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Research Article

Digestive coordination of the gastric function in Atlantic salmon *Salmo salar* juveniles

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ABSTRACT. Artificial diets have been reported to produce suboptimal pH values in the stomach of salmonid species. Therefore, it is interesting to investigate the gastric function of different species fed commercial diets. In the present work, two groups of Atlantic salmon juveniles were either fasted or fed with a commercial diet. The gastric and duodenal pH values were measured over a 25 h post-prandial period and, dry matter, moisture and protease activity in the gastric chyme of the fed group were also determined. In the fed group, the gastric pH dropped to 3.5, and duodenal pH increased from 7.0 to 7.5 at the 4th h post-feeding, whereas fasted fish showed no significant changes in the gastric pH or duodenal pH. Fed animals showed: i) two discrete events of evacuation, before the 2nd hand after the 8th h of digestion, ii) a change in the moisture of the gastric chyme from less than 10% in the diet to 60% at the 2nd h post-feeding, and iii) a maximal gastric proteases activity of 600 U mL^{-1} at the 2nd h followed by a nearly constant of value of ~300 U mL^{-1}. It can be concluded that the juveniles of the Atlantic salmon can suitably acidify a commercial diet to a value generally accepted as optimal for gastric proteases of fish, and to retain about 2/3 of the ingested diet under such conditions and at a nearly constant protease activity until the 8th h of digestion.

Keywords: *Salmo salar*, gastric pH, gastric proteases, gastric evacuation, commercial diet, diet buffering capacity.

INTRODUCTION

Understanding the mechanisms involved in the gastric function and their temporal coordination is necessary to design feeds for cultured fish with a stomach suitably. The digestive tract of most cultured species is not evolved to deal with commercial aquafeeds commonly high in dry matter, nutrient concentration and buffering capacity. Moisture of aquafeeds is usually below 10% (Kraugerud *et al*., 2011) whereas the water content of natural prey is over 70% (Ciancio *et al*., 2007). Therefore, the stomach of fish fed a commercial diet is challenged regarding moisturizing and acidifying capacities.

The stomach not only functions as an acidic-proteolytic organ in nearly all vertebrates (Koeltz, 1992) but also as a mixer and temporary storage of nutrients which feeds the intestinal reactor in an organized way (Schulze, 2015). In doing so, the first requirement is to turn the ingested food into a sufficiently fluidized mass (the chyme) that allows the regulated discharge of nutrients towards the duodenum. However, the dryness of aquafeeds forces the animal to get an extra quantity of water from gastric secretions or by drinking it (Kristiansen & Rankin, 2001), although the dry pellets used in aquaculture typically rehydrates up to 25-30% in 1-2 min before being eaten, depending on the culture system and the species produced (Kristiansen & Rankin, 2001; Misra *et al*., 2002; Bucking & Wood, 2006). This rehydration period may or may not cause a delay in the gastric evacuation in some species (Ruohonen *et al*., 1997; Chatzifotis *et al*., 2005) and also a change in the ingestion rate (Otterå *et al*., 2003) and/or the productive indexes (Chatzifotis *et al*., 2005). However, a general pattern associated with this process cannot be easily envisaged from the bibliographic data. Together with the dryness, aquafeed pellets are typically high in buffering capacity (BC).

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The BC is an essential characteristic of the diet in the field of livestock feed science (Mohammadpour *et al*., 2014). Low BC has been defined as a characteristic of gut-friendly diets for sustainable animal production (Celi *et al.*, 2017), and can be particularly important for animal nutrition and health during the weaning phase of terrestrial monogastrics (Partanen & Mroz, 1999). A list of BC values of ingredients used in diets for weaning pigs has already been published (Lawlor *et al*., 2005), and it is known that the BC of complex mixtures can be reasonably inferred from BC's of the ingredients (Lu *et al.*, 2016).

On the contrary, the BC of aquafeeds has been poorly studied despite the limited acidifying capacity of fish species (Sugiura *et al*., 2006). When juveniles of the rainbow trout *Oncorhynchus mykiss* were fed a commercial diet (41% crude protein, 11% crude lipid), suboptimal values (above 4.5) of the gastric pH were recorded for many hours post-feeding which, according to the researchers, was attributed to the BC of the diet (Bucking & Wood, 2009). Thus, these two factors, dryness and BC, simultaneously affect at least two physiological variables determining the gastric physiology in a tightly combined way: evacuation and pH patterns over time.

