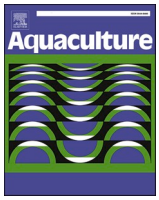
Aquaculture 580 (2024) 740331

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Salinity induced changes in the progression of water, ion and nutrient fluxes along the gastrointestinal tract of Atlantic salmon smolt (*Salmo salar*)

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ARTICLE INFO

Keywords:
Atlantic salmon
Water fluxes
Ion fluxes
Osmoregulation
Digestion kinetic

ABSTRACT

Water ingestion in fish increases with both water salinity and feeding. However, it is unclear whether, during feeding, water ingestion is intended to aid chyme liquefaction in the stomach or to maintain the osmotic homeostasis within the body of the fish. We investigated the effects of increasing water salinity (0, 10, 20, 35 ppt) on the progression of water, ion and nutrient fluxes in the gastrointestinal tract of Atlantic salmon smolt (*Salmo salar*) fed a commercial-like diet. Furthermore, the effect of water salinity on blood pH, plasma osmolality and ions was investigated. The experiment lasted for 8 weeks. Chyme was collected from 4 gastrointestinal tract (GIT) segments (stomach, proximal, middle and distal intestine) and analysed for dry matter, pH, osmolality, crude protein and mineral content. Water and electrolyte fluxes, kinetic of digestion and faecal digestibility were measured using yttrium oxide (Y_2O_3) as an inert marker. We found that between 0 and 35 ppt chyme dry matter decreased by 1.6% and 4.8% in the stomach and proximal intestine, respectively. Chyme pH was not affected by water salinity in the stomach, but it increased linearly ($p < 0.001$) with salinity in all intestinal segments. Chyme osmolality increased linearly ($p < 0.001$) with salinity in the stomach and it decreased in all intestinal segments. Water fluxes were similar among salinities in the stomach, but they increased nearly fivefold (6.2 versus 27.3 ml g^{-1} ingested DM) in the proximal intestine between 0 ppt and 35 ppt. An efflux of monovalent ions (Na^+ and K^+) increased linearly ($p < 0.001$) with salinity in the proximal intestine. An efflux of divalent ions (Ca^{2+} and Mg^{2+}) increased curvilinearly ($p < 0.001$) with salinity in the middle intestine. Plasma osmolality and ion levels increased with salinity. Crude protein digestibility and protease activity decreased significantly with water salinity in the intestine. Our study highlights that when Atlantic salmon moves from freshwater to higher water salinity environments, drinking of saltwater does not interfere with hydration of feed in the stomach, but instead bypasses to the proximal intestine to aid in osmoregulatory water uptake. Therefore, we suggest that water ingestion in seawater fish is intended for osmoregulation rather than to aid digestion by liquefying chyme in the stomach.

1. Introduction

Anadromous fish species have a life cycle that begins in freshwater (FW), followed by smoltification as juveniles, which allows them to migrate to seawater (SW). The physiological mechanism that contributes to the maintenance of water and ion balance (homeostasis) across membranes within the body is defined as osmoregulation. In most cases, fish osmoregulation has been studied in relation to environmental conditions and, in particular, to the transition from freshwater to the saltwater environment and vice versa (Boeuf, 1993; Hoar, 1976, 1988; McCormick et al., 2013, 1998; McCormick and Saunders, 1987). Freshwater fish have a higher concentration of solutes in their internal body fluids (~300 mmol kg^{-1}) than the surrounding water (~1 mmol kg^{-1}), thus they lose ions and gain water through osmosis. In contrast, SW fish have a lower concentration of solutes in their internal fluids than the surrounding water (~1000 mmol kg^{-1}), thus they gain ions and lose water through osmosis (Evans et al., 2005; Evans and Claiborne, 2008; Marshall and Grosell, 2005). Therefore, FW fish actively absorb ions and excrete water, whereas SW fish actively drink water and excrete ions to maintain osmotic homeostasis (Evans and Claiborne, 2008; Grosell,

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https://doi.org/10.1016/j.aquaculture.2023.740331
Received 19 July 2023; Received in revised form 24 October 2023; Accepted 7 November 2023
Available online 8 November 2023

2010; Marshall and Grosell, 2005). Smoltification allows the FW fish to prepare for the SW environment (McCormick, 2012; McCormick et al., 2013; Prunet et al., 1989). Different organs are involved in osmoregulation during smoltification: intestine, gills, kidney and skin, but the main one responsible for water and ion fluxes is the intestine (Sundell and Sundh, 2012). To maintain body homeostasis, water and ion flux occur in and out the gastrointestinal tract (GIT) (Larsen et al., 2014). This movement can be passive (as a result of osmosis) or active (due to specialized cells and transporters) (McCormick et al., 2013, 2009; Sundh et al., 2014). Moreover, during smoltification, drinking rate increases together with active ion and water transport across the intestine, becoming even greater during the SW stage (Sundell and Sundh, 2012). In contrast, drinking is minimal (< 2 ml kg^{-1} h^{-1}) in FW fish and it was mostly observed at fry stage or in association with feeding (Eddy, 2007; Fuentes and Eddy, 1997; Kristiansen and Rankin, 2001; Pyle et al., 2003; Ruohonen et al., 1997; Tytler et al., 1990). Bucking and Wood (2006) observed the presence of water influx into the stomach of FW rainbow trout within the first 12 h of feeding. Nevertheless, whether the water fluxes were of exogenous or endogenous remains to be explored.

Together with environmental parameters, feeding can alter salt and water balances along the gastrointestinal tract of fish during digestion (Bucking and Wood, 2006; Usher et al., 1988; Wood and Bucking, 2010). In aquaculture, where fish are fed dry pelleted diets, kinetic of digestion might be altered. It is hypothesized that consuming dry pelleted diet causes high osmotic pressure in the stomach and, as a result, water influx from the extracellular fluid and/or postprandial drinking (Kristiansen and Rankin, 2001; Ruohonen et al., 1997; Windell et al., 1969). Moreover, the ions present in the feed may pose an osmoregulatory challenge to the gastrointestinal tract of FW fish, driving intestinal ion transport mechanisms similar to those found in marine fish (Taylor et al., 2007). Furthermore, the liquefaction of the chyme in the GIT is fundamental for enzymatic activity during digestion (Buddington et al., 1997). As a result, the different strategies fish adopt to regulate their internal body fluids depending on water salinity may affect the activity of digestive enzymes (Usher et al., 1990). Dabrowski et al. (1986) investigated the effect of water salinity on protein digestion in rainbow trout and found that increasing water salinity had no negative effect. However, Silva-Brito et al. (2019) observed that as salinity increased, trypsin activity in the gut of European seabass (*Dicentrarchus labrax*) decreased. Therefore, salinity might change the digestive processes in the GIT of fish.

