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Effect of growth hormone overexpression on gastric evacuation rate in coho salmon

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Abstract Growth hormone (GH) transgenic (T) coho salmon consistently show remarkably enhanced growth associated with increased appetite and food consumption compared to non-transgenic wild-type (NT) coho salmon. To improve understanding of the mechanism by which GH overexpression mediates food intake and digestion in T fish, feed intake and gastric evacuation rate (over 7 days) were measured in size-matched T and NT coho salmon. T fish displayed greatly enhanced feed intake levels (~ 2.5-fold), and more than 3-fold increase in gastric evacuation rates relative to NT coho salmon. Despite the differences in feed intake, no differences were noted in the time taken from first ingestion of food to stomach evacuation between genotypes. These results indicate that enhanced feed intake is coupled with an overall increased processing rate to enhance energy intake by T fish. To further investigate the molecular basis of these responses, we examined the messenger RNA (mRNA) levels of several genes in appetite- and gastric-regulation pathways (*Agrp1*, *Bbs*, *Cart*, *Cck*, *Glp*, *Ghrelin*, *Grp*, *Leptin*, *Mc4r*, *Npy*, and *Pomc*) by qPCR analyses in the brain (hypothalamus, preoptic area) and pituitary, and in peripheral tissues associated with digestion (liver, stomach, intestine, and adipose tissue). Significant increases in mRNA levels were found for *Agrp1* in the preoptic area (POA) of the brain, and *Grp* and *Pomc* in pituitary for T coho salmon relative to NT. *Mch* and *Npy* showed significantly lower mRNA levels than NT fish in all brain tissues examined across all time-points after feeding. *Mc4r* and *Cart* for T showed significantly lower mRNA levels than NT in the POA and hypothalamus, respectively. In the case of peripheral tissues, T fish had lower mRNA levels of *Glp* and *Leptin* than NT fish in the intestine and adipose tissue, respectively. *Grp*, *Cck*, *Bbs*, *Glp*, and *Leptin* in stomach, adipose tissue, and/or intestine showed significant differences across the time-points after feeding, but *Ghrelin* showed no significant difference between T and NT fish in all tested tissues.

Keywords Appetite · Growth hormone · Transgenic · Coho salmon · Stomach · Feed intake · Gastric evacuation rates

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Introduction

Multiple studies have demonstrated noticeable increases in appetite and food intake associated with enhanced growth of growth hormone (GH) transgenic (T) coho salmon relative to non-transgenic (NT) coho salmon

(Devlin 2011; Devlin et al. 2004; Higgs et al. 2009; Kim et al. 2015a; Oakes et al. 2007; Raven et al. 2006; White et al. 2016). GH T fish exhibit highly elevated growth, metabolic rate, and altered feeding behaviour that is often coupled with modified physiological processes due to changes in gene expression, enzyme activities, and peripheral tissue architecture (Devlin 2011; Devlin et al. 2004, 2001; Kim et al. 2015a; Lohmus et al. 2008; Raven et al. 2008). To elucidate the molecular mechanisms that drive this enhanced growth in transgenic fish, both central and peripheral satiety signals were explored (Abernathy et al. 2015; Garcia de la Serrana et al. 2015; Lohmus et al. 2008; Raven et al. 2008). Appetite is regulated by an intricate and complex coordination between the central and peripheral nervous systems. These systems relay information on the energy status of the body through a set of complex endocrine signals. Nutrient uptake is stimulated or inhibited through orexigenic or anorexigenic factors, respectively. Neuropeptide Y (NPY) and agouti-related protein (AGRP) are examples of central orexigenic signals, whereas cocaine and amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC) are examples of central anorexigenic signals (Belgardt et al. 2009; Cornejo et al. 2016; Volkoff 2014; Volkoff et al. 2005). Cholecystokinin (CCK) has an inhibitory effect on gastric emptying in rainbow trout (Olsson et al. 1999), whereas gastrin-releasing protein (GRP) and bombesin (BBS) act as stimulators of stomach mobility in fish (Holmgren and Jönsson 1988; Holmgren and Nilsson 1983). In our previous study, we described that the hyperphagic tendency (chronic compulsive overeating) of T coho salmon is in association with the dysregulation of several neuroendocrine genes in the brain, notably *Agrp1*, *Cart*, and α-melanocyte-stimulating-hormone (*α-Msh*, processed from POMC) (Kim et al. 2015a). Differences in peripheral signals from the gastrointestinal tract, liver, and adipose tissue of T and NT coho have not been well explored at the gene expression level, but gut *Cck* messenger RNA (mRNA) levels have been found to not be affected in T relative NT (Kim et al. 2015a). It was also recently demonstrated that feed intake levels in T and NT salmon responded differently to the treatment with anorexigenic peptides CCK-8, BBS, α-MSH, and glucagon-like peptide-1 (GLP-1), with the action of the latter two ineffective in T animals (White et al. 2016).

Fish growth and appetite regulation by the GI tract are mediated through detection of meal volume, nutrition composition, and gastrointestinal motility (Windell 1978). To improve understanding of nutrition and fish growth in aquaculture, many studies have determined species-specific gastric evacuation rate (GER) (Baker et al. 2014; Basimi and Grove 1985; Bromley 1994; Caruso et al. 2014; Jimenez-Martinez et al. 2012). Thus, to complement our previous study of appetite regulation in the brain and further our understanding of GH transgenic coho salmon as a growth model, the present study examines how GH overexpression affects food intake and GER. Furthermore, we have compared the levels of mRNAs for several neuronal and peripheral appetite-regulatory genes during the process of digestion and gastric evacuation.

Materials and methods

Experimental animals

Experiments were performed between February 24 and March 03, 2015, at the Centre for Aquaculture and Environmental Research (CAER), Fisheries and Oceans Canada (DFO), West Vancouver, Canada. This facility is equipped with containment facilities designed to prevent the escape of genetically modified fish to the natural environment. All experimental procedures were carried out in compliance with the Canadian Council for Animal Care guidelines and were approved by the DFO Pacific Region Animal Care Committee. Two size-matched groups of coho salmon (*Oncorhynchus kisutch*; 160.9 ± 25 g) were examined: (1) Non-transgenic (NT) wild-type coho salmon hatched in February 2013, and (2) GH transgenic (T) coho salmon hatched in March 2014. All fish were derived from the same genetic background (Chehalis River hatchery coho salmon from Fisheries and Oceans Canada Chehalis River Enhancement Facility Agassiz, BC). Transgenic coho salmon (T) contain the OnMTGH1 gene construct (Devlin et al. 1994) in strain M77, produced at the CAER facility (Devlin et al. 2004), and maintained in a wild-type genetic background by crossing T at each generation to NT coho salmon collected from nature. Due to their difference in growth rate, T and NT genotypes became matched in size in February 2015. Both groups of fish were reared as separate

populations under standard conditions (400 fish/4000-L fibreglass tanks, one group of fish per tank, 10 ± 1 °C well water, and natural photoperiod). Fish were fed stage-appropriate commercial salmonid diets (Skretting Ltd., Canada) at fixed times of day (9 a.m. and 2 p.m.) to satiety throughout their lifespan.