To improve the understanding of the gastric function in cultured salmonids, it seems prudent to investigate the coordination among the most relevant variables impacting the gastric processing of proteins in fish of different species under experimental conditions resembling that of industrial cultures. The present work was designed attending that aim, and provide precise data on the coordination among the patterns of evacuation, pH and protease activity in the gastric chyme of the Atlantic salmon *Salmo salar* juveniles fed a commercial diet.

MATERIALS AND METHODS

Animal handling

Fish were stocked at the hatchery facilities of the Escuela de Acuicultura (Universidad Católica de Temuco, Chile) in 500 L tanks (<6 kg m^{-3}). Animals were reared in a run-away freshwater system (13-15°C) and fed a commercial diet (Ewos Parr: 43% crude protein, 17% lipid, 10% ash and 3% crude fiber) once a day in the morning to apparent satiation. The tanks were regularly cleaned from feces and food debris, covered with a plastic cap and maintained under non-stressful conditions. Before the experimental procedures, two batches of juvenile fish (58.3 ± 0.9 g, mean ± standard error) were stocked in separate tanks (500 L) and subject to a fasting period according to Bucking & Wood (2009) to assure the complete emptying of the digestive tract. Fish were deep anesthetized with benzocaine 0.03% and subject to euthanasia by cranial puncture to extract digestive organs animals were handled in compliance with Chilean legal requirements for experimentation with live animals (Ley 20.380, MINSAL).

Experimental design and sampling procedures

The experiment followed the usual design published by researchers monitoring digestive physical or chemical variables by sequential sampling (Deguara *et al*., 2003; Bucking & Wood, 2009; Márquez *et al*., 2012). After the fasting period, the first tank (40 fish) were sampled sequentially (without being fed) from 9:00 am and at the 2^{nd}, 4^{th}, 6^{th}, 8^{th} and 25^{th} h from the first sampling; 5-7 fish were netted and anesthetized per sampling point. This tank was used as a control to discard circadian rhythms in the gastric and duodenal pH in the absence of food.

The second tank (50 fish) was similarly sampled but fed one meal (50 g of the Ewos Parr diet, ~1.7% of the total biomass) in the morning (09:00 am). After feeding, fish were sequentially sampled at the 2^{nd}, 4^{th}, 6^{th}, 8^{th}, and 25^{th} h; 8 fish were netted and anesthetized per sampling point. The second tank contained more fish just in case some of them ingested no feed; nevertheless, all the sampled animals presented evidence of feed in the gut. The temperature was recorded during the sampling procedures and varied from 13.8-14.0°C at 9:00 am to 14.8-14.9°C at 17:00 pm.

Fish were anesthetized and euthanized, and the abdominal cavity immediately exposed. The values of gastric and duodenal pH were measured *in situ* using a narrowly-tipped pH probe (Hanna Instruments, HI 1083) calibrated at the temperature of the tank water. Gastric pH was taken at the antral stomach, whereas duodenal pH was recorded between the pylorus and the first pyloric caeca. Subsequently, the whole stomach together with the contents was dissected, weighed and stored at -20°C until further processing. Subsequent handling of the stomach and gastric contents was always done on the ice. Stored stomachs were cut open from the cardiac to the pyloric sphincter while thawing. The contents were extracted as a single block whenever possible and weighed, homogenized by hand, and a sample separated into a weighed Eppendorf. The average moisture of the gastric chyme was calculated from the loss in weight of the sample after being dried for 24 h at 105°C. An additional sample of gastric chyme was separated, mixed with deionized water in proportion 1:1 (w:vol), centrifuged at 15.800 g and 4°C for 5 min, and the supernatant fluid phase aliquoted and stored at -20°C until the measurement of acid protease

activity. The dilution of the gastric fluid phase due to the addition of water (before the centrifugation of the sample) was calculated from the moisture of the gastric content:

Dilution factor = (% moisture + 100)/(% moisture).

The dilution factor was then used to estimate back the protease activity for 1 mL of undiluted gastric fluid.