Overall, previous research proposed that fish drinking rate increases with water salinity (Wood, 2019) and feeding (Bucking et al., 2011; Eddy, 2007; Kristiansen and Rankin, 2001; Ruohonen et al., 1997; Usher et al., 1988; Wood and Bucking, 2010). However, it is unclear whether the ingestion of water is primarily intended to moisturize the pellet in the stomach or to maintain osmotic balance within the body of the fish. To study this, we measured water and ion fluxes in the gastrointestinal tract of Atlantic salmon smolts fed a commercial-like diet and reared at increasing water salinities (from freshwater to full-strength seawater). Further, digestion kinetics, blood pH, plasma osmolality, and ions were investigated.

2. Material and methods

The feeding trial and sampling were conducted at Matre Research Station of Institute of Marine Research (IMR, Bergen, Norway). All the sampling procedures were performed on euthanized fish. The study was evaluated by the animal experimentation administration of IMR (Forsksdyrforvaltningen) and approved as a non-invasive animal study conducted in accordance with the Norwegian regulations on the use of animals in research, in line with the EU directive 2010/63/EU. This trial was exempt from an animal ethics approval (FOTS application) to the Norwegian Food Safety Authority, according to the regulation "FOR-2015-06-18-761 Regulation concerning the use of animals for scientific purposes, § 6. Godkjenning av forsøk". The approval requirement does not apply to experiments involving only the killing of animals to use organs or tissues from them.

Table 1
Ingredients and analysed nutrient composition of the experimental diets.

Ingredients	%
Fish meal	25.00
Soy protein concentrate	18.00
Wheat gluten	15.00
Corn gluten	3.00
Wheat	9.11
Fish oil	12.70
Rapseed oil	11.00
Lecithin from rapeseed	0.50
Choline chloride	0.50
Vitamin premix	0.50
Monosodiumphosphate	2.30
Carophyll Pink	0.05
Mannan oligosaccharides	0.50
L-Lysine	0.20
L-Threonine	0.05
DL-Methionin	0.15
Mineral premix	0.50
Yttrium oxide	0.01
Water adjustment	0.93
Sum	100.00
Proximate composition	**%**
Dry matter	92.9
Protein	49.1
Fat	28.6
Calcium	0.95
Sodium	0.98
Potassium	0.90
Magnesium	0.24
Phosphorus	1.51

2.1. Experimental design, animal housing and feeding

The experiment followed a dose response design with increasing salinity levels from freshwater to full strength seawater. Four different salinities, 0, 10, 20 and 35 ppt, were used in quadruplicate tanks for each salinity level. The experiment was performed with a mixed sex population of Atlantic salmon (*Salmo salar*) smolts ($n = 480$) ready for seawater transfer. All fish came from the same population, AquaGen Atlantic InnOva Prime strain (AquaGen AS, Norway). The fish were randomly allocated to 16 tanks (1 m^3) with 30 fish per tank. The tanks were supplied with flow through water (8 l min^{-1}), which had the salinity according to the assigned treatment. The photoperiod set at 12:12, L:D. Water temperature was kept at 12 °C and oxygen saturation in the outlet was kept above 80%. At the start of the experiment, average fish weight was 188 ± 5 g (mean ± SD). All fish were fed the same diet (produced by Nofima AS, Bergen, Norway, 3.0 mm extruded sinking pellets), which mimics a commercial type of diet for Atlantic salmon smolts (Table 1). According to Austreng et al. (2000), yttrium oxide (Y_2O_3) was used as an inert marker to measure digestion kinetic and water/ion fluxes in the gastrointestinal tract (GIT). Fish were fed twice a day (2 h each feeding) for 8 weeks using automatic feeders until apparent satiation and feed intake was monitored through collection of feed spill. The uneaten feed pellets were collected 15 min after each meal, weighed and quantified to estimate feed intake according to Helland et al. (1996). The adaptation period of the experiment lasted for 8 weeks to ensure that fish were well adapted to the different salinities and that the feed intake stabilized overtime.

2.2. Sampling

Fish were sampled at 6 h post-prandial. This post-prandial time point was chosen to standardize the amount of chyme present in the

gastrointestinal tract following physiological gut transit at the water temperature of 12 °C. Feed refusal was also recorded during the last day of the experiment as described above. During this last feeding all fish were fed 2.8 g at all water salinities. Due to the labor intensive work, the final sampling was carried out during two days (days 56–57). Each day, eight tanks were randomly sampled. All fish were euthanized in tricaine methanesulfonate (Finquel, MS-222, 0.5 g l^{-1}) and batch weighed to measure the final biomass. Subsequently, four fish in each tank were sampled for blood from the caudal vein using 2 ml heparinized syringes (24G, 0.8 × 40 mm needle). Blood was then collected in 2 ml Eppendorf tubes, and pH was measured immediately after blood collection using a pH-meter (Seven2Go S2-Basic). Following the measurement of blood pH, the Eppendorf tubes were centrifuged at 10,000 RPM for 5 min (Eppendorf® Centrifuge 5430/5430R) for plasma separation, which was used to determine plasma osmolality (Micro-Osmometer, Fiske, Model 210) and ions (Ca^{2+}, Na^{+}, K^{+} and Cl^{-}) concentration (Radiometer, ABL90 FLEX plus). All fish per tank (n = 30) were then dissected for collection of chyme samples from the GIT. The method was adapted from Bucking and Wood (2009). In brief, The GIT was divided into the stomach, the proximal intestine (including the pyloric caeca), the middle intestine, and the distal intestine based on visual identification of anatomical structures. Clippers were placed at the junctions of the different segments before collecting the chyme to ensure that the contents did not mix. Each segment was squeezed into a separate 150 ml plastic container, where the chyme samples collected were pooled per tank. From these pooled chyme samples, a subsample of 2 ml was taken in an Eppendorf tube, centrifuged at 10,000 RPM for 5 min (Eppendorf® Centrifuge 5430/5430R) to separate the fluid and solid phase of chyme for the analysis of osmolality and ions in the liquid phase. One more subsample (~ 3 g) of the chyme from each GIT segment was collected into 50 ml plastic tubes and diluted with cold distilled water in a 1:1 (*w/v*) ratio to allow homogenisation (Homogeniser, POLYTRON® PT 2100, Kinematica). After homogenisation, the mixture was centrifuged at 3220 rpm for 30 min at 4 °C (Centrifuge 5804/5804R) and the supernatant (enzyme extract) were collected into 2 ml Eppendorf in triplicates and stored at −80 °C for further measurement of digestive enzyme activity (method modified from Yasumaru and Lemos, 2014). The remaining pooled chyme samples were then freeze-dried for 72 h, homogenised by pestle and mortar into a fine powder, and stored at 4 °C until analysis to determine chyme nitrogen, mineral and yttrium content.

