

## Dietary acidification enhances phosphorus digestibility but decreases H<sup>+</sup>/K<sup>+</sup>-ATPase expression in rainbow trout

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### Summary

Oxynticopeptic cells of fish stomach are thought to secrete less acid than the specialized parietal cells of mammalian stomach. Gastric acidity, however, has not been directly compared between fish and mammals. We therefore fed rainbow trout and rats the same meal, and found that the lowest postprandial pH of trout stomach was 2.7, which was only transiently sustained for 1 h, whereas that of rat stomach was 1.3, which was sustained for 3 h. Postprandial pH of the small intestine was slightly higher in trout (~8.0) than in rats (~7.6), but pH of the large intestine was similar (~8.0). Addition of acids to fish feeds, in an attempt to aid the weak acidity of fish stomach, has been known to improve phosphorus digestibility, but its physiological effect on fish stomach is not known. Exogenous acids did improve phosphorus digestibility but also decreased steady-state mRNA expression of trout H<sup>+</sup>/K<sup>+</sup>-ATPase (ATP4A, the proton pump) as well as Na<sup>+</sup>/bicarbonate cotransporter (NBC), and had no effect

on gastrin-like mRNA and somatostatin (SST) mRNA abundance. Gastrin-like mRNA and SST-2 mRNA were equally distributed between corpus and antrum. ATP4A mRNA and NBC mRNA were in the corpus, whereas SST-1 mRNA was in the antrum. Trout gastrin-like EST had modest homology to halibut and pufferfish gastrin, whereas trout ATP4A mRNA had ≥95% amino acid homology with mammalian, *Xenopus* and flounder ATP4A. Although ATP4A seems highly conserved among vertebrates, gastric acidity is much less in trout than in rats, explaining the low digestibility of bone phosphorus, abundant in fish diets. Dietary acidification does not reduce acidity enough to markedly improve phosphorus digestibility, perhaps because exogenous acids may inhibit endogenous acid production.

Key words: gastrin, oxynticopeptic cells, proton pump, somatostatin, stomach pH.

### Introduction

The mammalian stomach has two specialized cell types to acidify the lumen and to digest proteins. Parietal or oxyntic cells secrete hydrochloric acid (HCl), whereas chief or peptic cells secrete pepsinogen. In contrast, the stomach of fish and other non-mammalian vertebrates have only one cell type, appropriately referred to as oxynticopeptic (oxyntopeptic) cells, to accomplish both functions. Oxynticopeptic cells contain abundant zymogen granules (Garrido et al., 1993) or large intracellular vacuoles (Bomgren et al., 1998) or cavities (Garrido et al., 1996) and secrete both hydrochloric acid and protein. Oxynticopeptic cells are located mainly in the corpus of the stomach and are embedded in glands within the lamina propria as described in the winter flounder, European eel, rainbow trout and Atlantic stingray (Garrido et al., 1996; Garrido et al., 1993; Gawlicka et al., 2001; Smolka et al., 1994). Because oxynticopeptic cells are not specialized, they are thought to be less efficient in secreting acid than mammalian parietal cells (Koelz, 1992; Vial and Garrido, 1979). Even

though acid secretory output or gastric acidity has not to our knowledge been directly compared between fish and mammals, the notion that fish stomach is less acidic than that of mammals has persisted since the discovery of oxynticopeptic cells many years ago (Vial and Garrido, 1979). Support for this view arose mainly from circumstantial evidence, as follows. First, postprandial gastric acidity tends to be low in fish. Our laboratory has shown that when the rainbow trout stomach was filled with food, the pH of the chyme was high and remained high (~4.0) even 6–9 h after feeding (Sugiura and Ferraris, 2004). It is well known that postprandial pH of mammalian stomach can reach 2 or less (Berne and Levy, 2000). Second, phosphorus (P) in fish meal is poorly digested by fish (Cho and Bureau, 2001), but is well-digested by mammals (Soares, Jr, 1995). The main form of dietary P in fish food is hydroxyapatite or bone phosphate, which requires strong acidity to be solubilized in the stomach for subsequent absorption in the intestine. Inefficient digestion of dietary P in trout stomach can be explained by the high pH, whereas the

almost complete digestion of dietary P in mammals can be ascribed to the low pH in stomach lumen.

Because undigested dietary P is excreted by fish and pollutes the aquatic environment, a variety of methods has been introduced to improve P digestibility, one of which is incorporating acids into diets. Dietary acidification has been demonstrated to markedly improve dietary P digestibility in fish (Vielma et al., 1999). Not surprisingly, the pH of gastric chyme of rainbow trout fed diets acidified with citric acid became 0.5–1.0 pH units lower than that of fish fed the control non-acidified diet, suggesting that the supplementary acid compensated for the low gastric acidity of fish, thereby improving digestion of P. Dietary acidification is now one of the emerging research areas in fish nutrition. Unfortunately, there has been no study, even in mammals, on the effects of dietary acidification on regulatory mechanisms modulating gastric acid secretion.

In mammals, gastric acid secretion is tightly regulated by the stimulatory effects of gastrin, histamine and acetylcholine, and the inhibitory actions of somatostatin (SST) on their respective receptors located in the basolateral membrane of parietal cells (Samuelson and Hinkle, 2003). Gastrin, histamine and an acetylcholine analog, carbamoylcholine (carbachol) induce rapid and coordinated increases of H<sup>+</sup>/K<sup>+</sup>-ATPase (ATP4A/B, proton pump) mRNA in parietal cells (Dockray, 1999). When gastric pH becomes too low, SST secretion increases to inhibit not only acid production by parietal cell but also gastrin secretion by G cells (Berne and Levy, 2000). This feedback regulation stemming from acute changes in endogenous gastric acidity is well known; what is not known is the response of these regulatory systems to chronic consumption of exogenous acids.

There is scant information on physiological responses of fish to dietary acid intake. Previous studies indicated that fish might tolerate exogenous acid incorporation into their diet. For example, trout fed acidified diets had higher P and Ca contents in the body (Hardy et al., 1983) or similar gastrointestinal protease activities, as well as growth rates (Rungruangsak and Utne, 1981) as fish fed non acidified diets. One possible mechanism underlying this tolerance to exogenous acids is that fish can increase bicarbonate secretion from the apical membrane of the gastric mucous cells to maintain normal pH in the gastric mucosal barrier overlying the cells.

In this study, we compared the postprandial gastric and intestinal pH of rainbow trout and rats fed the same diet, to test the hypothesis that gastric acidity of fish stomach is lower than that of mammals. We also compared effects of different supplementary dietary acids on P digestibility, to determine whether dietary acidification indeed increases dietary P utilization by fish. Finally, we examined the steady state mRNA abundance of gastrin-like, H<sup>+</sup>/K<sup>+</sup>-ATPase, the Na(+)/bicarbonate cotransporter (NBC), and several SST genes in both antral and corpus stomach, to test the hypothesis that mRNA expression of gastric acid secretagogues such as gastrin decreases with dietary acid, whereas that of gastric acid inhibitors such as SST increases with dietary acid.

