

# Digestion of a single meal affects gene expression of ion and ammonia transporters and glutamine synthetase activity in the gastrointestinal tract of freshwater rainbow trout

Carol Bucking · Chris M. Wood

Received: 25 July 2011 / Revised: 23 September 2011 / Accepted: 27 September 2011 / Published online: 13 October 2011  
© Springer-Verlag 2011

**Abstract** Experiments on freshwater rainbow trout, *Oncorhynchus mykiss*, demonstrated how digestion affected the transcriptional expression of gastrointestinal transporters following a single satiating meal (~3% body mass ration) after a 1-week fast. Quantitative real-time polymerase chain reaction was employed to measure the relative mRNA expression of three previously cloned and sequenced transporters [ $\text{H}^+\text{-K}^+\text{-ATPase}$  (HKA),  $\text{Na}^+\text{/HCO}_3^-$  cotransporter (NBC), and the Rhesus glycoprotein (Rhbg1; an ammonia transporter)] over a 24-h time course following feeding. Plasma total ammonia increased about threefold from pre-feeding levels to  $288 \mu\text{mol l}^{-1}$ , whereas total ammonia levels in chyme supernatant reached a sixfold higher value ( $1.8 \text{ mmol l}^{-1}$ ) than plasma levels. Feeding did not appear to have a statistically significant effect on the relative mRNA expression of the gastric HKA or Rhbg1. However, the relative mRNA expression of gastric NBC was increased 24 h following the ingestion of a meal. Along the intestinal tract, feeding increased the relative mRNA expression of Rhbg1, but had no effect on the expression of NBC. Expression of the gastric HKA was undetectable in the intestinal tract of freshwater rainbow trout. Digestion increased the activity of glutamine synthetase in the posterior intestine at 12 and 24 h following feeding. This study is among the first to show that there are

digestion-associated changes in gene expression and enzyme activity in the gastrointestinal tract of teleost fish illustrating the dynamic plasticity of this organ. These post-prandial changes occur over the relative short-term duration of digesting a single meal.

**Keywords** Ammonia · Rhesus glycoprotein · Glutamine synthetase · Proton pump · NBC

## Introduction

The digestion of a typical meal has been shown to affect plasma ammonia concentrations in fish (Kaushik and Teles 1985; Wood 1993; Gelineau et al. 1998; Wicks and Randall 2002; Bucking and Wood 2008; Zimmer et al. 2010), doubling or even tripling concentrations beyond unfed levels, with even greater increases in the hepatic portal vein draining the intestinal circulation (Karlsson et al. 2006). The excess ammonia is generated by the catalysis of dietary proteins. This generates an amino acid surplus, which in fish are deaminated and used for energy (Ballantyne 2001; Stone et al. 2003). The plasma ammonia surplus results in an increased ammonia excretion to the environment (e.g. Handy and Poxton 1993; reviewed by Wood 2001; Bucking and Wood 2008; Zimmer et al. 2010) through branchial (Beamish and Thomas 1984), and to some extent renal (Bucking et al. 2010), excretion. In fact, ammonia excretion to the water is proportional to meal ration size (Alsop and Wood 1997).

While the exact mechanism(s) of ammonia excretion are still somewhat controversial, the recent discovery of a group of proposed ammonia transporters (Rhesus glycoproteins; Rh proteins) in aquatic animals (e.g. Weihrach et al. 2009; Nakada et al. 2007; Nawata et al. 2007, 2010;

---

Communicated by I.D. Hume.

C. Bucking · C. M. Wood  
McMaster University, 1280 Main Street, West Hamilton,  
ON L8S 4K1, Canada

C. Bucking (✉)  
University of Ottawa, 30 Marie Curie PVT, Ottawa,  
ON K1N 6N5, Canada  
e-mail: carolbucking@gmail.com

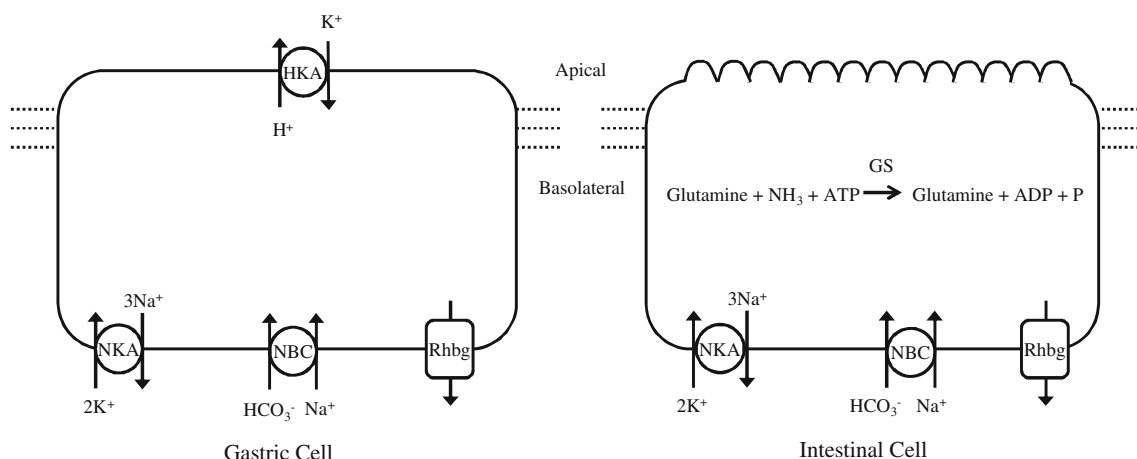
Tsui et al. 2009) has cast new light on the situation (reviewed by Wright and Wood 2009; Weihrauch et al. 2009). Briefly, Rh proteins form channels through which ammonia can be transported (reviewed by Wright and Wood 2009). There are several isoforms of Rh proteins: Rhag is typically associated with red blood cells, Rhbg is thought to be a basolateral channel and Rhcg an apical channel (e.g. Wright and Wood 2009; Nakada et al. 2007). Regulation of branchial Rh gene expression in response to various factors (high external ammonia, Nawata et al. 2007; Hung et al. 2007; elevated internal ammonia by infusion, Nawata and Wood 2009; feeding, Zimmer et al. 2010; or by blockade of ammonia excretion by water-buffering, Nawata and Wood 2008) has been found. To date, study of Rh genes has focused on the gill, the primary location of Rhcg1 and 2 expression (Nawata et al. 2007). However, Rhbg has been found in most tissues of the freshwater rainbow trout (blood, brain, eye, gill, heart, intestine, kidney, liver, muscle, skin, spleen) and was found to be responsive to high external ammonia (Nawata et al. 2007). Thus, the increase in plasma ammonia following feeding may also potentially trigger changes in the expression of Rhbg in these other tissues.

Digestion not only alters ammonia metabolism, but also acid–base balance (Wood et al. 2005, 2007a, b; Bucking and Wood 2008; Bucking et al. 2009, 2010) through the generation of an alkaline tide (reviewed by Hersey and Sachs 1995; Niv and Fraser 2002). Briefly, the secretion of HCl (acid) into the stomach lumen ultimately results in the secretion of  $\text{HCO}_3^-$  (base) into the blood generating a systemic metabolic alkalosis—the alkaline tide. Acid secretion in the stomach is facilitated by the gastric  $\text{H}^+$ – $\text{K}^+$ -ATPase (HKA) located in the apical membrane of the acid-secreting cell. This enzyme is a member of the P-type ATPase family, which includes  $\text{Na}^+$ – $\text{K}^+$ -ATPase,  $\text{Ca}^{2+}$ -

ATPase and colonic  $\text{H}^+$ – $\text{K}^+$ -ATPase (MacLennan et al. 1985; Shull et al. 1985; Crowson and Shull 1992), and as such shares structural and enzymatic identity with other members of the family (Hersey and Sachs 1995; Munson et al. 2000). The effect of feeding on the expression of HKA is not known in fish.