2.3. Analyses and calculations

The diets were homogenised and analysed for dry matter, ash, lipid and protein following standard procedures. Briefly, dry matter was measured after drying at 105 °C for 24 h; ash content determined by combustion in a muffle furnace at 550 °C for 16–18 h (NMKL, 1991). Total lipid was determined by ethyl-acetate extraction of tissue and acid-extraction in feeds (NS 9402, 1994). Total nitrogen was measured with a nitrogen analysed (Vario Macro Cube, Elementary Analysensysteme GmbH, Germany), according to AOAC official methods of analysis and crude protein calculated as N x 6.25 (AOAC, 1995). The concentration of minerals and yttrium in diets and chyme were analysed using a microwave assisted digestion (UltraWAVE, Milestone, Sorisole, Italy) (Julshamn et al., 2007) and an inductively coupled plasma mass spectrometry (iCapQ ICP-MS, Thermo Scientific, Waltham, USA) equipped with an auto sampler (FAST SC-4Q DX, Elemental Scientific, Omaha, USA) (Silva et al., 2019). In practice, 0.2 g of diet was digested using 2 ml of HNO_3 (69% *w*/w) and 0.5 ml of H_2O_2 (30% w/w) in a Milestone-MLS-1200 microwave oven (Milestone Inc., Shelton, CT, USA). The digested samples were subsequently diluted to 25 ml with Milli-Q® water. A similar procedure was applied to digest the ingredients and the faeces samples. Approximately 0.2 g of sample was digested using 2 ml of HNO_3 in an ultrawave digestion system (UltraWAVE, Milestone, Sorisole, Italy). The samples were capped and placed in the ultrawave system with a container of 130 ml Milli-Q® water and 5 ml H_2O_2. The extracts were then diluted to 25 ml with Milli-Q® water. Chyme pH and osmolality were measured on fresh samples using a pH-meter (Seven2Go S2-Basic) and osmometer (Micro-Osmometer, Fiske, Model 210), respectively. Fish performance was measured during the 8 week feeding period, as described by (Saravanan et al., 2013). Briefly, feed intake per fish (FI, g $fish^{-1}$) was calculated as FI = (total offered feed - uneaten feed)/ (number of fish) (on dry matter basis, DM). To determine the weight gain (Wg, g $fish^{-1}$), the difference between the average individual final (Wf) and initial (Wi) body weight per fish was calculated. Specific growth rate (SGR, % d^{-1}) was calculated using the formula (ln(Wf) - ln(Wi))/t)*100. The feed conversion ratio (FCR, on DM basis) was obtained by FI Wg^{-1}.

Crude protein (CP) digestion (%), water fluxes (ml g^{-1} of ingested DM feed) and ion fluxes of Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+} (mg g^{-1} of ingested DM feed) were calculated in the stomach, proximal, middle, and distal intestine using yttrium oxide (Y_2O_3) as a marker as described by Harter et al. (2013). Briefly, the relative water or ion content measured in the stomach chyme were subtracted from that in the diet and divided by the relative ingested feed dry matter. In the proximal, middle, and distal intestine, the relative water or ion content in the chyme of each intestinal segment was subtracted from that in the chyme of the previous segment and divided by the relative ingested feed dry matter. The relative ingested feed dry matter (g DM mg^{-1} yttrium) was calculated by dividing the ingested dry matter on the sampling day by the yttrium content of the ingested feed.

Spectrophotometric (colorimetric) assays were performed for enzyme activity using enzyme-specific substrates. Pepsin activity (U ml^{-1}) in the stomach chyme was measured using hemoglobin as substrate (Anson and Mirsky, 1932). Pepsin activity was defined as the amount of enzyme that produces an increase in absorbance (at 280 nm) of 0.001 per minute at a temperature of 37 °C and pH of 3 (Andreeva and Rumsh, 2001). However, real stomach pH was not taken into account to measure the real pepsin activity. Then, it was defined as the total putative pepsin activity. Alkaline protease activity (U ml^{-1}) of intestinal chyme was measured using casein as substrate, according to Walter (1984). The pH at which the chyme from each intestinal segment was analysed was the standard pH = 8 according to the method of Alarcón et al. (2002). One unit of protease activity was defined as 1 mg tyrosine released in 1 min using the extinction coefficient for tyrosine at 280 nm of 0.005 ml mg^{-1} cm^{-1} (Alarcón et al., 2002). Total putative pepsin and alkaline protease activity in the chyme (U mg^{-1} ww) of each GIT segment were calculated by dividing the enzyme activity (U ml^{-1}) by the chyme wet weight (ww, mg).

2.4. Statistical analyses

Fish tanks (n = 16) were used as experimental units for all analysed parameters and data are expressed as the mean ± pooled standard errors of the mean (pSEM) or standard deviations (pSD) per treatment of four replicates. All parameter were tested for the effect of salinity by regression analysis as well as one-way ANOVA. Treatment means and results of one-way ANOVA are given in the supplementary tables. When the salinity effect was significant ($p < 0.05$) by ANOVA, treatment means were compared using a Tukey HSD (honest significant difference) with multiple comparisons and a 95% level of significance. For all parameters, linear regression and quadratic regression analyses were performed using salinity levels as the dependent variable. Only the significant relationships were presented in all figures and tables of the results section. All statistical analyses were carried out using the IBM Statistical Package for the Social Sciences (SPSS) program (version 27.0.1; New York, NY, USA). Figures were made using GraphPad Prism version 8.

