5.8-fold increase in circulating IGF-I. There is some evidence that the growth of GH transgenic coho salmon may not be responding normally to plasma IGF-I levels. Weight did not correlate with IGF-I concentrations in transgenic fish and the decreased size of transgenic fish fed the 15 MJ/kg diet (Chapter 2 Fig. 2.1) was not accompanied by a change in IGF-I plasma levels. In contrast, non-transgenic coho salmon showed a significant relationship between weight and plasma IGF-I. Unfortunately, in this trial sufficient plasma was not available to determine GH concentrations and how they may have related to fish growth. Despite the impact that these two diets had on growth, feed intake, and feed efficiency (Chapter 2), I found no modifications to the GH-axis based upon mRNA levels or CCK expression.

3.5 Conclusions

Growth hormone expression was higher in transgenic coho than that of non-transgenic fish in the muscle and liver and in all brain regions tested, except the pituitary. No differences were seen between the feed-restricted and full-ration transgenic fish except in the liver. The higher GH expression in transgenic fish was generally accompanied by higher GHR expression, although these differences, as well as those between the transgenic groups, were tissue specific. IGF-I expression was higher in full-ration transgenic individuals, and in most tissues the feed-restricted group had IGF-I levels reduced to that of non-transgenic fish. This expression corresponded to the reduced growth seen in both the non-transgenic and feed-restricted transgenic fish. No conclusive differences were seen in GHRH, SS or CCK expression between the treatment groups suggesting that the large difference in growth and feed utilization in transgenic fish did not result from these GH regulating hormones or a deficiency in satiety signaling. The change in dietary energy intake between a 21 MJ/kg and 15 MJ/kg diet did not appear to be great enough to show mRNA expression differences in any of the hormones analyzed in transgenic fish, despite significant differences in growth and feed intake. Plasma IGF-I levels were greatly impacted by GH transgenesis with a 5.8 fold increase in the transgenic fish but diet had no effect on plasma IGF-I in either genotype. Furthermore, growth in weight may have been uncoupled with plasma IGF-I in transgenic individuals. Thus, growth hormone production, GHR expression and IGF-I expression were increased in both body tissues and the brain with no response by the central regulators of GH, GHRH and SS, to reduce this expression. Reduced dietary energy intake did not influence gene expression in the brain but had a marked effect on GH and IGF-I expression in the liver.

3.6 Acknowledgements

I would like to thank Mitchell Uh for his help analyzing muscle and liver samples, as well as Dionne Sakhrani, Carlo Biagi, and Geordia Rigter for their technical assistance.

Table 3.1
Quantitative PCR primer and probe sequences for coho salmon, *Onchorynchus kisutch*[1]

Gene	Forward Primer	Reverse Primer	TaqMan MGB Probe
ß-actin	ACGGCCGAGAGGGAAATC	CAAAGTCCAGCGCCACGTA	CACAGCTTCTCCTTGATGT
GH	CAAGATATTCCTGCTGGACTT	GGGTACTCCCAGGATTCAATCA	CAGTCCTGAAGCTGC
GHR	CACTGTGGAAGACATCGTGGAA	CAAAGTGGCTCCCGGTTAGA	AACTGGACCCTGCTGAA
IGF-I	GGCATTTATGTGATGTCTTCAAGAGT	CCTGTTGCCGCCGAAGT	TCTCACTGCTGCTGTGC
GHRH	TCATCTATGGAATCATAATGCACTACAG	CCATCCTCGTCATAAACCTCATT	CTATCCTAACCTTAGACTTGAA
SS	CCGGCTTTGCGCTCAC	ATTATATCCCAACTGTCCTTGCCT	CCACTGAGCGCTCCA
CCK	GCCTACCTCAGTGAATTGTTGGC	CGGGGTAGTCTCGGTCTTTTATC	AACTCAACAGTGAACAGCAGA

[1] Sequences are written 5' to 3' left to right

Table 3.2
Correlations of GH, GHR and IGF-I mRNA expression ratios in body tissues of coho salmon, *Oncorhynchus kisutch*

		NT-Full		T-Full		T-Restricted	
Tissue	Correlation	r (rs)[1]	p value	r (rs)[1]	p value	r (rs)[1]	p value
Telencephalon[2]	GH v. GHR	-	-	0.159	0.662	**0.770**	**0.006**
	GH v. IGF-I	-	-	-0.187	0.604	0.392	0.233
	GHR v. IGF-I	**0.686**	**0.028**	0.325	0.360	0.602	0.0501
Hypothalamus[2]	GH v. GHR	-	-	0.277	0.438	0.566	0.069
	GH v. IGF-I	-	-	0.252	0.482	0.415	0.205
	GHR v. IGF-I	**0.796**	**0.003**	0.196	0.586	**0.706**	**0.015**
Pituitary	GH v. GHR	-0.126	0.747	0.117	0.742	0.440	0.203
	GH v. IGF-I	0.308	0.420	-0.183	0.612	0.483	0.158
	GHR v. IGF-I	0.143	0.714	0.600	0.077	**0.831**	**0.003**
Muscle	GH v. GHR	0.500	0.109	0.217	0.521	0.248	0.468
	GH v. IGF-I	0.145	0.653	**0.676**	**0.023**	-0.018	0.946
	GHR v. IGF-I	-0.173	0.595	0.227	0.502	-0.333	0.327
Liver[2]	GH v. GHR	-	-	0.436	0.168	0.338	0.374
	GH v. IGF-I	-	-	-0.127	0.707	0.639	0.064
	GHR v. IGF-I	0.001	0.999	0.164	0.631	**0.740**	**0.023**
Pituitary v. Liver[3]	p GH v. l GHR	-0.234	0.516	-	-	-	-
	p GH v. l IGF-I	0.293	0.411	-	-	-	-
Pituitary v. Muscle[3]	p GH v. m GHR	0.0455	0.881	-	-	-	-
	p GH v. m IGF-I	0.164	0.614	-	-	-	-

[1] Pearson product moment correlation coefficients, r, are given for parametric data and Spearman rank correlation coefficients, rs, for non-parametric data

[2] GH values were too low in NT-Full group for correlation analysis

[3] Correlations of pituitary GH against liver and muscle GHR and IGF-1 were only analyzed in NT-Full fish because GH is produced by all tissues in the other two groups and any trends should be apparent in the intra-tissue correlations.