The metabolic base load generated by the activity of the HKA in freshwater rainbow trout is corrected mostly through increased base excretion at the gill (Bucking and Wood 2008) while the kidney appears to relieve a small portion of the metabolic alkalosis (Bucking et al. 2010). A potential additional route of base excretion is the intestine. Recently, studies have suggested that entry of  $\text{HCO}_3^-$  across the basolateral membrane of gulf toadfish (*Opsanus beta*) intestinal tissue is regulated by the basolateral  $\text{Na}^+$ / $\text{HCO}_3^-$  cotransporter (NBC; Grosell and Genz 2006; Kurita et al. 2008; Taylor et al. 2010). The increases in plasma  $[\text{HCO}_3^-]$  following feeding may hence trigger an increase in basolateral NBC expression in the intestine. Together with branchial base excretion, intestinal  $\text{HCO}_3^-$  secretion would act to relieve metabolic alkalosis. In fact, recent evidence suggests that the intestine indeed plays a role in base excretion of the alkaline tide in seawater-acclimated rainbow trout but this has not been shown in freshwater fish (Bucking et al. 2009).

The mRNA expression levels of the HKA, NBC and Rhbg1 in each section of the gastrointestinal (GI) tract were examined in freshwater rainbow trout before, and at various time points up to 24 h following feeding using quantitative real-time polymerase chain reaction (qRT-PCR). The putative cellular locations of each transporter examined in both the gastric and intestinal cells are shown in Fig. 1. The expression of HKA was expected to increase, in accord with previous evidence for an increase in gastric acid secretion during digestion of the meal



**Fig. 1** Putative cellular locations of the HKA, Rhbg and NBC transporters in both gastric and intestinal cells as well as role of glutamine synthetase (GS) in detoxifying ammonia

(Bucking and Wood 2009). mRNA expression levels of NBC in various parts of the intestine were not expected to change due to the lack of evidence of intestinal involvement in relieving the alkaline tide (Bucking et al. 2009). Finally, *Rhbg1* expression was expected to decrease to prevent excess ammonia entering the bloodstream from the chyme. Intestinal glutamine synthetase activity was also measured as it aids in converting ammonia into glutamine and potentially into urea, through the use of glutamine in the ornithine-urea cycle, both of which are much less toxic nitrogen products. We hypothesized that increases in enzyme activity would correlate with changes in relative gene expression of ammonia transporters.

Based on the evidence presented above, that the digestion of a meal results in transient changes in overall ammonia metabolism and acid/base balance in fish, the aim of the current study was to examine the relatively immediate effect of digesting a meal on gene expression of several transporters, as well as enzyme activity, related to these functions along the GI tract.

## Materials and methods

### Animals

Freshwater rainbow trout (*Oncorhynchus mykiss*; 234–313 g), obtained from Humber Springs Trout Hatchery (ON, Canada) were used for all experiments. The fish were acclimated to laboratory conditions and water (dechlorinated Hamilton tap water;  $\text{Na}^+ = 0.6$ ,  $\text{K}^+ = 0.05$ ,  $\text{Cl}^- = 0.8$ ,  $\text{Ca}^{2+} = 0.8$ ,  $\text{Mg}^{2+} = 0.3 \text{ mmol l}^{-1}$ ; pH = 8.0; hardness =  $140 \text{ mg l}^{-1}$  as  $\text{CaCO}_3$  equivalents; temperature 11–13°C) and were fed every other day at a consistent time with commercial fish feed (Martin Mills, ON, Canada) for a 6 week period to synchronize feeding-associated processes.

### Sampling

Following the 6-week acclimation and feeding schedule, the fish were fasted for 1 week to remove any undigested food from the GI tract. Fish were then sampled 3 h before the scheduled feeding (–3 h), fed to satiation (~3% body mass ration) and sampled at 6, 12 and 24 h after feeding. During sampling, fish were rapidly sacrificed by cephalic concussion, and a blood sample obtained through caudal puncture with an iced pre-heparinized #22 gauge needle. The plasma was immediately separated from the red blood cells through centrifugation (13,000g, 30 s) and placed in liquid nitrogen for further analysis of plasma ammonia levels. The GI tract was then exposed through a mid-line incision from mouth to anus. Chyme was extracted from

each section (when present) and centrifuged (13,000g, 60 s) to obtain the supernatant fraction which was also placed in liquid nitrogen for further analysis of total ammonia concentrations. A tissue sample of each section of the tract (stomach, anterior intestine including caeca, mid-intestine, and posterior intestine) was then obtained, with a conscious effort to collect samples from the same general area of the tissue at each time point. The tissue samples were immediately placed in liquid nitrogen for later quantification of mRNA expression. All fish sampled had evidence of recently consuming a meal.

The remaining fish were placed back on the original feeding schedule for 3 weeks, before being fasted again for 1 week and subsequently fed to satiation as before. Trout were then sacrificed in the same manner as above following the same sampling schedule. The GI tract was exposed as before and each GI tract section identified and sampled as above to determine tissue glutamine synthetase activity.

### Analysis

Plasma and chyme samples were analyzed for total ammonia ( $T_{\text{amm}}$ ;  $\mu\text{mol l}^{-1}$ ) by an enzymatic assay based on the glutamate dehydrogenase/NAD method using a commercial kit (Raichem; San Diego, CA, USA). Samples were analyzed on a microplate reader (SpectraMax 340PC) at 590 nm. Both plasma and chyme samples were deproteinized using 4% perchloric acid and neutralized using KOH prior to measuring ammonia.

Total RNA was extracted from ~15 mg of each GI tract tissue sample using TRIzol (Invitrogen, Burlington, ON, Canada) and quantified spectrophotometrically. The integrity of the RNA was verified through electrophoresis on 1% agarose gel stained with ethidium bromide. First strand cDNA was subsequently synthesized from 1  $\mu\text{g}$  total RNA using an oligo(dT17) primer and Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was then performed using the cDNA synthesized above and primers listed in Table 1. Reactions (20  $\mu\text{l}$ ) containing 4  $\mu\text{l}$  of DNaseI-treated (Invitrogen) cDNA, 4 pmol of each primer, 10  $\mu\text{l}$  of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), and 0.8  $\mu\text{l}$  of ROX (1:10 dilution) were performed at 50°C (2 min), 95°C (2 min), followed by 40 cycles of 95°C (15 s) and 60°C (30 s) using a Mx3000P QPCR System (Stratagene, Cedar Creek, TX). Melt-curve analysis confirmed production of a unique product. Relative expression of mRNA was determined using  $\beta$ -actin as a reference gene in the stomach tissue, while 18s rRNA was used for intestinal tissues, employing the  $\Delta\Delta C_t$  method (Livak and Schmittgen 2001).