## Materials and methods

### Feeding trial

Two feeding experiments were conducted. In the first experiment, we compared the pH of the gastrointestinal (GI) tract between rats *Rattus norvegicus* Berkenhout (120.3±3.2 g body mass) and rainbow trout, *Oncorhynchus mykiss* Walbaum (small size: 10.24±0.54 and mid-size: 211.4±2.4 g mass). The small-sized fish were approximately the same age as rats (~1–2 months), but the mid-sized fish had similar stomach sizes (~4 g) to those of the rat. The 10 and 211 g fish were stocked in two separate 500 l-capacity tanks filled with dechlorinated municipal (fresh) water (15°C) at the density of 120 and 50 fish per tank, respectively. Rats were maintained in 20 l-capacity Plexiglas cages. Both rainbow trout and rats were fed the same diet (Table 1) as much as they would consume for at least 7 days before being killed and the GI tract removed. The GI chyme was collected by dissection at various post-prandial intervals for pH measurements. To initiate an experiment, a number of animals were starved overnight (to ensure stomachs were completely empty), then pre-prandial pH was determined at 08:00 h prior to feeding (0 h), and the 0.5 h pH was measured 30 min after feeding, and so on.

In the second experiment, physiological effects of various dietary acids were studied. Twenty-eight rainbow trout (body mass 234.4±24.2; N=24) were stocked in each of six circular tanks (volume: 1 m<sup>3</sup>) located at Ed Weed Fish Culture Station, Vermont Fish & Wildlife Department, Grand Isle, Vermont, USA. Each tank was continually supplied with lake water (11.1–14.3°C; mean 13.2°C) at 20 l min<sup>-1</sup>. Fish were hand-fed twice daily for 11 days at 2% of their collective body mass. Since there were no studies on the effect of acidification on expression of genes involved in gastric acid secretion, we chose

Table 1. *Composition of basal diet (first experiment)*

Ingredients	%
Menhaden fish meal	25.0
Wheat gluten meal	20.0
Wheat flour	23.0
Fish oil	25.0
Vitamin mix (#30)*	1.0
Trace mineral mix (#3)*	0.1
Other micronutrients <sup>†</sup>	5.9
Total	100.0

Constituents	% (dry basis)
Crude protein	37.3
Crude fat	28.5
Crude fiber	3.4
Ash	7.5

\*Standard USFWS pre-mixtures (see Halver, 1989).  
<sup>†</sup>As dietary %: choline chloride, 0.2; myo-inositol, 0.05; ascorbic acid, 0.05; lysine hydrochloride, 0.2; NaCl, 0.3; KCl, 0.6; MgSO<sub>4</sub>, 0.2; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.015; SiO<sub>2</sub>, 1.0; alpha-cellulose, 3.285.

this feeding duration to determine steady state levels of gene expression after chronic consumption of acidified diets. At the end of the feeding period, fish were anesthetized with MS222, and the GI tissues and fecal samples were collected for analyses. Fish were not fed overnight (12 h) prior to killing.

All fish and rats were treated according to the guidelines of the Institutional Animal Care & Use Committee (IACUC) of the University of Medicine & Dentistry of New Jersey, UMDNJ, USA.

#### Experimental diets

Six test diets were prepared in the second experiment, using the basal diet described in Table 1. Diet 1 contained 5% (w/w) concentrated hydrochloric acid (~12 mol l<sup>-1</sup> HCl), the gastric acid of vertebrates. Diets 2 and 3 contained concentrated sulfuric acid (~18 mol l<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub>) at 3.5 and 1.0%, respectively. Diet 4 contained 5% glacial acetic acid (CH<sub>3</sub>COOH), the common food acidulant, vinegar. These acid concentrations have previously been found to increase P digestibility (unpublished data). Diet 5 was the basal diet, containing no acid. Diet 6 was a negative control, containing a common antacid, calcium carbonate (CaCO<sub>3</sub>) at 5%, which neutralizes gastric acid in the stomach. To make the diets, acid was dissolved in 300–350 ml of tap water and mixed with fish meal. The dough was left at room temperature for 1 h, and then mixed with the other ingredients to make pellets. The pellets were dried at room temperature for 1.5 days, stored at 4°C, and fed within 2 weeks. Diets were analyzed for proximate compositions (Table 1) and P and calcium (Ca) concentrations (Table 2) according to the method described previously (Sugiura and Ferraris, 2004).

#### Chemical analyses

In the acidification (second) experiment, fecal samples were collected at the end of the feeding period by dissection from the rectum from six fish per dietary treatment, and analyzed individually for P, Ca and acid-insoluble ash (AIA) contents to determine digestibility of P, Ca and dry matter according to the method described previously (Sugiura and Ferraris, 2004).

Table 2. Analytical composition of test diets (second experiment)

Test diets*	%Water	%Ash	%P	%Ca	Ca/P ratio
+HCl 5%	7.69	6.75	0.732	1.16	1.59
+H <sub>2</sub> SO <sub>4</sub> 3.5%	8.95	7.50	0.716	1.17	1.64
+H <sub>2</sub> SO <sub>4</sub> 1%	8.64	7.05	0.742	1.21	1.62
+Acetic acid 5%	8.65	6.90	0.756	1.21	1.59
No acid (0%)	8.51	6.84	0.730	1.14	1.57
+Antacid 5%	8.30	10.29	0.672	2.96	4.41

\*One of the following acids or antacid was added to the basal diet of Table 1 (w/w). Concentration of acids: HCl, 12 mol l<sup>-1</sup>; H<sub>2</sub>SO<sub>4</sub>, 18 mol l<sup>-1</sup>; acetic acid, glacial; No acid = basal diet, see Table 1. Antacid, CaCO<sub>3</sub>.

#### pH measurements

For the first experiment, the pH of stomach, pyloric caeca, small intestine and large intestine was measured at different post-prandial periods (0, 0.5, 1, 2, 3, 6, 12 and 24 h) to compare the GI pH between rainbow trout and rats, and between two different sizes of rainbow trout, using multi-range pH indicator strips (0.2 unit-interval multi-range pH indicator strips; colorpHast, EMD Chemicals, New Jersey, USA).