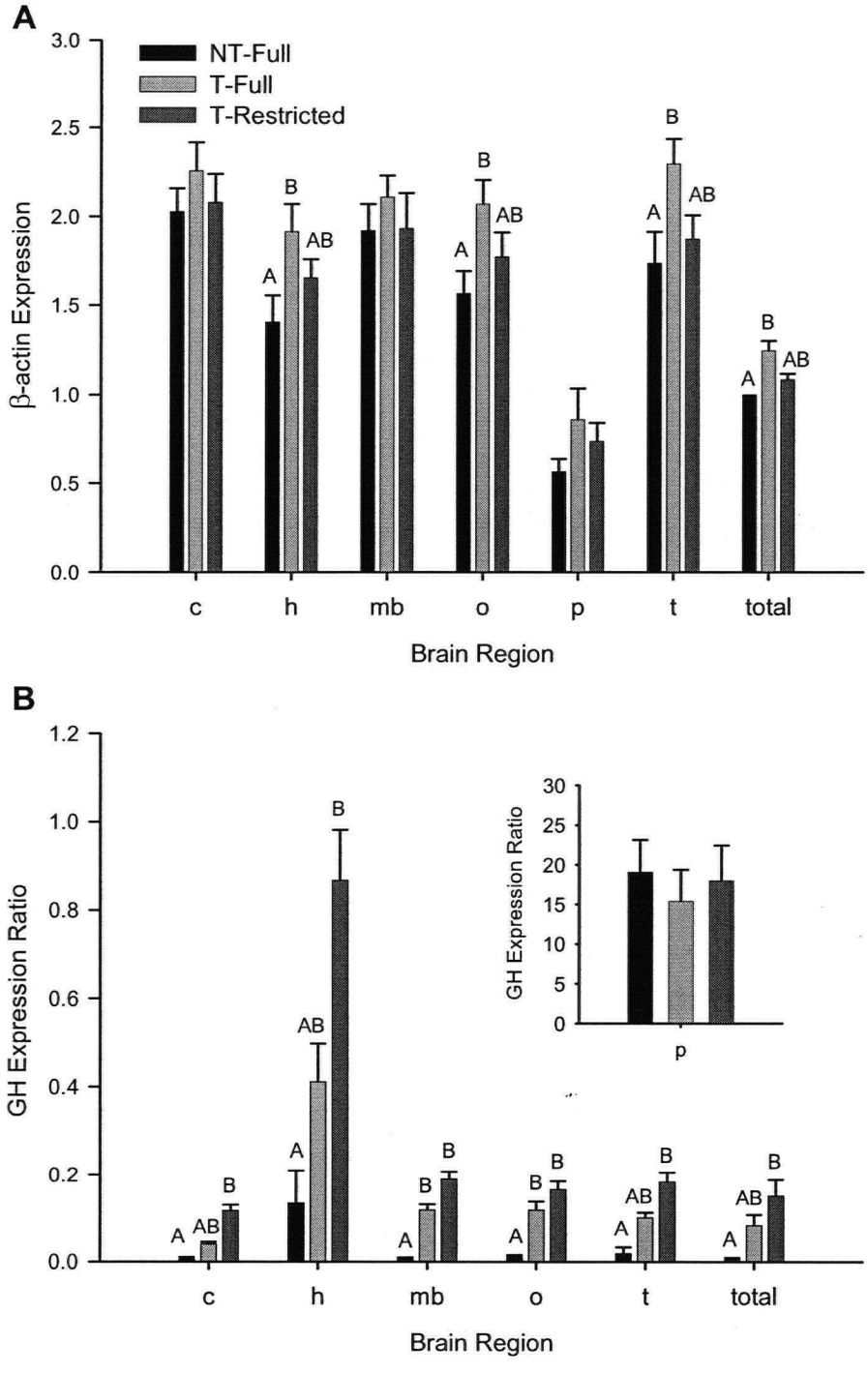

Fig. 3.1. Gene expression of A: ß-actin and B: growth hormone (GH) in the cerebellum (c), hypothalamus (h), midbrain (mb), optic tectum (o), pituitary (p), telencephalon (t) and whole brain without pituitary (total) of full-ration non-transgenic (NT-Full), full-ration transgenic (T-Full) and pair-fed, restricted-ration transgenic (T-Restricted) coho salmon. Values for ß-actin and GH in each tissue were standardized to NT-Full fish that were assigned a ratio of 1 and 0.01 respectively. These values were then combined for comparison (total). Comparisons between groups were made within each brain region. Comparisons between tissues are not shown and cannot be made with pituitary GH expression (inset). Letters that differ mark significant differences where they occur. Bars are means ± SE, N = 10 to 11 (GH T-Full c has an N of 5).