Glutamine synthetase (GS) activity was determined according to Webb and Brown (1980). Briefly, samples of intestine were homogenized (using a Brinkman Polytron

**Table 1** Primers used for quantitative real-time PCR

Gene	Sequence 5'–3'	
	Forward primer	Reverse primer
HKA (Sugiura et al. 2006)	GCC ACT GAC ATT TTT CCC TCT G	TTG CGC CAA TCT GGA AGT AGG
NBC1 (Grosell et al. 2007)	TGG ACC TGT TCT GGG TAG CAA	AGC ACT GGG TCT CCA TCT TCA G
Rhbg1 (Nawata et al. 2007)	CGA CAA CGA CTT TTA CTA CCG C	GAC GAA GCC CTG CAT GAG AG
18s (Grosell et al. 2007)	TCT CGA TTC TGT GGG TGG T	CTC AAT CTC GTG TGG CTG A
$\beta$ -Actin (Nawata et al. 2007)	ACT GGG ACG ACA TGG AGA AG	AGG CGT ATA GGG ACA ACA CG

HKA  $H^+-K^+$ -ATPase, NBC  $Na^+/HCO_3^-$  cotransporter 1, Rhbg1 Rhesus glycoprotein bg

homogenizer) in five volumes (w/v) of ice-cold homogenization buffer containing 20 mmol  $l^{-1}$   $K_2HPO_4$ , 10 mmol  $l^{-1}$  HEPES, 0.5 mmol  $l^{-1}$  EDTA, 1 mmol  $l^{-1}$  dithiothreitol, 50% glycerol adjusted with NaOH to pH 7.5 at 24°C. The homogenates were centrifuged at 10,000g and 4°C for 20 min. After centrifugation, the resulting supernatant was used for the determination of GS activities. The GS activity was expressed as  $\mu\text{mol } \gamma\text{-glutamyl hydroxamate formed min}^{-1} \text{g}^{-1}$  tissue. Freshly prepared glutamic acid monohydroxamate solution was used as a standard for comparison.

#### Statistics

Changes in plasma and chyme ammonia concentrations ( $T_{\text{amm}}$ ), mRNA expression, and intestinal GS activity over time were examined using a one-way ANOVA followed by a Tukey's post hoc test (SPSS 12). All values have been presented as mean  $\pm$  SEM ( $N = 6$ ).

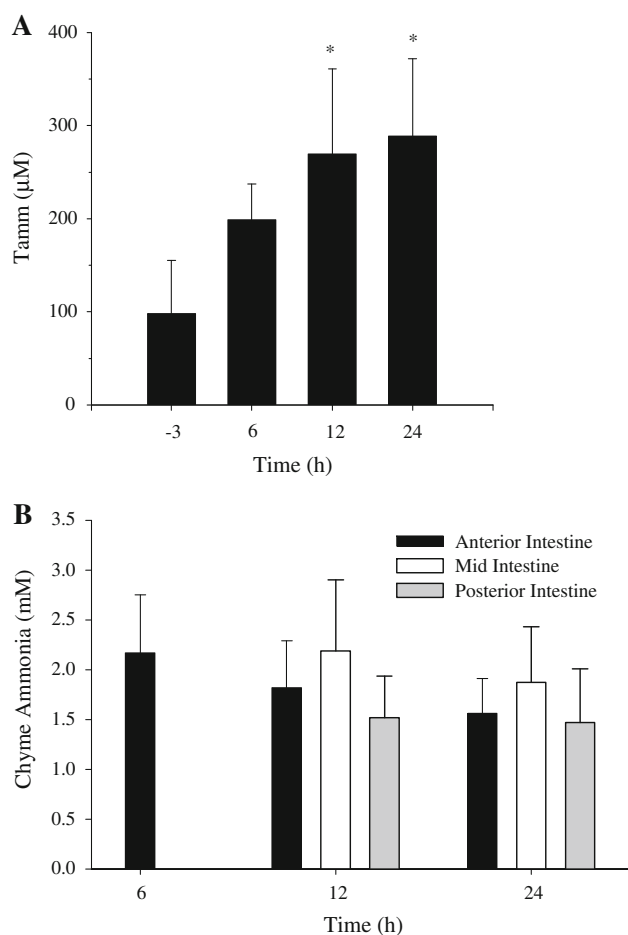
## Results

### Plasma and chyme composition

Plasma  $T_{\text{amm}}$  increased approximately threefold following feeding, with the highest measured value at 24 h post-feeding ( $288 \pm 83 \mu\text{mol } l^{-1}$ ; Fig. 2a). In contrast,  $T_{\text{amm}}$  in the supernatant of chyme was similar in all sections of the gastrointestinal tract and was unchanged with time (Fig. 2b; excluding the mid and posterior intestine at 6 h where no chyme was present). The average  $T_{\text{amm}}$  in the supernatant of chyme found along the entire gastrointestinal tract was  $1.8 \pm 0.6 \text{ mmol } l^{-1}$  ( $N = 18$ ), over sixfold higher than concentrations found in the plasma.

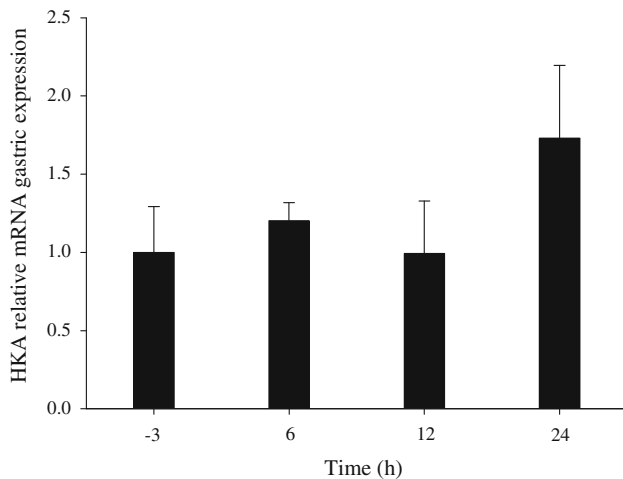
### Relative mRNA expression

The relative mRNA expression of HKA in the stomach of rainbow trout was unaffected by feeding (Fig. 3).



**Fig. 2 a** Total ammonia plasma concentrations ( $T_{\text{amm}}$ ;  $\mu\text{mol } l^{-1}$ ) in response to feeding. Feeding occurred at 0 h. Values are mean  $\pm$  SEM ( $N = 6$ ). Asterisk indicate a significant increase from control values ( $-3$  h = pre-feeding). **b** Total ammonia chyme supernatant concentrations ( $\text{mmol } l^{-1}$ ) following feeding (0 h). Values are mean  $\pm$  SEM ( $N = 6$ )

In contrast, the gastric expression of NBC1 mRNA was increased post-feeding, rising fourfold at 24 h following the ingestion of a meal (Fig. 4a). The relative expression of Rhbg1 mRNA in the stomach of rainbow trout was unaffected by feeding (Fig. 5a).