In the second experiment, dietary pH was determined by making slurry of the food with distilled water and using a pH electrode (Orion, New York, USA). The pH of various sections of the GI tract of fish (*N*=4 fish per test diet) was determined using the pH strips ~12 h after feeding. After calibrating with the pH electrode, the pH strips allowed rapid determinations of pH of fluids adhering to the tissue surface which may be different from that of the lumen (Berne and Levy, 2000). Preliminary work showed gastric pH to change by ~2.0 pH units as a function of postprandial time, and to differ by as much as 3 pH units between rats and mice, differences readily detectable by strips. The following GI sections were studied for luminal (chyme) and tissue adherent fluid pH: stomach (corpus area); pyloric caeca; pyloric small intestine (immediately posterior to the pyloric sphincter with many caecal junctions); small (proximal) intestine; and large (distal) intestine. Tissue definitions were described previously (Sugiura and Ferraris, 2004).

#### Determination of mRNA abundance

To determine physiological responses of fish to dietary acid intake, tissues of corpus stomach (greater curvature, ~25 mm<sup>2</sup> in excised size) and antral stomach (~5 mm anterior to pyloric sphincter, ~25 mm<sup>2</sup> in excised size) were collected at the end of the feeding period, and stored in a RNA fixative (RNAlater, Ambion, Inc., Austin, TX, USA) for subsequent determinations of mRNA abundance of selected genes. Genes studied were of gastrin-like polypeptide, ATP4A, SST1, SST2, SST2 isoform and NBC. To normalize total RNA concentration, RNA quality, and RT efficiency among samples, hypoxanthine phosphoribosyl transferase (HPRT), which has recently been verified as a highly stable housekeeping gene, was used as the control (de Kok et al., 2005; Kim and Kim, 2003). Total RNA was extracted from the tissues (Trizol reagent, Invitrogen Co., Carlsbad, CA, USA), reverse transcribed (Stratascript, Stratagene, La Jolla, CA, USA) using oligo(dT)<sub>18</sub>, and quantified by a real-time quantitative PCR (QPCR; MX3000P, Stratagene) using a SYBR Green fluorescent detection system. Either one of the two primers was designed to intersect an exon–intron junction, and at least one intron was included in between the two primers. Primers were designed using a Primer3 software program, and are as follows: Gastrin-like EST (gi:40308333), forward 5'-ctggcactgagcattccattac-3', reverse 5'-atgtcaaccaacccccacta-3'; ATP4A-like EST (gi:42752688), forward 5'-gccactgacatttttccctctg-3', reverse 5'-ttgcgccaatctggaagtagg-3'; SST1 mRNA (PubMed ID:10094862), forward 5'-agaccagaagaagatgctctc-3', reverse 5'-attcctggcaagctctgtttg-3'; SST2-1 mRNA (gi:975344),

forward 5'-ctgctccataccgactgatcc-3', reverse 5'-ctcgcttactccactctctgtg-3'; SST2-2 mRNA (PubMed ID:10600899), forward 5'-tcgtccctgcaaacccaactc-3', reverse 5'-ctcgcttactccactctctgtg-3'; NBC1 mRNA (gi:24266569), forward 5'-tctcaacgtgtccagttcttg-3', reverse 5'-ctgtcgatgcttcttctctg-3'; HPRT1-EST (gi:27739555), forward 5'-aagagctactgcaatgaccaatc-3', reverse 5'-tgctctggaacctcaaatcctatg-3'. The kinships of the rainbow trout gastrin and ATP4A ESTs to gastrin and ATP4A genes from various species were examined using an online clustering program, *MultiAlin* (multiple sequence alignment with hierarchical clustering by F. Corpet).

### Statistics

Each fish and rat was considered a statistical unit. Differences between treatments were examined by one-way ANOVA followed by Newman-Keuls multiple comparison tests. Equal variances among treatments were verified by Bartlett's test. Digestibility data (%) were arcsine-transformed before ANOVA. Differences of mRNA abundance between antral and corpus stomach were identified by paired *t*-tests (all treatments inclusive). Correlations between different genes in mRNA abundance were tested based on linear regression. All statistical calculations were conducted using a software program (Prism 4, GraphPad Software, Inc., San Diego, CA, USA). Differences were considered significant at  $P < 0.05$ , unless otherwise stated.

## Results

### Comparative GI pH of fish and rats

In fish, there was no significant difference (paired *t*-test,  $P = 0.51$ ) in gastric pH between small and mid-size rainbow trout at almost all time points, hence, their gastric pH were pooled for comparison with rats. In both fish and rats, pre-prandial gastric luminal pH was 2.5–3.0 (Fig. 1). At 0.5 h after feeding, however, the stomach luminal pH increased ( $P < 0.001$  in fish;  $P = 0.02$  in rats) to 3.4–3.8. The difference in gastric pH between fish and rats became prominent ( $P < 0.001$ ) in the next 3–6 h, when luminal pH decreased dramatically at a rate of about 0.8 pH units per hour in rats, but not in fish. The reason why stomach pH in trout began to rise after 2 h is not clear. The lowest pH in rats was ~1.3 (6 h postprandial), whereas that in trout was ~2.6 (0, 2, 12 h postprandial). By 12–24 h postprandial, gastric pH returned to the baseline level in both fish and rats ( $P > 0.1$ ).

From 0 to 24 h after feeding, the intestinal pH ranged from 7.15 (mean of four fish each time point) to 8.25 in small fish, and from 7.38 to 8.65 in mid-size fish. Time of sampling was the same as that in Fig. 1 but there clearly was no effect of time on intestinal and caecal pH ( $P = 0.6$ ), indicating that luminal pH in these organs is regulated within tight limits. The caecal pH ranged from 7.03 to 7.58 in small fish, and from 7.10 to 7.53 in mid-size fish. There was no significant difference between small and mid-size fish in the intestinal and caecal pH ( $P = 0.1$  for intestinal pH;  $P = 0.7$  for caecal pH, by paired *t*-test). However, in both small and mid-size fish, caecal pH was

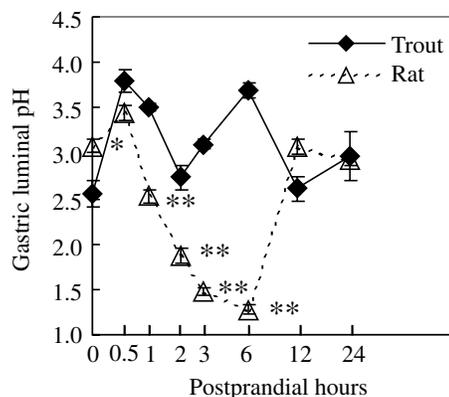


Fig. 1. Postprandial gastric luminal pH of fish and rats. The postprandial hours (x axis) are in log scale (0 h is the pre-prandial value), and gastric luminal pH (y axis) is shown as mean  $\pm$  s.e.m. (as error bars,  $N = 8$  for fish and  $N = 4$  for rats). Fish studied were four 2-month-old rainbow trout ( $10.24 \pm 0.54$  g; mean body mass  $\pm$  s.e.m.) and four 15-month-old rainbow trout ( $211.4 \pm 2.4$  g). Since there was no significant difference between these small and mid-sized fish, they were pooled for statistical comparison with rats. Rats studied were four 1–2-month-old rats ( $120.3 \pm 3.2$  g). Difference between fish and rats in gastric luminal pH at each postprandial hour is shown with asterisks: \* $P < 0.05$ ; \*\* $P < 0.01$ . Gastric luminal pH of rats was much lower than that of fish at 1–6 h postprandial.