The activity of gastric proteases

The activity of gastric proteases in the fluid phase of the chyme was measured following the protocol of Anson (1938) with modifications. To assay the protease activity, an aliquot of gastric fluid phase was thawed and maintained on ice; 10 µL of gastric fluid phase were assayed on 1 mL of bovine haemoglobin 0.5% (w/vol) in glycine 100 mM buffer at pH 2.5, which is very close to the optimum pH for protease activity of *S. salar* gastric extracts (Krogdahl *et al.*, 2015), and 25°C for 30 min. The reaction mixture was stopped with 0.5 mL of trichloroacetic acid (TCA) 20%, centrifuged at 15.800 g and 20°C for 15 min, and the concentration of TCA-soluble, tyrosine-containing peptides in the supernatant was evaluated spectrophotometrically at 280 nm. For each, two replicas and one blank assay (in which the gastric extract was added after the TCA) were performed. The protease activity was calculated from the difference in absorbance between the replicas and the blank assay. The extinction coefficient of L-tyrosine at 280 nm was set at 0.008 mL μg^{-1} cm^{-1}, and the unit of activity was defined as 1 µg of L-tyrosine released per minute and mL of gastric fluid phase.

Buffering capacity of the diet

The titration of the commercial diet was performed by mixing and continuously stirring 1 g of the diet with 10 mL of distilled water at 21°C until a stable pH was recorded. From this point, aliquots of 2.5 µL of a commercial HCl solution (~12.08 N) were added at 1 min intervals to the mixture, and the pH was also taken at 1 min intervals after each addition of HCl. The procedure was repeated in triplicate until reaching pH 2.8. The buffering capacity (BC) at a given pH was defined as the slope of the titration curve per gram of diet (mEq^{-1} g^{-1}). The acid-binding capacity (ABC) to a given pH value was defined as the cumulative addition of H^+ per unit of mass to get that pH (mEq g^{-1}).

Statistics

Average data in figures are expressed as means ± sem. Data for gastric and duodenal pH and weight, moisture and protease activity of gastric contents were explored to check the requirements of residual normality and homoscedasticity. Moisture percentages of the gastric contents were transformed with the arcsin(sqrt(x)) function before being statistically analysed. During the graphic representation of the titration curve (Fig. 1a) and the buffering capacity curve (Fig. 1b) of the diet, 4th and 3rd order polynomials were respectively fitted to the data for interpolation purposes.

Temporal patterns in the dependent variables were evaluated with a one-way ANOVA by time plus a Bonferroni *post-hoc* test for normal and homoscedastic data, a Kruskal-Wallis test by time plus a Bonferroni *post-hoc* test for non-normal and homoscedastic data, and with a robust Welch test by time plus a Games-Howell *post-hoc* test for non-homoscedastic data. The significance level was always set at 0.05.

RESULTS

Acid-binding and buffering capacities of the diet

The initial pH of the diet was slightly acidic, 6.03. The commercial diet was most easily acidified at the beginning of the titration process and gained in buffering capacity as pH dropped (Figs. 1a-1b). Thus, the titration curve showed a concave profile and a remarkable tendency towards linearity from pH 4.0

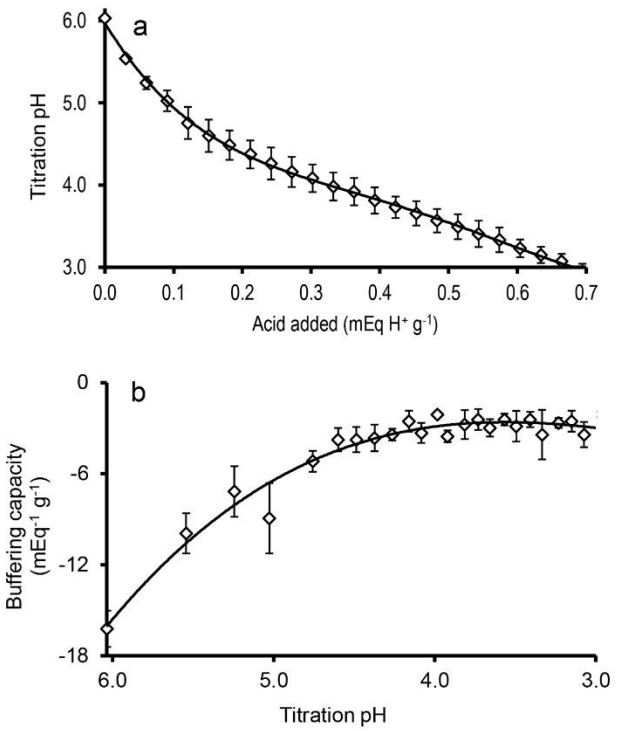

Figure 1. Titration and BC curve of the commercial diet used in the experiment. a) Titration curve of the diet; the continuous line represents a 4th degree polynomial fitting, b) buffering capacity (BC) of the diet as a function of the pH of the diet; the continuous line represents a 3rd degree polynomial fitting. Bars represent standard errors of the means.