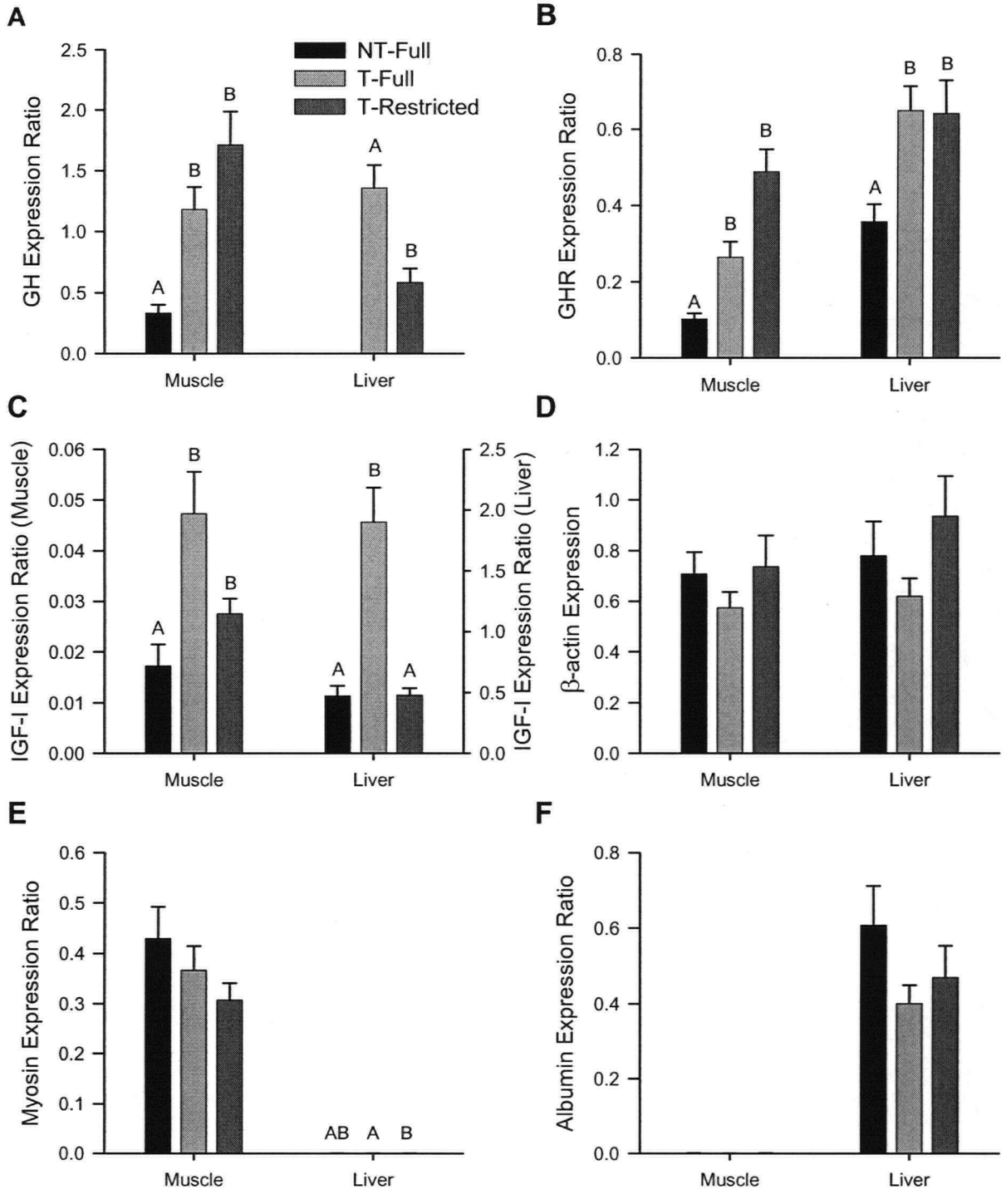

Fig. 3.2. Gene expression ratios of A: GH, B: GHR, C: IGF-I, D: ß-actin, E: myosin and F: albumin, in muscle and liver tissue of full-ration non-transgenic (NT-Full), full-ration transgenic (T-Full) and pair-fed, restricted-ration transgenic (T-Restricted) coho salmon. Comparisons between tissues were not made. Letters that differ mark significant differences where they occur. Bars are means ± SE, N = 9 to 11.

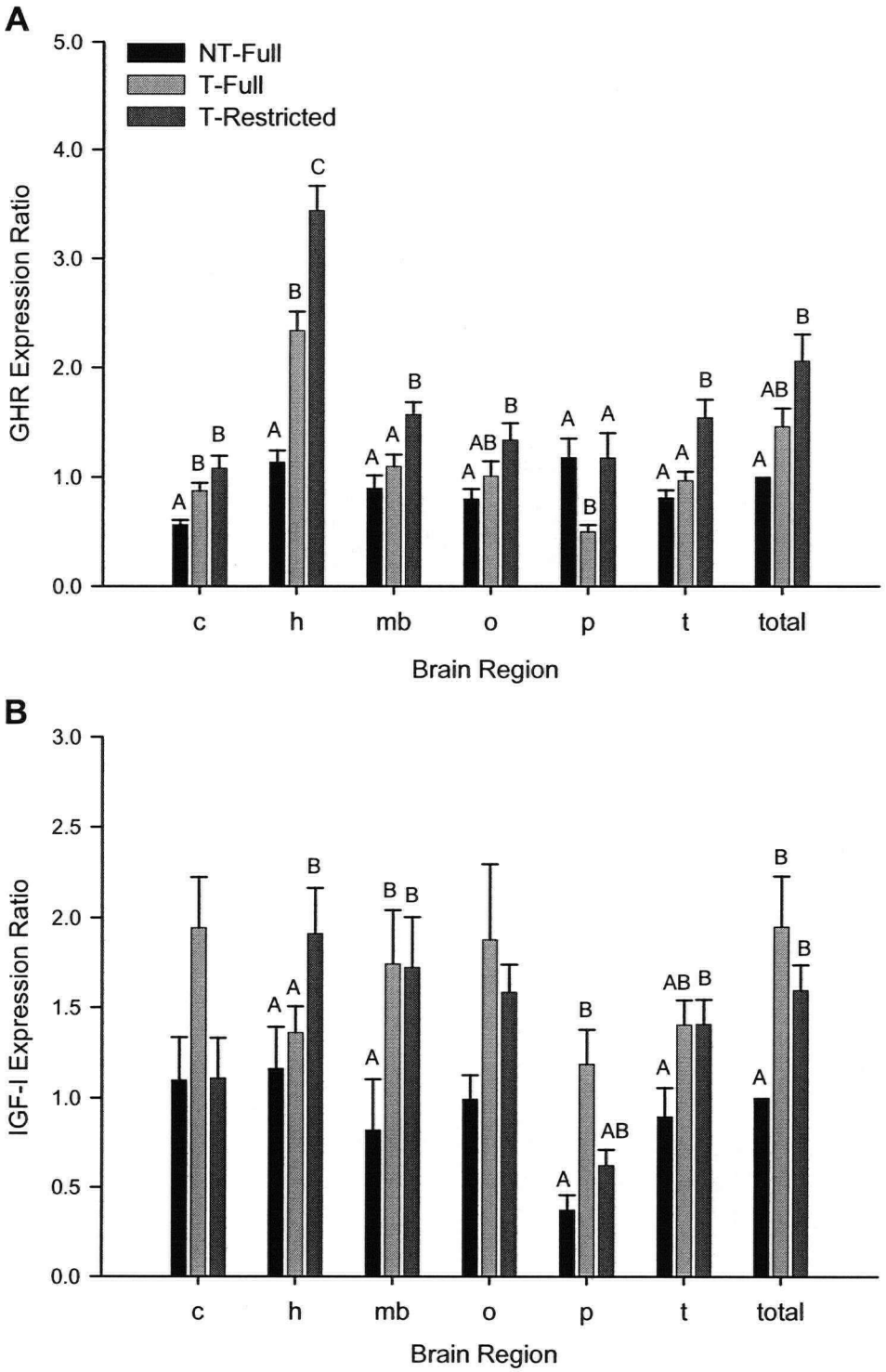

Fig. 3.3. Gene expression ratios of A: growth hormone receptor (GHR) and B: insulin-like growth factor I (IGF-I) in the cerebellum (c), hypothalamus (h), midbrain (mb), optic tectum (o), pituitary (p), telencephalon (t) and all regions except the pituitary (total) of full-ration non-transgenic (NT-Full), full-ration transgenic (T-Full) and pair-fed, restricted-ration transgenic (T-Restricted) coho salmon. GHR and IGF-I expression in both T groups were standardized to that of NT fish that were assigned a ratio of 1 for each tissue. These values for each tissue were then combined for comparison (total). Letters that differ mark significant differences where they occur. Bars are means ± SE, N = 10 to 11.