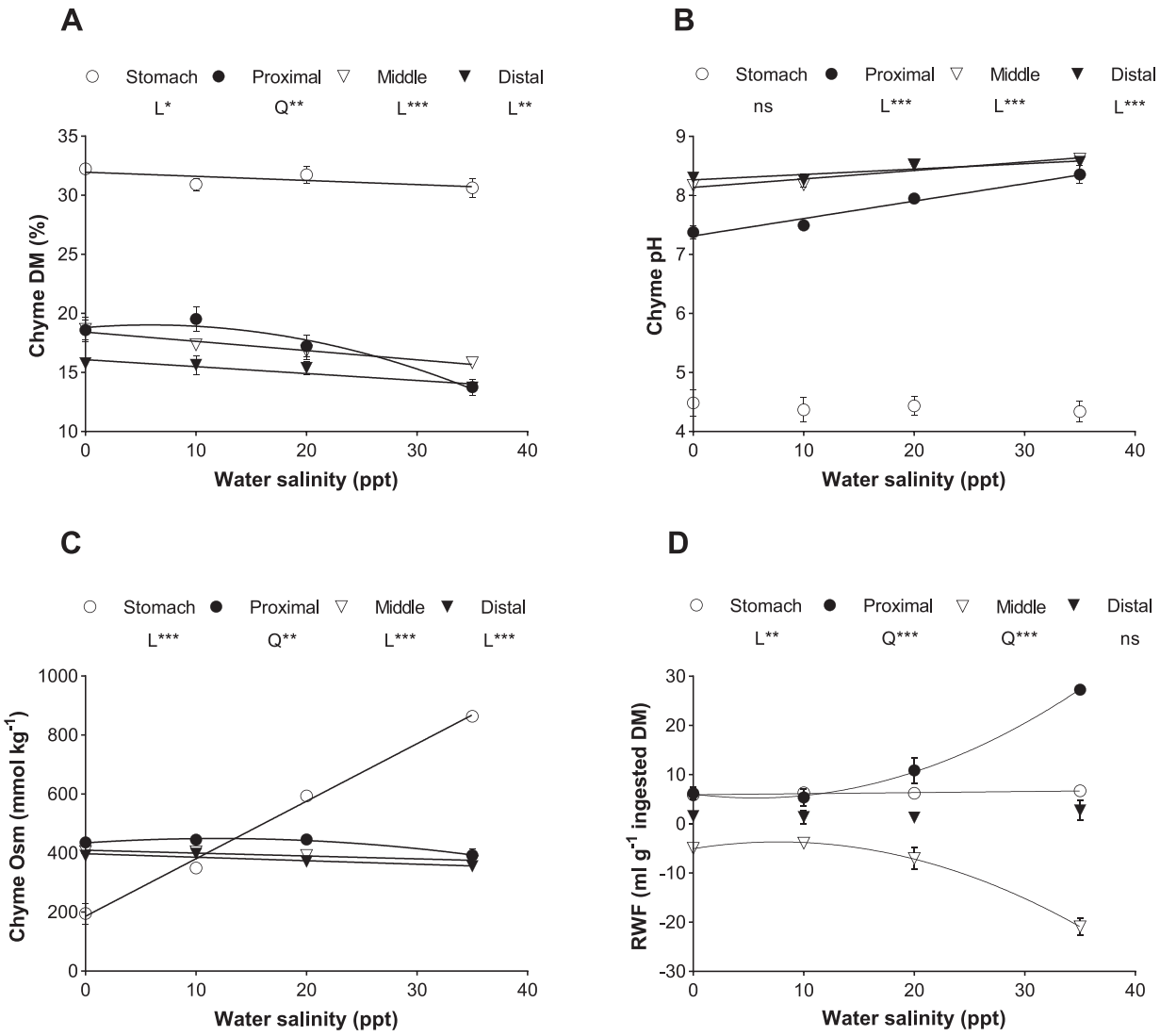

Fig. 1. (A) Chyme dry matter, DM, (B) pH, (C) osmolality, Osm and (D) relative water fluxes, RWF, as affected by increasing water salinity (0, 10, 20 and 35 ppt) in the stomach, proximal, middle and distal intestine of Atlantic salmon. Solid lines indicate a significant relationship, either linear (L) or quadratic (Q) (*, $p < 0.05$; **, $p < 0.001$, ***, $p < 0.001$), while no lines indicate a non-significant relationship (ns, $p > 0.05$). Values are expressed as the mean per treatment ($n = 4$) and pooled standard deviations (pSD). Estimations of the significant linear or quadratic relationships are given in Supplementary table S3.

3. Results

During the adaption period, feed intake was negatively affected by increasing water salinity ($p < 0.05$) (Supplementary Table S1). As a consequence, fish weight gain (Wg, g fish^{-1}) was also negatively affected ($p < 0.001$), being 200 ± 10.1, 161 ± 31.1, 105 ± 5.9 to 110 ± 12.8 g fish^{-1} (mean ± S.D.) in 0, 10, 20 and 35 ppt, respectively. Averaged across all salinities, Atlantic salmon grew from an initial weight of 188 ± 5 g fish^{-1} (mean ± S.D.) to a final weight of 332 ± 43 g fish^{-1} (mean ± S.D.) and fish survival was 99%.

3.1. Chyme characteristics and relative water fluxes

Chyme characteristics and relative water fluxes (RWF) as affected by water salinity are depicted in Fig. 1.

Chyme dry matter (DM) decreased linearly with salinity ($p < 0.001$) in all GIT segments except for the proximal intestine, where the relationship was curvilinear ($p < 0.01$) (Fig. 1A). In the stomach, chyme DM lowered by 1.6% between 0 and 35 ppt (Supplementary table S2). The proximal intestine had the largest decrease in chyme DM dropping by 4.8% between 0 and 35 ppt. Furthermore, between the stomach and the proximal intestine, chyme DM decreased by 13.7% and 16.9%, respectively (Supplemental table S2). Overall, water salinity had the least effect on chyme DM in the stomach compared to the middle and distal intestine, as indicated by estimated slopes of −0.04, −0.08, and − 0.06% ppt-1, respectively (Supplementary table S3).

Chyme pH was not affected ($p > 0.05$) by water salinity in the stomach (averaged over salinities 4.4) (Supplementary table S2). In all intestinal segments, chyme pH increased linearly ($p < 0.001$) with water salinity (Fig. 1B). In the proximal intestine, it increased from 7.4 to 8.3 between 0 and 35 ppt. In the middle and distal intestine, the effect of water salinity on chyme pH were comparable, as indicated by the estimated slope being 0.01 ppt^{-1} (Supplementary table S3).

Chyme osmolality (Osm) increased linearly ($p < 0.001$) with water salinity in the stomach, whereas it decreased curvilinearly ($p < 0.01$) in the proximal intestine and linearly ($p < 0.001$) in the middle and distal intestine (Fig. 1C). The largest change in chyme Osm occurred in the stomach, where it increased more than four times from 0 ppt (195 mmol kg^{-1}) to 35 ppt (864 mmol kg^{-1}), with an estimated slope of 20 mmol kg^{-1} ppt^{-1} (Supplementary table S3).

Relative water flux (RWF) was positive (water influx) in all GIT segments, except for the middle intestine, where water efflux occurred