onwards, where the BC value was nearly constant at -2.7 units of pH per mEq H^+ per g of diet. According to the 4th degree polynomial fitted in Figure 1a, the acid-binding capacities to pH values 4.0 (ABC-4) and 3.0 (ABC-3) can be estimated to be about 0.3 and 0.7 mEq of H^+ per g of diet respectively or, in relation to dietary proteins, 0.7 and 1.6 mEq of H^+ per gram of dietary protein respectively.

Temporal patterns in the gastroduodenal pH

The temporal patterns of gastroduodenal pH under fasting and feeding conditions are shown in Figure 2. Fasting animals did not produce any statistical difference in gut pH throughout a digestion cycle of 25 h (Figs. 2a-2c), average gastric and duodenal pH values being 3.20 ± 0.23 and 7.48 ± 0.05 respectively.

On the contrary, gastric and duodenal pH of fed animals presented statistical differences over time (Figs. 2b-2d). The gastric pH dropped during the first 4 h of digestion, from over 6.0 (diet pH) to about 3.44 ± 0.14, and this value was maintained for at least another 4 h. The duodenal pH was much less variable than the gastric one; however, it showed a significant increase from 7.05 ± 0.08 at the 2nd h post-feeding to 7.40 ± 0.03 at the 25th h post-feeding.

Temporal patterns in gastric dry matter content and moisture

Provided that no remains of food are detected after feeding, mean ingestion of 0.91 g of dry matter per fish was estimated. From the second to the eighth hour of digestion, the dry matter of the gastric contents remained statistically constant at 0.52 ± 0.03 g. The gastric dry matter content changed to 0.15 ± 0.04 g, between the eighth and the 25th h of digestion (Fig. 3a).

Temporal pattern in protease activity of the gastric chyme

The temporal changes of the protease activity present in the fluid phase of the gastric chyme are plotted in Figure 4. The maximal value was observed at the beginning of the digestion process (about 600 U mL^{-1}). Afterward, acid protease activity was maintained between 300-400 U mL^{-1}, although data pointed to a transient increase at the 6th h post-feeding.

DISCUSSION

The titration pattern of the commercial diet used in the present investigation was qualitatively similar to those reported for aquafeeds close in proximate composition (Márquez *et al*., 2012). The BC is low in the neutral zone of the titration curve, rose towards the acidic zone and finally level off from pH 4.0-4.5 onwards. This plateau most probably indicates the content of glutamic and aspartic residues in the proteins of the diet (Al-Dabbas *et al*., 2010), whose pKa's are in the range 3.0-4.0 (Toseland *et al*., 2006). It can be concluded that the general buffering characteristics of aquafeeds allow for fast acidification of the ingested diet during the first hours of digestion in *S. salar*. However, the importance of diet composition on the minimum pH reached in the digestive cycle is not evident. For example, in *Sparus aurata* juveniles, an experimental diet deficient in BC led to a minimal pH (*ca*. 4.6) very similar to that measured for a commercial-type diet (*ca*. 4.8, dietary BC 10 times higher), although the time to the minimal pH was 0.5 h in the former and over 4 h in the latter (Márquez *et al*., 2012). Additional research with *Sparus aurata* juveniles showed similar values for the pH of gastric chyme (Morales *et al.*, 2014). It is probable that the minimum gastric pH in fish is endogenously regulated to avoid mucosal damage, even at the expense of the pepsin activity. Indeed, a feedback mechanism controlling the gastric pH has been described in vertebrates (Goo *et al*., 2010). Within the salmonids, data previously published on juveniles of *Oncorhynchus mykiss* fed a commercial diet *ad libitum* in freshwater (10-13°C), indicating that the postprandial pH in the gastric lumen of the rainbow trout can be arrested at values between 4.0 and 5.0 for many hours (Bucking & Wood, 2009), which is far from the optimal pH for the activity of Atlantic salmon gastric extracts on hemoglobin (Krogdahl *et al.*, 2015). This fact raises the question of whether the gastric function always has a significant role in the digestion of dietary proteins in salmonids. However, recent work showed that some of the pepsin forms in the rainbow trout could retain most of the enzymatic activity at pH 5.0 (Wald *et al.*, 2016). Many fish species possess at least two types of pepsin with different isoelectric points and pH optima (Gildberg, 1988). One of the pepsin types may exhibit a relatively high pH optimum (*ca*. 5.0) when acting on myofibrillar proteins (Gildberg & Raa, 1983).