significantly lower than intestinal pH ( $P = 0.01$  for small fish;  $P = 0.001$  for mid-size fish). In rats, duodenal, jejunal and ileal pH were all similar ( $P = 0.03$  to  $0.9$ ) at all postprandial hours. The pH (mean of four rats each time point) ranged from 7.42 to 7.95 in duodenum, 7.41 to 7.83 in jejunum, and 7.52 to 7.85 in ileum. The intestinal pH of fish (range 7.5–8.3) did not differ significantly ( $P = 0.1$ ) from that of rats (7.5–7.8), but the caecal pH was significantly lower ( $P < 0.001$ ) than that of rat intestine. The pH of the large intestine was similar between fish and rats (7.2–8.9 in fish, 7.4–8.7 in rats;  $P = 0.1$ ).

### Feeding activity

In the first experiment, both rats and fish consumed the basal diet readily. In the second experiment, relative feeding activity of fish was highest with antacid (5%  $\text{CaCO}_3$ ) diet, and slightly less active among fish fed the basal (no acid) diet, 5% acetic acid diet, and 1%  $\text{H}_2\text{SO}_4$  diet, and least active among those fed 3.5%  $\text{H}_2\text{SO}_4$  and 5% HCl diets.

### Luminal pH and P digestibility

The dietary pH was only moderately low in acidified diets (3.5–4.8) compared with non-acidified basal diet (5.7) (Table 3). The luminal pH of the stomach and intestine, determined at 12 h postprandial, of fish fed the basal diet (control) in the second experiment was similar to that determined at 0, 12 and 24 h postprandial in the first experiment (Fig. 1), indicating consistent results from fish of different batches and fed the same diet. Dietary acidification tended to decrease gastric and caecal pH ( $P = 0.07$ – $0.3$ ), but the intestinal pH did not change significantly with diet. Dietary antacid

Table 3. The pH of test diets and fish digestive tract at 12 h post-prandial

Test diets	pH of diet	pH of tissue adherent fluid					pH of luminal content			
		St	PC	PI	SI	LI	St	PI	SI	LI
HCl (5%)	4.0	2.2 <sup>a</sup> ±0.4	7.7 <sup>b</sup> ±0.3	8.0 <sup>b</sup> ±0.0	8.1 <sup>b</sup> ±0.1	8.0 <sup>b</sup> ±0.0	2.0 <sup>a</sup> ±0.0	8.3 <sup>b</sup> ±0.2	8.6 <sup>bAB</sup> ±0.1	8.6 <sup>bA</sup> ±0.1
H <sub>2</sub> SO <sub>4</sub> (3.5%)	3.5	1.8 <sup>a</sup> ±0.2	7.8 <sup>b</sup> ±0.2	8.0 <sup>b</sup> ±0.0	8.1 <sup>b</sup> ±0.1	8.2 <sup>b</sup> ±0.2	2.0 <sup>a</sup> ±0.0	8.0 <sup>b</sup> ±0.0	8.3 <sup>bA</sup> ±0.1	8.5 <sup>bA</sup> ±0.1
H <sub>2</sub> SO <sub>4</sub> (1%)	4.8	1.7 <sup>a</sup> ±0.2	8.1 <sup>b</sup> ±0.1	8.0 <sup>b</sup> ±0.0	8.3 <sup>b</sup> ±0.1	8.5 <sup>b</sup> ±0.1	1.7 <sup>a</sup> ±0.2	8.5 <sup>b</sup> ±0.0	8.7 <sup>bAB</sup> ±0.1	8.8 <sup>bAB</sup> ±0.0
Acetic acid (5%)	4.7	1.8 <sup>a</sup> ±0.2	8.2 <sup>b</sup> ±0.1	8.0 <sup>b</sup> ±0.0	8.2 <sup>b</sup> ±0.2	8.6 <sup>b</sup> ±0.2	1.8 <sup>a</sup> ±0.2	8.2 <sup>b</sup> ±0.1	8.6 <sup>bAB</sup> ±0.1	8.8 <sup>bAB</sup> ±0.0
No-acid (0%)	5.7	2.5 <sup>a</sup> ±0.5	8.2 <sup>b</sup> ±0.2	8.0 <sup>b</sup> ±0.0	8.3 <sup>b</sup> ±0.1	8.3 <sup>b</sup> ±0.1	2.3 <sup>a</sup> ±0.3	8.2 <sup>b</sup> ±0.1	8.6 <sup>bAB</sup> ±0.2	8.7 <sup>bAB</sup> ±0.1
Antacid (5%)	6.0	2.0 <sup>a</sup> ±0.0	8.3 <sup>b</sup> ±0.0	8.0 <sup>b</sup> ±0.0	8.4 <sup>b</sup> ±0.1	8.5 <sup>b</sup> ±0.1	2.0 <sup>a</sup> ±0.0	8.3 <sup>b</sup> ±0.2	8.9 <sup>bB</sup> ±0.1	8.9 <sup>bB</sup> ±0.1

St, stomach; PC, pyloric caeca; PI, pyloric small intestine; SI, small intestine; LI, large intestine. Each value represents the average of three fish ( $\pm$  s.e.m.). Values in rows with different small letters are significantly different ( $P < 0.05$ ). Values in columns with different capital letters are significantly different ( $P < 0.05$ ).

tended to increase intestinal luminal pH ( $P \approx 0.06$ , compared with the basal diet;  $P = 0.007-0.02$ , compared with the 3.5% sulfuric acid diet), but the gastric pH did not change significantly with dietary antacid.

Fish fed a diet containing 3.5% H<sub>2</sub>SO<sub>4</sub>, or 5% acetic acid, excreted significantly less P in feces ( $P < 0.001$  and  $P = 0.008$ , respectively) compared with those consuming the basal (no-acid) diet (Table 4). However, those fed diets containing 5% HCl or 1% H<sub>2</sub>SO<sub>4</sub> excreted only slightly less fecal P compared with those fed the basal diet ( $P = 0.16$  and  $P = 0.20$ , respectively). Having less P in feces means more P was digested or utilized by fish. Indeed, fish fed diets containing 3.5% H<sub>2</sub>SO<sub>4</sub> had the highest P digestibility ( $P < 0.001$ ; Table 4). Fish fed 5% HCl-, 1% H<sub>2</sub>SO<sub>4</sub>- or 5% acetic acid-supplemented diets had slightly higher P digestibility (statistically borderline) than the basal diet. Fish fed the diet containing CaCO<sub>3</sub> had significantly lower P digestibility than those on any acidified diets, but not significantly different from the control diet. Fish fed the CaCO<sub>3</sub>-supplemented diet had markedly higher fecal Ca content, but the Ca digestibility did not differ from those of the other diets (Table 4). The dry matter digestibility (not shown) was not significantly different among diets, but there was a trend, with the acidified diets having the higher digestibilities whereas the no acid and antacid diets had lower digestibilities.