Feed intake and gastric evaluation measurements

Prior to experimentation, fish were fasted for a week to ensure the digestive organs were completely empty of food before re-feeding. Feed intake and gastric evacuation were examined at 11 time-points following re-feeding (1, 6, 12, 24, 36, 48, 60, 72, 96, 120, 168 h post feeding, hpf). At 0 hpf, both groups (NT and T) were heavily fed to satiation simultaneously by two persons for 40 min at 8:00 a.m. and not fed for the remainder of the experiment. Immediately after feeding, any uneaten food on the bottom of the tank and drain system was siphoned and collected, and the weight of this uneaten food was recorded to determine the food consumption of each experimental group. One hour after initiation of feeding (i.e. 9 a.m.), approximately 30 fish were transferred from their respective stock tanks and into 170-L experimental tanks. Aquacalm (1 mg/L, Syndel, Syndel Laboratories Ltd., Vancouver, BC, Canada) was used prior to sampling to partially sedate fish in an effort to minimise stress. After selecting fish of desired weighs and length to obtain size-matched fish, 12 fish of each group were rapidly euthanized in buffered tricaine methanesulphonate (200 mg/L; 400 mg/L sodium bicarbonate; Syndel Laboratories Ltd., Vancouver, BC, Canada). On cessation of ventilatory activity, six tissues (whole brain, pituitary, liver, stomach, intestine, and adipose tissue) were team dissected and rapidly collected (within 5 min) and placed into RNAlater (Ambion, Austin, TX, USA) for overnight storage at 4 °C, followed by long-term storage at − 20 °C.

The stomach contents of each fish were collected, weighed (wet weight), immersed in liquid nitrogen, and kept at − 80 °C until dry weight determination. The preoptic area (POA) and hypothalamus (HYP) were dissected from whole brain (fixed in RNAlater) under a dissecting scope following salmon anatomical descriptions and based on the methodology of our previous study (Billard and Peter 1982; Forlano and Cone 2007; Kim et al. 2015a).

RNA extraction and qPCR analysis

Total RNA from tissues was extracted at three time-points (6, 24, 96 hpf) using Qiagen RNeasy Mini kits (Valencia, CA, USA). RNA concentration and purity were measured with a NanoDrop (Thermo Scientific, Wilmington, DE, USA). For qPCR analysis, first-strand complementary DNA (cDNA) was synthesized from total RNA (0.5–1 μg) using High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, Foster City, CA, USA). An initial screening of expression effects was performed from pooled samples where 2 μg of total RNA from six individual fish from each group was combined to create two biological replicates (i.e. two pools of six fish for each group). For genes that showed significant differences between groups (NT and T) and/or time-points in specific tissue, additional qPCR analysis was performed with individual samples. Selection of appetite-related genes for qPCR was based on previous studies (Kim et al. 2015a; Penney and Volkoff 2014). Primers and/or probes used for qPCR are listed in Table 1, and the qPCR analysis was performed as previously described (Kim et al. 2015a, 2016). The primers for Ghrelin and gastrin-releasing peptide (Grp) were designed from the coding sequences of a coho salmon transcriptome (Kim et al. 2016). Levels of mRNA expression were calculated relative to the Ct value obtained for the housekeeping control gene (ubiquitin), which showed stable expression across all tissues (Kim et al. 2016) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

One-way ANOVA followed by Duncan's multiple range tests were used to evaluate differences of stomach contents across the time series and among genotypes (SPSS statistical package, version 18.0, SPSS Inc., Chicago, IL, USA). After detection of outliers using Grubbs' method (https//graphpad.com/quickcalcs/Grubbs1.cfm), two-way ANOVA tests (genotype and time as factors) were run to evaluate differences of mRNA expression level across the time series and among genotypes. If the dataset failed the tests on normality or equal variance (Kolmogorov-Smirnov test and Shapiro-Wilk test), variables were normalized by log transformation or by performing ANOVA on ranks. All results are expressed as the mean ± SEM, and statistical significance was determined at $P < 0.05$.

Table 1 Primers and probes used in this study

Genes	Oligo name	Sequences (5′ → 3′)
Agrp1	Agrp1-RT-F	ACCAGCAGTCCTGTCTGGGTAA
	Agrp1-RT-R	AGTAGCAGATGGAGCCGAACA
	Agrp1-RT-probe	CTGCCCTGCTGCGACCCCTG
Bbs	Bbs-RT-F	CAGAACGGGATGGGAAATCTC
	Bbs-RT-R	TTTTAGAGCGGTTCTCTGTGTCAT
	Bbs-RT-probe	CGCGTTGCAAGCCCAACTCAGA
Cart	Cart-RT-F	AGCATCAGGGTTCGCTCACT
	Cart-RT-R	TGGCAAACAACACTGAAGACAGA
Cck	Cck-RT-F	TCCTCTGAAGCACGTCTTGAAG
	Cck-RT-R	TGGCGGAGCGTGTCTGT
Glp	Glp-RT-F	AGTGGTGCTCCATCCAAACG
	Glp-RT-R	CGCCTGGTCCTGTAGGTAGGT
	Glp-RT-probe	CGATGGGACCTACACCAGCGACGT
Ghrelin	Ghrelin-RT-F	ACTGATGCTGTGTACTCT
	Ghrelin-RT-R	TTGTGTTTGTCTTCCTGGT
Grp	Grp-RT-F	GCTTGCTCCTCTCTCTGCG
	Grp-RT-R	CAAGGCTTTTCTTCCCCATC
Leptin	Leptin-RT-F	TGCTGGAGAACTGGATGATATCA
	Leptin-RT-R	GCCCTCCCTCTCCTGTCTGT
	Leptin-RT-probe	CTGCCCAGGCCGCCAACAGA
Mc4r	Mc4r-RT-F	CTCGCTCTACGTCCACATGTTC
	Mc4r -RT-R	GCAGCACGGCAATCCTCTT
	Mc4r -RT-probe	TGCTGGCCCGCCTGCACA
Mch	Mch-RT-F	GACTCTGGCCTGTGGATGAAC
	Mch-RT-R	GCTGCAGCTCTCAGCTTGTAGA
	Mch-RT-probe	TGAACAGAGGACTTCCT
Npy	Npy-RT-F	CAAGGCAGAGGTATGGGAAGAG
	Npy-RT-R	TCTCCTTTAGCAGCAGTTCTGAGA
	Npy-RT-probe	CCAGCCCTGACACACTGGATTCACTG
Pomc	Pomc-RT-F	ACCCATTGGGCACAAACG
	Pomc-RT-R	GGAGTCCCCCCCTTCCA
	Pomc-RT-probe	CCCTTCCAGACTGGAGGCA
Ubiquitin	Ubiquitin-RT-F	ACAGCTGGCCCAGAAGTACAA
	Ubiquitin-RT-R	GGCGAGCGTAGCATTTGC
	Ubiquitin-RT-probe	TGTGACAAAATGATCTGC

Agrp agouti-related protein, *Bbs* bombesin, *Cart* cocaine and amphetamine-regulated transcript, *Cck* cholecystokinin, *Glp* glucagon-like peptide, *Grp* gastrin-releasing peptide, *Mc4r* melanocortin 4 receptor, *Mch* melanin-concentrating hormone, *Npy* neuropeptide Y, *Pomc* proopiomelanocortin

Results

Gastric evacuation

The dissected stomachs of both coho salmon genotypes sampled at 1 hpf are shown in Fig. 1. In both genotypes, the stomachs of satiated fish are clearly distended. There were no significant differences in the length of the gastric organ from the oesophagus to the distal intestine among both genotypes (not shown). The distension of stomach in T fish was remarkably greater compared to NT fish; food was found even in the oesophagus of most T fish.