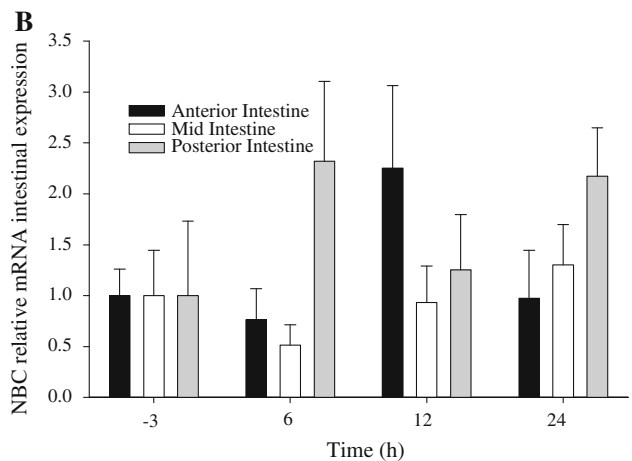
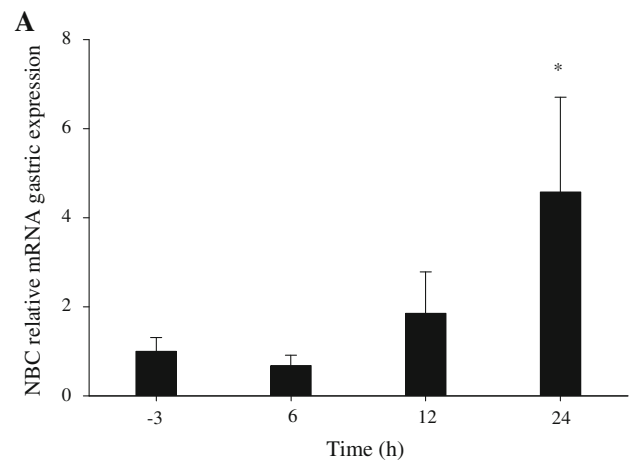


**Fig. 3** The effect of feeding on the relative mRNA expression of  $H^+-K^+$ -ATPase (*HKA*) in the stomach of the freshwater rainbow trout.  $\beta$ -actin was used as the reference gene. Feeding occurred at 0 h (-3 h = pre-feeding). Values are mean  $\pm$  SEM ( $N = 6$ ). There were no significant differences

The expression of *HKA* mRNA was below detectable levels in the intestinal tract of freshwater rainbow trout. The digestion of a meal had no effect on the relative expression of *NBC1* mRNA along the intestinal sections (Fig. 4b), in contrast to the increased expression observed in the stomach (Fig. 4a). Additionally, while digestion had no effect on gastric mRNA expression of *Rhbg1*, the relative expression of *Rhbg1* mRNA increased in the anterior intestine fivefold over unfed control values at 12 h post-feeding (Fig. 5b). Digestion of a meal also increased the relative expression of *Rhbg1* in the posterior intestine, rising sixfold over control values at 6 h following feeding (Fig. 5b). The relative expression remained fourfold elevated over control values in the posterior intestine up to 24 h (Fig. 5b). In contrast, *Rhbg1* expression in the mid-intestine did not exhibit a postprandial change.

**Enzyme activity**

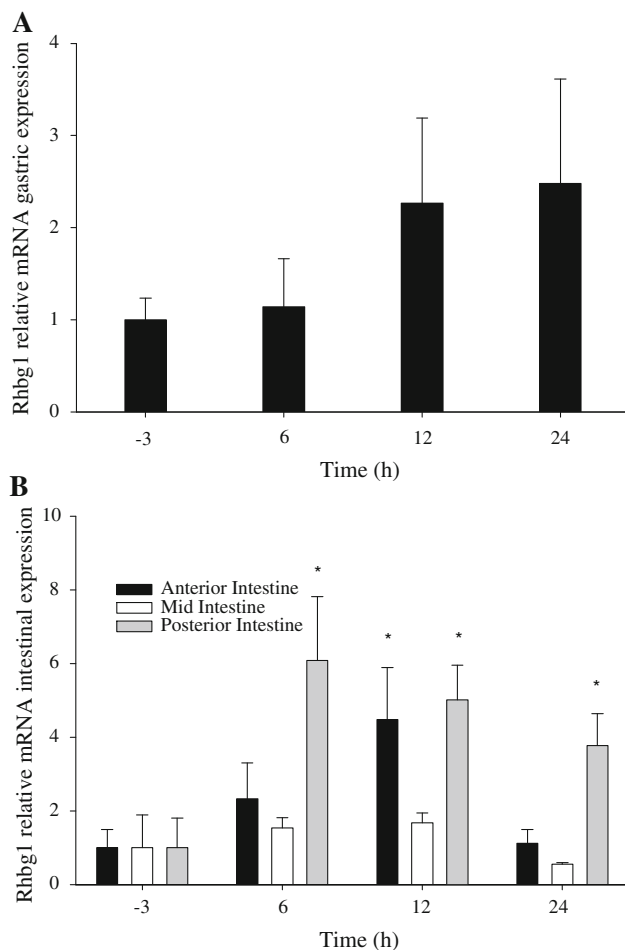
In the anterior intestine, glutamine synthetase (*GS*) activity did not mirror changes in mRNA expression of *Rhbg1* as there was no significant change with feeding (Fig. 6), despite increases in *Rhbg1* mRNA in the anterior intestine at 12 h (Fig. 5b). In contrast, the four- to sixfold increase in *Rhbg1* mRNA expression in the intestine at 6–24 h was accompanied by an increasing activity level of *GS* at 12 and 24 h following feeding (Fig. 6), increasing to approximately fourfold higher than pre-feeding values at 24 h. The mid-intestine did not show any significant changes in *GS* activity (Fig. 6), similar to the lack of change of *Rhbg1* expression in this segment (Fig. 5b).



**Fig. 4 a** The effect of feeding on the relative mRNA expression of *NBC1* ( $Na^+/HCO_3^-$  cotransporter 1) in the stomach of the freshwater rainbow trout.  $\beta$ -actin was used as the reference gene. Feeding occurred at 0 h. Values are mean  $\pm$  SEM ( $N = 6$ ). Asterisk indicate a significant increase from control values (-3 h = pre-feeding). **b** The effect of feeding on the relative mRNA expression of *NBC1* ( $Na^+/HCO_3^-$  cotransporter 1) in the anterior, mid, and posterior intestine of the freshwater rainbow trout. *18s* was used as the reference gene. Feeding occurred at 0 h (-3 h = pre-feeding). Values are mean  $\pm$  SEM ( $N = 6$ ). There were no significant differences within segments

**Discussion**

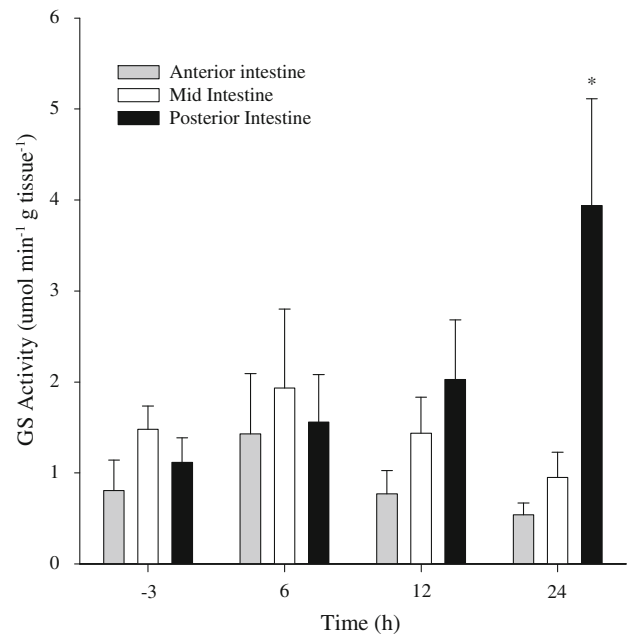
To our knowledge, this is the first evidence of the effects of feeding on the transcription levels of Rhesus glycoproteins, the *HKA* and the *NBC* transporters in the gastrointestinal tract of fish. In fact, this study adds to the relatively few glimpses at the gene expression responses of proteins to feeding and digestion in the GI tract of fish. Up to now, investigations have focused mostly at transporters or enzymes related to nutritional uptake (e.g. lipase-encoding mRNA, Klittilson et al. 2011; dipeptide transporters, Ostaszewska et al. 2010; Bakke et al. 2010; trypsin, Lui et al. 2007; Lilleeng et al. 2007; fatty acid desaturase, Leaver et al. 2006; Geay et al. 2010) or hormonal regulation of feeding (e.g. leptin, Ronnestad et al. 2010; Ostaszewska