#### Phylogenetic tree of gastrin and H<sup>+</sup>/K<sup>+</sup>-ATPase

Trout gastrin-like expressed sequence tag (EST) has 64%

similarity (in 117 amino acid sequence) to halibut gastrin, 57% similarity to pufferfish (fugu) gastrin (Kurokawa et al., 2003), but only ~40% similarity to trout CCKs (Fig. 2A). Trout H<sup>+</sup>/K<sup>+</sup>-ATPase-like EST is 98% homologous to flounder ATP4A (in 257 amino acid sequence), 95% homologous to human ATP4A, but only 75–78% homologous to trout Na<sup>+</sup>/K<sup>+</sup>-ATPase isoforms (ATP1A) (Fig. 2B). This EST has been reported as rainbow trout ATP4A (GenBank accession: DQ103514).

#### Molecular adaptations to exogenous acids

The steady state mRNA abundance of the gastrin-like gene did not vary with dietary acid intake (Fig. 3A). In addition, gastrin mRNA was equally distributed in the corpus (body) and antral regions of trout stomach. The SST1 mRNA was abundant in antrum, but rare in corpus (Fig. 3B). Dietary acid had no effect on SST1 expression. The antacid (CaCO<sub>3</sub>) tended to decrease SST1 mRNA abundance in the antrum. The mRNA abundance of ATP4A was high in corpus, and rare in antral stomach (Fig. 3C). The tissue distribution pattern of ATP4A was therefore opposite that of SST1. Dietary acid decreased ATP4A mRNA abundance, but dietary CaCO<sub>3</sub> did not increase ATP4A mRNA abundance. The mRNA of the two SST2 isoforms was abundant in both corpus and antral stomach (Fig. 3D,F), a distribution pattern different from that of SST1 located solely in the antrum. Expression of the two SST2 isoforms was independent of

Table 4. Fecal excretions and digestibility of phosphorus and calcium in fish fed test diets

Test diet fed	Fecal composition (% of dry feces)		Digestibility (% of intake)	
	P	Ca	P	Ca
HCl (5%)	1.047 <sup>a</sup> ±0.117	0.502 <sup>b</sup> ±0.115	61.3 <sup>b</sup> ±4.0	88.1±2.5
H <sub>2</sub> SO <sub>4</sub> (3.5%)	0.345 <sup>c</sup> ±0.037	0.438 <sup>b</sup> ±0.095	86.2 <sup>a</sup> ±1.4	89.4±2.1
H <sub>2</sub> SO <sub>4</sub> (1%)	1.063 <sup>a</sup> ±0.080	0.610 <sup>b</sup> ±0.145	56.7 <sup>b</sup> ±5.9	84.8±3.6
Acetic acid (5%)	0.874 <sup>b</sup> ±0.081	0.574 <sup>b</sup> ±0.133	58.9 <sup>b</sup> ±6.0	82.9±4.5
No-acid (0%)	1.169 <sup>a</sup> ±0.063	0.572 <sup>b</sup> ±0.118	40.3 <sup>b,c</sup> ±4.2	81.8±3.7
Antacid (5%)	1.189 <sup>a</sup> ±0.077	1.392 <sup>a</sup> ±0.211	30.1 <sup>c</sup> ±8.3	81.2±3.3

Each value represents the average of six determinations (6 fish)  $\pm$  s.e.m. Mean values in each column with different letters are significantly different ( $P < 0.05$ ). P, phosphorus; Ca, calcium.

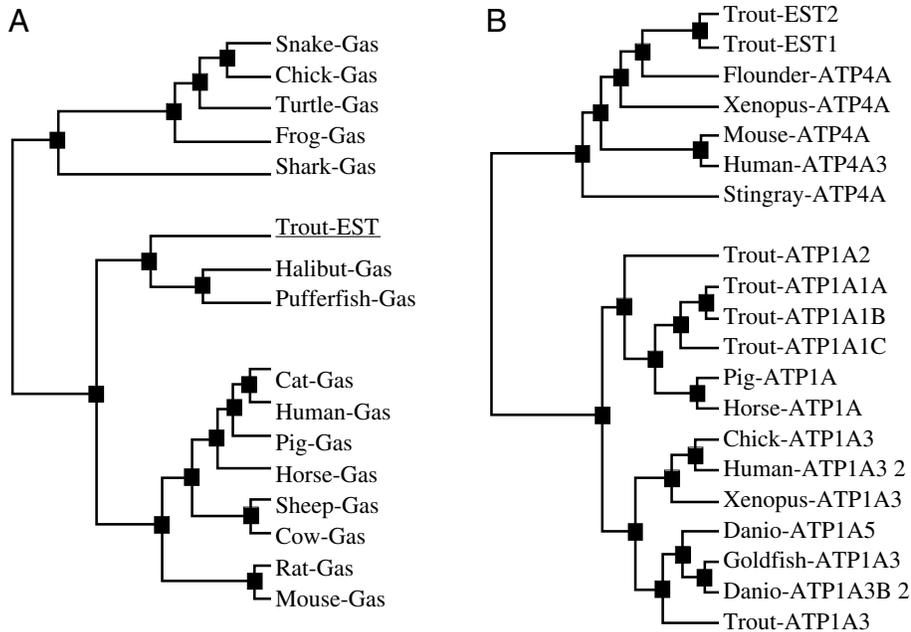


Fig. 2. Phylogenetic kinship of trout gastrin-like EST (A) and trout ATP4A-like EST (B) to those of other species. Based on the translated amino acid sequence, trout gastrin-like EST has 64% homology with halibut gastrin, and trout ATP4A-like EST is 98% homologous to flounder ATP4A. Gastrin and CCK belong to the same peptide family, but the trout gastrin-like EST belongs to a gastrin cluster that is sufficiently removed from the trout CCK cluster (not shown). Gas, gastrin; CCK: cholecystokinin; ATP4A:  $H^+/K^+$ -ATPase; ATP1A:  $Na^+/K^+$ -ATPase. Trout EST1 (gi:42752688) and trout EST2 (gi:42817423) in B are 97% homologous to one another in nucleotide base sequence.

dietary acid or antacid in the antrum and in the body of the stomach. The NBC mRNA was abundant in corpus stomach, whereas about one-third of that amount was also detected in antral stomach (Fig. 3E). In both corpus and antral stomach, the NBC mRNA abundance decreased in fish fed acidified diets. The acid-induced changes in expression of ATP4A and NBC mRNA were highly correlated to one another;  $y=0.784x+0.520$ ,  $R^2=0.708$  (Fig. 4).