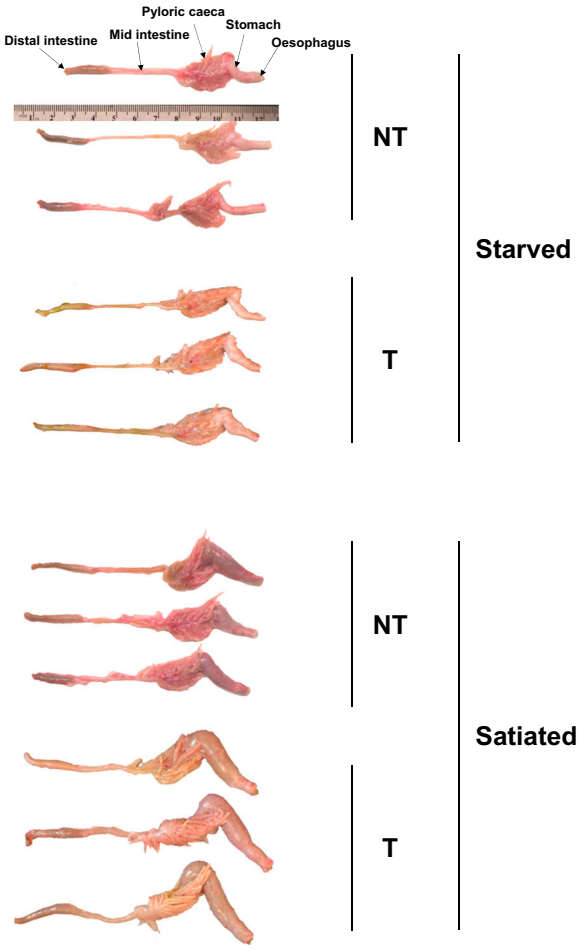


Fig. 1 Comparison of gastric distension on feeding state by dissected alimentary tract between genotypes. NT non-transgenic coho salmon, T transgenic coho salmon

The average amount of wet food in the stomach of both genotypes at each of 11 time-points post feeding (1, 6, 12, 24, 36, 48, 60, 72, 96, 120, 168 h post feeding, hpf) is shown in Fig. 2a. At 1 hpf, NT fish had on average 1.53 g of undigested food in the stomach, whereas T fish had 3.84 g of undigested food in the stomach. Subsequently, the amount of food in the stomach decreased overtime in both genotypes as digestion progressed. Moreover, it appears that the typical biphasic pattern of gastric emptying (lag phase from 1 to 12 h) followed by an emptying phase was evident in NT fish but not in T fish. The rate of stomach emptying in NT fish best fit a logarithmic curve ($Y = -0.925 \times \ln(X) + 4.292$, $R^2 = 0.91$), whereas the rate of stomach emptying in the T fish best fit an exponential curve ($\ln(Y) = -0.059 \times X + 1.595$, $R^2 = 0.97$). The greatest decline in stomach contents for NT fish was noted between 12 hpf (1.26 g) and 24 hpf (0.35 g), which accounted for approximately 70% of the total gastric evacuation. For T fish, the most dramatic change in gastric evacuation was noted between 6 hpf (3.61 g) and 24 hpf (0.98 g), which accounted for approximately 94% of the total gastric evacuation. For some NT fish, undigested food was still noted in the stomach 120 hpf; however, no undigested food remained in the stomach of T fish for longer than 72 hpf (Fig. 2b). The relationships between stomach contents and individual fish size (weight) between 1 hpf and 24 hpf are shown in Fig. 2c. NT fish showed a slightly higher positive correlation for stomach contents and fish size ($R^2 = 0.21$) than T fish ($R^2 = 0.11$). Gastric evacuation rates (GER) of both genotypes are shown in Fig. 2d, with NT fish showing an initial lag up to 6 hpf, whereas T fish showed their peak of GER at this time-point.

qPCR analysis of appetite-regulating transcripts

To examine which appetite-regulating genes are associated with modified gastric emptying of T fish, mRNA levels of central and peripheral appetite-regulatory signals were determined temporally using qPCR. Specifically, three time-points that represented key stages of gastric evacuation were examined (lag phase 6 hpf, gastric emptying 24 hpf, and fasted state 96 hpf). Statistical analyses of all qPCR results by two-way ANOVA are shown in Table 2.

For T fish, *Agrp1* mRNA levels were found to be significantly higher than NT fish in the preoptic area (POA) of the hypothalamus, but not in the remainder of the hypothalamus (HYP) or pituitary (PIT) (Fig. 3a and Table 2). The largest difference (31-fold) in *Agrp1* mRNA levels between T and NT was observed at 6 hpf in POA; however, it was not found to be significantly different from mRNA levels for other time-points examined for this tissue. There was a significant interaction between two factors of genotype and time-point.

Melanin-concentrating hormone (*Mch*) mRNA levels were significantly lower in T than in NT coho salmon in all three brain regions. High variance among the individual samples for these time-points was observed; in particular, 24 hpf NT *Mch* mRNA levels in the POA were on average 179-fold higher than in T fish (Fig. 3b and Table 2). In PIT, an interaction between genotype and time was observed (Fig. 3b and Table 2).