**Fig. 5** **a** The effect of feeding on the relative mRNA expression of Rhbgl (Rhesus glycoprotein bg1) in the stomach of the freshwater rainbow trout.  $\beta$ -actin was used as the reference gene. Feeding occurred at 0 h ( $-3$  h = pre-feeding). Values are mean  $\pm$  SEM ( $N = 6$ ). There were no significant differences. **b** The effect of feeding on the relative mRNA expression of Rhbgl (Rhesus glycoprotein bg1) in the anterior, mid, and posterior intestine of the freshwater rainbow trout. 18s was used as the reference gene. Feeding occurred at 0 h. Values are mean  $\pm$  SEM ( $N = 6$ ). Asterisk indicate a significant increase from control values ( $-3$  h = pre-feeding) for that particular segment

et al. 2010; peptide Y, Murashita et al. 2006; Gonzalez and Unniappan 2010; ghrelin, Terova et al. 2008; Xu and Volkoff 2009). However, the majority of these studies examine long-term adaptations to specific diets and/or fasting, and not short-term responses in gene regulation (i.e. within 48 h of consuming a meal) to the digestion of a meal. The analysis of short-term, post-prandial transporter expression in the GI tract provides a mechanistic basis for the interpretation of the physiological evidence provided in earlier studies of immediate alterations in homeostasis.

An interesting finding of the gene expression analysis was the increase in basolateral NBC1 expression levels in the gastric epithelium at 24 h post-feeding. At first glance,



**Fig. 6** Intestinal glutamine synthetase activity ( $\mu\text{mol min}^{-1} \text{g tissue}^{-1}$ ). Feeding occurred at 0 h. Values are mean  $\pm$  SEM ( $N = 7$ ). Asterisk indicate a significant increase from control values ( $-3$  h = pre-feeding) for that particular segment

this response appears to be counterproductive, as the mechanism of gastric acid formation results in elevated intracellular  $[\text{HCO}_3^-]$ , which is then removed from the cell across the basolateral membrane and eventually creates the alkaline tide in the systemic bloodstream (reviewed by Hersey and Sachs 1995; Niv and Fraser 2002). However, evidence exists that the basolateral loading of  $\text{HCO}_3^-$  into the gastric cells can be physiologically beneficial. Early in vitro studies in amphibians suggest that a basolateral NBC may provide  $\text{HCO}_3^-$  intended for secretion into the stomach lumen (Takeuchi et al. 1982) in order to preserve gastric epithelium integrity in the face of an acidic pH necessary for digestion by forming an alkaline mucus (reviewed by Allen et al. 1993). This theory has been supported by evidence from mammalian models as well (Seidler et al. 2000). In the trout, the HCl acid secretion associated with digestion has started to decline by 24 h following the ingestion of a meal based on gastric fluid and  $\text{Cl}^-$  secretion rates (Bucking and Wood 2006a, b, 2009). Hence, a secretion of an alkaline mucus would not counteract gastric acid secretion. Additionally, Bucking and Wood (2009) showed that gastric lumen pH had fallen below a pH of 4.0 by 24 h, and continued to fall to  $\sim 2.0$  by 48 h following ingestion of a meal. This continued fall in pH, despite an apparent reduction in gastric acid secretion, may be caused by the reduction of the buffering capacity of chyme as digestion proceeds. The alkaline mucous secretion could be triggered by the falling pH of

the stomach lumen as has been shown in mammals (Guha and Kaunitz 2002).

A further potential mechanism to explain the large increase in gastric NBC1, in addition to the lack of an observable post-prandial response in HKA mRNA expression, could involve the acid-secreting cells. In response to secretory stimulation in mammals, the gastric acid-secreting cell undergoes a morphologic transformation. Its apical membrane becomes highly infolded when secretory canaliculi are formed from the fusion of the tubulovesicles, which are membrane-bound structures that harbor the HKA (Forte et al. 1981; Agnew et al. 1999). Upon cessation of HCl secretion, the tubulovesicles are reformed, and the HKA is re-internalized. Thus, while there is an increase in the activity of HKA, it is as a result of pre-existing transporters, and not de novo synthesis. However, when the HKA transporters are endocytosed into the intracellular space, a continuous slow leak of protons out of the tubulovesicle lumen has been observed (Germino et al. 2004). The NBC transporter has been hypothesized as being important in neutralizing changes in intracellular pH of gastric cells themselves (reviewed by Guha and Kaunitz 2002) potentially caused by the reformation of tubulovesicles; a basolateral NBC could provide intracellular  $\text{HCO}_3^-$  to buffer this proton leak. Evidence for this is, however, controversial and when NBC is detected in acid-secreting cells, it is at levels much lower than in the mucus secreting cells (Seidler et al. 2000). Regardless, the timing of the increase in the expression of the NBC1 suggests that this could be occurring in rainbow trout.

The role of NBC in intestinal  $\text{HCO}_3^-$  secretion in fish has primarily been observed in seawater fish (Grosell and Genz 2006; Taylor et al. 2010), or fish transferred to seawater (Grosell et al. 2007), due to the pivotal (and unique) role of  $\text{HCO}_3^-$  secretion and precipitation in seawater osmoregulation (Wilson et al. 2002; Wilson and Grosell 2003; Marshall and Grosell 2006). The source of the luminal  $\text{HCO}_3^-$  is both endogenous and serosal (Wilson et al. 2002; Grosell 2006; Marshall and Grosell 2006) and recent evidence suggests that NBC plays a significant role in the basolateral transport mechanism for serosal  $\text{HCO}_3^-$  supply (Kurita et al. 2008; Taylor et al. 2010). Additionally, the intestine has demonstrated postprandial elevations in bicarbonate secretion (Taylor and Grosell 2006; Bucking et al. 2009) which may play a role in alleviating the alkaline tide. However, no such response has been observed in freshwater fish (Bucking et al. 2009) and the present lack of response of NBC to digestion, further reinforces a lack of acid–base regulation by the intestine in freshwater rainbow trout. However, there are temporal limitations to the current study. Transporter expression and enzyme activity were monitored for 24 h following the consumption of a meal, the period during which the most

profound changes in plasma  $T_{\text{amm}}$ , ammonia excretion rates, blood pH and  $\text{HCO}_3^-$ , and chyme pH along the intestine were seen in our previous studies (Bucking et al. 2010; Bucking and Wood 2006a, b, 2008). However, based on these and other previous studies (Bucking and Wood 2009; Bucking et al. 2010) using similar feeding protocols, digestion most likely would have continued for another 24 h. Therefore, it is possible that changes in gene expression or enzyme activity could have continued (or begun) after the cessation of the current study.