Dietary acidity generally increased ( $P<0.0001$ ) dietary P digestibility, but decreased ( $P<0.0001$ ) ATP4A mRNA expression in rainbow trout (Fig. 5). The effects of acids on P digestion and ATP4A expression seem to vary depending on the kind and quantity of acid used.

## Discussion

### *Species differences in GI pH*

Even though the proton pump seems highly conserved and is distributed in similar gastric regions of fish and mammalian stomach, there are marked differences in gastric pH. The lowest observed values of postprandial gastric pH (typically ~4) in trout (Sugiura and Ferraris, 2004) were considerably higher than corresponding values in mammals (typically <2 in rat and humans) (Berne and Levy, 2000; Gardner et al., 2002; Schade et al., 1994). However, these previously observed differences in gastric acidity may be due not to differences in rates of acid secretion, but to other factors, such as meal composition. Human and rat meals are generally higher in carbohydrate and lower in protein and ash contents when compared with trout feeds. Since carbohydrates have lower buffering capacity than proteins, the high acidity of mammalian stomach may be due to the low buffering capacity of its high carbohydrate meals. To our knowledge, this study is the first to directly compare the difference in gastric acidity between fish and mammalian

species using the same feed, and eliminated the contribution of differences in diet composition to species differences in gastric acidity. We found that the postprandial stomach pH decreased progressively in rats but not in trout, again suggesting that the innate acid secretory capacity of the trout stomach may be weaker than that of rats. Our conclusions are supported by studies indicating (1) mammalian parietal cells to be functionally more capable of secreting acid than oxynticoptic cells present in fish and lower vertebrates (Koelz, 1992; Vial and Garrido, 1979), and (2) trout gastric glands being extremely slow in secreting acid after stimulation by histamine (Bomgren et al., 1998).

Though we tried to minimize potentially confounding differences (e.g. in stomach size and in age), there are of course numerous differences between trout and rats that cannot be completely controlled for in a comparative study, but that still may account for differences in gastric pH, such as differences in esophageal and gastric anatomy, feeding behavior, and body temperature. Freshwater fish drink little water (Bomgren et al., 1998); hence, there is little possibility of the medium diluting gastric acidity of the trout stomach. Additional factors are diurnal rhythm, plasma osmolarity, admixture with other secretions, and the capacity of the pump.

Intestinal luminal pH in fish is 8.4–9.0 in non-fed marine fishes (Wilson et al., 2002) and ~8.5 in rainbow trout fed and kept in freshwater (Sugiura and Ferraris, 2004). Both values are much higher than that observed in human [pH 7.5 (Fallingborg, 1999)], rat [pH 6.4–7.8 (Ward and Coates, 1987) or ~6.4 (Vanhoof and De Schrijver, 1996)], pig [pH ~5.4 (Vanhoof and De Schrijver, 1996)] and chicken [pH 5.7–6.1 (Andrys et al., 2003)] intestines. The difference in intestinal pH between fish and homeotherms has been considered to be a result of species differences in rates of bicarbonate secretion and in composition of gut microflora that typically produces acidic compounds

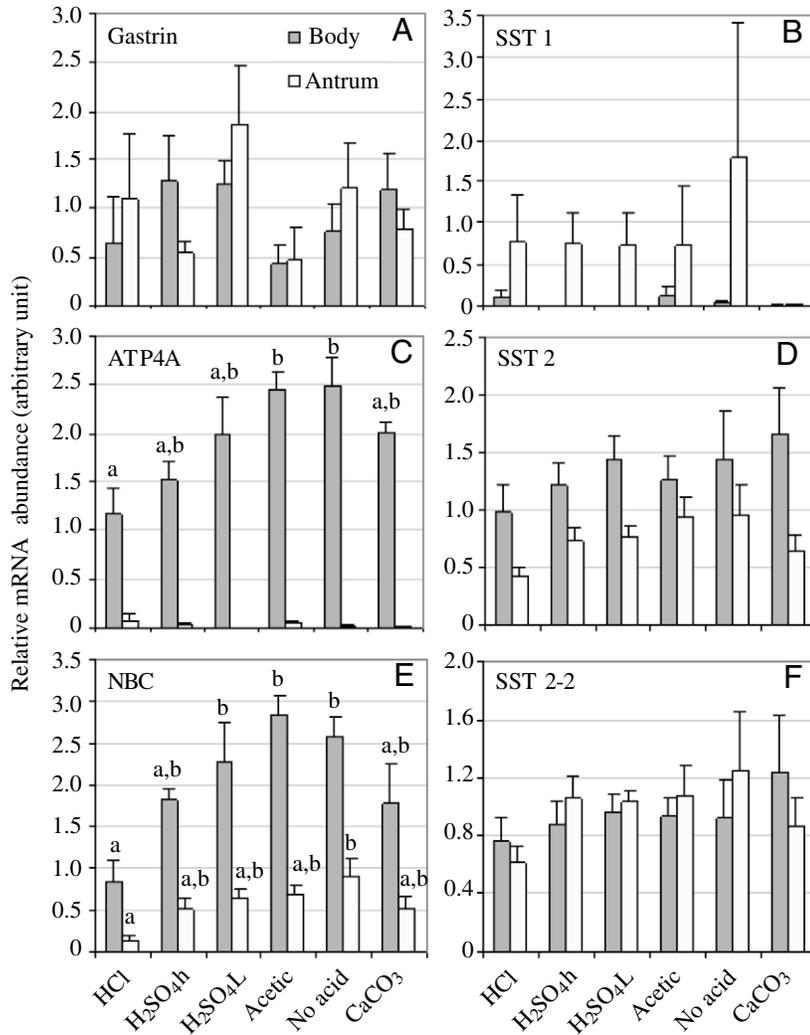


Fig. 3. Effects of various dietary acids (*x* axis) on relative gene expression (*y* axis) in the antral and corpus (body) regions of rainbow trout stomach. One of the following acids or antacid was added to the basal diet (No acid) (w/w). HCl: 12 mol l<sup>-1</sup> HCl 5.0%; H<sub>2</sub>SO<sub>4</sub>h, 18 mol l<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> 3.5%; H<sub>2</sub>SO<sub>4</sub>L: 18 mol l<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> 1.0%; acetic: glacial acetic acid 5.0%; antacid: CaCO<sub>3</sub> 5.0%. No acid: basal diet (see Table 1). Gray bars represent data of corpus stomach; white bars represent data of antral stomach. Each column represents the average (+ s.e.m. as error bar) of four fish. Columns within each stomach region labeled with different letters are significantly different (*P*<0.05). ATP4A, H<sup>+</sup>/K<sup>+</sup>-ATPase; SST, somatostatin; NBC, Na<sup>+</sup>/bicarbonate cotransporter). Differences of mRNA abundance between antral and corpus stomach (all treatments inclusive) were as follows: Gastrin-like gene (*P*=0.8); ATP4A (*P*=0.0003); SST1 (*P*=0.02); SST2 (*P*=0.002); SST2-2 (*P*=0.8); NBC (*P*=0.0007).

in homeotherms (Ward and Coates, 1987). Interestingly, when fish and rats were fed the same diet in the present study, they had similar intestinal pH. Additional research will be required to identify factors controlling the intestinal pH.