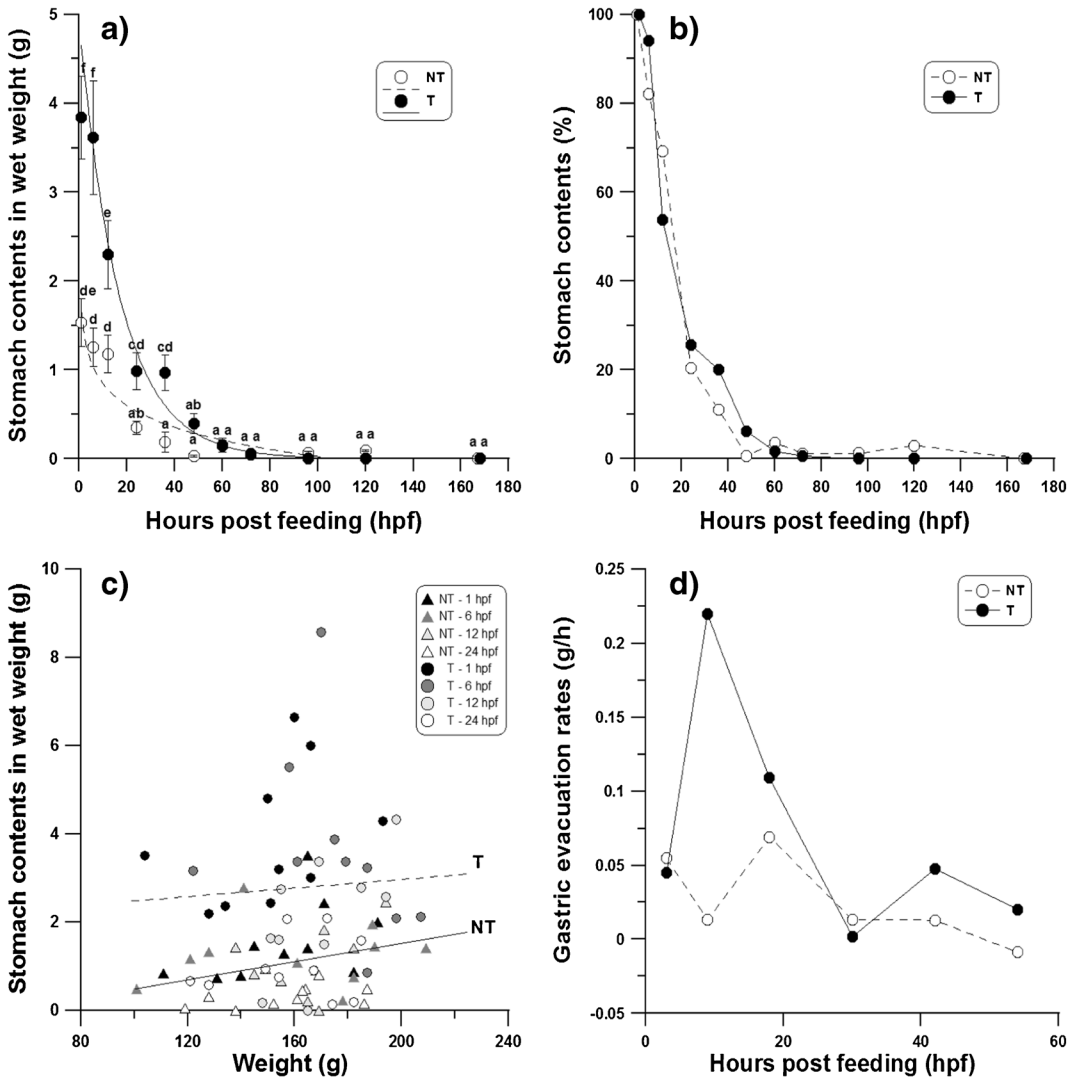


Fig. 2 Stomach contents in non-transgenic (NT) and transgenic (T) coho salmon at times post feeding (hours post feeding, hpf). **a** Wet food amount in stomach. **b** Percentage of stomach contents. **c** Correlation between stomach contents and fish size in two genotypes. **d** Gastric evacuation rates in two genotypes. Indicate what letters mean for stats (lower vs. upper case, are genotypes compared, or just data within genotypes, etc.)

Significantly higher melanocortin 4 receptor (*Mc4r*) mRNA levels in the POA were observed in NT than T salmon; however, there was no difference in *Mc4r* mRNA level between genotypes and among time-points in the HYP. For both genotypes, there was an increase in *Mc4r* mRNA levels in the POA at 96 hpf relative to 6 hpf (Fig. 3c and Table 2).

There were no significant differences found for *Pomc* mRNA levels in the POA when comparing T and NT genotypes, or among time-points. *Pomc* mRNA levels at 6 hpf were significantly higher than other time-points in the HYP tissues. In PIT, significantly elevated levels of *Pomc* mRNA levels were observed between genotypes at all time-points (Fig. 3d and Table 2).

Npy mRNA levels were reduced in T fish in both the POA and HYP tissues relative to NT fish. A significant interaction between genotype and time were noted in HYP tissue. *Npy* mRNA levels in NT fish at 96 hpf in POA were higher in comparison to other time-points (Fig. 3e and Table 2).

Cart mRNA levels in POA were not significantly different between genotypes and time-points. *Cart* mRNA levels in the HYP were significantly higher in

Table 2 A statistical analysis by two-way ANOVA test

Tissue	Gene	Significance level (*P* value)		
		Genotype	Time	Genotype × time
Preoptic area (POA)	*Agrp1*	0.000*	0.492	0.031*
	Mch	0.000	0.512	0.503
	Mc4r	0.032*	0.019*	0.546
	Pomc	0.460	0.761	0.648
	Npy	0.002*	0.002*	0.177
	Cart	0.208	0.068	0.253
Hypothalamus (HYP)	*Agrp1*	0.181	0.075	0.915
	Mch	0.000*	0.539	0.083
	Mc4r	0.530	0.594	0.942
	Pomc	0.407	0.002*	0.201
	Npy	0.000*	0.394	0.032*
	Cart	0.000*	0.747	0.837
Pituitary (PIT)	*Agrp1*	0.279	0.359	0.945
	Mch	0.000*	0.321	0.008*
	Pomc	0.000*	0.129	0.973
	Grp	0.000*	0079	0.731
Stomach	*Grp*	0.275	0.042*	0.923
	Cck	0.804	0.047*	0.665
	Bbs	0.131	0.000*	0.304
	Ghrelin	0.153	0.328	0.914
	Glp	0.670	0.014*	0.297
	Leptin	ND	ND	ND
Intestine	*Cck*	0.968	0.030*	0.429
	Bbs	0.950	0.025*	0.462
	Glp	0.047*	0.060	0.036*
Liver	*Ghrelin*	ND	ND	ND
	Leptin	0.228	0.420	0.898
Adipose tissue	*Ghrelin*	ND	ND	ND
	Leptin	0.000*	0.000*	0.999

Agrp agouti-related protein, *Bbs* bombesin, *Cart* cocaine and amphetamine-regulated transcript, *Cck* cholecystokinin, *Glp* glucagon-like peptide, *Grp* gastrin-releasing peptide, *Mc4r* melanocortin 4 receptor, *Mch* melanin-concentrating hormone, *Npy* neuropeptide Y, *Pomc* proopiomelanocortin, *ND* not detected

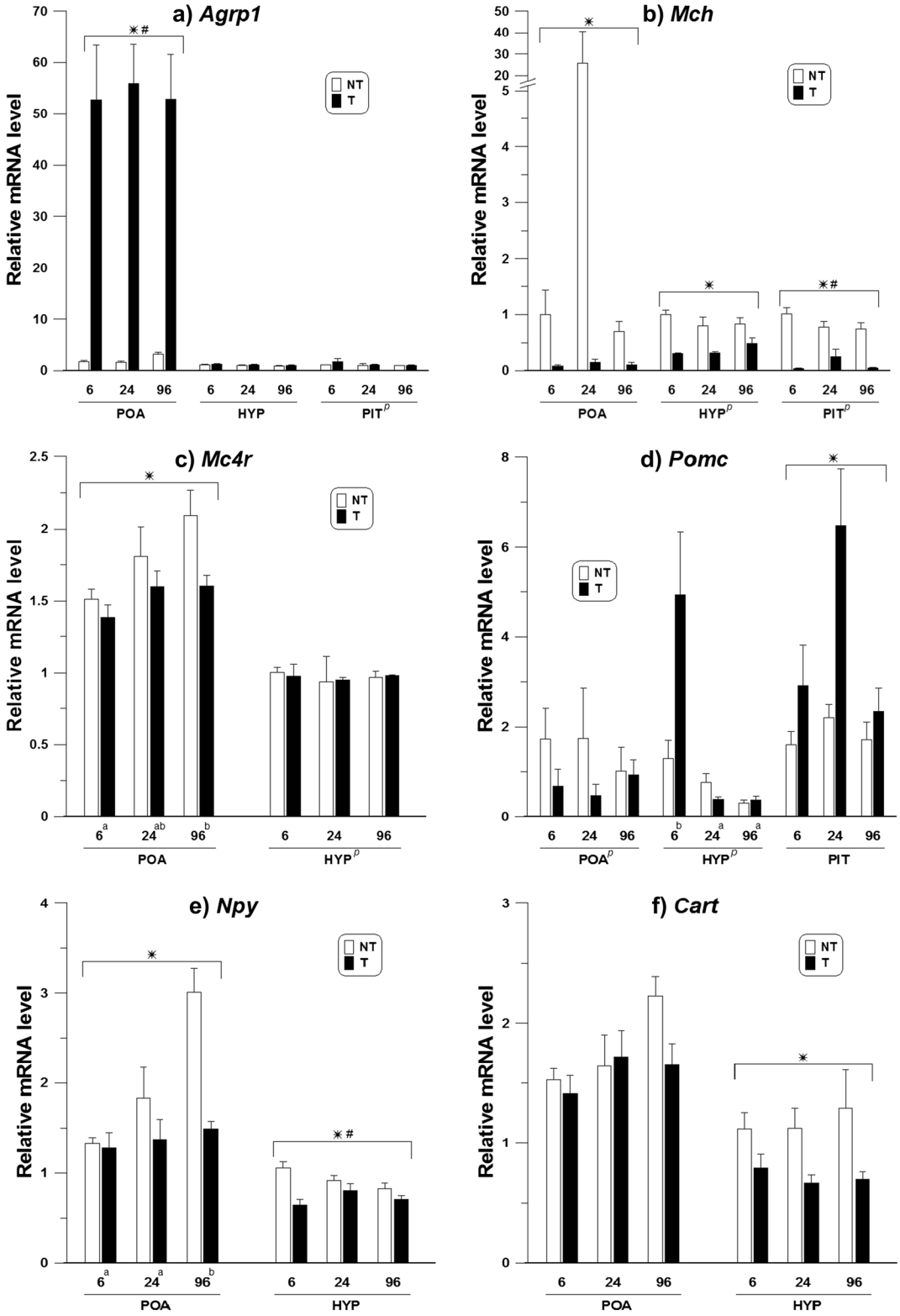
*Significant difference determined by *P* value ($P < 0.05$)