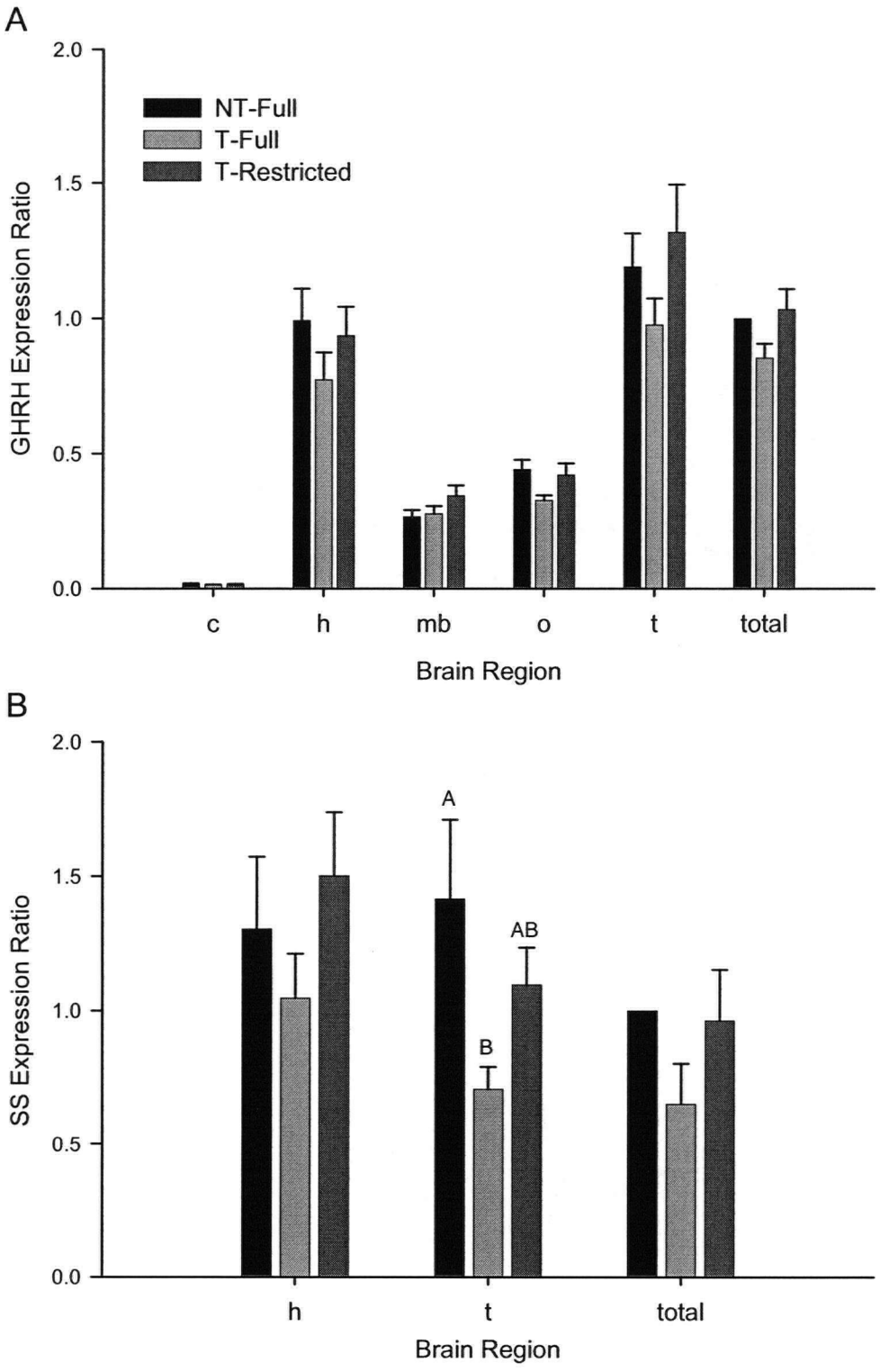

Fig 3.4. Gene expression ratios of A: growth hormone-releasing hormone (GHRH) and B: somatostatin (SS) in the cerebellum (c), hypothalamus (h), midbrain (mb), optic tectum (o) and telencephalon (t) and all regions (total) of full-ration non-transgenic (NT-Full), full-ration transgenic (T-Full) and pair-fed, restricted-ration transgenic (T-Restricted) coho salmon. GHRH and SS expression in both T groups were standardized to that of NT fish that were assigned a ratio of 1 for each tissue. These values were then combined for comparison (total). Letters that differ mark significant differences where they occur. Bars are means ± SE, N = 7 to 11.