#### Feeding activity and P digestibility

Although dietary organic acids improve P digestion in fish food (Vielma et al., 1999), inorganic acids are much less expensive, and are therefore the preferred solution to the feed industry problem of low P digestibility in fish diets. Inorganic acids, however, may depress food intake as was observed in this and previous studies. In pigs, use of inorganic acids as dietary supplements has not been successful because the acids severely depressed feed intake (Ravindran and Kornegay, 1993). In chickens, however, H<sub>2</sub>SO<sub>4</sub> did not appear to reduce feed intake at 1.2% (w/w) dietary level but significantly reduced intake at 2.4% or greater (Capdevielle et al., 1996; Pritzl and Kienholz, 1973). The pH of the 2.4% diet was reduced to 3.6. Chicks were also tolerant of dietary HCl up to 0.12 mol HCl kg<sup>-1</sup> feed (~1.18% w/w hydrochloric acid per feed), but at 0.24 mol kg<sup>-1</sup> feed, which decreased dietary pH to

4.6, depression of growth and feed conversion became significant (Pritzl and Kienholz, 1973). In rats, the feeding tolerance to dietary acidification appears to be much higher; i.e. up to 0.6 mol HCl kg<sup>-1</sup> feed (~5.2% hydrochloric acid) with dietary pH of 2.84 appears to cause no detectable reduction of feed intake (L'Estrange and Upton, 1976).

In fish, a preliminary study (unpublished) indicated that acidifying fish meal-based diet with sulfuric acid (3.75%) increased P digestibility by 25%, so that fecal P excretion decreased markedly. Fish consumed the acidified diet (pH 2.4) readily for 23 days of feeding with only a slight reduction in feed intake. In the previous study, however, moist diets were used, whereas in the present study, dry diets were used.

The dietary acids and antacid did not dramatically change the pH of the GI content at any section, suggesting that trout regulate endogenous acid and/or bicarbonate secretion to avoid marked deviations from normal gut pH. HCl supplementation increased P digestibility only modestly, probably because it might have reduced endogenous acid secretion by directly inhibiting ATP4A mRNA expression and the abundance of the proton pump. Other acids do not markedly decrease ATP4A

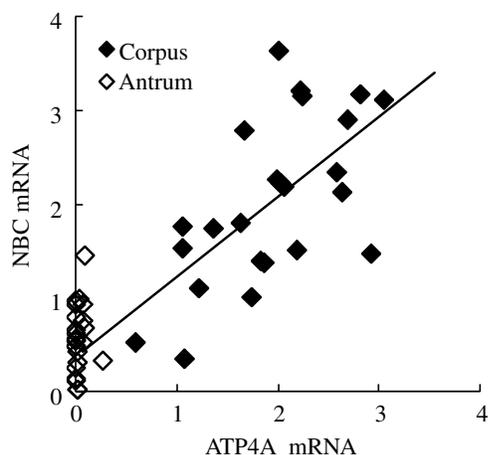


Fig. 4. Correlation between ATP4A ( $H^+/K^+$ -ATPase, the proton pump) and NBC ( $Na^+$ /bicarbonate cotransporter) mRNA abundance in the antral and corpus stomach of rainbow trout. Each dot represents one sample. One antral and one corpus sample was collected from each fish. In the antrum (open diamonds), ATP4A mRNA is nearly absent, whereas small amounts of NBC mRNA are present. In the corpus (filled diamonds), both ATP4A mRNA and NBC mRNA are abundant and their abundances are correlated to one another (regression of corpus ATP4A to corpus NBC:  $y=0.86x+0.36$ ;  $R^2=0.40$ ). Units of  $x$  and  $y$  axes are relative (average of all data points is 1).

mRNA levels, and therefore may be more effective than HCl in increasing P digestibility.

Apart from dietary pH, acidification might be more important in converting hydroxyapatite-P into more soluble (more absorbable) di- or mono-calcium phosphates. If this is the case, even though the dietary pH was not greatly different among treatments, the form of P in acidified diets might have changed into more readily available forms, explaining the enhanced P digestibility even as luminal pH did not change dramatically. Indeed our previous data indicate that acidification greatly increases the solubility of bone P in vitro (see Sugiura and Hardy, 2000).

Stomachless fish, such as carp, may even have a much lower ability to digest P in fish meal. Thus, dietary acidification could be more effective for stomachless fish than it is for trout.

#### *Molecular adaptation to exogenous acids*

In mammals, the key player in gastric acid secretion is  $H^+/K^+$ -ATPase (also known as proton pump or ATP4A/B) located in cytoplasmic tubulovesicles and apical secretory canaliculi of parietal cells. The number of proton pumps in the apical membrane of parietal cells is highly regulated, and changes markedly during a single meal (Samuelson and Hinkle, 2003). However, there may be long-term regulation of baseline levels of proton pumps in parietal cells, and this type of regulation should be distinguished from the well-known acute regulation of acid secretion during meals. Because tissues were collected from fish starved overnight, our findings reflect

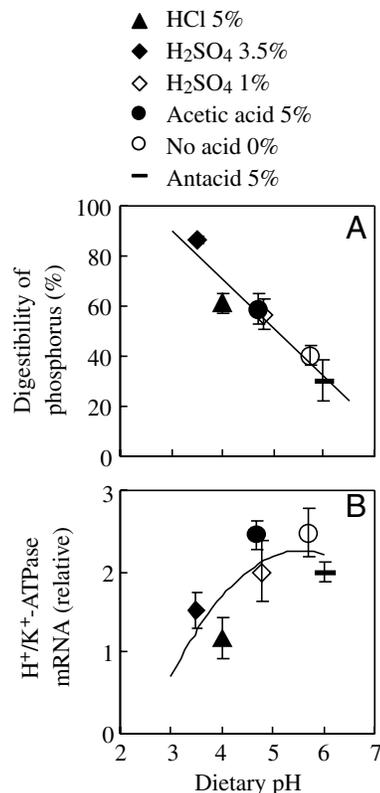


Fig. 5. Dietary acidification increases phosphorus digestibility (A) but decreases  $H^+/K^+$ -ATPase mRNA expression (B) in rainbow trout. Each dot represents the mean ( $\pm$  s.e.m.) of six fish for phosphorus digestibility measurements, and four fish for  $H^+/K^+$ -ATPase mRNA measurements. Legend shows dietary treatments (acids used). Significance of dietary acidification on phosphorus digestibility ( $P<0.0001$ ) and  $H^+/K^+$ -ATPase mRNA expression ( $P<0.0001$ ) was evaluated by linear regression.

chronic adaptations to acidification and are not confounded by observed differences in feeding activity.