NT fish than in T fish across the time series examined (Fig. 3f and Table 2).

Appetite-regulating gene expression was also examined in several peripheral tissues during gastric emptying. *Grp* mRNA levels in PIT were significantly higher in T fish than in NT fish across the times series. No significant differences in mRNA levels between genotypes were seen in stomach tissue; however, *Grp* mRNA levels overall at 24 hpf were significantly higher than at 96 hpf (Fig. 4a and Table 2).

For *Cck* mRNA levels, no significant differences were noted between genotypes in the intestine and stomach; however, overall mRNA levels of *Cck* differed for 96 hpf compared to 6 and 24 hpf in both tissues (Fig. 4b and Table 2).

In the intestine and stomach, *Bbs* did not show any differences in mRNA levels between genotypes, but


a) Agrp1
b) Mch
c) Mc4r
d) Pomc
e) Npy
f) Cart
Relative mRNA level
NT
T
6
24
96
POA
HYP
PIT
HYP^p
PIT^p
POA^p

◀ **Fig. 3** Quantitative PCR results of appetite-related mRNA levels in brain tissues (POA, HYP, and PIT) at 6, 24, and 96 h post feeding (hpf). All values are means ± SEM and were normalized to the value of NT at 1 hpf. An asterisk (*) at bar chart and a letter at a time-point indicates significant differences ($P < 0.05$) between genotypes and among times within tissues. A number symbol indicates that there is an interrelationship between two factors of genotype and time in the two-way ANOVA test ($P < 0.05$). *P* at a tissue name indicates that qPCR was performed with pooled samples

when compared among time-points, *Bbs* mRNA levels in both genotypes increased at 96 hpf in the intestine, and both genotypes showed significantly higher mRNA levels at 24 hpf in stomach (Fig. 4c and Table 2).

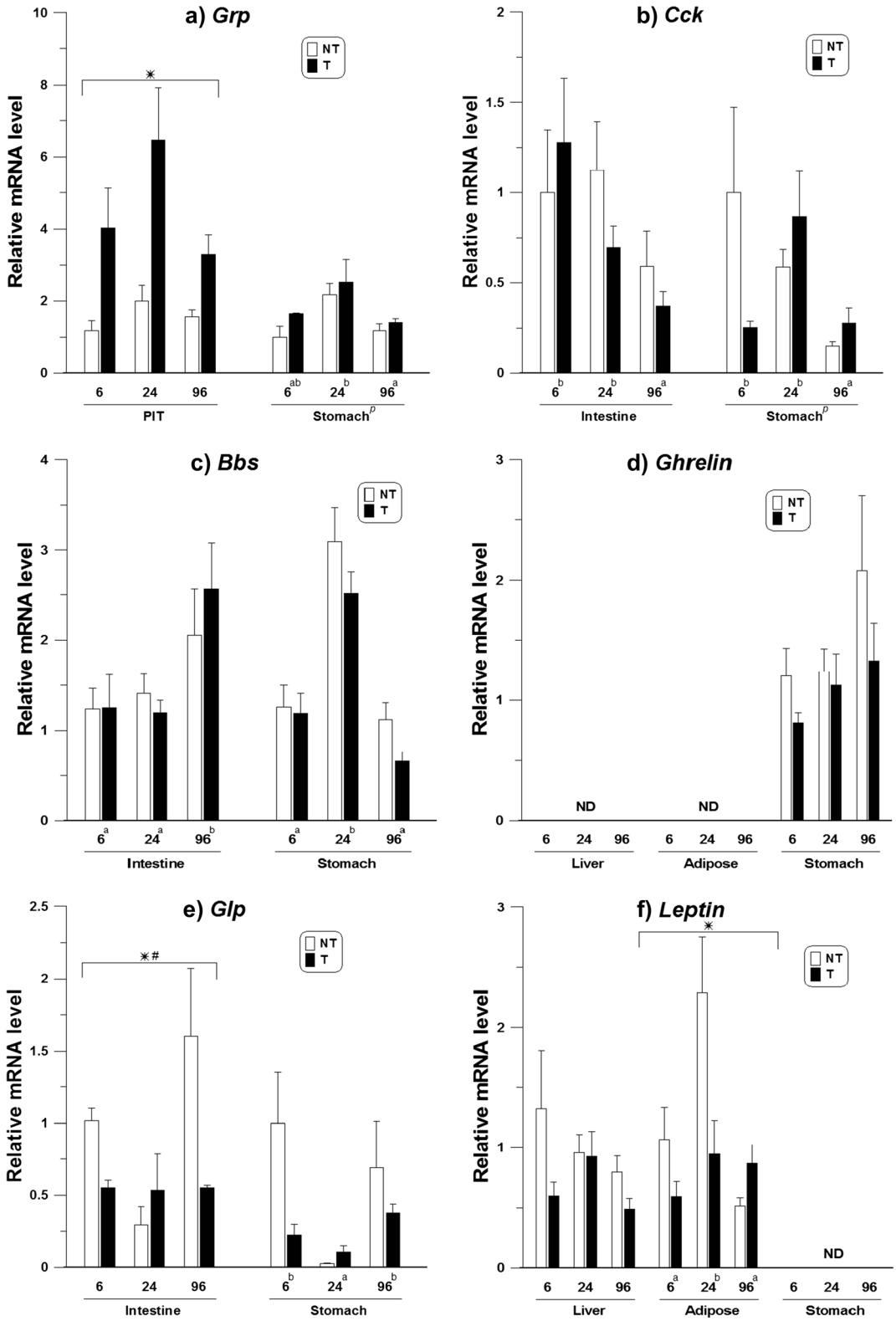
Ghrelin mRNA was not detected in the liver or adipose tissue. *Ghrelin* expression was only found in coho salmon stomach tissue and was not found to differ between genotypes or among time-points (Fig. 4d and Table 2).

Glp mRNA levels in the intestine were significantly lower in T fish than in NT fish with an interaction between genotypes and time-points. *Glp* mRNA levels in the stomach did not significantly differ between genotypes; however, reduced mRNA levels of *Glp* were noted for 24 hpf compared to other time-points in the stomach (Fig. 4e and Table 2).

Leptin mRNA levels were significantly lower in T fish than in NT fish in adipose tissue, and this was most noticeable at 24 hpf. *Leptin* mRNA levels did not differ in the liver between genotypes and time-points, and it was not detected in the stomach tissue (Fig. 4f and Table 2).