The intestinal expression of *Rhbg1* was expected to decrease in response to feeding based on the assumption that ammonia levels found along the GI tract would exceed those found in the plasma due to amino acid and adenylate catabolism within the chyme itself (Wood et al. 2009). This was confirmed by the present measurements (plasma  $T_{\text{amm}} = 288 \mu\text{mol l}^{-1}$  vs. chyme  $T_{\text{amm}} = 1.8 \text{ mmol l}^{-1}$ ; Fig. 2). A similar gradient has been seen in the GI tract of dogfish sharks during digestion where chyme supernatant levels rose to about  $2.0 \text{ mmol l}^{-1}$  relative to plasma levels of  $32 \mu\text{mol l}^{-1}$  (Wood et al. 2009). As *Rhbg1* transporters are channels, ammonia transport depends on the diffusive gradient. A decrease in *Rhbg* expression and a corresponding decrease in ammonia transport would then mitigate the potentially lethal rise in plasma ammonia. However, relative expression levels of *Rhbg1* mRNA actually increased, suggesting that ammonia transport was actually being facilitated by the intestinal tissue down this large gradient. Facilitated ammonia transport in the GI tract fits with the observations of Karlsson et al (2006) that  $T_{\text{amm}}$  was much higher in the hepatic portal vein than in the dorsal aorta following a force-fed meal in trout. The increase in *Rh* gene expression in the intestinal tissue corresponds to increased *Rh* gene expression (as well as  $\text{H}^+$ -ATPase) in the gill (Zimmer et al. 2010) although the time-frame of response does not match between the two investigations. A potential explanation may lie with the difference in the size of the trout between the two studies as digestion appears to progress more rapidly in smaller fish (i.e. shorter gastric evacuation time in smaller fish; Jobling et al. 1977).

The posterior intestine showed the greatest and most prolonged transcriptional increase in *Rhbg1* expression compared to all other intestinal segments, beginning 6 h following a meal and lasting for an additional 18 h (Fig. 5b). Notably, this segment also exhibited the highest chyme pH (close to 9.0; Bucking and Wood 2009), and therefore the greatest  $\text{NH}_3$  concentration, with the greatest potential for resultant toxicity. Interestingly, the only tissue to exhibit an increase in GS activity was the posterior intestine at 24 h (Fig. 6). This suggests that while the *Rhbg* channels are present along the entire intestinal tract, perhaps the posterior intestine is responsible for regulating the

amount of ammonia transported from the meal. Zonation in transporter expression along the gastrointestinal tract of fish has been observed for a variety of transporters from nutrient transporters (dipeptide transporters: Gonçalves et al. 2007; glucose transporters: Gonçalves et al. 2007) to  $\text{Na}^+$  and  $\text{Cl}^-$  transporters (Grosell et al. 2007; Cutler and Cramb 2008). As GS activity did not increase until 18 h following the up regulation of the ammonia channel, we hypothesize that the up-regulation in *Rhbg1* expression, presumably as a basolateral export channel, was great enough to prevent ammonia toxicity in the intestinal cells. However, as digestion progressed, the intracellular ammonia concentration increased beyond levels that could be removed by the basolateral channel and increased GS activity was required to prevent toxicity.

The present findings set the stage for future studies. First, natural daily rhythms in gene expression, independent of feeding, may have contributed to the results, and should be examined in future investigations. Additionally, transporter (HKA, NBC1, *Rhbg1*) activity remains to be investigated. Rapid and direct measurement of the activities of transporters tested in this study may not be possible due to the lack of assays tailored to these particular transporters. However, indirect evidence of activity can be relatively easily obtained. For example, the gastric pH of fed freshwater trout decreased over time, presumably due in part to gastric acid secretion as discussed above. This is circumstantial evidence suggesting that the activity of HKA is increased during digestion in freshwater rainbow trout (Bucking and Wood 2009). In vitro techniques such as gut sacs made at various times after a meal (e.g. Wood et al. 2010) can be used to further show the activity levels of the transporters under investigation. Examining differential regulation of transporter isoforms may also add some insight into transporter regulation along the GI tract. For example, while the gastric HKA has no known isoforms in fish, multiple variants of *Rhbg1* (*Rhbg1a* and *Rhbg1b*) have been observed in rainbow trout (Nawata et al. 2007), as has a *Rhbg2* isoform (itself with three variants, *Rhbg2a*, *Rhbg2b*, *Rhbg2c*; Nawata and Wood 2008). *Rhbg1a* and *Rhbg1b* have identical sequences except for a region in the 3'-UTR, which could relate to a regulatory difference in transcription (Nawata et al. 2007). However, with identical sequences, it would be difficult to identify which isoform was expressed. Finally, as mRNA is only itself an indirect measurement of transporter expression, protein expression analysis (through immunocytochemical studies; e.g. Perry et al. 2010) would reveal if the increase in mRNA expression relates to an increase in transporter number and not simply an increase in transporter turnover rate.

This study adds to the relatively few glimpses at the short-term response of transporters to feeding and digestion of a single meal in the GI tract of fish. While it has been

shown that digestion affects acid–base balance (Bucking and Wood 2008), ion regulation (Bucking and Wood 2006b, 2007; Taylor et al. 2007) and osmoregulation (Bucking and Wood 2006a) are also altered by digestion, areas that have yet to be investigated at a mechanistic level. To conclude, our results are suggestive of short-term, dynamic alterations in gene expression at the transcriptional level and enzyme activity in the GI tract triggered by digestion.

**Acknowledgments** Supported by an NSERC Discovery Grant to CMW. CMW is also supported by the Canada Research Chair Program. CB was supported by an NSERC Canada Graduate Scholarship. We thank Michele Nawata for molecular advice. The experiments were approved by institutional animal care committees at McMaster University.

## References

- Agnew BJ, Duman JG, Watson CL, Coling DE, Forte JG (1999) Cytological transformations associated with parietal cell stimulation: critical steps in the activation cascade. *J Cell Sci* 112:2639–2646
- Allen A, Flemstrom G, Garner A, Kivilaakso E (1993) Gastrointestinal mucosal protection. *Physiol Rev* 73:823–857
- Alsop DH, Wood CM (1997) The interactive effects of feeding and exercise on oxygen consumption swimming performance and protein usage in juvenile rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 200:2337–2346
- Bakke S, Jordal AEO, Gomez-Requeni P, Verri T, Kousoulaki K, Aksnes A, Ronnestad I (2010) Dietary protein hydrolysates and free amino acids affect the spatial expression of peptide transporter *PepT1* in the digestive tract of Atlantic cod (*Gadus morhua*). *Comp Biochem Physiol* 156:48–55
- Ballantyne JS (2001) Amino acid metabolism fish physiology. In: Anderson P, Wright P (eds) *Fish physiology*. Academic Press, Orlando, pp 77–107
- Beamish FWH, Thomas E (1984) Effects of dietary-protein and lipid on nitrogen losses in rainbow trout, *Salmo gairdneri*. *Aquaculture* 41:359–371
- Bucking C, Landman MJ, Wood CM (2010) The role of the kidney in compensating the alkaline tide, electrolyte load and fluid balance disturbance associated with feeding in the freshwater rainbow trout, *Oncorhynchus mykiss*. *Comp Biochem Physiol A* 156:74–83
- Bucking C, Fitzpatrick JL, Nadella SR, Wood CM (2009) Postprandial metabolic alkalosis in the seawater-acclimated trout: the alkaline tide comes in. *J Exp Biol* 212:2159–2166
- Bucking C, Wood CM (2006a) Water dynamics in the digestive tract of the freshwater rainbow trout during the processing of a single meal. *J Exp Biol* 209:1883–1893
- Bucking C, Wood CM (2006b) Gastrointestinal processing of  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{K}^+$  during digestion: implications for homeostatic balance in freshwater rainbow trout. *Am J Physiol* 291:R1764–R1772
- Bucking C, Wood CM (2008) The alkaline tide and ammonia excretion after voluntary feeding in freshwater rainbow trout. *J Exp Biol* 211:2533–2541
- Bucking C, Wood CM (2009) The effect of postprandial changes in the pH along the gastrointestinal tract on the distribution of ions between the solid and fluid phases of chyme in rainbow trout. *Aquac Nut* 15:282–296