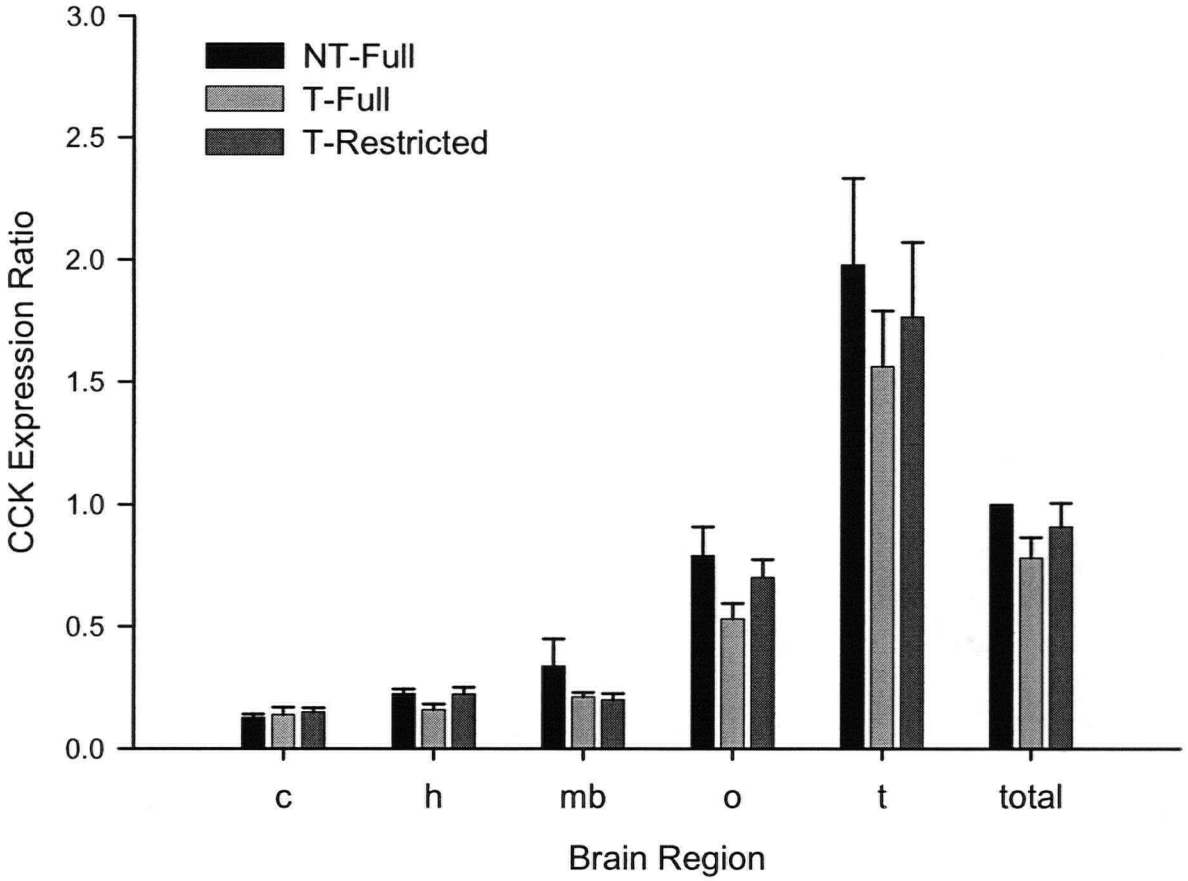

Fig 3.5. Gene expression ratio of cholecystokinin (CCK) in the cerebellum (c), hypothalamus (h), midbrain (mb), optic tectum (o), telencephalon (t) and all regions (total) of full-ration non-transgenic (NT-Full), full-ration transgenic (T-Full) and pair-fed, restricted-ration transgenic (T-Restricted) coho salmon. Gene expression in both T groups were normalized to that of NT fish that were assigned a ratio of 1 for each tissue. These values for each tissue were then combined for comparison (total). Letters that differ mark significant differences where they occur. Bars are means ± SE, N = 9 to 11.

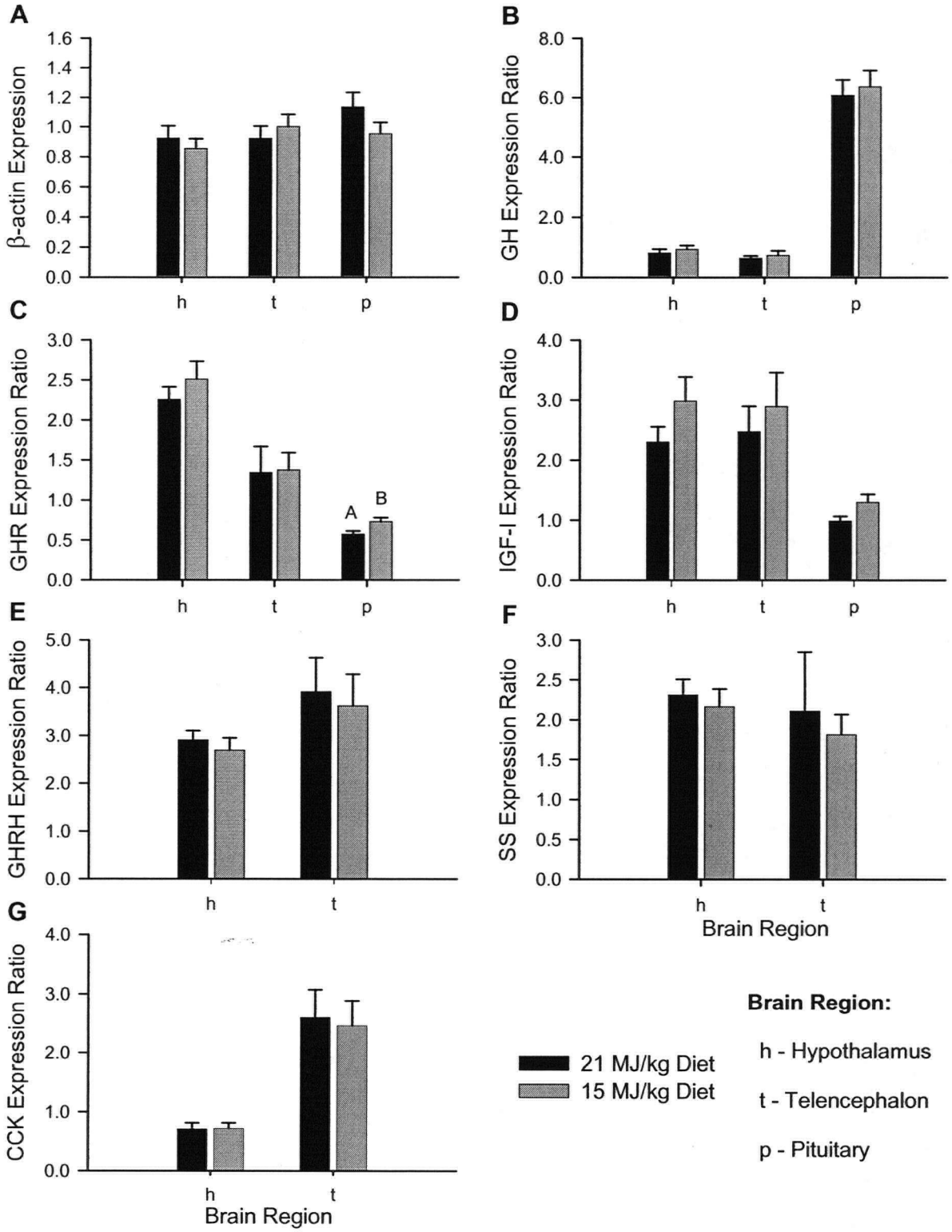

Fig. 3.6. Gene expression of A: ß-actin; and ratios of B: growth hormone (GH); C: growth hormone receptor (GHR); D: insulin-like growth factor I (IGF-I); E: growth hormone-releasing hormone (GHRH); F: somatostatin (SS); and G: cholecystokinin (CCK) in the hypothalamus (h), telencephalon (t) and pituitary (p) of transgenic coho salmon fed a 21 MJ/kg (black bar) or 15 MJ/kg (grey bar) diet. Comparisons between brain regions were not made. Letters that differ mark significant differences where they occur. Bars are means ± SE, N = 12.