Discussion

GH overexpression in fish has been associated with increased appetite and food intake partly due to modification of central appetite-regulating gene expression, including strong elevation of *Agrp1*expression resulting in antagonistic suppression of *Mc4r*-mediated anorexigenic signalling (Kim et al. 2015a; Zhu et al. 2013). However, it is likely that peripheral signals also play an important role, and as such it is hypothesized that the hyperphagic tendency of the transgenic coho salmon may also be partly due to rapid gastric emptying, thus shortening the duration of satiation. In order to assess this hypothesis, gastric evacuation rates (GER) were measured in both GH transgenic (T) and non-transgenic (NT) fish after satiation. Corresponding with previous studies, T fish consumed approximately 3-fold more food than NT fish (Kim et al. 2015a). Interestingly, T fish not only showed higher overall GER than NT fish; the trend of GER over time was also different among genotypes. NT fish displayed a significant difference of GER after 24 h post feeding (hpf) whereas T fish started to evacuate their stomach contents after 12 hpf. Many fish show a lag phase in GER which varies according to temperature and friability of the diet (Basimi and Grove 1985), and indeed, such a delay in GER was observed in NT. However, this typical biphasic pattern (a lag phase followed by gastric emptying) was not observed in T fish indicating they are in a continuous state of digestion and GER. In the present study, since both fish genotypes were examined under the same environmental condition and food composition, the difference in lag phase can be attributed to effects of GH overexpression. Such effects may be due to direct effects of GH and its consequent effects on endocrine and other metabolic signals, as well as secondary effects where elevated intake of food has resulted in modification of the gut to facilitate food processing. The pyloric caeca in the anterior intestine plays an important role as the major post-gastric absorptive surface area (Buddington and Diamond 1987) with provision of enzymatic function (Bishop and Odense 1966; Morrison 1987). In previous studies, T coho salmon were found to have increased intestinal surface area, and longer, more prolific caeca when compared to NT fish, which may be responsible in part for the differences in nutrient absorption between T and NT genotypes (Stevens and Devlin 2000, 2005). This morphological modification of gut size is also seen in GH transgenic Atlantic salmon (Stevens et al. 1999), and was concordant with growth enhancement. Gut enzyme activities, including trypsin and chymotrypsin in pyloric caeca and alkaline phosphatase in the intestine, have also been studied, but only small differences were observed for chymotrypsin (per unit weight of tissue) between T and NT coho salmon (Blier et al. 2002). These data suggest that enhancement of digestion by elevated enzyme activities arises from proliferation of gut tissue rather than stimulation of enzyme activity at the cell level (Stevens and Devlin 2000, 2005). Along with the proliferation of the gut surface, meal size of pelleted diets has been considered as a factor for different GERs, since gastric


a) Grp
b) Cck
c) Bbs
d) Ghrelin
e) Glp
f) Leptin
NT
T
Relative mRNA level
ND
6
24
96
PIT
Stomach
Intestine
Liver
Adipose

◀ **Fig. 4** Quantitative PCR results of appetite-related mRNA levels in peripheral tissues (liver, stomach, intestine, and adipose tissue) at 6, 24, and 96 h post feeding (hpf). All values are means ± SEM and were normalized to the value of NT at 1 hpf. An asterisk (*) at bar chart and a letter at a time-point indicates significant differences ($P < 0.05$) between genotypes and among times within tissues. A number symbol indicates that there is an interrelationship between two factors of genotype and time in the two-way ANOVA test ($P < 0.05$). *P* at a tissue name indicates that qPCR was performed with pooled samples. ND non-detection

evacuation is affected by variation in meal size and peristaltic action (Flowerdew and Grove 1979; Garber 1983; Persson 1981; Ruggerone 1989). Raising coho salmon requires supplying appropriate sizes of feed and ration level for different developmental stages. Growth-enhanced T coho salmon have experienced larger meals than NT all their life, likely enhancing the capability of their guts to process food more rapidly than NT salmon.

Neuropeptide control of feeding behaviour also plays a role in feed intake (Hoskins and Volkoff 2012; Ronnestad et al. 2017; Volkoff 2016; Volkoff et al. 2005) and GER (Morley 1987). Indeed, elevated *Agrp1* mRNA levels in POA were found for T fish relative to NT across all three stages of gastric emptying, consistent with this neuropeptide's role in enhancing appetite by acting as an antagonist for α-MSH stimulation of the melanocortin-4 receptor and appetite suppression. *Agrp* genes have been reported as an anorexigenic factor in several fish such as Atlantic salmon (Murashita et al. 2009), zebrafish (Song et al. 2003), goldfish (Cerdá-Reverter et al. 2003), carp (Wan et al. 2012), cyprinid fish (Wei et al. 2013), pufferfish (Murashita et al. 2009), and sea bass (Agulleiro et al. 2014). Among those, a dramatic increase in hypothalamic *Agrp1* mRNA levels during fasting was shown in zebrafish (Song et al. 2003), goldfish (Cerdá-Reverter et al. 2003), and sea bass (Agulleiro et al. 2014). In the present study, increased *Agrp1* mRNA levels in NT coho salmon were found in the preoptic area (POA) at 96 hpf compared to 6 and 24 hpf. However, there was no significant difference in hypothalamic *Agrp1* mRNA levels across the time-points. These findings are comparable with previous studies (Kim et al. 2015a) where GH transgenic salmon possessed a greater than 10-fold increase in *Agrp1* mRNA than in NT in both the POA and hypothalamus (HYP) of T fish. In comparison, the current study found significant differences in the expression level of *Agrp1* for transgenic salmon only in the POA (31-fold). We are not certain why *Agrp1* was not elevated in the non-POA portion of the hypothalamus in the present study, but differences between studies [current study vs. (Kim et al. 2015a)] may have arisen due to timing of sampling, variance in mRNA level for individual fish, fish size, and technical issues (e.g. dissection of POA from fresh vs. RNAlater-fixed samples, and/or seasonal variation). GH-transgenic common carp showed higher hypothalamic *Agrp1* mRNA levels and increasing feed compared to NT carp (Zhong et al. 2013). AGRP has been shown to play an important role in energy homeostasis correlated with the melanocortin system in teleosts (Ronnestad et al. 2017; Song and Cone 2007).

The present study found that there were differences in *Mc4r* mRNA levels in the POA with NT fish showing higher levels than T fish. This data suggests that increased *Mc4r* mRNA in NT fish may act to chronically decrease appetite, possibly by α-MSH stimulation. There were significant differences in overall *Mc4r* mRNA levels across time-points (6 vs. 96 hpf), whereas, in the previous study, T fish did not show consistent changes in *Mc4r* mRNA levels across tissues at 1 and 4 h post feeding (Kim et al. 2015a), indicating that this system is differently regulated by overexpression of GH in T relative to NT and may reflect long-term regulation of energy homeostasis. Similar findings have been observed in snakeskin gourami; the variation in *Mc4r* mRNA expression in the brain between feeding and fasting periods was suggested to be related to the circadian rhythm (Jangprai et al. 2011). In the present study, there were no significant differences in *Mc4r* mRNA levels in the HYP for both genotypes and for all time-points examined. Similar results were reported in barfin flounder and sea bass where hypothalamic *Mc4r* mRNA was not affected by fasting (Kobayashi et al. 2008; Sánchez et al. 2009). A dose-dependent inhibitory effect of intracerebroventricular injections of the MCR agonist was shown for food intake in rainbow trout (Schjolden et al. 2009) and in goldfish (Cerdá-Reverter et al. 2003), and a stimulating effect of MCR antagonists was also reported for food intake in rainbow trout and in goldfish (Cerdá-Reverter and Peter 2003). This previously reported data implies a complex but critical role for MCR in the regulation of appetite in fish, and corresponds to the time series data examined in the present study.