The decrease in steady state ATP4A mRNA abundance induced by chronic dietary acidification suggests that trout ATP4A synthesis could be inhibited by long-term increases in concentration of its product, luminal  $H^+$ . Consumption of the  $H^+/K^+$ -ATPase inhibitor omeprazole for 3 days reduces gastric acidity but increases transcription rate and abundance of rat  $H^+/K^+$ -ATPase mRNA (Tari et al., 1991). Expression and activity of the related transporter  $Na^+/K^+$ -ATPase in the gills and kidney of aquatic species appears to also be regulated by its substrate  $Na^+$  in the environment (Furriel et al., 2000; Lin et al., 2004).

It is not clear why dietary antacid ( $CaCO_3$ ) did not increase trout ATP4A mRNA abundance since it also alters  $H^+$  concentrations. In hypergastrinemic rats, chronic intake of antacid [ $Al(OH)_3$  and  $Mg(OH)_2$ ], for unknown reasons, seems to reduce the number of parietal cells (Koop et al., 1988). Gastric acid and fasting markedly decrease gastrin mRNA abundance in rats and humans (Dockray et al., 1993; Sandvik

et al., 1993). Conversely, refeeding of fasted rats dramatically increases gastrin and decreases SST mRNA abundance within 0.25 to 1 h (Wu et al., 1991). These observations in mammals indicate that gastrin and SST mRNA abundance is regulated in response to acute changes in gastric acidity or to feeding. In the present study, we examined steady state (or unfed) mRNA abundance, which could be one of the reasons why levels of gastrin-like, SST1, SST2' and SST2'' mRNA did not change.

Dietary acidification was expected to increase mucous bicarbonate secretion, which is necessary to protect the stomach wall from excessive acid in the lumen. However, NBC expression decreased with increasing acidification. The presence of NBC in the antrum, where oxynticopeptic cells are absent, suggests that NBC is in mucous cells in the antrum, or in both mucous and corpus-located oxynticopeptic cells. NBC is generally the basolateral bicarbonate transporter for the mucous cells to import bicarbonate from the interstitium into the cells for subsequent secretion into the mucous gel layer *via* an apical  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (Allen and Flemstrom, 2005). The lower expression of NBC induced by dietary acidification could result from decreased availability of bicarbonate in the interstitium caused by the suppression of endogenous acid secretion. In other words, having the endogenous acid production suppressed by dietary acid intake, as implied by the decrease in ATP4A mRNA, the oxynticopeptic cells now produce less bicarbonate and consequently less alkaline tide, decreasing the amount of bicarbonate in the interstitium. In this model, NBC expression must be coordinated with ATP4A. The apical bicarbonate transporter of gastric mucosal cells has been identified only recently in mammals (Xu et al., 2005). The putative transporter, SLC26A9, however, has no sequence homology to any of the available EST of trout or other fish species.

#### *Phylogeny of trout gastrin and $\text{H}^+/\text{K}^+$ -ATPase*

##### *Gastrin*

There is no gastrin sequence available for trout, but a gastrin-like sequence is present in a trout EST database. Our result shows that the mRNA was equally abundant in both antral and corpus stomach, which contrasts with the distribution of the gastrin-secreting G-cells in mammals. Comparative sequence analysis, however, reveals that this gastrin-like EST of trout is most closely related to the known gastrin sequences of two teleosts, halibut and pufferfish. Homology with trout CCK is much lower. Teleostean gastrin is more closely related to mammalian gastrin and to teleostean CCK than reptilian, amphibian, avian or elasmobranch gastrin.

##### *$\text{H}^+/\text{K}^+$ -ATPase*

The earliest phylogenetic appearance of gastric acid secretion occurs in cartilaginous fish. Despite the sizable phylogenetic distance, a C-terminal antibody against the pig proton pump ATP4A exhibited strong immunoreactivity against oxynticopeptic cells of the Atlantic stingray, suggesting structural similarities in ATP4A between primitive

cartilaginous fishes and mammals (Smolka et al., 1994). ATP4A sequence information described below supports this histochemical finding. There is no ATP4A sequence available for trout, but an ATP4A-like EST was identified and its unique distribution in the stomach verified to be similar to those of other species. Moreover, the mRNA abundance of this ATP4A-like EST was downregulated by exogenous (dietary) acid. Since the phylogenetic kinship to other ATP4A from different species was very high, this EST most likely represents the trout proton pump sequence. The ATP4A or  $\text{H}^+/\text{K}^+$ -ATPase sequences clearly belonged to a group markedly different from the ATP1A or  $\text{Na}^+/\text{K}^+$ -ATPase cluster. For this protein, homology seems to decrease as would be expected with increasing phylogenetic distance, as trout ATP4 was more closely related to that of flounder, followed by amphibian and then mammalian ATP4.

#### *Practical considerations and perspectives*

Most fish species, including rainbow trout, have only a limited ability (~10–80%) to digest P in fish meal (Cho and Bureau, 2001), whereas birds and mammals can digest nearly 100% of P in fish meal (Soares, Jr, 1995). Excreted P from aquaculture facilities is a major pollutant in freshwater environments. Improving the digestibility of P in fish feeds can reduce environmental pollution. The mechanism underlying the P digestibility problem was assumed, and is now confirmed, to be the weak acidity of fish stomachs; hence, dietary acidification was thought to overcome this limitation. However, we have now shown that the use of exogenous inorganic acids inhibits  $\text{H}^+/\text{K}^+$ -ATPase expression and may decrease endogenous acid secretion. Certainly, the acute effects of dietary acidification must be distinguished from chronic effects. Additional studies also need to be done at the protein and functional level to confirm that the consumption of exogenous acids does lead to decreases in acid secretion. The use of dietary organic acids may provide an alternative solution to this important industry problem.

Future studies immunolocalizing  $\text{H}^+/\text{K}^+$ -ATPase and determining its site density per oxynticopeptic cell, or the number of oxynticopeptic cells per stomach should provide detailed mechanisms underlying the low gastric acidity of trout stomach. Perhaps this physiological limitation, along with lower body temperatures, contributes to the slow digestion of food in fish.

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