- Crowson MS, Shull GE (1992) Isolation and characterization of a cDNA-encoding the putative distal colon  $H^+$ ,  $K^+$ -ATPase—similarity of deduced amino-acid-sequence to gastric  $H^+$ ,  $K^+$ -ATPase and  $Na^+$ ,  $K^+$ -ATPase and messenger-RNA expression in distal colon, kidney, and uterus. *J Biol Chem* 267:13740–13748
- Cutler CP, Cramb G (2008) Differential expression of absorptive cation-chloride cotransporters in the intestinal and renal tissues of the European eel (*Anguilla anguilla*). *Comp Biochem Physiol* 149:63–73
- Forte JG, Black JA, Forte TM, Machen TE, Wolosin JM (1981) Ultrastructural changes related to functional activity in gastric oxyntic cells. *Am J Physiol* 241:G349–G358
- Geay F, Culi ESI, Corporeau C, Boudry P, Dreano Y, Corcos L, Bodin N, Vandeputte M, Zambonino-Infante JL, Mazurais D, Cahu CL (2010) Regulation of FADS2 expression and activity in European sea bass (*Dicentrarchus labrax*) fed a vegetable diet. *Comp Biochem Physiol* 156:237–243
- Gelineau A, Medale F, Boujard T (1998) Effect of feeding time on postprandial nitrogen excretion and energy expenditure in rainbow trout. *J Fish Biol* 52:655–664
- Gerbino A, Hofer AM, McKay B, Lau BW, Soybel DI (2004) Divalent cations regulate acidity within the lumen and tubulovesicle compartment of gastric parietal cells. *Gastroenterology* 126:182–195
- Gonçalves AF, Castro LFC, Pereira-Wilson C, Coimbra J, Wilson JM (2007) Is there a compromise between nutrient uptake and gas exchange in the gut of *Misgurnus anguillicaudatus*, an intestinal air-breathing fish? *Comp Biochem Physiol D* 2(4):345–355
- Gonzalez R, Unniappan S (2010) Molecular characterization, appetite regulatory effects and feeding related changes of peptide YY in goldfish. *Gen Comp Endocrinol* 166:273–279
- Grossell M, Genz J (2006) Ouabain sensitive bicarbonate secretion and acidic fluid absorption by the marine teleost intestine play a role in osmoregulation. *Am J Physiol* 291:R1145–R1156
- Grossell M, Gilmour KM, Perry SF (2007) Intestinal carbonic anhydrase, bicarbonate, and proton carriers play a role in the acclimation of rainbow trout to seawater. *Am J Physiol* 293:R2099–R2111
- Guha S, Kaunitz JD (2002) Gastroduodenal mucosal defence: an integrated protective response. *Curr Opin Gastroenterol* 18:650–657
- Handy RD, Poxton MG (1993) Nitrogen pollution in mariculture: toxicity and excretion of nitrogenous compounds by marine fish. *Rev Fish Biol Fish* 3:205–241
- Hersey SJ, Sachs G (1995) Gastric acid secretion. *Physiol Rev* 75:155–189
- Hung CYC, Tsui KNT, Wilson JM, Nawata CM, Wood CM, Wright PA (2007) Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. *J Exp Biol* 210:2419–2429
- Jobling M, Gwyther D, Grove DJ (1977) Some effects of temperature, meal size and body weight on gastric evacuation time in the dab *Limanda limanda*. *J Fish Biol* 10:291–298
- Karlsson A, Eliason EJ, Mydland LT, Farrell AP, Kiessling A (2006) Postprandial changes in plasma free amino acid levels obtained simultaneously from the hepatic portal vein and the dorsal aorta in rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 209:4885–4894
- Kaushik SJ, Teles AD (1985) Effect of digestible energy on nitrogen and energy balance in rainbow trout. *Aquaculture* 50:89–101
- Klittitilsson JD, Reindi KM, Sheridan MA (2011) Rainbow trout (*Oncorhynchus mykiss*) possess two hormone-sensitive lipase-encoding mRNAs that are differentially expressed and independently regulated by nutritional state. *Comp Biochem Physiol* 158:52–60
- Kurita Y, Nakada T, Kato A, Doi H, Mistry AC, Chang M, Romero MF, Hirose S (2008) Identification of intestinal bicarbonate transporters involved in formation of carbonate precipitates to stimulate water absorption in marine teleost fish. *Am J Physiol* 294:R1402–R1412
- Leaver MJ, Tocher DR, Obach A, Jensen L, Henderson RJ, Porter AR, Krey G (2006) Effect of dietary conjugated linoleic acid (CLA) on lipid composition metabolism and gene expression in Atlantic salmon (*Salmo salar*). *Comp Biochem Physiol A* 145:258–267
- Lilleeng E, Froystad MK, Ostby GC, Valen EC (2007) Effects of diets containing soybean meal on trypsin mRNA expression and activity in Atlantic salmon (*Salmo salar* L). *Comp Biochem Physiol A* 147:25–36
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta Ct}$  method. *Methods* 25:402–408
- Lui ZY, Wang Z, Xu SY, Xu LN (2007) Two trypsin isoforms from the intestine of the grass carp (*Ctenopharyngodon idellus*). *J Comp Physiol B* 177:655–666
- MacLennan DH, Brandl CJ, Korczak B, Green NM (1985) Amino acid sequence of a  $Ca^{2+}$  and  $Mg^{2+}$ -dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. *Nature* 316:696–700
- Marshall WS, Grosell M (2006) Ion transport, osmoregulation and acid–base balance. In: Evans D, Claiborne JB (eds) *The physiology of fishes*. CRC Press, Boca Raton, pp 177–230
- Munson K, Lambrecht N, Shin JM, Sachs G (2000) Analysis of the membrane domain of the gastric  $H^+/K^+$ -ATPase. *J Exp Biol* 203:161–170
- Murashita K, Fukada H, Hosokawa H, Masumoto T (2006) Cholecystokinin and peptide Y in yellowtail (*Seriola quinqueradiata*): Molecular cloning, real-time quantitative RT-PCR, and response to feeding and fasting. *Gen Comp Endocrinol* 145:287–297
- Nakada T, Hoshijima K, Esaki M, Nagayoshi S, Kawakami K, Hirose S (2007) Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. *Am J Physiol* 293:R1743–R1753
- Nawata CM, Hung CYC, Tsui TKN, Wilson JM, Wright PA, Wood CM (2007) Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H-ATPase involvement. *Physiol Genomics* 31:463–474
- Nawata CM, Wood CM (2008) The effects of  $CO_2$  and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. *J Exp Biol* 211:3226–3236
- Nawata CM, Wood CM (2009) mRNA expression analysis of the physiological responses to ammonia infusion in rainbow trout. *J Comp Physiol B* 179:799–810
- Nawata CM, Wood CM, O'Donnell MJ (2010) Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis. *J Exp Biol* 213:1049–1059
- Niv Y, Fraser GM (2002) The alkaline tide phenomenon. *J Clin Gastroenterol* 35:5–8
- Ostaszewska T, Dabrowski K, Kamaszewski M, Grochowski P, Verri T, Rzepkowska M, Wolnick J (2010) The effect of plant protein-based diet supplemented with dipeptide or free amino acids on digestive tract morphology and PepT1 and PepT2 expressions in common carp (*Cyprinus carpio*). *Comp Biochem Physiol* 157:158–169
- Perry SF, Schwaiger T, Kumai Y, Tzaneva V, Braun MH (2010) The consequences of reversible gill remodelling on ammonia excretion in goldfish (*Carassius auratus*). *J Exp Biol* 213:3656–3665
- Rønnestad I, Nilsen TO, Murashita K, Angotzi AR, Moen AGG, Stefansson SO, Kling P, Björnsson BT, Kurokawa T (2010)