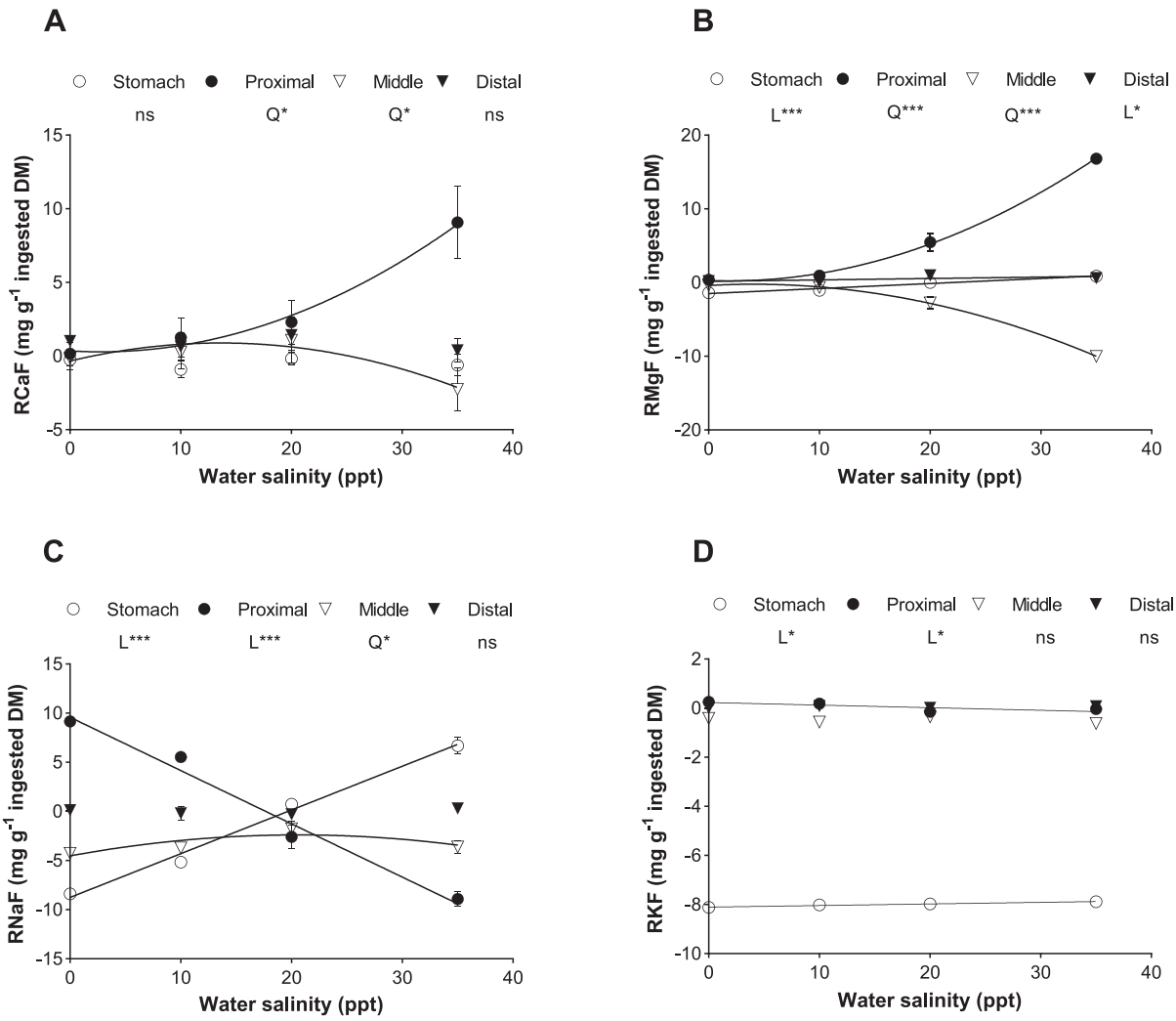

Fig. 2. Relative fluxes of (A) calcium, (RCaF); (B) magnesium, (RMgF); (C) sodium, (RNaF) and (D) potassium, (RKF) in the stomach, proximal, middle and distal intestine of Atlantic salmon, as affected by increasing water salinity (0, 10, 20 and 35 ppt). Solid lines indicate a significant relationship, either linear (L) or quadratic (Q) (*, $p < 0.05$; ***, $p < 0.001$), while no lines indicate a non-significant relationship (ns, $p > 0.05$). Values are expressed as the mean per treatment ($n = 4$) and pooled standard deviations (pSD). Estimations of the significant linear or quadratic relationships are given in Supplementary table S6.

(Fig. 2D). In the stomach, RWF increased linearly ($p < 0.01$) by 0.8 ml g^{-1} ingested DM between 0 and 35 ppt salinity (Supplementary table S2). The proximal intestine showed the largest change in RWF, with water influx increasing nearly 5-fold between 0 ppt (6.2 ml g^{-1} ingested DM) and 35 ppt (27.3 ml g^{-1} ingested DM). In the middle intestine, RWF had the same magnitude but opposite direction as in the proximal intestine, decreasing from 0 ppt (−5.0 ml g^{-1} ingested DM) to 35 ppt (−20.9 ml g^{-1} ingested DM). There was no significant effect of water salinity on water fluxes in the distal intestine.

3.2. Electrolyte fluxes

Ion fluxes along the GIT as affected by water salinity are depicted in Fig. 2.

Relative Ca^{2+} flux (RCaF) was not affected by water salinity in the stomach and in the distal intestine ($p > 0.05$). In the proximal intestine, a Ca^{2+} influx occurred at all water salinities increasing curvilinearly ($p < 0.05$) from 0.2 to 9.1 mg g^{-1} ingested DM between 0 and 35 ppt (Fig. 2A). In contrast to the proximal intestine, RCaF decreased curvilinearly ($p < 0.05$) with salinity in the middle intestine, but at a lower magnitude (from −0.1 to −2.3 ml g^{-1} ingested DM between 0 and 35 ppt) (Supplementary table S4, S5).

Water salinity affected relative Mg^{2+} flux (RMgF) in all GIT segments (Fig. 2B). RMgF increased with water salinity in all GIT segments, except for the middle intestine. The proximal intestine showed the largest change in RMgF, with Mg^{2+} influx increasing from 0.4 to 16.8 mg g^{-1} ingested DM between 0 and 35 ppt. In the middle intestine, RMgF had a similar magnitude but in the opposite direction (Mg^{2+} efflux). In the distal intestine, Mg^{2+} influx took place increasing linearly ($p < 0.05$) between 0 and 35 ppt (Supplementary table S4, S5).

Relative Na^{+} flux (RNaF) was affected by water salinity in all GIT segments, except for the distal intestine (Fig. 2C). RNaF increased and decreased linearly ($p < 0.001$) with water salinity in the stomach and proximal intestine, respectively. In the stomach, RNaF increased from 0 to 35 ppt (−8.4 to 6.7 mg g^{-1} ingested DM). In contrast, in the proximal intestine, RNaF declined from 0 to 35 ppt (9.2 to −2.6 mg g^{-1} ingested DM). The RNaF trends were relatively similar in absolute terms, as indicated by the estimated slopes being 0.4 and − 0.5 mg g^{-1} ingested DM ppt^{-1} in the stomach and proximal intestine, respectively. In the middle intestine, Na^{+} efflux occurred in all intestinal segments with a curvilinear trend ($p < 0.05$) as water salinity increased (Supplementary table S4, S5).

Relative K^{+} flux (RKF) was affected by water salinity in the stomach and proximal intestine, while no effect on the middle and distal intestine was detected (Fig. 2D; Supplementary table S4). The increase and decline of RKF in the stomach and proximal intestine were similar in

Table 2
Blood pH in the caudal vein, osmolality and ion concentration (mmol l^{-1}) in plasma of Atlantic salmon smolt as affected by increasing water salinity (0, 10, 20 and 35 ppt).