On the other hand, the results of the present work show that the temporal evolution of the gastric pH in *S. salar* can be different from that in *O. mykiss*. The most significant fact is the steady rate of gastric acidification during the first four hours of digestion in *S. salar* juveniles until reaching a stable value of ~3.5. Alternatively, these differences in the gastric physiology may also be the result of variations in the experimental conditions between both studies: 10-13°C *versus* 14-15°C, feeding *ad libitum versus* 1.7% BW, and 300-400 g trout *versus* 60 g salmon; further studies are needed to cover additional commercial diets and fish species.

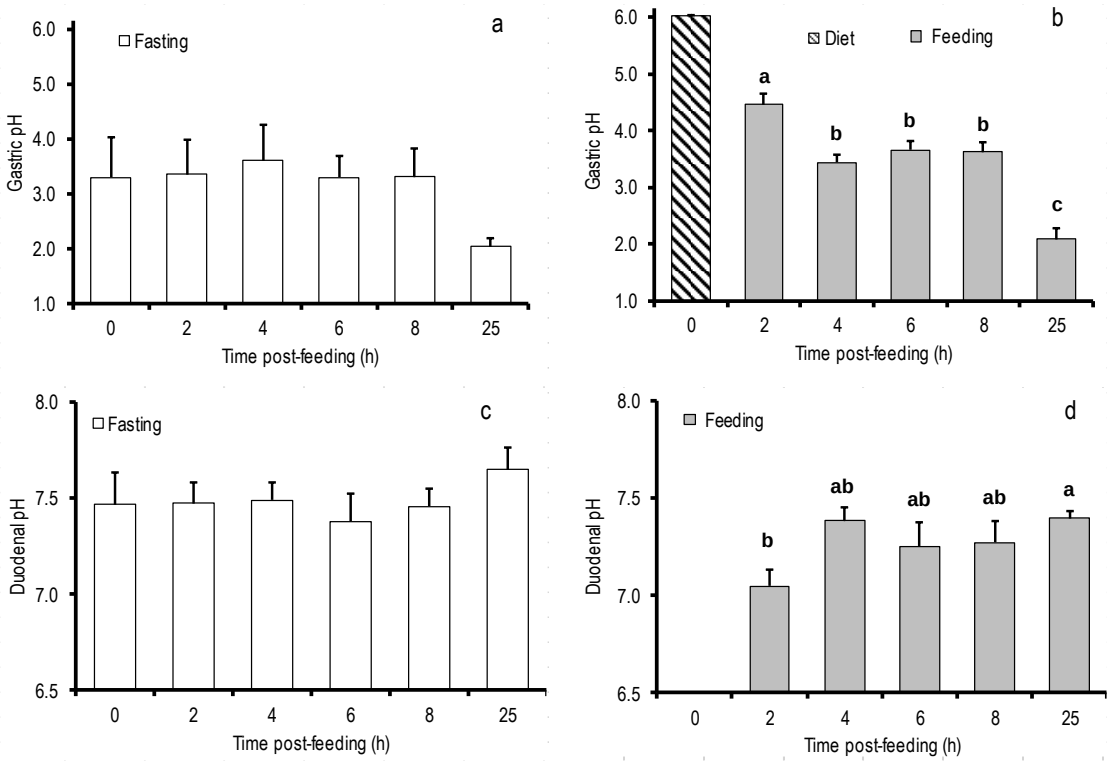

Figure 2. Temporal patterns of the gastroduodenal pH. a) Average gastric pH under fasting conditions, b) average gastric pH after feeding a commercial diet; the hatched column at time zero represents the pH of the diet before the feeding trial; grey columns represent the gastric pH; means of gastric pH significantly different at $P = 0.05$ do not share letters, c) average duodenal pH under fasting conditions, and d) average duodenal pH after feeding a commercial diet; duodenal means significantly different at $P = 0.05$ do not share letters. The composition of the diet used in the experiment was 47% crude protein and 17% crude lipid. Bars represent standard errors of the means.