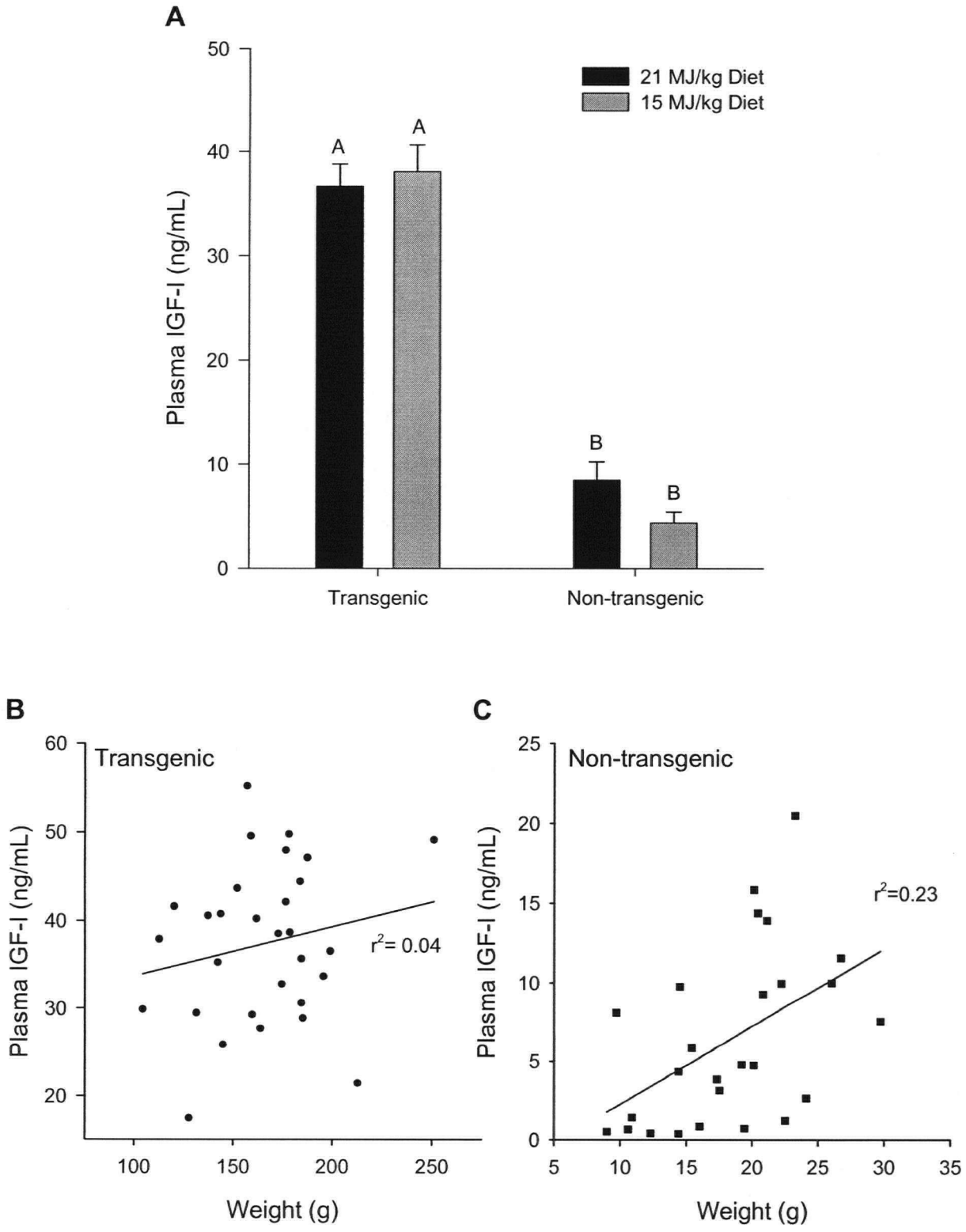

Fig. 3.7. Plasma insulin-like growth factor I (IGF-I) concentrations of transgenic and non-transgenic coho salmon fed a 21 MJ/kg and 15 MJ/kg diet (A). Correlations between weight and plasma IGF-I concentrations in transgenic (B) and non-transgenic (C) fish. In plot A, letters that differ mark significant differences where they occur, bars are means ± SE, N = 13 to 15. Note the difference in scale between plots B and C. The correlation between plasma IGF-I and weight was significant in the non-transgenic fish (C) only.

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CHAPTER 4

Thesis summary and future research.

4.1 Introduction

The enhanced production of growth hormone (GH) in GH transgenic coho salmon has marked effects on the growth and physiology of this species. Growth is increased 2-3 fold, resulting in early smolting and development of salt water tolerance (Devlin et al., 1995; Devlin et al., 2000). Both intestinal surface area and cartilage deposition are increased (Ostenfeld et al., 1998; Stevens and Devlin, 2000), and there is an elevation in the oxygen requirement of muscle tissue during exertion and following feeding of GH transgenic coho salomon (Hill et al., 2000; Lee et al., 2003). Furthermore, GH transgenesis may be disadvantageous as it increases the susceptibility of these fish to bacterial infections during the smoltification phase of their development (Jhingan et al., 2003). In addition to the above changes, feeding activity and feed intake have been observed to increase in transgenic coho salmon. Little is known, however, about their nutritional and energy needs and how appetite is controlled. Also, no studies have examined gene expression of the hormones regulating GH or appetite control and the manner in which gene expression changes in response to dietary energy source and level. The present thesis provides the first study on the dietary energy and nutrient use required to support enhanced growth in GH transgenic coho salmon. Furthermore, this is the first study on gene expression of the major GH regulating and appetite regulating hormones in a transgenic fish.

4.2 Digestible Energy and Protein Utilization

The results of the present study have shown that the physiology of growth hormone (GH) transgenic coho salmon has been altered in such a way that they are eating to a new energy demand and are more efficient at utilizing dietary protein than their non-transgenic counterparts. GH transgenic salmon can respond in growth and appetite to increases in dietary digestible energy in the range of 15 MJ/kg to 21 MJ/kg, whereas non-transgenic coho salmon on the other hand require 17 MJ/kg and preferably 19 to 21 MJ/kg for efficient growth during the winter season when this experiment was conducted. The increased feed efficiency and protein utilization that was observed in transgenic coho salmon in the present study is in accord with data from other GH transgenic and GH-protein-treated fish in which there tended to be an increase in feed conversion efficiency, protein synthesis and retention, amino acid transport and quantities, muscle growth, and lipid metabolism (Sun, 1990; Foster and Houlihan, 1991;

Chatakondi et al., 1995a; Fauconneau et al., 1996; Fauconneau et al., 1997; Fu et al., 1998; Farmanfarmaian and Sun, 1999; Krasnov et al., 1999b; Cook et al., 2000; Fu et al., 2000; Martinez et al., 2000; Rahman et al., 2001; Dunham et al., 2002). The ability of transgenic coho salmon to grow well on diets that were nutritionally insufficient for non-transgenic coho salmon (i.e. those diets with low digestible energy and high fibre content) may indicate that transgenic coho salmon, if introduced into the wild, would better utilize natural food sources that are nutritionally inferior to wild fish (e.g. those with high indigestible chitin content) and thus shift resource utilization in natural ecosystems.