- Leptin and leptin receptor genes in Atlantic salmon: cloning, phylogeny, tissue distribution and expression correlated to long-term feeding status. *Gen Comp Endocrinol* 168:55–70
- Seidler U, Rossman H, Jacob P, Bachmann O, Christiani S, Lamprecht G, Gregor M (2000) Expression and function of  $\text{Na}^+\text{-HCO}_3^-$  cotransporters in the gastrointestinal tract. *Ann NY Acad Sci* 915:1–14
- Shull GE, Schwartz A, Lingrel JB (1985) Amino acid sequence of the catalytic subunit of the  $\text{Na}^+\text{-K}^+$ -ATPase deduced from a complementary DNA. *Nature* 316:691–695
- Stone DAJ, Allan GL, Anderson AJ (2003) Carbohydrate utilization by juvenile silver perch, *Bidyanus bidyanus* (Mitchell). III. The protein-sparing effect of wheat starch-based carbohydrates. *Aquac Res* 34:123–134
- Sugiura SH, Roy PK, Ferraris RP (2006) Dietary acidification enhances phosphorus digestibility but decrease  $\text{H}^+/\text{K}^+$ -ATPase expression in rainbow trout. *J Exp Biol* 209:3719–3728
- Takeuchi K, Merhav A, Silen W (1982) Mechanism of luminal alkalization by bullfrog fundic mucosa. *Am J Physiol* 243:G377–G388
- Taylor JR, Grosell M (2006) Feeding and osmoregulation: dual function of the marine teleost intestine. *J Exp Biol* 209:2939–2951
- Taylor JR, Whittamore JM, Wilson RW, Grosell M (2007) Postprandial acid-base balance and ion regulation in freshwater and seawater-acclimated European flounder, *Platichthys flesus*. *J Comp Physiol B* 177:597–608
- Taylor JR, Mager EM, Grosell M (2010) Basolateral NBCe1 plays a rate-limiting role in transepithelial intestinal  $\text{HCO}_3^-$  secretion, contributing to marine fish osmoregulation. *J Exp Biol* 213:459–468
- Terova G, Rimoldi S, Bernardini G, Gornati R, Saroglia M (2008) Sea bass ghrelin: molecular cloning and mRNA quantification during fasting and refeeding. *Gen Comp Endocrinol* 155:341–351
- Tsui TKN, Hung CYC, Nawata CM, Wilson JM, Wright PA, Wood CM (2009) Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical  $\text{Na}^+/\text{NH}_4^+$  exchange complex. *J Exp Biol* 212:878–892
- Webb JT, Brown GW (1980) Glutamine synthetase: assimilatory role in liver as related to urea retention in marine chondrichthyes. *Science* 208:293–295
- Weihrauch D, Wilkie MP, Walsh PJ (2009) Ammonia and urea transporters in gills of fish and aquatic crustaceans. *J Exp Biol* 212:1716–1730
- Wicks BJ, Randall DJ (2002) The effect of sub-lethal ammonia exposure on fed and unfed rainbow trout: the role of glutamine in regulation of ammonia. *Comp Biochem Physiol A* 132:275–285
- Wilson RW, Grosell M (2003) Intestinal bicarbonate secretion in marine teleost fish—source of bicarbonate, pH sensitivity, and consequence for whole animal acid-base and divalent cation homeostasis. *Biochim Biophys Acta* 1618:163–193
- Wilson RW, Wilson JM, Grosell M (2002) Intestinal bicarbonate secretion by marine teleost fish—why and how? *Biochim Biophys Acta* 1566:182–193
- Wood CM, Kajimura M, Mommensen TP, Walsh PJ (2005) Alkaline tide and nitrogen conservation after feeding in the elasmobranch *Squalus acanthias*. *J Exp Biol* 208:2693–2705
- Wood CM, Bucking C, Fitzpatrick JL, Nadella S (2007a) The alkaline tide goes out and the nitrogen stays in after feeding in the dogfish shark, *Squalus acanthias*. *Respir Physiol Neurobiol* 159:163–170
- Wood CM, Kajimura M, Bucking C, Walsh PJ (2007b) Osmoregulation, ionoregulation and acid–base regulation by the gastrointestinal tract after feeding in the elasmobranch (*Squalus acanthias*). *J Exp Biol* 210:1335–1349
- Wood CM, Schultz AG, Munger S, Walsh PJ (2009) Using omeprazole to link the components of the postprandial alkaline tide in the spiny dogfish, *Squalus acanthias*. *J Exp Biol* 212:684–692
- Wood CM (1993) Ammonia and urea metabolism and excretion. In: Evans DH (ed) *The physiology of fishes*. CRC Press, Boca Raton, pp 379–425
- Wood CM (2001) The influence of feeding, exercise, and temperature on nitrogen metabolism and excretion. In: Anderson PA, Wright PA (eds) *Fish physiology*, vol 20. Academic Press, Orlando, pp 201–238
- Wood CM, Bucking C, Grosell M (2010) Acid-base responses to feeding and intestinal  $\text{Cl}^-$  uptake in freshwater and seawater acclimated killifish, *Fundulus heteroclitus*, an agastric euryhaline teleost. *J Exp Biol* 213:2681–2692
- Wright PA, Wood CM (2009) A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. *J Exp Biol* 212:2303–2312
- Xu MY, Volkoff H (2009) Molecular characterization of ghrelin and gastrin-releasing peptide in Atlantic cod (*Gadus morhua*): cloning, localization, developmental profile and role in food intake regulation. *Gen Comp Endocrinol* 160:250–258
- Zimmer A, Nawata CM, Wood CM (2010) Physiological and molecular analysis of the interactive effects of feeding and high environmental ammonia on branchial ammonia excretion and  $\text{Na}^+$  uptake in freshwater rainbow trout. *J Comp Physiol* 180:1191–1204