Another important consequence of the gastric pH is the impact on the duodenal pH following gastric emptying episodes. In the present work, over one-third of the ingested dry matter is discharged into the duodenum within the first two hours of digestion, when the gastric chyme dropped its pH from 6.0 to 4.5. The duodenal pH must be under control to prevent the irreversible denaturation of intestinal enzymes, *e.g*., trypsin from the rainbow trout is irreversibly inactivated at pH values below 5.0 (Kristjánsson, 1991). In the case of Atlantic salmon juveniles, the duodenal pH after at the second hour of digestion is about 7.0 which implies: i) an actual impact of the discharge of gastric chyme on the duodenal pH (to the best of our knowledge, this is the first time such a phenomenon is reported in fish); ii) a secretion rate of HCO_3^- into the duodenal cavity suitable to limit the change in pH to only 0.5 units in average. Since individual values of the duodenal pH at the 2nd h ranged from 6.7 to 7.4, it can be concluded that trypsin molecules are safe from being irreversibly denaturized in the duodenum and tryptic activity remained in the interval 30-70% of the maximal activity according to Kristjánsson (1991).

The postprandial pattern of the secretion of gastric proteases found in the present investigation is in close keeping with data reported by Einarsson *et al*. (1996) on juveniles of the Atlantic salmon weighing 22-45 g and fed a commercial diet (48:22, crude protein: crude lipid) in a single meal *ad libitum* and at 13°C. The authors used azocasein as pepsin substrate and set the pH of the protease assay at 4.25. In both studies, the activity in the chyme peaked two hours post-feeding, dropped to about 2/3 of the peak at the 4th h and approached to 2/5 of the peak after 25 h of digestion. In the present work, a possible but not statistically clear increment in the activity was detected at the 6th h post-feeding. This pattern suggests an initial injection of proteases just after food ingestion, followed by a dilution or washing-out period, perhaps accompanied by secondary events of protease secretion to the lumen. The dilution of the gastric juice can be explained by the entry of water into the gastric lumen from exogenous sources (Kristiansen & Rankin, 2001). Previous work

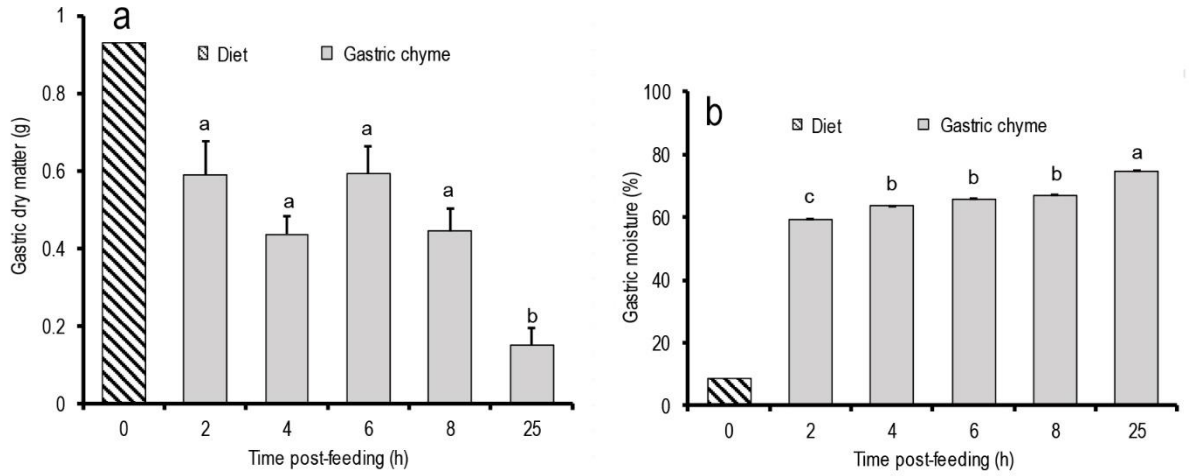

Figure 3. Temporal patterns in dry weight and moisture of the gastric contents. a) The dry weight of gastric contents (g); the hatched column at time zero represents the estimated mean ingestion of food per fish; means significantly different at $P = 0.05$ do not share letters, b) moisture of gastric contents (%); the hatched column at time zero represents the moisture of the commercial diet; means significantly different at $P = 0.05$ do not share letters. Bars represent standard errors of the means.