4.3 Plasma IGF-I

Transgenic fish from the diet experiment had greater insulin-like growth factor I (IGF-I) plasma concentrations. Fish weights (representing growth rate) were only correlated with plasma IGF-I in non-transgenic fish, which suggests that transgenic fish may not be responding to or inducing levels of plasma IGF-I using normal mechanisms. For example, transgenic coho salmon may be insensitive to small changes in plasma IGF-I at such an elevated level due to the saturation of growth hormone receptor signaling. The increased plasma IGF-I concentrations in the transgenic fish agree with previous studies in mice (Mathews et al., 1988a; Blackburn et al., 1997; Sotelo et al., 1998) and fish (Foucher et al., 1992; Shimizu et al., 1999) that have shown an increase in IGF-I with increases in GH. Among transgenic fish on different diets, despite changes in growth between the groups, there were no differences in plasma IGF-I concentrations. Although diet did not affect plasma IGF-I, decreases in energy intake due to fasting have previously been shown to cause reduced plasma IGF-I in coho salmon (Duan and Plisetskaya, 1993; Shimizu et al., 1999; Pierce et al., 2001).

4.4 Gene mRNA Expression

Gene expression data from size-matched transgenic and non-transgenic fish was found to be correlated with the presence/absence of the transgene in all transgenic body tissues tested and resulted in increased GH gene expression in transgenic fish. In some tissues, such as the hypothalamus, growth hormone receptor was increased in transgenic fish, a change which may have increased GH sensitivity of the neurons producing negative GH regulators. Increased IGF-I expression was accompanied by increased GH expression in transgenic fish, but a reduction in growth rate in these fish by decreasing the level of feed intake decreased hepatic IGF-I expression to that of non-transgenic controls. Little or no change occurred in the mRNA

expression of the other hormones in the GH axis viz., growth hormone-releasing hormone (GHRH), somatostatin (SS) and cholecystokinin (CCK). This result suggests that the major changes in growth likely stemmed from differences in GH and IGF-I interacting with other growth and appetite-regulating pathways. Furthermore, there were no changes in gene expression of any of the tested hormones within brain tissue from the diet experiment, with the exception of GHR in the pituitary.

The absence of significant differences (other than GHR and IGF-I) that were observed for the hormones of the GH axis generally does not agree with data from studies on transgenic rodents in which increases in circulating GH were found to increase somatostatin (Hurley et al., 1994; Szabo et al., 1995; Pellegrini et al., 1997) and decrease growth hormone-releasing hormone expression (Sato and Frohman, 1993; Flavell et al., 1996; Pellegrini et al., 1997). Furthermore, the difference in dietary energy intake between the different groups of transgenic coho salmon did not stimulate the suite of changes that would be expected in fish that had become GH resistant (Dauncey et al., 1994; Duan et al., 1995; Valente et al., 2003; Pierce et al., 2004). Some results are, however, concordant with other systems, such as the increase in GHR seen in the present experiment (Chen et al., 1991; Bartke et al., 1994; Chen et al., 1995; Gonzalez et al., 2001) and the rises in hepatic IGF-I mRNA expression and in plasma IGF-I concentrations (Du et al., 1992; Foucher et al., 1992; Shimizu et al., 1999; Pierce et al., 2004; Caelers et al., 2005; Pierce et al., 2005a). These data suggest that some but not all aspects of GH regulation are conserved among different vertebrate species.

4.5 Additional Research Questions

To my knowledge this is the first survey of the gene expression of hormones that are involved in GH regulation and appetite control in the brain of a GH transgenic fish. A central assumption of this analysis is that mRNA expression accurately reflects protein synthesis and that synthesis is proportional to protein release and a subsequent change in growth and appetite physiology. This may not always be the case. For example, a decrease in GH release by cultured pituitary cells has occurred without changes to GH mRNA expression (Melamed et al., 1996; Ran et al., 2004) which may in part be explained by the different intracellular responses regulating GH secretion that are stimulated by different forms of the somatostatin protein (Yunker et al., 2003; Yunker and Chang, 2004). A mRNA assay that detects changes to the prepro-mRNA will not accurately determine which form of the protein that will be produced to have a specific effect on GH secretion. Most hormones of the GH axis are neuropeptides and as

such require a depolarization of the neuron for secretion to occur. Changes in mRNA expression may indicate enhanced intracellular protein synthesis but not protein release. Furthermore, post-transcriptional regulation of mRNA, post-translation packaging of protein in cellular organelles, and protein turnover will also affect protein synthesis and secretion.

Despite the various stages controlling protein release after mRNA transcription, gene expression has been found to respond to hormone and diet manipulation in other studies on the GH axis (Duan et al., 1992; Duguay et al., 1994; Uchiyama et al., 1994; Pellegrini et al., 1997; Peyon et al., 1999; Fukada et al., 2004; Pierce et al., 2004; Saera-Vila et al., 2005), suggesting an immediate role of mRNA expression in hormonal change. Also, changes in gene expression are often associated with changes in the production of the hormones for which they encode (Devlin, 1984; Mathews et al., 1988a; Maniar et al., 1994; Duan et al., 1995; Pellegrini et al., 1997). The absence of significant differences in gene expression between groups in the present study, despite the observed differences in behaviour and growth, may have been due in part to changes in protein levels that are not apparent from mRNA expression. The development of salmonid radioimmunoassays for each hormone in the GH axis would be ideal but were not feasible (with the exception of IGF-I) in this experiment. Gene mRNA expression is an effective alternative to direct protein measurements and is commonly used in this field of study as a reliable indicator of hormonal change. Future studies on the protein levels of GH, GHR, GHRH, SS and CCK would be useful to complement the findings of this experiment.