	0 ppt	10 ppt	20 ppt	35 ppt	pSEM	ANOVA *p-value*	Regression *p-value*
Blood pH	7.18^{c}	7.04^{b}	6.96^{ab}	6.94^{a}	0.02	***	Q***
Plasma osmolality	323^{a}	331^{b}	327^{ab}	329^{b}	1.47	***	L*
Plasma Ca^{2+}	1.03^{a}	1.12^{ab}	1.11^{ab}	1.18^{b}	0.04	*	L**
Plasma Na^{+}	161^{a}	163^{ab}	165^{bc}	166^{c}	0.68	***	L***
Plasma K^{+}	3.5^{a}	5.4^{b}	3.2^{a}	3.7^{a}	0.26	***	ns
Plasma Cl^{-}	128^{a}	136^{b}	142^{d}	139^{c}	0.69	***	Q***

Ca^{2+}, calcium; Na^{+}, sodium, K^{+}, potassium, Cl^{-}, chloride.
L, linear effect; Q, quadratic effect. ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Values are means (n = 4) and pooled standard errors of the mean (pSEM). Equation, (intercept (SE) ± β (SE) ± β_1 (SE)): Blood pH, Y = 7 (0.02) – 0.02 (0.003) X + 0.00027 (0.000074) X^2 (R^2 = 0.59); plasma osmolality, Y = 325 (1.2) + 0.13 (0.062) X (R^2 = 0.06); plasma Ca^{2+}, Y = 1 (0.03) + 0.004 (0.001) X (R^2 = 0.11); plasma Na^{+}, Y = 161 (0.6) + 0.2 (0.03) X (R^2 = 0.33); plasma Cl^{-}, Y = 128 (0.7) + 1 (0.1) X – 0.02 (0.003) X^2 (R^2 = 0.87).

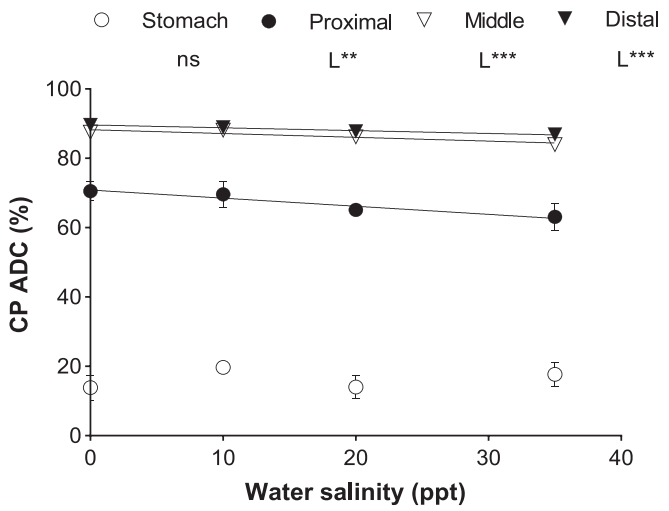

Fig. 3. Progression of digestion of crude protein (CP ADC) as affected by increasing water salinity (0, 10, 20 and 35 ppt) in the stomach, proximal, middle and distal intestine of Atlantic salmon smolt. Solid lines indicate a significant relationship, either linear (L) or quadratic (Q) (**, $p < 0.01$, ***, $p < 0.001$), while no lines indicate a non-significant relationship (ns, $p > 0.05$). Values are expressed as the mean per treatment ($n = 4$) pooled standard deviations (pSD). Equation, (intercept (SE) ± β (SE) ± β_1 (SE)): proximal, Y = 71.1 (1.25) – 0.22 (0.06) X (R^2 = 0.52); middle, Y = 88.1 (0.47) – 0.12 (0.03) X (R^2 = 0.67); distal, Y = 89.6 (0.22) – 0.07 (0.01) X (R^2 = 0.79).

absolute terms, as indicated by the estimated slopes being 0.01 and −0.01, respectively (Supplementary table S5).

3.3. Blood pH, plasma osmolality and ion content

The effect of water salinity on blood pH, plasma osmolality (Osm) and ion concentration are presented in Table 2. Blood pH decreased curvilinearly ($p < 0.001$) from pH = 7.2 in the 0 ppt group to pH = 6.9 in the 35 ppt group. Plasma osmolality (Osm) and ion content increased from 0 ppt to 35 ppt, except for K^{+}. Plasma Osm increased linearly ($p < 0.05$) from 0 ppt (323 mmol l^{-1}) to 35 ppt (329 mmol l^{-1}). Ca^{2+} and Na^{+} plasma concentration increased linearly ($p < 0.001$) with water salinity, while Cl^{-} plasma concentration increased curvilinearly ($p < 0.001$) (Table 2). Overall, the increase in ion concentration in the plasma was relatively stronger for Na^{+} than for Ca^{2+}, as indicated by the estimated slopes being 0.2 and 0.004 mmol l^{-1} ppt^{-1}, respectively (Table 2). In contrast, Cl^{-} concentration in the plasma increased between 0 (128 mmol l^{-1}) and 10 ppt (136 mmol l^{-1}) and decreased between 19.5 ppt (142 mmol l^{-1}) and 35 ppt (139 mmol l^{-1}) (Table 2).

3.4. Crude protein digestion kinetic

Crude protein (CP) progression of digestion is depicted in Fig. 3. In the stomach, CP digestion was not influenced by salinity ($p > 0.5$). In all other intestinal segments CP digestion was negatively affected by water salinity and this effect was linear ($p < 0.001$). In the proximal intestine, the difference in CP digestibility between 0 ppt (70.6%) and 35 ppt (63.1%) was nearly 7.5%, while this difference became much smaller in the middle (3.5%) and distal (2.7%) intestine (Supplementary table S6). Accordingly, the estimated slope of the line was bigger in the proximal intestine than in the middle and distal intestine being −0.22, −0.12 and − 0.07% ppt^{-1}, respectively (Fig. 3).

3.5. Proteolytic enzyme activity in the chyme

Water salinity did not affect protease activity in the stomach and in the proximal intestine ($p > 0.05$), whereas it linearly decreased ($p < 0.001$) with water salinity in the middle and distal intestine (Table 3). In the middle intestine, protease activity more than halved between 0 ppt (797.9 U mg^{-1} chyme ww) and 35 ppt (382.4 U mg^{-1} chyme ww). In the distal intestine, protease activity decreased almost three times between 0 ppt (367.4 U mg^{-1} chyme ww) and 35 ppt (136.9 U mg^{-1} chyme ww). Accordingly, estimated slopes of the line were − 14 and − 8 U mg^{-1} ppt^{-1} in the middle and distal intestine, respectively (Table 3).

Table 3
Total putative protease activity (U mg^{-1} chyme ww) in the chyme of stomach (pepsin), proximal, middle and distal intestine of Atlantic salmon smolt as affected by increasing water salinity (0, 10, 20 and 35 ppt).