POMC is synthesized mainly in PIT, and is processed into multiple neuropeptides including α-MSH. However, post-translational processing of the POMC is tissue-specific, and the functions of the multiple types of *Pomc*

genes still remain unclear (Ronnestad et al. 2017). While α-MSH is anorexigenic (acting via MC4R as described above), T coho salmon show higher *Pomc* mRNA levels in PIT than in NT fish, which would be expected to suppress feeding in T, the opposite of what is observed. Thus, GH overexpression may also affect appetite by other pathways. The extremely high levels of *Agrp1* in T may make any small changes in α-MSH and MC4R expression insignificant in terms of regulating appetite (i.e. MC4R signalling may be effectively fully inhibited). Other POMC-derived peptides such as ß-endorphin may also play roles in appetite regulation (Kim et al. 2015a). It is also noteworthy that *Pomc* mRNA levels in HYP showed a time-dependent reduction post feeding, indicating GH plays a role in temporal regulation of neuropeptides post feeding. In agreement with the present study, increased expression levels of *Pomc-a1* and *Pomc-b* in HYP of rainbow trout was seen due by fasting (Jørgensen et al. 2016), and *Pomc-2* mRNA levels increased with fasting in olive flounder (Kang and Kim 2015). Further studies regarding the isoform-specific response to *Pomc* would be beneficial (Ronnestad et al. 2017).

MCH is a neuropeptide mainly synthesized in the PIT but also is found in POA and other regions of the HYP, and it has known roles in pigmentation (Naito et al. 1985) and energy homeostasis (Pissios et al. 2006). The role of MCH related to appetite remains somewhat uncertain. An anorexigenic action was reported in goldfish (Matsuda et al. 2006, 2007), whereas orexigenic role was found in several fish such as Atlantic cod (Boswell and Takeuchi 2005), zebrafish (Berman et al. 2009), barfin flounder (Takahashi et al. 2004), and winter flounder (Tuziak and Volkoff 2012). The present study has found decreased *Mch* mRNA level in T fish compared to NT in all brain tissues (over 100-fold in POA at 24 hpf in POA). Further, *Mch* mRNA levels in T were not found to increase at 24 hpf as is seen in NT. This data suggests that MCH normally has an anorexigenic action in coho salmon, and that GH suppresses this action and normal elevation of *Mch* (as seen in NT), and thereby prevents suppression of feeding. Similar *Mch* mRNA levels between NT and ration-restricted T fish was previously observed (Kim et al. 2015a); this suggests *Mch* levels are primarily influenced by the chronic high feeding rates in fully fed T fish. Indeed, MCH has been implicated as an auxiliary factor in appetite regulation (Amano and Takahashi 2009; Date et al. 2001). In the present study, and that of Kim et al. (2015a), a large amount of variation was noted in *Mch* mRNA levels among individual fish for both genotypes. It would be valuable to examine in more detail to decipher the environmental and physiological factors associated with *Mch* variation among individuals.

NPY is a neuropeptide that is co-expressed in neurons of the POA, and is generally thought to have orexigenic action. However, this and our previous study (Kim et al. 2015a) found higher *Npy* mRNA expression levels in T compared to NT in both POA and HYP tissue contradicting its established role as the most potent orexigenic factors in vertebrates (Kojima et al. 2009). Our results suggest that an inhibitory and possibly compensatory response on NPY can arise from sustained GH overexpression (Kim et al. 2015b). Further studies are needed to fully understand the effect of GH on NPY, and its role in appetite regulation. We note that IGF-I protein and mRNA levels are elevated in T salmon, but only when full rations are provided (Kim et al. 2015a; Raven et al. 2008). T salmon grown on NT rations grow at the NT rate and do not possess elevated IGF-I; however, regardless of ration, T continues to possess dramatically increased feeding behaviour indicating that IGF-I may not play a major role in modulating *Npy* levels in the brain (Raven et al. 2008). The cyclic nature of *Npy* expression is seen in NT salmon, low levels during the lag phase, followed by a rise in *Npy* level as the gut empties to transmit signals for refeeding. The temporary reduction of *Npy* expression in the lag phase signifies satiation. However, this cyclical regulation of *Npy* mRNA expression was not noted in T coho salmon (present study and Raven et al. 2008) and T carp (Zhong et al. 2013); perhaps, GH overexpression is driving a heightened appetite condition that prevents increases in *Npy* expression.

CART is an anorexigenic peptide expressed in both PIT and POA. No effect of GH on *Cart* expression was seen in POA; however, T salmon possessed reduced *Cart* mRNA in HYP relative to NT, particularly at 96 hpf. Thus, chronic expression of GH can inhibit the anorexigenic actions of CART, resulting in promotion/stimulation of appetite in T salmon. T salmon do not display a change in *Cart* mRNA with time post feeding, indicating that a consistent effect of GH overexpression on *Cart* expression is independent on the degree of gastric emptying and unaffected by the normal controls that modulate *Cart* expression in NT salmon.

Gastric fullness is also regulated by multiple endocrine signals originating at the gut and other levels.

Gastrin-releasing peptide (GRP) is a neuropeptide which plays a major role in stimulating the release of gastrin from G cells of the stomach (Xu and Volkoff 2009). *Grp* mRNA is widely distributed, including brain, skin, gastrointestinal tract, gonad, and gill in goldfish (Volkoff et al. 2000) and rainbow trout (Jensen and Conlon 1992), and it is also known that GRP connects the gut and brain (Merali et al. 1999). *Grp* mRNA showed significant increases in T fish when compared to NT fish, but only in PIT and not in the stomach. In salmon, if PIT-derived GRP is playing a major role in gastric emptying (relative to stomach), then the elevated levels in T salmon relative to NT would be consistent with it being responsible for elevated GER in animals overexpressing GH. In cod, fish fed higher rations displayed higher *Grp* mRNA expression in the gut than smaller rations (Xu and Volkoff 2009), and in the present study, increased *Grp* mRNA is seen at 24 hpf both in PIT and stomach and in both genotypes, indicating that *Grp* mRNA expression is responsive to feeding and the level of gastric fullness. The difference in GER between T and NT fish during the lag phase may be due to elevated *Grp* mRNA levels in the PIT and may promote gastric acid secretion and enteric motor function via the vagus nerve (Debas and Carvajal 1994), thus enhancing gastric emptying.

There were few other significant differences found in peripheral tissues for any of the other appetite-related genes examined between genotypes. Intestinal *Cck* mRNA levels decreased with time in both genotypes post feeding, but did not differ between genotypes, consistent with previous studies (Raven et al. 2008). Reduced *Cck* mRNA levels correlated with the degree of gastric emptying and is consistent with its role as an appetite suppressor, but does not appear to be influenced by GH overexpression. Similarly, in both genotypes, increased *Bbs* mRNA levels were seen at 24 hpf in the stomach and at 96 hpf in the intestine, consistent with its anorexigenic role in the longer term and its insensitivity to GH levels. *Ghrelin* mRNA tends to be lower in T than NT in the stomach, consistent with feedback control arising from GH levels and degree of gastric fullness, but these differences were not significant.