The purpose of this study was to determine general expression patterns of each of the hormones of interest and thus the assays were designed to detect conserved areas in alignments of multiple transcripts within and between related species. In future studies, it would be beneficial to determine whether different forms of each of these transcripts are regulated differentially within the brain. Further, many genes present in salmonids are anticipated to be duplicated due to the semi-tetraploid nature of their genomes (Ohno et al., 1967; Allendorf and Thorgaard, 1984). GH is present in two forms in addition to the transgene in coho salmon, all of which may be expressed differently (Mori and Devlin, 1999). GHR is found in two forms in coho salmon (GenBank #: AF403539, AF403540), rainbow trout (Very et al., 2005) and sea bream (Saera-Vila et al., 2005) in which expression of the two forms changes in different tissues (Saera-Vila et al., 2005). Furthermore, both somatostatin (Lin et al., 1999; Canosa et al., 2005) and cholecystokinin (Jensen et al., 2001) may have three different transcripts in coho salmon. Determining if indeed these forms exist and their distribution across body tissues, will allow further research on their response to enhanced GH in transgenic salmon.

A considerable degree of growth regulation may be happening at the membrane-bound receptor level. GHRH receptor mRNA is reduced in somatotrophs of some GH transgenic mice (Szabo et al., 1995) but not others (Peng et al., 2001). SS receptors have been analyzed in the pituitary of GH transgenic mice where two forms, designated sst2 and sst5, did not change despite reductions in SS mRNA (Peng et al., 2001). Different forms of somatostatin trigger different intracellular responses in somatotrophs that affect GH release (Yunker et al., 2003; Yunker and Chang, 2004). Considerable research has also been done on the various NPY receptors in the teleost brain and their impact on feed intake (Berglund et al., 2000; Narnaware and Peter, 2001b). Growth hormone receptor and IGF-I receptors are important in the formation of growth hormone and IGF-I binding proteins. The extracellular regions of these transmembrane proteins cleave to carry bound protein throughout circulation (Bartke et al., 1994; Duan, 1998; Calduch-Giner et al., 2000; Liao and Zhu, 2004). It is possible that a wide range of cellular responses, including gene expression, can be modified at the receptor level. It would be beneficial to clone the genes encoding these receptors in order to study the mechanisms in which they operate in GH transgenic coho salmon.

Gene mRNA expression was analyzed for only a small selection of the hormones that influence GH production and regulate appetite. For example, it will be critical to complement the present studies with analyses of neuropeptide Y (NPY) mRNA expression in the brain of GH transgenic salmon. NPY is a negative regulator of GH expression that acts through stimulation of SS neurons (Minami et al., 1997) or through direct contact with somatotrophs in fish (Mathieu et al., 2002). It is also a potent appetite stimulator in fish and NPY gene expression is enhanced during starvation (Silverstein et al., 1996; Silverstein and Plisetskaya, 2000). As such, understanding the function of NPY and how it may respond to increased GH production is vital to understanding feeding and growth in transgenic coho salmon. The gut-derived hormone, ghrelin, is also important as it has been recently cloned from goldfish in which it increases GH release and stimulates feed intake (reviewed by Unniappan and Peter, 2005). Another major regulator of feed intake, growth and lipid metabolism is leptin (Cai and Hyde, 1998), which has recently been cloned in rainbow trout (Zou et al., GenBank #: AM042713). When leptin is injected into goldfish, appetite is increased (Volkoff and Peter, 2001) and the effect of CCK is attenuated (Volkoff et al., 2003). CCK production by the gut (CCK mRNA expression in the gut and the response of transgenic coho salmon to CCK antagonists is presently under investigation by collegues in my lab) (Gelineau and Boujard, 2001; Kamisaka et al., 2001; Kamisaka et al., 2002) and many other hormones that impact appetite (Himick and Peter, 1994; de Pedro et al.,

1995b; de Pedro et al., 1995a; Tritos and Maratos-Flier, 1999; Halford and Blundell, 2000) are likely affecting transgenic salmon as well and must be considered to fully understand appetite regulation in this system.

The absence of significant differences in gene expression within tissues other than the liver and between the full-ration and restricted-ration fish in trial 1, and the transgenic fish fed the 21 MJ/kg and 15 MJ/kg diets in trial 2, may have occured because the differences in dietary energy intake were too small. Most studies that investigate changes in hormone expression and plasma concentrations have analyzed fully-fed and fully-starved groups (Olivereau et al., 1984; Duan and Plisetskaya, 1993; Bjornsson et al., 1994; Hua and Lin, 2001; Uchida et al., 2003; Valente et al., 2003; Pierce et al., 2004). Presently we have obtained brain samples from transgenic and non-transgenic fish under fed and prolonged starvation conditions. Many appetite regulating hormones may not change during the first four weeks of starvation (Silverstein, personal communication), likely due to adaptations to natural periods of low food supply in salmon. The results from this study combined with the analysis of NPY (and potentially other appetite regulating hormones) will be very helpful in determining if there are any other differences in the way that GH transgenic coho salmon regulate growth and appetite control.

4.6 Conclusions

Transgenic coho salmon have greater feed intake and growth than non-transgenic counterparts which were respectively negatively and positively correlated with digestible energy of the diet. Dietary utilization of protein was more efficient in transgenic fish when they were fed diets that contained the 15 and 17 MJ/kg of digestible energy, suggesting that the enhanced growth seen in transgenic coho salmon may be supported on diets in which the digestible energy content is insufficient for growth of wild fish during the winter period when this study was conducted. Although growth was affected by dietary energy concentration, gene expression of the hormones involved in the GH-axis did not change between transgenic fish on the different diets. Plasma IGF-I was greater in transgenic fish but did not correlate with weight as in the non-transgenic coho salmon, possibly due to a reduced ability of the transgenic fish to respond to circulating IGF-I. In the size-matched study, GH expression was highest in transgenic fish, except in the pituitary where there were no differences. GHR expression generally followed that of GH but was tissue specific. IGF-I expression was highest in the transgenic fish and hepatic IGF-I levels in ration-restricted transgenic fish were reduced to that of non-transgenic fish, following the changes in dietary energy intake. There were no conclusive differences between

the size-matched groups in GHRH, SS or CCK expression. Transgenic fish, that had increased growth, feed intake and protein utilization, consumed food to meet an elevated energy demand possibly due to increased GH, GHR and IGF-I with little changes noted to the expression of other GH regulating hormones and the CCK satiety signal.

Although this study did not resolve the underlying physiological basis for the increased feeding motivation of transgenic coho salmon, the research furthered our understanding of energy and nutrient use and growth regulation in these fish in response to both elevated growth hormone and changes in dietary energy and these findings provide a foundation for future studies in this area.

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