U mg^{-1} chyme ww		0 ppt	10 ppt	20 ppt	35 ppt	pSEM	ANOVA *p-value*	Regression *p-value*
Pepsin	**Stomach**	120.5	246.4	148.5	142.1	33.5	ns	ns
Protease	**Proximal**	479.1	664.4	278.1	267.0	120.8	ns	ns
	Middle	797.9	865.4	374.3	382.4	131.2	*	L*
	Distal	367.4^{b}	403.7^{b}	107.0^{a}	136.9^{a}	41.9	***	L**

L, linear effect; ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$. Values are means (n = 4) and pooled standard errors of the mean (pSEM). Equation, (intercept (SE) ± β (SE) ± β_1 (SE)): middle, Y = 843 (112) – 14 (5) X (R^2 = 0.35); distal, Y = 388 (46) – 8 (2) X (R^2 = 0.50).

4. Discussion

The primary function of the stomach is temporary storage of feed to accommodate for large prey or meals. Consequently, it is the site of the initial physical and enzymatic breakdown of the meal into chyme (Bakke et al., 2010). When eating dry feed (pellets), this is also the location where pellets are moisturized (Usher et al., 1988). Previous research also investigated water dynamics in the GIT when fish were fed a moisturized feed (Kristiansen and Rankin, 2001; Ruohonen et al., 1997). In both cases, however, water influx to the GIT can originate from endogenous secretions or ingestion of exogenous water (drinking) (Bucking and Wood, 2006; Ciavoni et al., 2023; Elesho et al., 2022; Harter et al., 2015). Drinking rate in fish is affected by species, size, feeding state (fed versus starved), and environmental conditions (e.g., salinity, temperature) (Evans, 1968). Gaetano et al. (2023) found that both osmolality and Cl^- ion content were higher in plasma than in the intestinal chyme fluid of Atlantic salmon smolt in sea water and concluded that fish were able to process ingested seawater by absorbing ions and water through the GIT. Similarly, plasma Cl^- and Na^+ ion concentration in the present study (Table 2) were higher compared to the intestinal ion level in the chyme fluid phase at 35 ppt (Supplementary table S7), suggesting that fish were well adapted to water salinity. One of the important seawater adaptations in smolts is water absorption by the gut (Usher et al., 1988). Previous research found that unfed pre-smolt Atlantic salmon (fresh water) drink about 1.3 ml kg^{-1} h^{-1}, whereas fed fish drink nearly five times as much (about 6 ml kg^{-1} h^{-1}) (Eddy, 2007). In seawater, the difference in drinking rate between starved and fed post-smolt Atlantic salmon is less pronounced, ranging between 3.81- and 6.45-ml kg^{-1} h^{-1} in the former and 6 to 7.94 ml kg^{-1} h^{-1} in the latter (Usher et al., 1988; Smith et al., 1991; Eddy, 2007). In the current study, relative drinking rate in fed Atlantic salmon smolts increased with salinity from 0.78 to 4.11 ml kg^{-1} h^{-1} between 10 and 35 ppt salinity (Supplemental table S8), in comparison with FW (0 ppt). Further, Thodesen et al. (2001) proposed that large Atlantic salmon drink less than small salmon, which could explain the lower drinking rate measured in the current study. Nevertheless, the total volume of water ingested in seawater is much larger compared to freshwater condition. Therefore, we hypothesized that the ingestion of seawater would decrease the dry matter in the stomach, especially at high salinities. In contrast to our hypothesis, the DM of the chyme in the stomach was stable across all salinities, if any, slightly decreasing (by 1.6%) between 0 and 35 ppt (Fig. 1A). Furthermore, drinking rate and chyme osmolality increased with salinity without affecting the chyme DM in the stomach. Similar to chyme DM, water influx in the stomach slightly increased (0.8 ml g^{-1} ingested DM) between 0 and 35 ppt, which does not reflect the increasing magnitude of drinking occurring between freshwater and seawater conditions.

Water influx between the stomach and the proximal intestine increased from 5.9 to 6.2 ml g^{-1} ingested DM and from 6.7 to 27.3 ml g^{-1} ingested DM at 0 and 35 ppt salinity, respectively. The large increase in water influx in the proximal intestine at higher salinity reflects the increased drinking rate. Water influx in the proximal intestine of FW fish is primarily attributed to bile and intestinal wall secretions (Grosell, 2010). In contrast, in SW fish, the magnitude of increased water influx in the proximal intestine would necessitate a significant amount of metabolic energy to produce that enormous amount of endogenous fluid secretion, which would be disadvantageous to the fish (Grosell and Genz, 2006). As a result, we propose that most of the water ingested by SW adapted Atlantic salmon quickly moves to the proximal intestine (bypassing the stomach), where it combines with endogenous secretions resulting in a much higher water influx than in FW fish. Similar to our results, Hartviksen et al. (2014) observed a drop in chyme DM of more than half between the stomach and the proximal intestine in seawater-reared Atlantic salmon smolt fed various plant and animal based diets. Bergman et al. (2003) investigated water dynamics in the gastrointestinal tract of tilapia (*Alcolapia grahami*) living in an alkaline environment, Lake Magadi (carbonate alkalinity, pH = 9.85). They found that when the fish were not feeding, water almost entirely bypassed the stomach and moved directly into the intestine. Furthermore, they proposed that, while simultaneous intake of water with food into the stomach was unavoidable when the fish were feeding, at least some of the imbibed water was shunted past the stomach directly into the intestine, allowing the stomach pH to remain low during digestion. Increased gastric pH would result in increased gastric acid secretion for enzymatic digestion as well as a significant increase in blood HCO_3^- post-feed (alkaline tide phenomenon) (Goodrich et al., 2022). Furthermore, an increased gastric acidic secretion, would require more energetic cost of digestion which might affect fish growth. However, in the current study, the lower fish growth measured in SW fish is caused by the lower feed intake. In contrast, increased chyme pH in the proximal intestine promotes nutrient hydrolysis by activating pancreatic enzyme activity (optimum pH = 7–9) (Deguara et al., 2003; Fard et al., 2007). Similar to Bergman et al. (2003), we found that at higher water salinities, the increased seawater ingestion did not affect chyme pH in the stomach, suggesting that seawater is quickly moving to the proximal intestine, where chyme pH significantly increased with water salinity (Fig. 1B). Even though base secretions play a major role in increasing chyme pH in the intestine of marine fish (Grosell, 2006), we propose that the ingested seawater is shunted directly to the proximal intestine, bypassing the stomach, further contributing to the pH increase.

The water entering the proximal intestine at higher salinities was reabsorbed in the middle intestine (Fig. 1D). The addition of water into the proximal intestine and its reabsorption in the middle intestine describe the physiological role of the intestine in osmoregulation when fish move from freshwater to high saline water (Hoar, 1988; Sundell and Sundh, 2012). Based on our results, we propose that chyme liquefaction in the stomach is endogenous, whereas it is both endogenous and exogenous in the intestine. With regard to gut segment functionality, the distal intestine is commonly described to be the site for water reabsorption in fish (Whittamore, 2012; Wood and Bucking, 2010). However, our findings clearly show that the middle intestine plays a larger role in water reabsorption in Atlantic salmon.