In fish, GLP is mainly produced in the intestine and pancreas (Plisetskaya and Mommsen 1996), and GLP-1 has been known as an anorexigenic factor (Schroeter et al. 2015; Silverstein et al. 2001). Peripheral injection of GLP-1 strongly inhibits feed intake in coho salmon (White et al. 2016), indicating an anorexigenic action of GLP-1. An inhibiting role of GLP-1 for gastric emptying has been found in mammals (Imeryüz et al. 1997), whereas the function of GLP-1 on gastric evacuation in fish remains largely unknown (Ronnestad et al. 2017). In the present study, higher *Glp* mRNA levels were seen for NT relative to T, and different time-dependant expression patterns were found such that NT *Glp* decreased at 24 hpf in both intestine and stomach, whereas such an effect was not seen for T fish in the intestine. These data suggest GLP is differently regulated by overexpression of GH.

In fish, leptin is mainly secreted in the liver (Kurokawa and Murashita 2009; Rønnestad et al. 2010) with minor secretion from adipose tissue (Gong et al. 2013; Salmerón et al. 2015). No effect on hepatic *Leptin* mRNA levels was seen between genotypes and across time-points, except for 6 hpf. Variable responses to leptin expression have been reported, and reduced hepatic *Leptin* mRNA levels were reported during catabolic states in striped bass (Won et al. 2012); in contrast, no correlation between *Leptin* mRNA expression and feeding status was found in eel liver (Morini et al. 2015). Moreover, expression patterns of *Leptin* in adipose tissue appear to vary widely among species and show species-specific modulation to feeding status, such as mandarin fish (Yuan et al. 2016), Nile tilapia (Shpilman et al. 2014), eel (Morini et al. 2015), and salmonids (Angotzi et al. 2013; Fuentes et al. 2012; Jørgensen et al. 2013; Kling et al. 2009; Rønnestad et al. 2010). In the current study, adipose tissue *Leptin* mRNA levels increased at 24 hpf only in NT, suggesting that the role of leptin in satiation signalling is dysregulated in T fish.

Kim et al. (2015a) previously demonstrated multiple and complex responses in expression of appetite-regulating genes in the brain and pituitary in T coho salmon compared to NT salmon. The present study has extended these observations to include expression of appetite-regulating genes in peripheral tissues, and a similarly complex response has been observed. A time course of gastric evacuation point of views, *Agrp1*, *Mch*, and *Cart*, showed consistent expression patterns for their orexigenic and anorexigenic roles in appetite regulation (see summary in Fig. 5). *Grp* showed higher expression in T compared to NT fish during gastric emptying, indicating these genes may contribute to the strongly elevated gastric evacuation seen in T fish (Fig. 5). In T fish, overexpression of GH stimulated appetite-related genes including *Agrp1* in POA and

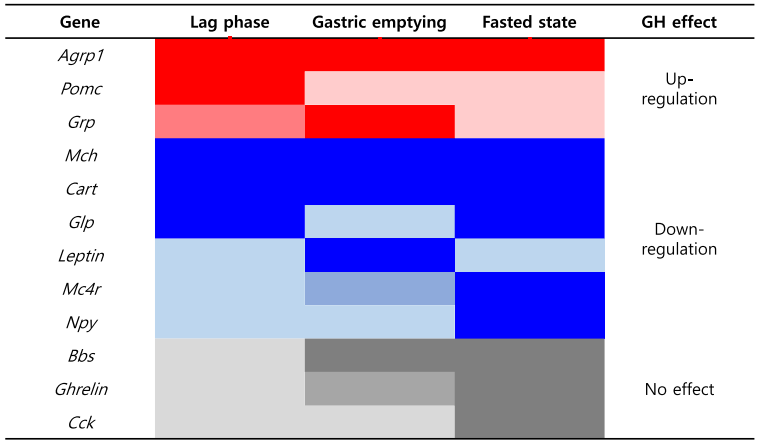


Fig. 5 Heatmap of appetite-related mRNA expressions of T coho salmon according to a time course of the gastric evacuation process. A red and a blue indicate higher and lower mRNA levels of T fish comparing the levels of NT fish, respectively. Each dark and light colour refers to increase and decrease of mRNA expression, respectively

Grp in PIT, or inhibited *Glp* in intestine and *Leptin* in adipose tissue, likely mediating the higher gastric evacuation rates seen in T (Fig. 6). Gastric evacuation is normally maintained by a balance between appetite regulation and release of gastric enzymes causing digestion; however, previous studies of gastric enzymes have only found small differences between T and NT (Blier et al. 2002). Further investigations are required to fully understand digestive processes in T fish, including which digestive enzymes are associated with rapid

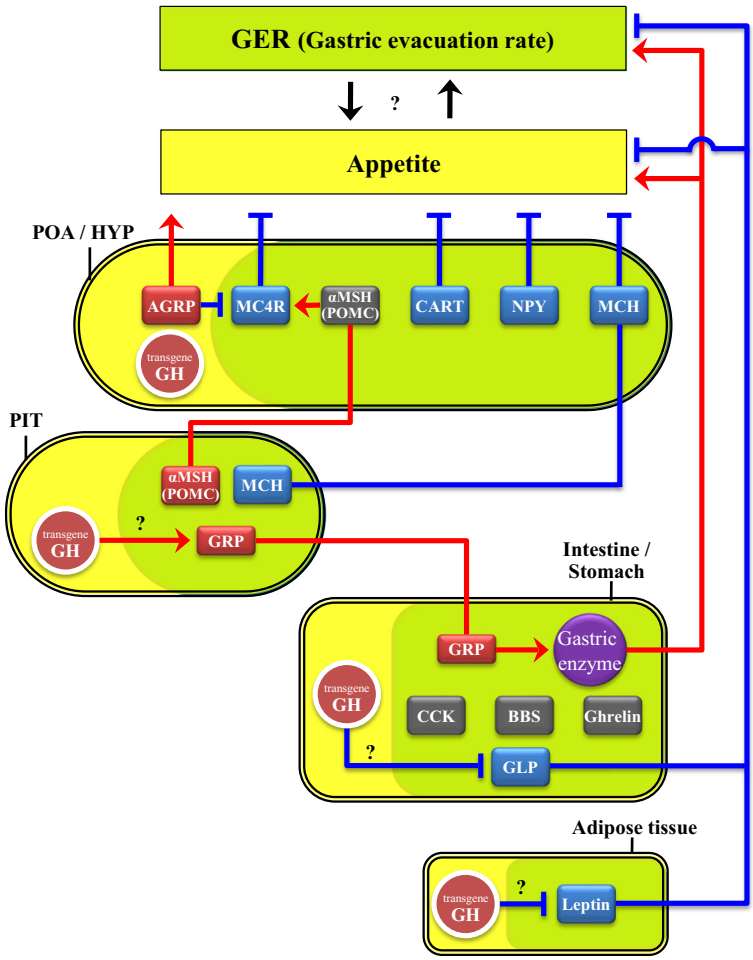


Fig. 6 Regulation of appetite-related genes and gastric evacuation-related genes mediated by overexpressed growth hormone. Genes and proteins/peptides that are increased in GH transgenic fish are shown in red, those that are decreased are shown in blue, and those that are unaffected by transgenesis are shown in grey. Red arrows indicate stimulation, and blue blunt-end lines indicate suppression. Data and pathway for regulation of appetite-related genes from Kim et al. (2015a)

gastric emptying, and whether there are significant differences in peristaltic and other mechanical actions of the digestive apparatus stimulated directly by GH and/or indirectly by the elevated feed intake observed in these animals.

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