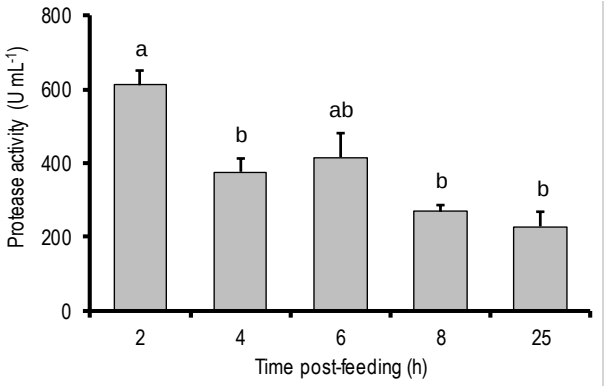

Figure 4. Temporal pattern in the activity of acid proteases from the fluid phase of gastric contents. Means significantly different at $P = 0.05$ do not share letters. Bars represent standard errors of the means.

pointed to a faster evacuation of the liquid phase of the gastric chyme when compared to the solid phase (Bucking & Wood, 2006), which can promote the washing-out of the protease secreted early in the digestion process. The significant reduction in protease activity by 1/3 between the 2nd and 4th h of digestion, when the increment in moisture is negligible, and no emptying of dry matter was detected, can be interpreted as evidence for such a process. At this point, it is interesting to note that the "gastric closure" phase (in terms of dry matter) extended for at least six hours (from 2nd to 8th h of digestion), so that 2/3 of the ingested solids was exposed to a rather constant protease activity under optimal or nearly optimal pH values (~3.5). Finally, a strong capacity of the juvenile salmon stomach for moisturizing dry diets in a short period of time is supported by the present data, from <10% to 60% in two hours, which is only a slightly lower moisturizing rate than that reported by Hughes & Barrows (1990) for *S. salar* juveniles (from 9% to 68% in two hours). This moisturizing capacity should be necessary for the mixing process of nutrients and gastric secretions.

CONCLUSIONS

To sum up, the data here presented to make it possible to build a minimum model about the coordinated function of the stomach of *S. salar* at an environmental temperature of 14°C (Fig. 5). Juvenile animals moisturize ingested feed to over 60% by the 2nd h of digestion while secreting an initial high dose of gastric proteases. Gastric emptying takes place in discrete phases, with an episode comprising about 1/3 of the ingested dry matter also before the 2nd h of the digestion, when the gastric pH is in the suboptimal range for pepsin. During this episode, the duodenal pH drops by 0.5 units. Afterward, a period of gastric closure regarding the dry matter is established for 6 h, throughout which the gastric pH reaches a value very close to the pepsin optimum. Finally, the bulk of the remaining gastric dry matter is evacuated from the 8th h of digestion onwards so that, by the 25th h it is possible to detect individuals with a wholly emptied stomach.

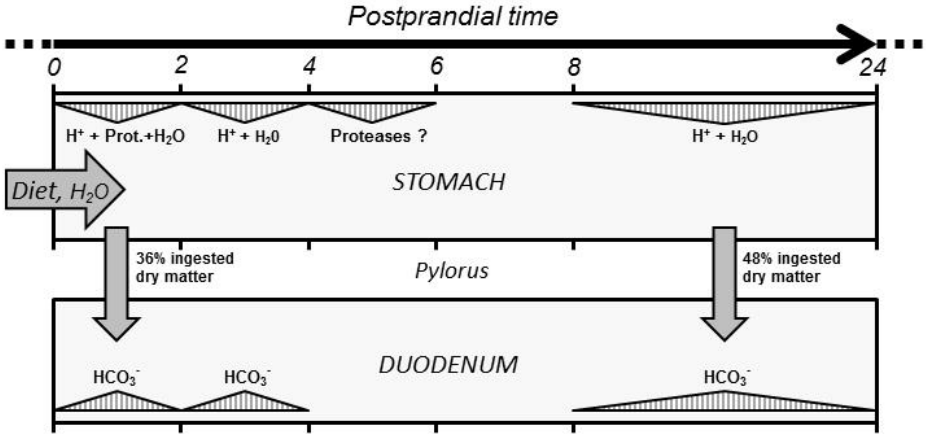

Figure 5. Minimal spatiotemporal model of the postprandial gastroduodenal function in *Salmo salar* juveniles fed a commercial diet in freshwater and compatible with the main findings reported in the present investigation

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