Along with water fluxes, ion fluxes can indicate if the water was of endogenous or exogenous origin. The high influx of divalent ions (Ca^{2+} and Mg^{2+}) in the proximal intestine (Fig. 2A, B) along with water, suggests that water influx derives from ingested seawater. Divalent ions are then partially re-absorbed (efflux) and partially precipitated and excreted in the middle intestine of SW fish (Grosell, 2010). When precipitation of divalent ions occurs, chyme osmolality decreases in the intestinal fluid, further aiding water absorption (Grosell, 2010). Seawater is also rich in monovalent ions (Na^+, K^+ and Cl^-), which are mostly desalinized in the esophagus (Hirano and Mayer-Gostant, 1976; Parmelee and Renfro, 1983). However, the high chyme osmolality measured in this study in the stomach of SW fish (864 mmol kg^{-1}) suggests that the desalinization process must continue beyond the esophagus. In fact, the efflux of monovalent ions increased with water salinity in the proximal intestine (Fig. 2C, D). Accordingly, previous research has shown that the first part of the esophagus and the intestine are essential for ion reabsorption in the GIT of SW teleosts (Kirsch, 1978; Kirsch and Meister, 1982).

The additional physiological roles of the GIT with regard to ion and water absorption can affect their prime function of digestion and nutrient absorption. Alkaline proteases are essential for proteolytic activity in the intestine. Alkaline protease activity in the chyme was negatively affected by salinity in the middle and distal intestine (Table 3). One possible explanation is that drinking rates increased with salinity, and because of more water ingestion, enzyme activity decreased due to dilution as there is less enzyme or substrate per unit of chyme. The proposed dilution effect is supported by the decreasing dry matter and apparent digestibility of crude protein in the respective segments with increasing salinity (Fig. 1A; Fig. 3). Usher et al. (1990) and Krogdahl et al. (2015), on the other hand, found no significant differences in

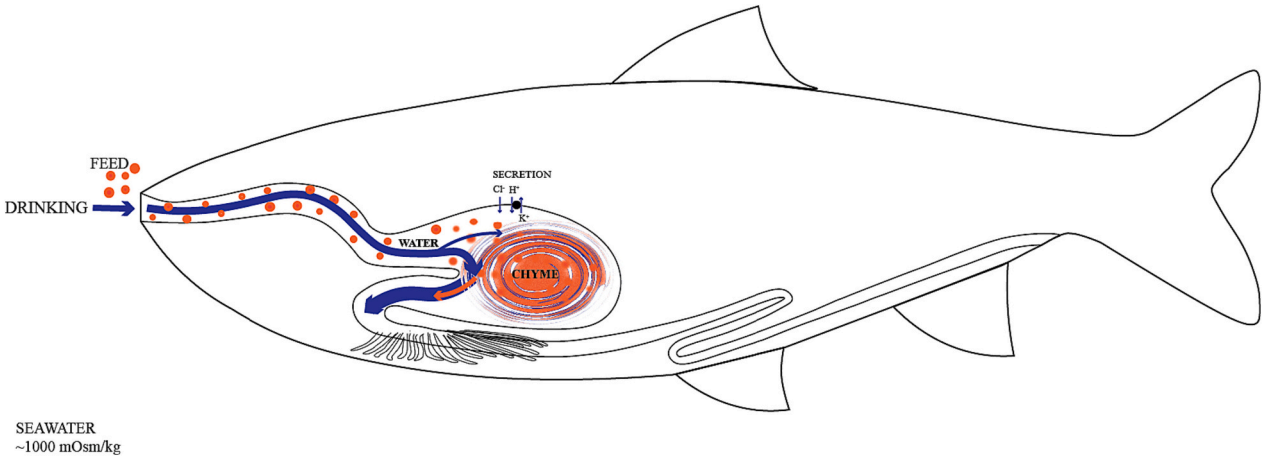

Fig. 4. Water and chyme dynamic in the stomach of seawater acclimatized Atlantic salmon (*Salmo salar*).

enzyme activity in the intestine chyme of freshwater and seawater Atlantic salmon. A similar result to Usher et al. (1990) and Krogdahl et al. (2015) was found for tilapia (*Oreochromis niloticus*) adapted to freshwater and seawater (Lee-Shing and Shu-Fen, 1989). However, the seawater-adapted tilapia appeared to have different salt-adapted proteolytic enzymes compared to the freshwater one. Furthermore, in the current study, proteolytic enzyme activity was highest in the middle intestine and lowest in the distal intestine at all water salinities. This is consistent with previous research that looked at the trend of digestive enzyme activity in the intestine of Atlantic salmon (Chikwati et al., 2013, 2012; Hartviksen et al., 2014; Krogdahl et al., 2015). With regards to protein digestibility, we found that salinity had a negative impact on CP digestibility in all intestinal segments (Fig. 3). This is consistent with other studies where CP digestibility was higher in FW compared to SW Atlantic salmon (Krogdahl et al., 2004; Usher et al., 1990). However, they only measured faecal digestibility, but not the progression of digestion. In this study instead, we observed that the difference in CP digestibility between 0 and 35 ppt was greater in the proximal intestine (7.5%) and decreased in the distal segment (2.8%) (Fig. 3). As a result, we could propose that, as water salinity rises, the magnitude of CP digestion shifts downstream in the intestine. Con et al. (2017) also reported a salinity-dependent shift in the localization of three peptide transporters along the intestine of the tilapia, another euryhaline species. Our findings suggest that the transition of salmon from FW to SW influences where the protein is absorbed in the intestine.

Overall, our results show that drinking rate increased with water salinity but the influx of water in the stomach was minimal compared to the proximal intestine at higher salinities. Moreover, water influx in the proximal intestine was followed by a re-absorption of water in the middle intestine. As a result, ingested water had an effect on chyme characteristics and digestion kinetics in the intestinal segments rather than the stomach, where the ingested food appears to stay longer (higher chyme DM). Therefore, we suggest that, at higher salinities, the majority of the ingested water is bypassing the stomach and moving to the proximal intestine more rapidly compared to the chyme in the stomach (Fig. 4). In conclusion, our results indicate that the exogenous water entering the GIT does not really mix with the chyme in the stomach. Therefore, we propose that ingested water by the fish is primarily used for osmoregulation processes rather than to moisturize the chyme in the stomach and aid digestion.

CRediT authorship contribution statement

Elisa Ciavoni: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Johan W. Schrama:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Gopika Radhakrishnan:** Methodology, Formal analysis. **Øystein Sæle:** Conceptualization, Methodology, Writing – review & editing. **Antony J. Prabhu Philip:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This study was funded by the Aquaculture and Fish Nutrition group (AFI) of Wageningen University and Research (Netherlands) and by the Institute of Marine Research (IMR) of Bergen (Norway). The authors wish to acknowledge the technical assistance rendered by Karen Anita Kvestad and the staff working at Matre experimental research station (IMR, Norway) for providing technical assistance during the experiment and laboratory work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.740331.

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